Fecal Findings: Investigating Eastern Massasauga Rattlesnake Diet Using DNA Metabarcoding

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Dedication

Dedicated to my grandpa, who inspired my love for science at a young age.

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Abstract

Characterizing the diet of imperiled species using minimally invasive methods is crucial to understanding their conservation requirements. DNA metabarcoding methods have been used to characterize the diet primarily in mammalian systems, while reptiles are heavily underrepresented in this literature. Here, we apply a DNA metabarcoding approach to study the diet of the eastern massasauga rattlesnake (*Sistrurus catenatus*); a Federally Threatened snake found throughout the Great Lakes Region. Eighty-three fecal samples collected across 10 different massasauga populations located in Michigan were sequenced. We use universal metazoan primers and develop a host-specific oligonucleotide blocker to uncover the full potential diet of the eastern massasauga. We identified at least 18 prey items. Non-target taxa and taxa from potential secondary consumption were also identified in fecal samples. Eastern massasaugas exhibited a strong preference towards small mammals, with meadow voles (*Microtus pennsylvanicus*) being the most common (69.4% of diet), along with occasional bird and snake prey. We did not find that younger snakes preferred other snake prey, but instead consumed smaller mammals such as masked shrews (*Sorex cinereus*) and northern short-tailed shrews (*Blarina brevicauda*). Adult individuals exhibited a more generalized diet, consuming a wider range of prey taxa, and appear to be opportunistic predators. We conclude that small mammals are a crucial part of eastern massasaugas diet and recommended this be taken into consideration when conservation strategies are developed. Additionally, we tested the efficiency of sample preservation methods with the fecal samples and suggest freezing samples as soon as possible following collection to prevent further degradation of DNA. This study is one of few to apply metabarcoding methods to study snake diet, and the first to study rattlesnake diet. We have demonstrated that DNA metabarcoding is a reliable, accurate approach to obtain quality dietary

information from snake fecal samples. As reptiles are currently facing global declines, the methods developed in this study can be applied to other reptile species, providing a way to study the diet of at-risk species minimally invasively.

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Abbreviations

μl- Microliters

μM- Micromolar

ASV- Amplicon sequence variants

BOLD- Barcode of Life Data System

Bp- Base pairs

C3- Carbon 3

cm- Centimeter

CO1- Cytochrome oxidase subunit 1 gene

DNA – Deoxyribonucleic acid

FOO- Frequency of Occurrence

GPS – Global Positioning System

LCA- Least common ancestor

NMDS- Non-metric multidimensional scaling

PCR- Polymerase chain reaction

PIT – Passive integrated transponder

pM- Picomolar

SVL – Snout-vent length

Chapter 1: Introduction

Introduction

Characterizing the diet of imperiled species has become a focus in ecological studies to improve conservation strategies (Pompanon et al., 2012). Although understanding dietary preferences is crucial for threatened species, characterizing them can quickly become complicated due to methodological restrictions. Chances of observing direct feeding events of wild individuals are rare for elusive predators. Additionally, diet analyses can be further complicated if a predator's diet differs among sex, age, time of year, and populations.

Diets have traditionally been assessed by examination of stomach contents or feces for identifiable remains of prey such as bones, hair, and scales. While these techniques can provide a starting point for diet characterization, they require time-consuming and specialized taxonomic expertise. Furthermore, traditional approaches have the potential for severe biases (Symondson, 2002), such as the inability to detect soft-bodied or easily digestible prey due to the lack of identifiable remains (Brown et al., 2012). Prey items that do have identifiable remains in stool are often limited to broad taxonomic identification, such as genus or family level, while precise species-level classification is rare. Traditional diet studies are further limited by the highly invasive collection process of stomach and gut contents, requiring stomach pumping or euthanasia to access and dissect the GI tract. These highly invasive techniques present ethical concerns if carried out on wild individuals and are not a feasible option for at-risk predators. Molecular analyses of feces are an alternative, non-invasive approach to assess the diet of predators (King et al., 2008; Valentini et al., 2009).

DNA barcoding is a means of identifying taxa from variable DNA sequences in a standardized region of the genome (Hebert, Cywinska, Ball, & deWaard, 2003). DNA

sequencing technology has vastly improved over time, increasing the ability to identify taxa from complex environmental samples, known as DNA metabarcoding (Shokralla et al., 2012). Next-generation sequencing amplifies thousands of DNA sequences in parallel, making it possible to obtain dietary information with increased accuracy and efficiency from fecal samples containing highly degraded prey DNA (King et al., 2008; Symondson, 2002). DNA metabarcoding methods can be used to analyze the diet of predators with limited *a priori* knowledge of potential prey by using universal primers to target a broad taxonomic range of prey (Pompanon et al., 2012). Additionally, the number of DNA reference sequences available in public databases such as BOLD (Barcode of life Database) and GenBank have greatly increased (Porter & Hajibabaei, 2018), further ensuring the success of pairing up an unknown sequence with reference taxa sequences.

DNA metabarcoding diet analyses using next-generation sequencing have been carried out successfully on a wide range of vertebrates, but are primarily focused on fish and mammalian systems (e.g. Bohmann et al., 2018; Deagle, Kirkwood, & Jarman, 2009; Sousa et al., 2016; Waraniak, Marsh, & Scribner, 2019). Certain groups, such as reptiles, are still heavily underrepresented in DNA metabarcoding studies, which have been carried out only in several lizard (Brown et al., 2012; Kartzinel & Pringle, 2015; Pereira et al., 2019) and snake species (Brown et al., 2014; Falk & Reed, 2015). However, most of these studies either use group-specific primers (e.g., Brown et al., 2012) or characterize the diet of insectivorous predators (e.g., Kartzinel & Pringle, 2015). Group-specific primers only target specific taxonomic groups of prey items, which can limit complete diets and the ecological significance of prey from being uncovered. Reptile diet characterization targeting the CO1 region to identify a generalized vertebrate diet has yet to be heavily explored.

The eastern massasauga rattlesnake (*Sistrurus catenatus*) is a pit viper species that occurs in wetlands throughout The Great Lakes region (Seigel, 1986). Massasauga populations have declined, and the species has been recently listed as Federally Threatened under the Endangered Species Act (U.S. Fish and Wildlife Service 2016), and Species at Risk Act (Committee on the Status of Endangered Wildlife in Canada, 2002). Loss of wetland habitat is the main contributor to this species' decline, due to fragmentation, conversion of wetlands for agricultural use, and vegetative succession. Road mortalities and direct human persecution have also contributed to population declines (Szymanski et al., 2015). Declines have resulted in disjunct populations, and populations appear to be continually declining throughout their range. Historical populations are either considered extirpated or at an unknown status, with the majority of remaining populations persisting in Michigan (Szymanski et al., 2015). Michigan is at the center of eastern massasauga geographic range and contains some of the most viable remaining populations (Jones et al., 2012), therefore conserving Michigan populations is critical for the persistence of the species.

Eastern massasaugas are ambush sit-and-wait predators, concealing themselves in a stationary position, striking, and injecting venom when a suitable prey item is in distance. Massasaugas additionally rely on heat-sensing pits located between their nares and eyes. Due to their elusive nature and current threatened status, direct feeding observations of massasaugas are rare in the wild. Current identification of eastern massasauga diet has been limited to opportunistic regurgitations (Tetzlaff et al., 2015), dissection of fecal samples for prey remains (Weatherhead et al., 2009), examination of gut contents (Hallock, 1991; Keenlyne & Beer, 1973; Ruthven, 1911), and feeding trials of neonate individuals (Shepard et al., 2004). Across their range, these analyses have revealed the majority of eastern massasauga diet consists of small mammals such as voles, shrews, and mice while occasionally feeding on birds and other snakes

(Hallock, 1991; Keenlyne & Beer, 1973; Shepard et al., 2004; Weatherhead et al., 2009). Geographic differences in diet preferences have been observed with this species (Shepard et al., 2004; Weatherhead et al., 2009), and other massasauga subspecies have been noted to have a generalist diet (Holycross & Mackessy, 2002). Previous studies have conflicting results on the possibility of ontogenetic dietary shifts occurring between age classes. In some parts of their range, neonate and juvenile massasaugas have been found to be the only consumer of other snake species (Keenlyne & Beer, 1973; Shepard et al., 2004), while no evidence of a dietary shift was found in Ontario or Ohio populations (Weatherhead et al., 2009). In feeding trials conducted with neonate eastern massasaugas, snakes preferred other neonate snake prey, but regurgitations from captured free-ranging individuals only consisted of shrews and voles (Shepard et al., 2004). With such limited information available on eastern massasauga diet, more accurate and minimally invasive means of identifying the prey species they consume is necessary to further understand their ecology.

Purpose

The purpose of this study is to apply a DNA metabarcoding technique to identify the prey that eastern massasaugas are consuming throughout the Lower Peninsula of Michigan. We also compare the dietary results between individuals and provide further information as to whether feeding preferences differ between age classes, sexes, and populations. Additionally, the methods we have developed in this study are applicable to other reptile species, particularly at-risk snakes.

Scope

Previous eastern massasauga diet studies have not focused on Michigan populations, despite Michigan containing the largest number of viable populations in the center of its range (Szymanski et al., 2015). Furthermore, previous dietary studies across the eastern massasauga range were carried out using traditional analysis of fecal or gut contents, leaving the potential for important prey items to be missed. Our research will inform the conservation of this species in Michigan by obtaining baseline ecological data. The methods we have developed here are relevant to similar reptile species to assess diet when a molecular method is desired.

Assumptions

1) We assume our recorded GPS locations of eastern massasauga locations are accurate.

2) We assume the collected fecal samples represent the diet of eastern massasaugas in each population.

3) We assume our selected reference database is up to date with correct taxonomic names and corresponding sequences.

4) We assume the programs used for our bioinformatic pipeline are suitable for our data and accurately processes our sequences.

Hypothesis

Our objectives in this study are to 1) create a DNA metabarcoding protocol capable of accurately identifying prey items consumed by eastern massasauga rattlesnakes, 2) identify what prey items eastern massasaugas are mainly consuming throughout the Lower Peninsula of Michigan, and 3) identify if there are differences in diet between age classes, sexes, gravidity, populations, and

seasons. We hypothesize that the bulk of eastern massasauga diet will consist of small mammal species, with the occasional occurrence of other snake and bird prey.

Significance

Our study is crucial to identifying the eastern massasaugas' preferred prey source in Michigan using a minimally invasive method. Traditional diet analyses have been carried out in Ohio, Ontario (Weatherhead et al., 2009), Wisconsin (Keenlyne & Beer, 1973), and Illinois (Shepard et al., 2004). Dietary data are currently lacking for Michigan populations, with only two known studies focusing on museum specimens (Hallock, 1991) and opportunistic regurgitations from wild individuals (Tetzlaff et al., 2015). In this study, we have developed a minimally invasive molecular method to study the diet of this species. Understanding how complex their feeding requirements are could provide insight into how management plans should be developed for Michigan populations. These minimally invasive methods are crucial given the threatened status of this species and can used as a guide in future studies to uncover diet across its range.

Our study will be the first to use a molecular method to characterize eastern massasuga diet and one of few to apply this method to a snake species. DNA metabarcoding approaches have yet to be commonly implemented in reptile diet studies. Reptiles are currently facing global declines (Gibbons et al., 2000); therefore, we aim for the methods developed in this study to be applicable to other snake species when traditional approaches are not possible.

Definitions

DNA metabarcoding- Identifying multiple taxa from complex environmental samples using variable DNA sequences by targeting a standardized region of the genome.

Gape limited- Predators who swallow their prey whole (such as snakes), are limited as to what they can ingest based on the prey size.

Blocking oligonucleotide- designed to have a specific preference to only bind to the predator or non-target DNA. Uses a modification on the 3' end of the primer to halt polymerase activity.

Polymerase chain reaction (PCR)- A laboratory method used to rapidly amplify DNA sequences using primers to target a designated region.

High fidelity polymerase- A polymerase that has a lower error rate compared to standard polymerases. Increases the ability for the polymerase to insert the correct base during polymerase chain reaction.

Amplicon sequence variants (ASVs)- distinct biological sequences, sequences with even one nucleotide difference will be classified as a unique feature.

Chapter 2.1

Fecal Findings: Investigating Eastern Massasauga Rattlesnake Diet Using DNA Metabarcoding

Abstract

Characterizing the diet of imperiled species using minimally invasive methods is crucial to understanding their conservation requirements. DNA metabarcoding methods have been used to characterize the diet primarily in mammalian systems, while reptiles are heavily underrepresented in this literature. Here, we apply a DNA metabarcoding approach to study the diet of the eastern massasauga rattlesnake (*Sistrurus catenatus*); a Federally Threatened snake found throughout the Great Lakes Region. Eighty-three fecal samples collected across 10 different massasauga populations located in Michigan were sequenced. We used universal metazoan primers and developed a host-specific oligonucleotide blocker to determine their potential diet. We identified at least 18 prey items, with eastern massasaugas exhibing a strong preference towards small mammals, with meadow voles (*Microtus pennsylvanicus*) being the most common (69.4% of diet), along with occasional bird and snake prey. Non-target taxa and taxa from potential secondary consumption were also identified in fecal samples. Our eastern massasauga-specific blocking primer was successful in allowing the amplification of rare prey items with the addition of an inverted dT at the 3' end. We did not find that younger snakes preferred other snake prey, but instead consumed smaller mammals such as masked shrews (*Sorex cinereus*) and northern short-tailed shrews (*Blarina brevicauda*). Adult individuals exhibited a more generalized diet, consuming a wider range of prey taxa. We conclude that small mammals are a crucial part of eastern massasauga rattlesnake diet and recommend this be taken into consideration when conservation strategies are developed. This study is one of few to apply metabarcoding methods to study snake diet, and the first to study rattlesnake diet. We have

shown that DNA metabarcoding of fecal samples is a reliable, accurate approach to obtain quality dietary information from snakes. As reptiles are currently facing global declines, the methods developed in this study can be applied to other reptile species, providing an accurate, minimally invasive, and thorough diet assessment for at-risk species

Introduction

Characterizing the diet of imperiled species is becoming increasingly common in ecological studies, as crucial food sources or feeding preferences may be identified. Obtaining dietary information can indicate the current state and health of the ecosystem and identify if a predator is a specialist or generalist consumer. Compared to generalist consumers, predators with specialist or limited diets are more vulnerable to declines due to suitable prey limiations. If a threatened predator's diet is limited, identifying its preferred food source can help guide conservation efforts of declining populations (Pompanon et al., 2012). Reptiles are currently facing global declines (Böhm et al., 2013; Gibbons et al., 2000; Zipkin et al., 2020), with dietary for at-risk species often lacking as a consequence. Characterizing diet becomes especially challenging for predatory reptiles, such as snakes, with cryptic and infrequent feeding events.

Snake diets have primarily been assessed by examination of stomach contents from wild individuals and museum specimens, or of feces for identifiable remains of prey (hair, scales, skulls, etc.). While these techniques can provide a starting point for diet characterization, they require specialized taxonomic expertise and have the potential for severe biases (Glaudas et al., 2017; Symondson, 2002). For example, many reptiles consume prey that are soft-bodied or easily digestible (e.g. invertebrates; Brown et al. 2014); therefore detection of these prey items would be impossible by relying solely on morphological identification of remains. Additionally, traditional methods requiring euthanasia for examination of stomach or gut contents are not an

option for at-risk wild individuals due to ethical considerations. An alternative, minimally invasive approach to assessing diet is through analysis of feces using DNA metabarcoding (King et al., 2008; Valentini et al., 2009).

Increased accessibility to next-generation sequencing technology, expansion of reference sequences in public databases, and the developments of universal primers have drastically improved the success of vertebrate DNA metabarcoding dietary studies (Porter & Hajibabaei, 2018). Obtaining dietary information from fecal samples containing highly degraded prey DNA is now feasible. The most commonly used barcode marker for targeting metazoan taxa is the mitochondrial cytochrome c oxidase subunit 1 gene (CO1) (Hebert et al., 2003). The CO1 region has faced criticism for not being truly universal due to potential taxonomic biases (Rubbmark et al., 2018), yet is still accepted as the most suitable barcode region for general metazoan metabarcoding down to the species level (Andújar et al., 2018; Sousa et al., 2019). Despite the promises of DNA metabarcoding-based diet analyses, these methods have yet to be commonly applied outside of mammalian systems or arthropod specialists (e.g., Bohmann et al., 2018; Deagle et al., 2009; Kartzinel & Pringle, 2015; Shehzad et al., 2012). Reptiles are heavily underrepresented in DNA metabarcoding studies, as only a few studies have implemented these approaches to study the diet of several lizards (Brown et al., 2012; Kartzinel & Pringle, 2015; Pereira et al., 2019) or snake species (Brown et al., 2012) with limited success in amplifying the CO1 region (Falk & Reed, 2015).

The eastern massasauga rattlesnake (*Sistrurus catenatus*) is a wetland species that occurs throughout the Great Lakes region (Seigel, 1986). Massasauga populations have declined across their range, and the species is listed as Federally Threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 2016) and the Canadian Species at Risk Act (Committee on

the Status of Endangered Wildlife in Canada, 2002). Habitat loss and fragmentation, vegetative succession, road mortalities, and human conflict have contributed to population declines (U.S. Fish and Wildlife Service 2016). Following drastic declines, the majority of remaining viable populations are located in Michigan. Characteristic of many reptiles, eastern massasaugas possess cryptic coloration and behavior, which, in combination with their threatened status, make them rare and difficult to detect.

Feeding observations of this species in the wild are rare, and identification of eastern massasauga diet composition has been limited to opportunistic regurgitations, analysis of fecal and gut contents, and feeding trials (Holycross & Mackessy, 2002; Keenlyne & Beer, 1973; Shepard et al., 2004; Weatherhead et al., 2009). Across their range, these analyses have revealed the majority of eastern massasauga diet consists of small mammals such as voles, shrews, and mice while occasionally feeding on birds and other snakes (Keenlyne & Beer, 1973; Shepard et al., 2004; Weatherhead et al., 2009). Dietary data are currently lacking for Michigan populations, with only two known studies focusing on museum specimens (Hallock, 1991), and opportunistic regurgitations from wild individuals (Tetzlaff et al., 2015). As gape-limited predators, Viperid snakes can exhibit ontogenetic shifts in diet (Glaudas et al., 2008). Juvenile individuals may feed on smaller ectothermic prey such as other snakes and invertebrates, later switching to a more mammal-dominated diet (Glaudas et al., 2008). However, there is conflicting evidence on the possibility of ontogenetic dietary shifts for massasaugas. In some parts of their range, only neonate and juvenile massasaugas consume other snake species, switching primarily to mammals as adults (Keenlyne & Beer, 1973); however, dietary shifts were not evident in Ontario and Ohio massasauga populations (Weatherhead et al., 2009). In feeding trials conducted with neonate

eastern massasaugas, snakes preferred other neonate snake prey, but regurgitations from captured free-ranging individuals only consisted of shrews and voles (Shepard et al., 2004).

