EXAMINING OCCURRENCE, LIFE HISTORY, AND ECOLOGY OF CAVEFISHES AND CAVE CRAYFISHES USING BOTH TRADITIONAL AND NOVEL APPROACHES

By

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Abstract: The persistence of cavefishes and cave crayfishes in the Ozark Highlands ecoregion is threatened due to narrow ranges coupled with declining groundwater quality. To adequately address the threats to cavefishes and cave crayfishes we need to understand their occurrence patterns and their basic life history and ecology. It is often difficult to obtain those data for stygobionts because they occur in locations inaccessible to humans. New techniques and improved study designs can increase our knowledge of where cavefishes and cave crayfishes occur and how to best facilitate their presence. Therefore, my objectives were: 1) assess the use of eDNA to detect the presence of cavefishes and cave crayfishes while accounting for imperfect detection, and 2) determine habitat use, morphological traits, and longevity of bristly cave crayfish *Cambarus setosus*. I developed single-season occupancy models to estimate occurrence probability of cavefishes and cave crayfishes while accounting for imperfect detection. Occurrence of stygobionts was related to geology series and anthropogenic disturbance at a fine scale (i.e., 500-m buffer). Water volume, water velocity, water turbidity, and substrate affected detection of stygobionts. I assessed habitat use and morphological characteristics of *C. setosus* in Smallin Civil War Cave (SCWC) using a long-term dataset. *Cambarus setosus* used different substrates, water velocities, and water depths in SCWC based on availability and life-history requirements. Female *C. setosus* were typically bigger than males and there was no difference in chelae size between the sexes. Finally, I investigated the effectiveness of the gastric mill for aging cave crayfishes. My results indicate that the gastric mill can be used to age some surface crayfishes, but not cave crayfishes. Further, temperature is one factor that contributes to gastric mill band formation. My project demonstrates that eDNA can be a useful tool for establishing occurrence of *Troglichthys rosae* and *C. tartarus*; however, more work needs to be done to understand other species genetic differences. Finally, while it is important to account for imperfect detection, long-term datasets from which it often cannot be estimated, are still important for obtaining trait data.

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CHAPTER I

INTRODUCTION

Stygobionts are diverse groups of organisms that are adapted to spend their entire lives in groundwater ecosystems, and many species are of conservation concern. Groundwater ecosystems are represented by the hyporheic zone of streams (i.e., the saturated interstitial areas beneath the streambed, White 1993), aquifers, caves, and springs (Gibert et al. 2009). In the United States, there are at least 469 species that are endemic to groundwater habitats, including worms, mollusks, insects, crustaceans, amphibians, and fishes (Niemiller et al. in press). For most organisms, adaptation to life in groundwater results in reduction, or complete loss, of pigment and eyes and improvements in the sensitivity of other sensory organs (Christiansen 2012). Additionally, many stygobionts have narrower ranges (Gibert et al. 2009, Tronelj et al. 2009), longer life spans (Culver 2012, Venarksy et al. 2012), and lay fewer and larger eggs (Culver 2012) compared to surface species. The life-history traits exhibited by stygobionts are often associated with elevated extinction risk in many taxa (Purvis et al. 2000, Larson and Olden 2010, Pearson et al. 2014). In fact, over half of the stygobionts in the United States are at elevated risk of extinction (Culver et al. 2000). Although the link

between human wellbeing and stygobionts is indirect, persistence of these populations confers several anthropogenic benefits.

Stygobionts provide many ecosystem services for groundwater, a resource that faces a variety of threats. Groundwater is important to human welfare because up to 2 billion people worldwide rely on it for drinking water, and 40% of the world's food production is supported by groundwater irrigation (Morris et al. 2003). Groundwater quality is threatened by overexploitation, contamination, and land-use changes (Morris et al. 2003). Stygobionts improve groundwater quality because they degrade pollutants, cycle nutrients, assist with water infiltration, and control diseases (Danielopol and Griebler 2008, Boulton et al. 2008, Griebler et al. 2014). For example, amphipod grazing on biofilms influenced the movement of energy through groundwater food webs (Cooney and Simon 2009). Additionally, Stumpp and Hose (2017) show how amphipods alter sediment structure through burrowing. In addition to these aforementioned ecosystem services, stygobionts can be important bioindicators of groundwater quality (Notenboom et al. 1994, but also see Dumas et al. 2001). Shifts to agricultural land use, for example, can alter groundwater assemblages due to associated changes in water quality (Stein et al. 2010, Español et al. 2017). Also, increases in heavy metals and sewage pollution can result in lower stygobiotic diversity (Malard et al. 1996). Understanding the distribution and traits of stygobionts is a necessary first step to developing conservation strategies that protect populations, and therefore, groundwater resources.

Distribution data can be used to address general threats to a species, whereas trait data can address population-specific threats. Documenting species' distributions is necessary to evaluate the effects of climate change on species (Thuiller 2004, Chu et al.

2005), predict invasion success (Caphina and Anastácio 2011, Tingley et al. 2018), and examine reintroduction and other recovery actions (Pearce and Lindenmayer 1998). Population responses to ecosystem threats may depend on population-level traits, such as diet, thermal tolerance, timing of reproduction, habitat use, and individual growth (Frimpong and Angermeier 2010). Knowledge of population traits can facilitate predictions of extinction and invasion risk (Larson and Olden 2010), be used to improve sampling designs (Pregler et al. 2015, Mollenhauer et al. 2017), and is useful to develop options to maintain stable ecosystems with proper functions (de Bello et al. 2010, Cadotte et al. 2011). Despite the importance of distributions and associated species' traits, our knowledge on the distributions and ecology of stygobionts is extremely limited (Hahn 2002, Gibert et al. 2009).

One of the difficulties in obtaining distribution and trait data relates to variable sampling detection (i.e., a species may not be detected on every sampling event, MacKenzie 2002). Stygobionts are emblematic of variable detection because they occupy habitats that are often inaccessible humans (Dole-Olivier et al. 2009). For example, it can often require numerous surveys of caves to detect stygobionts; thus, resulting estimates of species occupancy and richness are skewed to more heavily sampled locations (Culver et al. 2004, Krejca and Weckerly 2007). Promising new strategies such as sampling using environmental DNA (eDNA) may prove useful for detecting and monitoring biodiversity (Sigsgaard et al. 2015, Bergman et al. 2016, Stoeckle et al. 2016). Using eDNA as a sampling tool may be especially helpful for surveying cryptic stygobionts that occupy environments that are difficult to access and sample (Vörös et al. 2017, Niemiller et al. 2018).

The goal of my thesis was to improve our understanding of stygobiont distributions and traits, which are useful data for developing conservation actions. Traditional sampling for stygobionts is inherently difficult and often does not address issues related to detection; therefore, my first objective assessed the use of eDNA to detect the presence of cavefishes and cave crayfishes while accounting for imperfect detection. Using eDNA surveys may lead to improved knowledge of Ozark Highland cavefish and cave crayfish distributions, and thus facilitate targeted recovery and protection actions where necessary or desired (Rees et al. 2014). Establishing species occurrences is the first step in effective conservation, but associated species trait data allow for population-specific conservation actions. Therefore, my second objective determined habitat use, morphological traits, and longevity of bristly cave crayfish *Cambarus setosus*. Results of my second objective will be useful for facilitating monitoring efforts (e.g., knowledge of reproductive periods; de Souza et al. 2016), developing population models (e.g., size-specific demographics, Crouse et al. 1987), or examining the effects of the environment on hatch (e.g., back-calculating birth date from age data, Koehn and Harrington 2006).

Study area

My research focuses on the diverse karst habitats of the Ozark Highlands ecoregion in northeast Oklahoma, southwest Missouri, and northwest Arkansas. Average annual rainfall and air temperatures of the Ozark Highlands ecoregion range from 97–122 cm and 13–16 ˚C, respectively (Adamski 1995). The Ozark Highlands was historically a mix of prairie, oak, hickory, and pine forests, but many areas have been converted to agricultural uses (Woods et al. 2005). The Ozark Highlands is divided into 2 major

geological regions, the Springfield and Salem plateaus (Adamski 1995). The lithology of the Ozark Highlands ecoregion is primarily Mississippian limestone and Ordovician dolomite, which have been dissolved over time by groundwater, resulting in thousands of caves and springs (Unklesbay and Vineyard 1992).

Study species

Many stygobiont species that occur in the Ozark Highlands ecoregion face anthropogenic threats. There are at least 53 species of stygobionts that occur in the Ozark Highlands ecoregion, making it the fourth most diverse karst region in the United States (Niemiller et al. in press). Specifically, there are 2 species of cavefishes (i.e., the Ozark cavefish *Troglichthys rosae* and Eigenmann's cavefish *Typhlichthys eigenmanni*) and 7 species of cave crayfishes described from the Ozark Highlands ecoregion (i.e., Benton cave crayfish *C. aculabrum*, Salem cave crayfish *C. hubrichti*, bristly cave crayfish *C. setosus*, Delaware county cave crayfish *C. subterraneus*, Oklahoma cave crayfish *C. tartarus*, Hell Creek cave crayfish *C. zophonastes*, and Caney Mountain cave crayfish *Orconectes stygocaneyi*). My study specifically focused on both cavefish species and 5 of the cave crayfishes (i.e., *C. aculabrum*, *C. setosus*, *C. subterraneus*, *C. tartarus* and *O. stygocaneyi*). The American Fisheries Society (AFS) lists *C. aculabrum*, *C. subterraneus*, and *C. tartarus* as endangered; *O. stygocaneyi* as threatened; and *C. setosus* as stable (Taylor et al. 2007). In contrast, only *C. aculabrum* is federally listed as endangered (U.S. Fish and Wildlife Service 2019). The AFS lists *T. rosae* as threatened and *T. eigenmanni* vulnerable (Jelks et al. 2008). Only *T. rosae* is federally listed as threatened (U.S. Fish and Wildlife Service 2019). Threats to cavefishes and cave crayfishes include narrow

range of occurrence for most species coupled with declining groundwater quality, over-collection, and reservoir impoundment (Graening et al. 2006, Graening et al. 2010).

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CHAPTER II

COMPARING ENVIRONMENTAL DNA TO VISUAL SURVEYS FOR ESTABLISHING OCCURRENCE OF CAVEFISHES AND CAVE CRAYFISHES

Introduction

Variable detection is a major challenge when establishing species' occurrences and ecological relationships across the landscape and can be accounted for using appropriate study designs (Mackenzie et al. 2018). Detection varies among habitats (Price and Peterson 2010), sampling method (Pregler et al. 2015), species (Peterson and Paukert 2009), and through time (Hangsleben et al. 2013). For example, detection was higher via seining than backpack electrofishing for bridled shiner *Notropis bifrenatus* in Connecticut and led to inappropriate inferences about the status of the species (Pregler et al. 2015). Sampling standardization is useful for limiting some sampling variability (e.g., sampling at the same time of year; see Bonar et al. 2009 for an overview), but standardization alone does not account for environmental variability (e.g., habitat type) that is often of interest to ecologists (Mackenzie et al. 2004). For example, Mollenhauer et al. (2018) employed standardized sampling to estimate the occupancy of Great Plains fishes and showed that sand shiner *Notropis stramineus* occurrence was underestimated in 1 of 2 rivers due to differences in the environment (i.e., not controlled through standardization). The underlying species-environmental relationships of interest to ecologists often do not

emerge unless we first account for variable detection due to sampling (Gwinn et al. 2016). Variable detection can be accounted for by using a study design that allows detection probability to be related to environmental covariates (i.e., repeated sampling while measuring factors hypothesized to influence detection; MacKenzie et al. 2018). Accounting for species detection as part of the modeling process can improve estimates of extinction rates and associated environmental relationships (Kéry et al. 2006, Pregler et al. 2015), species richness (Tingley and Beissinger 2013), and distributions (Chen et al. 2013, Lahoz-Monfort et al. 2014).

Determining occupancy of cavefishes and cave crayfishes is difficult due to the challenges of sampling their environment. Cavefishes and cave crayfishes are typically surveyed by 1–3 people walking or snorkeling slowly upstream in caves while recording the number of organisms observed (Graening et al. 2006d, 2010). Visual surveys miss many stygobiotic organisms because caves are only snapshots of the entire groundwater ecosystem stygobionts might occupy (Means and Johnson 1995, Buhay and Crandall 2005). Additionally, visual surveys in caves can be expensive due to labor costs and the technical gear required (e.g., light sources, climbing equipment, helmets, SCUBA gear; Crandall 2016, Graening et al. 2006c). Visual surveys of caves can also be dangerous for the surveyors (e.g., cold and damp with rough terrain, White 2012) and can be lethal for both target and non-target organisms (e.g., accidental trampling, Graening et al. 2010). Traps are a viable option for sampling cave crayfishes because they are relatively simple and can be deployed in wells and springs; thus, increasing the amount of groundwater that can be sampled (Purvis and Opsahl 2005, Fenolio et al. 2017). Unfortunately,

sampling with traps can result in mortality (Fenolio et al. 2017) and be biased to certain individuals (e.g., large males, Stebbing et al. 2014).

Environmental DNA (eDNA) sampling is a relatively new technique in ecology and conservation biology that may improve detection of cavefishes and cave crayfishes; however, variable detection is still a concern. Environmental DNA surveys document species presence via the collection of DNA from the environment (Ficetola et al. 2008), which is derived from waste products, shed hair and skin, the slime coat of fishes and amphibians, shed exoskeletons of arthropods, and decomposing individuals (Tréguier et al. 2014, Thomsen and Willerslev 2015). Many taxa have been surveyed via eDNA, including fishes (Jerde et al. 2011), crayfishes (Tréguier et al. 2014), mollusks (Egan et al. 2013), reptiles (Piaggio et al. 2014), and the stygobiotic genera *Proteus* (Gorički et al. 2017, Vörös et al. 2017) and *Stygobromus* (Niemiller et al. 2018). Environmental DNA is even being used to search for the elusive bigfoot *Anthropoidipes ameriborealis* and Loch Ness monster (Meldrum 2017, Greshko 2018). In some cases, eDNA surveys have higher detection when compared to traditional survey methods (Ficetola et al. 2008, Jerde et al. 2011, Spear et al. 2015), are more cost-efficient (Sigsgaard et al. 2015), and are less detrimental to the study organisms (Tréguier et al. 2014, Sigsgaard et al. 2015). False positives (i.e., detecting a species when it is absent) for eDNA surveys can result from contamination (Thomsen and Willerslev 2015) and sampling DNA from the substrate (Tréguier et al. 2014). False positives from contamination can be detected by including negative controls at every step of the study (Ficetola et al. 2016). Further, false positives can be reduced through good laboratory protocol (Ficetola et al. 2016) and collecting

samples from the water column (Tréguier et al. 2014). False negatives (i.e., failing to detect a species when it is actually present) can be caused by several factors including DNA degradation from sunlight, pH, salinity, and temperature (Barnes et al. 2014), the transport of DNA from the collection site (Hyman and Collins 2012), and the presence of PCR inhibitors (Thomsen and Willerslev 2015). Variable detection of eDNA surveys can be accounted for through study designs where models that estimate detection probability can be developed.

Environmental DNA may be a viable option for surveying the cavefishes and cave crayfishes of the Ozark Highlands ecoregion and may address existing knowledge gaps on the distributions of these taxa. Eigenmann's cavefish *Typhlichthys eigenmanni* occurs in central and southeast Missouri and northeast Arkansas, whereas the Ozark cavefish *Troglichthys rosae* occurs in northwest Arkansas, southwest Missouri, and northeast Oklahoma (Niemiller and Poulson 2010). The bristly cave crayfish *Cambarus setosus* is known from 48 sites in southwest Missouri, a site in northwest Arkansas, and a site in northeast Arkansas (Graening et al. 2006b). The Delaware County cave crayfish *C. subterraneus* and Oklahoma cave crayfish *C. tartarus* are known from 3 and 2 caves in Delaware County, Oklahoma, respectively (Graening and Fenolio 2005, Graening et al. 2006a). The Caney Mountain cave crayfish *Orconectes stygocaneyi* occurs in a single cave in south-central Missouri (Hobbs III 2001). Finally, the Benton cave crayfish *C. aculabrum* is known from 4 sites in Benton and Washington counties, Arkansas (Graening et al. 2006d).

The specific objectives of this chapter were to 1) develop an eDNA protocol to detect the presence of cavefishes and cave crayfishes in the Ozark Highlands ecoregion, 2) compare the efficiency of eDNA to traditional visual surveys, and 3) determine some of the factors that drive occurrence of cavefishes and cave crayfishes. Both visual and eDNA surveys have inherent biases; therefore, I accounted for imperfect and variable detection in my study design and analysis. I hypothesized that eDNA surveys will have higher detection than visual surveys in springs and wells, as well as caves that are difficult to access. Human-induced changes (e.g., declining water quality, Graening et al. 2010) are often cited as a major threat to stygobiont distributions; thus, I hypothesized that anthropogenic disturbance will influence the occurrence of cavefishes and cave crayfishes. Results of this chapter will help managers focus conservation and monitoring efforts on particular locations of interest, choose the best approach for surveying those organisms, and provide a foundation for an eDNA monitoring protocol for the karst habitats of the Ozark Highlands ecoregion.

Methods

Both eDNA and visual surveys were conducted for cavefishes and cave crayfishes at 21 caves, 12 springs, and 9 wells (hereafter referred to as sites) across the Ozark Highlands ecoregion (Fig. 1). Carroll and Thunder rivers within Carroll Cave were considered separate sites due to extreme differences in the hydrologic regime (Miller 2010). Sites were chosen based on 3 criteria: 1) some sites were selected because of regular documentation of numerous cavefishes and cave crayfishes so these sites serve as

positive field controls, 2) other sites were selected because species occupancy was unknown, and 3) some sites were chosen because of accessibility.

