Using environmental DNA (eDNA) to assess the presence of cavefish and cave crayfish populations in caves of the Ozark Highlands

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FINAL REPORT

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Using environmental DNA (eDNA) to assess the presence of cavefish and cave crayfish populations in the Ozark Highlands

Photo: *Cambarus tartarus*

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Executive Summary

Many cavefishes and cave crayfishes are considered of conservation concern; however, sampling these species is inherently difficult given their occupied environments. The goal of our project was to verify the presence of select karst organisms while developing the foundation for sampling approaches that might be useful to conservation and management agencies. Our project objectives were to develop assays to amplify deoxyribonucleic acid (DNA) from several species of Ozark cavefishes and cave crayfishes and complete an initial surveillance of locations across the Ozark Highlands using environmental DNA (eDNA). Using DNA either provided by agency cooperators or that we extracted from tissue samples, we PCR amplified and then sequenced the Cytochrome Oxidase 1 (CO1) gene for cave crayfishes and the NADH Dehydrogenase Subunit 2 (ND2) gene for cavefishes. We developed species-specific primers and probes for five cave crayfishes and two cavefishes. From February 2017 to May 2017, we sampled 1–5 sampling units from 42 caves, wells, and springs (i.e., sites) using eDNA and traditional visual surveys. We measured physicochemical parameters at each sampling unit to estimate detection probability associated with both techniques. We also calculated two occupancy covariates for each site using geospatial data. We successfully amplified *Troglichthys rosae* DNA from the environment and detected DNA representing

this species at 24 of 40 sites. At 16 of the sites where we detected *T. rosae* DNA, we did not visually observe the species. Although our assay for *Typlichthys eigenmanni* successfully amplified the target DNA from the environment, it also resulted in false absences where the species was visually confirmed. Using eDNA to detect cave crayfishes was much more difficult. The assay for *Cambarus subterraneus* did not work for eDNA samples and we were unable to pick up DNA from the environment, even at locations where it was visually confirmed. Alternatively, the eDNA surveys worked well for *C. tartarus* and we were able to amplify DNA at every site where it was visually observed. Our assay for *C. aculabrum* was based on a single sample obtained from GenBank, and did not amplify eDNA from field samples. Lastly, our eDNA results from samples in the known range of *Orconectes stygocaneyi* suggested the species may be found at an additional cave. Detection using eDNA based on our *O. stygocaneyi* assay was likely low because it was designed from a pseudogene; however, positive eDNA samples were sequenced to confirm species-specific DNA. Detection probability of both cavefishes and cave crayfishes varied by survey technique and was influenced by water volume, water clarity, water velocity, and substrate. Detection of cavefishes and cave crayfishes via visual surveys decreased when water volume increased, whereas detection using eDNA increased with greater water volume. Detection between taxa using either sample method was highest in habitats classified by fine substrates, except for eDNA detection of crayfishes which was greatest in coarse substrates. Detection of cavefishes increased with water clarity, but detection of cave crayfishes increased with turbidity. Detection probability of both cavefishes and crayfishes using eDNA increased slightly with water velocity, but decreased with visual surveys as water velocity increased. Occupancy by both taxa was positively related to particular geologic series. Crayfish occupancy was negatively related to fine-scale anthropogenic disturbance (i.e., 500-m buffer around the site), whereas crayfish showed no relationship with disturbance. Our results suggest possible range extensions, provide insights to factors driving detection using both sample techniques, and suggest areas where recharge zones may be shared among caves. Future efforts focused on a comprehensive evaluation of genetic diversity among cave crayfishes to improve assay design could improve detection and the applicability of eDNA as a supplemental and non-invasive sampling approach.

Background

The Ozark cavefish *Troglichthys rosae* and Eigenmann's cavefish *Typhlichthys eigenmanni* are diminutive, depigmented, blind fishes that are endemic to karst habitats of the Ozark Highlands ecoregion and are difficult to sample using traditional sampling techniques. Several techniques are used to monitor cavefish populations including above-water counts (Willis and Brown 1985), snorkeling (e.g., Brown and Johnson 2001), and mark-recapture methods (Means and Johnson 1995; Brown and Johnson 2001). Each approach faces inherent disadvantages for several reasons, such as low visibility in aquatic cave habitats and difficulty accessing or effectively surveying in subterranean habitats (i.e., low detection rates). Densities of most cavefish populations are perceived to be low (Means 1993; Means and Johnson 1995; Niemiller and Poulson 2010; Niemiller et al. 2013); however, given the sampling challenges of many populations and our lack of understanding of emigration/immigration rates (e.g., may be as high as 75%; Means and Johnson 1995), population presence and abundance are unclear.

Historically, cavefish populations within individual cave systems have been treated as distinct, but recent molecular work has shown that these populations may be connected. Cavefishes can potentially disperse through groundwater aquifers that cross surface drainage divides (Woods and Inger 1957), making it difficult to assess the importance of individual caves to populations. Previous molecular studies of amblyopsid cavefishes uncovered considerable genetic differentiation among populations structured by surface hydrological basins (e.g., Dillman et al. 2011; Niemiller 2011; Niemiller et al. 2012). In the Ozark Highlands, genetic structure of the mitochondrial NADH dehydrogenase subunit 2 locus (ND2) in *T. rosae* corresponds with boundaries among the White, Neosho, and Illinois river drainages (Noltie and Wicks 2001). However, genetic structuring of mitochondrial (Noltie and Wicks 2001; Niemiller 2011; Niemiller et al. 2012) and nuclear loci (Niemiller 2011; Niemiller et al. 2012) in *T. eigenmanni* is less straightforward. Improving our knowledge of cavefish population genetics may reflect mismatch between surface and subsurface hydrology.

In addition to the cavefishes, several cave crayfishes are also known to occur within the Ozark Highland ecoregion including the Benton cave crayfish *Cambarus*

aculabrum, Hell Creek cave crayfish *C. zophonastes,* Oklahoma cave crayfish *C. tartarus,* Delaware County cave crayfish *C. subterraneus*, Salem cave crayfish *C. hubrichti*, bristly cave crayfish *C. setosus*, and Caney Mountain cave crayfish *Orconectes stygocaneyi*. There are perhaps more than 13 described and undescribed populations of cave crayfishes (Graening et al. 2006a). However, the distribution and abundance of these cave crayfishes are largely unknown and some species are considered endemic to only a few caves. (e.g., *C. subterraneus*, Graening and Fenolio 2005; *C. tartarus*, Graening et al. 2006a). There is limited information on the spatial and temporal extent of occurrence by these species and virtually nothing is known about their ecology or life history (Graening and Fenolio 2005; Graening et al. 2006a).

The detection and monitoring of rare and endangered species is an ongoing challenge for individuals tasked with conservation and management of cave and karst resources. Accurate and repeatable surveys of these taxa are problematic because their habitats are difficult to access or not amenable to traditional survey methods (Niemiller et al. 2018). Often, traditional approaches are not logistically possible, expensive, or are highly invasive or destructive to sensitive habitats. Our lack of knowledge on subterranean biodiversity is, in part, driven by the challenges associated with accessing, sampling, and studying organisms in subterranean habitats using traditional survey approaches. Consequently, most species, including some Ozark cave crayfishes, are known from just a few occurrences, are of great conservation concern, and are considered to be at an elevated risk of extinction. Survey efforts are limited largely to larger, human-accessible subterranean voids (i.e., caves) that are comparatively easier to access and study. The reality is that subterranean habitats are much more expansive in many areas, and individual cave systems represent merely a window into a vastly more complex and extensive series of cracks, fissures, and voids.

Several aquatic Ozark Highland cave species are listed as federally threatened (*T. rosae*), endangered (*C. aculabrum* and *C. zophonastes*), or have been petitioned for federal listing (i.e., *C. tartarus* and *C. subterraneus*). *Troglichthys rosae, C. tartarus,* and *C. subterraneus* are also Priority Species for the Gulf Coastal Plains and Ozarks Landscape Conservation Cooperatives. The United States Fish and Wildlife Service (USFWS) established national wildlife refuges within the Ozarks to protect important

habitat and further the recovery of the federally-listed aquatic cave species including the Ozark Plateau National Wildlife Refuge (NWR) in Oklahoma, Logan cave NWR in Arkansas, and Ozark Cavefish NWR in Missouri. The Recovery Plans for each of the listed species (USFWS 1988a, 1988b, and 1996) call for protecting important caves where the species are known to occur, monitoring known populations, and surveying for new populations. The USFWS works with partners to implement these important recovery actions and to implement conservation measures to reduce the need for future listings of species of concern. However, monitoring species with low detections is problematic for making conservation decisions. Developing a sampling protocol based on water collection would result in less cave disturbance and provide supplemental data on species presence.

Our project goal was to develop the foundation for environmental DNA (eDNA) monitoring methods that would benefit future monitoring efforts. In recent years, obtaining data on species' distributions and the composition of aquatic communities by leveraging DNA shed by an organism into its surrounding environment has become an attractive and viable complement to traditional sampling and monitoring approaches for many aquatic organisms, including stygobionts (e.g., Stankovic et al. 2016; Vörös et al. 2017; Niemiller et al. 2018). Emerging technologies and methods allow for the isolation, extraction, and analysis of DNA in environmental samples, termed eDNA, to detect and monitor biodiversity, providing a powerful new tool for the discovery and monitoring of biodiversity. Thus, our specific study objectives were: 1) to develop quantitative polymerase chain reaction (qPCR) assays to amplify species-specific DNA from cavefishes and several cave crayfishes of the Ozark Highlands ecoregion; and 2) survey caves, wells, and springs across the Ozark Highlands to test an eDNA protocol and determine detection using both eDNA and traditional visual surveys. Results from this project will support recovery and monitoring efforts of the USFWS and various conservation partners and help inform conservation decisions.

Methods

Reference DNA collection and sequencing. – Tissue samples, stored in 100% ethanol, and genomic DNA were obtained from several partner agencies to develop assays to amplify DNA via qPCR. Eleven fin clips of *T. rosae* were obtained from three caves: one from McGee's Cave in Oklahoma, and five each from Logan and Cave Springs caves in Arkansas (Table 1). We obtained tissue samples of six *C. subterraneus* from Twin Cave, six *C. tartarus* from January-Stansberry Cave, and one *C. tartarus* from McGee's Cave (Table 2). We also obtained genomic DNA samples of *C. hubrichti*, *C. subterraneus*, *C. zophonastes*, and *O. stygocaneyi* from the Missouri Department of Conservation (MDC; Table 3). We sequenced the genetic material of *C. hubrichti* and *C. zophonastes*; however, these species were not part of our biomonitoring effort as we did not sample caves within the ranges of these species (Table S1). Results from the *O. stygocaneyi* DNA was of particular interest to the MDC because: 1) it is the only known Ozark Highlands cave crayfish within that genus, and 2) nearby caves and springs are important locations for testing for the species presence and visual surveys have failed to detect the species. Finally, we collected walking legs from two *C. setosus* and three *O. stygocaneyi* to increase our knowledge of the intraspecific genetic variation within these two species.

Genomic DNA was extracted from the tissue samples and sequenced with the genomic DNA we obtained from the MDC. DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany; # 69504) following the manufacturer's protocol. For cavefishes, we PCR amplified a 500-base pair (bp) fragment of the mitochondrial ND2 using the forward primer MET: 5'-CATACCCCAAACATGTTGGT-3' and reverse primer ND2B: 5'-TGGTTTAATCCGCCTCAGCC-3' (Kocher et al. 1995). For cave crayfishes, we amplified a 710 bp fragment of the mitochondrial cytochrome oxidase subunit 1 locus (CO1) using the primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). PCR products were visualized on a 1.0% agarose gel then purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI; A9281). We Sanger sequenced products following standard protocols, using an ABI 3130 Genetic Analyzer. The forward and reverse sequences for each product were trimmed and aligned in Geneious 11.1.5 (Auckland, New Zealand). Our reference sequence database for cavefishes and cave crayfishes was

supplemented with ND2 and CO1 sequences accessioned in GenBank (Table 4). CO1 is one of the most frequently used genes (>10 000 nucleotides entries) for ecological and evolutionary studies of Decapoda (Lefébure et al. 2006).

qPCR assay design and validation. – We attempted to design species-specific qPCR assays for several cavefish and cave crayfish species (Table 5). We initially designed primers to use for a SYBR Green assay, but concerns about specificity and sensitivity led us to instead use Taqman® assays with primers and probes. Taqman® assays were synthesized using the program PrimerQuest (https://www.idtdna.com/PrimerQuest/Home/Index) and were designed to amplify a short (< 200 bp) fragment of the CO1 (crayfishes) and ND2 (cavefishes) genes via qPCR. Each assay consisted of forward and reverse primers and a hydrolysis probe. The 5'end of each probe was labeled with a fluorescent dye (6-FAM), the 3' end with a quencher (Iowa Black™ FQ), and there was an additional internal quencher (ZEN™). Probes were doubled quenched to reduce background fluorescence and increase signal intensity. The program Primer-BLAST (Basic Local Alignment Search Tool; NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to validate specificity of each assay in silico against the *nr* database. We further confirmed specificity of each assay by qPCR of available crayfish and fish DNA. Each qPCR reaction was conducted in a total volume of 20 µl, consisting of 10 µl TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA; Cat. # 4396838), 4.7 µl of ddH_2O, 0.9 µl of forward primer (20 µM), 0.9 µl of reverse primer (20 µM), 0.05 µl of probe (10 µM), and 3.0 µl of template DNA. Samples were run in 96-well optical plates on a LightCycler 480 (Roche, Pleasanton, CA). The thermal profile consisted of an initial denaturation step of 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for one min. For each of our assays, we determined the lower limit of detection for the target species' DNA by running a dilution series of the DNA that ranged from undiluted to 1:1,000,000. We were unable to test the assays *in vitro* for *C. aculabrum* and *T. eigenmanni* because we did not have genomic DNA for those species. Not all assays developed were species-specific, but we confirmed species identity of field samples via DNA sequencing of a subset of the positive samples.

