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MARFIN Project FINAL Report

Title: Regional Age Structure, Reproductive Biology and Trophic Patterns of Adult Goliath Grouper in Florida.

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Submitted by:

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Part I. Age, growth and reproduction (Debra Murie and Daryl Parkyn, leaders).

Draft manuscript title: Non-lethal Assessment of Age, Growth, and Reproduction of Atlantic Goliath Grouper from the Atlantic Coast of Florida **(**Debra J. Murie, Felicia C. Coleman, Jessica A. Cusick, Robert D. Ellis, Christopher C. Koenig, Christopher Malinowski, Daryl C. Parkyn, and Christopher D. Stallings (Note: Co-author names are in alphabetical order).

INTRODUCTION

Age, growth, and reproductive biological data are essential in assessing the recovery of fish stocks under protection (conservation closures or regulations) due to over-fishing and habitat loss, such as Atlantic Goliath Grouper (*Epinephelus itajara*), hereafter, Goliath Grouper. These data are used for estimating population parameters, such as age distributions, growth, age-specific reproductive potential, and mortality rates (Haddon 2001), that are used as input into stock assessments to predict recovery trajectories (Kingsley 2004). Although there is indication that the US stock of Goliath Grouper is undergoing a recovery from the time of the harvest ban in 1990 (Porch et al. 2006), the current lack of data on the Goliath Grouper's life history parameters makes it difficult to determine both the level of the stock's recovery and the level of harvest it can sustain, if any.

In particular, the sexual pattern (dioecious or hermaphroditic) of Goliath Grouper off the Atlantic coast of the U.S. is not known, whereas in the Gulf of Mexico it was concluded that they were dioecious (Bullock et al. 1992). In addition, the ovarian structure and oogenesis pattern of Goliath Grouper has not yet been described in either ocean (Sadovy and Eklund 1999). Sexual pattern can also affect how vulnerable a species is to fishing pressure. Sequential hermaphrodites may be more vulnerable to overfishing than gonochorists if there is sex-specific fishing mortality rates (Coleman et al. 1996). Estimation of sexual maturity also is linked to assessing stock productivity (Hunter and Macewicz 2003). These parameters have been estimated for Goliath Grouper but only at a time when the species was severely overfished (1980s), with over-exploitation resulting in a shift towards females maturing at smaller sizes and younger

ages (Law 2000). Current estimates of size- and age-at-maturity for Goliath Grouper during their recovery phases from fishing pressure will be necessary for recovery trajectories.

Our goal is to determine the size and age structure, growth and reproductive biology of Goliath Grouper to aid in plans for its recovery and management. Specific objectives of the proposed research are to: 1) determine the sex-specific size and age structure of Goliath Grouper on spawning aggregations off the east coast of Florida; 2) estimate contemporary sex-specific growth; and 3) determine the seasonal reproductive development of male and female Goliath Grouper, and in particular the potential occurrence of hermaphroditism.

MATERIALS & METHODS

Fish Sampling

Goliath Grouper were captured live by fishing off the east coast of Florida, USA, on known spawning aggregation sites (Figure 1) during their spawning season (August-October) in 2010-2015. Fish were captured using hand lines of braided nylon rope (9-mm, 60-m long) with monofilament leaders (1000-lb test, 5-m long) and 20/0 circle hooks. Hooks were baited with whole Hardhead Catfish (*Ariopsis felis*), or pieces of Great Barracuda (*Sphyraena barracuda*), Greater Amberjack (*Seriola dumerili*), Little Tunny (*Euthynnus alletteratus*), or stingrays (*Dasyatis sabina* and *D. americana*). Fish were captured in water depths less than 35 m to lessen the effects of barotrauma, with fish vented posterior to the pectoral fin when necessary. Fish were placed on a stretcher on the deck of the vessel, a seawater hose was inserted into the mouth to continuously irrigate the gills and a damp towel was used to over the eyes to protect it from direct sunlight and to reduce visual stimuli.

Size, Age, and Growth

All fish captured were measured for total length (TL) to the nearest cm, and tagged externally with cattle ear tags on the posterior ends of the soft dorsal and anal fins and internally with passive integrated transponder (PIT) tags to allow us to follow

recaptures of individual fish over time. Non-lethal samples of dorsal fin rays used for aging the fish were also collected from fish the first time they were captured. Fin rays were prepared for aging following the protocol outlined in Murie et al. (2009) and Artero et al. (2015). In brief, soft fin rays (rays 3-4) of the second dorsal fin were removed from the fish by cutting across the basal structures of the fins above the body of the fish. The fin rays were then cleaned of any tissue and allowed to air-dry. Dried fin rays were epoxied in thermoplastic resin (Hysol, Loctite Corporation, Bay Point, CA), thin-sectioned (~0.8-1.3 mm thick) using a variable high-speed sectioning saw (675 rpm) with a 152.4-mm diameter blade. Rays were sectioned from their base distally until the first annulus was observed to merge with the core, which provided multiple fin ray sections that could be used to clarify the position of the first annulus and the number of annuli compacted at the edge of the structure (Murie et al. 2009). Sections were mounted on glass slides using Flotexx® (Lerner Laboratories, Pittsburgh, PA) and examined using a zoom stereomicroscope (20-100X). A green filter (540 nm narrowband interference filter; Olympus, Tokyo, Japan) was used to enhance the contrast between opaque and translucent zones when necessary (i.e., if the sectioned ray was too thin).

As with otoliths, one complete annulus in a fin ray is the combination of one opaque growth zone and one translucent growth zone. Translucent zones are typically counted in fin ray aging because they are narrow and the most distinct zone (Chilton & Beamish 1982; Debicella 2005; Murie et al. 2009). However, Debicella (2005) observed that in Gag (*Mycteroperca microlepis*) the opaque zone in their fin rays was deposited at relatively the same time as the opaque zone in their otoliths (i.e., the translucent zone of the fin ray is deposited at the opposite time of the year as the opaque zone in their otoliths). The appearance of a translucent or opaque zone at the edge of the fin ray section was therefore recorded and used to assign an age class to the fish based on the number of opaque zones present to be comparable to otolith ages used by Bullock et al. (1992). Opaque zones form on the edge of Goliath Grouper otoliths during April to August, with limited translucent zone growth until October (Bullock et al. 1992). Goliath Grouper in the current study were captured during their spawning season from August to October, which was concurrent with the deposition of an opaque zone in their aging structures. Fish were therefore assigned to an age class based on: 1) fish collected in

August through October that had a translucent edge were demoted 1 year since it would be assumed that they had already started to deposit a translucent zone for current year end; and 2) all fish collected during August through October that had an opaque margin were assigned an age class equal to the number of translucent zones. Fin rays were aged without knowledge of size of the fish or date of capture.

Sex-specific length and age frequency distributions were compared between known female and male Goliath Grouper using Kolmogorov-Smirnov Tests to determine if there was any sex-specific skew to the length or age distributions, as would be expected if Goliath Grouper were protogynous hermaphrodites.

Growth of Goliath Grouper was analyzed by fitting age and total length to a von Bertalanffy growth curve using nonlinear regression with a Marquardt algorithm (PROC NLIN in SAS). The form of the von Bertalanffy growth curve was:

$$L_t = L_\infty (1\text{-}e^{-k(t-t_\circ)})$$

where L_t is the predicted TL (cm) at time t (age, in years), L_∞ is the estimate of the average maximum length (asymptotic length) (cm), k is Brody's growth coefficient, and t_0 is the theoretical age (years) when fish length would be 0. These models were compared to a pooled model using Likelihood Ratio tests for coincident curves to determine if females and males had significantly different growth (Haddon 2001). Growth curves were compared only over a similar range of fish age (Haddon 2001) and pooled when not significantly different from another.

For comparative purposes, the von Bertalanffy growth curve for Goliath Grouper from the Gulf of Mexico (Bullock et al. 1992) was overlain on the growth curve of Goliath Grouper from the Atlantic. This was also done to provide a qualitative comparison of the size at age for fish sampled in 1977-1990 (Bullock et al. 1992) versus the present study (2011-2015).

Reproduction

All captured Goliath Grouper were externally sexed using gonoduct/vent properties when possible. Smaller fish, approximately < 120 cm TL, were particularly difficult to sex externally using gonopores. Fish that were sexed externally but with doubt were excluded from all sex-specific analyses.

Gonad biopsies were also taken to determine the sex of the fish (or to confirm the external sex determination) and its reproductive condition when not obvious (i.e., reproductive males spewing milt were not biopsied); some smaller fish could not be biopsied due to the physical constraints of the method. Females were biopsied by inserting a polyethylene catheter (6.3-mm OD, 4-mm ID) through the oviduct into the lumen of the ovary. The catheter was gently moved back and forth to remove ovarian tissue using a hand-operated vacuum pump (Mityvac MV8000), with the tissue drawn into an in-line collection cup. Males were particularly difficult to biopsy due to the smaller diameter of the sperm duct and so a smaller diameter catheter (2-mm OD) and/or human uterine biopsy forceps was used to obtain the biopsies when possible. The extracted gonad tissue was fixed immediately in the field in 10% formalin. After several days of preservation, tissue from each sample was placed into a standard histological cassette, and then washed and stored in 70% ethanol until shipped for processing at Crowder Histology Consulting (Baton Rouge, LA). Tissues were processed using standard paraffin embedding, cross-sectioned at 5-6 μ thickness, followed by staining with hematoxylin and eosin.

To sex the fish using histology samples, the slides were scanned at 100X to note the presence of female and/or male gonadal tissue. Presence or absence of all oocyte types was noted and for females this included oogonia, primary growth (PG), cortical alveolar (CA), vitellogenic (Vtg1, Vtg2, Vtg3), germinal vesicle migration (GVM), germinal vesicle breakdown (GVB), hydrated (H), atresia (α- and β-), and post-ovulatory follicles (POF) (early/intermediate and late) (Wallace and Selman 1981; Hunter et al. 1992; Brown-Peterson et al. 2011). For males, cell types included spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ). For each fish, the most advanced cell type was noted, as well as the most prevalent cell type by area.

Phases in the female and male reproductive cycles were assigned according to Brown-Peterson et al. (2011) with modifications as noted, and for females included immature (never spawned), developing, spawning capable, regressing, and regenerating (Table 1). Females with only PG oocytes were designated as immature if there were no other signs of previous spawning (i.e., no POFs). Females with only PG and CA oocytes, and no other signs of previous spawning, were considered to be in the developing phase but were designated as immature since they would not be expected to spawn in the current spawning season (i.e., no vitellogenic oocytes, as per Brown-Peterson et al. 2011). Fish with Vtg1 and Vtg2 oocytes were in the developing phase and considered to be mature because it would be expected that they would be able to participate in the current spawning season. Females with Vtg3 or more advanced oocytes were considered to be spawning capable for the current spawning season. A female with GVM, GVBD and/or hydrated oocytes was close to ovulation or spawning (i.e., an active spawner). Females with a prevalence of POFs and atretic oocytes were considered as regressing, whereas females with a prevalence of PG oocytes and signs of previous spawning were considered to be in the regenerating phase. Males were considered immature if only SG were present in the sample. They were considered to be in the developing phase if SG, SC, and ST were present in the absence of any SZ. All males with SZ were considered to be mature and spawning capable, even though the biopsies did not regularly sample enough of a lobule to observe sperm in the lumen (as per required by Brown-Peterson et al. 2011). However, in many cases, this designation was confirmed by the male spewing milt when brought to the surface.

Since most groupers are known to be protogynous hermaphrodites, all fish with primarily female oocytes in the histological sections were thoroughly scanned for the presence of any male gonadal tissue to indicate a female transitioning into a male. Similarly, all fish with primarily male tissue were scanned for the presence of any remnant female gonadal tissue, which would indicate its transition from a female fish. Since the fish were biopsied and the gonads not sampled whole, histological slides that showed male and female gonadal tissue that could have arisen through contamination during the catheterization or through the histological processing were not designated as transitional fish. For example, the presence of a couple of Vtg2 ooctyes mixed in with

spermatozoa could possibly due to processing contamination. Fish were considered to be females in transition, or transitioned males, only if male and female gonadal tissue were integrated on the slide or could be followed through a continuous progression.

RESULTS AND DISCUSSION

Size and Age

In total, 679 Goliath Grouper were captured off the east coast of Florida (Figure 1) during 2011 to 2015. These captures represented 511 unique fish, as tracked through ear tag and PIT tag recoveries. Out of 656 fish measured for TL, the smallest fish captured was 102 cm TL and the largest was 225 cm TL (Figure 2), with the majority of fish between 140 and 180 cm TL. Females ranged from 123 cm to 225 cm TL and males from 102 cm to 222 cm TL. Females designated as transitioning into males (see below) ranged in size from 108 to 191 cm TL, and males designated as having transitioned from females ranged in size from 122 to 206 cm TL. Female size distribution was significantly different than the male size distribution (K-S Test: $D=0.1194$, $P<0.0001$), with females distributed in the larger size classes compared to males. Based on length distributions alone, the skewing of the female length distribution towards larger sizes lends support to the species being dioecious, or at least not a strictly a protogynous hermaphrodite. In this latter case, one would expect the male length distribution to be skewed to larger fish rather than females.

Goliath Grouper ranged in age from 4 to 20 years of age (Figure 3), with 403 fish aged using dorsal fin rays. Captured females were 5 to 19 years of age and males were 4 to 20 years old. Females that were transitioning into males were between 4 and 12 years of age, while males that had transitioned from females were between 5 and 14 years of age. The age distributions of females and males were not different from one another (K-S Test: $D=0.1194$, $P=0.120$). Again, based on age distributions alone, the similarity in the age distributions between males and females does not support a strictly protogynous hermaphroditic reproductive pattern, despite the occurrence of transitional fish. In the latter, one would expect the age distribution of the males to be skewed towards older fish.

The extant Goliath Grouper population on the east coast of Florida is younger than that encountered by Bullock et al. (1992) in the Gulf of Mexico, which was sampled during 1977-1990, with the fishery closed in 1990. Of the 382 fish Bullock et al. (1992) aged, 46% were ≤12 years old, ~78% were ≤ 18 years old, and ~22% of the fish were relatively old (> 18 years, up to a maximum age of 37 years). In contrast, most goliath grouper on the east coast of Florida are still ≤12 years old (85%), with 99% of the fish ≤ 18 years of age. Although in a 26 year, post-closure period, the population still appears to be skewed towards younger fish (i.e., juvenated) and will require more time to rebuild the older age classes.

Growth

Goliath Grouper sampled from the east coast of Florida did not exhibit sex-specific growth patterns, with growth models and individual length at age data overlapping (Figure 4A). All Goliath Grouper that had been measured and aged, regardless of whether their sex was known, were therefore pooled to estimate growth overall (Figure 4B). In comparison to the growth model for Goliath Grouper from the Gulf of Mexico in the 1980s reported by Bullock et al. (1992), the growth rate of Goliath Grouper from the Atlantic coast of Florida was greater for all ages through 4 to 20 years (Figure 4B). This faster growth rate suggests faster a density-dependent response to lower population size or increased food availability. Alternatively, the growth rates of Goliath Grouper in the Gulf of Mexico and off the Atlantic coast of Florida may be fundamentally different, as it is for other fish species that occur on both coasts of Florida (e.g., Black Seabass, *Centropristis striata*) (Watanabe 2011).

There has been some debate on the relative merits of using fin rays or spines to age protected fish species in a non-lethal manner. Brusher and Schull (2009) validated the use of the dorsal fin spines to accurately age juvenile Goliath Grouper < 6 years old, after which the central lumen of the spines became occluded with vascular tissue that prevented their use in accurate age estimation (Brusher & Schull 2009; Murie, pers. obs.). Although occlusion or resorption processes can also occur in fin rays (Chilton & Beamish 1982, McFarlane & King 2001), it does not occur in high frequency in Goliath Grouper

even in older fish and does not significantly impact our ability to use fin rays to age Goliath Grouper. Of more concern is the compaction of the annuli on the edge of the fin ray that can occur in older fish, leading to an underestimation of the age. The method has been validated for Goliath Grouper up to 18 years of age (Murie et al. 2009) and was not an obvious problem in the current study where Goliath Grouper were aged up to 20 years. However, the compaction of annuli on the edge of the fin ray structures could become more of a concern as the population continues to age.

Reproduction

The majority of Goliath Grouper sampled off the east coast of Florida were sexed as either a female or male using histological means, as well as gonopores. This, along with overlapping length and age distributions, supports the dioecious reproductive strategy reported by Bullock et al. (1992) for Goliath Grouper from the Gulf of Mexico. However, Goliath Grouper from the east coast of Florida clearly have the potential to be hermaphroditic, with protogyny supported by the occurrence of transitional fish. Based on all fish that could be measured and a sex assigned, 6.1% of Goliath Grouper sampled during the spawning season were in transition. Both fish with mostly female gonadal tissue with some testicular tissue (females in transition) (Figure 5), and fish with mostly male gonadal tissue with residual female tissue (males that had already transitioned), were present on the spawning grounds. Most females in transition were captured during August and September (9 of 15), but others were noted from May through to December. These females also spanned a large size range (108-191 cm TL) and age (4-12 years).

Using biopsies to determine the occurrence of hermaphroditism has limitations because males that are brought to the surface spewing milt are not biopsied even though these males may have evidence of residual female tissue in their testes. In addition, if the testicular tissue infiltrating the ovaries of a female starts at a specific place within the ovaries, for example the distal portion of the ovary, then the biopsy catheter may miss that particular portion of the ovary. Therefore, the occurrence of hermaphroditism noted in this study is the minimum expected; it may occur in greater frequency than determined through gonad biopsies.

It is perplexing that there is a relatively high occurrence of protogyny in the Goliath Grouper population on the east coast of Florida, yet a larger proportion of the fish are presumably not transitioning. Even for protogynous hermaphrodites it is commonly observed that not all females transition to males, and so there is usually the occurrence of some larger, older females in the population. However, the occurrence of small, young males would indicate that some males may be born male and not arise through transition from a female, indicating that Goliath Grouper may not be a monadric, protogynous hermaphrodite. It should be further explored whether Goliath Grouper could be diandric, protogynous hermaphrodite, where males can arise either directly from birth or through transition from a female. Complex reproductive strategies need to be addressed in stock assessments as the reproductive strategies can impinge on the species ability to withstand various harvest levels.

Although there have been some previous indicators that Goliath Grouper may be a protogynous hermaphrodite, the previous data have been inconclusive. Goliath Grouper testes have been reported as having a lumen and peripheral, sperm-collecting sinuses like the males of most protogynous hermaphrodites (Smith 1971) and at least one testes of a male Goliath Grouper captured in the Gulf of Mexico has been reported to have a few regressed oocytes (Bullock and Smith 1991). However, Bullock et al. (1992) collected males and females with substantially overlapping age compositions (males 3-26 years and females from 0-36 years). In addition, they did not find any sexual differences in growth patterns. Lastly, they report that males matured at slightly smaller and younger ages than females. None of these patterns are what would be expected if Goliath Grouper were in fact demonstrating protogynous hermaphroditism.

Given the observed difference in the reproductive strategies of Goliath Grouper off the east coast of Florida versus the Gulf of Mexico, it is important to sample Goliath Grouper in the Gulf of Mexico to determine if hermaphroditism occurs, and at what level. Reproductive characteristics of fish can change based on the level of harvesting, such as age and size at maturity, and so can their reproductive strategy. The former can be taken into account in population assessments, especially looking at changes over time.

However, we have no knowledge of any study that has taken into account an actual change in the reproductive strategy of a species over its exploitable time span.

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Table 1. Stages of Goliath Grouper associated with maturity, reproductive phase, and oocyte types, and any conditions or limitations for that reproductive phase.

Maturity	Reproductive Phase[1]	Oocyte Type(s)	Condition(s)
Immature	Immature	PG	No prior signs of spawning, such as POFs
	Developing	PG, CA	No prior signs of spawning, such as POFs; Probably will not spawn during the current spawning season
Mature	Developing	Vtg1, Vtg2	Will spawn during the current spawning season
	Spawning Capable	Vtg3, GVM, GVB, H	GVM, GVB, and H indicate active spawning
	Regressing	Atretic, POF	PG, CA, Vtg1, and Vtg2 may be present but not prevalent
	Regenerating	Oogonia, PG	Atretic and old POFs may be present

[1] The terminology for the reproductive phase is based on Brown-Peterson et al. (2011), however, the developing phase has been modified to reflect that females sampled during the current spawning season with only PG and CA have been designated as "Immature" fish since it is most probably that they will not spawn in the current spawning season.

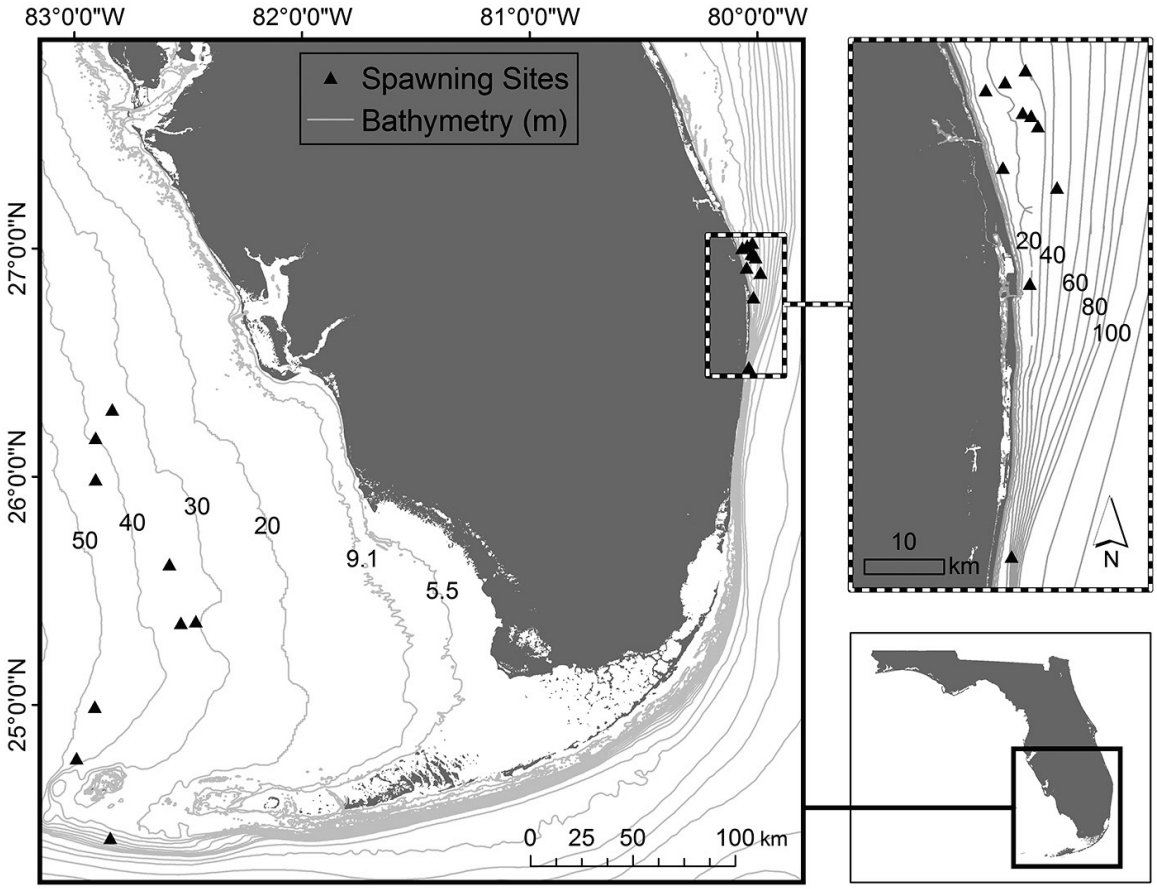


Figure 1. Sampling locations for Goliath Grouper from the east coast of Florida (dashed inset) during 2011-2015.

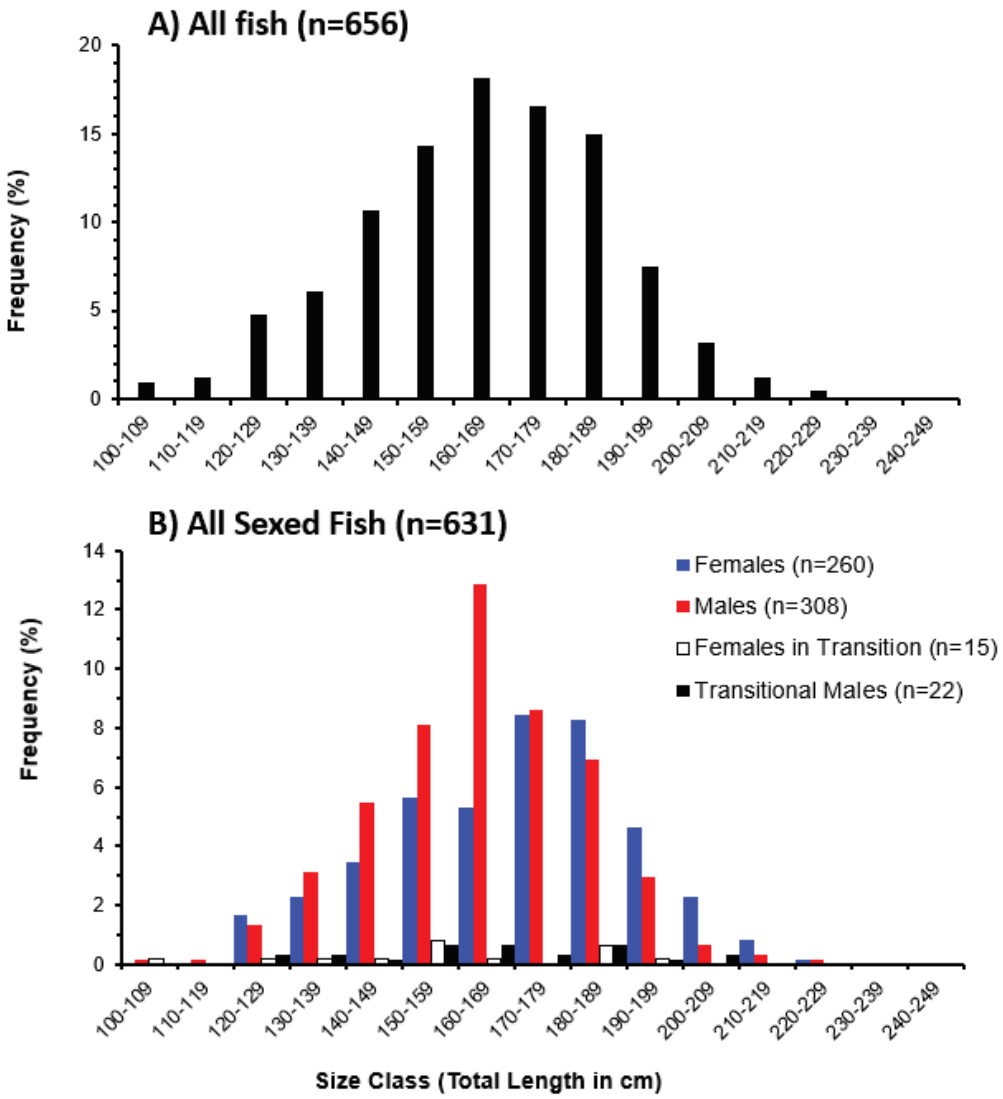


Figure 2. Length frequency distributions of Goliath Grouper sampled during the spawning season off the east coast of Florida for A) all fish captured and measured (n=631) and for B) fish that were sexed either by external gonopores or histological analysis.

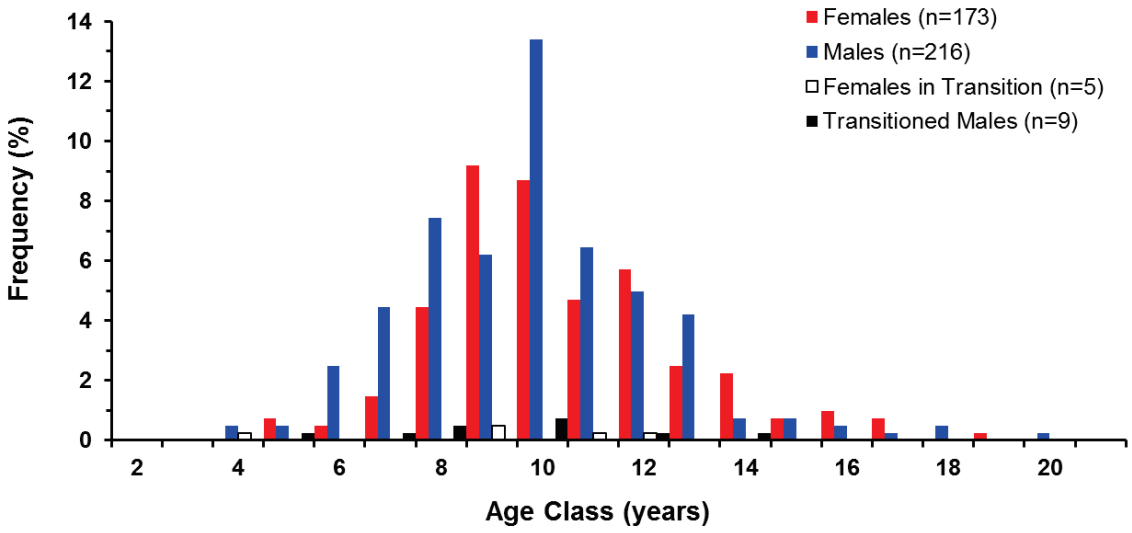
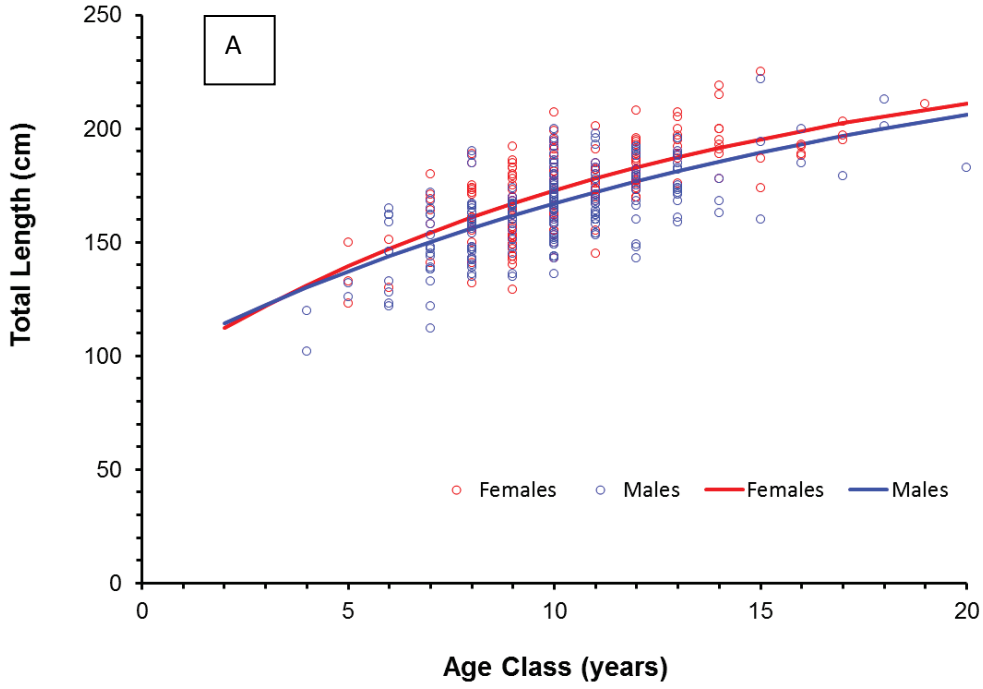


Figure 3. Sex-specific age frequency distribution of Goliath Grouper (n=403) sampled during the spawning season off the east coast of Florida.



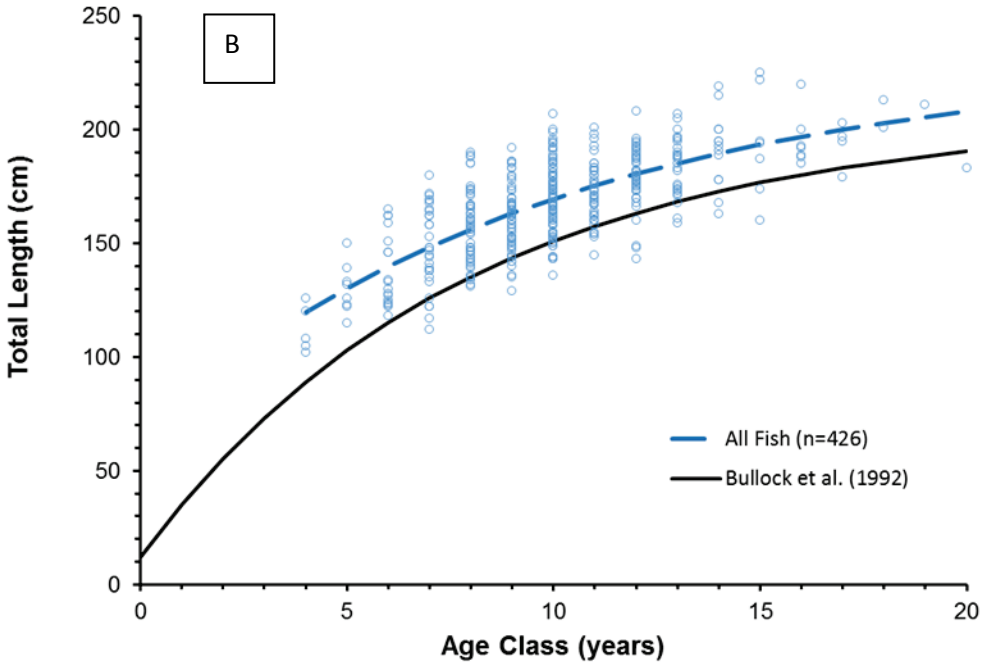


Figure 4. A) Growth of female and male Goliath Grouper sampled off the east coast of Florida, USA, during August to October 2011-2015; and B) Growth of all fish sampled, regardless of sex. Solid line is the von Bertalanffy growth curve for Gulf of Mexico Goliath Grouper from Bullock et al. (1992).

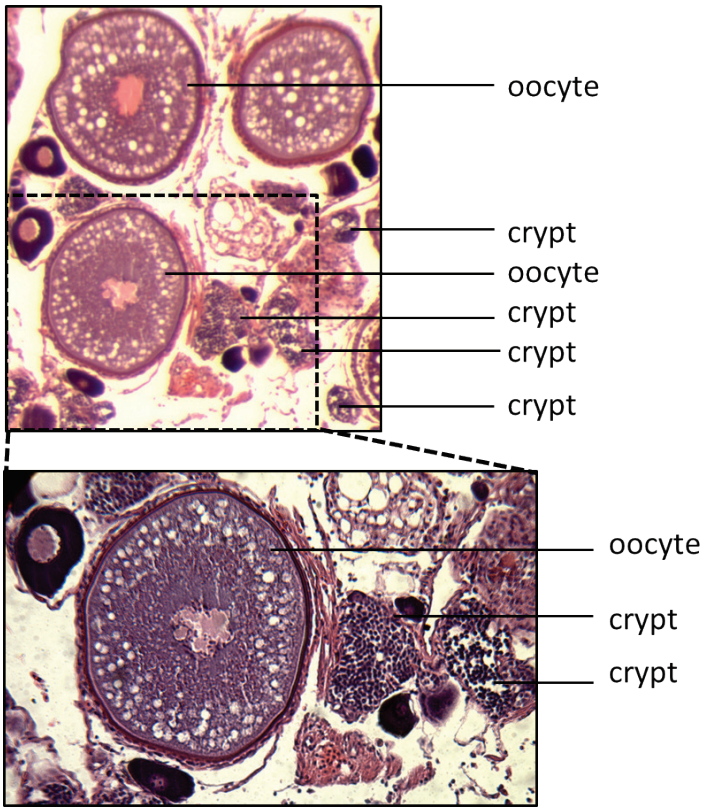


Figure 5. Histological slide showing a female Goliath Grouper transitioning to a male during the spawning season off the east coast of Florida. This was a 136 cm TL female caught in August of 2011. She was designated as spawning capable with Vtg3 oocytes.

Part II. Timing of Spawning (Christopher Koenig, leader).

Additional publication in the appendix, "Koenig, C. C., L. S. Bueno, F. C. Coleman, J. A. Cusick, R. D. Ellis, K. Kingon, J. V. Locascio, C. Malinowski, D. J. Murie, and C. D. Stallings. 2016. Diel, lunar, and seasonal spawning patterns of the Atlantic Goliath Grouper, *Epinephelus itajara*, off Florida, United States. Bull. Mar. Sci.: http://dx.doi.org/10.5343/bms.2016.1013.

Method of Identification of Goliath Grouper spawning sites (Christopher Koenig, leader):

Goliath Grouper spawning takes place on spawning sites (natural and artificial reefs) in South Florida during the months of August through October—that the fish forms spawning aggregations has been known since Colin (1990) attempted to determine the diel and lunar patterns of spawning off SW Florida. At the time, very few Goliath Grouper were present on the known spawning sites (information provided by former Goliath Grouper fisherman, Don DeMaria), so it was difficult to evaluate these patterns. From his observations of this fish and other reef fish, Colin (1990) suggested that Goliath Grouper spawn in pairs during the day on the full moon. Unfortunately, this suggestion directed many to assume those diel and lunar patterns while investigating the spawning behavior of the fish, while, the actual pattern and timing of spawning is exactly opposite—they group spawn primarily on new moons, during the night (Koenig et al. 2016, in appendix of this report).

Our strongest indication of this nocturnal new-moon pattern came when we recorded sounds produced by Goliath Grouper on their spawning sites (Mann et al. 2009) in 2005. The groupers produced choruses of single pulses (Figure 1) of sound ("booms", as they are often called) during the night, but the intensity of these sounds decreased during full moon nights (Figure 2).

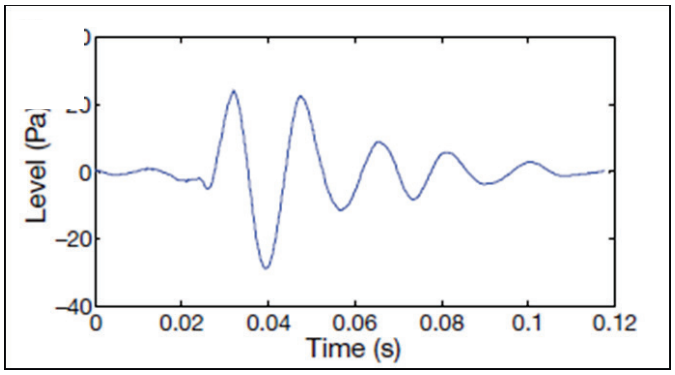


Figure 1. Single pulse sound made by Goliath Grouper. Maximum acoustic energy is at 60 Hz. (Mann et al. 2009)

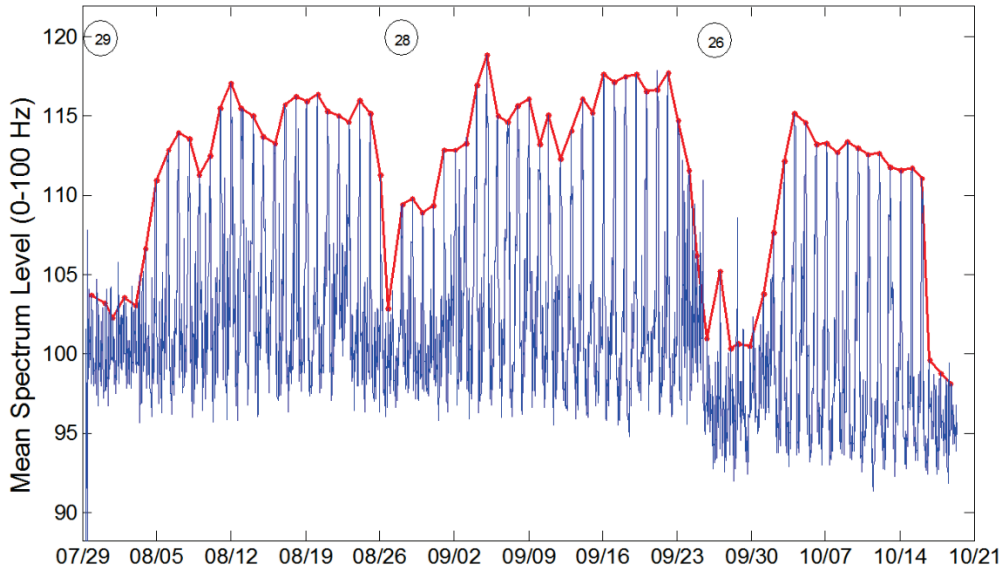


Figure 2. Spawning sounds produced by Goliath Grouper on a spawning aggregation throughout the spawning season. Each peak is produced at night, so peaks are 24 hrs apart. Note that the sound production decreases during full moons (indicated by circles at the top of the figure with dates of these moons indicated within the circles). These sounds are not present at non-spawning times of the year (Mann et al. 2009).

Subsequent to the work of Mann et al. (2009), we recorded on both spawning and non-spawning sites during the spawning season and outside of it and found that only fish on spawning sites during the spawning season produced these nocturnal sounds—no such

sounds were produced on non-spawning sites during the spawning season and no nocturnal sounds were produced outside of the spawning season.

Because spawning takes place on the darkest nights, identification and verification of spawning sites must be done using other methods than direct observation—we use both sound and histological evidence of spawning. Recording the sounds is straight forward, but it must be done around new moons of August and September. We recorded sounds using inexpensive hydrophones and recorders housed in a PVC housings (Figure 3) designed and built by Koenig.

To determine the exact time of sound production and (presumably) spawning, hydrophones were deployed on 12 known spawning sites around the time of the new moons of August and September—6 off SE Florida and 6 off SW Florida. In each case, night booms were maximal approximately between the hours of 1:00 AM to 5:00 AM (see Figure 4 as an example from a spawning site off Jupiter, FL).

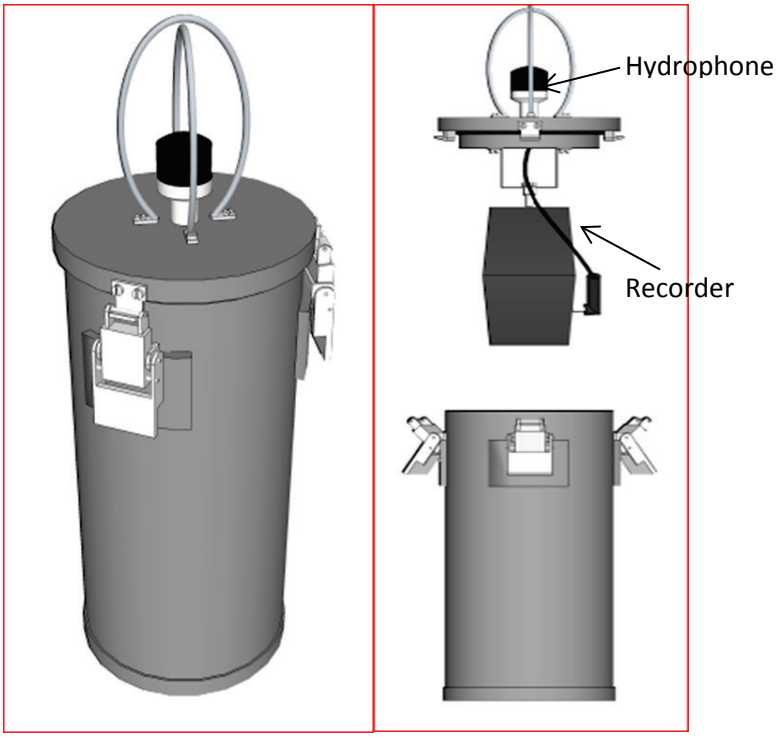


Figure 3. Hydrophone and H2n Zoom recorder in PVC housing, built for recording continuously for 20 hrs.

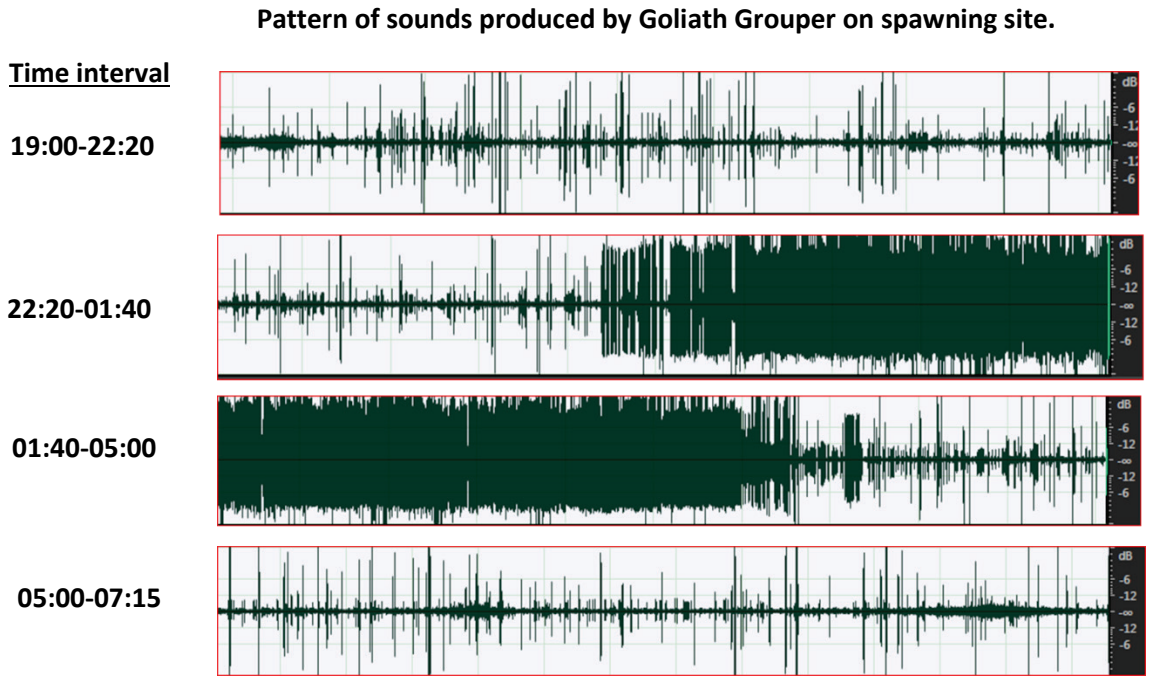


Figure 4. Sounds produced by Goliath Grouper on "Gary's Greys" natural reef spawning site off Jupiter , FL on 5-6 October 2013 (on the new moon). Fifty Goliath Grouper were counted on this spawning site. Vertical lines are booms produced by Goliath Grouper—note intense booming between about 1:00 AM and 4:30 AM. (Note, I have a sample of intense booming from this site in MP3 format if anyone would like me to send—Koenig)

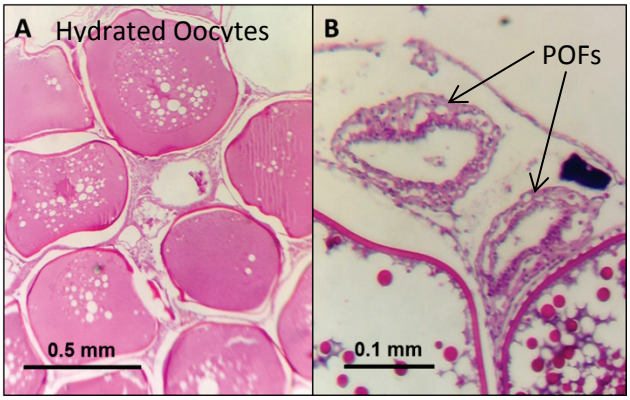


Figure 5. Histological section of Goliath Grouper ovary showing A. hydrated oocytes, and B. POFs (post-ovulatory follicles.

After spawning sites have been identified by characteristic nocturnal sounds, they can be verified by capturing female Goliath Grouper and taking ovarian biopsies (see

biopsy methods in Koenig et al. 2016 in appendix)—the presence of either hydrated oocytes or post-ovulatory follicles (Figure 5) is strong confirmation that spawning is about to take place (hydrated oocytes) or has taken place within the last 24 hrs (POFs). In the absence of direct observation, this method is the most straight-forward and direct determination of a spawning site.

Night-time sampling of Goliath Grouper fertilized eggs could also be done to confirm spawning sites, but this is a very difficult endeavor because it depends on the weather and current structure (direction, velocity and stability) overnight and whether or not there are jellyfish or other organisms that could foul the nets in the surface waters.

Other clues to spawning sites include: size and number of Goliath Grouper on a site, build-up of Goliath Grouper abundance during August (REEF data can be used to efficiently determine this).

Part III. Spawning site fidelity and movement patterns of acoustically tagged Atlantic Goliath Grouper (Robert Ellis, leader).

Additional publication in appendix: "Ellis, RD, CC Koenig, FC Coleman. 2014. Spawning-related Movement Patterns of Goliath Grouper (*Epinephelus itajara) o*ff the Atlantic Coast of Florida. Proc. 66th Gulf and Carib. Res Inst. p. 395-400").

Draft manuscript (below): Spawning site fidelity and movement patterns of acoustically tagged Atlantic Goliath Grouper (Robert Ellis, leader).

INTRODUCTION

Atlantic Goliath Grouper, *Epinephelus itajara* (Lichtenstein 1822) exhibit restricted home ranges and high site fidelity (Koenig et al., 2007; Koenig et al., 2011; Collins et al., 2015), but also form annual spawning aggregations (Colin 1990, Koenig et al. 2016). Spawning aggregations are defined as predictable, repeated concentrations of conspecific marine animals that gather for purposes of spawning at densities at least four times greater than outside aggregations and that result in a mass point source of offspring (Domeier 2012). Fish species that form spawning aggregations are highly vulnerable to fishing due to the predictable nature of the aggregations in time and space and the aggregations of many fish species have been severely disrupted by overexploitation, in some cases to the point of complete loss (Sadovy de Mitcheson et al., 2008). For species that form spawning aggregations, there remain significant knowledge gaps regarding the spatial and temporal patterns of aggregations, and the habitat linkages and trophic interactions that occur during fish migrations (Nemeth 2009). Furthermore, spawning aggregations present particular management challenges for ongoing fisheries sustainability. Goliath Grouper experienced significant population declines due to intense fishing pressure during the latter part of the 20th century, but the population has since shown signs of recovery following protection from harvest (Koenig et al., 2011). The extent of this recovery and the current status of Goliath Grouper in US waters remains unknown, in part due to the difficulty in data collection during the harvest moratorium (SEDAR 2016).

In addition to life history and population information, other information needed for Goliath Grouper stock assessments is lacking. Such information includes: spawning

movement patterns and distances, behaviors on residence and spawning sites, estimates of spawning population size, size of areas from which spawning fish migrate, distances individuals move to spawning sites, timing (e.g., seasonal, lunar, diel) of spawning, and the size and age structure of fish on spawning aggregations—these data are all necessary for fishery management (SEDAR 2004). Many of these data needs can be met through the use of passive acoustic telemetry. For species that migrate to spawning aggregations, the use of acoustic telemetry has provided researchers with the ability to determine: home sites, home ranges, migration distances and spawning migration corridors (Namami et al., 2013; Dahlgren et al., 2016).

We realized that we had a rare opportunity to monitor patterns of behavior related to Goliath Grouper reproduction in great detail by tagging fish with acoustic tags at spawning sites off the southeast Florida coast and tracking them through the Florida Atlantic Coast Telemetry (FACT) array of acoustic receivers. The FACT cooperative group makes use of compatible telemetry receiver hardware and a commitment to coordinate receiver spacing and share detection data to allow member researchers to track study animals over longer durations and over greater distances. Our involvement as a member of FACT allowed us to monitor movements of Goliath Grouper at both spawning sites and home sites along the Florida Atlantic coast. FACT members that maintained acoustic receivers on sites along the coast contacted us to report detections of transmitter-tagged Goliath Grouper; likewise, when we detected transmitter-tagged fish of other species, we reported the detections to the tag owners.

The information collected as tagged Goliath Groupers moved through the FACT array provided a tremendous advantage for us in terms of the amount and type of data that we could collect on Goliath Grouper movements. Specifically, we were interested in defining spawning site fidelity, the area of spawning aggregation sites (the areal extent of home ranges and migration routes of a spawning population; Nemeth 2012), and spawning related behaviors (e.g., single vs. multiple spawning, migratory patterns, etc.) that are critical management needs for species that form spawning aggregations. To that end, we tagged Goliath Groupers with acoustic tags and tracked them through the FACT array of stationary acoustic receivers to determine how Goliath Groupers move in

relation to spawning. Here we present the results of four calendar years of passive acoustic telemetry data collected from Goliath Groupers as they migrated along the Florida Atlantic coast.

MATERIALS & METHODS

Fish tagging

Starting in the fall of 2010 we tagged Goliath Groupers present at suspected spawning aggregation sites with Vemco V16-6H acoustic coded transmitter tags. Tags were set to produce a uniquely coded acoustic ping at 69 kHz randomly once every 60 to 180 seconds (nominal delay = 120 seconds) and had an expected battery life of 3033 days. Fish were captured using hook and line, hauled on deck and immediately strapped onto a stretcher frame modified with nylon tie-down straps in order to minimize fish movement. Once immobilized, a hose with running seawater was placed in the mouth to irrigate the gills and the eyes were covered with a wet towel to protect them from direct sunlight. Fish were measured for total length, the swim bladder was vented, and the soft dorsal fin rays number 6 and 7 (counting from anterior to posterior) were removed for aging. Sex was determined by visual examination of the vent region and a gonad biopsy was collected using a flexible plastic catheter attached to a hand-operated vacuum pump. Fish were tagged with a PIT (passive integrated transponder) tag injected into the dorsal musculature just below the juncture of the spinous and soft dorsal fins and with a pig-ear tag clipped into the base of the posterior part of the anal fin. Finally, an acoustic transmitter tag was implanted into the abdominal cavity by cutting a small incision anterior to the vent region, inserting the tag into the body cavity, and closing the wound with surgical staples or monofilament sutures. After surgery, fish were released at the site of capture.

In 2013 we tested an *in situ* method of externally attaching acoustic transmitter tags by a diver with a speargun. We attached a stainless steel T-bar anchor to the transmitter tag with 300 lb test monofilament and then attached the tag and anchor to a specially modified spear tip that was designed to implant the anchor about 10 cm into the dorsal musculature when shot from a posterior position by an experienced spear

fisherman. After impact the spear would penetrate into the dorsal musculature and the anchor would release from the spear, allowing the fish to swim away and the diver to collect the spear for further tagging.

Acoustic tracking and monitoring

To detect tagged fish, we used Vemco VR2W 69 kHz receivers mounted at specific sites thought to represent Goliath Grouper habitat. Receivers were mounted on a zinc-anode protected stainless steel cable (3/8 in, 9.5 mm) that was anchored to the bottom and suspended just above the bottom with a hard plastic float (Figure 1). From 2010 to 2015 we deployed ten VR2W receivers at 14 different sites located offshore of Palm Beach and Martin counties in southeastern Florida (Figure 2). During the study two receivers were lost, either due to structural failure of the mount or possible theft. Most of the sites we monitored were high relief natural reefs or artificial reefs where we had previous reports of Goliath Grouper from local fishers. We visited monitored sites in the spring and fall to download data, replace batteries, and to check on the integrity of the mooring system. Six of the sites we monitored over the course of the study off Jupiter were confirmed as spawning aggregation sites: Hole-in-the-Wall, Zion Train, Three-Holes, Sun Tug, MG-111, and Gary's Greys (see Koenig and Coleman 2013).

In addition to our spawning site monitoring, we also received location data from VR2W receivers maintained by the FACT cooperative group. FACT group members currently maintain over 700 acoustic receivers along more than 1000 km of the Atlantic coast from Ossabaw Sound, Georgia (31°52'N), to Riley's Hump in the Dry Tortugas National Park (24°30'N). In addition, cooperative partners with acoustic receivers located in the Everglades National Park and Ten Thousand Islands, the Bahamas, Puerto Rico and the U.S. Virgin Islands also contribute detection data to FACT members. Receivers are deployed along a continuum of coastal habitats from freshwater estuaries to marine waters of the adjacent continental shelf, including high relief natural and artificial reef sites preferred by Goliath Groupers (Koenig et al., 2011; Collins et al., 2015).

Figure 1. Goliath Groupers swimming near an acoustic receiver mooring consisting of a brake drum anchor, 3/8 in (9.5 mm) diameter stainless steel cable, and hard float buoys. Photo credit: Ellis.

Figure 2. Map of the main study area offshore of Palm Beach and Martin counties, Florida, USA, indicating sites that were monitored by us for this study (FSU), sites where we tagged fish (Tag), sites monitored by FACT group members where Goliath Grouper were detected (FACT), and sites that we previously confirmed as spawning aggregation sites (SPAG).

Data analysis

Data were downloaded into the Vemco VUE program and then exported into Excel (Microsoft, 2013, Redmond, Washington). In order to validate detection data, we used a false-detection filter that removed any single detections not associated with a second detection at the same location within 20 minutes (Pincock 2012; Young et al. 2016). From the validated detection data we calculated the number of days with at least one valid detection for each fish, hereafter "detection days". We limited the analysis of detection data to all records collected between 1 January 2011 and 31 December 2014 in order to maintain a consistent detection probability at the spawning aggregation sites that we monitored (our monitoring efforts concluded July 2015).

