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Impact of a CO2 Gradient on the Behavior of the Red Crayfish, Procambarus clarkii

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Abstract

With carbon dioxide levels on the rise, studies to investigate the possible detriment that climate change will have on our ecosystems and the organisms that live within them are essential. The field lacks an abundance of studies focusing on the effects of rising CO_2 levels on freshwater organisms. This study looks at the effects of a CO_2 gradient on the freshwater crayfish *Procambarus clarkii*. The gradient allows the crayfish to choose to avoid or prefer the higher carbon dioxide levels. Previous studies have looked at the effect of high CO_2 levels, decreased pH, on a variety of crustaceans, but did not use the gradient. Crayfish were introduced into a control environment and observed for normal behavior then introduced into a CO_2 gradient environment. The crayfish did not prefer a particular section of the tank in the control environment, making the CO_2 gradient experiment possible. When in the CO_2 gradient, the crayfish significantly preferred Sections 1, 2, and 3 over Section 4 (where the highest CO_2 levels were present). In the CO_2 gradient, the crayfish exhibited less hiding behavior and did not acclimatize to the CO_2 levels over time. The crayfish left themselves to be more vulnerable to their surroundings. However, exploratory and feeding behavior were surprisingly not affected by the CO_2 gradient environment. Rising carbon dioxide levels have the potential to negatively affect freshwater organisms such as the crayfish, but crayfish also may have the potential to adapt to these alterations brought about by climate change, especially if the change takes place over a significantly longer period of time.

KEY WORDS: crayfish, CO_2 gradient, behavior, climate change, plasticity

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Introduction

Crustaceans, especially crayfish, have the ability to be ideal organisms for behavioral studies. Their size allows them to be easily watched and distinguished from one another. Since crayfish in tanks have an average life span of over a year, crayfish can be used for multi-month studies with ease if kept in the proper conditions (Whiteley 2011). Crayfish are also well-known prominent organisms in many freshwater systems and can be obtained and shipped without difficulty. Behavioral characteristics such as feeding and exploring time can be monitored well in crustaceans. A study by Wu et al. (2017) looked at foraging behavior in Japanese stone crabs in decreased pH levels. This study not only showed that predation rates, foraging behavior, and feeding behavior are all able to be monitored successfully in crustaceans, but that decreased pH levels had a negative effect on the crustaceans' foraging behavior. The crustaceans spent an excessive amount of time leaving themselves vulnerable to others while feeding and foraging (Wu et al. 2017). Other studies involving crayfish range from changing temperature, growth conditions, and diet to investigation of crayfish neurons, showing the wide breadth of research that crayfish are useful tools for (Crawshaw 1974, Moody 1981, Kominoski et al. 2007, Adams et al. 2005). The use of crayfish as a means to research climate change is no exception.

The current rates of climate change are unparalleled. The biological responses of organisms to this rapid rate of climate change have been just as drastic at all levels. A lot of the research thus far has mainly looked on land and in oceans. A rise in CO_2 levels, accompanied by a decrease in the acidity of water, not only affects oceans but also freshwater systems. Since pH plays a significant role in biological response systems, this difference in the environment due to climate change has the potential to cause great detriment to many organisms (Wu et al. 2017, Whiteley 2011). The studies that have been completed in freshwater ecosystems have shown that freshwater organisms can be susceptible to the effects of climate change but that these effects can differ by species (Wu et al. 2017, Heino et al. 2008, Sand-Jensen et al. 2000, Saunders et al. 2002, Schindler 1997). Most of what we know about mechanisms involving acid-base balances in crustaceans are due to valuable laboratory-based studies where crustaceans are subjected to short periods of higher CO_2 levels. Through studies like this, we are given the ability to compare responses of different species. These studies also demonstrated the connection between acid-base balance and iono-regulation. Acidification has the potential to affect calcification rates of crustaceans by reducing alkaline pH or interfering with post-molt calcification (Whiteley 2011). Cameron (1985) showed that in a type of blue crab, post-molt calcification took twice as long in hypercapnia. Yet, most of these studies involved marine organisms.

Freshwater crustaceans have been shown to be strong iono-regulators and potentially have the capability to survive acidification and be significantly less vulnerable to it. In the short term, freshwater crustaceans may have the mechanisms that will allow them to withstand some disturbances in acid-base balance, but how they will fare in the long run remains unknown (Whiteley 2011). Physiological problems of crayfish in water with a more acidic pH than normal can differ across crayfish species; *Procambarus clarkia*, the organism being used for this study, showed a greater acid tolerance than the other crayfish studied in McMahon & Stuart (1989). Ricevuto et al. (2012) looked at multiple species of benthic invertebrates along a natural CO_2 gradient in the Tyrrhenian Sea over time in which some species' numbers dwindled and a few thrived, decreasing the biodiversity in the area. The studies of Ziveri et al. (2014) in coccolithophores in a natural CO_2 gradient reinforced these findings that a rise in CO_2 levels will cause biodiversity loss. A study that looked into the effects of acute CO_2 on *P. clarkii* showed that when these crayfish are introduced to high concentrations of CO_2 (not a CO_2 gradient), they demonstrate a strong avoidance response. This could be mainly due to a want to avoid the unresponsiveness and cessation that the crayfish first experienced in the experiment due to the high CO_2 levels (Bierbower & Cooper 2010).

