Using Environmental DNA (eDNA) to Determine Hellbender Distribution

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Executive Summary

Environmental DNA (eDNA) methods are non-invasive genetic sampling in which DNA from organisms is detected via sampling of water or soil, typically for the purposes of determining the presence or absence of an organism. In this project, we have evaluated the efficacy of eDNA sampling to detect populations of the eastern hellbender indirectly from their aquatic environments. We developed species-specific primers, validated their specificity and sensitivity, and assessed the utility of our methods.

Water samples were collected from 3 creeks and an aquaculture facility at the Columbus Zoo, the latter of which holds animals in captivity for breeding. Salt Creek and Scioto Brush Creek, both in southern-central Ohio were selected for positive control locations upon the recommendation of Greg Lipps, leader of the Hellbender Consortium, part of the Ohio Division of Wildlife. Due to the extremely low population numbers for the animal, coordinates for the release of captive bred animals is protected information, so these coordinates were invaluable in the work. A third waterway (Duck Creek) in the Eastern Cincinnati/Oakley Watershed was chosen as a negative control, due to an abundance of eDNA resulting from municipal wastewater impacting the water and the lack of animal habitat. The negative control sampling location was known to the investigators from previous research, and consists of a concrete raceway adjacent to a Combined-Sewer-Overflow. The structural engineering of the raceway allows for essentially no habitat for aquatic animal life, but the available environmental water contains an abundance of eDNA from sewage overflow, including human and canine mitochondrial DNA.

Due to the late start of the project samples were collected in early November, after the typical breeding season Aug-Oct. Thus, detection results represent a minimum detection range for the animal using the protocols detailed in the research section. From the positive detection results at Salt Creek, the animal detection range was found to be approximately 1/3 of a mile downstream of the animal at stream flow rate of approximately 11 ft^3/sec. Comparatively, Scioto Brush Creek had a slightly higher flow rate measured to be around 14 ft^3/sec and significantly wider stream bed width (about 1.5 times larger). The animal at this location was barely detectable at the coordinates provided. It is possible that the animal moved and/or the water volume provided a sufficient dilution to the animal eDNA resulting in only the eDNA detection threshold at the anticipated location. As expected, no animals were detected at the negative control site with the D-loop primers developed in this study. Primers from previous investigations provided little to no

animal specific eDNA amplification from the environmental water samples or positive control samples and yielded a variety of amplicons in the negative Duck Creek eDNA samples.

Project Background

The Eastern Hellbender is a largest amphibian aquatic salamander native to the state of Ohio. Hellbenders are found under rocks in clear moving water and their existence is dependent on clean, highly oxygenated water. Therefore, they have a major role as an indicator of water quality and health of the ecosystem [1]. Unfortunately in past decades, Hellbender's population has declined more than 80% in the state of Ohio and eastern Hellbender has been listed as an endangered species in Ohio by the US Fish and Wildlife Service [2]. The threat to Hellbenders is mainly siltation, pollution and degradation of stream habitat resulting from the conversion of forests to agriculture and human development [3]. Additionally, stream channelization, damming and removal of rocks are detrimental to the Hellbender's survival. In order to minimize these threats and conserve the species, the Ohio Department of Transportation (ODOT) has to perform habitat assessment at project sites prior to construction. Currently, the most effective method to assess Hellbender distribution is visually searching for the species by personnel wading in water and shifting large nest rocks which is disruptive to the Hellbender's habitat and harmful to their existence. Moreover, the physical hellbender survey could be very time consuming and requires manpower and cost.

These major downfalls of physical survey assessment foster a critical need for the concept of environmental DNA (eDNA) detection which has shown promising results as a reliable method to determine the presence/absence of target species from environmental samples[4,5]. In recent years, several research groups used eDNA to detect hellbenders in Indiana [6], Missouri [7], North Carolina [8] and Kentucky [7] streams and has shown a comparable results to the physical hellbender survey. Although using eDNA for non-invasive detection of imperiled species is a reliable scheme and could aid in species conservation, no concrete research has been established in the state of Ohio to identify the presence of hellbender via eDNA through Ohio streams and improve the documentation of Hellbender distribution through a comprehensive map. Thus, there is a clear and pressing need for development of an accurate, non-invasive species-specific detection methodology and tool to indicate the presence of Eastern Hellbender from environmental samples in the state of Ohio. In this project we have addressed the above need by developing an effective and highly accurate species-specific eDNA detection protocols for sampling and identifying the Eastern Hellbender. Such a system will equip ODOT with a powerful, comprehensive, and accurate field-based DNA screening technology which can be used by ODOT's Office of Environmental at project sites. Also, using this non-invasive screening

