Characterization of Prey Selectivity of Spotted Seatrout in South Central Louisiana via Molecular Gut Content Analysis

A Thesis

Submitted to the University Honors Program
Of Nicholls State University
In Partial Fulfillment
Of the Requirements
Of the University Honors Award

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B.S. in Cellular and Molecular Biology/ Marine Biology
Fall 2024

Fall 2024

I hereby certify all aspects of this thesis document, as well as all work it reports, as my original work.

Abstract:

In Louisiana, predatory fish species populations, such as the Spotted Seatrout (*Cynoscion nebulosus*), have declined in recent years (Smith et. al 2024). Suggested causes include overfishing of the predator species and declining prey availability (LDWF 2021; Berenshtein et. al. 2023). There have been preliminary taxonomic investigations, in which gut contents were visually analyzed for diet biomass ratios and predator selectivity. However, digestion and decomposition lead to gaps in the data and incomplete diet assessment (O'Dell et al. 2020). The purpose of this research was to identify the fish species present in the gut contents of Spotted Seatrout using Mifish DNA primers and high throughput sequencing. The hypothesis was that species diversity detected within Spotted Seatrout guts via molecular metabarcoding would elucidate prey preferences. Qiime 2 software was used for bioinformatics quality control. NCBI BLAST was used to obtain species ID, percent ID, and E-value. The results are under current analysis.

Introduction

Life History– Spotted seatrout (*Cynoscion nebulosus*) are pelagic marine fish that are characterized by an iridescent, dark silvery-gray appearance from the dorsal view and silvery-white ventrally. Black spots can be found on the back, fins, and tail of the fish. The species possesses a yellowish mouth and a few prominent, sharp canine teeth at the tip of the upper jaw. Individuals normally weigh 1-3 pounds when caught, primarily due to most fish landings containing fish between 1-2 years of age. However, fish larger than that are not rare, since individuals can live up to 10 years of age (West. 2021; Louisiana Fisheries- Fact Sheets 2020).

Spotted Seatrout reach sexual maturity after 2 years. Females can lay between 100,000 and 1 million eggs. Spawning occurs from April to September- sometime between dusk and dawn and in murky, freshwater, or saltwater marshes- making Louisiana waters a perfect destination. Adult Spotted Seatrout reside in shallow coastal and estuarine waters in the summers- normally over sandy bottoms, oyster reefs, and seagrass beds. They can tolerate a range of salinities, making the species very resistant to environmental fluctuation. In the winter, larvae feed on small crustaceans such as copepods- shifting to larger prey such as mysids and shrimp as they grow. Small trout generally eat large amounts of shrimp and other crustaceans while larger adults prefer a majority fish diet including anchovies, pinfish, silversides, Stripped mullet, Atlantic Croaker, and Gulf Menhaden (Horst, 2022).

Study sites– Louisiana's coastal wetlands are a favorable residence for Spotted Seatrout due to the abundance of estuarine ecosystems. This is comparable to more freshwater habitats and deep ocean habitats that do not support Spotted seatrout in any stage of their life history. This abundance is the primary driver in Louisiana's history of successful fisheries- 30 percent of the total volume of fisheries in the United States and 35 to 40 percent of the country's annual shrimp and oyster harvests (NOAA Fisheries). The Louisiana Wildlife and Fisheries divides Louisiana's coast into five coastal study areas (CSAs) representing the major river basins. According to the 2021 Spotted Seatrout Assessment, the CSAs and their boundaries are "CSA 1– Mississippi State line to South Pass of the Mississippi River (Pontchartrain Basin); CSA 3 – South Pass of the Mississippi River to Bayou Lafourche (Barataria Basin); CSA 5– Bayou Lafourche to the eastern shore of Atchafalaya Bay (Terrebonne Basin); CSA 6 – Eastern shore of Atchafalaya Bay to western shore of Freshwater Bayou Canal (Vermillion/ Teche/ Atchafalaya Basins); and CSA 7 –

western shore of Freshwater Bayou Canal to Texas State line (Mermentau/Calcasieu/Sabine Basins)" (West. 2021).

