BLINDLY SEEKING THE BLIND:

ASSESSING THE EFFICACY OF AN ENVIRONMENTAL DNA (eDNA) METABARCODING APPROACH FOR THE DETECTION OF SUBTERRANEAN CRAYFISH

by

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ABSTRACT

The state of Alabama has an incredible amount of aquatic biodiversity including a high number of endemic cave-dwelling species of conservation concern. Surveys for cave fauna present unique challenges, as subterranean habitats often extend well beyond what surveyors can physically or safely access. These obstacles can be overcome through collection and analysis of environmental DNA (eDNA) by providing information on occupying species without requiring extensive physical surveys and further anthropogenic disturbance of these delicate ecosystems. eDNA refers to genetic material that can be retrieved from bulk environmental samples and used for species detection. This study aimed to assess the efficacy of an environmental DNA (eDNA) metabarcoding approach for the detection of blind, depigmented, obligate cave-dwelling crayfishes using a custom general crayfish primer pair targeting the 16S mitochondrial rRNA gene and Illumina sequencing. As far as we know, this is the first study employing eDNA metabarcoding for the detection of subterranean crayfish. Crayfish eDNA was successfully detected at 15 of 22 sites, including four of the seven target subterranean crayfish species of the genera *Cambarus* and *Orconectes*, all of which are of conservation concern due to their presumed rarity and the specificity of their habitat. Detection was verified through phylogenetic analyses of amplicon sequence variants (ASVs), a custom reference database containing all publicly available crayfish 16S sequences, and full-length 16S sequences from six newly sequenced, complete mitochondrial genomes. Results not only indicate that these methods are effective for the detection of cave crayfishes, but also provide new information on the distributions of the cave species *Cambarus speleocoopi* and *C. jonesi*, which may influence

future conservation efforts. This research also provided insights on the taxonomy of the obligate cave crayfish *Orconectes sheltae*, and the cave associated species *C. tenebrosus*. Furthermore, results showed the utility of these methods for detection of various other crayfish species such as the widely invasive *Procambarus clarkii*, in addition to revealing potential future directions for research involving Alabama's remarkable subterranean biodiversity.

LIST OF ABBREVIATIONS AND SYMBOLS

ASV	Amplicon Sequence Variant
BIC	Bayesian Information Criterion
CO1	Cytochrome Oxidase Subunit I
eDNA	Environmental DNA
HDPE	High Density Polyethylene
ML	Maximum Likelihood
PCR	Polymerase Chain Reaction
SBMP	Sharp Bingham Mountain Preserve
WNWR	Wheeler National Wildlife Refuge
bp	Base pairs
L	Liter
µl	Microliter
µm	Micrometer
ml	Milliliter
nM	Nanomolar

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INTRODUCTION

The need for biological monitoring has never been greater. Evidence supports the growing consensus that Earth is currently experiencing its sixth mass extinction event because of anthropogenically driven climate change and environmental degradation (Barnosky et al., 2011; Ceballos et al., 2015; Pimm et al., 2014). Scientists call for enhanced conservation efforts and research to slow the rate of biodiversity loss (Pimm et al., 2014; Ripple et al., 2017). However, before conservation efforts can be initiated, accurate assessments of current species distribution, status, and risk are needed (Ficetola et al., 2019; Pimm et al., 2014; Stratton & DiStefano, 2021). Conservation efforts are wasted if the target species have already been extirpated from the area or have become extinct. Unfortunately, the amount of biological monitoring and surveying required to assess species presence or absence far exceeds current abilities to accomplish it. Moreover, not all species are equally easy to locate or sample, potentially clouding survey results (Ficetola et al., 2019). There is great need for a more efficient and cost-effective way to monitor fauna *in situ*, while minimizing additional anthropogenic disturbance (Geerts et al., 2018; Graening et al., 2010). This is especially true for hard to access, vulnerable, subterranean habitats (Boyd et al., 2020; Niemiller et al., 2018). Species detection through collection and analysis of environmental DNA (eDNA) may be a key part of the solution to this problem.

This project aimed to assess the efficacy of an eDNA metabarcoding approach for the detection of cave crayfish species, specifically those that occupy the caves of Alabama. The state of Alabama has an incredible amount of aquatic biodiversity with more species of freshwater

fishes, turtles, mussels, snails, and crayfishes than any other state (Duncan, 2013; Lydeard & Mayden, 1995; Shelton-Nix, 2017;). Amongst these are many endemic species and a high level of cave fauna diversity (Culver et al., 1999; Niemiller et al., 2019). There are over 99 crayfish species in Alabama, eight of which are obligate cave dwellers that are blind and without pigment (Shelton-Nix, 2017). All cave crayfishes in Alabama are of conservation concern due to their presumed rarity and the specificity of their habitat; several of which are endemic to Alabama or even a single cave (Boyd et al., 2020; Shelton-Nix, 2017). At the outset of this project, two endemic cave crayfish species of highest conservation concern were of unknown conservation status and thought to be possibly extinct (Shelton-Nix, 2017). These include the Shelta Cave Crayfish (*Orconectes sheltae*) and the White Spring Cave Crayfish (*Cambarus veitchorum*), which are known only from the cave for which they are named (Buhay & Crandall, 2009a; Buhay & Crandall, 2009b). Failure to locate an individual, however, does not necessarily mean these crayfish are not present somewhere within the ground water aquifer associated with a cave system, or even in another nearby aquifer or cave system. One of the initial goals of this project was to gain information on the status of these two crayfish species by attempting to detect their presence with eDNA metabarcoding while validating our methods through the detection of crayfish in Alabama. Unfortunately, permission to sample White Spring Cave was not granted by landowners, and all attempts to obtain DNA sequence data from the White Spring Cave Crayfish (*C. veitchorum*) from a museum specimen were unsuccessful. Consequently, without access to the habitat or a representative sequence with which compare eDNA results, *C. veitchorum* was not considered in this study and its status remains unknown. The target species for this study include all other obligate cave-dwelling crayfishes present in Alabama (Table 1).

Environmental DNA refers to genetic material that can be retrieved from bulk environmental samples (Barnes & Turner, 2016). Extracting and sequencing this DNA can provide valuable information about the species that exist within an ecosystem (Ficetola et al., 2008). Genetic material is released into the environment via several processes such as reproduction, decomposition, exfoliation, excretion, and egestion as well as blood or hemolymph loss and mucous secretion (Barnes & Turner, 2016; Ficetola et al., 2008; Livia et al., 2006). DNA shed by organisms can persist in the environment for varying amounts of time as determined by a variety of biotic and abiotic factors (Barnes & Turner, 2016). Recent studies indicate that DNA fragments degrade faster when exposed to ultra-violet (UV) radiation, unstable temperatures, low pH conditions, and high microbial activity (Barnes & Turner, 2016; Strickler et al., 2015). The reliability of using eDNA for species detection has been demonstrated for a variety of organisms in a wide range of ecosystems (Barnes & Turner, 2016; Ficetola et al., 2008). However, the majority of these studies have targeted amphibians and fish (Ficetola et al., 2008; Goldberg et al., 2011; Ruppert et al., 2019; Tréguier et al., 2014). In recent years more studies have utilized eDNA for the detection of surface (Chucholl et al., 2021; Dougherty et al., 2016; Geerts et al., 2018; Harper et al., 2018) and subterranean crayfish, with subterranean crayfish eDNA studies being limited to species-specific assays (Boyd, 2019; Boyd et al., 2020; DiStefano et al., 2020; Mouser et al., 2021). The usefulness of eDNA metabarcoding for the detection of various faunal groups has been established (Deiner et al., 2016; Valentini et al., 2016). However, few studies have employed eDNA metabarcoding in subterranean habitats, with those primarily focused on microbiota and non-crayfish invertebrates (Korbel et al., 2017; West et al., 2020). As far as we know this is the first study employing eDNA metabarcoding for the detection of subterranean crayfish.

Considering the factors influencing the persistence of DNA in the environment, an eDNA approach appears especially well-suited for the detection of cave fauna as subterranean habitats are characterized by lack of light, stable temperature regimes, and reduced microbial abundance in comparison to surface streams (Ellis et al., 1998; Venarsky et al., 2012). Additionally, the target caves for this study are formed from the dissolution of limestone bedrock resulting in a relatively stable, neutral pH, which should also facilitate the persistence of DNA within the groundwater (McGregor et al., 2008). An eDNA approach may also be preferable to traditional sampling methods due to the physical complications associated with cave exploration. Traditional surveys rely on direct observation of the fauna in question. This can be especially challenging or impossible in cave systems where the subterranean habitat extends well beyond what surveyors can physically or safely access (Ficetola et al., 2019; Mammola et al., 2019; Niemiller et al., 2018). These limitations can greatly obscure survey results causing species that are present to be overlooked due to our inability to observe them directly.

