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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

Disciplines

Ecology and Evolutionary Biology | Environmental Sciences | Genetics | Terrestrial and Aquatic Ecology

Comments

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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

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

44 *1.1. Environmental DNA and its uses*

45 Central to population monitoring is the need for a sensitive detection method. Recently,
46 environmental DNA (eDNA) has received attention for being able to sensitively reveal the presence
47 of target species, especially where traditional methods fall short [11,12]. We adopt Taberlet's (2018)
48 definition of eDNA as DNA extracted from environmental samples such as soil, water, air, and feces
49 [13]. Thus far, eDNA techniques have been applied to many environmental contexts, including leaf
50 litter, soil, and air. Although eDNA has been used to examine alpha and beta diversity through
51 metabarcoding (the use of "universal" primers to detect the presence of multiple taxa [14–19]), eDNA
52 has also been employed to detect single-species presence (e.g., DNA collected for target species from
53 water samples). Single-species eDNA techniques can be used widely, mainly because of the
54 sensitivity of eDNA methodology, compared with traditional methods [18,20,21]. Even so, how
55 eDNA is shed, degrades, travels, and interacts within specific environments varies with target species
56 and specific ecosystem (e.g., lentic vs lotic freshwater), thus methods continue to be refined for
57 obtaining eDNA in a variety of habitats [22–25].

58 Focusing on aquatic systems, single-species eDNA has been used in two main ways for
59 conservation: detecting invasive species and monitoring threatened species. Invasive species cause
60 environmental, ecological, and economic damage, incentivizing prevention and early detection
61 [26,27]. In some studies, eDNA is sensitive enough to detect the forefront of an invasion [28–30].
62 Knowing the range limits of the invasion can help reduce the cost of mitigation efforts. For example,
63 Asian carp (*Hypophthalmichthys* sp.) were one of the first targets for extensive eDNA monitoring of
64 an invasive species [29]. The presence of invasive carp was detected along a Chicago area waterway
65 above the previously defined invasion front [29]. Successful application of eDNA techniques allows

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66 carp behavior to be followed more easily than via traditional monitoring approaches; thus, eDNA
67 tools continue to be refined and used to inform monitoring efforts in the Great Lakes system for
68 multiple invasive carp species [31–37]. Because of the headway made in invasive carp biomonitoring,
69 many other biological invasions have been detected using eDNA. Since then, many invasive fish have
70 been targeted, and eDNA has been used for determining the efficiency of fish eradication efforts [38–
71 43]. Amphibian species have also been targeted [30,44,45], as have crustaceans [41,46–49], reptiles
72 [50,51], and molluscs [19,52,53]. The rapid adoption of eDNA for invasive aquatic species paved the
73 way for developing eDNA-based tools for other systems and continues to motivate advancing this
74 method for further genetic monitoring.

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

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

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

98 *1.2. Environmental DNA limitations*

99 Although monitoring populations with eDNA methods has clear benefits, the utility of the
100 information obtained from eDNA surveys beyond detecting species presence currently has limits. No
101 clear relationship seems to exist between organism biomass, density, or count and eDNA abundance
102 in a field setting [79,80]. Many measures of diversity (e.g., most biodiversity indices) require
103 abundance measurements, not simply presence [81]. Biomass can correlate with both sequence reads
104 and eDNA copy number/concentration, but these relationships may be species- and ecosystem-
105 specific [82–85]. Wide confidence intervals on quantification models can yield unreliable estimates
106 [86,87]. In addition, DNA may be shed at varying rates between individuals, diet, breeding season,
107 and life stage [34,80]. For example, at least one male hellbender (*Cryptobranchus alleganiensis*) shed
108 more eDNA during its mating season month than during other months [62]. Overall, variability
109 among species and individuals – mediated by environmental factors – can cloud our ability to relate
110 eDNA systematically to population or individual measures.

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

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118 of a predator [92,93]. Using eDNA methods alone could cause managers to initiate costly
119 management efforts when no action is needed. False negatives, where the target organism is present
120 but goes undetected, are also possible [28]. Small sample size, insufficient replication, or lack of a
121 sufficiently large sampling area can contribute to non-detection [12,89,90,94]. Employing a targeted
122 sampling design and species-specific PCR primers may increase the chance of species detection [80].
123 Increasingly, eDNA studies incorporate occupancy and species distribution models to robustly
124 confirm detection and mitigate false positives and negatives [56,95,96]. Like other sampling methods,
125 eDNA techniques can detect presence, whereas absence can never be detected. Therefore, species
126 occupancy modeling is used to determine the number of samples needed to have high (95%)
127 confidence of a true absence [97]. This probability can never be zero, but it can be minimized with a
128 high number of replicates and extensive sampling design coverage [91,95]. Species-distribution
129 models also can use information gathered from eDNA to determine the probability of presence [96].
130 Confidence in detection is essential, especially if managed species are targets, thus traditional
131 assessments of eDNA-identified localities may be necessary to confirm presence.

132 *1.3. Sampling Design and Workflow*

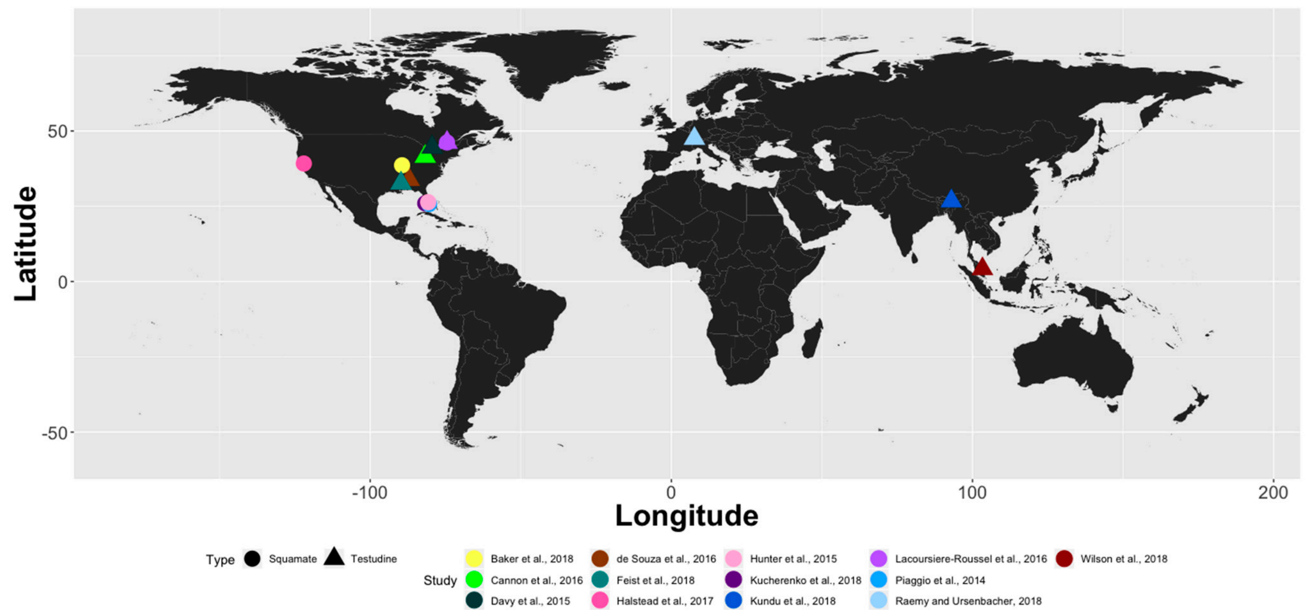
133 Sampling design is of paramount importance, as it often has a large impact on the results of
134 aquatic eDNA studies [98–101]. The biology of target organisms, water flow, and experimental design
135 can affect eDNA signal strength [22,37,102]. For instance, benthic marine species are best detected
136 with methods that target sediment and the lower water column, not surface water [41,56].
137 Furthermore, riverine systems may transport eDNA downstream from the actual location of target
138 species, which must be considered when designing sample sites and interpreting results [102–105].
139 The numbers of samples and replicates obtained directly affect occupancy probability (e.g., a large
140 number of replicates will likely yield higher detection probability) [89,106,107]. Larger volumes of
141 water and filter size also may increase probability of eDNA capture [108].
142 Extraction methodologies have been tested extensively, but may still require tailoring and
143 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

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



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

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

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

195 **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.

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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.

196
197 Previous work has assessed the ability of eDNA to detect presence of aquatic turtle species in a
198 variety of habitats. In a marine aquarium, a green sea turtle (*Chelonia mydas*) was present but not
199 detected when using eDNA metabarcoding methods [123]. Similarly, eDNA assays were developed
200 for multiple captive native Canadian turtles, and eDNA from red-eared slider turtles (*Trachemys*
201 *scripta*) was successfully detected in a small artificial pond (Table 1) [51]. Additionally, an eDNA

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

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

227 *1.5. Painted turtle eDNA case study*

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

239 **2. Materials and Methods**

240 *2.1. Experimental setup and eDNA collection*

241 We seeded four closed-system outdoor ponds with painted turtles at the Iowa State University
242 Horticulture Farm in 2016. These outside, uncovered ponds were natural with respect to abiotic
243 variables and water was not treated in any way. We lined the ponds with black polyethylene
244 laminated tarp and added three white water lily plants (*Nymphaea* sp.) to each pond. Ponds were
245 surrounded by an electric fence, preventing foreign turtles from entering. Although these ponds were
246 the same dimensions (19m L x 15m W x 1.5m D each), they varied in number of adult turtles (0, 11,
247 23, 38) and initial biomass (0g, 6088g, 9198g, and 12990g, respectively). We labeled these ponds as
248 zero (0 turtles at a density of 0kg/ha), low (11 turtles and a density of 6kg/ha), medium (23 turtles at
249 a density of 9kg/ha) and high (38 turtles at a density of 13kg/ha) density. In North American aquatic
250 systems, painted turtle densities can range between 7.2 and 106 kg/ha [130,131]. Our pond densities
251 most mimic low-density painted turtle populations, as these would most likely be relevant to co-

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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

278 and Tissue Kit spin column and spun the sample in a microcentrifuge for 2 min at 14,000 rpm [19].
279 We followed Qiagen's Manufacturer's instructions starting with the addition of 500 µL Buffer AW1
280 (step 5) until elution (step 7). We eluted the samples with 200 µL EDTA (low TE) buffer heated to
281 65°C [51]. We also filtered and extracted three negative laboratory control samples using Culligan
282 Nanopure water in this same way.

