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research paper

Nonlethal stable isotope analysis reveals consistent trophic growth of juvenile Atlantic goliath grouper *Epinephelus itajara* in Brazilian estuaries

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ABSTRACT.—Atlantic goliath grouper *Epinephelus itajara* (Lichtenstein, 1822) are classified as vulnerable by the IUCN and have decreasing local populations throughout their distribution due to overfishing and habitat degradation. Due to their protected status, basic life history information that can inform management and conservation is lacking for some local populations, including in Brazil. In the present study, we examined how $\delta^{15}N$ of juvenile Atlantic goliath grouper fin rays, a nonlethal method, varied with total length across two estuaries in Brazil. A total of 100 juvenile Atlantic goliath grouper (total length range: 95–505 mm) were analyzed, and we observed positive relationships between $\delta^{15}N$ and fish lengths (i.e., evidence of trophic growth). Among-estuarine slopes did not differ, suggesting trophic growth was consistent among sites despite different $\delta^{15}N$ values between the northernmost site and a group of southern sites, possibly reflecting different isotopic baselines. This study is the first effort to provide useful insights into the trophic ecology of juvenile Atlantic goliath grouper in Brazil, which could help address knowledge gaps and conserve this endangered species. The nonlethal methodology employed in this study could be used to advance our understanding of the trophic ecology of other vulnerable and endangered marine fishes and help inform conservation and management practices.

Atlantic goliath grouper *Epinephelus itajara* is the largest epinephelid in the Atlantic Ocean, living for at least 37 yrs and growing up to 3 m (total length, TL) and 400 kg (Sadovy and Eklund 1999). Populations of Atlantic goliath grouper have declined due to overfishing and habitat degradation. The species is currently classified as "Vulnerable - VU" by the International Union for Conservation of Nature (IUCN;

Bertoncini et al. 2018), and as "Critically endangered - CR" in Brazil (Ordinance N°13/2015). The Atlantic goliath grouper was the first marine fish species to have a national fishing moratorium in Brazil. However, life history information for this species in Brazil is generally lacking and is insufficient to assess its population status, especially during its juvenile phase.

Information about the Atlantic goliath grouper juvenile stage is limited throughout its distribution. In the eastern Gulf of Mexico, juvenile Atlantic goliath grouper remain in mangrove habitats for up to 6 yrs and up to about 1 m TL, after which they emigrate to reef habitats (Bullock et al. 1992, Koenig et al. 2007). Atlantic goliath grouper are generalist feeders with a wide trophic niche (Artero et al. 2015). They are an opportunistic ambush predator that feeds at a relatively low trophic position, mainly on slow moving teleosts and decapods (Koenig and Coleman 2009). Crustaceans are a dominant prey for juveniles (Artero et al. 2015), especially *Callinectes* sp. (Freitas et al. 2015). However, more work on the trophic ecology of juvenile Atlantic goliath grouper is needed to improve our understanding of its life history, especially from estuarine habitats that may serve as nurseries.

Stable isotope analysis (SIA) has become a common method to study trophic ecology (reviewed by Harrod and Stallings 2022), and particularly to estimate trophic growth (i.e., positive relationship between fish length and trophic position) of consumers (Minagawa and Wada 1984, Faletti and Stallings 2021). Indeed, nitrogen stable isotopes ($\delta^{15}N$) have been extensively applied to estimate trophic growth of organisms in food webs (Stricker et al. 2015, Kurth et al. 2019) because of its marked isotopic fractionation (i.e., the isotopic shift during the process of food digestion and assimilation), usually ranging from 2‰ to 4‰ with each trophic step (Post 2002). In addition, spatial variation in $\delta^{15}N$ may also reflect accumulation of anthropogenic biochemical inputs in aquatic systems (Cabana and Rasmussen 1994, Donadt et al. 2021). Thus, measuring $\delta^{15}N$ can be informative for both life history of focal species and background variation in anthropogenic influences (Curtis et al. 2020).

