A multi-method approach for assessing the distribution of a rare, burrowing North American crayfish species

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Primary burrowing crayfishes face high extinction risk, but are challenging to study, manage, and conserve due to their difficult-to-sample habitat (i.e., terrestrial burrows) and low population densities. We apply here a variety of methods to characterize the distribution, habitat associations, and conservation status of the Boston Mountains Crayfish Cambarus causeyi Reimer, 1966, an endemic burrowing crayfish found only in the Ozark Mountains of Arkansas, United States. We used species distribution modeling (SDM) on historic occurrence records to characterize the distribution and macro-scale habitat associations of this species. We then ground-truthed SDM predictions with conventional sampling, modeled fine-scale habitat associations with generalized linear models (GLM), and lastly developed and tested an environmental DNA (eDNA) assay for this species in comparison to conventional sampling. This represents, to our knowledge, the first successful eDNA assay for a terrestrial burrowing crayfish. Our MaxEnt-derived SDM found a strong effect of average annual precipitation on the historic distribution of C. causeyi, which occurred most frequently at locations with moderately high average annual precipitation (140-150 cm/yr) within our study region. Cambarus causeyi was difficult to detect by conventional sampling in 2019 and 2020, found at only 9 of 51 sites (17.6%) sampled by searching for and manually excavating crayfish burrows. Surprisingly, habitat suitability predicted from our MaxEnt models was not associated with contemporary C. causeyi occurrences per GLMs. Instead, C. causeyi presence was negatively associated with both sandy soils and the presence of other burrowing crayfish species. Poor SDM performance in this instance was likely caused by the omission of high resolution fine-scale habitat data (e.g., soils) and biotic interactions from MaxEnt models. Finally, our eDNA assay detected C. causeyi from 6 of 25 sites (24.0%) sampled in 2020, out-performing conventional surveys by burrow excavation for this species. Given the difficulty of studying primary burrowing crayfishes and their high conservation need, we propose that eDNA

may become an increasingly important monitoring tool for C. causeyi and similar species.

A MULTI-METHOD APPROACH FOR ASSESSING THE DISTRIBUTION OF A RARE, BURROWING NORTH AMERICAN CRAYFISH SPECIES

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Abstract

Primary burrowing crayfishes face high extinction risk, but are challenging to study, manage, and conserve due to their difficult-to-sample habitat (i.e., terrestrial burrows) and low population densities. We apply here a variety of methods to characterize the distribution, habitat associations, and conservation status of the Boston Mountains Crayfish *Cambarus causeyi* Reimer, 1966, an endemic burrowing crayfish found only in the Ozark Mountains of Arkansas, United States. We used species distribution modeling (SDM) on historic occurrence records to characterize the distribution and macro-scale habitat associations of this species. We then ground-truthed SDM predictions with conventional sampling, modeled fine-scale habitat associations with generalized linear models (GLM), and lastly developed and tested an environmental DNA (eDNA) assay for this species in comparison to conventional sampling. This represents, to our knowledge, the first successful eDNA assay for a terrestrial burrowing crayfish. Our MaxEnt-derived SDM found a strong effect of average annual precipitation on the historic distribution of *C. causeyi*, which occurred most frequently at locations with moderately high average annual precipitation (140-150 cm/yr) within our study region. *Cambarus causeyi* was difficult to detect by conventional sampling in 2019 and 2020, found at only 9 of 51 sites (17.6%) sampled by searching for and manually excavating crayfish burrows. Surprisingly, habitat suitability predicted from our MaxEnt models was not associated

41 **with contemporary *C. causeyi* occurrences per GLMs. Instead, *C. causeyi* presence was**
42 **negatively associated with both sandy soils and the presence of other burrowing**
43 **crayfish species. Poor SDM performance in this instance was likely caused by the**
44 **omission of high resolution fine-scale habitat data (e.g., soils) and biotic interactions**
45 **from MaxEnt models. Finally, our eDNA assay detected *C. causeyi* from 6 of 25 sites**
46 **(24.0%) sampled in 2020, out-performing conventional surveys by burrow excavation for**
47 **this species. Given the difficulty of studying primary burrowing crayfishes and their high**
48 **conservation need, we propose that eDNA may become an increasingly important**
49 **monitoring tool for *C. causeyi* and similar species.**
50

51 ## Introduction

52 Primary burrowing crayfishes are crayfish that spend the majority of their lives in burrows and
53 only leave occasionally to mate and find food (Hobbs 1942). Burrowing crayfish play an
54 important role as ecosystem engineers by disturbing the soil in which they burrow and by
55 creating habitat for a wide range of other taxa (Pintor and Soluk 2006; Welch et al. 2008).
56 Crayfish are understudied relative to other aquatic taxa (Reid et al. 2019), and even among
57 crayfishes, primary burrowing crayfish are particularly understudied (Bloomer et al. 2021;
58 Moore et al. 2013). About one third of all crayfish species globally are threatened with extinction
59 (Richman et al. 2015), and about 22% of North American crayfish species of conservation
60 concern are primary burrowing species (Taylor et al. 2007). The high rate of endemism and
61 narrow ranges among crayfish species exacerbates their imperilment rate (Dudgeon et al. 2006;
62 Taylor et al. 1996). Additionally, primary burrowing crayfish are known to inhabit a wide
63 diversity of habitats such as open-canopied grasslands (Rhoden et al. 2016), pitcher plant bogs
64 (Welch et al. 2008), and upland forested seeps (Loughman 2010), and can be habitat specialists
65 or generalists (Loughman et al. 2012). To effectively conserve a species, resource managers must
66 understand its distribution and habitat requirements in order to make informed decisions (Richter
67 et al. 1997; Taylor et al. 2007).
68

69 Species distribution modeling (SDM) is one tool for analyzing the habitat variables driving the
70 range of rare and endemic taxa (Fois et al. 2018; Peterman et al. 2013; Rhoden et al. 2017).
71 Species distribution models use species locality and environmental data to make correlative
72 models depicting relative habitat suitability for focal organisms (Warren & Seifert 2011). A
73 common and effective approach for creating SDMs is maximum entropy modeling or MaxEnt
74 (Elith et al. 2011; Phillips et al. 2006). MaxEnt is useful for rare and understudied species
75 because it performs well with small sample sizes and is capable of using presence-only data
76 (Elith et al. 2011; Hernandez et al. 2006, Rhoden et al. 2017).
77

78 While SDMs can be used for analyzing the drivers of a species' distribution, ground-truthing or
79 validation should be included as an important part of the process (Egly & Larson 2018; Peterman
80 et al. 2013; Rhoden et al. 2017; Stirling et al. 2016). Habitat modeling using traditional field
81 sampling can also incorporate variables influencing a species' distribution that are not easily

82   accounted for in large-scale spatial data (Hirzel et al. 2006). This is particularly true for sessile
83   organisms or organisms with a small home range, which benefit from finer grain analyses
84   (Guisan et al. 2007). Species distribution models can poorly account for the influence of biotic
85   interactions, lack fine-scale habitat information, or be limited by biased or unreliable historical
86   data (Fourcade et al. 2014; Guisan & Thuiller 2005; Peterman et al. 2013). These potential
87   limitations of SDMs illustrate the need for incorporating ground-truthing and utilizing traditional
88   habitat sampling when studying a species' habitat needs and distribution.
89
90   Another emerging method of detecting rare, endemic taxa is employing molecular approaches
91   collectively termed "environmental DNA" (eDNA). Environmental DNA refers to the capture
92   and identification of DNA from an environmental sample (air, soil, or water), generally without
93   the collection or identification of entire organisms (Rees et al. 2014). Environmental DNA may
94   detect some cryptic or low-abundance organisms better than conventional sampling methods
95   (Barnes & Turner 2016). As a recent example, Rice et al. (2018) found eDNA to successfully
96   detect a rare, large-river crayfish in the Missouri Ozarks. However, eDNA has not yet been used
97   to detect primary burrowing crayfish, which are often more difficult to collect than their stream
98   dwelling counterparts (Taylor et al. 2015). Environmental DNA offers a unique opportunity to
99   detect primary burrowing crayfish, while avoiding the high amounts of effort and low detection
100   rates associated with traditional methods, such as hand excavating burrows.
101
102   The Boston Mountains Crayfish (*Cambarus causeyi* Reimer, 1966) is an understudied and rarely
103   observed burrowing crayfish endemic to the Boston Mountains of Arkansas, United States of
104   America (USA). *Cambarus causeyi* spends the majority of its life in generally shallow, yet
105   complex, burrows that are often found underneath large rocks (Robison et al. 2009; Robison &
106   Leeds 1996). Occasionally *C. causeyi* occurs in "small springs and tiny creeks under rocks"
107   when reproducing, and was noted to be associated with intermittent mountain seeps and streams
108   (Robison et al. 2009; Robison & Leeds 1996). Between 1941 and 1986, only nine *C. causeyi*
109   individuals were captured over six surveys. These specimens were collected from four localities
110   in Pope County and one locality in Stone County (Robison & Leeds 1996). Subsequently,
111   Robison and Leeds (1996) reported the capture of 87 individuals from 39 separate localities
112   (erroneously reported as 40). Robison and Leeds (1996) expanded the known range of *C. causeyi*
113   to include Madison, Johnson, Franklin, Newton, and Searcy counties. However, they reported
114   that 67 of the individuals were collected from Johnson County. Robison et al. (2009) conducted a
115   follow-up study that located *C. causeyi* at four new localities, but otherwise could not recover the
116   species from the vast majority of sites sampled by Robison and Leeds (1996). The extreme
117   decrease in the number of individuals caught in the 2009 study caused *C. causeyi* to be updated
118   to the status of Vulnerable on the 2009 IUCN assessment (Robison et al. 2009). Only two
119   additional records of *C. causeyi* have been made by Arkansas Game and Fish Commission
120   (AGFC) since 2009; they are from Newton and Van Buren counties in 2017 and 2019,
121   respectively (B. Wagner, 2019, pers. comm.).

