

# Fine-scale geographic variation of stable isotope and fatty acid signatures of three fish species in the Indian River Lagoon, FL

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**FINE-SCALE GEOGRAPHIC VARIATION OF  
STABLE ISOTOPE AND FATTY ACID SIGNATURES OF THREE FISH SPECIES IN  
THE INDIAN RIVER LAGOON, FLORIDA**

by

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A thesis submitted in partial fulfillment of the requirements  
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## ABSTRACT

The Indian River Lagoon, Florida, is a unique closed “bar built” estuary system that has little interchange with the Atlantic Ocean and which is home to many resident species. Three fish species were investigated to see if their isotopic and fatty acid signatures differed based on geographic location. The goal was to assess the degree of resolution of spatial variation that is possible when using stable isotope and fatty acid signature analysis to interpret feeding habits and potential linkages between feeding habits and health status. Spotted seatrout (n=40), pinfish (n=60) and white mullet (n=60) were collected over a 4 week period at sites 30 km apart in two distinct biogeographic regions of the IRL. Fish were analyzed for stable isotope ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) and fatty acid composition. All three species were significantly different from each other in both isotopic and fatty acid signatures. In the North Indian River segment, spotted seatrout, white mullet and pinfish had mean values ( $\pm$  SE) for  $\delta^{13}\text{C}$  of  $-18.00 \pm 0.08$ ,  $-14.59 \pm 0.07$  and  $-16.88 \pm 0.04$  respectively and for  $\delta^{15}\text{N}$ , mean values were  $14.43 \pm 0.05$ ,  $8.30 \pm 0.04$  and  $10.43 \pm 0.03$  respectively. For the North-Central Indian River segment, spotted seatrout, white mullet and pinfish had mean values for  $\delta^{13}\text{C}$  of  $-18.98 \pm 0.02$ ,  $-16.25 \pm 0.06$  and  $-16.94 \pm 0.04$  respectively and for  $\delta^{15}\text{N}$ , mean values were  $14.21 \pm 0.02$ ,  $8.07 \pm 0.03$  and  $10.64 \pm 0.03$  respectively. When species and location interactions were examined using ANCOVA, a post-hoc Tukey’s HSD test showed that  $\delta^{13}\text{C}$  was significantly affected by sampling segment only for spotted seatrout and that there was no significant effect of location on  $\delta^{15}\text{N}$  values. Spotted seatrout was the only species that differed between segments for  $\delta^{13}\text{C}$ . Classification and Regression Tree (CART) analysis was then performed on the “extended dietary fatty acids” for each individual species and collection location. In this analysis, all three species were correctly identified to segment (white mullet - MR 2/53 p~ 0.03; spotted seatrout (MR 0/40), and pinfish (MR 0/56) indicating that

individual species were exhibiting significant differences in their fatty acid signature over distances of 30 km. The ability to discern fine-scale differences in potential prey allows for the possibility of better resolution of dolphin feeding habits and hence a better understanding of both habitat utilization and health impacts. Due to limited exchange of clean salt water, contaminants can theoretically become a problem and there are indications that the health of the resident population of bottlenose dolphins (*Tursiops truncatus*) has been declining over recent years.

I dedicate this thesis to my sister, Joanna, my parents Dorothy and Joe, my husband J.D. and my best friend Nicole. Without their help, support and dedication this thesis would have never come to be what it is today. I love them all and thank them from the bottom of my heart for everything they have done for me to see this dream through.

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## INTRODUCTION

Historically, feeding habits have been studied by either direct observation or by analyzing and identifying stomach and/or fecal contents. These analyses typically reveal the most recent meal(s) and do not necessarily reflect the entire breadth of an animal's diet (e.g. Worthy et al. 2008). While stomach and/or fecal analyses can be useful tools for studying feeding habits there are complications associated with them: 1) fecal collections are functionally restricted to terrestrial species or to those in captivity and 2) the inability to identify prey due to digestion (Jobling and Breiby 1986). Also, stomach lavage, which involves insertion of a tube into the subjects stomach and pumping water into it until the food is dislodged, may be harmful to the animal and potentially lead to death (e.g. Worthy et al. 2008). In marine species stomach content and/or fecal analyses can be problematic because the method usually involves identification of fish, detritus and/or invertebrate species where only non-digestible parts of the organism, such as vertebrae, scales, maxillary bones, skull cases, or otoliths, are present in the contents (e.g. Barros and Odell 1990, Pierce and Boyle 1991). To overcome this shortcoming, DNA found in the contents, have been used to identify prey species (Tollit et al. 2009). New approaches such as fatty acid signature and stable isotope analysis, using various mixing models, are being implemented as non-invasive, or limited invasive, procedures that reveal considerably more about feeding history of an individual than just the last meal.

Stable isotope analyses relies on the incorporation of the prey's isotopic composition into the predator to reveal feeding patterns without many of the pitfalls experienced with gastric lavage or fecal analysis (e.g. Hobson 1999). The usefulness of this technique is that the signatures of the studied animal reflect the indigenous food web (e.g. Peterson and Fry 1987,

Tieszen and Boutton 1988). Carbon and nitrogen are naturally found in the environment in two stable forms with the lighter forms,  $^{14}\text{N}$  and  $^{12}\text{C}$ , being more abundant than the heavier forms,  $^{15}\text{N}$  and  $^{13}\text{C}$ . The common vernacular is to refer to the heavier isotope concentration as a ratio in  $\delta$  notation (‰) as determined from:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}})-1] \times 1000$$

where X is  $^{15}\text{N}$  or  $^{13}\text{C}$  and R is the corresponding ratio of  $^{15}\text{N}/^{14}\text{N}$  or  $^{13}\text{C}/^{12}\text{C}$ . Standard reference materials are carbon from PeeDee Belemnite limestone and atmospheric nitrogen gas (Worthy et al. 2008). This technique uses differences in ratios of carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) to trace diet through their respective pathways. As isotopes move up the food chain, diet-tissue fractionation, the differential discrimination of the rarer heavier form, occurs thus allowing the trophic levels and individual predator/prey items to be traced through the food chain (e.g. Peterson and Fry 1987). Typically carbon and nitrogen have a fractionation rate of 0-1‰ and 3-4‰ respectively as you move up in trophic levels (McCutchan et al. 2003). The nitrogen isotope ratio indicates the trophic level of the sampled species and can be used to determine potential prey, while the carbon isotope ratio reflects the primary producers in the food web (e.g. DeNiro and Epstein 1978, Pauly et al. 1998). The initial fixation of carbon or nitrogen has a distinctive pathway that leads to each primary producer obtaining its' own signature. For example, the chemical pathways in  $\text{C}_3$  plants preferentially utilize  $^{12}\text{C}$  before using  $^{13}\text{C}$  while  $\text{C}_4$  plant chemical pathways utilize  $^{13}\text{C}$  before using  $^{12}\text{C}$ . This specific chemical utilization of carbon and nitrogen creates a signature which is unique to each plant and which is then translated into the herbivores and eventually to carnivores (Worthy et al. 2008).

Over the past 20-30 years, carbon and nitrogen isotope analyses have been used to study trophic webs (e.g. Abend and Smith 1995, Jennings et al. 1997, Hobson 1999, Stowasser et al. 2009, Wan et al. 2010, Zamzow et al. 2011), predator prey interactions (e.g. Schmutz and Hobson 1998, Worthy et al. 2008, Guest et al. 2010), seasonal prey shifts due to migration or prey availability (e.g. Nelson et al. 1982, Schmutz and Hobson 1998, Perga and Gerdeaux 2005, Wai et al. 2011) and spatial patterns of both predator, prey and primary producers (e.g. Nelson et al. 1982, Jennings et al. 1997, Guest and Connolly 2004, Guest et al. 2010). Stable isotopes have also been used to show potential dietary shifts that may have been missed otherwise (e.g. Darr and Hewitt 2008, Worthy et al. 2008). Several reviews of the literature on the use of stable isotopes have determined that this analysis can be useful for determining the overall food web structure within an ecosystem, as well as documenting changes within that food web (Hobson 1999, Layman 2007).

One major limitation to the application of stable isotopes is that turnover rate and diet-tissue fractionation values are not known for every species, As a result, these parameters must be estimated in order to recreate food webs (Hobson 1999, Layman 2007). Historically stable isotopes were used as a “coarse” evaluation of feeding history due to the limited ability of stable isotope variables to identify prey (i.e. carbon, nitrogen etc.). To refine this type of analysis, studies have shown that mixing models that take into account fractionation, enrichment, such as IsoSource (e.g. Phillips and Gregg 2003), can be used to give a more refined interpretation of feeding interactions by outlining the possible prey combinations that would give the predator its signature and then assigns a value as to how likely that scenario is (e.g. Worthy et al. 2008, Martinez del Rio et al. 2009, Wai et al. 2011). Another way to refine this type of analysis is to

study stable isotopes and fatty acids together to give a clearer look into a species role in the food web and food web interactions.

