

**The Relationship between Energy and Burrowing Behaviors of the Red Swamp Crayfish
(*Procambarus clarkii*)**

By

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Abstract

The Red Swamp Crayfish (*Procambarus clarkii*) is a globally invasive species whose burrowing behavior contributes to bank destabilization, increased sedimentation, and altered aquatic habitat structure. Despite the ecological significance of burrow construction, the energetic demands of this behavior have yet to be investigated. In the first chapter, I examined whole-body caloric density in crayfish that burrowed and crayfish that remained in individual tanks to fast. I found that neither burrowing to 32 cm nor fasting for 8 days resulted in a significant loss of energy, indicating that burrowing is not energetically expensive to this depth. However, individuals that failed to initiate a burrow showed significantly lower energy reserves after only two days, suggesting that there is a rapid decrease of energy at the surface due to some activity by the crayfish. In the second chapter, experimentally manipulating crayfish energetic condition revealed that high-energy crayfish dug deeper burrows than low-energy individuals and that the hepatopancreas' moisture content was negatively correlated with caloric density, supporting its use as a proxy for energetic health. These findings demonstrate that while burrowing does not substantially deplete caloric densities, the initial energetic state prior to burrowing has a strong influence over burrowing performance.

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Table of Contents

Abstract..... 2

Acknowledgements 3

List of Figures..... 5

Chapter 1: Energetic Cost of Burrowing in Red Swamp Crayfish 6

 INTRODUCTION 6

Methods..... 8

Results..... 13

Discussion 14

References..... 25

Chapter 2: Impacts of Energetic Health on Burrowing Behaviors in *Procambarus clarkii*. 30

Introduction 30

Methods..... 32

Results..... 36

Discussion 38

References..... 52

List of Figures

Figure 1.1 Burrow chamber diagram 188

Figure 1.2 Wet weight of crayfish in trials one and two 199

Figure 1.3 Initial caloric density of crayfish from trials one and two 200

Figure 1.4 Initial caloric density vs fasted/stationary crayfish211

Figure 1.5 Caloric density comparison of Initials vs 8cm burrowers vs 32cm burrowers 222

Figure 1.6 Caloric density comparison of Initials vs non-burrowing crayfish 233

Figure 1.7 Caloric density comparison of 8cm burrowers vs 32 cm burrowers vs stopped burrowers 244

Figure 2.1 Caloric density of crayfish after 1, 2, 3, and 5 weeks of fasting 421

Figure 2.2 Caloric density of fed/stationary vs fasted/mobile crayfish 432

Figure 2.3 Caloric density of fasted/ mobile crayfish at 2, 3, and 4 weeks of fasting 443

Figure 2.4 Caloric density of HE and LE initial groups 454

Figure 2.5 Final depth burrowed in cm for HE and LE groups 465

Figure 2.6 Caloric density comparison of HE and LE groups post burrowing..... 476

Figure 2.7 Percentage moisture content comparison in the HE and LE groups 487

Figure 2.8 Relationship between caloric density and hepatopancreas moisture content 498

Figure 2.9 Comparison of hepatopancreas wet weights from the HE and LE groups 49

Figure 2.10 Image of hepatopancreas taken from HE and LE crayfish 510

Chapter 1: Energetic Cost of Burrowing in Red Swamp Crayfish

INTRODUCTION

Procambarus clarkii (Red Swamp Crayfish) is native to the southeastern United States but has become widely distributed across multiple continents including North and South America, Asia, Europe, and Africa (Loureiro et al. 2015, Souty-Grosset et al. 2016). Its spread has been primarily due to intentional introductions by humans as a food source, research subject, and more recently the pet trade (Oficialdegui 2019). Once introduced, *P. clarkii* demonstrates rapid dispersal and high colonization success, especially in disturbed aquatic habitats like streams and ditches. This combination of human mediated dispersal and ecological plasticity facilitates its invasive success.

The ecological consequences of *P. clarkii* invasions are substantial. As a larger bodied, aggressive species it competes directly with native crayfish and other benthic macroinvertebrates for food and shelter (Taylor et al. 2007). In Europe, its role as a carrier of the crayfish plague has accelerated the decline of native populations of *Astacus* and *Austropotomobius spp* (Alderman and Poleglase 1986, Holdich et al. 2009). Outside of direct species interactions, *P. clarkii* alters aquatic food webs by consuming macrophytes, detritus, and other invertebrates which can lead to reduced plant cover, increased sedimentation, and altered nutrient cycling. Additionally, its ability to tolerate a wide range of water quality parameters makes *P. clarkii* a strong generalist species that can persist under sub-optimal conditions that may deter other specialist species. Preferred temperature of *P. clarkii* falls between 21° C and 27°C, theoretically restricting it to more temperate climates. However, in lab trials, *P. clarkii* does not show physiological stress until 38°C (Logarbo and Bonvillain 2020) and its relatively plastic reproductive ecology has allowed it to migrate northward into climates that were previously considered “safe” (Souty-Grosset et al. 2006, Pratt 2024).

Burrowing is one of the most critically important behaviors to *P. clarkii* ecology as the burrows provide protection against desiccation and a place to rear their young (Huner and Barr 1991, Herrmann and Martins 2024). However, these burrows can have a functional effect on the surrounding ecosystem. Excavated sediment can destabilize banks, accelerate erosion, and alter stream connectivity (Haubrock et al. 2019). *Procambarus clarkii* is a secondary burrower that digs simple 1-2 entrance burrows with a vertical shaft leading straight down to the groundwater that terminates in a slightly larger chamber. An occupied burrow typically houses one or occasionally two crayfish, with *P. clarkii* more likely to dig a new burrow after wandering or foraging behavior than to reoccupy old burrows in riparian zones (Barbaresi et al. 2004). This readiness to dig new burrows, combined with a high density of individuals along a stream bank, lends to cumulative impacts that exacerbate the effects of bank collapse and sediment resuspension (Bendoni et al. 2024).

Previous studies documented that crayfish burrow depth and structure can be affected by multiple environmental conditions; including groundwater depth, temperature, and soil type, but the energetic cost of burrowing remains unclear (Stoeckel et al. 2011, Barnes et al. 2024, Carlson 2024). In theory, burrowing should be energetically expensive for crayfish. Estimated *P. clarkii* burrow volumes range from 1.4 L/burrow ($1,299 \text{ burrows} / 917 \text{ m impacted riverbank} = 1.4 \text{ burrows} / \text{m}$; $2 \text{ L excavated soil per m of bank} / 1.4 \text{ burrows per m of bank} = 1.4 \text{ L} / \text{burrow}$; Faller et al., 2016) to a mean of 0.506 L / burrow (Bendoni et al., 2024). From this information, an adult crayfish with a mass of 30 g excavated a mean soil volume of 1 L / burrow, and the excavated soil had a mass of 1.4 g / ml (Faller et al. 2016), the crayfish would have excavated 1,400 g / burrow. This is equivalent to $\sim 47x$ its body mass. A 90 kg human would have to excavate 4,230 kg (9,325 lb) to dig an equivalent burrow relative to their body mass. This

suggests that burrowing should require a major energetic investment. However, even though *P. clarkii* are physiologically capable of finding their original burrows after foraging, they show low burrow fidelity and tend to create new burrows rather than re-occupying old burrows. This presents a paradox – why do *P. clarkii* show such low burrow fidelity if burrowing behavior is energetically expensive (Barbaresi and Gherardi, 2006)?

I hypothesized that burrowing activity is energetically expensive and that caloric density of crayfish would decline with increasing depth burrowed. Specific objectives of the study were to 1) determine whether crayfish that initiated burrows had a higher initial caloric density than those that remained on the surface 2) separate the energetic costs of fasting from costs of burrowing during the burrowing process, and 3) determine whether crayfish that stop burrowing have a lower caloric density than those that continue burrowing.

Methods

Burrowing Chamber Design

Burrowing chamber design was modified from Stoeckel et al. (2011) and Barnes (2024) (Figure 1.1). The front and back sides of chambers were made from plexiglass sheets (0.635 cm thick x 72 cm H x 91.5 cm W). The internal frame of each chamber was made from 3.8 cm x 8.9 cm (2 in x 4 in) untreated wood. The two vertical sides were 71.12 cm high and the bottom was 96.5cm long. The plexiglass sheets were secured to the internal frame with stainless steel bolts inserted through 6.35mm holes along the outer edges of three sides. Outside each piece of plexiglass was another external frame made of 4 x 2 cm wooden framing that measured 78.75 cm high x 80.0 cm long that was used as cushion to prevent the plexiglass from cracking as nuts and bolts were tightened to seal the chamber. Inside the wooden frames and plexiglass was a vinyl

gutter frame with three sides in a “U” shape that measured 73.0 cm long x 71.0 cm high x 3.81 cm wide. The gutter was sliced down its long sides and the resulting halves overlapped then backer rods were glued to either side of the gutter such that it could fit inside the chamber with the backer rod compressed against the plexiglass sides to retain soil within the inner gutter frame. Each internal vertical side of the gutter frame had 6.35mm holes drilled every 3 cm to allow water to perforate into the inner burrowing chamber where the soil was held. A strip of 500um Nitex mesh was glued to the inner sides of the gutter frame to keep the soil from moving out of the inner burrowing chamber but still allow water to flow in. A 1.27 cm internal standpipe was installed through the bottom of the wooden frame using a 1.27 cm uniseal that allowed us to manipulate the water level as needed. Another straight length of standalone gutter was constructed similarly to the main gutter frame and was inserted in the middle of the inner burrowing chamber to separate the inner chamber into two halves. This “middle divider” was a straight piece of overlapping gutter that measured 63.0 cm long by 2.5 cm wide with 1.2 cm backer rod glued along the length at either side of the short width. This gutter piece was sandwiched between the plexiglass to ensure crayfish on one side of the chamber could not burrow to the other side. Two plastic containers or “surface tanks” measuring 21 cm wide x 33 cm length x 13 cm tall were secured to the top of the chamber over the top of each half of the burrowing area. Burrowing chambers used in experiments were housed in a modified, 12.1 m (40 ft) long shipping container with photoperiod and temperature control.

Crayfish Collection and Holding

Experimental crayfish were collected from wild populations living in earthen ponds at E.W. Shell Fisheries Station at Auburn University using baited minnow traps. Crayfish for the first trial were collected in from mid-June to early July 2024 and crayfish for the second trial

were collected in August of 2024. After collection, crayfish were held in individual 1.5 L aquatic habitat systems (Pentair Aquatic Eco Systems, Apopka, Florida, USA). The system was filled with artificial freshwater (AFW: 0.189 g CaCO₃ , 0.189 g NaHCO₃ , 0.095 g CaCl₂ , 5.0 mL 35‰ saltwater·L⁻¹ of reverse osmosis/deionized water; pH = 8.05, total hardness = 52.5 mg·L⁻¹ CaCO₃ , total alkalinity = 40 mg·L⁻¹ CaCO₃ ; Gascho Landis et al. 2012) Water temperature was 25 °C and photoperiod was 12h:12h day: night cycle. Crayfish were fed 8-12 pellets of Ziegler shrimp feed every other day until a cumulative 40 males of the appropriate size (40-50 mm carapace length) had been captured and placed in the tanks. Feeding rate was then increased to once per day for seven days prior to the experiment to help ensure all crayfish had sufficient energy stores to burrow and to minimize differences in energy stores among individuals.