Additional studies need to be conducted on freshwater organisms so that increased knowledge can be massed and species distributions and responses can be predicted. The lack of this knowledge obstructs the ability to forecast the future of freshwater habitats and the responses of the significant organisms that all play roles within these ecosystems (Heino et al 2008, Sand-Jensen et al 2000, Saunders et al 2002, Schindler 1997). The study of the impact of rising CO_2 levels on various organisms is essential for determining the potential negative effects that climate change may bring. By examining freshwater organisms, such as the crayfish, we can begin to delve into how organisms will react to these changes through physiological and behavioral adaptations.

Our study's purpose was to gain information on the behavior of crayfish in response to rising

CO_2 levels. This behavior was analyzed to see if the crayfish are at all negatively affected by the higher acidity or if they have the potential to thrive. By the use of the CO_2 gradient, crayfish preference for higher or lower CO_2 levels will be able to be examined. If the crayfish do not move away from the higher CO_2 levels, this could signify vulnerability of the crayfish as they would not be able to sense the negative effects that the higher CO_2 levels have on them. Yet, this study will also investigate whether the higher CO_2 levels actually do affect crayfish behavior or if the crayfish are able to easily adjust. The following hypotheses are being investigated: (1) if crayfish are introduced into an environment containing a CO_2 gradient, the crayfish will avoid the portion of the gradient with the highest CO_2 levels (Sections 3 and 4) and will consistently spend the majority of their time in the part of the tank with the lowest CO_2 levels (Sections 1 and 2) and (2) if crayfish are exposed to a CO_2 gradient, then feeding, exploratory, and hiding behavior will be negatively affected. The crayfish will spend more time feeding and exploring and less time hiding, making themselves more vulnerable as the crabs did in the study by Wu et al. (2017).

Materials and Methods

Setup

A 122 cm x 38 cm x 30 cm aquarium was used as the crayfish environment. I measured the aquarium into four even sections, each one being 30.5 cm in length. Permanent marker was used to define the sections, no physical distinctions other than the markings were used to separate the sections. The aquarium was then set up to sustain the freshwater crayfish. Half distilled and half tap water were poured into the tank and de-chlorinated. We added two filters and two bubblers to the tank. Gravel was placed evenly across the bottom of the tank. Many hiding places, broken pot shards, along with three plastic climbing apparatuses, were spaced evenly across the four sections so that the crayfish would not favor a particular section for their hiding places. I marked the carapace of each of the twelve crayfish with a different color of nail polish in order to distinguish them from one another. I measured each crayfish for length and weight. The crayfish were introduced to their environment and allowed to acclimate to their new environment for a few days. During this time, I watched the crayfish in order to acquire a time period (330 minutes) that the crayfish could be monitored that would maximize their activity and behavior. For the CO_2 gradient environment, pH and temperature monitors were set up. Four pH meters along with four temperature probes were implemented one each into each of the four sections of the tank. We placed a bubbler attached to a CO_2 tank in the rightmost section of the tank (Section 4). After six trials, a CO_2 gradient was reached throughout the tank that maintained a slight difference (~0.1 U) in pH between each of the four sections of the tank. The gradient of the tank was kept at ~pH 7.00 in Section 1, ~pH 6.9 in Section 2, ~pH 6.8 in Section 3, and ~pH 6.7 in Section 4. The temperature remained between 24^oC and 25^oC at all times.

Data Collection

Our study was conducted by subjecting the same crayfish to two different environments, control and CO_2 gradient. The control environment was maintained in order to monitor the normal behavior of the crayfish for seven days. The seven days in which data were taken were not all consecutive. I recorded the number of crayfish in each of the four sections of the aquarium every half hour for 330 minutes. I also monitored the location and behavior of each of the crayfish at all times throughout this 330-minute period. Exploratory (E), hiding (H), feeding (F), climbing (C), leg and mouthpart movement (M), fighting (F), and retreating (Re) behavior were all noted for each crayfish for the entire time period. While I fed the crayfish, I recorded the number of crayfish that ate immediately (within one minute), ate after two minutes, did not eat, and showed aggression while feeding. At the end of the 330 minutes each day, the amount of time each crayfish spent in each of the four sections was calculated. After 7 days of data collection in the control environment, the CO_2 gradient was introduced. The pH and temperature monitors continuously detailed the state of the tank. The behavior of the crayfish was documented in the same steps as were taken for the control environment. The CO_2 gradient was only on for the 330-minute time period for each day; it was not maintained overnight. Behavioral data were also collected in the experimental environment for a total of seven days.