technique, ODOT could extend the monitoring time of the species based on the construction time frame and plan accordingly. Additionally, less environmental stress is expected to be induced on this species due to less invasive survey technique. Therefore, the eDNA sensing method will not only improve the health and survivor rate of this endangered species by protecting its habitat, but also eliminate the potential risk of disease transfer between the researchers and species.

Research Context

The specific objectives for this project (phase I) were to achieve the following 4 research tasks:

1) Development of a species-specific primer to be used in genetic sampling which is exclusive to Eastern Hellbender.

2) Collection of environmental samples from Ohio water streams.

3) Development of a standardized field collection protocol to ensure consistency, efficiency and reduce contamination.

4) Submission of the interim report to ODOT after validation and documentation of research results.

Task 1: Development of a species-specific primer to be used in genetic sampling

The detection and assessment of aquatic macroorganisms via environmental DNA (eDNA) has grown dramatically in recent years [9]. For Crytobranchus allegeniensis (Hellbender), this has taken the form of mitochondrial DNA amplification, specifically targeting the Cytochrome B (CytB) gene. In general, mitochondria are useful targets for Eukaryotes in the environment given that the genome is easily distinguished from prokaryotes, does not replicate independently in the environment once released and is several orders of magnitude higher in copy number than chromosomal DNA on a per cell basis. However, eDNA is made up of a variety of natural sources, the most abundant of these being microbial (usually 10^5 or more genomic copies of microbial 16S targets versus mitochondrial [10]) in nature.

To design the species-specific primers, Eastern Hellbender source water which contains Hellbender genetic material (sloughed epithelial cells, etc) can be analyzed for two separate markers: 1) Bacterial markers (16S) associated with the animal will be analyzed via high throughput sequencing (Illumina MiSeq), and 2) mitochondrial markers (CytB) will be analyzed through Sanger sequencing chemistry.

Bacterial indicators remain the most common molecular detection mechanism for human waste in the environment. This is based on the fact that fecal bacteria make up approximately one quarter of waste matter (per unit mass, dry weight). Other animal microbiomes have the same

salient characteristics: bacteria that populate the gut and skin are essential to animal digestion and survival in the environment, and as a result, remain abundant indicators. PCR can be used for ribotyping the bacterial populations that live on and in Eastern Hellbender populations by amplifying the 16S rRNA genes. Amplifying the mixed 16S genes from water samples collected from multiple sources and subjecting these to high throughput DNA sequencing will reveal bacteria closely associated with the animals. The goal will be to identify one or more bacterial indicators which may be indicative of Hellbender presence.

With the same water samples and resulting DNA extractions, previously reported mitochondrial primers for the CytB gene will be used to verify functionality [6-8]. While all existing eDNA Hellbender detection reports use the same gene, Olson's primers are expected to perform most efficiently based on existing work [6]. To these primer validation tests we will add our own eDNA mitochondrial primers designed that amplify the "D-loop" of the Hellbender mitochondrial genome, specifically to examine the variable regions. Like criminal forensics approaches, we will use the variable regions to attempt to distinguish individual animals via high throughput sequencing in an approach analogous to previous work from the Wendell lab [10-12].

We anticipate that microbial and D-loop primers will be valuable in the future for identifying sites where there is more than one animal in close proximity, preventing inadequate relocation efforts when only one is found and removed.

Task 2: Environmental sample collection

Task 2 will include collecting environmental samples during the Fall breeding season around month of September to collect the most abundant amount of mtDNA from known Hellbender locations and from streams with no animals for negative control. The team will work with ODOT and ODNR officials to identify a set of appropriate locations around Ohio as well as times of the year for field samples to be obtained in late summer through the Fall breeding season. Research team members will travel to these sites, and obtain samples using appropriate field sampling methodologies/protocols in order to ensure quality and integrity.

