Crayfish shelter-seeking behavior as an experimental system to teach experimental design and research skills to undergraduate biology students

Johnathan G. Davis*, Amelia Atwell, Stefanie Baker, Kelli Carroll, Matthew Crook, and Lori Cruz

* davisjg@wofford.edu; Department of Biology, Wofford College, Spartanburg, SC 29303

Abstract

During the 2023 – 2024 academic year, a research system based upon the study of crayfish shelter-seeking behavior was developed to facilitate an authentic research experience in a new course for second-year students focused on practicing scientific skills related to evaluating primary literature, designing and conducting experiments, analyzing results, and communicating research findings. Crayfish were utilized due to their availability from local streams and biological supply companies, hardiness and low mortality in aquaria, and their use as experimental models in studies of genetics, neuroscience, and animal behavior. Students evaluated shelter-seeking behavior in response to manipulations of shelter types, substrates, artificial light, and other variables. Crayfish were held in 10-gallon aquaria, provided with shelters, and tested in experimental arenas that students manipulated based upon their research hypothesis. More than 100 students conducted research across six course sections, developing more than 20 unique projects. Students reported results in both written and oral formats, describing substrate and shelter preferences under lab conditions that closely mimic their natural habitats and possible effects of artificial light at night on nocturnal behavior.

Keywords: crayfish, experimental design, undergraduate research

Introduction

The first-year course in biology at Wofford College (BIO 150 Biological Inquiry) exposes students to the process of biological inquiry (Goldey et al. 2012). Biology faculty designed a follow-up, second-year course (BIO 216 Experimental Design, Analysis, and Communication) to introduce students to experimental design and emphasize the practice of biological inquiry through designing and executing research experiences. Criteria for selecting a research system for this course included the ability to generate and test relevant and diverse hypotheses by sophomore-level students, a failure-tolerant system that allowed students to make and correct mistakes, and the capability to carry out experiments over a short (~4 week) time frame. Thus, the course implemented an experience similar to course-based undergraduate research experiences (CURE), which can increase student engagement, achievement, persistence, and inclusion (Kuh, 2008; Auchincloss et al., 2014; Freeman et al., 2014).

In Fall 2023 and Spring 2024, a research system was designed using crayfish, decapod crustaceans with a large global distribution, as experimental subjects. Crayfish are intriguing physiologically, ecologically, and behaviorally but are also imperiled across much of their range (Taylor et al., 2019). Although they are ecosystem engineers that increase nutrient availability in freshwater ecosystems (Reynolds et al., 2013; Albertson and Daniels, 2018), many species are not well studied (Loughman and Fetzner, 2015). Kubec et al. (2018) suggests their suitability for ethological studies and application to many disciplines. Investigation into species-specific behaviors may provide insight into topics such as the spread of invasive crayfishes and displacement of native crayfishes, extirpations from environmental degradation, resilience to climate change, and susceptibility to novel diseases. The marbled crayfish *Procambarus virginalis*, a parthenogenetic crayfish, has emerged as a new laboratory model in developmental biology, stem cell research, epigenetics, and evolutionary biology (Vogt, 2008). Recent analysis has investigated the crayfish's potential as a model for studies of drug addiction (Jackson and Staadan, 2019; Imeh-Nathaniel et al., 2019). Crayfish also have high sociality levels (Figler et al., 1995; Issa et al., 1999) with a high degree of specialization in sensory organs, particularly for communication (Basil and Sandeman, 2000; Kubec et al., 2018). Because of their behavioral and physiological responses to stress, crayfish are suggested as models to study emotional behavior and mechanisms for human affective disorders

(De Abreu et al., 2020).

Crayfish can be aggressive toward each other, resulting in stress, injury, and even cannibalism in captivity (Steele et al., 1997). Thus, when using crayfish as experimental models, proper conditions should be provided that prevent stress, which can improve the quality and validity of experiments (Alberstadt et al., 1995). Although the laboratory housing conditions needed to reduce stress of crayfish warrants further study, enrichment of the environment with shelters is known to reduce aggression and support shelter-seeking tendencies (Capelli and Hamilton, 1984). Various shelter characteristics, such as size, shape, and texture, may affect shelter use. Shelter occupancy can depend upon the darkness provided by the shelter (Alberstadt et al., 1995; Antonelli et al., 1999), the size of the shelter relative to a crayfish's size (Antonelli et al., 1999; Forsythe et al., 2011), the presence of conspecifics (Hill and Lodge, 1994; Quinn and Graves, 1998; Dunham, 1999), and previous occupancy of a shelter (Forsythe et al., 2011).