Data Analysis

We ran statistics for each of the eight graphs using Microsoft Excel 2013 with the Analysis Tool Pack Add-in. For all ANOVA and t-tests, 0.05 was used as the level of significance, a 95% confidence level. For Figures 1 and 2, standard deviation (+/-) error bars were calculated along with an ANOVA run to identify significance between the four data points. Figure 2 also employed the use of a multiple comparison post hoc test to see where the significance identified in the ANOVA was. The multiple comparison post hoc test was computed using GraphPad Prism Version 6.01. The average fraction of crayfish present in each section used the data recorded for how many crayfish were in each section every half hour for 330 minutes. Each half hour's data for each section was averaged together. Then, the data for all seven days were averaged to get the data points seen. Figure 3 used a regression test and the use of r^2 and significance F values from this test. Exploratory behavior was defined as crayfish movement, mostly in walking around the tank. Figures 4 and 6 used standard deviation bars and a two sample t-test assuming unequal variances. The one-tail value of the t-test was used since a certain outcome was hypothesized for both. For Figure 6, number of section switches represents how many times the crayfish crossed the line into a different section. All seven days' section switch averages were averaged together to get the bar seen. Figures 5, 7, and 8 employed the use of regression tests, two separate ones for the control environment and CO_2 gradient environment for each graph.

Results

Figure 1 shows that no section was preferred by the crayfish when in the control environment. In Figure 2, Sections 1, 2, and 3 were preferred over Section 4 when the crayfish are in the CO_2 gradient environment. The fraction of crayfish that showed exploratory behavior was not affected by the CO_2 levels, although the average number of section switches by the crayfish increased in the CO_2 gradient, as can be seen in Figures 3 and 4. Figures 5 and 6 show that hiding behavior in the crayfish did not acclimatize over time when in the CO_2 gradient, the crayfish had decreased hiding behavior when in the CO_2 gradient. Feeding behavior, shown in Figures 7 and 8, was not affected by the CO_2 gradient.

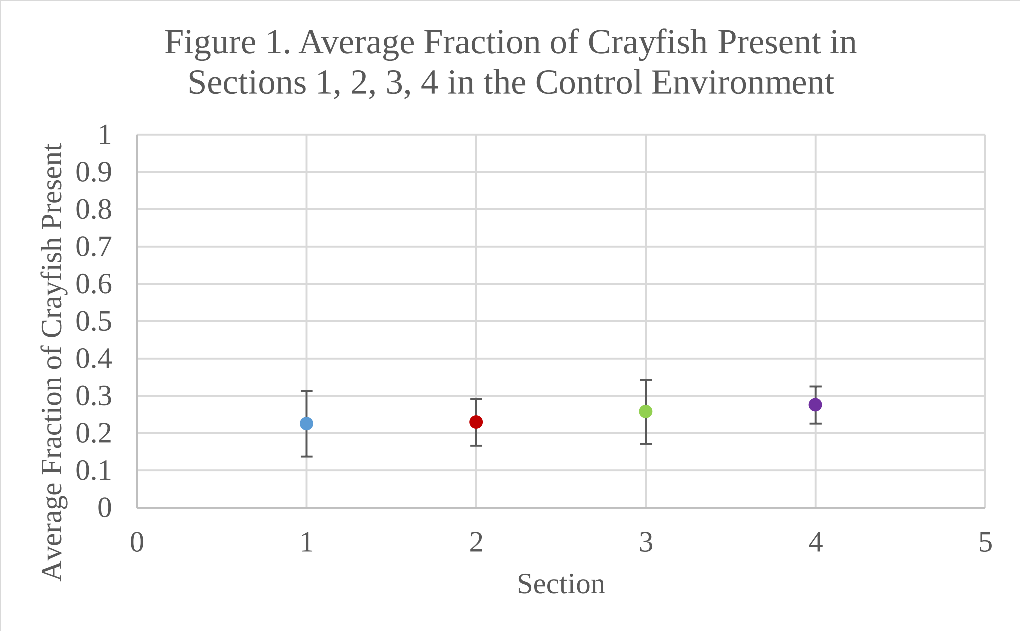

Figure 1. There was no significant difference between the average proportion of crayfish present in each section. No section was favored by the crayfish. The average fraction of crayfish was computed using the data recorded each day of how many crayfish were present in each section every half hour over the 330-minute period. To get the point seen, the data from all seven days were averaged. Standard deviation (+/-) error bars were calculated for each data point. An ANOVA test was run for the data giving a p value of 0.53; Therefore, there was no significant difference between the four points.

