CLOUDY WATERS: THE EFFECT OF FISH DENSITY AND SEDIMENT INPUT ON ENVIRONMENTAL DNA PERSISTENCE

by

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(Under the Direction of Byron Freeman and John Wares)

ABSTRACT

The use of environmental DNA for the detection of imperiled and/or difficult to detect species has seen a surge in popularity in recent years. In this study, we designed and tested a species-specific eDNA marker to aid in the detection and conservation of an imperiled Southeastern U.S fish, the Robust Redhorse (*Moxostoma robustum).* We investigated the effects of fish density and turbidity on eDNA detection to better understand how to use eDNA tools in highly turbid and dynamic aquatic environments like those in the Southeastern U.S. We found rapid eDNA degradation in a laboratory setting in agreement with previous studies, and that fish density, sediment, primer selection, and experimental vessel size all had significant effects on eDNA detection odds. With this study we hope to aid in the monitoring of an imperiled, charismatic Southeastern fish and inform future work on the capabilities of eDNA as a conservation tool.

INDEX WORDS: eDNA, Southeastern Biodiversity, degradation, conservation, monitoring, imperiled species

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CHAPTER 1

INTRODUCTION

The use of environmental DNA (eDNA) as a means of detection for imperiled or hard-to-find aquatic organisms is quickly becoming a common practice among researchers and management agencies alike. eDNA provides a wide range of information for a minimal time investment in the field, typically only requiring the filtration of a few liters of water. Recently, eDNA tools have been applied to categorize the species assemblage of areas **(Coghlan et al. 2021),** to detect invasive species and track their distribution **(Piaggio et al. 2014),** and to aid in the detection of rare or cryptic species **(Bonfil et al. 2021)**. While eDNA has the potential to be a powerful tool for species detection and management, species specific tools require extensive development to ensure specificity and effectiveness as bulk water samples can contain DNA from a wide host of organisms.

As important as a highly specific eDNA marker is for elimination false detections, if no eDNA is present in an environment then the application of such a tool would be useless. The question of eDNA persistence and degradation is one that has been explored extensively though not exhaustively. Temperature, UV radiation, and microbial activity have all been negatively associated with eDNA persistence time while sediment has been found to increase eDNA persistence time **(Barnes et al. 2014)**. Many prior eDNA studies have been done in clear, cold headwater streams where there is little to no sediment input **(Wilcox et al. 2013, Mckelvey et al. 2016, Izumi et al. 2017).** Sediment, which can be a primary contributor to high turbidity levels, was of particular interest to us as our target species, the Robust Redhorse (*Moxostoma*

robustum), inhabits the Altamaha, Pee Dee, and Savannah River systems which can have extremely high sediment input and turbidity levels. In order to effectively create a tool for the detection of *M. robustum* in its natural environment, the effect of sediment on eDNA detection and degradation was of the utmost interest to us.

Our first objective in this study was to develop a highly specific eDNA marker to aid in the detection and management of *M. robustum*. In conjunction with South Carolina Department of Natural Resources (SCDNR), we identified multiple unique segments of *M. robustum* mitochondrial DNA and developed unique primer/probe pairs to test against other closely related fishes in the family Catosotmidae that can be found alongside *M. robustum* in its natural environment. After testing, we selected one highly unique primer/probe pair developed at UGA and one developed by SCDNR to use to further our second objective.

Our second objective for this study was to obtain and compare the degradation rates of *M. robustum* eDNA under different conditions. We tested degradation rates in both tanks and bottles at two different captive fish densities, and in a separate trial introduced sediment in the form of river water to experimental samples in order to better understand its effect on eDNA detection. We predicted that degradation rates would not significantly differ between high and low fish densities, however samples from the high density treatments would be detectable longer due to more eDNA being present in the samples. Additionally, we predicted that sediment would increase the persistence of eDNA in our trials due to either binding to and protecting the eDNA molecules or deactivating extracellular nucleases **(Barnes et al. 2014).** An understanding of how long we can detect eDNA under differing conditions in addition to detailed natural history information about *M. robustum* gives us our best chance to utilize these tools to their maximum effectiveness.

CHAPTER 2

DESIGN AND USE OF A NEW ENVIRONMENTAL DNA TOOL FOR MONITORING AN IMPERILED SOUTHEASTERN FISH, THE ROBUST REDHORSE (*MOXOSTOMA ROBUSTUM)*

Introduction:

The use of environmental DNA (eDNA) markers is rapidly becoming the tool of choice for researchers to detect aquatic species quickly and efficiently. eDNA is organismal DNA that is available for capture in the natural environment and commonly takes the form of skin cells, mucous, or reproductive material. This material can be used in a variety of ways including categorizing the species assemblage of an area **(Coghlan et al. 2021)**, early detection of invasive species **(Piaggio et al. 2014)**, or detection of rare or cryptic species**. (Bonfil et al. 2021)** While the use of eDNA tools for species detection is becoming more and more common, there are still many uncertainties in their creation and deployment particularly for a focus on single species detection. Bulk water samples from the field can contain DNA from a host of organisms, and therefore if the goal is the detection of a single species, then marker specificity is of the utmost importance

Traditional methods for detection of fish species include seine nets, electroshocking, and/or visual surveys; however, these methods are heavily reliant on good field conditions and are time and labor intensive. Additionally, the risk of injury and/or mortality due to handling is a consistent possibility which is an important consideration when working with imperiled species. Despite this, traditional sampling methods ensure accurate identification of collections and can provide a wide

range of information including habitat use and local densities. Conversely, the use of highly specific eDNA markers allows for equal or greater sampling success with significantly less front end resource investment **(Ardura et al. 2015, Mckelvey et al. 2016)**, typically only requiring water samples from the field site for lab processing**.** In the case of rarely observed or captured organisms, the use of an eDNA marker provides a unique opportunity to improve on the detection success of traditional methods.

The primary goal of this project was to develop a highly specific eDNA marker to aid in the detection and management of an imperiled catostomid fish endemic to the southeastern United States. The Robust Redhorse (*Moxostoma robustum*) (**Cope, 1869)** is a large, full-bodied fish in the family Catostomidae. After collections in the 1980's and 1990's in Georgia and North Carolina, it was realized to be a fish described by Cope in 1870 as *M. robustum.* As part of an assessment of the status of southeastern fishes in 2000, *M. robustum* was thought to be endangered in part or all of its current range **(Warren et al. 2000)**. Currently, populations of *M. robustum* are known in the Ocmulgee and Oconee rivers (GA), the Savannah River (GA/SC), and the Pee Dee River (NC/SC). *M. robustum* is typically found in the mainstem of these rivers in deep water and is known to spawn on coarse gravel at a small number of known sites. (GADNR, 1999**)** These systems are large, turbid environments and make detection by traditional means difficult. The application of a highly specific *M. robustum* eDNA detection tool could greatly increase our ability to locate these cryptic fish and identify potential habitat usage in new areas.