Dorsal muscle is the most common tissue used for fish SIA analysis (Condini et al. 2015, Coletto et al. 2021), but requires lethal sampling (Hanisch et al. 2010). Thus, the use of muscle tissue for species of high conservation concern is often not appropriate. Tzadik et al. (2015) demonstrated the efficacy of a nonlethal approach in SIA studies involving Atlantic goliath grouper, with the use of dorsal fin rays that can be excised from the fish. Fin rays can be extracted without effects on fish survival and growth (Zymonas and McMahon 2006, Murie et al. 2009) and can regrow after being excised (Goss and Stagg 1957, Tzadik et al. 2015). Another advantage of using fin rays for trophic research is they allow the reconstruction of longer chronological histories of stable-isotope ratios (Estrada et al. 2006, Tzadik et al. 2015). Inner fin-ray layers lose vascularization and become encapsulated by growing outer layers, thus little to no turnover is expected (Sire and Huysseune 2003, Tzadik et al. 2017a). The ability to reconstruct life history information can further improve our understanding of the trophic dynamics of consumers, including those of conservation concern. Such information is generally lacking for juvenile Atlantic goliath grouper across its distribution, and especially in Brazil.

In the current study, we measured how $\delta^{15}N$ varied with length of juvenile Atlantic goliath grouper sampled across four different estuarine sites on the southeastern Atlantic coast of Brazil. Specifically, we asked: (1) Does $\delta^{15}N$ increase with total length of juvenile Atlantic goliath grouper? and (2) Are observed changes in $\delta^{15}N$

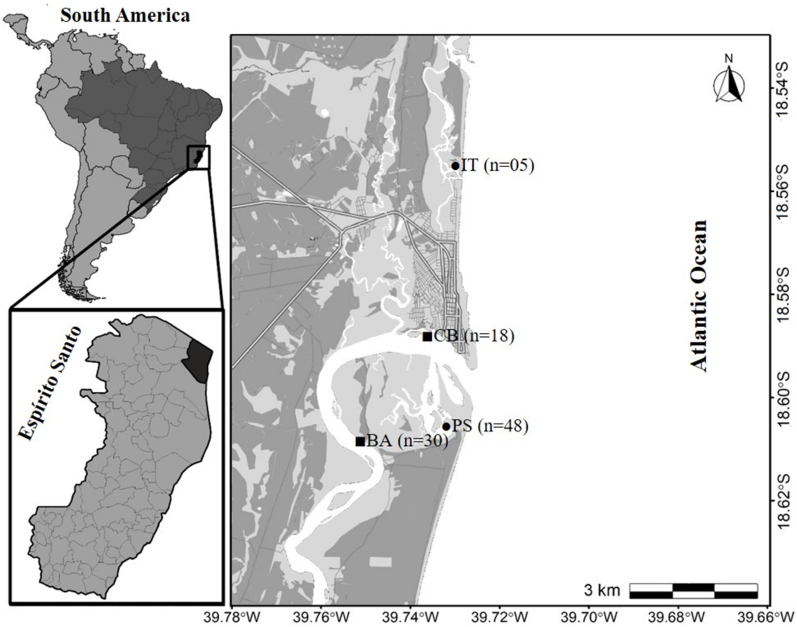

Figure 1. Map showing the study area in Conceição da Barra, Espírito Santo, Brazil, and the sampling sites Conceição da Barra (CB, *n* = 18), Barreiras (BA, *n* = 30), Pontal do Sul (PS, *n* = 48), and Itaúnas (IT, *n* = 5), where juvenile Atlantic goliath grouper *Epinephelus itajara* were (●) captured intentionally for this study and (■) captured accidentally by fishers and donated for the present study.

consistent across estuarine sites? Estimating trophic growth of Atlantic goliath grouper, its changes with fish ontogeny during critical phases of their life cycle (e.g., as juveniles in nursery grounds), and spatial variability can provide important information for better understanding the trophic role played by this endangered predator in coastal ecosystems. Our findings also provide baseline information for future monitoring programs and coastal management for this species.