Snake species that rely on venom for prey capture, such as ambush predators, may have a more specialized diet based on their prey-specific venom, while others with less complex venoms may follow a generalist diet (Gibbs et al., 2011, 2013; Lyons et al., 2020). Geographic differences in diet preferences have been observed in the eastern massasauga (Weatherhead et al., 2009), and other massasauga subspecies have been classified as dietary generalists (Holycross & Mackessy, 2002). With such limited information available on eastern massasauga diet, more accurate and minimally invasive means of identifying the prey species they consume is necessary to further understand their ecology and feeding preferences, and whether resource limitation may be contributing to their declines.

For the first time, we apply a DNA metabarcoding approach to identify what prey species eastern massasaugas are mainly consuming in populations distributed throughout the lower peninsula of Michigan. In addition, we compare diets between individuals to provide further insight as to whether feeding preferences differ between age classes, seasons, and populations. We hypothesize that the bulk of eastern massasauga diet will consist of a range of mammal species, while also opportunistically feeding on other taxa such as snakes and birds. Furthermore, we develop a pipeline in this study that is broadly applicable to any reptile species for future dietary studies. To our knowledge, this study is the first to apply a DNA metabarcoding diet analysis to a rattlesnake species, and the first to use a molecular method to analyze eastern massasauga diet.

Methodology

Sample collection and preservation

We carried out visual encounter surveys from May-September 2018-19 during the eastern massasauga active season (approximately April to October; Szymanski et al., 2015). To get an accurate representation of massasauga diet throughout the Lower Peninsula of Michigan, we selected sites that were distributed across the state (Figure 1). In addition, we collected samples from Bois Blanc Island (BBI), located off the northern coast of the Lower Peninsula. Considering the possibility that diet shifts may occur throughout the season, we visited each site multiple times during the active season whenever possible. If a snake was located, it was captured opportunistically using tongs, and safely secured in a cloth bag. All capture locations were recorded using handheld GPS units.

Snakes were secured in a clear plastic tube for safe handling. Newly captured individuals were marked using a passive integrated transponder (PIT) tag for permanent identification. Fecal material was directly extracted from the snake by placing its tail into a 50mL conical tube and was gently palpating until defecation occurred. All newly captured snakes and snakes that were recaptured more than 2 weeks apart were palpated, but not all captures produced samples. We collected mass (to the nearest g), snout-vent length (SVL) (cm), and tail length (cm). Sex was determined by probing of the cloaca, and gravidity was determined for adult females by gentle palpation. Collection attempts of gravid females were restricted to earlier in the active season (May-early July), while gravid females with well-developed embryos were limited to opportunistic collection. If snakes were unable to be probed, we determined sex based on the subcaudal scute count (≥ 25 subcaudals were considered male). Individuals were classified as adult, juvenile, or young (snakes born the previous year) based on SVL measurements. Females

with SVL ≥ 45 cm and males ≥ 43 cm were classified as adults (Bradke et al., 2018), and juveniles as ≥ 30 cm. Snakes with SVL < 30 cm that possessed one or fewer rattle segments, followed by a single complete terminal rattle segment (without breakage) were considered young. Following processing, each massasauga was returned to its initial capture site.

Samples collected in the summer of 2018 were temporarily placed in a cooler containing ice following collection and moved to long-term storage at -80°C as soon as possible. Due to field conditions, the length of time these samples were stored on ice greatly varied from a few hours to a few days until a freezer was accessible. In the following field season (May-August 2019), samples were immediately frozen using a dry ice-ethanol bath (see Chapter 2.2 for selection of best storage techniques). Each sample collection tube remained in the bath for a few minutes until frozen. These samples were preserved in a cooler with dry ice, where they remained frozen until being moved to a -80°C freezer for long-term storage. In total, 102 samples were collected across 10 populations (see Figure 1 and Table 2).

DNA extraction

Extraction from each fecal sample was carried out using QIAamp PowerFecal DNA Kits (Qiagen) following the standard protocol requiring 0.25 g of stool. A random subset was taken if the sample exceeded 0.25 g, while the entire sample was used if it was less than 0.25 g. DNA extractions took place in a laminar flow hood with UV sterilization to prevent contamination. Snakes excrete all wastes from a cloaca; therefore, urates were sometimes present in fecal samples. As dietary information cannot be obtained from urates, we avoided including them to the best of our ability during the extraction process. One negative control using double-distilled water containing only reagents was included during each extraction batch to test for contamination. Extraction success was confirmed by gel electrophoresis on a 1.5% agarose gel

by the presence of bright clear bands. Although we collected 102 fecal samples in total, sufficient DNA for amplicon sequencing was obtained from 83 samples. A random subset of extracted DNA samples was quantified using a NanoDrop™ OneC Spectrophotometer (ThermoFisher Scientific Inc.) with three replicates per sample. The quantity of DNA per sample ranged from 5.4-51.4 ng/µL (24.7 ng/µL on average), which we used to determine a suitable volume of DNA for PCR reactions (see prey amplification section below).

Primer selection

To identify all potential prey, we selected the universal metazoan forward mlCOIintF (Leray et al., 2013) and reverse jgHCO2198 (Geller et al., 2013) primer set, targeting a 313 bp fragment of the Cytochrome Oxidase subunit 1 (CO1 region) (see Table 3 for the list of primers used in this study). This primer pair is designed to amplify all metazoan taxa and is commonly used in DNA metabarcoding dietary assessments. The reverse primer jgHCO2198 is a redesign of the Folmer reverse primer HCO2198 (Folmer et al., 1994) corrected for mismatches and increased degeneracy to allow for broader taxonomic amplification (Geller et al., 2013). Additional Illumina index-specific overhangs were added onto the 5' end of the forward and reverse primer.

Predator blocking oligonucleotide design

When using universal primers, the non-target (predator) DNA will amplify at a larger scale and limit the amount of target prey DNA amplified successfully due to the degraded nature of the prey DNA. To increase the chances of identifying rare prey items, we designed an annealing inhibiting blocking oligonucleotide developed by Vestheim & Jarman, (2008). To design the blocking primer, eastern massasauga-specific sequences along with available sequences of previously recorded prey items from past diet studies (Holycross & Mackessy, 2002; Keenlyne

& Beer, 1973; Shepard et al., 2004; Tetzlaff et al., 2015; Weatherhead et al., 2009) were downloaded from GenBank (see Table 4 for list of aligned prey). Eastern massasaugas have been documented to consume other snake species, and so we treated all snakes with geographic ranges that overlap with the eastern massasauga as additional potential prey. Frogs have been recorded as occasional prey (Hallock, 1991; Ruthven, 1911), so geographically relevant frog species were also included. Massasauga, potential prey, and the forward mlCOIintF primer were aligned using ClustalW in MEGA X (Kumar et al., 2018). A region of variability between eastern massasauga and potential prey was visually identified as a suitable location to place the 3' end of the blocking primer. We designed the blocking primer based on the mlCOIintF forward primer that overlapped 10 bp at the 3' end of the primer and extended 19 bp into the massasauga-specific sequence (Table 3). Blocking oligonucleotides for diet studies are typically designed using a C3 spacer modification on the 3' end to prevent amplification (Vestheim & Jarman, 2008). However, we were unable to consistently block eastern massasauga DNA using this modification, likely due to our use of a high-fidelity polymerase, and instead opted for a 3' inverted dT modification. To test the specificity of the blocking primer, we performed PCR (see prey amplification section below for cycle conditions), on three mammal specimen (shrew, vole, and mouse), one sample containing strictly eastern massasauga DNA, and one eastern massasauga fecal sample to be used for downstream analyses. We determined the blocking primer as suitable when the band containing only eastern massasauga DNA was notably lighter (Figure 3).

This blocking primer is designed to compete with the universal primers and limit the amplification of the predator DNA. However, blocking primers may also block amplification of target prey DNA if they are closely related to the predator (Piñol et al., 2015; Shehzad et al.,

2012). Consequently, the blocking oligonucleotide may block the amplification of other snake species as well (Table 5).

Prey Amplification

To limit errors while generating amplicons during amplification, we selected the NEBNext® Q5U® Master Mix (New England Biolabs, USA) high-fidelity polymerase that is compatible with the inosine bases present in the jgHCO2198 reverse primer and possesses a 3'-5'exonuclease activity. The annealing inhibiting blocking primer was included at 15x the concentration of the universal primers. PCR was carried out using the following conditions: 12.5 μL of NEBNext Q5U Master Mix at 1x, 3 μL of genomic DNA, 1.25 μL of the forward and reverse primer (0.5 μM), 1.875 μL blocking oligonucleotide (7.5 μM), and 5.125 μL of nuclease-free water (NEB) for a 25 μL total reaction volume. We carried out an initial denaturation at 98°C for 30 s followed by 30 cycles: denaturation at 98°C for 10 s, annealing at 64°C for 30 s, extension at 72°C for 60s followed by a final extension at 72°C for 5 min. PCR amplification success was confirmed by gel electrophoresis on a 1.5% agarose gel by the presence of a clear band.

Library Preparation and Sequencing

To remove non-specific binding, the initial 25 μL of PCR product was cleaned using AMPure XP beads. The beads were washed with 200 μL of 80% ethanol twice, and DNA was eluted using 52.5 μL of 10mM Tris pH 8.5 buffer. Samples were run on a 2% agarose gel to confirm product was present. Amplicons were indexed using Nextera XT indexes (Illumina) using the following cycling conditions: 95°C for 3 min, followed by 8 cycles of 95°C for 30s, 55°C for

30s, 72°C for 30s, and a final step of 72°C for 5 min. Indexed amplicons were purified using the same process as described above. Purified libraries were quantified using a Qubit Fluorometer (ThermoFisher Scientific) and the average fragment size was determined in an Agilent 2100 Bioanalyzer. In total, 83 samples were prepared for sequencing. Libraries were then normalized at equal molarities and pooled. The pooled libraries at 10 pM concentration with 15% Phix were loaded onto an Illumina MiSeq v3 600-cycle cartridge for 2 x 300 bp paired-end read sequencing.

Sequence processing and taxonomic classification

All sequence processing and taxonomic classification were carried out using the program QIIME 2 v.2020.11 (Bolyen et al., 2019; see Figure 2 for general pipeline). The Cutadapt plugin (Martin, 2011) was used to trim the forward and reverse primers from the demultiplexed sequences using the cutadapt trim-paired command with the following parameters: –p-match-adapter-wildcards, --p-match-read-wildcards to allow matching of IUPAC wildcards, --p-discard-untrimmed to discard any reads in which the primers were not found, and the default –p-error-rate 0.1. The lengths to truncate the forward and reverse reads were based on sequence quality plots following trimming. We used DADA2 (Callahan et al., 2016) to truncate and denoise the trimmed sequences into amplicon sequence variants (ASVs), which corrects for amplicon errors from the sequencing run without clustering into OTUs. Compared to operational taxonomic units (OTUs), ASVs are distinct biological sequences providing more precise taxonomic identification, while such diversity can be missed by OTU clustering (Callahan et al., 2017). While ASVs have yet to be heavily adapted into dietary studies, this denoising method has been found to outperform OTU clustering with mock dietary datasets (O'Rourke et al., 2020). Additionally, the denoising step using DADA2 joins paired-end reads, and removes

singletons and chimeric sequences. To perform taxonomic classification, we used the MIDORI_UNIQ_GB240_CO1 database (Machida et al., 2017) consisting of unique sequences for all eukaryotes available in the GenBank 240 release. We first attempted taxonomic classification using classify-sklearn (Pedregosa et al., 2011) with a kmer-based Naive Bayes trained classifier. However, this classified method resulted in many ambiguous taxa along with taxa that did not fit the sampled geographic range. We instead opted for an alignment approach using the BLAST+ plugin (Camacho et al., 2009). This performs local alignments between the reference reads and query sequences and performs least common ancestor (LCA) classification. We used the classify-consensus-blast command for taxonomic classification with the following parameters: –p-maxaccepts 1000 as the maximum number of hits to keep for each query, --p-perc-identity 0.97 as the minimum percentage that the query sequence should match the reference sequence, --p-query-cov 0.89 as the percentage of the sequence to be aligned to the reference database, and –p-strand both to align the forward and reverse query sequences to the reference sequences (O'Rourke et al., 2020).

Following classification, we filtered out taxonomy that did not have a phylum level identification using the qiime taxa filter-table and filter-seqs commands. We filtered out taxa that we considered to be environmental contaminants or unlikely prey items, including any species under the phyla Mucoromycota, Apicomplexa, Discosea, Basidiomycota, Bacillariophyta, Rotifera, Zoopagomycota, Tubulinea, Chlorophyta, Heterolobosea or under the classes Oomycota, Eustigmatophyceae, and Chrysophyceae. In addition, we filtered out any remaining eastern massasauga sequences and human contaminant.

Statistical analyses

Using sequence counts to determine the overall abundance of prey taxa consumed can be prone to biases due to the degraded nature of prey DNA (Deagle et al., 2013), therefore we only relied on presence/absence data for our analyses. We calculated %FOO (frequency of occurrence) for each prey species as the total number of times each species appeared across individuals averaged over the number of samples. To determine if we captured the full dietary diversity in our dataset, a species accumulation curve of the presence/absence data was calculated in RStudio (v.4.0.3, RStudio Team, 2021) using the *specaccum* function in the vegan package (v.2.5-7, Oksanen et al., 2020) and the 'random' method. Species accumulation curves display the number of taxa that are detected within a dataset as the number of samples accumulates. To determine the differences in diets between age classes, seasons, and populations, a non-metric multidimensional scaling (NMDS) ordination was generated in a Jaccard matrix with 999 permutations with the *vegdist* function. A Permutational Multivariate Analysis of Variance (PERMANOVA) post-hoc test with 999 permutations was run for each separate analysis (age classes, season, populations, sex, and gravidity) using the *adonis2* function in the vegan package (v.2.5-7, Oksanen et al., 2020). If a significant p-value was obtained, we then ran a pairwise PERMANOVA using the function *pairwise.adonis2* in the pairwiseAdonis package (v.0.3, Pedro Martinez Arbizu, 2020) with 999 permutations and a Jaccard matrix to determine what variables were statistically different. P-values were Bonferroni corrected to account for multiple comparisons. To identify which species drove any significant differences, we ran a similarity percentage (SIMPER) test in the vegan package with 999 permutations. Due to a large number of single occurrence prey taxa, we had to limit the taxa included in the NMDS and PERMANOVA analyses to only those with more than

one occurrence across all samples. When site differences were compared, Crawford county (CAW) was removed as it only contained one sample.

Results

We successfully amplified DNA from all 83 samples that were sequenced. We obtained 6,016,360 raw sequence reads prior to any filtering steps. Read counts per sample ranged from 8,461 to 154,512, with a median of 69,913 reads per sample. The DADA2 pipeline in QIIME2 identified 6,102 ASVs belonging to 164 different taxa. 707,306 sequences were identified as eastern massasauga, meaning the host DNA compromised 11.8% of sequences before filtering. Additionally, 13,109 or 0.2% of sequences belonged to human contaminant DNA.

After unassigned taxa, taxa without a phylum level identification, eastern massasauga, and non-prey items (algae, fungi, etc.) were filtered out, we detected a number of metazoan taxa that we could not confidently determine as dietary items including larger mammals such as domestic dog (*Canis lupus familiaris*), domestic cat (*Felis catus*), and white-tailed deer (*Odocoileus virginianus*). We also detected numerous arthropod species (mites, ticks, etc.) that were present in small frequencies and represented by very low sequence counts (see Table 6 for all the arthropod taxa detected). Additionally, multiple nematode parasites in the Rhabditida (*Crossonema menzeli, Caenorhabditis remanei,* and *Baylisascaris procyonis*) were detected (Figure 4). We could not confidently label the above taxa to be dietary items and determined these taxa to be environmental contaminants or a result of secondary predation, therefore they were filtered out and excluded from future dietary analyses. All the metazoan orders detected in eastern massasauga fecal samples are presented in Figure 4. Following the above filtering steps, DADA2 identified 80 ASVs belonging to 18 metazoan taxa. During the filtering process, 11

samples were removed that did not obtain any dietary data, leaving 72 samples for downstream diet analyses.

The number of prey detected per sample ranged from 1-4 taxa. Frequency of occurrence (%FOO) data showed that small mammal species were the prey category most frequently consumed (Figure 5). Specifically, the meadow vole (*Microtus pennsylvanicus*) was the most common prey (69.4%) of eastern massasauga. The Northern short-tailed shrew (*Blarina brevicauda*), and masked shrew (*Sorex cinereus*) each made up 15.2% of eastern masasauga diet, followed by the woodland jumping mouse (*Napaeozapus insignis*; 11.1%), and the white-footed mouse (*Peromyscus leucopus*; 6.9%). The only reptiles consumed were one occurrence of a northern water snake (*Nerodia sipedon;* 1.4%), and Dekay's brown snake (*Storeria dekayi;* 1.4%) in two different individuals. Additionally, there was one occurrence each (1.4%) of a red-backed salamander (*Plethodon cinereus*), star-nosed mole (*Condylura cristata*), southern bog lemming (*Synaptomys cooperi),* field sparrow (*Spizella pusilla*), big brown bat, (*Eptesicus fuscus*), box turtle (*Terrapene carolina*), and land snail (*Oxyloma verrilli*) consumption. Multiple earthworm species (*Dendrodrilus* and *Lumbricus spp.)* were also detected (Figure 5).

All age classes consumed a range of mammal species, the most common being *Microtus pennsylvanicus* for young, juvenile, and adult age classes (66.67%, 84.62%, and 65.96%, respectively). Masked shrews (*Sorex cinereus*) were the second most common prey items for young (41.67%) and juvenile (30.77%), while only making up 4.25% of adult diets. The PERMANOVA partially explained the differences in diets between age classes (P= 0.068; Figure 6, 8).

The number of prey taxa detected at each site ranged from 2-13 different species. The PERMANOVA identified a significant difference of prey items consumed between the nine

populations (p=0.035). The pairwise PERMANOVA further revealed the Lenawee county site significantly differs from the Barry county site (p=0.018) and Bois Blanc Island (p=0.026). Montmorency and Kalamazoo county sites also significantly differed (p=0.023) There were no significant differences detected in prey items based on sex, gravidity, or season. Results of the SIMPER analysis indicated that northern short-tailed shrews were mainly responsible for driving the site differences.