We used the detection information to calculate site fidelity by tagged Goliath Groupers to spawning aggregation sites. We were not able to confirm one of the tagging sites as a spawning site and because we also observed that some fish visited multiple sites within a single spawning season. We defined spawning site fidelity as the ratio of the number of fish detected within the FACT array for a given year to the number detected at one of six confirmed spawning sites (see Figure 2) during the spawning season. We defined the spawning season from 1 July through 31 October each year in order to capture information on movements related to peak spawning that occurs during the August, September, and October new moons, as well as pre-spawning movements that we described previously and that appear correlated to the July full moon (Ellis et al., 2014; Koenig et al., 2016). We also used the detection information to describe movements of tagged Goliath Groupers both within the spawning season and during the rest of the year. Movements were calculated as the straight-line distance between two sites; this metric will under estimate the actual distance moved by individuals as long as fish deviate from linear paths during movements between sites.

We tested for differences in site visitation, spawning site fidelity, and movement behaviors based on fish size and sex with simple linear regressions and T-tests, respectively. Fish size was based on the measured total length (TL) at the time of capture during the first year after tagging. For subsequent years, fish size (L_t) was

estimated using a growth curve generated from the von Bertalanffy growth function,

$$L_t = L_\infty\left[1 - e^{-K(t-t_0)}\right]$$

where L_∞ is the asymptotic length (222.1 cm); t_0 is the theoretical age at a length of zero (0.67); and K is the growth parameter (0.0937). Parameter values were taken from the most recently completed stock assessment for Goliath Grouper (SEDAR 2016). Fish sex was based on visual examination at the time of tagging.

RESULTS

Fish capture & tagging

Between 4 September 2010 and 6 September 2013 we tagged 50 Goliath Groupers with V16 acoustic coded transmitters. The bulk of our tagging effort occurred during the fall of 2010 when we captured and tagged 38 fish (see Table 1). Two fish were captured and tagged in May 2011, followed by an additional five fish in September 2012. The final five fish were tagged externally by an experienced spearfisher (Capt. DeMaria) in September 2013; because these fish were tagged *in situ* and were not brought on board the fishing vessel, we were unable to obtain a length measurement or determine the sex of these five individuals.

Table 1. Summary information for the 50 Goliath Groupers fitted with acoustic tags. "n.d." indicates no data available. "I" indicates fish that were determined to be immature based on length at the time of tagging. Fish #024 and #030 were never detected after release; however #024 was recaptured in December 2010 with a failed transmitter tag.

Fish #	Sex	Length (cm)	Tag site	Tag date	Last detection	Days at liberty	Total days detected (2011-14)
024	F	153	Zion Train	9/4/2010	--	0	0
025	M	151	Zion Train	9/4/2010	11/9/2013	1162	725
027	M	156	Zion Train	9/4/2010	5/6/2013	975	370
028	F	136	Zion Train	9/4/2010	5/20/2013	989	603

029	M	190	Zion Train	9/4/2010	12/23/2012	841	246
030	F	136	3-Holes	9/5/2010	--	0	0
031	M	127	3-Holes	9/5/2010	5/4/2014	1337	633
032	F	125	3-Holes	9/5/2010	7/3/2013	1032	38
033	F	163	Gulfland	9/5/2010	9/17/2012	743	9
034	F	162	Gulfland	9/5/2010	8/29/2013	1089	130
035	F	167	Gulfland	9/5/2010	2/20/2014	1264	319
037	M	147	Gulfland	9/5/2010	7/27/2013	1056	70
038	F	150	Gulfland	9/5/2010	8/31/2011	360	1
039	M	135	Gulfland	9/5/2010	9/28/2011	388	76
040	M	136	Gulfland	9/5/2010	10/3/2012	759	169
041	I	117	Gulfland	9/5/2010	3/8/2013	915	369
042	I	104	Gulfland	9/5/2010	5/24/2015	1722	477
043	I	120	Gulfland	9/5/2010	12/22/2012	839	189
044	M	137	Zion Train	9/25/2010	12/15/2013	1177	351
045	F	189	Zion Train	9/25/2010	7/17/2013	1026	123
046	F	179	Zion Train	9/25/2010	9/19/2013	1090	55
048	M	172	Zion Train	9/25/2010	6/29/2013	1008	665
049	M	178	Zion Train	9/25/2010	1/29/2014	1222	290
051	F	130	Zion Train	9/25/2010	9/4/2013	1075	83
052	M	205	Zion Train	9/25/2010	5/25/2013	973	316
053	M	189	Zion Train	9/26/2010	7/12/2016	2116	968
055	F	174	Zion Train	9/26/2010	9/7/2015	1807	153
057	M	181	Zion Train	9/26/2010	11/26/2012	792	305
058	F	180	Zion Train	9/26/2010	9/27/2013	1097	88
059	M	182	Zion Train	9/26/2010	7/19/2014	1392	492
060	F	186	Zion Train	9/26/2010	9/1/2012	706	40

061	F	192	Zion Train	9/26/2010	10/9/2013	1109	240
062	F	156	Zion Train	9/26/2010	6/27/2015	1735	677
063	M	184	Zion Train	9/26/2010	3/11/2013	897	20
064	F	185	Zion Train	9/26/2010	9/16/2013	1086	74
065	M	127	Zion Train	9/26/2010	11/13/2013	1144	670
082	n.d.	151	Zion Train	12/12/2010	9/1/2013	994	711
083	M	162	Zion Train	12/12/2010	1/31/2013	781	59
089	F	184	3-Holes	5/25/2011	8/27/2016	1921	629
091	n.d.	166	3-Holes	5/25/2011	11/7/2013	897	167
417	F	194	Zion Train	9/15/2012	8/20/2016	1435	177
436	F	174	Zion Train	9/16/2012	9/23/2012	7	2
438	F	168	Zion Train	9/16/2012	9/7/2016	1452	125
439	F	177	Zion Train	9/16/2012	9/4/2016	1449	105
440	M	162	Zion Train	9/16/2012	10/7/2012	21	22
XT1	n.d.	n.d.	3-Holes	9/6/2013	5/5/2015	606	62
XT2	n.d.	n.d.	3-Holes	9/6/2013	4/29/2015	600	137
XT3	n.d.	n.d.	3-Holes	9/6/2013	9/26/2014	385	81
XT4	n.d.	n.d.	3-Holes	9/6/2013	5/30/2015	631	382
XT5	n.d.	n.d.	3-Holes	9/6/2013	3/6/2015	546	170

All fish were captured and tagged at three suspected spawning aggregation sites located offshore of Martin and Palm Beach counties (see Figure 2). During work funded by a previous MARFIN grant (Koenig and Coleman 2013) we confirmed that two of the three tag sites were also spawning sites for Goliath Grouper: Zion Train (artificial reef; 30 fish tagged) and Three-Holes (natural reef complex; 10 fish tagged). Ten fish were captured and tagged at the Gulfland, an artificial reef that we were not able to confirm as a spawning site, though we detected tagged fish at the site throughout the study period suggesting that it may be used by Goliath Groupers as a pre-spawning or staging site

(Koenig et al., 2016).

Tagged Goliath Groupers ranged in size from 104 to 205 cm TL (mean = 160.9 cm). We determined the sex of tagged fish by visual examination at the time of capture or from histology in the case of transitional individuals. The sex distribution of tagged fish was as follows: female = 22; male = 18; immature = 3; unknown = 2. In addition to the two individuals for which we could not resolve sex either visually or via histological examination of collected gonadal tissue, we do not know the sex of the five fish tagged *in situ*; these seven individuals were excluded from analyses which compare movement patterns by sex.

At least one of the tags that we implanted during our initial tagging effort failed to turn on. After its initial capture and release on 4 September 2010, this individual (fish #024; 153 cm TL; female) was recaptured on 11 December 2010 at a site approximately 3 km away. A VR2W receiver deployed at the recapture site did not detect the tag, nor did a handheld hydrophone used on-board the fishing vessel, indicating a malfunctioning transmitter tag. In addition, one fish was never detected again following its initial capture and release, nor was it recaptured later suggesting that it may have died. Excluding these two individuals, the 48 tagged fish were at large for an average of 1014 ± 63.4 days and were detected on 268 ± 35.5 days from 2011 to 2014.

General detection and movement patterns

Between 1 January 2011 and 31 December 2014, transmitter-tagged Goliath Groupers were detected at 58 different sites within the FACT array (Figure 3). All but two of the tagged fish were detected within the FACT array following release, one of these was due to a failed tag as described above, while the other we assume died shortly after release. The proportion of tagged Goliath Groupers detected at a FACT monitored site declined each year of the study: in 2011, 37 of the 40 tagged fish (92.5%) were recorded within the array; in 2012, 40 of the 45 tagged fish (88.9%) were detected in the array; in 2013, 38 of the 50 tagged fish (76.0%) were detected in the array, and in 2014 only 17 of the 50 tagged fish (34.0%) were detected in the array.

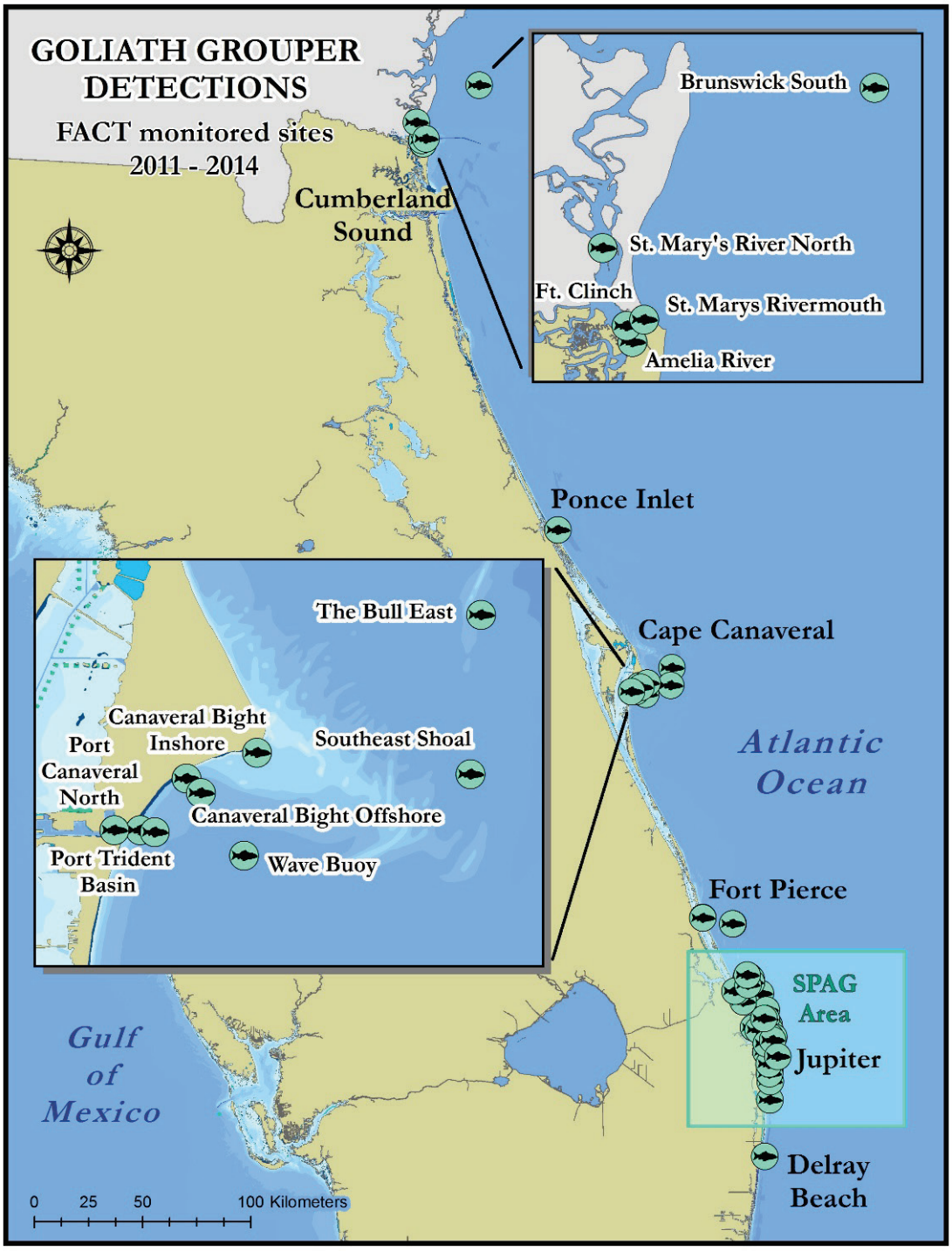


Figure 3. Sites along the Atlantic coast of Florida monitored by the FACT cooperative group where acoustic transmitter tagged Goliath Groupers were recorded from 2011 to 2014 (n = 58).

We examined detections temporally to determine patterns of site association. Seasonal patterns of detections were evident in both 2011 and 2012 (Figure 4), however this pattern was less evident in the last two years of the study, 2013 and 2014, when there were fewer tagged fish detected in the array overall (Figure 5). During the first two years of the study the number of fish detected in the array began to increase starting in early June and peaked in 2011 on 5 August (26 fish; day number 217). In 2012 the number of tagged fish detected in the array peaked on 12 September (22 fish; day number 256). The observed increase started and peaked earlier in 2011 compared to 2012. The maximum number of detected fish in 2013 occurred much earlier in the year, on 25 February (16 fish; day number 56), followed by a second peak later in the year on 28 August (15 fish; day number 240). In 2014 the peak in detected fish occurred on 9 September (10 fish; day number 252). The maximum number of fish detected within the FACT array on any single day declined each year of the study (Table 2), and was generally not aligned with any specific lunar phase; however the maximum number of detected fish in 2014 occurred on the day of the September full moon.

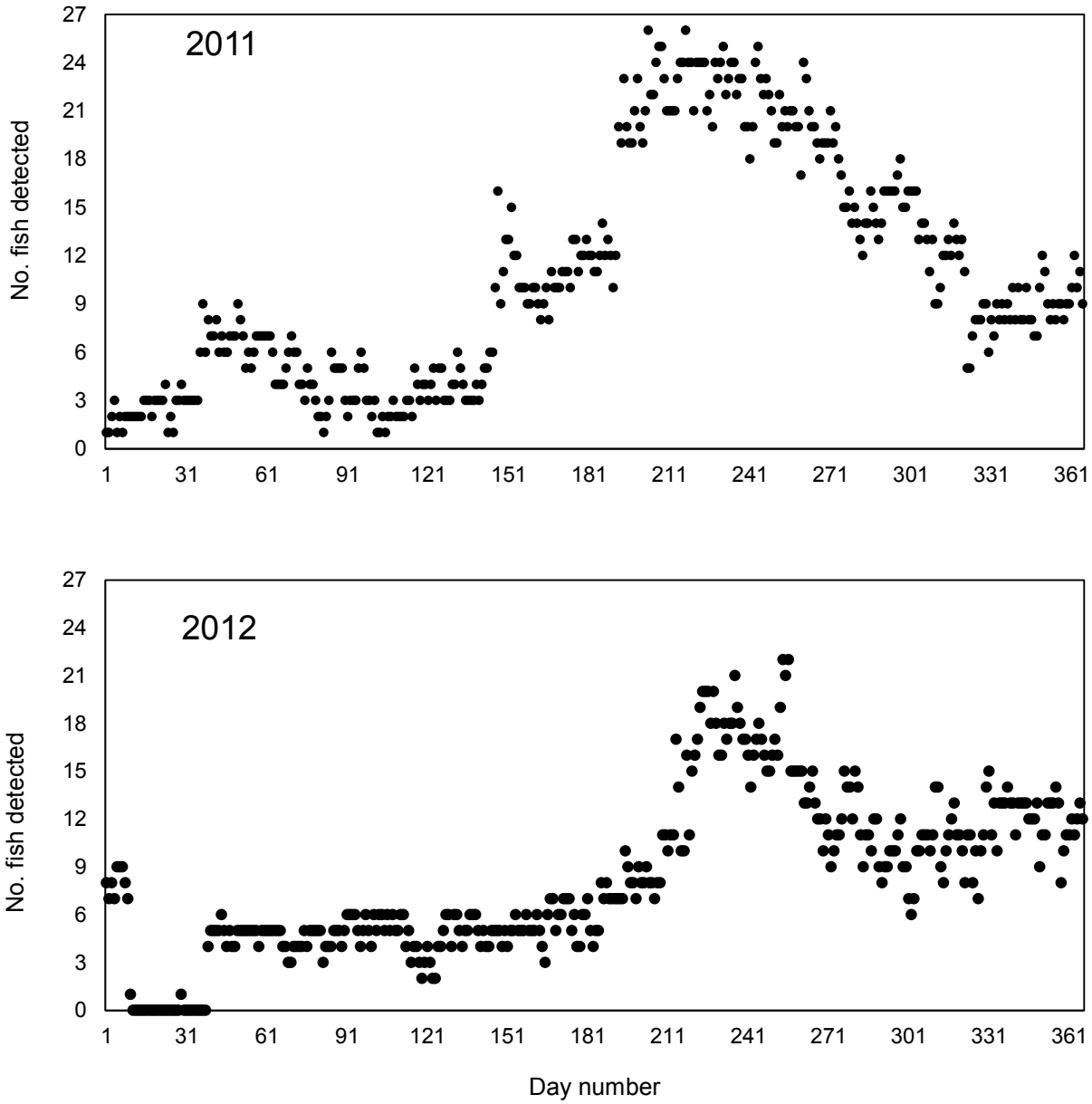


Figure 4. Number of tagged Goliath Groupers detected daily in the FACT array in 2011 (top) and 2012 (bottom).

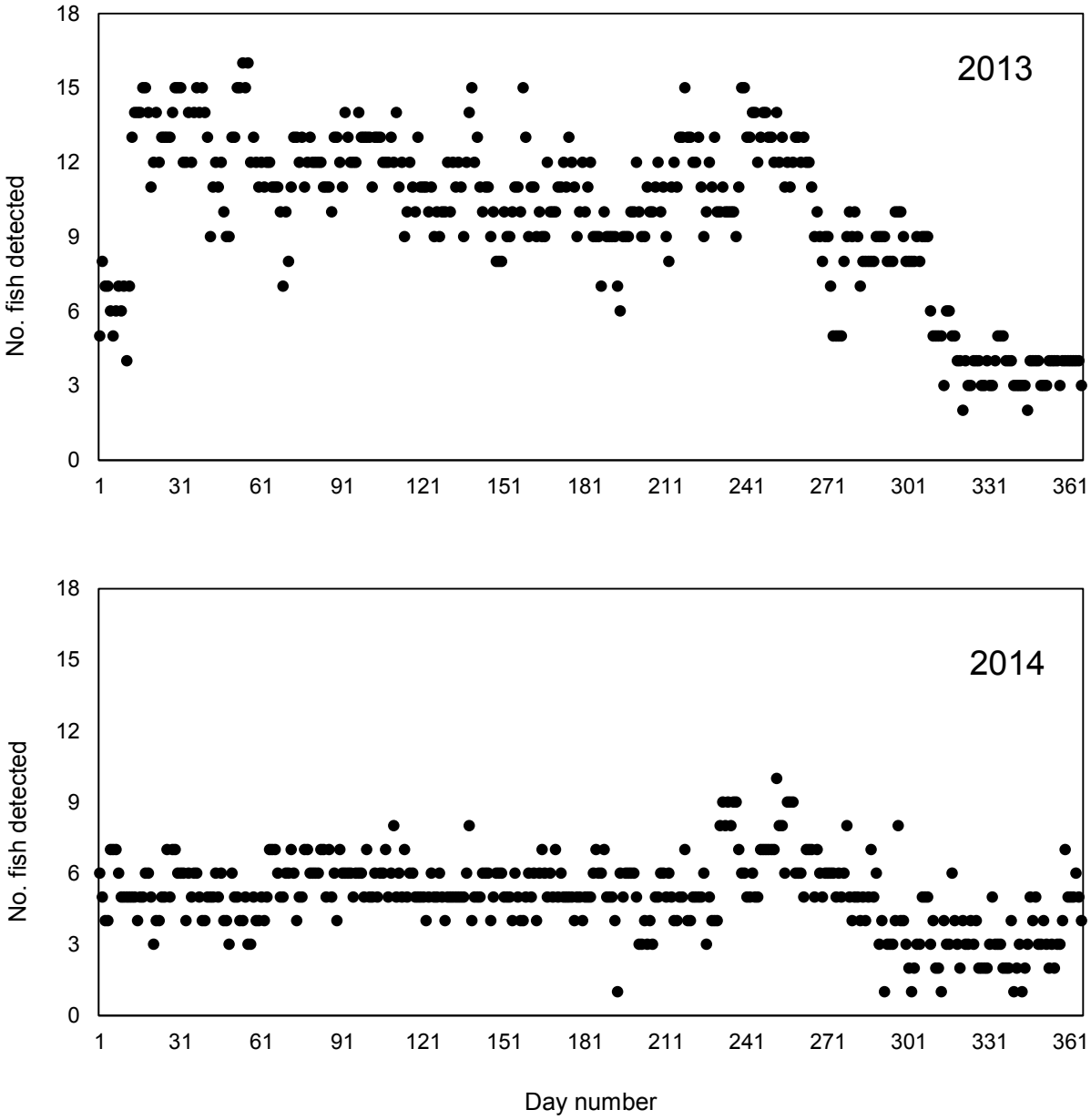


Figure 5. Number of tagged Goliath Groupers detected daily in the FACT array in 2013 (top) and 2014 (bottom).

Table 2. Maximum number of acoustically-tagged Goliath Groupers detected within the FACT array by day number, and the day numbers of the July, August, and September new moons for years 2011 to 2014.

	Year			
	2011	2012	2013	2014
Max fish number, day number	26, 217	22, 256	16, 56 (15, 240)	10, 252
July new moon	211	201	189	207
August new moon	241	230	218	237
September new moon	270	260	248	267

We described the movement patterns of tagged Goliath Groupers in various ways, first by examining patterns in detections by site where we summed the number of sites where each fish was detected during each month of the study from 2011 to 2014. This value varied between 0 (fish was not detected during a given month; multiple fish) and 12 (fish #045, 189 cm TL, female; detected at 12 sites in August 2011). Because the number of fish detected each month varied during the study period, we limited the dataset to include only fish that were detected during each month; thus the sites per month per fish parameter had a minimum value of 1 (fish was only detected at a single site during a given month). The mean number of sites where each tagged fish was detected during each month of the study varied seasonally and across study years (Figure 6). During most of the year, tagged fish remained at just one or a few nearby reefs, but during peak spawning in August and September, fish were detected at multiple sites. Across all months of the study, tagged fish were detected on average at 1.6 ± 0.05 sites per month, but individual months ranged from 1 site (multiple months), to a maximum of 2.58 sites in August 2011.

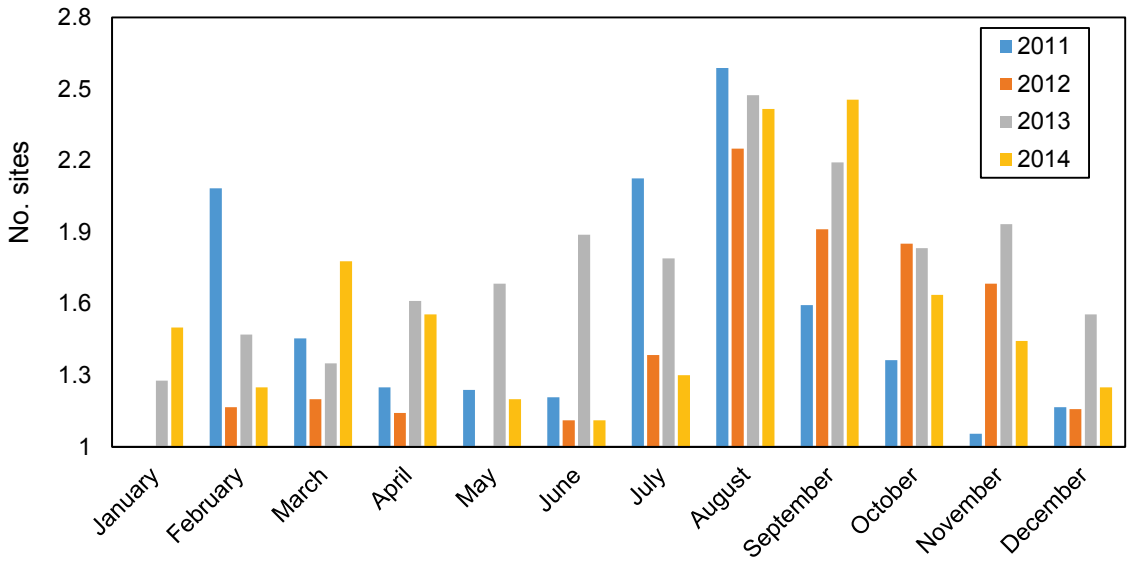


Figure 6. Number of sites where acoustically-tagged Goliath Groupers were detected each month from 2011 to 2014. Values shown represent mean number of sites with positive detections for all fish detected for each month of the study; minimum value for this dataset = 1 where each fish was only detected at a single site during a given month.

We then determined how many different sites were visited annually by each tagged Goliath Grouper for each year of the study. The number of sites visited each year did not vary significantly across years (d.f. = 130, $F = 2.67$, $p = 0.37$; Figure 7). Across the entire study, tagged fish were detected on average at just over 8 stations per fish (8.08 ± 0.82). Again, this varied across individuals and ranged from just a single site (6 fish) to 20 sites (fish #049, 178 cm TL, male; Figure 8).

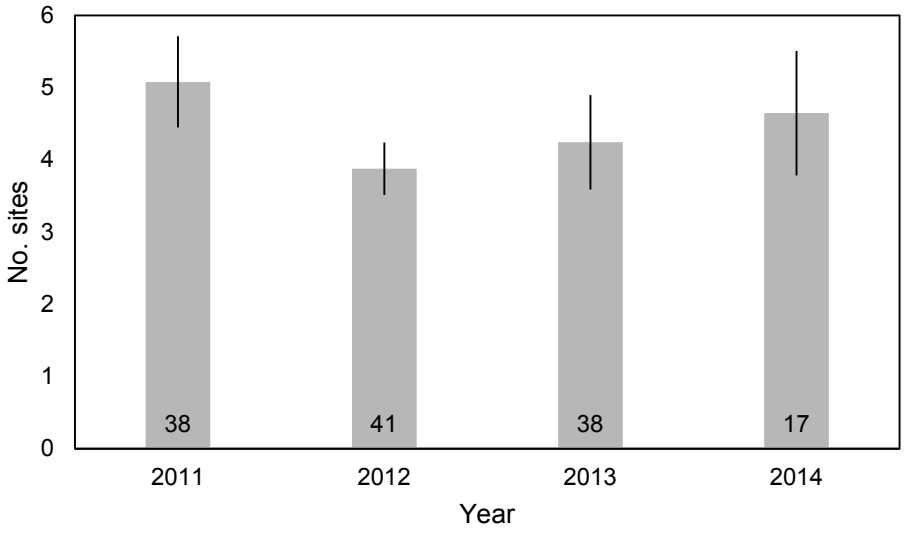


Figure 7. Mean number of sites visited annually by acoustically-tagged Goliath Groupers from 2011 to 2014. Numbers inside bars indicate the number of tagged fish detected during each year.

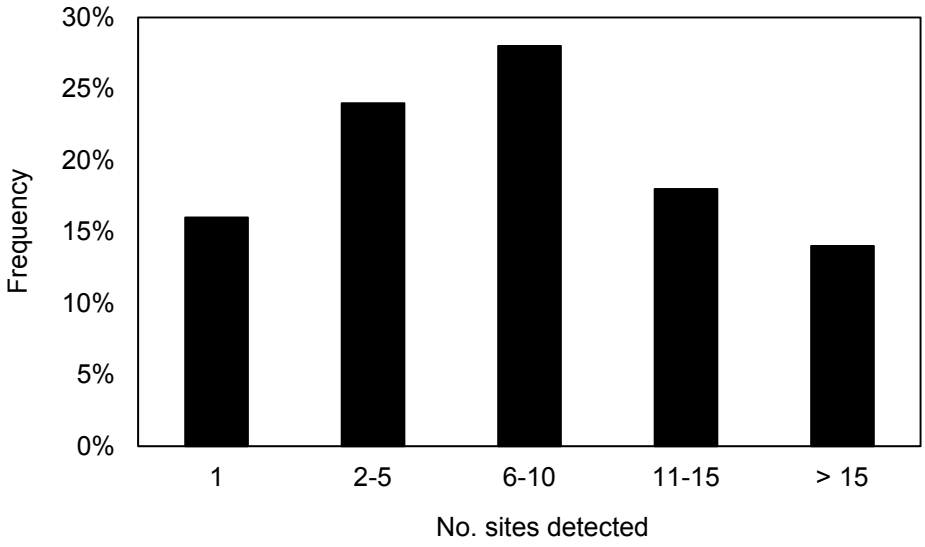


Figure 8. Frequency of total number of sites visited by all acoustically-tagged Goliath Groupers (n = 48) during the entire study period, 2011 to 2014.

In general, tagged Goliath Groupers did not move very often: over 90% of all detections occurred at the same site as the previous detection, indicating that tagged fish had not moved from the site. When tagged fish did move, they did not move far: 70.6% of all recorded movements between sites were less than 5 km, and 85.9% of all recorded movements were less than 10 km (Figure 9). However, tagged Goliath Groupers were also detected at sites that spanned over 500 km of the Florida and Georgia coast (see Figure 2) and we recorded multiple movements of more than 100 km between stations (see Figure 9). A few of these movements were particularly noteworthy. The maximum distance recorded between consecutive detections by any tagged fish in the study was 438 km over 10 days in August 2012 between a site in the Cumberland Sound, located near the Florida – Georgia border, and the spawning site MG-111, a movement of more than 40 km per day. In 2011 we detected a previous long distance movement by a fish (#060, 186 cm TL, female) of 252 km in July 2011 between Ponce Inlet and a natural reef site near the Jupiter Inlet over a period of 22 days (at least 12 km/day). Also in July 2011 we detected another tagged fish (#058, 180 cm TL, female) that moved 222 km between Ponce Inlet and an artificial reef near the St. Lucie Inlet in 9 days (at least 25 km/day).

We detected repeated long-distance migratory movements by another large female (#417, 194 cm TL) over multiple years of the study. In 2013 fish #417 was detected moving at least 175 km between Cape Canaveral and the Sun Tug spawning site over 12 days, at a speed of at least 37 km/day. In 2014 we again detected fish #417 migrating south between the Ft. Pierce inlet and the Sun Tug spawning site, moving over 100 km in 7 days. In 2015 we again detected fish #417 migrating south from sites near the Cumberland Sound to sites near the St Lucie Inlet, located at the northern edge of the spawning aggregation area, a distance of at least 435 km over 16 days (minimum speed = 27 km/day). Although we were no longer monitoring the SPAG sites in the fall of 2015 and thus cannot confirm that fish #417 returned to a SPAG site, this individual was detected at FACT monitored sites located within 10 km of confirmed spawning sites during the 2015 spawning season. Between late October 2015 and early August 2016, fish #417 was detected at sites near Cumberland Sound before again moving quickly south to an artificial reef near the St. Lucie Inlet on 20 August 2016, a distance

of at least 430 km over 17 days (minimum speed = 25 km/day).

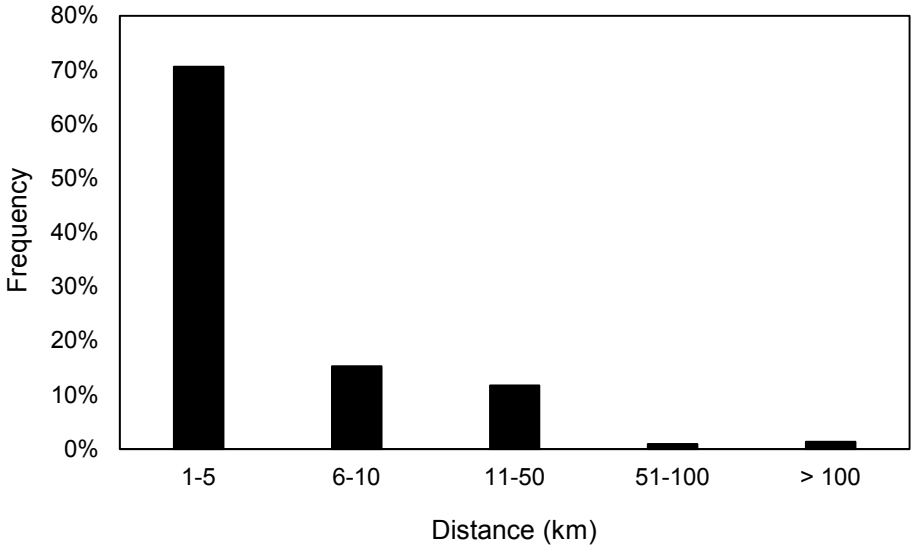


Figure 9. Frequency of recorded movements of acoustically-tagged Goliath Groupers along the Florida Atlantic coast from 2011 to 2014.

Temporal patterns of movement by tagged Goliath Groupers were consistent across all years of the study and showed that tagged fish were relatively sedentary during non-spawning months (December to June), and moved more during spawning months (July to October; Figure 10). We summed the cumulative movement distance for all fish for each month of the study and calculated mean monthly movements for each year of the study: on average Goliath Groupers moved 6.28 ± 0.58 km per month from 2011 to 2014. While this mean value does not fully capture the variability in movements (see Figure 10), it can provide a baseline with which to compare individual months to look for patterns or outliers. Tagged fish moved most during the month of August in three of the four years; in 2013 tagged fish moved most during the month of July. Monthly movements for the months of August and September were elevated during all four years of the study, while July, October, and November were the most variable months in terms of tagged fish movement. The movement data suggest that spawning may have occurred earlier in the year in 2011 and 2013 and later in the year in 2012 and 2014. Fish also appeared to move more during February 2011 and March

2014, both months that fall well outside of the predicted spawning period.

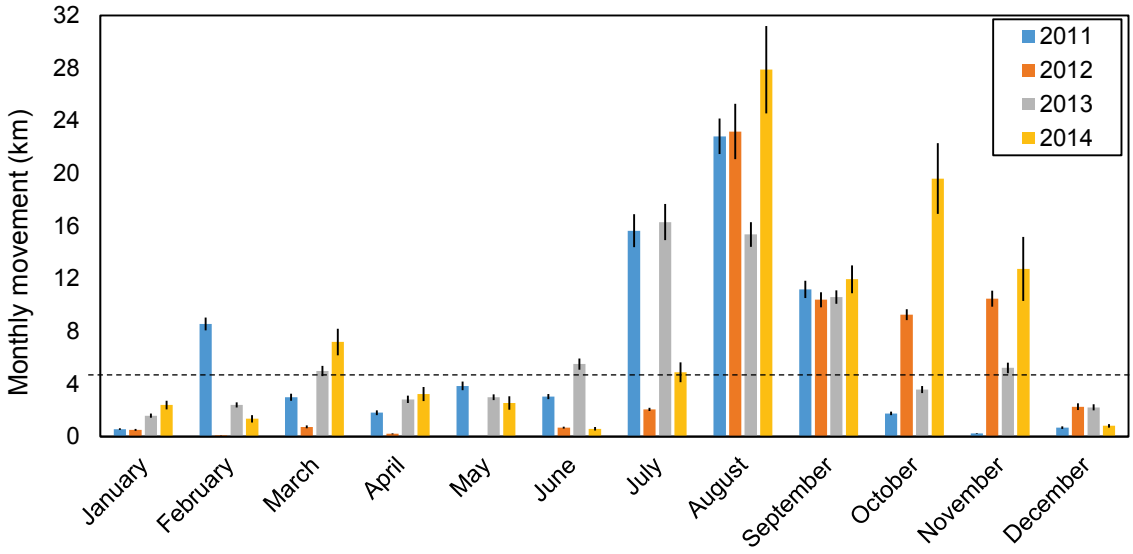


Figure 10. Cumulative movement distance (km) by month of acoustically-tagged Goliath Groupers from 2011 to 2014. Dashed line indicates the mean monthly movement distance for all fish across all four years of the study (6.28 km). Error bars are ± SE.

Spawning site fidelity

The number of tagged Goliath Groupers that were detected at spawning sites during spawning season (July to October) was high all four years of the study: annually more than 80% of tagged fish detected in the FACT array were also detected at one of the confirmed spawning aggregation sites (Table 3). On average, tagged fish visited 1.22 ± 0.07 spawning sites over the course of the study, however the mean number of spawning sites visited by tagged fish increased each year from 2011 to 2014 (Figure 11). Most fish (63.3%) only visited a single spawning aggregation site each year (Figure 12), but we detected one individual (fish #059, 185 cm TL, male) that visited four spawning sites in 2013.

Table 3. Inter-annual site fidelity by acoustically-tagged Goliath Groupers to the six spawning aggregation sites monitored during this study from 2011 to 2014.

	Year			
	2011	2012	2013	2014
No. fish detected in FACT array	38	36	26	14
No. fish detected at SPAG site	32	30	22	13
% of fish detected at SPAG site	84.2%	83.3%	84.6%	92.9%

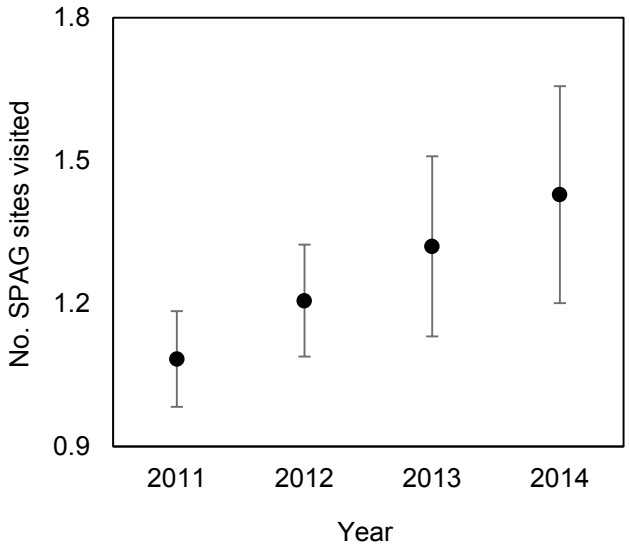


Figure 11. Number of spawning aggregation sites (mean ± SE) visited annually by acoustically-tagged Goliath Groupers from 2011 to 2014.

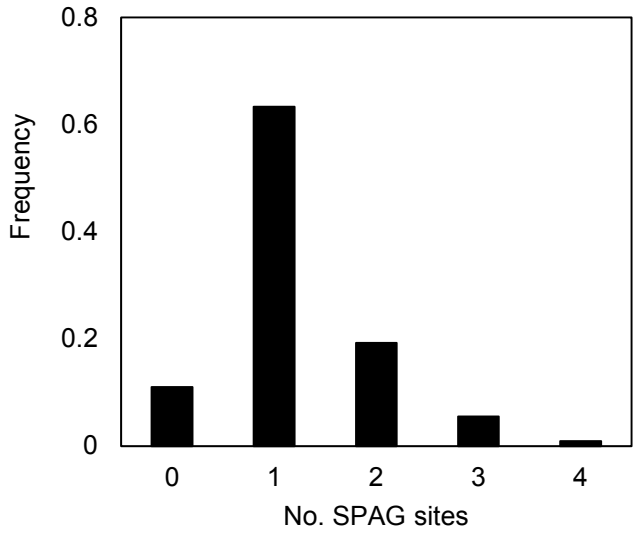


Figure 12. Frequency of the number of spawning aggregation sites visited annually by acoustically-tagged Goliath Groupers across all years of the study, 2011 to 2014 (n = 109).

On average, tagged Goliath Groupers were detected at one of the six spawning aggregation sites 39.4% of all detection days from 2011 to 2014 (Figure 12). However this value varied across individual fish, from two fish that were only detected at spawning aggregation sites to five fish that were never detected at a spawning site after their initial release. During the spawning season from July to October, tagged fish were detected at a spawning site on average for 45.5 ± 3.5 days each year. The number of detection days at spawning sites was highest in 2011 at 64.0 ± 7.1 days, but was between 37 to 42 days per spawning season for all other years (Figures 13 & 14).

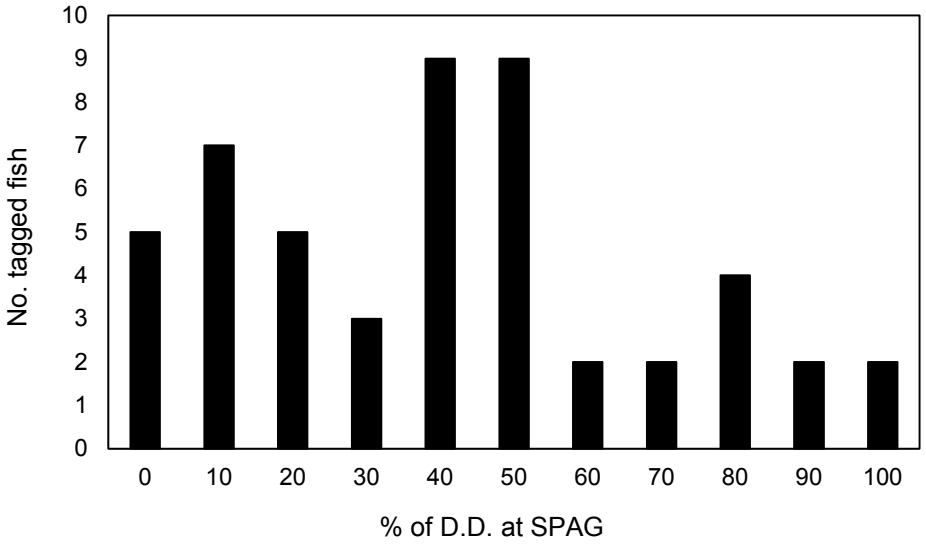


Figure 13. Percent of total detection days (DD) that were recorded at one of the six FSU-monitored spawning aggregation sites (SPAG) from 2011 to 2014.

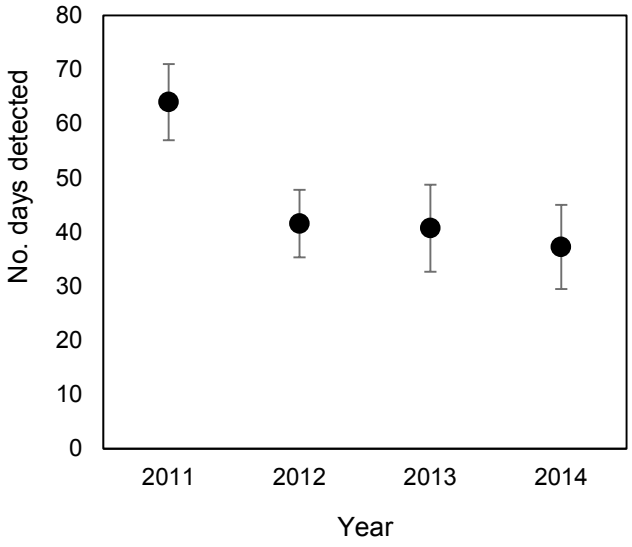


Figure 14. Mean number of days acoustically-tagged Goliath Groupers were detected at one of the six FSU-monitored spawning aggregation sites annually from 2011 to 2014. Error bars are ± SE.

The most frequently visited spawning aggregation site was Zion Train (ZT), which was also the site where we tagged the most fish and where divers observed the largest aggregations of Goliath Groupers each year (except for 2012, see below). In 2011, 28 of 38 (73.7%) tagged Goliath Groupers were detected at ZT; in 2012, 26 of 36 (72.2%) of tagged fish were detected at ZT; in 2013, 14 of 26 tagged fish (53.8%) were detected at ZT; and in 2014, just 5 of the 14 tagged fish at large visited the ZT site (35.7%). Over all four years of the study, the most visited spawning aggregation site was ZT, followed by Three-Holes (TH), the MG-111 wreck (MG), the Sun Tug wreck (ST), Gary's Greys (GG), and Hole-in-the-Wall (HIW; Figure 15). However, the relative rank importance of the six spawning aggregation sites, in terms of the total number of tagged fish detected at each site annually, varied during the study (Table 4). In 2012 the MG site was the second most visited site of the six spawning aggregation sites that we monitored, and we also observed the largest aggregation of Goliath Groupers at the MG site in 2012 (in all other years divers recorded the largest aggregation at the ZT site).

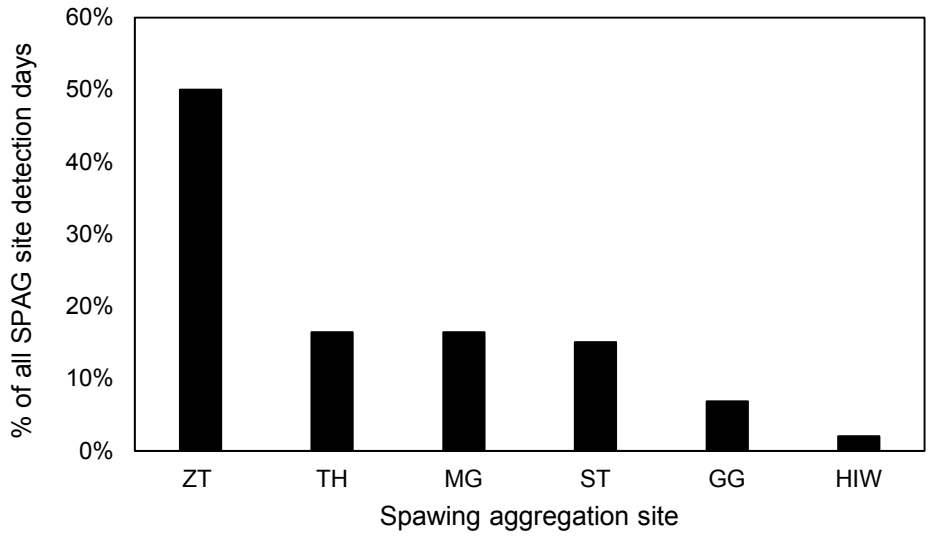


Figure 15. Percent of all spawning aggregation site detection days for acoustically-tagged Goliath Groupers that were recorded at each of the six monitored spawning aggregation sites from 2011 to 2014.

Table 4. Relative rank importance of the six FSU-monitored spawning aggregation sites in terms of the number of acoustically-tagged Goliath Groupers detected there from 2011 to 2014.

	SPAG site					
Year	ZT	TH	MG	ST	GG	HIW
2011	1	3	4	2	5	6
2012	1	4	2	3	6	5
2013	1	2	4	4	3	6
2014	1	2	4	3	4	6

Aggregating behavior

In order to visualize aggregation behaviors by tagged Goliath Groupers, we compared the number of FACT sites where tagged fish were detected from 30 May 2011 (day #150) to 16 November 2011 (day #330; Figure 16) to the number of tagged fish detected at the ZT site during this same time (Figure 17). In 2011, the number of sites where a tagged Goliath Grouper began to increase around the end of June (day #180) and peaked on day #199, 3 days after the July full moon that year, then stayed high until after the August full moon (day #225) when it started to decline again. The number of tagged fish at the ZT site began to increase around the August full moon, peaked on day #244, three days after the new moon in August, and stayed high until after the September new moon (day #270) when it began to decline. A similar pattern of fish aggregating at the ZT spawning site was clearly detectable in 2012, somewhat evident in 2013, and not evident at all in 2014 due to the reduced number of tagged fish detected in the array (Figure 18). These data suggest a strong lunar component of spawning aggregation behavior, where movements peak around the July and August full moons and aggregation formation peaks around the August and September new moons.

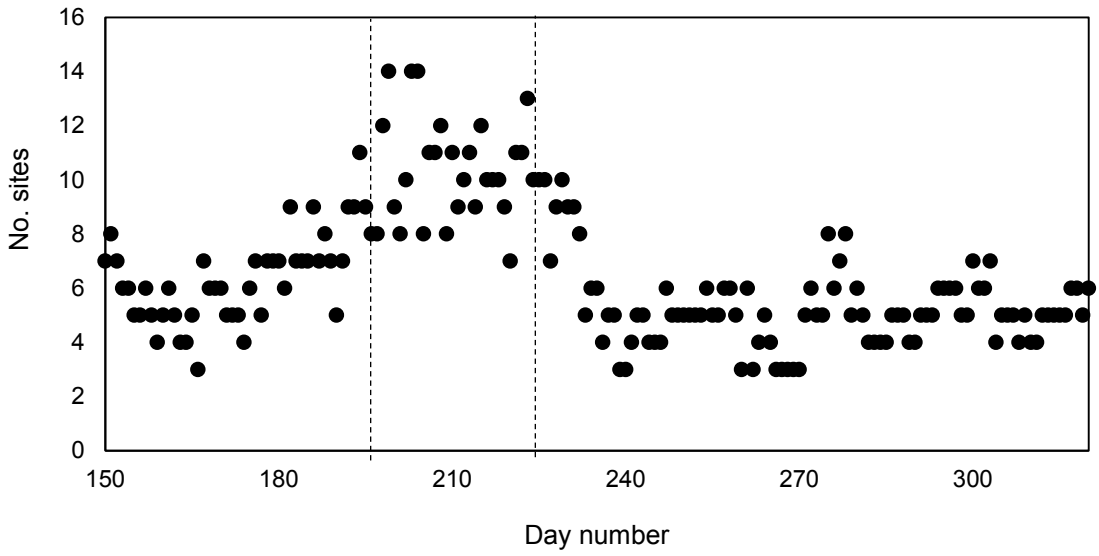


Figure 16. Number of FACT-monitored sites where acoustically-tagged Goliath Grouper were detected between 30 May and 16 November 2011. Vertical dashed lines indicate the approximate dates of the full moons in July and August.

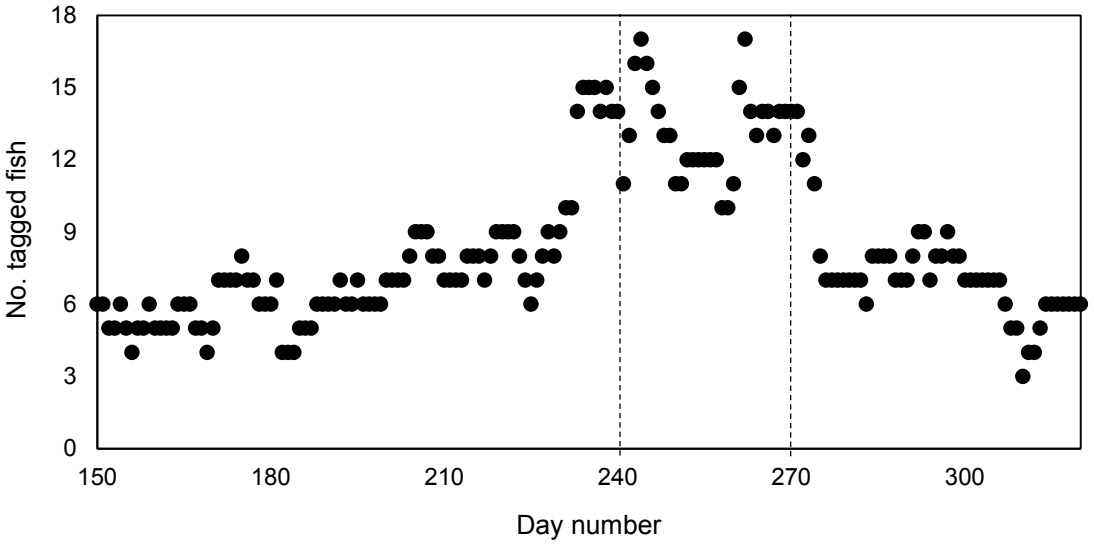


Figure 17. Number of acoustically-tagged Goliath Grouper that were detected at the Zion Train spawning site between 30 May and 16 November 2011. Vertical dashed lines indicate the approximate dates of the new moons in August and September.

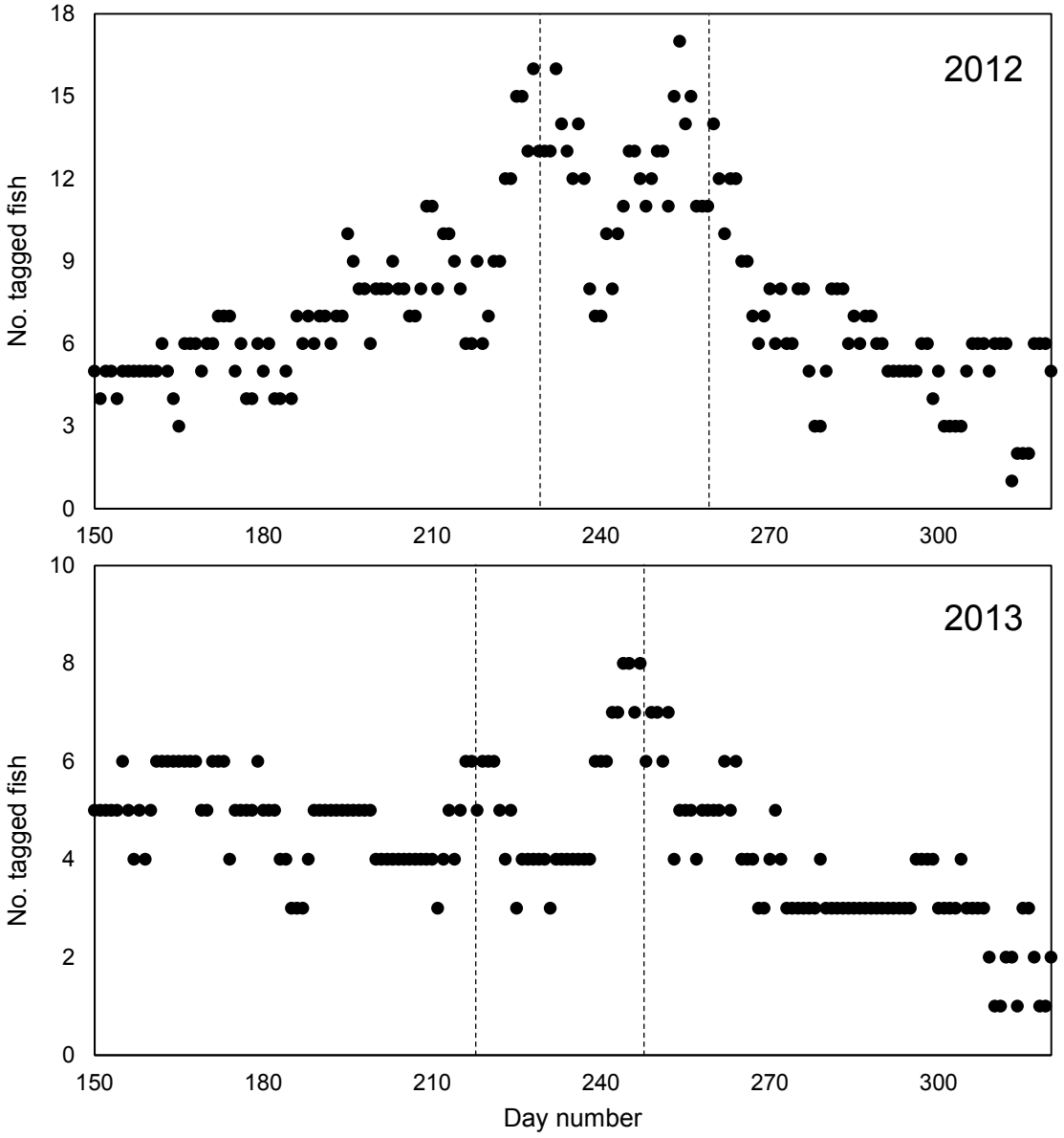


Figure 18. Number of acoustically-tagged Goliath Grouper that were detected at the Zion Train spawning site between 29 May and 15 November 2012 (top) and 30 May to 16 November 2013 (bottom). Vertical dashed lines indicate the approximate dates of the new moons in August and September in each year.

Size and Sex Differences

We analyzed the detection data to determine if any movement, residence, or

arrival patterns could be attributed to either fish size or sex. In general, larger fish visited more FACT-monitored stations during the study (Figure 19) and moved farther (Figure 20) than did smaller fish. Simple linear regressions performed on both of these metrics showed that both patterns are significant and positively correlated: number stations visited: d.f. = 120, $F = 31.4$, $p < 0.001$; distance moved: d.f. = 114, $F = 31.4$, $p < 0.001$. We calculated the regression of maximum distance moved relative to total length after discarding four observations we considered outliers based on the fact that they were more than 3 standard deviations greater than the mean (mean = 29.3 km; standard deviation = 59.9). We also found a significant positive relationship between fish size and the number of SPAG sites visited annually (d.f. = 104, $F = 7.00$, $p = 0.009$); the slope of the regression line was significantly positive, but the regression model explained just 6% of the variance in the spawning site detection data ($R^2 = 0.064$).

We tagged some fish that were determined to be immature during the time of tagging based on their size at capture relative to the published maturity at age schedule in the literature: Bullock et al. (1992) reported that male Goliath Grouper matured at 110 to 115 cm and females matured at 120 to 135 cm. Because the sex of immature fish was not apparent from visual examination during capture, we considered any fish less than 120 cm TL to be immature. Three fish were captured and tagged during the fall of 2010 that met these criteria: fish #041, 117 cm TL; fish #042, 104 cm TL; and fish #043, 120 cm TL. All three fish were captured and tagged on the same day at the Gulfland site, a sites where we tagged fish but were not able to confirm spawning. None of these individuals were detected at SPAG sites until they had grown to at least 135 cm TL. Fish #041 and #043 were both detected at SPAG sites in 2012 (approximate length 135 and 137 cm TL respectively), while fish #042 was not detected at a SPAG site until 2014 when it was approximately 141 cm TL.