Materials and Methods

This research was conducted in the São Mateus and Itaúnas estuaries on the southeastern Brazilian coast. We sampled juvenile Atlantic goliath grouper from four sites: (1) Barreiras (BA), (2) Pontal do Sul (PS), (3) Conceição da Barra (CB), and (4) Itaúnas (IT; Fig. 1). With the exception of IT, the other sites are located within the Environmental Protection Area of Conceição da Barra, which is a Sustainable Use Conservation Unit. CB is likely under substantial anthropogenic influence due to its adjacent proximity to a highly populated area (Conceição da Barra city with approximately 31,000 inhabitants, IBGE 2020). In contrast, IT is likely the least impacted site because it is within an Integral Protection Conservation Unit (Brasil 2000) and is located in a sparsely populated area with limited anthropogenic influences.

The sample collections were performed under license number 15080-7 provided by the Instituto Chico Mendes de Biodiversidade (ICMBio). Juvenile Atlantic goliath grouper were captured from March 2015 to November 2015 (*n* = 33) and from March

2018 to October 2019 (n = 68). Individuals were captured using a variety of gears including baited blue crab traps and setlines deployed close to mangrove roots and rocks. We also received donations of live juvenile Atlantic goliath grouper accidentally caught by artisanal fishers using a wide array of fishing gears, such as setlines, fishing rods, blue crab-traps, gill nets, cast nets, and surrounding nets.

All fish specimens (captured and donated) were measured for TL (mm), had their soft dorsal-fin rays 5–7 excised as close to the base as possible, and were released immediately following sampling procedures (Tzadik et al. 2017a). The excised rays were stored at −20 °C until further analyses as this has been shown to not impart offsets in SIA of groupers (Stallings et al. 2015). Later, frozen fin rays were thawed in a drying oven for 4 hrs at a temperature of 55 °C. After drying, fatty tissue was removed from the rays and each fin ray was soaked in 30% hydrogen peroxide (H_2O_2) for 5 min to help with the removal of skin and membranes (complete details in Tzadik et al. 2015). According to the growth curve proposed by Bullock et al. (1992), most individuals were young-of-year (i.e., <1 yr old); therefore, dried whole fin rays were turned into a fine homogeneous powder using a mortar and pestle, representing the whole life of individuals. A 200–1200 µg sample of each ray was weighed on a Mettler-Toledo precision micro-balance and encapsulated in a tin capsule (Tzadik et al. 2015).

Stable isotope ratios of nitrogen ($\delta^{15}N$) were determined by Continuous Flow Elemental Analyzer Isotope Ratio Mass Spectrometry at the University of South Florida, College of Marine Science, Marine Environmental Chemistry Laboratory (Werner et al. 1999). Isotope compositions were measured on a Thermo Finnigan Delta Plus XL IRMS and are reported in per mil (‰) notation, and scaled to Air calibration material ($d^{15}N$). Secondary reference materials [NIST 8574 $\delta^{15}N$ = +47.57‰ (SD 0.22‰), N = 9.52%; NIST 8573 $\delta^{15}N$ = −4.52‰ (SD 0.12‰), N = 9.52%] were used to normalize raw measurements to the Air scale (Werner and Brand 2001, Qi et al. 2003, Coplen et al. 2006). Measurement uncertainty, expressed as ±1 standard deviation of n = 10 measurements of a laboratory reference material [NIST 1577b $\delta^{15}N$ = 7.83‰ (SD 0.16‰), N = 9.95 ± 0.48) was 0.18‰. We did not analyze carbon stable isotope ($\delta^{13}C$) ratios because prior studies suggested bias in the determination of this isotope tracer in dorsal fin rays of Atlantic goliath grouper (Tzadik et al. 2015, 2017b).

Linear mixed models (Gaussian distribution) were used to estimate the relationship between $\delta^{15}N$ isotope values and both TL and collection location. Since collections were made in two time periods (2015 and 2018–2019), we first examined the effect of year and found it was not significant after accounting for both TL and collection location ($F_{1,93}$ = 2.76, P = 0.10). Next, we tested the interactive effect of TL and location (as fixed effects), which was not significant ($F_{3,91}$ = 0.52, P = 0.67), so we used an additive model with individual fish as a random effect. The models were conducted in R v4.1.0 (R Core Team 2021) and plotted with the ggplot2 package (Wickham 2016).