One to 5 sampling units (n = 63; Table 1), 2–200 m in length, were selected at each site based on presumed biological barriers. For example, sampling unit 10.1 was a cave with a single pool of water and no discernible change in habitat, and sampling unit 16.1 was a pool within a cave bounded by a waterfall downstream and shallow riffle upstream. Sampling units are referenced by the site number, then the sequential number of units within the site (e.g., 1.2 refers to the second sampling unit within site 1). Most sampling units were surveyed on 3–5 occasions from February–May 2017 to ensure that species would not have time to go extinct or colonize sampling units (i.e., the closure assumption of occupancy modeling is met). Although there was some localized flooding at the end of my sampling period, I 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). Further, defining my season to allow some changes in the physicochemical parameters at each sampling unit was preferred to examine relationships between detection and a range of physicochemical parameters using both sampling techniques. Sampling units 28.1 and 29.1 were only sampled once because they were flooded, and sampling unit 21.1 was only sampled once because it became dry. Therefore, those sampling units were excluded from the detection model but included in the occupancy model. I did not complete visual surveys at sampling units 10.1 and 18.1 on the last 2 survey dates due to the caves being flooded.

Sampling unit 3.4 was not surveyed on the third visit because it was dry. Sampling unit 4.4 was established after the first visit to the cave, so it was surveyed only twice. I did not complete full visual surveys of sampling unit 5.1 and sampling unit 6.2 due to sampling restrictions by the regulatory agency. Sampling units 38.1 and 39.1 were sampled via a different gear due to narrow openings, so they were excluded from my analysis.

eDNA surveys

I collected 2 water samples (≈ 1-L each) for eDNA analysis at each sampling unit during each visit. I collected 2 L of water to provide a replicate in case of error or contamination in subsequent steps. To avoid contamination, I immersed sampling equipment in 50% bleach for at least 30 s between sites, and if possible, between sampling units. Equipment was then rinsed in deionized water. Some caves were too difficult to navigate with more than 1 equipment set, so I used the same gear at multiple sampling units within the cave; however, I rinsed the gear thoroughly at new sampling units before water collection. I filtered distilled water in the field on 4 occasions to provide negative controls in the field, which were treated the same as field samples in subsequent steps. I collected water in two 1-L sample bottles (312187-0032, ThermoFisher Scientific, Waltham, MA) from approximately 10 cm above the substrate without disturbing the substrate. The water was too shallow at some sampling units to allow 10 cm between the sample bottle and substrate; thus, water was collected just above the substrate. To collect water from wells, I lowered a Van Dorn sampler (3-1920-H62, Wildco, Yulee, FL) to approximately 10 cm above of the substrate, closed the sampler, and returned it to the surface. I then transferred the water to two 1-L sample

bottles. I filtered the water immediately after collection, except for the samples collected from sampling units 4.1 through 4.4 on 21 March 2017, which were frozen and filtered later in the laboratory. While wearing nitrile gloves, I placed a 0.45-µm filter (14-555-624, Fisher Scientific, Waltham, MA) inside a filter funnel (09745, Fisher Scientific, Waltham, MA) attached to a vacuum flask via a rubber stopper (Fig. 2). I used a hand pump (AC3310, Advance Auto Parts, Raleigh, NC) to create a vacuum and pull water through the filter. Only 1 filter was typically needed to sample 1 L of water, but occasionally multiple filters (i.e., 2–6) were used due to clogging via sediment. Filters were stored at room temperature in vials of 900 µl of Longmire's buffer (Longmire et al. 1997), until extractions were completed (i.e., 1–18 months after collection).

Visual surveys

Visual surveys for cavefishes and cave crayfishes took place at each of the sampling units for later comparison to eDNA detection. For springs and caves, 2 observers walked or crawled the entire sampling unit while carefully searching the whole wetted area for cave crayfishes or cavefishes by overturning rocks and examining crevices (Graening et al. 2006d, 2010). Hand-dug wells were surveyed in their entirety using a spotlight (QBeam Max Million III, The Brinkmann Corporation, Dallas, TX), both before and after water samples were collected because disturbance from sampling sometimes caused stygobionts to emerge. I recorded the number of cavefishes and cave crayfishes observed and the time spent observing (min).

Detection covariates

Environmental covariates were collected at each sampling unit to explain variable detection of both of cavefishes and cave crayfishes. Detection covariates collected at each sampling unit were water turbidity (0.01 NTU), light (yes/no), water volume (1.0 m^3), approximate water-column velocity (hereafter referred to as water velocity, 0.01 m/s), and substrate (coarse/fine). I collected 250-ml water samples before the start of each visual survey to measure water turbidity using a turbidity meter (AQUAfast AQ4500, Thermo Fisher Scientific, Waltham, MA). The turbidity meter measures the light scattered by solid particles in the water sample as an indicator of turbidity. Light was recorded as ambient light visible (yes) or not visible (no) at the water-sample location. Survey length (1.0 m), wetted width (0.1 m), and maximum water depth (0.1 m) were multiplied to estimate water volume (0.1 m^3). Survey length was measured as the distance from the start to the end of the sampling unit. Wetted width and maximum water depth were measured at 3–5 points along the sampling unit to represent average conditions. Water velocity (Gordon et al. 2004) was visually estimated at each of the locations where wetted width and maximum water depth were measured. I visually estimated water velocity because it was unreasonable to bring a flow meter into many of the caves I sampled (e.g., narrow crawl spaces and deep water). Prior to the study, I compared my visual water velocity estimates to values measured with a Marsh-McBirney flow meter (Marsh-McBirney Inc., Frederick, MD) to ensure that my estimates were relatively accurate. I also distinguished between the prevalence of clay, silt, or bedrock substrates (hereafter referred to as “fine”), or pebble substrate, cobble substrates, or

woody debris of similar size or larger (hereafter references as “coarse”) at each sampling unit (see Wentworth 1922 for sizes of each substrate).

Occupancy covariates

I calculated an anthropogenic disturbance index at both fine (i.e., 500-m buffer) and coarse scales (i.e., recharge area) and recorded geology associated with each site (i.e., sampling units nested within sites received the same values) to estimate occupancy probability of cavefishes and cave crayfishes. I used land-use data to calculate site-specific anthropogenic disturbance indices, modified from Brown and Vivas (2005), following Mouser et al. (2019). Land-use data were acquired from the 2011 National Land Cover Database (https://www.mrlc.gov/). I used ArcMap (version 10.4, ESRI, Redlands, CA) 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 18 of my sites had known recharge areas, so I averaged the area and assigned the average to sites with unknown recharge area. The proportion of each land-use type within the buffers was calculated and multiplied by the following coefficients: 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 each scale. Finally, each site was assigned a geology category based on the underlying geological series data obtained from the United States Geological Survey (https://mrdata.usgs.gov/geology/us/).

Primer and probe development

Primers and probes were designed to amplify DNA for each of my study species (i.e., a species-specific Taqman® assay). I downloaded all published mitochondrial cytochrome oxidase subunit 1 (CO1) gene sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for *C. setosus* (*n* = 1), *C. aculabrum* (*n* = 1), and *C. tartarus* (*n* = 1). I downloaded all published mitochondrial NADH dehydrogenase subunit 2 (ND2) gene sequences for *T. eigenmanni* (*n* = 21) and *T. rosae* (*n* = 12). I created assays for *T. eigenmanni* from 2 of the downloaded sequences that covered the genetic variation of the population from which I collected water samples. I chose the CO1 and ND2 genes because they are relatively easy to isolate and purify (Billington 2003), have rates of divergence that allow species to be distinguished (Billington 2003), and are commonly used to amplify DNA of cave crayfishes (e.g., Buhay et al. 2007) and cavefishes (e.g., Niemiller et al. 2012). Collaborators provided tissue samples for *T. rosae* (*n* = 11), *C. subterraneus* (*n* = 6), *C. setosus* (*n* = 1), *C. tartarus* (*n* = 7; Table 2). I also collected tissue samples from *C. setosus* (*n* = 5) and *O. stygocaneyi* (*n* = 3; Table 2). Genomic DNA was extracted from those tissue samples using the Qiagen DNeasy® Blood and Tissue kit (Cat. # 69504, Qiagen, Hilden, Germany) according to the manufacturer's instructions. In addition to tissue samples, I obtained *C. subterraneus* (*n* = 2) and *O. stygocaneyi* (*n* = 2) genomic DNA from collaborators (Table 2). A 500-bp fragment of the cavefishes' ND2 gene was PCR amplified using the forward primer MET: 5'-CATACCCCAAACATGTTGGT-3' and reverse primer ND2B: 5'-TGGTTTAATCCGCCTCAGCC-3' (Kocher et al. 1995). For cave crayfishes, a 710-bp

fragment of the CO1 gene was amplified using the forward primer LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and the reverse primer 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 (A9281, Promega, Madison, WI). PCR products were Sanger sequenced in our laboratory with an ABI 3130 Genetic Analyzer (Applied Biosystems 3130, Thermo Fisher Scientific, Waltham, MA). All sequences were manually trimmed and aligned in Geneious (Version 11.1.5, Geneious, Auckland, New Zealand) to generate a consensus sequence for the CO1 locus of each cave crayfish species and the ND2 locus of each cavefish species. The consensus sequences were entered in PrimerQuest (https://www.idtdna.com/PrimerQuest/Home/Index) to generate species-specific Taqman® assays (Table 3). Specificity of both the primers and probe was checked using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

eDNA extraction

I extracted eDNA from the filters using a DNeasy® Blood and Tissue Kit by following the "purification of total DNA from crude lysates" protocol. All laboratory surfaces and equipment were sterilized with 10% bleach before extractions. DNA was initially extracted for only 1 filter collected at a sampling unit. Any additional filters were placed in fresh Longmire's buffer and set aside to use if the first filter was negative for the target species' DNA (see next section *quantitative PCR amplification*). Using forceps, each filter was halved, torn into pieces, and pieces from each half were added to separate 1-ml microcentrifuge tubes. Forceps were sterilized between filters by immersion into

100% ethanol and flaming. The Longmire's buffer was split into two 1-ml tubes, and if the volume was < 360 µl, fresh buffer was added. The tubes of Longmire's buffer were then centrifuged at 8,000 g for 30 sec. I then transferred 360 µl of the Longmire's buffer and the pellet to the respective tubes with the filter pieces. This resulted in a standard amount of filter pieces and buffer in each tube (i.e., 1 filter half and 360 µl). There were 2 tubes per sampling unit, and each tube was considered a subsample for that location. I added 40 µl of proteinase K to each tube and incubated the samples overnight (i.e., 8–12 h) at 56 ˚C with periodic vortexing. I then added 400-µl buffer AL and vortexed. If the pH was > 7.0, I added 1 µl of acetic acid. Next, 400-µl 100% ethanol was added, and samples were vortexed. The mixture was then pipetted off the filters and into a DNeasy® spin column. I centrifuged the DNeasy® columns at 6,000 g for 1 min. I then added 500-µl buffer AW1 to the column and centrifuged for 1 min at 6,000 g. Next, 500-µl buffer AW2 was added and the column was centrifuged for 3 min at 20,000 g. Finally, I placed the spin columns into clean 1-ml microcentrifuge tubes, added 125-µl buffer AE, incubated the samples at room temperature for 1 min, and then centrifuged for 1 min at 6,000 g. I stored my samples at 2 ˚C until amplification (i.e., up to 4 mo).

Quantitative PCR amplification

I amplified eDNA using quantitative Polymerase Chain Reaction (qPCR). Each amplification reaction had a total volume of 20 µl, consisting of 10-µl TaqMan® Environmental Master Mix 2.0 (4396838, ThermoFisher Scientific, Waltham, MA), 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 (BC3496, ThermoFisher Scientific, Waltham, MA) on a LightCycler 480 (Roche, Pleasanton, CA). The thermal profile consisted of an initial denaturation step of 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min. Each subsample was run in triplicate, which resulted in an initial 6 replicates for each sampling unit. If any replicates amplified, then the sampling unit was considered positive for the species. If none of the replicates amplified, I extracted eDNA from any remaining filters from the sampling unit and ran another qPCR. I repeated the above process until all filters were processed, or until any replicates amplified. If only 1 subsample amplified from 1 survey date, then I processed that subsample again. If the subsample still amplified, then the survey was considered positive for the species and if it was negative, then the survey was considered negative. I also ran 3 negative controls during each qPCR in which the template DNA was replaced by ddH_2O. If any of the negative controls amplified, then the qPCR run was discarded. A positive control that consisted of genomic DNA from the target taxa was included to ensure the reaction worked properly (i.e., the positive control should always amplify). I confirmed species identification of a subset of positive samples for each species by Sanger sequencing.

Statistical analysis

I developed single-season occupancy models (Mackenzie 2002) to estimate both detection and occurrence probability of cavefishes and cave crayfishes (i.e., 2 taxa with multiple species within each). I modeled both species of cavefishes together and all species of cave crayfishes together due to the relatively small samples sizes of certain species; thus, I assumed behavioral and trait differences among the cavefish and cave

crayfish species did not influence detection or occupancy. Occupancy modeling uses spatially or temporally repeated surveys and environmental covariates to estimate the probability of a site being occupied (Mackenzie et al. 2002). The assumptions of the basic occupancy model are that the sampling unit is closed to the species (i.e., species neither go extinct or colonize the sampling units), no false positives (hence why I controlled for those through sampling design), and detection at a sampling unit does not influence detection at other sampling units (Mackenzie et al. 2002). Visual and eDNA surveys were assumed to be independent (i.e., detecting an organism with a gear did not influence detection via the other gear), so I did not use the multi-gear method outlined by Nichols et al. (2008). Variation in detection probability was modeled as a Bernoulli process based on the species' capture histories from each sampling unit, conditional on the occupancy state, using multinomial likelihood with a logit link function (Fiske and Chandler 2011).

I modeled detection of cavefishes and cave crayfishes using the covariates light, substrate, water volume, water velocity, and water turbidity. Detection probability was modeled using only data from sampling units where I detected cave crayfishes or cavefishes (i.e., sampling units with all-zero capture histories do not inform the detection process). The continuous variables water volume and water turbidity were natural-log transformed due to right-skewed distributions and then standardized to a standard deviation of 1 and mean of 0 to improve interpretation. All continuous variables had a Pearson's pairwise correlation coefficient ($|r| \leq 0.11$. I also included 3 categorical detection covariates: water velocity, light, and substrate. Water velocity was treated as a binary variable (i.e., 0 = not flowing, 1 = flowing) because approximately half of the

surveys were conducted when water velocity was 0 m/s. Taxa-dependent and gear-dependent detection was accounted for by including three-way interaction terms between taxa, gear, and each environmental covariate. I calculated 90% confidence intervals for each parameter and removed all three-way interactions that overlapped zero. Next, I repeated the process for two-way interactions and then the main effects in the model. I fitted my models using the program Unmarked (Fiske and Chandler 2011) in the statistical software R (version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria).

After I determined the most parsimonious detection model, I repeated the selection process described above for the occurrence model using 3 occupancy covariates: geology, fine-scale disturbance, and coarse-scale disturbance. All-zero capture histories were included in the occupancy portion of the model. Disturbance values at both scales were natural-log transformed, standardized to a standard deviation of 1 and mean of 0 to improve interpretation. The Pearson's pairwise correlation coefficient between disturbance values at both scales was ($|r|$) = 0.70, so I ran separate models with each variable. My final model included only significant detection and occurrence covariates. Model fit was assessed with a Chi-square statistic using my most complex model (Mackenzie and Bailey 2004).

Results

eDNA surveys

I collected 183 water samples at 61 sampling units to later analyze for the presence of DNA from cavefishes (Tables 4) and cave crayfishes (Table 5). I detected *T.*

rosae DNA on 68 of 165 surveys at 31 of 55 sampling units and *T. subterraneus* on 4 of 18 surveys at 2 of 6 sampling units. At 21 sampling units, I detected cavefish DNA, but did not observe cavefish. I sequenced DNA from sampling units 5.1, 6.1, 10.1, 13.1, and 21.1, and all sequences closely matched *T. rosae* (i.e., ≥ 98%). I sequenced DNA from sampling unit 16.3 and it matched *T. subterraneus* by 100%. I amplified DNA from 3 of 15 surveys screened for *C. aculabrum*, but the sequences from all 3 surveys only matched DNA of the species by 95%. I was unable to detect *C. setosus* or *C. subterraneus* DNA from the environmental samples although I was able to amplify genomic DNA from both populations for which I screened water samples. I only processed water samples for *C. setosus* collected from sampling units 3.1–3.5 on a recent visit because they have the largest known population and should have represented a positive control. I was also able to detect *T. rosae* DNA from those sampling units, which suggests the absence of strong inhibitors. I detected *C. tartarus* on 11 of 17 surveys at all 6 sampling units, but it was visually observed at only 1 sampling unit. I sequenced DNA from sampling unit 19.1 and it matched the DNA of *C. tartarus* by 99%. I was able to amplify *O. stygocaneyi* DNA from water samples collected from only sampling unit 20.1, which is interesting because it has only been visually confirmed from sampling unit 10.1. However, DNA was amplified from water samples collected from sampling unit 10.1 on 2 subsequent visits (i.e., outside my season) for another project. All of the negative controls collected in the field were negative, suggesting our decontamination protocol was adequate.

Visual surveys

I conducted 179 visual surveys at 61 sampling units for both cavefishes (Table 4) and cave crayfishes (Table 5). I observed *C. setosus* and at a relatively large proportion (0.48) of sampling units compared to other species (Table 6). In contrast, *C. tartarus* and *T. rosae* were observed at a low proportion (0.16 and 0.18, respectively) of the sampling units (Table 5). I conducted 57 visual surveys at 19 sampling units where cave crayfishes have not been previously identified, and I did not observe any individuals.

Detection covariates

The environmental covariates I measured varied considerably during the sample season (Table 1). Water turbidity ranged from 0.20 to 41.5 NTU (mean = 2.97 ± 4.57 NTU). There was visible light at 26 sampling units, 34 sampling units were dark, and sampling unit 10.1 did not have visible light on the first 2 surveys but did on the last due to the cave being flooded. I surveyed a range of water volumes across sampling units (0.06 m^3–800.00 m^3, mean = 61.21 ± 132.00 m^3). Water velocity ranged from 0–0.53 m/s (mean = 0.06 ± 0.10 m/s) and 81 surveys were classified as 0 (i.e., no flow) and 102 as 1 (i.e., flow). Thirty-three sampling units were classified as having coarse substrate and 28 as having fine substrate.

