Article

A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA under field conditions

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Abstract: Environmental DNA (eDNA) is an increasingly used non-invasive molecular tool for detecting species presence and monitoring populations. In this article, we review the current state of non-avian reptile eDNA work in aquatic systems, as well as present a field experiment on detecting the presence of painted turtle (*Chrysemys picta*) eDNA. Thus far, turtle and snake eDNA studies have been successful mostly in detecting the presence of these animals in field conditions. However, some instances of low detection rates and non-detection occur for these non-avian reptiles, especially for squamates. We explored this matter by sampling lentic ponds with different densities (0 kg/ha, 6 kg/ha, 9 kg/ha, and 13 kg/ha) of painted turtles over three months, attempting to detect differences in eDNA accumulation using a qPCR assay. Only one sample of the highest density pond readily amplified eDNA. Yet, estimates of eDNA concentration from pond eDNA were rank-order correlated with turtle density. We present a "shedding hypothesis"–the possibility that animals with hard, keratinized integument do not shed as much DNA as mucus-covered organisms–as a potential challenge for turtle eDNA studies. Despite challenges with eDNA inhibition and availability in water samples, we remain hopeful that eDNA can be used to detect freshwater turtles in the field. We provide key recommendations for biologists wishing to use eDNA methods for detecting non-avian reptiles.

Keywords: Turtle; environmental DNA; eDNA; non-avian reptile; review; eDNA guidelines; *Chrysemys picata*; painted turtle, shedding hypothesis

1. Introduction

Monitoring changes in a target species, such as presence/absence in a given locality, is necessary to model future population trends and may illuminate important life-history traits of an organism [1]. Indeed, changes in population density have downstream demographic effects on range, metapopulation structure, and niche availability [2,3]. Stochastic environmental factors, anthropogenic pressures, or biotic interactions (e.g., disease, intrinsic growth and age class, fecundity, or predation) can change population density [4–8]. Thus, changes in population density can inform

researchers about fluctuations in environmental or biotic conditions. For example, novel habitat created by human activities could increase food resources, thereby expanding the area in which energy requirements can be met [9,10]. Thus, monitoring current species presence and abundance may aid in predicting future densities.

1.1. Environmental DNA and its uses

Central to population monitoring is the need for a sensitive detection method. Recently, environmental DNA (eDNA) has received attention for being able to sensitively reveal the presence of target species, especially where traditional methods fall short [11,12]. We adopt Taberlet's (2018) definition of eDNA as DNA extracted from environmental samples such as soil, water, air, and feces [13]. Thus far, eDNA techniques have been applied to many environmental contexts, including leaf litter, soil, and air. Although eDNA has been used to examine alpha and beta diversity through metabarcoding (the use of "universal" primers to detect the presence of multiple taxa [14–19]), eDNA has also been employed to detect single-species presence (e.g., DNA collected for target species from water samples). Single-species eDNA techniques can be used widely, mainly because of the sensitivity of eDNA methodology, compared with traditional methods [18,20,21]. Even so, how eDNA is shed, degrades, travels, and interacts within specific environments varies with target species and specific ecosystem (e.g., lentic vs lotic freshwater), thus methods continue to be refined for obtaining eDNA in a variety of habitats [22–25].

Focusing on aquatic systems, single-species eDNA has been used in two main ways for conservation: detecting invasive species and monitoring threatened species. Invasive species cause environmental, ecological, and economic damage, incentivizing prevention and early detection [26,27]. In some studies, eDNA is sensitive enough to detect the forefront of an invasion [28–30]. Knowing the range limits of the invasion can help reduce the cost of mitigation efforts. For example, Asian carp (*Hypophthalmichthys* sp.) were one of the first targets for extensive eDNA monitoring of an invasive species [29]. The presence of invasive carp was detected along a Chicago area waterway above the previously defined invasion front [29]. Successful application of eDNA techniques allows

carp behavior to be followed more easily than via traditional monitoring approaches; thus, eDNA tools continue to be refined and used to inform monitoring efforts in the Great Lakes system for multiple invasive carp species [31–37]. Because of the headway made in invasive carp biomonitoring, many other biological invasions have been detected using eDNA. Since then, many invasive fish have been targeted, and eDNA has been used for determining the efficiency of fish eradication efforts [38–43]. Amphibian species have also been targeted [30,44,45], as have crustaceans [41,46–49], reptiles [50,51], and molluscs [19,52,53]. The rapid adoption of eDNA for invasive aquatic species paved the way for developing eDNA-based tools for other systems and continues to motivate advancing this method for further genetic monitoring.

Another exponentially growing use for eDNA in aquatic systems is the detection of endangered and secretive taxa [20,54–60]. Many endangered species presences have been identified in this way, including in areas where presence had not been confirmed using traditional methods [11,61–64]. Endangered species distribution and migrations also have been monitored using eDNA [60,65,66], and seasonal spikes in eDNA may indicate spawning [62,67]. Recently, eDNA-obtained haplotypes for endangered species have helped identify relatedness between populations [68–70]. This non-invasive technique may even require fewer sampling permits compared to traditional methods, which can be difficult to obtain for protected species [71]. These benefits of eDNA detection could provide managers with important information on population presence, thereby aiding initial monitoring and conservation efforts.

Not surprisingly, eDNA could be both effective and useful for monitoring aquatic species in general [72]. One reason for increased efficiency is that eDNA can take fewer person-hours to sample biodiversity in a given area, as samples are easily obtained. One extreme example is when the presence of invasive carp was detected with eDNA, prompting 93 person-days of effort to find one individual carp using electrofishing [29]. The sensitivity of eDNA tools allows managers to target sites flagged by positive eDNA detection for more intensive sampling. Furthermore, eDNA does not harm target organisms (e.g., electrofishing may harm fish if used improperly) [73]. Other examples

include a 67% cost reduction and lower sampling effort for detecting fish species with eDNA, compared to triple-pass electrofishing [40, but see 74]. The ease of collecting samples has also enabled community science projects [75]. Genetic methods offer an advantage for identifying cryptic target species or species with small larval stages, which may be difficult even for expert taxonomists to identify[76–78]. Given the efficiency, cost, and analytical advantages, eDNA is an attractive tool for detecting species presence.

