Non-Invasive Detection and Identification of Eastern Hellbender in Ohio Surface Waters Using Environmental DNA


Image Courtesy of Greg Lipps

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16. Abstract

This research documents the development and application of an eDNA detection method for *Cryptobranchus allegeniensis*, or Eastern Hellbender in Ohio surface waters. The purpose of this method is to enable safe, non-invasive and convenient survey strategy for these endangered animals. Our DNA amplification target is the Hellbender mitochondrial genome, specifically the left D-loop hyper-variable region, which we use as a means of detecting and tracking the animals, and in some circumstances, distinguishing between individuals.

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Problem Statement

The Eastern Hellbender (*Cryptobranchus allegeniensis)* is an elusive salamander species that is native to the state of Ohio. This fully aquatic salamander was once found in great abundance in Ohio streams, but the population has declined rapidly in recent years. In the past few decades alone, the population of Eastern Hellbenders has decreased by nearly 80% in Ohio [5]. As a result, hellbenders have been listed as endangered by the Ohio Department of Natural Resources and have previously been a candidate for federal endangerment [11]. To combat the growing threat to hellbenders, conservation efforts throughout the region have been established and the demand for less harmful practices has grown.

Figure 1. Adult eastern hellbender, image from Greg Lipps Hellbender Consortium

Hellbenders are distinguished by their size as they can grow up to two feet in length making them the largest amphibian in North America. They serve as both predator and prey as they mainly feed on crayfish, but are preyed upon by raccoons, fish, turtles, and other hellbenders. They are brown to reddish-brown in color with folded outer layer of skin.

Hellbenders have traditionally existed in the eastern United States along the western portion of the Appalachian Mountains. They are known as "habitat specialists" as they seek out creeks and rivers with healthy ecosystems. Streams with swiftly flowing water characterized by high levels of dissolved oxygen and low levels of sediment are suitable habitats. Because these animals seek out markedly clean environments, their presence is an indication of clean water and the overall health of the ecosystem. Current threats to hellbenders are primarily from modern farming practices, including runoff, siltation, industrial pollution, and degradation of stream habitat from other anthropogenic development like conversion of forests to agriculture [6]. Additionally, common waterway engineering, including stream channelization, damming, and removal of rocks are detrimental to maintaining hellbender habitat.

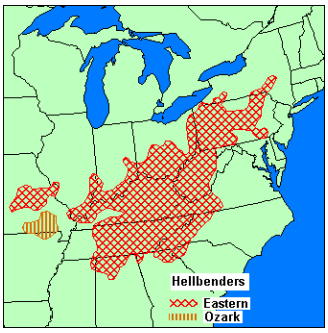


Figure 2. Eastern hellbender geographical distribution [7]

In order to minimize these threats and protect an already threatened species, the Ohio Department of Transportation (ODOT) performs habitat assessments at project sites prior to construction. Knowledge of whether endangered animals are present near project sites is vital to determining the constraints and scope of construction projects. The current method of assessing hellbender distribution is by visual survey wherein surveyors wade through

streams and manually search under rocks. This method is not only costly and time-consuming but detrimental to the animal's habitat, since it disturbs where they live and forage. These shortcomings foster a need for less invasive methods of detection. This research has sought to develop a detection method that relies on DNA produced by the animal in its aquatic environment (eDNA). Detection via eDNA provides a faster, less expensive, and most importantly, less invasive procedure for determining the presence of hellbenders in Ohio streams. By sampling surface water and amplifying target DNA, animal detection and proximity to sampling location can be determined with minimal human interactions.

Research Background

Environmental DNA (eDNA) can be defined simply as genetic material found in the environment. Just as forensic science uses DNA to identify suspected criminals, eDNA is used to identify animal, plant, and insect species in the environment using trace amounts of DNA left behind in waste, secretions, cell sloughing, and mating, among others. When used in aquatic settings, eDNA can provide information as to which species are present, the quantity of individual species, and the distribution of animals throughout the body of water.

Detection of aquatic animals by eDNA analysis has been practiced in rivers, streams, lakes, and other marine habitats. It has also been applied to examine fish, mollusks, amphibians, and crustaceans [4]. eDNA generally comes from epidermal cells, excrement, hair, body fluids, and germ cells. A standard workflow for eDNA analysis in aquatic settings involves the collection of water samples, then immediate analysis through biological tests including polymerase chain reactions. If tests show that DNA for a specific animal is present in the water, researchers can then perform additional tests to determine the quantity of the animal, the location of the animal relative to the testing sites, and the genetic sequence of the animal(s). Many variables affect the detection of eDNA such as rates of production and degradation, concentration, and transport from the locus of production. Factors such as streamflow, temperature, and time of year can greatly impact whether genetic material for an animal is detected.

This method has shown success in numerous areas of environmental research. A team led by Christopher Jerde from the Marine Science Institute at University of California Santa Barbara examined the effectiveness of eDNA in detecting two invasive species of Asian Carp [2]. These two species have invaded the Mississippi River Basin and there have been countless efforts to prevent these fish from invading the Great Lakes via the Chicago Sanitary and Ship Canal. Jerde's team successfully detected these species in Lake Michigan before they were eventually caught by local fisheries. Their detection results preceded those of conventional surveying thus providing valuable time and knowledge that helped advance the effort to quell the spread of the fish. Another project conducted in Japan sought to analyze the effectiveness of eDNA testing versus rudimentary hand capturing methods for the endangered Japanese clawed salamander, *O. japonicus* [4]. The group found that for nearly all sites where *O. Japonicus* was found by hand-capture surveying, eDNA for the animal was detected. Additionally, at sites where the animal was not found by hand-capture, eDNA was detected proving that this method may sometimes be more effective than conventional surveying strategies [4].

In the past several years, research has been conducted on eDNA detection of hellbenders in Indiana [8], Missouri [9], North Carolina [10], and Kentucky [9]. The results were comparable to physical hellbender surveys; yet no research has been performed for hellbender detection with eDNA in the state of Ohio. Thus, the competency of primer sets developed for detection in other states has not been verified for the Ohio population of eastern hellbenders. This project therefore sought to develop detection mechanisms of hellbenders in Ohio using eDNA and to test these methods on streams throughout the state. Additional and more detailed literature review can be found in Appendix B of this report.

The central objectives for this project included developing genetic testing protocols that are specific for Ohio hellbenders, establishing sampling procedures for the collection of Hellbender eDNA, and assessing the impact that different stream conditions have on detectability. The project was split up into two phases with the first phase focusing on the development of genetic testing protocols and a sampling procedure, and Phase II focusing on the further improvement of Phase I findings, identification of detection limits and inhibitors, and identification of Ohio streams where hellbenders are present.

Task 1 of Phase I consisted of developing a species-specific primer set to be used in genetic sampling. Published primers were tested but were unable to produce reliable detections so new primer sets were developed. Several iterations of new primers were tested, and the most successful primer set was then used during the remainder of the project. Task 2 of Phase I was the collection of environmental samples. During the first phase, Salt Creek and Scioto Brush Creek were chosen as control creeks on the advice of the Hellbender Consortium. A best practice sampling protocol was developed, then further perfected throughout the remainder of the project. Task 3 was development, validation, and modification of an eDNA protocol. This consisted of analyzing different stream conditions and their effect on detectability. Additionally, bacterial markers, and later viral indicators, were examined to determine their efficacy in detecting hellbenders. Finally, Task 4 of Phase I involved improvement of the established eDNA protocol and the submission of the interim report.

The objectives of Phase II consisted of improving and expanding the developed eDNA detection method. The first task of Phase II was to apply the D-loop primers developed in Phase I and develop bacterial primers for longer range detection. Bacterial indicators were initially thought to have potential in long-range detection of hellbenders, but this thinking eventually gave way to the use of viral indicators. Task 2 was to collect more environmental samples and test stream parameter influence on detection. Task 3 was the continued evaluation of eDNA detection limits through further testing of the long-range detection mechanisms and analysis of different stream conditions' effect on detectability. Task 4 of Phase II was the development of eDNA sequencing and bioinformatics protocols to distinguish individual animals.

The tasks outlined at the beginning and middle of this project were fulfilled through the development and continual improvement of the protocols. By sampling several streams throughout the region, the effectiveness of this protocol was reinforced and the conditions at which the protocol is most effective was determined. The research approach outlined below describes how these were fulfilled.

Research Approach

Sampling

During the first phase of the project, water samples were collected at Salt Creek and Scioto Brush Creek located in southern central Ohio. These were chosen as control creeks upon the recommendation of the director for the Hellbender Consortium. Multiple samples were taken at each site at varying distances from the animal habitat coordinates. In addition to these two sites, Hellbenders at an aquaculture facility at the Columbus Zoo were swabbed and tank water from the animal husbandry facility was sampled for genetic testing. Samples were also taken at channelized portion of Duck Creek in eastern Cincinnati which was selected as a negative control due to its abundance of eDNA from municipal combined sewer overflow leakage, and lack of aquatic life in the sampled section. These sources acted as important controls when developing the primer set and PCR protocol.

Figure 3. Salt Creek sampling locations

Figure 4. Scioto Brush Creek sampling locations

During the first part of Phase II, the Blue River was the focus of sampling efforts. A collaboration with Dr. Rod Williams (Purdue University) allowed us to sample water while his researchers released and tracked geo-tagged animals. With their assistance, two locations where captive bred hellbenders were released and known to inhabit were chosen for sampling. The results obtained from the Blue River trips helped determine the distance limit from a habitat that the animals can be detected by eDNA. In addition, several factors such as streamflow and water temperature were all analyzed for their effect on detectability of hellbender DNA. The Figure below depicts the locations along the Blue River where samples were taken during three different sampling trips in 2019.

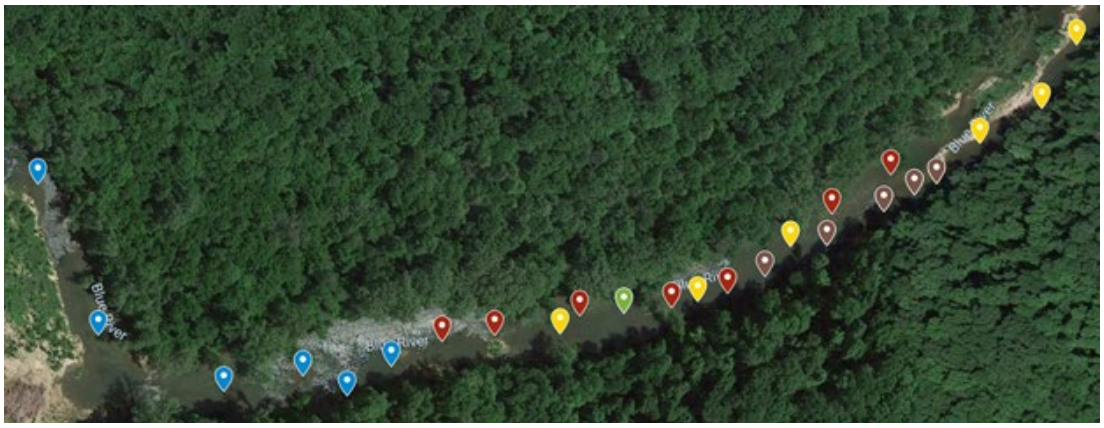


Figure 5 : Satellite View of sampling locations. 919 Samples (September 26, 2019) are colored in Blue. 1019 Samples (October 10, 2019) are colored in Red. 1119 Samples (October 23, 2019) are colored in Yellow. Common sampling locations for all three trips are colored in Brown. Sampling locations colored in Green are a common location for 2 trips. River flow is from right side of image to left.

After this, several new streams were selected for sampling in Ohio. These were chosen by considering factors such as streamflow, location, water quality, and water features. A thorough process for ranking streams in Ohio by suitability for hellbender habitation was developed. A full description of this procedure is included in the Appendix B. The selected streams were White Oak Creek, O'Bannon Creek, Big Darby Creek, the Kokosing River, and Jelloway Creek.

Figure 6. Streams selected for sampling in 2020

Figure 7. 8/6/20 Jelloway Creek sampling map

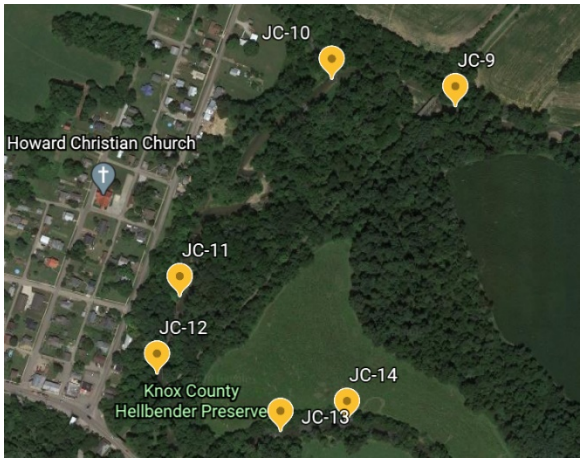


Figure 8. 10/2/20 Jelloway Creek sampling map

When taking environmental samples, it is important to maintain uniform practices. The process must be clean so that unwanted material like sediment/algae/detritus are avoided. Collection must be consistent so that certain samples do not appear to have more material than others simply because more water was collected. Further, certain protocols must be followed to preserve the samples. Over the course of the project, a sampling protocol that incorporated these standards was developed. A detailed description of this procedure can be found in Appendix A.

Samples were collected in 1L sterile Nalgene bottles. The samples were taken in the middle of the stream at a medium depth. Researchers used disposable nitrile gloves and positioned themselves downstream of the grab sample site. Water samples were then kept in a cooler of ice at 4 °C to ensure that bacterial organisms had minimal replication and that DNA in the water did not degrade. The samples were taken directly back to the lab and filtered with a 0.45 um nitrocellulose membrane. The genetic material in the filtrate was then extracted using Mo-Bio Power Water kits and kept at 4 °C.

Several stream conditions were analyzed during this project for their effect on hellbender detection. Streamflow was theorized to be the largest factor affecting detectability. With a high level of flow, any eDNA in the water would be much less concentrated and thus, more difficult to detect. The streamflow of the creeks and rivers where sampling occurred was recorded from the USGS. For the streams where the USGS does not record measurements, streamflow was calculated using a process suggested by the US EPA[12]. This involved measuring the width of the creek and taking depth measurements at roughly equal distances to obtain a cross section. The time it took a tennis ball to travel a given distance downstream was recorded several times and an average speed was calculated. Then, the streamflow was determined by multiplying the cross-sectional area by the speed of the water. Additionally, this value was multiplied by a correction factor for rocky-bottom creeks that accounts for the surface of the water traveling faster than other portions of the cross section [12].

In addition to the streamflow at the time of sampling, the streamflow pattern prior to sampling was analyzed. This was undertaken to ensure eDNA is not washed out by high stream flows prior to sampling. Water temperature was also considered, in order to determine the

effects on limit of detection downstream of a known animal location, and multiple samples were collected downstream of known animal locations at certain creeks to determine variability in quantitative eDNA results.

PCR Primer Development

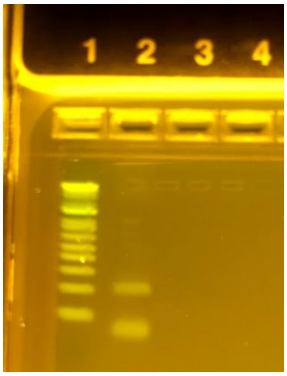


Figure 9. Gel picture of desired PCR product in lane 2 with primer

The protocol we developed for hellbender detection utilized polymerase chain reactions (PCR). This type of biological assay replicates DNA from a sample only if the desired DNA is present. Put another way, DNA replication will only occur in this test if hellbender DNA is present in the water sample. The results of the PCR reactions were then determined by gel electrophoresis. This method shows the size of the DNA fragments that amplified in the PCR. If fragments that are the size of the desired product are present, it can be said that the sample contains hellbender DNA. The image to the right depicts a sample in lane 2 that was determined to contain hellbender DNA due to its product size from the PCR.

In order to accurately and consistently detect/track the animals, the hyper-variable or "D-loop" region of the mitochondrial genome was the focus of this research. It is possible to identify individuals and maternally related relatives via the hyper-variable region of the mitochondrial DNA (mtDNA) in eDNA samples [3]. Recent studies into hellbender eDNA have not focused on the hyper-variable region of mtDNA as a target for individual identification. This project, therefore, sought to explore the use of the hyper-variable region of mtDNA to distinguish between different hellbenders.

During Phase I, the primer set and PCR conditions used by (Spear et al, Biological Conservation, 2014) [10] were tested on the positive controls (animal swabs, aquaculture tank water) and the negative control (Duck Creek) [10]. An Applied BioSystems 7500 Real Time PCR machine was used with identical parameters and reagents (primers, Taqman probe and Qiagen Quantitect Multiplex mastermix) as the published protocol. A variety of template DNA concentrations and qPCR cycling parameters were attempted but none of the tests detected hellbender DNA in the swab samples or animal tank water samples.