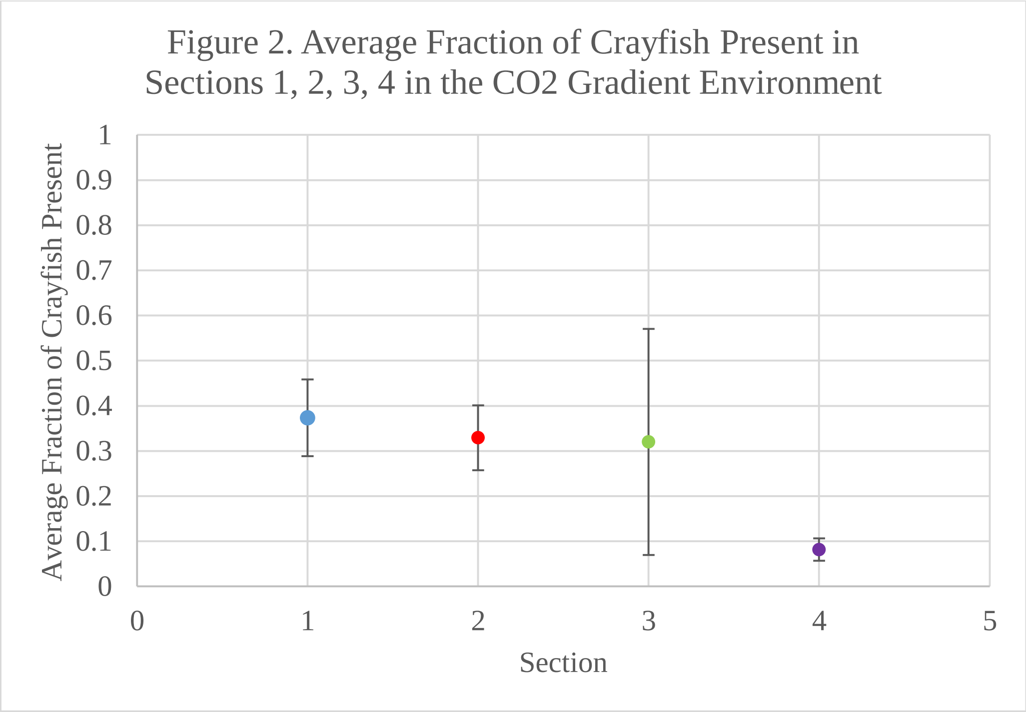

Figure 2. There was a significant difference between the average fraction of crayfish present in Section 4 and the average fraction of crayfish present in Sections 1, 2, and 3. The crayfish avoided Section 4. The average fraction of crayfish was computed using the data recorded each day of how many crayfish were present in each section every half hour over the 330-minute period. To get the point seen, the data from all seven days were averaged. Standard deviation (+/-) error bars were calculated for each data point. An ANOVA test was run and produced the p value of 0.0024, showing that there was a significant difference between at least two of the points. A multiple comparison post hoc test was then done and revealed that the average fraction of crayfish present in Section 4 is significantly lower than the average fractions of crayfish present in Sections 1, 2, and 3. There was no significant difference between Sections 1, 2, and 3.

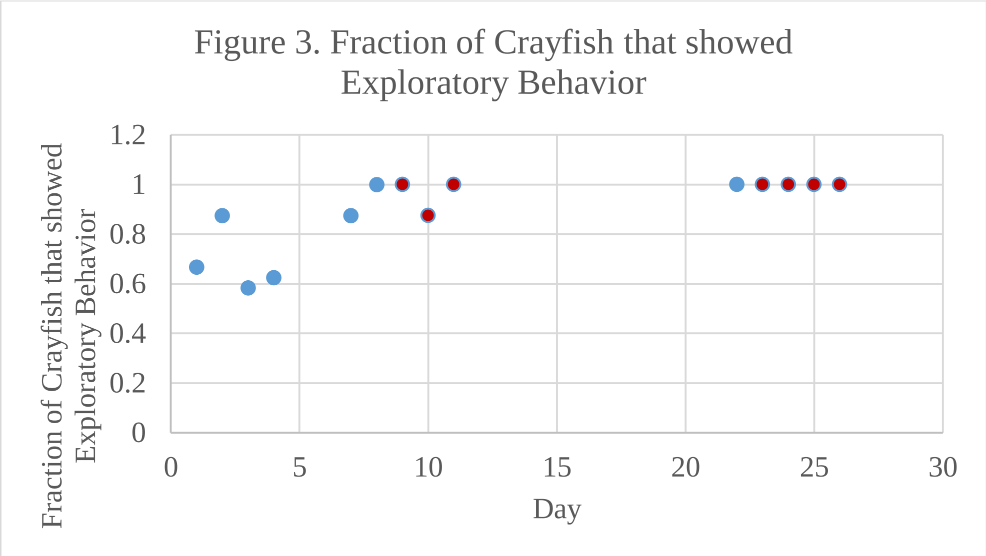

Figure 3. The CO_2 gradient environment did not have an effect on the exploratory behavior of the crayfish. A regression was done which showed an r^2 of 0.43 and a significance F of 0.11 for the control with N=7. For the CO_2 gradient with N=7, the regression gave an r^2 of 0.22 and a significance F of 0.29. This shows that there is not a relation between CO_2 levels and the exploratory behavior. The blue points represent the seven days of the control environment while the red points represent the seven days of the CO_2 gradient environment.