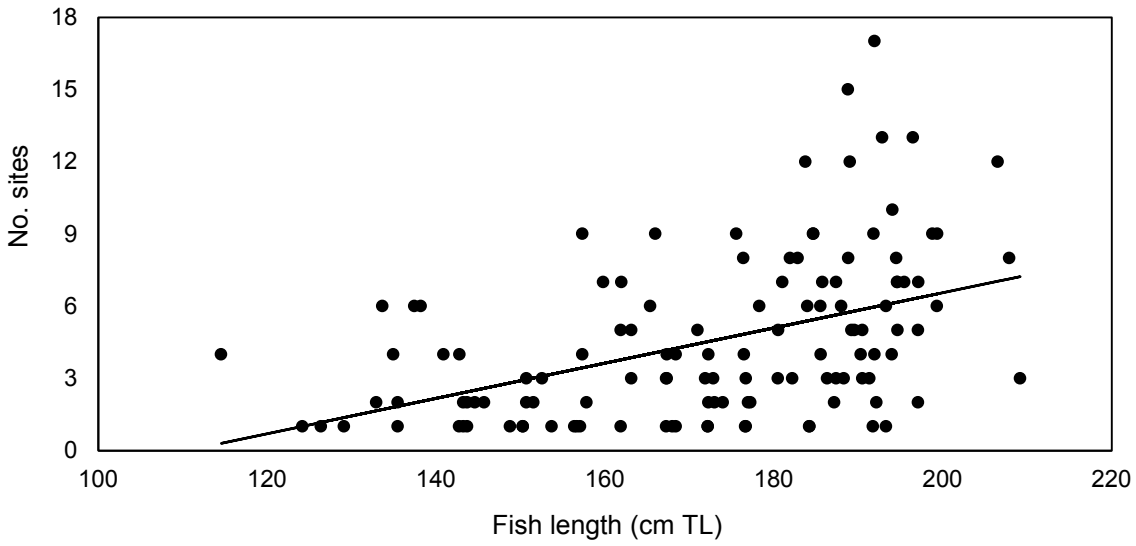


Figure 19. Number of sites where acoustically-tagged Goliath Groupers were detected plotted by fish length in cm TL. The slope of the linear regression line is significantly positive (number of sites = 0.073 * fish length – 8.07) and explained about 20% of the variance in the data ($R^2 = 0.207$).

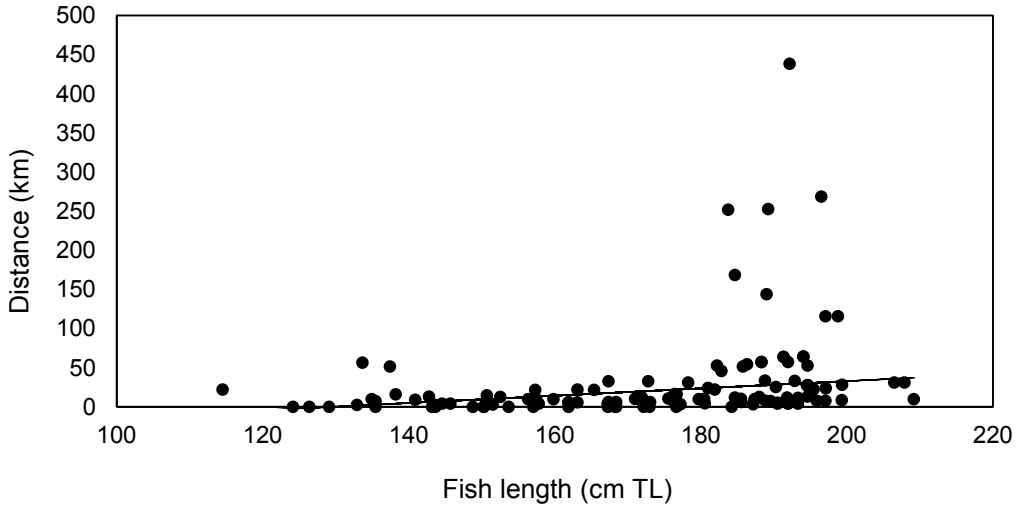


Figure 20. Maximum annual distance moved by acoustically-tagged Goliath Groupers plotted by fish length in cm TL. The slope of the linear regression line is significantly positive (distance = 0.47 * fish length – 60.3) and explained about 12% of the variance in the data ($R^2 = 0.121$).

We failed to find significant differences between females and males in terms of the number of sites visited annually (d.f. = 107, $t = 0.054$, $p = 0.957$; females = 4.58 ± 1.6 sites; males = 4.54 ± 1.7 sites), the number of sites visited over the entire study period (d.f. = 36, $t = 0.254$, $p = 0.801$; females = 9.05 ± 8.4 sites; males = 8.56 ± 8.1 sites), or the number of SPAG sites visited annually (d.f. = 93, $t = 0.668$, $p = 0.506$; females = 1.2 ± 0.08 sites; males = 1.3 ± 0.08 sites). However, we did find that females moved significantly farther than males: d.f. = 100, $t = 3.56$, $p < 0.001$; females = 29.9 ± 5.5 km; males = 10.5 ± 1.3 km. Movements by female Goliath Grouper were more variable (Figure 20), and accounted for all recorded movements greater than 50 km.

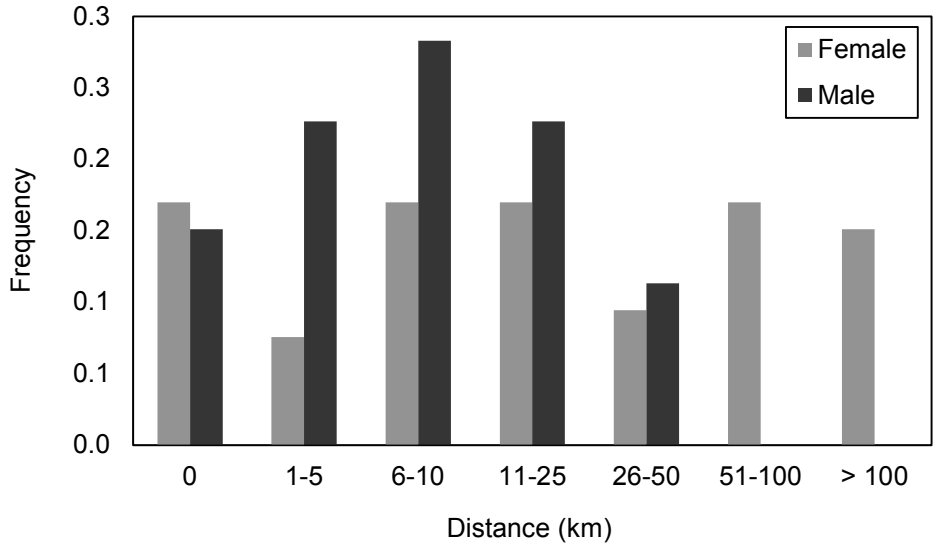


Figure 21. Frequency of maximum annual detected movements by female and male Goliath Groupers fitted with acoustic tags.

DISCUSSION

Based on the detection data collected from acoustically-tagged Goliath Groupers in this study, fish attending spawning aggregations located offshore of Martin and Palm Beach counties in southeastern Florida, appear to be derived from the entire Florida Atlantic coast north of the aggregation sites, including Georgia coastal waters.

To date we have no evidence that Goliath Groupers tagged on the Florida Atlantic coast move south from the aggregation sites to the Florida Keys or into the Gulf of Mexico. However, recent additions to the FACT array in those areas, combined with the extended battery life of the tags we used, which should continue to transmit through 2018 to 2020, may reveal that some spawning aggregation attendees are derived from home sites south of Palm Beach County.

The Goliath Groupers tagged for this study showed very high site fidelity to the spawning aggregation sites, with more than 80% of tagged fish at large returning to the spawning aggregation sites each year. Furthermore, Goliath Grouper appear to make a single migratory movement between home ranges and spawning aggregations, and remain on site at these aggregations long enough to spawn through at least two new moons. Based on the characteristics of fish spawning aggregations as defined by Domeier and Colin (1997), Goliath Grouper spawning aggregations are transitory: they peak at specific times during the year, last for days across multiple lunar cycles, are located well outside the home ranges of most individuals, and are derived from home sites spread over a large area requiring migrations lasting days to weeks. We observed a large variation in migration distance among the tagged fish, where some individuals appeared to use spawning sites as home ranges year-round at spawning aggregation sites, while other fish migrated long distances (> 100 km) between home ranges and spawning aggregation sites. Some fish appeared to use artificial reef sites located near the St. Lucie inlet (~ 30 km north of the spawning aggregation sites) as home ranges. These individuals made multiple trips to aggregation sites each spawning season that were timed to the new moon, returning to their home sites in between new moons.

The data suggest a strong association between Goliath Grouper reproductive behaviors and the lunar cycle. We detected increased movements by tagged fish that appeared to be triggered by the July full moon when fish became more active and moved more often between sites and into the aggregation area. Spawning is apparently centered on the new moon phase, as indicated by the high frequency of post-ovulatory follicles and hydrated oocytes found in ovarian biopsies collected during the new moons (relative to full moons) of August and September, and by increased night-time

sound production during these new moons (Koenig et al. 2016). Aggregation of tagged fish at specific spawning sites, specifically the ZT site, were evident from the detection data, further supporting the conclusion that peak spawning of Goliath Groupers occurs around the new moon.

Transmitter-tagged Goliath Grouper did not move very far or very often, except during migrations from home sites to spawning sites. Similar results have been reported previously by others (Koenig et al., 2011; Collins et al., 2015). The results from this study confirm this pattern of high home site fidelity and rapid long-distance migrations to spawning sites. These movement patterns have important implications for the management of this species: although the fishery remains under a complete harvest moratorium, a catch and release fishery has developed for adult Goliath Groupers in some parts of the state. Both recreational fishers and charter boats target Goliath Groupers for the experience of catching and landing a fish that can often exceed 300 lbs; photographs of landed adult Goliath Groupers are prevalent on social media, despite the illegality of such actions. Given the high site fidelity to home sites reported here, combined with relatively low densities of individuals at individual reefs (Koenig et al., 2011; Collins et al., 2015), likely means that individual Goliath Groupers are caught repeatedly by fishers who target specific sites. The consequences of frequent catch and release on fish health and reproductive resilience are unknown, as are reliable estimates of post-release mortality for Goliath Groupers.

The ability to estimate activity of Goliath Grouper, in terms of distance moved by tagged fish throughout the year, highlights the importance of using continuously monitored sites like those maintained by FACT group members. In addition to allowing us to describe migratory movements, nearly continuous monitoring of individuals that had monitored sites within their home ranges allowed us to estimate relative activity levels by summing all movements made during a given time period. This allowed us to graphically show movement behaviors related to spawning, both in terms of migrations to spawning sites before peak spawning and also increased movements between sites during the spawning period (see Figure 10). In February 2011 and again in March 2014 the cumulative average movements of tagged fish were

elevated over the group mean value during months when such movements would not be expected. It is possible that these elevated values represent fish moving in response to environmental variables such as those induced by cold-water upwelling events that occur seasonally in the study area (Smith 1983). However, despite deploying temperature loggers on all FSU-monitored sites starting in the fall of 2012, we cannot confirm this hypothesis. Elevated average movement values observed in March 2014 appear to be caused by two fish that moved between sites near the St. Lucie inlet and sites in the spawning aggregation area, but this result appears to be driven by sample size (only eight tagged fish were detected during March 2014) rather than environmental factors. The reasons why some Goliath Groupers moved frequently between nearby sites while others remained at single sites year round are unknown.

In general we found that female Goliath Groupers moved more than males. All of the repeated long distance (> 100 km) migrations between home ranges and spawning aggregation sites that we described were made by female fish. We also found that larger fish moved farther and visited more sites than did smaller fish. However, there was no significant difference in the size of female versus male fish that we tagged (female average length = 166.4 ± 4.4 cm; male average length = 162.3 ± 5.7 cm). To date the reproductive strategy of Goliath Groupers is as yet unresolved: while some evidence of protogynous hermaphroditism based on the collection of gonads from transitional individuals has been observed (Bullock and Smith 1991 and this study), there remains insufficient data to definitively conclude a single strategy (Sadovy and Eklund 1999). The movement patterns described here do not add any clarity to this issue (see Part I of this report on age, growth and reproduction).

We conducted the bulk of our tagging effort at a single spawning aggregation site: 30 of the 50 tags were implanted in fish captured at the Zion Train artificial reef. This was done primarily because it was the site of the largest aggregation of Goliath Groupers in the study area during the fall of 2010 based on diver observations and local knowledge. The ZT site had the largest aggregation of Goliath Groupers, again based on diver counts in 2010, 2011, 2013, and 2014; in 2012 the MG-111 wreck had the largest aggregation. Our estimates of inter-annual site fidelity to the general spawning

aggregation area were high, however this did not hold true for specific spawning sites. Most fish visited just a single spawning aggregation site each year, but not necessarily the same site where they were tagged. We found that the relative importance of spawning sites (in terms of the number of tagged fish detected at each site) changed from year to year, and that the site with the largest aggregation changed as well. With multiple aggregation sites in relatively close proximity, fish were not limited to a single "home" aggregation site. Similar patterns in the variability of specific spawning locations have been observed in other fish species that form spawning aggregations. For example, Nassau grouper in the Cayman Islands form annual spawning aggregations near the same reef promontory, however the exact location along the reef wall, as well as the size and shape of the aggregation, varies from year to year (Whaylen et al. 2006). The ability to vary the exact location of a spawning aggregation within a localized area may enable fish to react to favorable oceanographic conditions. Additional observations of the spatial variability of aggregations during spawning periods would be beneficial to determine the importance of multiple sites to reproductive success.

As the population of Goliath Groupers increases over time, individual fish may be forced to alter their movement patterns and behaviors in response to increased densities of fish at home and spawning sites. We did not detect any effect of year on the number of sites visited annually by Goliath Grouper; however, we did find that the number of spawning aggregations visited annually by tagged fish increased over time. This result may indicate such changes in behavior caused by increased densities of spawning individuals at aggregation sites. However, the variability in Goliath Grouper spawning aggregation size, as well as the spatial extent of aggregation sites along the Atlantic coast remains unknown. During the study the number of tagged Goliath Groupers detected in the spawning aggregation area declined over time, but these fish visited more sites each year. While we suspect that this pattern coincides with a similar increase in the total number of Goliath Grouper at the spawning aggregation sites, the variability in Goliath Grouper spawning aggregation size within our study area, as well as the spatial extent of aggregation sites along the Atlantic coast remain unknown. One of the main goals of this study was to determine the fidelity of individual fish to

spawning sites, and so we designed the study to focus on a fixed set of sites over time. Future studies should instead focus on the spatial distribution of spawning sites, which remains a critical knowledge gap for the management of the species. Night-time patterns of acoustic activity, especially during the new moons of August and September, may facilitate the location of spawning sites (Mann et al. 2009, Koenig et al. 2016, this study).

Finally, we did not test the detection range of the tags used in this study, but previous studies using similar tags reported maximum detection ranges between 250 to 750 m from the receiver (Humston et al., 2005, Kessel et al., 2015). Based on diver observations, Goliath Groupers tend to stay close to structure and well within the published detectable range of the transmitter tags. Furthermore, the tag detection range is much less than the distance between monitored sites, allowing us to assume that tagged individuals cannot be detected at multiple sites simultaneously. Kessel et al. (2015) also explicitly tested for and failed to find evidence of close proximity detection interference, CPDI or echoing of tag signals that can cause transmitters to be erroneously logged by receivers, at one of the spawning sites we monitored. The detection filter we used to eliminate false detections (at least two detections within 20 minutes) was designed to eliminate false detections that can occur due to code "collisions" and it follows the recommendations of the transmitter manufacturer. We observed a relatively low number of false detections (< 10%), further supporting the conclusions of Kessel et al. that CPDI is likely not a significant issue in our study area.

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Part IV. Non-fishing mortality (Robert Ellis and Claudia Friess, leaders)

Draft manuscript (below): Estimating survival of Goliath Grouper using conventional and acoustic tagging methods.

INTRODUCTION

As part of a broader investigation into the biology of Goliath Groupers, we captured and tagged Goliath Groupers using multiple methods in order to estimate survival and mortality and to detect patterns in individual fish movement with respect to spawning aggregations. For Goliath Grouper, the ongoing harvest moratorium presents a unique challenge to fishery managers because much of the data necessary for evaluating population size and associated benchmarks are fishery-dependent and thus not available for this population. Mark-recapture studies can be used to estimate population parameters such as survival, without sacrificing animals.

Various methods have been developed to estimate mortality rates of tagged animals in a mark-recapture format, depending on the population (e.g., open versus closed), study length (e.g., short, < 1 year, versus long, > 1 year), or even the type of tag used (e.g., uniquely identifying versus batch; Pine et al., 2003). The Goliath Grouper population found along the Florida Atlantic coast can be considered an open population, and thus the Jolly-Seber model is the most appropriate model to use to estimate mortality. The Jolly-Seber model makes the following assumptions: equal catchability; equal survival of tagged fish; no tag loss during the study; marked animals are released immediately following sampling; and sample periods have a short duration (Seber, 1982). Inherent in the assumption of equal catchability is that emigration from the study area is permanent, and there cannot be temporary migrations where the animal is present for some sampling periods but not others (Pine et al., 2003). This assumption is often violated unless alternate methods can be employed to understand the movements of tagged animals in relation to sampling events. Telemetry data can be used to collect complementary information about animal movements and can provide direct estimates of both natural mortality and relocation probabilities (Pollock et al., 2004). Recent studies that have combined mark-recapture and telemetry data have found that the addition of

acoustic telemetry information to traditional mark-recapture models results in reduced uncertainty of estimated parameters (Hightower and Pollock, 2013; Dudgeon et al., 2015).

MATERIALS & METHODS

Fish tagging

Starting in the fall of 2010, we captured fish using hook and line and tagged them with external and internal tags (see elsewhere in this report for more information on fish capture and sampling methodology). Initially, we tagged fish externally using dart tags inserted into the dorsal musculature immediately below the dorsal fin. In the fall of 2011, our second sampling season, we switched to pig-ear tags that were clipped into the posterior base of the anal fin. Also starting in the fall of 2011 we tagged all captured fish with a PIT (passive integrated transponder) tag injected into the dorsal musculature below the juncture of the spinous and soft dorsal fins. In addition, we removed two of the dorsal fin rays for use in aging individuals, which served as a non-specific tag that allowed us to identify recaptured individuals that had lost their external tag.

Some captured fish were also tagged with a coded acoustic transmitter tag (Vemco V16-6H) that was implanted into the abdominal cavity (n = 45) or attached externally by a diver (n = 5). These acoustic tags were set to produce a uniquely coded acoustic ping at 69 kHz randomly once every 60 to 180 seconds (nominal delay = 120 seconds) and had an expected battery life of 3033 days. Acoustically-tagged fish were tracked through a network of approximately 700 Vemco VR2W acoustic receivers located at sites along the Florida Atlantic coast. Detection information was collected and analyzed to determine the identity and location of acoustically-tagged fish over time.

Data analysis

We analyzed two data sets: one that included all mark-recapture information from the conventionally tagged fish and the other that included the telemetry data from fish

tagged with acoustic tags. We fit a Jolly-Seber model (Jolly 1965; Seber 1965) to both the Goliath Grouper mark-recapture (MR) and telemetry data in order to estimate annual survival rate S_t and recapture probabilities p_t for both data sets, tag retention rate T_t for the MR data, and detection probabilities d_t for the telemetry data. Individual capture-recapture (MR data) and capture-detection (telemetry) events during a given year i were pooled to generate total numbers of animals marked with conventional tags $M_{c,i}$ and acoustic tags $M_{a,i}$ for each sampling year as well as total number of animals recaptured or detected in each sampling year. Observed MR recaptures were separated into those individuals that had retained their tag between tagging year i and recapture year t, $r_{i,t}$, and those that were tagged during unknown tagging year i and had lost their tag by recapture year t, u_t. Tag loss recaptures were identifiable as having been previously tagged through permanent marks in the form of fin ray clips that were unique to the present study. Survival rates and tag retention and detection probabilities were estimated for each of the mark-recapture and telemetry data sets separately and then for both data sets combined.

Mark-recapture data analysis

The total number of tags from sampling period i that were present in the population during sampling event t was assumed to decrease in proportion to the survival rate S_t and tag retention rate T_t from time i to t:

$$M_{c,i,t} = S_t T_t M_{c,i}$$

Survival and tag retention parameters were fit to the number of observed marked recaptures, which included both individuals that retained and individuals that had lost their tags. Observation error was assumed to be Poisson distributed. The Poisson model for the probability of the observed data is:

$$P\left(r_{i,t}, u_t \middle| S_t, T_t\right) \propto e^{-p_t R_{i,t}} (p_t R_{i,t})^{r_{i,t}} e^{-p_t U_t} (p_t U_t)^{u_t},$$

where $R_{i,t}$ is the predicted number of recaptures of animals which retained their uniquely identifiable tag, marked during year i and recaptured during year t; U_t is the

predicted number of lost tag recaptures during sampling year t; and p_t is the capture probability. $R_{i,t}$ was calculated as:

$$R_{i,t} = S_{t-1}T_{t-1}p_tM_i.$$

Because marking occasion i is unknown for recaptured individuals that had lost their tag, the predicted number of these lost tag recaptures during sampling year t was calculated across all i as:

$$U_t = p_t \sum_i (S_t(1 - T_t)M_i),$$

where $(1–T_t)$ represents the proportion of individuals with lost tags between time i and t. We assumed that capture probability and survival did not differ between individuals that had retained their tags and those that had not.

The maximum likelihood estimate (MLE) of capture probability p_t was calculated as the ratio of the total number of observed marked individuals to the total number of predicted marked individuals:

$$p_t = \frac{\sum_i r_{i,t} + u_t}{\sum_i R_{i,t} + U_t}$$

The log likelihood function evaluated at the MLE estimate of p_t is:

$$\ln(L) \propto -R_{i,t} + r_{i,t}\ln(R_t) - U_t + u_t\ln(U_t)$$

Telemetry data analysis

The model for the telemetry data set was similar to the mark-recapture model, except that tag loss was not included; we assumed that because the majority of the acoustic tags were implanted internally and all were made with 8-year batteries that should last well beyond the duration of the present study, tag loss for these fish was not possible in the same way that conventional tags could be lost. Thus the number of predicted tags in the population was a function only of annual survival:

$$M_{a,i,t} = S_tM_{a,i}$$

The probability model for the observed data was:

$$P(l_{i,t}|S_t) \propto e^{-d_t L_{i,t}} (d_t L_{i,t})^{l_{i,t}},$$

where $l_{i,t}$ is the number of tagged fish detected in year t, and $L_{i,t}$ is the predicted number of animals with acoustic tag in the population. $L_{i,t}$ was calculated as:

$$L_{i,t} = S_{t-1} d_t M_{a,i}.$$

The detection probability here again is the ratio of number of observed to the ratio of predicted tagged individuals in the population:

$$d_t = \frac{\sum_i l_{i,t}}{\sum_i L_{i,t}}$$

The log likelihood function evaluated at the MLE estimate of d_t is:

$$\ln(L) \propto -L_{i,t} + d_{i,t} \ln(L_t)$$

Combined data analysis

For the combined data sets, the model variables and parameters were estimated as above, with the same estimate of S_t used to calculate predicted numbers of both conventional and acoustic tags in the population. The combined likelihood function is:

$$\ln(L) \propto -R_{i,t} + r_{i,t} \ln(R_t) - U_t + u_t \ln(U_t) - L_{i,t} + d_{i,t} \ln(L_t)$$

A non-linear search procedure was used to compute the MLE estimates of tag retention and annual survival. Parameters were estimated under both time-varying and time-invariant survival and tag-loss rates. For the time-invariant case, the joint likelihood profile for S and T was constructed using the likelihood ratio test (Sokal and Rolf, 1981) to evaluate uncertainty: two times the difference between the log of an estimate and the

log of the most likely estimate, known as the G statistic, is χ^2 distributed with 2 degrees of freedom for two estimated parameters. The negative log likelihood values were computed across a range of possible S and T values to estimate confidence bounds.

RESULTS

From 2010 to 2015 we captured and conventionally tagged a total of 700 Goliath Groupers, 151 of which were recaptured individuals (22.1%). We did not include individuals that were recaptured during the same year they were tagged in survival analysis, which limited our recapture data set to 124 individuals. Of the individuals that were recaptured during subsequent years, 86 had retained their tag (69.4%), and 38 recaptured individuals had lost their tag (30.6%). In some cases, the same individual was recaptured multiple times throughout the study period; when this occurred, it was listed as a recapture multiple times but only accounted for one marked individual. The number of marked and recaptured individuals varied over time (Table 1).

We also acoustically-tagged 50 Goliath Groupers across three main sampling events: fall 2010, 38 tags; fall 2012, 5 tags; and fall 2013, 5 tags externally attached. Two more acoustic tags were acoustically implanted in fish during the spring of 2011. For the telemetry data set, again the number of detected individuals (analogous to the recapture of a marked individual) varied across years of the study but generally declined over time (Table 2). We monitored spawning sites with acoustic receivers from the initiation of sampling in 2010 through July 2015, and so limited our survival analysis of acoustically-tagged fish from 2010 to 2014.

Table 1. Observed recaptures of conventionally-tagged Goliath Groupers that retained their unique tag (r), individuals that had lost their tag (u), and total tags released (M_c) for each year (i / t) from 2010 to 2014.

i / t	2011	2012	2013	2014	2015	$M_{c,i}$
2010	12	3	2	0	0	74

2011		18	7	2	1	133
2012			13	7	7	222
2013				6	3	86
2014					5	88
r_t	12	21	22	15	16	
u_t	5	9	10	7	7	

Table 2. Observed detections of acoustically-tagged Goliath Grouper (l), and the total number of acoustic tags implanted (M_a) for each year (i / t) from 2010 to 2014.

i / t	2011	2012	2013	2014	$M_{a,i}$
2010	37	33	25	8	38
2011		2	2	1	2
2012			3	3	5
2013				5	5
l_t	37	35	30	17	

Survival estimates from telemetry data

Annual survival rate estimates from the telemetry data were strongly dependent on starting values of survival, which were initially set equal for all years. This was true across a range of starting values (Table 3). Survival rates for the first two years, 2010 and 2011, were consistently estimated at or above 0.9, survival in the third year was consistently estimated at 0.70, while the survival estimates for the third and fourth year were essentially equal to the starting values. When we estimated a single survival rate value for all years, the maximum likelihood estimate was 0.89, and the 95% confidence region included values from 0.8 to 1 (Figure 1).

Table 3. Survival rate estimates from telemetry data of acoustically-tagged Goliath Groupers for various starting parameter values when survival rate was allowed to vary across years.

Starting value	2010	2011	2012	2013	2014
0.90	0.96	0.89	0.70	0.90	0.90
0.70	0.96	0.90	0.70	0.71	0.70
0.50	0.99	0.87	0.70	0.58	0.50

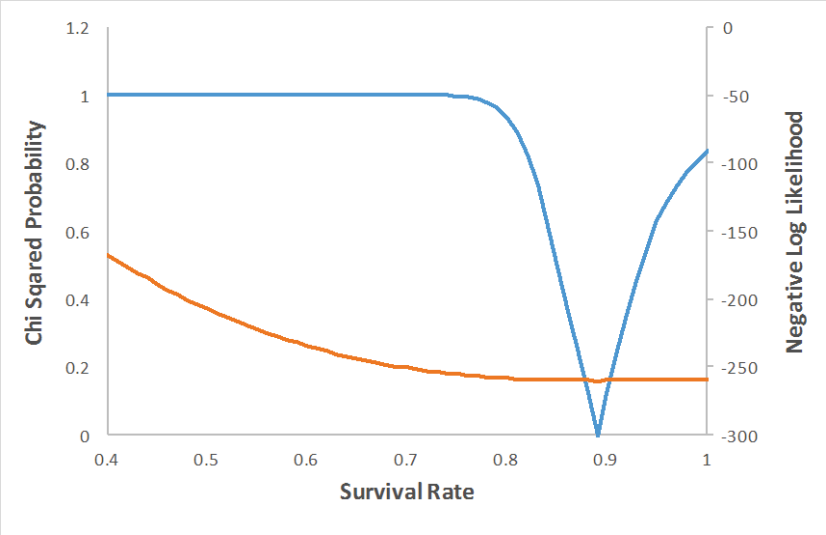

Figure 1. Likelihood profile for time-invariant survival rate estimated from telemetry data of acoustically tagged Goliath Groupers.

Mark-recapture survival estimates

For the MR data, when survival was allowed to vary across years, unconstrained searches resulted in annual survival rate estimates above 1 in some years. Therefore, we constrained survival to ≤ 0.96 for the MR data based on the results from the survival rate estimates generated from the telemetry data. For the constrained estimation, annual survival rate estimates ranged from 0.45 in 2010 to 0.96 in 2013 (Table 4). Tag retention rates varied between 0.58 and 0.71. For the constrained run, the MLE estimates of annual capture probability varied between 0.57 in 2010 and a low of 0.13 in 2014. We also

constrained annual recapture probability to be ≤ 0.3, which resulted in increased survival in 2010 (Table 4). The negative log likelihoods differences between the three runs were rather small: unconstrained estimation = -136.0; constrained survival, unconstrained recapture = -135.8; both survival and recapture probabilities constrained = -135.0.

Table 4. Annual survival and tag retention estimates for the mark-recapture data set under unconstrained parameter estimation, with annual survival constrained ≤ 0.96, and with survival constrained ≤ 0.96 and annual probability of recapture also constrained ≤ 0.3. The MLE estimates of recapture probabilities are also shown.

	2010	2011	2012	2013	2014	2015
Unconstrained estimation						
Survival rate	0.45	0.95	0.70	1.49	0.85	
Tag retention rate	0.71	0.68	0.64	0.58	0.55	
Recapture probability		0.57	0.23	0.17	0.09	0.13
$S_t \leq 0.96$						
Survival rate	0.45	0.95	0.77	0.96	0.85	
Tag retention rate	0.71	0.68	0.64	0.58	0.63	
Recapture probability		0.57	0.23	0.15	0.13	0.16
$S_t \leq 0.96$ & $p_t \leq 0.3$						
Survival rate	0.85	0.86	0.77	0.96	0.80	
Tag retention rate	0.65	0.68	0.64	0.58	0.63	
Recapture probability		0.30	0.23	0.15	0.13	0.17

When both survival, *S*, and tag retention, *T*, were assumed to be constant across years, the MLE estimate of survival rate was 0.80 and the tag retention rate was 0.64. The

95% confidence level for survival rate ranged from 0.53 to 1, and the tag retention rate ranged from 0.56 to 0.73 (Figure 2).

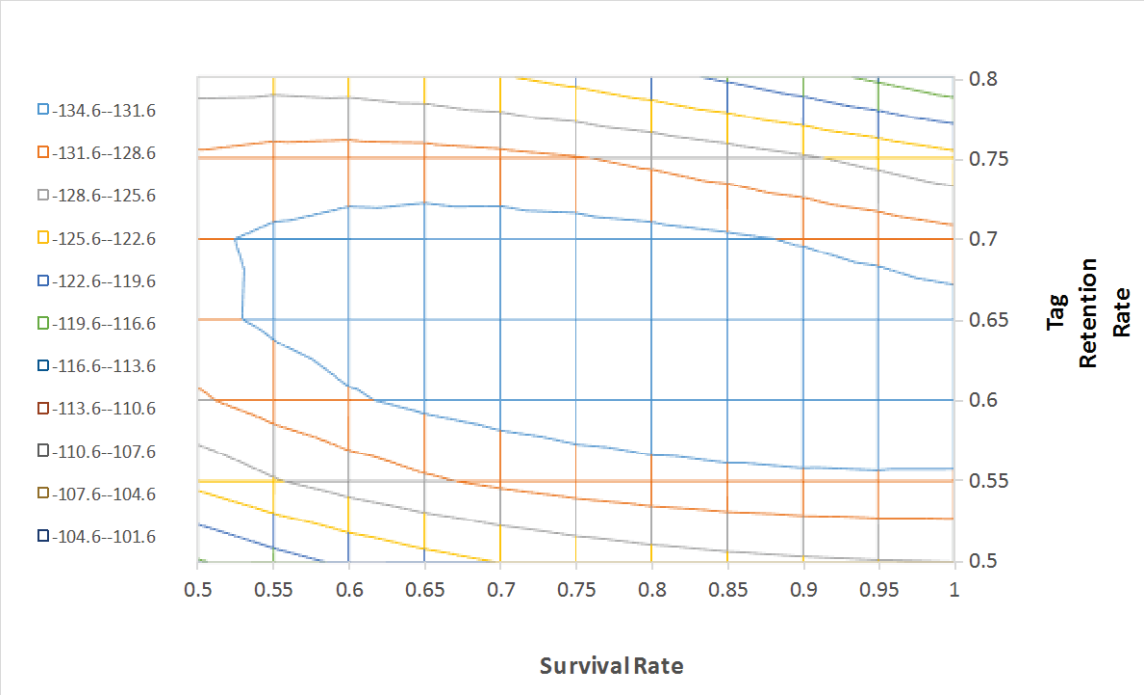

Figure 2. Approximate 95% confidence region for the joint likelihood profile for survival rate and tag retention rate for the mark-recapture data set.

Survival estimates from combined data

An unconstrained search on annual survival estimates using both the MR and telemetry data again resulted in survival estimates above 1 for some years. We therefore constrained annual survival to ≤ 0.96 as before, which resulted in survival rate estimates between 0.72 and 0.96 and tag retention rate estimates between 0.58 and 0.67 (Table 5). Recapture probabilities for hook and line sampling ranged from 0.13 to 0.29. Estimates of detection probabilities for acoustically tagged fish were close to 1 during the first three years and then declined sharply to 0.54 in the fourth year. The differences in the negative

log-likelihood between runs were, again, very small: unconstrained survival = -395.4; survival constrained to ≤ 0.96 = -395.3.

Table 5. Annual survival and tag retention estimates for the combined mark-recapture and telemetry data set for the unconstrained survival runs, and for runs with survival constrained to ≤ 0.96. Also shown are the MLE estimates of recapture (MR) and detection (telemetry) probabilities for both constrained and unconstrained survival runs.

	2010	2011	2012	2013	2014
Unconstrained Estimates					
Survival Rate	0.87	0.97	0.71	1.42	0.88
Tag Retention Rate	0.64	0.67	0.64	0.58	0.56
Recapture Probability*		0.29	0.21	0.16	0.09
Detection Probability**		0.999	0.999	0.999	0.370
$S_t \leq 0.96$					
Survival Rate	0.87	0.96	0.72	0.96	0.88
Tag Retention Rate	0.64	0.67	0.64	0.58	0.64
Recapture Probability*		0.29	0.21	0.16	0.13
Detection Probability**		0.999	0.999	0.999	0.541

When single time-invariant survival and tag retention rates were estimated, the maximum likelihood estimates were 0.88 for survival rate and 0.63 for tag retention rate. The 95% confidence region for survival rate ranged from 0.78 to 1, and for tag retention rate ranged from 0.56 to 0.70. Compared to the MR data alone, the MLE estimate of survival is higher for the combined data and the uncertainty in the estimate is lower.

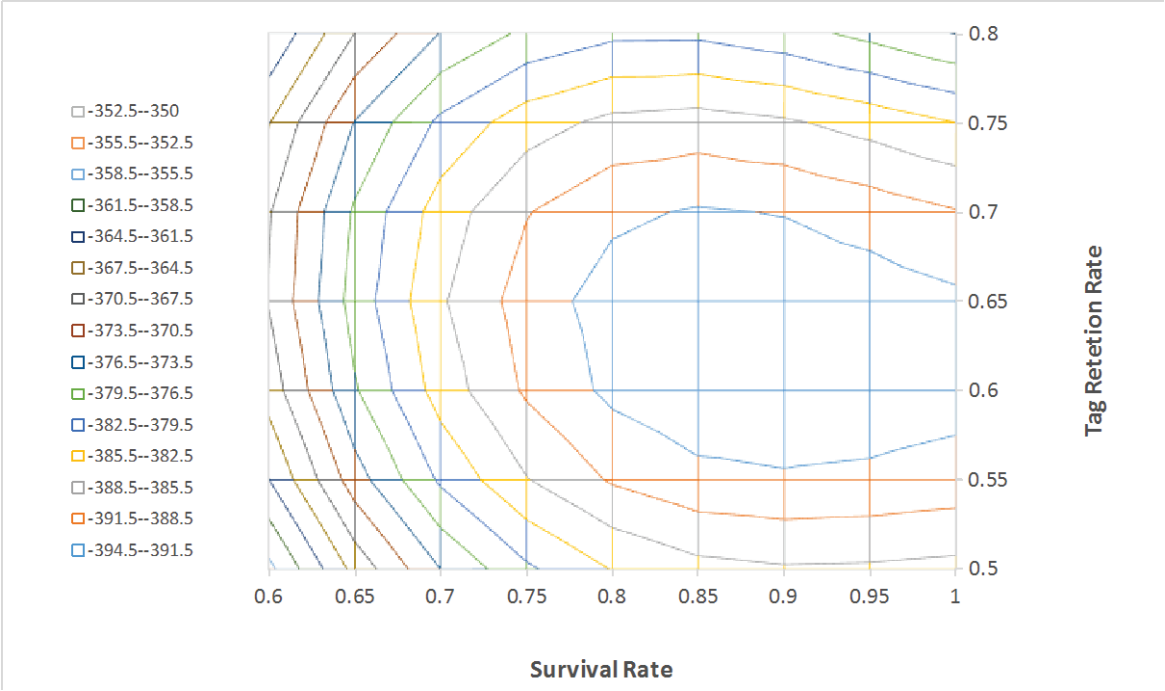

Figure 3. Approximate 95% confidence region for the joint likelihood profile for survival rate and tag retention rate estimated using the combined mark-recapture and telemetry data.

DISCUSSION

By combining data collected from conventional mark-recapture and acoustic telemetry based studies of Goliath Grouper, we found that survival rates were generally high (> 0.8) and that combining the two data sets reduced the uncertainty around the estimated survival rate for Goliath Grouper, as predicted by others (Pollock et al., 2004). Estimated survival rates generated from the MR data alone were lower compared to the telemetry and combined data estimates of survival rates. The survival estimates generated from this study are comparable to estimates of natural mortality used in recent stock assessments for Goliath Grouper. The most recent Goliath Grouper stock assessment used an average instantaneous natural mortality rate of 0.18, which corresponds to an annual survival rate = 0.835, while the previous stock assessment used an average instantaneous natural mortality rate of 0.12, which corresponds to an annual survival rate = 0.88

(SEDAR, 2016). Goliath Grouper are currently under a complete harvest moratorium, so it can be assumed that fishing mortality (F) effectively equals zero and that the only remaining source of mortality (Z) is natural mortality (M). Based on the MR analysis, the stock assessment estimates likely underestimate total mortality, while the telemetry alone and combined analyses are in line with the stock assessment estimates.

Tag loss can bias estimates of population size and mortality that are derived from the Jolly-Seber method (Cowen and Schwarz 2006). Here we used a model that accounted for conventional tag loss to avoid such biases when estimating survival rates for Goliath Grouper. In order to construct a model that would accept both data sets, the combined model also estimated detection probability for the telemetry data. The analyses showed that the detection probability for acoustically tagged fish dropped off dramatically during the last year of the study. In the analysis using telemetry data alone, this was reflected in the final year estimates equal to the initial starting estimate. That is, the loss of information was treated as mortality, and the starting condition worked as a bound for this estimate. However, for the combined data analysis, the information from the MR data supported a higher overall survival rate and the loss of information instead reduced the detection probability. Possible reasons for the low detection probability in the final year of the study could be transmitter failure, fish moving permanently out of the study area, or mortality. Additional telemetry data from 2015 or beyond may resolve this uncertainty. Alternative modeling frameworks (i.e. Bayesian hierarchical modeling) may also be able to more fully capture the information that the telemetry data includes regarding animal movement in relation to sampling events. We are continuing to work on both of these avenues by continuing to collect telemetry data from cooperative research partners and exploring additional modeling methods to improve survival rate estimates. As such, the estimates presented here should be considered preliminary.

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Part V. Diet and the dynamics of heavy metal contamination (Christopher Malinowski, leader.)

Draft manuscript: Patterns, Dietary Sources, and Effects of Mercury in Atlantic Goliath Grouper (*Epinephelus itajara*).

OVERVIEW:

The main goal of this project was to provide critical information for stock assessment on the dietary sources and effects of toxic mercury (Hg) on the health, reproductive success, and survival of Atlantic Goliath Grouper (*Epinephelus itajara*) (hereinafter referred to as "Goliath Grouper"). Goliath Grouper was an ideal model species for examining population dynamics, the dynamics of heavy metal bioaccumulation, and the physiological effects of Hg contamination on reef fishes because: (1) they are large (total length (TL)/mass ~3 m/400 kg), long-lived (age = 37+), and relatively easy to sample non-destructively; (2) they are fully protected in the southeastern U.S., making it plausible to estimate mortality rates for different sizes/ages, thus obtaining much needed information to support stock assessment; and (3) high Hg concentrations exist in all tissues (Evers et al. 2009, Adams and Sonne 2013, our unpublished data). In addition, there is considerable ecological information already available (Koenig et al. 2007, Koenig and Coleman 2009, Mann et al. 2009, Murie et al. 2009, Koenig et al. 2011, Ellis et al. 2014, Koenig et al. 2016) that provides details of adult habitat association, essential juvenile habitat, regional abundance, site density, survival, age and size structure, home range, recruitment, spawning, the mating system, migrations, and movement patterns.

INTRODUCTION:

Figure 1. Coastal regions of Florida used as sampling sites for this study (Koenig & Coleman 2013).

This work was conducted from 2010-2016 throughout the coastal waters of Florida in eight regions used by us in previous studies (Koenig and Coleman 2013). Within these regions, we targeted juvenile mangrove habitat and adult resident and spawning habitat. We used non-lethal capture and sampling methods developed during nearly 30 years of research on this species (with near 100% survival) to obtain stomach content and tissues for a suite of analyses. A considerable benefit of this approach was that the same individuals could be recaptured and resampled repeatedly over time, providing far greater information on changes associated with growth and aging than does one time destructive sampling.

Tissues sampled from live fish[1] included muscle, liver, and blood for Hg analysis, fin-rays for age, gonads for reproductive state, and blood plasma for health. To investigate the proximate source of Hg in Goliath Grouper, we used prey collected from their stomach contents (unique to Goliath Grouper, this includes whole, undigested prey) to determine which prey items contribute most to Hg levels measured in tissues.

Objectives:

(1) Demographic (size, sex) and temporal (year, season) diet patterns throughout all regions of this study (**Fig.1**).
(2) Dietary sources of mercury (Hg), including methylated mercury (MMHg) and inorganic mercury (iHg), by analyzing Hg concentration in Goliath Grouper prey found in stomach contents.

[1] Obtaining samples from live fish rather than dead fish avoids problems associated with tissue degradation and contamination.

(3) Demographic (size, sex) and temporal (year, month) patterns in Hg load and effects on health, reproductive success, and survival. We also investigated the capability of these long-lived fish to metabolize or shed significant amounts of Hg (using Hg concentration & isotopes) through various heavy metal offloading routes, including liver, blood, eggs, sperm, urine, and feces.

Coastal fish populations face unprecedented threats to their health and sustainability, and many have experienced substantial declines in recent decades (Hutchings and Reynolds 2004, Worm et al. 2006, Duffy 2009, Worm et al. 2009). Along with overfishing and habitat destruction, elevated levels of industrial contaminants are at the epicenter of this issue. Of particular concern is mercury (Hg), the levels of which have tripled in the upper ocean since the beginning of the Industrial Revolution, primarily through the burning of fossil fuels and mining (Lamborg et al. 2014).

Mercury has been used as an indicator of environmental contamination (Kružíková et al. 2013), with methylmercury (MMHg) generally considered the most toxic form. Methylmercury bioaccumulates and biomagnifies through the food chain, and therefore reaches its highest levels in older, larger, and higher trophic level (*e.g.*, piscivores) fishes (Folmar 1993, Heath 1995, Morel et al. 1998, Atli and Canli 2007). The most vulnerable species are often the most popular fishes targeted by commercial and recreational fishers. For large, long-lived fishes, like Goliath Grouper, high Hg levels can result in severe tissue damage, neurological impairment, reduced growth and development, reduced mobility, starvation, disrupted blood chemistry (e.g., immune system function), reduced offspring viability, and increased mortality rates (Bache et al. 1971, Kidd et al. 1995).

In humans and most animals, Hg is obtained primarily through dietary avenues, and seafood is the main source of Hg in the human diet (Organization 1990, Bank et al. 2007). High levels of exposure can cause a variety of neurological impairments, diseases, and developmental problems. Consequently, understanding the health risks associated with human consumption and global contamination of marine and aquatic systems

requires research into the factors affecting Hg accumulation in fishes (Trudel and Rasmussen 2006). Recent reports indicate that Hg concentrations for many fish species — including Goliath Grouper (Adams and Sonne 2013, our unpublished data) and other groupers (*e.g.*, Red Grouper) (Tremain and Adams 2012, Thera and Rumbold 2014) — exceed the human health advisory levels set by governmental and proposed by non-governmental organizations (EPA=0.3 ppm, NRDC=0.5 ppm) and legal action limits by the FDA (Hg=1.0 ppm) (USDA-USDHS 2010, Karimi et al. 2012, NRDC 2015, USFDA 2015).

Although fish can acquire Hg across their gills through the process of bioconcentration (uptake from surrounding water), dietary uptake accounts for more than 90% of MeHg in tissues (Gray 2002; Zillioux et al. 2015). This dietary connection is important because Hg has a tendency to biomagnify in the food web — with organisms at higher trophic levels having the highest Hg concentrations (Scheuhammer et al. 2007) — and investigation of the diet of predators and Hg levels of the prey community provides critical detail on the proximate sources of Hg to the predator. Diet differences often occur intraspecifically, as well as interspecifically, and can lead to variation in Hg exposure. These differences can be attributed to various factors including age-, size-, and sex-related morphological and physiological variations (Hoffman 1983; Collins 2014; McCormick 1998). In marine animals, diet shifts have been shown ontogenetically and between male and female conspecifics (Beck et al. 2007; Koen et al. 2002; Scharf et al. 2000). Size-dependent diet differences, including ontogenetically-mediated differences, were hypothesized for Goliath Grouper based on stage-specific patterns of habitat use: juveniles (up to age 6, total length (TL) < 110 cm) in inshore mangrove habitats and adults (age > 6 yr, TL >110 cm) on offshore reef habitat (see Koenig et al. 2007, Evers et al. 2009, Koenig et al. 2011).

The sectors of the fisheries that are affected— The concern about Hg contamination in Goliath Grouper is compounded by the state of its management. The Goliath Grouper fishery closed in the US in 1990 when the National Marine Fisheries Service (NMFS) declared the populations overfished. Despite 26 years of closure and recent signs of some recovery in south Florida, the actual status of the adult population

remains unknown (NMFS 2014), making it impossible for NMFS to develop management measures aimed at rebuilding the fishery, as required by the Sustainable Fisheries Act (SFA).

This conundrum has generated considerable public interest, with many fishers calling for reopening the fishery at some level, while non-governmental organizations (including Environmental Defense, Gulf Restoration Network, the Ocean Conservancy) and diving interests (PADI, recreational non-consumptive divers, and Reefkeeper International) request that NMFS adhere to the SFA mandate. The question arising from the new mercury data is whether a fishery should exist for a species whose tissues are considered too toxic to consume.

There is considerable recreational catch-and-release fishing for Goliath Grouper and growing interest in retained catch for science and for consumption (Lorenzen et al. 2010). It behooves us to mention another, relatively unstudied user group having an economic interest in the living marine resource of the United States: that is, the diving community. Interest in underwater viewing of unexploited marine populations is on the rise (Williams and Polunin 2000, Harrington et al. 2009, Shideler et al. 2015, Shideler and Pierce 2016), with a specific interest in Goliath Grouper, as indicated by diving excursions in south Florida.

MATERIALS AND METHODS (for each objective):

Objective 1: To characterize the demographic (size, sex) and temporal (year, month) diet patterns throughout all regions of this study (Figure 1).

To characterize the diet of adult Goliath Grouper, we sample stomach contents of live fish from the 8 regions of this study (Figure 1) during spawning and non-spawning seasons to document temporal variation in prey species. Although we recognize that stomach content analysis provides only an instantaneous picture of diet, we collected and analyzed 993 samples from 408 individual fish over the course of 7 years (2010-2016) to provide a comprehensive diet analysis for this species.

Capture methods - We have developed low-impact methods of capturing Goliath Grouper at depth and transferring them safely onto a research vessel for non-lethal tagging and tissue sampling. We capture Goliath Grouper using 20/0 circle hooks, 600-1000 lb monofilament leader, a 2 kg lead weight, and cut or live bait. The gear is attached to a 1.0 cm diam. braided nylon hand line and suspended above the bottom by a 60-cm diameter float. The fish are allowed to fight until exhaustion (about 3 to 5 minutes) before being hauled to the surface. We vent captured fish at the surface if caught at depths <25 m, or *in situ* at ~10 m if caught at depths >25 m. When fish reach the surface, they are placed on a stretcher and hoisted above the gunwale with two davits. The fish's gills are bathed with seawater by a hose attached to an overboard submersible 12 VDC pump; the eyes are covered to protect them from direct sunlight while the fish is held in place with Velcro straps for sample collection (**Fig. 2**).

Figure 2. Goliath Grouper *Epinephelus itajara* lifted from water on a stretcher. The fish's eyes are protected from direct sunlight and a gill irrigation hose is in its mouth.

Collection and analysis of stomach contents - Stomach contents of Goliath Grouper are obtained in two ways (1) by inserting a 15-cm diameter stainless steel tube into the grouper's mouth to hold the jaws open, and reaching down the esophagus to the stomach with a gloved hand to retrieve gut contents, or (2) using lavage (small fish), which entails pumping seawater at low pressure into the captured fish's stomach, and then collecting the stomach contents expelled with the water. The stomach contents are bagged and put on ice until returned to the laboratory where they are frozen for preservation. In the laboratory, the contents are thawed, blotted dry, weighed, measured, identified to the lowest possible taxon, and enumerated. Whole intact prey

items—often collected from Goliath Grouper stomachs—were used for Hg analysis (Objective 2) to investigate which prey serve as sources of Hg contamination.

Fin ray removal and aging methods: Goliath Grouper were aged in this study using fin rays (non-lethal) rather than otoliths (lethal), based on validation studies by Murie et al. (2009). We sampled both juveniles and adults[2], removing from each two dorsal fin rays (#s 6 and 7) at their point of articulation with pterygiophores (fin supports). Fin rays were placed in a plastic Whirl-Pak bag and placed on ice until returning to the laboratory. In the laboratory, fin rays were cleaned of any tissue or fat and allowed to air-dry. Dried fin rays were epoxied in resin, thin-sectioned (~0.5-0.8 mm thick) and mounted on glass slides. Fin ray sections were examined using a compound microscope with a green-filter to enhance contrast.

Tissue sampling (collected for components of Objectives 2 and 3). We used non-lethal techniques developed in our lab to collect various tissues (muscle, liver, blood, eggs and sperm), and excretions as potential heavy metal offloading routes (urine, feces). A small (<2 g) biopsy of liver was extracted through insertion of a biopsy tool at the base of the pectoral fin. Muscle tissue (~ 2 g) was obtained near the base of the excised fin ray. Gonad biopsies from live fish were collected using a hand-operated vacuum pump (see Koenig and Coleman 2009). Gonad tissue was separated into 10% formalin for histological preparation and frozen for heavy metal analyses. Urine and feces were collected by applying ventral pressure to the fish, near the urogenital and anal opening, and opportunistically collecting with a sterilized vial. Blood was collected via caudal venous puncture using a heparinized needle. Muscle, liver, and blood were frozen for Hg stable isotope and heavy metal analyses. Urine and feces were frozen for heavy metal analysis. Blood was additionally centrifuged to separate plasma and red blood cells, which were then frozen. Red blood cells and whole blood were used to measure concentrations of Hg. Plasma was frozen until it was analyzed for various health parameters.

[2] Other tissues collected from each fish include egg biopsies and sperm, stomach contents, blood, liver and muscle biopsies (Objective 3).

Objective 2: To characterize dietary sources of mercury (Hg), including methylated mercury (MMHg) and inorganic mercury (iHg), by analyzing Hg concentration in Goliath Grouper prey found in stomach contents.

Stomach content processing— Sample processing was conducted at the Florida State University Coastal and Marine Laboratory (FSUCML). Whole, undigested prey from stomach contents of individual fish were selected for Hg analysis (see Figure 5 for image of collected prey). The Hg concentrations were used to identify Hg contamination sources (which prey contained highest amounts of Hg). These selected stomach contents were thawed and identified to the lowest taxonomic level, then weighed (blotted wet weight), measured, and photographed. Fish were measured to total length and invertebrates by carapace length and width. Identified prey items were homogenized using a food processor. A subsample of the homogenate was stored at -20 C for later Hg analysis (**see Objective 3 for Hg analytical methods**). To prevent contamination between prey items, all materials were washed, rinsed three times with deionized water, and wiped off with methanol.

Objective 3: To investigate demographic (size, sex) and temporal (year, month) patterns in Hg load and effects on health, reproductive success, and survival. We also investigated the capability of these long-lived fish to metabolize or shed significant amounts of Hg (using Hg concentration & isotopes) through various heavy metal offloading routes, including liver, blood, eggs, sperm, urine, and feces.

Analysis of Hg species concentration and Hg isotopic composition — Analyses and sample preparation were carried out at the National High Magnetic Field Laboratory based at FSU. For measuring Hg species concentration, a mass of 0.1-0.2g of freeze-dried tissue was digested with 5mL of ultra-pure (distilled at the laboratory) 6M HNO_3 in an oven at 70°C for 6 hours. Samples were then centrifuged and the supernatant was recovered. Hg species concentrations were measured using Tekran®2700 Automated Methyl Mercury Analysis System following aqueous phase derivatization and detection via cold vapor atomic fluorescence spectrometry (CVAFS). Depending on the sample, from 0.5 to 30 ng/L of MMHg and iHg were derivatized. Sample were calibrated against

vials of known MMHg and iHg concentrations following the same protocol as samples. Certified reference materials for MMHg and total Hg were measured periodically between samples to ensure the accuracy of analysis. Duplicates of extractions, duplicates of derivatization, as well as samples spiked with MMHg and iHg standards, were analyzed periodically to ensure the robustness of the method. The precision error of the method, as relative standard deviation, was lower than 5%. Hg species concentrations were reported as μg Hg/g muscle on a dry weight basis.

For measuring Hg isotope ratios (IRs), a mass of ~0.2g dry tissue was digested overnight with ultra-pure concentrated HNO_3 (4mL) and HCL (1mL). Then 4 mL of deionized water was added and vials were heated at 80°C on a hot plate for 4h (1.5h of ramp and 2.5h of heating time). After that, 0.8 mL of BrCl were then added to ensure complete oxidation of Hg to Hg(II). An aliquot of the supernatant was pipetted and diluted to reach 2 ng/mL in 5% acid (HNO_3, HCl, BrCl) in a total volume of 10 mL, assuming total Hg concentration of a fish sample to be the sum of MMHg and iHg concentrations measured by Tekran® 2700. Just prior to analysis 0.4mL of 0.72M hydroxylamine (NH_2-HCl) was added to the sample to remove the excess of BrCl. For Hg IRs analysis, the sample was introduced in a multi-collector ICP-MS (ThermoFinningan® Neptune) using a cold vapor generator (CETAC® HGX-200) as an introduction system. The bracketing standard method was used to report the per mil (‰) deviation of the samples versus Hg international standard NIST 3133. The isotopic composition of the sample was reported as delta values (δ) for 5 Hg isotopes (199, 200, 201, 202, 204) versus isotope 198 (Blum and Bergquist 2007):

$$\delta^{xxx}Hg = (((^{xxx}Hg / {}^{198}Hg)_{sample} / (^{xxx}Hg / {}^{198}Hg)_{NIST3133}) - 1) \times 1000 \quad (1)$$

$\delta^{202}Hg$ is typically used as the signature of the Mass Dependent Fractionation (MDF) of Hg isotopes in fish tissues. To report the Mass Independent Fractionation (MIF) of Hg isotopes, capital delta values (Δ) represent the deviation from the theoretical MDF for each Hg isotope:

$$\Delta^{xxx}Hg = \delta^{xxx}Hg - (\delta^{202}Hg \times \beta) \quad (2)$$

where β is 0.2520, 0.5024, 0.7520, and 1.4930 for isotope 199, 200, 201 and 204, respectively.

Health effects of Hg – We submitted plasma samples to the University of Miami to measure various health parameters. Sublethal effects of Hg in Goliath Grouper were evaluated by comparing Hg concentrations in liver and muscle with health and immune system parameters examined using blood plasma assays, including superoxide dismutase activity (SOD), lysozyme activity (measure of immune system function), protein electrophoretic profiles (general health parameters), reactive oxygen species/reactive nitrogen species (ROS/RNS) activity (indicator of oxidative stress), glutathione-s-transferase activity (detoxification enzyme), glutathione peroxidase (detoxifying enzyme-protects against oxidative damage), and biochemistry panels (indicator of overall health, with certain enzymes indicative of specific organ function).

Reproductive histological examination - Gonad biopsies were processed using standard histological techniques and slides were evaluated microscopically at the University of Florida. Gonad tissue was immediately fixed in 10% neutrally-buffered formalin after collection. After at least 24 hrs fixation, samples were washed and stored in 70% ethanol. Samples were processed at Crowder Histology in Baton Rouge, LA. Tissue samples were embedded in paraffin, sectioned to 3-5 μm thickness, stained with hemotoxylin, and then counterstained with eosin. Histological analysis of gonads were used to determine sex and reproductive condition (Wallace and Selman 1981, Hunter et al. 1992).

