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Research Article

Noninvasive Method for a Statewide Survey of Eastern Hellbenders *Cryptobranchus alleganiensis* Using Environmental DNA

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Traditional survey methods of aquatic organisms may be difficult, lengthy, and destructive to the habitat. Some methods are invasive and can be harmful to the target species. The use of environmental DNA (eDNA) has proven to be effective at detecting low population density aquatic macroorganisms. This study refined the technique to support statewide surveys. Hellbender presence was identified by using hellbender specific primers (cytochrome b gene) to detect eDNA in water samples collected at rivers, streams and creeks in Ohio and Kentucky with historical accounts of the imperiled eastern hellbender (*Cryptobranchus a. alleganiensis*). Two sampling protocols are described; both significantly reduced the amount of water required for collection from the previously described 6 L collection. Two-liter samples were adequate to detect hellbender presence in natural waterways where hellbenders have been previously surveyed in both Ohio and Kentucky—1 L samples were not reliable. DNA extracted from 3 L of water collected onto multiple filters (1 L/filter) could be combined and concentrated through ethanol precipitation, supporting amplification of hellbender DNA and dramatically reducing the filtration time. This method improves the efficiency and welfare implications of sampling methods for reclusive aquatic species of low population density for statewide surveys that involve collecting from multiple watersheds.

1. Introduction

Hellbenders (*Cryptobranchus* spp.) are North America's largest amphibian species comprised of two subspecies. The Ozark hellbender (*Cryptobranchus alleganiensis Bishop*) is federally endangered, existing in fragment populations in southern Missouri and northern Arkansas, whereas the eastern hellbender, a species of special concern throughout its range, is found in Appalachian forests from southern New York to northern Alabama. Their native habitat consists of large slab rocks in clear and well aerated streams and rivers [1, 2]. In the last century, hellbender populations have declined due to anthropogenic factors [3, 4]. These include hunting and overcollecting [5], as well as degradation of habitat by pollution, dam construction, and sedimentation [6]. Collectively, these disturbances have had negative impacts on hellbender populations [1, 7, 8], particularly in the recruitment of juveniles [9]. Population trends of the hellbender have indicated an ~80% decrease in Missouri and Ohio when compared to data from the previous decades [4, 9].

Because of their sensitivity to environmental disturbances, hellbenders are considered important indicators of water quality and aquatic ecosystem health. However, due to their reclusive nature, finding hellbenders in streams is difficult and not finding hellbenders during physical surveys of natural waterways can provide inconclusive information [7]. The most reliable field method has been physically lifting large slab rocks in streams and rivers. Unfortunately, this type of sampling disturbs habitat and likely harms resident individuals [1]. Avoiding sampling altogether inhibits conservation efforts because the stability of natural populations, both in individual counts and genetic diversity, remains unknown in many areas where hellbenders were once reported.

Environmental DNA (eDNA) is genetic material found in a given environment from sources such as feces, urine, hair, feathers, shed skin, and egg tissue and has been described as a method to detect species presence [10]. The concept of eDNA has been used for over a decade in microbiological research and recently has been applied for larger aquatic species such as amphibians and invasive fish species [11, 12]. Recent work has shown this as a reliable method to determine the presence of these macroorganisms from environmental samples of streams, specifically the Rocky Mountain Tailed Frog (*Ascaphus montanus*) and the Idaho Giant Salamander (*Dicamptodon aterrimus*) [13]. Also, eDNA has been shown to be undetectable within two weeks after animals were removed from a freshwater mesocosm and demonstrated the avoidance of false positives. These experiments assayed eDNA from mesocosm water to detect the common spadefoot toad (*Pelobates fuscus*) and the great crested newt (*Triturus cristatus*) before and after metamorphosis and removal of these organisms [14]. Therefore, detection of eDNA provides evidence that the organism has recently inhabited the location. Recently, Olson et al. [15] used eDNA to detect hellbenders in Indiana and Missouri streams. Comparing their results to a recent physical hellbender survey, these authors were able to confirm this as a reliable method for detecting their presence, even at low population densities [15].

Using eDNA to detect the presence of imperiled species in aquatic environments could aid in species conservation by increasing survey accuracy, minimizing site visits, and expediting detection, while decreasing risk of animal stress and habitat destruction. However, the logistics are still difficult due to the amount of water collected for sampling (6 to 8-L per sterile filter). This amount of water can be logistically challenging due to transport and refrigeration needs. In areas where there are no access roads, sample handling can be extremely difficult, greatly increasing survey time. Therefore, the ultimate goal of our study was to reduce water collection and handling time for reliable surveys of aquatic species. Using information on hellbender sightings from field biologists and the general public, 27 sample sites across the state of Kentucky were assayed for hellbender eDNA.

