Katz, AD, Harper, LR, Sternhagen, EC, Pearce, SE, Melder, CA, Sperry, JH and Davis, MA

Environmental DNA is effective in detecting the federally threatened Louisiana Pinesnake (Pituophis ruthveni)

Received: 8 June 2020 | Revised: 23 July 2020 | Accepted: 24 July 2020

DOI: 10.1002/edn3.126

ORIGINAL ARTICLE

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

Environmental DNA is effective in detecting the federally threatened Louisiana Pinesnake (*Pituophis ruthveni*)

Aron D. Katz[1,2] | Lynsey R. Harper[2,3] | Elizabeth C. Sternhagen[1,4] | Sarah E. Pearce[4] | Christopher A. Melder[4,5] | Jinelle H. Sperry[1,6] | Mark A. Davis[2,6,7]

[1]Engineer Research and Development Center, Champaign, IL, USA

[2]Illinois Natural History Survey, Prairie Research Institute, University of Illinois Urbana Champaign, Champaign, IL, USA

[3]School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

[4]Joint Readiness Training Center and Fort Polk, United States Army, Fort Polk, LA, USA

[5]Center of Environmental Management of Military Lands, Colorado State University, Fort Collins, CO, USA

[6]Department of Natural Resources and Environmental Sciences, University of Illinois Urbana-Champaign, Urbana, IL, USA

[7]The Copperhead Institute, Spartanburg, SC, USA

Correspondence
Mark A. Davis, Illinois Natural History Survey, 1816 South Oak St., Champaign, IL 61820, USA.
Email: davis63@illinois.edu

Abstract

Successful conservation of rare, threatened, or endangered (RTE) species is dependent upon rapid and accurate assessment of their distribution and abundance. However, assessments are challenging as RTE species typically exist as numerically small populations in often fragmented habitats and can possess complex natural histories. Environmental DNA (eDNA) analysis may provide a rapid, cost-effective means of assessing RTE species presence/absence in viable habitat patches. We evaluated the efficacy of eDNA surveillance for the Louisiana Pinesnake (*Pituophis ruthveni*), an elusive, semi-fossorial, nonvenomous colubroid snake endemic to Louisiana and Texas, USA, that has dramatically declined in both distribution and abundance. We developed two quantitative polymerase chain reaction (qPCR) assays that target the mitochondrial cytochrome *c* oxidase subunit I (COI) and mitochondrially encoded ATP synthase membrane subunit 6 (ATP6) genes. We validated each assay *in silico*, *in vitro*, and *in situ*, and investigated the influence of eDNA extraction method and genetic marker on assay performance. Both assays were highly sensitive and successfully detected the Louisiana Pinesnake under artificial and field conditions, including bedding samples collected from captive snake enclosures (100%), soil samples from Louisiana Pinesnake release sites (100%), and soil samples from sites where Louisiana Pinesnakes were documented via radio telemetry (45%). Although differences between genetic markers were negligible, assay performance was strongly influenced by eDNA extraction method. Informed by our results, we discuss methodological and environmental factors influencing Louisiana Pinesnake eDNA detection and quantification, broader implications for management and conservation of the Louisiana Pinesnake and other terrestrial reptiles and provide recommendations for future research. We suggest that eDNA surveys can more effectively assess Louisiana Pinesnake occupancy than conventional sampling, highlighting the need for comprehensive eDNA monitoring initiatives to better identify suitable habitat that will promote persistence of this imperiled species going forward.

KEYWORDS
conservation management, eDNA, Endangered Species Act, gopher burrows, longleaf pine forests, quantitative PCR, radio telemetry, reptile, soil

1 | INTRODUCTION

Effective biodiversity conservation in the Anthropocene is reliant upon accurate delimitation of species' distributions and abundances (Niemiller et al., 2018). These data are essential to inform the designation of critical habitat (Camaclang, Maron, Martin, & Possingham, 2015; Rosenfeld & Hatfield, 2006), prescribe restorative action (Lindenmayer & Franklin, 2002), develop adaptive management strategies (Cushman & McKelvey, 2010), and, in some instances, target population reinforcements or reintroductions (Seddon, Griffiths, Soorae, & Armstrong, 2014). Yet accurate assessments of species' distributions and abundances are often difficult, if not impossible, to acquire (Amano, Lamming, & Sutherland, 2016).

Elusive species present particular challenges to detection (Chades et al., 2008), and imperfect detection of these species can bias occupancy estimates, ostensibly yielding detrimental adaptive management scenarios (Gu & Swihart, 2004). Numerically rare species are also at a disadvantage for detection, given the low probability that they actually occur (Cunningham & Lindenmayer, 2005; Gaston et al., 2000), and conventional sampling approaches are often ineffective for their capture (Guisan et al., 2006; Rushton, Ormerod, & Kerby, 2004). Thus, natural history (e.g., crypsis, semi-fossorial lifestyle, etc.) and rarity can have manifold effects on species detection. In certain contexts, these factors can negate our ability to detect species.

This is an acute problem for amphibians and reptiles (Durso & Seigel, 2015; Steen, 2010), and snakes present a particularly prickly case. Not only are they among the world's most imperiled taxonomic groups (Gibbons et al., 2000; Reading et al., 2010; Todd, Wilson, & Gibbons, 2010, but see Fitzgerald et al., 2018), they are often elusive and/or semi-fossorial. Consequently, detection and occupancy estimation is fraught with uncertainty (Durso & Seigel, 2015; Durso, Wilson, & Winne, 2011; Steen, 2010). Given the problematic nature of inventorying and monitoring elusive, semi-fossorial, and rare species, novel approaches to improve detection are imperative for accurate assessment of occupancy and quantification of distributions and abundances.

Environmental DNA (eDNA) analysis now stands firmly at the forefront of species monitoring as a viable alternative to conventional tools. eDNA is trace DNA released by organisms into their environment via secretions (Ficetola, Miaud, Popanon, & Tab erlet, 2008; Jerde, Mahon, Chadderton, & Lodge, 2011), excretions (Anderson et al., 2011; Martellini, Payment, & Villemur, 2005; Thomsen et al., 2012), or even decomposing carcasses (Merkes, McCalla, Jensen, Gaikowski, & Amberg, 2014, but see Curtis & Larson, 2020) that can be harnessed from environmental samples (e.g., soil, water) without observation or direct capture of the target organism itself (Thomsen & Willerslev, 2015). Over the past decade, the utility of eDNA analysis has grown rapidly, and its efficacy has been demonstrated from freshwater, marine, subterranean, terrestrial, and airborne samples (e.g., Franklin et al., 2019; Harper et al., 2020; Johnson, Cox, & Barnes, 2019; Niemiller et al., 2018; Thomsen et al., 2012). Since eDNA analysis was first applied to macrobiota (Ficetola et al., 2008), it has become an important conservation and management tool that is capable of detecting both invasive and imperiled species at low abundances in numerous contexts (e.g., Gasparini, Crookes, Prosser, & Hanner, 2020; Goldberg, Strickler, & Fremier, 2018; Kessler, Ash, Barratt, Larson, & Davis, 2020; de Souza, Godwin, Renshaw, & Larson, 2016; Tréguier et al., 2014; Valentin et al., 2020; Wacker et al., 2019).

Elusive, semi-fossorial, rare, and/or otherwise difficult to monitor snake species have recently come to the fore as yet another opportunity to apply eDNA surveillance, albeit with mixed results. The most successful case study is that of the invasive Burmese Python, *Python bivittatus* Kuhl, 1820, a large snake and capable swimmer in the Florida Everglades. eDNA analysis improved Burmese Python detection and occupancy estimates thereby providing an additional tool for managers tasked with eradication (Hunter, Meigs-Friend, Ferrante, Smith, & Hart, 2019; Hunter et al., 2015; Kucherenko, Herman, Everham, & Urakawa, 2018; Orzechowski, Ferderick, Dorazio, & Hunter, 2019; Piaggio et al., 2014). However, eDNA analysis has been more equivocal in detecting, semi-aquatic (Halstead et al., 2017; Rose, Wademan, Weir, Wood, & Todd, 2019) and/or semi-fossorial snakes (Baker et al., 2018; Crawford, Dreslik, Baker, Phillips, & Peterman, 2020; Ratsch, Kingsbury, & Jordan, 2020). An eDNA assay was also developed for Red Cornsnake, *Pantherophis guttatus* Linnaeus, 1766, and successfully used for laboratory-based eDNA accumulation/degradation experiments, but the assay was not tested under field conditions (Kucherenko et al., 2018). These studies illustrate the potential and pitfalls of eDNA analysis as an additional tool for surveying snakes.

Here, we sought to assess eDNA analysis as a viable alternative to detect the Louisiana Pinesnake, *Pituophis ruthveni* Stull, 1929. Listed as threatened under the United States Endangered Species Act in 2018, and classified as endangered by the International Union for Conservation of Nature (Hammerson, 2007), the Louisiana Pinesnake is considered to be among the rarest snakes in North America (Rudolph, Burgdorf, Schaefer, Conner, & Maxey, 2006; Thomas, Davis, & Culbertson, 1976; Young & Vanderventer, 1988). Semi-fossorial and ostensibly existing in numerically small populations, little is known about this species, due in part to its rarity and elusiveness (Conant, 1956; Rudolph & Burgdorf, 1997; Young & Vanderventer, 1988). It exhibits a bimodal activity pattern, with movement peaks in spring and fall, yet activity is generally low even during these peak movement periods (Ealy, Fleet, & Rudolph, 2004; Himes, Hardy, Rudolph, & Burgdorf, 2006). Given the above, conventional sampling methods (e.g., trapping, visual encounter surveys, road transects) are generally ineffective (Rudolph et al., 2006).

Consequently, critical information like occupancy in historic and/or suitable habitat patches remains obscured.

Our study objectives were to (1) develop a real-time quantitative polymerase chain reaction (qPCR) assay for the Louisiana Pinesnake, (2) validate the assay *in silico* to assess primer and probe specificity, (3) validate the assay *in vitro* using blood, tissue, and synthetic DNA from the Louisiana Pinesnake and available nontarget species, (4) validate the assay *in situ* on environmental samples taken under artificial and field conditions using both SYBR™ Green and TaqMan® probe qPCR chemistry, and (5) investigate potential factors influencing Louisiana Pinesnake eDNA detection by comparing two eDNA extraction methods and two genetic markers. Finally, we discuss the current state of eDNA knowledge for surveying semi-fossorial and semi-aquatic snakes. In concert, we use these results to inform the implementation of eDNA analysis as a viable means of surveying for the Louisiana Pinesnake, as well as other snakes with similar ecologies, and recommend potential management solutions.

2 | MATERIALS AND METHODS

2.1 | Study system

The Longleaf Pine forest, dominated by the eponymous Longleaf Pine tree, *Pinus palustris* Miller, 1768, endemic to the southeastern United States, was historically among the most extensive forested ecosystems in North America (Landers, van Lear, & Boyer, 1995). Once spanning an estimated 92 million acres, these forests have dwindled to less than three million acres (Frost, 2006; Oswalt et al., 2012), and remnant fragments are considered to be in generally poor condition. A changing fire regime, climate change, timber harvest, habitat fragmentation, development, and improper management have contributed to further degradation of these forests, placing their unique and highly endemic biodiversity at risk. More than thirty threatened or endangered species rely on Longleaf Pine ecosystems, including the Louisiana Pinesnake (Figure 1a), a large, heavy-bodied, nonvenomous colubrid endemic to the western Gulf Coastal Plain, with relictual populations existing in west Louisiana and east Texas. It is a Longleaf Pine savanna specialist, preferential to loose, sandy soils (Wagner, Pierce, Rudolph, Schaefer, & Hightower, 2014), heavily reliant upon Baird's Pocket Gopher, *Geomys breviceps* Baird, 1855, and spends the bulk of its time in gopher burrows (Figure 1b) (Ealy et al., 2004; Himes et al., 2006).