An eDNA tool for the detection of *M. robustum* would consist of three parts: a forward 5' primer, a reverse 3' primer, and a fluorescent probe. The primers would serve as a starting point for DNA synthesis to occur on the template strand of DNA, and the probe serves as a marker for a unique fragment of DNA within the sample. The most important feature of such a tool would be

by necessity its specificity. In a 2013 study by**(Wilcox et al. 2013)**, primer specificity was found to be more important to specificity than probes, and an increase in mismatches within the primer sequence led to reduced amplification of conspecifics. A large number of closely related fishes in the family Catostomidae also call the Altamaha, Savannah, and Pee Dee systems home **(Bagley et al. 2018) (Warren et al. 2000)**. To avoid the detection of non-target species, or false positives, a highly unique region of *M. robustum* DNA is needed to ensure the accuracy of the detection.

In order to identify a unique segment of *M. robustum* DNA, we analyzed three different mitochondrial genes (COi, ND2, and Cyt-B) using a combination of published sequences on GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and sequences that were amplified in the lab. Each primer set was tested against closely related confamilial species using both traditional PCR and more sensitive qPCR assays to determine which tool was most appropriate for use in the laboratory testing and field analysis. The final marker was tested in addition to a similarly created tool from SC DNR to determine its effectiveness and efficiency in the amplification of *M. robustum* DNA. The final products are two distinct mitochondrial markers that are both highly specific and provide the ability to quickly detect the presence of *M. robustum* at low concentrations, even in the company of its closely related conspecifics.

Methods:

Tissue extraction and amplification

In order to identify unique segments of *M. robustum* DNA for use in primer development, we began by extracting tissue from Georgia Museum of Natural History (GMNH) catalogued specimens of *M. robustum* as well as other co-occurring species. In addition, the Georgia Department of Natural Resources provided a collection of catostomid species from tributaries of both the Oconee and Savannah Rivers. Bodies were frozen and fin clips were stored in 95% ethanol

and received a GMNHTC catalog number. Tissues were extracted from 2-3 fin rays per specimen using the Puregene Method (Gentra Systems) and stored in dna-free water at -20 ℃. Genomic DNA was PCR amplified for 4 distinct mitochondrial gene regions using universal primer pairs including COI (**Folmer et. al 1994**), MIFISH **(Miya et al. 2015),** Cyt B (**Merritt et. al 1998)** and ND2. Successful amplicons were prepared for Sanger sequencing and sequence data generated at Psomagen. Returned sequences were trimmed and aligned using both Codon Aligner (https://www.codoncode.com/aligner/) and Geneious V 11.0.5 (https://www.geneious.com).

Primer/Probe Design

In total, 14 species of catostomids were included in the alignments in a mixture of sequences extracted in house and using sequences that were already available on GENBANK (https://www.ncbi.nlm.nih.gov/genbank/) including specimens of the same species from different river systems to account for intraspecific diversity. Alignments were scanned by eye to identify short (100-200bp) sites of high interspecific diversity among the sequences and then target regions were evaluated through Primer 3 software (https://primer3.ut.ee) to test for suitability. COI and Mifish were both removed from the selection process due to lack of divergence sites between *M. robustum* sequences and at least one other co-ocurring catostomid that were long enough to insert a fluorescent probe and new sets of primers.

Two sets of primers from ND2 and two sets from Cyt-B were selected for preliminary testing due to at least 3 mismatches between *M. robustum* and all other species in both the 5' and 3' primer sequences using the Primer 3 program. The forward and reverse sequences are in Table 1. In addition, SC DNR designed a set of forward and reverse primers for Cyt-B that were sent to UGA for testing alongside those developed in-house. This primer set can also be found in table 1.

For each primer sequence identified, a fluorescent probe region was also chosen in the identified region between the primers. Unique SNP's were identified in the selection regions by eye. Probe specificity has been shown to be significantly less important than primer specificity for overall tool specificity **(Wilcox et al. 2013)** and therefore areas with at least 1 SNP difference between *M. robustum* and the conspecific were considered suitable for use. Probe 5' CCTCCCTAGCCCTATTTTCACCTAA 3' was selected as it provided additional specificity for Cyt B_690 F/797 R.

Initial qualitative PCR was performed without the probe present in the master mix as the focus was solely on primer specificity. PCR was performed using a reaction volume of 25 uL containing 20 uL of TAQ man PCR master mix, 2 uL of the forward and reverse primers, and 1 uL of template DNA diluted to a concentration of ~20 ng/uL. Thermal cycling conditions of 95 ℃ for 5 s, then 40 cycles of 95 ℃ for 15 s, 55 ℃ for 30 s, and 72 ℃ for 30 s, and a final extension step at 72 ℃ for 5 minutes. A negative PCR blank was included in each run to control for contamination. PCR results were visualized on a 10% agarose gel and sent to PSOMAGEN for confirmation sequencing.

Confamilial testing using Qualitative PCR

To ensure primer specificity, each primer set was tested using DNA from each of the conspecific catostomid species in the laboratory. We prepared 25 uL reactions using 20 uL TAQman PCR mastermix, 2 uL forward and reverse primer each, and 1 uL of template DNA. The starting reaction was the same as what was used for initial testing; thermal cycling conditions of 95 ℃ for 5 s, then 40 cycles of 95 ℃ for 15 s, 55 ℃ for 30 s, and 72 ℃ for 30 s, and a final extension step at 72 ℃ for 5 minutes. Each reaction was repeated twice, and a negative PCR blank was used to test for contamination.

