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Persistence of Reptile DNA in a Terrestrial Substrate: A Case Study Using the Eastern Indigo Snake

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ABSTRACT

Environmental DNA (eDNA) analysis of terrestrial substrates, such as soil and sand, is a rapid and potentially cost-effective way to monitor rare wildlife species. A promising use-case in the southeastern United States is provided by the eastern indigo snake (*Drymarchon couperi*), for which accurate monitoring has been challenging due to large home ranges and low-density populations. However, knowledge gaps regarding eDNA deposition and persistence in this system currently limit our ability to apply eDNA sampling effectively at the landscape scale. To overcome some of these gaps, we used an optimized soil and sand eDNA extraction protocol and species-specific qPCR assay to conduct a full factorial experiment of eastern indigo snake DNA detection in sand as a function of the duration of snake presence and time since snake removal. We then used these data and a generalized linear mixed model to predict detection probability. Of the 224 total experimental samples, 68 (30.4%) tested positive for eastern indigo snake eDNA. Our model predicted that, with long periods in the enclosure and sampling soon after snake removal, eastern indigo snake eDNA is detectable 68.7% of the time. Eastern indigo snake DNA was detectable in as little as 100 s of snake presence in the enclosure (Pr = 21.1%) and for as long as 10 days after snake presence (Pr = 27.7%). These results suggest that DNA sampling in terrestrial systems may be an effective tool for increasing the temporal window of rare snake detection and a useful complement to existing sampling methods for eastern indigo snakes.

1 | Introduction

Conservation relies on high-quality monitoring, especially of invasive, rare, or endangered species. The most common form of wildlife monitoring involves obtaining distribution and abundance estimates over multiple time periods. These data impact nearly all conservation actions, including species status assessments, reintroduction programs, and designation of protected areas (IUCN/SSC 2013; Ranius et al. 2023; USFWS 2016). However, accurate monitoring can be challenging to achieve. Environmental DNA (eDNA) sampling—the inference of species presence from genetic material in the environment—is an emerging method of biomonitoring that is quick and cost-effective (Smart et al. 2016; Sternhagen et al. 2024; Wilcox

et al. 2016), making it feasible to monitor over large geographic areas and at finer-scales than conventional approaches. Further, because organisms may leave behind a genetic signal that lasts for hours or days (Andruszkiewicz et al. 2020; Collins et al. 2018; Thomsen et al. 2012), a single environmental sample integrates information about habitat use over an extended period of time. This makes eDNA sampling potentially useful for expanding the temporal window of detection, especially for highly mobile species.

Water is the most extensively sampled eDNA medium and has been used to detect a variety of taxa, including snakes (Hunter et al. 2019; Kronenberger et al. 2024; Sepulveda et al. 2019; Wilcox et al. 2013). However, inferences are limited to aquatic or semi-aquatic species (but see Deiner et al. 2016; Wilcox et al. 2021), restricting the species and environments for which sampling eDNA in water is effective. An alternative eDNA sampling medium for terrestrial species is soil or other terrestrial media, which—although not as extensively covered in the literature—has been used to detect eDNA from mammals and reptiles in terrestrial systems (e.g., Kyle et al. 2022; Leempoel, Hebert, and Hadly 2020). Effective eDNA sampling in terrestrial systems is limited due to the shortage of research on the "ecology" of eDNA (sensu Barnes and Turner 2016) in soil, sand, and sediment. Here, we use the eastern indigo snake as a case study of eDNA persistence under environmental conditions in sand.

The eastern indigo snake (*Drymarchon couperi*, hereafter EIS; Holbrook 1842) is the longest native snake in North America. These non-venomous apex predators are active, highly mobile hunters that inhabit large home ranges (Bauder et al. 2020; Breininger et al. 2011; Hyslop et al. 2014; Landers and Speake 1980). Their extant distribution includes peninsular Florida and southern Georgia, with populations currently being reintroduced into Alabama and the panhandle of Florida (Folt et al. 2019; Godwin et al. 2011; Piccolomini 2020; Stiles et al. 2013). In the northern portion of their range, EIS depends on gopher tortoise burrows (*Gopherus polyphemus*) to shelter during winter months (Diemer and Speake 1983; Hyslop, Cooper, and Meyers 2009; Lawler 1977; Stevenson et al. 2009). Gopher tortoise burrows are often located in xeric sandhills which constitute the majority of EIS overwintering habitat. However, the species can be found in a variety of other habitats including dry prairies, wetlands, and scrub habitats (Bauder et al. 2018; Hyslop et al. 2014). As generalist predators, EIS actively forage on a wide assortment of prey, including snakes, frogs, rodents, and juvenile tortoises (Stevenson et al. 2010).

The species faces substantial conservation challenges (IUCN RedList Status: Least Concern but Declining; Hammerson 2007; NatureServe Rank: G3, Declining; NatureServe 2024) and was listed by the U.S. Fish and Wildlife Service (USFWS) under the Endangered Species Act (ESA) in 1978 as Federally Threatened (Federal Register 43:4026). Primary threats to the EIS historically included habitat loss and alteration, collection for the pet trade, and gassing (i.e., the intentional introduction of diesel fuel) of gopher tortoise burrows to collect rattlesnakes (USFWS 2019a). While regulations and enforcement have reduced the threat posed by collection, and gassing gopher tortoise burrows is no longer legal, habitat loss, degradation, and fragmentation remain a substantial threat (USFWS 2019b). In 2019, the USFWS published an updated recovery plan for EIS, outlining recovery actions and delisting criteria (USFWS 2019c). This recovery plan stipulates that the collection of high-quality monitoring data across the species' range is a conservation imperative.

