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Environmental DNA (eDNA) Surveys for the Smalltooth Sawfish, Pristis pectinata, in the Chandeleur Islands, Louisiana

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by

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ABSTRACT

The Critically Endangered smalltooth sawfish, *Pristis pectinata*, was historically found throughout tropical and subtropical coastal waters of the Atlantic Ocean. As a result of mortalities in fisheries and habitat degradation, they became largely restricted to southwest Florida in the U.S. and the Bahamas by the 1980s. However, recent public encounter reports of sawfish in the Florida panhandle, Mississippi, and Louisiana suggest this species is occasionally present in northern Gulf of Mexico waters. Targeted species surveys are needed to improve our understanding of the occurrence and status of this species in these waters. This research used environmental DNA (eDNA) methods to assess the presence of *P. pectinata* in waters off the Chandeleur Islands, Louisiana. Water samples from 20 sites on the northwestern side of the Chandeleur Islands were collected and filtered in 2019. DNA was extracted from these samples, and these extracts were screened for target DNA using species-specific quantitative PCR and Droplet Digital™ PCR assays. Neither PCR assay confirmed the presence of target DNA from any of the 20 water samples, suggesting *P. pectinata* was not present in the vicinity of the collection sites during sampling. These results are inconclusive because they are based on a small number of samples collected at one timepoint. More comprehensive eDNA surveys are needed in the Chandeleur Islands to fully investigate their potential occurrence in these waters.

Keywords: eDNA, smalltooth sawfish, Chandeleur Islands, *Pristis pectinata*

DEDICATION

I dedicate this thesis to my grandfather, Joe Hebert. While you weren't able to see it to completion, I know you would be proud of me for finishing it. On the days when it was difficult to keep pushing forward, I would look at our "pink cookie" picture and remember just how much you loved and supported me through to the very end. You held out for me, so I had to hold out for you. To Popsie, love you always.

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Where do I even start? First, to my advisor, Dr. Nicole Phillips. Thank you endlessly for your support and patience with my seemingly infinite sleep-deprived drafts. Your guidance has gotten me this far, and I only strive to improve from here. Thanks to Ryan Lehman for spending his weekends to teach me extractions, reading one of my many drafts, and for collecting and filtering these samples to begin with. Thank you to Annmarie Fearing for showing me the ropes with qPCR, and to Claire Gauci for filtration and fieldwork experience. I truly appreciate all the help, support, and kind words you all have shown me. Thank you to Dr. Kelly and Zach Darnell for the opportunity to collect these samples. Thanks as well to Chris Graham for creating the sighting maps, Elora Pierce for filtration assistance, and Sammie Smith for the aid in field sample collection.

To Mom, Pop, Noah, Kyle, Gran, Mawmaw, and Pawpaw, thank you for your continuous support throughout my educational career (and for the spontaneous pictures of Creole and Gracie). Thank you, Dad, Marcie, and Travis, for the constant laughs and support, especially in the form of miniature cheesecakes. Lastly, never-ending thanks to Brittany for putting up with my lack of QGIS knowledge, and to Anna and Dayton for keeping me sane and smiling throughout this process. I love you all.

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LIST OF ABBREVIATIONS

bp	base pair
ddPCR™	Droplet Digital™ polymerase chain reaction
DI	deionized
DNA	deoxyribonucleic acid
DO	dissolved oxygen
eDNA	environmental DNA
ESA	Endangered Species Act
GPS	global positioning system
IUCN	International Union for the Conservation of Nature
LA	Louisiana
LoD	limit of detection
MT	manual threshold
ND2	NADH dehydrogenase 2
NMFS	National Marine Fisheries Service
PCR	polymerase chain reaction
pH	potential of hydrogen
qPCR	quantitative polymerase chain reaction
RFU	relative florescence unit
SE	standard error
SSRT	Smalltooth Sawfish Recovery Team
U.S.	United States
UV	ultraviolet

YOY	young-of-year

CHAPTER I: INTRODUCTION

1.1 Sawfishes

Five species of sawfish (Family: Pristidae) exist worldwide, each sporting a unique rostrum ('saw') lined with modified dermal denticles resembling teeth (Peverell, 2004). They use this saw, which is dotted with electro-receptive ampullae of Lorenzini, to swipe at and stun prey, and they use it for protection by slashing at predators (Wueringer et al., 2012). This unique appendage, however, renders the sawfish susceptible to entanglement in fishing gear. This detrimental interaction with humans has led to a decline in their range and abundance, and has made them one of the most threatened families of all marine fishes (Morgan et al., 2016). Currently, all five sawfish species are listed as Endangered or Critically Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (see Dulvy et al., 2016). Four of the five species of sawfish: largetooth sawfish *Pristis pristis*, dwarf sawfish *Pristis clavata*, green sawfish *Pristis zijsron*, and narrow sawfish *Anoxypristis cuspidata*, are primarily found in Australia, using the area as a stronghold (Peverell, 2004). The fifth species, the smalltooth sawfish, *Pristis pectinata*, was formerly found in the tropical and subtropical coastal waters of the Atlantic Ocean; however, now they mainly reside in south and southwest Florida in the United States and in the Bahamas (Carlson et al., 2007).

The most detrimental of threats faced by sawfishes include destruction of habitat, mortality via fishery bycatch, and trade of their fins and saws (Dulvy et al., 2016). Habitat loss has been the result of human development along coastlines, pollutant runoff, and storm activity damaging these areas (Norton et al., 2012). Sawfishes are benthic rays,

thus, trawling and other net entanglement has contributed to a monumental loss of individuals (Dulvy et al., 2016). Lastly, sawfish products such as meat and fins sell at a high price for Asian delicacies like shark fin soup, while rostra are traded, sold, or kept as trophy pieces (Dulvy et al., 2016).

1.2 Smalltooth Sawfish, *Pristis pectinata*

Pristis pectinata utilize tropical and subtropical shallow estuaries or bays that have muddy or sandy bottoms lined with red mangroves, *Rhizophora mangle,* with these habitats acting as nurseries (Simpfendorfer et al., 2016). *Rhizophora mangle* roots provide spaces where juveniles can hide from predators; aggregating fish and decay from mangroves leaves can then support other organisms in the food web, such as preferred prey for *P. pectinata* (Norton et al., 2012). During all stages of life, *P. pectinata* primarily feed on teleost fishes like mullets (*Mugil cephalus*) as well as other elasmobranchs including Atlantic stingrays, *Dasyatis sabina* (Poulakis et al., 2017). *Pristis pectinata* juveniles have an affinity for practical salinities of 18-30, and they will move up or down a freshwater stream in accordance with osmoregulation requirements or prey availability (Poulakis et al., 2011). Juveniles also prefer warmer waters (e.g., ≥30°C) with temperatures becoming lethal to sawfish at ~8°C (Poulakis et al., 2011). Sawfish do not sexually mature until ~10 years of age or when they reach 3.5 meters (m) in length (Seitz & Poulakis, 2006) (Brame et al., 2019). Upon reaching ~2.2 m in length (Scharer et al., 2017), the juvenile sawfish leave the nurseries and use coastal marine habitats (Poulakis & Grubbs, 2019). Adults largely remain in shallow coastal waters but can also enter waters as deep as 122 m (Seitz & Poulakis, 2006). Females reproduce biennially, display philopatric behavior when birthing pups, have a gestation period of approximately one

year, and can birth up to 7-14 live pups in estuarine waters (Brame et al., 2019). Parturition is highest during late spring or early summer, but it can occur year-round in some locations (Brame et al., 2019).

1.3 Decline of *Pristis pectinata*

Pristis pectinata are currently found in less than 20% of their estimated former range (Dulvy et al., 2016). Historically, *P. pectinata* were once found in the eastern Atlantic Ocean from Mauritania to Angola (Harrison & Dulvy, 2014). In the western Atlantic Ocean, the species could be found from the Rio de la Plata estuary between Argentina and Uruguay, northwards to Venezuela along the east coast of South America, throughout the Caribbean Sea and the Gulf of Mexico, and from South Carolina to Virginia in the United States (Harrison & Dulvy, 2014). Today, viable populations are restricted to south and southwest Florida in the United States and the Bahamas (Carlson et al., 2007), where *R. mangle* nursery habitats persist (Poulakis et al., 2017).