1.2. Environmental DNA limitations

Although monitoring populations with eDNA methods has clear benefits, the utility of the information obtained from eDNA surveys beyond detecting species presence currently has limits. No clear relationship seems to exist between organism biomass, density, or count and eDNA abundance in a field setting [79,80]. Many measures of diversity (e.g., most biodiversity indices) require abundance measurements, not simply presence [81]. Biomass can correlate with both sequence reads and eDNA copy number/concentration, but these relationships may be species- and ecosystem-specific [82–85]. Wide confidence intervals on quantification models can yield unreliable estimates [86,87]. In addition, DNA may be shed at varying rates between individuals, diet, breeding season, and life stage [34,80]. For example, at least one male hellbender (*Cryptobranchus alleganiensis*) shed more eDNA during its mating season month than during other months [62]. Overall, variability among species and individuals – mediated by environmental factors – can cloud our ability to relate eDNA systematically to population or individual measures.

Some technical and ecological considerations are required when using eDNA methods to detect species presence, since organisms will not be observed directly. More specifically, false positives and false negatives must be carefully considered [28,88,89]. Because the organism itself is not sampled, false positives may occur when a target is not truly present [90]. Negative controls throughout the eDNA sampling, extraction, and amplification process can help signal where contamination may occur [28,91]. Biologically, false positives may also occur when a signal is detected but comes from a nonviable source, such as eDNA from a decaying organism or eDNA from the gastrointestinal tract

of a predator [92,93]. Using eDNA methods alone could cause managers to initiate costly management efforts when no action is needed. False negatives, where the target organism is present but goes undetected, are also possible [28]. Small sample size, insufficient replication, or lack of a sufficiently large sampling area can contribute to non-detection [12,89,90,94]. Employing a targeted sampling design and species-specific PCR primers may increase the chance of species detection [80].

Increasingly, eDNA studies incorporate occupancy and species distribution models to robustly confirm detection and mitigate false positives and negatives [56,95,96]. Like other sampling methods, eDNA techniques can detect presence, whereas absence can never be detected. Therefore, species occupancy modeling is used to determine the number of samples needed to have high (95%) confidence of a true absence [97]. This probability can never be zero, but it can be minimized with a high number of replicates and extensive sampling design coverage [91,95]. Species-distribution models also can use information gathered from eDNA to determine the probability of presence [96]. Confidence in detection is essential, especially if managed species are targets, thus traditional assessments of eDNA-identified localities may be necessary to confirm presence.

1.3. Sampling Design and Workflow

Sampling design is of paramount importance, as it often has a large impact on the results of aquatic eDNA studies [98–101]. The biology of target organisms, water flow, and experimental design can affect eDNA signal strength [22,37,102]. For instance, benthic marine species are best detected with methods that target sediment and the lower water column, not surface water [41,56]. Furthermore, riverine systems may transport eDNA downstream from the actual location of target species, which must be considered when designing sample sites and interpreting results [102–105]. The numbers of samples and replicates obtained directly affect occupancy probability (e.g., a large number of replicates will likely yield higher detection probability) [89,106,107]. Larger volumes of water and filter size also may increase probability of eDNA capture [108].

Extraction methodologies have been tested extensively, but may still require tailoring and troubleshooting for particular systems. Shorter times between sample capture, filtration, and

144 extraction minimize eDNA degradation [99,100,109]. Multiple filters of varying material and pore
145 size and with preservation buffers have been tested, each yielding different amounts and qualities of
146 eDNA [100,109]. Numerous extraction techniques have been tested, commonly including variations
147 on the Qiagen Blood and Tissue kit, sodium acetate, or phenol-chloroform-isoamyl (PCI) extraction
148 protocols [54,99,100]. To clean up inhibited samples, a bead step, clean-up kits (e.g., Zymo one-step),
149 or dilution have all been successfully used [38,91,110]. It is possible to lose some extracted eDNA
150 while cleaning samples post-extraction, which may decrease detection of species presence [111].

151 Once extracted, samples are typically amplified with PCR and sequenced to confirm species
152 specificity and presence. In species-specific studies, primers must be sensitive to the species level,
153 often relying on a large number of mismatches between target and closely-related species or specific
154 probes, such as Taqman MGB or FAM probes [112,113]. Mitochondrial DNA (mtDNA) is often
155 chosen because of high copy number availability in the environment and commonality in databases
156 [114]. To simply detect presence, conventional PCR can be used if primers are specific. Beyond
157 presence, eDNA can be quantified via quantitative PCR (qPCR) to detect eDNA copy number in
158 samples [82]. However, for increased sensitivity and absolute quantification, droplet digital PCR
159 (ddPCR) has been used [69,98]. Once DNA is amplified, amplicons can be sequenced with Sanger
160 sequencing or next generation sequencing (e.g. Illumina) methods [115]. Matching eDNA-obtained
161 amplicons to known sequences (e.g., GenBank) confirms species DNA presence in a sample.

162 *1.4. Reptile eDNA*

163 Despite breakthroughs in assessing density in fish and amphibian species, there remains a
164 dearth of studies quantifying aquatic non-avian reptile populations with eDNA under field
165 conditions [116]. This lacuna is notable because turtles are among the most at-risk vertebrates, with
166 over 60% of modern species listed as threatened, endangered, or extinct [117,118]. To our knowledge,
167 most eDNA studies on non-avian reptiles that heavily use aquatic habitats focus on detecting the
168 presence of snakes and turtles (Figure 1). Attempts have also been made to find West African

crocodile (*Crocodylus suchus*) and Nile monitor (*Varanus niloticus*) with eDNA metabarcoding methods, but presence has not yet been detected successfully [119].