A common problem facing researchers who use eDNA to monitor imperiled species is the difficulty in determining whether the presence of eDNA represents a current, enduring population or remnant DNA from deceased organisms. Signals from deceased individuals may lead to wasted conservation efforts if the target species has already been extirpated or gone extinct. While residence times for crayfish DNA in aquatic systems are not fully understood, Harper et al. (2018) found that crayfish DNA was no longer detectable in tanks stocked with a high density of Signal Crayfish (*Pacifastacus leniusculus*) seven days after their removal, while crayfish DNA was no longer detectable 72 hours after removal from low density tanks. Additionally, a recent study published by Curtis & Larson (2020), determined that crayfish carcasses contribute negligible (non-detectable) amounts of mitochondrial DNA to the water

column indicating that positive detection can confidently be attributed to the existence of an active population.

Table 1. Cave Crayfishes of Alabama. State Conservation Status from Shelton-Nix, 2017. IUCN Conservation Status from IUCN, 2021. Type Locality information from Crandall & De Grave, 2017.

Crayfish Species	Common Name	Type Locality	State Conservation Status	IUCN Conservation Status
Orconectes australis	Southern Cave Crayfish	Shelta Cave, Alabama	Moderate Conservation Concern	Least Concern
Orconectes sheltae	Shelta Cave Crayfish	Shelta Cave, Alabama	Highest Conservation Concern	Critically Endangered
Cambarus hamulatus	Prickly Cave Crayfish	Nickajack Cave, Tennessee	High Conservation Concern	Least Concern
Cambarus jonesi	Alabama Cave Crayfish	Cave Spring Cave, Alabama	High Conservation Concern	Vulnerable
Cambarus laconensis	Lacon Exit Cave Crayfish	Lacon Exit Cave, Alabama	Highest Conservation Concern	Critically Endangered
Cambarus pecki	Phantom Cave Crayfish	McKinney Pit Cave, Alabama	Highest Conservation Concern	Endangered
Cambarus speleocoopi	Sweet Home Alabama Cave Crayfish	Kellers Cave, Alabama	High Conservation Concern	Endangered
Cambarus veitchorum	White Spring Cave Crayfish	White Spring Cave, Alabama	Highest Conservation Concern	Critically Endangered

METHODOLOGY

Sample Sites

With the goal of detecting all known cave crayfish species in Alabama, except *C. veitchorum* due to obstacles mentioned previously, at least one location with a historical record for each species was sampled, including the type locality (Table 1) for all target species except *C. hamulatus* whose type locality is in Tennessee. Crayfish occupancy records from Schuster et al. (in press). For the purposes of this project, “locations” and “sites” will be used

interchangeably. A number of target crayfish species are only known to exist in a single cave; thus the number of sampled sites per target species are not equally distributed. Beyond crayfish occupancy, these specific field sites were chosen based on ease of access and permitting guidelines. Sampled sites include four historical locations for *C. speleocoopi*: Beech Spring Cave, Cherry Hollow Cave, Kellers Cave, and Porches Spring Cave (Buhay & Crandall, 2009b); one location with records of *C. hamulatus*: Tumbling Rock Cave; and three locations with records of *C. pecki*: Cave Spring Cave, Key Cave, and McKinney Pit Cave, all of which are also reported to contain *C. jonesi*. Additional sites with reports of *C. jonesi* include Bobcat Cave, Hering Cave, Matthews Cave, Rockhouse Cave, and Shelta Cave, which is also the sole location of *O. sheltae*. At the start of this project, no molecular data were available from *O. sheltae*. During sample collection, however, a living specimen of *O. sheltae* was found, from which tissue was collected for mitochondrial genome sequencing before being released. Morphological identification of *O. sheltae* was performed by Dr. Matthew Niemiller of The University of Alabama in Huntsville (UAH). No previous DNA sequence data for *O. sheltae* existed for comparison but phylogenetic analysis of the mitochondrial 16S rRNA gene from the specimen identified as *O. sheltae* showed it to be a distinct, previously unsequenced species nested amongst other cave crayfish species, reinforcing its identification as the Shelta Cave Crayfish. The 16S gene sequence from *O. sheltae* was then used as a reference sequence for species detection. Samples targeting *C. laconensis* were collected at its only known location: Lacon Exit Cave. The seventh target species, *O. australis*, is the most widespread and least threatened of Alabama's cave crayfishes; of the sampled locations, it is reported from Hering Cave, Kennamer Cave, Limrock Blowing Cave, Matthews Cave, and Shelta Cave. Samples for Kennamer Cave were taken from a surface stream near the cave entrance due to lack of appropriate gear for cave

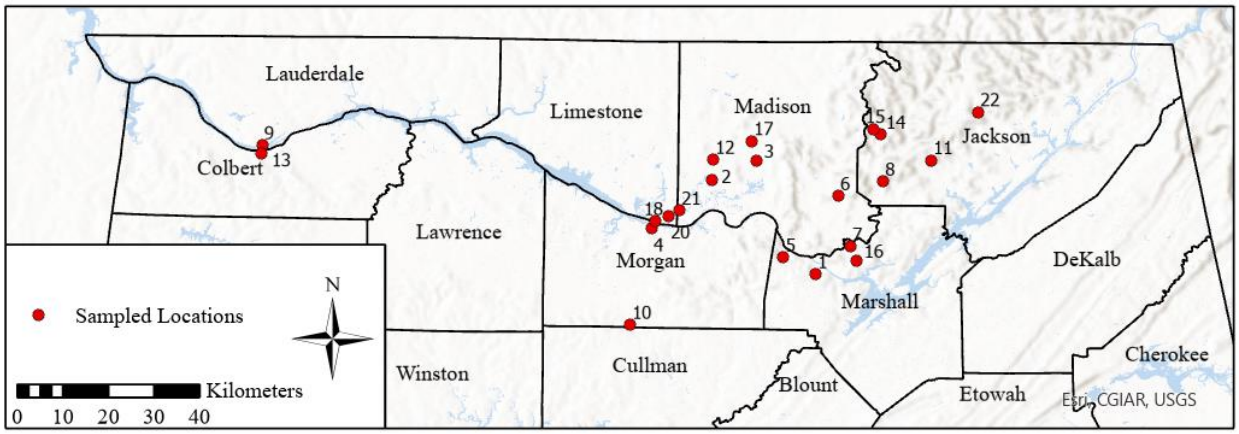
exploration at the time of sampling. Explicit permission to sample Kellers Cave and Rockhouse Cave could not be acquired, so samples were taken from a stream outflow near the entrance of Kellers Cave and from Blackwell Run, north and south of the entrance to Rockhouse Cave. Samples taken north of the entrance to Rockhouse Cave were treated as separate from samples taken south of the entrance to Rockhouse Cave for the purposes of this study.

Five surface stream/spring locations were also included to determine whether these methods would allow for detection of subterranean crayfish without sampling caves or cave outflows specifically. One surface spring sampled, Brahan Spring, is believed to be hydrologically connected to Shelta Cave (Rheams et al., 1992), and thus serves as an additional target site for the detection of *C. jonesi, O. australis, and O. sheltae*. Two surface streams were sampled on Sharp Bingham Mountain Preserve (SBMP) which contains numerous caves with records of *O. australis* (Godwin, 2008; Schuster et al., in press). The final two surface spring sites were on Wheeler National Wildlife Refuge (WNWR) in an area known as Rockhouse Bottoms which is believed to be hydrologically connected to Rockhouse Cave for which *C. jonesi* has been reported. Sites were sampled over the course of three years (2018–2020) in various seasons. The full list of sampled locations is presented in Table 2, along with reported crayfish inhabitants and notes on where the samples were taken. A map of sampled locations is presented in Figure 1, but the precise locations have been omitted to protect these sensitive habitats.

Table 2. All sampled locations for this project with notes on where samples were taken and what crayfishes have been reported to occupy the cave or caves hydrologically connected to the sample site. Cave crayfish occurrence records are from Schuster et al., in press.

Site Number	Cave/Spring/Stream	AL County	Collection Note	Inhabiting Cave Crayfish(es)
1	Beech Spring Cave	Marshall	Collected from cave	*Cambarus speleocoopi*
2	Bobcat Cave	Madison	Collected from cave	*Cambarus jonesi*
3	Brahan Spring	Madison	Collected from spring run	*Cambarus jonesi, Orconectes australis, Orconectes sheltae*
4	Cave Spring Cave	Morgan	Collected from cave	*Cambarus jonesi; Cambarus pecki*
5	Cherry Hollow Cave	Marshall	Collected from cave	*Cambarus speleocoopi*
6	Hering Cave	Madison	Collected from cave	*Cambarus jonesi; Orconectes australis*
7	Kellers Cave	Marshall	Collected from surface stream near cave entrance	*Cambarus speleocoopi*
8	Kennamer Cave	Jackson	Collected from surface stream near cave entrance	*Orconectes australis*
9	Key Cave	Lauderdale	Collected from cave	*Cambarus jonesi; Cambarus pecki*
10	Lacon Exit Cave	Morgan	Collected from cave	*Cambarus laconensis*
11	Limrock Blowing Cave	Jackson	Collected from cave	*Orconectes australis*
12	Matthews Cave	Madison	Collected from cave	*Cambarus jonesi; Orconectes australis*
13	McKinney Pit Cave	Colbert	Collected from cave	*Cambarus jonesi; Cambarus pecki*
14	Clear Creek on SBMP	Jackson	Collected from spring fed surface stream	*Orconectes australis*
15	Little Dry Creek on SBMP	Jackson	Collected from spring fed surface stream	*Orconectes australis*
16	Porches Spring Cave	Marshall	Collected from spring emerging from hillside	*Cambarus speleocoopi*
17	Shelta Cave	Madison	Collected from cave	*Cambarus jonesi, Orconectes australis, Orconectes sheltae*
18	Rockhouse Cave North	Limestone	Collected from Blackwell Run, north of cave entrance	*Cambarus jonesi*
19	Rockhouse Cave South	Limestone	Collected from Blackwell Run, south of cave entrance	*Cambarus jonesi*
20	Surface Spring #4 on WNWR	Limestone	Collected from spring pool	*Cambarus jonesi*
21	Surface Spring #6 on WNWR	Madison	Collected from spring pool	*Cambarus jonesi*
22	Tumbling Rock Cave	Jackson	Collected from cave	*Cambarus hamulatus*

Figure 1. Map of northern Alabama with all sampled locations. Site numbers correspond to those in Table 2.