In general, sampling will be accomplished using sterile 1L Nalgene bottles with collected surface water filtered through 0.45 um nitrocellulose membranes to collect available biomass. This biomass will then be extracted using Mo-Bio Power Water kits and held at -20C until PCR and/or sequencing analysis. All water samples will be transported on ice and kept at 4C to ensure that bacterial organisms have minimum replication and reduced degradation of any mitochondrial DNA sources. Filtration and DNA extraction will occur within 24 hours of water collection to minimize eDNA degradation. The effect of sample degradation will be examined with water sample

replicates, where one is collected and filtered as described above, while another is held at 4C for an additional 24 hours. Since surface water samples should always be kept on ice after collection, warmer temperatures will not be explored, since these are certain to degrade available eDNA.

In order to determine the limit of detection downstream of a known animal location, and the detection limits associated with stream flow rate and dilution, multiple samples of a known animal location will be collected from various downstream sites and will be analyzed. Also, at each site, the river flow rate will be measured and included in the analysis.

Past studies have shown [3-5] that sample storage time have little effect on Hellbender detection, mitochondrial DNA will certainly degrade over time due to environmental nucleases. We can examine eDNA decay by quantifying the target CytB copies over time via qPCR. As we examine PCR inhibition of sample locations (achieved through dilutions of DNA extractions subjected to qPCR) we will gain knowledge of the requisite amount of sample water necessary for robust eDNA detection and will have the time 0 concentrations for decay experiments.

Task 3: Development of eDNA protocol, validation and modification

In task 3 we will examine the limits of detection, including correlation between detected Hellbender biomass and any observed animals to improve the developed protocol.

Wet and dry weather flow regimes will be examined during the 1-2 month long sampling process to examine different surface water flow rates and dilution factors. DNA extraction and PCR reaction protocols will be optimized to match surface site specific water properties (ie turbidity, PCR inhibitors), as well as incorporate new bacterial markers identified through high throughput sequencing of microbial targets common to captive bred and wild animals. By applying these strategies to the sampling locations with known presence/absence of Hellbender animals, a comprehensive DNA based detection mechanism can be developed which identifies the best molecular markers or pairs of markers. This could take the form of a bacterial marker which is much more sensitive, but not a direct indicator, which can then inform the use of mitochondrial markers for areas of potential hellbender habitat. Following microbial marker detection, D-loop markers may be used to distinguish how many individuals may exist in that location, given that qPCR may be unreliable, particularly in the Fall breeding season. The proposed 16S microbial markers will provide a very fast and sensitive first round test for hellbender screening in unknown sites followed by the microbial mitochondrial detection scheme if the first test results were positive to ensure the presence of the hellbender.

Task 4: Improvement of the eDNA protocol and submission of the interim report to ODOT

With DNA gene targets identified and concentration detection limits known our 4th task will be to summarize this work as a written report, informing *Phase II* of the project sensor design. Results will be presented to ODOT officials and a *Phase II* proposal will be developed.

Research Approach

Water samples were collected from 3 creeks and an aquaculture facility at the Columbus Zoo, the latter of which holds animals in captivity for breeding. Salt Creek and Scioto Brush Creek, both in southern-central Ohio were selected for positive control locations upon the recommendation of Greg Lipps, leader of the Hellbender Consortium, part of the Ohio Division of Wildlife. Multiple samples were taken at each site at varying distances from the animal habitat coordinates (see figures 1 and 2). All samples were transported on ice, and purified using the Mo-Bio PowerWater system, as described above. Outputs from the DNA purification yielded average concentrations of 10.9ng/ul from Salt Creek, and 10.6ng/ul from Scioto Brush Creek. Samples held at 4C for an additional 24 hours did not yield a significant decrease in extracted eDNA.

A third waterway (Duck Creek) in the Eastern Cincinnati/Oakley Watershed was chosen as a negative control, due to an abundance of eDNA (14 ng/uL) resulting from municipal wastewater impacting the water and the lack of animal habitat. The negative control sampling location was known to the investigators from previous research, and consists of a concrete raceway adjacent to a Combined-Sewer-Overflow. The structural engineering of the raceway allows for essentially no habitat for aquatic animal life, but the available environmental water contains an abundance of eDNA from sewage overflow, including human and canine mitochondrial DNA, which are potentially valuable controls for the salamander mitochondrial target DNA.