2.2 | eDNA sampling

We sampled during the Louisiana Pinesnake active season in July 2018 at Fort Polk Military Installation (31°21′N, 93°15′W) in west-central Louisiana (Table SA1). Fort Polk main-post, comprising

FIGURE 1 Photographs of (a) a Louisiana Pinesnake, (b) a typical gopher burrow entrance, (c) a colleague sampling soil from a gopher burrow entrance, and (d) a captive Louisiana Pinesnake in an enclosure with wood chip bedding

26,931 hectares of mostly fire-maintained Longleaf Pine-Bluestem Grass, *Andropogon* spp. Linnaeus, 1753, savannah and mixed woodlands, has one of the largest extant populations of the Louisiana Pinesnake, with extensive trapping and monitoring efforts for the species occurring on the installation since 2007. Soil was sampled from eight sites with known Louisiana Pinesnake occupancy, including snake release sites ($n = 3$) and telemetry-confirmed sites ($n = 5$) where two individuals implanted with radio-transmitters were relocated between 8 June–19 July 2018 (Sperry, unpublished data). Soil was collected directly into sterile 50 ml Falcon tubes (Figure 1c) using sterile gloves that were changed between sampling events to minimize risk of contamination.

At the three release sites, a single Louisiana Pinesnake individual was released, and surface soil was collected at three different points (>1 m apart) as the snake moved across the ground and pooled together. Telemetry-confirmed sites primarily consisted of gopher burrows ($n = 4$), although an additional site was bare ground under dense herbaceous cover ($n = 1$). Soil was either collected on the same day as ($n = 3$), four days after ($n = 1$), or 25 days after ($n = 1$) telemetry relocation (Table SA1). At three telemetry-confirmed sites, three soil samples were collected from 2.5–15 cm below the surface after surface soil had been carefully removed by gloved hands. At two telemetry-confirmed sites, only one soil sample was collected (Table SA1). For gopher burrows, the surface soil was removed until an entrance hole was identified at which point soil was collected from inside the burrow.

Subsurface soil samples were also collected following the protocol above from unoccupied sites selected at a random direction and distance (mean = 40.58 m; range = 10.81–68.68 m) from telemetry locations ($n = 22$), and unsuitable sites identified within the Fort Polk area but outside of known Louisiana Pinesnake habitat ($n = 12$) that served as our negative field controls (Table SA1). All samples were immediately placed in labeled, sterile plastic bags on ice in a cooler, and transferred within 24 hr to a −20°C freezer until eDNA extraction.

Wood chip bedding material was sampled from two captive Louisiana Pinesnake enclosures (Figure 1d) and from two sealed, unopened bags of wood chips (bedding controls) for *in situ* assay validation. Sampling was conducted by filling three sterile 50 ml falcon tubes with wood chips from each enclosure ($n = 6$) and each control ($n = 6$). Cetyl trimethyl ammonium bromide (CTAB) was then added to each tube until full and stored at room temperature for 2–6 weeks until eDNA extraction.

2.3 | eDNA extraction

We compared two techniques for eDNA extraction from soil samples: (1) the commercial DNeasy PowerSoil Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol (hereafter QPS), and (2) a modified phenol–chloroform–isoamyl alcohol extraction (hereafter CIA) (Renshaw, Olds, Jerde, Mcveigh, & Lodge, 2015). Both methods are commonly used to isolate DNA from substrates containing high levels of PCR inhibitors (e.g., humic substances), which can inhibit downstream applications (Alaeddini, 2012; Eichmiller, Miller, & Sorensen, 2015; Turner, Miller, Coyne, & Corush, 2014). For both QPS and CIA extraction, 250 ± 25 mg of sample was used as starting material and extracts were stored at −20°C. Both QPS and CIA extractions were performed in triplicate for all soil samples ($n = 84$) and field controls ($n = 204$), but only CIA extractions were performed in triplicate on bedding samples ($n = 18$) and bedding controls ($n = 18$). An extraction negative containing only buffers was included each time eDNA extractions were performed ($n = 31$). Additional CIA extraction protocol details are provided in Supporting Information: Appendix B.

2.4 | Assay design and validation

We developed two novel qPCR assays that target the mitochondrial cytochrome *c* oxidase subunit I (COI) and mitochondrially encoded ATP synthase

TABLE 1 Primers and probes used to amplify COI and ATP6 gene fragments from tissue and environmental samples

Name		Sequence (5'–3')	Gene	Size (bp)	Reference
Primers used to amplify DNA from blood and tissue samples					
LCO1490	F	GGTCAACAAATCATAAAGATATTGG	COI	658	Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994)
HCO2198	R	TGATTTTTTGGTCACCCTGAAGTTTA			
PitATP-Fd	F	ATGCCACAACTTGATACDG	ATP	656	Present study
PitATP-Rd	R	GTRTTTTCTTGTAGRTAWAGG			
Primers and probes used to amplify eDNA					
LPS_COI_F	F	AATATAAGCTTCTGACTCCTACCC	COI	126	Present study
LPS_COI_R	R	GCCCGAGTGTACTAGATTTCC			
LPS_COI_P	Probe	FAM-AGACAGTTCATCCTGTACCAGCCC-MGBNFQ			
LPS_ATP_F	F	AAACCATCCATTACACTAGCC	ATP	144	Present study
LPS_ATP_R	R	ACCGGCTGTGATGTTAGC			
LPS_ATP_P	Probe	FAM-ACTACCAGAAGGCTCACCAACCC-MGBNFQ			

membrane subunit 6 (ATP6) genes for the Louisiana Pinesnake. Twenty-three COI and 58 ATP6 *Pituophis* Holbrook, 1842 sequences were obtained from NCBI GenBank to build reference sequence libraries to facilitate primer design. As of 24 September 2019, only a single COI sequence for the Louisiana Pinesnake (no ATP6) had been accessioned in GenBank. To supplement the reference sequence library, we used two sets of primers (Table 1) to generate COI (658 bp) and ATP6 (656 bp) sequences from blood and tissue samples from 56 Louisiana Pinesnakes (36 males, 20 females) and one male Bull Snake, *Pituophis catenifer sayi* (Schlegel, 1837) (Table SC1). Louisiana Pinesnake specimens were collected throughout the known range of the species, including populations both north and south of the Red River in Louisiana as well as in Texas (Katz, Pearce, Melder, Sperry, & Davis, 2020). Details of reference sequence generation are provided in Supporting Information: Appendix D. All sequences generated for this study were accessioned into GenBank (Table SC1).

Our Louisiana Pinesnake assays target 126 bp and 144 bp amplicons within the COI and ATP6 genes respectively (Table 1). Primers and TaqMan® probes were designed using our reference sequence library (Table SC1) with PrimerQuest® (Integrated DNA Technologies, http://idtdna.com/primerquest). Mismatches in the primer regions were maximized between Louisiana Pinesnake and congeners to increase specificity (Wilcox et al., 2013). Assay specificity was evaluated *in silico* against the full NCBI nucleotide database using BLAST (Zhang, Schwartz, Wagner, & Miller, 2000), and against custom ATP6 and COI reference databases for Louisiana reptiles (see Supporting Information: Appendix D) using ecoPCR (Ficetola et al., 2010). For BLAST, the megablast algorithm (max target sequences = 20,000, word size = 16, all other settings as default) was used with primers/probes submitted in pairs, and matched sequences with less than 100% query coverage were discarded. ecoPCR parameters allowed a 0–150 bp fragment and up to 3 mismatches between each primer/probe and each reference sequence. Forward and reverse primers, forward primer and probe, and reverse primer and probe for each assay were tested under these conditions as both primers and probe cannot be tested simultaneously using ecoPCR.

Assays were validated *in vitro* (1) using DNA extracted from Louisiana Pinesnake and Bull Snake blood and tissue samples with SYBR™ Green qPCR, (2) during optimization of primer and probe concentrations with synthetic Louisiana Pinesnake DNA for TaqMan® probe qPCR (see Supporting Information: Appendix E), and (3) when establishing the limits of detection (LOD) and quantification (LOQ) with synthetic Louisiana Pinesnake DNA using TaqMan® probe qPCR. The LOD and LOQ were established for both assays using gBlocks® Gene Fragments (Integrated Gene Technologies, Coralville, IA, USA), based on GenBank accessions for the Louisiana Pinesnake (COI: MN551796, 658 bp; ATP6: MN551856, 656 bp), as DNA template for the qPCR standards. Copy number for each gBlocks® stock was estimated by multiplying Avogadro's number by the number of moles, following which a 10-fold serial dilution was performed to generate a seven-point standard curve (1,000,000 to 1 copies/μL). We define the LOD as the lowest concentration where at least one technical replicate amplified, and the LOQ as the concentration at which all technical replicates consistently amplified (Agersnap et al., 2017). DNA extracts, standards, and negative controls (sterile molecular grade water [MGW]) were amplified in triplicate using TaqMan® probe qPCR conditions described below.

Assays were validated *in situ* using qPCR with SYBR™ Green and TaqMan® probe chemistry. SYBR™ Green qPCR (i.e., no probes or standard curves) was used to compare melt curve temperatures (Tm) for eDNA samples to positive controls (Louisiana Pinesnake and Bull Snake blood and tissue samples). SYBR™ Green qPCR was performed on 1) CIA extractions of wood chip bedding material sampled from two captive Louisiana Pinesnake enclosures (n = 18) and two sealed, unopened bags of wood chips as bedding controls (n = 18), and 2) a subset of QPS extractions of soil collected from four telemetry-confirmed sites (n = 30) and 14 field control sites (n = 42). Following the manufacturer's protocol, SYBR™ Green qPCR reactions used 20 μl volumes that included 10 μl of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 0.35 μl of each primer (10 μM), 3 μl of template DNA, and 6.3 μl of sterile MGW. Thermocycling conditions were as follows: 95°C for 10 min; 50 cycles of 95°C for 15 s then 60°C for 60 s; and a melt curve stage at 95°C for 15 s, 60°C for 60 s, and 95°C for 1 s.

TaqMan® probe qPCR was performed on all eDNA extractions, including bedding samples, bedding controls, soil samples, field controls, and extraction blanks, with standard curves for eDNA quantification. TaqMan® probe qPCR reactions used 20 μl volumes, consisting of 3 μl of template DNA, 10 μl of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), 4 μl of sterile MGW, and 1 μl of each primer and probe with optimized concentrations (COI: Forward 6 μM, Reverse 12 μM, Probe 2.5 μM [final reaction concentrations of 300 nM, 600 nM, and 125 nM respectively]; ATP6: Forward 6 μM, Reverse 12 μM, Probe 4 μM [final reaction concentrations of 300 nM, 600 nM, and 200 nM respectively]) (Supporting Information: Appendix E). Thermocycling conditions included a 95°C incubation step for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s.

For both SYBR™ Green and TaqMan® probe qPCR, three technical replicates were performed for each eDNA extraction replicate (including bedding samples, bedding controls, soil samples, field controls, and extraction blanks), standard, positive control, and negative control. All qPCRs were conducted on a 96-well QuantStudio™ 3 Real-Time PCR system (Applied Biosystems). Amplification, melt curve, and standard curve analysis was performed using Thermo Fisher Connect™ online software with default settings. qPCR products representing each eDNA sample that amplified were Sanger sequenced by the University of Illinois at Urbana-Champaign Core Sequencing Facility (Urbana, IL, USA) to confirm sequence identity. Forward and reverse sequences were assembled using Geneious Prime 2020.0.4. (https://www.geneious.com) and aligned to Louisiana Pinesnake reference sequences with MAFFT v7.450 (Katoh & Standley, 2013).

2.5 | Data analysis

We report the results of SYBR™ Green and TaqMan® probe qPCR, but only the TaqMan® probe qPCR results were analyzed statistically.

For TaqMan® probe qPCR, technical replicates for each qPCR standard that differed by > 0.5 C_q from the average of the three technical replicates performed were discarded to minimize bias induced by pipetting error. All technical replicates for eDNA samples were retained, and those that failed to amplify were classed as 0 copies/µL (Goldberg et al., 2016). The C_q values for each set of technical replicates were averaged and quantified to provide a single DNA copy number for each extraction replicate. Extraction replicates with no positive amplifications were assigned a DNA copy number of zero. However, all but three of the extraction replicates quantified at < 1 copy/µL, which was below our LOQ and LOD. Therefore, we conducted downstream analyses using C_q values as a relative measure of eDNA concentration.

