EFFECTS OF LOW pH WATER ON EUROPEAN GREEN CRABS *(Carcinus maenas):* AN ASSESSMENT OF SURVIVAL AND POST-ECDYSIS CUTICLE PROPERTIES

Honors Thesis

Presented in Partial Fulfillment of the Requirements
For the Degree of Bachelor of Science in Biology

In the College of Arts and Sciences
at Salem State University

By

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Commonwealth Honors Program
Salem State University
2018

ABSTRACT

Excess of carbon emissions drives not only global warming but also ocean acidification (OA). In addition to that, it has been established that OA negatively affects a variety of organisms that depend on calcium carbonate to build their shells. However, the effects of low pH water on crustaceans is not well understood. These marine organisms do not rely exclusively on calcium carbonate to build their carapaces, which are also composed of a wide range of organic and inorganic compounds. In this study, the effects of low pH water on the carapaces of European green crabs *(Carcinus maenas)* as well as on their survival were assessed. Twenty-two adult males between approximately 50mm and 60mm in length were included. Twelve were exposed to an initial decrease in pH from 8.0 to 7.8 over 50 days and then to a pH of 7.8 for 106 days, while the other ten were kept at a pH of 8.0 for the duration of the study (156 days). The thickness of the endocuticle and exocuticle was determined, and their quality assessed. Although the thickness appears to have been unaffected by the experimental conditions, the quality of the outer cuticle may have been compromised. In particular, structures such as tubercles and bristles were highly impacted. Survival analysis also suggests that drops in pH might lead to an increase in death that is not necessarily related to the process of ecdysis, a period in which the crabs are highly vulnerable. Negative impacts of OA on crustaceans could shed new insights on how this environmental problem indeed can impact not only calcifiers but possibly all marine life. A better understanding of the consequences of OA might lead to new initiatives on how to reduce carbon emissions.

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AKNOWLEDGEMENTS

I would like to thank Dr. Mark Fregeau for his unconditional support and endless feedback. Dr. Fregeau was the very first professor I had in freshman year when everything was new to me.

To Christopher Pelligra, because without his support none of this would have been possible.

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1. Background and Rationale

Atmospheric levels of carbon dioxide are steadily increasing due to human activities such as deforestation and burning fossil fuels. Studies show that the current levels of carbon dioxide are the highest levels within the last 66 million years (Zeebe at al. 2016). As carbon dioxide builds up in the atmosphere, there is a corresponding heating effect which causes an increase in the temperature of the Earth. Unfortunately, it is not only air temperature that is being affected by elevated amounts of carbon dioxide. Dissolved carbon dioxide can also affect sea water chemistry (Lauvset & Gruber, 2014). A few decades ago, scientists believed that the environment itself could take care of excess carbon dioxide through simple diffusion; therefore, decreasing the number of free molecules in the atmosphere and preventing global warming. However, the chemical reactions that happen in the oceans once the carbon dioxide is dissolved can be problematic to marine life which ultimately affects all living organisms including humans.

The ocean is home to a wide variety of animals, some of which heavily rely on optimal water chemistry. Calcifiers are animals that depend on aragonite and calcite to build their shells. Both of these compounds involve different forms of calcium carbonate which is depleted when carbon dioxide is dissolved. Carbon dioxide (CO_2) reacts with water (H_2O) to form carbonic acid (H_2CO_3), see equation below. Acids release a proton as they dissociate in water forming two different ions. In this case, carbonic acid dissociates into bicarbonate (HCO_3^-) and hydrogen ions (H^+).

$$H_2O + CO_2 \rightarrow H_2CO_3 \rightarrow HCO_3^- + H^+$$

The pH indirectly measures the concentration of hydrogen ions in the water as shown in the following definition:

$$pH \equiv -log\,[H^+]$$

The higher the concentration of hydrogen ions, the lower the pH is and the more acidic the solution. Hydrogen ions (H^+) strongly prefer to bind with carbonate (CO_3^{2-}), reducing the amount available for calcifiers to use to build their shells. First, calcium carbonate ($CaCO_3$) present in ocean water hydrolyzes into two ions:

$$CaCO_3 \xrightarrow{H2O} Ca^{2+} + CO_3^{2-}$$

Then, the free hydrogen ions released by the presence of carbon dioxide bind to the carbonate ion to form bicarbonate.

$$CO_3^{2-} + H^+ \rightarrow HCO_3^-$$

The overall reaction is shown below, where the carbon dioxide being dissolved into ocean water reacts with the carbonate present in the water column to form bicarbonate.

$$CO_2 + H_2O + CO_3^{2-} \rightarrow 2HCO_3^-$$

Calcifiers are not able to utilize bicarbonate in building their shells. Also, the lack of carbonate enables the excess of hydrogen ions to start taking up the carbonate present in the shells of the calcifiers. This issue leads to the dissolution of the animals' protective shells in acidic water. That is the reason why true calcifiers such bivalves and gastropods experience a significant negative effect when exposed to Ocean Acidification (OA) (Kroeker et al., 2010; Kroeker et al., 2013). However, many organisms may use other mechanisms, such as protein complexes, in order to build their exoskeletons. The carapace of decapods consists of a cuticle composed of 2 layers and a membranous internal layer (Travis, 1963). Both of these cuticle layers are made up of bundles of chitin

nanofilaments (Giraud-Guille, 1984). Amorphous calcium carbonate and calcite are forms of carbonate present within the chitin matrix (Dillaman et al., 2005; Roer & Dillaman, 1984). The decapod exoskeleton is made up of various components, as a result, the effects of Ocean Acidification is less evident. However, the depletion of carbonate forms caused by Ocean Acidification might have a direct impact on the formation of the new carapace after ecdysis. The functions of organic complexes such as chitin can also be drastically affected by conditions of low pH. The effect of acidic water on these organisms is, however, not as well-known as it is in true calcifiers.

If carbon dioxide levels keep raising at the current rate, the average pH of the ocean's surface is predicted to drop by 3 units in a period of less than 500 years (Paquay & Zeebe, 2013). The relationship between carbon emissions and ocean acidification has been well established. In pre-industrial times, the average pH of the ocean's surface was approximately 8.2 and it has been decreasing since then, reaching 8.1 today. The drop in pH is attributed to human-driven carbon emissions of which approximately 40% has dissolved in the ocean (Zeebe, et al., 2008). Remaining carbon emissions have led to global warming and the rising temperatures of the surface of the earth (Zeebe, et al., 2016).