Fatty acids have the potential for an even higher degree of resolution than when stable isotope mixing models are employed. Carbohydrates and proteins are quickly broken down during digestion, whereas, fatty acids are incorporated into a carnivore's tissues relatively unchanged (Ackman 1980). Fatty acids are named based on the number of carbon atoms and the number and location of their double bonds. For example, 16:1 n-11 has 16 carbons in the chain, and has a single double bond 11 carbons from the methyl end of the fatty acid. The combination of carbon length, number of double bonds, and the placement of those bonds allows for the huge variety of fatty acids found in nature. Fatty acid signature analysis (FASA) utilizes 60+ long chain fatty acids, 14 carbons or greater, to potentially identify the specific prey consumed by a predator (Iverson et al. 1997, Iverson et al. 2004). Lipids in marine organisms are characterized by their great diversity and high levels of long-chain and polyunsaturated fatty acids that originate in various unicellular phytoplankton and seaweeds (Ackman 1980). Marine food webs contain many fatty acid signatures that are specific to each prey species and depend on the origination of the fatty acid (i.e. plankton or seagrass) (Ackman 1980). Iverson et al. (1997, 2004) indicated that, of those 60+ fatty acids, there is a subset of "essential dietary fatty acids" that should be utilized when conducting FASA because these specific fatty acids cannot be endogenously produced and therefore must come from the consumed prey. Many studies have shown that these "essential dietary fatty acids" are incorporated into the predator's tissues without major modification and can then be used to trace feeding (e.g. Aguilar and Borrell 1990, Budge et al. 2006, Beck et al. 2007). Recent studies have used FASA to investigate the feeding

ecology of many predators such as the bottlenose dolphins (e.g. Samuel and Worthy 2004, Worthy et al. 2008), penguins (e.g. Tierney et al. 2008), marine fish (e.g. Perga and Gerdeaux 2005, Trushenski et al. 2008, Worthy et al. 2008, Surh et al. 2009, Wan et al. 2010, Wai et al. 2011), invertebrates (e.g. Budge et al. 2002, Guest et al. 2010) and pinnipeds (e.g. Beck et al. 2007). Fatty acid signature analysis has been shown to have potential fine scale sensitivity that can allow for the identification of individual consumers from a specific geographic area based on their dietary signature (Iverson et al. 1997, Smith et al. 1997). However, because FASA is such a fine scale tool it can be difficult to identify and profile all potential prey the predator can be consuming, especially if the trophic level at which that predator is feeding at is unknown. This limitation can lead to bias within the analysis for predators that utilize abundant easily accessible prey species.

Stable isotope analysis and FASA are now being used in conjunction with one another to reduce the limitations for each analysis (e.g. Hooker et al. 2001). The key advantage to using both techniques in concert is to get a long history of the prey consumed by any individual animal and reveal a species dietary history (Worthy et al. 2008). However, before food webs can be constructed there needs to be an understanding of what can cause variation within the species' signatures. Seasonal changes in the environment/prey availability (e.g. Perga and Gerdeaux 2005, Alves 2007, Worthy et al. 2008), ontogenetic shifts (e.g. Budge et al. 2002, Stowasser et al. 2009, Wai et al. 2011) and freshwater runoff (e.g. Wai et al. 2011) have been shown to have a significant effect on both stable isotope and fatty acid signatures. Having a clear understanding of the temporal and spatial variability of each of these factors and their impacts on the biochemical signatures of the potential prey is critical.

Geographic location has been shown to affect the carbon isotope (e.g. Abend and Smith 1995, Jennings et al. 1997, Boyce et al. 2001, Guest and Connolly 2004, Gerard and Muhling 2010, Guest et al. 2010), nitrogen isotope (e.g. Abend and Smith 1995, Jennings et al. 1997, Schmutz and Hobson 1998, Guest et al. 2010) and fatty acid signatures (e.g. Budge et al. 2002, Surh et al. 2009, Guest et al. 2010, Wan et al. 2010, Wai et al. 2011) of both aquatic and terrestrial taxa. Adams and Paperno (2012) conducted a study in three different sub-basins of the Indian River Lagoon (IRL) and determined that spotted seatrout (*Cynoscion nebulosus*) showed unique stable isotope ratios within these lagoon sub-basins and theorized that the hydrology, nutrient inputs and prey assemblages within each sub-basin, along with differences in the habitat, were responsible for significant difference between the isotopes signatures.

### *Study System*

The Indian River Lagoon (IRL) (Figure 1) is a unique closed “bar built” estuary system that extends 250 km north to south that has little interchange with ocean waters (Woodward-Clyde Consultants 1994). The IRL is home to many resident species that depend on this particular ecosystem for food, shelter, nursery habitat as well as many other needs. The IRL was designated an “Estuary of National Significance” by the EPA in 1990 due to its abundant biodiversity and need for protection. Due to the limited exchange of clean salt water within the IRL and the surrounding land use patterns, contaminants and fresh water runoff can be a problem and affect the biodiversity within this system (e.g. Defreese 1991, Gilmore 1995).



The hydrology within the IRL has been altered by man with the installment of hundreds of drainage canals and pipelines that deposit most of the fresh water that runs off from the surrounding land and with this influx comes pollutants and nutrients (Woodward-Clyde Consultants 1994). For the purposes of mapping hydrology, tracking biodiversity and many other management plans, the IRL was split into six segments based on several physical and chemical aspects (Figure 1). The present study focused on two segments of the IRL that show the greatest

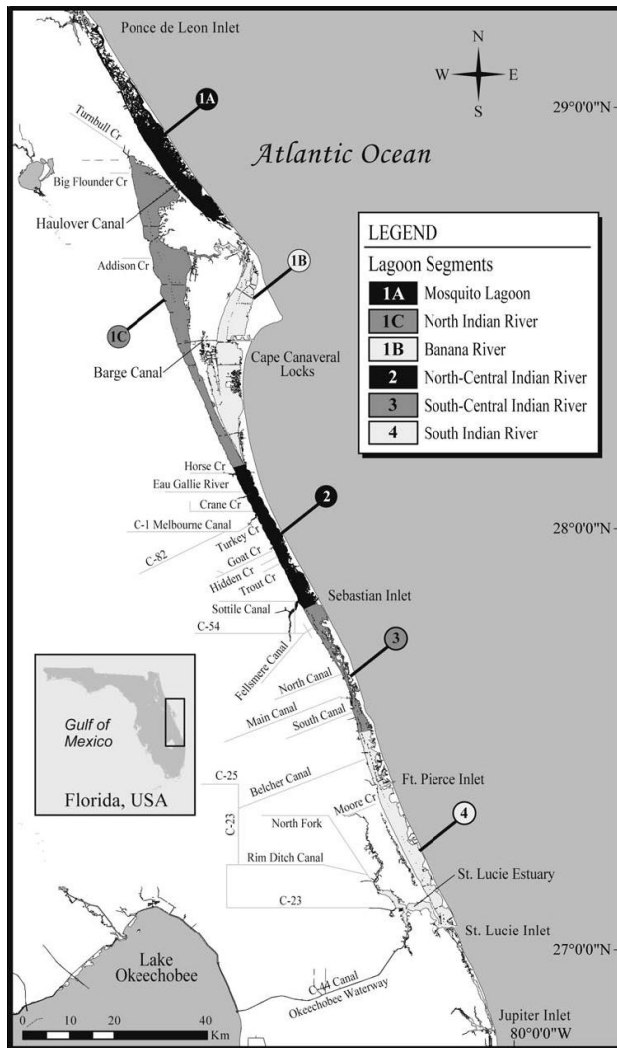


Figure 1: The Indian River Lagoon (IRL) is located on the east coast of Florida and was split into six segments to facilitate management projects (Mazzoil et al. 2008)

differences in their physical characteristics - the North Indian River (segment 1C) and North-Central Indian River (segment 2) segments (Figure 1). The North Indian River is characterized by limited alteration of its watershed with very few drainage canals and as a whole the topographic features of the land has not been extensively altered (Woodward-Clyde Consultants 1994). Smith (1993) found that the water exchange rates for this segment of the lagoon are very low, with the turnover rate typically exceeding a year. This segment has approximately double the seagrass coverage than the North-Central Indian River at approximately 7,778 ha (Steward et al. 2005) and is comprised of *Calerpa spp.*, *Halodule wrighti* and *Ruppia spp.* (Woodward-Clyde Consultants 1994).

