FINAL REPORT

South Carolina State Wildlife Grant SC-T-F19AF00780

South Carolina Department of Natural Resources
October 1, 2019 – December 31, 2022

Project Title: Development, Testing, and Field Validation of an eDNA Tool for Robust Redhorse

Principle Investigator:
Dr. Tanya Darden (SCDNR, MRRI)
Daniel Farrae (SCDNR, MRRI)

Project Collaborators:
Dr. Bud Freeman (University of Georgia)
Paula Marcinek (GADNR)

Project Goal: The overall goal of our project is to develop, test, and field validate an eDNA tool for Robust Redhorse for future application that will increase our understanding of distribution and habitat use for this species.

Project Summary: This project has provided a new, powerful environmental DNA (eDNA) tool that will increase our passive detection capabilities for Robust Redhorse, a species that has historically evaded routine field efforts outside of their spawning season. The rigorously designed eDNA assay was tested for species specificity, efficiency, and sensitivity. A laboratory experiment was conducted to evaluate the influence of both fish density and sediment on DNA persistence, and sampling of production ponds allowed us to evaluate DNA accumulation using the new eDNA assay. We also successfully detected Robust Redhorse with the new tool during field validation sampling in two areas with known Robust Redhorse present. Collectively, our project activities provide a solid interpretation foundation for the application of this new eDNA tool for Robust Redhorse in the field. The new eDNA tool will allow for the detection of juveniles and/or adults in habitat reaches that are challenging to sample with traditional gears/methods, increasing our understanding of distribution and habitat use for Robust Redhorse. Improvement of passive detection tools such as the new eDNA assay will provide critical data in making the best management decisions during a critical period for the species' survival and regulatory status.

<u>Objective 1:</u> Molecular Tool Development

One of the most important parts of an effective eDNA assay is ensuring that detection of non-target species is not occurring. For the Robust Redhorse, this is particularly challenging because of the number of closely related species that exist throughout its range. Following review of current phylogenetic studies and consultation at the 2020 Robust Redhorse Conservation Committee, we identified 10 priority closely related, co-occurring species for testing cross-species amplification with our primer/probe designs. These species included members of the genera *Moxostoma* (6 spp), *Hypentelium* (2 spp), *Erimyzon* (1 spp), and *Minytrema* (1 spp) and an additional secondary 6 co-occurring species including members of the genera *Erimyzon* (1 spp), *Carpiodes* (3 spp), *Catostomus* (1 spp), and *Ictiobus* (1 spp)

(Table 1). These species do not all occur throughout the entire Robust Redhorse range. For example, the Roanoke Hogsucker (*Hypentelium roanokense*) only inhabits the Pee Dee River system and is only thought to occur much further upstream in the system than Robust Redhorse. Though we originally planned on only testing 10 sympatric species, we expanded to 16 to account for some of the species only co-occurring in limited basins and due to the inability to obtain tissue samples for all species across all drainages. We compiled all data available from GenBank for the target species/genes and coordinated with field crews across the Robust Redhorse range to make additional field collections of needed specimens. All sequence data found on GenBank for these species was used during primer and probe design to maximize specificity.

Both SCDNR and UGA worked together to identify the most efficient and species-specific Robust Redhorse eDNA primer/probe combination (hereby called the eDNA assay). Forward and reverse primers were designed using Primer-BLAST and manually searching through available sequence data from GenBank. Based on a suite of primer characteristics and mismatches to other species, the top twelve primers were ordered for benchtop testing. All twelve of these targeted cytochrome B (CytB) or NADH dehydrogenase 2 (ND2) mitochondrial sequences. These regions are not only more likely to be species-specific, but also occur in higher number in a single cell because there are multiple mitochondria and are therefore more likely to be detected via eDNA collection methods. Based on exponential curves, low levels of variation among individual and technical replicates, and critical detection thresholds occurring in a low number of PCR cycles (i.e. qPCR primers and protocol are specific and efficient), two CytB primer and two ND2 primers were selected for probe design and further testing. Given the importance of ensuring that the markers are effective in different labs, UGA and SCDNR tested some of these on their own with the intent of identifying the best possible primer/probe combination and having the other lab test that as well. Each lab took a slightly different approach to further testing of these markers.

For those tested by SCDNR, probes were designed manually to maximize specificity and PCR efficiency. One probe design could be used with both ND2 primers due to overlapping sequences; therefore 4 assays were tested for amplifying Robust Redhorse DNA. Based on the results (Figure 1), one assay (CytB; black lines in Figure 1) was more efficient than the other three. This primer sequence amplifies a short, 79 base pair product and was chosen for further development.

A critical part of the development of an eDNA probe is to measure the qPCR efficiency by way of a 10-fold serial dilution. This test ensures that as DNA template concentration decreases, the amplification to a critical threshold still occurs at an exponential rate, albeit at an appropriate cycle in the PCR. The serial dilution test of our CytB assay (Figure 2) indicated that our qPCR efficiency was at 100.4% with an R^2 of 0.995 (Figure 3). Given the efficiency of the CytB assay (Table 2), these were chosen to continue developing as an eDNA tool for Robust Redhorse.

Table 1. Sympatric species by drainage for which DNA samples were obtained and tested for cross-species amplification of eDNA assay. Some tissue samples were obtained from the Cape Fear River in NC, rather than the Pee Dee River. Tissue samples were not obtained/tested for two species (*Carpiodes carpio*, River Carpsucker; *Ictiobus bubalus*, Smallmouth Buffalo), both of which were among the six secondary species to test based on discussions within the RRCC. Existing sequence data for these species was used during primer and probe design to maximize specificity.

Species	Common Name	Pee Dee (Cape Fear)	Santee	Savannah	Ogeechee	Oconee	Ocmulgee
Moxostoma collapsum	Notchlip Redhorse	X		X			
Moxostoma macrolepidotum	Shorthead Redhorse	X	X				
Moxostoma rupiscartes	Striped Jumprock					X	X
Moxostoma sp. 'Carolina'	Carolina Redhorse	X					
Moxostoma sp. cf. M. lachneri	Brassy Jumprock / Smallfin Redhorse	X			X		X
Moxostoma pappillosum	V-lip Redhorse	X					
Hypentelium nigricans	Northern Hog Sucker			X		X	
Hypentelium roanokense	Roanoke Hog Sucker	X					
Erimyzon oblongus	Creek Chubsucker				X	X	
Minytrema melanops	Spotted Sucker		X	X	X		X
Erimyzon sucetta	Lake Chubsucker		X				
Catostomus commersonii	White Sucker	X					
Carpiodes cyprinus	Quillback		X				
Carpiodes velifer	Highfin Carpsucker			X			

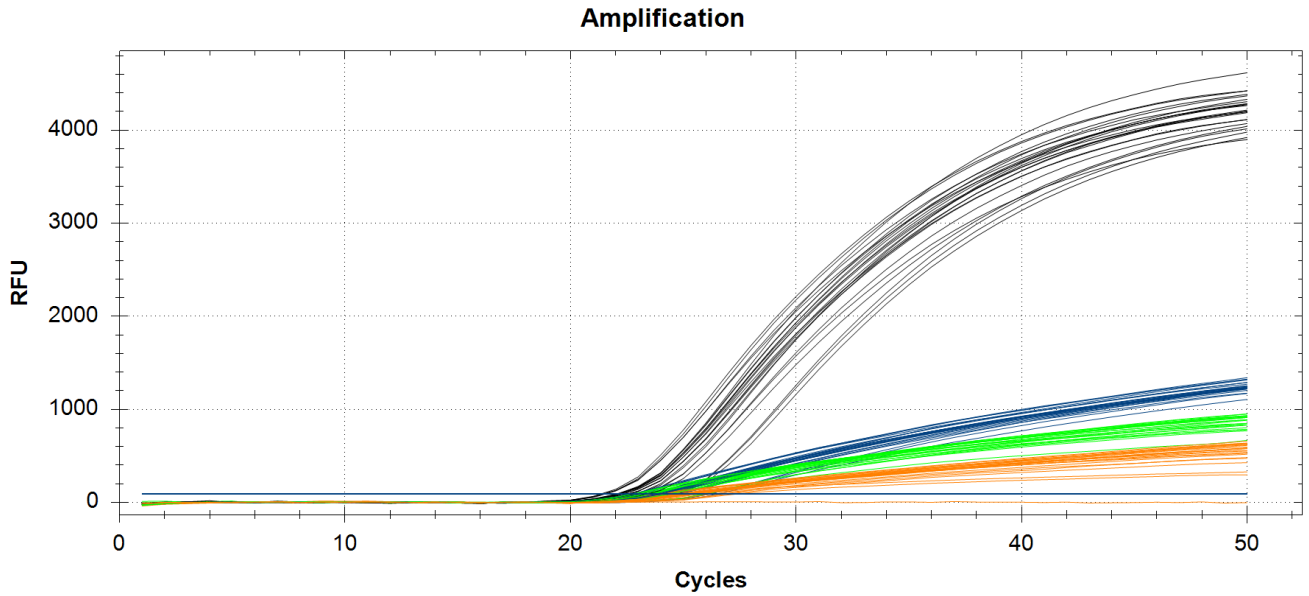

Figure 1. qPCR results indicating that the CytB assay (black lines) more efficiently amplified Robust Redhorse DNA than the other three combinations (all ND2; blue, green, and orange lines).

