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The use of otolith shape analysis for ageing juvenile red snapper, *Lutjanus campechanus*

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Abstract Morphological changes in otolith shape with age, of young (<age 3) red snapper were examined through shape analysis and tested as an objective method for age determination. Otoliths from two collections of juvenile fish (hatchery and wild) were used in the study. First, shape analysis was applied to a series of known-age otoliths from hatchery-reared age 0, 1 and 2 fish. Multidimensional scaling and non-parametric analysis of similarities showed significant shape differences among the three age classes of fish. Discriminant function analysis and cross-validation classification showed 65.6% correct age classification based on shape variables alone, and 86.7% correct age classification with inclusion of otolith weight in the discriminant function (n=90). Subsequently, the method was applied to otoliths from a series of age 0, 1 and 2 wild caught red snapper. Otoliths from wild fish showed a similar age classification success rate of 68.9% based on shape variables alone and 86.7% correct age classification with the inclusion of otolith weight in the discriminant function (n=90). Ageing of juvenile red snapper through otolith increment counts has been difficult in past studies and this study provides an alternative, objective method of otolith shape analysis for ageing young fish of this species.

Keywords Otolith shape analysis · Age determination · Discriminant function analysis · Red snapper

Introduction

Red snapper, *Lutjanus campechanus*, is an important marine fish species to both sport and commercial fisheries in the Gulf of Mexico. Management practices rely on age-based population assessments to assess the present status of the stock and health of the fishery. For management success, accurate age determinations are critical. However, the current ageing technique of counting annual growth increments on sectioned otoliths is often difficult and subjective in young red snapper. In particular, identification of the first annulus (typically diffuse and non-distinct) is subject to reader interpretation and is a source of considerable error in ageing of young fish (Holt and Arnold 1982; Wilson and Nieland 2001; Rooker et al. 2004; Allman et al. 2005; Morales-Nin and Panfili 2005). Difficulty in identifying annual growth increments in sectioned otoliths leads to reduced accuracy and precision by otolith readers. Therefore, the development of non-subjective methods for ageing of red snapper may increase accuracy and benefit management efforts for this species.

To date, otolith shape analysis (measuring morphological differences among otoliths) is considered an objective method to identify fish stocks; however,

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this method has not been widely explored as a possible method for ageing fish. Otolith shape analysis is most often used to identify fish species (Short et al. 2006) and to discriminate among fish stocks (Tuset et al. 2003). For example, by exploiting differences in otolith shape, individual stocks of Atlantic and Gulf of Mexico king mackerel, *Scomberomorus cavalla* were identified while being collected in mixed feeding grounds (DeVries et al. 2002), and multiple spawning stocks of Atlantic cod, *Gadus morhua*, were identified in the North Atlantic, a species previously managed as a single stock fishery (Cardinale et al. 2004). Significant age effects on otolith shape were noted in some studies employing otolith shape analysis to identify fish stocks (Begg and Brown 2000; Galley et al. 2006), but few studies have attempted to age fish with this method. Fossen et al. (2003) examined alternative methods to increase ageing accuracy of long rough dab, *Hippoglossoides platessoides* and found a significant correlation between otolith size characteristics and age. In that study, it was suggested that simple otolith measurements could be incorporated into a model to increase ageing accuracy and precision for that species. Doering-Arjes et al. (2008) provided the first successful age estimates from otolith shape analysis for known-age Atlantic cod and included otolith weight as a classification variable. In that study, classification error rates ranged from 11–54%, varying by the source of calibration material. Discriminant function analysis showed greater accuracy in age classification when known-age otoliths were used for calibration material as compared to reader-aged calibration material (Doering-Arjes et al. 2008).

In the present study, shape analysis was applied to a collection of otoliths from known-age hatchery fish and wild caught red snapper that were aged by traditional growth increment counts. The objectives were to examine otolith shape and weight differences among age 0, 1 and 2 red snapper, to identify growth patterns in otoliths from young red snapper, and to test otolith shape analysis as an objective method for ageing young red snapper. Objective and straightforward methods of age determination in young red snapper would benefit management practices for the fishery through increased ageing accuracy, efficiency and decreased costs involved with the traditional method of ageing red snapper otoliths.