122   We applied SDMs, fine-scale modeling, and eDNA to improve understanding of the distribution
123   and habitat associations for *C. causeyi*. Combined, these methods were intended to help us
124   determine if *C. causeyi* declined across its range as suggested in Robison et al. (2009) and
125   identify habitat needed to protect the species. We also evaluated whether SDM approaches like
126   MaxEnt are an effective means of predicting occurrences of rare, difficult to detect burrowing
127   crayfish. Additionally, we tested the efficacy of utilizing eDNA as a surveillance tool for *C.*
128   *causeyi* and other primary burrowing crayfish species. The results of this study will inform
129   managers looking to monitor *C. causeyi* and primary burrowing crayfish as a whole.
130

131   **Materials & Methods**

132

133   **Species Distribution Modeling**

134

135   Using presence-only historical occurrences, we created a SDM of suitable habitat for *C. causeyi*,
136   using MaxEnt (v. 3.4.1) with the methods previously described in Quebedeaux (2021). Due to
137   the vagueness of many of the historical site descriptions from Robison and Leeds (1996) and
138   Robison et al. (2009), we sorted the 44 historical sites by the precision of their location
139   descriptions. Out of the 44 sites there were two exact locations, eight good locations, 27 fair
140   locations, two poor locations, and five unusable locations (Fig. S1). Exact locations were sites
141   where geographic coordinates were provided by the original source. Good locations lacked
142   coordinates but could be narrowed down to a specific location and no other locations matched
143   the historical description. An example of a site that was classified as good was the "roadside
144   seepage on St. Hwy. 16, 3.1 mi. S of jct. of St. Hwys. 16 and 23 and 0.4 mi. W. of Dutton"
145   (Robison et al. 2009). Fair locations provided a township, range, and section, but an exact
146   location was not identifiable. Fair locations were either placed on a road crossing at a stream that
147   seemed to match the description, or in the center of the section if a specific stream crossing could
148   not be selected. Poor locations did not provide a township, range, and section and had vague site
149   descriptions. Unusable sites either had a description that made it unfeasible to locate the site, or
150   were in the same township, range, and section as another vaguely identified site. Only sites
151   labelled as exact, good, or fair were used in the model; this included 37 out of the 44 historical
152   sites in total. Although we had a small number of samples in our SDM, MaxEnt has been shown
153   to produce reliable models, even with smaller sample sizes (Fois et al. 2018, Galante et al. 2018).
154

155   Environmental variables included as predictors of *C. causeyi* habitat suitability in the SDM were
156   precipitation, Euclidean distance from a stream, elevation, slope, solar radiation, and average
157   available water storage for the top 150 cm of soil. Average annual precipitation data was taken
158   from the 30-year Parameter-elevation Regressions on Independent Slopes Model (PRISM) data
159   for 1981-2010 (Daly et al. 1997), and it was included in the model because of *C. causeyi*'s
160   association with ephemeral, precipitation dependent streams (Robison et al. 2009). The
161   Euclidean distance from a stream was calculated from the National Hydrography Dataset (NHD:

162 U.S. Geological Survey) and was selected because *C. causeyi* is associated with small streams.
163 Elevation data were taken from the National Elevation Dataset (U.S. Geological Survey & EROS
164 Data Center), and we predicted that *C. causeyi* may be associated with seepages in high elevation
165 areas. We predicted a connection between *C. causeyi* and higher elevation because stream
166 discharge and velocity vary with elevation. Elevation has been shown to be a driver of other
167 crayfish species' distributions, and *C. causeyi* has been noted to be found in higher elevation
168 areas (Dyer et al. 2013; Mouser et al. 2019; Robison & Leeds 1996). Slope and solar radiation
169 were calculated from the elevation dataset using ArcMap (v. 10.8.2) with the ESRI Spatial
170 Analysts Tools. Slope was included because we anecdotally noted that *C. causeyi* seemed to be
171 found on steeper inclines. Solar radiation was used because it was found to be positively
172 associated with other burrowing crayfish in Arkansas (Rhoden et al. 2017). Soil data were taken
173 from the gridded Soil Survey Geographic Database (gSSURGO). We included average available
174 water storage for the top 150 cm of soil because burrowing crayfish are associated with hydric
175 environments (Rhoden et al. 2016). All environmental layers had a 30 x 30 m grain size. We
176 identified an extent to model *C. causeyi*'s distribution by MaxEnt by selecting all Hydrologic
177 Unit Codes 8 watersheds (Watershed Boundary Dataset for HUC8 2019) that substantially
178 overlapped with counties of historical records, clipping them to the boundaries of Arkansas, and
179 omitting only one watershed with minimal overlap (Fig. S1).
180
181 We tested for correlations between all predictor variables using the Band Collection Statistics
182 tool in ArcMap 10.8.2, and all variable combinations had a correlation coefficient less than 0.5.
183 We next modeled suitable habitat for *C. causeyi* using a 45-replicate run in MaxEnt with
184 bootstrapping for replication and a 20% random test percentage. Variable importance was
185 measured with a jackknife analysis. We set a maximum of 5,000 iterations of the MaxEnt models
186 with background predictions. The above settings and the defaults were used in the final MaxEnt
187 model. Due to the narrow range of *C. causeyi* and the small number of presences, we did not use
188 a bias file. We calculated percent contribution and permutation importance in order to assess
189 variable importance. Percent contribution is how much training gain each variable provided to
190 the model, and permutation importance is how much the training area under the curve (AUC) is
191 impacted by a variable being permuted randomly. Area under the curve is a measure of how well
192 a model can differentiate between presences and background points within the data and scales
193 from 0 to 1, where values above 0.5 mean the model is better than random. Additionally, we
194 calculated AUC on the test data used to evaluate model support.
195
196 **Fine-scale Habitat Modeling**
197
198 Field Methods
199 In March 2019, we conducted a preliminary survey with hand excavation at 14 sites to ensure
200 that *C. causeyi* was still present and detectable in its known range with the methods previously
201 described in Quebedeaux (2021). We received a scientific collection permit from AGFC (permit

