EFFICACY OF USING ENVIRONMENTAL DNA (EDNA) TO DETECT KIRTLAND'S SNAKES (*CLONOPHIS KIRTLANDII*)

by

Rikki A. Ratsch

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STATEMENT OF COMMITTEE APPROVAL

Dr. Mark Jordan, Chair
Department of Biology
Dr. Bruce Kingsbury
Department of Biology
Dr. Tanya Soule
Department of Biology

Approved by:
Dr. Jordan M. Marshall
Head of the Graduate Program

For my fiancé Jessica Hinz and my parents Tom and Lisa.

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ABSTRACT

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Institution: Purdue University
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Committee Chair: Mark Jordan

Environmental DNA (eDNA) surveys utilize DNA shed from animals in order to detect their presence. Since it was developed, this technique has been applied to numerous species across several taxa. In some cases, it has been shown to be superior to traditional survey methods at detecting rare or cryptic species. It allows for the detection of animals in low numbers and does not require direct capture of an animal. This allows eDNA to be more effective at detecting rare or cryptic species that require high survey effort to find. This often reduces survey costs as many eDNA samples can be collected quickly with little equipment required.

The Kirtland's Snake (*Clonophis kirtlandii*) is a small Natricine snake endemic to the Midwest. It is a species of conservation concern since it is threatened throughout its range. Due to its cryptic and fossorial lifestyle, it is also a notoriously difficult snake to survey. This has resulted in a poor understanding of Kirtland's Snake life history and population status. Applying eDNA surveys to this species may increase detection probability, offering a more efficient way to survey for them.

In this study, a quantitative PCR (qPCR) assay was designed and tested alongside traditional coverboard surveys. The assay had a limit of detection of 166 copies of Kirtland's Snake DNA. In crayfish burrow sediment, eDNA was found to be detectable up to 10 days and may persist for up to 25 days. However, only one detection occurred out of 380 field samples. Coverboard surveys revealed temporal and spatial variation in Kirtland's Snake abundance. More snakes were captured in the spring, during the first field season, and at the south coverboard transects. Kirtland's Snake abundance was also found to be related to the presence of grass and herbaceous vegetation as well as close proximity to shrubs. Comparing survey methods, coverboards resulted in far better snake detection, suggesting that eDNA does not offer an advantage over traditional survey methods for this species.

INTRODUCTION

Environmental DNA

Detection of environmental DNA, or eDNA, is a survey technique that has seen increasing attention and use in wildlife management over the past few years. The method involves taking environmental samples such as water or soil and testing the material for the presence of short strands of DNA that were shed from organisms into the environment (Taberlet et al. 2012). The major advantage of this technique is that it does not require the direct observation and capture of organisms being studied. Although this is an indirect survey method, it can still retain a high detection probability that may exceed those of traditional surveys. Studies comparing detection probability using eDNA and traditional survey methods for invertebrates, amphibians, and fish found that eDNA can yield higher detection probabilities, in some cases nearing 100 percent (Rees et al. 2014a, Tréguier et al. 2014, Biggs et al. 2015, Sigsgaard et al. 2015, Spear et al. 2015). Another advantage of using eDNA is the potential reduction in costs. This occurs primarily through reducing person-hours required to reach adequate sampling effort. A cost analysis conducted on nine freshwater turtle species found that traditional surveys cost two-to-ten times higher than eDNA surveys to detect a single turtle species (Davy et al. 2015). Similarly, it was two-to-five times more cost efficient to achieve detection with eDNA compared to auditory and visual encounter surveys for invasive American Bullfrogs (*Lithobates catesbeianus*) in France (Dejean et al. 2012). The ability to effectively detect low numbers of organisms at a comparatively low cost makes eDNA a valuable tool for studying species that are rare, cryptic, or require high effort to achieve thorough sampling.

Environmental DNA has already been used for the detection of low numbers of individuals at the leading edge or initial invasion of an exotic species. Some of these species include the American Bullfrogs (Ficetola et al. 2008, Dejean et al. 2012) and Louisiana Crayfish (*Procambarus clarkii*) (Tréguier et al. 2014) in France, Asian Carp (*Hypophthalmichthys* spp.) (Jerde et al. 2011, Mahon et al. 2013, Turner et al. 2014b) and Zebra Mussels (*Dreissena polymorpha*) (Egan et al. 2013) in the Midwestern USA, and Burmese Pythons (*Python bivittatus*) in Florida (Piaggio et al. 2013, Moyer et al. 2014). Threatened and endangered species present another opportunity to apply eDNA as their rarity and legal protection can impede

standard survey methods (Thomsen et al. 2012b). Most traditional surveys have imperfect detection, require equipment placed in the environment, and are designed to capture as well as physically interact with the animals. That disturbance to them and the environment is considered during permit applications and may limit access to licensing for particular species or habitats. Listed as threatened or endangered in most areas they occur, the Eastern Hellbender (*Cryptobranchus alleganiensis*) is of high conservation concern and eDNA has already been used to detect their presence in streams (Olson et al. 2012, Spear et al. 2015). Similarly, eDNA has already been used for detection of the threatened Great Crested Newt (*Triturus cristatus*) in the UK (Rees et al. 2014a). Other threatened species that have been successfully detected with eDNA include the European Weather Loach, Eastern Massasauga Rattlesnake (*Sistrurus catenatus*), and the Bull Trout (*Salvelinus confluentus*) (Dejean et al. 2012, Wilcox et al. 2013, Wilcox et al. 2014, Baker et al. 2018).

Sampling methods and equipment are important considerations when collecting eDNA samples and must be tailored to the target species. Often, eDNA is collected from water as it offers advantages compared to other mediums like soil. Water suspends the DNA and allows for transportation away from the animal, increasing the potential for detection, especially in systems with flowing water (Pilliod et al. 2013, Deiner and Altermatt 2014, Pilliod et al. 2014, Jane et al. 2015, Spear et al. 2015, Wilcox et al. 2016). Water samples are easily processed by pumping water through a filter, allowing sample volume to be increased or decreased to fit study needs (Rees et al. 2014b). Though suspended eDNA is readily dispersed and processed, there is evidence of eDNA being more concentrated in sediment than in the water (Turner et al. 2015).

A major factor when using eDNA to detect a species is the persistence, or degradation rate, of DNA in the environment after it is shed from an animal. A short lifespan is ideal for up-to-date presence information. However, if the DNA degrades too quickly, it will reduce overall detection probability. A longer DNA lifespan allows for more chances for detection, but at the cost of accurate information relating to the current presence or absence of a target species. There are many environmental factors that can influence the persistence of eDNA. These include microbe activity, pH, temperature, and UV radiation (Strickler et al. 2015). Minimizing these influences will increase the longevity of eDNA, thus potentially increasing the probability of detection. Previous studies have shown a wide variation of eDNA persistence in the water column, ranging from less than one day to nearly 60 days (Dejean et al. 2011, Thomsen et al.

2012a, Thomsen et al. 2012b, Piaggio et al. 2013, Barnes et al. 2014, Pilliod et al. 2014, Strickler et al. 2015). Conversely, eDNA present in sediment has been shown to remain intact for months (Mao et al. 2013, Turner et al. 2015). Under extreme conditions, such as in permafrost layers or in caves, eDNA has been shown to survive for thousands of years (Thomsen and Willerslev 2015). The degradation rate of eDNA varies by species as each lives in a habitat with a unique combination of factors that preserve or degrade DNA in the environment.

Clonophis kirtlandii

Kirtland's Snake (*Clonophis kirtlandii*) is small and poorly understood due to its secretive, fossorial nature (Evers 1994, Ernst and Ernst 2003, Harding and Mifsud 2017). They are most often found under cover, buried in leaf litter, or in burrows underground. Observations peak during spring, followed by a drop off into summer before increasing once more in the fall (Gibson and Kingsbury 2004). It is believed that the snakes become nocturnal, retreat below ground to areas of higher moisture, or potentially go into aestivation during summer months (Conant 1943, Ernst and Ernst 2003, Harding and Mifsud 2017). Curiously, the majority of what is known about *C. kirtlandii* comes from studies and observations of urban or rural populations (Conant 1943, Minton 1972, Brown 1986, Minton 2001).

Clonophis kirtlandii is most often affiliated with open areas that are wet or prone to seasonal flooding. Their preferred habitats are moisture-rich open meadows, prairies, or grasslands (Conant 1943, Bavetz 1994). The presence of *C. kirtlandii* is strongly associated with nearby water bodies such as ponds, lakes, or streams (Wilsmann and Sellers 1988). These snakes can also be found in swamps, bogs, and forests containing pools or creeks (Conant 1943). *Clonophis kirtlandii* is also known to inhabit open urban environments with grassy areas, ample cover, a nearby water source, and crayfish burrows (Minton 1972, Brown 1986, Minton 2001). The existence of crayfish borrows is often linked with *C. kirtlandii* occurrence regardless of habitat and they likely use these burrows as refugia, hibernacula, and to seek prey or moisture (Wilsmann and Sellers 1988, Bavetz 1994, Anton et al. 2003).

Clonophis kirtlandii is endemic to the Midwest with populations currently residing in the states of Michigan, Illinois, Indiana, Ohio, and Kentucky (Wilsmann and Sellers 1988, Bavetz 1994). Their populations are patchy and reduced compared to historic records (Bavetz 1994, U.S. Fish and Wildlife Service 2017). They are listed as state endangered in Michigan, Indiana, and

Kentucky as well as having a state-threatened listing in Illinois and Ohio. Due to their cryptic and fossorial nature, it is difficult to assess the status of their populations. The most abundant information is restricted to simple presence or absence data. Traditional survey methods are difficult and expensive to properly conduct for this species, and the general lack of ecological knowledge restricts the ability to make informed management decisions (Gibson and Kingsbury 2004). This is underlined by the 2017 status assessment for *C. kirtlandii*, in which the species was unable to be federally listed due to the lack in overall understanding of both the health of their populations and the potential threats to them (U.S. Fish and Wildlife Service 2017).

Objectives

Established success in the application of eDNA to detect rare and cryptic species across various settings provides a foundation to move forward with eDNA use on other species such as *C. kirtlandii*. To understand eDNA distribution and potential for detection, various environmental samples of water, sediment, and soil were collected for this study. However, additional emphasis was placed on crayfish burrows, as they offer the greatest potential to detect *C. kirtlandii* eDNA. Several aspects of these burrows collect and protect eDNA, increasing the likelihood of detection compared to other microhabitats. *Clonophis kirtlandii* are known to be associated with crayfish burrows for most of the year and the longer an animal spends in an area, the more eDNA is likely to be deposited there (Anton et al. 2003). The burrows are rich in sediment, which is known to have higher concentrations of eDNA compared to the water (Turner et al. 2015). They limit degrative factors by insulating eDNA from high temperatures and UV radiation (Strickler et al. 2015). Crayfish burrows are also discrete and contained microhabitats that can be located and directly sampled. In this study I aimed to complete four primary objectives including 1) the development of an environmental DNA assay that is specific to *C. kirtlandii* that excludes other closely related and co-occurring snakes, 2) determination if *C. kirtlandii* eDNA is readily detectable and quantifiable, 3) determination of the degradation rate of *C. kirtlandii* eDNA in the environment, and 4) generation of a *C. kirtlandii* eDNA sampling protocol that can be used in future *C. kirtlandii* eDNA surveys.