Monitoring EIS populations is challenging due to their large home ranges and low-density populations in many areas throughout their range. The primary survey technique has been to conduct visual (active search) assessments of potential habitat. For example, Stevenson et al. (2009) conducted an 8-year study that included active searches of gopher tortoise burrows at Fort Stewart, during which they captured and marked 93 unique EIS. This method is used because it is relatively low cost and a large area can be surveyed during each visit; however, with this technique, occupancy information is limited to specific time points when the surveys are conducted (low temporal coverage; Bauder et al. 2017). To address this, pilot studies have explored camera trapping as an alternative approach (Erickson 2023). This passive technique deploys cameras at locations thought to be used frequently by EIS. Camera traps can be placed at gopher tortoise burrow entrances, or they can be coupled with drift fencing to funnel snakes into a single camera location from a larger area of suitable habitat (e.g., Erickson 2023). A benefit of this approach is that camera traps can collect occupancy data continuously across much longer time frames (high temporal coverage). However, costs associated with passive camera traps often result in surveying fewer locations (low spatial coverage) than active searches and require significant time investments to process camera photos.

Environmental DNA sampling may be a way to increase spatial and temporal coverage of EIS monitoring. Preliminary studies have been conducted using soil eDNA sampling to detect multiple species of snakes in situ and to test snake DNA persistence in soil and sediment (Katz et al. 2021; Kucherenko et al. 2018; Matthias et al. 2021; Ratsch, Kingsbury, and Jordan 2020). In field studies, soil eDNA sampling was able to detect snakes with known presence between 40% (Kucherenko et al. 2018) and 75% (Katz et al. 2021) of the time. Kucherenko et al. (2018) also found, in an experimental study, that corn snake (*Pantherophis guttatus*) eDNA is detectable in soil up to six days after snake removal. However, there are still knowledge gaps that limit our ability to apply terrestrial eDNA sampling effectively at the landscape scale—particularly the effect of duration of habitat use and time since snake occupancy on eDNA persistence and detectability.

Here, we addressed this knowledge gap using a full factorial experiment with a captive population of EIS. We controlled for (1) duration of snake contact with the sand (i.e., time in enclosure) and (2) time since snake contact with the sand after snake removal (i.e., sand collection time) to understand their influence on detection probability. We analyzed samples from this experiment using an optimized eDNA extraction method and a new, species-specific qPCR assay. Our results provide essential background information to support future use of eDNA in terrestrial substrates to monitor EIS and potentially other reptile species.

2 | Methods

2.1 | Study Design and Field Collection

We conducted the controlled study at the Central Florida Zoo's Orianne Center for Indigo Conservation (OCIC) in Eustis (Florida, USA) from August 18 to September 11, 2023. To test EIS eDNA detectability as a function of time in enclosure and sand collection time, we divided seven circular enclosures filled with sand (approximately 30 cm deep; sandy fill outbound; Mid Florida Materials; Zellwood, FL) by silt fencing and stakes to make quadrats of similar sizes (0.929–1.041 m^2; Figure 1). For each experimental replicate, we placed a captive-bred snake sequentially into four different quadrats for 100 s, 15 min, 1, and 2 h. We collected sand at seven time points after snake removal from each quadrat: 1, 4, 24, 48, 72 h, 7, and 10 days. We took multiple sub-samples to help integrate the eDNA signal from snakes which may have moved across the enclosure and used portions of the enclosure unevenly. At each sampling event we collected five sub-samples of 10 mL of sand. These were stratified randomly across the quadrat and were drawn from the top several millimeters of sand to catch eDNA on the sand surface. We used a sterile, 50 mL tube attached to a long pole in order to sample without entering quadrats used by snakes. For quadrats that were reachable without the use of a pole, samples were collected by holding the tube with gloved hands. Samples were immediately placed in plastic bags and transferred to a −20°C freezer within an hour. Samples were shipped overnight to the National Genomic Center for Wildlife and Fish Conservation (NGC; Missoula, Montana, USA) on dry ice and stored in another −20°C freezer prior to DNA extraction. This study design resulted in 28 sampling events per snake and was repeated across eight snakes for 224 total sampling events. Snakes weighed between 1680 and 3750 g with a median weight of 2800 g and were between 165.1 and 210.3 cm in total length, with a median total length of 185.5 cm (Table S1). We collected negative control samples from each enclosure prior to snake introduction and between experimental replicates ($n = 10$ over the course of the study).

We sterilized sampling equipment with a 20% household bleach solution (~1.2% sodium hypochlorite) and wore new nitrile gloves for each collection. Enclosures reused across replicates were cleaned of sand, sterilized with a 20% household bleach solution, rebuilt with new silt fencing, and refilled with fresh sand that had never been exposed to EIS. To measure environmental conditions during the experiment, we installed four light (lux) and temperature loggers (HOBO MX2202; Onset; Bourne, Massachusetts, USA) into four different enclosures and used precipitation data from the nearest National Oceanic and Atmospheric Administration (NOAA) weather station at Leesburg International Airport (approximately 14 km away).

All experiments were conducted with approval from the Central Florida Zoo and Botanical Gardens Research Committee (Project 2020–05) and cared for according to the Association of Zoos and Aquariums (AZA) Eastern Indigo Snake (*Drymarchon couperi*) Care Manual (AZA Snake TAG 2011).

