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Development and Use of an Environmental DNA Tool to Monitor Recovery of the Critically Endangered Smalltooth Sawfish, Pristis pectinata, in the Northwest Atlantic

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DEVELOPMENT AND USE OF AN ENVIRONMENTAL DNA TOOL TO MONITOR RECOVERY OF THE CRITICALLY ENDANGERED SMALLTOOTH SAWFISH, *PRISTIS PECTINATA*, IN THE NORTHWEST ATLANTIC

by

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ABSTRACT

The Critically Endangered Smalltooth Sawfish, *Pristis pectinata*, was once common in the tropical and subtropical waters of the Atlantic Ocean; however, following global declines in range and abundance over the past century, individuals were restricted to the waters of south and southwest Florida (SWFL) by about the 1980's. Recently, public encounter reports have emerged in historically occupied habitats in United States waters, suggesting individuals are present in, or re-occupying, these areas, although the status of *P. pectinata* outside of SWFL is not currently well understood. Targeted environmental DNA (eDNA) surveys were chosen to assess the occurrence of *P. pectinata* in these formerly inhabited waters due to the rapid, cost-effective, and non-invasive advantages of this technique over traditional survey methods for *P. pectinata* like gill nets. This research developed and validated a species-specific eDNA assay capable of targeting *P. pectinata* DNA in water samples at concentrations as low as 0.08 copies/μL using Droplet Digital™ PCR. This assay was then used in three formerly occupied estuaries in the western Atlantic that had recent, verified encounter reports: Tampa Bay and the Indian River Lagoon (IRL), Florida, and the Mississippi Sound, Mississippi. *Pristis pectinata* DNA was detected in water samples collected from the IRL in 2018 and 2019, and the Mississippi Sound in 2018, indicating at least one individual was present in the vicinity near the time of sample collection. These results provide another line of evidence for potential re-occupation; however, long-term, comprehensive eDNA surveys are needed to help foster future recovery.

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LIST OF ABBREVIATIONS

12S	12S Ribosomal RNA
bp	base pair
BLAST	Basic Local Alignment Search Tool
BRUVs	baited remote underwater video stations
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CMS	Convention on the Conservation of Migratory Species of Wild Animals
CO1	Cytochrome c Oxidase I
CO_2	Carbon Dioxide
CytB	Cytochrome B
ddPCR™	Droplet Digital™ polymerase chain reaction
DNA	Deoxyribonucleic acid
DO	dissolved oxygen
DI	Deionized water
dUTP	deoxyuridine triphosphate
eDNA	environmental DNA
EPBC	Environment Protection and Biodiversity Conservation Act
ESA	Endangered Species Act
FL	Florida
gDNA	genomic DNA

IDeA	Institutional Development Award
INBRE	Institutional Network of Biomedical Research Excellence
IRL	Indian River Lagoon
IUCN	International Union for Conservation of Nature
LA	Louisiana
LA-ICP-MS	laser ablation inductively coupled mass spectrometry
LEK	local ecological knowledge
LoD	limit of detection
MS	Mississippi
MT	manual threshold
mtDNA	mitochondrial DNA
NCBI	National Center for Biotechnology Information
ND2	NADH dehydrogenase 2
ND4	NADH dehydrogenase 4
NGS	Next Generation Sequencing
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
PCR	polymerase chain reaction
PVC	Polyvinyl chloride
qPCR	quantitative polymerase chain reaction
RED	rare event detection

RFUs	Relative Florescence Units
SRP	Species Recovery Plan
SWFL	southwest Florida
STL	stretch total length
TEK	traditional ecological knowledge
TX	Texas
UMMC	University of Mississippi Medical Center
USA	United States of America
USM	The University of Southern Mississippi
UV	ultraviolet
UVC	underwater visual census

CHAPTER I – INTRODUCTION AND LITERATURE REVIEW

1.1 The Sawfishes

Sawfishes (Pristidae) are large-bodied, benthic rays currently comprised of four species in the genus *Pristis*, and one species in the genus *Anoxypristis* (Faria *et al*. 2013). They are identifiable by their toothed rostrum, or saw, which is used for feeding and defense (Breder 1952; Wueringer *et al*. 2012). Sawfishes are among the most threatened marine fishes in the world (Dulvy *et al*. 2014), with all five species listed on the International Union for the Conservation of Nature's (IUCN) Red List of Threatened Species as either Critically Endangered or Endangered (see Dulvy *et al*. 2016). Historically, at least one species of sawfish inhabited the coastal, tropical, and subtropical waters of 90 countries within the Atlantic, Indian, or Pacific oceans, with only one circumtropically distributed species (Kyne *et al*. 2013); however, all species have undergone declines in range and abundance within the past century (Dulvy *et al*. 2016).

Declines in range and abundance of sawfishes are due to anthropogenic threats such as direct exploitation, bycatch in fisheries, and habitat alterations or destruction (Dulvy *et al*. 2016). The nature and severity of these threats vary spatially by geographic region, but are typically intensified in coastal areas (*e.g*., Seitz & Poulakis 2006) due to proximity to human activities (Dulvy *et al*. 2016). Bycatch mortality in commercial, recreational, and artisanal fisheries due to rostral entanglement is considered the top driver of the decline of sawfishes worldwide (Seitz & Poulakis 2006; Simpfendorfer 2014; Whitty *et al*. 2014; Dulvy *et al*. 2016). However, direct exploitation of sawfishes for their derivatives (*e.g*., fins, meat, and rostra) has also occurred historically in commercial fisheries, such as the Lake Nicaragua sawfish fishery that depleted a

Largetooth Sawfish, *Pristis pristis*, population in the late 20th century (Thorson 1982a, b), and through take in traditional artisanal fisheries (Simpfendorfer 2014). The majority of bycatch mortalities were intended to prevent sawfishes from injuring anglers or damaging fishing nets after becoming entangled (see Henshall 1895; Poulakis & Seitz 2004). However, the high value of sawfish parts in Asian markets for use in fin soups, ancient medicines, and as curios, prompts recreational and artisanal fishers to still retain captured sawfishes today, particularly in the Indo-Pacific (McDavitt 2014; Dulvy *et al*. 2014, 2016). Past and present modifications (commercial and private) and loss of nearshore habitats, have negatively affected all five species of sawfish (Seitz & Poulakis 2006; Norton *et al*. 2012; Dulvy *et al.* 2016). These developments are often accompanied by agricultural and industrial pollution, and ongoing fragmentation of critical nursery habitat for sawfishes (*e.g*., mangroves; see Section 1.2.3) that reduce habitat quality (Dulvy *et al.* 2016). Threats of accidental bycatch and habitat changes are exacerbated by sawfish life history traits (*e.g*., late maturity, low fecundity, and long life spans; see Section 1.2.2), which leave them susceptible to overexploitation, and makes population recovery a slow process (Stevens *et al*. 2000; Carlson & Simpfendorfer 2015).

Remaining viable populations of sawfishes are thought to be restricted to two global strongholds: the waters of the United States (U.S.) and northern Australia (Dulvy *et al*. 2016). Northern Australia has been identified as the global stronghold for viable populations of four of the five species of sawfishes, including the Narrow Sawfish, *Anoxypristis cuspidata* Latham 1794, the Green Sawfish, *Pristis zijsron* Bleeker 1851, the Dwarf Sawfish, *Pristis clavata* Garman 1906, and the Largetooth Sawfish, *Pristis pristis* (Linnaeus 1758) (D'Anastasi *et al*. 2013; Kyne *et al*. 2013; Simpfendorfer 2013).

South and southwest Florida (SWFL) and the Bahamas are believed to contain the remaining viable population(s) of the Smalltooth Sawfish, *Pristis pectinata* Latham 1794 (Carlson *et al*. 2013). Sawfishes may still be present outside of these strongholds (Dulvy *et al*. 2016), according to recent research and public encounter reports. The frequency of reports imply potentially one or more species may still occur at low levels within areas in proximity to the strongholds (*e.g*., northern Australia: Papa New Guinea and Bangladesh; SWFL: the Bahamas; see Dulvy *et al*. 2016; White *et al*. 2017; Poulakis & Grubbs 2019); however, the status, connectivity to stronghold populations, and viability of these reported populations is not well understood.

The severity of the declines that led to sawfishes being primarily restricted to two strongholds prompted international conservation action: trade of sawfishes and their derivatives became prohibited globally by 2013 via their listing under Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and in 2014 all five species were listed in Appendices I & II of Annex 1 of the Convention on the Conservation of Migratory Species of Wild Animals (CMS). Both Australia and the U.S. offer national protections for sawfishes (*i.e*., Environment Protection and Biodiversity Conservation (EPBC) Act of 1999 in Australia and Endangered Species Act (ESA) of 1973 in the U.S.); however, national legislation concerning the conservation of sawfishes outside of these strongholds varies greatly by country, and does not cover the total estimated range of all sawfish species (Dulvy *et al*. 2016).

1.2 The Smalltooth Sawfish, *Pristis pectinata*

1.2.1 Morphology and Distribution

Pristis pectinata reaches up to ~500 cm stretch total length (STL; from the tip of the rostrum to the tip of the upper lobe of the extended caudal fin; Brame *et al*. 2019), with the first dorsal fin origin in line with the pelvic fin origin (Faria *et al*. 2013), long, narrow pectoral fins (Compagno & Last 1999), and a small or non-existent lower caudal fin lobe (Bigelow & Schroder 1953). Depending on ontogeny, the rostrum can encompass up to 20–25% of the STL, and each individual has 21–30 fixed, non-replaceable teeth on each side of their saw (Bigelow & Schroeder 1953; Slaughter & Springer 1968; G. R. Poulakis unpubl. data). Fin placement, STL, and rostral tooth counts are commonly used to differentiate *P*. *pectinata* from other sawfish species, although it should be noted that a suite of morphometric measurements was found to be a more accurate method of identification for other sawfish species than tooth counts alone (Faria *et al*. 2013; Whitty *et al*. 2014).

Historically, *P*. *pectinata* inhabited the coastal waters of the eastern Atlantic from Angola to Mauritania (Carlson *et al*. 2013), the western Atlantic from Brazil to the U.S., and throughout the Caribbean (Bigelow & Schroder 1953) (Fig. 1.1A). As the threats to sawfishes grew in severity over the 20th century, evidence of declines in *P*. *pectinata* populations emerged in both the eastern and western Atlantic during the 1970's (Tamburello *et al*. 2014). A poor historical data repository from the eastern Atlantic prevents reconstruction of a finite timeline for declines; however, viable populations were considered extirpated from most African countries by the 1990's, although individuals may still be present from Guinea-Bissau to Liberia (Tamburello *et al*. 2014) (Fig 1.1C).

In the western Atlantic, populations were considered extirpated from all areas except the U.S. and several countries of the West Indies (*e.g.*, Bahamas, Cuba, Honduras, and Belize) by the late 1980's (Fig. 1.1B). Southwest Florida and the Bahamas are believed to retain the remaining viable populations for this species; however, recent research concerning habitat use (see Section 1.2.3) suggests individuals are not moving to or from either area, and Bahamian waters have not been extensively surveyed to date, making the U.S. population(s) of global conservation significance.

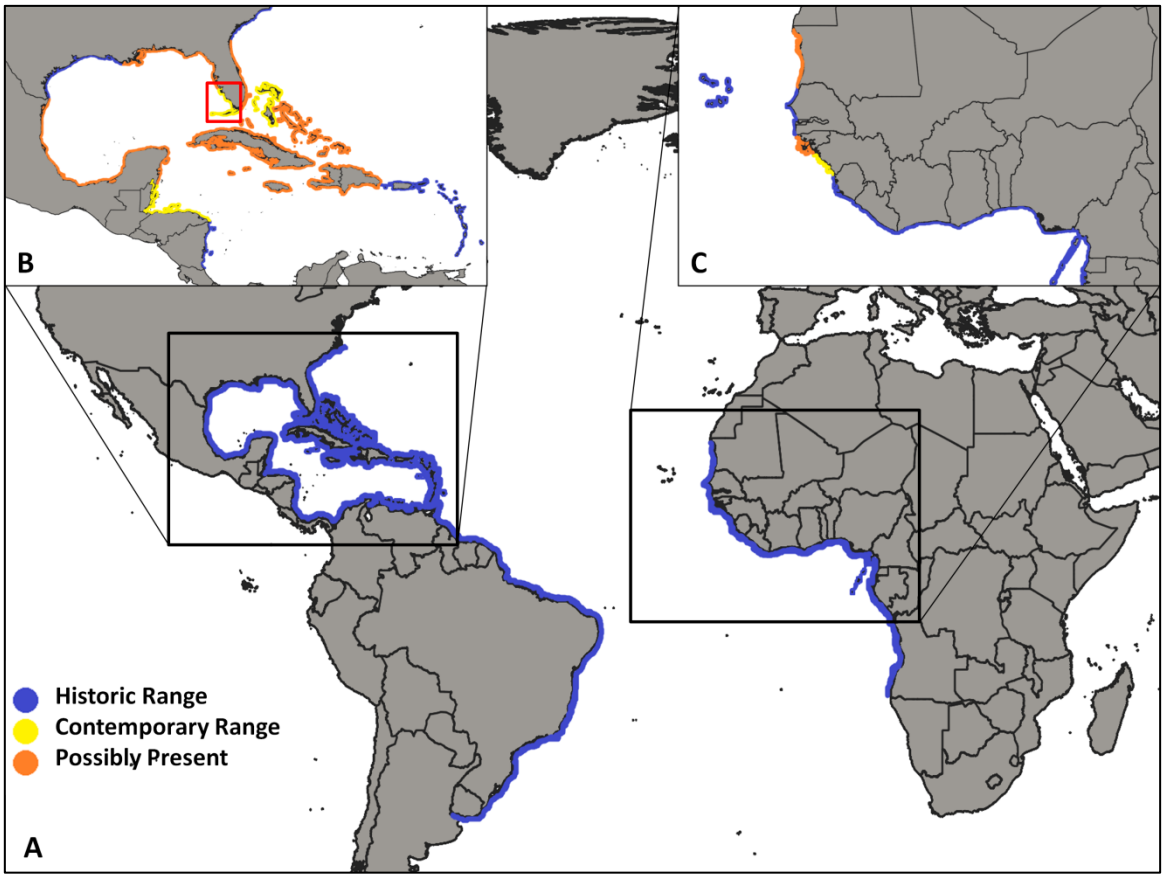

Figure 1.1 Pristis pectinata *global distribution*

Global historical distribution of the Smalltooth Sawfish, *Pristis pectinata* (A), with inserts depicting areas of interest in the northwest Atlantic (B) and the eastern Atlantic (C). Historic distribution is identified in blue, contemporary distribution in yellow, and areas where *P. pectinata* are possibly present in orange. The current core range is specified with a red box. Map modified from Dulvy *et al.* (2016).

In U.S. waters, *P. pectinata* was historically reported from New York to Texas; however, it remains unknown whether individuals farther north of Florida (*e.g.*, Virginia to New York) were vagrants (*i.e.*, "an individual found outside of the known distribution of its species, with no apparent biological context"; Grant *et al.* 2019) or seasonal migrants of a historic population (Wiley & Simpfendorfer 2010; Waters *et al.* 2014; Brame *et al.* 2019). Based on the small number of records above the Carolinas, the lack

of suitable habitat, and current knowledge of habitat use (see Section 1.2.3), it is likely that *P. pectinata* were only historically common from Texas to the Carolinas in U.S. waters (Brame *et al*. 2019). As populations declined in the mid 1900's, fewer encounters were reported in surveys outside of SWFL (*e.g*., Snelson & Williams 1981), and viable populations were restricted to south and SWFL by the 1980's (Poulakis *et al*. 2011) (Fig. 1.2). Hereafter, south and SWFL are referred to as the "core" population or range, and historically occupied areas outside of this portion of U.S. waters are referred to as "non-core". The higher abundance of reports from south and SWFL, combined with research concerning temperature tolerances (see Poulakis *et al*. 2011; Scharer *et al*. 2017) collected after their listing in 2003 as Endangered on the ESA of 1973, could suggest that southern Florida was also the historic core of *P. pectinata* in U.S. waters (Brame *et al*. 2019). Recent encounters between recreational anglers and *P. pectinata* have been reported sporadically in non-core habitats of their historic range, with marginally higher frequency on the Gulf of Mexico side (Wiley & Simpfendorfer 2010; Waters *et al*. 2014). Specifically, encounters have occurred in many areas of the Indian River Lagoon and Tampa Bay, FL, in the waters near Cedar Key, Apalachicola, and Pensacola, FL, Mississippi Sound, Mississippi (MS), and the Chandeleur Islands, Louisiana (LA) (National Sawfish Encounter Database, NMFS 2019, unpubl. data). However, as an indirect means of assessing species occurrence, encounter reports are unlikely to represent a complete understanding of this species' contemporary range and abundance due to an uneven distribution of public participants, signage, and public outreach (Wiley & Simpfendorfer 2010).

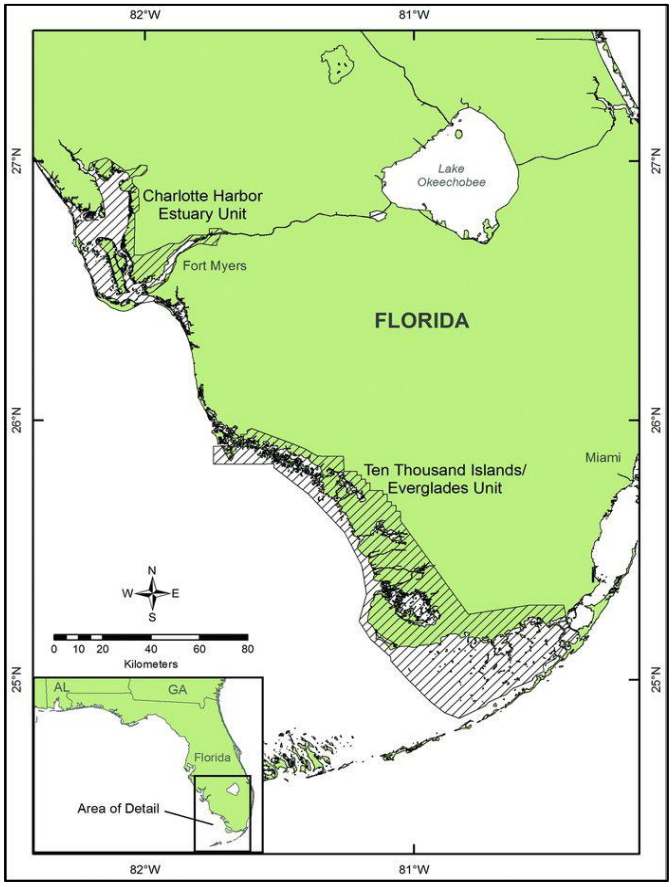

Figure 1.2 Pristis pectinata *core range*

A map of the core range and two critical habitat units for the Smalltooth Sawfish, *Pristis pectinata* (from Norton *et al*. 2012). Thatched areas represent critical habitat units for juveniles (as defined by the ESA), with their combined area and the space between comprising the contemporary core range.