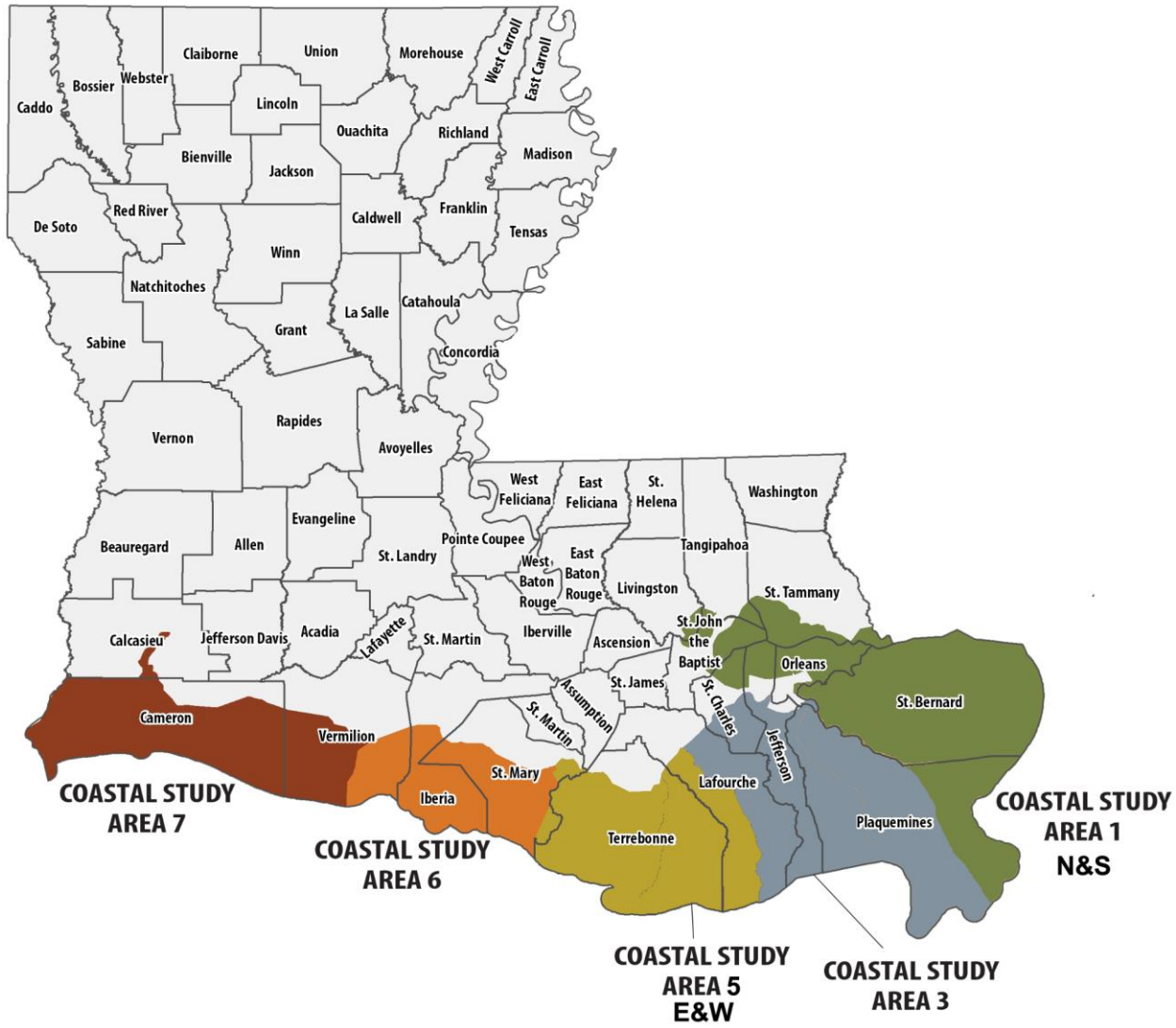

Figure 1. Depicts the 5 CSAs of Louisiana's Estuarine Coast. (LDWF 2024)

This study sought to compare the diet of Spotted seatrout in CSA 5 and 6. CSA 5 was characterized by a majority saline marsh with conversion to brackish followed by freshwater marsh when traveling inland. Further inland, the abandoned delta complex is characterized by a cypress swamp (Verret Sub basin). It consists of thick, loose sediments that are experiencing

dewatering and compaction. Consequently, this leads to high subsidence and subsequent distributary ridge development. This contributes to increased flooding and subsequent land loss.

The southern portion consists of low-lying barrier islands segregated from the mainland by lakes and bays. The basin has limited freshwater influx with more inland sub-basins receiving freshwater input via the Atchafalaya River and or rainfall. The southern end has the most limited freshwater resources and experiences the most sediment influx within the Delta Plain. The inactivity of the plain has contributed to an absence of freshwater overflow from multiple river sources and is the likely cause of the high salinity and low sediment characteristics of the region. Common species in this area include Brown Shrimp, Grass Shrimp, Crawfish, Blue Crab, Hermit Crab, Fiddler Crab, Spotted Sea Trout, Red Drum, Channel Catfish, Spotted Garfish, Stingray, Black Drum, Minnow, Shad, Mullet, Pinfish, Atlantic Croaker, and Largemouth Bass.

CSA 6 is split into two sections- the Vermillion/Cameron parishes and the Teche/ Vermilion parishes. The former is characterized by the Atchafalaya basin- also known as America's largest swamp forest- containing the largest continuous tract of freshwater marsh in the state. The basin also contains bottomland hardwood forests, cypress swamps, and open water. The area is experiencing a rare phenomenon of land gain due to the slowing of the Atchafalaya River as it filters nutrients and sediment from the water flowing downstream toward the Gulf of Mexico (CWPPRA 2024). Additionally, oil and gas industries have created canals through the wetlands and delta which have disconnected the river from the surrounding floodplains- preventing natural water overflow into the inland swamps. The river is losing its filtering ability which has contributed to decreased water quality and habitat health throughout the region (The Nature Conservancy 2024).

The latter section is primarily characterized by fresh, intermediate, and brackish marshes. It consists of 3 bays and an island (Marsh Island) that separates saltier waters and marshes from the more freshwater areas. The section is experiencing hotspots of land loss both inland and shoreline, which is attributed to water system construction, invasive species herbivory, and shoreline erosion. Impounded areas are susceptible to flooding while areas where current hydraulic barriers are being removed are vulnerable to saltwater intrusion (CWPPRA 2024). Current species residing in this CSA include Alligators, Red Snapper, Menhaden, Gar, Redfish, Drum, Catfish, Bass, Bluegill, Sheepshead, Jewfish, Blue, Jack, Blue Catfish, Yellow Catfish, Paddlefish or Spoonbill Catfish, Striped Bass, Mullet, Sea Trout, Blue Crab, and Grass Shrimp (LDWF 2024; U.S. EPA Calcasieu Estuary Remedial Investigation 2003).

CSAs 5 and 6 share many fish species and are undergoing varying yet significant habitat changes. In general, the complex estuarine food web found within all 5 CSAs is a product of a fresh to saltwater gradient- producing a variety of microhabitats. Some previous studies have elucidated the importance of low trophic level fish on transferring energy from producers to higher level consumers (predatory fish) using meta-analysis and others have indicated that many nearshore predatory fish species of the northern Gulf of Mexico, including Spotted Seatrout, are opportunistic generalists- feeding on whatever prey is available (Chee et. al. 2024). Figure 2 depicts the various organisms found on a typical Louisiana estuary and the complexity of the food web. Primary producers include bacteria, microbes, and phytoplankton. Primary consumers include zooplankton, macroinvertebrates, and meiofauna. Secondary consumers are primarily finfish, and tertiary consumers include marine mammals, large predatory fish, and sea birds (Rhodes et.al. 2021). This may be partially due to their life history pattern, in which they feed on different prey types based on life stage. Therefore, habitat conditions and subsequently prey

availability have also been proposed as contributing factors to Spotted Seatrout decline (Berenshtein et. al. 2023; Smith et. al. 2024). However, trophic interactions and their potential affective factors in any particular Louisiana CSA have yet to be researched.

