

Novel Techniques for Monitoring Freshwater Turtles in Ontario

Brynn Hickey, Hons B.Sc.

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AUTHOR: Brynn Hickey

SUPERVISOR: Dr. Patricia Chow-Fraser

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Preface

This Master of Science thesis is organized in sandwich format, as recommended by McMaster University. It consists of three chapters, including a general introduction and two data chapters. Chapter 1 is an overview of important background material. Both Chapters 2 and 3 are in manuscript form to be submitted for publication in a peer-reviewed journal.

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: USE OF ENVIRONMENTAL DNA TO CONFIRM BLANDING'S TURTLE
OCCUPANCY IN ONTARIO WETLANDS

Authors: Brynn Hickey, Steven Crookes, and Patricia Chow-Fraser

Status: manuscript in preparation for submission to a peer-reviewed journal

CHAPTER 3: PHOTO IDENTIFICATION OF PLASTRON PATTERNS: A TOOL TO UNIQUELY
IDENTIFY ENDANGERED SPOTTED TURTLES

Authors: Brynn Hickey, Kelsey Moxley, Jeff Hathaway, John Urquhart, Chantel Markle, and Patricia Chow-Fraser

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General Abstract

Freshwater turtles are struggling for their survival worldwide due to habitat loss and degradation, road mortality and illegal collection. To protect such at-risk species, we must establish accurate long-term monitoring programs to track changes in population abundance. For elusive freshwater turtles, tracking them requires trained researchers, specialized equipment, and an often prohibitively expensive field budget. Environmental DNA (eDNA) is a relatively new method that uses molecular techniques to detect the occupancy of elusive species from water samples and shows the potential to be a more accurate, cost and time-effective method. Pattern recognition software has been successfully used on many different aquatic species to uniquely identify individuals from photographs. The purpose of my thesis was to develop more accurate, non-invasive, and time-effective methods using eDNA and pattern recognition software to replace traditional methods for monitoring populations of at-risk turtles in Ontario. We collected eDNA samples in five wetland complexes at different times of the year, using various techniques to determine the best protocols for detecting the occupancy of Blanding's turtles in wetlands. Sampling for eDNA will produce more accurate results during the Active season (April-July), and pooling samples from a single wetland can help decrease costs while increasing accuracy. We used 600 photos of shell patterns from 500 different Spotted turtles in the Georgian Bay-Muskoka to uniquely identify Spotted turtles. When photos were pooled across all regions, there was a 90% probability of detecting the correct individual turtle. The use of eDNA and photo identification of turtle plastrons offer cost-effective, non-invasive methods to monitor at-risk freshwater turtle populations and individuals.

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Dedication

I would like to dedicate this thesis to my late cat and lifelong best friend Bitsey (2004 – September 10th, 2023). She taught me how to love unconditionally for 20 years and was there for me through everything, including sitting next to me as I submitted this thesis for oral defence. Bitsey was the reason I fell in love with animals at a young age, which grew into a passion for animal welfare and wildlife conservation. She gave me the strength to continue this thesis when I thought I had none left and I owe everything and more to her.

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Chapter 1: General Introduction

Brynn Hickey¹

Dr. Patricia Chow-Fraser¹

¹McMaster University, Department of Biology, Hamilton, ON L8S 4L8

1.1 Importance of freshwater turtles

Turtles have cultural and religious significance for many people, including Indigenous communities in North America. The freshwater turtle plays a key role in many First Nations Creation Stories, in which the turtle offered its shell as a home for humankind. Turtles have an incredibly old lineage, dating back 200+ million years, outliving the dinosaurs (Ernst and Lovich, 2009). The turtle and tortoise species together comprise the Order Testudines, otherwise known as chelonians, and are omnivorous or carnivorous. As noted by Iverson (1982), they have larger biomasses compared to other omnivores and carnivores, and therefore have a great impact on ecosystem processes through energy transfer from their prey (invertebrates, fish, other reptiles, and small mammals) to their predators (some large fish species, meso-predators, mammals, and herons/birds) (Lovich et al., 2018). For example, as predators, freshwater turtles have a direct and indirect impact on their prey's abundance, such as common snapping turtles and their amphibian prey (Garig, 2017). Considering nest predation can be as high as 78% within a year, freshwater turtle eggs provide a significant amount of energy to their vertebrate and invertebrate predators, making them very important to many ecosystems (Congdon et al., 2000; Lovich et al., 2018).

Turtles also contribute greatly to mineral cycling by consuming vegetation and mineral deposits, making them great environmental indicators for compounds that are a concern to human health (Hinton and Scott, 1990; Lovich et al., 2018). A substantial proportion of the world's Chelonians also consume fruits and/or seeds in

their diet, which are later dispersed in their excrement (Falcon et al., 2020). Such an example of seed dispersal is what makes them so important for the persistence of ecosystems (Wang and Smith, 2002; Howe and Smallwood, 1982). For all these reasons, the loss of freshwater or marine turtles from an ecosystem can lead to significant consequences for the environment.

1.2 Why are freshwater turtles at risk?

Habitat loss, degradation and other anthropogenic pressures have caused an immense decline in biodiversity and species abundance, causing a lot of concern for the long-term sustainability of ecosystems (Hoffman et al., 2010; Hooper et al., 2012). Approximately 22% of reptiles worldwide were assessed as a ‘Threatened’ species according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, which includes all the species in the ‘Vulnerable’, ‘Endangered’ and ‘Critically Endangered’ categories (Hoffman et al., 2010). As of 2017, 56% of the 356 turtle species worldwide were currently listed as Threatened in the IUCN Red List, making them one of the most at-risk large groups of vertebrates (Rhodin et al., 2018). The main factors driving turtle species decline include habitat transformation, as well as illegal collection for pet, food, and medicine trades (Stanford et al., 2020). The main threat to the most endangered turtles and tortoises is commercial exploitation followed by habitat loss, degradation, and fragmentation (Rhodin et al., 2018).

Habitat loss, fragmentation and degradation impacts are the leading causes of global biodiversity decline (Brooks et al., 2002). Habitat loss is most associated with land conversion for forestry, agriculture, urbanization, and wildfires (Curtis et al., 2018). Habitat degradation reduces the overall quality of lasting habitats and frequently occurs due to overexploitation from hunting and fishing, the introduction of invasive species, pollution, and climate change. The long lifespan of turtles (can be upwards of 80+ years) makes them especially vulnerable to degraded water quality and pollutants that can accumulate over time such as mercury and polychlorinated biphenyls (PCBs) (Hopkins et al., 2013; Ming-ch'eng Adams et al., 2016). Introduced invasive species have had severe impacts on many turtle species due to resource competition, predation, and parasites/diseases. For example, the European common reed (*Phragmites australis*) is an aggressive invasive plant species in North America that degrades wetland habitats and is heavily avoided by Blanding's turtles, reducing their effective habitat (Markle and Chow-Fraser, 2018).

Habitat fragmentation refers to human-modified landscapes that transform habitat into small, isolated patches (Banks-Leite et al., 2020). Roads and railways can act as barriers to travel within fragmented landscapes, decreasing population size and genetic diversity, and causing excess mortality from collisions (Forman and Alexander 1998; van der Ree et al., 2011). Fragmentation of landscapes impairs ecosystem functions and reduces biodiversity with effects increasing as isolated fragments become smaller and as time passes (Haddad et al., 2015). Fragmented ecosystems also subject populations to increased extinction risk due to inbreeding,

mutations, and restricted gene flow (Gallego-Garcia et al., 2019). In Northern Map Turtles (*Graptemys geographica*), habitat fragmentation caused a significant decrease in population abundance and growth rates. Turtles in fragmented habitats showed significantly female-biased sex ratios compared to slightly male-biased control sites (Bennett et al., 2009).

Turtles have been consumed by humans since at least the beginning of the Pleistocene (~2 million years ago) and were important for the diets of many early hominins (Steele, 2010; Rhodin et al., 2015). Many extinctions/extirpations of turtle species have been attributed to human exploitation such as the European Pond Turtle (*Emys orbicularis*) and Madagascar Big-headed Turtles (*Ermnochelys madagascariensis*) (Fritz, 2015; Kuchling, 1997). Their large biomass and extended survival in captivity with minimum effort make freshwater turtles a perfect subject for meat and egg harvest and subsequently their exploitation (Iverson, 1982; Rachmansah, 2020).

Due to their life history traits which include long lifespan, delayed sexual maturity, and low fecundity, any adult mortality, particularly that of females, threatens the viability of populations of freshwater turtles. For example, Blanding's turtles live to around 80 years in the wild and sexually mature at about 20 years (COSEWIC, 2016). This means Blanding's turtles must survive at least 20 years and have a successful nesting attempt to continue their population. Therefore, when a female turtle is killed by a car or stolen for illegal trade, it will take at least 20 years to replace her. Their delayed sexual maturity and long-life span result in severe

impacts on their ability to respond to any habitat change or disturbances (Congdon et al., 1993).

Despite the worldwide focus on conserving areas to protect biodiversity, only approximately 10% of the geographic ranges of freshwater turtles are protected. These conservation efforts do not adequately consider turtle vulnerability and their importance to various ecosystems (Roll et al., 2017). Land and water protection combined with site management is urgently needed for critically endangered turtles to address population decline (Hoffman et al., 2010, Ricketts et al., 2005). Establishing accurate, long-term population monitoring programs is essential in this case, to enhance the available data surrounding species-at-risk and to determine where protection is needed (Proença et al., 2017). Monitoring at-risk freshwater turtle species requires confirming occupancy or presence, determining their movements or behaviours, and identifying them individually for health using a variety of different methods (Table 1.1).

1.3 Monitoring at-risk populations of freshwater turtles

In Canada, the federal Species at Risk Act aims to prevent biodiversity loss by prohibiting human development on and surrounding critical habitats of threatened and endangered species (Government of Canada, 2002). To protect these habitats, however, managers must have evidence to confirm occupancy of target species on the landscape, and these locations must be mapped and monitored. Traditional monitoring methods to confirm the occupancy of freshwater turtle species include

Visual Encounter Surveys (VES), and trapping turtles while the use of radio telemetry has been used to track the movements of individual turtles and to determine their behaviour through the season (i.e., nesting, migrating, aestivating, brumating, etc.) (Table 1.2).

VES: is a non-invasive approach to spot turtles during spring when basking on logs or rocks and requires no tools other than binoculars for accurate identification. A proper VES, however, requires surveying multiple locations within extensive open-water wetlands, over a long period to ensure accuracy. VES are conducted through evenly spaced transects across all sections of the wetland. Researchers spot turtles as they bask on rocks or are travelling on land or in shallow water, and since they do not handle any at-risk species, they do not require specialized permits (Casper & Hecnar, 2011a; Mali et al., 2018).

Trapping: Turtles may also be caught by hand or with baited traps set in wetlands to determine information on population size or the sex, health, and size of individuals (Lagler, 1943). Common traps include hoop nets, basking traps, and dip-netting. Hoop nets are constructed of mesh with an opening on one end and facilitate entrance while also restricting escape (Lagler 1943). The nets are loaded with fish, insects, or cat food, submerged approximately two-thirds underwater in a suitable freshwater turtle habitat and must be checked multiple times a day. Once caught, turtles can be sexed, measured,

and marked individually, but special research permits are required for this activity (Casper & Hecnar, 2011b). All trapping techniques discussed show significant bias towards certain species, sizes, sexes, and ages of turtles they caught (Tesche and Hodges, 2015).

Telemetry: The conventional method to track individual movements of freshwater turtles is by affixing a radio transmitter on the turtle carapace that emits pulses of radio signals at known frequencies that can be detected by a receiver and antenna. Although radio telemetry is the tried-and-true tracking method to study the movement and habitat selection of many freshwater turtles, it has known limitations. First, it requires investigators to be on-site to search for transmitted signals and to record the locations of turtles manually. Sometimes, the exact location of the turtle can only be estimated by triangulation due to difficulties in accessing the turtle (i.e., if they are in the middle of a dense thicket swamp). Additionally, relocations are biased towards daylight hours due to the inherent risks to investigators if they sample in the dark. This is particularly problematic because many gravid turtles conduct their nesting activities after dusk and throughout the night (Congdon et al. 1983). Some analyses require many turtle relocations, and the increased cost to achieve this (salary for field staff and accommodation) is often prohibitively expensive, especially for remote field sites.