Thus, many hypotheses can be developed related to crayfish shelter-seeking behavior in captivity. We describe a research system utilizing crayfish for students to develop varying hypotheses and conduct research that is authentic, relevant, and iterative. Students read peer-reviewed papers related to crayfish shelter use, developed a hypothesis, designed and implemented an experiment, analyzed data, wrote a scientific manuscript, and presented results orally. We report examples of common hypotheses tested by students related to shelter-seeking behavior and shelter preference. The diverse hypotheses tested demonstrate the flexibility of this setup.

Course Structure and Design

The integration of the research system into the course is outlined in Table 1. Students were introduced to crayfish as experimental models by

Table 1. Course plan detailing the implementation of the course research experience.

Week	Activity
1	Introduce course learning objectives, project concept, crayfish as model organisms, view preserved crayfish, and rubrics for the final paper and presentation reviewed.
2	Read Kubec et al. (2019) as an introduction to review papers. Complete an activity that dissects the paper. Introduce students to conducting a literature review on a topic.
3	Read Alberstadt et al. (1995) and learn how to analyze a scientific paper. Analyze an additional paper in groups. Conduct a jigsaw activity to practice summarizing a research paper.
4	A science librarian introduces skills of searching scientific literature, and students develop an annotated bibliography on crayfish shelter-seeking behavior. Introduce scientific writing and the structure of Introduction and Methods sections. Begin writing an Introduction.
5	Review hypotheses/predictions, clarify the hypothesis/prediction for student experiments, and identify independent, dependent, and confounding variables. Introduce pseudoreplication and identify potential sources of pseudoreplication in their experiment.
6	Introduce randomized designs, blocking, and repeated measure designs and incorporate concepts into the development of experimental design for project.
7	Assessment of experimental design skills. Construct methods and set up experiment.
8	Test experimental design and begin data collection.
9	Continue data collection.
10	Practice with t-tests and box plots using project data. Continue data collection.
11	Practice with ANOVA and graphs. Continue data collection.
12	Practice with linear regression and scatter plots. Finish data collection and write Results section. Introduce structure of a Discussion section and complete guided activity for writing the Discussion.
13	Data analysis and visualization skills assessment. Submit Discussion draft. Introduce oral presentations.
14	Thanksgiving Break
15	Submit rough draft; develop oral presentation. Receive feedback on rough draft; finish presentation.
16	Submit final manuscript and give presentation.

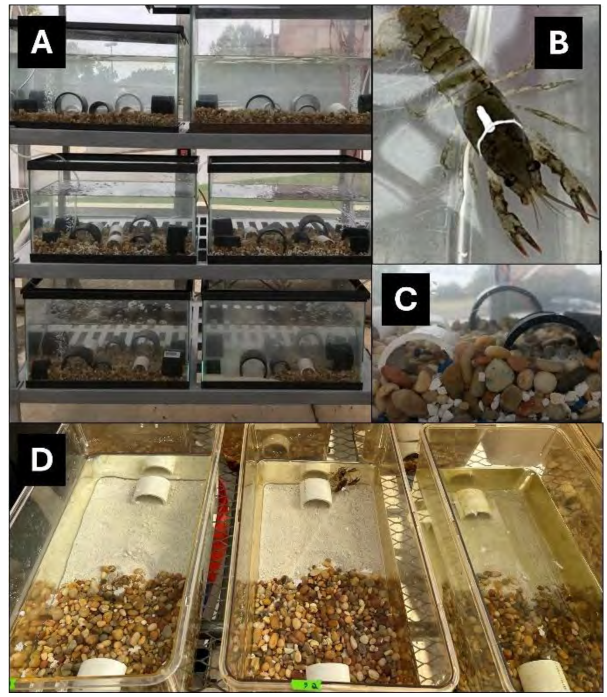
reviewing peer-reviewed literature, particularly Kubec et al. (2019), Alberstadt et al. (1995), and Antonelli et al. (1999). Students constructed an annotated bibliography to summarize relevant studies and then developed a hypothesis.

