5-2021

Assessing the Occurrence of Trichechus manatus latirostris in Mobile Bay, Alabama Using eDNA

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by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2020

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ABSTRACT

In the past several decades Florida manatees (*Trichechus manatus latirostris*) have begun to expand their summer range into the northern Gulf of Mexico. Because this is a recent occurrence, not much is known about their habitat use and distribution in this region. Citizen-sourced sighting data suggests that Florida manatees frequent subembayments of Mobile Bay, Alabama, reaching a sighting peak in August. To assess the occurrence of manatees in this area environmental DNA surveys were used from winter (February 19-20) and summer (August 21-22) of 2018. At each of the 21 sites ranging from the mouth of Mobile Bay, to the Mobile-Tensaw Delta, Mobile-Tombigbee River, and Tensaw-Alabama River, 5 × 1 L water samples were collected. An additional water sample was collected from an *ex situ* experiment to gain a positive eDNA sample. This was done by adding feces and flesh from a deceased Florida manatee then collecting the water sample 30 minutes later. All water samples were vacuum-filtered, extracted for DNA, and run on Droplet Digital™ Polymerase Chain Reaction. A previously developed ddPCR assay was used to amplify a 69-base pair segment of the *cytochrome b* gene. The assay was able to detect 77.2 copies/μL of target DNA in the positive eDNA sample, 1.180 copies/μL in the 1:10 dilution, and 0.211 copies/μL in the 1:100 dilution of this sample. One summer field sample met one out of three criteria while another met two out of three criteria for a positive detection. There was evidence of contamination in several negative control samples that highlights the importance of negative controls in eDNA experiments.

Keywords: eDNA, Manatee, ddPCR, Sirenia, Mammal, Conservation

DEDICATION

To my parents, for their neverending support.

ACKNOWLEDGMENTS

This research was funded by The University of Southern Mississippi and was supported by the Mississippi INBRE, funded under Institutional Development Award (IDeA) number P20-GM103476 from the National Institutes of General Medical Sciences of the National Institutes of Health. Thanks to Marcus Drymon, Katherine Schweiss, Ryan Lehman, Emily Seubert, Matthew Jargowsky, Katya Jagolta, Garrett Dunee, Sarah Cunningham, Ariel Williams, and Morghan Ellis for the collection and filtration of field water samples. Thanks to Joshua Speed, Michael Garrett, London Williams, Natalie Wilson, and Osvaldo Rivera Gonzalez for providing accommodations at University of Mississippi Medical Center (UMMC) and for use of Droplet Digital™ PCR equipment. Thank you to Ruth Carmichael and Elizabeth Hieb for providing the positive manatee sample as well as information on manatee sightings. Additional thanks to Ryan Lehman and Annmarie Fearing for their continued support and guidance. Special thanks to Nicole Phillips for always pushing me to do my best. Lastly, thank you to the Honors College for providing assistance every step of the way.

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LIST OF ABBREVIATIONS

ddPCR	Droplet Digital™ Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
eDNA	Environmental DNA
ESA	Endangered Species Act
IUCN	International Union for the Conservation of Nature
PCR	Polymerase Chain Reaction
U.S.	United States
USFWS	United States Fish and Wildlife Services

CHAPTER I: Introduction

Manatees

Family Trichechidae consists of three species of manatees: the Amazonian manatee *(Trichechus inunguis*), the African manatee (*Trichechus senegalensis*), and the West Indian manatee (*Trichechus manatus*) (Domning and Hayek 1986). The West Indian manatee is further divided into Antillean (*Trichechus manatus manatus*) and Floridian (*Trichechus manatus latirostris*) sub-species (Deutsch *et al.* 2008). Members of *T. m. manatus* are found in the Caribbean as far south as Brazil, whereas *T. m. latirostris* is found in coastal waters of the southeastern United States (Deutsch *et al.* 2008). All members of Family Trichechidae are considered Vulnerable by the International Union for the Conservation of Nature's (IUCN) Red List of Threatened Species (Deutsch et al. 2008, Keith Diagne 2015, Marmontel *et al.* 2016).

Manatees are aquatic mammals that typically have an 11-month gestation period after which they nurse their young for one to two years (Rathbun *et al.* 1995). Due to their life history, manatees have slow population growth rates. These mammals reach sexual maturity around 5 years of age and a female will typically give birth to one calf every 2-5 years (Rathbun *et al.* 1995). Manatees can live to be 60 years of age with the oldest known manatee living to be 69 years old in captivity (Allen *et al.* 2014). Individuals are typically solitary except for the years spent with their calves, during breeding, and when congregating in warm waters during winter (Rathbun *et al.* 1995, Laist *et al.* 2013). Mating occurs between one female manatee and a mating herd of a dozen or more males (Rathbun *et al.* 1995). There is not a strict breeding season although

the typical peak begins in March and ends in September of each year (Rathbun *et al.* 1995).