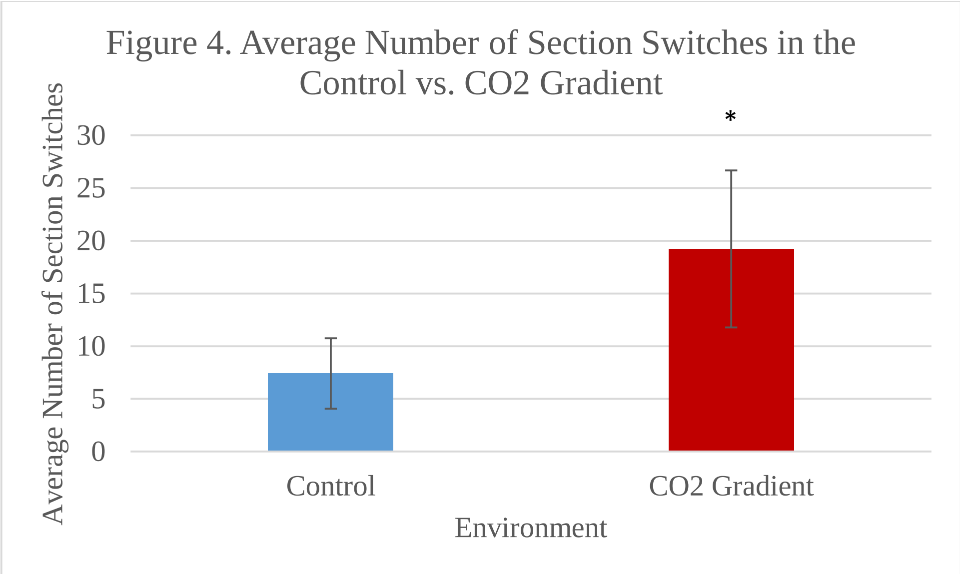

Figure 4. When the crayfish are in the CO_2 gradient, they have a significantly higher number of average section switches. To get the average number of times the crayfish explored into different sections, the number of times each crayfish crossed the line into a different section was recorded and totaled. The totals for all of the crayfish for a particular day were then averaged together, then the days were averaged together. Standard deviation (+/-) error bars were calculated for each bar. A two sample t-test assuming unequal variance yielded a one-tail p value of 0.0047. One-tail was used since we hypothesized that exploratory behaviors would increase in the CO_2 gradient. The same organisms were used for both environments.

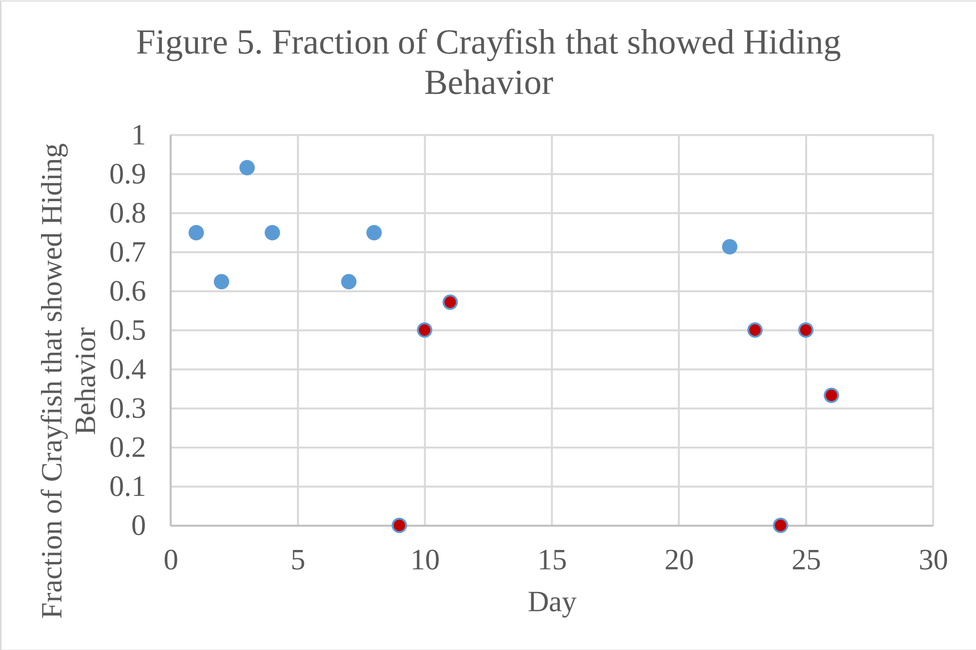

Figure 5. The crayfish hiding behavior in the CO_2 gradient did not acclimate to the CO_2 levels over time. Hiding behavior was affected by the CO_2 gradient. For the control environment with N=7, a regression was run which gave 0.021 for the r^2 value and 0.76 for the significance F. A regression gave an r^2 value of 3.14×10^{-6} and a significance F of 0.99 for the CO_2 gradient with N=7. This shows that when the crayfish were in the CO_2 gradient, their hiding behavior did not return to the control environment levels of hiding behavior. The blue points represent the seven days for the control environment while the red points represent the seven days for the CO_2 gradient environment.