To develop their methodology, students received instruction in experimental design and then set up experimental arenas and conducted pilot studies. Students completed four weeks of data collection, during which they were trained in data analysis (i.e., t-tests, analysis of variance, and linear regression), analyzed data using JMP Pro 16 (SAS Institute, Inc.), and designed figures for their results. All students developed a manuscript that followed the IMRAD (Introduction, Methods, Results, and Discussion) format that was written in sections throughout the course. Using the annotated bibliography, students wrote an Introduction (Week 4) and then wrote the Methods section after developing their experimental design (Week 8). The Results section was written after completing data analysis (Week 12) followed by the Discussion section (Week 13) with a rough draft due at the beginning of Week 15, which was dedicated to improving the draft and constructing an oral presentation. During the final week, students submitted the final draft and presented their research.

Common Methodology for Crayfish Research

Approximately two weeks before field collection (Week 5), aquaria were set up in a laboratory or greenhouse for housing crayfish (Figure 1). Aquarium gravel was added to 10-gallon aquaria to create a 2.5 – 5.0 cm layer of substrate. Water was treated with tap water conditioner to remove chlorine and received constant aeration. At least one shelter per crayfish was added to each tank. Shelters consisted of either small, hand-sized rocks taken from the source stream, or small lengths (~ 5 – 7.5 cm) of 3.8 – 7.6 cm diameter PVC pipe. PVC was buried into gravel substrates to provide a flat surface for crayfish. Because crayfish would excavate gravel from the PVC shelter, pipes were subsequently cut in half and placed on the substrate. In the laboratory, lighting was set to 16:8-hour schedule of light to darkness and provided by overhead fluorescent lighting. In the greenhouse, crayfish were subjected to the normal photoperiod of the environment.

Figure 1. Photographs of Experimental System. **A:** Ten-gallon source tanks with PVC shelters partially buried in gravel. **B:** Variable crayfish (*Cambarus latimanus*) individually marked on the dorsal surface of the cephalothorax with a paint pen. **C:** PVC shelters partially buried in gravel in source tanks. **D:** Experimental arenas divided into two halves with sand and gravel substrates and PVC shelters.

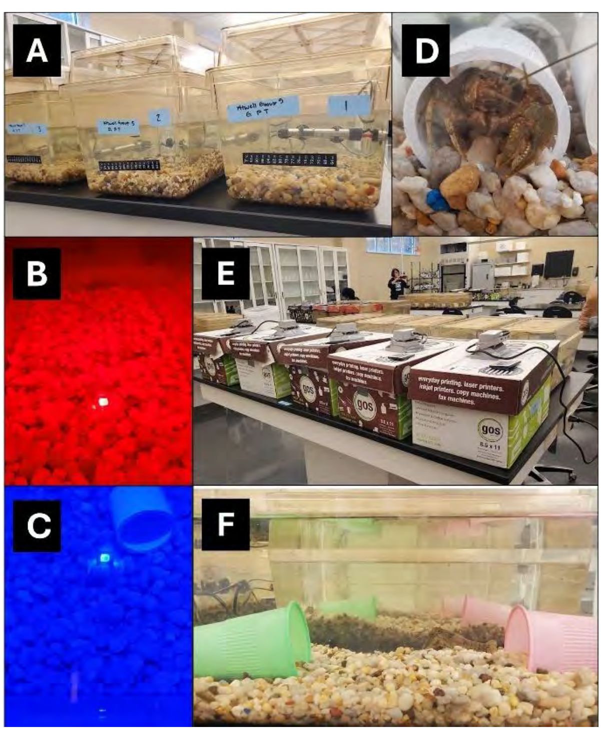


In Week 6 two crayfish species (*Cambarus latimanus* and *Cambarus chattahoochee*) were collected from a small (~2 – 3 m wide) stream in Spartanburg, South Carolina approximately 3 km from Wofford College. Approximately 12 – 15 crayfish were collected for each team of student researchers. During collection, crayfish were held in 5-gallon buckets of stream water and then transported to the college. Upon arrival, crayfish were acclimated to the system water source by adding ~ 1 quart of treated dechlorinated aquarium water every 15 minutes to buckets until water temperatures were similar between buckets and aquaria, and then crayfish were added to aquaria. Additionally, red swamp crayfish *Procambarus clarkii* were ordered from Carolina Biological Supply in Spring 2024 for use. Wire screens covered every tank, and a heavy object was placed on top of aquaria to prevent escape.