Discussion

Our results illustrate that DNA metabarcoding approaches are a robust, efficient way to assess snake diets. Despite the degraded nature of the DNA in fecal samples, we consistently identified a number of prey items, along with non-target items as well (e.g., fungi, algae and parasites). Our metabarcoding results demonstrate that eastern massasaugas strongly prefer small mammal prey, yet individuals occasionally consume other prey including amphibians, reptiles, and birds (Figure 5). Our metabarcoding approach identified prey species that had been previously documented in traditional eastern massasauga diet studies (Table 1) in addition to multiple new prey items that were not previously documented. New prey included the southern bog lemming, star-nosed mole, northern water snake, field sparrow, land snail, and multiple earthworm species (Figure 5). Identifying previously documented prey along with numerous new prey taxa, supports that minimally invasive metabarcoding techniques have a higher resolution and can be favorable compared to traditional approaches.

All age classes consumed a range of mammal species, the most common being the meadow vole (*Microtus pennsylvanicus*). Young and juvenile individuals tend to have a more limited prey base compared to adult individuals, with the younger age classes feeding mostly on

masked shrews (young 42%, juvenile 31%) northern short-tailed shrews (young 8%, juvenile 31%,), and meadow voles (young 66%, juvenile 85%) (Figure 6). Previous eastern massasauga dietary studies have suggested evidence of an ontogenetic diet shift occurring, with the younger snakes being the main consumer of other snake species (Keenlyne & Beer, 1973). However, we only identified two other snake species in our dietary dataset, with both species consumed by adult massasaugas (Figure 5) and did not find evidence of smaller snakes preferring other snake prey. Although a diet shift from snakes to mammals was not supported, we found that juvenile and young snakes targeted small mammal species including masked shrews and northern short-tailed shrews (Figure 6). Similarly, Shepard et al. (2008) found wild neonate individuals had largely consumed southern short-tailed shrews (*Blarina carolinensis*) in an Illinois population. Compared to *Microtus*, *Peromyscus*, and *Napaeozapus* species, Masked shrews and northern short-tailed shrews are among the smallest mammals observed in our dietary dataset, with maximum adult sizes around 10 cm and 12 cm respectively (Kurta, 2017). It is important to note that with metabarcoding techniques, determining the age class of the consumed prey is not feasible. Snakes are gape-limited predators, and it is likely the larger mammals consumed by smaller snakes, such as meadow voles, were younger individuals. Consistent with a gape-limited predator, we conclude that these snakes are more likely to consume smaller mammal prey during their first few years, later moving on to a larger, more generalized mammal diet during adulthood.

Similar to previous studies, we conclude that eastern massasaugas are somewhat opportunistic predators. Smaller individuals (young and juveniles) seemed to focus their diet on the smaller mammals (e.g., masked shrews). Adult individuals consumed a wider breadth of prey while retaining the small shrew species in their diet (Figure 6). Furthermore, the small

differences of prey consumed in different populations suggest that eastern massasaugas are somewhat opportunistic. These findings align with previous diet studies, where these snakes would consume what was most readily available (Keenlyne & Beer, 1973). Opportunistic feeding behavior is beneficial for these snakes from a conservation perspective, in that they will have food available as long as optimal prey species are abundant. Due to gape-limitations, smaller snakes during their first few years of growth may be more limited by the abundance of juvenile voles, or smaller shrews. Maintaining healthy small mammal populations should be considered when conservation strategies are developed for eastern massasaugas.

Based on our species accumulation curve, we may not have captured the full diversity of eastern massasauga rattlesnake diet (Figure 7). There were multiple instances of only one occurrence of a prey species (Figure 5). Showing evidence of opportunistic predation, a large amount of sampling may be required per site to fully capture the breadth of consumed prey.

When eastern massasauga diet was assessed in Ontario and Ohio populations by fecal sample dissection, the bulk of their diet consisted of mammals with the occasional snake prey (Weatherhead et al., 2009). Similar small mammal species were identified with our metabarcoding approach, including masked shrews, meadow voles, and meadow jumping mice. However, chipmunks (*Tamias striatus*) were classified as the most common prey item in both Ontario and Ohio populations by identification of hair samples from feces (Weatherhead et al., 2009). We did not identify any mammal species as large as chipmunks or squirrels using our metabarcoding approach. The discrepancies here could be a result of the methodological differences between traditional and metabarcoding techniques. Dissection of fecal samples may leave important prey items overlooked, or may result in morphological misidentification of prey, while as our metabarcoding diet characterization would avoid these issues. When traditional and

metabarcoding approaches were compared when studying the diet of the Selvagens gecko, the traditional methods resulted in overlooked diet items that were only identified through metabarcoding (Gil et al., 2020). We encourage future studies focusing on snake diet to take the differences between traditional and metabarcoding methods observed here into consideration.

Geographically, all the recorded prey ranges overlap with eastern massasauga range. However, there were 5 occurrences of woodland jumping mice (*Napaeozapus insignis*) that occurred outside of their current recorded distribution. Woodland jumping mice are historically distributed in the northern Lower Peninsula (Baker, 1983; Kurta, 2017). Our prey occurrences were located in Barry, Kalamazoo, and Lenawee County, further south than their currently recorded distribution. There are currently no records of recent shifts in distribution to the southern Lower Peninsula; however, recent surveys have primarily focused on the northern region (Myers et al., 2009). There is a limited amount of information available for the small mammal species available on Bois Blanc Island (Myers et al., 2009). The mammals we detected on Bois Blanc Island included the northern short-tailed shrew (*Blarina brevicauda*), Meadow vole (*Microtus pennsylvanicus*), and Woodland jumping mouse (*Napaeozapus insignis*). Considering we detected woodland jumping mice outside of their known range in independent fecal samples, these observations could also provide insight into the current distribution for these prey species. With the bulk of eastern massasauga diet consisting of a range of small mammals, our metabarcoding results could be complementary with field observations, and perhaps help overcome the limitations of detection with trapping techniques (Nørgaard et al., 2021).

A variety of non-target taxa were identified from eastern massasauga fecal samples along with the prey items. We decided to remove arthropods from analyses, as we could not confidently label them as prey items. DNA metabarcoding techniques are incapable of

differentiating between direct and secondary consumption, or accidental consumption of material that may occur during prey capture. The arthropod taxa we identified consisted of small taxa such as moths, flies, and ants (see Figure 4 for all orders detected). Eastern massasaugas are venomous ambush predators, and it is unlikely the small insect and arachnid occurrences are a result of direct consumption. Shrews, voles, and mice commonly feed on small insects and arachnids (Kurta, 2017). Arthropods were detected in 46 samples, 40 of which also contained mammal taxa. Arthropod detections were most often single occurrences with very low sequence counts, so we classified these taxa as secondary prey occurrences. It is also important to note that several of the identified taxa should be interpreted with caution. For example, land snails and earthworms are documented prey of numerous mammal prey species we identified in our dataset including star-nosed moles and northern short-tailed shrews (Kurta, 2017). Similar to the arthropods identified in our dataset, these taxa could also be a product of secondary consumption. We also detected several larger mammalian species that were unlikely prey items, including domestic dog (*Canis lupus familiaris*), domestic cat (*Felis catus*), and white-tailed deer (*Odocoileus virginianus;* Figure 4). One possible explanation for this could be due to scavenging events from the consumed small mammal species. Small mammals, such as *Peromyscus* and *Blarina* spp. have been observed to scavenge white-tailed deer carcasses (Jennelle et al., 2009). The presence of these larger mammals could also be a result of bloodmeals from ectoparasites we detected, such as ticks, chiggers, and mites that were then consumed by small mammal prey. Environmental contamination should also be considered as a possible source as well.

Three nematode species (*Crossonema menzeli*, *Caenorhabditis remanei,* and *Baylisascaris procyonis*) were detected in 10 samples. Numerous parasitic nematodes have been infect a variety of snake species (Bursey & Brooks, 2011; Hallinger et al., 2020; Lettoof et al.,

2020), including the eastern massasauga (Hallock, 1991). All of the nematode species we identified were Rhabdias nematodes, which have been previously documented to infect snakes (Hallinger et al., 2020). Snakes with poor body conditions have been noted to possess a larger number of parasites (Hallinger et al., 2020), although all the snakes we identified appeared healthy, and had no signs of snake fungal disease.

Rattlesnakes, including eastern massasaugas (Hallock, 1991; Ruthven, 1911), do occasionally exhibit cannibalism (Mociño-Deloya et al., 2009; Prival et al., 2002). If cannibalism had occurred in any of our collected samples, we would be unable to identify it as a prey item due to DNA metabarcoding limitations. Cannibalism cannot be excluded as a possibility for this species and may have occurred in the sampled massasauga populations.

The annealing inhibiting blocking oligonucleotide was successful in blocking sufficient eastern massasauga host DNA. When tested on an agarose gel, the well containing strictly eastern massasauga DNA was notably lighter compared to the three mammals (shrew, vole, and jumping mouse) and fecal DNA (Figure 3). Although a faint band was still present when only eastern massasauga DNA was amplified, the design of the blocking primer was enough to allow amplification of rare prey DNA. Although we detected two snake species, the use of the eastern massasauga blocking primer may have inadvertently blocked the amplification of other closely-related snake species (Table 5). Additionally, reptile species can be underrepresented in CO1 barcoding databases (Vences et al., 2012), therefore it is possible that potential reptile prey may be overlooked using metabarcoding approaches to study snake diet. As eastern massasaugas will sometimes consume snakes, we suggest future studies investigate more efficient primer design and a more specific blocking primer, such as the dual priming oligonucleotide method (Vestheim & Jarman, 2008). We also suggest that adding known reptile sequences to barcoding databases

be prioritized to improve the success of future dietary studies with a reptile host or potential reptile prey.

We conclude that DNA metabarcoding from feces is a reliable way to characterize snake diet. Our results complement, and expand upon, previous characterizations of eastern massasauga diet, and further demonstrate that small mammals are the ideal prey source. From a conservation perspective, the opportunistic feeding preferences we identified are beneficial for the long-term survival of the species. We suggest that the abundance of small mammal populations be taken into consideration when developing management plans for the species. This study has demonstrated the success of using minimally invasive methods to study the diet of threatened reptiles and can be used as a guide in future studies.

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Table 1. List of previously recorded prey items for the eastern massasauga rattlesnake from opportunistic regurgitations, gut content analysis, and fecal dissections. Records listed under N/A for common name were unable to be identified to species level due to the nature of these studies.

Class	Prey species	Location	Age Class	Source
Amphibia	Unidentified frog spp.	Michigan	N/A	Hallock 1991, Ruthven 1911
Aves	Brown thrasher	Michigan	Adult male	Tetzlaff 2015
Aves	Unidentified bird spp.	Michigan	N/A	Hallock 1991
Aves	Red-winged blackbird	Wisconsin	N/A	Keenlyne and Beer 1973
Insecta	Unidentified insect spp.	Michigan	N/A	Hallock 1991
Mammalia	Prairie vole	Illinois	Neonate	Shepard 2004
Mammalia	Southern short-tailed shrew	Illinois	Neonate (x7)	Shepard 2004
Mammalia	Rodent family	Michigan	N/A, Male	Hallock 1991, Tetzlaff 2015
Mammalia	Vole	Michigan	N/A	Hallock 1991
Mammalia	Woodland jumping mouse	Michigan	N/A	Hallock 1991
Mammalia	Deer mouse	Michigan, Ohio	N/A	Hallock 1991, Weatherhead 2009
Mammalia	American red squirrel	Michigan, Ontario	N/A, Adult Male	Tetzlaff 2015, Weatherhead 2009
Mammalia	Southern red-backed vole	Michigan, Ontario	N/A	Hallock 1991, Weatherhead 2009
Mammalia	Northern short-tailed shrew	Michigan, Ontario, Ohio	N/A	Hallock 1991, Weatherhead 2009
Mammalia	Meadow vole	Michigan, Ontario, Ohio, Wisconsin	N/A	Hallock 1991, Weatherhead 2009, Keenlyne and Beer 1973
Mammalia	Eastern cottontail	Ohio	N/A	Weatherhead 2009
Mammalia	Eastern fox squirrel	Ohio	N/A	Weatherhead 2009
Mammalia	Northern flying squirrel	Ontario	N/A	Weatherhead 2009
Mammalia	Snowshoe hare	Ontario	N/A	Weatherhead 2009
Mammalia	Eastern chipmunk	Ontario, Ohio	N/A	Weatherhead 2009
Mammalia	Masked shrew	Ontario, Ohio, Wisconsin	N/A	Weatherhead 2009, Keenlyne and Beer 1973
Mammalia	Meadow jumping mouse	Ontario, Ohio, Wisconsin	N/A	Weatherhead 2009, Keenlyne and Beer 1973
Mammalia	White-footed mouse	Wisconsin	N/A	Keenlyne and Beer 1973
Reptilia	Brown snake	Michigan	N/A	Hallock. 1991
Reptilia	Eastern massasauga	Michigan	N/A	Ruthven 1911, Hallock. 1991
Reptilia	Northern Red-bellied snake	Michigan	Adult female	Tetzlaff 2015
Reptilia	Unidentified snake spp.	Michigan, Ontario, Wisconsin	N/A	Hallock 1991, Ruthven 1911, Weatherhead 2009, Keenlyne and Beer 1973
Reptilia	Garter snake	Michigan, Wisconsin	N/A, Young of the year (x3), Adult male	Hallock 1991, Keenlyne and Beer 1973, Tetzlaff 2015

Table 2. Number of fecal samples collected at each site (n=102 total) and number of samples that had suitable PCR product for MiSeq sequencing (n=83 total). Site names indicate the county they were collected from (MNT= Montmorency, BBI= Bois Blanc Island, KAL= Kalamazoo, CAW=Crawford, IOS= Iosco, MAN= Manistee, LEN= Lenawee, BAR= Barry, OAK=Oakland, STJ= St. Joseph)

Site	Fecal samples collected	Fecal samples sequenced
MNT	3	2
BBI	13	10
KAL	12	8
CAW	2	1
IOS	7	6
MAN	10	6
LEN	7	7
BAR	33	29
OAK	10	9
STJ	5	5

Table 3. List of primers used in this study. The blocking primer was designed based on the mlCOIintF forward primer. It overlaps 10bp at the 3' end of the forward primer and extends 19bp into the massasauga-specific sequence. The inverted dT at the 3' end of the blocking primer halts the polymerase and prevents amplification of the host (massasauga) DNA.

Primer name	Sequence 5'-3'	Source
mlCOIintF (Forward)	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al. 2013
jgHCO2198 (Reverse)	TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. 2013
EMR_mlCOIintF_BLK	TTTATCCCCCCCTCTCCGGAAATCTAGTC-3InvdT	This study

Table 4. List of potential prey species along with their accession number, from which the annealing inhibiting blocking oligonucleotide was designed. Sequences were aligned against the eastern massasauga in MEGA to locate sources of mismatches at the 3' end.

Accession number	Species name	Common name
MG422537.1	*Sorex cinereus*	Masked shrew
JF456798.1	*Microtus pennsylvanicus*	Meadow vole
JF456964.1	*Napaeozapus insignis*	Woodland jumping mouse
JF457177.1	*Zapus hudsonius*	Meadow jumping mouse
JF435981.1	*Blarina brevicauda*	Northern short-tailed shrew
JQ601063.1	*Sylvilagus floridanus*	Eastern cottontail
JF457161.1	*Tamias striatus*	Eastern chipmunk
JF457151.1	*Tamiasciurus hudsonicus*	American red squirrel
JF457111.1	*Sciurus niger*	Fox squirrel
JF457030.1	*Peromyscus maniculatus*	Deer mouse
JF456922.1	*Myodes gapperi*	Southern red-backed vole
JF456597.1	*Glaucomys sabrinus*	Northern flying squirrel
GBMA1538-17	*Lepus americanus*	Snowshoe hare
MN135612.1	*Rana clamitans*	Green frog
MG422343.1	*Pseudacris crucifer*	Spring peeper
EF525895.1	*Rana septentrionalis*	Mink frog
EF5258861.1	*Rana catesbeiana*	American bullfrog
EF525818.1	*Hyla versicolor*	Gray treefrog
EF525740.1	*Bufo americanus*	American toad
AAY666391.1	*Agelaius phoeniceus*	Red-winged black bird
KU985793.1	*Plestiodon fasciatus*	Five-lined skink
MH273655	*Coluber constrictor*	Blue racer
MH274240	*Lamropeltis triangulum*	Eastern milk snake
KU985887	*Storeria dekayi*	Brown snake
KU986171	*Clonophis kirtlandii*	Kirtland's snake
KU985725	*Storeria occipitomaculata*	Red-bellied snake
KU985824	*Opheodrys vernalis*	Smooth green snake
MH273770	*Diadophis punctatus*	Ring-necked snake
KU986143	*Thamnophis sauritus*	Northern ribbon snake
MH274704	*Thamnophis sirtalis*	Eastern garter snake
MH274511	*Nerodia sipedon*	Northern water snake
MH274129	*Heteredon platirhinos*	Eastern hognose snake

Table 5. Alignment of the eastern massasauga specific blocking primer designed from the forward primer to potential snake prey. Dots indicate a shared nucleotide with the blocking primer, which may inadvertently block potential snake prey.

Accession number	Species name	Sequences (5'-3')
EMR_mlCOIintF_BLK		T T T A T C C C C C C C T C T C C G G A A A T C T A G T C
MH273655	*Coluber constrictor*	. . . . C . . . . . A . . A . . T . . . . . . T . . . . .
MH274240	*Lamropeltis triangulum*	. C . . . . . A . . . . . G . . T . . . . A
KU985887	*Storeria dekayi*	. A . . C . . . . . A . . . . . A . . . . . C . . . . . T
KU986171	*Clonophis kirtlandii*	. G . . C . . . . . A . . . . . A . . . . . C . . . . . A
KU985725	*Storeria occipitomaculata*	. A . . C . . . . . A . . T . . A . . . . . C . . . . . A
KU985824	*Opheodrys vernalis*	. . . . . A . . T T . G . . A . . . . . C . . . . . A
MH273770	*Diadophis punctatus*	. C . . . . . T T . A . . . . . C T . . . . A
KU986143	*Thamnophis sauritus*	. G . . C . . A . . T . . T . . A . . G . . . . A
MH274704	*Thamnophis sirtalis*	. A . . C . . G . . A . . T . . A . . G . . C . . . . . A
MH274511	*Nerodia sipedon*	. C . . C . . A . . A . . . . . A . . G . . C . . G . . A
MH274129	*Heteredon platirhinos*	. G . . C . . T . . T . . A . . A . . G . . C . . . . . T

Table 6. List of all arthropod taxa identified in our dataset. Arthropods were removed from our dietary analyses because we could not confidently label them as prey items due to likely secondary consumption from the small mammal prey.