Cave Monitoring. – In 2015, we collected 10 living *C. setosus* in conjunction with the Missouri Department of Conservation. The specimens were transported to the lab at Oklahoma State University where they were used to test filtering protocols before our cave monitoring began (see below). The purpose of holding the specimens in the laboratory was to determine how much water needed to be filtered to detect DNA. After completion of the filtering experiments, euthanized specimens were preserved in 70% ethanol.

In 2016, we worked with several agencies and private landowners to identify caves that could be sampled as part of the monitoring portion of our study. We worked with the Missouri Department of Conservation to identify four springs, nine wells, and three caves that we could sample in Missouri. We met with Missouri Department of Conservation personnel in November 2016 to visit these locations. During that visit, we identified the supplies we needed for our field efforts (i.e., how to sample water from a particular well depth and complete visual surveys). We acquired permission from several private landowners, The Nature Conservancy, and USFWS in Arkansas to sample additional caves in Missouri, Oklahoma, and Arkansas. We obtained sampling permits per the requirements in each of the respective states. Fieldwork began in February 2017.

During our predefined sampling "season" of February 2017 to May 2017 (see analyses), we sampled 42 caves, wells, and springs (hereafter referred to as "sites") in the Ozark Highlands ecoregion to compare the results of traditional visual and eDNA surveys. Carroll and Thunder rivers within Carroll Cave were considered separate sites due to extreme differences in the hydrologic regime (Miller 2010). One to five "sampling units" (i.e., individual survey locations within each cave) were visually surveyed and water samples for eDNA analysis were collected on 3–4 occasions within our predetermined season. We defined our season as Feb–May to avoid prolonged flooding (i.e., typically May–June) or drying (i.e., July–Oct) that could affect colonization or extinction by the species at each site. We hypothesized there would be a lag between the initiation of high-water or low-water events before there would be changes in species occupancy (i.e., it would take time for species to recolonize when a sampling unit either became wet or dry again, Adams and Warren Jr. 2005). Our season was chosen to meet the occupancy modeling assumption that each sampling unit was closed to the species

(i.e., no extinction or colonization during the season) while allowing physicochemical parameters to change at each sampling unit to better capture changes in detection using both sampling techniques (i.e., provide a range of detection covariates). Some of our sampling units were only surveyed on a single occasion or deviated from our standard protocol (reason in parentheses): springs 7 and 8 (flooding), cave 21 (dry on return visits), and wells 5 and 6 (a different gear was used due to narrow openings). We excluded the results associated with the aforementioned sampling units from our occupancy analyses, but report detection in our results (see below). Additionally, we did not process the water samples collected from well 5 because it contained dye for groundwater tracing. We also surveyed a few sampling units on additional occasions (e.g., Caney Mountain Conservation Area, Missouri Department of Conservation) that were outside of our predefined season. Lastly, we attempted to sample an additional well in Oklahoma in February 2018 at the request of the USFWS (Richard Stark, requesting entity); however, we were never able to obtain a sample after six site visits as the well remained dry.

Visual surveys of the cave organisms took place at each of the sampling units. With few exceptions, the status and trends of local populations of both cavefishes and crayfishes is based entirely on infrequent visual count surveys of aquatic habitat in cave systems. Visual surveys in both springs and caves consisted of two observers walking, crawling, or swimming the entire sampling unit while carefully searching the whole wetted area for cave fauna (overturning rocks and looking in possible hiding locations). We viewed hand-dug wells in their entirety using a spotlight, collected water samples (see below), and then viewed the area of the well again. We recorded the number of cavefishes and cave crayfishes observed at each sampling unit.

We collected two, 1-L water samples from each sampling unit for later eDNA analyses before conducting visual surveys. Nitrile gloves were worn during collections and changed between sampling units. All of our gear used to collect and filter water was sterilized between all sites and most sampling units (i.e., the latter was not always possible due to the amount of gear that could be taken into each cave), by immersing in a 50% bleach solution for at least 30 sec and thoroughly rinsed with distilled water. On occasions where we did not immerse our gear, we rinsed the gear using water at the next

sample unit. We filtered distilled water in the field on April 5, 2017 once between sampling units at OT-4 and once after, on April 6, 2017 between Bartholic and McMahan springs, and on September 4, 2018 before and after sampling Mud Cave to provide negative controls in the field, which were treated the same as field samples in subsequent steps. All of those samples were negative suggesting our decontamination protocol was adequate. For springs and caves, a 1-L Nalgene bottle was used to collect water near the bottom of the water column, but a minimum of 5 cm above the benthos to avoid humic acid contamination. To collect water from wells, we used a Van Dorn sampler that was lowered to approximately 5 cm above of the substrate and closed. Each 1-L water sample was pumped through a 0.45-µm cellulose nitrate filter (Thermo Scientific, Beverly, MA; 14-555-624) that was held in a Nalgene™ Polysulfone Filter Holder (Thermo Scientific, Beverly, MA; 09-745; Figure 1). We usually filtered the water immediately outside of the cave; however, for larger systems (e.g., Smallin Civil War Cave and Carrol Cave) we filtered within the cave. Typically, only one filter was needed to sample an entire liter of water, but multiple filters (i.e., 2–6) were used on approximately 10% of the surveys due to clogging via sediment. The filters were removed from the sampling device and stored in 900 µL of Longmire's Buffer (Longmire et al. 1997), at room temperature, until extractions were performed in the laboratory.

We measured physicochemical data at each sampling unit to estimate detection probability using both eDNA and visual surveys. We collected water samples before the start of each visual survey to measure water clarity (0.01 NTU) using a turbidity meter (Thermo Scientific, Beverly, MA; AQUAfast AQ4500). Because we hypothesized that water volume (0.1 m^3) would affect sampling detection using either survey method, we estimated water volume of each system sampled. Sample length was measured as the distance from the start to the end of the sampling unit. Wetted-width (0.1 m) was measured at the beginning of the sampling unit, end of the unit, and at 1–3 intervals in between to estimate average wetted width of the survey. We measured water depth (0.01 m) at the approximate deepest location, and we estimated water-column velocity (0.1 m/s) each time we measured sample width. Prior to sampling, we practiced estimating water velocity by comparing visual estimates with results obtained using the Marsh-McBirney flow meter (Marsh-McBirney Inc., Frederick, MD) as it was not reasonable to

take a flow meter into every cave sampled due to crawling space. Our estimates were accurate enough to easily distinguish between pool and transitional habitats. At the location where each water sample was collected, natural light was recorded as a binary variable: visible light or not visible. We also distinguished between the prevalence of mud, silt, and bedrock substrates (hereafter referred to as "fine" for simplicity) or pebble and cobble substrates or woody debris (hereafter references as "coarse" substrates) at each sampling unit.

We used existing geospatial data to estimate anthropogenic disturbance and geology to include as covariates in our occupancy models. We calculated a site-specific (i.e., multiple sampling units within a cave shared a value) anthropogenic disturbance index based on land use. Land-use data were acquired from the 2011 National Land Cover Database (https://www.mrlc.gov/) to create buffers around each site at a coarse scale (i.e., the recharge area) to assess the effect of cumulative disturbance, and at a fine-scale (i.e., 500-m buffer) to assess the effect of localized disturbance. Only twenty-eight of our sites have known recharge areas, so we averaged those values and assigned that value to each site with unknown recharge area. The proportion of each land-use type within the buffers was calculated and multiplied by the following coefficients modified from Brown and Vivas (2005): open-space development (1.83), low-intensity development (7.31), medium-intensity development (7.31), high-intensity development (8.67), pasture/hay (2.99), cultivated crops (4.54), and all other categories were considered undisturbed (1.00). The resulting values were summed across all land-use categories to obtain a final disturbance index for each site at both scales. Finally, each site was assigned a geologic category based on the underlying geological series data obtained via the U. S. Geological Survey (https://mrdata.usgs.gov/geology/us/). Our sites fell within four coarse geologic units: Smithville Dolomite (n = 15), Osagean Series (n = 28), Kinderhookian Series (n = 3), and Meramecian Series (n = 3). We condensed the Kinderhookian Series (n = 3) and Meramecian Series (n = 3) into the category "other" because they were close in proximity and outliers within the Springfield Plateau that is comprised mostly of Osagean Series.

eDNA filter extraction and quantification. – We first attempted a phenol extraction protocol, but determined the DNeasy® extraction kit would work better for our protocol. During every DNA extraction event we also included an extraction blank (i.e., an unused filter in Longmire's Buffer) that was treated the same as the field samples. If the extraction blank amplified during qPCR, then we discarded samples from that extraction. We initially extracted DNA from eight sampling units within three caves where *C. setosus* was present using a modified phenol-chloroform extraction method (Rendshaw et al. 2015). Those samples would not amplify unless spiked with high concentrations of genomic DNA, so we hypothesized phenol carryover was inhibiting the reactions and we switched to a DNeasy® extraction method. We extracted DNA from one water sample at each sampling unit, using the Qiagen DNeasy® Blood and Tissue Kit and following the protocol "purification of total DNA from crude lysates." The filters from the other water sample were retained in the event of error during the extraction process (e.g., contamination). The filter used was cut in half to provide two subsamples for each sampling unit.

We used qPCR to determine the presence of each cavefish or cave crayfish species. We followed the same amplification procedure outlined in the previous section "*qPCR assay design and validation"* of this report. Each subsample was run in triplicate, resulting in six replicates for each sampling unit. We chose to interpret our results conservatively due to the sensitive nature of the organisms (i.e., it would be better to obtain a false positive and conduct follow-up surveys than conclude an organism does not occur at a sampling unit). If any of the six replicates amplified, we considered the sampling unit positive for the target species tested (Figure 2). If there was amplification on only one date from a sampling unit, both subsamples were rerun in triplicate. We also ran three negative control replicates during each qPCR where the template DNA was replaced by ddH_2O. If any of the negative controls amplified, then the qPCR run was discarded. A positive control comprising genomic DNA from the target species also was included to ensure the reaction worked properly (i.e., the positive control should always amplify). We planned to use High Resolution Melt Analysis (HRMA) to confirm species identity, but 1) uncertainty in crayfish population differences made it more practical to use DNA sequencing, and 2) HRMA could not be completed with a Taqman® assay.

Therefore, we sent a subset of positive amplified samples to the Recombinant DNA and Protein Core Facility on the campus of Oklahoma State University for Sanger sequencing to confirm species identity.

Analyses. – We analyzed our biomonitoring data by developing single-season occupancy models (Mackenzie 2002), in a Bayesian framework, to estimate both detection and occurrence probability of cavefishes and cave crayfishes. Due to the relatively small sample sizes of certain species, we modeled both species of cavefish together and all species of cave crayfish together; thus, we are assuming that behavioral and trait differences among our fish and crayfish species do not influence detection or occupancy. Variation in detection probability was modeled as a Bernoulli process based on the species' capture histories from each sampling unit using multinomial likelihood with a logit link function (Fiske and Chandler 2011). We first modeled detection probability using only data from sampling units where we detected cave crayfish or cavefish (i.e., sampling units with all-zero capture histories do not inform the detection process). The detection model included three continuous detection covariates: water volume, water clarity, and water-column velocity. All continuous covariates were natural-log transformed due to right-skewed distributions. Continuous covariates were standardized to a standard deviation of one and mean of zero to improve interpretation. All of the continuous variables had a Pearson's pairwise correlation coefficient ($|r|$) ≤ 0.11. We also included two categorical detection covariates: light (yes/no) and substrate (coarse/fine) in the model. Three-way interaction terms using all combinations of taxa, technique, and environmental covariates were included in the model to determine how detection varies between taxa (i.e., cavefishes or cave crayfishes) and technique (i.e., eDNA or visual). For example, we hypothesized that eDNA and visual surveys would be differentially affected by the environment (e.g., water volume may make it difficult to see organisms, but not influence eDNA) and those relationships would be different for cavefishes and cave crayfishes (e.g., cave crayfishes may be more likely to be found in small volumes of water). We used uniform normal priors for all model parameters (Kéry and Royle 2016). Posterior distributions for parameters were estimated with Markov chain Monte Carlo methods using 60,000 iterations after a 10,000-iteration burn-in phase. We calculated

90% highest density intervals (HDIs) for each parameter and removed all three-way interactions that overlapped zero. We repeated the process for two-way interactions and then the main effects in the model. We fitted our models using the program JAGS (Plummer 2003) called from the statistical software R (version 3.5.1; R Core Team, 2018) with the package runjags (Denwood 2016).