It is fundamental to understand the negative impacts of excess atmospheric carbon dioxide and the consequences that it can bring to the overall health of the ecosystem which in turn always affects humans. A lack of shellfish could drastically impact many fish species by disrupting the marine food chains, leading to depressed fisheries and adversely affecting an important protein source in human diets. Shellfish populations including clams and oysters along with crustaceans such as crabs and lobsters which

constitute a major portion of seafood consumed throughout the world, would be threatened. In addition to altering marine food webs, there would be significant economic consequences with reduced shellfish populations. The decline in shellfish and finfish would also hurt other aspects of the fishing industry, processing and distribution companies would be depressed as these organisms become scarcer with time. A decline in shellfish would adversely impact seafood availability for consumers, employment opportunities for fishermen and the balance of life in the oceans. Previous studies have also looked at the effect of low pH water on fish. These studies have found that a disruption in the chemistry of the water induces great stress in the fish with irreversible behavioral and physical impairments such as foraging and swimming (Rodriguez-Dominguez, et al., 2018; Pimentel, et al., 2016). Fish failing to reach maturity, reduce the reproductive potential of the population, reducing species densities and therefore resulting in decreased fish availability for consumers.

The objective of this experiment was to investigate how the European green crab *(Carcinus maenas)* molting process responds to acidic conditions. I hypothesized that when green crabs are exposed to low-pH conditions, they would not be able to molt properly or experience higher mortality than those exposed to normal pH levels. This could be an important step in assessing how disruptive ocean acidification could be for the overall health of the marine organisms and chemical processes of the oceans.

2. Materials and Methods

2.1. Collection and Conditions Prior to Experimental Exposure

The animals studied in this experiment were European green crabs *(Carcinus maenas)* collected from Smith Pool, a saltwater pool connected to Salem Harbor,

Massachusetts and located at the Salem State University's Cat Cove Marine Laboratory on the northeast coast of the United States. The crabs selected for the study were chosen based on gender and size; specifically, males with an average carapace length between approximately 50mm and 60mm. The length, width, and rostral diagonal were measured in each crab (see Appendix A). The crabs were randomly divided into two groups: control (N=10) and experimental (N=12). The experimental group had more crabs to account for an anticipated increase in death due to the pH change. Prior to the experimental trial, all crabs were maintained in the same tank in large plastic breeder boxes. Each box had a divider in the middle allowing two crabs to be kept in the same container. The boxes were suspended in a 340-gallon round recirculating seawater tank in the marine laboratory. The tanks are maintained by a constant flow of filtered, ambient seawater; therefore, the water that the crabs were exposed to in the experiment matched the seawater they were taken from, except for the experimental condition described below. The crabs were kept in this tank for 16 days before the experimental group was taken out to be placed in the tank with the experimental condition. The crabs were fed sinking flounder pellets once a week from capture until the end of the experiment.

2.2. Experimental Exposure and pH Regulation

The pH was modified using an Apex Junior pH controller in conjunction with a laboratory grade Neptune Systems pH probe. The carbon dioxide controller was an ISTA controller (vertical type) that was attached to the bottle and connected to the Apex. The carbon dioxide controller would be turned on as needed by the Apex based on the readings of the probe. Once on, carbon dioxide would flow out of the bottle regulated by the controller passing through a bubble counters and then to the Aqua Medic carbon

dioxide reactor 1000. In this reactor, water pumped out of the tank by a Marineland ML90512 Maxi-Jet 1200 PRO, would be mixed with the incoming carbon dioxide using a countercurrent mechanism to ensure maximum carbon dioxide dilution (see Appendix B).

After 16 days, the experimental group was transferred to a sister tank where the pH conditions were altered. The initial pH was 8.1 and the target pH was 7.8. The target was achieved over a period of 50 days. The reason of the gradual change was to avoid high risk of mortality due to a rapid change in the acidity of the water which was also why juvenile crabs were not selected. This more accurately reflects conditions in the ocean where pH changes happen gradually over time. The crabs were exposed to the experimental condition for a total of 156 days (50 days before reaching the target pH and 106 days at pH of 7.8). If the crabs died without molting, their carapaces were not included in the SEM imaging analysis (see Section 2.4.). The exuviae was not taken into consideration. Only the carapace of crabs that underwent ecdysis were of interest. Structural analysis of these carapaces was a key aspect of the study. The specimens that were still alive at the end of the experiment were euthanized according to scientific protocol. If the crabs had gone through ecdysis, the carapace was removed and kept. Carapaces of crabs that did not molt during the study were discarded.

2.3. Survival Analysis

Crab survival times were analyzed in three different ways by using the Kaplan-Meier method. All statistical analyses were performed using IBM SPSS Statistics v. 24.0 (IBM Corp., Armonk, NY, USA). First, the survival of the experimental and control groups was compared without taking into account whether or not the crabs had molted. This was

important in order to assess the risk of death under low pH conditions without taking into account their vulnerability during and after ecdysis. Second, the survival time was compared between the crabs that underwent ecdysis and the ones that did not. This was done in order to determine if molting poses an increased risk of dying regardless of exposure to low pH conditions. Finally, for those who molted, the post-ecdysis survival was also compared between the experimental and control groups to examine if exposure to low pH levels influenced mortality of crabs after ecdysis. This analysis also has the potential to explain if low pH makes them even more vulnerable to die after ecdysis.

2.4. SEM Imaging and Elemental Analysis

Crab exoskeletons, specifically part of the carapace, was examined using SEM imaging analysis to reveal any potential changes in microstructure related to changes in pH levels (see Appendix C). These samples were cleaned with ethanol 200 proof prior to being placed in the sonicator. After the cleaning procedure, they were prepared for scanning electron microscope (SEM) imaging. The thickness was assessed using electron micrographs to determine whether or not the pH has an effect on it. A cross section of the sample was observed in order to obtain measurements along the same axis. The measurements were taken at magnification 200, spot size 39, acceleration voltage 4, working distance 20, and using FIJI software to measure the thickness of the endocuticle and exocuticle. Three measurements per sample were taken, averaged, and the results compared between the experimental and control groups by using a t-test, treating both layers of the cuticle independently (see Appendix D for an electron micrograph of a cross section where both layers, endocuticle and exocuticle, can be observed).

The health of the carapace was also qualitatively assessed for the inner side of the endocuticle and externally for the outer cuticle. The images were taken using spot size 39, acceleration voltage 10, and working distance 35 and magnification ranged between 50 and 200. The inner surface, tubercles and bristle where qualitatively assessed and classified in three categories, no damage, partial damage, and severe damage (see Appendix E for a representative image of the 3 structures in all categories of damage). For the inner side, the surface itself was taken into consideration. The inner surface of the endocuticle lacks structure and it appears flat and smooth in undamaged specimens. For the outer layer, the health of tubercles and bristles was assessed. Tubercles and bristles are structures unique to certain species of crabs and follow different patterns on different species and might be fundamental when determining the health of a specimen (Greco, 2014). These structures were specifically studied to assess the health of the outer layer. Once the samples were categorized, a t-test was performed to compare the differences in both groups regarding the health of the carapace.

3. Results

3.1. Baseline Analysis

A t-test was performed to compare the length, width, and rostral diagonal distances between the control and experimental groups. This was done to ensure the crabs in the two groups were comparable in size at the beginning of the study, baseline conditions, thus reducing potential bias when making comparisons. The p-values were 0.724, 0.476, and 0.835 for each of the comparisons, respectively, demonstrating that the length, width, and rostral diagonal are statistically not significantly different between the two groups, as expected due to the randomization process.