Recent advancements in detecting the DNA of wildlife in water samples have made it possible to confirm the occupancy of a target species or assemblage without using VES, trapping or telemetry. Environmental DNA or eDNA consists of short DNA fragments released into the environment by organisms and can include secreted urine, feces, mucous, shed skin cells, and dead carcasses. eDNA can be extracted from environmental samples including water, soil, or sediment (Ficetola et al., 2008). Extracted eDNA samples can be analyzed with either a species-specific approach or with DNA metabarcoding a multispecies approach that can detect the presence of species assemblages without prior knowledge (Rees *et al.* 2014).

To assess population trends and the health of turtles, investigators must be able to identify animals individually (Seber 1965; Table 1.2). Common methods for unique identification include capturing individuals and marking them by notching their shells at specific locations on their carapace (Cagle 1939). The pattern of shell notches will identify them when they are recaptured. Passive integrated transponder (PIT) tags, which are electronic microchips (10-14mm in length), are internally injected into the body cavity of freshwater turtles with 12-gauge needles to individually identify each animal (Buhlmann & Tuberville, 1998). A handheld reader can be used to scan the outside of each turtle in search of a tag; if a PIT tag is present, the reader will generate a close-range electromagnetic field that activates the tag, which transmits its unique number code. This unique alphanumeric code allows tagged turtles to be individually distinguished from one another like pet microchips (Gibbons and Andrews, 2004). Both the use of notches and PIT tags are

designed to be permanent identifiers (Table 1.3), and both involve highly invasive procedures; additionally, notches on shells can wear down over time while PIT tags can become damaged, or lost, or the tags may migrate in the tissue (Feldheim et al., 2002; Wyneken et al., 2010). Beyond these limitations, the techniques may subject turtles to an unknown degree of pain or shock.

An emerging non-invasive approach is to use the unique colour patterns of the turtle shell to identify individuals. It involves taking photographs of the turtle's plastron (ventral shell) when they are captured or encountered in field surveys. Various wildlife species, such as snow leopards have distinct patterns that can be used as an identifier for individuals (Jackson et al., 2006). This non-invasive option has been used to successfully identify individuals from photographs of the unique colour pattern of a turtle's plastron on several freshwater turtle species including Eastern Box turtles (*Terrapene carolina*) (Cross et al., 2014), Western Painted turtles (*Chrysemys picta bellii*) (Cooley et al., 2015) and Blanding's turtles (Markle et al., 2021). Photographs can be stored in a digital database and can help support monitoring freshwater turtle species across a large range while showing the potential to be a highly accurate, non-invasive, cost-effective method of identifying individual freshwater turtles for researchers and practitioners.

1.4 Aims and Objectives

The primary purpose of this thesis was to investigate novel techniques to monitor at-risk freshwater turtle species in Ontario that are non-invasive, cost-

effective, and highly accurate. For the first data-driven chapter, we examined the accuracy of using eDNA to determine Blanding's turtle occupancy during various activity seasons across Ontario. We explored various factors that could affect the accuracy of this novel technique, including sampling where only juveniles reside, pooling samples, and immediately preserving samples in the field. For the second data-driven chapter, we used open-source software to determine the efficacy of using photographs of Spotted turtle (*Clemmys guttata*) plastron patterns to uniquely identify them. These results will benefit the conservation of freshwater turtles by providing researchers and technicians with accurate techniques and protocols to monitor threatened species.

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1.6 Tables

Table 1.1: Summary of traditional monitoring methods to study freshwater turtle occupancy, movements, and habitat use.

Method	Brief Description	Purpose
Visual Encounter Surveys (VES)	Species identification is confirmed visually without the use of equipment (turtles are usually nesting, basking, or travelling)	Confirming occupancy of specific habitat and behavioural studies
Hoop nets or other traps	Baited or un-baited traps are set for multiple hours, and turtles are processed* and released	Confirming occupancy, population censuses and health assessments
Radio telemetry	Turtles caught in hoop nets or via visual encounters are tagged with radio transmitters so that they can be relocated regularly with a receiver during one or more seasons.	Behavioural studies, home ranges, distances travelled, habitat use and selection.
Capture -mark-recapture	Use of shell notching, passive integrated transponder (PIT) tags, leg banding, or unique coloration pattern of turtle plastron to identify individual turtles	Population census, health assessment, and individual monitoring

Table 1.2: Comparison of methods for occupancy detection including Visual Encounter Surveys (VES), use of hoop nets and analysis of environmental DNA (eDNA). Information is based on the number of people required to conduct fieldwork at an hourly wage of \$15, the cost of field equipment, and approximate analytical costs (Davy et al., 2015).

Parameter	VES	Hoop nets	eDNA
Length of time involved	Varies widely (10 to 410 hours)	Varies widely (10 to 669 hours)	Approximately 12 hours from sample collection to result*
Stress to animal	Low	High	None
Cost to Researchers (CAD)	Varies widely (\$150 to \$6150)	Varies widely (\$150 to \$10,035)	Estimated \$500 for a single species detection**
Are permits required?	Not required	Required	Not required
Accuracy of method	Low for rare, cryptic species	Low	High

*Samples can be preserved and processed later.

**Assumes that the sample is being analyzed by a commercial lab

Table 1.3: Comparison of methods for individual recognition including notching, Passive Integrated Transponder (PIT) tags,

Parameter	Notching	PIT tags	Pattern Recognition
Durability	Moderate, reduced by injuries and can fade	Low durability due to tag loss and migration	Depending on the species, usually high
Stress to animal	High	High	Low
Cost to Researchers (CAD)	Approximately \$30 - \$50	Tag readers range between \$350 and \$800. Tags range between \$5 and \$10	Free (requires a smartphone camera)
Training	Requires training from experienced personnel	Requires extensive training usually supervised by a veterinarian	Very easy to learn

**Chapter 2: Use of environmental DNA to confirm
Blanding's turtle (*Emydoidea blandingii*) occupancy in
Ontario wetlands**

Brynn Hickey¹

Dr. Patricia Chow-Fraser¹

¹McMaster University, Department of Biology, Hamilton, ON L8S 4L8

2.1 Abstract

Turtle species around the world are becoming threatened, endangered, or extinct, and there is a real urgency to find cost-effective ways to identify and protect turtle habitat, especially on public lands. Conventional methods for monitoring elusive freshwater turtle populations are labour-intensive, costly, and frequently invasive. Environmental DNA (eDNA) has recently gained popularity as a tool to confirm the occupancy of wildlife species or assemblages within a region. We investigated the use of eDNA to detect Blanding's turtle (*Emydoidea blandingii*) occupancy in a range of wetlands in southern Ontario throughout the seasons from winter 2020 to summer 2023. In total, 117 eDNA samples were taken from four positive control sites (confirmed with radio telemetry) and one negative control site. Preserving samples with ethanol followed by storage at 4°C until analysis produced no false negatives; this compares well with 80% false negatives when filters were preserved in dry silica beads and stored at -20°C until analysis. Using only ethanol-preserved samples, we found a significantly lower proportion of false negative results during the Active season (23.3%; April to August) than during either the Overwintering season (85.7%; December to March) or the Inactive season (100%; September to November). The effect of activity season was similar for a degraded urban site and a relatively undisturbed rural site; regardless of the preservative method, the percentage of true positive exceeded 70% during the Active season, whereas there was no true positive during the Inactive season and approximately 20% during the Overwintering season. We recommend pooling multiple water

samples within a wetland to increase the possibility of detection and using a syringe-based sampling technique to help reduce cost without compromising accuracy.

2.2 Introduction

Blanding's turtles (*Emydoidea blandingii*) are semi-aquatic freshwater turtles that have populations distributed south of the Great Lakes, Nova Scotia, Maine, New Hampshire, and Massachusetts (Ernst & Lovich, 2009). Their habitat includes a variety of wetlands (marshes, bogs, fens, coastal wetlands), slow rivers, vernal pools, lakes, bays, and upland forests (Edge et al., 2010). They have been assessed provincially and federally as threatened, due to habitat loss and degradation from invasive species, development, wetland modifications, collection for pet, food, and medicine trades, and increased predation of nests and juveniles by higher numbers of predators that have benefitted from increased human disturbance such as raccoons (COSEWIC, 2016; COSSARO, 2017). Since the habitat for this at-risk species varies greatly from site to site (Meng and Chow-Fraser, unpublished data), it is important to develop efficient surveying techniques to identify specific habitats that Blanding's turtles use throughout their geographic range.

Conventional surveying methods for freshwater turtle species include radio telemetry, visual surveys, trapping, and egg counts. These traditional approaches have many limitations including low detection rates, labour-intensive sampling, high costs and the invasiveness of the technique (Rees et al., 2014). Recent advancements

in molecular research show how DNA can be obtained directly from environmental samples to analyze the diversity of organisms within the environment, a method called environmental DNA (eDNA; Thomsen & Willerslev, 2015). Furthermore, this non-invasive approach has the potential for monitoring rare, at-risk, or invasive species with higher accuracy and at a lower cost (Schmelzle et al., 2016).

Environmental DNA is genetic material shed from target organisms such as feces, skin cells, or body secretions that can be detected with species-specific primers amplified and quantified through molecular techniques such as quantitative polymerase chain reactions (qPCR) (Jerde et al., 2011; Langlois et al., 2021; Goldberg et al., 2016; Hernandez et al., 2020). Advancements in eDNA methods have offered a non-invasive, less costly and more time-efficient method to detect elusive freshwater species at risk (Thomsen et al., 2012; Schmelzle et al., 2016; Jerde et al., 2011). Multiple studies have used this approach to detect the eDNA of many different species including amphibians (Dejean et al., 2011; Ficetola et al., 2008; Goldberg et al., 2011; Thomson et al., 2012), reptiles (Davy et al., 2015; Piaggio et al., 2013), fish (Dejean et al., 2011; Jerde et al., 2011; Thomson et al., 2012; Wilcox et al., 2013), and mammals (Foote et al., 2012; Thomson et al., 2012). Specifically for Blanding's turtles, two studies have recently confirmed that eDNA can be used to confirm their occupancy for a disjunct population in Nova Scotia during a short period in their active season (Loeza-Quintana et al., 2020), and for populations inhabiting 17 inland wetlands in four regions of Ontario during the early and late overwintering period (Tarof et al., 2021).

The probability of identifying the occupation of Blanding's turtles using eDNA depends heavily on many different abiotic and biotic factors including the source of the eDNA, as well as how the environmental samples are collected, handled, and analyzed (Tarof et al., 2021). The use of eDNA analysis is still in its infancy, and various protocols and approaches have been proposed to identify reptile occupancy, although there is a lot of variation in suggested protocols. Multiple steps include 1) field sampling and 2) DNA capturing, 3) preservation, 4) DNA extraction, 5) amplification, and 6) identification, which can vary greatly across studies. To maximize the accuracy of eDNA studies, we should not only consider the life cycle of the target species and characteristics of the ambient environment (wetlands, vernal pools, lakes, etc.), we must also scrutinize how, when and where samples are collected, how the DNA is extracted, and the factors affecting capture efficiency, inhibition probability, sources of contamination, and assay sensitivity (Goldberg et al. 2016; Schmidt et al., 2013).

For organisms with keratinized integument such as non-avian reptiles, rates of DNA shedding may be at a lower rate than organisms with mucous integument such as amphibians (Adams et al., 2019). This is especially important considering turtles most commonly shed scutes and integuments in small pieces rather than in cells, which are more likely to sink and not be detected by surface water samples (Ernst, 1971). Several studies also suggest that there is a positive relationship between the biomass of the organism and eDNA concentration (Takahara et al., 2012; Pilliod et al., 2013). For example, the eDNA release rate of adult bluegill

sunfish was three to four times higher than that of juveniles (Maruyama et al., 2014). Given that different eDNA capture and extraction protocols can also influence the detection rates of freshwater species, standardization of protocols must be a top priority to allow for cross-study comparisons (Deiner et al., 2015).