METHODS

Snake Surveys

Field Site

The field location for this study was Muscatatuck National Wildlife Refuge (MNWR) in Jackson and Jennings counties, Indiana (38° 55' 58" N, 85° 48' 32" W). Approximately half of the refuge's 7850 total acres are comprised of bottomland hardwood forest (Sieracki et al. 2002). Upland hardwood forest, agriculture areas, and wet herbaceous areas comprise another 40 percent of the refuge. The remaining 10 percent of the refuge land is composed of upland herbaceous plants, plantations, shrubs, and developed areas. About 2700 acres of MNWR is flooded on an annual basis.

Coverboard Surveys

Records of *C. kirtlandii* encounters through snake surveys conducted from 2008 to 2010 were used to select study sites within Muscatatuck (Evin Carter, pers. comm. 3/29/2017). My surveys utilized artificial cover objects, otherwise known as coverboards, placed in 30-meter intervals within stratified arrays across MNWR. These arrays, or transects, were strategically placed alongside bodies of water to capitalize on *C. kirtlandii* life history. They were also stratified spatially, with clusters of boards across different snake densities at the refuge. The coverboards were placed into 11 transects split into three groups, north (n=4), middle (n=4), and south (n=3) (Figure 1). Transects varied in length from seven to 36 boards, and a total of 193 coverboards were used across the entire study site. All boards were surveyed and maintained on a weekly basis from May into October of 2017 and April into July of 2018. Any coverboards that were lost or that had sustained a loss of 20 percent or more of the material were replaced. All board locations were entered into a Garmin 72H GPS to ensure relocation and accurate replacement of lost boards.

The coverboards were made of 0.95 cm thickness Saturn brand under carpet foam padding with an attached plastic moisture barrier (Figure 2). They were cut into 30x60 cm rectangles for easy handling while remaining large enough to be used as cover for *C. kirtlandii*.

The plastic covering retained moisture absorbed by the foam, creating a warm and moist microhabitat ideal for *C. kirtlandii* (Dr. Vicky Meretsky, pers comm. 3/17/2017). Each coverboard was held to the ground with two 10.80x3.18 cm yard staples to prevent them from being washed away by flooding. All cover objects were marked using a stencil and flat black Rust-oleum Painter's Touch 2x Ultra Cover Paint+Primer. Markings included the license number for this study, the name of the license holder, the university supporting this study, and the words "do not disturb". At the start of each transect, time, temperature, and cloud cover weather were recorded. Temperature was recorded via basic alcohol thermometer and cloud cover percentage was visually estimated. Time duration to complete the survey transect was also recorded.

During the first season, snakes encountered were marked by using stainless steel scissors to clip a square from the ventral scale anterior to the divided anal scale. Snakes encountered in the second season were individually marked with ventral scale clipping (Brown and Parker 1976). Additional tail clippings were taken from *C. kirtlandii* during 2018 by snipping off a half centimeter of tissue from the distal end of the tail. Clippings from marked snakes were placed in a sterile 5 mL tube filled with 95 percent ethanol, and stored at -80°C until needed for DNA extraction. Between individuals, the scissors were cleaned with alcohol wipes and flame sterilized. Tissue samples from laboratory collections were harvested and preserved in the same manner as tissue samples from living individuals. Samples were also collected from ventral scale clips of co-occurring close relatives of *C. kirtlandii*, including Dekay's Brownsnake (*Storeria dekayi*), Northern Watersnake (*Nerodia sipedon*), Copperbelly Watersnake (*Nerodia erythrogaster*), Gray Ratsnake (*Pantherophis spiloides*), and Gartersnakes (*Thamnophis sirtalis*, *T. sauritus*).

Feces Collection

At the start of each field season, a maximum of 10 *C. kirtlandii* were captured and held in captivity for up to two weeks and monitored closely with multiple daily checks in order to attain ejecta from them. Ejecta includes waste products such as urates and feces, shed skin, and material resulting from parturition. The material was collected using sterilized forceps or disposable pipettes and placed in sterile 10mL tubes containing 5 mL of distilled water. The samples were weighed and stored in a -80°C freezer until extraction or further use. Large samples were collected using disposable gloves and spatulas.

Snakes were housed individually in plastic tubs measuring 58x41x15 cm. The tubs, with weighted lids, were placed on metal wire shelving racks (Figure 3). A hide and a water dish were placed in each tub. Water dishes were cleaned as needed and water was provided ad libitum. ZooMed RH-7 mini heat pads were placed under one end of each tub and used to maintain a temperature of about 30°C. The other end of the tubs were left at room temperature, approximately 24°C. Once sufficient ejecta had been collected, or after a snake had been held for two weeks, snakes were returned and released at their capture site. All housing procedures were conducted according to Indiana DNR permits and approved Purdue Animal Care and Use Committee protocol (PACUC).

Habitat Data

A basic set of habitat variables were collected at coverboards during June of the second field season to see if any had a significant influence on the presence of *C. kirtlandii*, and thus, the presence of their eDNA. These variables included canopy cover, ground cover, dominant vegetation, and distance to a set of landmarks. Canopy cover was recorded using a densiometer and following standard protocols to average total coverage from all cardinal directions (Lemmon 1956). The distances to logs with a diameter greater than 10 cm, trees greater than 5 cm diameter at breast height (DBH), and shrubs were measured up to a distance of 30 meters. Ground cover categories included water, rock, bare ground, leaf litter, grass, herbaceous vegetation, and shrub. Ground cover percentage was estimated using a modified line transect method by extending a meter tape three meters in each cardinal direction and recording the ground cover in a 30 cm path for each (Herbeck and Larsen 1999, Greenberg 2001, Sutton et al. 2013). Similar to canopy cover, the total ground cover percentages were averaged between all cardinal directions. Habitat data was only collected at even-numbered coverboards, starting at the second one in each transect. This resulted in data only being collected from 96 coverboards instead of all 193. This was done to reduce effort needed to collect data, but still retain enough samples for statistical analysis.

Assay Design

A primary objective of this study was to develop a quantitative polymerase chain reaction (qPCR) assay that is species-specific to *Clonophis kirtlandii* and amplifies a fragment of

mitochondrial DNA (mtDNA). The assay developed must also be valid for use with environmental samples that contain low copy numbers of DNA. Following the recommendations of (Bustin et al. 2009), all qPCR assays were generated with observation of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. The purpose of these guidelines is to give qPCR results more validity and allow for replication of the experiment. Analysis of DNA sequences, primer selection, and qPCR assay design was aided by the use of GenBank, Primer-BLAST, and Primer3 from the National Center for Biotechnology Information (NCBI,) the program Geneious (Biomatters Ltd.), and the PrimerQuest Tool from Integrated DNA Technologies (IDT).

In order to design an assay specific to *Clonophis kirtlandii*, the cytochrome oxidase subunit I (COI) gene of the mitochondrial genome was selected to exclude other closely related and co-occurring Natricine snakes. COI has been shown to be highly variable between species, but far less so within, allowing for specificity between closely related co-occurring species. (Hebert et al. 2003, Hebert et al. 2004, Hajibabaei et al. 2007). *In silico* testing was conducted using snake COI sequences from GenBank. The sequences were aligned in Geneious and assessed for differences in base pairs. A mismatch of at least one base pair between aligned *C. kirtlandii* and non-target species was required for an assay to be considered for further testing. The species list and GenBank ascension numbers are as follows: *Clonophis kirtlandii* (KU986171.1), *Storeria dekayi* (KU985887.1), Redbelly Snake (*Storeria occipitomaculata*) (KU986005.1), *Nerodia sipedon* (KU985556.1), *Nerodia erythrogaster* (KU986256.1), *Pantherophis spiloides* (FJ627806.1), Eastern Foxsnake (*Pantherophis vulpinus*) (KU986157.1), Eastern Milksnake (*Lampropeltis triangulum*) (KU985694.1), Queensnake (*Regina septemvittata*) (KU985947.1), Smooth Earthsnake (*Virginia valeria*) (KU985953.1), *Thamnophis sauritus* (KU985906.1), Western Ribbonsnake (*T. proximus)* (KU986029.1), Plains Gartersnake (*T. radix*) (KU986268.1), *T. sirtalis* (KC750818.1), and Butler's Gartersnake (*T. butleri*) (KU985778.1).

Primer-BLAST and Integrated DNA Technologies (IDT) PrimerQuest Tool were used to generate 10 potential primer sets as well as their complimentary probes. Primer and probe design closely followed the NCBI and IDT guidelines for designing primers and probes. After the candidate primers were designed, they were put into the IDT OligoAnalyzer Tool to analyze their melting temperature (T_m), guanine and cytosine content (percent GC), primer hairpin (ΔG),

and primer dimerization (ΔG). Primers designed were 15-25 base pairs (bp), would create a 70-200 bp amplicon, had a GC percentage between 40-60, and had a T_m of 57- 63°C with a maximum of 2°C difference between primer pairs. They also had at least one mismatch between it and non-target species to limit cross amplification. Other selection parameters for primers were a ΔG (kcal/mol) more positive than -9.0 to limit hairpin formation and dimerization. Hairpins prevent primers from functioning properly and dimerization causes spurious amplification. Once a primer set was established, a complimentary probe was generated in PrimerQuest and evaluated by OligoAnalyzer for the same parameters as the primers. However, the probe parameters were slightly modified, with the T_m 6-8°C higher than the primers, GC content between 35-65 percent, annealing temperature (T_a) less than 5°C below the primers, and a ΔG (kcal/mol) more positive than -9.0. The probe was also not to overlap with either the forward or reverse primers. The primers and probe were ordered as DNA oligonucleotides from IDT and the probe utilized a FAM fluorescent dye and ZEN quencher.

Once a set of candidate primers were developed, *in vitro* testing was conducted with tissue derived DNA extracts from both *C. kirtlandii* as well as the other closely related Natricine snakes, including *S. dekayi, S. occipitomaculata, N. sipedon, T. sirtalis,* and *T. sauritus*. Primer specificity without a probe was first visualized using gel electrophoresis on a three percent agarose gel with a 100 bp ladder to test for proper amplification and specificity. The primer pair that exhibited the highest amplification and specificity on the gel then moved forward to be used with a complimentary probe to test the qPCR assay against the same species. PCR products from two positive field samples, tissue extractions, and a synthetic DNA template were also sent to be Sanger sequenced by Molecular Cloning Laboratories (MCLAB) to confirm assay efficacy and specificity. *In situ* assay performance was tested with DNA copy number standards included on most qPCR plates when processing eDNA samples. DNA standards consist of a series of known DNA copy numbers that allow for the assessment of assay efficiency and quantification of starting copy number in an eDNA sample.

