DNA Gut Analysis of Spotted Seatrout, *Cynoscion nebulosus*, in Coastal Regions of Louisiana with an 18s Primer

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

Abstract

In Louisiana, Spotted Seatrout*, Cynoscion nebulosus,* is a recreationally fished predatory fish in the Gulf of Mexico. In recent years there has been a decline in spotted trout populations and size. Spotted seatrouts are known to eat shrimp, crabs, mollusks, and small fish. While the broad categories are known for the diet of spotted seatrout, the prey items could vary based on the environment. The habitats along the coast of Louisiana vary in salinity and turbidity, which may change the feeding habits of Spotted Seatrout. For this study, metabarcoding data from Spotted Seatrout gut contents were bioinformatically analyzed using an 18s eukaryotic rRNA primer to compare diet differences along the coast of Louisiana. The coast was divided into five sections from the Texas to Mississippi border and the fish were categorized and compared based on where the fish were caught. This will take a nontraditional approach to dietary studies and use genetic sequences rather than observational identification of gut contents. Based on the results we will have a deeper understanding of the Spotted Seatrout diet along the coast of Louisiana. This may help design regulations on prey items to ensure there is no overfishing of important prey items. In addition, the results can be used to help structure protocols to help rebuild the diminishing spotted trout population in the Gulf of Mexico. Also, it could lead to a deeper understanding of the ecosystems that are present along the Louisiana coast, due to Spotted Trout being predatory fish, through the analysis of the prey available in each location.

Introduction

.Spotted seatrout (*Cynoscion nebulosus*) is a predatory fish that can be found from Massachusetts to Mexico. The species is nonmigratory and is generally found in estuary systems

(Rutherford, 1982). Spotted seatrout spawn in high-salinity areas and mature after one year. As a predator, spotted seatrout are carnivorous, generalist feeders. (Blaylock,2021) In a dietary study off the coast of Florida, in the Gulf of Mexico, it was found that the spotted seatrout had a diet of fish, shrimp, crabs, and mollusks. In this study, it was found that the prey species varied slightly depending on where they were caught, but all fell into those broad categories. (Rutherford, 1982)

On the coast of Louisiana in the Gulf of Mexico, there are many different saltwater fish that are recreationally fished. Throughout Louisiana's history, fishing has been a huge part of Louisiana culture. (Light,2014) Spotted seatrout (*Cynoscion nebulosus*), is one of the currently recreationally fished predatory fish in the Gulf of Mexico. In 2023, Louisiana Wildlife and Fisheries (LDWF) reported that 2,808,743 spotted seatrout were reported to have been caught along the Louisiana coast recreationally. To prevent overfishing, the Louisiana Department of Fisheries puts out yearly regulations to limit the amount being harvested and the sizes that can be harvested. The Louisiana Department of Wildlife and Fisheries also uses coastal study area locations to keep track of different population sizes and species within the state. (Lewin,2019) Five different coastal study area locations were designated by LDWF that span the coastline of the Gulf of Mexico. Each of the coastal study areas is along the boundaries of major basins lining the coast. Coastal study area seven consists of the Saine Basin, Calcasieu Basin, and Mermentau Basin. Coastal study area six consists of the Vermilion-Teche Basin and the Atchafalaya Basin. Coastal study area five consists of the Terrebonne basin. Coastal study area three consists of the Barataria Basin, part of the Mississippi Basin, and part of the Pontchartrain Basin. Coastal study area one consists of part of the Pontchartrain Basin and part of the Mississippi Basin.