After we determined the most parsimonious detection model, we repeated the selection process described above for the occurrence model using three occupancy covariates: geology and disturbance at two scales. The occurrence model was also modeled as a Bernoulli process using multinomial likelihood with a logit link function (Fiske and Chandler 2011). All-zero capture histories were included in the occupancy portion of the model. Disturbance values were standardized to a standard deviation of one and mean of zero to improve interpretation. We hypothesized that sampling units close in spatial proximity would be correlated, so we grouped them by the 10-digit watershed (https://nhd.usgs.gov/wbd.html) occupied. However, the estimates for all but one watershed overlapped zero, using 90% HDIs, indicating that the grouping factor was not important in our model and it was removed. Our final model included only significant detection and occurrence covariates (i.e., 90% HDIs that did not overlap zero).

We assessed both model fit and convergence. We assessed convergence of each model using both the Brooks-Gelman-Rubin statistic autocorrect (R hat), Gelman and Rubin 1992) and effective sample size (ESS; Kruschke 2015), where values <1.1 and >15,000, respectively, indicate adequate mixing of chains. Model fit was assessed with a Chi-square statistic using our most complex model (Kéry and Royle 2016).

Results

Reference DNA sequencing. – We completed the DNA sequencing for each of the samples provided by our partner agencies (Table S1). One of the *C. hubrichti* DNA samples (MDC10) appeared to be *Faxonius neglectus chaenodactylus* (i.e., using BLAST it matched published DNA sequences by 98%) suggesting an error occurred during collection, labeling, or transport. The other *C. hubrichti* sample (MDC22) matched published sequences of *Emballonura beccarii* (a species of bat from Indonesia and Papua

New Guinea) with 90% confidence. Although our genetics laboratory works with bats, we have never worked with this particular family. We also had issues obtaining quality sequences for most of the *C. setosus* samples because there was co-amplification with suspected pseudogenes, so those sequences should be interpreted with caution (i.e., there may be incorrect bases). The exception was ES1, which matched *C. setosus* by 98%. Only short fragments (i.e., less than 300 bases), based on DNA sequence using only one primer, were recovered using the specimens from Smallin Civil War Cave (SCWC) and Woody Cave (WC). The useable sequences from SCWC matched *C. setosus* (94–95%), and those from WC matched *Lacunicambarus ludovicianus* and *Cambarus diogenese* (93% and 94%, respectively). All of the sequences for *C. tartarus* matched published DNA sequences for the species by greater than 99%. We were unable to obtain quality sequences for the *C. subterraneus* samples, MDC11 and MDC12, likely because of their age (i.e., they were collected in 1989 and 1992). However, we were able to amplify the rest of the *C. subterraneus* samples, which all matched published *C. tartarus* DNA sequences by greater than 95%. These results were expected because *C. subterran*eus DNA has yet to be submitted to GenBank and *C. tartarus* is the closest related species. We were able to sequence both CA1 and CA7, which matched *C. aculabrum* by 93% and 94%, respectively. Again, these results for CA1 and CA7 would be expected because there is no record of *C. zophonastes* on GenBank and *C. aculabrum* is a closely-related species. We were unable to amplify the *O. stygocaneyi* DNA sample that was collected by MDC in 1982. The sequences MC58.1, MC58.2, MDC13 are all suspected pseudogenes from *O. stygocaneyi* (see discussion). The sequence MC7.1 matched published rotifer DNA sequences with 85% confidence, suggesting we amplified a non-target species. Finally, we were unable to sequence the *T. rosae* DNA from CSC02, and Logan01, but all other sequences matched published DNA sequences from the species with at least 99% confidence except McAr25, which matched by 95%.

Cave Monitoring. – The physicochemical covariates we measured varied greatly across our sampling units due to differences in both site type (i.e., wells versus caves) and local rainfall events that occurred at some locations during the field season (Table 6). On all survey visits, light was visible at 24 sampling units, but not visible at 33 sampling units.

Mud Cave, however, did not have visible light on the first two survey visits, but did on the last three visits (i.e., flooding required samples to be collected at the entrance of the cave during some visits). We classified 32 and 26 sampling units as having coarse and fine substrates, respectively. Velocity ranged from 0 to 0.5 m/sec across sampling units. Turbidity ranged from 0.20 to 21.10 NTU. Some sampling units contained comparatively small volumes of water (0.06 m^3) and others contained large volumes of water (800 m^3).

Geological series and disturbance at both fine and coarse scales were included as occupancy covariates in our analysis. Sites were located within four coarse geologic units: Smithville Dolomite (N = 15), Osagean Series (N = 28), Kinderhookian Series (N = 3), and Meramecian Series (N = 3). We condensed the Kinderhookian and Meramecian series into the category "other" because they were close in proximity and they were outliers in a larger region of Osagean series. Anthropogenic disturbance ranged 1.04–3.52 at the coarse scale and from 1.00–7.79 at the fine scale, where 8.67 would represent most highly disturbed via the index.

Our success amplifying eDNA varied among species. Our eDNA protocol was successful for *T. rosae* but additional work is needed to improve eDNA assays for *T. eigenmanni*. We detected *T. rosae* DNA at 24 of the 40 sites that were screened for that species (Table 7). At 16 of the sites where we detected *T. rosae* DNA, we did not visually observe the species. Some of the sites where we detected *T. rosae* DNA might represent range extensions for the species in Missouri (e.g., Bluff Dwellers Cave in McDonald County; caves and springs in Ozark County). The lower limit of detection for the *T. rosae* assay was 2.5 X 10^{-4} ng/µl. It is difficult to determine how well our assay for *T. eigenmanni* worked because we were unable to test it against genomic DNA. Although we had positive results at sites with visual confirmations, the assay may need to be improved as we also determined false absences at some sampling units where the species was visually observed (Table 8). Successful eDNA results for cave crayfishes were more variable. The assay for *C. subterraneus* did not work for eDNA samples and we were unable to pick up DNA from the environment, even at locations where it was visually confirmed during our surveys (Table 9). The lower limit of detection for the *C. subterraneus* assay was 3.9 X 10^{-4} ng/µl. The eDNA surveys worked well for *C. tartarus* and we were able to amplify DNA at every site we surveyed (but not every sampling

unit), including sampling units where we did not visually observe the species (Table 10). The lower limit of detection for the *C. tartarus* assay was 1.5 X 10^{-4} ng/µl . We amplified DNA from the *C. aculabrum* sites, but the resulting sequences only matched *C. aculabrum* DNA by 94–95%. Because our assay was based on a single sample obtained from GenBank, it may not be working properly (e.g., not binding properly to the *C. aculabrum* DNA); alternatively, we may not have a complete understanding of the genetic variation within the population. Finally, the assays for *O. stygocaneyi* suggest that the species may be found in more than the one cave where it is thought to occur (Table 11). We did not visually observe the *O. stygocaneyi* from one of the caves with the positive eDNA samples on any sample occasion so it may be that these two caves share recharge on some occasions or it may be that the species just was not observed. Detection with the *O. stygocaneyi* assays appears low because it was designed from a pseudogene (see discussion), which results in there being less genetic material available (i.e., mitochondrial DNA are more abundant than nuclear DNA), and/or poor binding of the primers and probes to the target DNA (Figures 2 and 3). The lower limit of detection for the *O. stygocaneyi* assay was 3.3 X 10^{-4} ng/µl. Our assays for *C. setosus* did not work for field samples (see discussion) and the lower limit of detection was 1.5 X 10^{-3} ng/µl.

Analyses. – Detection probability of both cavefishes and cave crayfishes varied by survey technique and was influenced by water volume, water clarity, water velocity, and substrate (Table 12). Our final model had a significant three-way interaction among species, gear, and water volume. Cavefishes detection via visual surveys decreased when water volume increased, whereas detection using eDNA increased slightly with greater water volume (Figure 4). We observed a more pronounced, but similar relationship, between detection of cave crayfishes and water volume (Figure 4). There was also a significant three-way interaction among species, gear, and substrate. Detection of both cavefishes and crayfishes was greatest using visual surveys from sampling units classified by fine rather than coarse substrates. Similarly, detection of cavefish was greatest using eDNA surveys in sampling units classified by fine rather than coarse substrates, and alternatively, detection of crayfishes was greatest in habitats classified by coarse substrates. Water clarity affected detection of cavefishes and crayfishes

differently. As expected, detection of cavefishes increased as water clarity increased (i.e., lower NTU). However, detection of cave crayfishes increased as turbidity increased (Figure 5). Although variation in water velocity across sample units was relatively small (i.e., 0–0.5 m/s), detection probability of both cavefishes and cave crayfishes using eDNA increased slightly as water velocity increased, and detection using visual surveys decreased as water velocity increased (Figure 6).

Geology and anthropogenic disturbance at the fine scale (i.e., 500-m buffer) affected occupancy of cavefishes and cave crayfishes (Figure 7, Table 13). Cavefishes were more likely to occur in the Smithville Dolomite and Osagean Series geologic units compared to the category we classified as "other." Alternatively, cave crayfishes were more likely to occur in Osagean and the category "other" relative to Smithville. Cavefishes had little relationship with anthropogenic disturbance, whereas cave crayfishes had a strong negative relationship.

Diagnostic tests indicated good model fit and each model in the backward selection process had adequate mixing of chains. We completed a goodness-of-fit test for the most-complex model which indicated that our model was not over dispersed (c hat = 1.09). Our highest R hat was less than 1.1 and our effective sample size was greater than 15,000 for all parameters.

Discussion

Environmental DNA surveys can provide supplemental data to traditional visual surveys for stygobionts. Environmental DNA is approximately of similar cost, can be less disruptive to cave organisms, and allows surveying of previously inaccessible areas, but does not replace traditional cave surveys. For documenting the presence of cavefishes, eDNA shows great promise; however, we also found eDNA surveys often resulted in false absences, especially for cave crayfishes. The effectiveness of both traditional surveys and eDNA were dependent on the habitat sampled making them complementary approaches under some circumstances. The deficiencies we encountered using eDNA surveys highlight the need to better understand the ecology of eDNA (i.e., the origin, state, fate, and transport of DNA in the environment, Barnes et al. 2016) in karst areas and the genetic structure of the populations of interest.

Detection of DNA in the environment can depend on the origin of the genetic material. For example, target organism abundance/biomass can relate to how much DNA will be released into the environment (Takahara et al. 2012) and can influence detection in some instances (Dougherty et al. 2016, Baldigo et al. 2017). However, in some cases target organism density can be poorly related to detection (Rice et al. 2018). We obtained false absences for *C. subterraneus* within Jail Cave, but maximum individual counts are lower when compared to similar caves within the same county where we were able to detect *C. tartarus* via eDNA (i.e., 4 vs. 17 individuals; Graening and Fenolio 2005; Graening et al. 2006a). The type of organism (e.g., fish or crayfish) can also be related to the amount of DNA present in the environment. For example, we detected cavefishes at every site where they were visually observed, and also in locations where they were not. Fishes would be expected to shed more DNA than crayfishes due to fishes having a slime coat and crayfishes having a hard exoskeleton (Tréguier et al. 2014). In contrast, we were often unable to detect cave crayfishes in some locations where they were visually observed. Timing of eDNA collection can also influence detection due to the release of more DNA during certain seasons when organisms are more active (de Souza et al. 2016).

The transport of eDNA in the environment can also have major implications in understanding the results of eDNA surveys. In surface waters, eDNA can travel horizontally downstream (up to 12.3 km; Deiner and Altermatt 2014) and can settle into the substrate (Turner et al. 2015). Asian carp DNA was detected near the Great Lakes, upstream of a fish barrier, with no evidence of live carp presence (Jerde et al. 2011), but later studies found that changes in flow direction were thought to be related to that phenomenon (Song et al. 2017). Karst environments are porous and water can flow in many directions underground (Aley and Kirkland 2012), making it difficult to understand how DNA may move through the environment. We observed increased detection of cavefishes and cave crayfishes increased as water velocity and water volume increased, which may be explained by movement of DNA from other locations. For example, we were able to detect *O. stygocaneyi* DNA in Onyx Cave, but it has never been visually observed in that cave. We hypothesize that *O. stygocaneyi* may not occupy that cave, but its DNA is present due to groundwater shared among systems during particularly wet

periods (i.e., Mud Cave is in close proximity to Onyx Cave). We also observed cavefish in certain caves only during periods when water was particularly high suggesting some of these systems are only used on particularly wet occasions (i.e., a cave on private land where S.K. Brewer observed a small cavefish in 2018). This highlights the need to focus conservation efforts on more than just the systems where organism have been historically observed. Environmental DNA can persist in terrestrial soil for at least six years (Andersen et al. 2012), and in cave soil for thousands of years (Hofreiter et al. 2003), complicating the use of eDNA as a monitoring tool. DNA may persist for years in the relatively stable underground aquifers, resulting in detections that are not indicative of the current population status. Alternatively, large floods can quickly move sediment and organisms out of caves (Van Gundy and White 2009, Graening et al. 2010), resulting in quick expulsion and dilution (Wilcox et al. 2016) of DNA.