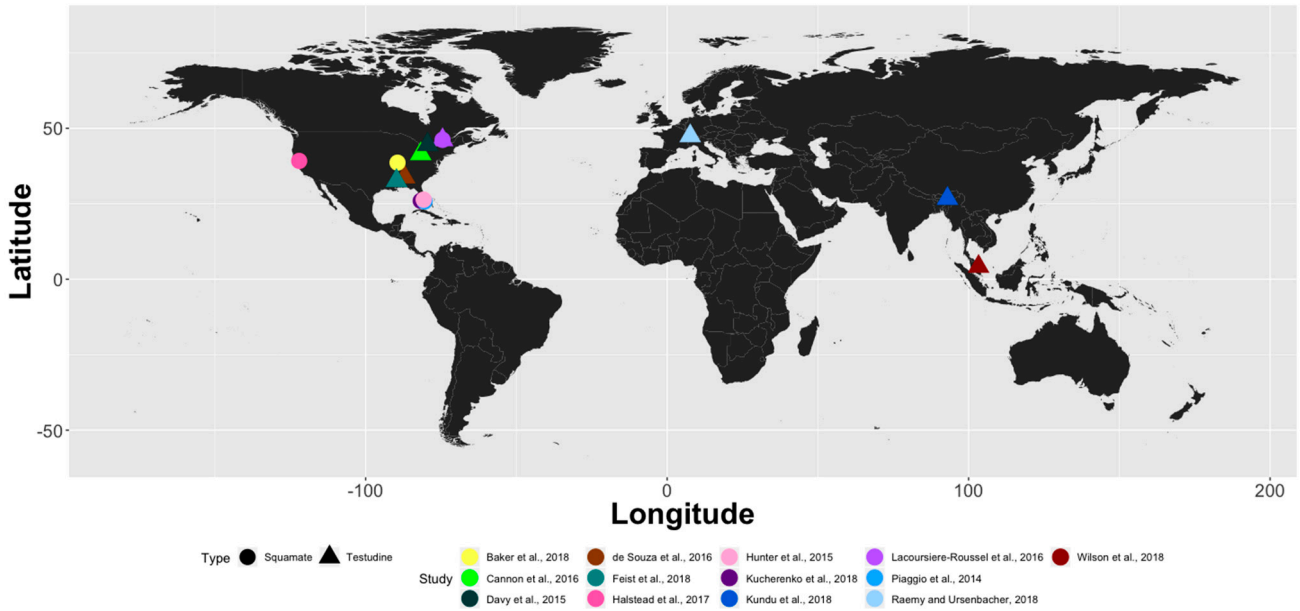

Figure 1. A global map of non-avian reptile studies using eDNA and metabarcoding methods mentioned in this paper. Each color denotes a different study. Circles indicate snake studies and triangles indicate turtle studies. Note that one study, Lacoursière-Roussel et al., 2016 found both snakes and turtles. Antarctica not pictured.

The first notable aquatic reptile eDNA study was on Burmese python (*Python bivittatus*) in south Florida [50]. After successfully detecting python presence from aquatic eDNA using penned snakes, field sites with previously sighted pythons were tested [50]. Field sites yielded positive eDNA detection where *P. bivittatus* had been detected previously, and no eDNA was detected at one site where a python had not been detected previously [50]. Further research detected eDNA in terrestrial samples under field conditions in sites monitored via radio telemetry [50]. Additional aquatic snake studies have focused on the threatened eastern massasauga rattlesnake (*Sistrurus catenatus*) [120]. Water was taken from crayfish burrows, typical *S. catenatus* overwintering refugia, in occupied field sites [120]. Despite known local abundance, only two of 100 environmental samples amplified positively with eDNA, compared to detecting 12 positive snake presences with traditional methods within a 2-m radius [120]. Similarly, giant garter snake (*Thamnophis gigas*) eDNA assays were created

for presence detection [121]. In this study, laboratory experiments detected *T. gigas* presence from skin and feces in water, but not live snakes in water [121]. Despite capturing snakes with traps at field locations, *T. gigas* eDNA was not detected in water at the same sites [121]. With metabarcoding primers, redbelly snake (*Storeria occipitomaculata*), northern watersnake (*Nerodia sipedon*) and milksnake (*Lampropeltis triangulum*) eDNA presence was detected in Canadian lakes and rivers [122]. Overall, results have been mixed for detecting the presence of snakes with eDNA (Table 1) and, to our knowledge, no studies have yet attempted to quantify snake eDNA. It is possible that the more time snakes spend in water, the more likely aquatic eDNA will be able to detect snake presence, however, more research is needed to support this relationship.

Table 1. Studies that include research on snake or turtle environmental DNA in aquatic systems.

Study	Order	Species	Country	Laboratory Detection?	Field Detection?	Consistent Field Detection?
Baker et al., 2018	Squamata	*Sistrurus catenatus*	U.S.A.	-	Yes	No, 2/100 samples amplified with *S. catenatus*.
Cannon et al., 2016	Testudines	*Terrapene carolina*	U.S.A.	-	Yes	2/91 samples amplified from universal "amphibian" primers.
Davy et al., 2015	Testudines	*Emydoidea blandingii, Clemmys guttata, Glyptemys insculpta, Chrysemys picta, Graptemys geographica, Sternotherus odoratus, Chelydra serpentina, Apalone spinifera, Trachemys scripta*	Canada	Yes	Yes	Yes, all PCR replicates of a field sample for *T. scripta* in a local pond. Other turtles not test for in a field setup.
de Souza et al., 2016	Testudines	*Sternotherus depressus*	U.S.A.	Yes	Yes	Yes, four water samples required in the warm season and 14 water samples required in the cold season for a 95% detection probability.

Feist et al., 2018	Testudines	*Macrochelys temminckii*	U.S.A.	Yes	Yes	2/3 to 1/6 replications amplified in the field when amplification occurred.
Halstead et al., 2017	Squamata	*Thamnophis gigas*	U.S.A.	Yes, limited.	No	No, no samples amplified.
Kelly et al., 2014	Testudines	*Chelonia mydas*	U.S.A.	No	-	-
Kucherenko et al., 2018	Squamata	*Pantherophis guttatus, Python bivittatus*	U.S.A.	Yes	Yes	66.7% successful detection rate.
Kundu et al., 2018	Testudines	*Nilssonia nigricans, Nilssonia gangetica, Chitra indica*	India	-	Yes	No information given on how many of the 10 replicates were successful.
Lacoursiere-Roussel et al., 2016	Testudines, Squamata	*Chelydra serpentina, Glyptemys insculpta, Nerodia sipedon, Lampropeltis triangulum, Storeria occipitomaculata*	Canada	Yes	Yes	Yes, targeted qPCR detected wood turtle in 9/9 locations. eDNA metabarcoding detected two turtle species in 3/9 locations, but 4/9 locations did not detect wood turtle otherwise detected with qPCR methodology. Snake species were found in 3/9 locations.
Piaggio et al., 2014	Squamata	*Python bivittatus*	U.S.A.	Yes	Yes	Yes, 5/5 field sites with known presence amplified.
Raemy and Ursenbacher, 2018	Testudines	*Emys orbicularis*	Switzerland	Yes	Yes	3/6 to 6/6 replications amplified in the field when amplification occurred.
Wilson et al., 2018	Testudines	*Batagur affinis*	Malaysia	Yes	Yes	Yes, with live individuals within 1km vicinity of turtle presence.