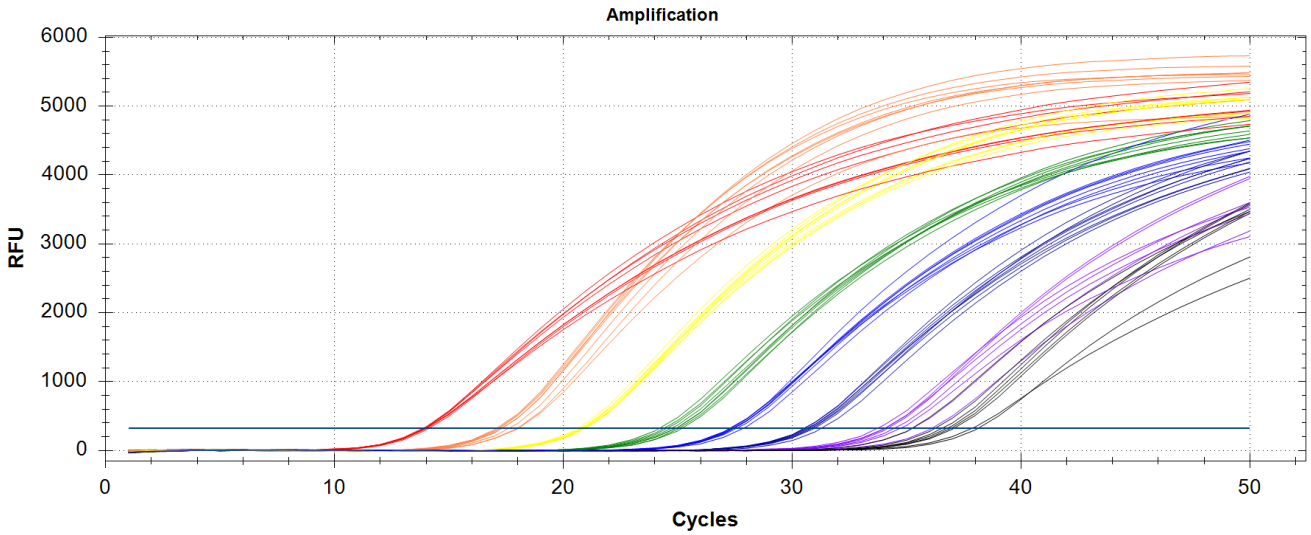

Figure 2. qPCR results from the CytB assay amplifying a 10-fold serial dilution of template Robust Redhorse DNA

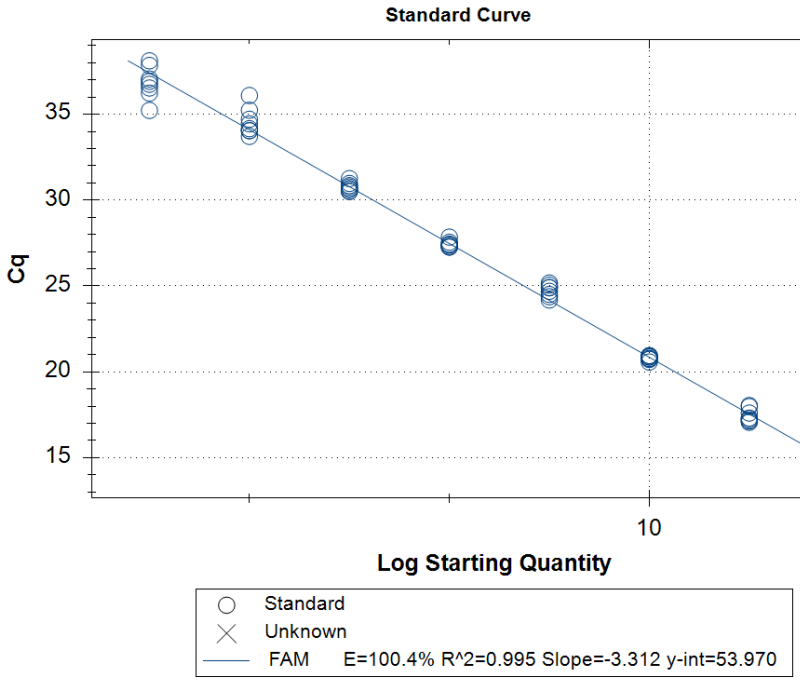

Figure 3. Efficiency and R^2 estimates for the standard curve from the CytB assay amplifying a 10-fold serial dilution of template Robust Redhorse DNA.

Table 2. Sequence data for the forward and reverse primers, standard probe, and LNA probe targeting a 79 base pair sequence of the mitochondrial genome of Robust Redhorse designed by SCDNR.

Primer	Sequence (5' – 3')
Cytb_1068_F	CGGACAAATTGCTTCCATC
Cytb_1108_R	AGCCTTGTTTTCCAGTCATCC
Cytb_1068_F_probe	TCCTGTTTCTAGCCCTAATCCCACTAGCG
Cytb_1079_probeLNA	***T**CCT***G**TTTCTAGCCCTAATCCCACT

***locked nucleic acid (LNA) nucleotides**; FAM used as reporter dye for probes

For those potential primer pairs tested by UGA, after examination and testing of three sets of forward/reverse primer combinations for CytB and one set for ND2, one ideal candidate was selected. Primer pair CytB_690_F- CytB_797_ R (Table 3) was selected due to a high degree of specificity for Robust Redhorse compared to other closely related, co-occurring Catostomid species (Table 4). This primer combination targets a 106 bp product. Additionally, a 25 bp probe region within this primer set starting at CytB_744 was selected based on its specificity against all other examined taxa (Table 5).

Design and testing of the primer/probe sequences revealed that even with multiple mismatches in the primer sequences, cross amplification of non-target species was still occurring for some taxa including *Moxostoma* sp. "Brassy" Jumprock and *Hypentelium nigricans*. Through a series of trials, it was determined that increasing the annealing temperature of the amplification reaction had little to no effect on the specificity of the reaction, but reducing the annealing time from 30s to 15s per cycle in combination with dilution of input DNA removed all cross amplification of taxa. Therefore, UGA began further testing with this putative marker as well.

Table 3. Sequence data for the forward and reverse primers and probe targeting a 106 base pair sequence of the mitochondrial genome of Robust Redhorse designed by UGA.

Primer	Sequence (5' – 3')
CytB_690_F	CCCCTATTTTTCATATAAAGACCTCCTA
CytB_797_R	AGAAAGTATCATTCTGGTTTAATGTGAG
CytB_690_F_probe	CCTCCCTAGCCCTATTTTCACCTAA

Table 4. Primer mismatches with co-occurring Catostomid species. Red letters indicate a mismatch.

Species and Drainage	**CytB_690_F Mismatches**
Moxostoma robustum (SAV and ALTA)	CCCCTATTTTTCATATAAAGACCTCCTA
Carpiodes cyprinus	CCCCTACTTCTCATATAAAGACCTATTA
Catostomus commersoni	CCCTTACTTTTCATACAAAGATCTCCTG
Erimyzon oblongus	CCCCTACTTCTCTTACAAGGACCTCCTA
Erimyzon sucetta	CCCATACTTCTCCTATAAAGACCTTTTA
Hypentelium nigricans (SAV)	TCCTTACTTTTCATATAAAGACCTTCTA
Minytrema melanops (SAV and ALTA)	CCCTTACTTTTCATACAAAGACCTCCTA
Moxostoma collapsum (SAV)	TCCTTATTTTTCATACAAAGATCTCCTA
Moxostoma rupiscartes (ALTA)	CCCCTATTTTTCATACAAGGGCCTCCTA
Moxostoma sp. "Brassy" Jumprock (ALTA)	TCCTTACTTTTCATACAAAGACCTCCTA
Species and Drainage	**CytB_797_R Rev Comp Mismatches**
Moxostoma robustum (SAV and ALTA)	AGAAAGTATCATTCTGGTTTAATGTGAG
Carpiodes cyprinus	AGGAAGTATCATTCTGGTTTAATATGGG
Catostomus commersoni	AGAAAATATCATTCTGGCTTAATATGAG
Erimyzon oblongus	AAAAAGTACCATTCTGGTTTAATATGGG
Erimyzon sucetta	AGAAAGTATCACTCTGGTTTGATATGAG
Hypentelium nigricans (SAV)	AGAAAGTATCATTCTGGCTTGATATGAG
Minytrema melanops (SAV and ALTA)	AGAAANTATCACTCAGGCTTGATATGGG
Moxostoma collapsum (SAV)	AGAAAGTACCACTCTGGTTTAATATGAG
Moxostoma rupiscartes (ALTA)	AAAAAGTATCATTCTGGTTTAATATGGG
Moxostoma sp. "Brassy" Jumprock (ALTA)	AGAAAGTACCATTCTGGTTTAATATGAG

Bold – tested with specimens from the GMNHTC
Plain Text – Genbank Sequence

Table 5. CytB_744 probe and mismatches with co-occurring Catostomid species.