1.2.2 Life History and Biology

Almost all of what is currently known about the biology and ecology (see Section 1.2.3) of *P. pectinata* was gained after its listing on the ESA in 2003 (Carlson *et al*. 2013) from the core population in two critical habitat units designated for juveniles following the Heupel *et al*. (2007) elasmobranch nursery guidelines: the Charlotte Harbor Estuary, and the Ten Thousand Islands/Florida Bay within the Everglades National Park (Norton *et al*. 2012) (Fig. 2). The dearth of data prior to 2003 is likely due to a historically low

demand in U.S. markets for commercialized *P. pectinata* products (Bigelow & Schroeder 1953), poor or non-existent bycatch monitoring programs, and a lack of available research funding (Poulakis *et al*. 2011). *Pristis pectinata* is considered a K-selected species, with a life history strategy characterized by low fecundity, slow growth, late maturation, and a long lifespan (Brame *et al*. 2019). Satellite tagging data showing sexual segregation of groups of *P. pectinata* in the spring and summer suggests copulation takes place during these seasons (Papastamatiou *et al*. 2015); however, due to a small sample size, no distinct breeding areas have been defined. Females are thought to have a biennial reproductive cycle, based on the reconstruction of parental genotypes from inferred siblings using 15 microsatellite loci (Feldheim *et al*. 2017). Gestation is thought to last approximately one year, and timeline estimates for both gestation and copulation are further supported by visual observations of mating wounds (Papastamatiou *et al*. 2015; Feldheim *et al*. 2017; Brame *et al*. 2019). The time of parturition is variable between the Charlotte Harbor (November–July) and Ten Thousand Islands (year-round except September) critical habitat units (Poulakis *et al*. 2011; Brame *et al*. 2019). Although the cause of this variation is not known, parturition peaks in both areas during the spring, further supporting one year of gestation after mating (see Poulakis *et al*. 2011; Brame *et al*. 2019). Embryos are aplacental viviparous, receiving nourishment from yolk rather than a placental connection to the mother, and after gestation, young are born alive in estuarine waters (Brame *et al*. 2019). The current estimate of brood size is 7–14, based primarily on the number of developing follicles observed during opportunistic necropsies (Brame *et al*. 2019). The exact brood size range is unknown; however, Feldheim *et al*. (2017) made a conservative estimate of eight, based on the highest number of pups

assigned to a single mother via reconstructed parental genotypes of inferred siblings. Additional genetic data generated for 190 individuals at 16 microsatellite loci suggests that parthenogenesis (*i.e.*, asexual reproduction) is possible in wild *P. pectinata*, based on the measure of internal relatedness from reconstructed parentage of juveniles (Fields *et al*. 2015).

In SWFL, the size at birth of *P. pectinata* ranges from 64 to 81 cm STL (Brame *et al*. 2019). In one of the first studies to examine growth rates, Simpfendorfer *et al.* (2008) compiled length-frequency and tag recapture data from multiple scientific sources and reported rapid growth from birth until individuals reach approximately 200 cm STL, with a von Bertalanffy growth coefficient of k= 0.14 y^{-1} where k equals the average rate of growth per year for an individual to reach its maximum size. When these data are compared to growth rates of other elasmobranchs (*e.g.*, see Cailliet & Goldman 2004), and other sawfishes (*e.g.*, k=0.06 $y^{-1}$for *P. pristis*; Tanaka 1991, k=0.10 y^{-1} for *P. clavata*, k=0.02 y^{-1} for *P. zijsron*, and k=0.27 y^{-1} for *A. cuspidata*; see Dulvy *et al*. 2016), *P. pectinata* is one of the fastest growing species studied to date. Scharer *et al*. (2012) expanded on *P. pectinata* growth rate research using vertebral band counts detected with laser ablation inductively coupled mass spectrometry (LA-ICP-MS) from opportunistically collected *P. pectinata* and found growth rates nearly double those of Simpfendorfer *et al*. (2008), determined with a von Bertalanffy growth coefficient of k=0.219 y^{-1}. The difference in reported growth rates between these studies may stem from variations in methodologies and a smaller sample size in Scharer *et al*. (2012). Sawfish growth rates are thought to slow as sexual maturity is approached; however, because the growth rates determined in these studies are largely based on data from

smaller individuals (*e.g.*, <300 cm STL) a data gap exists for large juveniles and adults (Simpfendorfer *et al*. 2008; Scharer *et al*. 2012).

Growth rates from Simpendorfer *et al*. (2008) and Scharer *et al*. (2012) combined with morphometric data from Simpfendorfer *et al*. (2005) have been used to estimate the size and age of maturity and the maximum lifespan for *P*. *pectinata* (Carlson & Simpfendorfer 2015). Size at maturity is thought be sexually dimorphic (>370 for females, >340 for males, cm STL; Brame *et al*. 2019). The von Bertalanffy estimates of length at maturity from Scharer *et al*. (2012) were used by Carlson & Simpfendorfer (2015) to extrapolate age at maturity as 7–11 years. Because the majority of samples and data used in these studies were comprised of juveniles, the exact ranges of size(s) and age(s) of sexual maturity for males and females remain unknown. Bigelow & Schroeder (1953) was the first study to estimate the maximum size adults can reach (≥550 cm STL); however, this value was likely overestimated due to inconsistencies in the sources of data. The largest individuals captured in any scientific research since the listing of *P*. *pectinata* on the ESA are approximately 500 cm STL, which is the currently accepted estimate of maximum size (Brame *et al*. 2019). The lifespan of *P*. *pectinata* is also unknown but using vertebral band count data from the oldest study organism in Scharer *et al*. (2012), Carlson & Simpfendorfer (2015) extrapolated a longevity of approximately 30 years in the wild (Brame *et al*. 2019). Using biological data primarily from juveniles may underestimate longevity (Simpfendorfer *et al*. 2008; Scharer *et al*. 2012), especially when compared to other sawfishes, which are reported to have similar, if not longer, life spans (see Table 2 in Dulvy *et al*. 2016).

1.2.3 Ecology and Habitat Use

Within the core range, *P. pectinata* exhibit ontogenetic changes in habitat use (*e.g.*, Wiley & Simpfendorfer 2010; Poulakis *et al*. 2011, 2013; Waters *et al*. 2014). Research regarding habitat use has largely been conducted on small juveniles, because they are relatively easy to encounter and handle in the shallow waters where they reside (Brame *et al*. 2019). Differences in habitat use by juveniles have been documented both between and within nursery habitats. Very small juveniles (*e.g.*, <100 cm STL) show strong associations with waters less than 1 m deep and Red Mangrove, *Rhizophora mangle*, habitat (Poulakis *et al*. 2011), likely to use mangrove prop roots as shelter from predators and for feeding (Lear *et al*. 2019; May *et al*. 2019). Juveniles make use of various nursery "hotspots" (see Poulakis *et al*. 2011) within these areas to escape from predators, hunt for prey, or in response to fluctuations of environmental variables such as temperature, salinity, and freshwater influx (Poulakis *et al*. 2011, 2013, 2016; Scharer *et al*. 2017). Larger juveniles (*i.e.*, 150–220 cm STL) also show association with *R. mangle* habitat, although vegetated areas are no longer used primarily for protection from predators (see Poulakis *et al*. 2011). Larger juveniles also remain associated with shallow waters (Poulakis *et al*. 2011); however, individuals begin to establish diurnal patterns of movement by moving up to five kilometers (km) away from shallow daytime refuge areas into deeper waters (2–3 m) at night to feed (Huston *et al*. 2017; Lear *et al*. 2019; May *et al*. 2019). Juveniles will remain in these shallow waters until reaching ~220 cm STL when they begin using deeper waters between 2–7 m within the estuaries and on the coastal shelf more frequently (Scharer *et al*. 2017).

Information regarding the habitat use of adult *P. pectinata* (>370 for females, >340 for males, cm STL) is limited to studies with small sample sizes, inferred from anecdotal evidence (*i.e.*, encounter reports), or from other sawfishes (see Brame *et al.* 2019). Satellite tagging studies suggest adults are associated with warm, coastal waters <10 m deep (Carlson *et al*. 2014; Guttridge *et al*. 2015), and appear to utilize various "hotspots" (*e.g.*, southern Charlotte Harbor and St. Lucie River Inlet, FL), likely due to habitat suitability, according to liner and additive modeling of encounter data (Waters *et al*. 2014). Although encounter reports are inherently biased by equipment and methodology, the deepest reported encounter suggests adults can reach depths of up to 122 m (Poulakis & Seitz 2004). Bycatch mortalities in commercial fishing trawls on the continental shelf were one of the factors in the decline of *P. pectinata* (see Wiley & Simpfendorfer 2010), which further supports adults using deeper waters; however, additional research on adult habitat use and movement patterns are needed to determine fine scale habitat use.

Electronic tagging data demonstrate that adult *P. pectinata* are capable of long-distance movements of up to an average of 80.2 km while tagged (Carlson *et al*. 2014) and may exhibit site fidelity over small spatial scales (see Papastamatiou *et al*. 2015). These data imply movement and fidelity may be related to sex. Carlson *et al*. (2014) discovered that females move greater distances in the autumn and winter compared to the spring and summer using satellite tags, although the cause of this is unknown. Papastamatiou *et al*. (2015) observed seasonal sexual segregation over a small spatial scale in Florida Bay using a combination of active tracking, acoustic tags, and satellite tags. Small sample sizes (n=12–23) in these studies limits the interpretation of these data;

however, differences in the movement of males and females, and the underlying cause(s) of sexual segregation, have both been linked to environmental factors (*e.g.*, tides, water temperature; Carlson *et al*. 2014; Papastamatiou *et al*. 2015).

Recent public encounter reports of adult *P. pectinata* are similar to historic literature (Bigelow & Schroeder 1953) and imply seasonal migrations northward, out of the core range during the warmer months as water temperatures become more suitable (Brame *et al*. 2019), although no directed studies have been able to confirm this. Potential seasonal migrations northwards have been linked to water temperatures (see Brame *et al*. 2019), and may also be influenced by female philopatry. Evidence of parturition site fidelity, wherein females return to the same areas for parturition each reproductive cycle, has been gathered from the reconstruction of parental genotypes from inferred *P. pectinata* siblings using 15 microsatellite loci (Feldheim *et al*. 2017). If mating takes place outside of suspected nursery habitats (*e.g.*, Florida Bay; see Papastamatiou *et al*. 2015) and females are linked to parturition sites on the northern edge of the core range (*i.e*. Charlotte Harbor), observed migration may be partially influenced via life history. Because data are lacking for the habitat use and movements of adult *P. pectinata*, finding adults of both sexes to conduct research on habitat use and movement patterns is logistically challenging (Carlson *et al*. 2014; Papastamatiou *et al*. 2015); however, the recent use of internal, long-term acoustic tags may help close data gaps (Brame *et al*. 2019).

Although *P. pectinata* show ontogenetic changes in habitat use, their trophic role in estuarine and coastal ecosystems and their prey items largely remain the same throughout each life stage (Poulakis *et al*. 2017). Like all sawfishes, *P. pectinata* use

electrically sensitive ampullae of Lorenzini, found in high concentrations on the ventral side of the rostra, to detect the electrical signals emitted by their prey (Wueringer *et al*. 2011, 2012). According to stable isotope analysis of fin clips, genetic analysis of fecal samples, and opportunistic necropsies, *P*. *pectinata* feed primarily on teleost fish (*e.g*., Engraulidae, Sciaenidae, Elopidae) as juveniles and adults, although predation on elasmobranchs and crustaceans has also been reported (Poulakis *et al*. 2013, 2017; Hancock *et al*. 2019).

1.2.4 Current Status

Recent evidence suggests that *P*. *pectinata* are stabilizing in SWFL, and preliminary data from both core and non-core range areas implies they may potentially be in the early stages of recovery (Brame *et al*. 2019). Within the core range, populations are thought to be genetically healthy, based on measures of allelic richness and observed heterozygosity, with no genetic signature of a genetic bottleneck, based on data at eight microsatellite loci for 137 individuals (Chapman *et al*. 2011). Assuming that the levels of genetic diversity at these eight microsatellite loci reflect genome-wide diversity, this suggests the survival outlook of the U.S. *P*. *pectinata* population(s) is promising, as high levels of genetic diversity allow populations to better resist disease and adapt to environmental changes (Frankham *et al*. 2002; Spielman *et al*. 2004; Chapman *et al.* 2011). Furthermore, the relative abundance of individuals in SWFL may be stable, or possibly increasing, according to encounter reports between anglers and *P*. *pectinata* stimulated by ongoing public outreach (see Waters *et al*. 2014), notwithstanding the caveats of these data (see Section 1.2.1).

In non-core areas of their range in U.S. waters, recent, verified (*i.e*., contains photographic evidence) encounter reports imply *P*. *pectinata* may be re-expanding into historically occupied waters (see Dulvy *et al*. 2016; NMFS 2018). However, due to the spatial bias of public outreach and reporting towards the core range (see Wiley & Simpfendorfer 2010; Waters *et al*. 2014), the status of *P*. *pectinata* outside of the core range, and in other areas of contemporary occurrence (*e.g*., Caribbean; see Section 1.1) remains unclear. Reports in these formerly occupied areas could stem from vagrant individuals or seasonal migrants from the core population, and, therefore, may not represent re-occupation. In contrast, if reports represent members of distinct, local populations, re-occupation is possible; however, any such interpretations are limited by the anecdotal nature of encounters, and the lack of a standardized database and analysis metric for reports. Gathering further information on the status of *P*. *pectinata* populations in non-core U.S. waters by using a combination of traditional survey techniques such as gill nets (see Poulakis *et al*. 2013 and Scharer *et al*. 2017), fisheries monitoring programs (see NMFS 2009, 2018), public encounter reporting (see Wiley & Simpfendorfer 2010 and Waters *et al*. 2014), and outreach will likely be logistically prohibitive due to the uncertainty of where to implement these techniques. These approaches have been shown to be effective in SWFL, where *P*. *pectinata* are encountered more frequently and signage is common; however, they may be less effective in non-core areas, where they occur less frequently (see Mangunson *et al.* 1994; Lewison *et al.* 2004). To effectively direct applicable research and management resources for *P*. *pectinata* in non-core range areas, rapid and effective directed species surveys are required.

1.3 Environmental DNA (eDNA)

1.3.1 Environmental DNA as a Species Detection Tool

Environmental DNA (eDNA) is a technique with the potential to quickly and effectively assess the presence of a species in aquatic habitats (Ficetola *et al*. 2008). DNA (*e.g*., cellular debris, skin cells, blood, feces, urine, gametes) recently shed by organisms can be suspended in an environmental media like water, sand, or soil, which can then be extracted and used to identify the source via genetic assays (Jerde *et al*. 2011; Turner *et al*. 2015). Environmental DNA methods were first applied to aquatic environments to detect the invasive American Bullfrog, *Lithobates catesbeianus*, in the wetlands of France (Ficetola *et al*. 2008) and the method has since grown into an established tool in ecological research (see Goldberg *et al*. 2016). Environmental DNA methods do not require the visualization, capture, or handling of the target species, making it an ideal, non-invasive choice for studies addressing listed species (Rees *et al*. 2014a). These methods also allow rapid surveys of habitats for species with logistically challenging survey windows, such as the Great Crested Newt, *Triturus cristatus* (Thomsen *et al*. 2012a). Protected under European law, currently permitted survey methods (*e.g*., netting, flashlight, egg counts) are often time consuming, and cannot detect the species during annual hibernation; however, in a comparative study, eDNA methods outperformed current survey methods in both speed and accuracy (Rees *et al*. 2014b).

Two broad approaches, metabarcoding or targeted species detections, are used in environmental DNA studies (Rees *et al*. 2014a), largely based on the research question(s) and study species being addressed. Metabarcoding uses Next Generation Sequencing (NGS) technologies to simultaneously identify DNA from multiple taxa in a single

environmental sample (Rees *et al*. 2014a). This approach has been used over multiple studies to assess the biodiversity of several freshwater and marine habitats by targeting numerous taxonomical groups such as amphibians, birds, insects, crustaceans, and fish (Minamoto *et al*. 2012; Thomsen *et al*. 2012a). Metabarcoding has been used to target a wide range of elasmobranch taxa (see Thomsen *et al*. 2016), providing a 44% increase in the detection of shark species compared to baited remote underwater video stations (BRUVs) and underwater visual census (UVC) (Boussarie *et al.* 2018). To detect multiple taxa at once, metabarcoding studies typically use partial or true universal primers (*i.e.*, primers that are not designed to target a single species or genus) (Wood *et al*. 2019). However, universal primers can fail to amplify all DNA isolated from a water sample due to primer bias, preferential amplification, or marker choice. Primer bias occurs when the primer sequences match more closely (*i.e*. one or more base pair (bp) matches) over another target sequence, making them not truly universal. Preferential amplification occurs when DNA of a low copy number is outcompeted by high copy number DNA for reagents in the reaction (Wood *et al*. 2019). Finally, a perfect marker does not exist for amplification of highly variable target sequences across species (see Stat *et al*. 2017). Regardless of the underlying mechanism, failure to amplify DNA can potentially result in false negatives: when the species is present in the study area, but is not detected in genetic assays (Wood *et al*. 2019).

Targeted species detection studies examine the presence or absence of DNA from a single species in an environmental sample (Rees *et al*. 2014a). This approach has been shown to be as sensitive, and sometimes more sensitive, of a method of species detections than well-established traditional techniques (see Rees *et al*. 2014a). Targeted species

eDNA surveys have detected a similar presence of Brook Trout, *Salvelinus fontinalis*, among sampling sites as electrofishing (Evans *et al.* 2017), and detected as many, or more, vertebrate species at study sites than nine traditional survey methods (Thomsen *et al.* 2012b). Targeted detection surveys have also been used to monitor the spread of invasive species such as the Asian Carp (*Hypohthalmichthy*s spp.; Jerde *et al*. 2011), as well as the occurrence of several threatened marine elasmobranchs including the Endangered Maugean Skate, *Zearaja maugeana* (Weltz *et al.* 2017), the Vulnerable Great White Shark, *Carcharodon carcharias* (Lafferty *et al.* 2018), the Vulnerable Chilean Devil Ray, *Mobula tarapacana* (Gargan *et al*. 2017), and the Critically Endangered Largetooth Sawfish, *Pristis pristis* (Simpfendorfer *et al*. 2016). Species-specific primers, primers designed to amplify DNA from only one specific target, are used in targeted species detection studies because they provide a greater affinity for target DNA sequences through 100% bp matches. This allows species-specific primers to bind to DNA that is degraded, or in low concentrations, even amongst high concentrations of non-target DNA (Wilcox *et al*. 2013). Wood *et al*. (2019) compare the sensitivity and specificity of this approach to metabarcoding and report that targeted methods almost double (0.43) the probability of detection in seawater samples than metabarcoding, concluding that this approach is preferred for invasive, rare, or threatened target species.