Class	Species	Common name
Arachnida	*Agyneta micaria*	Dwarf spider
Arachnida	*Atropacarus striculus*	Mite
Arachnida	*Chamobates cuspidatus*	Mite
Arachnida	*Dermacentor variabilis*	American dog tick
Arachnida	*Diapterobates humeralis*	Mite
Arachnida	*Eutrombicula splendens*	Chigger
Arachnida	*Kaestneria pullata*	Sheetweb spider
Arachnida	*Malaconothrus gracilis*	Mite
Arachnida	*Oribatula tibialis*	Mite
Arachnida	*Pardosa furcifera*	Wolf spider
Arachnida	*Pardosa moesta*	Wolf spider
Arachnida	*Philodromus rufus*	Crab spider
Arachnida	*Pholcus manueli*	Cellar spider
Arachnida	*Podoribates longipes*	Mite
Arachnida	*Punctoribates palustris*	Mite
Arachnida	*Radfordia lemnina*	Mite
Arachnida	*Rhizoglyphus robini*	Bulb mite
Arachnida	*Theridiosoma gemmosum*	Ray spider
Arachnida	*Tigrosa aspersa*	Wolf spider
Arachnida	*Trisetacus thujae*	Mite
Collembola	*Lepidocyrtus paradoxus*	Spring tail
Collembola	*Tipula hermannia*	Spring tail
Collembola	*Tipula latipennis*	Spring tail
Insecta	*Anagrus virlai*	Fairy fly
Insecta	*Anthrenus fuscus*	Carpet beetle
Insecta	*Apantesis phalerata*	Harnessed tiger moth
Insecta	*Aptinothrips elegans*	Thrip
Insecta	*Contacyphon laevipennis*	Marsh beetle
Insecta	*Crambus albellus*	Small white grass-veneer moth
Insecta	*Diaspidiotus perniciosus*	Tree bug
Insecta	*Docosia walpurga*	Fungus gnat
Insecta	*Drosophila suzukii*	Fruit fly
Insecta	*Ectopsocopsis cryptomeriae*	Large-winged psocid
Insecta	*Holcocephala calva*	Robber fly
Insecta	*Lucilia sericata*	Common great bot fly
Insecta	*Melanophthalma picta*	Beetle
Insecta	*Myrmica lobifrons*	Ant
Insecta	*Mythimna unipuncta*	Armyworm moth
Insecta	*Ochlerotatus canadensis*	Mosquito
Insecta	*Ochlerotatus excrucians*	Mosquito
Insecta	*Okanagana rimosa*	Say's cicada
Insecta	*Rhizoglyphus*	Bark lice
Insecta	*Rivellia variabilis*	Signal fly
Insecta	*Sciaphilus asperatus*	Weevil
Insecta	*Spilosoma latipennis*	Pink-legged tiger moth
Insecta	*Spilosoma virginica*	Yellow wooly bear moth
Insecta	*Strenaphis elongata*	Aphid
Ostracoda	*Cypridopsis vidua*	Seed shrimp

Figure 1. Map of eastern massasauga sampling locations. 10 sites were sampled, all in the lower peninsula except for Bois Blanc Island (BBI); located off the northern coast of the Lower Peninsula. Site names indicate the county they were collected from (MNT= Montmorency, BBI= Bois Blanc Island, KAL= Kalamazoo, CAW=Crawford, IOS= Iosco, MAN= Manistee, LEN= Lenawee, BAR= Barry, OAK=Oakland, STJ= St. Joseph)

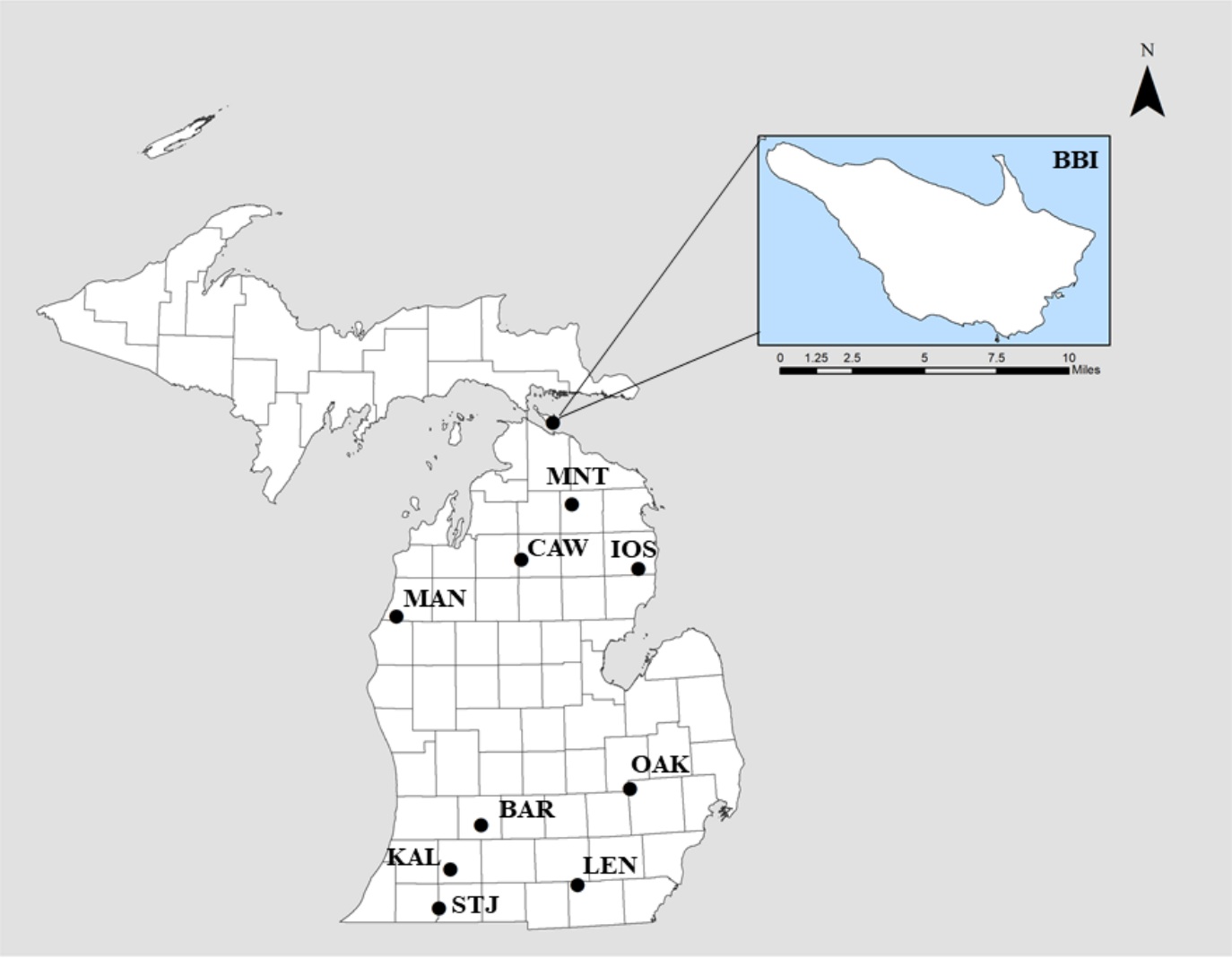


Figure 2. General bioinformatic pipeline carried out to classify eastern massasauga diet items. All steps were carried out using QIIME2 v.2020.11. Numbers present in a step represent the number of eastern massasauga fecal samples remaining after each filtering step.

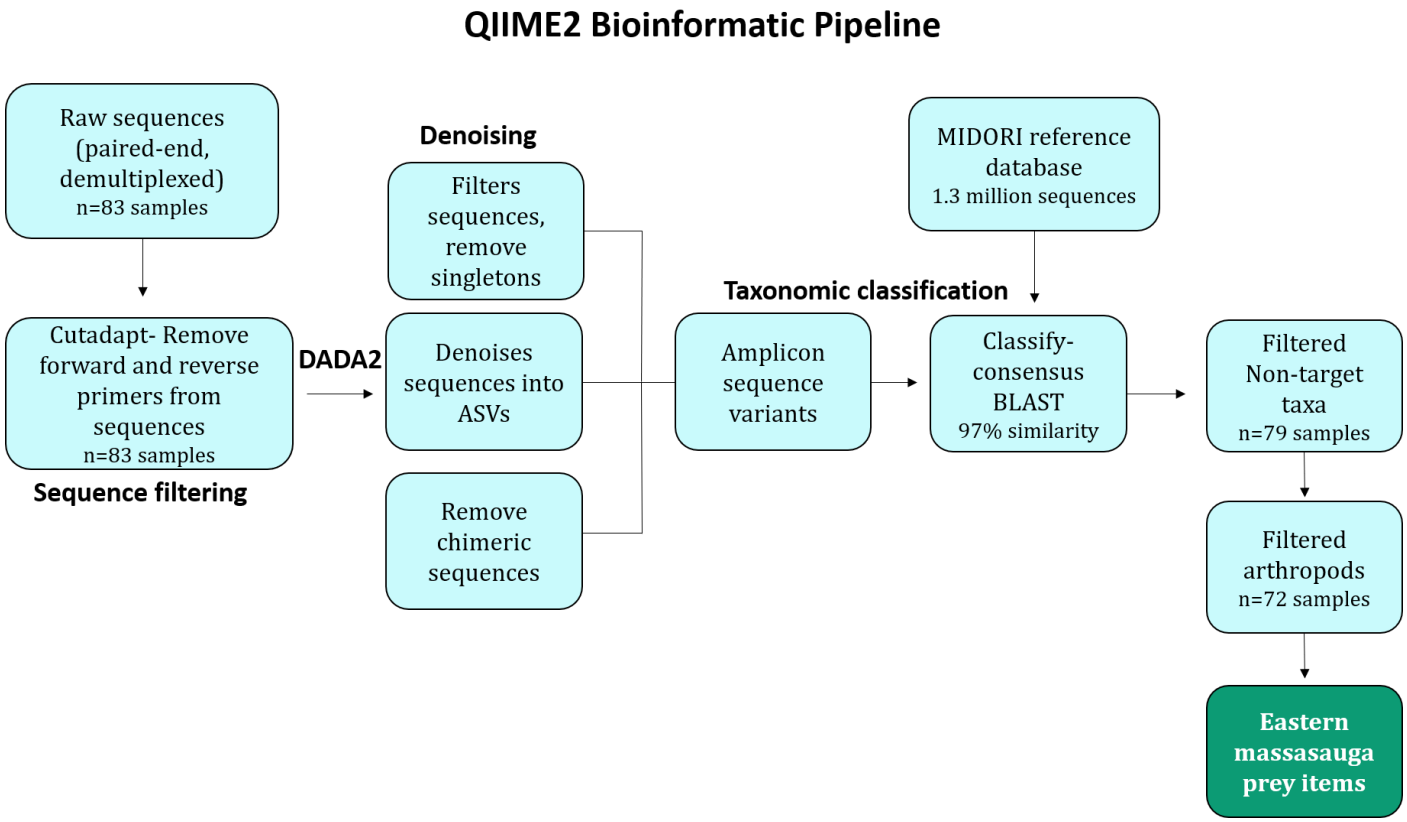


Figure 3. PCR amplifications with the blocking oligonucleotide EMR_mlCOIintF_BLK included. The first 3 wells are the respective potential mammal prey. The EMR well consists of only eastern massasauga rattlesnake DNA, and the last well is amplification of an eastern massasauga fecal sample. Note that due to primer size and tags added for future sequencing, the product size appears larger than the target 313 bp.

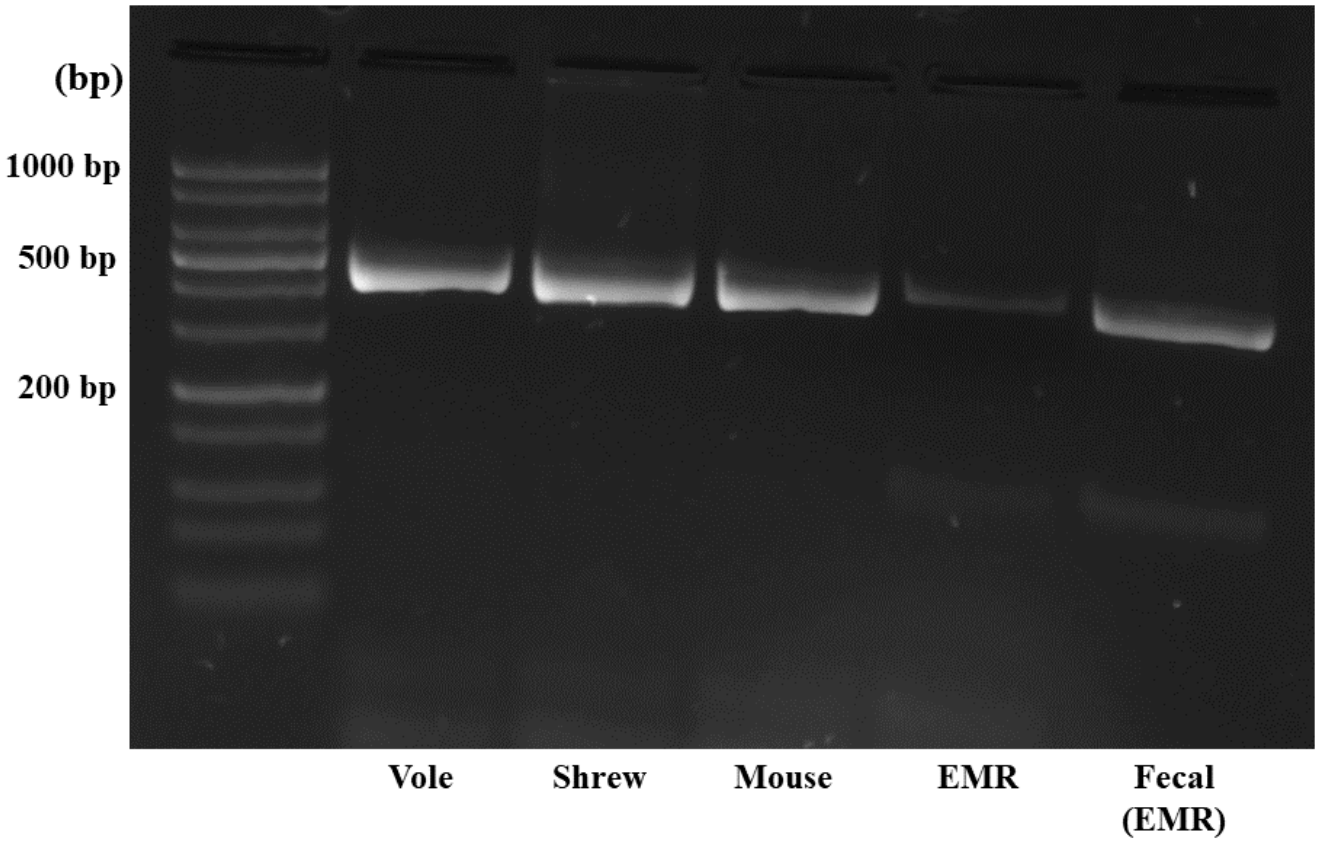


Figure 4. Frequency of occurrence (FOO) of metazoan orders identified in eastern massasauga fecal samples. FOO calculations were carried out using the presence/absence occurrences averaged across all samples. Arthropods, nematodes, and large mammals were removed from downstream diet analyses after being determined as unlikely prey items.

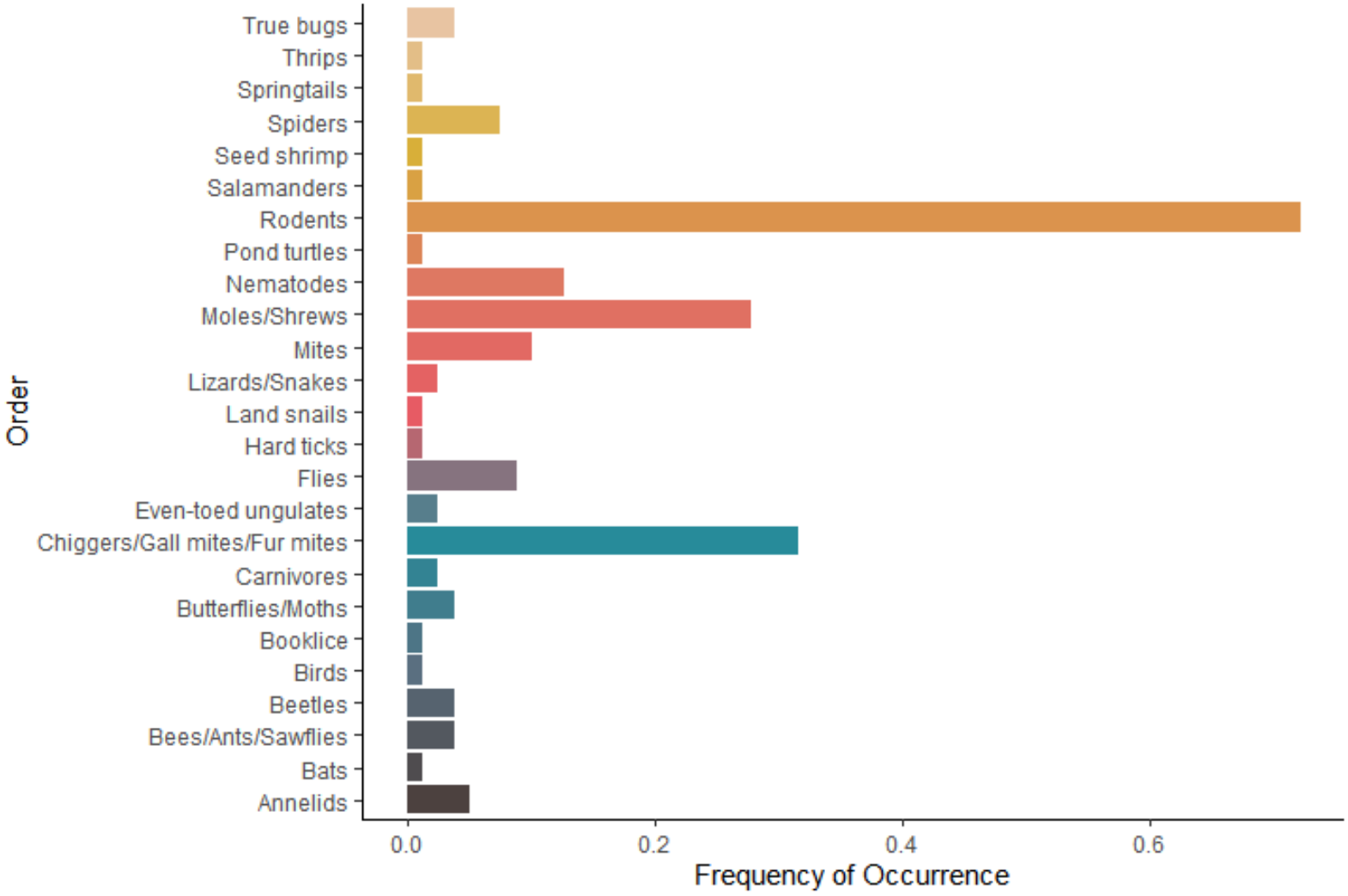


Figure 5. Frequency of occurrence (FOO) of all the prey items identified in our dataset down to the species level. FOO calculations were carried out using the presence/absence occurrences averaged across all samples.