3.2. Survival Analysis

Figure 1 shows the survival curves for crabs in the control and experimental groups for the entire length of the experiment (156 days). The control and experimental groups had 10 and 12 specimens, respectively. The tick marks on all the survival curves below signify censored subjects which had not died at the end of the study.

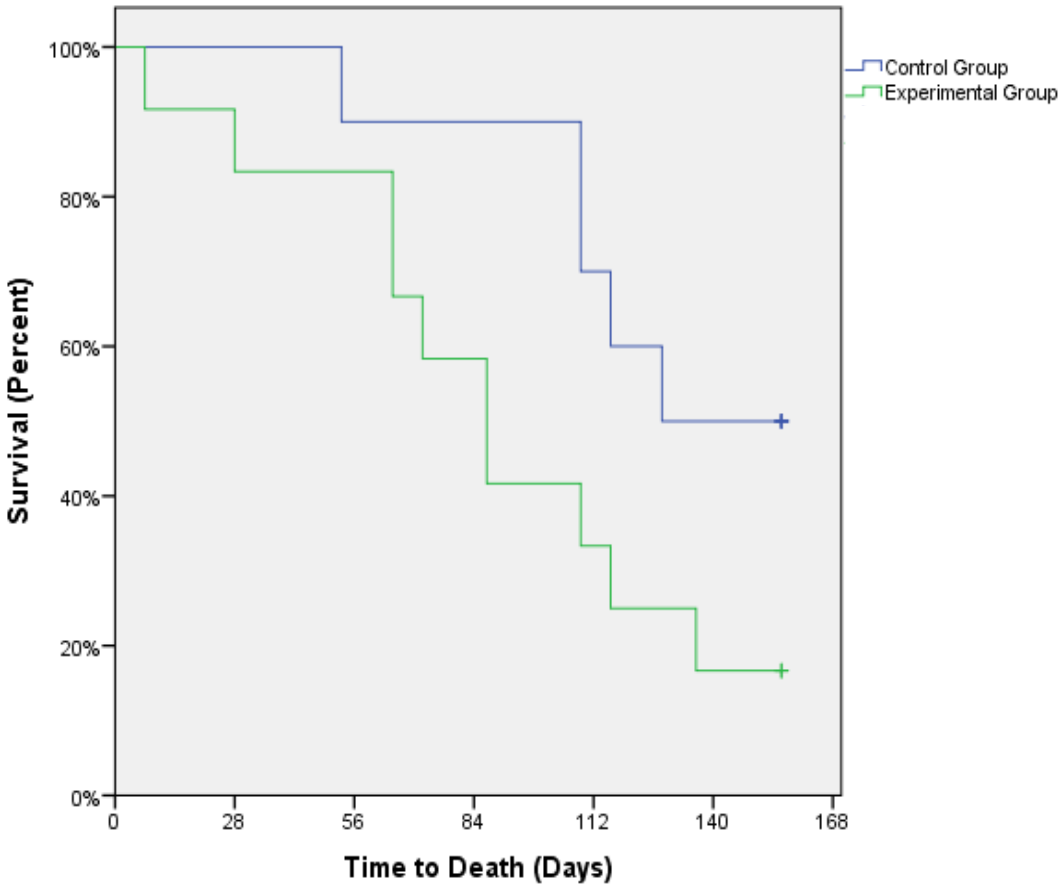

Figure 1: Survival curve of control and experimental groups over time

The group exposed to the experimental conditions showed a shorter survival time compared to the control. The survival distributions were significantly different with p-values of 0.055, 0.048, and 0.049 according to the log-rank, Breslow, and Tarone-Ware tests, respectively. Regardless of having experienced ecdysis or not, the crabs exposed to the experimental conditions seemed to have been adversely affected by low pH water.

The median survival time for the control and experimental groups were 128 and 87 days, respectively. 50% of crabs in the control group survived the entire study vs 16.7% in the experimental group.

Table 1 shows a summary of the crabs that experienced ecdysis and the ones that did not in the control and experimental groups.

Table 1: Summary of ecdysis events in control and experimental groups

	Control	**Experimental**
Ecdysis	6 (60%)	3 (25%)
No Ecdysis	4 (40%)	9 (75%)
Total	10	12

Figure 2 shows the survival between crabs that went through ecdysis (N=9) and the ones that did not (N=13) without making a distinction between control and experimental groups. Survival time distributions were similar between ecdysis groups (crabs that went through ecdysis in the control and the experimental groups), with a p-value of 0.997 according to the log-rank test. The survival distribution of these two groups showed no statistically significant difference; therefore, the molting process might not be a contributing factor to mortality. Median survival times were 116 days and 109 days for the crabs that experienced ecdysis and the ones that did not, respectively. 38.5% of the crabs died pre-molt vs 22.2% of the ones that died post-molt.

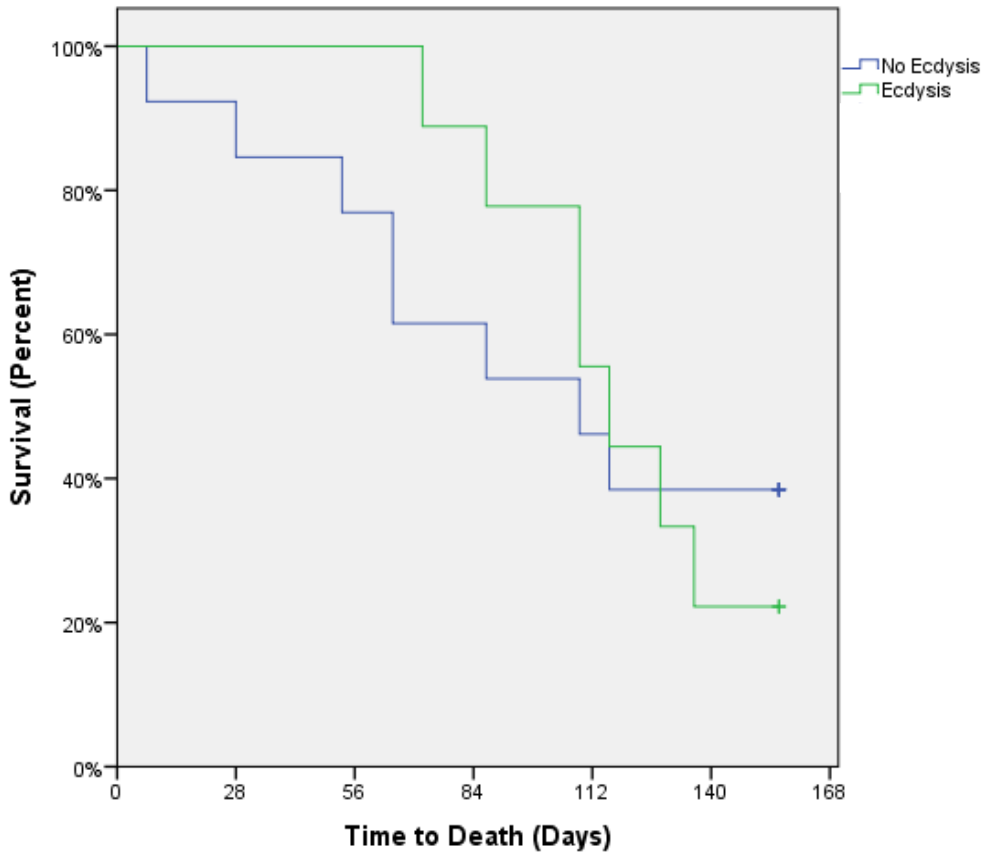

Figure 2: Survival of crabs that underwent ecdysis vs the ones that did not over time