2. Materials and Methods

2.1. Water Collection Locations for Positive Controls. Water from known hellbender populations was collected to test the efficiency of the cyt-b region hellbender specific primers from 1 L and 2 L water samples. The first water samples were drawn 12 cm from live hellbenders in water tanks used for conservation of captive hellbenders located at the Columbus Zoo, Columbus, Ohio (compliments of P. Johantgen). The second positive site used was located in a natural waterway in southeastern Ohio. A boulder/slab field that was harboring a known hellbender nest consisting of adults and juveniles, was documented on April 5th, 2012 (G. Lipps pers. comm.). A video borescope was used to confirm and document the hellbender nest at the site. Water samples were collected at 12 cm, 10 m, and 20 m downstream from that rock point. Water was filtered and processed as stated below.

2.2. Water Collection Locations for Kentucky Field Sites. Water samples were collected from moving water at a total of 27 sites in four Kentucky watersheds. Each sampling location had historical documentation of hellbender presence either by field biologists or the general public. Some reports of hellbenders were from the 1960s and the hellbender population size for each location was not known. At each site, a boulder/slab field in the stream was identified as an indicator of possible hellbender habitat. These boulder/slab fields typically occur downstream of bends in the river and were approximately 1-2 m^2 in footing. Water collection sites were set 1–5 meters downstream of the boulder/slab fields. The stream width and flow rate were determined and samples were collected at three equidistant points across its width. Facing upstream, a water sample (1 L or 2 L) was taken at each of the points labeled stream left (A), stream center (B), or stream right (C). All water collections were conducted during May 2012 through June 2012. Water was filtered and processed as stated below.

2.3. Processing and Filtering of Water Samples. To collect the water samples, an autoclaved 1 liter or 2 liter wide-mouth Nalgene bottle with lid intact was placed as close to the bottom of the waterway as possible without touching the bottom to avoid sediment which would impede filtration and opened until filled. The bottle was then resealed underwater. Hands were rinsed and alcohol sanitized, and equipment was cleansed using 10% bleach solution prior to taking samples at each site to avoid possible sample contamination and site-to-site transmission of disease. Water samples collected were stored on ice for <24 hours prior to filtration. The water samples were returned to the lab for filtering. Using a vacuum pump, each 1 L or 2 L water sample was filtered through a 0.45 μm cellulose filter (Mo Bio Laboratories, Inc., Carlsbad, CA). Each filter was then placed into a 50 mL PowerWater bead tube and stored at −20°C for four weeks or less.

While conducting this survey, a bottleneck was evident in the time required to filter 2 L of the stream water. Filtration alone would often take up to three hours or more for one sample. Clearly, this type of time investment would impede water sampling such as that needed in a statewide survey. Therefore, a second method was developed to alleviate the excessive time required in the filtration step. The first method employed collection of a 2 L water sample and collection of stream particulate onto one filter. The second method involved collection of three 1 L water samples from a single stream location. Water particulate from each 1 L water sample was filtered onto a separate filter. The DNA extracted from the three filters was later combined and concentrated through ethanol precipitation.

To confirm specificity, water samples representing negative controls were collected from streams and ponds where hellbenders have not been historically reported and were considered too small to support hellbenders. These samples were collected in waterways in Kentucky and in Cumberland, Ohio (at the Wilds Conservation Center). Recent surveys conducted in 2010 and 2011 from these waterways indicated various salamanders and a large population of fish (such as largemouth bass) but no known hellbenders. In addition,

a cooler blank was included in which three bottles were filled with tap water in the laboratory and placed in the cooler along with our samples throughout the trip. This was included to determine if mere handling of samples resulted in cross-contamination during sampling.

2.4. DNA Extraction and Amplification. The eDNA was extracted from each filter using the PowerWater DNA isolation kit according to manufacturer's recommendations (Mo Bio Laboratories, Inc., Carlsbad, CA). To increase concentration of eDNA for amplification, DNA extract from three 1 L water samples was combined and ethanol was precipitated. Briefly, in the laboratory, 1/10th volume 3 M sodium acetate (pH 5.2) was added to the sample. After inversion, 2.5 volumes of 100% cold ethanol was added and the sample was incubated overnight at −20°C. The samples were then centrifuged at 4°C at 12,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged as before and allowed to air dry. Lastly, the pellet was resuspended in 40 μL sterile water. The eDNA extracted from the 2 L water samples was not combined nor was it concentrated through ethanol precipitation.