Sample Collection

The eDNA sampling methodology involved filtering three 1L samples of water through a 0.8µm cellulose-nitrate filter contained in a sterile filter housing, using either a GeoPump™ (GeoPump Inc., Medina, NY, USA) or the ANDe™ integrated environmental DNA sampling system from Smith-Root (Smith-Root Inc, Vancouver, WA, USA). The ANDe™ sampling device acts to reduce contamination risk and improve collection of eDNA using a negative-pressure inline filtration system and replaceable filter housings, which were designed to minimize accumulation of large particulate matter and maximize the rate of sample filtration (Thomas et al., 2018). The design of the negative-pressure filtration system allows the components of the device beyond the filter housing to be reused without sterilization by preventing backflow contamination (Thomas et al., 2018). Filter housings were reused after being sterilized in a 40% bleach solution and triple rinsed with ultrapure water as was all other non-sterile equipment (forceps, collection bottles, and dipnets) prior to sample collection and between sites. Filter housings were reused no more than three times before being retired as recommended by the distributor. The ANDe™ sampling device was not acquired until after sample collection for this project had begun, thus earlier samples were filtered using a

GeoPump™. However, samples were treated the same regardless of filtration method and a negative equipment control was filtered prior to sample collection in all cases, unless a new sterile filter housing pack was used, rendering the negative equipment control unnecessary. Additionally, all caving gear was sanitized between sites in accordance with the Clean Caving Procedures outlined by the Southeastern Cave Conservancy (Southeastern Cave Conservancy, Inc., Signal Mountain, TN, USA). A sterile 1L high density polyethylene (HDPE) bottle was then filled with ultrapure water and brought into the field to act as the negative equipment control. When possible, filtration was performed *in situ* by suspending the intake tubing midway through the water column to avoid collection of sediment or surface films. If filtration could not occur on site, three sterile 1L HDPE bottles were used to collect cave water. In these cases, water collection bottles were placed on ice and filtered within 24 hours to avoid DNA degradation. In some cases, less than 1L of cave water was filtered due to particulate matter clogging the filter. In such instances, the volume of water filtered was recorded. The sample with the lowest filtered volume, 180ml, from Lacon Exit Cave still resulted in crayfish detection albeit from a non-target species (see Table 7), thus the inability to filter the entire liter of cave water does not appear to have impacted results. After filtration of the ultrapure water control, the filter was removed from the housing using sterile forceps and placed in a sterile 50ml tube. These forceps were then used to place a new filter in the housing for collection of the environmental sample. Forceps were not sterilized between collection of the three environmental samples taken at each site, as these samples are not considered independent of one another. Upon completion, tubes containing filters were transported on ice to the lab and stored at -80°C for later DNA extraction. In all cases, filter samples were placed in a -80°C freezer within four hours of filtration. These same

procedures were repeated for each of the 22 sampling sites regardless of whether filtration took place *in situ* or on The University of Alabama campus.

DNA Extraction

Prior to extraction, all laboratory surfaces were sterilized using 100% ethanol and four pairs of stainless-steel forceps (one for each sample including the control) were placed in a 50% bleach solution for at least 10 minutes before being rinsed with deionized water, then dipped in 100% ethanol and flamed until the metal glowed red. Filters were retrieved from the 50ml tube using sterile forceps before being placed in a sterile petri dish. Individual filters were then cut into fourths using a fresh razor blade and three of the four filter pieces were returned to the 50ml tube and quickly re-frozen should additional extractions need to take place. The remaining quarter filter was then cut into smaller pieces and placed in a labeled 1.5ml microcentrifuge tube. For each set of samples this resulted in four tubes i.e., one control, and three environmental samples, which underwent the extraction process separately. DNA extraction was performed using the Qiagen® DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) following a modified extraction protocol developed by the Niemiller lab at UAH using 360µL ATL Buffer and 40µL of Proteinase K (Boyd et al., 2020). The Qiagen® DNeasy Blood & Tissue Kit was chosen because it has been shown to outperform the Qiagen® DNeasy PowerWater Kit in terms of eDNA yield (Hinlo et al., in 2017). Following extraction, DNA yield was assessed using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) before being stored at -20°C until further analysis could take place.

Mitochondrial Genomes

To aid in designing a general crayfish PCR primer and ensure its suitability for species of interest, tissue samples from six target cave crayfish species, *Cambarus hamulatus*, *Cambarus*

jonesi, *Cambarus pecki*, *Cambarus speleocoopi*, *Orconectes australis*, and *Orconectes sheltae* were used to generate full mitochondrial genomes from which the 16S mitochondrial rRNA gene sequence was identified and added to a custom reference database. This was the first time full mitochondrial genomes for these species were sequenced. For mitochondrial genome sequencing, total DNA was extracted from crayfish leg tissue using either the Omega Bio-Tek MicroElute Genomic DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) or the DNeasy Blood & Tissue Kit from Qiagen® in accordance with the recommended protocol for tissue samples. Crayfish DNA yield was assessed using a NanoDrop™ Lite Spectrophotometer before being stored at -20°C for later use in Illumina library construction and as positive controls in polymerase chain reaction (PCR) experiments. Dual-indexed Illumina sequencing libraries were prepared with the NEB Next Ultra II FS kit (New England Biolabs, Inc., Ipswich, MA, USA) and were sequenced using 1/23 lane of an Illumina HiSeq 4000. Raw paired-end reads were assembled using SPAdes 3.14.0 with the –careful assembly setting (Bankevich et al., 2012), and complete mitochondrial genomes were identified using BLAST (Altschul et al., 1990) with the *Procambarus clarkii* mitochondrial genome (NCBI accession number JN991197.1) as the query sequence. Mitochondrial genomes were annotated with MITOS2 (Donath et al., 2019). Further examination of genomic data revealed that the specimen identified as *Cambarus jonesi* from Bobcat Cave was in fact *Cambarus speleocoopi*. Consequently, six mitochondrial genomes representing five distinct species of cave crayfish (plus publicly available sequences; see below) were used to aid in primer design.

Primer design

For the purposes of this study, a general crayfish PCR primer pair for the 16S mitochondrial rRNA gene was developed *in silico* based on a custom reference database

containing all publicly available crayfish 16S sequences from GenBank (Clark et al., 2016) and the six crayfish mitochondrial genomes generated for this project. Primers were designed manually in MEGA 7 (Kumar et al., 2016) after aligning all GenBank and novel crayfish sequences using MUSCLE (Edgar, 2004). Primers were compared to 16S sequences from several other arthropods (including cave-dwelling amphipods, various insects, shrimp, and lobster) to confirm crayfish specificity. Primers were optimized based on assessment of melting temperature, GC content, and propensity to form homo- and heterodimers with OligoAnalyzer 3.1 (Integrated DNA Technologies Inc., Coralville, IA, USA). The resulting primer pair produced is NS-Cray-F2 (5′- GGGACGATAAGACCCTATAAAAC-3′) and NS-Cray-R1 (5′-TTTAAAGGTCGAACAGACCTTCT-3′) with an average target amplicon length of 270 base pairs (bp). Additional information on primers is available in Table 3. Primers were ordered from Invitrogen™ (Thermo Fisher Scientific Inc., Waltham, MA, USA) and reconstituted upon arrival using molecular biology grade water to create a 100nM stock solution from which 10nM aliquots were made before storing at -20°C. Primers were subsequently validated using DNA acquired from crayfish tissue samples of the genera *Orconectes* and *Cambarus*. Primers were subsequently tested along an annealing temperature gradient from 58.8°C to 68.9°C to determine the temperature at which the primers performed best while minimizing non-target amplification. This temperature gradient test was performed using 12.5µl of AMRESCO's Hot Start Taq PCR Master Mix (VWR International, LLC., Radnor, PA, USA) in seven 25µl reactions with 1µl each of forward primer, reverse primer, and bovine serum albumin (BSA) and 7.5µl molecular grade water. Six of these reactions were spiked with 1µl of DNA extract from an *O. australis* specimen collected from Hering Cave in Madison County, Alabama, with the seventh receiving an additional 1µl of molecular grade water to serve as a negative control. The cycling parameters

for the temperature gradient test were 95°C for 30 seconds, followed by 40 cycles of 95°C for 15 seconds, a temperature gradient range of 58.8°C to 68.9°C for 15 seconds, and 72°C for 15 seconds with a final extension of 72°C for 5 minutes before being cooled to 10°C. PCR products resulting from the gradient test were visualized using gel electrophoresis, which indicated an optimized annealing temperature of 64.3°C based on the presence of a single clear band with minimal smearing. For downstream applications utilizing the Adapterama II protocol (Glen et al., 2019), new fusion primers consisting of the original crayfish primer and a 33bp TruSeq partial adapter sequence (Table 3) compatible with dual-indexed iTru indices for Illumina library construction were ordered from Invitrogen™ and used in all subsequent reactions. The Adapterama II protocol for dual or quadruple indexing is a useful and cost-effective strategy that allows large numbers of samples to be pooled (Glen et al., 2019). While pooling hundreds of samples was not necessary for this study, future projects utilizing these methods can make use of the Adapterama II protocol to keep costs low while sequencing eDNA from many sites and samples at once, which may be the best method to get a clear and accurate picture of species composition at locations of interest.