Occupancy covariates

Geological series and disturbance at both fine and coarse scales were included as occupancy covariates in my analysis. My sampling units were located within 4 coarse geology units: Smithville Dolomite (n = 19), Osagean Series (n = 37), Kinderhookian Series (n = 3), and Meramecian Series (n = 3). I 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 (mean = 2.00 ± 0.62) at the coarse scale and from 1.00–7.79 (mean = 2.02 ± 0.99) at the fine scale, where 8.67 would represent most highly disturbed via the index.

Statistical analysis

Detection probability of both cavefishes and crayfishes varied by survey technique and was influenced by water volume, water turbidity, water velocity, and substrate (Table 7). The final detection model was:

$$\text{logit}(p_{ij}) = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \beta_4 X_{4ij} + \beta_5 X_{5ij} + \beta_6 X_{6ij} + \beta_7 X_{1ij} X_{2ij} + \beta_8 X_{1ij} X_{3ij} + \beta_9 X_{2ij} X_{4ij} + \beta_{10} X_{1ij} X_{5ij} + \beta_{11} X_{2ij} X_{5ij} + \beta_{12} X_{1ij} X_{6ij} + \beta_{13} X_{2ij} X_{6ij} + \beta_{14} X_{1ij} X_{2ij} X_{6ij} + \beta_{15} X_{1ij} X_{2ij} X_{5ij};$$ where

$\text{logit}(p_{ij})$ = the detection probability of cavefishes and cave crayfishes at survey unit i during survey j,

β_0 = y-intercept

β_1 = slope for taxa

β_2 = slope for gear

β_3 = slope for water turbidity

β_4 = slope for water velocity

β_5 = slope for substrate

β_6 = slope for water volume

β_7 = slope for taxa and gear interaction

β_8 = slope for taxa and water turbidity interaction

β_9 = slope for gear and velocity interaction

β_{10} = slope for taxa and substrate interaction

β_{11} = slope for gear and substrate interaction

β_{12} = slope for taxa and water volume interaction

β_{13} = slope for gear and water volume interaction

β_{14} = slope for taxa, gear, and water volume interaction

β_{15} = slope for taxa, gear, and substrate interaction

X_1 = taxa

X_2 = gear

X_3 = average water turbidity (NTU)

X_4 = water velocity

X_5 = substrate

X_6 = average water volume (m^3)

My final model contained a significant three-way interaction among taxa, gear, and water volume. Detection of cavefishes and cave crayfishes via visual surveys decreased when water volume increased, whereas there was little relationship between water volume and detection using eDNA (Fig. 3). There was also a significant three-way interaction among taxa, gear, and substrate. Detection of both cavefishes and crayfishes was greatest for visual surveys when sampling units were classified by coarse rather than fine substrates. Similarly, detection of cavefishes was greatest using eDNA surveys in sampling units classified by fine rather than coarse substrates. Alternatively, eDNA detection of cave crayfishes was greatest in habitats classified by coarse rather than fine substrates. Water turbidity affected detection of cavefishes and crayfishes differently. As expected,

detection of cavefishes increased as water turbidity decreased. However, detection of cave crayfishes increased as turbidity increased (Fig. 4). When water was flowing detection probability of both cavefishes and cave crayfishes using eDNA increased and detection using visual surveys decreased.

My final model indicated that geology series and anthropogenic disturbance at a fine scale influenced occupancy of cavefishes and cave crayfishes (Table 8). My final occupancy model was:

$\text{logit}(\psi_i) = \alpha_0 + \alpha_1 X_{1i} + \alpha_2 X_{2i} + \alpha_3 X_{3i} + \alpha_4 X_{4i} + \alpha_5 X_{1i} X_{3i} + \alpha_6 X_{2i} X_{3i} + \alpha_7 X_{3i} X_{4i}$; where

$\text{logit}(\psi_i)$ = the occurrence probability of cavefishes and cave crayfishes at sampling unit i,

α_0 = y-intercept

α_1 = slope for Osagean Series

α_2 = slope for Smithville Dolomite

α_3 = slope for taxa

α_4 = slope for disturbance index

α_5 = slope for taxa and Osagean series interaction

α_6 = slope for taxa and Smithville Dolomite interaction

α_7 = slope for taxa and disturbance index interaction

X_1 = Osagean Series

X_2= Smithville Dolomite

X_3 = taxa

X_4 = average disturbance index

Cavefishes were more likely to occur in the Smithville Dolomite and Osagean Series geologic units compared to the category I classified as “other”. Alternatively, cave crayfishes were more likely to occur in Osagean and the category I classified as “other” relative to the Smithville Series. Anthropogenic disturbance, as represented by land use within the estimated recharge area, was not an important factor related to occupancy by cavefishes or cave crayfishes. However, cave crayfishes were negatively associated with anthropogenic disturbance within a 500-m buffer, and cavefishes showed little relationship with disturbance (Fig. 5). I completed a goodness-of-fit test for the most-complex model which indicated that my model was not over dispersed ($\hat{c}$ = 1.09).

Discussion

I found that occupancy of cavefishes and cave crayfishes was related to the site’s underlying geology series and disturbance at a fine scale. These patterns in occupancy would not be realized without accounting for variable detection which differed by gear type, changing environmental conditions, and the taxa being surveyed. This study serves as a base for eDNA surveys upon which karst researchers and managers can improve.

I found that cave crayfishes were negatively related to anthropogenic disturbance and conservation efforts may need to be directed towards protecting habitat for those species. The drivers of the higher disturbance values were urban and agricultural areas, which often result in decreased water quality and altered hydrology in surface streams (Allan et al. 1997, Paul and Meyer 2001). Surface streams are the primary recharge sources for aquifers in the Ozark Highlands ecoregion (Wicks et al. 2004), so impaired surface water will lead to poor water quality in the aquifers. The modeled relationship

between occupancy of cavefishes showed little relationship with anthropogenic disturbance, which is interesting because they are thought to have low tolerance to water quality degradation due to human changes (Graening et al. 2010). In contrast, we found that cave crayfishes had a strong negative relationship with anthropogenic disturbance. These results suggest focusing efforts to minimize disturbances near karst features would benefit cave crayfishes, many of which are federally threatened or endangered (U.S. Fish and Wildlife Service 2019). Some of the sites where cave crayfishes were observed are located just beyond the city limits of Neosho, Missouri, suggesting urban development may put these populations at risk of further decline.

The geology series associated with my sites also influenced occupancy of cavefishes and cave crayfishes, which is likely due to groundwater connectivity and water chemistry. I included the geology series Smithville Dolomite (i.e., the Salem Plateau groundwater region), Osagean Series (i.e., the Springfield Plateau groundwater region), and "other" (i.e., 2 isolated geology units within a larger region of Osagean Series) in my model. The underlying geology of a site controls the groundwater connection to other locations, which can affect stygobiont distributions (Noltie and Wicks 2001). The geology of the Salem Plateau suggests that suitable cavefish habitat lies deep beneath the surface, whereas cavefish are confined near the surface in the Springfield Plateau (Noltie and Wicks 2001). I detected *T. rosae* in both the Salem and Springfield plateaus and it would be expected that the extreme difference in geology would limit species to a region. However, these results may be explained by false absences because I detected *T. rosae* DNA in caves well outside of its known range. It is strange that I

detected cavefish DNA in McDonald County, which was a site categorized as "other" geology. The karst layer in McDonald County is absent, or extremely thin, which may exclude cavefish (Noltie and Wicks 2001). I never visually observed cavefishes within McDonald County and the Salem Plateau, suggesting that occurrence should be confirmed via alternative sampling methods. Cave crayfishes, however, have been visually observed within both isolated geology pockets (Graening et al. 2006b), and I encountered a species of cave crayfish in only one area of the Salem Plateau. This area of the Salem Plateau is hypothesized to be an isolated groundwater system (Hobbs III 2001), suggesting that cave crayfishes may require less groundwater connection than cavefishes. The underlying geology also controls the water chemistry (Hynes 1975), which plays a role in structuring the distributions of aquatic organisms (Neff and Jackson 2012). The water comprises mostly calcium bicarbonate in both the Springfield and Salem plateaus, although there are some minor differences among and within each region (Adamski 2000, Berndt et al. 2005) that may play a role in structuring the distributions of cavefishes and cave crayfishes.

Detection of eDNA can depend on the target organism and associated density. Detection for cavefishes was typically higher than for cave crayfishes, but under some environmental conditions, crayfishes were easier to detect. Although some of the discrepancy in detection between cavefishes and cave crayfishes can be explained by the amount of genetic data available, physiological and behavioral differences may also play a role. For example, fish have a slime coast and release more DNA in the environment than crayfish that have a hard exoskeleton (Tréguier et al. 2014); thus, making it easier to

detect fishes. However, when turbidity increased, I found detection was higher for cave crayfishes than cavefishes, which may be due to behavioral responses. It is difficult to speculate as to what behavioral responses caused the difference in detection, but organism behavior can influence detection of eDNA (e.g., spawning activity, de Souza et al. 2016, Erickson et al. 2016). The abundance/biomass of the target organism also relates 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, target organism density can also be poorly related to detection (Rice et al. 2018). I obtained false absences for *C. subterraneus* within sampling units 17.1 and 17.2, but historic maximum counts are lower in that cave compared to similar caves within the same county where I was able to detect *C. tartarus* via eDNA (i.e., 4 vs. 17 individuals; Graening and Fenolio 2005; Graening et al. 2006a). Further, within sampling units 15.1–15.3 and 16.1–16.3, I was unable to detect *T. eigenmanni* at sampling units where I only observed 1 fish across all surveys, but I detected them at sampling units where multiple individuals were observed.

The transport of eDNA in the environment can also influence detection. In surface waters, eDNA flows downstream (up to 12.3 km; Deiner and Altermatt 2014) and can settle vertically (Turner et al. 2015). For example, Asian carp DNA was detected upstream of a fish barrier near the Great Lakes (Jerde et al. 2011), but flow reversals and not fish presence were provided as the explanation (Song et al. 2017). In karst environment water flows in many directions because it is controlled by gravity and topography (Aley and Kirkland 2012) making it difficult to understand how eDNA

moves through the environment. For example, I detected *O. stygocaneyi* DNA in sampling unit 20.1, but it has never been visually confirmed at that location. I hypothesize that *O. stygocaneyi* may not live in that cave, but its DNA is present due to groundwater shared among systems during particularly wet periods (i.e., sampling unit 10.1 is located just downslope of sampling unit 20.1). Transport can further be complicated by the retention of eDNA in the environment. Environmental DNA persists across various time periods after a species has been removed depending on the environment: up to 25 d in experimental ponds (Dejean et al. 2011), in terrestrial soil for at least 6 y (Andersen et al. 2012), and in cave soil for thousands of years (Hofreiter et al. 2006). 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 of DNA. My detection model indicated that flowing water increased detection via eDNA, which would be expected because some flow would mix and transport eDNA that had been held in the soil or deeper groundwater, but the retention time is unknown.

Substrate also influenced detection of cavefishes and cave crayfishes. The substrate controls the chemicals and suspended particles found in the water, which can influence detection by inhibiting PCR reactions. Zymo OneStep PCR Inhibitor Removal columns (D6030, Zymo Research, Irvine, CA) and dilution of samples can reduce or remove inhibitions, but using these techniques can reduce the concentration of DNA in a sample, which may result in false negatives (Goldberg et al. 2016). Instead, I used

Environmental Master Mix 2.0 (C4396838, Thermo Fisher Scientific, Waltham, MA) because it is designed to work when inhibitors are present without the risk of diluting DNA. The Environmental Master Mix 2.0 seemed to effectively remove inhibitors because spiked environmental samples had the same quantification cycle as spiked negative controls (Goldberg et al. 2016).

I found that false negatives of cave crayfishes were often related to the presence of pseudogenes in some species' DNA and the lack of genetic data. Pseudogenes are mitochondrial genes that have moved into the nucleus, become nonfunctional, and therefore acquire mutations; thus, making it difficult or impossible to determine species when pseudogenes are co-amplified with the target mitochondrial gene (Buhay 2009). For example, pseudogenes were present in the DNA of *O. stygocaneyi*, which resulted in non-specific binding of the primers and probes and lower detection. Genetic techniques such as cloning, RT-PCR, long PCR, mtDNA enrichment, sequencing mitochondrial rich tissues, and targeting different genes during the eDNA analysis can all assist in isolating the actual mitochondrial gene, but those techniques all have associated difficulties (e.g., expense and technicality; Song et al. 2008, Buhay 2009). Covering the genetic variation of the population is critical when designing assays because assays need to match the species and not amplify non-target individuals (Furlan et al. 2015). For example, I had access to 23 sequences for *T. rosae*, 21 sequences for *T. eigenmanni*, and 8 for *C. tartarus*, which provides genetic material representing much of the known distribution of these species. The assays I developed for the aforementioned species worked well. I had 7 *C. setosus* DNA sequences, but because it has a broad distribution, more samples of

genetic material would be necessary to adequately capture the specie's genetic variation (Niemiller et al. 2018). We also do not have a comprehensive understanding of the genetic variation and species designations among cave crayfish populations. *Cambarus setosus* individuals that were collected from opposite ends of their range had a 6% difference in their genes. Further, an individual hypothesized to be *C. setosus* based on collection location, more closely matched a genetic sequence from *C. zophonastes*. We must first isolate the true CO1 gene from the pseudogene and understand intra- and inter-population levels of DNA sequence variation of the study species before a completely successful eDNA assay can be developed.

There are many options for how to conduct eDNA surveys (Rees et al. 2014); however, my protocol can serve as a starting point for those working in karst environments. Detection using an eDNA approach varies with environmental conditions and should be accounted for in a biomonitoring design. Additionally, it may be advantageous to try to improve detection of genetic material. Modifying the water collection protocol may improve detection. For example, collecting subsamples of water from multiple locations within a sampling unit, then pooling subsamples (Piaggio et al. 2014), or collecting greater volumes of water may improve detection (Schultz and Lance 2015). I used a Qiagen DNeasy® Blood and Tissue kit to extract DNA because it was relatively simple to use and recovered adequate amounts of DNA; however, other extraction methods may yield more DNA (Amberg et al. 2015, Renshaw et al. 2015). Future studies may also try different amplification protocols for eDNA to improve detection. For example, next generation sequencing can allow for multiple species to be

detected at once (i.e., metacommunity analysis; Deiner et al. 2016) and droplet digital PCR can detect lower concentrations of DNA in some cases (Doi et al. 2015). It will take additional time and effort to improve eDNA protocols.

Environmental DNA is a useful tool; however, it should not be viewed as a replacement for traditional surveys in karst environments, especially for cave crayfishes. Environmental DNA is a viable option for sampling for cavefishes from locations that provide access to groundwater but cannot be physically accessed easily (i.e., springs, wells, and flooded caves). In fact, I detected cavefishes' DNA in locations where they have not been previously identified (i.e., McDonald and Ozark counties). However, these detections could be false positives or the results of DNA transported through the groundwater; so it would be prudent to confirm presence via visual surveys. Follow up survey efforts at these locations should be relatively intense given the realization that visual surveys are also only a small snapshot of the underground environment. Environmental DNA, however, may not be sensitive enough to detect organisms if they are found deep within the groundwater or cave systems, preventing sampling at the cave entrance. Therefore, eDNA surveys are not completely non-invasive to cave or spring systems, but still less so than visual surveys that require intensive searching for organisms. The life history and ecological data gained from traditional surveys provide important information necessary for developing conservation strategies and are still necessary (see Chapter 3). Future efforts to improve the applicability of eDNA surveys should focus on understanding DNA movement through karst environments, evaluating

of genetic diversity among the Ozark cave crayfishes, isolating the actual CO1 (or other) gene of cave crayfishes, and working to improve the current protocol.

Tables

Table 1. I measured environmental covariates at each sampling unit to model detection probability of cavefishes and cave crayfishes. Sampling units are referenced by the site number, then the sequential number of units within the site (e.g., 1.2 refers to the second sampling unit within site 1). Sampling unit 10.1 was sampled a fourth time on 5/15/2017 and a fifth time on 5/17/2017. Sampling units 3.1–3.5 and 7.1 were also sampled on 4/1/2017. Values are reported as the average across survey dates ± standard deviation.

