Age frequency, growth, mortality and PAH levels of roughtongue bass, *Pronotogrammus martinicensis*, following the Deepwater Horizon oil spill.

by

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ABSTRACT

The 2010 Deepwater Horizon (DWH) oil spill and its immediate effects on the surrounding ecosystems have been extensively examined, but longer term effects are still widely unknown. Among the regions potentially affected by the DWH spill were the Pinnacle reefs located 54 to 111 km from the spill site. Roughtongue bass, *Pronotogrammus martinicensis*, is an ecologically important resident fish from these "mesophotic" reefs. The present study examined age frequency, growth rate, mortality, and PAH levels in roughtongue bass from the Pinnacle reefs. Sample sites within the Pinnacles included the Alabama Alps 54 km from the DWH site, and Roughtongue Reef 111 km from the DWH site. Fish were collected in September-October 2014 (n = 190), December 2014 (n = 249), March 2015 (n = 310) and June-July 2015 (n = 360). Size of roughtongue bass collected ranged from 54 to 135 mm standard length (SL; n = 1109). Resident fish (n = 1090) were dominated by the 2009 and 2010-year classes. The Von Bertalanffy (VB) growth rates (L_∞ = 103.5, K = 0.54, t_0 = 0.02, R^2 = 0.22) were similar to previous pre-oil spill estimates. The VB growth curve for the east site (L_∞ = 101.2, K = 0.73, t_0 = 0.22, n = 873, R^2 = 0.17), showed faster rates than the west site (L_∞ = 95.7, K = 0.32, t_0 = -1.48, n = 216, R^2 = 0.20), and comparisons of the linear portions of the curves confirmed significant differences in growth rates between east and west sites ($F_{3,1081}$ p < .02). Roughtongue bass mortality rate (M) = 1.7 and survival rate (S) = 18%. Polycyclic aromatic hydrocarbons were detected (> 5 ppb) in 76 % of the roughtongue bass tested (n=38; mean ± SE = 50 ± 52.2 ppb), but below the minimum 300 ppb level that had detectable effects in marine organisms established by the EPA in 1987. Levels of PAH in fish were not significantly different between collection sites and were not correlated with growth. The present study examined a mesophotic reef species where PAH contamination might be expected due to reefs proximity to the DWH oil spill site. Despite this close proximity, there was little effect detected in this ecologically important mesophotic reef fish species.

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INTRODUCTION

In April 2010, the Deep Water Horizon (DWH) Macondo well exploded and sank. The well was located 73 km off the southeast coast of Louisiana. For nearly three months, the well leaked oil from 1500 m below the ocean's surface (Thibodeaux et al. 2011). The spill was labeled as the worst oil spill recorded in U.S. history, with approximately 4.7-5.5 million barrels of oil entering the Gulf of Mexico (Thibodeaux et al. 2011).

There have been several studies documenting the negative effects of large quantities of oil entering the marine system. These studies have shown detectable effects of oil in the aquatic environment at the biochemical, organismal, population, and community levels (Capuzzo 1987). A few well known examples of oil spill effects in the marine environment include the 1989 EXXON Valdez spill and the 1979 Ixtoc-1 spill. In both events there were reports of immediate wildlife mortalities and detections of longer term negative effects on the surrounding ecosystems. Directly after the EXXON Valdez spill, an estimated 300,000 birds from 90 different species died due to initial oil exposure (Piatt et al. 1990). In addition, the EXXON Valdez spill was implicated in the herring collapse of 1993 by potentially causing immunodeficiency and disease susceptibility in the herring stock (Thorne and Thomas 2008; Incardona et al. 2015). Following the Ixtoc-1 spill, there were reports of reductions in crabs and declined abundance in two year classes of shrimp (Jernelöv 2010).

Oil residues have the potential to persist for many years in anaerobic benthic environments, which then may continuously expose the organisms that inhabit the sedimentary layer (Albers 1995; Jewett et al. 2002; Soto et al. 2014). Several years after the EXXON Valdez and Ixtoc-1 spills, traces of oil were still being found within the marine system (Jewett et al 2002; Jernelöv 2010). For example, ten years after the EXXON Valdez spill, new exposure to hydrocarbons was detected within the tissue of masked greenling, *Hexagrammos octogrammus* (Pallas), and crescent gunnel, *Pholis laeta* (Cope; Jewett et al. 2002).

Initial oil exposure and long term oil residue exposure have both been reported to cause reduced survival, reproductive loss and growth reduction in several different fish species. For example, in laboratory studies, reduced survival and growth rate were detected in pink salmon, *Onchorynchus gorbuscha* (Walbaum), that survived initial oil exposure as eggs (Heintz et al. 2000). Also, chronic exposure to low levels of hydrocarbons caused reduced growth and survival similar to a single short term high-hydrocarbon exposure (Heintz et al. 2007).

Many of these long term immunotoxicity effects (e.g., morphology impairment and genome expression changes) are because petroleum contains polycyclic aromatic hydrocarbons (PAHs; Albers 1995; Barron 2012; Whitehead et al. 2012). These PAH compounds have two or more benzene rings composed of hydrogen and carbon that can be arranged linearly, angularly or clustered with potentially substituted groups (Sims and

Overcash 1983; Albers 1995; Latimer and Zheng 2003). The significance of these compounds is that they can be highly toxic, carcinogenic and mutagenic to organisms in quantities as low as 1.5 ppm (Sims and Overcash 1983; Latimer and Zheng 2003). There are 16 identified PAHs established by the Environmental Protection Agency as being particularly toxic to aquatic organisms, and include acenaphthylene, acenaphthene, anthracene, benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenzo[ah]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene (EPA 1987; Latimer and Zheng 2003).

In recent studies on fish, PAHs have been connected with biochemical effects in the form of alteration to mixed-function oxygenase enzymes (Sims and Overcash 1983; Akcha et al. 2003). These enzymes are associated with increased free radical production and are linked to mutagenic and carcinogenic events (Albers 1995; Tuvikene 1995). Similarly, PAH contamination from a coking plant was associated with liver cancer in brown bullhead catfish, *Ameiurus nebulosus* (LeSueur; Baumann and Harshbarger 1995). Other studies have also shown PAH contamination had damaging effects on larvae formation. Specifically, phenanthrenes, flourenes, and dibenzothiophenes have been shown to cause heart dysfunction and malformation in several fish species including dolphinfish, *Coryphaena hippurus* (Linnaeus; Edmunds et al. 2015).

After the initial release into the ecosystem, approximately 4 % of the DWH oil consisted of PAHs, resulting in $2.1x10^{10}$ g of PAHs entering the Gulf of Mexico. These PAHs were detected at concentrations of 189 ppb in depths of 1320 m, within 13 km of the spill site, three weeks after the explosion (Diercks et al. 2010; Reddy et al. 2012). Petrogenic PAHs can degrade in marine environments, but within anaerobic subtidal sediments of aquatic environments, they can persist for many years after their initial release into the ecosystem, creating the possibility for continuous exposure for benthic species (Albers 1995; Diercks et al. 2010).

As a result of these potential effects of mass amounts of oil from the DWH spill entering the Gulf of Mexico ecosystems, many of its important fisheries were faced with uncertain ecological and economic damages (Mendelssohn et al. 2012). Numerous species from diverse environments in the northern Gulf of Mexico were possibly subject to different effects from the spill. Immediately after the DWH spill there were mortalities of 6,147 birds, 613 turtles and 157 mammals (U.S. Fish and Wildlife Service 2011). Since the spill there have been several studies focusing on the potential residual effects of the DWH oil, both from initial exposure as well as potential chronic exposure (Mendelssohn et al. 2012). Sub-lethal DWH oil concentrations have been reported to cause changes in genome expression and tissue morphology impairment in gulf killifish, *Fundulus grandis* (Baird and Gerard; Whitehead et al. 2012). However, other recent studies have not detected year class failures or recruitment failures associated with the DWH spill (Fodrie and Heck 2011; Szedlmayer and Mudrak 2014; Beyer et al. 2016).

One of the specific environments potentially affected by the DWH spill was the continental slope off Alabama-Mississippi coastline (the “Pinnacles”). This habitat has been called a “mesophotic” reef system, and is located approximately 54 to 111 km northeast of the DWH spill site (Weaver et al. 2001; Thurman et al. 2004; Sulak et al. 2008; McBride et al. 2009).

The Pinnacles reef system is a series of rock outcrops at depths ranging from 68 to 100 m on the continental slope of the Gulf of Mexico (Thurman et al. 2004). This system has a diverse array of at least 53 demersal fish species, including commercially important Serranidae, Carangidae and Lutjanidae (Thurman et al. 2004; Sulak et al. 2008). These deep slope habitats are the closest mesophotic reef structures to the DWH oil spill site and as such may have been exposed to PAH contamination in 2010 (Silva et al. 2016).

The roughtongue bass, *Pronotogrammus martinicensis* (Guichenot) is one of the most abundant fish species residing on these mesophotic reef habitats (Thurman et al. 2004; McBride et al. 2009). It is a small (< 200 mm) reef fish species in the family Serranidae (Guichenot 1868). This species has a bright iridescent coloration, with a reddish orange colored body, purple tipped fins and greenish yellow blotchy bands on the anterior portion of the body (Guichenot 1868). Roughtongue bass range throughout the

Gulf of Mexico and South Atlantic from North Carolina, Florida, Bahamas, Bermuda, Caribbean to Brazil, at depths ranging from 45 to 250 meters (Anderson and Heemstra 1980; Gilmore 1977; Dennis and Bright 1988). This species is a protogynous hermaphrodite, meaning they mature first as females and then switch to males around age 2 (Coleman 1981; Thurman et al. 2004; McBride et al. 2009). Spawning peaks from February to July and females spawn daily (Bullock and Smith 1991; Thurman et al. 2004; McBride et al. 2009).

The primary prey of roughtongue bass are zooplankton, and as such, roughtongue bass are considered secondary consumers in this mesophotic reef system (Bullock and Smith 1991; Bryan and Kilfoyle 2007). They are also prey for many larger reef predators such as yellowedge grouper, *Epinephelus flavolimbatus* (Valenciennes)*;* snowy grouper, *Epinephelus niveatus* (Valenciennes)*;* and almaco jack, *Seriola rivoliana* (Bloch; Bullock and Smith 1991; George et al. 2007). Roughtongue bass and other species of the subfamily Anthiinae may be considered important trophic links in these mesophotic reef systems, responsible for energy transfer to the deeper reef systems (Thurman et al. 2004; McBride et al. 2009). Therefore, understanding the DWH effects on this species has important ecological, economic and management implications (Bullock and Smith 1991; George et al. 2007).

Most studies attempting to examine DWH oil effects lack pre-oil spill data sets. Roughtongue bass is an exception in that extensive study occurred before the DWH oil spill (Continental Shelf Associates, Inc. and Texas A&M University 2001; Weaver et al. 2001; Thurman et al. 2004; Sulak et al. 2008; McBride et al. 2009). Thus, this species was well suited for assessing the potential effects of the DWH spill on fishes from the mesophotic reef zone.