Initial PCR & Gel Electrophoresis

Extracted DNA from field samples and equipment controls were subjected to PCR using the Titanium® Taq PCR Kit from Takara Bio (Takara Bio USA, Inc., Mountain View, CA, USA). For samples and equipment controls, 3µl of sample DNA was added to 22µl of PCR master mix to create 25µl reactions. Negative PCR controls using 3µl of molecular grade water and positive PCR controls using 1µl of spike-in crayfish DNA and 2µl H_2O were included to ensure a successful reaction and the absence of contamination. The thermocycling parameters were 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, 64.3°C for 15 seconds,

and 72°C for 30 seconds with a final extension of 72°C for 5 minutes before being cooled to 10°C. Successful amplification was determined through visualization of the amplicon via gel electrophoresis. In most cases, gel electrophoresis took place directly after PCR; if not, then PCR products were stored at -20°C until gel electrophoresis could take place. PCR products were visualized via gel electrophoresis on a 1.5% agarose gel containing SBYR Safe nucleic acid stain (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR products exhibiting successful amplification were purified using either AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, USA) or Kapa Pure Beads (Roche Sequencing Systems, Inc., Indianapolis, IN, USA) in a 2:1 bead to sample volumetric ratio as deemed appropriate based on the size of the target amplicon. DNA was eluted from the beads using 25µl of molecular grade water. Resulting DNA concentration was assessed using Qubit fluorometric quantification (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Crayfish were not detected at all sites. To determine whether the presence of PCR inhibitors was the cause, an additional PCR reaction was performed following the methods described above using 2µl of eDNA sample extract from each site spiked with 1µl of target crayfish DNA. PCR products from these inhibition tests were visualized via gel electrophoresis and revealed that PCR inhibition was not the cause of non-detection at sites without positive results as the spike-in template DNA was amplified successfully in each case.

Sequencing Library Preparation

Purified PCR products were subsequently used in Illumina sequencing library preparation. Unique combinations of iTru i5 and i7 indices were assigned to each sample. 3ul of each 10µM forward (i7) and reverse (i5) index primer were combined with 5µl of purified PCR product and 14µl of master mix, prepared using the Kapa HiFi PCR Kit from Roche Sequencing

Systems (formerly Kapa Biosystems), for a final reaction volume of 25µl. Samples underwent PCR following the thermocycling parameters outlined in the Kapa HiFi PCR Kit manual before being visualized via gel electrophoresis and purified in the same manner described earlier. Following the final purification, library concentrations were measured using Qubit fluorometric quantification. Libraries with concentrations greater than 50ng/µl were diluted by a factor of five to simplify pooling volumes. The pooled product was then sent to Psomagen (Psomagen, Inc., Rockville, MD, USA) for high-throughput sequencing on a MiSeq V3 flow cell using 300bp paired-end reads. Base-calling was performed by Illumina Real-Time Analysis and reads were de-multiplexed and converted to FASTQ format with bcl2fastq 2.20.

Table 3. General crayfish primers designed for this study and all iterations utilizing TruSeq partial adapter sequences from Glen et al., 2019.

General Crayfish Primers			
	5'-3'	Primer Length (bp)	GC Content %
Forward Primer (NS-Cray-F2)	GGGACGATAAGACCCTATAAAAC	23	44
Reverse Primer (NS-Cray-R1)	TTTAAAGGTCGAACAGACCTTCT	23	39
TruSeq Partial Adapter Sequence			
Forward Primer Adapter	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	33	52
Reverse Primer Adapter	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	34	55
Full Fusion Primers (General Crayfish Primer + TruSeq Partial Adapters**)**			
Forward Fusion Primer	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGG GACGATAAGACCCTATAAAAC	56	48
Reverse Fusion Primer	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT TTTAAAGGTCGAACAGACCTTCT	57	47
Fully Indexed Fusion Primers (with example indices) (General Crayfish Primer + TruSeq Partial Adapters + ITru i5 & i7 Indices[1]**)**			
Forward Indexed Fusion Primer	TTACCGAGACACTCTTTCCCTACACGACGCTCTTC CGATCTGGGACGATAAGACCCTATAAAAC	64	48
Reverse Indexed Fusion Primer	CTCGTTCTGTGACTGGAGTTCAGACGTGTGCTCTT CCGATCTTTTAAAGGTCGAACAGACCTTCT	65	48

1.Example i5 and i7 index sequences. Different combinations of indices were used for every sample.

Computational Analyses

Raw paired-end Illumina reads were analyzed using the Qiime2 platform (version 2020.6.0) (Bolyen et al., 2019). Within the Qiime2 platform, reads were denoised and amplicon sequence variants (ASVs) were generated using the DADA2 pipeline (version 2020.6.0) with a trim-left length of 23 to remove primers (Callahan et al., 2016). A total of 155 ASVs were recognized by DADA2. The full output from the DADA2 pipeline can be seen in Table S1 (supplementary). To determine the identity of each ASV, they were first queried using BLASTN version 2.12.0+ (Zhang et al., 2000). Results were organized by percent identity, with only those above 97% being considered for identification. This step was mainly to ensure ASVs corresponded to 16S from crayfish species known to occur in the Southeast. More robust identification of ASVs was conducted on the basis of phylogenetic analysis. To generate the phylogenetic tree (Supplementary Figure 1), ASV sequences were added to the reference database containing all publicly available crayfish sequence data from GenBank and the new crayfish 16S sequences generated for this project. All sequences in the database were then aligned in MAFFT 7.453 (Katoh & Standley, 2013) and the resulting FASTA file was submitted to the IQ-Tree web server for a maximum likelihood (ML) analysis using the best-fitting model with free-rate heterogeneity and 1000 rapid bootstrap replicates (Trifinopoulos et al., 2016). The Bayesian information criterion (BIC) determined that the best-fitting model was TPM+F+R4. The output bootstrap consensus tree was then visualized in MEGA X where the identity of ASVs was determined by their phylogenetic placement (Kumar et al., 2018). To ensure accuracy of species assignments, all sequences (representative and ASV) in each target species clade were subsequently used in pairwise distance calculations to determine their level of divergence. While not without shortcomings, a pairwise sequence dissimilarity value of 3% serves as a useful rough cutoff for species delineation based on the 16S rRNA gene (Nguyen et al., 2016). Pairwise

distance analyses were conducted in MEGA X using the Tamura 3-parameter model with empirical nucleotide frequencies in accordance with the best-fitting model determined by the IQ-Tree ModelFinder (Kumar et al., 2018; Tamura, 1992; Trifinopoulos et al., 2016;). Rate of variation among sites was modeled with a gamma distribution shape parameter of one and all ambiguous positions were removed for each sequence pair (pairwise deletion; Tamura & Kumar, 2002). *O. sheltae* was sequenced for the first time as a part of this study, thus pairwise distance analysis could not be performed for this species individually but its 16S rRNA sequence was included in pairwise distance calculations comparing all cave *Cambarus* species, and all target cave crayfish collectively. Pairwise distance calculations were not performed for non-target species. A threshold of 100 reads per ASV was set for inclusion in Table 6, which presents target species detections at each site, while Table 8 shows an unfiltered list of the ASVs and read counts. The 100 read count threshold was set for Table 6 to filter out potential false positives that can result from sequencing error.

RESULTS

Of the 22 sampled locations, crayfish eDNA was detected from fifteen. Of those fifteen locations, target cave crayfish species were detected from thirteen, while the other two contained eDNA from the non-target, albeit cave associated species, *Cambarus tenebrosus*. Beyond *C. tenebrosus*, three locations showed detection for the Red Swamp Crayfish (*Procambarus clarkii*), and two showed detection for what we estimate to be around three to four other *Cambarus* species whose identity we were unable to determine due to the limited sequence data available for crayfishes of Alabama (see Supplementary Figure 1). Average pairwise distance values for individual target species, minus *O. sheltae*, in addition to those calculated for all cave *Cambarus* species and all target cave crayfish collectively are presented in Table 4. The average

pairwise distance values for all sequences making up target species clades were well below 3%, bolstering our confidence that ASVs were correctly identified to the species level. A breakdown of the number of ASVs attributed to each species can be seen in Table 5. Locations with positive results and the target species detected therein are presented in Table 6, while Table 7 displays the non-target species detected. Criteria for inclusion in Table 6 indicating positive detection for a target species was set as the presence of at least one corresponding ASV with a read count greater than 100. However, when referencing positive detections on a sample-by-sample basis, ASVs with less than 100 reads were considered legitimate if that same ASV, or ASVs corresponding to the same species, had over 100 reads in a sample from the same location

Table 4. Average pairwise distance values for each crayfish species clade in addition to all cave crayfishes of the genus Cambarus and all cave crayfishes present in Alabama.