Increasing the annealing temperature of a reaction can be used to increase the specificity of the primer tool as increased mismatches between the primer and template are less likely to bind tightly enough to avoid being separated by the heat of the reaction (Hillis et. al 1996). In order to increase the specificity of our primer sets, I ran each set of conspecific samples at increasing annealing temperatures. Starting at 55℃ and increasing to 55 ℃, 60℃, 62 ℃, 62.5℃, 65 ℃ and 70℃. Results were visualized on a 10% agarose gel, and any amplification fragments of non-target species was considered a false positive result. Altering the annealing time of PCR reactions can also be used to increase reaction specificity as decreased annealing time requires a better primer/template match to be successful. After an initial annealing time of 30 s was used for the temperature increase reactions, the reactions were then repeated using a 15 s annealing time to further increase specificity of reactions.

qPCR

After initial testing with qualitative PCR to identify which primer sets were suitable, we switched our focus to quantitative testing using qPCR. After two suitable primer sets were identified, conspecific testing was conducted on an Abi 7500 machine (Applied Biosystems) in order to visualize results via a series of dilution curves. We prepared 20 uL reactions using 10 uL of iTAQ, 2.7 uL water, 0.8 uL of both forward and reverse primers, and 0.7 uL of probe. thermal cycling conditions of 95 ℃ for 300 s, then 65 cycles of 95 ℃ for 15 seconds, 64 ℃ for 12 s and 72 ℃ for 45 s were used. Annealing temperatures were also tested again during this stage based on the results from previous trials. Annealing temperatures were tested for specificity at 62℃, 65℃, 68℃, and 69℃.

Field Validation

In order to test the effectiveness of this eDNA tool for field assays, we used archived filters taken from below active spawning *M. robustum*. Samples were taken in 2018 from Juliette Dam on the Ocmulgee River (33.1060° N, 83.7928° W) and were frozen at 20 C for later extraction and use. 5 samples were taken below Juliette Dam where actively spawning *M. robustum* were visually observed. One sample was taken above Juilette Dam to act as a field control as Juliette Dam presents an impassible barrier to upstream passage. This upstream sample works as an ideal field control as the area should contain co-occurring fish species but without *M. robustum* present.

Results:

Primer Selection

Of the 4 primer sets we identified as suitable candidates, only Cyt-B 690F/797R and Cyt B 409F-595R were determined to be successful at amplifying *M. robustum* template DNA. Both sets of ND2 primers failed to amplify over 4 trials for each set. Cyt-B 690F/797R and Cyt-B 409F-595R amplified *M. robustum* template equally well across all temperatures except for 70 ℃. At 70℃ annealing temperature, PCR products from either primer set were not visible on a 1% agarose gel.

Conspecific Testing using Qualtitative PCR

Cyt-B 690f/797R out performed Cyt-B 409F-595R in species specificity testing. At all temperatures, Cyt-B 409F-595R amplified two different samples of *Hypentelium nigricans,* a common catostomid species found sympatric with *M. robustum*. In addition, in two trials at 60 degrees annealing temperature, Cyt-B 409F-595R also amplified template from *Moxostoma rupiscartes*, another closely related catostomid species. Cyt-B 690F/797R was highly specific at all temperatures.

qPCR Conspecific Testing

Quantitative PCR (qPCR) is more sensitive than qualitative PCR, therefore further testing was required with the addition of the designed probe. Using primer Cyt-B 690F/797R at 62 degrees, *Moxostoma sp.* Brassy and *M. rupiscartes* amplified late in the run around 31 cycles. At 65 degrees, *M. rupiscartes* still amplified late in the run, around 37 cycles. At 69 degrees, only *M. robustum* amplifies with no conspecific amplification. Using primer set Cyt-B 1068F/1108R from South Carolina DNR at a 60-degree annealing temperature, *M. rupiscartes* amplified late in the run around cycle 40. At a 62-degree annealing temperature, only *M. robustum* amplified with no non-target amplification.

Field Validation

Using the 6 samples archived from Juliette Dam in 2018, we detected *M. robustum* in 4/5 samples downstream of Juliette Dam. We also successfully showed no amplification of 1/1 samples from upstream of Juliette Dam where no *M. robustum* should be present.

Discussion:

In keeping with the original project goal, we successfully created a highly specific eDNA tool for the detection of *M. robustum*, an imperiled and difficult to detect a southeastern catostomid sucker. Our results demonstrate how it is possible to develop unique markers that are able to differentiate between closely related species, however they also demonstrate that this process is not without its difficulties. Many studies do not detail the extensive troubleshooting required to fully vet a tool of this nature for consistent effectiveness. This is exemplified by the fact that we

identified many potential markers during preliminary alignments and following robust testing only one proved to be suitable for our purposes after significant manipulation to reaction conditions.

Other studies have shown similar difficulties in refining primer specificity. In a 2016 study by **(Uchii et al. 2016),** a similar marker was created for the detection and distinction of two closely related haplotypes of common carp (*Cyprinus carpio)*. The tool used in this scenario was unique to *C. carpio* however during conspecific testing the authors identified non-specific amplification of a closely related carp species common in Japan's waters, *Carassius cuvieri,* despite mismatches in the target sequences. The authors found that by limiting the PCR cycles to 40, they effectively increased the specificity of their tool as *C. cuvieri* only amplified after 39 cycles. This illustrates the need for extensive testing against closely related conspecifics even with unique primer/probe combinations, and that specificity can be achieved through, e.g., manipulations of cycle number, temperature, and annealing times.

Upon testing both UGA and SC DNR developed tools on archived field samples, we successfully detected *M. robustum* in 4/5 samples taken downstream of actively spawning fish and had 0 detections upstream of Juliette Dam where no *M. robustum* should be present. While this is a small sample size, it is an encouraging start to the field testing and validation of both tools. It is important that successful detection of *M. robustum* in the field occurred at lower annealing temperatures (69 vs. 65) than needed to successfully eliminate co-occuring species using high quality laboratory DNA stock. This is likely because eDNA quantity and quality are low under field conditions and could be indicative of few problems with false detections. Further field testing and sequencing of positive samples is currently underway.

eDNA markers are successfully being employed for the detection of a wide range of aquatic taxa including anguillids **(Burgoa Cardás et al. 2020)**, trout **(Wilcox et al. 2013)**, sturgeon **(Dejean et al. 2011),** and many more. While success with these taxa is encouraging, many questions remain about persistence of eDNA in aquatic environments. Detection can be highly variable in adverse environmental conditions and in the presence of high non-target species abundance. Continued diligence on marker specificity and robust testing of markers in both the lab and field will allow for greater versatility and success of eDNA markers in the future.