As a consequence of declines in range and abundance within U.S. waters, *P. pectinata* was listed as Endangered on the Endangered Species Act (ESA) of 1973 in 2003 (NMFS, 2009). This ESA listing provided federal protection for *P. pectinata* from take (defined as harvest, slaughter, or harassment) by humans (NMFS, 2009). As the species was listed on the ESA, a recovery plan was developed to prioritize research needs, recommend solutions to prevent further decline, and define and promote recovery of *P. pectinata* (NMFS, 2009).

1.4 Measuring Recovery of *Pristis pectinata*

The National Marine Fisheries Service (NMFS) created the Smalltooth Sawfish Recovery Team (SSRT), and in 2009, this team released the Smalltooth Sawfish

Recovery Plan (NMFS, 2009). This plan sets out three goals to promote recovery of *P. pectinata*: 1) increase sawfish numbers in their 'core range' (i.e., south and southwest Florida) and historically occupied habitats, 2) safeguard and repair damaged sawfish habitat, and 3) reduce human interactions that could negatively impact sawfish health (NMFS, 2009). Increased protections offered by the ESA, conservation guidance from the recovery plan, public education initiatives, sawfish encounter reports, and net bans in important sawfish habitats have collectively contributed to the stabilization of *P. pectinata* populations within their core range (NMFS, 2018). In a review conducted in 2012, it was determined that with the core population of *P. pectinata* stabilizing, 'spill over' into other surrounding areas (i.e., historically occupied habitats) was possible (Carlson & Osborne, 2012). Over the last decade, *P. pectinata* sightings have been reported in historically occupied habitats in the northern Gulf of Mexico from Louisiana to the Florida panhandle, and as far north as North Carolina on that Atlantic coast (Figure 1A). Surveys are needed to understand the extent and seasonality of *P. pectinata* occurrence in historically occupied habitats.

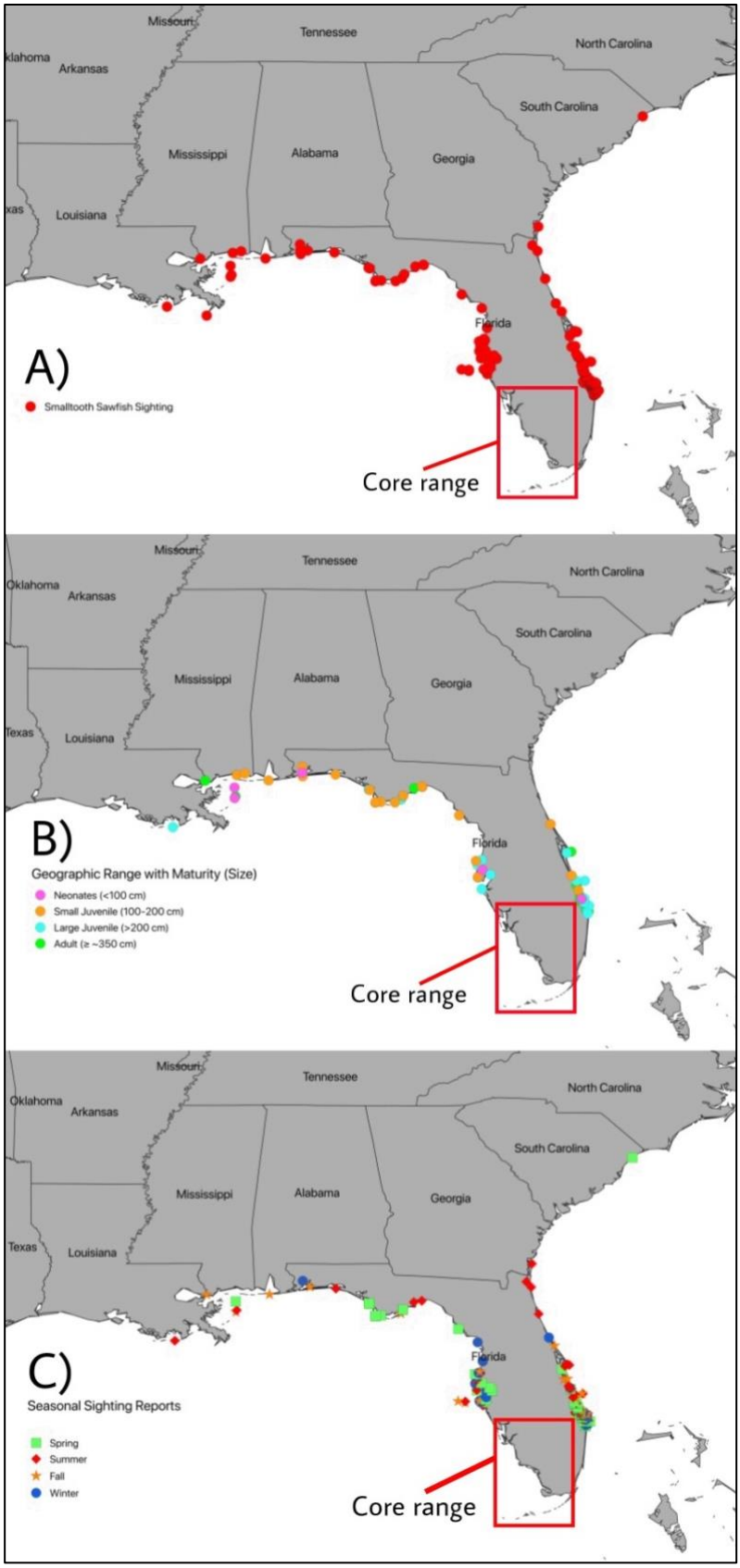
Missouri
Tennessee
North Carolina
Arkansas
South Carolina
Mississippi
Alabama
Georgia
Louisiana
Florida
A)
Smalltooth Sawfish Sighting
Core range
Missouri
Tennessee
North Carolina
Oklahoma
Arkansas
South Carolina
Mississippi
Alabama
Georgia
Texas
Louisiana
Florida
B)
Geographic Range with Maturity (Size)
Neonates (<100 cm)
Small Juvenile (100-200 cm)
Large Juvenile (>200 cm)
Adult (≥ ~350 cm)
Core range
Missouri
Tennessee
North Carolina
Oklahoma
Arkansas
South Carolina
Mississippi
Alabama
Georgia
Texas
Louisiana
Florida
C)
Seasonal Sighting Reports
Spring
Summer
Fall
Winter
Core range

Figure 1: Panel A shows sightings of smalltooth sawfish, Pristis pectinata, in historically occupied habitat outside of their core range from 2009-2019. Panel B shows the sightings in accordance with sawfish maturity, which is determined by length. In the Chandeleur Islands, Louisiana, two P. pectinata were considered neonates, while one was an adult. Panel C highlights the seasonality of the reports. In the Chandeleur Islands, LA, the three sighted P. pectinata were found during spring, summer, and fall months. Map created by Chris Graham. Data from the National Marine Fisheries Service (NMFS, 2019, unpublished data).

1.5 eDNA

Survey methods traditionally involve catching *P. pectinata* in nets; however, this process can be stressful for the sawfish, and they can be difficult to catch due to the rarity of the species (Poulakis & Grubbs, 2019). Environmental DNA (eDNA) offers a novel technique that involves collecting and filtering water samples, and extracting DNA from the particulate material (Hanfling et al., 2016). All aquatic organisms release DNA into their environment via fecal excretions, shed mucus or scales, or urine; this shed DNA settles in benthic sediments and/or remains suspended in the water column (Turner et al., 2015). This method allows detection of the target species in an area without the need to capture individual organisms. Environmental DNA methodologies have been successful in detecting other threatened elasmobranchs such as whale sharks, *Rhincodon typus*, and largetooth sawfish, *Pristis pristis* (Simpfendorfer et al., 2016; Sigsgaard et al., 2016). The aim of these surveys (e.g., species presence assessment) is to evaluate the effectiveness of eDNA methodologies in accordance with population genetics studies. Environmental DNA approaches to study the distribution and ecology of Critically Endangered

elasmobranchs negates the need to acquire permits for research activities and eliminates the risk of inducing stress or harm to animals, since the animal does not have to be physically available for the study (Simpfendorfer et al., 2016).