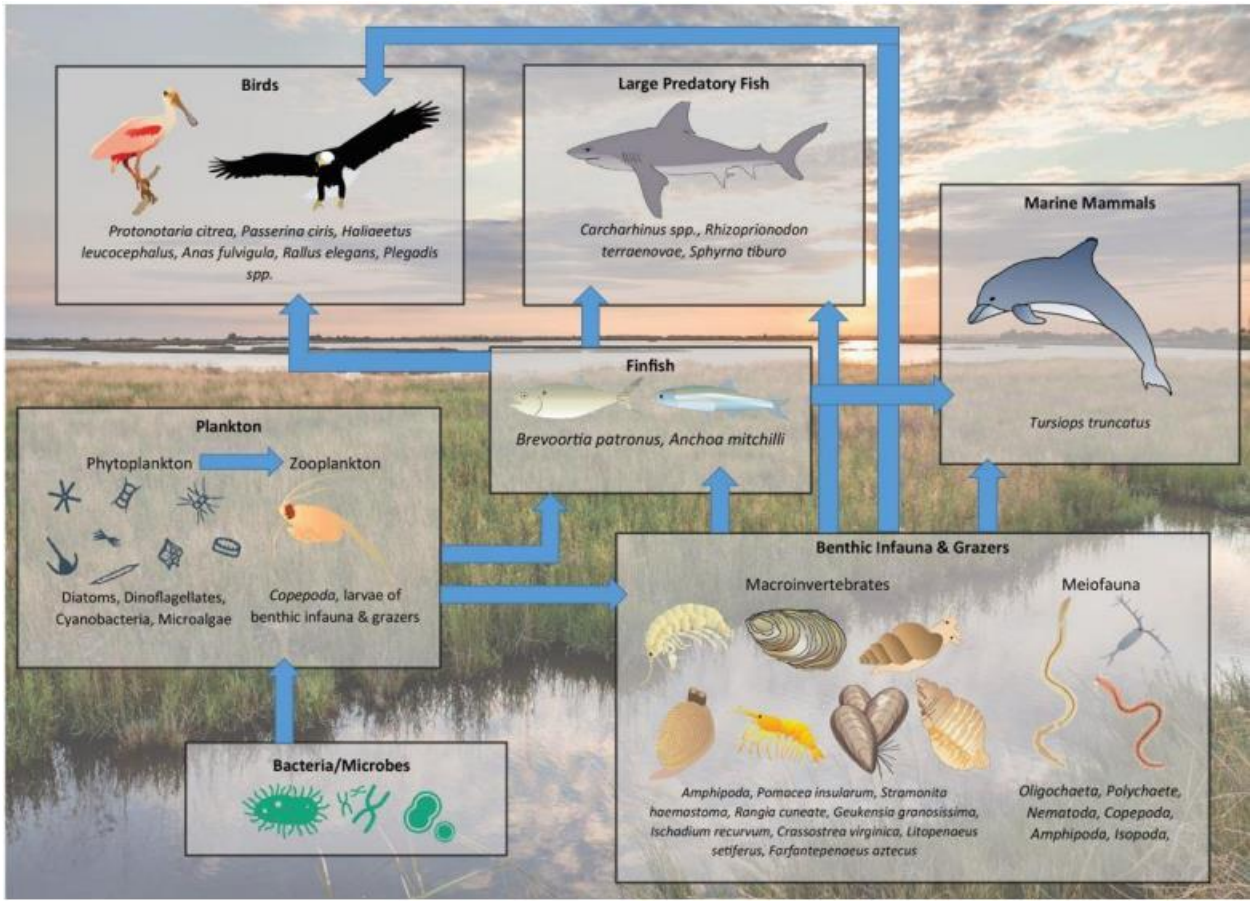

Figure 2. Typical Louisiana estuarine food web (Rhodes et al. 2021).

Spotted Seatrout in Decline— In a recent fish stock assessment by the Louisiana Department of Wildlife and Fisheries (LDWF), indicated that the Louisiana Spotted Seatrout population has been overfished since 2016 (West. 2021). The LDWF Louisiana Wildlife and Fisheries Commission drafted a Notice of Intent (NOI) to be reviewed by the Louisiana House of Representatives Committee on Natural Resources and the Senate Committee on Natural Resources- proposing updated management regulations for Spotted Seatrout Fisheries to achieve

female spawning stock biomass (total weight of individuals capable of reproduction) of 6.2 million pounds in 5 years. Proposed changes included- changing the current allowed harvest fish size of a 12-inch minimum to a 13-20 in range and 2 oversize fish allowance, maintenance of the 15 fish per day bagging limit, prohibition of the personal bagging of charter guides during tours, and the requirement to conduct a stock assessment report by April 2027 followed by the development of a new Notice of Intent (NOI) by January 2028. Other studies attribute population decline to overfishing of key prey species (Berenshtein et. al. 2023).

Molecular Metabarcoding— This is the collection, amplification, sequencing, and analysis of eDNA sequences (DNA collected from within environmental samples including water, soil, feces, or air from all the living and dead organisms in an environment). Molecular metabarcoding targets specific variable regions (barcodes) on DNA that are taxa-specific (Helbing and Hobbs, 2019). They are found between universally congruent segments of the DNA chain. Primers are derived from the universal segments so that the barcodes are amplified in PCR reactions. Next Generation Sequencing Technology (NGS) sequences the fragments. Previously, NGS has been used in food authentication research to replace Sanger Sequencing Technology. This is because NGS sequences as the DNA chain is synthesized by tracking the addition of fluorescently tagged nucleotides, whereas Sanger sequencing determines the order of nucleotides post-amplification. Recently, several platforms have used NGS to demonstrate its capacity to identify 15 or more different fish species in a single highly processed fish product (Franco et al. 2021). This provides a cost-effective alternative for multiple species to be rapidly and concurrently detected and differentiated (Rouker et al. 2021). The compilation of these techniques allows for optimization of the detection of ecosystem biomass and population

densities within a given spatial-temporal zone. Therefore, the use of metabarcoding and NGS was ancillary in the experimental design of this research.

