FINAL PERFORMANCE REPORT
South Carolina State Wildlife Grant SC-T-F21AF03627
South Carolina Department of Natural Resources
Reporting period: October 1, 2021 – December 31, 2025

Project Title: Determining the effects of human disturbance on the ecology and conservation status of populations of at-risk crayfish in the genus *Distocambarus*

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Project Goal: The goals of this study were to understand the ecology of at-risk *Distocambarus* species and to investigate how land use and management practices impact their distribution, trophic ecology, and genetic diversity.

Objectives:
1) Identify and characterize critical habitats of *Distocambarus* (focal species *D. carlsoni*)
2) Conduct phylogenetic analysis of the genus *Distocambarus*
3) Compare morphologies of individuals collected from across the range of *Distocambarus*
4) Determine, through the use of stable isotope analyses, whether sources of nutrition for these crayfishes are related to land use

Progress: Completed

Abstract: The southeastern United States is a global hotspot for crayfish diversity, with more than 300 described species in the region. Unfortunately, nearly one fifth of the North American crayfish species are currently threatened with extinction, and efforts to protect crayfishes have been hindered by a lack of biological and ecological knowledge. Crayfishes can have significant impacts on their environment and can function as ecosystem engineers due to their ability to create habitat for other species and redistribute sediments and nutrients through their burrowing activities. Burrowing crayfishes have been understudied, in part, due to the effort required to sample and observe these species. In SC, the genus *Distocambarus* represents a group of burrowing crayfishes that are referred to as 'semi-terrestrial' crayfish because they inhabit areas like Piedmont prairies and forests. *Distocambarus* species are restricted to a small portion of the South Carolina and Georgia Piedmont and are of conservation concern due to their small ranges that make them hypersensitive to anthropogenic habitat modifications. Two *Distocambarus* species have been petitioned for federal listing (*D. carlsoni* and *D. youngineri*), and all species are designated as highest or high conservation priority by the State Wildlife Action Plans of South Carolina and Georgia. A better understanding of this group is essential for developing effective long-term conservation strategies. Research from this project has identified critical habitat characteristics using distribution models, characterized the genetic makeup of *Distocambarus* crayfishes through genomic and mitochondrial sequencing, quantified and compared body dimensions across species, and assessed nutritional patterns of *Distocambarus* crayfishes across land uses. These results show the important role of wetlands for these crayfishes, demonstrate previously unrecognized genetic diversity within this genus, show high variability in body dimensions, and demonstrate that *Distocambarus* can function as predators

high in the food chain. Overall, this research has been critical to better understanding the taxonomy, ecology, and conservation status of this important group of burrowing crayfishes.

Accomplishments:

Objective 1. Identify and characterize critical habitats of *Distocambarus*

Introduction

The southeastern United States is a global hotspot for crayfish diversity, with more than 300 described species in the region. Unfortunately, nearly one fifth of the North American crayfish species are currently threatened with extinction, and efforts to protect crayfishes have been hindered by a lack of biological and ecological knowledge (Richman et al. 2015). Crayfishes can occupy both semi-terrestrial and aquatic ecosystems, and thus their conservation and management are directly related to prairie and moist woodland habitats, as well as strictly aquatic ecosystems. Burrowing crayfishes comprise only 15% of total crayfish species diversity, while representing 32% of the critically-imperiled crayfish species (Welch and Eversole 2006). Burrowing crayfishes have been understudied with respect to their ecology, population dynamics, and genetic diversity, in part, due to the substantial effort often required to sample and observe these species.

Distocambarus is a genus composed of five described species, all of which are primary burrowers. *Distocambarus* is restricted to a small portion of the South Carolina (SC) and Georgia (GA) Piedmont region and are of conservation concern due to their small ranges that make them hypersensitive to anthropogenic habitat modifications (Richman et al. 2015). Two *Distocambarus* species have been petitioned for federal listing (*D. carlsoni* and *D. youngineri*), and all species are designated as highest or high conservation priority by the State Wildlife Action Plans of South Carolina and Georgia. Information on *Distocambarus* species is limited to the original species descriptions (Hobbs 1981; Hobbs 1983; Hobbs and Carlson 1983, 1985; Fitzpatrick and Eversole 1997), and studies on distribution and habitat use of *D. crockeri* and *D. youngineri* (Welch et al. 2007; Eversole and Welch 2010, 2013). *Distocambarus* species can be found in Piedmont prairie habitats, a habitat that has greatly declined over the past century (Welch and Eversole 2006). Since habitat degradation and loss are known to be some of the greatest threats to crayfish in general (Richman et al. 2015), it is essential to identify and characterize critical habitats of *Distocambarus* crayfishes to develop effective long-term conservation and management strategies.

This objective was met through multiple approaches including field sampling for *Distocambarus* crayfishes, multiple analyses of habitat characteristics (i.e. burrow temperatures, sediment characteristics around burrows, and habitat associations), and the development of distribution models for the genus *Distocambarus*. Much of the work for this objective was derived from the masters' thesis of West Liberty University Student Kathryn Schulz.

Methods

Field sampling began February 2022 and lasted through April of 2024, sampling a variety of habitats including roadside ditches, fields, and forested areas like those shown in Figure 1. In 2022, we sampled 169 sites over 11 counties in SC. For each site, data collections included crayfish species identifications, date, drainage basin, habitat type, coordinates, current weather conditions. Of the 169 sites, 86 contained historic records of *Distocambarus* while 83 sites were not previously known to have *Distocambarus*. In 2023, collections occurred in late February and early April during peak *Distocambarus* activity. Fifty new sites were sampled. Additional

sampling was conducted in February and April of 2024, including a total of 241 sites, some of which had previously been sampled.

Figure 1. A collage of images showing habitats and sampling activities for *Distocambarus* crayfishes.

Burrow conditions

To quantify burrow environments, 11 temperature probes (HOBO Pendant MX Temp/Light, MX2202) were set to record hourly temperature data from July 2022 to April 2023. Loggers were placed at 5 sites inside known *Distocambarus* burrows and on the surface near known burrows. Soil samples were collected between 2022 and 2023 from three habitat types: forests, fields, and roadside ditches, as well as from chimneys created by *Distocambarus youngineri*. Samples were sent to the West Virginia University Soil Testing Laboratory (Morgantown, WV) for routine soil analysis, soil organic matter, soil electrical conductivity, and microelement analyses. There, the following elements and chemical properties were quantified for each sample: pH, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), aluminum (Al), sodium (Na), boron (B), zinc (Zn), manganese (Mn), nickel (Ni), copper (Cu), percent organic matter (% OM), electrical conductivity (EC dS/m), and percent saturation ratio (PSAT). All data from 2022 and 2023 were combined into one final dataset which was then used for analysis.

Distribution modelling and habitat associations

Historic and contemporary records of *Distocambarus* spp. were queried from the National Museum of Natural History, Smithsonian Institution online database, as well as West Liberty University's Astacology Lab and USDA Forest Service Southern Research Station records, totaling 261 presence records from 1978 to 2023. These records were from the following HUC8 subbasins: Stevens, Upper Savannah, Seneca, Saluda, Enoree, and the Lower Broad River drainages. We created three separate SDMs with separate variables and the presence-only

data. The Maximum-entropy (Cunha 2020) tool in ArcGIS Pro (Version 3.2.0) was used to develop distribution models.

To determine which bioclimatic variables were informative for the SDMs, we ran the Maximum-entropy tool with all 19 bioclimatic variables from WorldClim (Table 1, Fick and Hijmans 2017) and presence-only data for the first model (Model A). In the second model (Model B), we filtered out the bioclimatic variables that were not contributing to predictability and retained those that were, following recommendations from Liu (2022). Model B contained a Digital Elevation Model (DEM) of South Carolina, informative bioclimatic variables from Model A, and the Soil Survey Geographic Database (SSURGO) Soil Hydrologic layer. In the third model (Model C), we included a digital elevation model, all bioclimatic variables, SSURGO Soil Hydrologic layer, and the Southeast Conservation Adaption Strategy's (SECAS) Grassland layer. The grassland layer is composed of 5 categories: 4 = Known grassland buffer, 3 = Potentially compatible management within grassland geology (undeveloped powerline right-of-way or perennial forbs and grasses), 2 = Potentially compatible management outside of grassland geology (undeveloped powerline right-of-way or perennial forbs and grasses), 1 = Grassland geology, and 0 = grassland less likely. For models B and C, the Maxent output was classified into five categories based on relative occurrence rate; 0-60%, 61%-70%, 71%-80%, 81%-89%, and 90%-100%. A fourth model (Model D) was a compilation of the three other models.

We conducted an initial round of field sampling to add additional data to our distribution model and then conducted a second round of field sampling to test the distribution model we had developed. Phase one was conducted from February 21 - April 6 2024 where we sampled based on site accessibility, suitable habitat, and abundance of the target species. Phase two was conducted from April 7 - April 8, 2024, where predetermined sites were selected using the random point toolbox in ArcGIS. For this process, 5,000 random points were overlayed onto our study area, where we then chose 15 sites from each relative occurrence rate category: totaling 75 chosen sites. Sites were composed of roadside ditches (RSD), powerline rights-of-way (PROW), fields, and forests.

For both phases of field sampling, A 30 person-minute timed search for burrows was conducted at each site. When burrows were encountered, the substrate was mechanically excavated as outlined by Loughman (2010). If water was not reached during excavation, a one-gallon jug of water was used to flood the burrow, which was then plunged to dislodge the crayfish from the resting chamber. We identified fine-scale habitat characters at each site, noting the presence of the following variables: low-lying depressions, perched water at the surface, standing and moving water, and *Juncus* spp. and sedges (*Carex* spp.). If the site had standing or running water present, dipnets were used to survey for *Distocambarus* spp.

During phase two, we implemented a transect and quadrat method to standardize the habitat characterization. We collected habitat data from $1m^2$ polyvinyl chloride (PVC) pipe quadrats and flags along a 10m × 10m transect. We placed the first quadrat down haphazardly, following Quebedeaux et al. 2023 methodologies, then proceeded to take quadrat data at each end of the transects and the center. We searched everywhere in the quadrats as well as along the transects due to low species abundance at each site. A 30 person-minute timed search (e.g. 15 minutes x 2 people = 30 person minutes) was conducted at each site to aid in the detection of *Distocambarus* species.

Data analysis

Daily average temperatures from inside burrows and from surface areas for three sites that successfully logged temperature were plotted throughout the 9-month deployment period to

visualize temperature differences between habitats. Means and standard deviations of soil chemistry data were compared across habitats.

We used the field validation results produced using model D to isolate informative variables. The Maxent output produced a partial response of both categorical and continuous variables to test each variable's influence on the probability of presence. Through this analysis, variables not contributing to the probability of presence could be identified and removed. To evaluate a model's performance, a Receiver Operating Characteristic curve (ROC) was generated to produce area-under-the-curve (AUC). An AUC from 0.7-1 was considered to indicate a well-performing model (Mandrekar 2010). Standard deviation (SD) was also calculated to observe the variability of the model's predictions.

Crayfish assemblages were based on site-level presence and compared with the habitat variables collected. We developed a Jaccard dissimilarity matrix because species data was presence/absence based (Oksanen et al. 2024). We used a non-metric multidimensional scaling (nMDS) analysis to visualize general community structure patterns as a non-constrained approach. This non-constrained method tests the differences and similarities of the species data without the influence of environmental variables to explain the dissimilarities (Faith et al. 1987). The nMDS plot was created using the Vegan (Version 2.6-8) package in R (Oksanen et al. 2024). All significant values were plotted in the nMDS ordination space and further examined through distance-based redundancy analysis to model how environmental variables explain community structure (dbRDA, Oksanen et al. 2024). Distance-based redundancy analysis (dbRDA) is a constrained ordination approach that directly explains the dissimilarities in species composition by examining their relationship with specific variables (Faith et al. 1987). To assess how variation in the community composition is attributed to the environmental variables, we conducted a permutational multivariate analysis of variance (PERMANOVA, 2000 permutations) based on Jaccard dissimilarity indices (presence-absence matrix).

Results

Burrow conditions

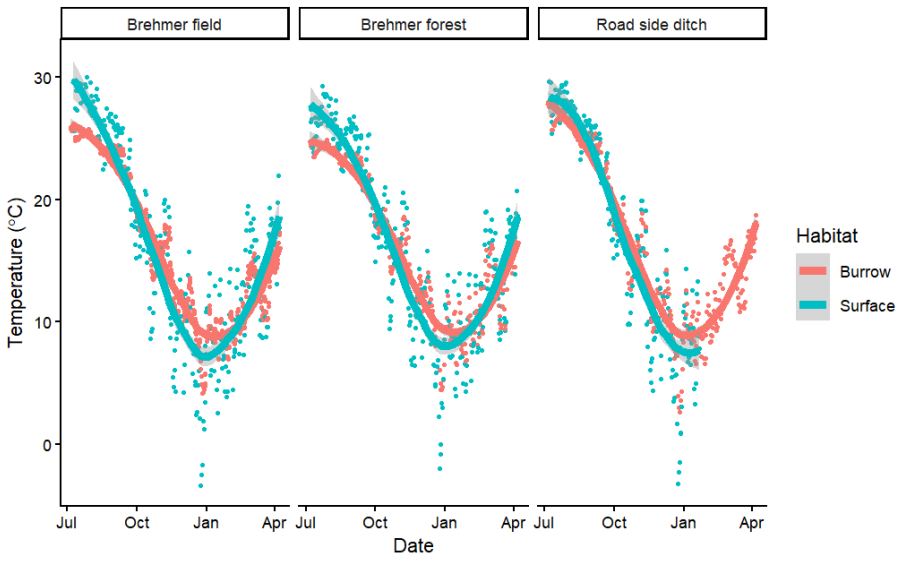

Figure 2. Temperature patterns for burrow and surface conditions from July 2022 to April 2023 showing the moderating effect of *Distocambarus* burrows on temperature.

For comparison of burrow and surface temperature, burrow temperatures tended to stay cooler than surface conditions in the summer. In the winter, burrows stayed warmer than surface conditions (Figure 2). A total of 793 samples were collected and analyzed over the two years. The means and standard deviations for each microelement and chemical parameter were calculated and grouped by habitat type, chimney sample, and year (Table 1).

pH: The mean pH across all samples was slightly acidic (mean = 5.1). Mean pH was comparable across habitat types, with forests, fields, and RSDs averaging 5.2 (SD = 0.4), 5.1 (SD = 0.5), and 5.0 (SD = 0.4), respectively. Chimney samples showed elevated pH compared to non-chimney samples across all habitats, most notably in forests (chimney: mean = 5.6, SD = 0.3; non-chimney: mean = 5.2, SD = 0.4) and RSDs (chimney: mean = 5.2, SD = 0.3; non-chimney: mean = 5.1, SD = 0.4), with a smaller difference in fields (chimney: mean = 5.3, SD = 0.4; non-chimney: mean = 5.1, SD = 0.5).