Blanding's turtles exhibit distinctive behaviours throughout the year that may influence the detectability of their eDNA, depending on whether or not these activities decrease or increase the shedding rate of eDNA. For example, Buxton et al., (2017) demonstrated that eDNA detection rates were likely to vary seasonally due not only to temperature changes but also to species activity, with increasing temperatures leading to increased degradation rates and increased seasonal activity leading to increased eDNA concentrations. During the breeding season, eDNA concentration of the great crested newt (*Triturus cristatus*) increased with ambient temperature, but this was not the case during the non-breeding season, when both eDNA concentration and ambient temperature decreased, suggesting that seasonal activity may have a larger influence on eDNA shedding than environmental temperature. Similarly, the Blanding's turtle exhibits different behaviours seasonally that may affect shedding rates. During brumation, which commences in December and ends in March, reptiles stop eating and their heart and respiratory rates drop significantly as does their digestion. Tarof et al., (2021) found that individuals during the Overwintering period decreased shedding rates compared to those who were induced to become active for more than a week.

Despite the promising results of recent studies that confirmed the utility of eDNA from Blanding's turtles living in inland wetlands of Ontario and a disjunct population in Nova Scotia (Loeza-Quintana et al., 2020) we do not know if the efficacy of this technique varies throughout the active (mating, foraging, nesting), inactive summer (aestivation), and inactive overwintering (brumation) seasons. In addition, this technique has not yet been attempted along the Great Lakes coast, where there are relatively pristine and high-quality coastal marshes and in the highly urbanized region of Lake Ontario where there are turbid and degraded wetlands (Cvetkovic and Chow-Fraser, 2011). There is some urgency to identifying habitat for threatened turtle species in these Great Lakes coastal wetlands given that the loss rate of remaining wetland areas in southern Ontario between 2011 and 2015 is considerably higher than that previously assessed for the decade between 2000 and 2011, and most of these are occurring along the coast (Ontario Biodiversity Council, 2015).

The overall goal of this study is to investigate factors that influence the detectability of Blanding's turtle eDNA in Great Lakes coastal marshes, focusing on areas where there are still relatively pristine wetland habitats as well as in regions with degraded wetlands, to fully test the capability of this approach under contrasting environmental conditions. We wanted to specifically test the effect of the timing of sampling on the rate of detection. We hypothesize that eDNA shedding rates would be greater during the Active period and predict that detection rates would be highest during April to August when turtles are mating, foraging, and

nesting, than during the Overwintering period (December to March) or the Inactive season, following nesting and pre-brumation (September to November) (Rowe, 1987; Millar et al., 2011). Since this technique had only been tested for adults, we also wanted to test its applicability for juvenile Blanding's turtles. To develop a cost-effective and widely applicable field protocol, we also wanted to compare the efficiency of two common preservation methods (silica gels versus ethanol) and pooling multiple samples from one wetland to save sampling time. By field-testing our methods across multiple sites with different environmental conditions, and across the three behavioural seasons, we will provide an improved eDNA protocol that can be used by future researchers and volunteers to conduct routine sampling of wetlands to determine occupancy by this at-risk species throughout their geographic range.

2.3 Methods

2.3.1 Sampling sites

We selected four positive controls, with three sites located on the Ontario Shield, along the shoreline of Georgian Bay, and one along the shore of Lake Ontario; we also selected a negative control wetland in the city of Guelph, which had not been known to support Blanding's turtle for the past 30 years (Figure 2.1). The most northerly positive sites will be referred to as Whitefish River First Nation (WRFN), which include relatively undisturbed coastal marshes in the McGregor Bay archipelago, and more disturbed palustrine wetlands on Birch Island, within WRFN. Further east and south along Georgian Bay is the second positive site that we will refer to as Henvey Inlet First Nation (HIFN), which includes wetland complexes that exist in a moderately developed landscape. The third positive site is referred to as Moose Deer Point First Nation (MDPFN), which includes minimally disturbed wetlands on a large nature reserve as well as more disturbed wetlands within the MDPFN. The last positive sites are marshland and rivers within the highly developed Rouge National Urban Park (RNUP) in Toronto. The occupancy of Blanding's turtles in all positive sites has been confirmed through radio telemetry. Eighteen radio-tagged adult Blanding's turtles have been monitored by McMaster University in HIFN since 2020, 26 turtles in the WRFN and 22 in the MDPFN since 2021, while several adults and dozens of juvenile Blanding's turtles have been monitored by the Toronto Zoo in RNUP since the Blanding's reintroduction project in 2014. Therefore,

before the start of eDNA sampling, we had very good knowledge of the movement patterns and distribution of the target species at all four positive sites.

2.3.2 eDNA sample collection

Influence of season

We only collected eDNA in wetlands after confirming the presence of at least one Blanding's turtle in a specific wetland using radio telemetry or visual survey. This required more coordination between the tracking team and the eDNA sampling team, but the extra effort greatly increased our chance of obtaining a true positive result. To address the influence of season on eDNA detectability, we collected 111 eDNA samples at the four positive sites over the three seasons (the Overwintering period (December to March), the Active period (April to August), and the Inactive period (September to November)). Sampling of the positive sites occurred between September 2021 to July 2023, while 6 samples were taken in the negative control site once in July 2023 (Table 2.1).

Filtering methods

We contacted the lead author of previous studies at U of Guelph (R. Hanner, pers. comm.), who recommended that we use the OSMOS eDNA sampler to filter water samples. The OSMOS is a portable backpack pump designed and manufactured by Halltech (Guelph, ON) with an extension pole and reusable cartridges where filters are housed. In all cases, Millipore Sigma 0.8 μm cellulose nitrate filters (47-mm diameter) were used. The OSMOS was programmed to intake 1.5 L of water per filter, but due to turbid water clogging the filter, the amount that

could be filtered at some sites was considerably less than 1 L. This meant that for those wetlands, we had to double the amount of time filtering water to reach a volume of 1.5 L. To obtain spatially representative samples, we filtered water from 5 to 10 cm below the surface every 15 to 20 m along the shoreline near the confirmed Blanding's turtle for each filter.

Because of problems we encountered with the OSMOS freezing during the winter, and the unit clogging up, we decided to try a more low-tech method. We took grab samples of water with bleached 0.5 or 1 L glass Mason jars or Whirlpaks. Aliquots of grab samples were then filtered through a reusable 47-mm filter holder (Cole-Parmer) containing the cellulose nitrate filter. Using a 30-mL single-use syringe, we pulled up an aliquot of water and then attached the syringe to the filter holder and pushed the sample through (SYRINGE). We continued this process many times until all the water from the Mason jar had been pushed through and filtered. All reusable materials were bleached and/or autoclaved after use to ensure no cross-contamination. During winter months, we used a hand-held auger to drill a hole (30 cm diameter) through the ice, where grab samples were collected and filtered either by the OSMOS or SYRINGE method.

We were also interested in knowing the effect of pooling grab samples in a bucket before filtering them through a syringe in replicates. For this comparison, we randomly collected one grab sample at a random location within a wetland that had a confirmed Blanding's turtle. Each of these grab samples was filtered individually (INDIVIDUAL). We then collected 18 grab samples in the same wetland every 60 m,

pooled the samples, ensured it was well mixed and then filtered from the pooled water (POOLED). Therefore, both methods resulted in one filter that needed to be processed, but the POOLED method collected more water from multiple locations around the wetland.

Preservation methods

After filtration was complete, we used sterile forceps to extract the filters from the OSMOS or the reusable filter holder. They were either preserved in dry silica beads (SILICA) or ethanol (ETHANOL). In the SILICA method, the filter was placed in a small paper coin bag with silica beads, and this was placed inside another plastic bag or immediately put on ice; after returning from the field, the sample was frozen at -20°C. In the ETHANOL method, the filter was placed in approximately 0.5 mL of 96% ethanol inside a 1.5 mL microcentrifuge tube and subsequently refrigerated at 4°C upon returning from the field. A direct comparison between the two preservation methods was performed by taking identical replicates at one location near a confirmed Blanding's turtle and preserving three replicates using 'SILICA' and three using 'ETHANOL'. A field-negative control sample was taken with 0.5 – 1 L of tap water and filtered with the same protocols used for the field-positive samples.

2.3.3 DNA Extraction and Target Detection

After filtration, we extracted eDNA with the DNeasy Blood and Tissue Kit produced by QIAGEN (Toronto, ON) following the manufacturer's protocol. Extracted DNA was stored at -20°C until further analysis could be performed. We

performed qPCR with the Quantitect Probe Master Mix (QIAGEN) in the Bio-Rad CFX384 Real-Time PCR Detection System following QIAGEN's protocols using a final reaction volume of 50 μ L with 5 μ L of DNA template. We set the thermocycler conditions for activation at 95°C for 120 s followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Positive and negative qPCR controls were included for each analysis with the Bio-Rad thermocycler. Proprietary species-specific TripleLock™ qPCR assays and gBlock© Gene Fragments for Blanding's turtles were designed by Precision Biomonitoring (Guelph, ON). The Taqman™ probe proprietary assays detect a 128 bp amplicon region of mitochondrial genome specific to Blanding's turtles. The assay was validated with DNA sequences from six of eight native freshwater turtle species in Ontario to test for amplification of any closely related species (Tarof et al., 2021). We followed strict protocols and performed all qPCR analyses in a room separate from where DNA was extracted to reduce the potential for contamination. Samples were considered negative if there was no exponential curve before cycle 38 ($C_q = 0$) or if the sample was amplified before the fourth cycle ($C_q < 4$).

2.3.4 Statistical Analysis

We performed chi-square analyses to test for associations between eDNA detection and activity season (Overwintering, Active, or Inactive), sampling method (OSMOS or SYRINGE, POOLED vs INDIVIDUAL), preservation method (ETHANOL vs SILICA), and juvenile sampling activity only (Overwintering, Active, or Inactive). Means are presented and the significance level was set to 0.05. Chi-square analyses

were performed with JMP 13.0 for PC. Wilcoxon Signed Rank test was used to compare mean Cq values associated with pooled and individual samples processed with the SYRINGE method.

2.4 Results

As expected, none of the negative controls (qPCR or field; true negatives) for *E. blandingii* amplified, while positive detections (true positives) for Blanding's turtles associated with positive controls were found at least once, thus confirming that the TripleLock™ qPCR assays developed by Precision Biomonitoring were accurate for detecting Blanding's turtles in coastal marshes. Also, all positive controls for qPCR runs showed amplification with $Cq > 0$, indicating good assay and reaction performance.

E. blandingii DNA was detected in only 38.7% of the expected positive samples (43 of 111; Table 2.1). eDNA detection for each positive sample ranged from 1 per 3 replicates to 3 per 3 replicates. There were 61.3% false negatives (where $Cq = 0$), which were disproportionately higher in the degraded wetlands of the RNUP (71%), and lowest in the WRFN (52%). In the RNUP wetlands, *E. blandingii* DNA was detected in only 29% of the samples where a Blanding's turtle had been confirmed, and it should be noted that six of the seven positive samples were taken when only juvenile Blanding's turtles had been present at the time of sampling. These data are, however, not directly comparable since they have not yet

been standardized for potential effects of differences in activity season, preservation method, and filtration methods.

We standardized the data to compare results when either the OSMOS or SYRINGE method was used and found no significant differences (Chi-square; $P > 0.20$). We also carried out a direct comparison between SILICA and ETHANOL as the preservation method and found significantly better results with ETHANOL than with SILICA (Figure 2.2). Whereas 100% of replicates preserved with ETHANOL amplified and showed true positive results, only 20% of replicates preserved with SILICA amplified properly ($X^2_{125,3} = 20.32, P < 0.0001$). Given the significant effect of the preservation method on our results, we excluded all samples preserved with SILICA to determine the influence of behavioural season on the accuracy of detection.

