Thermal Tolerance and Burrowing Behaviors of Red Swamp Crayfish (*Procambarus clarkii)*

By

Nicholas Spencer Barnes

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama

Keywords: Crayfish, Invasive, Thermal Tolerance, Burrowing, *Procambarus clarkii*

Approved by

James Stoeckel, Chair, Associate Professor of Fisheries, Aquaculture, and Aquatic Science
Matthew Catalano, Associate Professor of Fisheries, Aquaculture, and Aquatic Science
Christopher Bonvillain, Associate Professor of Biological Studies

Abstract

Red Swamp Crayfish (*Procambarus clarkii)* are a globally invasive species whose burrowing habits contribute to damn and levee failures, erosion, and resistance to control measures. I used artificial burrowing chambers to investigate the effects of groundwater drainage rates and soil type on burrow initiation and morphology. I also examined the relationship between caloric density of crayfish and burrowing. Burrowing was more strongly controlled by groundwater declines than by soil type. Crayfish that did not burrow had a significantly lower caloric density than crayfish that actively burrowed. In the thermal tolerance assays, I acclimated crayfish to two different temperatures (15 and 25°C) then tested response to acute thermal shock. Thermal tolerance was strongly affected by acclimation temperature, with crayfish acclimated to 25°C having a higher thermal tolerance than those acclimated to 15°C. Results will help farmers and managers promote or discourage burrowing behavior based on drainage rates as well as develop non-chemical control techniques for crayfish in burrows based on application of hot water treatments across a range of latitudes.

Acknowledgments

I'd additionally like to thank Oliver Williams and Billy Moore for providing heavy machinery operation and construction assistance that was crucial to completing aspects of this study. Thanks to Colin Nuun, Patrick McGill, and Josiah Gullate for helping to dig holes in the hottest parts of the summer. Huge shout out to my technician James Rodgers for dealing with me and my crazy ideas for a year, digging countless holes when there was no end in sight, and being my seeing eye dog. Thank you to my committee members, Chris Bonvillain and Matt Catalano, for input on study design and invaluable data analysis assistance. The biggest thank you to my advisor, Dr. Jim Stoeckel, for sticking with me through the end. And finally, thanks to Nick Barnes for being the best, couldn't have done it without you.

Table of Contents

Chapter One: **Thermal Tolerance of Red Swamp Crayfish**

Chapter Two: Burrowing Behaviors of Red Swamp Crayfish

List of Tables

List of Figures

List of Abbreviations

RSC - Red Swamp Crayfish

LT_x - Lethal Temperature(Percentile)

LOE – Loss of Equilibrium

PPD – Presumed Physiological Death

CL – Carapace Length

Chapter One: Thermal Tolerance of Red Swamp Crayfish

Introduction

Red Swamp Crayfish (*Procambarus clarkii*, Girard 1852) is a large crayfish species native to the southern United States and Northeastern Mexico and is found on all continents except Antarctica and Australia (Dalosto et al., 2015; Gherardi, 2006). Red Swamp Crayfish are one of the most economically important crayfish taxa due to their prominence in aquaculture, wild harvest, and research (Schuster et al., 2022). Additionally, global aquaculture practices, the pet trade, and shipping contribute to their increased range expansion (Oficialdegui et al., 2020) and they have been documented as a disease vector for pathogens such as crayfish plague (Holdich et al., 2009) and White Spot Syndrome Virus (Bruce et al., 2024). These diseases can have devastating effects on crayfish species within and outside of North America that lack a previous exposure history (Holdich et al., 2009; Jiravanichpaisal et al., 2001).

Red Swamp Crayfish are as a secondary burrowing species that leave surface waters and dig burrows to during environmental extremes and reproductive periods (Huner et al., 2002). Control methods for RSC are typically divided into treatments focused on surface waters or burrows. Surface water control techniques such as trapping have historically been the preferred method for both harvest and control because RSC typically live in turbid water, limiting removal by hand (Paillisson et al., 2011). However, each type of surface water control technique is associated with specific drawbacks. Collection with minnow or basket traps is size and sex selective which favors collection of larger crayfish (Paillisson et al., 2011) and leaves smaller crayfish free to grow and reproduce. The use of carbon dioxide diffused into surface waters is somewhat effective in small ponds but requires substantial effort in larger ponds (Abdelrahman et al., 2021). Chemical control requires control agents to be dispersed across the entire water

body, may kill non-target species, and are negatively influenced by wind and rain events that reduce effectiveness by diluting or carrying the chemical into non-target areas. For example, pyrethrin is an organic insecticide that has been applied to surface waters to target invasive crayfish but can also impact native crayfish and insect populations (Centers for Disease Control and Prevention, 2014). Furthermore, pyrethrin is ~60% effective killing RSC (K. Quebedeaux, Michigan DNR, pers. comm). Chemical treatments also require a large amount of paperwork and approval for application.

Because female RSC typically extrude eggs and produce broods in terrestrial burrows, applying treatment methods to burrows allows for a stronger focus on the reproductive stage of the RSC life cycle. Such treatments include filling burrows with bentonite clay or expanding foam to plug the entrance and trap the crayfish underground. This treatment has been shown to cause 60-80% mortality (Bates et al., 2023) but is affected by burrow morphology and the treatment's ability to reach the groundwater. In straight, vertical burrows the treatment can follow the main shaft down to the terminal chamber, however, curved burrows can reduce ability of the treatment to reach the groundwater and reduce treatment effectiveness (Bates et al., 2023). Additionally, under certain conditions, some individuals may dig around or through the plug and escape the treated burrow (K. Quebedeaux, Michigan DNR, pers. comm.).

Hot water may be a useful, alternative control agent for crayfish in burrows. Hot water (<45 °C) may reduce or eliminate strict permitting processes associated with chemical treatments and doesn't require large amounts of bentonite clay to be brought to a treatment site and applied by hand. Heated pressure washers are commercially available and don't require advanced training or applications to use. Units with a self-contained water tank can be brought into the field on a truck without requiring a water or electrical hookup. They can be equipped with a long

(i.e., 33 m) hose to treat burrows within a wide radius of the transport system. Hot water (45 °C) is currently used to kill invasive species like zebra mussels (*Dreissena polymorpha,* Pallas 1771) and killer shrimp (*Dikerogammarus villosus,* Sowinksy 1894) on fishing equipment in the UK (Anderson et al., 2015; Bradbeer et al., 2020) and might be a useful tool for invasive crayfish control.

Use of hot water as a treatment requires knowledge of critical thermal maximum (CTmax) and the effects of temperature acclimation for RSC. Acclimation temperature can have a major effect on CTmax of some crayfish species like Rusty Crayfish (*Faxonius rusticus,* Girard 1852), with CTmax increasing by as much as 10.8°C when acclimated to high (33°C) as opposed to low (2.5°C) temperatures (Mundahl, 1989). In contrast, acclimation temperature had no effect on CTmax of RSC or White River Crayfish (*Procambarus zonangulus,* Hobbs and Hobbs 1990) (Logarbo and Bonvillain, 2020), but this lack of an effect may have been due to testing a narrower range of acclimation temperatures (24-30°C) compared to the *F. rusticus* study.

Because Red Swamp Crayfish has a large global distribution encompassing a wide range of latitudes, a wide range of acclimation temperatures needs to be tested to estimate lethal water temperatures required to kill invasive crayfish at different latitudes or seasons within a relatively short period of time. The goals of this study were to determine 1) the temperatures required to cause functional death (loss of equilibrium) and presumed physiological death (loss of movement in response to probing in ≤ 10 min), 2) whether these upper thermal limits are affected by acclimation temperature (15, 25°C), and 3) whether RSC are able to recover from endpoints used to indicate functional death and presumed physiological death as temperature decreases after acute thermal shock.

Methods

Crayfish Collection and Maintenance

Acclimation happened within two 305 x 70 x 60 cm fiberglass troughs, filled with 640.5 L water obtained from an 8.1 ha earthen reservoir on the station. Water was aerated and constantly circulated through shredded PVC ribbon to maintain active biofiltration. Water changes were implemented if water quality parameters fell outside of the following suitable ranges for RSC: pH 6.5-8.5, Total Ammonia Nitrogen (TAN) < 0.5 mg/L, Nitrite < 5 mg/L, and DO > 2 mg/L (Lutz et al., 2005). One trough was held at 15°C using a chiller unit, and the other was held at 25°C using a combined chiller/heater unit to represent the large range in latitude that RSC occupy (Oficialdegui et al., 2020). Once water parameters in each trough were within the acceptable range, 180 Red Swamp Crayfish (90 males+90 females) were collected from wild populations living in earthen ponds at the EW Shell Fisheries Center of Auburn University (Auburn, Alabama) using minnow traps. Crayfish sex and carapace length (mm) were recorded prior to acclimating crayfish to baseline temperatures. Only crayfish with hardened exoskeletons, indicating intermolt to early premolt stages, ranging from 25 mm – 44 mm carapace length, were used for experiments. Crayfish were individually placed in 16.5 x 11.5 x 7.5 cm Tupperware containers with 0.6 cm drainage holes drilled on all sides to allow for water exchange. Acclimation containers were randomly distributed in equal numbers between the two acclimation troughs. 90 RSC (45 males + 45 females) were added to each acclimation trough, with 80 being assigned to thermal shock assays, and the remaining 10 being extras to replace any deaths or crayfish that molted. Any RSC that molted within a week prior to the experiment were discarded and not used for thermal assays (Logarbo and Bonvillain, 2020). During the one week

acclimation period, RSC were fed commercial shrimp pellets ad libitum every other day prior to experiments.

Thermal Tolerance Experiments

10 RSC (five males and five females) from each acclimation treatment (15°C and 25°C) were randomly assigned to one of eight thermal shock treatments (Control, 30, 36, 38, 40, 42, 44, or 46°C), resulting in a total of 20 crayfish assigned to each thermal shock treatment. In the control treatment, crayfish were simply exposed to the same temperature to which they had been acclimated (15 or 25°C). Crayfish in the 15°C acclimation group had a higher molting and mortality rate during acclimation, reducing the number of animals available for the thermal shock treatments. Thus only eight crayfish from the 15°C acclimation group were exposed to the 30°C thermal shock treatment. Crayfish were exposed to thermal shock in water baths consisting of 66 x 33 x 35 cm coolers, each equipped with a 300 watt Finnex heater bar and small submersible pumps to circulate water and prevent a temperature gradient from forming. Water temperature was verified using a calibrated reference thermometer (Thermco Dual Probe PT100 Platinum Digital Thermometer). To induce heat shock, each crayfish was transferred from its acclimation container and immediately placed in a 13 cm diameter by 10 cm tall cylindrical glass dish submerged in a water bath at the appropriate heat shock temperature. Control exposures were conducted in the acclimation troughs, using the same glass jars and mortality endpoint criteria as the treatment groups. Crayfish were exposed to a given treatment temperature for 9 minutes and tested for functional death (loss of equilibrium: LOE) and physiological death (lack of response to probing) every 3 minutes. 9 minutes was chosen to represent the maximum amount of time managers would reasonably allot for treating individual RSC burrows. If the

thermal tolerance assays found hot water wasn't an effective control method within 9 minutes, alternative control methods should be investigated that may be more time and labor effective. To test for LOE, RSC were flipped using a spatula until they were dorsum-down. If they were not able to right themselves within 30 seconds they were recorded as exhibiting LOE and were considered functionally dead-disorganization of locomotory activity such that the animal loses its ability to escape from lethal conditions (Lutterschmidt and Hutchison, 1997). Crayfish exhibiting LOE were then poked with a stainless-steel probe. If they exhibited LOE and did not respond to probing, they were presumed to be physiologically dead (Barbee et al., 2010). To test for ability to recover from endpoints used to indicate functional death and/or presumed physiological death, all crayfish were transferred back to their original acclimation temperature after 9 minutes of exposure to a given temperature shock treatment, regardless of response. After 24 hours of recovery, each crayfish was again checked for righting response and response to probing at its original acclimation temperature (15 or 25 °C).

Data analysis

Lethal temperature (LT_x) values, confidence intervals, and regression lines were calculated using Toxicity Relationship Analysis Program (TRAP) Version 1.21. (U.S. Environmental Protection Agency, Washington, DC, EPA/600/C-11/002, 2010). Graphs were created from the data generated by TRAP using SigmaPlot Version 15.0.0.13 (Grafiti LLC, Palo Alto, CA 94301). Differences in response to acute thermal shock between the two acclimation temperatures were considered significant if there was no overlap between the 95% confidence intervals calculated for LT_x values at each exposure time interval (3, 6, and 9 minutes).

Results

Loss of Equilibrium

Results were analyzed to find the lethal temperature values (LT_x) at which 5, 25, 50, and 95% of crayfish exhibited either LOE or loss of response to probing. At 3 minutes of exposure, the software package was not able to accurately calculate confidence intervals for the crayfish acclimated at 25 °C so we were not able to determine whether differences were significant between 3 min and the other exposure times. LT_x estimates at 3 min ranged from 31.6°C (LT_{05}; 15°C acclimation) to 37.3°C (LT_{95}; 25°C acclimation) (Fig. 1.1A, Table 1).

Within a given acclimation temperature and LT_x category, LT_x estimates associated with LOE did not typically differ between 6 and 9 min exposure times, as indicated by overlapping 95% confidence intervals. For crayfish acclimated to 15°C, only the LT_{95} differed between 6 and 9 min as indicated by non-overlapping 95% CI. For crayfish acclimated to 25°C, there were no differences in $LT_{05, 25, 50, 95}$ values between 6 and 9 min exposure times (Table 1).

However, $LT_{05, 25, 50, 95}$ values did significantly differ between 15 and 25°C acclimation temperatures after 6 and 9 minutes of exposure as indicated by non-overlapping 95% CI. After 6 minutes of thermal shock exposure, the $LT_{05, 25, 50, 95}$ estimates for crayfish acclimated to 15°C were consistently lower than for crayfish acclimated to 25°C. The difference was greatest at the lowest LT_x (2.4°C for LT_{05}) and lowest at the highest LT_x (0.8°C for LT_{95}). LT_x estimates ranged from 34.4°C (LT_{05}; 15°C acclimation) to 37.3°C (LT_{95}; 25°C acclimation) (Fig. 1.1B, Table 1).

