Chapter 10

Modern Toolbox to Address a Decades-old Problem in the Conservation of Freshwater Turtles: Blanding's Turtles (*Emydoidea blandingii*) as a Case Study

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Abstract −Traditional monitoring methods to study the movement patterns and habitat use of freshwater turtles include visual encounter surveys and radio telemetry. Both approaches are labour-intensive and time-consuming, and studying behaviours such as nesting attempts is challenging due to infrequent direct observation, requiring inference from limited data. In this chapter, we introduce several emerging approaches that address some limitations of traditional techniques. These include using 1) multi-sensor biologgers to explore movement and behaviour, 2) environmental DNA to determine occupancy, and 3) pattern recognition software to enhance traditional individual identification methods. We summarize existing traditional approaches, show the advantages and disadvantages of these new tools, and illustrate how they support research to protect Blanding's turtles (*Emydoidea blandingii*) in Ontario, Canada.

Introduction

Globally, freshwater turtles, members of the order Testudines, face many threats (Lovich *et al.* 2018) that have caused widespread population declines and extirpations (Rhodin *et al.* 2021). Their disappearance harms ecosystem health because they play a significant role in seed dispersal and exchange of biomass and nutrients between aquatic and terrestrial systems (Congdon and Gibbons 1989; Moss 2017; Lovich *et al.* 2018). The Blanding's turtle (*Emydoidea blandingii*), for example, is a semi-aquatic freshwater species found in various habitats centred around the North American Great Lakes, with a few disjunct populations in northeastern North America (Congdon *et al.* 2008). Their habitat includes a variety of wetlands (marshes, bogs, fens, coastal wetlands, swamps), slow rivers, vernal pools, lakes, bays, and upland forests (Edge *et al.* 2010). Blanding's turtles are at risk of habitat loss and degradation due to invasive species, human development, wetland modifications, collection for the pet, food, and medicine trades, and increased subsidized predation of nests and juveniles by numerous mesopredators, such as northern raccoons (*Procyon lotor*) (COSEWIC 2016).

The Great Lakes/St. Lawrence's population of Blanding's turtles has declined by more than 60% over the last 120 yrs, and without effective mitigation, road mortality by itself will reduce the adult population by approximately 50% over the next 120 yrs (COSEWIC 2016). The Blanding's turtle was assessed provincially as threatened, prioritizing long-term population monitoring for this species (Ministry of the Environment, Conservation and Parks 2019). A vital research avenue is to examine how turtles behave and interact with their environment in different ecosystems, information that is crucial for developing effective recovery strategies for populations with home ranges that contain altered land uses. In this chapter, we

review current technologies to study freshwater turtle behaviour, movements, and habitat selection, and then highlight emerging innovative technologies, including multi-sensor biologgers, environmental DNA (eDNA), and pattern recognition software. We discuss how these tools could increase knowledge and reduce the effort required to collected data on turtle ecology and behaviours using Blanding's turtles as a case study. This new toolkit could be easily modified for other semi-aquatic turtle species to advance the conservation goals of freshwater turtles worldwide.

Available research methods for different investigative purposes

Turtles are inherently cryptic and are challenging to locate in their natural environments. Studying species such as the Blanding's turtle can be complicated because turtles have large home ranges that include several types of wetlands and upland habitats, and they are often underwater during the day (Edge *et al.* 2010). Existing research methods to study turtles have focused on ways to confirm their occupancy/presence, trap them, determine their movements or behaviours, and identify them individually (see Table 1). With many freshwater turtle species being at risk, permits must be acquired to handle or perform research on the species.

Confirming occupancy

Visual Encounter Surveys (VES) are a non-invasive approach to spot turtles during spring when basking on logs or rocks and require no tools other than binoculars for accurate identification. A proper VES, however, requires surveying multiple locations along the shorelines of extensive open-water wetlands; in shallow wetlands, VES are conducted through evenly spaced transects across all sections of the wetland. This time-consuming approach confirms species presence and cannot yield information regarding habitat use, movements, or population census. No specialized research permits are required.

Trapping turtles

For information on population size or the sex, health and size of individuals, turtles must be caught by hand or with a trap (baited or not). A common trap is the hoop net, constructed of mesh netting strung over circular metal hoops with a funnel opening at one end that facilitates entry while restricting escape (Casper and Hecnar 2011). Traps are semi-submerged (~60% submerged) and anchored in densely vegetated areas in wetlands and are often checked once to multiple times a day to ensure animal welfare. One can sex, measure, and mark captured individuals, and then released them so they can be monitored as desired. Special research permits must be acquired for this activity.

Tracking movements

Tracking the individual movements has evolved from following threads on spools that are mounted on turtle carapaces (e.g., Breder 1927; Iglay *et al.* 2010) to affixing radio transmitter on carapace that emits pulses of radio signals at known frequencies that can be detected by a receiver and antenna. Spool-thread tracking has limited efficacy except for small spatial scales and has not been used for Blanding's that make extensive migrations during nesting season. Although radio telemetry is the tried-and-true tracking method to study the movements and habitat selection of many freshwater turtles (Obbard and Brooks 1981; Rowe and Moll 1991; Aebischer *et al.* 1993), 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 determining where the animal is situated (e.g., if they are in the middle of a dense thicket swamp). Additionally, relocations are biased towards daylight hours due to investigator preference and the possible hazards 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 accommodations) is often prohibitively expensive, especially for remote field sites. The greatest limitation when radio telemetry is used on its own is that the location and movement data only can be gathered when the researchers are present. Recording activities such as swimming, resting, nesting, and other fine-scale habitat uses require direct visual observations that

are either very time-consuming, altered by researchers' presence or, in some settings, logistically unmanageable. Special research permits must be acquired for this activity.

Table 1. Comparison of research methods available to study turtle occupancy, movements, and habitat use. Purposes: Confirm occupancy of specific habitat types/locations (OCC), Species identification (ID), Behavioural study (BS), Population census (POPCen), Radio telemetry or biologging program (RT/BL), Health assessment (HA), Individual turtle movements (MOV), Individual or population home ranges (HR), Average daily distance travelled (DDT), and Habitat use and selection (HS).

Method	Brief Description	Purpose
Visual encounter surveys	Species identification or enumeration confirmed visually without the use of equipment.	OCC, ID, BS
Hoop nets or other traps	Baited or un-baited traps are set for multiple hours, and turtles are processed [a] and released.	OCC, POPCen, RT/BL, HA, ID, (and MOV & HS to some extent with proper design).
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.	OCC, RT/BL, HA, MOV, HR DDT, HS
Single sensor biologger	A single animal-borne sensor that collects data on an aspect of a turtle's movement, behaviour, physiology, or ambient environment.	OCC, BS, RT/BL , HA, MOV HR, DDT, HS [b]
Multi-sensor biologger	An animal-borne unit that contains multiple sensors, including inertial measurement units (IMU, e.g., accelerometers, magnetometers and gyroscopes) that collect data on one or multiple aspects of a turtle's movement, behaviour, physiology, or ambient environment.	OCC, BS, RT/BL , HA, MOV HR, DDT, HS [b]
Environmental DNA	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.	OCC, ID
Individual identification	Use of shell notching, passive integrated transponder (PIT) tags, leg banding, or unique coloration pattern of turtle plastron to identify individual turtles	OCC, ID, BS, POPCen, HA, MOV, HR

[a] Processing can include species identification, determination of sex, weight, carapace length, and eventual tagging for use in a tracking program.
[b] Purposes vary based on sensors employed, frequent purposes reported.

