DEVELOPMENT OF AN ENVIRONMENTAL DNA ASSAY FOR EASTERN MASSASAUGA

by

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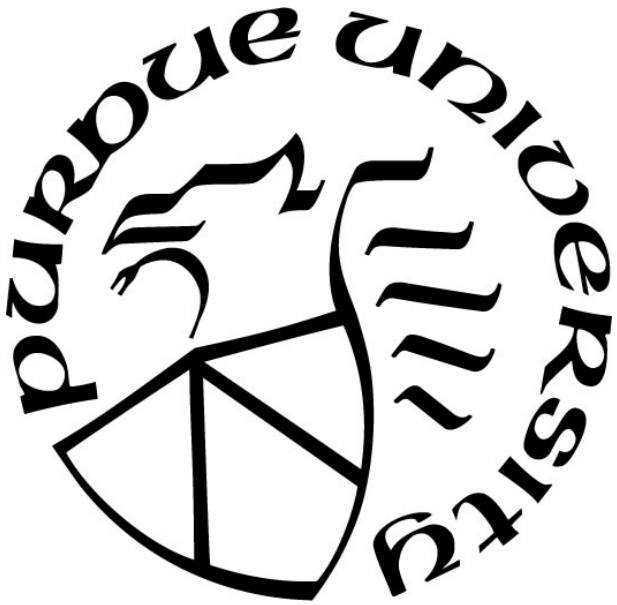

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ABSTRACT

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Utilizing environmental DNA (eDNA) for the detection of species in the field is a potentially cost-effective and time-saving technology that may be useful in understanding the distribution and abundance of threatened or endangered species such as the Eastern Massasauga (*Sistrurus catenatus*). I describe the development of an eDNA assay for the species and evaluate its ability to detect eDNA in laboratory and field conditions. In the field samples, I also investigated the potential for abiotic conditions to influence eDNA detection. Species-specific primers and probe were designed to amplify a 152 bp segment of the massasauga COI gene. Target eDNA could be detected in samples containing as little as 100 copies of target DNA/µL. Water samples collected from laboratory housed snakes indicated that eDNA can be detected in water 56 days after massasauga removal. Field samples were taken from crayfish burrows, known overwintering habitat for the species, from four sites that vary in snake use as ascertained by traditional visual surveys. Of the 60 burrows sampled, seven had a positive detection for massasauga eDNA with no difference in detection rate between DNA extracted from burrow water and burrow sediment. Occupancy models fitted to burrow water indicated that larger amounts of total DNA in a sample may increase the probability of detection of a massasauga eDNA. Large confidence intervals in site occupancy (ψ) and burrow detection (Θ) values suggest that a larger sample size is needed for more reliable occupancy models. Abiotic conditions within crayfish burrows varied among sites but correlation with eDNA detection was not supported. Estimates of qPCR detection within a burrow with eDNA (ρ) suggest that up to 10 qPCR replicates per burrow sample may be necessary. Further studies need to examine eDNA degradation in the field, improve upon the limit of detection, and sample a larger number of sites for eDNA sampling to be a stand-alone survey method for Eastern Massasaugas.

INTRODUCTION

A common issue with traditional field surveys of animals is that rare or cryptic species can go undetected during visual encounter surveys or be missed by traps. A further manifestation of the problem is if such species have low population numbers that warrant conservation concern. Environmental DNA (eDNA) surveying is a comparatively new, non-invasive, sampling approach that can be an alternative to conventional surveying.

Environmental DNA is DNA that is found in water or soil occupied by organisms and accumulates through the shedding of skin, fecal or nitrogenous waste elimination, or the release of reproductive products such as sperm and eggs. This type of DNA has also been found in feathers, urine, saliva, and other secretions (Davy *et al.* 2015, Hunter *et al.* 2015, Olson *et al.* 2012, Schultz and Lance 2015). Eastern hellbenders (*Cryptobranchus alleganiensis*), long-finned pilot whales (*Globicephala melas*), and European eels (*Anguilla anguilla*) are all threatened taxa that are cryptic and have been successfully monitored using eDNA methods (Foote *et al.* 2012, Olson *et al.* 2012, Thomsen *et al.* 2012). As such, eDNA is gaining use for species detection and monitoring including the evaluation of population sizes, dispersal, and survival (Davy *et al.* 2015, Olson *et al.* 2012).

Many potential benefits are associated with the use of eDNA surveys. First, it limits the amount of stress that a species may experience via handling and capture procedures. Second, it may decrease the risk of transporting diseases among populations (Olson *et al.* 2012). Thirdly, eDNA procedures can aid in the rapid detection of rare or cryptic species which would allow the development of more effective conservation plans (Davy *et al.* 2015, Hunter *et al.* 2015). Another benefit that Olson *et al.* (2012) found when using eDNA to sample for Eastern hellbenders was the monetary savings that came from the reduction of person hours spent for field surveying. It takes only one researcher to collect and filter water samples as compared to having teams of biologists assembling to safely sample hellbender habitats. Freshwater turtles provide another example of cost effectiveness of eDNA. Assuming a successful eDNA reading and an hourly wage of $7.30 for workers, it would cost approximately $365 to identify a single species of turtle at a site while conventional methods ranged from around $75 to $3,000 (Davy *et al.* 2015).

In the laboratory, quantitative real-time polymerase chain reaction (qPCR) has become the gold standard for eDNA detection. The amplification of a target gene and fluorescence detection occur simultaneously during each cycle and thus allows for quantitative measurements of the starting amount of DNA in a sample (Pérez *et al.* 2013). Essentially, the null hypothesis for any eDNA analysis is that if the marker concentration is zero, it indicates a lack of target DNA in a sample. The alternate hypothesis is that if the marker concentration is greater than zero, it suggests that the DNA of interest is present in a sample (Schultz and Lance 2015).

One of the advantages of using qPCR for eDNA analyses is that it does not rely on the number of copies of the target gene in a sample. Other benefits include the speed of recognizing the presence of a species, sensitivity to identification, and the reduced risk of contamination (Pérez *et al.* 2013). Species-specific eDNA assay utilizing real-time quantitative PCR (qPCR) have been used for the detection of snakes including Burmese python (*Python bivittatus*), Northern African python (*Python sebae*), boa constrictor (*Boa constrictor*), green anaconda (*Eunectes murinus*), and the yellow anaconda (*Eunectes notaeus*) in the Florida Everglades (Hunter *et al.* 2015).

Eastern Massasaugas (*Sistrurus catenatus*) are an excellent candidate for eDNA surveying. The species has a historic range in the Great Lakes Region, within ten states, and in southern Ontario. However, populations are currently in steady decline in all locations and now are considered extirpated from some of the states in which it once occurred. Anthropogenic factors have decimated historic populations. Living in wet areas including prairies, marshes, and low areas near water, the draining of these places has reduced the availability of suitable habitats (Durbian *et al.* 2008, U.S. Fish & Wildlife Service 2016). Road construction in particular is also a key threat to populations, this contributes to habitat loss, direct mortality of the species, resource inaccessibility, and the separation of current populations (Rouse *et al.* 2011). Additionally, massasaugas do not travel long distances and the increasing presence of anthropogenic barriers are isolating small groups, further decreasing their numbers (Cushman *et al.* 2013, U.S. Fish & Wildlife Service 2016). Furthermore, people's aversions to snakes have led to many deaths (Durbian *et al.* 2008, U.S. Fish & Wildlife Service 2016). Due to these factors, Eastern Massasaugas are considered either threatened or endangered in every state and providence they inhabit and is currently listed under the Federal Endangered Species Act

(Durbian *et al.* 2008, Gibbs *et al.* 2011, Robilliard and Johnson 2015, Smith 2009, U.S. Fish & Wildlife Service 2018).

There are management techniques in place to facilitate the recovery of the species, but most procedures depend on thorough information about many different aspects of massasauga ecology (Smith 2009). Understanding the distribution and current status of populations is crucial for this species (Davy *et al.* 2015, U.S. Fish & Wildlife Service 2016). Despite efforts to manage massasauga habitats and increase population numbers, the spatial requirements of the snakes are still largely unknown (Durbian *et al.* 2008). Traditionally, data collection for this species is conducted using visual encounter surveys in places where the species has been historically sited. Several challenges are associated with this type of assessment. First, survey locations are often far apart from one another. Secondly, massasaugas are notoriously difficult to observe due to their cryptic nature and their activity periods are often limited to particular seasons or times of day. Finally, their densities, even in ideal habitats, are relatively low. Because of these difficulties, considerable person hours are needed to get reliable measures of occurrence which can be costly in time and funding (Hunter *et al.* 2015).

Not only are massasaugas rare and difficult to detect in the field, but they also have a behavior that should make the collection of eDNA comparatively simple and reliable due to their overwintering habits (Cushman *et al.* 2013, Goldberg *et al.* 2015, Smith 2009). Massasaugas, being ectotherms, can spend up to half of their lives overwintering (Smith 2009). Eastern Massasaugas overwinter in crayfish burrows that hold water below the freeze line of the soil and spend most of their winters submerged. Staying in groundwater below the frost line protects the snakes from freezing, drying out, and insulates them from temperature changes. They choose sites that are close to the water line but are still safe from flooding. Crayfish dig burrows below the frost line which hold and maintain a reservoir of water for crayfish survival, making their abandoned burrows a preferred place to overwinter (Goldberg *et al.* 2015, Grow and Merchant 1980, Smith 2009).

Studies have shown that eDNA can persist in nature for at least 14 and up to 60 days (Goldberg *et al.* 2015, Santas *et al.* 2013), and cold water in dark conditions like as is found in crayfish burrows, should further prevent its natural degradation. Moreover, while many eDNA analyses are made in large bodies of water (streams, rivers, or lakes) a crayfish burrow is

relatively small, and should aid in the concentration of cellular materials of the resident snakes (Santas *et al.* 2013).

While many recent studies observe the efficacy of eDNA surveillance for target species, few have observed the environmental factors that contribute to eDNA degradation. Studies indicate that DNA degrades more quickly when exposed to higher temperatures, higher levels of oxygen, and more acidic conditions (Barnes *et al.* 2014, Corinaldesi *et al.*2008, Matsuo *et al.* 1995, Poté *et al.* 2009, Zhao and Fleet 2003).

Beyond the potential utility of crayfish burrows for eDNA detection, the overwintering ecology of snakes in these habitats is incompletely understood. Correctly identifying and protecting overwintering sites can facilitate the management and recovery of *S. catenatus* (Smith 2009). Smith (2009) and Sage (2005) observed that massasaugas prefer overwintering sites that are ≥100 meters from the edge of a water source's edge, where there is more nearby cover including live trees or logs and contain higher amounts of sand. Additionally, observations were made that chosen overwintering sites had a lower pH and anoxic conditions. As long as an individual snake could keep its head above water, the oxygen content of the water should not be a factor.

By varying sampling areas, I anticipate finding higher rates of positive eDNA detections in those sites that have environmental measurements more often preferred by massasaugas including more cover in the form of trees, logs, and shrubs. Further, if positive detections are found, I anticipate those sites that are cooler, high in oxygen, and more alkaline should have higher starting amounts of eDNA.

My research has two objectives. First, I describe the development and testing of an eDNA assay that identifies *S. catenatus* in environmental samples from crayfish burrows. My approach includes the identification of a minor groove binding assay implemented in qPCR that is specific to Eastern Massasauga. This assay is verified using tissue extracted DNA, water samples from captive snakes, and analysis of environmental samples from crayfish burrows collected across a gradient of overwintering use by snakes. Second, I correlate abiotic characteristics of crayfish burrows with eDNA detection and overwintering site selection of massasaugas. The results of this analysis will aid in the selection of sample locations for *S. catenatus* eDNA.

METHODS

Sample Collection

Laboratory-Housed Massasauga

Environmental DNA sampling relies on target species being recently present in a location. As a way to measure relative amount of eDNA in a sample over time, massasaugas were temporarily housed in a lab to provide environmental water samples used to assess DNA degradation. Specimens that were being held for implantation of radio-transmitters for use in another study on spatial ecology were brought in from Grayling, Michigan.