Mortality estimates– We used mark-recapture data for Goliath Grouper (as a model species) to distinguish between male and female mortality rates, and mortality rates as a product of age, because our data show that males have significantly higher Hg levels than females for all tissues. These same patterns were found for large individuals over smaller

individuals, with larger individuals having higher Hg. From 2010-2016, we have tagged 588 adult Goliath Grouper in the south Atlantic (primarily zones 6 &7, see Fig.1) with unique tag numbers—using internal and external tags for redundancy. Of these 588 fish, recaptures have occurred 176 times (recapture rate = 30%). Goliath Grouper have a high degree of site fidelity and a majority of the adult sample population return to the same sites each spawning season—which allows us to estimate mortality for the entire U.S. south Atlantic adult population. Mortality was therefore estimated and compared between sexes and size classes of Goliath Grouper to determine how Hg may impact mortality rates differently, combined with its impacts on health and reproduction.

RESULTS:

Diet characterization—Diet analysis revealed that Goliath Grouper are generalist predators, but that only a few prey items make up a majority of their diet. Crab species were the most consumed prey [28% occurrence], both by %occurrence and %weight (Figure 3, Table 1). Of the crabs, the most common were box crabs *Calappa flammea* and other *Calappa.* spp., followed by calico crab *Hepatus epheliticus* and speckled swimming crab *Arenaeus cribrarius* (Table 1). The next most important prey [next highest prey item by %occurrence] were scad, including *Decapterus punctatus*, *D. tabl,* and other *D.* spp. Although small in size, they occurred frequently in the diet and comprised 14.1% of the total diet. Burrfish *Chilomycterus reticulatus* and other *C.* spp. were fairly common prey items [7% occurrence], with spines and jaws often dominating stomach content samples, even when other parts of the body had been digested. Fishing gear of all types occurred often [12.1% occurrence] in the mouth and stomach, and included lines, sinkers, hooks, leaders, and lures of all shapes and sizes. Such gear is evidence of high rates of interaction with fishers, who often target the same sites where Goliath Grouper occur. However, very few fish species of high economic value— that is, those species most often targeted by fishers— were observed. Of the grouper-snapper complex, Gag, *Mycteroperca microlepis,* occurred in only 0.1% of sampled stomach contents, and *Lutjani*s spp. in 0.3% of sampled stomach contents. Whole lobster included Caribbean spiny lobster *Panulirus argus* and slipper lobster *Scyllarides nodifer*, and although they

are large and comprised 11.8% total weight, they only made up 2.4% of total occurrence in the diet.

This comprehensive diet study dispels the myth that Goliath Grouper feed primarily on prey that are of commercial and recreational interest. Our data do support high rates of interaction with fishing gear, but this is merely opportunistic. Unlike the occasional prey obtained through such opportunistic foraging, the fishing gear does not break down through digestion and is instead accumulated in the mouth and gut of Goliath Grouper.

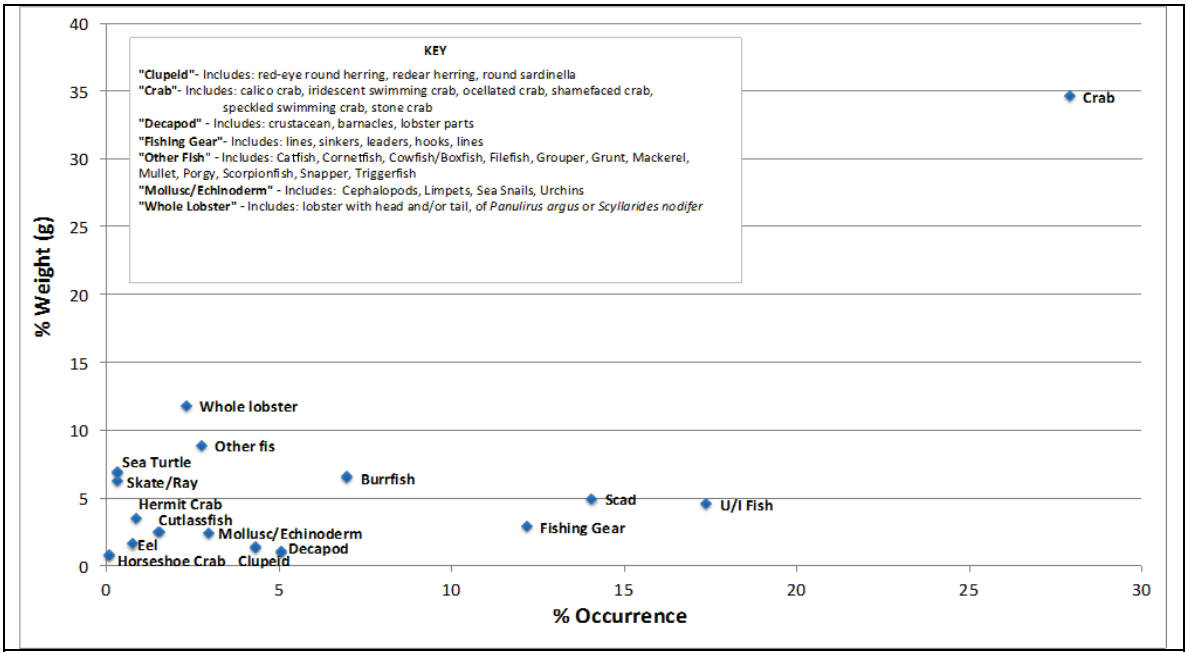


Figure 3. Diet categories by %occurrence and %weight of prey items collected from the stomachs of 408 individual fish, of which 993 prey items were identified, over the course of 7 years (2010-2016), caught primarily off Palm Beach and Martin counties during spawning (mid-July through early Oct.) and non-spawning months. The "Key" defines how prey items are grouped by major categories for ease of interpretation. "Whole lobster" were included as a separate group because it is likely that Goliath Grouper ingest heads and body parts of discarded lobster during lobster season, which coincides with a majority of our sampling. "U/I" includes all unidentified prey items for each specified category.

Table 1. Diet by %occurrence and %weight of prey items collected from the stomachs of 408 individual fish, of which 993 prey items were identified, over the course of 7 years (2010-2016), caught primarily off Palm Beach and Martin counties during spawning (mid-July through early Oct.) and non-spawning months. Species groups correspond to those of Fig.3, and are organized by the group with highest %occurrence at the top and lowest at the bottom. Within categories, specific prey items are organized similarly.

Species	% Occurrence	% Weight
Crab		
Calappa flammea/*C.* spp.	13.6	22.8
Hepatus epheliticus	4.7	4.0
Arenaeus cribrarius	4.7	5.8
U/I crab	3.7	0.9
Menippe mercenaria	0.6	0.6
Portunus gibbesii/*P.* spp.	0.3	0.2
Hexapanopeus hemphillii	0.2	0.0
Ovalipes floridanus	0.1	0.1
Cronius tumidulus	0.1	0.1
U/I fish	17.4	4.5
Scad		
Decapterus punctatus/*D. tabl*/*D.* spp.	14.1	4.9
Fishing Gear		
Fishing gear	12.1	2.6
Burrfish		
Chilomycterus reticulatus/*C.* spp.	7.0	6.5
Decapod		
Panulirus argus	3.1	0.5
U/I decapod	1.4	0.4
U/I lobster	0.3	0.1
Balanus amphitrite	0.2	0.1
Clupeid		
Sardinella aurita/*S.* spp.	3.9	1.0
Etrumeus teres	0.2	0.2
Clupeid spp.	0.1	0.0
Harengula humeralis	0.1	0.1
Mollusc/Echinoderm		
U/I bivalve	1.0	0.1
Crepidula plana	0.7	0.0
U/I cephalopod	0.2	0.0
Strombus pugilis	0.1	0.2
Olividae spp.	0.1	0.0
Cassis flammea	0.1	0.5
Costoanachis spp.	0.1	0.0
Crepidula fornicata	0.1	0.0
Arbacia punctulata	0.1	0.0
Cidaridae spp.	0.1	0.0

Busycon contrarium	0.1	1.3
Busycon spp.	0.1	0.2
U/I urchin	0.1	0.0

Table 1 (continued).

Species	% Occurrence	% Weight
Other Fish		
Ostraciidae spp.	0.3	0.0
Acanthostracion polygonius	0.3	0.6
Scorpaena spp.	0.3	0.4
Lutjanis spp.	0.3	1.4
Haemulon aurolineatum/*H.* spp.	0.3	0.3
Aluterus spp.	0.2	0.5
Balistidae spp.	0.2	0.7
Ariidae spp.	0.1	0.0
Mycteroperca microlepis	0.1	4.7
Trachurus lathami	0.1	0.0
Mugil spp.	0.1	0.0
Sparidae spp.	0.1	NA
Archosargus probatocephalus	0.1	0.1
Fistularia spp.	0.1	0.2
Whole Lobster		
Panulirus argus	1.8	9.0
Scyllarides nodifer	0.6	2.8
Cutlassfish		
Trichiurus lepturus	1.6	2.4
Hermit Crab		
Petrochirus diogenes	0.9	3.5
Eel		
Ophichthidae spp.	0.3	0.3
Gymnothorax spp.	0.2	1.3
U/I eel	0.2	0.0
Sea Turtle		
Caretta caretta	0.1	0.0
Chelonia mydas	0.1	6.8
U/I turtle	0.1	0.0
Skate/Ray		
U/I stingray	0.2	0.0
Dasyatis spp.	0.1	6.3
Horseshoe Crab		
Limulus polyphemus	0.1	0.7

Demographic (size and sex) and temporal (year, month) diet patterns.

Diet analysis, using chi-square tests on %occurrence, indicated a significant diet difference between males and females (χ^2=32.46, df=105, p<0.005). The largest differences were observed for fishing gear, *Arenaeus cribrarius*, and *Calappa flammea*, respectively (**Fig.4**). Similarly, we found a significant difference between different size groups (χ^2=222.81, df=17, p=0.013), with the largest differences between unidentified fish and crabs, *Decapturus punctatus* and other *D. spp.*, *Arenaeus cribrarius,* fishing gear, and *Chilomycterus reticulatus* and other *C. spp*. (Table 2). Diet analysis also revealed a difference between years (χ^2=610.55, df=300, p<0.005) and between months (χ^2=561.6, df=150, p<0.005).

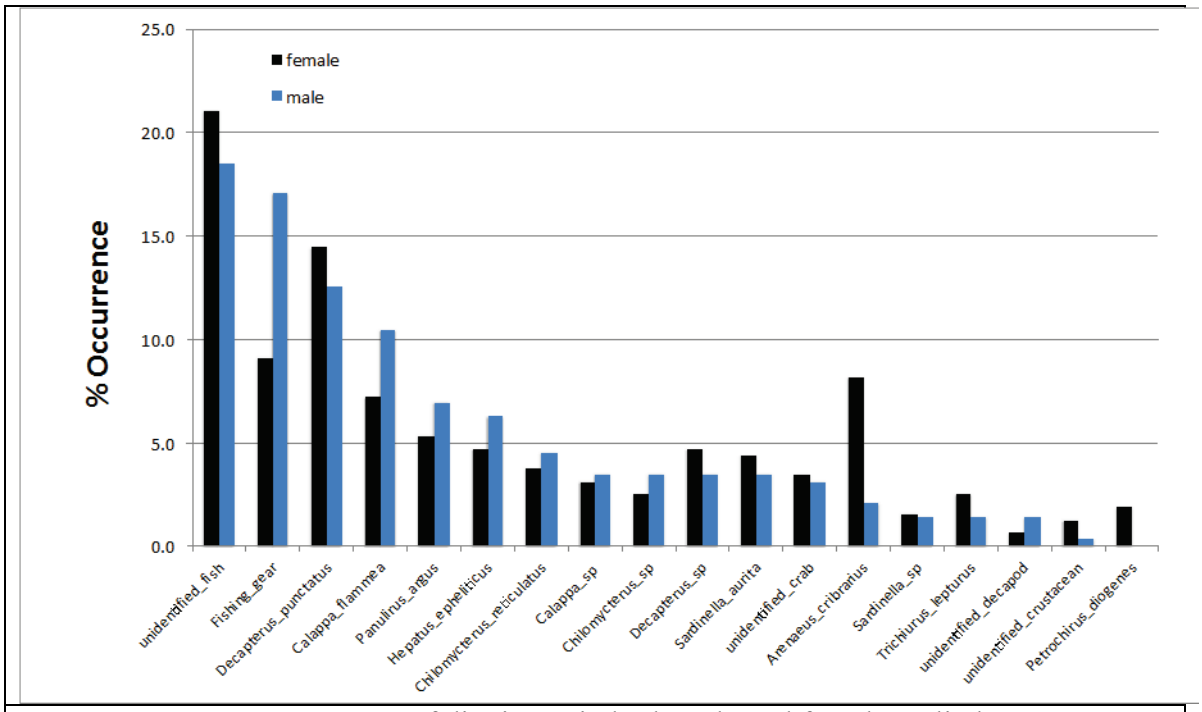


Figure 4. Percent occurrence of diet items in both male and female Goliath Grouper. Sex differences were significant (p<0.05), with fishing gear and *Arenaeus cribarius* showing the most striking differences.

Table 2. Diet of Goliath Grouper organized by size categories, with %occurrence of the top 6 prey items for each size category (note: juveniles are <100 cm). Dashes (--) indicate no additional prey items identified per size category. Diet differences were significantly different by size ($p<0.05$).

Size Category (cm)	Sample Size (n)	Most Abundant Prey Items (% Occurrence)					
		1	2	3	4	5	6
0-100	5	Unidentified crab (40.0)	Unidentified fish (20.0)	Decapterus punctatus (20.0)	Chilomycterus sp. (20.0)	–	–
101-120	23	Decapterus punctatus (26.1)	Fishing gear (17.4)	Unidentified fish (8.7)	Arenaeus cribrarius (8.7)	Calappa flammea (8.7)	Calappa sp (8.7)
121-140	122	Decapterus punctatus (24.6)	Unidentified fish (16.4)	Fishing gear (12.3)	Calappa flammea (9.8)	Hepatus epheliticus (8.2)	Panulirus argus (5.7)
141-160	191	Unidentified fish (22.0)	Fishing gear (16.8)	Decapterus punctatus (14.1)	Chilomyterus reticulatus (9.4)	Calappa flammea (8.9)	Decapterus sp. (7.9)
161-180	253	Unidentified fish (19.0)	Fishing gear (15.8)	Calappa flammea (11.5)	Decapterus punctatus (10.3)	Hepatus epheliticus (6.7)	Panulirus argus (6.7)
181-200	153	Unidentified fish (19.0)	Calappa flammea (17.0)	Arenaeus cribrarius (11.8)	Fishing gear (9.2)	Unidentified crab (6.5)	Sardinella aurita (6.5)
201-220	42	Unidentified fish (28.6)	Areneaus cribrarius (23.8)	Calappa flammea (14.3)	Calappa sp. (7.1)	Chilomyrectus sp. (7.1)	Fishing gear (4.8)
221-240	4	Unidentified fish (75.0)	Fishing gear (25.0)	–	–	–	–

Mercury in prey:

Mercury levels analyzed in a subset of common Goliath Grouper prey collected from stomach contents suggest that *H. epheliticus* (a benthic crab) contained the highest levels of Hg, and that a majority of the Hg in these prey exist as inorganic Hg (**Figs. 5 & 6**). Subsequent respective Hg levels in prey include speckled crab (256.53 ppb dw), spiny lobster (227.17 ppb dw), grunt (174.69 ppb dw), shameface crab (114.60 ppb dw), and scad (114.09 ppb dw). The variability in %MMHg in invertebrates can be fairly high, with major interspecific and trophic level differences (Andersen and Depledge 1997), which suggests many more samples need to be analyzed for this study before interpreting sources and patterns of contamination in Goliath Grouper prey. This work is ongoing, and more prey samples are in the process of Hg analysis.

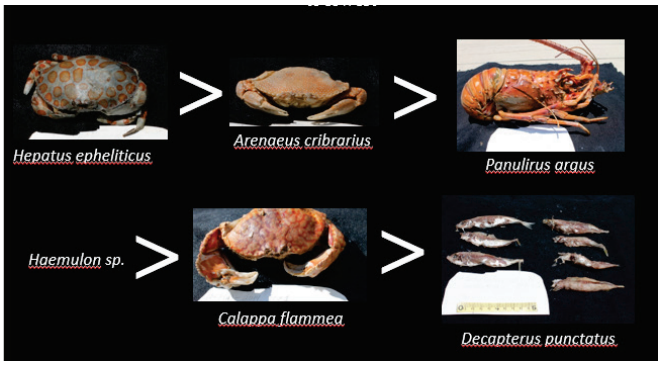


Figure 5. Whole, unprocessed Goliath Grouper *Epinephelus itajara* stomach contents prior to Hg analysis in order of highest to lowest Hg concentration.

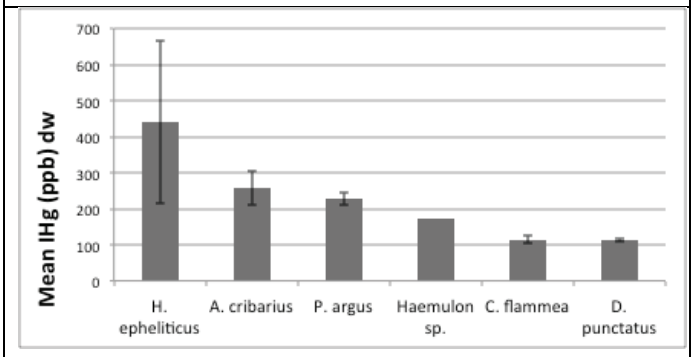


Figure 6. Mean inorganic Hg concentration recorded in parts per billion dry weight (ppb dw). *Hepatus epheliticus* n = 2, *Arenaeus cribrarius* n = 9, *Panulirus argos* n = 3, *Haemulon sp.:* n = 1, *Calappa. flammea:* n = 5, *Decapterus punctatu* n = 14. Error bars represent standard error.

Hg levels and isotopic composition in Goliath Grouper -- Hg analyses conducted on Goliath Grouper tissue samples collected from the U.S. South Atlantic during 3 sampling trips between August and October 2014 indicate that all Hg levels in Goliath Grouper muscle tissue (fish fillet) exceeded human health advisory levels for the consumption of fish (EPA=0.3 ppm, NRDC=0.5 ppm) and most are above the legal action limits by the FDA (Hg=1.0 ppm) (USDA-USDHS 2010, Karimi et al. 2012, USFDA 2015) (Figure 7). Hg levels were positively correlated with fish length (Figure 7), and males had significantly higher Hg levels than females (t-test: $p=0.0209$). This indicates that Hg bioaccumulates in the muscle over time and that females are better able to evacuate MMHg—possibly via transfer to their offspring, which we will measure in egg biopsies. For liver and muscle, results showed significantly higher Hg levels ($p<0.001$) in liver.

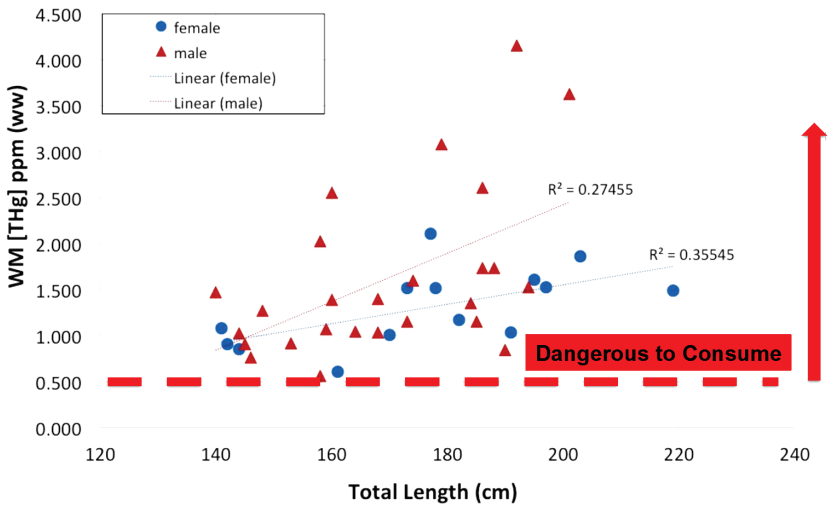


Figure 7. Linear regression of total Hg (THg), which is primarily methylmercury (MMHg), levels in muscle (ppm) versus total length (cm) of Goliath Grouper *Epinephelus itajara.* Both sexes (males and females) show a significant relationship between Hg concentration and length ($p<0.01$), with males containing significantly more Hg than females($p< 0.05$). All levels exceed U.S. EPA's highest risk for human consumption (dangerous to eat): Hg = 0.3 ppm ww (Karimi et al. 2012), and NRDC health advisory limit; Hg = 0.5 ppm ww. Note: ww=wet weight, ppm=parts per million.

than muscle (Figure 8). While muscle contained predominantly MMHg (>90% of total Hg), liver contained mostly inorganic Hg (1 to 22% of MMHg). The levels of MMHg in the muscle increased with the levels of iHg in the liver (Figure 9), while the %MMHg in the liver decreased when MMHg level in the muscle increased (Figure 9b). An exponential decrease of the ratio Hg_{muscle}:Hg_{liver} was observed as MMHg levels in the liver increased (Figure 10**)** suggesting that the liver stores, with time and in addition to iHg, higher quantities of MMHg than the muscle, which can result in higher rates of MMHg degradation and excretion. Hg isotopic composition was measured in liver and muscle from a subsample of the fish we characterized for Hg species concentrations. We observed that total Hg in the muscle was always enriched in heavier Hg isotopes (i.e. higher δ^{202}Hg values) than Hg in the liver (Figure 11a).

Since the muscle contains mostly MMHg and the liver contains mostly iHg, we suggest that the Hg isotopic compositions of iHg and MMHg are significantly different, and that the total Hg isotopic composition in each tissue depends on the respective fractions of MMHg and iHg. Our results show that, as a function of the difference of δ^{202}Hg values between muscle and liver, there is progressive depletion of heavier isotopes in the liver concurrent with enrichment of heavier isotopes in the muscle (Figure 11b). These trends are similar to what was observed in marine mammals (Perrot et al. 2012), suggesting that *in-vivo* demethylation of MMHg occurs in Goliath Grouper, leading to mass-dependent fractionation (MDF) of Hg isotopes (enrichment of lighter isotopes in the product, iHg).

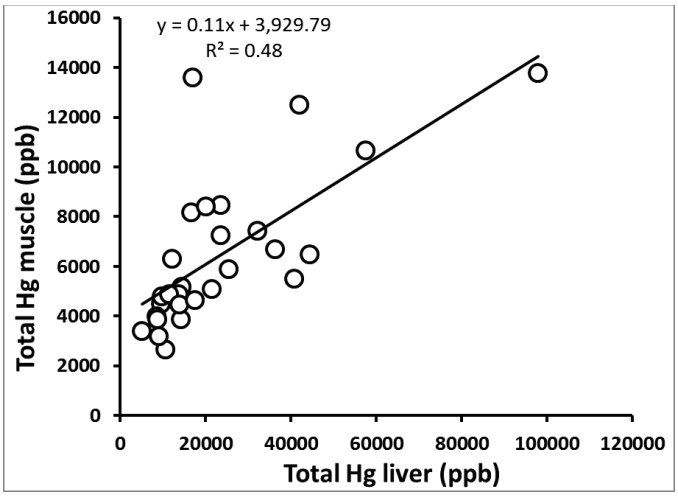


Figure 8. Regression analysis between total Hg in the muscle and liver of 28 Goliath Grouper *Epinephelus itajara* ($p<0.001$) sampled in coastal waters of Florida, USA. Note: ww=wet weight, dw=dry weight, ppm=parts per million, ppb=parts per billion, 0.3 ppm ww ~1500 ppb dw.

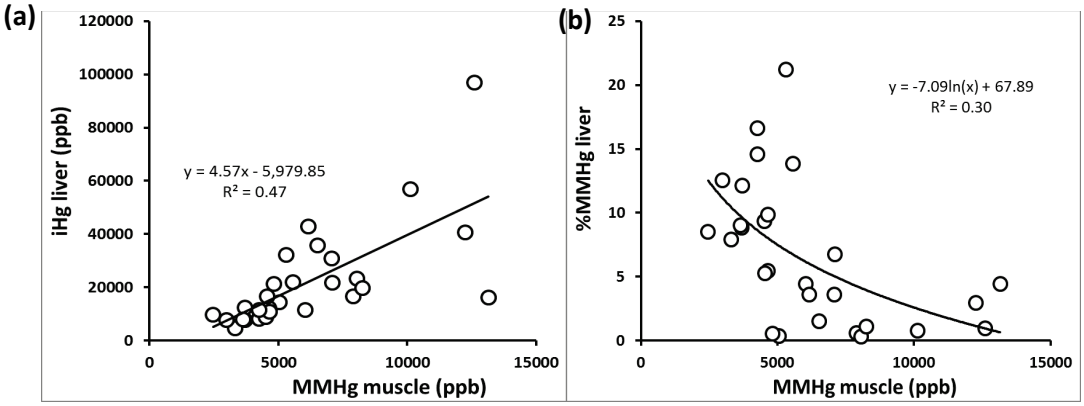


Figure 9. Inorganic Hg levels (a) and %MMHg (b) in the liver as a function of MMHg levels in the muscle ($p<0.001$ and $p<0.005$, respectively) of Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA.

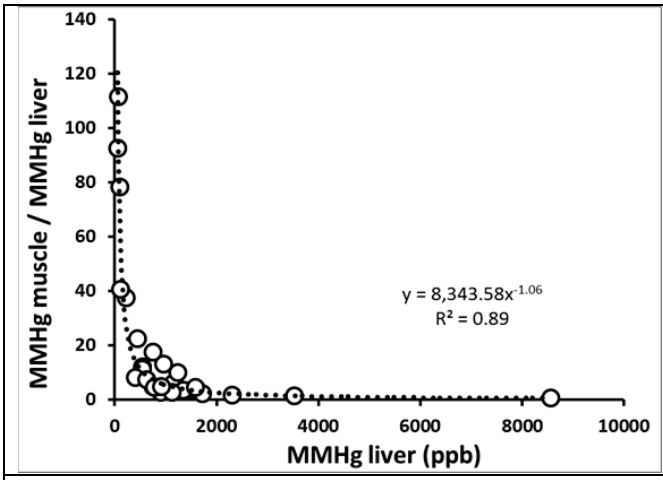


Figure 10. Regression analysis between the ratio of total Hg between muscle and liver as a function of MMHg levels in the liver ($p<0.001$) of Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA.

We observed significant ($p<0.001$) and positive mass-independent fractionation signatures (MIF) in muscle and liver (i.e. positive Δ^{199}Hg values), indicating that Goliath Grouper have incorporated MMHg that has been partially photodegraded in the water column before entering the food web (Bergquist and Blum 2007). Interestingly, and contrary to MDF, we found similar Δ^{199}Hg values and Δ^{199}Hg/Δ^{201}Hg ratios in both liver and muscle of each individual fish (**Fig. 12**). This further suggests that both iHg and MMHg in fish tissues have the same origin, i.e. MMHg from diet, and that an important part of the iHg fraction in the liver is due to *in-vivo* MMHg demethylation.

Overall, these results indicate that MMHg and iHg accumulation in muscle and liver are linked. We also measured Hg species in the blood (whole blood and separated red blood cells), and found Hg levels up to 500 µg/L, of which 95% was in the form of MMHg. Interestingly, we observed 2 to 4-time higher Hg levels in red blood cells than in the whole blood (Figure 13). This indicates that hemoglobin might be a key molecule responsible for the transport and distribution, through the bloodstream, of MMHg in fish organs—which has been suggested in a recent publication on marine mammals (Zayas et al. 2014), but is apparently unknown for fish.

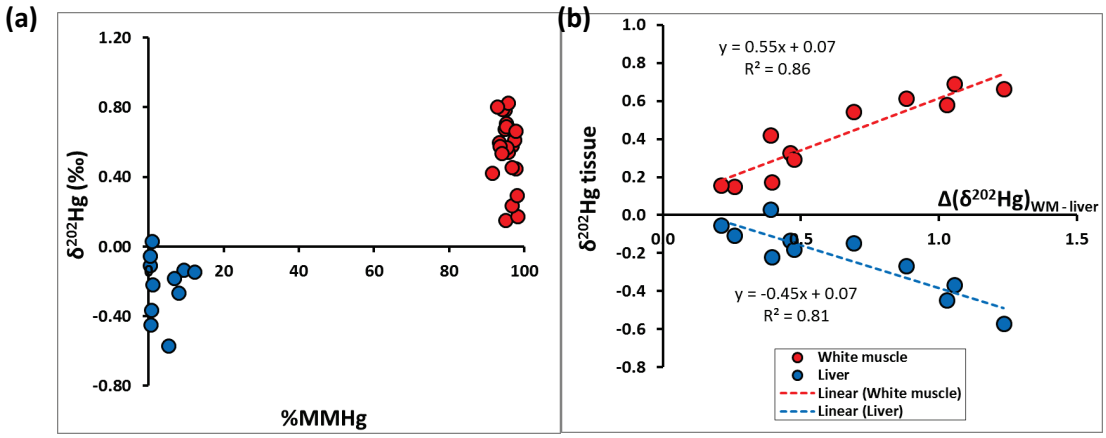


Figure 11. δ^{202}Hg in liver (blue dots) and muscle (red dots) as a function of %MMHg (a) and as function of the difference (Δ) of δ^{202}Hg values between the muscle and the liver (b) of Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA.

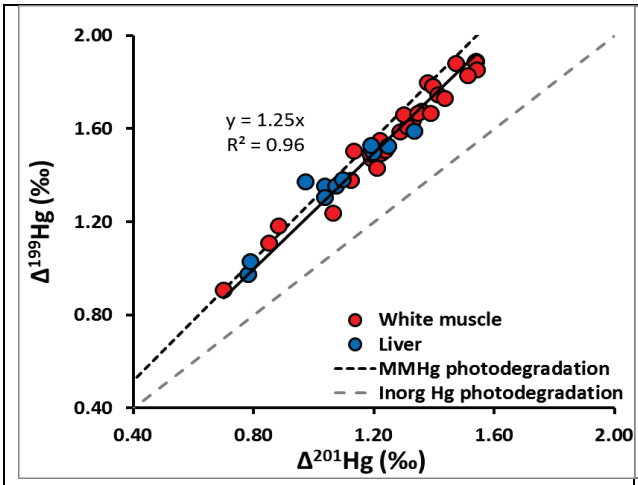


Figure 12. Δ^{199}Hg as a function of Δ^{201}Hg in liver (blue dots) and muscle (red dots) of Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA. This shows that the signature of MMHg residual from photodegradation (1.3) and not from iHg photodegradation (1.0) (Bergquist and Blum 2007). The Hg fraction of iHg measured in liver is the product of MMHg demethylation.

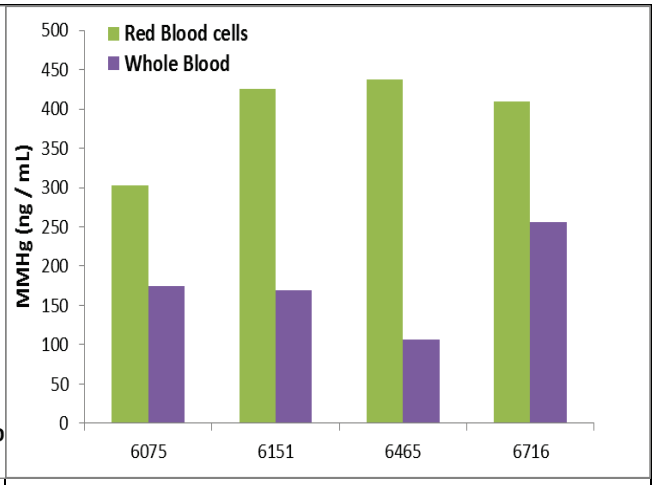
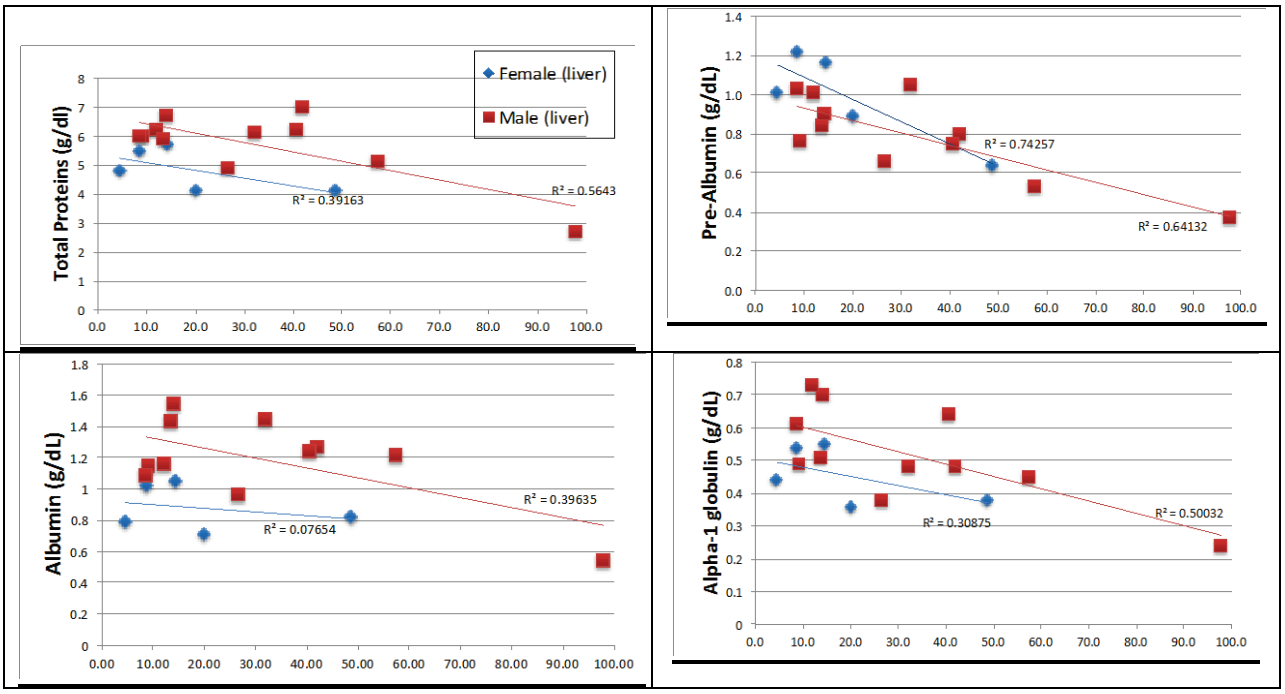


Figure 13. Concentration of methylmercury in the red blood cells and whole blood of 4 individual Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA.

Mercury accumulation, health parameters, and mercury offloading--

A number of health parameters for Goliath Grouper sampled during 2014 have been measured, using blood plasma, and compared with Hg in the muscle and liver. Mercury appears to have a significant effect ($p < 0.05$) on a number of health parameters, and is impacting males and females differently (Figures 14 & 15, Tables 3 & 4). We suggest that such health results are directly related to dietary differences between sexes and differences in Hg contamination between sexes. Further measurements of Hg in offloading routes, including eggs, sperm, feces, and urine, indicate that females are capable of offloading more Hg (MMHg and iHg) than males, and that non-sex specific routes (*i.e.,* feces, urine) may enable offloading that does not adversely impact offspring survival and development (Figure 16).



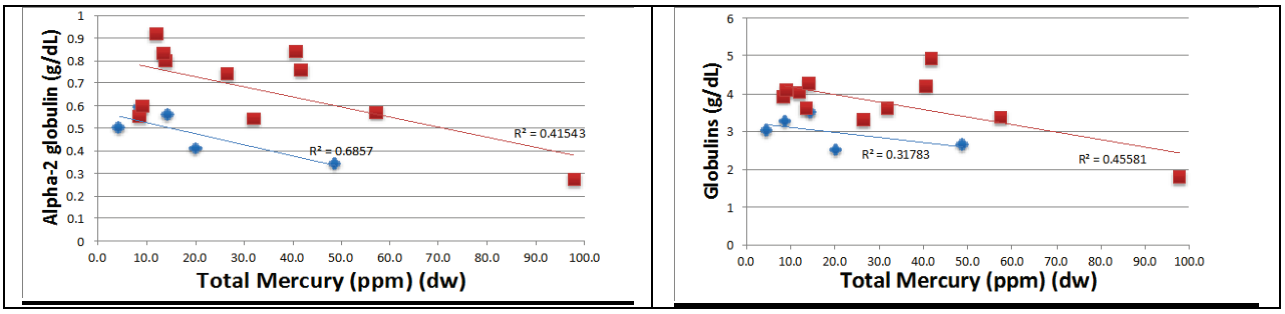
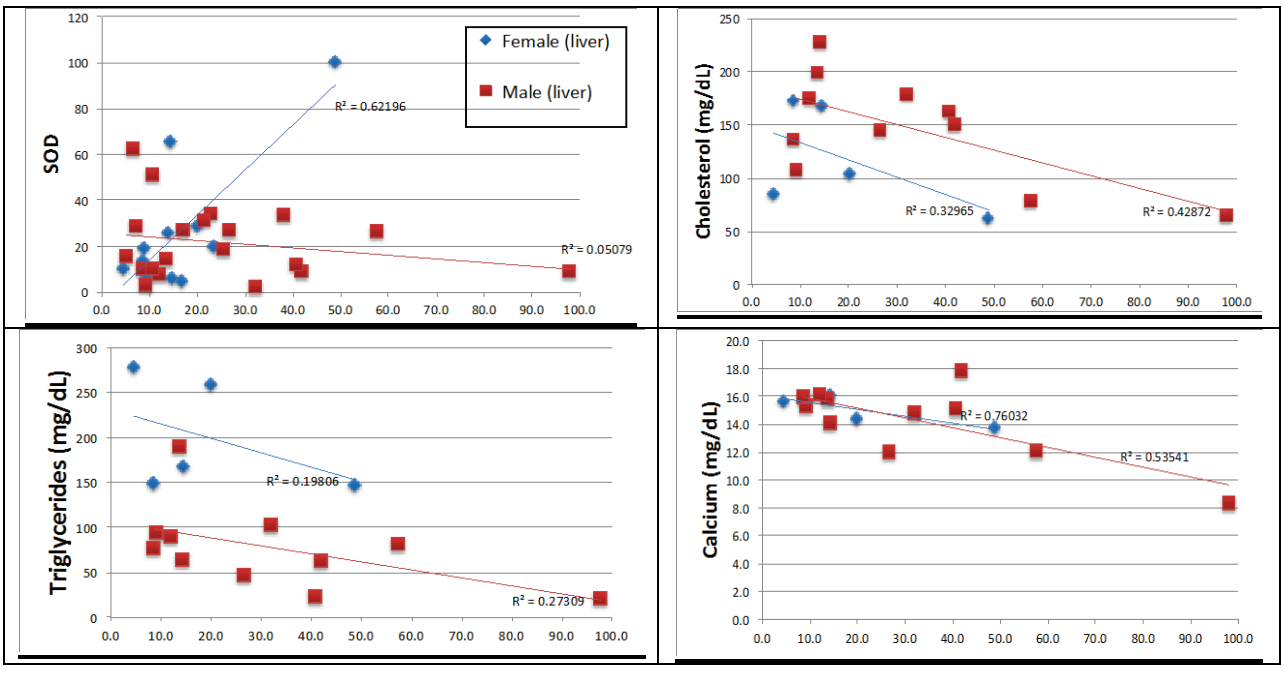


Figure 14. Blood protein concentration as a function of total mercury in the liver (ppm=parts per million) (dw=dry weight). Linear regression equations relate to each sex (red squares and lines=males, blue diamonds and lines=females) of Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA.



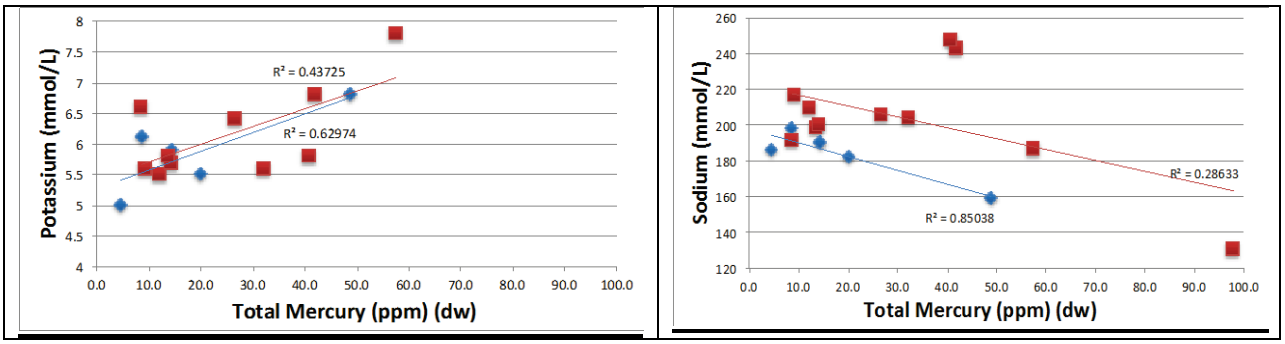


Figure 15. Health parameters and plasma electrolytes as a function of total mercury in the liver (ppm=parts per million) (dw=dry weight). Linear regression equations relate to each sex (red squares and lines=males, blue diamonds and lines=females) of Goliath Grouper *Epinephelus itajara* sampled in coastal waters of Florida, USA.

Table 3. MANOVA results for plasma protein levels (dependent variable) tested for significance with mercury (Hg) concentration (independent variable), fish length, sex, and tissue type (liver and muscle). Significant results ($p<0.05$) are in bold. Reported effects relate to the relationship between mercury concentration and plasma protein levels (*e.g.*, negative relationship=protein decreases as Hg increases).

									Correlation with Total Hg (R^2)				
									Liver		Muscle		
Analyses	n	Df	num Df	den Df	Sum Sq	Pillai	F-value	p-value	Male	Female	Male	Female	Reported effects/Notes
Total Proteins	49												Lower protein levels, as correlated with Hg for this population, can indicate: liver, kidney, or other organ disorder/disease; malnutrition; GI disease (inability to digest proteins).
Tot Prot~Hg*Length*Sex*Tissue		1	4	44		0.41339	7.7518	**8.09E-05**	(-) 0.5643	(-) 0.39163	(-) 0.19867	(-) 0.23914	
Tot Prot~Hg		1			257.7		0.7938	0.3775					
Tot Prot~Sex		1			0.5945		2.1432	0.1499					
Tot Prot~Length		1			10521		31.789	**9.46E-07**					
Pre-Albumin	49												A protein produced mainly by the liver with a shorth half-life (2-3 days) and is a good indicator of nutritional status and acute phase response. Declines, as measured in this population, indicate infection, malnutrition, digestive disorder, chronic illness, and/or liver disease.
PreAlb~Hg*Length*Sex*Tissue		1	4	44		0.35371	6.0203	**6.000E-04**	(-) 0.64132	(-) 0.74257	(-) 0.32468	(-) 0.065	
PreAlb~Hg		1			2015.3		7.0149	**0.01097**					
PreAlb~Sex		1			1.3126		5.0073	**0.03002**					
PreAlb~Length		1			1816.3		3.5188	0.0669					
Albumin	49												The main protein produced in blood plasma and is produced primarily by the liver. Lower correlated levels of this protein corroborates with lower levels of total protein, and can indicate liver disease, malabsorption, diseases related to protein loss, and can result in edema.
Alb~Hg*Length*Sex*Tissue		1	4	44		0.45775	9.2857	**1.57E-05**	(-) 0.39635	(-) 0.07654	(-) 0.30279	(-) 0.37768	
Alb~Hg		1			83.3		0.2536	0.6169					
Alb~Sex		1			2.5704		10.921	**1.83E-03**					
Alb~Length		1			10562		31.994	**8.88E-07**					
Alpha-1 globulins	49												Along with albumins, are the major proteins within blood, and are produced by the liver and immune system. Low correlated levels, as measured for this population, can signal: kidney disease, liver dysfunction, diseases causing malnutrition and GI dysfunction, acute hemolytic anemia.
Alpha1~Hg*Length*Sex*Tissue		1	4	44		0.35696	6.1063	**5.35E-04**	(-) 0.50032	(-) 0.30875	(-) 0.37597	(-) 0.00948	
Alpha1~Hg		1			339.5		1.0511	0.3105					
Alpha1~Sex		1			0.6045		2.1807	0.14640					
Alpha1~Length		1			8723.5		23.627	**1.35E-05**					
Alpha-2 globulins	49												Along with albumins, are the major proteins within blood, and are produced by the liver and immune system. Low correlated levels, as measured for this population, can signal: kidney disease, liver dysfunction, diseases causing malnutrition and GI dysfunction, acute hemolytic anemia.
Alpha2~Hg*Length*Sex*Tissue		1	4	44		0.47296	9.8715	**8.66E-06**	(-) 0.41543	(-) 0.6857	(-) 0.15969	(-) 0.13551	
Alpha2~Hg		1			66		0.2006	0.65630					
Alpha2~Sex		1			1.1318		4.2551	**0.04468**					
Alpha2~Length		1			12268		41.753	**5.37E-08**					
Globulins	49												Along with albumins, are the major proteins within blood, and are produced by the liver and immune system. Low correlated levels, as measured for this population, can signal: kidney disease, liver dysfunction, diseases causing malnutrition and GI dysfunction, acute hemolytic anemia.
glob~Hg*Length*Sex*Tissue		1	4	44		0.37655	6.6438	**2.84E-04**	(-) 0.4581	(-) 0.31783	NA	NA	
glob~Hg		1			51.4		0.1561	0.69450					
glob~Sex		1			0.7873		2.8807	0.09626					
glob~Length		1			9791.8		28.26	**2.87E-06**					

Table 4. MANOVA results for health parameters and plasma electrolytes (dependent variable) tested for significance with mercury (Hg) concentration (independent variable), fish length, sex, and tissue type (liver and muscle). Significant results ($p<0.05$) are in bold. Reported effects relate to the relationship between mercury concentration and plasma protein levels (*e.g.,* negative relationship=health parameter decreases as Hg increases).

Analyses	n	Df	num Df	den Df	Sum Sq	Pillai	F value	p-value	Correlation with Total Hg (R^2) Liver Male	Liver Female	Muscle Male	Muscle Female	Reported effects/Notes
ROS/RNS	57												
ROS/RNS~Hg*Length*Sex*Tissue		1	4	52		0.22634	3.8033	**8.713E-03**	(-) 0.09713	0.07445	(-) 0.07685	0.03597	High rates of oxidative and nitrosative stress can result from an increase in ROS/RNS levels. Although increases in ROS/RNS can be an effect of chromium (Cr), it has not been reported as an effect of Hg in fish (Sfakianakis et al. 2015).
ROS/RNS~Hg		1			174.9		0.6216	0.4338					
ROS/RNS~Sex		1			2.9267		14.355	**3.773E-04**					
ROS/RNS~Length		1			1492.7		2.9191	0.09317					
SOD	78												
SOD~Hg*Length*Sex*Tissue		1	4	73		0.16762	3.6751	**8.792E-03**	0.05079	0.62196	(-)0.00291	0.0006	Protects cells against harmful effects of superoxide free radicals (Malmstrom *et al.* 1975) by catalyzing their destruction, thus fighting against many types of cell damage. An increase in SOD may indicate a heightened response to damages caused by reactive O^{2-}.
SOD~Hg		1			16.4		0.0714	0.79					
SOD~Sex		1			1.084		4.1632	**0.04478**					
SOD~Length		1			4900.7		11.998	**8.785E-04**					
Cholesterol	49												
chol~Hg*Length*Sex*Tissue		1	4	44		0.46938	9.7304	**9.99E-06**	(-) 0.42872	(-) 0.32965	(-) 0.27053	(-) 0.1379	A type of fat used to build cells and certain hormones. Lower levels, such as those correlated for this population, have been shown for fish with lowered disease resistance, increases rate of parasitic infection, and increased rates of mortality due to parasitic infection (Gatlin, 2007).
chol~Hg		1			241.2		0.742	0.39340					
chol~Sex		1			0.7858		2.8748	0.09659					
chol~Length		1			12066		40.472	**7.61E-08**					
Triglycerides	49												
Trig~Hg*Length*Sex*Tissue		1	4	44		0.57187	14.693	**1.07E-07**	(-) 0.27309	(-) 0.19806	(-) 0.10125	0.25738	A type of fat used to store and supply energy. Reduced levels are indicative of malnutrition or the inability to digest and absorb nutrients from food.
Trig~Hg		1			1281.2		4.2295	**0.0453**					
Trig~Sex		1			7.0283		50.016	**6.35E-09**					
Trig~Length		1			2781.5		5.6117	**0.022**					
Calcium (Ca)	49												
Ca~Hg*Length*Sex*Tissue		1	4	44		0.34204	5.7182	**8.534E-04**	(-) 0.53541	(-) 0.76032	(-) 0.18493	(-) 0.11083	Low correlated levels, as measured for this population, can lead to: reduced nerve function and muscle contraction; reduced blood clotting ability; and reduced heart function. Low levels have also been correlated with cadmium levels (see Sfakianakis, 2015).
Ca~Hg		1			1018.1		3.3002	0.07565					
Ca~Sex		1			0.663		2.4027	0.1278					
Ca~Length		1			3863		8.1745	**0.006316**					
Sodium (Na)	49												
Na~Hg*Length*Sex*Tissue		1	4	44		0.41474	7.7952	**7.71E-05**	(-) 0.28633	(-) 0.85038	(-) 0.12601	0.0015	Has a major role in maintaining normal blood pressure, nerve and muscle functioning, and regulating the body's fluid balance (i.e. osmoregulation). Lower correlated levels, as measured for this population, impact this functioning and can indicate heart, liver, and kidney problems.
Na~Hg		1			21.2		0.0641	0.8012					
Na~Sex		1			1.2148		4.5979	**0.03721**					
Na~Length		1			10688		32.642	**7.29E-07**					
Potassium (K)	49												
K~Hg*Length*Sex*Tissue		1	4	43		0.28476	4.2798	**0.005289**	0.43725	0.62974	(-) 0.00392	(-) 0.19974	A delicate balance of potassium is necessary for proper heart and muscle functioning. Elevated levels, as correlated for this population, are indicative of reduced kidney function, breakdown of red blood cells, and tissue breakdown or injury.
K~Hg		1			1268.2		8.5894	**5.250E-03**					
K~Sex		1			0.0333		0.1139	0.7372					
K~Length		1			1450.6		2.7862	0.1019					

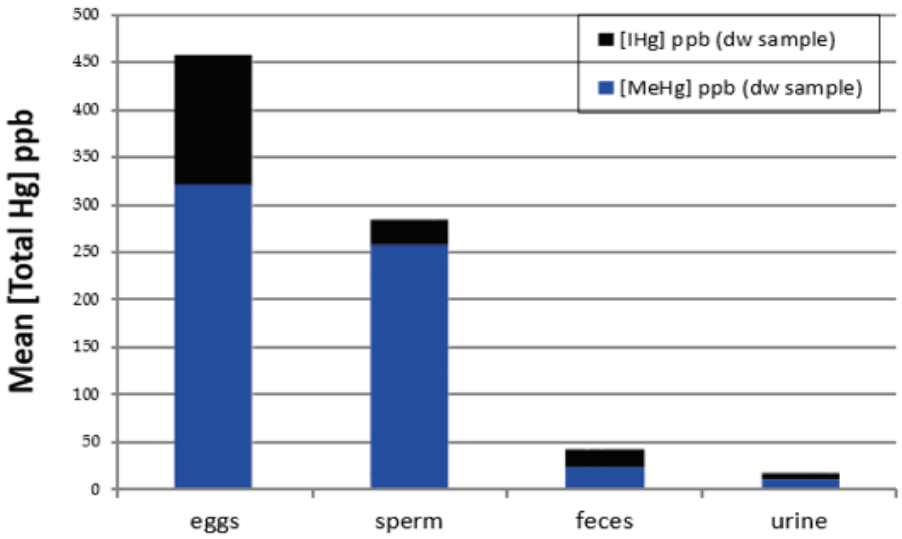


Figure 16. Summary of total Hg (THg) and ratios of iHg to MeHg (MMHg) in various offloading routes in parts per billion (ppb). Eggs n = 10, sperm n = 3, feces n = 11, urine n = 16.

Mortality estimates

One would expect that in this protogynous species, males that develop from functional females would be larger and older than females in the population, and as larger fish would have higher Hg levels. Our data indicate that larger fish have significantly higher mercury loads than smaller fish of both sexes. However, overall, males captured were smaller than females ($\bar{x}_{TLmale}$ = 169.2 cm, n = 312; $\bar{x}_{TLfemale}$ = 177.2 cm, n = 285) and had significantly ($p < 0.05$) higher Hg burdens. Further, our studies indicate that in large ($\geq$190 cm) Goliath Grouper, recapture rates are significantly lower in males than in females ($p < 0.0001$, n=16), whereas the recapture rates of smaller (< 190 cm TL) males and females differed little ($p > 0.05$, n=105) (Figure 17**)**. **This strongly suggests a loss of large males from the population, potentially due to high Hg levels.**

DISCUSSION:

General diet patterns

Much misinformation exists on the diet of Goliath Grouper, with a common one being that because of their large size they are eating everything on the reef. This includes the opinion that Goliath Grouper are eating reef fish like snapper and other grouper, in direct competition with fishers. Previous studies by us (Koenig and Coleman 2009, Coleman et al. 2011, Koenig and Coleman 2013) and others (Longley and Hildebrand 1941, Randall 1967, Randall 1983, Bullock and Smith 1991, Randall and Heemstra 1993, Yeiser et al. 2008) have shown such opinions to be incorrect. Yet, these opinions persist and have become mainstream opinions within some groups-- perhaps greatest with the fishing community. The current study is the most comprehensive diet study to-date that has been conducted on this species. Results show that Goliath Grouper are indeed opportunistic predators, but consume primarily prey at lower trophic levels than opined by some groups. Benthic invertebrates, mostly crab species, make up a majority of the Goliath Grouper diet. Scad, also common in their diet, are a small shoaling species in the family Carangidae that often occur near the sea floor near immobile reef structure or surrounding Goliath Grouper, likely for protection. It is probable that some individuals are ingested accidently through feeding events on other targeted prey; but many stomach samples included only scad, thus indicating that they also target this small prey.

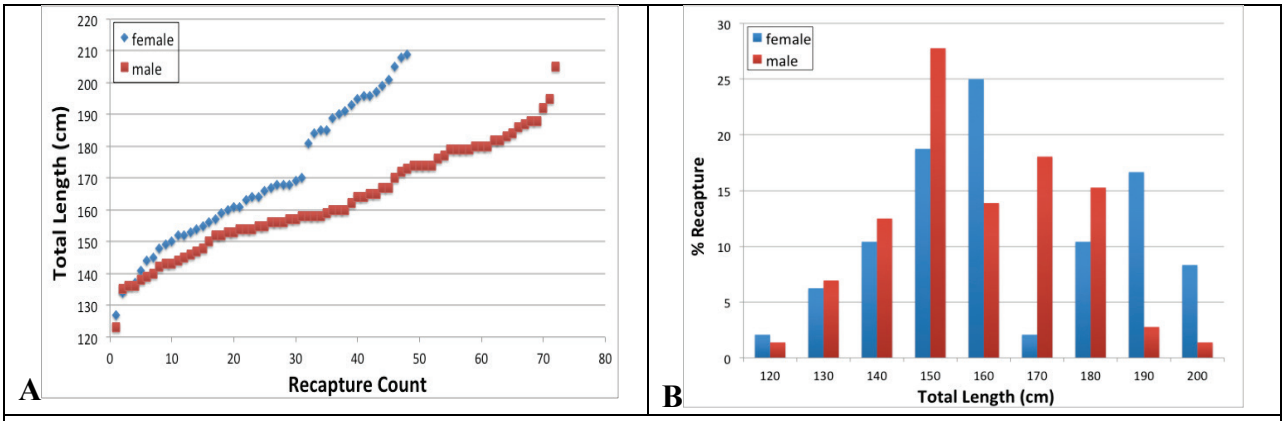


Figure 17. Goliath Grouper recaptures showing higher recapture rates for large females (>190 cm), separated by male and female A) recapture count (each point represents an individual fish) by total length (cm), and B) Percent recapture by total length (cm). Recapture is a function of # of recaptures per size divided by the total # of recaptures per sex. The black outlined rectangle highlights the higher recapture rates for large females over males ($p<0.05$).

Interaction between Goliath Grouper and fishers has also been increasing, due to a positive response of Goliath Grouper to protection from harvest since 1990 (GMFMC 1990, SAFMC 1990), and has been a source of contention among some groups (Collins 2014). The source of most of this contention is that anglers are increasingly reporting that Goliath Grouper are becoming a 'nuisance' species due to their presumed propensity to 'steal' hooked fish from anglers (Fleshler 2011, Kelly 2011, Frias-Torres 2012). Our data show that although Goliath Grouper will opportunistically target speared or hooked fish, such prey items do not occur often enough to be detected in stomach contents, and prey obtained in this manor are typically not a normal part of their diet. Goliath Grouper are opportunistic predators, and like any predator, will target easy meals. When a fish is caught by an angler or spearfisher, these fish have the appearance of being injured and become an easy target for Goliath Grouper. The outcome of these interactions leaves frustrated fishers and often adversely impacted Goliath Grouper. Fishing gear from these interactions often gets broken off into the mouth or stomach of Goliath Grouper. This gear then tends to accumulate in the digestive system of Goliath Grouper over time. Depending on the type and number of individual interactions with fishers, this can lead to feeding impediments (see Figure 18).