Sampling unit	Type	County	State	Survey date 1	Survey date 2	Survey date 3	Water turbidity (NTU)	Water velocity (m/s)	Water volume (m^3)
1.1	Cave	Lawrence	MO	2/25/2017	3/9/2017	3/18/2017	0.77 ± 0.63	0	4.2 ± 0
1.2	Cave	Lawrence	MO	2/25/2017	3/9/2017	3/18/2017	1.01 ± 0.74	0	11.6 ± 0
2.1	Cave	Lawrence	MO	2/25/2017	3/9/2017	3/18/2017	1.30 ± 0.07	0	16.9 ± 0
3.1	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	1.03 ± 0.92	0.06 ± 0.06	104.3 ± 36.8
3.2	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	1.09 ± 1.29	0.10 ± 0.12	12.4 ± 4.9
3.3	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	1.18 ± 1.10	0.05 ± 0.06	18.4 ± 0
3.4	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	1.19 ± 1.03	0.08 ± 0.10	17.3 ± 14.1
3.5	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	1.24 ± 0.91	0.02 ± 0.02	26.2 ± 9.2

4.1	Cave	Delaware	OK	2/21/2017	3/10/2017	3/21/2017	0.55 ± 0.07	0.02 ± 0.01	358.0 ± 0
4.2	Cave	Delaware	OK	2/21/2017	3/10/2017	3/21/2017	0.60 ± 0.02	0.02 ± 0.02	504.8 ± 0
4.3	Cave	Delaware	OK	2/21/2017	3/10/2017	3/21/2017	0.66 ± 0.00	0.02 ± 0	96.5 ± 0
4.4	Cave	Delaware	OK	NA	3/10/2017	3/21/2017	0.68 ± 0.06	0.02 ± 0.01	143.6 ± 0
5.1	Cave	Benton	AR	2/22/2017	3/1/2017	3/7/2017	0.41 ± 0.20	0.01 ± 0	1.3 ± 0
5.2	Cave	Benton	AR	2/22/2017	3/1/2017	3/7/2017	0.79 ± 0.88	0.15 ± 0	17.3 ± 0
5.3	Cave	Benton	AR	2/22/2017	3/1/2017	3/7/2017	1.05 ± 0.57	0.12 ± 0	47.5 ± 0
6.1	Cave	Benton	AR	2/22/2017	3/1/2017	3/7/2017	1.55 ± 0.86	0	73.8 ± 0
6.2	Cave	Benton	AR	2/22/2017	3/1/2017	3/7/2017	2.33 ± .033	< 0.01 ± 0.01	1.5 ± 0
7.1	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	0.85 ± 0.42	0.01 ± 0.01	5.6 ± 0
8.1	Cave	Christian	MO	2/27/2017	3/9/2017	3/18/2017	0.37 ± 0.04	0.03 ± 0	19.2 ± 0
9.1	Cave	Taney	MO	2/28/2017	4/12/2017	5/17/2017	1.29 ± 0.85	0.06 ± 0.05	114.1 ± 6.9
9.2	Cave	Taney	MO	2/28/2017	4/12/2017	5/17/2017	1.15 ± 0.40	0.24 ± 0.12	7.2 ± 1.1
9.3	Cave	Taney	MO	2/28/2017	4/12/2017	5/17/2017	1.31 ± 0.72	0.07 ± 0.06	53.2 ± 9.3
10.1	Cave	Ozark	MO	3/1/2017	4/12/2017	4/25/2017	15.57 ± 3.61	0	368.7 ± 327.2
11.1	Cave	McDonald	MO	3/8/2017	3/17/2017	3/31/2017	1.08 ± 0.65	0	1.5 ± 0

11.2	Cave	McDonald	MO	3/8/2017	3/17/2017	3/31/2017	1.64 ± 1.11	0	37.5 ± 0
12.1	Cave	McDonald	MO	3/17/2017	3/31/2017	4/6/2017	3.05 ± 1.13	0	0.6 ± 0
13.1	Cave	Ottawa	MO	3/17/2017	3/29/2017	4/5/2017	3.68 ± 3.05	0.04 ± 0.02	1.0 ± 0.2
14.1	Cave	Ottawa	OK	3/19/2017	3/29/2017	4/5/2017	1.9 ± 0.94	0.11 ± 0.08	4.1 ± 2.0
14.2	Cave	Ottawa	OK	3/19/2017	3/29/2017	4/5/2017	2.97 ± 2.44	0.15 ± 0.14	4.8 ± 1.9
14.3	Cave	Ottawa	OK	3/19/2017	3/29/2017	4/5/2017	3.56 ± 2.58	0.10 ± 0.07	11.1 ± 2.5
15.1	Cave	Camden	MO	3/25/2017	4/15/2017	5/13/2017	2.11 ± 1.90	0	3.9 ± 1.5
15.2	Cave	Camden	MO	3/25/2017	4/15/2017	5/13/2017	3.65 ± 4.21	0.01 ± 0	163.5 ± 52.4
15.3	Cave	Camden	MO	3/25/2017	4/15/2017	5/13/2017	4.43 ± 3.57	0	98.7 ± 32.6
16.1	Cave	Camden	MO	3/25/2017	4/15/2017	5/13/2017	4.77 ± 2.68	0.27 ± 0.23	21.1 ± 5.7
16.2	Cave	Camden	MO	3/25/2017	4/15/2017	5/13/2017	3.82 ± 0.18	0.23 ± 0.16	16.4 ± 6.3
16.3	Cave	Camden	MO	3/25/2017	4/15/2017	5/13/2017	4.41 ± 4.00	0.06 ± 0.04	1.8 ± 0.9
17.1	Cave	Delaware	OK	3/30/2017	4/5/2017	4/24/2017	3.54 ± 2.47	0	33.3 ± 15.3
17.2	Cave	Delaware	OK	3/30/2017	4/5/2017	4/24/2017	3.79 ± 3.54	0	4.3 ± 6.0
18.1	Cave	Delaware	OK	4/4/2017	4/24/2017	5/19/2017	0.54 ± 0	0	693.3 ± 184.8
19.1	Cave	Delaware	OK	4/4/2017	4/24/2017	5/19/2017	3.95 ± 4.37	0	54.4 ± 25.7

20.1	Cave	Ozark	MO	4/25/2017	5/15/2017	5/17/2017	17.52 ± 3.79	0	163.3 ± 80.9
21.1	Cave	Ozark	MO	4/25/2017	NA	NA	41.50	0	3.1
22.1	Spring	Ozark	MO	3/1/2017	4/12/2017	4/25/2017	2.05 ± 0.00	0.05 ± 0.05	0.1 ± < 0.0
23.1	Spring	Newton	MO	3/8/2017	3/16/2017	3/31/2017	1.61 ± 1.39	0	150.7 ± 16.3
24.1	Spring	Newton	MO	3/16/2017	3/31/2018	4/6/2017	2.55 ± 1.21	0.08 ± 0.04	37.2 ± 24.6
25.1	Spring	Newton	MO	3/16/2017	3/31/2018	4/6/2017	3.59 ± 2.43	0.02 ± 0.03	3.4 ± 1.9
26.1	Spring	Newton	MO	3/16/2017	3/31/2018	4/6/2017	1.62 ± 1.14	0.13 ± 0.03	12.0 ± 3.5
27.1	Spring	Newton	MO	3/16/2017	3/31/2018	4/6/2017	3.38 ± 1.53	0.13 ± 0.04	24.6 ± 5.3
28.1	Spring	Taney	MO	4/13/2017	NA	NA	1.85	0	6.0
29.1	Spring	Taney	MO	4/13/2017	NA	NA	0.50	0.30	0.2
30.1	Spring	Taney	MO	2/28/2017	4/13/2017	4/26/2017	3.10 ± 4.04	0.04 ± 0.06	112.0 ± 86.7
31.1	Spring	Taney	MO	4/13/2017	4/26/2017	5/16/2017	2.79 ± 3.96	0.04 ± 0.05	1.3 ± 0.5
32.1	Spring	Taney	MO	4/13/2017	4/26/2017	5/16/2017	3.10 ± 1.95	0.40 ± 0.10	2.3 ± 2.31
33.1	Spring	Taney	MO	4/13/2017	4/26/2017	5/16/2017	4.51 ± 7.00	0.04 ± 0.05	1.0 ± 0.5
34.1	Well	Newton	MO	2/25/2017	3/8/2017	3/17/2017	0.82 ± 0.85	0	0.9 ± 0
35.1	Well	Newton	MO	2/26/2017	3/8/2017	3/17/2017	2.48 ± 2.71	0	0.2 ± 0

36.1	Well	Newton	MO	2/26/2017	3/10/2017	3/17/2017	3.26 ± 3.56	0	0.2 ± 0
37.1	Well	Newton	MO	2/26/2017	3/8/2017	3/16/2017	0.36 ± 0.22	0	1.2 ± 0
38.1	Well	Taney	MO	4/13/2017	4/26/2017	5/16/2017	99.77 ± 116.25	0	0.1 ± < 0.0
39.1	Well	Taney	MO	4/13/2017	4/26/2017	5/16/2017	14.82 ± 15.71	0	0.2 ± 0.1
40.1	Well	Lawrence	MO	5/14/2017	5/18/2017	5/20/2017	2.63 ± 1.51	0.22 ± 0.17	2.2 ± 0
41.1	Well	Newton	MO	2/25/2017	3/8/2017	3/17/2017	4.39 ± 3.68	0.08 ± 0.03	1.0 ± 0
42.1	Well	Newton	MO	5/14/2017	5/18/2017	5/20/2017	7.16 ± 6.12	0	0.2 ± 0.1

Table 2. I obtained DNA sequences for each of my study species from various sources. Tissue samples and genomic DNA of cavefishes and cave crayfishes were provided by the Missouri Department of Conservation (MDC) and United States Fish and Wildlife Service (USFWS). Some tissue samples were also collected for this study. I also downloaded DNA sequences from Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

Species	Sequences	Source
Cambarus aculabrum	1	GenBank
Cambarus setosus	7	MDC (1), this study (5), GenBank (1)
Cambarus subterraneus	8	USFWS (6), MDC (2)
Cambarus tartarus	8	USFWS (7), GenBank (1)
Orconectes stygocaneyi	5	MDC (2), this study (3)
Troglichthys rosae	23	USFWS (11), Genbank (12)
Typhlichthys eigenmanni	21	Genbank

Table 3. – I designed Taqman® assays to amplify DNA for each of my target species. 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 4. Results of the eDNA surveys for cavefishes (i.e., *Troglichthys rosae* and *Typhlichthys eigenmanni*) are reported as the number of positive replicates over the total number of replicates and the visual surveys (Vis) are the total number of cavefishes observed. Sampling units (SU) are referenced by the site number, then the sequential number of units within the site (e.g., 1.2 refers to the second sampling unit within site 1). Sampling unit 10.1 was not visually surveyed for cave crayfishes on the fifth visit.

SU	eDNA 1	eDNA 2	eDNA 3	eDNA 4	eDNA 5	Vis 1	Vis 2	Vis 3	Vis 4	Species
1.1[a]	3/6	1/6	1/6	NA	NA	2	4	3	NA	*T. rosae*
1.2[a]	1/6	2/6	0/6	NA	NA	3	3	4	NA	*T. rosae*
2.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
3.1[b]	1/6	1/6	0/6	0/6	NA	0	0	0	0	*T. rosae*
3.2	0/6	0/6	0/6	0/6	NA	0	0	0	0	*T. rosae*
3.3[b]	2/6	0/6	0/6	0/6	NA	0	0	0	0	*T. rosae*
3.4[b]	1/6	1/6	NA	0/6	NA	0	0	NA	0	*T. rosae*
3.5[b]	0/6	0/6	0/6	1/6	NA	0	0	0	0	*T. rosae*
4.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
4.2[b]	0/6	1/6	0/6	NA	NA	0	0	0	NA	*T. rosae*

4.3	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
4.4	NA	0/6	0/6	NA	NA	NA	0	0	NA	*T. rosae*
5.1[a]	4/6	4/6	1/6	NA	NA	1	0	1	NA	*T. rosae*
5.2[a]	2/6	4/6	1/6	NA	NA	0	1	0	NA	*T. rosae*
5.3[b]	2/6	1/6	2/6	NA	NA	0	0	0	NA	*T. rosae*
6.1[b]	1/6	1/6	1/6	NA	NA	0	0	0	NA	*T. rosae*
6.2[b]	0/6	0/6	3/6	NA	NA	0	0	0	NA	*T. rosae*
7.1[b]	0/6	1/6	1/6	1/6	NA	0	0	0	0	*T. rosae*
8.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
9.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
9.2	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
9.3	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
10.1[b]	1/6	1/12	1/12	1/12	1/12	0	0	0	NA	*T. rosae*
11.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
11.2[b]	1/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
12.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*

13.1[b]	1/6	2/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
14.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
14.2	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
14.3	0/3	0/3	0/6	NA	NA	0	0	0	NA	*T. rosae*
17.1[a]	2/6	0/6	1/6	NA	NA	1	1	0	NA	*T. rosae*
17.2[b]	1/6	1/6	1/6	NA	NA	0	0	0	NA	*T. rosae*
18.1[a]	0/6	0/6	3/6	NA	NA	1	NA	NA	NA	*T. rosae*
19.1[a]	6/6	0/12	6/6	NA	NA	0	0	1	NA	*T. rosae*
20.1[b]	2/6	2/6	1/6	NA	NA	0	0	0	NA	*T. rosae*
21.1[b]	1/6	NA	NA	NA	NA	0	NA	NA	NA	*T. rosae*
22.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
23.1[b]	1/6	0/6	2/6	NA	NA	0	0	0	NA	*T. rosae*
24.1	0/6	0/12	0/6	NA	NA	0	0	0	NA	*T. rosae*
25.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
26.1[b]	2/6	2/6	2/6	NA	NA	0	0	0	NA	*T. rosae*
27.1[b]	1/6	1/6	0/6	NA	NA	0	0	0	NA	*T. rosae*

28.1	0/3	NA	NA	NA	NA	0	NA	NA	NA	*T. rosae*
29.1	0/3	NA	NA	NA	NA	0	NA	NA	NA	*T. rosae*
30.1[b]	0/6	1/6	2/18	NA	NA	0	0	0	NA	*T. rosae*
31.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
32.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
33.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. rosae*
34.1[b]	1/6	0/6	1/6	NA	NA	0	0	0	NA	*T. rosae*
35.1[a]	2/6	6/6	2/3	NA	NA	0	2	1	NA	*T. rosae*
36.1[b]	4/6	0/12	0/6	NA	NA	0	0	0	NA	*T. rosae*
37.1[a]	1/6	2/6	6/6	NA	NA	1	0	1	NA	*T. rosae*
40.1	0/6	0/6	0/12	NA	NA	0	0	0	NA	*T. rosae*
41.1[a]	1/6	0/6	1/6	NA	NA	0	3	0	NA	*T. rosae*
42.1	0/6	0/6	0/12	NA	NA	0	0	0	NA	*T. rosae*
15.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. subterraneus*
15.2[c]	0/6	0/6	0/6	NA	NA	1	0	0	NA	*T. subterraneus*
15.3[c]	0/6	0/6	0/6	NA	NA	0	0	1	NA	*T. subterraneus*

16.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*T. subterraneus*
16.2[a]	0/6	1/6	0/6	NA	NA	1	1	1	NA	*T. subterraneus*
16.3[a]	3/6	1/6	4/6	NA	NA	4	2	3	NA	*T. subterraneus*

a. Sampling units positive via eDNA and visual surveys

b. Sampling units positive via eDNA only

c. Sampling units positive via visual surveys only

Table 5. Results of the eDNA surveys for cave crayfishes (i.e., *Cambarus aculabrum*, *C. setosus*, *C. subterraneus*, *C. tartarus*, and *Orconectes stygocaneyi*) are reported as the number of positive replicates over the total number of replicates, and visual surveys (Vis) are the total number observed. Sampling units (SU) are referenced by the site number, then the sequential number of units within the site (e.g., 1.2 refers to the second sampling unit within site 1). Sampling unit 10.1 was not visually surveyed for cave crayfishes on the fifth visit.

SU	eDNA 1	eDNA 2	eDNA 3	eDNA 4	eDNA 5	Vis 1	Vis 2	Vis 3	Vis 4	Species
5.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*C. aculabrum*
5.2	0/6	0/6	0/6	NA	NA	0	0	0	NA	*C. aculabrum*
5.3	0/6	0/6	0/6	NA	NA	0	0	0	NA	*C. aculabrum*
6.1[c]	0/6	0/6	0/6	NA	NA	0	1	1	NA	*C. aculabrum*
6.2[c]	0/6	0/6	0/6	NA	NA	2	1	2	NA	*C. aculabrum*
1.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
1.2	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
2.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
3.1[c]	NA	NA	NA	NA	NA	1	1	0	0	*C. setosus*
3.2[c]	NA	NA	NA	NA	NA	2	2	2	1	*C. setosus*

3.3[c]	NA	NA	NA	NA	NA	2	0	1	0	*C. setosus*
3.4	NA	NA	NA	NA	NA	0	0	NA	0	*C. setosus*
3.5[c]	NA	NA	NA	NA	NA	0	1	1	1	*C. setosus*
7.1[c]	NA	NA	NA	NA	NA	0	0	0	1	*C. setosus*
8.1[c]	NA	NA	NA	NA	NA	10	6	7	NA	*C. setosus*
11.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
11.2	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
12.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
23.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
24.1[c]	NA	NA	NA	NA	NA	0	2	0	NA	*C. setosus*
25.1[c]	NA	NA	NA	NA	NA	6	3	7	NA	*C. setosus*
26.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
27.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
34.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
35.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
36.1[c]	NA	NA	NA	NA	NA	2	1	1	NA	*C. setosus*

37.1[c]	NA	NA	NA	NA	NA	1	2	1	NA	*C. setosus*
40.1[c]	NA	NA	NA	NA	NA	1	2	2	NA	*C. setosus*
41.1[c]	NA	NA	NA	NA	NA	5	3	3	NA	*C. setosus*
42.1	NA	NA	NA	NA	NA	0	0	0	NA	*C. setosus*
17.1[c]	0/6	0/6	0/6	NA	NA	1	1	1	NA	*C. subterraneus*
17.2	0/6	0/6	0/6	NA	NA	0	0	0	NA	*C. subterraneus*
18.1[b]	0/6	1/6	0/6	NA	NA	0	NA	NA	NA	*C. tartarus*
19.1[a]	3/6	1/6	1/6	NA	NA	0	0	1	NA	*C. tartarus*
4.1[b]	2/6	2/6	0/6	NA	NA	0	0	0	NA	*C. tartarus*
4.2[b]	1/6	0/6	1/6	NA	NA	0	0	0	NA	*C. tartarus*
4.3[b]	6/6	1/6	1/6	NA	NA	0	0	0	NA	*C. tartarus*
4.4[b]	NA	0/6	1/6	NA	NA	NA	0	0	NA	*C. tartarus*
10.1[c]	NA	0/12	0/18	0/12	0/12	3	4	0	NA	*O. stygocaneyi*
20.1[b]	0/6	2/12	0/6	NA	NA	0	0	0	NA	*O. stygocaneyi*
21.1	0/12	NA	NA	NA	NA	0	NA	NA	NA	*O. stygocaneyi*
22.1	0/6	0/6	0/6	NA	NA	0	0	0	NA	*O. stygocaneyi*

9.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
9.2	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
9.3	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
13.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
14.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
14.2	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
14.3	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
15.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
15.2	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
15.3	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
16.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
16.2	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
16.3	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
27.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
28.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
29.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown

30.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
31.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown
32.1	NA	NA	NA	NA	NA	0	0	0	NA	Unknown

a. Sampling units positive via eDNA and visual surveys

b. Sampling units positive via eDNA only

c. Sampling units positive via visual surveys only

Table 6. The number of surveys and sampling units (SU) at which I visually observed cave crayfishes (i.e., *Cambarus aculabrum*, *C. setosus*, *C. subterraneus*, *C. tartarus*, and *Orconectes stygocaneyi*) and cavefishes (i.e., *Troglichthys rosae* and *Typhlichthys subterraneus*). The mean (± standard deviation) and maximum counts for cave crayfishes and cavefishes are also reported.