In contrast, the North-Central Indian River is the most significantly altered segment found within the IRL (Woodward-Clyde Consultants 1994) with several drainage canals that flush fresh water, soil particles and nutrients into the lagoon (Steward et al. 2005). In this segment, water exchange is also very low with a turnover rate exceeding one year (Smith 1993). Steward et al. (2003) found that this segments' runoff volume was three times greater than that found in the North IRL segment. Turbidity, color, and chlorophyll have been shown to be most responsible for attenuating downwelling light and therefore decreasing the density and coverage of seagrass species in this basin (e.g. Philips et al. 2002, Steward et al. 2003). Shoreline hardening has also occurred with the additions of seawalls and riprap which has reduced the shoreline vegetation. The main primary producers in this segment are *H. wrighti*, *Syringodium filiforme* and *Halophila engelmannii* (Woodward-Clyde Consultants 1994).

### *Fish Species Life Histories*

The three species chosen for this project were selected because they: 1) are abundant in the IRL, 2) represent three distinct trophic levels, 3) have previously been shown to differ from each other in stable isotope and fatty acid signatures, and 4) because they represent known IRL bottlenose dolphin prey (e.g. Barros and Odell 1990, Worthy et al. 2008).

White mullet (*Mugil curema*) are benthic detritivores that inhabit coastal bays, estuaries and lagoons (Aguirre and Gallardo-Cabello 2004). Adult white mullet reportedly feed on sediment particles, detritus, diatoms, green algae, and blue-green algae (Phillips et al. 1989). Although white mullet can be found in close proximity with striped mullet (*Mugil cephalus*), sometimes in the same schools, white mullet exhibit a unique stable isotope and fatty acid signature that is distinct from all other species found within the IRL (Worthy et al. 2008). Current research has found that male and female white mullet reach sexual maturity at a mean length of  $274 \pm 9.3$  and  $278 \pm 8.3$  mm total length (TL)  $\pm$  SE respectively (Aguirre and Gallardo-Cabello 2004). Once sexual maturity is reached (February-May), white mullet migrate to their spawning grounds outside of the IRL into higher salinity waters.

Pinfish (*Lagodon rhomboides*) are omnivores and as adults generally consume minnows, crustaceans, amphipods, shrimps, and mollusks, occasionally eating seaweed and organic debris (Hansen 1970, Muncy 1984, Darcy 1985). Juvenile pinfish are known to feed on shrimp, mysids, and amphipods (Muncy 1984, Pattillo et al. 1997). Pinfish are numerically dominant among fish species found in seagrass habitats in shallow subtidal areas (Muncy 1984, Pattillo et al. 1997, Nelson 1998). Pinfish exhibit an ontogenetic diet shift at standard lengths of 80-100

mm (Darcy 1985). Sexual maturity occurs at approximately 135-150 mm SL (Hansen 1970, Nelson 2002) and spawning generally occurs during the months of October-February on near-shore reefs (Darcy 1985).

Spotted seatrout (*Cynoscion nebulosus*) are members of the drum family (*Sciaenidae*) and spend most of their time on grass flats, around sandbars, near mangroves, or around man-made structures (Nelson and Leffler 2001). Spotted sea trout are carnivores feeding primarily on crustaceans and fish (e.g. McMichael and Peters 1989, Pattillo et al. 1997). Penaeid shrimp and blue crabs were the most important prey in fish of 150-275 mm SL (Laussy 1983), whereas adult spotted seatrout are opportunistic carnivores feeding primarily on fish (79%) and macroinvertebrates (13%) (Laussy 1983). Young-of-the-year spotted seatrout reach sexual maturity around mid-summer (Nelson and Leffler 2001) indicating spawning most likely occurs in the late spring with individuals reaching sexual maturity at 200-250 mm SL.

### *Project Objectives*

The overall objective of the present study was to determine if the documented biotic and abiotic differences between the North and North-Central IRL translate up the food web into these three fish species of differing trophic levels. Within the Indian River Lagoon there has been a growing concern over the health of the apex predators that reside in the lagoon. Mazzoil et al. (2008) have indicated that in recent years the fungal infection, *lobomycosis*, has become a problem in the IRL resident bottlenose dolphin, *Tursiops truncatus*, populations which can lead to the infected individuals stranding and possibly dying. Barros and Odell (1990) have indicated that the three species examined in the present study are putative prey for bottlenose dolphins in

the IRL. If it can be determined that these fish differ in their stable isotope and fatty acid signatures dependent on location, they could potentially be utilized to track the foraging patterns of these dolphins and determine if there is a correlation between habitat utilization and health decline. It could be possible to create a mixing model that would allow researchers to take a sample from a stranded animal and determine feeding habits and movement patterns of that animal before its stranding. The hypotheses of the present project were:

- 1) Given that these three species exhibit unique feeding habits, it is hypothesized that these species will have different carbon ( $^{13}\text{C}/^{12}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and fatty acid signatures.
- 2) As a result of the recognized biotic and abiotic differences between segments of the Indian River Lagoon, it is hypothesized that the North and North-Central segments will have different carbon ( $^{13}\text{C}/^{12}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and fatty acid signatures.
- 3) Given hypothesis 1 and 2, it is further hypothesized that individual species will have different carbon ( $^{13}\text{C}/^{12}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and fatty acid signatures between segments.

## MATERIALS AND METHODS

### *Fish Collection*

White mullet (145-195 mm), seatrout (200-250mm) and pinfish (110-135mm) were collected within a specific total length range. The selection of these specific length ranges and a narrow six week timeframe for the collections were used to minimize differences in biochemical signatures that could be affected by changes in diet through ontogeny or temporal changes in habitat use or prey availability. Sampling was conducted at collection sites located in the North Indian River and North-Central Indian River segments in habitats that reflected each species' specific life histories. In order to control for potential overlap, the collection grid within each segment was 10 km in length. To reduce the possibility of fish moving from one segment to another, the collection month was chosen because none of the three species are known migrate during that period (e.g. Hansen 1970, Moore 1974, Nieland et al. 2002). A minimum distance of 30 km between sites was maintained between the two segments' collection grids to reduce the potential for these fish species to move from one segment to another (e.g. Tremain et al 2004).

Fish were collected either in coordination with Dr. Richard Paperno of the Florida Fish & Wildlife Conservation Commission (FFWCC) in Melbourne FL or through personal sampling collections (Special Activities License # SAL-09-1139-SR and UCF IACUC 09-17W).

Collections were undertaken by FFWCC using 3 m deep x 183 m long haul seines to collect the fish. During personal collections, standard hook and line techniques were used with either artificial DOA<sup>®</sup> ½ oz. gold shrimp or artificial DOA<sup>®</sup> ¼ oz. silver shrimp used as bait. Pinfish traps were left at a local marina for no more than 24 h, baited with a frozen homogenized fish bait block and/or frozen shrimp blocks. Gut contents of samples were not removed, to be

consistent with methods currently being used in the Physiological Ecological and Bioenergetics Laboratory (PEBL) as part of a larger dolphin feeding ecology study, and were assumed to be non-significant for the purposes of the present study since most stomach contents only accounted for an average 0.01% of total sample weight. Fish were collected during the period March 13, 2009 to April 20, 2009. Fish collected by FFWCC were immediately stored at  $-20^{\circ}\text{C}$  until processing. In compliance with UCF-IACUC protocol, samples collected by the primary investigator were immediately placed into a cooler filled with dry ice and water until death at which time they were transported to UCF and frozen at  $-20^{\circ}\text{C}$  until processing.

#### *Fish Processing*

Individual fish were identified to species, then sexed, weighed (g) and standard length (mm) and total length (mm) recorded before being ground using a commercial grade blender to a homogenous consistency. Homogenized fish were then separated into two bags (for stable isotope and fatty acid analysis) and placed into a  $-20^{\circ}\text{C}$  freezer before further processing.

#### *Stable Isotope Analysis*

One subsample bag was lyophilized (LabConco) for 96 hours, then ground in a Spex mixer mill (Spex model 8000) and placed into an air tight sample vial. An approximately 1g sample of homogenate was placed into a 7 cm diameter microfiber glass filter (Whatman type GF/A) and lipid extracted using petroleum ether in a Soxhlet extractor for 24-48 hours. Samples were then placed in an oven at  $60^{\circ}\text{C}$  for 24 hours to remove any remaining solvent. Freeze-

dried, lipid-extracted tissues were ground to fine powder by hand, and a portion (0.9-1.5 mg) sealed in 5 by 9 mm tin capsules. These samples were analyzed in a Delta Finnigan Mat stable isotope ratio mass spectrometer (IRMS) at the University of Georgia. Quality assurance of stable isotope ratios were tested by running one known standard sample (bovine tissue) for every 12 unknown (fish) samples (Worthy et al. 2008).