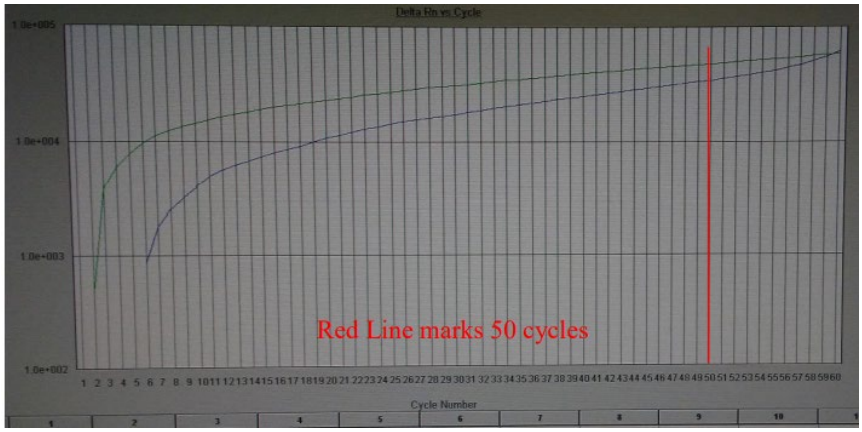


Figure 10. qPCR of positive controls using published

Shown above is a typical qPCR for Positive Control DNA (animal swab) using the originally published protocols. Results are considered positive when the amplification plot becomes exponential instead of linear and occurs between 20 and 50 cycles. Any amplification after 50 cycles is unreliable and signal amplitude (y-axis) remains orders of magnitude below efficient PCR amplification. This indicated the published primers were returning non-detects from the tank water and the swab of the live animal. While we were unsuccessful generating positive qPCR reactions using the published conditions, it is possible that PCR inhibitors co-purifying with sample DNA is responsible. We tried different dilutions of template DNA to circumvent these inhibitors without success. Given this result, we explored if our reactions were generating any product at all via PCR reactions with additional polymerase kits. To our surprise only poorly amplified, non-specific targets were generated in either the negative or positive controls (Figure 11).

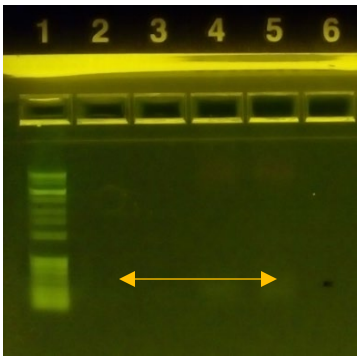
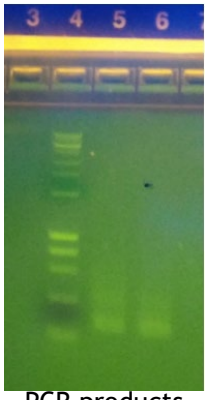


Figure 11. Lanes 2 and 3 represent positive control (animal husbandry tank water) and lanes 4,5 negative controls

Figure 11 shows PCR results for positive and negative control template DNA with (Spear et al., 2014) primers using 2 DNA polymerases (Q5-NEB and Qiagen Quantitect). Lanes 2 and 3 are PCR products from New England Biolabs Q5 Hi-Fi DNA Polymerase, with Positive Control (Tank Water) in Lane 2 and Negative Control in Lane 3. Lanes 4 and 5 are PCR products from Qiagen Quantitect Multiplex DNA Polymerase which was used in the published protocol. The positive control (tank water) is in Lane 4 and Negative Control is in Lane 5. Lane 1 contains the DNA Marker Ladder. Positive results would be visible as a clear bright band around 150bp (yellow line with arrows). Faint amplicon below this expected size was seen in both positive and negative controls, indicating reaction byproducts instead of amplified target DNA. This confirmed the non-specific (no detect), low amplitude results of the qPCR reactions. However, it should be noted that qPCR relying on a TaqMan probe for amplicon detection could circumvent non-specific amplification products, thus in the event the Columbus Zoo animal aquaculture tank water produced PCR inhibitors that co-purified and other, non-specific amplicons typically amplify in greater quantities, but these do not react with the established TaqMan probe, it is possible that the previously published primers are viable under other water conditions. However, we cannot explain why animal swabs did not amplify, unless the same inhibitors in the tank water were present on the animal skin. Given the lack of amplicon with positive controls and a desire to explore Hellbender mtDNA Hypervariable region sequence complexity, we explored new D-loop primer sets.

It has been suggested by reviewers that *Cryptobranchus allegeniensis* could inhabit our negative control location in Duck creek, so to provide additional clarification, we wish to point out that the negative control sample location, as shown in Figure 3 of the Phase I report, was taken from a portion of the Duck Creek watershed that was completely channelized (no rocks), occasionally running dry in the summer, with the source water for the sampled portion of the channel, at the time of sampling, coming from a leaking combined sewer overflow (CSO). This was considered a negative control, as stated above, due to lack of habitat, no sign of animals (fish or amphibians) and source of input waters (municipal wastewater). It is possible that some very hardy animals inhabit some other portions of Duck Creek as suggested by reviewers, but given the authors past work documenting human waste impact to Duck Creek and Deerfield Creek (Duckcreek Watershed), from CSO input, the chance of survival in much of this watershed seems very remote.



PCR products

Given the difficulties with published primer sets; namely, no amplification in positive control qPCR's and non-specific amplification visible in our PCR reactions (Figures 10-11), new primers were designed and tested starting with the left D-loop of the Hellbender mtDNA. The original left D-loop covered a region from the Cytochrome B gene into the beginning of Variable Region I of the mitochondrial D-loop; and in our hands this target produced more reliable detection when used with the positive control and environmental samples then available primer sets. Our primers were then coupled with a nested PCR primer set to provide additional specificity and detection sensitivity. Figure 12 to the left depicts the results of the left D-loop Nested PCR products. A clear band can be seen around the 130bp area, with very little non-specific resulting from the nested PCR reaction.

Some homology to other salamander species was discovered in the original left D-loop primers by way of primer BLAST on NCBI, so two new species-specific primer set variations were designed. The new primers were designed to amplify regions of the D-loop hypervariable region that are 503bp and 609bp, respectively. Figure 13 on the right shows the results of New England Biolabs Q5 HiFi Polymerase with these primers. Lane 6 is the DNA Marker Ladder. Lane 7 is the 503bp primer reaction on the zoo tank water. Lane 8 is the 503bp primers on the Negative Control sample. Lane 9 is the 609bp primers on the animal swab Positive Control. Lane 10 is the 609bp primers on the Negative Control Sample. While these results show additional amplification, the nested strategy was designed to eliminate unwanted product while also providing a future substrate for sequencing and animal identification. Nested amplicon specificity was verified via Sanger sequencing (plasmid library) and NGS using Oxford Nanopore's MinIon (figures 30-40).

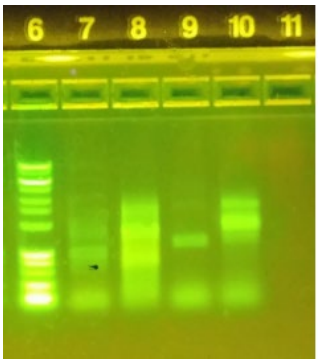


Figure 13. 503 bp and 609 bp products with Q5 polymerase mastermix

Both new primer sets showed improved ability to detect target DNA. A bright band at 609bp can be seen in Lane 9 and 503 bp product (among others) in lane 7. Non-specific amplification (and no 503 bp product) can be seen in the negative controls (lane 8 and 10) samples. That is, there appeared to be little to no amplification of analogously-sized product in these controls, indicating these primer sets could be useful template for subsequent nested rounds.

An amplification protocol for positive control and environmental samples was developed using the 609bp primers and optimized for amplicon yield and specificity. Unfortunately, this primer set was ultimately discarded because it occasionally showed faint product (at an analogous 600 bp length) in negative control samples, thus further effort was applied to the D-loop primers producing the 503 bp product. These primers may hold promise for future nanopore sequencing, described later in the report.

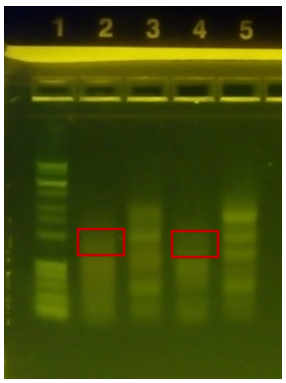


Figure 14. Initial 503 bp PCR reactions with environmental samples and negative control

A large variety of experimental conditions were tested to increase amplification specificity and yield, varying the volumes and concentrations of all thermocycling and PCR mix parameters, and a detailed protocol was established for these primers. While animal specific amplicon was generated in positive control samples and the 503 band was absent from negative control samples, some non-specific amplification occurred. This necessitated a nested reaction to ensure PCR outputs could reliably identify hellbender presence or absence in environmental samples, as well as provide potential sequence information about individual animals.

Figure 14 shows the results of the 503bp target primers tested on 3 environmental samples and the negative control sample, all using New England Biolabs Q5 HiFi DNA Polymerase. While there is non-specific amplification occurring in all the environmental samples and the negative control, bands can be seen in 2 of the environmental samples at the 503bp length that can serve as template for subsequent rounds, while the negative control has no product at that size. Like the left D-loop primer set, nested primers were designed for the 503 bp target, producing bright and specific amplicon, demonstrating a slightly larger amplicon that appeared to amplify more efficiently. The resulting gel electrophoresis PCR clearly shows detection of the animal in Positive Controls and no detection in Negative Controls (Figure 15).

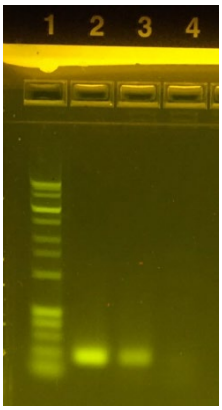


Figure 15. Nested PCR products derived from 503 PCR template

Figure 15 shows the gel electrophoresis of products of the nested PCR. Lane 2 contains positive control Hellbender DNA extracted from aquaculture water/ animal swabs. Lane 3 contains eDNA from Salt Creek (determined positive for Hellbender from Phase I) as template. Lane 4 contains products from the Negative Control as template. The Nested PCR target is approximately 200bp. As seen in the image, both the Positive control and the Salt Creek sample show clear bright bands at 200bp with almost all non-specific amplification having been eliminated. The negative control sample in Lane 4 showed no appreciable amplification eliminating the non-specific products of the previous “503” reaction. Encouraged by these results, more PCR reactions for the initial 503 primer set were explored on eDNA and positive control samples, resulting in relatively clean product shown in Figure 16 on the right, using only 1 primer set. Thus, for some samples we were able to have

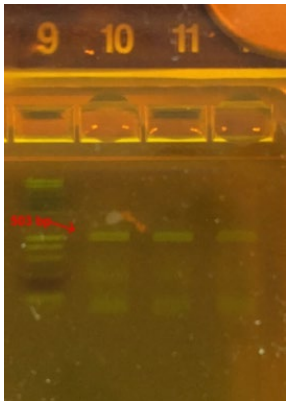


Figure 16. Results of optimized PCR reaction with 503 bp product

strong evidence of animal presence after the 503 target reaction, with the nested round serving only as an additional positive control.

Digital Droplet PCR

After identifying a successful PCR method for detection of animal presence in environmental waters with a nested PCR, we explored the downstream distance dependent detection limits. To accomplish this, digital droplet PCR (ddPCR) technology was used, since this form of DNA amplification is the currently the most sensitive form of quantifying DNA amplification. ddPCR quantizes a conventional PCR reaction into tens of thousands of femtoliter sized individual reactions. The emulsion volume is designed to produce, on average, about one strand of template DNA per droplet. Therefore, up to 20,000 simultaneous reactions occur, each in individual droplets, while excluding misprimed inter-amplicon interactions.

The aim for this method was to amplify and quantify the nested 503 product. For ddPCR quantification of our target, we used Bio-Rad QX200 Digital Droplet PCR system with Quantasoft software for analysis.

The fluorescence of the ddPCR product is displayed on a graph with the droplets arranged in order of droplets counted (see figure 17)

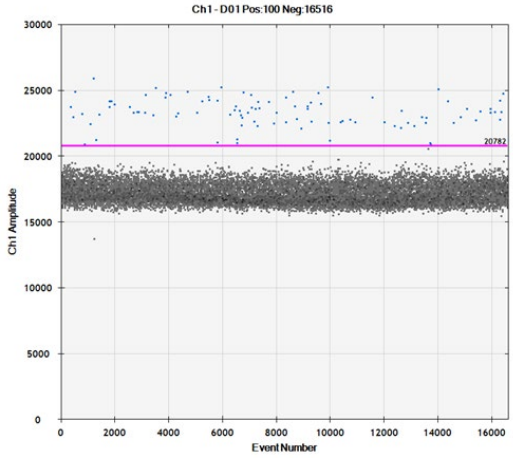


Figure 17. ddPCR fluorescence plot with manually

By comparing the nested amplicon concentrations from different sample sites obtained in a survey stream, the relative location of hellbenders can be identified. The distance dependent decay/ detection limit of our method was identified in Phase I at approximately 1/4 – 1/3 of a river mile (Figure 23). While this detection limit distance will be highly location (water quality, flow rate, temperature, inhibitors) specific, it should be pointed out that the multi-mile detection limit of past work [10] was never achieved with any primer set we tried. Given the lack of efficient amplification of animal skin swabs with these [10] primer sets, it may be that some of these detection events were non-specific. Likewise, detection limits can be derived from a ddPCR by taking multiple samples at intervals downstream of a known hellbender location. The concentrations decrease according to the distance from the eDNA

source and the maximum distance for detection can be found. This principle was employed for the Salt Creek samples to obtain a downstream detection limit for that creek.

Amphibian Specific Ranavirus

As part of Phase II, secondary PCR targets were investigated to improve the distance detection limitations. One method that was considered was tracing bacterial markers from the microbiome of Hellbenders. However, research conducted by Dr. Williams research lab found a paucity of unique bacterial targets on the skin microbiome of hellbenders that might enable specific identification. As a result, we shifted focus to potentially more abundant microorganism: an amphibian specific version of the Ranavirus. Ranavirus are one of the most abundant microorganisms found in environmental water. The Ranavirus genus can infect amphibians and reptiles, including frogs, turtles, and salamanders. Knowing this, a primer set specific to Ranavirus that infects only amphibians was developed. While a Ranavirus specific to amphibians could potentially produce false positives for Mudpuppies, frogs, and other non-specific targets, our long-range strategy was considered only as a preliminary step, in conjunction with subsequent 503/nested rounds. Thus, a positive Ranavirus amplicon did not equate to Hellbender detection, only that it could be useful to then resample and/or run subsequent PCR rounds closer or upstream of a Ranavirus positive location if a related 503 PCR produced no amplicon.

The Ranavirus primers provided a broad net for potential long-range detection. Although Ranavirus is not exclusive to hellbenders, the results indicated a relationship between sample positivity and Ranavirus presence (Figure 27). This PCR was also performed as a ddPCR and the results were compared to those from the nested PCR reactions.

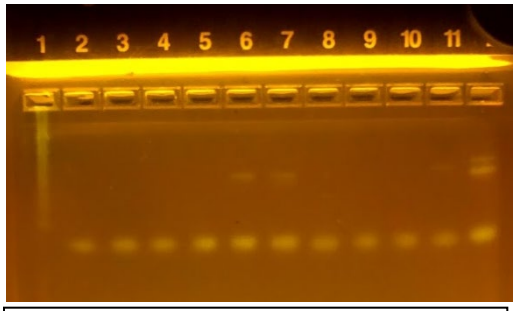


Figure 18: Amphibian specific Ranavirus PCR products. Lanes 6,7 11, and 12 contain the desired length DNA.

Sequencing

Assays performed during Phase I and much of Phase II found that two separate, but interdependent PCR rounds were necessary for the isolation of clean mtDNA product. This product was accurate enough to reliably determine presence/absence of hellbenders but longer mtDNA sequence information was desirable to potentially reveal single nucleotide polymorphisms (SNPs) present for distinguishing animals. Therefore, a new PCR protocol was developed which used the 503 forward primer along with the nested reverse primer. This reaction maintains some the specificity of the nested reaction while producing a longer product. Since sequence data allows one to identify/exclude an amplicon as homologous to the animal's reference genome, the goal with the sequencing PCR product was to produce enough clean product to have successful plasmid library preparation. The image on the right shows the products of this reaction in lanes 2, 3, 5, 6, and 7. The DNA shown in these wells are dilutions of a gel purified product of the new sequencing PCR.

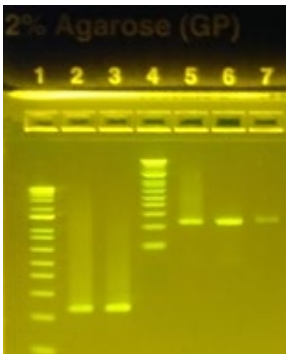


Figure 19. PCR products of new sequencing reaction

The products of this reaction (amplicons or amplicons transferred to pETBlue2) were sequenced using the Sanger method. This was performed as the most cost-effective method for sequencing, and it confirmed the amplified DNA was hellbender mitochondrial DNA (Figures 30-32). The samples sequenced were compared to the National Center for Biotechnology Information (NCBI) nucleotide collection to analyze the homology between the samples and the hellbender mitochondrial D-loop region (Figures 30-32). Divergences between sample sequences and the NCBI sequence helped determine if there were different animals present (Figures 30-32).