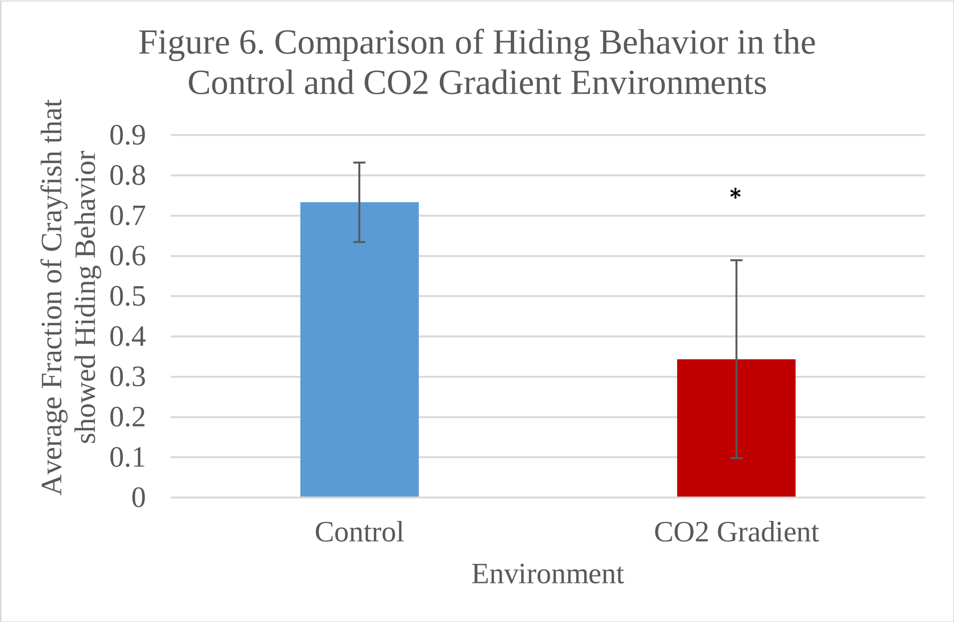

Figure 6. The crayfish in the CO_2 gradient demonstrated significantly less hiding behavior than when in the control environment. A two sample t test assuming unequal variance gave a p value of 0.0023. Since it was hypothesized that the crayfish would spend less time hiding when in the CO_2 gradient, the one-tail value for the t test was used. There was a significant difference between the average fraction of crayfish demonstrating hiding behavior between the control and CO_2 gradient environments since the p value is below 0.05. Standard deviation (+/-) error bars were calculated for each bar.

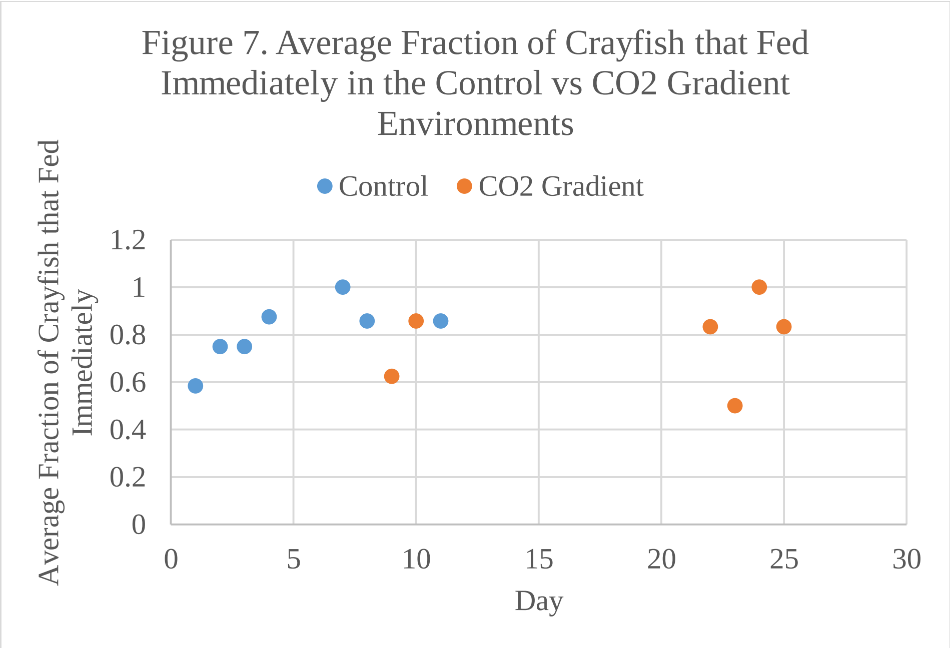

Figure 7. The average fraction of crayfish that fed within the first minute was not affected by the CO_2 gradient. A regression gave an r^2 of 0.039 and a significance F of 0.71 for the CO_2 gradient with N=6. A regression for the control environment with N=7 gave an r^2 of 0.46 and a significance F of 0.092. This shows that neither time nor CO_2 levels were affecting the feeding behavior of the crayfish.