Figure 18. Interactions with fishing gear that are harmful to the fish. The **photo on the left** is of a nest of fishing line and multiple lead weights removed from the mouth and gut of an adult Goliath Grouper off of West Palm Beach, FL. This line was so entangled in the fishes' mouth that food was blocked from entering its stomach, which was evidenced by multiple prey items in its mouth being blocked from entering down into the stomach for digestion. This fish was presumably undersized and malnourished as a result. The **photo on the right** is of a separate Goliath Grouper caught in the northern Gulf of Mexico, off the coast of Florida, that had its lower jaw damaged and tongue/throat ripped from its body, where it can be seen here protruding out. This injury was likely sustained as a result of fishing gear snagging the fish and subsequently being ripped out of its mouth under the power of a vessel. Although the open wounds were healing, this fish appeared thin and was presumably malnourished. Because Goliath Grouper engulf a majority of their prey through ram and suction feeding, injuries sustained through fishing interactions like these impede their ability to feed.

Demographic and temporal feeding patterns in relation to mercury

Goliath Grouper are mid-level benthic predators as both juveniles and adults and they consume primarily benthic prey that are closely associated with the substrate where Hg is methylated (*e.g.*, various crab species, skates/rays, mollusks) (Koenig et al. 2011, Koenig and Coleman 2013)--which along with their large size and longevity, make them susceptible to high levels of Hg exposure. Differences in spatio-temporal patterns and diet differences between juvenile and adult Goliath Grouper, including residency on different habitat types (juveniles=mangroves, adults=offshore reefs) (Koenig et al. 2007, Evers et al. 2009, Koenig et al. 2011) likely contributed to differences found in Hg concentration between age groups— both ontogenetically and between different size classes of adults. As fish get larger, so do the size of their gape. Along with prey selection as a function of size, optimal foraging may differ between size classes based on optimal allocation of time spent searching for and handling prey (Werner and Hall 1974, Scharf et al. 2000). Such consideration may also support the high

rate of interaction between larger individuals [221-240 cm] and fishing gear. Large fish are often the target of fishers, and larger prey were more likely targeted and obtained by Goliath Grouper when opportunistically foraging on such fish.

Goliath Grouper differed in diet across months and years. Prey community abundance and distribution can change based on season, year class, and anthropogenically-induced stressors (*i.e.* pollution, overfishing, climate change, habitat destruction) (Cushing 1990, Babcock et al. 1999, Frederiksen et al. 2006, Walther 2010), leading to diet differences in predators. Densities of Goliath Grouper during spawning (mid-July through early October) over non-spawning seasons increase dramatically over specific spawning sites. This increased density has the propensity to change prey communities over the course of the season, which could result in the diet differences observed in Goliath Grouper between months (Thrush et al. 1994). Because Goliath Grouper are generalists, they can likely overcome any changes in prey abundance and density by switching to alternative prey.

Our study showed that the calico crab (*H. spp.*) had the highest per gram amount of THg, thus contributing the highest amount of dietary Hg to Goliath Grouper per prey item. This crab species lives on sandy and muddy substrates, which is the region where Hg is methylated. Although prey in this study contained primarily iHg, Goliath Grouper may ingest additional MMHg by foraging in the benthos on benthic-associated species. Depending on environmental conditions, THg and MMHg fractions can be fairly high in sediment (*e.g.,* 2.84 ng/g in a California wetland downstream of Hg or gold mining) (Domagalski 2001).

Differences in diet between male and female Goliath Grouper, and subsequent differences in tissue Hg levels, may in part be due to sex-related foraging behavior. For example, physiological differences due to energy demands for growth and reproduction, as a consequence of age and reproductive status, are also known to result in differences in intraspecific and interspecific prey use in fishes and other marine organisms (Grossman 1980, Stein et al. 1984, Malinowski and Herzing 2015). This has not been observed directly for Goliath Grouper, but males in many species minimize foraging time as a tradeoff with time spent on reproductive activities, while females spend less time engaged in social and mating activities and instead maximize time foraging (Hoffman 1983). Goliath Grouper have the capacity to adapt their foraging behavior between suction and ram feeding depending on prey availability (Collins 2014), and if males indeed spend less time foraging than females, it seems likely that they would feed more opportunistically on available and more easily obtained prey. It would then

follow that our observed difference in fishing gear interaction between males and females could be a product of males targeting more easily attained prey as they struggle at the end of a fishing line or spear tip.

Patterns in Hg load and effects on health, reproductive success, and survival—

Although recent reductions in nation-wide Hg emissions may be resulting in general declines in deposition of Hg, no such decline appears to be occurring in Florida. In fact, areas in south Florida, including Miami and Tampa, have some of the highest Hg emission concentrations in the state while the levels of Hg in fish from the Everglades National Park exceed those found anywhere else in the southeastern U.S. (Strom and Graves 2001). These high levels can be attributed in part to such point sources as municipal and medical incinerators, and electrical power plants (Strom and Graves 2001). Rates will likely rise even higher with the addition of a new commercial garbage incinerators in Palm Beach County, which will burn up to an estimated 3,000 tons of trash each day (Williams 2015).

Higher or lower rates in Hg deposition and environmental concentration, of course, do not necessarily reflect rates of bioaccumulation and biomagnification. Factors involving rates of methylation by sulfur- and iron- reducing bacteria are the major players in the process of bioaccumulation of MMHg, which can vary substantially depending on a variety of environmental factors (*e.g.*, pH, DOC/DOM, temperature, geographic region, chlorophyll a, other nutrient cycling) (Di Giulio and Hinton 2008, Sackett et al. 2010). For many reef fish species, fishing is the primary threat considered to affect their sustainability while habitat loss and pollution are rarely considered in stock assessments.

In this study, we addressed these questions: (1) Are there cryptic mortality or other unknown threats to the sustainability of these stocks? (2) Are there threats to the health of these fish and to human consumers? Recovery of protected populations requires that they have high quality habitat, high quality offspring, and high survival rates. We contend that Goliath Grouper recovery is compromised in some habitats, and must be considered in the management and conservation of this species. Data collected thus far (Adams and Sonne 2013, our unpublished data) suggest that Goliath Grouper's health, immune systems, reproductive success, and survival of all life stages are compromised by having heavy Hg loads.

Mercury occurs in the environment in elemental and methylated forms, the latter being the most toxic form. But tracing the sources or pathways of transfer through the environment is not an easy task. Recent studies reveal that Hg isotopes, however, opens an entirely new avenue of investigation using Hg isotopic systematics to trace the sources and processes of Hg transfer and transformation (Bergquist and Blum 2007, Sonke 2011)—much like how N and C isotopes are used to investigate trophic level and carbon source, respectively. Mercury stable isotopes can fractionate either as a function of their mass (MDF) or independently of their mass (MIF). While MDF has the potential to occur during all chemical transformations—lighter isotopes react faster than heavier ones, and consequently the product of the reaction is enriched in lighter isotopes relative to the remaining reactant—MIF has been identified only for odd Hg isotopes (199 and 201 amu) predominantly during photochemical transformations (Bergquist and Blum 2007).

In the aquatic environment, Hg isotopic fractionation occurs both before and after entering the food chain. Before entering the food chain, fractionation occurs in response to MMHg formation (Rodríguez-González et al. 2009, Perrot et al. 2015), to MMHg and inorganic Hg (iHg) degradation via photochemical (Bergquist and Blum 2007, Chandan et al. 2014) or microbial processes (Kritee et al. 2009), or by sorption/desorption to/from particulate material (Wiederhold et al. 2010, Jiskra et al. 2012). After entering the food chain – that is, once it has entered an organism's tissues, fractionation can be triggered by bioaccumulation, trophic transfer, and metabolism of Hg.

While this process has been reported in mammals (Perrot et al. 2012, Sherman et al. 2013), its occurrence in fishes is less certain (Kwon et al. 2012, Kwon et al. 2013, Xu and Wang 2015) – although recently published research shows that some fishes (*e.g.,* gag *Mycteroperca microlepis* and tilefish *Lopholatilus chamaeleonticeps*) have specific isotopic composition in their organs (muscle and liver) indicative of *in-vivo* MMHg demethylation (Kružíková et al. 2013, Yamashita et al. 2013). Combining the measurement of Hg species (MMHg and iHg) concentrations and Hg stable isotopic composition in target tissues (predominantly liver and muscle) of Goliath Grouper has provided valuable information on: (1) Hg levels of exposure, (2) possible organ dysfunction due to Hg poisoning, (3) Hg transformations, and (4) the distribution, transfer and metabolization of Hg species.

Why the difference between sexes in Goliath Grouper? It may be that females — which are batch spawners, releasing hundreds of millions of eggs during each spawning event — are reducing

their Hg burdens by offloading Hg into gamete production, transferring some of the maternal Hg burden to the embryo during synthesis and incorporation of a yolk protein. Mercury exposure can adversely affect reproductive potential through changes to ovarian morphology, delaying oocyte development, and through inhibition of steroid hormone synthesis--although it remains unclear whether these effects of Hg are direct or indirect (Hammerschmidt and Sandheinrich 2005, Crump and Trudeau 2009) .

We tested this hypothesis by directly measuring Hg in collected egg samples. Indeed, our results from evaluating Hg levels in gonad biopsies (~0.2 g tissue) of reproductively active females (n = 10) revealed mean total Hg levels of 0.15 ppm and maximum total Hg levels of 0.45 ppm wet weight. Exposure to levels this high are known to diminish reproductive potential of maternal fish by reducing egg viability and hatching success of embryos in experimental studies (e.g., Hammerschmidt and Sandheinrich 2005). For example, increased mortality rate in Rainbow Trout (*Onchorhynchus mykiss*) was associated with egg Hg levels as low as 0.07-0.10 ppm wet weight (Birge et al. 1979). Thus both for large males and larval fishes, we suggest that one repercussion of high Hg loads, is higher mortality.

It is critical for stock assessment that we understand if higher mortality rates exist among males and if they are indeed a consequence of higher Hg levels. This loss of large males from the population over the last 6 years requires further investigation. Additionally, these attributes need further, more detailed study, with expansion to include other species, additional tissues for Hg analysis, and better estimates of mortality.

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Part VI. Non-lethal analyses of stabile isotopes and micro-constituents in fin rays.

Two publications in the appendix:

"Tzadik, OE, EB Peebles and CD Stallings. 2016. Life-history studies by non-lethal sampling: using microchemical constituents of fin rays as chronological recorders. J Fish Biol. doi:10.1111/jfb.13156",

"Tzadik, O.E., E. A. Goddard, D.J. Hollander, C.C. Koenig, C.D. Stallings. (2015). Non-lethal approach identifies variability of δ 15 1 N values in the fin rays 2 of Atlantic Goliath Grouper, *Epinephelus itajara*. *http://dx.doi.org/10.7717/peerj.1010*."

OVERVIEW:

The study of diet and movements in fishes is often logistically challenging. Trace element and stable isotope analyses have advanced these fields considerably, but are still constrained by methodological impediments, such as lethal sampling, which is inappropriate for threatened or endangered species. Studying endangered fishes is particularly challenging as representative samples are difficult to obtain. However, the information gained from such studies is often critical to the recovery of endangered fishes as knowledge of life history attributes has the potential to greatly influence the success of management strategies.

Doctoral student (USF), Orian Tzadik, tested the applicability of using fin rays in fishes as a non-lethal approach to study diet and movement patterns over time. He then applied the methods to study life history aspects of Goliath Grouper. Fin ray analyses have traditionally been used in age and growth studies, as well as in a limited number of projects that study the chemical constituents of the ray itself. Therefore, fin rays were first tested as chronological recorders of chemical properties over time by using aquarium fish in which the time of capture was known. Based on the assumption that a chronological series of trace elements and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) are conserved in otoliths (much data from other studies support this assumption), comparisons were made between fin rays and otoliths of these fishes to determine differences between wild and captive phases in each individual. Divalent ions and positively-charged transition metals (e.g., Fe, Co) levels, in particular, were in close correspondence between the two structures, indicating conservation of incorporated materials in the fin rays and suggesting that fin rays could be used to document historical patterns of diet and movement.

Tzadik then tested and modeled the differences in $\delta^{15}N$ values over time between the populations of Goliath Groupers on the west and east coasts of Florida. In general, individuals on the west coast had lower overall values and a larger difference between juvenile and adult values. The mechanism that caused the differences between coastal populations may have been an artifact of the environment, rather than different feeding behaviors.

Upon the establishment of the use of fin rays as a potential non-destructive method to trace chronological life history changes, Tzadik investigated Goliath Grouper nursery habitats in southwest Florida. Fin rays from captured juvenile Goliath Groupers were excised and analyzed to determine if patterns of trace elements in the rays could be used to characterized juvenile nurseries (juvenile Goliath Grouper have a very long sojourn of about 5 years in the juvenile mangrove habitat, so it was not necessary to discern among annuli in the fin rays.) Nursery habitats could be distinguished with considerable accuracy. Thus, this non-destructive method can be used to identify nursery habitat for this, and potentially for other species, threatened or not, with a juvenile estuarine phase. Such information would be useful in determine the most useful habitats for various species.

Overall, Tzadik's research demonstrated the efficacy of novel techniques—fin ray micro-constiutent analyses— used to gather life history information on an threatened/endangered fish. Results from this work are useful to fishery management in the US and in other countries where this and other endangered species require information on nursery habitat use.

Manuscript title: Tzadik, OE, DL Jones, EB Peebles, CC Koenig, CD Stallings. In prep. The effects of spatial scale on assigning nursery habitats in Atlantic Goliath Groupers (*Epinephelus itajara*) using non-lethal analyses of fin rays.

Abstract We evaluated Atlantic Goliath Groupers, *Epinephelus itajara*, in their nursery habitats via microchemical analyses of fin rays. Juveniles were sampled from known nursery habitats off southwest Florida, and adults were primarily sampled from a spawning aggregation off southeast, Florida. We collected fin rays using a non-lethal technique that is minimally invasive with no known negative effects on growth or survival. Trace-metal constituents in the fin rays were quantified with an

inductively coupled plasma mass spectrometer via laser ablation (LA-ICP-MS). Two spatial scales were quantified to test the limitations of grouping individuals based on elemental compositions. On a small spatial scale (i.e., 100's of meters), individuals were correctly classified within individual watersheds 64% of the time. On a larger spatial scale (i.e., 10's-100's of kilometers), juveniles were classified with 100% accuracy. Trace metals in adults were analyzed by back-tracking across fin-ray annuli to a year in which our previous studies have shown they occupied their juvenile habitats (i.e., 2006). These fish were grouped using a measure of dissimilarity, and then analyzed to test whether we could re-classify them into these same groupings based solely on the chemical components in their fin rays, which was done with over 84% accuracy. Although juvenile habitats of the adults could not be determined due to the lack of baseline data, classifications were driven by similar elements to those that drove the juveniles, suggesting similar physiological mechanisms. The results highlight the importance of spatial scale in the interpretation of microchemical analyses on calcified structures in fishes.

Key Words Fin Ray Chemistry · Ten Thousand Islands · Natural Tags · Nursery of Origin · Trace Element Analysis · Chemical Fingerprints

INTRODUCTION

Estuarine habitats have long been assumed to be important nurseries for many fishes and invertebrates based on the observed high abundances of juveniles associated with them (Beck et al. 2003). However, the relative contribution of juveniles from a particular habitat to the adult population, by way of ontogenetic migrations, is a more meaningful criterion for "essential nursery habitats (ENH)," than abundance alone (sensu Beck et al. 2001, Stoner 2003, Dahlgren et al. 2006). ENHs are particularly relevant to recovering stocks of depleted species, as recruitment success can be paramount to the persistence of their populations (Sheaves et al. 2006).

The ability to track members of the adult population to their juvenile habitats offers a quantifiable metric to assess ENH and can direct the management of endangered species by suggesting preservation sites at nursery grounds. Until recently, the research on ENHs has been largely theoretical due to the difficulties associated with tracking individuals throughout the course of their ontogenetic migrations (i.e., measuring movement between juvenile and adult habitat). Tagging studies that aim to quantify the contributions of juvenile habitats are costly and often suffer from low return rates (Pine et al. 2003). Studies that use natural tags offer a viable alternative, but still require the characterization of individual nurseries on multiple spatial scales so that adults can be traced back to their nursery origin (Gillanders et al. 2003). Spatial heterogeneity confounds background levels of trace elements in the marine environment and may result in unique background signatures, in time and space, at very small spatial scales that cannot then be characterized on larger scales (e.g., Gao et al. 2009). Chemical heterogeneity in the marine environment may limit the quantification and characterization of habitats on different spatial scales, depending on the ecosystem. Traditionally, the study of ontogenetic movements of marine fishes has relied on otolith microchemistry (Gillanders and Kingsford 2000, Hobbs et al. 2010, Mercier et al. 2011), requiring sacrifice of the study organisms.

The Atlantic Goliath Grouper (*Epinephelus itajara*) is critically endangered throughout its range (Pusack and Graham 2009) and is extirpated in waters off western Africa (Craig et al. 2009). As a result of their exceptionally low abundances, a federal moratorium in the United States has prohibited landings of the species since 1990 in US continental waters (primarily off Florida). In the early 2000s, the *E. itajara* population in Florida waters began showing early signs of recovery, initially off the southwest coast, and more recently throughout the state (Koenig et al. 2011).

The ongoing recovery of *E. itajara* in Florida highlights the role that an ENH can play in the restoration of a depleted population (Koenig et al. 2007). Postlarval juveniles of the species settle into leaf litter in mangrove lagoons (Lara et al. 2009). They remain in the mangrove ecosystem for the initial 4-7 years of their lives, where they typically inhabit deep undercuts and submerged structure such as mangrove roots (Koenig et al. 2007). Indeed, the extensive and intact mangrove habitat off the southwest coast of Florida in the Ten Thousand Islands region (TTI) is the presumed ENH for the species and is thought to be largely responsible for its recovery (Koenig et al. 2007). The TTI borders the Big Cypress National Preserve, which prohibits development and limits anthropogenic influences. As a result, the mangrove habitat in the TTI has relatively high water quality (Fourqurean et al. 2003), which may produce ideal conditions for the ENH of *E. itajara*. However, the information currently available regarding nursery use is based on tagging studies with tag returns of less than 5% for juveniles that were tagged and then recaptured as adults (Eklund and Schull 2001, Koenig et al. 2011).

Our objectives were to characterize the *E. itajara* juvenile habitats at multiple spatial scales within the TTI region and the surrounding areas in order to measure future contributions to the adult population. Specifically, we identified chemical indicators, or "fingerprints," of juvenile habitats by sampling multiple individuals within each location. Due to the endangered status of *E. itajara*, we employed a non-lethal and minimally invasive technique to study microchemical trends among individuals as an alternative method to examine nursery habitats at multiple spatial scales. Specifically, we identified the chemical fingerprints of juvenile habitats embedded in fin rays of both juvenile and adult Goliath Grouper. Our approach was possible because the annuli within the fin rays of *E. itajara* correspond to yearly depositions (Clarke et al. 2007, Murie et al. 2009), which retain chemical properties over time (Tzadik et al. 2015).

MATERIALS & METHODS

Sample Collection and Study Area

We collected fin rays from 40 juveniles in southern Florida. Sampling occurred during June through August, 2014 in the TTI region, Pine Island Sound, and the Lower Florida Keys, which are all areas where elevated levels of *E. itajara* juveniles have been previously documented (Fig. 1). Due to the ENH-role previously suggested in the TTI region for *E. itajara* (Koenig et al. 2007, Lara et al. 2009), we focused more effort there compared to Pine Island Sound and the Lower Florida Keys.

Sampling sites within TTI were categorized by watershed and drainage basin (Fig. 1). All juvenile sites had high-relief, subtidal structure. Most sites were natural habitats such as mangrove prop-roots or rock undercuts, but three sites were artificial structures such as ship wreckage and concrete pilings. We also sampled 54 adults from known spawning aggregations (Koenig et al. In Press) at offshore locations in southeastern Florida during August through September, 2013, and an additional 11 adult samples collected in the TTI were donated from collaborating fishermen. All adult sites had high structural relief, whether the reef habitats were natural or artificial.

Juvenile Sampling

Juveniles were captured using traps, set lines, and hand lines. Blue-crab traps (61 cm x 61 cm x 46 cm) were used based on previously documented effectiveness (Koenig et al. 2007). They were constructed of coated-wire mesh with two funnels (proximal openings of 19 cm x 12.5 cm and distal openings of 18 cm x 7.5 cm) leading into the lower chamber and another two funnels (both proximal and distal openings of 18 cm x 7.5 cm) leading into the upper chamber. Traps were placed next to mangrove roots, primarily in low-current canals, and weighted using l kg lead weights. Roughly two thirds of all traps were baited (using dead baitfish, e.g., *Ariopsis felis*, *Bagre marinus*, *Lagodon rhomboides*, *Orthopristis chrysoptera*), while the remaining traps were un-baited.

Set lines were made using 14/0 or 15/0 circle hooks that were attached to 50 cm of 400kg test monofilament. The monofilament sections were attached to 3-4 m of 0.16 cm stainless steel cable with a 170 g weight to keep the line taught. The end of the cables were attached to an 8/0 gangion clip. Lines were baited with either live or dead *A. felis*, *B. marinus*, *L. rhomboides*, or *O. chrysoptera*. We attached all lines to mangrove prop-roots in areas with deep undercuts and high currents.

We used hand lines opportunistically in locations where set lines were not practical, such as areas of exceptionally high currents or where water clarity allowed snorkelers to place the bait directly in front of the fish. Hand lines comprised a 15/0 circle hook and two 170 g weights attached to 135 kg test monofilament that was wrapped around a hand reel. Hooks were baited with either live or dead *A. felis*, *B. marinus*, *L. rhomboides*, or *O. chrysoptera*.

After capture, juveniles were tagged ventrally with individually numbered stainless-steel-core internal-anchor tags (Floy Tag Company) and measured for total length. We excised soft-dorsal fin-rays six and seven to maintain consistency with a companion study (Koenig et al. 2015). Fin membranes on

the anterior and posterior sides of the two rays were cut with a knife to the base of the fin and then excised as close to the base as possible, using 15 cm cutting pliers. Juveniles were never held out of the water for more than 3 minutes.

Adult Sampling

Adults were captured using hand lines in collaboration with a companion study to determine the age structure of *E. itajara* in Florida (Koenig et al. 2015). After capture, adults were measured for total length and tagged both externally (livestock tag) and internally (Passive Integrated Transponder). Again, we removed the soft-dorsal fin-rays six and seven in the same manner as described for the juveniles. Sampling adults typically took 5-10 minutes, so we flushed ambient water over the gills and placed a damp towel over the eyes while the fish was on deck being processed. Individuals were released immediately following sampling.

Fin Ray Analysis

Immediately after excision, fin rays were bagged, labelled, and stored on ice. Samples were stored in a freezer at -20^0 C until further processing. Fin rays were thawed by removing them from the freezer and then immediately placed in a drying oven for 3 hours at 55^0 C. Once thawed, the fat, membrane, and muscle tissues were removed using rubber-tipped forceps. We then soaked the rays in trace-metal grade 30% hydrogen peroxide (H_2O_2) for 5 minutes to loosen any remaining tissues, which were removed using rubber-tipped forceps and paper towels.

Once cleaned, each ray was attached to a petrographic microscope slide using Crystalbond™ adhesive (SPI Supplies, Westchester, Pennsylvania, USA). Two cross sections, each 0.5 mm thick, were cut from the ray as close to the base as possible using a Beuhler IsoMet™ slow-speed saw (Beuhler, Lake Bluff, Illinois, USA). We used one cross section for aging and the other for chemical analysis. Cross sections did not typically require polishing to expose the annuli, but when necessary, we polished the section using 800-grit wet sandpaper. Cross sections were independently aged by two readers. If there was disagreement between age estimates, a third reader was used. All adult samples were also sent to the age and growth lab at the University of Florida for additional age verification.

The second cross section from each fish was mounted on petrographic slides using Crystalbond™ and sonicated in ultrapure Milli-Q™ water for 5 minutes. After sonication, samples

were air dried for 24 hours in a class-100 laminar flow clean hood. The second sections were attached to acid-washed petrographic slides so that roughly 20 samples were attached to a single slide. All samples were assayed using an Agilent Technologies 7500 ICP-MS coupled with a Photon Machines Analyte 193 excimer UV laser ablation system (LA-ICP-MS).

We used a sequence of replicate spot samples (n = 3) of 64 μm diameter at the outer-most annulus for juvenile samples and the annulus corresponding to the year 2006 for the adult samples. The year 2006 was chosen for analysis as the majority of the adults were believed to still be in their nursery habitats at that time, based on their ages. The laser system operated at a wavelength of 193 nm and a set point of 7.0 mJ. Fin-ray ablations were conducted with 86% power and a 5 Hz frequency. Background levels were collected for 60 seconds between each spot scan. We used a single glass standard (NIST 612) with known isotopic compositions to calibrate the instrument. The NIST 612 standard was analyzed prior to and after each sample slide. We also analyzed the standard after every two samples to account for instrument drift. Measurements were made for 26 unique isotopes[3] to quantify the trace elemental compositions within the structure. An internal standard is essential to these measurements due to biases in yield that are apparent during the ablation process over an irregular surface such as fin-ray sections. Calcium (Ca) was used as the internal standard due to its abundance and stoichiometric consistency in hydroxyapatite (Wopenka and Pasteris 2005). During a prior analysis using solution-based methods (SB-ICP-MS), Ca concentrations in fin rays were measured via digestion in 16 N HNO_3 within polypropylene vials at 180^0 C for 2 hours. Samples were diluted with 2% HNO_3. These solutions were then quantitatively analyzed in the ICP-MS to obtain Ca concentrations. Drift of the SB-ICP-MS was monitored and corrected using scandium (Sc) added as an internal standard,. The calibration line measured from 5 to 50 ppm for Ca. Based on our previous analysis, Ca concentration was measured as 27.5% of the molecular weight of fin rays.

The Agilent Technologies Instrument control software was used for data collection. Isotopic values of each element of interest were recorded as counts per second. These counts were then converted to concentration (ppm) using Matlab version R2015a, with functions created in the Fathom Toolbox for Matlab (Jones 2014). We used ppm values in all subsequent analyses.

[3] Li^7, Na^{23}, Mg^{24}, P^{31}, Ca^{43}, Sc^{45}, V^{51}, Cr^{53}, Mn^{55}, Fe^{57}, Co^{59}, Ni^{60}, Cu^{63}, Zn^{64}, Cu^{65}, Ge^{72}, Rb^{85}, Sr^{88}, Y^{89}, Cd^{114}, Sn^{118}, Ba^{137}, Au^{197}, Pb^{208}, Th^{232}, U^{238}

Statistical Analyses

We classified juvenile samples according to the location in which they were captured to test whether we could reassign them based on their chemical properties. Two separate groupings were created based on relevant spatial scales: 1) sites separated by 100s of meters (hereafter "small scale") and 2) sites separated by 10s of kilometers (hereafter "large scale"). Sites with less than three individuals were not considered due to the small sample size. Given that absolute concentrations of elements naturally varied by up to three orders of magnitude, we standardized them to z-scores to equally weight them (Legendre and Legendre 2012). All variables with measurements that were below the limits of detection were removed prior to further analysis. In order to test and visualize the differences among groups at each spatial scale, we used a canonical analysis of principal coordinates (CAP) based on a Euclidean distance matrix (Anderson and Willis 2003). The CAP generated a leave-one-out (LOO) cross-validation matrix, and we used a proportional chance criterion (PCC) to assess the performance of the CAP model and the probability that it performed better than a null model generated by random chance (Morrison 1969). Indicator values were calculated for elements with significant influences on the groupings (at $\alpha < 0.05$) via the indicator value method (IndVal, Dufrene and Legendre 1997).

For adults, we calculated a dissimilarity matrix for all samples because their nursery locations were unknown. A similarity profile analysis (SIMPROF) based on Ward's minimum variance method (Ward's Cluster Analysis) and a Euclidean distance matrix was implemented via the dissimilarity profile analysis (DISPROF) function in the Fathom Toolbox (Jones 2014). The DISPROF identified groups that were formed based on the dissimilarities of elemental compositions among individuals (Clarke et al. 2008). The IndVal method was used to identify indicator elements for each group. For Sr, a natural break existed at one standard deviation above the mean, between the 12 highest values and the remaining samples. High Sr values are representative of high salinity water, due to the influence from marine limestone and other sediments. These 12 individuals with the highest Sr values were presumed to have moved out of their nursery habitat, or still occupying up-river locations, by the year 2006, possibly due to size-driven egress (Koenig et al. 2007), and were therefore not representative of the juvenile habitats of interest (Elsdon and Gillanders 2003). We excluded these 12 individuals from further analysis.

Last, we used a random forest analysis on the remaining adult samples to model the relationship among elemental concentrations in fin rays and the DISPROF groups, while also re-classifying unknowns to assess the accuracy of the model (Breiman 2001, Cutler et al. 2007, Mercier et al. 2011). The forest was a collection of unique classification trees, each originating from a root node of a bootstrapped training dataset derived from the elemental concentration data. Data from each root node were successively divided into progressively smaller and more homogenous nodes (i.e., branches). At each node, a random set of predictor variables was analyzed to find the one that minimized the sum-of-squared errors among the remaining observations, which was then used to split the data. Trees were grown until the data at the terminal nodes could not be split into more homogenous groups. Once the trees were grown, fitted values of the categorical variable (i.e., the grouping vector) were assembled from their terminal nodes and weighted to produce the final predicted response of the forest. For the adult samples, we used a non-linear random forest model instead of a linear CAP model due to the better fit of the data.

RESULTS

Age estimates based on the cross sections of fin rays ranged from 2.0 to 6.2 years old for juveniles (median = 4.2) and from 5.0 to 14.0 years old for adults (median = 10.0). Total lengths ranged from 33.2 cm to 124.0 cm for juveniles (median = 62.0 cm) and from 122.0 cm to 222.0 cm for adults (median = 171.0). In the present study, we classified fish by habitat (juveniles in mangroves and adults on offshore reefs) instead of by age or total length.

Chemical Fingerprints in Juvenile Habitats

When juveniles were evaluated on a small spatial scale, six areas were classified into a grouping vector based on location. The chemical data from the juvenile fin rays were classified correctly 64% of the time with the output model created by the CAP (as compared to 18% by the PCC null, $p = 0.001$). Locations as close as 200 m apart were distinguished to be different by the CAP and were largely influenced by the relative concentrations of cobalt (Co) and barium (Ba) (Fig. 2 and Table 1).

When we categorized the juveniles into groups at the larger spatial scale, three areas were identified. The three groups comprised two sites within TTI and a third from the Lower Florida Keys. The classification success rate for the output model produced by the CAP was 100% (as compared to 42% by the PCC null, $p = 0.001$). Groupings at this spatial scale were precise with no apparent among-

group overlap (Fig. 3). The primary drivers of these classifications were Co and manganese (Mn) (Table 2). The majority of sites in the TTI region grouped together (largely driven by iron; Fe), while those from two southern TTI sites in Pumpkin Bay grouped on their own (driven by zinc; Zn, Ba, and magnesium; Mg). A third group was identified as samples from the Lower Florida Keys and was characterized by elevated levels of tin (Sn).

Adult Classification

The DISPROF clustering method identified four groups ($p < 0.05$) from the adult samples (Fig. 4). These groups varied in size (i.e., $n = 25$, $n = 23$, $n = 13$, $n = 4$). Note that all individuals that were subsequently removed from further analyses due to high Sr values came from a single group (Fig. 4, Group B). The output model produced by the random forest clustered samples with a classification rate of 85% (as compared to 32% by the PCC null, $p = 0.001$) and was significantly driven by six elements: Mn, Fe, Sr, Sn, Ba, and lead (Pb) (Fig. 5 and Table 3).

DISCUSSION

Juvenile habitats used by *E. itajara* can be accurately distinguished at varying spatial scales in the state of Florida using the microchemical analyses of fin rays. The chemical fingerprints that were incorporated into the fin rays of *E. itajara* acted as natural tags that allowed us to classify specific locations where individuals were sampled. The use of these natural tags may be used to augment tagging studies that commonly experience low return rates. Using a baseline of chemical fingerprints (composed of juvenile fin-rays, sampled yearly via a random-stratified sampling design), individuals of unknown origins can be classified by nursery location.

The current application of our methodology was used to identify and characterize juvenile habitats for *E. itajara*. However, the methods are applicable to studying movements and ontogenetic migrations in other fishes. Indeed, our methods were largely derived from studies that tracked movements in diadromous fishes over long periods, in some cases over 30 years (Allen et al. 2009, Jaric et al. 2012). Due to the preservation of chemical properties, as previously documented in fin rays (Tzadik et al. 2015, Tzadik et al. *in press*), we suggest that juvenile habitats can be assigned to species of interest over long time periods.

Chemical Fingerprints of Juvenile Habitats

Chemical fingerprints of juvenile habitats were distinguishable at two spatial scales. At the small spatial scale, the relatively high correct classification rate demonstrated that the chemical fingerprints in our study system were distinctive even for closely located sites. Indeed, individuals from two sites (groups 1 and 2 in Fig. 2) that were separated by only 157 m (Fig. 1) were distinguishable based on the concentrations of trace elements in their fin rays. Most trace element concentrations in the body parts of fishes are thought to derive primarily from ambient water chemistry (Kerr and Campana 2014), and previous tagging studies have clearly demonstrated high site fidelity of juvenile *E. itajara* at similarly small spatial scales (Eklund and Schull 2001, Koenig et al. 2007, Koenig et al. 2011). Thus, the differences in fin-ray chemistry may have derived directly from differences in ambient water chemistry at these two sites. The high indicator value for Ba in the division between the two sites may suggest that it was driven in some part by haloclines in the mangrove lagoons, particularly as Ba is derived almost exclusively from the ambient water as opposed to diet (Walther and Thorrold 2006). Regardless of mechanism, the presence of small-scale microhabitats may have some utility for informing management (e.g., determining boundaries of nursery reserves), and the life history of *E. itajara*. Sequential microhabitat use and strong site fidelity occur in the juvenile phases of other estuarine fishes (e.g., Brame et al. 2014) and have been suggested for *E. itajara* based on observational and tagging studies (Koenig et al. 2007, Lara et al. 2009). Using natural tags in fin rays, future studies can expand upon current knowledge of microhabitat use by *E. itajara* in its juvenile phase. However, the unique chemical fingerprints among microhabitats may confound results of future studies, as comprehensive sampling across all locations may not be feasible.

The groups that formed at the large spatial scale, which had reclassification accuracy of 100%, are likely more relevant for management and conservation purposes under most circumstances. The mechanisms that drive different chemical fingerprints at this scale are more interpretable than at a small scale (due to a stronger signal-to-noise ratio), and may be directly influenced by both natural and anthropogenic processes in the vicinity.

The main TTI group was largely driven by Fe concentrations (Fig. 3), which are physiologically regulated (Gauldie and Nathan 1977). Importantly, the TTI group was not characterized by elements with anthropogenic sources (e.g., Zn, Cu), which suggests the juvenile habitats had minimal anthropogenic influence. In contrast, the combination of elements from the samples collected at Pumpkin Bay may have resulted from the upstream water source, the Faka Union Canal, which is dredged and has more boat traffic (Browder et al. 1986). Indeed, the downstream water of the two bays

that neighbor Pumpkin Bay (i.e., Faka Union Bay and Fakahatchee Bay), as well as that of Pumpkin Bay, is influenced by the outflow of the Faka Union Canal (Browder et al. 1986). The Faka Union Canal effectively starts at a dam location that traps freshwater from the Everglades and periodically flushes into the canal. The dam location also houses a marina and a frequently used boat ramp. The freshwater input over the dam could contribute to the high concentrations of Ba, while the heavy boat traffic could contribute to the elevated levels of Zn and Mg, which are commonly used as sacrificial anodes on boat engines (Shanmugam et al. 2007).

The elements that were characteristic of the group from the Lower Florida Keys may be reflective of heavy boat traffic in the area, as Sn is a common alloy used in the forging of industrial metals, particularly aluminum, a common material used in the marine industry (Li and Feng 2003, Yan et al. 2013, Naeem et al. 2014). High levels of Sn may also result from use of illegal anti-fouling agents containing the element in the region, even though such agents (primarily tributyltin) have been banned in the United States for several decades (Yebra et al. 2004).

Adult Stock Origins

Five of the six significant indicator elements that were the most influential in clustering the adult samples (i.e., n = 53) were also drivers in the juvenile analyses, suggesting a similar mechanism of elemental substitution and retention (Tables 1, 2 and 3). Pb, which is often associated with fuel docks (Duarte et al. 2012), was the only element unique to the adult analysis, possibly as a result of individuals living near fueling stations. Three other ordinated groups were evident from the random-forest classification of the adults. One was characterized by the abundance of Sr and Sn, a second by Ba and Mn, and a third group by lower abundances of most of the elements measured. The lack of a baseline from 2006 (i.e., fin rays from juveniles sampled in all possible nursery habitats from that year) precludes the possibility of re-classifying adults into their nursery habitats. However, the grouping of adults, as influenced by nearly the same elements as the juveniles in 2014, suggests that similar mechanisms may be driving the groupings found in both adults and juveniles.

Conclusions and Implications

The technique used in the current study can be used to study ENHs of endangered fishes and others of management concern. The present study is the first of which we are aware to use fin rays to establish chemical fingerprints with the objective of discerning ENH in a marine fish. Future

applications include long-term monitoring projects that could be used to re-classify members of adults stocks into their nursery habitats. Chemical fingerprints can act as natural tags and are imprinted onto every individual in a population, thereby increasing inference to the entire population, rather than only the ones with implanted tags. However, the microchemical variability on exceptionally small scales can present challenges to future work on essential nursery habitats in marine ecosystems. Specific to our study, the ability to assign individuals to nursery habitats among the northern TTI bay system, the Faka Union Bay system (i.e., Pumpkin Bay, Faka Union Bay and Fakahatchee Bay), and the Lower Florida Keys, suggests that the role of spatial scale in habitat classification is paramount to studies that aim to quantify nursery habitats. Future research for *E. itajara* should aim to classify additional habitats, possibly via an annual random-stratified sampling design to minimize the possibility of type I errors (i.e., a false positive) in the reclassification of adults.

Similar techniques, using otoliths, have been used for the same purposes, but require lethal sampling. The use of fin rays allows non-lethal sampling and can be used in a manner similar to otoliths to differentiate among nursery habitats. The process outlined in the current study is particularly relevant for recovering stocks, such as *E. itajara*, that must depend on their ENHs to help rebuild their population.

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TABLES

Table 1. Indicator values for the significant elements when juvenile samples were grouped on a small spatial scale. IndVal = Indicator Value, P-Value = Significance based on 1000 permutations.

Element	Atomic Weight	IndVal	P-Value
Li	7	22.59	0.001
Na	23	20.40	0.001
Mg	24	19.09	0.001
V	51	31.00	0.013
Fe	57	19.65	0.001
Co	59	31.73	0.001
Zn	64	22.49	0.004
Rb	85	22.41	0.009
Sr	88	18.13	0.026
Ba	137	26.98	0.005

Table 2. Indicator values for the significant elements when juvenile samples were grouped on a large spatial scale. IndVal = Indicator Value, P-Value = Significance based on 1000 permutations.

Element	Atomic Weight	IndVal	P-Value
Na	23	38.03	0.003
Mg	24	35.50	0.016
Mn	55	44.99	0.014
Fe	57	39.20	0.001
Co	59	69.32	0.001
Zn	64	40.72	0.007
Ba	137	43.27	0.029

Table 3. Indicator values for the significant elements when the adult samples were grouped. IndVal = Indicator Value, P-Value = Significance based on 1000 permutations.

Element	Atomic Weight	IndVal	P-Value
Mn	55	30.05	0.033
Fe	57	18.14	0.029
Sr	88	17.68	0.006
Sn	118	26.64	0.010
Ba	137	30.62	0.006
Pb	208	27.36	0.045

Figure Captions

Fig. 1 Map of sampling locations for juveniles in southern Florida, with a diagram of the life history of Goliath Groupers (inset). Numbers on the map correspond to sample sites for Fig. 2. The large "X" represents the sampling location of adults in southeastern Florida

Fig. 2 The canonical analysis of principal coordinates (CAP) for juveniles that were analyzed on a small spatial scale. The length of each vector corresponds to its relative importance in grouping individuals in the direction in which it is pointing. Correlation vectors directly correlate and are proportional to the ordination plot. Site numbers correspond to those presented in Fig. 1

Fig. 3 The canonical analysis of principal coordinates (CAP) for juveniles that were analyzed on a large spatial scale. The length of each vector corresponds to its relative importance in grouping individuals in the direction in which it is pointing. Correlation vectors directly correlate and are proportional to the ordination plot. Site labels run from north to south, (i.e., T = TTI northern bay system, sites 1, 2 & 3; P = Pumpkin bay system, sites 4 & 5; K = Lower Florida Keys, site 6)

Fig. 4 A DISPROF-based cluster analysis for the adult samples in the study. Solid lines indicate significant divisions for classification. These groupings were used in the subsequent random forest

Fig. 5 Adults that were analyzed via a random forest from Fig. 4. The length of each vector corresponds to its relative importance in grouping individuals in the direction in which it is pointing. Correlation vectors directly correlate and are proportional to the ordination plot

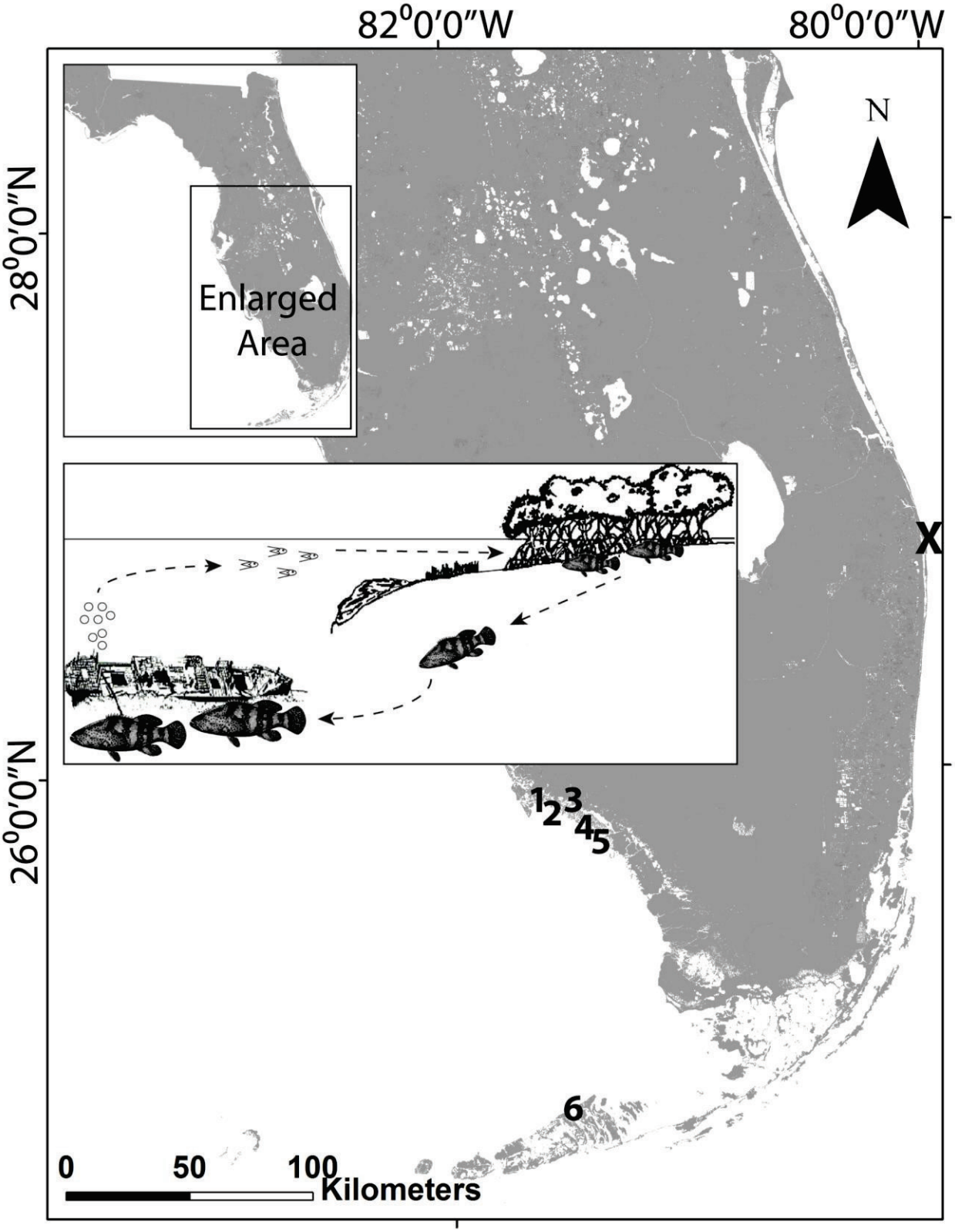
82°0′0″W
80°0′0″W
28°0′0″N
26°0′0″N
N
Enlarged Area
X
1
2
3
4
5
6
0
50
100
Kilometers

Fig 1

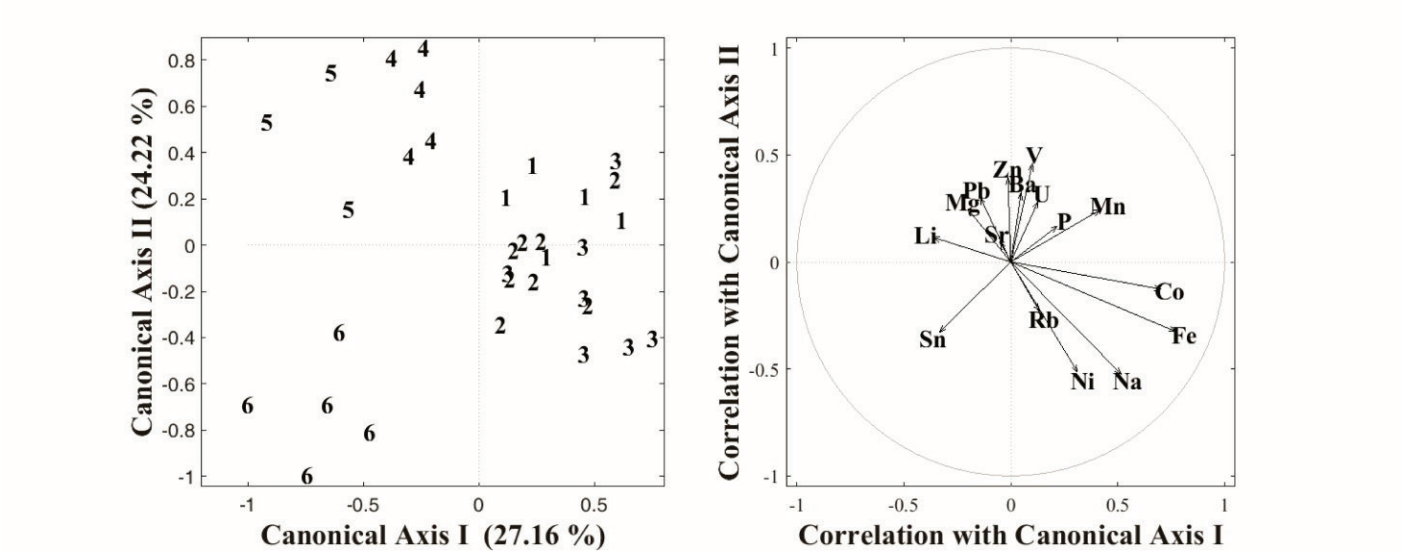
Canonical Axis II (24.22 %)
Canonical Axis I (27.16 %)
Correlation with Canonical Axis II
Correlation with Canonical Axis I
V
Zn
Ba
U
Pb
Mg
Mn
P
Li
Sr
Co
Rb
Sn
Fe
Ni
Na

Fig. 2.

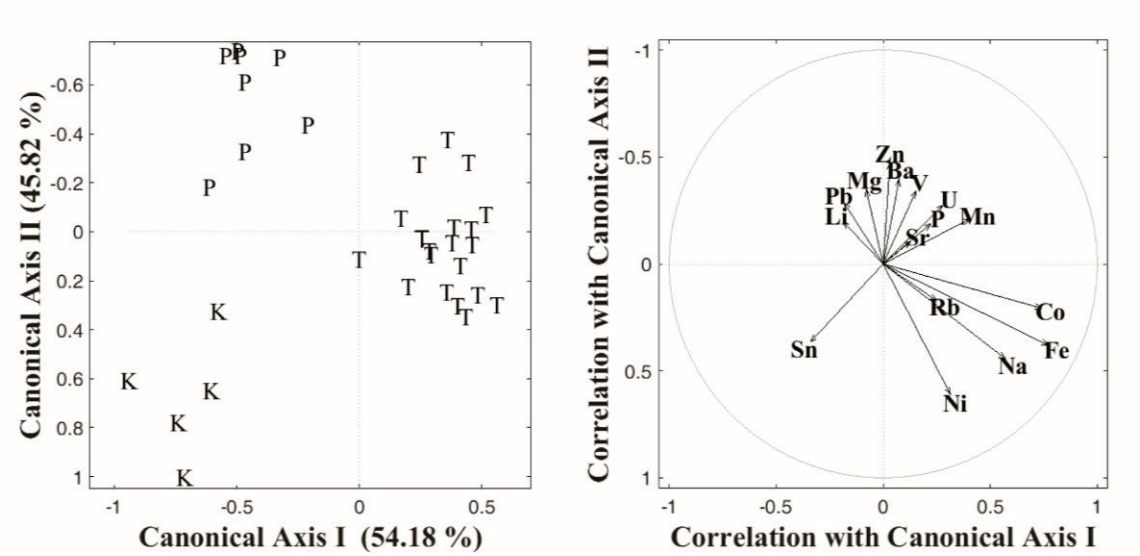
Canonical Axis II (45.82 %)
Canonical Axis I (54.18 %)
Correlation with Canonical Axis II
Correlation with Canonical Axis I
Zn
Mg
Ba
V
Pb
U
Li
P
Mn
Sr
Rb
Co
Sn
Fe
Na
Ni

Fig. 3.

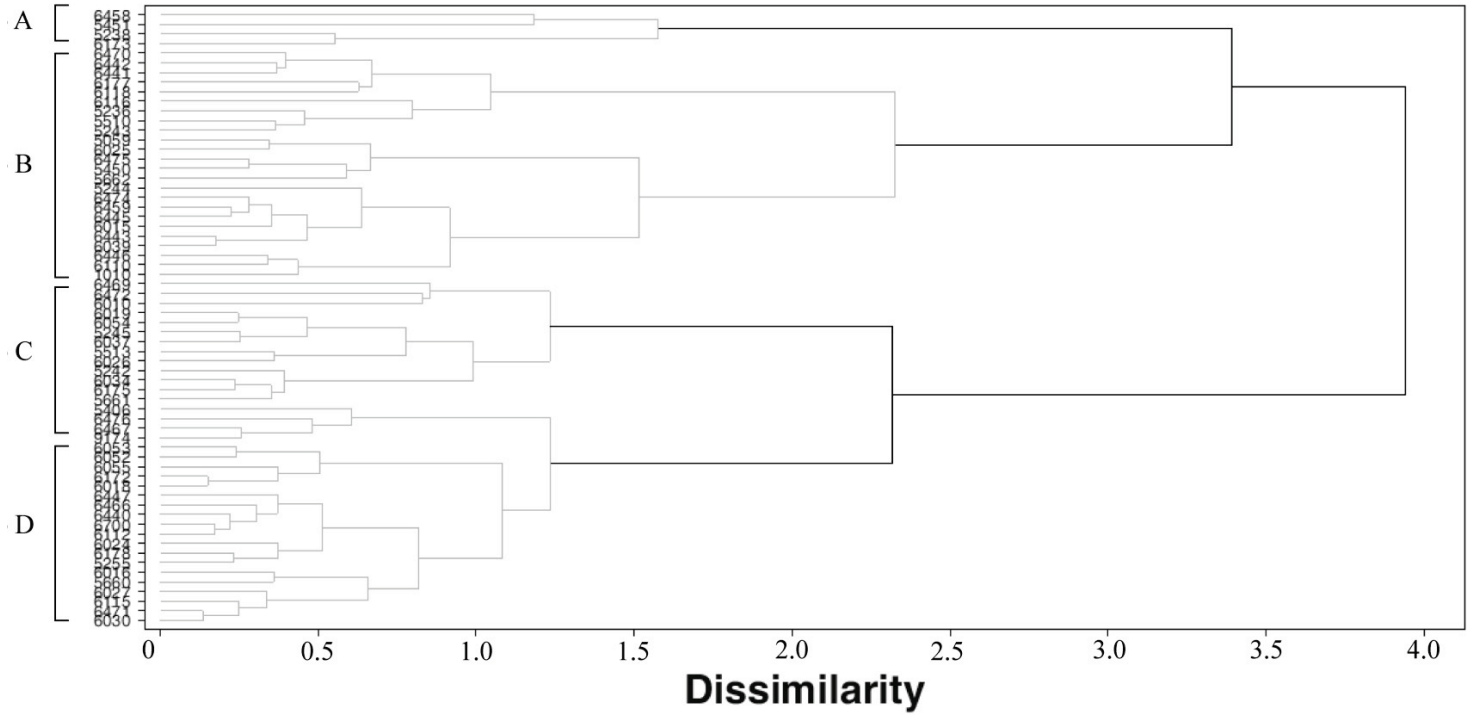

Fig 4

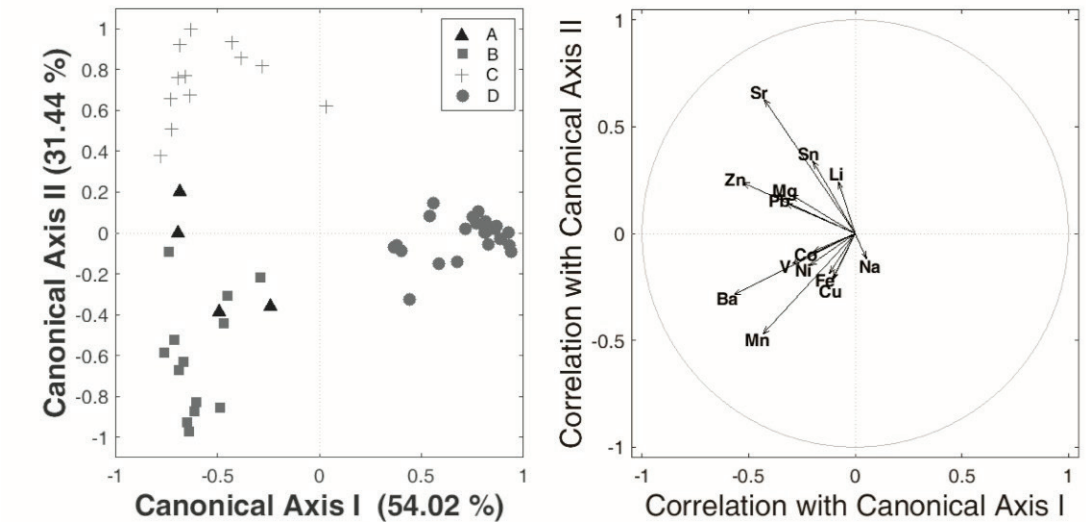
A
B
C
D
Canonical Axis II (31.44 %)
Canonical Axis I (54.02 %)
Correlation with Canonical Axis II
Correlation with Canonical Axis I
Sr
Sn
Li
Zn
Mg
Pb
Co
V
Ni
Fe
Cu
Na
Ba
Mn

Fig. 5.

Part VII. Concluding remarks:

Management of the Goliath Grouper fishery (Christopher Koenig, leader).

INTRODUCTION

The Atlantic Goliath Grouper (*Epinephelus itajara*) is a unique reef fish species that requires innovative methods of management. It is the largest reef fish in the western Atlantic, obtaining sizes of nearly 3 m and weights of 455 kg (1000 lbs; Robins and Ray 1986). The fish is valuable ecologically and economically—ecologically because it creates and/or enhances reef habitat structure through excavating behaviors that lead to higher biodiversity and abundance (Koenig et al 2011), and economically as a potential recreational fishery species and a dive ecotourism species (Lorenzen et al. 2013, Shideler et al. 2015, Shideler and Pierce 2016). Goliath Grouper is highly vulnerable to overexploitation as witnessed by its near extinction in the SE US during the 1980s and its designation as critically endangered throughout its range by the IUCN (International Union for the Conservation of Nature). In the US they were exposed to intense recreational and commercial fishing by both hook and line and spear fishing which quickly depleted the Gulf and Atlantic populations during the 1970s and1980s. Spawning aggregations occurring off SE and SW Florida, primarily in August and September, were favorite targets of the commercial fishery (Don DeMaria, personal communication). Complete protection was afforded by the Gulf and South Atlantic Fishery Management Councils in 1990, and the population has responded by continuing to recover in the SE US over the last 26 years. Any reoccurrence of the extreme exploitation levels of the 1970s and 1980s would rapidly undo gains in recovery made since 1990. Another factor in the decline of Goliath Grouper throughout its range is the loss of its essential juvenile habitat— mangrove forests (Valiela et al 2001, Ueland 2005, Koenig et al. 2007) by over one-third. And poor water quality has reduced the amount of effective habitat by even more (Koenig et al. 2007). This situation still exists in south Florida resulting in a bottleneck in the rate of recruitment, production and recovery.