202 number 021520193) to sample in Arkansas, USA, and a permit waiver letter from The National
203 Forest Service (file number 2600; 2700) to sample in the Ozark National Forest. Following this
204 preliminary survey, we conducted standardized crayfish and habitat sampling over the summers
205 of 2019 and 2020. We did not conduct conventional sampling during spring 2020, peak time for
206 *C. causeyi* reproduction (Robison et al. 2009), due to COVID-19 pandemic travel restrictions.
207 All historical sites that could be located (including known sites from March 2019 sampling) and
208 new sites were sampled for crayfish and habitat. New sites were selected from across the entire
209 anticipated range of *C. causeyi,* located near streams, and spanned a gradient of habitats
210 predicted to range from unsuitable to highly suitable from our MaxEnt SDM model.
211
212 We sampled crayfish and gathered habitat data from 27 sites during May and June 2019 and 24
213 sites during June 2020 (51 total sites). We collected habitat data and crayfish from five, 1 m^2
214 quadrats placed at each site. We marked quadrats with a 1 m^2 polyvinyl chloride (PVC) pipe
215 quadrat sampler and flags. We placed the first quadrat within the stream channel, or the dried
216 stream channel, haphazardly. Then we placed a quadrat 10 m from the central quadrat upstream,
217 downstream, on the left ascending bank, and on the right ascending bank. In situations where
218 there was a barrier preventing a quadrat being placed exactly 10 m away from the central
219 quadrat, we placed the quadrat as close to 10 m away as possible. Additionally, we conducted a
220 30 min timed search to aid in detection of *C. causeyi*, due to its low abundance at most sites. We
221 searched anywhere in the vicinity of the quadrats, excluding the areas within the five quadrats.
222 All crayfish were collected with hand excavation or dip net, which was used if there was
223 standing water present and the burrows were found empty.
224
225 We characterized habitat within our 20 m x 20 m sites by recording the following variables:
226 average forest canopy cover across quadrats, proportion of quadrats with large rock present,
227 proportion of sand in soil at the central quadrat, presence of another primary burrowing species
228 at the site (*Procambarus liberorum* Fitzpatrick, 1978), and the presence of stream dwelling
229 crayfishes at the site (*Faxonius meeki* Faxon, 1898, and *Faxonius williamsi* Fitzpatrick, 1966).
230 Low canopy cover was shown to be an important positive driver for occurrence of other
231 burrowing crayfishes (Rhoden et al. 2016). We measured percent canopy cover at each quadrat
232 using a Model-A spherical densiometer (Forestry Suppliers, Inc., Jackson, Mississippi, USA),
233 and averaged canopy cover from all five quadrats to represent canopy cover at the site scale. We
234 expected the presence of large rock to have a positive effect on the presence of *C. causeyi* due to
235 this species' association with large rocks in past surveys (Robison et al. 2009). We considered
236 any rocks larger than 128 mm x 128 mm, including both cobble and boulders, to be large. Sandy
237 soils have been shown to have a negative association with other burrowing crayfish species
238 because they are not suitable for constructing burrows, and we expected to find the same with *C.*
239 *causeyi* (Dorn & Volin 2009; Grow 1982; Grow & Merchant 1980). Sand proportion was
240 calculated from a soil core collected at the central quadrat at each site. We took only one soil
241 core from the center of each site, anticipating little site-scale variability in soil attributes within

242   this 20 m x 20 m area. The soil samples were analyzed with laser diffraction by the Illinois State
243   Water Survey (Champaign-Urbana, Illinois, USA) (Table S1). We expected the presence of other
244   crayfish species to have a negative effect on likelihood of *C. causeyi* occurrence due to
245   competition (James et al. 2015; Reynolds et al. 2013). We separated crayfish species into other
246   primary burrowing species or stream dwelling species as these organisms differ in their ecology
247   and life history, and considered these crayfishes present or absent at the site if they were detected
248   in any quadrat or by the timed search.
249
250   Modeling
251   We modeled our fine-scale data with zero-inflated Poisson generalized linear models with the
252   methods previously described in Quebedeaux (2021) in the program R (v. 3.5.2) with the
253   package glmmTMB (v. 1.0.2.1; Brooks et al. 2017). Our response variable was the number of
254   quadrats within a site occupied by *C. causeyi*. We treated the timed search for *C. causeyi* as a
255   sixth quadrat in this analysis because we were interested in modeling *C. causeyi* habitat at the
256   site, rather than quadrat or micro-habitat, scale. We had intended to estimate *C. causeyi*
257   occupancy while correcting for detection probabilities from our replicated quadrat samples
258   (Durso et al. 2011; MacKenzie et al. 2003), but had too few detections for these models to
259   converge. Accordingly, we instead used zero-inflated models to account for the large number of
260   absences in our data. We used the habitat data collected from the quadrat sampling and the
261   interpolated MaxEnt output to create a suite of potential models of *C. causeyi* relative abundance
262   at a site scale. We ground-truthed the MaxEnt model by using the interpolated MaxEnt habitat
263   suitability for the site as a predictor variable. Candidate models included the null model, sand
264   only, interpolated MaxEnt habitat suitability, canopy cover only, proportion of quadrats
265   containing a large rock, presence/absence of *P. liberorum*, presence/absence of stream dwelling
266   crayfish species, sand and *P. liberorum*, interpolated MaxEnt output and sand and *P. liberorum*,
267   and sand and large rock. We had no global model because the model would not converge. We
268   selected the most supported model with Akaike's information criterion corrected for small
269   sample size (AICc). Additionally, we analyzed the accuracy of our MaxEnt predictions by
270   calculating the AUC of the receiver operating characteristic of *C. causeyi* occupied sites
271   compared to unoccupied sites from our fine-scale habitat sampling (Fawcett 2006; Rhoden et al.
272   2017) with the pROC package in program R (Robin et al., 2011).
273
274   **Environmental DNA**
275
276   Environmental DNA Field Collection
277   During March 16-18, 2020, we sampled 25 stream sites across northern Arkansas for *C. causeyi*
278   eDNA. We intended on sampling all 51 sites where habitat data was collected, in addition to
279   some of our preliminary sampling sites, but we were unable to do so as a consequence of
280   COVID-19 pandemic restrictions on travel. Within sites sampled for eDNA, we included a
281   combination of *C. causeyi* detections and non-detections from field sampling to evaluate assay

282 performance for this species. We used one field blank of distilled water per site to assess for
283 potential contamination in supplies. At every site, we triple-rinsed water bottles with stream
284 water prior to water collection. We then collected four 250 mL surface water samples, spaced 10
285 m apart, along a 40 m reach of the stream by submerging the water bottle until full or by using
286 the bottle cap to assist with collecting enough water in shallower streams. We targeted surface
287 waters during the spring to detect *C. causeyi* in adjacent, submerged burrows or crayfish using
288 surface water. We did not collect eDNA soil or water samples directly from crayfish burrows
289 because the burrows were either in or next to the stream channel during the spring months in
290 which we sampled, and *C. causeyi* are often found outside of burrows within the streams when
291 they are reproductively active (Robison et al. 2009; Robison & Leeds 1996). Additionally,
292 studies have found that reproductive events may increase the eDNA concentrations in water
293 samples, making target species easier to detect (Curtis et al. 2021b; de Souza et al. 2016; Spear et
294 al. 2015). Also, many of the ephemeral streams that *C. causeyi* utilize become dry during the
295 summer. Ephemeral stream drying combined with the reproductive behavior of *C. causeyi* makes
296 our eDNA sampling design specifically adapted for surface water sampling during spring
297 months. After samples were collected, we sealed the bottles in a clean plastic bag and placed
298 them on ice in a cooler until filtration (<12 hours of sample collection) (Curtis et al. 2021a).
299
300 Prior to filtration of water samples, we cleaned the filtering space with 50% bleach solution. We
301 then vacuum filtered water samples onto 1.0 μm cellulose nitrate filters (Whatman™) and
302 submerged the filters in ~1 mL of cetyl trimethylammonium bromide (CTAB; Teknova©) in a 2
303 mL centrifuge tube. We kept filters in the dark and at room temperature for about one month to
304 increase eDNA concentrations by cell lysis (Renshaw et al. 2015), and then froze the filters at -
305 80°C until we extracted DNA. All bottles and filtration supplies had been washed with 50%
306 bleach prior to use and we wore nitrile gloves to collect all water samples, filter samples, and
307 during all subsequent lab procedures with frequent glove changes.
308
309 *Cambarus causeyi* Assay
310 We designed a single-species qPCR assay for *C. causeyi* by using the OligoAnalyzer™ Tool and
311 the program Sequencher 5.4 (Gene Codes Corporation, Ann Arbor, Michigan, USA). We used *C.*
312 *causeyi* sequences (accession numbers: JX514477.1 and DQ113443.1) from GenBank and newly
313 sequenced individuals of the species (accession numbers: OP673577, OP673578, OP673579,
314 OP673580, OP673581, OP673583, OP673584) to design the following primer-probe assay to
315 amplify a 119 bp region of the cytochrome oxidase subunit one (COI) DNA bar coding region:
316
317 F-primer: 5'-GCGGGTATAACTATAGATC-3'
318 R-primer: 5'- CTATGCTATTAACAGATCG-3'
319 Probe: 5'-FAM-ACTATTGTTATCTTTGCCTGTG-MGB-NFQ-3'.
320