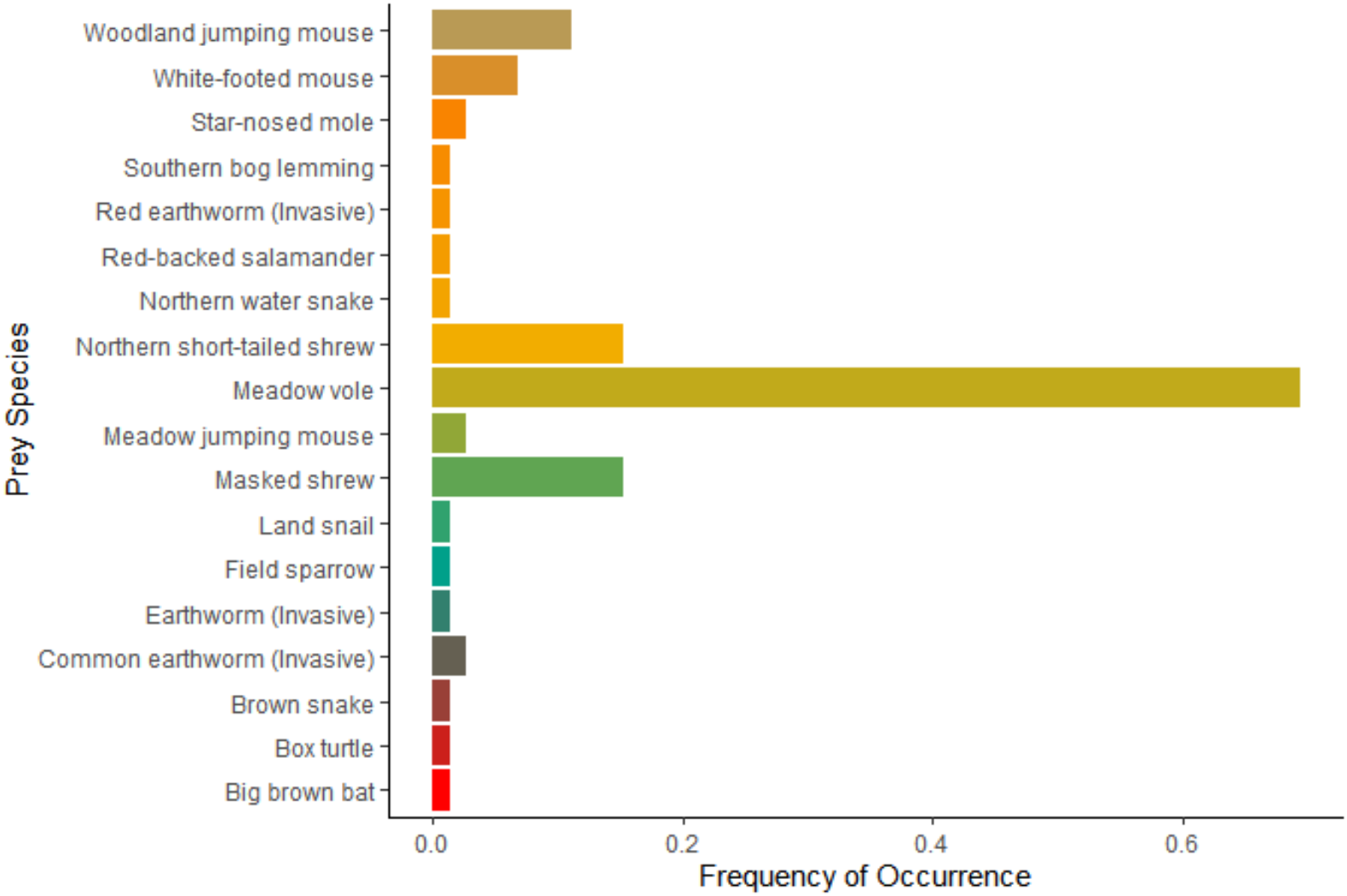


Figure 6. Frequency of occurrence (FOO) of all the prey items identified in our dataset down to the species level separated by adult, juvenile, and young age classes. FOO calculations were carried out using the presence/absence occurrences averaged across the number of samples for each age class category.

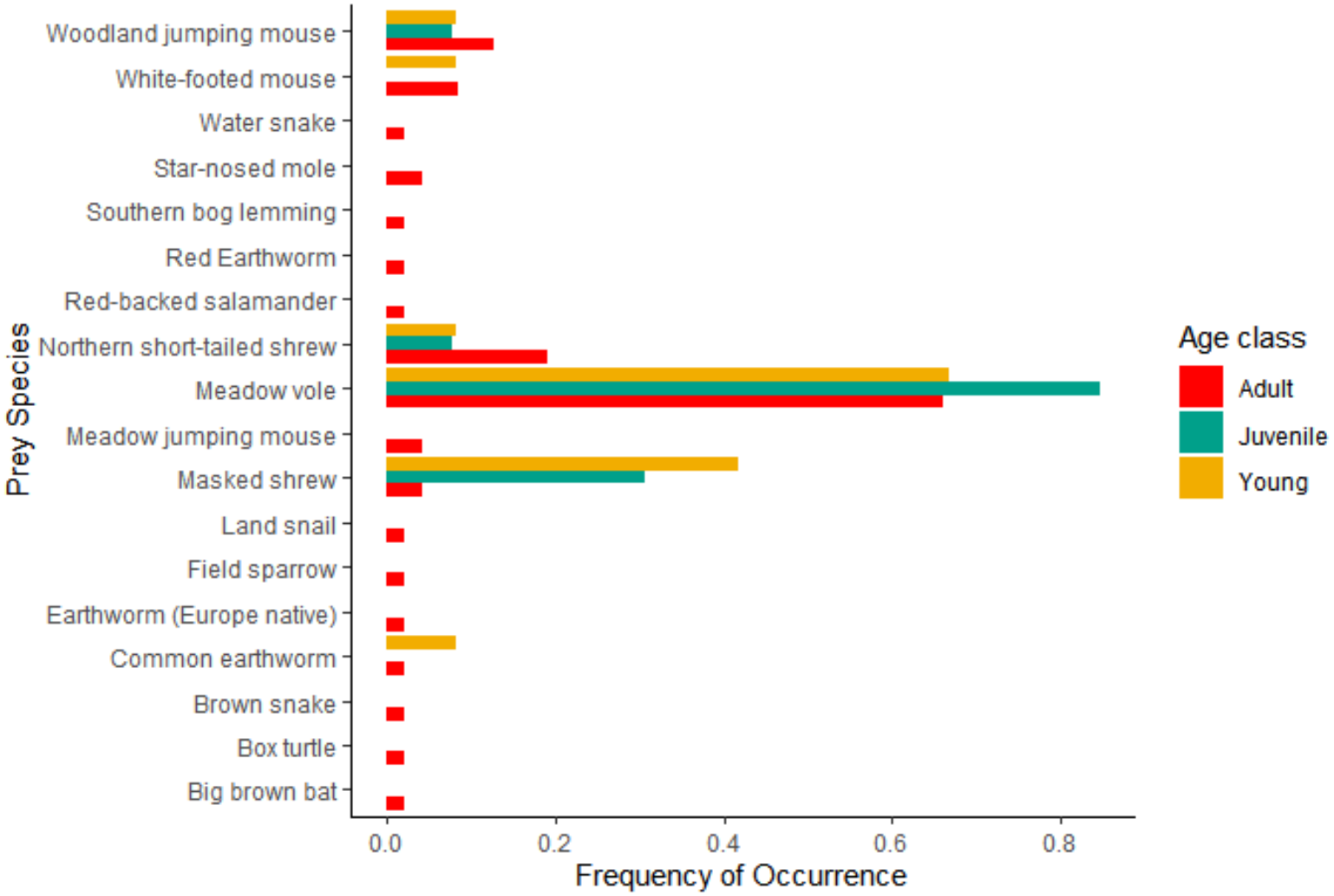


Figure 7. Species accumulation curve based on the number of fecal samples and presence/absence of prey taxa detected in the eastern massasauga dietary dataset. Vertical bars indicate confidence intervals.

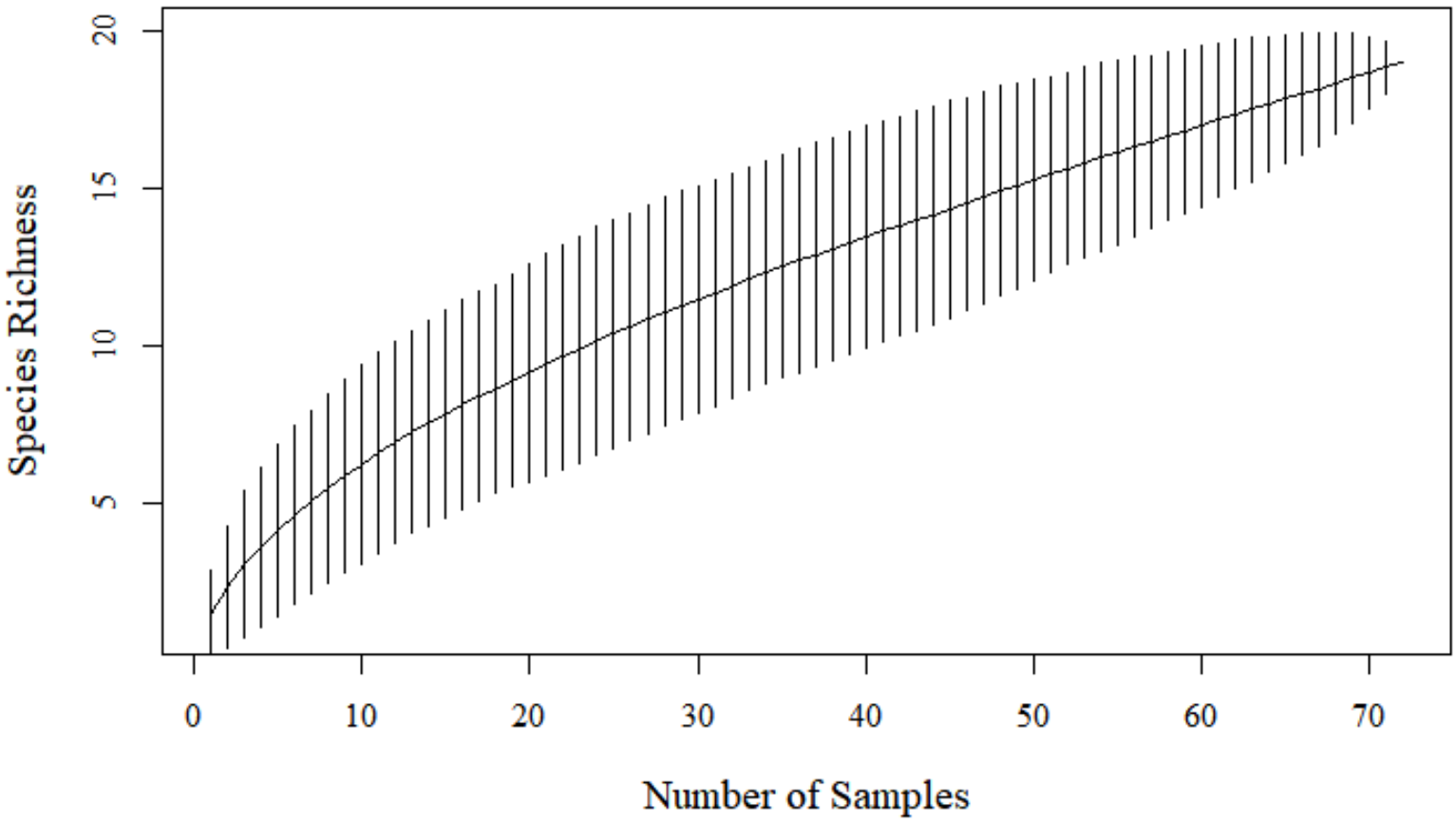
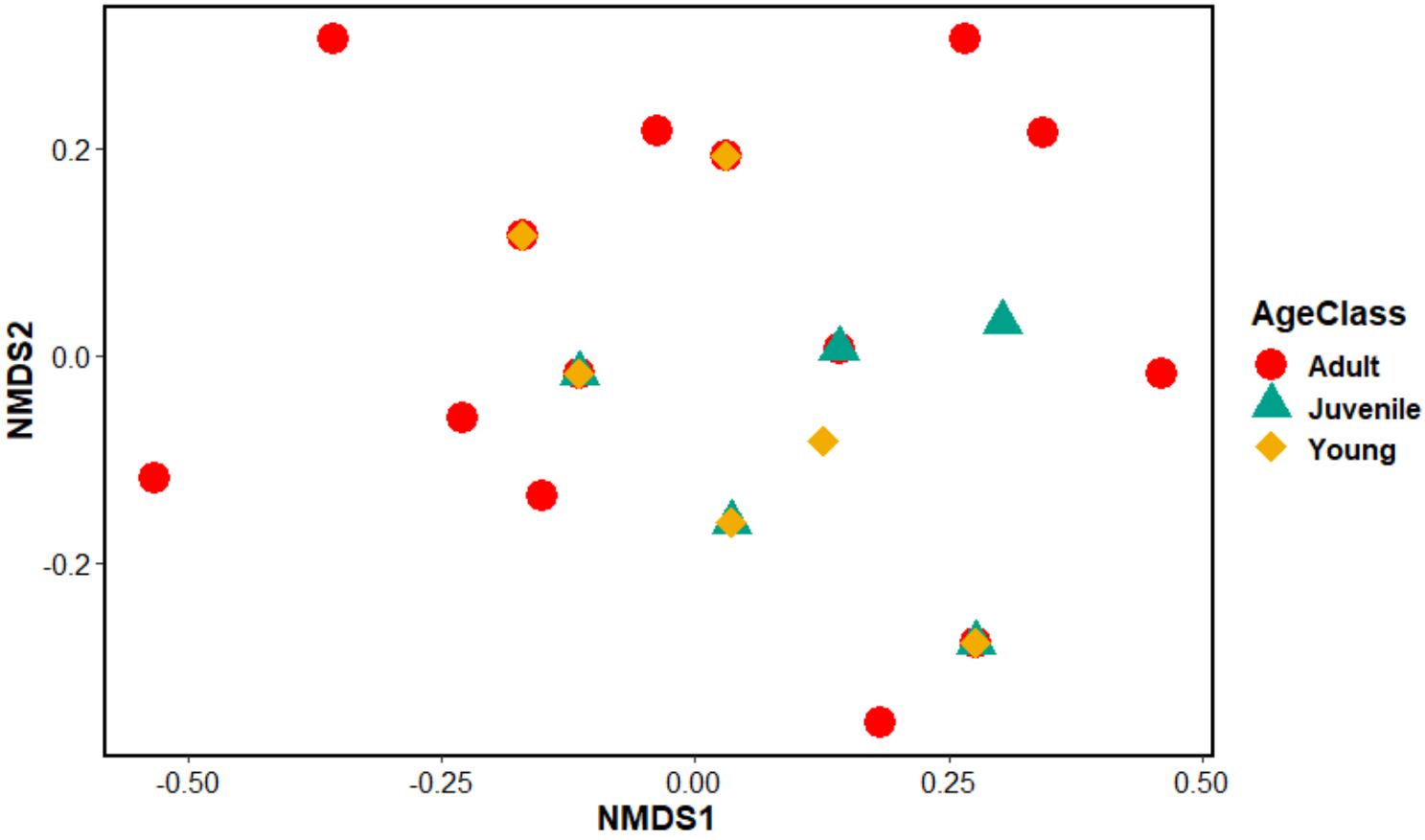


Figure 8. NMDS Jaccard matrix of diet items among young, juvenile, and adult age classes (P=0.068, stress= 0.04). We limited this analysis to prey items that had >1 occurrence across all samples (*Zapus hudsonius*, *Peromyscus leucopus*, *Napaeozapus insignis*, *Blarina brevicauda*, *Condylura cristata*, *Sorex cinereus*, *Microtus pennsylvanicus*).



Chapter 2.2

Sample preservation for downstream molecular diet analyses in reptiles: a case study focusing on a threatened rattlesnake

Abstract

Molecular identification of prey items from fecal samples is a minimally invasive way to study diet. This offers new opportunities to assess the diets of species facing declines, such as reptiles, in which there is a lack of information on dietary preferences. Although sequencing technologies have vastly improved, sampling methods can greatly impact DNA amplification success. Our aim with this study was to determine which fecal sample preservation methods produced high-quality genomic DNA from diet items suited for downstream molecular analyses. We tested the efficiency of sample preservation methods with fecal samples collected from the eastern massasauga rattlesnake; a federally threatened snake found throughout the Laurentian Great Lakes region. We tested samples that were 1) stored in ethanol at ambient temperature (n=7), 2) temporarily stored dry on ice until being frozen at -20°C (n=7), or 3) immediately frozen dry in the field using dry ice until being stored at -80°C (n=7). We were unable to obtain DNA suitable for polymerase chain reaction (PCR) from ethanol stored samples, while samples that were frozen with dry ice produced consistent successful amplification. Although field conditions may be unpredictable, we recommend freezing samples as soon as possible to prevent further degradation of DNA. In addition, our results suggest that reptile fecal samples can provide suitable DNA for molecular-based studies.