### *Fatty acid Analysis*

The second subsample bag was used to undertake fatty acid signature analysis. Lipids were extracted from a 1-2 g subsample of homogenized ground fish using a 2:1 chloroform/methanol solution and drying over nitrogen (Iverson 1993, Samuel and Worthy 2004). Fatty acid methyl esters (FAME) were made from the extracted lipid using a Hilditch solution (0.5 N sulfuric acid in methanol) and dichloromethane and then placing the resultant solution in the dark for 72-96 h. FAMEs were suspended in hexane then analyzed using a gas-liquid chromatograph (Perkin-Elmer Autosystem XL) connected to a computerized integration system (Totalchrom version 6.3.1, Perkin Elmer). Resultant chromatograms were compared to known standard mixtures (Nu-Chek Prep, Elysian MN) and secondary external reference standards to determine fatty acid composition (Worthy et al. 2008). Fatty acids were converted to percent amount of the total sample, and standardized by dividing each fatty acid detected by the total percent amount of all identified fatty acids (excluding unidentified peak noise).



### *Statistical analysis*

Stable isotope data were transformed to improve parametric assumptions for normality. A square root transformation was used for  $\delta^{15}\text{N}$  and a constant was added to  $\delta^{13}\text{C}$  values (to create a positive integer) and then transformed using  $1/\text{integer}$ . Transformed data were tested for normality using Kolmogorov-Smirnov tests. Leven's F was used to test homogeneity of variance between factors. Differences in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were tested using two-way ANCOVAs with sample length being the covariate. Significant two-way results were then analyzed with Tukey's HSD post hoc tests. All statistical analyses were run using JMP 8 and plotted using Sigma Plot 10 (Systat Software Inc). All statistical analyses were judged to be significant at  $p < 0.05$ .

A subset of the 65 assessed fatty acids ( $\geq 0.5\%$ ) that were identified, referred to as the "extended dietary fatty acids" (Iverson *et al.* 2004), were used in the statistical analysis. Fatty acids were analyzed using Classification and Regression Tree (CART) analysis (S-Plus 8, Insightful Corporation). CART analysis identifies the individual fatty acid that will produce the most homogenous nodes. From there the program generates a graph of the frequency of a particular % area on the y-axis and the % area on the x-axis. At that point the program determines the point where the frequency drops off as the "split point". This means that all samples that have a value less than that split point will go into one node and all those above the split point value goes into another node. The program then identifies if the terminal nodes are homogenous, if they not the program will identify another fatty acid within that heterogeneous sample node. This goes on until the nodes are either homogenous or the program cannot find another fatty acid that will produce homogenous nodes. Significance was determined if the

misclassification rate (# misclassifications/# samples) was  $<0.05$ . Multiresponse Permutation Procedure (MRPP) is another non-parametric test that was used to identify if there were significant differences between groups. MRPP requires no assumption of normality or heterogeneous variances (PCORD 5).

## RESULTS

In the North Indian River segment, 30 pinfish, 30 mullet and 10 spotted seatrout were collected, while in the North-Central Indian River segment, 30 of each fish species were collected (Table 1 and Figure 2). In the North Indian River segment, spotted seatrout, white mullet and pinfish had mean values ( $\pm$  SE) for  $\delta^{13}\text{C}$  of  $-18.00 \pm 0.08$ ,  $-14.59 \pm 0.07$  and  $-16.88 \pm 0.04$  respectively and for  $\delta^{15}\text{N}$ , mean values were  $14.43 \pm 0.05$ ,  $8.30 \pm 0.04$  and  $10.43 \pm 0.03$  respectively (Figure 3, 4, 5). For the North-Central Indian River segment, spotted seatrout, white mullet and pinfish had mean values for  $\delta^{13}\text{C}$  of  $-18.98 \pm 0.02$ ,  $-16.25 \pm 0.06$  and  $-16.94 \pm 0.04$  respectively and for  $\delta^{15}\text{N}$ , mean values were  $14.21 \pm 0.02$ ,  $8.07 \pm 0.03$  and  $10.64 \pm 0.03$  respectively (Figure 3, 4, 5). Isotopic signatures for carbon were significantly different between the three fish species (ANCOVA,  $F= 26.79$ ,  $P<0.0001$ ) (Figure 2, Table 2). Nitrogen values were also significant for these species (ANCOVA,  $F=213$   $P<0.0001$ ) (Figure 2, Table 3). A Tukey's post hoc HSD test confirmed that these species were significantly different for both carbon and nitrogen (Table 4, 5).

CART analysis showed that spotted seatrout ( $n=40$ ), white mullet ( $n=53$ ) and pinfish ( $n=56$ ) were individually identifiable based on their fatty acid profiles (misclassification rate (MR) =  $0/150$ ,  $p=0.00$ ) (Figure 6). CART analysis showed that fatty acids 16:1 n-11 and 18:3 n-6 were the major split points. Multiresponse Permutation Procedure confirmed the CART's findings ( $A=0.3465$ ,  $T= -80.57$ ,  $P< 0.0001$ ).

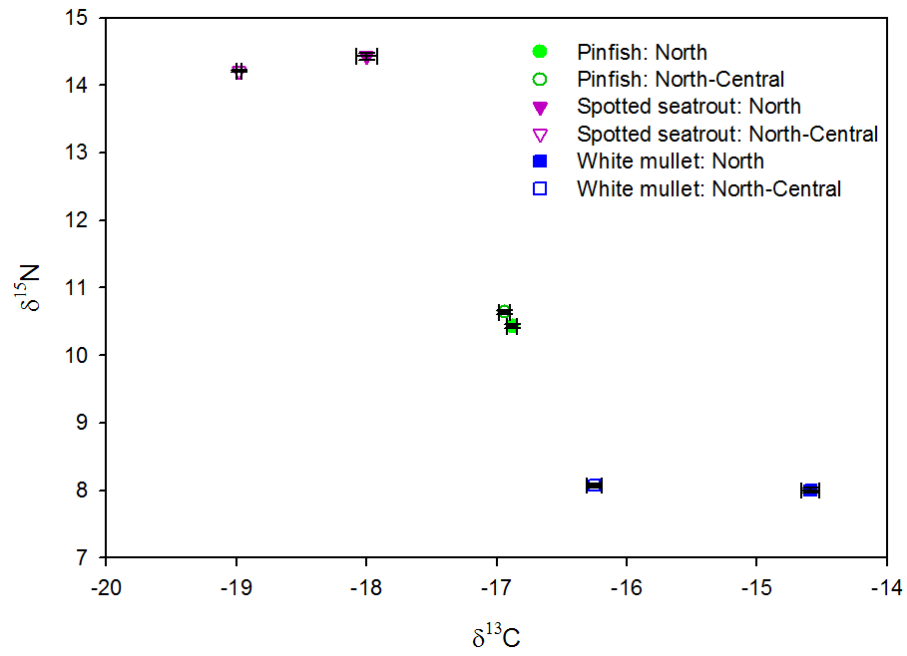


Figure 2: Variability in  $\delta^{15}\text{N}$  (‰) as a function of  $\delta^{13}\text{C}$  (‰) for all three species in each of the two IRL segments. Mean and standard error represented

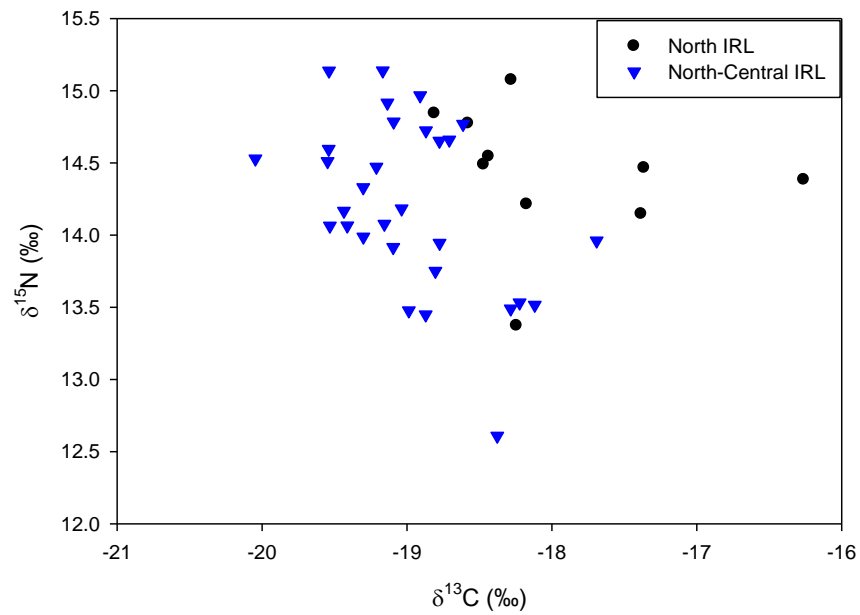


Figure 3: Variability in  $\delta^{15}\text{N}$  (‰) as a function of  $\delta^{13}\text{C}$  (‰) for spotted seatrout in each IRL segment.