Species	**Probe Mismatches**
Moxostoma robustum	CCTCCCTAGCCCTATTTTCACCTAA
Carpiodes cyprinus	CATCCCTGGCCCTATTCTCACCAAA
Catostomus commersonii	CCTCCCTCGCGCTATTTTCACCTAA
Erimyzon oblongus	CCTCGCTAGCCTTGTTCTCACCTAA
Erimyzon sucetta	CCTCCCTAGCATTATTCTCACCCAA
Hypentelium nigricans	CCGCCCTAGCTCTATTTTCACCTAA
Minytrema melanops	CCTCCCTAGCACTATTTTCACCTAA
Moxostoma collapsum	CCTCCCTAGCCCTATTTTCACCCAA
Moxostoma rupiscartes	CCTCCCTAGCCCTATTTTCCCCAA
Moxostoma lachneri	CCTCCCTAGCCCTATTTTCACCCAA
Moxostoma sp. "Brassy" Jumprock	CCTCCTTAGCCCTATTTTCACCCAA

Significant effort was expended ensuring that at least one assay was species-specific. As noted in some of the earlier UGA tests, reducing the annealing time may prevent some non-target amplification. However, SCDNR did not document the same results when testing this with their assay. Most sympatric species tested did not show any signs of amplification with our assay. However, two species (Shorthead Redhorse and Carolina Redhorse) continually amplified with our assay across many individual tissue samples and technical replicates. Because the eDNA assay is extremely sensitive, contamination of tissue samples with Robust Redhorse DNA was a concern that would potentially lead to false indications of cross-species amplification. To rule this out, we ordered synthetic DNA sequences of the Shorthead Redhorse and Carolina Redhorse CytB mitochondrial sequences that our assay targets. Synthetic Shorthead Redhorse DNA showed limited signs of amplification; however, the synthetic Carolina Redhorse DNA still amplified, albeit not in an exponential manner.

Any cross-species amplification complicates interpretation during the future use of the new eDNA tool. Therefore, we adapted our probe to incorporate a locked nucleic acid (LNA) to improve specificity. This probe included two LNA nucleotides. Amplification protocols were optimized with the new LNA probe. The final test included a qPCR run testing 23 different samples from 13 different species (*Hypentelium roanokense* was included in a separate run), including synthetic DNA of Shorthead Redhorse and Carolina Redhorse in which none of the non-target species showed any signs of amplification (Figure 4).

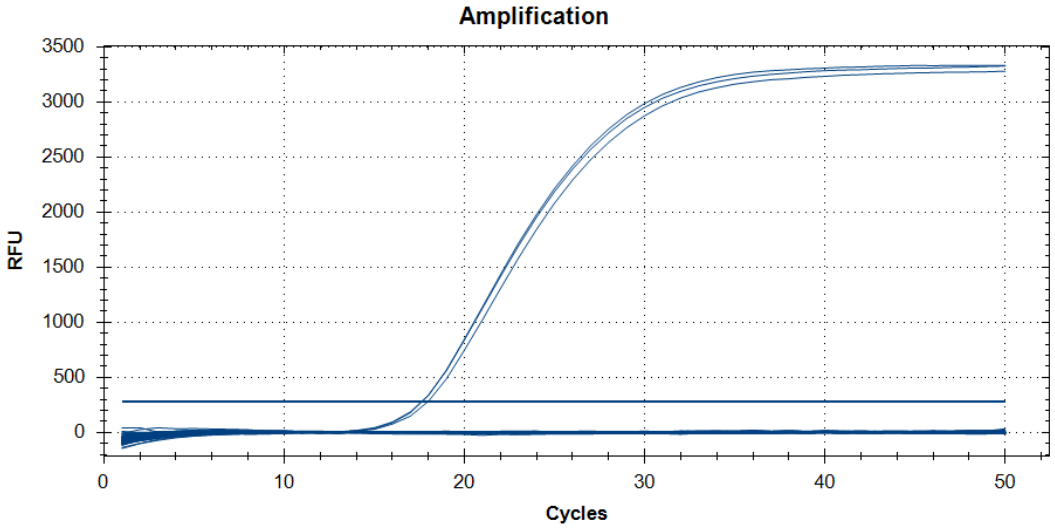

Figure 4. qPCR plot showing lack of amplification from other Catostomid species with final LNA-based assay qPCR conditions. Analysis included 3 technical replicates each of 23 different samples from 13 different species (*Hypentelium roanokense* was included in a separate run, but likewise did not amplify), including synthetic DNA of Shorthead Redhorse and Carolina Redhorse. The only samples showing any amplification were the 3 technical replicates of positive control DNA from Robust Redhorse.

Therefore, this CytB assay with the LNA probe design was tested again for efficiency using a ten-fold serial dilution using a known quantity of synthetic Robust Redhorse DNA (Figure 5), with the qPCR efficiency remaining high at 90.9% (Figure 6). Starting with a known quantity of synthetic Robust Redhorse DNA allowed us to estimate the number of copies of our target sequence in our serial dilutions and test the limits of detection relative to the number of copies. DNA copy number was estimated using the following formula:

$$DNA\ copy\ number = \frac{DNA\ mass\ (g) * 6.022x10^{23}}{fragment\ length\ (bp) * 650}$$

The concentration of our serial dilutions ranged from 10 ng/µl (7.4 billion copies/µl) to 10^{-11} ng/µl (0.07 copies/µl). These dilutions amplified in 100% of technical replicates down to 10^{-9} ng/µl (7.4 copies/µl). Dilutions to 10^{-10} ng/µl (0.7 copies/µl) amplified in only 1 of 8 technical replicates, and dilutions beyond that did not amplify in any of 8 technical replicates. In short, dilutions estimated to contain > 1 copy of our target sequence were always detectable. Dilutions with < 1 copy were sometimes detectable, which is likely based just on chance whether a copy of the target sequence is added to the PCR from the dilution. The Cq value for the single replicate with < 1 copy was 37.4, which likely represents a rough threshold of detection if all aspects of the assay are held constant. Any "positive" detections with Cq > 37.4 should be considered questionable.

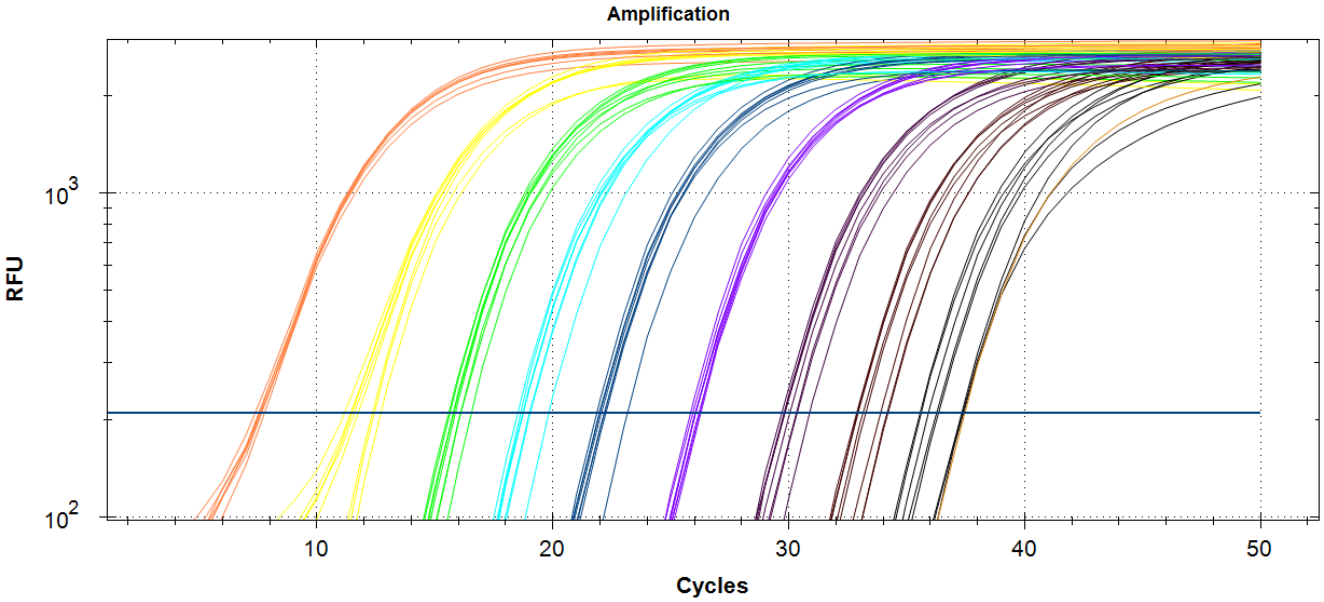

Figure 5. qPCR plot with serial dilution of synthetic Robust Redhorse eDNA with final LNA-based assay and conditions.

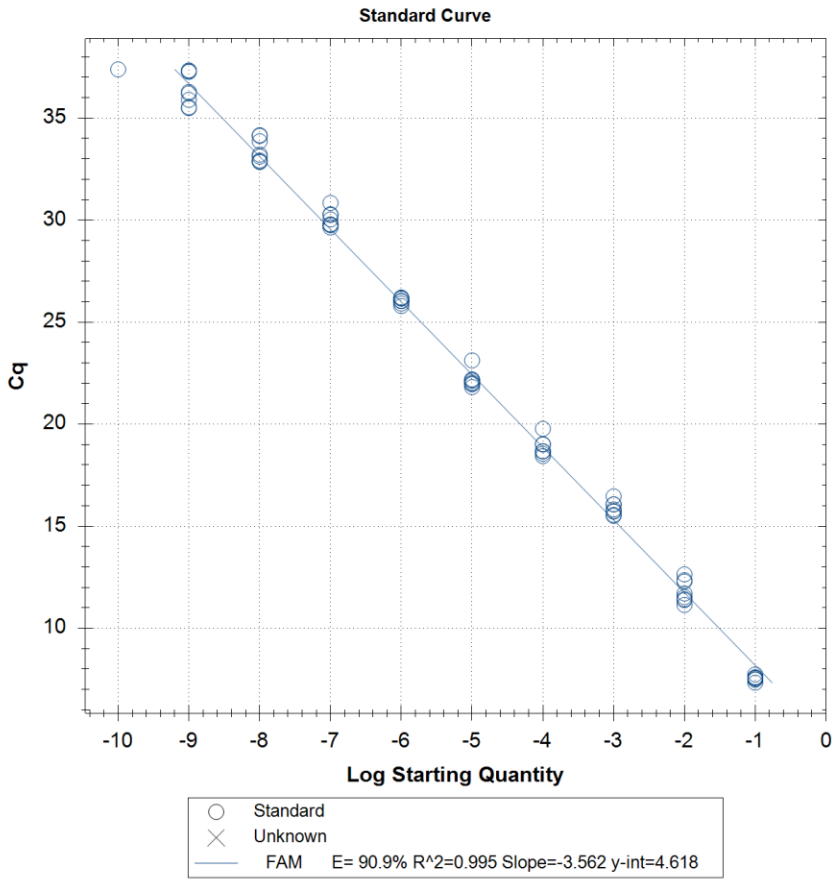

Figure 6. Standard curve of the 10-fold serial dilution qPCR results with final LNA-based assay and conditions, showing the efficiency (E=90.9%) of the reaction conditions (R^2 = 0.995).

