The effect of animal size on the carbonic anhydrase activity of the sea anemone *Anthopleura elegantissima*

by
Allison P. Tep

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Public Health
(Honors Scholar)

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AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____

Virginia Weis

The sea anemone *Anthopleura elegantissima* is a model organism for the study of temperate symbiosis. *Anthopleura elegantissima* can engage in symbiosis with two different algal symbionts: the dinoflagellate *Breviolum muscatinei* and the chlorophyte *Elliptochloris marina*. One host enzyme that has been shown to be important in cnidarian-algal symbioses is carbonic anhydrase (CA). Carbonic anhydrase catalyzes the interconversion of bicarbonate and carbon dioxide and assists in the transportation of inorganic carbon from the host cnidarian to the photosynthetic algae. Factors such as temperature, light intensity, latitude, and size influence the productivity of symbiotic cnidarians. In this study, I hypothesized that *A. elegantissima* with small column crown diameters would have lower CA activity than larger animals. Anemones with a smaller column crown diameter have increased surface area to allow for the diffusion of materials, such as carbon dioxide, to occur and present a decreased need for CA activity. To test this, I measured and compared the CA activity of anemones of varying column crown diameters and developed a method to measure the surface area to volume ratio of *A. elegantissima*. Linear regression of carbonic anhydrase analysis showed that CA activity was negatively correlated with column crown. Additionally, the smooth regression of CA activity as a function of column crown diameter followed a bell-shaped curve, where the peak of the curve was at a crown diameter of 20 mm. These results suggest an optimal energetic size of *A. elegantissima* and that column crown diameter of *A. elegantissima* negatively influences rates of CA activity. This study furthers the knowledge of the principal enzyme CA and provides an exploration of the effects of varying physical characteristics of *A. elegantissima* on CA activity.

Key Words: *Anthopleura elegantissima*, carbonic anhydrase, column crown diameter, surface area, volume

Corresponding e-mail address: allisontep@gmail.com

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<u>Honors Baccalaureate of Science in Public Health</u> project of Allison P. Tep presented on June 4, 2019.

APPROVED:

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Virginia Weis, Mentor, representing Department of Integrative Biology

.   _____

Nathan Kirk, Committee Member, representing Integrative Biology

.   _____

Jason Presnell, Committee Member, representing Integrative Biology

.   _____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes the release of my project to any reader upon request.

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Allison P. Tep, Author

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1.   INTRODUCTION

1.1   Symbiosis

Symbioses between different organisms significantly impact ecological processes and increase the growth and diversity of ecosystems worldwide (Wolters et. al., 2000). Many marine cnidarians, such as the sea anemone *Anthopleura elegantissima*, form symbioses in temperate environments (Weis & Reynolds, 1999). *Anthopleura elegantissima* is found as a conspicuous member of the intertidal zone ecosystem on the west coast of North America from southeast Alaska to Baja California, Mexico (Secord & Augustine, 2000). This anemone forms symbioses with two distantly related algal symbionts: the brown unicellular dinoflagellate *Breviolum muscatinei* and the green unicellular chlorophyte *Elliptochloris marina*. These symbionts live in gastrodermal cells within host derived vacuoles called symbiosomes and provide photosynthetically-derived sugars to the anemone in return for inorganic carbon (C_i) (Trench, 1971). These anemones can exist without symbionts (aposymbiotic), with one symbiotic algae, or in rare instances, with both symbiotic algae. My study focused on *A. elegantissima* engaging in symbiosis with the brown unicellular dinoflagellate *B. muscatinei*, which was formerly known as *Symbiodinium muscatinei* (LaJeunesse et al., 2018). These symbiotic states are driven by ecological factors such as irradiance, temperature, and latitude (Muscatine 1971; O'Brien & Wyttenbach 1980).Animals containing *B. muscatinei* are more abundant in areas of bright light and warm temperatures; accordingly, animals harboring this dinoflagellate are commonly found at lower latitudes (Secord & Augustine, 2000).

1.2   *The Enzyme Carbonic Anhydrase*

At the current seawater pH of 8.20, most C_i is in the form of bicarbonate (HCO_3^-). However, the enzyme responsible for carbon fixation during photosynthesis, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), requires CO_2. The abundance of HCO_3^- presents an issue to the photosynthetic productivity of the anemone symbionts, which provide photosynthetically-derived sugars to the anemone in exchange for inorganic carbon in the form of CO_2. This issue is further remedied by the activity of an enzyme called carbonic anhydrase (CA). Carbonic anhydrase catalyzes the interconversion of HCO_3^- and CO_2 and assists in the transportation of C_i to the site of photosynthesis, which is important for the success of cnidarian-algal symbioses (Lucas & Berry, 1985). Additionally, work by Weis, Smith, and Muscatine (1989) found that the activity of CA was significantly greater in symbiotic anemones than in aposymbiotic anemones. Furthermore, the inhibition of CA activity lead to a significant decrease in the photosynthetic productivity of the algal symbionts (Weis, Smith, & Muscatine, 1989).