Species-specific eDNA assays vary in design, but typically target a small fragment (see Jo *et al*. 2017) of mitochondrial DNA (mtDNA) via amplification on a polymerase chain reaction (PCR) platform (see Chapter 2). Targeting short DNA fragments (*e.g.*, ~50–250 bp) may increase the chances of detecting target eDNA (see Goldberg *et al*. 2016; Gargan *et al*. 2017; Jo *et al*. 2017), due to the constant degradation

of eDNA molecules by abiotic and biotic factors (see Section 1.3.2). MtDNA is commonly used in eDNA studies to increase the chances of recovering target DNA in water samples since individuals have a higher abundance in their cells than nuclear DNA (Bogenhagen & Clayton 1974). MtDNA gene choice varies between eDNA studies, primarily due to the rates of mutation across genes, which can vary from conserved across taxa (*e.g.*, 12S Ribosomal RNA (12S), Cytochrome Oxidase 1 (CO1)) to variable within taxa (*e.g.*, Control Region (CR), NADH dehydrogenase subunit 4 (ND4). For example, the high intra-specific variation of the non-coding mtDNA CR (see Zhang & Hewitt 1997) could make it less appropriate for eDNA studies targeting all individuals of the same species over a wide spatial scale (*e.g.*, global), compared to a more conserved protein coding gene like the CO1 marker (see Hwang & Kim 1999). In contrast, conserved genes may not evolve fast enough to allow sufficient specificity from closely related, non-target species. In particular, the molecular evolution of mtDNA in elasmobranchs is slow compared to many other vertebrates (Martin *et al*. 1992; Dudgeon *et al*. 2012), making marker choice (*i.e.*, variable or conserved) within this class challenging. Species detection studies targeting elasmobranchs must choose a target gene for primer development that offers enough variability from other, closely related species, but not so highly polymorphic that it excludes any individuals of the target species. Previous elasmobranch eDNA studies have used mtDNA protein coding genes that are more conserved within species, such as Cytochrome B (Cyt b) (Lafferty *et al.* 2018) and CO1 (Simpfendorfer *et al.* 2016), and genes that are more variable within species to exclude genetically similar taxa, such as NADH dehydrogenase subunit 2 (ND2) (Schweiss *et al.* 2019).

1.3.2 The Ecological Relationship of Environmental DNA Molecules to Detectability

To link to organismal presence in near real-time, eDNA research requires a thorough understanding of how the "ecology" (*i.e.*, origin, state, transport, and fate; see Barnes *et al*. 2016) of eDNA molecules affects their detectability in each study area (Andruszkiewicz *et al*. 2017). Environmental DNA molecules originate from the source either intracellularly (*e.g.*, enclosed) or extracellularly (*e.g.*, "naked" DNA; see Nielsen *et al*. 2007), and a fast release rate can increase detectability (see Sassoubre *et al*. 2016). Release rate is variable between species (Sassoubre *et al*. 2016), individuals (Klymus *et al*. 2015), reproductive status (Spear *et al*. 2015), and life stages (Maruyama *et al*. 2014). Once eDNA is released from the source, molecules can bind with suspended particulate matter in the water column, and mechanical forces such as currents, waves, and tides disperse molecules both vertically and horizontally (Deiner & Altermatt 2014; Turner *et al*. 2015). Vertical transport allows eDNA matter (*e.g.*, feces) to settle to the bottom, and this matter can be re-suspended via local water flow (Turner *et al*. 2015). Settling and re-suspension suggests eDNA studies should sample from areas of the water column based on target species' habitat use (*e.g.*, pelagic vs. benthic; see Chapter 3). Local currents and tides can facilitate horizontal dispersion of eDNA matter, with fast currents (*e.g.*, 3.60 m^3/s) transporting eDNA ~12 km from the source (see Deiner & Altermatt 2014). This suggests dispersion can quickly transport eDNA outside of the study area, or dilute it below detectable concentrations; however, this is dependent on local water flow (see Beng *et al*. 2020). The influence of coastal marine transport will likely be minimal on *P. pectinata* eDNA, as comparative studies have found the quantifiable diversity within eDNA samples to be relatively stable within 100 m of defined study areas nearshore

marine habitats (see Port *et al*. 2016; O'Donnell *et al*. 2017). Furthermore, in marine tidal zones, Kelly *et al*. (2018) report that the most likely fate of eDNA molecules is to degrade due to water quality before they can be dispersed long distances via tides.

In aquatic systems, eDNA molecules begin to degrade immediately after being shed, and *ex situ* research indicates eDNA generally persists longer in cold, dark, alkaline waters with limited microbial activity (see Table 1 in Barnes *et al*. 2016). Estimates of molecular decay vary, and occasionally conflict, between studies, likely due to variations in experimental design and manipulation (*e.g*., univariate vs. co-factorial). However, implications across all studies suggest that it is typically not one ecological factor driving decay rates, but rather a combination of factors that vary per region and season, working in concert to break down eDNA molecules and influence their detectability (see Strickler *et al*. 2015). Environmental DNA is thought to degrade rapidly in natural marine environments (Dell'Anno & Corinaldesi 2004; Thomsen *et al*. 2012b), and the rate of molecular decay (k) is reportedly 1.6 times faster in coastal waters (k=0.029 hr^{-1}±0.03) than in offshore (k=0.019 hr^{-1}±0.02), likely due to freshwater input reducing salinity and increasing microbial activity (Collins *et al*. 2018). These results imply that *P. pectinata* eDNA may only persist in nearshore coastal environments for approximately 48 hours or less after release (Collins *et al*. 2018). In marine systems, the production of exogenous nucleases via microbial action is the most widely implicated factor on eDNA decay rates (see Paul *et al*. 1989; Torti *et al*. 2015; Collins *et al*. 2018). Microbial action can be exacerbated in nearshore waters by acidic conditions mediated by anthropogenic influences (see Strickler *et al*. 2015; Collins *et al*. 2018), such as freshwater runoff. In the absence of freshwater input, it is likely the relative stability seen in the world's oceans as

a result of the buffering (or storage) of atmospheric CO_2 (Middelburg *et al*. 2019), could limit the combined effects of pH and microbial action on eDNA molecules in marine systems unless external processes (*e.g*., ocean acidification) drive acidic conditions. However, the reverse may be true in nearshore waters where freshwater influx is high, causing pH and salinity to drop regularly based on seasonal freshwater inflows. Research indicates higher salinities (*e.g*., >27) have a preservative effect on eDNA molecules and may serve as an indicator of slower decay rates overall (Collins *et al*. 2018), suggesting molecules may degrade faster in certain areas of the historic range (*e.g*., Mississippi Sound, Tampa Bay) where salinity commonly ranges from moderate to low. The majorities of these formerly occupied areas are within nearshore waters rather than open ocean, and, as such, should be subject to an increased decay rate (see Collins *et al*. 2018). Decay rates have been estimated for temperate, inshore marine waters, and no difference was determined between summer (16.9°C) and winter (9.8°C) treatment groups (see Collins *et al*. 2018); however, this conclusion may not be applicable to warmer water habitats. In warmer waters (*e.g*., >20°C), such as those primarily used by *P*. *pectinata*, eDNA decay rates may be greater, based on an *ex situ*, freshwater microcosm study that determined temperatures greater than 20°C significantly increased decay rates (Strickler *et al*. 2015). While temperature is associated with light, ultraviolet (UV) radiation may not strongly influence molecular decay throughout the water column in mid-latitude marine systems (Andruszkiewicz *et al*. 2017). Molecular decay due to UV radiation may be greater in the equatorial latitudes of the historic range as a result of increased exposure and intensity; however, more research is needed to determine this. Taken together, the findings of ecological eDNA research suggest that the habitat preferences of the target

organism are directly linked to the rate of molecular decay, and *P. pectinata* eDNA will likely only be detectable for a short period of time (*e.g.*, ~48 hours) after release in U.S. waters (see Chapter 3).

1.3.3 Using Environmental DNA to Conduct Occurrence Surveys for *P. pectinata*

The advantages of eDNA approaches (*e.g.*, non-invasive, rapid, high sensitivity) over traditional survey methods indicate it could be used as a survey tool to better understand the occurrence of *P. pectinata* outside of its core range in U.S. waters. Given that this study will target an unknown, and likely a low number of *P. pectinata* individuals within non-core areas of their historic range in U.S. waters, a targeted species detection approach that employs a species-specific assay capable of identifying *P. pectinata* DNA among DNA of closely related, co-occurring species (Wilcox *et al.* 2013), is required. The ability of a targeted species detection survey to detect low quantities of target DNA makes it ideal for use in non-core range areas, where individuals are thought to be present, based on local and traditional ecological knowledge (LEK and TEK; see Poulakis & Grubbs 2019) and public encounter reports (Wiley & Simpfendorfer 2010), but their status is unknown. Environmental DNA surveys can build on these anecdotal data by rapidly determining areas of the non-core range where *P. pectinata* are present, providing an updated estimate of their total contemporary occurrence geographically. Data from robust eDNA surveys could then be used to more effectively direct additional field research (Poulakis & Grubbs 2019) and educational initiatives.

1.4 Project Aims

This study aims to develop and use an eDNA tool for the detection of *P. pectinata* DNA in water samples collected from non-core areas of their historic range in U.S. waters. This project will:

1) Develop an eDNA assay to detect *P. pectinata* DNA from water samples, and
2) Validate this assay using a positive water sample in an *ex situ* tank experiment, and
3) Investigate the occurrence of *P. pectinata* in non-core, but historically occupied, areas of their historic range including: Tampa Bay, Florida, the Indian River Lagoon, Florida, and Mississippi Sound, Mississippi.

CHAPTER II – AN ENVIRONMENTAL DNA TOOL FOR MONITORING THE STATUS OF THE CRITICALLY ENDANGERED SMALLTOOTH SAWFISH, *PRISTIS PECTINATA*, IN THE WESTERN ATLANTIC

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2.1 Abstract

The Critically Endangered Smalltooth Sawfish, *Pristis pectinata*, was once widespread in the tropical and subtropical waters of the Atlantic Ocean, but following substantial declines over the past century, the remaining population(s) are currently confined to Florida in the U.S., and the Bahamas. Recent research and verified public encounter reports suggest that the core population in south and southwest Florida may be stabilizing and, potentially expanding into formerly occupied areas of their historic range in the

western Atlantic; however, the status of this species outside of core waters is not well understood. Environmental DNA (eDNA) methods provide a relatively cost effective and rapid assessment tool for monitoring species occurrence in aquatic habitats. Here, we have developed an eDNA tool: a species-specific Droplet Digital™ PCR (ddPCR™) assay targeting a 100-base pair portion of the mitochondrial NADH dehydrogenase subunit 2 gene in *P. pectinata*, with the ability to reliably detect as little as 0.25 pg of target DNA. The assay was validated by analyzing a water sample from an occupied nursery in southwest Florida, which was found to contain an average of 11.54 copies of target DNA/µL (SE = 0.72) in the reaction. The assay was then further tested by placing a juvenile sawfish in an *ex situ* tank and analyzing water samples collected at time intervals. The implementation of this eDNA tool into field surveys will provide additional, reliable data to assess species recovery and aid in prioritizing localities in which to focus new research, conservation, and education initiatives.

2.2 Introduction

Sawfishes (family Pristidae) are among the most threatened families of marine fishes worldwide (Dulvy et al. 2014), with all five species listed as Critically Endangered or Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (see Dulvy et al. 2016). All sawfishes have undergone global declines in range and abundance due to direct exploitation, bycatch in fisheries, and habitat loss (Dulvy et al. 2016). Juveniles are of particular concern as they inhabit coastal waters, including estuaries, mangrove shorelines, and rivers, increasing their exposure to these anthropogenic activities. These threats are exacerbated by their life history traits (e.g., late maturity, low fecundity, and long life spans), which leave sawfishes susceptible to

overexploitation, and makes population recovery a slow process (Stevens et al. 2000; Carlson and Simpfendorfer 2015). The Critically Endangered Smalltooth Sawfish, *Pristis pectinata*, is thought to have experienced the largest global range contraction of all sawfishes and is currently found in less than 20% of its former range (Dulvy et al. 2016). Once widespread in the tropical and subtropical waters of the Atlantic Ocean, remaining population(s) are thought to be limited to Florida in the U.S., and the Bahamas (Carlson et al. 2013), making these population(s) of global conservation significance (Dulvy et al. 2016). In U.S. waters, *P. pectinata* were historically found from Texas to the Carolinas (Brame et al. 2019); but after experiencing reductions in range and abundance over the past century, became restricted to south and southwest Florida (hereafter referred to as the "core" population or range) by the 1980's (Norton et al. 2012).

Due to the dramatic declines in range and abundance, in 2003, *P. pectinata* was listed as Endangered under the U.S. Endangered Species Act of 1973 by the National Marine Fisheries Service (NMFS) (NMFS 2003), and a Species Recovery Plan (SRP) was developed to promote recovery and long-term viability of the species in U.S. waters (NMFS 2009, 2018). One characteristic of a full species recovery is the re-establishment of the species in some or all of their former range (Akçakaya et al. 2018); therefore, the SRP designated 15 recovery regions throughout their historic range in U.S. waters, wherein recovery efforts should be implemented if species presence is confirmed (NMFS 2009, 2018). As a result of over 15 years of U.S. federal and state protections, scientific advances in the understanding of the biology and ecology of the species, and public education initiatives, the core population of *P. pectinata* in southwest Florida is believed to be stabilizing (NMFS 2018). One line of evidence for this potential stabilization is the

emergence of relatively recent sawfish encounter reports in historically occupied portions of their range in U.S. waters (hereafter referred to as the "non-core" population or range), including in SRP designated recovery regions (NMFS 2018); however, the status of *P. pectinata* in these non-core areas is unknown.

Traditional survey methods for monitoring the status of threatened species can be expensive and time-consuming (Lewison et al. 2004). Environmental DNA (eDNA) methods provide a relatively cost effective and rapid assessment tool for monitoring species occurrence in aquatic habitats (Rees et al. 2014; Evans et al. 2017). Water provides a medium for traces of DNA recently shed by organisms (e.g. skin cells, blood, feces, urine), which can be collected and analyzed via genetic assays (Jerde et al. 2011). Environmental DNA has been shown to be as sensitive, and sometimes more sensitive, in rare aquatic species detections compared to survey methods such as electrofishing (Evans et al. 2017), baited remote underwater video systems (BRUVs) and underwater visual censuses (UVCs) (Boussarie et al. 2018), and traditional net surveys (Thomsen et al. 2012b). Environmental DNA methods also negate the need to capture and handle the target species, making it an ideal tool to assess the presence or absence of a threatened species (Rees et al. 2014a), and have been used in targeted, single species studies for a growing number of threatened elasmobranchs, including the Endangered Maugean Skate, *Zearaja maugeana* (Weltz et al. 2017), the Vulnerable Great White Shark, *Carcharodon carcharias* (Lafferty et al. 2018), the Vulnerable Chilean Devil Ray, *Mobula tarapacana* (Gargan et al. 2017), and the Critically Endangered Largetooth Sawfish, *Pristis pristis* (Simpfendorfer et al. 2016).

Here, we developed and validated an eDNA assay to detect *P. pectinata* DNA in water samples, for use as a tool for monitoring their presence and extent of recovery in the western Atlantic. This eDNA assay uses Droplet Digital™ PCR (ddPCR™), which has greater sensitivity and precision when compared to other PCR platforms (see Doi et al. 2015a; Wood et al. 2019), making it the preferred approach for detecting threatened or rare species in eDNA studies. This tool will allow scientists and managers to better understand the status of *P. pectinata* in non-core areas and provide quantitative baseline data from which to measure progress towards recovery. Such data can also aid in further prioritizing recovery regions in which to focus research and education initiatives, playing an important role in adaptive management strategies as *P. pectinata* expands into its former range.

2.3 *Pristis pectinata* eDNA Assay Development

2.3.1 Field and Laboratory Controls

To reduce the risk of contamination by exogenous DNA or cross-contamination between samples, rigorous controls were used throughout all stages of this research (see Ficetola et al. 2016; Goldberg et al. 2016; Port et al. 2016; Schweiss et al. 2019). All water collection bottles and filtering equipment were cleaned prior to each use using a combination of two methods of sterilization; cleaning with 10% bleach followed by either autoclaving at 120°C for 20 min or exposure to UV light for 20 min, depending on the materials. To prevent contamination between the stages of sample processing, water filtration, DNA extractions, and PCR amplifications were conducted in physically isolated laboratories. Furthermore, water samples were filtered in laboratories where contemporary *P. pectinata* tissue had never been present (see Deiner et al. 2015). During

water filtration and DNA extraction, designated sterile forceps were used to handle filters for each water sample and gloves were changed between water samples to prevent cross-contamination (see Pilliod et al. 2013; Goldberg et al. 2016). During DNA extractions and PCR, aerosol barrier pipette tips were used to prevent cross-contamination between samples (Schweiss et al. 2019). Additionally, no positive control DNA templates were included in any PCRs due to the risk of contamination from the positive itself, as per ancient DNA PCR protocols (see Mulligan 2005).

To test for the possibility of contamination, negative control samples were incorporated into water sample collection and each stage of laboratory processing and analyzed through PCR (Jerde et al. 2011; Bakker et al. 2017). To test for contamination during water sample collection, three sterile 1 L Nalgene® bottles filled with autoclaved deionized (DI) water were brought onto the boat and stored on ice until filtration. To test for contamination during filtration, 3 L of autoclaved DI water were filtered and processed through to PCR. Negative controls for DNA extractions contained no particulate matter or filters, and PCR negatives contained no DNA template. Analysis of all negative control samples, using the optimized protocols described below, found no evidence of target DNA across any PCR replicates.

2.3.2 Water Collection, Filtration, and DNA Extraction

For all aspects of this study, 3 L water samples were collected in three sterile, 1 L high-density polyethylene Nalgene® bottles. All water samples were kept on ice in pre-cleaned marine coolers until filtration, which occurred within 24 hours of collection. All water samples were vacuum filtered using Whatman® (Maidstone, United Kingdom) 47 mm 0.8 µm nylon filters, which were replaced when they became clogged every ~350 ml

(e.g. ~9 filters were used for each 3 L water sample). Used filters were rolled and preserved in 95% ethanol at room temperature. Total eDNA was extracted from a ¼ portion of each filter using the QIAGEN® DNeasy® Blood & Tissue Kit (Hilden, Germany) following the Goldberg et al. (2011) protocol incorporating QIAshredder™ spin columns. The qualities of DNA extracts were visualized using 2% agarose gels and the quantities of DNA were assessed using Thermo Fisher Scientific™ NanoDrop™ technology.