Materials and methods

Sample collection

Hatchery red snapper were spawned in May 2002 and 2003 at the Claude Peteet Mariculture Center in Gulf Shores, Alabama U.S.A. Juvenile fish were reared in circular tanks (1.5 m diameter, 0.7 m depth) within an 11000 L recirculating seawater system. Fish were sampled at age 0, 1 and 2 from September through December 2002, April through December 2003, and January through July 2004. Sagittal otolith pairs were dissected, dried and stored in individual plastic vials. A total of 750 otolith pairs were taken from hatchery red snapper, including 518 pairs from age 0, 124 pairs from age 1 and 108 pairs from age 2 fish (Chapin et al. 2009).

A total of 7507 age 0 wild snapper were collected by trawls 13 km south of Mobile Bay, Alabama U.S.A. in 1994 and 1995 (Szedlmayer and Conti 1999). Age 0 fish used in the study were sampled from the December 1995 survey. A total of 547 age 1 and 1178 age 2 wild red snapper were caught by hook-and-line 20 to 40 km south of Mobile Bay, Alabama U.S.A. from 1999 to 2003 (Szedlmayer 2007). Age 1 and age 2 otoliths used in the study were randomly sampled from the 1999 collection (March through June). Otoliths from wild caught red snapper were dissected and stored the same as described for hatchery fish. Ages of wild red snapper were determined by thin-sectioning one otolith from each pair and counting growth increments with knowledge of capture date, location and fish size. Annual growth increments were counted in age 1 and age 2 fish and daily microincrements counted for age 0 fish (Szedlmayer and Conti 1999). Ages of wild fish were assumed to be accurate for this study by incorporating data on individual fish size, date of capture and capture location along with visual counts of annual growth increments in sectioned otoliths to determine age for each fish. An annual increment formation rate was validated from mark and recaptured red snapper for older fish (Szedlmayer and Beyer unpublished data); however, visual identification of the first growth increment in young red snapper remains a source of error in age estimates for the species. To directly compare shape analysis to traditional ageing techniques, one otolith from each of the age 1 and age 2 (n=56) hatchery-reared fish was

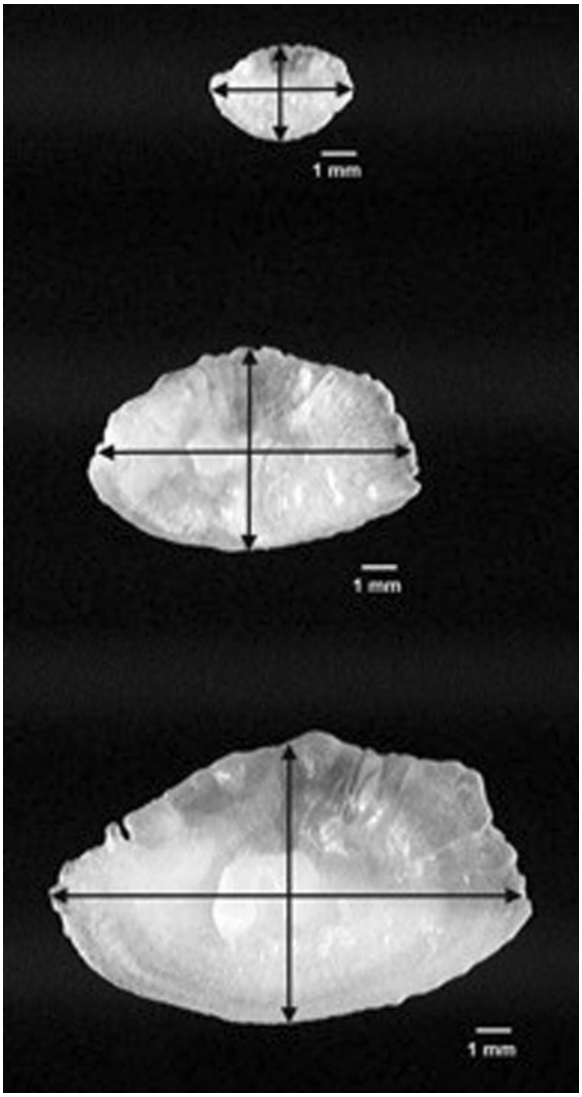

Fig. 1 Whole otoliths from age 0 (**a**), age 1 (**b**), and age 2 (**c**) hatchery-reared red snapper. Arrows show measurements used in the calculation of aspect ratio