A species-specific Droplet Digital™ PCR (ddPCR™) eDNA assay was recently developed for *P. pectinata* for use in U.S. waters (Lehman et al., 2020). Environmental DNA surveys conducted using this tool in historically occupied habitats in Mississippi and Florida successfully detected the presence of *P. pectinata* DNA in the waters surrounding Deer Island in the Mississippi Sound and in the Indian River Lagoon (Lehman, In Press). These eDNA survey results corroborate recent sawfish encounter reports from the general public in these two estuaries, and provide additional evidence that *P. pectinata* are present in at least some historically occupied waters. Wider use of this eDNA assay across all historically occupied waters, and especially in those with recent reports of sawfish presence from the general public, are needed to monitor recovery of this species in U.S. waters (Lehman, In Press).

1.6 The Chandeleur Islands

Sawfish encounters have recently been reported in the Chandeleur Islands, Louisiana (Figure 1). These reports include one adult and two young-of-year (YOY), based on estimated lengths of the animals (Figure 1B). The adult was sighted during summer, while one YOY was sighted during spring and the other during fall (Figure 1C). Stretching 72 kilometers (km) in the Gulf of Mexico southwest of Louisiana, the Chandeleur Islands are barrier islands containing turtle seagrass (*Thalassia testudinum*) and black mangrove (*Avicennia germinans*) habitats (Poirrier & Handley, 1940; Scheffel et al., 2018). These types of habitats are commonly used as nursery grounds for marine

species (Moore et al., 2014), and mangroves in particular provide protection and food sources for numerous juvenile sharks and rays (McKenzie, 2013). For instance, lemon sharks, *Negaprion brevirostris*, are known to use the Chandeleur Islands as a nursery site (McKenzie, 2013). The Chandeleur Islands are the only known nursery site for *N. brevirostris* in the northwest Gulf of Mexico (McKenzie, 2013). Considering that the Chandeleur Islands support mangrove and seagrass habitats, which are used as nursery areas for other elasmobranchs, it is possible these habitats also support young *P. pectinata*, based on sawfish encounter report data from the National Marine Fisheries Service (Figure 1B).

Mangrove and seagrass habitats have rapidly been disappearing from the northern Gulf of Mexico due to hurricanes, sea level rise, and human pollution (e.g., the Deepwater Horizon oil spill) (Moore et al., 2014), making these remnant habitats in the Chandeleur Islands critical for numerous marine species (McKenzie, 2013). The loss of these unique habitats could threaten the survival of species of conservation concern (McKenzie, 2013) and hinder local recovery of *P. pectinata*. Surveys for *P. pectinata* in the Chandeleur Islands are needed to better understand the extent of sawfish occurrence, and their potential reliance on these habitats. The aim of this research was to conduct eDNA surveys to assess the presence of *P. pectinata* in the Chandeleur Islands, Louisiana.

CHAPTER II: METHODS

All laboratory controls and collection, filtration, extraction, qPCR, and ddPCR™ methods are those of Lehman et al. (2020) and Lehman (In Press).

2.1 Laboratory, Field, and Negative Controls

In order to mitigate the possibility of contamination by external DNA, all materials used (water sample collection bottles, filtering systematics, microcentrifuge tubes, pipette tips, forceps, tube racks, etc.) were sterilized via autoclaving at 121°C for 20 minutes, soaking in 10% bleach for 15 minutes, and/or treating with UV light for 15 minutes. The sterilization methods used depended on the materials, but two cleaning methods were combined for all materials. For example, work benches were soaked with 10% bleach for 15 minutes and treated with UV light for 15 minutes. Water filtration, DNA extractions, and the PCRs were all conducted in separate laboratory spaces to reduce the risk of contamination across the stages of sample processing. Water filtration also occurred in a lab that never had contemporary *P. pectinata* tissue present.

Various negative controls were implemented, and all were treated with the same protocol as field samples and processed through to PCRs to check for contamination and reagent performance. The collection negative controls consisted of 3 liters (L) of autoclaved deionized (DI) water that were stored on ice on the field boat. The filtration negatives consisted of 3 L of autoclaved DI water that were filtered in a lab. The extraction negatives received all reagents from the extraction process, but they did not contain filters. Lastly, PCR negatives did not have a DNA template.

2.2 Water Collection, Filtration, and DNA Extraction

A total of 20, 3 L water samples were opportunistically collected on the western side of the Chandeleur Islands across two days in September 2019, following the field protocols described in Lehman (In Press) (Figure 2). Abiotic data on water depth, pH, salinity, temperature, and dissolved oxygen were also taken during each sampling day. The water samples were stored on ice in the field and frozen upon return to the lab. Samples were later thawed at room temperature and vacuum filtered using Whatman® 47 millimeter 0.8 micrometer nylon filters. After approximately 350 milliliters, new filters were applied, totaling ~9 filters for each 3 L water sample. These filters were rolled with sterile forceps and stored in 95% ethanol at room temperature. During DNA extractions, filters were unrolled, and eDNA was extracted from the particulate material of one half of each filter. Gloves, forceps, and cutting boards were changed between each sample to reduce the risk of cross-contamination across samples. DNA was extracted using the QIAGEN® DNeasy® Blood & Tissue Kit with Qiashredder™ spin columns following the protocols in Goldberg et al. (2011). Minor modifications to this protocol were made including: the use of barrier pipette tips, DNA was eluted with 50 microliters (μL) of heated elution buffer, and the inclusion of extraction negatives. Quality of the DNA extracts was observed via electrophoresis with a 2% agarose gel. The concentration of DNA was quantified via Thermo Fisher Scientific™ NanoDrop™ One Spectrophotometer.

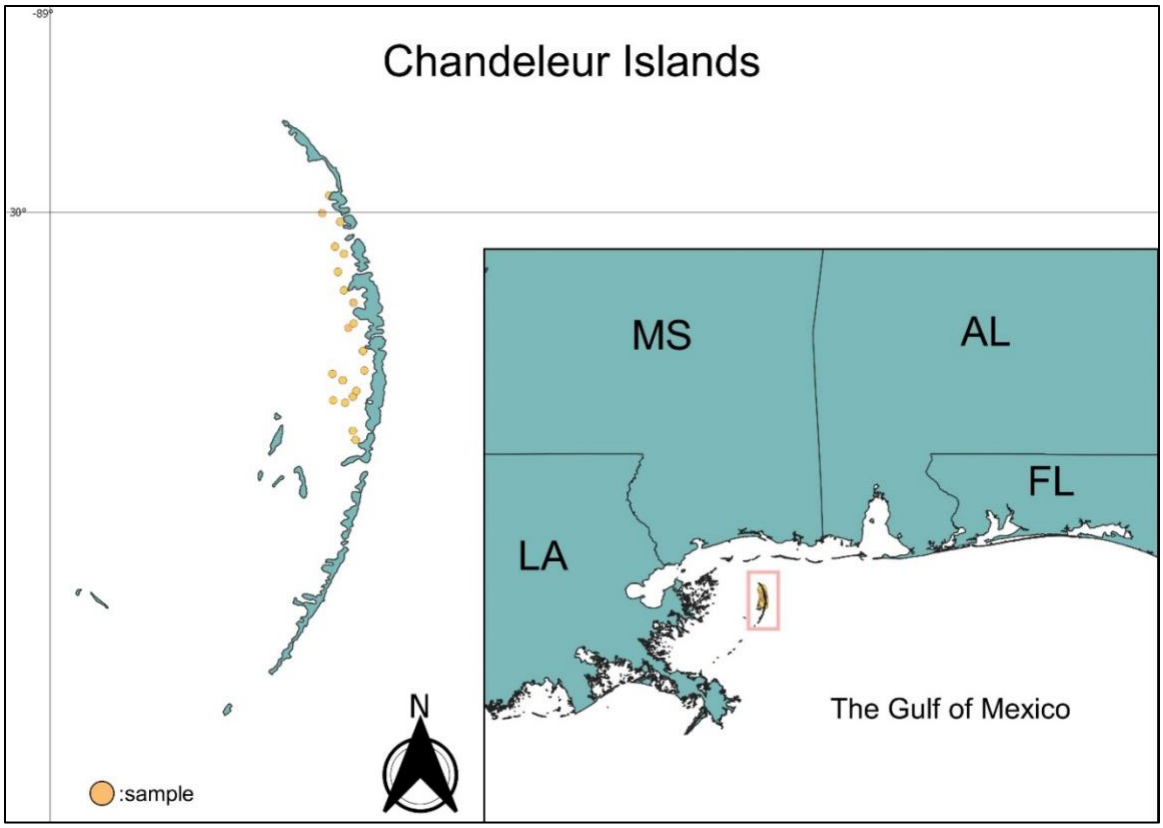

Figure 2: Locations of water samples collected in the Chandeleur Islands, Louisiana.