All statistical analyses were conducted in R v3.6.3 (R Core Team, 2020). qPCR plate amplification efficiency and R^2 values were compared between samples subjected to each eDNA extraction method and each genetic marker (hereafter assay) using an unpaired two-samples Wilcoxon test or *t* test after checking the data for normality (Shapiro–Wilk normality test) and equal variances (*F*-test). Amplification success was assessed by calculating the percentage of sites, samples, and eDNA extractions that amplified according to site category (soil collected from release versus telemetry-confirmed sites), extraction method (CIA versus QPS), and assay (COI versus ATP6).

We examined the influence of site category, extraction method, and assay on detection rate (inferred by number of positive qPCR replicates per soil eDNA extraction) and eDNA concentration (inferred by C_q value) with linear mixed-effects models using the package *lme4* v1.1–23 (Bates, Mächler, Bolker, & Walker, 2015). This allowed us to account for the residual variance of eDNA samples by assigning them as random effects. First, detection rate, for all extractions from each soil sample with at least one positive qPCR replicate regardless of treatment (n = 42), was modeled as the response variable using a Poisson generalized linear mixed-effects model fit by maximum likelihood (Laplace approximation). Second, C_q value, for only qPCR reactions with at least one positive qPCR replicate (n = 46), was modeled as the response variable using a linear mixed-effects model fit by restricted maximum likelihood. All possible model combinations of fixed effect predictor variables (site category, extraction method, and assay), and interactions between predictors, were compared and ranked using the compare_performance() function in the package *performance* v0.4.5.1 (Lüdecke, Makowski, Waggoner, & Patil, 2020). The highest ranked models that included all predictors of interest were used to estimate marginal means with the package *emmeans* v1.4.6 (Lenth, 2020). Marginal mean C_q values for bedding samples were estimated independently from soil samples using a linear mixed-effects model fit by restricted maximum likelihood with assay as the sole predictor variable (QPS extractions were not performed on bedding samples).

Significance of predictors relating to detection rate and eDNA concentration was assessed with Wald z-test and Satterthwaite t-test approximations respectively, using the model_parameters() function in the package *parameters* v0.6.1 (Makowski, Ben-Shachar, & Lüdecke, 2019). Validation checks were performed for selected models using the check_model(), model_performance(), check_overdispersion(), check_zeroinflation(), and check_singularity() functions in the package *performance* to ensure all model assumptions were met (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). All figures were produced using the package *ggplot2* v3.2.1 (Wickham, 2009).

3 | RESULTS

3.1 | Assay validation

Development of the Louisiana Pinesnake ATP6 and COI reference sequence libraries revealed two distinct haplotypes (A and B) of each gene (Table SC1), with each haplotype differing at a single polymorphic nucleotide position. Using NCBI BLAST and ecoPCR for *in silico* assay validation, nine (Table SF1) and 27 (Table SF2) nontarget species respectively matched ATP6 and COI primer/probe combinations. Only Gopher Snake, *Pituophis catenifer* (Blainville, 1835), and Pine Snake, *Pituophis melanoleucus* (Daudin, 1803), had 100% sequence identity (0 mismatches) with primers and probes. Gopher Snake matched all primer/probe combinations for both ATP6 and COI, whereas Pine Snake only matched all COI primer/probe combinations. With NCBI BLAST, seven other nontarget species matched single primer/probe combinations, each with 1–3 mismatches (Table SF1). With ecoPCR, 25 other nontarget species matched primer/probe combinations with 1–3 mismatches, of which three species matched all primer/probe combinations and 22 species matched single primer/probe combinations (Table SF2). None of the nontarget species, including Bull Snakes and Gopher Snakes, returned by BLAST have range overlap with the Louisiana Pinesnake (Fig. SF1), and species returned by ecoPCR that overlap with the Louisiana Pinesnake were freshwater and marine turtles. Importantly, many reptiles that potentially co-occur with the Louisiana Pinesnake have no ATP6 sequence representation on GenBank and thus remain untested. The COI region possesses better coverage for reptiles, but some data deficiency remains (Fig. SF2).

In vitro assay validation with SYBR™ Green qPCR confirmed that both assays would amplify Louisiana Pinesnake DNA. The nontarget Bull Snake amplified with both assays but had a higher Tm (ATP6: 79.61–79.62°C; COI: 80.90–81.20°C) than the Louisiana Pinesnake (ATP6: 78.60–79.17°C; COI: 80.12–80.90°C). TaqMan® probe qPCR of gBlocks® standards using the optimized primer and probe concentrations revealed that our assays are highly sensitive with a LOD of 1 copy/µl for both ATP6 and COI, and a LOQ of 10 copies/µl for ATP6 and 100 copies/µl for COI.

In situ assay validation with SYBR™ Green qPCR confirmed the *in silico* results and demonstrated that primers would amplify Louisiana Pinesnake eDNA from bedding and soil samples. Amplification was observed for all bedding samples, and Tm of amplicons was within the range of the Louisiana Pinesnake positive controls indicating no nontarget amplification. Amplification was observed for

34 qPCR reactions from soil samples (ATP6: $n = 40$, COI: $n = 17$); however, only seven reactions, representing four samples from telemetry-confirmed site TS4 (ATP6: $n = 4$, COI: $n = 2$) and one sample from telemetry-confirmed site TS2 (COI: $n = 1$), produced amplicons with Tm in the range of the Louisiana Pinesnake positive control (ATP6: 78.81–79.15°C; COI: 80.27–80.77°C). All other amplicons had lower Tm and smaller peaks that were indicative of primer dimer. Nonetheless, melt curve analysis indicated that both assays were able to detect Louisiana Pinesnake eDNA from two telemetry-confirmed sites (TS2 and TS4). For all *in vitro* and *in situ* tests using SYBR™ Green and TaqMan® probe qPCR, no amplification was observed for any controls.

3.2 | Assay performance with TaqMan® probe qPCR

Based on standard curves, mean amplification efficiency (Table 2) and R^2 (Table 3) were similar between all treatments (amplification efficiency range = 88.73%–109.88%; R^2 range = 0.992–1.000), but R^2 for the COI assay was significantly higher than the ATP6 assay ($p = .003$) (Table 3). Only one ATP6 plate had efficiency < 90%, but it was not repeated due to high R^2 (0.992). No amplifications were observed for bedding, field, extraction, or qPCR negative controls.

All bedding samples amplified with the COI and ATP6 (excluding two qPCR reactions) assays. We detected target eDNA in 75% of confirmed Louisiana Pinesnake sites ($n = 6$), including 100% of release sites ($n = 3$) and 60% of telemetry-confirmed sites ($n = 3$). In total, 100% of samples from release sites ($n = 3$) and 45% of telemetry-confirmed sites tested positive for the Louisiana Pinesnake ($n = 5$), including 72% ($n = 13$) and 26% ($n = 17$) of eDNA extractions from release and telemetry-confirmed sites, respectively (Figure 2). All eDNA amplicons submitted for Sanger sequencing shared 100% sequence identity with Louisiana Pinesnake reference sequences (COI: $n = 24$; ATP6: $n = 22$), confirming species identity.

3.3 | Factors influencing Louisiana Pinesnake eDNA detection and concentration

Highest ranked models selected to investigate potential factors (i.e., site category, extraction method, and assay) influencing soil eDNA detection rate and concentration with TaqMan® probe qPCR (Table SG1) explained substantial variance (conditional $R^2 = 0.32$ and 0.57 respectively), as did the model used to evaluate the influence of assay on bedding eDNA concentration (conditional $R^2 = 0.92$) (Table SG1). Overall, QPS extraction produced higher eDNA detection rates (beta = 1.86, $p < .001$). However, extraction method interacted with site category, where QPS extractions from telemetry-confirmed sites possessed lower eDNA detection rates than QPS extractions from release sites (beta = −1.80, $p = .002$) (Table 4). This explains the large difference in estimated detection rates for Louisiana Pinesnake release samples extracted with QPS and CIA (marginal mean rate ± SE = 1.75 ± 0.34 and 0.27 ± 0.12 respectively) compared to the negligible difference in estimated

TABLE 2 TaqMan® probe qPCR performance statistics for each treatment, including tests for differences between extraction method (CIA and QPS) and assay (ATP6 and COI)

Treatment	*n*	Mean amplification efficiency (%) ± SE	Amplification efficiency (%) range	Shapiro test		*F*-test		*t* test	
				W	*p*	*F*	*p*	*t*	*p*
Extraction									
CIA	17	100.43 ± 0.84	93.70–109.88	0.920	0.151	0.910	0.858	2.020	0.051
QPS	18	98.01 ± 0.91	88.73–102.95	0.930	0.233				
Assay									
ATP6	18	98.33 ± 1.07	88.73–109.88	0.960	0.521	4.080	0.005	−1.600	0.123
COI	19	100.47 ± 0.49	96.03–103.64	0.930	0.201				

TABLE 3 TaqMan® probe qPCR R^2 statistics for each treatment, including tests for differences between extraction method (CIA and QPS) and assay (ATP6 and COI)

Treatment	*n*	Mean R^2 ± SE	R^2 range	Shapiro test		*F*-test		Wilcoxon test	
				W	*p*	*F*	*p*	*W*	*p*
Extraction									
CIA	17	0.998 ± 0.001	0.987–1.000	0.430	<0.001	2.960	0.032	175.500	0.400
QPS	18	0.999 ± 0.000	0.992–1.000	0.500	<0.001				
Assay									
ATP6	18	0.998 ± 0.001	0.987–1.000	0.540	<0.001	44.260	<0.001	87.500	0.003
COI	19	0.999 ± 0.000	0.999–1.000	0.590	<0.001				