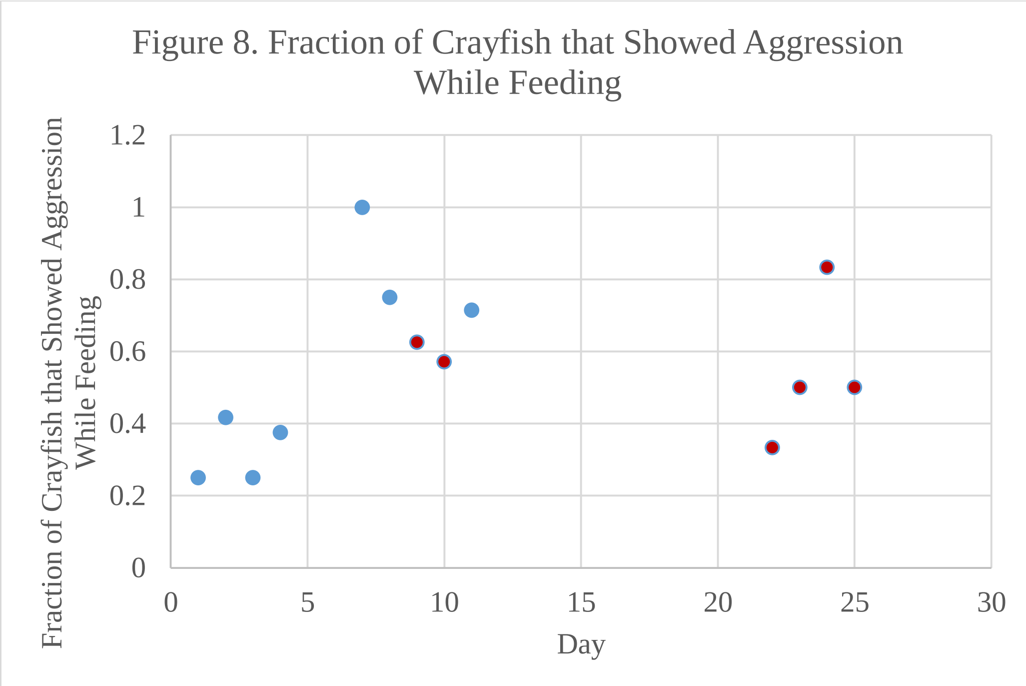

Figure 8. Crayfish aggression while feeding was not significantly affected by the CO_2 gradient environment. When a regression was done for the CO_2 gradient data with N=6, an r^2 value of 0.012 and a significance F of 0.84 were obtained. For the CO_2 gradient, the slope is 0 and since the r^2 is so low, there are likely other factors affecting the aggression of the crayfish while feeding, it is not time or CO_2 affecting their feeding behavior. The regression run for the control environment with N=7 gave an r^2 of 0.61 and a significance F of 0.039. The blue points represent the seven points for the control environment while the red points represent the six days for the CO_2 gradient environment.

Discussion

We noted crayfish behavior in two different environments. I first introduced the crayfish into the control environment with data being recorded for a total of seven days after acclimation of the crayfish to the environment. I then introduced the same crayfish into a CO_2 gradient environment where data were collected for another total of seven days. Previous studies have investigated the effects of high CO_2 levels and decreased pH on crustaceans including crayfish, but there have not been studies involving a CO_2 gradient with crayfish where the crayfish have the ability to choose to move away from the highest CO_2 levels (Bierbower & Cooper 2011, Cameron 1985, McMahon & Stuart 1989, Whiteley 2011, Wu et al. 2017). I watched behavior for the control environment in order to see what normal behavior for the crayfish is like so that it could be compared to the behavior of the crayfish when subjected to the CO_2 gradient. This way, the data would be able to clearly show whether or not the CO_2 gradient had a significant effect on the crayfish behavior or whether the crayfish were able to adapt. With climate change on the rise, research to see whether freshwater crustaceans such as the crayfish are able to adjust to changes in CO_2 levels is vital (Whiteley 2011).

Section Preference

I watched the crayfish over a period of 330 minutes each day. During this time, I recorded the number of crayfish in each section every half hour. Since the number of crayfish dwindled from twelve to six throughout the course of the project, fractions were used so that the data could be compared with more ease. In the control environment, the crayfish did not prefer any particular section out of the four sections over all seven days as can be seen in Figure 1. Since no section was preferred, this made the CO_2 gradient experiment possible. If a section was preferred in the control environment, then preference of the crayfish to a particular section in the CO_2 gradient would not be able to be identified to be caused by higher CO_2 levels. Figure 2 shows the section preference of the crayfish in the CO_2 gradient environment. The crayfish in the CO_2 gradient preferred Sections 1, 2, and 3 over Section 4. The crayfish avoided the section with the highest CO_2 levels, the lowest pH. This could be due to the fact that pH plays such a significant role in biological response systems (Cameron 1985, Whiteley 2011, Wu et al. 2017). The results only partially support the hypothesis (1) that the crayfish will avoid Sections 3 and 4 and consistently spend more time in Sections 1 and 2. Unexpectedly, there is no difference in crayfish preference for Sections 1 and 2 and Section 3 even though the pH of Section 3 is very close to Section 4's pH. Yet, the crayfish do avoid Section 4, the section with the lowest pH levels, which supports the hypothesis (1). The crayfish were repelled from the section with the highest CO_2 levels.