We found, similar to other efforts using eDNA, the environment also influences eDNA detection. Inhibitors present in the environment (e.g., humic acid) may result in false absences when using eDNA surveys (Thomsen and Willerslev 2015). We used Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA; Cat. # 4396838) because it works in the presence of inhibitors. Zymo OneStep PCR Inhibitor Removal columns (Zymo Research, Irvine, CA; D6030) can also remove inhibitors, but using the columns can also reduce the DNA yield of a sample, which may be problematic when working with very low concentrations of DNA. We acknowledge that inhibitors are certainly present in cave systems (i.e., humic acid, limestone) and thus, may be one factor that increased the rate of false negative results (e.g., *C. subterraneus*).

We found the presence of pseudogenes limited detection for some species of cave crayfishes. Pseudogenes, or numts, are mitochondrial genes that have moved into the nucleus, become nonfunctional, and thus, acquire mutations (Buhay 2009). The numt sequences of CO1 can be highly divergent from the actual CO1, which complicates species identification based on sequence similarity (Song et al. 2008; Buhay 2009). Pseudogenes have been discovered in more than 82 eukaryote species and can be especially prevalent in cave crayfishes (e.g., up to 60 were found in *O. australis*; Song et al. 2008). It can be difficult or impossible to determine species when pseudogenes are co-amplified with the target mitochondrial gene because many different PCR products are

present. For example, pseudogenes were present in *O. stygocaneyi* DNA, resulting in poor binding of the primers and probes (Figures 2 and 3). The presence of putative pseudogenes also limited our ability to obtain clean sequences for *C. setosus* and design a useable assay for that species. Genetic techniques such as cloning, RT-PCR, long PCR, and mtDNA enrichment can assist in isolating the actual mitochondrial gene, but those techniques can be expensive, time consuming, and are not guaranteed to be effective (Song et al. 2008, Buhay 2009). Sequencing mitochondrial rich tissue (i.e., the gills) may help avoid the pseudogene (Buhay 2009), but requires sacrificing the organism. Finally, we could focus on different genes, but pseudogenes can still potentially be found with no way of easily identifying them because other commonly used genes (e.g., 16s) are not protein coding genes. Therefore, the presence of stop codons in the DNA sequence that would typically indicate that a gene is non-functional cannot not be used to differentiate between pseudogenes and the target genes (Buhay et al. 2009).

Geology series related to occupancy of cavefishes and cave crayfishes. Estimating actual recharge area of these system would be beneficial to improving our estimate (and some of these recharge areas are currently being delineated). However, the geologic series at each site influenced occupancy by both cavefishes and cave crayfishes. The underlying geology of a site controls the groundwater connection to other locations, which can affect stygobiont distributions (Noltie and Wicks 2001). We characterized geology using the series Smithville Dolomite, Osagean Series, and "Other". The geologic series generally corresponded to the Salem and Springfield plateau groundwater regions, and two isolated geological units within the Springfield Plateau, respectively. The geology of the Salem Plateau may reflect suitable cavefish habitat deep beneath the surface, whereas cavefish are confined near the surface within the Springfield Plateau (Noltie and Wicks 2001). It is surprising that we detected *T. rosae* in both the Salem and Springfield plateaus because we suspected the extreme difference in geology would confine the species to one plateau region. The karst layer in one of the isolated geology pockets (i.e., McDonald County) is absent, or extremely thin, which may exclude cavefish (Noltie and Wicks 2001). We never visually observed cavefish within the isolated geology pockets and only detected DNA in McDonald County, which suggests the presence of the species should be considered, but verified before implementing any

conservation or management actions. Cave crayfishes, however, have been visually observed within both of the isolated geology pockets (Graening et al. 2006b), suggesting they may require less groundwater connection compared to fishes. We also encountered cave crayfishes in one area of the Salem Plateau, which is hypothesized to be an isolated groundwater system (Hobbs III 2001). Future examination of these isolated pockets is warranted to ensure these populations receive adequate protections if needed.

We found that anthropogenic disturbance at the coarse scale (i.e., recharge area) was unimportant in our model; however, at a fine scale (500-m buffer) it affected species occurrence. It is interesting that cavefishes showed little relationship with disturbance, whereas crayfish displayed a strong negative relationship. Both cavefishes and cave crayfishes are thought to be negatively influenced by changes in groundwater quality (Graening et al. 2010). Our results suggest that cave crayfishes may be even more sensitive to human disturbance (i.e., primarily urban and agriculture lands) than cave fishes.

Conservation and Management Implications

Environmental DNA can be a valuable tool for surveying stygobiont populations, particularly in areas not easily accessed (i.e., wells and springs). For example, we used eDNA to identify new locations where *T. rosae* potentially occurs (e.g., McDonald, Taney, and Ozark counties in Missouri). Environmental DNA studies are best viewed as supplemental to visual surveys because the biological data gained from visual surveys are crucially important in conservation decisions. Environmental DNA surveys, although useful, may not be sensitive enough to be used successfully at cave entrances to avoid entering a cave, especially when cave organisms occupy deep portions of the cave or karst region, or there is little water flow. Lastly, eDNA is useful when examining systems that have never been sampled as a means to identify sites where traditional survey efforts may be used as a follow up.

Collection and processing of eDNA can be conducted at a similar cost to traditional surveys once the methods are well established, eDNA surveys may be less damaging to the habitat, and use of eDNA can increase the number of areas that we can survey relatively quickly. Our cost was approximately $15–$35 per sample when

factoring in all costs except collection. Visual surveys may end up being just as expensive when considering the time necessary to survey a cave using the required specialized equipment (i.e., headlamps, helmets, and rappelling gear, etc.). Baldigo et al. (2016) found that the cost of an eDNA survey ($20–50) was much less expensive than traditional electrofishing surveys ($500–$3000). Further, visual surveys may pose a greater risk to cave inhabitants because of trampling (Graening et al. 2006b) and introduction of diseases such as white-nose syndrome. Lastly, eDNA can allow us to easily sample areas such as wells and springs where we cannot see organisms that are underground. However, traditional surveys remain the best method if agencies are interested in morphometric, habitat use, movements, or population dynamics.

Our results suggest range extensions for both cave crayfishes and cavefishes and eDNA results should be confirmed by surveys with other techniques. We sequenced DNA from a single walking leg collected from a crayfish in Mitchell Cave, OK and our results confirm that *C. tartarus* occupies that cave. We amplified *T. rosae* DNA from caves that may represent range extensions for the species. We amplified DNA from caves in McDonald, Taney, and Ozark counties in Missouri, which are not known to harbor *T. rosae* (Graening et al. 2010). McDonald County does not have documented occurrences of *T. rosae*, but it is present in the five surrounding counties and other stygobionts (e.g., *C. setosus*) are known to occur in the cave where it was detected via eDNA. The positive results in Ozark County are intriguing because it is adjacent to the range of *T. eigenmanni* and well outside of the known range of *T. rosae*. False positives are quite common when conducting eDNA surveys and we chose to analyze our results conservatively, which might increase the chances of false positives. Therefore, future surveys should confirm the presence of *T. rosae* in both McDonald and Ozark counties.

Future efforts focused on expanding our knowledge of the genetic variation in the stygobiont populations of the Ozarks would be beneficial, especially for wide-ranging species such as *C. setosus*. We were limited in our ability to develop species-specific assays due to the sparse data available for certain species. For example, there are genetic sequences for only one *C. setosus* individual on GenBank. For a wide-ranging species, that is not nearly enough information to incorporate all of the genetic variation and design assays. We supplemented the available information with tissue samples and genomic

DNA shared by collaborators, but the resulting sequences revealed a limited understanding of cave crayfish species. For example, we sequenced a specimen for which species was unknown, that we assumed to be *C. setosus* because the collection locality was within that species known range (and only ~3 km from two caves we sampled), but the specimen was genetically closer to *C. zophonastes* (5.2% different) than *C. setosus* (6.5% different). A 6% difference would suggest that those sequences may be derived from a different species (i.e., based on the differences typically found for crayfish, Sinclair et al. 2004) or the diversity within the species is quite high. Further, sequences from opposite ends of the species' range (i.e., Newton and Greene counties, MO) were only 93.5% similar. However, we also recognize that the differences observed may be due to the presence of pseudogenes within the DNA of *C. setosus*. We did not observe stop codons in the sequences, but they had double peaks, suggesting that we were co-amplifying both the mitochondrial gene and the pseudogene. It seems prudent to better identify the genetic diversity within some of the more wide-ranging populations using an adequate representation of individuals. Obtaining genetic specimens across the geographic range (or perceived range) could be accomplished with minimal to no mortality by removal of a single walking leg. These data would be necessary to identify possible intergrade zones where species will not have sufficient variation to appear distinctly different (Buhay 2009), additional species, and the natural genetic heterogeneity within a species. Molecular studies of amblyopsid cavefishes uncovered considerable genetic differentiation among populations (e.g., Dillman et al. 2011; Niemiller 2011; Niemiller et al. 2012).

Use of eDNA by conservation agencies to direct more intensive and targeted sampling with other gears would be a valuable preliminary survey approach. Our results indicate select environmental factors affected detection when using either eDNA or visual surveys; thus, some bias would be reduced in survey results if detection covariates were included as part of survey efforts and analyses. For example, water clarity, water volume, substrate, and water velocity were all important detection covariates in our models, so future monitoring or research efforts might consider measuring these covariates as part of the sampling approach. Additionally, use of our eDNA protocol would be most beneficial for surveying *C. tartarus* and *T. rosae* until more work can be

completed to provide more genetic data for the other species. If the goal is to establish an eDNA monitoring effort for these species, collecting water samples during the autumn season would avoid interactions with the federally-endangered gray bat *Myotis grisescens* (MDC 2000). Typical precipitation events that occur during autumn would also increase the chances of some moving water within these karst systems, thereby facilitating the mixing and transport of DNA. Also, extreme flooding is less likely during the autumn season; thus, sampling during that period would minimize false positives caused by hydrologic connections among systems at high flows. Lastly, collecting a greater volume of water (Schultz and Lance 2015) and subsampling water across the sampling unit (i.e., pooling water across the sampling unit, Piaggio et al. 2014) might increase detection of small amounts of DNA in the water during low-flow conditions.

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Table 1. – Ozark cavefish *Troglichthys rosae* tissue samples obtained from USFWS were used to develop assays to amplify DNA from the environment. The samples listed were sequenced (see Table S1).

Sample ID	Location	County	State	Species
McAr25	McGee's Cave	Delaware	OK	*Troglichthys rosae*
Logan01	Logan Cave	Benton	AR	*Troglichthys rosae*
Logan02	Logan Cave	Benton	AR	*Troglichthys rosae*
Logan03	Logan Cave	Benton	AR	*Troglichthys rosae*
Logan04	Logan Cave	Benton	AR	*Troglichthys rosae*
Logan05	Logan Cave	Benton	AR	*Troglichthys rosae*
CSC01	Cave Springs Cave	Benton	AR	*Troglichthys rosae*
CSC02	Cave Springs Cave	Benton	AR	*Troglichthys rosae*
CSC03	Cave Springs Cave	Benton	AR	*Troglichthys rosae*
CSC04	Cave Springs Cave	Benton	AR	*Troglichthys rosae*
CSC05	Cave Springs Cave	Benton	AR	*Troglichthys rosae*

Table 2. – Delaware County cave crayfish *Cambarus subterraneus* and Oklahoma cave crayfish *Cambarus tartarus* tissue samples obtained from USFWS were used to develop assays to amplify DNA from the environment. The samples listed have been sequenced (see Table S1).

Sample ID	Location	County	State	Species
TC10	Twin Cave	Delaware	OK	*Cambarus subterraneus*
TC19	Twin Cave	Delaware	OK	*Cambarus subterraneus*
TC20	Twin Cave	Delaware	OK	*Cambarus subterraneus*
TC34	Twin Cave	Delaware	OK	*Cambarus subterraneus*
TC35	Twin Cave	Delaware	OK	*Cambarus subterraneus*
TC39	Twin Cave	Delaware	OK	*Cambarus subterraneus*
JS2	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
JS7	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
JS8	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
JS14	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
JS15	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
JS17	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
Mc26	McGee's Cave	Delaware	OK	*Cambarus tartarus*

Table 3. – Genomic DNA of four cave crayfish species was provided to us by the Missouri Department of Conservation to develop assays to amplify DNA from the environment. The samples listed have been sequenced (see Table S1).

Sample ID	Location	County	State	Species
MDC10	Lewis Cave	Ripley	MO	*Cambarus hubrichti*
MDC22	Medlock Cave	Shannon	MO	*Cambarus hubrichti*
MDC11	Jail Cave	Delaware	OK	*Cambarus subterraneus*
MDC12	Star Cave	Delaware	OK	*Cambarus subterraneus*
CA7	Nesbit Spring Cave	Stone	AR	*Cambarus zophonastes*
CA1	Hell Creek Cave	Stone	AR	*Cambarus zophonastes*
MDC13	Mud Cave	Ozark	MO	*Orconectes stygocaneyi*
MDC171	Mud Cave	Ozark	MO	*Orconectes stygocaneyi*

Table 4. – We supplemented the reference sequence database we created from obtained tissue and genomic DNA samples with sequences accessioned in GenBank. NA is listed if the sample location was not reported.