The Florida Manatee

Distribution

Trichechus manatus latirostris is endemic to the southeastern United States (U.S.) (Bossart *et al.* 2003). These animals do not tolerate cold waters and prefer water temperatures above 20 °C (Bossart et al. 2003). They inhabit the coastal waters of Florida all year round due to the warmer temperatures in this region (Bossart *et al.* 2003). From March to November some individuals identified through scar patterns on their back are known to travel all along the Gulf of Mexico coast from Texas to Florida (Aven *et al.* 2016, Deutsch *et al.* 2008). They also travel along the east coast in these non-winter months and have been seen as far north as Rhode Island (Deutsch *et al.* 2003). Little is known about the spatial use patterns of manatees in the warmer months although some hypotheses have been made that they are searching for seagrass meadows or mates (Deutsch *et al.* 2003, Bengston 1981, Rathbun *et al.* 1995). Studies have shown that adult males have higher daily travel rates and lower site fidelity than adult females in the months of March through September, which is the main breeding season (Rathbun et al. 1995). In winter months, migratory manatees return to the warmer waters of Florida due to temperature stress (Laist *et al.* 2013). If water temperatures fall below 20 °C, manatees experience cold stress that can result in emaciation or mortality (Bossart *et al.* 2003). Within Florida, many manatees seek shelter in freshwater artesian springs or around power plant thermal outfalls (Laist *et al.* 2013).

Four regional subpopulations of *T. m. latirostris* are delineated in the Florida Manatee Recovery Plan based on distribution in summer and use of winter warm-water refugia (USFWS 2001). Studies indicate that there is little exchange between the subpopulations based on telemetry and photo-identification (Rathbun *et al.* 1990, Weigle *et al.* 2001, Deutsch *et al.* 2003). Research suggests that there are high rates of gene flow between geographic regions of Florida although studies of gene flow have not yet been done for the specific subpopulations (McClenaghan and O'shea 1988). The northwest subpopulation ranges from Pasco-Hernando County (on the west central coast of Florida) along the Florida panhandle into Louisiana (Deutsch *et al.* 2008). In the past 10 years, this subpopulation has seen an annual growth rate of 3.7% (Deutsch *et al.* 2008).

Threats and Status

Historically, *T. m. latirostris* faced threats from direct exploitation through hunting for their meat, bones, and hide (Nowacek *et al.* 2004). Today, protections have been put in place although manatees are still threatened by cold stress, habitat loss, algal blooms, natural disasters, and boat strikes (Nowacek *et al.* 2004). Over 30% of annual manatee deaths are caused by collisions with boats (Nowacek *et al.* 2004). The manatee photo-identification database also shows that 97% of individuals had scar patterns from boat strikes (O'shea *et al.* 2001). Red tides, which are caused by a neurotoxin producing dinoflagellate, *Karenia brevis*, can be ingested by manatees and cause mortality (Deutsch *et al.* 2008). Nutrient run-off from excessive fertilizer use and livestock can worsen red tides when nutrients are loaded into the system and increase phytoplankton blooms (Deutsch *et al.* 2008). The U.S. population of *T. m. latirostris* has also been shown to

have relatively low genetic diversity compared to other placental mammals based on 18 microsatellite markers in 362 manatees (Tucker *et al.* 2012). This may have been caused by a recent genetic bottleneck or colonization of manatees from the West Indies (Cantanhede *et al.* 2005). Reduced levels of genetic diversity could make *T. m. latirostris* more susceptible to anthropogenic and stochastic events (Tucker *et al.* 2012).

In the United States, *T. m. latirostris* was listed as Endangered under the Endangered Species Act (ESA) of 1973 (Adimey *et al.* 2016). Aerial surveys of Florida manatees began in 1991 and estimated the population to be 1,267, which has since increased to 6,300 (USFWS 2019). As a result, *T. m. latirostris* was downlisted by the U.S. Fish and Wildlife Service to Threatened under the ESA in 2017 (Department of the Interior, Fish and Wildlife Service 2017). They are also legally protected in U.S. waters under the Marine Mammal Protection Act of 1972 and the Florida Manatee Sanctuary Act of 1978 (Adimey *et al.* 2016). Under the ESA, which requires the implementation of a recovery plan, the U.S. Fish and Wildlife Service's recovery plan has begun efforts to minimize boat collisions (USFWS 2001). These efforts have included education, scientific research, and increased enforcement strategies to ensure boater compliance in manatee speed zones in areas where there has been a high rate of collisions (USFWS 2001). Rehabilitation programs have become prevalent for manatees and they may be rescued for reasons including: cold stress, boat strikes, or exposure to red tide toxins (Adimey *et al.* 2016). A study used telemetry tags to evaluate Florida manatees that had been rehabilitated from 1988 to 2013 (Adimey *et al.* 2016). It found that 72% of wild-born manatees were successful at least one-year post-rehabilitation (Adimey *et al.* 2016).