283 *2.3. Amplification and quantification*

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

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

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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

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

332 **3. Results**

333 From our qPCR dataset, we obtained 27 Cq sample values from seven sampling days by
334 averaging triplicates. One sample—from the zero-turtle density pond on Julian day 167 —was below
335 our detection limit and did not yield a Cq value. Our negative controls amplified at an average Cq of
336 40.07 (SD = 0.39, SE = 0.11) and our blood positive control Cq was 21.43 (SD = 0.39; SE = 0.11).
337 Background signal in the negative controls were always detected. The mean of all samples (excluding
338 positive and negative controls) was 38.27 Cq (SD = 0.86; average SE = 0.48). The lowest mean value
339 (i.e. highest eDNA abundance) for any sample was the high turtle density pond on Julian day 122,
340 with 31.06 Cq (SD = 0.39; SE = 0.11). This reading is more than 7 Cq values away from the mean of
341 our negative controls, rendering it able to be considered for analysis [137]. The next highest eDNA
342 abundance was for the medium turtle density pond on Julian day 167, with 33.92 Cq (SD = 0.08; SE =
343 0.04), which is not more than 7 Cq values away from the negative control and therefore not
344 sufficiently distinguishable from background amplification. Thus, with only one sample meeting
345 detection criteria, we could not statistically analyze individual Cq values (Figure. 2). That we detected
346 background signal, however, indicates our amplification assay was sensitive and that potential turtle-
347 specific eDNA concentrations in our samples were simply too low.

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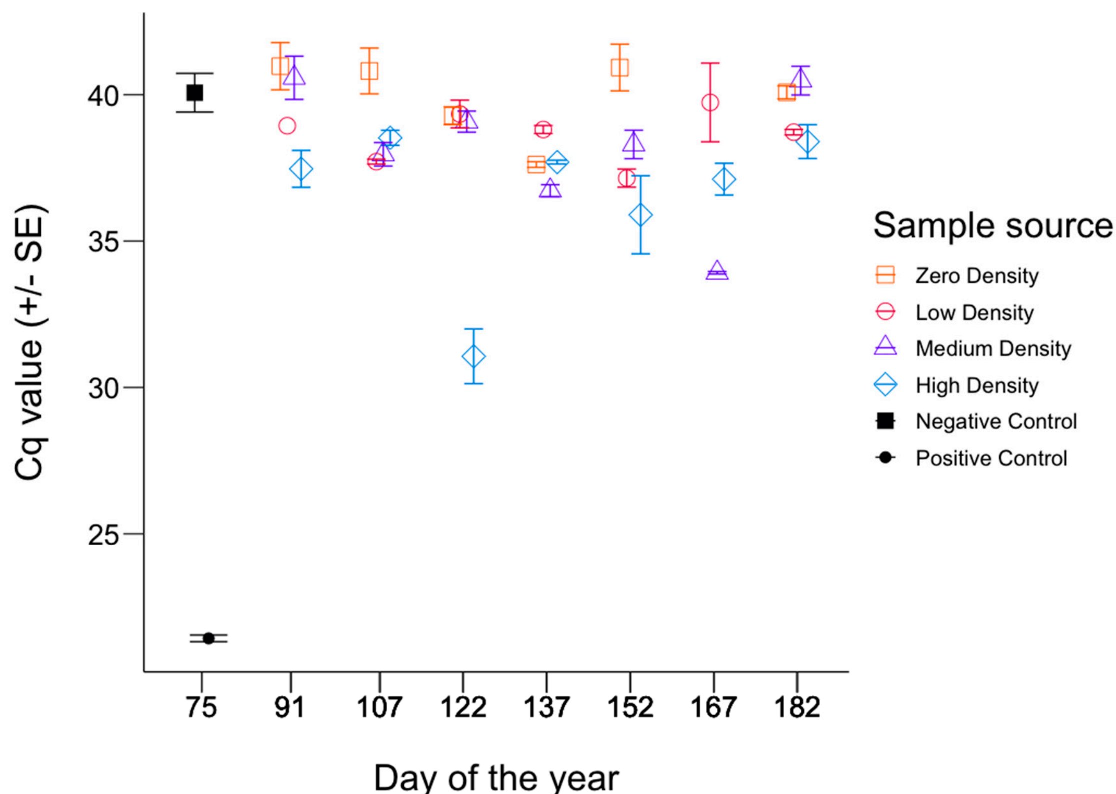
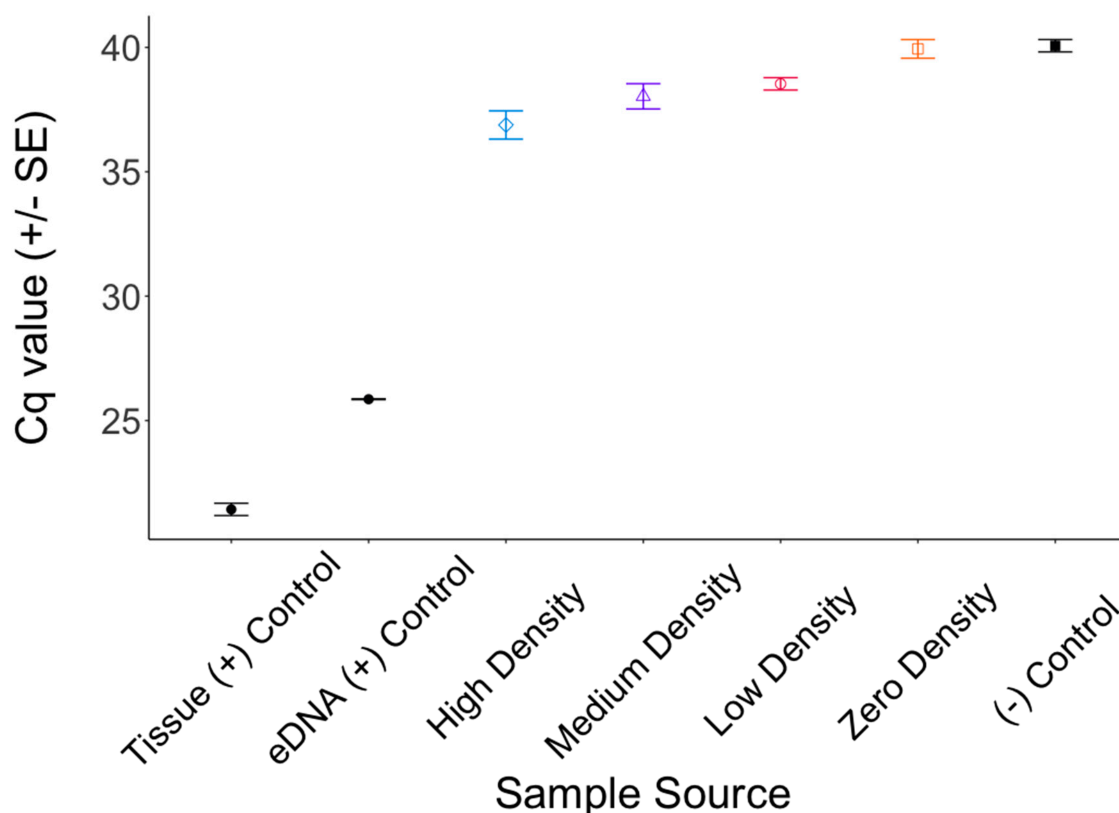


348 **Figure 2.** Amplification (quantification cycle = Cq) of adult painted turtle eDNA as a function of
349 sample source and date. Higher Cq values indicate less eDNA. Varying colors and symbols represent
350 pond treatments: the zero-density pond had 0 turtles (orange squares), the low-density pond 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

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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

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402 [127]. Still, advances in technology may soon realize the full potential of eDNA for monitoring the
403 density of turtle populations. One promising avenue is ddPCR, a sensitive PCR tool that absolutely
404 quantifies template copy number [83,84,98]. This technology has already shown a correlation
405 between density and eDNA copy number in a variety of environments and could be used to aid in
406 quantifying reptile eDNA [84]. As ddPCR technology becomes more widely available and decreases
407 in cost, it may be an attractive alternative to current qPCR methods, especially as it can be more robust
408 to inhibition than qPCR [143,144]. That fish and amphibians have well developed eDNA techniques
409 lends optimism to the view that eDNA eventually can be used to monitor populations of aquatic
410 turtles.