Tracking behaviours

Biologgers are animal-borne archival devices that contain one or more sensors to collect physical or biological data that can be used to study fine-scale movements and behaviours (Hooker *et al.* 2007). Single-sensor biologging devices have been used in conjunction with radio telemetry for over a decade and are often either temperature sensors (Edge *et al.* 2009; Millar *et al.* 2012) or global positioning system (GPS) loggers (Kydd 2010). Temperature sensors have been used to describe thermoregulation regimes (Millar *et al.* 2012) and overwintering thermal coping mechanisms (Edge *et al.* 2009), while GPS loggers have been used to increase the frequency of relocations without additional labour involved in manually relocating individuals. The use of GPS loggers led to the identification of novel critical habitats and travel corridors (e.g., Christensen and Chow-Fraser 2014), and with increased battery life and larger data storage capacity,

GPS loggers can track fine-scale movements throughout the day and night as long as the turtle is above water. A common limitation of this method is the inability of GPS units to obtain a satellite signal underwater (Hjort Toms *et al.* 2022). This results in a serious constraint for use of species that do not fully emerge out for the water regularly and leading to relocations only occurring when turtles are basking or conducting overland travels (Christensen and Chow-Fraser 2014). If the turtle is often submerged when the unit is attempting to establish a satellite connection, it can lead to a depletion of valuable battery life. A workaround is to schedule the loggers to obtain a fix at a time when turtles are out of the water (e.g., between 10:00 and 16:00), but this can also be a problem if the target species often moves at night. For example, using only GPS loggers, Hjort Toms *et al.* (2022) could not distinguish between activity and inactivity during the night for Blanding's and spotted turtles (*Clemmys guttata*) that were submerged during this period. Though turtles could have been active during the night, their movements could not be recorded, and this represents a huge limitation since Blanding's turtles are semi-aquatic and spend a large portion of their time underwater and out of sight. This activity requires research permits.

New tools to study movement, habitat selection and behaviour

Recent technological advancements have paved the way for some exciting upgrades to old approaches to studying movement, habitat selection, and fine-scale behaviours of freshwater turtles. We will highlight 3 novel approaches in our toolbox to address decades-old problems in turtle conservation with some distinctive advantages, including multi-sensor biologgers, environmental DNA, and identification of individuals through their plastron coloration pattern.

Multi-sensor biologgers

The use of multiple sensors in biologgers can solve some of the problems associated with the use of single sensors. For example, additional sensors that measure conductivity or partial pressure could be integrated into GPS loggers to trigger the device to stop attempting satellite fixes and thus save battery life. Multi-sensor biologgers have also been used successfully on large-bodied sea turtles to investigate their patterns of movement, behaviour, and activity (Tyson *et al.* 2017), and have recently been used on small-bodied turtles (Marchand *et al.* 2021). These devices typically include a combination of bi- or tri-axial accelerometers, magnetometers, gyroscopes, ambient conductivity, ambient pressure, and ambient temperature sensors (Table 2). Devices typically include a mechanism to log the locations of the turtle either via a GPS logger for species that can be retrieved periodically (as discussed earlier) or via a satellite tag that transmits locations of the turtles in real time for species such as sea turtles that have global migration routes. The incorporation of multiple sensors can enhance data collection when individuals are submerged underwater and GPS loggers are unable to acquire signals, thereby expanding their utility across multiple freshwater species.

Accelerometers and magnetometers are considered Inertial Measurement Units (IMU) that, together with additional sensor information, can classify individual behaviour patterns and activity levels of turtles. For example, by calculating the overall dynamic body acceleration (ODBA) or vectorial sum of the dynamic body acceleration (VeDBA) from the tri-axial accelerometer data, activity can be investigated across different habitat types or important behavioural seasons (Wilson *et al.* 2006). Robichaud *et al.* (2023) used a multi-sensor biologger on northern map turtles (*Graptemys geographica*) and found that they maintained locomotor activity throughout the winter. While the maintenance of locomotor activity throughout the winter was previously hypothesized based on radio telemetry studies (Litzgus *et al.* 1999) and short-term visual observations (Graham and Graham 1992), this type of fine-scale data in situ would have been impossible to collect without the use of multi-sensor biologgers. The availability of such sensors has expanded our capability to track fine-scale movements of aquatic and semi-aquatic turtles throughout the day and night to understand how distinct species adjust their behaviour to meet physiological demands.

A large volume of data at fine temporal scales can be collected with IMUs. These data can be analyzed with simple decision-tree classification techniques (Auge *et al.* 2022) or more complex machine learning methods such as Hidden Markov Models (HMM; Leos-Barajas *et al.* 2017) and neural networks (Jeantet *et*

al. 2022) to reveal behaviours and broad activity/behavioural states. Supervised classification involves synchronizing annotated data segments with the in-situ observation of behaviours or behavioural states, and

Table 2. Ecological applications and data for common sensors used in multi-sensor biologging studies of marine and freshwater turtles.

Sensor	Data	Ecological applications	Publications
Global positioning system (GPS)	Location	Obtains high spatial accuracy to enhance studies of habitat use and movement of freshwater species.	Kydd 2010; Christensen and Chow-Fraser 2014; Hjort Toms *et al.* 2022
Temperature sensor	Ambient temperature	Records ambient temperatures to investigate thermal ecology.	Edge *et al.* 2009; Millar *et al.* 2012
Water sensor	Ambient conductivity	Detects if a turtle is in or out of water; triggers turning on/off of integrated GPS loggers as appropriate.	Wilmers *et al.* 2015
Pressure	Ambient pressure, depth	Records ambient pressure, and when calibrated with a barometer, depths can be extracted and investigated.	Hays *et al.* 2000; Hazel *et al.* 2009; Iverson *et al.* 2019
Tri-axial accelerometer	Animal acceleration	Assesses energy expenditure, activity patterns, and movement to classify behaviours.	Wilson *et al.* 2006; Tyson *et al.* 2017; Jeantet *et al.* 2020; Auge *et al.* 2022
Magnetometer	Heading	Classifies specific behaviours or behavioural states to provide information on geomagnetic cues.	Narazaki *et al.* 2009; Tyson *et al.* 2017; Jeantet *et al.* 2020
Gyroscope	Angular velocity	Provides additional information for behaviour identification.	Tyson *et al.* 2017; Jeantet *et al.* 2020
Video camera	Video or audio	Allows investigator to confirm what animal is doing at the time that sensors are logging activities; also used to estimate distribution and density of prey.	Narazaki *et al.* 2013; Chakravarty *et al.* 2019
Hydrophone	Ambient sound	Records ambient sound and can be used in conjunction with activities and locations to infer specific behaviours	Tyson *et al.* 2017