Molecular Gut content assessments— Preliminary biomass gut content studies have shown that comparing visual observations and molecular analyses allowed for optimal gut biodiversity assessment. One study conducted a food web analysis of Rainbow Trout (Oncorhynchus mykiss (Walbaum); Salmoniformes: Salmonidae) and Prickly Sculpin (Cottus asper (Richardson); Scorpaeniformes: Cottidae) using gut content samples obtained from a Canadian stream in British Columbia. The study compared results from 3 approaches- visual, individual prey item sequencing, and using NGS to sequence homogenated gut content samples. The study concluded that taxonomic identification of predator diet through visual observation typically led only to order-level assignments of invertebrates that were not subject to digestion due to chitinous exoskeletons and other proteinaceous or fibrous structures. Comparably, identification to the genus and species level was possible for individually sequenced specimens across nine orders, mainly Ephemeroptera, Plecoptera, Trichoptera, Diptera, and Hemiptera. However, the greatest number of genera and species were identified from the NGS analysis. The analysis resulted in the identification of 79 unique taxonomic signatures - with 92% classified to the genus level. In comparison, 53 unique taxa were achieved from individually barcoding specimens (Odell et al. 2020). These results were synonymous with other Next Generation Sequencing literature. The anticipated result of this study was that the use of NGS would elucidate a wide range of prey species that otherwise would not have been detected via visual studies alone. It was anticipated that the results would align closely with previous literature, except for the types of prey species identified- more likely low tropic fish commonly found in the Gulf of Mexico-since Mifish

primers (specifically designed to identify fish species) were used in DNA amplification within the project methodology.

Rationale

This research was funded by the Science Center for Marine Fisheries (SCEMFIS). The goal was to provide credible data concerning Spotted Seatrout diet preferences. There have been preliminary investigations by private companies, in which gut contents of pelagic fish were analyzed, using visual observations, to assess prey biomass ratios and predator selectivity. However, results were limited due to the digestion and decomposition of gut contents. This led to gaps in the data and an incomplete assessment of the pelagic fish diet (O'Dell et al. 2020). A complementary assessment using molecular metabarcoding was proposed to fill those gaps. For this study, the hypothesis was that species diversity detected within Spotted Seatrout guts via molecular analysis would exhibit numerous and varied prey preferences of Spotted Seatrout between CSAs 5 and 6.

Methodology

Collections—The goal of this study was to conduct a multiple-approach analysis of Spotted Seatrout prey specificity through gut content biomass surveying. Spotted Seatrout specimens were collected by the Louisiana Department of Wildlife and Fisheries (LDWF) Saltwater Sampling. The department conducts yearly, standardized assessments to improve the understanding of physical conditions (water quality), the status of the fish stocks, and the effects of man on freshwater and saltwater ecosystems. (Fish Sampling, LDWF). The samples were collected from the Inshore and Nearshore waters of CSAs 5 and 6- two of the five total.

Specimens will be collected using Gillnets and Trammel Nets. Logbooks containing the date and location of catches, water temperature, salinity(ppt), dissolved oxygen levels(mg/L), turbidity(ft.), gear code, taxonomic identification codes; and weight, length, sex, stage, and condition upon the catch of individuals were recorded. Google Sheets was utilized for the documentation of this information. Each CSA sampling group had a unique methodology for recording data, so there was a disparity in information recorded within the datasheet.

Dissections—Dissection procedures began in the morning with specimen removal from freezers to thaw until the afternoon. Then, fish length measurements were taken and matched to corresponding data in the provided logbook to ensure the accuracy of logged information and to ID the provided specimens. The workstation was sterilized with bleach and left to dry while fish measurements were underway. Digestive tracts were extracted from fish carcasses using dissection kits. Scissors (for smaller fish) and tin snips (for larger fish) were used to make lateral incisions on the ventral side of fish and cut out the digestive tract from the esophagus to the end of the large intestine. Digestive tracts were placed in sterilized metal trays followed by longitudinal cuts along the surface layer of the digestive tube. Notations of identifiable stomach contents (fish, shrimp, or crab) were recorded in the datasheet for preliminary reportable data. Tweezers were used to collect solid stomach content in conical tubes (15ml or 50ml depending on size and amount of contents). Sterile cotton swabs were used to collect liquid, stomach, and fecal samples in microcentrifuge tubes. Fecal samples were placed in separate microcentrifuge tubes from the stomach swabs because fecal material could impede the result accuracy of sample PCR analysis. Scissors were also used to collect fin clip samples of predator specimens, which were placed in microcentrifuge tubes. All samples were filled with EtOH (ethanol) and labeled

with the following information- sample name (species ID and specimen number), location of specimen collection, and date of dissection. Dissection data from these specimens were inserted into the Google datasheet.

<A>Sterile Techniques—All tools were placed in conical tubes filled with bleach followed by tubes filled with DI water for 1 minute each to ensure proper sterilization, to prevent sample cross-contamination and chemical removal, and to prevent DNA degradation from the bleach- between specimens and between digestive tract openings. Gloves were also replaced at these points in the dissection process. Gloves were always worn to prevent human DNA contamination.

Tissue Preparations and DNA Extraction—The purpose of molecular analysis in this study was to confirm and refine the understanding of prey preference and gut content biomass of Spotted Seatrout. A percentage of recorded contents in primary observation studies was notated as “unidentifiable Clupididae ". Unlike other studies that extracted eDNA from water samples on filters (Govindarajan et al. 2022) or sediment samples (Ogata et al. 2021), mixed sample DNA extractions, from the tissue found in the stomachs of Spotted Seatrout, were performed so that multiple prey species identification could be determined from the DNA within the fish stomach content samples. For each Spotted Seatrout specimen, all the associated gut content samples were combined into one sub-sample. DNA was extracted from the sub-sample using the Dneasy Blood and Tissue Extraction Kit Protocol.

<*A*>*Sterile Techniques*—Before starting, all equipment was sterilized with a 50/50 bleach-DI water solution, and the bench space was sterilized with bleach followed by a DI water rinse.