2.3 PCR Amplification

Species-specific forward (5′-CTGGTTCACATTGACTCTTAATTTG-3′) and reverse primers (5′-GCTACAGCTTCAGCTCTCCTTC-3′) and a PrimeTime® double-quenched ZEN™/IOWA Black™ FQ probe (Integrated DNA Technologies) labeled with 6-FAM probe (5′-TACCATAGCCATCAT CCCATTATTATTC-3′) were used to amplify a 100-bp fragment of the NADH dehydrogenase subunit 2, or ND2 gene, in *P. pectinata* (see Lehman et al., 2020). Lehman (2020; In Press) developed the *P. pectinata* eDNA assay using a ddPCR™ platform, however, quantitative PCR (qPCR) is a more widely available PCR platform. Therefore, DNA extracts from water samples were screened using both PCR platforms to assess whether qPCR provides sufficient

sensitivity to be used in eDNA surveys for this species, which could facilitate eDNA surveys in areas that do not have such technologies available. All samples, including negatives, were run on each PCR platform with five replicates.

Prior to running field samples, a 'positive control' qPCR was run with the Bio-Rad® C1000™ Thermal Cycler using a verified positive *P. pectinata* eDNA sample to ensure successful amplification (see Lehman et al., 2020). Each reaction mixture contained 1X Bio-Rad® iTaq supermix, 900 nanomolar (nM) of each primer, 170 nM of probe, 1 μL of the positive eDNA extract, and was adjusted to 20 μL with PCR-grade water. This mixture was cycled at 95°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds and 64°C for 2 minutes, and finished with 98°C for 10 minutes, all at a ramp rate of 1°C/second (Lehman et al., 2020). Upon confirmation the assay was working, all eDNA extracts, including negative controls, were run using the qPCR (using the described protocols) and the ddPCR™ platforms. Droplet Digital™ PCRs used the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System, Droplet Generator instrument no. 773BR1456 and Droplet Reader instrument no. 771BR2544. Each ddPCR™ reaction mixture contained 1X ddPCR™ supermix, 900 nM of each primer, 170 nM of probe, 1.1 μL of DNA extract, and was adjusted to 22 μL with PCR water. The automated droplet generator added ~70 μL automated droplet generation oil for probes to 20 μL of the reaction mixture. This mixture was then partitioned into ~15,000-20,000 droplets, and PCR-amplified using the protocol of 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 64°C for 2 minutes, ending with 98°C for 10 minutes, using a ramp rate of 1°C/second. After cycling, the plate was set into the Droplet Reader where each droplet was screened for the presence of *P. pectinata* DNA.

2.4 Data Analysis

The qPCR data were analyzed with the Bio-Rad® C1000™ Thermal Cycler software using two criteria for a positive detection: 1) logarithmic amplification beginning at 20 cycles, and 2) amplification between 1,200 – 1,600 relative florescence units (RFUs) (Figure 3). These criteria were defined based on the amplitude and timing of the positive *P. pectinata* eDNA sample. The ddPCR™ data, which were analyzed using Rare Event Detection in the Bio-Rad® QuantaSoft™ software, had three criteria for positive detection, as defined by Lehman et al. (2020): 1) droplet amplitude must be greater than or equal to the manual threshold (MT) of 3,000 RFUs, 2) droplet amplitude is within a range of 5,000-7,000 RFUs, as seen with the positive target DNA collection in Lehman et al. (2020), and 3) the concentration of target DNA is greater than or equal to the Limit of Detection (LoD) for the assay, 0.08 copies/µL (Figure 4). Only one replicate needed to meet these criteria to be considered a positive detection. Negative control samples were considered free from contamination when collection, filtration, extraction, qPCR and ddPCR™ negatives did not meet any of the defined criteria.

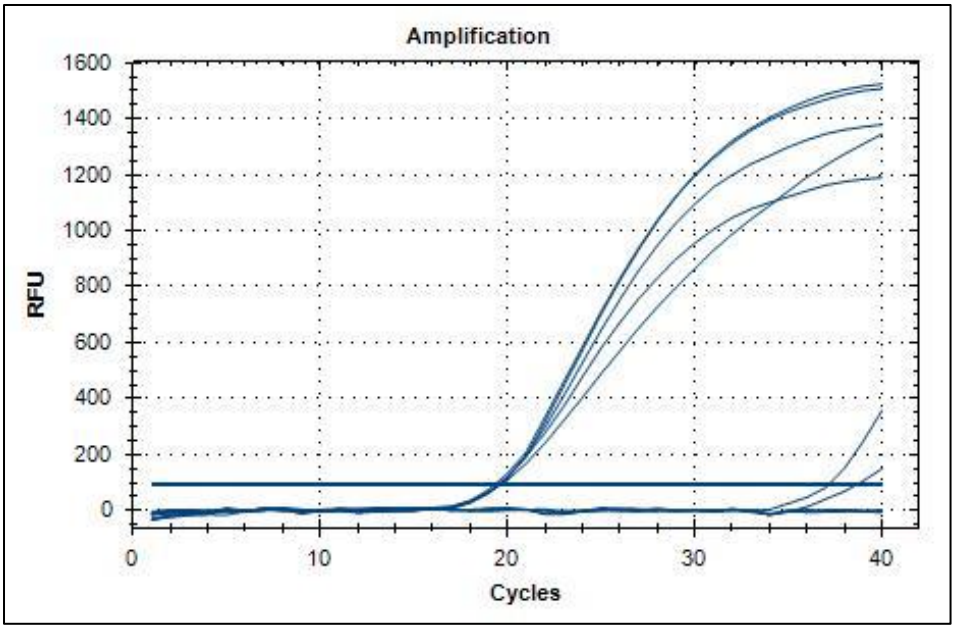

Figure 3: Successful amplification of a 100 base pair fragment of the NADH dehydrogenase subunit 2 gene for a positive smalltooth sawfish, Pristis pectinata, eDNA sample, demonstrating the assay was functioning on the qPCR platform. The five lines that form the amplification curve illustrate amplification in each of the five sample replicates. The five lines that are <400 RFUs are the qPCR negative replicates. Figure created using Bio-Rad® C1000™ Thermal Cycler software. RFU is relative florescence units, and cycles are the amplification rounds.