thin-sectioned and annual growth increments read independently by two readers. The only data provided to the readers was date of sacrifice for each fish. Actual age and fish size were unknown by the otolith readers when determining age.

Imaging and shape analysis

Only whole otoliths without chips or damage were used in the analysis. Otoliths were viewed under a dissecting microscope against a black background, sulcus down and rostrum positioned to the left for standardization. Magnification varied from 1 to 4 X to ensure detailed photographs among varying otolith sizes. Images were captured with a Sony CCD video camera and Flashpoint 128-4 M digitizing board (Integral Technologies, Inc), and analyzed in Image-Pro Plus version 4.5. Measurement calibrations were based on a micrometer image in both the vertical and horizontal axes at all magnifications. An automated trace function of the otolith perimeter in the imaging program collected size and shape data for each otolith (Fig. 1). Shape variables measured were aspect ratio, rectangularity, box x/y, radius ratio, roundness and perimeter ratio (Table 1). Dry weights of whole otoliths were measured with an Ohaus CT10 balance to the nearest 2 mg. We randomly selected 30 otoliths from each age class (left side only, age 0, 1 and 2), from both hatchery and wild red snapper collections to use in the shape analysis.

Multivariate analysis

Shape variables were used to calculate a similarity index (Czekanowski) for all possible pairs of otoliths: similarity $= 100\left(1 - \left(\sum\left|\mathrm{Y_{ij}} - \mathrm{Y_{ik}}\right| / \sum\left(\mathrm{Y_{ij}} + \mathrm{Y_{ik}}\right)\right)\right)$ where $\mathrm{Y_i}$=a specific shape variable and j and k are the otoliths being compared (Field et al. 1982; Yoshioka

Table 1 Description of shape variables used in otolith image analysis

Shape variable	Description
Aspect ratio	Ratio of major axis length and minor axis length of an ellipse enclosing the object
Rectangularity	Ratio of object area and area of its enclosing box
Box x/y	Ratio of width and height of object's enclosing box
Radius ratio	Ratio of maximum radius and minimum radius
Roundness	Perimeter^2 / (4 *π* area)
Perimeter ratio	Ratio of convex portion of perimeter to total perimeter

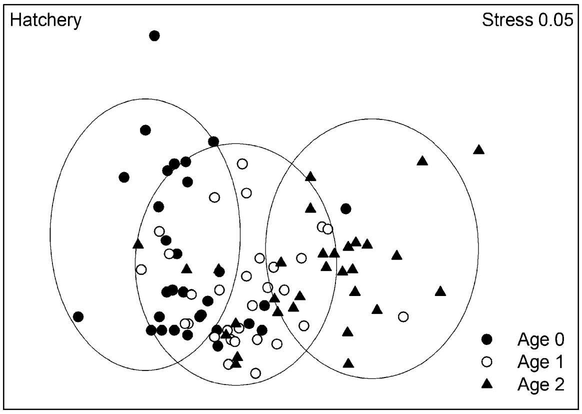

Fig. 2 Multidimensional scaling plot showing separation of age 0, 1 and 2 hatchery-reared red snapper based on shape variables alone

2008). The similarity indices were then used in a multidimensional scaling analysis (MDS; Schiffman et al. 1981). The MDS provided a two-dimensional "map" of the distances among otoliths in Euclidian space based on the similarity index. Thus, comparisons of red snapper otoliths were based on all shape variables, yet independent of age class. A stress value of <0.05 indicated an "excellent" representation of the similarity rankings in the MDS plot (Clarke and Warwick 2001). A non-parametric analysis of similarity (ANOSIM) identified the significance of shape differences among age classes with the Global *R* statistic.