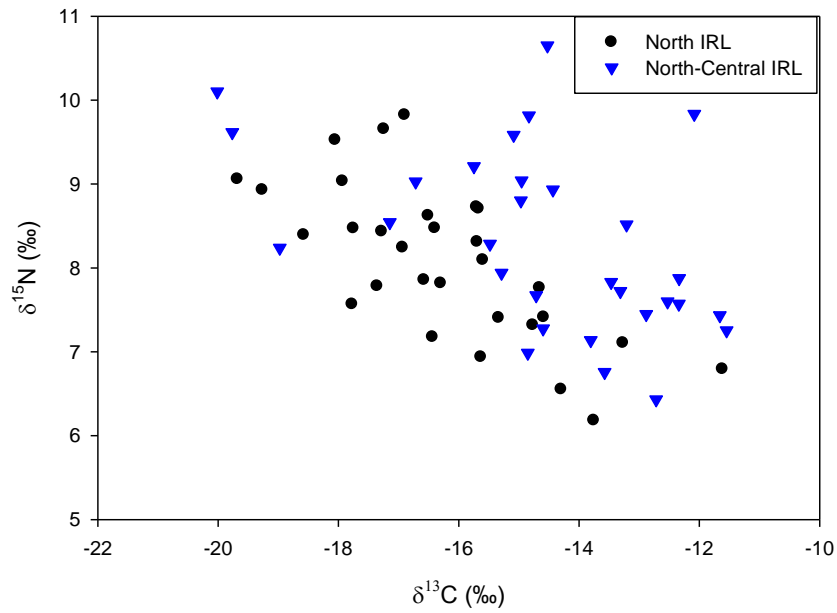


Figure 4: Variability in  $\delta^{15}\text{N}$  (‰) as a function of  $\delta^{13}\text{C}$  (‰) for white mullet in each IRL segment.

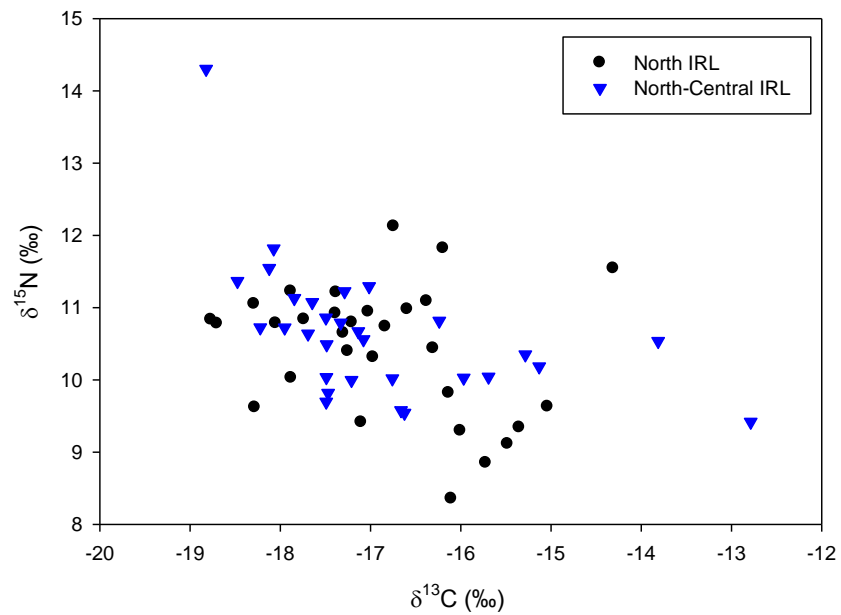


Figure 5: Variability in  $\delta^{15}\text{N}$  (‰) as a function of  $\delta^{13}\text{C}$  (‰) for pinfish in each IRL segment.

Table 1: Summary statistics for all three species for both IRL segments

	<b>Spotted Seatrout</b>		<b>Pinfish</b>		<b>White Mullet</b>	
	<b>North IRL</b>	<b>North-Central IRL</b>	<b>North IRL</b>	<b>North-Central IRL</b>	<b>North IRL</b>	<b>North-Central IRL</b>
<b>Mean Standard Length (mm)</b>	232	213	127	126	127	143
<b>Standard Error</b>	0.3	0.4	0.5	0.4	0.2	0.5
<b># Samples</b>	10	30	30	30	30	30



Table 2: Results of Two-Way ANCOVA for  $\delta^{13}\text{C}$  (‰)

	<b>df</b>	<b>Sum of squares</b>	<b>F<sub>value</sub></b>	<b>P</b>
<b>Species</b>	2	0.02818	26.79	<0.0001
<b>Segment</b>	1	0.0009	4.55	0.0343
<b>Length (covariate)</b>	1	0.0027	6.19	0.0138
<b>Species*Segment</b>	2	0.0152	17.36	0.0004
<b>Error</b>	153	0.073		

Table 3: Results of Two-Way ANCOVA for  $\delta^{15}\text{N}$  (‰)

	<b>df</b>	<b>Sum of squares</b>	<b>F<sub>value</sub></b>	<b>p</b>
<b>Species</b>	2	313.87	213	<0.0001
<b>Segment</b>	1	0.02989	1.44	0.2318
<b>Length (covariate)</b>	1	0.0009	0.0012	0.9721
<b>Species*Segment</b>	2	0.008332	0.2196	0.8031
<b>Error</b>	153	123.7755		

Table 4: Tukey's HSD Test for  $\delta^{13}\text{C}$  with all tested combinations being significant ( $p < 0.05$ ).

<b>Mean Difference Standard Error Lower Confidence Limit Upper Confidence Limit P<sub>value</sub></b>	<b>Spotted seatrout</b>	<b>White mullet</b>
<b>Pinfish</b>	-0.0536 0.01149 -0.0808 -0.0264 <0.0001	0.01304 0.00393 0.00374 0.02234 0.0032
<b>Spotted seatrout</b>	- - - - -	-0.0666 0.01056 -0.0916 -0.0417 <0.0001

Table 5: Tukey's HSD Test for  $\delta^{15}\text{N}$  with all combinations being significant

<b>Mean Difference Standard Error Lower Confidence Limit Upper Confidence Limit Pvalue</b>	<b>Spotted seatrout</b>	<b>White Mullet</b>
<b>Pinfish</b>	-3.65	2.35
	0.16	0.16
	-4.02	1.90
	-3.27	2.70
	0.0001	0.0004
<b>Spotted seatrout</b>	-	6.00
	-	0.16
	-	5.60
	-	6.40
	-	0.000001

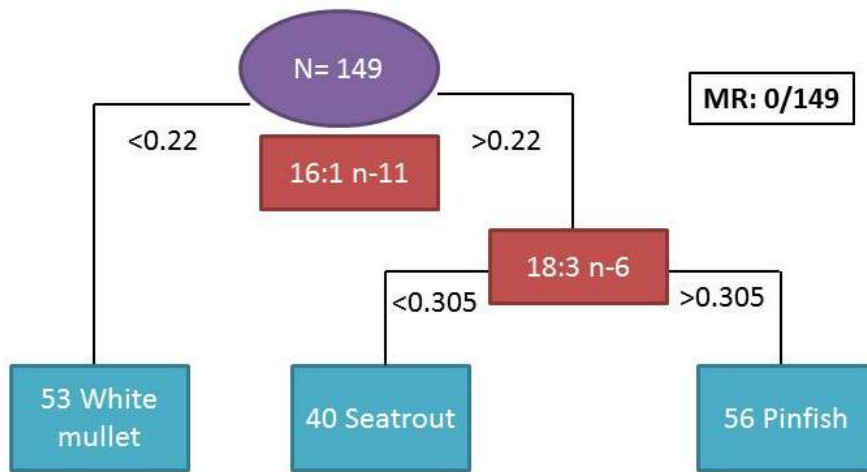


Figure 6: CART analysis for all three species – all fish were correctly classified to species with no misclassifications.