Phosphorus (P): Mean P was highest in fields (mean = 27.2 ppm, SD = 22.7) and RSDs (mean = 22.0 ppm, SD = 37.8), and lowest in forests (mean = 18.3 ppm, SD = 15.5). High SDs throughout indicate considerable variability within groups. Chimney samples had higher P in forests (mean = 23.2 ppm) and fields (mean = 30.6 ppm) compared to non-chimney samples (forests: mean = 13.5 ppm; fields: mean = 23.8 ppm), though this pattern was reversed in RSDs (chimney: mean = 17.7 ppm; non-chimney: mean = 26.3 ppm).

Potassium (K): Mean K was similar across habitat types: forests (mean = 42.1 ppm, SD = 18.2), fields (mean = 37.4 ppm, SD = 24.0), and RSDs (mean = 35.9 ppm, SD = 18.9). Chimney samples consistently showed elevated K across all habitats, with the largest difference in forest chimneys in 2023 (chimney: mean = 61.3 ppm, SD = 20.9; non-chimney: mean = 36.4 ppm, SD = 22.1).

Calcium (Ca): Forests had substantially higher Ca (mean = 449.4 ppm, SD = 258.9) than fields (mean = 213.6 ppm, SD = 90.0) and RSDs (mean = 258.4 ppm, SD = 122.6), with high variability in all groups. Chimney samples were elevated relative to non-chimney samples in forests (chimney: mean = 536.5 ppm; non-chimney: mean = 362.2 ppm) and RSDs (chimney: mean = 283.5 ppm; non-chimney: mean = 233.3 ppm), with minimal difference in fields (chimney: mean = 232.5 ppm; non-chimney: mean = 194.7 ppm).

Magnesium (Mg): Forests had considerably higher Mg (mean = 131.7 ppm, SD = 82.1) than fields (mean = 57.8 ppm, SD = 33.3) and RSDs (mean = 63.8 ppm, SD = 33.8). The chimney effect on Mg was modest across habitats, with slightly elevated values in RSD chimneys (chimney: mean = 68.7 ppm; non-chimney: mean = 58.9 ppm) and minimal differences in forests and fields.

Iron (Fe): Fe showed strong year-to-year variation, with 2023 means consistently higher than 2022 across all habitats. Overall means were comparable across forests (mean = 246.1 ppm, SD = 105.3), fields (mean = 218.3 ppm, SD = 90.4), and RSDs (mean = 225.7 ppm, SD = 136.2). Chimney samples had markedly higher Fe than non-chimney samples across all habitat types, most pronounced in forest chimneys in 2023 (chimney: mean = 423.0 ppm, SD = 137.0; non-chimney: mean = 213.1 ppm, SD = 137.9).

Aluminum (Al): Mean Al was similar across forests (mean = 365.7 ppm, SD = 117.3), fields (mean = 319.6 ppm, SD = 85.8), and RSDs (mean = 326.3 ppm, SD = 137.8). Chimney samples consistently showed lower Al than non-chimney samples across all habitats, with the largest reduction in RSD chimneys (chimney: mean = 263.9 ppm; non-chimney: mean = 388.8 ppm) and forest chimneys (chimney: mean = 274.9 ppm; non-chimney: mean = 356.5 ppm).

Sodium (Na): Na was slightly higher in fields (mean = 61.4 ppm, SD = 24.0) and RSDs (mean = 59.0 ppm, SD = 11.6) than in forests (mean = 48.8 ppm, SD = 26.0). The chimney effect on Na was minimal and inconsistent across habitat types and years, with overlapping means and SDs throughout.

Boron (B): Forests had notably lower B in non-chimney samples (2022: mean = 0.5 ppm, SD = 1.5; 2023: mean = 5.0 ppm, SD = 4.8) compared to fields and RSDs, though 2023 values were substantially higher across all groups. Chimney samples had higher B than non-chimney samples in forests (chimney: mean = 3.4 ppm; non-chimney: mean = 2.8 ppm) and RSDs (chimney: mean = 7.8 ppm; non-chimney: mean = 6.8 ppm). The large year effect in B warrants attention in downstream analyses.

Zinc (Zn): Mean Zn was highest in RSDs (mean = 5.9 ppm, SD = 5.9), followed by forests (mean = 3.3 ppm, SD = 2.8) and fields (mean = 3.3 ppm, SD = 3.1). High SDs relative to means indicate substantial within-group variability. Chimney samples had higher Zn than non-chimney samples in forests (chimney: mean = 4.3 ppm; non-chimney: mean = 2.3 ppm) and RSDs (chimney: mean = 6.9 ppm; non-chimney: mean = 5.5 ppm), but lower Zn in fields (chimney: mean = 3.9 ppm; non-chimney: mean = 2.7 ppm).

Manganese (Mn): Forests had the highest Mn (mean = 87.4 ppm, SD = 80.4), followed by fields (mean = 62.9 ppm, SD = 57.3) and RSDs (mean = 40.2 ppm, SD = 49.0), with high variability throughout. Chimney samples had lower Mn than non-chimney samples in RSDs (chimney: mean = 32.8 ppm; non-chimney: mean = 47.7 ppm) and forests (chimney: mean = 72.3 ppm; non-chimney: mean = 102.5 ppm), but higher Mn in fields (chimney: mean = 66.7 ppm; non-chimney: mean = 59.1 ppm).

Nickel (Ni): Ni levels were low across all groups. Forests had the highest mean Ni (mean = 1.0 ppm, SD = 2.2), while fields (mean = 0.3 ppm, SD = 0.6) and RSDs (mean = 0.3 ppm, SD = 0.5) were comparable. No consistent chimney effect was observed, and SDs exceeded means in most groups, indicating highly skewed distributions likely driven by outliers.

Copper (Cu): Cu was low and comparable across habitat types: forests (mean = 0.9 ppm, SD = 0.4), fields (mean = 0.8 ppm, SD = 0.5), and RSDs (mean = 1.2 ppm, SD = 1.1). Chimney samples had slightly elevated Cu in RSDs (chimney: mean = 1.3 ppm; non-chimney: mean = 1.1 ppm) with minimal differences in forests and fields.

Organic Matter (%OM): Forests had the highest organic matter (mean = 3.3%, SD = 1.4), followed by RSDs (mean = 1.9%, SD = 0.8) and fields (mean = 2.0%, SD = 0.8). Chimney samples had slightly elevated %OM compared to non-chimney samples in forests (chimney: mean = 3.9%; non-chimney: mean = 2.7%) and fields (chimney: mean = 2.1%; non-chimney: mean = 1.8%), with minimal difference in RSDs.

Electrical Conductivity (EC ds/cm): EC values were uniformly very low across all groups (mean = 0.0–0.1 ds/cm, SD ≤ 0.1), with no meaningful differences across habitat types, chimney presence, or years.

Phosphorus Saturation (PSAT): PSAT was highest in fields (mean = 6.0%, SD = 5.3) and RSDs (mean = 4.1%, SD = 5.7), and lowest in forests (mean = 3.9%, SD = 3.3). Chimney samples showed notably higher PSAT than non-chimney samples in fields (chimney: mean = 7.2%; non-chimney: mean = 4.8%) and forests (chimney: mean = 5.2%; non-chimney: mean = 2.7%), though this pattern was weaker in RSDs. The large SDs throughout indicate high within-group variability in phosphorus saturation.

Distribution Modeling

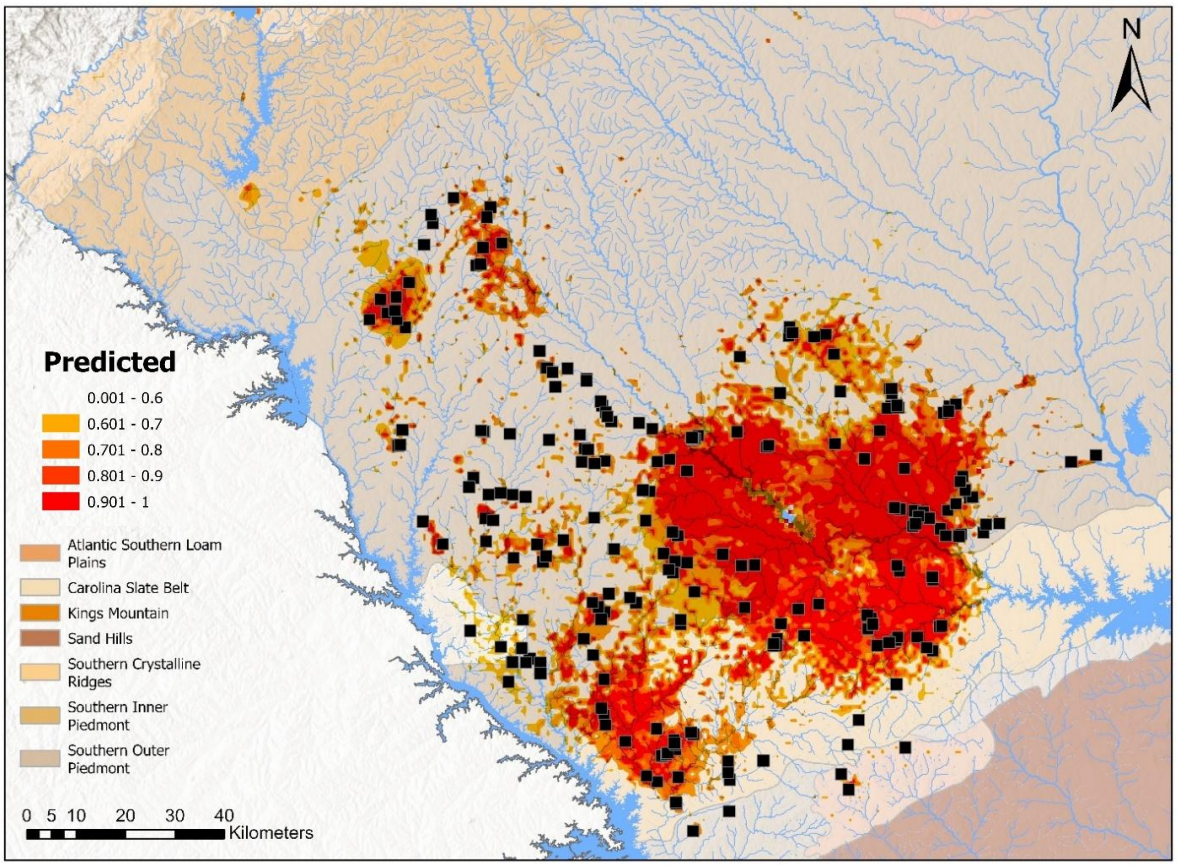

Figure 3. Distribution model for *Distocambarus* crayfishes in SC showing each of the 241 sampling sites as black squares.

The model predicted 70 new sites supporting *Distocamabarus* populations, resulting in a 29% predictive success rate for the combined SDMs. The AUC for the first Maxent model was 0.9346 (Model A) with a Standard deviation of 0.1951. Model B had an AUC of 0.9402, with a standard deviation of 0.1905. Model C had an AUC of 0.9090 with a standard deviation of 0.2425. All three models indicated a good fit with the dataset and displayed low variation from the mean. Model D (Figure 3) represents a compilation of all three models.

Environmental variable modeling

Distocambarus crockeri was most present in SECAS grassland group 1 (55%, grassland geology) but was near equally as present in group 0 (42%, grassland not likely). *Distocambarus carlsoni* was most present in group 0 (64.3%) followed by group 2 (14.3%, Potentially compatible management outside of grassland geology). *Distocambarus hunteri* and *D. youngineri* were both mainly in group 1 (50%, 4 occurrences total), and *D. youngineri* was most present in group 1 (Table 4). The lowest elevation with *Distocambarus* presence was 105.5 meters while the highest was 260.1 meters with a mean occurrence at 160.5 meters. The most dominant (most frequent) SSURGO hydrology groups for all species were groups B and C (46.4% and 30.4%, respectively). For *Distocambarus crockeri* and *D. hunteri*, the most frequent

group was group B (71.9% and 75%, respectively). The most frequent group for *D. carlsoni* and *D. youngineri* was group C (50% for both species, Table 5).

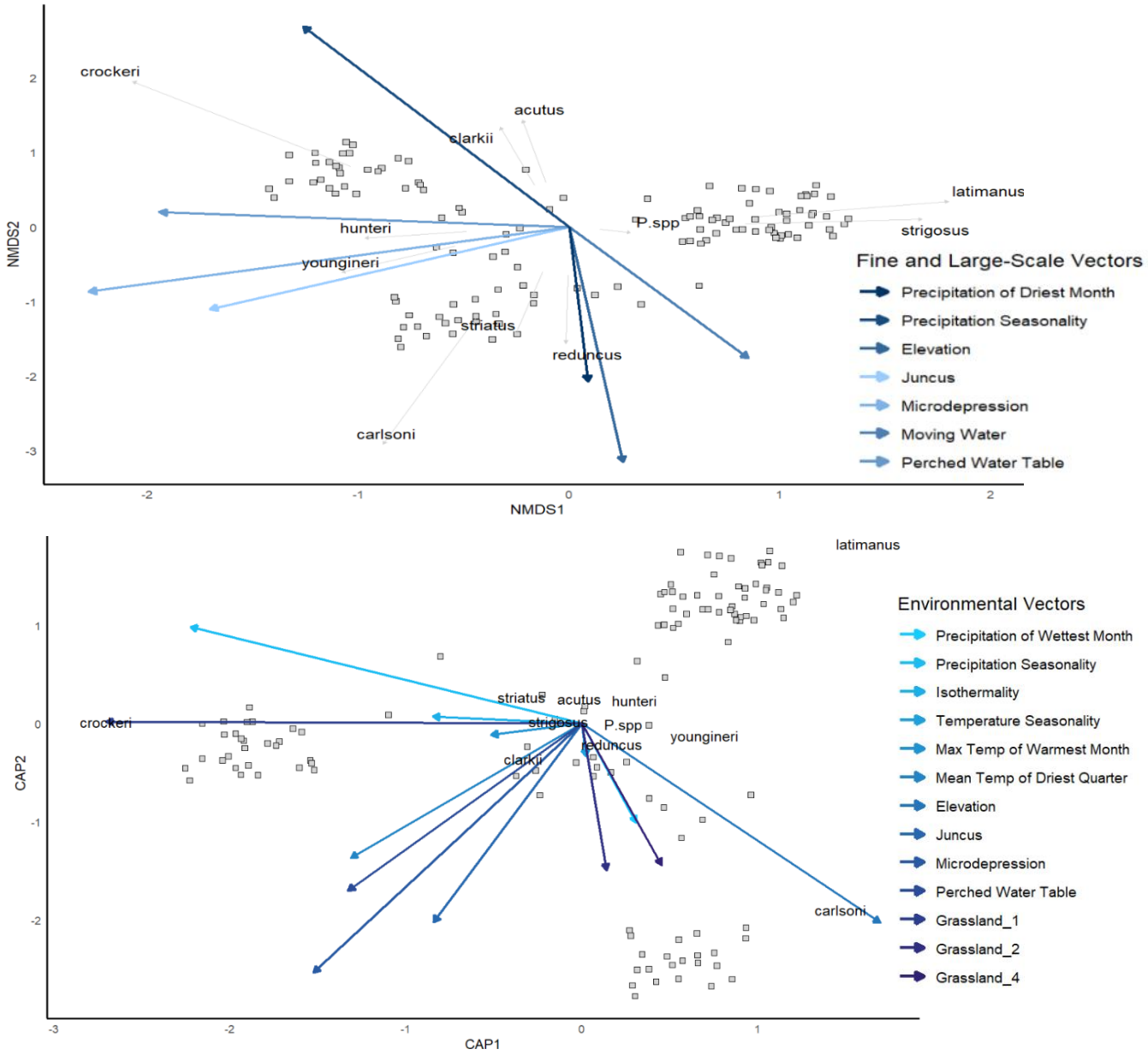

Figure 4. Non-metrics multidimensional scaling (nMDS; Top) and distance-based redundancy analysis (Bottom), visualizing significant variables as vectors with each point representing species composition at each site.