Crayfish were allowed to adjust to source tanks for at least 48 hours before any experimentation. Crayfish were fed every 2 days, water temperature and dissolved oxygen were checked daily, and water changes were conducted once per week over a 4-week experimental period. After 48 hours, crayfish were removed from their tank, measured for carapace length, and had a unique number written on the dorsal side of their carapace with a paint pen (Ramalho et al., 2010).

Students tested hypotheses by removing crayfish from source tanks and placing them into experimental arenas that students could manipulate (Figure 2). These arenas were subjected to the same environmental conditions, including air temperature, lighting, and water temperature, unless the hypothesis necessitated manipulation of one of these. Arenas were plastic containers approximately 46–cm x 25–cm x 20.5–cm with fitted covers and a layer of substrate. Substrate composition and shelter characteristics varied based upon experimental manipulations. In some cases, barriers were placed between adjacent arenas to reduce the influence of crayfish in nearby arenas on behavior. Crayfish were placed into an arena and observed over a defined period by students.

Figure 2. Photographs of Experimental System II. A: Experimental arenas in the laboratory with varying water temperatures. **B:** Red swamp crayfish (*Procambarus clarkii*) inhabiting a PVC shelter in a source tank. **C:** Red light manipulation in an experimental arena. **D:** Blue light manipulation in an experimental arena. **E:** Laboratory setup of experimental arenas showing cardboard covers embedded with lights. **F:** Experimental arena providing crayfish with choice between two color options.



Examples of Student Experiments

In Fall 2023 and Spring 2024, six sections of the BIO 216 course implemented this experimental system with ~110 students collaborating to complete 28 research projects. Using experimental arenas, many students designed choice experiments, and observed choices made by crayfish. The following are examples of experimental designs developed by students and their results.

Alberstadt et al. (1995) and Antonelli et al. (1999) tested shelter choice of crayfish when manipulating thigmotactic (i.e., shelter size in comparison to crayfish size) and darkness cues provided by shelters by using clear or opaque glass beakers of varying sizes. For experimentation, students typically introduced crayfish into an arena, observed them over an 8-hour period, and scored them as occupying a particular shelter type. Shelter arrangement and position in each arena was randomized in most experiments. One team of student researchers provided crayfish with the choice of two shelters – a clear glass beaker half-buried in gravel substrate and a clear beaker completely covered by gravel substrate to provide darkness Another team implemented a repeated measures design that randomly assigned crayfish to three experimental arenas, which included shelter choices among a flat, hand-sized rock, a PVC pipe, and a dark glass beaker.

Activities such as deforestation, urbanization, and agriculture contribute excessive sediment to stream substrates, covering rocky and gravel substrates, and filling interstitial spaces and crevices utilized by crayfish (Swank et al., 2001; Taylor et al., 2007; Richman et al., 2015). Therefore, students manipulated substrates in experimental arenas, providing crayfish choices between gravel, sand, and mixed sand-gravel substrates, to make inferences about the effect of sedimentation on crayfish behavior. Students standardized the shelter type, and the arena was divided into left and right sections with each section containing a different substrate. Other students approached the issue of sedimentation by manipulating water quality and increasing the turbidity of the water column. Students designed an experiment with treatments consisting of three levels of suspended sediment in the water column.

As nocturnal animals, crayfish typically emerge from shelters at night, but in the presence of artificial light at night (ALAN), they may spend more time under shelter, decreasing foraging time and interaction with other crayfish (Thomas et al., 2016; Fischer et al., 2019). Various colors of light and the use of LED lights can alter behavior also (Ruokonen et al., 2021; Suryanto et al., 2023). To evaluate the effects of ALAN, students exposed crayfish to varying light intensities and colors at night, placing crayfish in covered arenas fitted with LED lights with PVC shelters at either end of the arena (Figure 2). During nighttime darkness, experimental arenas containing a single shelter were exposed to four treatments of light, no light, white light, red light, and blue light.

Discussion

A research system for studying crayfish shelter-seeking behavior was utilized to prepare second-year biology majors for more intensive research experiences as third- and fourth-year students. The research system provided the opportunity for students to develop authentic, relevant, and diverse research questions, and many other questions can be asked in this system related to animal behavior, light pollution, sedimentation of streams, urbanization, and climate change through manipulations of shelters, substrates, light, water temperature and other variables.