Sample ID	Location	County	State	Species
JX514482	NA	Benton	AR	*Cambarus aculabrum*
JX514464	NA	Dade	MO	*Cambarus setosus*
JX514465	January-Stansberry Cave	Delaware	OK	*Cambarus tartarus*
JN592335	Carroll Cave	Camden	MO	*Typhlichthys eigenmanni*
JN592328	Norfolk Lake	Baxter	AR	*Typhlichthys eigenmanni*

Table 5. – We designed Taqman® assays to amplify DNA for each of our target species. We chose to use Taqman® assays to increase specificity and sensitivity. The 5′ end of the probe was labeled with the fluorescent dye (6-FAM), the 3′ primer end with a quencher (Iowa Black™ FQ), and there was an additional internal quencher (ZEN™). Probes were doubled quenched to reduce background fluorescence and increase signal intensity.

Species	**Forward primer**	**Reverse primer**	**Probe**
Cambarus	CAA GAG GGA TAG	CCG GCT AAG TGC	ACC CAC CTT TAG CTT
aculabrum	TAG AGA GAG G	AAA GAA	CAG CAA TTG CTC A
Cambarus	CAG ACC AAA CAA	GCA CGG GAT	AGC ATG AGC AAT TGC
setosus	ATA ATG GTA TCC	GAA CTG TTT	CGA AGC CAA
Cambarus	GCA TTC GAT CCA	CTT AGC TGG AGT	CCG CCG CAC GTA TAT
subterraneus	TGG TCA TAC	GTC TTC TAT TT	TAA TAG CTG TTG T
Cambarus	TCC GAT CCG TTA	GTA CTG CAG GYA	ATC TTT GCC TGT GCT
tartarus	GTA GCA TAG	TGA CAA TGG	AGC GGG AGC
Orconectes	CAT GAG CTG TCA	TTT GGT ACT TGG	TCC GAT TAA CCT ACC
stygocaneyi	CTA CCA CAT TA	GCT GGA ATA G	TAC CTG GCC T
Troglichthys	GGT GRT GYT GAT	ACC CWC TCA TCC	TTG CGA AGG TGA TAG
rosae	GAG CTA TG	TAG TAR CC	TRG TGC CCA
Typhlichthys	CTG GCT ACT AGC	TTG CGC TGG CGA	CCC GCG CAG TAG AAG
eigenmanni	ATG AAT GG	ATA AG	CCA CAA CAA

Table 6. – Variability of physicochemical conditions at each sampling unit included in our occupancy model. The mean (± standard deviation) of each variable across survey dates is provided.

Site	County	State	Water clarity (NTU)	Water volume (m^3)	Water Velocity (m/s)
Bartholic Spring	Newton	MO	3.59 ± 2.43	3.35 ± 1.93	0.02 ± 0.03
Billies Creek Cave	Lawrence	MO	1.30 ± 0.07	16.87 ± 0	0
Bear Hollow Cave	Benton	AR	1.55 ± 0.86	73.83 ± 0	0
Bear Hollow Cave	Benton	AR	2.33 ± .033	1.5 ± 0	0.00 ± 0.01
Bluff Dwellers Cave	McDonald	MO	1.08 ± 0.65	1.5 ± 0	0
Bluff Dwellers Cave	McDonald	MO	1.64 ± 1.11	37.5 ± 0	0
Buddy Well	Newton	MO	0.36 ± 0.22	1.23 ± 0	0
Capps Creek Well # 2	Newton	MO	0.82 ± 0.85	0.91 ± 0	0
Capps Creek Well #1	Newton	MO	4.39 ± 3.68	0.98 ± 0	0.08 ± 0.03
Carroll River	Camden	MO	2.11 ± 1.90	3.87 ± 1.54	0
Carroll River	Camden	MO	3.65 ± 4.21	163.47 ± 52.42	0.01 ± 0
Carroll River	Camden	MO	4.43 ± 3.57	98.72 ± 32.57	0
Elm Spring	Newton	MO	2.55 ± 1.21	37.24 ± 24.63	0.08 ± 0.04
Fielden Cave	Christian	MO	0.85 ± 0.42	5.64 ± 0	0.01 ± 0.01
Harrison Cave	Lawrence	MO	0.77 ± 0.63	4.2 ± 0	0
Harrison Cave	Lawrence	MO	1.01 ± 0.74	11.64 ± 0	0

Hearrell Spring	Newton	MO	3.38 ± 1.53	24.62 ± 5.33	0.13 ± 0.04
Jail Cave	Delaware	OK	3.54 ± 2.47	33.33 ± 15.3	0
Jail Cave	Delaware	OK	3.79 ± 3.54	4.27 ± 6	0
January-Stansberry Cave	Delaware	OK	0.55 ± 0.07	357.96 ± 0	0.02 ± 0.01
January-Stansberry Cave	Delaware	OK	0.60 ± 0.02	504.79 ± 0	0.02 ± 0.02
January-Stansberry Cave	Delaware	OK	0.66 ± 0.00	96.52 ± 0	0.02 ± 0
January-Stansberry Cave	Delaware	OK	0.68 ± 0.06	143.64 ± 0	0.02 ± 0.01
Johnson Well	Lawrence	MO	2.63 ± 1.51	2.16 ± 0	0.22 ± 0.17
Karst Window	Taney	MO	3.10 ± 4.04	111.95 ± 86.69	0.04 ± 0.06
Leopold Spring	Ozark	MO	2.05 ± 0.00	0.11 ± 0.04	0.05 ± 0.05
Logan Cave	Benton	AR	0.41 ± 0.20	1.25 ± 0	0.01 ± 0
Logan Cave	Benton	AR	0.79 ± 0.88	17.33 ± 0	0.15 ± 0
Logan Cave	Benton	AR	1.05 ± 0.57	47.5 ± 0	0.12 ± 0
Long's Cave	Delaware	OK	0.54 ± 0	693.33 ± 184.75	0
McGee Cave	Delaware	OK	3.95 ± 4.37	54.38 ± 25.66	0
McMahan Spring	Newton	MO	1.62 ± 1.14	12 ± 3.46	0.13 ± 0.03
Mud Cave	Ozark	MO	15.57 ± 3.61	368.69 ± 327.22	0
Onyx Cave	Ozark	MO	17.52 ± 3.79	163.3 ± 80.89	0
OT-4	Ottawa	OK	1.9 ± 0.94	4.11 ± 1.98	0.11 ± 0.08
OT-4	Ottawa	OK	2.97 ± 2.44	4.86 ± 1.91	0.15 ± 0.14

OT-4	Ottawa	OK	3.56 ± 2.58	11.12 ± 2.5	0.10 ± 0.07
Peter Well	Newton	MO	7.16 ± 6.12	0.16 ± 0.05	0
Poor Well	Newton	MO	2.48 ± 2.71	0.24 ± 0	0
Protem Spring	Taney	MO	3.10 ± 1.95	2.33 ± 2.31	0.40 ± 0.10
Slaughter Well	Newton	MO	3.26 ± 3.56	0.16 ± 0	0
Smallin Civil War Cave	Christian	MO	1.03 ± 0.92	104.34 ± 36.79	0.06 ± 0.06
Smallin Civil War Cave	Christian	MO	1.09 ± 1.29	12.36 ± 4.94	0.10 ± 0.12
Smallin Civil War Cave	Christian	MO	1.18 ± 1.10	18.4 ± 0	0.05 ± 0.06
Smallin Civil War Cave	Christian	MO	1.19 ± 1.03	17.25 ± 14.12	0.08 ± 0.10
Smallin Civil War Cave	Christian	MO	1.24 ± 0.91	26.19 ± 9.18	0.02 ± 0.02
Spring House	Taney	MO	2.79 ± 3.96	1.27 ± 0.46	0.04 ± 0.05
Sugar Bowl Cave	McDonald	MO	3.05 ± 1.13	0.6 ± 0	0
Thunder River	Camden	MO	3.82 ± 0.18	1.02 ± 0.16	0.23 ± 0.16
Thunder River	Camden	MO	4.41 ± 4.00	21.08 ± 5.73	0.06 ± 0.04
Thunder River	Camden	MO	4.77 ± 2.68	16.35 ± 6.31	0.27 ± 0.23
Tumbling Creek Cave	Taney	MO	1.29 ± 0.85	1.77 ± 0.87	0.06 ± 0.05
Tumbling Creek Cave	Taney	MO	1.15 ± 0.40	114.61 ± 6.85	0.24 ± 0.12
Tumbling Creek Cave	Taney	MO	1.31 ± 0.72	7.23 ± 1.07	0.07 ± 0.06
Tumbling Creek Spring	Taney	MO	4.51 ± 7.00	53.2 ± 9.31	0.04 ± 0.05
Unnamed Cave	Ottawa	MO	3.68 ± 3.05	1 ± 0.5	0.04 ± 0.02

Walbridge Spring	Newton	MO	1.61 ± 1.39	150.72 ± 16.32	0
Woody Cave	Christian	MO	0.37 ± 0.04	19.2 ± 0	0.03 ± 0

Table 7. – We detected *Troglichthys rosae* using eDNA at every site where it was observed via visual surveys, and 16 additional sites where it was not visually observed. However, at some sites, we did not detect a species at every sampling unit (SU), even when it was visually detected. Some of these detections may represent range extensions (e.g., Bluff Dwellers Cave and the caves/springs in Ozark County). Each site was sampled at 1–5 sampling units, on multiple occasions, using traditional visual surveys and an eDNA survey technique where 2-L of water were collected. For eDNA and visual surveys, 'Yes' represents a positive detection and 'No' reflects negative detection.

Site	County	State	Date	SU	eDNA	Visual
Bartholic Spring	Newton	MO	3/16/2017	1	No	No
Bartholic Spring	Newton	MO	3/31/2018	1	No	No
Bartholic Spring	Newton	MO	4/6/2017	1	No	No
Bear Cave	Ozark	MO	4/25/2017	1	Yes	No
Bear Hollow Cave	Benton	AR	2/23/2017	1	Yes	No
Bear Hollow Cave	Benton	AR	3/1/2017	1	Yes	No
Bear Hollow Cave	Benton	AR	3/7/2017	1	Yes	No
Bear Hollow Cave	Benton	AR	2/23/2017	2	No	No
Bear Hollow Cave	Benton	AR	3/1/2017	2	No	No
Bear Hollow Cave[a]	Benton	AR	3/7/2017	2	Yes	No
Bluff Dwellers Cave	McDonald	MO	3/8/2017	1	No	No
Bluff Dwellers Cave	McDonald	MO	3/17/2017	1	No	No
Bluff Dwellers Cave	McDonald	MO	3/31/2018	1	No	No
Bluff Dwellers Cave[a]	McDonald	MO	3/8/2017	2	Yes	No
Bluff Dwellers Cave	McDonald	MO	3/17/2017	2	No	No
Bluff Dwellers Cave	McDonald	MO	3/31/2018	2	No	No
Buddy Well	Newton	MO	2/26/2017	1	Yes	Yes
Buddy Well	Newton	MO	3/8/2017	1	Yes	No
Buddy Well[b]	Newton	MO	3/16/2017	1	Yes	Yes
Capps Creek Well #1[b]	Newton	MO	5/14/2017	1	Yes	No
Capps Creek Well #1	Newton	MO	5/18/2017	1	No	Yes

Capps Creek Well #1[b]	Newton	MO	5/20/2017	1	Yes	No
Capps Creek Well #2	Newton	MO	2/25/2017	1	Yes	No
Capps Creek Well #2	Newton	MO	3/8/2017	1	No	No
Capps Creek Well #2[b]	Newton	MO	3/17/2017	1	Yes	No
Elm Spring	Newton	MO	3/16/2017	1	No	No
Elm Spring	Newton	MO	3/31/2018	1	No	No
Elm Spring	Newton	MO	4/6/2017	1	No	No
Fielden Cave	Christian	MO	2/27/2017	1	No	No
Fielden Cave	Christian	MO	3/9/2017	1	Yes	No
Fielden Cave	Christian	MO	3/18/2017	1	Yes	No
Fielden Cave	Christian	MO	4/1/2017	1	Yes	No
Fielden Cave	Christian	MO	8/2/2017	1	No	No
Harrison Cave	Lawrence	MO	2/25/2017	1	Yes	Yes
Harrison Cave	Lawrence	MO	3/9/2017	1	Yes	Yes
Harrison Cave[b]	Lawrence	MO	3/17/2017	1	Yes	Yes
Harrison Cave	Lawrence	MO	2/25/2017	2	Yes	Yes
Harrison Cave	Lawrence	MO	3/9/2017	2	Yes	Yes
Harrison Cave	Lawrence	MO	3/17/2017	2	No	Yes
Hearrell Spring	Newton	MO	3/16/2017	1	Yes	No
Hearrell Spring[b]	Newton	MO	3/31/2018	1	Yes	No
Hearrell Spring	Newton	MO	4/6/2017	1	No	No
Jail Cave	Delaware	OK	3/30/2017	1	Yes	Yes
Jail Cave	Delaware	OK	4/5/2017	1	No	Yes
Jail Cave[b]	Delaware	OK	4/24/2017	1	Yes	No
Jail Cave[b]	Delaware	OK	3/30/2017	2	Yes	No
Jail Cave[b]	Delaware	OK	4/5/2017	2	Yes	No
Jail Cave[b]	Delaware	OK	4/24/2017	2	Yes	No
January-Stansberry Cave	Delaware	OK	2/21/2017	1	No	No
January-Stansberry Cave	Delaware	OK	3/10/2017	1	No	No
January-Stansberry Cave	Delaware	OK	3/23/2017	1	No	No