Environmental DNA

Environmental DNA Sample Collection

To assess the detection of *C. kirtlandii* eDNA in the field, two survey transects were selected for use as eDNA sampling sites. Approximately two months of coverboard surveys were conducted in 2017 to assess rough *C. kirtlandii* abundance at each site before the high and low eDNA transects were chosen. The two eDNA sample sites included one of relative high snake abundance (S3) and the other with low snake abundance (M1). Environmental DNA samples were then taken from every other coverboard at both transects during both field seasons. Due to low snake captures in the 2018 season, a parallel set of samples were also taken spaced 30 meters away from the coverboards. The eDNA samples included burrow water and sediment, soil from under artificial cover objects, and open water. During 2017, spring, summer, and fall sampling events were conducted to test for seasonal variation in eDNA presence and a spring resampling event took place in 2018. Samples were collected May 30th, July 26th, and October 25th in 2017 and on May 23rd in 2018.

To draw water and sediment from crayfish burrows, a sterile 60 mL Luer-Lok syringe was used (Merkling 2018). This was achieved by using an Addto Inc. catheter adapter to attach a one meter section of 3.175 mm diameter flexible Fisherbrand clear PVC tubing to the syringe (Figure 4). The tube was guided as far as possible down the burrow and used to draw a 50 mL sample of water and sediment. The sample was then placed into a sterile 50 mL Thermoscientific conical nunc tube. At the same borrows as the water samples, a 5-gram sample of sediment was collected using a sterile Fisherbrand 22.86 cm length spatula and placed into a separate 50 mL tube. A 5-gram sample of soil was also scooped from under artificial cover objects and placed into a 50 mL tube. All tubes, syringes, and spatulas were disposed after their first use. Finally, 500 mL of water was sampled from open surface water. Open water samples were collected by immersing a sterile 500 mL polypropylene Nalgene sample bottle into the water until full. Care was taken to minimize perturbation of the sediment when collecting open water samples. After collection, all samples were placed into a cooler filled with ice for transportation back to the lab where they would be refrigerated and filtered within 24 hours. Between sites, footwear was cleaned, then sterilized by immersion in a 10 percent bleach solution for 10 minutes. Whenever

possible, walking in the water and mud was avoided to limit the potential collection and transport of materials containing eDNA to the next site.

Negative and positive field control samples were included with each day of sampling effort. The negative field control consisted of a 50 mL sample tube of autoclaved reverse osmosis water brought into the field along with empty sample tubes. From that point on, it was treated as an environmental sample. The positive field control was created by placing the collected feces from one *C. kirtlandii* into a crayfish burrow water and sediment sample.

Habitat data was collected from around each eDNA sample. The predominant vegetation in a 10 meter radius was recorded. Distance to the nearest shrub, trees greater than 5 cm diameter at breast height (DBH), road, and open water were recorded up to 20 meters away using a meter tape. The number of crayfish burrows in a 1.5-meter radius was also recorded. A ruler was used to measure crayfish burrow diameter and chimney height. Burrows were also labelled as capped or open. After each water sample, a second sample of water was taken and placed into a plastic cup and a Xylem Pro1020 YSI meter was used to collect data on temperature, pH, and dissolved oxygen. All eDNA sampling points were marked with a flag and the location was logged in the Garmin 72H GPS.

Environmental DNA Degradation Study

Clonophis kirtlandii eDNA degradation was field tested using previously collected snake feces to spike artificial crayfish burrows. A coverboard transect with no records of *C. kirtlandii* presence was selected as a degradation study site (M4). Following the coverboard transect along the edge of the water, a meter tape was used to place 10 artificial crayfish burrows at 10-meter intervals, skipping points that aligned with the 30-meter coverboard spacing. To create the artificial burrows, 3.81 cm diameter PVC piping was cut to lengths of 76.2 cm and then a post-hole digger was used to bury those pipes in the ground (Figure 5). Opposing pairs of 3 mm holes were drilled into the pipes at 8 cm intervals and the bottom of the pipe was left open to allow for water to fill the pipe. The top of the pipes remained exposed, resembling the chimney of a crayfish burrow. Vents were added to keep animals from entering and becoming trapped in the pipes, but still allow for rainwater to enter. The pipes were numbered and their GPS location was logged. They were allowed to settle and fill with water for two weeks before the degradation experiment began. A collected sample of *C. kirtlandii* feces was added, or spiked, to each of the

pipes before nine samples sets were collected from each of the 10 pipes. They included a pre-spike sample and a post-spike sample three hours after the addition of feces. Subsequent samples were collected at day one, two, three, 10, 17, 25, and 31 after spiking the artificial burrows. Sampling followed the same methods used with crayfish burrows to collect paired water and sediment samples. Temperature, pH, and dissolved oxygen were also recorded. Sediment samples were analyzed both before and after being concentrated in a Zymo DNA Clean & Concentrator-5 kit.

DNA Extraction

DNA extraction from environmental samples utilized a modified CTAB (cetyltrimethylammonium bromide) and Sevag (chloroform:iso-amyl 24:1 alcohol) protocol developed by Coyne et al. and used by Turner et al. (Coyne et al. 2001, Coyne et al. 2005, Coyne et al. 2006, Turner et al. 2014b, Turner et al. 2015). Five grams of 360,000 molecular weight polyvinyl-pyrrolidone (PVP) was added per 500 mL of CTAB to mitigate potential inhibition through plant-based polyphenols during qPCR (Renshaw et al. 2014). Once in the lab, crayfish burrow environmental samples were centrifuged at 3000 RPM at 4°C for 10 minutes to separate the suspended sediment from the water (Merkling 2018). The supernatant was then passed through a Nalgene 150 mL rapid flow filter unit with a 0.2 μm PES membrane to separate eDNA from other suspended materials (Turner et al. 2014a). The 500 mL open water samples were passed through a Nalgene 500 mL rapid flow filter unit with a 0.45 μm PES membrane in order to capture eDNA, but prevent clogging issues with 0.2 μm filters at larger volumes (Coyne et al. 2001, Eichmiller et al. 2015). All filtration utilized a Barnant Company vacuum pressure station generating 10-35 PSI to pass the total volume of water through the filtration membrane. Forceps were used to tear, fold, and place the membranes into a 2 mL microcentrifuge tube before adding enough CTAB buffer solution to completely cover them. Between filters, forceps were immersed in 10 percent bleach, rinsed with distilled water, and flame sterilized via Bunsen burner. Sediment and soil samples were processed by the addition of 2 mL of CTAB buffer per gram of sediment (Coyne et al. 2001).

All samples remained immersed in CTAB for two weeks at room temperature to maximize DNA extraction (Renshaw et al. 2014). After two weeks, the samples were run through their respective sediment or water extraction process (appendices A and B). After

extraction, samples were placed into a -80°C freezer for storage until needed for qPCR analysis. Negative controls of autoclaved reverse osmosis water were added to each extraction set to determine potential contamination occurrence and distinguish between collection-based and extraction-based contamination. Crayfish burrow water and sediment samples were also passed through a Zymo DNA Clean & Concentrator-5 kit. I followed the 5:1 binding buffer to sample ratio DNA fragment protocol in order to concentrate DNA present in those samples. The kit utilized a 95 percent ethanol wash buffer solution and final sample elution was 15 µL of buffer.

Tissue samples of *C. kirtlandii* and non-target species were included to test for cross amplification of the eDNA assay (described below). These extraction were done with a Qiagen DNeasy Blood and Tissue Kit following the standard tissue extraction protocol. After extraction, they were all subsequently diluted to 0.1 ng/µL for use in PCR and qPCR. This allows for comparable amplification, since all tissue extracted DNA was at the same starting concentration. A set of extractions were assessed for DNA copy number with a Qubit 3.0 fluorimeter, using double stranded broad range (dsBR) reagents and standard Qubit protocol.

Quantitative PCR Protocol

All 15 µL qPCR reactions were conducted on 96 well Thermoscientific AB-2800/W qPCR plates and contained 0.3 µM of each primer and 0.2 µM of the probe. A 1x concentration of reaction mix (BioRad SSoAdvanced Universal Probes Supermix), Internal Positive Control master mix, and IPC DNA (Appliedbiosystems TaqMan Exogenous Internal Positive Control) was also added. To this, 2 µL of DNA was injected to reach the desired volume in each well. The IPC was added to detect the presence of any inhibitors in the reaction that would reduce the replication efficiency, potentially causing false negatives. All reactions were done in triplicate to improve detection and negate the effects of well-to-well variation. The reaction conditions started with a 95°C denaturation for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The reactions were conducted on a BioRad CFX96 Real-Time System and FAM (probe) and VIC (IPC) fluorophores were used.

Negative and positive controls were added to each plate to determine contamination and to check proper reaction function respectfully. The qPCR plates also contained a series of DNA standards made from a 135 bp synthetic dsDNA oligo ordered from Integrated DNA Technologies diluted to known quantities in triplicate. There were two standard dilution series

used. The first contained dilutions at the following copy number per µL: $1x10^0$, $1x10^2$, $1x10^4$, $1x10^6$, and $1x10^8$. The dilution series was later altered to contain the following copy number per µL: $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, and $1x10^6$. The addition of DNA standards at known concentrations allow for the calculation of reaction efficiency and R^2 values. The limit of detection (LOD) is defined by the MIQE guidelines as the lowest concentration that 95 percent of samples are positive (Bustin et al. 2009). At $1x10^2$ starting copies, there was 50 percent detection (93 replicates), while there was 100 percent detection at $1x10^3$ starting copies (9 replicates). Thus, a theoretical LOD was calculated from the standards using the following calculation: LOD=3.3 x standard deviation of regression line/slope of regression line (Shabir 2003, Shrivastava and Gupta 2011).

All surfaces were decontaminated with 10 percent bleach prior to use. All qPCR tubes and pipettes were comprised of low DNA binding material and pipettes were fitted with aerosol barriers to minimize DNA lost during qPCR preparation (Ellison et al. 2006). In order to prevent errors in pipetting small volumes of replicate reactions, all of the reagents needed for qPCR reaction for an entire plate was first mixed in a microcentrifuge tube. The appropriate volume of reagent mix was then transferred into each well using a repeating pipettor to minimize well-to-well variation (Turner et al. 2014b). Handling of qPCR reagents and DNA were conducted in separate rooms across the building from reach other. The qPCR machine was also located in a separate room.

Statistical Analyses

Binary logistic regression was used to test if any of the habitat variables measured had a significant impact on the presence of *C. kirtlandii* at the 96 coverboards examined. Before the regression was applied, variables with low sample sizes were removed from further analysis. These were ground cover water, bare dirt, and rock. The remaining variables (canopy cover, distances to log, tree, shrub, and ground cover of leaf, grass, herbaceous, and shrub) were then placed into the regression using backward stepwise (Wald) removal. This analysis removed the least influential variable one at a time until a best fit model was found. The binary response was zero if a snake was never captured at a board and one if a snake was captured at a board.