Introduction

Molecular analyses using minimally invasively collected fecal samples are becoming increasingly common in ecological studies. For example, the information that can be obtained

from fecal samples includes the DNA of prey items in addition to that of the consumers (King et al., 2008; Symondson, 2002). Non-invasively collected fecal samples offer an opportunity to obtain valuable dietary information from imperiled species, which is often lacking due to the ethical concerns associated with traditional (e.g. stomach content analysis) techniques (Valentini et al., 2009). Despite the promises of molecular-based diet analyses, these methods have yet to be heavily applied outside of mammalian systems (Pompanon et al., 2012; Vo & Jedlicka, 2014). Reptiles are one taxonomic group in which these techniques are particularly underused, so applying these methods can provide further insight on feeding preferences.

Reptile diet is typically assessed by examination of stomach contents or feces for identifiable remains of prey (e.g., bones, hair, or scales) and by opportunistic wild observations of feeding behavior. While these techniques can provide a starting point for diet characterization, they require specialized taxonomic expertise and have the potential for severe biases (Symondson, 2002). For example, reptiles have been documented to consume prey that are soft-bodied or easily digestible (e.g., invertebrates; Brown et al. 2014); therefore, detection of these prey items would be impossible by relying on morphological identification. In addition, the collection of stomach contents is highly invasive, requiring either euthanasia or stomach pumping. Furthermore, reptiles have cryptic feeding behavior, making observations of feeding rare in the wild. Reptiles are currently facing global declines (Böhm et al., 2013; Zipkin et al., 2020), often with limited ecological information on feeding preferences. Minimally invasive methods are crucial to minimizing the stress and handling of at-risk species. An alternative, less invasive approach to assessing diet is through molecular analysis of feces via DNA metabarcoding (King et al., 2008; Valentini et al., 2009).

DNA barcoding is a means of identifying taxa from variable sequences in a standardized region of the genome (Hebert et al., 2003). When used in combination with next-generation sequencing, thousands of DNA sequences are amplified in parallel with increased accuracy and efficiency to identify multiple taxa from complex environmental samples (DNA metabarcoding). This makes it possible to obtain genetic information from fecal samples containing highly degraded DNA (King et al., 2008; Shokralla et al., 2012; Symondson, 2002). The number of taxa available in reference databases (e.g. Barcode of Life Data, GenBank) has rapidly grown, increasing the accuracy of taxonomic classification for diet studies (Porter & Hajibabaei, 2018). The most commonly used barcode marker for targeting animals is the mitochondrial cytochrome c oxidase subunit 1 gene (CO1) (Hebert et al., 2003). This region has been generally accepted to be reliable in identifying most animals down to the species level and has become the standard barcode marker used in dietary studies with animal prey (Sousa et al., 2019).

Although DNA metabarcoding is becoming a common tool for dietary assessments, working with fecal samples often presents challenges. Despite the ability of next-generation sequencing to detect rarer sequences, the quality and quantity of genomic DNA obtainable from fecal samples can be unpredictable (Vo & Jedlicka, 2014). There are multiple criteria to consider before performing downstream molecular diet techniques, one of which is the method of sample collection and preservation. The best preservation methods can greatly differ across taxonomic groups and sample types (Vo & Jedlicka, 2014), and so it is crucial this step be considered prior to sample collection. Although molecular diet analyses have been carried out successfully in few reptile species (Brown et al., 2012; Kartzinel & Pringle, 2015; Pereira et al., 2019), the impacts of sample preservation have yet to be discussed. While freezing is typically the preferred method of sample preservation, conditions in the field often constrain some storage techniques. As DNA

metabarcoding diet studies become an increasingly common method to characterize diet, it is crucial to establish the most effective sample preservation method to produce suitable quality genomic DNA for polymerase chain reaction (PCR).

Here, we assess three different sample preservation methods intended for molecular analyses of reptile diet. These include storage in ethanol at ambient temperature, temporary storage on ice until frozen at -20°C, and immediate freezing using dry ice and storage at -80°C. We aim to determine the optimal preservation method to obtain high-quality genomic DNA. In addition, we test extraction kits and one primer set commonly implemented in DNA metabarcoding animal diet studies. Experiments were conducted using fecal samples from the Eastern Massasauga Rattlesnake (*Sistrurus catenatus*), a cryptic species listed as threatened across its range with limited information on feeding preferences (Szymanski et al., 2015).

Materials and Methods

Study species

The eastern massasauga rattlesnake is a wetland species that occurs throughout The Great Lakes region (Seigel, 1986). Eastern massasauga populations have declined, and the species has been recently listed as Federally Threatened under the Endangered Species Act (U.S. Fish and Wildlife Service, 2015). They are mainly threatened by habitat loss and fragmentation, road mortalities, and vegetative succession (U.S. Fish and Wildlife Service, 2015). Characteristic of many reptiles, eastern massasaugas possess cryptic coloration, making them rare and difficult to detect. As a consequence, feeding observations of this species are rare in the wild, and identification of eastern massasauga diet composition has been limited to opportunistic

regurgitations, visual analysis of fecal and gut contents, and feeding trials (Holycross & Mackessy, 2002; Keenlyne & Beer, 1973; Shepard et al., 2004; Weatherhead et al., 2009).

Field sampling

We carried out visual encounter surveys from May-September 2018 and 2019 during the active season (approximately April to October) (Szymanski et al., 2015) from populations located throughout the Lower Peninsula of Michigan. If a massasauga was located, the snake was captured opportunistically using tongs and safely secured in a cloth bag. All capture locations were recorded using handheld GPS units. Prior to processing, we secured snakes in clear plastic tubes for safe handling. Newly captured individuals were marked using a passive integrated transponder (PIT) tag for permanent identification. While the snake was safely restrained in a plastic tube, we collected fecal samples opportunistically by gentle palpation or voluntary excretion. Fecal material was directly extracted from the snake by placing its tail into a 50mL plastic tube until defecation occurred. Following processing, each Massasauga was returned to its initial capture site.

Sample preservation and collection

Following collection, fecal samples were either 1) stored in ethanol at ambient temperature, 2) stored dry and temporarily cooled on ice, or 3) stored dry and immediately frozen using dry ice. Samples collected in the summer of 2018 were temporarily placed in a cooler containing ice following collection and moved to long-term storage at -20°C as soon as possible. Due to field conditions, the length of time these samples were stored on ice greatly varied from a few hours to a few days until a freezer was accessible. In the following field season (May-August 2019),

samples were immediately frozen using a dry ice ethanol bath. Each sample collection tube remained in the bath for a few minutes until frozen. These samples were preserved in a cooler with dry ice, where they remained frozen until moved to a -80°C freezer for long-term storage. In addition, samples collected from an Ohio population were stored in ethanol at ambient temperature. During the active season in 2018 and 2019, we collected 101 total fecal samples across 10 sampling locations in Michigan. From May-July 2019, 24 total fecal samples were collected from one population in Ohio. We randomly selected seven samples from each sample preservation method: immediate freezing with dry ice, cooled on ice, and stored in ethanol at ambient temperature for this study.

DNA extraction and quantification

We tested two DNA extraction kits for extraction efficiency. Extraction of fecal samples was carried out using QIAamp PowerFecal DNA Kit (Qiagen) and the QIAamp DNA Stool Mini Kit (Qiagen) following the standard protocol. If possible, urates were removed from samples prior to extraction, as no dietary information can be obtained from them. One negative control containing only reagents was included during each extraction batch to test for contamination. Successful DNA extraction was confirmed by bright clear bands on a 1% agarose gel.

Primer selection testing and amplification

Primer selection is a crucial part of DNA metabarcoding studies and must fit several criteria in order for the prey DNA to be amplified successfully. Prey DNA in feces is highly degraded after passing through the predators digestive system (Deagle et al., 2006), therefore the selected primers must target a short DNA region to increase amplification success (Pompanon et al.,

2012). If the predator is a generalist, or there is a lack of prior knowledge of potential prey species, the barcoding primers must also cover a broad taxonomic range yet be variable enough to discriminate among closely related species. In cases such as this, universal metazoan primers are the best option. The 658 bp fragment of the region encoding for the mitochondrial cytochrome c oxidase 1 (COI) gene is the generally accepted standard barcode to target animals (Hebert, Ratnasingham, & de Waard, 2003; Valentini et al., 2009).

We tested the universal metazoan forward mlCOIintF 5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' (Leray et al., 2013) and reverse jgHCO2198 5'-TAIACYTCIGGRTGICCRAARAAYCA-3' (Geller et al., 2013), targeting a 313 bp fragment of the CO1 region. This primer pair has successfully amplified a wide range of metazoan taxa and has performed well for previous metabarcoding and diet assessments (e.g. Riccioni et al., 2018; Bohmann et al., 2018). The reverse primer jgHCO2198 is a redesign of the commonly used Folmer reverse primer HCO2198 (Folmer et al., 1994) corrected for mismatches and with increased degeneracy to allow for broader taxonomic amplification (Geller et al., 2013). PCR was carried out using illustra™ puReTaq Ready-To-Go PCR Beads with the following conditions: 0.6 μL of the forward and reverse primer (0.24 μM), 1 μL BSA, 1 μL of genomic DNA, and 21.8 μL of water for a 25 μL total reaction volume.

We used a touchdown PCR protocol modified from Leray et al. (2013) to minimize non-specific amplification. We carried out an initial denaturation at 95°C for 10 min followed by 16 cycles: denaturation at 95°C for 10s, annealing at 62°C for 30s (-1°C per cycle), extension at 72°C for 60s followed by 25 cycles: denaturation at 95°C for 10s, annealing at 46°C for 30s, extension at 72°C for 60s followed by a final extension at 72°C for 7min. PCR amplification success was confirmed by clear bands on a 2% agarose gel.

Results

DNA extraction and quantification

We were unable to extract amplifiable DNA successfully using the QIAamp DNA Stool Mini Kit. However, extraction was successful using the QIAamp PowerFecal DNA Kit (Fig. 1). DNA extraction from fecal samples collected in 2019 and stored in ethanol at ambient temperature was either entirely unsuccessful (3 of 7 extracts) or appeared extremely smeared on the gel (1 of 7 extracts; Fig. 1A). We successfully extracted DNA from both ice-stored (7 of 7 extracts) and dry ice frozen (7 of 7 extracts) samples (Fig. 1 B, C). For the purpose of this study, we decided to include all samples for further analysis even if they did not yield visible DNA on the gel.

Primer selection testing and amplification

Amplification success of the universal metazoan primers mlCOIintF (Leray et al., 2013) and jgHCO2198 (Geller et al., 2013) differed across sample preservation methods (Fig. 2). Specifically, amplification was rarely successful for samples stored in ethanol (Fig. 2A). PCR amplification was not consistently successful for ice-stored samples (Fig. 2B), while all dry ice frozen samples were amplified with the selected primers (Fig. 3B).

Discussion

The success of DNA extraction and PCR amplification varied between storage preservation methods. Although a few faint bands are visible on the gel from fecal samples stored in ethanol, we were unable to isolate DNA from these samples reliably. The remaining DNA extractions from ethanol-stored samples ranged from unsuccessful (no visible gel bands) or appeared extremely smeared on the gel (Fig. 1A), indicating the DNA was highly degraded. PCR

amplification attempts with these samples were rarely successful. This suggests the presence of inhibitors that were coextracted in the ethanol-stored samples that prevented efficient DNA extraction and/or amplification (Schrader et al., 2012). Samples containing degraded DNA templates have previously been shown to limit amplification success, which is also a likely cause for the lack of PCR amplification (Deagle et al., 2006). Based on these results, ethanol stored fecal samples likely will not produce high enough quality genomic DNA for successful diet analyses, and this method should be avoided.

Preserving samples on ice or immediately freezing was more effective compared to storage in ethanol. DNA extraction and PCR amplification were most successful when samples were immediately frozen. Although PCR amplification was successful from fecal samples temporarily stored in ice or flash frozen, it is unknown until sequencing whether this is only predator DNA. This is a common problem for dietary studies when a universal primer is selected, inadvertently amplifying the predator (non-target) DNA. During passage through the lower digestive system, cells of the predator are shed, resulting in the fecal extracts containing less prey DNA compared to the predators (Deagle et al., 2006). To prevent predator DNA from swamping out the amplification of rare prey sequences, designing a predator-specific blocking primer should be considered for future diet assessments (Vestheim & Jarman, 2008).

Previously, non-invasive sampling of reptile feces proved unsuccessful when a larger amplicon size (~500 bp mtDNA) was targeted, producing low-quality sequences unsuitable for further analyses (Jones et al., 2008). However, these samples were only cooled on ice and later stored at -20°C. In the present study, we have demonstrated that targeting a relatively small amplicon around 313 bp is possible when samples are stored correctly. It is possible that PCR

amplification of larger amplicons can be successful when samples are immediately frozen, and future studies should focus on this.

There are multiple biological characteristics reptiles possess which should be considered as potential roadblocks when attempting a molecular diet analysis. Reptiles tend to have extremely efficient digestive systems (Secor, 2008), which can further degrade prey DNA and severely impact the ability to obtain quality DNA suitable for amplification. For example, Falk and Reed (2015) attempted a molecular analysis to determine the prey consumed by the Burmese Python using samples collected directly from the stomach and intestine. The majority of samples returned poor quality or non-target sequences. The low quality or lack of prey DNA was likely a result of the extremely long digestive time in this species compared to most reptiles (Lillywhite et al., 2002). Therefore, we stress that reptile study species should be selected with caution for diet analyses.

We were unable to extract any DNA using the Qiagen Stool Mini Kit although this kit has performed well for mammalian systems (e.g. Hájková et al., 2006; Zeale et al., 2011). The lack of successful extraction may be attributed to inhibitors present in the feces. Compared to mammals, reptiles possess a cloaca and do not excrete nitrogenous waste (uric acid) and feces separately (Shoemaker & Nagy, 1977). Uric acid may act as a potential inhibitor in successful DNA extraction and amplification if coextracted (Schrader et al., 2012). Studies that attempted to isolate DNA from avian systems have had similar results. Jedlicka et al. (2013) were unable to obtain high-quality DNA suitable for PCR amplification from the Western Bluebird using the Qiagen Stool Mini Kit. They attributed this to the possible coextraction of PCR inhibitors, as bird fecal matter contains high levels of uric acid (Jedlicka et al., 2013). This potentially puts

molecular studies of reptile diet at a disadvantage; extra steps may need to be considered to avoid sample contamination from uric acid.

Here, we have shown that usable DNA intended for downstream molecular applications can be obtained from reptile feces if the samples are frozen as soon as possible to minimize further DNA degradation. If possible, removal of uric acid in the field following collection of fecal samples may also increase DNA extraction and PCR success. Although field conditions may not always allow for contamination-free and precise sample collection, choosing an effective sample preservation protocol is crucial for reptile diet analyses. Although the samples used in this study are intended to be used for future diet analyses, reptile fecal samples are greatly underused and have the potential for numerous other molecular applications. In addition to diet analyses, we suggest fecal samples can replace blood samples to genotype individuals to monitor populations of protected species. Future work should focus on quantifying DNA yields and focus on the success of downstream molecular applications. To minimize both contamination and DNA degradation of reptile fecal samples, we suggest the above criteria be considered to obtain high-quality dietary data.

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Figure 1. DNA extractions of (A) ethanol stored (B) ice stored and (C) dry ice frozen samples. Numbers denote lane numbers the samples were loaded into.