Figure 3 shows post ecdysis survival involving crabs that died only after having gone through ecdysis (N=9). It compares the survival from the time of ecdysis between the control (N=6) and experimental (N=3) groups. Time zero corresponds to the first molting event. One crab that belonged to the control group died while molting, hence the drop at time zero in the control curve.

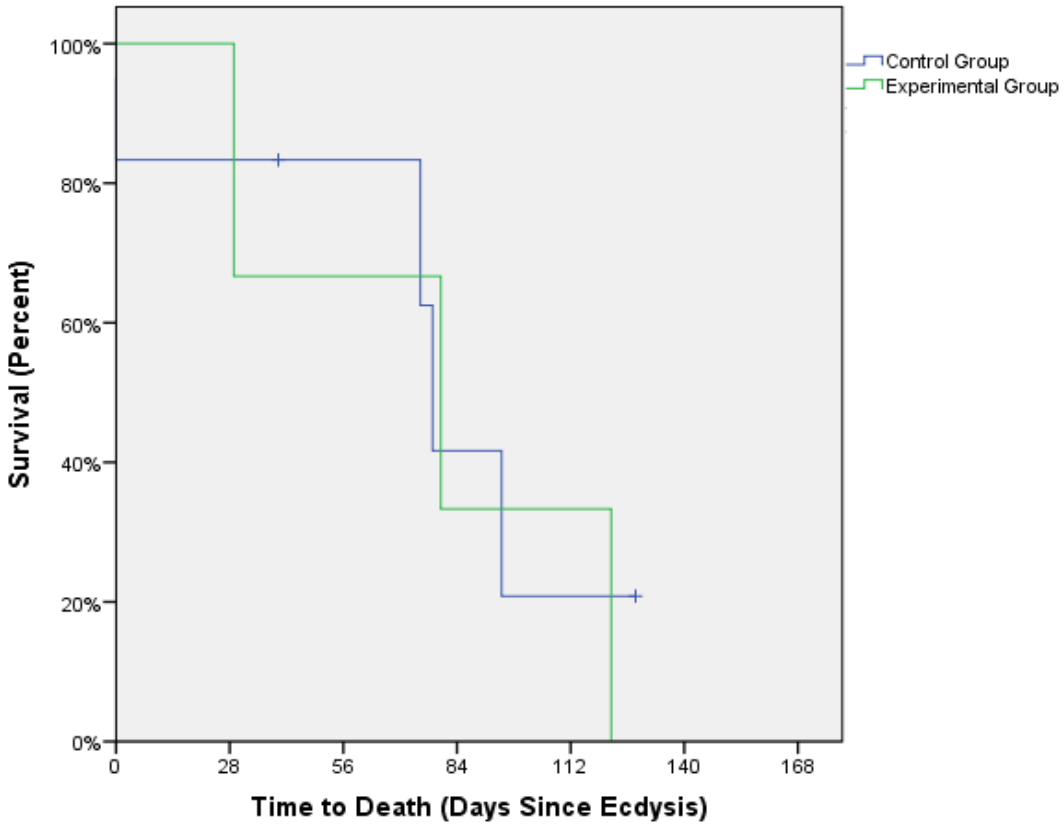

Figure 3: Survival of control vs experimental groups after ecdysis

Comparing the survival distributions between the control and experimental groups, a p-value of 0.271 was obtained using the log-rank test. Thus, these distributions were not statistically significantly different, which means that low pH water might not have a direct impact on the time of survival after the first molting event. Median survival times for the control and experimental groups were 78 days and 80 days, respectively. 20.8% of the crabs in the control group remained alive vs the experimental group in which none survived post-ecdysis.

3.3. Thickness Analysis

A t-test was also performed to analyze the difference in the carapace thickness of the crabs that survived in both groups after molting. The thickness of the endocuticle and

exocuticle between control and experimental groups were not significantly different at the 0.05 significance level (p-values of 0.994 and 0.324, respectively). Seawater pH seemed to have no effect on the mineralization of the endocuticle nor did it have an impact on the formation of the less mineralized exocuticle. This conclusion can only be made regarding the first molting event.

3.4. Carapace Quality Analysis

The quality of the inner side of the endocuticle as well as the outer side of the exocuticle were classified in three categories qualitatively, no damage, partial damage, and severe damage. **Figure 4** shows the number of crabs with each level of damage. In the control group, 60%, 40%, and 0% were classified as having no damage, partial damage, and severe damage, respectively, vs 33% in each category in the experimental group. The quality of the inner layer of the endocuticle was compared between the two groups using the likelihood ratio chi-square test, which resulted in a p-value of 0.322. Thus, the difference in damage was not statistically significant at a 0.05 level.