A general estimating equation (GEE) was used to detect any significance that transect group, date, time of day, temperature, and cloud cover had on the number of snakes captured.

Generalized estimating equations are an extension of generalized linear models that allow for repeated measures of subjects. I chose to use the negative binomial model due to large numbers of zeros in the dataset, which resulted in overdispersion (large variance compared to the mean count). An AR(1) correlation matrix was used with the model, which assumes higher correlation between elements that are closer to each other. This matrix works for variables like temperature that are related to time of day or seasonal variation, as they become less correlated over time. Transect group (n=3) and year (n=2) were used as factors. The south transect group and year 2018 were used as reference categories, so they were set to zero in the model. Day of year, survey start time, temperature, and cloud cover were used as covariates. Total snake captures were used as the dependent variable.

Only a single positive detection was recorded from all the eDNA field samples, thus no statistical analyses were performed on those samples. For eDNA degradation testing, a general estimating equation was used with a binary logistic model to test what variables had a significant impact on eDNA detection in the concentrated sediment samples. Again, a GEE was used since repeated samples were collected from the artificial burrows. Before the model was ran, a correlation matrix of independent variables was created to assess multicollinearity. Temperature, pH, and dissolved oxygen were removed from further analysis due to high correlation with water volume. Pipes were used as the subject in the model (n=10) while days, feces weight, and water volume were used as covariates. The dependent variable used was positive eDNA detection. An unstructured working correlation matrix was selected for this model to allow for correlation between repeated measures, but not constrain the relationships between the variables. All statistical analyses were performed on IBM's statistical package for the social sciences (SPSS) (IBM Corporpation 2017).

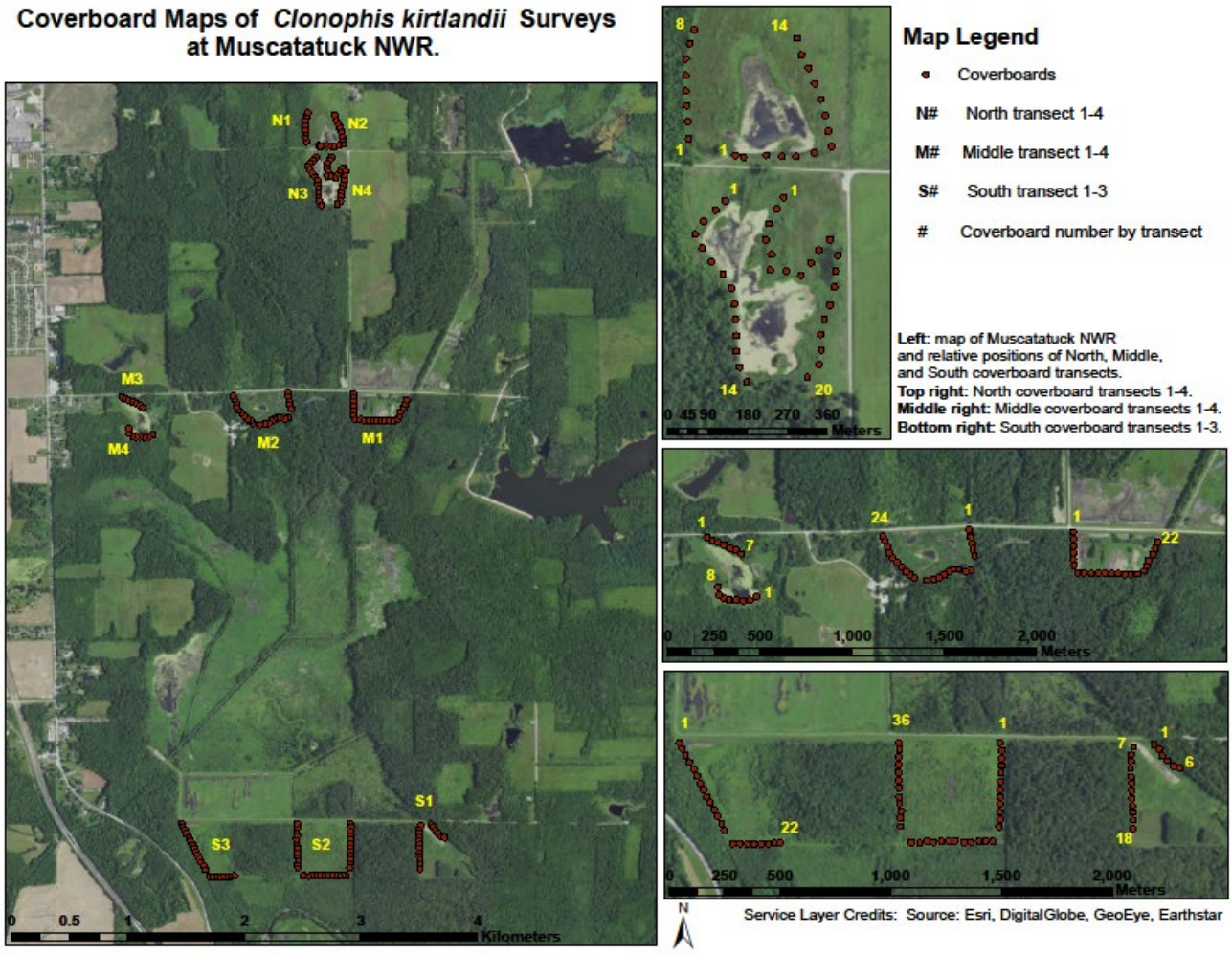

Figure 1. Aerial map of coverboard transects at Muscatatuck NWR. Transects are in northern (top right), middle (middle right), and southern (bottom right) groups. The south transect group had the greatest number of snake captures. The large open wetland complex just north of the south transect sets may be a cause of higher snake abundance at those transects.

Figure 2. Foam coverboards used for *C. kirtlandii* surveys.

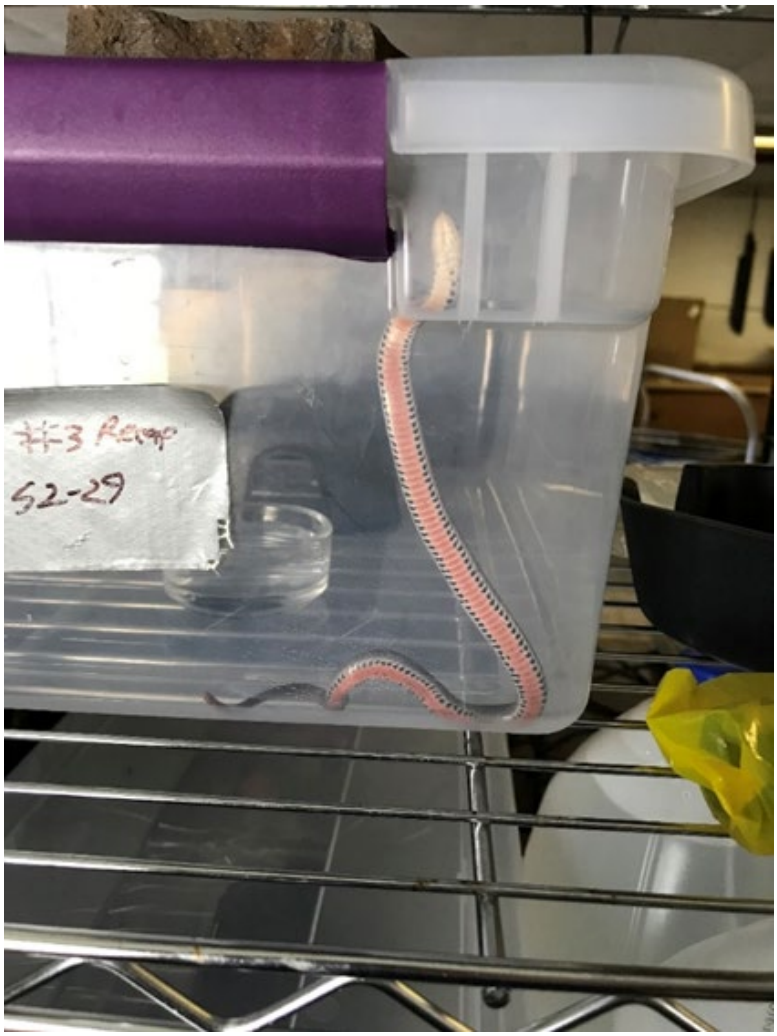

Figure 3. *Clonophis kirtlandii* housing setup for feces collection. All snakes were provided with a hide, water bowl, and heat pad. Sterilization occurred before and after each use.

Figure 4. Syringe with catheter adapter and hose used to collect crayfish burrow samples.

Figure 5. Artificial crayfish burrow used for eDNA degradation testing. To simulate real crayfish burrows, they were positioned alongside water and placed as deep as possible into the ground. Each pipe was capped with a vent to prevent animals from entering, but allow for air and water to move freely.

RESULTS

Coverboard Surveys

Clonophis kirtlandii abundance varied both temporally and spatially in this study (Figures 6 and 7). Transect group ($p<0.0001$), year ($p<0.0001$), and day of year ($p<0.0001$) were related to the number of snakes captured in the GEE of coverboard survey variables (Table 1). The middle and north transect groups, in addition to day of year, had negative coefficients with snake captures while year 2017 had a positive coefficient with captures.

In 2017, 25 surveys were conducted from May to October. This resulted in 134 total *C. kirtlandii* being captured. Of that total, only 22 snakes (16.4 percent) were recaptures. In 2018, 17 surveys were conducted, resulting in only 20 snakes captured. Of that, only one snake (5 percent) was a recapture. Late spring was the most successful time of the year. The greatest number of snakes found in a single day was 42 on the 23 of May in 2017. Due to low overall snake numbers in 2018, no peak was observed that year.

Spatially, *C. kirtlandii* were relatively more abundant in the south group of transects compared to the middle and north transect groups. Between the two survey seasons, 134 snakes were captured in the three south transects while only 20 snakes were captured in the other eight transects. Within the south group, transects two and three had the greatest overall number of snake captures at 57 and 49 respectfully.

Before use in the binary regression, three habitat variables were removed due to high zero counts out of the 96 boards measured. These were ground cover percentage of water (n=4), bare ground (n=10), and rock (n=6). In the final model, only three habitat variables remained statistically significant, with a higher probability of snake presence with closer distance to shrub ($p=0.041$), and higher ground cover of grass ($p=0.044$) and herbaceous vegetation ($p=0.048$) (Table 2). Distance to log and percent ground cover shrub were included in the final model, but were not statistically significant ($p=0.065$, $p=0.055$).