411 *4.1. Inhibition*

412 As with other eDNA studies, our experiment likely suffers from DNA inhibition in the
413 environmental samples. When standard curves were run, 1:4 and 1:8 sample dilutions had a lower
414 Cq value than the full sample itself, signaling the presence of inhibitors [110]. With non-inhibited
415 DNA extracted from painted turtle blood and painted turtle laboratory water, this was not the case.
416 Despite the troubleshooting with Environmental Master Mix 2.0 and the use of ToughMix
417 (QuantaBiosciences), specifically designed to reduce the effects of PCR inhibition, we were unable to
418 amplify enough turtle eDNA to quantitatively relate to turtle density. Inhibition is common in eDNA
419 field studies and is addressed through various protocols. Employing special buffers during extraction
420 (e.g. CTAB), applying clean-up kits (e.g. Zymo One Step), using BSA in PCR reactions, and diluting
421 template for PCR reactions are common ways of minimizing the effect of inhibitory compounds
422 [51,100,109,145,146]. Common environmental inhibitors include plant secondary compounds such as
423 polysaccharides, pectin, xylan, phenols and tannins [147,148]. Soil also contains known PCR
424 inhibitors including humic acids, minerals such as calcium, and inorganic compounds [147,148].
425 Proteases, urea, and competing DNA may additionally inhibit reactions or decrease reaction
426 efficiency [148]. While inhibitors are well documented in the literature, it may be difficult to ascertain

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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

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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

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

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

493 To obtain eDNA, many filtration, extraction, and amplification methods have been used. It may
494 be best to test various filter types systematically, but protocols often use cellulose nitrate filters with
495 0.45μM pores to capture eDNA [100,160]. Larger pore size may be needed if clogging occurs,
496 especially with water containing high concentrations of algae or sediments [161]. Generally, larger
497 volumes (>1L) of water increase the chance of detecting organisms, though increasing replicates can
498 allow for smaller volumes to be used [106,108,162]. Once filtered, samples are extracted, such as with
499 Qiagen's Blood and Tissue Kit or via a phenol-chloroform isoamyl solution [100]. To decrease sample
500 inhibition, Zymo's One-Step PCR Inhibitor Removal Kit can be helpful, although dilution can work
501 as well [110,111]. Both methods may decrease inhibition, but potentially risk decreasing extracted
502 DNA concentration or yield [111]. Turbid aquatic environments can be more prone to inhibition, yet
503 it may still be possible to obtain eDNA from them [111,160]. To increase PCR reaction efficiency,
504 bovine serum albumin (BSA) may also be added to PCR reactions [51]. Once successful, Sanger

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

509 5. Conclusions

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

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

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524 project administration, L.A.H. and F.J.J.; visualization, C.I.M.A. and L.A.H.; writing—original draft preparation,
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535

536 **References**

537 1. Caswell, H. *Matrix population models : construction, analysis, and interpretation*; Sinauer Associates, 1989;
538 ISBN 0878930930.
539 2. Wells, J.; Richmond, M. Populations, metapopulations and species populations - What are they and who
540 should care? *Wildl. Soc. Bull.* **1995**, *23*, 458–462.
541 3. Holt, R.D. Bringing the Hutchinsonian niche into the 21st century: Ecological and evolutionary
542 perspectives. *Proc. Natl. Acad. Sci.* **2009**, *106*, 19659–19665, doi:10.1073/pnas.0905137106.
543 4. Kendall, D.G. Stochastic processes and population growth. *J. R. Stat. Soc. Ser. B* **2018**, *11*, 230–264,
544 doi:10.1111/j.2517-6161.1949.tb00032.x.
545 5. Sutcliffe, O.L.; Thomas, C.D.; Moss, D. Spatial synchrony and asynchrony in butterfly population
546 dynamics. *J. Anim. Ecol.* **2006**, *65*, 85, doi:10.2307/5702.
547 6. Saether, B.-E.; Bakke, O. Avian life history variation and contribution of demographic traits to the
548 population growth rate. *Ecology* **2000**, *81*, 642, doi:10.2307/177366.
549 7. Frankham, R. Genetic adaptation to captivity in species conservation programs. *Mol. Ecol.* **2008**, *17*, 325–
550 333, doi:10.1111/j.1365-294X.2007.03399.x.
551 8. Caswell, H. Matrix models and sensitivity analysis of populations classified by age and stage: a vec-
552 permutation matrix approach. *Theor. Ecol.* **2012**, *5*, 403–417, doi:10.1007/s12080-011-0132-2.
553 9. Marzluff, J.M.; McGowan, K.J.; Donnelly, R.; Knight, R.L. *Causes and consequences of expanding American*
554 *Crow populations*; Marzluff, J.M., Bowman, R., Donnelly, R., Eds.; Springer US: Boston, MA, 2001; ISBN
555 978-1-4613-5600-4, 978-1-4615-1531-9.
556 10. Mills, C.E. Jellyfish blooms: Are populations increasing globally in response to changing ocean
557 conditions? *Hydrobiologia* **2001**, *451*, 55–68, doi:10.1023/A:1011888006302.
558 11. Gargan, L.M.; Morato, T.; Pham, C.K.; Finarelli, J.A.; Carlsson, J.E.L.; Carlsson, J. Development of a
559 sensitive detection method to survey pelagic biodiversity using eDNA and quantitative PCR: a case
560 study of devil ray at seamounts. *Mar. Biol.* **2017**, *164*, 112, doi:10.1007/s00227-017-3141-x.
561 12. Tucker, A.J.; Chadderton, W.L.; Jerde, C.L.; Renshaw, M.A.; Uy, K.; Gantz, C.; Mahon, A.R.; Bowen, A.;
562 Strakosh, T.; Bossenbroek, J.M.; Sieracki, J.L.; Beletsky, D.; Bergner, J.; Lodge, D.M. A sensitive
563 environmental DNA (eDNA) assay leads to new insights on Ruffe (*Gymnocephalus cernua*) spread in
564 North America. *Biol. Invasions* **2016**, *18*, 3205–3222, doi:10.1007/s10530-016-1209-z.
565 13. Cilleros, K.; Valentini, A.; Allard, L.; Dejean, T.; Etienne, R.; Grenouillet, G.; Iribar, A.; Taberlet, P.;
566 Vigouroux, R.; Brosse, S. Unlocking biodiversity and conservation studies in high diversity
567 environments using environmental DNA (eDNA): a test with Guianese freshwater fishes. *Mol. Ecol.*
568 *Resour.* **2018**, doi:10.1111/1755-0998.12900.
569 14. Andersen, K.; Bird, K.L.; Rasmussen, M.; Haile, J.; Breuning-Madsen, H.; Kjær, K.H.; Orlando, L.; Gilbert,
570 M.T.P.; Willerslev, E. Meta-barcoding of “dirt” DNA from soil reflects vertebrate biodiversity. *Mol. Ecol.*
571 **2012**, *21*, 1966–1979, doi:10.1111/j.1365-294X.2011.05261.x.
572 15. Drummond, A.J.; Newcomb, R.D.; Buckley, T.R.; Xie, D.; Dopheide, A.; Potter, B.C.; Heled, J.; Ross, H.A.;
573 Tooman, L.; Grosser, S.; Park, D.; Demetras, N.J.; Stevens, M.I.; Russell, J.C.; Anderson, S.H.; Carter, A.;
574 Nelson, N. Evaluating a multigene environmental DNA approach for biodiversity assessment.
575 *Gigascience* **2015**, *4*, 46, doi:10.1186/s13742-015-0086-1.
576 16. Kraaijeveld, K.; De Weger, L.A.; Ventayol, M.; Ia, G.; Buermans, H.; Frank, J.; Hiemstra, P.S. Efficient
577 and sensitive identification and quantification of airborne pollen using next-generation DNA
578 sequencing. *Mol. Ecol. Resour.* **2015**, *15*, 8–15, doi:10.1111/1755-0998.12288.

579 17. Craine, J.M.; Barberán, A.; Lynch, R.C.; Menninger, H.L.; Dunn, R.R.; Fierer, N. Molecular analysis of
580 environmental plant DNA in house dust across the United States. *Aerobiologia.* **2017**, *33*, 71–86,
581 doi:10.1007/s10453-016-9451-5.
582 18. Rees, H.C.; Bishop, K.; Middleditch, D.J.; Patmore, J.R.M.; Maddison, B.C.; Gough, K.C. The application
583 of eDNA for monitoring of the great crested newt in the UK. *Ecol. Evol.* **2014**, *4*, 4023–4032,
584 doi:10.1002/ece3.1272.
585 19. Goldberg, C.S.; Sepulveda, A.; Ray, A.; Baumgardt, J.; Waits, L.P. Environmental DNA as a new method
586 for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshw. Sci.* **2013**, *32*, 792–800,
587 doi:10.1899/13-046.1.
588 20. Olson, Z.H.; Briggler, J.T.; Williams, R.N. An eDNA approach to detect eastern hellbenders
589 (*Cryptobranchus a. alleganiensis*) using samples of water. *Wildl. Res.* **2012**, *39*, 629–636,
590 doi:10.1071/WR12114.
591 21. Hunter, M.; Meigs-Friend, G.; Ferrante, J.; Kamla, A.; Dorazio, R.; Keith-Diagne, L.; Luna, F.; Lanyon, J.;
592 Reid, J. Surveys of environmental DNA (eDNA): a new approach to estimate occurrence in Vulnerable
593 manatee populations. *Endanger. Species Res.* **2018**, *35*, 101–111, doi:10.3354/esr00880.
594 22. Barnes, M.A.; Turner, C.R. The ecology of environmental DNA and implications for conservation
595 genetics. *Conserv. Genet.* **2016**, *17*, 1–17, doi:10.1007/s10592-015-0775-4.
596 23. Shogren, A.J.; Tank, J.L.; Andruszkiewicz, E.A.; Olds, B.; Jerde, C.; Bolster, D. Modelling the transport of
597 environmental DNA through a porous substrate using continuous flow-through column experiments. *J.*
598 *R. Soc. Interface* **2016**, *13*, 20160290, doi:10.1098/rsif.2016.0290.
599 24. Buxton, A.S.; Groombridge, J.J.; Griffiths, R.A. Is the detection of aquatic environmental DNA influenced
600 by substrate type? *PLoS One* **2017**, *12*, e0183371, doi:10.1371/journal.pone.0183371.
601 25. Salter, I. Seasonal variability in the persistence of dissolved environmental DNA (eDNA) in a marine
602 system: The role of microbial nutrient limitation. *PLoS One* **2018**, *13*, e0192409,
603 doi:10.1371/journal.pone.0192409.
604 26. Pimentel, D.; Zuniga, R.; Morrison, D. Update on the environmental and economic costs associated with
605 alien-invasive species in the United States. *Ecol. Econ.* **2005**, *52*, 273–288,
606 doi:10.1016/j.ecolecon.2004.10.002.
607 27. Ricciardi, A. Are modern biological invasions an unprecedented form of global change? *Conserv. Biol.*
608 **2007**, *21*, 329–336.
609 28. Darling, J.A.; Mahon, A.R. From molecules to management: Adopting DNA-based methods for
610 monitoring biological invasions in aquatic environments. *Environ. Res.* **2011**, *111*, 978–988,
611 doi:10.1016/j.envres.2011.02.001.
612 29. Jerde, C.L.; Mahon, A.R.; Chadderton, W.L.; Lodge, D.M. "Sight-unseen" detection of rare aquatic
613 species using environmental DNA. *Conserv. Lett.* **2011**, *4*, 150–157, doi:10.1111/j.1755-263X.2010.00158.x.
614 30. Dejean, T.; Valentini, A.; Miquel, C.; Taberlet, P.; Bellemain, E.; Miaud, C. Improved detection of an alien
615 invasive species through environmental DNA barcoding: The example of the American bullfrog
616 *Lithobates catesbeianus*. *J. Appl. Ecol.* **2012**, *49*, 953–959, doi:10.1111/j.1365-2664.2012.02171.x.
617 31. Erickson, R.A.; Rees, C.B.; Coulter, A.A.; Merkes, C.M.; McCalla, S.G.; Touzinsky, K.F.; Walleser, L.;
618 Goforth, R.R.; Amberg, J.J. Detecting the movement and spawning activity of bigheaded carps with
619 environmental DNA. *Mol. Ecol. Resour.* **2016**, *16*, 957–965, doi:10.1111/1755-0998.12533.
620 32. Erickson, R.A.; Merkes, C.M.; Jackson, C.A.; Goforth, R.R.; Amberg, J.J. Seasonal trends in eDNA
621 detection and occupancy of bigheaded carps. *J. Great Lakes Res.* **2017**, *43*, 762–770,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