Experimental Protocol

I ran two trials of this experiment, the first beginning on 17 July 2024 and the second beginning on 29 August 2024. Each trial used 40 *P. clarkii* collected from E.W. Shell station ponds. Air temperature was held at 25 °C and 12h:12h day: night photoperiod was maintained. Black plastic sheeting was placed over the front and back of each chamber to prevent ambient light from shining into burrows from the chamber sides.

On day 0 of a given trial, ten *P. clarkii* were randomly removed from the AHAB tanks, placed in individual 4"x 6" Ziplock bags, and stored in a -80°C ultracold freezer for subsequent calorimetric analysis. Ten additional crayfish were randomly chosen to remain in the AHABs, without food, for the duration of the experiment to determine loss in caloric density due to fasting. The remaining twenty crayfish were randomly assigned to a burrow chamber (two crayfish per chamber) that had been prepared for the trial. During each burrowing trial, air temperature was held at 25 °C, and photoperiod to a 12hr:12hr day: night cycle.

Prior to adding the crayfish, each chamber was filled with soil collected from Auburn University E.W. Shell Fisheries and sieved through 6-mm mesh. The chamber groundwater level was raised to the soil surface for 24 h to fully saturate the soil (Ames et al. 2015). After 24 h, one crayfish was added to each surface tank (two surface tanks per burrowing chamber) and the groundwater was immediately lowered to 4 cm below the surface to induce burrowing. (Barnes et al. 2024). Every 24 h thereafter the shades on the front were lifted to determine current burrow depth and the standpipe adjusted to lower the water level by an additional 4cm/day. At the 8 cm water level (end of day 2) I removed and froze all the crayfish that had not yet initiated a burrow, and 4 of the crayfish that had burrowed down to the water level (8 cm). All other crayfish remained in the chambers until the water level reached 32 cm (day 8) at which time burrow depth of each was recorded and the crayfish were frozen for later analysis. All crayfish that were removed were placed in individual 4"x 6" Ziplock bags for storage at -80°C.

Energetic content of *P. clarkii* was measured using bomb calorimetry following the traditional whole-body approach (Glover et al. 2010). Within 60 days of collection and freezing, each crayfish was placed in a drying oven for 24 h at 100°C to remove all moisture. Each dried crayfish was then ground into a powder using an analytical mill (IKA A11 basic analytical mill, 2635 Northchase Parkway SE Wilmington, NC 28405 USA). Two subsamples of the powder were then pressed into pellets weighing 0.1-0.2 g each. The pellets were ignited in one of two Parr calorimeters (Parr 6725, Parr 1425) following the procedures described in the manual. Caloric values obtained from the two pellets per crayfish were then averaged to obtain a mean caloric density for that individual (calories/ gram of dry mass).

Data analysis

Sigma Plot 15 (Grafiti, Palo Alto California) was used for all data analyses. Data from trial 1 and trial 2 were combined for all analyses except for the non-burrower analysis from trial 1 and the analysis using the crayfish that stopped burrowing in trial 2. A t-test was done to compare the weights from crayfish in both trials.

To test for differences in caloric density between initial crayfish and the fasted crayfish that remained in water for eight days, I used a t-test followed by a Mann-Whitney rank sum test due to data failing assumptions of normality.

To test for differences in caloric density between initial crayfish and crayfish that had burrowed down to the groundwater level on day two and on day eight I ran a one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis one way analysis on ranks due to data failing assumptions of normality.

To test for differences in caloric density between initial crayfish and crayfish that had not initiated a burrow by day two, I used a t-test followed by a Mann-Whitney Rank Sum test due to the data failing assumptions of normality.

ANOVA was used to examine differences in caloric density between crayfish that burrowed to the groundwater (32 cm) on day eight and those that stopped burrowing before reaching 32 cm by day eight. This test passed normality and equal variance. I only used data from trial 2 in this analysis because all crayfish had burrowed to 32 cm by day eight in trial 1.

To estimate the mass of soil crayfish excavated to create the deepest burrows observed in this study (32) cm deep burrow, I assumed a burrow diameter of 3.8 cm and a soil density of 1.34 g/cm³ (USDA 2025). Soil volume excavated was then calculated as $\pi r^2 \cdot \text{depth (cm)}$. To estimate the equivalent soil mass that would be excavated by a human, proportional to size, I assumed an average crayfish mass of 0.0238 kg and an average human mass of 70kg and used the formula $ESM = M_h(M_s/M_c)$

where

ESM = equivalent soil mass excavated by a human

M_h = mass of human (kg)

M_s = mass of soil excavated by crayfish (kg)

M_c = mass of crayfish (kg)

Results

In trials 1 and trial 2 we observed < 10% mortality in the chambers. The mean initial weight of *P. clarkii* in the first trial was 23.5 g ± 4.69 and 24.1 ± 5.5 in the second trial. There was no significant difference in crayfish initial weight between trials ($p = 0.69$, $t_{78} = -0.39$, Figure 1.2). There was no significant difference ($p = 0.650$; U statistic = 44) in *P. clarkii* caloric density between the initial groups in Trial 1 and Trial 2 (Figure 1.3).

I found no evidence of energetic costs of fasting during the experiment. There was no significant difference ($p = 0.256$; U statistic = 158) in *P. clarkii* caloric density between individuals collected from holding tanks just prior to initiating a burrowing trial and crayfish that

remained submerged in holding tanks and were fasted for the burrowing trial duration (Figure 1.4).

There was no significant difference ($p = 0.925$; $H = 0.156$) in caloric density among initial crayfish, crayfish that had burrowed to 8 cm on day two, and crayfish that had burrowed to 32 cm on day eight (Figure 1.5). However, crayfish that had not initiated burrows by day two had significantly lower caloric density than the initial crayfish ($p = 0.02$) (Figure 1.6).

There was no significant difference ($F_{2, 12} = 0.419$; $p = 0.667$) in caloric density among crayfish that had burrowed to 8 cm on day 2 ($2761.2 \text{ cal/g} \pm 185.8$), crayfish that had burrowed to 32 cm on day 8 ($2869.1 \text{ cal/g} \pm 77.1$) and crayfish that had burrowed to less than 32 cm on day 8 ($3000.5 \text{ cal/g} \pm 250.9$) (Figure 1.7).

I estimated that *P. clarkii* burrowing to 32 cm in our study excavated 628.32 cm^3 of soil weighing a total of 0.78 kg. This would be equivalent to a 70 kg man excavating 2,294 kg of soil over an 8-day period.

Discussion

Procambarus clarkii is one of the most invasive species around the world and a major key to their success is their ability to utilize a combination of aquatic and terrestrial habitats (Barbaresi et al. 2004). Burrows allow them to escape stressors such as high temperatures, direct sunlight, and predators (Ilhéu et al. 2003). The burrows are also where they brood eggs and rear their young (Huner and Barr 1991). The rapid construction of many burrows can cause damage to levees and harm invaded ecosystems by increasing sediments in nearby surface waters

(Haubrock et al. 2019). Therefore, it is critical to investigate their burrowing behaviors and factors that contribute to their burrowing.

It is often assumed that excavation of a burrow is energetically expensive to crayfish (Barbaresi and Gherardi, 2006). However, there have been no studies explicitly examining the energetic costs of burrowing activities in crayfish. We estimated that crayfish burrowing to 32 cm in our study excavated 0.78 kg soil, which would be equivalent to a 70 kg man excavating 2,300 kg of soil over an 8 day period while fasting (i.e. no caloric intake). However, neither fasting nor burrowing appeared to incur substantial energetic costs for crayfish in terms of a reduction in whole-body caloric density.

We found no evidence that fasting for 8 days, while remaining in water, resulted in a decrease in whole-body caloric density of crayfish. This finding is supported by previous studies that show ectotherms, such as crayfish, have a low metabolic rate compared to endotherms and can survive long periods without food (Huner and Barr 1991, Gillooly et al 2017, Iqbal et al. 2024). Because crayfish are ectotherms and do not need to expend energy to maintain a constant body temperature, their baseline metabolic demands are lower than that of similarly sized endotherms. This physiology allows them to tolerate extended periods of food deprivation with relatively little reduction in energy reserves (Powell and Watts 2010). The tolerance to fasting likely provides an advantage in fluctuating environments and within the burrows where food availability may be highly variable or consistently low.

We expected that excavating a volume of soil that was an estimated ~ 33 times their body weight would incur significant energetic costs, depleting energy reserves and result in a reduction in whole-body caloric density. However, this was not the case. Burrowing to 32cm did not appear to be energetically expensive as evidenced by a lack of a decrease in caloric density

over the course of 8 days. This finding contrasts with previous studies of burrowing endotherms. Vleck (1979) demonstrated that burrowing in pocket gophers requires a disproportionately high energetic investment compared to aboveground movement with the cost of moving soil being several times greater than that of walking or running.

Burrowing also appears to be energetically costly for several ectotherms. For semi-fossorial skinks, the net energetic cost of burrowing was 350 times more expensive than the estimated cost of terrestrial locomotion (Wu et al., 2015). For the giant burrowing cockroach (*Macropanesthia rhinoceros*), burrowing activities induced a 17-fold increase in metabolic rate (Xu et al. 2014). The discrepancy between our study and previous studies may be based in part on differences in methodology. Previous studies used respirometry whereas we used caloric density to estimate energetic costs. It is likely that respirometry is a more sensitive indicator of energetic costs than changes in caloric density. Still, our results suggest that any increases in metabolic rate associated with burrowing in crayfish were insufficient to reduce internal energy stores to the point where caloric density declined.

If burrowing in crayfish was more energetically costly than activity on the surface, we would expect to have seen no decrease in caloric density of crayfish that remained on the surface and did not burrow. However, caloric density of crayfish that did not burrow within 2 days was significantly lower than estimated caloric density of crayfish on Day 0. Because measurements of caloric density were lethal, we were not able to directly measure the caloric density of crayfish on Day 0, prior to them being placed in the burrowing chambers. Rather, we measured caloric density of a subset of crayfish and assumed that this represented initial caloric density of the remaining crayfish that were transferred to the burrowing chambers. It is possible that the initial caloric density of non-burrowers was uncharacteristically low and these crayfish simply did not

have enough energy to initiate burrowing. It is also possible that energetic demands were high for non-burrowers remaining at the surface due to increased scaphognathite activity as a result of hypoxia (Burggren and McMahon 1983). They may also have expended energy wandering the upper chamber and trying to escape. Additional studies are needed to tease out the mechanism behind the low caloric density of non-burrowers remaining at the surface.

In conclusion, we found that neither fasting nor burrowing over an 8 day period was energetically expensive as measured by changes in caloric density. This may help explain the results of previous studies showing a tendency of *P. clarkii* to dig new burrows after exploratory behavior rather than simply re-occupying initial burrows (Barbaresi et al., 2004). Future research should focus on the underlying mechanisms causing rapid energy loss in non-burrowers at the surface and exploring how burrow construction interacts with environmental stressors in natural field conditions. Understanding these dynamics will improve predictions of crayfish impact on invaded systems and inform management strategies aimed at mitigating the effects of the invasive *P. clarkii* populations.