Assay Design

All primer pair candidates showed either high dimerization or cross amplification with non-target species. To find an effective primer pair, all forward and reverse primers were cross

matched to design a new primer set. The 20-base forward primer selected was 5′-TCC CCT TGT TCG TTT GGT CA-3′ (T_m=59.5°C) and the 19-base reverse was 5′-CAC CTC CGC ATG GAT CGA A-3′ (T_m=59.8°C). This set had the lowest dimerization, with the most negative ΔG at -7.13 kcal/mol, and showed the least cross amplification during gel visualization. They produced a 135 bp amplicon spanning from 426-560 of *C. kirtlandii* COI gene. Within this, the 25-base probe selected was 5′-ACC GAC CGA AAC ATT AAC ACC TCC TT-3′ (T_m=68.0°C) and fit at the three prime end without overlapping the reverse primer.

During *in vitro* testing with gel electrophoresis, the *C. kirtlandii* primer set had cross-amplification with *S. dekayi*, *T. sauritus*, and *T. sirtalis* (Figure 8). The addition of the probe during qPCR did not fully resolve this (Figure 9). However, the non-target species amplified at a far later cycle than *C. kirtlandii*. With 0.1 ng/μL tissue samples, the non-target species were crossing the detection threshold at approximately 40 cycles. To eliminate false positives in qPCR results, a cutoff point was set at cycle 40. Any amplification after this cycle would be classified as spurious, thus still considered a negative.

The average amplification efficiency of the assay was 80 and the assay had an R^2 of 0.899. A standard curve equation of y=-3.9774x+46.498 was created by plotting starting quantities of the DNA standards against the Cq values for all of the plates ran (Figure 10). Utilizing the standard curve equation and the limit of detection calculation, the LOD was estimated to be 166 starting copies of *C. kirtlandii* DNA. Sanger sequencing results confirmed amplification of *C. kirtlandii* DNA.

Environmental DNA Surveys

A total of 380 eDNA field samples were collected from transects S3 and M1 between the two field seasons. In 2017, three sampling events (May, July, October) collected 20 samples of each of the five sample types (burrow water, burrow sediment, open water, coverboard soil, and burrow soil), resulting in 300 samples. An additional 40 samples of both crayfish burrow water and sediment were collected in May 2018. Out of all samples, only one crayfish burrow sediment in May of 2017 at the S3 transect resulted in a positive detection. Two open water samples in July of 2017 had one amplification out of three, but these were not considered positive detections. Since the only detection occurred in a crayfish burrow sediment sample, they were concentrated in a Zymo DNA Clean & Concentrator-5 kit and reanalyzed. The lone positive

remained as such and no new detections occurred. Over 50 percent of the two soil sample types, under coverboard and burrow lip, resulted in inhibition. Due to the high number of samples and the high rate of inhibition, these samples were considered not fit for concentration and reanalysis. All field positives of feces resulted in positive detections in both water and sediment samples. Additionally, no detections occurred in any field or lab negatives.

Environmental DNA Persistence

During the degradation study using artificial crayfish burrows, eight sample sets over time were collected, including one prior to spiking *C. kirtlandii* feces into the pipes. Due to natural water fluctuation, the day 17 sample set was not able to be collected as the pipes completely dried out that week. One feces sample did not have a recorded weight, so the average weight from the other nine feces samples was used as a substitute (Table 3). This allowed for all 10 pipes to be used in the statistical analysis. No water samples or pre-spike samples had a positive detection. Among all 10 pipes, sediment samples had nine positive detections from post-spike to day three (Table 4). Sediment samples were subsequently selected to be concentrated with the Zymo DNA Clean & Concentrator-5 kit and reanalyzed. This increased positive detections to 22 and detections occurred up to 10 days post-spike. While not considered positive detections, one or two replicate amplifications out of three did occur at low numbers up to 31 days post-spike for both concentrated sediment and water samples. The correlation matrix revealed high correlation between water volume, temperature, dissolved oxygen, and pH (Table 5). Water volume seemed to be the driving factor for the other three variables, so the others were excluded. Of the variables in the GEE, only days post-spike was a was found to predict eDNA presence ($p=0.017$, $\beta=0.094$). Water volume ($p=0.837$) and feces weight ($p=0.544$) did not have an influence on detection in the concentrated sediment samples. Detection probability was plotted using detection means and the β coefficient (-0.225) from a binary logistic regression containing only days post spike as a factor. This found exponential decay in the probability of detection (Figure 12). Detection of eDNA started at 60 percent on day zero, dropped to 50 percent by day two, was only 10 percent on day 10, and reached zero on day 25.

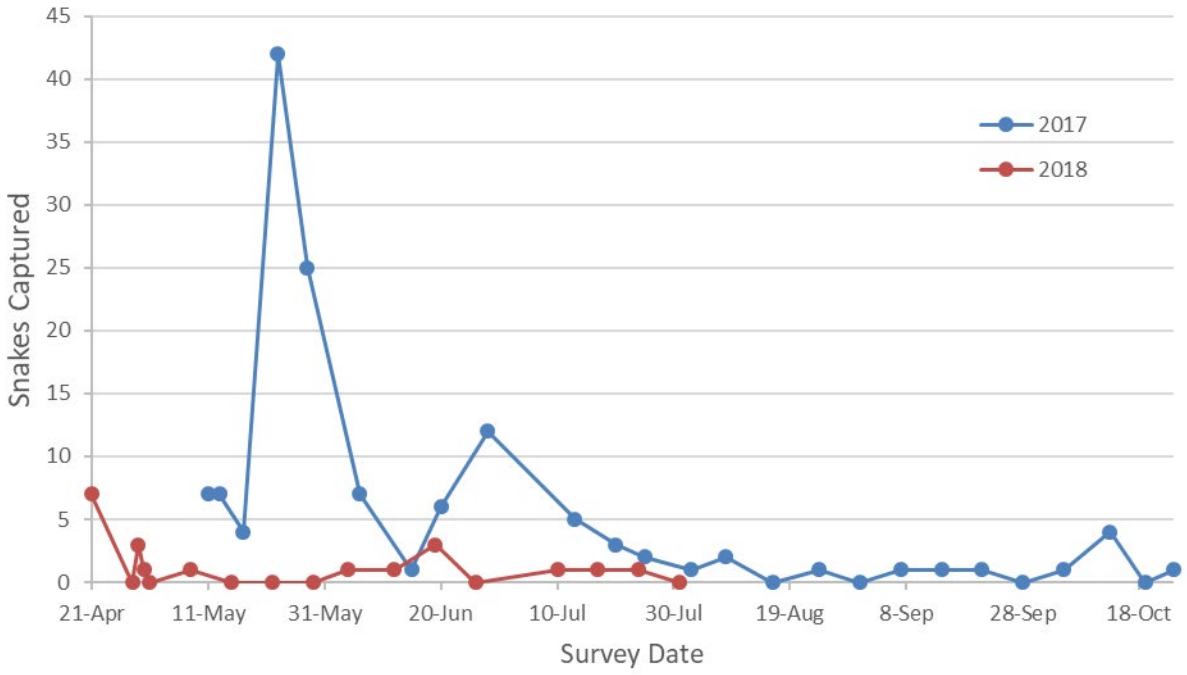

Figure 6. *Clonophis kirtlandii* captures over time from coverboard surveys. A peak in snake abundance was observed on May 23rd of 2017. No peak in snake activity occurred in 2018.

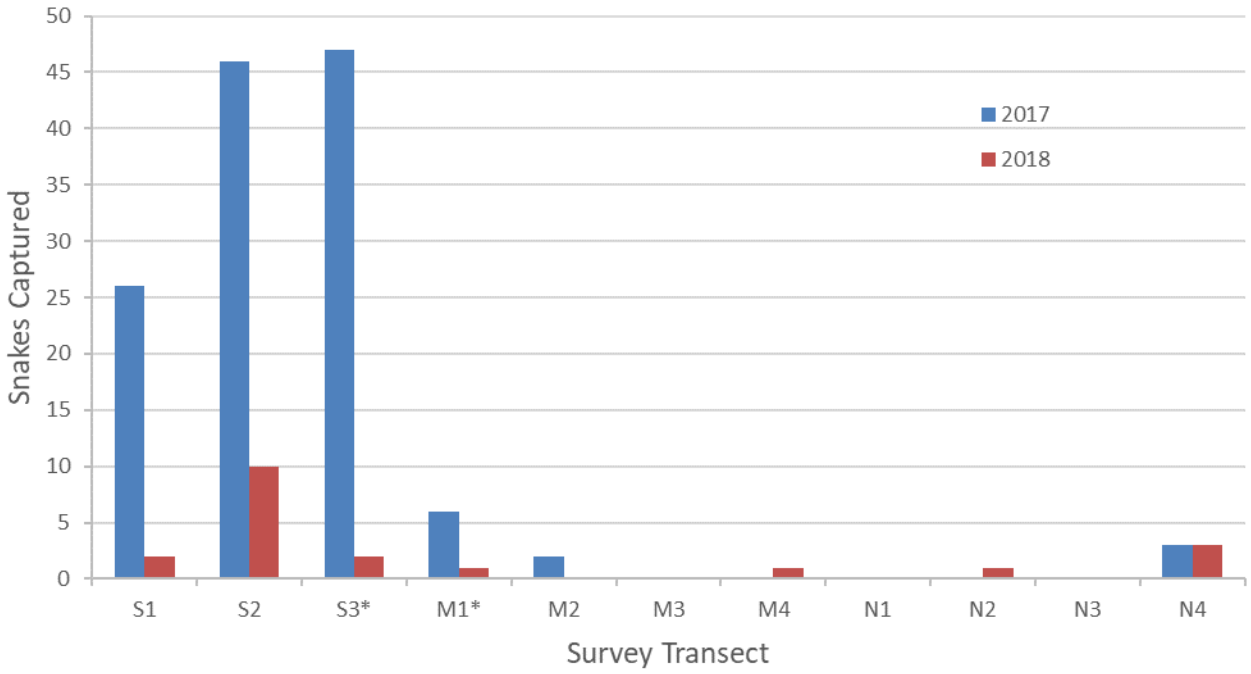

Figure 7. *Clonophis kirtlandii* captures between coverboard transects. More snakes were captured in 2017 and the majority of snakes were captured from the S transects. Asterisks mark transects used for eDNA sampling.

Table 1. Results of generalized estimating equation analysis using a negative binomial model to test the effect of survey variables on snake encounters. Transect group south and year 2018 were set as reference categories, thus were set to zero.

			Interval		Hypothesis Test		
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square	df	Sig.
(Intercept)	0.921	0.9357	-0.913	2.755	0.970	1	0.325
Middle Transect Group	-2.714	0.7335	-4.152	-1.277	13.694	1	<0.0001
North Transect Group	-3.241	0.7053	-4.623	-1.859	21.117	1	<0.0001
South Transect Group	0[a]						
Year 2017	1.977	0.4794	1.038	2.917	17.015	1	<0.0001
Year 2018	0[a]						
Day of Year	-0.020	0.0052	-0.030	-0.009	14.224	1	<0.0001
Survey Start Time	0.123	0.0734	-0.021	0.267	2.793	1	0.095
Cloud Cover (%)	0.002	0.0019	-0.002	0.006	0.890	1	0.345
Air Temperature (°C)	-0.013	0.0157	-0.044	0.018	0.658	1	0.417

a. Set to zero because this parameter is redundant.