Due to the late start of the project samples were collected in early November, after the typical breeding season Aug-Oct.

Water flow rates on the date of collection include an estimated 14 ft^3/sec at Scioto Brush Creek and approximately 11 ft^3/sec for Salt Creek. The positive Control samples acquired through support from the Columbus Zoo, were in the form of skin swabs of several different Eastern Hellbender animals, and samples of the tank water in which the animals live. These positive control samples were then filtered (.45 um) and purified using the Mo-Bio PowerWater system as described previously. Yields from DNA purification of skin swabs averaged 2.5ng/ul, and DNA yields from tank water purifications averaged 3.4ng/ul. Serial dilutions up to 1:1000 were performed on Positive Control samples to aid in determining the sensitivity of the detection protocol and the presence of PCR inhibitors.

Negative control water samples were taken from an area adjacent to Cincinnati that is unsuitable as an Eastern Hellbender habitat. The Duck Creek sampling site was chosen due to an abundance of eDNA resulting from municipal wastewater impacting the water and the lack of animal habitat. The negative control sampling location was known to the investigators from previous research, and consists of a concrete raceway adjacent to a Combined-Sewer-Overflow (see Figure 3). These samples were also purified using the Mo-Bio PowerWater system with yields from averaging 12 ng/ul.

Fig 1. *Map of sampling locations taken from Salt Creek*

Fig 2. *Map of sampling locations taken from Scioto Brush Creek*

Fig 3. *Image of sampling location taken from Duck Creek*

Initial analysis of positive and negative control eDNA samples, was carried out with conventional PCR and qPCR, the latter using an Applied BioSystems 7500 Real Time PCR machine, with the cycling parameters and Quantitect multiplex mastermix previously described by (Spears et al, Biological Conservation, 2014) [8]. We were unable to replicate the detection results previously

described on any of the environmental samples or the positive control samples (animal tank water or the animal swab), with the same thermocycler, qPCR mastermix and primers, indicating that there may be an error in the primer sequence listed. A variety of template DNA concentrations and qPCR cycling parameters were attempted in a significant effort to repeat the published findings. See figures 4-6 for more detailed analysis of these trials.

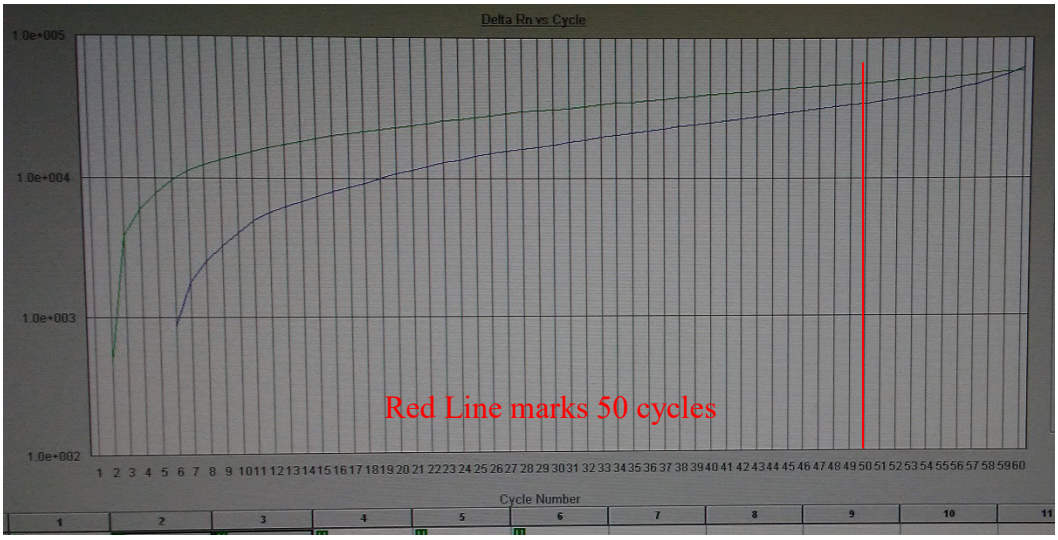

Fig 4. *qPCR of Positive Controls using published protocols*

Shown above is a typical qPCR for Positive Control DNA (animal swab) using the originally published protocols. Results are considered positive when 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.