January-Stansberry Cave	Delaware	OK	2/21/2017	2	No	No
January-Stansberry Cave[a]	Delaware	OK	3/10/2017	2	Yes	No
January-Stansberry Cave	Delaware	OK	3/23/2017	2	No	No
January-Stansberry Cave[a]	Delaware	OK	2/21/2017	3	No	No
January-Stansberry Cave	Delaware	OK	3/10/2017	3	No	No
January-Stansberry Cave	Delaware	OK	3/23/2017	3	No	No
January-Stansberry Cave	Delaware	OK	3/10/2017	4	No	No
January-Stansberry Cave	Delaware	OK	3/23/2017	4	No	No
Johnson Well	Lawrence	MO	5/14/2017	1	No	No
Johnson Well	Lawrence	MO	5/18/2017	1	No	No
Johnson Well	Lawrence	MO	5/20/2017	1	No	No
Karst Window	Taney	MO	2/28/2017	1	No	No
Karst Window	Taney	MO	4/13/2017	1	Yes	No
Karst Window	Taney	MO	4/26/2017	1	Yes	No
Leopold Spring	Ozark	MO	3/1/2017	1	No	No
Leopold Spring	Ozark	MO	4/12/2017	1	No	No
Leopold Spring[a]	Ozark	MO	4/25/2017	1	No	No
Leopold Spring	Ozark	MO	8/3/2017	1	No	No
Leopold Spring	Ozark	MO	5/8/2018	1	No	No
Leopold Spring	Ozark	MO	9/4/2018	1	No	No
Logan Cave	Benton	AR	2/22/2017	1	Yes	Yes
Logan Cave	Benton	AR	3/1/2017	1	Yes	No
Logan Cave	Benton	AR	3/7/2017	1	Yes	Yes
Logan Cave	Benton	AR	2/22/2017	2	Yes	No
Logan Cave	Benton	AR	3/1/2017	2	Yes	Yes
Logan Cave[b]	Benton	AR	3/7/2017	2	Yes	No
Logan Cave	Benton	AR	2/22/2017	3	Yes	No
Logan Cave	Benton	AR	3/1/2017	3	Yes	No
Logan Cave	Benton	AR	3/7/2017	3	Yes	No
Long's Cave	Delaware	OK	4/4/2017	1	Yes	Yes

Long's Cave[c]	Delaware	OK	4/24/2017	1	Yes	NA
Long's Cave[c]	Delaware	OK	5/19/2017	1	No	NA
Long's Cave	Delaware	OK	4/4/2017	2	No	No
McGee Cave	Delaware	OK	4/4/2017	1	Yes	No
McGee Cave	Delaware	OK	4/24/2017	1	No	No
McGee Cave	Delaware	OK	5/19/2017	1	Yes	Yes
McMahan Spring	Newton	MO	3/16/2017	1	Yes	No
McMahan Spring[b]	Newton	MO	3/31/2018	1	Yes	No
McMahan Spring[b]	Newton	MO	4/6/2017	1	Yes	No
Mud Cave	Ozark	MO	3/1/2017	1	Yes	No
Mud Cave	Ozark	MO	4/12/2017	1	Yes	No
Mud Cave	Ozark	MO	4/25/2017	1	Yes	No
Mud Cave	Ozark	MO	5/15/2017	1	Yes	No
Mud Cave	Ozark	MO	5/17/2017	1	Yes	No
Mud Cave	Ozark	MO	8/3/2017	1	No	No
Mud Cave	Ozark	MO	5/8/2018	1	No	No
Mud Cave	Ozark	MO	9/4/2018	1	No	No
Onyx Cave	Ozark	MO	4/25/2017	1	Yes	No
Onyx Cave	Ozark	MO	5/15/2017	1	Yes	No
Onyx Cave	Ozark	MO	5/17/2017	1	Yes	No
Onyx Cave	Ozark	MO	5/8/2018	1	No	No
OT-4	Ottawa	OK	3/19/2017	1	No	No
OT-4	Ottawa	OK	3/29/2017	1	No	No
OT-4	Ottawa	OK	4/5/2017	1	No	No
OT-4	Ottawa	OK	3/19/2017	2	No	No
OT-4[a]	Ottawa	OK	3/29/2017	2	No	No
OT-4	Ottawa	OK	4/5/2017	2	No	No
OT-4	Ottawa	OK	3/19/2017	3	No	No
OT-4	Ottawa	OK	3/29/2017	3	No	No
OT-4	Ottawa	OK	4/5/2017	3	No	No

OT-4	Ottawa	OK	3/29/2017	4	No	No
Peter Well	Newton	MO	5/14/2017	1	No	No
Peter Well	Newton	MO	5/18/2017	1	No	No
Peter Well	Newton	MO	5/20/2017	1	No	No
Poor Well[b]	Newton	MO	2/26/2017	1	Yes	No
Poor Well	Newton	MO	3/8/2017	1	Yes	Yes
Poor Well	Newton	MO	3/17/2017	1	Yes	Yes
Billies Creek Cave	Lawrence	MO	2/25/2017	1	No	No
Billies Creek Cave	Lawrence	MO	3/9/2017	1	No	No
Billies Creek Cave	Lawrence	MO	3/17/2017	1	No	No
Protem Spring	Taney	MO	4/13/2017	1	No	No
Protem Spring	Taney	MO	4/26/2017	1	No	No
Protem Spring	Taney	MO	5/16/2017	1	No	No
Slaughter Well	Newton	MO	2/26/2017	1	Yes	No
Slaughter Well	Newton	MO	3/10/2017	1	No	No
Slaughter Well	Newton	MO	3/17/2017	1	No	No
Smallin Civil War Cave	Christian	MO	2/27/2017	1	Yes	No
Smallin Civil War Cave	Christian	MO	3/9/2017	1	Yes	No
Smallin Civil War Cave	Christian	MO	3/18/2017	1	No	No
Smallin Civil War Cave	Christian	MO	4/1/2017	1	No	No
Smallin Civil War Cave	Christian	MO	8/2/2017	1	Yes	No
Smallin Civil War Cave	Christian	MO	2/27/2017	2	No	No
Smallin Civil War Cave	Christian	MO	3/9/2017	2	No	No
Smallin Civil War Cave	Christian	MO	3/18/2017	2	No	No
Smallin Civil War Cave	Christian	MO	4/1/2017	2	No	No
Smallin Civil War Cave	Christian	MO	8/2/2017	2	No	No
Smallin Civil War Cave	Christian	MO	2/27/2017	3	Yes	No
Smallin Civil War Cave	Christian	MO	3/9/2017	3	No	No
Smallin Civil War Cave	Christian	MO	3/18/2017	3	No	No
Smallin Civil War Cave	Christian	MO	4/1/2017	3	No	No

Smallin Civil War Cave	Christian	MO	8/2/2017	3	No	No
Smallin Civil War Cave	Christian	MO	2/27/2017	4	Yes	No
Smallin Civil War Cave	Christian	MO	3/9/2017	4	Yes	No
Smallin Civil War Cave	Christian	MO	4/1/2017	4	No	No
Smallin Civil War Cave	Christian	MO	8/2/2017	4	Yes	No
Smallin Civil War Cave	Christian	MO	2/27/2017	5	No	No
Smallin Civil War Cave	Christian	MO	3/9/2017	5	No	No
Smallin Civil War Cave	Christian	MO	3/18/2017	5	No	No
Smallin Civil War Cave	Christian	MO	4/1/2017	5	Yes	No
Smallin Civil War Cave	Christian	MO	8/2/2017	5	No	No
Spring House	Taney	MO	4/13/2017	1	No	No
Spring House	Taney	MO	4/26/2017	1	No	No
Spring House	Taney	MO	5/16/2017	1	No	No
Sugar Bowl Cave[a]	McDonald	MO	3/17/2017	1	No	No
Sugar Bowl Cave	McDonald	MO	3/31/2018	1	No	No
Sugar Bowl Cave	McDonald	MO	4/6/2017	1	No	No
Tumbling Creek Cave	Taney	MO	2/28/2017	1	No	No
Tumbling Creek Cave	Taney	MO	4/12/2017	1	No	No
Tumbling Creek Cave	Taney	MO	5/17/2017	1	No	No
Tumbling Creek Cave	Taney	MO	2/28/2017	2	No	No
Tumbling Creek Cave	Taney	MO	4/12/2017	2	No	No
Tumbling Creek Cave	Taney	MO	5/17/2017	2	No	No
Tumbling Creek Cave	Taney	MO	2/28/2017	3	No	No
Tumbling Creek Cave	Taney	MO	4/12/2017	3	No	No
Tumbling Creek Cave	Taney	MO	5/17/2017	3	No	No
Tumbling Creek Spring #1	Taney	MO	4/13/2017	1	No	No
Tumbling Creek Spring #2	Taney	MO	4/13/2017	1	No	No
Tumbling Creek Spring #3	Taney	MO	4/13/2017	1	No	No
Tumbling Creek Spring #3	Taney	MO	4/26/2017	1	No	No
Tumbling Creek Spring #3	Taney	MO	5/16/2017	1	No	No

Tumbling Creek Well	Taney	MO	4/13/2017	1	No	No
Tumbling Creek Well	Taney	MO	4/26/2017	1	Yes	No
Tumbling Creek Well	Taney	MO	5/16/2017	1	No	No
Unnamed Cave	Ottawa	OK	3/17/2017	1	Yes	No
Unnamed Cave	Ottawa	OK	3/29/2017	1	Yes	No
Unnamed Cave	Ottawa	OK	4/5/2017	1	No	No
Walbridge Spring	Newton	MO	3/8/2017	1	Yes	No
Walbridge Spring	Newton	MO	3/16/2017	1	No	No
Walbridge Spring	Newton	MO	3/31/2018	1	Yes	No
Woody Cave[a]	Christian	MO	2/27/2017	1	No	No
Woody Cave	Christian	MO	3/9/2017	1	No	No
Woody Cave	Christian	MO	3/18/2017	1	No	No

a. Replicates from only one sampling occasion amplified, so the samples were run a second time.

b. Weak amplification compared to other samples (i.e., fluorescence occurred after 40 cycles and less than half of the intensity of most samples).

c. Long's Cave was filled with water on the last two sampling dates, so one sample was collected at the mouth of the cave.

Table 8. – Our assays for *Typhlichthys eigenmanni* worked properly, but there were false absences using eDNA surveys (i.e., we observed the species, but did not pick up its DNA from the environment). Carroll and Thunder rivers both flow through Carroll Cave. Each site was sampled at 1–5 sampling units (SU), on multiple occasions, using traditional visual surveys and an eDNA survey technique where 2-L of water were collected. For eDNA and visual surveys, 'Yes' represents a positive detection and 'No' reflects negative detection.

Site	County	State	Date	SU	eDNA	Visual
Carroll River	Camden	MO	3/25/2017	1	No	No
Carroll River	Camden	MO	4/15/2017	1	No	No
Carroll River	Camden	MO	5/13/2017	1	No	No
Carroll River	Camden	MO	3/25/2017	2	No	Yes
Carroll River	Camden	MO	4/15/2017	2	No	No
Carroll River	Camden	MO	5/13/2017	2	No	No
Carroll River	Camden	MO	3/25/2017	3	No	No
Carroll River	Camden	MO	4/15/2017	3	No	No
Carroll River	Camden	MO	5/13/2017	3	No	Yes
Thunder River	Camden	MO	3/25/2017	1	No	No
Thunder River	Camden	MO	4/15/2017	1	No	No
Thunder River	Camden	MO	5/13/2017	1	No	No
Thunder River	Camden	MO	3/25/2017	2	No	Yes
Thunder River	Camden	MO	4/15/2017	2	Yes	Yes
Thunder River	Camden	MO	5/13/2017	2	No	Yes
Thunder River	Camden	MO	3/25/2017	3	Yes	Yes
Thunder River	Camden	MO	4/15/2017	3	Yes	Yes
Thunder River	Camden	MO	5/13/2017	3	Yes	Yes

Table 9. – We were unable to detect *Cambarus subterraneus* DNA from our field-collected water samples even though the species was observed (i.e., false negative). Amplification of genomic DNA suggested that the assay should work, so inhibitors in the water or too little DNA in the environment are likely explanations for the lack of amplification. Each site was sampled at 1–5 sampling units (SU), on multiple occasions, using traditional visual surveys and an eDNA survey technique where 2-L of water were collected. For eDNA and visual surveys, 'Yes' represents a positive detection and 'No' reflects negative detection.