2.3.3 Droplet Digital PCR Assay

Primers were designed to amplify a 100-base pair (bp) fragment of the mitochondrial NADH dehydrogenase subunit 2 (mtDNA ND2) gene in *P. pectinata*, but not in those of other elasmobranchs that could co-occur with this species in U.S. waters, or in other *Pristis* sawfishes. To design these primers, mtDNA ND2 sequences for *P. pectinata* (GenBank accession no. KP400584.1) and 17 genetically similar or co-occurring exclusion species were downloaded from GenBank (Online Resource 1) and aligned in CodonCode v. 6.0.2 (CodonCode Corporation, Dedham, U.S.A.). Forward (PpecF: 5'-CTGGTTCACATTGACTCTTAATTTG-3') and reverse (PpecR: 5'-GCTACAGCTTCAGCTCTCCTTC-3') primers (Eurofins Scientific, Luxemburg) and an internal PrimeTime® double-quenched ZEN™/IOWA Black™ FQ probe (Integrated DNA Technologies, Coralville, U.S.A.) labeled with 6-FAM (PpecIBQF: 5'-TACCATAGCCATCATCCCATTATTATTC-3') were designed to amplify DNA in only *P. pectinata* by including bp differences in the primers and the probe in all exclusion species (see Online Resource 1). To initially confirm that the combination of primers and probe amplified the desired locus, quantitative PCR (qPCR) was conducted using a Bio-

Rad® C1000™ Thermal Cycler and total genomic DNA (gDNA) from four *P. pectinata*, in duplicate reactions. Reaction mixtures contained ~25 ng of DNA, 1X Bio-Rad® iTaq™ universal probe supermix, 900 nM of each primer, and 170 nM of probe, adjusted to 22 µL using PCR-grade water. Cycling conditions consisted of enzyme activation at 95°C for 10 min, followed by 40 cycles of: 94°C for 30 s and 64°C for 2 min, followed by enzyme deactivation at 98°C for 10 min, using a ramp rate of 1°C/s. All replicates for all four *P. pectinata* successfully produced amplicons, one of which was sequenced to verify the locus identity. The amplicon was cleaned using a QIAGEN® QIAquick PCR Purification Kit using the manufacturer's protocol, with the exception that all centrifugation steps were conducted at 12,000 rpm for 2 min. Forward and reverse sequences were generated using a BigDye™ Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, U.S.A.) on an Applied Biosystems™ 3730XL DNA Analyzer. A consensus sequence was assembled in CodonCode v. 6.0.2 and its identity was verified as *P. pectinata* using the NCBI BLAST search function; the generated sequence was 99.3% similar to *P. pectinata* GenBank accession no. KP400584.1 (Chen et al. 2016).

The PCR reaction and cycling conditions were optimized for the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System (Droplet Generator instrument no. 773BR1456, Droplet Reader instrument no. 771BR2544) by systematically adjusting seven variables (i.e., primer and probe concentrations, cycle number, ramp rate, annealing temperature, denaturation time, and elongation time) to produce positive results with high relative florescence units (RFUs) and little to no "droplet rain" (i.e., droplets, or clusters of droplets, that lie between the positive and negative droplet bands on the

ddPCR™ scatter plot) (see Online Resource 2). All ddPCR™ optimization reactions were performed using ~0.20 ng gDNA derived from fin clips from four *P. pectinata*, with five replicates per individual. Optimized ddPCR™ reaction mixtures contained 1.1 µL of extracted DNA, 1X Bio-Rad® ddPCR™ supermix for probes (no dUTP), 900 nM of each primer, and 170 nM of probe, adjusted to 22 µL using PCR-grade water, as per the manufacturer protocol for automated droplet generation (Bio-Rad® Laboratories 2014). Using an automated droplet generator, 20 µL of each of these ddPCR™ reaction mixtures was combined with ~70 µL of automated droplet generation oil for probes to create up to 20,000 nanoliter-sized droplets prior to PCR cycling (Bio-Rad® Laboratories 2014). Optimal ddPCR™ cycling conditions consisted of enzyme activation at 95°C for 10 min, followed by 40 cycles of: 94°C for 30 s and 64°C for 2 min, with a final enzyme deactivation step at 98°C for 10 min, using a ramp rate of 1°C/s. To ensure the assay was species-specific for *P. pectinata* in U.S. waters, the optimized ddPCR™ reaction and cycling conditions were tested using ~0.20 ng gDNA from four *P. pectinata* and one individual for each of 12 representative exclusion species (Table 2.1), with three replicates per individual. The target DNA fragment was amplified in all ddPCR™ replicates for each *P. pectinata* individual, but was not amplified in any of the ddPCR™ replicates for any representative species from five genetically similar ray genera and two shark genera that could co-occur with *P. pectinata* in the study area, or in other *Pristis* sawfishes.

Table 2.1

List of genetically similar species for which tissue samples were acquired for assay development.

List of 12 exclusion species and country of origin for each tissue sample that was tested to ensure species-specificity of the primers and probe developed for the mitochondrial NADH dehydrogenase subunit 2 gene in the Smalltooth Sawfish, *Pristis pectinata*, using the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System. [a]Ray nomenclature follows Last et al. (2016).

Species[a]	**Origin**
Green Sawfish, *Pristis zijsron*	Australia
Dwarf Sawfish, *Pristis clavata*	Australia
Largetooth Sawfish, *Pristis pristis*	Australia
Freckled Guitarfish, *Pseudobatos lentiginosus*	USA
Atlantic Stingray, *Hypanus sabinus*	USA
Bluntnose Stingray, *Hypanus say*	USA
American Cownose Ray, *Rhinoptera bonasus*	USA
Whitespotted Eagle Ray, *Aetobatus narinari*	USA
Clearnose Skate, *Rostroraja eglanteria*	USA
Roundel Skate, *Rostroraja texana*	USA
Bigeye Thresher, *Alopias superciliosus*	USA
Atlantic Sharpnose Shark, *Rhizoprionodon terraenovae*	USA

2.3.4 Data Analysis

Data were analyzed using three criteria for positive *P. pectinata* detections: 1) droplets fell above a manual threshold (MT) defined for this assay, 2) droplets above the MT fell within the prescribed range of the positive droplet population for this assay (5000–7000 RFUs; Fig. 3.1), and 3) the concentration of target DNA, determined using Bio-Rad® QuantaSoft™ software using the Rare Event Detection (RED) setting, was at or above the Limit of Detection (LoD) determined for the assay (see Klymus et al. 2019).

Defining an assay-specific MT minimizes the likelihood of incorrectly calling artifact droplets (i.e., droplets that fall above the negative band population in the absence

of target DNA, positive detections (see Online Resource 3; Hunter et al. 2017). To define an appropriate MT for the *P. pectinata* eDNA assay, 162 reactions containing no DNA template were analyzed on the ddPCR™ platform, using the described reaction and cycling conditions. The highest amplitude of an artifact droplet across the 162 reactions was 2,700 RFUs; therefore, to be conservative, 3,000 RFUs was chosen to minimize the risk of false positives.

To determine the LoD of the assay, ddPCR™ reactions were performed using gDNA from three *P. pectinata* with a 6-fold series of 10X dilutions from starting concentrations of 20 ng/μL (i.e., 1:10 to 1:1,000,000), with five ddPCR™ replicates per individual and dilution. Target DNA was reliably detected in all replicates for all individuals and dilutions up to 1:10,000, but not in the 1:100,000 or 1:1,000,000 dilutions (Fig. 2.2a). The standard errors of the 1:100,000 and 1:1,000,000 dilutions also included zero, making detection at these concentrations unreliable. To further refine the LoD, ddPCR™ reactions were performed on subsequent 3-fold series of 2X dilutions from the 1:10,000 dilutions. Target DNA was detected in all replicates for all individuals in the 1:80,000 dilutions, corresponding to ~0.25 pg of target DNA in the reactions (Fig. 2.2b-c). The standard errors of the 1:80,000 dilutions did not include zero, nor did they overlap with the standard errors of the 1:100,000 dilutions. Using the average number of copies of target DNA/μL in the 1:80,000 dilutions and applying the lower standard error as the relaxed detection threshold (see Baker et al. 2018; Schweiss et al. 2019), the LoD of the assay was determined to be 0.08 copies/μL.

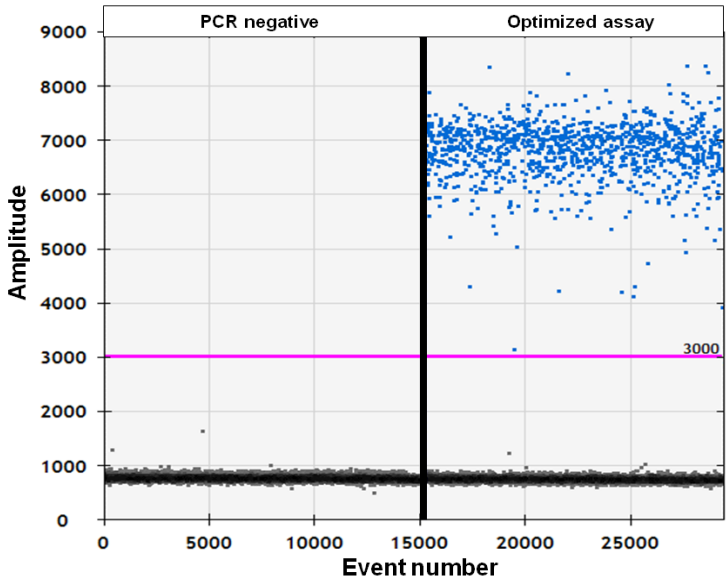

Figure 2.1 *Optimized Droplet Digital™ PCR reaction for* P. pectinata

Raw droplet scatter plot of Droplet digital™ PCR products using a negative control (PCR negative) and genomic DNA from one Smalltooth Sawfish, *Pristis pectinata,* with the optimized assay conditions (Optimized assay). Each droplet in each well was classified as either negative (below 3000), or positive (between 5000 and 7000) for target DNA, based on a manual threshold amplitude of 3000 relative florescence units, detected using a Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software with the Rare Event Detection analysis setting. Each well is separated by vertical lines, and is labeled to correspond with the sample it represents. Bio-Rad® QuantaSoft™ Software and MSOffice® Suite were used to create this figure.

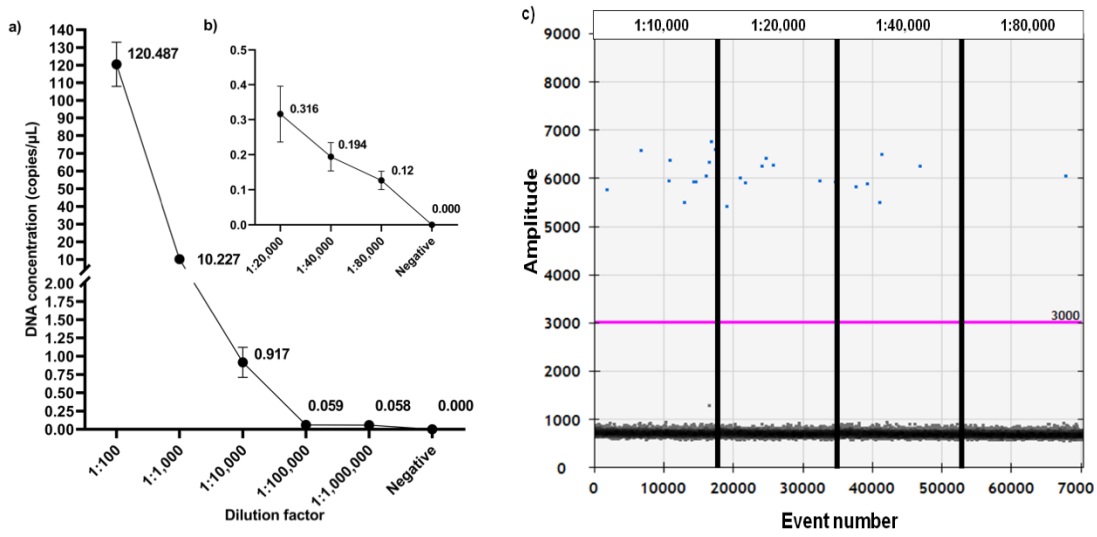

Figure 2.2 *Dilution Series DNA concentration and Droplet Digital™ PCR wells for LoD testing*

Average target DNA concentrations (copy number/µL) of the Limit of Detection dilution series, using genomic DNA from three Smalltooth Sawfish, *Pristis pectinata*, with five replicates each in: a) a 6-fold series of 10X dilutions from a starting concentration of 20 ng/µL, b) a 3-fold series of 2X dilutions from the 1:10,000 dilution, and c) a corresponding raw Droplet digital™ PCR scatter plot of serial dilution reactions from one replicate of one Smalltooth Sawfish, *Pristis pectinata*. Average DNA concentrations are indicated next to each point with corresponding standard error bars. The Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software with the Rare Event Detection analysis setting was used across all samples, and each droplet in each well was classified as either negative (below 3000), or positive (between 5000 and 7000) for target DNA based on a manual threshold amplitude of 3000 relative florescence units. GraphPad Prism® version 8.0.2 for Windows, Bio-Rad® QuantaSoft™ Software, and MS Office® Suite were used to create this figure

2.4 Validation of the *Pristis pectinata* eDNA Assay

To validate the ddPCR™ assay, positive *P. pectinata* eDNA samples were acquired via analysis of a water sample from an occupied nursery and through an *ex situ* tank experiment. To prevent contamination of the eDNA equipment by field equipment, two boats were used for this validation experiment. The surfaces of the boat used for water collection and the tank experiment were pre-cleaned twice with bleach and this

boat was used only by eDNA personnel for the duration of the experiment. A second boat held the necessary field equipment and personnel to capture and handle a live sawfish. To collect the positive water samples, on 16 May 2018, a pre-cleaned ~160 L tank was filled with ambient surface water from a known *P. pectinata* nursery, the Caloosahatchee River, Florida (Poulakis et al. 2011), approximately 330 m outside of Harbour Isles Marina. A 3 L water sample was immediately collected from the tank to assess whether *P. pectinata* eDNA was present in the Caloosahatchee River water. Shortly after this water sample was collected, one juvenile female *P. pectinata*, measuring 786 mm stretch total length, was captured by gill net inside Harbour Isles Marina and then placed into the tank. An aerator was added to the tank and dissolved oxygen and water temperature were monitored for the duration of the experiment. A 3 L water sample was collected from the tank immediately after the juvenile was added (time zero) and again after 30 min. After 30 min, the sawfish was removed from the tank and released at the capture site.

All positive *P. pectinata* water samples were filtered, DNA was extracted, run on ddPCR™ in replicates of five (e.g. screening 5% of the total DNA extract), and analyzed using the methods developed in this study. Total eDNA extracts contained high molecular weight when viewed on agarose gel, with nanodrop concentrations ranging from 13.9 ng/µL for ambient water, to 17.1 ng/µL for the 0 min sample, and 29.3 ng/µL for the 30 min sample. Applying all three criteria for a positive detection of target DNA, the ddPCR™ reactions containing DNA extracted from ambient nursery water contained an average of 11.54 copies/µL (SE = 0.72) of *P. pectinata* DNA (Fig. 2.3), with all replicates producing positive results. The amount of target eDNA increased to an average of 739.4 copies/µL (SE = 38.31) immediately after the juvenile was added to the tank

(time zero) and increased to an average of 3,175.8 copies/µL (SE = 589.3) after 30 min (Fig. 3), with all ddPCR™ replicates producing positive results. At 30 min, the large quantity of target DNA isolated from the water sample oversaturated the PCR product, resulting in a high standard error.

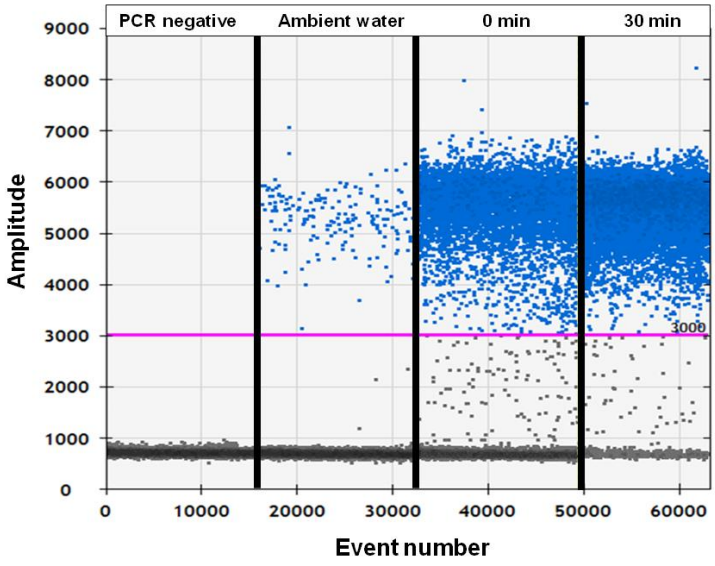

Figure 2.3 Pristis pectinata *Assay Validation Droplet Digital™ PCR Scatterplot*

Raw droplet scatter plot of Droplet digital™ PCR products from a negative control (PCR negative), a water sample collected from the Caloosahatchee River, a nursery area for Smalltooth Sawfish, *Pristis pectinata* (Ambient water), and positive water samples collected from the *ex situ* tank containing a live *P. pectinata* at times 0 (0 min) and 30 (30 min). Each droplet in each well was classified as either negative (below 3000), or positive (between 5000 and 7000) for target DNA, based on a manual threshold amplitude of 3000 relative florescence units, detected using a Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software using the Rare Event Detection analysis setting. Each well is separated by vertical lines and is labeled to correspond with the sample or time stage it represents. Note that "droplet rain" (i.e., droplets, or clusters of droplets, that lie between the positive and negative droplet bands on the ddPCR™ scatter plot) is seen at 0 min and, to a greater extent, 30 min due to an oversaturation of target DNA. Bio-Rad® QuantaSoft™ Software and MS Office® Suite were used to create this figure

2.5 Discussion

The developed eDNA assay provides a rapid-assessment tool to conduct targeted surveys to investigate the occurrence and infer the status of *P. pectinata* beyond their contemporary core range in south and southwest Florida. This assay has been validated in the Caloosahatchee River, Florida, where *P. pectinata* is the sole species of sawfish; however, because the assay did not amplify DNA in other *Pristis* sawfishes, it could be used in locations where the other western Atlantic sawfish, the Largetooth Sawfish, *Pristis pristis*, has been known to historically co-occur, such as Texas (Brame et al. 2019). In addition, there were no differences in the primer and probe sequences reported in this study when compared to a mtDNA ND2 sequence from a *P. pectinata* collected recently in Mexico (GenBank accession no. MF682494.1; Diaz-Jaimes et al. 2018), indicating that the developed assay should amplify the target gene in this species broadly in the western Atlantic.