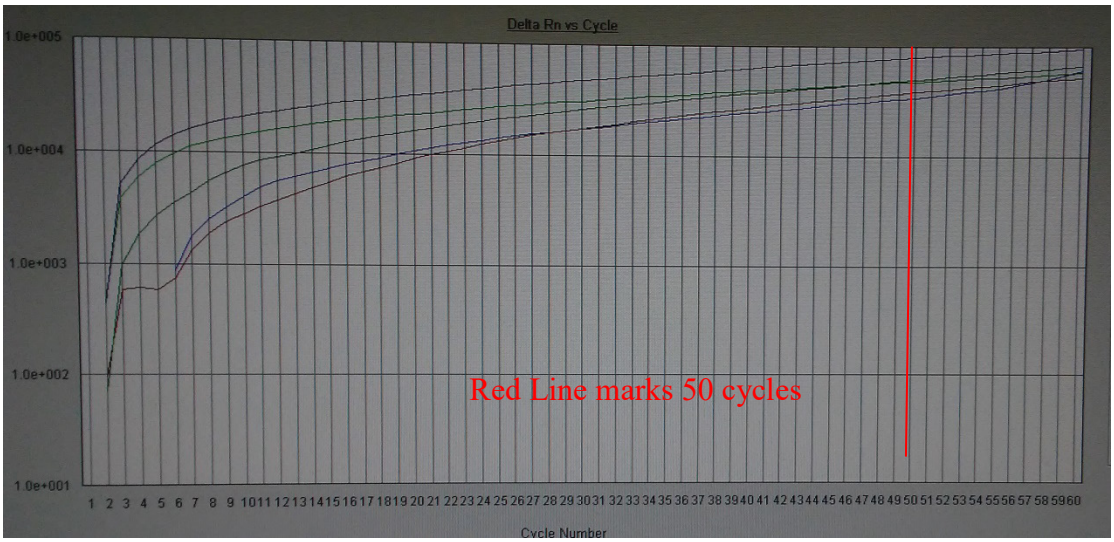

Fig 5. *Additional qPCR results with Negative Control samples overlaid*

Figure 5 shows the same PCR reaction as Figure 4, with the addition of Negative Controls in the graph. The two highest overall amplification plots are both from Negative Control samples, indicating that the published primers were returning a non-detect result from the tank water or a swab of the live animal.

Additionally, the same PCR reactions were investigated via gel electrophoresis. Figure 6 below shows the results of the Spears primers on positive and negative control template DNA for two different DNA polymerases. Lanes 2 and 3 are PCR products from New England Biolabs Q5 Hi-Fi DNA Polymerase, with Positive Control (Tank Water) DNA in Lane 2 and Negative Control DNA in Lane 3. Lanes 4 and 5 are PCR products from Qiagen Quantitect Multiplex DNA Polymerase (used in published protocol), with positive control (Tank Water) DNA in Lane 4 and Negative Control DNA in Lane 5. Lane 1 contains the DNA Marker Ladder. Positive results would be visible as a clear bright band around 150bp. Faint amplicon occurring near the expected target size was seen in both positive and negative controls, indicating reaction byproducts instead of amplified target DNA were the result, confirming the non-specific (no detect), low amplitude results of the qPCR reactions.