The nMDS and dbRDA results were congruent and indicated that Precipitation Seasonality (bio 15), SECAS grasslands, and elevation exert the strongest influence on site-level species composition (p = 0.001, nMDS; p < 0.006, dbRDA; Table 3). Bio 15 had the lowest coefficient of variation (14.31). Contradictory results between nMDS and dbRDA were observed for Precipitation of Driest Month (bio 14), which was significant (p = 0.003, R^2 = 0.0671) in the nMDS but not significant (p > 0.05) in dbRDA. Similarly, the dbRDA indicated that Temperature Seasonality (bio 4) (p = 0.0001, F = 8.8751), followed by Max Temperature of Warmest Month (bio 5) (p = 0.0003, F = 3.3538), then by Mean Temperature of Driest Quarter (bio 9) (p =

0.0017, F = 5.2051), then Isothermality (bio 3) (p = 0.0024, F = 4.6153), and lastly Precipitation of Wettest Month (bio 13) (p = 0.0034, F = 4.3678) were all highly significant, while nMDS did not support those variables as significant (Table 2).

Fine Scale Habitat Modeling

The most significant fine-scale variable in nMDS analyses was low-lying depression presence (p = 0.004, R^2 = 0.0929), followed by the presence of *Juncus* (p = 0.021, R^2 = 0.0643), then perched water table presence (p = 0.023, R^2 = 0.0598), and lastly moving water (p = 0.024, R^2 = 0.0597 (Figure 4). Distance based redundancy analyses suggested low-lying depressions were the most significant variable in influencing overall species composition (p = 0.0013, F = 5.3136), followed by the presence of *Juncus* spp. (p = 0.0105, F= 3.6001, Table 3; Figure 4). All other fine-scale habitat variables were not significant for the dbRDA analysis. The significant values for the presence of low-lying depressions and *Juncus* were congruent in both nMDS and dbRDA analyses (p < 0.05, Table 3).

Permutational analysis of variance results indicated that Max Temperature of Warmest Month (bio 5) (p = 0.004, F = 7.1989, R^2 = 0.03802), Temperature Seasonality (bio 4) (p = 0.004, F = 6.9423, R^2 = 0.03666), and Isothermality (bio 3) (p = 0.004, F = 6.9740, R^2 = 0.03683) were the most significant environmental variables (p = 0.004 for all variables). The only significant fine-scale habitat variables were the presence of a low-lying depression (p = 0.0030, F = 5.1096, R^2 = 0.02698) and a perched water table (p = 0.0095, F = 3.8433, R^2 = 0.02030, Table 6). The residual values from our PERMANOVA suggest that approximately 49.3% of the variation in species composition is explained by the environmental variables we used in our analysis.

Discussion

Our analyses of burrow conditions highlight the important role that burrows play in moderating temperatures for these crayfish. These moderating effects were especially important during cold winter periods when burrows remained above freezing while surface conditions dropped below freezing. Our distribution models for *Distocambarus* proved to be effective, evidenced by strong model performance metrics. While an overall predictive success rate of 29% was not ideal, it highlights the difficulties in accurately predicting species distribution for highly endemic groups like *Distocambarus*. This lower predictive accuracy could be explained by the broad-scale nature of the environmental variables used, which may overgeneralize the specific microhabitats that these species require.

Our nMDS and dbRDA analyses indicated that precipitation seasonality is a significant climatic factor influencing the distribution of *Distocambarus* species. However, the strength and direction of this association appear to vary among species—potentially placing some in areas with lower rainfall variability and more consistent water availability, and others in contrasting hydrological conditions. Regions with a stable precipitation pattern support wetland habitats and stream environments (Kovářová & Pokornỳ 2010), which in turn sustain the reproductive cycles of primary burrowers by ensuring a steady flow of water into burrows for egg incubation (Reynolds 2002).

Additionally, both nMDS and dbRDA identified SECAS grassland groups as significant predictors of community composition. The significant grassland categories—1 (Grassland geology), 2 (Undeveloped powerline right-of-way or perennial forbs and grasses), and 4 (Known grassland buffer)—may support burrowing crayfish populations by providing habitats that retain soil moisture, influenced by higher stem densities and soil type. (Barden et al. 2002; Szakacs

2020). Surprisingly, the 'grasslands not likely' group (0) was common across all species, including *D. crockeri* and *D. youngineri*, despite previous suggestions that these species have significant associations with grassland and prairie-like habitats. This finding supports the idea that the genus *Distocambarus* exhibits little, if any, prairie habitat specificity. For example, we expected *D. crockeri* to show a preference for the grassland geology group, as it has been suggested to be a terrestrial prairie habitat specialist (see Welch & Eversole 2006a, 2006b; Welch et al. 2007; Welch 2010; Eversole & Welch 2013). However, *D. crockeri* appears to show little preference between grassland and non-grassland habitats, suggesting greater plasticity than previously thought. In contrast, *D. carlsoni* exhibited a strong preference for the 'grasslands not likely' group, indicating a potential partitioning of habitat preference between *D. carlsoni* and *D. crockeri*. The developers of the SECAS grasslands layer have noted that roadside edges—often serving as refugia for many grassland and savanna species—can be overprioritized in analyses as grasslands, potentially convoluting results (Allen 2024).

Furthermore, both analyses identified elevation as a significant factor influencing community composition. This suggests that the genus may be geographically influenced by elevation within the Piedmont region, potentially due to its impact on water infiltration and hydrology (Condon & Maxwell 2015), which are both crucial landscape properties for terrestrial crayfish (Eversole & Welch 2010, 2013; Welch et al. 2007). The association with physiographic features (i.e. precipitation seasonality and elevation) and soil hydrology highlights the importance of conservation efforts in areas with little precipitation variability at elevations between 105 m and 260 m.

Soil hydrology was only important in nMDS and not dbRDA. Because *Juncus* presence was significantly important in both, we deemed it biologically relevant to include soil hydrology as it is known to heavily influence *Juncus* presence (McCurdy et al. 2022; Syranidou et al. 2017). As SSURGO hydrologic group preference was species specific, conservation areas should be designated at sites supporting SSURGO hydrologic groups B for *D. crockeri* and *D. hunteri*, and C for *D. carlsoni* and *D. youngineri*. Group B soils are characterized as deep and well drained, with moderately fine to moderately coarse textures and a moderate rate of infiltration and runoff, while group C soils have a slow rate of infiltration caused by a horizon that decreases water permeability (Natural Resources Conservation Service 2023). In many areas where we found *Distocambarus*, such as roadside ditches and powerline rights-of-ways, the soil may have been compacted by management using heavy machinery, such as ditch dredging, which, during periods of heavy precipitation, lessen the rate of water infiltration and increases water availability for reproduction and respiration. Interestingly, the hydrologic group with the least number of *Distocambarus* occurrences (1) was C/D, which are soils with a very slow infiltration rate caused by a high water-table (Natural Resources Conservation Service 2023).

Both nMDS and dbRDA analyses agreed that low-lying depressions and *Juncus* presence were the most significant variables influencing community composition. When sampling, we often discovered *Distocambarus* species in small depressions lower in elevation than the area immediately surrounding the colony. The difference in elevation in the depression was typically minute. In addition, these areas often exhibited higher soil moisture, potentially due to slower evaporation and higher water inundation as runoff from areas of higher elevation. Low-lying depressions in the landscape can also be likened to roadside ditches. Roadside ditches are dredged, which can lower the distance from the surface to the apparent water table (Buchanan et al. 2013; Dollinger et al. 2015). Likewise, these crayfish may associate with low-lying depressions for easier access to groundwater and surface runoff inundation, as freshwater is still necessary for reproduction, egg extrusion, and juvenile development (Bloomer 2021; Richardson, 2007). *Creaserinus fodiens*, another primary burrowing crayfish, has been observed occupying roadside ditches, with a preference for the lowest elevation points within

them (Norrocky 1991). Similar to, for example *Distocambarus crockeri*, the reproductive activity of *C. fodiens* coincided with the regional hydroperiod (Eversole & Welch 2013; Norrocky 1991). Additionally, *Cambarus dubius* has been documented using multiple habitat types, but were specifically concentrated around forested seeps where soil moisture was the highest (Loughman 2010b). Based on this evidence, it can be inferred that low-lying depressions are significant to *Distocambarus* species, as these microhabitat features provide better access to freshwater in a terrestrial environment, particularly when surface water is absent.

The presence of *Juncus* validates the observations made by Hobbs & Carlson (1983). The authors noted that sedges were present and dominant at all but one site supporting *Distocambarus crockeri*. Both *Juncus* (rushes) and sedges are helophytes, and their presence often indicates a waterlogged environment, which aligns with the habitat descriptions for all other species in the genus *Distocambarus*. *Distocambarus devexus* was described from a marshy area adjacent to a floodplain containing two helophytes and a water-loving tree: sedges, *Typha* sp. (cattails), and *Salix nigra* (Black Willow; Hobbs 1981). *Distocambarus carlsoni* was described from a swamp-like area adjacent to a tributary with common wetland trees and shrubs present, notably *Liquidambar styraciflua* (Sweetgum; Evans et al. 2022) and *Alnus rugosa* (Speckled Alder; Hobbs & Carlson 1983; Hurd & Raynal 2004). The *D. youngineri* type locality was moist and bordered a wooded pool, which supported a common wetland woody plant, *Nyssa* sp. (Tupelo; McCarron, McLeod & Conner 1998; Hobbs & Carlson 1985). Fitzpatrick & Eversole (1997) described *D. hunteri* from a wet runoff drainage with *Quercus phellos* (Willow Oak) present, another common wetland tree (Schlaegel et al. 1990). In each species, the presence of helophytes or water-loving woody plants at sites indicates that their soils remain wet or lose water at a much slower rate than other areas, further highlighting the need for moisture to satisfy life-history requirements. Interestingly, crayfish may aggregate near sedges due to the sedges' ability to store atmospheric oxygen in the rhizosphere (Syranidou, Christofilopoulos & Kalogerakis 2017). This may supplement oxygen for crayfish when conditions in the freewater of burrows become hypoxic or anoxic (Neculae et al. 2024). While maintaining moist gills, crayfish may still obtain oxygen from the air through diffusion (Grow & Merchant 1980; Stoeckel et al. 2021). Thus, the significance of helophyte presence for *Distocambarus* indicates the importance of consistently moist conditions to support reproduction, egg extrusion, and respiration.

The insights gained from this study are valuable for guiding future conservation strategies. Identifying fine-scale habitat features, such as *Juncus* presence and low-lying depressions, provides critical information for selecting sites most likely to support *Distocambarus* populations. Additionally, the strong influence of precipitation seasonality and elevation on species distribution suggests that conservation efforts could prioritize regions with stable moisture levels and elevation ranges between 105–260 m in the Piedmont region.

Moving forward, applying species distribution models alongside fine-scale habitat analyses will be instrumental in identifying areas where *Distocambarus* populations might be. Future studies should refine these models by incorporating additional fine-scale variables and exploring the role of climatic factors in shaping crayfish populations over time. As more data becomes available, future modeling should focus on the species level rather than the genus level to avoid over-generalization.

Objectives 2 & 3: Conduct phylogenetic analysis of the genus Distocambarus / Compare morphologies of individuals collected from across the range of Distocambarus

Introduction

When paired with morphological variability, an in-depth understanding of phylogenetic relationships among individuals can help inform species delimitations. To characterize the phylogenetic diversity and relationships among crayfish species in the genus *Distocambarus* we used two approaches. First, we generated sequence data for the mitochondrial cytochrome oxidase I (*COI*) gene for all collected samples. The *COI* locus, often referred to as the 'barcode' gene, is widely used in studies for species-level identification, taxonomy, and biogeography. We also generated a larger genomic dataset using a restriction enzyme associated DNA sequencing (RADseq) approach. The larger RADseq genomic dataset was used to provide higher phylogenetic resolution in cases where the *COI* locus appeared to have limited phylogenetic informativeness. A dedicated effort to elucidate *Distocambarus* systematics was initiated in the spring of 2022, with efforts focused on *Distocambarus carlsoni* and *D. youngineri*.

Methods

We isolated genomic DNA from ethanol-preserved tissue samples (gill tissue or pleopod segments) using Qiagen DNeasy Blood and Tissue kits, following manufacturer's protocols.

COI sequencing and phylogenetic analysis- For all 328 *Distocambarus* genetic tissue samples analyzed here, we amplified the mitochondrial *COI* region using primers designed to amplify the gene region in metazoan invertebrates (Folmer et al. 1994) and the following thermocycler reaction conditions: an initial 5 minutes at 95° C; 35 cycles of 95° C for 30 seconds, 30 seconds at 51° C, and 45 seconds at 72° C; followed by 10 minutes at 72° C. After PCR, excess nucleotides and primers were enzymatically removed using ExoSAP-IT, following manufacturer's protocols. Sequencing was conducted in two reactions (each using forward- and reverse- primers, respectively). Forward and reverse sequence chromatograms were assessed for quality and sequence agreement using Sequencher v. 5.4.6. Final sequences were aligned using the ClustalW algorithm in UGENE v5.2.1 (Okonechnikov, et al. 2012). Appropriate models of molecular evolution were determined with MEGA v11.0.13 (Tamura et al. 2021) and gene trees inferred using MrBayes 3.2.7 (Ronquist et al. 2012). Tree analyses were run for 10 million generations, with 2.5 million generation burn-in removed before constructing the final summary consensus tree.