Objective 2: Laboratory Stream Testing

During the Dennis Wildlife Center (DWC; Bonneau, SC) pond harvest on 28 October 2021, 200 juvenile Robust Redhorse were transported to the UGA Warnell School of Forestry and Natural Resources Aquaculture Lab and transferred to three 300-gallon recirculating tank systems in preparation for a DNA degradation experiment to inform our eDNA detection interpretations. 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 placed in tanks following treatment specifications. All experiments, lab processing, and analyses were conducted as part of a Master of Science thesis project; full details are available in Bennett 2022.

For the fish 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; the first eDNA samples were then taken to evaluate DNA degradation rate. The original holding tanks were left stagnant after fish were removed. On day 0 of the experiment, four sterilized 2 L bottles were filled 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. Bottles were sampled daily for the next 7 days and then on day 14, 21, and 28.

Upon arrival in the molecular lab, bottles were placed on shakers and kept constantly in motion at ~100 rpm to keep any eDNA suspended in the water column and relatively evenly distributed. DNA capture was achieved via centrifugation following modified methods from Uchii et al. (2016) and Bockrath et al. (2022). Prior to each extraction, each 2 L bottle was shaken vigorously, then uncapped and ~100 mL was poured into a sterile conical tube. In addition to the four lab treatment samples, two 2 L bottles were filled daily from the treatment tanks and transported to the molecular lab for capture and extraction to explore detection differences in larger volumes of water in a more uncontrolled setting than the closed 2 L bottle system. An extraction blank consisting of a 2 L 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 2 L bottles reused for daily tank sampling. All samples were then centrifuged for 30 min at 5000 rpm at 4 °C. After centrifugation was complete, the supernatant was poured off each sample tube into a waste container until the liquid was just below the conical tip of the tube. Samples were then transported to a different lab room for extraction.

In addition to the fish density experiment, we also conducted an experiment to evaluate the influence of sediment on DNA degradation. 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 approximately 70 juvenile *M. robustum*, capped and rinsed, then placed on ice. 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* juveniles. 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*. Turbidity was measured twice for each 2 L sample and averaged to obtain a turbidity measurement (Table 6). Bottles were sampled daily for the next 7 days and then on day 14, 21, and 28. The sediment experiment followed the same protocol for capture of eDNA.

DNA from all experimental samples was extracted using the DNeasy Blood and Tissue Kit (Qiagen) protocol for animal tissue extraction after a ProK digestion. Extracted eDNA was stored in labeled 1.5 mL centrifuge tubes at -20 °C for later use. All samples were amplified using a MyGo Mini Real Time Thermocycler using the non-LNA SCDNR-developed CytB 1068F/1108R assay protocol.

In the fish density trials, although initial Cq values were lower in both high-density treatments (31.2 in high density vs 34.8 in low density), rapid DNA degradation occurred across all treatments making comparative interpretation challenging. However, evaluation of percent positive detections of replicates indicated the source tank samples showed a faster degradation rate of DNA compared to the bottle samples (Figure 7); perhaps genetic material present in the tanks settled to the bottom and sides of the tank which lowered the likelihood of successfully collecting eDNA from the mid-water column. A general pattern was observed in both sample sources of longer DNA retention in the high-density fish treatment, but DNA was not reliably detected in any samples past Day 7. Our results follow a similar pattern to other eDNA degradation literature (Darden et al. 2019, Watson et al. 2022) in that DNA degrades exponentially over time though at a variable rate between different fish density and environmental treatments.

As volumes from source DNA was not constant across our sediment experiments, evaluation of percent positive detections of replicates provided an interpretation of relative DNA degradation over time (Figure 8). The presence of sediment decreased the rate of DNA degradation in the experiments as

compared to the control samples. 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. During our experiment, all samples maintained at least one replicate of positive detection through the entire 28-day sampling period, likely due to the higher fish density in the tanks used for the initial source water as compared to the fish density experiments (70 vs 5 and 45 juvenile robust redhorse).

Table 6. Turbidity measurements for all samples across three treatments in the sediment experiment. All measurements are in Nephelometric Turbidity Units (ntu). For each sample, turbidity was measured in two separate replicates, then averaged to obtain the value for that sample.

Sample	Turbidity Average
Control 1	0.98
Control 2	1.36
Low Sediment 1	3.79
Low Sediment 2	3.47
High Sediment 1	6.14
High Sediment 2	6.55

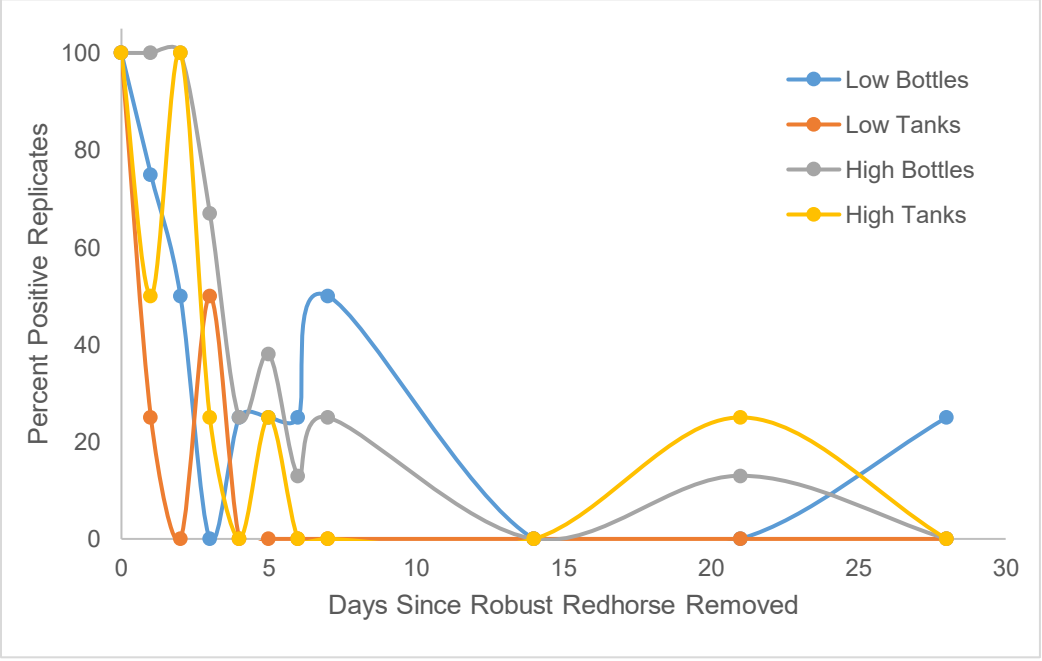

Figure 7. Percent positive detection replicates over time in the fish density experiments sampled in both tanks directly and sequestered bottles maintained in the lab. Low density included 5 juvenile Robust Redhorse per tank; high density included 45 juvenile Robust Redhorse. All samples represented 100 ml water processed.

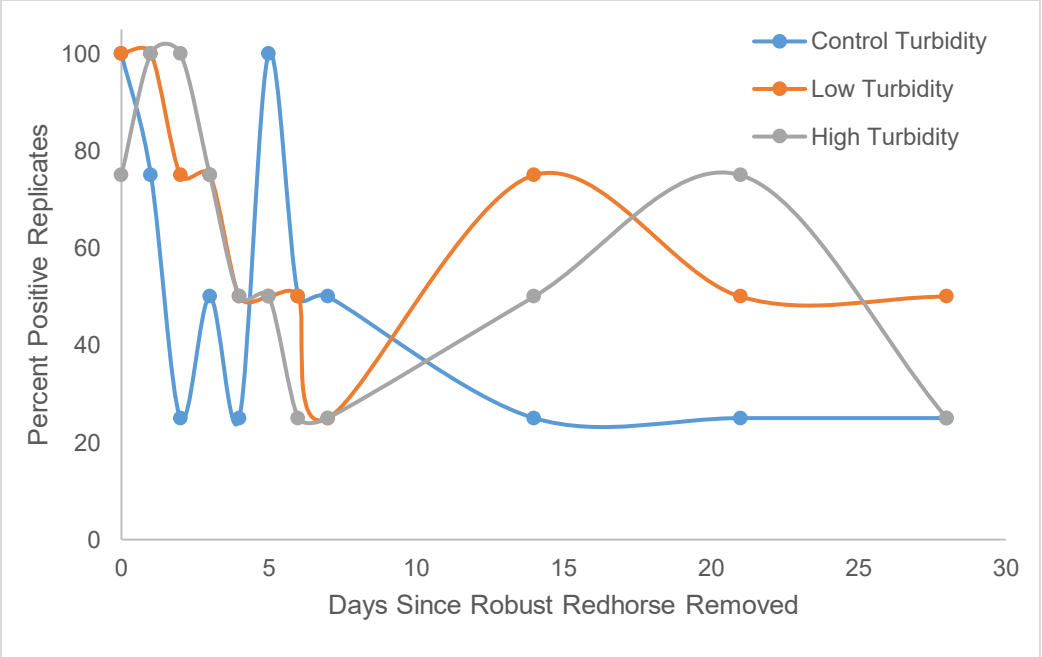

Figure 8. Percent positive detection replicates over time in the sediment experiments sampled from sequestered bottles maintained in the lab. Source water was from a tank with 70 juvenile Robust Redhorse, but initial DNA concentrations were not constant across treatments. All samples represented 100 ml water processed.