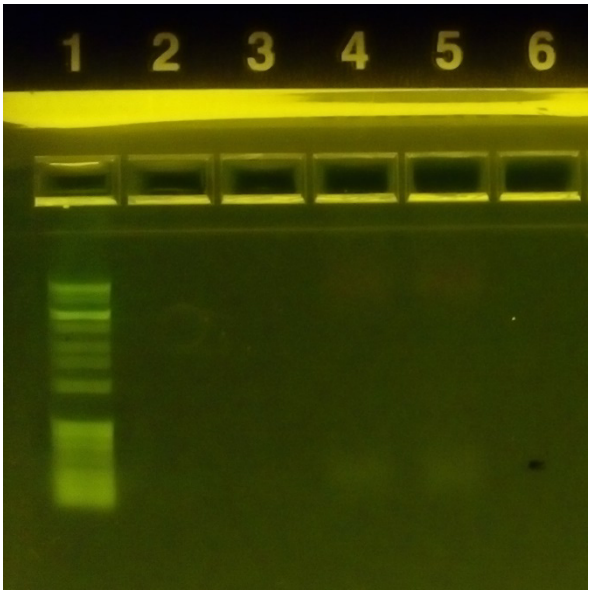

Fig 6. *Gel Electrophoresis of PCR Products of Positive and Negative Control DNA using published primers*

With the published primers unable to produce reliable detections, 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 Varible Region I of the mitochondrial D-loop. The intitial D-loop primers produced more reliable detection from Positive Control and environmental samples, which we then coupled with a nested PCR to help eliminate non-specific product from environmental samples. Figure 7 below 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 product after the Nested PCR reaction.

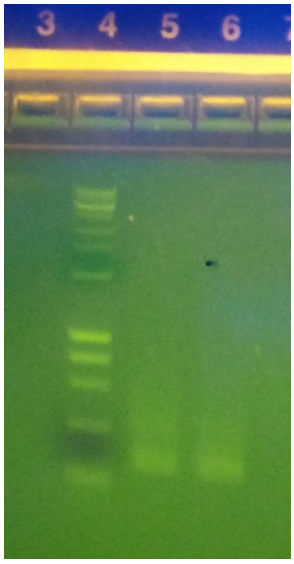

Fig 7. *Gel Electrophoresis of Left D-loop Nested PCR*

Some homology to other salamander species was discovered in the original Left D-loop primers, so two new variations were designed, this time to be species-specific. The new primers were designed to amplify regions of the Left D-loop that are 503bp and 609bp, respectively. Figure 8 below 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 primers on the animal swab Positive Control. 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.

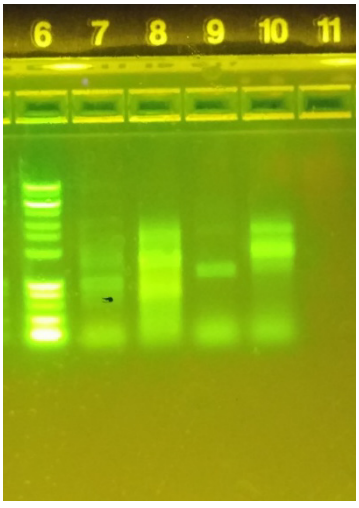

Fig 8. *New species-specific Left D-loop primers for Positive and Negative Controls*

Both new primers showed improved ability to detect target DNA. A bright band at 503bp can be seen in Lane 7, and a bright band at 609bp can be seen in Lane 9. The primers also amplified environmental samples non-specifically – especially in the Negative Controls – but there appeared to be little to no amplification of analogously-sized product from either negative control sample. Because the 609bp primers appeared to amplify target DNA more specifically, we moved forward with these for further optimization.

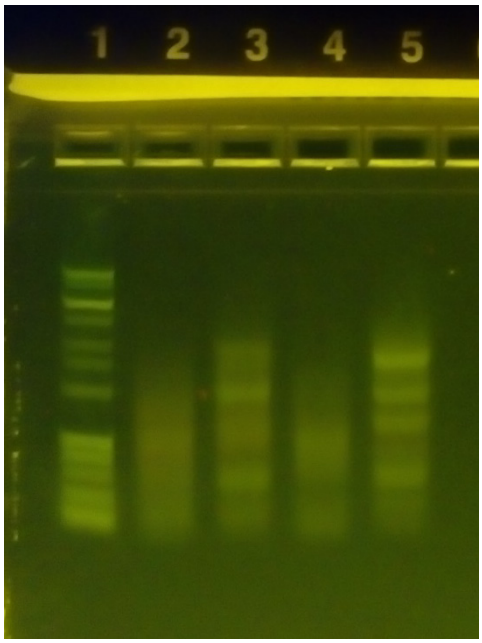

Fig 9. *Initial 503bp PCR reactions with environmental samples and Negative Control*

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 in negative control samples, thus further effort was applied to the D-loop primers producing the 503 bp product.