Using only samples preserved with ETHANOL, we found the lowest percentage of false negatives (30.4%) associated with the Active season and by implication, the highest percentage of true positives (69.6%) (Figure 2.3). By comparison, during the Overwintering period, 82.8% of the samples were false negatives with only 17.2% true positives, while during the Inactive season, 100% of the samples analyzed were false negatives. Therefore, consistent with our prediction, the probability of detection was significantly higher for Blanding's turtle during the Active period compared with the Overwintering or Inactive periods ($X^2_{125,6} = 22.0, P = 0.0012$). Due to the small sample size, however, we could not test for any significant effect of activity season on the results of juvenile Blanding's

turtles ($N = 10$, $P > 0.25$). We further investigated the effect of activity season on individual sites. There was sufficient data for MDPFN and RNUP, but only when we combined preservation methods. Given that the data were not directly comparable because of the combination of preservation methods, we did not perform any statistical comparison. Nevertheless, the seasonal trends for both sites were remarkably similar and were consistent with the overall trend in Figure 2.3.

For both the POOLED and INDIVIDUAL methods, 100% of the samples were true positive; however, when we compared the Cq values of replicates from respective sampling protocols, mean Cq values for the POOLED method were significantly higher than those of the INDIVIDUAL method (Wilcoxon Signed Rank test; $P=0.0495$; Figure 2.5). This indicates that although percent accuracy was the same, the INDIVIDUAL samples had greater amplification and may be more desirable than the POOLED samples. That said, the POOLED method can cover a greater area of the target wetland at a lower cost and may be better for routine monitoring purposes unless the exact location of each sample needs to be preserved.

2.5 Discussion

Our objective was to determine a set of field sampling methods that would increase the detection of Blanding's turtles and decrease the likelihood of false negatives by investigating various factors and methods and their effect on detection. We have demonstrated that eDNA detection can be made for Blanding's turtles under many field conditions. Our samples had been collected during harsh winter

days in February as well as exceedingly hot summer days in August. We have used expensive dedicated sampling equipment such as the OSMOS back-pack filter, as well as a low-tech inexpensive syringe and filter. We have sampled wetlands where several juvenile Blanding's turtles were found, as well as where the carcass of a single dead adult Blanding's was found. We also used silica beads and ethanol to preserve samples. The variable that had the greatest effect on our results was the timing of the sampling. Regardless of field protocol and preservative, when we sampled during the Active season (between April and August), the likelihood of detection of adult Blanding's increased significantly, while the corresponding likelihood of false negatives decreased. That said, we were able to provide evidence for eDNA detection of juvenile Blanding's turtles in both a winter sampling environment as well as during their Active period; however, this was a very small sample size, and further research should be conducted to verify our results.

The ideal equipment used to collect environmental samples varies widely across eDNA studies, where some studies used peristaltic pumps to evenly pump water through a filter (Akre et al., 2019; Goldberg et al., 2011), whereas others used syringes (Buxton et al., 2017), vacuum pumps (Kessler et al., 2020), manual hand pumps (Goldberg et al., 2018), expensive commercial eDNA samplers such as Smith-Root (Tarof et al., 2021), large glass jars (De Souza et al., 2016), or single-use sampling scoops (Vimercati et al., 2020). Single-use field sampling equipment can help reduce the risk of contamination, although reusable sampling equipment such as glass jars can be used but should be decontaminated with a bleach solution

(Goldberg et al., 2016). Based on our experience, it is not necessary to use an expensive commercial eDNA sampler (OSMOS; Guelph, ON), since we found no association between eDNA detection and sampling equipment used. Syringes, glass jars or Whirlpaks are much less expensive to use and would be cost-effective for researchers with small budgets or monitoring programs where a large number of samples must be collected. Additionally, commercial eDNA samplers are relatively heavy (40-50 lbs) and may not be ideal for remote field work that requires long treks.

Precipitation and filtration are the two most common methods of capturing DNA from environmental samples such as soil or water (Ficetola et al., 2008; Goldberg et al., 2011). Filtration involves filtering relatively large volumes of water (250 mL to 10 L) through filters with pore sizes ranging from 0.4 μm to 10 μm , which are made most commonly from glass fibre (Jerde et al., 2011), cellulose nitrate (Goldberg et al., 2011), polycarbonate (PC) (Takahara et al., 2012), nylon (Thomson et al., 2012), and polyvinylidene fluoride (PVDF) (Brys et al., 2021). Capturing eDNA through precipitation involves adding sodium acetate and ethanol to small samples of water (i.e., under 100mL) and storing the sample in a -20°C freezer. The precipitated DNA can then be centrifuged for recovery (Ficetola et al., 2008). Filters made out of cellulose nitrate often outperformed other filter materials in terms of both the cost and efficiency of DNA capture (Hinlo et al., 2017; Liang & Keeley, 2013). Peixoto et al., (2021) found that the use of capsule or disc filters

outperformed precipitation for water samples, thereby detecting higher amounts of captured eDNA of the amphibian species *Salamandra salamandra* with filters. Filter capsules such as the Sterivex-GP capsule filter are commonly used to reduce contamination risks (Tsuji et al., 2019) and have been shown to yield a higher amount of eDNA (Spens et al., 2017).

Filtration through a cellulose nitrate filter, with pore sizes ranging from 0.4 μm to 1.0 μm seems to be the most common method for capturing eDNA to detect reptiles from water samples. Turner et al., 2014 found that the optimal filter pore size to maximize eDNA detection was 0.2 μm . It is important to note that smaller filter pore sizes can get clogged more easily than larger-sized pores because of suspended particles in turbid waters (e.g., in wetlands) (Tsuji et al., 2019). Therefore, the type of study system should be used as an indicator to decide which filter pore size should be used to capture eDNA. Tarof et al., 2021 used 1.0 μm cellulose nitrate filters to detect Blanding's turtles and considered that there was a possibility that some of their false negatives may be attributed to using filters with a 1 μm pore as opposed to smaller sizes. In our study, we decided to use 0.8 μm cellulose nitrate filters which performed extremely well, and we found that filter clogging was not an issue when filtering volumes between 0.5 and 1 L.

The captured eDNA on a filter is usually preserved by using one or a combination of different techniques including freezing at -20°C to -80°C (Takahara et al., 2012), refrigeration at 0°C to 4°C (Osathanukul & Minamoto, 2021), immersion in ethanol (Goldberg et al., 2012), immersion in cell lysis buffer (Renshaw et al.,

2015), or even just keeping them dry in a sealed bag with silica gel beads (Allison et al., 2021). The addition of a solvent (either ethanol or a cell lysis buffer) immediately after collection has been highly recommended to ensure maximal eDNA yield (Spens et al., 2016). Storage in a freezer has also been shown to maximize the recovery of eDNA of pond loaches (*Misgurnus anguillicaudatus*) from water samples (Hinlo et al., 2017). We found that filtering and preserving directly in the field with 96% ethanol and then refrigerating samples at 4°C after returning from the field can help increase eDNA detection and decrease false negatives.

Since eDNA in surface waters degrades rapidly, taking anywhere from a few hours to a few days, a positive detection should be an accurate indication of recent species occupancy (Thomsen et al., 2012). However, since eDNA in sediment can last much longer and in higher concentrations, water samples that are contaminated with sediment may lead to false positives, otherwise known as an identified positive occupancy of a species even though they are no longer present in the area (Shaw et al., 2016). Therefore, Shaw et al., (2016) demonstrated that sampling eDNA from surface water rather than from the sediment yielded a more accurate species composition and reduced the number of false positives. Goldberg et al., (2018) showed that an optimized sampling design should include multiple sampling locations within a single wetland, especially since the acidic environment can contribute to false negatives. They suggested that the maximum detection distance from an organism was inversely related to the dispersion and degradation rate and recommended that sample collection within wetlands occur at least every 60 m (e.g.,

wetlands with a surface area of 1200 m²). We collected water samples that were only 15m away from an adult Blanding's turtle (confirmed with radio telemetry) that did not amplify when analyzed. Such false negatives could be due to several factors, including the relatively low pH, degradation of DNA, chemical inhibition, etc. We therefore suggest that the maximum distance between samples in highly acidic environments be at most 15 m.

For the effective conservation and management of amphibians and reptiles, scientists must determine their occupied habitats, which usually entails costly and labour-intensive tracking methods such as radio telemetry, surveying, or trapping. Advancements in eDNA methods offer a more non-invasive, cost and time-efficient method to detect the occupation of at-risk species. Overall, eDNA is still a relatively new method that has limitations that need to be considered. For instance, eDNA analysis cannot distinguish between DNA from a living versus deceased animal, and this can lead to a false conclusion regarding occupancy. Moreover, the monitoring method cannot distinguish between the life stages of animals or yield micro-habitat information for the species of interest. The greatest limitation is the lack of information on how an abundance of reptile species affects eDNA concentrations. A great deal more research is required on how reptiles and freshwater turtles with hard exteriors shed their eDNA under different environmental conditions. Therefore, eDNA should not entirely replace traditional methods but rather become a routine tool for researchers and practitioners. Although using eDNA to monitor Blanding's turtles may present some challenges, our study confirms that it can be

used as a non-invasive, cost-effective, and efficient method to detect the occupancy of adults during the summer months in coastal marshes of the Great Lakes.

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2.7 Tables and Figures

Table 2.1: Detection results for Blanding’s turtles at 4 positive control sites and 1 negative control site in Ontario, Canada based on environmental DNA surveys. Expected positives or negatives were determined based on field surveys conducted with hoop nets, hand catching, and radio telemetry. Positive detection is based on amplification of target eDNA shown by Cq values > 0 for at least one replicate while negative detection was based on Cq values of 0 for all replicates.

Sampling Site	Expected results	No. of samples taken	No. of positives	No. of negatives
Whitefish River First Nation (WRFN)	Positive	25	12/25 (48%)	13/25 (52%)
Henvey Inlet First Nation (HIFN)	Positive	23	10/23 (43%)	13/23 (57%)
Moose Deer Point First Nation (MDPFN)	Positive	39	14/39 (36%)	25/39 (64%)
Rouge National Urban Park (RNUP)	Positive	24	7/24 (29%)	17/24 (71%)
All positive	Positive	111	43/111 (38.7%)	68/111 (61.3%)
Guelph	Negative	6	0/6 (0%)	6/6 (100%)



Figure 2.1: Location of sampling sites for environmental DNA to determine occupancy of Blanding's turtle (*Emydoidea blandingii*) in southern Ontario.