After 9 minutes of exposure to thermal shock, the $LT_{05, 25, 50, 95}$ estimates for crayfish acclimated to 15°C were consistently lower than for crayfish acclimated to 25°C. The difference was greatest at the lowest LT_x (2.5°C for LT_{05}) and lowest at the highest LT_x (1.0°C for LT_{95}).

LT_x values ranged from 34.3°C (LT_{05}; 15°C acclimation) to 37.3°C (LT_{95}; 25°C acclimation) (Fig. 1.1C, Table 1).

Presumed Physiological Death

Within a given acclimation temperature, LT_x estimates associated with presumed physiological death significantly decreased with increasing time of exposure within each LT_x category. $LT_{05, 25, 50, 95}$ estimates were always significantly higher at 3 min than 6 or 9 min regardless of acclimation temperature, as evidenced by non-overlapping 95% CI (Table 1.2).

LT_x estimates also significantly differed between acclimation temperatures at each time of exposure. After 3 minutes of exposure to thermal shock, $LT_{05, 25, 50, 95}$ estimates for RSC acclimated to 15°C were lower than for crayfish acclimated to 25°C as evidenced by non-overlapping 95% CI. The difference in $LT_{05, 25, 50, 95}$ estimates between the two acclimation temperatures remained constant at 2.0°C. LT_x estimates ranged from 41.4°C (LT_{05}; 15°C acclimation) to 44.4°C (LT_{95}; 25°C acclimation) (Fig. 1.2A, Table 1.2).

After 6 minutes of thermal shock exposure, $LT_{05, 25, 50, 95}$ values for crayfish acclimated to 15°C were lower than for crayfish acclimated to 25°C as evidenced by non-overlapping 95% CI. The difference was greatest at the lowest LT_x (3.3°C for LT_{05}) and lowest at the highest LT_x (2.8°C for LT_{95}). LT_x estimates ranged from 39.5°C (LT_{05}; 15°C acclimation) to 43.3°C (LT_{95}; 25°C acclimation) (Fig. 1.2B, Table 1.2).

After 9 minutes of thermal shock exposure, $LT_{05, 25, 50, 95}$ estimates for crayfish acclimated to 15°C were consistently lower than for crayfish acclimated to 25°C as evidenced by non-overlapping 95% CI. The difference was greatest at the lowest LT_x (1.2°C for LT_{05}) and lowest at

16

the highest LT_x (0.7°C for LT_{95}). LT_x values ranged from 39.6°C (LT_{05}; 15°C acclimation) to 41.3°C (LT_{95}; 25°C acclimation) (Fig. 1.2C, Table 1.2).

24-hour recovery

Within the 15°C acclimation group, all ten control crayfish and all ten crayfish exposed to 30°C remained alive after the 24h recovery period. Of the nine crayfish exposed to 36°C that exhibited LOE after 9 min, four died and five recovered after being cooled back down to 15°C for 24 h. Of the ten crayfish exposed to 38°C that exhibited LOE after 9 min, eight died and two recovered after being cooled back down to 15°C for 24 h. Of the 6 crayfish exposed to 40°C that exhibited LOE after 9 min, four died and two recovered after being cooled back down to 15°C for 24 h. The remaining four crayfish were scored as physiologically dead at 9 min and remained dead after 24 h. Similarly, at the exposure temperatures of 42, 44, and 46°C, all ten crayfish within each exposure group were scored as physiological dead at 9 min and remained dead after being cooled back down to 15°C for 24 h (Fig. 1.3).

Within the 25°C acclimation group, all control crayfish and all crayfish exposed to 30 and 36°C remained alive after the 24 h recovery period. Of the 10 crayfish exposed to 38°C that exhibited LOE after 9 min, one continued to exhibit LOE and nine recovered after being cooled back down to 25°C for 24 h. Of the 10 crayfish exposed to 40°C that exhibited LOE after 9 min, all recovered after being cooled back down to 25°C for 24 h. All crayfish exposed to 42, 44, and 46°C were scored as physiologically dead at 9 min and remained dead after being cooled back down to 21°C for 24 h (Fig. 1.4).

17

Discussion

Procambarus clarkii have a global distribution ranging from tropical conditions in central America and Africa to high latitude temperate regions in Canada and Europe (Barbaresi et al., 2004; Buhay and Crandall, 2005; Oficialdegui et al., 2020). As one of the most invasive crayfish species in the world, control techniques must account for how invasive populations may adapt to local conditions and how environmental tolerances may change among regions. As I showed in this chapter, effectiveness of control techniques using lethal temperatures may vary depending upon temperatures to which local crayfish have become acclimated. In this study, we compared the response of adult RSC to acute thermal shock after acclimation to cool or warm temperatures. Results provide baseline data for development of hot water treatment methods for crayfish occupying warm and cold environments across differing latitudes or seasons.

Studies estimating upper thermal limits of crayfish historically haven't followed a standardized methodology, making it difficult to compare results from one paper to another. Study objectives often supersede following a set protocol in order to answer species specific questions about acclimation and life history, and the diversity of crayfish species means the questions being asked in these studies vary as much as the crayfish themselves. Thermal tolerance assays are typically conducted using either a static or dynamic approach. The static approach can be further subdivided into a "plunge" approach whereby crayfish are acclimated to a set temperature and then immediately plunged into a different temperature, or an acclimated chronic exposure (ACE) whereby crayfish are acclimated to a given temperature that is then gradually raised (i.e., 1°C/day) until reaching the target temperature which is then held constant. The dynamic approach involves altering water temperature at a steady rate until a defined tolerance endpoint is reached. The rate of increase in the dynamic approach is not standardized

but varies among different studies. Dynamic assays tend to be more common in the literature, while the static plunge approach is the least common (Westhoff and Rosenberger, 2016).

Because our goal was to identify lethal water temperatures that could be used to quickly kill crayfish in burrows, we used the static plunge approach. The temperatures at which crayfish reached LOE or presumed physiological death were strongly affected by acclimation temperature (15 or 25°C) but not by time of exposure (3-9 min). Temperature at LOE did not significantly differ between 6 and 9 minutes for any LT_x category but were lower by as much as 2.5°C for crayfish acclimated to a cool (15°C) compared to a warm (25°C) temperature. In contrast, temperature at presumed physiological death was significantly affected by exposure time as well as acclimation temperature. Within a given LT_x category, temperature at presumed physiological death could differ by as much as 3.1°C between 3 and 9 minute exposure times, and by as much as 3.3°C between cool and warm acclimation groups.

Although differences in methodology make direct comparisons difficult, previous studies provide strong support that upper thermal limits (as indicated by cessation of scaphognathite movement and lack of response to prodding) of crayfish are affected by acclimation temperature. Among the three studies identified by Westhoff and Rosenberger (2016) that used the plunge approach, Becker et al. (1975) showed that the upper lethal temperature of Signal Crayfish (*Pacifastacus leniusculus,* Dana 1852) changed by 3.0°C over an acclimation range of 5-30°C after 48 hours exposure. Similarly, Mirenda and Dimock (1985) showed that the upper lethal temperature of Acuminate Crayfish (*Cambarus acuminatus,* Faxon 1884) changed by 3.0°C over an acclimation range of 4-30°C after 48 hours. The similarities in results (i.e., an ~3.0°C difference among acclimation temperatures) suggests that effects of acclimation on acute "plunge" thermal tolerance may be conserved across families (Astacidae, Cambaridae) and

regions (Southeastern, Pacific Northwest) in North America. The third plunge study (Austin, 1995) did not examine the effects of acclimation temperature.

Evidence for effects of acclimation on thermal tolerance when using a dynamic approach are contradictory and more difficult to compare. Mundahl (1989) examined Rusty Crayfish (*Orconectes rusticus*, Girard 1852) acclimated to stream temperatures from 2.5-33°C and increased temperatures at a relatively rapid rate of 1°C/minute. Crayfish collected from warmer streams had higher temperature at which LOE was observed compared to those from cooler streams. In contrast, Logarbo and Bonvillain (2020) and Bone et al. (2014) found no effect of acclimation on LOE when using slower ramping rates (1°C/hour and 1°C/week, respectively) and a narrower range of acclimation temperatures (24-30°C and 11.8 -14.1°C in laboratory tanks and outdoor ponds, respectively). Taxa studied included *Orconectes rusticus* (Cambaridae; Mundahl, 1989), *Procambarus clarkii* (Cambaridae; Logarbo and Bonvillain, 2020) and *Euastacus sulcatus* (Riek, Parastacidae; Bone et al. 2014). It is possible that the effects of acclimation temperature on thermal tolerance are more pronounced the more rapid the change in temperature, but differences in taxa studied and range of acclimation temperatures tested may also be responsible for differences in results among studies.

Regardless of the mechanisms driving the differences among studies, static plunge studies (this study, Becker et al. 1975, and Mirenda and Dimock, 1985) and the most rapid dynamic approach (Mundahl 1989) showed an effect of acclimation temperature that suggests acclimation temperatures (i.e. natural burrow temperatures) are an important consideration when using hot water to control crayfish in burrows. If the burrow contains little to no natural groundwater prior to treatment, the change in temperature will be nearly instantaneous and similar to the static plunge approach. If the burrow contains moderate to large volumes of

groundwater, the change in temperature will not be instantaneous but would still be rapid and more likely to resemble the 1°C/m rate (Mundahl, 1989) than the 1°C/h rate (Logarbo and Bonvillain, 2020). It is likely that hot water treatments would be most effective at higher latitudes and/or during the cooler months compared to lower latitudes during mid summer.

A potential concern when applying laboratory results to control measures in the field is that crayfish that were still alive at the end of the laboratory exposure period were so stressed that they would eventually die even after temperatures cooled. In this case managers might be investigating or recommending higher application temperatures than actually needed. This scenario occurred in the cool acclimation group after exposure to 36 - 40°C. Twenty to forty percent of crayfish exhibiting LOE after 9 minutes of exposure died within 24 h of being cooled back down to 15°C indicating that they were unable to adjust to the acute thermal shock. In contrast, the warm group did not exhibit any deaths during the 24 h recovery period. In colder environments (e.g. higher latitudes or winter months), 36-40°C might be hot enough to kill 40% of crayfish, whereas these temperatures would not be effective in warmer environments.

A larger concern is that RSC might recover from functional or presumed physiological death after temperatures cooled, resulting in field application temperatures being less effective than anticipated. Functional death (LOE) is not true death, and aquatic taxa such as fish may recover from LOE if provided a 24 hr recovery period as temperatures cool (Bard and Kieffer 2019). This scenario occurred in both the cool and warm acclimation groups exposed to 36-40 °C for 9 minutes. Within the cool group, 20-80% of crayfish recovered from LOE within 24h whereas in the warm group, 90-100% of crayfish recovered from LOE. Thus, LOE may not be an appropriate endpoint to use when assessing upper lethal limits for purposes of invasive species control, especially when applying hot water in warmer environments (e.g. lower latitudes or

summer months). Presumed physiological death, as defined by a lack of response to probing, is frequently used as a lethal endpoint in ecotoxicology studies (e.g. Barbee et al.,2010; Wigginton and Birge, 2007) and was a more stable endpoint. No crayfish in the cool or warm acclimation groups recovered from presumed physiological death within 24 hours of being cooled back down to their original acclimation temperature – indicating that loss of response to probing was a reliable indicator of physiological death for crayfish. For both groups, 100% of RSC exhibited physiological death after exposure to 42-46°C for 9 minutes.

Taken together, these results suggest that applying water $\geq 42\,°C$ to burrows for 9 minutes would be sufficient to kill 100% of RSC, regardless of acclimation temperature. As most managers are looking at the quickest and most effective control method, treating an individual burrow for 9 minutes may be unrealistic when trying to eradicate thousands of RSC. In order to reduce treatment time, increasing treatment temperature above $46\,°C$ may allow for 100% mortality in less than 9 minutes. Commercially available heated pressure washers can easily heat water to $100\,°C$, which is more than double the temperature required to kill 100% of crayfish within 9 minutes. They are frequently used to control invasive fouling organisms on boats (Shannon et al., 2018). We are not aware of previous studies using heated pressure washers to control invasive species in burrows, but hot water is frequently used as a treatment to control nematodes burrowed into plant roots (e.g. Tsang et al., 2003).

Additional research using a similar approach but with warmer temperatures (i.e., up to $100\,°C$) is required to determine whether RSC can be killed at < nine minutes with minimal risk of recovery. Anecdotal evidence from low country crawfish boils have shown RSC only need 5-10 minutes in boiling water to be fully cooked (Huner and Barr, 1991). Assuming they die before being fully cooked, death should occur in < 5 minutes. Spraying $100\,°C$ water directly into

a burrow would be similar to boiling them in a pot. However, because acclimation temperature can affect tolerance to acute thermal shock, treating crayfish in southern Alabama during late summer may require a few extra minutes to reach 100% mortality than treating crayfish in Canada during the cooler months.

As with all controlled lab studies, these results may not translate directly in the field due to multiple variables in the natural environment. The efficiency of using hot water as a control agent is likely dependent on factors such as burrow depth, soil composition, volume, number of entrances, size and status of the occupying crayfish, environmental temperature, and burrow temperature. For example, because it takes longer to fill up a 2m burrow than a 0.5m burrow, the water has more potential to cool before it fills the burrow. Burrow morphology can also influence results, as multiple entrances may act as thermal vents and not concentrate the heat as much as in a single entrance burrow. Secondary entrances might also provide an escape route for the occupant while water is being applied to a different entrance. Percent mortality is improved as temperature and treatment time increases, but it is only practical to heat water up to 100 °C and burrow treatment for longer than five minutes may be more laborious than employing another control technique such physical blockers (Bates et al., 2023) or chemical control (Cecchinelli et al., 2012). This study suggests hot water may be a valuable tool to consider for integrated pest management. However, empirical studies are needed to test the application of hot water in the field and compare its efficiency to other techniques.