Results

In total 100 juvenile Atlantic goliath grouper were analyzed due to the exclusion of one outlier value ($\delta^{15}N$ = 7.49 from CB), although none of the statistical interpretations were influenced by its inclusion. Fish lengths ranged from 95 mm to 505 mm TL, with the lowest mean length observed in the PS [236.7 (SE 15.9)] and the highest in the IT sites [373.6 (SE 40.1); Table 1].

Table 1. Mean (SE) total length (TL) and δ15N of sampled *Epinephelus itajara* (n = 101) by site. Pontal do Sul (PS), Barreiras (BA), Conceição da Barra (CB), and Itaúnas (IT). The far-right column reports the TL coefficients (SE) for its modeled relationship with δ15N.

Sites	n	TL (mm)		δ15N		TL coef (SE)
		Mean (SE)	Range	Mean (SE)	Range	
PS	48	236.7 (15.9)	95–505	12.45 (0.17)	10.03–14.97	0.005 (0.001)
BA	30	239.0 (10.3)	156–431	12.24 (0.20)	10.63–14.59	0.008 (0.003)
CB	17	311.1 (6.0)	140–500	13.18 (0.30)	11.18–15.62	0.003 (0.003)
IT	5	373.6 (40.1)	249–460	11.11 (0.24)	10.17–12.77	0.005 (0.002)

Measured values of δ15N in juvenile Atlantic goliath grouper ranged from 10.03‰ to 15.62‰, with the highest and lowest mean values observed in the CB and IT sites, respectively (Table 1). A positive relationship was observed between juvenile Atlantic goliath grouper length and δ15N ($F_{1,94}$ = 19.33, P < 0.01) and the rate of change was consistent across areas (interaction term: $F_{3,91}$ = 0.52, P = 0.67; Fig. 2). The δ15N values differed among areas ($F_{3,94}$ = 8.78, P < 0.01), and a posthoc Tukey test revealed this was largely due to lower values at IT compared to the other three areas (all pairwise tests with IT: P < 0.01).

Discussion

In this study, we have shown that δ15N scaled positively with length of juvenile Atlantic goliath grouper and that this relationship was consistent across spatially separated locations. This work fills a gap in our understanding about the trophic ecology of juvenile Atlantic goliath grouper and is the first effort to do so in Brazil. Although ontogenetic increases in trophic position in fish is common (Estrada et al. 2006, Kurth et al. 2019) and is often correlated with increasing body length due to expected 15N enrichment in their tissues as they feed on larger prey up the food webs (Condini et al. 2011, Iitembu et al. 2012, Park et al. 2018, Faletti and Stallings 2021), the increase in this study was rather slow, being less than one trophic level across the entire length range of sampled fish. This result suggested that juvenile Atlantic

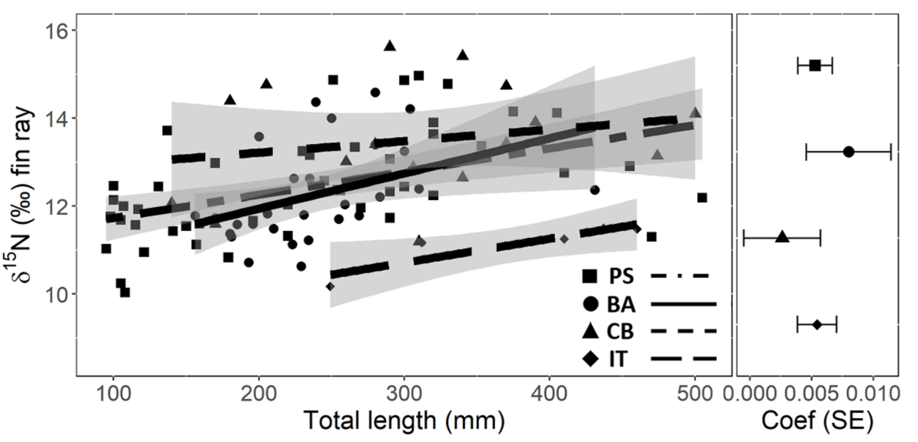

Figure 2. Values of δ15N from juvenile Atlantic goliath grouper fin rays plotted against total length (TL, mm) with fitted generalized linear mixed model trendline (gray envelope = 95% CI), separated by study sites from which fish were caught.