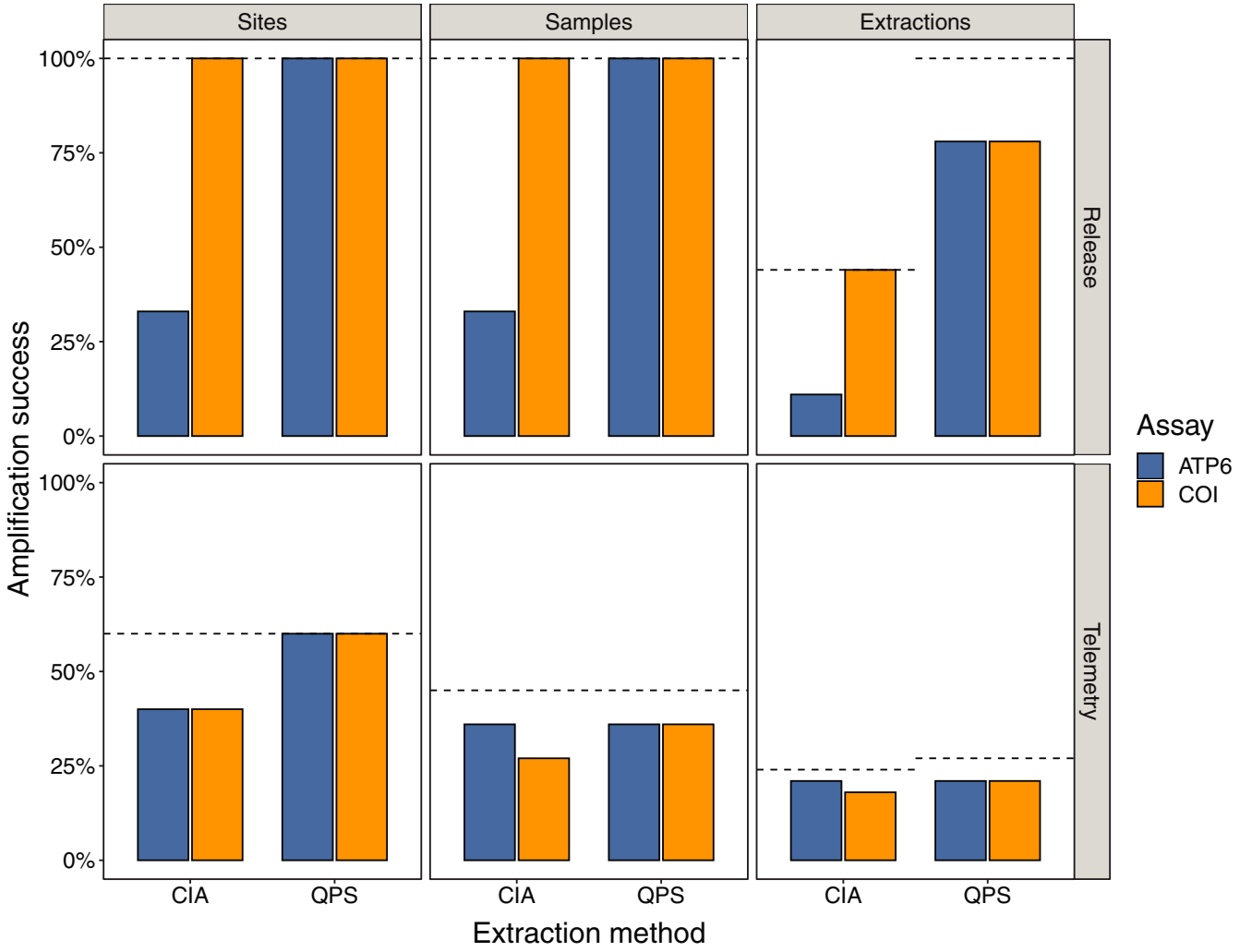

FIGURE 2 Percentage of sites, soil samples, and extractions with Louisiana Pinesnake eDNA detections by site category (release and telemetry-confirmed sites), extraction method (CIA and QPS), and assay (COI and ATP6). Overall amplification success, regardless of extraction method (for sites and samples) or assay treatment, is indicated by dashed lines

TABLE 4 Model parameter statistics for linear mixed-effects models used to investigate factors influencing eDNA detection rate (inferred by number of positive qPCR replicates per soil eDNA extraction) and eDNA concentration (inferred by C_q value)

Model parameter	Beta	SE	95% CI	*Z*	*t*	*df*	*p*
Soil eDNA detection rate							
(Intercept)	−1.35	0.47	[−2.27, −0.43]	−2.88		78	0.004
method [QPS]	1.86	0.48	[0.91, 2.80]	3.86		78	<0.001
sitecat [Telemetry]	0.99	0.51	[−0.01, 2.00]	1.94		78	0.052
assay [COI]	0.11	0.23	[−0.35, 0.56]	0.47		78	0.642
method [QPS] * sitecat [Telemetry]	−1.80	0.58	[−2.94, −0.66]	−3.10		78	0.002
Soil eDNA concentration							
(Intercept)	39.11	0.71	[37.72, 40.50]		55.10	35.76	<0.001
method [QPS]	−1.30	0.50	[−2.28, −0.32]		−2.59	33.73	0.014
sitecat [Telemetry]	1.47	0.71	[0.08, 2.86]		2.07	18.25	0.052
assay [COI]	−0.62	0.45	[−1.49, 0.25]		−1.39	27.05	0.176
Bedding eDNA concentration							
(Intercept)	29.91	0.59	[28.75, 31.07]		50.42	18.60	<0.001
assay [COI]	−1.25	0.25	[−1.74, −0.75]		−4.95	17.00	<0.001

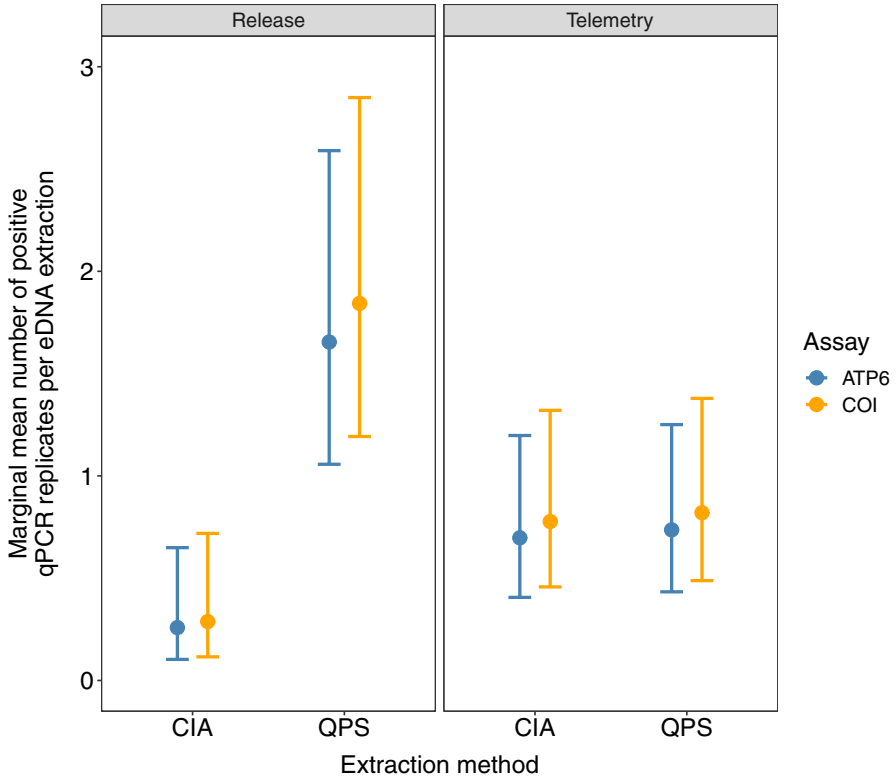

FIGURE 3 Marginal mean number of positive qPCR replicates per eDNA extraction (i.e., detection rate) for release and telemetry-confirmed sites by extraction method (CIA and QPS) and assay (ATP6 and COI). Error bars indicate the 95% confidence interval of the marginal mean

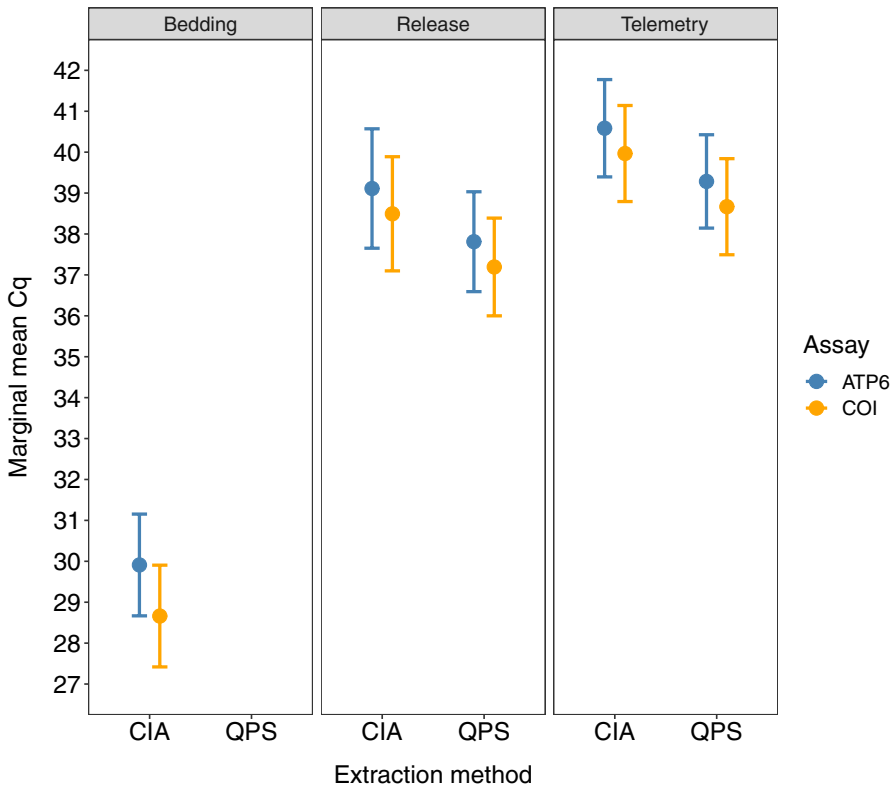

FIGURE 4 Marginal mean Cq value (i.e., eDNA concentration) for bedding, release, and telemetry-confirmed environmental samples by extraction method (CIA and QPS) and assay (ATP6 and COI). Error bars indicate the 95% confidence interval of the marginal mean

detection rates for telemetry-confirmed samples extracted with QPS and CIA (marginal mean rate ± SE = 0.78 ± 0.19 versus. 0.74 ± 0.19 respectively) (Figure 3). QPS extraction generated lower soil C_q values (beta = −1.30, p = .014) (Table 4), explaining the lower C_q value estimates for soil extracted with QPS (marginal mean C_q ± SE = 38.24 ± 0.39) relative to soil extracted with CIA (marginal mean C_q ± SE = 39.54 ± 0.49). Lower C_q values represent higher eDNA concentration, thus QPS extraction positively influenced eDNA concentration (Figure 4). Site category and assay did not influence soil eDNA detection rate or C_q value ($p > 0.05$), but

the COI assay produced lower C_q values for bedding samples (beta = −1.25, $p < 0.001$) (Table 4), explaining the lower C_q values estimated for COI (marginal mean Cq ± SE = 28.66 ± 0.59) than ATP6 (marginal mean C_q ± SE = 29.91 ± 0.59). Therefore, assay positively influenced eDNA concentration of bedding samples (Figure 4).

4 | DISCUSSION

We have developed two eDNA assays that target the cryptic and semi-fossorial Louisiana Pinesnake (see Figure 5 for a flowchart outlining all components of this study). These assays are highly sensitive and capable of detecting this species in artificial and field conditions, both at release sites and where telemetered snakes were recorded. Our results have implications for the application of eDNA analysis to Louisiana Pinesnake conservation and management as well as terrestrial reptiles more broadly. We discuss factors potentially influencing Louisiana Pinesnake eDNA detection and quantification, review the current state of eDNA knowledge for surveying snakes, and provide recommendations for future study.

4.1 | Methodological considerations for Louisiana Pinesnake eDNA detection and concentration

Although we successfully detected Louisiana Pinesnake eDNA in soil samples, additional considerations may aid future sampling efforts. We collected three soil samples in 50 ml conical tubes from gopher burrows, with three extraction replicates performed on each using 250 mg of sample as starting material. However, it may be necessary to process more soil for DNA extraction via greater amounts of starting material or additional extraction replicates (Leempoel, Hebert, & Hadly, 2020; Zinger et al., 2020). Alternatively, it may be more efficient to combine the lysate from extraction replicates during the spin column centrifugation step. Our results indicate that QPS performed better than CIA extraction for Louisiana Pinesnake eDNA detection and concentration (Figures 2–4), but the QPS approach may be cost prohibitive for some conservation efforts (USD 5.86 per extraction as of April 2020). Cheaper, modular DNA extraction methods are now available that may also provide greater eDNA yield and purity (Sellers, Di Muri, Gómez, & Hänfling, 2018),