Site	County	State	Date	SU	eDNA	Visual
Jail Cave	Delaware	OK	3/30/2017	1	No	Yes
Jail Cave	Delaware	OK	4/5/2017	1	No	Yes
Jail Cave	Delaware	OK	4/24/2017	1	No	No
Jail Cave	Delaware	OK	3/30/2017	2	No	No
Jail Cave	Delaware	OK	4/5/2017	2	No	No
Jail Cave	Delaware	OK	4/24/2017	2	No	No

Table 10. – We obtained positive amplification from every site sampled for *Cambarus tartarus* and from some sampling units where it was not visually observed. *Cambarus tartarus* was previously observed at all of the sites we sampled. Each site was sampled at 1–5 sampling units (SU), on multiple occasions, using traditional visual surveys and an eDNA survey technique where 2-L of water were collected. For eDNA and visual surveys, 'Yes' represents a positive detection and 'No' reflects negative detection.

Site	County	State	Date	SU	eDNA	Visual
January-Stansberry Cave	Delaware	OK	2/21/2017	1	Yes	No
January-Stansberry Cave	Delaware	OK	3/10/2017	1	Yes	No
January-Stansberry Cave	Delaware	OK	3/23/2017	1	No	No
January-Stansberry Cave	Delaware	OK	2/21/2017	2	Yes	No
January-Stansberry Cave	Delaware	OK	3/10/2017	2	Yes	No
January-Stansberry Cave	Delaware	OK	3/23/2017	2	Yes	No
January-Stansberry Cave	Delaware	OK	2/21/2017	3	Yes	No
January-Stansberry Cave	Delaware	OK	3/10/2017	3	No	No
January-Stansberry Cave	Delaware	OK	3/23/2017	3	Yes	No
January-Stansberry Cave	Delaware	OK	3/10/2017	4	Yes	No
January-Stansberry Cave	Delaware	OK	3/23/2017	4	Yes	No
Long's Cave	Delaware	OK	4/4/2017	1	No	No
Long's Cave[a]	Delaware	OK	4/24/2017	1	Yes	NA
Long's Cave[a]	Delaware	OK	5/19/2017	1	No	NA
Long's Cave	Delaware	OK	4/4/2017	2	Yes	No
McGee Cave	Delaware	OK	4/4/2017	1	Yes	No
McGee Cave	Delaware	OK	4/24/2017	1	Yes	No
McGee Cave	Delaware	OK	5/19/2017	1	Yes	Yes

a. Long's Cave was flooded on the last two sample dates, so water samples were collected only at the mouth of the cave.

Table 11. – We amplified *O. stygocaneyi* DNA from the only cave where they are known to occur and from a nearby cave. Each site was sampled at one sampling unit, on multiple occasions, using traditional visual surveys and an eDNA survey technique where 2-L of water were collected. Each site only had one sampling unit because of the relatively small size of the caves wetted areas (i.e., multiple surveys would violate the closure assumption of occupancy modeling). For both eDNA and visual surveys, 'Yes' represents a positive detection and 'No' reflects negative detection. If detection is listed as 'NA', the sample was contaminated during the genetic analyses.

Site	County	State	Date	eDNA	Visual
Bear Cave	Ozark	MO	4/25/2017	No	No
Leopold Spring	Ozark	MO	3/1/2017	NA	No
Leopold Spring	Ozark	MO	4/12/2017	NA	No
Leopold Spring	Ozark	MO	4/25/2017	No	No
Leopold Spring	Ozark	MO	8/3/2017	No	No
Leopold Spring	Ozark	MO	5/8/2018	No	No
Leopold Spring	Ozark	MO	9/4/2018	No	No
Mud Cave	Ozark	MO	3/1/2017	NA	Yes
Mud Cave	Ozark	MO	4/12/2017	No	Yes
Mud Cave	Ozark	MO	4/25/2017	No	Yes
Mud Cave	Ozark	MO	5/15/2017	No	No
Mud Cave	Ozark	MO	5/17/2017	No	No
Mud Cave	Ozark	MO	8/3/2017	Yes	Yes
Mud Cave	Ozark	MO	5/8/2018	Yes	Yes
Mud Cave	Ozark	MO	9/4/2018	Yes	Yes
Onyx Cave	Ozark	MO	4/25/2017	No	No
Onyx Cave	Ozark	MO	5/15/2017	Yes	No
Onyx Cave	Ozark	MO	5/17/2017	No	No
Onyx Cave	Ozark	MO	5/8/2018	Yes	No

Table 12. – We used single-season occupancy modeling in a Bayesian framework to estimate detection probability of both cavefishes and cave crayfishes. Our final detection model indicated that volume and substrate influenced detection, but varied by species and the gear used to survey. Further, water clarity and velocity affected detection and varied by gear and species, respectively. HDIs references highest density intervals.

Parameter	**Mean ± SD**	**90% HDIs**
Detection intercept	0.42 ± 0.48	-0.37 – 1.20
Taxa-cavefishes	0.09 ± 0.55	-0.81 – 1.00
Gear-visual	-0.23 ± 0.57	-1.20 – 0.68
Water clarity (NTU)	0.22 ± 0.19	-0.09 – 0.54
Water velocity (m/s)	0.45 ± 0.20	0.11 – 0.78
Substrate-fine	-1.52 ± 0.94	-3.01 – 0.45
Water volume (m^3)	0.11 ± 0.34	-0.44 – 0.67
Taxa-cavefishes X gear-visual	-1.68 ± 0.72	-2.86 – -0.50
Taxa-cavefishes X water clarity (NTU)	-0.38 ± 0.23	-0.76 – < 0.01
Gear-visual X water velocity (m/sec)	-0.82 ± 0.27	-1.26 – -0.37
Taxa-cavefishes X substrate-fine	2.66 ± 1.02	0.99 – 4.34
Gear-visual X substrate-fine	0.71 ± 1.07	-1.04 – 2.45
Taxa-cavefishes X water volume (m^3)	-0.19 ± 0.39	-0.83 – 0.44
Gear-visual X water volume (m^3)	-1.42 ± 0.47	-2.19 – -0.66
Taxa-cavefishes X gear-visual X substrate-fine	-2.86 ± 1.23	-4.85 – -0.81
Taxa-cavefishes X gear-visual X water volume (m^3)	1.00 ± 0.55	0.11 – 1.90

Table 13. Parameter estimates of a single-season occupancy modeling developed using a Bayesian framework to estimate occurrence probability of both cavefishes and cave crayfishes. Of the variables we included in our model, only geology influenced occupancy and varied by taxa. HDIs references highest density intervals.

Parameter	**Mean ± SD**	**90% HDIs**
Occurrence intercept	-0.35 ± 0.87	-1.77 – 1.08
Geology-Smithville	0.96 ± 0.99	-0.66 – 2.59
Geology-Meramecian	-2.60 ± 1.57	-5.11 – -0.06
Disturbance index	-1.89 ± 0.70	-2.99 – -0.75
Taxa-cavefishes	-1.39 ± 1.65	-4.03 – 1.26
Disturbance X taxa-cavefishes	2.30 ± 0.78	1.01 – 3.51
Geology-Meramecian X taxa-cavefishes	1.78 ± 1.77	-1.13 – 4.55
Geology-Smithville X taxa-cavefishes	4.13 ± 2.16	0.65 – 7.67

Figure 1. – Filtration setup for eDNA collection. Two, 1-L water samples were collected from each sampling unit. While wearing nitrile gloves, a 0.45-µm microbial filter was placed inside a filter funnel that was attached to a vacuum flask via a rubber stopper. A hand pump was used to create a vacuum and pull water through the filter. Filters were stored in 900 µl of Longmire's buffer (Longmire et al. 1997).

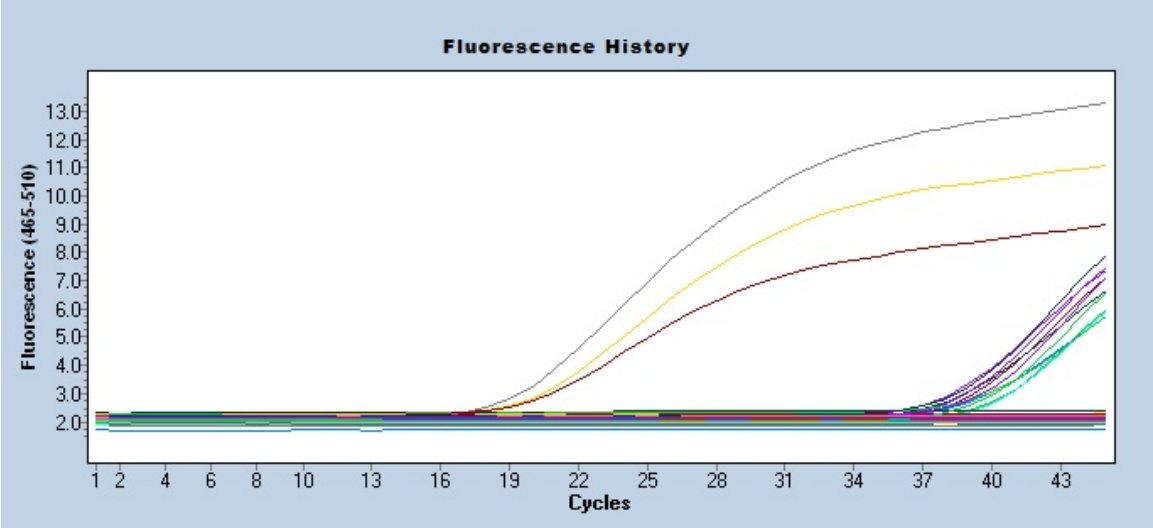

Figure 2. – During the qPCR, DNA was replicated and replication was tracked via a fluorescent dye (i.e., higher fluorescence = more DNA replication). If any of the six replicates from a survey amplified, the survey was considered positive for a species. This figure shows amplification of the positive controls (i.e., first three curves from the left) and several field samples (i.e., curves on the far right).

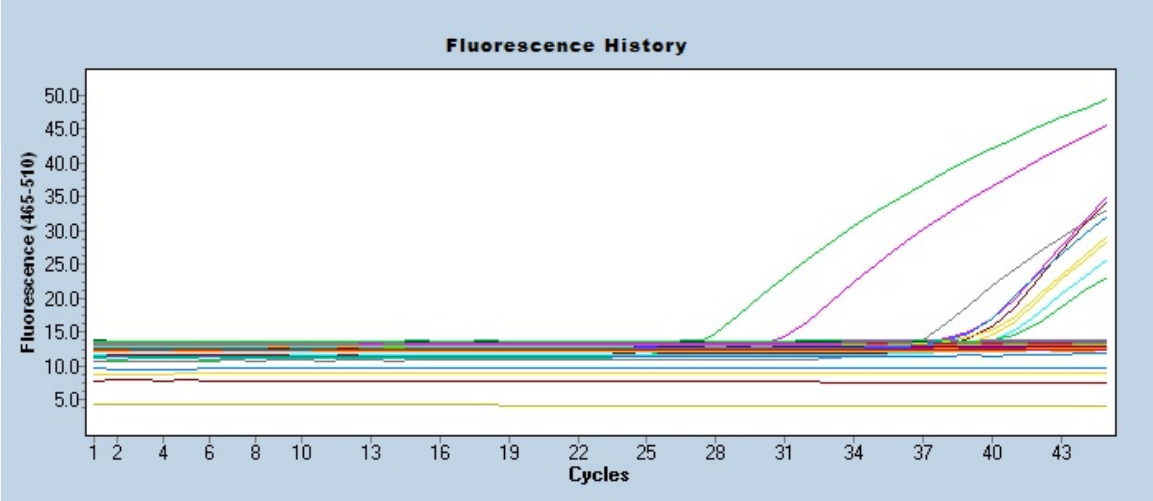

Figure 3. – The shape of the positive control curves (i.e., first two curves from the left) suggest poor binding of the primers and probes to *Orconectes stygocaneyi* DNA. In Figure 2, the curves of the positive control are sigmoidal (i.e., the typical shape resulting from DNA amplification) and in Figure 3 the curves are closer to straight lines. Our assay for *O. stygocaneyi* was designed based on a pseudogene, which resulted in the curves shown and possibly poor detection. The large difference in the amount of fluorescence shown in Figures 2 and 3 is due to different types of plates being used.