<u>Objective 3:</u> Production Pond Testing

Robust Redhorse production was ongoing at the SCDNR Dennis Wildlife Center for the Pee Dee River supplementation stocking project before and during our eDNA project. With Robust Redhorse naturally occurring in larger river systems, production ponds provide an additional opportunity to validate the eDNA tool in a semi-controlled but larger experimental unit. Starting in the 2018 year class production year, ponds were sampled at the DWC by collecting 2 L water samples via glass bottles at 2 locations per pond. The samples were kept on ice until they were returned to the SCDNR Population Genetics lab and pumped through 1.6 µm glass fiber filters. Two ponds were sampled on 05 June 2018, two days prior to Robust Redhorse fry being released into these ponds for grow-out. The same two ponds were sampled on 08 June 2018, approximately 20 hours after the fry were released and then at approximately 1 week, 1 month, 2 months, 3 months, and 4 months post-stocking. During the project, we purchased a new Smith-Root eDNA sampler which utilized a different filter media. For comparative purposes, we repeated pond sampling from a single production pond (2021 year class) on 01 October 2021 and 22 October 2021, roughly four months after Robust Redhorse fry were released into this pond to test whether the sampler or filter influenced our assay's detectability.

For all sample processing, we utilized the final, optimized SCDNR-developed LNA assay protocol. Samples collected before Robust Redhorse were released into the ponds all tested negative with our eDNA assay (Figures 9 and 10), as did the samples collected approximately 20 hours after they were released. Given the large volume of the ponds (1 acre) and the small size of the fry, this is not a surprising result after only 20 hours. Beginning with samples collected one week after release, we begin to detect some Robust Redhorse eDNA in one pond, with 2 and 3 positive technical replicates from the two samples and average Cq values ~ 36. The other pond still tested negative at the one-week sampling

time. Samples collected one month after release all tested positive, though not all technical replicates tested positive. Two months after release, all samples and technical replicates tested positive. We also detect a gradual decrease in average Cq value over time from ~ 36 to ~33 (Figure 10), which may indicate the accumulation of eDNA in the ponds which is roughly equal to a ten-fold difference according to our standard curve (Figure 6). The samples collected by our eDNA sampler in 2021 (116 and 137 days after fry were released) all tested positive and with similar Cq values to samples collected via glass bottles and manually filtered.

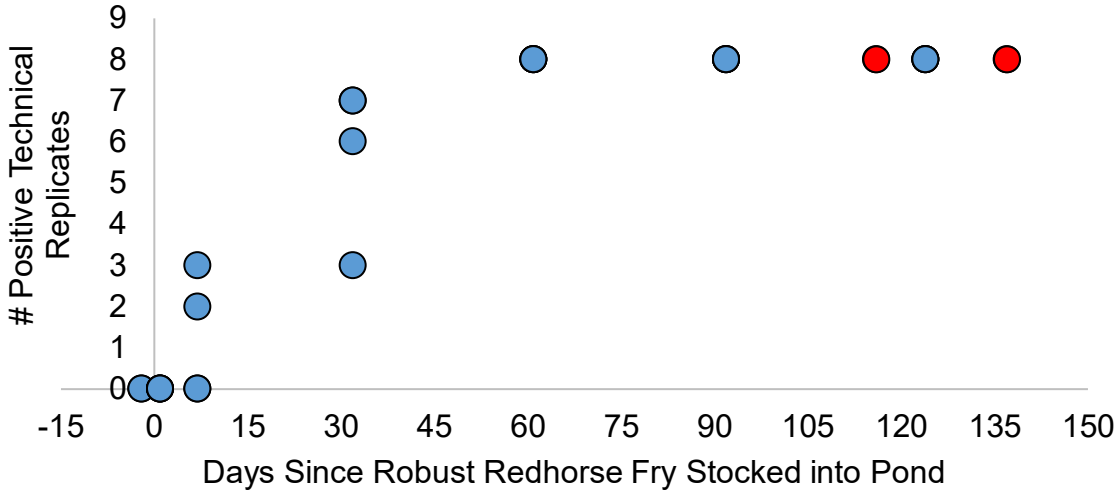

Figure 9. Number of eDNA assay positive technical replicates (all samples were run with 8) versus days since Robust Redhorse fry were released into ponds at the DWC for growth. Collections from 2018 are shown in blue, and collections from 2021 are shown in red.

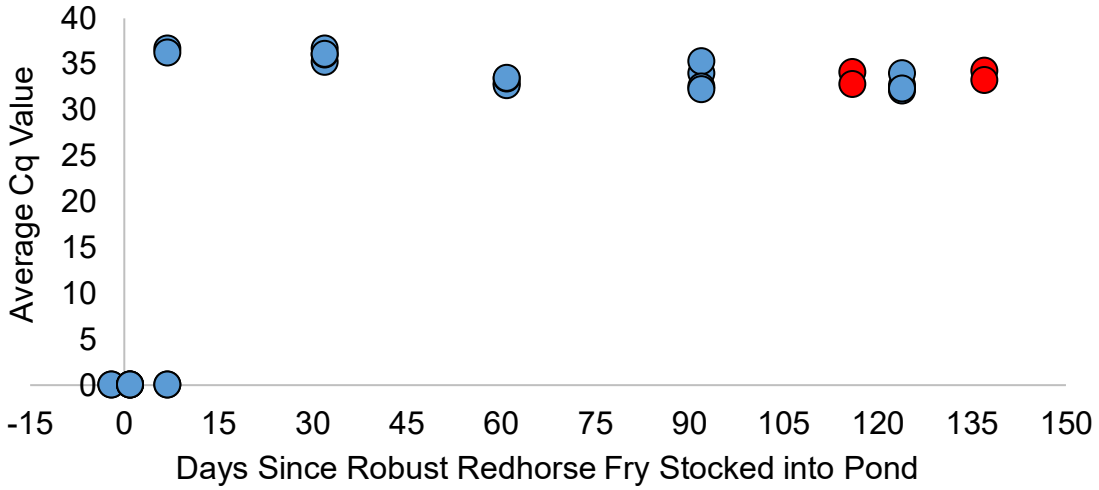

Figure 10. Average Cq value of eDNA assay replicates versus days since Robust Redhorse fry were released into ponds at the DWC for growth. Note that Cq values of 0 are null values indicating that amplification did not occur. Collections from 2018 are shown in blue, and collections from 2021 are shown in red.

<u>Objective 4:</u> Field Validation

Water collection sampling trips were conducted in the Savannah, Ocmulgee, and Broad (GA) rivers to provide a field validation of the new eDNA assay for Robust Redhorse (Figure 11). Ten 2 L water samples were collected from the Augusta Shoals area of the Savannah River on 21 May 2019 (https://www.youtube.com/watch?v=VWH9Hc9U1fU). The sampling area is known to have a gravel bar that mature Robust Redhorse use for spawning. At the time of our collections, two Robust Redhorse were located upstream on the same day via acoustic telemetry of previously tagged individuals (J. Roberts, pers. communication). The water samples were kept on ice until they were returned to the SCDNR Population Genetics Lab and later that day filtered through 1.6 µm glass fiber filters. These filters were kept frozen until DNA was extracted. The extracted DNA was amplified with the final, optimized LNA qPCR eDNA assay to determine if Robust Redhorse could be detected via eDNA in the field. One filter from each water sample was amplified with eight technical replicates and appropriate positive and negative controls. All eight replicates for each of the ten water samples tested positive for Robust Redhorse eDNA, with an average Cq value of 35.3 (Figure 12).

A similar test was conducted in Georgia by UGA by collecting one water sample from the Ocmulgee River below the Juliette Dam during spawning season in 2018 and one sample from upstream of Juliette Dam, which serves as a barrier to upstream passage, on the same day. Five additional samples were collected near adult Robust Redhorse that were visually observed in spawning behaviors in the Broad River in 2018. All water samples consisted of 900 ml of water filtered on site. The extracted DNA was amplified with the final, optimized LNA qPCR eDNA assay. From the initial qPCR results, only a single technical replicate from one site in the Broad River tested positive (Figure 13). We also repeated the qPCR replacing 1 µL of water in the reaction with an additional 1 µL of the DNA isolation (2 µL total). The increase in DNA resulted in more positive detections, with 3 of the 4 Broad River sites having at least one technical replicate test positive (Figure 14). However, no samples (in any replicates) from the Ocmulgee River amplified. These results highlight the importance of considering the expected Robust Redhorse density or expected lack of detection in a given area, and how an increase in the number of technical replicates or an increase in the volume of DNA isolation included in the qPCR may be appropriate measures to take.