Figures

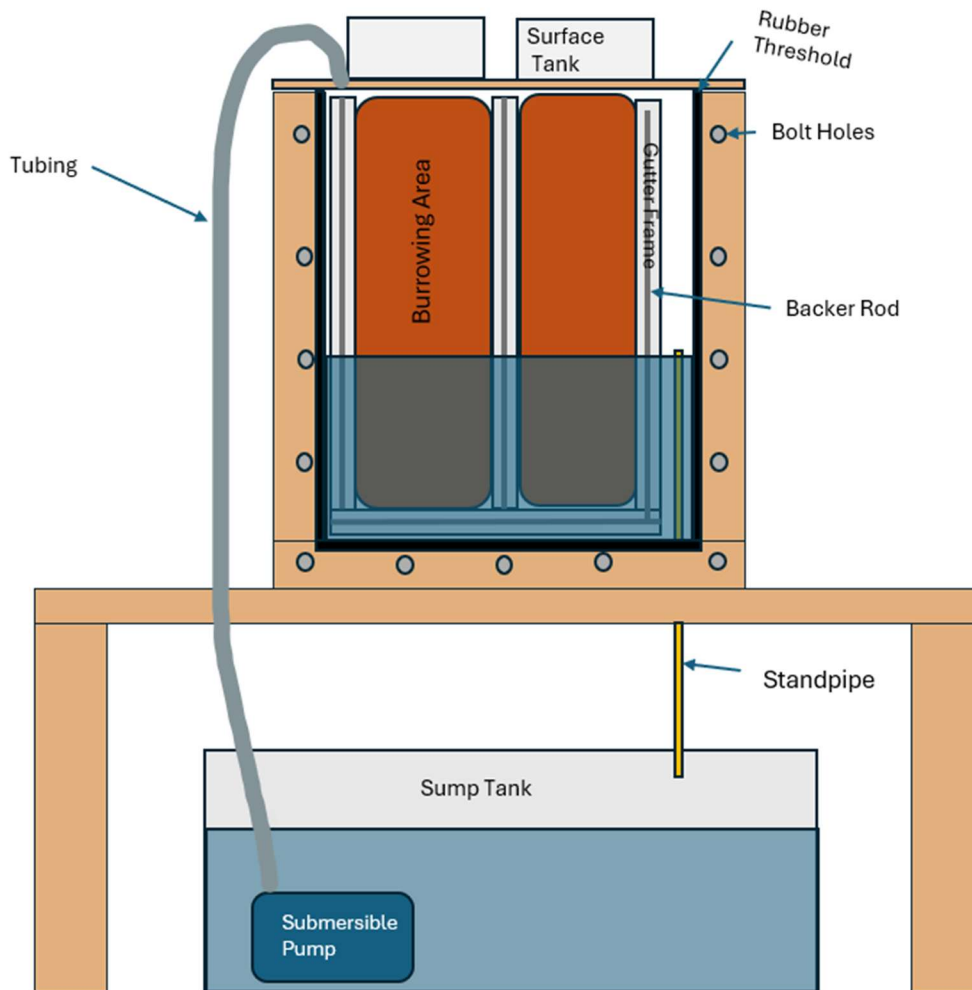


Figure 1.1 Diagram of burrow chambers used in the experiments in both chapters.

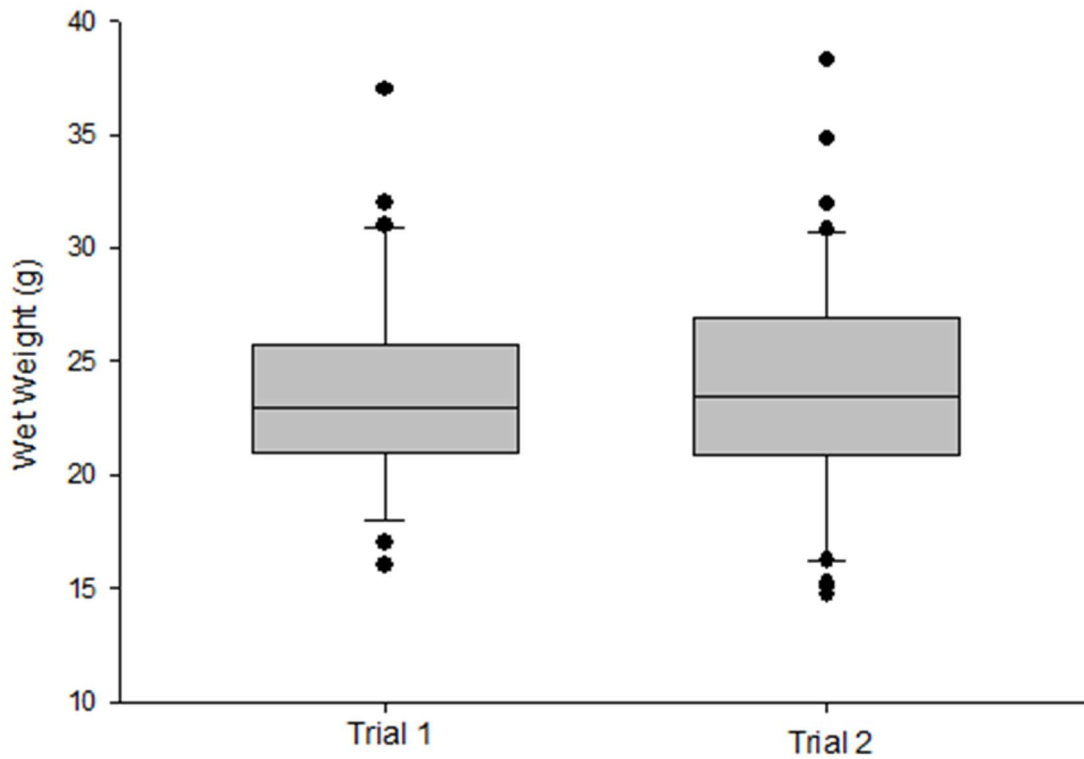


Figure 1.2 Wet weight of crayfish from trial one and two. Horizontal lines in boxes represent the median value, the upper and lower limits of boxes represent the 25th and 75th percentiles and whiskers represent the 10th and 90th percentiles.

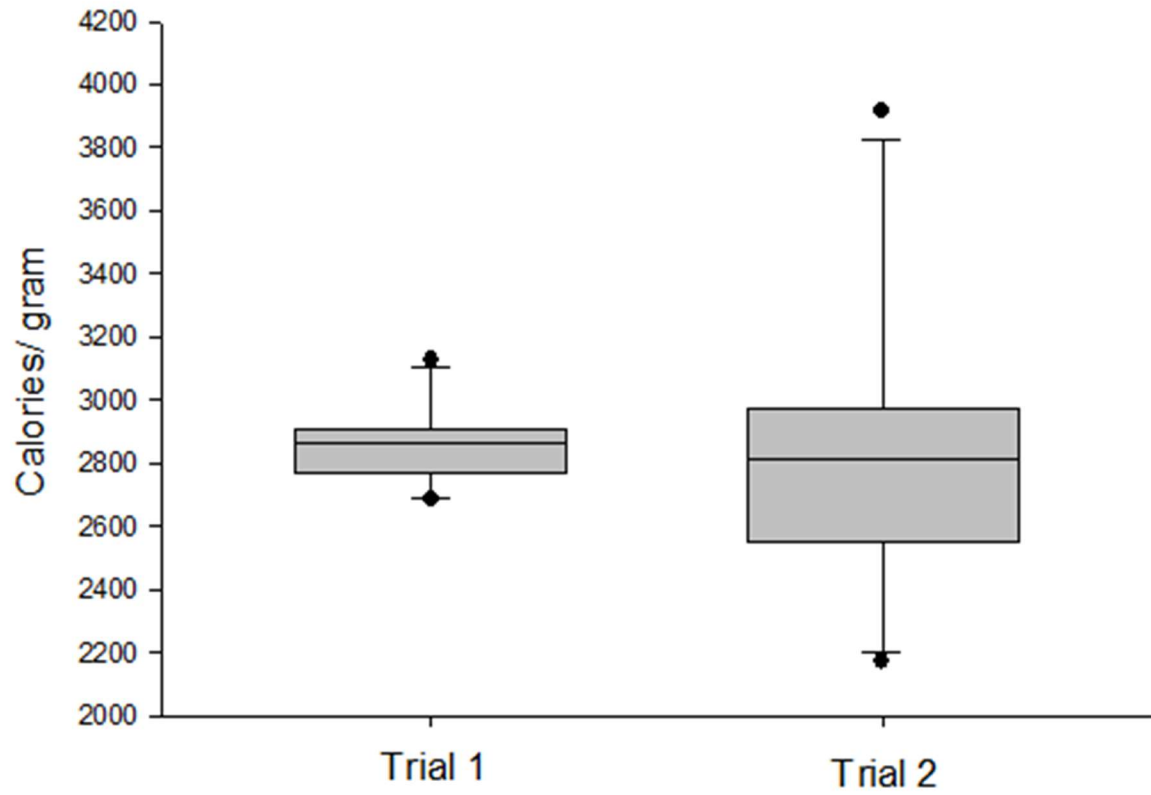


Figure 1.3 Initial caloric density of crayfish in trial 1 and trial 2. Horizontal lines in boxes represent the median value, the upper and lower limits of boxes represent the 25th and 75th percentiles and whiskers represent the 10th and 90th percentiles.

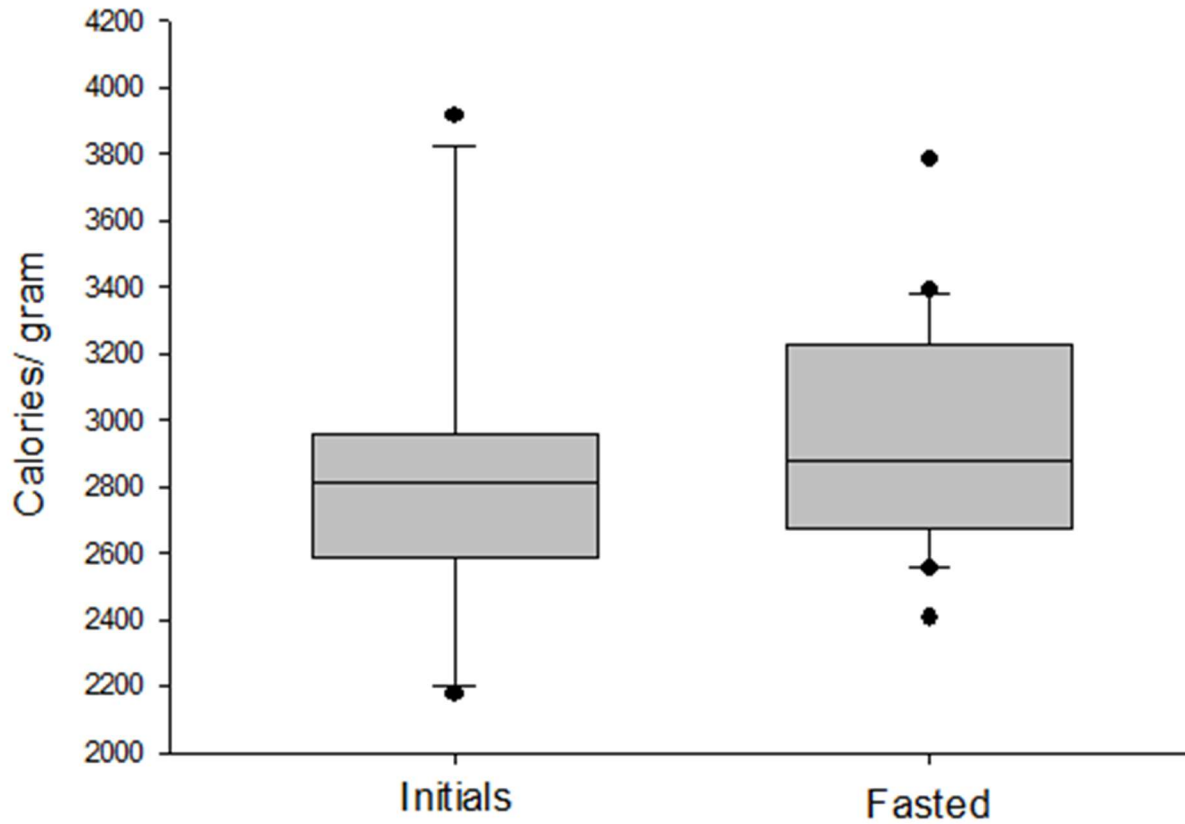


Figure 1.4 Initial crayfish caloric density, and caloric density of crayfish after one week of fasting in surface water for trials 1 and 2 combined. Horizontal lines in boxes represent median values, upper and lower limits of boxes represent 25th and 75th percentile and the error bars represent the 10th and 90th percentiles.