The present study measured the effects of the DWH oil spill through comparisons of pre-spill to post-spill year class abundances, growth rates and mortality rates. The use of year class abundance as a measure of pollution effects is a well-documented assessment technique. For example, year class abundance has been used to specifically assess the effects of stream degradation and pollution through recruitment and population abundance (Siligato and Böhmer 2001). It has been shown that significant reductions in fish recruitment and population abundances occur in highly polluted streams, while less polluted streams show higher fish recruitment and higher adult abundance (Siligato and Böhmer 2001). In addition to direct mortalities, another well documented indicator of pollution is reduced growth rates. For example, significantly reduced growth rates of juvenile sole, *Solea solea* (Linneaus), were caused by decreased environmental quality (Amara et al. 2007). Thus, comparison of pre to post DWH spill measures of these selected life history parameters in this deep water Serranidae is a suitable method of evaluating oil spill effects on this mesophotic reef ecosystem.

The present study also measured PAH content in roughtongue bass. Different levels of the various PAHs from fish tissues can be used to identify sources of pollution as well as magnitude of exposure (Yunker et al. 2002). Ratios of specific PAHs help distinguish between pyrogenic and petrogenic sources (Yunker et al. 2002). There are multiple sources of PAHs within the marine environment, including run off, boat exhaust and diffusion from the atmosphere (Latimer and Zheng 2003). Therefore, quantifying the different PAHs is critical for estimating the extent of exposure, as well as the identification of whether or not roughtongue bass were specifically exposed to DWH oil. In addition, PAH quantification within fish tissue can be compared with life history parameters (e.g., year-class strength, mortality, and growth rates) for a more comprehensive evaluation of potential DWH oil spill effects.

The present study examined life history parameters and measured PAH levels in roughtongue bass to evaluate the potential effects of the DWH oil spill on this relatively unknown mesophotic reef fish species.

METHODS

Study sites:

Study sites were located within the Pinnacles reef area on the continental slope approximately 70 km south of the Mississippi-Alabama coastline. Particular locations are defined as the Alabama Alps (West site: N 29.2518, W 88.3373) and Roughtongue Reef (East site: N 29.4415, W 87.5785; Thurman et al. 2004). These sites have shown high abundances of roughtongue bass prior to the DWH spill and are within close proximity to the spill site (Alabama Alps is 54 km and Roughtongue Reef is 111 km from the DWH site; Figure 1). Each site has been previously characterized for habitat and fish diversity (Thurman et al. 2004). Roughtongue Reef has a diameter of approximately 400 m, with a depth of 68 to 78 m and Alabama Alps has a diameter of approximately 1000 m, with a depth of 72 to 88 m (Figure 2; Thurman et al. 2004).

Fish collections:

Roughtongue bass were randomly sampled from reef tops, reef slopes and reef base at both Alabama Alps and Roughtongue Reef. Collections were made for four seasons over one year (fall = September-October 2014, winter = December 2014, spring = March 2015, summer = June-July 2015). After arrival at a reef site, the research vessel anchored at random locations and drifted back and forth over the reef as fish were collected. The exact positions (latitude and longitude) for each fish collected were recorded. The research vessel was repositioned several times (> 20) at each reef site over 2 to 5 day periods for each seasonal survey.

Roughtongue bass were collected with hook and line, sabiki rigs (number 4-6 hook size) with six hooks for each fishing gear. Weights were varied from 227 to 454 g depending on current and sea state. The fishing gear included Daiwa deep drop rods and Tanacom Bull 750 electric reels. Reels were filled with 50 # braided line. Four deep drop gears were fished simultaneously at each site.

When fishing started the time and location were recorded. Once the lines reached the sea floor, the fishing depth was recorded from the electric reels. The fishing continued until a fish was caught or a 5 min limit was reached at which time the line was retrieved and checked (for fish or lost hooks) and then fishing continued. If there were less than 5 hooks when checked, the sabiki rig was replaced with a new 6 hook rig. After capture, fish were removed with a de-hooker and placed in ice chilled seawater. All captured fish were measured and identified on the research vessel. Fish were labeled with waterproof paper and placed into Ziploc bags and frozen on the research vessel for transport back to the laboratory.

AGE ESTIMATION AND ANNUAL GROWTH RATES

Otolith Extraction:

In the laboratory roughtongue bass were weighed to the nearest 0.01 g on an Ohaus Scout-Pro 200 g scale and otoliths were extracted by dorsal dissection. Both sagittal otoliths were removed, rinsed with de-ionized water and stored in sealed vials. Vials contained an internal label with capture date and fish ID number, and an external label with the fish ID number.

Otolith preparation, image capture and increment counting:

Otoliths were stored and dried for a minimum of two months before age estimates. After the drying period, otoliths were viewed with a Leica® MZ6 dissection microscope and digital images of the whole otolith were captured at 2046 x 1536 pixel resolution (5165 x 3878 µm) with an Infinity I Lumenera® digital camera and Lumenara® Infinity Capture program version 6.1.0. All otolith images were captured at 2x magnification on the Leica microscope.

After otolith images were captured, readers were assigned a random set of otoliths to count increments. The following counting protocol for whole otoliths was developed after unsuccessful attempts to count sectioned otoliths. In the present study (n = 17) sectioned otoliths from fish ranging from 72 to 85 mm SL showed diffuse or missing opaque bands that were much clearer and distinct in whole otoliths. Initial examination of whole otoliths from small sized fish (< 90mm SL) showed that the first increment was formed within 1767-2271 µm diameter across the core, but in larger fish (> 100mm SL) this first band would sometimes become obscure due to otolith increasing thickness. Subsequently, the distance spanning across the core to the first visible increment was measured, if this distance was > 2398 µm a count was added for an assumed obscured first increment at 1767-2271 µm diameter in larger otoliths. Also, the smallest fish (54 to 90 mm SL) sometimes showed an apparent false increment that was visible at 1010-1262 µm. This increment was assumed a false increment based on previous age and growth studies, i.e., the observed small size was difficult to accept as an age-2 fish (McBride et al. 2009; Thurman et al. 2004). Annuli were assumed to be deposited in winter (McBride et al. 2009; Thurman et al. 2004). Marginal increment analysis was used to verify that increments were formed annually (Thurman et al. 2004).

When an increment count was completed, it was used along with date of capture to estimate year class. For example, a fish captured in the fall of 2014 with an increment count = 4 was from the 2010-year class, a fish that was captured in spring 2015 with an increment count = 4 was also from the 2010-year class, while a fish captured in summer 2015 with an increment count = 4 was from the 2011-year class. All otoliths were independently counted by two readers. Counts and year class were then compared and any disagreements were reexamined by both readers. If consensus was not reached the otolith count was not used in further analyses.

Length–age relations:

A length at age Von Bertalanffy growth model was used to estimate growth rate for all roughtongue bass, and separately for east and west sites. Parameters were estimated with the non-linear regression procedure in Statistical Analysis Software (SAS) for the equation:

$$L_t = L_\infty[1 - e^{-K(t-t_o)}]$$

Where L_t = the mean standard length (mm) at age t, L_∞ = the asymptotic standard length predicted by the equation, K = the growth coefficient, and t_0 = the age at which standard length was equal to zero. Non-linear regression and pseudo R^2 (1-[sums of square error/corrected sums of square total]) were used to estimate the variation around the Von Bertalanffy growth curve. Von Bertalanffy growth curves were compared between sites as well as pre and post DWH oil spill by visual and standard deviation comparisons. Linear regression analysis was also used to compare SL at age between east and west sites (Zar 2010). Linear comparisons were limited to the portions of the growth curves that were linear (age < 8). Differences were considered significant if $P \leq 0.05$.

MORTALITY AND SURVIVAL

Linear regression of log abundance on age was used to estimate total mortality (Z) for roughtongue bass pooled over all sites, and for east and west sites (Beverton and Holt 1957). Year class, rather than age was used in this mortality estimation. The 2011, 2012, and 2013-year classes were not fully recruited to the fishing gear, therefore mortality estimations were based on all 2010 and prior year classes. Mortality *Z* (regression slope) estimates were compared between sites with analysis of covariance (ANCOVA). Mortality estimates were significantly different between sites if there was a significant interaction effect between site and age. Roughtongue bass are not caught by either sport or commercial fishers, therefore total mortality was equal to natural mortality ($Z = M$). Annual survival was estimated as $S = e^{-Z}$.

PAH's

PAH Extraction:

Roughtongue bass (n = 171) were randomly selected for PAH measurement from the earliest sampled season (Fall 2014) to minimize the time between collections and the DWH spill. Similar sample sizes were measured from Alabama Alps (n = 86) and Roughtongue Reef (n = 85). A modified NOAA method was used for PAH extraction (Sloan et al. 2004; Roberts and Szedlmayer 2015). In the present study the entire fish was used for the PAH extraction, because individual tissue weights were too small for the present extraction method.

Quantification:

Gas chromatograph mass spectrometer (GC/MS) analysis was used to detect and identify potential PAHs after the extraction procedure of the fish samples. The limit of detection for the GC/MS (Waters GCT Premier™) was 5 ppb PAH. Control blanks were used to detect and correct for analytes not associated with the sample. Control blanks were considered acceptable when PAH's measured were < 2x the detection limit of the GC/MS (Roberts and Szedlmayer 2015). An analyte was considered significant in a sample if the peak presented was at least 3X the limit of detection. Peak PAH areas were quantified with Waters MassLynx™ version 4.1 SCN 569.

The present study tested for eight different PAH's: naphthalene, acenaphthylene, acenapthene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene. Two samples in each analysis were spiked with 10 µl of PAH solution, that was at a concentration of 10 ppm, as standards for the any PAH's detected in the samples and for conversion of peak areas into ppb. Total PAH's and average PAH's were then calculated for each fish. Total PAH's were pooled quantities of all eight PAH's. If a particular PAH signal was below the limit of detection it was not included in the Total PAH estimate. Fish PAHs were compared between sites with a ttest. Analysis of variance was used to compare PAH among year class and PAH among size (SL; Zar 2010).

Marine environmental PAHs are not limited to petrogenic sources (oil source) and can also be pyrogenic (combustion source), therefore, the present study also analyzed PAHs in fish tissues for source identifiers. Source of PAH's can be identified from ratios of phenanthrene to anthracene (PHEN: ANTH) and flouranthene to pyrene

(FLOU:PYRE) within fish samples (Budzinski et al. 1997; Ke et al. 2002; Yunker et al. 2002). If PHEN:ANTH was > 10 and FLUO: PYRE < 1 then the PAHs detected were considered petrogenic (Budzinski et al. 1997; Ke et al. 2002).

RESULTS

Length-Frequency Distributions

A total of 1109 roughtongue bass were collected from the Pinnacles reef area from September 2014 to July 2015. Mean size ± SD was 93 ± 10.5 mm SL and ranged from 54 to 135 mm (Figure 4). Mean sizes of roughtongue bass were significantly different between east (mean SL = 96 ± 7.8 mm; n = 873) and west (mean SL = 79 ± 9.4 mm, n = 219; T_{df} = 1090, p < 0.001; Figure 5).

Age and Year-Class Abundance Estimates

There was an 82% agreement between readers in initial otolith increment counts (n = 1090). After simultaneous review by both readers of all disagreements, a consensus was reached for all otolith counts (100% agreement). The most abundant age = 4 and year class = 2010 (Figure 6). There were significant differences in age (T_{1088} = 8.2, p < .001) and year class (T_{1088} = -7.5, p < .001) between the east (n = 873) and west sites (n = 216; Figure 7). In the present study few juvenile roughtongue were collected, e.g., no age-1 and only six age-2.