Clear, plastic, Sterilite® containers (38 centimeters x 29.3 centimeters x 15.25 centimeters) with locking lids were obtained, cleaned with 10% bleach solution, rinsed with distilled water, and dried thoroughly. Holes were drilled in the lids to allow for air flow. Approximately 500 mL of autoclaved, double distilled water was added to the containers 24 hours prior to the addition of snakes. Three different snakes were left in individual containers with water for approximately 24 hours at room temperature (Figure 1).

Using the same methods as described below for field samples, 50 mL of mixed water was collected in conical tubes for each sampling and was filtered and prepared for extraction (see below for filtration details). Prior to each sample collection, water was gently mixed to help disperse any solid particles and potential DNA. No new water was added to the containers after sampling. The first control sample was taken from each container prior to the addition of snakes to serve as a negative control. Immediately after removal, a sample was taken to represent zero days after snake removal from each container. Other samples were taken at 1, 2, 7, 14, 28, and 56 days after snake removal. In between sampling days, the containers were covered with plastic wrap to prevent evaporation and kept in a dark closet at room temperature.

Field Sites

Field sampling took place at Pierce Cedar Creek Institute (PCCI), a 742 acre nature preserve in south central Michigan near Hastings (http://www.cedarcreekinstitute.org). Permits from the state of Michigan were obtained for all field sampling. This location was chosen for

several reasons. While Eastern Massasaugas are endangered in Indiana and populations are very sparse, Michigan is a relative stronghold for the species. PCCI has a robust population of *S. catenatus* that has been monitored since 2004 (Bailey *et al.* 2011, Bailey *et al.* 2012), and has habitats that are classified as prairie fen and wet meadow, both of which are wetlands conducive to fostering massasauga populations (Bartman and Kudla 2014). Specific areas on the property are known as overwintering sites and allow for on-site comparison of areas with varying degrees of snake activity. Four different sites were selected for sampling at PCCI (Figure 2). Additionally, the Devil Crayfish (*Cambarus diogenes*), Calico Crayfish (*Orconectes immunis*), Digger Crayfish (*Fallicambarus fodiens*), and the White River Crawfish (*Procambarus acutus*) are all examples of species of crayfish found in Michigan that demonstrate primary or secondary burrowing behavior (Taylor *et al.* 2015) and can contribute to potential sampling sites at PCCI where field work and sample collections occurred.

Four different areas at PCCI were chosen for comparison of expected snake abundance. The first site (Area 1) is divided by a road. Area 1 is an open-wetland prairie fen, a type of wetland located on peat and dominated mainly by grasses and sedges with occasional shrubs. The portion of Area 1 to the north of the road has a soil composition that is predominately loamy sand. On the south side of the road, the first 18 centimeters of soil are comprised of loam and then change to clay loam. This type of habitat is conducive to thriving massasauga populations, and massasaugas have been regularly spotted and monitored in this area since 2004 (Bartman and Kudla 2014, Bradke 2017, Howell 2014, Soil Survey Staff 2018).

Area 2 is a forested upland with a mix of young oak and hardwood species including red oak (*Quercus rubra*), black oak (*Q. velutina*), white ash (*Fraxinus americana*), bitternut hickory (*Carya cordiformis*), black cherry (*Prunus serotine*), red maple (*Acer rubrum*), sugar maple (*A. saccharum*), American elm (*Ulmus americana*), and American beech (*Fagus grandifolia*). Massasaugas have also been observed in this area during spring emergence albeit with slightly less frequency than Area 1. As on the southern side in Area 1, Area 2 is predominately comprised of loamy sand (Bradke 2017, Howell 2014, Soil Survey Staff 2018).

The third site (Area 3) sampled at PCCI is situated on the edge of a prairie fen, butting up to a forested tamarack swamp. This area does not have heavy foot traffic from the public compared to other sites. Massasaugas have been observed venturing into this area, but due to the extremely wet characteristics of the swamp, it is likely too wet for overwintering. Most of the

area is comprised of muck down to two meters and is very poorly drained (Bradke 2017, Howell 2014, Soil Survey Staff 2018).

The final area sampled for eDNA was along the southern edge of Aurohn Lake (Area 4), a relatively recent addition to the institute that transitions from a reconstructed native grass and forb prairie to a forested wetland similar to Area 2. The soil is comprised predominately of loamy sand. Snakes have not been seen in this area since monitoring efforts for massasaugas started in 2004 (Bradke 2017, Howell 2014, Soil Survey Staff 2018).

It has been observed that massasaugas start moving towards their overwintering sites in the middle of September and begin using their burrows by the first week of October. By late March, the snakes start to come to the surface of their hibernacula, are fully emerged in April, and start to disperse by mid-May (Smith 2009). Environmental samples were taken May 5, 9, 11, and 13, 2016 to coincide with dispersal from overwintering sites. Sampling at this time was aimed at maximizing eDNA detections due to massasaugas being recently present in burrows over a period of months in cool microenvironments. These conditions were expected to limit DNA degradation.

Crayfish Burrow Sampling

Water samples were collected from 15 randomly selected crayfish burrows in each of the four aforementioned areas. Because DNA contamination is a concern in any PCR-based protocol (Taberlet *et al.* 1996, Thomesen and Willerslev 2015), prior to entering the field on any of the sampling days, 50 mL of distilled water was placed in a conical tube and then into the sample cooler (previously bleached and rinsed) to provide a negative "collection" control. These controls were subsequently treated the same as all other environmental samples. To eliminate cross-contamination between sampled burrows, a new set of gloves and new tubing (see below) was used at each burrow. Additionally, all sites were photographed and marked with flagging and GPS to be used in analysis.

Water was drawn from a burrow using a hand-made apparatus. A pre-packaged, sterile 60 mL syringe was connected to a one-meter section of new vinyl tubing 0.32 centimeters in diameter and 0.16 centimeters in thickness. Tubing was connected to the syringe using a pre-packaged, sterile catheter adapter (Figure 3). The tubing was gently lowered into the burrow entrance and the syringe was pulled to extract water. The collected water was deposited into a

50 mL, labeled, pre-packaged, sterile conical tube, wiped down on the outside with an alcohol swab, and then placed on ice in a cooler during transportation to the laboratory. Samples were in the cooler for approximately three hours and then refrigerated in the laboratory overnight to preserve eDNA until filtration the following day (Deiner *et al.* 2015). Water collection was the first task completed at each site to help prevent cross-contamination of samples. Furthermore, care was given to not collapse the burrow and to minimize disturbance. Used gloves and sampling equipment were immediately discarded so that they could not be used at a following burrow.

A range of habitat characteristics of the burrow were also collected. First, additional water was drawn from the burrow and placed in a new plastic cup for measurement of pH, temperature (°C), and dissolved oxygen (mg/L) using a YSI® Pro 1020 meter. Sufficient water was added to the cup to ensure the meter probe was submerged for accurate readings. Before moving to the next burrow, the meter was rinsed with distilled water and used cups were discarded. Second, a tape measure was used to measure the diameter of the burrow at its widest point (cm), and the distances to the nearest burrow, tree, shrub, open water, and log (m). If any of the structures were farther than 20 meters away, it was recorded as >20 m. A tree was defined by stem diameter ≥5 cm at breast height (DBH) even if a species was technically a shrub such as the invasive autumn olive (*Elaeagnus umbellata*). ArcMap 10.5.1 was used to determine the distance (m) to the nearest open road or trail using straight transects from the uploaded GPS coordinates of the burrow to the edge of the trail, road, or path. The shortest transect that did not cross a body of water was selected. Third, a core sampler was used to take a soil sample immediately adjacent to burrow openings. The pulled core was used in conjunction with a ribbon test and the Web Soil Survey from the National Resources Conservation Services (NRCS) (2017) to confirm soil type in an area.

Filtration

It was imperative that eDNA samples were kept separate from extracted DNA samples and PCR products to prevent potential contamination. Therefore, filtration and extraction of field samples took place in a lab separate from labs used for the extraction of DNA from snake tissue.

Preserving the eDNA until extraction was an important step. Filtration is the primary method currently employed for acquiring eDNA (Renshaw *et al.* 2015). To prevent breakdown of DNA, samples were kept on ice until reaching the lab and were then placed at 4°C until filtration. To further prevent DNA degradation, water samples were filtered within 24 hours. During the filtration process, new gloves were used for each sample. The filtration area was wiped down with 10% bleach and covered with new bench paper prior to the start of each filtration process. Forceps used for the filter paper were soaked in 10% bleach, rinsed, and dried between samples.

Water collected from the burrows was turbid with suspended sediments. Consequently, prior to filtration for each sample, the samples were spun in a pre-cooled centrifuge at 4°C for ten minutes at 3,000 RPM. This was done to separate the larger sediment particles from the water sample and to prevent clogging the filter. The sediment and filtered water samples were retained to ascertain which sample type was better suited for eDNA detection.

After centrifugation, the water portion was carefully decanted into a pre-packaged, sterile, disposable 0.2 µm nitrocellulose filter unit which was attached to a peristaltic pump following Hunter *et al.* (2015). The vacuum pump remained on until all water was filtered. Using the bleached forceps, the nitrocellulose filter was immediately placed into a clean, 1.7 mL tube with 1,450 µL of cationic detergent cetyltrimethylammonium bromide (CTAB) lysis buffer solution with added polyvinylpyrrolidone (PVP). The CTAB has shown to maximize lysis and to preserve eDNA at room temperature for up to two weeks. Polyvinylpyrrolidone was used to absorb polyphenols found in plants that may act as an inhibitor later during PCR (Renshaw *et al.* 2014). Autoclaved, double distilled water was added to remaining sediment (after decanting the burrow water) to bring the volume up to 5 mL and then CTAB buffer (with PVP) was added to bring the final volume up to 15 mL.

Both the filter papers and the sediment samples were allowed to breakdown in the CTAB buffer for 14 days at room temperature. The sediment samples tubes were regularly inverted by hand to keep the CTAB mixed throughout the sediment. After two weeks, DNA extraction was conducted. Water samples collected from housed snakes (described above) were treated in the same way except that water was filtered and CTAB was added the same day, and there was no associated sediment to extract.

DNA Extraction from Environmental Samples

Protocols for DNA extraction from water and sediment water samples mimicked those found in Deiner *et al.* (2015), Renshaw *et al.* (2014), Turner *et al.* (2014), and Turner *et al.* (2015). All extractions were performed inside a hood that had been cleaned with 10% bleach and covered in bench paper at the start of extractions. DNA concentrations from all extractions were measured with a Qubit® 3.0 machine using double stranded, High Sensitivity (ds HS) reagents (Qubit®).

Water Samples

At the beginning of extractions, the outsides of the tubes containing filter paper samples were wiped down with 10% bleach solution. The 1,450 µL filter samples were placed in a water bath at 60°C for ten minutes. Subsequently, 1,450 µL of Sevag solution (24:1 chloroform-isoamyl alcohol) was added to each sample and vortexed at a low speed for five minutes. Samples were then centrifuged at 14,000 RPM for 15 minutes at room temperature. The aqueous phase was then pipetted into a new, bleached 1.7 mL microcentrifuge tube. Care was taken to only extract the top layer and not the intermediate nor bottom layers. An equal amount of ice-cold 100% isopropanol was added to the sample followed by a half volume of ice-cold 5M NaCl. The samples were then chilled in a freezer at -20°C overnight.

The following day, the supernatant was decanted after being centrifuged at 14,000 RPM for 15 minutes at room temperature. Taking care to wash down the sides of the tube, 300 µL of 70% ethanol was added to the tube and then centrifuged at 14,000 RPM for five minutes at room temperature. The ethanol was decanted, and the step was repeated. After the second centrifugation, the ethanol was poured off and the pellet allowed to dry completely. The pellet was then re-suspended in 100 µL of LoTE buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA) and heated for ten minutes in a water bath at 55°C. The tube was then gently vortexed and centrifuged at 14,000 RPM for five minutes at room temperature to completely re-dissolve the pellet. All liquid was then transferred to a new 1.7 mL centrifuge tube and stored at -80°C until qPCR.

Sediment Samples

Extraction of DNA from sediment samples followed the same protocol as for water filter samples; however, the amount of Sevag solution added to the sample was 15 mL and centrifugations occurred at 8,000 RPM.