Currently, the population of spotted trout is being closely monitored by LDWF because the population has been declining since 2015. In recent years the size range of the spotted trout has also been on the decline as well and fewer are reaching maturity to reproduce. This population decline is due to overfishing of spotted seatrout in past years. Due to this decline, in recent years Louisiana wildlife and fisheries have been monitoring the population and lowering the limits. In 2024, LDWF is following a plan to reduce the limit by 20% from previous years, placing more regulations, and preventing crew from catching Spotted trout while they are out on a charter with clients. LDWF's current goal is to reach a target population by 2027 while following the 20% reduction of the available limit. (LDWF, 2024)

Traditional dietary studies can be difficult to conduct and result in limited findings as they are focused on the appearance of the prey item. In studies, prey items have been identified by analyzing the gut contents. When analyzing the gut contents there are three main resulting gut categories. The stomach contents can be placed into empty, intact prey items, or prey items are present but unidentifiable. () The unidentifiable prey items can sometimes be identified as the type of prey item (ex. fish or shrimp), but prey items with soft tissue and smaller bodies tend to digest in the stomach faster leaving liquid remains or unidentifiable parts. This leads to fish in dietary studies not being able to be analyzed because the stomach contents were not in a state at which they could be analyzed. This limits the data able to be collected and restricts the information that is known. ()

Next generation sequencing DNA meta-barcoding is a nontraditional analysis of dietary samples to analyzed by next generation sequencing DNA meta-barcoding. Next generation sequencing is DNA sequencing where a mixture of DNA is running at the same time parallel

allowing for multiple DNA sequences to be sequenced without separation. (Berry et. al, 2017) In 2017 this process was used for analyzing the diet of Australian sea lions through their fecal DNA. In that study by Berry, the main limitation with genetically analyzing is reference barcodes to compare the DNA sequences to. (Berry et. al., 2017)

In all eukaryotic cells, the 18s rRNA gene is present. The 18s rRNA codes for the small subunit of eukaryotic cytoplasmic ribosomes. Because of its presence in all eukaryotic cells, it can be used as a primer that will work on any eukaryote and can be used as a standard reference sequence for the taxonomic classification of organisms. ()

The goals of this present study are to examine the dietary habits of spotted seatrout through the use of next generation sequencing using an 18s eukaryotic rRNA primer across four CSA locations along the Louisiana coast. Based on spotted trout being an opportunistic predator, we hypothesized that spotted trout would have common prey items across all four locations with specific prey items that are unique to each coastal study area.

Rationale

This study will be conducted to provide information regarding the eating habits of spotted seatrout, a predatory fish on the Louisiana coast. In the Gulf of Mexico, spotted seatrout, recreationally fished predatory fish. Depending on the preference of prey in each area, the fishing regulations may need to be evaluated to make sure the fishing of other species is not impeding the prey available to these predatory fish.

This study could also potentially create a deeper understanding of the dietary habits of spotted seatrout by using a nontraditional approach to dietary analysis. In traditional dietary

analysis, there can be empty stomachs where no information can be collected, or unidentifiable objects are present in the stomachs. By genetically analyzing the stomach contents, there could be prey items that are part of the diet of spotted seatrout, that were potentially not originally able to be identified.

The data collected will also help to gain a better understanding of the predatory tendencies of fish in the varying coastal study area locations. By analyzing each coastal study location and the overall differences between the four coastal study areas, commonalities and variations of prey types can be discovered. This can potentially be used to better understand the tendencies of predatory fish and how different environments can affect prey choice in spotted seatrout.

Altogether, the results of this data can demonstrate the variations and similarities of prey preference in different coastal environments. If there are similarities between the prey item choices across all four coastal study area locations, it could potentially be used to analyze the fishing limits of that prey item to ensure that it is not being overcaught, putting a limiting pressure on the predatory fish.

The data from this experiment can also be used to gain a deeper understanding of the prey in the varying areas of the Louisiana coast. This could potentially be used in the future to predict the types of predatory fish that may be in the varying coastal study area locations if they are following a specific type of prey.