<*B*>*Tissue Preparations*—The sample prep protocol has a maximum tissue weight of 25mg and suggests cutting the tissue into small pieces or homogenizing for 1 minute- to increase tissue and cell lysis efficacy when adding reagents in later steps (QIAGEN 2023). The step was modified to perform mixed DNA extractions. This was done by pouring the sample contents, stored in ethanol, into a sieve that rested on top of a glass bowl. DI water was sprayed over the sample to obtain as much of the tissue DNA as possible in the bowl. The DNA, water, and ethanol mixture was then pipetted in and out of the bowl to further homogenize the mixture. Next, a subsample of 1000 ul was collected and transferred to a sterile microcentrifuge tube. After twenty-one samples were prepped, all were centrifuged at max speed for 60 seconds and the supernatant was poured off. The microcentrifuge tubes were placed into a vacufuge at 37 ℃, with the lids off, to dry the pellet. The protocol suggested a 3-minute dry time, but it was modified by an extension of the drying time to a full 24 hours for the gut content samples. The dried pellets served as the "tissue sample" for DNeasy Blood & Tissue extractions.

<*C*>*DNeasy DNA Extraction Protocol: Tissue Lysis*—The eDNA extraction samples contained a variety of DNA regions, but the focus was on the sequence that codes for 12S ribosomal RNA(rRNA)- a fragment responsible for making ribosomal subunits. The extraction protocol was a two-step process- split over 2 days. Day one procedures were as follows. First, 180 uL of Buffer ATL (degraded tissue) was added to each of the 21 microcentrifuge tubes containing the dried pellets. Then, 20 uL of Proteinase K (digests proteins) was added to each tube. The

samples were placed in the vortexing incubator overnight at 56 °C. On Day 2, the samples were removed from the incubator. Then, 200 ul of Buffer AL (cell lysis buffer) was added and each microcentrifuge tube was hand vortexed for 3-5 seconds to homogenize the samples. Next, 200 ul of Ethanol was added followed by another round of hand vortexing. Ethanol facilitated alcohol-water hydrogen bond interactions to free DNA from water hydration. This allowed DNA to precipitate out of the solution. The presence of ethanol promoted DNA aggregation into the pellet form-post vortexing.

<C>DNeasy DNA Extraction Protocol: Binding—The DNA-reagents mixtures were pipetted into individual Mini spin columns, which contained a filter to capture the DNA, and placed in 2 mL collection tubes. The tubes were placed in the centrifuge and spun for 1 minute at ≥ 6000 x g (8000 rpm). The flow-through and collection tubes were discarded and the spin columns were placed into new collection tubes.

<C>DNeasy DNA Extraction Protocol: Washing—Next, 500 uL of Buffer AW1, which cleans other biomolecular material off of DNA, was pipetted into each spin column. The tubes were centrifuged for 1 minute at ≥ 6000 x g (8000 rpm). Then, the flow through and collection tubes were discarded and the spin columns were placed in new collection tubes. The process was repeated using Buffer AW2, except the tubes were centrifuged for 3 minutes at 20000 x g (14,000rpm). Additionally, the

spin columns were placed into 1.5 or 2 mL microcentrifuge tubes after removal from the collection tubes.

The purpose of centrifugation and the discarding of the collection tubes containing flow-through solution at the end of the binding and washing steps was to ensure that all ethanol was removed from the DNA samples. Precautions were taken to ensure that the flow through did not seep upward through the spin column filter during removal.

<C>DNeasy DNA Extraction Protocol: Eluting—Next, 200 uL of Buffer AE were added to each spin column. Buffer AE detached the DNA from the spin column filter membrane- allowing it to flow into the microcentrifuge tube. The solution also provided a stable environment for DNA storage. The DNA was incubated at room temperature for 1 minute followed by centrifugation for 1 minute at ≥ 6000 x g (8000 rpm). This last step could have been repeated for greater DNA yield, but the step was unnecessary for this study. The spin columns were discarded. Lastly, the microcentrifuge tubes, containing the DNA extracts, were stored in the −20℃ freezer until library preparations began.

Library preparations—Library preparations are the PCR amplification productions that were shipped off to be sequenced using Next Generation Sequencing (NGS). The qPCR reaction

amplified the DNA similar to regular PCR but also contained Illumina DNA Prep protocol for NGS. Illumina DNA Prep, previously known as NextEra DNA Flex, is more efficient than traditional PCR because it uses bead-linked transposomes to combine gDNA fragmentation and sequencing primer annealing steps- minimizing the potential for species identification bias or errors. Additionally, sequencing-ready libraries that are compatible with a variety of DNA sizes and types make the prep universal. Lastly, shortened processes within other stages in the workflow such as DNA extraction, DNA quantification, and library prep QC and library quantification increase efficiency (Illumina 2024). In this prep, extra-long primers were used so that when the samples underwent Illumina Sequencing, the adaptor sequences matched and annealed to the oligonucleotides on the flow cell (i.e. glass plate primers).

A complete library was composed of the DNA fragment of the genomic DNA from the samples (DNA insert) and adapters (P5 and P7) on either side of the fragment- P5 represented the forward adapter on the 5' end and P7 represented the reverse adapter on the 3' end. Each adapter had 3 components: primer-binding sites for sequencing, unique index (barcoding) sequences used for multiplexing (allowed for individual ID of each sample during sequencing so that multiple samples could be analyzed within a run) adapter sequences for clustering in the flow cell (P5 or P7). Each adapter had a unique index so the library was dual-indexed.

Selecting Primers—Mifish primers are universal primers for metabarcoding fish DNA samples. The primers were designed using mitogenome sequences from 880 fish species (Miya et al. 2015). The first 33 nucleotides were for primer binding, while the following 6 were used for the MiSeq machine calibration. The sample extracts were kept in the minus 20-degree freezer until library preparations began.

Primer Name	Primer Sequence	No. of Bases
MiFish-U-F-PCR1_TS	**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**GTCGGTAAAACTCGTGCCAGC	54
MiFish-U-R-PCR1_TS	**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**CATAGTGGGGTATCTAATCCCAGTTTG	61

Table 1. Mifish Primers for Illumina library preparation using TruSeq tags (denoted in bold).