Florida Manatees in the Northern Gulf of Mexico

In recent years the northern Gulf of Mexico has begun to experience tropicalization (Heck *et al.* 2015), with an increase in seasonal mean water temperatures (Fodrie *et al.* 2010). Summer bottom-water temperatures have seen an average increase of 0.051 $^{o}C\ y^{-1}$ from 1985 to 2015 (Turner *et al.* 2017). Many tropical species that were not historically found in this region have become established, including tropical species like the emerald parrotfish (*Nicholsina usta*), green turtles (*Chelonia mydas*), black mangroves (*Avicennia germinans*), and warm-water coral species (e.g., *Acropora palmata*) (Heck *et al.* 2015). The first members of *T. m. latirostris* were sighted in the Gulf of Mexico in the early 1900's (Heck *et al.* 2015). The introduction of tropical species into this region may begin to impact food-web structure (Heck *et al.* 2015). Members of *T. m. latirostris* are herbivorous and primarily feed on seagrass and macroalgae (Lefebvre *et al.* 1999). Many invader species moving into the area also feed on seagrass, which could potentially cause top-down effects on these seagrass systems (Heck *et al.* 2015).

Sightings of *T. m. latirostris* have also become more frequent in the northern Gulf of Mexico in the last several decades (Hieb *et al.* 2017). In Alabama, members of *T. m. latirostris* frequent local river systems and sub-embayments of Mobile Bay (Hieb *et al.* 2017). Further, in recent years, more manatees have also been reported using rivers and subembayments of the nearby Mississippi Sound (Hieb *et al.* 2017). Sightings in these areas are reported year-round with peaks in Alabama occurring in August for live sightings and in December through February for carcasses (Hieb *et al.* 2017). In the northern Gulf of Mexico, seagrasses are not common so manatees must find an alternative food source (Sturm *et al.* 2007, Vittor *et al.* 2016). Instead, they feed on plants

such as coontail (*Ceratophyllum demersum*), milfoil (*Myrophyllum spicatum*), and southern naiad (*Najas guadalupensis*) (Sturm *et al.* 2007, Vittor *et al.* 2016).

Little is known about the temporal occurrence, or of frequently used 'hotspots' (i.e. high-use sites), of *T. m. latirostris* in the northern Gulf of Mexico (Hieb *et al.* 2017). Brackish near-shore waters in this region are typically highly turbid, making aerial surveys challenging and prone to sampling bias (Hunter *et al.* 2018). Environmental DNA (eDNA) is a non-invasive monitoring technique that could fill these knowledge gaps and allow us to understand travel corridors, hotspots, range limits, and distribution of manatees in the northern Gulf of Mexico (Hunter *et al.* 2018). An eDNA assay detects genetic material that organisms have shed into the environment, which can be anything from skin cells, to blood, gametes, saliva, or feces (Hunter *et al.* 2018). This eDNA can be collected in water samples and screened for DNA from species of interest (Thomsen and Willerslev 2015). These eDNA approaches are often much more sensitive than other methods (i.e. quantitative PCR) which is important for analyzing turbid waters with low concentrations of DNA (Evans *et al.* 2017, Goldberg *et al.* 2011). Here, eDNA methods were used to conduct surveys for *T. m. latirostris* in estuarine and freshwater habitats in Mobile Bay to (1) utilize an eDNA assay to assess the presence of *T. m. latirostris* in Mobile Bay, AL and (2) understand if eDNA is a tool that can be used to study changing distributions of *T. m. latirostris*.

CHAPTER II: Methods

Laboratory & Field Controls

To minimize the risk of cross-contamination between samples or from outside sources of DNA, strict laboratory controls were used (see Lehman *et al.* 2020, Schweiss *et al.* 2020). A combination of sterilizing techniques was used depending on the materials; cleaning with 10% bleach, autoclaving at 120° C, and/or exposure to ultraviolet (UV) light for 15 minutes. To further prevent contamination between stages of sample processing, water filtration, DNA extractions, and PCR amplifications were performed in separate laboratories. Negative controls were implemented at every stage and analyzed through to PCR. Autoclaved deionized water was brought onto the boat and stored on ice until filtration as field negatives (Drymon *et al.* 2021). Filter negatives contained autoclaved deionized water and were filtered and processed through PCR (Drymon *et al.* 2021). Aerosol barrier filter pipette tips were used during DNA extractions, and ddPCR reactions used them to add eDNA to the reaction with designated eDNA pipettes. Additionally, DNA extraction negatives did not contain particulate matter and PCR negatives contained no DNA. Negatives were only considered free from contamination if they met zero criteria for a positive detection (see Data Analysis).