Materials, Tools, and Supplies:

- Spotted Seatrout (*Cynoscion nebulosus)*
- -20° C Freezer
- Scissors
- Fish ruler
- Bleach
- Gloves
- 50 mL Large Conical Tubes
- 15 mL Medium Conical Tubes
- 1.5 ml Microcentrifuge tubes
- Ethanol
- DI water
- Incubator
- Vacufuge
- Sieve
- DNeasy Blood and Tissue DNA extraction kit
- 5X Platinum TM Super Fi Buffer
- 10 mM dNTPs
- 5µM 18s rRNA Eukaryotic Forward and Reverse primer
- Platinum Super Fi DNA polymerase
- Nuclease free water
- Agarose powder
- TAE solution
- Ethidium Bromide
- Loading Dye
- Gel Rig
- Electrophoresis
- Filtered pipette tips and pipettes
- Bio Rad Chemi Doc Imager
- Excel
- GenBank

Methodology

Samples

Spotted seatrout was collected from coastal study area 1, coastal study area 3, coastal study area 5, and coastal study area 6 by Louisiana Wildlife and Fisheries and kept frozen until

dissection. The spotted seatrout will be left at room temperature to thaw. Each fish was then assigned a sample number and dissected by Nicholls State University students. For dissection, the fish were placed in dissection trays and cut from the end of the large intestine to the jaw. The digestive tract was removed and all instruments were bleached. The stomachs were then cut open and the contents were removed and placed into 25-50 mL plastic centrifuge tubes with pure methanol for preservation. After each sample the work area was cleaned with bleach and gloves were changed. The samples were then placed into the -20° C freezer until sequencing.

Sequencing

The samples for each individual fish were placed onto a sieve and 10 mL of diH_2O was poured over the sample. The runoff was then collected into 15 mL plastic centrifuge tubes. 1000 µL of the runoff was placed into microcentrifuge tubes and centrifuged at maximum speed for 1 minute. The resulting supernate was poured off and the pellet was dried in the vacuum at 37° C for 24 hours. The resulting pellet was then according to DNeasy Blood and tissue kit instructions for DNA purification.

For the DNeasy protocol, we added 180 µL of buffer ATL to each of the samples. Then 20 µL of Proteinase K was added and the samples were left in the vortexing incubator at 56 ° C for 12 hours. When removed, 200 µL of buffer AL was added to each sample and vortexed for five seconds. Then 200 µL of Ethanol is added followed by 5 seconds of vertexing. This mixture is then pipetted into a new microcentrifuge tube with a mini spin column and centrifuged at 8000 rpm for 1 minute. Then the spin column is placed in a new microcentrifuge tube and 500 µL of Buffer AW1 is added to the spin column. This is then centrifuged at 800 rpm for 1 minute. The spin column is placed into a new microcentrifuge tube and 500 µL of buffer AW2 is added to the

spin column. This is then centrifuged at 14,000 rpm for three minutes. The spin column is then discarded and the microcentrifuge tubes are labeled as extracted DNA. The samples were then labeled and placed into the 5° C refrigerator until PCR amplification.

The samples then underwent polymerase chain reaction (PCR) using 18s rRNA eukaryotic primers (Table 1). The tubes for PCR each contained 25µL of mixture that consisted of 2 µL of extracted DNA, 5µL 5x Platinum SuperFi Buffer, 1.25 µL 2.5 µM forward primer, 1.25 µL 2.5 µM reverse primer, 0.5 µL 10mM dNTPs, 0.25 µL Platinum SuperFi DNA polymerase, and 14.75 µL nuclease free water. The PCR was run for 35 cycles after an initial three minutes at 94°C under the following conditions: denaturing at 94°C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72°C for 30 seconds, and then one final annealing at 72°C for 7 minutes. A 1% agarose gel was then run using gel electrophoresis to check for band presence at approximately 900 bp. Using 2µL of PCR sample and 2 µL of loading dye. The samples were then sent off to the University of New Hampshire Hubbard Center for Genome Studies to undergo Next Generation Sequencing (NGS).