using the annotated data to create a dataset to train the models. The model can then be used to classify the remaining raw data. These classification approaches allow scientists to obtain a more accurate estimation of time allocation to certain activity states or behaviours (e.g., being active, resting, etc.) (Auge *et al.* 2022), information which is often limited by the temporal resolution of the data, separability among states, as well as the variables being classified and quality of classifier training. For example, when accelerometer data were recorded at exceptionally fine scales (>20hz), coupled with robust training data and a robust classification model, the reproductive outputs (number of eggs laid) from green sea turtles (*Chelonia mydas*) could be estimated with high accuracy (95%) (Jeantet *et al.* 2022). Such classification models can be used to address hard-to-observe behaviours, such as estimating the frequency of nesting attempts by gravid females (Marchand *et al.* 2021). To accomplish this with traditional approaches would have required an investigator to continuously observe the turtle through all its nesting attempts, something that is often too time-demanding and may often disturbed the turtle.

Integrating relevant abiotic and biotic covariates directly into classification models, such as with HMM, can help us explore how each covariate potentially drives the behavioural state (Leos-Barajas *et al.* 2017).

This is often completed with an unsupervised classification method, with which the number of behavioural/activity states are selected and the model used. For example, Byrnes *et al.* (2021) used an HMM to measure the change in the probability of sicklefin lemon sharks (*Negaprion acutidens*) being in a specific activity state based on temporal (time of day and tide) and environmental characteristics (temperature).

Despite their exceptional capabilities, multi-sensor biologgers also suffer from limited battery life, which constrains the temporal resolution of the data. Multi-sensor biologgers like the AxyTrek (Technosmart eu, 10g) offer a water sensor to disable GPS logging when individuals are submerged, and a solar panel for recharging, which can help conserve battery power. Nevertheless, investigators must be prepared to limit the number of sensors and frequency of data collection. Additionally, multi-sensor biologging devices are 3 to 4-fold more expensive than standard radio telemetry transmitters but can be priced lower than some single-sensor GPS loggers (Table 3).

Table 3. Comparison of methods for monitoring Blanding's turtle movement, habitat use and behaviour for populations and individuals with attached devices.

Parameter	Radio telemetry	Single-sensor Biologger	Multi-sensor Biologger
Time of data acquisition	Immediately upon relocation	Upon device removal/download	Upon device removal/download
Volume of data	Low (Manual relocations)	Medium (Programmable remote relocations)	Very high (programable remote relocations and collection of other sensor data at programable frequencies)
Battery Life	12-30 mo	20-36 mo	Hours to ~5 m [a]
Cost to researchers (Units only; CAD)	~ $250 AI-2F [b] (Holohil systems Ltd.)	~ $ 1700 W510 [b] Wildlink GPS logger (Advanced Telemetry Systems)	~ $1000 AxyTrek (Technosmart)

[a] Rechargeable battery whose depletion depends on device configuration and solar radiation.
[b] Units can be refurbished at a reduced cost (company/model dependent).

The weight of the device is another factor that needs to be considered. Animal Use Protocols often dictate that the total weight of all devices must not exceed 5% of the body mass of turtles (CCAC 2023). The type of biologgers (either single or multi-sensor) discussed earlier often must be retrieved before data can be downloaded, and therefore we must also account for the weight of radio transmitters that need to be attached (or in some cases integrated into the sensor) as a method of relocating individuals for device retrieval. While some devices weighing less than 10 g are easy to use on spotted turtles, painted turtles (*Chrysemys picta*) and Blanding's turtles (Auge *et al.* 2022; Hjort Toms *et al.* 2022), it may be impractical to include additional sensors such as hydrophones (Tyson *et al.* 2017) or video cameras (Narazaki *et al.* 2009) on anything smaller than common snapping turtles (*Chelydra serpentina*). These latter devices will require further miniaturization for inclusion in studies of smaller-bodied freshwater turtles.

New tools to detect habitat occupancy

One of the most important pieces of information required to develop recovery plans for at-risk freshwater turtles is the confirmed occupancy of the species within the study area. As discussed earlier, traditional occupancy detection methods such as VES (Table 1) have several limitations, including low detection rates, the prohibitive cost in terms of salaries and time associated with conducting intensive sampling (Table 4), and handling disturbances associated with trapping and releasing turtles (Rees *et al.* 2014). Recent advancements in barcoding show how DNA can be obtained directly from environmental samples to analyze the diversity of organisms within the environment (Willerslev *et al.* 2003). Furthermore, this non-invasive approach has the potential for monitoring rare, at-risk species more rapidly and at a lower cost (Table 4).

Table 4. Comparison of Visual Encounter Surveys (VES), use of hoop nets and analysis of environmental DNA (eDNA) to confirm turtle occupancy. Information is based on the number of people required to conduct fieldwork at an hourly wage of $15.60, the cost of field equipment, and approximate analytical costs (adapted from Davy *et al.* 2015).

Parameter	VES	Hoop nets	eDNA
Length of time involved	Varies widely (10 to 410 hrs)	Varies widely (10 to 669 hrs)	Approximately 12 hrs from sample collection to result [a]
Relative stress to animal [b]	Low	High	None-Low [c]
Cost to Researchers (CAD)	Varies widely ($156 to $6,396)	Varies widely ($156 to $10,436)	Estimated $500 for a single species detection [d]

[a] Samples can be preserved and processed at a later time.
[b] Defined by the disturbance caused by the presence of researchers and the time required for capture and handling.
[c] Varies based on protocol and the number/location of sample collected.
[d] Assumes that the sample is being analyzed by a commercial lab.

Environmental DNA

Environmental DNA consists of short DNA fragments released into the environment by organisms and can include secreted urine, feces, mucous, shed skin cells, and dead carcasses. Environmental DNA can be extracted from environmental samples, including water, soil, or sediment (Ficetola *et al.* 2008) and can be analyzed with either a species-specific approach or with eDNA metabarcoding, a multispecies approach that can detect the presence of species assemblages without prior knowledge (Rees *et al.* 2014). Next-generation sequencing techniques, such as high-throughput DNA metabarcoding, have allowed researchers to detect entire faunas using environmental samples (Thomsen *et al.* 2011). Species-specific usages of eDNA analysis include surveying for endangered species (Jerde *et al.* 2011; Wilcox *et al.* 2013), detecting invasive species (Dejean *et al.* 2012; Ficetola *et al.* 2008; Jerde *et al.* 2011), as well as routine population monitoring of a target species. Multiple studies have used this approach to detect the genetic material of many different species, including amphibians (Ficetola *et al.* 2008; Dejean *et al.* 2011; Goldberg *et al.* 2011; Thomsen *et al.* 2012), reptiles (Piaggio *et al.* 2013; Davy *et al.* 2015; Akre *et al.* 2019), fish (Dejean *et al.* 2011; Jerde *et al.* 2011; Thomsen *et al.* 2012; Wilcox *et al.* 2013), and mammals (Foote *et al.* 2012; Thomsen *et al.* 2012).