2.2 | DNA Extraction

We optimized the extraction protocol by comparing DNA yields from variations on three different extraction methods (see Supporting Information). Our top extraction protocol utilized filtration of solubilized DNA released from sediments using the method of Taberlet et al. (2012; hereafter the filter method). To extract each sample, the five 10 mL sub-samples were pooled and then mixed 1:1 by volume with sodium phosphate buffer (0.12 M, pH ~8; Sigma-Aldrich; St. Louis, Missouri, USA) and agitated for 20 min to displace primarily extracellular DNA. This mixture was then centrifuged for 5 min at 1100 × *g* and the supernatant filtered through a 0.1 μm pore size, 47 mm diameter mixed cellulose ester membrane filter (MilliporeSigma; Burlington, Massachusetts, USA) using an electronic peristaltic pump. Filters were preserved in silica desiccant and frozen at −20°C until DNA extraction using the modified DNeasy Blood and Tissue Kit (QIAGEN; Germantown, Maryland, USA) protocol described in Franklin

FIGURE 1 | Eastern indigo snake experimental enclosures while (a) an OCIC staff member collects sand samples and (b and c) eastern indigo snakes are present in the enclosure. One quadrat in each enclosure was exclusively used by staff members in order to collect sand samples in the remaining quadrats (and, therefore, was never occupied by snakes).

et al. (2019). To avoid contamination, we conducted all work in pre-PCR laboratory spaces and extractions were performed in a sterile hood that had been cleaned with a 20% household bleach solution and irradiated with ultraviolet (UV) light for 1 h between sets, in accordance with the Standard Operating Procedures of the NGC. Extraction sets varied between 23 and 48 samples and were randomized to reduce operator and technical bias. Sand handling and disposal was done in compliance with Animal and Plant Health Inspection Service (APHIS) Agreement # MT-SL-2024-01.

2.3 | qPCR

We designed a species-specific quantitative PCR (qPCR) assay with hydrolysis probe for detection of EIS mitochondrial DNA at the cytochrome b (*cytb*) locus; forward primer: 5′-GCATCCTTCGATCAATTCCAAAT-3′, reverse primer: 5′-GGGCGGAAYGTTATYGATCGA-3′, and probe: 5′-CTTATA ATCATACCATTCACCCA-MGB-NFQ-3′. We optimized primer concentrations by testing all possible combinations of forward and reverse primers at 100, 300, 600, and 900 nM while holding probe concentrations at 250 nM ($n = 16$ combinations total). We retained the combination with the lowest primer concentrations that generated the earliest C_t value and highest end-point fluorescence. We created a serial dilution of 15625, 3125, 625, 125, 25, 5, 1, 0.5, 0.25, and 0 copies/reaction with six replicates at each dilution level, made from a gBlock gene fragment (IDT; Coralville, Iowa, USA). We used this standard dilution to parameterize the eLowQuant model (Lesperance et al. 2021) to estimate limit of blank (LOB), limit of detection (LOD; the lowest copy number an assay can detect in 95% of replicates; Bustin et al. 2009; Klymus et al. 2020), and limit of quantification (LOQ), assuming three replicates per sample to match the level of replication for samples in this study.

We tested the generality of this assay in silico by comparing oligonucleotide sequences with 76 EIS *cytb* sequences from across the species' range (as produced by Krysko et al. 2016). We tested generality in vitro by testing DNA extracted from 25 EIS tissues. Twenty-four tissue-derived DNA samples were from a regional assessment of genetic structure within EIS, with tissues from across both "Gulf" and "Atlantic" lineages in Florida and Georgia, as described by Folt et al. (2019). Nineteen samples were from the Auburn University Museum of Natural History (Loan# AUM-H 2021–008) and five were from the Museum of Natural History Genetic Resources Repository (Voucher# 123800, 150061, 157531, 159826, 169236). We also tested one tissue sample from a 2018 road mortality collected in Florida (Angy Chambers; *personal communication*).

We tested specificity of the assay in silico using the framework of Kronenberger et al. (2024) and the eDNAssay classifier—a highly accurate machine learning tool that predicts qPCR cross-amplification from sequence alignments (Kronenberger et al. 2022). This tool assigns each sequence a probability of belonging to the "amplify" class (hereafter, assignment probability). While assignment probabilities do not equate to amplification probabilities, prior work indicates that DNA templates with assignment probabilities ≥0.3 are very unlikely to cross-amplify (Kronenberger et al. 2022; Kronenberger et al. 2024). We tested all 47 confamilial species that occur in Georgia and Florida, of which all but two had *cytb* sequence data available on GenBank (Sayers et al. 2019; Table 1). For the two confamilials that did not have publicly available *cytb* data (*Storeria victa* and *Tantilla oolitica*; Table 1) we used the framework described in Wilcox et al. (2024) to estimate cross-amplification risk for an unsequenced confamilial species based on the distribution of eDNAssay assignment probabilities for sequenced confamilials. In addition, we identified 13 of the confamilial species in Georgia and Florida that would be most likely to co-occur with EIS and tested the highest eDNA assignment probability haplotype in vitro using synthetic DNA fragments (Twist Bioscience; San Francisco, California, USA). Finally, we conducted a Primer-BLAST assessment of GenBank to identify taxa which may cross-amplify and were not previously identified.