Differences in fish length (mm) and otolith weight (mg) among age classes of both hatchery and wild fish were tested with an analysis of variance (ANOVA). Multiple a posteriori ANOVA's followed by Tukey HSD tested individual shape variables against age class to further define and identify patterns in otolith growth with age.

Quadratic discriminant function analysis followed by the formulation of cross-validation classification matrices showed the number of correctly aged fish and provided age classification success and error rates for each discriminant function. Cross-validation procedures omitted the individual object (otolith) being classified (i.e. n-1) to classify each otolith into an age class, thus creating an unbiased age classification matrix (Friedman 1989). A significance level of $P \leq 0.05$ was used for all statistical tests unless specified.

Results

Hatchery fish

Hatchery red snapper otoliths showed significant differences in shape among age 0, 1 and 2 fish (ANOSIM, R=0.287, P=0.001). Age class separation based solely on otolith shape variables was shown in an MDS plot, stress level=0.05 (Fig. 2). The shape variables aspect ratio, box x/y and radius ratio significantly increased across all three age classes (Table 2). Also, fish length and otolith weight significantly increased with age (Table 2). Classification success of otoliths into correct age groups was 65.6% with shape variables alone and increased to 86.7% with the inclusion of otolith weight in the discriminant function (Table 3). Accuracy of age readings for sectioned otoliths were 80.4% (reader 1)

Table 2 ANOVA comparisons of fish size, otolith weight and otolith shape variables (mean±SD) across age classes of otoliths from known-age, hatchery red snapper. Different letters show significant ($P \leq 0.05$) differences

Variable	Fish age class				
	Age 0	Age 1	Age 2	F value	*P* value
Fish total length (mm)	68±30 (a)	219±29 (b)	284±36 (c)	311.11	<0.0001
Otolith weight (mg)	13±17 (a)	157±46 (b)	293±58 (c)	307.85	<0.0001
Aspect ratio	1.47 ± .06 (a)	1.53 ± .06 (b)	1.60 ± .08 (c)	32.95	<0.0001
Rectangularity	0.71 ± .01 (a)	0.72 ± .01 (ab)	0.72 ± .01 (b)	4.23	0.0176
Box x/y	1.43 ± .05 (a)	1.49 ± .07 (b)	1.55 ± .08 (c)	24.15	<0.0001
Radius ratio	1.59 ± .06 (a)	1.65 ± .06 (b)	1.76 ± .10 (c)	31.02	<0.0001
Roundness	1.15 ± .04 (a)	1.15 ± .03 (a)	1.18 ± .03 (b)	5.99	0.0037
Perimeter ratio	0.98 ± .01 (ns)	0.99 ± .01 (ns)	0.98 ± .01 (ns)	2.22	0.1152

Table 3 Number (and percent) of red snapper otoliths assigned to the three age groups by cross-validation of the quadratic discriminant function analysis for known-age, hatchery-reared fish

Group	Assigned age (%)			
	Age 0	Age 1	Age 2	Total classification success (%)
Shape variables				65.6
Age 0	23 (76.7)	6 (20.0)	1 (3.3)	
Age 1	7 (23.3)	17 (56.7)	6 (20.0)	
Age 2	3 (10.0)	8 (26.7)	19 (63.3)	
Shape variables with otolith weight				86.7
Age 0	28 (93.3)	2 (6.7)	0 (0)	
Age 1	0 (0)	25 (83.3)	5 (16.7)	
Age 2	0 (0)	5 (16.7)	25 (83.3)	

and 75.0% (reader 2) for age 1 and age 2 hatchery fish.

Wild fish

Similar to hatchery fish, shape analysis showed significant differences in otolith shape among age 0, 1 and 2 wild red snapper (ANOSIM, R=0.181, P=0.001). The MDS plot showed otolith shape differences by age class at a stress level=0.06 (Fig. 3). Although fewer significant differences in otolith shape were detected across age classes in wild compared to hatchery red snapper; aspect ratio, rectangularity, box x/y, and radius ratio all showed significant differences across certain age groups (Table 4). Similar to hatchery fish, fish length and otolith weight significantly increased with age. Age classification success of wild fish was 68.9% based on shape variables alone and increased to 86.7% with the inclusion of otolith weight in the discriminant function (Table 5).