### *Location*

North Indian River (n=70) and North-Central Indian River (n=90) samples, pooled for all species, were analyzed for their stable isotope ratios for both carbon and nitrogen. For North Indian River, mean and standard deviation for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  was  $-16.77 \pm 0.021$  and  $9.99 \pm 0.033$  respectively. For North-Central Indian River,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  was  $-16.84 \pm 0.026$  and  $11.05 \pm 0.029$  respectively. A two-way ANCOVA for  $\delta^{15}\text{N}$  showed no significant differences for segment (ANCOVA F= 1.44, p=0.23), however there was an effect for  $\delta^{13}\text{C}$  (ANCOVA, F=4.55, p=0.034) (Table 3, 2). CART analysis of the fatty acid signature for all fish in North (n=88) and North-Central (n=74) segments showed that segment was correctly identified based on their fatty acid profiles (MR= 7/149, p=0.05) (Figure 7). Of those misclassified, six were white mullet and one was a spotted seatrout. MRPP confirmed the CART analysis (A=0.0326, T= -10.762, P=  $1.54 \times 10^{-6}$ ).

### *Location x Species Interaction*

A two-way ANCOVA of  $\delta^{13}\text{C}$  showed that the interaction between species and segment was significant (ANCOVA, F=17.3575, p=0.0004) (Table 2). A post-hoc Tukey's HSD test was performed which showed that  $\delta^{13}\text{C}$  was significantly affected by sampling segment for spotted seatrout but was not significant for white mullet or pinfish (Table 6). Seven white mullet and four pinfish were not included in this analysis due to processing error when samples were made into FAMEs and there was insufficient tissue to redo the FAME part of the chemical processing. Seatrout (MR=0/40), white mullet (MR=2/53, p=0.04) and pinfish (MR=0/57) could be correctly

classified to their segment of origin using CART analysis (Figure 8, 9, 10). MRPP analysis of spotted seatrout ( $A=0.278$ ,  $T= -21.252$ ,  $P<0.0001$ ), mullet ( $A=0.0456$ ,  $T= -5.174$ ,  $P=0.002$ ) and pinfish ( $A=0.12$ ,  $T= -18.415$ ,  $P<0.0001$ ) showed results are consistent with the CART analysis.

Table 6: Tukey's HSD Test for  $\delta^{13}\text{C}$  with the species and segment interaction \*Indicates a significant result.

	<b>Pinfish</b>	<b>White Mullet</b>	<b>Spotted Seatrout*</b>
<b>Difference in means</b>	-0.0013	0.01259	-0.0325
<b>Standard Error</b>	0.00538	0.00565	0.00581
<b>Lower Confidence limit</b>	-0.0168	-0.0037	-0.0493
<b>Upper Confidence limit</b>	0.01421	0.02889	-0.0157
<b>P<sub>value</sub></b>	0.9999	0.2303	<0.0001



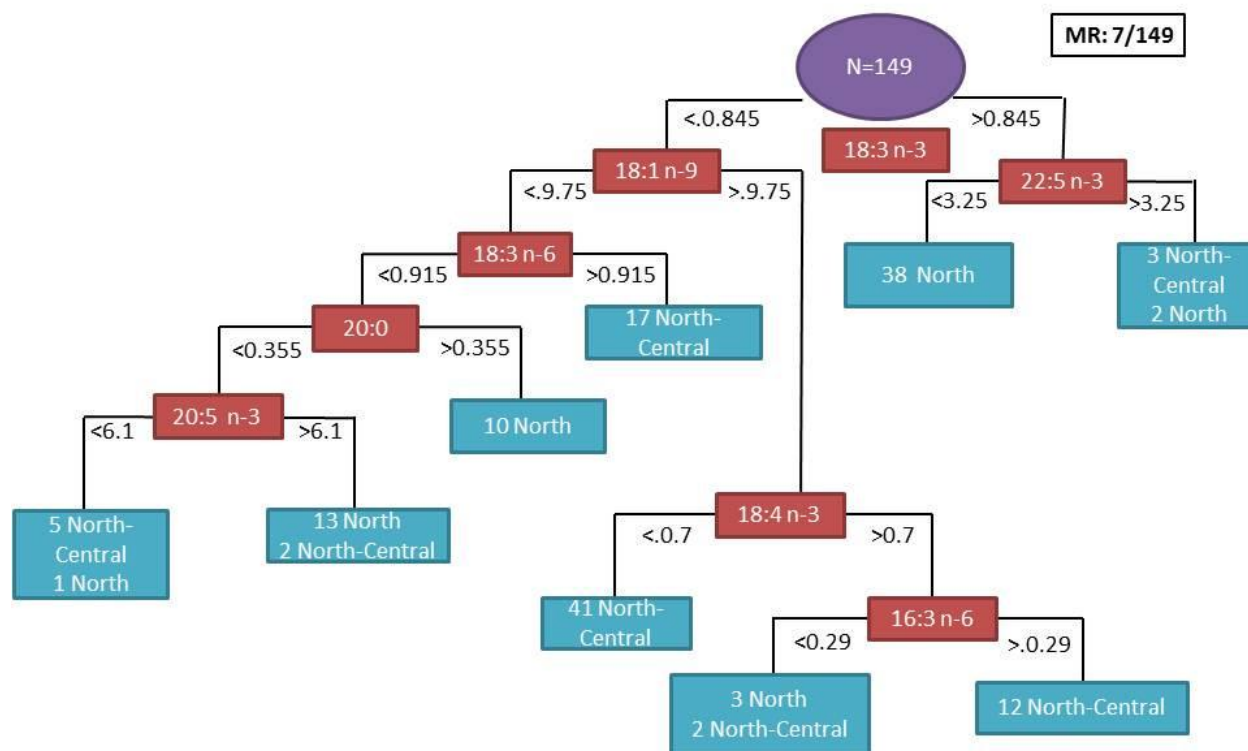


Figure 7: CART analysis of the fatty acid signatures of fish sorted by IRL segment. Segment of origin was correctly classified (MR=7/149,  $p=0.05$ ), regardless of species. Three fish samples collected in the North IRL and 4 fish from the North-Central IRL were incorrectly classified.

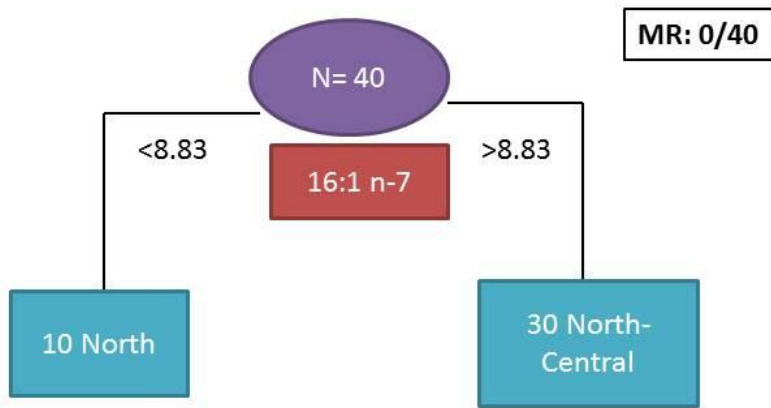


Figure 8: CART analysis of spotted seatrout by IRL segment (MR=0/40).