References

Abdelrahman, H. A., Gibson, R. L., Fogelman, K. J., Cupp, A. R., Allert, A. L., and Stoecke, J. A. (2021). Evaluation of dissolved carbon dioxide to stimulate emergence of red swamp crayfish (Decapoda: Cambaridae) from infested ponds. *Management of Biological Invasions*, *12*(4), 952-974. https://doi.org/10.3391/mbi.2021.12.4.11

Anderson, L. G., Dunn, A. M., Rosewarne, P. J., and Stebbing, P. D. (2015). Invaders in hot water: a simple decontamination method to prevent the accidental spread of aquatic invasive non-native species. *Biol Invasions*, *17*(8), 2287-2297. https://doi.org/10.1007/s10530-015-0875-6

Austin CM. (1995). Effect of temperature and salinity on the survival and growth of juvenile redclaw (*Cherax quadricarinatus*). *Freshwater Crayfish* 10(1):419-426.

Bates, B. L., Allert, A. L., Wildhaber, M. L., and Stoeckel, J. A. (2023). Use of physical blockers to control invasive red swamp crayfish in burrows. *Management of Biological Invasions*, *14*(4), 709-729. https://doi.org/10.3391/mbi.2023.14.4.09

Barbaresi, S., Santini, G., Tricarico, E., & Gherardi, F. (2004). Ranging behaviour of the invasive crayfish, *Procambarus clarkii* (Girard) [Article]. *Journal of Natural History*, *38*(22), 2821-2832. https://doi.org/10.1080/00222930410001663308

Barbee, G. C., McClain, W. R., Lanka, S. K., & Stout, M. J. (2010). Acute toxicity of chlorantraniliprole to non-target crayfish (<i>Procambarus clarkii</i>) associated with rice-crayfish cropping systems [Article]. *Pest Management Science*, *66*(9), 996-1001. https://doi.org/10.1002/ps.1972

Bard, B., & Kieffer, J. D. (2019). The effects of repeat acute thermal stress on the critical thermal maximum (CTmax) and physiology of juvenile shortnose sturgeon (<i>Acipenser brevirostrum</i>) [Article]. *Canadian Journal of Zoology*, *97*(6), 567-572. https://doi.org/10.1139/cjz-2018-0157

Becker, C. D., Genoway, R. G., and Merrill, J. A. (1975). Resistance of a Northwestern Crayfish, *Pacifastacus leniusculus* (Dana), to elevated temperatures [Article]. *Transactions of the American Fisheries Society*, *104*(2), 374-387. https://doi.org/10.1577/1548-8659(1975)104<374:Roancp>2.0.Co;2

Bone JWP, Wild CH, Furse JM (2014) Thermal limit of *Euastacus sulcatus* (Decapoda: Parastacidae), a freshwater crayfish from the highlands of central eastern Australia. Mar Freshw Res 65:645–651

Bradbeer, S. J., Coughlan, N. E., Cuthbert, R. N., Crane, K., Dick, J. T. A., Caffrey, J. M., Lucy, F. E., Renals, T., Davis, E., Warren, D. A., Pile, B., Quinn, C., and Dunn, A. M. (2020). The effectiveness of disinfectant and steam exposure treatments to prevent the spread of the highly invasive killer shrimp, . *Scientific Reports*, *10*(1). https://doi.org/ARTN 191910.1038/s41598-020-58058-8

Bruce, T. J., Abernathy, J. W., Tripp, N., Barnes, N., Harrison, C. E., Oladipupo, A. A., Krol, J. D., Wise, A. L., Warg, J. V., and Stoeckel, J. A. (2024). White spot syndrome virus in Alabama red swamp crayfish (*Procambarus clarkii*). *Journal of Fish Diseases*, *47*(2). https://doi.org/10.1111/jfd.13873

Buhay, J. E., & Crandall, K. A. (2005). Subterranean phylogeography of freshwater crayfishes shows extensive gene flow and surprisingly large population sizes [Article]. *Molecular Ecology*, *14*(14), 4259-4273. https://doi.org/10.1111/j.1365-294X.2005.02755.x

Cecchinelli, E., Aquiloni, L., Maltagliati, G., Orioli, G., Tricarico, E., and Gherardi, F. (2012). Use of natural pyrethrum to control the red swamp crayfish *Procambarus clarkii* in a rural district of Italy. *Pest Management Science*, *68*(6), 839-844. https://doi.org/10.1002/ps.2335

Centers for Disease Control and Prevention. (2014, March 25). *Pyrethrins and Pyrethroids*. Centers for Disease Control and Prevention. https://wwwn.cdc.gov/TSP/PHS/PHS.aspx?phsid=785&toxid=153#bookmark11

Charles Lutz, M. G. S., Robert P Romaire, William R MCclain. (2005). Crawfish Production Manual.

Dalosto, M. M., Palaoro, A. V., Souty-Grosset, C., Bueno, S. L. D., Loureiro, T. G., Almerao, M. P., de Araujo, P. B., and Santos, S. (2015). One step ahead of the enemy: investigating aggressive interactions between invasive and native crayfish before the contact in nature. *Biological Invasions*, *17*(12), 3503-3515. https://doi.org/10.1007/s10530-015-0974-4

Gherardi, F. (2006). Crayfish invading Europe:: the case study of. *Marine and Freshwater Behaviour and Physiology*, *39*(3), 175-191. https://doi.org/10.1080/10236240600869702

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowledge and Management of Aquatic Ecosystems*(394-95). https://doi.org/ARTN 1110.1051/kmae/2009025

Huner, J. V., Jeske, C. W., and Norling, W. (2002). Managing agricultural wetlands for waterbirds in the coastal regions of Louisiana, USA. *Waterbirds*, *25*, 66-78. <Go to ISI>://WOS:000180065700008

Huner, J. V. and Barr, J. E. (1991). Red Swamp Crawfish: Biology And Exploitation.

Jiravanichpaisal, P., Bangyeekhun, E., Söderhäll, K., and Söderhäll, I. (2001). Experimental infection of white spot syndrome virus in freshwater crayfish. *Diseases of Aquatic Organisms*, *47*(2), 151-157. https://doi.org/DOI 10.3354/dao047151

Logarbo, J. R., and Bonvillain, C. P. (2020). Thermal stress responses of two sympatric crayfishes in Louisiana, Hobbs and Hobbs, 1990 (Decapoda: Astacoidea: Cambaridae). *Journal of Crustacean Biology*, *40*(6), 734-738. https://doi.org/10.1093/jcbiol/ruaa066

Lutterschmidt, W. I., & Hutchison, V. H. (1997). The critical thermal maximum: history and critique [Review]. *Canadian Journal of Zoology*, *75*(10), 1561-1574. https://doi.org/10.1139/z97-783

Mirenda, R. J., and Dimock, R. V. (1985). Temperature tolerance of the crayfish cambarus - *Acuminatus faxon,* 1884 (Decapoda, Astacidea) [Article]. *Crustaceana*, *48*(MAY), 249-259. https://doi.org/10.1163/156854085x00963

Mundahl, N. D. (1989). Seasonal and Diel Changes in Thermal Tolerance of the Crayfish *Orconectes rusticus*, with Evidence for Behavioral Thermoregulation. *Journal of the North American Benthological Society*, *8*(2), 173-179. https://doi.org/Doi 10.2307/1467635

Oficialdegui, F. J., Sanchez, M. I., and Clavero, M. (2020). One century away from home: how the red swamp crayfish took over the world. *Reviews in Fish Biology and Fisheries*, *30*(1), 121-135. https://doi.org/10.1007/s11160-020-09594-z

Paillisson, J. M., Soudieux, A., and Damien, J. P. (2011). Capture efficiency and size selectivity of sampling gears targeting red-swamp crayfish in several freshwater habitats. *Knowledge and Management of Aquatic Ecosystems*(401). https://doi.org/ARTN 0610.1051/kmae/2011015

Shannon, C., Quinn, C. H., Stebbing, P. D., Hassall, C., & Dunn, A. M. (2018). The practical application of hot water to reduce the introduction and spread of aquatic invasive alien species [Article; Proceedings Paper]. *Management of Biological Invasions*, *9*(4), 417-423. https://doi.org/10.3391/mbi.2018.9.4.05

Schuster, G. A., Taylor, C. A., & McGregor, S. W. (2022). *Crayfishes of Alabama*. The University of Alabama Press.

Tsang, M. M. C., Hara, A. H., & Sipes, B. (2003). Hot-water treatments of potted palms to control the burrowing nematode, <i>Radopholus similis</i> [Article]. *Crop Protection*, *22*(4), 589-593. https://doi.org/10.1016/s0261-2194(02)00235-1

Westhoff, J. T., and Rosenberger, A. E. (2016). A global review of freshwater crayfish temperature tolerance, preference, and optimal growth [Review]. *Reviews in Fish Biology and Fisheries*, *26*(3), 329-349. https://doi.org/10.1007/s11160-016-9430-5

Wigginton, A. J., & Birge, W. J. (2007). Toxicity of cadmium to six species in two genera of crayfish and the effect of cadmium on molting success [Article]. *Environmental Toxicology and Chemistry*, *26*(3), 548-554. https://doi.org/10.1897/06-186r.1

Table 1.1 Lethal temperatures (LTx) that caused functional death (loss of equilibrium) for x percent of *Procambarus clarkii* after 3, 6, and 9 minutes. 95% confidence intervals are listed underneath each LT_x where available. Grey and white fill patterns demarcate different LTx categories. Superscript letters indicate significant differences in LTx estimates within each LTx category.

Acc Temp	LTx Category	LTx estimate (℃) 3-minute LOE	LTx estimate (℃) 6-minute LOE	LTx estimate (℃) 9-minute LOE
15	LT_{05}	31.6 (N/A)	34.4^b (34.2-34.8)	34.3^b (33.9-34.6)
25	LT_{05}	36.8 (N/A)	36.8^a (36.6-36.9)	36.8^a (36.6-36.9)
15	LT_{25}	33.2 N/A	35.1^b (34.9-35.3)	34.9^b (34.7-35.1)
25	LT_{25}	36.9 (N/A)	36.9^a (36.8-37)	36.9^a (36.8-37)
15	LT_{50}	34.1 (N/A)	35.5^b (35.4-35.6)	35.3^b (35.1-35.4)
25	LT_{50}	37 (N/A)	37^a (36.9-37.1)	37^a (36.9-37.1)
15	LT_{95}	36.6 (N/A)	36.5^c (36.4-36.7)	36.3^b (36.2-36.3)
25	LT_{95}	37.3 (N/A)	37.3^a (37.1-37.4)	37.3^a (37.2-37.4)

Table 1.2 Lethal temperatures (LTx) that caused presumed physiological death (lack of response to probing) for x percent of *Procambarus clarkii* after 3, 6, and 9 minutes. 95% confidence intervals are listed underneath each LT_x where available. Grey and white fill patterns demarcate different LTx categories. Superscript letters indicate significant differences in LTx estimates within each LTx category.

| Acc Temp	LTx Category	LTx estimate (℃) 3-minute Death	LTx estimate (℃) 6-minute Death	LTx estimate (℃) 9-minute Death
15	LT_{05}	41.4^c (41.2-41.4)	39.5^e (39.4-39.5)	39.6^e (39.5-39.6)
25	LT_{05}	43.4^a (43.4-43.5)	42.8^b (42.6-42.9)	40.8^d (40.6-40.9)
15	LT_{25}	41.7^c (41.6-41.7)	39.8^e (39.8-39.8)	39.9^e (39.9-39.9)
25	LT_{25}	43.7^a (43.7-43.8)	42.9^b (42.8-43)	40.9^d (40.8-41)
15	LT_{50}	41.9^c (41.8-41.9)	40^f (40)	40.1^e (40.1-40.1)
25	LT_{50}	43.9^a (43.9-43.9)	43^b (42.9-43.1)	41^d (40.9-41.1)
15	LT_{95}	42.4^c (42.3-42.4)	40.5^e (40.5-40.6)	40.6^e (40.5-40.6)
25	LT_{95}	44.4^a (44.4-44.5)	43.3^b (43.2-43.6)	41.3^d (41.2-41.4)
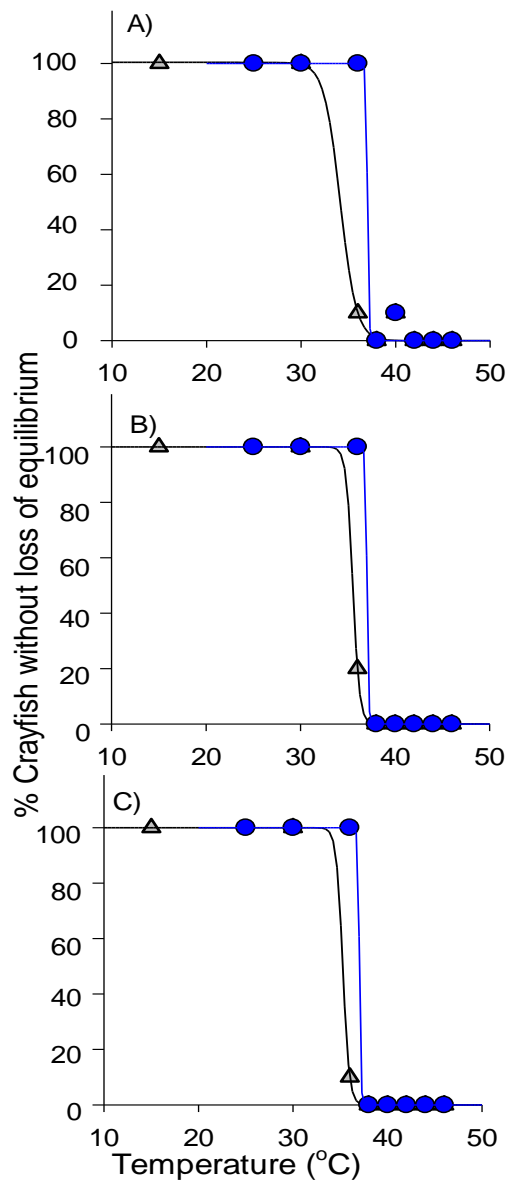
Figure 1.1 Percentage of *Procambarus clarkii* without loss of equilibrium after 3 minutes (A), 6 minutes (B), and 9 minutes (C) exposure to heat bath treatments. Gray triangles and black lines represent the 15ºC acclimation group. Blue points and lines represent the 25ºC acclimation group.