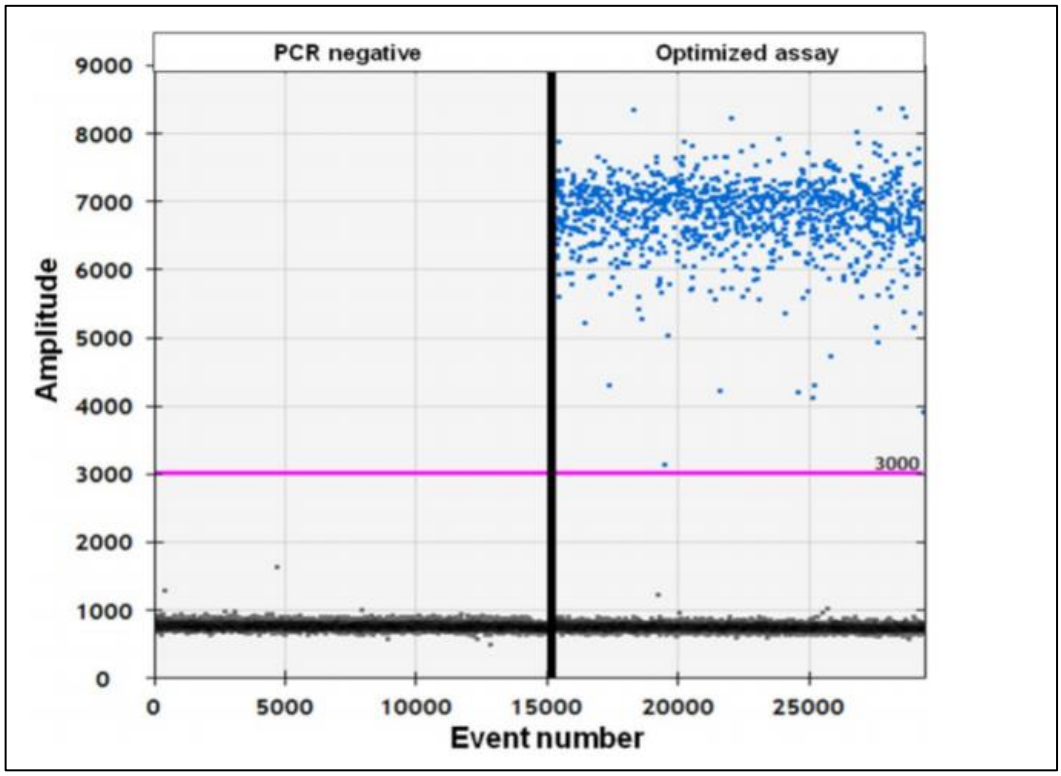

Figure 4: A comparison of a Droplet Digital™ PCR negative control (left) and an optimized assay for smalltooth sawfish, Pristis pectinata, using genomic DNA (right). Droplet amplitude is measured in relative florescence units (RFUs), and event number is the droplets created. Rare event detection was used to analyze droplets via the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software. To be considered a positive detection, each droplet must 1) reach the manual threshold of 3,000 RFUs (pink line), 2) be between 5,000-7,000 RFUs, and 3) contain a concentration of DNA greater than or equal to 0.08 copies per µL. Blue dots represent droplets positive for target DNA, while gray dots are negative for target DNA. Figure is from Lehman et al. (2020).

CHAPTER III: RESULTS

3.1 Environmental Data

Water samples were collected from shallow, warm, and estuarine waters. A high dissolved oxygen content of 7.9 milligram (mg)/L was present along with a slightly basic pH of 8.5 (Table 1). The bottom type throughout the sampling area consisted of seagrass with mangroves and sandy beach nearby.

Table 1: Mean environmental data with standard errors for sampling sites in the northwest area of Chandeleur Islands, Louisiana, during September 2019.

	Depth (m)	**Water temperature (°C)**	**Salinity**	**Dissolved oxygen (mg/L)**	**pH**
Chandeleur Islands N=20	1.2 (SE=0.1)	28.01 (SE=0.2)	24.3 (SE=1.1)	7.9 (SE=0.4)	8.5 (SE=0.02)

3.2 Negative Controls

Most of the negative controls had <2.0 nanogram (ng)/µL concentration of DNA, which is the limit of reliable readings on the Nanodrop. The average DNA concentration was -3.6 ng/µL (SE=3.7). DNA from *P. pectinata* was not detected in any of the negative controls when using the qPCR platform. This is evidenced by none of the negative controls meeting either of the two criteria for positive detection. The collection and first extraction negative control for day one of sampling met two out of three of the required ddPCR™ criteria. The second ddPCR™ negative met two out of the three criteria as well (Appendix A).

3.3 eDNA Field Surveys

The average DNA concentration for samples collected during eDNA field surveys was 36.2 ng/µL (SE=3.5). No eDNA field samples met the criteria for a positive detection using qPCR (Figure 5) or ddPCR™ (Figure 6). None of the replicates for any samples met either criteria for a positive detection with qPCR (Appendix B). However, three samples from day two of collection met two out of three of the criteria for positive detection with ddPCR™ (Appendix C). None of the day one samples met the ddPCR™ criteria (Appendix C). The closest samples were 1.6 km from each other, while the samples farthest apart were 7.2 km from each other (Figure 7). These three samples had DNA concentrations ranging from 24.1 to 49.5 ng/µL.

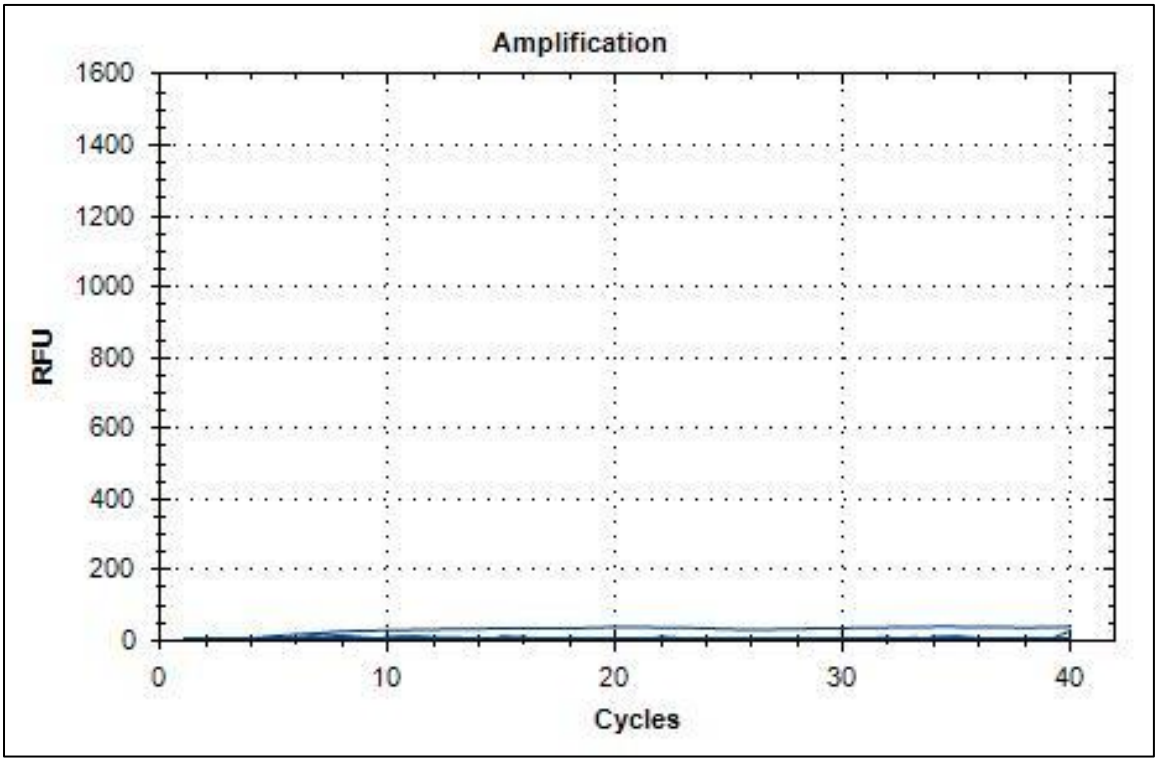

Figure 5: Quantitative PCR analysis completed on field samples from the Chandeleur Islands, Louisiana. While this figure does not contain all field samples analyzed via

qPCR, it represents how all of the field samples appeared on qPCR. For samples to be positive for smalltooth sawfish, Pristis pectinata, DNA, logarithmic amplification beginning at 20 cycles and amplification between 1,200 – 1,600 relative florescence units (RFUs) is needed. However, no field samples met either of these requirements for positive detection. No samples met the requirements for positive smalltooth sawfish detection.