622 doi:10.1016/J.JGLR.2017.06.003.
623 33. Wozney, K.M.; Wilson, C.C. Quantitative PCR multiplexes for simultaneous multispecies detection of
624 Asian carp eDNA. *J. Great Lakes Res.* **2017**, *43*, 771–776, doi:10.1016/J.JGLR.2017.05.001.
625 34. Klymus, K.E.; Richter, C.A.; Chapman, D.C.; Paukert, C. Quantification of eDNA shedding rates from
626 invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biol.*
627 *Conserv.* **2015**, *183*, 77–84, doi:10.1016/j.biocon.2014.11.020.
628 35. Strickler, K.M.; Fremier, A.K.; Goldberg, C.S. Quantifying effects of UV-B, temperature, and pH on
629 eDNA degradation in aquatic microcosms. *Biol. Conserv.* **2015**, *183*, 85–92,
630 doi:10.1016/j.biocon.2014.11.038.
631 36. Song, J.W.; Small, M.J.; A. Casman, E. Making sense of the noise: The effect of hydrology on silver carp
632 eDNA detection in the Chicago area waterway system. *Sci. Total Environ.* **2017**, *605–606*, 713–720,
633 doi:10.1016/J.SCITOTENV.2017.06.255.
634 37. Turner, C.R.; Uy, K.L.; Everhart, R.C. Fish environmental DNA is more concentrated in aquatic
635 sediments than surface water. *Biol. Conserv.* **2015**, *183*, 93–102, doi:10.1016/J.BIOCON.2014.11.017.
636 38. Adrian-Kalchhauser, I.; Burkhardt-Holm, P. An eDNA assay to monitor a globally invasive fish species
637 from flowing freshwater. *PLoS One* **2016**, *11*, e0147558, doi:10.1371/journal.pone.0147558.
638 39. Davison, P.I.; Copp, G.H.; Créach, V.; Vilizzi, L.; Britton, J.R. Application of environmental DNA analysis
639 to inform invasive fish eradication operations. *Sci. Nat.* **2017**, *104*, 35, doi:10.1007/s00114-017-1453-9.
640 40. Hinlo, R.; Furlan, E.; Suitor, L.; Gleeson, D. Environmental DNA monitoring and management of
641 invasive fish: comparison of eDNA and fyke netting. *Manag. Biol. Invasions* **2017**, *8*, 89–100,
642 doi:10.3391/mbi.2017.8.1.09.
643 41. Carim, K.J.; Christianson, K.R.; McKelvey, K.M.; Pate, W.M.; Silver, D.B.; Johnson, B.M.; Galloway, B.T.;
644 Young, M.K.; Schwartz, M.K. Environmental DNA marker development with sparse biological
645 information: A case study on opossum shrimp (*Mysis diluviana*). **2016**, *11*, e0161664,
646 doi:10.1371/journal.pone.0161664.
647 42. Balasingham, K.D.; Walter, R.P.; Mandrak, N.E.; Heath, D.D.D. Environmental DNA detection of rare
648 and invasive fish species in two Great Lakes tributaries. *Mol. Ecol.* **2018**, *27*, 112–127,
649 doi:10.1111/mec.14395.
650 43. Banks, J.C.; Demetras, N.J.; Hogg, I.D.; Knox, M.A.; West, D.W. Monitoring brown trout (*Salmo trutta*)
651 eradication in a wildlife sanctuary using environmental DNA. *New Zeal. Nat. Sci.* **2016**, *41*, 1–13,
652 doi:10289/10302.
653 44. Ficetola, G.F.; Miaud, C.; Pompanon, F.O.; Taberlet, P. Species detection using environmental DNA from
654 water samples. *Biol. Lett* **2008**, *4*, 423–425, doi:10.1098/rsbl.2008.0118.
655 45. Secondi, J.; Dejean, T.; Valentini, A.; Audebaud, B.; Miaud, C. Detection of a global aquatic invasive
656 amphibian, *Xenopus laevis*, using environmental DNA. *Amphib. Reptil.* **2016**, *37*, 131–136,
657 doi:10.1163/15685381-00003036.
658 46. Ardura, A.; Zaiko, A.; Martinez, J.L.; Samulioviene, A.; Semenova, A.; Garcia-Vazquez, E. eDNA and
659 specific primers for early detection of invasive species – A case study on the bivalve *Rangia cuneata*,
660 currently spreading in Europe. *Mar. Environ. Res.* **2015**, *112*, 48–55,
661 doi:10.1016/J.MARENVRES.2015.09.013.
662 47. Dougherty, M.M.; Larson, E.R.; Renshaw, M.A.; Gantz, C.A.; Egan, S.P.; Erickson, D.M.; Lodge, D.M.
663 Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances.
664 *J. Appl. Ecol.* **2016**, *53*, 722–732, doi:10.1111/1365-2664.12621.

665 48. Forsström, T.; Vasemägi, A. Can environmental DNA (eDNA) be used for detection and monitoring of
666 introduced crab species in the Baltic Sea? *Mar. Pollut. Bull.* **2016**, *109*, 350–355,
667 doi:10.1016/j.marpolbul.2016.05.054.
668 49. Larson, E.R.; Renshaw, M.A.; Gantz, C.A.; Umek, J.; Chandra, S.; Lodge, D.M.; Egan, S.P. Environmental
669 DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes
670 of North America. *Hydrobiologia* **2017**, *800*, 173–185, doi:10.1007/s10750-017-3210-7.
671 50. Piaggio, A.J.; Engeman, R.M.; Hopken, M.W.; Humphrey, J.S.; Keacher, K.L.; Bruce, W.E.; Avery, M.L.
672 Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and
673 an assessment of persistence of environmental DNA. *Mol. Ecol. Resour.* **2014**, *14*, 374–380,
674 doi:10.1111/1755-0998.12180.
675 51. Davy, C.M.; Kidd, A.G.; Wilson, C.C. Development and validation of environmental DNA (eDNA)
676 markers for detection of freshwater turtles. *PLoS One* **2015**, *10*, e0130965,
677 doi:10.1371/journal.pone.0130965.
678 52. Klymus, K.E.; Marshall, N.T.; Stepien, C.A. Environmental DNA (eDNA) metabarcoding assays to detect
679 invasive invertebrate species in the Great Lakes. *PLoS One* **2017**, *12*, e0177643,
680 doi:10.1371/journal.pone.0177643.
681 53. Xia, Z.; Zhan, A.; Gao, Y.; Zhang, L.; Haffner, G.D.; MacIsaac, H.J. Early detection of a highly invasive
682 bivalve based on environmental DNA (eDNA). *Biol. Invasions* **2018**, *20*, 437–447, doi:10.1007/s10530-017-
683 1545-7.
684 54. Thomsen, P.F.; Kielgast, J.; Iversen, L.L.; Wiuf, C.; Rasmussen, M.; Gilbert, M.T.P.; Orlando, L.;
685 Willerslev, E. Monitoring endangered freshwater biodiversity using environmental DNA. *Mol. Ecol.*
686 **2012**, *21*, 2565–2573, doi:10.1111/j.1365-294X.2011.05418.x.
687 55. Rees, H.C.; Baker, C.A.; Gardner, D.S.; Maddison, B.C.; Gough, K.C. The detection of great crested newts
688 year round via environmental DNA analysis. *BMC Res. Notes* **2017**, *10*, 327, doi:10.1186/s13104-017-2657-
689 y.
690 56. Schmelzle, M.C.; Kinziger, A.P. Using occupancy modelling to compare environmental DNA to
691 traditional field methods for regional-scale monitoring of an endangered aquatic species. *Mol. Ecol.*
692 *Resour.* **2016**, *16*, 895–908, doi:10.1111/1755-0998.12501.
693 57. Ikeda, K.; Doi, H.; Tanaka, K.; Kawai, T.; Negishi, J.N. Using environmental DNA to detect an
694 endangered crayfish *Cambaroides japonicus* in streams. *Conserv. Genet. Resour.* **2016**, *8*, 231–234,
695 doi:10.1007/s12686-016-0541-z.
696 58. Piggott, M.P. An environmental DNA assay for detecting Macquarie perch, *Macquaria australasica*.
697 *Conserv. Genet. Resour.* **2017**, *9*, 257–259, doi:10.1007/s12686-016-0666-0.
698 59. Brozio, S.; Manson, C.; Gourevitch, E.; Burns, T.J.; Greener, M.S.; Downie, J.R.; Hoskisson, P.A.
699 Development and application of an eDNA method to detect the critically endangered Trinidad golden
700 tree frog (*Phytotriades auratus*) in bromeliad Phytotelmata. *PLoS One* **2017**, *12*, e0170619,
701 doi:10.1371/journal.pone.0170619.
702 60. Laramie, M.B.; Pilliod, D.S.; Goldberg, C.S. Characterizing the distribution of an endangered salmonid
703 using environmental DNA analysis. *Biol. Conserv.* **2015**, *183*, 29–37, doi:10.1016/j.biocon.2014.11.025.
704 61. Sigsgaard, E.E.; Carl, H.; Møller, P.R.; Thomsen, P.F. Monitoring the near-extinct European weather
705 loach in Denmark based on environmental DNA from water samples. *Biol. Conserv.* **2015**, *183*, 46–52,
706 doi:10.1016/j.biocon.2014.11.023.
707 62. Spear, S.F.; Groves, J.D.; Williams, L.A.; Waits, L.P. Using environmental DNA methods to improve