A variety of experimental conditions were tried to increase reaction specificity and yield, varying the volumes and concentrations of all input parts, and a detailed protocol was determined for use with 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, necessitating a nested reaction to ensure PCR outputs can be obtained that will reliably detect the presence or absence of the Eastern Hellbender in environmental samples. More detailed analysis of the process is discussed below, along with figures 8-9 for clarification.

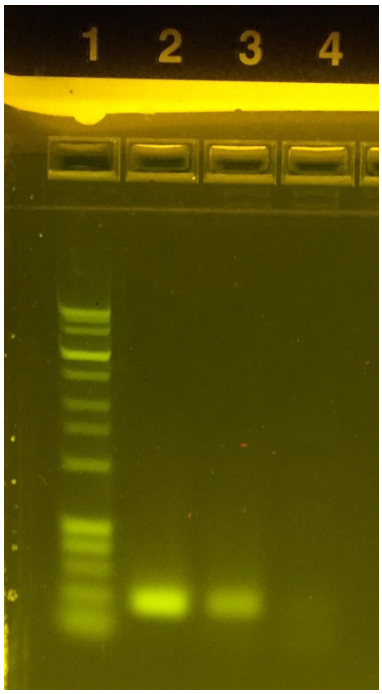

Fig 10. *Results of Nested PCR from 503bp target*

Figure 9 above 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, bright bands can be seen in the environmental samples at the 503bp locus, while the negative control has no product of that size. In order increase specificity and exclude non-target amplification, a set of nested PCR primers was developed for this reaction, to be run on the products of the original PCR. The Nested PCR clearly shows detection of the animal in Positive Controls and no detection in Negative Controls. Figure 10 below shows the gel electrophoresis of products of the Nested PCR. Lane 2 contains Positive Control DNA template products. Lane 3 contains products from a Salt

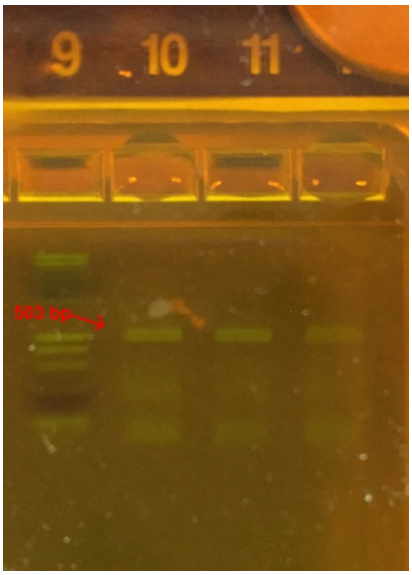

Fig 11. *Results of Optimized PCR reaction for 503bp target*

Creek environmental sample as template. Lane 4 contains products from the Negative Control as template. The Nested PCR target is approximately 175bp. As shown below, both the Positive control and the Salt Creek sample show clear bright bands at 175bp, with almost all non-specific amplification having been eliminated. The Negative Control sample in Lane 4 shows no appreciable amplification of any kind, after the non-specific products are no longer amplified by the Nested primers. Encouraged by these results, more PCR reactions for the initial 503 primer set were tried on eDNA and positive control samples, resulting in relatively clean (primer dimers [below 100 bp], and a faint intermediate band can also be seen), visible product shown in figure 11, using only 1 primer set.

Further analysis was performed by using ddPCR, an extremely sensitive absolute quantification method for DNA targets. These experiments were carried out on the C1000 Thermocycler and QX200 Droplet Generator and Reader, all manufactured by Bio-Rad technologies. Results from ddPCR (see figure 12) confirmed the results of the 503bp target nested and optimized PCR reactions as investigated via gel electrophoresis.