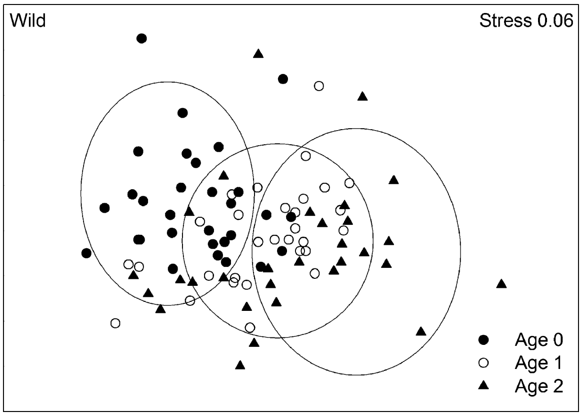

Fig. 3 Multidimensional scaling plot showing separation of age 0, 1 and 2 wild red snapper based on shape variables alone

Discussion

Otolith shape analysis identified significant shape differences among age 0, 1 and 2 red snapper otoliths from both hatchery-reared and wild caught fish. Thus, otolith shape, independent of size or weight can be used to correctly assign age classes to young red snapper. Doering-Arjes et al. (2008) found similar results using otolith shape to assign ages of known-age Atlantic cod and similar studies have used external features such as otolith size and weight measurements to estimate age of fish (Fossen et al. 2003; Petursdottir et al. 2006).

The cross-validation classification matrices showed 65.6% correct age classification of otoliths from known-age, hatchery fish and 68.9% correct age classification of otoliths from wild fish. These results are similar to findings reported by Doering-Arjes et al. (2008) where classification success in assigning age through otolith morphometric measurements ranged from 46–89%. Also, classification rates in the present study were comparable to success rates from other studies that used otolith shape analysis to identify fish stocks (Tuset et al. 2003; Cardinale et al. 2004; Stransky 2005). The addition of otolith weight in the discriminant function increased classification success to 86.7% for both hatchery and wild fish indicating that otolith weight is a valuable characteristic for age determination. Boehlert (1985) and Mc Dougall (2004) also found otolith weight to be a useful measure of age in

Table 4 ANOVA comparisons of fish size, otolith weight and otolith shape variables (mean±SD) across age classes of otoliths from wild red snapper. Ages were based on otolith increment counts. Different letters show significant ($P \leq 0.05$) differences

Variable	Fish age class				
	Age 0	Age 1	Age 2	F value	*P* value
Fish total length (mm)	90±27 (a)	242±19 (b)	285±27 (c)	530.60	<0.0001
Otolith weight (mg)	24±15 (a)	172±27 (b)	231±53 (c)	270.28	<0.0001
Aspect ratio	1.50±0.05 (a)	1.57±0.06 (b)	1.59±0.08 (b)	16.63	<0.0001
Rectangularity	0.70±0.01 (a)	0.70±0.02 (a)	0.72±0.02 (b)	5.97	0.0037
Box x/y	1.43±0.05 (a)	1.50±0.06 (b)	1.54±0.09 (b)	19.36	<0.0001
Radius ratio	1.60±0.07 (a)	1.66±0.06 (b)	1.71±0.13 (b)	10.35	<0.0001
Roundness	1.16±0.03 (ns)	1.17±0.03 (ns)	1.17±0.04 (ns)	.30	0.7398
Perimeter ratio	0.98±0.01 (ns)	0.98±0.01 (ns)	0.99±0.01 (ns)	2.20	0.1172

fish. In the present study, independent age readings of sectioned otoliths from known-age, hatchery fish showed 75.0% and 80.4% accuracy in identifying annual growth increments, thus providing a direct comparison of ageing accuracy between the two techniques. However, greater costs involved in the time consuming process of thin-sectioning and polishing otoliths for production ageing, and continued difficulty in identification of the first annulus remains problematic and a source of error for ageing of young red snapper. Thus, otolith shape analysis along with otolith weight may provide a better method for age estimation in fish species with indistinct first annuli.