Previous work has assessed the ability of eDNA to detect presence of aquatic turtle species in a variety of habitats. In a marine aquarium, a green sea turtle (*Chelonia mydas*) was present but not detected when using eDNA metabarcoding methods [123]. Similarly, eDNA assays were developed for multiple captive native Canadian turtles, and eDNA from red-eared slider turtles (*Trachemys scripta*) was successfully detected in a small artificial pond (Table 1) [51]. Additionally, an eDNA

assay was developed to detect alligator snapping turtle (*Macrochelys temminckii*) presence in both lentic and lotic environments in the southeastern USA [124]. In India, several imperiled turtle species (*Chitra indica*, *Nilssonia gangetica*, and *N. nigricans*) were detected in a temple pond using eDNA methodology [125]. In Southeast Asia, the southern river terrapin (*Batagur affinis*) was detected in river samples in Malaysia [126]. This eDNA detection corresponded to the presence of at least one radio-tracked individual within one km (Table 1).

Beyond presence detection, site-occupancy models in slow-flowing streams in the southeastern USA quantified the minimum number of eDNA samples needed to determine presence of the endangered flattened musk turtle (*Sternotherus depressus*) [106]. This study found the warm season (May-September) yielded higher eDNA detection rates for *S. depressus*, which likely corresponds to turtle activity [106]. Four replicate samples were needed in the warm season for a 95% detection probability versus 14 during the cool season. Density dependence of threatened European pond turtles (*Emys orbicularis*) in natural ponds was also investigated using eDNA in Switzerland [127]. No correlation was found between turtle density, number, or biomass and eDNA abundance, although sites with shallow waters and vegetation yielded more turtle eDNA [127]. In Canadian riverine environments, the sensitivity of eDNA detection of at-risk wood turtles (*Glyptemys insculpta*) was tested [122]. With qPCR methodology, presence of *G. insculpta* was detected and correlated with turtle abundance from visual surveys. Furthermore, when using eDNA-metabarcoding methodology and "universal" primers, both *G. insculpta* and common snapping turtles (*Chelydra serpentina*) were detected. However, these metabarcoding methods did not detect *G. insculpta* eDNA in all rivers where qPCR eDNA methods detected this species [122]. Finally, eastern box turtle (*Terrapene carolina*) presence was detected using metabarcoding methods on an Illinois river, though turtle presence was not confirmed with an actual specimen [128]. These studies illustrate successes in detecting turtle eDNA in aquatic systems, indicating promise for using this population monitoring technique in this increasingly imperiled group.

1.5. Painted turtle eDNA case study

At the conception of this experiment in 2015, essentially no turtle eDNA studies had been published (Table 1). Thus, we conducted a field experiment to quantify relationships between turtle density and turtle eDNA over time in a lentic pond system. We used painted turtles (*Chrysemys picta*) as a model because they exist in the same aquatic habitats as multiple endangered turtle species, such as the yellow mud turtle (*Kinosternon flavescens*) and Blanding's turtle (*Emydoidea blandingii*) [129]. We populated semi-natural ponds with varying numbers of adult turtles and correlated painted turtle eDNA in water samples with painted turtle biomass in this enclosed system over a three-month period. We hypothesized the amount of total eDNA and turtle eDNA would linearly increase with time and turtle density. Establishing a relationship between eDNA concentration and turtle density between ponds and throughout time could deliver an eDNA-based monitoring tool for the painted turtle and other imperiled freshwater turtles.

2. Materials and Methods

2.1. Experimental setup and eDNA collection

We seeded four closed-system outdoor ponds with painted turtles at the Iowa State University Horticulture Farm in 2016. These outside, uncovered ponds were natural with respect to abiotic variables and water was not treated in any way. We lined the ponds with black polyethylene laminated tarp and added three white water lily plants (*Nymphaea* sp.) to each pond. Ponds were surrounded by an electric fence, preventing foreign turtles from entering. Although these ponds were the same dimensions (19m L x 15m W x 1.5m D each), they varied in number of adult turtles (0, 11, 23, 38) and initial biomass (0g, 6088g, 9198g, and 12990g, respectively). We labeled these ponds as zero (0 turtles at a density of 0kg/ha), low (11 turtles and a density of 6kg/ha), medium (23 turtles at a density of 9kg/ha) and high (38 turtles at a density of 13kg/ha) density. In North American aquatic systems, painted turtle densities can range between 7.2 and 106 kg/ha [130,131]. Our pond densities most mimic low-density painted turtle populations, as these would most likely be relevant to co-

252 occurring imperiled species. We placed turtles in the ponds on 1 April 2016, which coincides with
253 extensive painted turtle post-hibernation activity [132].

254 We sampled 250mL of water at randomized locations around the perimeter of each pond
255 approximately 0.75m from the edge once every three days starting 1 April through 30 June 2016,
256 which corresponds to Julian days 91 thru 182. To process samples within 48 hours, we chose small
257 water sample volumes due to frequent filter clogging and high turbidity. We took samples in 10%
258 bleach sterilized, autoclaved glass Nalgene jars. When sampling, we used sterile gloves and did not
259 touch the water's edge with our feet to prevent pond-to-pond contamination. We immediately
260 transported samples to Iowa State University, stored them in a 4°C refrigerator, and filtered and
261 extracted DNA within 48 hours. Samples were filtered with 0.45µm cellulose nitrate filters in a room
262 never used for amplifying turtle DNA (however, they were carried to a room with PCR products
263 from past testudine and squamate experiments for extraction and amplification).

264 Painted turtle eDNA was also extracted from laboratory water containing captive turtles for use
265 as an eDNA positive control ("turtle lab water"). Four adult turtles were placed in a bin (0.59 m x 0.42
266 m x 0.27 m, 47L) about 1/3rd full of water for two weeks during their hibernation period. Water was
267 sampled as above on 15 January 2016 and immediately filtered using 0.45µm cellulose nitrate filters
268 in a room never used for amplifying turtle DNA.