Sensor Development

We also examined the feasibility of a sensor prototype that was tested in the laboratory setting to determine the presence or absence of specific mtDNA sequence. The majority of the technologies for mtDNA detection require laborious enzymatic digestion, DNA denaturation, and PCR amplification prior to detection [13-17] which are not desirable for being deployed in the field. The prototype that we developed and tested with mtDNA and circular plasmid DNA aimed to be simple and low-cost and also minimize the sample preparation steps while maintaining the specificity, sensitivity, and reproducibility of the results. The device is miniaturized and electrokinetic-based that can operate with low-voltage and thus can be powered with batteries for deployable purpose. This sensor omits the need for enzymatic digestion, DNA denaturation, and PCR-amplification. It provides “yes” or “no” answer in the presence or absence of the target mtDNA sequence which could be easily digitalized and transmitted remotely. The sensor is based on nanopore sensing scheme combined with a highly sensitive peptide nucleic acid (PNA) conjugated to the bead-based hybridization assay for detection of sequence-specific circular double stranded DNA. To amplify the signal intrinsically, the bead upon hybridization with the target is moved under the controllable electrokinetic forces toward a nanopore and blocked the pore which resulted in an amplified ionic current signal. Upon the pore blockade by the bead harboring the target DNA, the ionic current blockade shows distinguishable conductance signals when compared to the control experiments with non-complementary DNA sequences (Figure 20). Also, as the bead blocks the pore, the signal duration is extended to tens of milliseconds which can be easily detected with a simple and low-cost operational amplifier. This semi-automated, sensor is capable of detection of circular mtDNA at 10 picomolar (pM) concentration [18]. Therefore, in future, this miniaturized, low-power and PCR-independent sensor can be integrated with microfluidics to be evolved as a rapid, cost-effective, and portable mtDNA detection tool for a complete deployable environmental sensing purposes.

Procedure of the Sensor Development

Probe Coupling and Characterization of Microspheres

100 µL of carboxylic acid polystyrene beads (6.78 ×109/mL) were conjugated with 10 nmol of amine-functionalized PNA probe. To evaluate the conjugation chemistry procedure zeta potential of the negatively charged carboxylic acid polystyrene beads was measured prior and after the immobilization of the neutral PNA probes utilizing the NanoBrook Omni (Brookhaven Instruments Corp, Holtsville, NY). Zeta potential for carboxylic acid beads was -60 ± 2.2 mV, followed by -15 ± 4.0 mV for PNA-beads.

Hybridization Assay

The probe conjugated beads were washed three times with 0.4×SSC buffer (60 mM NaCl, 6 mM trisodium citrate, 0.1%Triton X-100 in DI water, pH 8) and were re-suspended into 50 µL of hybridization buffer (10 mM Na3PO4, 1 mM EDTA in DI water, pH 7). The PNA-beads were incubated with serially diluted target and control circular DNA from 100 pM to 1 pM at 42 °C overnight. After hybridization, the samples were washed three times with 0.4×SSC buffer and suspended into 10 mM KCl, 10 mM HEPES, pH 7 solution for sensing measurements.

Sensor Apparatus and Electrical Measurements

Micropipettes with 2 µm diameter were fabricated utilizing the laser-assisted puller-Sutter P 2000 with a pre-set program (Heat 350, Filament 4, Velocity 40, Delay 200, Pulling 0; Heat 350, Filament 4, Velocity 25, Delay 200, Pulling 0). 50 µL of electrolyte (10 mM KCl, 10 mM HEPES, pH 7) was back filled into a micropipette and 30 Volts DC bias was applied across the pipette. The baseline current was stabilized and recorded for 1 minute prior to the injection of probe-conjugated microspheres hybridized with target and control oligonucleotides into the pipettes. The conductance across the pipette was recorded using the LabVIEW program and the trajectory of the beads was monitored and recorded at the capturing frequency of 100 frames/sec utilizing a high-resolution camera (Andro Neozyla 5.5) under the inverted microscope (Nikon Eclipse TE2000-E). Every experiment was repeated for at least 10 times by reversing the voltage polarity in a freshly pulled pipette.

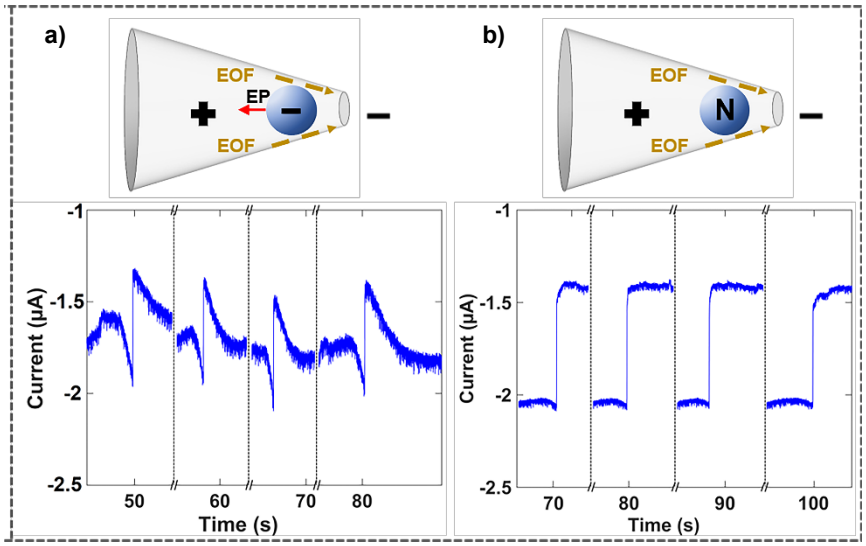


Figure 20: **a)** target circular DNA-PNA-bead complex was driven to the pore under the electroosmotic force (EOF) and the serrated shaped electrical signals were obtained. **c)** Control PNA-bead incubated with the non-complementary circular DNA was driven under EOF and the right angled electrical signals were obtained.

While circular DNA isolation was achieved with the sensor, portability and field applications are limited. Instead, the commercially available nanopore sequencer, MinIon, made by Oxford Nanopore, was utilized at the end of Phase II as an alternative to plasmid library preparation and Sanger sequencing.

Research Findings and Conclusions

503 nested PCR

The protocol that was developed using the 503 bp primers and the corresponding nested primers was effective in determining hellbender presence or absence at all streams that were sampled. 25 uL reactions were typically performed with these primers. The ideal conditions for the first round (503 round) involved 1 uL of extracted eDNA template, .75 uL of forward/reverse 503 bp primer, 10 uL of water, and 12.5 uL of New England Biolabs Q5 Hi-Fi DNA Polymerase. A 3 step 35 cycle protocol was found to be the most successful in amplifying the desired amount of product. The reaction produces smearing around 500 bp, necessitating a nested round to isolate a specific band. The image below depicts the gel electrophoresis of the first round for the Jelloway Creek samples:

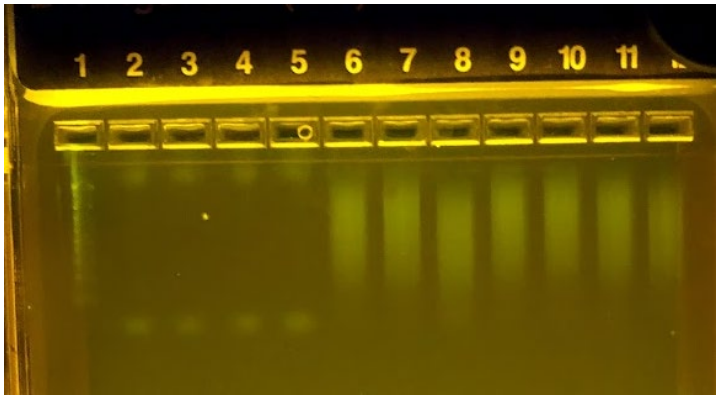


Figure 21. 503 PCR products. JC-9 through JC-14 are located in lanes 6 through 11 with a positive control in lane 12.

The product from the 503 round was cleaned using the Qiagen MinElute PCR Purification kit and the concentrations of the DNA were observed. These typically ranged from 30 – 100 ng/uL. The variability in these concentrations is dependent on the amount of unwanted biological material in the original extracted DNA template as well as the abundance of hellbender DNA in the samples. The cleaned products were diluted so that the concentrations were reduced to less than 5 ng/uL. The subsequent nested round was optimized with primers that generated a product around 175 bp. This reaction was also performed at 25 uL sizes and was a two-step 35 cycle protocol. The conditions for this reaction varied between different streams. For the Blue River, the optimal conditions were found to include 1.2 uL of diluted 503 round product as template, .625 uL of each forward and reverse primers, 10.05 uL of water, and 12.5 uL of New England BioLabs OneTaq master mix polymerase. In contrast, for the Jelloway Creek samples, 0.8 uL of each primer was determined most optimal. The image below is the gel of the nested round product for the Jelloway Creek samples. While faint, bands are seen in lanes 3, 4, 5, and 6 which are JC-10, JC-11, JC-13, and JC-14 respectively. The positive control is the Blue River 919-1 sample located in lane 8.

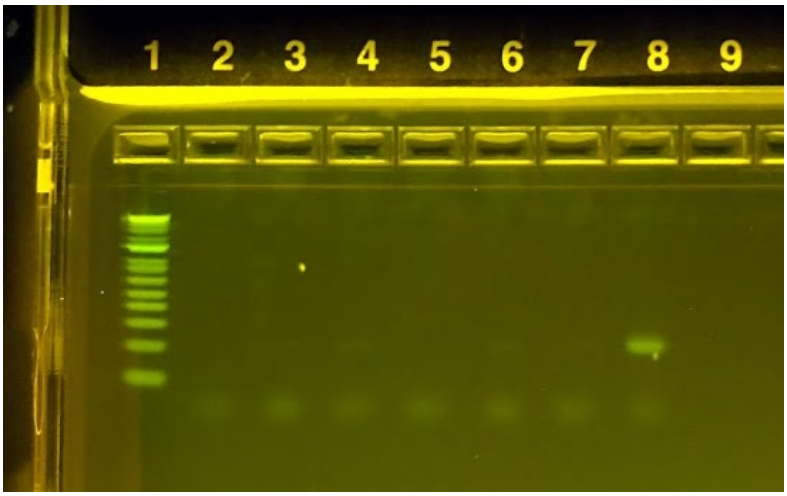


Figure 22. Nested PCR products for JC-9 through JC-14 in lanes 2-7. A positive control is in lane 8.

With any environmental DNA testing protocol, many factors can affect the results. For this nested PCR, variability existed between samples of different creeks showing that one stringent protocol cannot be strictly applied to all hellbender eDNA samples. The Blue River samples likely had a higher concentration of hellbender DNA because the samples were taken at locations where hellbenders were known to inhabit. As a result, the nested PCR amplified a more concentrated product that showed more visibly on the agarose gel. In contrast, at Jelloway Creek, the exact location of animals was unknown so the nested PCR, while effectively indicating hellbender presence, produced less pronounced results.

In employing this primer set, several conditions can be altered. It was found that 1.2 uL of each primer was most successful in attaining the desired amplicon for the 503 round but in some circumstances, less primer may produce a better result. The ideal amount of primer in the nested round fluctuated between samples from different streams. The Blue River samples amplified best when 0.625 uL of nested primer was used while the Jelloway Creek samples favored 0.8 uL of primer. Furthermore, the same thermocycler conditions and number of cycles was effective for all samples, but slight variation may produce better results. The recommended protocol is included in Appendix A.

Digital Droplet PCR

The samples from Salt Creek, Jelloway Creek, and the Blue River were analyzed with digital droplet PCR (ddPCR) for absolute quantification of the eDNA present. This method presents a more detailed insight into the concentration of hellbender eDNA in each sample. The experimental workflow closely followed the workflow suggested in the Bio-Rad Digital Droplet PCR Manual [1]. 20 uL reactions were performed. Typically, 1 uL of template DNA was used with 1.1 uL each of the forward and reverse nest PCR primers. 11 uL of Bio-Rad QX200 Evagreen supermix was used for these assays. The reactions were prepared in 22 uL amounts so 7.8 uL of water was added. 20 uL of each sample reaction mixture was transferred to a Bio-Rad DG8 cartridge where oil was then added. The oil emulsified the sample mixture in a Bio-Rad QX200 Droplet Generator. The emulsified mixture was then transferred to a 96-well plate and ran in a Bio-Rad C1000 Thermocycler. The PCR followed similar conditions as the

conventional nested PCR with a two-step protocol with slight variations (see Appendix A). After completion, the well plate was transferred to the QX200 Droplet Reader and analyzed with QuantaSoft software.

The Salt Creek samples were the first to be analyzed by this method and the results were used to contrive a detection distance limit. These samples were collected in November after the hellbender mating season of August through October 2017. Downstream detection of the animals was found to be approximately 1/3 mile and detection limits were thought to be lower than normal due to the time that these samples were taken.

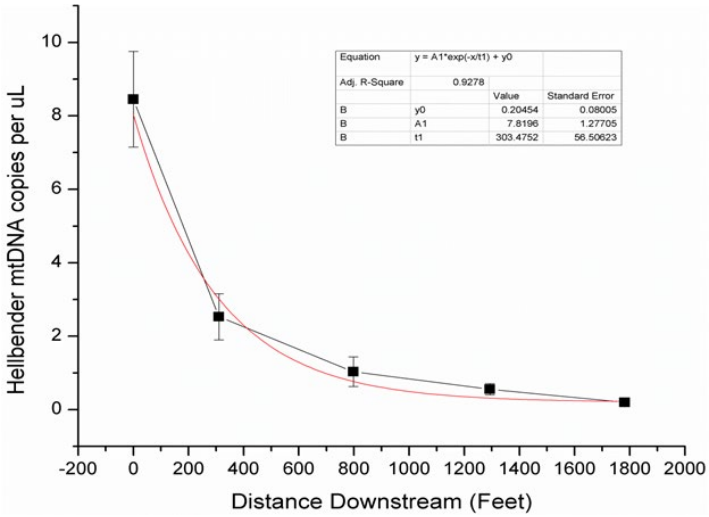


Figure 23. Salt Creek ddPCR results for determining downstream detection limit

Absolute quantification of the Blue River eDNA samples was achieved through several ddPCR trials. To develop a standard curve, known concentrations of the products from sample locations 919-1 through 919-6 503 round products were used as template. These included an undiluted 503 product, a 1:10 dilution, and a 1:100 dilution. All of the results were consistent with the findings of the nested PCR except for 919-6 which did not amplify. A 1:1000 dilution was then made for all six samples and used as template for another ddPCR. Many environmental samples may improve target PCR output by dilution, due to PCR inhibitors present in the purified eDNA sample. Indeed, diluting template DNA is a common method for PCR inhibition detection in quantitative PCR. With this dilution 919-6 amplified along with 919-1, 919-4, and 919-5 which were the same four samples that amplified in the nested PCR. Because the Bio-Rad system relies on proprietary mastermixes for use with the instrument, we had to adjust our nested PCR reaction to work with the Taq-polymerase based mastermixes sold by Bio-Rad. We did this by using a 503 PCR product as template with 20 PCR cycles instead of 35 for template and once again, all four samples that were positive. The cause of the discrepancy between these assays is likely due to an unequal amount of PCR inhibitors in the samples. The figure below depicts the average concentrations of these samples normalized on a scale of 0 to 1 with 1 denoting 2000 copies/uL.

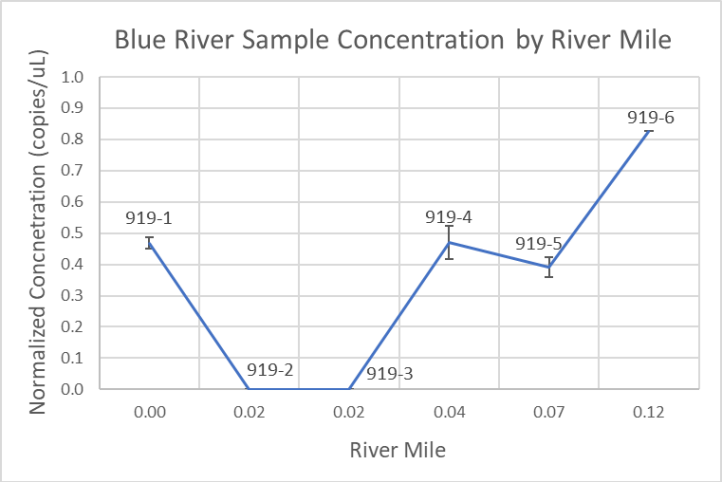


Figure 24. Blue River sample concentration by river mile.
A value of 1 denotes 2000 copies/uL

Isolating an effective and repeatable ddPCR protocol for the two sets of Jelloway Creek samples proved a greater challenge. The protocols developed for each set were similar but not identical to each other and were slightly different than the protocols for the Blue River and Salt Creek. The first set of Jelloway Creek samples, JC-1 through JC-8, were ran with several different dilutions of the 503 products, likely due to PCR inhibitors. However, the bimodal distribution was less distinguishable because the products were similar in fluorescence to the primer dimers and unreacted template. Therefore, the focus was set on the 35 cycle 503 product. This product was then diluted and showed successful amplification of the nested product in the ddPCR (using Taq-based mastermix), which was consistent with the results using the Q5 mastermix.