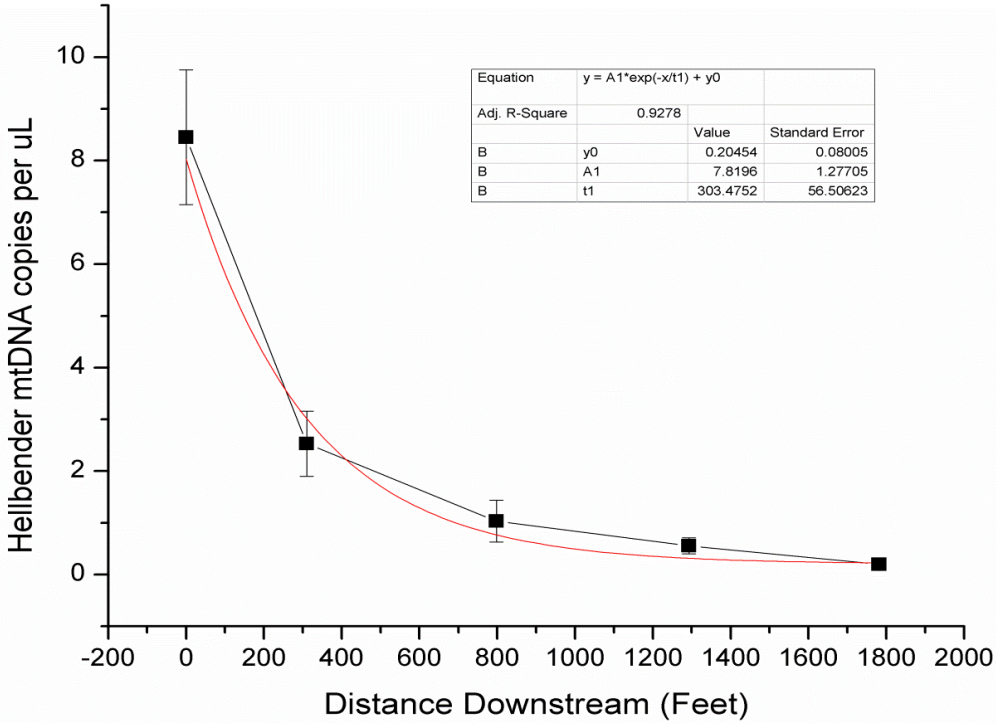

Fig 12. *ddPCR Absolute Quantification of Hellbender mtDNA from Salt Creek eDNA*

Research Findings and Conclusions

The most important results from the ddPCR results indicate a limit of detection that is approximately 1/3 of a mile on Salt Creek. While this is lower than published results indicated during breeding season and summer months, we expect that the winter months are a dormant time of year for the animal with the least amount of eDNA available. It was curious that the previously published CytB primer set were not useful or specific, even on positive controls including swabs from the animals skin, we attribute this to a possible mistake in the published primer sequence or complications with inhibitors from the various DNA sources. While an error could be determined via sequence alignment to the animal mitochondrial genome, the degenerate nature of the primer design makes this more difficult to determine using the available published Hellbender mtDNA sequences.

Based on the result discussed above, a primer set specific to the left D-loop of the Hellbender mitochondrial genome was developed which enabled indirect animal detection from eDNA. Digital PCR allowed for convenient absolute quantification of this genome target from Salt Creek in southern-central Ohio, with the results indicating a minimum detection limit distance of 0.33 miles. Bacterial markers were not investigated given the positive results with mtDNA alone, although the distance limit of detection will undoubtedly improve with season, and potentially a bacterial marker or the addition ddPCR of the nested reaction; however, the 503 amplicon is a useful stopping point, since this can be DNA sequenced directly to determine SNP/indel variation and potentially individual animal counts.

Recommendations for Implementation of Research Findings

Recommendations for these findings include rigorous investigation into published primer sets for Hellbender mtDNA before concluding eDNA results. While other CytB primer sets (in addition to the Spears et al, 2014 [8]) may be useful, a CytB primer set established by Santas et al, 2013 [7] was also briefly investigated without dramatically better results (nonspecific amplification was observed). Since most published qPCR reactions for Hellbender rely on Taqman probes, nonspecific amplification is less of a problem, but clearly additional, nonspecific amplification will decrease the efficiency of the whole qPCR reaction, and ultimately the radius of detection for the animal. The D-loop primer set provided a larger (qPCR targets are usually 100-150 bp) and potentially valuable amplicon that may be sequenced to examine animal number in a particular habitat. Although the d-loop variation within animals of the Ohio River valley has yet to be determined.

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