Growth rate

Von Bertalanffy (VB) growth parameters were estimated for all roughtongue bass and for each site. The VB parameters for all roughtongue bass were L_∞ = 103.5, K=0.54

and t_0 = 0.02 (n = 1090; R^2 = 0.22), and were similar to pre-spill collections (L_∞ = 106.3, K = 0.64, t_0 = 0.65, R^2 = 0.49; McBride et al. 2009; Figure 8). The VB growth curve for the east site (L_∞ = 101.2, K = 0.73, t_0 = 0.22, n = 873, R^2 = 0.17), showed faster rates than the west site (L_∞ = 95.7, K = 0.32, t_0 = -1.48, n = 216, R^2 = 0.20; Figure 9), and comparisons of the linear portions of the curves (age < 8) confirmed significant differences in growth rates between east and west sites ($F_{3,1081}$ $p < .02$; Figure 10).

Mortality and Survival Estimates

Annual mortality and survival were calculated for all roughtongue bass and separately by sites. Total mortality and survival based on catch curve analysis were Z = 1.7 and S = 18% (n = 1090; Figure 11). At the east site Z = 1.7 and S = 18% (n = 873), while at the west site Z = 2.0 and S = 14% (n =216), but significant differences were not detected between sites ($F_{1,4}$ = 0.4, p = 0.57; Figure 12).

PAH's

PAH Content and Ratios

Out of 171 roughtongue bass samples that were extracted and analyzed for PAH, only 38 produced acceptable results (east n = 19, west n = 19). Failure to detect PAH's in standardized samples was the principle cause of sample elimination. Total PAH content detected in these roughtongue bass (n = 38) ranged from 0 to 220 ppb. Among the valid PAH extractions 76 % (29/38) contained detectable levels of PAHs. Mean ± SD total PAH content in all fish tested (n = 38) was 50 ± 52.2 ppb. Mean ± SD total PAH for fish from the west was 44 ± 87.9 ppb (n = 19) and the east site was 56 ± 120. 2 ppb (n = 19), but no significant differences were detected between sites (T_{38} = 0.7, p = 0.47).

Comparisons of PAH with fish size and year class

No significant differences were detected for each type of PAH among year classes (n = 38, $F_{3,34}$ = 0.58, p = 0.33), or among size classes ($F_{1,37}$= 0.23*;* p = 0.64). There were also no significant correlations between total PAH and size (n = 38, R^2 = 0.2, p = 0.26; Figure 13), or total PAH and year class (n = 38, R^2 = 0.25, p = 0.13).

DISCUSSION

Size, age, growth and mortality

The present study showed many similarities to previous pre-spill studies. Roughtongue bass size frequency distributions in the present study were similar to previous studies (Thurman et al. 2004; McBride et al. 2009). Size ranges were similar, but truncated (55 - 135 mm SL) compared to previous studies (31 – 143 mm SL, Thurman et al. 2004; 15 – 143 mm SL, McBride et al. 2009). The size ranges in these earlier studies were greater, most likely because they used multiple sampling methods (hook and line and trawls) compared to only hook and line in the present study. Thus, it is difficult to assess for real differences in size range between pre-spill and post spill studies.

Age frequencies in the present study were also similar to pre-spill studies. The modal age 4 in the present study was the same as in Thurman et al. (2004), but one year older than age 3 in McBride et al. (2009). In contrast, maximum age differed among studies: maximum age = 8 (present study), maximum age = 9 (Thurman et al. 2004) and maximum age = 15 (McBride et al. 2009). These differences in maximum ages between pre-spill and post-spill were again likely due to sampling and otolith analysis methods. The present study and Thurman et al. (2004) both used whole otoliths for increment counting, while McBride et al. (2009) used sectioned otoliths. Sectioned otoliths that are clear will provide a more accurate estimate of maximum age (age =15; McBride et al.

2009), but most sectioned otoliths in the present study showed difficulties with viewing increments. In contrast, whole otolith increments were clear and counting comparisons among different readers were consistent (100 %) and thus more appropriate for age frequency distribution comparisons. It might be argued that sectioned otoliths are more appropriate for older aged fish, but in the present study all whole otoliths were clear even up to the maximum age 8.

Roughtongue bass mortality estimates in the present study were $Z = 1.7$, and greater than a previous estimates of $Z = 0.5$ (McBride et al. 2009), but more similar to an earlier estimate of $Z = 1.3$ (Thurman et al. 2004). It is likely that mortality and survival differences observed in the present study compared to these previous studies were at least in part due to differences in otolith increment counting methods. The present study used whole otoliths for aging and spent a considerable amount of time and effort in establishing a method and validating counting precision. As part of that effort it was determined that roughtongue bass otoliths would often show a false increment (2 opaque bands in the first year). If these false increments were counted it would result in reduced mortality rates. Also as mentioned above, sectioned roughtongue bass otoliths were not useable in the present study and not recommended for future age estimation of roughtongue bass. In previous studies both sectioned otoliths (McBride et al. 2009), and whole otoliths (Thurman et al. 2004) were used, but neither study reported a false first increment. Thus, differences in mortality rates between the present and previous studies were likely due to otolith counting methods rather than pre-spill versus post-spill mortality rates.

Age and Year Class Frequency Distribution

Roughtongue bass year classes were dominated by 2009 and 2010 recruits (age 4 and 5; Figure 6). This 2010-year class dominance was the exact opposite of what would be predicted if there was a significant effect of the DWH oil spill in 2010. The lack of year class failure or any indication of reduction in 2010 implies that the DWH spill did not affect roughtongue bass early stages. There are several possible explanations why these young of year were unaffected.

It is possible that roughtongue bass from the Pinnacles reefs had minimal exposure to DWH oil, i.e., the oil did not reach the sampling sites. This explanation is consistent with a study performed by Silva et al. (2016) that showed sediment samples from 2010 and 2011, taken from the same sites as the present study, had only slight increases in total PAH and no actual significant differences. In fact, the sediment samples analyzed by Silva et al. (2016) showed that PAH sediment detections in some samples actually had lower total PAH values in 2010 and 2011 than what were present in 2000. This suggest that little to no oil exposure from the DWH spill occurred at the Pinnacle reef sample sites in the present study.

Another more complicated explanation is that the roughtongue bass recruited from distant unoiled areas. Abundant populations of roughtongue bass have been identified in surrounding regions at much greater distances from the DWH spill site compared to the Pinnacle reefs, and most likely these distant areas were not exposed to oil in 2010 (Nuttall et al. 2014). Also, small numbers of roughtongue bass larvae have been collected in ichthyoplankton surveys of the loop current, which was largely uncontaminated by DWH oil spill (Richards et al. 1993; Liu et al. 2011). Distant populations and planktonic larvae both open up the possibility that roughtongue bass on the Pinnacle reefs may have recruited from areas distant from the DWH oil spill. However, there are many unanswered question that need to be addressed for this remote recruitment to function and at present there is little information on the early life history of roughtongue bass.

Another possibility for the abundance of the 2010-year class could be natural biological resilience due to conditioning and even if traces of oil had reached the Pinnacles, resident fish may have already been resistant to any such exposure. Petroleum has been present in the Gulf of Mexico from other sources prior to the DWH event. There are natural petroleum seeps in the Gulf of Mexico as well as land runoff sources and atmospheric deposition (Board and Board 2003; Latimer and Zheng 2003). Thus, it should be recognized that these fish and their environments have most likely been previously exposed to petroleum and its derivatives for some time, and may have the genetic capability to metabolize petroleum sources and PAHs more readily without experiencing negative effects (Board and Board 2003). For example, mummichog, *Fundulus heteroclitus* (Linnaeus), from chronically polluted sites showed heritable resilience to PAH toxicity and were also resistant to multiple insecticides (Meyer and DiGiulio 2002; Clark and DiGiulio 2012). Similarly, in another study on the DWH spill, it was concluded that preadaptation to oil exposure was the reason for not detecting differences in fish from oiled versus non-oiled sites (Able et al. 2015). This resilience would likely need to be present in roughtongue bass larval stages to explain the 2010-year class dominance, if in fact they were exposed to excess oil. At present it is difficult to validate an oil resistant hypothesis especially for roughtongue bass as they are difficult to retrieve from deep colder waters, keep alive, and hold captive for oil exposure studies.

At the opposite end of this oil spill effect on roughtongue bass is the question, did the oil spill actually enhance the 2010-year class? Was there a possible increase in prey sources for larvae from the oil spill? Roughtongue bass feed on zooplankton and zooplankton feed on phytoplankton and other microbes (Bullock and Smith 1991; Zöllner et al. 2009). There are microbes in the Gulf of Mexico, including bacteria and fungi that are capable of degrading petroleum source PAHs both in soils and within the water column (Latimer and Zheng 2003; Gutierrez et al. 2013; Joye et al. 2014). One study suggested that several microbial taxa connected with hydrocarbon degradation were enriched by the surface and plume waters associated with the DWH spill (Gutierrez et al. 2013). In that study, both *Cycloclasticus* sp. and *Oceanospirillales* sp., were enriched due to competitive advantages. Increases in microbes, can increase zooplankton populations (Zöllner et al. 2009). Increases in zooplankton would increase the food sources for roughtongue bass, which may have helped generate a particularly strong 2010-year class. Similar increases in biota was also suggested during the EXXON Valdez oil spill, where an increase in young of the year mummichog, *Fundulus heteroclitus,* was attributed to an increased prey source of pollution-resistant polychaetes, *Capitella* sp. (Brzorad and Burger 1994). Evaluation of roughtongue bass population abundancies in connection with microbial abundance and zooplankton would be necessary to more fully understand this explanation.

Site differences in growth rates

Growth rates in the present study were also similar to previous studies (Thurman et al. 2004; McBride et al. 2009). Variance around the VB growth relation in the present study (R^2 = 0.22), was greater than previous a growth relation (R^2 = 0.49 McBride et al. 2009). This greater variance may be that all fish lengths at age were used in the present study VB growth relation, rather than just the mean lengths at age, which is a typical approach, but details of regression were not reported in McBride et al. (2009). Also, R^2 values are not as accurate for nonlinear regressions as they are for linear regression and should be interpreted with caution (Kvålseth 1985). However, this earlier VB growth curve was within one SD of the present study curve (Figure 8).

Roughtongue bass growth rates were significantly different between east and west sites (Figure 9 and Figure 10). Similarly, Thurman et al. (2004) also detected slower growing roughtongue bass from the Alabama Alps (west site). In the present study collection methods were the same at both sites, ruling out gear selectivity as a possible cause. There are several possible factors that may explain growth rate site differences.