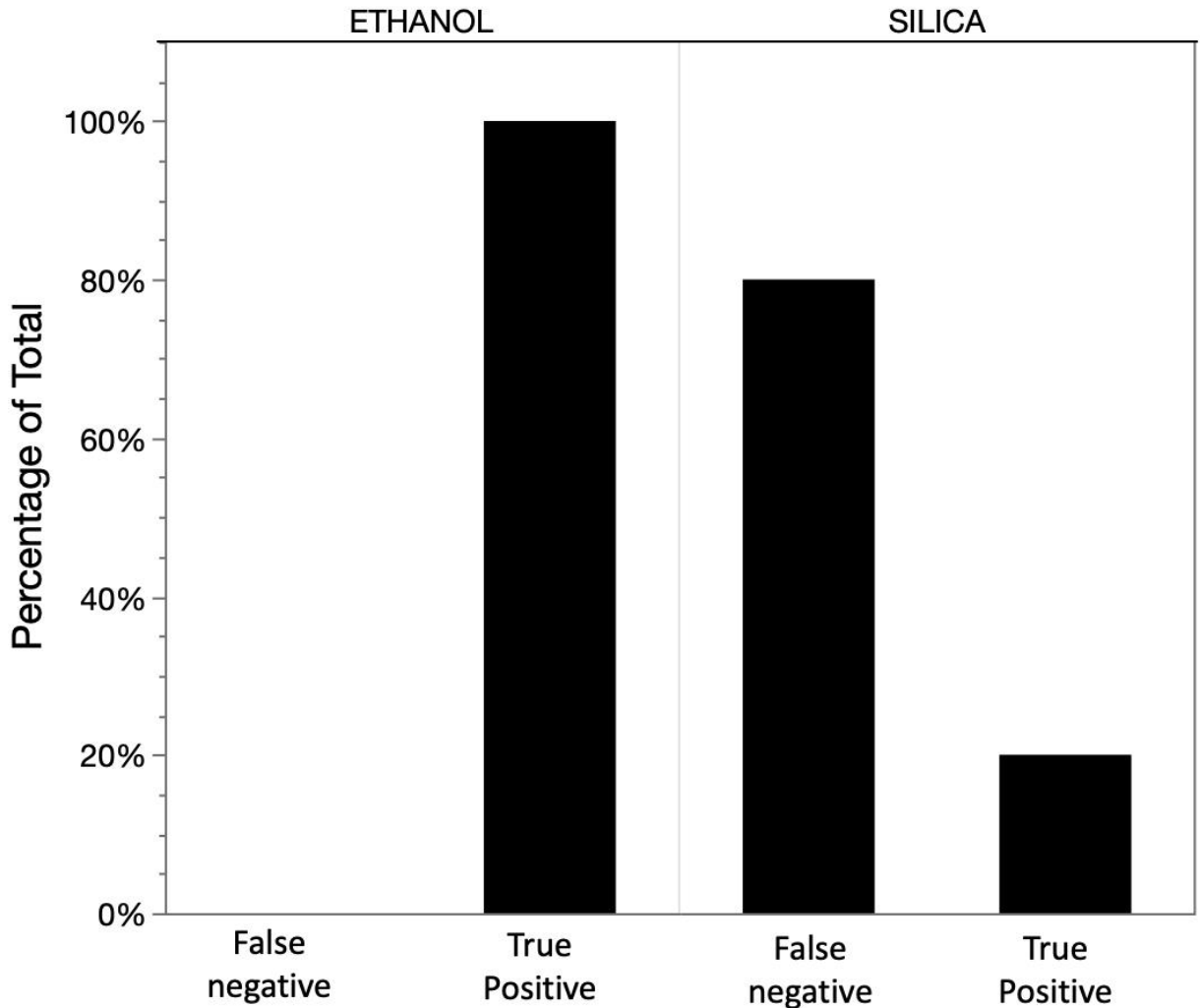


Figure 2.2: Direct comparison between the percentage of total samples that resulted in false negatives (known positive location was negative after analysis) or true positives (known positive location was positive after analysis) for determination of Blanding's turtle (*Emydoidea blandingii*) occupancy using environmental DNA. 'ETHANOL' refers to eDNA filters preserved ~0.5mL of 96% ethanol and stored at 4°C while 'SILICA' refers to eDNA filters preserved in dry silica beads and stored at -20°C after returning from the field ($X^2_{125,3} = 20.32, P < 0.0001$).

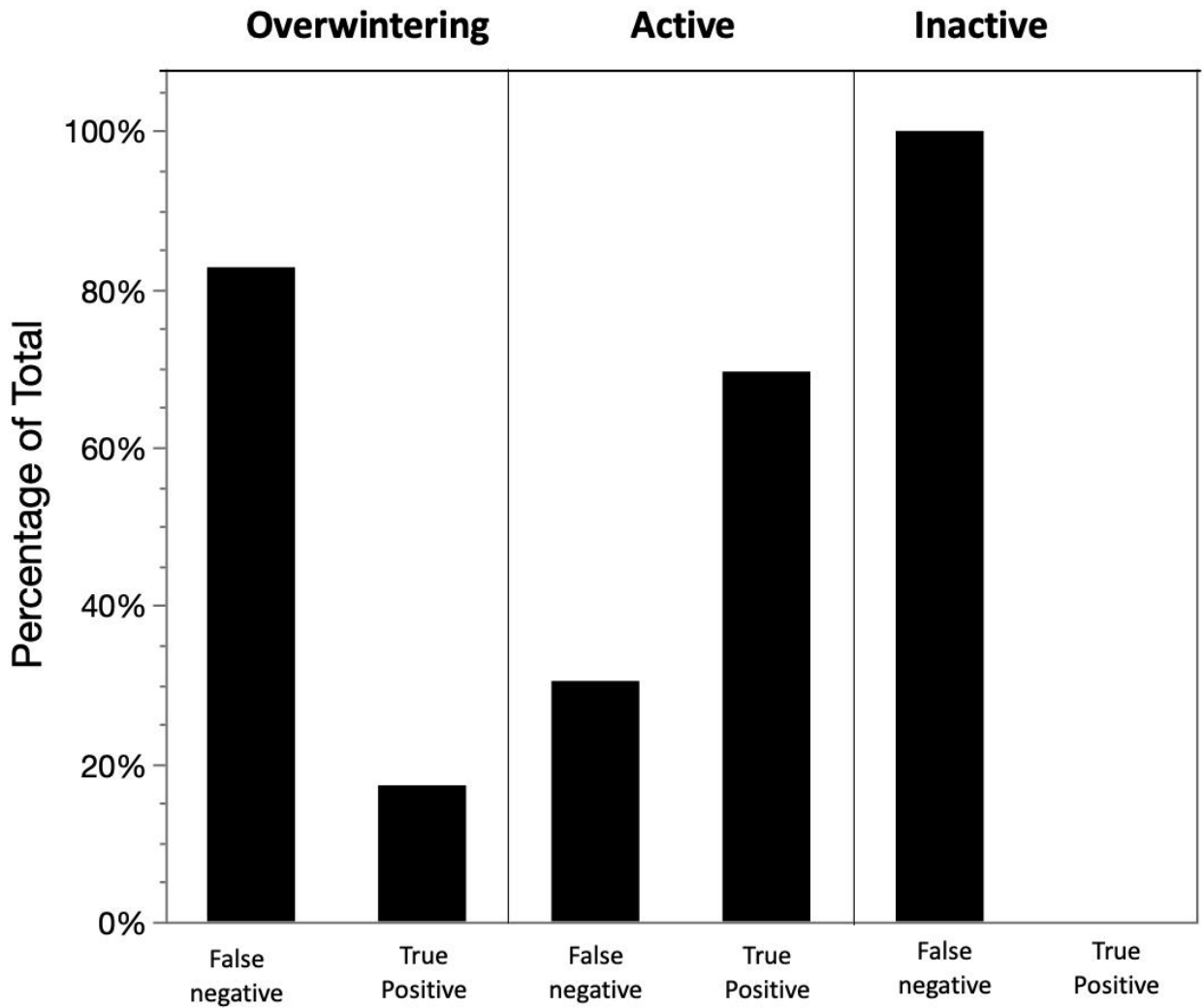


Figure 2.3: Percentage of total samples that resulted in false negatives (known positive location was negative after analysis) or true positives (known positive location was positive after analysis) for the detection of Blanding's turtle (*Emydoidea blandingii*) occupancy using environmental DNA ($X^2_{125,6} = 22.0$, $P = 0.0012$). The Overwintering season defines samples taken from December to March ($n = 29$), the Active season defines samples taken from April to August ($n = 64$) and the Inactive season defines samples taken from September to November ($n = 18$).

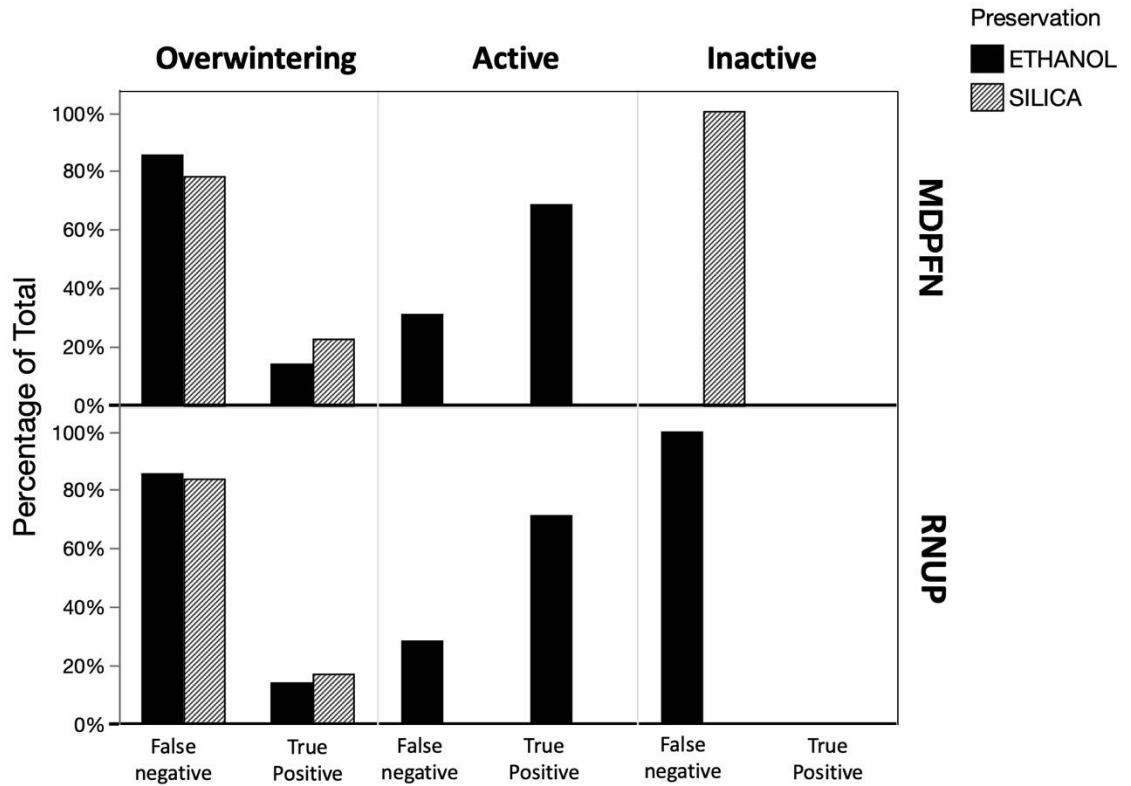


Figure 2.4: Percentage of total samples that resulted in false negatives or true positives for the detection of Blanding's turtle (*Emydoidea blandingii*) occupancy using environmental DNA at minimally disturbed wetlands in MDPFN and a highly disturbed wetland in RNUP during three seasons. Data for both preservation methods are compared. The duration of the Overwintering season was December to March (n = 16 for MDPFN, n = 16 for RNUP), the duration of the Active season was April to August (n = 16 for MDPFN, n = 7 for RNUP) and the duration of the Inactive season was September to November (n = 7 for MDPFN and n = 5 for RNUP) ($X^2_{125,12} = 135.3, P < 0.001$).

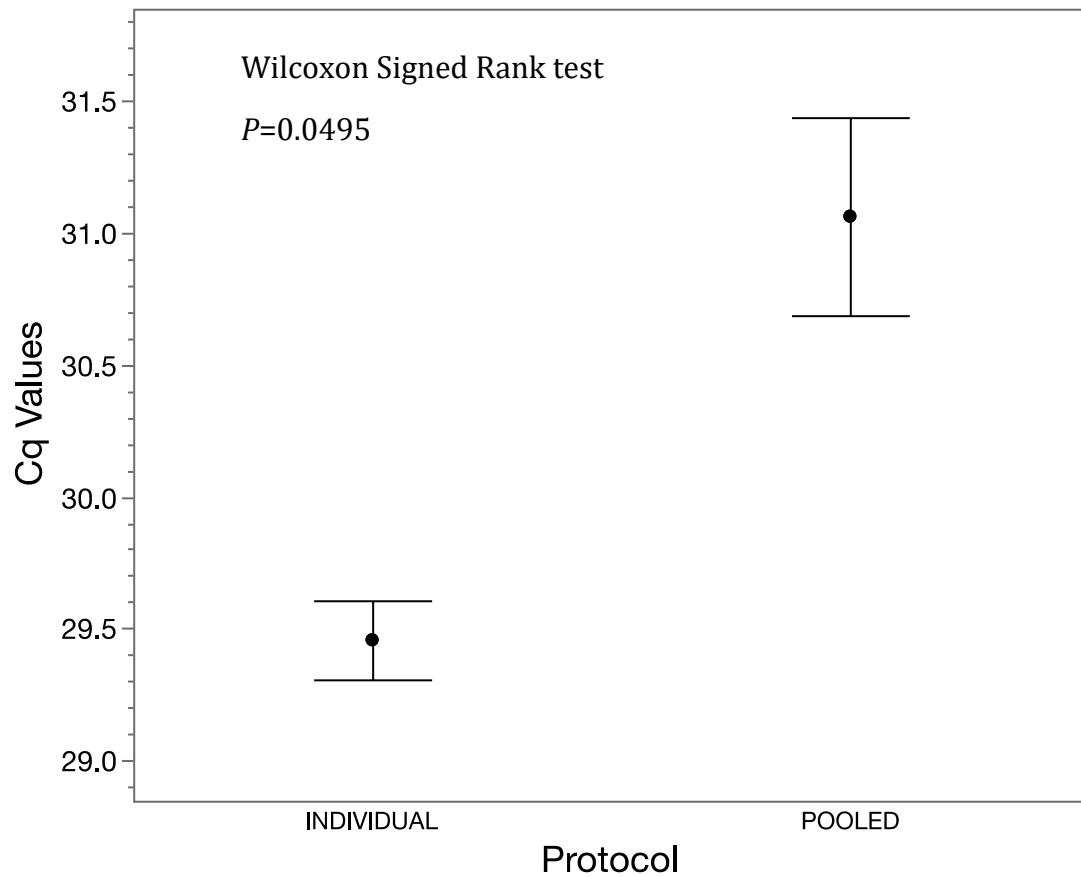


Figure 2.5: Mean Cq values associated with a random sample taken in a wetland with a Blanding's turtle confirmed at the time of sample (INDIVIDUAL) versus 18 samples taken every 60m in the same wetland that were pooled and then filtered for analysis (POOLED) (Wilcoxon Signed Rank Test, $P=0.0495$).