321 We tested this assay *in silico* against potential co-occurring non-target crayfish species
322 (*Cambarus hubbsi* Creaser, 1931, *P. liberorum*, *F. williamsi*, *F. meeki meeki*, and *F. meeki brevis*
323 Williams, 1952) with less than 95% similarity in sequence match (Table 1). Next, we tested the
324 assay *in vitro* using synthetic COI gBlock fragments of *C. causeyi* and the four potential non-
325 target species (Table S2).
326
327 We optimized the assay by testing different primer and probe concentrations and annealing
328 temperatures until we produced the lowest Cq values, the highest efficiency and best R^2 using
329 serial dilutions of the *C. causeyi* gBlock. We then tested the assay against high concentrations
330 (0.57–1.07 ng/μL confirmed concentration on Qubit™) of non-target species DNA to examine if
331 there was non-target amplification. *Faxonius williamsi* (0.79 ng/μL) and *P. liberorum* (0.57
332 ng/μL) DNA did not amplify, but there was delayed amplification in *F. meeki meeki* (0.69 ng/μL
333 amplified in 3/3 plate replicates at Cq 27) and *C. hubbsi* (1.07 ng/μL amplified in 2/3 plate
334 replicates at Cq 22). We then diluted the *F. meeki meeki* and *C. hubbsi* gBlock to a more
335 environmentally realistic concentration (10^{-4} ng/μL) for an eDNA sample and DNA samples did
336 not amplify within the 40 qPCR cycles.
337
338 Environmental DNA Extraction and qPCR
339 Prior to eDNA extraction or qPCR preparation, we cleaned the laboratory space with a 50%
340 bleach solution and treated hoods with UV for 20 min. We extracted DNA from filters using a
341 chloroform–isoamyl alcohol extraction procedure (Renshaw et al. 2015) in a clean room free
342 from high-copy DNA and isolated from the PCR laboratory (Goldberg et al. 2016). At the end of
343 the extraction procedure, we eluted eDNA samples with 100 μL of TE buffer (Invitrogen™) and
344 froze at -80°C until qPCR. One extraction blank was used for every ~12 eDNA samples to assess
345 the level of lab carry-over contamination.
346
347 We ran 20 mL qPCR reactions with the following: 10 μL of iTaq Universal Probes Supermix
348 (Bio-Rad©), 4.0 μL of molecular grade nuclease-free water (Invitrogen™), 1.0 μL (10 μM) of
349 each primer, 1.0 μL (10 μM) of the probe, and 3 μL of eDNA. For the negative plate controls
350 included on every plate, we replaced the 3 μL of eDNA with 3 μL of molecular grade nuclease-
351 free water. We prepared all plates (qPCR strip tubes) in an isolated and spatially separate PCR
352 product-free clean room. We then transported our samples to our PCR room where we added our
353 *C. causeyi* gBlock standard using a serial dilution (1:10) from 10^{-4} to 10^{-10} ng/μL to create a
354 standard curve for each plate. We next ran the plate on a QuantStudio 3 Real-Time PCR
355 (Applied Biosystem®) with the following parameters: 95°C for 5 min denaturation and 40 cycles
356 of 95°C for 30 s, 52°C for 1 min, and 72°C for 1 min. We ran all samples in triplicate and
357 considered a true amplification if 1/3 plate replicates amplified before cycle 40. In order to
358 confirm that there were no false positives, all eDNA samples that amplified were cleaned with
359 ExoSap-It Express, Sanger sequenced at the University of Illinois' W.M. Keck Center, and then
360 confirmed as *C. causeyi* using NCBI's BLAST (Altschul et al. 1990). To test whether samples

361 displayed evidence of inhibition, we randomly selected an eDNA sample that did not amplify *C.*
362 *causeyi* eDNA and where no crayfish were found (KBQ 20-77) (Table S3), spiked the sample
363 with 10^{-5} ng/μL *C. causeyi* gBlock standard, and confirmed that there was no evidence of
364 inhibition (<1 average ΔCq between the spiked sample and the standard).
365
Results
367 **Species Distribution Modeling**
368
369 Our MaxEnt model's test AUC was 0.911, indicating good fit, with a standard deviation of
370 0.0342 (Fig. 1). Average annual precipitation was the best predictor of *C. causeyi* presence
371 estimated from a jacknife analysis (Fig. 2). All other variables performed poorly. Percent
372 contribution and permutation importance mirrored results of the jackknife analysis. Precipitation
373 had a contribution of 65.0%, Euclidean distance from a stream had a contribution of 13.4%,
374 elevation had a contribution of 12.0%, and all remaining variables had a contribution under 5%.
375 We found a unimodal relationship between average annual precipitation and *C. causeyi* presence,
376 which peaked at approximately 140 cm/yr (Fig. 3). *Cambarus causeyi* habitat suitability declined
377 below this value, plateauing around 150 cm/yr.
378
379 **Fine-scale Habitat Modeling**
380
381 We detected *C. causeyi* at nine of 51 sites during our habitat sampling (Fig. 4; Table S4). We
382 failed to find *C. causeyi* at three sites where the species had been found during our March 2019
383 preliminary survey, and these preliminary detections were omitted from models (Table S3). We
384 found male and female pairs sharing burrows during the spring preliminary sampling, and found
385 one ovigerous female and many young instars during the summer habitat sampling. No models
386 were particularly well-supported, and our most-supported model (proportion of sand and
387 presence of *P. liberorum*) was only marginally better than the null model (Table 2). Both the
388 proportion of sand and the presence of *P. liberorum* had negative associations with *C. causeyi*
389 relative abundance (Fig. 5). Proportion of sand and presence of *P. liberorum* were also the only
390 single variable models to perform better than the null model (Table 2). Interpolated MaxEnt
391 habitat suitability generally performed poorly in predicting *C. causeyi* relative abundance from
392 conventional sampling. Surprisingly, MaxEnt habitat suitability had a negative effect on *C.*
393 *causeyi* relative abundance in all models including this variable (Table 2). Similarly, AUC for
394 the field validation of our MaxEnt model was only 0.548, indicating that this model only
395 performed marginally better than random.
396
397 **Environmental DNA**
398
399 No field, extraction, or plate blanks amplified. Standard curves using *C. causeyi* serial dilutions
400 had R^2 values between 0.980–0.998 and efficiency between 98–110%. Our limit of detection

401 (LOD), or the concentration where 1/3 plate replicates amplified, was 1×10^{-10} ng/μL (~0.77
402 copies). Our limit of quantification (LOQ), or the concentration where 3/3 of the plate replicates
403 amplified, was 1×10^{-9} ng/μL (~7.73 copies). When detecting *C. causeyi* eDNA from field
404 samples, concentrations were generally low (near LOD; Table S5).
405
406 We detected *C. causeyi* eDNA at six of the 25 stream sites sampled in March 2020 (Fig. 4; Table
407 S3). These six detections included three sites where *C. causeyi* was collected during our fine-
408 scale habitat survey, two sites where *C. causeyi* was collected in our March 2019 preliminary
409 survey but not by fine-scale habitat sampling, and one site where *C. causeyi* was not collected by
410 any conventional sampling. Non-detections of *C. causeyi* eDNA included six sites where the
411 species was collected by our fine-scale habitat survey, and 13 sites where the species was not
412 collected by any conventional sampling (Fig. 4). Accordingly, eDNA and conventional sampling
413 for *C. causeyi* had moderate agreement, with a Cohen's Kappa statistic of 0.403 (5 shared
414 detections, 1 eDNA detection without conventional detection, 6 conventional detections without
415 eDNA detection, and 13 shared non-detections). Environmental DNA detected *C. causeyi* more
416 frequently (24% of sites) than conventional sampling overall (16.7% of sites excluding
417 preliminary surveys, 17.7% including preliminary surveys).
418