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

708 detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biol. Conserv.* **2015**, *183*,
709 38–45, doi:10.1016/j.biocon.2014.11.016.
710 63. Vörös, J.; Márton, O.; Schmidt, B.R.; Gál, J.T.; Jelić, D. Surveying Europe's only cave-dwelling Chordate
711 species (Proteus anguinus) using environmental DNA. *PLoS One* **2017**, *12*, e0170945,
712 doi:10.1371/journal.pone.0170945.
713 64. Goricki, Š.; Stankovic, D.; Snoj, A.; Kuntner, M.; Jeffery, W.R.; Trontelj, P.; Pavic, M.; Grizelj, Z.; Naparus-
714 Aljancic, M.; Aljancic, G. Environmental DNA in subterranean biology: Range extension and taxonomic
715 implications for *Proteus*. *Sci. Rep.* **2017**, *7*, 45054, doi:10.1038/srep45054.
716 65. Gustavson, M.S.; Collins, P.C.; Finarelli, J.A.; Egan, D.; Conchúir, R.; Wightman, G.D.; King, J.J.;
717 Gauthier, D.T.; Whelan, K.; Carlsson, J.E.L.; Carlsson, J. An eDNA assay for Irish *Petromyzon marinus*
718 and *Salmo trutta* and field validation in running water. *J. Fish Biol.* **2015**, *87*, 1254–1262,
719 doi:10.1111/jfb.12781.
720 66. Stewart, K.; Ma, H.; Zheng, J.; Zhao, J. Using environmental DNA to assess population-wide
721 spatiotemporal reserve use. *Conserv. Biol.* **2017**, doi:10.1111/cobi.12910.
722 67. Hardy, C.M.; Gleeson, D.M.; McGuffie, P.; Lintermans, M.; Bylemans, J.; Furlan, E.M. An environmental
723 DNA-based method for monitoring spawning activity: a case study, using the endangered Macquarie
724 perch (*Macquaria australasica*). *Methods Ecol. Evol.* **2016**, *8*, 646–655, doi:10.1111/2041-210x.12709.
725 68. Sigsgaard, E.E.; Nielsen, I.B.; Bach, S.S.; Lorenzen, E.D.; Robinson, D.P.; Knudsen, S.W.; Pedersen, M.W.;
726 Jaidah, M. Al; Orlando, L.; Willerslev, E.; Møller, P.R.; Thomsen, P.F. Population characteristics of a large
727 whale shark aggregation inferred from seawater environmental DNA. *Nat. Ecol. Evol.* **2016**, *1*, 0004,
728 doi:10.1038/s41559-016-0004.
729 69. Baker, C.S.; Steel, D.; Nieukirk, S.; Klinck, H. Environmental DNA (eDNA) from the wake of the whales:
730 droplet digital PCR for detection and species identification. *Front. Mar. Sci.* **2018**, *5*, 133,
731 doi:10.3389/fmars.2018.00133.
732 70. Parsons, K.M.; Everett, M.; Dahlheim, M.; Park, L. Water, water everywhere: environmental DNA can
733 unlock population structure in elusive marine species. *R. Soc. Open Sci.* **2018**, *5*, 180537,
734 doi:10.1098/rsos.180537.
735 71. Witmer, G.W. Wildlife population monitoring: some practical considerations. *Wildl. Res.* **2005**, *32*, 259,
736 doi:10.1071/WR04003.
737 72. Hoffmann, C.; Schubert, G.; Calvignac-Spencer, S. Aquatic biodiversity assessment for the lazy. *Mol.*
738 *Ecol.* **2016**, *25*, 846–848, doi:10.1111/mec.13535.
739 73. Snyder, D.E. Invited overview: Conclusions from a review of electrofishing and its harmful effects on
740 fish. *Rev. Fish Biol. Fish.* 2004, *13*, 445–453.
741 74. Evans, N.T.; Shirey, P.D.; Wieringa, J.G.; Mahon, A.R.; Lamberti, G.A. Comparative cost and effort of
742 fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries* **2017**, *42*, 90–99,
743 doi:10.1080/03632415.2017.1276329.
744 75. Biggs, J.; Ewald, N.; Valentini, A.; Gaboriaud, C.; Dejean, T.; Griffiths, R.A.; Foster, J.; Wilkinson, J.W.;
745 Arnell, A.; Brotherton, P.; Williams, P.; Dunn, F. Using eDNA to develop a national citizen science-based
746 monitoring programme for the great crested newt (*Triturus cristatus*). *Biol. Conserv.* **2015**, *183*, 19–28,
747 doi:10.1016/j.biocon.2014.11.029.
748 76. Hebert, P.; Penton, E.H.; Burns, J.M.; Janzen, D.H.; Hallwachs, W. Ten species in one: DNA barcoding
749 reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci.* **2004**,
750 *101*, 14812–14817, doi:10.1073/pnas.0406166101.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

751 77. Fletcher, L.M.; Zaiko, A.; Atalah, J.; Richter, I.; Dufour, C.M.; Pochon, X.; Wood, S.A.; Hopkins, G.A.
752 Bilge water as a vector for the spread of marine pests: a morphological, metabarcoding and experimental
753 assessment. *Biol. Invasions* **2017**, *19*, 2851–2867, doi:10.1007/s10530-017-1489-y.
754 78. Ardura, A.; Zaiko, A.; Martinez, J.L.; Samuiloviene, A.; Borrell, Y.; Garcia-Vazquez, E. Environmental
755 DNA evidence of transfer of North Sea molluscs across tropical waters through ballast water. *J.*
756 *Molluscan Stud.* **2015**, *81*, 495–501, doi:10.1093/mollus/eyv022.
757 79. Iversen, L.L.; Kielgast, J.; Sand-Jensen, K. Monitoring of animal abundance by environmental DNA - An
758 increasingly obscure perspective: A reply to Klymus et al., 2015. *Biol. Conserv.* 2015, *192*.
759 80. Lacoursière-Roussel, A.; Rosabal, M.; Bernatchez, L. Estimating fish abundance and biomass from eDNA
760 concentrations: variability among capture methods and environmental conditions. *Mol. Ecol. Resour.*
761 **2016**, *16*, 1401–1414, doi:10.1111/1755-0998.12522.
762 81. Pielou, E.C. The measurement of diversity in different types of biological collections. *J. Theor. Biol.* **1966**,
763 *13*, 131–144, doi:10.1016/0022-5193(66)90013-0.
764 82. Eichmiller, J.J.; Bajer, P.G.; Sorensen, P.W. The relationship between the distribution of common carp
765 and their environmental DNA in a small lake. *PLoS One* **2014**, *9*, e112611,
766 doi:10.1371/journal.pone.0112611.
767 83. Doi, H.; Uchii, K.; Takahara, T.; Matsuhashi, S.; Yamanaka, H.; Minamoto, T. Use of droplet digital PCR
768 for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS One* **2015**, *10*,
769 e0122763, doi:10.1371/journal.pone.0122763.
770 84. Uthicke, S.; Lamare, M.; Doyle, J.R. eDNA detection of corallivorous seastar (*Acanthaster cf. solaris*)
771 outbreaks on the Great Barrier Reef using digital droplet PCR. *Coral Reefs* 2018, *37*, 1229–1239.
772 85. Sassoubre, L.M.; Yamahara, K.M.; Gardner, L.D.; Block, B.A.; Boehm, A.B. Quantification of
773 environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environ. Sci. Technol.* **2016**,
774 *50*, 10456–10464, doi:10.1021/acs.est.6b03114.
775 86. Nevers, M.B.; Byappanahalli, M.N.; Morris, C.C.; Shively, D.; Przybyla-Kelly, K.; Spoljaric, A.M.; Dickey,
776 J.; Roseman, E.F. Environmental DNA (eDNA): A tool for quantifying the abundant but elusive round
777 goby (*Neogobius melanostomus*). *PLoS One* **2018**, *13*, e0191720, doi:10.1371/journal.pone.0191720.
778 87. Baldigo, B.P.; Sporn, L.A.; George, S.D.; Ball, J.A. Efficacy of environmental DNA to detect and quantify
779 brook trout populations in headwater streams of the Adirondack Mountains, New York. *Trans. Am. Fish.*
780 *Soc.* **2017**, *146*, 99–111, doi:10.1080/00028487.2016.1243578.
781 88. Takahara, T.; Minamoto, T.; Doi, H. Effects of sample processing on the detection rate of environmental
782 DNA from the common carp (*Cyprinus carpio*). *Biol. Conserv.* **2015**, *183*, 64–69,
783 doi:10.1016/j.biocon.2014.11.014.
784 89. Wilcox, T.M.; McKelvey, K.S.; Young, M.K.; Sepulveda, A.J.; Shepard, B.B.; Jane, S.F.; Whiteley, A.R.;
785 Lowe, W.H.; Schwartz, M.K. Understanding environmental DNA detection probabilities: A case study
786 using a stream-dwelling char *Salvelinus fontinalis*. *Biol. Conserv.* **2016**, *194*, 209–216,
787 doi:10.1016/j.biocon.2015.12.023.
788 90. Lahoz-Monfort, J.J.; Guillera-Arroita, G.; Tingley, R. Statistical approaches to account for false-positive
789 errors in environmental DNA samples. *Mol. Ecol. Resour.* **2016**, *16*, 673–685, doi:10.1111/1755-0998.12486.
790 91. Goldberg, C.S.; Turner, C.R.; Deiner, K.; Klymus, K.E.; Thomsen, P.F.; Murphy, M.A.; Spear, S.F.; McKee,
791 A.; Oyler-McCance, S.J.; Cornman, R.S.; Laramie, M.B.; Mahon, A.R.; Lance, R.F.; Pilliod, D.S.; Strickler,
792 K.M.; Waits, L.P.; Fremier, A.K.; Takahara, T.; Herder, J.E.; Taberlet, P. Critical considerations for the
793 application of environmental DNA methods to detect aquatic species. *Methods Ecol. Evol.* **2016**, *7*, 1299–