Data Analysis

The results were then converted into a fasta file which was then merged with a feature table that was created with the unique sample OTI and the readings for each sample. This merged document was then used with GenBank to blast each sequence for the species. The results were sorted by E-value and then percent identity. Any results with a percent identity less than ninety-five percent were not included in the analysis. The feature table was then sorted by coastal study area location and each species sequence was analyzed. For each sample, if DNA for that species was present it was counted once as being present. If multiple DNA sequences are

coded for the same species; the species was only counted once despite multiple DNA strands coding for the singular species. Then the species prevalence across the four coastal study area locations was compared based on the amount of samples per species and by the species diversity in each CSA location.

Results

Meta-barcoding data from all coastal study area locations were sorted by location and blasted using GenBank to associate the DNA sequences with their associated species. Each sample was analyzed based on if the species was present in the gut contents. The total number of samples with each species present was then calculated. It was found that in coastal study area 1 (n=24), 44 different species were identified within the gut contents of the spotted samples. The highest species present within the gut contents of the Spotted trout analyzed were *Barbatia virescens, Tresus nuttallii*, and *Tucetona pectunculus* which was present in 23 out of the 24 samples. It was found that in coastal study area 3 (n=35), 42 different species were identified within the gut contents of the spotted trout samples. The highest species present within the gut contents of the Spotted trout analyzed were *Barbatia virescens, Tresus nuttallii, and Squilla empusa* that was present in all 35 samples. It was found that in coastal study area 5 (n=93), 54 different species were identified within the gut contents of the spotted trout samples. The highest species present within the gut contents of the Spotted trout analyzed was *Squilla empusa* which was present in 91 out of 93 samples. It was found that in coastal study area 6 (n=63), 50 different species were identified within the gut contents of the spotted trout samples. The top two highest species present within the gut contents of the Spotted trout analyzed were *Barbatia virescens* and *Tresus nuttallii* which was present in all 63 samples.

Coastal Study Area 1

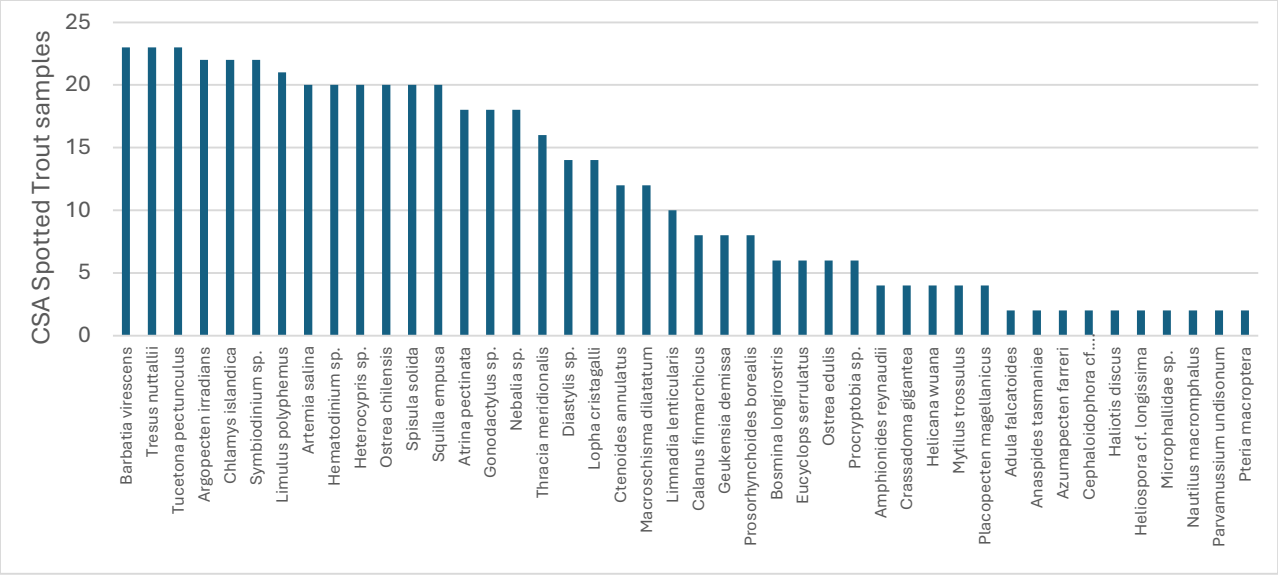

Figure 1. The total amount of samples with each species present in the gut contents in CSA 1