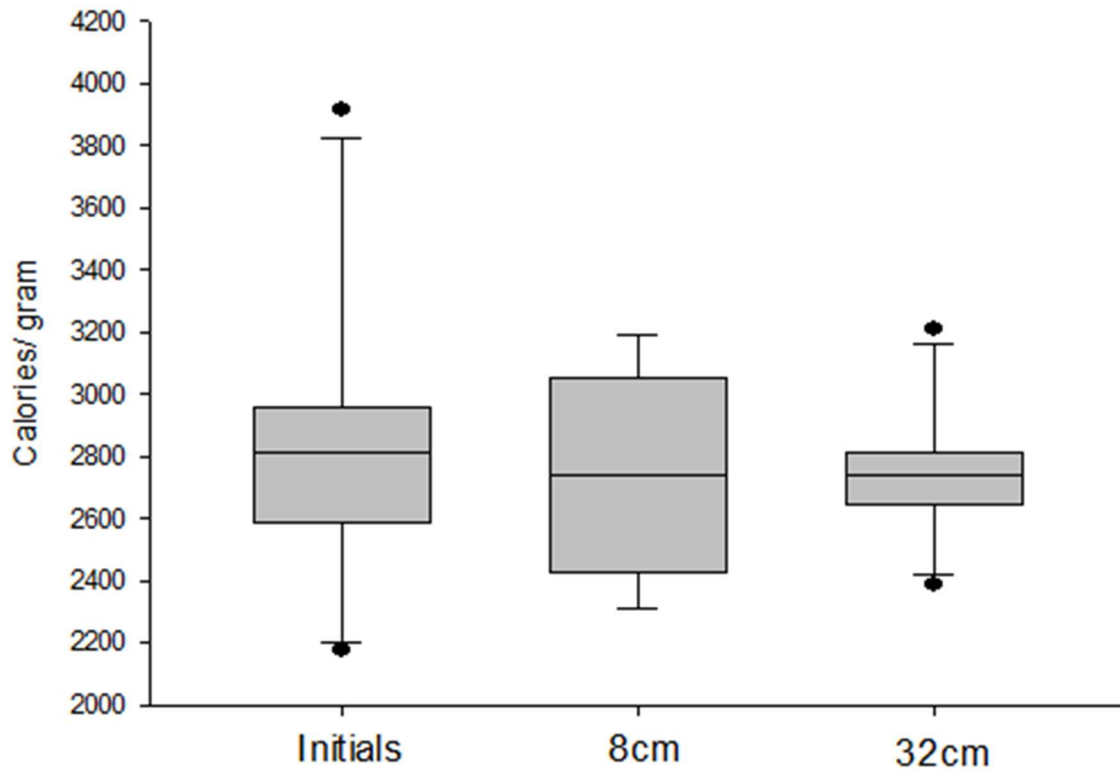


Figure 1.5 Initial crayfish caloric density (n=20) and caloric density of crayfish after burrowing to 8 cm (n = 8) on day 2, and to 32 cm on day 8 (n = 11) for trials 1 and 2 combined. Horizontal lines in boxes represent median value, upper and lower margins of boxes represent 25th and 75th percentile and the error bars represent the 10th and 90th percentiles.

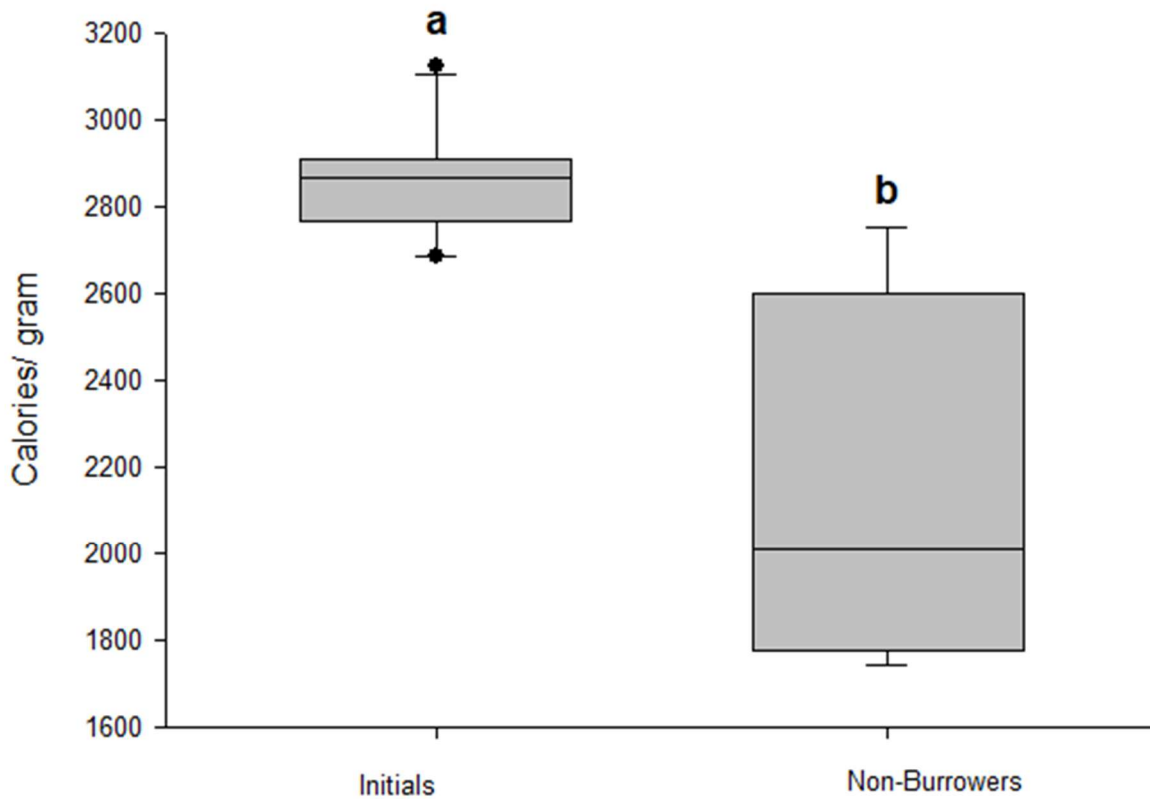


Figure 1.6 Initial caloric density of crayfish and caloric density of crayfish after spending two days on the soil surface without burrowing. Horizontal lines in boxes represent median values, upper and lower margins of boxes represent 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Letters indicate significance between the groups. Data is from trial 1 only.

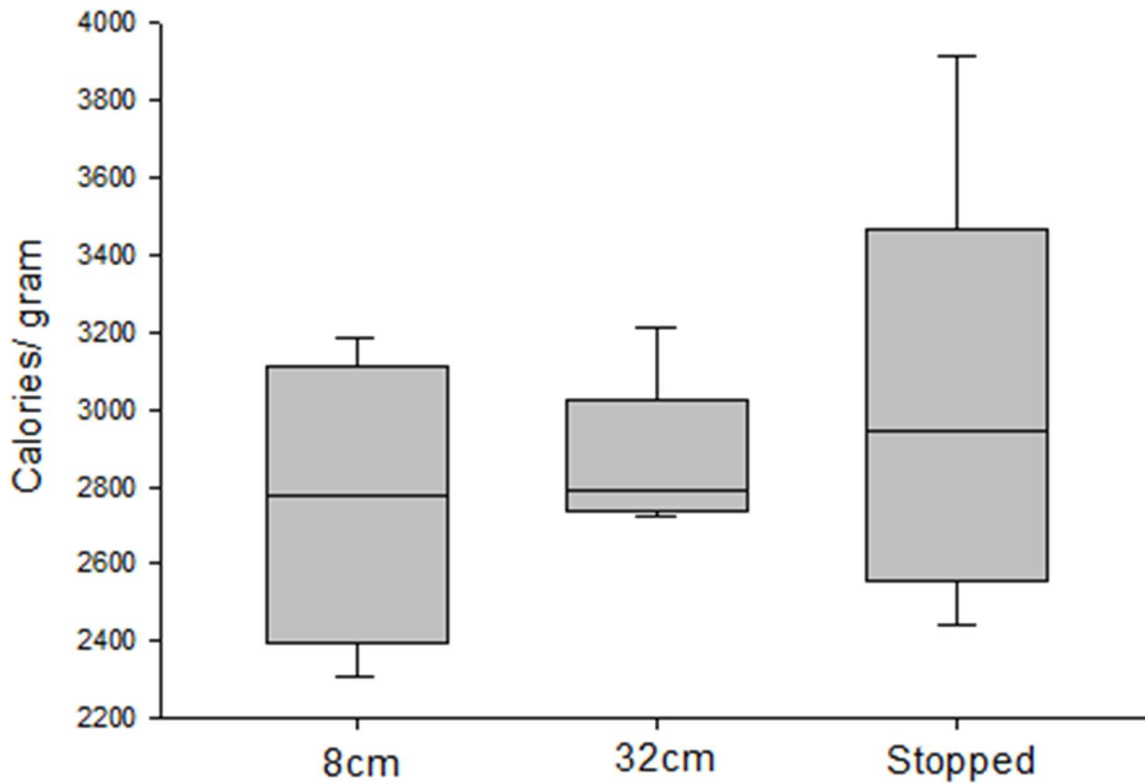


Figure 1.7 Caloric density of actively burrowing crayfish collected at 8 and 32 cm and those that initiated burrowing but were collected 3-5 days after they stopped burrowing. Horizontal line in the box represents the median values, the upper and lower margins of the box represent the 25th and 75th percentiles, and the whiskers represent the 10th and 90th percentiles. Plots represent data from Trial 2 only.

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Chapter 2: Impacts of Energetic Health on Burrowing Behaviors in *Procambarus clarkii*

Introduction

Procambarus clarkii or the Red Swamp Crayfish (RSC) is a globally invasive species with established populations on multiple continents including North and South America, Asia, Europe, and Africa. Its native range encompasses northeastern Mexico and the southern USA (Loureiro et al. 2015, Souty-Grosset et al. 2016). It is a generalist species that tolerates a wide range of environments, exhibits high growth rates, and has a robust feeding strategy (Alcorlo et al. 2024, Hobbs et al. 1989). This combination of traits favor its establishment in new habitats. Dispersal outside of its native range is primarily intentional as it is a popular food item, research subject, and aquarium pet (Officialdegui et al. 2019). *Procambarus clarkii* can be detrimental to native crayfish populations as a predator, through competition for resources, and as a vector for crayfish plague and other diseases (Alderman and Poleglase 1986, Holdich et al. 2009, Taylor et al. 2007).