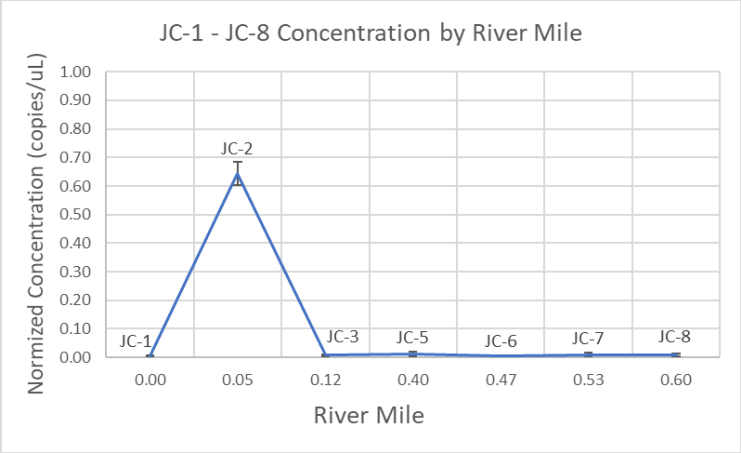


Figure 25. Jelloway Creek 8/6/20 sample set concentration by river mile. A value of 1 denotes 100 copies/uL. JC-4 is not included because it was taken from a tributary to Jelloway Creek.

On a different date, an overlapping section of Jelloway Creek was sampled, labeled as JC-9 through JC-14, were examined in a similar fashion. It was determined that a 1:100 dilution of the 503 product was the best form of template. JC-9 and JC-11 showed little amplification while JC-10, 12, 13, and 14 amplified. This result differed from the nested PCR where JC-10,

11, 13, and 14 were positive; thus, JC-11 and JC-12 were inconsistent between the two assays. The inconsistency could be attributed to certain samples having more PCR inhibitors. Nevertheless, the ddPCR offers a relative quantification of the different concentrations of eDNA that aids in determining hellbender distribution.

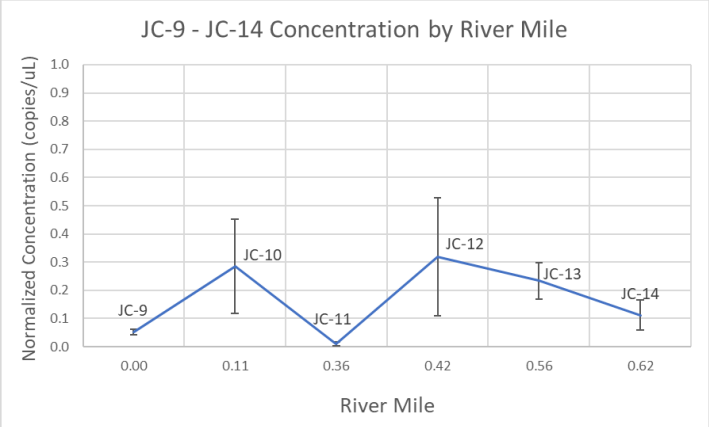


Figure 26. 10/2/20 Jelloway Creek samples concentration by river mile. A value of 1 denotes 3000 copies/uL.

Amphibian Specific Ranavirus

The amphibian specific Ranavirus primers (AmphSpecRana) were tested on the samples collected from the three Blue River trips in the fall of 2019. These primers were positive for Ranavirus DNA in the same samples that produced positive results with the mitochondrial primers for the Blue River samples, as well as in lower concentrations in the other samples where no mitochondrial DNA was detected. The reactions using the amphibian specific Ranavirus primers were also negative on 87.5% of the samples collected from the two October trips that resulted in negative hellbender mtDNA detection.

The DNA that was amplified using these primers on positive samples was isolated and sequenced. The results were compared to the NCBI nucleotide collection and were found to match the sequence for the Ranavirus genus with over 40 base pairs of direct homology. This data provided evidence that the template amplified by the primers from the environmental samples is that of Ranavirus DNA. When a ddPCR was run with the AmphSpecRana primers on the six samples from September 2019, the resulting DNA concentrations appeared to correlate with the concentrations of the mtDNA for the same samples as shown in Figure 27. This data shows that a direct correlation may exist between the concentration of Ranavirus DNA and hellbender mtDNA.

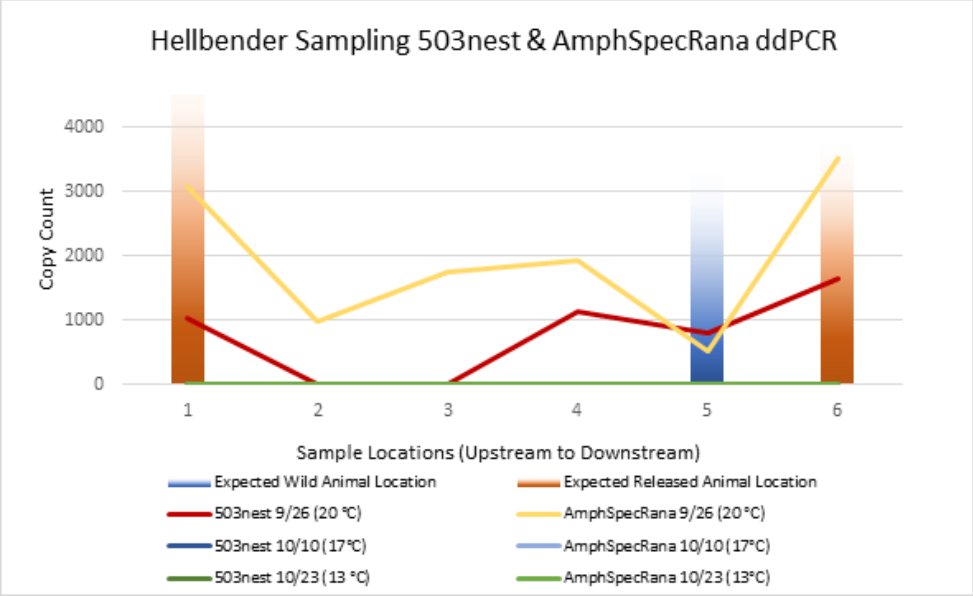


Figure 27: Samples used for the ddPCR runs include the 919 Samples (9/26), 1019 Samples (10/10), and 1119 Samples (10/23). 503nest indicates mtDNA targets, and AmphSpecRana indicates the Amphibian Specific Ranavirus as the target. The vertical bars indicate suspected Hellbender individual locations. Water temperatures for when each sample set were taken are also noted.

The most abundant organisms present in surface water for aquatic environments are microbial in nature, and viral particles are represented by an order of magnitude over their microbial hosts. While the Ranavirus targeted infects animal hosts, it is hypothesized that the Ranavirus DNA will be similarly abundant. As such, the viral DNA should serve as a longer-range guide for samples when compared to the eDNA released from Hellbender individuals. The results from the ddPCR tend to corroborate this, as the environmental concentration of original viral template DNA is higher than the Hellbender DNA in five out of the six sampling sites chosen and is able to be detected upstream of positive mtDNA samples. A notable factor when considering Ranavirus detection, however, is water temperature. Ranavirus replication is limited to temperatures above 14 °C, thus long range detection using this target, particularly during breeding season in late fall, may not be an option. Even with this supporting evidence, it is the opinion of the authors that AmphSpecRana primer set requires further study to be a reliable long range test. Significant effort was dedicated to previous primer sets that did not work for us as they had been described by other researchers, and to avoid this potential scenario and provide a method as repeatable and robust as possible PCR work targeting the mtDNA 503bp portion of the Hellbender Hypervariable region should remain the focus, with the goal of producing both detection and a DNA target for sequencing.

Sequencing

Genetic sequencing of the eDNA samples was a major aspect of this project because it is a reliable method in confirming the accuracy of the PCR results. While the nested PCR accurately determines the presence or absence of hellbenders, the product is not the ideal length for obtaining inter-animal sequence specific information. That is, with stocked Eastern Hellbender in Ohio rivers coming from common clutches of eggs (same maternal line), mtDNA diversity may be limited, thus the longest amplicon possible from 503 product is desired for animal specific SNPs and identification.

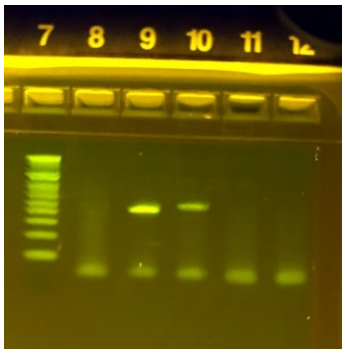


Figure 28. Sequencing PCR product

To increase the size of the nested product, specifically for sequencing, a combination of the 503 bp primers and the nested primers was utilized to isolate a longer product. This provided the increase in target DNA (relative to non-specific product) of the nested round, while maintaining a longer fragment. This nested-hybrid 503 product was made with the typical conditions except that only initial 503 cycling was limited to 20 rounds. This product was cleaned and concentrated on a Qiagen MinElute column, and 1 uL was used as template for the following hybrid PCR round. The 503 forward primer and nest reverse primer were paired for this reaction. The product, seen in the image to the right in lanes 9 and 10, is larger than the nested product (~400 bp) but smaller than the 503 bp product. These products were gel purified and sequenced using the Sanger Method. Though the 503 round with 20 cycles is not necessary to produce a band in the second round, it helps reduce amplification of unwanted products (smearing) during the second round.

Figure 29. Map of the September 2019 Blue River samples. Positive samples are blue and negative samples are yellow.

The four positive samples from the September 2019 Blue river sampling trip are seen in the map above. Several different products were generated for sequencing by using the PCR protocol described above. In addition to these environmental samples, skin swab samples obtained at the release of the captive-bred hellbenders at the Blue river were also sequenced. The sequences of the environmental samples were compared to the NCBI nucleotide reference mitochondrial genome (accession: GQ368662.1). This showed a 98% homology to the hellbender mitochondrial d-loop region, confirming that the positive amplicons from the nested PCR did correctly identify the presence of Eastern Hellbenders.

Comparing the sequences of the samples obtained at sites 1 and 4 revealed that there was complete homology. This is shown in the first four rows in figure 30 below. In contrast, the two sequenced samples from sites 5 and 6 showed individual divergences from the samples at sites 1 and 4. The differences in the sequenced DNA from these samples was sufficient to identify different animals present at those locations. In Figure 30 below, the sample from site 6 contained variations indicating a different but closely related animal to sites 1 and 4.

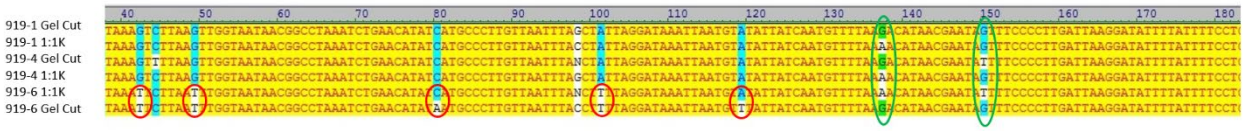


Figure 30. Complete homology between sample 919-1 and four different swab sequences. Those highlighted in red are sequences that have been selected to be repeated by the sequencing team. When removed, the only remaining SNPs were revealed to be miscalls after further analysis. This indicates that the samples 919-1 and 919-4 have identical sequences to four different swab samples, confirming the sample's identity as one of the released animals.

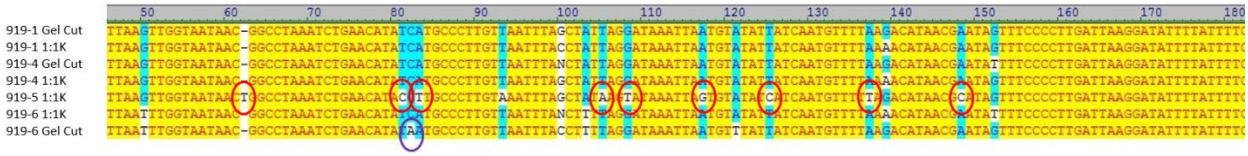


Figure 31: Variations in sample 5 vs. Samples 1, 4, and 6. SNPs highlighted in red are distinctly different in sample 5 than in all other samples sequenced. The purple circle at bp 80 of sample 6 shows a distinct difference between each of the other positive samples that is not present in the 919-6 1:1k sequence.

The sequence of sample 5 possessed several different nucleotides from the animal located at sites 1 and 4 as seen above in Figure 31. This includes a base pair addition, as well as multiple single nucleotide polymorphisms (SNPs) that are not present in the other three positive samples or any of the swab samples that were sequenced. These differences pointed to the presence of a different animal than sites 1, 4 and 6.

All the captive-bred hellbenders released at the Blue River share the same mother and therefore were expected to possess the same mitochondrial DNA. The sequences of the swabs were compared to all four positive samples. These swab samples showed complete homology with samples 1 and 4, confirming the presence of a released animal at those locations. This homology also confirms that the sequences of the D-loop region of individuals from the same clutch are homologous. In the figure below, sample 1 is compared to the swab sequences. The variations in sample 6 discussed above and seen in Figure 30 indicate a different but closely related animal than the released animal(s) detected at sites 1 and 4. Furthermore, while sample 5 was 90% homologous to hellbender DNA, these distinct differences in sample 5's sequence seem to indicate the presence of an animal unrelated to the captive bred/ released maternal line.

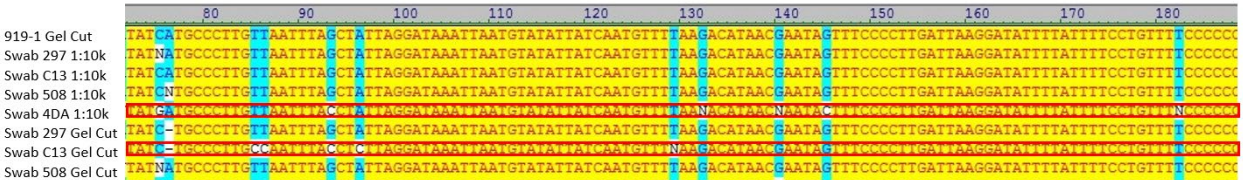


Figure 32. Complete Homology between samples 919-1 and four different swab sequences. Those highlighted in red are sequences that have been selected to be repeated by the sequencing team. When removed, the only remaining SNPs were revealed to be miscalls after further analysis. This indicates that the samples 919-1 & 919-4 have identical sequences to four different swab samples, confirming the sample's identity as one of the released animals.

In addition to the Blue River environmental samples and skin swabs, samples taken from Salt Creek and the Columbus Zoo were also sequenced. The Columbus Zoo samples, taken in July of 2018, consisted of skin swabs and filtered tank water from different clutches of Hellbenders. One group is associated with individuals released in Salt Creek in southern Ohio, and the other is associated with Cross Creek in eastern Ohio. Environmental samples had been collected from Salt Creek in 2016 based on the location of a single released individual associated with the first of these groups. Because of this, the sequencing data from the Salt Creek environmental samples and the tank samples for that parental group at Columbus Zoo were expected to be homologous, which was confirmed. This homology was also shared with the Blue River environmental samples and swabs as seen below in Figure 33.

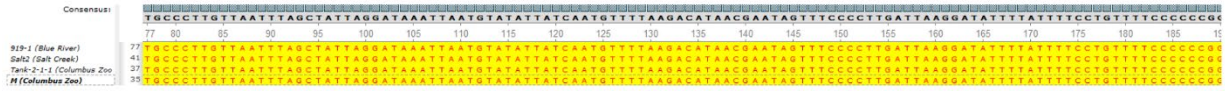


Figure 33: Comparison between Blue River environmental (919-1), Salt Creek environmental (Salt2), and two Columbus Zoo filtered tank water samples (Tank 2-1-1 and M). All sequences show complete homology.

If the individuals at the Columbus Zoo that are associated with Cross Creek had different maternal origins than those associated with Salt Creek, then the sequences obtained from the swab and tank water samples may have been distinguishable. This was also shown to be the case, as the sequences obtained from the Hellbenders belonging to the Cross Creek lineage were not completely homologous to those of the Salt Creek lineage, featuring a single base pair substitution (Figures 34, 35, and 36). This polymorphism does not appear in any of the Salt Creek environmental samples or any of the Blue River environmental samples or skin swabs.

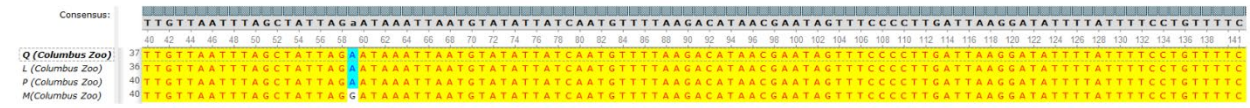


Figure 34. Alignment of sequencing results from Columbus Zoo samples collected in 2018. The only significant polymorphism is present at base pair 59 of the sequence related to Salt Creek (M), where a base call for G is present rather than A.

According to the sequencing data above, captive bred animals do not exhibit heterogeneity in the section of the mtDNA D-loop sequenced. Therefore, any variation in the sequencing data from a sample would result from different Hellbender individual's DNA being present. The variation of the sequence between the two Columbus Zoo parental groups indicates that there may be enough divergence to identify different parental lineages of Hellbenders, even between captive bred animals. When the sequences of samples 919-5 and 919-6 are compared to those originating from captive bred populations, the number of differences is even more significant. As such, this data seems to indicate that the DNA collected from those points did not originate from a captive Hellbender population, but instead from wild, native Hellbenders.

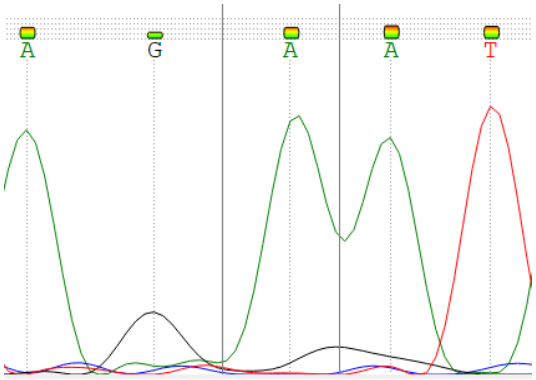


Figure 35: Highlight of raw sequencing data for Sample Q, associated with Cross Creek, at base pair 59. The strong green peak at this location indicates an A base; this is shared by samples P and L, also associated with the Cross Creek.