Species	**Number of Reference Sequences in Calculation**	**Number of ASVs in Calculation**	**Average Pairwise Distance**
C. hamulatus	14	0	0.8%
C. jonesi	10	0	0.5%
C. laconensis	7	7	0.7%
C. pecki	3	7	0.7%
C. speleocoopi	8	15	1.5%
All Cave *Cambarus* Species	42	29	5.4%
O. australis	48	18	1.5%
All Cave Crayfishes	90	47	7.0%

Table 5. All species detected (target and non-target) and the number of ASVs assigned to each.

Species Detected	**Corresponding ASVs**
Cambarus laconensis	7
Cambarus pecki	7
Cambarus speleocoopi	15
Cambarus tenebrosus	18
Orconectes australis	71
Procambarus clarkii	28
Unknown (3-4)	9
Total	**155**

Table 6. Results table. All site locations exhibiting crayfish detection with read counts over 100. Checkmarks indicate detection of target species. Sites with no checkmarks indicate detection of crayfish DNA for non-target species only.

Sample Site	**Target Crayfish Species**						
	Cambarus hamulatus	***Cambarus jonesi***	***Cambarus laconensis***	***Cambarus pecki***	***Cambarus speleocoopi***	***Orconectes australis***	***Orconectes sheltae***
Beech Spring Cave	-	-	-	-	✓	-	-
Cherry Hollow Cave	-	-	-	-	✓	-	-
Cave Spring Cave	-	-	-	-	-	-	-
Kellers Cave	-	-	-	-	✓	-	-
Kennamer Cave	-	-	-	-	-	✓	-
Lacon Exit Cave	-	-	✓	-	-	-	-
Limrock Blowing Cave	-	-	-	-	✓	-	-
Matthews Cave	-	-	-	-	-	✓	-
McKinney Pit Cave	-	-	-	✓	-	-	-
Porches Spring Cave	-	-	-	-	-	-	-
Clear Creek on SBMP	-	-	-	-	-	✓	-
Little Dry Creek on SBMP	-	-	-	-	-	✓	-
Rockhouse Cave North	-	-	-	-	✓	-	-
Rockhouse Cave South	-	-	-	-	✓	-	-
Surface Spring #4 on WNWR	-	-	-	-	✓	-	-

Table 7. All site locations exhibiting crayfish detection with checkmarks indicating detection of non-target species. Sites with no checkmarks indicate detection of crayfish DNA for target species only.

Sample Site	Additional Crayfish Detections		
	Cambarus tenebrosus	***Procambarus clarkii***	**Unknown Species**
Beech Spring Cave	✓	-	✓
Cherry Hollow Cave	✓	-	-
Cave Spring Cave	✓	-	-
Kellers Cave	-	-	-
Kennamer Cave	✓	-	-
Lacon Exit Cave	✓	-	✓
Limrock Blowing Cave	✓	-	-
Matthews Cave	-	-	-
McKinney Pit Cave	✓	-	-
Porches Spring Cave	✓	-	-
Clear Creek on SBMP	✓	-	-
Little Dry Creek on SBMP	-	-	-
Rockhouse Cave North	-	✓	-
Rockhouse Cave South	-	✓	-
Surface Spring #4 on WNWR	-	✓	-

Cambarus hamulatus

C. hamulatus was not detected in any samples, including those from Tumbling Rock Cave where it is known to occur and was observed during sample collection.

Cambarus jonesi

C. jonesi was not detected in any samples despite being listed as occupying eight of the caves sampled and four additional sample locations were believed to be hydrologically connected to caves occupied by *C. jonesi*. However, the morphologically similar species, *C. speleocoopi* was detected at a number of these sites.

Cambarus laconensis

The critically endangered species, *C. laconensis*, considered of highest conservation concern in Alabama, was successfully detected in 2 out of 3 samples collected from its sole known location, Lacon Exit Cave. Positive detection included seven ASVs with 74; 76; 421; 508; 677; 727; and 55,057 reads respectively. These are promising results, as no individuals were spotted during recent surveys but eDNA indicates the presence of an active population.

Cambarus pecki

C. pecki, another endangered species of highest conservation concern in Alabama, was successfully detected at its type locality, McKinney Pit Cave, in one of the three samples. Five different ASVs were detected with read counts of 39; 56; 150; 322; 369; 13,165; and 44,614. However, *C. pecki* was not detected at Key Cave or Cave Spring Cave despite multiple individuals being found whilst sampling.

Cambarus speleocoopi

C. speleocoopi, an endangered species of high conservation concern in Alabama, had more detections than any other target species. A number of these detections occurred at sites with no

previous records of *C. speleocoopi* occupancy. Of the previously recorded locations with *C. speleocoopi*, only Porches Spring Cave did not result in a detection while this species was detected in two out of three samples from Beech Spring Cave representing two ASVs; two out of three samples from Cherry Hollow Cave representing one ASV; and one out of three samples from Kellers Cave representing seven ASVs (see Table 8 for read counts). *C. speleocoopi* was also detected in one out of three samples from Limrock Blowing Cave representing one ASV; one out of three samples from Blackwell Run, north of the entrance to Rockhouse Cave representing one ASV; two out three samples from Blackwell Run, south of the entrance to Rockhouse Cave representing two ASVs; and two out of three samples taken from Surface Spring #4 on Wheeler National Wildlife Refuge (WNWR) representing one ASV.

Orconectes australis

O. australis was successfully detected at four locations, two of which were caves with historical records of *O. australis* occupancy. The other two positive detections came from spring fed surface streams on Sharp Bingham Mountain Preserve which is in a core region of subterranean biodiversity and contains numerous caves, many of which are known to contain *O. australis* (Schuster et al., in press). No positive detections for *O. australis*, however, occurred at Shelta Cave, Limrock Blowing Cave, or Hering Cave despite individuals being spotted while sampling the latter two. *O. australis* was initially detected at eight locations, however, upon closer inspection it was found that four of those locations, Beech Spring Cave, Cherry Hollow Cave, Lacon Exit Cave, and McKinney Pit Cave showed singular detections, all corresponding to the same ASV with read counts of 7; 3; 6; and 6 respectively. This same ASV was present in samples from the other four locations, Kennamer Cave, Matthews Cave (two samples), Clear Creek, and Little Dry Creek with corresponding read counts of 31,403; 83,265; 76,202; 35,143;

and 74,871. These four locations also had additional ASVs attributed to *O. australis* (Kennamer Cave: six ASVs; Matthews Cave: nine ASVs in both samples; Clear Creek: five ASVs; Little Dry Creek: ten ASVs) while the other five locations did not.

Orconectes sheltae

O. sheltae was not detected in any samples, including those from its sole known location, despite encountering an individual whilst sampling.

Table 8. ASVs from target species by site, with read counts per ASV in each sample. Samples with a dash (-) exhibited no PCR amplification and were not sequenced.

Species ***Cambarus laconensis***				
Site	Lacon Exit Cave	Read Count in Sample 1	Read Count in Sample 2	Read Count in Sample 3
ASVs	CLA1	0	0	74
	CLA2	0	0	76
	CLA3	0	0	421
	CLA4	0	0	508
	CLA5	0	0	677
	CLA6	0	0	727
	CLA7	0	1142	55057
Species ***Cambarus pecki***				
Site	McKinney Pit Cave	Read Count in Sample 1	Read Count in Sample 2	Read Count in Sample 3
ASVs	CPE1	-	39	0
	CPE2	-	56	0
	CPE3	-	150	0
	CPE4	-	322	0
	CPE5	-	369	0
	CPE6	-	13165	0
	CPE7	-	44614	0
Species ***Cambarus speleocoopi***				
Site	Beech Spring Cave	Read Count in Sample 1	Read Count in Sample 2	Read Count in Sample 3
ASVs	CSP1	3255	0	242
	CSP2	468	0	0
Site	Cherry Hollow Cave			
ASVs	CSP3	5371	-	11852
Site	Kellers Cave			