269 *2.2. Extraction*

270 We optimized our eDNA protocol by testing multiple published eDNA methods and
271 commercially available extraction kits before settling on the following methods. We processed all
272 samples under a UV-sterilized hood to ensure sterility. We vacuum-filtered water samples through
273 a 0.45µm-pore cellulose nitrate filter. Once filtration was finished, we immediately folded the filter
274 inward and put it into a QIAshredder with 350µL buffer ATL and 25µL proteinase K
275 [19,45,99,133,134]. We then incubated the sample overnight at 65°C [135,136]. After the overnight
276 incubation, we spun down the QIAshredder column for 2min at 14,000 rpm and added 200 µL buffer
277 AL and 200 µL 95% ethanol to the elute. After vortexing, we put the solution into a DNeasy Blood

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and Tissue Kit spin column and spun the sample in a microcentrifuge for 2 min at 14,000 rpm [19]. We followed Qiagen's Manufacturer's instructions starting with the addition of 500 µL Buffer AW1 (step 5) until elution (step 7). We eluted the samples with 200 µL EDTA (low TE) buffer heated to 65°C [51]. We also filtered and extracted three negative laboratory control samples using Culligan Nanopure water in this same way.

2.3. Amplification and quantification

No species-specific qPCR protocol existed at the time of sampling for the painted turtle; therefore, we developed our own. Thermo Fisher Scientific designed a primer-probe combination from painted turtle mtDNA using GenBank Accession numbers KF874616.1, NC_023890.1, NC_002073.3, and AF069423.1. Primer and probe sequence can be ordered using Taqman Assay APMFWY7_C_PICTA_V2 from Thermo Fisher Scientific. These were custom designed to have at least six mismatches over both primers and probe from five other sympatric turtle species (*Chelydra serpentina* (GenBank Accession Numbers EF122793.1, NC_011198), *Trachemys scripta* (GenBank Accession Numbers NC_011573.1, FJ392294.1), *Apalone spinifera* (GenBank Accession Numbers NC_021371.1, JF966197.1), *Graptemys ouachitensis* (GenBank Accession Number JN993985.1 (incomplete mtDNA genome), and *Graptemys geographica* (GenBank Accession Number JN993982.1 (incomplete mtDNA genome)). We tested species-specificity of the primer/probe set by amplifying DNA from blood samples from these five sympatric turtle species. These turtle species and negative controls all yielded quantification cycle (Cq) values ≥5 higher than painted turtle amplification, denoting species specificity [137,138]. Due to cost and time constraints, we ran a subset of our field samples, using samples from all ponds from dates spaced at roughly two-week intervals: 30 March (Julian day 91), 16 April (Julian day 107), 1 May (Julian day 122), 16 May (Julian day 137), 31 May (Julian day 152), 15 June (Julian day 167), and 30 June (Julian day 182).

We performed a qPCR assay composed of 20µL PerfeCTa qPCR ToughMix (Quanta Biosciences, MD), 10µL nanopure water and 2 µL of the Taqman primer/probe reaction mix, and 8µL of 1:4 diluted template for a final reaction volume of 40µL. Reaction conditions were as follows: 10 minutes initial

304 denaturation at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 45 seconds. We ran
305 qPCR reactions in triplicate and averaged the Cq values for each sample. We ran standard curves
306 using DNA extracted from painted turtle blood and painted turtle eDNA from laboratory water in a
307 1:2 dilution series. We ran one sample (31 May, high density pond) alongside these standard curves
308 at the same dilutions. Due to non-linear eDNA amplification likely from inhibitor presence, we chose
309 a 1:4 dilution for all samples [110]. Using more concentrated eDNA consistently failed to improve
310 eDNA amplification, indicating the presence of inhibitors.

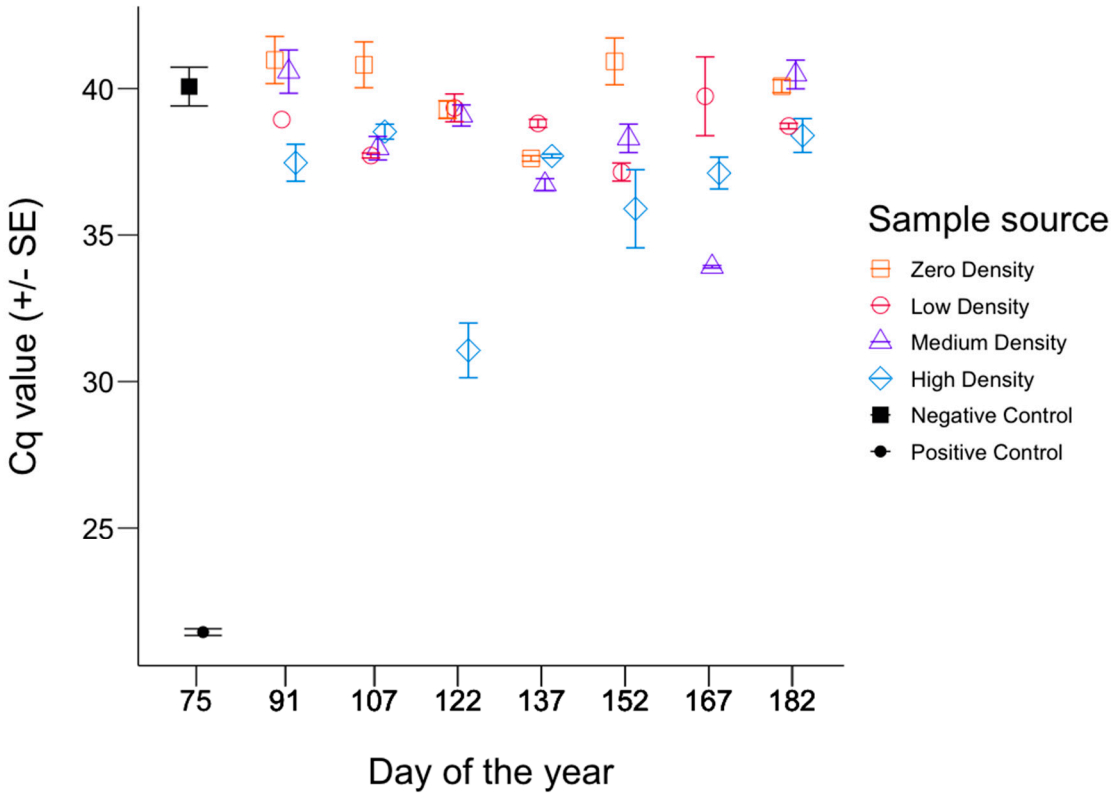
311 We assumed replicates that did not return a Cq value were below detection limit and excluded
312 them from Cq averages, standard deviation (SD), and standard error of the mean (SEM) for the
313 sample. Samples without Cq values also were excluded from future analysis. All qPCR runs
314 contained no template controls in triplicate and all were prepped in a UV-sterilized hood treated with
315 10% bleach. We only considered values <33 Cq to ensure our samples were distinct from background
316 amplification (i.e. turtle DNA amplifying that was not derived from pond samples) [137,138].
317 Assuming exponential amplification, less than one percent (0.95%) of signal contribution would be
318 non-target DNA contribution when efficiency is 100% ($10^{-1/m}$, m = -3.497 = slope of eDNA lab water
319 standard curve, E_{AMP} = 1.932, intercept = 25.888) [137].