Burrowing is critical to the ecology of *P. clarkii*. Burrows provide shelter from predators, protection from desiccation during dry weather, and most importantly a place to rear their young (Huner and Barr 1991, Herrmann and Martins 2024). These burrows can have an effect on the surrounding ecosystem by modifying sediment loading into nearby waterbodies and causing bank erosion (Haubrock et al. 2019). *Procambarus clarkii* are secondary burrowers that typically dig burrows with 1-2 entrances sometimes accompanied by a chimney and burrows typically have 1-2 occupants. When crayfish leave a burrow to forage, and surface waters are available, they will dig a new burrow more readily than they will return to the old burrow, w (Barbaresi et al. 2004). This phenomenon combined with a high density of individuals along a bank culminates in a cumulative effect that increases bank collapse and sedimentation.

Burrowing in other taxa, such as mammals and reptiles, has been shown to be energetically expensive. The Namib Desert golden mole (*Eremitalpa granti namibensis*), a specialized burrower, has been shown to exert 26 times more energy when burrowing (80 J m^{-1}) compared to running on the surface (3 J m^{-1}) (Seymour et al. 1998). Species that burrow to forage like *Liopholis* skinks have been shown to use almost 350 times more energy burrowing compared to running across the same distance (Wu et al 2015). Despite the considerable energetic cost, there is much benefit to burrowing for these species including thermoregulation, protection from predators, and access to other food sources. Determining how burrowing and energetic health are correlated in *P. clarkii* can reveal key insights into their persistence and adaptability as they spread into new environments.

In Chapter 1, I used whole body caloric density measured by bomb calorimetry as a proxy for energetic health. The hepatopancreas serves as the main energy storage reserve for crayfish (Lindqvist and Louekari, 1975; Holdich and Reeve 1988) and it is possible that hepatopancreas caloric density is a more sensitive indicator of energetic requirements for burrowing than whole body caloric density. Additionally, because there is a significant linear relationship between hepatopancreas moisture content and hepatopancreas caloric density (Jussila et al. 1997) in some crayfish taxa, moisture content may provide a less laborious, and more rapid approach to assessing crayfish energetic health.

In previous experiments, I noted that non-burrowing *P. clarkii* had significantly less energy after two days compared to the initial group. However, our experimental design did not allow us to determine if *P. clarkii* lost energy at the surface or if there was a specific energy threshold necessary before burrowing would commence. In this study, I investigated the relationship between initial energy content and burrowing behaviors in *P. clarkii*, with a

particular focus on the effects of energetic health on initiation and construction of burrows. My main objectives were to 1) establish a methodology to create low and high energy crayfish groups, 2) determine whether *P. clarkii* with a lower caloric density were less likely to initiate and dig burrows, and 3) to determine if the moisture content of *P. clarkii* hepatopancreas can be used as an indicator of whole body caloric density and/or a predictor of burrowing activity.

Methods

Effects of Fasting and Activity on Caloric Density

Effect of fasting alone

Forty male *P. clarkii* with carapace length (CL) between 40-50 mm were collected from pond F-14 at the E.W. Shell Fisheries Center in Auburn, Alabama. These crayfish were held in individual 1.5 L aquatic habitat systems (Pentair Aquatic Eco Systems, Apopka, Florida, USA) tanks in two recirculating systems (20 in each system) beginning in March of 2024 in the CAMEL (Crayfish and mussel ecology lab) at Auburn University. Each recirculation system had a separate sump from which water was recirculated among the tanks of that system. Each system was filled with artificial freshwater (AFW: 0.189 g CaCO₃ , 0.189 g NaHCO₃ , 0.095 g CaCl₂ , 5.0 mL 35‰ saltwater·L⁻¹ of reverse osmosis/deionized water; pH = 8.05, total hardness = 52.5 mg·L⁻¹ CaCO₃ , total alkalinity = 40 mg·L⁻¹ CaCO₃ ; Gascho Landis et al. 2012) and was held at 25 C° with a 12h on 12h off light cycle. Crayfish were fed every day for seven days for acclimation to artificial feed and to minimize differences in energetic content before the fasting treatments began. The crayfish were then fasted for five weeks. At the end of weeks one, two,

three, and five, five crayfish were randomly removed from each system each week and frozen for bomb calorimetry to test for a decline in caloric density over time when fasted.

Effects of Fasting Coupled with Forced Activity

Forty *P. clarkii* of 40-50 mm CL were collected from a wild population in pond F-14 at E.W. Shell in late June 2024 and held in the AHAB systems filled with AFW at 25°C for one week. During this time, I fed each crayfish 8-12 pellets per day. Photoperiod was held constant at 12 h:12h day night cycle.

On 27 July 2024, after one week of feeding, all 40 crayfish were transferred to a trough (3.0 m long x 0.7 m wide x 0.6 m max depth) set up as a recirculating system with a biofilter (0.9 m long rectangular box with bio-balls and pvc ribbon) fed by a Pondmaster pump. Two additional pumps (Pondmaster Magnetic drive utility pump model 12 B 500 GPH) were placed in either corner, opposite one another, causing the water to circulate. Each crayfish was placed in an individual, submersed, 5” hamster ball (HB; Kaytee 5” clear Run-about exercise ball for hamsters and mice) to prevent aggressive interactions and cannibalism. Ten *P. clarkii* were randomly selected for the Fed/Stationary treatment and placed in individual HBs suspended above the bottom on a threaded longline to keep them stationary. Crayfish were fed 8-12 pellets per day. The remaining crayfish were placed in HBs that sank to the bottom of the troughs and were not fed (Fasted/Mobile Treatment). Currents created by the two recirculating pumps helped keep the HBs in frequent motion and prevented them from getting stuck in corners. Crayfish also moved the HBs by their own activity as they crawled up the inside walls of the HBs. After two weeks, ten crayfish from the Fasted/Mobile treatment were removed along with the ten

Fed/Stationary crayfish, frozen and stored at -80°C for later caloric density analyses. The remaining Fasted/Mobile crayfish were removed in batches of 10 each week for an additional two weeks and prepped similarly.

Effect of Initial Caloric Density on Burrowing

Forty male *P. clarkii* between 40-50 mm CL were collected from pond F-14 during the month of February 2025 and fed and acclimated for one week in AHAB systems at 25°C as in the previously described experiment. To create lower and higher caloric-density groups, I used the same procedure as previously described in the *Effects of Fasting Coupled with Forced Activity* section. Crayfish were randomly assigned to one of two groups: 20 were fed in stationary HBs, while the other 20 were fasted in mobile HBs. After three weeks, 10 crayfish from each treatment were randomly selected for initial caloric-density analysis, frozen and stored at -80°C for future analysis. The remaining 10 crayfish from each treatment were transferred to artificial burrowing chambers with two crayfish per chamber, one from each treatment, in a randomized block design. Chamber design and preparation are described in Chapter 1. Photoperiod was held at 12L:12D and temperature was maintained at 25°C. Ground water was brought up to the soil surface for 24h before crayfish were introduced into each chamber. Following introduction, groundwater level was drawn down at a rate of 4 cm per day for eight days to a final depth of 32 cm and crayfish were allowed to burrow undisturbed. Final burrow depth was measured for each crayfish on day eight.

After completion of the experiment, all crayfish were removed and prepped for bomb calorimetry. Before the prep, each crayfish was dissected and hepatopancreas wet weight was

recorded using a scale (Mettler Toledo XS204, $d = 0.1$). Each hepatopancreas was then dried at 100°C for 24h (VWR Drying oven model number 1321F) and dry weight recorded. Moisture content was calculated as $M = ((H_w - H_d) / H_w) \times 100$ to express it as a percentage where H_w is hepatopancreas wet weight and H_d is dry weight. The samples were then returned to the mixture of the rest of the individual's sample that were prepped in the same way as described in chapter 1, which were then ground and taken for energetic analysis. *Procambarus clarkii* energetic content was measured using bomb calorimetry following the traditional whole-body approach (Glover et al. 2010) Within 60 days of collection and freezing, each crayfish was placed in a drying oven for 24 h at 100°C to remove all moisture. Each dried crayfish was then ground into a powder using an analytical mill (IKA A11 basic analytical mill, 2635 Northchase Parkway SE Wilmington, NC 28405 USA). Two subsamples of the powder were then pressed into pellets weighing 0.1-0.2 grams each. The pellets were ignited in one of two Parr calorimeters (Parr 6725, Parr 1425) following the procedures described in the manual. Caloric values obtained from the two pellets per crayfish were then averaged to obtain a mean caloric density for that individual (calories/ gram of dry mass).

Data Analysis

Data was analyzed using SigmaPlot 15.0 Grafiti. I used an analysis of variance (ANOVA) to test for differences in caloric content over time for fasted crayfish in the initial fasting trial and in the Fasted/Mobile treatment of the second fasting trial. Because data failed assumptions of equal variance for both ($p < 0.05$), I then used a Kruskal-Wallis test on rank-transformed data.

I used t-tests to test for differences between Fed/Stationary and Fasted/Mobile crayfish caloric densities in the second fasting trial and in the burrowing trial. I also used t-tests to test for differences in, final burrow depth, final whole-body caloric content, final hepatopancreas moisture content and hepatopancreas wet weight between Fed/Stationary and Fasted/Mobile crayfish in the burrowing experiment. In all cases, data passed assumptions of normality and constant variance ($p > 0.05$).

I used a linear regression to test for a relationship between whole body caloric density and hepatopancreas moisture content.

Results

Effects of Fasting and Activity on Caloric Density

Effect of Fasting Alone

I saw no significant difference in *P. clarkii* caloric density among groups collected after one, two, three, and five weeks of fasting in AHAB tanks ($p = 0.097$, $H_3 = 6.323$) (Figure 2.1).

Effect of Fasting Coupled With Forced Activity

P. clarkii that were fasted and forced to move for two weeks had a significantly lower mean caloric density than crayfish fed and held stationary ($t_{18} = 2.598$, $p = 0.018$) (Figure 2.2).

When crayfish were fasted and forced to move for longer than two weeks, there was no subsequent significant change in energy content ($H_2 = 1.019$, $p = 0.6$) (Figure 2.3).

Effect of Initial Caloric Density on Burrowing

Procambarus clarkii fed for three weeks had a significantly higher mean caloric density (3123.1 ± 94.6 cal/g) compared to crayfish fasted and forced to move for three weeks ($2,674.9 \pm 114.7$ cal/g) ($t_{18} = -3.103, p = 0.007$) (Figure 2.4) Crayfish from these treatments will hereafter be referred to as high energy (HE) and low energy (LE) crayfish.

All ten *P. clarkii* in the HE group initiated burrows with eight of ten burrowing to the final groundwater depth of 32 cm. Similarly, all ten crayfish in the LE group initiated burrows, but only two of ten burrowed to the final groundwater depth of 32 cm. One crayfish in the HE group and two crayfish in the LE group died before the trial ended on day eight. Of the surviving crayfish (9 HE and 8 LE), those in the LE group dug significantly shallower burrowers (15.5 ± 4.5 cm) compared to the HE group (30.44 ± 3.6 cm) ($t_{15} = -2.584, p = 0.021$) (Figure 2.5).