Figure 1.2 Percentage of *Procambarus clarkii* with a response to probing after 3 minutes (A), 6 minutes (B), and 9 minutes (C) exposure to heat bath treatments. Gray triangles and black lines represent the 15°C acclimation group. Blue points and lines represent the 25°C acclimation group.
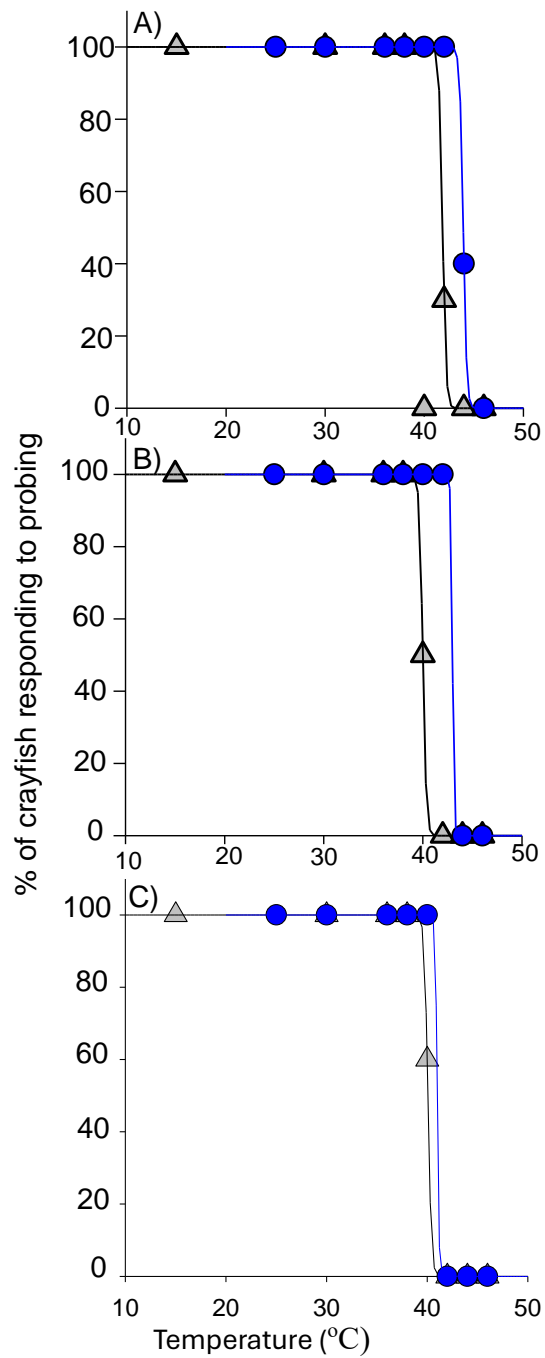
Figure 1.3. Status of 15°C acclimated crayfish after a 24h recovery period compared to their original status after 9 min exposure to a given thermal shock temperature. During the recovery period, crayfish were returned to their original acclimation temperature of 25°C. The leftmost bar in this graph represents control Red Swamp Crayfish.
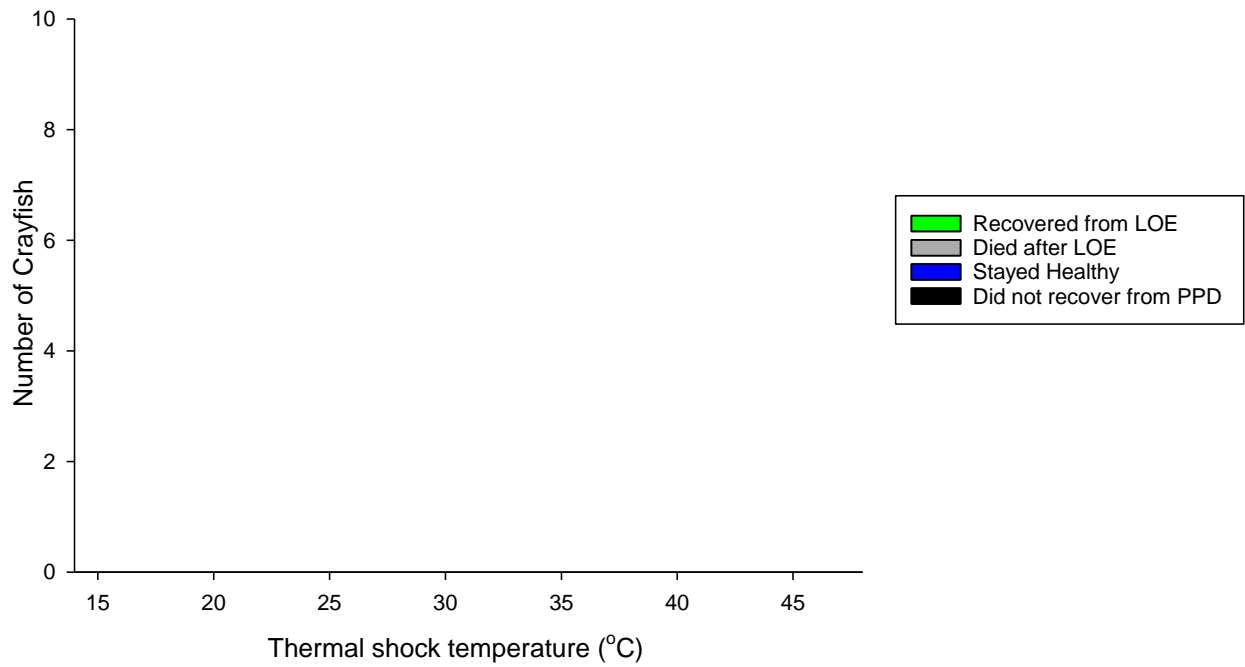
Figure 1.4. Status of 25°C acclimated crayfish after a 24h recovery period compared to their original status after 9 min exposure to a given thermal shock temperature. During the recovery period, crayfish were returned to their original acclimation temperature of 15°C. The leftmost bar in this graph represents control Red Swamp Crayfish.
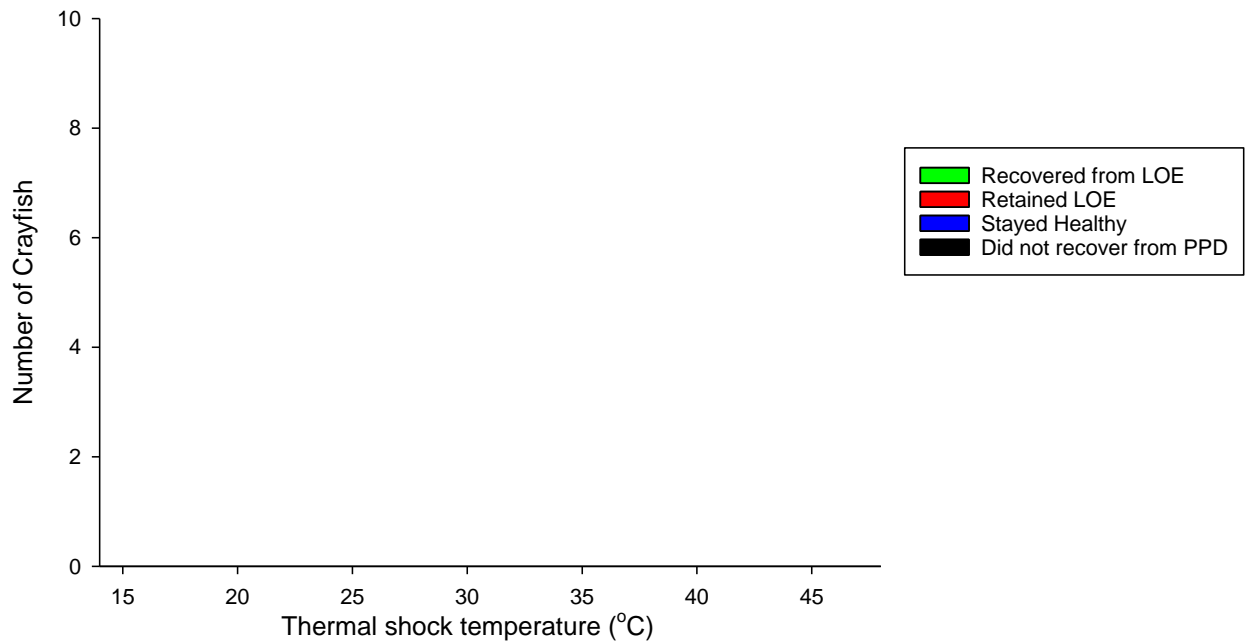
Chapter Two: Burrowing Behaviors of Red Swamp Crayfish

Introduction

Crayfish are a very diverse taxa within the infraorder Astacidea, represented by over 600 species of crayfish worldwide (Crandall and De Grave, 2017). The environments to which they have adapted are correspondingly diverse and vary from surface waters to underground burrows and caves, freshwater to brackish, tropical to arctic, and fully aquatic to nearly terrestrial (Barbaresi et al., 2004; Buhay and Crandall, 2005; Mouser et al., 2022). The southeastern United States is a diversity hotspot for crayfish, with over 400 different species (Crandall and De Grave, 2017). Compared to the five native species in Europe that primarily live in open water (Trouilhé et al., 2007), crayfish in the United States have evolved to fit into many niches and to modify their local environments, including construction of ventilated underground burrows (Stoeckel et al., 2021). A quarter of the threatened or endangered crayfish species in the U.S. are primary burrowers and life-history information is often lacking (Taylor et al., 2007; Bloomer et al., 2021). To develop conservation plans, life history cycles of these species need to be better understood. Additionally, understanding the ecology of secondary burrowers, like the Red Swamp Crayfish (RSC: *Procambarus clarkii*) that are invasive in many parts of the world, requires a solid understanding of both the open water and burrowing portion of their lives to develop an effective treatment plan. Understanding the life cycle of crayfish that live underground is often more challenging than studying stream dwelling species, as observing subterranean behavior and collecting crayfish are difficult without excavating and destroying the burrow microhabitat (Bloomer et al., 2021).

Red Swamp Crayfish are highly prolific due to their relatively large size, rapid growth rates, high fecundity, tolerance to hypoxic waters, and the ability to live in a range of aquatic and semi-terrestrial (i.e. burrows) habitats (Lutz et al., 2005). Mature adult RSC can exceed 65 mm total carapace length (TCL) and females can reproduce twice a year in warmer environments, with large females able to produce over 600 eggs per brood (Schuster et al., 2022). Fecundity and growth rate are higher than many other crayfish species allowing RSC to rapidly increase in population size (Schuster et al., 2022). They can live in areas with poor water quality such as the hypoxic waters of the Atchafalaya River Basin (Bonvillain et al., 2012), however, individuals found in these areas have a smaller clutch and egg size (Ortman, 2021). Additionally, RSC have been found molting and reproducing in estuarine habitats with salinities up to 25 ppt (Bissattini et al., 2015).

Aquaculture activities, production and/or shipment of live animals to consumers, represent a major dispersal pathway for RSC. In 2019, U.S. production was 72,000 metric tons (mt) of RSC, with 93% of farms located in Louisiana. Global production of RSC reached 2.2 million mt, driven by the increase in production from China (2.12 mt) (Tucker, 2022). Production typically occurs in outdoor ponds connected to a nearby waterway used to fill and drain ponds (Lutz et al., 2005) which allows for opportunities to escape production facilities and invade regional ecosystems. Red Swamp Crayfish aren't limited to dispersing through waterways, but can also move across terrestrial environments (Thomas et al., 2019). They can also be dispersed via shipments for the pet trade, food trade, and research, making control especially difficult. Invasive RSC populations are now established globally (Oficialdegui et al., 2020).

Red Swamp Crayfish are considered to be a secondary burrower in many regions, spending around half the year underground to brood eggs and rear young (Huner and Barr,

1991). This presents both a challenge and an opportunity. Control efforts need to focus not only on populations in surface waters but also on burrows in surrounding terrestrial habitat. However, control of crayfish in burrows may present a valuable opportunity to focus control efforts on brooding females and/or young, thus directly reducing the reproductive capacity of an invasive population.

To develop an effective RSC management plan, more information is needed about the burrowing portion of their life cycle and the factors that trigger a shift from surface water to terrestrial habitat. For research purposes, a protocol that effectively promotes burrowing is needed to generate a sufficient number of burrows for experiments (Ames et al., 2015; Bates et al., 2023). Many crayfish species, including RSC, show a preference for burrowing in substrates with small particle sizes such as clay, while coarser particles (e.g. sand) and rocky substrates can restrict burrow depths (Correia and Ferriera, 1995; Stoeckel et al., 2011; Barnes, pers. obs). Even when optimal soil types are available, water withdrawal rates seem to be an important factor with slow drainage rates in ponds inducing burrowing behavior better than rapid drawdowns (Lutz et al., 2005).

Burrowing chambers provide a valuable tool to study burrowing crayfish by allowing for controlled manipulation of variables such as soil composition, drainage rate, temperature, and photoperiod while burrow construction can be viewed through clear walls and quantitative endpoints measured through time via burrow tracings (Stoeckel et al., 2011; Helms et al., 2013). The objectives of this study were to (1) determine the effect of ground water drainage rates on burrowing, (2) determine the effect of soil composition on burrowing, and (3) test for differences in burrowing behaviors between males and females.