ASVs	CSP4	387	-	-
	CSP5	291	-	-
	CSP6	78492	-	-
	CSP7	71	-	-
	CSP8	15666	-	-
	CSP9	573	-	-
	CSP10	416	-	-
	CSP11	46	-	-
	CSP12	537	-	-
Site	Limrock Blowing Cave			
ASVs	CSP13	-	137	-
Site	Rockhouse Cave North			
		231	0	0
Site	Rockhouse Cave South			
ASVs	CSP14	156	0	0
	CSP15	0	0	155
Site	Surface Spring #4 WNWR			
ASVs	CSP14	35	0	647
Species ***Orconectes australis***				
Site	Beech Spring Cave	Read Count in Sample 1	Read Count in Sample 2	Read Count in Sample 3
ASVs	OAU1	7	0	0
Site	Cherry Hollow Cave			
ASVs	OAU1	0	-	3
Site	Lacon Exit Cave			
ASVs	OAU1	0	6	0
Site	McKinney Pit Cave			
ASVs	OAU1	-	0	6
Site	Kennamer Cave			
ASVs	OAU1	31403	-	0
	OAU2	222	-	0
	OAU3	2757	-	0
	OAU4	373	-	0
	OAU5	11	-	0
	OAU6	314	-	0
Site	Matthews Cave			
ASVs	OAU1	83265	-	76202
	OAU2	445	-	427
	OAU3	6485	-	3961

	OAU7	416	-	355
	OAU8	453	-	431
	OAU9	2693	-	2690
	OAU10	29	-	21
	OAU11	38	-	37
	OAU12	3070	-	2731
Site	Clear Creek SBMP			
ASVs	OAU1	35143	-	0
	OAU2	215	-	0
	OAU3	3021	-	0
	OAU10	17	-	0
	OAU13	74	-	0
Site	Little Dry Creek SBMP			
ASVs	OAU1	-	-	74871
	OAU2	-	-	363
	OAU3	-	-	6548
	OAU10	-	-	21
	OAU11	-	-	34
	OAU14	-	-	1207
	OAU15	-	-	115
	OAU16	-	-	805
	OAU17	-	-	493
	OAU18	-	-	921

DISCUSSION

The purpose of this study was to assess the efficacy of using eDNA metabarcoding to glean useful information on cave crayfish occupancy. Essentially, can we detect the presence of cave dwelling crayfishes through these quick, low-impact sampling methods and do they produce valuable information to better our understanding of cave crayfish occupancy and distribution? Based on the results of this study we can say yes, these methods for eDNA detection of subterranean crayfishes are effective in their ability to detect species known to occupy certain caves, even if we were unable to detect all species present or were unsuccessful in acquiring amplifiable eDNA from all sample sites. Four obligate cave-dwelling crayfish species

of conservation concern, of which three are considered endangered by the IUCN (IUCN, 2021), were successfully detected from environmental samples using the general primer developed for this study.

Detections were examined for potential false positives but only four detections for *O. australis*, all corresponding to the same ASV with very low read counts, were excluded. When dealing with the incredibly high number of reads associated with high-throughput sequencing, there is a non-zero chance of error. Thus, it is likely that the detections from the four locations corresponding to a single ASV with very low number of reads are a result of "bleed-through" from another sample with a very high read count for that same ASV. This can occur during sequencing of multiplexed samples with low sequence diversity (relatively low number of barcodes used) when sequences are incorrectly binned and attributed to another sample (Mitra et al., 2015). It is important to closely inspect multiplexed Illumina data for such inaccuracies, specifically if a single ASV that is highly abundant in one or more samples appears across many different samples. For these reasons, positive detection results for *O. australis* at those four locations are not considered credible and were excluded. Fortunately, this is only likely to occur when an ASV is extremely abundant in one or more samples, as was the case here. Apart from the four detections associated with a single ASV for *O. australis*, no other detections for target species at any sites exhibited potential for falsity in this way. All other reported detections exhibited at least one ASV with read counts greater than 100, many of which showed multiple ASV detections for the target species. Based on the results of Harper et al. (2018), which found that crayfish DNA persisted no longer than 72 hours when present in low densities, as we believe is the case for most subterranean crayfish, and Curtis & Larsen (2020), which found carcasses

contribute negligible amounts of DNA to the system; we are confident these detections correspond to current, active populations of cave crayfish species in the habitats sampled.

It is difficult to accurately determine the reason why certain sites with known crayfish communities did not result in eDNA detection. For some, this is likely a result of the hydrologic conditions that characterize the sample location. Shelta Cave and Key Cave are largely phreatic with water levels mainly controlled by the local water table. In general, both caves often exhibit very little flow and high volumes of water. While all the factors influencing eDNA transport and detectability are not fully understood, high water volumes have been shown to dilute eDNA concentrations, reducing detectability (Curtis et al., 2020) and lack of flow has been found to inhibit the transport of eDNA causing it to remain near its source (Dunker et al., 2016; Jane et al., 2015). Additionally, lack of flow can result in DNA settling out of the water column (Harrison et al., 2019). These conditions can make it harder to get a clear signal if DNA is not distributed throughout the system or retained in the water column. Lack of detection for *O. sheltae* in Shelta Cave was likely compounded by its extremely low abundance, with only 2 individuals having been found since 1988 (Dooley et al., in review; Hobbs & Bagley, 1989). Other possible causes for non-detection include the influence of sampling conditions, as water was collected over the course of three years (2018-2020) in various seasons without accounting for discharge. Finally, there is the stochastic nature of environmental sampling, even within the three samples collected moments apart during a site visit, there is variation in what we were able to detect. Sometimes the eDNA content necessary to produce a signal was not acquired in a given sample. Many of these issues may be resolved by repeat sampling under varying conditions until adequate eDNA is acquired or by further optimizing laboratory techniques to detect species from very low DNA input. Additionally, future attempts to detect cave crayfish

may benefit from using filters with a larger pore size as crayfish have been successfully detected at low eDNA concentrations when using pore sizes of 2µm (Strand et al., 2019), which would facilitate filtration of larger volumes of water as may be necessary in habitats such as Shelta and Key Cave where water levels are high and numbers of target organisms are low. Repeated sampling may currently be our best option for acquiring the clearest picture of crayfish occupancy when using eDNA. If such a route is taken, the methods developed in this study can be readily used in combination with the Adapterama II protocol to analyze hundreds of samples while keeping costs relatively low (Glen et al., 2019).

While obligate subterranean crayfish species were the target of this project, the general crayfish primer pair developed for this study can be used in future eDNA metabarcoding studies targeting a wide range of subterranean and surface crayfishes. Results have already shown the usefulness of this primer for the detection of *C. tenebrosus* and various other *Cambarus* species, whose identity we were unable to determine. The inability to determine the species-specific identity of some of the crayfish detected in this study highlights the necessity of continued contributions of crayfish sequence data to publicly available databases so that these gaps may be filled. The Red Swamp Crayfish (*P. clarkii*), a common target for eDNA studies focused on early detection of invasive species (Geerts et al., 2018; Mauvisseau et al., 2017; Riascos et al., 2018; Tréguier et al., 2014) due to its standing as one of the mostly widely introduced species on the planet (Nagy et al., 2021; Souty-Grosset et al., 2016), was also successfully detected using this methodology. This means future studies targeting *P. clarkii* can use these approaches for its detection while also gaining information on other occupying crayfish species.

Beyond establishing the efficacy of an eDNA metabarcoding approach for detection of subterranean crayfishes, these methods provided new information regarding the presence and

distribution of *Cambarus speleocoopi*. Results indicate that the distribution of *C. speleocoopi* is more extensive than previously thought, not being limited to Marshall County but rather extending into Limestone, Madison, and Jackson counties. A number of these detections were from locations with historical records of *C. jonesi* occupation; specifically, Rockhouse Cave and Limrock Blowing Cave. Detections for *C. speleocoopi* at these sites were independently corroborated by Dooley (2021) using species-specific assays. Agreement between species-specific assays and eDNA metabarcoding results further validates our methodology and findings. It is likely that several populations attributed to *C. jonesi* are instead *C. speleocoopi* as was the case for Kellers Cave, Beech Spring Cave, and Cherry Hollow Cave before Buhay and Crandall (2009b) revealed the existence of *C. speleocoopi*. The presence of *C. speleocoopi* in Limrock Blowing Cave is surprising, as up until now there had been no record of *C. speleocoopi* co-occurring with any other obligate cave-dwelling crayfish. However, considering genomic analysis revealed that the crayfish identified as *C. jonesi* in Bobcat Cave is *C. speleocoopi*, we suspected, given its proximity to Matthews Cave, that the population of *C. jonesi* reportedly coexisting with *O. australis* in that locale was also *C. speleocoopi*. This was recently confirmed through phylogenetic analysis of sequence data extracted from purported *C. jonesi* specimen collected in Matthews Cave, showing it to be *C. speleocoopi* (Dooley, 2021). This makes two new records of *C. speleocoopi* co-occurring with *O. australis*. Ultimately, the results of this project indicate the need to reassess current populations of *C. jonesi* from a molecular standpoint to determine which subterranean habitats genuinely contain this species as opposed to its cryptic lookalike *C. speleocoopi*. The range of *C. jonesi* may in fact be much more restricted in Alabama than previously believed, potentially requiring increased conservation measures and reclassification as a species of higher conservation concern, though the results from this study

alone cannot justify those claims. Further research is needed to resolve the distributions of *C. speleocoopi* and *C. jonesi* so that informed management decisions can be made to protect these unique organisms and their specialized habitats.