After burrowing for eight days, *P. clarkii* in the HE group retained a higher caloric density ($2,939.4 \pm 61.8$ cal/g) than crayfish in the LE group ($2,545.1 \pm 89.3$ cal/g) ($t_{15} = -3.699, p = 0.002$) (Figure 2.6). *Procambarus clarkii* hepatopancreas moisture content in the HE group ($68.4\% \pm 1.5\%$) was significantly lower than crayfish in the LE group ($84.8\% \pm 1.6\%$) after burrowing for eight days ($t_{35} = 7.306, p < 0.001$) (Figure 2.7). Among all HE and LE crayfish combined, there was a significant negative relationship between hepatopancreas moisture content and whole-body caloric density. For every 1% increase in hepatopancreas moisture content there was a decrease of 16.9 ± 4.9 calories/ gram in whole body caloric density ($p < 0.0001, R^2 = 0.25, y = 4,128 - 16.9x$) (Figure 2.8). *Procambarus clarkii* hepatopancreas wet weight in the HE group (1.14 ± 0.25 g) was significantly different from the LE (1.69 ± 0.37 g) ($t_{35} = -5.28, p < 0.00001$, Figure 2.9). Additionally, *P. clarkii* hepatopancreas tissues from the LE crayfish were noticeably darker in color than in the HE crayfish (Figure 2.10).

Discussion

I found that energetic health, as measured by whole-body caloric density, significantly impacted *P. clarkii* burrowing activity, supporting the idea that burrowing in natural populations may be physiologically constrained (Haubrock et al 2019, Barbaresi 2004). Although initial caloric density did not affect initiation of burrows, crayfish with a lower caloric density dug shallower burrows, on average, than higher caloric density crayfish. Burrow construction likely requires sustained muscular exertion for sediment displacement and posturing (Grow 1981, Dorgan 2015, Laidre et al 2018), and crayfish with higher energy reserves may be better at maintaining this activity over longer durations. This has important implications for invaded systems where crayfish with better access to food may create deeper burrows, accelerate bank destabilization, increase turbidity and alter sediment composition. Conversely, in systems where crayfish have poor nutrient availability, they may burrow more shallow, potentially reducing physical effects on the environment.

In other species burrowing is energetically expensive so I expected to see that in these species as well. This hypothesis was supported by what I observed in this experiment. Previous studies have shown burrowing to be energetically expensive in mammals and reptiles (Wu et al. 2015, Seymour et al. 1998) that dig temporary burrows for foraging and in other species like *Macropesthia rhinoceros* which digs more permanent burrows (Xu et al 2014). However, I investigated this in my first chapter and found no evidence that burrowing is energetically expensive to *P. clarkii*, which was not what I expected to see based on these previous studies. The *P. clarkii* HE group in this chapter maintained higher caloric density than the LE initial individuals even after burrowing. Therefore, when compared to other facultative burrowers, *P.*

clarkii displays a remarkable ability to burrow without much energetic investment. This suggests that although it is not as energetically expensive to burrow as initially expected, there is still a threshold in caloric density below which crayfish begin to dig shallower burrows.

The two preliminary experiments showed that crayfish are capable of a torpor like state where they expend little energy while fasting for several weeks. This corroborates previous claims that adult *P. clarkii* can survive for long periods, up to one year, under simulated burrow conditions without food. (Huner and Barr 1991). Another study found up to 65% survival where *P. clarkii* females were kept in artificial terminal chambers for four - seven months while fasting (Mcclain et al. 2010). Crayfish in burrows likely slow their metabolism throughout the reproductive season to ensure their energy stores carry them until they can emerge.

The hepatopancreas investigations gave us some interesting insight into the physiological effects of constant movement and agitation on energy storage where moisture content, color and caloric content of the hepatopancreas all changed when fasting crayfish were forced out of torpor. The negative relationship between hepatopancreas moisture content and whole-body energetic content is supported by a similar study done by Jussila et al. (1997). Hepatopancreas moisture content may be a good proxy for whole body caloric density. This would be useful because bomb calorimetry can be an expensive and laborious method for energy estimates and calculating moisture content can be much easier. Samples of hepatopancreas from wild populations might also yield valuable information as to condition and energetic health of crayfish in natural systems. However, in this study, moisture content explained only 25% of the variation in whole-body caloric content of crayfish. In Jussila's study hepatopancreas energy content was used instead of whole body caloric content and found 44% of the variation was due to moisture content. In both cases this relationship, although significant, is not a great predictor. Additional

research is needed to determine if this relationship can be improved before hepatopancreas moisture content can be recommended as a useful predictor of energetic health.

We also noted a significant difference in hepatopancreas moisture content between the LE and HE groups. The LE group had significantly higher moisture content. This result is corroborated by a study that showed increased moisture content in starved *Cherax destructor* (Jones and Obst 2000). They also noted that about seven months of starvation at 10 °C resulted in a nutritionally depleted hepatopancreas. This can be taken to support the earlier claims of Huner and Barr that crayfish can survive large periods of time without food. However, at higher temperatures the nutritional depletion may occur sooner because *P clarkii* metabolism is suppressed at low temperatures (Ding et al. 2025) Overall, a diminished hepatopancreas as indicated by an increased moisture content can be used as a sign of a poor state of energetic health even if the linear relationship is not strong enough to use as a direct predictor of whole body caloric density.

Previous studies that showed high energetic costs of burrowing used respiration rate as a proxy for energetic demand. This may be a more sensitive metric than whole-body caloric density. I chose to use bomb calorimetry because respirometry would not have been practical to conduct on crayfish in the burrowing chambers. Possible future projects include designing large scale respirometry systems to measure respiration of crayfish while actively burrowing.

Our results demonstrate a clear energetic threshold influencing burrow construction in *P clarkii*. This finding underscores the importance of considering individual energetic health in behavioral ecology, especially in invasive species where energy uptake and allocation can shape ecological impacts. Future work is needed to learn how to integrate this knowledge into the field

and long-term observation will be necessary to fully understand how energy plays a role in burrowing.

Figures

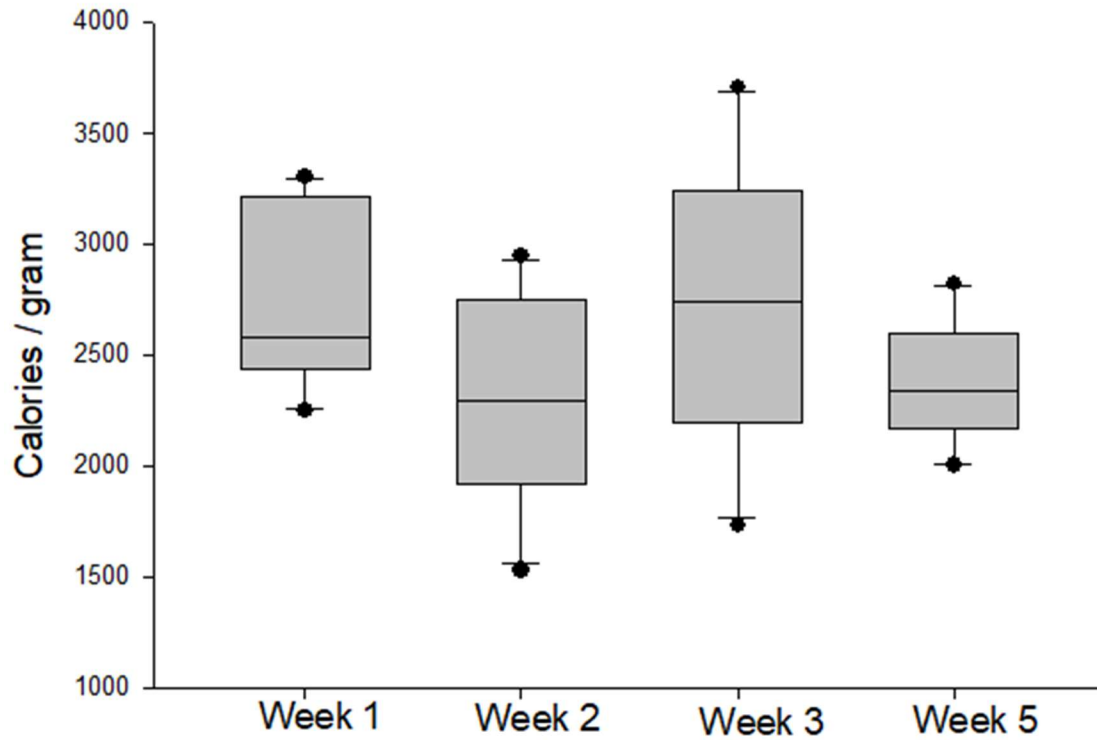


Figure 2.1 Whole-body caloric density of crayfish after 1, 2, 3, and 5 weeks of fasting in the *Effects of Fasting Alone* trial. Horizontal lines in the boxes represent median values, upper and lower margins of boxes represent the 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles.

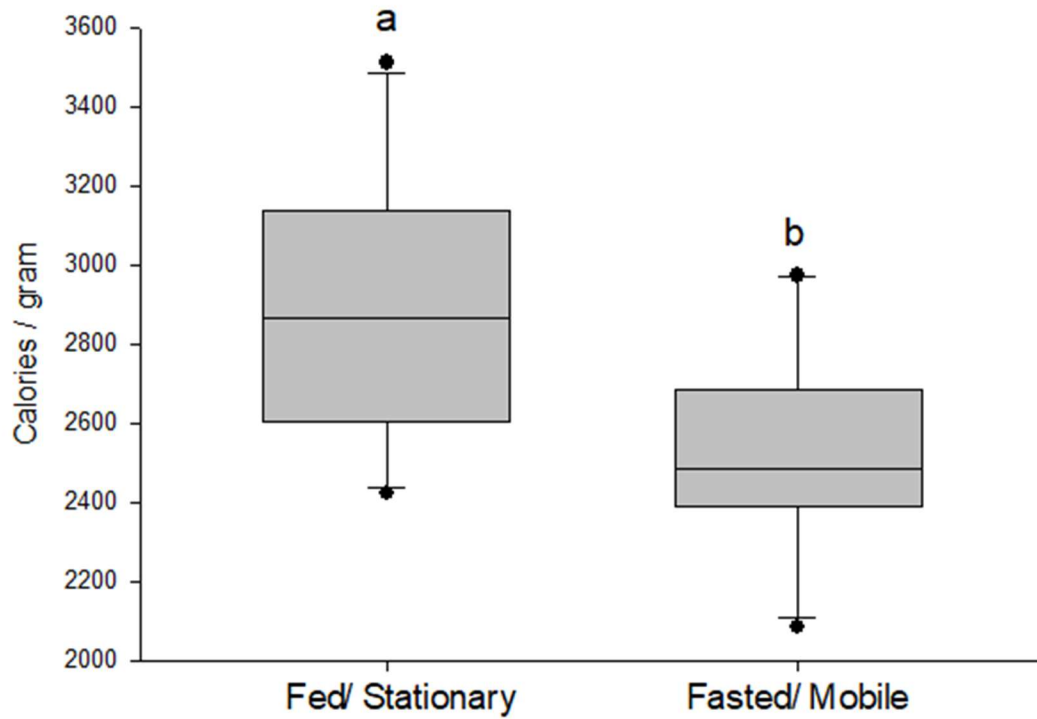


Figure 2.2 Whole-body caloric density of Fed/Stationary and Fasted/Mobile crayfish in the *Effects of Fasting plus Forced Activity* trial at 2 weeks. Horizontal lines in boxes represent median values, the upper and lower margins of the box represent 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Lowercase letters indicate significant differences between the two treatments.