For the second day, centrifugations occurred at 8,000 RPM, and after the supernatant was carefully decanted, 2 mL of 70% ethanol was added to the samples. The samples were then centrifuged at 8,000 RPM for two minutes at room temperature after which the ethanol was poured off and the pellet was allowed to dry entirely. The wash step was not repeated for sediment samples. After drying, 1 mL of LoTE buffer was added to re-suspend the pellet. The remaining steps were the same as for water filter samples, except for centrifuging at 8,000 RPM.

Quantitative PCR

All extracted environmental samples were subject to the same real-time quantitative polymerase chain reaction (qPCR) protocol. Technical qPCR replicates from each sample from water and sediment were run in triplicate on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). The final volume for each reaction was 20 µL with the following composition: 10 µL TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems™), 2 µL TaqMan™ Exogenous Internal Positive Control (EXO IPC) Mix, 0.4 µL EXO IPC DNA, 0.2 µL of 5µM probe, 0.4 µL each of 10 µM forward and reverse primers, and 4.6 µL of molecular-grade water. 18 µL of this working master mix was combined with 2 µL of extracted environmental DNA in a qPCR low-profile, skirted plate and pipet mixed. Each plate was covered with a clear plate film and briefly centrifuged at 8,000 RPM to help concentrate the mixture to the bottom of the plate wells and to remove air bubbles that could potentially interfere with the qPCR fluorophore reading. Thermocycling conditions were: an initial denaturation step of ten minutes at 95°C followed by 50 cycles of 95°C for 30 seconds of denaturation, and an annealing phase at 62.5°C for 30 seconds. The software program allowed for the reading of both FAM and VIC fluorophores (FAM was for the designed targeted probe, and VIC was for the IPC).

The internal positive control (IPC) was included to help detect false negatives due to inhibitors from particles in the environment such as plant debris that can hinder a PCR reaction.

If any of the three replicates for a sample did not register a positive response for the IPC, samples were cleaned using DNA Clean & Concentrator™ 5 from Zymo Research following manufacturer protocol and retested. All plates contained a negative, non-target control sample to test for contamination.

As only massasauga DNA was targeted for extraction from environmental samples, quantification was used to try to determine initial concentrations. This was achieved by comparison of 1:10 serial dilutions of purified PCR product from a custom-ordered ultramer (Integrated DNA Technologies®) comprised of the targeted 152 bp segment. The dilution series contained $1x10^8$, $1x10^6$, $1x10^4$, $1x10^2$, and $1x10^0$ copies of target DNA/μL. This allowed for the determination of the minimum number of copies of target DNA required for a positive detection and provided a standard for designating a sample as positive for the presence of massasauga eDNA.

Primer and Probe Design for Quantitative PCR

Because an eDNA assay had not been developed for the Eastern Massasauga at the start of the project, it was necessary to test novel molecular primer and probe combinations for use in a real-time quantitative polymerase chain reaction (qPCR). TaqMan™ primers and probes were developed from the mitochondrial cytochrome oxidase subunit I (COI) gene, a growing standard for the barcoding of vertebrate taxa due to its low within, but high among, species variation (Hebert *et al.* 2003, Ratnasingham and Hebert 2007). TaqMan™ hydrolysis probes are designed to fit within a segment of DNA that is defined by specific primers. The probe consisted of a fluorescent dye and a quencher. With each qPCR cycle, the dye and quencher are separated and illumination of a dye at a specific wave length is released and measured by a qPCR machine. This measured fluorescence signal allows a user to both add specificity to a target DNA sequence and to quantify amounts of target DNA between samples (Nagy *et al.* 2017).

Cytochrome oxidase subunit I sequences of Eastern Massasaugas and other Michigan snake species were downloaded from the Barcode of Life© (BOLD) and GenBank® databases and aligned using MEGA7 to create a consensus sequence (Appendix). Primers were selected on this sequence using Primer-BLAST (Ye *et al.* 2012) to yield a 75 - 200 bp (base pair) product, a target size that increases the probability of detecting degraded DNA from an environmental site. Primers were chosen only if they annealed to sites that were not polymorphic within Eastern

Massasaugas, had melting temperature (T_m) between 57 and 63°C with a maximum difference of 3°C between primers, a near random mix of nucleotides with a CG composition of 50-60%, and ended in a C or G residue. A final condition was that primers had more than one mismatch with other Michigan snake species such that any primers that returned products homologous to other snake species were excluded from testing (Goldberg *et al.* 2015).

Based on specificity and initial testing on co-occurring species (see below), the forward and reverse primers were each 21 base pairs in length and amplified a 152 base pair product. The forward primer was two base pairs different from other snake species while the reverse primer differed by one base pair. The forward primer was 5′ – CCC CTT TTC GTG TGG TCT GTA – 3′ (T_m= 56.9˚C) and the reverse primer was 5′ – TGA AAT AGA ACT GGG TCA CCG – 3′ (T_m= 54.5˚C). Following the recommendations from Prediger (2016), the probe was designed to be located near the forward primer without overlapping. It was intended to be ~20 bp in length to fit within the 152 bp segment that was amplified from the primers. The T_m of the probe was also targeted to be 6-8°C higher than both primers, and the annealing temperature (T_a) no more than 5°C lower than either primer. The probe was designed to have a CG concentration between 35-65% and a G at the end was not avoided so as not to quench the -5' fluorophore. The 24 bp probe used had the sequence 5′ – ACT TCT AGC ACT ACC CGT GCT TGC – 3′ (T_m=61.0˚C) ordered from Integrated DNA Technologies® which utilized a FAM dye with a ZEN quencher.

To check for possible complimentary and secondary structures that may be formed between either primer or probe individually or with each other, both primers and probe were checked using the OligoAnalyzer Tool (Integrated DNA Technologies). A primer or probe was accepted for use if the ΔG value was more positive than -9.0 kcal/mole for any potentially forming heterodimers, homodimers, or hairpin formations.

The specificity of the primer and probe combination was tested against likely or potential co-occurring snake species from the study site using touchdown PCR (Figure 4). DNA from Kirtland's Snake (*Clonophis kirtlandii*), Dekay's Brownsake (*Storeria dekayi*), Red-bellied Snake (*S. occipitomaculata*), Eastern Gartersnake (*Thamnophis sauritus*), Black Ratsnake (*Pantherophis obsoletus*), and Eastern Hog-nosed Snake (*Heterodon platirhinos*), as well as Eastern Massasauga (*Sistrurus catenatus*), was extracted using Qiagen DNeasy® Blood and Tissue Kit following the manufacturer's protocol and diluted to between approximately 12.5 and

25 ng/µL. Extracted DNA was run on PCR machine using touchdown PCR as opposed to traditional PCR to increase specificity of the desired product. The program was set up for a 25 µL reaction. The master mix consisted of 12.5 µL of 1X Qiagen® Mulitplex PCR Master Mix, 0.5 µL of 10 µM of both the forward and reverse primers, and 9.5 µL of autoclaved, double distilled H2O. 23 µL of the working master mix was pipetted into PCR strips and 2 µL of extracted DNA from each snake species was added.

The steps of the touchdown PCR program were as follows: an initial denaturation step at 94°C for three minutes, followed by cycles of 45 second denaturation steps at 94°C, and annealing cycles of 30 seconds starting at 65°C and going down to 50°C. Each cycle contains a primer extension step at 72°C for one minute. There was a final extension at 72°C for ten minutes and then the samples are held at 4°C.

PCR product was mixed with 5X Green GoTaq® Flexi Buffer and run on a 2% agarose gel with ethidium bromide alongside a negative water control and Thermo Scientific™ GeneRuler 100 bp DNA Ladder mixed with Thermo Scientific 6x DNA Loading Dye. Once specificity was confirmed with PCR, it was again confirmed using qPCR (see below).

qPCR Product Sequencing

A subset of positive samples was sent to Genewiz® for Sanger sequencing. Because the designed probe can interfere with sequencing methods and IPC reagents could also interfere with sequencing, the samples were run through qPCR with the same concentrations of reagents as described above; however, the IPC reagents and probe were excluded. Additionally, the qPCR volume was adjusted to run a 25µL reaction.

Real-time quantitative PCR products were first run on a 2% agarose gel along with a ladder and negative control to test for contamination. Afterward, qPCR products were analyzed on a Qubit® 3.0 machine using ds HS reagents to determine DNA concentration. Samples were diluted to contain 10-20 ng of DNA in 10 µL, placed in PCR tubes, and sent along with 5 µM forward primer mixture for sequencing. Sequences that were sent back were entered back into the initial alignment to confirm that the sequences fell within the desired 152 bp product. These sequences were also searched in GenBank®.

Statistical Analysis

Burrow Characteristics

Using R© and packages "mvnormtest" and "dplyr", the average values of pH, dissolved oxygen, and temperature, and distances to various types of cover were compared via MANOVA to determine if there were differences in the mean values among sites. Further, ANOVA analysis was used to determine which environmental measurements were statistically significant, and finally, a multiple comparison using TukeyHSD was used to see which sites were most similar to each other.

Probability of Occurrence and Detection

For cryptic species, such as Eastern Massasaugas, individuals may be missed during surveys even though they may be present. To account for these errors in detection, occupancy models can be used to estimate the occurrence of a species. Utilizing the R package, EDNAOCCUPANCY, the qPCR detection data along with environmental measurements taken at the various burrows was able to be fitted to Bayesian, multiscale occupancy models following Dorazio and Erikson (2017). With this package, positive detections could be analyzed along with or without covariate measures of the environment such as pH, dissolved oxygen, and temperature. This program and type of modelling are appropriate for sampling that has three nested levels of sampling such as the samples taken at PCCI.

The R program, EDNAOCCUPANCY was used to try to determine the parameters of the probability of occurrence of massasauga eDNA in any given area, the probability of detection of massasauga eDNA in a burrow given that there was a positive detection of eDNA in the area, and the probability of eDNA detection in a qPCR replicate given that target eDNA was in a burrow using the hierarchal occupancy model with the following levels (Dorazio and Erikson 2017):

$$Z_i \sim \text{Bernoulli}\ (\psi)$$

Where Z_i is the presence or absence of eDNA in an area (i^{th} location, Areas 1-4), and ψ is a function of parameters and regressors.

$$A_{ij}|Z_i = z_i \sim \text{Bernoulli}\ (z_i\Theta_{ij})$$

Where A_{ij} is the presence or absence of eDNA in a burrow (sample) and Θ_{ij} is the conditional probability that eDNA is present in the j^{th} sample, and z_i is the realized probability of Z_i.

$$Y_{ijk}|A_{ij} = \alpha_{ij} \sim \text{Bernoulli}\ (\alpha_{ij}\rho_{ijk})$$

Where Y_{ijk} is the presence or absence of eDNA in a qPCR technical replicate and ρ_{ijk} is the conditional probability that eDNA is present in the k^{th} replicate.

This model was fitted to Eastern Massasauga eDNA using Bayesian methods of computation including a Markov Chain Monte Carlo (MCMC) method using 20,000 iterations to ensure that the chains converged to a stationary distribution. With this information, the probability of detection in an area, burrow, or qPCR replicate could be determined and examined to observe which factors influence the positive detection of massasauga eDNA. The posterior-predictive loss criterion (PPLC) and widely applicable information criterion (WAIC) were used to determine which model best estimated eDNA detection within a burrow (Dorazio and Erikson 2017).

A model was fitted for both filter water and sediment samples using: the four sample areas, burrow samples within an area, and three qPCR replicates for each burrow. Covariates of eDNA occurrence in a burrow sample included: pH, temperature (°C), dissolved oxygen (DO; mg/L), and DNA concentration (ng/µL). Using summaries of the posterior distributions of each covariate parameter, the mean values of the probabilities of eDNA detection in a burrow were compared.

Figure 1. *Sistrurus catenatus* housed in laboratory in container with ~ 500 mL autoclaved, double distilled water.