**Chapter 3: Photo identification of plastron patterns to
uniquely identify endangered Spotted turtles (*Clemmys
guttata*)**

Brynn Hickey¹

Dr. Chantel Markle²

Dr. Patricia Chow-Fraser¹

¹ McMaster University, Department of Biology, Hamilton, ON, L8S 4K1

²University of Waterloo, Dept. of Geography and Environmental Management
and School of Environment, Resources and Sustainability

3.1 Abstract

Spotted turtles (*Clemmys guttata*) are native to Canada and the United States and are currently endangered along their entire range. Uniquely identifying individual freshwater turtles is essential for monitoring population health and abundance. Traditional techniques to mark and identify individual turtles include shell notching and passive integrated transponder (PIT) tags, but notches can wear down over time and PIT tags can migrate, while both methods are invasive. Spotted turtles have unique black patterns on their plastron that can be photographed for an accurate method to identify individuals. We used the I³S Pattern software to annotate Spotted turtle plastron patterns, extract key points, and subsequently identify individuals. We compiled 549 images from 425 individuals from four study areas in Ontario taken between 2015 and 2022. Using customized parameters and the simple evaluation tool in I³S Pattern, we determined a 90.56% probability of identifying the correct individual turtle and an identification accuracy of 97.42% within the top three suggested turtle matches. Accuracy was increased when we excluded photos for the following reasons: 1) the image was improperly illuminated (shadows or reflections from sunlight obscured details in the photo), 2) the image had not been acquired at right angles to the camera, 3) the turtle plastron was iron-stained and 4) individuals were not mature (since plastron patterns of hatchlings and juveniles change as they age). For adult Spotted turtles located in areas without iron staining, plastron pattern identification offers a highly accurate, cost-effective,

and non-invasive method to uniquely identify individuals and help support population monitoring and conservation for this endangered species.

3.2 Introduction

The Spotted turtle (*Clemmys guttata*) is a semi-aquatic freshwater turtle in Canada and the United States with populations occurring in southern, central, and eastern Ontario. Their range extends from Florida to Atlantic Canada, with isolated populations in Michigan and Southern Ontario. Approximately 80% of the Canadian population occurs in small patches throughout Ontario (Environment and Climate Change Canada, 2018). Spotted turtles primarily use shallow wetlands, vernal pools, creeks, and sheltered bays (Ernst and Lovich, 2009). It is a smaller freshwater turtle species with a maximum length of 14.25 cm, named after its arched black carapace (top of shell) with yellow-scattered spots. Spotted turtles also have a seemingly unique dark pigmented pattern on their plastron (bottom of shell) similar to Blanding's turtles (Gray, 2008; Ernst and Lovich, 2009). They currently face many threats including road mortality, illegal collection for trade, exotic/invasive species introduction, and habitat loss, degradation, or fragmentation (Environment and Climate Change Canada, 2018). Federally, the conservation status of the Spotted turtle is endangered, and recovery strategies for this species rely on accurate tracking of individuals in remnant populations (COSEWIC, 2014).

Uniquely identifying individuals is essential for monitoring populations and estimating sizes. Multiple ways of estimating population sizes, survival rates, and

immigration rates, have been suggested that all rely on capture-mark-recapture models and being able to accurately identify individuals (Darroch, 1961; Jolly, 1965). For mark-recapture estimations, reliable marking techniques are critical to ensure accuracy. It is also essential that marked and unmarked individuals have the same probability of being caught as estimation models frequently rely on this assumption (Seber, 1965). Traditional marking techniques for the unique identification of freshwater turtles specifically include painting numbers on shells (Koper and Brooks, 1998), shell notching (Cagle, 1939), steel tags attached to marginal carapace scutes or legs (Koper and Brooks, 1998), and passive integrated transponder (PIT) tags (Buhlmann & Tuberville, 1998), which are all meant to be permanent identifiers. Nevertheless, paint and notches are known to fade throughout time, steel tags can become damaged or lost, and PIT tags can be shed or may migrate within the turtle (Limpus, 1992; Hoper and Brooks, 1998; Feldheim, 2002; Wyneken et al., 2010; Pfaller et al., 2019). Beyond these limitations, the techniques are also invasive and may subject turtles to an unknown degree of pain or shock through handling (e.g., PIT tags causing infection) (Dutton and McDonald, 1994; Wyenken et al., 2010).

Photographic individual identification of natural markings on animals has been used for decades in many long-term studies of long-lived species such as cetaceans and is gaining popularity due to it being a safe and painless alternative identification method (Wursig and Wursig, 1977; Pennycuick, 1978; Katona et al., 1979). More recently, this non-invasive option to identify individuals from photographs has been used with the unique colour pattern of a turtle's plastron

(ventral shell) successfully on several freshwater turtle species including Eastern Box turtles (Cross et al., 2014), Western Painted turtles (Cooley et al., 2015), Blanding's turtles (Markle et al., 2021), Red-eared slider (Janzen et al., 2000), and the Common Snapping turtle (Kolbe and Janzen, 2001). Since the Spotted turtle also has a unique pigmented pattern on its plastron that remains relatively unchanged (Gray, 2008), we hypothesize that this photo-identification approach can be applied successfully to this species. The purpose of this chapter is to determine if the plastron pattern on Spotted turtles can be accurately used to identify individuals. For this study, we acquired hundreds of plastron photos from four organizations in Ontario to 1) establish the accuracy of using plastron patterns as a tool to uniquely identify Spotted Turtles, and 2) determine the limitations of using 2D images to delineate 3D patterns.

3.3 Methods

3.3.1 Photograph Database

We collected 549 Spotted turtle plastron photographs from 425 individuals taken by staff of Blazing Star Environmental, Scales Nature Park, and C. Markle at the University of Waterloo between 2015 and 2022 within their Ontario range between Georgian Bay and Muskoka (Table 3.1). The Spotted turtles were captured either by hand or baited hoop traps and subsequently photographed with the technicians' phone camera before being released into their original habitat. All plastrons that were photographed showed the individual's notches which corresponded to a notch

code system (based on Cagle, 1939) to ensure that individual identifications were accurate.

3.3.2 Data Analysis

We used the Pattern software (Version 4.02) in the Interactive Individual Identification System (I³S) to identify patterns on Spotted turtle plastrons (den Hartog and Reijns, 2014). The software package Interactive Individual Identification System (I³S) Pattern is an open-source program developed to automatically extract key points within a pattern to match animals for identification purposes (den Hartog and Reijns, 2014). The algorithm used by I³S Pattern is based on key point extraction, reference points and key point comparison. To help recognize individual characteristics within a pattern, I³S automatically extracts a set number of key points within a region of interest. This region of interest is determined by three set reference points that are visible and consistent for all individuals. The reference points help correct differences in viewing angle, rotation, and scaling. With reference points selected, various photos can be compared within the same 2D coordinate system.

We identified the 3 reference points as the top of the plastron where the gular scutes meet and the bottom of the left and right anal scutes (Figure 3.1). I³S Pattern then converts the photo to a grayscale which is created from a sum of luminance values from the red, green, and blue channels, each weighted by a conversion value summed to 1. If the weights of each channel are adjusted, the focus on different colours will vary. The Pattern software in I³S automatically extracts 35

key points from each plastron to use as a comparison with other photos (Figure 3.1). Key point pairs are compared in a point cloud and matched if the nearest key point is at a sufficient distance from the current match (Figure 3.2). From the pairs, a distance metric is calculated based on the sum of the distances between each key point pair, divided by the square of the number of key point pairs (Figure 3.2). The distance metric is used to rank each image on the most likely match. All photos and individual matches are stored in an identification database that can also store metadata relating to each individual, including length, size, scars, or other important information you may want to store. When a new photo is uploaded, potential matches are scored with the most likely match receiving the lowest score. The most likely matches represent the individuals with the lowest scores and are presented in ascending order to allow users to visually compare matches.

We altered the default red, green, and blue conversion scale values as well as the number of key points extracted to identify the custom settings that produced the highest accuracy for Spotted turtles. We adjusted the key points in intervals of 5 starting at 25 and ending at 50 and performed a simple evaluation with the entire database. The simple evaluation tool gives one an indication of how often the same individual matches with each other by comparing all photos in the database with all other photos. When a match between two photos of the same individual yields a score outside the top 10 best matches, it is logged under “Result outside top 10”. The “top x” defines the percentage of comparisons that resulted in a correct match ranking within the top x results. The accumulated ranking score represents the sum

of all rankings between two photos of the same individual. For the conversion scale values, we changed each channel weight in intervals of 5% starting with the default values and ending when the accumulated score no longer decreased. Two channels were changed at a time while the third channel was held constant. We also tested the custom conversion values calibrated for Blanding's turtles by Markle et al., (2021) due to their similarity to the pattern colouring of the Spotted turtle's plastron. We recorded the percentage of correctly identified turtles within the top 1, top 3, and top 5, and the accumulated score to see which custom setting yielded the highest accumulated score and accuracy.

Once the ideal settings were determined, we used the simple and elaborate evaluation tools in I³S Pattern to identify photos that yielded a score outside the top 10 best matches. The majority of these photos were excluded from our final 'Refined Database' (532 images of 415 individuals) because of poor quality due to shadows and reflections from sunlight that obscured patterns (poor illumination), iron staining, presence of leeches on the plastron, and the plastron not being photographed at a 90-degree angle from the camera (Figure 3.3). The elaborate evaluation tool elicits a more realistic scenario that separates reference images from test images, and the test images are then compared against the reference set 1000 times in a comparison matrix.

3.4 Results

Using the simple evaluation tool and all default values of plastron photos of Spotted turtles compiled in the database (549 photographs of 425 individuals; Table 3.1), we obtained an 89.2% probability of correctly identifying an individual to the top-rank match. Since increasing or decreasing the number of key points beyond the default of 35 did not improve accuracy, we continued to use this default setting for the remainder of the data analysis (Table 3.2). For comparison purposes, we obtained a lower accuracy (87.45%) using Markle et al. (2021) gray-scale conversion values (red = 0.466, green = 0.333, blue = 0.201) that had been used to identify Blanding's turtles (Table 3.3). We also developed a custom gray-scale conversion with red values of 0.318, green values of 0.537 and blue values of 0.145 (instead of default values of 0.299, 0.587, and 0.114 for the red, green, and blue colours, respectively), and this further increased the accuracy to 91.0% for the top-rank match and 97.9% for the third-rank match (Table 3.3). We, therefore, used the custom gray-scale conversion parameters to analyze the entire dataset.