We included an internal positive control (IPC) to check for PCR inhibitors, with inhibited samples being characterized by a Ct shift of ≥1 relative to the negative control. Any inhibited samples were treated with an inhibitor removal column ($n = 4$, Zymo Inhibitor Removal Kit; Zymo Research; Irvine, California, USA) and then re-analyzed. Each qPCR well contained 7.5 μL of 2× TaqMan Environmental Master Mix (ThermoFisher Scientific; North Logan, Utah, USA), 0.75 μL of 20× assay mix (optimized primer concentrations; IDT; Coralville, Iowa, USA), 0.95 μL of sterile water, 4 μL of template DNA, 1.5 μL of 10× exogenous IPC assay, and 0.3 μL of 50× exogenous IPC template (TaqMan Exogenous IPC Kit; ThermoFisher Scientific; North Logan, Utah, USA). Each plate included a negative control, and a standard curve serially diluted fivefold, from 15,625 to 1 copy per reaction. Each sample was run in triplicate and deemed positive if there was linear amplification in at least one replicate. Quantification was performed per sample by averaging across all replicates that amplified. Thermocycling conditions were 95°C 10 min, (95°C 15 s, 60°C 1 min)×45. All PCR preparations were conducted in a dedicated space, physically isolated from the post-PCR laboratory, and in a hood that was irradiated with UV light for 1 h between each set, in accordance with the Standard Operating Procedures of the NGC.

2.4 | Modeling

We modeled detection probability of EIS eDNA using a binomial generalized linear mixed-effects model (GLMM) with the logit link function. We chose to use presence/absence data (i.e., binomial model) because eDNA abundance distribution was strongly right-skewed and zero-inflated (see Results). Main effects in the global model were time in the enclosure and sand collection time, with individual snake ID included as a random intercept to account for repeated measures. To this global model we separately added the following environmental covariates: mean daily light exposure, temperature, and precipitation between the time the snake was removed from the enclosure and when the sand sample was taken. Environmental covariates were added separately, and mean values were used instead of cumulative and maximum values due to collinearity. We tested the impact of each of these covariates using Akaike Information Criterion (AIC) and Likelihood Ratio Tests (LRT). Models were fit with the *lme4* package (Bates et al. 2015) in R (R Core Team 2008) using the *glmer* function.

TABLE 1 | Name and eDNAssay assignment probability (AP) for all 47 confamilial species that occur in Georgia and Florida.

Common	Latin	# of sequences tested	Max AP
Common wormsnake	*Carphophis amoenus*	1	0.242
Scarletsnake	*Cemophora coccinea*[b]	5	0.228
North American Racer	*Coluber constrictor*[b]	262	0.403
Ring-necked snake	*Diadophis punctatus*	5	0.289
Eastern indigo snake	***Drymarchon couperi***	**73**	**0.792**[a]
Red-bellied mudsnake	*Farancia abacura*	3	0.247
Rainbow snake	*Farancia erytrogramma*	1	0.17
Eastern hog-nosed snake	*Heterodon platirhinos*[b]	1	0.19
Southern hog-nosed snake	*Heterodon simus*[b]	1	0.159
Scarlet kingsnake	*Lampropeltis elapsoides*[b]	5	0.165
Short-tailed kingsnake	*Lampropeltis extenuate*[b]	1	0.254
Eastern kingsnake	*Lampropeltis getula*[b]	5	0.21
Eastern black kingsnake	*Lampropeltis nigra*	4	0.21
South Florida mole kingsnake	*Lampropeltis occipitolineata*	3	0.149
Northern mole kingsnake	*Lampropeltis rhombomaculata*	5	0.149
Striped swampsnake	*Liodytes alleni*	1	0.202
Black swampsnake	*Liodytes pygaea*	1	0.231
Glossy swampsnake	*Liodytes rigida*	1	0.260
Coachwhip	*Masticophis flagellum*[b]	2	0.207
Saltmarsh snake	*Nerodia clarkii*	5	0.215
Mississippi green watersnake	*Nerodia cyclopion*	2	0.206
Plain-bellied watersnake	*Nerodia erythrogaster*	5	0.218
Southern watersnake	*Nerodia fasciata*	5	0.224
Florida green watersnake	*Nerodia floridana*	4	0.192
Diamond-backed watersnake	*Nerodia rhombifer*	3	0.219
Common watersnake	*Nerodia sipedon*	3	0.176
Brown watersnake	*Nerodia taxispilota*	2	0.219
Rough greensnake	*Opheodrys aestivus*	5	0.152
Eastern ratsnake	*Pantherophis alleghaniensis*[b]	2	0.234
Red cornsnake	*Pantherophis guttatus*[b]	1	0.193
Gray ratsnake	*Pantherophis spiloides*	2	0.234
Eastern pinesnake	*Pituophis melanoleucus*[b]	5	0.193
Queensnake	*Regina septemvittata*	2	0.207
Pine Woods littersnake	*Rhadinaea flavilata*	2	0.190
Dekay's brownsnake	*Storeria dekayi*	2	0.182
Red-bellied snake	*Storeria occipitomaculata*	1	0.175
Florida brownsnake	*Storeria victa*	—	—
Southeastern crowned snake	*Tantilla coronata*[b]	1	0.201

(Continues)

TABLE 1 | (Continued)

Common	Latin	# of sequences tested	Max AP
Rim Rock crowned snake	*Tantilla oolitica*	—	—
Florida crowned snake	*Tantilla relicta*[b]	2	0.209
Eastern ribbonsnake	*Thamnophis saurita*	2	0.242
Common gartersnake	*Thamnophis sirtalis*	1	0.211
Rough earthsnake	*Virginia striatula*	2	0.242
Smooth earthsnake	*Virginia valeriae*	2	0.226

Note: Sequence data were not available for *Storeria victa* and *Tantilla oolitica*. Bold text indicates the target species for this assay.
[a]Minimum AP instead of maximum.
[b]Species tested in vitro and did not cross-amplify.