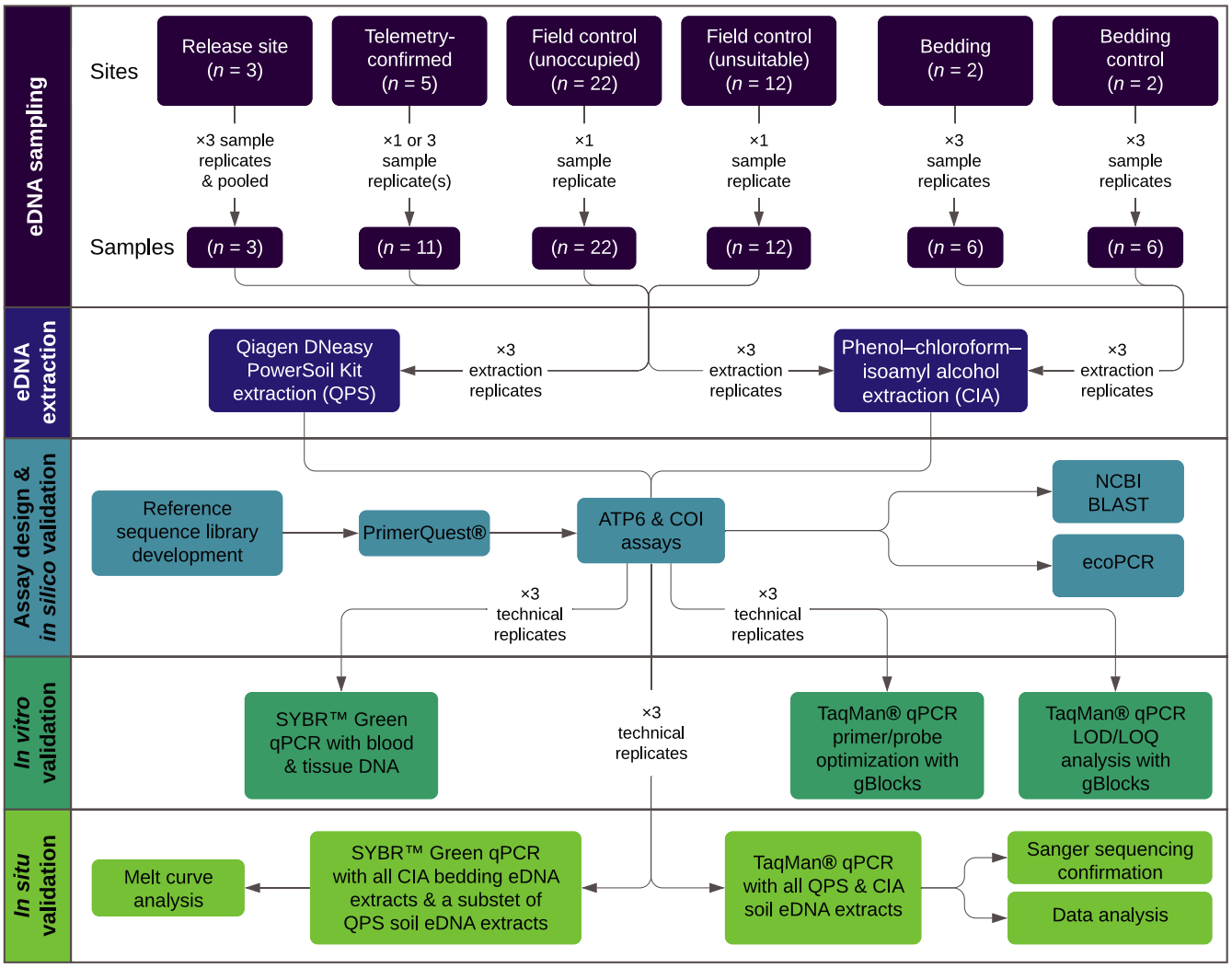

FIGURE 5 Flowchart diagram outlining all components of this study, including eDNA sampling, eDNA extraction, and assay design and validation

thus future studies should compare these homebrew alternatives to more expensive commercial kits. The COI and ATP6 assays were comparable in terms of Louisiana Pinesnake eDNA detection and concentration in soil samples (Figures 2–4), but our results highlight the importance of assay optimization and TaqMan® probe chemistry for qPCR. The SYBR™ Green assays exhibited primer dimer based on Tm from soil eDNA amplicons, but this was mitigated by optimizing our assays using primer-probe concentration matrices (Bustin et al., 2009; Wilcox, Carim, McKelvey, Young, & Schwartz, 2015).

Although we obtained amplicons from eDNA samples, concentrations were exceptionally low and less than the LOQ and LOD for both assays. We could not quantify eDNA concentration, but detections were not discarded as they came from sites where Louisiana Pinesnakes were released or recorded via radio telemetry. Detections that quantify below the LOD are not unexpected for terrestrial and/or rare species that deposit trace amounts of DNA in their environment. It has been suggested that these detections be considered true positives if they occur within 40 cycles, curve morphology is uniform, and no amplification is observed in negative controls (Klymus et al., 2019). Our soil samples conformed to these criteria, and Sanger sequencing confirmed species identity as Louisiana Pinesnake. An LOD of 1 copy/μL is highly sensitive (Harper, Griffiths, et al., 2019; Rodgers, Olson, Klobucar, & Mock, 2018; Wilcox et al., 2013), thus we did not dilute our standard curves further. However, the standard curve ranges may need to be widened for future analyses, with the lowest standard at a concentration of 0.01 copies/μL or less for confidence in detections (Klymus et al., 2019).

Of three technical replicates performed for each of our soil eDNA extractions, typically only one would amplify, especially for extractions from telemetry-confirmed sites (Figure 3). Despite relatively high amplification success for samples from release sites, the majority of samples from telemetry-confirmed sites did not produce any positive technical replicates (Figure 2). Detection sensitivity for the Louisiana Pinesnake could be improved by increasing qPCR technical replication (Klymus et al., 2019). Other eDNA studies have used as many as five (Harper, Griffiths, et al., 2019), eight (Buxton, Groombridge, & Griffiths, 2018), or twelve technical replicates (Tréguier et al., 2014), and only one may amplify. Yet, greater replication may be unnecessary if the steps discussed above and below are taken to improve initial sample concentration.

4.2 | Abiotic and biotic factors influencing Louisiana Pinesnake eDNA detection and concentration

Louisiana Pinesnake eDNA concentrations were often below the LOQ for both assays. Occupancy modeling can estimate detection probability in cases where eDNA concentrations fall below the LOQ (Buxton et al., 2018), and hierarchical occupancy models are particularly suited to eDNA data as they capture occurrence and detection probabilities at each level of the eDNA sampling hierarchy, that is, site, sample, PCR (Harper, Griffiths, et al., 2019; Orzechowski et al., 2019). However, our environmental covariate data for each sampling site were too sparse to enable occupancy modeling, and a null model would be of limited value for understanding detection probability. Consistent and repeated measurements of environmental covariates are necessary to robustly model and understand Louisiana Pinesnake eDNA detection probabilities. Here, we highlight the abiotic and biotic factors that may have influenced our results and that future studies should account for where possible.

A myriad of abiotic factors may impact eDNA persistence in soil, including pH, temperature, UV-B exposure, and precipitation. The effects of temperature are well understood, with aquatic studies frequently reporting greater eDNA production at higher temperatures due to increased physical and metabolic activity, but faster degradation due to more microbial growth (Buxton, Groombridge, Zakaria, & Griffiths, 2017; Goldberg et al., 2018; Strickler, Fremier, & Goldberg, 2015; Tsuji, Ushio, Sakurai, Minamoto, & Yamanaka, 2017). eDNA degradation is hastened under acidic conditions, as acids catalyze chemical hydrolysis, but the effects of pH are intertwined with temperature and microbial activity (Goldberg et al., 2018; Strickler et al., 2015). UV-B exposure is similarly reported to increase eDNA degradation in combination with temperature (Kessler et al., 2020; Strickler et al., 2015); however, effects of UV-B exposure can be inconsistent (Buxton et al., 2017). Based on studies from aquatic systems, we predict that eDNA should persist longer and experience slower degradation in cooler soil with neutral or slightly alkaline pH that does not experience high UV-B exposure or heavy rainfall. In particular, reduced UV-B exposure in burrows may facilitate persistence and subsequent detection of eDNA from burrow dwelling animals, such as the Louisiana Pinesnake, but further investigations incorporating soil temperature and pH are needed.

In addition to the many abiotic factors influencing eDNA persistence and degradation, the role of biotic factors cannot be underestimated. Microbial activity and abundance are driven by several of the abiotic factors that influence eDNA persistence and degradation. Bacterial abundance is higher at warmer temperatures, neutral pH, and moderate UV-B exposure (Strickler et al., 2015; Tsuji et al., 2017). Bacteria feed on eDNA as a dissolved organic phosphorus substrate by producing enzymes that break down DNA thus contributing to reduced eDNA concentration and eventual degradation (Tsuji et al., 2017; Zinger et al., 2020). However, bacteria-mediated eDNA decay may be slowed in environments containing humic substances that bind DNA (e.g., soil) due to the protection they offer from enzymatic degradation (Alaeddini, 2012; Tsuji et al., 2017). Problematically, humic substances can be coextracted with eDNA and inhibit PCR, but the extraction methods we used included an inhibitor removal step. In our study, microbial activity may have contributed to imperfect Louisiana Pinesnake detection from soil eDNA samples. Therefore, future studies should evaluate microbial communities, preferably via high-throughput sequencing, in order to quantify bacterial abundance and potential effects on eDNA detection and concentration.

The target organism itself can exacerbate low eDNA detection rates and concentration. eDNA can have an extremely heterogeneous spatial distribution in the environment if the target organism does not readily release eDNA (Adams, Hoekstra, Muell, & Janzen, 2019; Harper et al., 2020; Hunter et al., 2015; Tréguier et al., 2014), is at low density (Gasparini et al., 2020; Wacker et al., 2019), exhibits periods of lower activity (Buxton et al., 2018; Hunter et al., 2015; de Souza et al., 2016), and utilizes microhabitats or has a large home range, both of which limit opportunities for eDNA deposition (Goldberg et al., 2018; Harper, Handley, et al., 2019). Spatial heterogeneity is further influenced by the ecosystem the target organism occupies, with eDNA being more patchily distributed in static systems with little mixing or flow/wave dynamics to enable eDNA transport, for example, ponds, lakes, soil (Goldberg et al., 2018; Harper, Handley, et al., 2019; Lawson Handley et al., 2019; Zinger et al., 2020). Louisiana Pinesnakes are rare and sparsely distributed across the landscape, which combined with the patchy distribution of soil eDNA may explain low detection rates. Nonetheless, greater snake activity and probable eDNA deposition within gopher burrows can increase sampling efficiency and detection probability. Likewise, other species that concentrate in known dwellings (e.g., hibernacula, burrows, caves) may be good candidates for eDNA surveillance.

Although burrow dwelling behavior of the Louisiana Pinesnake presumably enhanced eDNA detection, it is important to note that we did not achieve detection at all sites where snakes were documented via radio telemetry. Sampling may have missed soil containing eDNA because the snake used a different entrance or went deeper in the burrow. Alternatively, the snake may not have occupied the site long enough for eDNA to accumulate to detectable levels, or eDNA may have degraded by time of sampling (Kucherenko et al., 2018). In one laboratory study, eDNA from three Red Cornsnake individuals accumulated within a few hours, but degraded rapidly after snake removal (Kucherenko et al., 2018). Conversely, Halstead et al. (2017) observed relatively slow (~21 days) eDNA release from skin shed by Giant Garter Snake, *Thamnophis gigas* Fitch, 1940, and highest detection rates from feces in water, but false negatives were still produced under laboratory conditions. Here, we successfully detected Louisiana Pinesnake eDNA in soil collected up to 25 days after telemetry confirmation, but sampled burrow entrances may have been utilized throughout this 25-day period.

Seasonal activity and behavior can have pronounced effects on eDNA detection, especially for species that do not readily produce or shed DNA, such as invertebrates with exoskeletons (Tréguier et al., 2014) or reptiles with keratinized exterior integuments (Adams et al., 2019; Harper et al., 2020; Hunter et al., 2015; Kucherenko et al., 2018; de Souza et al., 2016). For example, Halstead et al. (2017) observed negative eDNA detections from water for Giant Garter Snakes, which spend long periods on land (nearly 60% for females) during the active season and overwinter terrestrially in small mammal burrows. Careful consideration of species ecology and behavior is key to maximize eDNA detection probabilities. In our study, Louisiana Pinesnake burrow use and associated soil sampling occurred during summer when snakes are more active and exhibit movement between sites (Himes et al., 2006), thus it is unclear how interseasonal sampling may impact detection. In winter, snakes are largely dormant in gopher burrows (Pierce et al., 2014; Rudolph, Schaefer, Burgdorf, Duran, & Conner, 2007), potentially increasing eDNA accumulation in burrow soil. However, snakes also hibernate deeper in burrows during winter than the active season (Rudolph et al., 2007; Sperry, unpublished data), which may complicate soil sampling. Comprehensive investigations of eDNA accumulation and degradation under laboratory and field conditions are needed to determine optimal timing for Louisiana Pinesnake eDNA sampling strategies.