TABLES

Table 1.1: Forward and reverse primer pairs selected for further testing due to high specificity to M. robustum and the forward and reverse primer pair Cyt-B 1068F/1108R created by SC

Primer Pair	Forward Seq (5'-3')	Reverse Seq (5'-3')
CytB_690_F – CytB_797_R	CCCCTATTTTTCATATAAAGACCTCCTA	CTCACATTAAACCAGAATGATACTTTCT
CytB 409F – CytB 595R	CAGCTACTATCATTCATCTTCTCTTTC	GGA TCTCCTAATAGGTTAGGTGAAAATA
ND2 F1 – ND2 R1	GTTCCTTGCACTAAAACTATCATCAG	AAGTCATTTAGGTATGAATCCTGTTAGG
ND2 F2 – ND2 R2	CCTAACAGGATTCATACCTAAATGACTT	ACGATGTATCCCAATACAGTCAATTCTA
SC CytB 1068 F – SC CytB 1108 R	CGGACAAATTGCTTCCATC	AGCCTTGTTTTCCAGTCATCC

CHAPTER 3:

CLOUDY WATERS: THE EFFECT OF FISH DENSITY AND SEDIMENT INPUT ON EDNA PERSISTENCE

Introduction

The use of environmental DNA (eDNA) as a tool for the detection of hard-to-find aquatic organisms has quickly become a common practice for researchers looking to improve on traditional detection methods. Bulk water samples can be taken from the field in a fraction of the time required to effectively sample an area of similar size with seine nets or electroshocking. These water samples can effectively give us a snapshot of the species assemblage of an area, including the presence or absence of a target species **(Coghlan et al. 2021)**. This offers an attractive alternative to researchers who may face logistical challenges during their sampling period including short timeframes, adverse field conditions, or limited number of available personnel. Despite these advantages, many questions remain about the efficacy of eDNA persistence time especially under non-ideal conditions such as high flows or high turbidity.

With the surge of popularity in eDNA studies has come a similar increase of investigations into the best methods for refining the use of eDNA tools in both the lab and field. The method of extraction of eDNA has been a particularly targeted topic as the effectiveness of different capture and extraction methods seems to vary from laboratory to laboratory. While

most approaches will successfully capture and extract useable eDNA, many disagree on which method is best. In a 2016 study, the authors found that filtration of water samples through glass fiber filters yielded the most eDNA **(Eichmiller et al. 2016)** when compared to centrifugation and precipitation. Another study in 2017 similarly found that filtration yielded the most eDNA but asserted that cellulose nitrate filter paper of the same pore size resulted in better yields **(Hinlo et al. 2017)**Yet another study in 2022 published contrary results stating that centrifugation, not filtration, captured more starting material and yielded a higher overall recovery of eDNA regardless of extraction method **(Bockrath et al. 2022).**While these studies disagree on which method is most efficient, all prove that useable eDNA can be captured and successfully extracted using a variety of different methods.

The goal of this experiment was not to test which collection and extraction methods are most efficient, but rather to better understand how long eDNA can be used to successfully detect a target species. eDNA has been shown to persist anywhere from less than 1 day to over 58 days in some conditions **(Barnes et al. 2014, Strickler et al. 2015)**however in all cases it clearly follows an exponential decay rate. Additionally, the organisms we seek do not always exist in ideal conditions. UV light, microbial activity, temperature and more have all been shown to influence eDNA decay rates**(Barnes et al. 2014).** In the southeastern United States historic and current land use practices have had significant impacts on water conditions, and in large rivers with many inputs such as the Savannah and Altamaha high turbidity levels are commonplace. This could potentially confound the use of eDNA tools in these habitats, and warrant investigation.

To aid in our ability to detect an imperiled southeastern catostomid fish, *Moxostoma robustum,* we experimentally tested the degradation rate of *M. robustum* DNA under multiple

conditions including low density of fish, high density of fish, and differing treatments of turbidity. Samples were taken from vessels containing M. robustum DNA daily for 7 days, then once per week for a month. Using two highly specific eDNA markers for *M. robustum* detailed in the previous chapter, we tested detection success over time in order to assess the potential time windows required for the successful application of these tools in the field.

Methods:

High and Low Density Tank Studies

To build up eDNA for this study, approximately 200 *M. robustum* juveniles were received from SC DNR and transported to Whitehall aquatics lab at the University of Georgia where they were placed in approximately even numbers in 3, 300-gallon circular tanks. Fish were held for approximately 9 months prior to the start of the experiment at which point the tanks were fully cleaned, and fish were replaced following treatment specifications. For the high density treatment, 45 fish were placed in a single tank, and for the low density treatment 5 fish were placed in a separate tank. Tanks were covered with foam coverboards to prevent splashing and transfer of aerosolized droplets between tanks. Fish were kept in the treatment tanks for two weeks prior to their removal and the first eDNA samples being taken.

Sampling Methods

eDNA Capture

On day 0 of the experiment, we filled four, 2 liter bottles with tank water from each treatment. Water was taken from the top third of the water column, then the bottles were immediately capped and rinsed with city service tap water and covered in ice for transport to the laboratory. Upon arrival in the lab, each 2L bottle was sprayed with a 20% bleach solution and wiped dry after 10 minutes with a clean paper towel.

Bottles were placed on shakers and were kept constantly in motion at ~100 rpm in order to keep any eDNA suspended in the water column and relatively evenly distributed in the bottle. DNA capture was achieved via centrifugation following modified methods from **(Uchii et al. 2016, Bockrath et al. 2022).** Prior to each extraction, each 2L bottle was shaken vigorously, then uncapped and ~50 mL was poured into a sterile 50 mL falcon tube. For each sample, two 50 mL aliquots were taken, and the 2L bottle was capped and shaken between aliquots. Once all samples had been aliquoted into two falcon tubes each, the tubes were wiped down with 20% bleach. 2L bottles were also wiped down with 20% bleach again and placed on a shaker at room temperature to shake continuously. In addition to the 4 lab treatment samples (T1-T4), two 2L bottles were filled daily from the treatment tanks and transported to the lab for capture and extraction. This was to explore detection differences in larger volumes of water in a more uncontrolled setting than the closed system of a 2L bottle. An extraction blank consisting of a 2L bottle filled with tap water was also extracted along with each batch of samples to ensure that no contamination was present in the bleach treated 2L bottles reused for daily tank sampling. Samples were then centrifuged for 30 min at 5000 rpm at 4 °C. After centrifugation was complete, each sample was removed, and the supernatant was poured off into a waste container until the liquid was just below the conical tip of the falcon tube. Samples were then transported into a different room for extraction.

The sediment experiment followed the same protocol for capture of eDNA, however the sample set up was distinct from the high and low fish density trials. Three treatments were used: no sediment (control), low sediment, and high sediment. Two 2 L bottles were used for each treatment. The control samples were filled directly from a tank containing around 70 *M. robustum*, capped and rinsed, then placed on ice for transport back to the lab. For the low

sediment treatment, 500 mL of water was taken from the North Oconee River and added to a sterile 2 L bottle for each of the two samples, then the remainder of the bottle was filled from the same tank containing the *M. robustum* fingerlings. The North Oconee River was chosen because there are no *M. robustum* and few confamilial species present in the system, and the river has high sediment levels through most of the year. The high sediment treatments consisted of 1 L of water from the North Oconee River and 1 L from the tank containing the *M. robustum* fry. Turbidity measured in nephelometric turbidity units (NTU's) was taken two times for each 2 L sample and averaged to obtain a turbidity measurement. These values can be found in Table 1.