Use of this assay outside the western Atlantic requires careful consideration and, likely, further *a priori* testing. Mitochondrial DNA genes are often variable among populations within a species (Rubinoff et al. 2006); therefore, before using this assay to conduct eDNA surveys in other geographic regions (e.g. eastern Atlantic), the primers and probe should ideally be tested with *P. pectinata* tissue samples obtained from the local population. Where fresh or archived *P. pectinata* tissue samples are not available due to the possibility of local extinctions, historic rostra could be used as an alternative source of DNA (Phillips et al. 2009). Finally, the primers and probe developed here were cross-tested with representative species from closely related genera found in U.S. waters; testing with additional exclusion species would likely be required to ensure that the assay

remains species-specific in other geographic regions (see Wilcox et al. 2013), highlighting the need for local fisheries knowledge (Poulakis and Grubbs 2019).

The use of ddPCR™ for single species detections is gaining popularity in eDNA research due to its unparalleled ability to detect minute quantities of target DNA amongst high concentrations of non-target DNA and in the presence of natural inhibitors found in water samples (Evans et al. 2017; Hunter et al. 2018). Droplet Digital™ PCR assays developed for species such as the Bull Shark, *Carcharhinus leucas* (Schweiss et al. 2019) and Killer Whale, *Orcinus orca* (Baker et al. 2018), have found this platform to be capable of detecting less than 1 pg of target DNA in a reaction. Such highly sensitive assays are especially important for eDNA surveys targeting Critically Endangered or Endangered species, where there can be substantial conservation outcomes based on the results of such surveys (Hunter et al. 2018; Poulakis and Grubbs 2019). The use of ddPCR™ could reduce the risk of false negatives (i.e., where target DNA is present but not detected) stemming from the use of less sensitive PCR methodologies, which are unlikely to detect such minute quantities of target DNA (Doi et al. 2015a). In a comparison of metabarcoding, qPCR, and ddPCR™ approaches for species detections from water samples, detection probabilities using qPCR and ddPCR™ were almost double that of metabarcoding, and ddPCR™ had a 8–10% higher detection rate than qPCR (Wood et al. 2019), making ddPCR™ the preferred approach for the detection of rare target species. Conservation and management strategies developed on the basis of false negatives as a consequence of using a less sensitive PCR platform could lead to slower implementation and inadequate protections along with incomplete habitat designations for threatened species, ultimately hindering their recovery.

Using the three-criteria approach described here to define positive detections on the ddPCR™ platform provides a rigorous approach for interpreting the results of eDNA surveys, reducing the risk of incorrectly calling PCR artifacts as positive species detections (i.e., false positives). For example, using only a MT, an artifact droplet just above the threshold could be incorrectly interpreted as a positive detection. Ensuring that the quantity of target DNA is also within the detection capabilities of an assay allows for more robust and confident positive detections. Positives are often confirmed via DNA sequencing of amplicons in eDNA studies (e.g. Simpfendorfer et al. 2016); however, sequencing ddPCR™ amplicons is challenging and may not always accurately reflect the results of the Bio-Rad® QuantaSoft™ RED analysis, especially when there is little target DNA present. This is due to: 1) the need to run parallel samples that are not analyzed by the droplet reader, so sequenced amplicons may not be identical to quantified products (i.e., analyzed samples are discarded as waste by the droplet reader), 2) the need to breakdown the oil emulsion prior to sequencing (see Bio-Rad® Laboratories, 2014), and 3) the potential for small concentrations of target DNA from multiple individuals, which can require additional PCRs prior to sequencing (e.g. Baker et al. 2018). However, when the primary purpose of DNA sequencing is to confirm species identity to avoid false positives, this step can be ameliorated by rigorous primer testing with co-occurring exclusion species, as performed here. False positives can also result from contamination between eDNA samples or from exogenous DNA. Given the detection capabilities of ddPCR™ assays, strict protocols to prevent contamination (see Goldberg et al. 2016; Schweiss et al. 2019) coupled with testing for contamination at every stage in sample processing are critical for producing reliable data from eDNA surveys that may be used

for conservation planning. This is especially important when the results of eDNA surveys could be used to prioritize research and management initiatives as well as in the allocation of resources (Poulakis and Grubbs 2019).

With a well-designed water sampling regime, strict field and laboratory controls, and a highly sensitive ddPCR™ assay, targeted species eDNA surveys provide a powerful tool to improve our knowledge of the occurrence of *P. pectinata*. The eDNA tool developed here can be used to provide quantitative baseline data in non-core ranges from which to measure future progress towards species recovery. Recovery in *P. pectinata* populations is expected to be a slow process due to their life history characteristics. Range re-expansion during recovery is predicted to begin in locations closest to the core population(s) as a result of spillover from adjacent areas, in a stepping-stone fashion (see Saura et al. 2014). There is, however, the possibility that because female *P. pectinata* exhibit philopatry (Feldheim et al. 2017), occurrence and encounter reports of juveniles in non-core areas further away from southwest Florida (e.g. northern or southern Gulf of Mexico) may represent remnant *P. pectinata* populations scattered over portions of their former range. Under such a scenario, patterns of recovery could be more complex and would ultimately depend on the availability of suitable habitat and the mitigation of threats from anthropogenic sources, including climate change (Seitz and Poulakis 2006; Poulakis et al. 2011; Norton et al. 2012; Scharer et al. 2017). Conducting targeted eDNA surveys for *P. pectinata* across all historically-occupied regions in U.S. waters could not only aid in conservation planning and prioritizing areas for research, but could also increase our understanding of patterns of recovery in a highly threatened marine species.

2.6 Supporting Information

2.6.1 *Online Resource 1:* P. pectinata *Assay Development*

Table 2.2

Base pair differences between P. pectinata *and 17 exclusion species used for assay design*

Online Resource 1. List of 17 exclusion elasmobranch species and mitochondrial NADH dehydrogenase subunit 2 (mtDNA ND2) gene GenBank accession numbers that were used to manually design species-specific primers and an internal probe for the Smalltooth Sawfish, *Pristis pectinata*. Columns include the number of base pair mismatches between *P. pectinata* and each exclusion species within each primer and probe used in the assay. Ray nomenclature follows Last et al. (2016)

Species	GenBank Accession Number	Forward primer	Reverse primer	Probe
Green Sawfish, *Pristis zijsron*	JQ519151.1	5	3	0
Dwarf Sawfish, *Pristis clavata*	KF381507.1	4	2	5
Largetooth Sawfish, *Pristis pristis*	NC_039438.1	5	3	2
Narrow Sawfish, *Anoxypristis cuspidata*	KP233202.1	3	7	3
Common Guitarfish, *Rhinobatos rhinobatos*	JQ518913.1	5	4	5
Southern Stingray, *Hypanus americanus*	xJN184288.1	7	8	8
Atlantic Stingray, *Hypanus sabinus*	JQ518787.1	6	5	6
Bluntnose Stingray, *Hypanus say*	JQ518788.1	5	4	5
Roughtail Stingray, *Bathytoshia centroura*	KY909632.1	5	5	6
Pelagic Stingray, *Pteroplatytrygon violacea*	KJ641617.1	5	5	8

Table 2.2 (continued)

Species	GenBank Accession Number	Forward primer	Reverse primer	Probe
Bullnose Eagle Ray, *Myliobatis freminvillei*	JQ518847.1	6	5	5
American Cownose Ray, *Rhinoptera bonasus*	JX241056.1	8	4	6
Giant Manta Ray, *Mobula birostris*	KM364991.1	8	7	5
Yellow Round Ray, *Urobatis jamaicensis*	JQ518941.1	7	6	6
Whitespotted Eagle Ray, *Aetobatus narinari*	KX151649.1	8	5	4
Clearnose Skate, *Rostroraja eglanteria*	JQ518889.1	6	5	7
Bigeye Thresher, *Alopias superciliosus*	MF374733.1	2	7	4

2.6.2 *Online Resource 2: Droplet Digital™ PCR Scatterplot of "droplet rain"*

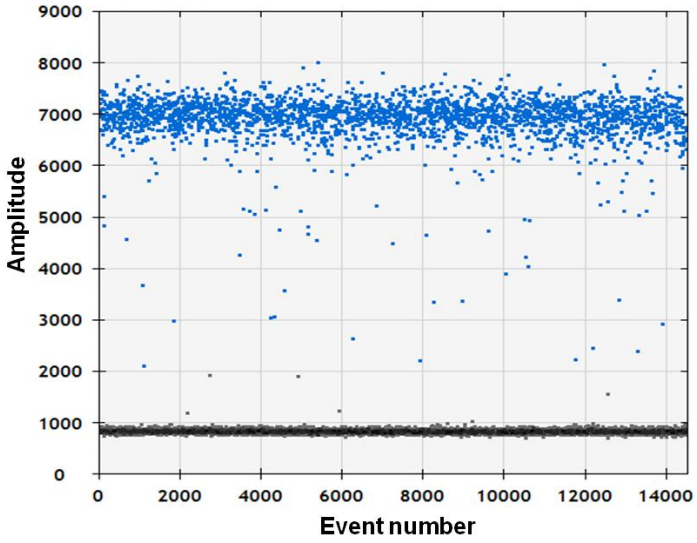

Figure 2.4 *Droplet Digital™ PCR Scatterplot of "droplet rain"*

Online Resource 2. Raw droplet scatter plot of Droplet Digital™ PCR products using genomic DNA from one Smalltooth Sawfish, *Pristis pectinata*, during assay optimization depicting "droplet rain" (i.e., droplets, or clusters of droplets, that lie between the positive and negative droplet bands on the ddPCR™ scatter plot). Each droplet was classified as either positive (blue) for target DNA, or negative (grey) for target DNA using the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software with the Rare Event Detection analysis setting. Because the assay was not fully optimized at this point, droplet rain can be seen as the droplets that fell outside of the positive droplet population to the manual threshold amplitude of 3000 Relative Fluorescence Units (RFUs)

2.6.3 *Online Resource 3: Droplet Digital™ PCR Scatterplot of artifact droplets*

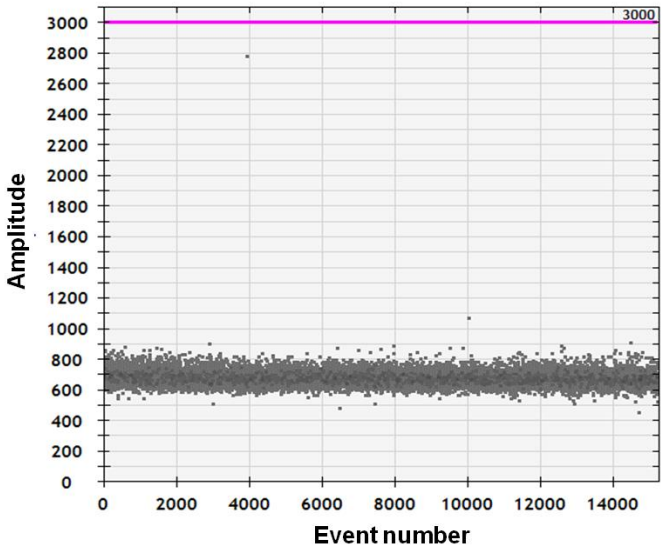

Figure 2.5 *Droplet Digital™ PCR Scatterplot of artifact droplets*

Online Resource 3. Raw droplet scatterplot of ddPCR™ products from one replicate of No Template Control (NTC) depicting "artifact droplets" (i.e., droplets, or clusters of droplets, that lie between the negative droplet band and the manual threshold). Each droplet was classified as negative due to the absence of target DNA, as detected by the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software using the RED analysis setting. Artifact droplets are shown as the errant droplets that fall between the negative droplet population (700–900 Relative Fluorescence Units; RFUs) and the manual threshold amplitude of 3000 RFUs

CHAPTER III – ENVIRONMENTAL DNA EVIDENCE OF THE CRITICALLY ENDANGERED SMALLTOOTH SAWFISH, *PRISTIS PECTINATA*, IN HISTORICALLY OCCUPIED UNITED STATES WATERS

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3.1 Abstract

1. Formerly common in tropical and subtropical waters of the Atlantic Ocean, the Critically Endangered smalltooth sawfish, *Pristis pectinata*, underwent severe declines over the past century, restricting population(s) to south and southwest Florida (SWFL) in the U.S., and Bahamian waters.
2. Anecdotal evidence (e.g. encounter reports) suggests that *P. pectinata* have recently been observed in historically occupied habitats in U.S. waters; however, no directed surveys have been conducted to verify their occupancy.
3. Here, eDNA surveys were used to investigate the occurrence of *P. pectinata* in three formerly occupied estuaries outside of the core range in SWFL. Water samples were collected in the summers from Tampa Bay and the Indian River Lagoon (IRL), Florida in 2018 and 2019, and in the Mississippi Sound, Mississippi in 2018, and screened for target DNA using a species-specific Droplet Digital™ PCR assay.
4. Target DNA was detected at four sites in the IRL in 2018, and one site in 2019 (average concentration: 0.086 copies/µL; SE = 0.004), but was not detected in either year in Tampa Bay. Target DNA was also detected at three sites near Deer Island in Mississippi in 2018 (average concentration: 0.090 copies/µL; SE = 0.005). These surveys provide an additional line of evidence that *P. pectinata* is present, or is re-occurring, within two historically occupied estuaries in U.S. waters.
5. More comprehensive eDNA surveys in historically occupied habitats, combined with clearly defined post-survey management actions, can direct additional

research and public outreach initiatives in emerging priority areas, fostering recovery for this Critically Endangered species.

KEYWORDS: estuary, coastal, endangered species, recovery, survey, fish, elasmobranch, ray, eDNA, conservation

3.2 Introduction

Sawfishes (Pristidae) are large-bodied, benthic rays that occur primarily in tropical and subtropical nearshore, estuarine, or riverine habitats (Dulvy et al., 2014), which increases their exposure to anthropogenic activities (Seitz & Poulakis, 2006). Direct exploitation, bycatch mortality in fisheries, and habitat degradation and loss have caused dramatic declines in the ranges and abundances of all five species over the past century, and all are listed as Critically Endangered or Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (see Dulvy et al., 2016). Sawfishes have been lost from at least 20 of the 90 countries where they formerly occurred across the Atlantic, Pacific, and Indian oceans, and an estimated 23 additional countries have lost at least one species where two or more once co-occurred (Dulvy et al., 2016). Viable populations of sawfishes persist in the waters of northern Australia, the southeastern United States (U.S.), and the Bahamas (Dulvy et al., 2016). The outlook for recovery in Australia and the U.S. is promising; however, the status of most species outside of these global strongholds remains largely uncertain (Harrison & Dulvy, 2014).

The Critically Endangered smalltooth sawfish, *Pristis pectinata*, has undergone the most dramatic decline of all five sawfishes, and presently only inhabits ~20% of its historic range in the tropical and subtropical waters of the Atlantic Ocean (Dulvy et al., 2016). In the eastern Atlantic, *P. pectinata* inhabited coastal waters from Mauritania to

Angola, but no viable populations are thought to still occur there (Carlson, Wiley & Smith, 2013). In the western Atlantic, historic literature indicates that *P. pectinata* occurred from Uruguay to the U.S., and throughout the Caribbean (Bigelow & Schroeder, 1953). The only remaining viable populations are thought to be restricted to the southeastern U.S. and the Bahamas (Carlson, Wiley & Smith, 2013).

Within the U.S., *P. pectinata* once occurred in coastal waters from Texas (TX) to the Carolinas, but by about the 1980's, were restricted to south and southwest Florida (SWFL), specifically Charlotte Harbor to the Florida Keys (hereafter the "core range") (Bigelow & Schroeder, 1953; NMFS, 2009). Historical faunal surveys documented the widespread presence of both juveniles and adults in the western and northern Gulf of Mexico from Laguna Madre, TX to Pensacola, Florida (FL), noting Galveston Bay, TX, the mouth of the Mississippi River, Lake Ponchartrain, Louisiana (LA), Pascagoula Bay, Mississippi (MS), and Mobile Bay, Alabama, (AL) as areas where specimens were collected, or large numbers of individuals were observed (see Goode, 1884; Bigelow & Schroeder, 1953). In peninsular Florida, all age classes were reported from Cedar Key to Cape Canaveral (Goode, 1884; Bigelow & Schroeder, 1953), with specific mention of large numbers in Tampa Bay and the Indian River Lagoon (IRL) (Jordan & Swain, 1884; Henshall, 1891; Henshall, 1895; Evermann & Bean, 1898). Individuals have been reported on the east coast, north of Florida (e.g. Chesapeake Bay, Cape Lookout, Cape May); however, the presence of a sustained historic population in these waters is considered unlikely, based on the lack of available mangrove habitat and low average winter water temperatures (e.g. ≤12°C; Brame et al., 2019). These historic reports also make specific mention of gravid females or pups in Galveston Bay, TX, Biloxi Bay, MS,

and the IRL, FL (see Evermann & Bean, 1898; Bigelow & Schroeder, 1953). Museum specimens and photographic records of *P. pectinata* further support the presence of all life history stages in these areas (e.g. Mississippi Museum of Natural History specimen #5881.0, Smith KL, 2020, unpublished data).

Decades of undocumented bycatch mortalities in fisheries, and a lack of understanding of the biology and ecology of this species, has led to the loss of *P. pectinata* from much of its historic range in U.S. waters; consequently, the species was listed as Endangered in 2003 under the U.S. Endangered Species Act (ESA) of 1973 by the National Marine Fisheries Service (NMFS) (NMFS, 2003). While historic surveys documented a large range for *P. pectinata* in U.S. waters, the decline of this species went largely unnoticed in scientific research until shortly before the petition for listing, aside from brief mentions in specific regions (e.g. the IRL; Evermann & Bean, 1898; Snelson & Williams, 1981). Evidence of species decline was supported by sawfish encounter data from citizen scientists, which was instrumental in detailing the extent of decline (NMFS, 2009). Ultimately, these data also became integral in identifying critical habitat for juveniles (as defined by the ESA; Norton et al., 2012), and developing strategies to facilitate recovery (Norton et al., 2012; Poulakis & Grubbs, 2019). Recent data from scientific research and numerous public outreach initiatives suggest that the core population of *P. pectinata* in SWFL may be stabilizing (NMFS, 2018), likely as a result of almost two decades of federal protection.

A species is considered fully recovered when they are present in all parts of their estimated historic range, populations are viable (e.g. genetically healthy), and the species is fulfilling its functional role in the ecosystem (Akçakaya et al., 2018; Akçakaya et al.,

2020). Full recovery of *P. pectinata* in U.S. waters is predicted to be a slow process due to their life history, which is characterized by late maturity, low fecundity, and a long-life span (Brame et al., 2019). Recently, verified (i.e. photographic evidence) public sawfish encounter reports have emerged outside of their core range in SWFL (hereafter referred to as "non-core range"), spanning from Terrebonne Bay, LA to the mouth of the Satilla River, Georgia (National Sawfish Encounter Database; NMFS, 2019, unpublished data). These reports fall within 9 of the 15 designated 'recovery regions', geographic units of the historic range defined in the smalltooth sawfish Species Recovery Plan as areas where management efforts should occur, if individuals re-occupy these areas (NMFS, 2009). Citizen science encounter reporting can be advantageous by directly involving and giving the general public ownership of, or investment into, recovery progress (see Foster-Smith & Evans, 2003). However, these data can be spatially biased based on differences in public education and participation efforts (e.g. Crall et al., 2011), making it a non-comprehensive account of contemporary occurrence (Wiley & Simpfendorfer, 2010). Complementary scientific surveys are needed to independently assess the contemporary occurrence of *P. pectinata* in their historically occupied, non-core range.