Growth rate differences in fish can result from changes in prey type or other environmental habitat characteristics (Fry 1972; Abrahams 2011). In the present study there are several environmental factors that potentially differ between sites. The first and probably "the major factor" was the Mississippi river outflow and the potential difference in its influence between sites due to its closer proximity to the west site. The Mississippi river is one of the top ten largest rivers in the world and carries sediment in quantities of 150 x 10^{9} kg^{-yr}, that are then deposited into the Gulf of Mexico (Meade 1996; Dagg and Breed 2003). The closer proximity of the west sites (68 km) to this outflow likely caused reduced water quality for roughtongue bass that inhabit the west sites compared to east sites that are further away (142 km). For example, the Mississippi river plume has been shown to extend out 60 km to the northeast of the Pass a Loutre, which would put the west sites near the boundaries of the sediment outflow from the Mississippi river (Walker 1996). In support of this contention, ROV video surveys during September-October 2014 showed the west sites were much more turbid and "degraded" compared to the east site (Szedlmayer, unpublished data). Similarly, previous studies have reported silt deposition up to 0.5 m at the same west study sites as the present study (Continental Shelf Associates, Inc. and Texas A&M University 2001). Roughtongue bass are visual feeders, and reduced visibility alone can significantly decrease feeding success in fish (Kestemont and Baras 2008). Thus, reduced growth rates at the west site may simply be reduced feeding success due to decreased water clarity and sedimentation that results from proximity to the Mississippi river outflow.

In addition to reduced water clarity caused by the Mississippi outflow there is also nitrogen loading and phosphorus influxes due to fertilizers and fresh water volumes of 350 km^3 per year (Meade 1996; Dagg and Breed 2003). The Mississippi is responsible for 1.8×10^9 kg of nitrogen entering the Gulf of Mexico each year (Howarth et al. 1996; Dagg and Breed 2003). This nitrogen loading results in eutrophication of waters and ultimately causes hypoxia events and dead zones in the Gulf of Mexico (Goolsby et al. 1999). Reduced oxygen has been shown in many studies to cause decreases in growth (Kestemont and Baras 2008). Steady increase in fertilizers and nutrient inputs in the last 50 years has coincided with increasing dead zones in the Gulf of Mexico (Goolsby et al. 1999; Galloway et al. 2004). In addition, freshwater influx from the Mississippi river has been shown to effectively reduce salinity over the entire Gulf of Mexico (Grimes and Finucane 1991; Dagg and Breed 2003). Salinity variation away from the optimal ranges has been shown to affect fish growth rates (Kestemont and Baras 2008). Again, closer proximity of the west site to the Mississippi outflow suggest that this site may experience lower oxygen levels and lower salinity levels at more frequent intervals than the east site, which ultimately could have reduced growth rates at the west site.

A secondary factor that likely results from proximity to Mississippi river runoff is predation risk. This again may be related to a greater influence of the Mississippi outflow on the west site compared to the east site. With increased nutrient input, decreased water clarity, and increased salinity fluxes, coral cover (shelter) may be reduced. Increased sediment loads and eutrophication have been specifically documented to harm corals by reducing light penetration, smothering, and increasing competition for space with filter feeders (Gabric and Bell 1993). For example, a reduction of coral encrusting communities was shown with closer proximity to riverine inputs in Rio Bueano, north Jamaica (Mallela 2007), and decreased coral taxa has been linked to increasing proximity to the Mississippi that directly corresponded with the sites in the present study (Continental Shelf Associates, Inc. and Texas A&M University 2001). These types of structures provide shelter to roughtongue bass and their reduction would increase predation rates as well as reduce foraging time and subsequently growth rates.

A final environmental factor to consider is presence of chemical contaminants. Exposure to contaminants can cause an organism to expend energy otherwise used for growth in attempting to eliminate or avoid certain toxins (Meador et al. 2006; Kestemont and Baras 2008). Additionally, some toxins may inhibit the uptake of certain nutrients in food sources which can further reduce growth rates (Kestemont and Baras 2008). There are several possible sources of toxins in the Gulf of Mexico that could vary significantly in west sites compared to east sites.

Again proximity to the Mississippi outflow, which contains runoff from 3.2×10^9 km^2 of the United States, is likely an overwhelming source of contaminants in the Gulf of Mexico (Meade 1996; Howarth et al. 1996). Studies in estuaries have shown that there is an extensive list of contaminants in riverine discharge that are capable of causing deleterious effects to marine organisms (Kennish 1991). When tested for contaminants the Mississippi river outflow showed evidence of substantial concentrations of wastewater sewage contaminants, agriculture contaminants and industrial point source contaminants (Meade 1996). Within these groupings are specific contaminants such as alkyl benzene sulfonate and ammonia which have been shown to cause reduced swimming capability and reduced growth in rainbow trout fry, *Oncoryhnchus mykiss* (Walbaum; Hofer et al. 1995). Again due to closer proximity to the Mississippi mouth, reef fish residing at the west site may receive greater exposure to such contaminants.

The DWH spill and PAH's:

An important question to consider is the effect of the DWH spill. The west site was closer (54 km) to the spill site than the east site (111 km), potentially increasing the possibility of oil exposure. There is some evidence that levels of PAH's in sediments were higher at the west site compared to the east site, but significant differences were not detected (Silva et al. 2016). The present study showed detectable PAH levels in roughtongue bass with a maximum of 220 ppb in one fish, but no significant difference between east and west sites. Unfortunately, there are no baseline data for PAH thresholds for toxicity in roughtongue bass. However, it appears that PAH levels in the present study were below minimum levels for toxic effects. The US EPA (1987) establishes 300 ppb as the lowest level of PAHs that had any effect on organisms in the marine environment. This is 70 ppb greater than the highest recorded total PAH content for a single roughtongue bass, and 250 ppb above the mean total PAH content in all roughtongue bass in the present study. Also, previous studies have shown that PAH levels of at least 630 ppb in sediments were required to cause reproductive disruption in English sole, *Pleuronectes vetulus* (Girard; Johnson et al. 2002). Also, PAH levels in the present study were well below the minimum level of 180,000 to 220,000 ppb of PAH concentration needed to affect growth in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum; Meador et al. 2006). Finally, in a review study on PAH effects, total PAH content needed to be at least 4,000 ppb before it had low effects on multiple fish species (Long et al. 1995). Thus, the low levels of PAHs detected in roughtongue bass in the present study were unlikely to have affected this important fish species from the mesophotic reef habitats.

Although the PAHs in the present study were low, a potential difficulty was the use of plastic bags for storing the roughtongue bass prior to PAH extraction. Plastic bags have been implicated in PAH leaching and are generally not used when collecting samples for PAH analysis (Law and Biscaya 1994). In the original methods of the present study the intention was to only analyze internal fish tissue that had not come into contact with the plastic bags. However, after initial trials it became clear that there was not sufficient tissue to allow for adequate extraction procedures, thereafter the entire fish was used in all extractions. Most of these fish had been stored in plastic bags for 1.5 years prior to being analyzed for PAHs. Thus, even the low values of PAHs detected in the present study may simply be an artifact of plastic bag fish storage and not related to the DWH oil spill.

Lastly, PAH analyses have limitations in measuring oil exposure in fish. First, PAHs are rapidly metabolized by most fish and are often out of the system before a fish can be examined (Altenburger et al. 2003; Van der Oost et al. 2003). Therefore, PAH detection or lack of detection may not adequately represent actual fish exposure. Additionally, when PAH levels begin to fall below ppm and reach the ppb category their detection is difficult and often inconsistent. A simple change in GC/MS analysts or machine variation can result in variations > 200 ppb. The present study encountered difficulties in PAH detections and after extracting and analyzing 171 roughtongue bass samples only 38 were considered usable. Variations such as this were also apparent in the study performed by Silva et al. (2016) that showed several inconsistencies in PAH detections. Thus, the PAH levels detected in the present study as well as other studies may be compromised due to this inherent variation when measuring at ppb levels and should be interpreted with caution. The use of PAH biliary metabolites as biomarkers as an alternative quantification of exposure has shown promise and is suggested as a future method of oil exposure assessment in roughtongue bass (Van der Oost et al. 2003).

PAH source

Despite the potential problems with the PAH values, this study attempted to analyze PAH ratio values for source identification. Low ratios of PHEN:ANTH would suggest that detected PAHs were mostly non-petrogenic sources. Unfortunately, only one fish had detectable levels of both phenanthrene and anthracene, and although the ratio indicated a non-petrogenic source results were inconclusive due to sample size.

CONCLUSIONS

The present study has provided important ecological information on a little studied reef fish species, roughtongue bass, from mesophotic reef habitats after the DWH oil spill. This species showed a dominant 2010-year class that appears to have recruited the same year as the DWH spill. Also, post-spill growth rates were similar to pre-spill studies. There were significant growth rate differences detected between sites, but these were more likely linked to Mississippi River discharge rather than the DWH oil spill.

Different mortality rates between pre-spill studies and the present study were most likely linked to otolith aging methods rather than actual differences in mortality rates. Detected PAH levels in roughtongue bass were well below toxicity levels, but these low levels should be interpreted with caution due to the difficulty of measuring contaminants at the ppb level. Based on these present study results, there was little effect of the DWH oil spill on roughtongue bass from these mesophotic reef habitats.

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All applicable international, national and institutional guidelines for the care and use of animals were followed.

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Table 1 Comparison of the age, growth, mortality and survival of roughtongue bass, *Pronotogrammus martinicensis*, collected in the present study to the pre-oil studies of McBride et al. (2009) and Thurman et al. (2004)

Study	Aging Structure	N	Max Age (yr)	Modal Age (yr)	L_∞ mm (SL)	K	t_0	M	S
Present Study	Whole sagittal otoliths	1092	8	4	102.2	0.60	0.25	1.7	18% ($t_r = 4$)
McBride et al. (2009)	Sectioned sagittal otoliths	490	15	3	106.3	0.64	0.65	0.5	60.1 ± 4% ($t_r = 3$)
Thurman et al. (2004)	Whole sagittal otoliths	667	9	3-4	.	.	.	1.3	27% ($t_r = 4$)

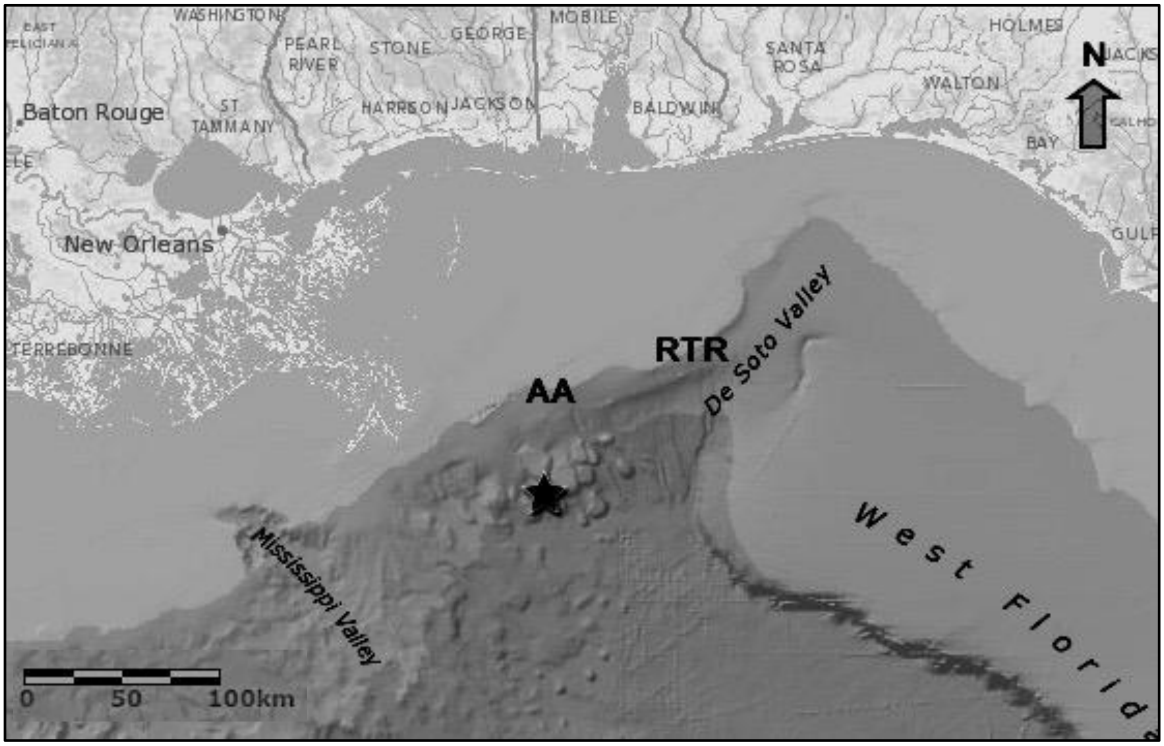

Fig 1 Sampling sites for roughtongue bass, *Pronotogrammus martinicensis*, from the Pinnacles reefs in the northern Gulf of Mexico; Alabama Alps = AA (N 29.2518, W 88.3373) and Roughtongue Reef = RR (N 29.4415, W 87.5785); DWH spill site (N 28.738139, W 88.365944) indicated by the black star; (Map layers courtesy of ESRI, GEBCO, DeLorme, NaturalVue, IHO-IOC, StoryMaps)