RADseq sequencing and phylogenetic analysis- Using a subset of genetic tissue samples described above (n=104) that represented each of the described *Distocambarus* species and major groupings identified in the mitochondrial *COI* gene tree, we used a reduced representation genomic sequencing approach (3RAD) following the study design of Bayona-Vasquez et al. (2019). Genomic library preparation included normalization of isolated DNA (approximately 100 ng of DNA for each sample); enzymatic digestion (using *Xba*I, *Eco*R1, and *Nhe*I restriction enzymes), and adapter ligation with unique combinations of iTru5-8N and iTru7 adapters. After ligation, genomic libraries were purified using a magnetic bead clean-up and pooled equimolar for sequencing. These multiplexed libraries were size-selected using a Pippen Prep system and amplified using Kapa HiFi Hotstart and iTru primers. The multiplexed library was quantified with a Qubit fluorometer and submitted for sequencing on an Illumina platform.

Sequence data manipulation was conducted using the Stacks2 software pipeline (Catchen et al. 2013; Rochette et al. 2019). These steps included: demultiplexing raw sequence reads (via Stacks2's *process_radtags* program); denovo sequence assembly; filtering dataset to include

samples with varying levels of sequencing coverage; and alignment generation (via Stacks2's *populations* program). We explored phylogenetic signal in the dataset through assembly of data matrices based on sequence read thresholds on a per-sample basis (e.g. all samples; samples with at least 200,000 reads; samples with more than 2.5 million reads) as well as thresholds in taxonomic completeness in locus sequence alignments (e.g. each locus with at least 40%, 60%, and 80% taxonomic completeness). Phylogenies were inferred on concatenated sequence matrices using a maximum likelihood approach in IQTree2 (Minh et al. 2020). Appropriate models of molecular evolution were determined with IQTree2's implementation of ModelFinder (Kalyaanamoorthy et al. 2017), and maximum likelihood tree search implemented with 1,000 bootstrap replicates and node support assessed with ultrafast bootstrap support (Hoang et al. 2018).

We conducted a geometric morphometric analysis of select *Distocambarus* crayfishes from across their range. Measurements associated with these analyses included 27 standardized morphologies measured on the chelipeds (e.g. palm width, PalmW), carapace (e.g. post-orbital carapace length, POCL), and abdomen (abdomen width). Measurements were made on 117 individuals across 6 phylogenetic clades. Putative species were assigned based on where these individuals, or individual of similar color and morphology, were placed in the phylogenetic trees (see Objective 2). Morphological measurements were then used to calculate 14 metrics by taking the ratios of measured morphologies (e.g. carapace width:carapace length; rostrum width:rostrum length; etc.).

Results and Discussion

We generated mitochondrial *COI* sequence data for 328 *Distocambarus* individuals, which yielded 143 unique haplotypes. The *COI*-inferred gene tree showed complex patterns in which some species and interspecific relationships are strongly resolved (i.e. node support higher than 0.95 Bayesian posterior probability), while other species appear to be paraphyletic, and many lineages form a multi-species polytomy (Figure 5). The *COI* gene tree strongly supports the monophyly of *D. youngineri* and two lineages that are currently identified as *D. carlsoni*, (labeled *D. carlsoni* and *Distocambarus* species 1, in Figure 5). These three species form a strongly supported clade, although the interrelationships of the three lineages are weakly supported, with *D. youngineri* sister to the *Distocambarus* species 1 group. The weak support at this node reflects the tree search analysis' uncertainty in the relationship between the three lineages (e.g. whether *D. youngineri* is sister to *D. carlsoni*, *D.* species 1, or sister to a clade containing *D. carlsoni* and *D.* species 1). These three groups are geographically distributed in more northern localities relative to the remaining species of *Distocambarus*. The two *D. carlsoni* lineages have a northwest versus southeastern split, with a 'northern' *D. carlsoni* population in Anderson, Abbeville, and Laurens counties, and the 'southern' population (e.g. *D.* species 1) in Laurens, Greenwood, Newberry, and Saluda counties. *Distocambarus youngineri* is distributed adjacent and northeastern of the 'southern' *D. carlsoni* clade in Laurens and Newberry counties. An individual sample of *D. carlsoni* (Dca_00199), collected near the South Carolina-Georgia border area in Abbeville County, appears as a phylogenetic outlier, and is resolved with strong support sister to the larger *D. carlsoni* – *D. youngineri* clade.

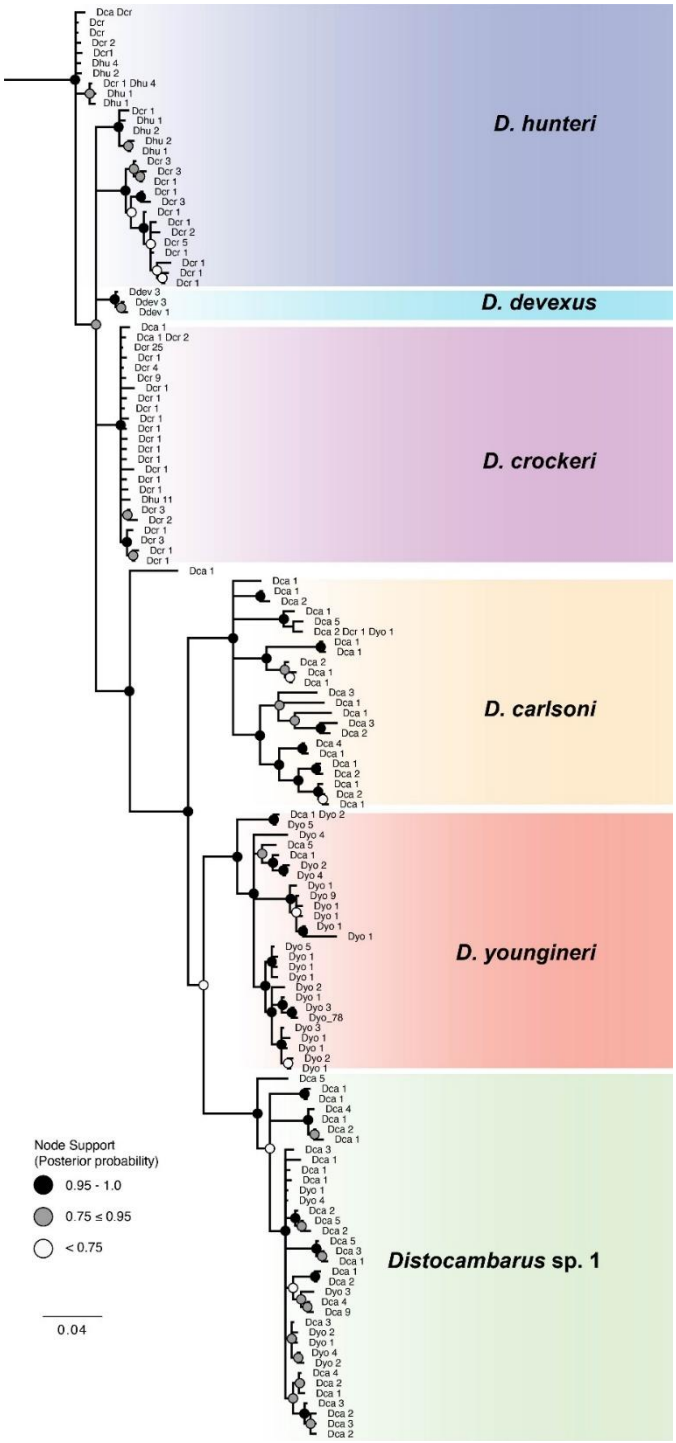

Figure 5. *Distocambarus* gene tree inferred with Bayesian analysis of COI sequence data. Duplicate sequences were removed prior to analysis, and numbers adjacent to tip labels indicate the number of individuals bearing that haplotype. Bayesian posterior probability (BPP) support values are indicated by shaded circles on each node, with black indicating strong support (BPP > 0.95), gray indicating modest support (BPP between 0.75 and 0.95), and white weak support (BPP lower than 0.75). Sequence data from a *Procambarus* species was used as a taxonomic outgroup in tree inference analysis and was pruned out of the figure.

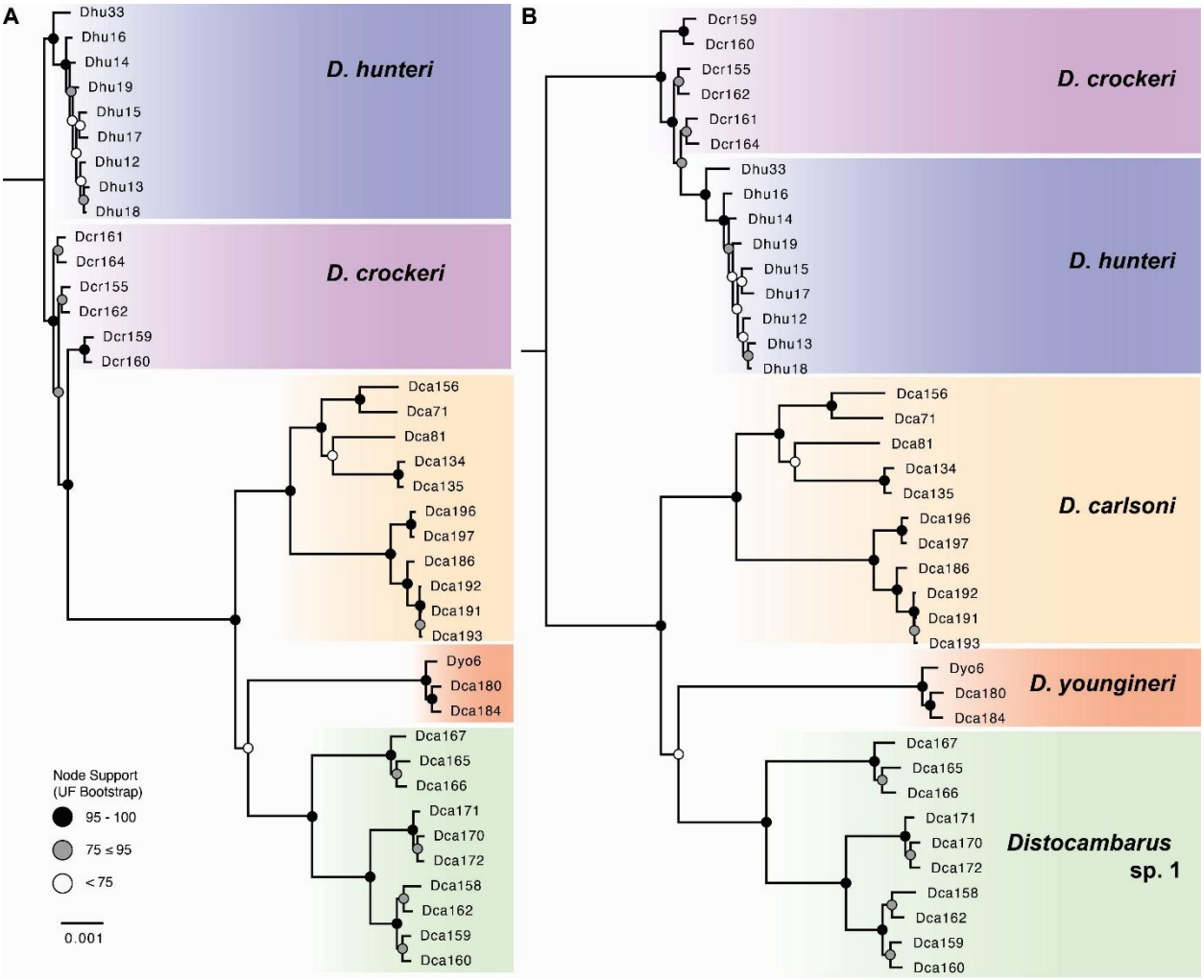

Figure 6. Phylogeny of *Distocambarus* inferred through maximum likelihood analysis of concatenated 3RAD sequence data, illustrated with a root at two alternate locations: A) rooted on the branch subtending *D. hunteri*, and B) rooted on the branch subtending the *D. carlsoni* and *D. youngineri* clade. Ultrafast bootstrap support is indicated by shaded circles on each node, with black circles indicating strong (UFBoot > 95), gray indicating modest (UFBoot between 75 and 95), and white weak support (UFBoot below 75).

In the *COI* gene tree, *Distocambarus hunteri* is resolved as paraphyletic, with a group of samples forming an unresolved polytomy at the base of the gene tree, and two additional *D. hunteri* sample groups in a moderately supported polytomy with *D. devexus*, *D. crockeri*, and the lineage containing *D. carlsoni* and *D. youngineri*. The geographic distributions of *D. hunteri* and *D. crockeri* are south of the *D. carlsoni-youngineri* clade, with some geographic overlap between collections of the two species.

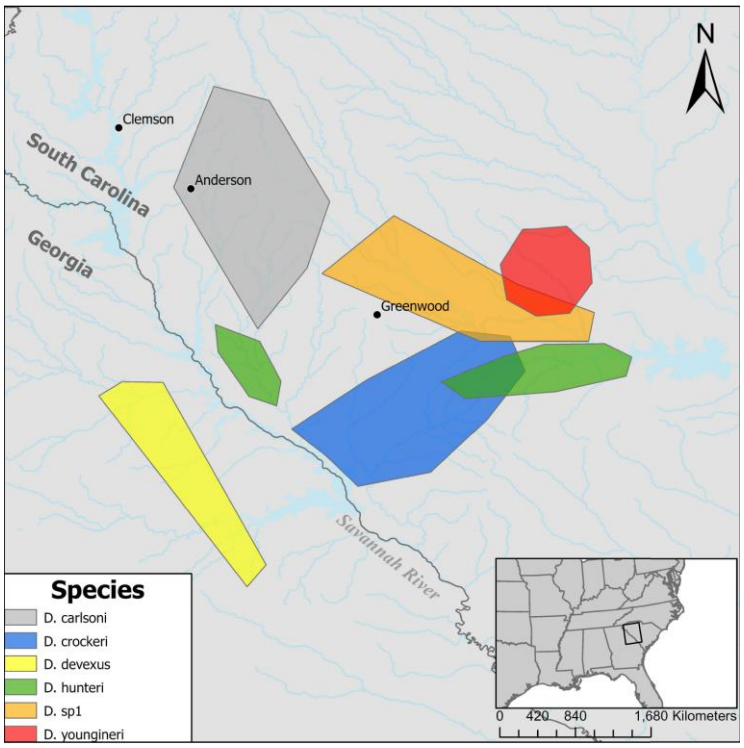

Figure 7. Generalized distribution of *Distocambarus* crayfishes based on COI-derived phylogenetic clades.