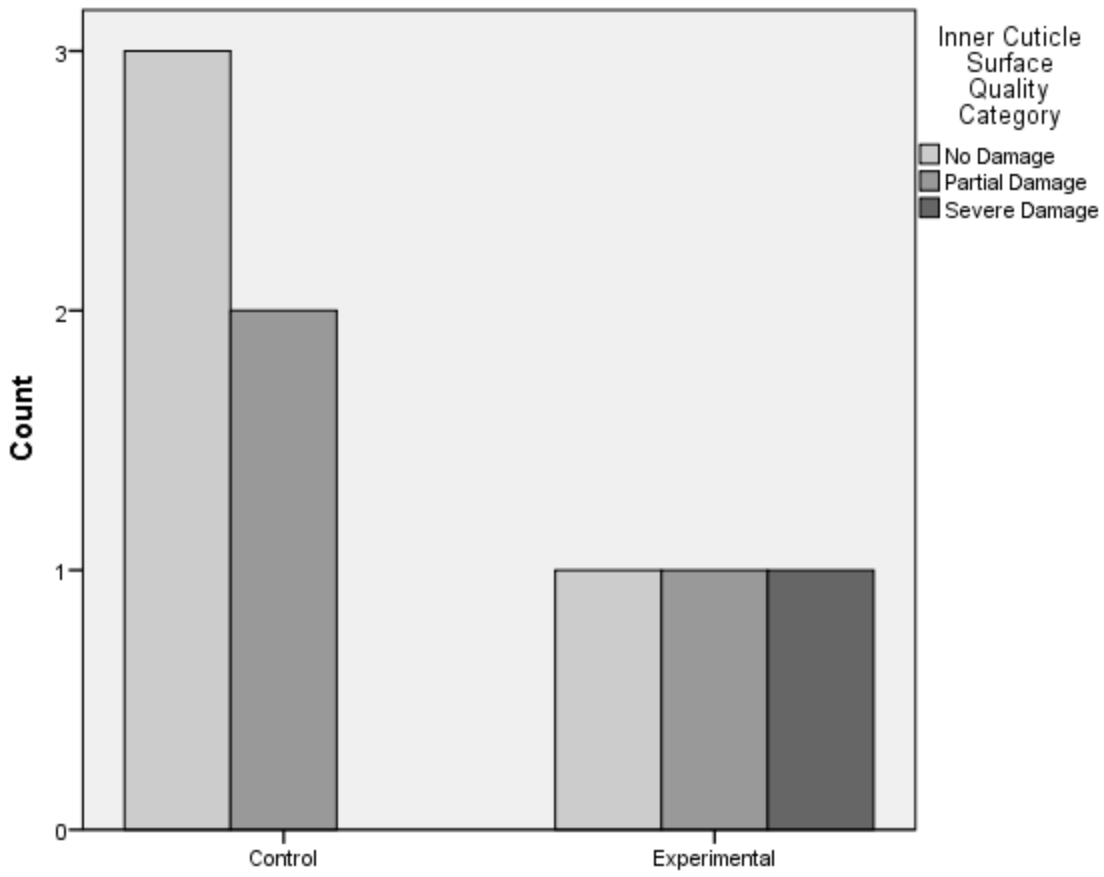

Figure 4: Endocuticle inner surface quality of control vs experimental groups

The quality of the bristles and tubercles on the outer side of the exocuticle was also assessed. **Figure 5** shows the number of crabs with each level of damage to the bristles. All specimens in the control group remained undamaged, while 33% and 66% in the experimental group were partially and severely damaged, respectively. The likelihood ratio chi-square test comparing the two groups resulted in a p-value of 0.018, demonstrating statistical significance at the 0.05 level.

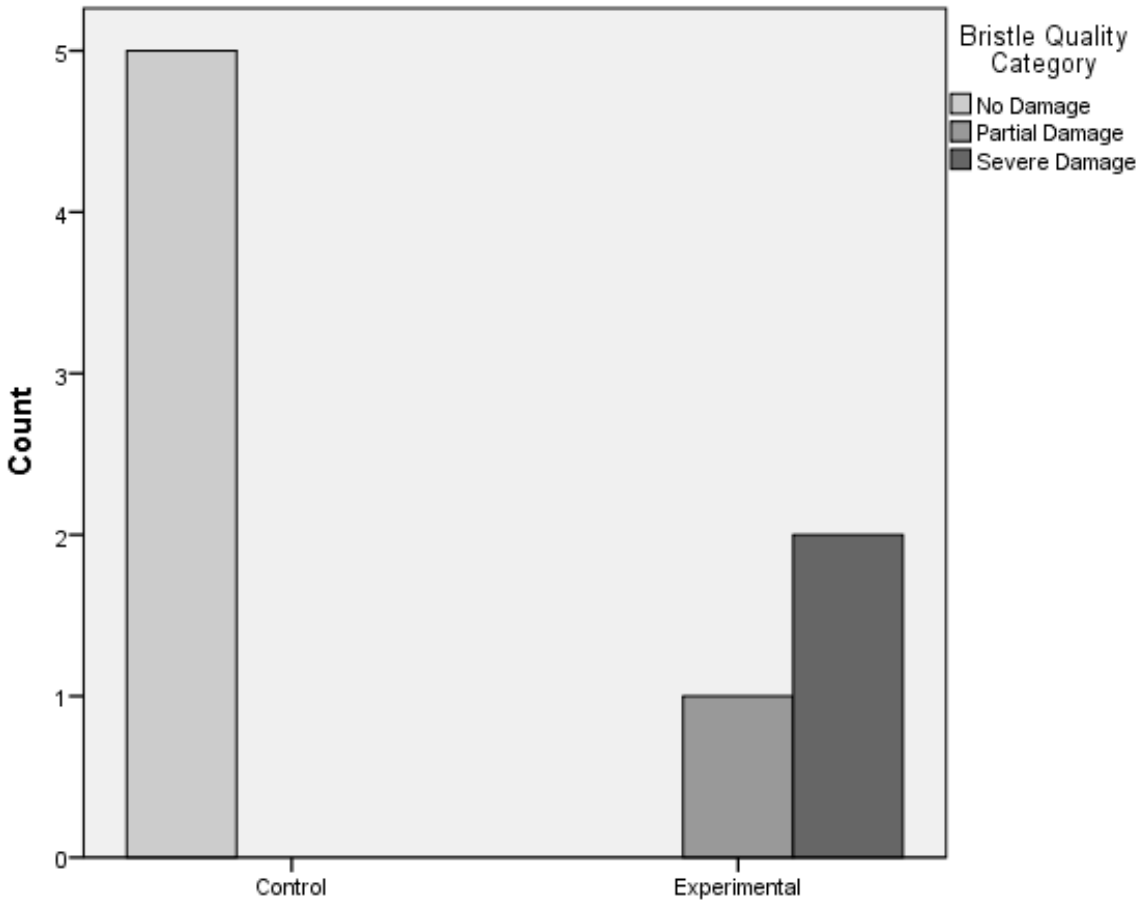

Figure 5: Bristle quality of control vs experimental groups

Figure 6 shows the number of crabs with each level of damage to the tubercles. All crabs in the control group were undamaged, while crabs were spread evenly across the three categories in the experimental group (33% in each). The likelihood ratio chi-square test gave a p-value of 0.075, lacking statistical significance at the 0.05 level. Taking these results together, the pH of seawater might indeed have an impact on the microstructure of the outer layer of the exocuticle.