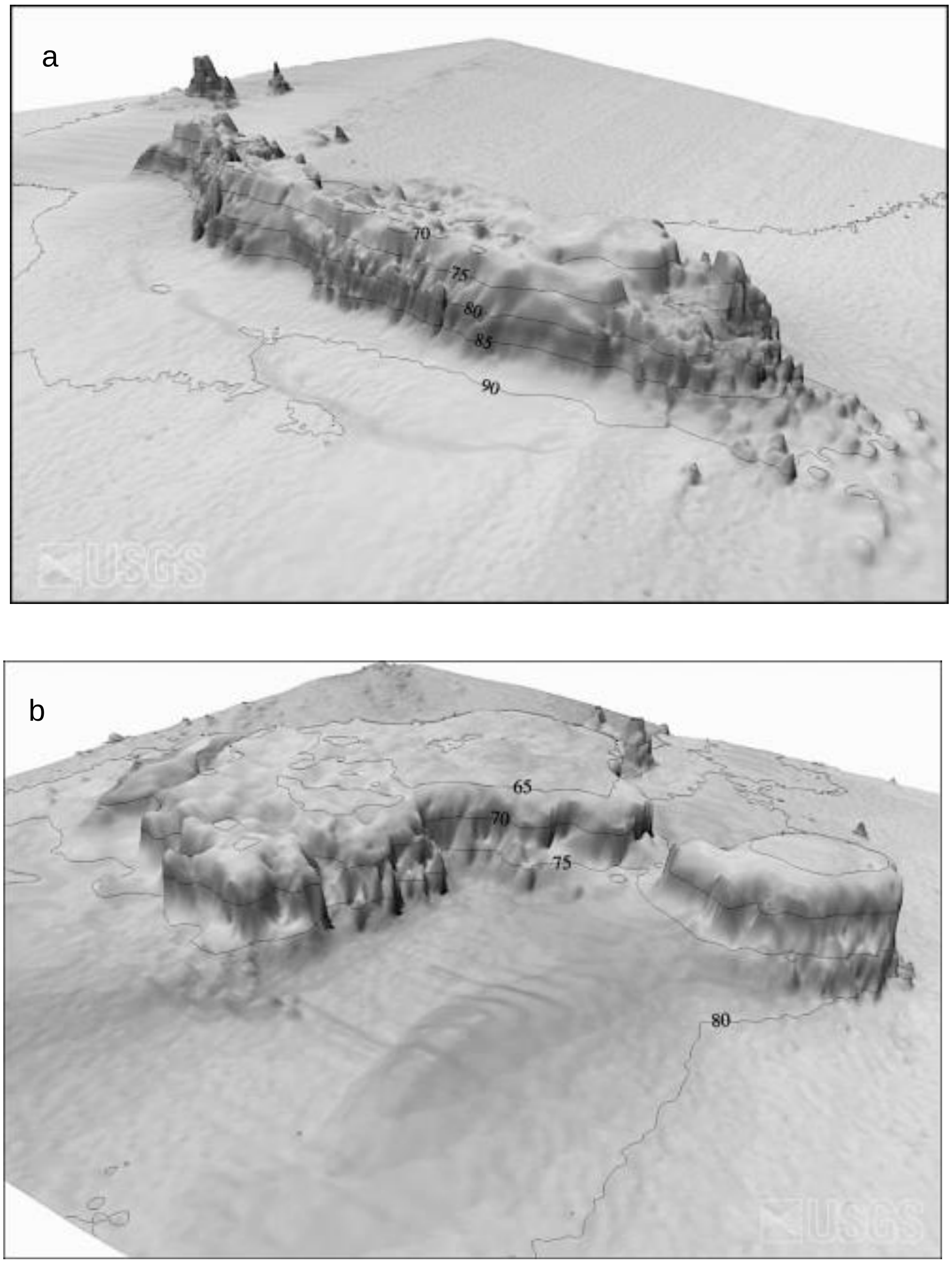

Fig 2 Three dimensional bathymetry of (a) Alabama Alps (West site) and (b) Roughtongue Reef (East site; Multibeam map courtesy of USGS)

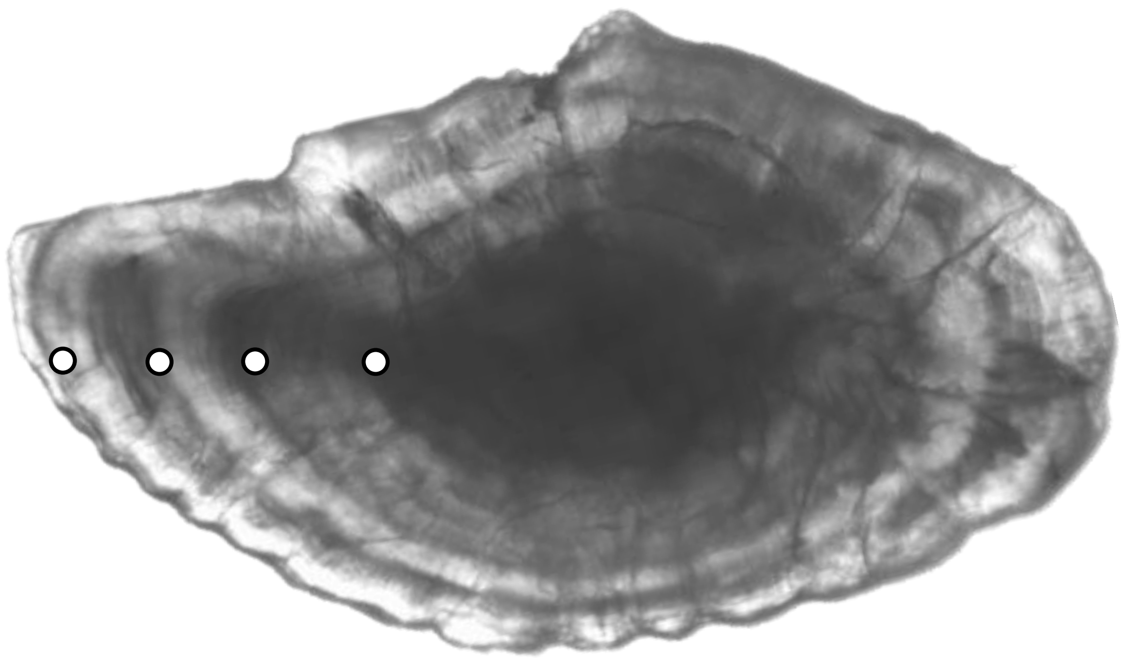

Fig 3 Whole sagittal otolith from 4-year old roughtongue bass, *Pronotogrammus martinicensis* (SL = 94mm); this fish was captured in March of 2015 with a newly formed opaque edge and was from the 2011-year class

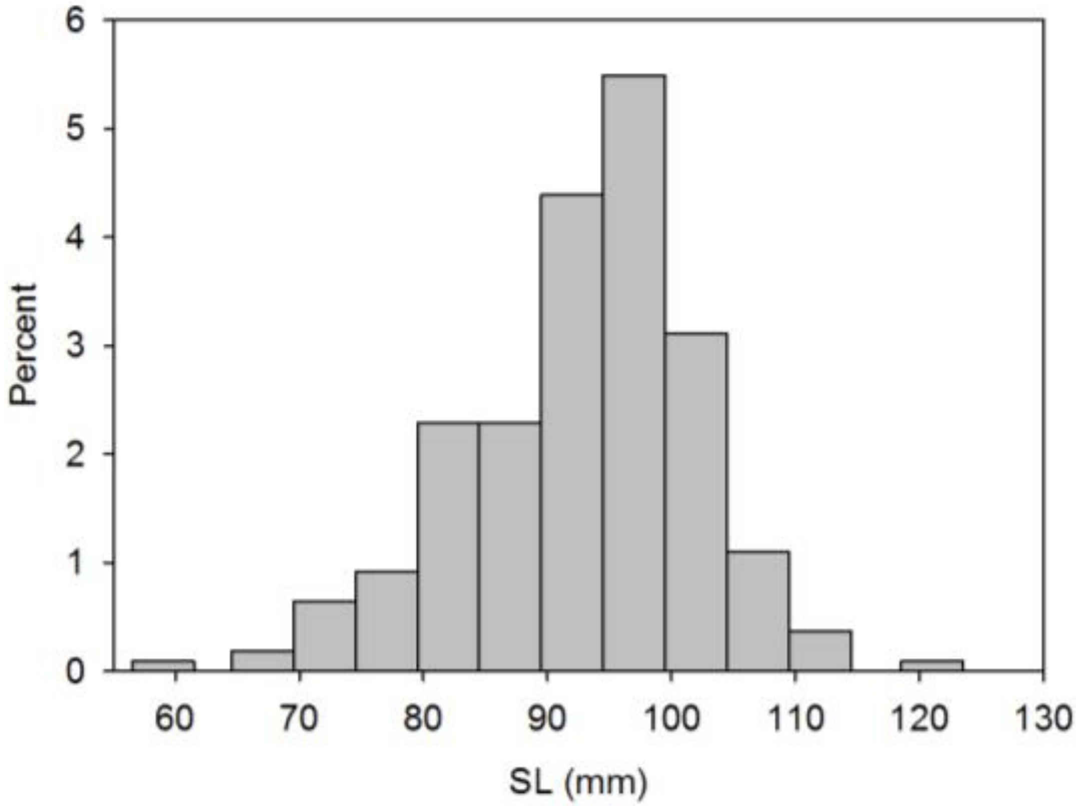

Fig 4 Size (SL mm) distribution of roughtongue bass, *Pronotogrammus martinicensis*, collected from the Pinnacle reefs in the northern Gulf of Mexico