Scientific survey methods such as gill nets, bottom longline, and electronic tagging have been used to assess the contemporary occurrence of *P. pectinata* in SWFL (see Poulakis et al., 2011; Carlson et al., 2014), but these methods may not be effective in the non-core range where the species is rare (see Magnuson, Benson & McLain, 1994; Lewison et al., 2004). A species-specific environmental DNA (eDNA) tool was recently developed and validated for *P. pectinata* for use in U.S. waters (Lehman et al., 2020), taking advantage of trace sources of DNA (e.g. cellular debris, feces) suspended in the

water column (Ficetola et al., 2008). This trace DNA can be captured in water samples, which are filtered, the DNA extracted, and then screened for the presence of target DNA using the Droplet Digital™ PCR (ddPCR™) platform (e.g. Nathan et al., 2014). The sensitivity of ddPCR™ eDNA assays (Baker et al., 2018; Schweiss et al., 2019; Lehman et al., 2020) makes them ideal for "early detection" surveys targeting threatened species (see Wood et al., 2019), especially in turbid waters that make other technologically advanced methods, such as drones, ineffective (Kelaher et al., 2019). Additionally, eDNA methods do not require observation, capture, or handling of the target species, making them an ideal, non-invasive choice for studies addressing rare species (Weltz et al., 2017).

Here, we assessed the presence of *P. pectinata* in non-core range U.S. waters using the recently developed *P. pectinata* eDNA tool (Lehman et al., 2020). EDNA field surveys were conducted in estuaries that once supported this species and had recent, verified public sawfish encounter reports. Through *in situ* application, the goal of these surveys was to demonstrate the utility of this eDNA tool as a viable option for documenting early detection of this Critically Endangered species. Results of such surveys could be used to prioritize locations for future research and outreach efforts, allowing for more effective allocation of limited resources (see Poulakis & Grubbs, 2019), and to serve as a metric to measure recovery progress (see Campbell et al., 2002).

3.3 Methods

3.3.1 Study Areas

Water samples were collected from sites in three non-core range estuaries: Tampa Bay and the IRL in Florida, and the Mississippi Sound in Mississippi. These estuaries once

supported *P. pectinata*, with historic reports documenting the presence of all life history stages, and each has recent (i.e. within five years of sampling) verified public sawfish encounter reports. Tampa Bay is a coastal estuary totaling ~846 km^2 of the central portion of the west coast of Florida (Simon, 1974), and is the first major estuary north of the core range. The bay is split into five sections based on freshwater inflow including: Old Tampa Bay, which is fed via a series of minor creeks, Hillsborough Bay, fed via the Hillsborough, Palm, and Alafia rivers, and Tampa Bay proper, Boca Ciega Bay, and Terra Ceia Bay, which all receive freshwater through the Little Manatee and Manatee rivers. The IRL is the proximal estuary outside of the core range on the east coast, and stretches ~217 km. It includes five ocean inlets (i.e. Ponce de Leon, Sebastian, Fort Pierce, St. Lucie, Jupiter) and receives freshwater from several relatively small rivers with greatly expanded watersheds (e.g. Sebastian, St. Lucie, Jupiter, Loxahatchee) (Evermann & Bean, 1898; Snelson & Williams, 1981). The Mississippi Sound is a ~160 km estuary spanning the Mississippi and Alabama coastline out to a series of natural barrier islands (i.e. Cat, Ship, Horn, Petit Bois, Dauphin). Two additional islands are present within the sound (i.e. Round and Deer), and there is continuous freshwater input via the Pearl and Pascagoula rivers, as well as drainage from the inland Biloxi Bay and Bay St. Louis systems (see Eleuterius, 1978).

3.3.2 Field and Lab Controls

Rigorous controls were used in each stage of this research (i.e. field sampling, water filtration, DNA extraction, and PCR-amplification) to minimize contamination occurring across samples or from exogenous sources (see Goldberg et al., 2016). All equipment was sterilized using a two-step process; first cleaned with 10% bleach, and then, depending on

the materials, either autoclaved at 120°C for 20 min or exposed to UV light for 20 min. In the field, the benthic water sampler (model: VDBS-3L; Deep South Samplers, LLC; see Supplementary Materials Figure 1) was sterilized after each sample using Clorox Healthcare® Bleach Germicidal Wipes, followed by rinsing the sampler collection tubes with 10% bleach and then rinsing three times with autoclaved deionized (DI) water. New latex or nitrile gloves were used at each collection site.

Water filtration, DNA extraction, and PCR amplification were conducted in physically isolated laboratories where contemporary *P. pectinata* tissue had never been present (see Deiner et al., 2015). In the filtration lab, the exterior of the PVC filtration rigs were cleaned using Clorox Healthcare® Bleach Germicidal Wipes before use. Designated sterile forceps were used to handle filters from different collection sites and gloves were changed between each sample during water filtration and DNA extractions (see Goldberg et al., 2016). During DNA extraction and PCR amplification, aerosol barrier pipette tips were used to prevent contamination via DNA aerosols (see Schweiss et al., 2019; Lehman et al., 2020). As per ancient DNA protocols (see Mulligan, 2005), positive control templates were not used in PCRs to eliminate the risk of cross contamination from the positive itself.

Negative control samples were incorporated into each stage of water sample collection and laboratory processing and analyzed through to PCRs, which were conducted in replicates of five, to test for contamination. A field collection negative, comprised of 3 L of autoclaved DI water, was poured through the benthic sampler to simulate the collection of a water sample and stored in three sterile 1 L Nalgene® bottles on ice (as per field sample protocols) until filtration, to test for contamination during each

sample collection day. A filtration negative was used to test for contamination during water filtration by filtering 3 L of autoclaved DI water and processing it through to PCRs. No particulate matter or filters were used in negative controls for DNA extractions, and no DNA template was included in PCR negative controls. Negative controls were considered free from contamination (i.e. negative) if none of the three criteria for positive detection were met (Lehman et al., 2020).

3.3.3 Field Sampling

Water sample collection occurred in the summers of 2018 and 2019 for Florida locations, and in the summer of 2018 in Mississippi. In 2019, the Bonnet-Carré Spillway was open through the summer, introducing a vast volume of freshwater into the estuary and causing extensive algal blooms, hypoxic conditions, and other water quality issues (Hendon, Wiggert & Hendon, 2019), effectively prohibiting eDNA surveys in Mississippi that year. Summers were chosen for sampling based on a higher frequency of sawfish encounter reports and current knowledge of *P. pectinata* temperature affinity (see Poulakis et al., 2011; Brame et al., 2019). General study areas for eDNA surveys within each estuary were defined using the numbers and locations of recent verified sawfish reports from the National Sawfish Encounter Database (NMFS, 2019, unpublished data). Samples were collected from most sections of Tampa Bay, the IRL between the Sebastian and Jupiter inlets, and the waters surrounding Deer Island in the Mississippi Sound (Figure 1). Within each defined study area, directed and random sampling approaches were used to identify sites for water collection, as described by Poulakis et al. (2011). This approach allowed for targeting promising locations, while still covering as broad an area as possible with minimal bias. Directed sampling locations comprised up to 20% of all sites

sampled, and were determined using GPS coordinates of recent, verified sawfish encounter reports from the National Sawfish Encounter Database (NMFS, 2019, unpublished data). For random sampling, each study area was divided into 1 x 1 nautical mile grids along each minute of latitude and longitude. Each grid was equally subdivided into 100 numbered microgrids, which represented potential sample sites. Only microgrids with a depth of ≤3 m were included in the sampling "universe" (see Poulakis et al., 2011) to target habitats where primarily small juveniles are known to occur (Norton et al., 2012). A random number generator was used to select microgrids to sample within.

Across all three estuaries, 150 water samples were collected in this study in 2018 and 2019. A total of 30 samples were collected each year in July in the IRL and in August in Tampa Bay. Due to logistical challenges collecting samples in Mississippi, 30 samples were collected over the span of three summer months (June, July, and August) in 2018 only. Ten, 3 L water samples could be collected and filtered in a single day; therefore, the 30 water samples from Tampa Bay and the IRL were collected over three days in one week each year. The 30 water samples from MS were collected on one day of each sampled month.

Environmental data (i.e. temperature, salinity, dissolved oxygen, pH) were collected at the surface and the bottom of the water column at each site prior to water sample collection. Turbidity was assessed at each site using a Secchi disk on the shaded side of the vessel. At each site, 3 L bottom water samples were collected using a custom designed and fabricated watertight benthic sampler (model: VDBS-3L; Deep South Samplers, LLC) from ~30 cm above the substrate (see Supplementary Materials Figure 1 & Item 2). The average depth of sites where water samples were collected was 1.63 m

(SE = 0.09) and the depths sampled varied by less than a meter (0.61 m) across all study areas. Water samples were transferred into sterile, 1 L high-density polyethylene Nalgene® bottles, and stored on ice in clean marine coolers until filtration, which occurred within 24 hours of collection.

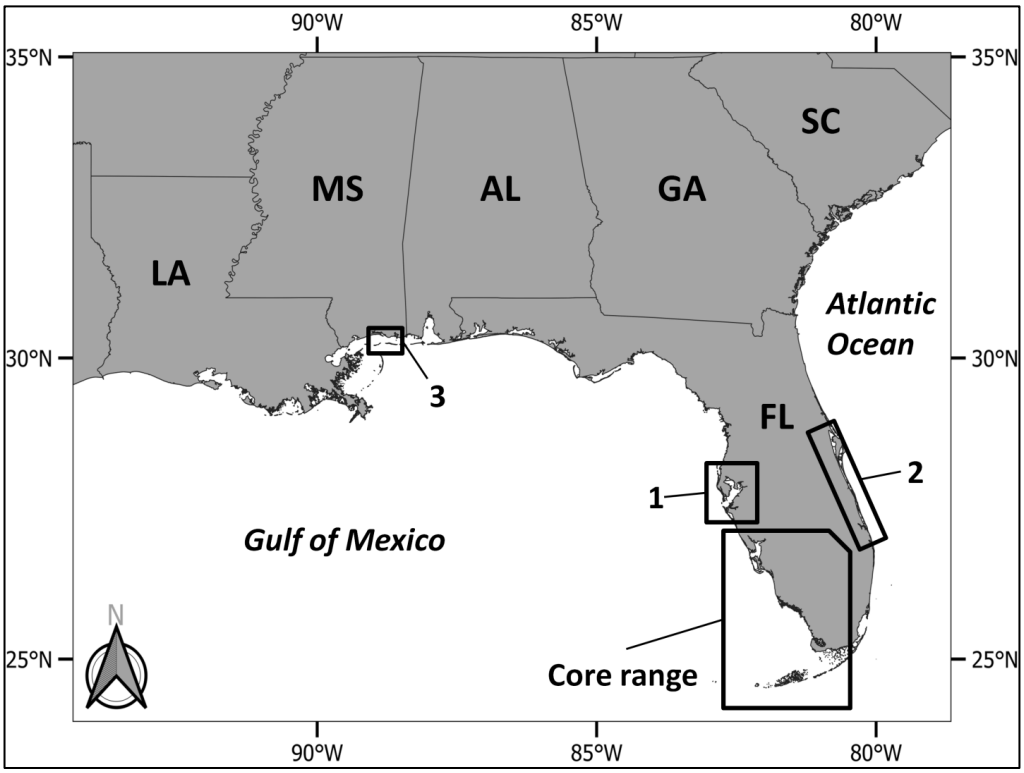

Figure 3.1 *Environmental DNA study areas*

Study areas in United States waters and their relation to the core range (black rectangle minus one corner) of the smalltooth sawfish, *Pristis pectinata*, in southwest Florida. Each study area is outlined by a black box and labeled based on proximity to the core range: 1) Tampa Bay, 2) the Indian River Lagoon, and 3) the Mississippi Sound. States that border the Gulf of Mexico or the Atlantic Ocean are indicated by their abbreviation. General locations of each estuary are indicated by latitude and longitude on the borders of each map.

3.3.4 Water Filtration and DNA Extraction

Water samples were vacuum-filtered using primarily Whatman® (Maidstone, United Kingdom) brand 0.8 µm, 47 mm diameter nylon filters. However, depending on product availability, Steriltech® (Washington, USA) and Cole Parmer® (Illinois, USA) brand filters were occasionally used as needed. Filters were replaced when they became clogged every ~350 mL, therefore ~9 filters were used for each 3 L water sample. After filtration, filters were rolled, folded, and preserved in 95% ethanol at room temperature. For samples collected in 2018, total eDNA was extracted from a ¼ portion of each filter using the QIAGEN® DNeasy® Blood & Tissue Kit (Hilden, Germany) following the Goldberg et al. (2011) protocol incorporating QIAshredder™ spin columns, and eluted with 100 µL of elution buffer. To allow for screening more of the samples in 2019, DNA was extracted from ½ of each filter and eDNA was eluted with 50 µL of elution buffer. Following DNA extraction, 2% agarose gels were used to assess the quality of extracts, and Thermo Fisher Scientific™ NanoDrop™ technology was used to quantify the concentration of each DNA extract.

3.3.5 PCR Amplification

A species-specific eDNA assay was used to screen samples for the presence of *P. pectinata* DNA by amplifying a 100 base pair fragment of the mitochondrial NADH dehydrogenase subunit 2 (mtDNA ND2) gene, following Lehman et al. (2020). Samples were run on the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System (Droplet Generator instrument no. 773BR1456, Droplet Reader instrument no. 771BR2544) platform in replicates of five (i.e. screening 5% of the total DNA extract for samples collected in 2018 and increasing to 10% for samples collected in 2019).

3.3.6 Data Analysis

Each sample was analyzed using the three criteria described in Lehman et al. (2020). These criteria were: 1) droplets fall above a manual threshold (MT) of 3000 Relative Florescence Units (RFUs), 2) droplets above the MT must also fall within the range of the positive droplet population for the assay (5000–7000 RFUs), and 3) the concentration of target DNA must also be at or above the Limit of Detection (LoD) of 0.08 copies/µL for the assay using the Bio-Rad® QuantaSoft™ software and the Rare Event Detection (RED) setting. Samples were defined as positive detections for *P. pectinata* DNA if at least one replicate per sample met all three criteria.

3.4 Results

Environmental data collected at each sample site were similar within estuaries, and salinity and dissolved oxygen (DO) demonstrated the largest differences between study areas (Table 1). On average, the IRL had the highest salinity of the three study areas. Average DO concentrations ranged from 4.7 to 8.24 mg/L, with sites in Mississippi generally having the lowest DO values. Average water temperatures in each estuary were warm (e.g. >28°C), and pH remained constant. The average turbidity was highly variable between estuaries, and the waters of the Mississippi Sound were generally the most turbid (Table 1).

Table 3.1

Environmental data from eDNA surveys

Mean (SEM) environmental data collected within Tampa Bay and the Indian River Lagoon in Florida and the Mississippi Sound in Mississippi.

	Depth (m)	Turbidity (m)	Temperature (°C)	Salinity	Dissolved Oxygen (mg/L)	pH
Tampa Bay, FL						
2018 (n = 20)	1.6 (0.1)	1.9 (37.4)	31.1 (0.1)	21.9 (0.9)	7.66 (0.3)	7.9 (0.1)
2019 (n = 30)	1.2 (0.3)	0.5 (6.5)	30.7 (0.0)	12.9 (0.0)	6.0 (0.1)	7.8 (0.0)
Indian River Lagoon, FL						
2018 (n =30)	1.9 (0.1)	1.1 (9.9)	30.9 (0.2)	25.9 (0.0)	8.24 (0.8)	8.0 (0.0)
2019 (n = 30)	1.5 (0.1)	1.3 (22.5)	31.6 (0.2)	33.7 (0.5)	5.7 (0.3)	8.0 (0.0)
Deer Island, Mississippi						
June (n = 10)	2.3 (0.4)	0.7 (3.5)	29.7(0.3)	15.1 (0.6)	5.8 (0.9)	8.0 (0.1)
July (n = 10)	1.7 (0.3)	0.7(5.4)	29.5(0.0)	18.8 (0.4)	4.7 (0.5)	7.9 (0.0)
August (n = 10)	1.7 (0.3)	0.9 (5.3)	30.3 (0.4)	25.0 (0.8)	6.5 (0.8)	7.7 (0.0)

Negative control tests for samples collected from sites in the IRL, Mississippi Sound, and for five of the six sample collection days from Tampa Bay did not meet any of the three criteria for positive detections, indicating that no contamination occurred. However, target DNA was detected in both the water collection and filtration negative controls for one sampling day in Tampa Bay in 2018 and therefore, data for those 10 field samples were discarded from the analysis.

Total eDNA extracts from each estuary contained high molecular weight DNA, with average nanodrop concentrations of 28.64 ng/µL (SE = 4.10) for samples from Tampa Bay and 52.82 ng/µL (SE = 8.08) for samples from IRL in 2018. In 2019, sample processing protocols were modified to increase DNA yields, and the average concentrations were 163.60 ng/µL (SE = 12.52) and 125.39 ng/µL (SE = 18.74) for samples from Tampa Bay and the IRL, respectively. In the Mississippi Sound, the average concentration of total eDNA extracts varied across each month: June, 34.58 ng/µL (SE = 2.95), July, 63.90 ng/µL (SE = 9.72), and August, 48.86 ng/µL (SE = 6.03).

When all three positive detection criteria were applied, *P. pectinata* DNA was detected in at least one ddPCR™ replicate for water samples collected from the IRL and the Mississippi Sound. In the IRL, *P. pectinata* DNA was detected in samples collected from five of the 60 sites (average concentration: 0.086 copies/µL; SE = 0.004); four in 2018 and one in 2019, all generated via random sampling. Two of the four positive detections in 2018 were in close proximity, ~5–8 km north of Fort Pierce Inlet, one was ~20 km north of St. Lucie Inlet, and another was <1 km west of the mouth of the St. Lucie Inlet (Figure 2a). In 2019, the only positive detection was ~6 km southwest of Fort

Pierce Inlet (Figure 2b). In Tampa Bay, none of the samples collected from any of the 50 sites met all three criteria for a positive detection (Figure 3). In the Mississippi Sound, samples collected from three of the 30 sites near Deer Island contained *P. pectinata* DNA (average concentration: 0.090 copies/µL; SE = 0.005). All three of these positive detections occurred in August on the southern side of the island (Figure 4c). The positive on the eastern end of the island was a directed sampling site based on a verified sawfish encounter report from 2014, while the other two were from randomly selected sites.