Table 2. Binary logistic regression of habitat variables related to snake presence. Percent ground cover water, rock, and bare ground were omitted from the analysis due to high zero counts. The Wald backward method removed canopy cover and distance to tree. In the final model (Step 3), distance to shrub, ground cover grass, and ground cover herbaceous were retained.

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Canopy cover (%)	0.008	0.020	0.156	1	0.693	1.008
	Tree distance (m)	0.028	0.078	0.126	1	0.723	1.028
	Shrub distance (m)	-0.543	0.266	4.168	1	0.041	0.581
	Log distance (m)	-0.103	0.084	1.505	1	0.220	0.902
	Grass (%)	0.340	0.168	4.072	1	0.044	1.404
	Herbaceous (%)	0.331	0.167	3.950	1	0.047	1.393
	Shrub (%)	0.317	0.165	3.706	1	0.054	1.373
	Constant	-31.775	16.597	3.665	1	0.056	0.000
Step 2[a]	Canopy cover (%)	0.006	0.019	0.087	1	0.768	1.006
	Shrub distance (m)	-0.534	0.261	4.198	1	0.040	0.586
	Log distance (m)	-0.083	0.063	1.769	1	0.183	0.920
	Grass (%)	0.322	0.160	4.048	1	0.044	1.380
	Herbaceous (%)	0.314	0.159	3.911	1	0.048	1.369
	Shrub (%)	0.300	0.157	3.669	1	0.055	1.351
	Constant	-29.925	15.715	3.626	1	0.057	0.000
Step 3[a]	Shrub distance (m)	-0.531	0.259	4.187	1	0.041	0.588
	Log distance (m)	-0.094	0.051	3.415	1	0.065	0.910
	Grass (%)	0.316	0.157	4.050	1	0.044	1.372
	Herbaceous (%)	0.309	0.156	3.919	1	0.048	1.362
	Shrub (%)	0.298	0.155	3.684	1	0.055	1.347
	Constant	-29.120	15.264	3.640	1	0.056	0.000

a. Variable(s) entered on step 1: Canopy cover (%), Tree distance (m), Shrub distance (m), Log distance (m), Grass (%), Herbaceous (%), Shrub (%).

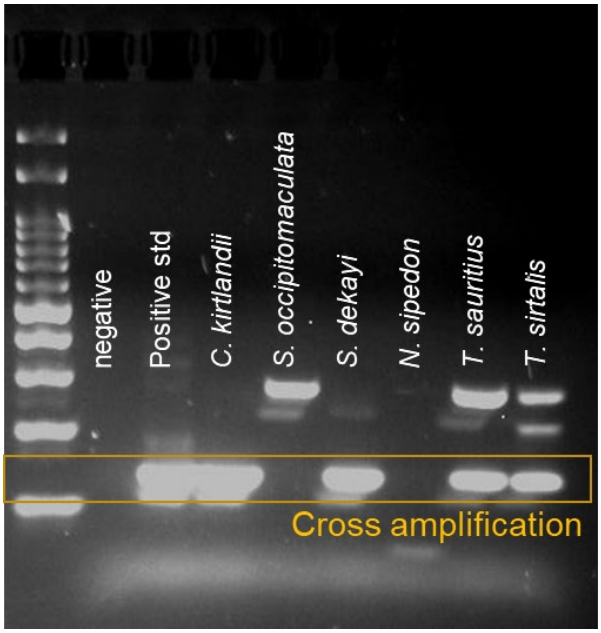

Figure 8. Agarose gel showing amplification of the 135 bp mitochondrial COI locus *in C. kirtlandii* as cross amplification with *S. dekayi, T. sirtalis*, and *T. sauritus*. From the left are a 100 bp ladder, a negative control of double distilled water, a positive control of a double stranded target sequence, and tissue extracted DNA from various snake species. The ladder on the left starts at 100 bp on the bottom and increases 100 bp for each band going up.

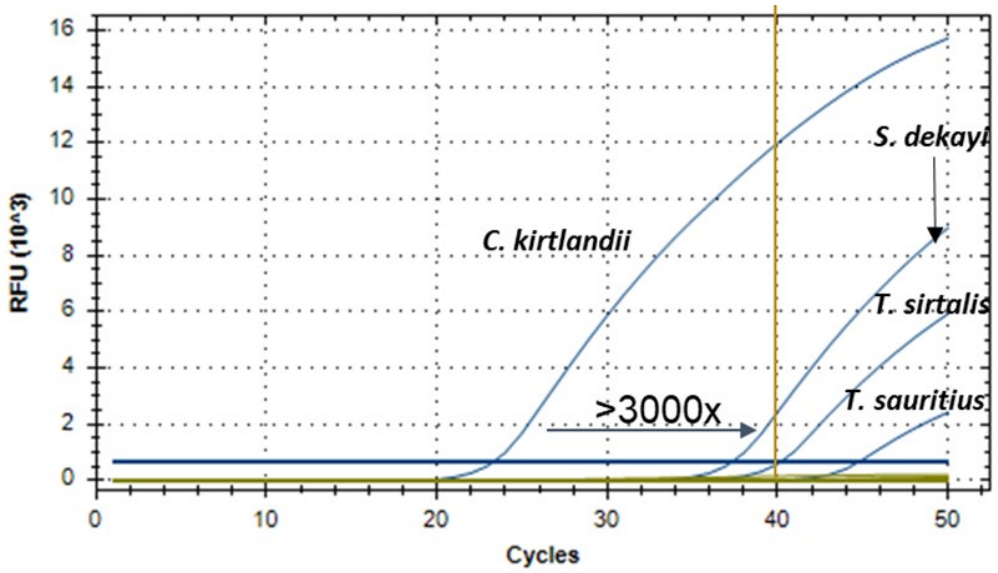

Figure 9. Quantitative PCR amplification of 0.1 ng/µL concentration tissue extracted snake DNA. *Clonophis kirtlandii* DNA amplification occurred well before that of the non-target species *S. dekayi*, *T. sirtalis*, and *T. sauritus*.

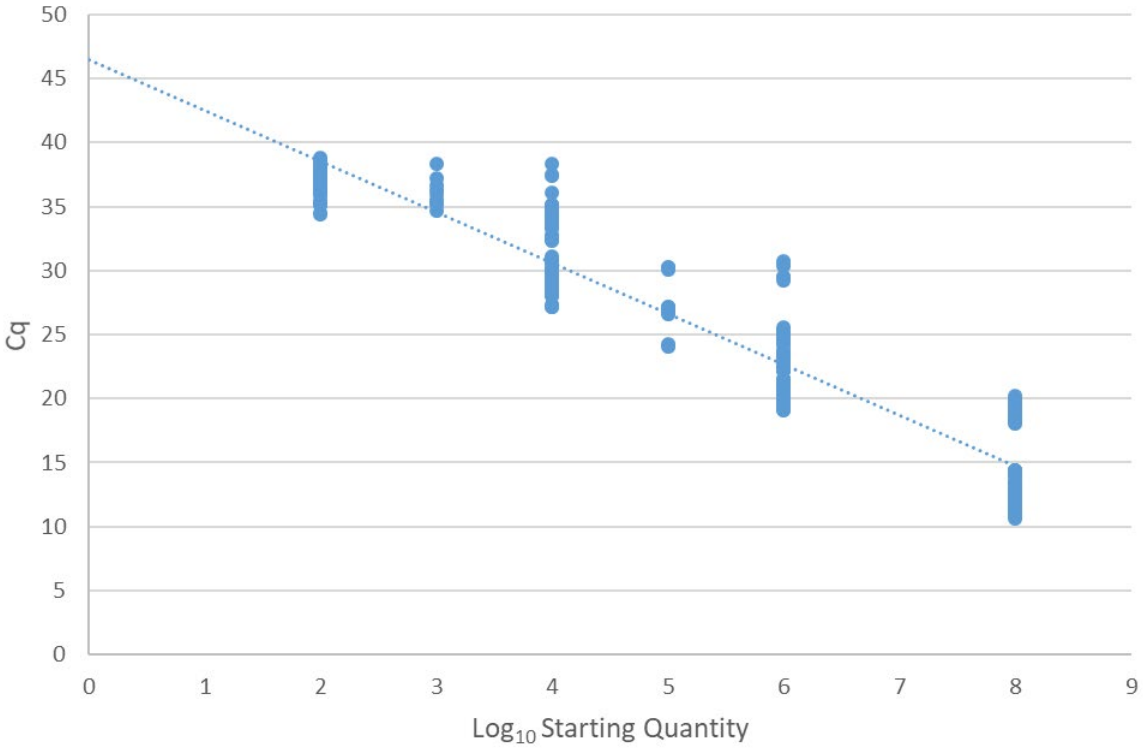

Figure 10. Cumulative standard curve from all qPCR standards. No detections were recorded at or below $1x10^1$ copy number standards. The equation from this standard curve was used in the limit of detection calculation.

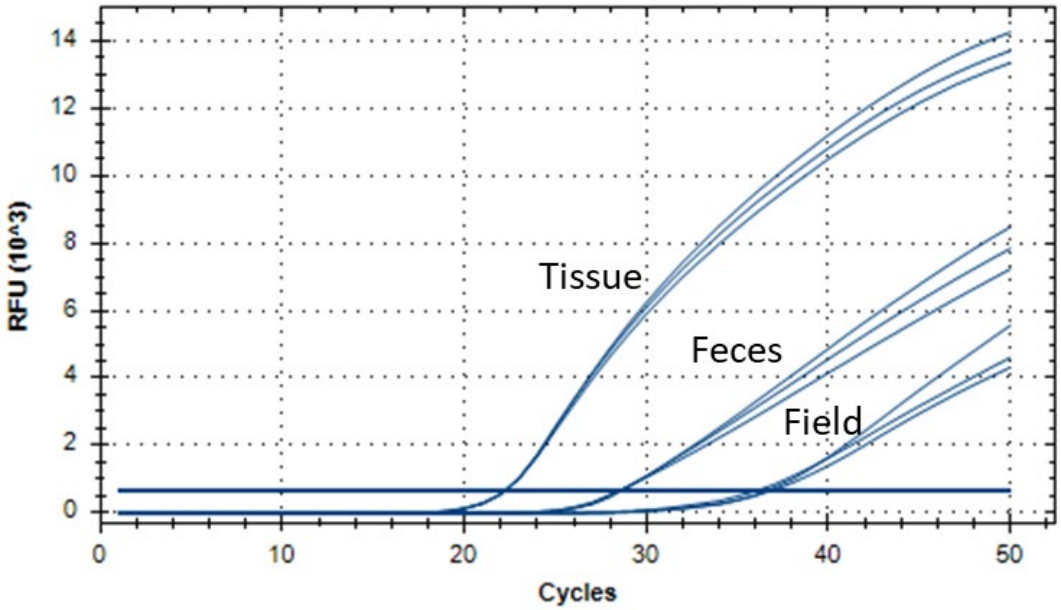

Figure 11. Quantitative PCR amplification of *C. kirtlandii* tissue and feces positives as well as a positive detection of *C. kirtlandii* eDNA from crayfish burrow sediment in spring 2017.