To amplify the cytochrome b (cyt-b) region of the hellbender mitochondrial DNA, primers Cytb-CA-R4: 5′GGCAATTAAGGCCAGAACACCACCG and Cytb-CA-F4: 5′CCCAACCTTGGAGACCCAGAAA were used at a final concentration of 0.3 μM in conjunction with both AmpliTaq Gold Mastermix (Applied Biosystems, Foster City, CA, USA) and Multiplex Mastermix (Qiagen, USA). Of the two, the Multiplex yielded reliable results. The maximum volume of extracted eDNA was included in each sample (24 μL). The positive control used was DNA extracted directly from Eastern Hellbender blood. Negative controls included sterile water combined with the primers and mastermix. The thermal cycler was programmed to the following regime: one cycle at 95°C for 15 minutes, 55 cycles at 94°C for 30 seconds, 69.5°C for 3 minutes, 72°C for 60 seconds, and one cycle at 60°C for 30 minutes.

PCR products were separated by electrophoresis on 12% polyacrylamide gel in 1xTBE. DNA was stained using SYBR green (Invitrogen, Grand Island, NY). To confirm that the PCR products were those of hellbender, samples were purified using Wizard SV 96 Genomic DNA Purification System (Promega, Madison, WI) and sequenced at the Ohio State Plant Genomic Facility. Generated sequences were then compared to those present in GenBank for hellbender identity [16]. The primers and method developed supported detection of a region of the hellbender cytochrome b gene (150 bp) from as little as 1.5×10^{-5} ng/μL DNA extracted from hellbender blood.

3. Results

This method supported detection of hellbender eDNA from both 1 L and 2 L samples of static aquarium water housing hellbenders (Table 1). Although each 2 L water sample supported hellbender eDNA detection (3 of 3), only a subset of the 1-L water samples were positive for hellbender eDNA (2 of 3) (Table 1). Therefore, even in static water housing hellbenders, collection of 1 L water samples did not allow for detection of hellbender eDNA in all of the trials (Table 1).

Table 1: Detection of Hellbender cyt b gene eDNA in static zoo water and stream water harboring a documented Hellbender in a Southeastern Ohio watershed.

Location	Site number	Liters	Hellbender cyt b DNA +/−
Columbus Zoo Hellbender Tank	A1	1	+
	A2	2	+
	B1	1	+
	B2	2	+
	C1	1	−
	C2	2	+
Southeastern Ohio Watershed	A1	1	−
	B2	1	−
	C2	1	−
	A3	1	−
	A1	2	+
	A2	2	+
	B1	2	+
	B2	2	+
	C1	2	+
	C2	2	+
Environmental negative control		2	−

Detection of hellbender eDNA was achieved using water collected from a natural flowing stream which had confirmed hellbender presence. This location is the only one in Ohio confirmed as a successful site for hellbender reproduction based on the presence of juveniles and identification of a hellbender nest (G. Lipps pers. comm.). From this location, eDNA was only detected using 2 L water samples and not 1 L water samples (Table 1). No hellbender eDNA was detected in the negative control samples (Table 1).

3.1. Survey Samples for Hellbenders in Kentucky Waterways. To determine if this method could detect hellbenders in natural waterways, water sampling was performed on moving water in which historical observations of hellbenders had been documented. Considering that recent confirmation of hellbender presence was not available for the locations sampled, it was not anticipated that hellbender eDNA would be detected in all natural waterways analyzed. Of the 27 sites sampled in Kentucky, hellbender eDNA was amplified from 23 of the sites (85%), which represented all four watersheds (Table 2). No hellbender eDNA was detected in the negative control samples (Table 2).

Hellbender eDNA was also detected when analyzing samples generated by ethanol precipitation of the combined 1 L filtered samples (3-L). Therefore, both filtering methods

Table 2: Detection of Hellbender cyt b gene eDNA in multiple Kentucky watersheds using 2 L samples as well as using ethanol precipitation of DNA resulting from three 1 L water samples denoted as 3E.