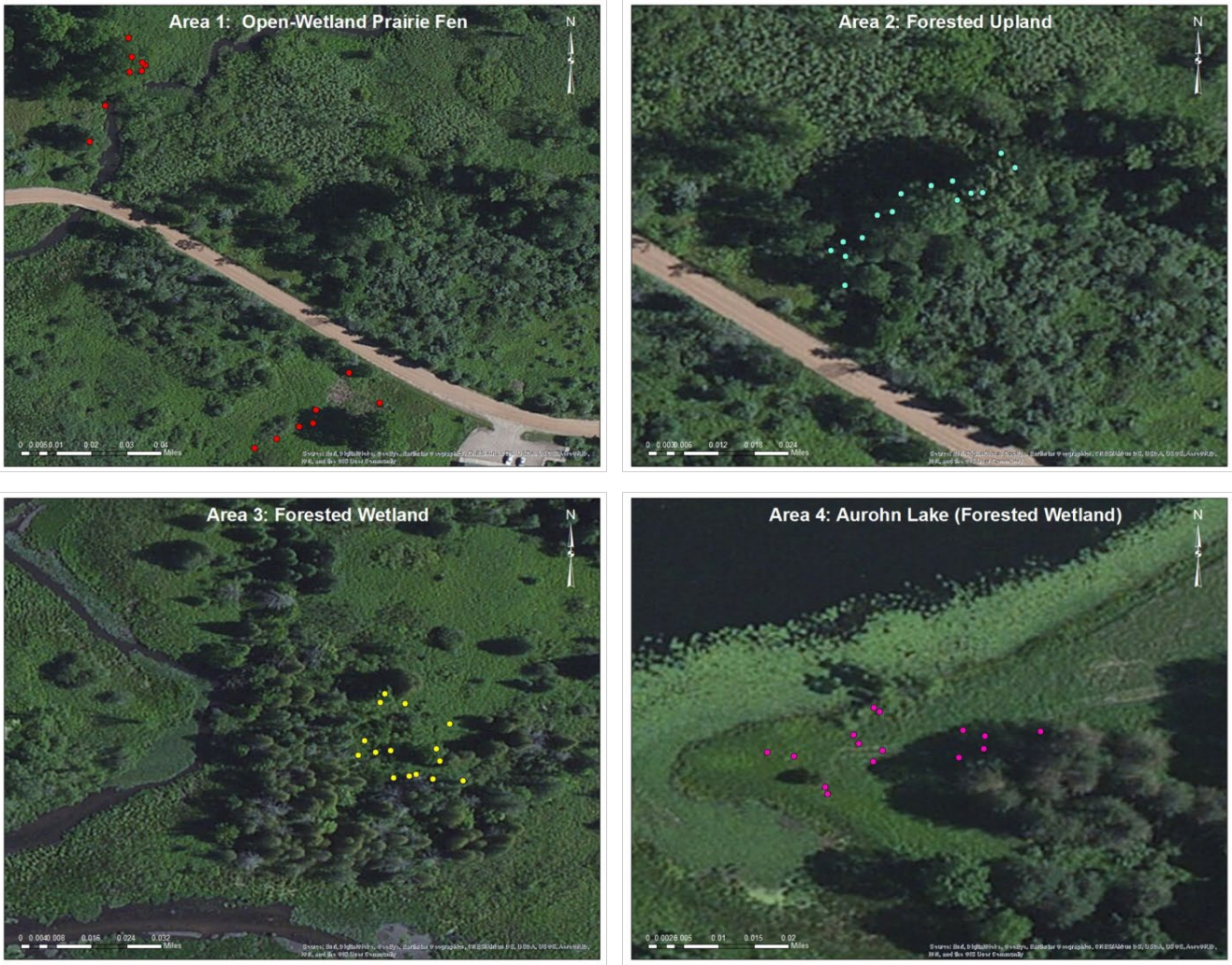

Figure 2. Aerial map of sampled sites at Pierce Cedar Creek Institute and sampled burrow locations.

Figure 3. Hand pump constructed from tubing, a catheter adapter, and a syringe inserted into crayfish burrow to draw water from crayfish burrows.

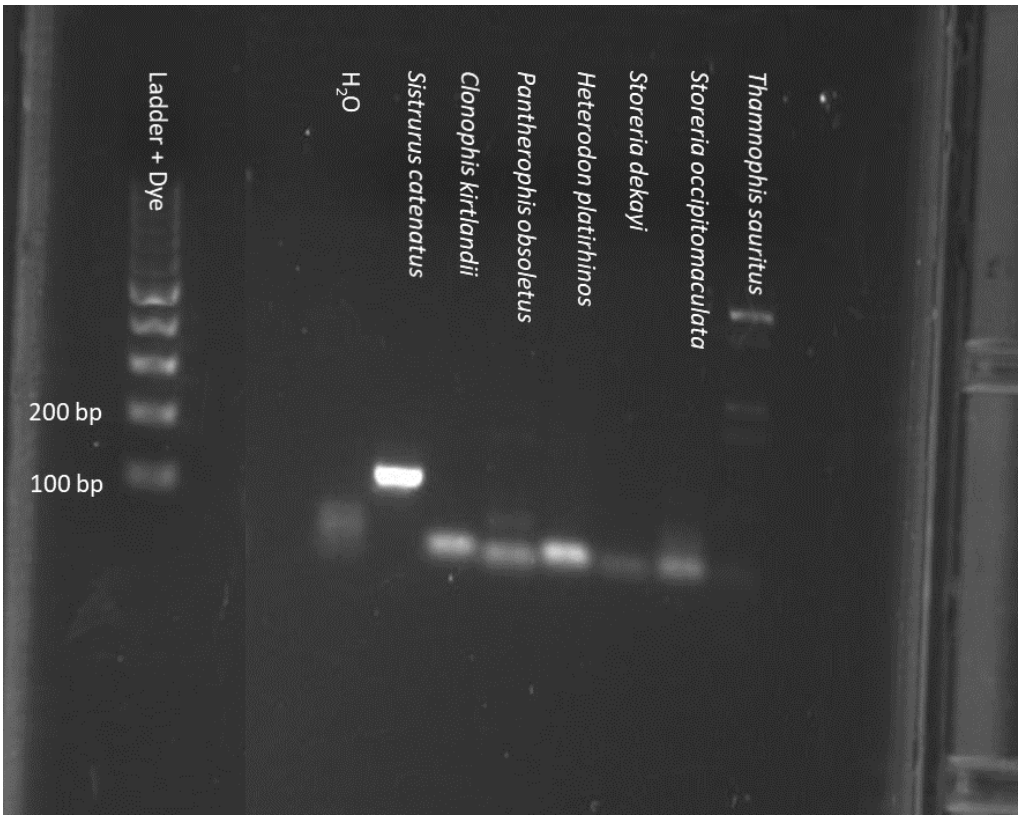

Figure 4. 2% agarose gel of PCR product from Eastern Massasauga and possible co-occurring snake species in southern Michigan. Product was run against 100 bp ladder. Primers targeted 152 bp segment of *S. catenatus* mitochondrial COI gene. The brightest band indicates target sequence. Faint product in other lanes is believed to be the result of primer-dimer formation.

RESULTS

Burrow Habitat Analysis

MANOVA showed that there were statistically significant differences among sites in habitat variables associated with crayfish burrows (Pillai = $P<0.05$). ANOVA results indicated that all variables were significantly different between sites (Table 1). TukeyHSD analyses were used to determine which sites were different from one another for each habitat variable (Table 2).

Overall, Areas 1 and 2 were consistently the most different from each other although both sites were most similar in historical reports of Eastern Massasaugas overwintering sites. Area 1 was more acidic, warmer, and had higher amounts of dissolved oxygen in burrows than burrows in Area 2. The only variable in which Areas 1 and 2 remained similar was in the distances from burrows to the nearest path. Both sites were much closer to various pathways than other sites.

Areas 1 and Area 4 were highly similar for most habitat measurements. Mean dissolved oxygen was higher in Areas 1 and 4 and distances to various types of cover were farther from the nearest tree, log, and water compared to the other sites. Areas 1 and 4 were most different in the distances to the nearest path. Area 1 was closer to pathways than Area 4.

Temperature differences were largest between Areas 1 and 2 followed by Areas 1 and 3. For both comparisons, Area 1 had higher temperatures than Areas 2 and 3.

Area 2 had lower amounts of dissolved oxygen than Area 4 and this difference was the largest. Less drastic comparisons of amounts of dissolved oxygen were between Areas 3 and 4, Areas 1 and 2, and Areas 1 and 3. Overall, Areas 2 and 3 were lower in amounts of dissolved oxygen compared to Areas 1 and 4.

Regarding physical dimensions and habitat measurements, Areas 1 and 3 were most different in burrow diameter followed by Areas 1 and 2. Burrow diameter average was largest in Area 1. Distances to the nearest burrow were greatest in Area 1 and lowest in Area 2. The average distance from a burrow entrance to the nearest tree was farthest in Areas 1 and 4. The closest shrubs to burrow openings were found in Area 2 and the farthest shrubs were in Area 1. The distance to the nearest open water source was much greater in Areas 2 and 3 compared to Areas 1 and 4. Nearest logs to burrow openings were found in Areas 2 and 3. Finally, Areas 1 and 2 were much closer to a pathway than Areas 3 and 4.

Laboratory-Housed Massasauga Samples

Environmental DNA was positively detected in 15 of the 21 post-incubation samples taken from the lab. A positive detection was recorded if the estimated starting quantity was ≥1 copy of target DNA/µL. The average quantification cycle (C_q) values ranged from 19.8 to 47.0 cycles and average starting quantities (SQ) of eDNA estimated from standard curves and qPCR ranged from 2.3 to $9.9x10^5$ copies of target DNA/µL and were plotted against days since snake removal (Figure 5). All negative control samples had no amplification of DNA, and the positive controls from massasauga tissue extractions amplified as expected. The exogenous IPC spiked into the qPCRs of all the laboratory-housed massasauga water samples amplified indicating that none of the laboratory samples were subject to inhibition.

Specimen Eastern Massasauga 1 (EM1) had the highest starting quantity of DNA in a sample on day zero compared to EM2 and 3. The samples from this snake depict a decline in starting amount of eDNA from days zero to seven. After day seven, the amount of starting eDNA in this sample increase on 14 and 28 days after snake removal and decrease again by day 56. EM1 defecated in its water, and it was noted that fecal matter dissolved by day seven.

Observations of EM2 were similar to those of EM1. This snake also defecated in the water and fecal matter was noted to also have dissolved by day seven. The amount of eDNA detected in this water sample increase from days zero to one, followed by a decrease from day one to day two, with a spike in eDNA from days seven to 28 and then a decline from day 28 to day 56.

The third snake, EM3, did not have fecal matter in its container. After day 2, eDNA could not be detected.

Species-Specific qPCR Marker Design

From the aligned sequences, unique primers and a probe were designed using the COI gene sequence. When tested against other conspecific snake species using standard PCR and the following qPCR, no amplification of non-target species was detected.

Using serial dilutions created from a synthesized ultramer, the lower limit number of copies detected by qPCR could be determined. Triplicates of standard dilutions of $1x10^0$, $1x10^2$, $1x10^4$, $1x10^6$, $1x10^8$ copies of target DNA/µL created from the diluted ultramer were used to

create a standard curve with which to compare starting target DNA amounts in samples. From these standards, Cq values were plotted against the log starting quantities of the standard curve dilution series. Using the regressions determined from ten plates of three replicates of each of the standard dilutions, it was determined that the limit of detection (LOD) for this study was reliably 100 copies of target DNA/µL. While there were detections at lower concentrations, all standard curves on all plates amplified 100 copies of target DNA/µL.