Table 3. Feces weights used for spiking artificial crayfish burrows for degradation study. There was no recorded feces weight for pipe 10, so the average of the other nine feces weights was used.

Pipe	1	2	3	4	5	6	7	8	9	10
Feces Weight (g)	0.05	1.94	0.24	2.5	0.09	0.02	0.11	0.47	1.1	*0.72

Table 4. *Clonophis kirtlandii* detections from artificial crayfish burrows (n=10) in degradation study. Average (+-standard deviation) values of water volume, temperature, dissolved oxygen, and pH are given for each sample day. Samples were unable to be collected at 17 days.

Day of Sample	Water Volume (mL)	Temperature (°C)	Dissolved Oxygen (%)	pH	Burrow Water Detections	Burrow Sediment Detections	Concentrated Burrow Sediment Detections
Pre-Spike	523.4 (+-60.5)	14.1 (+-0.7)	37.4 (+-7.6)	5.9 (+-0.2)	0	0	0
Post-Spike	473.4 (+-60.5)	14.1 (+-0.7)	37.4 (+-7.6)	5.9 (+-0.2)	0	2	4
1	355.7 (+-98.9)	18.3 (+-2.7)	22.3 (+-6.7)	6.1 (+-0.3)	0	3	6
2	296.1 (+-80.1)	18.5 (+-1.9)	22.1 (+-6.1)	6.2 (+-0.3)	0	4	6
3	225.9 (+-65.4)	24.8 (+-1.6)	14.7 (+-5.4)	6.2 (+-0.2)	0	0	5
10	181.7 (+-64.8)	22.1 (+-1.2)	16.4 (+-3.2)	6.3 (+-0.2)	0	0	1
17	-	-	-	-	-	-	-
25	430.3 (+-55.6)	20.1 (+-0.7)	22.2 (+-5.8)	5.7 (+-0.3)	0	0	0
31	251.9 (+-85.9)	30.2 (+-1.1)	15.7 (+-7.8)	6.2 (+-0.2)	0	0	0

Table 5. Correlation matrix of artificial crayfish burrow variables measured (*p< 0.05, **p<0.01).

	Feces (g)	Days Post Spike	Water Volume (mL)	pH	Dissolved Oxygen (%)	Temperature (°C)	Air Temperature (°C)
Feces (g)	1	0.000	0.126	0.050	-0.067	0.018	0.000
Days Post Spike	0.000	1	-0.181	-0.106	-.308**	.640**	.793**
Water Volume (mL)	0.126	-0.181	1	-.556**	.577**	-.695**	-.702**
pH	0.050	-0.106	-.556**	1	-.317**	.403**	.255*
Dissolved Oxygen (%)	-0.067	-.308**	.577**	-.317**	1	-.708**	-.693**
Temperature (°C)	0.018	.640**	-.695**	.403**	-.708**	1	.939**
Air Temperature (°C)	0.000	.793**	-.702**	.255*	-.693**	.939**	1

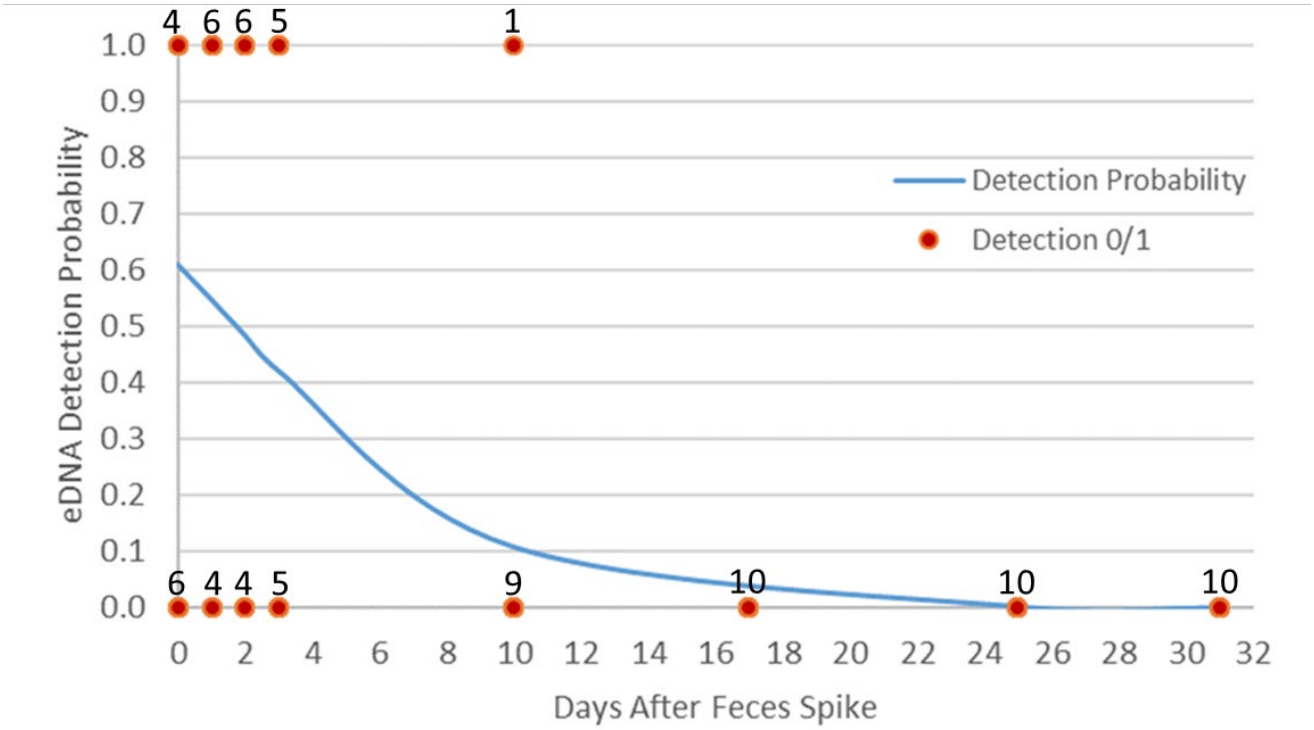

Figure 12. Probability of detecting *C. kirtlandii* eDNA over time from artificial crayfish burrow sediment. Probability was plotted using the mean detection (number of detections out of 10) per day and the β coefficient (-0.225) from a binary logistic regression containing only days post spike as a predictor. Numbered points at zero and one indicate non-detections and detections respectfully.

DISCUSSION

Coverboard Surveys

Temporal Variation

During coverboard surveys, snake abundance varied both temporally and spatially at MNWR. Day of year had a significant influence on seasonal abundance, with a peak during late May. *Clonophis kirtlandii* have been documented mating during spring when water levels are high (U.S. Fish and Wildlife Service 2017). During this time, they are more mobile while searching for mates, which would increase their potential for detection in surveys. This time period aligned well with the peak of snake activity observed in my surveys. Further support that snakes were mating was the common occurrence to find multiple snakes under a single coverboard at this time. In these cases, one snake was often larger than the others. While not definite, this does support the idea that *C. kirtlandii* were mating as females are larger than the males in the species.

Snake abundance was also significantly different from year to year, with far more snakes captured in 2017. This variation may also be tied to water level variation at MNWR. Water levels were higher in 2017, thus closer to the coverboard transects. As with many Natricine snakes, *C. kirtlandii* are often associated with close proximity to water (Wilsmann and Sellers 1988). Dry years or during later summer when water levels are usually low, *C. kirtlandii* are likely to follow the receding water or spend more time in refugia like crayfish burrows. In this case, seasonal and annual low water levels likely caused snakes to move away from the stationary coverboard transects.

Spatial Variation

When analyzing habitat variables, close shrub proximity and the presence of nearby grass as well as herbaceous vegetation were associated with snake presence at any given coverboard. *Clonophis kirtlandii* are generally defined as a wet meadow or marshland species (Conant 1943, Bavetz 1994). These habitats are dominated by grasses and herbaceous vegetation, with shrubs interspersed though it. The *C. kirtlandii* at Muscatatuck seem to follow this general habitat

choice. The survey variable analysis found that coverboard transect group varied in snake captures, with the south group having the greatest chance of encountering a snake. However, the driver for this was not found in the analyses conducted. The habitat variables that influence *C. kirtlandii* distribution are most likely of a larger macrohabitat scale not measured in this study. When viewing satellite imagery of MNWR, there is a large open wetland that encompasses the south transect group as well as between the south and middle transect groups (Figure 1). The middle and north transect groups have far less of this habitat for *C. kirtlandii* presence. As *C. kirtlandii* are so poorly understood, MNWR should provide a valuable field location to further study macrohabitat choice in this species.

Environmental DNA Surveys

Assay Design

Assay design is one of the most vital parts of species detection with eDNA. Cross amplification with non-target species, amplification efficiency, and limits of copy number detection must be considered in order to be successful in the application of this technique. The assay design for this study was not completely optimized as can be seen by the relatively low efficiency, which was only 80 percent. An efficiency of at least 90 percent is desired to ensure the greatest potential for eDNA detection (Svec et al. 2015). Primer and probe concentrations were varied as were annealing temperatures and times, but this rigorous testing to improve efficiency of the assay was unsuccessful. When an efficiency above 80 percent was achieved, there was either primer dimerization or greater cross amplification with non-target species. Redesigning a new primer and probe set would resolve this issue, but it is unknown how much it would improve detection for this species.

eDNA Detection

The results of this study indicate that eDNA does not offer any advantage compared to traditional survey methods. While difficult to find, 154 *C. kirtlandii* were captured in 42 coverboard surveys across the two seasons. Compare that to 380 eDNA samples in four sampling events with only a single detection. Looking at per day effort, there were three to four snakes captured per survey on average using coverboards while there was only one detection in four

sample events with eDNA. Focusing on transects with eDNA samples taken (S3 and M1) and only using survey dates closest to sampling events (May 30th, July 26th, and October 25th in 2017 and May 23rd in 2018), coverboard surveys still remain more effective. On May 28th, 13 snakes were captured at S3 and one from M1. On July 25th, one snake was captured at M1. No snakes were captured at S3 or M1 on October 24th. In the following year, one snake was captured at S3 on May 21st. Out of the four eDNA sampling events, only one detection occurred at S3 on May 30th of 2017. Neither method had high success, but this certainly suggests that coverboards were more effective at detecting *C. kirtlandii*.