419 **Discussion**
420 We assessed the distribution and habitat preferences of a rare, understudied crayfish species
421 using SDMs, fine-scale modeling, and eDNA. Our MaxEnt model for this species appeared to
422 perform well with a high test AUC and identified average annual precipitation as a factor in
423 defining the distribution of *C. causeyi*. However, ground-truthing revealed poor performance of
424 the MaxEnt model, as predicted habitat suitability was not included in any of our subsequent
425 models explaining *C. causeyi* relative abundance. Instead, we found site-specific measures of
426 soil texture and presence of another burrowing crayfish (*P. liberorum*) best explained *C. causeyi*
427 relative abundance, albeit in relatively poor-performing GLM models. Large-scale analyses like
428 MaxEnt and other SDMs often lack fine-resolution data and biotic interactions that can affect
429 distributions (Anderson et al. 2002; Guisan & Thuiller 2005; Peterman et al. 2013). Lastly, our
430 eDNA assay detected *C. causeyi* from the field at a greater rate than, and in moderate agreement
431 with, conventional sampling. Although SDMs and fine-scale habitat modeling from conventional
432 surveys had moderate or weak performance explaining *C. causeyi* habitat associations, eDNA
433 appears to be a promising approach to sampling for this and similar burrowing crayfish in the
434 future.
435
436 We encountered several limitations during the course of our study. Our most supported
437 generalized linear model from conventional field sampling performed better than the null model
438 but had a mediocre fit (Fig. 5). We initially designed our field sampling data to be analyzed with
439 occupancy models using replicated quadrats or timed searches for estimating detection
440 probabilities (Durson et al. 2011; MacKenzie et al. 2003). However, we did not detect *C. causeyi*

441   at enough sites for our models to converge. Instead, we employed zero-inflated Poisson
442   generalized linear models on relative abundance of *C. causeyi*, but this approach was still limited
443   by our infrequent crayfish detections. We had planned to improve detection of *C. causeyi* by
444   sampling intensively in spring of 2020, but COVID-19 travel restrictions reduced our sampling
445   in this season to only one week of eDNA surveys and no conventional surveys. We suggest that
446   future sampling for *C. causeyi* be conducted in the spring when this species is most actively
447   exhibiting mating behaviors (Robison et al. 2009), supported by our finding of male and female
448   crayfish sharing burrows in March 2019 preliminary sampling but not in subsequent summer
449   sampling. Rhoden et al. (2017) had better success than our study in ground-truthing SDM models
450   for burrowing crayfish species in Arkansas, but had sampled for these species in spring rather
451   than summer. We expect this seasonal sampling recommendation also applies to eDNA sampling
452   for *C. causeyi* and other burrowing crayfishes, as timing of eDNA sampling affects detection
453   probabilities relative to life history and organism activity (e.g., de Souza et al. 2016; Troth et al.
454   2021). For example, Dunn et al. 2017 found that ovigerous crayfish produce more eDNA than
455   other individuals in a laboratory experiment. We suspect that *C. causeyi* would be most likely to
456   be ovigerous in the middle to late spring, as we found many juveniles during our summer
457   sampling but only one female with eggs.
458
459   Our MaxEnt model had a high testing AUC when validated on historic *C. causeyi* occurrences,
460   but did not predict detections of this species well during ground-truthing in 2019 and 2020.
461   MaxEnt identified average annual precipitation as the best predictor of *C. causeyi* presence, and
462   this climate factor may limit the distribution of *C. causeyi* to wetter locations at a broad scale.
463   However, our MaxEnt model seemingly missed factors affecting *C. causeyi* presence and relative
464   abundance at the site or local scale. For example, in our fine-scale modeling, proportion of sand
465   in soil was negatively associated with *C. causeyi* relative abundance, but water storage capacity
466   of soil was not identified as an important variable in our MaxEnt model. Given these disparate
467   results, we subsequently compared water storage capacity of soil from gSSURGO to our field-
468   observed sand proportion at sites. Sandier soils should have low water holding capacity, but we
469   found a very weak, noisy relationship between proportion of sand in soil and estimated water
470   holding capacity from gSSURGO (Fig. S2). Soil conditions are important to burrowing crayfish
471   (Dorn & Volin 2009; Grow 1982), but our MaxEnt model seemingly lacked high resolution or
472   accurate soil data for our study sites. Improved spatial data on soil attributes (i.e., Vergopolan et
473   al. 2022) may lead to better SDM models for burrowing crayfish in the future.
474
475   Biotic interactions also appear to affect *C. causeyi* presence and relative abundance. *Cambarus*
476   *causeyi* was never found at the same site as the primary burrowing crayfish *P. liberorum* (Fig. 5),
477   which may be excluding *C. causeyi* by competition. Egly & Larson (2018) found poor ground-
478   truthing performance of an SDM for crayfish to be partially explained by biotic interactions, in
479   which native species were absent from suitable habitat occupied by invasive species. Biotic
480   interactions can be incorporated into SDMs (Anderson et al. 2002; Guisan & Thuiller 2005;

481   Peterman et al. 2013), but we lacked a priori data to know that *P. liberorum* may be an effective
482   competitor excluding *C. causeyi* from otherwise suitable sites. Our study emphasizes the
483   importance of having familiarity with a study species' biology and ecological interactions when
484   selecting variables for SDMs.
485
486   We designed and validated a robust qPCR assay for *C. causeyi* that does not amplify the DNA of
487   non-target crayfishes at realistic, field concentrations. Five of the six eDNA detections for *C.*
488   *causeyi* were at locations where the species was detected by conventional sampling, whereas
489   only one eDNA detection was at a location where *C. causeyi* was not directly collected in our
490   study. In this one instance, eDNA might be interpreted as out-performing conventional sampling
491   for *C. causeyi*, particularly as we documented no evidence of field or laboratory contamination in
492   our study commonly associated with false positives for eDNA studies. However, eDNA may
493   have been advantaged relative to conventional sampling because eDNA samples were taken in
494   the spring during the presumed active, reproductive season of *C. causeyi* (Robison & Leeds
495   1996). Future comparisons of eDNA and conventional sampling for burrowing crayfish would
496   ideally be done during the same season and year. Alternatively, we failed to detect *C. causeyi*
497   eDNA from six sites where the species was detected by conventional sampling in either 2019 or
498   2020. These apparent non-detections or false negatives could be the consequence of mismatches
499   between years of sampling eDNA and conventional surveys, especially because only one of the
500   potential false negatives was conventionally sampled in 2020 (Table S3). For example, *C.*
501   *causeyi* may have experienced population declines at some sites between conventional sampling
502   in 2019 and eDNA sampling in 2020 (Fournier et al. 2019). Our *C. causeyi* eDNA samples had
503   low copy numbers, near the LOD, when the species was detected, which may have been caused
504   by low local abundance or biomass at most sites. Therefore, the performance of our eDNA assay
505   for *C. causeyi* might also be improved by increasing either the volume of water filtered or
506   number of field replicates taken (Dougherty et al. 2016; Sepulveda et al. 2019). Additionally,
507   future burrowing crayfish monitoring could be done by testing burrow water or soil for eDNA
508   (Baker et al. 2020), although sampling *C. causeyi* associated surface water (ephemeral streams)
509   during the reproductive season appears to be a good approach for this particular species. Given
510   the difficulty of detecting *C. causeyi* by conventional sampling, eDNA appears to be an
511   encouraging approach for monitoring the presence of this species and other primary burrowing
512   crayfish.
513

Conclusions

515   Our study was motivated by the possibility that *C. causeyi* had experienced severe range and
516   population declines between Robison and Leeds (1996) and Robison et al. (2009), which resulted
517   in elevated IUCN conservation status for this species. Our results suggest that *C. causeyi* still
518   occurs throughout its historic range (Fig. 4), but the species is difficult to detect. Further,
519   detection probabilities for *C. causeyi* are likely higher in spring than in summer, and may be
520   higher in wetter than drier years. For example, MaxEnt revealed a positive association between