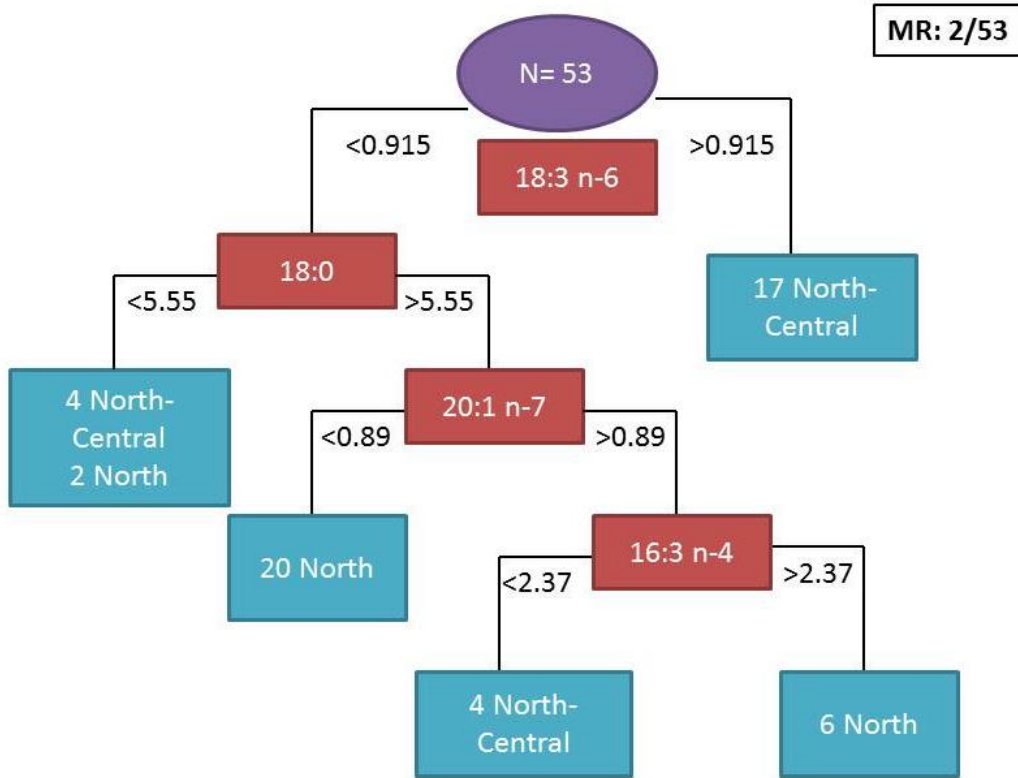


Figure 9: CART analysis of white mullet by IRL segment (MR=2/53).

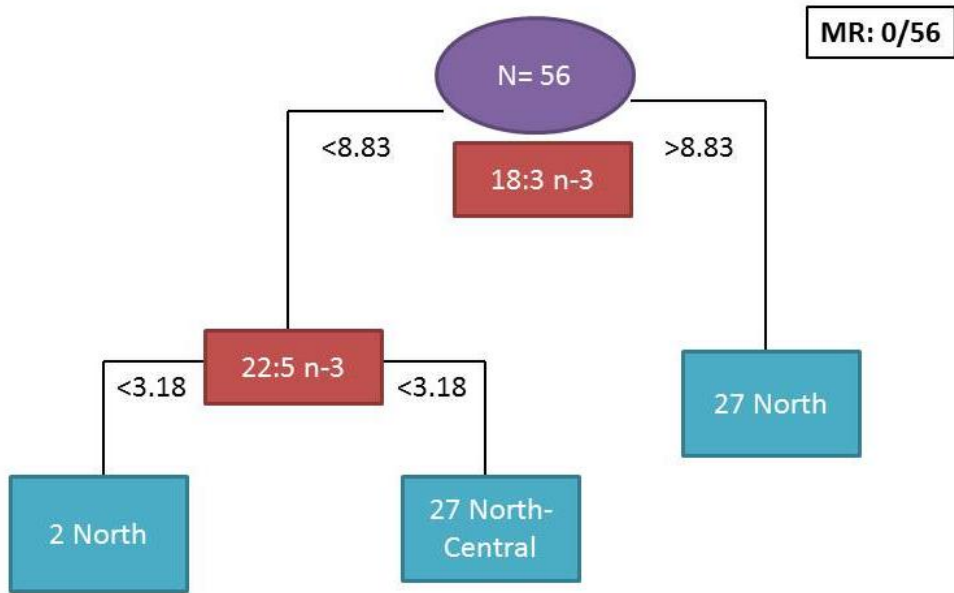


Figure 10: CART analysis of pinfish by IRL segment (MR=0/56).

## DISCUSSION

Biochemical analysis of prey tissues have been used to reconstruct food webs (e.g. Castell et al. 1995, Guest and Connolly 2004, Iverson et al. 2004, Wan et al. 2010), shed light on feeding relationships and how those relationships can vary temporally (e.g. Schmutz and Hobson 1998, Perga and Gerdeaux 2005), geographically (e.g. Vander Pol et al. 2011, Wai et al. 2011) or with age (e.g. Schmutz and Hobson 1998, Wai et al. 2011). Stable isotope and fatty acid analysis have also been used to define the link between animals and their initial primary producer source within an ecosystem (e.g. Hemminga and Mateo 1996, Layman 2007, Bouillon et al. 2008). The results of the present study indicate that fine-scale regional biotic and abiotic differences significantly affect the stable isotope and fatty acid signatures of these three fish species and that those differences transfer up the food web.

Each species studied in the present project were found to have unique carbon, nitrogen and fatty acid profiles despite possibly consuming similar prey and the potential for aggregation of multiple species of fish into one foraging school. This study compliments the work done by Worthy et al. (2008, 2011) which confirmed that these three species, regardless of season, can be separated by their biochemical signatures with 100% accuracy due to their distinct feeding habits and life histories. These species feed on different trophic levels where mullet are known to feed on detritus, pinfish are omnivores, and spotted seatrout are strict carnivores (Aquirre and Gallardo-Cabello 2004, Muncy 1984 and Nelson and Leffler 2001). These differences in feeding habits are ultimately reflected in the fatty acid, nitrogen and, carbon isotope ratios (e.g. Jennings et al. 1997, Hobson 1999, Darr and Hewitt 2008, Koiadinovic et al. 2008, Stowasser et al. 2009, Gerard and Muhling 2010). Though carbon is typically used to determine the primary producer

it is also affected by enrichment as energy is moved up trophic levels. In addition to these internal biochemical reactions, Peterson and Fry (1987) proposed that the primary producers' signature is incorporated into the food web structure and reflected in the predator giving a unique regional carbon signature even if species feed within a relatively similar trophic level (DeNiro and Epstein 1978, McCutchan et al. 2003, Guest and Connolly 2004, Guest et al. 2004, Gerard and Muhling 2010). These factors also affect the fatty acid profiles of each species were significantly different as well.

The different segments of the IRL have been documented to have differences in nitrogen loading, primary producer species composition, and temperature (e.g. Virnstein 1995, Woodward-Clyde Consultants 1994, Phlips et al. 2002, Steward et al. 2003, Steward et al. 2005). Light penetration, temperature (e.g. Hemminga and Mateo 1996, Budge et al. 2002), carbon dioxide levels (Burkhardt et al. 1999) and turbidity have also been documented to affect seagrass, algae and phytoplankton productivity and abundance. Badylak and Phlips (2004) found that, in each segment of the IRL, there were differing species compositions of phytoplankton potentially as a result of limited water exchange, differing light attenuation values and other environmental factors (Phlips et al. 2002, Phlips and Badylak 2010). Budge et al. (2002) found that light, depth and CO<sub>2</sub> alter the fatty acids profiles of plankton within the marine environment. Consistently, studies have also shown that sea grass, mangroves and algae have radically different carbon signatures within the Indian River Lagoon (Alves 2007, Vaslet et al. 2011). Typically algal based food webs are more depleted in <sup>13</sup>C than seagrass or detritus based food webs (Hemminga and Mateo 1996). The North segment has a higher abundance of seagrass

then the North-Central segment. Collectively these influences could be the basis for the observed fatty acid signatures differences amongst the fish species examined in the present study.

When comparing individual study species between the two segments it becomes clear that segment differences are reflected in these fish. Documented differences within the various habitat components highlighted above are hypothesized to cause these differences and result in the significant results in the carbon and fatty acid biochemical signatures (e.g. Phlips et al. 2002, Steward et al. 2003, Steward et al. 2005, Virnstein 1995 and Woodward-Clyde Consultants 1994). Results of the present study also suggest that these species are physically segregating themselves into specific segments of the IRL at the non-reproductive life stages even though there are no apparent physical barriers present.

Spotted seatrout are estuarine residents that utilize estuaries like the Indian River Lagoon for their entire life history (McMichael and Peters 1989). Although adults have been documented to occasionally move large distances for spawning opportunities (Tremain et al. 2004), most studies have indicated that spotted seatrout are non-migratory. Regardless, in the present study only subadults were used to control for the potential movement due to spawning. Significant differences in the  $\delta^{13}\text{C}$  and fatty acid signatures between segments, seen for spotted seatrout either reflects the lack of movement of subadults and/or the sedentary nature of this species during the late winter/early spring months. Adams and Paperno (2012) conducted a similar study on a larger size range of spotted seatrout within the Indian River Lagoon and also found that these animals had unique stable isotope signatures dependent on their collection segment consistent with the findings of the present study.