4.3 | Broader applicability of the Louisiana Pinesnake eDNA assays

The range of the Louisiana Pinesnake does not overlap with any congener ranges, thus nontarget amplification was unconcerning and should not prevent eDNA surveillance being used for this species. Nonetheless, we verified that the Louisiana Pinesnake could be discriminated from the closely related Bull Snake via melt curve analysis with SYBR™ Green qPCR, and Sanger sequencing of amplicons produced by TaqMan® probe qPCR. Primer mismatches with nontarget species are more crucial for specificity than probe mismatches (Wilcox et al., 2013), but it is not always possible to design primers containing mismatches for congeners. In these scenarios, a probe with mismatches to congeners is essential for specificity (Rodgers et al., 2018). We designed species-specific TaqMan® probes for both assays to remove nontarget amplification (Wilcox et al., 2013) and Sanger sequenced amplicons to confirm species identity. Although we recommend TaqMan® probe qPCR to ensure species-specific detection of the Louisiana Pinesnake, the COI and ATP6 primer sets could enable detection of other species when used with SYBR™ Green chemistry.

Across North America, there are many burrow-dependent snakes (e.g., the threatened Indigo Snake and Eastern Diamondback Rattlesnake, *Crotalus adamanteus* Palisot de Beauvois, 1799) that face similar threats to the Louisiana Pinesnake. eDNA analysis could enable more effective conservation management for these semi-fossorial reptiles through cost-effective, noninvasive surveillance (Baker et al., 2018; Kucherenko et al., 2018; Ratsch et al., 2020). The primers developed here will amplify other species in the *Pituophis* genus and potentially species whose DNA was not tested *in vitro* (see *in silico* results). These primers could be used for preliminary broad-scale monitoring of at-risk snake species to determine where to focus targeted survey efforts and maximize resource allocation. However, there are caveats to this genus-specific approach.

If information on species identity is desirable when using SYBR™ Green qPCR, then differences in Tm are essential for species discrimination. We were able to distinguish the Louisiana Pinesnake and Bull Snake with SYBR™ Green qPCR based on Tm, albeit the differences were small. Small differences in Tm (even $< 0.5°C$)

have been satisfactory for species determination in other eDNA studies using SYBR™ Green qPCR (Berry & Sarre, 2007; Cowart et al., 2018; Harper et al., 2020; Smith, Wood, Mountfort, & Cary, 2012), but future studies could also perform high-resolution melting (HRM) analysis for further verification (Robinson, Uren Webster, Cable, James, & Consuegra, 2018). Where Tm is highly similar and cross-reactive species are sympatric, amplicons should always be Sanger sequenced to confirm species identity (Cowart et al., 2018; Harper et al., 2020).

4.4 | eDNA surveillance for semi-fossorial or semi-aquatic snake species

To date, few studies have evaluated the viability of eDNA surveillance for rare, elusive, semi-fossorial and/or semi-aquatic reptiles, and those that have yielded equivocal results (Adams et al., 2019). Concerning snakes, target taxa have included semi-fossorial crayfish burrow commensals, such as the Eastern Massasauga (Baker et al., 2018; Merkling, 2018) and Kirtland's Snake (Ratsch et al., 2020), as well as semi-aquatic snakes, including the Giant Garter Snake in California wetlands (Halstead et al., 2017), the introduced Banded Water Snake, *Nerodia fasciata* (Linnaeus, 1766), and Northern Water Snake, *Nerodia sipedon* (Linnaeus, 1758), in California waterways (Rose et al., 2019), and the invasive Burmese Python in the Florida Everglades (Hunter et al., 2015, 2019; Orzechowski et al., 2019; Piaggio et al., 2014).

Both Eastern Massasauga studies observed poor eDNA-based detection and occupancy estimation. Baker et al. (2018) recovered only two positive detections, despite sampling when snakes were directly adjacent to or partially in crayfish burrows. Merkling (2018) also recorded few detections from crayfish burrow water and sediment. Conventional surveys (e.g., coverboards, trapping) provided higher detection rates than eDNA surveys for Kirtland's Snake, Giant Garter Snake, Banded Water Snake, and Northern Water Snake (Halstead et al., 2017; Ratsch et al., 2020; Rose et al., 2019). In all studies, snakes were known to occur (sometimes at high abundance) at sampled sites, but eDNA sample processing and qPCR performance may have influenced detection rates.

The Eastern Massasauga and Kirtland's Snake studies centrifuged 50 ml water samples collected from crayfish burrows to separate water from sediment before filtration, but two studies did not extract the resultant pellet (Baker et al., 2018; Ratsch et al., 2020). Centrifugation may spin down eDNA with sediment reducing eDNA yield from water samples and subsequently detection rates. Furthermore, the qPCR assays used by Merkling (2018) and Ratsch et al. (2020) performed suboptimally, with average R^2 and amplification efficiencies outside the recommended ranges (Bustin et al., 2009). The assay used by Ratsch et al. (2020) also exhibited nontarget amplification, and samples were not treated for confirmed inhibition. Assays for Giant Garter Snake, Banded Water Snake, and Northern Water Snake all required a preamplification step with endpoint PCR before qPCR to achieve detection, which is indicative of low sensitivity (Halstead et al., 2017; Rose et al., 2019).

In contrast, all Burmese Python eDNA investigations have been successful with moderate to high detection rates, and eDNA records complementing or outpacing conventional methods, for example, visual searches, trapping, telemetry, or cameras (Hunter et al., 2015, 2019; Kucherenko et al., 2018; Orzechowski et al., 2019; Piaggio et al., 2014). Success may be associated with large population densities and associated high biomass of these snakes. However, these studies also collected more water (~1 L), took soil or sediment, and qPCR average R^2 and amplification efficiencies were within the recommended ranges (Bustin et al., 2009).

Both species-specific and metabarcoding studies have highlighted the importance of sampling strategy for eDNA detection in aquatic and terrestrial systems (Gasparini et al., 2020; Goldberg et al., 2018; Harper, Handley, et al., 2019; Lawson Handley et al., 2019; Leempoel et al., 2020; Valentin et al., 2020). Concerning crayfish burrow commensals, such as the Eastern Massasauga and Kirtland's Snake, crayfish burrows are often complex (Hasiotis & Mitchell, 1993), and snake movements within them are poorly understood. Standard eDNA sampling methodologies may be inappropriate for these snakes if they are using burrow antechambers. Furthermore, these groundwater systems may be particularly prone to high levels of PCR inhibitors (Niemiller et al., 2018). Water chemistry in the study system for the Giant Garter Snake was similarly characterized by inhibitory substances that may have impacted DNA extraction or qPCR (Halstead et al., 2017).

Likewise, abiotic and biotic factors are likely to influence the success of eDNA surveillance for snakes. Time of day (proxy for water temperature) was associated with reduced Burmese Python eDNA detection and concentration in the Florida Everglades (Orzechowski et al., 2019). Heat and UV-B exposure may also have contributed to low eDNA detection rates for the semi-aquatic Giant Garter Snake, Banded Water Snake, and Northern Water Snake in California (Halstead et al., 2017; Rose et al., 2019). Rainfall may facilitate eDNA transport and concentrate or dilute eDNA signals depending on sampling locality. Improved detections of Northern Water Snake were linked to drought conditions that shrank available habitat thereby concentrating snakes and ostensibly eDNA (Rose et al., 2019).

These elements individually or combined may explain the low eDNA detection rates observed for semi-fossorial and/or semi-aquatic snakes in previous investigations (Baker et al., 2018; Halstead et al., 2017; Merkling, 2018; Ratsch et al., 2020; Rose et al., 2019). As such, snake eDNA detection may be improved through sampling strategies that consider species' ecology and behavior, enable greater and continuous soil coverage at higher replication (Leempoel et al., 2020; Zinger et al., 2020) or aggregate eDNA on various terrestrial substrata (Valentin et al., 2020), and account for abiotic and biotic factors.

4.5 | Conclusions

Elusive, rare, and semi-fossorial reptiles are notoriously difficult to survey, requiring intensive time and resource investments, often with

diminished results, and the Louisiana Pinesnake perfectly exemplifies this challenge. To that end, we successfully developed and validated two eDNA assays using TaqMan® probe qPCR chemistry to improve Louisiana Pinesnake detection. Our study revealed that: (1) both assays were highly sensitive and successfully detected Louisiana Pinesnake eDNA in soil collected from release and telemetry-confirmed sites (although we note that the assay is genus-specific as opposed to species-specific, the Louisiana Pinesnake is not sympatric with any congeners); (2) method of eDNA extraction can substantially influence detection success; (3) Louisiana Pinesnake eDNA concentrations in soil were exceptionally low despite sequence-confirmed positive detections; and (4) the Louisiana Pinesnake can be distinguished from congeners with SYBR™ Green qPCR using Tm, but amplicons should be Sanger sequenced to confirm species identity. Based on our own results and those we synthesized from the current snake eDNA literature, we provide recommendations for future studies implementing soil eDNA analysis and highlight the need to include comprehensive eDNA monitoring initiatives to better manage and conserve the Louisiana Pinesnake and other threatened and/or endangered semi-fossorial reptiles. Ultimately, our results are encouraging and suggest that the Louisiana Pinesnake, relative to other snakes, is an excellent candidate for a rangewide eDNA occupancy assessment, which could improve strategic targeting of capacities and resources to improve conservation outcomes for this iconic Longleaf Pine forest endemic.

DATA ARCHIVING STATEMENT

All sequence data generated for this study have been accessioned to GenBank (see Appendix C for accession numbers).

ACKNOWLEDGMENTS

Louisiana Pinesnake blood and tissue samples were collected by Christopher A. Melder under permit number LNHP-16-053, and Josh B. Pierce under permit number SPR-0490-059, and graciously provided by Dr. Matthew Kwiatkowski (Stephen F. Austin University). Bull snake (*Pituophis catenifer sayi*) tissue samples were collected and provided by Dr. Matthew Smith (North Dakota State University) under North Dakota Department of Game and Fish permit numbers GNF02388267 and GNF02599962, and South Dakota Department of Game, Fish, and Parks permit numbers 37 and 51. We also thank Dr. Sasha Tetzlaff for his assistance with statistical modeling, and Mary Best for editing for content and clarity. Finally, we are deeply grateful to two anonymous reviewers, whose recommendations and insights were invaluable to improving this paper for publication.

AUTHOR CONTRIBUTIONS

SP, JHS, and MAD conceived and designed the study. ADK, LRH, ES, CM, SP, JHS, and MAD acquired, analyzed, and interpreted the data. ADK, LRH, ES, CM, SP, JHS, and MAD wrote the manuscript.