Observed Behaviors

When I was still determining the CO_2 gradient levels, Section 4's pH was lower than wanted. The crayfish demonstrated some interesting behaviors. The three crayfish being most exposed to the low pH levels flipped over on their backs. Two of the crayfish after a lengthy struggle were able to flip themselves over while one eventually stopped fighting and went into an unresponsive state as was displayed in the study by Bierbower & Cooper (2010). I placed the unresponsive crayfish in Section 1, the section with the lowest CO_2 levels, and after 40 minutes the crayfish recovered and became much more aggressive than previously. Increased aggression by this particular crayfish was noted for the entirety of the rest of the study. By 120 minutes on Day 1 of the CO_2 gradient, six of the eight crayfish were present in Section 1 while two were present in Section 2. The crayfish chose to be in closer proximity to one another rather than be in the higher CO_2 levels in Sections 3 and 4. At 150 minutes, all eight of the crayfish were crowded into Section 1. By 330 minutes, two crayfish were in Section 1, four in Section 2, and one each in Sections 3 and 4. Although the crayfish were still showing preference for Sections 1 and 2, by the end of the day they seemed more willing to venture back into the other sections.

Exploratory and hiding behavior were both recorded for the control and CO_2 gradient environments. Figures 3 and 4 depict different measures of the exploratory behavior of a crayfish. Exploratory behavior was characterized as movement of the crayfish mostly in the form of the crayfish walking around the tank. During the 330-minute period each day, I noted any exploratory behavior. Figure 3 shows the fraction of crayfish that showed exploratory behavior over all of the days of the study, including control and CO_2 gradient. Exploratory behavior increases in the beginning then seems to even out. The regression computed shows that CO_2 levels are not affecting the exploratory behavior of the crayfish. The increase in exploratory behavior at the beginning could possibly be due to the crayfish still getting used to being in the environment or getting used to the observer, but it is known not to be caused by the CO_2 levels. This data does not support the hypothesis (2) that the crayfish will be negatively affected by the CO_2 gradient. On the other hand, Figure 4, a different way of quantifying exploratory behavior in the crayfish, does support this hypothesis (2). Figure 4 shows that the crayfish in the CO_2 gradient have a significantly higher average of section switches than when in the control environment. The crayfish could also have been more willing to spend more time exploring different sections once there were less crayfish in the aquarium, the population having decreased from twelve to six throughout the study.

Figure 5 shows that the hiding behavior of the crayfish does not acclimatize over time, as is supported by the regression test. The CO_2 levels do have an effect on hiding behavior. Figure 6 demonstrates that the average fraction of crayfish that showed hiding behavior is significantly less in the CO_2 gradient than in the control environment. The crayfish are leaving themselves to be more vulnerable by not hiding and protecting themselves. The decreased amount of time spent hiding by the crayfish mimics the results of the study by Wu et al. (2017) in Japanese stone crabs. This supports the hypothesis (2) that the crayfish would be negatively affected by the CO_2 gradient, exhibit less hiding behavior. This vulnerability of the crayfish in the CO_2 gradient could be concerning for the future. However, as can be seen in Figures 8 and 9, the feeding behavior of the crayfish was not affected by the CO_2 gradient. Neither the fraction of crayfish that fed within one minute nor the fraction of crayfish that showed aggression while feeding was affected by the CO_2 levels as supported by the results of the regressions run on each. These results do not support the hypothesis (2) that feeding behavior would be negatively affected by the CO_2 gradient environment. Since feeding behavior was not affected, this shows that these crayfish, even in higher CO_2 levels, should be able to find food quickly and fight for it properly despite the changes in climate.

Conclusion

The outcome that the crayfish did not prefer any particular section in the control environment was anticipated. For the CO_2 gradient environment, the crayfish preferred Sections 1, 2, and 3 over Section 4, which only partially supports the hypothesis (1). The result that Section 3 was not avoided went against the hypothesis (1), but the crayfish did avoid Section 4, the section with the highest CO_2 levels. Feeding and overall exploratory behavior were not affected by the CO_2 gradient. Hiding behavior and the average number of times the crayfish switched sections, another measure of exploratory behavior, were affected by the CO_2 gradient. These results only partially support the hypothesis (2) that feeding, exploratory, and hiding behavior would be negatively affected by the CO_2 gradient environment. Overall, rising CO_2 levels have the potential to negatively affect freshwater organisms such as the crayfish, but crayfish also have the potential to adapt to these alterations brought about by climate change, especially if the change takes place over a significantly longer period of time. Although, the crayfish do thrive in and prefer normal CO_2 and pH levels. Future experiments could focus on other species that are a part of the freshwater ecosystems that crayfish play a role in, including their food sources.

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