Several samples collected from sites in Tampa Bay and the Mississippi Sound had at least one ddPCR™ replicate meet two of the three criteria required for positive detections (n = 9). Four of the 9 sites were in Tampa Bay (one in 2018 and three in 2019) and five were in Mississippi Sound in June (n = 1), July (n = 2), and August (n = 2) (see Figures 3 & 4).

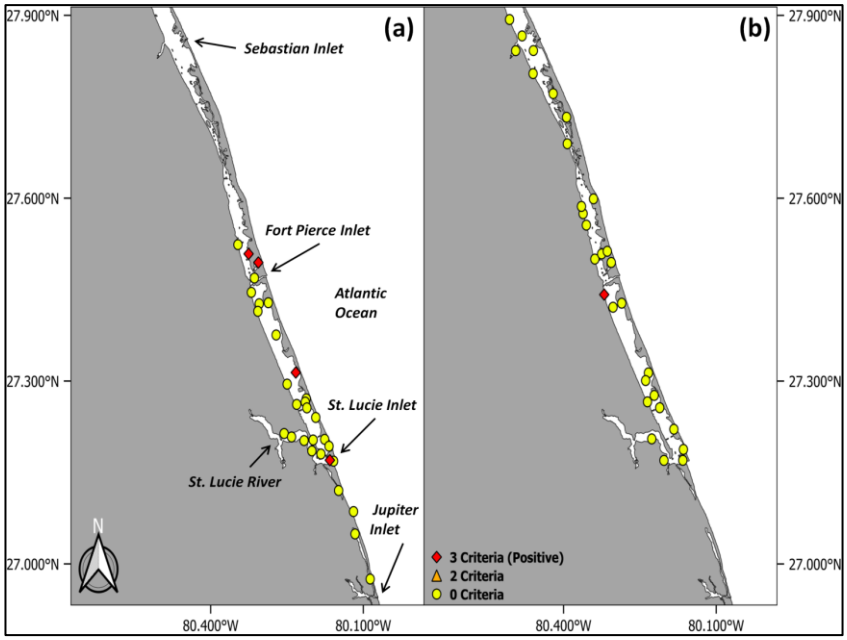

Figure 3.2 *Results of eDNA surveys in the Indian River Lagoon*

Water sample collection sites within the Indian River Lagoon, Florida during July a) 2018 and b) 2019. The four major inlets and the St. Lucie River are indicated by arrows. Positive samples (diamonds) had at least one ddPCR™ replicate that met all three criteria for a positive detection of target *Pristis pectinata* DNA (see methods and Lehman et al., 2020). Samples that did not meet any of the criteria (circles) did not contain target DNA. No samples only met one or two (triangles) of the three criteria. The general locations where each sample was collected within the estuary are indicated by latitude and longitude on the borders of each map.

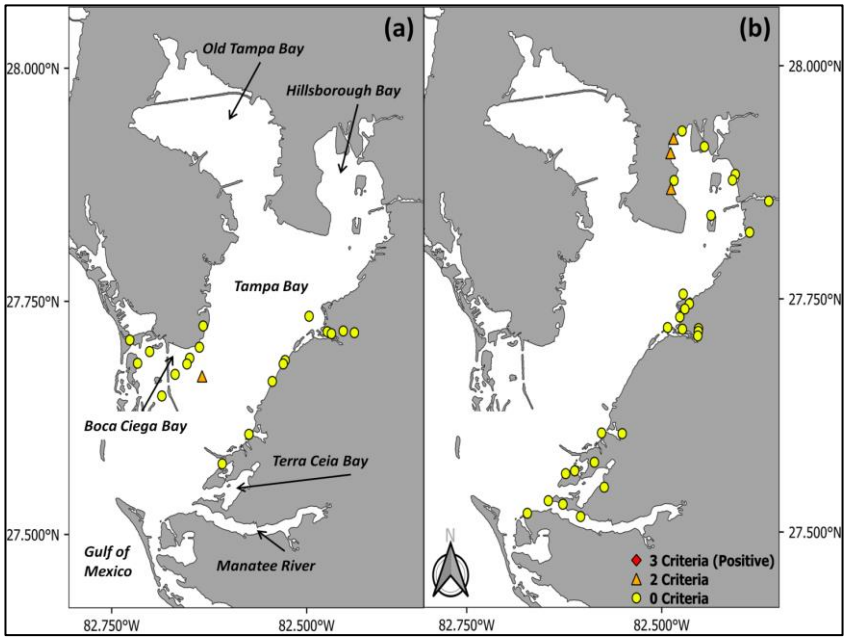

Figure 3.3 *Results of eDNA surveys in Tampa Bay*

Water sample collection sites within Tampa Bay during August a) 2018 and b) 2019. The five major subunits of the bay and the Manatee River are indicated by arrows. No samples had at least one ddPCR™ replicate that met all three criteria for a positive detection of target *Pristis pectinata* DNA (diamonds) (see methods and Lehman et al., 2020). Samples where at least one ddPCR™ replicate met two criteria (triangles) and those that did not meet any of the criteria (circles) did not contain target DNA. No samples only met one of the three criteria. The general locations where each sample was collected within the estuary are indicated by latitude and longitude on the borders of each map.

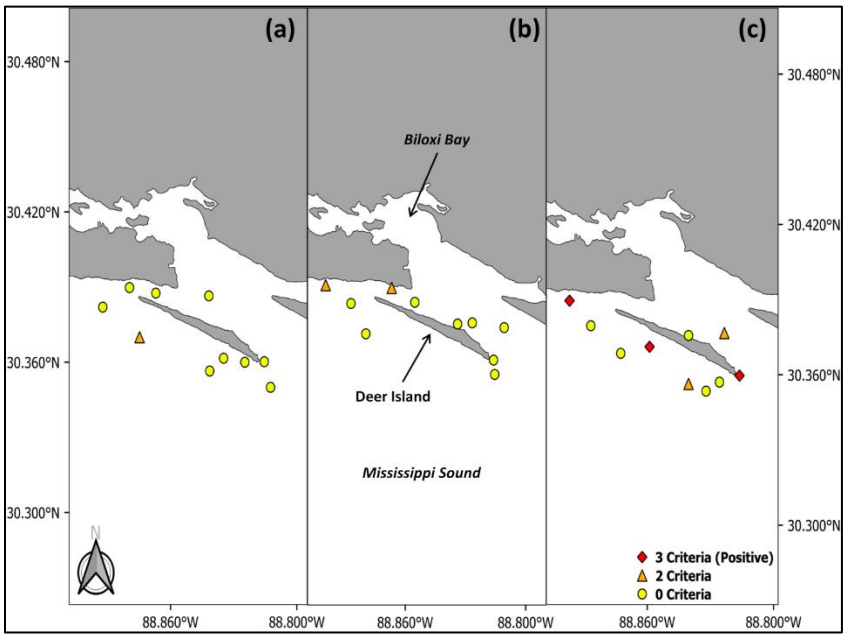

Figure 3.4 *Results of eDNA surveys in Mississippi Sound*

Water sample collection sites within Mississippi Sound during a) June, b) July, and c) August 2018. The major islands and bays of the sound are marked by arrows. Positive samples (diamonds) had at least one ddPCR™ replicate that met all three criteria for a positive detection of target *Pristis pectinata* DNA (see methods and Lehman et al., 2020). Samples where at least one ddPCR™ replicate met two criteria (triangles) and those that did not meet any of the criteria (circles) did not contain target DNA. No samples only met one of the three criteria. The general locations where each sample was collected within the estuary are indicated by latitude and longitude on the borders of each map.

3.5 Discussion

The results of this study demonstrate that eDNA field surveys can be successfully implemented to detect *P. pectinata* DNA in historically occupied, non-core range habitats in U.S. waters. *Pristis pectinata* DNA was detected in water samples collected from five sites in the IRL in Florida in two years (2018, 2019) and from three sites near Deer Island in Mississippi in August 2018. However, the eDNA tool did not detect *P. pectinata* DNA in Tampa Bay in Florida despite its proximity to the core range, and an analysis of 50 samples collected in two years with good spatial coverage. The rigorous data analysis

used to define positive detections in this study reduces the risk of false positives (Lehman et al., 2020), and when combined with thorough testing for potential contamination, provides robust evidence of target DNA at collection sites.

The verified presence of *P. pectinata* DNA in water samples collected from the IRL and the Mississippi Sound indicate that at least one individual was recently present in, or within the vicinity of, each of these estuaries, although the location of the DNA source (i.e., an animal) remains uncertain. After release from its source, eDNA molecules are immediately subject to degradation (Barnes & Turner, 2016) and dispersion, either free floating in the water column or attached to particulate matter (Turner, Uy & Everhart, 2015). In *ex situ* experiments, DNA decay rates were higher in warm (e.g. >20ºC), low salinity (e.g. <27) waters with high levels of microbial activity, which tends to be intensified in coastal areas due to anthropogenic activities (Strickler, Fremeir & Goldberg, 2015; Collins et al., 2018). Based on the results of these *ex situ* studies, the persistence time of target eDNA in the three study areas during sample collection was likely to be a maximum of ~48 hours (Collins et al., 2018). However, this timeframe could have been much shorter in areas or times of lower salinity (e.g. observed salinities in Tampa Bay during 2019 sampling). As eDNA molecules are degrading, they are also transported both vertically (e.g. settling to the bottom) and horizontally, depending on local water circulation patterns (Deiner & Altermatt, 2014; Andruszkiewicz et al., 2019). All positive detections of *P. pectinata* in the IRL and the Mississippi Sound were from shallow (i.e. <2 m) sites that were not subject to strong currents or waves during sampling (Beaufort ≤1). In the Mississippi Sound, all water samples with positive detections were collected during outgoing tides, (average tidal range (SE): 0.03 (0.05)–

0.45 (0.00) m), while in the IRL, there were positive detections on incoming and outgoing tides, as well as high tide (average tidal range (SE): -0.04 (0.08)–0.34 (0.04) m)), which would have influenced dispersion of eDNA molecules. Regardless, the prospect of target eDNA traveling large distances, such as from the core range (~260 km away from the IRL) in the 48-hour period prior to water collection is unlikely. Modeling of water circulation and particle movement patterns in the IRL and the Mississippi Sound are needed to facilitate interpretations of positive detections by estimating the potential locations of eDNA sources (e.g. Andruszkiewicz et al., 2019). Such analyses could inform on whether the *P. pectinata* DNA detected in the IRL, in particular, originated from sources within the estuary or from adjacent coastal waters, particularly since some positive detections were within 5 km of inlets.

The frequencies of positive detections were similar in the IRL (~13%) and the Mississippi Sound (10%) in 2018. However, within the IRL, there was only a single positive detection in 2019, despite processing a larger proportion of each filter and screening a higher percentage of the DNA extract. These relative frequencies may reflect and be influenced by the locations sampled, or by the number of DNA sources (i.e. individuals) present, since in theory, multiple sources should increase the amount of target DNA present. The relationship between DNA concentrations and organismal abundance has been explored in *ex situ* freshwater mesocosm studies, where a positive correlation has been identified (e.g. Nathan et al., 2014; Doi et al., 2015). However, this relationship remains largely unexplored in marine systems, and is complicated by factors such as the shed rate of eDNA molecules from the source and time since its release (Maruyama et al., 2014; Klymus et al., 2015; Spear et al., 2015). Therefore, at present, it

is unknown whether the multiple positive detections in each of the IRL and the Mississippi Sound in 2018 represent DNA from single or multiple sources, or if the detections in IRL in 2018 reflect the presence of more individuals compared to the single detection in 2019.

Temporal patterns of positive detections (i.e. presence of *P. pectinata*) in the waters surrounding Deer Island, MS could be linked to environmental variables and water quality, specifically salinities and DO levels. Here, sampling spanned three summer months, but positive detections only occurred in August, when salinities and DO were also within the affinity ranges of *P. pectinata* (Poulakis et al., 2011). The lack of positive detections in June and July may reflect the absence of *P. pectinata* from the area, possibly due to sub-optimal environmental conditions. In June, salinities were slightly below the typical range for juveniles (18–30, Poulakis et al., 2011); in July salinities were within this range, but DO levels were at about the cutoff (6 mg/L^{-1}). The short sampling windows (i.e. one week) in Tampa Bay and the IRL prohibit any interpretation of temporal patterns of occurrence or relationships between environmental variables and positive detections. More comprehensive eDNA surveys are required to assess temporal patterns in the presence of *P. pectinata* in non-core range habitats, and whether presence is linked to environmental parameters or water quality; particularly since some of these estuaries and their watersheds are designated as Impaired Waters under the Clean Water Act (U.S. Environmental Protection Agency, 2020) as a result of anthropogenic threats including oil and gas pollution (e.g. *Howard Star*, Getter, Scott, & Michel, 1981; *Deepwater Horizon*, Balmer et al., 2018), hydrological changes (Dybas, 2002; Day et al.,

2003), and rising levels of pathogenic microorganisms (e.g. Chigbu, Gordon & Strange, 2004; Lapointe et al., 2015).

All aspects of this study were designed to maximize the likelihood of capturing and detecting target DNA while maintaining rigorous data analysis standards to allow for high confidence in positive detections. However, eDNA surveys did not provide evidence of *P. pectinata* DNA at sites in Tampa Bay, which has been suggested to be one of the first locations where recovery could occur given its proximity to the core range (Brame et al., 2019). Interpretation of negative results such as these requires careful consideration of the caveats associated with the sampling regime, genetic assay, and proportion of the samples that were ultimately screened for target DNA (e.g. Pinfield et al., 2019). The inability of the eDNA tool to detect target DNA may indicate that *P. pectinata* was not present in the estuary at the time of sampling, however, positive detection relies on the successful capture and detection of target DNA in water samples. To increase the chances of capturing target DNA in these surveys, water samples were collected from the bottom of the water column since *P. pectinata* is a benthic species (see Supplementary Material Item 2). The sampling was also designed to target the shallow depths primarily used by juveniles in their critical habitat (Norton et al., 2012), due to their representation in recent sawfish encounter reports in the surveyed estuaries (National Sawfish Encounter Database; NMFS, 2019, unpublished data). The volume of each water sample in this study (3 L) was smaller than some other studies that have targeted threatened elasmobranchs (e.g. 5 L, Gargan et al., 2017), and some eDNA studies also collect duplicate water samples at a single site (see Table 1 in Rees et al., 2014). In theory, the larger the volume of water collected, the greater the chances that it contains target DNA

(Sepulveda et al., 2019); however, the time involved with collecting and filtering large volumes of water or duplicate samples at single sites reduces the number of sites that can be sampled in a single day (see Mächler et al., 2016). Here, forgoing duplicate samples at single sites, and instead collecting smaller volumes of water at each site, allowed for sampling more sites across each estuary while remaining within the 24-hour time limit for filtration. This spatial coverage is especially important when eDNA surveys are designed for rare species based on limited or anecdotal (i.e. encounter derived) data, which can introduce bias when selecting potential areas for surveys.

If *P. pectinata* DNA is successfully captured in water samples, it may still evade detection due to sampling error stemming from screening only a portion of each filter and subsequent DNA extract for target DNA. Despite increasing the proportion of each sample screened for target DNA from samples collected in 2019, there were still no positive detections in Tampa Bay and the frequency of positive detections did not increase for the IRL. While it might be considered ideal to analyze the entire sample for each site, the increased cost would be prohibitive. It was also preferred to retain some of the filter for archiving purposes in case contamination occurred during DNA extraction or for future use, when more advanced technologies become available. Finally, while this study used a highly sensitive ddPCR™ assay to detect minute quantities of *P. pectinata* DNA (see Lehman et al., 2020), the assay was designed to only target a single locus (mtDNA ND2; Lehman et al., 2020). Therefore, the inability to detect target DNA in some samples reflects the absence of this locus; other *P. pectinata* genes may be present in water samples and DNA extracts. There is little information available on the relative decay rates of different genes, although eDNA studies have shown that the frequencies of

positives can differ depending on the target locus (Zhan et al., 2014; Wood et al., 2019). Future studies should consider targeting multiple loci for *P. pectinata* to accommodate this bias and potentially increase detection probabilities (e.g. Dobnik et al., 2016).

Samples that met two of the three criteria for positive detections highlight a critical consideration when analyzing ddPCR™ results for eDNA studies focused on threatened or listed species: the potential trade-offs of false negatives (i.e. where target DNA is present in the sample, but is not detected) and false positives (i.e. where target DNA is "detected", despite not being present). The possibility that samples that met two of the three positive detection criteria could represent false negatives cannot be ruled out. However, these samples either had target DNA concentrations that fell below the limit of detection of the assay, or had droplet patterns that were difficult to differentiate from possible errant PCR artifact droplets (i.e. droplets that did not fall within the normal range for the assay), making the data unreliable. Further, the ddPCR™ eDNA assay used here has the ability to detect ~0.25 pg of target DNA, inherently reducing the frequency of false negatives (Lehman et al., 2020) compared to other PCR platforms (see Wood et al., 2019). Applying such rigorous thresholds for positive detections increases the confidence in the data and reduces the likelihood of false positives (Klymus et al., 2019). Since the primary purpose of conducting eDNA surveys for *P. pectinata* in U.S. waters is to monitor and assess recovery of this species, positive detections from eDNA surveys need to be supported by rigorous methods and data analyses to reduce the possibility of false positives, ultimately increasing the utility of these data in management (see Sepulveda et al., 2020). False positives in historically occupied non-core range habitats could have substantial implications for the management of *P. pectinata*; for example,

they could be used as a line of evidence to prematurely support down-listing this species on the ESA. A high frequency of samples in a study area of interest that meet some, but not all three analysis criteria, may merit further investigation, through additional eDNA surveys.

3.6 Conservation Implications

This study provides evidence of *P. pectinata* DNA in the IRL in consecutive years (2018–2019) and in the Mississippi Sound in 2018. When these data are combined with recent sawfish encounter reports in both areas, as well as recently available fishery-independent survey data from the IRL (see Roskar et al., 2020), this indicates that *P. pectinata* is likely present in these historically occupied, non-core range habitats. Robust outreach efforts (e.g. events, signage) should be implemented in these areas to inform the public about the presence of this Critically Endangered species (see Wiley & Simpfendorfer, 2010; Waters et al., 2014).

Prior to initiating surveys using traditional gear types, Sepulveda et al. (2020) recommends conducting eDNA surveys until multiple positive detections are observed across multiple surveys. There were multiple positive detections in both the IRL and Mississippi Sound during 2018 surveys, but these results were not replicated in additional surveys (e.g. only one positive detection in the IRL in 2019); therefore, further eDNA surveys are warranted in both the IRL and Mississippi Sound. Comprehensive and long-term eDNA surveys in each of these estuaries could reveal any seasonal patterns of occurrence of *P. pectinata*. When combined with particle modeling scenarios, which could allow for estimates of the source locations of eDNA, these data could potentially be used to investigate spatial patterns of occurrence within each area. Results of such studies

could then be used to direct surveys using traditional gear types to conduct telemetry studies and collect tissue samples for genetic analyses. Comparable comprehensive eDNA surveys should also be conducted in other historically occupied habitats in the non-core range. As in this study, survey locations could be prioritized based on recent public sawfish encounter reports and/or historic reports of small juveniles or gravid females, which are indicative of possible nurseries, and the presence of suitable habitat. Alternatively, annual or biennial eDNA surveys could be conducted across all recovery regions in the summer to generate baseline occurrence data to better support early detection and re-emergence of *P. pectinata*.