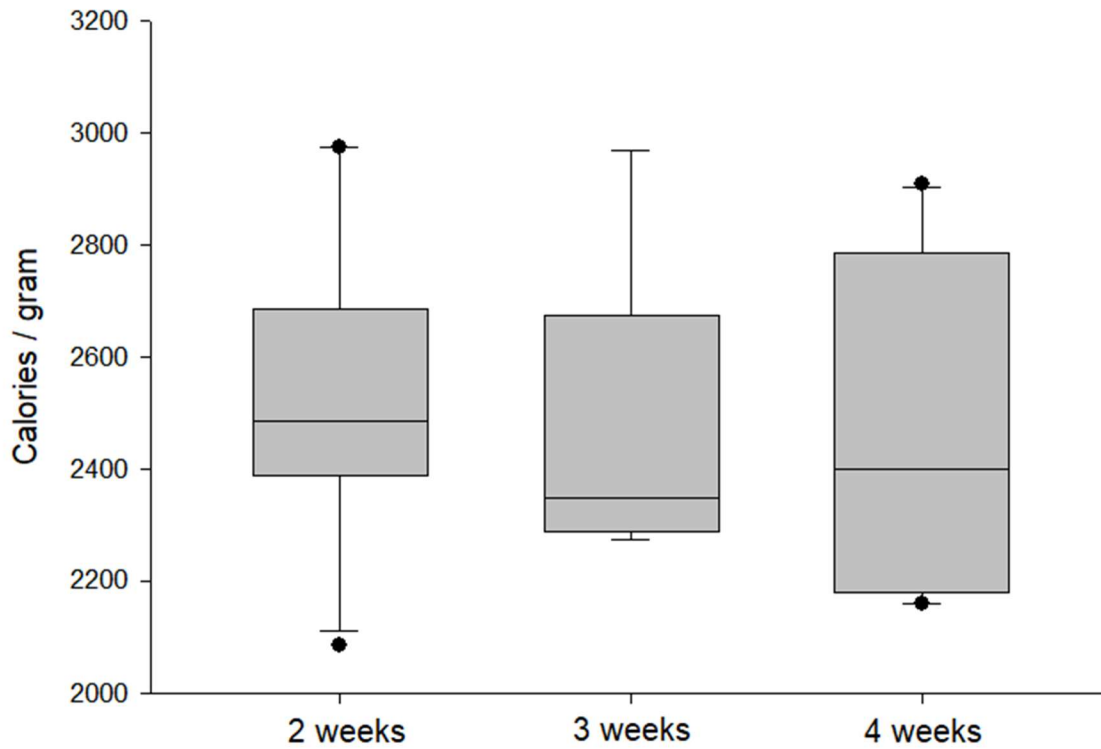


Figure 2.3 Whole-body caloric density of crayfish in the Fasted/Mobile treatment after 2, 3, and 4 weeks of forced activity. Horizontal lines in boxes represent the median values, the upper and lower margins of the boxes represent the 25th and 75th percentiles, and the whiskers represent the 10th and 90th percentiles.

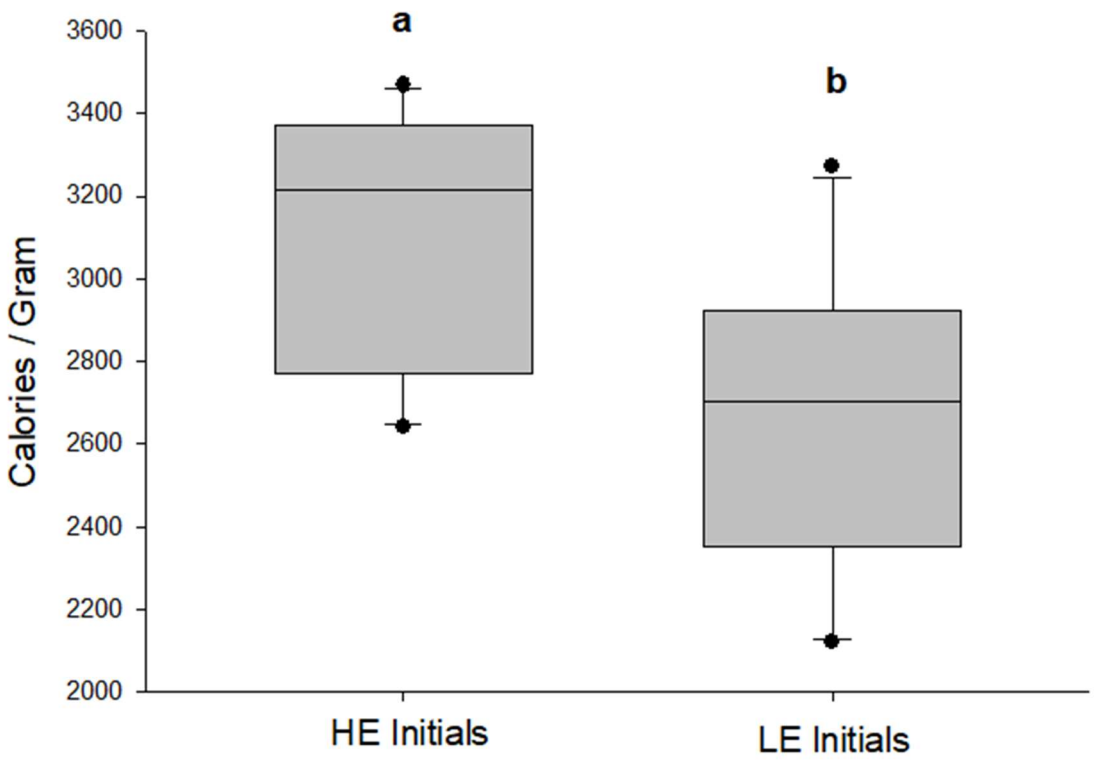


Figure 2.4 Whole-body caloric density of high energy (HE) and low energy (LE) crayfish just prior to initiation of the burrowing trial. Horizontal lines in boxes represent median values, upper and lower margins of boxes represent 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Lower case letters indicate significant differences between groups.

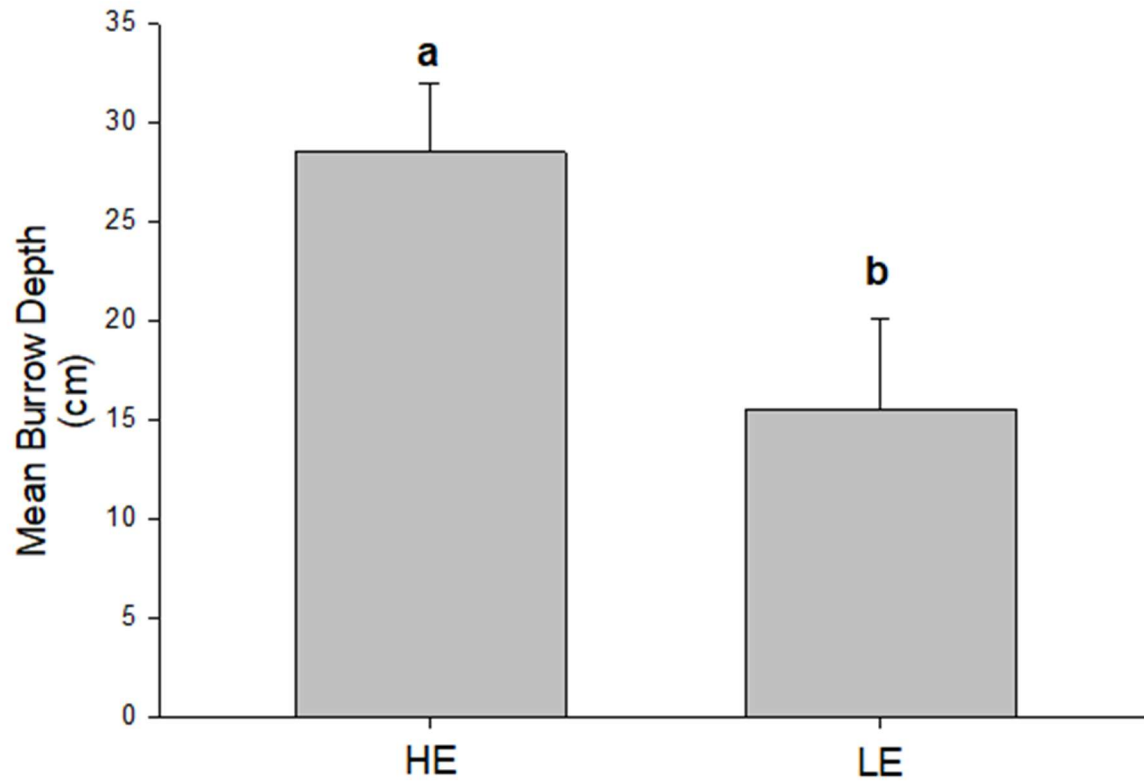


Figure 2.5 Final depth of high energy (HE) and low energy (LE) crayfish at the end of the burrowing trial (day 8). Lowercase letters indicate significant differences between groups.

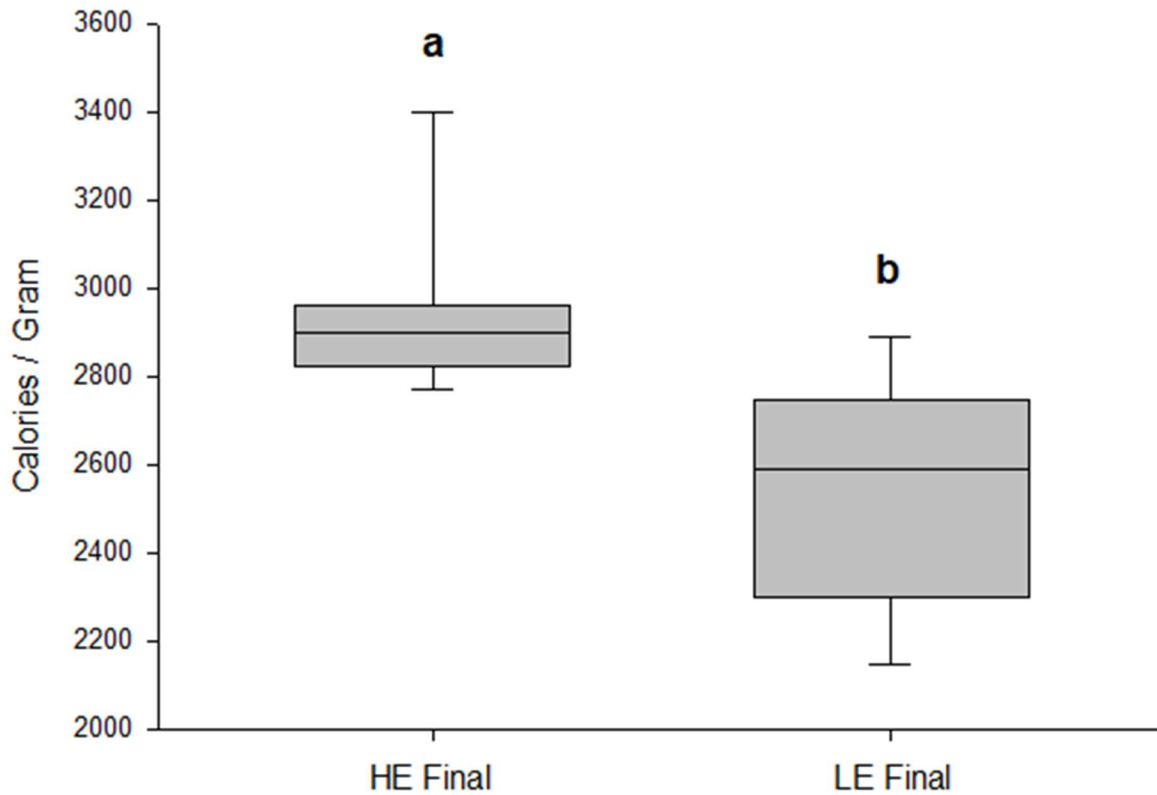


Figure 2.6 Whole-body caloric density of high energy (HE) and low energy (LE) crayfish at the end of the burrowing trial (day 8). Horizontal lines in boxes represent the median values, upper and lower margins of boxes represent the 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Lowercase letters indicate significance between groups.