3 | Results

3.1 | qPCR Assay Validation

Optimized primer concentrations were 900 nM for both forward and reverse primers. Using the eLowQuant model, we estimated that the LOB is 0.00 copies/reaction (no amplification of negative controls over the course of the study). We used the Binomial-Poisson linear model with intercept (selected based on likelihood ratio test) to estimate LOD and LOQ. Assuming three replicates and 5% false negative rate (Bustin et al. 2009), the LOD is 1.56. Assuming three replicates and a maximum tolerable coefficient of variation (CV) of 0.1, the LOQ is 2.29. The in silico and in vitro tests of generality indicated that the assay had no mismatches with 76 published EIS *cytb* sequences and amplified all 25 EIS tissues collected in Florida and Georgia. The in silico assessment of specificity indicated all but one of the 45 non-target confamilials with sequence data had maximum eDNAssay assignment probabilities <0.3 (range $=0.147$–0.403, mean $=0.211$). We did not observe any amplification from any of the 13 nontarget species tested in vitro (Table 1). Although sequence data were unavailable for two potentially co-occurring taxa (*Storeria victa* and *Tantilla oolitica*), the distribution of eDNAssay assignment probabilities for sequenced non-target confamilials (*sensu* Wilcox et al. 2024) suggests a very low probability of an unsampled confamilial exceeding the 0.3 assignment probability threshold ($<0.1\%$). Primer-BLAST did not identify any extra-familial species that may cross-amplify and co-occur with EIS.

3.2 | Study Design and Field Collection

Over the course of the experiment, several rain events occurred in the area; five days received over 7.6 mm of rain, one of which received 25.4 mm (Figure 2). To limit the impacts of precipitation on eDNA persistence, we put tarps over the enclosures after snakes were removed; however, this led to temperatures as high as 56.5°C, so we proceeded without covering enclosures. Even without the tarps, temperatures ranged from 29.3°C to 54.5°C, and the enclosures were exposed to direct sunlight (Figure 2). Over the course of the study, mean daily light exposure ranged from 2264 to 6307 lx, mean daily temperatures ranged from 27.3°C to 33.8°C, and mean daily precipitation ranged from 0 to 10.9 mm. There was one change in scheduling to protect human and animal safety: eight sample sand collection dates were shifted one day earlier to avoid a hurricane warning on August 30 (four 10-day samples and four 7-day samples).

Of the 224 total experimental samples, 68 (30.4%) tested positive for EIS eDNA (Table 2). There was no amplification in any of the nine negative control samples tested (one control sample was lost in transit) nor the 13 no-template PCR controls. Standard curve efficiency ranged from 84% to 109% (mean $=92\%$, $r^2>0.98$). Eastern indigo snake eDNA concentrations were low on average; excluding three outliers, the range was 0–9 copies/reaction (Figure 3). The three outliers were from snake IDs 627 and 620 and ranged from 44 to 139 copies/reaction ($z=3.97$–12.65). The outliers were taken from 2 h and 1 h snake presence in enclosure and from sand collection times of 7 days and 4 h. The median number of copies per reaction for each of the eight replicates varied from 0 to 0.3 (Table S1; *Kruskal–Wallis*, $\chi^2=14.87$, $p=0.038$).

3.3 | Modeling

Our model included time in enclosure and sand collection time as fixed effects and snake ID as a random intercept. The model had a reasonable fit (area under the curve [AUC] $=0.776$), and all the coefficient estimates were statistically significant (Table 3). Eastern indigo snake detection probability was positively correlated to time in enclosure and negatively correlated with sample collection time (Figure 4).

Adding mean light or rainfall to the global model did improve model fit; however, only mean light was statistically significant (Table 4). Light was negatively correlated with detection, and temperature and precipitation were positively correlated with detection (Table 4).

4 | Discussion

We optimized a soil and sand eDNA extraction protocol and designed, validated, and deployed a new species-specific qPCR assay to detect EIS DNA in sand samples. We used the framework from Kronenberger et al. (2024) to evaluate assay specificity. None of the confamilial species tested using a highly accurate in silico