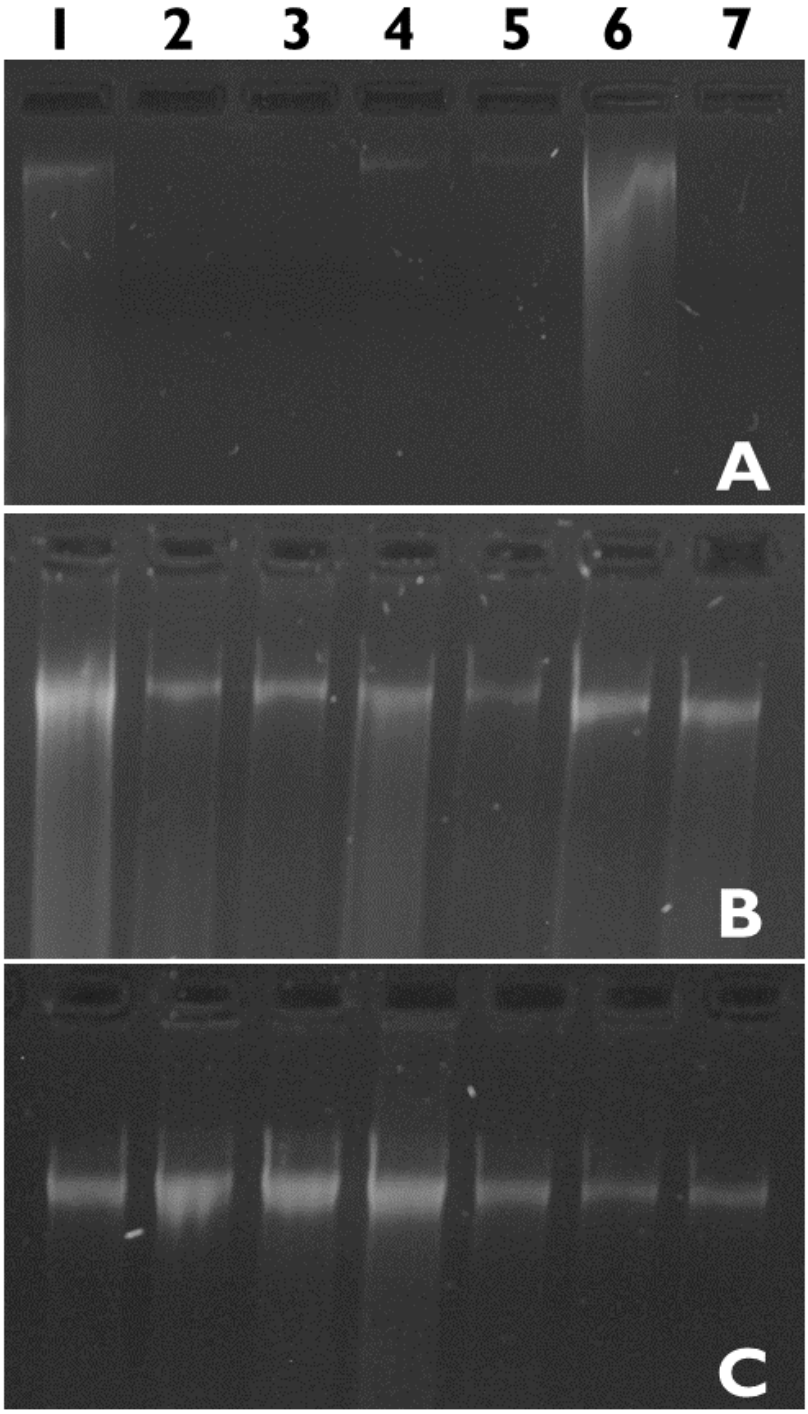
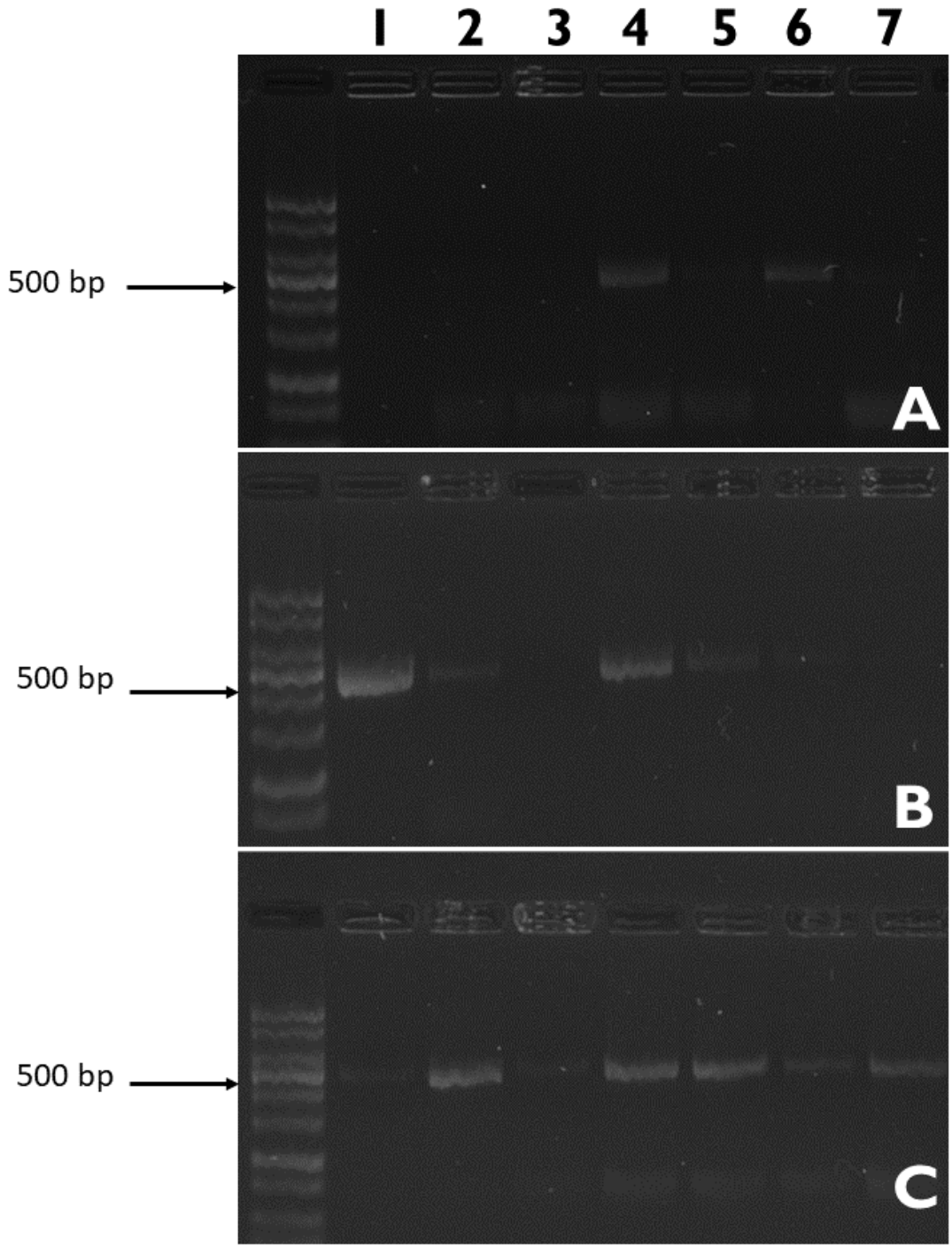


Figure 2. PCR amplification of (A) ethanol stored (B) ice stored and (C) dry ice frozen samples. Numbers denote lanes, with samples loaded in the same order as the extraction gel. Arrows denote the target region for amplification. Note that due to primer size and tags added for future sequencing, the product size appears larger than the target 313 bp.



Chapter 3: Extended Review of Literature and Extended Methodology

Extended Review of Literature

Trophic interactions are critical to understanding ecosystems and the ecology of the species which inhabit them (Estes et al., 2011). Predator-prey interactions are often the driving force of changes within an ecosystem, but food webs are complex and difficult to identify (Pompanon et al., 2012). Diet studies have identified important aspects of a species feeding ecology, such as if the predator is a specialist or generalist (Clare et al., 2009), how human-modified landscapes influence feeding behavior (Cristóbal-Azkarate & Arroyo-Rodríguez, 2007), or variation across seasons (Kartzinel & Pringle, 2015). Understanding the feeding behavior of threatened species is particularly important, with this information being considered for the development of management plans (Pompanon et al., 2012; Symondson, 2002; Valentini et al., 2009). Although knowledge of a species diet has critical conservation implications, it is often difficult to identify for predators with cryptic feeding behavior. Species that are elusive in nature such as reptiles are often underrepresented in diet studies. Reptiles are facing global declines (Gibbons et al., 2000), therefore it is critical that these knowledge gaps are filled for threatened species to increase conservation efforts.

Diet Study Methods

Diets are most commonly assessed by opportunistic feeding observations or regurgitations, examining prey remains in gut or fecal contents, and stable isotope analyses (Pompanon et al., 2012). Typically, one method is chosen depending on which is the most appropriate for the species of interest. Wild feeding observations are rare, and it is unknown whether the observed foraging behavior is typical for that predator. Stable isotopes are better suited when energy flow

through an ecosystem is the focal point of the study with *a priori* diet knowledge, as species-specific identifications cannot always be made (Pompanon et al., 2012; Sousa et al., 2019). Dissection of fecal contents relies on identifiable remains of prey such as bones, hair, or scales. While these methods have been successful, they are labor-intensive and heavily rely on taxonomic expertise and accurate reference material. There is also the opportunity for biased or false-negative results by relying solely on morphological identification (Sheffield et al., 2001). If the consumed prey item is soft-bodied or easily digestible, there may be no identifiable remains and detection would be impossible by hard-part analysis alone. In addition, biased results may occur if the prey remains are digested at different rates. Another factor to consider is what methods are possible for the specific study species. For example, examining gut contents is invasive, requiring euthanasia to access a predator's GI tract, therefore this method would not be realistic for studying threatened vertebrate species. Using these methods, the ecological importance of certain prey species could be easily overlooked (Brown et al., 2012; Symondson, 2002). Increased accessibility to next-generation sequencing technology, expansion of reference sequences in public databases, and the developments of universal primers have drastically improved the success of vertebrate DNA metabarcoding dietary studies (reviewed by (King et al., 2008; Sousa et al., 2019; Symondson, 2002).

DNA Metabarcoding in Dietary Studies

DNA barcoding identifies taxa from unique DNA sequences in a standardized DNA region (Hebert et al., 2003). These standardized DNA regions must be highly conserved, but divergent enough among species to allow identification (Hebert et al., 2003). Following sequencing, the unknown DNA sequences are compared to those from known taxa in a reference sequence

database to identify the prey taxa consumed. The most commonly used barcode marker for targeting metazoan taxa is the mitochondrial cytochrome c oxidase subunit 1 gene, consisting of 658 base pairs (CO1; Hebert et al., 2003). This region has been generally accepted to be the most reliable in identifying metazoan to the species level and is heavily used in dietary studies with animal prey (Sousa et al., 2019).

DNA barcoding abilities have increased along with developments in sequencing technology. Compared to the traditional Sanger sequencing approach, next-generation sequencing platforms allow multiple species to be identified from complex environmental samples (e.g., soil, feces, and water), known as DNA metabarcoding. The traditionally used Sanger sequencing methods (Sanger et al., 1977) can only sequence one long fragment of DNA with individuals samples, while next-generation sequencing can process thousands of sequence reads in parallel (Mardis, 2008). As DNA metabarcoding has increased in popularity, the number of taxa available in reference databases (e.g. Barcode of Life Database, GenBank) continues to grow, making them a reliable resource for diet studies (Porter & Hajibabaei, 2018). Ecologists have recently taken advantage of DNA metabarcoding techniques to assess biodiversity levels, analyze diets, and assess the presence of rare or invasive species (reviewed by Kress et al., 2015; Valentini et al., 2009). This has been particularly useful for diet studies, providing a minimally-invasive method to identify prey consumed using fecal samples (Casper et al., 2007; King et al., 2008; Valentini et al., 2009). Although DNA metabarcoding has greatly increased the ability to characterize diets, it is currently the most reliable for occurrence data. Previous studies have attempted quantitative assessments of diets by relating the amount of prey consumed to its DNA sequence abundances in metabarcoding analyses of fecal samples (Deagle, Chiaradia, McInnes, & Jarman, 2010). However, the ability to quantitatively measure prey items is still in its infancy

and has limited accuracy (Jarman et al., 2013; Pompanon et al., 2012; Thomas et al., 2014), as the number of sequence reads can vary depending on differing digestion rates and PCR bias when using universal primers.

DNA metabarcoding diet studies have been attempted using multiple different barcode regions (Valentini et al., 2009). Universal primers targeting a 76-100 base pair range of the 12S region (Riaz et al., 2011), and the around 100 base pairs of the 16S region (Barba et al., 2014) have been applied to metabarcoding studies. The CO1 target region size has varied, and developed primers target shorter fragments around 160 base pairs (Zeale et al., 2011), or larger fragments around 300 base pairs (Leray et al., 2013). These barcoding markers target smaller sequences of DNA and are occasionally used in metabarcoding diet studies (e.g. Barba et al., 2014; Kartzinel and Pringle, 2015; Shehzad et al., 2012). Prey DNA found in fecal samples is highly degraded due to passing through the predators digestive system (Deagle et al., 2006), therefore primers which target shorter sequences are necessary so rare prey items can be sequenced. While these primer regions are commonly used to characterize the diets of predators, they have yet to be heavily tested with reptile species.

Inhibiting Predator DNA in Dietary Studies

When using universal primers for diet analyses, the non-target (predator) DNA will amplify at a larger scale and limit the amount of target prey DNA amplified successfully due to the degraded nature of the prey DNA. As next-generation sequencing technology often specifically focuses on sequencing short DNA fragments, the slightly degraded DNA from feces can be effectively analyzed. However, due to passing through a digestive system, the prey DNA will be highly degraded compared to predator DNA (Deagle et al., 2006). To prevent the predator DNA from

amplifying at a larger scale than the prey DNA, Vestheim & Jarman (2008) developed a blocking oligonucleotide. By minimizing the amount of predator DNA, rare prey items are less likely to be swamped out. Blocking primers are designed to have a specific preference to only bind to the predator or non-target DNA. Once the primers are effectively bound, the 3' end of the primer is typically modified with a C3 spacer. This modification contains a three-carbon chain attached to the terminal 3' hydroxyl group that halts polymerase extension. Blocking oligonucleotides are commonly incorporated into metabarcoding diet analyses. When previous studies have compared prey items identified with and without blocking oligonucleotides, the blocker was successful in limiting the amount of predator DNA, allowing more sequence reads to be assigned to prey (Kumari et al., 2019; Shehzad et al., 2012). For example, when leopard cat prey DNA was amplified under blocking oligonucleotide conditions, several new prey items were detected that were not previously when leopard cat DNA was amplified (Shehzad et al., 2012).

Eastern Massasauga Rattlesnake

The eastern massasauga rattlesnake (*Sistrurus catenatus*) described by Rafinesque in 1818 is a pit viper species found throughout the Great Lakes Region. Its range extends from western New York to southern Ontario, eastern Missouri, and Iowa, and southeast Minnesota (United States Fish and Wildlife Service, 2016). Eastern massasaugas select for a combination of wetland and upland habitats, ranging seasonally (Moore & Gillingham, 2006). Wetland habitats provide suitable habitats for foraging, hibernation sites, and thermoregulation (Szymanski, 1998). During the winter months, massasauga will retreat in crayfish or small mammal burrows for brumation, later moving into more open canopy areas during the summer months (Moore & Gillingham, 2006).

The eastern massasauga was listed as Federally Threatened under the Endangered Species Act in 2016 (United States Fish and Wildlife Service, 2016) and the Species at Risk Act (Committee on the Status of Endangered Wildlife in Canada, 2002). Loss of wetland habitat is the main contributor to this species decline due to fragmentation, conversion of wetlands for agricultural use, and vegetation succession. Road mortalities and direct human persecution have also contributed to population declines (Szymanski et al., 2015). Wetlands are becoming increasingly fragmented due to human activities further fragmenting massasauga populations (United States Fish and Wildlife Service, 2016). Wetlands are also at risk of invasion from invasive woody plant species, which occupy important open canopy areas that massasaugas require. Populations appear to be continually declining throughout their range, with historical populations either extirpated or at an unknown status, with the majority of remaining populations persisting in Michigan (Szymanski et al., 2015). Michigan is at the center of eastern massasauga geographic range and contains some of the most viable remaining populations (P. C. Jones et al., 2012), therefore conserving Michigan populations is critical for the persistence of the species. Due to their cryptic behavior, there are aspects of eastern massasauga ecology in which knowledge is limited, one of which includes diet.

Eastern Massasauga Diet Studies

Knowledge on massasauga diet is limited, with prey items being identified through opportunistic regurgitations, hard-part analysis of fecal or gut contents, and captive feeding trials (Keenlyne & Beer, 1973; Shepard et al., 2004; Tetzlaff et al., 2015; Weatherhead et al., 2009). With consumed prey records so few, there are a wide range of identified prey species throughout their range which are labeled into broad taxonomic categories. Previous dietary studies suggest massasaugas

are opportunistic predators with a generalist diet (Weatherhead et al., 2009) with variability across their range. Small mammals such as voles, shrews, and mice are suggested to make up the bulk of their diet (Keenlyne & Beer, 1973; Shepard et al., 2004; Weatherhead et al., 2009). In Wisconsin populations, the highest occurrence of prey consisted of meadow voles (*Microtus pennsylvanicus*) for adult individuals (Keenlyne & Beer, 1973), while shrews (genus *Blarina*) were the most commonly predated in Ontario and Ohio populations (Weatherhead et al., 2009). Eastern massasaugas have also been observed feeding upon larger mammals such as squirrels (*Glaucomys, Tamiasciurus, Sciurus;* Tetzlaff et al., 2015, Weatherhead et al., 2009).

Eastern massasaugas have been reported to consume other snake species through stomach analyses and opportunistic regurgitations including *Thamnophis spp*., *Storeria dekayi*, and *Storeria occipitomaculata*. Along with these analyses, multiple unidentifiable snake species have been documented as well (Hallock, 1991; Keenlyne & Beer, 1973; Ruthven, 1911; Tetzlaff et al., 2015). In addition, few cases of cannibalism have been documented via gastro-intestinal content dissection (Hallock, 1991; Ruthven, 1911). Cannibalism is not an unknown dietary habitat for rattlesnakes, as it has also been observed with numerous other rattlesnake species in the *Crotalus* genus (e.g. Mociño-Deloya et al., 2009). In more rare occurrences, they have been noted to birds (*Agelaius spp.*) and other snake species (Keenlyne & Beer, 1973).

There are discrepancies in the literature on whether ontogenetic diet shifts occur. In feeding trials, neonates consumed snake prey every time it was offered (Shepard et al., 2004). However, regurgitations from captured free-ranging individuals only consisted of shrews and voles (Shepard et al., 2004). When gut contents were examined, young snakes were mostly the only feeders of other snake prey in Wisconsin (Keenlyne & Beer, 1973). While no evidence of

an ontogenetic diet shift was observed in Ontario and Ohio populations, young snakes also commonly fed on small mammals, with rare snake occurrences (Weatherhead et al., 2009).

Techniques in the previous diet studies described above are either invasive (examining gut contents) or have the potential for biased results from easily digestible prey (morphological identification from feces). With the massasauga recently listed as Federally Threatened, the techniques to carry out diet analysis are limited and must be minimally invasive. Molecular techniques have yet to be used to identify eastern massasauga diet, and will provide an accurate, minimally invasive means to identify their diet in Michigan. By identifying what prey are consumed by eastern massasaugas, these species can also be considered when management plans are developed.

Reptile DNA Metabarcoding Assessments

Many reptile species have cryptic feeding behavior, making observations of feeding rare in the wild. Molecular diet analyses using a DNA metabarcoding approach in combination with next-generation sequencing have been carried out in few reptile species (Brown et al., 2012; Falk & Reed, 2015; Kartzinel & Pringle, 2015; Pereira et al., 2019), ranging in success. The majority of these studies characterize the diets of predators with insectivorous or specialist diets, while rarely focusing on generalists that feed on a wide breadth of taxonomic groups. As a result, the majority of previous studies have been restricted to the use of group-specific primers. Insectivorous lizard species have been the most extensively studied group using DNA metabarcoding methods (Gil et al., 2020; Kartzinel & Pringle, 2015). Additionally, the slow worm lizard diet was studied using earthworm-specific primers targeting the mtDNA 12S gene (Brown et al., 2012). The diet of *Anolis* lizards, considered an insect generalist predator, was successfully characterized when

only targeting arthropods in the 16S region (Kartzinel & Pringle, 2015). A diet analysis attempted with Moroccan rock lizards targeting the 16S and CO1 region returned sequences of varying quality and different prey items identified depending on the barcode region (Pereira et al., 2019). Falk and Reed (2015) attempted a molecular analysis targeting the CO1 region to determine the prey consumed by the Burmese python using samples collected directly from the stomach and intestine. The majority of samples returned poor quality or non-target sequences. The selected mini-barcoding primers; miniBarF and miniBarR (Meusnier et al., 2008) only returned successful prey item identification from 15% of samples (Falk & Reed, 2015). The low quality or lack of prey DNA was likely a result of the extremely long digestive time in this species compared to most reptiles (Lillywhite et al., 2002).

When compared to traditional hard-part analyses, DNA metabarcoding approaches have uncovered that some reptile predators follow a more generalist diet. For example, Gil et al. (2020) applied both approaches when characterizing the diet of the Selvagens gecko (*Tarentola boettgeri bischoffi)* which was previously thought to follow a specialist arthropod diet. When a vertebrate-specific primer was tested on fecal samples, additional prey such as seabirds, reptiles, and fish were identified (Gil et al., 2020).