On average, across all four study areas and including contributions from all three collaborators as well as photos of the same individual taken in different years (i.e., Entire database), we obtained a 91.0% probability of correctly identifying turtles when using the simple evaluation tool, and a slightly lower probability of 88.6% when using the elaborate evaluation tool with 1000 repetitions (Table 3.4). Accuracy was increased slightly to 91.4% for the top-rank match with simple evaluation if we excluded photos corresponding to plastrons with leeches or were

darkly stained with iron or improperly illuminated images that had dark shadows, or reflections of bright sunlight that obscured the plastron patterns (i.e., Refined database; see Figure 3.3). For both databases, we obtained slightly lower accuracies when we used the elaborate evaluation tool (Table 3.4). Expanding the pool of turtles to include the top 5 matches, we obtained 100% accuracy for the refined database using the simple evaluation tool and 99.8% using the elaborate evaluation tool (Table 3.4).

3.5 Discussion

When all photographs in the refined database were analyzed, we found a 91.4% chance that different photos of the same individual Spotted turtle would appear as the top first-ranked match (Table 3.4). Meanwhile, there was a 98% chance of the correct turtle being identified within the top three ranked matches (Table 3.4). For a more realistic scenario, when we added an unidentified turtle into the refined database, the correct Spotted turtle would be identified as the first-ranked match 90% of the time, and within the top three ranked matches 99% of the time (Table 3.4). In wetlands with high iron content in the water, however, plastron patterns in photographs were almost entirely obscured (Figure 3.3C-D); excluding these photographs, along with poor-quality photographs as mentioned previously (Figure 3.3), increased individual accuracy for correct matches of top rank by 0.4 - 1.40%, depending on the evaluation tool used.

We recommend that photos of Spotted turtles be taken in the shade to reduce the likelihood of reflections and shadows from sunlight obscuring plastron patterns. The turtle should be lying on the ground and the camera should be held at 90° angle to the turtle to minimize distortion of the size/shape of pattern markings. To maximize image resolution, the camera lens should be placed as close as possible to the plastron so that the entire turtle is captured in the image without much background. Whenever possible, a high-quality phone camera or a digital camera such as a DSLR (digital single-lens reflex camera) should be used. Researchers or technicians should ensure that the plastron pattern is completely unobstructed by items such as leeches, dirt, or plant material. Taking high-quality photographs (e.g., Figure 3.1) once a year will improve individual identification accuracy and is essential for the long-term monitoring of Spotted turtles.

Pattern stability is critical for the use of photo identification as a sole means for individual long-term monitoring (Vincent et al., 2001). Spotted turtle plastrons are known to undergo pigmentation changes which start in hatchlings as a dark central pattern that slowly spreads outward towards the marginal scutes leaving a central light-coloured area and eventually entirely black as the turtle ages (Ernst et al., 1994; Gray, 2008). These changes in patterns occur slowly over their long lifespan of up to 110 years for females and 65 years for males and remain relatively unchanged for up to 5-10 years (Litzgus, 2006; Carroll, 1999; Gray, 2008). Photos of individuals in this study covering a timespan of up to 10 years were accurately identified with this approach. This confirms the great potential of using only photos

of Spotted turtle plastrons to accurately identify individuals of an endangered population in long-term monitoring programs across their geographic range in Ontario.

Similar to Blanding's turtles (Markle et al., 2021) and Wood turtles (Cowin and Cebek, 2006), juvenile and hatchling Spotted turtles will require the use of additional individual identification methods such as notching, or PIT tags since patterns change too drastically during this life stage. Therefore, we recommend that individual pattern recognition only be used for adults since changes progress much more slowly after they reach maturity at 11 years of age (Gray, 2008). Future research should investigate the age when plastron patterns become relatively stable, and accuracy associated with photos of adults recaptured more than 10 years apart. Although I³S Pattern greatly exceeded our predictions in terms of accuracy, all photos used in the database were taken within Ontario. We suggest sampling in other provinces and states where Spotted turtles are found to ensure that this approach is accurate for all animals within their geographic range.

Spotted turtles have light-yellow spots scattered throughout their carapace (dorsal shell) that also appear to be relatively unique to each individual turtle. We briefly investigated using the Spotted turtle's carapace for photo identification but quickly ruled it out, as the number of spots on the carapace has been shown to change from year to year (Gray, 2008). Hatchlings usually start with one spot per scute and will accumulate spots as the turtle ages (Ernst and Lovich, 2009). As adults, Spotted turtles have been recorded to lose up to eight spots after only one

year (Gray, 2008). The lack of contrast from the predominantly dark pigmentation of the carapace, as well as sunlight reflection due to the curvature of the carapace, can both compromise the quality of the photo for the pattern recognition software. In addition, Spotted turtle carapaces have been shown to sustain extensive peeling and flaking of the keratinized layer which obscures spots and makes pattern annotation much more difficult (Gray, 2008). Therefore, we recommend solely using the plastron pattern to individually identify Spotted turtles.

Currently, I³S Pattern is only available for download on Windows or Mac computers. In addition, images require a few pre-processing steps that take several minutes per photo, and these can be cumbersome to accomplish in the field. The source code for the software is freely available and can be modified to create an iOS or Android app that can be used with a field tablet to immediately identify Spotted turtle individuals in the field. The software requires the user to complete a manual identification of the correct turtle within the suggested matches. With our custom values and refined database, we found that the correct match was located in the top 5 suggested matches 100% of the time. This manual step ensures the user can create accurate database references of individual Spotted turtles despite the use of lower-quality photos or photos where the plastron pattern is slightly obscured. The program also allows the storage of metadata for individual turtles such as age, sex, deformities, health issues, and any other data that might be useful for identifying individuals. Coordinated image-identification databases with existing conservation

programs (e.g., Ontario Turtle Conservation Centre, Scales Nature Park, Ontario Nature) also have the potential to help support community science initiatives.

With a good database of images, I³S Pattern is a non-invasive, inexpensive, and efficient way to identify individual Spotted turtles in long-term monitoring programs and mark-recapture studies. This approach does not suffer from notching and the use of PIT tags that are invasive and that can fade or become lost over time. For freshwater turtle species such as the Spotted turtle whose patterns do not fully develop until many years into their sub-adult life, pattern-recognition software should be coupled with additional techniques, until the long-term viability has been verified. Entirely replacing techniques such as notching may also not be feasible for species that are threatened by illegal exotic trades since notches can make individuals less desirable to poachers. Pairing pattern recognition with traditional marking methods will help to ensure the most accurate data are used for monitoring this endangered freshwater turtle population. Therefore, we encourage researchers and conservation technicians to take photos of the plastron of all Spotted turtles in their routine processing of turtles they encounter in their research and monitoring.

3.6 Acknowledgement

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3.8 Figures and Tables

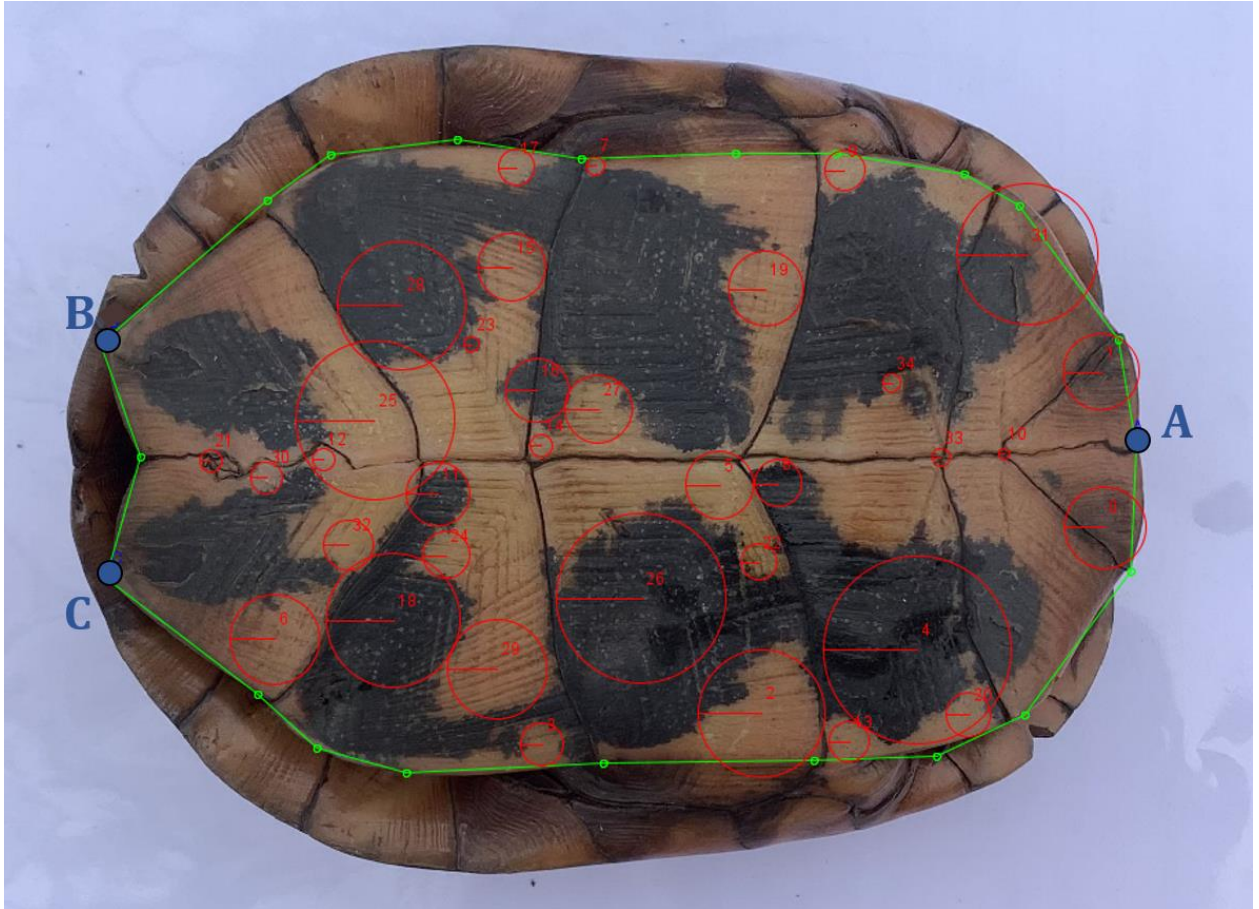


Figure 3.1: Fully annotated photograph in I³S of a Spotted turtle (*Clemmys guttata*) plastron. Reference point A is located at the top of the plastron between the gular scutes, while B and C are at the bottom left and right anal scutes, respectively. The plastron's outer edge must be manually traced to delineate the region of interest (green lines and circles). In total, 35 key points are automatically extracted by I³S Pattern (red circles).