DNA Amplification—DNA extraction samples for PCR were prepared using 96 well plates; working in columns to keep the sample order aligned with sequencing facility equipment standards. The facility machines analyzed 8 library preparations at a time, and there are 8 wells per column on the 96-well plate. The PCR technique is a process that uses DNA primers to harness the molecular machinery of DNA polymerase to make numerous copies of a target DNA fragment. The product was observed at the end of the amplification process using agarose gel electrophoresis with ethidium bromide staining. The aim of using this technique was to detect the presence or absence of a target fragment. The reagents that were used in PCR were combined in the Invitrogen Platinum SuperFi II PCR Master Mix (5X), which consolidated reaction prep steps in a ready-to-use mixture. According to Thermo Fisher Scientific, the Platinum Superfi II DNA Polymerase, SuperFi II Buffer, and dNTP combination ensures optimal primer annealing for successful amplification (PlatinumTM SuperFi II PCR Master Mix). The library prep protocol represented the first PCR reaction- which used primers with Illumina adaptors annealed to them to amplify target DNA sequences.

<A>Sterile Techniques—Tip extraction occurred in the order and direction of sample preparations to keep track during pipetting. Pipette tips were observed before and after drawing up liquid to ensure that the correct amount was acquired.

<B>Primer and Master Mix Preparation—The primers will come in 100uM stock, so they will need to be converted to a 25ul working solution of 5uM using the following equation:

(100uM) (X) = (2.5 uM) (100 ul)

X = (2.5 x 100)/100

X = 2.5 ul of primer stock + 97.5 ul of sterile water

To make a master mix, the following reagents were combined- 5x Platinum SuperFi Buffer (5ul), 10 mM dNTPs (0.5 ul), 2.5 uM F primer (1.25 ul), 2.5 uM R primer (1.25 ul), Platinum SuperFi DNA polymerase (.25 ul), and Nuclease Free Water (14.75 ul)- per sample. The master mix volume and reagent concentrations were adjusted so that there were enough microliters of reagents for every sample despite potential pipetting and calibration errors. This was achieved via the use of conversion factors equations (Table 2) and adjustment of the regent ratio requirements to accommodate X + 2 samples.

Reagent	Per rxn volume	65 rxns	Final Concentration
5x Platinum SuperFi Buffer	5 ul	325 ul	1 X

10 mM dNTPs	0.5 ul	32.5 ul	0.2 mM each
2.5 uM F primer	1.25 ul	81.25 ul	0.125 uM
2.5 uM R primer	1.25 ul	81.25 ul	0.125 uM
Platinum SuperFi DNA polymerase	0.25 ul	16.25 ul	0.02 units/ul
Nuclease Free Water	14.75 ul	955.50 ul	

Table 2. Reagents Conversion Factors and Final Concentrations for Master Mix Prep

The next step required the addition of the 23ul master mix to individual 0.2 mL polypropylene PCR tubes. Then, 2 ul of DNA at 1-10 ng/ul were added to each tube. The tubes were sealed, placed in the thermocycler, and run under the following conditions: 94C/3 mins (1 cycle) followed by 94C/30s, 55C/30s, 72C/30s (35 cycles), followed by 72C/7 mins (1 cycle), held at 4C.

<C>Reaction Process—In the first reaction, a region of interest-specific primer, connected to an overhang adapter sequence, annealed to the designed DNA fragment derived from the genomic DNA in the DNA extract samples. The next step was completed by the sequencing facility. In the second PCR reaction, the dual indices were attached by a complementary primer of the overhang adapter sequences. Each index was unique and had either a P5 or P7 adapter sequence connected to it. The 5'-end adapters were useful for library product binding to the oligonucleotides on the

Illumina flow cell surface. The 3'-end adapters served as priming sites for MiSeq sequencing (Miya 2015). These procedures were followed for each set of samples processed.

Gel Electrophoresis—Once the first PCR amplification was completed, the products were analyzed via Agarose Gel Electrophoresis to ensure that the PCR reaction was successful in amplifying the DNA before being shipped off for sequencing. An agarose (sugar) gel was infused with ethidium bromide (EtBr)- which denatured and stained DNA. The goal of using electrophoresis was to send negative charges through the buffer so that DNA fragments (negatively charged) flowed from the negative end to the positive end to visualize the length and concentration of DNA fragment samples. DNA fragment bands were separated by size- short sequences were lighter and traveled faster through the gel than longer ones. More concentrated bands appeared brighter and thicker in the BioRad ChemiDoc Imager. The protocol began with the placement of the gel rig vertically in the electrophoresis chamber along with the insertion of the appropriately sized well (holes to insert PCR products) combs into the mold. Gloves were worn since EtBr is a potential mutagen/carcinogen. Large gels were made using the reagent measurements in the highlighted row in Table 3.

Mold size	Agarose	1x TBE (or TAE)
Large	1	100 mL

Table 3. Gel solution reagents measurements based on rig (mold) size.

<*A*>*Making the Gel*—The agarose and 1X TBE (or TAE) were added to a flask and microwaved for 30 seconds until completely dissolved- followed by sufficient time for cooling. Once the flask was cooled to the touch, 2ul of Ethidium Bromide was added to the solution. Then, the

solution was poured into the gel rig and allowed to cool and solidify for approximately 10-20 minutes.

<B>*Loading the gel*—The gel rig required reorientation in the electrophoresis chamber so that the wells were near the cathode. Then, 1.5ul of loading dye was added to 5ul of PCR product. Each PCR product and loading dye solution was added into individual wells- starting at the second well. Then, 1-2 ul of DNA ladder- containing DNA fragments of different lengths- were added into the left-most well. The DNA ladder served as a reference for fragment size to compare the unknown DNA. Fragments moved from cathode to anode at a velocity proportional to their size and charge- the smaller, more negatively charged fragments traveled the fastest.

<C>*Running the electrophoresis*—The power source and the electrophoresis chamber were connected using electrode wires- black for the cathode and red for the anode. On the power source, the manual button was selected followed by constant volts. The strength of the electrical current was set at 120 volts and the gel was run for 40 minutes.

<D>*Visualizing the gel*—At the end of the electrophoresis procedure, the power was turned off and disconnected from the gel rig. The gel was removed from the rig and placed in the BioRad ChemiDoc Imager. A photograph of the results was taken and saved to the computer hard drive. These procedures were repeated for each gel run throughout the study.