In a sterile reaction hood, we prepped one 2mL tube per sample by adding 300 uL of Cell Lysis Solution (CLS) and 30 uL of proteinase K. Next, a long wooden handled cotton swab was dipped into the CLS and Pro-K solution, then used to swab 1 first 50 mL aliquot vigorously and placed back into the CLS Pro-K solution. This was repeated using the same swab for the second 50 mL aliquot of the same sample. The swab was then placed back into the CLS and Pro-K solution and cut as close to the cotton end of the swab as possible. This was repeated for each of the samples using a new swab and new set of gloves for each sample in order to avoid cross contamination. Once all samples were swabbed they were placed on a shaker and incubated at 60 °C for 30 minutes. Upon removal from the shaker, the tubes were centrifuged for 3 minutes at 6000 rpm, and the supernatant was pipetted off into a clean 1.5 uL tube containing 200 ul of buffer AL and 200 uL of 95% ethanol. This mixture was then extracted using DNeasy spin columns following the DNeasy Blood and Tissue Kit protocol for animal tissue extraction exactly. Extraction eDNA was stored in labeled 1.5 uL centrifuge tubes at -20 °C for later use. In total, 2 individual samples were omitted from the low fish density experiment (bottle samples 2 and 3 from day 0) and 1 from the high fish density experiment (bottle sample 1 from day 3) due

to erroneous pipetting. For the low fish density trial, bottle samples 2 and 3 from day 0 were removed, and for the high fish density trial lab bottle sample 1 from day 3 was removed.

qPCR

All samples were amplified using a MyGo Mini Real Time Thermocycler. The reaction profile was 300s at 95 °C, then 95 °C for 10 seconds and 62 °C for 10 seconds for 65 cycles. This allowed for quick and efficient amplification of *M. robustum* DNA. For all experiments except for the low fish density trial, all samples were run two separate times using two sets of highly specific primer/probe pairs, Cyt B 690F/797R and Cyt B 1068F/1108R, in order to obtain technical replicates during the qPCR process. For the low density fish trial, limited reagent resources limited us to running only one replicate of each sample using both primer sets. A PCR master mix containing 5 uL PrimeTime Taq Polymerase, 2 uL water, .4 uL of both forward and reverse primers, and .2 uL of probe was used for each sample preparation. 2 uL of DNA template from extracted samples was used in each reaction.

Analysis

Statistical analysis was conducted using R studio (Version 1.2.5033 © 2009-2019 RStudio, Inc.) using packages "glm", "ggplot2" (Wickham H 2016) and "dplyr" (Wichham H 2022). One data point was removed from sample T4 on day 28 of the low fish density trial because of an assumed false positive detection. Sample T4 had previously shown its last detection on day 4, and although the negative control was blank on day 28 we assumed that this was an anomalous false positive.

We used logistic regression to fit in package glm to estimate probability of eDNA detection in relation to treatment factors. For the high and low fish density trials we modeled detections of eDNA as an additive function of time (in days), primer selection (Cyt B 690F/797R

or Cyt B 1068F/1108R), fish density (high or low treatments), and vessel (tank samples or 2L bottle samples). We also fit an alternate model with an interaction between time (days) and fish density (high or low; Table 2.2). For the sediment experiment, we fit three alternative models to estimate sediment effects on eDNA detection. The first two models allowed for additive effects of day and either sediment level (none, low, and high) or sediment presence (control vs. any sediment added) on detection probability. The third model allowed for an interaction between sediment presence and day on detection (Table 2.2). Fish density and vessel were held even during the sediment trial and were not included in our model. We used the best-supported models from the density and sediment trials with the "predict" function in package glm to derive the probability of eDNA detection over time across different treatments.

Results:

Fish Density

In the fish density trials, we saw a clear decrease in detection odds over time with significant effects on detection resulting from time, primer choice, fish density, and vessel choice. We saw no significant support for an effect from the interaction between time and fish density. (Table 2.2) We observed a 0.55 times decrease in the odds of detection per day over the course of the trial. Samples from the high fish density treatments had odds of detection that were 3.32 times higher at any given time than those from the low fish density trial.

The use of primers Cyt B 1068F/1108R rather than primers Cyt B 690F/797R increased the odds of detection by 2.64 times. Using primers 1068F/1108R on samples taken from the aquatic lab tanks, detection probability dropped below 10% for a single sample after day 3 for the low fish density trial and after day 5 for the high fish density trial. (Figure 3) When using primers Cyt B 690F/797R on the same samples, detection probability dropped below 10% after

1.5 days for the low fish density trial and 3.5 days for the high fish density trial. (Figure 3) Conversely, samples taken from the 2L bottles kept in the laboratory had odds of detection that were 6.62 times higher than those taken from the tanks. When using primers Cyt-B 1068F/1108R, detection probability dropped below 10% on day 6 for the low fish density treatment and day 8.5 for the high fish density treatment (Figure 1). Using primers Cyt-B 690F/797R, detection probability dropped below 10% by day 4.5 for the low fish density treatment and day 6.5 for the high fish density treatment (Figure 2).

Sediment Trials

Similarly in our sediment experiment, we saw a decrease in detection odds over time with significant effects on detection from time, primer choice, and sediment. There was little support for an effect from the interaction between time and sediment or for differences in sediment treatment (high or low; Table 2.2) The presence of sediment regardless of amount increased the odds of detection by 1.88 times. Using primers Cyt-B 11068F/1108R increased detection odds by 4.59 times on any given day. When using primers Cyt B 1068F/1108R with no sediment present (control), detection probability of a single sample dropped below 10% on day 28, and with sediment present the detection probability did not get lower than 20% by day 28. (Figure 1) In the sediment trials using primers Cyt B 690F/797R with no sediment present (control) detection probability dropped below 10% on day 20, while for samples with sediment present detection probability only reached 10% on day 28, the last day of our experiment. (Figure 2)

Discussion:

Our results follow a similar pattern to other eDNA degradation literature in that detection odds decay exponentially over time though at a variable rate between different treatments**(Strickler et al. 2015)**. However, our results also demonstrate the high variability of

eDNA detection even in similar experimental setups with the same target species. In our study, the decision to use one eDNA marker over another and the vessel in which samples are kept had strong effects on the detection odds of a given sample. Additionally, we found that the presence of sediment in the form of river water from a turbid southeastern river increases the odds of detection in a single sample by up to 1.88 times. This is an encouraging result for the successful application of eDNA tools in a wider range of habitats than the clear, cold environments where they have been successfully applied in the past **(Wilcox et al. 2013, Mckelvey et al. 2016, Izumi et al. 2017).**