Species	Positive surveys	Total surveys	Positive SU	Total SU	Mean	Max
C. aculabrum	5	15	2	5	0.47 ± 0.74	2
C. setosus	30	80	12	25	1.01 ± 1.91	10
C. subterraneus	3	6	1	2	0.50 ± 0.54	1
C. tartarus	1	15	1	6	0.06 ± 0.25	1
O. stygocaneyi	2	10	1	4	0.70 ± 1.49	4
T. rosae	18	161	10	52	0.21 ± 0.70	3
T. subterraneus	8	18	4	6	0.78 ± 1.17	4

Table 7. I used single-season occupancy modeling to estimate detection probability of both cavefishes and cave crayfishes. Both species of cavefishes and all species of cave crayfishes were modeled together due to small samples sizes of some species. The detection probability estimates for each parameter is reported as the mean ± standard deviation. CI refers to the 90% confidence interval.

Parameter	Mean ± SD	CI
Detection intercept	0.43 ± 0.48	-0.39 – 1.22
Taxa-cavefishes	-0.06 ± 0.57	-0.98 – 0.89
Gear-visual	-0.23 ± 0.58	-1.15 – 0.75
Water turbidity (NTU)	0.23 ± 0.19	-0.09 – 0.54
Water velocity-flowing	0.46 ± 0.20	0.12 – 0.78
Substrate-fine	-1.55 ± 0.94	-3.10 – < 0.00
Water volume (m^3)	0.12 ± 0.34	-0.44 – 0.67
Taxa-cavefishes X gear-visual	-1.63 ± 0.73	-2.81 – -0.41
Taxa-cavefishes X water turbidity (NTU)	-0.40 ± 0.23	-0.77 – 0.02
Gear-visual X water velocity-flowing	-0.81 ± 0.27	-1.25 – -0.38
Taxa-cavefishes X substrate-fine	2.79 ± 1.03	1.10 – 4.48
Gear-visual X substrate-fine	0.73 ± 1.08	-1.02 – 2.50
Taxa-cavefishes X water volume (m^3)	-0.04 ± 0.38	-0.65 – 0.59
Gear-visual X water volume (m^3)	-1.43 ± 0.47	-2.20 – -0.66
Taxa-cavefishes X gear-visual X substrate-fine	-2.73 ± 1.23	-4.71 – -0.69
Taxa-cavefishes X gear-visual X water volume (m^3)	1.02 ± 0.53	0.12 – 1.88

Table 8. I used single-season occupancy modeling to estimate occurrence probability of both cavefishes and cave crayfishes. Both species of cavefishes and all species of cave crayfishes were modeled together due to small samples sizes of some species. The occupancy probability estimate for each parameter is reported as the mean ± standard deviation. CI refers to the 90% confidence interval.

Parameter	Mean ± SD	CI
Occurrence intercept	-0.31 ± 0.86	-1.71 – 1.11
Geology-Smithville	0.91 ± 0.97	-0.70 – 2.49
Geology-Meramecian	-2.67 ± 1.58	-5.23 – -0.10
Disturbance index	-1.89 ± 0.71	-3.01 – -0.74
Taxa-cavefishes	-1.46 ± 1.62	-3.98 – 1.21
Disturbance X taxa-cavefishes	2.21 ± 0.78	0.95 – 3.45
Geology-Meramecian X taxa-cavefishes	1.88 ± 1.73	-1.00 – 4.59
Geology-Smithville X taxa-cavefishes	4.52 ± 2.16	1.00 – 8.03

Figures

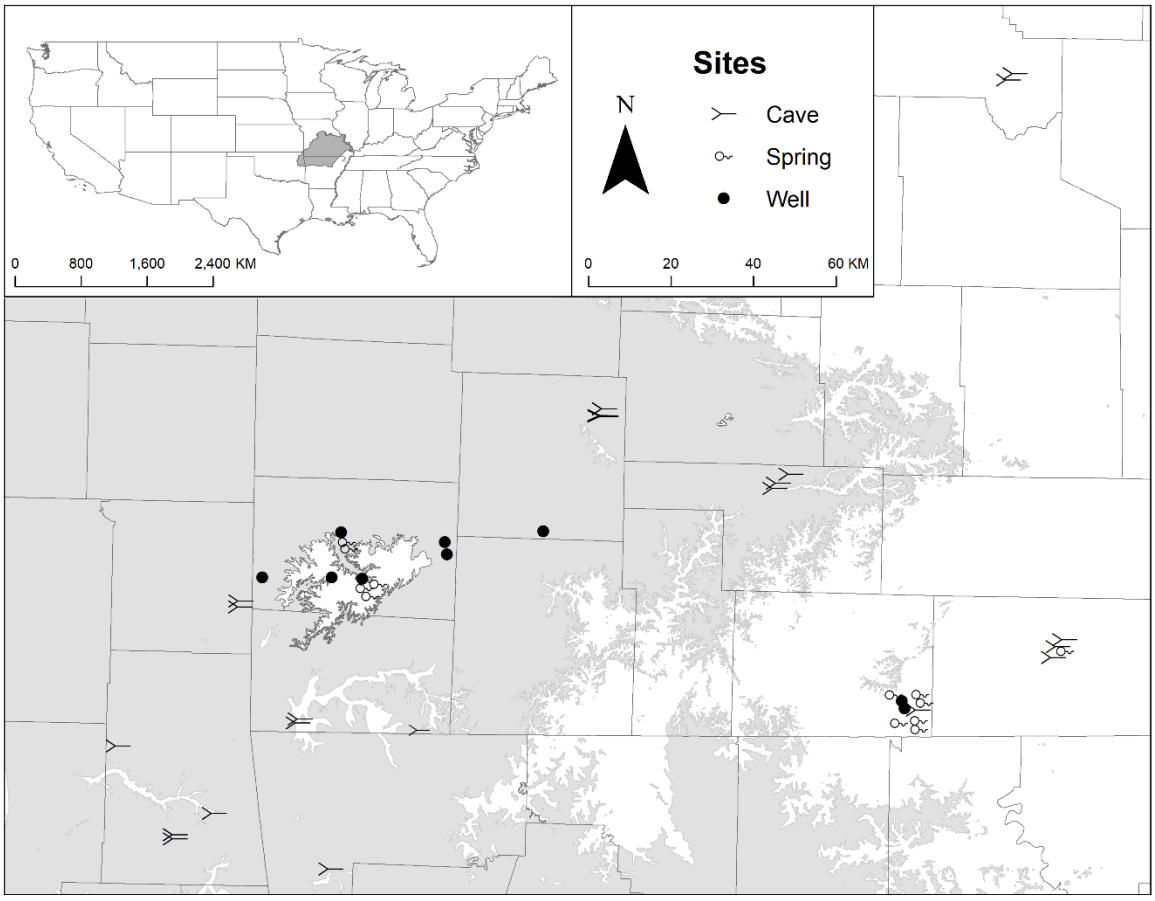

Figure 1. I conducted eDNA and visual surveys for cavefishes and cave crayfishes from 42 caves, wells, and springs across the Ozarks Highlands ecoregion (shaded in the upper inset). The gray region of the map is the Springfield Plateau and the white region represents the Salem Plateau. The cavefishes and crayfishes surveyed were: *Cambarus aculabrum*, *C. setosus*, *C. subterraneus*, *C. tartarus*, *Orconectes stygocaneyi*, *Troglichthys rosae*, and *Typhlichthys subterraneus*.

Figure 2. Filtration setup for eDNA collection. While wearing 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 at room temperature in vials of 900 µl of Longmire's buffer (Longmire et al. 1997).

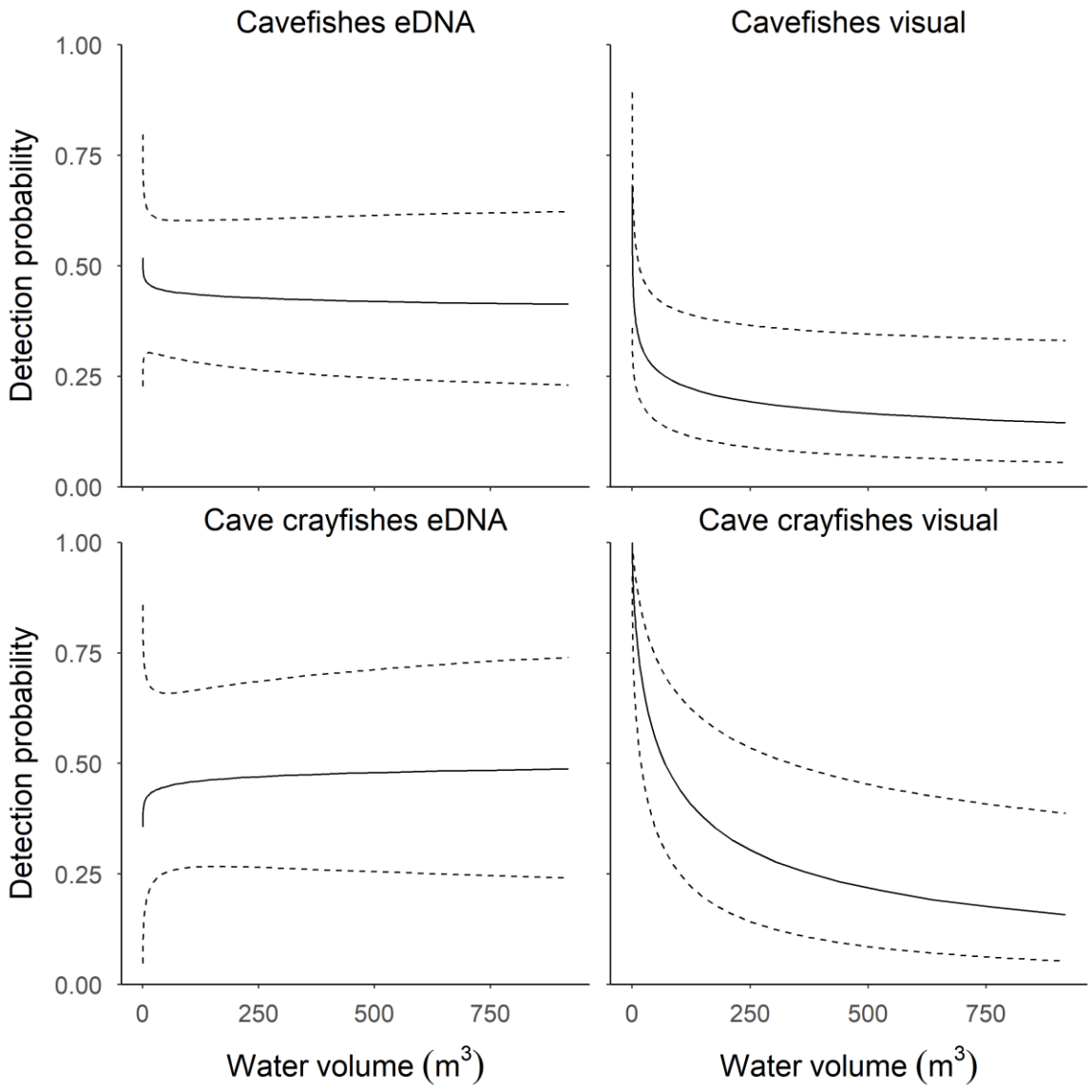

Figure 3. – The modeled relationship between water volume and detection probability of cavefishes and cave crayfishes. Solid lines depict the modeled relationship and dotted lines reflect 90% confidence intervals. Detection estimates used to create the figures were derived through the development of an occupancy model in the R package unmarked (Fiske and Chandler 2011). To represent this modeled relationship, I held water turbidity at mean levels and the categorical variable substrate and water velocity and were set to "coarse" and "1", respectively.

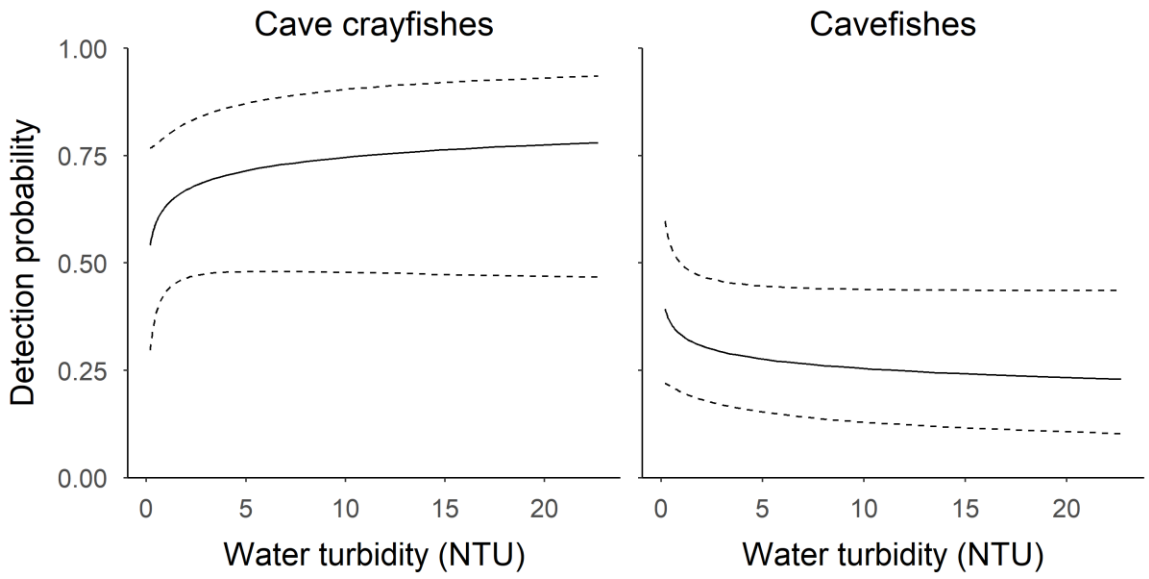

Figure 4. – The modeled relationship between water turbidity and detection probability of cavefishes and cave crayfishes. Solid lines depict the modeled relationship and dotted lines reflect 90% confidence intervals. Detection estimates used to create the figures were derived through the development of an occupancy model in the R package Unmarked (Fiske and Chandler 2011). To represent this modeled relationship, I held water turbidity at mean levels and the categorical variable substrate and water velocity and were set to "coarse" and "1", respectively.

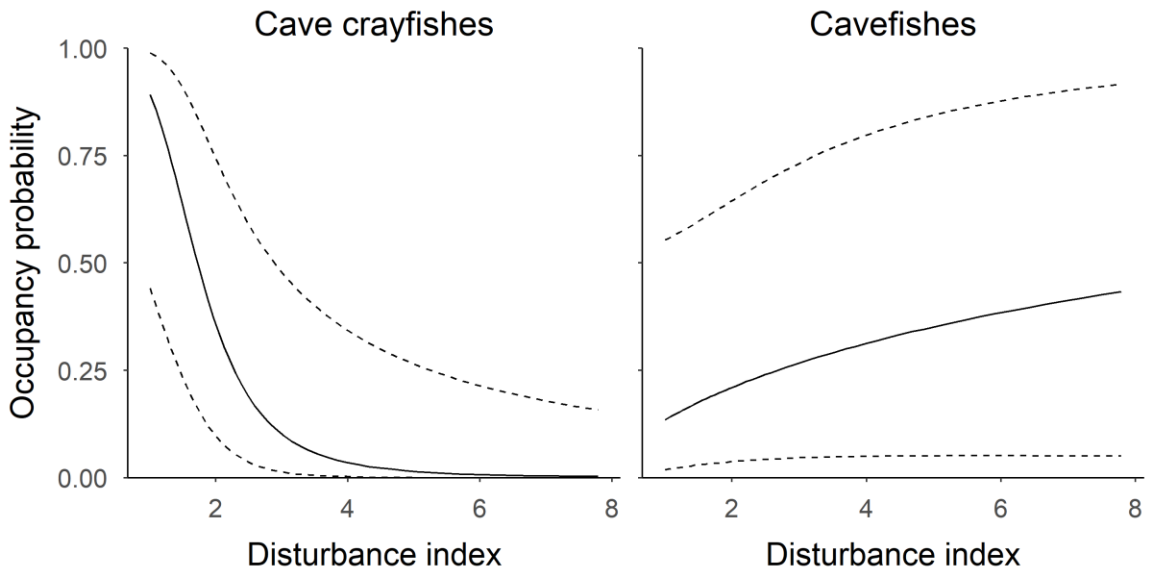

Figure 5. – The modeled relationship between anthropogenic disturbance and occupancy probability of cavefishes and cave crayfishes. Solid lines depict the modeled relationship and dotted lines reflect 90% confidence intervals. Detection estimates used to create the figures were derived through the development of an occupancy model in the R package Unmarked (Fiske and Chandler 2011). To represent this relationship, the categorical variable geology was set to "other."

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CHAPTER III

LIFE HISTORY AND ECOLOGY OF THE BRISTLY CAVE CRAYFISH *CAMBARUS SETOSUS*

Introduction

Crayfishes are ecologically important and threatened species, especially those that occupy groundwater habitats. Crayfishes are keystone species because they shape the functioning and structure of the aquatic ecosystems where they occur (Paine 1969). For example, both native and invasive crayfishes alter plant, invertebrate, fish, and amphibian populations (James et al. 2015). Almost 50% of North American crayfishes are at some risk of extinction (Taylor et al. 2007), and the loss of crayfishes may cause drastic changes to aquatic ecosystems. Cave crayfishes are especially threatened, and out of the 7 species that occur in the Ozark Highlands ecoregion, 4 are listed as endangered and 1 as threatened (Taylor et al. 2007). Major threats to Ozark Highlands cave crayfishes include groundwater pollution, over-collection, and incidental trampling (Graening et al. 2006a, 2006b).