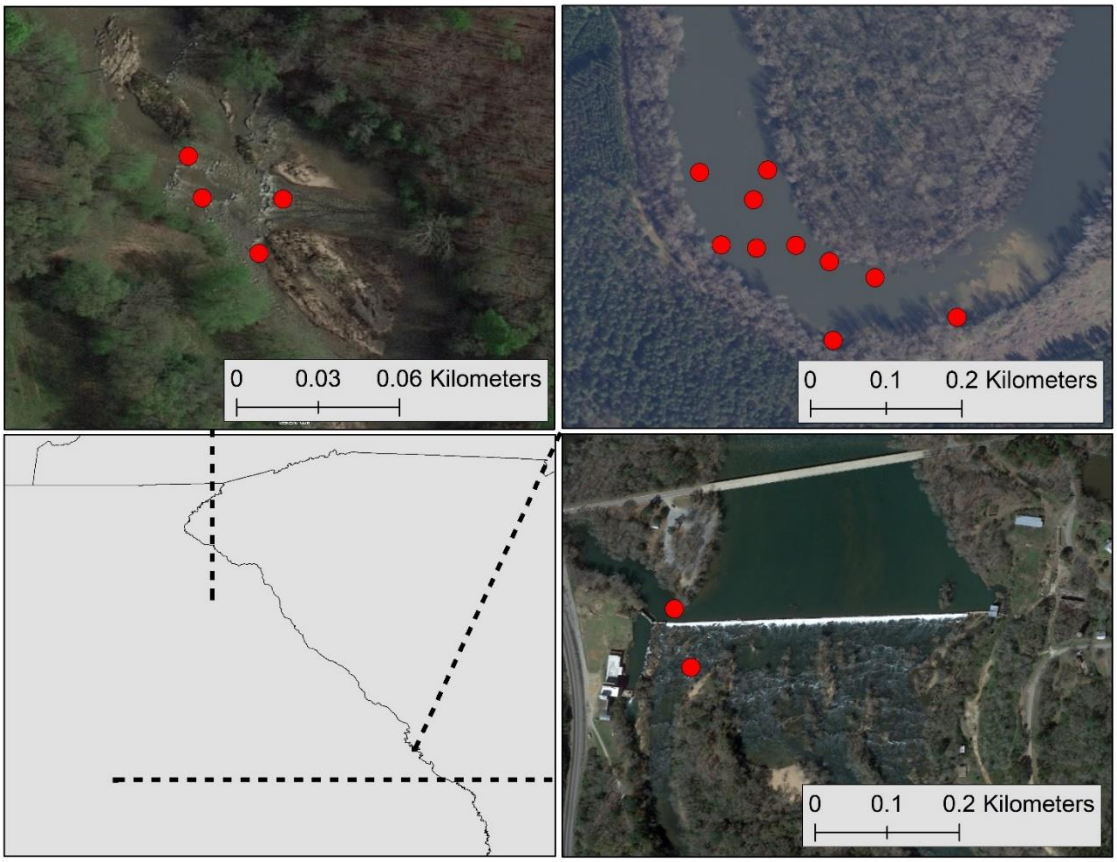

Figure 11. eDNA water samples collected from the Broad River (Savannah River tributary; top left), Savanah River (top right), and Ocmulgee River (bottom right) to test for the ability to detect Robust Redhorse using the new eDNA assay.

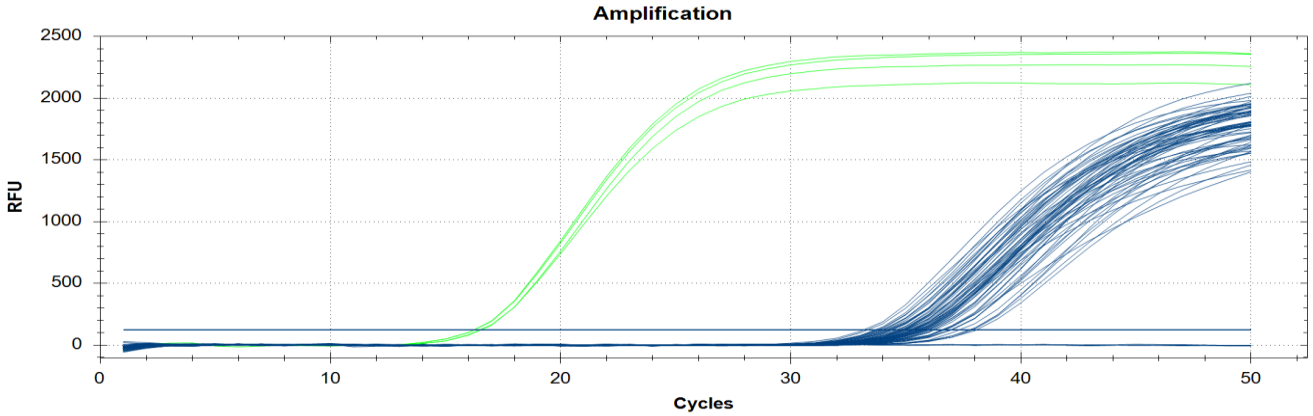

Figure 12. qPCR plot showing positive detections of Robust Redhorse at all technical replicates of eDNA samples collected from the August Shoals area of the Savanah River in May 2019. Positive controls of Robust Redhorse DNA are shown in green, and the field-collected eDNA samples are shown in blue.

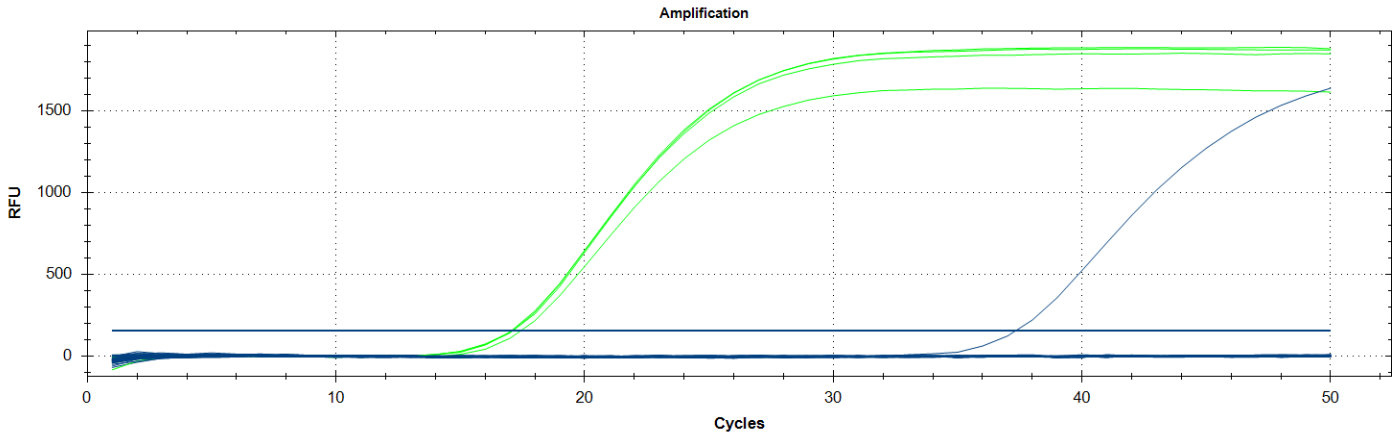

Figure 13. qPCR plot showing one positive detection of Robust Redhorse at one technical replicate of eDNA samples collected from the Ocmulgee and Broad rivers. Positive controls of Robust Redhorse DNA are shown in green, and the field-collected eDNA samples are shown in blue. 1 µL of isolated DNA was included in these reactions.

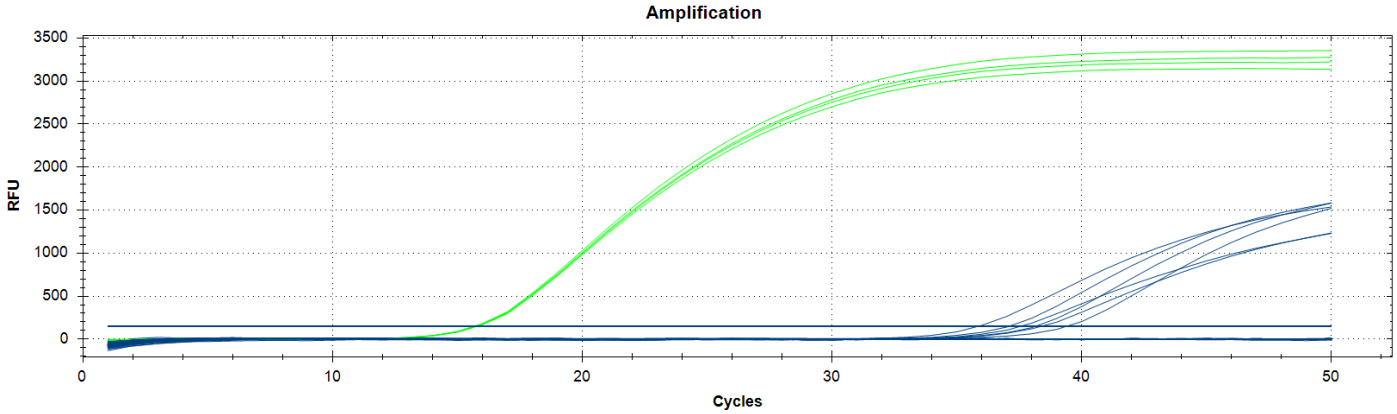

Figure 14. qPCR plot, with 2 µL of isolated DNA, showing positive detections of Robust Redhorse at 3 of 4 eDNA samples collected from the Broad River. All samples/replicates from the Ocmulgee River did not amplify. Positive controls of Robust Redhorse DNA are shown in green, and the field-collected eDNA samples are shown in blue.