Additional insights into crayfish taxonomy were attained through the analyses conducted as a part of this project. Phylogenetic analysis of the 16S rRNA gene indicates that *O. sheltae* is likely a member of the genus *Cambarus* as opposed to *Orconectes* as it is nested within the clade containing all other cave *Cambarus* and appears to be a sister species to *C. laconensis*. These findings were independently corroborated through ML and Bayesian analyses of the 16S and cytochrome oxidase subunit I (CO1) genes conducted by Dooley et al. (in review), further indicating the need to reclassify *O. sheltae* as a member of the genus *Cambarus*. In another case, *C. tenebrosus* was referred to as a singular species in this publication for the sake of simplicity, however, the phylogenetic analyses in this study used all publicly available sequences for *C. tenebrosus* and revealed it to be polyphyletic and better described as a species flock. Additional research is needed to establish the boundaries between *C. tenebrosus,* and other potentially unnamed species currently classified under *C. tenebrosus.*

Ultimately, the results of this study establish the validity and efficacy of an eDNA metabarcoding approach for the detection of subterranean crayfish, reveal insights beyond our initial goals regarding our understanding of the distributions and evolutionary relationships of crayfishes in Alabama, and illuminate numerous directions for future research to resolve unanswered and yet unasked questions about eDNA and Alabama's staggering aquatic and subterranean biodiversity.

LITERATURE CITED

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol*. 215:403-410

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., & Pevzner, P.A. (2012). SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *Journal of Computational Biology*. 19:5:455-477

Barnes, M., & Turner, C. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*. 17:1:1-17.

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., & Ferrer, E.A. (2011). Has the Earth's sixth mass extinction already arrived? *Nature.* 471:7336:51–57.

Boyd, S.H. (2019). Development of an environmental DNA assay for detection and monitoring of the troglobitic crayfishes *Cambarus speleocoopi* and *Cambarus laconensis*. M.S. thesis. The University of Alabama in Huntsville, Huntsville, Alabama. 31 pp.

Boyd, S.H., Niemiller, K.D.K., Dooley, K.E., Nix, J., & Niemiller, M.L. (2020). Using environmental DNA methods to survey for rare groundwater fauna: Detection of an endangered endemic cave crayfish in northern Alabama. *PLoS One.* 15(12): e0242741. https://doi.org/10.1371/journal.pone.0242741

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R.…& Caporaso, J.G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology.* 37:852–857. https://doi.org/10.1038/s41587-019-0209-9

Buhay, J.E., & Crandall, K.A. (2009a). Taxonomic Revision of Cave Crayfish in the Genus *Cambarus*, Subgenus *Aviticambarus* (Decapoda: Cambaridae) with Descriptions of Two New Species, *C. Speleocoopi* and *C. Laconensis*, Endemic to Alabama, U.S.A., *Journal of Crustacean Biology*. 29:1:121–134, https://doi.org/10.1651/08-3089.1

Buhay, J.E., & Crandall, K.A. (2009b). Taxonomic Revision of Cave Crayfish in the Genus *Cambarus*, Subgenus *Aviticambarus* (Decapoda: Cambaridae) with Descriptions of Two New Species, *C. Speleocoopi* and *C. Laconensis*, Endemic to Alabama, U.S.A., *Journal of Crustacean Biology*. 29:1:121–134, https://doi.org/10.1651/08-3089.1

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., & Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*. 13:7:581–583. https://doi.org/10.1038/nmeth.3869

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., Garcia, A., Pringle, R.M., & Palmer, T.M. (2015). Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Science Advances*. 1:5:1400253–1400253.

Chucholl, F., Fiolka, F., Segelbacher, G., & Epp, L.S. (2021). eDNA Detection of Native and Invasive Crayfish Species Allows for Year-Round Monitoring and Large-Scale Screening of Lotic Systems. *Frontiers in Environmental Science*. 9:23. DOI:10.3389/fenvs.2021.639380. ISSN:2296-665X.

Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., & Sayers, E.W. (2016). GenBank. *Nucleic acids research*, 44(D1), D67–D72. doi:10.1093/nar/gkv1276

Crandall, K., & De Grave, S. (2017). An updated classification of the freshwater crayfishes (Decapoda: Astacidea) of the world, with a complete species list. *Journal of Crustacean Biology*. 37:10.1093/jcbiol/rux070.

Culver, D.C., Hobbs, H.H. III, & Mylroie, J.E. (1999). Alabama: a subterranean biodiversity hotspot. *J Ala Acad Sci*. 70:97–104.

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

Curtis, A.N., Tiemann, J.S., Douglass, S.A., Davis, M.A., & Larson, E.R. (2020). High stream flows dilute environmental DNA (eDNA) concentrations and reduce detectability. *Divers. Distrib*. 00:1–14. doi:10.1111/ddi.13196

Deiner, K., Fronhofer, E., Mächler, E., Walser, J.-C., & Altermatt, F. (2016). Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nat Commun.* 7:12544. https://doi.org/10.1038/ncomms12544

DiStefano, R.J., Ashley, D., Brewer, S.K., Mouser, J.B., & Niemiller, M.L. (2020). Preliminary investigation of the critically imperiled Caney Mountain cave crayfish *Orconectes stygocaneyi* (Hobbs III, 2001) (Decapoda: Cambaridae) in Missouri, USA. *Freshwater Crayfish*, *25*:1:47–57.

Donath, A., Jühling, F., Al-Arab, M., Bernhart, S.H., Reinhardt, F., Stadler, P.F., Middendorf, M., & Bernt, M. (2019). Improved annotation of protein-coding genes boundaries in metazoan mitochondrial genomes. *Nucleic Acids Research*; 47:20:10543–10552

Dooley, K.E. (2021). Phylogenetics of cave crayfish in the genus *Cambarus* and using environmental DNA to detect populations of *Cambarus jonesi* and *Cambarus speleocoopi*. M.S. thesis. The University of Alabama in Huntsville, Huntsville, Alabama. 49 pp.

Dooley, K.E., Niemiller, K.D.K, Sturm, N.D., & Niemiller, M.L. (In Review). Rediscovery and phylogenetic analysis of the Shelta Cave Crayfish (*Orconectes sheltae* Cooper & Cooper, 1997), a decapod (Decapoda: Cambaridae) endemic to Shelta Cave, Madison County, Alabama, USA.

Dougherty, M.M., Larson, E.R., Renshaw, M.A., Gantz, C.A., Egan, S.P., Erickson, D.M. & Lodge, D.M. (2016). Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *J Appl Ecol*. 53:722-732. https://doi.org/10.1111/1365-2664.12621

Duncan, R.S. (2013). Southern wonder: Alabama's surprising biodiversity. The University of Alabama Press.

Dunker, K.J., Sepulveda, A.J., Massengill, R.L., Olsen, J.B., Russ, O.L., Wenburg, J.K., & Antonovich, A. (2016). Potential of Environmental DNA to Evaluate Northern Pike (*Esox lucius*) Eradication Efforts: An Experimental Test and Case Study. *PLoS One.* 11:9:e0162277. https://doi.org/10.1371/journal.pone.0162277

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 32:1792–1797. https://doi.org/10.1093/nar/gkh340 PMID: 15034147

Ellis, B.K., Stanford, J.A., & Ward, J.V. (1998). Microbial assemblages and production in alluvial aquifers of the Flathead River, Montana, USA. *Journal of the North American Benthological Society*. 17:382–402.

Ficetola, G.F., Canedoli, C., & Stoch, F. (2019). The Racovitzan impediment and the hidden biodiversity of unexplored environments. *Conservation Biology*. 33:1:214-216.

Ficetola, G.F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters.* 4:4:423–425.

Geerts, A.N., Boets, P., Van den Heede, S., & Goethals, P. (2018). A search for standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): A lab and field evaluation. *Ecological Indicators*. 84:564-572.

Glenn, T.C., Pierson, T.W., Kieran, T.J., Hoffberg, S.L., Thomas, J., Bayona-Vasquez, N.J., Lefever, D. E., Finger Jr., J.W., Gao, B., Bian, X., Louha, S,. Kohli, R., Rushmore, J., Wong, K., Shaw, T.I., Rothrock, M., McKee, A. M., Guo, T.L., Mauricio, R., Molina, M., Lu, K. & Faircloth, B.C. (2019). Adapterama II: Universal amplicon sequencing on Illumina platforms (TaggiMatrix). *PeerJ.* 7:e7786 https://doi.org/10.7717/peerj.7786

Godwin, J.C. (2008). Biological Inventory of the Cave and Karst Systems of The Nature Conservancy's Sharp-Bingham Mountain Preserve. Alabama Natural Heritage Program. Environmental Institute, Auburn University. Auburn, AL

Goldberg, C.S., Pilliod, D.S., Arkle, R.S. & Waits, L.P. (2011). Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS One*. 6:e22746.

Graening, G.O., Fenolio, D.B., Niemiller, M.L., Brown, A.V., & Beard, J.B. (2010). The 30-year recovery effort for the Ozark cavefish (*Amblyopsis rosae*): Analysis of current distribution, population trends, and conservation status of this threatened species. *Environmental Biology of Fishes.* 87:55–88. https://doi.org/10.1007/s10641-009-9568-2

Harper, K.J., Anucha, P., Turnbull, J.F., Bean, C.W., & Leaver, M.J. (2018). Searching for a signal: Environmental DNA (eDNA) for the detection of invasive signal crayfish, *Pacifasticus leniusculus* (Dana, 1852). *Management of Biological Invasions*. 9:2:137-148.