Goliath Grouper is a valuable member of the shelf reef community of Florida. It feeds at a relatively low trophic level mostly on non-fishery species of crustaceans (crabs) and fishes (slow moving species such as burrfish), and it excavates sediment-smothered reefs thereby increasing habitat complexity and biodiversity (Koenig and Coleman 2009, Malinowski, this

report). High adult densities are positively correlated with high species diversity and high abundance of some economically important reef fish (Koenig et al. 2011). In addition, the great size of adults and their tendency to aggregate in relatively accessible, shallow-water locations during spawning times creates a unique spectacle for the eco-tourist dive industry. An entire dive-tourist industry is building in south Florida around the easy accessibility of these large fish, and sustained economic benefits to this industry depend on a healthy population of adult Goliath Grouper.

Divers from all over the world come to south Florida to see and photograph aggregations of this harmless fish. Divers can approach very closely for dramatic photographs of dozens of individuals, each weighing several hundred pounds—nowhere else in the world can divers have this experience. Shideler and Pierce (2016, Figure 2), in a survey of divers (n = 1537) in SE Florida, showed that non-resident divers were willing to pay on average around $300 to dive with 10 to 40 Goliath Groupers (larger aggregations occur on spawning sites) while Florida residents were willing to pay around $150 to $200 for the experience. On the other hand, Shideler et al. (2015) surveyed recreational fishermen and found that the mean willingness-to-pay to harvest a single Goliath Grouper was between $35 and $79. The important point here is that once a fish is harvested, it produces no more revenue, but live fish, easily accessible to the dive tourism industry, continue producing revenue year after year.

Most fishers want to see a fishery for adult Goliath Grouper because their perception of this fish is that they are depleting reef fish populations (Shideler et al. 2015). The truth is that Goliath Grouper, like many other species, is opportunistic predator and may take any struggling fish at the end of a fisher's line or spear, but they do not normally feed on these species. In that sense, Goliath Grouper are a nuisance to many fishers, but many fishers do not understand the difference between opportunistic feeding—taking an injured or struggling fish—and feeding within the ecological context of the fish's natural environment. Thus, erroneous perceptions arise among many fishers from the misinterpretations of their observations—many videos on the internet misleadingly show Goliath Grouper eating everything from sharks to snook, but in all such cases the depredation occurs because the prey species is either injured, exhausted or tethered at the end of a line or spear.

Of the respondents to the Shideler et al. (2015) survey over half thought that Goliath Grouper were eating "all the fish on the reef" despite extensive scientific research showing otherwise (e.g., Koenig and Coleman 2009, Koenig et al. 2011, Malinowski, this report). Many recreational fishermen do not realize that Goliath Grouper is a native species, and as such, has had millions of years to adapt to the reef ecosystems of the SE US. Non-native species, such as the invasive lionfish, heavily impact native species precisely because they have not evolved on the reef ecosystems of the SE US, and therefore, are not adapted to these systems. Lionfish truly do eat "all the fish on the reef" when those resident reef species arrive as new recruits (Albins and Hixon 2008).

At the time of the Shideler et al. (2015) survey of recreational fishermen (2013) and the similar survey of Lorenzen et al. (2013)—both showed similar results—it was not widely known that adult Goliath Grouper were heavily contaminated with mercury. The first comprehensive report of mercury contamination of Goliath Grouper (Adams and Sonne 2013) showed that all adults sampled from the Gulf of Mexico had mercury concentrations in their white muscle above the toxic limit of 0.5 ppm set by the US Food and Drug Administration (FDA) and the National Resource Defense Council (NRDC). These agencies recommend that all fish above this limit be avoided. Malinowski (this report) found similar heavy mercury contamination in Goliath Grouper adults from the Atlantic population. And both studies showed liver concentrations up to 10 time higher than white muscle levels and clear increases in mercury concentration with fish size and age, so juveniles (fish less than about 1200 mm (4 ft TL and about 70 lbs) have mercury levels below the critical levels set by the FDA and NRDC. Levels of mercury are so high in both Atlantic and Gulf adult populations that impacts on the health of the older fish are evident (Malinowski, this report). But, in terms of fishery considerations, adult Goliath Grouper are dangerous for humans to eat because mercury is a neurotoxin with strong teratogenic effects. More information at http://enhs.umn.edu/current/5200/mercury/intro.html .

Thus, it is clear that adult Goliath Grouper should not be fished, but there are other reasons besides unacceptably high levels of mercury, including: 1. the dive eco-tourism industry benefits greatly from large aggregations of adults, 2. a fishery for adults would diminish large old breeders, a segment of the population of all fishery species that should be preserved to maximize production—these old adults, known as BOFFFFs or Big Old Fat Fecund Female Fish

(Berkland and Dayton 2005, Hixon et al. 2014) contribute greatly to the reproductive potential of the population and 3. habitat structuring activities of adults are beneficial for biodiversity and abundance of reef ecosystems (Koenig et al. 2011).

Management considerations: economic value to divers and fishers.

Traditional stock assessments for Goliath Grouper have proven difficult. Some reasons are: 1. there are no catch data available for the past 26 years because the species has been and is completely protected, 2. the catch history prior to 1990 is sketchy at best. Thus, there is a poor understanding of the population in an unfished state, knowledge of which is necessary to derive management benchmarks. Thus, to establish reference points such as MSY and SPR50, stock assessment biologists must make unwarranted assumptions to support their selection of reference points—this is an unacceptable basis for managing fish stocks, so all earlier assessments have been rejected under peer review. Other complicating factors for sustainable management include temporal changes in habitat conditions for juveniles and adults. Primary juvenile habitat—mangrove forest (Koenig et al. 2007)—has suffered severe losses in both coverage and quality over the last century world-wide (Valiela 2001) including South Florida (Ogden et al. 2005, Ueland 2005 and many other studies), thus limiting the recruitment capacity for the species. Conversely, adult habitat has increased since the 1950s as the state of Florida continues to deploy high-relief artificial structures, a preferred habitat for adults (Koenig et al. 2011, Collins et al. 2015). Clearly, this species cannot be managed effectively by traditional stock assessment methods, but there are other options; reliable bench marks for the extant Goliath Grouper population may be obtained directly.

Ecological research has shown clear opportunities for assessing relative stock size and recruitment directly based on habitat and aggregating behavior of the species. For juveniles, we have previously shown that Jolly-Seber mark-recapture methods may be used to estimate absolute abundance and survival of juveniles during their 5-year sojourn in mangrove forests, a time when their home-range movements are highly restricted and survival is high (Koenig et al. 2007). Even more efficient estimations of juvenile relative abundance may be gained from use of the Everglades National Park annual creel survey data. For adults, diver surveys may be used, especially on fish aggregated on spawning sites, to obtain estimations of the relative abundance

of the spawning stock (using REEF methods http://www.reef.org/programs/volunteersurvey). Our studies (see Ellis et al., Part III, this report) show that adults are capable of migrating great distances (e.g., up to 500 km off east Florida, Ellis et al. 2014) to join spawning aggregations which are concentrated in relatively small areas in SE Florida (mostly off Palm Beach County). Similar spawning migrations occur off SW Florida (Koenig et al. 2011, Collins 2015). Thus, spawning aggregations represent spawning stock biomass from broad geographical regions. Size structure could be estimated using underwater video-lasers and age structure may be obtained by non-destructive fin-ray methods for juveniles (Brusher and Schull 2009) and adults (Murie et al. 2009, Murie et al., this report). Thus, direct measures of the relative abundance of juveniles and adults, as well as size and age may be obtained directly and efficiently.

How do we work from direct estimates of population size to establish fishery reference points for Goliath Grouper that will allow some removal of juveniles but maintains the population at some acceptable level for ecological and economic benefits? Wade (1998) introduced the concept of Potential Biological Removal (PBR) to establish limiting reference points for marine mammals. PBR is the maximum number of animals that may be removed from the population while still achieving recovery of a depleted population or subsequent maintenance of the recovered population near its carrying capacity. Wade (1998) found through extensive simulations that a very robust estimate of this limiting reference point is:

$$PBR = N_{min} \cdot \frac{1}{2} R_{max} \cdot F_r$$

where *N min* is the minimum population estimate of the stock, ½ *R max* is one-half the maximum net productivity (recruitment) rate, and *Fr* is a recovery factor between 0.1 and 1. A value of 1 for *Fr* allows no extra margin for error. Lower values of *Fr* are considered precautionary, for example an *Fr* of 0.5 would allow marine mammal populations to reach or maintain their carrying capacity with high probability (Wade 1998). Simulations where mortality was consistently greater than PBR had a 5% chance of depleting the population. The PBR reference point has not yet been rigorously tested for fish populations (Sainsbury 2008), but its characteristics suggest it could be used as an effective precautionary catch limit for data-poor species such as Goliath Grouper.

Therefore, limiting reference points— i.e., those reference points that indicate a population level at which fishing should be halted— should be established before any consumptive harvest is allowed. Such limiting reference points have previously been developed for marine mammals (Wade 1998) and proposed for use in other threatened, endangered or protected species (Sainsbury 2008). Because of the high vulnerability of Goliath Grouper combined with its high ecological and live-economic value, it is necessary to use extreme precautionary measures. The purpose of this work is to examine alternate approaches which involve precautionary and adaptive qualities to managing this extremely vulnerable, but highly valuable species.

Target reference points are acceptable levels of catch or mortality that do not jeopardize recovery or maintenance of the population. This reference point may be adjusted over time depending on the response of the juvenile and adult population, as determined by direct estimations as described above. Of course, the success of this or any management approach is dependent upon accurate measure of mortality (including the legal and illegal catch, incidental catch mortality, and natural mortality; see Ellis & Friess and Malinowski of this report), continued monitoring of both juvenile and adult populations and effective management intervention if a limiting reference point is exceeded. The Reef Environmental Education Foundation (REEF) provides automatic and reliable measures of Goliath Grouper population status within zones in Florida (Figure 1) and other coastal areas within the SE US and could be used to augment direct density measures on spawning sites (northern part of zone 6 and southern part of zone 7 in the Atlantic and zone 4 in the Gulf of Mexico). Based on these data, regional carrying capacities could be estimated from equilibrium conditions in REEF data (Koenig et al. 2011). That is, equilibrium conditions could be defined as conditions when an increasing trend in regional site density levels off. Spawning biomass could also be estimated from REEF data using the relative increase in numbers of adults in spawning areas during August and September (Koenig et al. 2016).

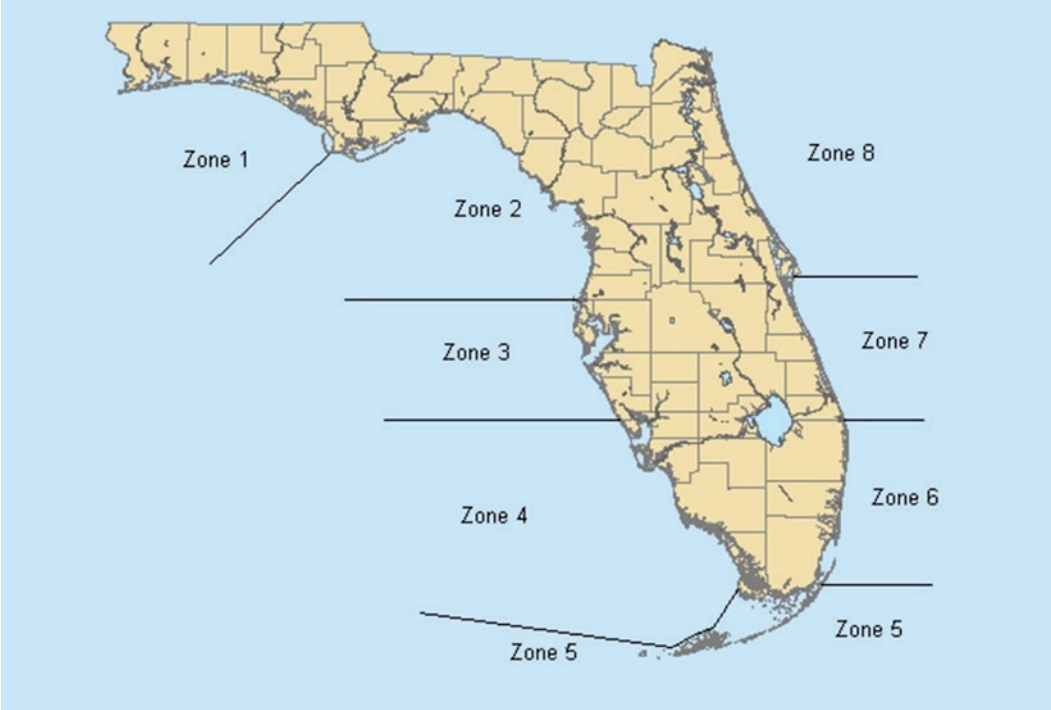

Figure 9. Map of REEF (Reef Environmental Education Foundation) survey zones of Florida.

Direct measures of juvenile abundance will also be useful in detecting population declines caused by events such as red tide and cold events. A dramatic example of cold-event mortality occurred in January 2010 when there was a juvenile mortality that exceeded 90% (based on 'catch-per-unit-effort' in Everglades National Park (ENP) creel surveys). Such a pervasive impact on a species that requires a 5-year sojourn in the shallow mangrove nursery translates to near-zero recruitment to the adult population from 2010 to 2015. If this event had gone unnoticed, i.e., no monitoring of the juvenile Goliath Grouper catch per effort in the mangrove, then recruitment, and therefore allowable catch, would have been vastly overestimated because recruitment would have essentially stopped for 5 years. Further surveys in other mangrove habitats (e.g., Pine Island Sound, Florida Bay, Indian River Lagoon, etc.) should be implemented to determine the spatial extent of the juvenile population, provided that these habitats are not too heavily impacted by anthropogenic impacts. While the creel surveys of the ENP have previously given the best information on the state of the juvenile population, it is less than ideal for two reasons. First, creel surveys are by definition indirect measurements of population size. That is, the relationship between juvenile abundance and catch-per-unit-effort of

the recreational fishery depends on the behavior of the fishermen. If fishermen target Goliath Grouper for catch-release sport, then catch-per-effort (and thus presumed abundance) will be higher than if the catch is purely incidental to the targeting of other species. An actual example of this happened in the late 2000s—apparent juvenile abundance increased dramatically (see Cass-Calay and Schmidt 2009, Fig. 4) after sport-fishing magazines and television fishing shows touted the sport of targeting juvenile Goliath Grouper for catch-release. Second, the ENP survey does not determine size or age structure. Further development of a juvenile survey should include expanded range, standardized effort, and record size and age structure to ensure adequate information to best inform recruitment estimates.

Management approach and data needs*:*

In this paper we are suggesting that adult Goliath Grouper should remain off limits for any fishery, but that a fishery should be established for juveniles based on a slot limit and fishing take based on a conservative PBR model. The following information and actions should be done to construct the model.

Monitor juvenile abundance and recruitment: The ENP creel surveys should be used to estimate relative juvenile abundance in south Florida mangroves. Additional data, if needed, may come from mark-recapture estimates of absolute density per linear mangrove shoreline in major mangrove habitat including the Ten Thousand Islands (TTI), a mangrove habitat known to be of high quality for Goliath Grouper juveniles (Koenig et al. 2007), Florida Bay, Biscayne Bay, and Indian River Lagoon. Juvenile size and age structure may be developed non-destructively using fin spines or rays (Brusher and Schull 2009, or Murie et al. 2009) to estimate net recruitment to the adult population.

Monitor adult population densities: This information may also come from REEF data in the various regions. Population density data on spawning aggregations may also be derived from REEF surveys from areas off Palm Beach and Martin Counties on the east coast and Lee and Collier Counties on the west coast of Florida—spawning fish are derived from broad regions of the east coast and west coast (Koenig et al. 2011, Ellis et al. 2014 and Ellis et al. this report), so their size and age structure should be representative of broad areas. Spawning sites, other than

those we identified, may be initially identified through the detection of nocturnal acoustic activity (Mann et al. 2009, Koenig et al. 2016, Koenig et al. this report), then later verified by capture of a sample of Goliath Grouper around new-moon nights and subsequent analysis of ovarian biopsies for presence of post ovulatory follicles (POFs; Koenig et al. 2016).

REEF data could be used to evaluate regional population stability. Koenig et al. (2011) validated REEF data and showed that the adult Goliath Grouper population reached saturation off southwest Florida in the late 1990s, but continued to increase in other parts of the state. A similar adult abundance probably exists now off southeast Florida, but recovery in other parts of the state should also be evaluated using REEF data.

Fishing allowances. Catch of Goliath Grouper should be limited to recreational fishing for juveniles within a specified slot limit only, for example, a length of 600 mm to 1200 mm TL (about 2 ft to 4 ft.)—these sizes have a corresponding weight of 4 kg to 33.6 kg (about 10 lbs to 75 lbs). Juveniles should be safe, but mercury levels in muscle tissue of adults > 1200 mm (4 ft) TL of both the Atlantic and Gulf are too high for human consumption (see Adams and Sonne 2013 and Malinowski, this report).

Catch of juvenile Goliath Grouper should be carefully regulated and verified—for example, through the use of a stamp system. In this system a fisher buys a stamp for a single fish and only a limited amount of stamps are issued each year, the number issued depending on adjustments to the target reference point. Money from the stamps could be used to continue monitoring the Goliath Grouper adult and juvenile populations.

Time-area closures and gear restrictions: Gears such as bottom long lines (for sharks) inadvertently catch Goliath Grouper and may produce significant incidental mortality—these gears should be outlawed in Goliath Grouper spawning areas during July through October, the period when spawners start to amass on spawning sites.

Reference points (limit and target): Limit reference points should be calculated from PBR where the recovery factor *Fr* is 0.5 or some other best estimate. This reference point limit (i.e., point at which fishing should be halted) can be adjusted as more information becomes available

(e.g., if no significant effect on the spawning population can be shown, *Fr* and the allowable catch may be raised). The value can lie between 0.1 and 1.0. The lower the value of *Fr*, the higher the precaution. A limit reference point of 0.5 provides significant precaution for other protected species (Wade 1998). A target reference point is one in which a constant removal can be maintained without significant loss of population recovery or maintenance at carrying-capacity. The target limit should be modest initially, but then, depending upon the juvenile and adult population response, this limit could be raised.

Mangrove habitat: Significant steps should be taken to increase mangrove habitat cover and improve water quality in that habitat in south Florida. Significant mangrove habitat loss has occurred over the last 50 years (Ueland 2005, Koenig et al. 2007), but more importantly, there has been significant degradation in mangrove water quality from pollutants and from eutrophication (Ogden et al. 2005). Because of the long-term juvenile sojourn in the mangroves, juvenile Goliath Grouper require long-term (5 years) stability of dissolved oxygen, salinity, and temperature. As such they may be considered important indicator species for water quality in the mangrove habitat, but improvements in the quality of this habitat quickly translates to improved production of other species as well, species such as snook and red drum which support major recreational fisheries in Florida.

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APPENDIX

FastTrack publication

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research paper

Diel, lunar, and seasonal spawning patterns of the Atlantic goliath grouper, *Epinephelus itajara*, off Florida, United States

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ABSTRACT.—The diel, lunar, and seasonal timing of spawning in Atlantic goliath grouper *Epinephelus itajara* (Lichtenstein, 1822) in the United States is highly specific, occurring at night during new moon phases of August, September, and October. We derive these patterns from four lines of evidence apparent on spawning sites during the known spawning season: (1) from the transitory appearance of fish aggregations; (2) from simultaneous recordings of goliath grouper nighttime calls and nighttime vertical ascents that were far more frequent during the new moon phase than on the full moon; (3) from collections of goliath grouper eggs (genetically verified) at night downstream from known spawning sites; and (4) from significantly higher frequencies of both hydrated oocytes (indicating imminent spawning) and postovulatory follicles (indicating recent spawning) in ovarian biopsies taken from goliath grouper captured on spawning sites during new moon phases relative to full moon phases. We suggest that dark-night spawning is an adaptation minimizing egg predation by several species of scad [*Decapterus punctatus* (Cuvier, 1829), *Decapterus tabl* Berry, 1968, and *Decapterus macarellus* (Cuvier, 1833)] and herring [*Sardinella aurita* Valenciennes, 1847 and *Etrumeus teres* (DeKay, 1842)] that are abundant on goliath grouper spawning sites. The seasonal spawning of goliath grouper, late summer–early fall, coincides with habitat conditions considered ideal for settlement of early juveniles in mangrove nurseries.

Many reef fishes, including groupers (Epinephelidae), exhibit reproductive patterns that parallel lunar cycles with spawning occurring near dusk (Johannes 1978, Thresher 1984, Colin et al. 1987, Shapiro 1987, Sadovy 1996, Domeier and Colin 1997, Rhodes and Sadovy 2002, Sadovy de Mitcheson and Colin 2012). Many large groupers tend to make extensive seasonal migrations to form spawning aggregations on traditional sites to attend reproductive events that persist for days to months (Colin et al. 1987, Shapiro 1987, Coleman et al. 1996, Zeller 1998, Rhodes and Sadovy 2002, Ellis et al. 2014). This behavior is common among larger species, but not smaller ones (Sadovy 1996). Aggregation size among grouper species varies, with some forming many small (<100 individuals) aggregations distributed over a wide area (Coleman et al. 1996), including gag [*Mycteroperca microlepis* (Goode and Bean, 1879)] and scamp (*Mycteroperca phenax* Jordan and Swain, 1884), whereas others, such as Nassau grouper [*Epinephelus striatus* (Bloch, 1792)], form very large aggregations (thousands of individuals) at only a few sites (Smith 1972).

Spawning aggregation characteristics of Atlantic goliath grouper *Epinephelus itajara* (Lichtenstein, 1822) (hereafter "goliath grouper")—the largest grouper in the Atlantic Ocean—has been difficult to determine until recently. The first directed observations of their aggregations off the southeastern US, made by Colin (1994), occurred when the population was at an all-time low (due to intensive overfishing) and aggregation size was quite small (<10 individuals), making it difficult to discern either diurnal or lunar reproductive patterns. It was not until the population started to recover, which we recently documented (Koenig et al. 2011), that a realistic evaluation could be conducted. In addition to documenting the initiation of population recovery, we have elucidated aspects of spawning migrations, spawning locations, and timing using acoustic telemetry and passive acoustics (Koenig and Coleman 2013). We now know that goliath grouper form multiple small (<100 individuals) spawning aggregations drawn from areas sometimes hundreds of kilometers away from spawning sites (Koenig et al. 2011, Ellis et al. 2014).

In this paper, we describe the seasonal, lunar, and diel timing of spawning of goliath grouper in Florida and discuss these temporal patterns as driven by various aspects of adult, larval, and juvenile biology (review by Robertson 1991).

Materials and Methods

Spawning Sites.—We relied heavily on the local knowledge of commercial fishers and divers to locate spawning sites of goliath grouper. Some of these fishers had previous experience targeting this species prior to the fishery closure in 1990. We confirmed spawning aggregation sites through: (1) direct observation of increases in population size via scuba diving; (2) passive acoustic monitoring; (3) histological analysis of ovarian biopsies; and (4) nighttime net sampling of goliath grouper eggs. We also used acoustic telemetry with the involvement of the Florida Atlantic Coast Telemetry (FACT) group initiated and coordinated by the Florida Fish and Wildlife Conservation Commission (see Ellis et al. 2014 for details), and through the use of the Reef Environmental Education Foundation (REEF) volunteer dive surveys (http://www.reef.org/programs/volunteersurvey) (Koenig and Coleman 2009, 2013).

Passive Acoustic Monitoring.—We recorded goliath grouper sounds (low frequency sounds that occur within the range of 0–100 Hz) using DSG-Ocean acoustic

recorders (http://loggerheadinstruments.com/) placed on known or suspected goliath grouper spawning aggregations off southwest and southeast Florida during the 2010, 2011, and 2012 spawning seasons. The DSG-Ocean, a low-power underwater acoustic recorder that makes high-quality acoustic recordings over long periods, records to SD memory cards using a FAT32 file system. The DSG-Ocean can be programmed to either sample continuously, at rates approaching 80 kHz, or intermittently to conserve battery power.

We made all acoustic recordings on well-known shipwrecks, most of which were intentionally deployed as artificial reefs by state and county agencies. On the Atlantic coast (off Palm Beach County, Florida), recordings were made on the MG111, Zion Train, and Gulfland wrecks, all located within 10 km of each other. Water depths for these sites are 9 m (Gulfland), 20 m (MG111), and 28 m (Zion Train). In the Gulf of Mexico, we made recordings off Lee County on the Fantastico (35 m deep, a freighter that sank in a storm in 1993) and the Stoney wreck (40 m deep), both known spawning sites (Koenig and Coleman 2013).

We attached acoustic recorders directly to the wrecks during the spawning season (late August or early September), where they remained for several months (through late November), recording intermittently for 10 s every 10 min within a frequency range of 0–10 kHz. Analysis using a Fast Fourier Transform allowed us to examine the concentration of acoustic energy in the 0–100 Hz range and analyze using MATLAB R2009b and Adobe Audition 2.0.

Capture and Sampling of Live Fish.—Collecting samples to determine reproductive condition, age structure, diet, and degree of contamination of various toxicants required that we capture live goliath grouper. We tagged captured fish externally with cattle tags and internally with PIT (passive integrated transponders) tags. Some individuals were additionally tagged with acoustic tags (Vemco VR16 tags; 69 kHz, with 8-year batteries) surgically implanted intraperitoneally for determination of movements related to spawning activity (see Ellis et al. 2014). We followed federal and state laws for handling a protected species[1] that require the release of captured animals alive and in good condition. We captured goliath grouper during the spawning season on known or suspected spawning aggregation sites using hand lines (9-mm braided nylon, 60-m long) with monofilament leaders (1000-lb test, 5-m long) and circle hooks (20/0). Bait included whole live hardhead catfish [*Ariopsis felis* (Linnaeus, 1766)], and 1–2 kg pieces of great barracuda [*Sphyraena barracuda* (Walbaum 1792)], greater amberjack [*Seriola dumerili* (A. Risso, 1810)], little tunny [*Euthynnus alletteratus* (Rafinesque, 1810)], or stingrays [*Dasyatis sabina* (Lesueur, 1824) and *Dasyatis americana* Hildebrand and Schroeder, 1928]. The leader was connected to a hand line and to a 2-kg lead weight with longline snap (8/0 model 148, 1000-lb test). At times, we used a polyball buoy (69-cm diameter) to suspend the bait off the bottom and transfer the force of the powerful pull of the grouper to the buoy rather than the fisher's hands.

Each captured fish was hauled through a door in the vessel's transom onto a stretcher and tied down with straps to keep the fish from thrashing about the deck, protecting both fish and field personnel. We covered the exposed eye with a damp towel protecting it from direct sunlight and from responding to visual stimuli. A

[1] Goliath grouper are protected from all forms of fishery extraction in all United States continental and Caribbean territories.

hose with constantly flowing seawater inserted into the mouth irrigated the gills continuously.

We vented each fish on deck just posterior to the pectoral fin and below the midline with a stainless steel trocar (9.5-mm diameter) and cannula (http://www.scbt.com/datasheet-362154.html). The trocar and cannula were inserted through the body wall into the swimbladder, then the trocar was removed leaving the cannula inserted for about 1 min or until the gas had fully escaped the swimbladder. All fishing occurred at depths less than approximately 35 m to limit the effects of barotrauma, which can cause hemorrhage and death at greater depths of capture.

Gonad Biopsies.—We took gonad biopsies of all captured fish during the spawning seasons of 2010, 2011, and 2012 to determine sex and reproductive condition. The gonad biopsy method used on captured females involved inserting a polyethylene catheter (6.3-mm OD, 4-mm ID) through the oviduct into the lumen of the ovary and removing tissue with a hand-operated vacuum pump (Mityvac MV8000). Drawing the inserted tube back and forth in the lumen of the ovary allowed continuous removal of ovarian tissue. We withdrew the tube while still under vacuum to draw the ovarian tissues into a collection cup inserted in the vacuum line. We preserved half of the gonad tissue in 10% formalin for histological studies and half on ice for subsequent freezing followed by analysis of toxicants, such as mercury. Formalin-preserved samples were allowed to fix for several days, after which the individual tissue samples were placed in plastic tissue cassettes, washed with 70% ethanol, put into plastic bags, sealed, and shipped to Crowder Histology Consulting (4952 Alvin Dark Ave., Baton Rouge, Louisiana 70820) for preparation of histological slides.

We modified the biopsy method for males by using a smaller diameter catheter (2-mm OD) and/or human uterine biopsy forceps. The small diameter of the sperm duct made biopsies particularly difficult to obtain from males.

Only one reader (DJM) analyzed all of the histological sections. For our study, the most important features identified were hydrated oocytes and recent (i.e., newly formed) post-ovulatory follicles (POFs). The hydrated egg and POF data were standardized to percent occurrence and displayed relative to moon phase at the time of capture (representing time of the biopsy). We analyzed the frequency of occurrence across moon phases using a chi-square test and tested for significant differences of POFs among lunar phases using the Marascuilo comparison procedure (Marascuilo 1966).

Nighttime Egg Collection.—We set plankton nets (0.5-mm mesh) downstream from active spawning sites (location of fish determined by echosounder or diver observation) off Palm Beach County, Florida, to capture eggs of goliath grouper. To locate downstream deployment sites, we released a drogue (a weighted 1-m diameter sea anchor attached to a float) where the aggregated fish were observed, and then followed it 50–100 m downstream, anchoring mooring lines with nets attached at the end point and noting coordinates.

Three mooring lines were separated at 10–15 m intervals; buoyed at the surface, anchored to the bottom, and aligned perpendicular to the current, these served as attachment sites for several nets on each line distributed from the surface to about mid-depth in the water column. Mooring line anchors were rigged to break away from the bottom with sufficient tension from the vessel; this facilitated retrieval of the nets in the strong Florida Current.

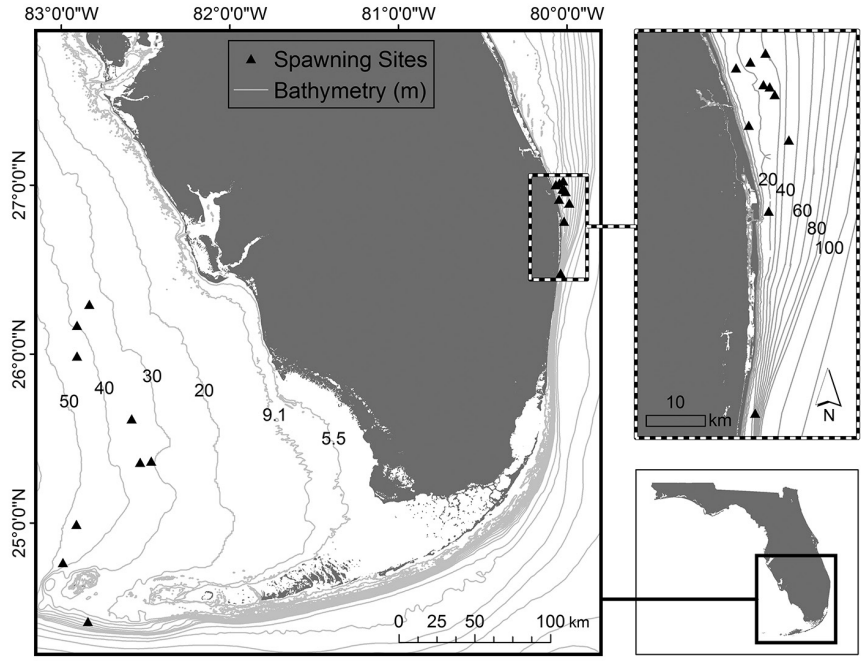

Figure 1. Map of confirmed Atlantic goliath grouper, *Epinephelus itajara*, spawning sites in Florida. Left panel: sites off southwest Florida and southeast Florida coasts. Right upper panel: off Palm Beach County. Right lower panel: entire state of Florida.

We deployed nets soon after sundown and left them out all night during the first deployment. We stopped using this approach when we determined that nets fouled quickly with plankton and debris, decreasing their capture efficiency. Subsequent deployments occurred at sundown with retrieval just before midnight, approximately 4–6 hrs after deployment.

Once retrieved, nets were held over a 5-gallon bucket and washed gently on the inside to avoid dislodging plankton and small jellyfish embedded in the mesh (intense washing of the nets made separation of eggs from plankton very difficult). The samples were then poured through three nested sieves of different mesh sizes: 3-mm mesh and 1.5-mm mesh to remove unwanted material; and a 0.5-mm mesh to collect eggs (approximately 0.95 mm diameter). Washing eggs off the 0.5-mm sieve into a 5-gallon bucket filled with clean, high-salinity (35) seawater resulted in live eggs floating to the surface while most dead plankton and eggs sank. After about 20 min, we swept a small fine-mesh (0.5 mm) aquarium net around the inside edge of the buckets at the water surface and transferred the net contents to a second bucket filled with clean high-salinity seawater, repeating the process at least two times to remove dead plankton and debris from the sample of developing eggs.

Results

Spawning Aggregations.—We confirmed 20 locations as actual spawning sites based on fish densities, sound production, ovarian biopsies, observations of commercial fishers working prior to the fishing moratorium, and/or egg collection. This evidence was collected during and outside of the spawning season during this and

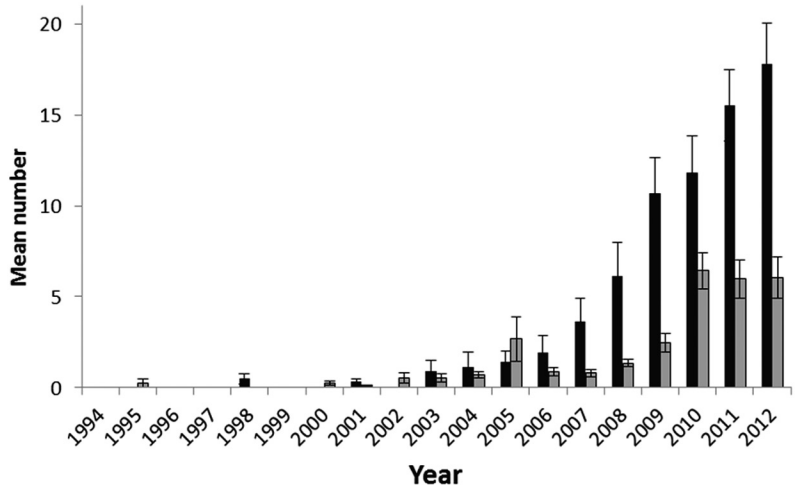

Figure 2. Annual mean abundance (±SE) of Atlantic goliath grouper, *Epinephelus itajara,* based on repeated surveys of all sites off the southeastern coast of Florida, from the southern Martin County through Palm Beach County, Florida, during (black bars) and outside (grey bars) of the spawning season (August through September). Because spawning and non-spawning sites were included in these REEF surveys the spawning season abundance (black bars) is underestimated.

our previous studies. Verified spawning sites occurred off the southeastern (SE) and southwestern (SW) coasts of Florida (Fig. 1). Spawning habitats consisted of relatively high-relief rocky reefs and artificial reefs (including wrecks and towers) in water depths of 15–50 m (Koenig et al. 2011). We found no spawning sites on or near coral reefs along the Florida Keys reef tract, an area in which the abundance of goliath grouper is relatively low (Koenig et al. 2007, 2011). There were sufficient data available from the REEF database to show the buildup of the goliath grouper population in the spawning area off SE Florida (Fig. 2), but not enough to demonstrate this pattern off SW Florida. Because all sites reported to REEF were used in Figure 2, including both spawning and non-spawning sites, the graph underestimates population increases during the spawning season.

Sound Production.—We recorded distinct patterns of nighttime calls ("booms", as described in Mann et al. 2009) off SW and SE Florida sites during the spawning seasons of 2010 and 2011. These calls were observed on spawning sites—Fantastico and Stoney wrecks off SW Florida and the MG111 off southeast Florida—but not on the non-spawning site, Gulfland wreck off SE Florida, where fish were less abundant and generally smaller than on known spawning sites (Fig. 3A–D).

Ovarian Biopsies.—We sampled 253 live goliath grouper captured off Palm Beach County, Florida, during the spawning seasons of 2010–2012 and analyzed histological preparations of ovarian biopsies for frequency of occurrence of hydrated oocytes and POFs (Fig. 4A, B) on all females: 2010 (n = 30 females), 2011 (n = 37 females), and 2012 (n = 94 females) (total n = 161, including recaptures). Ovarian biopsies indicated significantly higher frequency of occurrence of POFs (χ^2 test: $P < 0.0001$) and hydrated oocytes (χ^2: $P < 0.05$) in samples collected during the new moon phase than in those collected during the full moon phase (Fig. 5A, B). Because of the small sample size of females captured and examined during first quarter (n = 9) and

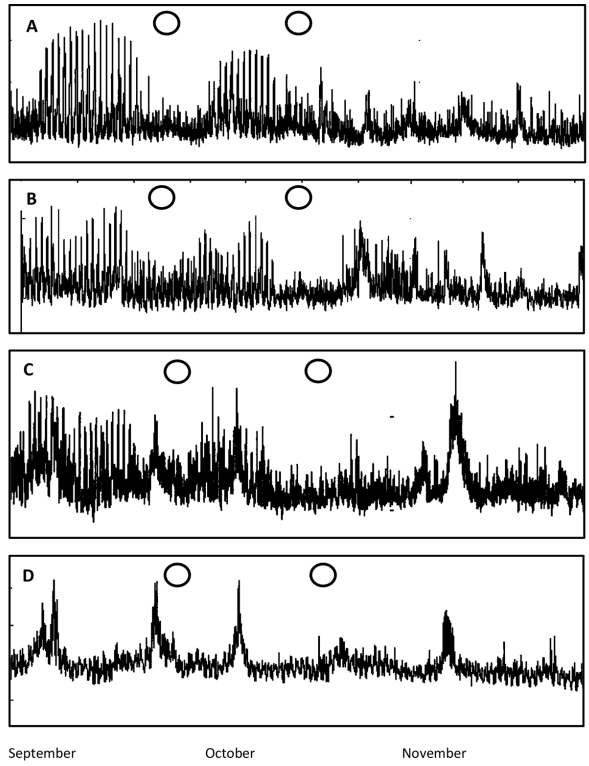

Figure 3. Patterns of nocturnal sound production in Atlantic goliath grouper, *Epinephelus itajara,*on three spawning sites: (A) Stoney Wreck, 2010, Gulf of Mexico; (B) Fantastico Wreck, 2010, Gulf of Mexico; (C) MG111 wreck, 2011, Atlantic Ocean; and one non-spawning site, (D) Gulfland wreck, 2011, Atlantic Ocean. Open circles = full moons of September and October. Lunar phases of August are not represented. Each new moon peak represents the nightly maxima of sound production levels (band sound pressure level dB re 1µPa (0–100 Hz); therefore periods associated with peak to peak intervals are approximately 24 hrs.

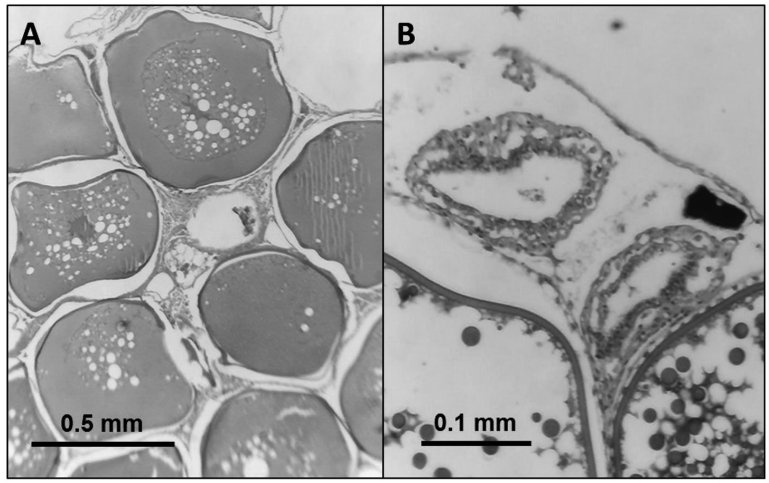

Figure 4. Photomicrographs of histological sections of ovarian tissue in Atlantic goliath grouper, *Epinephelus itajara,* captured off the southeastern coast of Florida, during the new moon of spawning season. Shown are (A) hydrated oocytes and (B) recent postovulatory follicles (POFs)—note double cell layers on POFs.

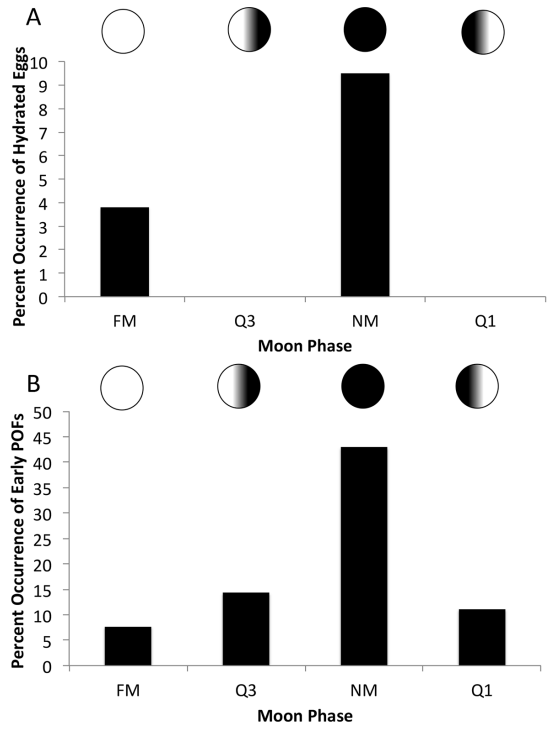

Figure 5. Comparison of changes in gonad characteristics relative to moon phase in female Atlantic goliath grouper, *Epinephelus itajara,* sampled in 2010, 2011, and 2012 on spawning aggregations off southeastern Florida (near Jupiter). Shown are (A) percent occurrence of hydrated oocytes and (B) percent occurrence of early postovulatory follicles (ePOFs). Description of moon phases are as follows: New Moon (NM; closed circle) = NM ± 3.5 d; and First Quarter (Q1) = NM ± 3.5 to NM ± 11.5 d. Full Moon (FM; open circle) = FM ± 3.5 d; Third Quarter (Q3) = FM ± 3.5 FM+11.5 d. New moon occurrences were significantly greater than full moon: χ^2 tests for POFs, $P < 0.0001$; for hydrated oocytes, $P < 0.05$.

third-quarter moon phases ($n = 7$), we could not distinguish them from new moon or full moon samples (Marascuilo comparison: $P > 0.05$).

Male Biopsies.—Male biopsies were largely unsuccessful. Identifying reproductively active males, however, was not. All mature males captured on spawning aggregations released copious quantities of milt. When the expansion of gas in the swimbladder caused fish to roll on their backs as they surfaced, milt spewed upward a meter or more in the air.

Egg Collection.—We collected several thousand goliath grouper[2] eggs during the new moon of September 2008 (Fig. 6) downstream from spawning sites off SE Florida (Palm Beach County, Hole-in-the-Wall—a natural reef) and off SW Florida (Lee County, Fantastico). Similarly, we collected many eggs downstream of a spawning site off SE Florida (Palm Beach County, MG111) during the new moon phase of August 2012, providing additional evidence that these sites served as active spawning

[2] Species identification verified genetically by M Craig using the methods described in Craig et al. (2009).

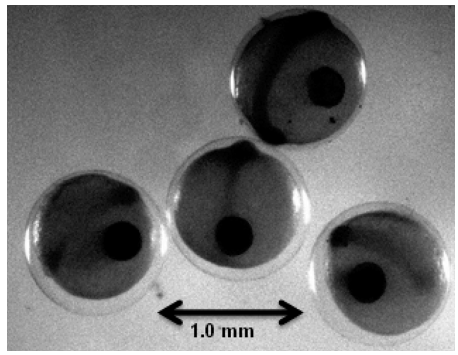

Figure 6. Photomicrograph of Atlantic goliath grouper, *Epinephelus itajara*, eggs in the neurula stage.

sites. All eggs were in early stages of development indicating that they were derived from nighttime spawns.

Direct Observation of Spawning.—J Hays (National Geographic underwater photographer) is the only person we know who has observed goliath grouper spawning. Notes on her observations, taken soon after sunset at a confirmed spawning site off Palm Beach County, Florida, in the new moon phase of September 2011, state that "The large presumptive female was tightly surrounded by large numbers of small planktivorous fish (scad and herring) [...] The presumptive female appeared to rub its vent on a vertical surface of the wreck, then swam away from the wreck and slammed its body against the sand then swam about erratically, in an apparent attempt to evade the many little fish, then she ascended straight up into the water column while followed by two smaller presumptive males. At the apex of their ascent, a cloud was seen (apparently a sperm cloud), then all three goliath groupers swam down to the sand and dispersed—the entire spawning event took less than 20 s."

Discussion

We know from previous studies that during their juvenile and adult life stages, goliath grouper show strong site attachment to their home ranges and to spawning sites (Koenig et al. 2007, 2011, Collins et al. 2015). For juveniles, the primary habitats are the mangrove forests that border south Florida rivers and islands, whereas for adults, they are rocky reefs, wrecks, or artificial reefs typically in shallower water for home sites and in deeper water for spawning sites. Home sites can also serve as spawning sites, as evidenced by fish numbers increasing dramatically during the spawning season. Our data and that collected by REEF bear this out (Koenig et al. 2011).

Behavioral and Morphological Characteristics of Active Spawners.—Goliath grouper reproduction in Florida strongly paralleled lunar patterns—migration to spawning sites occurred during the full moons of July, whereas spawning occurred with new moons of August, September, and October (Ellis et al. 2014, present study). During new moons, goliath grouper displayed distinct behavioral and, in the gonad, morphological characteristics. These included: (1) intense nocturnal calling (Mann et al. 2009, and present study); (2) the presence of hydrated eggs,

Figure 7. Photograph of Atlantic goliath grouper, *Epinephelus itajara*, adult on a spawning site off Palm Beach County, Florida surrounded by round scad (*Decapterus punctatus*) and Spanish sardines (*Sardinella aurita*). Photo credit: L Bueno, printed with permission.

which are indicators of imminent spawning (i.e., <12 hr); and (3) the presence of new POFs—indicators of immediate post-spawning[3].

We suspect for goliath grouper that in the absence of visual cues, calling serves as a means of aggregating potential spawners into a cohesive unit and providing critical behavioral cues that spawning is imminent, leading to synchronized hydration of eggs, gamete release, and maximized fertilization success (Lobel 2002). Sound production may also serve to stimulate hormone production associated with spawning condition (Locascio and Mann 2011). These findings support previous research on goliath grouper and other epinephelids demonstrating the relationship between fish sound production and seasonal patterns of reproduction (Mann et al. 2009, 2010, Nelson et al. 2011, Rowell et al. 2011, 2012, Schärer et al. 2012, 2014, Locascio and Burton 2016). They also suggest that nighttime calling is an effective means of identifying active spawning sites. Still unknown is whether the frequency of nighttime calls during new moon phases is a good indicator of the size of the spawning population. A recent study by Rowell et al. (2012) demonstrated this relationship holds true for red hind [*Epinephelus guttatus* (Linnaeus, 1758)] in Puerto Rico. If true for goliath

[3] POF degeneration occurs quite rapidly in most fishes in warm water temperatures: within 10–12 h in the northern anchovy, *Engraulis mordax* Girard, 1854 (Hunter and Macewicz 1985), <24 hrs in Atlantic menhaden *Brevortia tyrannus* (Latrobe, 1802) (Fitzhugh and Hettler 1995); 24 hrs in red snapper *Lutjanus campechanus* (Poey, 1860) (Jackson et al. 2006). If goliath grouper have similar POF degeneration times, then full disappearance of POFs would occur well within a lunar phase.

grouper, then acoustic monitoring could be an extremely useful tool not only for determining on-site spawner density, but also for determining relative spawning stock biomass throughout its range in the southeastern United States.

While night spawning is clearly the dominant pattern in goliath grouper, we cannot exclude the possibility that daytime spawning also occurs. It seems extremely unlikely, however, because we have not observed a single spawning event during the thousands of hours we dived with goliath grouper in the daytime on spawning sites during the spawning season from 1994 through 2015. Nor have we received a single report from the many other divers we know, especially off Palm Beach County, Florida. If some daytime spawning does occur, it would likely be minor relative to nighttime spawning.

We also suspect that goliath grouper are group spawners, meaning multiple males ascend with a single female during a spawning event. We draw on two lines of evidence from this and our previous studies: (1) the observation that more than two fish participate in spawning rushes; and (2) the observation that males produce copious quantities of sperm. Sperm production of such a large magnitude is indicative of group spawning rather than pair spawning as sperm competition dominates over individual competition for fertilization (Petersen and Warner 1998). This is borne out in groupers by the difference in size of testes of pair spawners such as red grouper, *Epinephelus morio* (Valenciennes, 1828), and gag, which have relatively small testes (Coleman and Koenig, pers obs) and group spawners, which tend to have large testes.

Egg Collection.—An additional approach to determine unequivocally whether the suspected goliath grouper aggregation sites were in fact spawning sites was to capture eggs during spawning events and genetically verify egg species identity. We assumed that this would be relatively straightforward because of our success in collecting fertilized goliath grouper eggs from the Hole-in-the-Wall natural reef on the first try in 2008 using a downstream passive sampling approach (Koenig and Coleman 2009). However, upon repeated attempts, we encountered numerous problems, including highly unpredictable current speeds and directions, and rapid fouling of nets caused by high densities of plankton, blooms of moon jellyfish (*Aurelia aurita* Linnaeus, 1758), and floating debris. Because this technique required ideal conditions, which rarely occurred, we determined that egg collection in this manner had limited applicability for verifying spawning sites.

Determinants of Seasonal Timing of Reproduction.—The timing of spawning in fishes (seasonal, lunar, and diel) is critical to reproductive success (Lowerre-Barbieri et al. 2011, Donahue et al. 2015). In broadcast spawners with pelagic larvae, a broadly accepted hypothesis is that reproductive success requires a match between the spawning season and optimal conditions for larval survival (Hjort 1914, Cushing 1943, and others). That is, larval biology controls reproductive timing. This idea has dominated analyses of seasonal patterns of spawning of temperate and tropical marine fishes (Robertson 1991). This belies the fact that natural selection also occurs on juveniles and spawning adults, not just larvae, in the scheduling of reproduction that ultimately maximizes reproductive success.

Successful recruitment to and survival in suitable settlement habitat is an important determinant of reproductive success (Lowerre-Barbieri et al. 2011). Juvenile habitat requirements include availability of refuge, abundant food, and suitable environmental conditions for optimal growth and survival. If the quality and quantity

of settlement habitat varies seasonally, then the seasonal timing of spawning may be strongly influenced by the timing of optimum settlement conditions.

For goliath grouper off Florida, larvae derived from August through mid-October spawning events settle as juveniles from September to early January (pelagic larval duration is 30–80 d; Lara et al. 2009) in mangrove nurseries primarily in submerged mangrove leaf litter (Koenig et al. 2007, Lara et al. 2009). Litter fall in south Florida occurs year round, increasing in the autumn (Lugo and Snedaker 1974, Twilley et al. 1986, Dawes et al. 1999) and during the dry season (December through May) when salinity increases. Under conditions of increased salinity, leaf breakdown is faster and the community of macroinvertebrates more abundant (Odum et al. 1982). Settlement of goliath grouper just before or during the dry season may confer a survival advantage because: (1) they occur only in waters with higher salinities (Odum and Heald 1972, Koenig et al. 2007); and (2) the abundance of potential prey for the early stage juveniles is higher (Odum et al. 1982, Sadovy and Eklund 1999).

A similar relationship between the timing of spawning and settlement of juveniles occurs with gag in northwest Florida seagrass meadows (Koenig and Coleman 1998). Gag spawn offshore on the shelf-edge primarily during February and March (Coleman et al. 1996), and pelagic juveniles settle in shallow seagrass in the spring (Koenig and Coleman 1998, Fitzhugh et al. 2005) during a period of rapid seagrass growth that reaches its peak of biomass and productivity in August (Zieman and Zieman 1989). They egress from the seagrass beds in autumn, moving to offshore reefs during late September to mid-October, when the aboveground biomass and productivity of the seagrass is rapidly declining (Stallings et al. 2010). Thus, juvenile habitat requirements likely contribute to seasonal timing of gag spawning, reminiscent of the pattern observed in goliath grouper. This may be a general pattern among fish species with highly specific settlement habitat requirements.

Determinants of Diel Patterns of Spawning Behavior.—We considered the life history patterns in goliath grouper that represent trade-offs between predation risk and spawning activity to address the question of whether dark-night spawning confers a selective advantage on various life stages of goliath grouper.

Adult goliath grouper spawning on dark nights might fall prey to large sharks feeding at night. It would appear that the abundant low-frequency pulses emitted by spawning groupers (Mann et al. 2009, present study) would attract sharks as several species of shark have been shown to be attracted to pulsed low-frequency sounds (Myrberg 2001), and the distributions of large sharks overlaps the distribution of goliath grouper spawning sites (Graham et al. 2016). However, the limited data available show that large sharks tend to feed during crepuscular times (Hammerschlag et al., this issue) rather than at night. In addition, on all of our dives on goliath grouper spawning aggregations during day and night— hundreds of dives from 1994 through 2015 in the Atlantic Ocean and Gulf of Mexico—we have never observed a non-human-predator-mutilated or injured goliath grouper, although we have observed injuries and partial carcasses from illegal fishing. It would seem with a fish as large as an adult goliath grouper, there would be some physical indication of a natural predation event, but none were observed. We conclude, then, that dark-night spawning is either inconsequential to shark predation or that it confers a selective advantage for reduced risk by temporally separating the activities of predators and spawners.

While it is unlikely that dark-night spawning confers any selective advantage on juveniles settling in the mangroves or on larval survival, it probably is an important factor in minimizing egg predation by the abundant planktivorous fishes present on goliath grouper spawning sites. The planktivorous fishes include round scad, mackerel scad, redtail scad [*Decapterus punctatus* (Cuvier, 1829), *Decapterus macarellus* (Cuvier, 1833), and *Decapterus tabl* Berry, 1968, respectively], and Spanish sardines and round herring [*Sardinella aurita* Valenciennes, 1847 and *Etrumeus teres* (DeKay, 1842)]. Several of these species are known egg predators (Hales 1987, Donaldson and Clavijo 1994). This hypothesis is consistent with the single direct observation of goliath grouper spawning—numerous scad and herring surrounded the presumptive female just prior to the spawning ascent (Fig. 7).

In summary, we used a variety of approaches to evaluate the reproductive locations, timing, and behavior of goliath grouper. These included passive and active acoustics, multiple types of tagging, in situ diver counts made by us and by volunteer divers submitting surveys to REEF, histological analysis of gonad biopsies, and downstream collection of fertilized eggs. All these approaches proved informative, expanding our knowledge of goliath grouper life history and contributing to their conservation. Such approaches would be useful to researchers working in other parts of their range where they remain endangered.

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Spawning-related Movement Patterns of Goliath Grouper (*Epinephelus itajara)* Off the Atlantic Coast of Florida

Patrones de Movimiento Relacionados al Desove del Mero Guasa (*Epinephelus itajara*) en las Afueras de la Costa Atlántica de Florida

Modèles de Mouvement Liés à la Reproduction des Mérous Géant (*Epinephelus itajara*) de la Côte Atlantique de la Floride

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ABSTRACT

Goliath Grouper (*Epinephelus itajara*), the largest reef fish in the western Atlantic, was once relatively common throughout Florida and the Caribbean. Due to overfishing and loss of juvenile habitat, it is considered critically endangered (Craig 2011). However, under total protection since 1990, population recovery is occurring is the southeastern US. Spawning aggregations are now forming on the shelf off southeast and southwest Florida. Aggregations of 20 to over 100 individuals occur on specific sites, both artificial and natural sites, from late July through October. In an effort to determine the nature of spawning migrations, we implanted 40 adult Goliath Grouper with ultrasonic transmitter tags (VEMCO 69 kHz V16-P coded transmitters) on known spawning sites in 2010 and 2012. Tagged fish were tracked as they moved through the Florida Atlantic Coast Telemetry array of VEMCO VR2 and VR2W ultrasonic receivers. Results indicate that adult Goliath Grouper are relatively sedentary during non-spawning months (mean monthly distance moved = 1.98 km ± 0.6) but moved significantly more prior to aggregation formation in July (18.5 km ± 8.56). Tagged fish moved more during spawning months compared to non-spawning months. Multiple individuals were tracked moving long distances (> 300 km) between residence reefs and spawning sites. Site fidelity to aggregations was high: 84.2% of tagged fish returned to the site of tagging after one year and 77.8% returned after two years. Our study utilizes long-term tagging data of individual fish to aid in understanding the movement patterns of a large reef fish species of special conservation concern.