For the high and low fish density experiments, daily samples were concurrently taken from 2L vessels held under laboratory conditions and from large 300 gallon tanks held in the Whitehall aquatics lab at UGA. While we did expect to see some differences between the tank and bottle treatments, the results were much stronger than anticipated. Samples held in the 2L bottles in the laboratory had detection odds that were 6.22 times higher on any given day than those taken from the aquatic lab tanks. This is likely due to the fact that the bottle samples were kept on a rotating shaker to keep particulate in the water suspended, while the tank samples were left as is. Genetic material present in the tanks may have quickly settled to the bottom and sides of the tank which lowered the likelihood of successfully collecting DNA from the mid water column.

Primer choice also had a significant effect on detection odds. During the fish density trials, when using primer pair Cyt-B 1068F/1108R which was designed by SC DNR, detection odds were increased by 2.64 times higher on a given day than when using the UGA designed primer pair Cyt-B 690F/797R. In the sediment trials, this effect was even higher with detection odds increased by 4.59 times when using the SC DNR designed primer. These results highlight

the importance in the design and testing of multiple primer sets for a sample. Many studies designing species specific primers only report on the design and testing of a single set of primers **((Piaggio et al. 2014)(Ardura et al. 2015, Uchii et al. 2016)** with high detection success on their target species. However without multiple primer sets to use in comparison, studies may be missing an opportunity to select the best possible tool for detection.

The presence of sediment in the form of North Oconee River water in our samples also had a positive effect on detection success although the mechanisms by which this occurs are unclear. eDNA degradation rates have been shown to be affected by a wide range of abiotic variables including temperature, UV radiation, and sediment **(Barnes et al. 2014)**. Sediment has been linked to increased eDNA persistence. The presence of sediment in a sample may shield or protect the eDNA from other factors like UV or microbial degeneration by decreasing light penetration or binding to eDNA and making it less available to microbes **(Barnes et al. 2014)**. This is particularly encouraging for the application of eDNA tools in the southeastern United States due to high numbers of endemic, imperiled species coupled with highly turbid and dynamic waterways **(Elkins et. al 2016).**

While these results are encouraging, more work must be done to fully validate this tool, as well as others used for single species detections. When using a tool that does not allow you to visually confirm the identify of an organism, false positives and false negatives are a critical issue. This study gives the probability of detection of eDNA in a single sample during a single qPCR repetition however this does not inform us of the probability of false positives which could occur due to contamination at any step during the collection, extraction, or qPCR process. In order to have full confidence in a positive result, complementary data are required. In a study by **(Guillera-Arroita et al. 2017)**, the authors suggest that inclusions of field data collection using

an unambiguous detection method such as seine netting or electroshocking as well as a calibration experiment to provide laboratory qPCR contamination rates will allow for higher confidence in positive detections using an eDNA tool such as the one we designed for this study.

False negatives also pose a significant problem for the effectiveness of eDNA tools. The use of occupancy modeling can significantly increase confidence in detecting eDNA when it is present in a site **(Hunter et al. 2015, Guillera-Arroita et al. 2017)**. In a study by **(Hunter et al. 2015)** to detect invasive Burmese pythons, they estimated detection probabilities with their eDNA tool to range between 0.59-0.87 given that a sample contained eDNA, and therefore that three qPCR replicates per eDNA sample were effective at detecting eDNA when it was present. A similar approach could be used with our data to better inform our protocol for the application of this tool in the field for detection *M. robustum*.

TABLES

Table 2.1: Turbidity measurements for all samples across three treatments in the sediment experiment. All measurements are in Nephelometric Turbidity Units. For each sample, turbidity was measured in two separate replicates, then averaged to obtain the value for that sample.

Sample	Turbidity 1 (ntu)	Turbidity 2 (ntu)	Average (ntu)
Control 1	1.11	0.85	0.98
Control 2	1.28	1.44	1.36
Low Sediment 1	3.53	4.04	3.79
Low Sediment 2	3.61	3.32	3.47
High Sediment 1	5.99	6.28	6.14
High Sediment 2	6.33	6.76	6.55

Table 2.2: Structure and relative support (AIC) of alternative models used to estimate treatment and primer effects on detections of eDNA in two laboratory experiments testing effects of fish density and sediment additions. All models were fit as logistic regressions to detections by day. Bolded AIC values indicate most supported model in each experiment.

Model	AIC
Density Trials	
Additive model: Day, Density (High or Low), Primer (690F/797R or 1068F/1108R), Vessel (Tank or Bottle)	**271.41**
Interaction model: Day* Density (High or Low), Primer (690F/797R or 1068F/1108R), Vessel(Tank or Bottle)	272.81
Sediment Trials	
Additive Model 1: Day, Primer (690F/797R or 1068F/1108R), Sediment Treatment (None, Low, High)	306.6
Additive Model 2: Day, Primer (690F/797R or 1068F/1108R), Sediment (Present or Absent)	**304.09**
Interaction Model: Day*Sediment (Present or Absent), Primer(690F/797R or 1068F/1108R)	305.91

Table 2.3: Treatment and primer effects on detections of eDNA in the high and low fish density trials. Model outputs are from the Additive Model in Table 2.2 under Density Trials.

Variable	Coefficient	Std. Error	P value	Effect on Odds of Detection
Intercept	-1.35726	0.47097	0.003954	-
Day	-0.59174	0.08133	3.45e-13	0.55x
Primer (SC)	0.97498	0.31605	0.002036	2.64
Vessel (Bottle or Tank)	1.89354	0.39156	1.33e-06	6.62
High Fish Density	1.20382	0.33694	0.000353	3.32x

Table 2.4: Treatment and primer effects on detections of eDNA in the sediment trial. Model outputs are from Additive Model 2 in Table 2.2.