The RADseq sequencing yielded a range of 2,200 to 21.5 million reads per sample. The range of reads-per-sample was skewed towards a low number of reads for many samples: 21 samples with fewer than 10,000 reads; 29 had between 10,000 and 200,000 reads; 11 samples between 200,000 and 2.5 million reads; and the remainder had more than 2.5 million. We generated multiple data matrices based on a range of sequence read thresholds (e.g. alignments with all samples; alignments only containing samples with more than 200,000 reads; and dataset containing only samples with more than 2.5 million reads). For each of these datasets, we assessed the robustness of phylogenetic signal in the dataset by analyzing concatenated sequence alignments that were 40%, 60%, and 80% complete (i.e. each locus with at least 40, 60, or 80 percent of individuals represented with sequence data). We found that including samples with fewer than 2.5 million reads resulted in phylogenies that had inconsistent topologies across 40%, 60%, and 80% complete alignments, and therefore we present results for the more conservative dataset of samples with more than 2.5 million reads per sample. The final conservative dataset included 39 samples and yielded identical topologies across the range of 40%, 60%, and 80% complete matrices, which were composed of 7,316 loci, 3,120 loci, and 1,101 loci, respectively. All three samples of *Distocambarus devexus* and the *Cambarus* specimens intended to function as phylogenetic outgroups did not meet sequencing thresholds to include in our final tree.

Although the RADseq phylogeny that we infer is unrooted due to lack of a non-*Distocambarus* outgroup, most aspects of the topology are strongly supported. Regardless of the selection of root location in the RADseq tree, the topology based on the 80% complete alignments is characterized by several relationships that are strongly supported and concordant with those in the *COI* gene tree. For instance, samples of *Distocambarus carlsoni*, *D. youngineri*, and *D.* species 1 each form reciprocally monophyletic groups that are subtended by relatively long branches. As in the *COI* gene tree, these three species form a strongly supported clade, with *D. youngineri* weakly supported as the sister lineage of *Distocambarus* species 1, although their relationship receives moderately strong support (UFbootstrap support of 84) from analyses of the 40% complete alignment. Samples of *D. hunteri* are inferred as monophyletic, although the

choice of root in the phylogeny can influence the interpretation of its placement relative to *D. crockeri.* If rooted along the branch subtending the *D. carlsoni-youngineri* clade, the monophyletic *D. hunteri* group is nested within a paraphyletic *D. crockeri* (illustrated in Figure 6B). However, if rooted on the branch subtending *D. hunteri*, it is sister to all remaining *Distocambarus,* but *D. crockeri* appears paraphyletic relative to the remaining *Distocambarus* species (Figure 6A).

Our phylogenetic results strongly support the presence of two distinct lineages of *Distocambarus carlsoni,* and their close relationship with *D. youngineri.* These lineages comprise adjacent allopatric ranges that are north of collection localities of both *D. hunteri* and *D. crockeri* in South Carolina. Both datasets resulted in some degree of uncertainty regarding the relationships and distinctiveness of *D. hunteri* and *D. crockeri*, which may potentially represent a single species. Taxonomic conclusions drawn from these phylogenetic analyses of these two species should be considered preliminary until further data can provide an appropriate outgroup and phylogenetic rooting option, as well as the inclusion of *D. devexus* in the genomic dataset, which in the mitochondrial gene tree is resolved in a polytomy with populations of *D. hunteri* and *D. crockeri*. Results from the COI-based gene tree and the 3RAD phylogeny were used to develop a generalized distribution map for *Distocambarus* species (Figure 7).

There was substantial overlap in visualizations of multivariate morphometric data (Figure 8). While some differences in morphological characteristics were detected using the PERMANOVA approach (P = 0.004), dispersion was also significantly different among groups (P = 0.03), suggesting that differences in sample sizes could be contributing to statistically significant findings. These results may suggest that the lack of strong differences in morphological characteristics across phylogenetic clades could be related to a convergence of morphological traits in burrowing *Distocambarus* crayfishes.

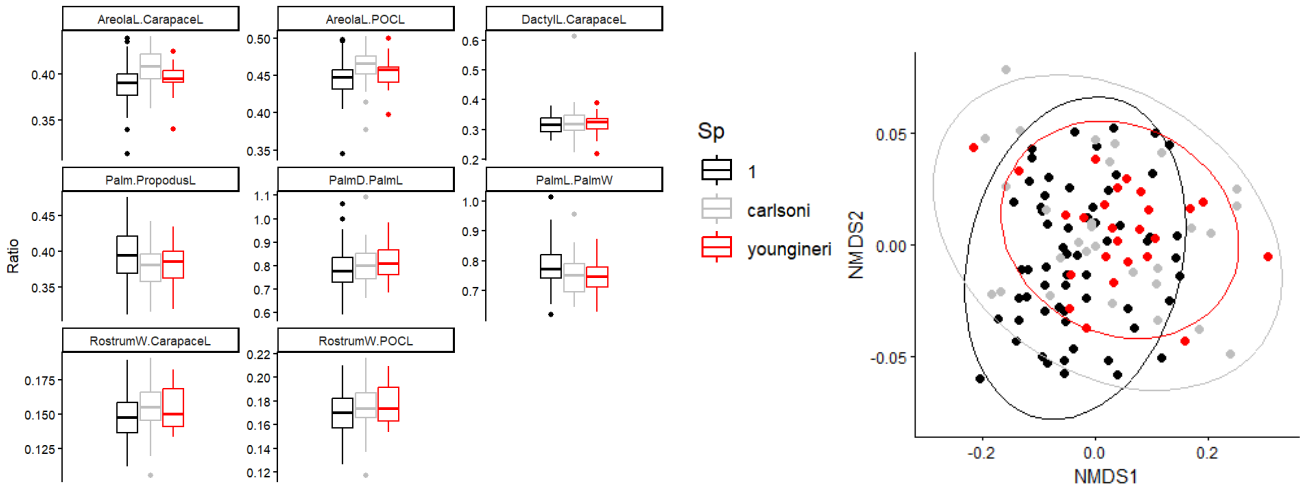

Figure 8. Morphometric analyses of select *Distocambarus* species. nMDS stress = 0.08; PERMANOVA P = 0.004.

Objective 4. Determine, through the use of stable isotope analyses, whether sources of nutrition for these crayfishes are related to land use

A manuscript addressing this objective has been submitted to a peer-reviewed journal for potential publication.

Does Habitat Matter? Assessing Food Resource and Trophic Niche Differences of Primary Burrowing Crayfishes in Multiple Habitats

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Introduction

Understanding species resource use and functional roles are invaluable in conservation planning and effective ecosystem management. Trophic interactions can greatly influence population sizes, community structure, and ecosystem processes (Estes et al. 2011; Frisch et al. 2014), thus understanding these interactions is key to effective ecosystem management. Resource use and functional roles of species are useful in understanding trophic interactions because they provide information such as diet and trophic level, which gives insight on energy transfer from producer to consumer. This information is imperative in understanding species' roles within ecosystems (Ginzberg & Arditi 2012), as well as the plasticity of those roles in different habitats. Despite the importance of understanding trophic dynamics, it remains poorly understood for many groups, including highly imperiled crayfishes.

Crayfish are an important component of freshwater and terrestrial ecosystems, often acting as keystone species and ecosystem engineers as well as playing a major role in food web dynamics (Creed Jr. & Reed 2004; Reynolds et al. 2013; Richman et al. 2015). Crayfish have been described as detritivores, omnivores, and carnivores, fulfilling several functional roles (e.g. primary consumers, scavengers, and predators; Thoma & Armitage 2008; Usio & Townsend 2008; Taylor & Soucek 2010; Stites et al.,2017). Nonetheless, our understanding of crayfishes functional roles are primarily based off of crayfishes within lotic habitats, with only two studies assessing burrowing crayfish living in semiterrestrial habitats (Bloomer et al. 2022; Graham et al. 2022). Comparisons between aquatic and terrestrial ecosystems have demonstrated clear differences in food web structure, with top-down control greater in water than on land (Shurin et al. 2002). Additionally, less herbivory, more decomposers, and more detrital accumulation often occur in terrestrial than aquatic ecosystems (Shurin et al. 2006). Thus, our current understanding of stream crayfish trophic roles cannot be extended to burrowing crayfishes.

Burrowing crayfish diets have been explored through foraging behavior, gut content and stable isotope analyses. Foraging studies have found reduced vegetation around burrow entrances (Loughman et al. 2015; Loughman et al. 2019), plant matter in resting chambers (Foltz II et al. 2018; Loughman et al. 2019), and remains of small vertebrates in burrows (Thoma & Armitage 2008). Studies also report burrowing crayfish exhibiting sit-and-wait predation at the entrance of their burrows (Thoma & Armitage 2008; Diehl et al. 2022; Graham et al. 2022), with prey varying from small worms and ants (Diehl et al. 2022; Graham et al. 2022) to larger vertebrates (e.g. salamanders and frogs; Thoma & Armitage 2008; McCormack & Coughran 2011). Similarly, studies assessing gut content and stable isotope analyses highlight the importance of invertebrates in crayfish diets (Bloomer et al. 2022; Graham et al. 2022), with crayfish trophic positions similar to other invertebrate primary consumers (Bloomer et al. 2022). Despite these recent gains in understanding burrowing crayfish diets, conducting comprehensive diet studies of burrowing crayfishes presents several challenges, including assessing all prey items, due to the small size, heterogeneity, and cryptic nature of some prey, and having to take into account the influence of seasonal changes, habitat variation, and environmental stressors. For example, techniques such as gut content analyses are only able to assess what is currently in crayfish guts, and crayfish gastric mills crush food items making them unidentifiable or biased towards food items that take longer to break down (Vannote & Ball 1972; Lorman & Magnuson 1978; Momot 1995). Stable isotope approaches can help remedy some of these issues with comprehensive diet studies determining the functional trophic role of a given crayfish in an ecological system. Specifically, stable isotope approaches integrate food sources over time and reveal what the animal has assimilated, allowing for a more inclusive diet estimation than other techniques. Because relative abundance of ^{13}C to ^{12}C ($\delta^{13}C$) changes little from prey to predator, and relative abundance of ^{15}N to ^{14}N ($\delta^{15}N$) increases by 3–4 ‰ per trophic level (Sweeting et al. 2007a, b), estimations can be made on the source of primary production ($\delta^{13}C$)

and organisms trophic levels ($\delta^{15}N$), providing an integrative food web perspective of diet. Nonetheless, the type of tissue used in stable isotope analyses can impact detection of recent changes in food sources, with different types of tissue having different turnover rates. For example, muscle tissue has a slower turnover rate (months to years) than crayfish digestive glands (days) (Hamilton et al. 2004). Additionally, most studies assess a limited number of sites and habitats, thus species variation within habitats and trophic plasticity based on species use of various habitat types has not been assessed.

Past studies have connected the trophic position of crayfishes with aspects of trophic ecology theory, such as trophic position typically increasing when crayfish body size increases (Roth et al. 2006; Larson et al. 2010; Kreps et al. 2016; Larson et al. 2017; Stites et al. 2017). In addition, trophic ecology theory predicts that a species trophic position will differ when habitat and resources change (Carscallen et al. 2012; Dixon et al. 2012; Sánchez-Hernández & Amundsen 2018). However, no studies have assessed variation in the trophic position of burrowing crayfishes among habitats. Burrowing crayfishes occur across many habitat types from wetlands that are frequently inundated (e.g. roadside ditches) to semiterrestrial habitats that experience surface water inundation more rarely (e.g. prairies), with single species populations occurring over a heterogeneous mix of habitats. Many crayfish are also omnivores, and most omnivore studies highlight their flexibility in feeding (Fagan 1997; Lancaster et al. 2005; Anderson & Cabana 2007; Olsson et al. 2008). Understanding the differences in food resources and trophic positions of species utilizing numerous ecosystem types is a basis for understanding food web dynamics and ecosystem function.

We used stable isotope approaches to examine the flexibility of nutritional resources, energy pathways, and functional role (i.e. trophic position) of two burrowing crayfish across two distinct habitat types. The Newberry Burrowing Crayfish, *Distocambarus youngineri* Hobbs & Carlson 1985 and the Piedmont Prairie Burrowing Crayfish, *D. crockeri* Hobbs & Carlson 1983 are both endemic to the Piedmont region of South Carolina, USA. Both species are often found in historical prairie habitats, poorly drained areas where the ground is saturated during wet parts of the year (Eversole & Welch 2010). However, they have distinct ranges, with *D. youngineri* found only in Newberry County, South Carolina and *D. crockeri* found within 5 counties throughout the Caolina Slate Belt ecoregion. Burrowing crayfish are a critically imperiled group, accounting for 15% of the United States crayfish species but representing 32% of critically imperiled crayfish (Welch & Eversole 2006; Bloomer et al. 2021). Additionally, *Distocambarus youngineri* is ranked as critically imperiled globally and subnationally (G1/S1), and at the highest level for South Carolina species of greatest conservation needs (SGCN). *Distocambarus crockeri* is ranked as vulnerable globally and subnationally (G3/S3), and at a high level for South Carolina SGCN. Despite their conservation status, their nutritional and energetic ecology are poorly understood. Understanding the trophic position of imperiled species is essential for effective conservation. These species may occupy unique roles in food webs, and changes in their trophic interactions could signal ecosystem instability or other threats. By identifying their trophic position, managers can predict cascading effects of their decline and design strategies that maintain ecological balance and resilience. Furthermore, there is an urgent need to improve management of these species and gain a better understanding of their ecological functions to guide management actions and preserve or enhance terrestrial ecosystems.

Materials and Methods

Study Area / Site Selection

To select habitat types and sites, we performed surveys of *Distocambarus* species throughout areas of the southern outer piedmont and slate belt ecoregions of South Carolina in February–March 2020 and 2022. Sites were sampled in the late winter/early spring due to the water table being highest during this time of year (Welch & Eversole 2006), increasing the chances of collecting primary burrowing crayfish without causing great destruction to their habitats. Our surveys identified *D. youngineri* and *D. crockeri* within numerous habitat types including fields, vernal pools, roadside habitats, and forests. For both species, several field and roadside populations were found. Thus, sites were selected for isotopic analysis in both field and roadside habitats. Field sites included areas maintained for powerline rights-of-ways, managed as wildlife openings (i.e. agriculture-like lands that are plowed and managed to grow crops and/or to attract wildlife for hunting), or farmlands. Roadside habitats refer to habitats that appear to have been created by the construction of roadways (e.g. roadside ditches).