521   *C. causeyi* presence and wetter locations as represented by average annual precipitation. Robison
522   et al.'s (2009) *C. causeyi* survey was preceded by and coincided with the 2006-2007 extreme
523   drought in Arkansas (National Drought Mitigation Center et al. 2022). Although *C. causeyi* is
524   associated with higher elevation ephemeral and intermittent streams, which dry during a typical
525   year, the species may still be negatively affected by severe drought. Robison et al. (2009) may
526   have detected widespread, drought-associated population declines of *C. causeyi* that the species
527   has since recovered from. Alternatively, *C. causeyi* may also have been difficult to detect during
528   Robison et al. (2009) due to drought conditions. *Cambarus causeyi* uses shallow burrows, often
529   under large rocks, but may have burrowed deeper to follow the receding water table during the
530   2006-2007 drought (Novakowski & Gillham 1988; Robison et al. 2009). Future work could
531   investigate seasonal and annual effects of precipitation on both *C. causeyi* occupancy and
532   detection probability.
533
534   Although we did not find a decrease in the range of *C. causeyi*, there are still reasons this species
535   may be vulnerable to population declines or range contraction. Future work might investigate
536   biotic or competitive interactions with other burrowing crayfishes like *P. liberorum*, which could
537   displace *C. causeyi* if they spread due to factors like climate or land use change. *Cambarus*
538   *causeyi* could also be directly vulnerable to climate change if severe weather events like droughts
539   become more common in Arkansas over time (Mukherjee et al. 2018), particularly given the
540   apparent preference of this species for locations with high average annual precipitation. Despite
541   our success finding *C. causeyi* at more sites than Robison et al. (2009), we suggest the species
542   still deserves its vulnerable conservation status and increased management attention due to its
543   narrow range, low local abundances (Table S3), and the threats described above. Furthermore,
544   eDNA was effective at detecting *C. causeyi,* and future monitoring for this and other burrowing
545   crayfishes might benefit from incorporating eDNA into sampling programs, although this
546   emerging methodology should continue to be tested against conventional field sampling and
547   distributional predictions from models like SDMs.
548

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Figure 1

Predicted distribution of *Cambarus causeyi* from MaxEnt model.

Predicted distribution of *Cambarus causeyi* in Arkansas, USA from MaxEnt, all historical *C. causeyi* sites, and our modeling extent. HUC8 watersheds visualized in the inset map were used as the study extent because they overlapped with counties (black lines) with historical records.

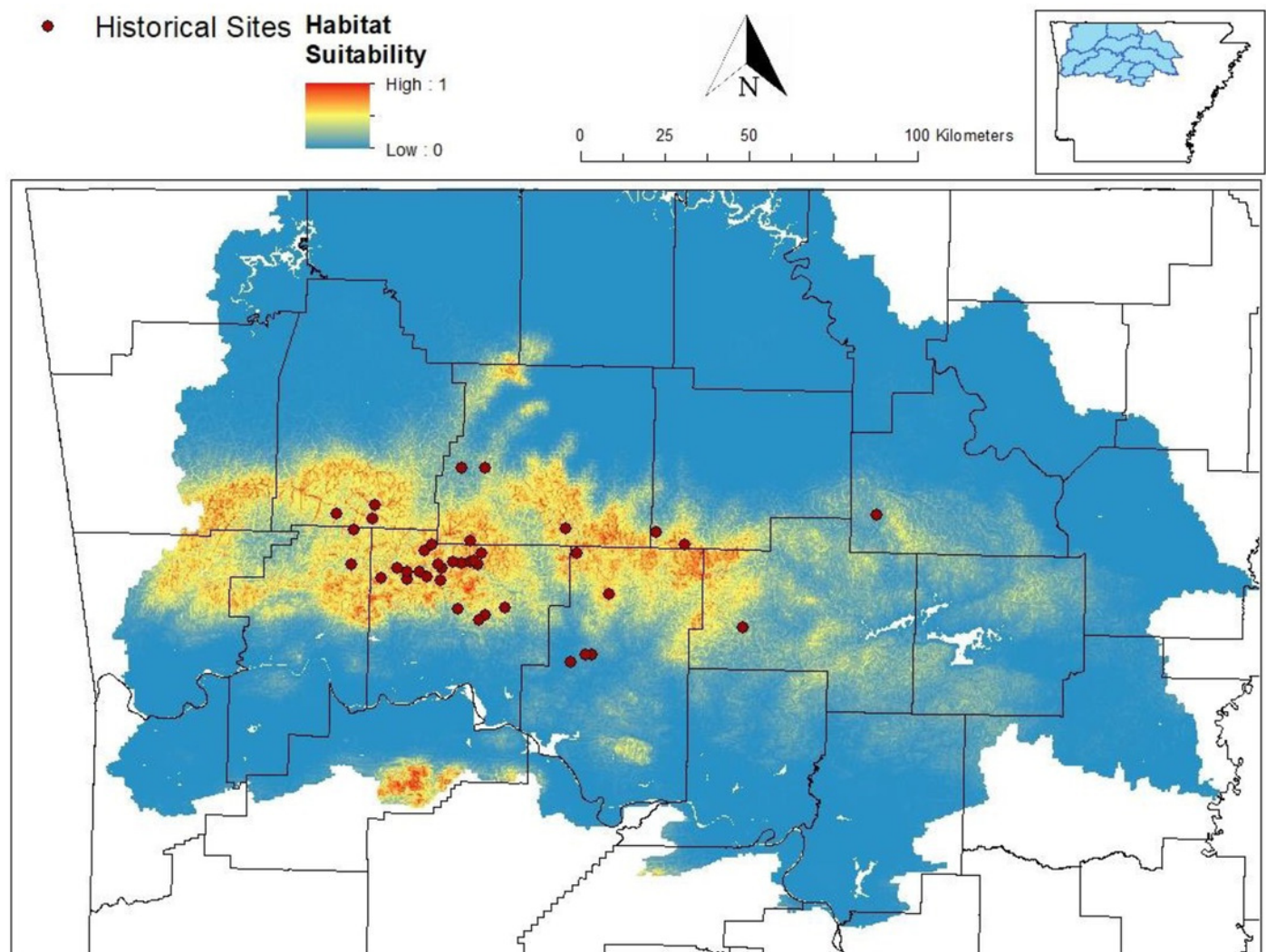



Figure 2

MaxEnt jackknife analysis results.

MaxEnt jackknife analysis on the area under the curve (AUC) for our *Cambarus causeyi* model. "30melevupdate" = elevation, "euclidmax" = Euclidean distance from a stream, "h2Ostormax" = average available water storage for the top 150 cm of soil, "precipmax" = 1981-2010 annual average precipitation, "radiationmax" = solar radiation, and "slopemax" = slope. Precipitation is the only variable that, when it was the only variable included in a model, had a higher AUC than in a model without that variable. Additionally, the AUC of the model with all variables was approximately equal to the AUC of the model with just precipitation.

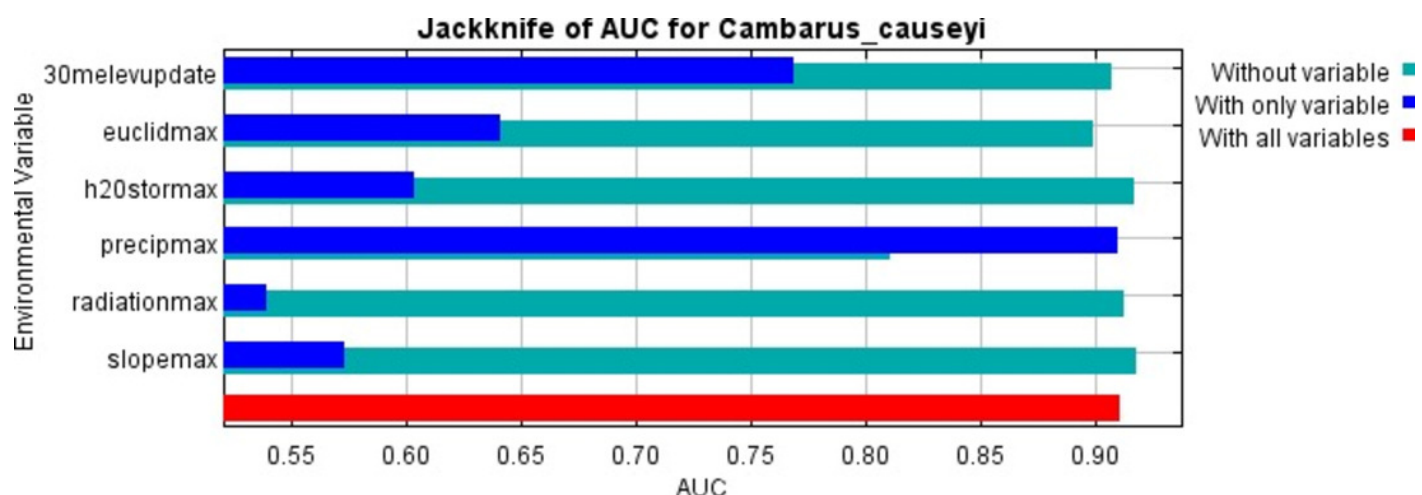



Figure 3

Response of estimated suitability for *Cambarus causeyi* to precipitation.