Given that eDNA analyses are new, there are diverse protocols to detect the occupancy of reptiles, but researchers have begun to develop standardized protocols for the basic steps involved in eDNA sample

analysis. The 7 basic steps include sample collection, DNA capture, sample preservation, extraction, amplification, sequencing, and species identification (Figure 1). The most common approach to detect the occupancy of reptiles is to use species-specific short fragments of mitochondrial DNA markers, mostly from the cytochrome b gene and amplify these with polymerase chain reactions (PCR).

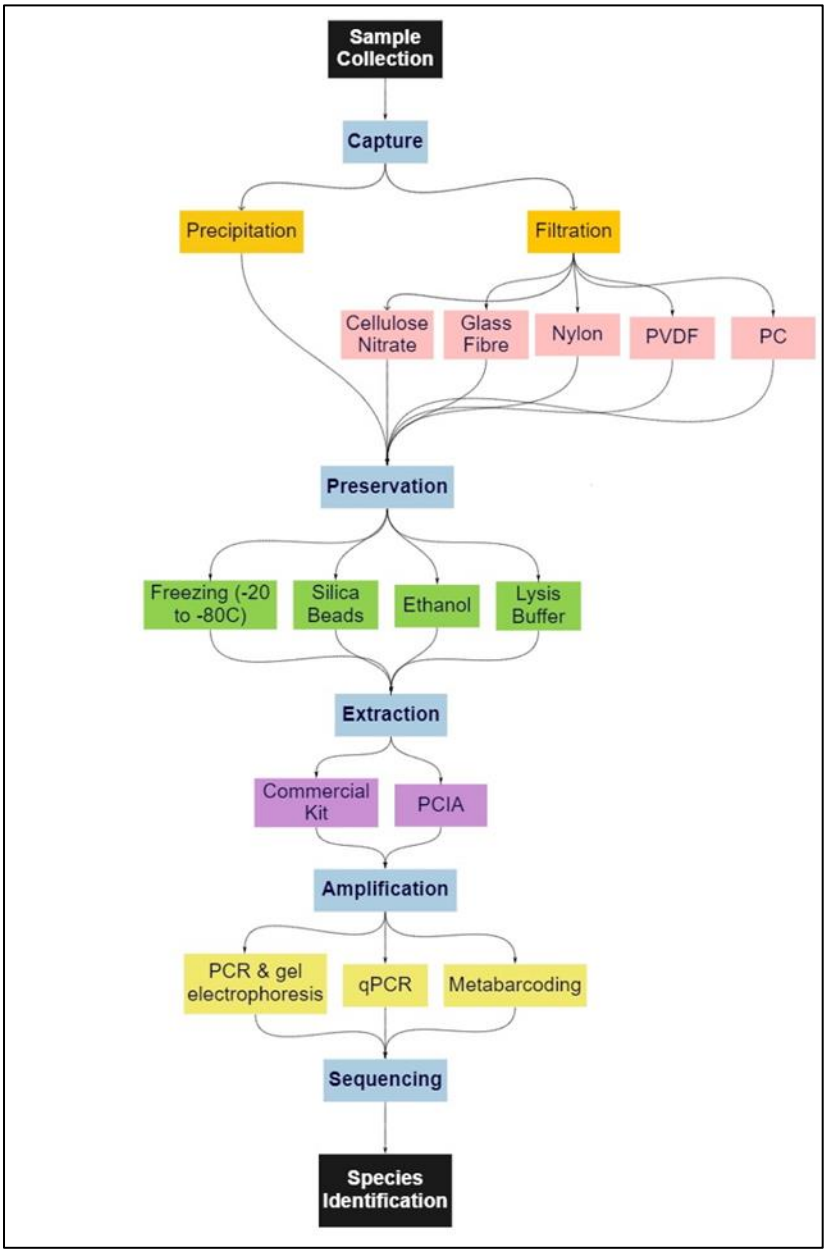

Figure 1. Schematic of common eDNA methods for capture, preservation, extraction, and amplification for monitoring reptiles and amphibians. Abbreviations: polyvinylidene fluoride (PVDF), polycarbonate (PC), phenol-chloroform isoamyl alcohol (PCIA), quantitative polymerase chain reactions (qPCR).

Sample collection

Environmental samples for eDNA studies have been collected by peristaltic pumps to evenly pump water through a filter (Akre *et al.* 2019; Goldberg *et al.* 2011; Feng *et al.* 2020), while others have used syringes (Buxton *et al.* 2017), vacuum pumps (Kessler *et al.*, 2019), manual hand pumps (Goldberg *et al.* 2018), commercial eDNA samplers such as Smith-Root (Tarof *et al.* 2021), large glass jars (Adams *et al.* 2019; De Souza *et al.* 2016), or single-use sampling scoops (Vimercati *et al.* 2020). The amount of water collected can range from 100 ml of water (Vimercati *et al.* 2020) to 10-l buckets of water (Goldberg *et al.* 2011).

Single-use field sampling equipment can help reduce the risk of contamination but are costly and wasteful. To reduce waste and cost, reusable sampling equipment such as glass jars can be used, but they must be decontaminated with a 50% bleach solution between samples (Goldberg *et al.* 2016). The number of environmental samples taken, the number of replicates, and the distance between each sample will depend on the study system; lentic (e.g., wetlands, ponds, lakes) and lotic freshwater systems (e.g., streams or rivers) require different protocols (Akre *et al.* 2019; Feng *et al.* 2020). Separate negative controls at each stage are also usually included in eDNA studies to determine the confidence level of results and to reject the possibility of contamination at every step.

DNA capture

Precipitation and filtration are the 2 most common methods of capturing DNA from environmental samples such as soil or water (Ficetola *et al.* 2008; Goldberg *et al.* 2011). For filtration, volumes of water (0.25 to 10.0 l) are filtered through filters (pore sizes from 0.4 to 10 μm) made of cellulose nitrate (Goldberg *et al.* 2011), glass fibres (Jerde *et al.* 2011), nylon (Thomsen *et al.* 2012), polyvinylidene fluoride (PVDF) (Brys *et al.* 2020) or polycarbonate (PC) (Takahara *et al.* 2012) (Figure 1). For precipitation, sodium acetate and ethanol are added to small (< 100 ml) aliquots of sample water and stored in a -20ºC freezer. The precipitated DNA can then be centrifuged for recovery (Ficetola *et al.* 2008). The most common method for capturing eDNA to monitor reptiles is often filtration through a cellulose nitrate filter, with pore sizes ranging from 0.4 to 1.0 μm. Filters made from cellulose nitrate often outperformed other filter materials in terms of both the cost and efficiency of DNA capture (Liang and Keeley 2013; Hinlo *et al.* 2017). Peixoto *et al.* (2021) found that the use of capsule or disc filters outperformed precipitation for water samples and detected higher amounts of captured eDNA of the amphibian species *Salamandra salamandra* than with regular filters. Brys *et al.* (2020) and Vimercati *et al.* (2020) used filter capsules over the more popular choice of filter discs. 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.* 2016). When sampling in wetlands, investigators should avoid using filters with the smallest pore size since the sample is likely to contain algae and suspended solids that will clog the filters (Tsuji *et al.* 2019). Therefore, the choice of pore size should be appropriate for the system under study and should be advised by pilot studies conducted under similar conditions (e.g., Goldberg *et al.* 2012).