goliath grouper fed within approximately the same level in the local food webs, making gradual changes to slightly higher positions. This slow trophic growth was expected for juvenile Atlantic goliath grouper, given that previous studies have shown their diets consist primarily of small, low trophic-level benthic organisms, such as crustaceans throughout their life (Koenig and Coleman 2009, Artero et al. 2015, Freitas et al. 2015). We also sampled a relatively small size range of juvenile Atlantic goliath grouper since they can be found in mangrove habitats at total lengths up to 1000 mm (Koenig et al. 2007), thus our results do not capture their full trophic ontogeny during this stage of their life history. Future work should target larger juveniles to address this knowledge gap.

In addition to slow but consistent trophic growth, we also observed notably lower $\delta^{15}N$ values at IT compared to the other three areas. These observed differences in trophic baselines likely reflected a stronger influence of nutrient inputs from anthropogenic activities at the southern sites. Anthropogenic activities associated with urbanization and industrialization create potential problems in coastal areas such as mangroves and estuaries (Elliott and Whitfield 2011). Untreated anthropogenic effluents usually have higher values of $\delta^{15}N$ compared to nearby locations free of them or where human activities are relatively low (Lassauque et al. 2010). For instance, Souza et al. (2018) evaluated anthropogenic impacts on two contrasting mangrove food webs by using multiple stable isotopes measured in sediments, mangrove trees, plankton, shrimps, crabs, oysters, and fish. They observed higher $\delta^{15}N$ across the food web in the estuary closest to intensive human activities, apparently influenced by sewage. Our findings are consistent with Souza et al. (2018). For example, the São Mateus River receives a significant number of pollutants along its course, mostly as runoff from agricultural areas upstream and domestic wastewater downstream (Cotta et al. 2017). The problem is worsened when the river waters reach Conceição da Barra City (adjacent to the CB sampling site), which has a deficient sewage treatment system (Conceição da Barra 2017). Coincidently, lower $\delta^{15}N$ values were observed in the site surrounded by a sparsely populated area with limited anthropogenic influences (IT). Prior work elsewhere has suggested that nutrient pollution could be a reason for $\delta^{15}N$ enrichment in the juvenile phase of Atlantic goliath grouper (Lapointe et al. 2005, Tzadik et al. 2015) and similar conclusions have been drawn for other groupers and mesopredators (Curtis et al. 2020). Further investigation is needed to identify the potential influence of human impacts (untreated sewage input and other nitrogen pollution sources) to avoid overestimation in trophic enrichment of juvenile Atlantic goliath grouper in estuaries.

The nonlethal methodology of using fin rays applied in this study causes minimal effects to fish. Tzadik et al. (2015) recaptured several individuals with baited hooked gears the same day after having their fin rays excised, suggesting maintenance of their feeding behavior within hours of being sampled. This has also been corroborated in the current study by the recapture of individuals, using baited blue crab traps, the same day and weeks after sampling, confirming their recovery and survival to the procedure (L Lopes Almeida, unpubl data).

Although a small sample size is a limitation commonly observed when working with endangered and protected species (Frias-Torres 2006), this study was possible due to the combination of using a nonlethal method and a long-standing and reliable partnership with artisanal fishers from traditional communities, who donated live accidentally captured Atlantic goliath grouper individuals. This partnership between researchers and local citizens shows that local engagement is an important asset toward successful management (Silva et al. 2021) and the protection of biodiversity. Furthermore, this was the first study to our knowledge on the trophic ecology of juvenile Atlantic goliath grouper off South America, an important region for the southern extent of the species distribution. Future studies are needed to further evaluate the trophic role of this endangered predatory fish, especially during its early development in nursery habitats.

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