Conclusions

Using molecular methods such as DNA barcoding with the addition of next-generation sequencing can dramatically increase knowledge on a species diet. Threatened species will particularly benefit from these abilities to use these minimally invasive methods to fill in gaps of their foraging behavior, resulting in more effective conservation efforts. These methods haven’t yet been applied to any rattlesnake species, and the eastern massasauga rattlesnake will be the

first. Identifying prey which eastern massasaugas eat will allow for better management of their wetland habitats, and therefore will protect other species which rely on wetlands as well.

Extended Methodology

Fieldwork and sample collection (Chapter 2.1, 2.2)

We selected survey sites based on previously recorded eastern massasauga rattlesnake locations During the selection process, we aimed to evenly distribute survey locations across the lower peninsula of Michigan to accurately capture any diet variation which may be occurring among populations. Ten sites in total were surveyed, including Bois Blanc Island, located above the lower peninsula and represents the northern edge of eastern massasauga range.

We carried out visual encounter surveys from May-September 2018-19 during the eastern massasauga active season (approximately April to October; Szymanski et al., 2015). Considering the possibility that diet shifts may occur throughout the season, we visited each site multiple times during the active season whenever possible, excluding Bois Blanc Island. If a snake was located, it was captured opportunistically using tongs and safely secured in a cloth bag inside of a bucket. All capture locations were recorded using handheld GPS units. Upon capture, environmental data such as ambient and substrate temperature, cloud cover, precipitation were recorded.

Prior to processing, we handled snakes by encouraging them to move up a clear plastic tube until we could secure them for safe handling. Newly captured individuals were marked using a passive integrated transponder (PIT) tag for permanent identification. We collected mass (to the nearest g), snout-vent length (SVL) (cm), subcaudal scute count, and tail length (cm). Gravidity was determined for adult females by gentle palpation. If gravid, we attempted to

estimate the number of embryos present while palpating. Sex was determined by probing of the cloaca. If snakes were unable to be probed, we determined sex based on the subcaudal count (≥ 25 subcaudals were considered male). Individuals were classified as adult, juvenile, or young based on SVL measurements. Females with SVL ≥ 45 cm and males ≥ 43 cm were classified as adults (Bradke et al., 2018), and juveniles as ≥ 30 cm. Snakes with SVL < 30 cm that possessed one or fewer rattle segments, followed by a single complete terminal rattle segment (without breakage) were considered young. Fecal material was directly extracted from the snake by placing its tail into a 50mL plastic tube and was gently palpated until defecation occurred. Following processing, each massasauga was returned to its initial capture site. In total, 102 samples were collected across 10 populations (see Figure 1, Table 2; Chapter 2.1).

Sample preservation (Chapter 2.1, 2.2)

Following collection, fecal samples were either (1) stored in ethanol at ambient temperature, (2) stored dry and temporarily cooled on ice, or (3) stored dry and immediately frozen using dry ice. Samples collected in the summer of 2018 were temporarily placed in a cooler containing ice following collection and moved to long-term storage at -20°C as soon as possible. Due to field conditions, the length of time these samples were stored on ice greatly varied from a few hours to a few days until a freezer was accessible. In the following field season (May-August 2019), samples were immediately frozen using a dry ice ethanol bath. Each sample collection tube remained in the bath for a few minutes until frozen. These samples were preserved in a cooler with dry ice, where they remained frozen until moved to a -80°C freezer for long-term storage.

To compare sample preservation methods (Chapter 2.2), 24 additional fecal samples collected from an Ohio population and stored in ethanol at ambient temperature were included.

We randomly selected seven samples from each sample preservation method: immediate freezing with dry ice, cooled on ice, and stored in ethanol at ambient temperature.

DNA extraction (Chapter 2.1, 2.2)

We tested two DNA extraction kits for extraction efficiency. DNA extractions from fecal samples were tested using the QIAamp DNA Stool Mini Kit (Qiagen) and QIAamp PowerFecal DNA Kit (Qiagen) following the standard protocols. All attempted DNA extractions using the QIAamp DNA Stool Mini Kit were unsuccessful, therefore the QIAamp PowerFecal DNA Kit was used for all extractions. Extraction from each fecal sample was carried out following the standard protocol requiring 0.25 g of stool. A random subset was taken if the sample exceeded 0.25 g, while the entire sample was used if it was less than 0.25 g. DNA extractions took place in a laminar flow hood with UV sterilization to prevent contamination.

Snakes excrete all wastes from a cloaca; therefore, urates were sometimes present in fecal samples. As dietary information cannot be obtained from urates, we avoided including them to the best of our ability during the extraction process. One negative control using double-distilled water containing only reagents was included during each extraction batch to test for contamination. Extraction success was confirmed by gel electrophoresis on a 1.5% agarose gel by the presence of bright clear bands.

Although we collected 102 fecal samples in total, sufficient DNA for amplicon sequencing was obtained from 83 samples. To determine a suitable volume of DNA for PCR reactions in Chapter 2.1, a random subset of DNA samples was quantified using a NanoDrop™ OneC Spectrophotometer (ThermoFisher Scientific Inc.) with three replicates per sample. The quantity of DNA per sample ranged from 5.4-51.4 ng/μL (24.7 ng/μL on average).

Primer and reagent selection (Chapter 2.1)

There are multiple criteria to consider when selecting the most appropriate barcoding primer and increase the success of prey DNA amplifying successfully. Prey DNA in feces is highly degraded following passage through the predators digestive system. (Deagle et al., 2006). To compensate for the degraded nature of fecal samples, primers must target a short DNA region (~100-400 base pairs) to increase amplification success. If the predator is a generalist, or there is a lack of *a priori* knowledge of potential prey species, the barcoding primers must also cover a broad taxonomic range yet be variable enough to discriminate among closely related species. In cases such as this, universal metazoan primers are the best option. The 658 bp fragment of the region encoding for the mitochondrial cytochrome c oxidase 1 (COI) gene is the generally accepted standard barcode to target animals (Hebert et al., 2003; Valentini et al., 2009). The CO1 region has faced criticism due to potential taxonomic biases yet is still accepted as the most suitable barcode region for metazoan metabarcoding.

To identify as many prey taxa as possible, we selected the universal metazoan forward mlCOIintF 5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' (Leray et al., 2013) and reverse jgHCO2198 5'-TAIACYTCIGGRTGICCRAARAAYCA-3' (Geller et al., 2013), targeting a 313 bp fragment of the CO1 region. This primer pair is designed to amplify all metazoan taxa and is commonly used and has performed well in previous DNA metabarcoding dietary assessments. The reverse primer jgHCO2198 is a redesign of the commonly used Folmer reverse primer HCO2198 (Folmer et al., 1994) corrected for mismatches and increased degeneracy to allow for broader taxonomic amplification (Geller et al., 2013). Primers were further modified for Illumina

MiSeq sequencing by adding overhangs at the 5’ end of the forward and reverse primer to allow for indexing.

For this primer set, it is recommended to use a high-fidelity polymerase with proofreading activity to limit errors during PCR for next-generation sequencing (Leray et al., 2013). Attempts of generating any amplicons using KAPA HiFi HotStart ReadyMix PCR kit (KAPA Biosystems Inc., USA) and Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs, USA) were unsuccessful. This is due to the inosine bases present in the reverse primer (jgHCO2198), which is incompatible with many high-fidelity polymerases and halts amplification (e.g. (Clarke et al., 2017; Zhang et al., 2018). Amplicons were successfully generated using NEBNext® Q5U® Master Mix (NEB) which can read inosine bases and possesses 3’-5’ exonuclease proofreading ability. PCR products were checked via gel electrophoresis on a 1.5% agarose gel for amplification success. PCR success was determined by the presence of bright clear bands at the target amplicon size, while failure resulted in no bands with only primer dimer present.

Predator blocking oligonucleotide design (Chapter 2.1)

When using universal primers, the non-target (predator) DNA will amplify at a larger scale and limit the amount of target prey DNA amplified successfully due to the degraded nature of the prey DNA. To increase the chances of identifying rare prey items, we designed an annealing inhibiting blocking oligonucleotide developed by Vestheim & Jarman, 2008. Blocking primers are designed to have a specific preference to only bind to the predator or non-target DNA. Once the primers are effectively bound, the 3’ end of the primer is typically modified with a C3 spacer.

This modification contains a three-carbon chain attached to the terminal 3’ hydroxyl group that halts polymerase extension.

To design the blocking primer, eastern massasauga-specific sequences along with available sequences of previously recorded prey items from past diet studies (Holycross & Mackessy, 2002; Keenlyne & Beer, 1973; Shepard et al., 2004; Tetzlaff et al., 2015; Weatherhead et al., 2009) were downloaded from GenBank (see Table 4 for list of aligned prey). Eastern massasaugas have been documented to consume other snake species, and so we treated all snakes with ranges that overlap with the eastern massasauga as additional potential prey. Sequences were aligned using Clustal W in MEGA X (Kumar et al., 2018). Using the alignments, a suitable 3’ end was selected by identifying a variable region where there were a number of mismatches between the eastern massasauga with potential prey. We designed the blocking primer based on the mlCOIintF forward primer that overlapped 10 bp at the 3’ end of the primer and extended 19 bp into the massasauga-specific sequence (Table 3). Blocking oligonucleotides for diet studies are typically designed using a C3 spacer modification on the 3’ end to prevent amplification (Vestheim & Jarman, 2008). However, we were unable to consistently block eastern massasauga DNA using this modification, likely due to the use of a high-fidelity polymerase, and instead opted for a 3’ inverted dT modification. To test the specificity of the blocking primer, we performed PCR (see prey amplification section below for cycle conditions), on three mammal specimen (shrew, vole, and mouse), one sample containing strictly eastern massasauga DNA, and one eastern massasauga fecal sample to be used for downstream analyses. The success of the blocking primer was validated by viewing all the above PCR products on a 2% agarose gel. The three mammals, along with the fecal sample produced bright clear bands, while the sample containing only massasauga DNA produced a

faint band (Figure 3). We determined the blocking primer a success due to the clear visual differences in PCR product.

This blocking primer is designed to compete with the universal primer and limit the amplification of the predator DNA. However, blocking primers have been noted to block amplification of target prey DNA if they are closely related to the predator (Piñol et al., 2015; Shehzad et al., 2012). Consequently, the blocking oligonucleotide may block the amplification of other snake species as well (Chapter 2.1; Table 5).

Prey Amplification (Chapter 2.1)

To limit errors while generating amplicons during amplification, we selected the NEBNext® Q5U® Master Mix (New England Biolabs, USA) high-fidelity polymerase that is compatible with the inosine bases present in the jgHCO2198 reverse primer and possesses a 3'-5'exonuclease activity. The annealing inhibiting blocking primer was included at a 15:1 ratio compared to the forward and reverse primer. PCR was carried out using the following conditions: 12.5 uL of NEBNext Q5U Master Mix at 1x, 2 uL of DNA, 1.25 uL of the forward and reverse primer (0.5 uM), 1.25 uL blocking oligonucleotide (5 uM), and 7.75uL of nuclease-free water (NEB) for a 25uL total reaction volume. We carried out an initial denaturation at 98°C for 30 s followed by 30 cycles: denaturation at 98°C for 10 s, annealing at 64°C for 30 s, extension at 72°C for 60s followed by a final extension at 72°C for 5 min. PCR amplification success was confirmed by gel electrophoresis on a 1.5% agarose gel by the presence of a clear band.

Universal primers may result in better amplification of some taxa over others, resulting in potential PCR bias. We opted for *in vitro* testing of the above primer set, to ensure that the

potential prey DNA would be successfully amplified with such a universal primer. We obtained successful PCR product with the vole, shrew, and mouse DNA. As massasaugas have been documented to consume other snake species, we also tested these primers with eastern garter snake (*Thamnophis sirtalis*), a previously documented prey item (Tetzlaff et al., 2015). However, we were unable to successfully obtain any PCR product with this species. Reptile species were not heavily considered during the design of many universal primers and may result in unsuccessful amplification due to mismatches at the priming region.

Primer testing and amplification of fecal samples (Chapter 2.2)

To evaluate how storage methods influenced amplification success in Chapter 2.2, PCR was carried out using illustra™ puReTaq Ready-To-Go PCR Beads with the following conditions: 0.6 μL of the forward and reverse primer (0.24 μM), 1 μL BSA, 1 μL of genomic DNA, and 21.8 μL of water for a 25 μL total reaction volume. We used a touchdown PCR protocol modified from Leray et al. (2013) to minimize non-specific amplification. We carried out an initial denaturation at 95°C for 10 min followed by 16 cycles: denaturation at 95°C for 10s, annealing at 62°C for 30s (-1°C per cycle), extension at 72°C for 60s followed by 25 cycles: denaturation at 95°C for 10s, annealing at 46°C for 30s, extension at 72°C for 60s followed by a final extension at 72°C for 7min. PCR amplification success was confirmed by clear bands on a 2% agarose gel.

Library Preparation and Sequencing (Chapter 2.1)

To remove non-specific binding, the initial 25 μL of PCR product was cleaned using AMPure XP beads. The beads were washed with 200uL of 80% ethanol twice, and DNA was eluted using

52.5 μL of 10mM Tris pH 8.5 buffer. Samples were run on a 2% agarose gel to confirm product was present. Amplicons were indexed using Nextera XT indexes (Illumina) using the following cycling conditions: 95°C for 3 min, followed by 8 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s, and a final step of 72°C for 5 min. Indexed amplicons were purified using the same process as described above. Purified libraries were quantified using the Qubit Fluorometer (ThermoFisher Scientific Inc.) and the average fragment size was determined in an Agilent 2100 Bioanalyzer. In total, 83 samples were prepared for sequencing. Libraries were then normalized at equal molarities and pooled. The pooled libraries were loaded onto an Illumina MiSeq v3 600-cycle cartridge for 2 x 300 bp paired-end read sequencing.

Sequence processing and taxonomic classification (Chapter 2.1)

All sequence processing and taxonomic classification were carried out using the program QIIME 2 v.2020.11 (Bolyen et al., 2019; see Figure 2 for pipeline). The Cutadapt plugin (Martin, 2011) was used to trim the forward and reverse primers from the demultiplexed sequences using the cutadapt trim-paired command with the following parameters: –p-match-adapter-wildcards, --p-match-read-wildcards to allow matching of IUPAC wildcards, --p-discard-untrimmed to discard any reads in which the primers were not found, and the default –p-error-rate 0.1. The lengths to truncate the forward and reverse reads were based on sequence quality plots following trimming. We used DADA2 (Callahan et al., 2016) to truncate and denoise the trimmed sequences into amplicon sequence variants (ASVs), which corrects for amplicon errors from the sequencing run without clustering into OTUs. Compared to operational taxonomic units (OTUs), ASVs are distinct biological sequences providing more precise taxonomic identification, while such diversity can be missed by OTU clustering (Callahan et al., 2017). While ASVs have yet to be heavily adapted into dietary studies, this denoising method has been found to outperform OTU

clustering with mock dietary datasets (O'Rourke et al., 2020). Additionally, the denoising step using DADA2 joins paired-end reads, and removes singletons and chimeric sequences. To perform taxonomic classification, we used the MIDORI_UNIQ_GB240_CO1 database (Machida et al., 2017) consisting of unique sequences for all eukaryotes available in the GenBank 240 release. We first attempted taxonomic classification using classify-sklearn (Pedregosa et al., 2011) with a kmer-based Naive Bayes trained classifier. However, this classified method resulted in many ambiguous taxa along with taxa that did not fit the sampled geographic range. We instead opted for an alignment approach using the BLAST+ plugin (Camacho et al., 2009). This performs local alignments between the reference reads and query sequences and performs least common ancestor (LCA) classification. We used the classify-consensus-blast command for taxonomic classification with the following parameters: –p-maxaccepts 1000 as the maximum number of hits to keep for each query, --p-perc-identity 0.97 as the minimum percentage that the query sequence should match the reference sequence, --p-query-cov 0.89 as the percentage of the sequence to be aligned to the reference database, and –p-strand both to align the forward and reverse query sequences to the reference sequences (O'Rourke et al., 2020).

Following classification, we filtered out taxonomy that did not have a phylum level identification using the qiime taxa filter-table and filter-seqs commands. We filtered out taxa that we considered to be environmental contaminants or unlikely prey items, including any species under the phyla Mucoromycota, Apicomplexa, Discosea, Basidiomycota, Bacillariophyta, Rotifera, Zoopagomycota, Tubulinea, Chlorophyta, Heterolobosea or under the classes Oomycota, Eustigmatophyceae, Chrysophyceae. In addition, we filtered out any remaining eastern massasauga sequences and human contaminant.

Statistical analyses (Chapter 2.1)

Using sequence counts to determine the overall abundance of prey taxa consumed can be prone to biases due to the degraded nature of prey DNA (Deagle et al., 2013), therefore we only relied on presence/absence data for our analyses. We calculated %FOO (frequency of occurrence) for each prey species as the total number of times each species appeared across individuals averaged over the number of samples. To determine if we captured the full dietary diversity in our dataset, a species accumulation curve of the presence/absence data was calculated in RStudio (v.4.0.3, RStudio Team, 2021) using the *specaccum* function in the vegan package (v.2.5-7, Oksanen et al., 2020) and the 'random' method. Species accumulation curves display the number of taxa that are detected within a dataset as the number of samples accumulates. To determine the differences in diets between age classes, seasons, and populations, a non-metric multidimensional scaling (NMDS) ordination was generated in a Jaccard matrix with 999 permutations with the *vegdist* function. A Permutational Multivariate Analysis of Variance (PERMANOVA) post-hoc test with 999 permutations was run for each separate analysis (age classes, season, populations, sex, and gravidity) using the *adonis2* function in the vegan package (v.2.5-7, Oksanen et al., 2020). If a significant p-value was obtained, we then ran a pairwise PERMANOVA using the function *pairwise.adonis2* in the pairwiseAdonis package (v.0.3, Pedro Martinez Arbizu, 2020) with 999 permutations and a Jaccard matrix to determine what variables were statistically different. P-values were Bonferroni corrected to account for multiple comparisons. To identify which species drove any significant differences, we ran a similarity percentage (SIMPER) test in the vegan package with 999 permutations. Due to a large number of single occurrence prey taxa, we had to limit the taxa included in the NMDS and PERMANOVA analyses to only those with more than

one occurrence across all samples. When site differences were compared, Crawford county (CAW) was removed as it only contained one sample.

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