Of the subset of samples sent for sequencing, ten of the 12 sequences aligned with the target sequence in the expected region. The other two samples had failed sequencing reactions. Additionally, for re-confirmation of specificity, all sequenced products were run in BLAST and returned the target sequence for *Sistrurus catenatus* as the top species match with the identity ranging from 97 to 100%. Additionally, the Expect value (E value) ranged from $3x10^{-42}$ to $1x10^{-50}$. The next five top species matches include Black-tailed Rattlesnake (*Crotalus molossus*), Timber Rattlesnake (*Crotalus horridus*), Brahm's Dwarfgoby (*Eviota brahmi*), Purple Dwarfgoby (*Eviota dorsopurpurea*), and Yellow-faced Pocket Gopher (*Cratogeomys castanops*) with the top identities ranging from 87 to 91% and E value ranging from $2x10^{-8}$ to $3x10^{-32}$. The Black-tailed and Timber Rattlesnakes are not likely found in Michigan, being distributed in the south western United States and in the eastern United States respectively. Both dwarfgobies are found in the southwestern Pacific Ocean near Papua New Guinea and also not likely to be found in Michigan. Finally, the Yellow-faced Pocket Gopher is also found in the southwestern United States and not a likely inhabitant of southern Michigan (Desai 2004, Falk 2002, Greenfield and Randall 2011, Greenfield and Tornabene 2014, Neufeld 2017).

eDNA in Water Samples

As with laboratory samples, a positive detection was recorded if the estimated starting quantity was ≥1 copy of target DNA/µL. In Area 1, four burrows had a positive detection (Table 3). Of these four burrows, the positive detection was only found in one of the three qPCR replicates. The C_q values ranged from 42.1 to 45.5 cycles and had an estimated starting quantity from 12.7 to 74.7 copies of target DNA/µL. Area 2 had one positive detection in one replicate of a burrow. This sample had a C_q value of 44.5 and an estimated starting quantity of 69.8 copies of target DNA/µL. Lack of a fluorescent reading in two of the water samples from burrows at Area 3 indicated that there was inhibition of the sample. These samples were cleaned and re-ran.

A positive detection was found in one of three replicates at one burrow and in all three replicates of another. The C_q values ranged from 38.3 to 39.6 with a range of starting quantity from 5.2 to 10.0 copies of target DNA/µL. In Area 4, there were no positive detections of eDNA in any of the water samples taken from burrows. There was an overall detection in filter water samples in seven of 60 burrows and nine out of 180 replicates. After inhibited samples were cleaned, all samples had amplification of IPC and all negative control samples did not have amplification. All replicates of the positive control amplified.

eDNA in Sediment Samples

Again, positive detections were recorded at an estimated starting quantity ≥1 copy of target DNA/µL. There were no positive detections in any replicates from sediment samples taken from Area 1 (Table 4). In Area 2, two sediment samples from two burrows indicated inhibition. When cleaned and re-ran, positive detections were found in three total replicates from two burrows in Area 2. These sample had a C_q value range of 37.1 to 38.8 cycles and an estimated starting quantity ranging from 7.3 to 22.6 copies of target DNA/µL. Area 3 had one positive detection in one replicate of one burrow. This had a C_q of 43.3 and an estimated starting quantity of 136.3 copies of target DNA/µL. Area 4 yielded a positive in four different replicates of three different burrows. Quantification cycles ranged from 14.0 to 43.7 and estimated starting quantities ranged from 7.5 to 1.03×10^9 copies of target DNA/µL. There was an overall detection in sediment samples in six of 60 burrows and a detection rate of eight out of 180 replicates. After inhibited samples were cleaned, all samples had amplification of IPC and all negative control samples did not have amplification. All replicates of the positive control amplified.

Probability of Occurrence and Detection

Five occupancy models of the conditional probability of eDNA detection from a water sample taken from a burrow were compared based on measurements taken at burrows (Table 5). The models assumed that the probability of massasauga occurrence is constant across sites and the probability of detecting eDNA in a qPCR replicate is constant; however, the models are testing the hypothesis that the conditional probability of eDNA detection in a burrow (Θ) changes due to the influence of covariates including pH, temperature, dissolved oxygen, and amount of total DNA from a burrow sample. Due to low detection rates and low biological

replicates with a small number of study sites, all samples that estimated a starting quantity ≥1 copy of target DNA/µL were included although the limit of detection is only as low as 100 copies of target DNA/µL.

Posterior-predictive loss criterion (PPLC) and widely acceptable information criterion (WAIC) values were compared to see which covariate most accurately fit the water burrow data. The lowest values for these criteria implicate which covariate, if any, affects the probability of eDNA detection in a burrow given that it is present at a location. The PPLC values for water samples ranged from 8.50 to 8.67 and WAIC values ranged from 0.204 to 2.10. The model without covariates had the lowest PPLC value (8.50) in water samples; however, the effect of DNA concentration in a sample had the lowest WAIC value (0.204).

The model without any covariates suggests that physical characteristics measured at the burrow are not likely to increase or decrease the probability of detection in a burrow at a site where massasaugas are present.

Alternatively, a low WAIC value suggested that of the other covariates, the total amount of DNA in a sample may affect the probability of detection in a burrow water sample (Θ) (Figure 6). With an increase in the concentration of total DNA (ng/µL) in a sample, there is an increase in the probability of massasauga in a burrow. Although this model had the lowest WAIC value, the confidence intervals for the detection probabilities were extremely large and overlapping. For example, the highest value of Θ was 0.733 in Area 1, but confidence intervals suggest the value could have been as low as 0.232 and as high as 0.999. Further, while there is a positive correlation trend with an increase in Θ with increased total DNA concentration, all confidence intervals for all Θ values at all four sites overlap.

As with the water samples, five occupancy models were compared for the probability of eDNA detection from sediment samples taken from crayfish burrows (Table 6).

The PPLC values for sediment samples ranged from 12.50 to 13.91 and WAIC values ranged from 0.263 to 0.299. Model selection criteria indicate that the occupancy model without covariates had the lowest PPLC and WAIC values at 12.50 and 0.263 respectively. There may have been slight trends in other covariates; however, greatly overlapping confidence intervals suggest that there is no statistically significant trend (Figure 7).

It was anticipated that there would be a higher detection probability in a burrow that was less acidic, cooler, and had lower levels of oxygen, and with higher amounts of total DNA in a

sample. While the expected trend was observed in water samples and amount of total DNA, it was not observed in sediment samples. Other trends are noticeable, but large, overlapping confidence intervals do not allow for highly conclusive results.

Table 1. MANOVA test statistics for variation among sample localities on individual burrow habitat variables (n=60, $df_{3,56}$).

Variable	Mean=	SD Value=	Mean sq =	F=	P=
pH	7.083	0.304	0.343	4.345	0.008
Temperature (˚C)	13.627	2.109	30.512	10.004	<0.0001
Dissolved Oxygen (mg/L)	1.595	0.719	4.94	17.661	<0.0001
Burrow Diameter (cm)	3.200	1.592	21.378	14.007	<0.0001
Nearest Burrow (m)	1.466	1.511	11.125	6.152	0.001
Nearest Tree (m)	4.176	3.912	141.883	16.649	<0.0001
Nearest Shrub (m)	0.938	0.831	3.717	7.037	0.0004
Nearest Water (m)	16.121	6.155	344.270	16.038	<0.0001
Nearest Log (m)	5.355	6.542	369.23	14.585	<0.0001
Nearest Path (m)	106.399	74.691	103,573	314.770	<0.0001

Table 2. Tukey HSD results of ANOVA analysis for similarity among sites for the site variables that are statistically significant.

Variable	Sites Compared	Mean difference	P value =	95% CI	
				lower	upper
Temperature (˚C)	Area 2 - Area 1	-3.34	<0.0001	-5.028	-1.651
	Area 3 - Area 1	-2.52	0.001	-0.817	0.001
	Area 4 - Area 1	-2.23	0.005	-0.544	0.005
	Area 3 - Area 2	0.82	0.563	2.522	0.563
	Area 4 - Area 2	1.11	0.315	2.795	0.315
	Area 4 - Area 3	0.29	0.973	1.962	0.973
Dissolved Oxygen (mg/L)	Area 2 - Area 1	-0.99	<0.0001	-1.503	-0.480
	Area 3 - Area 1	-0.96	<0.0001	-1.468	-0.445
	Area 4 - Area 1	0.04	0.100	-0.473	0.550
	Area 3 - Area 2	0.03	0.100	-0.477	0.546
	Area 4 - Area 2	1.03	<0.0001	0.519	1.541
	Area 4 - Area 3	1.00	<0.0001	0.484	1.507
Burrow Diameter (cm)	Area 2 - Area 1	-2.33	<0.0001	-3.528	-1.139
	Area 3 - Area 1	-2.67	<0.0001	-3.861	-1.472
	Area 4 - Area 1	-1.40	0.015	-2.594	-0.206
	Area 3 - Area 2	-0.33	0.881	-1.528	0.861
	Area 4 - Area 2	0.93	0.176	-0.261	2.128
	Area 4 - Area 3	1.27	0.034	0.072	2.461
Nearest Burrow (m)	Area 2 - Area 1	-1.93	0.001	-3.232	-0.632
	Area 3 - Area 1	-1.41	0.029	-2.706	-0.106
	Area 4 - Area 1	-0.56	0.667	-1.860	0.741
	Area 3 - Area 2	0.53	0.708	-0.774	1.826
	Area 4 - Area 2	1.37	0.035	0.072	2.673
	Area 4 - Area 3	0.85	0.321	-0.454	2.147
Nearest Tree (m)	Area 2 - Area 1	-5.97	<0.0001	-8.794	-3.149
	Area 3 - Area 1	-5.14	<0.0001	-7.960	-2.315
	Area 4 - Area 1	-0.55	0.955	-3.371	2.274
	Area 3 - Area 2	0.83	0.862	-1.989	3.657
	Area 4 - Area 2	5.42	<0.0001	2.600	8.245
	Area 4 - Area 3	4.59	0.0004	4.589	7.411
Nearest Shrub (m)	Area 2 - Area 1	-1.14	0.0004	-1.847	-0.441
	Area 3 - Area 1	-0.49	0.255	-1.197	0.208
	Area 4 - Area 1	-0.21	0.859	-0.912	0.493
	Area 3 - Area 2	0.65	0.080	-0.053	1.352
	Area 4 - Area 2	0.93	0.005	0.232	1.637
	Area 4 - Area 3	0.29	0.706	-0.417	0.988

Table 2 (cont.). TukeyHSD results of ANOVA analysis for similarity among sites for the site variables that are statistically significant.

Variable	Sites Compared	Mean difference	P value =	95% CI lower	95% CI upper
Nearest Water (m)	Area 2 - Area 1	5.68	0.007	1.196	10.155
	Area 3 - Area 1	5.68	0.008	1.196	10.155
	Area 4 - Area 1	-4.16	0.077	-8.644	0.315
	Area 3 - Area 2	0.00	1.000	-4.480	4.480
	Area 4 - Area 2	-9.84	<0.0001	-14.320	-5.360
	Area 4 - Area 3	-9.84	<0.0001	-14.320	-5.360
Nearest Log (m)	Area 2 - Area 1	-11.20	<0.0001	-16.060	-6.330
	Area 3 - Area 1	-9.39	<0.0001	-14.253	-4.524
	Area 4 - Area 1	-5.40	0.024	-10.267	-0.537
	Area 3 - Area 2	1.81	0.759	-3.058	6.672
	Area 4 - Area 2	5.79	0.013	0.928	10.658
	Area 4 - Area 3	3.99	0.144	-0.878	8.852
Nearest Path (m)	Area 2 - Area 1	-10.57	0.389	-28.107	6.970
	Area 3 - Area 1	170.44	<0.0001	152.983	187.98
	Area 4 - Area 1	63.07	<0.0001	45.532	80.610
	Area 3 - Area 2	181.01	<0.0001	163.475	198.55
	Area 4 - Area 2	73.64	<0.0001	56.101	91.179
	Area 4 - Area 3	-107.37	<0.0001	-124.912	-89.835

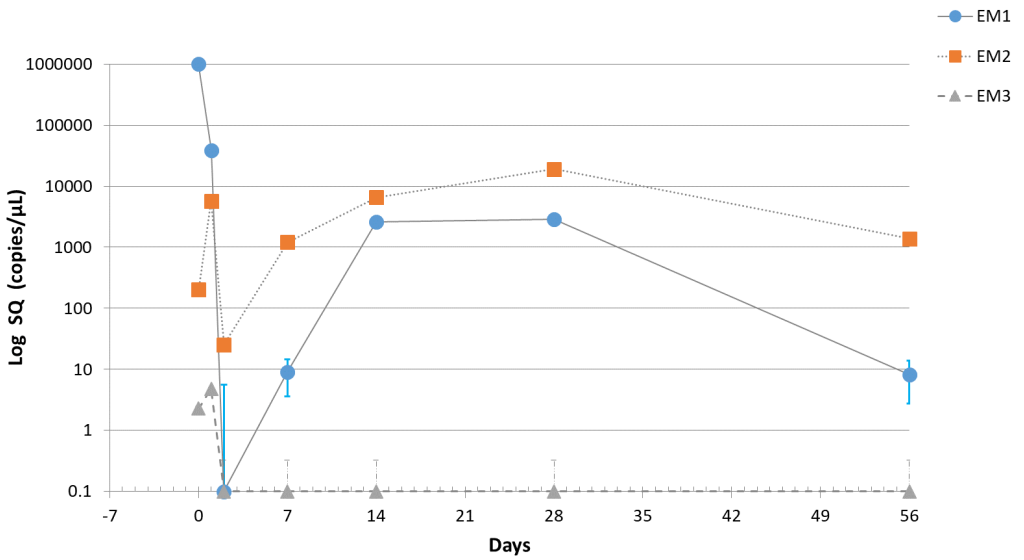

Figure 5. Average amount of estimated starting quantity (SQ) of eDNA (copies of target DNA/µL) from three laboratory-housed massasaugas (EM1-EM3) versus the number of days the snake was removed from the container. Error bars represent 95% confidence intervals for the average of the three replicates of each sample.