Water Sample Collection, Filtration, & DNA Extraction

Forty-two water samples were collected as described in Drymon *et al.* (2021) from Mobile Bay, Alabama estuarine and riverine systems in the winter (February 19-20, 2018) and summer (August 21-22, 2018). Environmental data was also taken at all sites (Table 1). There were five estuarine sample sites in Mobile Bay, six sites in the Mobile-Tensaw delta and ten freshwater sites in the Mobile-Tombigbee river and the Tensaw-

Alabama river (Figure 1). Each sample site was 15-25 km apart and 5 x 1 L water samples were collected at each site. Water samples were collected 0.5 m below the surface of the water using 1 L high-density polyethylene Nalgene bottles that had been cleaned in 10% bleach solution and exposure to ultraviolet (UV) light (Schweiss *et al.* 2020). Water samples were stored on ice until filtration using a vacuum pump, that occurred within 24 hours of collection, or were frozen until filtration could occur. Samples were then filtered in a precleaned laboratory space where *T. m. latirostris* tissue had never been present. Water samples were inverted three times to ensure they were evenly mixed before being vacuum-filtered with 47-mm-diameter, 0.8-um nylon filters (Schweiss et al. 2020). Filters were replaced every ~350 mL when they became clogged (i.e. ~3 filters per 1 L) and then preserved using 95% ethanol at room temperature (Schweiss *et al.* 2020). Extractions for total eDNA used ¼ of each filter following the Goldberg *et al.* (2016) QIAGEN® DNeasy™ Blood & Tissue Kit protocol, which incorporated the QIAshredder spin columns (Schweiss *et al.* 2020). In order to determine the quality of the DNA extracts, 2% agarose gel was assessed, and DNA quantities were measured using Thermo Fisher Scientific NanoDrop™ spectrophotometer technology (Schweiss *et al.* 2020).

Table 1. Environmental Data Environmental data collected from each site in the winter (February 19-20, 2018) and summer (August 21-22, 2018).

Station	Latitude	Longitude	Depth (m)	Temperature (°C)		Salinity (psu)		Dissolved Oxygen (mg/L)	
				Winter	Summer	Winter	Summer	Winter	Summer
1	30.256	−88.0510	4.7	14.00	28.5	2.61	23.86	9.66	6.04
2	30.438	−88.0110	5.2	14.80	28.1	1.28	14.84	9.89	6.52
3	30.538	−87.9970	5.6	13.10	27.6	0.33	13.00	9.50	7.15
4	30.666	−88.0250	1.6	11.80	28.6	0.74	4.17	9.44	6.65
5	30.771	−88.0250	1.4	12.10	30.0	0.08	1.49	9.23	6.75
6	30.914	−87.9630	5.1	11.60	30.3	0.07	0.08	9.09	6.94
7	31.056	−87.9860	4.6	11.50	29.7	0.07	0.09	9.20	6.87
8	31.246	−87.9467	4.8	11.70	29.5	0.07	0.10	9.33	6.84
9	31.340	−87.9215	8.2	11.30	29.0	0.06	0.10	9.38	6.92
10	31.447	−87.9172	5.9	11.50	30.0	0.06	0.12	9.23	7.65
11	31.587	−88.0569	5.4	11.50	30.4	0.06	0.12	9.32	8.08
12	31.757	−88.1290	4.3	11.40	30.7	0.06	0.12	9.27	7.82
13	31.611	−87.5505	4.9	11.50	29.2	0.06	0.07	10.67	8.50
14	31.499	−87.5505	7.5	11.70	29.1	0.06	0.07	10.50	7.81
15	31.405	−87.6931	2.8	11.70	29.7	0.07	0.07	10.62	7.56
16	31.296	−87.7651	5.0	12.40	29.4	0.07	0.07	9.97	7.50
17	31.200	−87.8731	5.0	12.10	29.8	0.06	0.07	9.67	6.87
18	31.027	−87.9560	5.0	12.40	29.2	0.07	0.08	9.00	6.50
19	30.930	−87.9220	1.7	13.70	31.1	0.07	0.09	8.98	7.88
20	30.734	−87.9340	6.2	13.20	30.2	0.07	0.12	9.24	7.06
21	30.644	−87.9270	5.1	13.10	30.5	0.07	0.20	9.20	7.56