1.3   *Surface Area to Volume Ratio*

When cells grow larger, the internal volume increases at a greater rate than the expanding surface membrane. Likewise, when organisms grow larger, the surface area available for diffusion of materials per unit volume decreases (Blamire, 2001). In fact, when the volume is too large in relation to surface area, diffusion cannot support physiological processes (Beals, 2000). The effects of this surface area to volume (SA:V) ratio lead to further questions regarding the relationship between morphology and body processes, such as the effect of SA:V ratio on the photosynthetic productivity of cnidarians or the feeding habits of cnidarians.

Weis, Smith, and Muscatine (1989) studied the relationship of SA:V in symbiotic cnidarians of varying morphologies, including the anemone *Condylactis gigantea* and the coral *Stylophora pistillata*. They found that cnidarians with high SA:V ratios had low CA activity compared to the extremely high carbonic anhydrase (CA) activity measured in cnidarians with low SA:V ratios. The proposed explanation was that animals with high SA:V ratios have shorter diffusion distances and surfaces more suitable for diffusion relative to their total volume. Additionally, it is likely that an increased surface area allows for more CO_2 to diffuse across cell membranes, ultimately leading to an increase in the level of CO_2 needed for photosynthesis by the symbionts. Because of the increase in CO_2 levels, there is a decreased need for CA within the host, as a decreased need for the interconversion of CO_2 and HCO_3^- is presented (Weis, Smith, & Muscatine, 1989).

1.4   *Computed Tomography Scanners*

Computed tomography (CT) scanning is computerized x-ray imaging in which a narrow beam of x-rays is aimed and rotated around an object (National Institute of Biomedical Imaging and Bioengineering, n.d.). The x-rays produce signals that are processed by the CT scanner and mathematical techniques are used to create two-dimensional (2D) cross-sectional images of the body. Once an adequate number of 2D cross-sectional images are generated, they are digitally "stacked" together to create a three-dimensional (3D) image. The 3D image provides simple identification and location of basic anatomical structures and allows for surface area and volume to be measured. Within the field of x-ray imaging, denser structures are imaged easily, while soft tissues are not scanned and produced with as much clarity (National Institute of Biomedical Imaging and Bioengineering, n.d.).

A study by Chen (2011) aimed to compare traditional anatomy visualization with 3D reconstruction in clinical studies. Traditional anatomy visualization is completed by performing a dissection on a cadaver and recording measurements and observations during the dissection. Chen (2011) found that the 3D reconstruction could successfully visualize the bones, vessels, skin, and muscles of the lower leg of a human body. This reconstruction also allowed for the virtual dissection and data acquisition of different anatomical layers (Chen et al., 2011). The use of CT scanning in the field of marine biology is currently being used to further explore animal anatomy and physiology. For marine invertebrates, CT technology can provide useful information regarding surface area, volume, and internal anatomical structures. A recent study successfully CT scanned and visualized fixed shrimp muscle morphology and their circulatory system. Analysis and processing of these images allowed for body surface area to be calculated (Bagge et. al., 2017).

1.5   Purpose and Predictions

This paper presents an examination of the effects of animal size, as measured by column crown diameter, on the carbonic anhydrase (CA) activity of *A. elegantissima*. I predicted to find that *A. elegantissima* with small crown diameters would have lower CA activity than larger anemones. Anemones that have a small crown diameter have a corresponding higher SA:V ratio and therefore a greater capacity for diffusion and thus potentially a decreased need for CA. Additionally, anemones were observed with two assumptions: 1. anemones with small column crown diameters have high surface area to volume (SA:V) ratios, and 2. all anemones that were sampled had a similar height, which resulted in comparable SA:V ratios across anemone samples. In addition, I aimed to develop a method for measuring the relationship between column crown diameter and SA:V ratio.

2.   MATERIALS AND METHODS

2.1   Anemone Collection and Preparation

Anthopleura elegantissima were collected under ODFW permit number 22015 from Seal Rock, Oregon (44.4972ºN, 124.0827ºW) in summer 2018 for CA activity analysis. A total of 62 anemones were collected from two different colonies; 31 anemones were collected from each of two colonies. The anemones were collected and organized into groups based on their column crown diameter measurement: twenty-one sea anemones ranging from 1.0 to 15.0 mm (small), 21 sea anemones ranging from 15.1 to 30.0 mm (medium), and 20 sea anemones ranging from 30.1 to 45.0 mm (large) were collected. Column crown diameters were measured in the field using calipers. Anemones were removed from the field using a microspatula, labeled, and then frozen immediately with liquid nitrogen for later CA analysis. Anemones were transported in liquid nitrogen to Oregon State University and stored at -80ºC.

2.2   Carbonic Anhydrase Activity Assay

Carbonic anhydrase (CA) activity analysis was performed as in Weis and Reynolds (1999) with 62 samples of varying size. Briefly, anemones were each homogenized for at least 1 minute over ice in a Teflon-glass tissue grinder in extraction buffer to extract the protein found within the host anemone. The extraction buffer was composed of 25 mM veronal buffer, 5 mM ethylenediaminetetraacetate (EDTA), 5 mM dithiothreitol (DTT), and 10 mM $MgSO_4$, adjusted to pH 8.2. Extraction buffer was added in increments to the tissue grinder until 7 ml of extraction buffer had been added in total. The homogenate was centrifuged at 4ºC for 10 minutes at 4,300 RCF. Following the centrifugation, 3 ml of the host anemone supernatant were kept as a native

sample and another 3 ml were subsequently boiled for 5 minutes (denatured sample). The last 1 ml was kept for future protein analysis in a 1.5 ml micro-centrifuge tube.