The bristly cave crayfish *Cambarus setosus* is the most common cave crayfish of the Ozark Highlands ecoregion (Graening et al. 2006a); however, the limited ecological information available for *C. setosus* is based on qualitative observations. For example, Pflieger (1996) noted that *C. setosus* was collected most often in sand and silt habitats

and were also observed near bat guano piles, whereas Marquat (1979) found *C. setosus* in silty substrates with rock, gravel, and organic debris. *Cambarus setosus* is currently known from 48 sites (i.e., caves, wells, and springs) in Missouri and 2 sites in Arkansas with a population of at least 164 individuals (Graening et al. 2006a). The largest population of *C. setosus* is estimated as at least 47 individuals in Smallin Civil War Cave (hereafter referred to as SCWC) in Christian County, Missouri (Graening et al. 2006a). *Cambarus setosus* can reach 120-mm total length (TL), males can be reproductively active (i.e., form I) at 53-mm TL, and there is little difference in size between the sexes (Pflieger 1996). *Cambarus setosus* is currently listed as stable (Taylor et al. 2007) and vulnerable in Missouri (Missouri Department of Conservation 2009), but proactive management is important to ensure sustainable population levels persist (Graening et al. 2006a).

Effective conservation and management of crayfish populations are based on data derived from both life-history and ecological traits (Moore et al. 2013, DiStefano et al. 2016). Data are often lacking to support development of management options for crayfishes, especially for the species most at risk. For example, only 13% of crayfish species in the United States and Canada have published life-history information even though the status of 79% of those species is currently stable (Moore et al. 2013). Examples of important life-history traits include age, fecundity, and recruitment (Moore et al. 2013). These life-history traits can be used for crayfishes to predict at-risk and invasive species (Larson and Olden 2010), choose appropriate sampling approaches (Crandall 2016), and promote habitat restoration based on life-stage requirements (Dyer et al. 2016). In addition to life-history data, management decisions also benefit from an

understanding of a species' ecology. Species-habitat associations are particularly useful to help direct restoration efforts (Smith et al. 1996), control invasive crayfish (Light 2003), and determine potential reintroduction sites (Renai et al. 2006).

The objective of my third chapter is to provide quantitative information about the ecology (i.e., substrate use) and life history (i.e., age and morphological traits) of *C. setosus*. Although it is preferable to assess ecological relationships via models that account for detection probability (MacKenzie et al. 2018, see also Chapter 2), it is not always possible when using previously collected and valuable data sources (Tingley and Beissinger 2009, Lele et al. 2012). I specifically focused on determining 2 traits: age and size, because they can be used to calculate important life-history metrics (i.e., growth, mortality, and recruitment, Panfili et al. 2002). Further, validating the longevity of cave crayfishes is needed because estimates of cave crayfish longevity range from 20–100+ years (Venarksy et al. 2012).

Methods

Crayfish aging

I aged crayfish via counting bands on thin sections of the gastric mill. The gastric mill is a food grinding structure in decapod crustaceans, and it most prominently comprises the paired zygocardiac ossicles, the paired pterodcardiac ossicles, and the mesodcardiac ossicle (Fig. 1). The gastric mill has been used to obtain yearly age estimates for several species (Kilada et al. 2012), including crayfishes (Leland et al. 2015), without the same limitations of commonly used techniques (Kilada et al. 2012). Leland et al. (2015) marked redclaw crayfish *Cherax quadricarinatus* gastric mills with calcein, allowed them to grow for 1 y, and observed 1 growth band despite individuals

molting several times. Leland et al. (2105) studied parasticid crayfishes, which are subject to contrasting climate and morphologically different from the cambarid crayfishes of North America (Scholtz 2002). Differences between the 2 crayfish taxa suggest that research is needed to assess band formation on North American crayfishes. Further, certain species of crustaceans molt their gastric mill suggesting the structure cannot retain a record of annual age (Becker et al. 2018, Sheridan et al. 2018).

Due to the questions surrounding the use of the gastric mill for aging North American crayfish, I conducted 2 experiments prior to applying this technique to age *C. setosus*. I first compared gastric mill age estimates to those obtained from a length-frequency analysis and determined the ossicle that provided the most precise age estimates (see *Age estimate comparison and ossicle precision*). Results from the age comparison were promising, but suggested more work was needed to understand band formation, so I conducted a controlled laboratory trial to assess how temperature influenced band formation (see section *Laboratory trials*). I applied the knowledge gained from the first 2 experiments to age *C. setosus* from SCWC (see *Cave crayfish aging*).

Aging process. – To age each crayfish, the gastric mill was removed, mounted, and bands were counted. I cut away the carapace to expose the stomach and removed the gastric mill with forceps (Fig. 2). I placed the gastric mill in water, separated each ossicle, removed any tissue with forceps, and each ossicle was mounted separately in epoxy resin (105-B & 205-B, West System Epoxy, Bay City, MI). I sectioned ossicles to a thickness of 180–220 µm using an IsoMet™ low-speed saw (11-1280, Buehler, Lake Bluff, IL) with a diamond blade (M4D10/20MIC-N50M9-1/8, Norton, Worcester, MA).

Transverse cross sections were made on the pterocardiac and zygocardiac ossicles, whereas the mesocardiac ossicles were cut longitudinally (Fig. 3). Sections were viewed for growth bands with a light transmission microscope under 10X–40X magnification. A year of growth was defined as a wide lighter section (i.e., hypothesized period of rapid growth) followed by a thin darker section (i.e., hypothesized period of slow growth; Fig. 4). Dark bands were enumerated to estimate the age of crayfish.

Age estimate comparison and ossicle precision. – I compared gastric mill and length-frequency analysis age estimates for a common North American crayfish species. I collected 710 ringed crayfish *Faxonius neglectus neglectus* via hand netting and seining from several streams in the Ozark Highlands ecoregion. I constructed a length-frequency histogram and used the Bhattacharya method to distinguish age classes (Bhattacharya 1967) with the R package TropFishR (Mildenberger et al. 2017; Fig. 5). For a subset of 51 crayfish, I mounted gastric mill sections from multiple ossicles of the same individual on a single slide with a unique identifier. I included multiple sections from each individual crayfish because some sections were more difficult to read than others (i.e., too thin or thick). Three readers independently provided age estimates for each slide. If age estimates provided across the readers did not agree, the 3 readers examined the slides together and provided a consensus age estimate. I compared the consensus estimates from the gastric mill to the age predicted from the length-frequency analysis. In cases where the length-frequency analysis showed overlap between age classes (i.e., a 15-mm crayfish could be either age 0 or age 1), I used the age closest to the gastric mill age estimate.

I compared age estimates between each of the gastric mill structures for 25 *F. n. neglectus* to determine which provided the most precise age estimates. Each of the 5

structures (i.e., mesocardiac ossicle, paired zygocardiac ossicles, and the paired pterocardiac ossicles) were sectioned 2–5 times, and the multiple sections from each structure were mounted on 1 slide. I gave each slide a unique identifier, and I aged each slide. For each individual crayfish, I first determined the coefficient of variation (CV; Campana 2001) for each of the paired structures (i.e., to determine congruence between the paired structures). Next, I calculated the CV across the 5 age estimates for each individual crayfish: 2 estimates for each paired structure and 1 for the mesocardiac ossicle. Lastly, I averaged the 3 CVs calculated above across all crayfish examined to provide 1 estimate for the paired pterocardiac ossicle, the paired zygocardiac ossicle, and all structures combined.

Laboratory trials. – I collected juvenile *F. n. neglectus* from the Ozark Highlands ecoregion to investigate how temperature influences band formation. Crayfish were collected from Spavinaw Creek on 9 January 2018 (n = 57), 5 February 2018 (n = 324), and 16 February 2018 (n = 62). Crayfish were held for at least 2 weeks to acclimate to laboratory conditions, and then I measured CL (1.0 mm) before beginning the trials on 1 March 2018. I only included crayfish that were < 16-mm CL in the laboratory trials to ensure that all individuals were age 0 (Price and Payne 1984a).

Crayfish were held in 2 laboratories to assess the effect of thermal regime on band formation. I housed 138 crayfish in laboratory A that had a relatively stable temperature regime (i.e., mean ± SD water temperature ≈ 17 ± 1.5 °C). I also housed 138 crayfish in laboratory B that had a thermal regime that fluctuated relatively naturally (i.e., mean water temperature ≈ 25 ± 3 °C). Each laboratory held 10 crayfish each in twelve 75-L glass tanks (n = 120), and 12 crayfish in one 150-L plastic tank (n = 18). Daily care

included removing molted exoskeletons, cleaning out uneaten food, and feeding crayfish protein pellets *ad libitum*. Water quality parameters (i.e., chlorine, ammonia, and nitrite) were checked every 2 weeks, and recorded.

The gastric mill of the crayfish held in the laboratories were processed upon death or after a 1-yr period when they were humanely euthanized. I processed the gastric mills as described above (*Aging process)* and I mounted 1 section from each zygocardiac ossicle on each slide (i.e., those ossicles provided the most precise age estimates). The slides were aged independently by 2 readers, disagreements were discussed, and a consensus age was reached.

I assessed the differences in estimated crayfish age estimates between the 2 laboratories (i.e., thermal conditions). I used a two-sample *t*-test to determine if the crayfish in each laboratory had significantly different average band counts. I also used 2, one-sample *t*-tests to assess if each laboratory had average band counts that differed significantly from 1 y (i.e., the hypothesized number of bands). My data met the assumptions of equal variances but not normality; however, *t*-tests are robust to departures from normality (Heeren and D'Agostino 1987). Finally, I used a Kolmogorov-Smirnov test to determine if the age estimates from both laboratories were distributed differently, and I plotted empirical cumulative distribution functions for both labs to determine where the distributions varied. My analyses were conducted using the statistical software R (version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria) and $\alpha \leq 0.10$ was chosen *a priori* as my level of significance.

Cave crayfish aging. – *Cambarus setosus* collected from SCWC were aged via the gastric mill to determine the longevity of the species. Eight *C. setosus* were collected

that died naturally in SCWC. I aged sections from multiple ossicles because the recovered crayfish were in various states of decomposition and not all of the ossicles could be located. The best section from each zygocardiac ossicle and pterocardiac ossicle, and the 2 best sections from the mesocardiac ossicle were mounted on each slide. The slides were aged independently by 2 readers, disagreements were discussed, and a consensus age was reached.

Habitat Use

Crayfish surveys. – Visual surveys were conducted to assess habitat use and morphology of *C. setosus* within SCWC from 19 November 2006 to 27 November 2018. Each crayfish was captured, distance into the cave from the dripline (i.e., cave entrance) was recorded, and a 100-cm^2 grid was placed at the capture location to estimate used microhabitat conditions. The gender of each crayfish was noted and TL (1.0 mm), carapace length (CL; 1.0 mm), carapace width (1.0 mm), and length of both chelae (1.0 mm) were measured. Water depth (1.0 cm) was measured in the center of each occupied grid and water velocity was visually ranked as 0 = not flowing, 1 = slow flow, 2 = moderate flow, and 3 = fast flow. I combined slow (n = 29), moderate (n = 9), and fast flow (n = 1) into the category ("flowing") because there were relatively few measurements in each category when compared to not flowing (n = 117). Substrate was visually estimated as the proportion of the quadrat that was composed of (approximate particle diameter in parentheses): mud (< 0.1 mm), sand (0.01–0.2 cm), pebble (>0.2–6 cm), cobble (>6–20 cm), large rock (>20–26 cm), and bedrock (> 26 cm). I condensed the substrate estimates into 4 categories that I hypothesized were ecologically relevant based on homogeneity or mixing of fine or coarse materials (Table 1). For example,

quadrats that were 100% bedrock, sand, silt, or clay were assigned the category "bedrock/fine" because those substrates would not provide adequate shelter for a crayfish (i.e., crayfish cannot burrow into bedrock and too much fine substrate results in suffocation; Dyer et al. 2015). I visually estimated availability of each substrate category within the cave on 8 August 2017.

Statistical analysis. – A multinomial logit model was used to estimate the probability that crayfish were found in bedrock/fine, heterogeneous rock, homogenous rock, and heterogeneous fine substrates. Crayfish often select habitat based on sex (e.g., DiStefano et al. 2013), size (e.g., Dyer et al. 2016), and season (e.g., DiStefano et al. 2013). Therefore, I predicted the substrate in which crayfish were found using the variables sex, CL, season, and distance into the cave. I also included interaction terms between sex and all other variables as predictor terms in the model. A step-wise approach was used to determine the combination of predictor variables that produced the lowest Akaike information criterion adjusted for a small sample size (AICc; Burham and Anderson 2001). I assessed model fit using a chi-squared test to compare the observed probability crayfish were found in each substrate category to the probability predicted by the model (Yau et al. 2013).

I used a multiple linear regression model to predict water depth in which crayfish were located. Water depth was natural-log transformed due to a right-skewed distribution. One crayfish was found out of the water and that datum was removed due to its high influence on the model. I included the same predictor terms and selected the best model as described for the multinomial model. Model fit was visualized via a Q-Q plot and plotting the residuals against the fitted values.

Finally, I developed a generalized linear model with a binomial distribution to predict whether crayfish were more likely to be found in flowing or non-flowing water. I included the same predictor terms and selected the best model as described for the multinomial model. I assessed model fit using binned residual plots because traditional residual plots are uninformative for models with binary response variables (Gelman and Hill 2007).

I also assessed differences in morphometric characteristics between the sexes. I compared average CL between the sexes using a *t*-test. I also compared chelae size divided by CL (i.e., adjusted for overall size) between the sexes. I hypothesized that a larger difference in left and right chelae size would reflect aggressive behavior that results in the loss and partial chela regeneration. Therefore, I compared the size difference between the left and right chelae via 3 *t*-tests: 1) males only, 2) females only, and 3) all individuals in the population. The assumptions of normality and equal variances were met for all *t*-tests. My analyses were conducted using the statistical software R (version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria) and $\alpha \leq 0.10$ was chosen a priori as my level of significance.

Results

Crayfish Aging

Age estimate comparison and ossicle precision. – Gastric mill age estimates showed good congruence with length-frequency analysis estimates (Fig. 6), which suggests that bands align with annual growth. The range of individual crayfish CL included in our length-frequency analysis was 7–46 mm, whereas the individuals aged via gastric mill were 12–46 mm. The same age estimate was provided by both techniques

about half of the time (43%, 22/51); however, most (78%, 40/51) of our age estimates were no more than 7 y different (e.g., age 2 vs. age 1). In the cases where the discrepancy exceeded 1 y, the crayfish were all > 30-mm CL, indicating more disagreement in older individuals when comparing these aging techniques. Age estimates provided by the gastric mill agreed with the length-frequency analysis as follows (number in agreement/total number in the age class; percent agreement): age 0 (1/1; 100%), age 1 (1/6; 17%), age 2 (7/12; 58%), age 4 (7/12; 58%), age 5 (4/12; 33%), and age 6 (2/8; 25%).

Age estimates obtained from the paired zygocardiac ossicles were the most precise. Average CV was 27% across all ossicles but decreased to 20% when the pterocardiac ossicles were excluded from the calculation. Estimating age using the mesocardiac ossicle often (10 of 14 crayfish) resulted in estimates that were lower than suggested using the zygocardiac ossicle. For 19 crayfish, we obtained age estimates from both zygocardiac ossicles and there was generally good agreement between the 2 structures (CV = 14%). In 2 cases, however, the age estimates from the 2 zygocardiac ossicles were more than two-fold different (8 vs. 4 years and 6 vs. 3 years). We were only able to obtain age estimates for the paired pterocardiac ossicles from 11 crayfish due to difficulties processing the structure (i.e., obtaining the correct thickness on the smallest structure). The CV was 24% for the 11 individuals examined, and for 4 individuals, age estimates were more than 2 years different.

Laboratory trials. – Crayfish grew and molted in both laboratories across the 21–305 days that they were held in captivity. My study began with 138 crayfish of similar sizes in both laboratories A (9.0–16.0 mm, mean = 12.7 ± 1.9 mm) and B (9.0–16.0 mm,

mean = 13.2 ± 1.8 mm). I recovered 92 crayfish from laboratory A (CL range = 10.9–33.9 mm; mean CL = 20.1 ± 4.9 mm) and 87 crayfish from laboratory B (CL range = 10.0–38.0 mm; mean CL = 21.7 ± 5.7 mm). The missing crayfish were presumed to be cannibalized. Crayfish in both laboratories grew quickly from March through August (Fig. 7) due to repeated molting (i.e., average of ≈ 2 molts per tank each month). I found 7 of the recovered crayfish were undergoing molting and had 2 set of gastric mills.

I discovered that gastric mill bands may be at least partially controlled by thermal conditions. Mean age estimates of crayfish from laboratory A (0.97 ± 0.94 years) and laboratory B (1.38 ± 1.07) were significantly different (t_{170} = -2.49, p = 0.01). Crayfish ages in laboratory A did not differ significantly from 1 (t_{91} = -0.11, p = 0.91). Alternatively, mean crayfish age estimates from laboratory B were greater than 1 (t_{79} = 3.16, p < 0.01). The range of age estimates for crayfish from both laboratories was 0–4 y, and the age distributions from the two laboratories were different (D_{170} = 0.22, p = 0.04). Laboratory A tended to have more age-1 crayfish than laboratory B (Fig. 8).

Cave crayfish aging. – I used gastric mill sections to age 8 *C. setosus* that were collected from SCWC. The carapace length of the aged crayfish ranged from 13.0–31.0 mm (mean = 19.1 ± 5.7 mm) for the recovered *C. setosus*. None of the crayfish displayed yearly growth bands.

Habitat Use

Crayfish surveys. – Male and female *C. setosus* of various CLs (range = 3.0–43.0 mm, mean = 22.5 ± 7.0 mm) were captured in all seasons from a variety of habitats in SCWC. Location of capture ranged 23–420 m from the dripline. Both male (n = 159, mean CL = 22.1 ± 6.1 mm) and female (n = 131, mean CL = 24.81 ± 6.9 mm) crayfish

were collected during all seasons. Reproductively-active males (i.e., Form 1) were collected during the spring (n = 9), summer (n = 5), autumn (n = 5), and winter (n = 7) seasons, whereas females carrying eggs were captured only during the spring (n = 2) and autumn (n = 5) seasons. Crayfish were sampled from a variety of microhabitats characterized by different substrate compositions: bedrock/fine substrate (n = 16), heterogeneous fine (n = 21), heterogeneous rock (n = 21), and homogenous rock (n = 104). The approximate proportion of each substrate found within the cave was: bedrock/fine (15%), heterogeneous rock (15%), heterogeneous fine (5%), and homogenous rock (65%). Crayfish were found in 0–106 cm of water (mean = 20.7 ± 18.0 cm) and mostly in non-flowing water (n = 117) compared to flowing (n = 39).