Droplet Digital PCR Assay

Forward (5’-CGCTAACCGCATTCTCTTCAG-3’) and reverse (5’-GGTAGCGAATGA TYCAACCATAGTT-3’) primers and an internal PrimeTime® 106 double-quenched ZEN™/IOWA 107 Black™ FQ probe (5’-CCCACATTTGCCGAGAC-3’) labeled with 6-FAM at the 5’ designed by Hunter *et al.* (2018) were used to amplify a 69 base pair portion of the *cytochrome b* gene in *T. manatus*. The assay had previously been optimized as described in Hunter *et al.* (2018), with the addition of an automated droplet generator step. The total reaction volume of the mixture was 25 ul, with 4 ul of each DNA extract, 12.5 uL of ddPCR mix (BioRad), 250

nM of the probe, and 800 nM of each of the forward and reverse primers. DdPCRs were run using the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System (Droplet Generator instrument no. 773BR1456, Droplet Reader instrument no. 771BR2544). An automated droplet generator combined 70 ul of automated droplet generation oil for probes (BioRad) with 20 ul of sample PCR mix to create up to 20,000 nanofluidic droplets. Optimal ddPCR cycling conditions included 1 cycle at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 60°C for 1 min, and finally 1 cycle at 98°C for 10 min. The ddPCR amplifications were performed for five plates in replicates of five, except for one plate of control negatives in which only three replicates were performed.

Positive Control Water Samples

A positive *T. m. latirostris* eDNA sample was obtained via an *ex situ* experiment to ensure the eDNA assay was fully optimized prior to screening field samples. A surface water sample was collected from coastal Mobile Bay, Alabama waters in October 2020 and placed in a pre-cleaned tub. A 1 L water sample was collected and stored in a high-density polyethylene Nalgene bottle from this tub prior to the addition of any genetic material. Feces collected in October of 2014 from the Mobile Tensaw Delta and tissue collected from a *T. m. latirostris* carcass in January of 2019 was added to the water. After 30 minutes the positive eDNA sample was collected and placed in a 1 L high-density polyethylene Nalgene bottle. The samples were immediately frozen and thawed completely at room temperature prior to filtration, which occurred three weeks later. These samples were processed using the protocols described above. The ddPCR also followed the protocols described above and five replicates of each sample were run.

Additionally, five replicates of a 1:10 dilution and 1:100 dilution of the positive eDNA water sample were run in order to determine the positive droplet range for data analysis.

Data Analysis

Analysis of ddPCR results were done using QX200™ Droplet Reader and QuantaSoft™ Rare Event Detection (RED) analysis. Positive detections for *T. m. latirostris* DNA had to meet three criteria: (1) droplets fell above the manual threshold of 3,472 (see Hunter *et al.* 2018), (2) droplets above the manual threshold were within the positive droplet range for the assay (5,000-7,000 RFUs), defined using the positive eDNA control and (3) the concentration of target DNA was at or above the Limit of Quantification (LoQ) of 0.185 molecules/μL (see Hunter *et al.* 2018). A positive detection was defined for *T. m. latirostris* at least one ddPCR replicate for a sample met all three of these criteria.