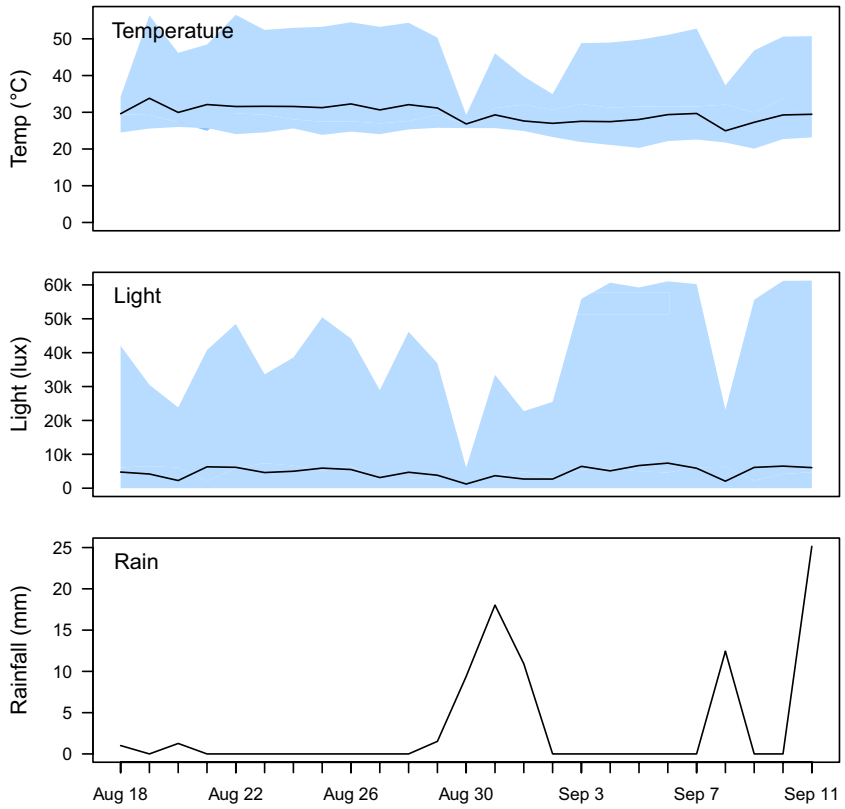

FIGURE 2 | Temperature, light, and precipitation over the course of the experimental study. Temperature and light were averaged from four loggers placed in different enclosures; rainfall information was taken from the nearby NOAA station. Black lines indicate daily averages for temperature and light, and daily total for rainfall. Blue shading indicates the daily ranges. Gray lines indicate days on which sampling took place.

TABLE 2 | Number of detections of eastern indigo snake eDNA in sand samples by time in enclosure (columns) and sample collection time after snake removal (rows) ($n = 8$).

	Time in enclosure			
Sand collection time	**100 s**	**15 min**	**1 h**	**2 h**
1 h	6	2	5	7
4 h	3	3	3	7
24 h	1	2	2	4
48 h	0	0	2	2
72 h	0	0	1	5
7 days	0	2	2	2
10 days	1	0	1	5

PCR model (eDNAssay; Kronenberger et al. 2022; Kronenberger et al. 2024) were predicted to cross-amplify, and the risk of untested confamilials cross-amplifying (*sensu* Wilcox et al. 2024) was statistically estimated to be approximately zero. We cross-checked eDNAssay predictions for 13 of the most common nontarget species by conducting in vitro testing using synthetic DNA fragments (see Table 1) and indeed none cross-amplified. On the validation scale of Thalinger et al. (2021)—which provides a framework for assessing the readiness of eDNA assays—this assay has reached complete validation (Level 5); therefore, this assay is fully operational throughout the range of EIS.

When applied to experimental samples, our modeling revealed that detection probability varied from just over 0% to 70%, depending on time the snake spent in the enclosure and time since snake contact with sand. When snakes spent two hours in the enclosure, detection probability was greater than 50% for four and a half days after presence. Snake DNA was still detected up to ten days after snake removal (estimated detection probability = 27%) and, according to our model, may be detectable as long as 20 days (< 5%). Users should interpret our model extrapolation of maximum eDNA persistence time with caution. Empirical testing beyond 10 days after exposure would be more accurate and reliable, but at the initiation of this study, it was not known that snake eDNA in sand and soil was potentially detectable beyond this time period (Kucherenko et al. 2018). Even when EIS spent short periods of time in the enclosures (100 s and 15 min), eDNA was detected, especially within the first 24 h after snake presence (18%–25%).

Sand samples from short-duration presences yielded substantially lower detection probabilities, indicating that eDNA sampling may not be ideal for transient snakes. Eastern indigo snakes exhibit site fidelity, often returning to the same sandhills or even tortoise burrows during winter (Stevenson et al. 2009), providing a targeted focus for sampling efforts. Kucherenko et al. (2018) conducted a field study looking for Burmese python eDNA in soil from one meter into a gopher tortoise burrow (an area of higher exposure time) and on the apron of the burrow (an area of lower exposure time). No detection was recorded on the burrow apron; however, Burmese