The CA activity was determined by measuring the proton production, and subsequent pH decrease, of the anemone homogenate by adding CO_2 saturated DI water. The CO_2 saturated DI water was made by bubbling CO_2 gas into 300 ml of DI water until the pH was less than 4.0. For each assay, 1 ml of protein extract and 1 ml of veronal buffer were initially stirred and mixed together. The veronal buffer was composed of 5 mM Barbital and 145 mM NaCl, adjusted to pH 8.2. This step was followed by the addition of 1 ml of CO_2 saturated DI water. The decrease in pH of a constantly stirred assay mixture was measured by a YSI pH probe connected to a Hach pH meter. Three replicates of both native and denatured samples were completed. Figure 1 shows the predicted trends in pH levels of both native and denatured anemone samples during a CA assay.

2.3   Bradford Protein Assay

Bradford protein analysis was performed as in Bradford (1976) using a BioRad Bradford Assay Kit and following the manufacturer's instructions. The anemone protein extracts were diluted in DI water to a 1:20 protein mixture. Solutions of 0.125, 0.25, 0.50, 0.75, 1.0, 1.50, and 2.0 mg/ml protein standards were made following the manufacturers' instructions. The Bradford assay was performed in a 96-well clear bottom microplate. Five µl of each standard solution and five µl of each protein extract were added in triplicate to the well plate. Two hundred and fifty ml of dye reagent were added to each well of the microplate and gently mixed via pipetting.

The SpectraMax® M3 Multi-Mode Microplate Reader was used to calculate absorbance at 595 nm of the protein samples. The clear bottom microplate was shaken in the machine for 10

seconds prior to the scan. A standard curve was created using the average absorbance of each standard to calculate a linear equation. The average absorbance was calculated for each sample and then converted to protein concentration using the linear equation. Protein-specific carbonic anhydrase activity (ΔpH/min/mg protein) was calculated based on the amount of protein in each sample.

2.4   Micro-Computed Tomography Scanning

One anemone was relaxed and fixed in a two-step process. First, 250 ml of a 1:1 relaxant solution of 0.37 M $MgCl_2$ and artificial seawater was made. The anemone was submerged in the $MgCl_2$ solution for 15-20 minutes to relax it. Second, a 3:1 fixation solution of 4%

paraformaldehyde (PFA) and 0.37 M $MgCl_2$ was made to fix the anemone. The 1:1 relaxant solution was removed while the 3:1 fixation solution was slowly added to the anemone.

The micro-computed tomography scanning was completed with assistance from the Oregon State University Micro-CT laboratory (http://microct.oregonstate.edu/). The sea anemone was transferred with tweezers to a low-density aluminum sample holder that was 19 mm in diameter and 55 mm in length. The 3:1 fixation solution was not added to the low-density sample holder and thus, the sea anemone was scanned dry. The sea anemone was scanned for 2 hours and 22 minutes at an acquisition voxel size of 19.5862 μm.

2.5   Image Processing

The OSU Micro-CT laboratory assisted with the reconstruction of the data, preliminary visualization, and the transfer of data. A total of 1,297 2D images of the one anemone sample was produced by the CT scanner, which were analyzed and edited for preliminary visualization using Python and then used for 3D visualization in the program ImageJ.

In ImageJ, the file was opened as a 16-bit unsigned file with width and height of 1,318 pixels (Figure 2A). By clicking through *Stack>3DProject…>3D Viewer*, a three-dimensional series of images was produced (Figure 2B). To measure surface area and volume, the plugin 3D_Convex_Hull.jar was used. For any set of points within a plane, the convex hull is the smallest convex set to encompass all of the points within a plane (Chan, 1996). This plugin calculates 3D shape descriptors based on solidity and convexity. Solidity is defined as "*Solidity = Area / Convex Area*" and convexity is defined as "*Convexity = Convex Perimeter / Perimeter*." Additionally, the 3D equivalents of those measurements are "*Solidity3D = Volume / Convex Volume*" and "*Convexity3D = Convex Surface Area / Surface Area*" (Sheets, 2011). The plugin measurements are utilized as follows:

Analyze>3D Convex Hull>Convex Measures>Measure Convex Volume

Analyze>3D Convex Hull>Convex Measures>Measure Convex Surface Area

The measurements appeared in a new window called "Results." These measurements were then added to a Microsoft Excel spreadsheet and the SA:V ratio was calculated.

2.6   Statistical Analysis

Linear and smooth regression models were used to compare the relationship of column crown diameter to CA activity in *A. elegantissima.* All analyses were completed using R statistical software. The linear regression model was produced by using the Linear Model function and the smooth regression model was produced by using the Locally Weighted Scatterplot Smoothing function in R Studio (Data Science Central, 2019). The linear regression data were further analyzed by using a one-way analysis of variance (ANOVA) comparing CA activity in small, medium, and large anemones.