Methods

Groundwater drainage rate

Burrowing chambers were modified from Stoeckel et al., (2011). For ground water drainage experiments (Fig. 2.1A), chamber sides were made from 6.35 mm thick plexiglass sheets measuring 72 cm H x 91.5 cm L. The internal frame of the chambers was made of 5.08 cm x 8.89 cm wooden boards (2" x 4"s) with each side 96.5 cm long and the internal width between the plexiglass chamber sides was 5.08 cm. The plexiglass sheets were secured to the internal frame with stainless steel bolts inserted through 6.35 mm holes along the outer edge. An additional external wooden frame measuring 78.75 cm H x 80 cm L was used to tighten the seal and keep water contained within the chambers. Inside the wooden frames sat a vinyl gutter frame measuring 73 cm L x 71 cm H x 5.08 cm W. Either side of the gutter frame had 6.35 mm holes drilled every 3 cm to allow water to permeate into the inner burrowing chamber where the substrate was contained. On the inner side of the vinyl gutter frame a 500 µm Nitex mesh was glued to keep the soil from flowing out of the inner burrowing arena while still allowing water to flow in. A 1.27 cm internal standpipe was installed through the wooden frame on the bottom edge using a 1.27 cm uniseal that allowed water levels to be raised and lowered as needed to meet experimental conditions. A 41 cm H x 28.5 cm L x 29 cm W plastic tub "surface chamber" was secured to the top of each burrowing chamber. Each surface chamber had a 5 cm diameter hole in the center to allow crayfish access the burrowing chamber. The soil used to fill burrowing chambers was high clay content (36.7% fine particles, Stoeckel et at., 2011) collected from the Auburn University EW Shell Fisheries Station where RSC frequently create burrows. Within the burrowing chamber laboratory, temperature was held constant at 25°C and photoperiod at 12:12 L:D to mimic early summer temperatures and photoperiod when RSC were frequently observed

to burrow at the collection site (Barnes, pers. obs.). To control photoperiod, light timers were set to 12 hours on and 12 hours off. During the day period, the burrowing portion of each chamber was shaded by placing black plastic sheeting over the front and black plexiglass sheets.

Prior to adding crayfish, each burrowing chamber was filled with soil and ground water raised to the surface via the internal standpipe for 24 hours in order to saturate the soil (Stoeckel et al. 2011). RSC were trapped from a pond on station with a high population of RSC, selecting males with 45-55 mm carapace lengths, and transported directly into the burrowing chamber lab. After 24 h of soil saturation, a single male RSC ranging from 45-55 mm CL was added to each acclimation chamber above the burrowing chamber (Fig. 2.1A) and the groundwater immediately drained to the prescribed level associated with a particular treatment. Each chamber was assigned to one of three groundwater drainage treatments: 4 cm/day for 5 days, 8 cm/day for 3 days, or 24 cm/day for 1 day. Each run contained nine burrowing chambers, with nine RSC being randomly assigned to one of the three treatments. After each run, all chambers were broken down, had dirt replaced, and set up again in the same manner, saturating the soil 24 hours prior to the introduction of the next RSC. The nine burrowing chambers were run four times, ultimately having 12 RSC assigned to each treatment. Regardless of treatment, all crayfish were allowed to burrow for a total of 5 days. Study initiation time was recorded as soon as all crayfish were placed in chambers and water levels were dropped. At that time on each subsequent experimental day, the shade panel was lifted from the front of each chamber and burrow depth was recorded for each crayfish as the linear distance from the soil surface to the bottom of the burrow.

Groundwater data analysis

Data were rank-transformed to meet assumptions of normality (SigmaPlot version 15.0.0.13).To determine the relationship between burrowing rate and groundwater drainage rate,

we fit a linear regression to a plot of mean burrow depth vs. day for the sole treatment (4 cm/day) where crayfish burrowed on a daily basis (SigmaPlot Version 15.0.0.13, Grafiti LLC, Palo Alto, CA 94301). To test for differences in final burrow depth (cm) among the drainage rate treatments, we used a Kruskal-Wallis analysis of variance on ranks followed by a *post hoc* Tukey Test.

Soil Composition

For the soil composition experiment, burrowing chambers were modified to house two crayfish in each chamber (Fig. 2.1B). The outer wooden and gutter frames were narrowed to 3.81 cm deep. A gutter divider was inserted down the center of each chamber to separate it into two halves. The single surface tank on top of each burrowing chamber was replaced with two plastic containers measuring 21 cm W x 33 cm L x 13 cm T that sat on the top of each half of the burrowing chamber. The soil for these studies was the ambient hillside soil used in the previous ground water drainage rate study, mixed with increasing amounts of Quick-Crete Play Sand. 10 different ambient soil:sand ratios were tested: 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90. Both sides of a given burrowing chamber were filled with the same soil:sand mixture. Red Swamp Crayfish for this study were collected on site from the E.W. Shell Fisheries Station at Auburn University and all measured between 45-55mm total carapace length. One male and one female were randomly assigned to one of two surface tanks on each burrowing chamber (one crayfish per surface tank). Based on the previous groundwater drainage experiment (see Results Section) we used a drainage rate of 4 cm/day in all soil types to provide favorable conditions for burrowing. After drawing down the groundwater for five days, tracings of the final burrow made by each crayfish were made by shining a light through the back plexiglass and placing a sheet of tracing paper over the front plexiglass.

Soil composition data analysis

To test for effects of soil composition, sex and carapace length on total depth burrowed, burrow area, and burrow perimeter we used a one-way ANCOVA (SigmaPlot version 15) where soil composition was the factor, and sex and carapace length were covariates. Burrow area and perimeter were calculated to the nearest centimeter by analyzing burrow tracings using ImageJ version 1.54g.

Energetics

Because not all crayfish burrowed in the soil composition experiment and a lack of burrowing activity was not related to soil type (see results), we decided to assess the energetic health of each crayfish and test whether non-burrowing crayfish had a lower energy density than burrowers. We used bomb calorimetry to measure energetic content of each individual following the traditional approach of Glover et al. (2010). After being in the burrowing chambers for 5 days, crayfish were euthanized via freezing and stored at -80°C. Within 60 days of collection all crayfish were thawed and then dried at 100 °C for 12 hours. After drying, the entire crayfish was ground into a fine powder using an analytical mill (IKA A11 basic analytical mill, 2635 Northchase Parkway SE Wilmington, NC 28405 USA) then pressed into a pellet weighing 0.1-0.2 grams. Each pellet was then ignited in a Parr 6725 semimicro calorimeter following the procedures described in the manual. Caloric values obtained for two pellets per crayfish were then averaged to obtain a caloric density for that individual (cal/g dry mass). A T-test (SigmaPlot v.15) was used to test for differences in caloric density between burrowing and non-burrowing crayfish.

Results

Groundwater drainage rate

A majority of RSC (11/12) burrowed at the slowest groundwater drainage rate (4 cm/day for 5 days). Mean burrow depth increased linearly with time ($r^2 = 0.996$, $P < 0.001$) at a rate of 3.3 cm/day based on the slope of the regression (Figure 2.2A). At the intermediate groundwater drainage rate (8 cm/day for 3 days), 7/12 crayfish initiated burrows on day one. The remaining five crayfish remained at the surface for the remainder of the trial. Mean burrow depth on day one was 5.6 cm (+/- 1.22 cm) and remained relatively constant for the remaining four days despite the groundwater declining to 24 cm below the surface (Fig. 2.2B). At the fastest drainage rate (24 cm /day for 1 day), none of the 12 crayfish initiated burrows on day one and all remained at the surface for the duration of the trial (Fig. 2.2C) with three crayfish dying. Mean burrow depth on day five significantly differed among treatments (Kruskal Wallis; $H = 23.730$, $d.f. = 2$, $P < 0.001$), with depth significantly greater in the slow drainage treatment compared to the moderate (Tukey: $P = 0.018$) and fast treatments (Tukey; $P < 0.001$) but not significantly different between the moderate and fast treatments (Tukey; $P = 0.175$) (Fig. 2.3).

Soil composition

Of the 20 RSC tested in this study, 12 exhibited burrowing activity (six males and six females), while the other eight did not construct any type of burrow (four males and four females). Non-burrowers were observed in the following ambient:sand soil mixtures: 100:0 (both), 90:10 (male), 80:20 (female), 60:40 (female), 30:70 (male), and 20:80 (both). Only two chambers had both crayfish not construct any burrow, two chambers had only one crayfish burrow, and the remaining six chambers had both crayfish construct burrows.

Total Depth Burrowed.

When analyzing data for effects of sex, carapace length, and soil type on burrow depth, the Shapiro-Wilk normality test and the Levene equal variance tests passed (P = 0.220 and 0.416, respectively). There were no significant interactions between sex and soil type (P = 0.488) or sex and carapace length (P = 0.273). The equal slopes assumption passed, and the equal slopes model showed no significant effect of sex or carapace length on burrow depth (P = 0.33 and 0.441 respectively). However, there was a significant effect of soil type on burrow depth (P = 0.006).

For those crayfish that exhibited burrowing activity, burrow depth showed a negative relationship with increasing percent sand in the soil mixture. When fitted with a quadratic regression, the studentized residual test identified the 50% sand (male) burrow depth as an outlier. After removing this outlier, the quadratic regression passed the Shapiro-Wilk normality test (P = 0.2905) and exhibited a $P < 0.001$ and $r^2 = 0.9472$ (Fig. 2.4).

Burrow Area.

When analyzing data for effects of sex, carapace length, and soil type on burrow area, the Shapiro-Wilk normality test and the Levene equal variance tests passed (P= 0.427 and 0.333, respectively). There were no significant interactions between sex and soil type (P= 0.769) or sex and carapace length (P= 0.612). The equal slopes assumption passed, and the equal slopes model showed no significant effect of sex, soil type, or carapace length on burrow area (P= 0.609, 0.079, and 0.436 respectively). There was no significant linear relationship between burrow area (cm^2) and percent sand added to soil (P = 0.073; Fig. 2.5).

Burrow Perimeter.

When analyzing data for effects of sex, carapace length, and soil type on burrow perimeter, the Shapiro-Wilk normality test and the Levene equal variance tests passed ($P= 0.185$ and 0.482, respectively). There were no significant interactions between sex and soil type ($P= 0.712$) or sex and carapace length ($P= 0.581$). The equal slopes assumption passed, and the equal slopes model showed no significant effect of sex, or carapace length on burrow area ($P= 0.606$, and 0.254 respectively). However, there was a significant effect of soil type on burrow perimeter ($P= 0.020$).

For those crayfish that exhibited burrowing behavior, burrow perimeter showed a significant, negative linear relationship with increasing percent sand in the soil mixture ($P=0.005$, $r^2=0.5494$). For each 10% increase in sand in soil composition, we observed a 1.5 (+/- 0.9; 95% CI) cm decrease in burrow perimeter. (Fig. 2.5).

Energetics

Mean (±SD) caloric density (1948.6 ± 472.6) of crayfish that did not burrow was significantly lower than that of crayfish that did burrow (2452.2 ± 427.4) in the soil composition experiment ($t(18) = -2.476$, d.f. $= 18$, $P = 0.023$; Fig. 2.6a). The t-test passed the Shapiro Wilk Normality Test and Brown-Forsythe Equal Variance Test ($p= 0.440$ and 0.855, respectively). For those crayfish that did burrow, there was no significant linear relationship between depth burrowed and energy (Fig. 6b), or between soil composition and energy (Fig. 2.6c) ($p = 0.0716$ and 0.7202, respectively).

Discussion

Procambarus clarkii have established populations globally in environments ranging from freshwater to brackish, tropical to arctic, coastal swamps to desert springs (Barbaresi et al., 2004; Buhay and Crandall, 2005; Mouser et al., 2022). Within invaded regions such as Europe, RSC may be better at burrowing than native species. They are able to construct vertical burrows in sandy-clay soils while many European indigenous species do not (Kotovska et al., 2016). Due to the diversity of successfully invaded habitats and the natural tendency of RSC to brood young in terrestrial burrows, RSC may burrow readily in a wide range of terrestrial habitats. However, there is evidence that RSC burrowing is more constrained than their wide range would suggest. The presence of rocks appears to decrease burrowing activity and RSC tend to burrow less frequently in natural banks compared to non or semi-natural banks presumably due to reduced steepness of the natural banks. Reduced movement from water and up onto banks was also observed in bare sandy soils, presumably reducing burrowing activity (Souty-Grosset et al. 2014; Lemmers et al. 2022).

With the large variance in precipitation among native and invaded habitats worldwide, initial expectations were that groundwater drainage rates from the soil surface would not strongly affect burrowing behavior. When comparing average rainfalls, RSC native range in Houma, Louisiana, near where much of the RSC harvest in Louisiana happens, averages 166.12 cm per year, which is much greater than some invaded regions such as the Portugal which averages 81.28 cm per year (climatestotravel.com) and has an invasive population of RSC (Correia and Ferriera, 1995). However, we observed a strong decrease in burrowing rate when drainage rate increased from 4 cm/day to 8 cm/day, and total avoidance of burrowing behavior at 20 cm/day. In the 8 cm/day treatment group, seven crayfish initiated burrows on the first day but didn't

follow the water line farther than 8 cm, suggesting that there is a maximum rate at which crayfish will follow the water, and if they fall behind, they won't continue to burrow like a primary burrowing species might (Helms et al., 2013).