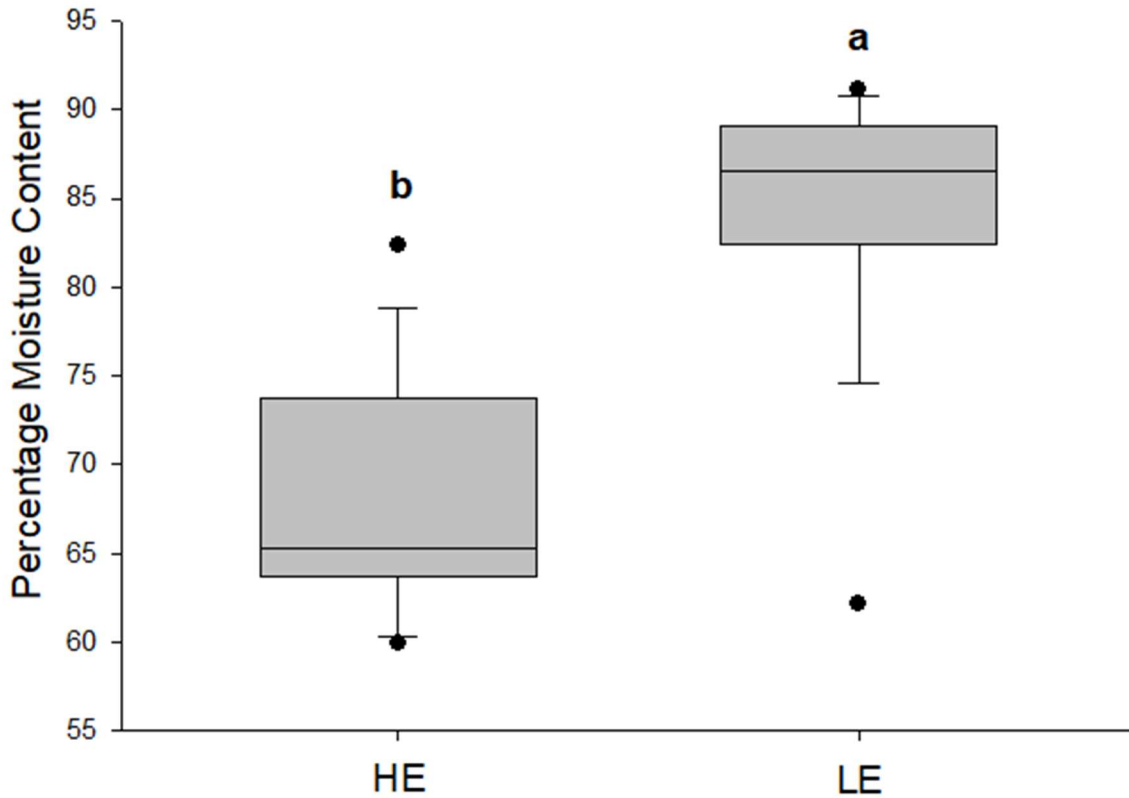


Figure 2.7 Percent moisture content of the hepatopancreas in high energy (HE) and low energy (LE) crayfish at the end of the burrowing trial (Day 8). Horizontal lines in boxes represent median values, upper and lower margins of boxes represent the 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Lowercase letters indicate significance between groups.

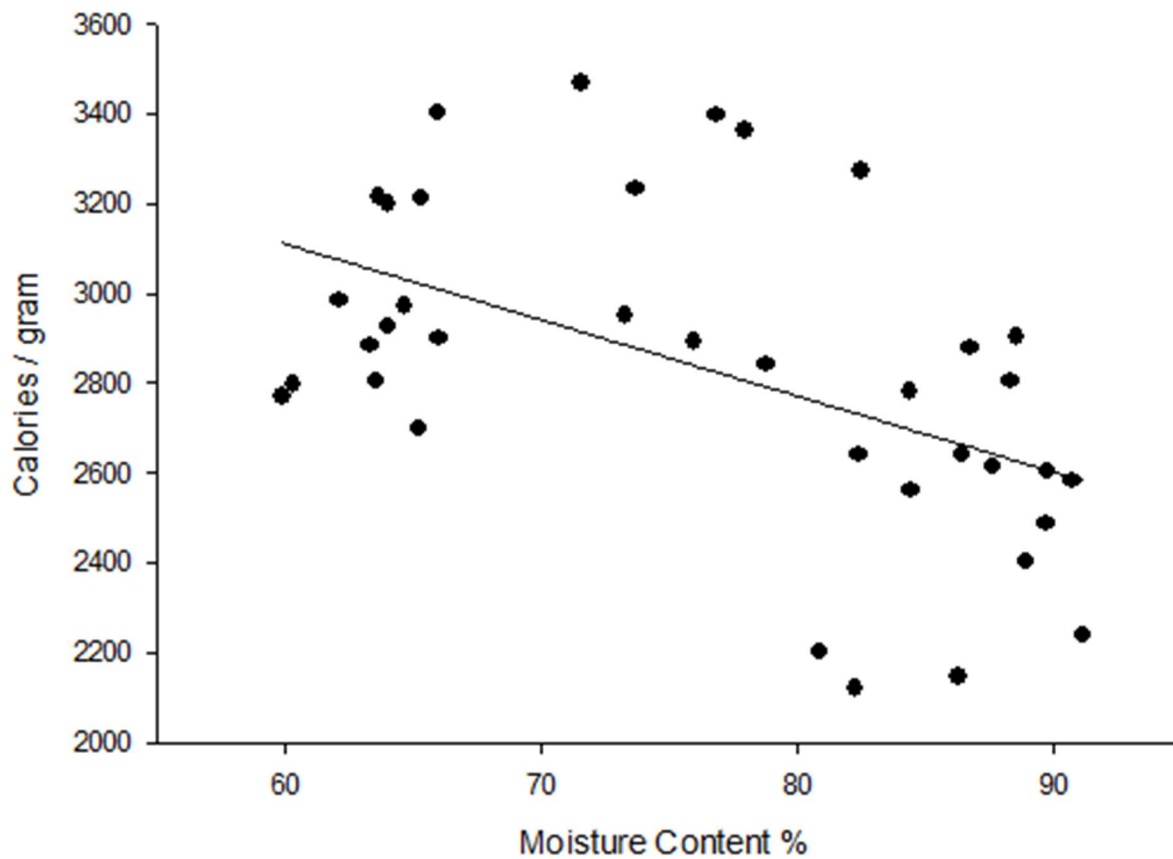


Figure 2.8 Relationship between whole-body caloric density and hepatopancreas moisture content of all surviving crayfish at the end of the burrowing trial (day 8). Black circles represent data from individual crayfish. Solid line represents a linear regression through the data where $y = 4128.2542 - 16.9595x$.

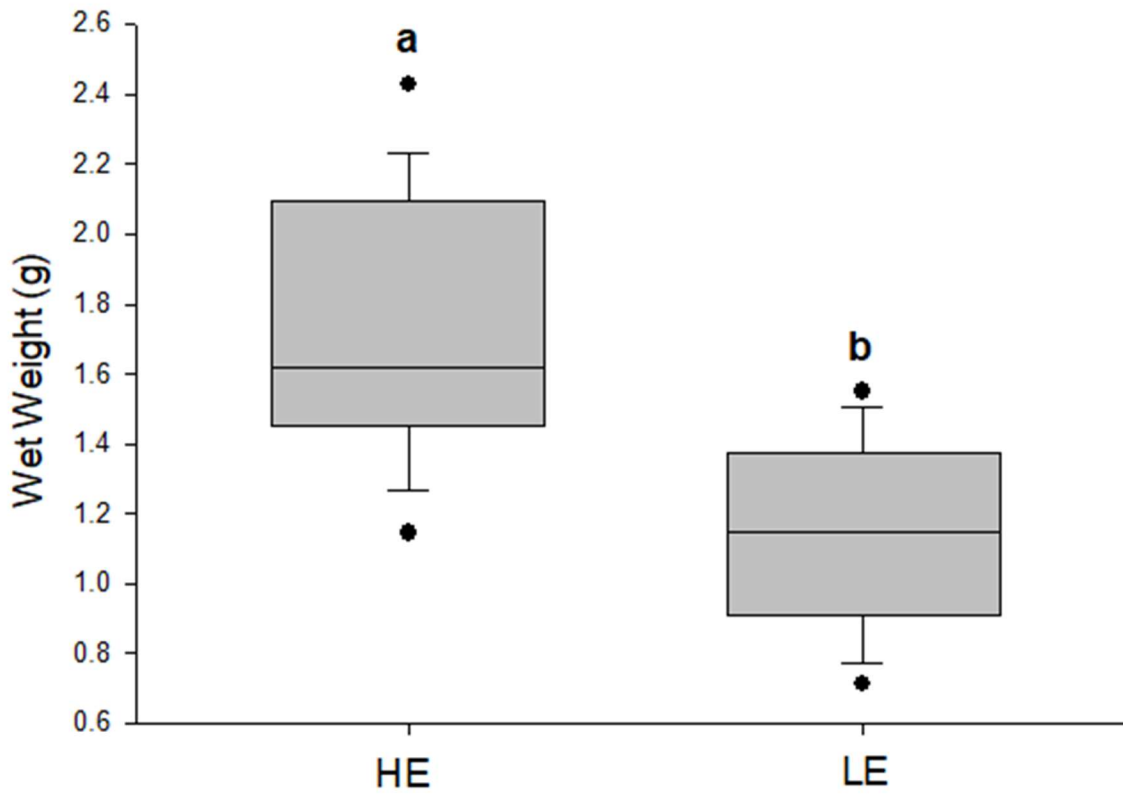


Figure 2.9 Comparison of wet weight of dissected hepatopancreas in the HE and LE groups. Horizontal lines in boxes represent median values, upper and lower margins of boxes represent the 25th and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Lowercase letters indicate significance between groups.



Figure 2.10 Images of dissected hepatopancreas from LE (left) and HE (right) crayfish

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