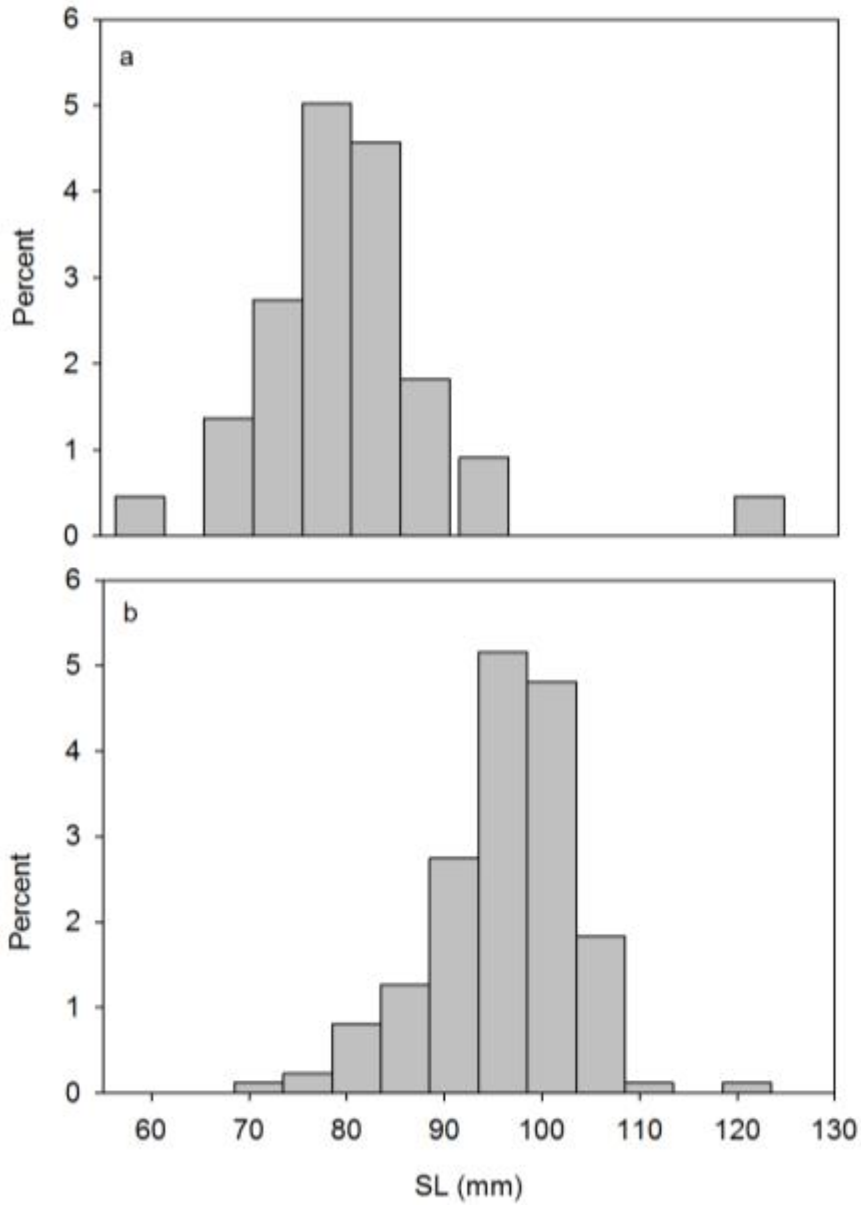

Fig 5 Comparison of size distribution for roughtongue bass, *Pronotogrammus martinicensis*, between the East site (a), and the West site (b) of the Pinnacle reefs in the northern Gulf of Mexico

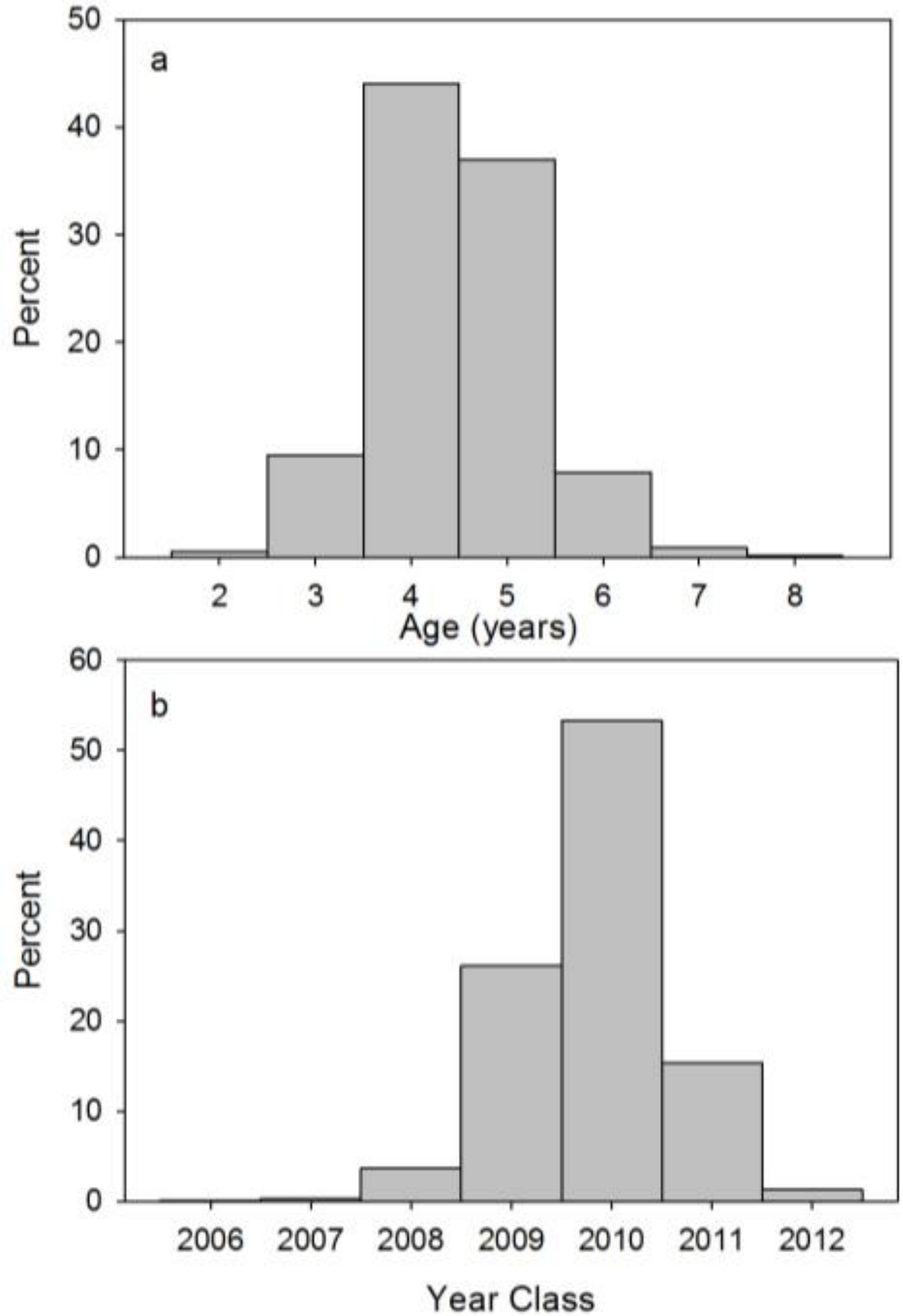

Fig 6 Roughtongue bass, *Pronotogrammus martinicensis*, total age distribution (a), and year class distribution (b) from the Pinnacle reefs in the northern Gulf of Mexico

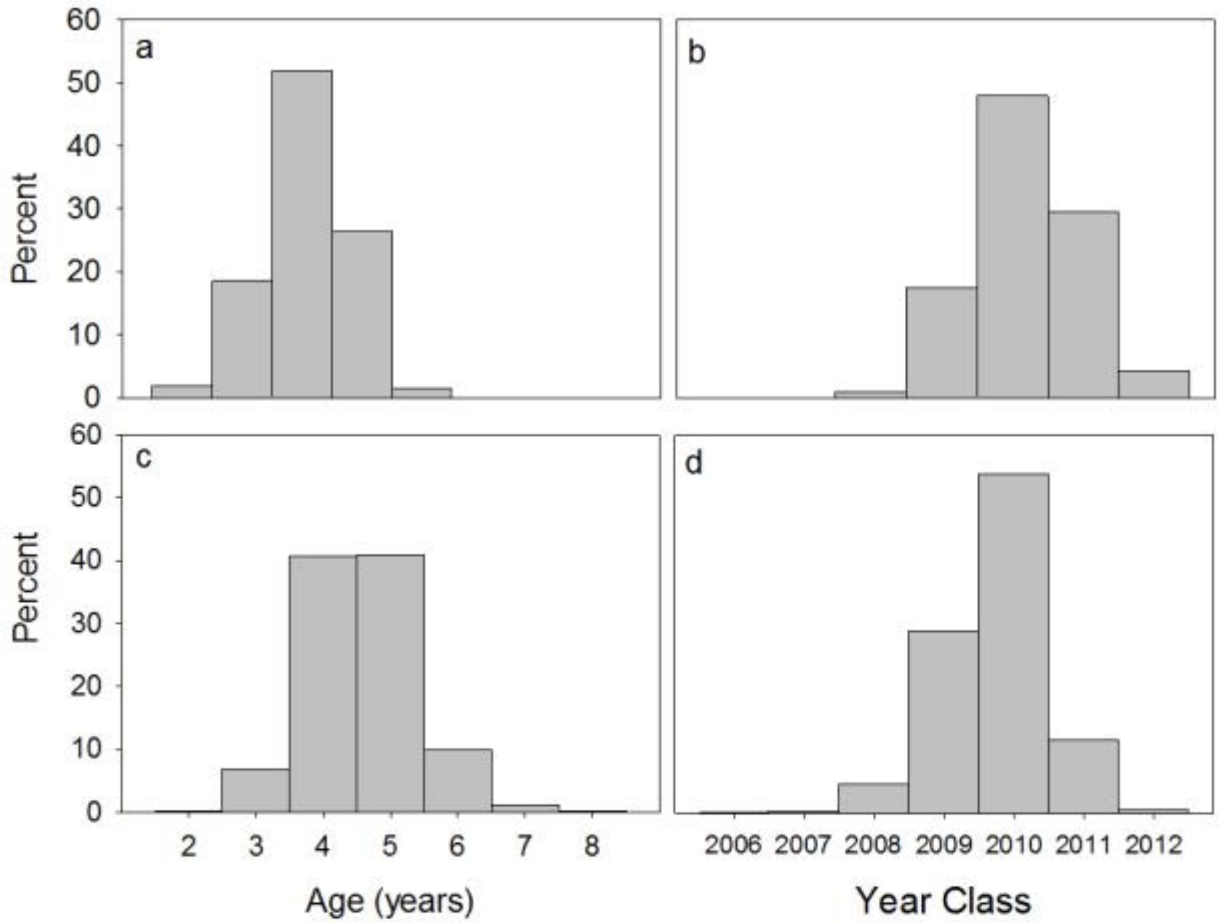

Fig 7 Comparison of age and year class distribution for roughtongue bass, *Pronotogrammus martinicensis*, between sites from the Pinnacles reefs in the northern Gulf of Mexico; West site age distribution (a) and year class distribution (b); East site age distribution (c) and year class distribution (d)