There is some supporting evidence for the importance of groundwater depth and/or drainage rate to burrow initiation in natural and semi-natural settings. In their native range, RSC are observed to burrow in warm summer months when water is receding beneath ground level, then entering the open water once fall floods occur (Lutz et al., 2005). Although burrowing is part of their life cycle, RSC often will not initiate burrows. Researchers trying to induce RSC burrowing in freshly drained earthen ponds during the summer months have observed only ~10% of stocked crayfish creating burrows (Ames et al., 2015; Bates et al., 2023). This is also a re-occurring problem in RSC aquaculture, as farmers are unsure of the proper flooding cycle that provides optimal conditions for burrowing (Lutz et al., 2005). It is unknown whether crayfish are able to directly sense the distance to the groundwater or are responding to secondary cues such as soil moisture. Future experiments using soil moisture probes would be very useful in determining whether hesitancy to burrow is driven by soil moisture levels and whether RSC require a specific moisture threshold to initiate and/or continue to burrow. The effects of soil particle size on crayfish burrowing has been previously documented (Stoeckel et. al., 2011). Burrows of RSC in the southern United States, where rainfall is relatively high and soils contain a high percentage of clay, are often vertical, ranging from 30 to 190 cm deep (N. Barnes, pers. obs). High clay content has been hypothesized to allow for the construction of deeper burrows without collapsing like they would in sandier soils. Fine-grained soils like clay have more malleability, allowing crayfish to handle larger pieces than those in sandier soils (Helms et al., 2013). The clay can also retain water for longer than sand, keeping the burrow moist to prevent desiccation, which can occur in

only 21.5 hours (Banha and Anastácio, 2014). Burrow density (#/m^2) of RSC may increase with increasing proportion of fine sediments (silt/clay) whereas the presence of sand/gravel typically reduces the ability of crayfish to burrow (Correia and Ferriera, 1995; Grow and Merchant, 1979; Ilhéu et al., 2003).

In artificial habitats a primary/secondary burrower (*Cambarus striatus*) tested in chambers similar to those used in the current study found that only ~20% of crayfish burrowed in 100% sand with mean burrow depth decreasing by ~50% compared to burrows in soils containing < 50% sand (Stoeckel et al., 2011). Because RSC are considered to be secondary/tertiary burrowers (Haubrock et al., 2019) we expected the effect of soil type to be even stronger for this species, however, this was not the case. Each crayfish in the 90% sand treatment group initiated a burrow. Average burrow depth in 90% sand was only ~33% shallower than the maximum burrow depth in ambient soil. Thus, in natural habitats with a high, stable water table, sandy soils are not likely to deter *Procambarus clarkii* from burrowing, although burrows may be somewhat shallower.

Although sand did not strongly deter burrowing, soil composition did have a significant effect on burrow morphology with burrow depth and perimeter, but not area, significantly decreasing with increasing sand content. In high clay soils, indentations the crayfish made into the burrow wall tended to be conserved, making the walls less smooth. In sandier soils those irregularities tended to fall or wash away, making the burrow walls smoother. It is likely that the decrease in burrow perimeter with increasing sand was due more to burrow wall irregularities than the decrease in burrow depth. If changes in burrow depth were a major factor, we would have expected a stronger negative relationship between burrow area and percent sand, but this

relationship was only marginally significant. We found no evidence that crayfish sex or size affected burrow morphology.

As it likely requires substantial energy to dig large, subterranean burrows, researchers have been studying burrowing behaviors across the animal kingdom to understand the factors that affect the degree of energy investment required. Semifossorial degus (*Octodon degus,* Molina 1782*)* are a specialized fossorial rodent found in South America that spend much of their life underground. (Ebensperger and Bozinovic, 2000) used open-flow respirometry system to calculate respiration rates of burrowing *O. degus*, which were then converted to energy units. (Schmidt-Nielsen 1990). Burrowing in compact soil required significantly more energy compared to looser, wet soil. To the best of our knowledge, similar studies have not yet been conducted for crayfish. However, there is a strong linear relationship between caloric content and moisture content of the crayfish hepatopancreas, suggesting that moisture content of the hepatopancreas may provide a rapid method of determining energy content of this organ (Jussila and Mannonen, 1997). Because the hepatopancreas serves as the main energy reserve of crayfish (Lindqvist and Louekari, 1975) this relationship may be a useful method for assessing the relative amount of energy expended by burrowing crayfish under various field and laboratory conditions.

We expected to find a negative relationship between crayfish caloric content and percent sand added to ambient soils because increasing sand content would presumably lead to more frequent burrow collapse and require more excavate and maintain burrows. However, we only drew the water down to 24 cm and differences may only become apparent as crayfish burrow even deeper and continue to expend energy. Conversely, we did find that non-burrowing crayfish had a significantly lower caloric density than crayfish that actively burrowed, suggesting either

that crayfish needed sufficient energy stores to initiate burrowing, or that remaining on the surface was more energetically expensive than burrowing. Further investigations into relationship between energy and burrowing behavior is crucial, as it can fill in knowledge gaps of how factors such as temperature, soil composition and sexual maturity interact to affect decisions to initiate burrowing as well as subsequent burrow morphology.

In conclusion, RSC burrowing activities appeared to be more strongly constrained by groundwater level and drainage rates than soil type. Knowledge of rainfall patterns and manipulation of groundwater levels may be more important to predicting and controlling the burrowing/reproductive phase of RSC than soil type. Adding sand to banks and levees of invaded aquatic systems may not be an effective control measure but reducing the frequency of flooding events and limiting access to low-lying areas with a high water-table may be more effective. However, even this latter strategy comes with some risk. Steeply inclined banks – which would increase the distance from the soil surface to the groundwater – may actually promote burrowing activity if RSC switch from vertical to lateral burrows into the steep banks (i.e. Lemmers et al., 2022) with burrow water supplied from the connected surface waters rather than groundwater. The ability to withstand different environmental conditions is key in RSC's ability to spread and thrive outside of their native range and knowledge of factors that inhibit or encourage burrowing and reproduction are key to predicting further range expansion as well as controlling populations within their currently invaded range.

References

Abdelrahman, H. A., Gibson, R. L., Fogelman, K. J., Cupp, A. R., Allert, A. L., & Stoecke, J. A. (2021). Evaluation of dissolved carbon dioxide to stimulate emergence of red swamp crayfish (Decapoda: Cambaridae) from infested ponds. *Management of Biological Invasions*, *12*(4), 952-974. https://doi.org/10.3391/mbi.2021.12.4.11

Ames, C. W., Helms, B. S., & Stoeckel, J. A. (2015). Habitat mediates the outcome of a cleaning symbiosis for a facultatively burrowing crayfish [Article]. *Freshwater Biology*, *60*(5), 989-999. https://doi.org/10.1111/fwb.12559

Anderson, L. G., Dunn, A. M., Rosewarne, P. J., & Stebbing, P. D. (2015). Invaders in hot water: a simple decontamination method to prevent the accidental spread of aquatic invasive non-native species. *Biol Invasions*, *17*(8), 2287-2297. https://doi.org/10.1007/s10530-015-0875-6

Barbaresi, S., Santini, G., Tricarico, E., & Gherardi, F. (2004). Ranging behaviour of the invasive crayfish, *Procambarus clarkii* (Girard) [Article]. *Journal of Natural History*, *38*(22), 2821-2832. https://doi.org/10.1080/00222930410001663308

Banha, F., & Anastácio, P. M. (2014). Desiccation survival capacities of two invasive crayfish species [Article]. *Knowledge and Management of Aquatic Ecosystems*(413), 01P01-01P05, Article 01. https://doi.org/10.1051/kmae/2013084

Bates, B. L., Allert, A. L., Wildhaber, M. L., & Stoeckel, J. A. (2023). Use of physical blockers to control invasive red swamp crayfish in burrows. *Management of Biological Invasions*, *14*(4), 709-729. https://doi.org/10.3391/mbi.2023.14.4.09

Becker, C. D., Genoway, R. G., & Merrill, J. A. (1975). Resistance of a northwestern crayfish, *Pacifastacus leniusculus* (Dana), to elevated temperatures [Article]. *Transactions of the American Fisheries Society*, *104*(2), 374-387. https://doi.org/10.1577/1548-8659(1975)104<374:Roancp>2.0.Co;2

Bloomer, C. C., Distefano, R. J., & Taylor, C. A. (2021). A global review of the life history studies on burrowing crayfish [Review]. *Crustaceana*, *94*(3), 357-379. https://doi.org/10.1163/15685403.bja10098

Bissattini, A. M., Traversetti, L., Bellavia, G., & Scalici, M. (2015). Tolerance of Increasing Water Salinity in the Red Swamp Crayfish Procambarus Clarkii (Girard, 1852). *Journal of Crustacean Biology*, *35*(5), 682-685. https://doi.org/10.1163/1937240x-00002366

Bonvillain, C. P., Rutherford, D. A., Kelso, W. E., & Green, C. C. (2012). Physiological biomarkers of hypoxic stress in red swamp crayfish *Procambarus clarkii* from field and laboratory experiments [Article]. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, *163*(1), 15-21. https://doi.org/10.1016/j.cbpa.2012.04.015

Bradbeer, S. J., Coughlan, N. E., Cuthbert, R. N., Crane, K., Dick, J. T. A., Caffrey, J. M., Lucy, F. E., Renals, T., Davis, E., Warren, D. A., Pile, B., Quinn, C., & Dunn, A. M. (2020). The

effectiveness of disinfectant and steam exposure treatments to prevent the spread of the highly invasive killer shrimp, . *Scientific Reports*, *10*(1). https://doi.org/ARTN 1919

10.1038/s41598-020-58058-8

Bruce, T. J., Abernathy, J. W., Tripp, N., Barnes, N., Harrison, C. E., Oladipupo, A. A., Krol, J. D., Wise, A. L., Warg, J. V., & Stoeckel, J. A. (2024). White spot syndrome virus in Alabama red swamp crayfish (*Procambarus clarkii*). *Journal of Fish Diseases*, *47*(2). https://doi.org/10.1111/jfd.13873

Buhay, J. E., & Crandall, K. A. (2005). Subterranean phylogeography of freshwater crayfishes shows extensive gene flow and surprisingly large population sizes [Article]. *Molecular Ecology*, *14*(14), 4259-4273. https://doi.org/10.1111/j.1365-294X.2005.02755.x

Cecchinelli, E., Aquiloni, L., Maltagliati, G., Orioli, G., Tricarico, E., & Gherardi, F. (2012). Use of natural pyrethrum to control the red swamp crayfish *Procambarus clarkii* in a rural district of Italy. *Pest Management Science*, *68*(6), 839-844. https://doi.org/10.1002/ps.2335

Charles Lutz, M. G. S., Robert P Romaire, William R MCclain. (2005). Crawfish Production Manual.

Correia, A. M., & Ferreira, O. (1995). Burrowing behavior of the introduced Red Swamp Crayfish (*Procambarus clarkii)* (Decapoda, Cambaridae) in Portugal [Article]. *Journal of Crustacean Biology*, *15*(2), 248-257. https://doi.org/10.2307/1548953

Crandall, K. A., & De Grave, S. (2017). An updated classification of the freshwater crayfishes (Decapoda: Astacidea) of the world, with a complete species list [Article]. *Journal of Crustacean Biology*, *37*(5), 615-653. https://doi.org/10.1093/jcbiol/rux070

Dalosto, M. M., Palaoro, A. V., Souty-Grosset, C., Bueno, S. L. D., Loureiro, T. G., Almerao, M. P., de Araujo, P. B., & Santos, S. (2015). One step ahead of the enemy: investigating aggressive interactions between invasive and native crayfish before the contact in nature. *Biological Invasions*, *17*(12), 3503-3515. https://doi.org/10.1007/s10530-015-0974-4

Ebensperger, L. A., & Bozinovic, F. (2000). Energetics and burrowing behaviour in the semifossorial degu *Octodon degus* (Rodentia: Octodontidae) [Article]. *Journal of Zoology*, *252*, 179-186. <Go to ISI>://WOS:000165134400004

Gherardi, F. (2006). Crayfish invading Europe:: the case study of. *Marine and Freshwater Behaviour and Physiology*, *39*(3), 175-191. https://doi.org/10.1080/10236240600869702

Glover, D. C., DeVries, D. R., Wright, R. A., & Davis, D. A. (2010). Sample Preparation Techniques for Determination of Fish Energy Density via Bomb Calorimetry: An Evaluation Using Largemouth Bass [Article]. *Transactions of the American Fisheries Society*, *139*(3), 671-675. https://doi.org/10.1577/t09-110.1

Grow L, Merchant H (1979) The burrow habitat of the crayfish, *Cambarus diogenes diogenes* (Girard). Am Midl Nat 103:231–237

Haubrock, P. J., Inghilesi, A. F., Mazza, G., Bendoni, M., Solari, L., & Tricarico, E. (2019). Burrowing activity of *Procambarus clarkii* on levees: analysing behaviour and burrow structure [Article]. *Wetlands Ecology and Management*, *27*(4), 497-511. https://doi.org/10.1007/s11273-019-09674-3

Helms, B., Budnick, W., Pecora, P., Skipper, J., Kosnicki, E., Feminella, J., & Stoeckel, J. (2013). The influence of soil type, congeneric cues, and floodplain connectivity on the local distribution of the devil crayfish (*Cambarus diogenes*, Girard) [Article]. *Freshwater Science*, *32*(4), 1333-1344. https://doi.org/10.1899/12-160.1

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., & Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowledge and Management of Aquatic Ecosystems*(394-95). https://doi.org/ARTN 11

10.1051/kmae/2009025

Huner, J. V., Jeske, C. W., & Norling, W. (2002). Managing agricultural wetlands for waterbirds in the coastal regions of Louisiana, USA. *Waterbirds*, *25*, 66-78. <Go to ISI>://WOS:000180065700008

Huner, J. V. and Barr, J. E. (1991). Red Swamp Crawfish: Biology And Exploitation.