3.   RESULTS

3.1   The Effect of Crown Diameter on Carbonic Anhydrase Activity

Figures 3A and 3B respectively show the linear and smooth trends of carbonic anhydrase (CA) activity as a function of *A. elegantissima* size, as measured by column crown diameter. Carbonic anhydrase activity decreased as the column crown diameter increased and the SA:V ratio decreased (Figure 3A). The p-value of the linear regression slope was statistically significant; this finding indicates that there is a slight negative correlation between column crown diameter and CA activity (Table 1). A 'bell-shaped' curve was fit to the data and suggested that CA activity increased up to a crown diameter of approximately 20 mm and then decreased as diameters increased from 20 to 45 mm (Figure 3B). Smooth regression models provide a flexible approach to the representation of data but cannot obtain an equation for this set of data (Data Science Central, 2019). Unlike the linear regression model, an R^2 value and a p-value also cannot be deduced from the smooth regression model. A one-way ANOVA was run in order to determine the significance of column crown diameter on the rate of CA activity. There were no statistically significant differences between group means of column crown diameter (p = 0.436*).*

Multiple linear regression models of the CA activity as a function of column crown diameter were created for each group size in order to visualize the individual trends of CA activity found within each group size (Figure 4). The linear regression slopes, R^2 values, and p-values indicated that none of the regressions were significant (Table 1). The linear regression slope for the small group (1.0 – 15.0 mm) was positive, while the linear regression slopes for the medium (15.1 – 30.0 mm) and large (30.1 – 45.0 mm) groups were negative.

3.2   The Effect of Colony Identity on the Carbonic Anhydrase Activity

The linear regression plots of colonies 1 and 2 did not follow trends similar to each other (Figure 5A). Colony 1 had a slightly positive linear regression slope while colony 2 had a negative linear regression slope (Table 2). Additionally, the smooth regression models of colonies 1 and 2 have peak diameter values that differ by 9 mm (Table 2). The peak values are the column crown diameters that had the highest rates of CA activity, as indicated by the smooth regression curve. A one-way ANOVA found that there were no statistically significant differences between group means of column crown diameters from different colonies ($p = 0.151$).

Multiple linear regression models of the CA activity as a function of column crown diameter for each colony were created and organized by group size (Figure 6). These regression models allowed for a visualization of the individual trends of CA activity within each group size. The linear regression slopes, R^2 values, and p-values were organized in Table 3.

3.3   Computed Tomography Scanner Measurements

The measurements of surface area and volume of the sea anemone were calculated based on the convex hull constructed from a 16-bit grayscale image stack. One sample of *A. elegantissima* was scanned and measured; this anemone had a crown diameter of 17 mm. The surface area of the anemone was 2.77×10^5 mm^2 and the volume of the anemone was 7.02×10^5 mm^3. Thus, the SA:V ratio was 0.40.

4.   DISCUSSION

4.1   Anemone Size and Metabolism

The findings of this study contrast with my original hypothesis that anemones with larger crown diameters would result in a decreased capacity for the diffusion of inorganic carbon to the anemone and thus an increased need for CA, in comparison to anemones with smaller crown diameters. Additionally, these findings are also inconsistent with the findings of Weis, Smith, and Muscatine (1989), who found that SA:V ratios were inversely related to CA activity in multiple cnidarian species. The discrepancy between my findings and the Weis, Smith, and Muscatine results may be due to the fact that the latter study investigated multiple species with a diversity of shapes, including anemones and corals. The SA:V ratio findings may not be applicable to my study, which only sampled *A. elegantissima* in symbiosis with *B. muscatinei.*

Within the intertidal zone, energetic costs of anemones are influenced by size and morphology (Sebens, 1982). The smooth regression curve may suggest that there is a limit to the rate of CA activity and thus photosynthetic productivity needed by *A. elegantissima* based on their column crown diameter (Figure 3B). As marine invertebrates grow larger, the metabolic cost per unit body mass decreases as the total metabolic rate increases (Kleiber, 1932; Zeuthen, 1953). This, in turn, leads to less energy per unit mass needed to fulfill demands of metabolism in larger animals (Sebens, 1982). Furthermore, as found in Sebens (2002), the difference between energy intake and cost as a function of body mass followed a 'bell-shaped' curve. Sebens concluded that all animals have an energetic optimal size, which is the mass that provides the greatest surplus of energetic activity (Sebens, 2002). The findings of my study suggest that an energetic optimal size can be determined by measuring column crown diameter; anemones

with a column crown diameter above 20 mm may have low metabolic demands and thus, a low need for CA. Additionally, the CA activity of small and medium anemones was more variable than the CA activity of large anemones. After a crown diameter of approximately 20 mm, there was less variability found in the negative trend of CA activity. The variability found in the CA activity of small and medium anemones could potentially contribute to the 'bell-shaped' curve that the smooth regression shows (Figure 3B).