Eight *D. youngineri* sites (4 field and 4 roadside habitats) were selected within Newberry County, SC based on accessibility and abundance of target species (Fig. 1, Online Resource 1). Two field sites were farmlands actively managed for agriculture (*D. youngineri* Field 2 and 3) and two field sites were managed powerline right-of-way. Surface water was not observed at the roadside habitats, all of which were actively maintained by mowing. Ten *D. crockeri* sites (5 field and 5 roadside habitats) were selected within Edgefield, McCormick, Greenwood, and Saluda counties, SC based on accessibility and abundance of the target species (Fig. 1, Online Resource 1). All field sites were managed as wildlife openings and all roadside habitats were managed as roadside ditches, retaining water during the winter and spring months and during heavy rains events.

Stable Isotope Sampling

Distocambarus crockeri and *D. youngineri* sites were sampled for stable isotope collections March 2021 and March 2022, respectively. Mean temperature and rainfall data were checked from the National Oceanic and Atmospheric Administration, National Weather Service (https://www.weather.gov/wrh/Climate) and were similar in both sampling years, indicating that data from the two years should be comparable with regard to interannual variations in weather. Crayfish collection occurred via burrow excavation at all *D. youngineri* sites and all *D. crockeri* field sites. Crayfish were collected with dipnets at *D. crockeri* roadside habitat sites because all *D. crockeri* roadside sites were ditches filled with water. Target species were the only or most abundant crayfish species collected at sites. At each site, 6–12 adult (total carapace length [CL] ≥ 15 mm) crayfish of the target species were captured, with a minimum of 3 individuals of each sex (Online Resource 1, 2). Carapace length was measured for most collected *D. youngineri* and *D. crockeri*. Samples of potential food sources such as the dominant live vegetation (grasses, *Plantago major*, *Solidago* spp., *Taraxacum officianalis*) detrital leaves (*Quercus* spp., *Carya* spp., *Nyssa* spp., etc.) and terrestrial invertebrates (Carabidae, Hymenoptera, Isopoda, Lycosidae, Oligochaeta, etc.) were also collected from the same sites. Samples of all potential food sources were made by hand at all *D. youngineri* sites and all *D. crockeri* field sites. At *D. crockeri* roadside habitat sites, vegetation was collected by hand and all other food sources were collected with dipnets. We classified all invertebrates into primary and secondary consumer groups. All herbivore invertebrates were classified as primary consumers, and all carnivore or omnivore invertebrates were classified as secondary consumers. All crayfish and food sources were kept on ice in the field and frozen for later analysis.

Laboratory Analysis

Samples were prepared for stable isotope analysis at the U.S. Forest Service, Southern Research Station, Center for Bottomland Hardwoods Research, Aquatic Conservation and Ecology Team lab (Oxford, MS). At least 10 mg of each sample was dried at 60 ºC for at least 48 hours. Abdomen tissue samples from tail muscle were used from individual crayfish. Vegetation and detritus were rinsed to remove debris before drying, while guts of large invertebrates (e.g. crickets and grasshoppers) were removed to avoid gut contamination. Whole bodies of small invertebrates (e.g. isopods) were used in analyses. Numerous invertebrates were grouped in one sample to meet the 10 mg required weight. After drying, samples were shipped to Washington State University Stable Isotope Core Laboratory (Pullman, WA) for stable isotope measurements of carbon and nitrogen isotopic ratios. Samples were ground to homogenous consistency and approximately 2 and 3 mg of animal and plant tissue were used in analyses.

For carbon 13:12 and nitrogen 15:14 isotopic analyses, samples were converted to N_2 and CO_2 with an elemental analyzer (ECS 4010, Costech Analytical, Valencia, CA). These gases were separated with a 3m gas chromatography column and analyzed with a continuous flow isotope ratio mass spectrometer (IRMS) (Delta PlusXP, Thermofinnigan, Bremen) (Brenna et al. 1997; Qi et al. 2003). Isotopic reference materials were interspersed with samples for calibration. Contribution of ^{17}O was corrected by the IRMS software using the Santrock correction (Santrock et al. 1985). Quality control was performed using reference materials with similar composition to samples and interspersed with samples in all analytical sequences. Reference materials of NIST1547, a peach leaf, and NIST1577b, a bovine liver, were used to assure both repeatability and reproducibility of isotope results and element compositions. Analytical precision for samples were less than 0.3 parts per mil.

Data Analysis

Isotope signatures were used to calculate crayfish trophic positions (TP) using the equation:

$$\mathrm{TP}_{\mathrm{crayfish}} = \mathrm{TP}_{\mathrm{base}} + (\delta^{15}\mathrm{N}_{\mathrm{crayfish}} - \delta^{15}\mathrm{N}_{\mathrm{base}}) / \Delta\mathrm{N}$$

where base refers to invertebrate primary consumers (Post 2002), TP_{base}= 2, and $\delta^{15}N_{base}$ refers to the average $\delta^{15}N$ of primary consumers at the site. The ΔN was set at 3.40 based on an established average fractionation rate (Vander Zanden & Rasmussen 2001; Post 2002;). Taxa groups were combined into categories: detritus, live vegetation, invertebrate primary consumers (e.g. Hymenoptera, Isopoda, Euryuridae, Lumbricidae, Gastropoda, and Oligochaeta), and invertebrate secondary consumers (e.g. Carabidae, Lycosidae, Geophilidae, and Pisauridae), as well as male and female crayfishes shown separately.

To assess if *D. youngineri* and *D. crockeri* within field and roadside habitats share a similar trophic niche, we assessed the percentage of stable isotope niches overlap among groups. We calculated standard ellipse areas corrected for small sample sizes (SEAc) as a measure of the stable isotope niche size for each species within each habitat type using the Stable Isotope Bayesian Ellipses (SIBER) R package (Jackson et al. 2011). Standard ellipses contain ~40% of the individuals in the stable isotope space (Jackson et al. 2011). The degree of stable isotope niche overlap (percentage of standard ellipse overlapped) between field and roadside habitats for *D. youngineri* and *D. crockeri,* separately, was calculated as the proportion of the non-overlapping area between the two standard ellipses.

To assess if crayfish resource use differed between habitat types or sex, we used linear mixed effect (lme) models for *D. youngineri* and *D. crockeri* separately. In each model, trophic position or $\delta^{13}C$ was the response variable, habitat and sex were fixed effects, and site was the random effect. In addition, we used linear mixed-effect models to assess if there were shifts in crayfish resource use with size. In each model, trophic position or $\delta^{13}C$ were response variables,

crayfish size was the predictor variable, and site was the repeated measure. Because we did not measure the CL of all crayfish collected, linear models assessed the subset of the specimens measured. Crayfish trophic positions and sizes were log-transformed so that data was normally distributed. All tests were conducted using the *lmer* package in R (Kuznetsova et al. 2015). Tukey's post-hoc analysis was used to assess which habitat type or sex differed for any significant ($p < 0.05$) results.

To determine relative contribution of resources (detritus, vegetation, and other invertebrate) to crayfish diets, we used Bayesian mixing models in R package *simmr* (Parnell & Inger 2016) for *D. youngineri* and *D. crockeri* separately. Models were run with unconverted $\delta^{13}C$ and $\delta^{15}N$ values of individual crayfish in mixing models. We used corrected values of 2.4‰ for $\delta^{15}N$ and 0.40‰ for $\delta^{13}C$ for vegetation (McCutchan Jr. et al. 2003) and 3.23‰ for $\delta^{15}N$ and 0.47‰ for $\delta^{15}N$ for invertebrates (Vander Zanden & Rasmussen 2001). Values of food sources must be corrected before being used in mixing models to account for differences in isotope ratios between a consumer and its food source due to metabolic processes (Ben-David et al. 1997; Phillips 2012). Using corrected values ensures more accurate interpretation of the data.

Results

In total, 92 and 75 *D. youngineri* and *D. crockeri* were collected, respectively (Table 1). For *D. youngineri*, 40 males and 52 females were collected, with 47 and 45 specimens collected from fields and roadside habitats, respectively. For *D. crockeri*, 37 males and 38 females were collected, with 34 and 41 specimens collected from fields and roadside habitats, respectively.

Trophic positions

Distocambarus youngineri had a broad trophic position (Table 1), ranging from 1.89–4.25. *Distocambarus youngineri* trophic niche was similar within fields and roadside habitats for both the carbon and nitrogen stable isotope axes (Fig. 2). There was little variation in niche size between habitat types (SEA_c: field = 24.47; roadside habitat = 27.82), with 38% overlap observed between *D. youngineri* trophic niche in each habitat (Fig. 2). There was no variation in *D. youngineri* trophic position between sex (lme model: $F_{1,89} = 0.06$, $p = 0.80$), habitat type (lme model: $F_{1,89} = 0.67$, $p = 0.44$), or size ($F_{1,90} = 1.29$, $p = 0.26$). Additionally, there was no variation in *D. youngineri* $\delta^{13}C$ levels between sex (lme model: $F_{1,89} = 0.18$, $p = 0.68$), habitat type ($F_{1,89} = 0.24$, $p = 0.64$), or size (lme model: $F_{1,90} = 0.00$, $p = 0.99$). We observed similar structure among sites, with male and female *D. youngineri* $\delta^{15}N$ levels similar to secondary consumers in both field and roadside habitat sites (Fig. 3ab; Online Resource 2).

Distocambarus crockeri had a broad trophic position (Table 1), ranging from 1.47–4.80. *Distocambarus crockeri* trophic niche differed between field and roadside habitats along both the carbon and nitrogen stable isotopes axis (Fig. 2), with *D. crockeri* in fields having more enriched carbon and nitrogen levels. *Distocambarus crockeri* trophic niche size was also larger in fields than roadside habitats (SEA_c: field = 17.36; roadside habitat = 6.85). Similarly, *D. crockeri* trophic position and $\delta^{13}C$ levels in fields was higher than those from roadside habitats (lme model of TP: $F_{1,69} = 9.36$. $p = 0.02$; lme model of $\delta^{13}C$: $F_{1,69} = 27.50$. $p < 0.001$) (Fig. 2). *Distocambarus crockeri* within fields had $\delta^{15}N$ levels above our sampled secondary consumers in 40% of field sites, indicating that they can serve as tertiary consumers in field habitats (Fig. 4a; Online Resource 2). *Distocambarus crockeri* within roadside habitats had $\delta^{15}N$ levels with similar values as secondary consumers (Fig, 4b; Online Resource 3). There was no variation between male and female *D. crockeri* trophic positions (lme model: $F_{1,69} = 0.09$, $p = 0.76$) or $\delta^{13}C$ levels (lme model: $F_{1,69} = 0.99$, $p = 0.32$) (Fig. 4ab; Online Resource 3). There was also no relationship between *D. crockeri* size and trophic position (lme model: $F_{1,69} = 1.19$, $p = 0.28$).

Food resources

Our Bayesian mixing models suggest that invertebrates contributed up to 82% of the tissue composition of *D. youngineri*, ranging from 77–82% among males and females in fields and roadside habitats (Table 2). Results indicated that *D. youngineri* relied heavily on primary (e.g. Euryuridae, Lumbricidae, Gastropoda) and secondary consumers (e.g. Carabidae, Geophilidae, Pisauridae), attributing up to 43% and 38% of their tissue composition, respectively. Mixing models also indicated that detritus and vegetation contributed up to 13% and 17% of crayfish tissue, respectively.

Our Bayesian mixing models suggest that invertebrates contributed up to 84% and 59% of *D. crockeri* tissue composition within fields and roadside habitats, respectively (Table 2). Results indicated that *D. crockeri* within field habitats relied heavily on secondary and primary consumers, attributing up to 50% and 34% of their tissue composition, respectively. Conversely, *D. crockeri* within roadside habitats relied primarily on primary consumers (up to 42% of tissue composition), with all other groups (e.g. secondary consumers, vegetation, detritus) contributing evenly (~20%) to its tissue composition. Mixing models also indicated that detritus and vegetation are a more important part of the tissue composition of *D. crockeri* in roadside habitats (up to 42%) than those in fields (up to 21%).

Discussion

Understanding the trophic position of imperiled species is essential for effective conservation and management to maintain ecological balance and resilience within ecosystems. Thus, our goal was to understand the trophic and nutritional ecology of two imperiled crayfishes, *D. youngineri* and *D. crockeri*, within two habitat types. Results of our stable isotope analyses show a high degree of flexibility in nutritional resources, energy pathways, and trophic position for two burrowing crayfishes. As one of the first isotopic analyses performed on primary burrowing crayfishes, our results indicate that burrowing crayfish may operate at higher trophic levels than expected, with previous studies indicating that while crayfish are omnivores they prefer eating vegetation and serve similar roles as primary consumers or levels between primary and secondary consumers (Gherardi et al. 2004; Johnston et al. 2011). While *D. youngineri* and *D. crockeri* have omnivorous diets, invertebrates comprise a large portion of their energetic and nutritional needs, and both species appear to serve as secondary or tertiary consumers within their ecosystems. Omnivory is advantageous because it provides flexibility to feed at both lower and higher trophic levels, allowing individuals to survive periods when high-quality food is not available, but consuming higher-quality food when it is available (Diehl 2003; France 2012; Kratina et al. 2010). In the current study, up to 84% of *Distocambarus* spp. tissue originated from invertebrate prey and up to 40% of *Distocambarus* spp. diets included vegetation and detritus. Burrowing crayfish are known to exhibit predatory behaviors (Loughman 2010; Graham et al. 2022) and, combined with our results, suggest that *D. youngineri* and *D. crockeri* actively target primary and secondary consumers when foraging for food. Variability in feeding activity likely has implications for crayfish roles in controlling invertebrate populations (Ushio & Townsend 2004, 2008) and detrital processing (Momot 1995; Usio 2000). Historically, crayfishes were thought to have an opportunistic omnivore lifestyle, sustaining themselves on plant material and, if the opportunity arises, consuming animals (Momot 1995; Bloomer et al. 2022). Our results highlight that energy and nutrition for burrowing crayfish are derived mainly from invertebrate food sources, although plant matter and detritus are still an important component of their diets.

In the current study, differences were observed between habitats for both isotopes, with higher isotopic values and larger trophic niche breadth detected for *D. crockeri* in fields (wildlife openings) than roadside habitats (roadside ditches). Differences in *D. crockeri* $\delta^{13}C$ and $\delta^{15}N$ levels are likely due to a wider variety of plants (i.e. C_3 and C_4 plants) and invertebrates available in fields. The average $\delta^{13}C$ levels of *D. crockeri* in fields (mean $\delta^{13}C$ levels = -16‰)

were higher than most C_3 vegetation $\delta^{13}C$ levels (mean $\delta^{13}C$ levels = -27‰). C_4 plants (i.e. millet, and corn) generally have higher $\delta^{13}C$ values (i.e. -13‰; Fry 2006) and were planted in field sites during spring and summer months. Due to our sampling timing, these vegetation sources were not present and thus not collected and used in data analyses. Nonetheless, high $\delta^{13}C$ levels indicate C_4 vegetation may be an essential diet attribute of *D. crockeri* in fields. Similarly, differences in $\delta^{15}N$ levels are likely due to differences in invertebrate diversity present within each habitat type, with at least 7 and 4 invertebrate families present within field and roadside habitats, respectively (Online Resource 1, 2). While invertebrate abundance was not formally accessed, there were more secondary consumers present at field than roadside sites, which may impact crayfish feeding strategies.