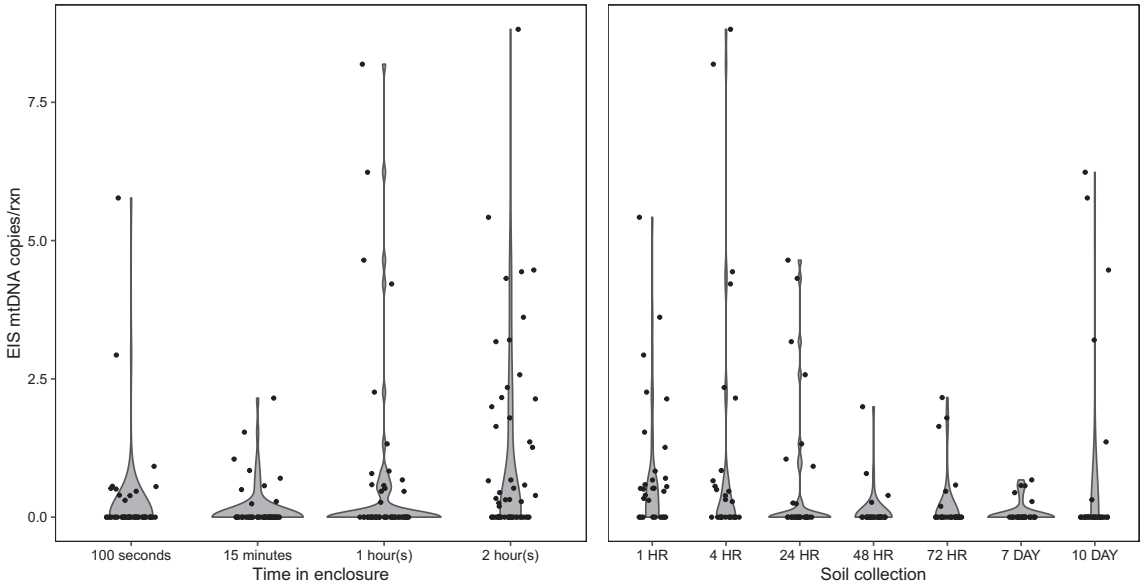

FIGURE 3 | The distribution of eastern indigo snake DNA copies per reaction by time in enclosure and sand collection time. Three outliers (44, 72, 139 copies) were excluded for ease of visualization.

TABLE 3 | Fixed effect estimates from model predicting eastern indigo snake DNA amplification as a function of time in enclosure and sand collection time.

Parameter	Estimate	95% CI	*p*
Intercept	−1.335	−2.032 to −0.676	<0.001
Time in enclosure	0.018	0.011–0.026	<0.001
Sand collection	−0.007	−0.013 to −0.004	0.001

Note: The 95% CI around each estimate was determined from 500 parametric bootstraps.

python DNA was detected within the burrow. This discrepancy is likely attributed to shorter exposure time to soil and higher UV exposure. These findings, alongside our own, advocate for directing field sampling efforts towards areas where snakes have prolonged exposure and minimal environmental exposure. However, in southern portions of their range, EIS are less likely to inhabit gopher tortoise burrows because warmer winters do not necessitate thermal refugia. Further research is needed to assess sampling efforts in these areas to enhance detection efficacy.

It is important to note that our estimates of detection probability are based on captive animals, and model generality for wild populations remains untested. The experimental conditions differed from typical field conditions in several ways, likely affecting our model estimates. First, time in enclosure was a period in which snakes roamed the enclosure and therefore were not exposed exclusively to one location of sand for the entire duration. We attempted to account for this via sub-sampling; however, this does not guarantee that sand samples capture precise locations of snake presence, and the increased volume of the samples may dilute the concentration of any DNA that was captured. It was also noted that, while at shorter periods of time in the enclosure the snakes were very active, however, as they acclimated, snakes seemed to settle into a single location. If eDNA shedding rates were relatively constant, and these consequently concentrated DNA at unsampled locations, the detection rates would be lower than otherwise expected.

Another difference between field sampling and our experimental study is the substrate. EIS often overwinter in gopher tortoise burrows that are located in xeric sandhills, making the substrate a sandy soil. We used sand in our experiments which approximates natural conditions; however, sand has a lower organic carbon content than the sandy soil of EIS habitats. Soils with higher organic carbon content have an increased rate of eDNA degradation and qPCR inhibition, due to the presence of microbes and humic substances (Sirois and Buckley 2019; Tsai and Olson 1992). These differing conditions might either reduce detectability in the field or simply decrease the length of time in which EIS is detectable in the sampled substrate.

Further, the sand was exposed to UV radiation (i.e., direct sunlight), moisture (i.e., rain), and high temperatures, which have been shown to increase the rate of DNA degradation (Johnson et al. 2023; Sirois and Buckley 2019; Strickler, Fremier, and Goldberg 2015). During winter months, when EIS monitoring usually takes place, there are lower light and lower temperatures than in our experimental trials. Additionally, the protective nature of the gopher tortoise burrows, and canopy cover typical in EIS habitats might decrease the effect of environmental exposure, ostensibly increasing eDNA residence time. However, microbial activity associated with eDNA degradation may increase in protective areas where low UV and high moisture increase microbial activity, therefore possibly nullifying the benefits of the protective areas (Guthrie et al. 2024).

Although previous research has shown a negative effect of rain and temperature on eDNA persistence (e.g., McCartin et al. 2022; Valentin et al. 2021), our modeling showed a positive, though non-significant effect, for both rain and temperature. Temperature and rainfall were highly correlated ($r = -0.80$), consequently rainfall

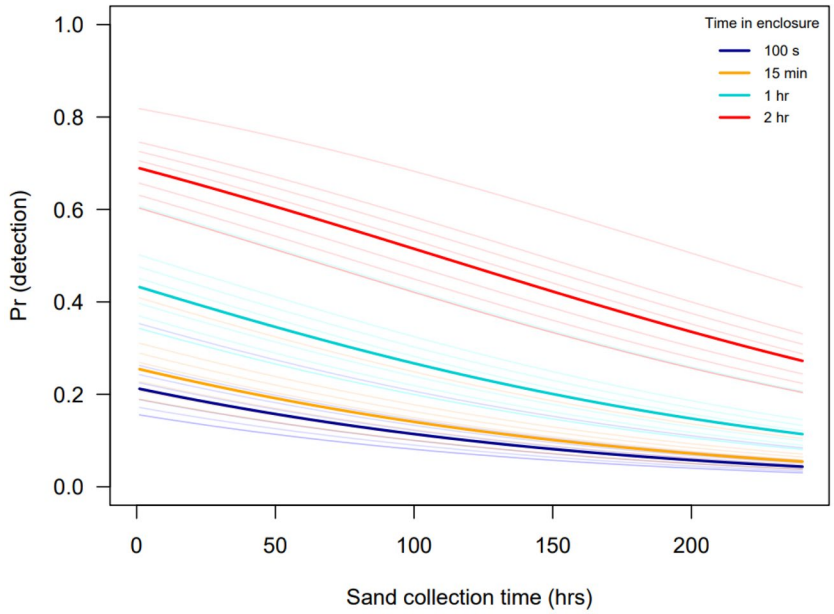

FIGURE 4 | Modeled probability of eastern indigo snake DNA detection in samples as a function of sand collection time (hours) and time in enclosure. Each experimental time in enclosure curve has a solid line representing the model mean, and surrounding lines represent individual snakes as modeled by the random effect term.