Sequencing— A secondary gel electrophoresis analysis was conducted by the sequencing facility to ensure that the right size DNA was used for sequencing. The target fragment size of the 12s

rRNA region of interest was 175 base pairs (bp). PCR products underwent Next Generation Sequencing (NGS), using Illumina MiSeq technology, at the University of New Hampshire Hubbard Center for Genome Studies. NGS allows scientists to sequence hundreds of millions of molecules at a time- reducing the time and cost of obtaining sequencing data.

Bioinformatics—Once the sequencing was completed, Google Sheets was used to create a manifest file that sorted the sequences in the following format: sample-id, absolute-file path, and direction. The sample ID consists of a 4-letter code (SPTR) followed by the sample number. The absolute-file path was the exact location in QIIME2 in which the sequences could be found. The sequences were produced in the forward and reverse directions. The manifest file was used by the QIIME2 program to search and import the sequences into the system. The manifest was saved as a .csv file for formatting purposes. Next, a metadata (mdat) file was generated in the following format: sample-id, total length (mm), sample site, sex of specimen, coastal study area where the sample site is located, gear type used to catch specimen, water temperature (degrees Celsius), water salinity (parts per thousand), dissolved oxygen in the water (mg/L) and the turbidity of the water (distance of visibility while looking down into the water [measured in ft]). The mdat file was used to sort the sample in future data comparisons. Then, the manifest file was imported into the research folder within FileZilla software so the sample information could be extracted and analyzed within a terminal (MobaXterm). Using the HCGS Metabarcoding Tutorials: Workflow for QIIME2 workshop by Joseph7e in GitHub, the protocol for the bioinformatic pipeline was typed into the terminal in the following order:

1)This showed all environments. The latest QIIME2 environment was desired.

conda info --envs

2) This step activated QIIME2. It was important to remember to exclude parentheses when this step was typed into the terminal.

conda activate (latest qiime version)

3) These commands were used to import the manifest data.

qiime tools import

--type 'SampleData[PairedEndSequencesWithQuality]'

--input-path manifest_fb56.csv

--output-path demux

--input-format PairedEndFastqManifestPhred33

4) The demux step was used for quality control.

qiime demux summarize

--i-data demux.qza

--o-visualization demux

5) The denoising step, or truncating (cleaning) step was the use of DADA2 by QIIME2 to convert the raw sequence reads into "real" sequence format. DADA2 accomplished this by learning the error rates for each transition between bases at each quality score. It lined up all of the sequences and determined points of difference to separate real DNA from errors. Sequences that were 100% percent identical and above the error threshold were aggregated together, while sequences that did not meet the error threshold were segregated from the main group. Segregated sequences that exhibited 99% identity to the main group sequences were reinserted, while the rest were discarded. DADA2 also assembled paired-end reads (forward and reverse sequence

reads of the DNA insert) by a 10-base pair overlap and filter for unusually long amplicons or ambiguous sites. Lastly, it filtered for chimeric sequences (artifacts formed by the incorrect merging of two or more sequences) and removed primer sequences.

```
qiime dada2 denoise-paired\
  --i-demultiplexed-seqs demux.qza\
  --p-trim-left-f 17 --p-trim-left-r 21\
  --p-trunc-len-f 200 --p-trunc-len-r 200\
  --p-n-threads 18\
  --o-denoising-stats dns\
  --o-table table\
  --o-representative-sequences rep-seqs
```

6) This step allowed visualization of the denoising results using DADA2. DADA2 created a metadata table to organize the sequences for efficient analysis. It also created a FASTA file containing the sequences.

```
## Metadata on denoising
qiime metadata tabulate\
  --m-input-file dns.qza\
  --o-visualization dns
## Unique sequences across all samples
qiime feature-table tabulate-seqs\
  --i-data rep-seqs.qza\
```

```
  --o-visualization rep-seqs
## Table of per-sample sequence counts
qiime feature-table summarize\
  --i-table table.qza\
  --m-sample-metadata-file mdat.tsv\
  --o-visualization table
```

After coding was complete, an output of operational taxonomic units (OTUs), with associated FASTA files, was obtained for each species present with any combination of samples. The list of OTUs and the number of instances that each was found within any particular sample was generated in a feature table. This feature table and the fasta file were merged into one Excel spreadsheet with the following categories: species name/scientific name, E-value, Percent ID, sequence, OTUID, and Spotted Seatrout sample number (SPTR#) from both CSAs. Sequences that did not have a corresponding OTU ID were deleted from the merged file. The e-value indicated the probability that a given sequence alignment matched the input sequence (query) by chance. The percent identity represented the percentage of nucleotides of the sequence alignment that matched the query. Each sequence was copied and pasted into the NCBI Nucleotide BLAST database and the best sequence alignment was selected for 100 sequence alignments given based on the following criteria: the lowest e-value, the highest percent ID. Other parameters were used to distinguish between alignments with similar scores: the highest query cover (which indicated what percentage of the query length was used by each sequence alignment) and the instance of the species associated with the alignment within the Gulf of Mexico (the sample locations were off the coast of Louisiana). Species identifications were completed using the World Register of

Marine Species (WoRMS), United States Fish and Wildlife Services (USFWS), and FishBase databases. When all parameters were exhausted and a particular OTU sequence and a few possible sequence alignments remained indistinguishable, the OTU sequence was assigned the next highest taxa classification. Additionally, sequences that were imputed into NCBI Blast and had output sequence alignments of Spotted seatrout (*Cynoscion nebulosus*), other *Cynoscion spp., or Homo spp.* were deleted from the data set due to contamination.