Ilhéu M, Acquistapace P, Benvenuto C, Gherardi F (2003) Burrowing of the red swamp crayfish (*Procambarus clarkii*) in a temporary stream of south Portugal. Arch Hydrobiol 157:535–546

Jiravanichpaisal, P., Bangyeekhun, E., Söderhäll, K., & Söderhäll, I. (2001). Experimental infection of white spot syndrome virus in freshwater crayfish. *Diseases of Aquatic Organisms*, *47*(2), 151-157. https://doi.org/DOI 10.3354/dao047151

Jussila, J., & Mannonen, A. (1997). Marron (*Cherax tenuimanus*) and noble crayfish (*Astacus astacus*) hepatopancreas energy and its relationship to moisture content [Article]. *Aquaculture*, *149*(1-2), 157-161. https://doi.org/10.1016/s0044-8486(96)01425-1

Lemmers, P., van der Kroon, R., van Kleef, H. H., Verhees, J. J. F., van der Velde, G., & Leuven, R. (2022). Limiting burrowing activity and overland dispersal of the invasive alien red swamp crayfish *Procambarus clarkii* by sophisticated design of watercourses [Article]. *Ecological Engineering*, *185*, 8, Article 106787. https://doi.org/10.1016/j.ecoleng.2022.106787

Lindqvist, O.V. and Louekari, K., 1975. Muscle and hepatopancreas weight in Astacus astacus L. (Crustacea, Astacidae) in the trapping season in Finland. Ann. Zool. Fenn., 12: 237-243.

Logarbo, J. R., & Bonvillain, C. P. (2020). Thermal stress responses of two sympatric crayfishes in Louisiana,

 Hobbs & Hobbs, 1990 (Decapoda: Astacoidea: Cambaridae). *Journal of Crustacean Biology*, *40*(6), 734-738. https://doi.org/10.1093/jcbiol/ruaa066

Huner, J. V. and Barr, J. E. (1991). Red Swamp Crawfish: Biology And Exploitation.

Kotovska, G., Khrystenko, D., Patoka, J., & Kouba, A. (2016). East European crayfish stocks at risk: arrival of non-indigenous crayfish species [Article]. *Knowledge and Management of Aquatic Ecosystems*(417), 8, Article 37. https://doi.org/10.1051/kmae/2016024

Mirenda, R. J., & Dimock, R. V. (1985). Temperature tolerance of the crayfish Cambarus-*Acuminatus faxon,* 1884 (Decapoda, Astacidea) [Article]. *Crustaceana*, *48*(MAY), 249-259. https://doi.org/10.1163/156854085x00963

Mouser, J. B., Ashley, D. C., Zentner, D. L., & Brewer, S. K. (2022). Seasonal context of Bristly Cave Crayfish *Cambarus setosus* Habitat use and life history [Article]. *Journal of Cave and Karst Studies*, *84*(3), 85-95. https://doi.org/10.4311/2021lsc0110

Mundahl, N. D. (1989). Seasonal and Diel Changes in Thermal Tolerance of the Crayfish *Orconectes-Rusticus*, with Evidence for Behavioral Thermoregulation. *Journal of the North American Benthological Society*, *8*(2), 173-179. https://doi.org/Doi 10.2307/1467635

Ortman, A. (2021). *Effects of environmental hypoxia on Red Swamp Crayfish Procambarus clarkii life history, fecundity, and trophic ecology in the Atchafalaya River Basin.* [Unpublished master's thesis]. Nichols State University

Oficialdegui, F. J., Sanchez, M. I., & Clavero, M. (2020). One century away from home: how the red swamp crayfish took over the world [Review]. *Reviews in Fish Biology and Fisheries*, *30*(1), 121-135. https://doi.org/10.1007/s11160-020-09594-z

Paillisson, J. M., Soudieux, A., & Damien, J. P. (2011). Capture efficiency and size selectivity of sampling gears targeting red-swamp crayfish in several freshwater habitats. *Knowledge and Management of Aquatic Ecosystems*(401). https://doi.org/ARTN 06

10.1051/kmae/2011015

Schuster, G. A., Taylor, C. A., & McGregor, S. W. (2022). *Crayfishes of Alabama.* The University of Alabama Press.

Souty-Grosset, C., Reynolds, J., Gherardi, F., Aquiloni, L., Coignet, A., Pinet, F., & Cisneros, M. D. M. (2014). Burrowing activity of the invasive red swamp crayfish, <i>Procambarus clarkii</i>, in fishponds of La Brenne (France) [Article]. *Ethology Ecology & Evolution*, *26*(2-3), 263-276. https://doi.org/10.1080/03949370.2014.892538

Stoeckel, J. A., Helms, B. S., & Cash, E. (2011). Evaluation of a crayfish burrowing chamber with simulated groundwater flow [Article]. *Journal of Crustacean Biology*, *31*(1), 50-58. https://doi.org/10.1651/09-3271.1

Stoeckel, J. A., Szoka, M., Abdelrahman, H. A., Davis, J. D., Blersch, D. M., & Helms, B. S. (2021). Crayfish chimneys function as burrow-ventilation structures [Article]. *Journal of Crustacean Biology*, *41*(3), 9, Article ruab045. https://doi.org/10.1093/jcbiol/ruab045

Taylor, C. A., Schuster, G. A., Cooper, J. E., DiStefano, R. J., Eversole, A. G., Hamr, P., Hobbs, H. H., Robison, H. W., Skelton, C. E., & Thoma, R. E. (2007). Feature: Endangered species - A reassessment of the conservation status of crayfishes of the united states and Canada after

10+years of increased awareness [Article]. *Fisheries*, *32*(8), 372-389. https://doi.org/10.1577/1548-8446(2007)32[372:Arotcs]2.0.Co;2

Thomas, J. R., Masefield, S., Hunt, R., Wood, M. J., Hart, A. G., Hallam, J., Griffiths, S. W., & Cable, J. (2019). Terrestrial emigration behaviour of two invasive crayfish species [Article]. *Behavioural Processes*, *167*, 4, Article 103917. https://doi.org/10.1016/j.beproc.2019.103917

Trouilhé, M. C., Souty-Grosset, C., Grandjean, F., & Parinet, B. (2007). Physical and chemical water requirements of the white-clawed crayfish (*Austropotamobius pallipes*) in western France [Article]. *Aquatic Conservation-Marine and Freshwater Ecosystems*, *17*(5), 520-538. https://doi.org/10.1002/aqc.793

Tucker, L. (2022, July 5). *Red Swamp Crayfish*. Monterey Bay Aquarium SeaFood Watch. https://www.seafoodwatch.org/globalassets/sfw-data-blocks/reports/c/seafood-watch-crayfish-us-696.pdf