ORCID

Aron D. Katz https://orcid.org/0000-0002-7810-9773
Lynsey R. Harper https://orcid.org/0000-0003-0923-1801
Elizabeth C. Sternhagen https://orcid.org/0000-0002-4599-1448
Sarah E. Pearce https://orcid.org/0000-0003-4822-6896
Jinelle H. Sperry https://orcid.org/0000-0003-0929-1900
Mark A. Davis https://orcid.org/0000-0001-9034-9430

REFERENCES

Adams, C. I. M., Hoekstra, L. A., Muell, M. R., & Janzen, F. J. (2019). A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA Under Field Conditions. *Diversity*, *11*(4), 50. https://doi.org/10.3390/d11040050

Agersnap, S., Larsen, W. B., Knudsen, S. W., Strand, D., Thomsen, P. F., Hesselsøe, M., … Møller, P. R. (2017). Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. *PLoS One*, *12*(6), e0179261. https://doi.org/10.1371/journal.pone.0179261

Alaeddini, R. (2012). Forensic implications of PCR inhibition—A review. *Forensic Science International: Genetics*, *6*(3), 297–305. https://doi.org/10.1016/j.fsigen.2011.08.006

Amano, T., Lamming, J. D. L., & Sutherland, W. J. (2016). Spatial gaps in global biodiversity information and the role of citizen science. *BioScience*, *66*(5), 393–400. https://doi.org/10.1093/biosci/biw022

Anderson, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kjaer, K. H., … Willerslev, E. (2011). Meta-barcoding of 'dirt' DNA from soil reflects vertebrate diversity. *Molecular Ecology*, *21*, 1966–1979. https://doi.org/10.1111/j.1365-294X.2011.05261.x

Baker, S. J., Niemiller, M. L., Stites, A. J., Ash, K. T., Davis, M. A., Dreslik, M. J., & Phillips, C. A. (2018). Evaluation of environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces ophiodiicola* in crayfish burrows. *Conservation Genetics Resources*, *12*, 13–15. https://doi.org/10.1007/s12686-018-1053-9

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01.

Berry, O., & Sarre, S. D. (2007). Gel-free species identification using melt-curve analysis. *Molecular Ecology Notes*, *7*(1), 1–4. https://doi.org/10.1111/j.1471-8286.2006.01541.x

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., … Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Buxton, A. S., Groombridge, J. J., & Griffiths, R. A. (2018). Seasonal variation in environmental DNA detection in sediment and water samples. *PLoS One*, *13*(1), e0191737. https://doi.org/10.1371/journal.pone.0191737

Buxton, A. S., Groombridge, J. J., Zakaria, N. B., & Griffiths, R. A. (2017). Seasonal variation in environmental DNA in relation to population size and environmental factors. *Scientific Reports*, *7*, 46294. https://doi.org/10.1038/srep46294

Camaclang, A. E., Maron, M., Martin, T. G., & Possingham, H. P. (2015). Current practices in the identification of critical habitat for threatened species. *Conservation Biology*, *29*(2), 482–492. https://doi.org/10.1111/cobi.12428

Chades, I., McDonald-Madden, E., McCarthy, M. A., Wintle, B., Linkie, M., & Possingham, H. P. (2008). When to stop managing or surveying cryptic threatened species. *Proceedings of the National Academy of Science*, *105*(37), 13936–13490. https://doi.org/10.1073/pnas.0805265105

Conant, R. (1956). A review of two rare pine snakes from the Gulf coastal plain. *American Museum Novitates*, *1781*, 1–31.

Core Team, R. (2020). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Cowart, D. A., Renshaw, M. A., Gantz, C. A., Umek, J., Chandra, S., Egan, S. P., … Larson, E. R. (2018). Development and field validation of an environmental DNA (eDNA) assay for invasive clams of the genus

Corbicula. *Management of Biological Invasions*, *9*(1), 27–37. https://doi.org/10.3391/mbi.2018.9.1.03

Crawford, J. A., Dreslik, M. J., Baker, S. J., Phillips, C. A., & Peterman, W. E. (2020). Factors affecting the detection of an imperiled and cryptic species. *Diversity*, *12*, 177. https://doi.org/10.3390/d12050177

Cunningham, R. B., & Lindenmayer, D. B. (2005). Modeling count data of rare species: Some statistical issues. *Ecology*, *86*(5), 1135–1142. https://doi.org/10.1890/04-0589

Curtis, A. N., & Larson, E. R. (2020). No evidence that crayfish carcasses produce detectible environmental DNA (eDNA) in a stream enclosure experiment. *PeerJ*, *8*, e9333. https://doi.org/10.7717/peerj.9333

Cushman, S. A., & McKelvey, K. S. (2010). Data on distribution and abundance: Monitoring for research and management. In S. A. Cushman, & F. Huettmann (Eds.), *Spatial Complexity, Informatics, and Wildlife Conservation* (pp. 111–130). New York: Springer Publishing. https://doi.org/10.1007/978-4-431-87771-4_6

de Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One*, *11*(10), e0165273. https://doi.org/10.1371/journal.pone.0165273

Durso, A. M., & Seigel, R. A. (2015). A snake in the hand is worth 10,000 in the bush. *Journal of Herpetology*, *49*(4), 503–506. https://doi.org/10.1670/15-49-04.1

Durso, A. M., Wilson, J. D., & Winne, C. T. (2011). Needles in haystacks: Estimating detection probability and occupancy of rare and cryptic snakes. *Biological Conservation*, *144*(5), 1508–1515. https://doi.org/10.1016/j.biocon.2011.01.020

Ealy, M. J., Fleet, R. R., & Rudolph, T. C. (2004). Diel activity patterns of the Louisiana Pine Snake (*Pituophis ruthveni*) in East Texas. *Texas Journal of Science*, *56*(4), 383–394.

Eichmiller, J. J., Miller, L. M., & Sorensen, P. W. (2015). Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular Ecology Resources*, *16*(1), 56–68. https://doi.org/10.1111/1755-0998.12421

Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., … Pompanon, F. (2010). An *in silico* approach for the evaluation of DNA barcodes. *BMC Genomics*, *11*, 434. https://doi.org/10.1186/1471-2164-11-434

Ficetola, G. F., Miaud, C., Popanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*, 423–225. https://doi.org/10.1098/rsbl.2008.0118

Fitzgerald, L. A., Walkup, D., Chyn, K., Buchholtz, E., Angeli, N., & Parker, M. (2018). The future for reptiles: Advances and challenges in the Anthropocene. *Encyclopedia of the Anthropocene*, *3*, 163–174. https://doi.org/10.1016/B978-0-12-809665-9.10291-5

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*(5), 294–299.

Franklin, T. W., McKelvey, K. S., Golding, J. D., Mason, D. H., Dysthe, J. C., Pilgrim, K. L., … Schwartz, M. K. (2019). Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved noninvasive techniques. *Biological Conservation*, *229*, 50–58. https://doi.org/10.1016/j.biocon.2018.11.006

Frost, C. (2006). History and future of the longleaf pine ecosystem. In S. Jose, E. J. Jokela, & D. L. Miller (Eds.), *The longleaf pine ecosystem: Ecology, silviculture and restoration* (pp. 9–48). New York: Springer Science. https://doi.org/10.1007/978-0-387-30687-2_2

Gasparini, L., Crookes, S., Prosser, R. S., & Hanner, R. (2020). Detection of freshwater mussels (Unionidae) using environmental DNA in riverine systems. *Environmental DNA*, *2*(3), 321–329. https://doi.org/10.1002/edn3.71

Gaston, K. J., Blackburn, T. M., Greenwood, J. J. D., Gregory, R. D., Quinn, R. M., & Lawton, J. H. (2000). Abundance-occupancy relationships. *Journal of Applied Ecology*, *37*(s1), 39–59. https://doi.org/10.1046/j.1365-2664.2000.00485.x

Gibbons, J. W., Scott, D. E., Ryan, T. J., Buhlmann, K. A., Tuberville, T. D., Metts, B. S., … Winne, C. T. (2000). The global decline of reptiles, déjà vu amphibians. *BioScience*, *50*(8), 653–666. https://doi.org/10.1641/0006-3568(2000)050[0653:TGDORD]2.0.CO;2

Goldberg, C. S., Strickler, K. M., & Fremier, A. K. (2018). Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of sampling designs. *Science of the Total Environment*, *633*, 695–703. https://doi.org/10.1016/j.scitotenv.2018.02.295

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., … Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299–1307. https://doi.org/10.1111/2041-210X.12595

Gu, W., & Swihart, R. K. (2004). Absent or undetected? Effects of non-detection of species occurrence on wildlife-habitat models. *Biological Conservation*, *116*(2), 195–203. https://doi.org/10.1016/S0006-3207(03)00190-3

Guisan, A., Broennimann, O., Engler, R., Vust, M., Yoccoz, N. G., Lehmann, A., & Zimmermann, N. E. (2006). Using niche-based models to improve sampling of rare species. *Conservation Biology*, *20*(2), 501–511. https://doi.org/10.1111/j.1523-1739.2006.00354.x

Halstead, B. J., Wood, D. A., Bowen, L., Waters, S. C., Vandergast, J. S. E., Skalos, S. M., & Casazza, M. L. (2017). An evaluation of the efficacy of using environmental DNA (eDNA) to detect Giant Gartersnakes (Thamnophis gigas): U.S. Geological Survey Open-File Report 2017–1123, 41 p. doi: 10.3133/ofr20171123.

Hammerson, G. A. (2007). *Pituophis ruthveni*. *The IUCN Red List of Threatened Species 2007* (p. e.T63874A12723685.). https://doi.org/10.2305/IUCN.UK.2007.RLTS.T63874A12723685.en

Harper, K. J., Goodwin, K. D., Harper, L. R., LaCasella, E. L., Frey, A., & Dutton, P. H. (2020). Finding crush: Environmental DNA analysis as a tool for tracking the Green Sea turtle *Chelonia mydas* in a marine estuary. *Frontiers in Marine Science*, *6*, 810. https://doi.org/10.3389/fmars.2019.00810

Harper, L. R., Griffiths, N. P., Lawson Handley, L., Sayer, C. D., Read, D. S., Harper, K. J., … Hänfling, B. (2019). Development and application of environmental DNA surveillance for the threatened crucian carp (*Carassius carassius*). *Freshwater Biology*, *64*(1), 93–107. https://doi.org/10.1111/fwb.13197

Harper, L. R., Handley, L. L., Carpenter, A. I., Ghazali, M., Di Muri, C., Macgregor, C. J., … Hänfling, B. (2019). Environmental DNA (eDNA) metabarcoding of pond water as a tool to survey conservation and management priority mammals. *Biological Conservation*, *238*, 108225. https://doi.org/10.1016/j.biocon.2019.108225

Hasiotis, S. T., & Mitchell, C. E. (1993). A comparison of crayfish burrow morphologies: Triassic and Holocene fossil, paleo- and neo-ichnological evidence and the identification of their burrowing signatures. *Ichnos*, *2*(4), 291–314. https://doi.org/10.1080/10420949309380104

Himes, J. G., Hardy, L. M., Rudolph, D. C., & Burgdorf, S. J. (2006). Movement patterns and habitat selection by native and repatriated Louisiana Pine Snakes (*Pituophis ruthveni*): Implications for conservation. *Herpetological Natural History*, *9*(2), 103–116.

Hunter, M. E., Meigs-Friend, G., Ferrante, J. A., Smith, B. J., & Hart, K. M. (2019). Efficacy of eDNA as an early detection indicator for Burmese Pythons in the ARM Loxahatchee National Wildlife Refuge in the greater Everglades ecosystem. *Ecological Indicators*, *102*, 617–622. https://doi.org/10.1016/j.ecolind.2019.02.058

Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., … Hart, K. M. (2015). Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese Pythons. *PLoS One*, *10*(4), e0121655. https://doi.org/10.1371/journal.pone.0121655

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*, 150–157. https://doi.org/wo.wwww/j.1755-263x.2010.00158.x. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Johnson, M. D., Cox, R. D., & Barnes, M. A. (2019). Analyzing airborne environmental DNA: A comparison of extraction methods, primer type, and trap type on the ability to detect airborne eDNA from terrestrial plant communities. *Environmental DNA*, *1*(2), 176–185. https://doi.org/10.1002/edn3.19. https://doi.org/10.1002/edn3.19

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Katz, A. D., Pearce, S., Melder, C., Sperry, J. H., & Davis, M. A. (2020). Molecular sexing is a viable alternative to probing for determining sex in the imperiled Louisiana Pine Snake (*Pituophis ruthveni*). *Conservation Genetics Resources*, https://doi.org/10.1007/s12686-020-01145-9

Kessler, E. J., Ash, K. T., Barratt, S. N., Larson, E. R., & Davis, M. A. (2020). Radiotelemetry reveals effects of upstream biomass and UV exposure on environmental DNA occupancy and detection for a large freshwater turtle. *Environmental DNA*, *2*(1), 13–23. https://doi.org/10.1002/edn3.42

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., … Richter, C. A. (2019). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271–282. https://doi.org/10.1002/edn3.29

Kucherenko, A., Herman, J. E., Everham, E. M. III, & Urakawa, H. (2018). Terrestrial Snake Environmental DNA Accumulation and Degradation Dynamics and its Environmental Application. *Herpetologica*, *74*(1), 38–49. https://doi.org/10.1655/Herpetologica-D-16-00088

Landers, J. L., van Lear, D. H., & Boyer, W. D. (1995). The longleaf pine forests of the southeast: Requiem or renaissance? *Journal of Forestry*, *93*(11), 39–44.