Trophic position and niche differences were detected between field and roadside habitats for *D. crockeri,* but not for *D. youngineri*. Differences in trophic position and niche of the same species may result from differences in types of food consumed, food chain length, or environmental and ecological conditions (Beatty 2006; Olsson et al. 2008; Sánchez-Hernández & Amundsen 2018). Field and roadside ditch management can directly impact vegetation and invertebrate composition, changing the ecosystems' trophic structure (Wilson et al. 2005; Dollinger et al. 2017; Jakobsson et al. 2018). For example, herbicide use can reduce the diversity and abundance of plants and invertebrates within habitats (Haughton et al. 1999; Sullivan & Sullivan 2003; Wilson et al. 2005), while planting crops may increase food type availability if both naturally occurring and planted vegetation are present within the system. Food availability also shifts throughout the year. Food may be limited in habitats where food is clumped or patchily distributed (Fero et al. 2007), as well as in managed fields where herbicide use and agricultural practices may remove large portions of vegetation and invertebrates during periods of the year (House 1989; Brust 1990; Haughton et al. 1999; Sullivan & Sullivan 2003). Unlike *D. crockeri* roadside habitats, which were often ditches inundated with water during portions of the year, *D. youngineri* roadside habitats were more like fields (i.e. grassy open areas without a trench). Thus, differences between species may be due to environmental and community composition differences among sampling sites, and not species-specific traits. Because species ranges do not overlap, we could not assess sites with both species to understand if differences are due to species-specific traits or site composition.

Although differences were not detected between *D. youngineri* trophic positions or niches in field and roadside habitats, management of sites did impact them. *Distocambarus youngineri* in farmed fields (Field 2 and 3) had higher nitrogen levels than non-farmed fields, likely due to the fertilization of these fields. Both isotopic niche and dietary contributions of *Procambarus clarkii* were affected by the introduction of fertilizers in rice-crayfish coculture systems (Sun et al. 2023). Because vegetation was nitrogen-enriched due to fertilization, crayfish isotopic signatures showed high levels of nitrogen similar to levels that would be present if high amounts of invertebrates were eaten. Nonetheless, dietary contribution analysis indicated that crayfish in fertilized fields did not consume more animal material, but rather they consumed more particulate organic matter (POM). Furthermore, increased $\delta^{15}N$ values in *D. youngineri* habitats may explain why analyses showed similar $\delta^{15}N$ values between all *D. youngineri* and field *D. crockeri* but consumptions of more vegetative food sources by *D. youngineri*.

Even though secondary consumers were present in both field and roadside habitats, *D. crockeri* populations in roadside habitats relied more on detrital and vegetation sources than secondary consumers. This could be due to crayfish foraging style (Bloomer & Taylor 2022; Diehl et al. 2022; Graham et al. 2022). *Distocambarus crockeri* within fields may exhibit predatory behavior, similar to the Little Brown Mudbug (*Lacunicambarus thomai*; Graham et al. 2022), while those in roadside habitats may exhibit an opportunistic foraging behavior, similar to the Slenderwrist Burrowing Crayfish (*Fallicambarus petilicarpus*; Bloomer et al. 2022). Foraging behavioral

differences may be impacted by the presence of predators (Pecor & Hazlett 2003; Diehl et al. 2022; Graham et al. 2022). Crayfish in fields without surface water may have higher predation risks, exhibiting more anti-predator behavior and less foraging, eating mostly plants and animals near their burrow, while crayfish in roadside habitats with surface water may have less predation risks and thus, spend more time foraging. These factors may also explain why field crayfishes had a larger trophic niche.

Competition can cause shifts in diets and trophic positions, with a reduction in niche size often occurring (Harrington et al. 2009; Jackson & Britton 2014; Baudry et al. 2024). *Cambarus latimanus* co-occurred with *D. crockeri* at all roadside sites, but *D. crockeri* was the only crayfish collected from all field sites. Additionally, *D. crockeri* had a smaller trophic niche in roadside than field sites. Conversely, *D. youngineri* co-occurred with *C. latimanus* at roadside sites, yet, trophic position and stable isotope analysis support that *D. youngineri* could target invertebrates as its main food sources and operate as secondary consumers in both habitat types. Because we did not collect large numbers of *C. latimanus* from each site, we did not include them in our analyses. Nonetheless, the shift in *D. crockeri*'s diet from field to roadside sites may be due to niche partitioning (Stites et al. 2017). Similarly, Stites et al. (2017) showed that *Barbicambarus cornutus* and *B. simmonsi* occupied a higher trophic level than co-occurring crayfish species in the same habitat. *Barbicambarus spp.* were secondary consumers or predators, while co-occurring crayfish species were primary consumers. Furthermore, competition for food sources could be occurring at sites where *D. crockeri* and *C. latimanus* are both present, with *D. crockeri* shifting to a lower trophic level and *C. latimanus* potentially occupying the higher tertiary consumer level. Further analyses of these sites are required to determine the effect of *C. latimanus* on *D. crockeri* diet and trophic position.

Contrasting results of the relationship between body size and trophic position have been documented for crayfishes, with most studies assessing differences between juveniles and adults (Roth et al. 2006; Taylor & Soucek 2010; Larson et al. 2017; Stites et al. 2017), and no studies assessing burrowing species. We did not see any correlations between size (range, *D. crockeri* = 16–29 mm CL; *D. youngineri* 16–35 mm CL) and trophic position. Similar to Stenroth et al. (2006), larger crayfishes were just as reliant on animal tissue as smaller crayfishes, with crayfish potentially obtaining their carbon and nitrogen from different sources (i.e. consuming other invertebrates for nitrogen and detritus for carbon). We considered all *Distocambarus* species used in our study adults (CL ≥ 16 mm), based on the smallest form 1 *Distocambarus* species (*D. carlsoni*) collected having a 16 mm CL (unpublished data). Thus, we were not able to assess if diets changed between juvenile and adult *Distocambarus* spp. Including smaller individuals in future isotopic analyses could provide a better insight into diet changes, as juveniles were more commonly found occupying surface water of both roadside ditches and fields.

While isotopic analyses can integrate food sources over several months to years, dietary contribution and trophic positions are dependent on samples from our single collection. Thus, our dietary contribution and trophic positions analyses are limited to only food available during our March collections. For example, our isotopic analyses identify that C_4 plants were a key vegetation source for crayfish diets, but they were not represented at most sites. In addition, other key resources (i.e. primary and secondary consumers) that were present outside of our sampling window may not be represented in our data set. Nonetheless, *D. youngineri* and *D. crockeri* are often in their burrows (average depth of *D. crockeri* burrows 1.5 m; Welch & Eversole 2006) during summer and fall months when rainfall is reduced and the water table is low (Eversole & Welch 2010; Eversole & Welch 2013). Thus, our sampling likely represents most of the resources these species would encounter when foraging. Additionally, yearly differences were not observed for crayfishes or food sources available within sites, and

precipitation and temperatures were similar between years, reducing the likelihood of interannual differences between species collected in 2021 and 2022. Trophic position can also be impacted by the baseline $\delta^{15}N$ values used in trophic analyses. Because we did not collect primary consumers from the same family at every sampling site, $\delta^{15}N$ values of all primary consumers collected from a sight were averaged to assess trophic positions. Primary consumers collected were grazers that may present a certain degree of omnivory, and thus may have a TP higher than 2 (Vander Zanden & Fetzer 2007). This discrepancy could lead to higher than normal TP values for our target species. Nonetheless, Letourneur et al. (2024) points out that a weakness of TP estimates is the use of a single baseline value, while we still used a single baseline value averaging multiple types of primary consumers allows us to incorporate different sources of nitrogen within a system. Additionally, C:N biplots show that primary consumer $\delta^{15}N$ values fell between detritus and secondary consumers at all sites.

Despite the gain in understanding of the trophic interactions of burrowing crayfish, comprehensive food resource studies of burrowing crayfishes remain limited. Results of our analysis indicated there may be considerable variation in trophic position, niche space, and dietary contribution among primary burrowing crayfish species. Further, our analyses indicate the ecological distinctness of primary burrowing crayfishes, occupying similar habitats but serving different roles within their ecosystems. Studies have shown that primary burrowing crayfish often act as omnivores feeding on invertebrates, vegetation, and detritus, but the extent of species' reliance on these food sources and how habitat, competition, or predators impact food selection is poorly understood. Our findings highlight the important role primary burrowing crayfishes play in these ecosystems, by regulating herbivore numbers, which helps ensure ecosystem stability, as well as facilitating energy flow to higher trophic levels. Further, understanding a species' food source and factors impacting food sources will allow conservationists to identify threats to the species' survival, protect and restore necessary habitats and food sources, and ensure ecosystem stability.

Acknowledgements

We thank the following people for assistance with field collections: G. McWhirter, M. Bland, C. Smith, S. Santiago, W. Hammond, J. Whalen (United Stated Department of Agriculture [USDA] Forest Service [USFS]); and M. Stubbs (West Liberty University). We thank landowners, B. Merchant, C. Merchant, C. Metz, and C. Smith, for granting us access to their property. We also thank C. Sabatia (USFS Contractor) for statistical advice and C. Smith (USFS) for GIS assistance. Products mentioned do not constitute endorsement by USFS, West Liberty University or the State of South Carolina. Findings and conclusions in this publication are those of the authors and do not necessarily represent the views of the USFS. This is contribution number [*TBD*] of SCDNR's Marine Resources Research Institute.

Table 1. *Distocambarus* species mean (± SD) trophic position (TP), carbon and nitrogen stable isotope values, and carapace length (CL), as well as number of sampled individuals between sexes per site.

	Males					Females				
	N	*TP*	δ^{15}C	δ^{15}N	CL	*N*	*TP*	δ^{15}C	δ^{15}N	CL
D. youngineri										
Field 1	5	2.98 (0.10)	-26.45 (0.33)	6.00 (0.33)	20.7 (3.49)	7	2.80 (0.11)	-26.23 (1.14)	5.41 (0.36)	27.2 (3.28)
Field 2	5	3.24 (0.08)	-26.08 (0.58)	10.57 (0.29)	24.6 (2.45)	6	3.23 (0.13)	-26.01 (0.41)	10.52 (0.44)	26.6 (2.26)
Field 3	4	3.47 (0.35)	-18.52 (2.80)	7.65 (1.19)	26.4 (3.79)	8	3.46 (0.42)	-20.45 (3.48)	7.60 (1.42)	29.3 (4.00)
Field 4	6	3.00 (0.16)	-27.06 (1.24)	5.53 (0.55	24.6 (3.40)	6	3.14 (0.32)	-24.80 (4.66)	6.01 (1.09)	31.9 (2.31)
Total	**20**					**27**				
Roadside 1	5	3.59 (0.49)	-25.02 (0.71)	9.26 (1.68)	24.8 (4.16)	7	3.83 (0.21)	-23.34 (1.85)	10.09 (0.72)	25.4 (2.41)
Roadside 2	6	2.38 (0.15)	-25.62 (1.52)	3.85 (0.51)	22.9 (4.24)	6	2.19 (0.29)	-25.89 (2.41)	3.18 (0.97)	25.3 (4.47)
Roadside 3	4	2.65 (0.17)	-17.72 (1.14)	4.70 (0.57)	26.1 (0.06)	5	2.89 (0.15)	-18.24 (1.27)	5.50 (0.50)	24.3 (7.34)
Roadside 4	5	2.80 (0.24)	-25.37 (0.88)	4.23 (0.80)	21.0 (3.61)	7	2.81 (0.22)	-25.97 (0.65)	4.26 (0.76)	27.8 (2.96)
Total	**20**					**25**				
D. crockeri										
Field 1	3	3.69 (0.11)	-16.33 (1.34)	7.20 (0.39)		3	3.62 (0.08)	-16.46 (0.99)	6.95 (0.28)	
Field 2	4	3.98 (0.31)	-16.04 (0.42)	7.79 (1.04)		3	4.17 (0.59)	-17.74 (1.00)	8.43 (2.01)	
Field 3	3	2.26 (0.19)	-21.92 (0.53)	3.97 (0.63)		3	2.42 (0.16)	-22.57 (0.59)	4.52 (0.56)	

Field 4	3	3.34 (0.14)	-19.69 (3.56)	5.92 (0.49)		3	3.26 (0.21)	-20.81 (3.61)	5.67 (0.73)	
Field 5	4	3.19 (0.18)	-14.30 (0.57)	8.32 (0.61)		5	3.20 (0.06)	-14.81 (1.44(	8.33 (0.20)	
Total	**17**					**17**				
Roadside 1	4	2.14 (0.17)	-24.58 (0.11)	3.77 (0.57)	17.5 (1.98)	5	1.96 (0.16)	-24.79 (0.44)	3.17 (0.54)	17.3 (4.07)
Roadside 2	4	2.11 (0.46)	-24.92 (2.45)	2.79 (1.58)	20.2 (3.89)	4	2.01 (0.52)	-24.67 (2.93)	2.46 (1.75)	15.2 (0.64)
Roadside 3	3	2.60 (0.03)	-20.62 (0.23)	3.41 (0.09)	19.5 (1.92)	4	2.69 (0.11)	-21.39 (1.34)	3.70 (0.39)	24.9 (0.80)
Roadside 4	4	2.22 (0.09)	-26.72 (0.29)	1.70 (0.32)	19.7 (3.46)	3	2.28 (0.12)	-26.68 (0.33)	1.91 (0.40)	17.0 (3.18)
Roadside 5	5	2.15 (0.31)	-24.95 (1.39)	2.85 (1.07)	20.4 (3.32)	5	2.11 (0.22)	-24.61 (0.95)	2.72 (0.74)	17.3 (1.18)
Total	**20**					**21**				

Table 2. *Distocambarus* species proportional contribution of food sources (± SD) for males and females at field and roadside habitats.