Preservation

The captured eDNA on a filter should be preserved with 1 or a combination of techniques, such as freezing them at -20°C to -80°C (Takahara *et al.* 2012), refrigeration at 0°C to -4°C (Osathanukul and Minamoto 2021), immersion in ethanol (Goldberg *et al.* 2012), in cell lysis buffer (Renshaw *et al.* 2015), or even keeping them dry in a sealed bag with silica gel beads (Allison *et al.* 2021). Addition of a buffer (either ethanol or a cell lysis buffer) immediately after collection is 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).

Extraction

DNA extraction is most often performed with a commercial kit such as the DNeasy Blood and Tissue Kit (Qiagen, Germany), M1 Sample DNA Extraction Kit (Biomeme, USA), or PowerWater DNA Isolation Kit

(MO BIO, USA). Another common method of extraction is a modified phenol-chloroform isoamyl alcohol (PCIA) technique (Sambrook *et al.* 1998), which involves using lysis buffers, or sodium dodecyl sulphate and proteinase K to enzymatically digest proteins and non-nucleic cellular components (McKiernan *et al.* 2017). Qiagen's DNeasy Kit for DNA extraction has been the preferred extraction method in both lentic and lotic systems (Deiner *et al.* 2015; Djurhuus *et al.* 2017; Hinlo *et al.* 2017).

Amplification, Sequencing and Species Identification

Following extraction, the recovered eDNA is amplified with either the classic polymerase chain reaction (PCR) analysis and gel electrophoresis or with quantitative real-time PCR (qPCR) analysis for species-specific detection. Metabarcoding, on the other hand, amplifies universal primers with sufficient variation that enables identification across multiple taxa to identify the biodiversity of entire ecosystems (Thomsen *et al.* 2012) and can estimate intraspecific genetic diversity (Elbrecht *et al.* 2018).

The probability of detecting occupancy of turtles using eDNA depends on many abiotic and biotic factors (Table 5), including the likelihood of eDNA persisting in source waters, how water samples are handled and analyzed, the concentration of eDNA, sampling methods, extraction protocols, capture efficiency, inhibition probability, contamination, and assay sensitivity y (Goldberg *et al.* 2016; Schmidt *et al.* 2013).

Table 5. Abiotic and biotic factors that can affect eDNA accuracy. Adapted from Tarof *et al.* (2021).

Category	Factors affecting accuracy
eDNA and species ecology	- Species life history stage (sexual maturity) - Organism size and mass - The activity level of the organism - Keratinized integument vs mucous membranes - Time spent in a location - UV light exposure - Water temperature and pH - eDNA fragment size - Microbial or enzymatic activity - Open or closed system - Water flow and depth - Habitat size and distance from the source
Field sampling design	- Time of sampling - Volume of sample - Number of replicates - Sampling from sediment vs topwater - Filter type and pore size - Sampling equipment malfunctioning (clogging, freezing) - Water temperature, pH, turbidity, and conductivity - Sampling during extreme weather (rain, winter, etc.), - eDNA degradation during transport - Contamination of sample - Interference in sample
Molecular analysis	- Sample storage - eDNA extraction method - Number of PCR replicates - PCR method - Assay specificity and optimization - PCR inhibition - Lack of controls and contamination

Given that different eDNA capture and extraction protocols can 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). Although short DNA fragments have been shown to persist in dark, dry, cold environments for a very long time (even 10,000-year-old DNA extracted from sediment and amplified to observe extinct vertebrates; Willerslev *et al.* 2003), Pereira *et al.* (2010) demonstrated that greater upstream UV exposure or eDNA significantly decreased the detection of alligator snapping turtles (*Macrochelys temminckii*) in water samples. In addition to UV light, increased degradation and dispersion of eDNA associated with acidic conditions (pH < 5) and warm water temperatures (>25°C) can also decrease the efficiency and accuracy of detecting occupancy of amphibians in wetlands (Goldberg *et al.* 2018).

Spatial and temporal variability are important considerations when designing an effective eDNA sampling program. Goldberg *et al.* (2018) showed that sampling at multiple locations within a single wetland was crucial to achieving accurate detection of amphibians in acidic environments. They also determined that the maximum detection distance from an organism was inversely related to the dispersion and degradation rate of DNA; therefore, Goldberg *et al.* (2018) determined that samples should be collected at least every 60 m within wetlands. A fine-scale sampling design has the potential to reveal spatial distribution patterns of viable eDNA and an abundance of amphibians in a lotic system (Brys *et al.* 2020). Buxton *et al.* (2017) also demonstrated that eDNA detection rates were likely to vary seasonally because greater activity by animals during the summer can increase eDNA concentrations. During the breeding season of the great crested newt (*Triturus cristatus*), eDNA concentration increased when compared to the levels measured during the non-breeding season (Buxton *et al.* 2017).

Since eDNA in surface waters degrades rapidly (a few hours to a few days) when detected, they are an accurate indication of recent species occupancy (Thomsen *et al.* 2012). However, eDNA in sediment can last much longer and in higher concentrations and can 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). Based on this, sampling eDNA from surface water provides a more accurate reflection of recent species occupancy than sampling in sediment. By contrast, floods and heavy rainfall can produce false negatives (when species is present but cannot be detected) due to the dilution of eDNA concentration (Curtis *et al.* 2021). This can also occur in lotic systems, as streams with high flow rates were associated with decreased eDNA yields from the invasive freshwater clam (*Corbicula fluminea*) (Curtis *et al.* 2021). Kessler *et al.* (2019) also found that the detection of alligator snapping turtle eDNA was accurate for the stretch of stream within 1 km of the sampling site, but the size of this buffer depended on streamflow rates, target species, and other environmental conditions.

The primary approach to validate the use of eDNA is to initially ensure that the primer accurately detect individual when they are present (Kirtane *et al.* 2019), and subsequently, to compare this method with traditional approaches (Akre *et al.* 2019). A meta-analysis conducted by Fediajevaite *et al.* (2021) indicated that, in cases of direct comparison, eDNA methods exhibit greater accuracy and efficiency than traditional survey methods for most taxa, with the exception of reptiles and annelids. These differences may be the result of species-specific shedding rate of eDNA, disproportionate research efforts or attributed to differences in habitat conditions. Adams *et al.* (2019) formulated the "shedding hypothesis," which predicts that organisms with keratinized integuments (e.g., non-avian reptiles) would shed eDNA at a lower rate than organisms with mucous integuments (e.g., amphibians). While there are very few studies that focus directly compare traditional and eDNA methods for turtles, Akre *et al.* (2019) using concurrent VES surveys and eDNA sampling, found that the methods were directly comparable for estimating wood turtle occupancy in streams and at half the cost.