Table 3. Positive amplification of *S. catenatus* eDNA from water collected from crayfish burrows at Pierce Cedar Creek Institute.

Site	Water samples positive for *S. catenatus* at least once in a burrow	Positive qPCR Replicates
Area 1	4/15	4/45
Area 2	1/15	1/45
Area 3	2/15	4/45
Area 4	0/15	0/45

Table 4. Positive amplification of *S. catenatus* eDNA from sediment collected from crayfish burrows at Pierce Cedar Creek Institute.

Site	Sediment samples positive for *S. catenatus* at least once in a burrow	Positive qPCR Replicates
Area 1	0/15	0/45
Area 2	2/15	3/45
Area 3	1/15	1/45
Area 4	3/15	4/45

Table 5. Parameter estimates (posterior means) for occupancy (ψ), covariate dependent burrow detection (Θ), and conditional probability of detection in a replicate (ρ) with model-selection criteria (PPLC and WAIC) for each model fitted to massasauga eDNA detections in burrow water samples. Each model was fitted using MCMC algorithm for 20,000 iterations. Bold font indicates the lowest values of PPLC and WAIC.

Covariate	Site	Probability of Occupancy at a Site (ψ)	ψ 95% Confidence Interval (lower, upper)	Conditional Probability of DNA Detection in a Sample (Θ)	Θ 95% Confidence Interval (lower, upper)	Conditional Probability of DNA Detection in a Replicate (p)	ρ 95% Confidence Interval (lower, upper)	PPLC	WAIC
None	Any	0.566	0.163, 0.954	0.548	0.142, 0.970	0.128	0.029, 0.360	**8.50**	0.210
pH		0.617	0.185, 0.976			0.123	0.032, 0.326	8.67	0.207
	Area 1			0.751	0.246, 0.998				
	Area 2			0.297	0.015, 0.939				
	Area 3			0.448	0.077, 0.971				
	Area 4			0.351	0.033, 0.015				
Temperature (˚C)		0.601	0.174, 0.971			0.129		8.58	0.205
	Area 1			0.728	0.233, 0.999		0.032, 0.335		
	Area 2			0.282	0.012, 0.926				
	Area 3			0.390	0.510, 0.924				
	Area 4			0.432	0.072, 0.931				
DO (mg/L)		0.586	0.179, 0.957			0.125	0.028, 0.337	8.63	0.210
	Area 1			0.707	0.224, 0.998				
	Area 2			0.362	0.022, 0.970				
	Area 3			0.373	0.027, 0.969				
	Area 4			0.717	0.225, 0.999				
DNA concentration (ng/µL)		0.612	0.185, 0.968			0.129	0.033, 0.334	8.61	**0.204**
	Area 1			0.733	0.232, 0.999				
	Area 2			0.297	0.020, 0.918				
	Area 3			0.239	0.006, 0.922				
	Area 4			0.520	0.119, 0.955				

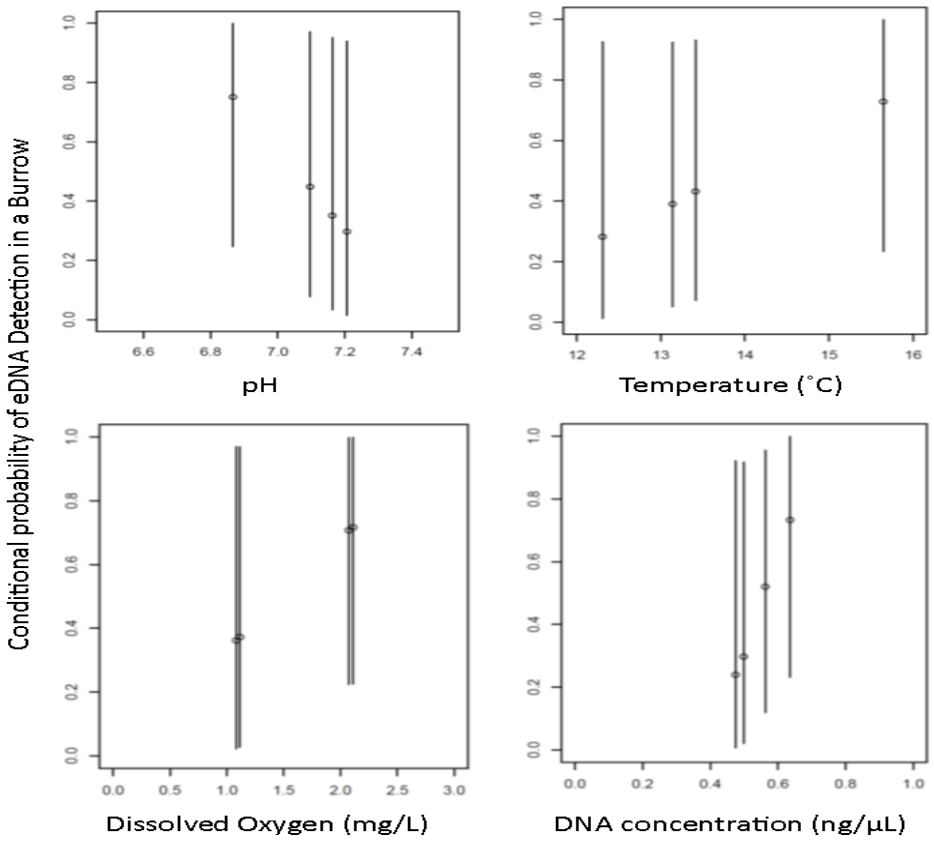

Figure 6. Conditional probabilities of eDNA detection in burrows from water samples from areas of different pH, temperature (˚C), dissolved oxygen (mg/L), and DNA concentration (ng/µL). Symbols are estimates of posterior means with 95% confidence intervals.

Table 6. Parameter estimates (posterior means) for occupancy (ψ), covariate dependent burrow detection (Θ), and conditional probability of detection in a replicate (ρ) with model-selection criteria (PPLC and WAIC) for each model fitted to massasauga eDNA detections in burrow sediment samples. Each model was fitted using MCMC algorithm for 20,000 iterations. Bold font indicates the lowest values of PPLC and WAIC.

Covariate	Site	Probability of Occupancy at a Site (ψ)	ψ 95% Confidence Interval (lower, upper)	Conditional Probability of DNA Detection in a Sample (Θ)	Θ 95% Confidence Interval (lower, upper)	Conditional Probability of DNA Detection in a Replicate (p)	ρ 95% Confidence Interval (lower, upper)	PPLC	WAIC
None	Any	0.723	0.315, 0.985	0.281	0.083, 0.737	0.264	0.067, 0.547	**12.50**	**0.263**
pH		0.767	0.360, 0.991			0.243	0.056, 0.534	13.24	0.282
	Area 1			0.165	0.000, 0.934				
	Area 2			0.401	0.099, 0.950				
	Area 3			0.273	0.053, 0.828				
	Area 4			0.346	0.098, 0.897				
Temperature (˚C)		0.740	0.322, 0.989			0.242	0.055, 0.540	13.36	0.285
	Area 1			0.308	0.001, 0.988				
	Area 2			0.364	0.058, 0.976				
	Area 3			0.332	0.094, 0.891				
	Area 4			0.323	0.080, 0.864				
DO (mg/L)		0.708	0.304, 0.983			0.230	0.055, 0.515	13.70	0.289
	Area 1			0.434	0.083, 0.975				
	Area 2			0.284	0.052, 0.813				
	Area 3			0.288	0.057, 0.809				
	Area 4			0.439	0.384, 0.980				
DNA concentration (ng/µL)		0.733	0.320, 0.987			0.222	0.052, 0.522	13.91	0.299
	Area 1			0.301	0.045, 0.896				
	Area 2			0.430	0.061, 0.998				
	Area 3			0.321	0.068, 0.855				
	Area 4			0.330	0.074, 0.882				

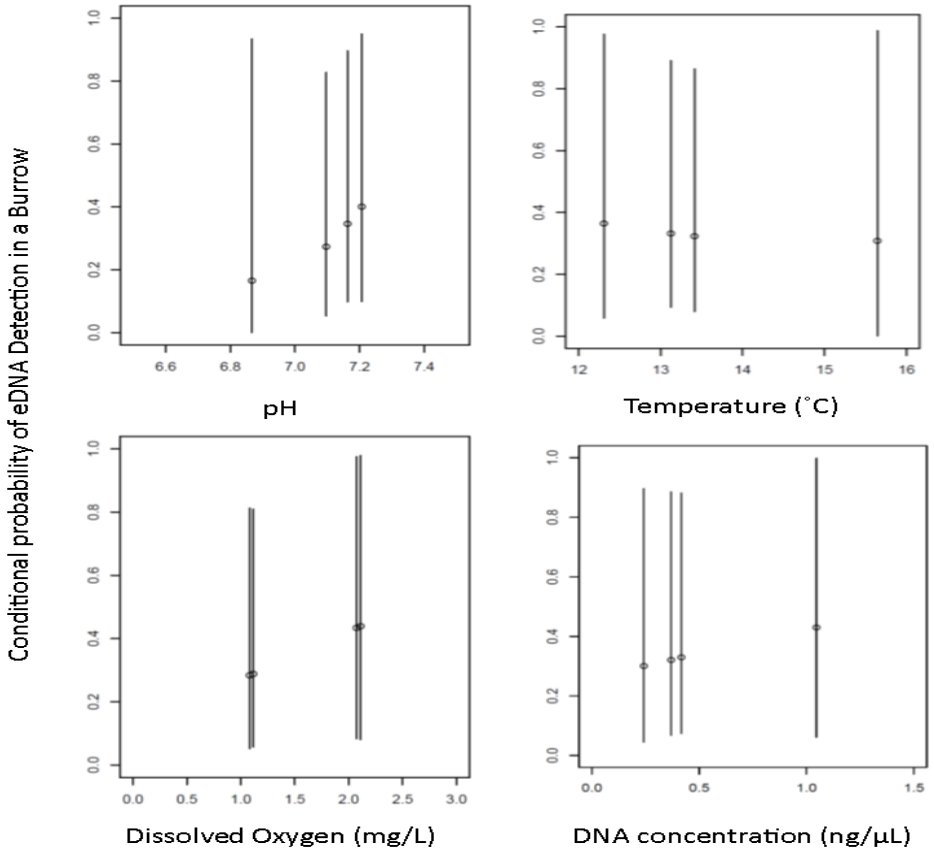

Figure 7. Conditional probabilities of eDNA detection in burrows from sediment samples from areas of different PH, temperature (˚C), dissolved oxygen (mg/L), and DNA concentration (ng/µL). Symbols are estimates of posterior means with 95% confidence intervals.