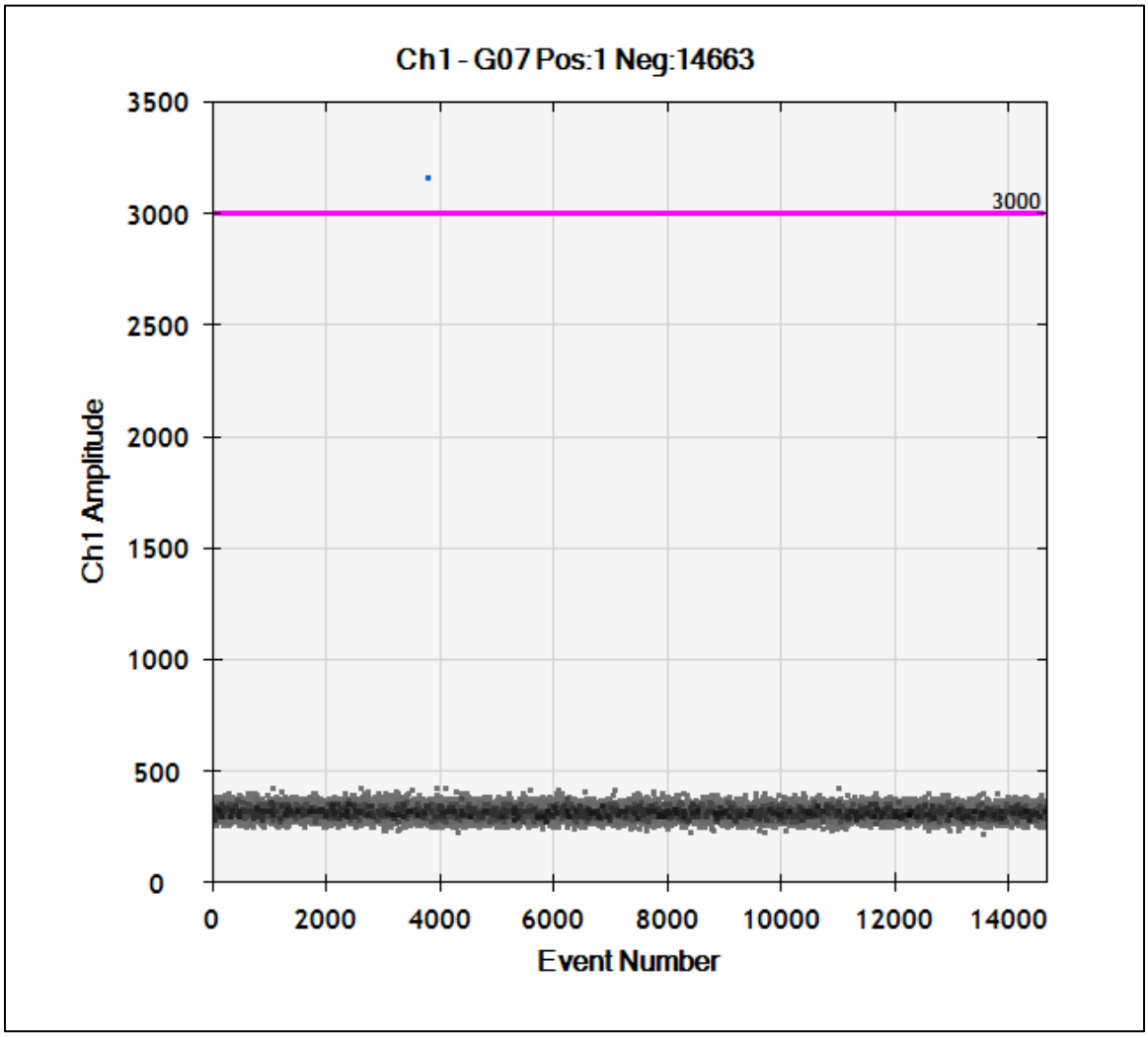

Figure 6: Environmental DNA sample replicate that meets two of the three criteria for positive smalltooth sawfish, Pristis pectinata, on Droplet Digital™ PCR detection.

Droplet amplitude is measured in relative florescence units (RFUs), and event number is the droplets created. The droplet amplification is greater than the manual threshold of 3,000 RFUs and falls within the normal droplet range for positive samples. The concentration of target DNA was 0.08 copies/µL. However, this replicate did not meet the third criterion of the 5,000-7,000 RFUs range. Figure was created via Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software.

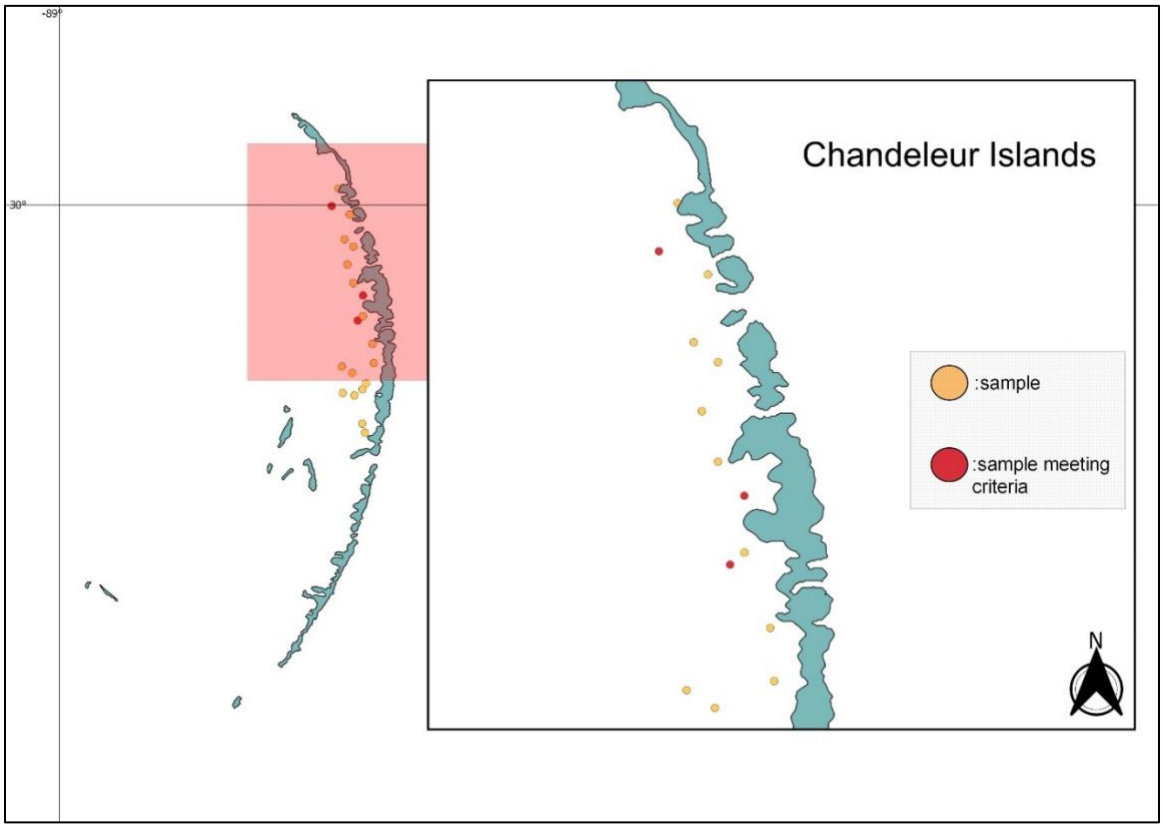

Figure 7: Three samples from day two collection in the Chandeleur Islands, Louisiana, that met two out of three criteria for positive smalltooth sawfish, Pristis pectinata, detection on Droplet Digital™ PCR. The red points are the samples that met two out of three criteria, while the orange points met no criteria. The red points on the

southernmost end were located 1.6 kilometers from each other. The northernmost point and southernmost point were located 7.2 kilometers from each other.