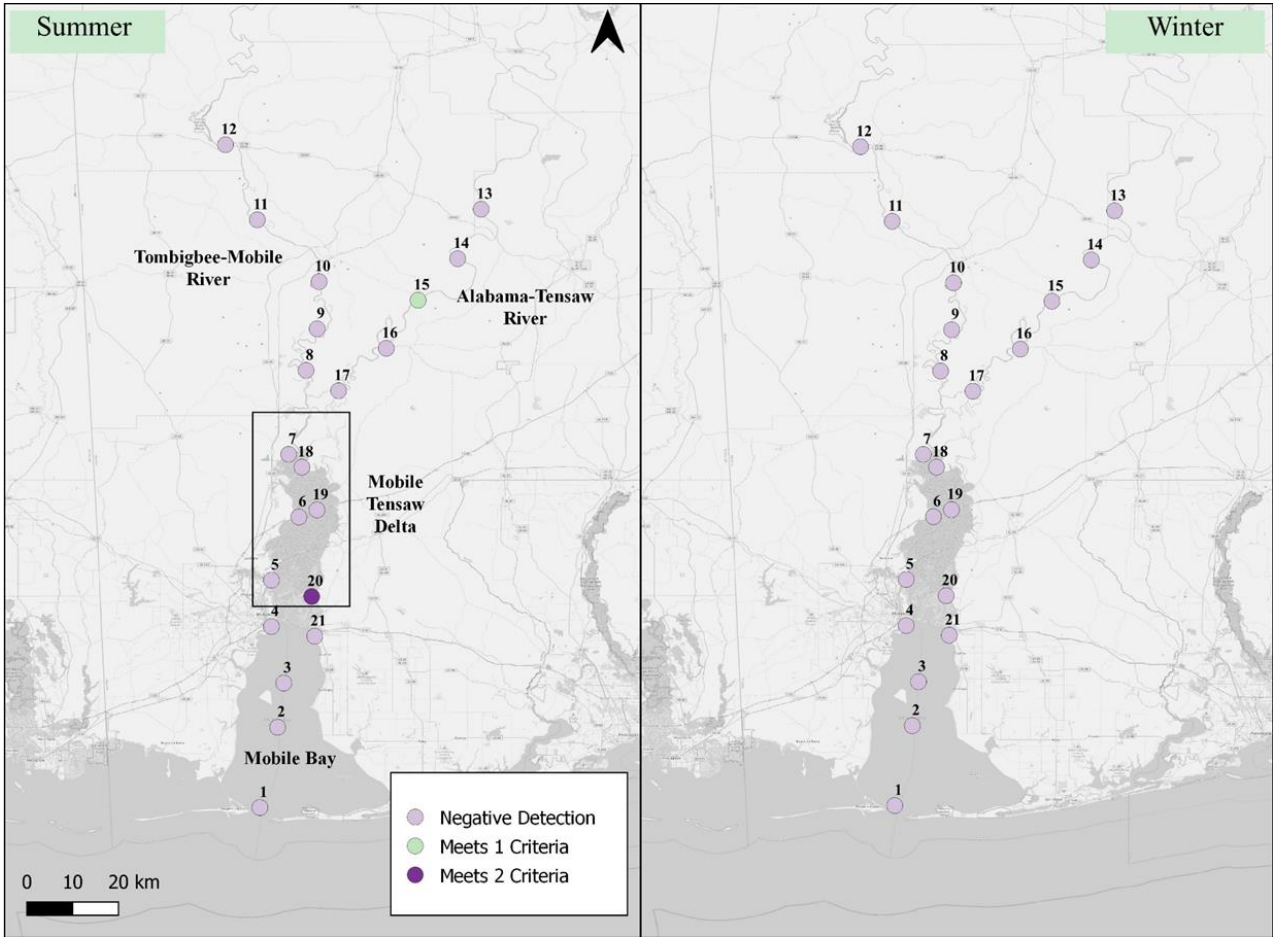

Figure 1. eDNA Sample Sites The surveyed environmental DNA sites are shown for manatees from the Mobile Bay, Mobile Tensaw Delta (black box), Alabama-Tensaw Delta, and Tombigbee-Mobile River. Summer (August 2018) and winter (February 2018) water sample sites are represented by circles. Negative detection sites are light purple, samples that met one out of three criteria to merit a positive detection are green, and samples that met two criteria are dark purple.

CHAPTER III: Results

Positive Control Water Sample

In the *ex situ* positive eDNA experiment, the sample taken before eDNA was added met zero criteria for a positive detection (Figure 2). An average of 77.2 copies/ μL (SE = 5.33) was found from the sample taken 30 minutes after *T. m. latirostris* flesh and feces had been added. For the 1:10 dilution there were 1.180 copies/μL (SE = 0.528) and 0.211 copies/μL (SE = 0.094) for the 1:100 dilution of the positive eDNA water sample. All replicates of the positive *T. m. latirostris* eDNA sample as well as the 1:10 dilution and 1:100 dilution met all three criteria for a positive detection. Although, one replicate of the filtration negative from the processing of the positive control water sample met two criteria for a positive detection (criteria 1 and 2). A ddPCR negative replicate from the positive eDNA sample plate also met two criteria (criteria 1 and 2). The extraction negative met zero criteria for a positive detection.

eDNA Field Samples

In the winter samples (February 2018) none of the field samples met any of the three criteria for positive detection. Negative controls for the filtration, and all DNA extraction negatives were evidenced to be free from contamination. However, a collection negative replicate met two criteria (criteria 1 and 2). Additionally, one ddPCR negative replicate, which contained the filtration negative control, also met two criteria (criteria 1 and 2).

In the summer (August 2018) no field samples met all three criteria for a positive detection. Negative controls for the collection, filtration and DNA extraction negatives were all free from contamination. One ddPCR replicate from a field water sample (Site

15; see Table 1) in the Alabama-Tensaw River met one criterion (criterion 1) for a positive detection (Figure 1). Additionally, two replicates for a second field sample met two criteria for a positive detection (criteria 1 and 2) (Figure 1). This sample was taken from Site 20 in the southernmost region of the Mobile Tensaw Delta (Figure 1; Table 1). One ddPCR negative control replicate, which was run with the summer samples from Sites 15 and 20, met one of the criteria for a positive sample detection (criterion 1).