Harrison, J.B., Sunday, J.M. & Rogers, SM. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B.* 286:20191409. https://doi.org/10.1098/rspb.2019.1409

Hinlo, R., Gleeson, D., Lintermans, M., & Furlan, E. (2017). Methods to maximise recovery of environmental DNA from water samples. *PLoS One.* 12:6:e0179251

Hobbs, H.H. III, & Bagley, F.M. (1989). Shelta Cave management plan. Unpublished report prepared by Biological Subcommittee of the Shelta Cave Management Committee, Huntsville, 78 pp.

IUCN. (2021). The IUCN Red List of Threatened Species. Version 2021-2. https://www.iucnredlist.org.

Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., Letcher, B.H. & Whiteley, A.R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Mol Ecol Resour*. 15:216-227. https://doi.org/10.1111/1755-0998.12285

Katoh, K., & Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution.* 30:4:772-780

Korbel, K., Chariton, A., Stephenson, S., Greenfield, P., & Hose, G.C. (2017). Wells provide a distorted view of life in the aquifer: implications for sampling, monitoring and assessment of groundwater ecosystems. *Sci Rep.* 7:40702. https://doi.org/10.1038/srep40702

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. *Molecular Biology and Evolution.* 33:7:1870-4. doi: 10.1093/molbev/msw054.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.

Livia, L., Antonella, P., Hovirag, L., Mauro, N. & Panara, F. (2006) A nondestructive, rapid, reliable and inexpensive method to sample, store and extract high-quality DNA from fish body mucus and buccal cells. *Molecular Ecology Notes*. 6:257–260.

Lydeard, C. & Mayden, R.L. (1995), A Diverse and Endangered Aquatic Ecosystem of the Southeast United States. *Conservation Biology*. 9:800-805. https://doi.org/10.1046/j.1523-1739.1995.09040800.x

Mammola, S., Cardoso, P., Culver, D.C., Deharveng, L., Ferreira, R.L., Fiser, C., Galassi, D.M.P., Griebler, C., Halse, S., Humphreys, W.F., Isaia, M., Malard, F., Martinez, A., Moldovan, O.T., Niemiller, M.L., Pavlek, M., Reboleira, A.S.P.S., Souza-Silva, M., Teeling, E.C., Wynne, J.J., & Zagmajster, M. (2019). Scientists' warning on the conservation of subterranean ecosystems. *BioScience.* 69:641–650.

Mauvisseau, Q., Coignet, A., Delaunay, C., Pinet, F., Bouchon, D., & Souty-Grosset, C. (2017). Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. *Hydrobiologia*. 805:163–175. doi:10.1007/s10750-017-3288-y

McGregor, S.W., O'Neil, P.E., & Wynn, E.A. (2008). Water quality and biological monitoring in Bobcat and Matthews Caves, Redstone Arsenal, Alabama, 1990-2008. Geological Survey of Alabama: Water Investigations Program. Open-File Report 0813.

Mitra, A., Skrzypczak, M., Ginalski, K., & Rowicka, M. (2015). Strategies for Achieving High Sequencing Accuracy for Low Diversity Samples and Avoiding Sample Bleeding Using Illumina Platform. *PLoS One.* 10:4:e0120520. https://doi.org/10.1371/journal.pone.0120520

Mouser, J.B., Brewer, S.K., Niemiller, M.L., Mollenauer, R., & Van Den Bussche, R.A. (2021). Refining sampling protocols for cavefishes and cave crayfishes to account for environmental variation. *Subterranean Biology.* 39:79–105.

Nagy, R., Fusaro, A., Conard, W., & Morningstar, C. (2021). *Procambarus clarkii* (Girard, 1852): U.S. Geological Survey, Nonindigenous Aquatic Species Database, Gainesville, FL, https://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=217

Nguyen, N.P., Warnow, T., Pop, M., White, B. (2016). A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. *npj Biofilms and Microbiomes.* 2:16004. https://doi.org/10.1038/npjbiofilms.2016.4

Niemiller, M.L., Bichuette, E., & Taylor, S.J. (2018). Conservation of cave fauna in Europe and the Americas. *Ecological Studies: Cave Ecology.* 451–478 (Moldovan, O.T., Kovac, L., & Halse, S., eds). Springer.

Niemiller, M.L., Taylor, S.J., Slay, M.E., & Hobbs III, H.H. (2019). Biodiversity in the United States and Canada. *Encyclopedia of Caves*, 3rd edition:163-176 (Culver, D.C., White, W.B., & Pipan, T., eds.). Elsevier.

Pimm, S.L., Jenkins, C.N., Abell, R., Brooks, T.M., Gittleman, J.L., Joppa, L.N., Raven, P.H., Roberts, C.M., & Sexton, J.O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science.* 344:1246752–1246752.

Rheams, K.F., Moser, P.H., & McGregor, S.W. (1992). Geologic, hydrologic, and biologic investigations in Arrowwood, Bobcat, Matthews, and Shelta Caves and selected caves, Madison County, Alabama. Geological Survey of Alabama. Report prepared for the U.S. Fish and Wildlife Service, Jackson, 262 pp.

Riascos, L., Geerts, A.N., Oña, T., Goethals, P., Cevallos-Cevallos, J., Vanden Berghe, W., Volckaert, F.A.M., Bonilla, J., Muylaert, K., Velarde, E., Boets, P., & Van der heyden, C. (2018). DNA-based monitoring of the alien invasive North American crayfish *Procambarus clarkii* in Andean lakes (Ecuador). *Limnologica*. 70:20-25

Ripple, W.J., Wolf, C., Newsome, T.M., Galetti, M., Alamgir, M., Crist, E., Mahmoud, M.I., & Laurance, W.F. (2017). 15,364 scientist signatories from 184 countries, World Scientists' Warning to Humanity: A Second Notice. *BioScience*. 67:12,1026–1028, https://doi.org/10.1093/biosci/bix125

Ruppert, K.M., Kline, R.J., & Rahman, M.S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: a systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*. 17:e00547, 10.1016/j.gecco.2019.e00547

Schuster, G.A., Taylor, C.A., & McGregor, S.W. (In Press). Crayfishes of Alabama. Tuscaloosa, The University of Alabama Press.

Shelton-Nix, E. (2017). Alabama wildlife. Vol. 5. Tuscaloosa, AL: University of Alabama Press.

Souty-Grosset, C., Anastácio, P.M., Aquiloni, L., Banha, F., Choquer, J., Chucholl, C., & Tricarico, E. (2016). The red swamp crayfish *Procambarus clarkii* in Europe: Impacts on aquatic ecosystems and human well-being. *Limnologica.* 58:78-93. https://doi.org/10.1016/j.limno.2016.03.003.

Strand, D.A., Johnsen, S.I., Rusch, J.C., Agersnap, S., Larsen, W.B., Knudsen, S.W., Møller, P.R., & Vrålstad, T. (2019). Monitoring a Norwegian freshwater crayfish tragedy – eDNA snapshots of invasion, infection and extinction. *J. Appl. Ecol.* 56:1661–1679. doi: 10.1111/1365-2664.13404

Stratton, C.E., & DiStefano, R.J. (2021). Is Native Crayfish Conservation a Priority for United States and Canadian Fish and Wildlife Agencies? *Freshwater Crayfish*. 26:1:25-36. doi:10.5869/fc.2021.v26-1.25

Strickler, K.M., Fremier, A.K., & Goldberg, C.S. (2015). Quantifying the effects of UV, temperature, and pH on degradation rates of eDNA in aquatic microcosms. *Biological Conservation.* 183:85–92.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution.* 9:678-687.

Tamura, K. & Kumar, S. (2002). Evolutionary distance estimation under heterogeneous substitution pattern among lineages. *Molecular Biology and Evolution.* 19:1727-1736.

Thomas, A.C., Howard, J, Nguyen, P.L., Seimon, T.A., & Goldberg, C.S. (2018). ANDe™: A fully integrated environmental DNA sampling system. *Methods in Ecology and Evolution*. DOI: 10.1111/2041-210X.12994

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

Trifinopoulos, J., Nguyen, L.T., von Haeseler, A., & Minh, B.Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucl. Acids Res*. 44(W1): W232-W235. doi: 10.1093/nar/gkw256

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F…., & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology.* 25:929–942.

Venarsky, M.P., Benstead, J.P., & Huryn, A.D. (2012). Effects of organic matter and season on leaf litter colonization and breakdown in cave streams. *Freshwater Biology.* 57:773–86.

West, K.M., Richards, Z.T., Harvey, E.S., Susac, R., Grealy, A., & Bunce, M. (2020). Under the karst: detecting hidden subterranean assemblages using eDNA metabarcoding in the caves of Christmas Island, Australia. *Sci Rep.* 10**:**21479. https://doi.org/10.1038/s41598-020-78525-6

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J Comput Biol*. 7:1-2:203-14.