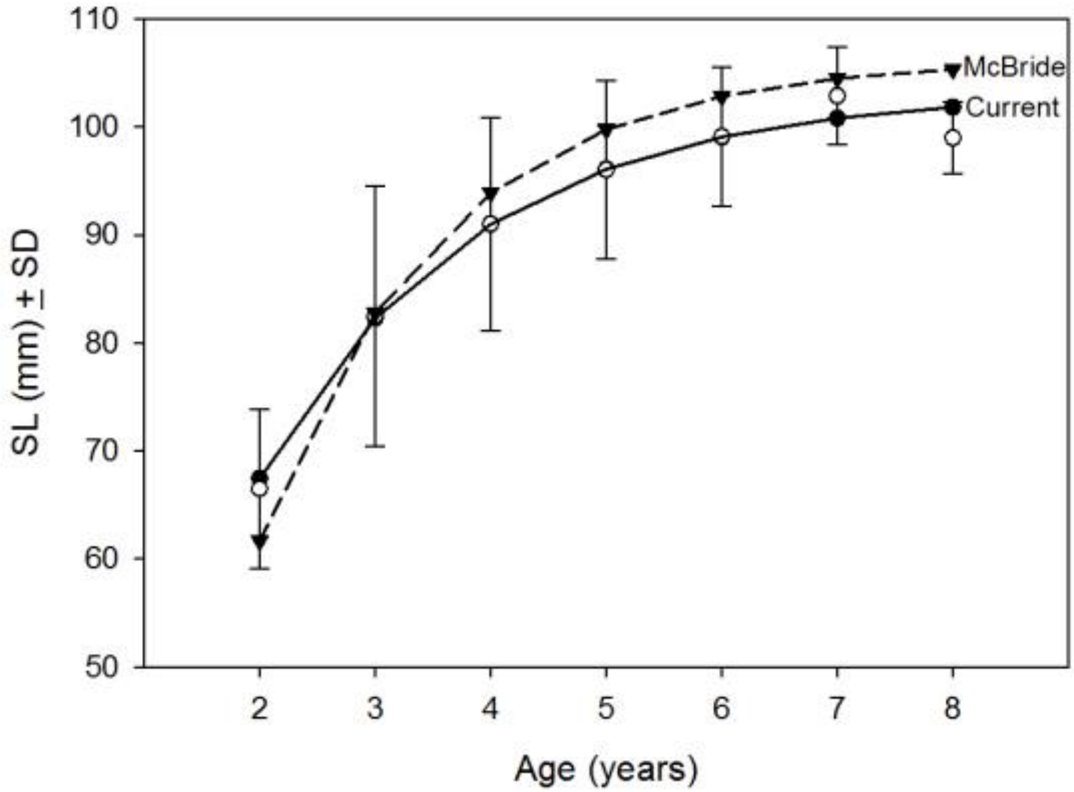

Fig 8 Comparison of Von Bertalanffy growth curves for roughtongue bass, *Pronotogrammus martinicensis*, collected in the present study (black dots) to pre-oil spill collections (black triangles; McBride et al. 2009); Unfilled circles correspond to actual observed SL means ± SD for the present study

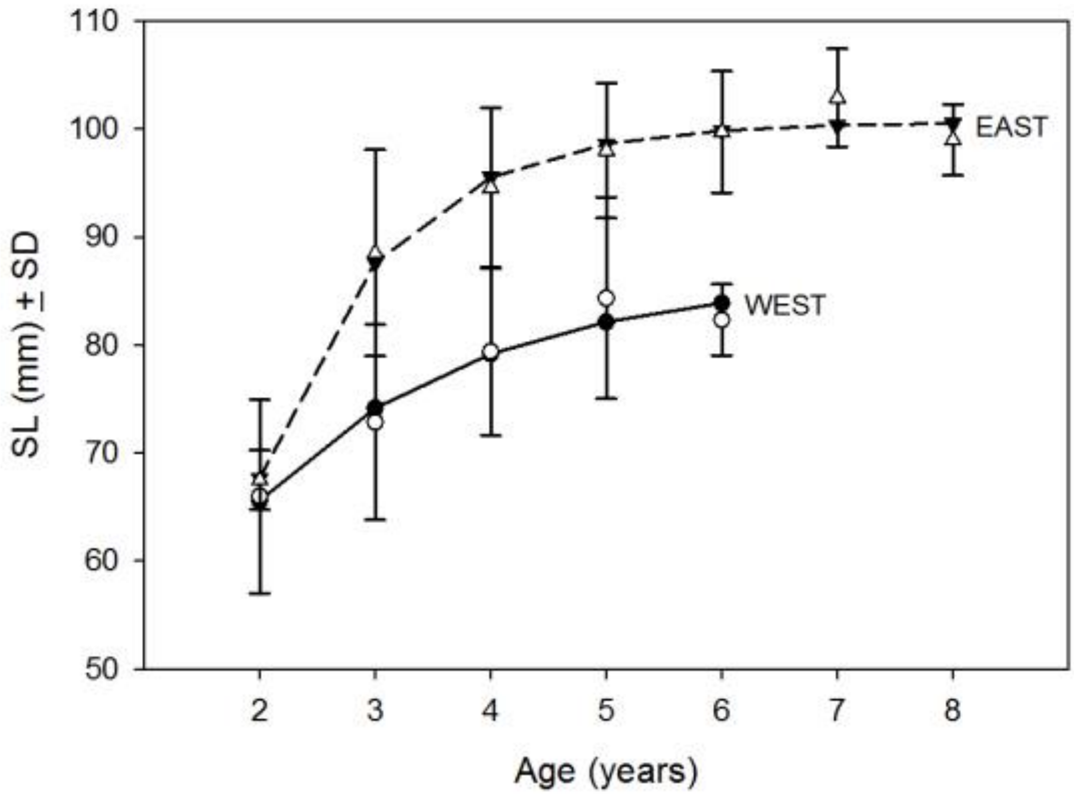

Fig 9 Comparison of Von Bertalanffy growth curves for roughtongue bass, *Pronotogrammus martinicensis*, between east and west sites from the Pinnacle reefs in the northern Gulf of Mexico; Black triangles = east site and black dots = west site; Unfilled shapes correspond to actual observed SL means ± SD for each site

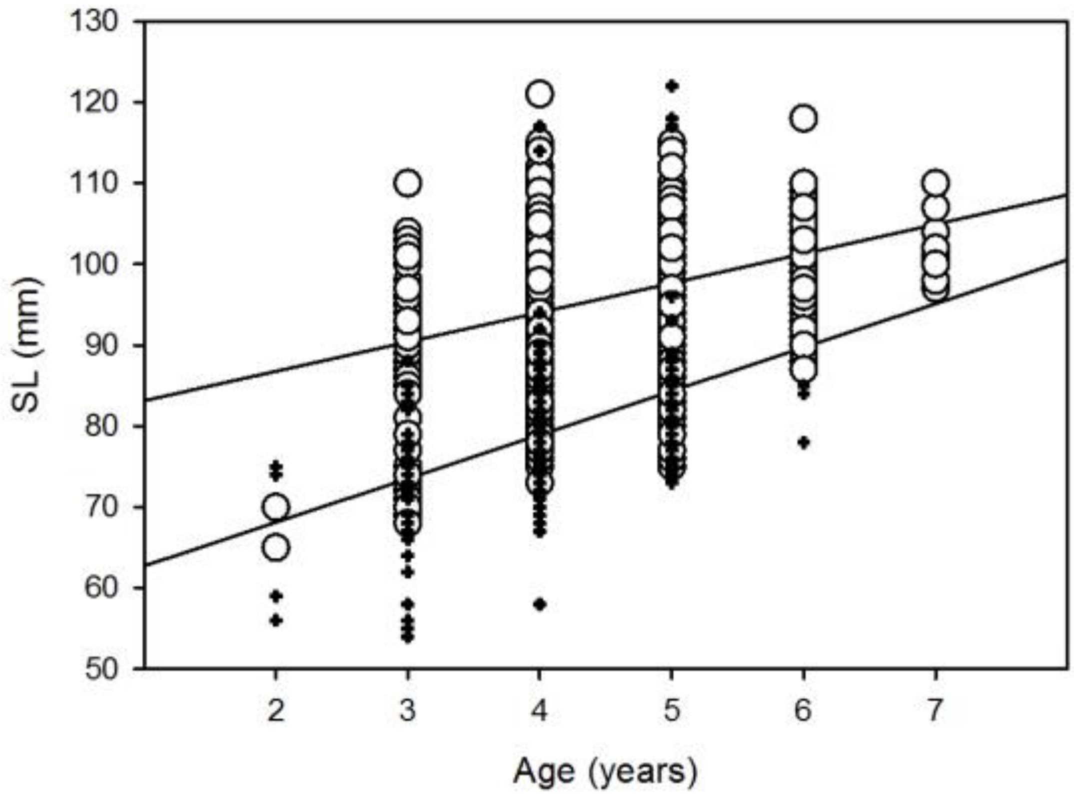

Fig 10 Roughtongue bass *Pronotogrammus martinicensis* growth rate comparisons of SL on age between sites from the Pinnacle reefs in the northern Gulf of Mexico; East site = open circles, and west site = black crosses

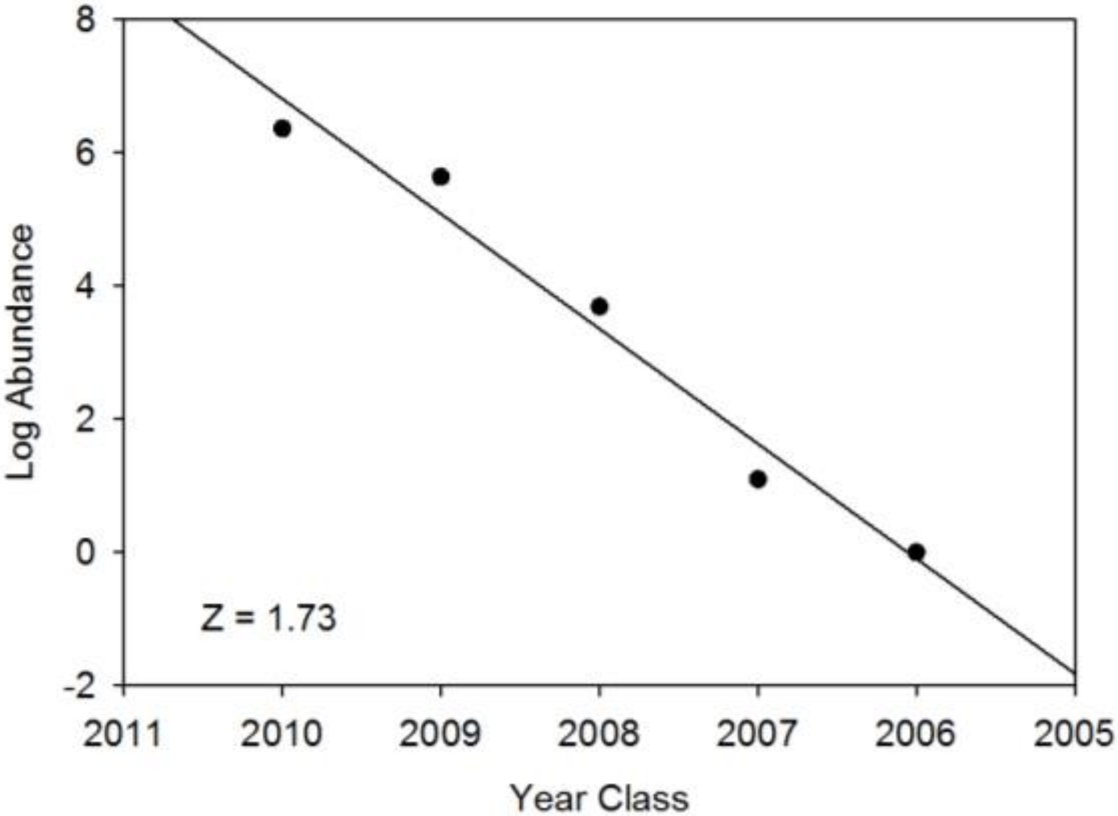

Fig 11 Mortality estimate (Z = 1.73) from year class distribution of roughtongue bass, *Pronotogrammus martinicensis* collected for the Pinnacle reefs in the northern Gulf of Mexico