CHAPTER IV: DISCUSSION

The eDNA surveys in the Chandeleur Islands, Louisiana did not provide evidence of *P. pectinata* DNA from water samples collected in September 2019. This suggests that sawfish were not present in the vicinity of the collection sites during sample collection. The abiotic conditions on the days the water samples were collected suggest the habitat was suitable for *P. pectinata*. The depths sampled and the salinities, temperatures, and DO levels were all within the affinity range for *P. pectinata* (Poulakis et al., 2011; Brame et al., 2019). Further, the bottom type where samples were collected consisted of the turtle grass *T. testudinum,* which *P. pectinata* have been historically associated with (Poulakis & Seitz, 2004), and there were black mangroves, *A. germinans,* in the vicinity of the sites sampled.

In the past 10 years, three sawfish encounters have been reported by the public in the Chandeleur Islands, suggesting *P. pectinata* do occasionally occur in these waters. These sawfish encounters consisted of one adult and two YOY sawfish, based on the total length, and they were reported in spring, summer, and fall months (Figure 1B and Figure 1C). The report of one adult and one juvenile *P. pectinata* was further south by ~6 km and ~11.5 km, respectively, from where the water samples were opportunistically collected in this study; the northern YOY was ~5 km from the nearest sampled area (Figure 8). The southern YOY was sighted in fall, while the northern YOY was sighted in spring. Temporally, the YOY sightings were not aligned with the samples opportunistically collected from the Chandeleur Islands. However, the adult was sighted during summer, and these samples were collected in late summer. Spatially, all *P. pectinata* sightings were located much further north and south than the area sampled.

Sawfish may be present in the area but could have been left undetected due to seasonal occurrence. Therefore, future eDNA surveys would benefit from water sample collection in fall, summer, and spring months, and the collection sites should include the northernmost and southernmost ends on the Chandeleur Islands.

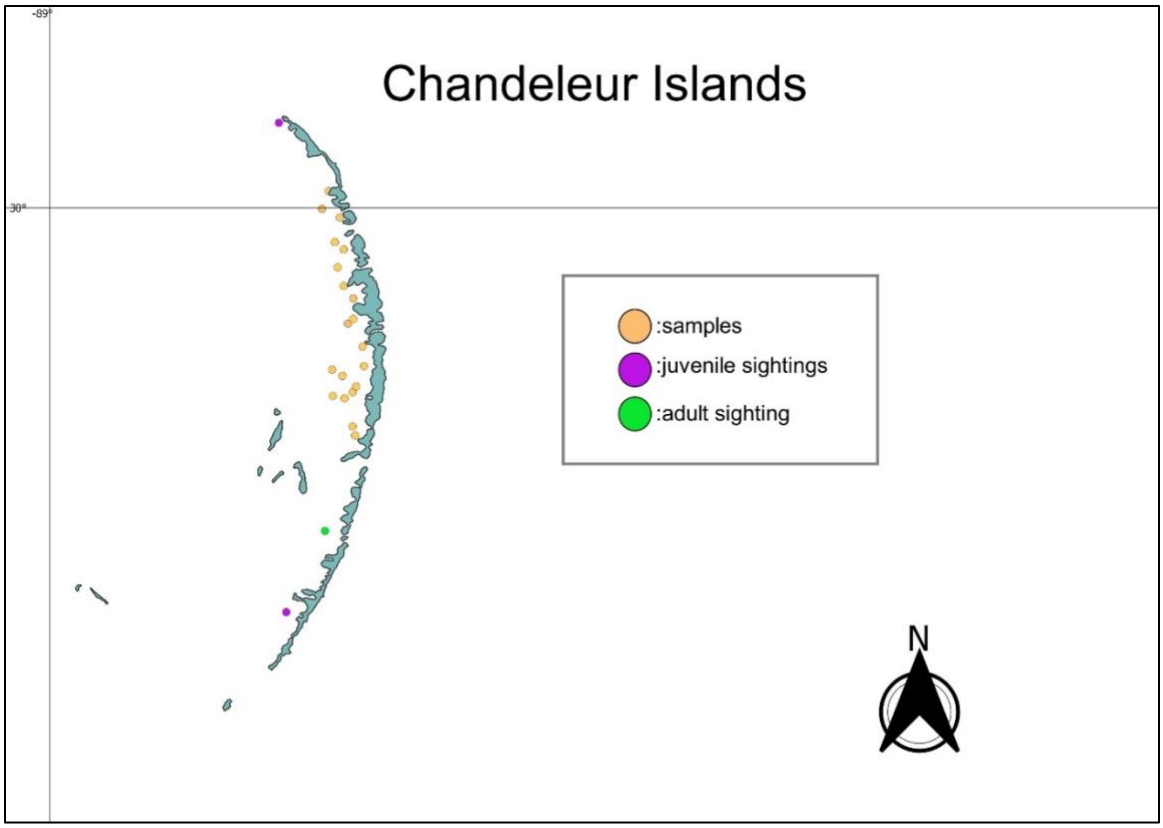

Figure 8: Combined map of opportunistically collected water samples from this study in the Chandeleur Islands, Louisiana, and the reported smalltooth sawfish, Pristis pectinata sightings in the same area from 2009-2019. The northernmost juvenile was located ~5 kilometers (km) from the closest site sampled. The sighted adult was located ~6 km from the sample area, and the southernmost juvenile was located ~11.5 km from the sample area. Sighting data from NMFS (2019, unpublished data).

Different variables affect dispersal and degradation as eDNA is dispersed into the water column. Once shed by an organism, eDNA does not spread out evenly. It can also be pushed by currents or settle on the ground sediment (Shogren et al., 2017). Environmental DNA degradation occurs 1.6 times faster in estuarine waters than coastal waters (Collins et al., 2018). Temperature, salinity, and pH are steadier in the marine waters than freshwater or nearshore coastal waters, which contributes to a slower eDNA degradation rate in marine environments (Collins et al., 2018). Salinities >27 tend to preserve eDNA, while temperatures >20°C may degrade *P. pectinata* eDNA within ~48 hours (Collins et al., 2018). Therefore, sawfish may have present or nearby the islands, but their DNA was not detected as *P. pectinata* may have been too far away from the northwest site, leading to eDNA decay by the time the site was sampled. Additional eDNA surveys should be conducted across the Chandeleur Islands, and include sampling sites near the locations where sawfish encounters have been reported as well as areas of optimal habitat, such as those habitats with *A. germinans* and *T. testudinum* (Poirrier & Handley, 1940; Scheffel et al., 2018). Such eDNA surveys should also be conducted on multiple days from the spring through fall when *P. pectinata* have been encountered in the Chandeleur Islands (see Figure 1C).

Sawfish may not have been detected in the samples due to the amount of filter extracted, gene targeted, and water volume collected. As only one half of each filter was extracted for DNA, sampling error could have resulted from solely analyzing part of the filter and the extracted DNA. The whole filter was not utilized and extracted from the start of the experiment in case the issue of contamination arose, and DNA needed to be reextracted. A single gene (ND2) was targeted on qPCR and ddPCR™, and other *P.*

pectinata genes may have been present in the Chandeleur Islands samples but were left undetected due to the locus screened. Also, only 3 L of water was collected per sample. While collecting a larger volume of water could have resulted in more eDNA being captured (Sepulveda et al., 2020), this would have increased the amount of time spent filtering, so fewer sites would have been sampled. The resulting tradeoff for this project was a smaller volume of water for more sites sampled.

Contamination in eDNA studies warrants concern, and its occurrence is underreported in the literature (Sepulveda et al., 2020). Although five of the water samples met two of the three criteria for a positive detection of *P. pectinata* eDNA, evidence of contamination was present. These samples that met only two criteria may reflect cross-contamination, either during DNA extraction, or PCR amplification. No contamination was detected on the qPCR platform, but the ddPCR™ detected contamination in three negative controls. When analyzed on the ddPCR™ platform, the collection and extraction negatives from the first day of sampling met two of the three criteria for a positive detection, and the PCR negative for the second day of sampling met two of the criteria. This difference in the ability to detect contamination in eDNA studies stems from the relative sensitivities of the qPCR and ddPCR™. Droplet Digital™ PCR reactions are partitioned into 10,0000-20,000 nanodroplets, and the PCR reaction occurs within each droplet (Doi et al., 2015). This allows for unparalleled precision in detecting and quantifying target DNA among non-target DNA (Hunter et al., 2016), and overall, is a much more sensitive platform when compared to qPCR (see Doi et al., 2015). Therefore, eDNA studies that only use qPCR assays for rare species may not only be

missing positive detection, but they may also be missing evidence of sample contamination that ddPCR™ could otherwise detect.