New tools to identify individuals

To assess population trends and the health of turtles, investigators must be able to identify animals individually (Seber 1965; Table 1). Common methods for unique identification include marking individuals by shell notching (Cagle 1939), passive integrated transponder (PIT) tags (Buhlmann and Tuberville 1998), or leg bands (Marion and Shamis 1977), which are all designed to be permanent identifiers. Nevertheless, shell notches may be damaged through time or may be misread, while PIT tags can become damaged, lost, or the tags may migrate in the tissue (Feldheim *et al.* 2002; Wyneken *et al.* 2010). Beyond these limitations, the techniques are also invasive and may subject turtles to an unknown degree of pain or risk of infection. An emerging non-invasive approach, used in conjunction with traditional methods, involves the use of unique colour patterns on a turtle's shell to identify individuals when traditional methods may be obscured or removed. This approach involves taking photographs of the turtle's plastron (ventral shell) when they are captured or encountered in field surveys for subsequent comparison with previous images. The matching of plastron patterns to a photo catalogue has been used on several freshwater turtle species, including eastern box (*Terrapene carolina carolina*) (Cross *et al.* 2014), western painted (*Chrysemys picta bellii*) (Cooley *et al.* 2013), red-eared slider (*Trachemys scripta elegans*) (Janzen *et al.* 2000), Rio Grande cooter (*Pseudemys gorzugi*) (Suriyamongkol and Mali 2018), spotted turtles (Hickey and Chow-Fraser, unpublished data), and the common snapping turtle (Kolbe and Janzen 2001).

Traditionally, investigators identified the colour patterns visually using catalogues of photographs, but this is time-consuming and may not be accurate when there are too many photographs in the catalogue (Jackson *et al.* 2006). The software package I^3S (Interactive Individual Identification System; http://www.reijns.com/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 is based on key point extraction, reference points and key point comparison. To help recognize individual characteristics within a pattern, I^3S automatically extracts a set number of key points within a region of interest (Figure 2). This region of interest is determined by 3 set reference points that are visible and consistent for all individuals. The reference points help correct for differences in viewing angle, rotation, and scaling. With reference points selected, various photos can be compared within the same 2D coordinate system. Key point pairs are matched if the nearest key point is at a sufficient distance from the current match. 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. The distance metric is used to be able 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 any other valuable information you may want to store. Unpublished data from Hickey and Chow-Fraser showed that using customized parameters and the simple evaluation tool in I^3S Pattern led to a 90.56% probability of identifying the correct individual turtle and an identification accuracy of 97.42% within the top 3 suggested turtle matches.

Another similar software is Wild-ID (Bolger *et al.* 2012), a stand-alone, open-source software that uses a Scale Invariant Feature Transform Operator (SIFT) to find distinctive features (Lowe 2004). The major stages of the software include (*i*) a grey-scale space extrema detection which detects small changes in the grey-scale; (*ii*) Taylor expansions (an infinite sum of terms expressed in terms of the derivatives at a single point) are used to interpolate subpixel locations for key point localization; (*iii*) dominant orientations are assigned to each key point according to the pixel intensity gradient around the point; and (*iv*) additional local gradients are measured at the selected scale surrounding each key point to generate a key point descriptor. Wild-ID then uses a modified version of the random sample consensus (RANSAC) algorithm (Fischler and Bolles 1981) which measures outliers in a data set and identifies model parameters to find geometrically consistent matches. The goodness-of-fit between images is assessed, and Wild-ID assigns a score based on a scale from 0.0000 to 1.0000, where 1.0000 indicates a strong match. Cross *et al.* (2014) found that Wild-ID was able to identify individual Box turtles correctly every time and did not mismatch turtles from different states or sampling locations.

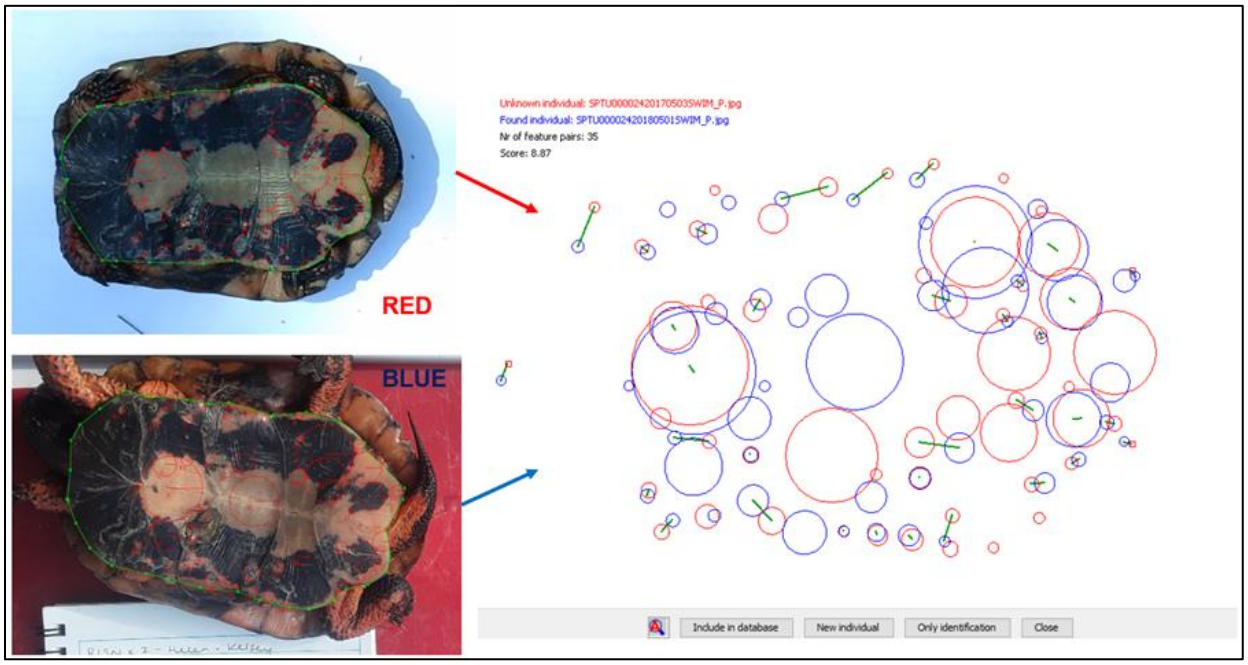

Figure 2. Key point extraction and comparison between 2 photos of the same spotted turtle individual using the software I^{3}S Pattern.