Three of the otolith shape variables including aspect ratio, box x/y and radius ratio significantly increased across all three age classes of hatchery fish otoliths. This indicated that otoliths from juvenile hatchery red snapper grew faster along the anterior-posterior axis compared to the dorsal-ventral axis, or became more elongate with age. Patterns were less distinct in the wild red snapper otoliths, but significance differences were still detected across at least one age group. Red snapper otolith growth and shape patterns were similar to patterns shown for long rough dab (Fossen et al. 2003), and Atlantic cod (Galley et al. 2006). Both studies showed faster otolith growth along the anterior-posterior axis.

Differences in observed variation between hatchery and wild fish may have been caused by differences in sampling periods. All wild fish in this study were sampled across wide seasonal recruitment periods and greater population pool size of wild fish may have caused larger variance in otolith shape measures compared to hatchery fish. For example, hatchery-

Table 5 Number (and percent) of red snapper otoliths assigned to the three age groups by cross-validation of the quadratic discriminant function analysis for wild fish

Group	Assigned Age (%)			
	Age 0	Age 1	Age 2	Total classification success (%)
Shape variables				68.9
Age 0	22 (73.3)	6 (20.0)	2 (6.7)	
Age 1	3 (10.0)	22 (73.3)	5 (16.7)	
Age 2	5 (16.7)	7 (23.3)	18 (60.0)	
Shape variables with otolith weight				86.7
Age 0	30 (100)	0 (0)	0 (0)	
Age 1	0 (0)	24 (80)	6 (20.0)	
Age 2	0 (0)	6 (20.0)	24 (80.0)	

reared fish were spawned over a single month (May) and reared under controlled environmental conditions over the course of three years, whereas wild fish were sampled in the month of December (age 0) and March through June (age 1, 2), and individual fish were probably exposed to greater environmental variability compared to hatchery fish. Also, red snapper in the wild have a protracted spawning season ranging from April to October (Fitzhugh et al. 2004), which results in greater variation in size at age when fish are pooled into year groups. These factors including sampling period, protracted spawning and natural environmental variance, most likely increased variance of shape variables in wild caught red snapper. However, significant differences in shape were still observed among age 0, 1 and 2 otoliths from both hatchery and wild fish resulting in successful age classification based on objectively measured variables.

This study showed potential for otolith shape analysis to serve as a new, objective method for ageing juvenile wild red snapper. One advantage over increment count methods is that age classification error is clearly defined within classification success matrices as compared to reader interpretation error, which can vary substantially across individuals. Accuracy of increment counting is subject to experience and is especially difficult in young red snapper otoliths considering that the first annual growth increment may be diffuse or non-existent (Buckmeier 2002; Allman et al. 2005). Also, shape analysis is considerably less time consuming and less expensive than traditional ageing techniques, eliminating the costs of sectioning and counting growth increments on individual otoliths. Reducing ageing error through automated computer image analysis could potentially benefit large-scale population studies requiring production ageing of young red snapper.

Only otoliths from the first three age classes of red snapper were available for shape analysis even though this species may live to 40 or 50 years (Szedlmayer and Shipp 1994; Patterson et al. 2001; Wilson and Nieland 2001). Red snapper undergo rapid growth within the first 10 years of life and otolith shape differences among age classes are probably more distinct over this early time period. Considering this rapid growth period, we suggest that otolith shape ageing may be possible for a number of years past age 2, but further validation with older fish is required. The method of otolith shape analysis to age fish may be most useful for fast-growing, short-lived species, in which the opportunity for large changes in otolith shape may be greatest.

In conclusion, otolith shape analysis may be a more accurate and cost effective method for ageing juvenile red snapper and provides a new, objective method of ageing independent of otolith increment counts. Less subjective methods of ageing could benefit the fishery, especially considering that management of red snapper relies heavily on accurate ageing practices.

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