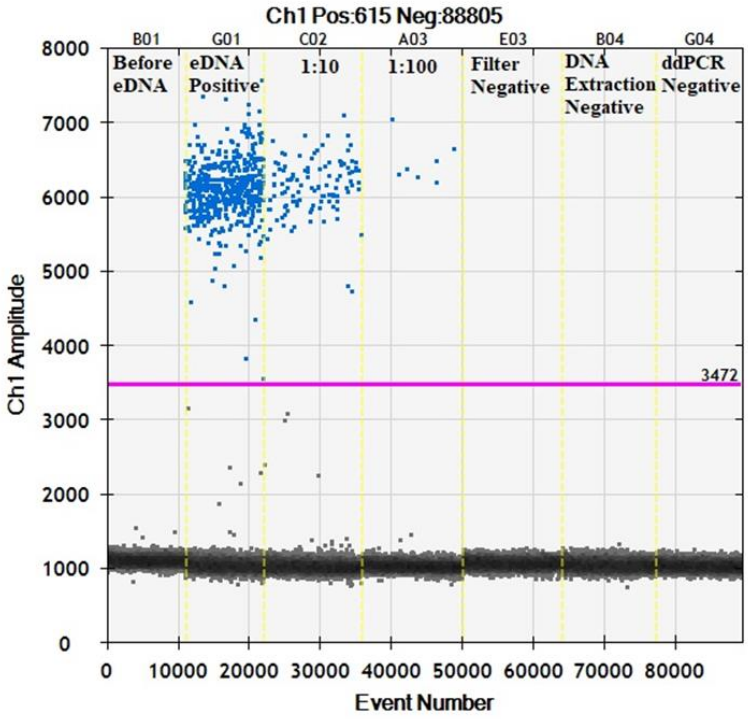

Figure 2. eDNA Positive Experiment ddPCR™ Raw data of ddPCR™ products from the eDNA positive experiment of *Trichechus manatus latirostris* is shown. An *ex situ* experiment was performed using a water sample collected from Mobile Bay (October 2020) which was the before eDNA sample. The positive eDNA sample corresponds to the sample collected 30 minutes after manatee tissue and feces were added to the sample. The corresponding 1:10 dilution of the positive sample, 1:100 dilution, filter negative, DNA extraction negative, and ddPCR negative are also included. Wells are separated by vertical lines and each sample is labeled. Droplets were considered positive (blue droplets) or negative (gray droplets) based on a manual threshold (above 3,472 amplitude) using the QuantaSoft™ Rare Event Detection analysis.

CHAPTER IV: Discussion

Developed eDNA assays have proven valuable in detecting target DNA to assess the occurrence of different taxa in previous studies (Lehman *et al.* 2020, Schweiss *et al.* 2020, Drymon *et al.* 2021). The eDNA assay previously designed by Hunter *et al.* (2018) was demonstrated to be working via the validation experiment where a positive control eDNA sample and the 1:10 and 1:100 dilutions of this sample met all three criteria for a positive sample. There was evidence of contamination in the filtration negative from the positive control eDNA sample and in one collection negative from the winter samples, which both met two criteria for a positive detection. Summer field samples from Site 15 met one criterion (criterion 1) and from Site 20 met two criteria (criteria 1 and 2) for a positive detection (Figure 1). There was evidence of contamination in ddPCR negatives associated with the eDNA positive sample, the summer field samples, and the winter samples filtration negative. All winter field samples, filter negatives, and extraction negatives met zero criteria for a positive detection. Contamination in this study most likely occurred due to cross-contamination between samples and potentially improper cleaning of field sample materials. Future studies should understand that even with the implementation of strict protocols and negative controls contamination can still occur and time should be left over for re-filtration, re-extraction, and re-run of samples on ddPCR if needed.

The water samples used in this study were previously collected and filtered for an eDNA project studying bull sharks (*Carcharhinus leucas*) (see Schweiss *et al.* 2020). The boat used to collect field samples was ensured to not have been in recent contact with *C. leucas* eDNA but could have been with *T. m. latirostris* eDNA. This could have been a

source of contamination which may have contaminated the collection negative for the winter samples. The collection negative may have then contaminated the associated ddPCR negative. The collection negative would need to be re-extracted and re-run on ddPCR to ensure contamination did not occur during the first extraction. Sources of contamination should be considered from multiple taxa during eDNA field surveys and boats should be thoroughly cleaned. This may also highlight the need for boats that are only used for eDNA work to ensure proper cleaning protocols are followed.

The Mobile Bay habitat is unique in that it offers manatees desirable conditions (e.g., high freshwater input, large quantities of fresh and brackish vegetation) for a portion of the year although they cannot stay there year-round due to temperature limitations (Aven *et al.* 2016, Dingle and Drake 2007). Because of this, peak sightings in Alabama of *T. m. latirostris* occur in August. Summer field samples taken from sites 15 and 20 both met two criteria for a positive detection (criteria 1 and 2) and were associated with a contaminated ddPCR negative. The ddPCR negative was most likely contaminated from the field samples through poor pipetting technique. To ensure this was the source of the contamination the ddPCR would need to be re-run. Although, the field samples did not meet all three criteria to warrant a positive detection, this would align with the peak sighting data as the samples were also collected in August (Drymon *et al.* 2021, Hieb *et al.* 2017). Citizen-sourced sighting data in Alabama does not include sightings further north than the Mobile Tensaw Delta (Figure 1). Although, Site 20 which met one criteria for a positive detection (criterion one) is in the Mobile Tensaw Delta region which has had high numbers of sightings of *T. m. latirostris* from 2007-2014 (Figure 1; Hieb *et al.* 2017).