Another unexpected result was the difference in CA activity trends between the two colonies, especially since the colonies experienced similar ecology and were approximately 5 meters apart from each other. This may be explained by two models that Frances (1976) found to potentially address the characteristic differences between *A. elegantissima* in varying clonal aggregations. First, the growth of individual anemones of varying colonies may be affected by their respective microhabitats. For instance, colonies of *A. elegantissima* can be found on open rock surfaces or in the crevices of boulders, and each are subject to different levels of irradiance and wave velocities (Frances, 1979; Sebens, 1981). Second, unequal divisions and poor regeneration of clones may lead to further variation within colonies (Frances, 1976). These developmental models may account for the different rates of CA activity found between the two colonies. Overall, this finding leads to further questions regarding the effect of colony on the CA activity of varying clonal aggregations.

4.2   SA:V Image Processing

There were several plugins available for calculating the volume and surface area of an object in ImageJ, including Volume Viewer, 3D Object Counter, and Convex Hull. Volume Viewer is advantageous in that it provides 3D visualization, but unfortunately does not allow for

measurements of the scanned object to be taken (Barthel, 2012). The plugin 3D Object Counter was also able to provide 3D visualization but only offered area measurements for the individual 2D images. The convex hull plugin was ultimately used because it allowed for 3D visualization and incorporated the individual tentacles into the surface area and volume calculations accurately. For future studies, it would be ideal to CT scan sea anemones before they are used in a CA assay, in order to better analyze the influence that SA:V ratio has on the CA activity of *A. elegantissima.* The SA:V ratio was calculated to be 0.40 for an anemone with a column crown diameter of 17 mm, but comparable SA:V ratios are needed in order to understand the value of this ratio.

4.3   Limitations

4.3.1   Computed Tomography Scanning

I was unable to scan the sea anemones live, as was my initial plan. My first approach was to scan an anemone while it was submerged in the 3:1 fixation solution, in order to stabilize and leave the anemone open for the scan and to simulate an anemone in low tide. This led to a blank scan of the container that the anemone was in. Because the CT scanner utilized x-ray imaging, I believe the scan of the anemone was not present because PFA and sea anemones are predominantly carbon structures and there was a lack of contrast in molecular composition between the PFA and the anemone. For this reason, my second approach was to submerge the sea anemone in a potassium iodide (KI) solution of varying concentrations, including 0.20% KI (w/v), 0.50% KI (w/v), 1.0% KI (w/v), and 5.0% KI (w/v). This did not prove successful either; the KI concentration was unable to provide a significant contrast between the sea anemone and the KI solution that the anemone was submerged in. I later discovered that the motion of the CT scanner during the scan disturbed the liquid surrounding the anemone, as the scanner moved in a

helical fashion. It was ultimately necessary that the sea anemone was not submerged in solution and thus not moving during the scan. Our third and final approach was to scan the anemone without solution. Though the anemone was out of solution for two hours and 22 minutes, this approach proved successful.

4.4   Conclusions

The results of the CA activity study suggested that the increasing column crown diameters of *A. elegantissima* may negatively influence the rates of CA activity. Additionally, there may be a relationship between body size and an optimal energetic size. CT scanning fixed anemones in the absence of solution resulted in successful 3D visualization and measurement of one anemone. Future studies of 3D anemone reconstruction could sample a larger number of anemones to allow for a method to be properly tested and developed, and to allow for further exploration of the relationship between SA:V ratio and column crown diameter.