Local and traditional ecological knowledge (see Poulakis & Grubbs, 2019) and public encounter reports (see Dulvy et al., 2016) suggest that *P. pectinata* may still occasionally occur from Guinea-Bissau to Liberia in the eastern Atlantic, and in the waters of several countries of the western Atlantic, including Cuba, Honduras, and Belize (Carlson, Wiley & Smith, 2013). In the Bahamas, Andros and Abaco islands have been identified as priority areas for research (see Harrison & Dulvy, 2014) due to recent encounters with *P. pectinata* and the presence of large areas of suitable habitat (e.g. mangrove shorelines) for both juveniles and adults (Guttridge et al., 2015). With careful consideration of methodological caveats (Lehman et al., 2020), conducting eDNA surveys in these areas could improve our understanding of the occurrence and status of *P. pectinata* more widely in the Atlantic, and aid in identifying and prioritizing locations that warrant additional research, ultimately fostering recovery of this Critically Endangered species.

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3.7 Supporting Information

3.7.1 Supplementary Figure 1: Benthic Sampler

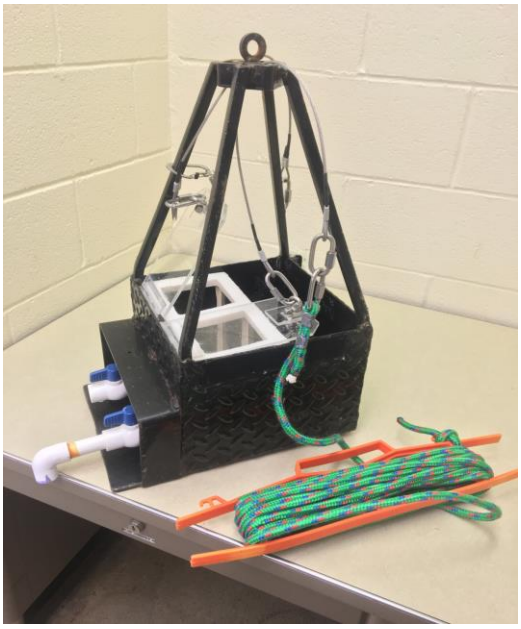

Figure 3.5 *Benthic Sampler*

Supplementary Material Figure 1. Watertight, custom designed and fabricated 3 L bottom-water sampler (model: VDBS-3L, Deep South Samplers, LLC). The 3 L sample compartment is divided in half, each with its own door and flow valve as a failsafe in the event of a malfunction of one side in the field.

3.7.2 Supplementary Item 2: Environmental DNA Yields in Bottom vs. Surface Waters

To date, many eDNA studies have collected and analyzed water samples from surface waters (see Rees et al., 2014) due to the ease of collection, or the ecology (i.e. life history, habitat use) of the study species. However, by binding to particulate matter in the water column, eDNA can sink and potentially settle into the benthos (see Turner et al., 2015). Due to this transport, collecting bottom water samples may increase the amount of DNA captured during targeted species surveys, and may be the preferred approach when targeting a benthic species (e.g. smalltooth sawfish, *Pristis pectinata*).

To compare total eDNA yields from water samples collected from surface and bottom waters, 3 L water samples were collected, in triplicate, from ~30 cm above the substrate and ~30 cm below the surface (9 L total at each depth) using a watertight, custom fabricated benthic sampler (model: VDBS-3L; Deep South Samplers, LLC; see Supplementary Material Figure 1). Water samples were collected from one site (depth ~1 m) ~1.2 km off the southeastern side of Deer Island, Mississippi in November 2018. Samples were filtered and DNA extracted from filters using the protocols described in Lehman et al. (2020). Analysis of the negative controls found no evidence that contamination occurred during any stage of sample processing.

The mean quantity of total eDNA recovered from bottom water samples was significantly higher (67.5 ng/µL; SE = 3.70) than those from surface samples (22.7 ng/µL; SE = 1.45) (Student's t-test; $P<0.001$) (Supplementary Material Figure 2). When viewed on an agarose gel, eDNA extracts from benthic samples contained high molecular weight DNA, but surface samples did not. While the reported NanoDrop™ values may not be accurate in absolute terms (see O'Neill et al., 2011), there was a substantial difference (e.g. >40 ng/µL) between average concentrations (Supplementary Material Figure 2). Although these data are limited, the finding of higher concentrations of DNA in bottom water samples, combined with the benthic behavior of the target species, suggests targeting bottom waters may be the preferred approach for eDNA surveys targeting *P. pectinata*. It remains unclear, however, whether sampling bottom water increases the probability of capturing *P. pectinata* DNA in the sampled study areas, as this may ultimately depend on local water circulation patterns (see Turner, Uy & Everhart, 2015).

3.7.3 Supplementary Figure 2: Bottom vs. Surface DNA Concentrations

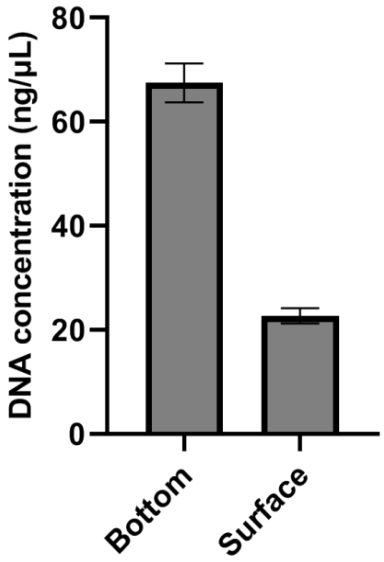

Figure 3.6 *Bottom vs. Surface DNA Concentrations*

Supplementary Material Figure 2. Mean (SEM) environmental DNA concentration yields (ng/µL) for 3 L water samples collected from ~30 cm above the bottom (depth ~1 m; n = 3) and ~30 cm below the surface (n = 3) near Deer Island, Mississippi.

CHAPTER IV – CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

4.1 Conclusions

This research developed and validated an eDNA tool capable of detecting Smalltooth Sawfish, *Pristis pectinata*, DNA from water samples and then applied the tool to investigate the occurrence of this species in non-core, but historically occupied, areas of their former range in United States (U.S.) waters. Specifically, a species-specific genetic assay capable of targeting only *P. pectinata* DNA at concentrations as low as 0.08 copies/µL was developed and optimized for the Droplet Digital™ PCR (ddPCR™) platform (see Chapter 2; Lehman *et al*. 2020). The ability of this assay to detect *P. pectinata* DNA in water samples was validated using samples collected from the Caloosahatchee River, a known, occupied, *P. pectinata* nursery in southwest Florida (SWFL) (Lehman *et al*. 2020). This assay was also capable of detecting an increase in the concentration (copies/µL) of *P. pectinata* DNA in water samples collected from beside a live juvenile in a time series (0 and 30 minutes) during an additional *ex situ* experiment in the same nursery (Lehman *et al*. 2020). A rigorous, three-criteria analysis protocol was developed to increase the confidence of positive detections and minimize the likelihood of false positives. Collectively, the specificity and sensitivity of the developed assay, combined with rigorous data analysis, indicate it can provide robust evidence of *P. pectinata* DNA in water samples collected during eDNA surveys in U.S. waters (Lehman *et al*. 2020).

The developed eDNA tool was used to investigate the occurrence of *P. pectinata* in non-core range areas of their former range in U.S. waters, where anecdotal evidence (*e.g.*, encounter reports from the public) suggested individuals were present (see Chapter

3). During the summer, eDNA surveys were conducted in Tampa Bay and the Indian River Lagoon (IRL) in Florida (FL) in 2018 and 2019 and in the Mississippi Sound, Mississippi (MS) during only 2018. *Pristis pectinata* DNA was detected in water samples collected during 2018 in the IRL and Mississippi Sound, and the IRL in 2019, demonstrating that that at least one individual was recently present within the survey window in the vicinity of the sample site, and that the eDNA tool can be used to detect *P. pectinata* in formerly occupied habitats of their historic range. Ultimately, these eDNA survey data provide another line of evidence supporting that *P. pectinata* are re-occurring in two formerly occupied areas in U.S. waters.

4.2 Caveats and Limitations of the Data

The resultant data may be influenced by aspects of the study design implemented here (*i.e.*, sampling regime, sample processing methods, assay design). Logistics and cost associated with using random sampling limited the sample size (n = 30) per season in this study, potentially under-representing positive detections. Based on observed site fidelity exhibited by juvenile sawfish (see Poulakis *et al*. 2011; Scharer *et al*. 2017), directed sampling was incorporated to potentially increase the chances of positive detections by targeting the locations of recent verified encounter reports. However, only one directed site yielded a positive detection, suggesting that random sampling may be the optimal approach for regime design in non-core range areas. During sample processing, variable filtration times were observed across three different brands of nylon filters (*i.e.*, Whatman®, Steriltech®, Cole-Parmer®), suggesting not all filters of the same type are manufactured equally. It is unknown if this affected the DNA capture efficiency, and therefore the frequency of detection; however, it is strongly recommended that future

research use primarily Whatman® brand filters, as these processed water most efficiently. Marker choice may also introduce bias into the conclusions reported here by limiting amplification to only one small fragment of *P. pectinata* DNA (see Beng *et al*. 2020). Therefore, the absence of a detectable target fragment in water samples collected during this study does not necessarily reflect the absence of the species. This study targeted a small (100 bp) fragment of the mitochondrial DNA NADH dehydrogenase subunit 2 gene (mtDNA ND2); however, it is possible that only *P. pectinata* mtDNA sequences from other genes (*e.g.*, CO1, ND4) remained intact in some samples at the time of amplification (see Stat *et al*. 2017).

Perhaps the most notable limitation of our eDNA survey data is the inability to draw any spatial or temporal links to the target DNA source (see Eble *et al*. 2020). In marine systems, advection can transport eDNA large distances from the source (2–4 km; see Baker *et al*. 2018; Andruszkiewicz *et al*. 2019), or quickly diffuse the initial concentration below detectable limits over a wide geographic area. These effects are thought to be less pronounced in nearshore and intertidal areas (O'Donnell *et al*. 2017; Kelly *et al*. 2018) like those sampled in this study, and our eDNA assay was designed for maximum sensitivity; however, we cannot determine the locations of the DNA source(s). Temporally, target DNA may persist for up to ~48 hours after being shed in estuarine study areas (see Strickler *et al*. 2015; Collins *et al*. 2018). Current research indicates *P. pectinata* can move an average of ~1.5 km per day, depending on life history stage (Carlson *et al*. 2014). Should the size of the DNA source be ≥2 m, it could be absent from the vicinity of the study area by the time of sample collection, limiting the management utility of these data.

4.3 Future Research Directions

To address caveats and limitations, future research should consider incorporating improvements to study design, and exploring additional techniques that improve the utility of eDNA data. Due to the uncertainty surrounding the distribution of *P. pectinata* in non-core range study areas, the number of sites to be sampled was maximized; however, more sites necessitated smaller sample volumes (3 L) due to excessive filtration times and sample processing costs. Recent research has shown that processing larger volumes of water (*e.g.*, ≥5 L) may increase detection probabilities (see Schabacker *et al*. 2020; Sepulveda *et al*. 2019); therefore, future eDNA research targeting *P. pectinata* in these study areas may benefit from selecting sites based on prior survey data and collecting larger sample volumes at fewer sites. In contrast, eDNA surveys targeting large geographic areas or investigating previously unexplored non-core range areas may benefit from collecting replicate samples of smaller volumes at each site, increasing the total number of sites that can be sampled and the overall volume of water collected in the study area, while still increasing detection probabilities. Furthermore, eDNA surveys for *P. pectinata* may benefit from the use of a multiplexing approach targeting two or more sequences on different genes, which has been shown to improve detection probabilities (Stat *et al*. 2017). These surveys should also consider targeting fragments of varying length to account for unequal molecular decay and variation in gene copy number between individuals (Bylemanns *et al*. 2018; Beng *et al*. 2020).

Future surveys targeting *P. pectinata* can potentially benefit from the addition of Langragian particle monitoring, a spatio-temporal computer modeling exercise (see Andruszkiewicz *et al*. 2019), to predict the transport of *P. pectinata* eDNA in the

presence of flow and advection in marine systems. Additional testing to determine decay and settling rates will be required for eDNA molecules in each study area (see Andruszkiewicz *et al*. 2019); however, by "backtracking" molecules to their estimated point of origin, spatio-temporal modeling stands to revolutionize the field of threatened species eDNA research by potentially allowing scientists to comment on habitat use and the "age" of the DNA.

Future research could potentially use eDNA methods to estimate abundance, genetic diversity, and population structure of *P*. *pectinata*, which are key data for determining the status of populations in conservation studies (Begon *et al*. 2005; Adams *et al*. 2019). *Ex situ* eDNA studies have shown species relative abundance is positively correlated with eDNA concentration on the ddPCR™ platform (Doi *et al*. 2015b; Uthicke *et al*. 2018). Population structure has been quantified using eDNA, identifying previously known haplotypes from Whale Shark, *Rhincodon typus*, eDNA (Sigsgaard *et al*. 2016), and successfully assigning Killer Whale, *Orcinus orca*, eDNA to known cetacean ecotypes (Baker et al. 2018). Recent research has identified ddPCR™, the highly sensitive platform the *P*. *pectinata* eDNA assay is optimized to, as optimal for these types of studies via its accuracy in quantifying species abundance or allelic diversity (Uthicke *et al*. 2018; Adams *et al*. 2019). However, before eDNA can be routinely used in these capacities, additional knowledge is required to determine the influence of primer design (Piñol *et al*. 2019), eDNA shed rate (e.g., Sassoubre *et al*. 2016), and eDNA ecology (see Barnes *et al*. 2016).

Recent encounter reports suggest eDNA surveys should be expanded to encompass more areas of their historic range. Within non-core range areas in U.S. waters,

encounters with *P. pectinata* have been reported over the last ~5 years in the waters near Apalachicola, Panama City, and Pensacola, FL, and near the Chandeleur Islands, Louisiana in the northern Gulf of Mexico (National Sawfish Encounter Database, NMFS 2019, unpubl. data). Given the historical importance of these locations (see Goode 1884; Bigelow & Schroeder 1953), these areas are the logical next steps in expanding eDNA surveys for *P. pectinata* in U.S. waters.

Encounter reports also suggest *P. pectinata* are present in other areas of their historic range outside of U.S. waters (see Dulvy *et al*. 2016). At present, the Bahamas represents the only other area besides SWFL where *P. pectinata* can be reliably encountered (Brame *et al*. 2019), and the availability of suitable habitat (Guttridge *et al*. 2015), combined with largely unenforced national protections, marks it as a high priority area for research (see Harrison & Dulvy 2014) and is well suited for comprehensive eDNA surveys. Local and traditional ecological knowledge (LEK, TEK) suggests individuals may also still occur in Mexico, Belize, Honduras, and Cuba in the western Atlantic, and from Guinea-Bissau to Liberia in the eastern Atlantic (Carlson *et al*. 2013). The frequency of reports varies within each of these areas, but combining eDNA surveys targeting *P. pectinata* with outreach or citizen science can help direct sampling efforts in these areas, and increase the geographic reach of research (see Biggs *et al*. 2015; Poulakis & Grubbs 2019). With additional *a priori* testing, the eDNA assay developed for *P. pectinata* may be suitable in the aforementioned areas (see Lehman *et al*. 2020), and in any additional area(s) LEK or TEK identifies for surveys.

4.4 Conservation Implications

Currently, eDNA detections center around presence/absence determinations, and the uncertainty surrounding false detections makes management decisions based on these data alone difficult, especially for protected species like *P. pectinata* (Sepulveda *et al*. 2020). Environmental DNA survey data can become more useful in management via the incorporation of larger sample volumes , multiplexing PCRs, and Langragian particle monitoring, by adopting strict contamination control protocols (see Goldberg *et al*. 2016), and giving careful consideration to methodological caveats(Lacoursière-Roussel & Denier 2019). Additionally, the creation of a decision-making tree (see Figure 1 in Sepulveda *et al*. 2020) may also serve to increase the utility of eDNA data in management, and serve as a guide for conservation strategies concerning eDNA research and *P. pectinata*.

This study provides evidence of *P. pectinata* in two of three formerly occupied areas with recent encounter reports and suitable habitat via eDNA surveys (*e.g*., Chapter 3); however, to avoid prematurely allocating funds or effort in these areas based on these data alone, positive detections should serve as a "trigger" to begin additional sampling to acquire multiple lines of evidence. First, a second round of eDNA surveys should be implemented in the IRL and Mississippi Sound to attempt to duplicate positive detections and, optimistically, increase the spatial coverage of surveys within each estuary. However, rather than be repetitive, these surveys should build on prior data by incorporating a comprehensive outcome (*e.g*., temporal or spatial). For example, in the IRL, monthly surveys across the estuary for one year would serve to accomplish both aforementioned goals, while providing insight on seasonal use of the estuary (if any).

Any areas with multiple positives in multiple eDNA surveys should serve as targets for preliminary non-molecular sampling (*e.g.*, netting). In Tampa Bay, where preliminary eDNA surveys did not provide evidence of *P. pectinata* presence, reports should be monitored for a period of 1–2 years, and surveys should be re-initiated after that time, or if a major increase in report volume is identified.

All additional eDNA surveys targeting *P. pectinata* in the IRL and Mississippi Sound, and in other non-core range waters, should begin to incorporate new or existing techniques (*e.g.*, spatio-temporal modeling, outreach) and existing datasets (*e.g.*, Florida Atlantic University Land/Ocean Biogeochemical Observatory), when appropriate, that bolster the utility of eDNA data. Outreach efforts (*i.e.*, angler education) should continue in these two estuaries, prioritizing locations with reports and eDNA evidence. Outreach efforts (*i.e.*, signage) should be developed and implemented in any additional historic areas in U.S. waters where evidence of *P. pectinata* occupation is probable through existing reports. These additional areas could also serve as targets for wide scale surveys to assess the occurrence of *P. pectinata* across all formerly occupied habitats in U.S. waters, providing the ability to measure patterns of recovery (if any) as management proceeds. Ultimately, the combination of robust data from comprehensive eDNA surveys and clear management actions, similar to those mentioned above, delineated through decision trees (see Figure 1 in Sepulveda *et al*. 2020) can assist in guiding recovery efforts for the Critically Endangered Smalltooth Sawfish throughout its historic range.

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