	Field		Roadside	
	Females	Males	Females	Males
D. youngineri				
Secondary consumer	36.9 (14.5)	37.0 (15.4)	37.7 (20.5)	31.6 (17.8)
Primary consumer	39.8 (19.5)	35.0 (20.1)	43.8 (24.0)	43.4 (22.1)
Vegetation	13.6 (9.0)	16.5 (11.0)	9.0 (6.6)	12.0 (8.2)
Detritus	9.7 (6.0)	11.5 (6.9)	9.5 (6.2)	13.1 (7.7)
D. crockeri				
Secondary consumer	50.0 (14.7)	47.4 (17.7)	18.5 (7.6)	17.9 (7.9)
Primary consumer	34.1 (17.3)	31.4 (19.6)	40.3 (13.7)	41.7 (13.1)
Vegetation	9.9 (6.8)	12.9 (8.9)	19.8 (10.5)	20.3 (11.3)
Detritus	5.9 (3.7)	8.3 (5.2)	21.5 (10.9)	20.1 (10.6)

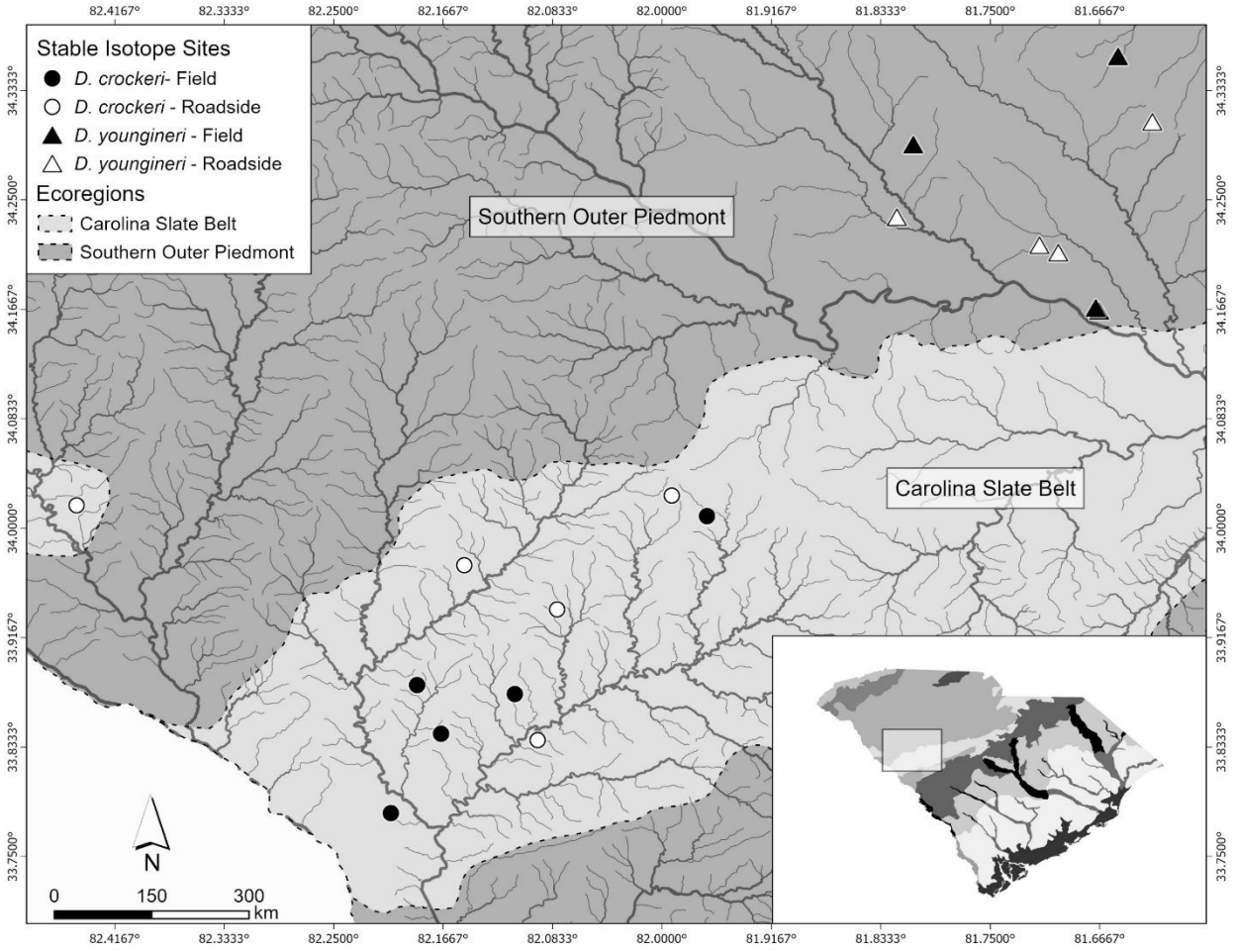

Fig. 1 Map of *Distocambarus youngineri* and *D. crockeri* field and roadside habitat sampling sites

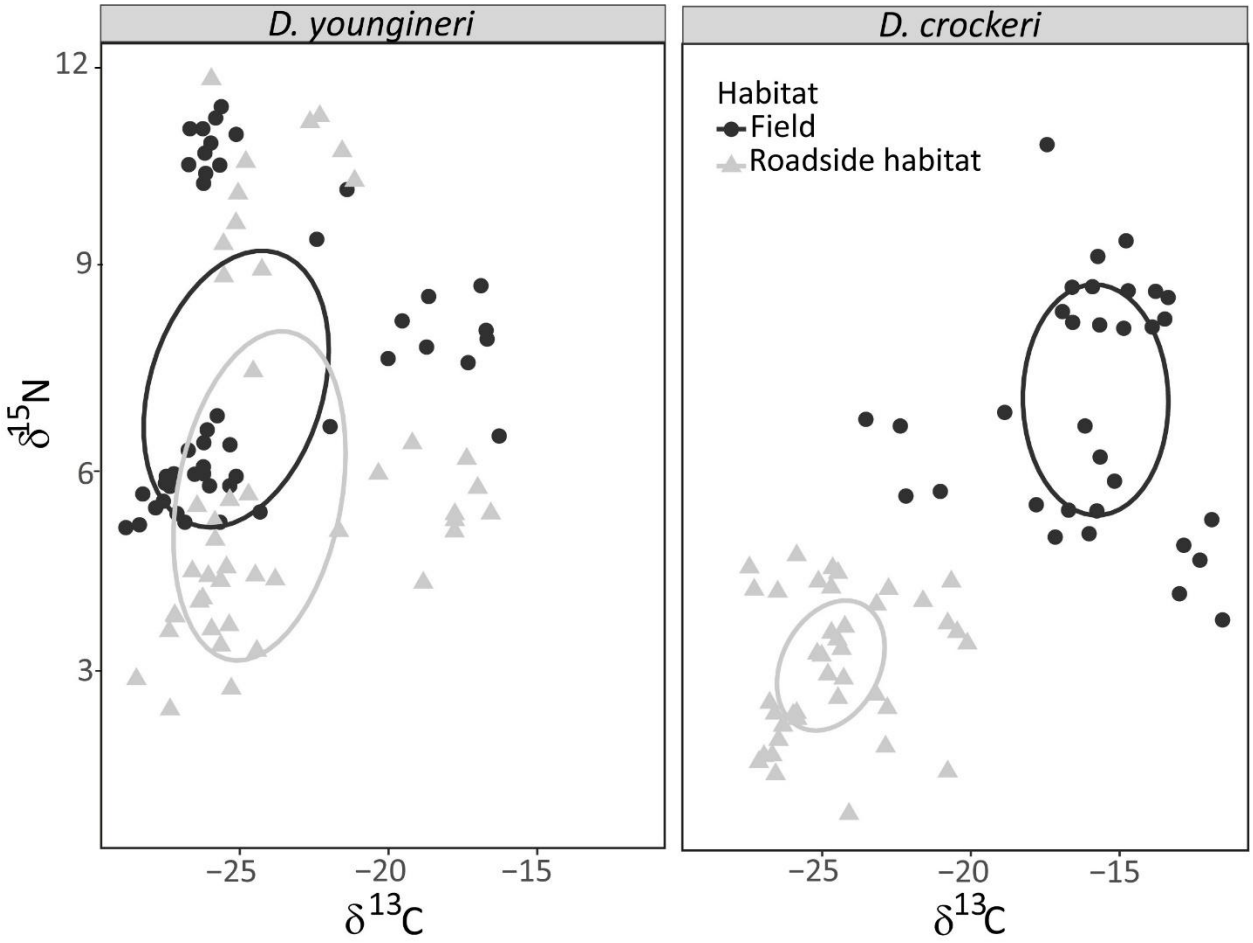

Fig. 2 Stable isotope values of *Distocambarus youngineri* and *D. crockeri* with associated isotopic niche (correct standard ellipses (SEAc)) for field and roadside habitat populations

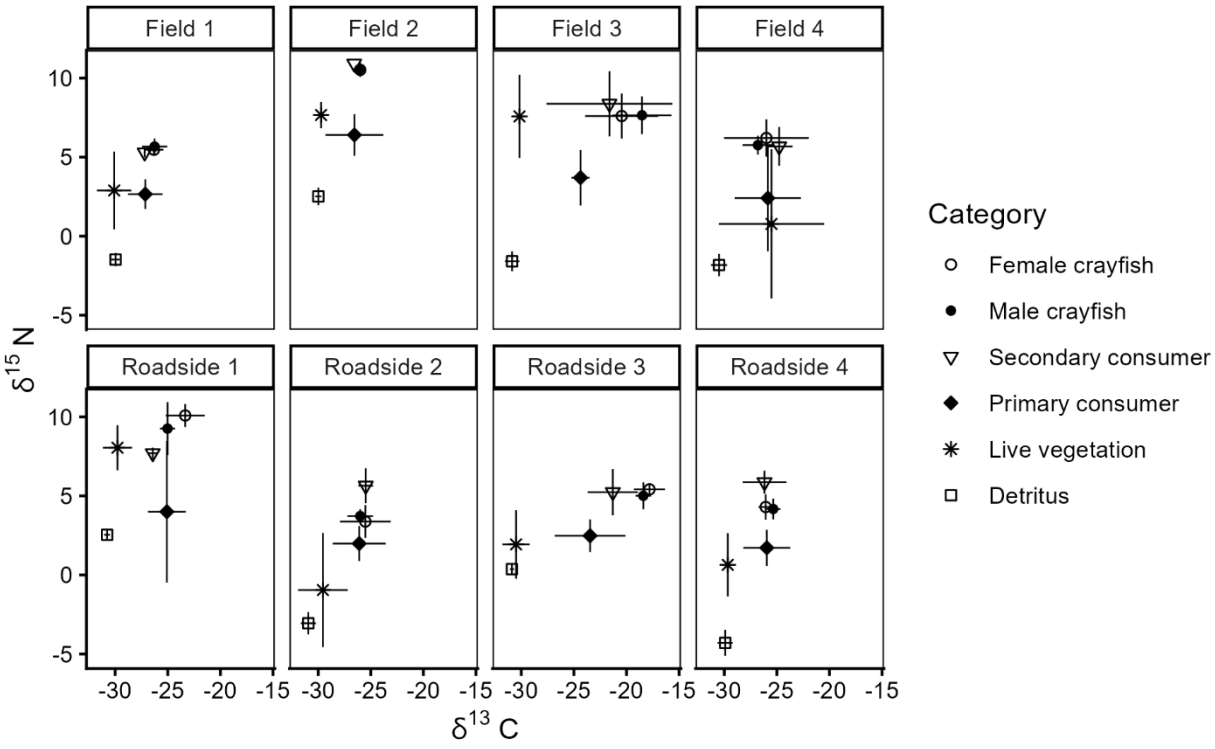

Fig 3 δ^{15}N and δ^{15}C isotopic signatures for *Distocambarus youngineri* male and females captured in all (A) field and (B) roadside sites. Error bars show the standard deviation of isotope signatures of all individuals and vegetative samples. Y axes differ between graphs of field and roadside habitats

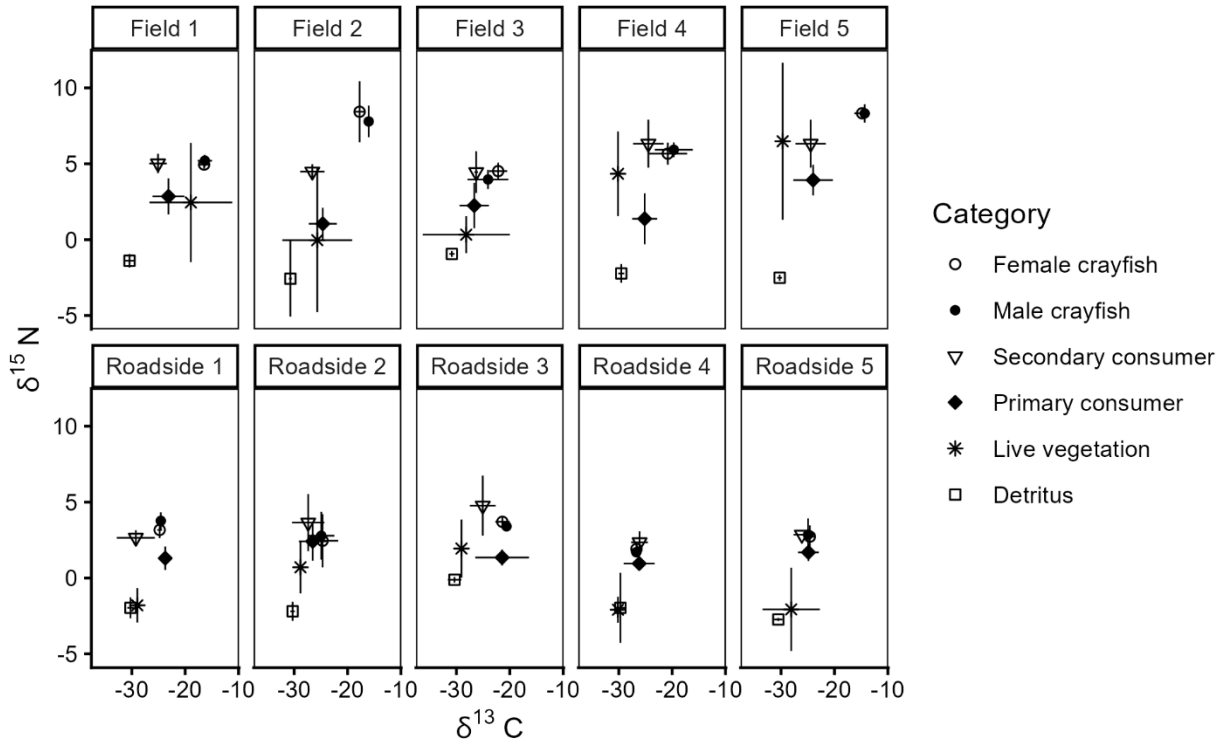

Fig. 4 δ^{15}N and δ^{15}C isotopic signatures for *Distocambarus crockeri* male and females captured in all (A) field and (B) roadside sites. Error bars show the standard deviation of isotope signatures of all individuals and vegetative samples. Y axes differ between graphs of field and roadside habitats

Significant deviations: None.

Final Federal Cost: See 425.

Recommendations: Close the grant.

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