<u>Objective 5:</u> Standard Operating Protocol Development

Based on the results of the development, optimization, and testing of the final LNA-based eDNA assay, we have written a Standard Operating Protocol (SOP) for both field and lab implementation of the new eDNA tool for Robust Redhorse. We have provided guidance for both automated on site and delayed lab filtering of water samples. The finalized SOP is included as Appendix A.

Significant Deviations: There have been no significant deviations for this project.

Estimated Project Federal Cost: All project funds were expended to complete the project objectives.

Project Recommendations: The grant has been completed; close the grant.

Prepared by: Daniel Farrae, Tanya Darden; 21 April 2023

Literature Cited

Barnes, M. A., C. R. Turner, C. L. Jerde, M. A. Renshaw, W. L. Chadderton, and D. M. Lodge. 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science and Technology 48:1819–1827.

Bennett, J. 2022. Cloudy waters: the effect of fish density and sediment input on environmental DNA persistence. Master of Science Thesis, University of Georgia.

Bockrath, K. D., M. Tuttle-Lau, E. L. Mize, K. Von Ruden, and Z. Woiak. 2022. Direct comparison of eDNA capture and extraction methods through measuring recovery of synthetic DNA cloned into living cells. Environmental DNA:1–11.

Darden, T, B. Albanese, M. Scott, K. Kubach, M. Walker, and D. Farrae. 2019. Development and implementation of an environmental DNA (eDNA) monitoring tool for Blackbanded Sunfish (*Enneacanthus chaetodon*) populations in South Carolina and Georgia with determination of relative abundance, genetic health, and connectivity of extant populations. Final SC SWG Report; USFWS SC-U2-F14AP00997.

Uchii, K., H. Doi, and T. Minamoto. 2016. A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes. Molecular Ecology Resources 16:415–422.

Waston, A. J. Broach, M. Walker, J. Yost, E. Levesque, J. Allen, L. Yong, and T. Darden. 2023. Cobia stock enhancement and population assessment. Final DJ Report; USFWS F21AF01877.

Appendix A: Robust Redhorse eDNA Standard Operating Protocol

Robust Redhorse eDNA Water Sample Collection Protocol

Safety Information

DANGER

Hazardous substances (see SDS):
- 10% bleach

Personal Protective Equipment (PPE) required:
- Gloves (any variety, all steps)
- Safety glasses (steps involving bleach)
- Lab coat (steps involving bleach)

Personnel: If three personnel are available for sampling, tasks are assigned to the roles described below. If two personnel are available, the Notetaker and Filter handler roles are combined, and the Pump Operator roles remain the same. Care should always be taken to avoid contamination at all steps.

- Notetaker (NT): This person is responsible for opening and closing filter containers, sealing and labeling filter freezer bags, and keeping accurate notes of sampling sites and filter samples and controlling/monitoring the vacuum pump while sampling.
- Pump Operator (PO): This person is responsible for holding the filter sample tube submerged in the water during sampling.
- Filter Handler (FH): This person is responsible for handling and loading the filter on the sampling pole, unloading and placing the filter in a freezer bag, and maintaining proper sterile technique to avoid contamination.

Equipment:

- eDNA sampler, telescoping pole (charge battery)
- eDNA field box (charge remote batteries)
- Self-preserving filters
- Field control water samples
- Cooler with ice, used filter storage box
- Filter labels/markers
- Gloves
- 5 gallon bucket
- Weather appropriate PPE (masks, hats, sunscreen, sunglasses, etc.)

Procedure:

Prepping the eDNA sampler machine

1) Once at a sample site, the eDNA sampler is turned on for the first time (the machine will remain on for the duration of the sampling period). To turn on the machine, flip the "on/off" toggle switch, and the machine will power on with a series of beeps. At this point, the machine will be ready to take its first sample (all settings should be verified), so the clear cover box can be latched closed to minimize exposure to water.

2) The collection hose (longer) is attached to the input valve on the side of the sampler, and the outflow hose (shorter) can be secured to the opposite side of the sampler (and placed to discharge overboard). Note: the red valve covers need to be safely stowed to avoid losing them during sampling.
3) If using the remote control, turn on close to the eDNA sampler to allow for Bluetooth connection to establish. The machine can now be controlled via the remote (the NT will control). Attach the collection hose to the sampling pole.

Taking a sample

4) The NT will set up a new trash bag in the 5 gallon bucket for each sampling site. The NT will open the new filter storage box for the FH. The FH, with a clean pair of gloves, will get one filter out of the box before it is then closed back by the NT.
5) The FH will then carefully tear open the seal (and discard the top piece) and assemble the filter casing inside the bag. This includes removing the filter casing and maneuvering it to connect the large side (white) of the filter casing to the clear tube that remains inside the bag until it is attached to the sampling pole. The storage bag that the filter was in gets handed to the NT to capture appropriate data on the outside of the freezer bag that is then subsequently stored inside the field clipboard. NT should add target species, filter #, and site or station abbreviation/# (ex: AugShoals2) to the label. On the eDNA Field Data Sheet, NT should record date, staff, and weather/water conditions for the day at each station, the time sampling started, role of staff at each station, range of filter #s for the station (these should be sequential and continuous, and include the control), and range of eDNA sampler record #s for the station (these may or may not match), and any noteworthy deviations or observations.
6) After the filter unit is assembled inside the bag, the filter casing can then be mounted to the sampling rod. The PO and the FH work together to place the filter onto the sampling pole, where the FH holds the filter in gloved hands, while the PO secures the filter with the latch mechanism on the sampling pole.
7) Once the filter casing is secured to the sampling pole, the PO lowers the filter to submerge the clear tube in the water while avoiding as much as possible getting the filter housing wet. The NT then engages the pump to begin filtering. When the filter becomes saturated (or 2 L volume threshold is reached), a signal beep will sound. At that point, the PO will remove the sampling pole from the water and wait for the 2nd beep from the sampling unit. Following the 2nd beep, the sampling pole should be turned upside down and the pump turned off. If sampling for a target volume, invert filter assembly immediately after the 1st beep and allow to filter water until the 2nd beep. The PO will then bring the sampling pole back on board and maneuver the apparatus toward the FH.
8) The FH will grab the filter casing, and the PO will help unscrew the filter from the sampling pole. The FH will then pop the seal on the filter casing and allow the pump to dry the filter. This step should be completed carefully and as quickly as possible. The filter casing is resealed, and the FH then removes the entire casing from the collection tube, removes the clear sampling tube (and discard in trash bag) and place sample filter housing in the appropriately labeled storage bag (being held by the NT).

Storing the sample

9) The NT seals the freezer bag and ensures it is appropriately labelled before being placed in the cooler/used filter storage bin.
10) After each filter, the FH should change gloves.

At any point, if the FH's hands become contaminated, they should change gloves.

Field Controls

1) Equipment controls are created by filling a 10% bleach sterilized Nalgene bottle with 500mL of DI water. Gloves, safety glasses, and lab coats are required during sterilization of the Nalgene bottles, which should be completed prior to sampling. Bottles should be soaked in 10% bleach solution for 10 minutes and then flushed thoroughly with DI water prior to being filled with 500mL of DI water.
2) Equipment controls will be taken at the end of each sampling transect.
3) The field sampling procedure remains exactly the same as above for processing the controls, except the clear sample tube is lowered into the control bottle until all control water has passed through the filter. The PO holds and opens the bottle of control water for filtering.

After the completion of field sampling, the eDNA sampler should be flushed with 10L of freshwater. The flushing should be recorded in the eDNA sampling field notebook (log record # and source of water). The eDNA sampler and exterior of hoses should be wiped down with disinfectant wipes. Replace the red valve covers on the in/out ports **with water remaining in the sampling unit** to keep seals from drying out. Charge the sampler battery following each sampling day.

Storage after sampling

1) After returning to the lab after field sampling, place all field and control filters (in their individual storage bags) into a -20° C freezer for storage until processing.
2) Data (including GPS sampling track, filtered volume, and date/time of collection) should be downloaded from the eDNA sampler, reconciled with field notes, and stored in an appropriate database.

Note for sampling water without an eDNA sampler:

The same level of care to avoid contamination should be followed. The general procedure can still be followed, though water will be collected in sterilized glass/plastic 2L bottles. These should be placed on ice immediately following collection and filtered as soon as possible (but within 12 hours of collection) utilizing appropriate sterilization protocols. Freezing/thawing of water samples is not recommended as the DNA will quickly begin to degrade over the time it takes such a large volume of ice to thaw.

Robust Redhorse eDNA Filter Isolation Protocol with the Mo Bio PowerSoil Kit

Hazardous substances (see SDS):

- Solution C1 (contains sodium lauryl sulfate, irritant only)
- Solution C2 (contains ammonium acetate, irritant only)
- Solution C3 (proprietary components, irritant only)
- Solution C4 (contains guanidinium chloride)
- Solution C5 (contains ethanol)
- 10% bleach

DANGER

Personal Protective Equipment (PPE) required:

- Gloves (any variety) (all steps)
- Safety glasses (all steps)
- Lab coat (steps involving bleach & Solution C4)

This protocol is adapted from the Mo Bio PowerSoil standard protocol to use with eDNA filters rather than soil samples and with some added information about preventing contamination.