Statistical analysis. – Cave crayfish in SCWC were found in varying substrates, water depths, and water velocities (Tables 2–4). Male *C. setosus* were more likely to use homogenous and heterogeneous rock substrates (Fig. 9), shallower water depths, and non-flowing water compared to females. However, near the cave entrance the probability of females occurring in bedrock/fine was much greater and males were more likely to use deeper and flowing water. Crayfish were found in shallower water depths more often in autumn compared to spring. Similarly, crayfish were less likely to use flowing water in the summer and autumn compared to the spring. Results of the chi-square goodness of fit test indicated multinomial model fit was appropriate ($X^2_9 = 12$, $p = 0.21$). The Q-Q plot and the residual plot showed no concerning trends, suggesting adequate linear model fit. Finally, the binned residual plot indicated good generalized linear model fit because 95% of the binned residual were contained in theoretical error bounds, and the plot did not reveal any concerning trends.

I found morphological differences between male and female *C. setosus*. Female crayfish were significantly larger than males ($t_{248} = 3.30$, $p < 0.01$). Among all individuals sampled, there was no significant difference between chelae size ($t_{263} = 1.17$, $p = 0.24$). Likewise, right and left chelae were not significantly different for males ($t_{136} = 1.50$, $p = 0.14$) or females ($t_{112} = -0.41$, $p = 0.68$).

Discussion

I found that counting gastric mill bands may be useful for aging some surface crayfishes in older year classes; however, more work needs to be completed before cave crayfishes and younger individuals can reliably be aged. I also found that male and female *C. setosus* vary morphologically and have different ecological relationships (i.e., they were found in varying substrates, water depths, and water velocities). My results provide life history and ecological data that will help develop conservation strategies for *C. setosus*.

Crayfish aging

The factors governing band formation on the gastric mill are unclear but may relate to seasonal fluctuations. Yearly cycles (i.e., temperature and food variation) are thought to control annual ring formation on fish hard structures (Panfili et al. 2002) and could explain band formation on gastric mills. I observed that gastric mill bands in surface species appeared to reflect annual age for older individuals. In contrast, the cave crayfishes from SCWC that I aged did not display gastric mill bands. Caves are generally thought to be stable compared to surface environments (i.e., without major changes in temperature and food; Simon 2012). In SCWC, for example, water temperature fluctuations are limited, and the food supply is relatively small because large bat colonies

are absent (D. Ashley, Missouri Western State University, personal communication). Other cave environments may have seasonal environmental fluctuations. For example, Mouser et al. (2009) found that epigean *F. n. neglectus* in caves still displayed bands that seemed to reflect annual conditions; however, crayfish movement or seasonal food fluctuations in food derived from maternal colonies of gray bats *Myotis grisescens* may be contributing factors. Using the gastric mill to age cave crayfishes would be valuable because it is difficult to determine the longevity of cave crayfishes through traditional methods (Venarsky et al. 2012). However, there are many factors we do not understand in relation to gastric mill band formation and my study begins to address those knowledge gaps.

Results of my laboratory trials show that temperature fluctuations are a factor that contributes to the formation of growth bands on crayfish gastric mills, and molting does not control band formation. I multiple found bands on the gastric mills of age-1 crayfish although the mean number of bands examined from thermally-stable environments did not differ from 1. These bands could be secondary growth zones, which have been observed in fishes, especially for age-0 individuals. The secondary growth zones observed in fishes are hypothesized to be formed by temperature, food intake, and developmental transitions (Panfili et al. 2002). Previous crustacean aging using the gastric mill indicated that the structure was molted (Becker et al. 2018, Sheridan et al. 2018, my study), which would most easily explain the formation of gastric mill bands. The bands I quantified in my laboratory crayfish could not reflect molting alone because I aged many crayfish to be age 0, and I regularly observed molting as the crayfish grew. Further, every crayfish in my study likely molted at least once during the trials and

probably multiple times before capture (i.e., *F. neglectus* molt approximately 8 times in their first year, Price and Payne 1984b). Temperature fluctuations are a driver of band formation, but band formation may also relate to other factors (e.g., food consumption) that I did not measure in my study.

Habitat use

Cambarus setosus reproduction occurred primarily in the spring and autumn in SCWC; however, reproduction may occur all year. Although my sample size was limited, like many cave studies, I found reproductively-active males (i.e., Form I) occupied the cave during all seasons. Many species of lotic crayfish at this latitude appear to molt twice annually for reproductive purposes and generally reproduce during the spring (Pflieger 1996). Less is known about the reproductive habits of cave crayfishes; however, it appears they follow similar, although not identical, patterns as surface crayfish. Mouser et al. (2019) found that surface crayfish reproduced all year in a cave; however, most reproduction did occur in the spring. Jegla (1966) observed that *O. pellucidus inermis* followed reproductive patterns similar to surface species; however, flooding events shifted reproductive timing (Jegla and Poulson 1970).

Male and female *C. setosus* are found in different habitats depending on the location within the cave. Male crayfish tended to use homogenous and heterogeneous rock substrates, slower water velocities, and shallower water more often than females. The observed differences in habitat use between the genders could be explained by more dominant males excluding females from ideal habitats (Fero and Moore 2008). However, chelae size differences did not suggest aggressive behavior in the population, but other forms of behavior (e.g., pheromone releases; Schneider et al. 1999) may cause females to

avoid males. Females were typically larger than male crayfish and body size can influence cover use (Streissl and Hödl 2002, Dyer et al. 2016). For example, male *C. setosus* may avoid faster water because smaller crayfish are more likely to be swept downstream (Hobbs III 1978, Caine et al. 1978). Female crayfish could be associated with bedrock because they are too large to take refuge under other substrates and crevices in the bedrock may provide cover.

I also found that season affected the water depth and water velocity associated with occupied habitat. Crayfish are more likely to be found in non-flowing and shallow water during autumn. This pattern may relate to reproductive requirements because autumn is when *C. setosus* typically reproduce. Food availability may be greater in shallower riffle habitats (e.g., Flinders and Magoulick 2007), which would be especially important when crayfish require additional energy for gamete production.

My results reflect the life history and basic ecology of a single population of *C. setosus* but adds to the growing body of literature necessary to conserve cave crayfishes. It is important to recognize that populations have genetic differences (see Chapter 2), which may translate into phenotypic or life-history differences. Future studies should investigate cave crayfish population traits to assess generalizations that can be extended to other cave systems. I found the males and females can reproduce when they reach 20-mm CL, and these data can be used in population models to predict changes when different management options are applied (e.g., Crouse et al. 1987). Further, my results support that autumn (i.e., the time most reproduction is occurring) may be a useful time period for monitoring these populations using eDNA because reproduction may increase eDNA in the environment (de Souza et al. 2016; see also Chapter 2). Conservation efforts

focused on maintaining the natural habitat within the cave are advantageous given the crayfish appear to use many habitat components relative to their availability.

Tables

Table 1. At the crayfish capture location in Smallin Civil War Cave, the proportion of substrate was estimated within a 100-cm^2 grid. I condensed the substrate proportions into ecologically-relevant categories for modeling purposes.

Category	Description
Homogenous rock	Substrate that was not bedrock, but larger than sand
Heterogeneous rock	Substrate larger than sand with bedrock present
Heterogeneous fine	Mix of any substrate type with sand, silt, or clay present
Bedrock or fine	100% bedrock, or 100% sand, silt, or clay

Table 2. *Results* of the multinomial model used to predict the substrate where bristly cave crayfish *Cambarus setosus* wehre found in Smallin Civil War Cave. SE = standard error, HEF = heterogenous fine, HER = heterogenous rock, HOR = homogenous rock

Parameter	Mean	SE
HEF-intercept	-1.66	1.40
HER-intercept	-7.83	0.67
HOR-intercept	-1.60	1.55
HEF-female	-0.12	1.05
HER-female	7.82	0.50
HOR-female	2.30	1.29
HEF-male	0.88	1.19
HER-male	9.58	0.53
HOR-male	3.80	1.38
HEF-meter	0.01	5.25×10^{-3}
HER-meter	-2.05×10^{-3}	6.31×10^{-3}
HOR-meter	4.62×10^{-3}	4.88×10^{-3}

Table 3. Results of the mulitple linear regression model used to predict the association between bristly cave cryafish *Cambarus setosus* and water depth. SE = standard error

Parameter	Mean	SE	*p*
Intercept	2.51	0.28	3.29×10^{-15}
Summer	0.21	0.18	0.24
Autumn	-0.42	0.22	0.07
Winter	-0.29	0.21	0.18
Male	0.53	0.36	0.14
Meter	1.67×10^{-3}	1.45×10^{-3}	0.25
Male X meter	-3.82×10^{-3}	1.92×10^{-3}	0.05

Table 4. Results of the generalized linear model used to predict the association between bristly cave cryafish *Cambarus setosus* and flowing or not flowing water. SE = standard error

Parameter	Mean	SE	*p*
Intercept	-1.11	0.72	0.13
Male	-0.49	0.98	0.62
Meter	4.9×10^{-3}	3.71×10^{-3}	0.18
Summer	-1.43	0.59	0.02
Autumn	-2.33	1.07	0.03
Winter	0.63	0.47	0.19
Male X meter	7.77×10^{-3}	5.38×10^{-3}	0.15

Figures

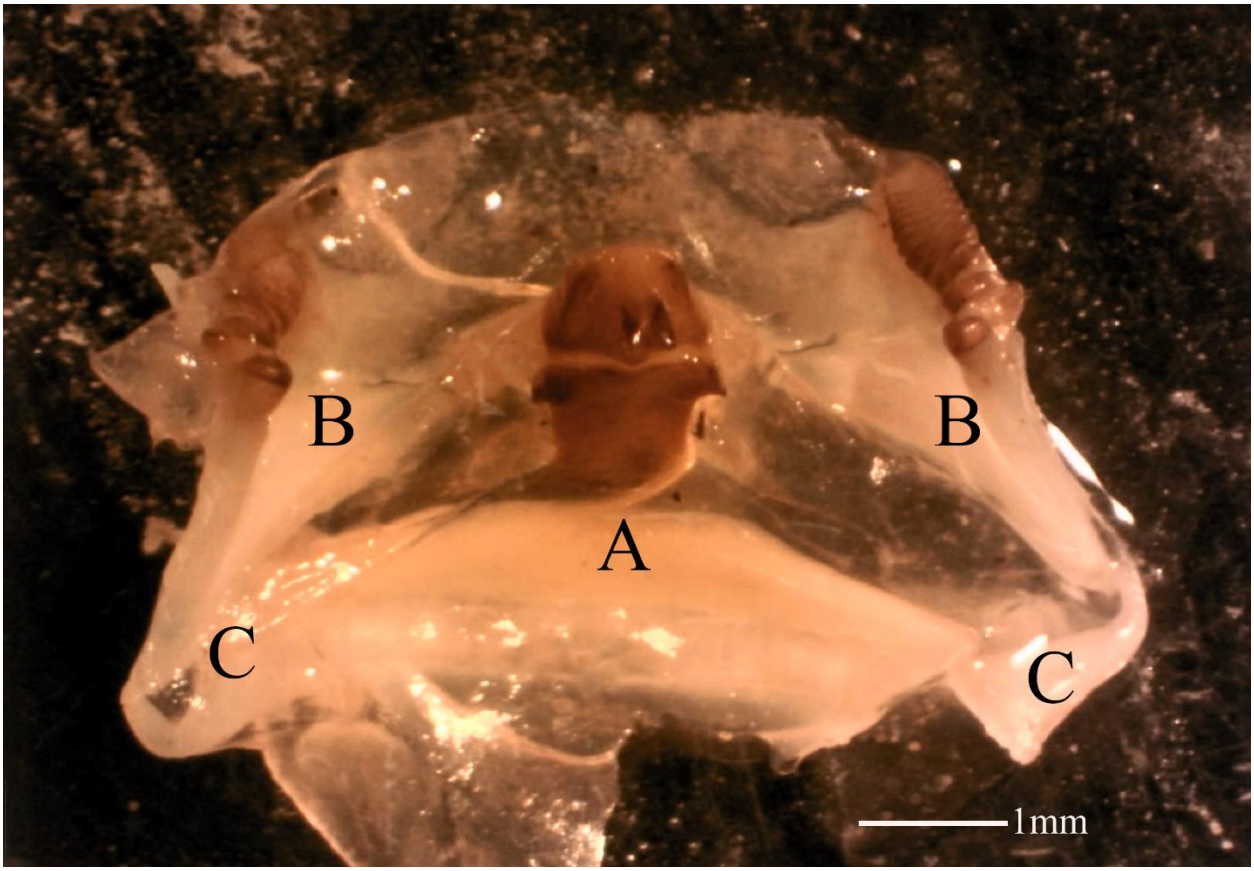

Figure 1. The gastric mill is located in the stomach of decapod crustaceans and is used for grinding food. The gastric mill comprise 5 prominent structures: the mesocardiac ossicle (A), the paried zygocardiac ossicles (B), and the paried pterocardiac ossicles (C).

Figure 2. The location of the gastric mill within a crayfish is shown by the **X**. I extracted the gastric mill by cutting away the carapace and then carefully removed it using forceps. Gastric mills were then placed in water, separated, and the remaining tissue was removed with forceps.

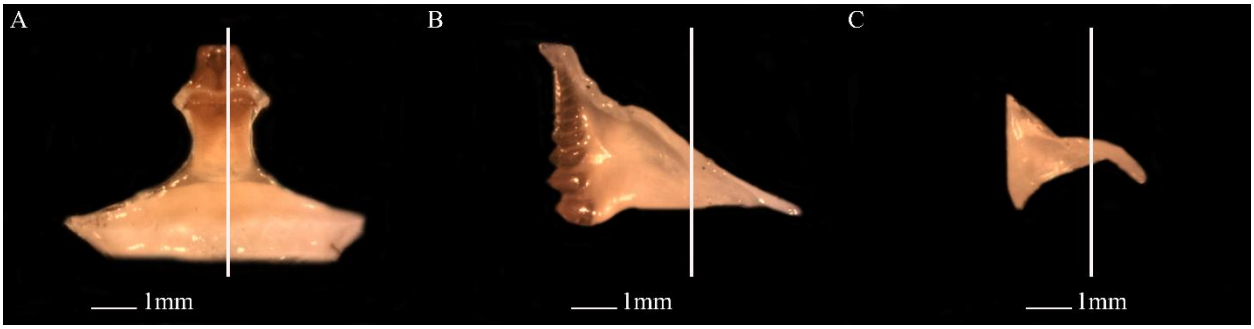

Figure 3. The approximate location of the sections taken from the gastric mill. The mesocardiac ossicles were sectioned longitudinally (**A**), and the zygocardiac (**B**) and pterocardiac ossicles were sectioned transversely (**C**).

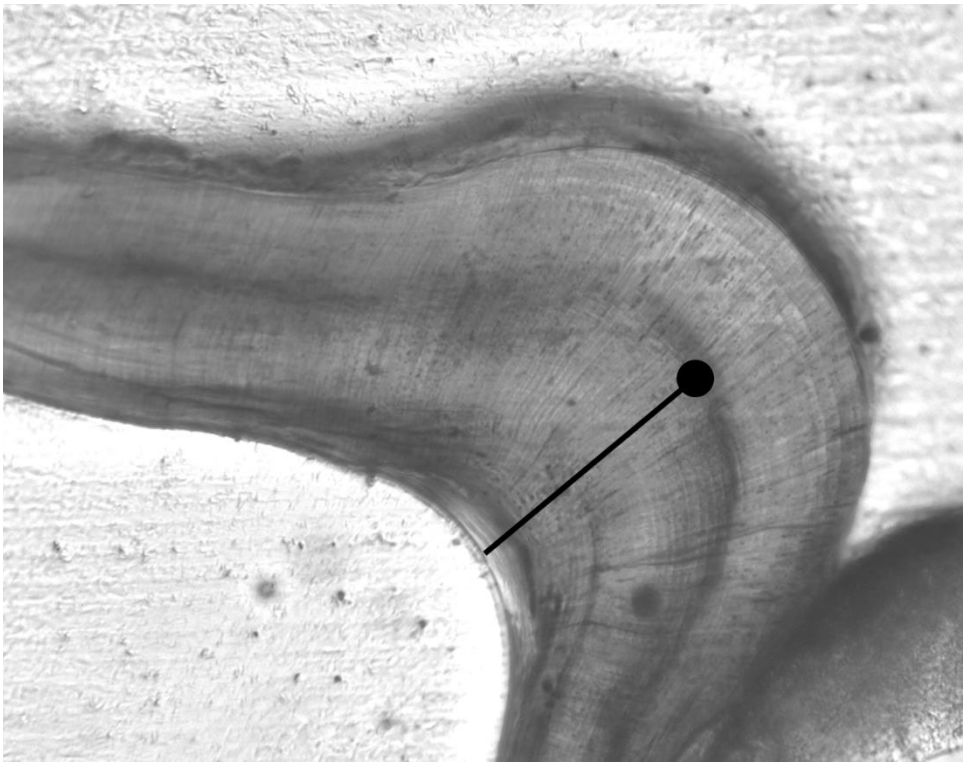

Figure 4. Image of a hypothesized one-year-old crayfish. A year of growth was defined as a wide lighter section (i.e., hypothesized period of rapid growth, shown by the line) followed by a thin darker section (i.e., hypothesized period of slow growth, shown by the dot).