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

794 1307, doi:10.1111/2041-210X.12595.
795 92. Kamoroff, C.; Goldberg, C.S. An issue of life or death: using eDNA to detect viable individuals in
796 wilderness restoration. *Freshw. Sci.* **2018**, *37*, 685–696, doi:10.1086/699203.
797 93. Guilfoyle, M.P.; Schultz, M.T. The contribution of double-crested cormorants (*Phalacrocorax auritus*) to
798 silver carp (*Hypophthalmichthys molitrix*) DNA loads in the Chicago Area Waterway System. *J. Great Lakes*
799 *Res.* **2017**, *43*, 1181–1185, doi:10.1016/j.jglr.2017.09.008.
800 94. Ficetola, G.F.; Pansu, J.; Bonin, A.; Coissac, E.; Giguet-Covex, C.; De Barba, M.; Gielly, L.; Lopes, C.M.;
801 Boyer, F.; Pompanon, F.; Rayé, G.; Taberlet, P. Replication levels, false presences and the estimation of
802 the presence/absence from eDNA metabarcoding data. *Mol. Ecol. Resour.* **2015**, *15*, 543–556,
803 doi:10.1111/1755-0998.12338.
804 95. Willoughby, J.R.; Wijayawardena, B.K.; Sundaram, M.; Swihart, R.K.; DeWoody, J.A. The importance of
805 including imperfect detection models in eDNA experimental design. *Mol. Ecol. Resour.* **2016**, *16*, 837–844,
806 doi:10.1111/1755-0998.12531.
807 96. Muha, T.P.; Rodríguez-Rey, M.; Rolla, M.; Tricarico, E. Using Environmental DNA to improve species
808 distribution models for freshwater invaders. *Front. Ecol. Evol.* **2017**, *5*, 158, doi:10.3389/fevo.2017.00158.
809 97. Mackenzie, D.I.; Nichols, J.D.; Hines, J.E.; Knutson, M.G.; Franklin, A.B.; Knu, M.G.; Franklin, A.B.
810 Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly.
811 *Ecology* **2003**, *84*, 2200–2207.
812 98. Nathan, L.M.; Simmons, M.; Wegleitner, B.J.; Jerde, C.L.; Mahon, A.R. Quantifying environmental DNA
813 signals for aquatic invasive species across multiple detection platforms. *Environ. Sci. Technol.* **2014**, *48*,
814 12800–12806, doi:10.1021/es5034052.
815 99. Deiner, K.; Walser, J.C.; Mächler, E.; Altermatt, F. Choice of capture and extraction methods affect
816 detection of freshwater biodiversity from environmental DNA. *Biol. Conserv.* **2015**, *183*, 53–63,
817 doi:10.1016/j.biocon.2014.11.018.
818 100. Renshaw, M.A.; Olds, B.P.; Jerde, C.L.; Mcveigh, M.M.; Lodge, D.M. The room temperature preservation
819 of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol
820 DNA extraction. *Mol. Ecol. Resour.* **2015**, *15*, 168–176, doi:10.1111/1755-0998.12281.
821 101. Alberdi, A.; Aizpurua, O.; Gilbert, M.T.P.; Bohmann, K. Scrutinizing key steps for reliable
822 metabarcoding of environmental samples. *Methods Ecol. Evol.* **2018**, *9*, 134–147, doi:10.1111/2041-
823 210X.12849.
824 102. Deiner, K.; Altermatt, F. Transport distance of invertebrate environmental DNA in a natural river. *PLoS*
825 *One* **2014**, *9*, doi:10.1371/journal.pone.0088786.
826 103. Cerco, C.F.; Schultz, M.T.; Noel, M.R.; Skahill, B.; Kim, S.-C. A fate and transport model for Asian carp
827 environmental DNA in the Chicago area waterways system. *J. Great Lakes Res.* **2018**,
828 doi:10.1016/j.jglr.2018.04.010.
829 104. Carraro, L.; Hartikainen, H.; Jokela, J.; Bertuzzo, E.; Rinaldo, A. Estimating species distribution and
830 abundance in river networks using environmental DNA. *Proc. Natl. Acad. Sci.* **2018**, *115*, 11724–11729,
831 doi:10.1073/pnas.1813843115.
832 105. Nukazawa, K.; Hamasuna, Y.; Suzuki, Y. Simulating the advection and degradation of the
833 environmental DNA of common carp along a river. *Environ. Sci. Technol.* **2018**, *52*, 10562–10570,
834 doi:10.1021/acs.est.8b02293.
835 106. de Souza, L.S.; Godwin, J.C.; Renshaw, M.A.; Larson, E. Environmental DNA (eDNA) detection
836 probability is influenced by seasonal activity of organisms. *PLoS One* **2016**, *11*, e0165273,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

837 doi:10.1371/journal.pone.0165273.
838 107. Furlan, E.M.; Gleeson, D.; Hardy, C.M.; Duncan, R.P. A framework for estimating the sensitivity of
839 eDNA surveys. *Mol. Ecol. Resour.* **2016**, *16*, 641–654, doi:10.1111/1755-0998.12483.
840 108. Mächler, E.; Deiner, K.; Spahn, F.; Altermatt, F. Fishing in the water: Effect of sampled water volume on
841 environmental DNA-based detection of macroinvertebrates. *Environ. Sci. Technol.* **2016**, *50*, 305–312,
842 doi:10.1021/acs.est.5b04188.
843 109. Wegleitner, B.J.; Jerde, C.L.; Tucker, A.; Chadderton, W.L.; Mahon, A.R. Long duration, room
844 temperature preservation of filtered eDNA samples. *Conserv. Genet. Resour.* **2015**, *7*, 789–791,
845 doi:10.1007/s12686-015-0483-x.
846 110. McKee, A.M.; Spear, S.F.; Pierson, T.W. The effect of dilution and the use of a post-extraction nucleic
847 acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biol.*
848 *Conserv.* **2015**, *183*, 70–76, doi:10.1016/j.biocon.2014.11.031.
849 111. Williams, K.E.; Huyvaert, K.P.; Piaggio, A.J. Clearing muddied waters: Capture of environmental DNA
850 from turbid waters. *PLoS One* **2017**, *12*, e0179282, doi:10.1371/journal.pone.0179282.
851 112. Wilcox, T.M.; McKelvey, K.S.; Young, M.K.; Jane, S.F.; Lowe, W.H.; Whiteley, A.R.; Schwartz, M.K.
852 Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS*
853 *One* **2013**, *8*, e59520, doi:10.1371/journal.pone.0059520.
854 113. Wilcox, T.M.; Carim, K.J.; McKelvey, K.S.; Young, M.K.; Schwartz, M.K. The dual challenges of
855 generality and specificity when developing environmental DNA markers for species and subspecies of
856 *Oncorhynchus*. *PLoS One* **2015**, *10*, e0142008, doi:10.1371/journal.pone.0142008.
857 114. MacDonald, A.J.; Sarre, S.D. A framework for developing and validating taxon-specific primers for
858 specimen identification from environmental DNA. *Mol. Ecol. Resour.* **2017**, *17*, 708–720, doi:10.1111/1755-
859 0998.12618.
860 115. França, L.T.C.; Carrilho, E.; Kist, T.B.L. A review of DNA sequencing techniques. *Q. Rev. Biophys.* **2002**,
861 *35*, 169–200, doi:DOI: 10.1017/S0033583502003797.
862 116. Hunter, M.E.; Oyler-McCance, S.J.; Dorazio, R.M.; Fike, J.A.; Smith, B.J.; Hunter, C.T.; Reed, R.N.; Hart,
863 K.M. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive
864 Burmese pythons. *PLoS One* **2015**, *10*, e0121655, doi:10.1371/journal.pone.0121655.
865 117. Böhm, M.; Collen, B.; Baillie, J.E.M.; Bowles, P.; Chanson, J.; Cox, N.; Hammerson, G.; Hoffmann, M.;
866 Livingstone, S.R.; Ram, M.; Rhodin, A.G.J.; Stuart, S.N.; van Dijk, P.P.; Young, B.E.; Afuang, L.E.;
867 Aghasyan, A.; García, A.; Aguilar, C.; Ajtic, R.; Akarsu, F.; Alencar, L.R. V; Allison, A.; Ananjeva, N.;
868 Anderson, S.; Andrén, C.; Ariano-Sánchez, D.; Arredondo, J.C.; Auliya, M.; Austin, C.C.; Avci, A.; Baker,
869 P.J.; Barreto-Lima, A.F.; Barrio-Amorós, C.L.; Basu, D.; Bates, M.F.; Batistella, A.; Bauer, A.; Bennett, D.;
870 Böhme, W.; Broadley, D.; Brown, R.; Burgess, J.; Captain, A.; Carreira, S.; Castañeda, M. del R.; Castro,
871 F.; Catenazzi, A.; Cedeño-Vázquez, J.R.; Chapple, D.G.; Cheylan, M.; Cisneros-Heredia, D.F.;
872 Cogalniceanu, D.; Cogger, H.; Corti, C.; Costa, G.C.; Couper, P.J.; Courtney, T.; Crnobrnja-Isailovic, J.;
873 Crochet, P.A.; Crother, B.; Cruz, F.; Daltry, J.C.; Daniels, R.J.R.; Das, I.; de Silva, A.; Diesmos, A.C.;
874 Dirksen, L.; Doan, T.M.; Dodd, C.K.; Doody, J.S.; Dorcas, M.E.; Duarte de Barros Filho, J.; Egan, V.T.; El
875 Mouden, E.H.; Embert, D.; Espinoza, R.E.; Fallabrino, A.; Feng, X.; Feng, Z.J.; Fitzgerald, L.; Flores-
876 Villela, O.; França, F.G.R.; Frost, D.; Gadsden, H.; Gamble, T.; Ganesh, S.R.; Garcia, M.A.; García-Pérez,
877 J.E.; Gatus, J.; Gaulke, M.; Geniez, P.; Georges, A.; Gerlach, J.; Goldberg, S.; Gonzalez, J.C.T.; Gower, D.J.;
878 Grant, T.; Greenbaum, E.; Grieco, C.; Guo, P.; Hamilton, A.M.; Hare, K.; Hedges, S.B.; Heideman, N.;
879 Hilton-Taylor, C.; Hitchmough, R.; Hollingsworth, B.; Hutchinson, M.; Ineich, I.; Iverson, J.; Jaksic, F.M.;