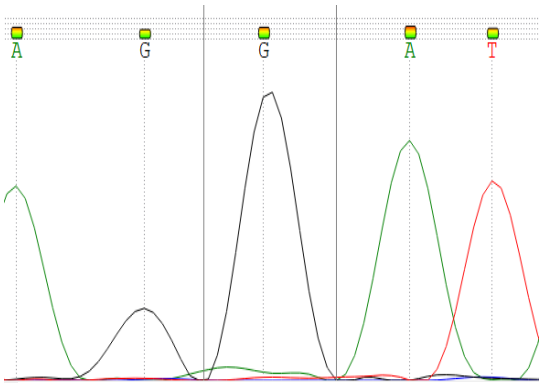


Figure 36: Highlight of the raw sequencing data for Sample M, [released] at Salt Creek, for base pair 59. The data indicates a strong call for the base G.

MinIon Next-Gen Sequencing

While Sanger sequencing can produce reliable results for a low cost relative to high-throughput sequencing strategies like Illumina and Ion Torrent, Sanger chemistry does not tolerate multiple simultaneous sequence reads per reaction. As a result, DNA sequence targets must be single amplicon, without any contaminating mis-primed PCR product, or a plasmid library sample where amplicons are inserted into a carrier vector for sequencing. The downside of the plasmid library is time and resources dedicated to relatively few reads. Even when non-transformants are eliminated, plasmid purification can add to costs, and when non-target DNA is inserted, library reads are wasted. To circumvent this waste, we explored next-gen Nanopore sequencing using the MinIon from Oxford Nanopores. For many years nanopore sequencing technology suffered from poor base calling, and with current best practices producing 7-12% error rates from in an independent laboratory setting[20], this still appears to be an issue. However, with a large number of redundant reads (10^5-10^6) statistically significant basecalling can be achieved that is insensitive to contaminating sequences, since these contaminants can simply be eliminated by length, quality score or lack of homology. Indeed, this screening can now be done in real-time, as the sequences are generated, using open-source software (Uncalled).

To eliminate the labor and resources required for plasmid library preparation (Sanger sequencing) we prepared the Jelloway Creek samples for sequencing using the MinIon from Oxford Nanopore. The PCR of the nested reactions for the samples from this creek produced

relatively clean bands (although not clean enough for direct amplicon sequencing with Sanger) with some smearing indicating a small amount of contaminating DNA. While these non-target DNA products were included in the sequencing run, they are easily eliminated by the aforementioned screening parameters, making the MinIon a powerful and relatively inexpensive approach for eDNA species identification and by way of read counts per sample DNA concentration, an estimation of quantification.

The sequencing device is small (shown in Figure 37, red circle), portable and can be used in

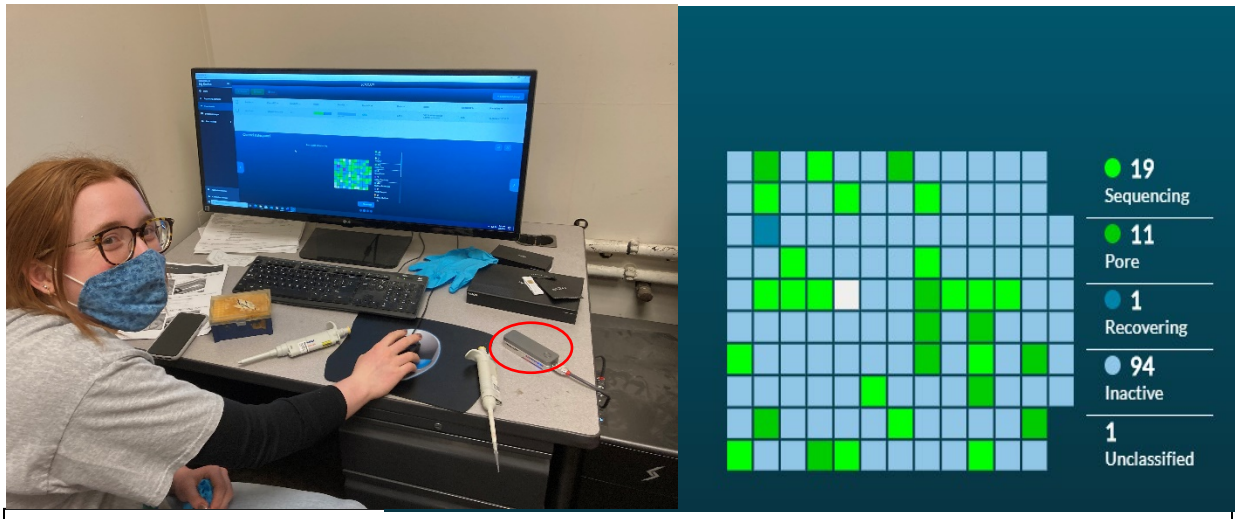


Figure 37. Operation of MinIon in the Wendell lab sequencing eDNA samples (left), real-time sequencing information from nanopore sequencer, with green squares representing active nanopore sequencing events for JC13 (right).

the field, allowing eDNA animal identification to move out of the lab, identical to the original goal of our mtDNA sensor.

DNA samples slated for sequencing require about 3 hours of preparation, once prepared and applied to the flow cell, a sequencing run can last up to 24 hours or as little as 1 hour depending on the desired scale of output. For our samples we ran each for approximately 18 hours, producing over 100,000 reads in both attempts.

Given the large of amount of redundant coverage for a small segment of the reference genome (less than 500 bp) for JC13 we decided to pool JC2 and JC10/11, since these sights were approximately equivalent in location, but sampled on different days. The JC13 sample location was approximately 1/3 of a mile downstream from the JC2-JC10 location, and outside of our predicted animal detection distance, so it was assumed to be a different animal. Quality scores and number of reads were excellent with over 100,000 reads for the same 220 bp target. This allowed us to set a lower limit of mutation frequency at 18%, given that past work demonstrated a 7-12% error rate, while still maintaining a pool of reads for both strands in excess of 50,000 respectively. Using the aforementioned mitochondrial reference genome, we aligned our reads in Epi2ME, a GUI provided with MinIon sequencer. This created a series of alignment files (BAM) from our sequencing data that required indexing using Samtools. Samtools and IGVtools were run on a Linux OS, combining BAM files for each

respective sequencing run into “merged-Bam”. The most frequently occurring mutations for the nested DNA target were tabulated and can be seen in Figures 38 and 39.

JC2 and JC10 Locations Mutations			SNP frequency relative to sequence GQ368662.1			
BP #	reference	count	A	C	G	T
15959	A	100747	0.25	0	0	0.74
15961	T	95059	0.94	0	0	0.05
15974	G	100781	0.57	0	0.28	0.15
16012	T	99986	0	0.95	0	0.05
16018	A	101222	0.05	0	0.92	0.03

Figure 38. SNP frequency for nested amplicon relative to reference genome GQ368662.1 for PCR reactions from JC2 and JC10/11.

JC 13 Major Mutations			SNPs frequency relative to sequence GQ368662.1			
BP #	reference	count	A	C	G	T
15959	A	134837	0.29	0	0	0.7
15961	T	126776	0.92	0	0	0.07
15974	G	134665	0.55	0	0.25	0.19
16003	A	131597	0.79	0	0	0.2
16012	T	133208	0	0.94	0	0.06
16018	A	135768	0.06	0	0.91	0.03

Figure 39. SNP frequency for nested amplicon relative to reference genome GQ368662.1 for the PCR reaction from JC13.

The IGVtools graphical user interface, with default settings, displays mutations as colored lines at the top of the alignment, allowing for a quick reference to display that JC13 appears to have 6 SNPs and JC2/10 has 5 for the same region (see figures 40 and 41 respectively).

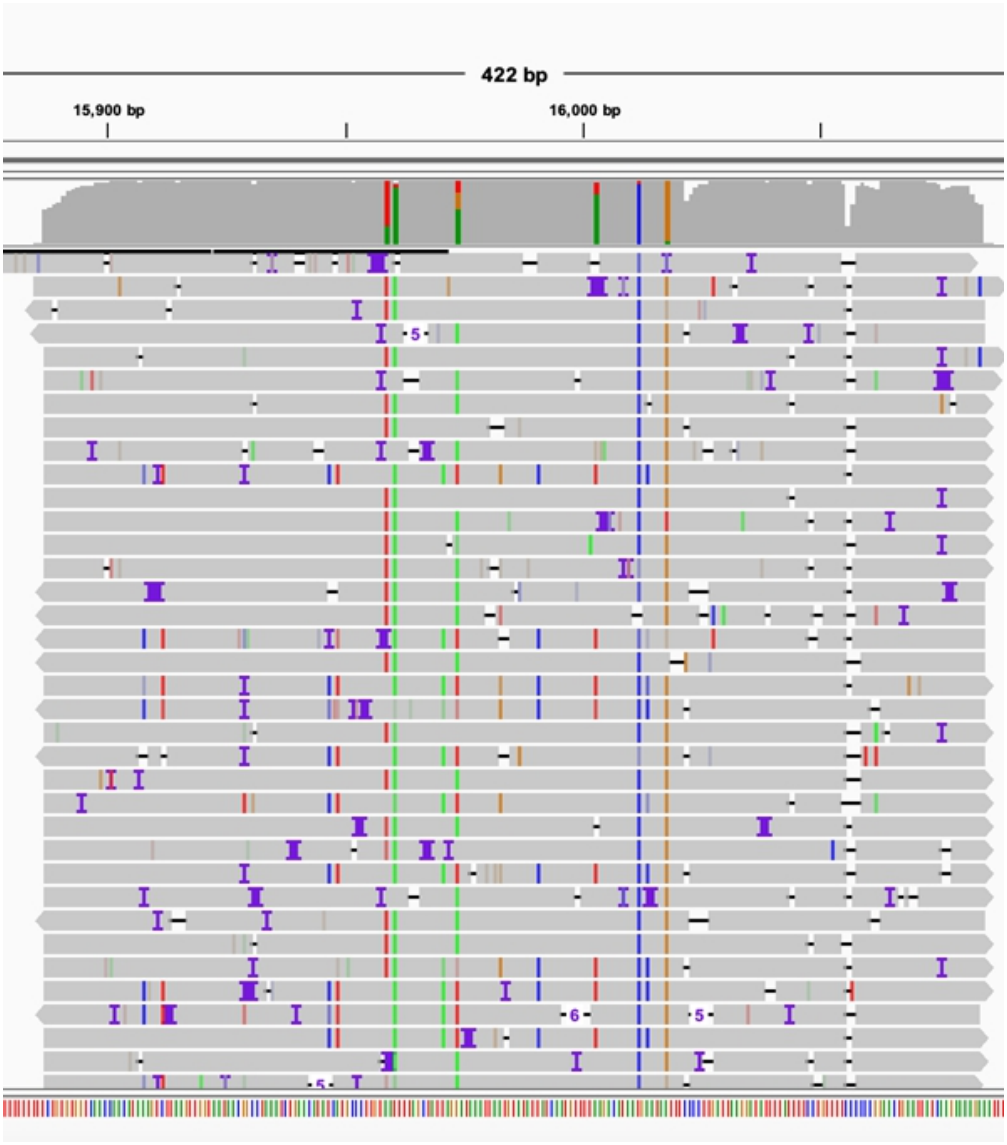


Figure 40. IGVtools GUI displaying JC13 sample mtDNA aligned to reference sequence accession# GQ368662.1, 6 major mutations are visible as noted by the colored lines at the top.

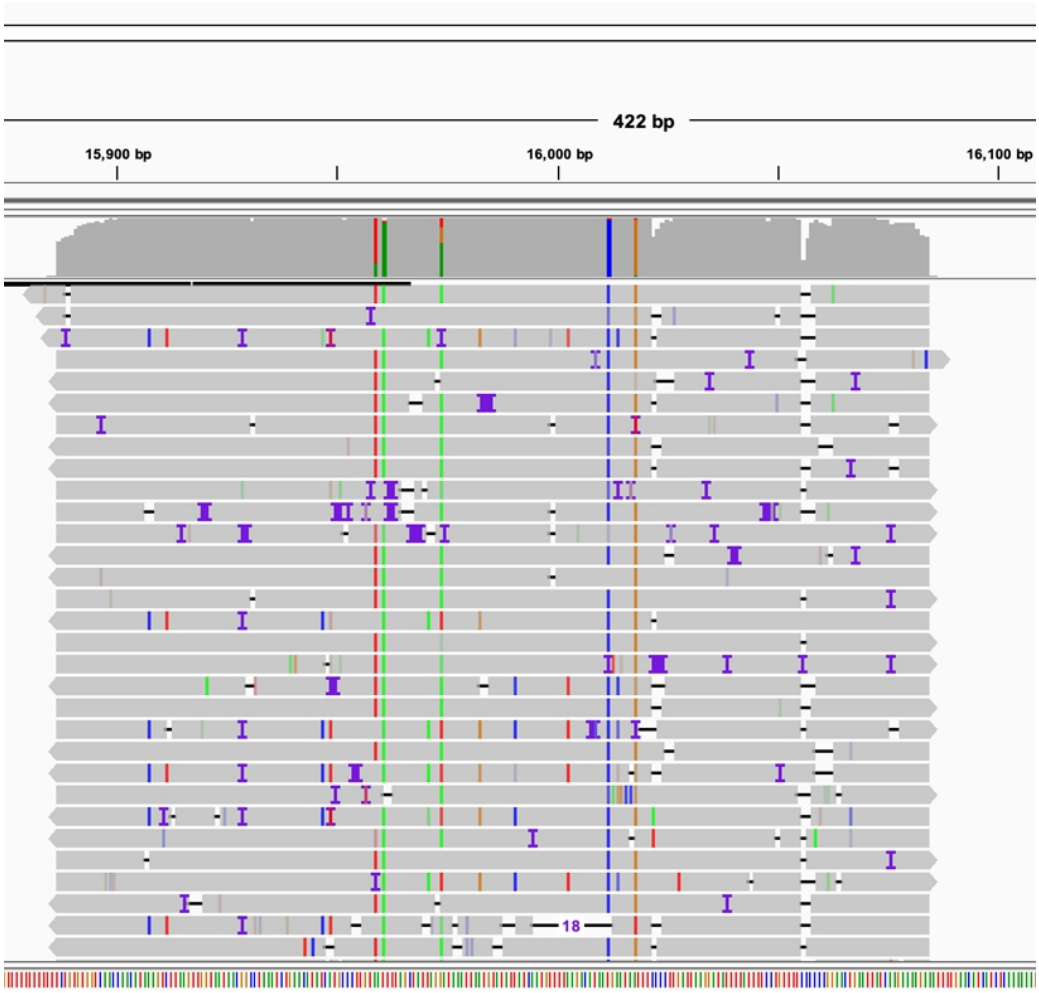


Figure 41. IGVtools GUI displaying JC2 sample mtDNA aligned to reference sequence accession# GQ368662.1, 5 major mutations are visible as noted by the colored lines at the top

Based on the basic difference of 5 SNPs at JC2-10/11 and 6 SNPs at JC13, coupled with the rate of decay/dilution of DNA over the distance of river miles separation we feel the data supports the presence of different animals at these locations. For our analysis, we merged BAM files produced by the EPI2ME software and indexed them using SAMtools and displayed this merged BAM in IGVtools. The alignment showed sequencing coverage of both strands, similar to a paired-end read in Illumina. Due to our concerns about sequencing error as a significant point of misinformation we excluded low frequency mutation events (18% and down). This cutoff was based on published work showing most error was within the 7-12% range, with the MinIon more accurate than other options from Oxford [20]. This eliminated all of the frameshift mutations (insertions/deletions) and all other mutations with the exception of those displayed in Figure 38 and 39. The main difference in Figures 40 and 41 can be seen at bp 16003 of the reference genome, where one JC13 animal sequence has “T” mutation (highlighted green on Figure 39). It is not clear how meaningful these frequencies are at this time, as nanopore sequencing was only recently explored for this research effort,

primarily to overcome difficulties with plasmid library preparation/ Sanger sequencing. As a result, several questions arise: does a 20% "T" mutation equate to more than 1 animal, with the lower frequency SNP representing an animal more distant, or equally close but shedding less mtDNA in the form of gametes/sloughed cells/feces? These questions are particularly important for SNP 15974 highlighted yellow in both figures 38 and 39. This position has 2 SNPs along with the expected basepair at the referenced position with all possessing frequencies above 18% at *both sampling sites*. It cannot be ruled out that these divergent calls are artifacts of the sequencing process but given there are over 134,000 reads at this position for JC13, the authors would expect this to be a statistically significant result. If these are correct it would appear there are at least 3 animals at both JC2-10/11 and JC13, with more of the "15974-T" animal DNA found at the JC13 location, or upstream animal DNA adding to the downstream fraction. These samples were also taken during mating season, so it is possible the detected collection of SNPs represent different egg DNA circulating in the creek. To define a more accurate frequency cutoff for SNPs, positive control DNA from animal swabs and other well defined mtDNA sources, spiked into the sequencing method analyzed over similar megabasepair sequencing yields would provide a framework for future guidance on how to interpret nanopore high throughput sequencing results for eDNA.