This system allowed students to test hypotheses related to shelter-seeking behavior while also building their skills in experimental design, quantitative analysis, and communication. In the research system students applied experimental design concepts of hypothesis development, use of controls, randomization, replication, and blocking. Students designed a randomized process to select crayfish from source tanks using a list of computer-generated numbers after many initially proposed haphazard sampling methods (i.e., attempting to grab a crayfish out of a source tank without bias). Pseudoreplication was considered by randomizing locations of arenas in labs or greenhouses or the location of shelters in arenas and treating results from arenas as experimental units rather than individual crayfish. Some students applied concepts of blocking by equally dispersing crayfish of varying sizes among treatments or arenas. In some cases, students designed repeated measures studies in which individual crayfish were exposed to multiple treatments, randomizing the order of treatment exposure.

This research system provided students with autonomy in hypothesis development and experimental design, but students were steered toward viable designs through reading assignments early in the course, including Alberstadt et al. (1995), Antonelli et al. (1999), Forsythe et al. (2003), Capelli and Hamilton (1984), and Fischer et al. (2020). This avoided impractical research experiences due to space, time, and financial constraints and helped students navigate indecisiveness that could delay the project. Even so, some students procrastinated through group indecision or by displaying perfectionist tendencies. Instructors may need to facilitate group decision-making and encourage pilot studies to test the experimental design.

An emphasis on experimental design theory was necessary prior to the start of data collection and encompassed 3 – 4 weeks of the course. To avoid a disconnect between the theory and skill of experimental design and the forthcoming research project, it is recommended to integrate the two. For example, when teaching randomization, students completed an activity which incorporated randomization into their design and the crayfish research system. This resulted in piecemeal construction of the experimental design, leading to a completed course unit focused on analyzing data with common statistical methods (e.g. t-test, ANOVA, regression, etc.) and visualizing the data, and class sessions were designed to incorporate the analysis of available data from the research projects into the lesson. Because this unit coincided with the completion of data collection, students were able to produce their results and figures quickly and efficiently. A flipped learning approach that utilized pre-class video lectures and assessments was implemented such that in-class meetings could be dedicated to guided activities on experimental design and data analysis.

With class enrollments capped at 16 students, group sizes were limited to 3 or 4 students, and each group was provided with one source tank containing 12 – 15 crayfish and 3 – 4 experimental arenas. Larger classes or small groups sizes result in more groups which greatly expands the size of the research operation, including the equipment, space, and crayfish needed. In both semesters, a shared lab space and a small area (3m x 3m) in a greenhouse was dedicated to accommodating the research setup.

The necessity of reviewing peer-reviewed literature and introducing experimental design theory prior to data collection resulted in the start of data collection coinciding with either fall break or spring break, creating logistical problems in the timing of acquiring crayfish and maintaining them in aquaria during the break. Starting data collection after these breaks either reduces the data collection period or limits the time for composing the final paper and presentation and for addressing any issues with crayfish mortality or equipment malfunction. In the next iteration of the course, some experimental design concepts may be excluded to allow for an earlier start to research. Teaching assistants were critical to maintaining the research system and addressing any issues, limiting the workload for faculty.

An authentic research experience for undergraduates was provided using a research system focused on the shelter-seeking behavior of crayfish. Undergraduate research is a high-impact practice where students practice inquiry, analyze primary literature, generate ideas, apply knowledge through experimentation, develop communication skills, and connect with faculty and peers (Kuh, 2008; Brownell and Kloser, 2015). Students experience gains in resilience, academic identity (Mraz-Craig et al., 2018), self-confidence (Bascom-Slack et al., 2012; Prunuske et al., 2013), content knowledge (Makarevtich et al., 2015; Ward et al., 2014), and self-efficacy (Frantz et al., 2006). Participation in research also improves retention, completion rates, and graduate school enrollment (Junge et al., 2010; Brew and Mantai, 2017). Multiple course sections successfully implemented the experience over two semesters with different instructors, and students developed diverse hypotheses to test with success leading to further implementation in Fall 2024 and students building on previous research. The experience allowed students to develop competence in hypothesis development, experimental design, data analysis, and oral and written communication and emphasized practice of these skills. Lastly, crayfish are an active invertebrate subject for students to study and can serve as an engaging entry point for students new to research.

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