Variable	Coefficient	Std. Error	P value	Effect on Odds of Detection
Intercept	-0.77958	0.31438	0.0131	-
Day	-0.10354	0.02081	6.53e-07	.90x
Primer (SC)	1.52379	0.28924	1.38e-07	4.59x
Sediment	0.62886	0.30483	0.0391	1.88x

FIGURES

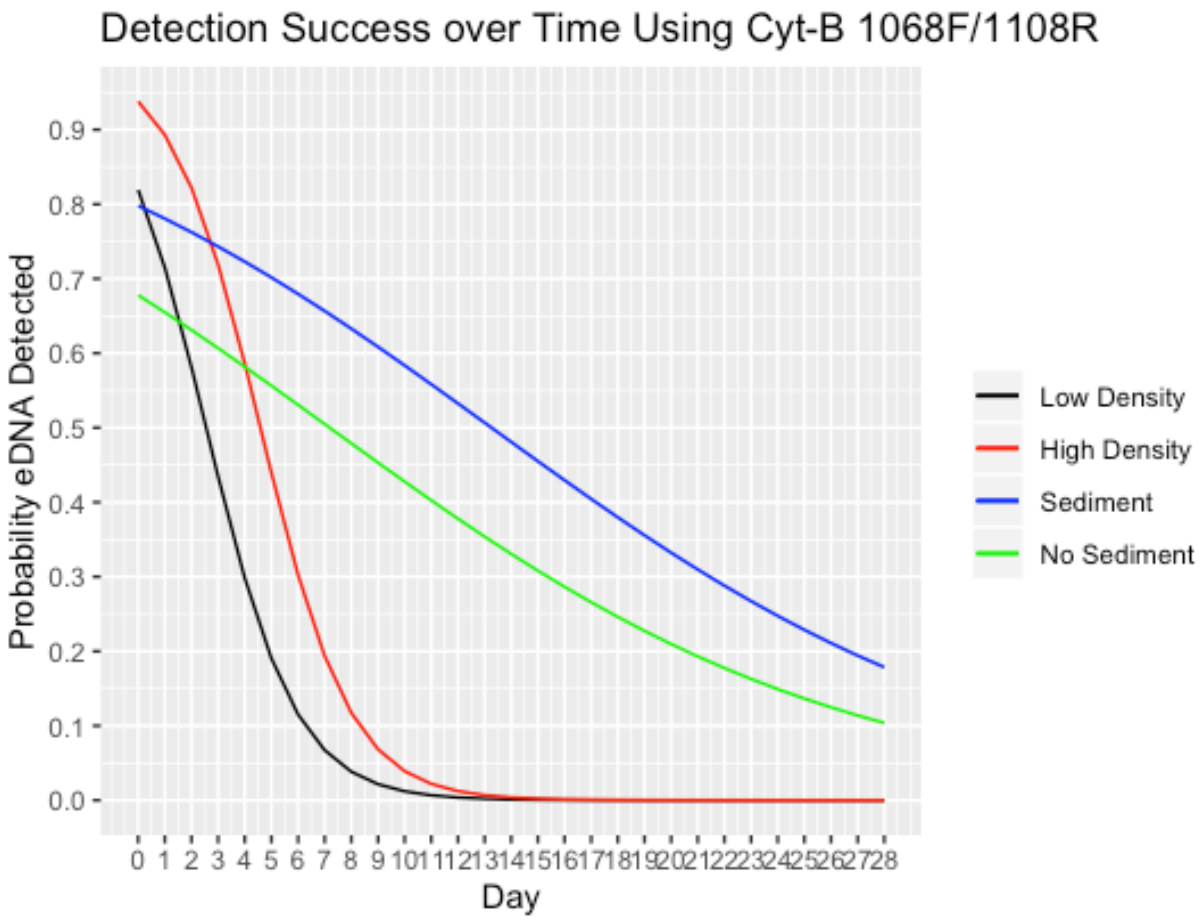

Figure 1: Detection success over time using Cyt-B 1068F/1108R showing low fish density treatment (black), high fish density treatment (red), sediment present (blue), and no sediment present (green). All samples represented were taken from 2L bottles.

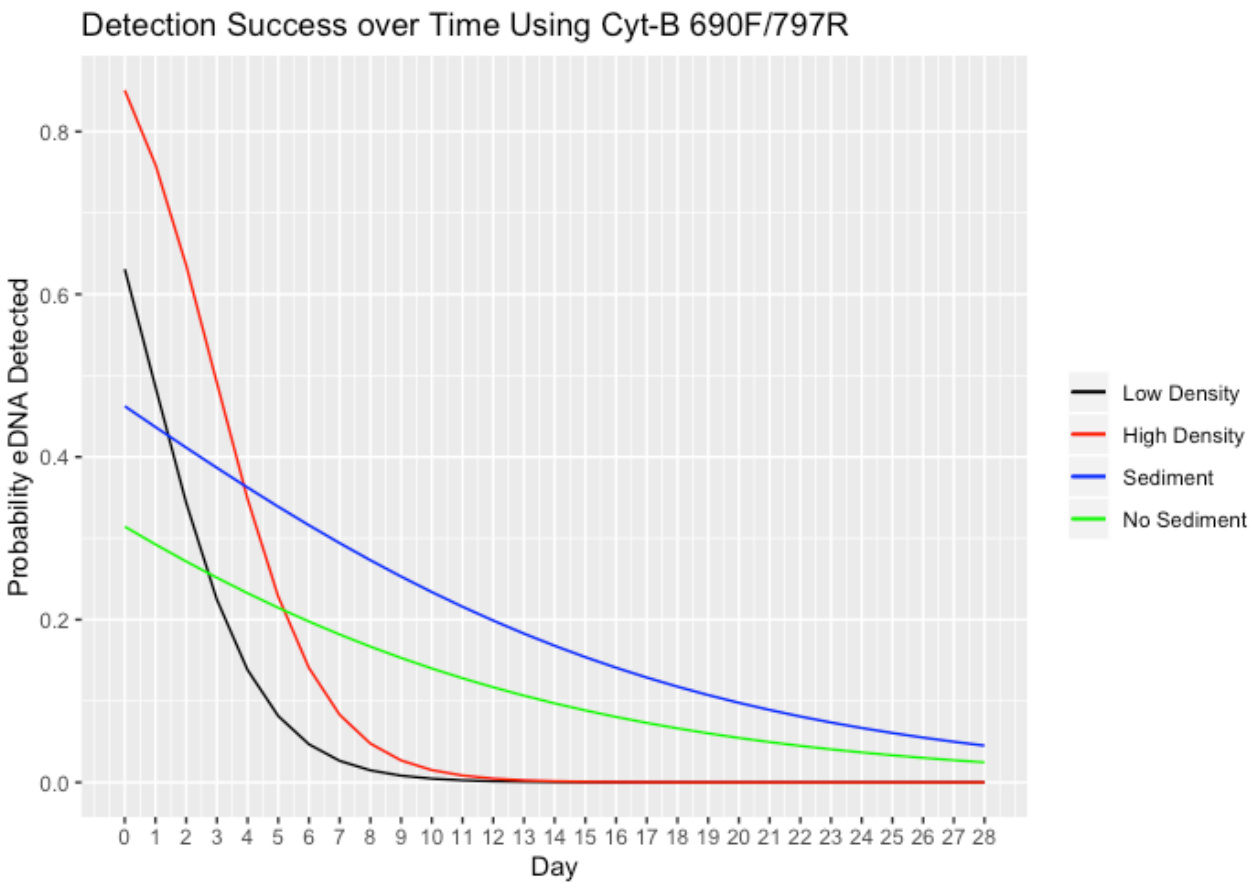

Figure 2: Figure 1: Detection success over time using Cyt-B 690F/797R showing low fish density treatment (black), high fish density treatment (red), sediment present (blue), and no sediment present (green). All samples represented were taken from 2L bottles.