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

880 Jenkins, R.; Joger, U.; Jose, R.; Kaska, Y.; Kaya, U.; Keogh, J.S.; Köhler, G.; Kuchling, G.; Kumlutaş, Y.;
881 Kwet, A.; La Marca, E.; Lamar, W.; Lane, A.; Lardner, B.; Latta, C.; Latta, G.; Lau, M.; Lavin, P.; Lawson,
882 D.; LeBreton, M.; Lehr, E.; Limpus, D.; Lipczynski, N.; Lobo, A.S.; López-Luna, M.A.; Luiselli, L.;
883 Lukoschek, V.; Lundberg, M.; Lymberakis, P.; Macey, R.; Magnusson, W.E.; Mahler, D.L.; Malhotra, A.;
884 Mariaux, J.; Maritz, B.; Marques, O.A. V; Márquez, R.; Martins, M.; Masterson, G.; Mateo, J.A.; Mathew,
885 R.; Mathews, N.; Mayer, G.; McCranie, J.R.; Measey, G.J.; Mendoza-Quijano, F.; Menegon, M.; Métrailler,
886 S.; Milton, D.A.; Montgomery, C.; Morato, S.A.A.; Mott, T.; Muñoz-Alonso, A.; Murphy, J.; Nguyen, T.Q.;
887 Nilson, G.; Nogueira, C.; Núñez, H.; Orlov, N.; Ota, H.; Ottenwalder, J.; Papenfuss, T.; Pasachnik, S.;
888 Passos, P.; Pauwels, O.S.G.; Pérez-Buitrago, N.; Pérez-Mellado, V.; Pianka, E.R.; Pleguezuelos, J.; Pollock,
889 C.; Ponce-Campos, P.; Powell, R.; Pupin, F.; Quintero Díaz, G.E.; Radder, R.; Ramer, J.; Rasmussen, A.R.;
890 Raxworthy, C.; Reynolds, R.; Richman, N.; Rico, E.L.; Riservato, E.; Rivas, G.; da Rocha, P.L.B.; Rödel,
891 M.O.; Rodríguez Schettino, L.; Roosenburg, W.M.; Ross, J.P.; Sadek, R.; Sanders, K.; Santos-Barrera, G.;
892 Schleich, H.H.; Schmidt, B.R.; Schmitz, A.; Sharifi, M.; Shea, G.; Shi, H.T.; Shine, R.; Sindaco, R.; Slimani,
893 T.; Somaweera, R.; Spawls, S.; Stafford, P.; Stuebing, R.; Sweet, S.; Sy, E.; Temple, H.J.; Tognelli, M.F.;
894 Tolley, K.; Tolson, P.J.; Tuniyev, B.; Tuniyev, S.; üzüm, N.; van Buurt, G.; Van Sluys, M.; Velasco, A.;
895 Vences, M.; Veselý, M.; Vinke, S.; Vinke, T.; Vogel, G.; Vogrin, M.; Vogt, R.C.; Wearn, O.R.; Werner, Y.L.;
896 Whiting, M.J.; Wiewandt, T.; Wilkinson, J.; Wilson, B.; Wren, S.; Zamin, T.; Zhou, K.; Zug, G. The
897 conservation status of the world's reptiles. *Biol. Conserv.* **2013**, *157*, 372–385,
898 doi:10.1016/j.biocon.2012.07.015.
899 118. van Dijk, P.P.; Iverson, J.; Rhodin, A.; Shaffer, B.; Bour, R. Turtles of the World, 7th Edition: Annotated
900 checklist of taxonomy, synonymy, distribution with maps, and conservation status. *Chelonian Res.*
901 *Monogr.* **2014**, *5*, 329–479, doi:10.3854/crm.5.000.checklist.v7.2014.
902 119. Egeter, B.; Peixoto, S.; Brito, J.C.; Jarman, S.; Puppo, P.; Velo-Antón, G. Challenges for assessing
903 vertebrate diversity in turbid Saharan water-bodies using environmental DNA. *Genome* **2018**, 1–8,
904 doi:10.1139/gen-2018-0071.
905 120. Baker, S.J.; Niemiller, M.L.; Stites, A.J.; Ash, K.T.; Davis, M.A.; Dreslik, M.J.; Phillips, C.A. Evaluation of
906 environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces ophiodiicola* in crayfish burrows.
907 *Conserv. Genet. Resour.* **2018**, 1–3, doi:10.1007/s12686-018-1053-9.
908 121. Halstead, B.J.; Wood, D.A.; Bowen, L.; Waters, S.C.; Vandergast, A.G.; Ersan, J.S.; Skalos, S.M.; Casazza,
909 M.L. An evaluation of the efficacy of using environmental DNA (eDNA) to detect giant gartersnakes
910 *(Thamnophis gigas)*; *USGS Open-File Report*. **2017**, 41p.
911 122. Lacoursière-Roussel, A.; Dubois, Y.; Normandeau, E.; Bernatchez, L.; Adamowicz, S. Improving
912 herpetological surveys in eastern North America using the environmental DNA method. *Genome* **2016**,
913 *59*, 991–1007, doi:10.1139/gen-2015-0218.
914 123. Kelly, R.P.; Port, J.A.; Yamahara, K.M.; Crowder, L.B. Using environmental DNA to census marine fishes
915 in a large mesocosm. *PLoS One* **2014**, *9*, e86175, doi:10.1371/journal.pone.0086175.
916 124. Feist, S.M.; Jones, R.L.; Copley, J.L.; Pearson, L.S.; Berry, G.A.; Qualls, C.P. Development and validation
917 of an environmental DNA method for detection of the Alligator Snapping Turtle (*Macrochelys*
918 *temminckii*). *Chelonian Conserv. Biol.* **2018**, *17*, 271–279, doi:10.2744/CCB-1315.1.
919 125. Kundu, S.; Kumar, V.; Tyagi, K.; Chandra, K. Environmental DNA (eDNA) testing for detection of
920 freshwater turtles in a temple pond. *Herpetol. Notes* **2018**, *11*, 369–371.
921 126. Wilson, J.-J.; Sing, K.-W.; Chen, P.-N.; Zieritz, A. Tracking the southern river terrapin (*Batagur affinis*)
922 through environmental DNA: prospects and challenges. *Mitochondrial DNA Part A* **2017**, *29*, 862–866,

923 doi:10.1080/24701394.2017.1373109.

924 127. Raemy, M.; Ursenbacher, S. Detection of the European pond turtle (*Emys orbicularis*) by environmental
925 DNA: Is eDNA adequate for reptiles? *Amphibia-Reptilia* **2018**, *39*, 135–143, doi:10.1163/15685381-
926 17000025.

927 128. Cannon, M. V.; Hester, J.; Shalkhauser, A.; Chan, E.R.; Logue, K.; Small, S.T.; Serre, D. In silico
928 assessment of primers for eDNA studies using PrimerTree and application to characterize the
929 biodiversity surrounding the Cuyahoga River. *Sci. Rep.* **2016**, *6*, 22908, doi:10.1038/srep22908.

930 129. Ernst, C.H.; Lovich, J.E. *Turtles of the United States and Canada*; 2nd ed.; JHU Press, 2009; ISBN 0801891213.

931 130. Iverson, J.B. Biomass in turtle populations: A neglected subject. *Oecologia* **1982**, *55*, 69–76,
932 doi:10.1007/BF00386720.

933 131. Congdon, J.D.; Greene, J.L.; Gibbons, J.W. Biomass of freshwater turtles: A geographic comparison. *Am.
934 Midl. Nat.* **1986**, *115*, 165–173, doi:10.2307/2425846.

935 132. Ernst, C.H. Population dynamics and activity cycles of *Chrysemys picta* in southeastern Pennsylvania. *J.
936 Herpetol.* **1971**, *5*, 151–160, doi:10.2307/1562736.

937 133. Dunker, K.J.; Sepulveda, A.J.; Massengill, R.L.; Olsen, J.B.; Russ, O.L.; Wenburg, J.K.; Antonovich, A.
938 Potential of Environmental DNA to Evaluate Northern Pike (*Esox lucius*) Eradication Efforts: An
939 Experimental Test and Case Study. *PLoS One* **2016**, *11*, e0162277, doi:10.1371/journal.pone.0162277.