320 In addition to assessing absolute Cq values, we examined the ordered trend of lowest Cq value
321 to highest Cq value among ponds and controls, with abundance corresponding to 1/Cq. Thus, we
322 expected the pond with the highest turtle density to have the lowest Cq value followed by ponds
323 with medium, low, and zero densities of turtles. We also included positive controls (DNA extracted
324 from blood and turtle laboratory water) and negative controls, expecting extracts from blood to have
325 the highest concentration of turtle DNA, followed by turtle lab water, and the negative controls. We
326 evaluated the statistical significance of this ordering with Jonckheere's trend test. This test is similar
327 to the Kruskal-Wallis test, but is used specifically to assess a priori ordering hypotheses [139]. Our
328 null hypothesis was that there was no trend order, whereas our alternative hypothesis dictated the
329 following strict trend: turtle blood, turtle laboratory water, high turtle density pond, medium turtle

density pond, low turtle density pond, zero turtle density pond, then negative controls. To perform these tests, we used the packages *ggplot2*, *clinfun* and base R statistical software (version 3.2.3) [140].

3. Results

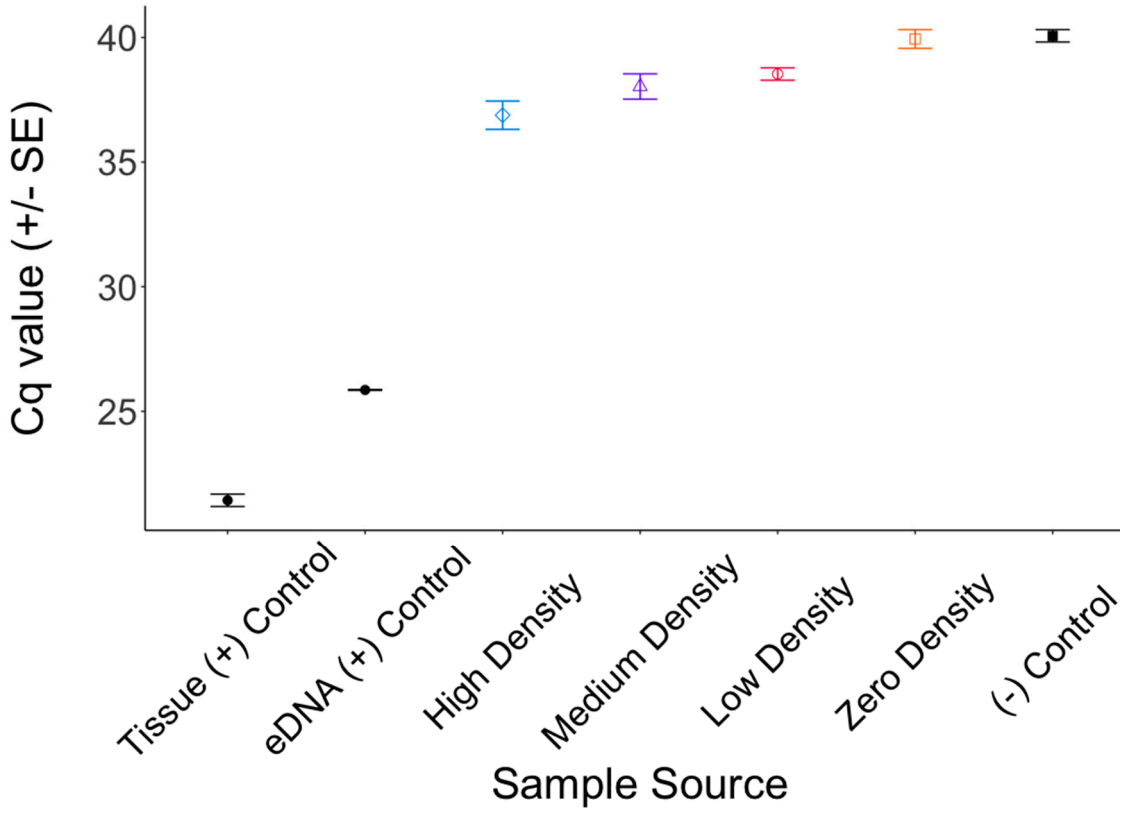
From our qPCR dataset, we obtained 27 Cq sample values from seven sampling days by averaging triplicates. One sample—from the zero-turtle density pond on Julian day 167 —was below our detection limit and did not yield a Cq value. Our negative controls amplified at an average Cq of 40.07 (SD = 0.39, SE = 0.11) and our blood positive control Cq was 21.43 (SD = 0.39; SE = 0.11). Background signal in the negative controls were always detected. The mean of all samples (excluding positive and negative controls) was 38.27 Cq (SD = 0.86; average SE = 0.48). The lowest mean value (i.e. highest eDNA abundance) for any sample was the high turtle density pond on Julian day 122, with 31.06 Cq (SD = 0.39; SE = 0.11). This reading is more than 7 Cq values away from the mean of our negative controls, rendering it able to be considered for analysis [137]. The next highest eDNA abundance was for the medium turtle density pond on Julian day 167, with 33.92 Cq (SD = 0.08; SE = 0.04), which is not more than 7 Cq values away from the negative control and therefore not sufficiently distinguishable from background amplification. Thus, with only one sample meeting detection criteria, we could not statistically analyze individual Cq values (Figure. 2). That we detected background signal, however, indicates our amplification assay was sensitive and that potential turtle-specific eDNA concentrations in our samples were simply too low.



348 **Figure 2.** Amplification (quantification cycle = Cq) of adult painted turtle eDNA as a function of
349 sample source and date. Higher Cq values indicate less eDNA. Varying colors and symbols represent
350 pond treatments: the zero-density pond had 0 turtles (orange squares), the low-density pond had 11
351 turtles (red circles), the medium-density pond had 23 turtles (purple triangles), and the high-density
352 pond had 38 turtles (blue diamonds). Points indicate the average triplicate value of each sample and
353 points are jittered for readability. The positive controls from extracted painted turtle blood and the
354 negative controls were plotted at Julian day 75 to facilitate comparisons. The zero density pond on
355 Julian day 167 failed to amplify, and only one replicate of the low density pond on Julian day 91
356 amplified.