The response of *Cambarus causeyi*'s estimated suitability, shown with the cloglog (complementary log-log) transformation, to the annual average precipitation in centimeters from 1981-2010 from the PRISM climate data (Daly et al. 1997) generated in our MaxEnt analysis.

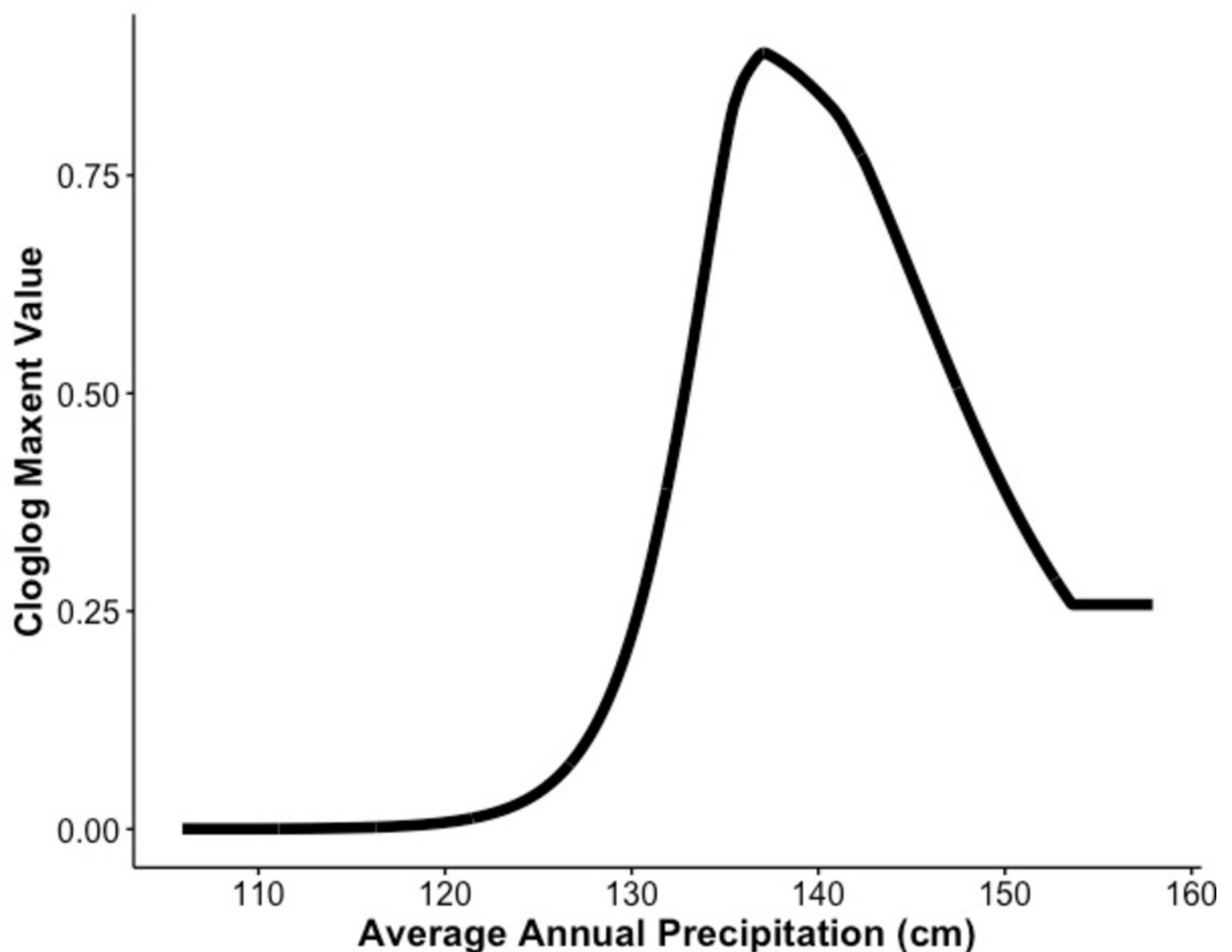



Figure 4

Map of sites sampled for fine-scale habitat modeling and eDNA.

All sites included in our fine-scale habitat modeling and eDNA sampling of *Cambarus causeyi* in Arkansas, USA. Red triangles were habitat sampling absences, and blue circles were presences during habitat sampling. Blue squares indicate sites where we detected *C. causeyi* in March 2019, but not during the course of our habitat sampling during the following summers. The squares were treated as absences in our fine-scale habitat analysis. Red diamonds are sites where *C. causeyi* was not detected with eDNA. Blue diamonds are sites where *C. causeyi* was detected with eDNA.