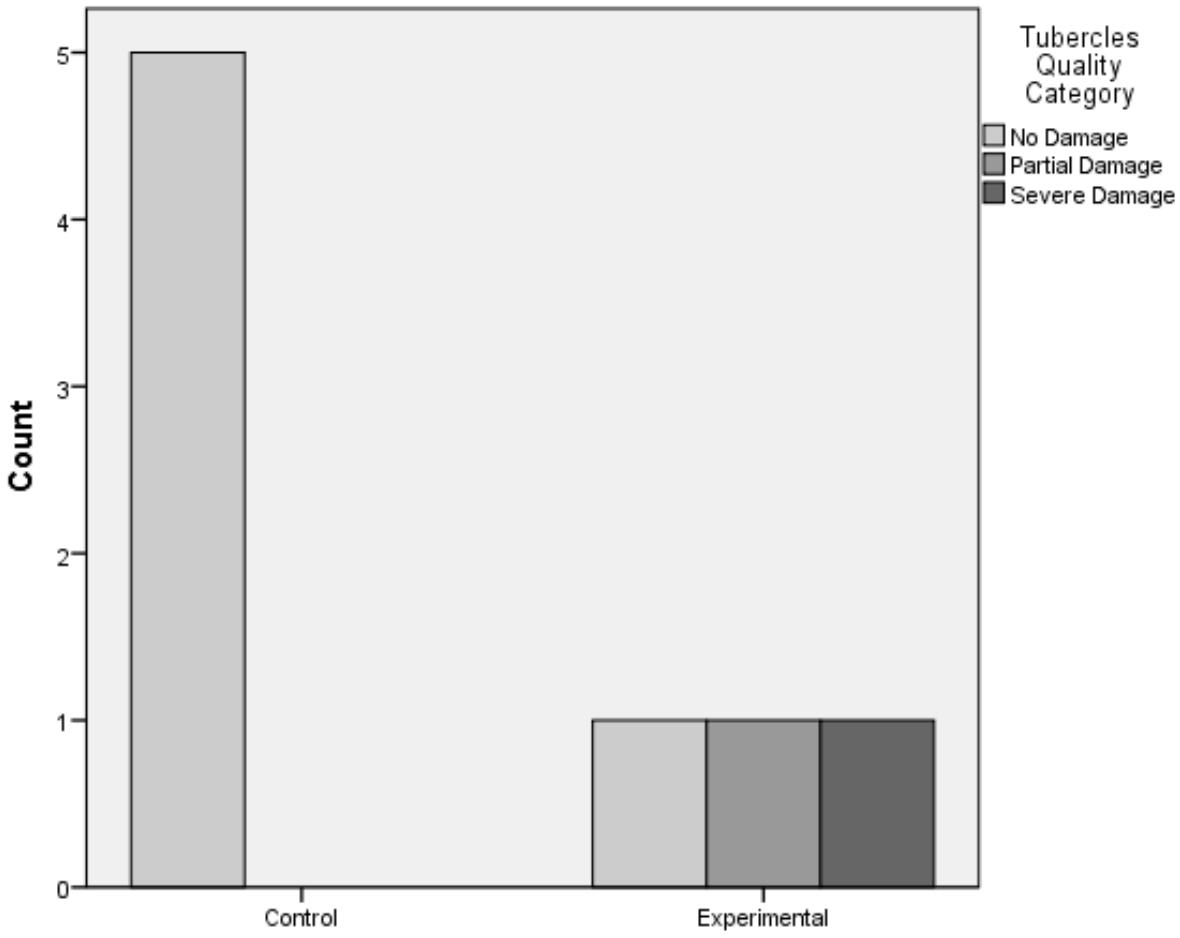

Figure 6: Tubercle quality of control vs experimental groups

5. Discussion

The effect of low pH on crustaceans varies widely as opposed to the adverse effects seen in true calcifiers. Previous studies have shown that low pH water can have a negative impact on growth and survival in certain species whereas others might be unaffected (Whiteley, 2011; Sokolova et al., 2016). However, based on the results of the current study, European Green crabs seem to be vulnerable at a pH of 7.8. Although these

crabs are believed to be a very hardy species due to their long invasive status in the North East of the United States, they tend to face hardship when living under these conditions.

Ecdysis is a period of high vulnerability where the crab molts its old exoskeleton and makes a new one. The new carapace usually takes a couple of days to harden and be used as a protection armor. Based on the results obtained, having gone through ecdysis does not make the crabs more vulnerable to dying than those that did not experience ecdysis. In this part of the experiment, the low pH conditions were not taken into account; only the survival between molting crabs was assessed.

The survival after ecdysis was also analyzed. This process does partially depend on the readiness and availability of carbonate in the water. It is then expected that decapods would have hardship synthesizing the new carapace under low pH conditions since carbonate ions are being depleted from the water column. Interestingly, the survival of European Green crabs after ecdysis seems to be unaffected by low pH conditions. It is possible that the sample size was not big enough to detect any difference in the survival of both of these groups. Therefore, future studies should consider including more specimens when analyzing the effects of low pH on the decapod carapace. There is a significant correlation between the experimental conditions and the low survival rate; therefore, it is necessary to increase the number of individuals subject to the experimental conditions to ensure a bigger sample size of live specimens after ecdysis.

Other studies have also looked at the relationship between OA and thickness of the both layers of the cuticle. Although the thickness of the carapace and chela in juvenile red king crabs was unaffected by pH of 7.8 and 7.5, there is a slight significant reduction in exocuticle thickness in blue king crabs (Coffey et al., 2017). The thickness of the chela in

Carcinus maenas also remain intact under OA (Landes & Zimmer, 2012). In other decapods, the carapace remained also unaffected at pH of 7.53 (Taylor et al., 2015). The results of other studies support the idea about different species having different reactions under acidic conditions. The current study shows that the thickness of neither the endocuticle nor the exocuticle was affected by the experimental conditions.

Most studies have looked at the overall composition of the carapace including calcium, magnesium, and various components. However, few to no experiments have analyzed specific physical structures such tubercles and bristles which are characteristic of *Carcinus maenas* (Travis, 1963). In the current study, these structures together with the surface of the inner cuticle have been analyzed using Scanning Electron Imaging. Because of the constraints of the sample size any minute detail either strongly deviates or supports the hypothesis. Nevertheless, the current study shows that the damages of the inner surface of the endocuticle and the bristles were not significant to attribute them to the experimental conditions. Even though the bristles did not show significant different at 0.05 level, it is believed that with a bigger sample size, it could be actually relevant. The damage of the tubercles was indeed significant with all of the specimens in the control groups showed no damage whereas the experiment showed partial and severe damage.

6. Conclusions

The effects of OA in true calcifiers has been widely studied and they seem to be equally harmful in this type of organisms. However, the effects in decapods vary within species. Other studies have shown the thickness of the carapace remains unaffected by OA, but there seems to be a strong correlation between low pH conditions and low survival rate in *Carcinus maenas*. Although the surface quality of the endocuticle and the

bristles were not significantly different between both groups, there were apparent damages on the outer bristles. However, there is a strong possibility that increasing the sample size would give stronger evidence to support the results reported in this study. Assessing the effects of low pH water on decapods is an important step in understanding the potential damages to all marine life. Currently, OA together with global warming are the human-driven problems that threaten ocean life the most. Therefore, it is imperative to shed light on possible consequences to marine ecosystems in order to come up with counteracting solutions.