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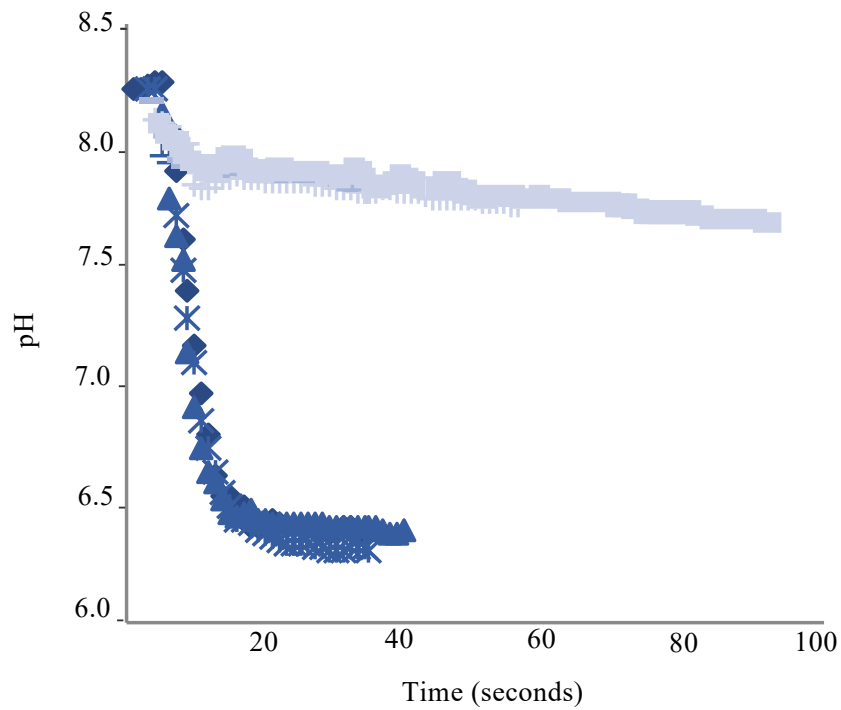
Figure 1. Recorded pH of three native and three denatured trials of a CA assay. The native and denatured samples are dark and light blue respectively. The different shapes represent the three trials that were completed for both the native and denatured samples. Low CA activity led to a very slow decrease in pH level, as shown in the denatured trials, while high CA activity led to a rapid decrease in pH level, as shown in the native samples.
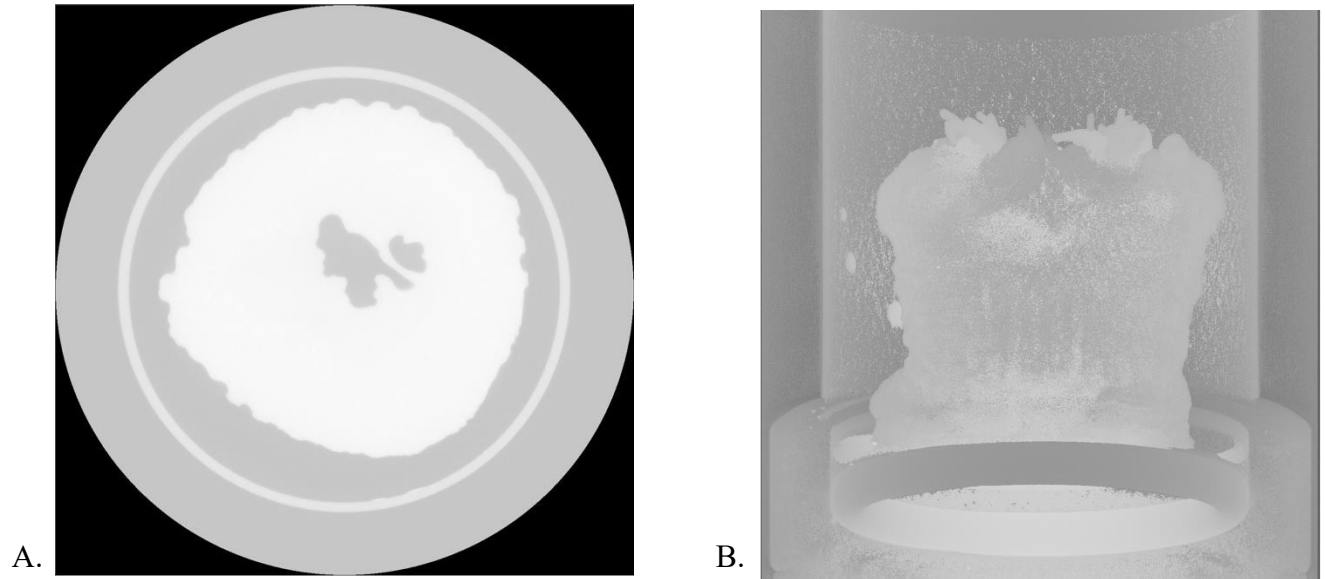
Figure 2. Micro-computed tomography scan of *A. elegantissima*.

A. A preliminary stack of 1297 two-dimensional images that were created by the software Python prior to the 3D reconstruction. The gray that appears in the middle of the anemone accounts for the inside cavity of *A. elegantissima*.

B. The 3D reconstruction of *A. elegantissima*. This image was created from the preliminary stack of images shown in Figure 2A with the use of the Convex Hull plugin in the software ImageJ. A gif of the 3D visualization can be found here.
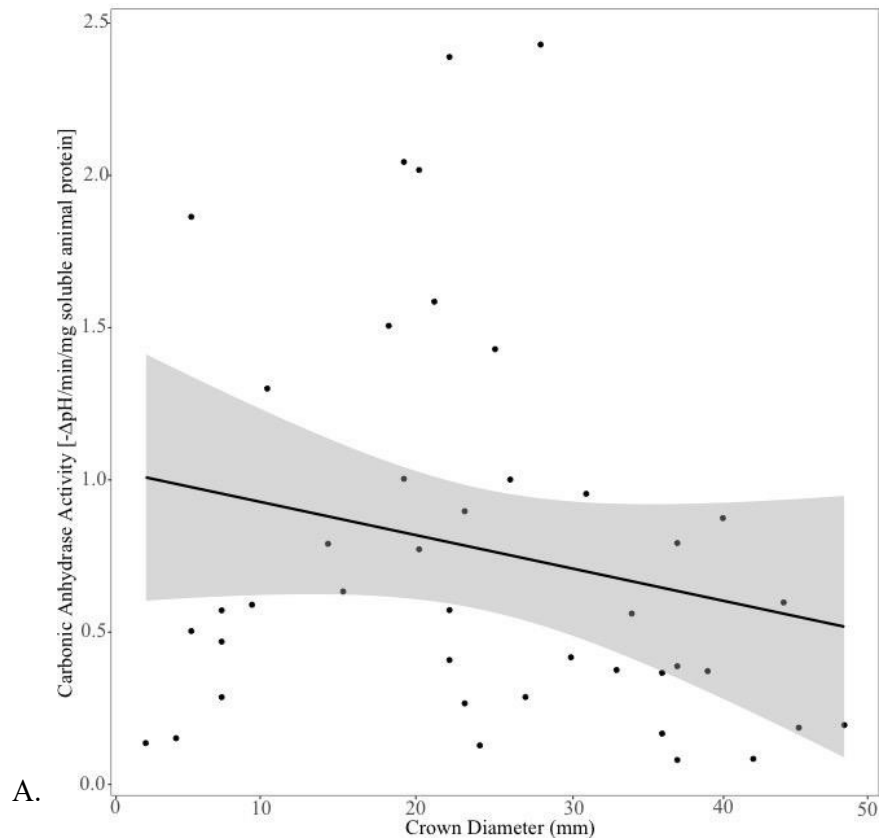
A.