TABLE 4 | Impact of adding environmental covariates to the base model.

Covariate	Estimate	95% CI	ΔAIC	*p*
Mean light	> −0.001	−0.001 to 0	2.390	0.033
Mean temp	0.163	−0.112 to 0.494	−0.602	0.244
Mean rainfall	0.219	< 0.001–0.549	2.342	0.063

Note: Each model includes fixed effects of sand collection time, time in enclosure, and one environmental covariate, as well as a random intercept of snake ID. The 95% CI around estimates was determined from 500 parametric bootstraps.

may have washed DNA further into the sand or increased degradation, and heavy rain days were also the coolest days. However, this does not explain why rainfall did not negatively affect detection. One possibility is that our mean precipitation metric fails to capture the inconsistent pattern of rain; several days had substantial rain and on all other days there was no rainfall, potentially generating a spurious relationship in the model. Another possibility is that rainfall perturbed the sand enough to cause the spreading of DNA which otherwise might be too localized to show up in subsamples. Studies have also shown the negative effect of UV light on DNA persistence (Kessler et al. 2020; Pilliod et al. 2013). We used loggers to track lux as a proxy variable for UV due to the expense of UV loggers. However, lux may not be an accurate proxy of UV, and therefore interpreting the effect of UV based on lux should be given caution. Average light did have a negative effect on detection rates, with lower light days having greater detection probabilities, although the effect size was small.

The only other study assessing snake eDNA degradation in a terrestrial substrate published to date was conducted indoors and therefore did not include environmental effects (Kucherenko et al. 2018). They found that eDNA became undetectable 7 days after 7 days of snake presence. Contrastingly, our study found eDNA still detectable up to 10 days after 2 h of snake presence. This difference may be explained by different collection and extraction techniques; Kucherenko et al. (2018) used a modified phenol-chloroform method on less than 2 mL of soil, compared to our method of filtering sodium phosphate buffer that had been mixed with 50 mL of sand. Another explanation could be the differing species and sizes of the two study organisms; the corn snakes used in Kucherenko et al. (2018) weighed on average 350 g compared to the 2160 g average of the EIS used in this study. However, there is little research on factors that impact DNA shedding rates and larger species are not guaranteed to have a higher shedding rate.

Soil and sand sampling and handling can be logistically difficult. Samples are often collected in 50 mL tubes and therefore are not space efficient. Unlike water eDNA samples, we lack established protocols for ambient temperature preservation of soil and sand, which could make sampling in remote settings particularly challenging. Future studies should explore alternative sample preservation options to freezing such as silica, ethanol, and preservative buffers such as Longmire's (Chen et al. 2024; Smenderovac et al. 2024).

DNA extraction of terrestrial substrates can also be difficult. Our extraction method was optimized to increase DNA yield but was labor-intensive. The hybrid protocol we used (the filter method), which combines the phosphate buffer protocol of Taberlet et al. (2012) with filtration of solubilized DNA takes more than three times as long as the second highest yielding method (referred to as the PB method; see Supporting Information). The phosphate buffer used in both methods is meant to displace extracellular DNA (which is potentially the most abundant form of DNA in soil; Pietramellara et al. 2008), although intracellular DNA is undoubtedly still present in

the buffer. Unlike the PB method, which does not include a lysis step, the filter method does, increasing the likelihood of capturing intracellular DNA along with extracellular DNA. Another difference between the two protocols is that the filter method uses all the buffer mixed (~50 mL in this study), while the PB method uses only 700 μL regardless of the total final volume of the buffer. The filter method ended up having higher rates of detection, but DNA copy number was still low, with only three samples having more than ten copies per reaction. The three outliers in our eDNA quantifications were likely due to aggregates of DNA being left by the snake (e.g., skin or feces; Turner et al. 2014).

Despite these logistical challenges, soil and sand eDNA sampling may be a viable approach for terrestrial animal detection. Even with imperfect detection, the potential to increase the temporal window for animal detection could be valuable for very low density, mobile animals like the EIS. The next step in this work is to extend comparisons of detection methods, including this optimized eDNA sampling protocol, to natural field systems.

Author Contributions

L.R.N.S., H.C.C., M.H., M.E., R.A., B.S.S., J.E.B., M.A.D., S.H., and T.W. conceptualized and designed the study. M.H. set up the experiment and collected all samples with support from J.E.B. L.R.N.S. performed all laboratory work with samples. L.R.N.S. and J.A.K. optimized the DNA extraction protocol. J.A.K. and T.W. designed the qPCR assay. L.R.N.S. analyzed data, interpreted results, and drafted the manuscript with support from T.W. L.R.N.S., H.C.C., M.H., J.A.K., M.E., R.A., B.S.S., J.E.B., M.A.D., S.H., M.K.S., and T.W. edited and reviewed the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data and scripts underlying this manuscript are available at: https://github.com/NationalGenomicsCenter/EasternIndigoSnake.

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Supporting Information

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