To be clear, SNP analysis and distinct animal identification remains difficult, but it appears relatively certain, based on sequence homology, that both of these sampling locations have Hellbenders present, and because of their separation, at least 2 animals should be expected. Based on the SNP analysis, we propose that more are present. As an example, we would apply this knowledge as a recommendation to future ODOT construction projects by stating the expectation of relocating 2 animals and retesting the water after their removal for additional animals, if more are not observed during the relocation process.

While our nanopore sequencing approach requires further refinement, it is possible to provide 2 global metrics for the technology: the quality of the reads (11-12), which are analogous to Phred scores, and indicate expected error in the 11-12% range; and a 95% homology to the reference sequence, using the sequencing software (EPI2ME). A graphical output of this first stage of analysis is shown below for both locations:

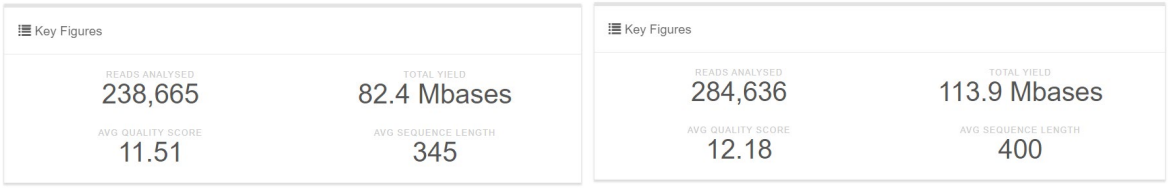


Figure 42. Total sequencing quantity and quality scores before trimming and analysis (left JC13, right JC2-10/11).

Alignment output from EPI2ME for sites JC 13 and JC 2-10/11 shows greater than 95% identity for both (see Figure 43). Sequence identity percentage is defined as the ratio of the number of matching residues to the total length of the alignment. From the literature we expect an error rate of at least 7% with best practices [20], and from the software derived quality score an error rate of about 11-12%, so a 95% identity (5% error) from our results is slightly better than expected, with several SNPs common among both locations, but different from the

reference, this identity could be even higher to the consensus sequence for local Ohio animals.

The total number of reads has been reduced by about half (see Figure 42 “reads analyzed” relative to “alignments” in Figure 43) as a result of trimming reads of incorrect length (excluding DNA equating to the smear on the gel image shown Figure 19).

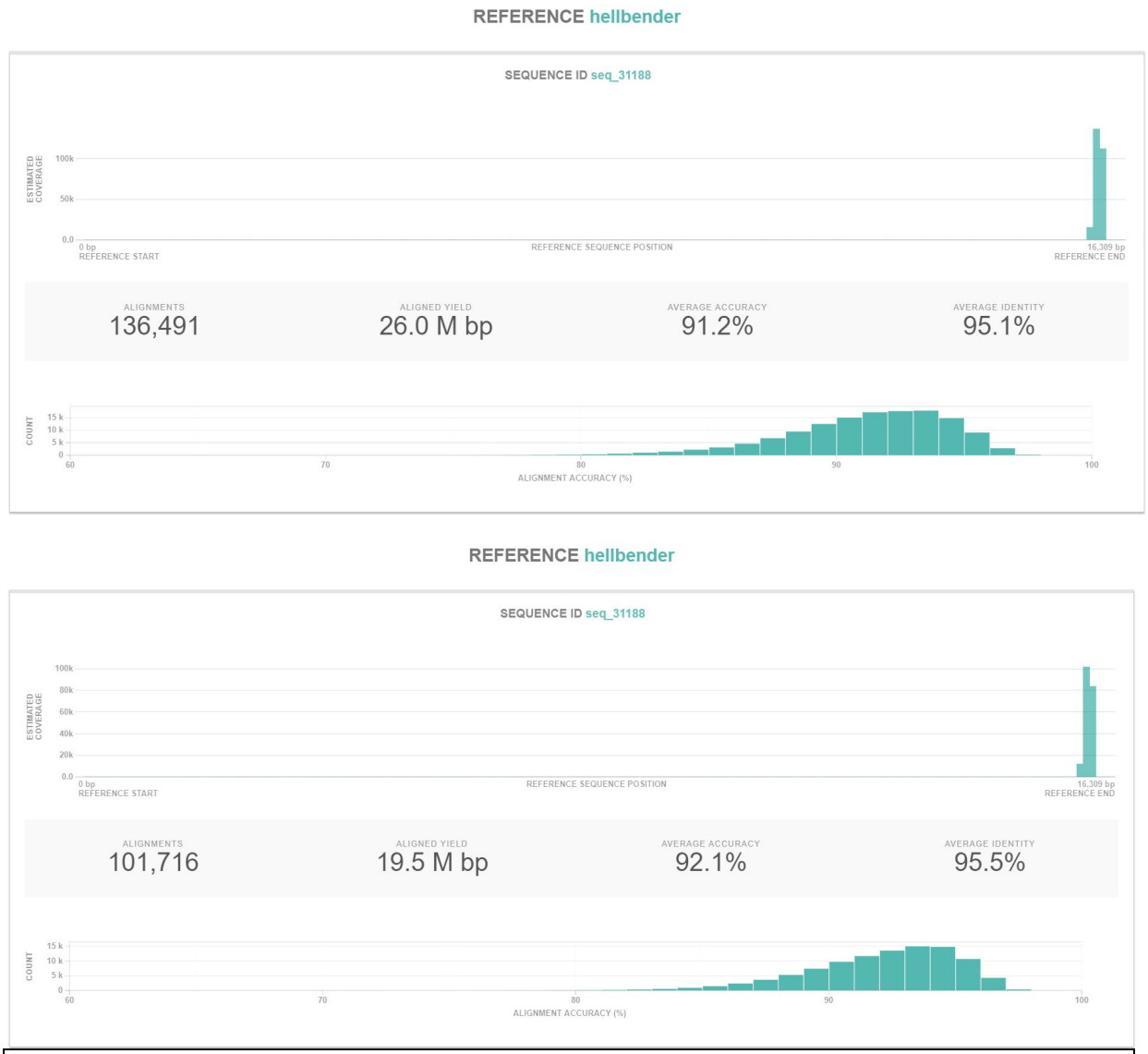


Figure 43. EPI2ME alignment of trimmed sequencing results for JC 13 (top) and JC 2-10/11 (bottom).

Figure 30-32, 36, 40 and 41 support the theory that mtDNA found in surface waters, more specifically eDNA amplified for the hypervariable region of Hellbenders, can be a useful and informative target for species identification and distinguishing different animals in said waters. While early Sanger sequencing identified the difficulty in distinguishing captive bred animals, which come from the same egg clutch/ maternal line, and thus have identical mtDNA. With this difficulty in mind, it appears possible to obtain some informative SNP

information from the hypervariable region, despite claims that wild animal mtDNA may be too closely related to produce valuable insights. Similarly, is has been documented within the last decade that genetic diversity of Eastern Hellbenders in the environment is greater than previously expected [21], challenging past data showing a paucity of genetic diversity among these animals [22].

Method Limitation Discussion

Detection of aquatic animals from eDNA is sensitive to several factors. The time of year when sampling occurs plays a role in the amount of eDNA that is likely to be collected. For hellbenders, August through October is the most ideal time for sampling to occur as this is the animal's breeding season and stream flow rates are generally lower. Animal activity is highest during this time so eDNA is shed in greater amounts than during other times of the year. This project mainly focused on this period in order to maximize time spent perfecting the genetic detection protocol. Yet, hellbender DNA may still be detected at other times of the year, albeit in lower concentrations.

The most significant limitations of eDNA detection in our experience pertains to stream conditions: streamflow, or discharge relative to average flow rates. Higher levels of flow dilute any DNA in stream water and often make the water more turbid, making it more difficult to filter and extract potential target eDNA. Positive detections of hellbender DNA at Salt Creek, Scioto Brush Creek, the Blue River, and Jelloway Creek occurred when streamflow was 11 cfs, 14 cfs, 40 cfs, and 18 cfs, respectively. These streamflows are all lower than typical flow levels for each of these streams respectively. Several Blue River sampling trips that failed to detect hellbender DNA occurred when the streamflow was significantly higher indicating that there may be a detection threshold for stream flow and eDNA based detection. Furthermore, the streamflow pattern prior to sampling can also affect detectability. Heightened streamflow levels could act to flush the river of settled or suspended eDNA, or if flow is excessive, move or push animals from their established habitat. Therefore, for the best practices, sampling should occur when streamflow is low and ideally after a period of time where that flow has remained low.

Water temperature was also considered as a detection factor. However, positive detections occurred when the water temperature was at both high and low points indicating that temperature does not play as significant of a role as streamflow. A full discussion of detection factors is included in Appendix B.

Recommendations for Implementation

Traditional methods of detecting the endangered hellbender salamander are costly, time-consuming, and detrimental to the animal's habitat. The protocol we have developed for Hellbender detection - by water sampling and genetic testing- was curated to mitigate these factors. With success observed through continued trial of these developed methods, it is recommended that ODOT implement this protocol when surveying for the Eastern Hellbender. One of the most important benefits attained from this adoption is non-invasive detection and preservation of animal habitat. Another clear benefit is reduction in survey costs. With our

method, costs of surveying may be reduced by several ordered of magnitude to approximately $3500- $5000 per survey, depending on sequencing needs. The health and safety of ODOT biologists in the field is also improved because much of the physical surveying practices can be avoided. Similarly, the staff required to perform these analyses may be reduced from an entire team of surveyors to one or 2 individuals. In our implementation at least 2 researchers were always present for safety reasons. It is also beneficial to have more than one person perform and review the results; however, only one lab member is required for all of the methods and procedures. Among the remaining benefits, and certainly one of great importance, is the reduced impact to the species. This is due to less invasive survey techniques and elimination of transfer of disease between surveyor and species. With these benefits and the observed success of the protocol, it is recommended that the protocol documented in this report be considered by ODOT for future survey application needs.

The Wendell lab estimates that the cost of each stream survey will be $3,425-$5,100. This number was determined through a cost analysis of materials, labor, and travel. The material costs and testing time required were evaluated assuming 10 samples are taken for each stream survey. The total material costs are estimated to be $857.87 per 10 sample survey. It is estimated that the time required for these assays is 76 hours and the associated labor cost is $1,140. Lastly, the travel expense, determined from an average trip distance and the federal gas mile cost, is $287.50, with additional expenses going to sequencing if animals are detected. A full itemized list of materials is included in Appendix A.

As with any scientific testing protocol, constraints exist. This testing method provides an accurate assessment of hellbender presence, but several factors limit its use in certain areas. First, the ideal sampling period is during the fall breeding season, when animal eDNA may be highest and stream flow rates low. However, this may not always align with the timeline of construction or survey projects. It is not impossible to detect hellbender eDNA during other times of the year; however, most sampling trips occurred during the Fall period to minimize time spent sampling and testing. This constraint can be overcome through detailed planning of construction project timelines.

Another factor when employing this protocol is that lack of detection is distance and water quality dependent. Samples may be taken at an unfavorable time when hellbender eDNA is not detectable but the animal is present. In addition, PCR inhibitors can produce unforeseen non-detects in the DNA amplification phase of our protocol. No method, whether traditional surveying or eDNA testing, is without uncertainty. However, the success of this method and the associated benefits may outweigh traditional survey operations; for example, when habitat is particularly sensitive to human disruption, survey resources are limited or animals successfully avoid visual detection.

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Appendix A

Environmental Sampling and Genetic Testing Workflow

Water Sample Collection

Materials

- Ice cooler
- 6-10x 1 Liter sterilized Nalgene bottles
- Gloves
- Labelling tape
- Waders or flotation device (if necessary)
- Field notebook
- GPS unit

When to Sample

For the most accurate results, water sampling should occur between August and October during the hellbender mating season when eDNA concentrations are highest. Sampling should occur when the streamflow is low. Avoid days that follow wet weather events. Streamflow data may be obtained from the USGS website (refer to the sampling site determination procedure in Appendix B for more detailed instructions on how to access this data). Hellbenders are mostly nocturnal but do have some reported activity during the day. Therefore, sampling can take place at any time during the day without appreciable impact.

Preparations before sampling

The 1 L Nalgene bottles that are used for water collection must be completely sterilized. The bottles need to be cleaned by soap and water or by dishwasher before final sterilization. The bottles should be sterilized by autoclave at 121 °C at 15 psig/1 bar for at least 20 minutes. If an autoclave is unavailable, sterilizing with a diluted (at least 10% concentrate) bleach solution can work but an autoclave is recommended, but only to be performed in a laboratory setting. If bleach is used the bottles must be thoroughly rinsed with DI or RO water. Do not bleach the bottles near the streams as bleach is tremendously harmful to aquatic organisms. Finally, make sure that the coolers that the samples are to be stored in are full of shaved ice before sampling. The water samples must be kept cold from the time they are collected until they are filtered in the lab or DNA degradation can occur.

Collection of Samples

Ideally, water samples should be taken at the center of the stream so that the maximum amount of genetic material is collected. The water collector should position his or herself downstream of where the sample will be taken so that there is no contamination caused by the sampler's body or river sediment disturbed by feet. Once positioned, the sampler should wait one minute to allow any disturbed organic matter to settle or be flushed downstream. Sediment in samples often inhibits the ability for filtering and thus detecting DNA so results may be inaccurate if too much sediment is collected. Once the researcher is ready to collect the water, a grab sample is taken by dipping the mouth of the 1L Nalgene bottle straight down until the opening is mid-depth in the water column. Then, rotate the opening of the

bottle to face upstream so that water flows into it. Once completely full, screw the cap firmly on the bottle and place it in a cooler with ice. Make sure to label the bottles and note the stream conditions at that location and the global position with GPS unit. Samples should be collected no farther than 1/3 mile between one another. For this project, samples were often collected 300 ft apart.

Water Sample Filtration

Materials

- 0.45 micron cellulose nitrate filters
- Sterile 1L Erlenmeyer vacuum flasks
- Number 8 MilliporeSigma Silicone stoppers
- Stainless steel filter heads
- Vaccuum pump
- 3/8 inch Nalgene 180 PVC autoclaveable tubing
- Tweezers
- Conical tube

Filtration

All equipment must be sterilized before use. This can be accomplished by autoclaving the autoclavable materials at 121 °C at 15 psig/1 bar for at least 20 minutes. The materials may also be bleached in 30% concentrate bleach then rinsed with water. After all materials are sterilized, assemble the filtration setup. One 0.45 micron cellulose filter should be used, these are available as individual filters or disposable filter/funnel combinations. Once everything is set up, turn on the vacuum and pore half of the 1L sample into the basin. Some samples may take longer than others to filter due to the amount of sediment. After the first half has filtered through, pour the remaining half liter into the filter funnel. Often, the water will not completely run through because there is too much sediment contained on the filter. In order for enough genetic material to be collected, at least 400 mL of water needs to be filtered. This may take up to half an hour per sample but collecting enough material is necessary for accurate PCR detection. After all the water has run through, disassemble the configuration and, using sterilized tweezers, transfer the filter to a conical tube. Label the tube and place in a –20 °C freezer until DNA extraction can occur.

DNA Extraction

Materials

- Qiagen DNeasy PowerWater kit
- Razor blades
- 15 mL capable Centrifuge (example: Eppendorf 5810)
- Microcentrifuge (example: Eppendorf 5425)
- Pipets
- Pipet tips

- Microcentrifuge tubes

Procedure

The procedure for extracting the DNA from the filtrate that was used is the protocol from the Qiagen DNeasy PowerWater kit. The first step of the protocol involves cutting the filter into strips with a razor blade. It was found that the filters were most easily cut when they were completely frozen. Additionally, if there are many samples (12 or more), it is advised that the extractions are subdivided into manageable numbers (6-12) so that all of the steps are consistent and correctly practiced. Once the extraction is performed, measure the DNA concentration. If the eDNA concentration was below 1 ng/uL, the extraction may have been unsuccessful. Ensure that the tube caps are firmly sealed so that DNAses or other contaminants do not enter the tubes and degrade the DNA. Extracted DNA samples need to always be stored in a 4 °C refrigerator while in use, and -20 or -80C for long term storage. Avoid freeze/thaw with your samples as this will degrade the samples.

PCR Testing

Materials

- PCR tubes
- Microcentrifuge tubes
- 10 uL, 20 uL, 100 uL, 1000 uL autoclavable pipets
- Pipet tips
- Nano filtered water
- 503 primer set
- 503 nest primer set
- New England Biolabs Q5 high Fidelity DNA Polymerase
- New England Biolabs OneTaq Polymerase
- Qiagen PCR Purification kit
- Thermocycler
- 2% E-gel Agarose gels
- Gel electrophoresis System
- Gel Electrophoresis DNA marker
- Nitrile gloves

Genetic Sequence of Primers

503 fwd: 5’ - ACGCATAACTGAGTCTGCCC – 3’

503 rev: 5’ – AGCATTTTCAGTGCTGTGCT – 3’

503 nest fwd: 5’ – CTGTGTGGTCAACCAACATAAA – 3’

503 nest rev: 5’ – CTTTTCTTGATAATTCAGTTCCGG – 3’

PCR Protocols

A nested PCR is the most accurate way of isolating an mtDNA product which can determine if there is hellbender mitochondrial DNA in the sample. The conditions for these two interdependent rounds are seen below. The conditions outlined are for 25 uL reactions. After the first round, the products should be PCR purified, the concentrations should be measured, and the products need to be diluted so that the concentration is lower than 5 ng/uL. The gels will look similar to those depicted below for the two respective rounds. The 503 round has more variability in the gel electrophoresis because the product will contain unwanted genetic material which is different for each sample. However, a distinct band should be seen around 175-200bp for the nested product. If the band is not seen, hellbender DNA is not present. These are the recommended conditions; however, they may be adjusted accordingly. Different samples have different levels of DNA and PCR inhibitors so the quantities and thermocycler conditions may need to be adjusted for different samples.