940 134. Tsuji, S.; Yamanaka, H.; Minamoto, T. Effects of water pH and proteinase K treatment on the yield of
941 environmental DNA from water samples. *Limnology* **2017**, *18*, 1–7, doi:10.1007/s10201-016-0483-x.

942 135. Taberlet, P.; Griffin, S.; Goossens, B.; Questiau, S.; Manceau, V.; Escaravage, N.; Waits, L.P.; Bouvet, J.
943 Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res.* **1996**, *24*,
944 3189–3194, doi:10.1093/nar/24.16.3189.

945 136. Olds, B.P.; Jerde, C.L.; Renshaw, M.A.; Li, Y.; Evans, N.T.; Turner, C.R.; Deiner, K.; Mahon, A.R.;
946 Brueseke, M.A.; Shirey, P.D.; Pfrender, M.E.; Lodge, D.M.; Lamberti, G.A. Estimating species richness
947 using environmental DNA. *Ecol. Evol.* **2016**, *6*, 4214–4226, doi:10.1002/ece3.2186.

948 137. Sow, F.B.; Gallup, J.M.; Sacco, R.E.; Ackermann, M.R. Laser capture microdissection revisited as a tool
949 for transcriptomic analysis: Application of an excel-based qPCR preparation software (PREXCEL-Q).
950 *Int. J. Biomed. Sci.* **2009**, *5*, 105–124.

951 138. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl,
952 M.W.; Shipley, G.L.; Vandesompele, J.; Wittwer, C.T. The MIQE Guidelines: minimum information for
953 publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622,
954 doi:10.1373/clinchem.2008.112797.

955 139. Jonckheere, A.R. A distribution-free k-sample test against ordered alternatives. *Biometrika* **1954**, *41*, 133,
956 doi:10.2307/2333011.

957 140. R Core Team R: A language and environment for statistical computing. *R Found. Stat. Comput. Vienna,
958 Austria* 2013.

959 141. Shaffer, H.B.; Minx, P.; Warren, D.E.; Shedlock, A.M.; Thomson, R.C.; Valenzuela, N.; Abramyan, J.;
960 Amemiya, C.T.; Badenhorst, D.; Biggar, K.K.; Borchert, G.M.; Botka, C.W.; Bowden, R.M.; Braun, E.L.;
961 Bronikowski, A.M.; Bruneau, B.G.; Buck, L.T.; Capel, B.; Castoe, T.A.; Czerwinski, M.; Delehaunty, K.D.;
962 Edwards, S. V; Fronick, C.C.; Fujita, M.K.; Fulton, L.; Graves, T.A.; Green, R.E.; Haerty, W.; Hariharan,
963 R.; Hernandez, O.; Hillier, L.W.; Holloway, A.K.; Janes, D.; Janzen, F.J.; Kandoth, C.; Kong, L.; de Koning,
964 A.P.J.; Li, Y.; Literman, R.; McGaugh, S.E.; Mork, L.; O'Laughlin, M.; Paitz, R.T.; Pollock, D.D.; Ponting,
965 C.P.; Radhakrishnan, S.; Raney, B.J.; Richman, J.M.; St John, J.; Schwartz, T.; Sethuraman, A.; Spinks,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2019

966 P.Q.; Storey, K.B.; Thane, N.; Vinar, T.; Zimmerman, L.M.; Warren, W.C.; Mardis, E.R.; Wilson, R.K. The
967 western painted turtle genome, a model for the evolution of extreme physiological adaptations in a
968 slowly evolving lineage. *Genome Biol.* **2013**, *14*, R28, doi:10.1186/gb-2013-14-3-r28.
969 142. Jiang, J.-J.; Xia, E.-H.; Gao, C.-W.; Gao, L.-Z. The complete mitochondrial genome of western painted
970 turtle, *Chrysemys picta bellii* (Chrysemys, Emydidae). *Mitochondrial DNA Part A* **2016**, *27*, 787–788,
971 doi:10.3109/19401736.2013.873900.
972 143. Yang, R.; Paparini, A.; Monis, P.; Ryan, U. Comparison of next-generation droplet digital PCR (ddPCR)
973 with quantitative PCR (qPCR) for enumeration of *Cryptosporidium oocysts* in faecal samples. *Int. J.*
974 *Parasitol.* **2014**, *44*, 1105–1113, doi:10.1016/j.ijpara.2014.08.004.
975 144. Rački, N.; Dreo, T.; Gutierrez-Aguirre, I.; Blejec, A.; Ravnikar, M. Reverse transcriptase droplet digital
976 PCR shows high resilience to PCR inhibitors from plant, soil and water samples. *Plant Methods* **2014**, *10*,
977 42, doi:10.1186/s13007-014-0042-6.
978 145. Barnes, M.A.; Turner, C.R.; Jerde, C.L.; Renshaw, M.A.; Chadderton, W.L.; Lodge, D.M. Environmental
979 conditions influence eDNA persistence in aquatic systems. *Environ. Sci. Technol.* **2014**, *48*, 1819–1827,
980 doi:10.1021/es404734p.
981 146. Pierson, T.W.; McKee, A.M.; Spear, S.F.; Maerz, J.C.; Camp, C.D.; Glenn, T.C. Detection of an enigmatic
982 Plethodontid salamander using environmental DNA. *Copeia* **2016**, *104*, 78–82, doi:10.1643/CH-14-202.
983 147. Wilson, I.G. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **1997**, *63*,
984 3741–3751, doi:0099-2240/97/$04.00?0.
985 148. Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR inhibitors - occurrence, properties and removal.
986 *J. Appl. Microbiol.* **2012**, *113*, 1014–1026, doi:10.1111/j.1365-2672.2012.05384.x.
987 149. Weldon, P.J.; Demeter, B.J.; Rosscoe, R. A survey of shed skin-eating (Dermatophagy) in amphibians and
988 reptiles. *J. Herpetol.* **1993**, *27*, 219, doi:10.2307/1564942.
989 150. Ernst, C.H. Growth of the painted turtle, *Chrysemys picta*, in Southeastern Pennsylvania. *Herpetologica*
990 **1971**, *27*, 135–141.
991 151. Parmenter, R.R. Digestive turnover rates in freshwater turtles: The influence of temperature and body
992 size. *Comp. Biochem. Physiol. Part A Physiol.* **1981**, *70*, 235–238, doi:10.1016/0300-9629(81)91451-1.
993 152. Northmore, D.; Granda, A. Ocular dimensions and schematic eyes of freshwater and sea turtles. *Vis.*
994 *Neurosci.* **1991**, *7*, 627–635.
995 153. Collins, R.A.; Wangensteen, O.S.; O'Gorman, E.J.; Mariani, S.; Sims, D.W.; Genner, M.J. Persistence of
996 environmental DNA in marine systems. *Commun. Biol.* **2018**, *1*, 185, doi:10.1038/s42003-018-0192-6.
997 154. Taberlet, P.; Bonin, A.; Zinger, L.; Coissac, E. *Environmental DNA: For Biodiversity Research and Monitoring*;
998 Oxford University Press, 2018; ISBN 0198767226.
999 155. Deiner, K.; Bik, H.M.; Mächler, E.; Seymour, M.; Lacoursière-Roussel, A.; Altermatt, F.; Creer, S.; Bista,
1000 I.; Lodge, D.M.; de Vere, N.; Pfrender, M.E.; Bernatchez, L. Environmental DNA metabarcoding:
1001 transforming how we survey animal and plant communities. *Mol. Ecol.* **2017**, *26*, 5872–5895,
1002 doi:10.1111/mec.14350.
1003 156. Takahashi, M.K.; Meyer, M.J.; Mcphee, C.; Gaston, J.R.; Venesky, M.D.; Case, B.F. Seasonal and diel
1004 signature of eastern hellbender environmental DNA. *J. Wildl. Manage.* **2018**, *82*, doi:10.1002/jwmg.21349.
1005 157. O'Donnell, J.L.; Kelly, R.P.; Shelton, A.O.; Samhouri, J.F.; Lowell, N.C.; Williams, G.D. Spatial
1006 distribution of environmental DNA in a nearshore marine habitat. *PeerJ* **2017**, *5*, e3044,
1007 doi:10.7717/peerj.3044.
1008 158. Jane, S.F.; Wilcox, T.M.; Mckelvey, K.S.; Young, M.K.; Schwartz, M.K.; Lowe, W.H.; Letcher, B.H.;

1009 Whiteley, A.R. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Mol. Ecol.*
1010 *Resour.* **2015**, *15*, 216–227, doi:10.1111/1755-0998.12285.
1011 159. Wilson, C.C.; Wozney, K.M.; Smith, C.M. Recognizing false positives: Synthetic oligonucleotide controls
1012 for environmental DNA surveillance. *Methods Ecol. Evol.* 2015, *7*, 23–29.
1013 160. Li, J.; Lawson Handley, L.-J.; Read, D.S.; Hänfling, B. The effect of filtration method on the efficiency of
1014 environmental DNA capture and quantification via metabarcoding. *Mol. Ecol. Resour.* **2018**,
1015 doi:10.1111/1755-0998.12899.
1016 161. Turner, C.R.; Barnes, M.A.; Xu, C.C.Y.; Jones, S.E.; Jerde, C.L.; Lodge, D.M. Particle size distribution and
1017 optimal capture of aqueous macrobial eDNA. *Methods Ecol. Evol.* **2014**, *5*, 676–684, doi:10.1111/2041-
1018 210X.12206.
1019 162. Hinlo, R.; Gleeson, D.; Lintermans, M.; Furlan, E. Methods to maximise recovery of environmental DNA
1020 from water samples. *PLoS One* **2017**, *12*, e0179251, doi:10.1371/journal.pone.0179251.
1021 163. Beans, C. Core Concept: Environmental DNA helps researchers track pythons and other stealthy
1022 creatures. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 8843–8845, doi:10.1073/pnas.1811906115.