The degradation experiment suggests that *C. kirtlandii* eDNA can persist for approximately 25 days in crayfish burrow sediment. However, only one out of 10 replicate burrows resulted in a positive detection at 10 days. For a 50 percent chance of detecting *C. kirtlandii* feces in crayfish burrow sediment, the time is reduced to only one day after feces is deposited. Even on the same day as feces deposition, there is only a 60 percent chance of detection. The degradation rate of *C. kirtlandii* eDNA in artificial crayfish burrows are similar to results from other eDNA degradation studies. A study using Idaho Giant Salamanders (*Dicamptodon aterrimus*) in aquaria found a 99 percent drop in eDNA concentration three days after salamanders were removed and shaded samples only had a 20 percent chance of detection 11 days after salamander removal (Pilliod et al. 2014). A similar aquaria study using American Bullfrogs found an 80 to 90 percent drop in eDNA concentration three days after removal of individuals (Strickler et al. 2015). Another degradation study using captive Burmese Pythons found that their eDNA began to degrade two days after snake removal and was 60 percent degraded after seven days (Piaggio et al. 2013). Low detection probability and high degradation rate of *C. kirtlandii* severely limits the application of eDNA for this species as evidenced by only a single positive detection in 380 field samples.

The low detection rate in this study is also supported by similar results from two recent Eastern Massasauga eDNA studies. These two species have the most similarity among eDNA studies as they have overlapping ranges and are both associated with wetlands as well as crayfish burrows (Szymanski et al. 2015, U.S. Fish and Wildlife Service 2017). Both of the Massasauga studies focused on crayfish burrows and one had two positive detections out of 100 samples (Baker et al. 2018). The other study only had one positive detection out of 60 paired crayfish burrow water and sediment samples (Merkling 2018). While the detection probability was

extremely low, the use of eDNA to detect *C. kirtlandii* should not be completely discarded. Snake and reptile eDNA has shown to be detectable in other field trials in aquatic environments. Both turtles and snakes have been successfully detected with eDNA in field samples (Piaggio et al. 2013, Davy et al. 2015, Hunter et al. 2015).

While low detection in this study could be due to the assay design, it may also be caused by the target species. *Clonophis kirtlandii* is a small reptile, which appears to be unfavorable to eDNA production (Halstead et al. 2017). Reptiles have keratinized scales covering their bodies and snakes shed all of their skin in one event. Compared to amphibians or fish with wet skin that produce copious amounts of mucus, the production of reptilian eDNA from the integument is far less. Feces is another way animals can deposit DNA into the environment. However, *C. kirtlandii* are quite small. Per individual, smaller animals should produce smaller amounts of feces. The largest fecal sample recorded from a *C. kirtlandii* in this study was 2.5 grams and the smallest was only 0.02 grams. With such small amounts of feces being produced by *C. kirtlandii*, the chances that it will degrade or be diluted beyond the ability to detect is likely increased. That alone may lead to the poor ability to detect this species. The influence of body size, and by extension, feces size should be researched further to better understand how it relates to eDNA production and detectability. Additionally, understanding where animals such as snakes prefer to defecate will aid in targeting optimal microhabitats to sample for their eDNA.

Understanding when, where, and how many samples to collect in order to establish thorough surveying is vital to the success of an eDNA study. Time of year with the greatest eDNA output and microhabitats that protect and concentrate eDNA should determine when and where to sample for a species. This has been established for aquatic species such as the Eastern Hellbender, which were found to be most readily detected in September during their mating season (Spear et al. 2015). Similarly, Great Crested Newt eDNA concentrations peak at the end of mating season in June and during the height of larval abundance in July and August (Buxton et al. 2017). An eDNA study on Black Warrior Water Dog (*Necturus alabamensis*) and Flattened Musk Turtle (*Sternotherus depressus*) in an Alabama river basin found a strong seasonal variation in eDNA detectability that correlated with the active season of both species (de Souza et al. 2016). Interestingly, these two species have opposing active seasons, with the water dog active from October to April and the turtle active from May to September. The facets important to maximizing eDNA detection are not yet known and should be the focus of further snake

eDNA studies. Many Natricine snakes overwinter in crayfish burrows and some continue to use them as refugia in the active season, so there is a wide range of times that may provide the optimal chance to detect their eDNA. Perhaps sampling in late winter or early spring when the snakes have been in the burrows for an extended period of time would be the most effective. Maybe sampling during the mating season in spring when individuals are more active would be better. It is also possible that waiting until later summer when water levels are low and eDNA is more concentrated would be best. Unfortunately, the temporal eDNA sampling in this study did not provide any information on that aspect. However, it does highlight the importance of timing when collecting eDNA samples.

Choosing a microhabitat to sample from presents another difficulty when applying eDNA to semi-aquatic species. Species density has been shown to affect detection probability and temperature, pH, UV radiation, and microbe activity have been shown to significantly affect eDNA degradation. High densities, low temperature, low UV radiation, and alkaline conditions all function to extend the persistence of eDNA (Dejean et al. 2011, Pilliod et al. 2014, Strickler et al. 2015). Furthermore, eDNA has been shown to persist far longer in sediment compared to suspended in the water column (Mao et al. 2013, Turner et al. 2015). In theory, crayfish burrows seem to be ideal contained aquatic habitats that capture, preserve, and concentrate eDNA. They are used by snakes, are insulated from temperature fluctuations, shield DNA from UV radiation, and contain a high amount of sediment. When actually tested, these burrows seem to be more hit-or-miss for attaining detections. While sampling both real and artificial crayfish burrows, the water levels fluctuated greatly, sometimes preventing samples from being collected at all. It is feasible that the fluctuating water levels in burrows and the associated physiochemical changes in pH, dissolved oxygen, temperature, and so on actually increase rates of degradation. There was a high correlation found between water volume, temperature, and dissolved oxygen in the artificial crayfish burrows used for the degradation study. Microbe activity has also been shown to have a strong influence on eDNA persistence and crayfish burrows likely contain high number of microbes, but their influence on eDNA in this microhabitat is not known. At this point, little has been done to understand the mechanics of crayfish burrow eDNA. Additionally, we are not even sure if snakes defecate in crayfish burrows, perhaps they prefer to do so elsewhere. That alone could dramatically limit the effectiveness of using this microhabitat to sample from.

The type of sample must also be considered when sampling for eDNA. I attempted to account for this by collecting several different kinds of samples, including two types of water, a sediment, and two different soil samples. Sediment did perform better than water in the degradation portion of this study, which aligns with what is known about eDNA behavior in aquatic environments (Turner et al. 2015). Open water samples did not have positive detections, but there were a couple amplifications recorded during July of 2017. During this time water levels were quite low, which would concentrate DNA. Under certain circumstances, open water samples may be viable for *C. kirtlandii* detections, but that would need to be further studied to understand what those are. Soil samples from burrow lips and under coverboards had high rates of inhibition, thus were not suitable for eDNA use in this study. This was partly due to the large number of samples collected, which made soil a lower priority compared to the other sample types. However, soil can be a viable sample medium if extracted properly (Taberlet et al. 2012).

Conclusions

Few studies have paired eDNA with such extensive surveying by means of traditional methods. To my knowledge, no studies have simultaneously applied eDNA and standard surveys (e.g. coverboards or drift fences) to a species of snake in order to compare their detection efficacy. Doing so in this study allowed for a direct comparison between survey techniques. It revealed that utilizing eDNA surveys on a poorly understood species like *C. kirtlandii* resulted in similar outcomes as traditional survey methods.

Coverboard surveys also allowed for testing variables that influence *C. kirtlandii* abundance. Temporally, more snakes were captured in the spring and during the first field season. Spatially, snakes were far more abundant at the south coverboard transects at MNWR. Snake occurrence was also related to the presence of grass and herbaceous vegetation as well as close proximity to shrubs.

While eDNA may offer advantages over standard surveys in more well-known species, the poor understanding of *C. kirtlandii* life history made surveying for them using eDNA equally as difficult as conducting traditional surveys. This study highlights the need for further research to understand all aspects of *C. kirtlandii* as well as the application of eDNA on semi-aquatic snake species. Whether using traditional methods or eDNA, it seems the ability to detect this cryptic species remains challenging.

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APPENDIX A

eDNA Extraction from Water (Filter) Samples

1. Heat samples in 65°C water bath for 10 minutes.
2. Cool briefly, then add 700µL of Sevag (choloform:iso-amyl alcohol 24:1).
3. Vortex tubes in fume hood and shake at low speed for 5 minutes (until filter dissolves).
4. Centrifuge at 15,000g for 15 minutes at room temperature to separate phases.
5. Pipette of aqueous supernatant (~500µL), being careful to avoid subnatant, into a new 2mL low bind microcentrifuge tube.
6. Add equal volume of 100 percent ice-cold isopropanol (~500µL).
7. Add half volume of chilled 5M NaCl solution (~250µL).
8. Freeze at -20°C for ~1 hour (or overnight)
9. Centrifuge at 15,000g for 15 minutes at room temperature to form DNA pellet.
10. Carefully pour/pipette off the supernatant (pour very slowly if there is no pellet).
11. Add 150µL of 70 percent ethanol, being sure to wash down sides of tube.
12. Centrifuge at 15,000g for 5 minutes, then pour/pipette off ethanol.
13. Repeat steps 11 and 12 once.
14. Air dry residual ethanol.
15. Add 100µL of 60°C warmed low TE buffer
16. Heat for 10 minutes in a 55°C water bath.
17. Vortex gently to resuspend DNA.
18. Store samples at -80°C until needed for qPCR analysis.

APPENDIX B

eDNA Extraction from Soil/Sediment Samples

1. Vortex 50mL nunc tube at highest speed for 30 seconds to mix and suspend sediment.
2. Heat samples in a 60°C water bath for 10 minutes.
3. Add 10mL Sevag (chloroform:iso-amyl alcohol 24:1).
4. Vortex briefly and shake at low speed (vortex setting 4) for 5 minutes.
5. Centrifuge at 3220g for 15 minutes at room temperature to separate phases.
6. Avoiding the intermediate layer, carefully transfer supernatant to new 50mL nunc tube using a 10mL pipette for the first 8-12mL and a 1mL pipette for the last 2-3mL.
7. Add equal volume of 100 percent ice cold isopropanol (~10mL).
8. Add half volume of chilled 5M NaCl solution (~5mL).
9. Chill at -20°C for ~1 hour (or overnight).
10. Centrifuge at 3220g for 15 minutes at room temperature and pour off supernatant.
11. Add 2mL of 70 percent ethanol, being sure to wash down sides of tube.
12. Centrifuge at 3220g for 2 minutes at room temperature.
13. Pour/pipette of ethanol and allow to air dry.
14. Add 1mL low TE buffer
15. Heat briefly in a 45°C water bath to resuspend DNA.
16. Use a 1mL pipette to transfer DNA into a new 2mL low bind centrifuge tube.
17. Store samples at -80°C until needed for qPCR analysis.