According to citizen-sourced data, sightings in Alabama have increased 8-fold from 2007-2014 to historical data from 1978-2004 (Hieb *et al.* 2017). The greatest number of Alabama sightings of *T. m. latirostris* occurred in rivers and subembayments of Mobile Bay (Hieb *et al.* 2017). Therefore, it would have been expected that more samples in these areas (e.g., the Mobile Tensaw Delta) met all three criteria for positive detection. According to the environmental data (Table 1) the average temperature in summer was 29.6 °C (SE = 0.192) and 12.3 °C (SE = 0.215) in winter. This evidence supports the fact that all winter samples met zero criteria for a positive detection as *T. m. latirostris* only tolerates temperatures 20 °C and above and were more likely to be absent during this sampling period (Bossart *et al.* 2003). Environmental parameters (i.e., salinity and DO) were considered normal based on recorded parameters from 2003-2011 (Tetra Tech 2012). The environmental data also indicates that conditions were more favorable for *T. m. latirostris* in the summer months. Lack of samples meeting all three criteria may then have been due to sampling bias.

Sampling methods could be improved in order to ideally gain more positive detections for target DNA. Water samples could be taken from subembayments such as Dog River, Fowl River, Weeks Bay, and Wolf Bay in Alabama which were not sampled in this study. Manatees are also known benthic feeders meaning they spend most of their time at the bottom of the water column (Marshall *et al.* 2003). In order to increase the chances of capturing target DNA, water samples should be taken from the bottom of the water column (see Lehman *et al.* 2020). Additionally, the detection of *T. m. latirostris* DNA may not occur due to only ¼ of the filter and DNA extract being screened for target DNA. Although, this is preferred especially in cases of contamination so there is a chance

to re-extract samples. The ddPCR assay designed by Hunter *et al.* (2018) only targeted a 69 base pair of the *cytochrome b* gene. Water samples may have had other *T. m. latirostris* genes present which is why target DNA was not detected.

False eDNA positives have the ability to undermine the credibility of eDNA as a means of detecting imperiled taxa (Ficetola *et al.* 2016). A false positive may occur for a number of reasons including from contamination during sampling, filtration, DNA extraction, or ddPCR (Ficetola *et al.* 2015). Because this method is so sensitive it is very important to have measures to prevent false positives, which is why three criteria must be met for a sample to be considered positive for target DNA (Goldberg *et al.* 2011). In this case, a false positive could give inaccurate information on the ability of eDNA to detect manatee DNA as well as potentially present false information on their spatial occurrence.

Human error most likely caused the contamination in this study. Because the DNA concentration for the positive eDNA experiment was so high (77.2 copies/μL) the possibility of contamination of the filtration negative was higher and could have easily occurred during filtration, extraction, or PCR. Contamination during filtration could have come from spray or drips of water from other samples. This can be avoided by cleaning surfaces frequently with 10% bleach and cleaning immediately if any obvious drips occur. To minimize chances of contamination, fully encapsulated filter membranes have also been developed that minimize the risk of DNA transferring from one sample to another (Thomas *et al.* 2019). Some are even partially biodegradable, which reduces single-use plastic waste (Thomas *et al.* 2019). Although, due to the PCR negative from the positive eDNA experiment meeting criteria 1 and 2 there was some cross-contamination that occurred during the ddPCR. Poor pipetting technique could have

resulted in contamination of this step (e.g., droplets on the side of the pipette falling onto the plate). This could be prevented by re-extracting the filtration negative and ensuring clean lab protocols are met as well as ensuring proper pipetting technique to make sure there is no spray or cross-contamination from droplets during the ddPCR.

Variability in seasonal temperatures is resulting in the changing distribution of *T. m. latirostris* in the northern Gulf of Mexico (Hieb *et al.* 2017). Because these mammals are listed as Threatened under the Endangered Species Act (ESA) of 1973 their movements and distribution are of concern (Adimey *et al.* 2016). Although, their population has increased in recent years they face many anthropogenic and biological threats (Nowacek *et al.* 2004). Manatees may rely on habitats in the northern Gulf of Mexico in warmer months which would make this ecosystem extremely important to their survival. Further eDNA studies in the northern Gulf of Mexico could allow us to learn more about their distribution and habitat-use in this area.

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