Coastal Study Area 3

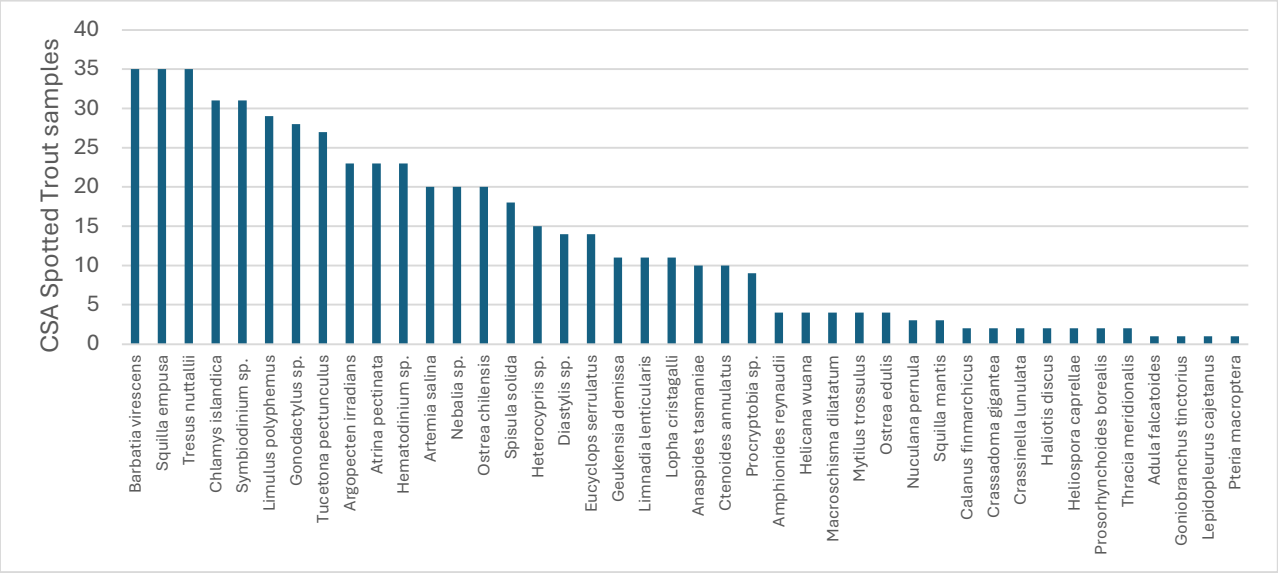

Figure 2. The total amount of samples with each species present in the gut contents in coastal study area 3