While this tool has found applications across multiple species of freshwater turtles, it is important to acknowledge its inherent limitations for use with all species or as a replacement for traditional techniques. Some turtle species, such as midland painted turtle (*Chrysemys picta marginata*) and Rio Grande cooter, exhibit distinct plastron patterns when individuals are young; however, as growth occurs the pattern becomes obscured and can result in lower accuracy for when comparing photos at lengths greater than a year (Suriyamongkol and Mali 2018). Some freshwater turtle species such as the spotted turtle's patterns do not fully develop until they are sub-adults (Gray 2008). Therefore, this method cannot be accurately used on juvenile spotted turtles. Some species such as the common snapping turtles lack distinct individual patterns, and this methodology would be less applicable to such species. This suggests that it may be best to limit the use of this methodology to species that have distinct, consistent patters across multiple years.

Additionally, limitations can occur as the result of the environmental conditions or species-specific behaviours. It has been documented that turtle's plastrons can be stained by tannins and other deposits from their aquatic environment (Wright and Andrews 2002; Markle *et al.* 2021), which can result changes in their integument colouration, particularly in areas with high concentrations of these substances (Surasinghe *et al.* 2019). In cases where turtles have experienced heavy staining from their environment, the reliability of the pattern recognition software is reduced (Markle *et al.* 2021). If environments are highly productive, there may also be an accumulation of algae on an individual's plastron (Beau and Brischoux 2021), possibly resulting in additional stress for animals when cleaning the surface prior to photographing. Despite the limitations of this method, coordinated image-identification databases have the potential to enhance the accuracy of individual identification of freshwater turtles at a low to no cost.

Using the new toolkit to improve the study of Blanding's turtles

Traditional tracking methods, such as radio telemetry, have been invaluable for studying the ecology of Blanding's turtles. Early investigators described Blanding's turtles as being strictly terrestrial (Garman 1892), and later described as primarily aquatic, with occasional forays onto land in the spring and fall (Gibbons 1968). Since the early 1990s, radio telemetry has been used extensively across the geographic

range of Blanding's turtles to answer questions about their ecology and behaviour. Radio telemetry has been used to investigate the habitat selection and movement of Blanding's turtles in both natural (Edge *et al.* 2009; Markle and Chow-Fraser 2014) and disturbed sites (Rubin *et al.* 2001), monitoring reintroduction efforts (Carstairs *et al.* 2019), and to investigate nest-site selection (Wilson 1998; Hughes, and Brooks 2006). The inclusion of GPS loggers in research improved the temporal resolution of data and allowed Markle and Chow-Fraser (2014) to capture the nesting migration of 2 gravid females to an upland rocky outcrop, a novel nesting habitat. It also allowed us to obtain more detailed information on Blanding's turtle habitat use than radio telemetry alone (Christensen and Chow-Fraser 2014) and was important in obtaining locations late at night while turtles were nesting.

Blanding's turtles exhibit distinctive behavioural periods (Figure 3). During each period, certain behaviours and activities are expressed more readily to fulfill life history requirements, such as thermoregulation and terrestrial nesting activity (Millar and Blouin-Demers 2011). Beyond this, Blanding's turtles are also diurnal, with increased activity during the day and reduced activity at night (Hjort Toms *et al.* 2022). As ectotherms, ambient temperature also influences behaviour because turtles adjust their behaviour to maintain certain body temperatures required for physiological needs (Millar *et al.* 2012). Using a multi-sensor biologger, these variables can be recorded simultaneously. Multiple sensors measure the ambient environment around individuals, including temperature, pressure, and conductivity (Table 2). In addition, time of day, season, and habitat type can be derived from the internal clock and the GPS logger.

The type of sensor data collected simultaneously can allow researchers to estimate the drivers of specific activities and behaviours (Patterson *et al.* 2009). Wetland types have differing habitat structures and heterogeneity, which may influence the expression of certain behaviours. Specifically, we are investigating how habitat type and relevant covariates affect the probability of an individual exhibiting specific behavioural states (e.g., resting in water, active out of water). To achieve this, we are collecting sensor data from multiple turtle populations occurring in differing types of habitats, such as coastal marshes, inland fens, and coastal zone of an undisturbed archipelago. This approach could also aid in understanding how species with more cryptic or complex activity periods may be influenced by the abiotic and biotic variables within their environments. Understanding the relationship between animal behaviour and their environments is crucial for comprehending how disturbances to natural environments can affect behavioural states.

Classification of sensor data into behavioural states allows us to record hard-to-observe behaviours such as nesting attempts (Figure 4). Gravid female turtles often attempt to nest in multiple locations to test for nest-site adequacy and ensure offspring success (Hughes and Brooks 2006; Mui *et al.* 2016). Through classifying biologger data to various behavioural states, we plan to quantify the frequency and duration of nesting attempts in different habitat classes. Specifically, we hypothesize that habitat characteristics are important cues of nest-site adequacy to turtles and that females will spend more time attempting to nest in suitable habitats than in unsuitable habitats. This work will increase the knowledge of how individuals use habitat characteristics as cues to initiate nesting attempts leading to increased knowledge for better artificial nest site creation and enhanced protection. This type of research which could not have been achieved with traditional methods, can advance our understanding of Blanding's turtle ecology.

Blanding's turtles in the Great Lakes are currently threatened mostly by habitat loss and fragmentation due to urban development. To protect such species at risk, we must establish monitoring programs and methods to know what habitats they occupy. For elusive freshwater turtles such as the Blanding's and spotted turtles, tracking them requires trained researchers, specialized equipment, and an often prohibitively expensive field budget (Davy *et al.* 2015). Advancements in eDNA methods have offered a non-invasive, less costly and more time-efficient method to detect elusive species at risk that does not require permits (Jerde *et al.* 2011; Thomsen and Willerslev 2012). Researchers in 2 studies have recently confirmed that eDNA can be used to confirm the occupancy of Blanding's turtles in overwintering lake habitats, but we still do not know the efficacy for determining the occupancy in a large number of wetlands under a range

of environmental conditions and during the turtle's active season (Loeza-Quintana *et al.* 2021; Tarof *et al.* 2021). From the previous review of eDNA analysis, it was clear that the sampling system, target species' life history, and environmental conditions should all be taken into consideration when creating protocols to maximize the accuracy of eDNA studies.

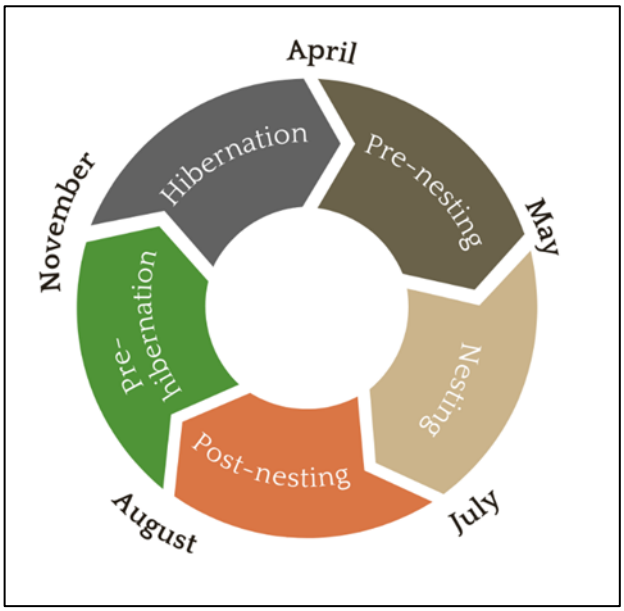

Figure 3. Blanding's turtle distinctive behavioural seasons.