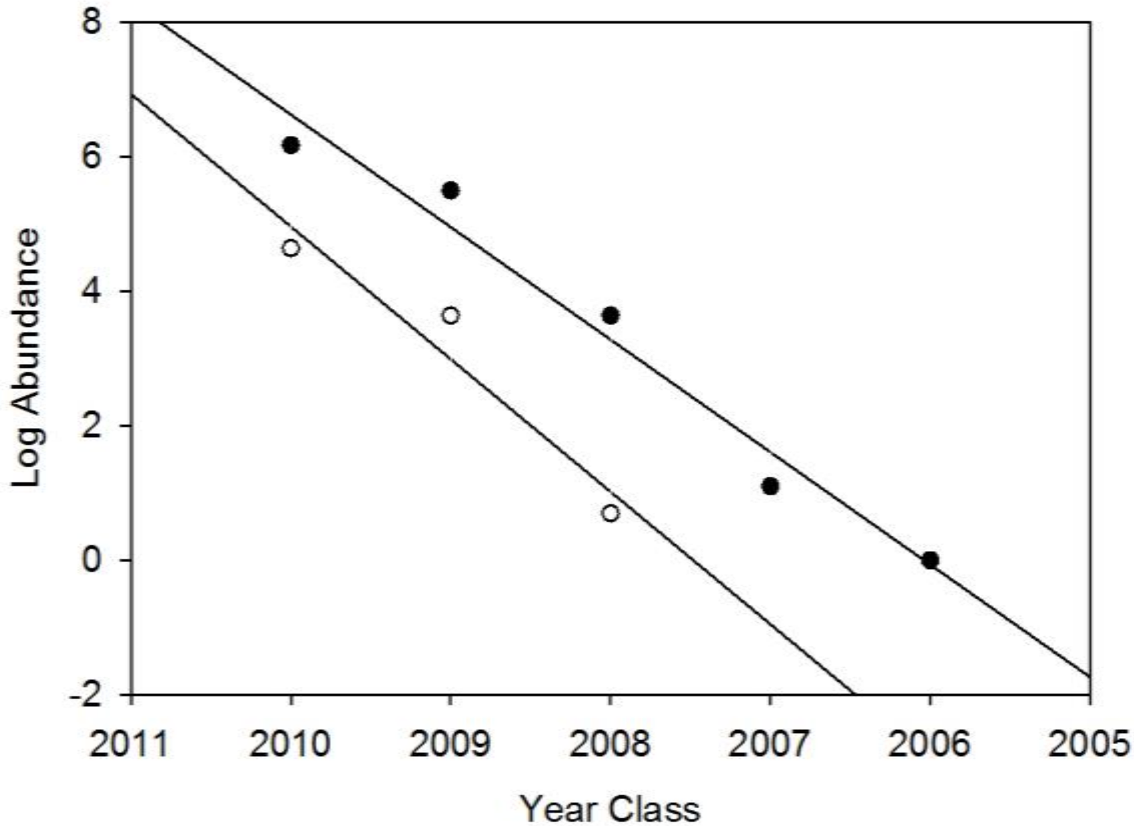

Fig 12 Comparisons of mortality estimates (*Z*) for roughtongue bass, *Pronotogrammus martinicensis*, between east and west sites from the Pinnacle reefs in the northern Gulf of Mexico; East site = black dots (Z = 1.67), west site = open circles (Z = 1.97)

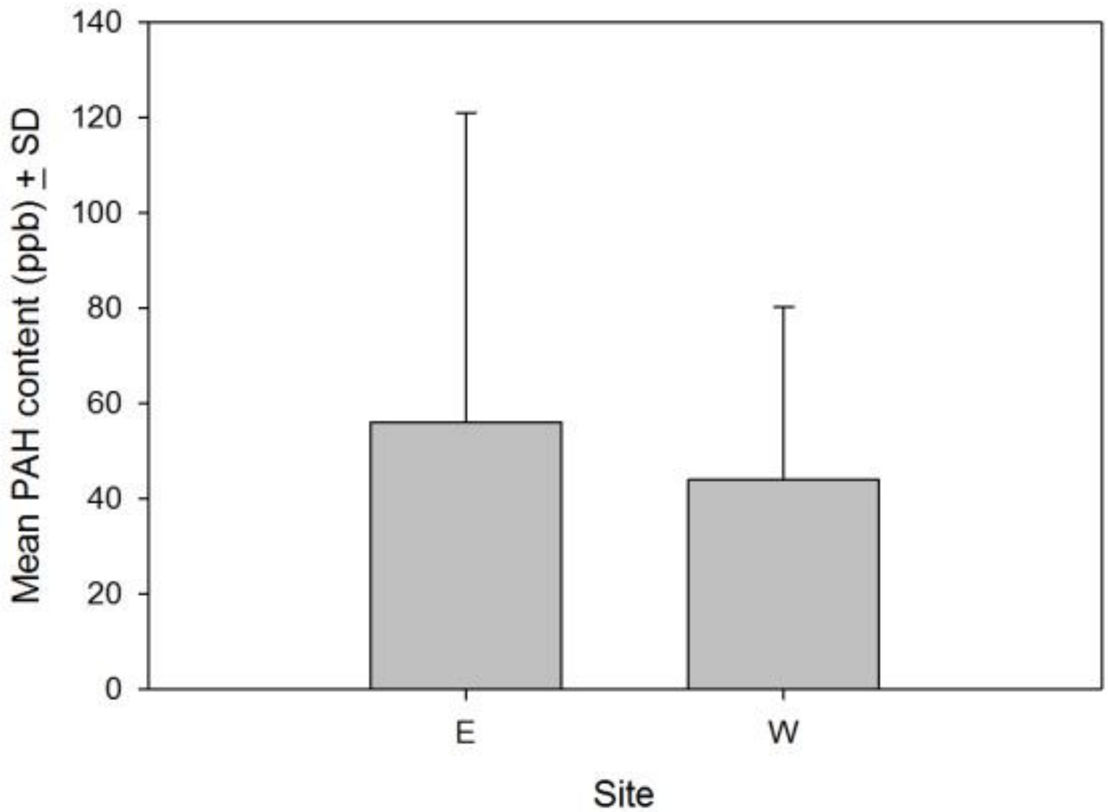

Fig 13 Mean total PAH content ± SD for roughtongue bass, *Pronotogrammus martinicensis*, in east sites vs. west sites

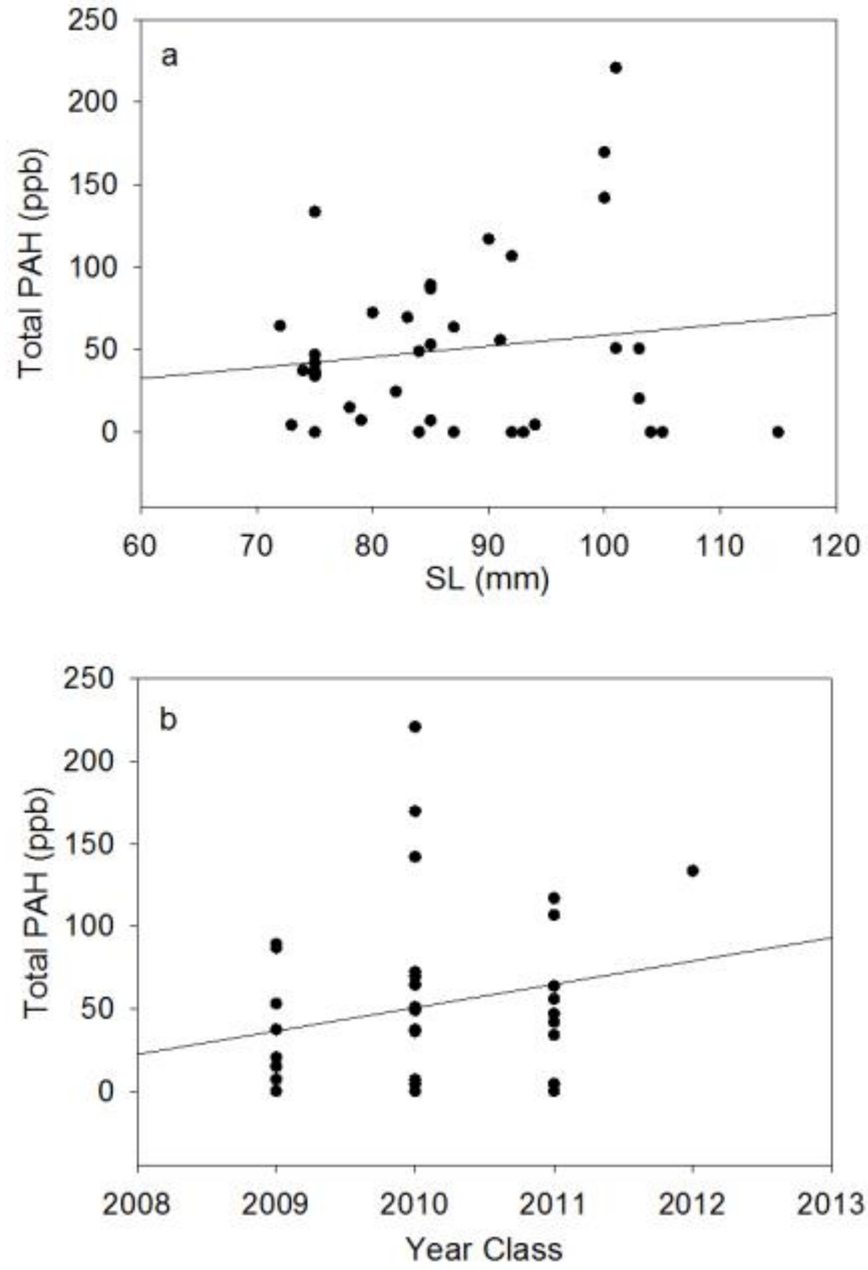

Fig 14 Comparison of total PAH level on (a) size (SL) and (b) year class of roughtongue bass, *Pronotogrammus martinicensis*