Coastal Study Area 5

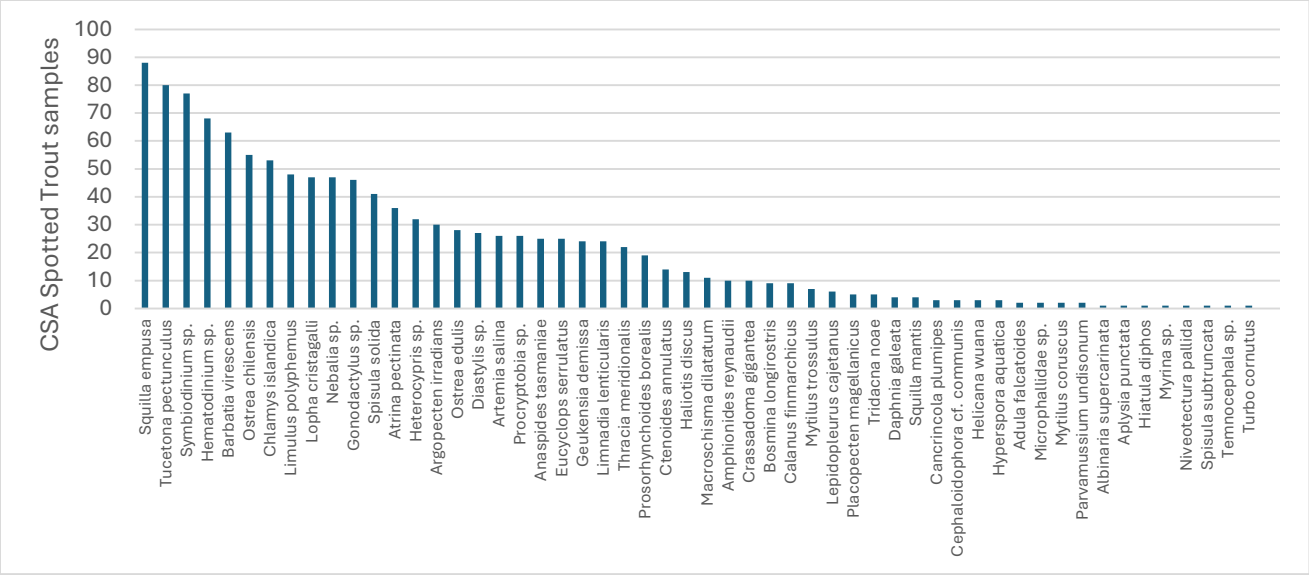

Figure 3. The total amount of samples with each species present in the gut contents in the coastal study area 5\

Coastal Study Area 6

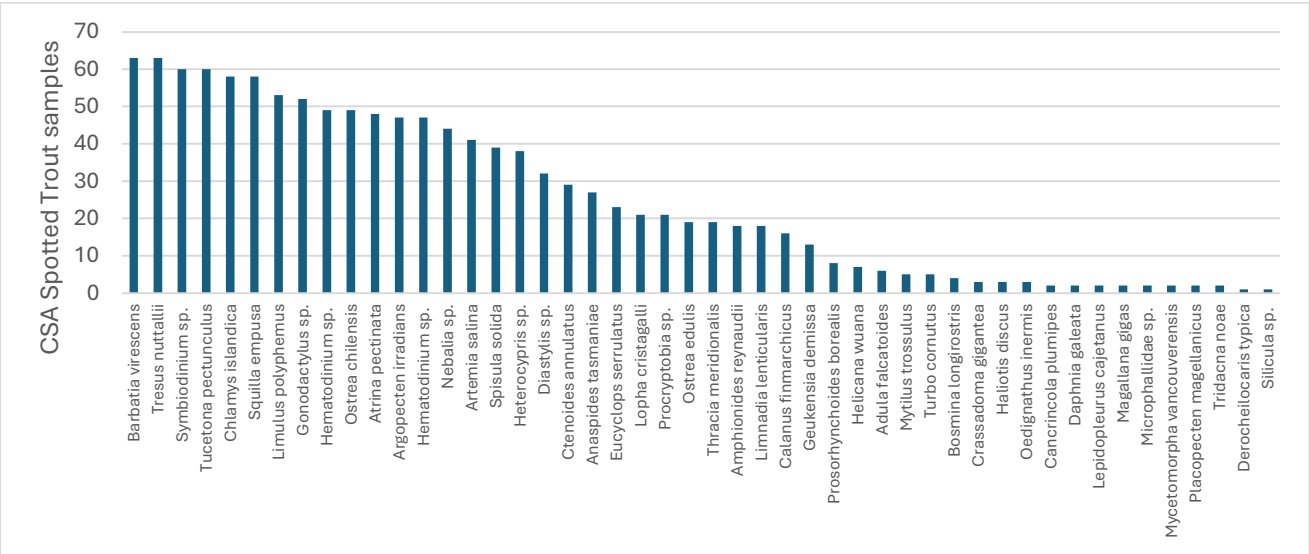

Figure 4. The total amount of samples with each species present in the gut contents in coastal study area 6