Legend

Sampling Sites for *Cambarus causeyi*

eDNA Sites

◆ No Amplification

◆ Amplified

Habitat Sites

● Present

▲ Absent

■ Present Only in March 2019

⬭ Border of Counties

National Forest

N

0   12.5   25   50 Kilometers

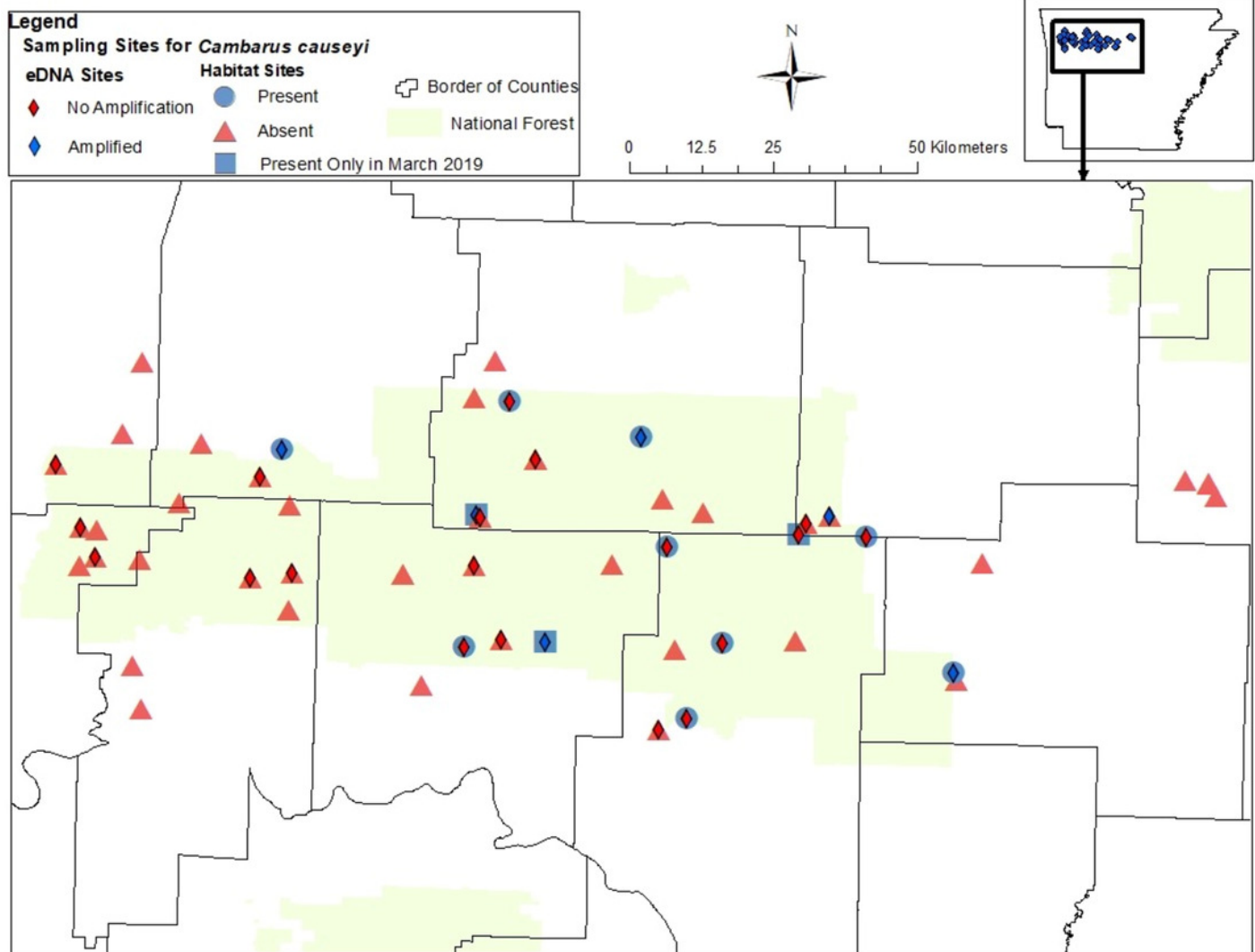
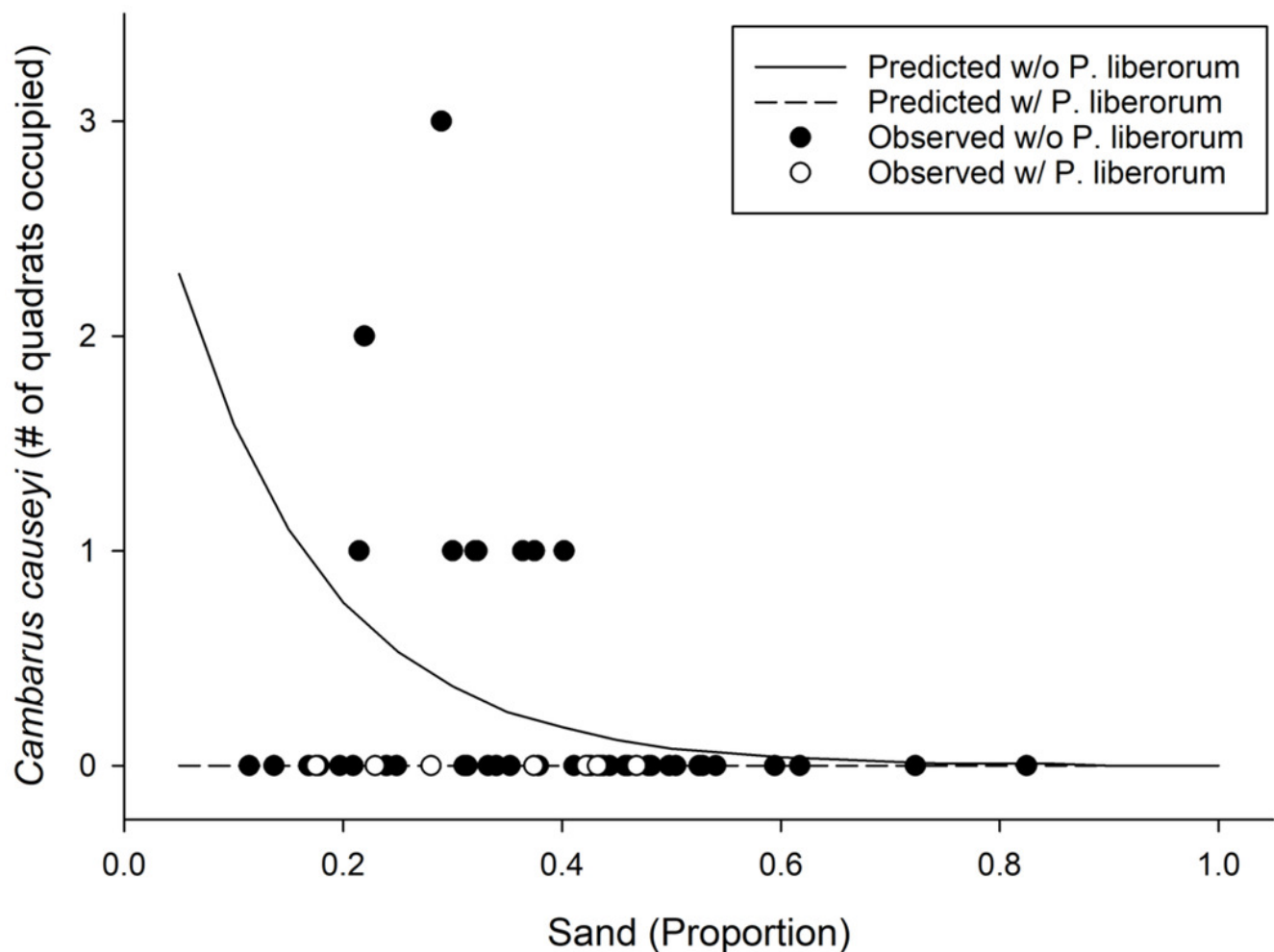



Figure 5

Results from our most supported model in the fine-scale habitat analysis.

Observed (points) and predicted (lines) results from our most supported model of *Cambarus causeyi* relative abundance using *Procambarus liberorum* presence and proportion of sand as predictor variables (Table 2).

Comparison of primer-probe assay to non-target crayfish species.

Comparison of *Cambarus causeyi* primer-probe assay against non-target crayfish species.

Locations of mismatches are underlined and in bold.

Species	GenBank accession numbers	Forward primer mismatch	F primer percent identity	Reverse primer mismatch	R primer percent identity	Probe mismatch	Probe percent identity
Cambarus causeyi	JX514477.1 DQ113443.1 OP673577 OP673578 OP673579 OP673580 OP673581 OP673583 OP673584	GCGGGTATAA CTATAGATC	100%	CTATGCTATT AACAGATCG	100%	ACTATTGTTAT CTTTGCCTGTG	100%
Cambarus hubbsi	MG872957.1	G**TA**GGTATAA CTAT**G**GATC	84%	CTATG**TTG**TT **G**AC**G**GATCG	79%	A**T**TATT**A**TTAT CTTT**A**CCTGTG	86%
Procambarus liberorum	KF827978.1 KF827979.1	G**TA**GG**A**ATAA CTATAGATC	84%	CTAT**A**CTATT AACAGA**C**CG	89%	A**T**G**T**TGTTAT CT**CT**A CCTGTG	82%
Faxonius williamsi	OP673582	GC**T**GG**A**ATAA CTAT**G**GATC	84%	CTAT**AT**TATT AAC**T**GATCG	84%	A**T**G**T**TA**C**TAT C**A**TT**A**CCTGTG	73%
Faxonius williamsi-	AY701252.1	GC**T**GG**A**ATAA CTAT**G**GATC	84%	CTAT**AT**TATT AAC**T**GATCG	84%	A**T**G**T**TA**T**TAT CTTT**A**CCTGTG	82%
Faxonius meeki meeki	AY701213.1	G**TA**GGTATAA CTAT**G**GATC	84%	CTAT**AT**TATT AAC**T**GATCG	84%	A**T**TATTGTTAT CTTT**A**CCTGTG	91%
Faxonius meeki brevis	AY701212.1	G**TA**GG**A**ATAA CTAT**G**GATC	79%	CTAT**AT**TATT AAC**T**GATCG	84%	A**T**TATT**A**TTAT CTTTGCCTGTG	91%

Candidate models for our fine-scale habitat analysis.

Candidate models relating *Cambarus causeyi* relative abundance to predictor variables (Sand, *P. liberorum*, Large Rock, Interpolated Maxent, Stream Dwelling Crayfish, Canopy Cover) with the number of parameters (K), delta corrected Akaike Information Criteria (AICc), and log likelihood (LL) values representing model performance. Estimates of parameter coefficients with standard errors in parentheses are given for each predictor variable when it is included in a model, whereas predictor variables omitted from a model are represented by (-).

Sand	*P. liberorum*	Large Rock	Interpolated Maxent	Stream Dwelling Crayfish	Canopy Cover	K	ΔAICc	LL
-7.331 (5.001)	-20.401 (16810.675)	-	-	-	-	4	0.00	-27.107
-	-20.860 (19040.000)	-2.196 (1.189)	-	-	-	3	0.89	-28.729
-6.290 (3.634)	-	-2.084 (1.285)	-	-	-	4	0.95	-27.582
-7.252 (4.977)	-	-	-	-	-	4	1.02	-27.616
-7.832 (5.224)	-20.904 (19922.950)	-	-0.827 (1.430)	-	-	3	1.71	-29.144
-	-20.443 (19897.237)	-	-	-	-	5	2.12	-26.938
-	-	-	-	-	-	2	2.40	-30.618
-7.728 (5.260)	-	-	-0.568 (1.468)	-	-	3	2.83	-29.701
-	-	-1.631 (1.174)	-	-	-	4	3.09	-28.653
-	-20.457 (19168.973)	-	-0.551 (1.357)	-	-	4	3.91	-29.060
-	-	-	-	-0.271 (0.916)	-	3	4.57	-30.574
-	-	-	-0.244 (1.373)	-	-	3	4.63	-30.602
-	-	-	-	-	-0.128 (1.158)	3	4.65	-30.612