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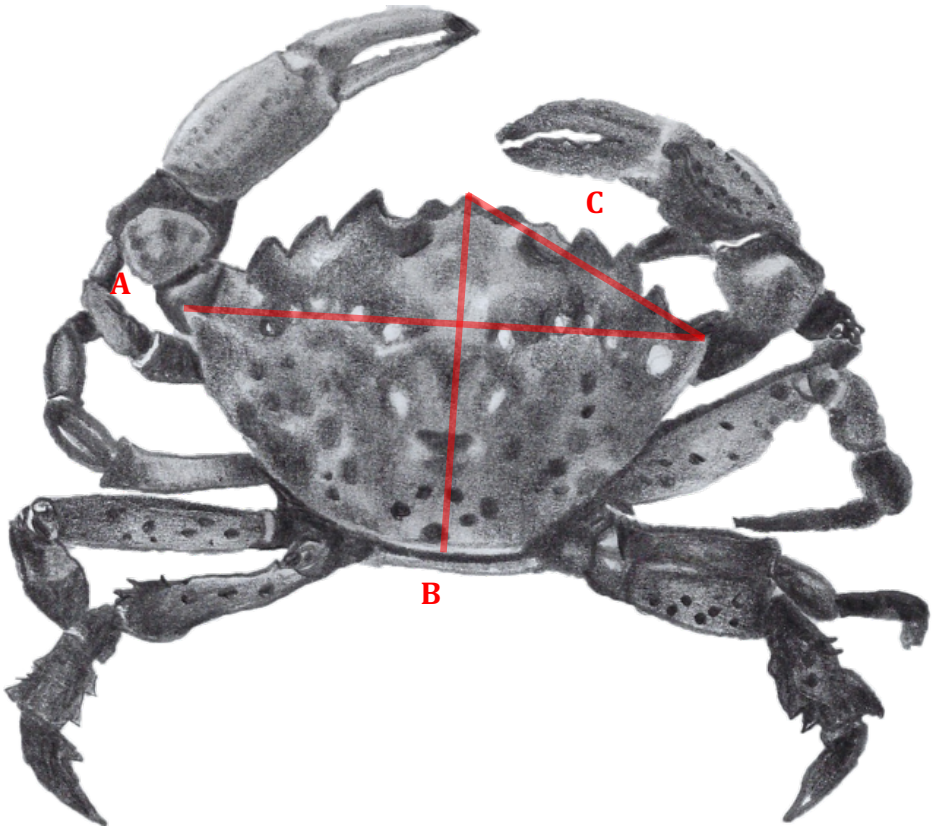
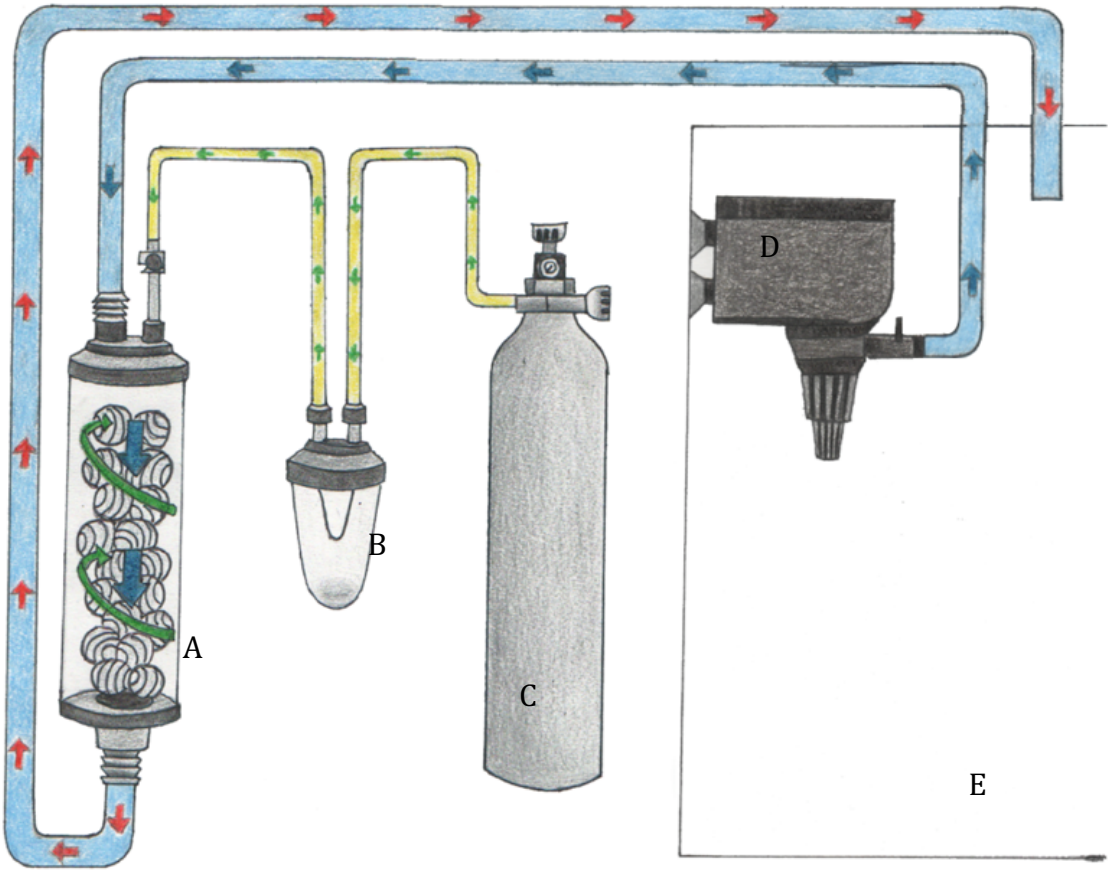
APPENDICES