357 Regardless of sample Cq values relative to background amplification, we assessed whether
358 sampled Cq values followed an expected trend of turtle-specific eDNA concentrations. The rank-
359 order obtained for highest to lowest amplification of turtle-specific eDNA was: turtle blood, turtle
360 lab water, high turtle density pond, medium turtle density pond, low turtle density pond, zero
361 turtle density pond, and our negative control (Figure 3). This ranking of turtle-specific eDNA

362 concentrations exactly matched our alternative hypothesis, and Jonckheere's test suggested a
363 meaningful order to these samples ($P < 0.001$).



364 **Figure 3.** Plot of Cq mean per sample source with the standard error of the mean (SE) for painted
365 turtle eDNA from water samples obtained from experimental ponds during the 2016 field season.
366 Higher Cq values indicate lower eDNA. Varying colors and symbols represent pond treatments: the
367 zero-density pond had 0 turtles (orange squares), the low-density pond had 11 turtles (red circles),
368 the medium-density pond had 23 turtles (purple triangles), and the high-density pond had 38 turtles
369 (blue diamonds). See Figure 2 for more information.

370 **4. Discussion**

371 Overall, we could not discern quantitative patterns of painted turtle-specific eDNA in individual
372 samples from semi-natural ponds, indicating potential detection limitations. This result occurred
373 despite known abundances of turtles in the water we sampled and a sensitive qPCR assay. We
374 conclude that our qPCR protocol for painted turtle-specific eDNA did not effectively detect turtles or
375 quantify turtle density, because only 1 of 27 field samples amplified substantial turtle-specific eDNA

376 (the high-density pond on Julian day 122). Even so, our rank-order analysis supported the expected
377 trend of increased turtle-specific eDNA with increased turtle density.

378 We developed an eDNA amplification assay for detecting and quantifying turtle eDNA. We
379 detected background painted turtle signal despite thorough use of UV-sterilizing equipment before
380 qPCR amplification, isolation of qPCR preparation from DNA extraction, and much care to prevent
381 contamination. Although the majority of our turtle-specific eDNA samples did not differ enough
382 from the persistent background noise to allow quantitative analysis, the raw abundances do
383 qualitatively follow the expected rank-order pattern from highest-turtle density pond to lowest-turtle
384 density pond. Thus, if we had detected a higher concentration of painted turtle eDNA in our samples,
385 we would expect to have obtained enough copies of eDNA for quantitative analysis. Turtle eDNA
386 possibly has a stochastic nature at low concentrations, exemplified by one clear amplification and
387 several others which fall short of the cutoff (Figure 2). Larger water samples passing through multiple
388 filters may have mitigated this issue by increasing the chance of turtle eDNA capture [108]. Because
389 our negative control amplified, and painted turtle mtDNA has been amplified in our laboratory space
390 before, perhaps targeting another region, such as a nuclear portion not targeted by previously used
391 primers or restriction enzymes, of the painted turtle genome would aid in eliminating the DNA signal
392 in the negative control [141,142]. Despite an abundance of turtles in the sample water, we were unable
393 to collect and extract enough turtle eDNA to reliably exceed the detection limit of qRT-PCR.

394 Currently, we cannot recommend our particular eDNA quantification assay for monitoring
395 aquatic turtle density under field conditions. We obtained just one substantially amplifiable sample
396 of turtle eDNA from pond water despite successfully amplifying turtle-specific eDNA from lab water
397 and developing a sensitive qPCR amplification assay. On the other hand, we did observe the expected
398 positive relationship between turtle density and turtle-specific eDNA, hinting at a possible
399 correlation between turtle density and eDNA extracted. Still, this study highlights some limitations
400 of detecting aquatic reptile eDNA density under field conditions. Indeed, other studies have reported
401 similar difficulties of not being able to relate known turtle density to eDNA under field conditions

[127]. Still, advances in technology may soon realize the full potential of eDNA for monitoring the density of turtle populations. One promising avenue is ddPCR, a sensitive PCR tool that absolutely quantifies template copy number [83,84,98]. This technology has already shown a correlation between density and eDNA copy number in a variety of environments and could be used to aid in quantifying reptile eDNA [84]. As ddPCR technology becomes more widely available and decreases in cost, it may be an attractive alternative to current qPCR methods, especially as it can be more robust to inhibition than qPCR [143,144]. That fish and amphibians have well developed eDNA techniques lends optimism to the view that eDNA eventually can be used to monitor populations of aquatic turtles.

4.1. Inhibition

As with other eDNA studies, our experiment likely suffers from DNA inhibition in the environmental samples. When standard curves were run, 1:4 and 1:8 sample dilutions had a lower Cq value than the full sample itself, signaling the presence of inhibitors [110]. With non-inhibited DNA extracted from painted turtle blood and painted turtle laboratory water, this was not the case. Despite the troubleshooting with Environmental Master Mix 2.0 and the use of ToughMix (QuantaBiosciences), specifically designed to reduce the effects of PCR inhibition, we were unable to amplify enough turtle eDNA to quantitatively relate to turtle density. Inhibition is common in eDNA field studies and is addressed through various protocols. Employing special buffers during extraction (e.g. CTAB), applying clean-up kits (e.g. Zymo One Step), using BSA in PCR reactions, and diluting template for PCR reactions are common ways of minimizing the effect of inhibitory compounds [51,100,109,145,146]. Common environmental inhibitors include plant secondary compounds such as polysaccharides, pectin, xylan, phenols and tannins [147,148]. Soil also contains known PCR inhibitors including humic acids, minerals such as calcium, and inorganic compounds [147,148]. Proteases, urea, and competing DNA may additionally inhibit reactions or decrease reaction efficiency [148]. While inhibitors are well documented in the literature, it may be difficult to ascertain

exactly what mixture of inhibitors are responsible for decreased PCR yield. Therefore, general methods such as clean-up kits and dilution are commonly used for eDNA samples.