DISCUSSION

This study examined the development of an environmental DNA assay for the Eastern Massasauga (*Sistrurus catenatus*). To accomplish this, a unique set of primers and probe were designed using the COI gene to specifically target a 152 bp segment of massasauga DNA. Specifically, eDNA was extracted from both water and sediment samples extracted from abandoned crayfish burrows, known overwintering sites for massasaugas. In addition to extracting eDNA from crayfish burrows, environmental measurements were taken to be used as covariates in occupancy models to observe the effect, if any, they have on the probability of detection at a site, burrow, or qPCR replicate. Finally, massasaugas were housed in water-filled containers in the laboratory to observe the ability to detect eDNA in water samples at 0, 1, 2, 7, 14, 28, and 56 days after snakes have been removed from a water source.

eDNA Assay

The primers and probe designed for this study are sufficient for detecting *S. catenatus* eDNA in both laboratory and field settings in both water and sediment samples. Detection of eDNA was found in 21 lab samples, seven water burrow samples and in six sediment burrow samples.

The theoretical limit of detection of 100 copies of target DNA/µl is higher than the 4.2 copies of target DNA/µL that was calculated by Baker *et al.* (2018), however fit within limits of detection determined in snake species detections in the Florida Everglades which ranged from 0.4 to $4.2x10^7$ copies of target DNA/µL. The average slope, R^2 value, and qPCR efficiency for the standard curves in this study were -3.98, 0.96, and 85.86% respectively. The target values for accurate quantification of target DNA should have a slope between -3.1 to -3.6, an R^2 value $\geq$ 0.99, and a qPCR efficiency between 90 to 110% (Hunter *et al*. 2015). While the methods used successfully detected massasaugas in the field and the lab, improvements need to be made for accurate quantification of the starting amounts of target DNA in samples.

Interestingly, a positive detection of massasauga DNA was identified in six out of 60 water samples compared to two out of 100 samples as was the case in the study conducted by Baker *et al.* (2018). As with Baker *et al.*, the sequence designed for this study was sequenced

and confirmed to be *Sistrurus catenatus* DNA; however, this study had a 152 bp target sequence compared to 137 bp. Due to low detection rates at sites where massasaugas are known to overwinter, both studies indicate that more research needs to be done to improve eDNA methods for detecting massasaugas to make it a more reliable practice than traditional surveying methods.

Laboratory eDNA Persistence

Results from laboratory-housed massasaugas showed that after snake removal, eDNA could be detected for up to 56 days for two of three housed snakes which falls within the 14 to 60 day detection window found in other studies (Goldberg *et al.* 2015, Santas *et al.* 2013). This observation supports the potential validity of using eDNA as a survey method for massasaugas in the field. Because of the persistence of eDNA, snakes would not need be immediately present in an area to be detected so long as they had been in an area within 56 days, nor would researchers have to visually confirm the presence of this cryptic species on the day of sampling. Moreover, a general decline in the starting concentration of eDNA in a sample over time further bolsters argument for using eDNA surveying. The decline demonstrates that not only could larger amounts of starting DNA mean that there are potentially more snakes in an area as was found in Asian carp (Takahara *et al.* 2012), but also that higher amounts of starting DNA in a sample indicate a shorter time frame has passed between sampling and when snakes were present in an area.

Further, those housed snakes (EM1, EM2) that had fecal matter in their containers were easier to detect with the eDNA assay. Studies have shown that DNA extracted from fecal matter in reptiles has reliably been used for genotyping and sequencing (Pearson *et al.* 2015). Fecal matter in the containers of two lab snakes likely contributed to eDNA detection in laboratory samples even though snakes were in the water for 24 hours and no skin was visibly shed. The lack of fecal matter in EM3 may explain why DNA could not be detected after day two. Hunter *et al.* (2015) noticed a nonlinear, positive correlation between DNA concentration and time. They believed variations in snake activity and defecation may have contributed to their observed relationship which is may explain why DNA was not as easily detected in EM3.

In addition to the massasaugas in the lab defecating, there is a higher estimated starting amount of DNA after day seven when it is noted for both EM1 and EM2 that feces had broken down completely. This suggests that in the first seven days, water collection may not sample

fecal material well; however, once the material was more broken down and presumably more evenly distributed in the water, eDNA was more likely to be detected using qPCR. Although larger particle sizes are more likely to be detected because they are more likely to be captured in a filter (Turner *et al.* 2014), the particles still must be widely distributed to be represented in the sample apparatus.

While a spike in initial DNA concentration was noticed in two specimens after seven days, another contribution to the increase may be due to fecal matter becoming more concentrated in the containers. The water volume was declining with each sampling and no new water was being added. Keeping the volume of water consistent throughout sampling maybe beneficial for future studies.

The observations in this study regarding eDNA persistence and the concentrations of DNA based on the break down of biological material only took place in a laboratory setting at room temperature. It would be beneficial to observe these interactions when DNA is inserted into burrows in a natural setting. Additionally, it has been observed that overwintering snakes exert very low amounts of energy as well as low and inactive metabolic rates (Beaupre and Duvall 1998). Because of this, it is possible that sampling crayfish burrows during the overwintering periods of massasauga activity may not yield high amounts of DNA to be detected during eDNA surveying.

Field Application of Assay

Four sites were sampled at Pierce Cedar Creek Institute in May 2016 which coordinated with the timing when it was likely that massasaugas were dispersing from their overwintering sites (Smith 2009). It is well known that Pierce Cedar Creek Institute has a relatively large population of *S. catenatus* that has been well studied, making it an ideal location to test the validity eDNA sampling and multiscale occupancy modeling.

Study sites were labeled according to the historical use of sites by massasaugas for overwintering sites with Areas 1 and 2 being most likely to support overwintering massasaugas due to their routine observation in visual surveys. Area 3 has occurrences of massasaugas but is too wet for overwintering while in Area 4 massasaugas have never been documented. Analyses of sampling sites using MANOVA and ANOVA indicated that there were differences at the sites

in the habitat features associated with burrows including acidity, temperature, and dissolved oxygen.

Because of this, it was expected that the highest amount of detections would be in Area 1 and lowest in Area 4 for both water and sediment samples. This expected trend was observed in water samples from burrows, but in sediments, the highest number of detections was in Area 4 and the lowest was in Area 1. Area 4, on the southern side of Aurohn Lake, is a relatively new addition to the PCCI property (since 2015) while Brewster Lake (another lake on PCCI property) has been a part of the institute since 2001. Since massasauga surveying methods began in 2008, Brewster Lake has not had documented populations of massasaugas (Bradke 2017, Howell 2014, Kuyt 2015). Even if Area 4 is not a current overwintering site, positive detections from soil samples in the area may suggest historical massasauga use, and the need for future surveying or restoration efforts in this location.

Site occupancy was expected to be greater in Areas 1 and 2 and lower in Areas 3 and 4; however, site occupancy (ψ) remained very similar among water and sediment samples. Although site occupancy was as high as 0.767, confidence intervals were vey large. Despite similarity in ψ, physical characteristics of the burrows at each were still expected to affect the likelihood of detection of eDNA in a burrow. Previous studies indicated that massasaugas choose overwintering sites that were more acidic and more anoxic. Being ectothermic, it was also assumed that higher temperatures would also have an affect on site selection. Studies have also shown that DNA is more stable in anoxic, alkaline, and cooler conditions (Barnes *et al.* 2014, Corinaldesi *et al.* 2008, Matsuo *et al.*1995, Poté *et al.* 2009, Sage 2005, Smith 2009, Zhao and Fleet 2003). These past observations led to the hypothesis that the probability of detection of eDNA in a burrow would be most greatly affected by one of the covariates including pH, dissolved oxygen, and temperature.

When the eDNA detection data and the environmental variables of burrows were fit to occupancy models with and without covariates, the model with the total amount of starting DNA in a sample seemed to have some support in when using the water sample data. With an increase in total DNA, the probability of detection in a burrow increased as was observed in Asian carp (Turner *et al.* 2015). Additionally, other trends were noticed including a decrease in burrow detection with an increase in pH, an increase in burrow detection as temperature increased, and an increased probability of burrow detection with an increase in dissolved oxygen. These

relationships were opposite of what was excepted. While trends were observed, large, overlapping confidence intervals, as well as a model without covariates having the lowest criteria values indicates that covariates do not have a large effect on the probability of detection of eDNA in a burrow from a water sample.

In sediment samples, expected trends were observed. These trends included an increase in detection with increased pH and decreased detection with increased temperatures. As with water samples, an increase in dissolved oxygen led to an increase in the probability of detection in a burrow which was not what was predicted. Again, large confidence intervals do not allow for concrete inferences to be made.

Confidence intervals were smallest for the probability of detection in a qPCR replicate (ρ) given that massasaugas were present at a location and DNA was in a burrow. While the highest value for ρ was 0.264, it is reliable among all samples that eDNA will be detected in a qPCR replicate about a quarter of the time.

The differences in selection criteria may be due to the small sample size. I took one sample from each burrow and separated divided the sample into a water and a sediment sample. Other studies suggest that a minimum of two samples per site (burrow) is necessary for reliable occupancy estimation while other studies used up to four samples (Dorazio and Erikson 2017, Schmidt *et al.* 2013). In addition to having two biological replicates at each burrow, it is recommended to sample from 20 different areas to more accurately estimate the probability of occurrence in a site (Schmidt *et al.* 2013). This study only observed four different sites. Improving these sample sizes may help to decrease overlap in the confidence intervals in the probability of occurrence in an area and detection in a burrow. Finally, confidence intervals were relatively small in the probability of detection in a qPCR replicates (ρ) across water and sediment samples and ranged from 0.123 to 0.264. This study ran qPCR replicates in triplicate. Future studies should consider running up to ten replicates with this assay.

Recommendations for Assay Use

Although this study followed the minimum reporting guidelines for an environmental DNA study and made strides in providing an alternative surveying method for massasaugas, improvements can be made to make an massasauga eDNA survey more reliable.

A minimum of 20 different sites would decrease the size of the confidence intervals of ψ, and at least two biological replicates per burrow would decrease the variance of probability in Θ. Additionally, at least 10 qPCR replicates are necessary to detect eDNA in a qPCR replicate if massasaugas are present at a site, and eDNA is in a burrow. Admittedly, while the dollar amount of plastics is not greatly increased for sampling more burrows in more sites, the increase in replicates may get expensive with regards to reagents including the IPC and reaction mixes. The increased expense for the reagents may negate any monetary savings from the use of only one researcher in the field. Because there was no difference in the number of detections in either water or sediment samples but there was a difference in possible trends in occupancy models, it is still suggested that both types of samples be tested.

After collection of samples, the filtration of samples and placement in CTAB may need altered in the future. While this study supported that CTAB may help to preserve extracted DNA for up to two weeks, Renshaw *et al.* (2015) found that copy number decreased from week one to week two. Being that eDNA is extracted at extremely small amounts and in very short fragments, future studies might benefit from placing samples in buffers for a shorter time frame so that any possible eDNA can be extracted more quickly without risking degradation over time.

In this study, the theoretical limit of detection was only 100 copies of target DNA/µL, but in my analysis, all positive detections, even as low as 1 copy of target DNA/µL, were included to account for a low number of samples. Being more conservative in the parameters for a positive detection would lower the amount of positive detections in both field and laboratory settings, indicating that improvements would need to be made to the standard curve to increase the sensitivity of a qPCR assay. Future studies would greatly benefit from running more qPCR reactions and creating a standard curve that covers a lower limit range.

As this study supported eDNA degradation over time in lab samples as was observed in other studies, it is important to examine if this degradation occurs in the field when samples are exposed to various environmental conditions. Additionally, spiking known sources of massasauga DNA into burrows or using radio telemetry to confirm the presence of snakes in burrows may be beneficial in confirming eDNA assay observations.

Conclusions

The design of primers to isolate a 152 bp fragment of the COI gene DNA sequence in conjunction with a 24 bp probe can be used to identify *Sistrurus catenatus*-specific DNA extracted from both water and sediment environmental samples taken from crayfish burrows and can be used to differentiate from other co-occurring snake species in southern Michigan. Although the designed primer is unique to species of snakes occurring in Michigan, further comparisons should be made of Eastern Massasaugas against other species of snakes especially the Timber Rattlesnake that may overlap massasauga distribution at the western and eastern edges (Falk 2002).