During the initial design of the present project, it was expected that white mullet and pinfish would show no effect of segment because these species are known to be highly mobile and vary in the habitat in which they forage, particularly as they reach sexual maturity (Hansen 1970, Darcy 1985, Aguirre and Gallardo-Cabello 2004). However while there was no significant difference for individual species between segments for  $\delta^{13}\text{C}$ , analysis of their fatty acid signatures indicated significant differences. Stable isotope analysis is known to be a coarse tool in this area of study (Hobson 1999) which is why fatty acid signature analysis is frequently being incorporated into these studies; so that “finer” scale differences can be seen (Budge et al. 2002, Iverson et al. 2004, Worthy et al. 2008, Wan et al. 2010, Wai et al. 2011).

With stable isotope analyses, there are only two variables that can be utilized for statistical purposes whereas with FASA there are potentially 60+ variables that can be utilized. It has been suggested that using both methods together could allow for a more complete understanding of feeding habits (e.g. Guest et al. 2010, Worthy et al. 2008). This limitation was best illustrated by the pinfish results where differences in stable isotopes were not evident, but were seen in the fatty acid results. Pinfish are opportunistic omnivores and these results indicate it is possible for a pinfish to consume differing types or quantities of prey in each segment but have the same stable isotope signature.

It is assumed that the results for white mullet and pinfish were observed because only juveniles were used which controlled for the possibility of migration to ecosystems outside of the Indian River Lagoon for spawning. Previous studies have indicated that water temperature as well as tidal fluctuations can be a driving force for movement patterns in juveniles of these species and can be a deterrent to juvenile fish migration (Ditty and Shaw 1996). In addition,



man-made structures such as causeways and bridges have also been shown to inhibit larval movement (Irlandi et al. 2002). It is possible that during the present study abiotic factors and/or man-made barriers deterred these juvenile fish from migration thus producing the significant fatty acid signature differences that reflected the segment from which the fish were collected. It is also possible that these species were not moving within the Indian River Lagoon because there was enough food available in the segment where they were born. This concept that both segments had ample prey but that the North Indian River Lagoon segment had more estuarine prey assemblages while the North-Central region had a more pelagic prey assemblage was suggested as one of the reasons differences in stable isotope signatures were found in spotted seatrout (Adams and Paperno 2012).

In the present study, stomachs and their contents were not removed before processing. This was done to keep collected samples consistent with previous work within the PEBL lab. Unlike carnivores, the microbial flora of gut fermenting species can potentially alter fatty acid composition of consumed food items and thus negate, or at least complicate, the potential application of fatty acid signature analysis for determining feeding habits. The goal of the present study was to simply compare the composition of individual fish within and between segments and, since gut contents were small to non-existent in most fish, the analysis was still able to separate a species into its' respective collection segment with extreme accuracy. It is the conclusion of this work that while the gut contents may have added variation, it is presumed to be an insignificant amount and did not render the study invalid because the significant differences between the segments resonated through in the signatures.

Previous work has indicated that these different segments of the IRL receive different volumes of nitrogen input from point and non-point sources (Steward et al. 2003, Steward et al. 2006) and therefore it was assumed that nitrogen would be a significant isotopic variable. This was not seen in the present study, and one possible cause for this result was that the samples were collected during March, the dry-season, when run-off entering the IRL would be typically low. This run-off is the primary source of anthropogenic nitrogen which, in turn, is the main driving force behind the nitrogen differences (Steward et al. 2006). It is conceivable that if samples had been collected during the wetter months, the results for nitrogen in this experiment might have been different due to the higher volume of run-off entering the IRL from terrestrial sources.

In order to construct an accurate food web of this important ecosystem, the interaction between the biochemical signatures and spatial scale needs to be understood. Proper management, conservation and restoration efforts rely on the understanding of the interactions between various habitat components in relation to the function of the IRL ecosystem (Nobriga et al. 2005). The present study showed that there is a significant impact of segment on the stable isotope and fatty acid profiles for these fish species inhabiting the IRL. Each of these studied segments has unique land use patterns that affect the composition of the water run-off that enters the IRL (Woodward-Clyde Consultants 1994, Defreese 1995, Larson 1995, Duncan et al. 2004). The SWIM (surface water improvement and management) plan was enacted in 1989 to deal with the rising concern over the water quality and management of the extensive watershed that flows into the IRL. With the recent efforts to try and restore the IRL back to a more pristine condition, the impact of these restorations on the food web need to be considered. As the influx of fresh

water and nutrients is altered this will in turn affect the primary producers and radiate up the food web to the top predators. One of the main concerns outlined in the SWIM project plan was the nutrient loading from both point and non-point sources surrounding the lagoon (Steward et al. 2003). Urbanization such as shoreline hardening, and septic systems and, surrounding agriculture are one of many factors that contribute to anthropogenic nitrogen and phosphorus into the watershed thus affecting the surrounding ecosystems (Sigua 2010). These inputs have been documented to alter the nitrogen stable isotope ratios of marine species (Badylak and Phlips 2004). These anthropogenic influences in the region have not only impacted the abundance and species richness of the primary producer in each segment (e.g. Defreese 1991, Fletcher and Fletcher 1995, Schmalzer 1995, Virnstein 1995, Phlips et al. 2002, Badylak and Phlips 2004, Steward et al. 2005, Steward et al. 2006) but potentially the health and reproduction of the top predators (e.g. Mazzoil et al. 2005, Mazzoil et al. 2008, Worthy et al. 2008).

Within the IRL there has been a growing concern regarding the long-term viability of the sub-population of one of the top predators, the bottlenose dolphin (*Tursiops truncatus*), as their health has been declining over recent years (e.g. Mazzoil et al. 2005, Mazzoil et al. 2008). Dolphins inhabiting ecosystems similar to the IRL are exposed to an increasing variety of pollutants from anthropogenic sources that degrade their habitat, limit their food resources, and increase their susceptibility to diseases (e.g. Fair and Becker 2000, Berrow et al. 2002, Irwin 2005). Many studies within the IRL have indicated that bottlenose dolphins (e.g. Mazzoil et al. 2005, Mazzoil et al. 2008, Murdoch et al. 2008) and West Indian manatees (e.g. Deutsch et al. 2003, Alves 2007) segregate into several collective segments within the IRL which increases their risk of being impacted from these harmful disturbances. Bossart et al. (2003) found

pathological evidence that the resident dolphins of the IRL showed a high incidence of infectious and inflammatory diseases, such as lobomycosis and leishmaniasis (Reif et al. 2006a, Reif et al. 2006b, Murdoch et al. 2008, Murdoch et al. 2010), which he concluded was evidence of immunologic dysfunction. Organochlorines and algal blooms have also been implicated in many marine mammal deaths which can be directly linked to the degradation of the Indian River Lagoon (e.g. Berrow et al. 2002, Flewelling et al. 2005, Walsh et al. 2005). Unfortunately it can be difficult to track the progression of these negative health impacts because marine mammals can be difficult to track. When an animal becomes ill and strands itself it is unclear whether that animal was confused and stranded far from its resident basin or if it was a permanent resident of that segment. The utility of the results from this study will be in our ability to combine the methods of fatty acid and stable isotope analysis to create a biochemical map of the IRL. With this “map” it could be possible to identify the biochemical of each prey species dependent on its segment of origin and then trace those small spatial differences ultimately into the predator signature using a mixing model. This information could shed light on habitat utilization and movement patterns and therefore better assess disease progression, and general of threatened and endangered species that inhabit the IRL.

## CONCLUSIONS

The main goal of the present study was to determine whether there are stable isotope and fatty acid signature differences between segments of the Indian River Lagoon. My study suggests that there are differences in the stable isotope and fatty acid signatures within a particular species that are dependent on the segment in which they inhabit. The next step will be to examine what causes those differences and sample other segments of the Indian River Lagoon to see if they follow the same pattern. It is unclear as to whether these significant differences between segments for each species are a result of a) the primary producer, b) prey availability, c) abiotic variables or d) a combination of the aforementioned variables. What is clear is that biotic/abiotic properties of each segment are translating up the food web into the fish. Future research should be geared towards determine what combination of these variables translate up the food web from prey to predator, thus affecting the stable isotope and fatty acid signatures of the species examined in this project.

Although there is potential for this method of research, one should be cautious about moving forward on a bigger scale. Other researchers have cautioned that in order for the results of this type of study to be utilized properly that turnover rate and fractionation of the stable isotopes needs to be quantified for predator, prey and primary producer; and the effects of season and growth period need to be taken into account when using fatty acids or stable isotopes (Hobson 1999, Guest et al. 2010). When considering doing research of this kind the results of the present study indicate the need for researchers to take into consideration the effects of fine scale latitudinal patterns. In addition, the present study shows that there are limitations to stable isotope research that were not experienced when using fatty acid signature analysis. Future

research should considering using both methods in conjunction with one another in order to get a finer resolution of the spatial variation.

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