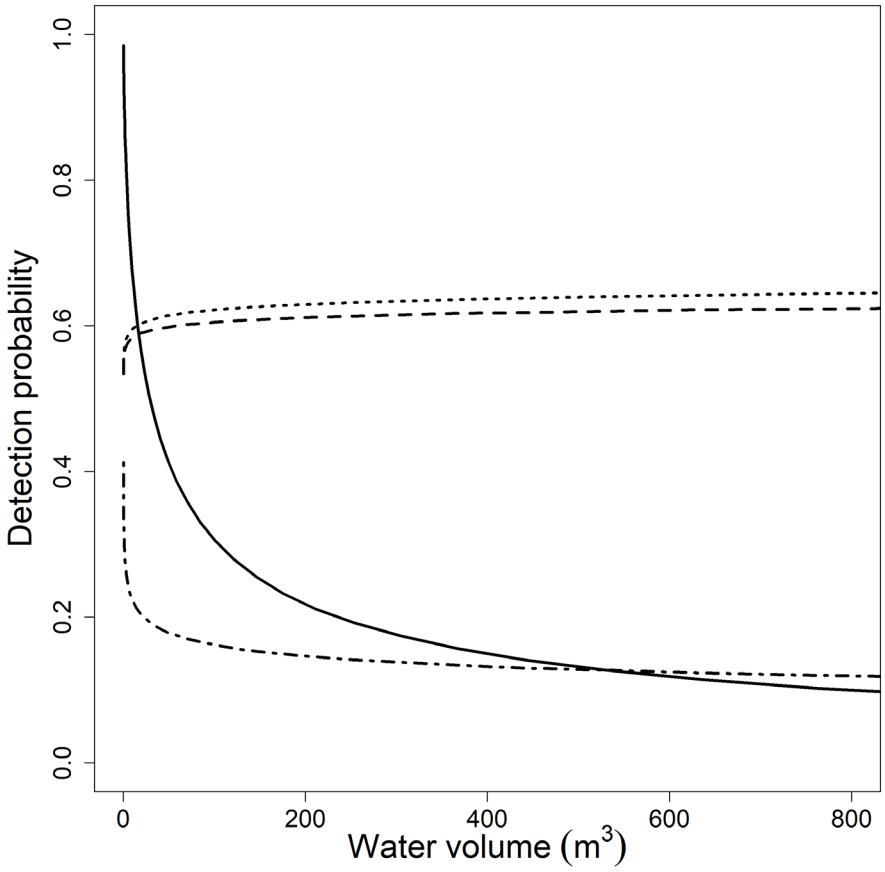

Figure 4. – The relationship between water volume and detection probability of cavefishes and cave crayfishes. Detection probability via each technique and organism are represented by: the dashed line, eDNA surveys for cavefishes; the dotted line, eDNA surveys for cave crayfishes; the solid line, visual surveys for cave crayfishes; and the dotted-dashed line, visual surveys for cavefishes. Detection estimates were derived through the development of an occupancy model. To represent this relationship, we held water velocity and water clarity at mean levels and the categorical variable "substrate" was set to "coarse."

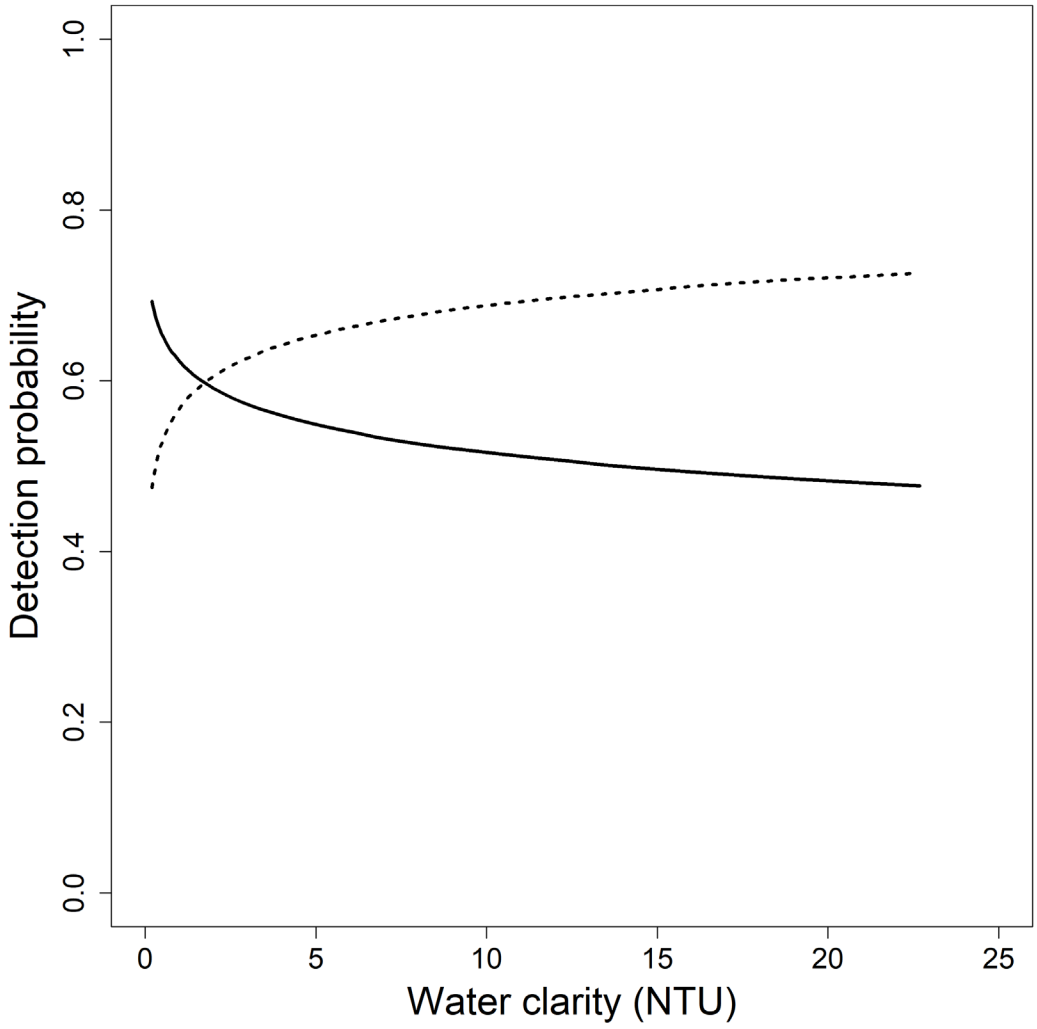

Figure 5. – The relationship between detection probability and water clarity by cavefishes (solid line) and cave crayfishes (dashed line). Estimates were derived using an occupancy model to estimate occurrence and detection probability of cave organisms. To represent this relationship, we held water volume and water velocity at mean levels and the categorical variable "substrate" was set to "coarse."

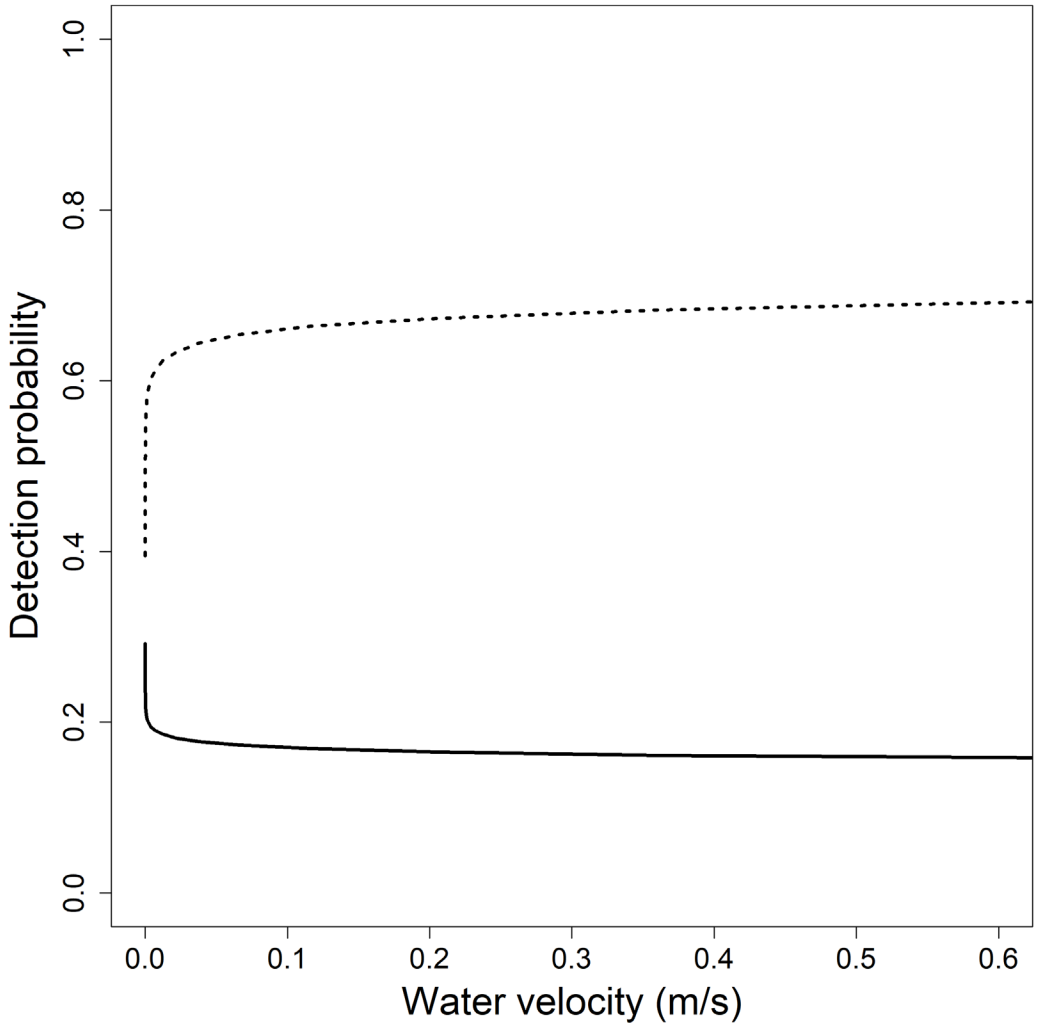

Figure 6. – The relationship between detection probability and water velocity differed by survey method: traditional visual surveys, solid line; and eDNA surveys, dashed line. Detection estimates were derived from an occupancy model used to estimate occurrence and detection probability of cavefishes and cave crayfishes. To represent this relationship, we held water volume and water clarity at mean levels and the categorical variable "substrate" was set to "coarse."

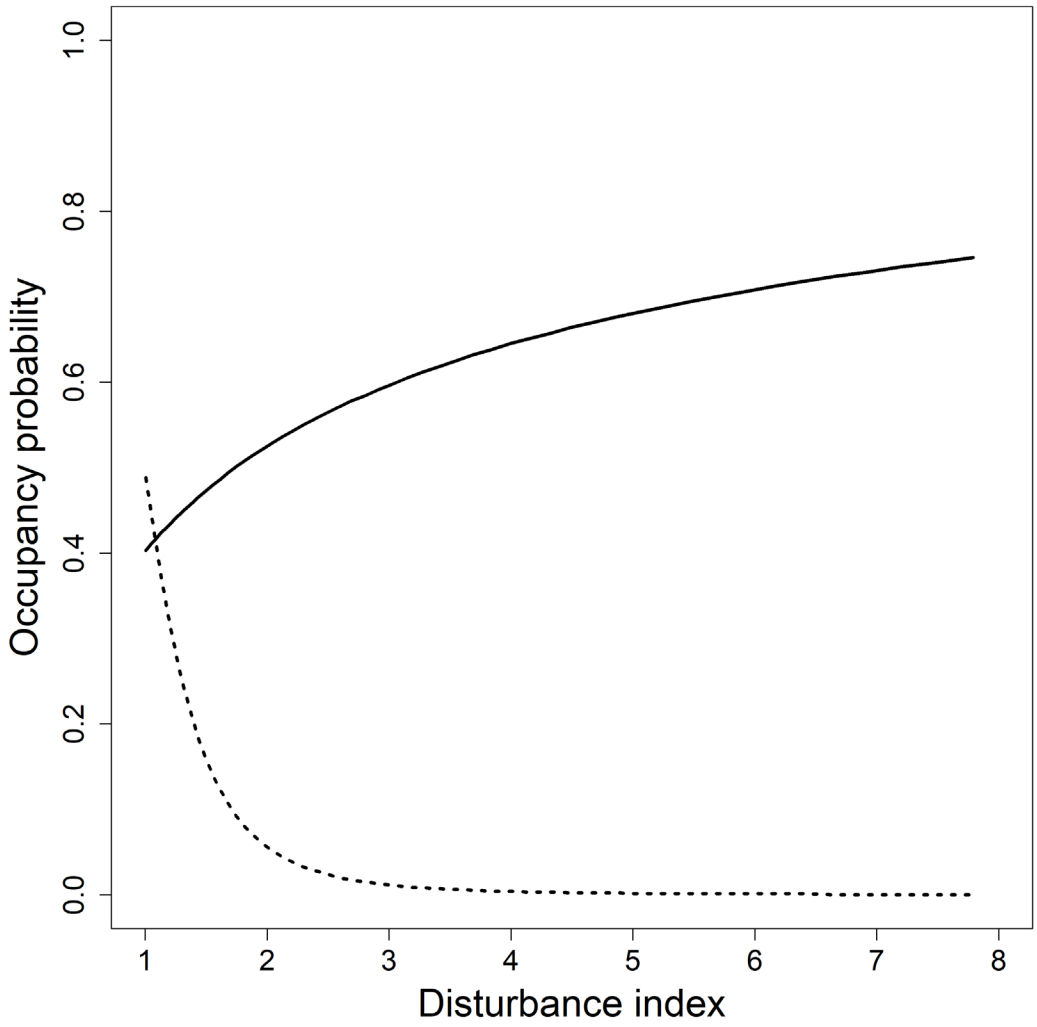

Figure 7. – The relationship between occupancy probability and anthropogenic disturbance differed by taxa: cavefishes, solid line; and cave crayfishes, dashed line. Occupancy estimates were derived from an occupancy model used to estimate occurrence and detection probability of cavefishes and cave crayfishes. To represent this relationship, the categorical variable geology was set to "other."

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