Appendix A: Carapace Measurements



Appendix A: *measurements of the crabs A: length, B: width, C: rostral diagonal*

Appendix B: CO_2 Injection System

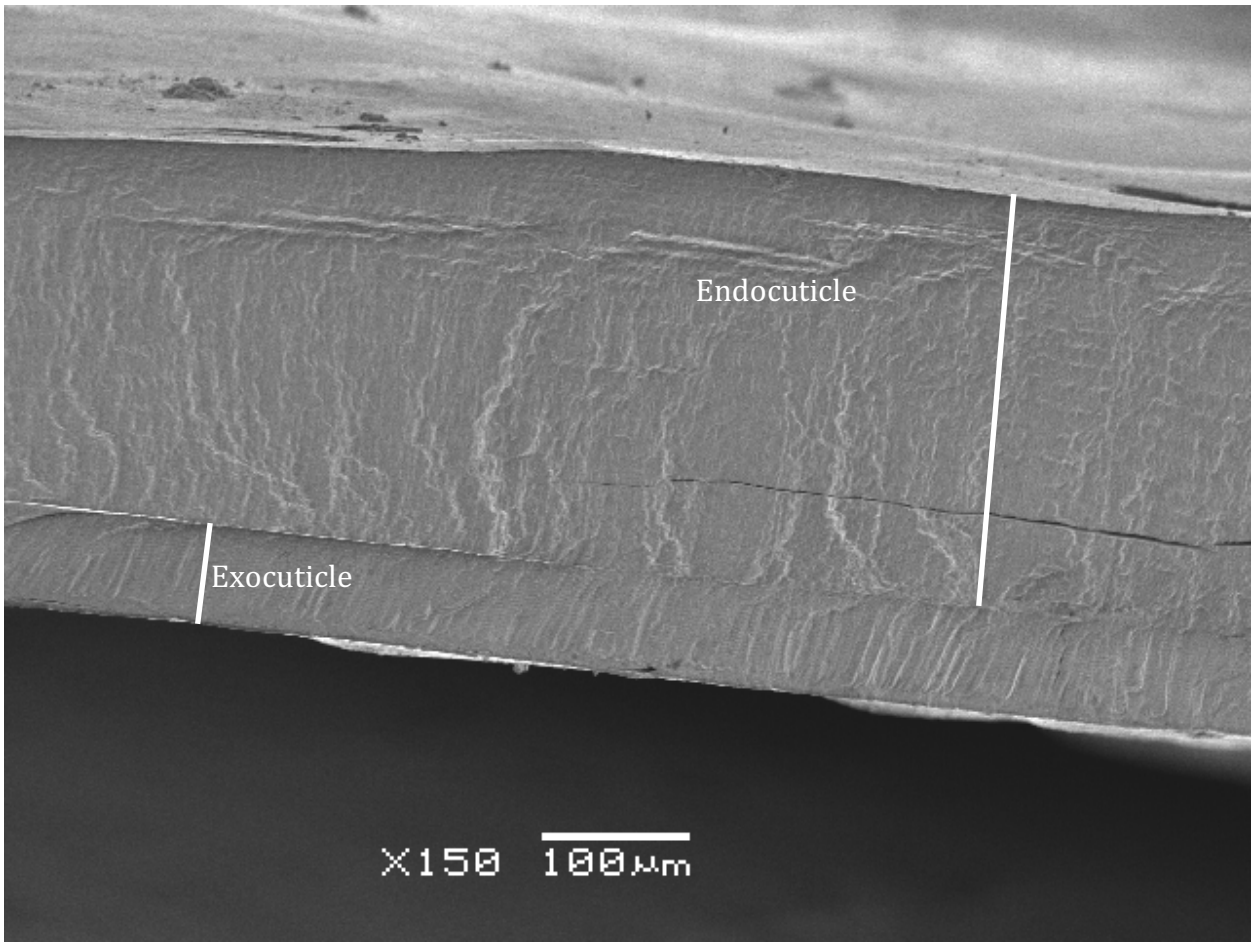


Appendix B: Carbon dioxide diffusion system. A: carbon dioxide reactor B: bubble counter C: carbon dioxide bottle D: water pump E: tank

Appendix C: Carapace Sections for SEM Imaging

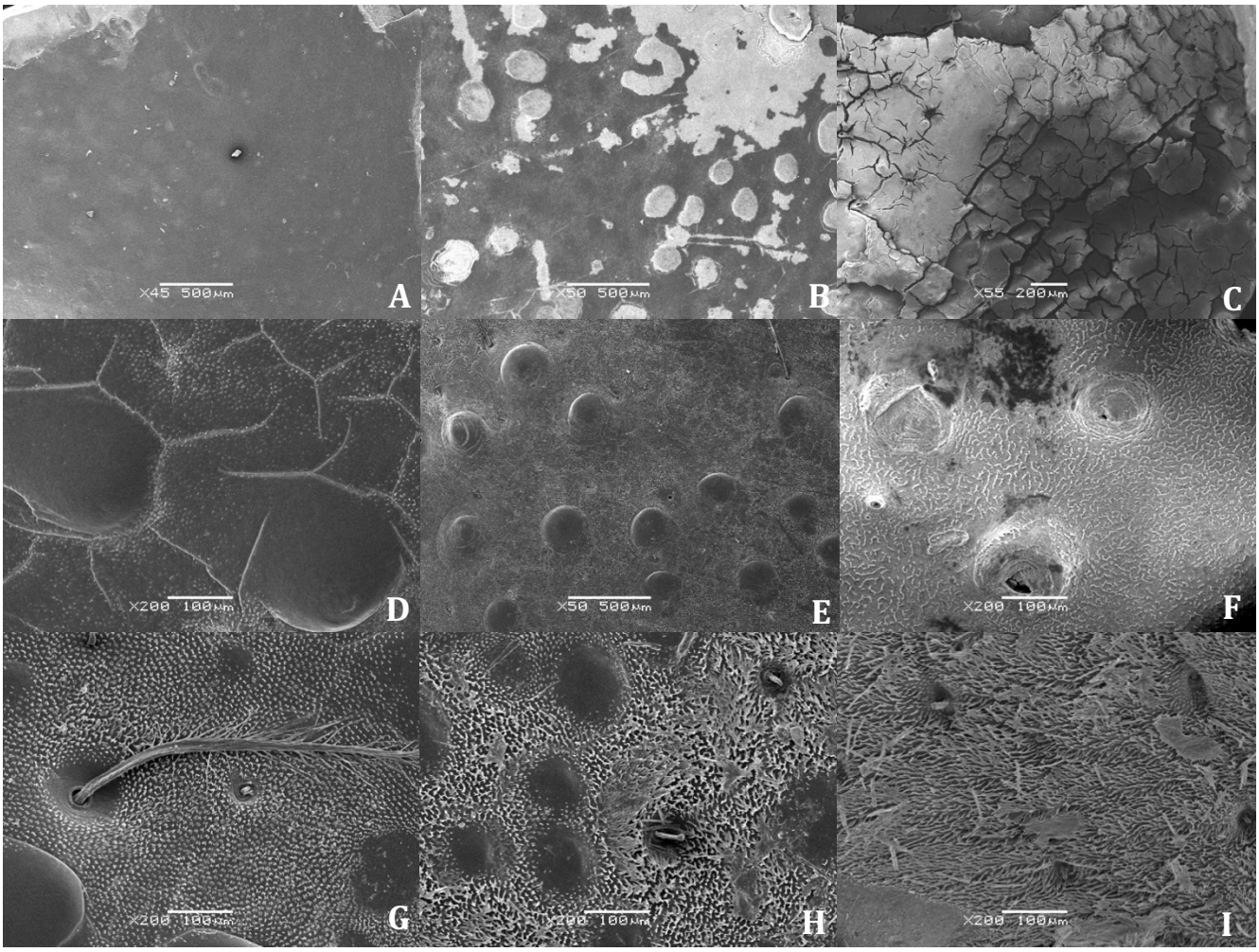
Appendix C: Specific area extracted in red for SEM imaging

Appendix D: Carapace Cross Section and Thickness



Appendix D: Electron micrograph of a carapace cross section showing the mineralized endocuticle and the exocuticle

Appendix E: SEM Imaging and Carapace Quality



Appendix E: Representatives of categories of damage. A: normal inner endocuticle B: partial damage present in inner endocuticle C: severe damage present in inner endocuticle D: normal tubercles E: partial damage in tubercles F: severe damage in tubercles G: normal bristles H: partial damage in bristles I: severe damage in bristles