Westhoff, J. T., & Rosenberger, A. E. (2016). A global review of freshwater crayfish temperature tolerance, preference, and optimal growth [Review]. *Reviews in Fish Biology and Fisheries*, *26*(3), 329-349. https://doi.org/10.1007/s11160-016-9430-5
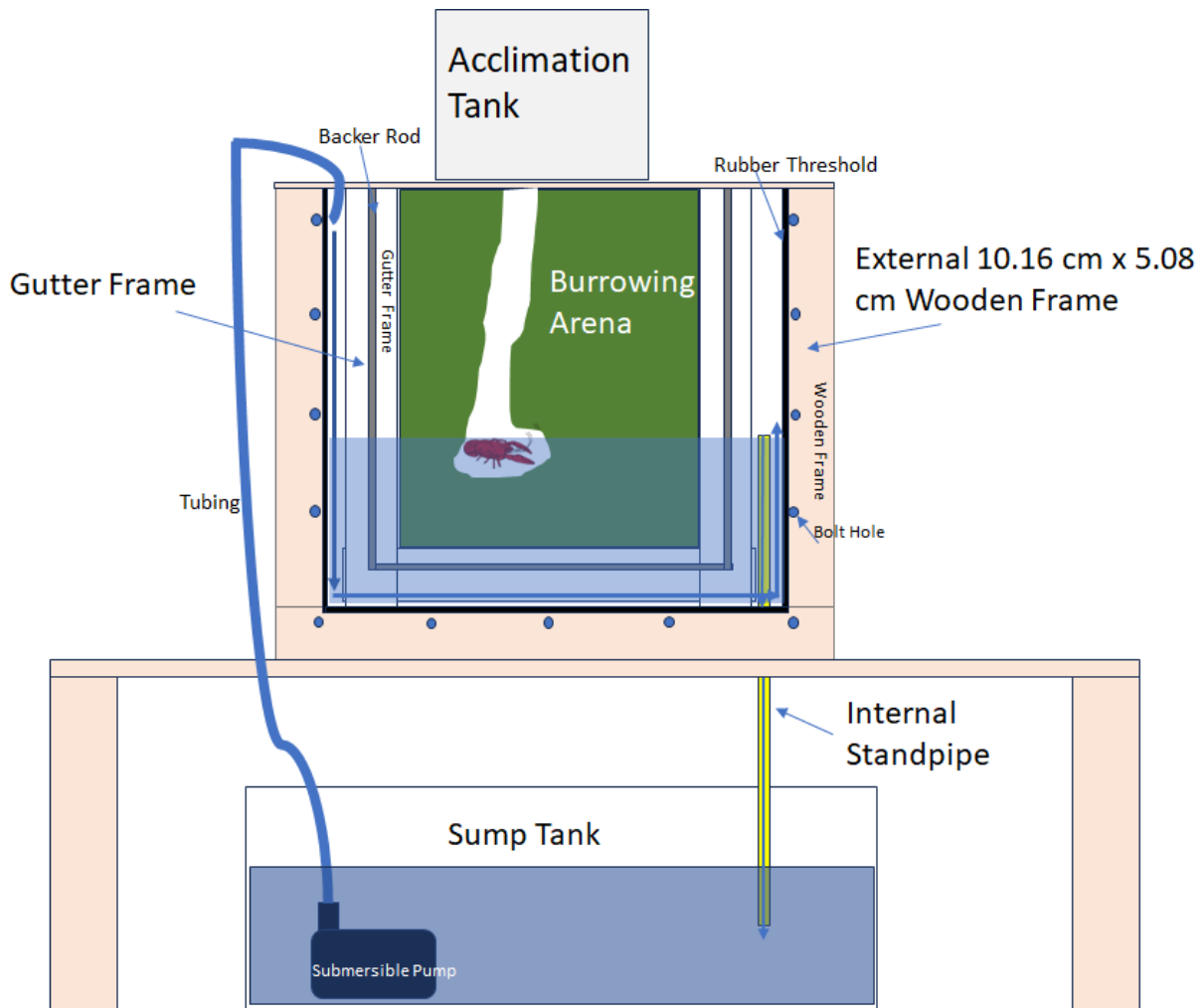
Figure 2.1.A Schematics of the burrowing chamber setup for the groundwater drainage rate experiment.
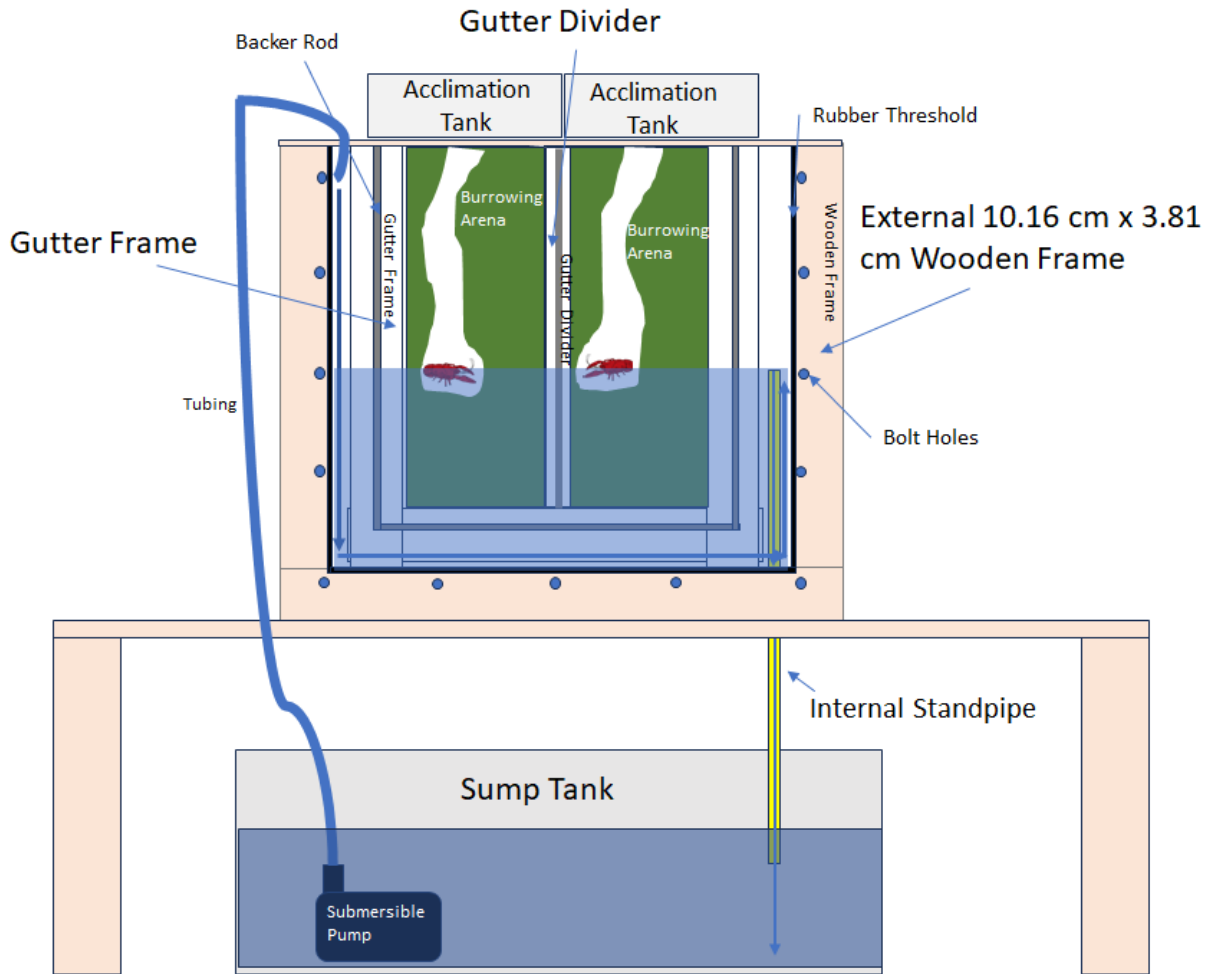
Figure 2.1.B Schematics of the burrowing chamber setup for the soil composition experiment.
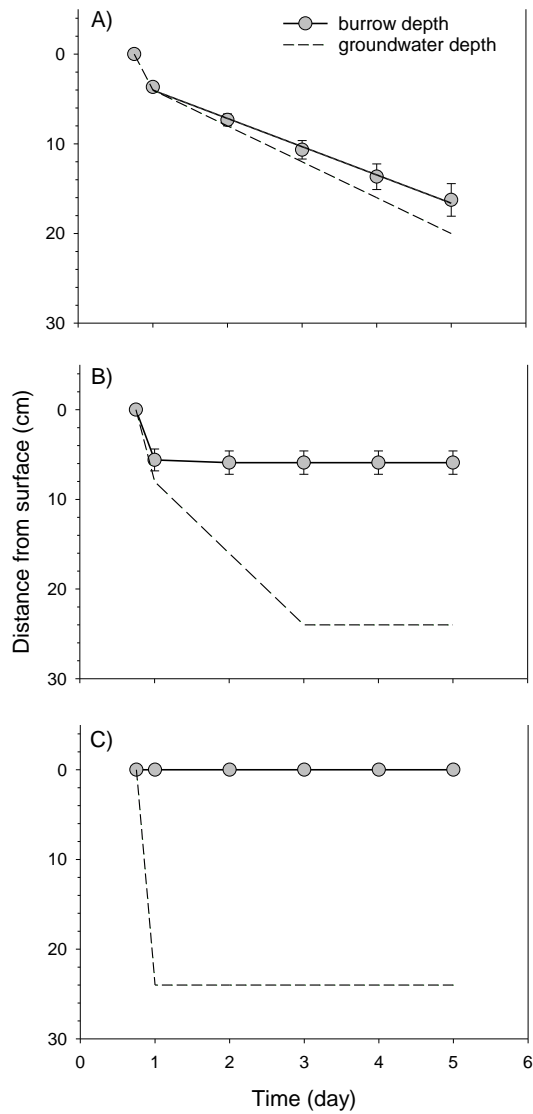
Figure 2.2. Change in burrow and groundwater depth over time for crayfish exposed to groundwater drainage rates of A) 4 cm/day for 5 days, B) 8 cm/day for 3 days and C) 24 cm/day for 1 day. In the top panel, the burrow depth data from day 1-5 was fitted with a linear regression. In the other two panels, data points are connected by a line. Error bars represent ± 1 SE.
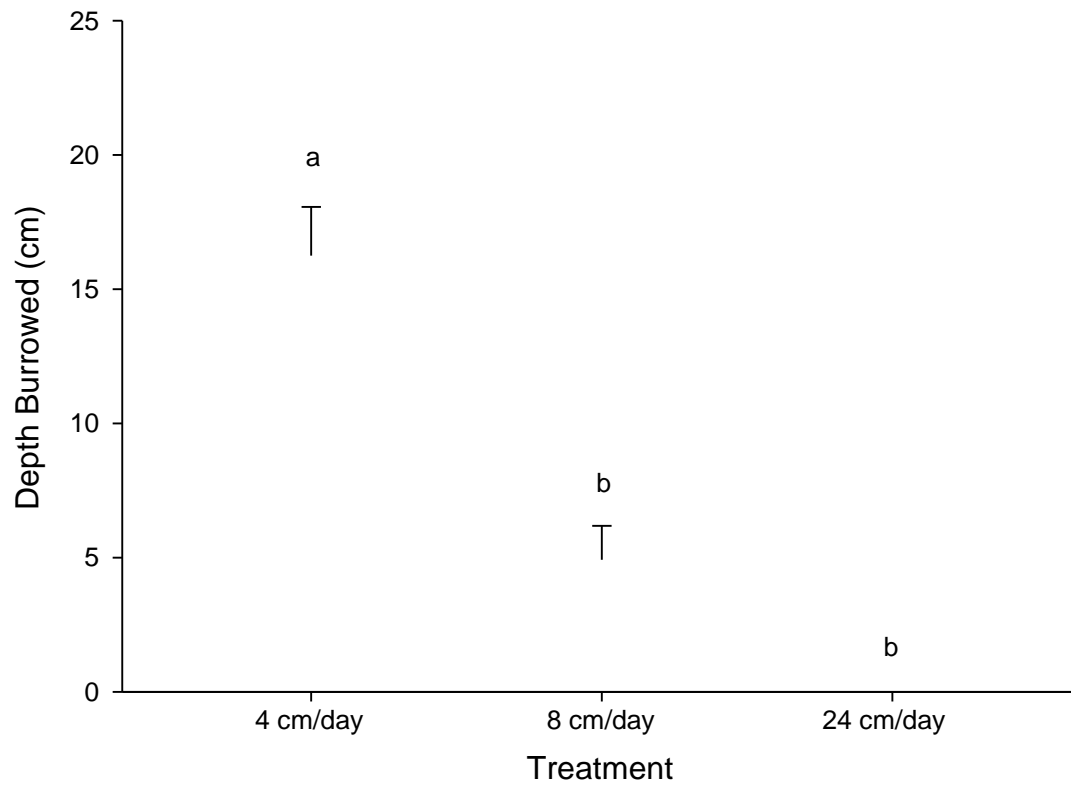
Figure 2.3. Average burrow depth after 5 days of burrowing for each groundwater drainage rate. Error bars represent ± 1 SE. Lower case letters indicate significant differences between treatments. Note that an ANOVA was run on rank-transformed data in order to meet assumptions of normality, but the figure shows untransformed data.
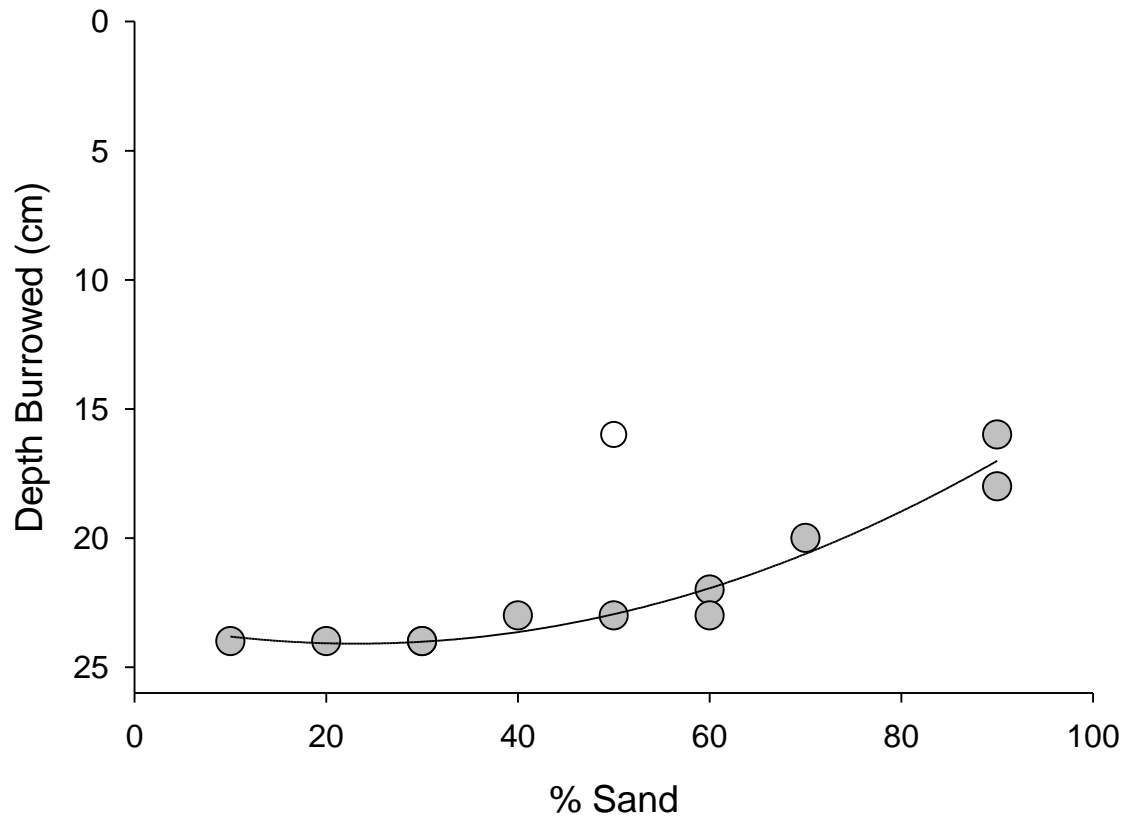
Figure 2.4. Relationship between total depth burrowed and soil composition for crayfish that burrowed. The open circle shows the data point identified as an outlier and removed from regression analysis. Solid line represents a quadratic regression through the remaining data.
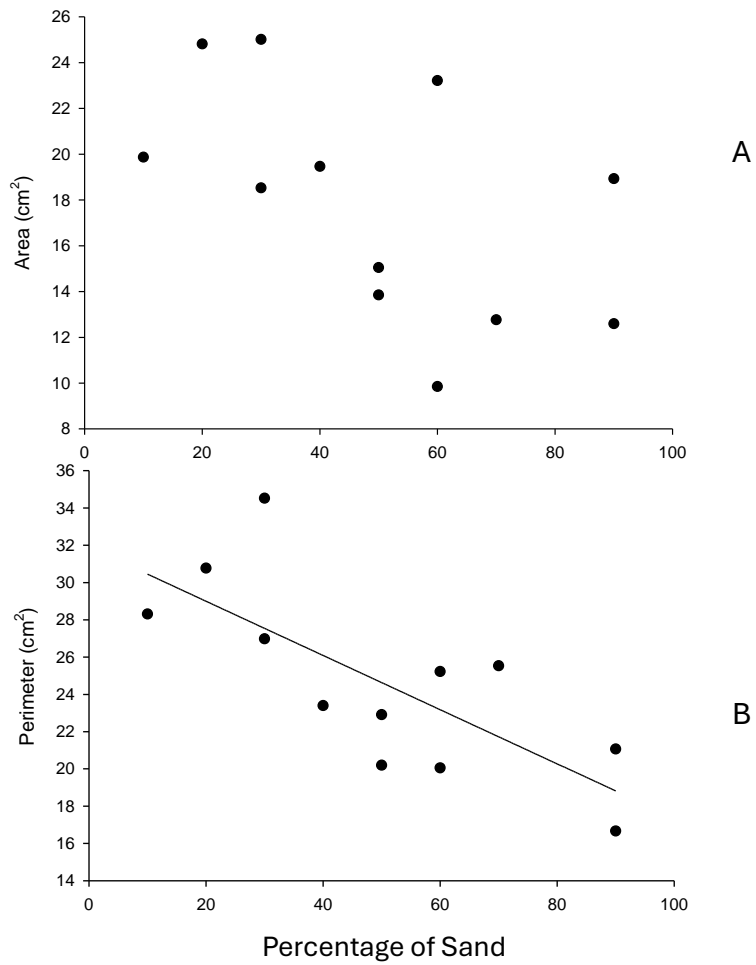
Figure 2.5 Relationship between soil composition and burrow area (A) and burrow perimeter

(B). There was no significant relationship between soil composition and burrow area (p=0.0728).

There was a negative linear relationship between soil composition and burrow perimeter for each

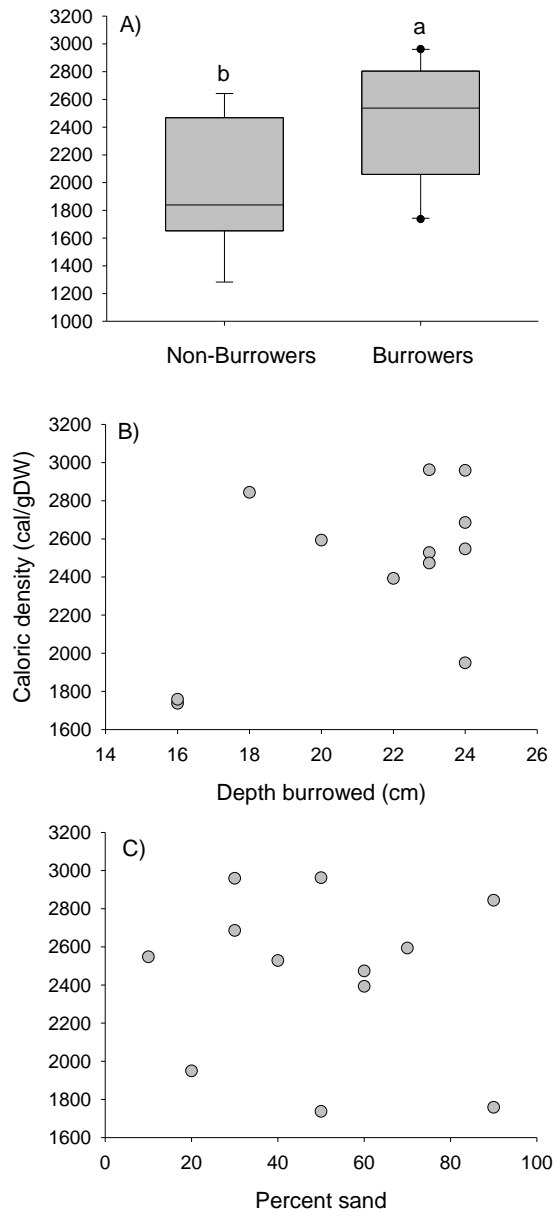crayfish that burrowed (p= 0.020)

Figure 2.6. A) Comparison of caloric density between non-burrowers and burrowers. Box plot horizontal line represents the median value, box represents the 25th and 75th percentiles, and error bars represent the 10th and 90th percentiles. Different letters indicate significant difference between categories. There was no significant linear relationship between caloric density and B) depth burrowed or C) percent sand mixed with ambient soil (p = 0.0716 and 0.7202, respectively)