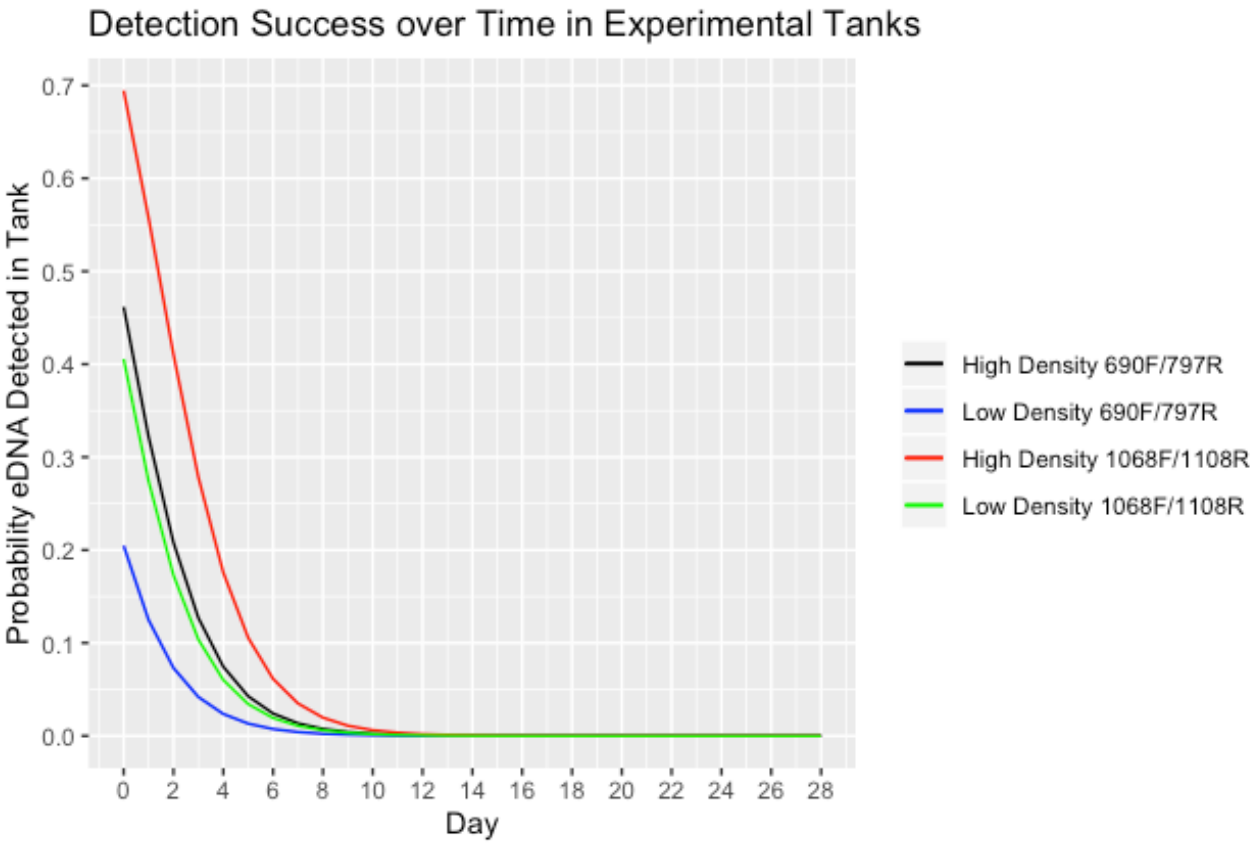

Figure 3: Probability of eDNA detection over time in samples taken from experimental tanks in UGA Whitehall aquatics lab showing high fish density using primers 690F/797R (red), low fish density using primers 690F/797R (green), high fish density using primers 1068F/1108R

CHAPTER 4

CONCLUSION

This study demonstrates that while an eDNA marker can be successfully created and used for single species detection, a wide array of factors can have strong effects on detection success and timeframe and therefore thoughtful testing and implementation is required for effective use. During all phases of design and testing, small change in treatments and procedures resulted in important changes in the effectiveness of our tool. During the design phase of the tool, we showed that increases in temperature and decreases in annealing times during the PCR process can result in higher primer specificity and can exclude closely related con-familial species from amplifying in a sample. During our degradation trials, we demonstrated how choice of primer, experimental vessel, fish density, and sediment can all affect the detection odds and degradation of DNA to differing degrees. These results show us how regardless of target species, thorough testing of an eDNA tool can allow for optimization under differing environmental conditions and species densities.

Moving into the future, more work remains to fully utilize the potential of this tool for use in the monitoring and conservation of *M. robustum*. Between SCDNR and the Georgia Museum of Natural History, dozens of archived water filters are awaiting extraction and sequencing. Many samples were taken directly below active *M. robustum* spawning beds where the presence of spawning suckers was directly observed. Samples were also taken in years where high flows

prohibited visual confirmation of *M. robustum* spawning and therefore the application of this new eDNA tool becomes particularly relevant as other sampling methods were unable to detect fish during these years. The extraction and subsequent amplification of the eDNA collected on these filters will allow us to put together a better idea of spawning habitat usage and frequency for *M. robustum*, and additionally will allow gaps in visual detections to be filled. Using these archived filters will also allow us to further test the effectiveness and contamination rate/false positive rate in the field. Samples taken at Juliette Dam like those mentioned in Chapter 2 offer a unique site to test our tool using water samples taken below the dam at active *M.robustum* spawning sites and water samples taken above the dam where no *M. robustum* should be present. Those data, combined with a site occupancy modeling approach such as that used in Guillera-Arroita et al. 2017, would allow for further elimination of uncertainty in a positive result, and would reinforce a powerful new tool for the conservation and monitoring of *M. robustum.*

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