Positive detections in areas previously recognized as void of massasaugas may warrant a greater survey effort in these areas; however, rates of detection in qPCR were low even in areas known to host over-wintering snakes. The probability of detection in a technical qPCR replicate is low but with small confidence intervals. Because of this, future sampling should require running ten qPCR replicates for each sample to more consistently have positive detections if it is believed that eDNA is in a burrow. Due to low detection rates, eDNA surveying likely not useful as a stand-alone survey tool, but rather a way to observe a site with the suspected presence of snakes prior to more extensive, traditional surveying methods.

With data obtained from environmental measurements and eDNA detection, occupancy models can be created to aid in detection probabilities of this cryptic snake to better protect, conserve, and restore necessary habitat to protect this species (U.S. Fish & Wildlife Service 2018). Detection of favorable massasauga habitat and the subsequent protection of these locations will be vital in the preservation of the species. With more cost and time effective studies such as eDNA surveillance methods, it is hopeful that more locations with positive massasauga detections can be identified more quickly and protected so that this species, and other species with similar status can hopefully be prevented from being driven to extinction.

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APPENDIX

Alignment of mitochondrial COI gene sequences of target Eastern Massasauga and other potentially co-occurring snake species. Sequences were acquired from BOLD© and GenBank® databases. If available, multiple sequences of the COI gene from the same species was aligned. Underlined portions indicate forward and reverse primers while doubly underlined portions indicate probe. Highlighted nucleotides in red indicate a difference between non-target and target DNA sequences.

Species	Sequence (5' - 3')	Accession ID
Sistrurus catenatus	CCC CTT TTC GTG TGG TCT GTA ATA ATT ACA GCA ATT ATA CTA CTT CTA GCA CTA CCC GTG CTT GCA GCA GCA ATT ACC ATA CTC CTG ACG GAC CGG AAC TTA AAC ACA ACC TTT TTC GAC CCG TGC GGA GGC GGT GAC CCA GTT CTA TTT CA	KU985958
Sistrurus catenatus	CCC CTT TTC GTG TGG TCT GTA ATA ATT ACA GCA ATT ATA CTA CTT CTA GCA CTA CCC GTG CTT GCA GCA GCA ATT ACC ATA CTC CTG ACG GAC CGG AAC TTA AAC ACA ACC TTT TTC GAC CCG TGC GGA GGC GG- --- --- --- --- --- --	KU985651
Sistrurus catenatus	CCC CTT TTC GTG TGG TCT GTA ATA ATT ACA GCA ATT ATA CTA CTT CTA GCA CTA CCC GTG CTT GCA GCA GCA ATT ACC ATA CTC CTG ACG GAC CGG AAC TTA AAC ACA ACC TTT TTC GAC CCG TGC GGA GGC GGT GAC CCA GTT CTA TTT CA	KU986216
Coluber constrictor	CCA CTA TTC GTC TGA TCT GTA CTA ATT ACC GCC ATT ATA CTT CTC CTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACA ATA CTA TTA ACA GAC CGA AAT ATC AAC ACC TCA TTC TTC GAC CCC TGT GGA GGA GGG GAC CCC GTA CTA TTT CA	KU985813
Coluber constrictor	CCA CTA TTC GTC TGA TCT GTA CTA ATT ACC GCC ATT ATA CTT CTC CTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACA ATA CTA TTA ACA GAC CGA AAT ATC AAC ACC TCA TTC TTC GAC CCC TGT GGA GGA GGG GAC CCC GTA CTA TTT CA	KU985863
Coluber constrictor	CCA CTA TTC GTC TGA TCA GTA CTA ATT ACC GCC ATT ATA CTT CTT CTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACA ATA CTA TTA ACA GAC CGA AAT ATC AAC ACC TCA TTC TTC GAC CCC TGT GGG GGA GGG GAC CCT GTA CTA TTT CA	KU986055
Coluber constrictor	CCA CTA TTC GTC TGA TCA GTA CTA ATT ACC GCC ATT ATA CTT CTT CTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACA ATA CTA TTA ACA GAC CGA AAT ATC AAC ACC TCA TTC TTC GAC CCC TGT GGG GGA GGG GAC CCT GTA CTA TTT CA	KU986094
Heterodon platirhinos	CCC TTA TTC GTC TGA TCA GTG CTA ATT ACT GCC ATT ATG TTG CTC CTA GCT CTA CCC GTG CTG GCA GCA GCT ATT ACC ATG CTA CTT ACA GAC CGA AAT CTT AAC ACC TCT TTC TTT GAC CCG TGC GGT GGA GGG GAC CCT GTT TTA --- --	KU986007
Heterodon platirhinos	CCC TTA TTC GTC TGA TCA GTA CTA ATT ACT GCC ATT ATG TTG CTC CTA GCT CTA CCC GTG CTA GCA GCA GCT ATT ACC ATG CTA CTT ACA GAC CGA AAT CTT AAC ACC TCT TTC TTT GAC CCA TGC GGT GGA GGG GAC CCT GTC TTA TTC CA	KU985930
Heterodon platirhinos	CCC TTA TTC GTC TGA TCA GTG CTA ATT ACT GCC ATT ATG TTG CTC CTA GCT CTA CCC GTG CTG GCA GCA GCT ATT ACC ATG CTA CTT ACA GAC CGA AAT CTT AAC ACC TCT TTC TTT GAC CCA TGC GGT GGA GGG GAC CCT GTC TTA TTC CA	KU986023
Lampropeltis triangulum	CCC CTA TTT GTC TGA TCA GTA CTT ATT ACC GCT ATT ATA CTA CTT TTA GCC CTG CCT GTA TTA GCA GCA GCA ATT ACT ATA CTT CTG ACT GAT CGA AAT CTA AAT ACC TCT TTC TTT GAC CCC TGT GGA GGG GGG GAC CCT GTA CTA TTC CA	KU986088
Lampropeltis triangulum	CCC CTA TTT GTC TGA TCA GTA CTT ATT ACC GCT ATT ATA CTA CTT TTA GCC CTG CCT GTA TTA GCA GCA GCA ATT ACC ATA CTT CTG ACT GAT CGA AAT CTA AAT ACC TCT TTC TTT GAC CCC TGT GGA GGG GGG GAC CCT G-- --- --- --	KU985601
Lampropeltis triangulum	CCC CTA TTT GTC TGA TCA GTA CTT ATT ACC GCT ATT ATA CTA CTT TTA GCC CTG CCT GTA TTA GCA GCA GCA ATT ACT ATA CTT CTG ACT GAT CGA AAT CTA AAT ACC TCT TTC TTT GAC CCC TGT GGA GGG GGG GG- --- --- --- --- --	KU985694
Nerodia sipedon	CCC TTG TTC GTT TGG TCA GTC CTA ATT ACA GCC ACT ATA CTA CTG TTA GCC CTA CCA GTA CTA GCG GCA GCA ATT ACC ATG TTA CTA ACC GAC CGA AAC ATC AAC ACC TCA TTT TTC GAC CCT TGT GGA GGC GGA GAC CCG GTT TTA --- --	KU985915
Nerodia sipedon	CCC TTG TTC GTT TGG TCA GTC CTA ATT ACA GCC ACT ATA CTA CTG TTA GCC CTA CCA GTA CTA GCG GCA GCA ATT ACC ATG TTA CTA ACC GAC CGA AAC ATC AAC ACC TCA TTT TTC GAC CCT TGT GGA GGC GGA GAC CCG GTT TTA TTC CA	KU985556

Species	Sequence (5' - 3')	Accession ID
Pantherophis obsoletus	CCT TTG TTT GTC TGA TCA GTA CTT ATC ACC GCC ATT ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACT ATA CTT TTA ACT GAT CGA AAT CTA AAT ACC TCT TTC TTT GAT CCT TGT GGA GGA GGG GAC CCT GTA CTA TTC C-	KU986043
Pantherophis obsoletus	CCT TTG TTT GTC TGA TCA GTA CTT ATC ACC GCC ATT ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACT ATA CTT TTA ACT GAT CGA AAT CTA AAT ACC TCT TTC TTT GAT CCT TGC GGA GGG GGG GAC CCT GTA CTA TTC CA	KU985879
Pantherophis obsoletus	CCT TTG TTT GTC TGA TCA GTA CTT ATC ACC GCC ATT ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCA ATT ACT ATA CTT TTA ACT GAT CGA AAT CTA AAT ACC TCT TTC TTT GAT CCT TGC GGA GGG GGG GAC CCT GTA CTA TTC CA	KU985940
Pantherophis obsoletus	CGC TT- --- -TA ATT ATA TTA TTA ACT --- --	KU985993
Storeria dekayi	CCT CTA TTC GTC TGG TCA GTT TTA ATC ACC GCC ATC ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCG ATT ACC ATA TTA CTA ACT GAC CGA AAC ATT AAC ACC TCC TTC TTT GAT CCT TGT GGT GGC GGA GAC CCA GTC CTA TTC CA	EF417389
Storeria dekayi	CCT CTA TTC GTC TGG TCA GTT TTA ATC ACC GCC ATC ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCG ATT ACC ATA TTA CTA ACT GAC CGA AAC ATT AAC ACC TCC TTC TTT GAT CCT TGT GGT GGC GGA GAC CCA GTC CTA TTC CA	KU985887
Storeria dekayi	CCT CTA TTC GTC TGG TCA GTT TTA ATC ACC GCC ATC ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCG ATT ACC ATA TTA CTA ACT GAC CGA AAC ATT AAC ACC TCC TTC TTT GAT CCT TGT GGT GGC GGA GAC CCA GTC CTA TTC CA	KU986037
Storeria dekayi	CCT CTA TTC GTC TGG TCA GTT TTA ATC ACC GCC ATC ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCG ATT ACC ATA TTA CTA ACT GAC CGA AAC ATT AAC ACC TCC TTC TTT GAT CCT TGT GGT GGC G-- --- --- --- --- --- --	KU986092
Storeria dekayi	CCT CTA TTC GTC TGG TCA GTT TTA ATC ACC GCC ATC ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCG ATT ACC ATA TTA CTA ACT GAC CGA AAC ATT AAT ACC TCC TTC TTT GAT CCT TGT GGT GGC GGA GAC CCA GTC CTA --- --	KU986264
Storeria dekayi	CCT CTA TTC GTC TGG TCA GTT TTA ATC ACC GCC ATC ATA CTA CTA TTA GCC CTA CCA GTA CTA GCA GCA GCG ATT ACC ATA TTA CTA ACT GAC CGA AAC ATT AAT ACC TCC TTC TTT GAT CCT TGT GGT GGC GGA --- --- --- --- --- --	KU985755
Thamnophis sauritus	CCC TTG TTT GTT TGG TCA GTC TTA ATC ACC GCC ATC ATA CTA CTG TTA GCC CTA CCA GTA CTT GCG GCA GCG ATT ACT ATA CTA CTA ACC GAC CGT AAC ATC AAC ACC TCA TTT TTT GAT CCT TGT GGG GGT GGA GAC CCG GTT TTA TTC CA	KU985906
Thamnophis sauritus	CCC TTG TTT GTT TGG TCA GTC TTA ATC ACC GCC ATC ATA CTA CTG TTA GCC CTA CCA GTA CTT GCG GCA GCG ATT ACT ATA CTA CTA ACC GAC CGT AAC ATC AAC ACC TCA TTT TTT GAT CCT TGT GGG GGT GGA GAC CCG GTT TTA TTC CA	KU985691
Thamnophis sauritus	CCC TTG TTT GTT TGG TCA GTC TTA ATC ACC GCC ATC ATA CTA CTG TTA GCC CTA CCA GTA CTT GCG GCA GCG ATT ACT ATA CTA CTA ACC GAC CGT AAC ATC AAC ACC TCA TTT TTT GAT CCT TGT GGG GGT GGA GAC CCG GTT TTA TTC CA	KU986143
Thamnophis sirtalis	CCC CTG TTT GTT TGG TCA GTC TTA ATC ACC GCC ATT ATA CTA CTG TTA GCC CTA CCA GTA CTA GCG GCA GCG ATT ACT ATG CTA CTA ACC GAC CGA AAC ATC AAC ACC TCA TTC TTT GAT CCT TGT GGG GGC GGA GAC CCG GTT TTA TTC CA	KC750818