In a review by Sepulveda et al. (2020), 91% of eDNA studies implemented at least one negative control during the experiment, but many studies did not use negative controls throughout each stage of eDNA sample processing (e.g., water collection, filtration, extraction, PCR). DNA extraction negatives were only used in 36% of studies, and only 25% of studies used negatives during water collection in the field (Sepulveda et al., 2020). Incorporating negative controls during the entirety of an eDNA study is critical to identifying and remedying potential sources of contamination. If contamination is detected early on, this could avert wasting resources and prevent the contaminant from amplifying.

Sources of contamination must be mitigated throughout the eDNA process. During sample collection in the field, water capture devices must be thoroughly cleaned with 10% bleach between each sampling. Improper cleaning could result in negative control or sample cross-contamination. During sample filtration, contamination could have transpired via improperly sterilized equipment or when rolling and transferring filters to vials containing ethanol for storage. During extraction, droplet spray may arise when moving filter pieces or using buffers with a thick consistency. When pipetting reagents such as viscous buffers, bubbles may form and pop, spraying DNA onto surfaces and other samples. Contamination could have also occurred via aerosolized DNA when PCR tubes were opened and manipulated (Hebsgaard et al., 2005). Future research should re-analyze the samples in this study to determine if the contamination can be remedied. As only half of each filter was used during extractions, re-extracting the samples with the

remaining half of a filter is possible. As only the ddPCR™ negative was contaminated during day two, re-running the ddPCR™ could resolve the issue. For the day one samples with multiple contaminated negatives, aliquoting fresh stock DNA and repeating the PCRs could remedy the problem.

Positive detection data should not be used when there is evidence of contamination during sample processing. Before this data can be used, it must be reanalyzed and shown to be free of contamination. In this study, false positives were guarded against by incorporating negative controls at each stage of sample processing and a rigorous, three-criteria approach to data analysis. False positives in eDNA surveys of historically occupied habitats, such as the Chandeleur Islands, could erroneously suggest *P. pectinata* is re-occurring in these waters. Signs of recovery of *P. pectinata* evidenced by eDNA surveys could be premature if contamination is present, because such data could be used to partially meet criteria for downlisting or delisting the species. A hasty downlisting or delisting could negatively impact the full recovery of this species.

In conclusion, while sighting reports from 2009 to 2019 in the Chandeleur Islands suggested the presence of *P. pectinata* in this historically occupied habitat, this study's eDNA surveys were not able to detect *P. pectinata* DNA in the water samples. Contamination in various negative controls prevented field samples from being considered positives, as rigorous analyses are needed to protect research on rare species. Reanalysis of this project's samples could be warranted, and future eDNA studies in the Chandeleur Islands would benefit from sampling the northernmost and southernmost areas of the islands in multiple seasons. Non-invasive eDNA surveys are important, as they assist in understanding the recovery of *P. pectinata* to historically occupied habitats.

APPENDIX A: NEGATIVE CONTROL DATA

Table 2: Results of negative controls analyzed via Nanodrop, Quantitative PCR, and Droplet Digital™ PCR. Two extractions were needed for each day's samples. The two qPCR criteria were: 1) logarithmic growth at 20 cycles, 2) amplification between 1,200 – 1,600 relative florescence units (RFUs). The three ddPCR™ criteria were: 1) must reach the manual threshold of 3,000 RFUs, 2) sit between 5,000-7,000 RFUs, and 3) contain a concentration of DNA greater than or equal to 0.08 copies per µL.

Negative Controls	DNA concentrations (ng/µL)	qPCR criteria met (out of 2)	ddPCR™ criteria met (out of 3)
Day 1 collection	-0.1	0	2
Day 1 filtration	11.3	0	0
Day 1 extraction	4.2	0	2
Day 1 second extraction	2.0	0	0
Day 2 collection	-13.2	0	0
Day 2 filtration	-16.8	0	0
Day 2 extraction	-16.5	0	0
Day 2 second extraction	0.5	0	0

APPENDIX B: QUANTITATIVE PCR DATA

Table 3: Results of the Quantitative PCR data, including GPS coordinates, and whether field samples met the two criteria for positive detections: 1) logarithmic growth at 20 cycles, 2) amplification between 1,200 – 1,600 RFUs.

	Latitude	Longitude	Criterion 1: Logarithmic growth at 20 cycles	Criterion 2: 1,200 – 1,600 RFU amplification	Number of criteria met
Day 1: sample 1	29.8771	-88.8348	No	No	0
Day 1: sample 2	29.8822	-88.8363	No	No	0
Day 1: sample 3	29.9005	-88.8362	No	No	0
Day 1: sample 4	29.9036	-88.8343	No	No	0
Day 1: sample 5	29.9093	-88.8417	No	No	0
Day 1: sample 6	29.9146	-88.83	No	No	0
Day 1: sample 7	29.9251	-88.8308	No	No	0
Day 1: sample 8	29.9128	-88.8473	No	No	0

Table 3 (continued).

Day 1: sample 9	29.8985	-88.8469	No	No	0
Day 1: sample 10	29.8972	-88.8405	No	No	0
Day 2: sample 11	29.9376	-88.8387	No	No	0
Day 2: sample 12	29.94	-88.8359	No	No	0
Day 2: sample 13	29.9512	-88.8359	No	No	0
Day 2: sample 14	29.9579	-88.8411	No	No	0
Day 2: sample 15	29.9679	-88.8443	No	No	0
Day 2: sample 16	29.9776	-88.8411	No	No	0
Day 2: sample 17	29.9815	-88.8459	No	No	0
Day 2: sample 18	29.9949	-88.8431	No	No	0

Table 3 (continued):

Day 2: sample 19	29.9995	-88.8528	No	No	0
Day 2: sample 20	30.009	-88.8491	No	No	0

APPENDIX C: DROPLET DIGITAL™ PCR DATA

Table 4: Results of the Droplet Digital™ PCR data, including GPS coordinates, and whether field samples met the three criteria for positive detection: 1) must reach the manual threshold of 3,000 RFUs, 2) sit between 5,000-7,000 RFUs, and 3) contain a concentration of DNA greater than or equal to 0.08 copies per µL.

	Latitude	Longitude	Criterion 1: ≥3,000 RFUs for MT	Criterion 2: 5,000-7,000 RFUs amplification	Criterion 3: ≥ LoD of 0.08 copies/µL	Number of criteria met
Day 1: sample 1	29.8771	-88.8348	No	No	No	0
Day 1: sample 2	29.8822	-88.8363	No	No	No	0
Day 1: sample 3	29.9005	-88.8362	No	No	No	0
Day 1: sample 4	29.9036	-88.8343	No	No	No	0
Day 1: sample 5	29.9093	-88.8417	No	No	No	0
Day 1: sample 6	29.9146	-88.83	No	No	No	0
Day 1: sample 7	29.9251	-88.8308	No	No	No	0
Day 1: sample 8	29.9128	-88.8473	No	No	No	0

Table 4 (continued):

Day 1: sample 9	29.8985	-88.8469	No	No	No	0
Day 1: sample 10	29.8972	-88.8405	No	No	No	0
Day 2: sample 11	29.9376	-88.8387	Yes	No	Yes	2
Day 2: sample 12	29.94	-88.8359	No	No	No	0
Day 2: sample 13	29.9512	-88.8359	Yes	No	Yes	2
Day 2: sample 14	29.9579	-88.8411	No	No	No	0
Day 2: sample 15	29.9679	-88.8443	No	No	No	0
Day 2: sample 16	29.9776	-88.8411	No	No	No	0
Day 2: sample 17	29.9815	-88.8459	No	No	No	0
Day 2: sample 18	29.9949	-88.8431	No	No	No	0

Table 4 (continued:

Day 2: sample 19	29.9995	-88.8528	Yes	No	Yes	2
Day 2: sample 20	30.009	-88.8491	No	No	No	0

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