Discussion

Our data supported our hypothesis that the spotted trout at the four coastal study area locations would have the same main primary prey items with some individual prey items specific to each coastal study area location. In our findings, we found that in coastal study area 1, coastal study area 3, and coastal study area 6 *Barbatia virescens* was one of the highest prey items found in the gut contents. In coastal study area 5 *Barbatia virescens* was not present in the highest samples in comparison to other species, but out of the samples tested, it was present in 67.7% of the spotted seatrout. When looking at coastal study area 5 and coastal study area 3, *Squilla empusa* was one of the most prevalent species in the spotted seatrout samples tested. In coastal study area 1 and coastal study area 6 it was still found to be present in 83.33% and 92.06% respectively. In the four coastal study areas, it was also found that the top ten species commonly were *Barbatia virescens, Tresus nuttallii, Symbiodinium sp., Tucetona pectunculus, Chlamys islandica, Squilla empusa, Limulus polyphemus, Gonodactylus sp., Hematodinium sp, Ostrea chilensis, Artemia salina.*

In the coastal study area location, we also found that there were species that were isolated only in the gut contents of that coastal study area. In coastal study area 1, *Azumapecten farreri, Heliospora cf. longissimi, and Nautilus macromphalus* were found in the gut contents. In coastal study area 3, *Crassinella lunulate, Goniobranchus tinctorius, Heliospora caprellae, and Nuculana pernula* were found in the gut contents. In coastal study area 5, *Albinaria supercarinata, Aplysia punctata, Hiatula diphos, Hyperspora aquatica, Myrina sp., Mytilus coruscus, Niveotectura pallida, Spisula subtruncata, and Temnocephala sp.* in the gut contents. In coastal study area 6, Derocheilocaris typica, Magallana gigas, Mycetomorpha vancouverensis, Oedignathus inermis, and Silicula sp. were found in the gut contents. This indicates that in these

four coastal study area locations, those species were only isolated in that coastal study area location. This could be due to that species only being present in that portion of the Louisiana coast, the species has a higher presence in those coastal study area locations than the others. However further testing would need to be conducted to see if the prey item is present in the other coastal study area locations but not a prey item in those locations.

When analyzing the four locations for prey variety, the four coastal study area locations had a median of 47.25 prey species per coastal study area location with coastal study area five having the most at 55 prey species and CSA 3 having the least at 42 prey species. Based on our results, this indicates that coastal study area 5 has a larger biodiversity than the other coastal study area locations. This can also be seen in the prey species that was only in coastal study area 5. Coastal study area 5 also had the largest amount of prey species that was unique to the coastal study area.

Analyzing the species, the species that resulted from this study were bivalves, crustaceans, and gastropods. This is comparable to the results of the study in Florida. However, in this study, there were not any sequences that matched with any types of fish. Fish is a known prey item for spotted trout. It is possible that the 18s rRNA was not specific enough for the DNA to be matched with the database. It also could be due to PCR bias where the primer amplifies one gene and others get covered up. Further testing would need to be conducted to better understand the full diet.

Potential limitations of this study would include the sample size, location size, and the extent of GenBank's database. In this study, 215 spotted seatrout were analyzed. (coastal study area 1: n=24, coastal study area 3: n=35, coastal study area 5: n=93, coastal study area 6: n=63) The small sample size of each coastal study area location limits how representative of the whole

population the data is. In future studies, a larger sample size will help get a more accurate understanding of the population. The location of the coastal study area includes many different environments within each coastal study area. Because of the wide environments covered, using smaller grouping in future studies may help to gain a better understanding of spotted seatrout dietary habits when it comes to the environment that they are located in. Lastly, this study is limited by the data in GenBank. When analyzing the DNA sequences, we compared them to the known sequences of GenBank. Due to it being a growing database, some species are not included in the database or there is only limited data. This limits the specificity of the data being collected to the limits of their database. Future studies would also need to analyze the samples using more specific primers to be able to better understand the dietary habits of spotted seatrout.

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