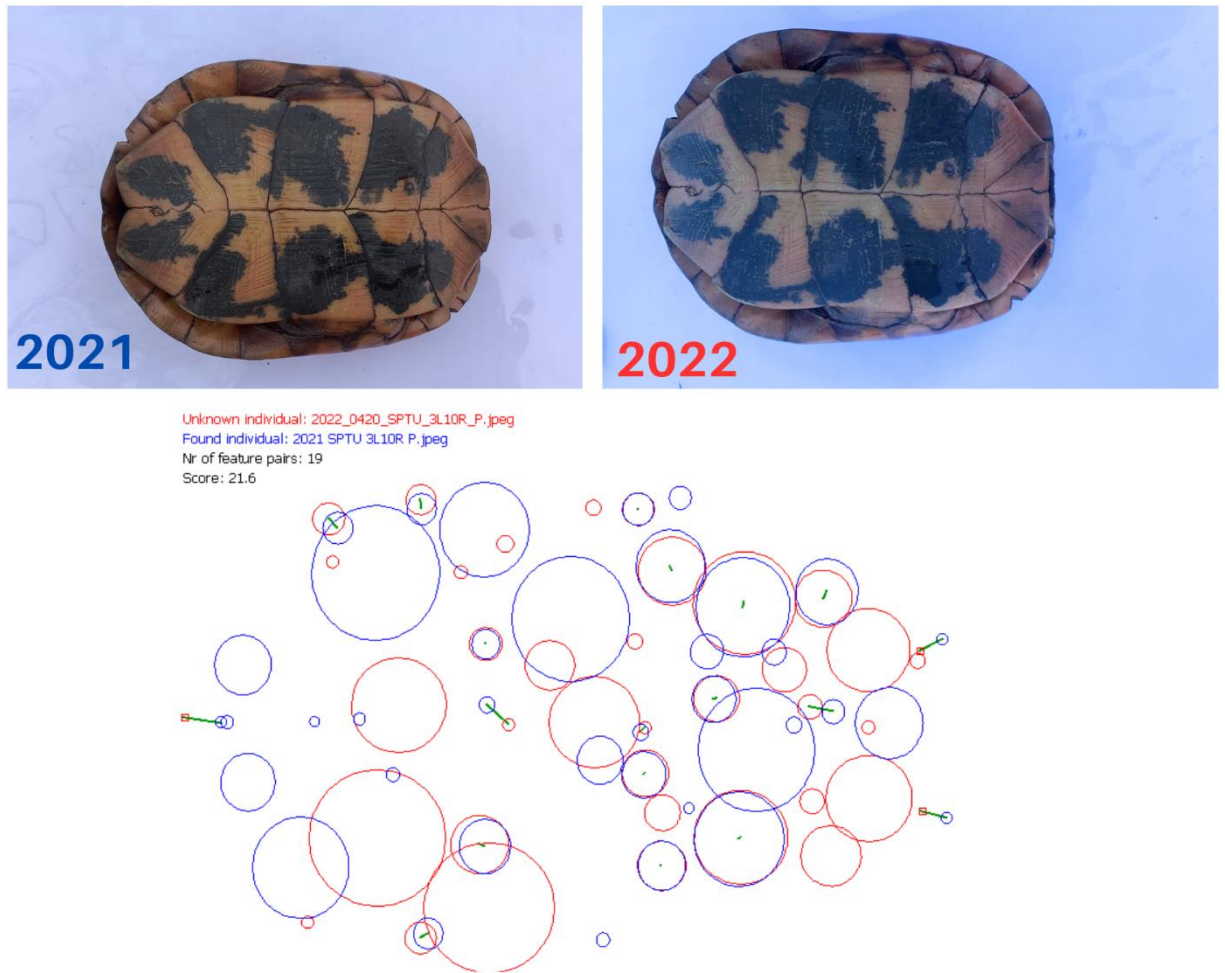


Figure 3.2: Comparison of key-point clouds corresponding to two photographs of the same Spotted turtle (*Clemmys guttata*) acquired in 2021 (blue circles) and in 2022 (red circles). The green lines connect two key points and indicate they are a matching feature pair (n=19). The score is calculated as the sum of the distances between each key point pair divided by the square of the number of key point pairs.

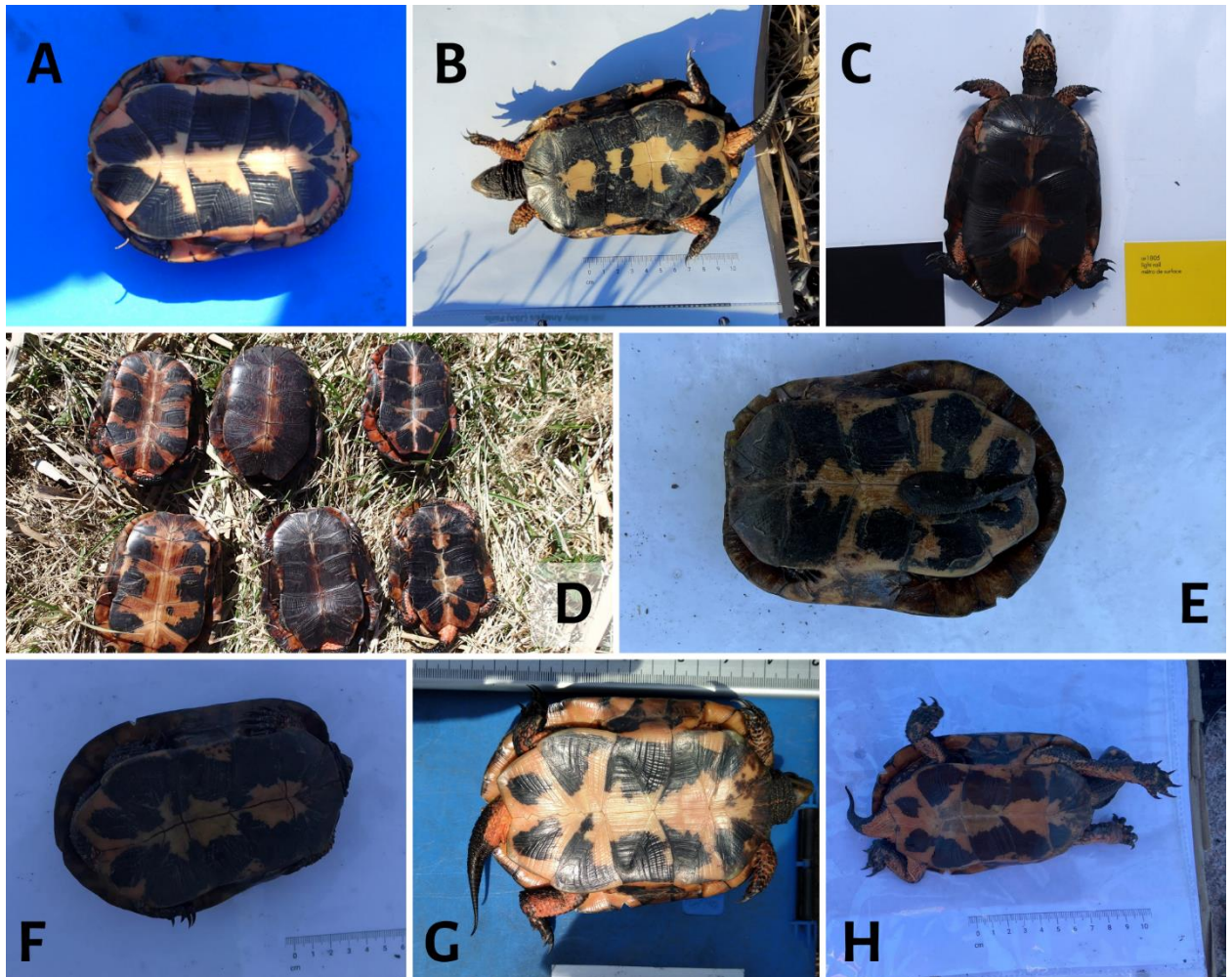


Figure 3.3: Examples of Spotted turtle (*Clemmys guttata*) plastron photos that did not produce accurate results (lower than the top 10th rank) due to shadows obscuring patterns (A-B), iron staining (C-D), leeches (E), dark shade (F), sunlight reflections (G), and the plastron not being at a 90-degree angle from the camera (H).

Table 3.1: Organizations and researchers who contributed to the 549 images in the study, the number of Spotted turtles (*Clemmys guttata*) photographed (425 individuals), the location where turtles were caught, and the duration when photos were acquired of turtles.

Organization/ Researcher	Number of turtles photographed	Location	Duration when photos were acquired
Blazing Star Environmental (John Urquhart, Principal)	230	Eastern Georgian Bay	2020-2022
University of Waterloo (Prof. Chantel Markle)	77	Muskoka	2017-2019
Scales Nature Park (Jeff Hathaway, Founder)	119	Muskoka, Eastern Georgian Bay	2015 - 2022

Table 3.2: Percentage of individual Spotted turtles (*Clemmys guttata*) correctly identified with the simple evaluation tool in I³S Pattern, where plastron patterns were analyzed with various key points and default gray-scale conversion values.

Number of Key Points	Top first rank	Top third rank	Top fifth rank	Accumulated ranking score
25	81.82%	92.21%	93.07%	3605
30	84.42%	96.54%	96.97%	3337
35	89.18%	96.97%	97.84%	2793
40	87.45%	96.97%	97.84%	3220
45	85.71%	95.67%	96.97%	3186
50	84.42%	96.54%	97.40%	3262

Table 3.3: Percentage of individual Spotted turtles (*Clemmys guttata*) correctly identified with the simple evaluation tool in I³S Pattern, where plastron patterns were analyzed with various default red, green, and blue conversion values compared with custom values developed for Spotted turtles in this study.

Protocol	Gray-Scale Conversion Values			Percentage of Correctly Identified Individual Turtles		
	Red weight	Green weight	Blue weight	Top #1	Top #3	Top #5
Default setting	0.299	0.587	0.114	89.18%	96.97%	97.84%
Markle et al., 2021	0.466	0.333	0.201	87.45%	96.97%	98.27%
Custom for this study	0.318	0.537	0.145	90.99%	97.85%	99.14%

Table 3.4: Percentage of correctly identified Spotted turtles (*Clemmys guttata*) with the simple and elaborate (1 reference image, 1000 repetitions) evaluation tools in I³S Pattern software. The entire database refers to all photographs (549 images of 425 individuals) obtained for this study, while the refined database excludes photographs that could not be identified correctly with fewer than 10 options due to iron staining, sunlight reflections and shadows obscuring plastron patterns (532 images of 415 individuals).

Database	Evaluation type	Percentage of correctly identified turtles		
		Top #1	Top #3	Top #5
Entire Database	Simple Evaluation	90.99%	97.85%	99.14%
	Elaborate Evaluation	88.58%	97.66%	98.24%
Refined Database	Simple Evaluation	91.40%	98.64%	100%
	Elaborate Evaluation	89.98%	99.33%	99.79%

Chapter 4: Conclusion

Brynn Hickey¹

Dr. Patricia Chow-Fraser¹

¹ McMaster University, Department of Biology, Hamilton, ON, L8S 4K1

4.1 Conclusion

For the effective conservation and management of threatened species, scientists must determine their occupied habitats, which usually entails costly and labour-intensive tracking methods such as visual surveying, or trapping. Technicians and researchers also monitor freshwater turtle population and health trends (e.g., fecundity or growth rates), requiring individual identification methods such as mark-recapture. Many of these traditional monitoring techniques are invasive, inaccurate, labour-intensive, or costly. Advancements in novel techniques to reduce the cost and invasiveness associated with occupation detection and individually identifying at-risk freshwater turtles are essential to their conservation. Coordinated image-identification databases and environmental DNA monitoring programs with existing conservation programs (e.g., Ontario Nature) have the potential to transform community science initiatives.

As yet, federal, and provincial agencies have avoided basing any regulatory decisions on data obtained with eDNA methods. For example, surveying protocol for threatened species such as Blanding's turtles in Ontario includes visual encounter surveys, traps, nest surveys and road surveys (OMNRF, 2015). Meanwhile, Environment and Climate Change Canada (2018) only considers the identification of occupancy or critical habitat from data obtained through professional surveys, incidental sightings, telemetry studies, nest site and overwintering site observations, dead individuals, and/or observations in unsuitable habitats. For eDNA to become an accepted methodology to confirm occupancy of at-risk species for management

and conservation purposes, government agencies must establish protocols and processes to certify eDNA labs. Without government uptake for regulatory decisions, the potential for eDNA to improve wildlife conservation will continue to be unused (Lodge, 2022).

Unfortunately, there is also a niche market of specialized eDNA equipment and lab services provided by for-profit businesses that charge more than 100 times the actual cost of processing. Greater reliance on eDNA for species at risk monitoring would help incentivize market demand for universities, non-profit organizations, and environmental consulting companies to provide these services. Adopting eDNA into policy and regulation by Canadian and provincial agencies would unlock the potential for more affordable, less invasive methods for ecosystem management.

We have shown that updating traditional monitoring techniques with novel methods has the potential to enhance our understanding of habitat use and population trends for at-risk freshwater turtle species in Ontario. Specifically, the use of eDNA has extended the traditional survey season into the fall and winter months, while pattern recognition software to identify individuals will reduce stress to individuals. The use of non-invasive techniques would not require restrictive research permits, in turn expanding the participation of technicians and volunteers in research and monitoring for the effective conservation of freshwater turtles.

4.2 References

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