4.2. The shedding hypothesis

Biologically, non-avian reptiles may not shed eDNA into the environment at the same rates as other organisms. This we dub the "shedding hypothesis," or the hypothesis that organisms with a keratinized exterior integument may shed eDNA at lower rates compared to those with a mucus integument, such as teleost fish and amphibians. For example, turtles lack gills and most integument is keratinized, thus they may not shed eDNA as readily as organisms with a mucus layer [129,149]. Indeed, one study noted that when eDNA metabarcoding is used for non-avian reptiles and amphibians, > 95% of read abundance was comprised of amphibian DNA for that specific primer set [122]. Potentially, amphibian DNA is more abundant in environmental samples than non-avian reptile eDNA and thus contributes to a larger percentage of read abundance. Furthermore, turtles commonly shed scutes and skin in pieces (rather than as rafts of cells), which, due to their mass, may sink into substrate and be unlikely to be detected in the water column as readily by our eDNA methodology [150]. Thus only excrement, tears, and saliva may be primary shedding mechanisms for detecting turtle eDNA [151,152]. As a result, turtle eDNA may not be overly abundant in the water column. For example, when detecting alligator snapping turtle presence, Cq values were larger than the usual <35 Cq, ranging from 39.06 to 44.89 Cq, indicating low quantities of eDNA [124]. Additionally, despite detection, that study had a low rate of replicates amplifying in a field setting, with most amplifications occurring at a 16% to 33% rate with no 100% replication rates [124]. In studies of European pond turtles, some ponds with known turtle presence did not yield eDNA, resulting in false negatives [127]. Further evidence comes from a previous mesocosm study, specifically targeting marine vertebrates in a semi-controlled environment, where no turtle eDNA was found with vertebrate metabarcoding primers although a sea turtle was present [123].

Along with turtles, other animals with hard exteriors may have reduced shedding of eDNA. For example, European green crab eDNA (*Carcinus maenas*) was about an order of magnitude lower than

that of shanny fish eDNA (*Lipophrys pholis*) in a laboratory marine setup, despite comparable biomass added to tanks [153]. Shedding of large skin fragments, rather than numerous small bits of tissue containing DNA, also may have contributed to non-detection in previous studies of non-chelonian reptiles in aquatic systems. Despite the aquatic nature of West African crocodiles, the species was not detected in a metabarcoding study [119]. Additionally, giant garter snake individuals placed in water were not detected with eDNA in a laboratory setting, suggesting live snake presence may not be enough to shed sufficient eDNA [121]. However, given that substances such as fecal matter can yield DNA [121] (and some successful snake detections have occurred using eDNA in the wild [50,122]), animals with non-mucus integument ultimately may be detectable via eDNA sources other than skin. The shedding hypothesis presented here may be applicable beyond turtles and other vertebrates with keratinized skin, but likely only reduces environmentally available DNA rather than prevents eDNA shedding altogether. We present the shedding hypothesis as just one potential explanation for why eDNA may be less available in the water column for organisms with relatively rigid exteriors.

4.3. Best practices

Both the system and the particular target should be considered when sampling. Different targets require different considerations. It is usually best to carry out a small-scale proof-of-concept experiment in conjunction with traditional methods for comparison before widely applying eDNA methods for monitoring. Here, we outline a few considerations when designing a species-specific eDNA study and recommend additional reviews of eDNA study design [91,154].

Before obtaining samples, planning a robust experimental design as well as having a clean, DNA-free space where experiments will be carried out is important [91,155]. Target species' biology can be used to optimize sample timing. Periods of increased activity, such as breeding seasons, can elevate eDNA availability in the water [62,156]. For example, painted turtle eDNA may be taken while animals are not hibernating and during times of day when they are most active and not basking. For these species, as they are in shallow waters and regularly climb out to bask, surface water may be sufficient. Samples should be taken with an appropriate number of replicates [106], which may

vary depending on season and target biology. Regardless, replicates may increase the chance of detection and confirm positive detection beyond stochasticity [106,107]. Field site(s) should also be considered, as eDNA travels downstream in lotic systems or can have different spatial distribution in lentic systems [82,102,105,157,158]. Water samples need to be filtered, extracted and have PCRs set up in a PCR-free room, preferably in another building, floor, or lab. These practices will prevent contamination, especially if the target species DNA has been amplified before in the same lab.

Beyond planning, sampling and laboratory workflows should be considered. Multiple negative controls (e.g., field, extraction, amplification, and sequencing) are needed to determine at what step contamination is introduced, if at all [28]. During amplification and sequencing, positive controls should be used for comparison, such as laboratory eDNA or DNA tissue extract from the target species [91,159]. At times, synthetic positive controls have been used to distinguish positive controls from potential contamination [59,159]. Furthermore, primers should be tested with closely related, sympatric species to ensure species specificity. Probe-based qPCR for closely-related taxa can increase amplicon specificity [112] to discern single base pair mismatches.

To obtain eDNA, many filtration, extraction, and amplification methods have been used. It may be best to test various filter types systematically, but protocols often use cellulose nitrate filters with 0.45μM pores to capture eDNA [100,160]. Larger pore size may be needed if clogging occurs, especially with water containing high concentrations of algae or sediments [161]. Generally, larger volumes (>1L) of water increase the chance of detecting organisms, though increasing replicates can allow for smaller volumes to be used [106,108,162]. Once filtered, samples are extracted, such as with Qiagen's Blood and Tissue Kit or via a phenol-chloroform isoamyl solution [100]. To decrease sample inhibition, Zymo's One-Step PCR Inhibitor Removal Kit can be helpful, although dilution can work as well [110,111]. Both methods may decrease inhibition, but potentially risk decreasing extracted DNA concentration or yield [111]. Turbid aquatic environments can be more prone to inhibition, yet it may still be possible to obtain eDNA from them [111,160]. To increase PCR reaction efficiency, bovine serum albumin (BSA) may also be added to PCR reactions [51]. Once successful, Sanger

sequencing of amplicons can be used to confirm target species DNA. A number of positive identifications across replicates may be needed to support the presence of a target organism, depending on how dilute the eDNA is expected to be and habitats sampled (e.g. lentic, lotic, or marine) [91,124].

5. Conclusions

Beyond solving eDNA technical difficulties, there is no stand-in for knowing the biology of the target organism. To maximize the probability of success of using eDNA, sampling should be targeted to the life history and ecology of the particular species. Without this basic research, genetic knowledge, and rigorous testing of methodology, eDNA monitoring may not easily yield useful results. As in our case study, painted turtle eDNA may be difficult to obtain in the field. Even so, eDNA could be a powerful tool for detecting presence of non-avian reptiles in lentic habitats [163], as it is already being used successfully for fish and amphibians. Although employing eDNA for studying reptiles in aquatic systems presents challenges, such as decreased eDNA shedding, we remain hopeful that more sensitive technological advancements and robust study design will mitigate these issues.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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