503 bp Amplicon:

PCR Conditions for 503bp amplification of Hellbender Hypervariable region:

Quantity	Material
1 uL	Template: Extracted eDNA sample
.75 uL	503 fwd primer
.75 uL	503 rev primer
10 uL	Nano purified water
12.5 ul	Q5 polymerase mastermix

Thermocycler Conditions

Temperature °C	98	98	62	72	72	4
Time	:30	:10	:10	:11	:20	infinite
# of cycles	1	35			1	1

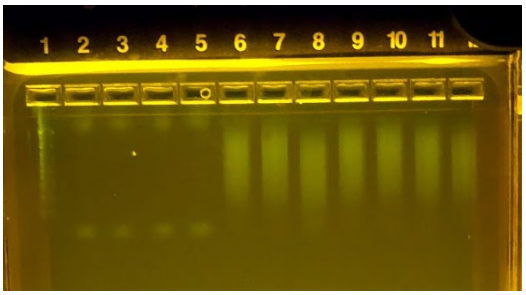

Figure 44. Typical gel picture of 503 round product in lanes 6 through 12. A clear singular amplicon length is not necessary for this round, as the nested will use this collection of amplified DNA as template, making the 2nd PCR reaction product more specific.

503 nested PCR reaction

PCR Conditions for 503 nested round

Quantity	Material
1.2 uL	Template: cleaned diluted 503 round product
0.8 uL	503 nest fwd primer
0.8 uL	503 nest rev primer
9.7 uL	Nano purified water
12.5 uL	OneTaq polymerase

Thermocycler Conditions

Temperature °C	94	94	55	4
Time	:30	:25	1:00	infinite
# of cycles	1	35		1

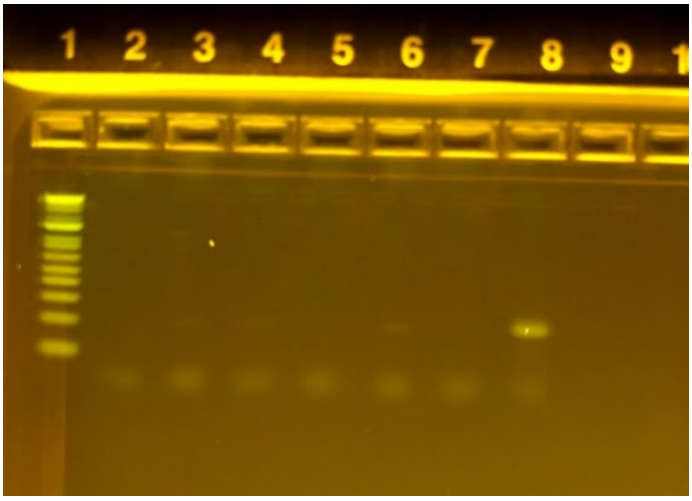

Figure 45. A typical gel picture of the nested amplicon generated from 503 template with bands in lanes 3, 4, 6, 7, and 8

Amphibian Specific Ranavirus

Genetic Sequence of primer:

AmpSpecRana fwd: 5' – GTCCTTTAACACGGCATACCT – 3'

AmpSpecRana rev: 5' – ATCGCTGGTGTTGCCTATC – 3'

PCR Conditions

Quantity	Material
1 uL	Template: eDNA sample
1 uL	AmphSpecRana fwd primer
1 uL	AmphSpecRana rev primer
9.5 uL	Nano purified water
12.5 uL	Q5 polymerase mix

Thermocycler Conditions

Temperature °C	98	98	61	72	72	4
Time	:30	:10	:05	:10	:30	infinite
# of Cycles	1	40			1	1

Sequencing

- The 503bp product should be generated with 20 cycles
- The product should be cleaned but not diluted

PCR Conditions for generating sequencing-ready product

Quantity	Material
1 uL	Template: 503x20 product
1 uL	503 fwd primer
1 uL	503 rev primer
9.5 uL	Nano purified water
12.5 uL	Q5 polymerase

Thermocycler Conditions

Temperature °C	98	98	55	72	72	4
Time	:30	:10	:12	:12	:20	60 minutes
# of cycles	1	35			1	1

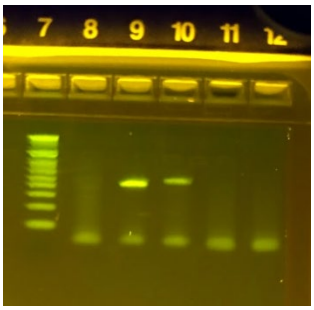

Figure 46. Gel picture of sequencing PCR product. Lanes 9 and 10 have desired bands.

- To eliminate unwanted amplified DNA, a get cut may be performed on the sequencing PCR products.
- The samples must then be processed and sequenced with the Sanger method.

Digital Droplet PCR

Equipment

- PCR tubes
- Pipets
- Pipet tips
- 5-100 uL multichannel pipet
- 200 uL pipet filter tips
- Digital droplet generation oil
- Bio-Rad DG8 cartridge
- QX200 Droplet Generator
- Gasket
- Foil
- 96-well plate
- Bio-Rad cartridges
- Cartridge holder
- C1000 Thermocycler
- QX200 Droplet Reader
- QuantaSoft software
- QX200 ddPCR Evagreen supermix
- 503 nest primer set
- Nano purified water
- 96 well plate heat sealer

ddPCR Protocol

- The workflow described in the Bio-Rad Digital Droplet PCR Manual is to be followed.
- Consistency is key in these reactions so that the samples are assessed equally.
- A 22 uL reaction is set up then 20 uL of it is multi-pipetted into the cartridges.

- 70 uL of oil is added to each cartridge well.
- 45 uL of the emulsified mixture is multi-pipetted to the 96 well plate.
- Positive and no template controls should always be used to ensure that the reactions worked properly.
- The concentration of the template may need to be adjusted according to the samples to reduce the effect of PCR inhibitors.
- If the QuantaSoft program does not count positive droplets when there is a clear bimodal distribution, a threshold may be assigned. The threshold should clearly mark the separation between unreacted material and amplified droplets. Using the positive and no template controls, one may decipher a fluorescence amplitude at which positive droplets can be assigned. The image below shows an instance where the software did not designate droplets as positive but a clear bimodal distribution existed. A threshold was therefore added above the unreacted material.

Suggested ddPCR Conditions

Quantity	Material
1 uL	Template: 503bp template (diluted)
1.1 uL	503 nest fwd primer
1.1 uL	503 nest rev primer
7.8 uL	Nano purified water
11 uL	Evagreen

Thermocycler Conditions

Temperature °C	95	95	55	4	90	12
Time	5:00	:30	1:00	5:00	5:00	infinite
# of cycles	1	40		1	1	1

Material Cost List

Material	**Maker**	**Item Number**	**Cost Per Unit**	**Quantity per 10 samples**	**Cost per 10 samples**
Medium Nitrile Gloves (100)	Kimberly Clark	50578	$13.50	1	$13.50
.45 micron cellulose nitrate filters (100)	MF-Millipore	HAWP04700	$153	10	$15.30
60 mm x 15 mm Polystyrene Petri Dish (500)	Fisherbrand	0875713A	$56	10	$1.12
Dneasy PoweWater kit (100)	Qiagen	166016973	$1,006	10	$100.60

Razor blades (100)	Razor Blade Company	94-0186	$5.25	10	$0.53
.5 uL - 10 uL pipette tips (1000)	TipOne	P1000-10	$484.00	200	$96.80
200 uL pipette tips (1000)	TipOne	P1000-200-R	$277.03	200	$55.41
1000 uL pipette tips (1000)	TipOne	P1000-1000	$161.62	50	$8.08
1.7 mL Microcentrifuge tubes (5000)	Costar	3621	$472	100	$9.44
PCR tubes (1000)	Thermo Fisher	AB0620	$110	100	$11.0
503 primer set	Thermo Fisher	-	$10	0.5	$5.0
503 nest primer set	Thermo Fisher	-	$10	0.5	$5.0
Q5 high Fidelity DNA Polymerase (500 reactions)	New England BioLabs	M0492L	$441	50	$44.10
New England Biolabs OneTaq Polymerase (1000 reactions)	New England BioLabs	M0480L	$170	20	$3.40
MinElute PCR Purification kit (250)	Qiagen	28204	$633	30	$75.96
E-Gel Agarose Gels 2% (20)	ThermoFisher	A45204	$212.96	5	$53.24
Trackit 1 Kb Plus DNA Ladder	Invitrogen	903616	$73.50	0.2	$14.70
200 uL pipet filter tips (960)	Biotix	63300001	$103	150	$16.09
Digital droplet generation oil (10 bottles)	Bio-Rad	1864006	$324	0.05	$16.20
Bio-Rad DG8 cartridge	Bio-Rad	1864008	$272	0.5	$136.0
Gasket	Bio-Rad	1863009	$68	0.3	$20.40
Foil	Bio-Rad	1814040	$98	0.2	$19.60
96-well plate	Bio-Rad	12001925	$162	0.2	$32.40
QX200 ddPCR Evagreen supermix	Bio-Rad	1864034	$520	0.2	$104.0
				Total:	$857.87

Sequencing costs will range per total basepair, for a few Sanger samples (approximately $25/ sample), for megabase pair amounts of data with an Oxford Nanopore run ($400-500). Note multiple samples can be combined using bar coded primers and run on a single Oxford Nanopore flow cell.

Appendix B

Literature Review Extended

The analysis of environmental DNA (eDNA) from waterways is a valuable tool that can be used to detect organisms in aquatic environments. Compared to traditional methods of animal surveying in waterways, eDNA sampling is less invasive to animal habitat. This method also serves to reduce the required labor and cost of surveying for aquatic animals. eDNA analysis is an effective means for early discovery of invasive species and can assist in identifying the presence of threatened or endangered species in waterways.

Detection of aquatic animals by eDNA sampling has been practiced in rivers, streams, lakes, and other marine habitats [4]. This method has been applied to identify fish, mollusks, amphibians, and crustaceans [4]. eDNA generally comes from epidermal cells, excrement, hair, body fluids, and germ cells that are emitted from aquatic animals. Several abiotic factors have been tested for their effects on eDNA detection. These include streamflow, sediment presence, and animal density [5]. In one study, the presence of sediment and flow were found to decrease the detection rate of eDNA, which was confirmed from our experience, whereas increased animal densities resulted in greater rates [5]. The temperature of the water may also have an effect on the detectability of eDNA. In addition to these conditions, biotic inhibitors such as algae and humic acid contamination have been found to interfere with PCR processes and result in decreased detection rates [5].

An experiment similar to our own was conducted in Japan to test for the presence of the endangered Japanese clawed salamander, *Onychodactylus Japonicus*, in the headwaters of the Ibo river [4]. *O. japonicus* tend to dwell under rocks, stones, tree roots, and logs in locations with cool and clear water. The primary purpose of the project was to analyze the effectiveness of eDNA testing versus rudimentary hand capturing methods [4]. The tributaries where the sampling took place were no greater than 8 meters in width and 70 cm in depth, dimensions comparable to the waterways where we have tested for the Eastern Hellbender [4].

Two questions that the experiment examined were whether sampling at two different positions in the streams made any difference in eDNA concentration and if the eDNA collected matched the results from the hand-capture surveys [4]. A similar procedure to ours was followed as 1L samples were collected using propylene bottles [4]. 5L samples were also collected [4]. The samples were taken from the center of the streams at the water's surface and under rocks at the bed of the streams [4]. The water samples were filtered and analyzed using qPCR with primers that were developed to amplify a portion of the 12S ribosome of mitochondrial DNA [4].

The group compared the eDNA extracted to other types of salamanders and found that it belonged solely to *O. japonicus* [4]. In comparing the samples taken in the center of the waterways to those taken under rocks, there was no meaningful difference in the eDNA concentrations between samples [4]. The group also found that for most of the sites where *O. japonicus* was discovered by hand-capture, eDNA for the animal was detected [4]. Further, eDNA was also found at sites where the animal was not discovered by hand-capture [4].

Despite these encouraging results, *O. japonicus* eDNA was not recorded at a site where the animal was physically observed indicating a false negative [4]. Nevertheless, these findings illustrate the effectiveness of eDNA sampling in detecting amphibians in aquatic environments.

Another experiment of interest was performed in an effort to detect the presence of freshwater mussels, *Sinanodonta* spp. The preservation of mussels is important as they filter water, and many other species depend on them as a food source. Additionally, invertebrates and algae use their shell as a habitat [7]. Their presence is an indicator of ecological biodiversity. Therefore, it is important to be able to efficiently monitor for them as well. The aim of this study was to compare the results from the traditional, time consuming process of hand surveying to eDNA-based detection [7]. The sources for this data collection were rice patty and other artificial ponds, so stream flow was not an issue [7]. Something to note from this study is that they used the Qiagen DNeasy Blood and Tissue Kit for DNA purification [7].

PCR detection for this study was done using real-time PCR to amplify a 111 base pair fragment of the COI region of mitochondrial DNA [7]. NCBI was utilized to search for other bivalves with homologous sequences using Primer-BLAST. This study confirmed that eDNA could be used as a means of detection for bivalves; however, it failed to detect low-density populations, and bodies of water containing only dead specimens. Therefore, there may be difficulty in a stream setting for other studies when streamflow is high.

Another eDNA research project endeavored to detect the presence of the quagga mussel, *Dreissena rostriformis bugensi*s, in the River Wraysbury in the United Kingdom [1]. The focus was the comparison of metabarcoding, a more general/multispecies approach, to PCR. The founding population for this study was in the Wraysbury Reservoir in Berkshire. Samples were taken 0.61 km downstream, 1.10 km downstream, and 2.75 km downstream, along with an upstream sample as a negative control [1]. The gene fragment utilized for the PCR was the COI gene, as it did not amplify other genes and detection was successful in all samples [1].

More recently, a study was performed to detect *Necturus maculosus*, as known as the mudpuppy. This indicator species of salamander, like the Hellbender, was once widespread, but has suffered population decline due to habitat loss, pollutants, and contamination [8]. In this experiment, trapping surveys were compared to eDNA surveys. It was found that eDNA surveys provided a higher rate of detection than the trapping methods, which demonstrates the value in using eDNA detection for secretive organisms [8].

In addition to amphibians and mollusks, eDNA has been used to test for the presence and abundance of fish in aquatic environments. Traditional methods of determining fish abundance including weirs, walking and aerial surveys, counting towers, and hydroacoustics are costly and labor intensive. A study by the University of Washington examined eDNA of sockeye salmon in Hansen Creek in Alaska and sought to determine the utility of eDNA as a means to determine fish abundance [6]. The group sampled during the sockeye spawning season of mid-July through August at locations along the creek and in tributaries. Daily visual counts of live and dead sockeye salmon were also performed as a part of an independent research program at the creek.

The position of the sampling sites enabled the group to draw conclusions about the transportation of eDNA and whether it settles or degrades as it travels through water. They

found that eDNA concentration was similar between sites located within 10's of meters, similar to our Phase I findings. In contrast, a site located 1.5 km downstream from all three of these sites the concentration of eDNA was significantly less. This shows that the eDNA concentrations diminish over longer distances due to settling or degradation and that nearly all detected eDNA is found in close vicinity to the source. This study also analyzed the difference in eDNA emissions between live, naturally dead, and killed fish and found that naturally dead fish emitted significantly more eDNA than the other two states [6]. Nevertheless, these results show that there is potential for eDNA to be used to estimate fish abundance.

eDNA detection is a science that has grown considerably over the last several decades and continues to evolve. Despite this, uncertainty remains among certain parties regarding the accuracy of eDNA testing. It is true that detection of invasive or endangered species via eDNA can have significant impacts on businesses and public practices as it can spur the cancellation of development plans, limit water use, alter regulation of recreational practices, and produce financial harm to fisheries. Christopher Jerde from the Marine Science Institute at UCSB conducted ample research of eDNA for detecting aquatic species during the late 2000s and early 2010s. His work on the invasive silver carp and bighead carp in the Chicago Area Waterway System (CAWS) garnered backlash from skeptical fishery experts as his data was not originally substantiated by traditional surveying methods [2,3].

There have been many efforts to prevent the two species of Asian carp that have already invaded the Mississippi River basin from entering the Great Lakes via the Chicago Sanitary and Ship Canal (CSSC) [3]. When sampling for the Asian carps during 2009-2011, Jerde's team detected the species in both Lake Erie and Lake Michigan. Though bighead carp had previously been captured in Lake Erie, the detection of both species in Lake Michigan caused concern and fisheries began searching for the fish. The initial failure to capture any Asian carps in Lake Michigan led to the question of whether the eDNA results were accurate. The fish were eventually caught and Jerde's findings were backed up by other studies that detected the Asian carps in Lake Michigan (Amberg et al., 2015; Klymus et al., 2015; Turner et al., 2014b; USFWS, 2019; Wilson et al., 2014) [3].