Location	Site number	Liters	Hellbender cyt b eDNA +/−
Four rivers	1-1	3E	+
Four rivers	1-2	3E	+
Four rivers	1-3	3E	+
Licking	2-1	3E	+
Licking	2-2	3E	+
Licking	2-3	3E	+
Salt	3-1	2	−
Salt	3-2	2	+
Salt	3-3	2	+
Salt	3-4	2	−
Salt	3-5	2	+
Upper green	4-1	3E	+
Upper green	4-2	3E	+
Upper green	4-3	3E	+
Upper green	4-4	3E	+
Upper green	4-5	3E	+
Upper green	4-6	3E	−
Upper green	4-7	3E	+
Upper green	4-8	3E	+
Upper green	4-9	3E	+
Upper green	4-10	3E	+
Upper green	4-11	3E	−
Upper green/river number 1 system	5-1	2	+
Upper green/river number 1 system	5-2	2	+
Upper green/river number 1 system	5-3	2	+
Upper green/river number 1 system	5-4	2	+
Upper green/river number 1 system	5-5	2	+
Cooler blank		3E	−
Environmental negative control		2	−

supported detection of hellbender eDNA (Table 2). No difference in sensitivity of either approach can be made using this data set. These results demonstrated an alternative filtering method that supports the detection of eDNA present in natural waterways, while alleviating the excessive filtration time.

4. Discussion

It has been documented that hellbender populations have undergone a massive decline (~80%) in the states of Missouri and Ohio. Although data are available to document the decline in these states, it is likely that the overall population of hellbenders has decreased throughout its geographical distribution. The remaining hellbender populations likely have become more reclusive and sought out only the largest rock structures under which to dwell. As these rock structures are immobile, traditional physical surveys of the hellbenders which involve turning over large rocks to locate the hellbender would become ineffective at locating existing populations.

This study is one of few that employed eDNA detection in moving waters [13] and the second study to show the ability to detect the presence of hellbenders [15]. This project used environmental DNA (eDNA) to detect hellbenders in natural waterways in Kentucky and Ohio and presents an efficient method which supports statewide surveying. Identification of eDNA from moving water downstream of a documented hellbender nest required a 2 L water sample. And, the method supported the detection of hellbender eDNA in Kentucky waterways (85%) with reported but unconfirmed hellbender presence. Not surprisingly, hellbender eDNA was not amplified from each location sampled as these reports dated back to the 1960s and many lacked photo documentation to verify the presence of hellbender. Considering that recent confirmation of hellbender presence is not available at this time, it was not anticipated that hellbender eDNA would be detected in all natural waterways analyzed.

Recently Olson et al. [15] identified eDNA as a technique to detect hellbenders from water samples as a model for species that occur in low densities. Using 8 L of water per filter, the authors were able to detect the presence of hellbenders, even at low densities. They also found density and detection to be correlated: the lower the known population, the fewer positive amplifications they found. Our protocol was intended to be applied to statewide surveys and therefore present alternate methods to accommodate the difficulties of managing large amounts of water and multiple sites. The 2 L or 3 L water sample in place of the 8 L sample was adequate to detect hellbenders in moving water. Therefore the experimental design presented here provides a method requiring reduced water volume collection that could be conducted as a statewide survey of waterways.

Several natural factors could impact the ability to detect hellbender eDNA. Increased stream flow or stream width could result in a reduction of the tissue present in each water sample, thereby diluting the amount of tissue in an increased volume of water [13]. The streams analyzed within this study had a stream flow which ranged from 0.04 m^3/s to 2.04 m^3/s with a median of 0.25 m^3/s. The stream width varied considerably from 1.9 m to 49.2 m with a median of 13.2 m. Despite this variability, hellbender eDNA was detected throughout these ranges of stream and width flow. Further study is required to better characterize these variables when using eDNA detection.

Olson et al. [15] utilized a protocol which collected water during times of relatively low stream levels (October–December). In our study, positive samples were detected in water collected in Ohio and Kentucky in May and June when water levels are typically increased during the spring. If the

hellbender population remains the same in a given stream, this increase in water levels will result in a dilution of the eDNA present in any given water sample.

Increased stream flow could alter the behavior of the hellbender resulting in the hellbender seeking out more protected areas within the waterway. This in turn could hinder detection of hellbender eDNA. Also, the possibility exists that the presence of chemicals within the stream water could impede the process of DNA extraction. Many of these factors cannot be controlled and may represent limitations of this line of experimentation. Therefore, this technique is not intended to serve as a method to determine the absence of hellbender but rather to provide evidence for the presence of hellbenders within a stream. The possibility always exists that the water sampled did not flow directly past a hellbender especially since a hellbender nest often exists in sheltered areas of the stream. Also, even if the water was passing over a hellbender, release of hellbender cells may be minimal at the time of collection and therefore preclude detection. Given these experimental constraints the methodology presented is intended to provide evidence for presence of the hellbender and not to demonstrate hellbender absence in a waterway.