Before getting started:

1. Prepare the samples or filters to be processed. Select them randomly from the freezer to minimize batch effects. Note: Don't do more than 12 samples in an extraction period.

2. Put on gloves. Get out the Mo Bio PowerSoil Isolation Kit.

3. **Check Solution C1.** If precipitated, place on hot block at 55°C until dissolved.

4. **Wipe down bench with 10% bleach (caution: corrosive).**

5. Get out supplies:
   a. two beakers: small beaker (fill with 10% bleach) and medium beaker (empty).
   b. squirt bottle filled with water.
   c. three 2ml tube racks (bleach after use).
   d. forceps (use the long ones with smooth edges) and dissecting scissors; wipe these down with water and then place them in the beaker with 10% bleach.

6. Line up solutions C1-C6 on the bench (during the isolation, be sure not to touch the pipettes to the sides of these solution bottles when pipetting them out).

7. Get out the number of PowerBead Tubes (provided) that you will need. Spin or tap the PowerBead Tubes down to get all of the liquid in the bottom of the tube. Place the PowerBead Tubes in one of the 2ml tube racks and label them.

8. Place a paper towel beside the 2ml tube rack (cut the filters over this towel, to catch the pieces of the filter if they fall).

9. Get a filter pouch ONE AT A TIME from the freezer and place them on a clean paper towel. You can isolate a maximum of twelve filters during a whole extraction session.

10. **Change gloves.** Rinse off the scissors, use the squirt bottle and the medium beaker to collect the water. Wipe off the scissors with a Kimwipe and place them down on the bench. Rinse and wipe off the forceps in the same manner.

Experienced User Protocol

1. To each of the **PowerBead Tubes** (provided), add a single filter. **Note:** always use your one hand to touch the filter and your other hand (handling with forceps) to hold the instruments.
    a. using the forceps, remove the filter from the tube. Make sure to take only the filter piece (cloth-like) and not the plastic piece that is found between the filter and funnel.
    b. make sure that the filter is folded into an even semi-circle, and that the edges of the filter are crimped together over the clean paper towel
    c. hold the filter vertically with the forceps so it forms a half-moon shape
    d. cut the filter into three equal strips with the scissors, placing half of each strip into the PowerBead tube at a time (e.g. cut each strip until it is almost separated from the rest of the filter, cut off the bottom half of the strip and guide it into the tube with the scissors, disconnect the top half of the strip from the rest of the filter and guide it into the tube with the scissors, repeat for all three strips).
    e. when you are adding the pieces of the filter to the PowerBead tubes, be sure to:
        i. use the scissors to press each piece of the filter up against the side of the tube to make room for more pieces
        ii. make sure all of the pieces of the filter are vertically straight in the tube and are relatively the same height with one another
        iii. make sure that none of the pieces of the filter are laying against the beads at the bottom of the tubes
    f. between each sample:
        i. place the scissors and forceps into the beaker with the 10% bleach
        ii. change your gloves and the paper towel
        iii. rinse the scissors, use the squirt bottle and the empty beaker to collect the water
        iv. wipe the scissors off and place them down on the bench; rinse and wipe off the forceps in the same manner
    g. repeat steps a. - g. for all of the sample filters

2. Add 60µl of **Solution C1 (caution: irritant)** to the side of the tube opposite the filter pieces.

3. Secure the PowerBead Tubes in a bead beater (Specific to QIAGEN TissueLyser; the Mo Bio protocol is written for using an adapter on a vortexer; using a PowerLyzer is also an option). Beat at maximum speed for 10 mins.
    a. turn on the bead beater, open the lid, and take out the two metal plates
    b. add the appropriate number of balance tubes to the short side of the metal plates; place the balance tubes at the ends of the plates
    c. add the sample tubes to the long side of the metal plates; place the sample tubes at the ends of the plates
    d. put the lids on the metal plates and place them in the bead beater; make sure that the plates sit correctly in the slots
    e. tighten the plates down, flip the lock, and continue to tighten (hand-tighten only!)

f. close the lid, lock it, and start the beater (10 mins)
g. double check for any weird noise that may arise from improper plate locking
*during the beating, get the collection tubes ready for the rest of the isolation procedure; you will need to label 4 sets of **2ml Collection Tubes** and 1 set of **Spin Filters** (provided).

4. Centrifuge tubes at 10,000 x *g* for 1 min at room temperature (use the outer row of holes in the centrifuge).

5. Using the filter tips, transfer the supernatant to a clean **2ml Collection Tube** (provided).
Note: Expect ~700µl of supernatant but get as much out of the tube as possible. The supernatant may contain some bits of ground filter, but do not remove any of the beads.

6. Add 250µl of **Solution C2 (caution: irritant)** and vortex for 5 sec. Incubate at 4°C for 5 mins.
*use this time to clean and dry the forceps and scissors; **change gloves**

7. Centrifuge the tubes for 1 min at 10,000 x *g* (use the inner row of holes in the centrifuge).

8. Avoiding the pellet, use filter tips to transfer supernatant to a clean **2 ml Collection Tube**.

9. Add 200µl of **Solution C3 (caution: irritant)** and vortex briefly. Incubate at 4°C for 5 mins.

10. Centrifuge the tubes for 1 min at 10,000 x *g* (use the inner row of holes in the centrifuge).

11. Completely avoiding the pellet (try to get the clearest liquid possible), transfer up to, but no more than, 750µl of supernatant into a clean **2ml Collection Tube** using the filter tips.

12. Shake to mix Solution C4 before use. Add 1100µl of **Solution C4 (caution: irritant)** to the supernatant and vortex for 5 sec. **Note: tubes will be very full after this step, so be very careful when opening/closing them**. Because the C4 bottle is tall, the pipette will be dirty after and will need cleaning after.

13. Using filter tips, load approximately 620µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 min at room temperature (use the inner row of holes on the centrifuge for step 13). Discard the flow through and add an additional 620µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 min at room temperature. Discard the flow through and load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 min at room temperature. Discard the flow through. **Note**: A total of three loads for each sample are required.

Change gloves any time they come in contact with the liquid or use different KIMWIPES as an extra physical barrier to handle the filters to make sure that no liquid gets on the gloves.

14. Add 500µl of **Solution C5 (caution: contains ethanol, flammable)** and centrifuge at room temperature for 30 sec at 10,000 x *g* (use the inner row of holes on the centrifuge). Discard the flow through. **Change gloves.**

15. Centrifuge again at room temperature for 1 min at 10,000 x *g* (inner row of centrifuge).
*get the collection tubes for step 17 open and ready during the spin.

16. Carefully place the spin filter in a clean **2ml Collection Tube**. Avoid splashing any **Solution C5** onto the **Spin Filter**.

17. Add 100µl of **Solution C6** to the center of the white filter membrane.

18. Centrifuge at room temperature for 30 sec at 10,000 x *g* (inner row of centrifuge).

19. Discard the **Spin Filter**. Store DNA frozen (-20° to -80°C).

20. Clean-up. Place the 2ml tube racks in the sink and dump the 10% bleach from the small beaker into the racks; allow the racks to soak in the bleach before rinsing. Put the PowerSoil Kit and all components away. Rinse the bench and all pipettes with bleach.

Robust Redhorse eDNA qPCR Protocol

Safety Information

Hazardous substances (see SDS):
- none

Personal Protective Equipment (PPE) required (all steps):
- Gloves (any variety)

Primer/probe type	Name	Sequence (5′-3′)
Forward	Cytb_1068_F	CGGACAAATTGCTTCCATC
Probe	Cytb_1079_probeLNA	***T**CCT***G**TTTCTAGCCCTAATCCCACT
Reverse	Cytb_1108_R	AGCCTTGTTTTCCAGTCATCC

***locked nucleic acid (LNA) nucleotides**; FAM as reporter dye

qPCR Amplification

All qPCR performed in 11 µL reactions: 5.5 µL iTaq Universal Probes Supermix (BioRad), 2.0 µL water, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.3 µL probe (10 µM), 0.2 µL MgCl2 (25 mM), and 1 µL of DNA. Reactions should be performed on a qPCR machine (such as a BioRad CFX Opus 96) with the following protocol: 30 s at 95° C denaturation, then 50 cycles of 15 s at 94° C and 30 s at 62° C, with a plate read after each of the 50 cycles.

Every qPCR run should have appropriate positive (to verify the PCR worked) and negative/no template DNA (to detect contamination) controls. Any qPCR showing contamination in negative controls should not be used and samples should be re-run.

Repeated runs of the same sample (technical replicate) should be included during all qPCR runs to assess repeatability via the number of replicates that result in positive or negative results. A standard run should include eight technical replicates per analysis.

If all technical replicates of a given sample are negative, the sample should be tested for PCR inhibition. Spiking environmental sample DNA with positive control DNA (1 µL) in place of an equal volume of water (1 µL) can be used to determine if there is a decrease in the Cq value of the positive control during a qPCR.

Analysis and Interpretation

Any “positive” detections with Cq values > 37.5 should be considered questionable and re-run with a greater number of technical replicates or a greater volume of isolation. If same resolution, samples should also be assessed for any inhibition effects that may result in higher Cq values by spiking environmental sample qPCR with positive control DNA to determine if there is a decrease in the Cq value of the positive control. If any samples that are coming from an expected low Robust Redhorse

density area test positive at a single technical replicate, then the sample should be re-run with a greater number of technical replicates or a greater volume of isolation in the PCR. Samples from an expected low density (or unlikely to occur) area can also be run with more technical replicates or a greater volume of isolation initially, though this should be done judiciously since there is a limited volume of isolation available (100 µL when following this protocol).