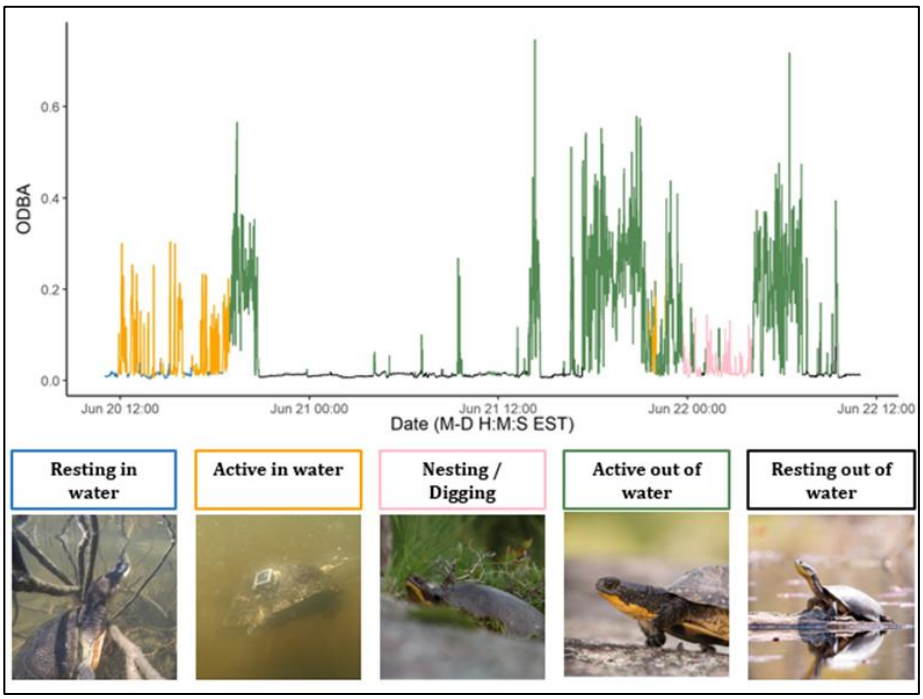

Figure 4. Example of Hidden Markov Model inferred states for a Blanding's turtle between 20-22 June. Behavioural states are classified using a 5-state unsupervised hidden Markov model. Overall dynamic acceleration (ODBA) was averaged over a 1-min period.

We took eDNA samples in 4 study sites in Ontario (Whitefish River First Nations, Moose Deer Point First Nation, Henvey Inlet First Nation, and Rouge National Urban Park) during both inactive and active periods and using 3 basic field sampling protocols. These are 1) grab samples filtered with the OSMOS eDNA sampler (Halltech, Guelph; OSMOS), 2) grab samples filtered with a syringe technique (SYRINGE), and 3) in-situ filtration with the OSMOS eDNA sampler (INSITU). Environmental DNA samples were taken in wetlands with radio telemetry-confirmed Blanding's turtles during 3 different seasons based on Blanding's turtle activity and life cycle; these are the overwintering season (November to March), active season (April to July), and inactive season (August to October).

A larger proportion of samples that positively detected Blanding's turtle eDNA were taken during their active season rather than the overwintering or inactive seasons, suggesting that turtle activity can increase the probability of detection (Hickey and Chow-Fraser, unpublished data). In one trial, we took multiple samples within a single wetland and pooled them before filtration to increase the probability of detection, while decreasing the cost of processing multiple filters. We were also able to prove that eDNA can be used in areas with only juvenile Blanding's turtles. Since freshwater turtles most commonly shed scutes and integuments in small pieces rather than in single cells and are, therefore, more likely to sink to the sediment (Ernst 1971), eDNA from these shed pieces is less likely detectable in samples collected from the water column. Instead, turtles and other animals with hard exteriors may primarily be detected from eDNA consisting of tears, saliva, and excrement, making detection during their active seasons more likely (Adams *et al.* 2019). Studies have successfully, sampled eDNA in the winter; however, often recommend increasing the number of samples in more locations to ensure detection (Tarof *et al.* 2021).

Reliable marking techniques for mark-recapture are essential for the long-term monitoring of freshwater turtle populations and individuals. Photo identification of patterns has been used to identify multiple freshwater turtle species uniquely. Blanding's turtles have unique plastron patterns that are a viable candidate for individual pattern recognition. Markle *et al.*, (2021) used the I^3S Pattern software package to determine if plastron patterns accurately identify individual Blanding's turtles within a distinct study area and among all sampled study areas. For each plastron photo, they identified 3 reference points the top of the plastron where the gular scutes meet and the bottom of the left and right anal scutes. I^3S 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 one. Adjusting the weight values of each channel can help increase the emphasis on distinct colours, subsequently increasing identification accuracy (Markle *et al.* 2021; den Hartog and Reijns 2014). Thirty-five key points were automatically extracted, and each set of key points was used as a fingerprint to compare individual turtles. Markle *et al.* (2021) found an 84% probability of correctly identifying an individual Blanding's turtle within the top 3 suggested matches using I^3S Pattern. Photographic identification of individuals relies on 3 important conditions: (1) an individual's patterns can be photographed while free ranging or after being captured, (2) individuals have patterns on some region of themselves that are variable among individuals, and (3) an individual's pattern is stable throughout the study/survey (Bolger *et al.* 2012).

For long-lived species such as Blanding's turtles, whose patterns do not fully develop until after sexual maturity, pattern-recognition software alone may not be sufficient and should be used in conjunction with additional marking techniques until the validity of the method has been verified (Cross *et al.* 2014). Low image quality, shadows and iron staining on turtle shells will lower the individual identification accuracy (Markle *et al.* 2021). Shell damage is another large potential source of error, especially with large burn scars that destroy generous portions of shell patterns (Cross *et al.* 2014). For adult populations without significant iron staining or burn damage, plastron pattern recognition software offers a cost-effective, non-invasive method to individually identify and monitor freshwater turtle species such as the Blanding's turtle.

Conclusion

We have shown that updating the traditional toolkit with novel techniques has enhanced our understanding of the movements, habitat use, and behaviours of Blanding's turtles and will allow us to delineate and conserve their critical habitats. Specifically, integrating multi-sensor biologgers into research programs has enabled us to classify behavioural patterns, while the use of eDNA has extended the survey season into the fall and winter months. The use of pattern recognition software to identify individuals can reduce stress to individuals as well as provide a probabilistic measure if the notches on scutes have been damaged. While the traditional methods have been widely used for many years, we believe these tools show promise in expanding our knowledge and reducing the effort required to collected data on turtle ecology and behaviours. The use of minimally invasive and non-invasive techniques would not require research permits and should therefore expand the participation of researchers and volunteers in research and monitoring for the effective conservation of freshwater turtles.

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