Through the development of this approach, it became clear that the largest bottleneck in performing a statewide survey would be the time required to filter environmental material from the 2 L water sample onto the filter prior to extracting the DNA. In an attempt to decrease the amount of time spent filtering, DNA was first extracted from filter particulate collected from a 1 L water sample. However, identification of hellbender eDNA was only successful in using 1 L samples from static water and not moving water inhabited by hellbenders. To ensure sufficient eDNA would be present in each sample, extracted DNA from the three 1 L samples (A, B, and C) were collected, pooled, and concentrated through a routine ethanol precipitation method. Combination and concentration of these three eDNA samples (A, B, and C) produced one sampling point that contained sufficient eDNA to support detection of hellbender eDNA. Although this required more time to manipulate the sample in the laboratory, the overall time invested was significantly decreased as the time to filter a 1 L water sample through a 0.45 μm filter was much less (30 minutes to 1 hour) than the time required to filter a 2 L sample through a single 0.45 μm filter (1 to 8 hours). Although some 2 L samples could be efficiently filtered within one hour, most of the samples from natural waterways required an entire day to filter. Therefore, even if three separate 1 L samples were filtered, the total time investment was at most 3 hours (1 hour per sample) and often less than that.

Larger diameter filters could be employed to decrease the filtration time required; however, the downstream DNA extraction procedure requires a 47 mm filter size and therefore would necessitate cutting of the larger filter. This increased manipulation could result in loss of eDNA and increased chance of contamination, while providing no benefit over the ethanol precipitation method. Although our laboratory has not tested the benefit, Mo Bio Laboratories has developed the Sterivex Filter Unit specifically for DNA isolation from turbid waters. This filtration unit may also improve the efficiency of water filtration. Also, the filter used in the current study may have yielded more DNA due to its smaller pore size (0.45 μm) when compared to other methods that used a 1.5 μm pore-size glass filter [15].

PCR amplification was further optimized by using Qiagen's Multiplex PCR Mastermix. The unique biochemical components of this mastermix aided in the amplification of hellbender DNA from our positive water samples (Columbus Zoo and water collected in southeastern Ohio). Along with Goldberg et al. [13], our data support that the Qiagen Multiplex mastermix was most reliable for the amplification of eDNA. All sequenced products confirmed that the amplified product was 97–100 percent identical to *C. alleganiensis* mitochondrial genes or genome within the NCBI database. Detection sensitivity was at least $1.5 * 10^{-5}$ ng/μL of DNA when using purified genomic DNA from hellbender blood. Using similar molecular methods, Jerde et al. [12] reported the detection of rare Silver Carp in streams. They collected much larger water samples (5 and 10 L) which increased the sensitivity to $3.3 * 10^{-8}$ ng/μL of DNA. However, our method demonstrates reliable amplification of hellbender eDNA from 2 L or 3 L water samples using Qiagen's Multiplex Mastermix.

Use of eDNA has proven to be effective as a method for detection of aquatic macroorganisms, even at low population densities, in both still and flowing waters. This study reported the presence of hellbender eDNA through a method which requires much less water than Olson et al. [15] and minimizes filtering time. Follow-up studies are currently coupling field surveys to better understand the population densities that were detected. Questions remain including what the proximity of hellbenders is in relation to point sources of eDNA, what the half-life of hellbender eDNA is, and determining the impact of temperature and stream flow on the sensitivity of this approach. In addition, quantitative PCR (qPCR) protocols may further our understandings of population density by quantifying DNA concentrations in waterways. Indeed, qPCR has been demonstrated to detect the presence of fish (bluegill sunfish, *Lepomis macrochirus*) and amphibian species (common spadefoot toad (*Pelobates fuscus*) and the great crested newt (*Triturus cristatus*)) in ponds [14, 17]. Additional work has demonstrated that qPCR could be used to estimate fish biomass for the common carp (*Cyprinus carpio* L.) when analyzing water from aquaria and experimental ponds [17]. Therefore, it may be possible to extend this technology and utilize qPCR procedures to determine density of macrovertebrates within moving waterways. Future research utilizing eDNA in moving waterways may also help in the investigation of the presence and abundance of other organisms that may threaten the survival of this species.

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