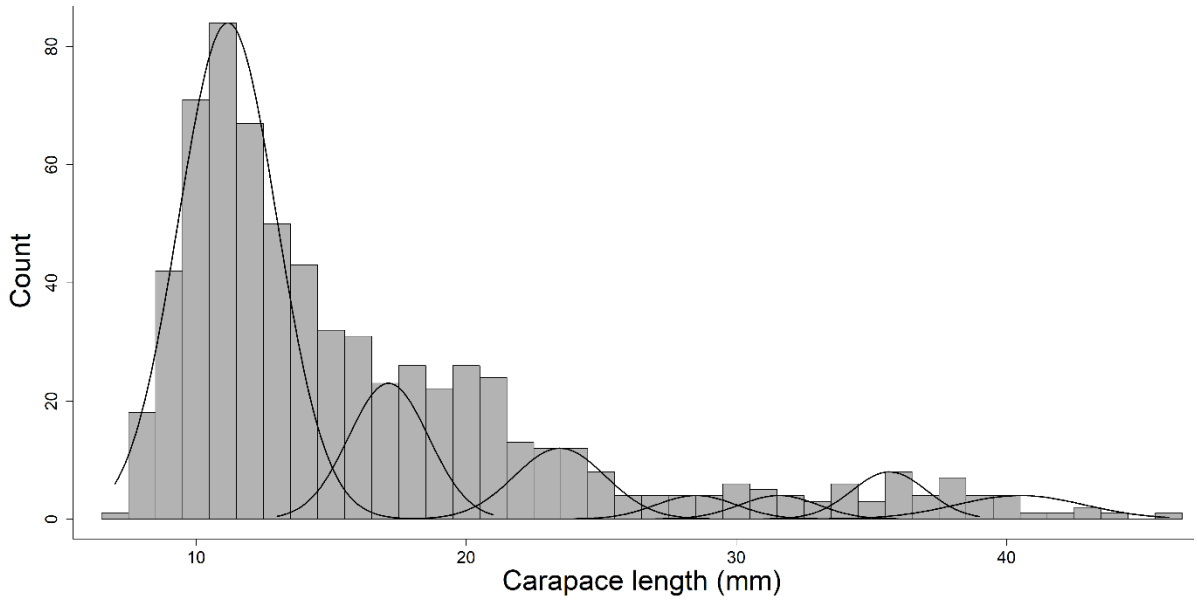

Figure 5. I generated a length-frequency histogram from the carapace lengths of 71 *Faxonius neglectus neglectus*. Age classes were determined using the Bhattacharya method (Bhattacharya, 1967). I compared this length-frequency histogram to age estimates obtained from the gastric mill.

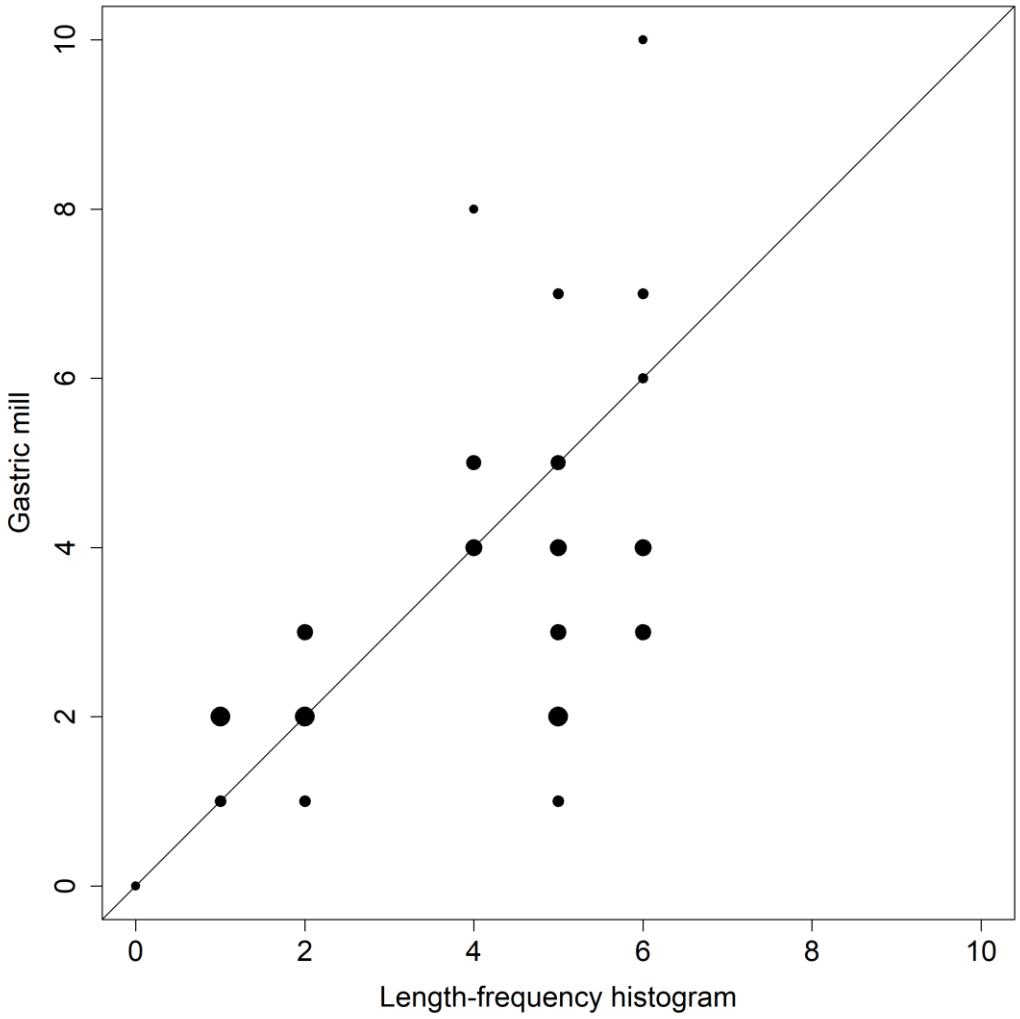

Figure 6. Gastric mill age estimates generally matched (i.e., ± 1 year) the predicted age via the length-frequency analysis for younger age classes (i.e., 0–4 years of age), but the 2 methods disagreed for older age classes. The line shows agreement between the 2 methods, where points above the line indicate when the gastric mill overestimated the length-frequency histogram and points below the line show underestimation. Further, the size of each point represents how many individuals were estimated to be that age (i.e., bigger points = more crayfish and smaller points = less crayfish).

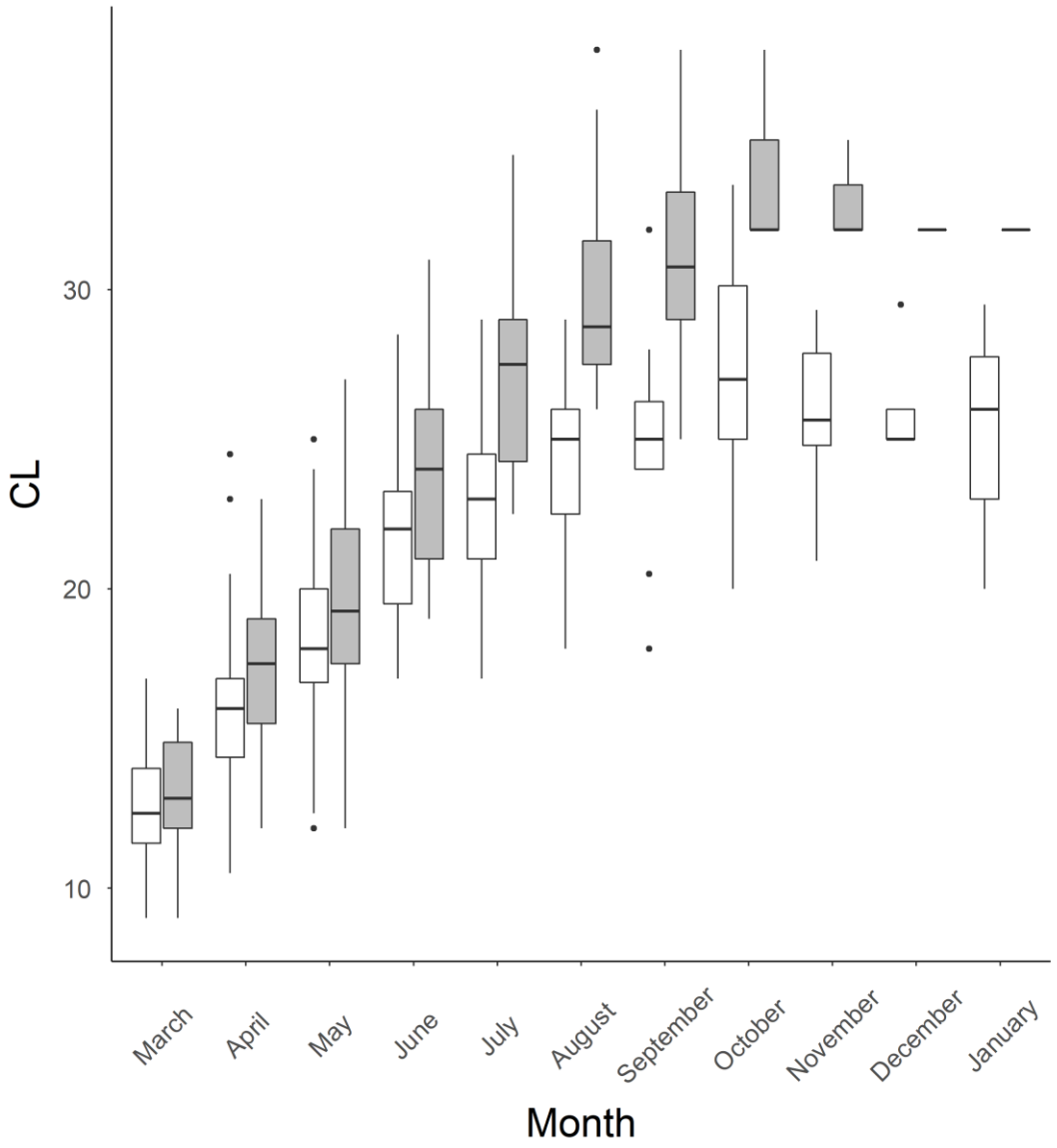

Figure 7. Crayfish grew quickly in both laboratories A (white boxes) and B (grey boxes) until September. Average water temperature in laboratory A was 17 ± 1.5 ˚C and in laboratory B was 25 ± 3 ˚C. The decrease in growth around October was the result of bigger crayfish being removed from the trials due to predation. The boxes represent the interquartile range (IQR; i.e., 25th to 75th percentile), the black horizontal line is the median carapace length, the vertical lines are the values 1.5 X the IQR, and the black dots are outliers.

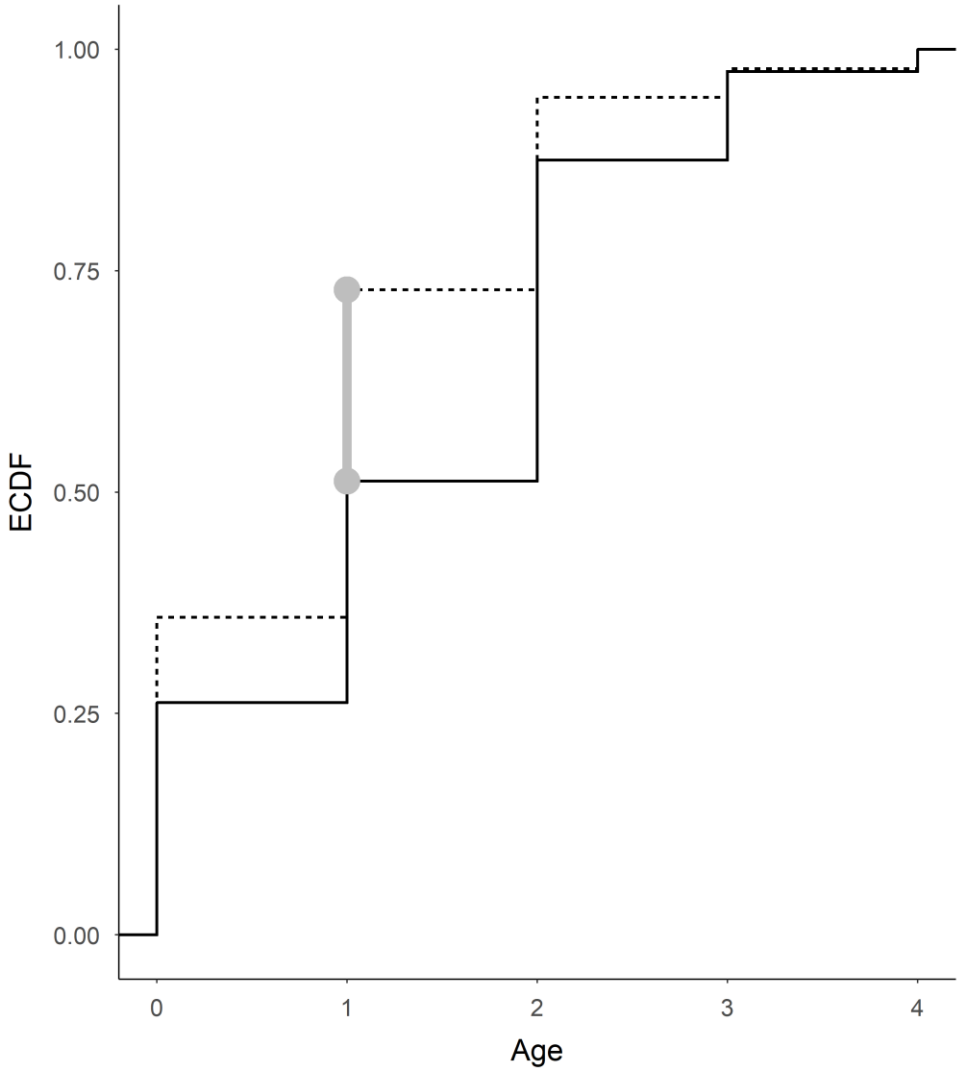

Figure 8. The age estimates from a thermally-stable environmental (laboratory A: dashed line) and thermally-variable environment (laboratory B: solid line) were distributed differently with the greatest difference being for estimates of 1 year (gray line). The x-axis represents the estimated age and the y-axis represents the empirical cumulative distribution function (ECDF).

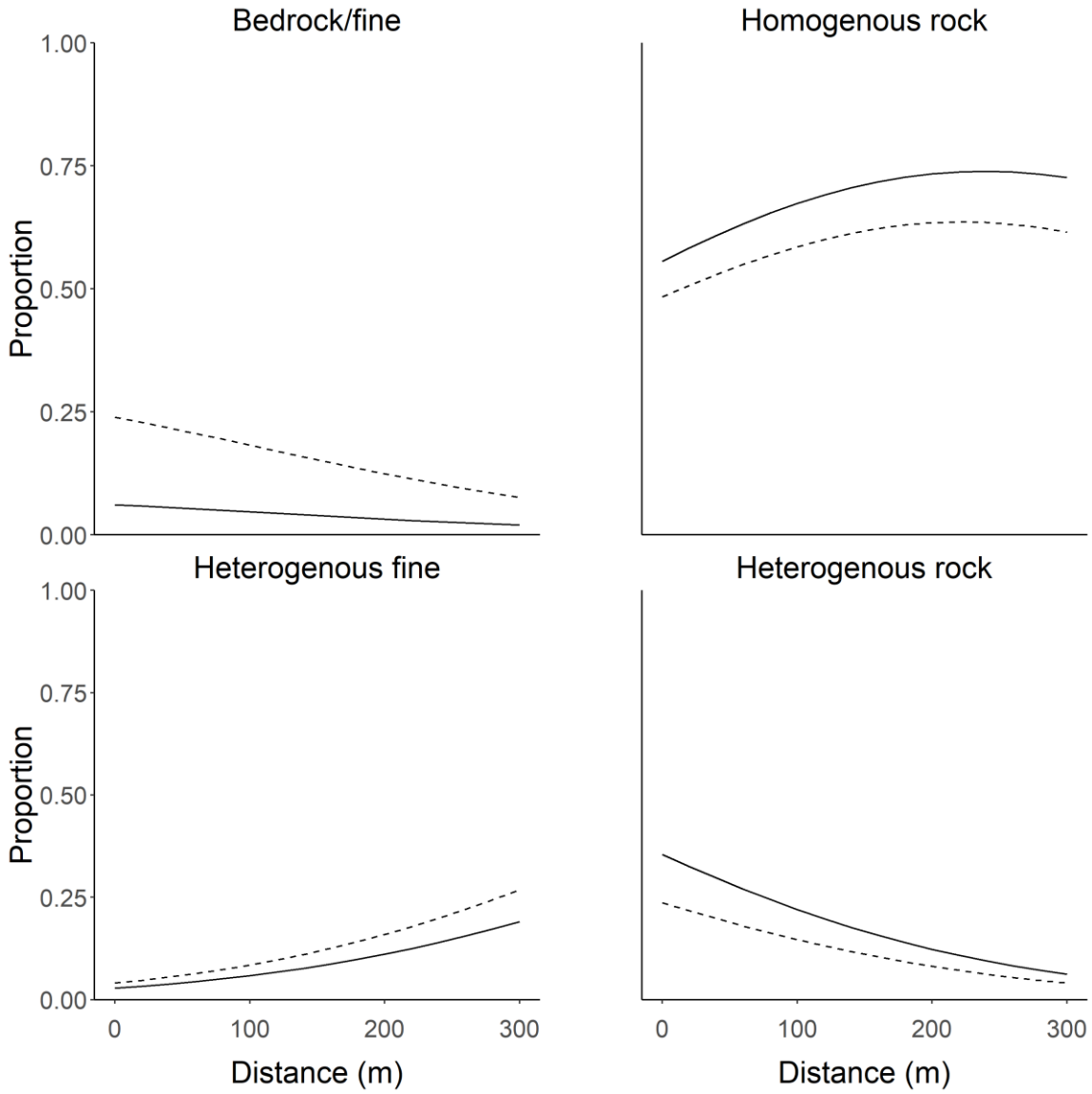

Figure 9. Results of a multinomial model assessing substrate use by male (solid line) and female (dashed line) bristly cave crayfish *Cambarus setosus* in Smallin Civil War Cave.

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