KEY WORDS: Grouper, spawning, movement, aggregation, Goliath Grouper

INTRODUCTION

The Atlantic Goliath Grouper (*Epinephelus itajara*) is the largest reef fish in the western Atlantic but has been over-fished to the extent that the IUCN has classified it as 'critically endangered' (Craig 2011). In the southeastern U.S., the population of Goliath Grouper has been steadily recovering following a recreational and commercial fishing moratorium enacted in 1990 by both the South Atlantic and the Gulf of Mexico Fishery Management Councils (Koenig et al. 2011). Currently spawning aggregations (SPAGs) of Goliath Grouper form annually during the late summer and early fall off both the Gulf and Atlantic coasts of Florida. Although Goliath Grouper form SPAGs, they are also known to exhibit a restricted home range and show high site fidelity to residence or home reefs (Koenig et al. 2007, Koenig et al. 2011). However, details about individual movement patterns of Goliath Grouper between home sites and spawning sites and specific behaviors during aggregation periods are unknown. Diver reports of Goliath Grouper sightings are increasingly common in areas not associated with spawning activity (Koenig et al. 2011). It is well known that SPAGs are composed of individuals derived from broad geographical areas; however, it is important to know how large that geographical area is and the consistency with which the fish move to spawning sites and back to home sites. Therefore, information on migration dynamics (distances, patterns, pathways and spawning site fidelity) is important for effective management.

We realized that we had a rare opportunity to monitor patterns of behavior related to Goliath Grouper reproduction in great detail by joining the Florida Atlantic Coast Telemetry (FACT) Array cooperative research group initiated and coordinated by Florida Fish and Wildlife Conservation Commission (FWC). The FACT group makes use of compatible telemetry technology and a commitment to coordinate receiver spacing and to share detection data which allows member researchers to track study animals over long durations and great distances. As of early 2013, the 16 member organizations of FACT maintained 201 Vemco VR2 and VR2W receivers over a 500-km span of Florida's Atlantic coast deployed along a continuum of coastal habitats from freshwater estuaries (e.g. Indian River Lagoon) to marine waters of the adjacent continental shelf. This cooperative effort allows us to monitor movements of Goliath Grouper over a very large area of the east coast of Florida, from Palm Beach County to the Florida-Georgia border. In 2010 we added ten Vemco VR2W receivers to the FACT array on suspect Goliath Grouper spawning sites offshore of Jupiter, FL. By capturing and tagging Goliath Groupers while they were at the SPAGs we were able to track them as they moved through the FACT array of acoustic receivers back to home sites and then again when they returned to SPAGs in following years. This study gives us critical insight into the behaviors of Goliath Grouper during spawning while they are aggregating, but also during the rest of the year when they return to their home reefs. It also allows us to estimate site fidelity to home sites and spawning sites.

Here, we present some preliminary results of the first two years of the study, 2011 and 2012, showing how Goliath Groupers move along the Florida Atlantic coast in relation to SPAGs.

METHODS

Beginning in September 2010, we tagged Goliath Groupers intraperitoneally with acoustic transmitter tags (Vemco V16-4H, Vemco Ltd.). The cylindrical tags (16-mm (diameter) x 68-mm) came equipped with an 8-year battery. Fish were captured at known and suspected SPAG sites offshore of Jupiter, FL, during the annual late-summer/early-fall SPAG. The Vemco acoustic tags "ping" a unique identifier code once every 5-minutes which is recorded by Vemco VR2W-69 kHz receivers anchored to the bottom. During the study we deployed ten VR2W receivers at 14 different known or suspected SPAG sites offshore of Jupiter, FL (see Figure 1). Additional acoustic receivers maintained by the Florida Atlantic Coast Telemetry (FACT) Array group greatly expanded our sampling area.

Detection data were downloaded into the Vemco VUE program (Vemco Ltd., Halifax, NS, Canada) and exported into Excel (Microsoft, 2007, Redmond, Washington). All detections were first scanned for false detections using a 2-detection within 20-minute filter criteria. False detections were eliminated and the remaining detection data were entered into an Excel database. Using these data we calculated a number of different metrics to determine annual site fidelity and individual movement patterns of tagged Goliath Groupers, including the number of individuals returning to tagging site annually, the number of individuals detected in the SPAG area, maximum distance moved per month or year (defined as the maximum distance moved by a transmitter-tagged fish between any two stations during a given time period), and the number of SPAG sites visited per spawning season. We tested for differences in these metrics attributable to sex (ANOVA) and size (ANCOVA) using the R statistical software (R Core Development Team, 2013, Vienna, Austria).

RESULTS

We tagged 40 Goliath Groupers with coded transmitter tags from 4 September 2010 to 25 May 2011. The majority of tagging effort took place in the fall of 2010 (38 of 40 fish tagged), with the remaining two transmitters implanted in May 2011. Tagging was conducted at three suspect SPAG sites off Jupiter, FL (Figure 1), two of which have since been confirmed as spawning sites: Zion Train (artificial reef, 28-meters depth; 25 fish tagged) and Three-Holes (natural reef complex, 17-meters depth; 5 fish tagged). Ten fish were tagged at the Gulfland wreck (artificial reef, 10-meters depth), which has been confirmed

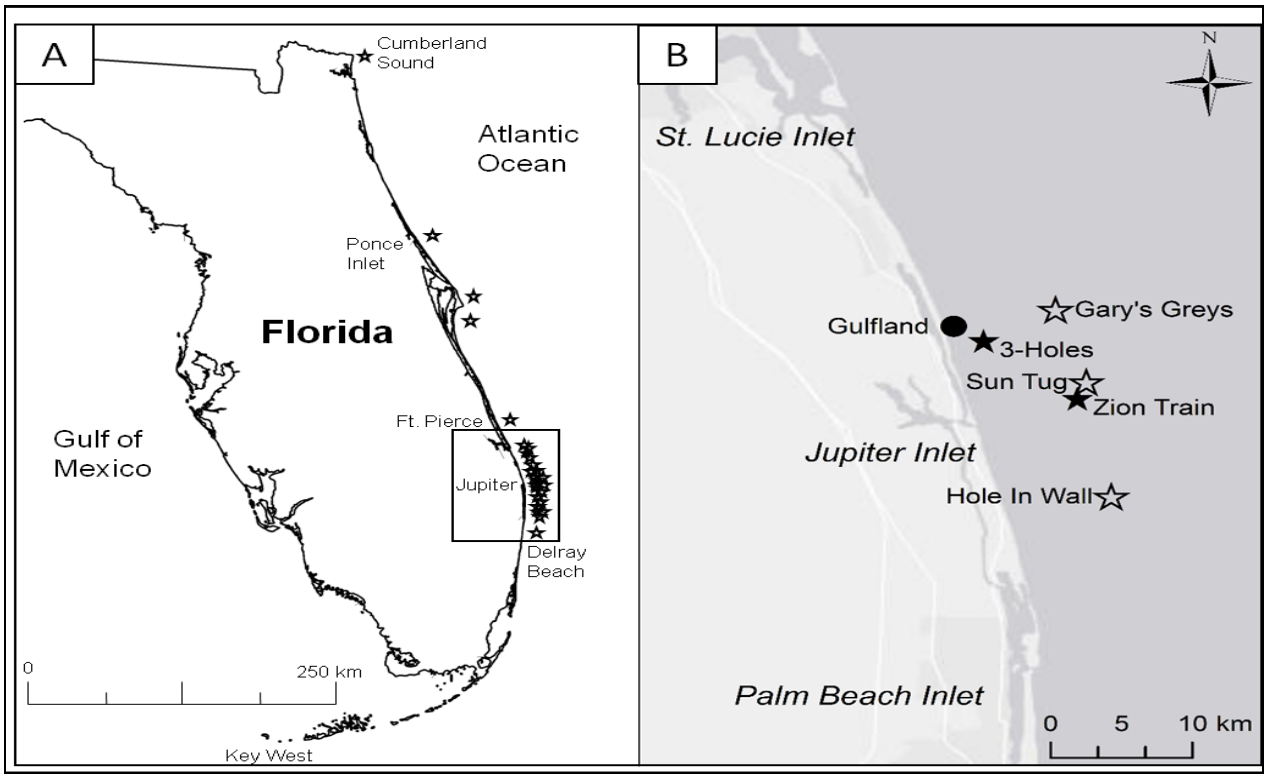

Figure 1. A: Locations of FACT monitored sites where acoustically tagged Atlantic Goliath Grouper were detected in 2011 and 2012; B: Locations of Goliath Grouper SPAGs (stars) and sites where Goliath Grouper were captured and tagged with acoustic transmitters (closed circle & closed stars).

as a non-spawning site. Tagged Goliath Groupers ranged in size from 104 to 205 cm total length (TL; mean TL = 159.1 cm). Sex distribution of tagged fish (as determined histologically from gonad biopsies or visually for males emitting sperm) was as follows: female = 17; male = 13; transitional = 6; immature = 3; unknown = 1 (the single "unknown" individual was excluded from analyses which compare movement patterns of males and females).

Between 1 January 2011 and 31 December 2012, transmitter-tagged Goliath Groupers were detected at 43 unique stations monitored by FACT (see Figure 1). During 2011, 37 of the 40 tagged Goliath Groupers (92.5%) were recorded at one or more sites within the array. In 2012, 35 of the 40 tagged Goliath Groupers (87.5%) were recorded at one or more sites within the array. Two individuals were never detected in the array subsequent to tagging. However, one of these individuals was recaptured during sampling approximately 4 months after being tagged; an acoustic receiver deployed at the site of recapture did not detect the fish, thus indicating a malfunctioning transmitter. The majority of detections occurred within 10-km of the tagging site, but tagged goliaths were detected at sites that spanned the entire range of the FACT array, a total distance of approximately 500 km.

Site fidelity of transmitter-tagged fish was high: 75% of fish tagged in 2010 and 2011 (30 of 40 fish) returned to the site where they were tagged within one year, and 25 of 38 (65.8%) returned to their tagging site in both 2011 and 2012 (we had only one full year of data for the two fish tagged in 2011 so these are not included here). All tagged fish, with the exception of the two that were lost since tagging, were detected at one of the five confirmed SPAG sites during the study (95%, 38 of 40 fish). Each year the number of tagged fish detected at spawning sites was the same - 85% or 34 of the 40 tagged fish visited a SPAG each year. Tagged fish visited an average of 1.78 ± 0.141 (mean ± SEM) SPAG sites over the course of the study. Tagged fish were detected at slightly more spawning sites in 2012 relative to 2011 (1.88 [± 0.212] vs. 1.70 [± 0.187]). A single tagged individual visited four SPAG sites in 2011; in 2012 a single individual was detected at all five confirmed spawning sites. All five confirmed spawning sites were visited by one or more transmitter-tagged fish during both 2011 and 2012.

The most frequently visited spawning site was "Zion Train" (ZT) which was the site where most fish were tagged. In 2011, 28 of 40 (70%) tagged Goliath Groupers were detected at ZT; in 2012, 21 of 40 (52.5%) of tagged Goliath Grouper were detected at ZT. Over both years, 29 of the 40 (72.5%) tagged Goliath Groupers were detected at the ZT site, followed by "Sun Tug" (26 tagged fish), "MG-111" (17 tagged fish), "3-Holes" (12 tagged fish), and "Gary's Greys" (12 tagged fish).

The number of FACT-monitored stations visited by transmitter-tagged Goliath Grouper during the study varied seasonally. In both years of the study the number of stations visited peaked during July – September, indicating increased activity during the spawning season. Most individuals moved little outside of the late-summer spawning season, remaining at one or few nearby reefs. The number of sites visited each month for tagged Goliath Groupers ranged from 0.2 (± 0.07) sites in January 2011 to 2.65 (± 0.44) sites in September 2012. Over the two-years reported here, the average transmitter-tagged fish was detected at just over 5 monitored stations (5.03 [± 0.441]), with slightly more detections during 2012 compared to 2011 (5.53 [± 0.661] vs. 4.53 [± 0.582]). The maximum number of monitored stations visited by any tagged fish over the course of the study was 20.

Consistent with the patterns described above, tagged fish moved more often and farther during months associated with spawning activity than the rest of the year (Figure

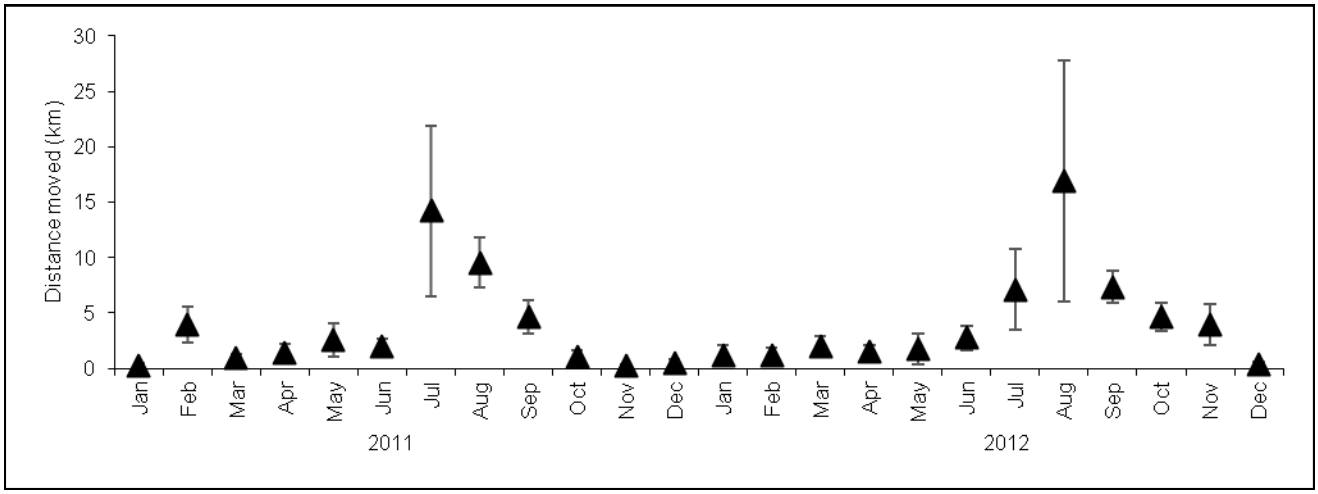

Figure 2. Distance moved along the east coast of Florida during each month of 2011 and 2012 by transmitter-tagged Atlantic Goliath Grouper. Error bars [± SEM].

2). Movement was generally above average from July – September each year with slight differences between the two years of the study: in 2011 peak movement occurred in July and was above the annual monthly mean (3.8-km per month) for August and September, while in 2012 tagged fish moved furthest in August and showed above average movement from July through November. Movement of tagged fish was also above average in February 2011.

The maximum distance moved between consecutive detections by a tagged fish in the study occurred between 11 and 21 August 2012 between Cumberland Sound near the Florida – Georgia state border and the SPAG site "MG-111" a total straight-line distance of 437.8-km. This same individual accounted for the second longest movement of 252.3-km over 22 days in July 2011 between a site located offshore of Ponce Inlet and "Tunnels", a natural reef site near the Jupiter, FL SPAG area. A different individual moved 222.1 km between Ponce Inlet and an artificial reef near Port St. Lucie, FL in 9 days, also during July 2011.

We analyzed the movement data to determine if any differences could be attributed to either fish size or sex. In general, larger fish visited more FACT-monitored stations during the study (Figure 3) and moved farther compared to smaller individuals. Linear regressions performed on the movement data showed a significant positive relationship between fish size (measured as total length) and the number of sites visited in 2011 (R^2 = 0.294; $F(1, 38)$ = 17.2; p = 0.00018) and 2012 (R^2 = 0.248; $F(1, 38)$ = 13.9; p = 0.00063). ANCOVA results showed these regressions were not different from each other ($F(2, 77)$ = 16.8; p = 0.19), suggesting these trends were consistent between the two years of the study. We found no differences between sexes in terms of the number of stations visited. However, the maximum distance moved by females was significantly greater than the distance moved by either males or transitional fish ($F(2, 69)$ = 3.22; p = 0.046).

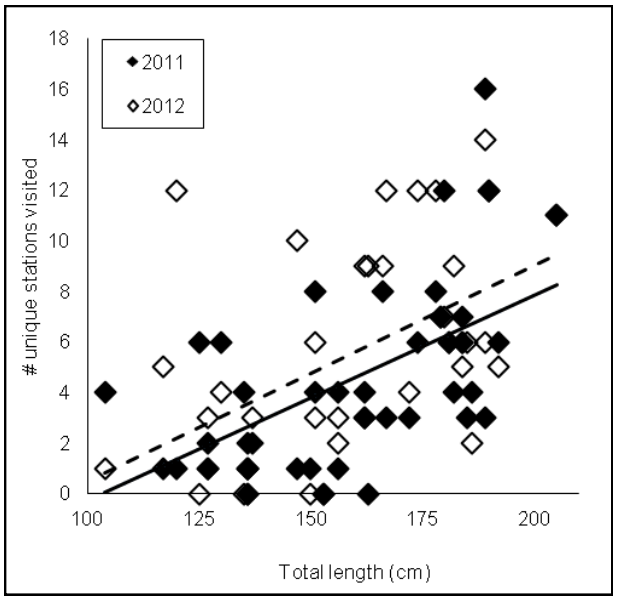

Figure 3. Number of unique FACT-monitored stations visited annually by transmitter-tagged Atlantic Goliath Groupers during 2011 (closed marks) and 2012 (open marks). Linear regressions were performed separately for each year (2011 = solid line; 2012 = dashed line), but were not significantly different from each other (ANCOVA: [$F(2, 77)$ = 16.8; p = 0.186]).

Analysis of transmitter-tagged fish movements suggests a strong lunar component to spawning site fidelity (Figure 4). In both years the number of transmitter-tagged goliaths recorded at the ZT site peaked during the new moons of August and September.

DISCUSSION

Monitoring of transmitter-tagged Goliath Grouper revealed that they do not, on average, move very far or very often, except around spawning time. This point was suggested by Koenig et al. (2011) from mark-recapture data and is confirmed by this study. By transmitter-tagging fish caught during the SPAG in 2010 we were able to passively track fish as they moved back to home sites and then returned to the SPAG area in 2011 and 2012. Some fish never left the SPAG area: on average 4 to 6 tagged individuals were detected daily at the ZT spawning site year-round. Likewise, detection data from the "3-Holes", "MG-111", and "Sun Tug" spawning sites all recorded the presence of resident individuals that remained at these sites all year. Another group of Goliath Groupers (7 individuals) was detected in the vicinity of a group of artificial reefs offshore of St. Lucie Inlet, approximately 25 km north of the spawning area. Individuals from this group made multiple movements between their home sites and the SPAG sites during the spawning season (July – September). After spawning, these fish returned to the St. Lucie reefs and did not return to Jupiter until the spawning season of the following year.

We concentrated most of our tagging effort on the ZT site because in both 2010 and 2011 this was the site of the largest aggregation in the offshore Jupiter area and was a presumed spawning site when the study started in 2010. Our estimates of site fidelity suggest that most fish return to the same sites year after year. However, in this area there are multiple aggregations in relatively close proximity and fish not only visited the aggregation where they had been tagged, but multiple others as well. Six of the 15 fish that were tagged at other sites visited the ZT site at some point during the study.

The tagging data suggest that Goliath Groupers move from residence or home sites to SPAG sites starting in July and remain relatively active throughout the spawning period. Our data suggest a strong connection between reproductive behavior of Goliath Grouper and the lunar cycle. We observed increased movement of tagged fish coincident with the July full moon. During this time fish

were actively moving between sites and into the spawning area offshore of Jupiter, FL. This was followed by aggregations that peaked in density during the August and September new moon (see Figure 4). It appears that the July full moon triggers Goliath Grouper to begin moving into the aggregation area, while spawning is triggered by the new moon. Complementary data on the occurrence of early POFs indicates that peak spawning occurs on new moons of August and September (Koenig and Coleman 2013).

We also observed what may be environmentally forced movements, such as those potentially induced by cold-water upwelling events that are known to occur in the study area. In February 2011 the monthly movement data was greater than average at a time when such movements would not be expected. It is possible that the elevated movements recorded in February 2011 represent tagged fish moving in response to variable environmental conditions. Unfortunately, we do not have corresponding temperature records to confirm this hypothesis. Since late 2012 we have attached temperature loggers to our receivers to evaluate the influence of cold temperatures on movements. It is well known that Goliath Grouper are sensitive to cold temperatures—temperatures below 15^{o}C can be lethal (Sadovy and Eklund 1999).

Detection range was not explicitly tested for this study. However, other studies using these same acoustic tags report detection ranges between 50 and 750 m, with peak efficiency occurring between 250 and 500 m (Humston et al. 2005, Whitty et al. 2009). Based on diver observations, Goliath Groupers tend to stay close to structure, well within the detectable range of the transmitters. Furthermore, even the maximum detection range of 750-m is much less than the distance between sites, allowing us to assume that single individuals cannot be detected at multiple sites simultaneously. The detection filter we used to eliminate false detections (i.e., 2 detections within 20 minutes) was designed to eliminate false detections that can occur when multiple transmitter codes arrive at the receiver at the same time, sometimes causing the receiver to record anomalous tag identifiers. Our detection filter follows the recommendations of the transmitter manufacturer to reduce the likelihood of such "collisions" from being recorded in the data.

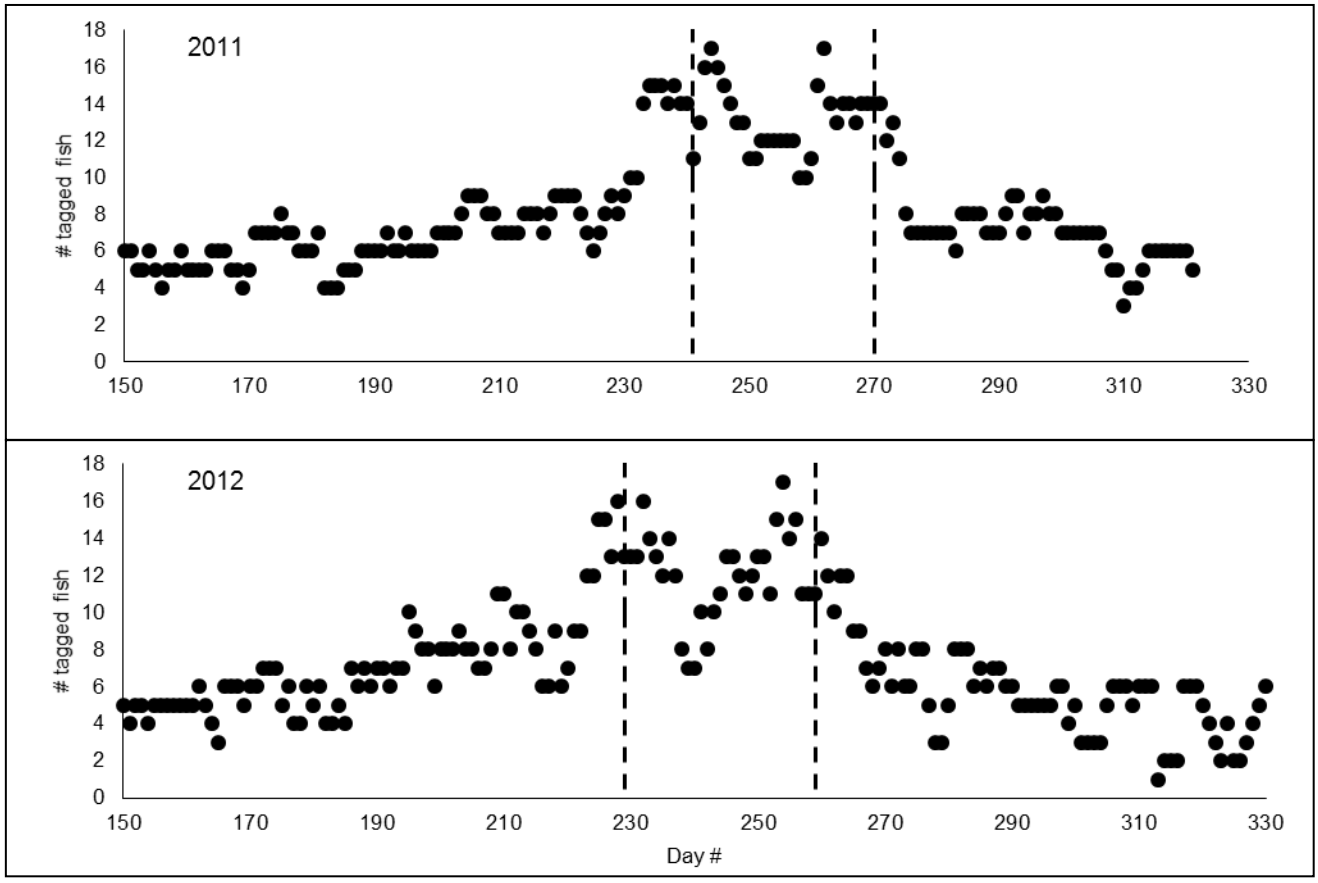

Figure 4. Number of acoustically-tagged Goliath Groupers detected at the Zion Train SPAG site located offshore of Jupiter, FL, during the spawning seasons of 2011 (top) and 2012 (bottom). The dashed vertical lines indicate the approximate dates of the new moons in August and September of each year.

This study highlights the importance of continuously monitored reef sites, such as the FACT Array, for the study of movements of reef-associated species. Little detail on movements can be gained without such a cooperative system, but the FACT system, among other things, allowed us to confirm a single spawning area off Palm Beach and Martin Counties and extensive migrations from the full extent of the FACT array. Thus a main conclusion from this work is that the SPAG sites located off the east coast of Florida are composed of Goliath Groupers derived from the entire east coast of Florida and probably also include fish from Georgia. That these individuals were found to return to the same spawning sites over consecutive years is an important insight into the aggregating behavior of this critically endangered species.

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Non-lethal approach identifies variability of δ^{15}N values in the fin rays of Atlantic Goliath Grouper, *Epinephelus itajara*

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ABSTRACT

The Atlantic Goliath Grouper, *Epinephelus itajara*, is critically endangered throughout its range but has begun to show initial signs of recovery in Florida state waters. As the population continues to rebound, researchers face a pressing need to fill the knowledge gaps about this iconic species. Here, we examined the δ^{15}N isotopic records in fin rays collected from Atlantic Goliath Grouper, and related changes of isotopic ratios over time to life history characteristics. Fin-ray analysis was used as a non-lethal technique to sample individuals from two locations at similar latitudes from the west and east coasts of Florida, USA. δ^{15}N data were acquired by mechanically separating the annuli of each fin ray and then analyzing the material in an Irradiance Elemental Analyzer Mass Spectrometer. The δ^{15}N values were consistent among individuals within populations from each coast of Florida, and mirrored the expected changes over the lives of the fish. Overall, differences were found between δ^{15}N values at juvenile life history phases versus adult phases, but the patterns associated with these differences were unique to each coastal group. We demonstrated, for the first time, that δ^{15}N values from fin rays can be used to assess the life histories of Atlantic Goliath Grouper. The non-lethal strategies outlined here can be used to acquire information essential to the management of species of concern, such as those that are threatened or endangered.

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Subjects Aquaculture, Fisheries and Fish Science, Biochemistry, Ecology, Marine Biology
Keywords Isotope chronology, Fin-ray chemistry, Ontogeny, Trophic shifts, Nursery, Mangrove habitat, Food web, Diet

INTRODUCTION

The Atlantic Goliath Grouper (*Epinephelus itajara*) is the largest epinephelid in the Atlantic Ocean and the second largest in the world, weighing up to 400 kg and reaching lengths of up to 3.0 m (*Bullock et al., 1992*; *Robbins, Ray & Douglass, 1999*; *Sadovy & Eklund, 1999*). They are a long-lived, slow growing fish that can remain in their juvenile habitat (primarily mangroves) for up to 7 years before moving to reef habitats as adults (*Bullock et al., 1992*; *Koenig et al., 2007*). Unlike other large reef fishes which tend to be upper trophic-level piscivores (*Romanuk, Hayward & Hutchings, 2011*), invertebrates make up approximately 70% of the diet of the Atlantic Goliath Grouper (*Koenig & Coleman, 2010*). These large

fish can serve as ecological engineers by exposing and expanding reef overhangs and ledges through their excavating activities. This behavior enhances structural complexity of the habitat thereby increasing abundance and diversity of the reef community (*Koenig, Coleman & Kingon, 2011*; *Macieira et al., 2010*).

Similar to many large-bodied reef fishes which are vulnerable to overfishing (e.g., due to slow maturation, aggregation behavior, limited juvenile habitat; *Stallings, 2009*), Atlantic Goliath Grouper are overfished throughout their range (*Aguilar-Perera et al., 2009*; *McClenachan, 2009*) and are classified as "critically endangered" by the International Union for Conservation of Nature (*Pusack & Graham, 2009*). However, the population of Atlantic Goliath Grouper has shown early signs of recovery in Florida state waters, in large part due to a federal fishing moratorium instituted in the United States in 1990 (*Cass-Calay & Schmidt, 2009*; *Koenig, Coleman & Kingon, 2011*). While this initial recovery is encouraging, more basic research on life history traits is needed to enhance and inform management. However, the slow maturation, large size, behavior, and long lifespans of Atlantic Goliath Groupers limit our ability to infer processes from controlled experimentation and short observational studies. To date, movement patterns and trophic shifts from nursery to adult habitats are still poorly understood, and warrant further investigation (*Lara et al., 2009*; *Koenig & Coleman, 2010*). The study of these processes in fishes typically requires lethal sampling, however due to the endangered status of the Atlantic Goliath Grouper, a non-lethal sampling technique is needed.

Stable isotope analysis (SIA) has become a common method to study fish movements and diets. In fishes, muscle tissue is most commonly used to quantify basal resources (δ^{13}C; *Hobson, 1999*; *Dierking et al., 2012*) and trophic level (δ^{15}N; *Vanderklift & Ponsard, 2003*; *Galvan, Sweeting & Reid, 2010*). Because these isotopic ratios integrate chemical information about an animal's diet across time scales beyond the "snapshot" scale from examining stomach contents, they can be used to quantify dietary patterns over a period of weeks to months (*Nelson et al., 2011*), before tissue turnover (*Ankjaero, Christensen & Gronkjaer, 2012*; *Hobson & Bond, 2012*). However, the study of long-lived fishes requires knowledge of longer time frames, often on the order of years.

To understand life history characteristics over annual time scales, researchers have recognized the need to analyze a conserved organic matrix that retains isotopic ratio values over the entire lifetime of the individual (*Caut, Angulo & Courchamp, 2008*). To our knowledge, a chronology of isotopic ratios continuously from birth to time of capture for an individual fish has only been accomplished via lethal sampling methods. *Wallace, Hollander & Peebles (2014)* measured both δ^{13}C and δ^{15}N across sequentially deposited layers of the eye lenses. However, this method has only recently been validated and the time scale over which eye-lens layers are deposited remains unclear. The sagittal otolith has been suggested as another possibility due to its chronological deposition of a metabolically inert matrix (*Campana, 1999*). However, the otolith contains miniscule amounts of organic material that may be conserved chronologically. To date, otoliths have only been analyzed at the bulk level for the entire structure, thus destroying the time series of interest (*Gronkjaer et al., 2013*). Otolith sampling also requires the fish to be sacrificed,

thus confounding conservation and management efforts for threatened and endangered species. Cartilaginous vertebrae in elasmobranches (*Estrada et al., 2006*; *Borrell et al., 2011*; *Polo-Silva et al., 2013*) have been used to document life history characteristics, however sampling is also lethal, and annuli banding in elasmobranch vertebrae are often difficult to interpret or absent for many species (*Cailliet et al., 2006*). While scales in teleosts (*Kennedy et al., 2005*; *Kelly et al., 2006*; *Sinnatamby, Dempson & Power, 2008*; *Woodcock & Walther, 2014*) represent non-lethal sampling, they may present inaccurate age estimations as the annuli of older fish tend to compress at the edge of the scales (more than other calcified structures). Additionally, scales are often lost and replaced, and the formation of scales may not occur at the larval stage (*Helfman et al., 2009*).

Fin rays of fishes can record the chronology of isotopes and may allow for non-invasive sampling as they can be excised non-lethally. Indeed, fin rays have the capability to regrow once they are excised (*Goss & Stagg, 1957*) and can be removed with minimal effects on both survival and growth on the individual (*Zymonas & McMahon, 2006*). The organic matrix of fin rays is largely composed of proteins, mostly collagen, while the inorganic matrix is carbonated hydroxyapatite (*Mahamid et al., 2010*). Chemical tracers from an individual's diet have been recorded over time within these matrices, suggesting that they are at least partially derived from the animal's food source (*Woodcock, Grieshaber & Walther, 2013*). Annuli conservation over time and the encapsulation of the organic matrix suggest that isotopic values of organic elements (e.g., δ^{13}C, δ^{15}N) are retained within these matrices. Initially, aging studies concentrated on the analysis of fin rays for fishes in temperate regions, such as salmonids and hexagrammids, aided by clear banding of annuli due to strong seasonality (*Bilton & Jenkinson, 1969*; *Beamish & Chilton, 1977*). However, more recent studies have demonstrated the effectiveness of the technique on fishes at lower latitudes, including Gag and Goliath Groupers (*Murie & Parkyn, 2005*; *Brusher & Schull, 2009*; *Murie et al., 2009*; *Koenig et al., 2011*). The mineral deposition in fin rays occurs on a similar time scale as in otoliths, although the two structures are formed via different metabolic pathways (*Helfman et al., 2009*). Indeed, age estimates from cross sections of fin rays corresponded to those from otoliths of the same fish (*McFarlane & King, 2001*; *Muir et al., 2008*; *Khan & Khan, 2009*; *Murie et al., 2009*; *Glass, Corkum & Mandrak, 2011*). The correspondence of annuli between otoliths and fin rays suggests minimal turnover or reabsorption in fin rays since previous layers are encapsulated and non-vascularized after new ones are added. The fin ray comprises both organic and inorganic chemical matrices, with a robust organic component (∼40%) compared to other calcified structures (*Mahamid, 2010*).

The documentation of a conserved organic matrix over time via fin-ray analysis may provide essential information regarding ontogenetic dietary and movement patterns of fishes. The method is suited to study life history characteristics for endangered species, such as the Atlantic Goliath Grouper, and those of management concern, due to its non-lethal nature and conserved chemical history. We tested whether δ^{15}N values were retained over time in fin rays of Atlantic Goliath Grouper, and if these changes were consistent with life history characteristics documented in previous studies. Changes in δ^{15}N values over time within an individual can be caused by movement to areas with

different isotopic baselines and dietary shifts. The documentation of these changes can be used to guide strategies that minimize the impact to the local population via habitat restoration and responsible fishing practices. Considering the lack of information on these ontogenetic characteristics of Atlantic Goliath Grouper, the technique presented in this study may lay the foundation for future research as well as inform management.

MATERIALS AND METHODS

Study area

We obtained samples of fin rays from adult Atlantic Goliath Grouper from mid-Peninsular regions of Florida on both the Gulf of Mexico (hereafter, "west coast fish") and Atlantic Ocean sides (hereafter, "east coast fish"). West coast fish ($n = 13$) were acquired from the Florida Fish and Wildlife Research Institute (FWRI) when opportunistic "fish-kill" samples (e.g., red tide casualties, discard mortalities) were reported from May 2012 to September 2013 (Site 1, Fig. 1). East coast fish ($n = 17$) were collected at known spawning aggregation sites during spawning seasons (July–September) in 2012 and 2013 (Site 2, Fig. 1). All samples were obtained through the procedure approved by the Institutional Animal Care and Use Committee (IACUC), approval number 4193W. In addition, all field sampling was permitted on both the state (Florida Fish and Wildlife Conservation Commission, permit number SAL-13-1244A-SRP) and federal levels (National Oceanic and Atmospheric Administration, permit number F/SER24:PH). Sites with elevated Atlantic Goliath Grouper abundances were chosen based on local knowledge and later confirmed by SCUBA surveys during the spawning season. Sites were typically artificial reefs (sunken wrecks) or natural ledges with high structural relief.

Tissue selection and sample collection

The soft-dorsal fin rays of the Atlantic Goliath Grouper were chosen for analysis over other calcified structures for several reasons. First, fin rays will grow back once they are excised (*Goss & Stagg, 1957*). The effects of fin ray removals do not significantly alter survival or growth of individuals (*Zymonas & McMahon, 2006*). Indeed, during the current study, several individuals were recaptured the same day after having their fin rays excised (indicating feeding behavior within hours of being sampled) and we have recaptured several fish over 1,100 days after initial sampling (C Koenig et al., 2014, unpublished data). Our methodology for capturing, excising the fin rays and release of the Atlantic Goliath Grouper has resulted in approximately 100% survival (*Koenig et al., 2011*). When compared to other calcified structures such as fin spines and scales, fin rays have the highest correspondence to ages obtained from otoliths in Atlantic Goliath Groupers (*Murie et al., 2009*). In addition, the organic matrix in fin rays is proportionally larger than any other reliable chronological recorder in Atlantic Goliath Grouper and many other fishes. Last, fin rays were being used for aging in a collaborative study, and were already being excised for analysis (*Koenig et al., 2011*).

Excision of dorsal fin-rays was deemed preferable to other fins due to the relative low usage of this fin during locomotion in Atlantic Goliath Groupers. Dorsal fin rays 5 to 7

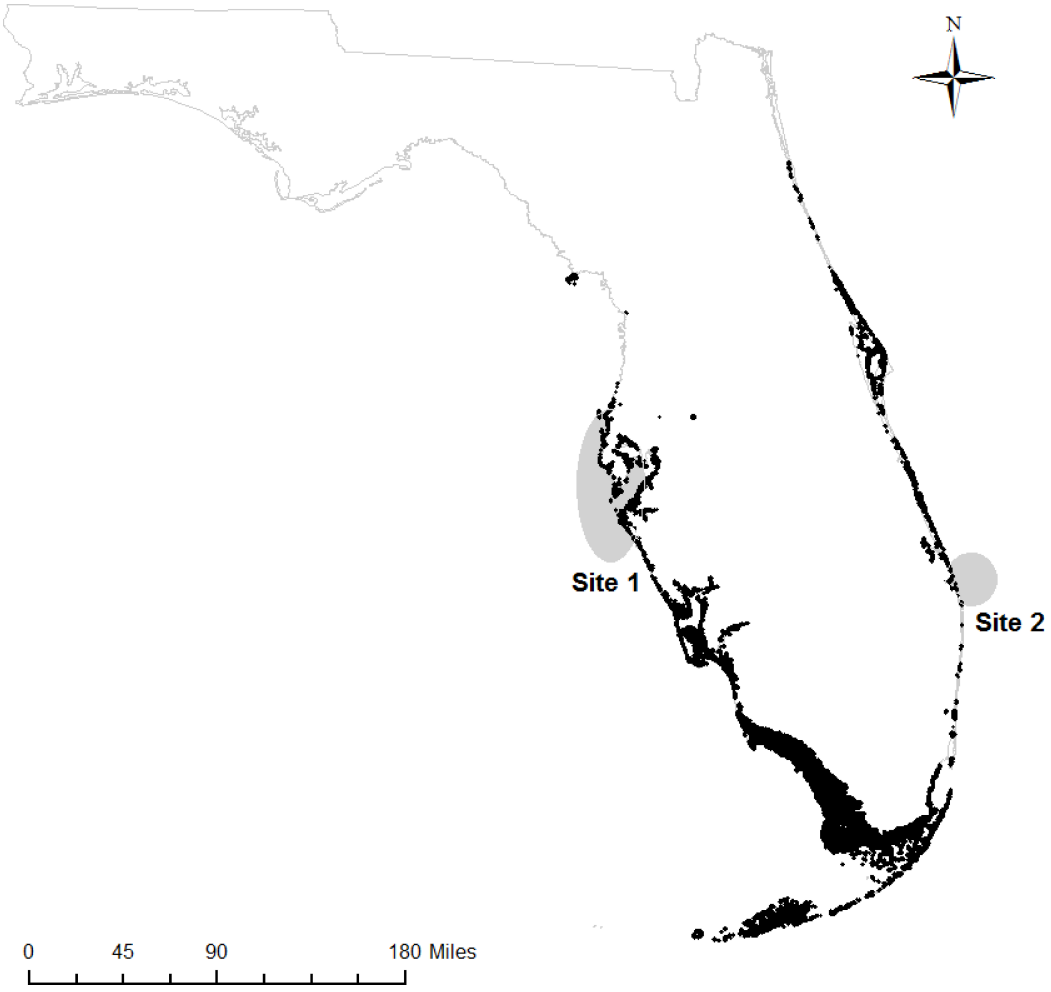

Figure 1 Sampling regions. Regions on the west and east coasts of Florida where Atlantic Goliath Grouper (*Epinephelus itajara*) were sampled (gray); mangroves are shown in black (*Florida Fish and Wildlife Research Institute, 2013*).

from the west coast fish were excised to include the entire ray structure (including distal pterygiophores) or as close to the base as possible. Fin rays with prior damage exhibit scar-like markings at the point of damage. None of the samples used in this study exhibited such markings. The excised rays were placed in labelled plastic bags on ice and ultimately stored in a freezer prior to processing. Whenever possible, total length (TL), total weight and age estimates based on otoliths were determined for these individuals.

East coast fish were captured using hook and line in collaboration with an on-going study to determine their age structure throughout Florida using non-lethal techniques (*Koenig et al., 2011*). Once onboard, the total length was measured and the fish was doubly tagged with uniquely-numbered external (live-stock) and internal (Passive Integrated Transponder) tags. Dorsal fin-rays 5 to 7 were collected and processed as described above. Stomach contents were also collected non-lethally by manually removing partially digested prey items.

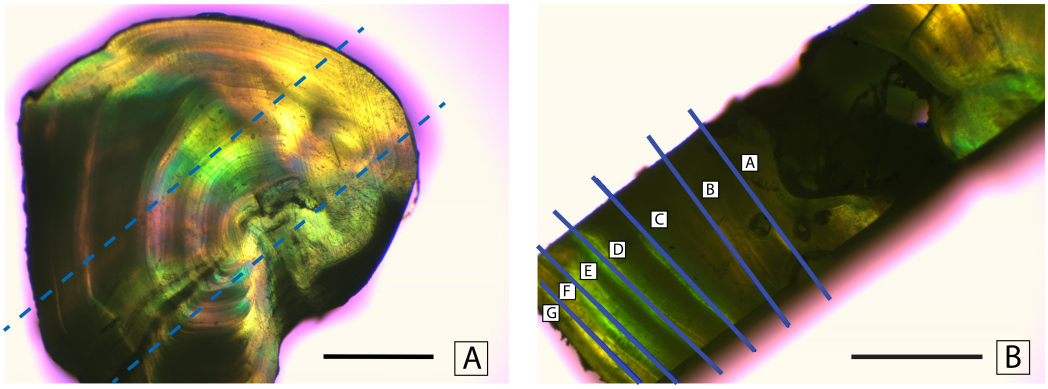

Figure 2 Mechanical separation of annuli. Separation of annuli from a cross section of a dorsal fin ray of Atlantic Goliath Grouper, *Epinephelus itajara*. Dashed lines in (A) show where a rectangular section is taken from the cross section. Solid lines in (B) indicate excision lines separating individual annuli. Scale bars represent 1 mm.

Sample processing

Fin rays were thawed in a drying oven for 4 h at a temperature of 55 °C. Once the samples had thawed, fatty tissue was removed using forceps. Each fin ray was then soaked in 30% hydrogen peroxide (H_2O_2) for 5 min to loosen the soft tissue surrounding the rays. Skin and membranes were cleaned from the rays using forceps and paper towels. The cleaned rays were glued to a petrographic microscope slide using Crystalbond (SPI Supplies, West Chester, Pennsylvania, USA). A set of two cross sections (1.5 mm thick) were cut from the fin rays using a Buehler IsoMet slow-speed saw (Buehler, Lake Bluff, Illinois, USA). The purpose of these cross sections was to isolate individual annuli for stable isotope analysis. These cross sections were then sliced perpendicular to the first cut to create rectangular bands that represented the time series of the entire life of the fish (Fig. 2A). The slices were cut using a modified feather-blade guillotine. By inserting a spacer and a second parallel blade, the rectangular slices were cut from the initial cross sections of the fin ray. The rectangular slice was then cut using the single blade of the feather-blade guillotine to mechanically separate the rectangular slice into smaller pieces, each of which comprised a single annulus (or two annuli if the sample was too small to separate individual annuli, Fig. 2B). When the smaller pieces comprised two annuli the mean of the two ages was used, and the associated values were presented as such.

Annuli were analyzed for bulk molar concentrations of carbon and nitrogen (C, N, C:N), and stable isotope abundance values, calculated as defined below ($\delta^{13}C$, $\delta^{15}N$). A 200 to 1,200 µg sample of each cross section was weighed on a Mettler-Toledo precision micro-balance (Mettler-Toledgo, Columbus, Ohio, USA), encapsulated in tin and loaded into a Costech Technologies Zero-Blank Autosampler (Costech Technologies, Montréal, Quebec, Canada). Samples were combusted at 1,050 °C in a Carlo-Erba NA2500 Series-II Elemental Analyzer (EA) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled in continuous-flow mode to a Finnigan Delta Plus XL isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at the

University of South Florida, College of Marine Science. Stable isotopic compositions were expressed in per mil (‰) using delta notation: e.g., $\delta^{15}N = (Rsample/Rstandard)-1]$; where $R={}^{15}N/{}^{14}N$. We calibrated the C:N measurements and $\delta^{13}C$ and $\delta^{15}N$ were normalized to the AT-Air and VPDB scales, respectively, using NIST 8573 (USGS 40; $\delta^{15}N = -4.52‰ \pm 0.12‰$; $\delta^{13}C = -26.39‰ \pm 0.09‰$) and NIST 8574 (USGS 41; $\delta^{15}N = 47.57‰ \pm 0.22‰$; $\delta^{13}C = 37.63‰ \pm 0.10‰$) L-glutamic acid Standard Reference Materials. All reference materials were sourced from the National Institute of Standards and Technology, U.S.A. Analytical precision, estimated by replicate measurements of a laboratory working standard (NIST 1577b Bovine Liver SRM, $N = 31$; $\delta^{15}N = 7.83‰ \pm 0.16‰$; $\delta^{13}C = -21.69‰ \pm 0.14‰$), was ± 0.13 $\delta^{13}C$, 0.18‰ $\delta^{15}N$, and ± 0.25 C:N.

De-mineralization of fin rays

In an attempt to eliminate the carbon noise associated with the inorganic matrix (due to unpredictable substitutions between carbonate and phosphate), we tested whether de-mineralization of the fin rays was a feasible preparation technique to obtain values for both $\delta^{13}C$ and $\delta^{15}N$ that only measured concentrations in the organic matrix. While demineralization is often performed to isolate an organic matrix, recent studies suggest that the chemical process may alter the organic components of a sample, specifically $\delta^{13}C$ and $\delta^{15}N$ values (*Rude, Smith & Whitledge, 2014*). Fin rays ($n = 21$) were initially cleaned as described above, split into two halves and then sectioned at a 1.5 mm thickness. One of the two sections from each fin ray was then chosen at random for the de-mineralization process. Samples that were demineralized were sonicated in "ultra-pure," milli-Q (Millipore, Billerica, Massachusetts, USA) water for 5 min and then submerged in 2% HCl for 24 h. After 24 h, the HCl was replaced and the samples were soaked for an additional 24 h. Samples were then rinsed thoroughly with distilled water, dried and sectioned (1.5 mm thick). All cross sections from both de-mineralized and control samples were powdered using a mortar and pestle to ensure uniformity within each sample. Samples were then weighed, encapsulated, and run on the EA-IRMS, as described above.

Data analysis

All statistical analyses were performed using MATLAB version R2012b. Age and size distributions between the two sample sets (west and east coasts) were plotted and analyzed using a two sample Kolmogorov–Smirnov test. Paired de-mineralized and control samples were analyzed for differences in two ways. A paired, two-tailed t-test was used to test overall differences of $\delta^{13}C$ and $\delta^{15}N$ values between the de-mineralized and control data sets. A procrustes analysis was used as an orthogonal least-squares analysis between the de-mineralized and control data sets by minimizing the sum of squares between the two. The symmetric orthogonal procrustean statistic (m^2) was calculated as a goodness of fit between the control data set, and the de-mineralized data set. Values of m^2 can range between 0 and 1, with lower values indicating a better fit between two sets of data. The procrustes analysis was able to test differences between each individual paired-sample.

In order to test whether the isotopic ratios were conserved over time, a two-tailed t-test was conducted using the $\delta^{15}N$ values of the annuli corresponding to age 4 for two age

groups of individuals within each study location. Here, we focused on age 4 to maximize sample size ($n = 27$), by choosing an annulus that was most commonly represented among samples (27 out of a possible 30). Samples within coastal groups were split into "young" ($\leq$8 years old) and "old" fish (>8 years old) based on age at time of capture. The two groups were analyzed for differences in δ^{15}N values to test whether the fin rays of older fish displayed signs of isotopic change over time. If δ^{15}N values degraded over time in fin rays, then we would expect to see differences between the two groups as the signals in older fish would have had more time to change.

The chronologies of δ^{15}N were created for each of the 30 individuals and grouped by coastal origin. Isotopic values were plotted against age (as determined by annuli) to investigate whether life-history shifts were indicated by changes in the δ^{15}N values of individuals over time. Isotopic shifts were theorized to be most evident when the fish moved out of their nursery habitat at roughly 5 to 7 years of age (*Koenig et al., 2007*), due to either dietary shifts, shifts in background δ^{15}N levels, or a combination of both. Given the repeated measures aspect of these data, a non-linear mixed-effects model based on the logistic equation was generated to model the distribution of data between δ^{15}N values and age. The model predicted three parameters (response coefficient, y-intercept and horizontal asymptote) for each fish. These values were averaged to produce parameters for each population. F-ratios were calculated to compare between sample sets as a measure of goodness-of-fit. A permutation based p-value was calculated based on the F-ratio. In addition to the model comparison over the entire lives of each individual, a paired t-test was used to compare the "nursery habitat" life stage ($\leq$6 years) and the "adult habitat" life stage (>6 years) to test ontogenetic shifts during a presumed migration period. Last, δ^{15}N values were plotted against total lengths and age at time of capture for all fish and linear least squares regressions were calculated for both comparisons. Individual δ^{15} N values at time of capture were compared to total length of each specimen via a linear least squares regression of TL with the outer-most annulus to test for differences in adult feeding patterns between coasts. Correlations were then compared between the two sampling regions to test whether δ^{15}N values consistently changed with size or age among all individuals.

RESULTS

Total lengths of west coast fish ranged from 62 to 205 cm, that of east coast fish from 122 to 211 cm (Table 1). West coast samples were 2 to 18 years old and east coast samples ranged from 6 to 19 years old (Fig. 3). A two-sample Kolmogorov–Smirnov test verified that age structure did not differ between the two sample sets (ks = 0.3, $p = 0.43$), although size structure did due to several smaller individuals among the west coast samples (ks = 0.56, $p = 0.01$). None of the individuals analyzed were recaptures from previous sampling efforts.

De-mineralization

De-mineralization introduced strong and non-systematic artifacts, with samples having inconsistent loss of the light or heavy isotope for both δ^{13}C ($t = 2.02$, df = 20 $p = 0.009$) and δ^{15}N ($t = 2.02$, df = 20, $p = 0.004$). The procrustes analysis (Fig. 4) further

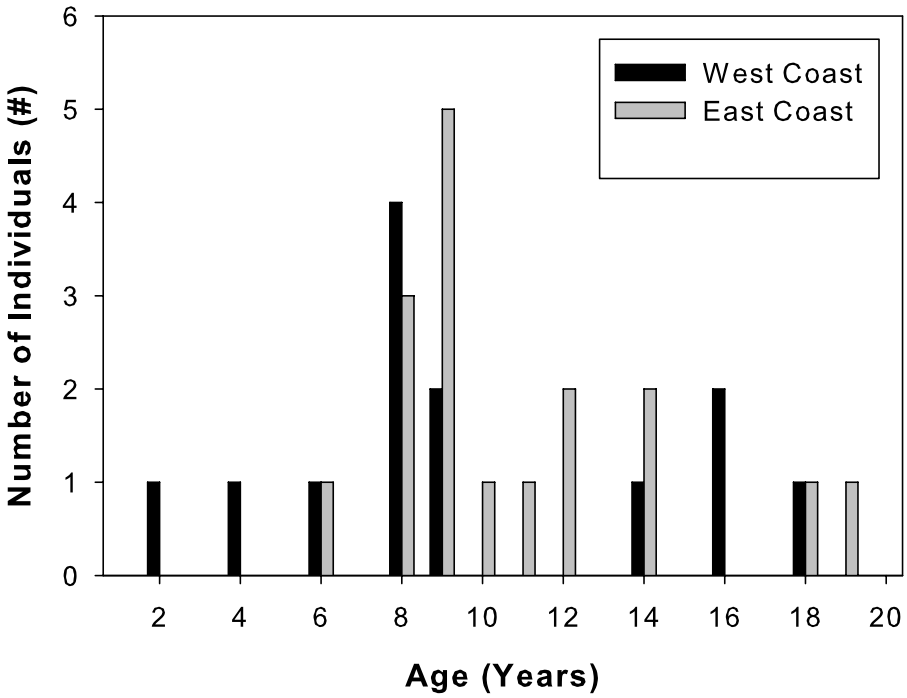

Figure 3 Sample age distributions. Percent frequency distributions of ages of Atlantic Goliath Grouper, *Epinephelus itajara*, sampled from the east coast (gray, $n = 17$) and west coast (black, $n = 13$) of Florida.

supported that alterations during the de-mineralization process were variable to both δ^{13}C and δ^{15}N values ($m^2 = 0.64$, $p = 0.001$). Differences in isotopic values between paired samples ranged from ±0.01 to ±3.14 for δ^{13}C values and from ±0.05 to ±1.19 for δ^{15}N values. The variable effects of demineralization to both δ^{13}C and δ^{15}N values precluded the use of a correction factor for treated samples. Due to these differences, demineralization was deemed inappropriate for this study, and carbon values were dismissed.

Isotopic conservation and δ^{15}N values at age

The δ^{15}N values at the annulus corresponding to age 4 did not differ between young and old fish for either west coast ($t = 2.26$, df = 9, $p = 0.46$) or east coast samples ($t = 2.14$, df = 14, $p = 0.20$; Fig. 5). An *ad hoc* power analysis demonstrated high power for both west (power = 0.99) and east (power = 0.96) coast samples.

The values of δ^{15}N for west coast samples varied from 8.39 to 12.61‰ (range = 4.22) and from 9.71 to 14.41‰ (range = 4.70) for east coast samples. However, one outlier (>3 SD from the mean) was responsible for the larger range associated with east coast samples. Once the outlier was removed, the values ranged from 12.16 to 14.41‰ (range = 2.25), roughly half that of west coast samples.

The δ^{15}N values of both sample sets increased as the fish aged (Fig. 6). A general increase was observed during the presumed nursery life stage (i.e., at approximately ages 0–7 years) which then leveled off once the fish moved into their adult-habitat life stage. A paired *t*-test confirmed higher values for adult compared to juvenile stages ($t = 2.16$, df = 12, $p < 0.001$

Table 1 Goliath grouper samples. Age, length and location of all samples of Atlantic Goliath Grouper, *Epinephelus itajara*, included in the study.

Sample location	Total length (cm)	Age (years)
West coast	62	2
West coast	83	4
East coast	122	6
West coast	112	6
East coast	174	8
East coast	146	8
East coast	162	8
West coast	120	8
West coast	126	8
West coast	145	8
West coast	124	8
East coast	157	9
East coast	147	9
East coast	162	9
East coast	168	9
East coast	178	9
West coast	130	9
West coast	151	9
East coast	171	10
East coast	180	11
East coast	185	12
East coast	182	12
East coast	195	14
East coast	200	14
West coast	198	14
West coast	190	16
West coast	190	16
East coast	197	18
West coast	205	18
East coast	211	19

for west coast samples and $t = 2.12$, df $= 16$, $p = 0.004$ for east coast samples). However, the non-linear mixed effects model highlighted differences between the two populations with regard to the response coefficients and horizontal asymptotes ($F = 6.34 \times 10^3$, $p = 0.001$, coef $= 2.35$, asymptote $= 11.98$ for west coast samples and $F = 5.57 \times 10^4$, $p = 0.001$, coef $= 1.44$, asymptote $= 13.33$ for east coast samples, Fig. 7).

δ^{15}N values at time of capture

δ^{15}N values were positively related to total length for both west (coef(se) $= 0.07(0.03)$; $t = 2.3$, $r^2 = 0.33$, $p = 0.05$) and east coast fish (coef(se) $= 0.15(0.02)$; $t = 6.5$, $r^2 = 0.75$, $p = 0.001$; Fig. 8A). δ^{15}N values were positively related to age for east coast fish

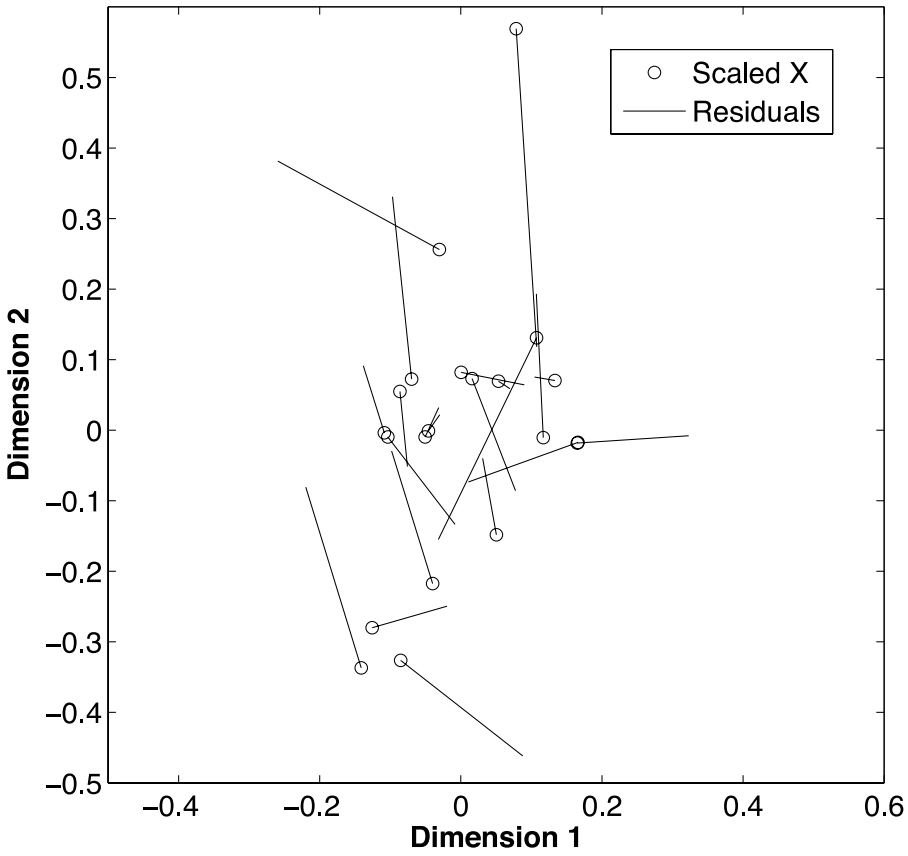

Figure 4 Effects of de-mineralization. Procrustean superimposition plot of de-mineralized and control paired samples of fin rays from Atlantic Goliath Grouper, *Epinephelus itajara*. The dimensions describe the rotated data of the δ^{13}C and δ^{15}N values before and after transformation. The "scaled X" points are the initial values of the rotated data, of each non-demineralized sample. Residual lengths indicate the amount of difference between the samples.