Results

Through visual observations, prey items identified in the Spotted seatrout digestive tracts included shrimp, crab, and fish. Visual observations were unable to achieve further specificity. A total of 116 Spotted Seatrout samples were sequenced and analyzed using Qiime 2 and NCBI Blast. A total of 37 species, 6 genera, one subfamily, and one clade were associated with at least one OTU. They are listed as follows- *Funulus similis* (Longnose killifish), *Micropogonias undulatus* (Atlantic croaker), *Micropogonias furnieri* (Whitemouth croaker), *Brevoortia partronus* (Gulf Menhaden), *Brevoortia gunteri* (Finescale menhaden), *Anchoa mitchilli* (Bay anchovy), *Membras martinica* (Rough silverside), *Anchoa hepsetus* (Broad-striped anchovy), *Syngnathus scovelli* (Gulf pipefish), *Leiostomus xanthurus* (Spot croaker), *Ctenogobius shufeldti* (American freshwater goby), *Paralichthys lethostigma* (Southern flounder), *Gobionellus oceanicus* (Highfin goby), *Atractosteus spatula* (Alligator gar), *Lagodon rhomboides* (Pinfish), *Ophidion marginatum* (Striped cusk-eel), *Stellifer lanceolatus* (American stardrum), *Gobiosoma bosc* (Naked goby), *Ctenogobius boleosoma* (Darter goby), *Menidida menidia* (Atlantic silverside), *Alosa chrysochloris* (Skipjack shad), *Dorosoma petenense* (Threadfin shad), *Trichiurus lepturus* (Atlantic cutlassfish), *Euthynnus alletteratus* (Little tunny), *Harengula*

jaguana (Scaled sardine), *Ariopsis felis* (Hardhead catfish), *Scomberomorus maculatus* (Spanish mackerel), *Percina vigil* (Saddleback darter), *Symphurus plagiusa* (Balckcheek tonguefish), *Clupea harengus* (Atlantic Herring), *Sciaenops ocellatus* (Red drum), *Menidia beryllina* (Inland silverside), *Micropterus punctulatus* (Spotted bass), *Alosa alabamae* (Alabama shad), *Lepisosteus oculatus* (Spotted gar), *Menticirrhus americanus* (Southern kingcroaker); Genera: *Fundulus spp., Brevoortia spp, Lepisosteus spp., Alosa spp., Paralichthys spp., Anchoa spp.; Alosinae spp.* (subfamily); and *Percomorphaceae spp.* (clade). Many taxonomic classifications corresponded with multiple OTUs, which totaled to 176 OTUs with taxonomic assignments. The top four species identified throughout all Spotted seatrout (SPTR) samples included *Micropogonias undulatus* (Atlantic croaker) at 19.32%, *Brevoortia gunteri* (Finescale menhaden) at 18.18%, *Anchoa mitchilli* (Bay anchovy) at 15.91%, and *Leiostomus xanthurus* (Spot croaker) at 6.82%. OTUs not classified to the species level accounted for 6.82%.

Then, CSAs 5 and 6 were analyzed. A total of 63 SPTR gut content samples were processed from CSAs 5 and 6- 54% of all SPTR samples. In CSA 5, 28 gut-content samples were processed with 16 taxonomic classifications absent- including the genuses *Anchoa spp.* and *Alosa spp.* Out of all 28 samples, sample SPTR041 was the highest consumer with 11 different species within its gut contents. The top four taxonomic classifications present within the gut contents of the CSA 5 samples included *Micropogonias undulatus* (Atlantic croaker) at 14%, *Anchoa mitchilli* (Bay anchovy) at 11%, *Fundulus spp.* (killfish genus) at 10%, and *Brevoortia gunteri* (Finescale menhaden) at 9%. In CSA 6, 35 gut-content samples were processed with 29 taxonomic classifications absent- including the genuses *Alosa spp., Anchoa spp., Fundulus spp., Paralichthys spp.*, and the clade *Percomorphaceae spp.* Samples SPTR143, SPTR144, SPTR152, SPTR175, SPTR213, SPTR214, SPTR215, SPTR216, SPTR223, SPTR239,

SPTR240, and SPTR242 did not have any taxa associated with their gut contents. The top four taxonomic classifications present within the gut contents of CSA 6 species included *Micropogonias undulatus* (Atlantic croaker) at 21%, *Anchoa mitchilli* (Bay anchovy) at 20%, *Brevoortia gunteri* (Finescale menhaden) at 18%, and *Brevoortia partronus* (Gulf Menhaden) at 16%.

Comparisons- CSA 5 had 19 prey species that were absent in CSA 6, while CSA 6 had 7 prey species absent in CSA 5. Lastly, the highest consumer in CSA five had more taxa present in its gut contents than the highest consumer in CSA 6. Overall, CSA 5 has a more even distribution of prey taxa than CSA 6.

Conclusion

Over half of the SPTR samples were collected from Spotted seatrout in CSAs 5 and 6. Additionally, three out of four of the top prey species in all SPTR samples were also in the top four prey species of both CSA 5 and CSA 6, and nine of all 45 taxa were not found in either CSA. This indicated that both CSAs are highly representative of the total Spotted Seatrout sample set. The low instance of taxonomic classifications deferred to higher levels and the wide diversity in taxa detected in the gut contents of the SPTR samples supported the hypothesis that molecular analysis would effectively detect a diverse and large quantity of prey within the gut content samples to supplement visual gut content observations and provide more conclusive data concerning prey selectivity diversity. The absence of taxa in some SPTR samples in CSA 6 could be attributed to three reasons: 1) the digestive tracts of the fish associated with the samples were empty, 2) all detected OTUs were deleted due to contamination or indistinguishability, 3) PCR

and sequencing were limited in their ability to elucidate the prey species in their digestive tracts. CSA 6 was also less diverse in prey selectivity than CSA 5. A probable explanation could be that the slowing of the Atchafalaya River from backed-up nutrient and sediment filtration and inhibition of water overflow into swamps due to construction contributed to decreased water quality and habitat health which affected the diversity of prey available rather than the previous hypothesis concerning the decline in preferred prey species. However, more research in more numerous, smaller sectioned locations in each CSA from the gulf to inland, conducted over a multi-year period is needed to develop evidence to support this claim. Overall, The effectiveness of this study in obtaining conclusive diet data supports the claim that molecular diet studies are more effective than visual observation studies, as reported by previous studies.

Other future implications of this study included providing the results to LDWF to assist committees during hearings for the next set of Spotted Seatrout regulations and using metabarcoding and Next-Generation Sequencing in molecular diet studies. Lastly, comprehensive diet data could be added to ecological surveys to contribute to understanding trophic interactions and energy flow within the CSAs of Louisiana.

Manuscript Format

This document was formatted according to the American Fisheries Society style with websites formatted using the American Psychology Association style.

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