Jerde offered a simple statistical prediction for detecting Asian carp eDNA compared to traditional capturing methods in his paper: "Can we manage fisheries with the inherent uncertainty from eDNA?"[2]. Citing his own findings in Jerde et al. (2011) that eDNA detection occurred every time it was used, and over a 31 day period of electrofishing only one fish was found, he calculated that the probability of detecting an Asian carp using eDNA before electrofishing was 97% [2]. This result, along with the eventual detection of Asian Carps in Lake Michigan by the aforementioned studies, points at the fact that eDNA is a useful and reliable method of aquatic species detection and predicting where animals are even though they are not seen directly by an observer. Though eDNA detection cannot provide problem-free species detection, the net benefits of this method have been demonstrated by a strong body of literature over the last 2 decades and lend support to ODOT adopting this strategy for Eastern Hellbender and other species in the future.

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Sampling Site Determination Procedure

During the first several years of the project, the Blue River in Indiana was used as a positive control sampling site because hellbenders have been released there by Purdue University. In July 2020, after three consecutive Blue River sampling trips failed to detect any hellbenders, new sites in Ohio were selected for sampling.

Hellbenders are known to inhabit streams with clean, swiftly flowing water. These streams typically have high levels of dissolved oxygen and low levels of sediment. They tend to avoid streams that are wide with slow moving water as well as those with slab rock bottoms. Streams with intermittent, medium sized rocks are favorable because hellbenders live under rocks where water is flowing. With these characteristics in mind, streams were ranked according to five criteria of streamflow, location, water quality, surrounding environment, and water features.

The United States Geological Survey collects data on streams by remote monitoring and this data is available to the public on their website. The most common metric that is measured in Ohio is streamflow, also known as discharge. The first step of the sample site determination process involved collecting all the available mean monthly streamflow data for Ohio streams on the USGS database. 220 sites contained data until at least 2017 so these sites were listed in a document for consideration. Because future sampling was going to occur during August and September, the mean streamflow amounts for these two months were averaged to give one number that could be used to rank the monitoring sites. USGS sites that had mean August/September streamflow levels between 20 and 200 cfs were selected for further consideration. 123 sites remained in consideration after this step. In some cases, there was more than one monitor on a specific stream so less than 123 streams remained at this point.

YEAR	00060, Discharge, cubic feet per second, Monthly mean in ft3/s (Calculation Period: 2012-04-01 -> 2020-04-30)								
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
2012				103.3	438.9	54.9	81.4	14.6	25.1
2013	333	203.9	508.3	254.6	119.3	108	108.3	75	18.2
2014	300.5	752.3	204.8	906.4	219.6	295.4	14.1	18.4	3.82
2015	144.5	63.6	1,264	1,011	25.9	237.8	783.4	94.7	7.65
2016	136.5	704.5	437.4	488.5	274.5	38.8	378.4	315.1	8.64
2017	562	320.8	776.3	486.8	479.9	423	216.8	9.76	5.31
2018	287.1	1,348	446.7	730.2	337.8	236.1	101.8	154.5	786.1
2019	850.3	1,630	731.3	690.7	309	844.9	46.7	258.3	13.7
2020	503.8	701.2	509.4	392.7					
Mean of monthly Discharge	390	716	610	563	276	280	216	118	109

Figure 47. Mean Monthly streamflow for White Oak Creek above Georgetown OH

USGS site #03238495

Hellbenders are known to be more prevalent in the Ohio River basin in eastern and southern parts of the state. Thus, the previously selected streams were organized by their location and further narrowed down. The county for each monitoring site was identified and only sites located in eastern and southern counties were selected for further consideration. The image below depicts the counties that were considered. 51 monitoring sites remained in consideration after this step.

Figure 48. Counties selected for consideration

The next step was to assess the water quality of the remaining sites. The Ohio EPA (OEPA) publishes a water quality assessment report every two years and as a part of this initiative, the OEPA surveys and assesses creeks and rivers throughout Ohio [4]. These surveys have occurred over the last few decades, so the assessments are not always up to date, but they offer a relative gauge of the health of the stream. Several thousand sites have been assessed, many of which are on the same stream [4]. The OEPA assesses the stream's health by assigning scores for the Index of biologic Integrity (IBI), Qualitative Habitat Evaluation Index (QHEI), Invertebrate Community Index (ICI), and the Modified Index of Well Being (MIWB) [4]. Because hellbenders are vertebrates, the IBI and QHEI were deemed most appropriate for consideration.

The IBI assesses the relative health of an aquatic environment [5]. It scores a body of water by how much of an impact anthropogenic influences and biological activity have had on the water. The IBI is composed of 12 metrics and the score falls anywhere from 12 to 60 with 60 denoting a very healthy water body [5]. The QHEI judges the physical environment at a specific point by considering such traits as substrate quality, instream cover, channel quality, riparian attributes, and pool/riffle quality [6]. The QHEI is scored from 0 to 100 with 100 denoting an ideal setting for aquatic species habitation [6].

Station: 609110
WHITE OAK CREEK DST. GEORGETOWN WTP DAM, ADJ ST. RT. 221
Full Attainment

Aquatic Life Use: EXCEPTIONAL WARMWATER
Drainage Area: 218.00 sqm
Rivercode: 10-400-000
Ecoregion: INTERIOR PLATEAU

Aquatic Life Use Metrics:

Metric	Score/Narrative
IBI:	52 /
MIWB:	10 /
ICI:	/
Invertebrate Narrative:	Exceptional
QHEI:	74 /
Year Fish Monitored:	2006
Year Invertebrates Monitored:	2006

IBI: Index of Biologic Integrity (fish)
QHEI: Qualitative Habitat Evaluation Index
ICI: Invertebrate Community Index (invertebrates)
MIWB: Modified Index of Well Being (fish)

Figure 49. Ohio EPA water quality assessment of the White Oak Creek near Georgetown, OH

The IBI and QHEI scores were collected from the EPA's *Water Quality: Assessment Unit Summaries (2020)* GIS map at the closest monitoring point to the USGS monitoring location for each site [1]. The image above shows the scoring list for an individual monitoring point [1]. Since higher IBI and QHEI scores reflect better water quality, the sites were ranked by the average of these two scores. The highest 15 sites were then selected for further consideration.

The final step in determining new sampling locations was analyzing the surrounding area and observable water features using Google Maps. Streams that are surrounded by mostly farmland were less desirable since farmland can cause high amounts of sediment to runoff into streams. Furthermore, by using the street view feature on bridges and viewing the streams by satellite view, the water features were assessed. Streams with intermittent rocks and visibly flowing water were favored. A full listed of creeks sampled can be found below in Figure 50.

Date	Stream	Animals Detected	Notes
6/10/2016	Duck Creek	No	Concrete raceway
6/16/2016	Salt Creek	Yes	Average flow
6/20/2016	Scioto Brush Creek	Yes	Average flow
5/14/2018	Blue River	Yes	Average flow
6/5/2018	Salt Creek	No	Very high flow
6/19/2018	Scioto Brush Creek	No	Very high flow
7/3/2018	Little Beaver Creek	No	
8/9/2018	Cross Creek	No	
8/15/2018	Blue River	Yes	Release of tagged animals
9/8/2018	Mohican River	No	
9/26/2019	Blue River	Yes	Low flow
10/10/2019	Blue River	No	High flow
10/23/2019	Blue River	No	High flow
7/1/2020	Blue River	No	High flow
7/27/2020	White Oak Creek	No	
7/27/2020	O'Bannon Creek	No	
8/6/2020	Jelloway Creek	Yes	Average to low flow
8/6/2020	Kokosing River	No	
8/12/2020	Big Darby Creek	No	
10/2/2020	Jelloway Creek	Yes	Average to low flow

Figure 50. List of Creek sample locations used in this research report.

Jelloway Creek which feeds into the Kokosing River was also chosen for sampling after it was discovered that the creek runs through the Knox County Hellbender Preserve. Eventually, Captina Creek was deemed too far from the laboratory to effectively serve as a sampling site. Moreover, the five streams below were sampled and the eDNA was tested for hellbender presence. While this method of selecting sampling sites was affirmed by these articles, the

exact location where animals lived or have been released was not known. Therefore, the sampling sites often did not produce positive detections.

Figure 51. Locations of the five selected sampling sites

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Stream Conditions Affecting Hellbender Detection

Using eDNA to detect aquatic animals is highly sensitive to many factors. Sampling at improper times may lead to false negatives in streams where hellbenders are present. Several conditions have been analyzed for their effect on detection of hellbender eDNA and the most optimal conditions for sampling have been concluded.

The breeding season of hellbenders occurs from August through October. The animals shed more DNA during this period than the rest of the year, so this is the optimal period for sampling to occur. When sampling at any time of the year, several stream conditions must be considered. The first and most important is streamflow. The amount of water flowing affects the concentration of DNA in the water. Heightened streamflows dilute the DNA in the water and make detection more difficult.

Because it was known that the Blue River contained hellbenders, this stream acted as a control when examining the effect of streamflow. In 2019, three sampling events took place at the Blue River on September 26, October 10, and October 23 with varying river conditions. From these three events, only the September 26 event resulted in positive Hellbender detection. On July 1, 2020, another sampling trip occurred at the same site. Like the two October trips of the previous year, no Hellbender eDNA was detected. In order to quantify the different levels of streamflow for these four events, data has been obtained from the United States Geological Survey's (USGS) Blue River monitoring site located 3 miles downstream from the sampling location. Because there are no significant tributaries into the Blue river between the sampling site and the monitoring site, the streamflow at the sampling site is assumed to be the same as the monitored streamflow downstream.

Table 1: Streamflow of the Blue River for the four sampling dates.

Sampling date	Streamflow (cfs)
9/26/19	40.6
10/10/19	42.1
10/23/19	40.9
7/1/19	1,170.0

As seen in the chart above, the three sampling trips in 2019 had relatively similar levels of streamflow. With a streamflow of 40.6 cfs, the September 26 trip—the trip with positive detection—had the lowest streamflow. However, the difference in streamflow between the September trip and the two October trips was only 1.5 cfs and 0.3 cfs. While this minor change could be the cause of the negative results, it is more likely that the other factors had a greater effect. The July 1 sampling event took place when the river was extremely blown out as the streamflow was more than 28 times greater than the level on September 26, 2019. It can be deduced that the level of streamflow was too high for eDNA detection because the concentration of any Hellbender eDNA would have been much too low for detection.

When the Salt Creek samples were collected, the stream flow was estimated to be 11 cfs. Likewise, the stream flow at the Scioto Brush Creek was 14 cfs on the day sampled and for the Jelloway Creek, the level was 18.4 cfs. Sampling occurred during periods of lower flow for these three creeks improving the detection probability.

Another factor that has been considered to potentially affect the detectability of Hellbender eDNA is the streamflow of the river prior to sampling. Figure 52 shows that the successful 9/26/19 sampling trip took place after the river was at a lower point. In contrast, Figures 53 and 54 indicate that the 10/10/19 and 10/23/19 trips occurred after the river had been at higher levels. Heightened streamflow levels could act to flush the river of settled or suspended eDNA. Since the streamflow for these two October trips was similar to the September trip, the difference in prior streamflow pattern could be the reason why eDNA was not detected. Figure 55 shows just how high the river was on July 1. Compared with the days preceding and following July 1, this was the least ideal day to sample.

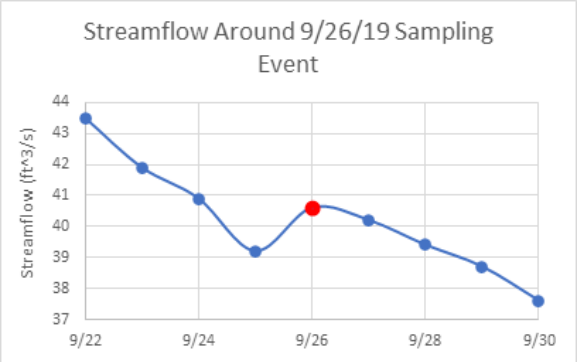

Figure 52.

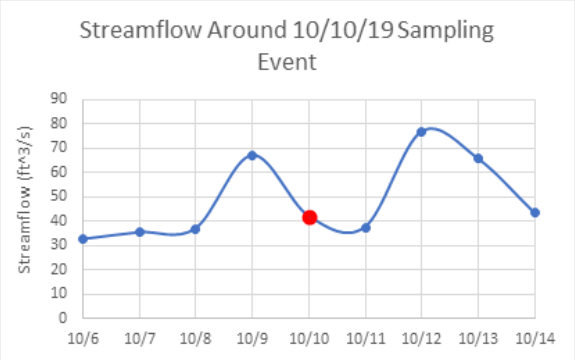

Figure 53.

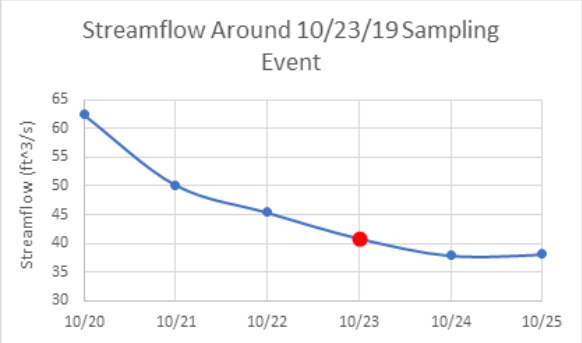

Figure 54.

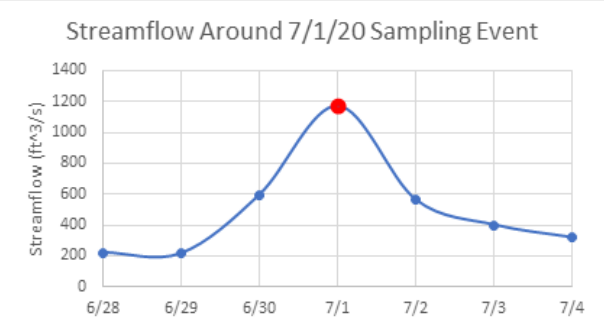

Figure 55.

Table 2. Water temperature of the Blue River and average daily air temperature on sampling days. Air temperatures obtained from Weather Underground.

Sampling Date	Water Temperature (˚C)	Air Temperature (˚C)
9/26/19	20	24
10/10/19	17	21
10/23/19	13	13
7/1/19	22	27

A third possible factor is the temperature of the water. Some studies have suggested that an increase in water temperature decreases the concentration eDNA in water. Tillotson *et al.* found that a 1 °C increase in temperature decreased the concentration of sockeye salmon in Hansen Creek, AK, by around 14 DNA copies measured in DNA copies flowing past the sampling site per second [1]. Yet, our data from 2019 suggest the opposite. The water temperature on 9/26 was higher than the water temperature on 10/10 and 10/23. Since the streamflows were similar, this could account for the negative results for the latter trips. While this may be true, the conditions from the two 2020 Jelloway Creek trips reflect differently. The August 2020 trip occurred when the water was 20 °C and the October trip occurred when the water temperature was 12 °C. Both of these trips produced positive detections. Therefore, temperature was clearly not a significant factor in detection.

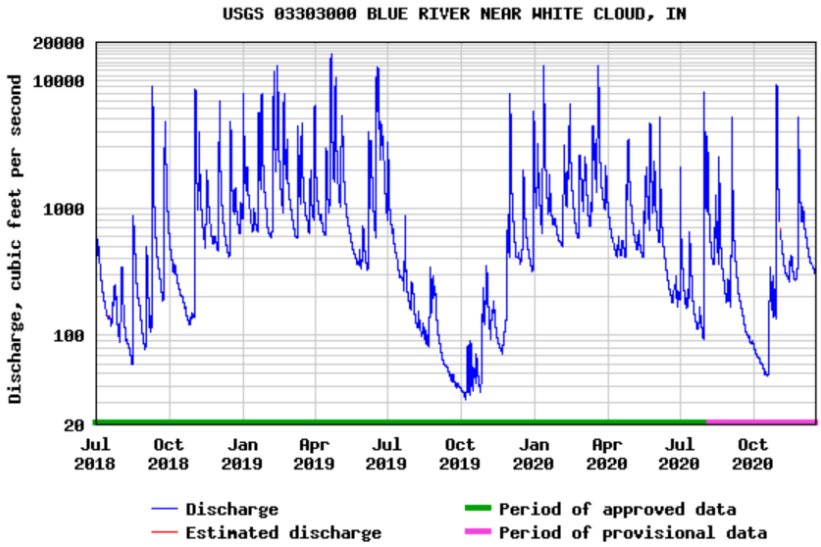

Figure 56. Blue River streamflow near the sampling location from July 2018 to December 2020

It is unclear which factor has a greater effect on the detectability of Hellbender eDNA. Based on the results of the four Blue River sampling trips, the most ideal conditions involve a low streamflow, warmer temperatures, and a prior period of low or steady streamflow. The figure above is the streamflow obtained by USGS monitor for the Blue River from July 2018 until December 2020. The graph shows two evident troughs around October of 2019 and 2020. The positive samples from 2019 occurred in September at the height of the hellbender breeding season. Clearly, this also occurred during a period of low flow which is caused from season precipitation patterns. This further reinforces that the fall is the ideal time for sampling.

References

[1] Tillotson, M. D., Kelly, R. P., Duda, J. J., Hoy, M., Kralj, J., & Quinn, T. P. (n.d.). *Concentrations of environmental DNA (eDNA) reflect spaqning salmon abundance at fine spatial and temporal scales* (Tech.). doi:https://doi.org/10.1016/j.biocon.2018.01.030