Lawson Handley, L., Read, D. S., Winfield, I. J., Kimbell, H., Johnson, H., Li, J., … Hänfling, B. (2019). Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA*, *1*(1), 26–39. https://doi.org/10.1002/edn3.5

Leempoel, K., Hebert, T., & Hadly, E. A. (2020). A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings of the Royal Society B*, *287*(1918), 20192353. https://doi.org/10.1098/rspb.2019.2353

Lenth, R. (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means. *R Package Version*, *1*(4), 6. https://CRAN.R-project.org/package=emmeans .

Lindenmayer, D. B., & Franklin, J. (2002). *Conserving forest biodiversity*. Covelo, California: Island Press.

Lüdecke, D., Makowski, D., Waggoner, P., & Patil, I. (2020). *performance: Assessment of Regression Models Performance*. R package version 0.4.5.1. https://easystats.github.io/performance/.

Makowski, D., Ben-Shachar, M., & Lüdecke, D. (2019). *Describe and understand your model's parameters*. R package: CRAN. https://github.com/easystats/parameters.

Martellini, A., Payment, P., & Villemur, R. (2005). Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Resources*, *39*, 541–548. https://doi.org/10.1016/u.watres.2004.11.012

Merkes, C. M., McCalla, S. G., Jensen, N. R., Gaikowski, M. P., & Amberg, J. J. (2014). Persistence of DNA in carcasses, slime, and avian feces may affect interpretation of environmental DNA data. *PLoS One*, *9*(11), e113346. https://doi.org/10.1371/journal.pone.0113346

Merkling, J. L. (2018). *Development of an environmental DNA assay for Eastern Massasauga*. M.S. Thesis, Purdue University. https://doi.org/10.25394/PGS.7495952.v1.

Niemiller, M. L., Porter, M. L., Keany, J., Gilbert, H., Fong, D. W., Hobson, C. S., … Taylor, S. J. (2018). Evaluation of eDNA for groundwater invertebrate detection and monitoring: A case study with endangered *Stygobromus* (Amphipoda: Crangonyctidae). *Conservation Genetics Resources*, *10*, 247–257. https://doi.org/10.1007/s12686-017-0785-2

Orzechowski, S. C. M., Ferderick, P. C., Dorazio, R. M., & Hunter, M. E. (2019). Environmental DNA sampling reveals high occupancy rates of invasive Burmese pythons at wading bird breeding aggregations in the central Everglades. *PLoS One*, *14*(4), e0213943. https://doi.org/10.1371/journal.pone.0213943

Oswalt, C. M., Cooper, J. A., Brockway, D. G., Brooks, H. W., Walker, J. L., Connor, K. F., …Conner, R. C.(2012). History and current condition of longleaf pine in the southern United States. United States Department of Agriculture, United States Forest Service Southern Research Station General Technical Report SRS-166. https://www.fs.usda.gov/treesearch/pubs/42259.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacker, K. L., Bruce, W. E., & Avery, M. L. (2014). Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Molecular Ecology Resources*, *14*(2), 374–380. https://doi.org/10.1111/1755-0998.12180

Pierce, J. B., Rudolph, D. C., Burdgorf, S. J., Schaefer, R. R., Conner, R. N., Himes, J. G., … Fleet, R. R. (2014). Winter movements of Louisiana Pine Snakes (*Pituophis ruthveni*) in Texas and Louisiana. *Southeastern Naturalist*, *13*, 137–145. https://doi.org/10.1656/058.013.s513

Ratsch, R. A., Kingsbury, B. A., & Jordan, M. A. (2020). Exploration of environmental DNA (eDNA) to detect Kirtland's Snake (*Clonophis kirtlandii*). *Animals*, *10*, 1057. https://doi.org/10.3390/ani10061057

Reading, C. J., Luiselli, L. M., Akani, G. C., Bonnet, X., Amori, G., Ballouard, J. M., … Rugiero, L. (2010). Are snake populations in widespread decline? *Biology Letters*, *6*(6), 777–780. https://doi.org/10.1098/rsbl.2010.0373

Renshaw, M. A., Olds, B. P., Jerde, C. L., Mcveigh, M. M., & Lodge, D. M. (2015). The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. *Molecular Ecology Resources*, *15*(1), 168–176. https://doi.org/10.1111/1755-0998.12281

Robinson, C. V., Uren Webster, T. M., Cable, J., James, J., & Consuegra, S. (2018). Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biological Conservation*, *222*, 241–252. https://doi.org/10.1016/j.biocon.2018.04.009

Rodgers, T. W., Olson, J. R., Klobucar, S. L., & Mock, K. E. (2018). Quantitative PCR assays for detection of five arctic fish species: *Lota lota*, *Cottus cognatus*, *Salvelinus alpinus*, *Salvelinus malma*, and *Thymallus arcticus* from environmental DNA. *Conservation Genetics Resources*, *10*, 859–865. https://doi.org/10.1007/s12686-017-0883-1

Rose, J. P., Wademan, C., Weir, S., Wood, J. S., & Todd, B. D. (2019). Traditional trapping methods outperform eDNA sampling for introduced semi-aquatic snakes. *PLoS One*, *14*(7), e0219244. https://doi.org/10.1371/journal.pone.0219244

Rosenfeld, J. S., & Hatfield, T. (2006). Information needs for assessing critical habitat of freshwater fish. *Canadian Journal of Fisheries and Aquatic Science*, *63*(3), 683–698. https://doi.org/10.1139/f05-242

Rudolph, D. C., & Burgdorf, S. J. (1997). Timber rattlesnakes and Louisiana pine snakes of the west Gulf Coast plain: Hypotheses of decline. *Texas Journal of Science*, *49*(3), 111–122.

Rudolph, D. C., Burgdorf, S. J., Schaefer, R. R., Conner, R. N., & Maxey, R. W. (2006). Status of *Pituophis ruthveni* (Louisiana Pine Snake). *Southeastern Naturalist*, *5*(3), 463–472. https://doi.org/10.1656/1528-7092(2006)5[463:SOPRLP]2.0.CO;2

Rudolph, D. C., Schaefer, R. R., Burgdorf, S. J., Duran, M., & Conner, R. N. (2007). Pine snake (*Pituophis ruthveni* and *Pituophis melanoleucus lodingi*) hibernacula. *Journal of Herpetology*, *41*, 560–565. https://doi.org/10.1670/06-235.1

Rushton, S. P., Ormerod, S. J., & Kerby, G. (2004). New paradigms for modelling species distributions? *Journal of Applied Ecology*, *41*(2), 193–200. https://doi.org/10.1111/j.0021-8901.2004.00903.x

Seddon, P. J., Griffiths, C. J., Soorae, P. S., & Armstrong, D. P. (2014). Reversing defaunation: Restoring species in a changing world. *Science*, *345*(6195), 406–412. https://doi.org/10.1126/science.1251818

Sellers, G. S., Di Muri, C., Gómez, A., & Hänfling, B. (2018). Mu-DNA: A modular universal DNA extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics*, *2*, e24556. https://doi.org/10.3897/mbmg.2.24556

Smith, K. F., Wood, S. A., Mountfort, D. O., & Cary, S. C. (2012). Development of a real-time PCR assay for the detection of the invasive clam, *Corbula amurensis*, in environmental samples. *Journal of Experimental Marine Biology and Ecology*, *412*, 52–57. https://doi.org/10.1016/j.jembe.2011.10.021

Steen, D. A. (2010). Snakes in the grass: Secretive natural histories defy both conventional and progressive statistics. *Herpetological Conservation and Biology*, *5*(2), 183–188.

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Stull, O. G. (1929). The description of a new subspecies of *Pituophis melanoleucus* from Louisiana. Occasional Papers of the Museum of Zoology, University of Michigan,*205*, 1–3.

Thomas, R. A., Davis, B. J., & Culbertson, M. R. (1976). Notes on variation and range of the Louisiana Pine Snake, *Pituophis melanoleucus ruthveni* Stull (Reptilia, Serpentes, Colubridae). *Journal of Herpetology*, *10*(3), 252–254. https://doi.org/10.2307/1562989

Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., … Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA–an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Todd, B. D., Wilson, J. D., & Gibbons, J. W. (2010). The global status of reptiles and causes of their decline. In D. W. Sparling, S. A. Bishop, & S. Krest (Eds.), *Ecotoxicology of Amphibians and Reptiles*, 2nd ed. (pp. 47–67). Pensacola: CRC Press.

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., & Roussel, J.-M. (2014). Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, *51*(4), 871–879. https://doi.org/10.1111/1365-2664.12262

Tsuji, S., Ushio, M., Sakurai, S., Minamoto, T., & Yamanaka, H. (2017). Water temperature-dependent degradation of environmental DNA and its relation to bacterial abundance. *PLoS One*, *12*(4), e0176608. https://doi.org/10.1371/journal.pone.0176608

Turner, C. R., Miller, D. J., Coyne, K. J., & Corush, J. (2014). Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys* spp.). *PLoS One*, *9*(12), e114329. https://doi.org/10.1371/journal.pone.0114329

Valentin, R. E., Fonseca, D. M., Gable, S., Kyle, K. E., Hamilton, G. C., Nielsen, A. L., & Lockwood, J. L. (2020). Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects. *Molecular Ecology Resources*, *20*(3), 746–755. https://doi.org/10.1111/1755-0998.13151

Wacker, S., Fossøy, F., Larsen, B. M., Brandsegg, H., Sivertsgård, R., & Karlsson, S. (2019). Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration. *Environmental DNA*, *1*(1), 64–73. https://doi.org/10.1002/edn3.10

Wagner, R. O., Pierce, J. B., Rudolph, D. C., Schaefer, R. R., & Hightower, D. A. (2014). Modeling Louisiana Pine Snake (*Pituophis ruthveni*) habitat use in relation to soils. *Southeastern Naturalist*, *13*(5), 146–158. https://doi.org/10.1656/058.013.s514

Wickham, H. (2009). ggplot2: *Elegant graphics for data analysis*. New York: *Springer*. https://doi.org/10.1007/978-0-387-98141-3

Wilcox, T. M., Carim, K. J., McKelvey, K. S., Young, M. K., & Schwartz, M. K. (2015). The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of Oncorhynchus. *PLoS One*, *10*(11), e0142008. https://doi.org/10.1371/journal.pone.0142008

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS One*, *8*(3), e59520. https://doi.org/10.1371/journal.pone.0059520

Young, R. A., & Vanderventer, T. L. (1988). Recent observations on the Louisiana Pine Snakes, *Pituophis melanoleucus ruthveni* (Stull). *Bulletin of the Chicago Herpetological Society*, *23*(12), 203–207.

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*, *7*(1–2), 203–214. https://doi.org/10.1089/10665270050081478

Zinger, L., Donald, J., Brosse, S., Gonzalez, M. A., Iribar, A., Leroy, C., … Lopes, C. M. (2020). *Advances and prospects of environmental DNA in neotropical rainforests*. *Advances in ecological research*. London: Academic Press.

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects Models and extensions in ecology with R*. New York: Springer. https://doi.org/10.1007/978-0-387-87458-6

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Katz AD, Harper LR, Sternhagen EC, et al. Environmental DNA is effective in detecting the federally threatened Louisiana Pinesnake (*Pituophis ruthveni*). *Environmental DNA*. 2020;00:1–17. https://doi.org/10.1002/edn3.126