(coef(se) = 2.93(1.16); $t = 2.53$, $r^2 = 0.31$, $p = 0.02$), but not for west coast fish (coef(se) = 0.05(0.03); $t = 1.73$, $r^2 = 0.21$, $p = 0.14$; Fig. 8B).

DISCUSSION

Isotope chronologies

Fin-ray analysis is a non-lethal methodology that can be used to track isotopic chronologies in fishes. Organic isotopic-chronologies have previously been explored via annuli chronology in living tissues, such as trees and corals (*McCarroll & Pawellek, 2001*; *McCarroll & Loader, 2004*; *Risk et al., 2009*; *Andreu-Hayles et al., 2011*). To our knowledge, the current study represents the first to use a non-lethal method to derive an organic isotope chronology from a calcified tissue in fishes, without aging biases. The shift in isotopic values over time generated in the current study coincide with presumed life history events for Atlantic Goliath Grouper and may indicate that little to no tissue-turnover occurred as annuli were deposited in fin rays.

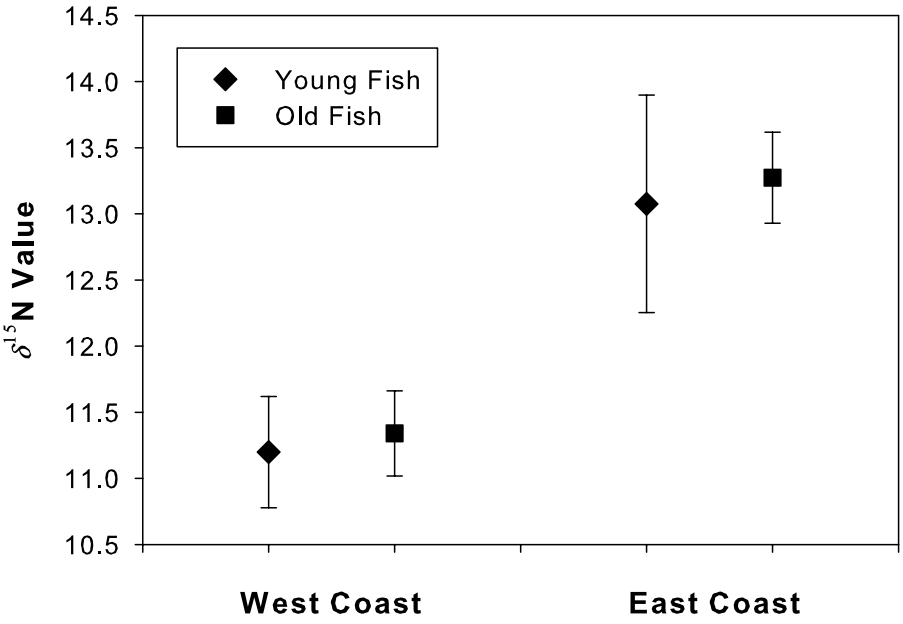

Figure 5 Degradation test for "young" and "old" fish. Mean (se) δ^{15}N values for the annulus corresponding to age 4 in both young and old Atlantic Goliath Grouper, *Epinephelus itajara*, from east and west coasts of Florida. Note differences in mean δ^{15}N values from fish sampled from the east and west coasts of Florida.

The use of isotopic analysis on fin rays required several assumptions, most notably, that fin-ray annuli corresponded with the age of the fish. This correspondence has been demonstrated for Atlantic Goliath Grouper (*Brusher & Schull, 2009*; *Murie et al., 2009*; *Koenig et al., 2011*) and other fishes (*McFarlane & King, 2001*; *Sun, Wang & Yeh, 2002*; *Debicella, 2005*; *Murie & Parkyn, 2005*; *Muir et al., 2008*; *Khan & Khan, 2009*; *Glass, Corkum & Mandrak, 2011*). Second, we assumed that the measured δ^{15}N corresponded directly to the age associated with each annulus within individuals. Similar to the assumptions made in otolith micro-chemical analysis (*Campana, 1999*), we assumed that the chemical constituents of each annulus were representative of the fish's diet at that age.

The limitations of the technique we used were classified as either mechanical or isotopic. The mechanical limitations were largely due to the size and composition of cross sections of fin rays. Curved annuli were cut with a straight blade, which made separating annuli difficult. Even with the relatively large samples used in this project, roughly ten samples had annuli that were coupled. No samples were excluded from the analysis due to this constraint. However smaller fishes with narrower annuli than Atlantic Goliath Grouper would present a new challenge and would require instrumentation with higher precision. In addition, two main isotopic limitations were apparent in the current study. The absence of δ^{13}C values from the study represented an important constraint, given its relevance to understanding basal resources. The carbonated hydroxyapatite that makes up the inorganic matrix of fin rays (*Mahamid et al., 2008*; *Mahamid et al., 2010*) introduced carbonate analytes into control samples. These carbonates replaced the phosphate molecules that make up the structural component of fin rays. This carbonate replacement

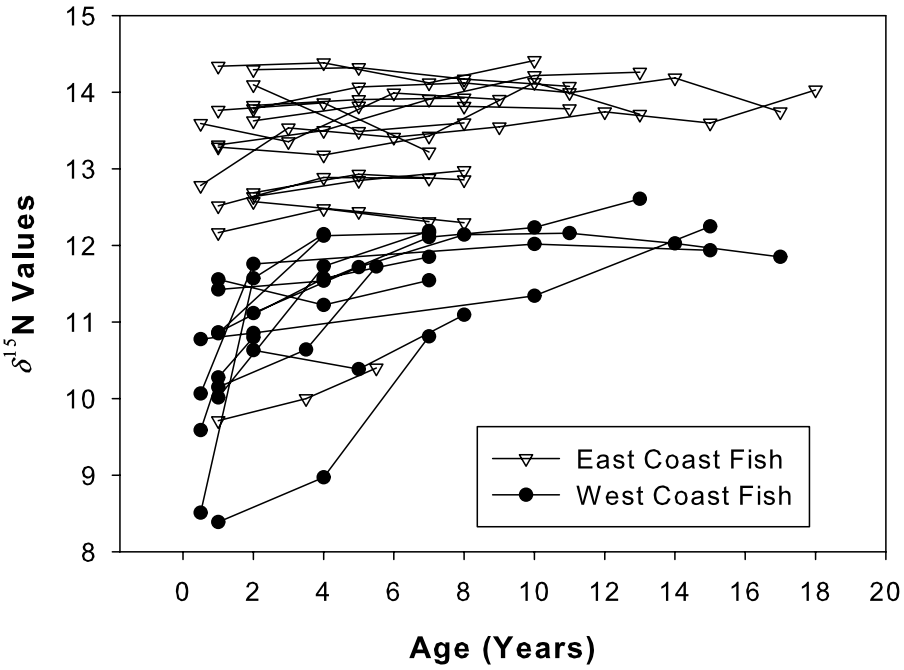

Figure 6 Isotope chronologies for each individual. δ^{15}N values for all sampled annuli (one annulus = one data point) excised from dorsal fin rays of all sampled Atlantic Goliath Grouper (*Epinephelus itajara*) from both east ($n = 17$; open triangles) and west ($n = 13$; filled circles) coasts of Florida. Each line represents annuli from a single individual. When the separation of individual annuli was not possible, the average of the two was presented.

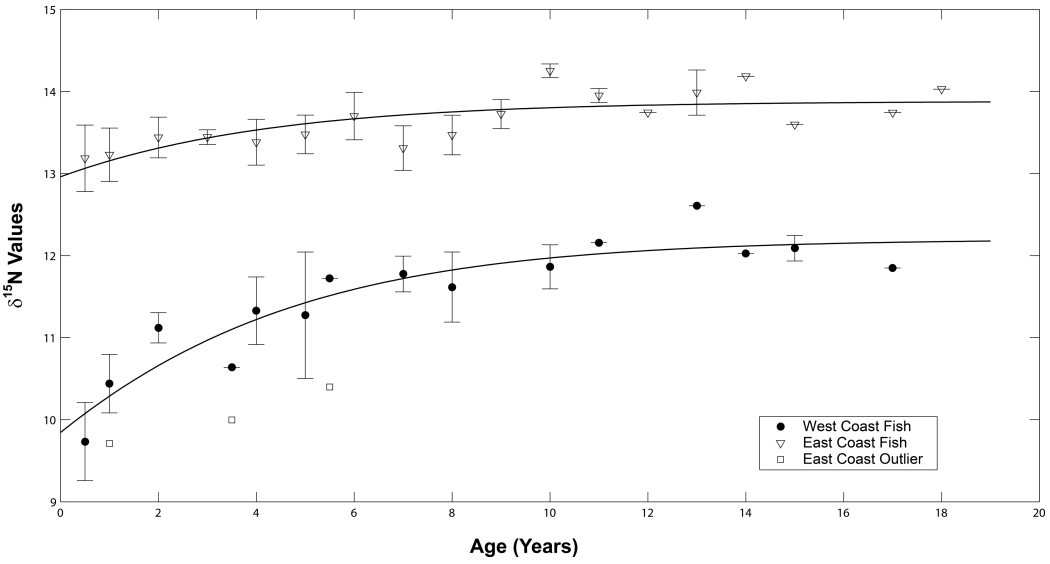

Figure 7 Average isotope chronologies by coast. Mean (se) values of δ^{15}N at age for west (filled circles) and east coast (open triangles) Atlantic Goliath Grouper, *Epinephelus itajara*. The single outlier (open squares) from the east coast is presented separately from the mean values for east coast fish. Trend lines represent the average non-linear mixed effects model for each population.

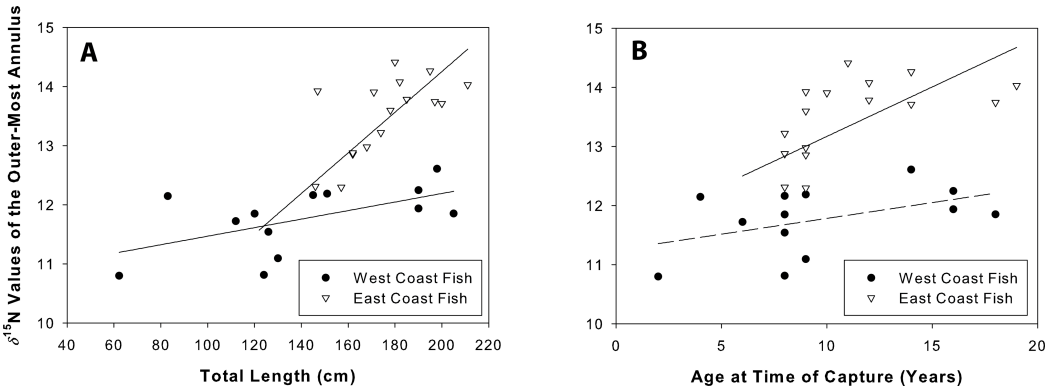

Figure 8 Nitrogen values at time of capture. δ^{15}N values of the outer-most annulus for both sample sets of Atlantic Goliath Grouper, *Epinephelus itajara*. Isotopic values were plotted against length (A) and age (B) at time of capture. δ^{15}N was positively related to total length on both coasts, and age for east coast fish.

is in non-stoichiometric equilibrium, and cannot be quantified consistently. Intact fin rays will thus have inorganic-carbon components that are variable among samples. De-mineralization was deemed inappropriate for the current study, based on the altered and inconsistent offsets of both δ^{13}C and δ^{15}N values as highlighted in the procrustes analysis. Consequently, δ^{13}C values were excluded from the analysis. Future studies may choose to test the de-mineralization process further by powdering samples prior to acid treatments. Powdering the sample prior to de-mineralization may facilitate a more complete digestion of all the inorganic carbon in the sample. The second isotopic limitation we faced dealt with the source of the observed δ^{15}N shifts over time. The δ^{15}N chronologies in this study match the life-history characteristics of Atlantic Goliath Grouper observed in previous studies, although the magnitude of the shifts differed between coasts. The difference in δ^{15}N values between the juvenile and adult stages was consistent with the ontogenetic movement patterns and dietary shifts previously documented (*Eklund & Schull, 2001*; *Koenig et al., 2007*). The composition of prey differ taxonomically between juveniles (mainly crabs) and adults (mix of crabs, lobsters, fishes, mollusks and echinoderms), largely due to species composition at the different habitats (*Koenig & Coleman, 2010*). The observed isotopic variations may have been due to an altered diet at sequential ontogenetic events, to a shift in isotopic baseline values at different locations or a combination of both. Isotopic background values differ between locations, and can thus influence measured isotopic values if a fish moves from one area to another (*McMahon, Hamady & Thorrold, 2013*; *Radabaugh, Hollander & Peebles, 2013*). Further study using compound-specific analysis of amino acids could potentially differentiate between δ^{15}N variation due to diet or to background isotopic differences (*McClelland & Montoya, 2002*; *Chikaraishi et al., 2007*; *Loick, Gehre & Voss, 2007*; *Ellis, 2012*).

Population differences

The relationship between δ^{15}N values and age differed between the two sampling regions. Similar-aged Atlantic Goliath Groupers exhibited 0.5 to 6.0 δ^{15}N value enrichment on

the east coast compared to the west coast. Without an isoscape effect (e.g., *Radabaugh, Hollander & Peebles, 2013*), east coast Atlantic Goliath Groupers would be expected to feed at 1 to 2 trophic levels higher than west coast fish, which is unlikely. To our knowledge, such a drastic difference in trophic level has never been documented between local populations of a conspecific, nor has concurrent research found differences between the stomach contents of individuals between the two coasts (*Koenig et al., 2011*). The clear division in overall δ^{15}N values can most likely be attributed to isoscape effects, meaning that isotopic background levels change among locations depending on ambient conditions (*Graham et al., 2010*). The data presented here suggest that an isoscape effect may be detectable between the two coasts of Florida. Isoscape effects naturally occur over large spatial scales, such as open ocean environments (*Graham et al., 2010*), but river outflows can influence isotopic baselines of ocean basins (*Radabaugh, Hollander & Peebles, 2013*) while near-shore environments can be directly influenced by anthropogenic activities (*Seitzinger et al., 2005*). Elevated δ^{15}N values in the east coast fish may be partially due to the higher abundance and density of the human population than on the west coast of Florida (*U.S. Census Bureau, 2010*). Anthropogenic inputs, such as treated sewage released into east coast waters has been documented to elevate background δ^{15}N values, particularly near centers of high human population density (*Lapointe et al., 2005a*; *Lapointe et al., 2005b*; *Risk et al., 2009*).

Both west and east coast fish exhibited a shift in isotopic values from juvenile to adult life stages, but west coast individuals did so faster and with a lower asymptote. The elevated δ^{15}N signals from the isoscape effect may once again contribute to the observed patterns. One pragmatic explanation may be that both populations exhibit an isotopic shift over time, but the patterns observed in east coast samples were swamped by isoscape effects. Indeed, the δ^{15}N values of several individuals in their juvenile phases are similar to that of their adult values. Future studies should aim for a community-level assessment to test whether lower values exist throughout the food web on the west coast of Florida in comparison to the east coast. High baseline δ^{15}N values would enrich all the organisms within a food web, particularly those close to shore, where the juvenile Atlantic Goliath Grouper reside. The individual outlier on the east coast may have migrated from a west coast nursery, as rare instances (<5%) have been observed for individuals making migrations of those distances (*Koenig, Coleman & Kingon, 2011*). In contrast, a larger area of mangrove habitat, with a potentially different isoscape, exists on the west coast (Fig. 1). Extensive mangrove habitat has been documented as the primary nursery habitat for Atlantic Goliath Grouper (*Eklund & Schull, 2001*; *Koenig et al., 2007*; *Gerhardinger et al., 2009*; *Lara et al., 2009*). The southwest coast of Florida is dominated by mangrove habitat that has been suggested as the most densely populated nursery habitat for Atlantic Goliath Grouper in their range (*Koenig et al., 2007*; *Koenig, Coleman & Kingon, 2011*).

The δ^{15}N values associated with total length may have also been affected by differences in ecosystem dynamics between the two coastal shelves. Previous studies have documented advanced sexual maturity at shorter total lengths of several fish species in the eastern Gulf of Mexico compared to other nearby regions (*Gartner, 1993*). If this trend holds true for Atlantic Goliath Groupers, then we would not expect to observe a significant relationship

between total length and $\delta^{15}N$ values on the west coast, because development within individuals would be variable. The mechanism for this phenomenon of unpredictable development in the region is not clearly understood, and warrants further investigation.

Implications and significance

The chemical analysis of fin rays represents a powerful method to better understand life-history movements and trophic shifts of fishes. Moreover, this approach is non-lethal and uses fewer samples than other, common techniques. These benefits help to promote a methodology that can facilitate important studies on endangered fishes around the world, as exemplified here with the Atlantic Goliath Grouper. Although a controlled laboratory experiment would be ideal to test isotopic chronologies in fin rays of teleosts, such an experiment is not practical for longer lived fishes. One viable alternative would be to test fin rays of fishes that have been in captivity for an extended period of time. A direct comparison could be made between life history stages that occurred in the wild versus those that occurred while in captivity. Unless the isotopic background levels as well as the isotopic values of the food source were exactly the same, then these two life history stages should differ within individual fishes. Our efforts here represent an observational basis to justify such a study. Studies that examine life history processes via non-lethal sampling can be used in turn to influence management strategies by gaining a better understanding of age-specific characteristics of species of interest. Changes in such characteristics over time are the result of altered movements, diet or both. This knowledge can be used in turn to direct both habitat and community-level conservation practices.

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The authors declare there are no competing interests.

Author Contributions

- Orian E. Tzadik conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Ethan A. Goddard performed the experiments, reviewed drafts of the paper.
- David J. Hollander contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Christopher C. Koenig contributed reagents/materials/analysis tools, reviewed drafts of the paper, guidance on methodology.
- Christopher D. Stallings conceived and designed the experiments, reviewed drafts of the paper.

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Life-history studies by non-lethal sampling: using microchemical constituents of fin rays as chronological recorders

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Chemical properties of fin rays were investigated in nine fish species to test whether life-history characteristics can be analysed using a non-lethal and minimally invasive methodology. Fish specimens from public aquariums were acquired after fishes died in captivity. Analyses concentrated on exploring the differences between the wild and captive life periods of each fish, which were known from aquarium records. Differences between the two life periods were observed in both the trace-element and stable-isotope compositions of the chemical matrix of the fin ray. Trace-element concentrations in fin rays were compared with those in otoliths using measures of resolved variance and cross-correlation to test the assumption of conserved matrices in the fin ray. Divalent ions and positively charged transition metals (*i.e.* Fe and Co) had strong associations between the two structures, suggesting conservation of material. Stable-isotope values of δ^{13}C and δ^{15}N differed between the wild and captive life periods in most of the fishes, also suggesting conserved matrices. δ^{13}C and δ^{15}N were derived from the organic matrix within the fin ray, which may present a stable-isotope chronology. Future studies can use these chronologies to study diet and movement trends on a temporal scale consistent with the entire lifetime of an individual.

Key words: microchemistry; mineral matrix; otolith chemistry; proteinaceous matrix; trophic chronology.

INTRODUCTION

Microchemical techniques can provide complementary information to existing methods used for fish movement and diet studies. Trace-element analysis (TEA) of calcified structures in fishes has not been used extensively in diet studies, but instead has been used most commonly to study movement in fishes (Elsdon *et al.*, 2008). Using TEA, elemental fingerprints help researchers map the movements of fishes by first tracing elemental concentrations along chronological landmarks within the calcified structures and then comparing the observed trends with known variation in the ambient environment (Dierking *et al.*, 2012). In comparison, stable-isotope analysis (SIA) has been used to both obtain trophic information (Galvan *et al.*, 2010) and to infer movement (Gillanders *et al.*, 2003; Dierking *et al.*, 2012). More specifically, δ^{13}C has been used to identify basal-resource dependence (Hobson, 1999; March & Pringle, 2003; Solomon

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et al., 2011) and δ^{15}N has been used to estimate trophic level (Vanderklift & Ponsard, 2003; Galvan *et al.*, 2010). δ^{13}C and δ^{15}N can also be used to track movements of fishes against background isotopic levels, which are mapped as isoscapes (Graham *et al.*, 2010; Radabaugh & Peebles, 2014).

Stable-isotope ratios in fishes have primarily been measured in muscle tissue. Muscle tissue, however, has a turnover rate of weeks to months and thus SIA of muscle provides short-term perspectives (Nelson *et al.*, 2011; Ankjaero *et al.*, 2012). To date, five types of fish tissue (otoliths, eye lenses, vertebral cartilage, scales and fin rays) have been used to reconstruct longer chronological histories of stable-isotope ratios (Estrada *et al.*, 2006; Elsdon *et al.*, 2008; Wallace *et al.*, 2014; Woodcock & Walther, 2014; Tzadik *et al.*, 2015). While all five structures appear to be effective recorders of stable isotopes, only the analysis of scales and fin rays is non-lethal; however, it can be difficult or impossible to obtain age-specific isotope measurements from fish scales (Hutchinson & Trueman, 2006; Helfman *et al.*, 2009). Fin rays, as in other calcified structures (*e.g.* otoliths, fin spines, scales and cleithra), are incremental structures that can be used for age and growth determination in many fishes (McFarlane & King, 2001; Murie & Parkyn, 2005; Muir *et al.*, 2008; Khan & Khan 2009; Murie *et al.*, 2009; Glass *et al.*, 2011), although limitations exist in fishes with high metabolic rates such as billfishes (istiophorids and xiphiids), which cannot be aged using fin rays due to resorption of annuli (Antoine *et al.*, 1983). Despite this, fin rays offer a potential structure for non-lethal ageing and chemical profile mapping.

In a variety of species, studies have documented conserved trace-element concentrations (especially divalent ions such as Ba and Sr, which have similar ionic radii to Ca) within otoliths and fin rays that correlate with concentrations in ambient water (Clarke *et al.*, 2007; Woodcock *et al.*, 2013). While elements deposit into otoliths and fin rays through different internal pathways, the correlation of certain elements with the ambient environment suggests layers retain their chemical properties over time in both structures, rather than being re-worked (Clarke *et al.*, 2007; Allen *et al.*, 2009; Smith & Whitledge 2010; Jaric *et al.*, 2011; Phelps *et al.*, 2012; Woodcock & Walther, 2014). Inner fin-ray layers become encapsulated by growing outer layers, after which the encapsulated inner layers lose their vascularization, thus inhibiting tissue turnover within the inner layers (Sire & Huysseune, 2003).

Direct comparisons of elemental chronologies (either by TEA or SIA) between otoliths and fin rays have not been made, nor has the conservation of organic material within the fin ray been tested. The present study uses fishes with known histories of wild and captive life periods to investigate whether the annuli of fin rays retain chemical characteristics over time. Specifically, the study tests the assumption of conservation of trace elements within the inorganic matrix of fin rays (primarily $CaPO_4$) by comparing values in fin rays with those in otoliths and conservation of stable-isotope ratios within the organic matrix of fin rays (primarily collagen) to test whether changes occur when ambient water conditions are altered.

MATERIALS AND METHODS

SAMPLE COLLECTION

Fishes were obtained from public aquariums after they had died in captivity. All fishes donated to the study were wild before being captured and raised in captivity. These conditions

TABLE I. Species list of all specimens (n) used in the study and the number of fishes that had otoliths available for trace element analysis (n_{TEA}), as donated by: Mote Marine Laboratory and Aquarium, Sarasota, FL; Guy Harvey Rum Fish Grill restaurant, St Petersburg, FL; Vancouver Aquarium, Vancouver, BC; Rookery Bay Learning Center, Naples, FL; Pier Aquarium, St. Petersburg, FL

Species	Family	n	n_{TEA}	Donor
Centropomus undecimalis	Centropomidae	12	6	Mote Marine Lab
Epinephelus morio	Epinephelidae	5	4	Rum Fish Grill
Sebastes pinniger	Sebastidae	1	1	Vancouver Aquarium
Sebastes caurinus	Sebastidae	4	4	Vancouver Aquarium
Sebastes melanops	Sebastidae	1	1	Vancouver Aquarium
Sebastes flavidus	Sebastidae	3	3	Vancouver Aquarium
Sebastes ruberrimus	Sebastidae	1	1	Vancouver Aquarium
Pogonias cromis	Sciaenidae	1	0	Rookery Bay Learning Centre
Sciaenops ocellatus	Sciaenidae	2	0	Pier Aquarium

allowed for comparison between known wild and captive life periods over longer time frames (*i.e.* years in the present study) than would be feasible *via* laboratory-controlled experimentation (Table S1, Supporting Information). The specimens originated from different families (Table I), thus offering better inference on the generalities of whether the annuli of fin rays retain chemical characteristics over time. Thirty individuals were obtained, of which 20 were used for comparison of otoliths (due to tissue availability) with fin rays by TEA. Fin rays from all 30 individuals were used in comparisons with SIA. Each fish was aged using otoliths and fin rays and an estimate of aquarium residency time was established based on aquarium records, which allowed estimation of the location of the wild-to-captive transition on the calcified structures.

FIN-RAY AND OTOLITH PREPARATION

Fin rays were excised from all individuals to include the distal pterygiophores and then frozen at −20° C or colder. Once removed from the freezer, the fin rays were defrosted in a drying oven for 3 h at 55° C. Plastic forceps were used to peel away as much skin and membrane as possible. Fin rays were soaked in 30% hydrogen peroxide (H_2O_2) for 5 min to loosen any remaining adhering tissue, which was then removed using plastic forceps and paper towels. Once the fin rays were cleaned, they were secured to a petrographic slide using Crystalbond (Aremco; www.aremco.com). Fin rays were sectioned as close to the base of the ray as possible at a 1·5 mm thickness using stacked diamond wafering blades on a Buehler IsoMet low-speed saw (www.buehler.com), producing two or three cross sections. Two readers aged all cross sections under a dissecting microscope before further processing.

Sagittal otoliths were removed using rubber-tipped forceps. Otoliths were rinsed in ultrapure Milli-Q (Millipore; www.emdmillipore.com) water upon removal and soaked in 30% H_2O_2 for 5 min before being mounted and sectioned as above, except with 1·0 mm section thickness. Transverse sections were taken from all otoliths across all families. Cross-sections were repositioned and mounted onto a single slide.

Prepared slides with samples were sonicated in ultrapure Milli-Q (Millipore) water for 5 min using an FS30H sonicator (Fisher Scientific; www.fishersci.com). Samples were placed in a class-100 laminar flow clean hood where they were air-dried for a minimum of 24 h before analysis. All trace-element and stable-isotope analyses were conducted at the University of South Florida, College of Marine Science.

TABLE II. Mean resolved variance (β) and cross correlation (r_{CC}) values for 12 elements that were above the limits of detection* within three families of fishes. Higher values of β and r_{CC} indicate stronger matches between datasets, with a maximum value of 1·0. *%P*, the percentage of individuals in each family where $P < 0{\cdot}05$, based on the Monte Carlo simulations; Σ *%P*, the total percentage of fishes with significant values see Table SII for full list of β, r_{CC}, and *%P* values

Element		Centropomidae ($n = 6$)	*%P*	Sebastidae ($n = 10$)	*%P*	Epinephelidae ($n = 4$)	*%P*	Σ *%P*
Li	β	−668·49	0	−17·92	0	−365·46	0	0
	r_{CC}	−0·05	0	0·04	0	0·16	25	5
Na	β	−3·27	0	−1·87	30	−432·76	25	20
	r_{CC}	−0·11	0	−0·25	20	−0·13	0	10
Mg	β	−4·33E+04	0	−6·75E+04	0	−5·92E+05	0	0
	r_{CC}	0·54	0	−0·23	0	−0·27	0	0
P	β	−1·34E+06	0	−1·59E+06	0	−2·20e+06	0	0
	r_{CC}	−0·31	17	0·26	10	−0·12	25	15
V	β	−22·42	83	−955·38	40	−537·60	50	55
	r_{CC}	−0·04	0	−0·05	0	−0·10	0	0
Mn	β	−152·54	0	−760·35	0	−2292·21	0	0
	r_{CC}	0·24	33	−0·17	0	−0·23	0	10
Fe	β	0·90	100	0·47	100	0·85	100	100
	r_{CC}	0·04	0	0·08	0	0·09	0	0
Co	β	0·72	100	−936·72	100	−1·86	100	100
	r_{CC}	0·00	0	0·01	0	0·03	0	0
Zn	β	−3·50E+04	0	−6·83E+03	10	−7·73E+03	0	5
	r_{CC}	0·42	17	0·23	10	−0·29	0	10
Cu	β	−1·98E+02	83	−9·91E+04	40	−1·06E+07	25	50
	r_{CC}	0·01	0	−0·21	10	0·06	25	10
Sr	β	0·78	100	0·78	100	0·72	100	100
	r_{CC}	0·33	33	−0·03	30	0·39	50	30
Ba	β	0·42	100	−566·29	80	−967·47	75	85
	r_{CC}	0·06	0	−0·10	30	−0·53	50	25

*Elements analysed: Li^{7}, Na^{23}, Mg^{24}, P^{31}, Ca^{43}, Sc^{45}, V^{51}, Cr^{53}, Mn^{55}, Fe^{57}, Co^{59}, Ni^{60}, Cu^{63}, Zn^{64}, Cu^{65}, Ge^{72}, Rb^{85}, Sr^{88}, Y^{89}, Cd^{114}, Sn^{118}, Ba^{137}, Au^{197}, Pb^{208}, Th^{232}, U^{238}.

TRACE-ELEMENT ANALYSIS

Core-to-edge transects were ablated on each structure using a Photon Machines Analyte 193 excimer laser ablation system (Evisa; www.speciation.net) that was connected to an Agilent 7500 ICP-MS (Agilent Technologies; www.agilent.com). The laser system operated at a wavelength of 193 nm and a maximum output of 8 mJ. The ablations of otolith and fin-ray samples were conducted with 86% power, a 5 Hz frequency and a 108 µm spot size. The laser moved across each structure at a speed of 10 µm s^{-1}. Background counts were monitored for 60 s between laser transects to ensure sufficient removal of residue from the previous transect. Measurements were made for 26 unique isotopes. Out of these, 12 were above detection limits for both structures and used in statistical analysis (Table II). Calcium was used as an internal standard for the other 25 analytes being measured due to its stoichiometric abundance within the $CaCO_3$ (primarily aragonite) and $CaPO_4$ (primarily hydroxyapatite) inorganic matrices, where Ca was 40·0% of the molecular mass of $CaCO_3$ (Campana, 1999) and 27·5% of $CaPO_4$. [Ca] of fin rays was verified after acid digestion within polypropylene vials at 180 ° C in 16 M HNO_3 for 2 h. Digested samples were diluted with 2% HNO_3 and quantitatively analysed in the ICP-MS

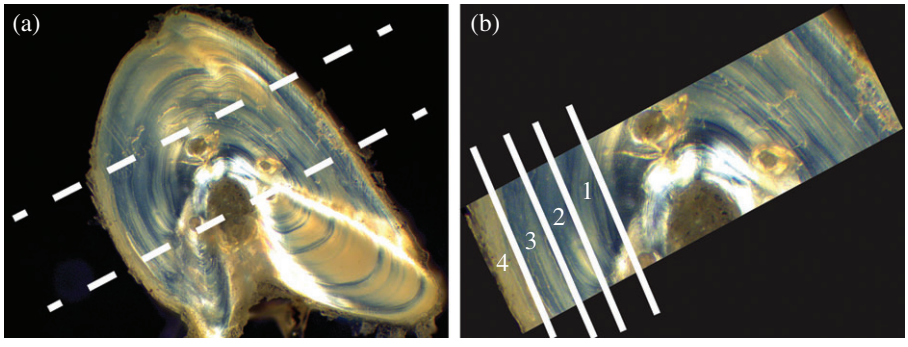

FIG. 1. Separation of annuli from a cross section of a dorsal-fin ray. (a) ---, line along which a rectangular section was taken from the cross section; (b) ——, excision lines separating individual annuli.

to obtain [Ca]. Drift of the ICP-MS during the solution-based analysis was monitored and calibrated using scandium as an internal standard. The calibration line, which establishes a transfer function from original measurements to a scale-normalized quantity, ranged from 5 to 50 mg l^{-1} for Ca.

Agilent Technologies instrument-control software was used for data collection. One external glass and one synthetic calcium carbonate standard with known isotopic compositions (NIST 612 and MACS-4) were used to calibrate the instrument. The MACS standard was analysed prior to and immediately following the analysis of all samples. The U.S. National Institute of Standards and Technology (NIST) standard was analysed both prior to and following all analyses, as well as in between each sample, allowing external drift correction. Concentrations were recorded as counts s^{-1} and then converted to mg l^{-1} using MATLAB R2015a (www.mathworks.com), with functions created in the Fathom Toolbox (Jones, 2014). Concentrations (mg l^{-1}) were used in all subsequent analyses.

STABLE-ISOTOPE ANALYSIS

A second cross section from each fin ray was used to isolate individual annuli for SIA. Each cross section was further cut to create a rectangular slice comprising radial bands that collectively represented the entire lifetime of the fish [Fig. 1(a)]. Slices were cut using a modified feather-blade guillotine. By inserting a second blade and a 0·10 mm spacer, segments were sliced without using mounting adhesive. Each slice was then cut into smaller, perpendicular subsections using the single blade of the guillotine to mechanically separate the annuli [Fig. 1(b)]. Each fin-ray subsection was representative of a different life period. The same procedure was not applied to the otolith samples because the quantification of organic material within incremental sections was beyond the capabilities of modern instrumentation.

Fin-ray sections were then classified as wild period or captive period. Each subsection was then analysed for bulk molar concentrations of carbon and nitrogen (C, N and C:N) and stable isotope ratios (δ^{13}C and δ^{15}N). A 200–1200 µg sample of each cross section was weighed on a precision micro-balance (Mettler-Toledo; www.mt.com), encapsulated in tin and loaded into a zero-blank autosampler (Costech Technologies; www.costech.com). Samples were combusted at 1050 °C in a Carlo-Erba NA2500 Series-II elemental analyser (EA; ThermoScientific) coupled in continuous-flow mode to a Finnigan Delta Plus XL isotope ratio mass spectrometer (IRMS; ThermoScientific). Stable-isotope compositions were expressed as ‰ using delta notation: *e.g.*, $\delta^{15}N = (R_{sample}R^{-1}{}_{standard}) - 1]1000$, where $R = {}^{15}N^{14}N^{-1}$. The C:N measurements were calibrated and δ^{13}C and δ^{15}N were normalized to the AT-Air and Vienna peedee belemnite (VPDB) scales, respectively, using NIST 8573 and NIST 8574 L-glutamic acid standard reference materials. Analytical precision, estimated by replicate measurements of a laboratory working standard (NIST 1577b bovine liver SRM, $n = 30$), was ± 0·25 δ^{13}C, 0·10‰ δ^{15}N and ± 0·43 C:N.

STATISTICAL ANALYSIS

In order to compare trace element concentrations between otoliths and fin rays, a Gaussian filter was applied to the otolith data, which were then standardized to match the smaller dataset associated with the fin ray for each fish. This methodology was created specifically for this project and differs from other techniques that correlate unknown concentrations in calcified structures (*i.e.* otoliths and fin rays) to ambient water concentrations. A measure of resolved variance (Mann *et al.*, 1998) was used as well as cross-correlation values (Legendre & Legendre, 2012) to compare the two structures. The resolved variance statistic measures how effectively the variance of one data series (*i.e.* otolith) is explained by the other (*i.e.* fin ray);

$$\beta = 1 - \left[\sum \left(y_{oto}\text{-}y_{fin}\right)^2\right]\left[\sum \left(y_{oto}\right)^{-2}\right],$$

where y_{oto} is a series of elemental concentrations in the otolith (after Gaussian standardization) of a single fish from time of birth to time of death and y_{fin} is the series of the same element over the same time period in the fin ray. The resolved variance (β) was calculated for each element that was above the limits of detection (Tables II and SII,) in each fish and compared with values of cross-correlation using the same time series. The resolved variance statistic was chosen as the primary metric for comparison because it is a more robust comparison of datasets than traditional correlations, as it measures the correspondence based on the relative departure from the mean, the mean itself and the absolute variances of the two datasets. The use of the resolved variance statistic allowed for the exploration of matches between datasets that were not apparent from the cross-correlation values. Higher values of both β and cross-correlation values indicate stronger matches between datasets, with a maximum value of 1·0. In addition, a two-sample Kolmogorov-Smirnov test was used to test whether either correspondence variable (*i.e.* β or cross-correlation) differed between life periods.

Significance levels for both β and cross-correlation values were estimated by Monte Carlo simulations ($n = 1000$ permutations) that took serial correlation into account. The serial correlation derived from a null model of AR(1) red noise was used (*i.e.* an auto-regressive model with a lag of one). Degrees of freedom were based on the autocorrelation coefficients with lag-one of the two series.

Stable-isotope comparisons were made for both $\delta^{13}C$ and $\delta^{15}N$ values in fin-ray sections between the captive and the wild life periods for each fish by calculating absolute differences. Absolute differences were used instead of signed differences because there was not an *a priori* reason to expect that one life period would have a higher or lower isotopic value than another and thus the magnitude of differences was of concern rather than the sign of differences. In order to assess the significance of this magnitude between the two life periods, a bootstrapping technique was used to estimate the range of average differences (99% C.I.) around the observed mean value. In addition, a Procrustes analysis was used to test how well the data from the two periods matched (Peres-Neto & Jackson, 2001). Specifically, how well did captive and wild-period data agree? Differences between periods should be expected, given associated differences in background levels and feeding history. All statistical analyses were conducted using MATLAB version R2015a (www.mathworks.com/).

RESULTS

TRACE-ELEMENT CHRONOLOGIES

The comparison statistics of core-to-edge transects between otoliths and fin rays of the same fishes varied among elements and individual fishes (Table II). Significant values of β were consistently observed for the concentrations of Fe, Co, Ba and Sr between structures (Fig. 2 and Table SIII). No other element had values that were significant in more than 55% of the samples. The cross-correlation values were not consistently

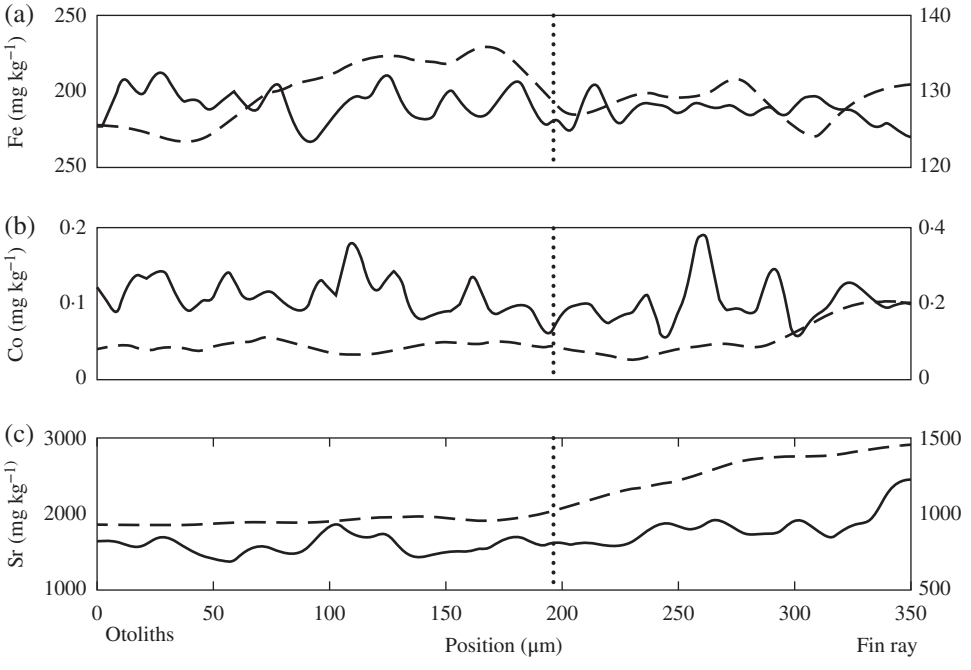

FIG. 2. Concentrations of elements with a 2+ charge, *i.e.* (a) iron (Fe), (b) cobalt (Co) and (c) strontium (Sr) over time in the otolith after smoothing (——) *via* a Gaussian filter and fin-ray concentrations (– – –) of a copper rockfish *Sebastes caurinus.* ....., the documented time of capture (*c*. 200 μm). This sample represents a fish with particularly strong β values for elemental concentrations, but is representative of the general trend observed among all samples. Profiles were run from the core of the structure (on the left, corresponding to birth) to the edge of the fin ray (on the right, corresponding to end of life).

significant for any single element, but had the highest occurrence of significance for Ba and Sr (Table II). Neither measure of correspondence differed between life period for the elemental concentrations of Fe, Co, Ba and Sr ($\overline{ks} = 0{\cdot}35$, $\overline{P} > 0{\cdot}05$), thus further analyses focused on entire lifetime comparisons of each fish instead of by life period.

Differences among families for each element were apparent for some elements, but not others. While β was consistently significant for Fe, Co, Ba and Sr across all families, other elements such as V and Cu were more often significant for centropomids than for other families (Table II). Significant values of cross-correlation in most elements were unevenly distributed among families. For example, while no centropomids had significant values of cross correlation for Ba, half of the epinephelids did (Table II).

STABLE-ISOTOPE CHRONOLOGIES

The values of δ^{13}C in all fishes ranged from −24·65 to −11·33 (mean ± S.E., −18·29 ± 0·44) for the wild periods and −21·63 to −11·24 (mean ± S.E., −16·29 ± 0·29) for the captive periods (Table III). Values of δ^{15}N ranged from 7·98 to 13·47 (mean ± S.E., 10·53 ± 0·19) for the wild periods and 7·86 to 13·62 (mean ± S.E., 10·63 ± 0·19) for captive ones. All families showed δ^{13}C enrichment and a more narrow range of values after being put into captivity (Table III). The mean absolute difference between wild

TABLE III. Mean values (‰) of $\delta^{13}C$ and $\delta^{15}N$ between wild and captive life periods in all fishes. Absolute differences are presented with lower (−C.L.) and upper confidence limits (+C.L.) for the 99% confidence interval as calculated by the bootstrapping technique

				Difference		
		Wild	Captive	−C.L.	Mean	+C.L.
Centropomidae	$\delta^{13}C$	−17·05	−15·18	1·63	2·47	3·41
	$\delta^{15}N$	10·33	10·12	0·46	0·71	0·97
Sebastidae	$\delta^{13}C$	−19·74	−17·51	1·23	2·24	3·63
	$\delta^{15}N$	11·90	12·00	0·25	0·40	0·55
Epinephelidae	$\delta^{13}C$	−19·32	−16·63	1·93	3·01	4·02
	$\delta^{15}N$	9·11	9·92	0·65	1·14	1·66
Sciaenidae	$\delta^{13}C$	−18·15	−16·63	1·88	3·31	4·74
	$\delta^{15}N$	12·08	12·40	0·54	0·69	0·83

and captive periods for individual fishes was 2·55‰ (14%) for $\delta^{13}C$ and 0·71‰ (7%) for $\delta^{15}N$. All values were significant at $P < 0{\cdot}01$.

The Procrustes analysis of all fishes further illustrated the differences between wild and captive periods *via* the comparison of paired samples ($m^2 = 0{\cdot}47$, $P < 0{\cdot}001$) (Fig. 3). When separated by family, the centropomid ($m^2 = 0{\cdot}46$, $P < 0{\cdot}001$) and sebastid samples ($m^2 = 0{\cdot}34$, $P < 0{\cdot}001$) differed strongly between wild and captive periods. While the Procrustes analyses performed on the epinephelids produced a high procrustean statistic ($m^2 = 0{\cdot}77$, $P > 0{\cdot}05$), the two periods were not significantly different. The sciaenids that were analysed were also not significantly different, although only three individuals were tested ($m^2 = 0$, $P > 0{\cdot}05$) (Fig. 4).

DISCUSSION

The data presented in this study were generated to address the hypothesis that trace elements and stable-isotope values were conserved over time in the inorganic and organic matrices of fin rays in fishes. The correspondence of certain divalent cations between otoliths and fin rays in the same fishes is consistent with the assumption of conserved inorganic-matrices (Fig. 2). Similarly, the differences in $\delta^{13}C$ and $\delta^{15}N$ values between wild and captive periods are consistent with organic-matrix conservation. The conservation of matrices in fin rays is one parsimonious explanation for the observed trends in the data, but further testing is necessary to verify the actual mechanism behind these trends. The data presented provide a framework from which to further investigate the uses and applications of trace element and stable-isotope chronologies in the fin rays of fishes.

TRACE-ELEMENT CHRONOLOGIES

The high β values generated from comparisons of divalent cations (alkaline earth and transition metals) between otoliths and fin rays in all fishes that were analysed suggest a high level of correspondence among elemental values between the two structures.

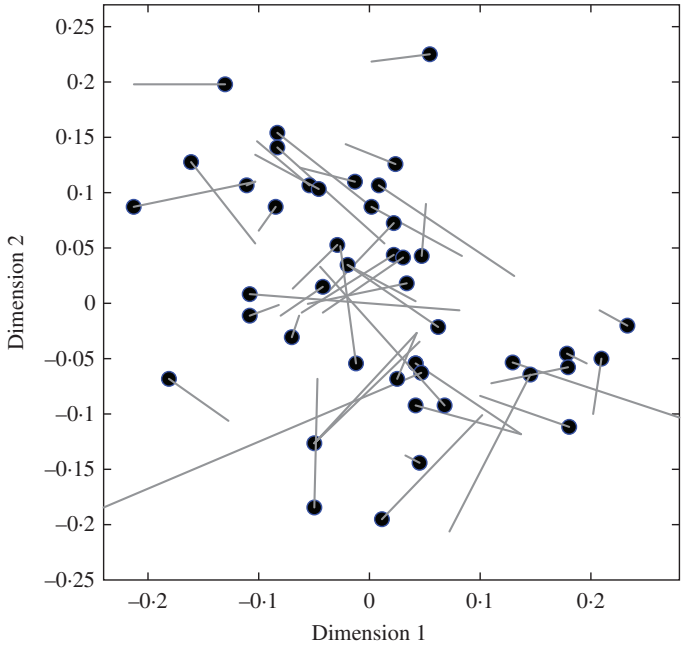

FIG. 3. Procrustean superimposition plot of wild and captive life-period paired samples of fin rays from all fishes. The *m*-statistic (m^2) for this analysis can range from 0 to 1 where smaller values represent more similarity between datasets; here, the $m^2 = 0{\cdot}47$. The dimensions describe the rotated data of the δ^{13}C and δ ^{15}N values prior to and after capture. ●, scaled wild life-period values of the rotated data; ——, residual lengths indicating the amount of difference between the samples.

This metric should not be confused with the correlation term (CC), as it measures a different aspect of the data. While CC is a measure of how well the departures from the mean correspond between two datasets, the β term offers a more robust comparison by accounting for the mean itself, the relative departure from the mean and the variances of the two datasets. Owing to the charge and size of atomic radii, the elements with high levels of correspondence can substitute for the Ca-cation in the inorganic matrices of both calcium carbonate and hydroxyapatite. Interestingly, other elements with 2+ charges (*i.e.* Mg and Mn) did not show strong correspondence between structures, possibly due to different substitution rates in $CaCO_3$ (otoliths) compared with $Ca_5(PO4)_3(OH)$ (fin rays) in the marine environment, as is common with Mg (Martens & Harriss, 1970). Trace elements, acquired from either diet or ambient water, are absorbed into the bloodstream of a fish and are incorporated into mineral matrices at the time of osteogenesis (Mahamid *et al.*, 2010; Woodcock *et al.*, 2012). The different elements tested could be expected to have different incorporation pathways, bioavailabilities or substitution affinities for each of the two calcified structures analysed. It could therefore be assumed that individual trace elements would have different rates of incorporation into each calcified structure within the fish. The correspondence of certain elements, however, is suggestive of similar incorporation processes and may indicate the conservation of material, as both structures retained similar values over time. Overall, the TEA was consistent with the hypothesis of conserved inorganic

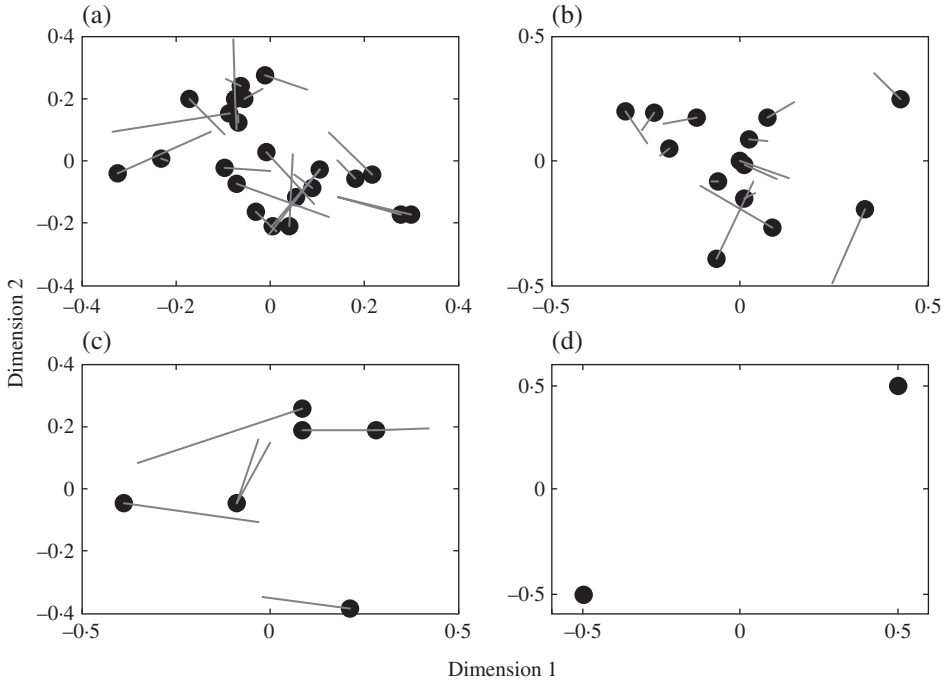

FIG. 4. Procrustean superimposition plot of wild and captive life-period paired samples of fin rays from all fishes, separated by family: (a) Centropomidae, $m^2 = 0{\cdot}46$; (b) Sebastidae, $m^2 = 0{\cdot}34$; (c) Epinephilidae, $m^2 = 0{\cdot}0$; (d) Sciaenidae $m^2 = 0{\cdot}77$. ●, scaled wild life-period values of the rotated data; ——, residual lengths indicating the amount of difference between the samples.

matrices in fin rays, as the concentrations corresponded strongly to those of the otolith, which has been documented to be a conserved matrix (Campana, 1999).

STABLE-ISOTOPE CHRONOLOGIES

Stable-isotope analysis in fin rays required several assumptions with regard to chemical stability. Most notably, the assumption of little to no turnover is critical to the effective use of stable-isotope chronologies for the inference of life-histories in individual fishes. The high level of resolved variance in divalent cation concentrations between otoliths and fin rays was consistent with this assumption, as the inorganic matrix in fin rays is embedded within the organic one (Mahamid *et al.*, 2010). Thus, as one matrix is encapsulated and its properties are conserved over time, the same could be expected for the other matrix. In addition, encapsulated layers are not vascularised, thus little to no turnover would be expected. The differences in $\delta^{13}C$ and $\delta^{15}N$ values between wild and captive periods could have resulted from differences in ambient water, differences in diet, tissue turnover or tissue decay. The reason for the unidirectional enrichment of $\delta^{13}C$ values in the captive period remains unclear, but may have resulted from carbon-based filtration systems in aquarium tanks, such as those that use activated carbon. Differences in water chemistry and diet between the natural environment and an artificial one (*i.e.* public aquariums) are suggestive of the most

parsimonious explanation for the observed differences. Tissue turnover would have probably resulted in equal values across both life periods, while tissue decay would have exhibited consistent directional differences (most likely depletion) in both $\delta^{13}C$ and $\delta^{15}N$ values. Other explanations for the observed differences include differential turnover rates among annuli, incomplete turnover (*i.e.* where turnover only occurs in one part of the ray) and different incorporation rates among annuli.

A last assumption of chronological SIA involved the time period of deposition. In this study, the organic matrix within each annulus was assumed to originate at the corresponding age of the fish. Further studies are necessary to verify this assumption as there may be a depositional lag due to metabolic pathways.

The stable-isotope trends observed in this study were consistent among three families and seven species. It should be noted, however, that differences among families cannot be differentiated from differences among ambient conditions, as samples within families all came from the same aquariums. Centropomids and sebastids had stable-isotope values that were significantly different between wild and captive periods based on the Procrustes analysis (Fig. 4). The epinephelids also showed a strong trend towards differences between life periods (*i.e.* the highest observed *m*-statistic); however, no significant difference was observed, possibly as a result of a low sample size ($n = 5$). The only family that had outlying trends was Sciaenidae, which did not show differences in stable-isotope values between wild and captive periods in the Procrustes analysis (Fig. 4). No sciaenid otoliths were available for analysis, so the TEA trends were undocumented. The anomalous trend among SIA in sciaenids may be due to small sample size ($n = 3$) or a true homogeneity between life periods. A larger sample size should be used to identify the cause of this anomaly.

METHODOLOGICAL LIMITATIONS AND POTENTIAL USES

Microchemical analyses in fin rays have several limitations that are primarily related to the chemical composition of each sample. Values of $\delta^{13}C$ need to be interpreted carefully, as inorganic carbon noise can affect the final output from the mass spectrometer. Even though fin rays are primarily composed of hydroxyapatite, carbonate molecules commonly substitute for phosphate and the resulting carbon noise in the analysis of the organic component in fin rays (and all bones) will obstruct the signal (Peroos *et al.*, 2006). Limitations also exist due to the size of cross sections in fin rays and the annuli therein. Cutting curved annuli with a straight blade is challenging. Owing to mechanical limitations and the small sizes of the samples used in this study, temporal resolution was limited to two, multi-year periods. Further refinement with this method and instrumentation with higher precision (*e.g.* micro-elemental analysis mass spectrometry) could lead to stable-isotope chronologies that are representative of smaller time gaps. Fishes with larger fin rays can be used with existing instrumentation (Tzadik *et al.*, 2015).

In its current form, the methods presented here could be used to track migratory and trophic patterns across ontogeny for individual fishes. If matrices are indeed conserved in fin rays, then life-history attributes for each fish are recorded continuously so that a complete record is available for each individual. These records can be used to test actual life-history trends in individuals as opposed to assumed life periods (that are ultimately researcher defined), such as ontogenetic migrations (Allen *et al.*, 2009). Traditional SIA using muscle tissue is limited in temporal inference due to relatively fast

turnover rates. A conserved matrix of organic material could be used instead to infer trends over time with much smaller sample sizes than would be necessary through the use of muscle tissue. Combining stable-isotope chronologies with existing isoscapes can lead to detailed studies on individual movements by recording how baseline values change over time. Inferring movements from isoscapes is relatively common in terrestrial ecology, but has yet to be used extensively in the marine environment. Chronological recorders of stable isotopes in fishes (*e.g.* fin rays and eye lenses) can be used to bridge the gap between these two fields. The use of fin-ray analysis for management purposes should be considered especially when endangered species are in question, as fin-ray removal is minimally invasive and does not affect growth or survival (Zymonas & McMahon, 2006).

The results presented here are consistent with the hypothesis that chemical matrices in fin rays are conserved over time. These matrices can be used to measure trace-element and stable-isotope values over time that are representative of the inorganic and organic matrices, respectively. These types of analyses appear to present viable alternatives to lethal techniques to study life-history characteristics in fishes where culling activities are inappropriate.

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Supporting Information

Supporting Information may be found in the online version of this paper:

TABLE SI. Ages, time spent in captivity, and date of death (collection date) for all samples used in the study. Age estimates were derived from fin rays, and verified by otoliths when available.

TABLE SII. A list of mean values of the limits of detection (LOD) and the per cent relative standard deviation (%RS.D.). Each value was calculated as a mean value across each sample and each run of the instrument.

TABLE SIII. A complete list of resolved variance statistics (β), cross correlation (r_{CC}) values and associated P-values ($^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$) for each specimen among all elements tested.

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