B.
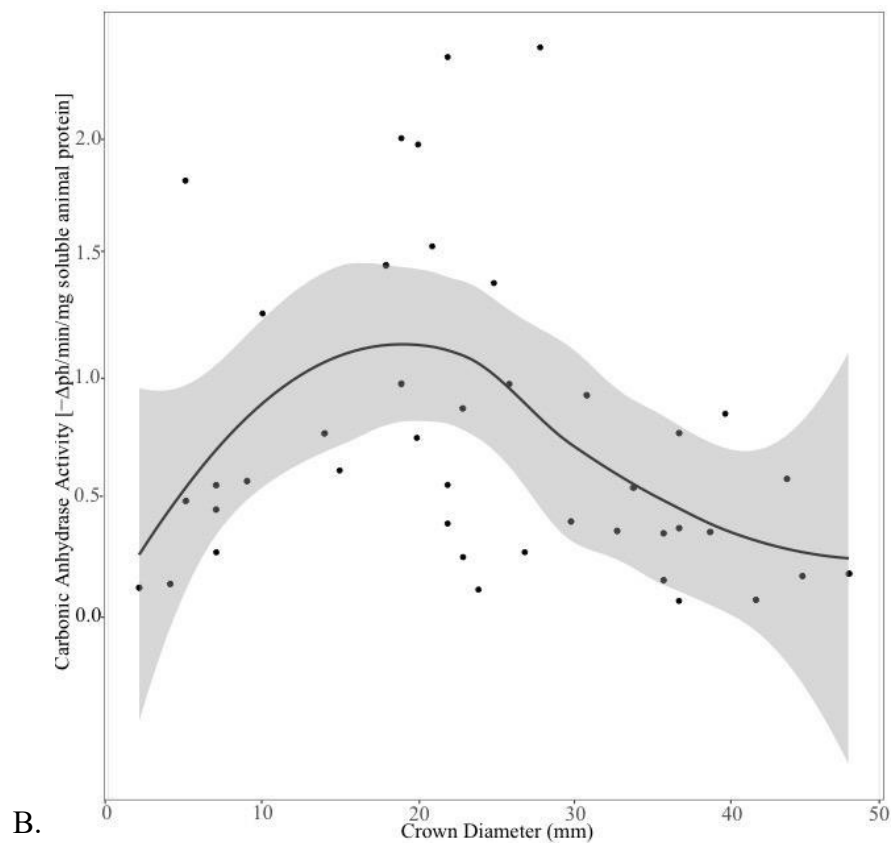
Figure 3. Linear and smooth trends of CA activity as a function of anemone size, as measured by crown diameter. **A.** The linear trend of CA activity vs. anemone crown diameter. The linear regression slope, R^2 values, and p-value are organized in Table 1. The one-way ANOVA test found that there was no significance between column crown diameter and CA activity (F = 0.618). **B.** The smooth trend of CA activity vs. anemone crown diameter. Carbonic anhydrase activity was highest at the column crown diameter value of 20 mm.
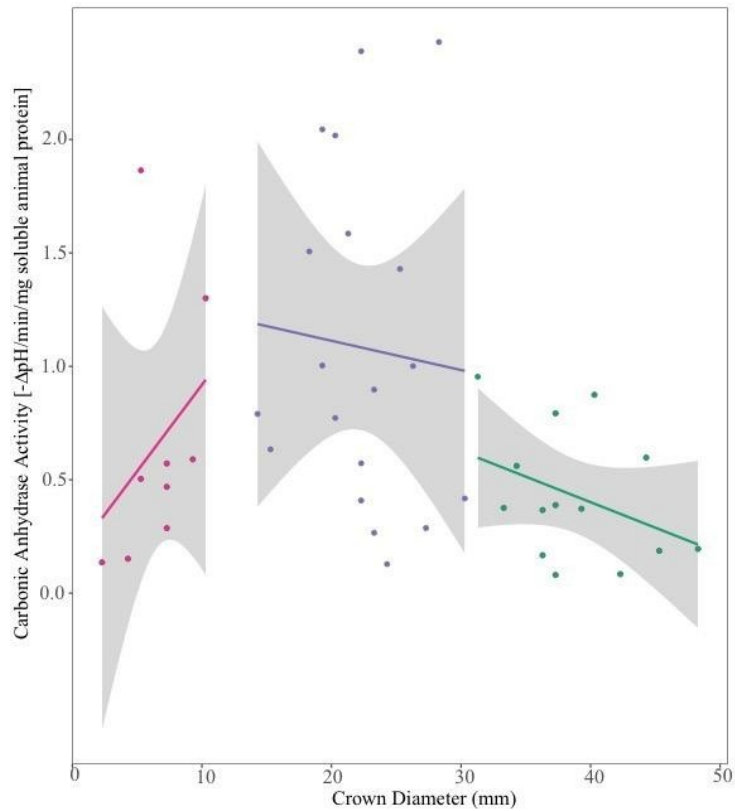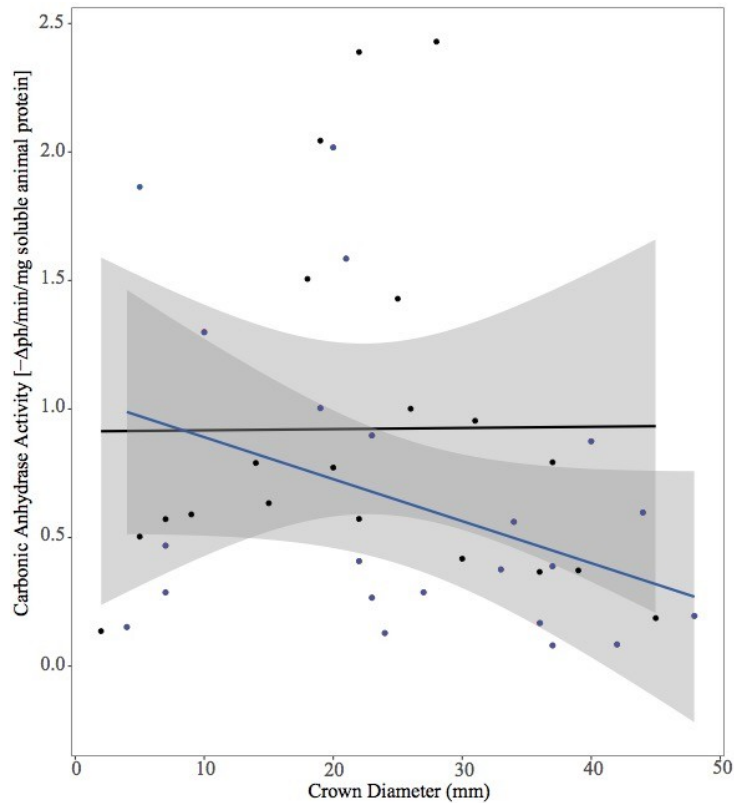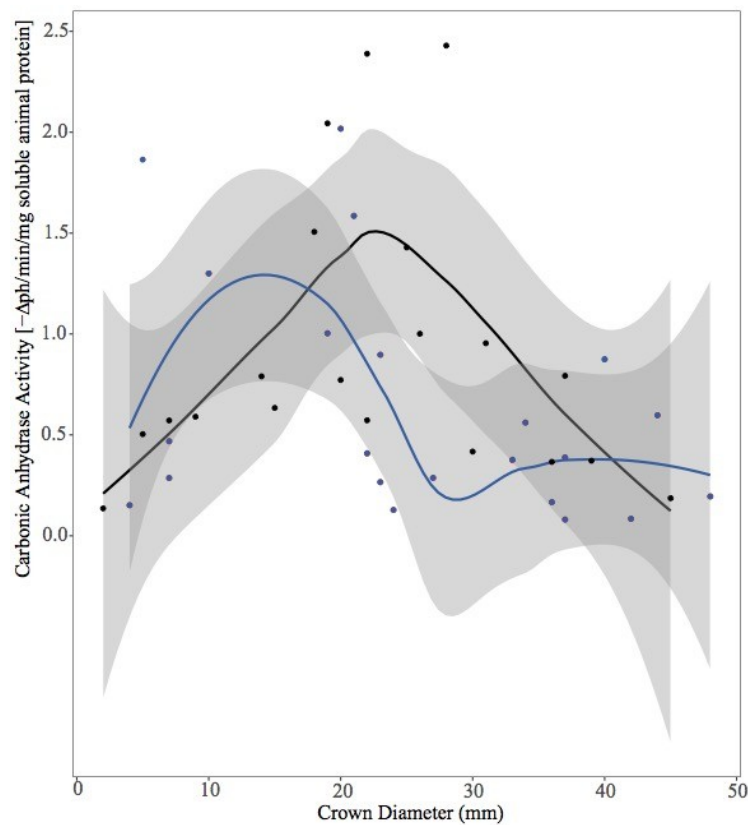
Figure 4. Multiple linear trends of carbonic anhydrase activity as a function of anemone size, as measured by crown diameter, organized by group size. The magenta, purple, and green data points respectfully represent the small group (1.0 – 15.0 mm), the medium group (15.1 – 30.0 mm), and the large group (30.1 – 45 mm) of anemones. The respective linear regression slopes, R^2 values, and p-values are organized in Table 1.

Figure 5. Linear and smooth trends of carbonic anhydrase activity as a function of anemone size, as measured by crown diameter, within two different colonies. **A.** The linear trends **B.** The smooth trends. Colony 1 and 2 are respectfully represented by the black and blue points. The respective linear regression slopes, R^2 values, and p-values of Figure 5A and the peak values of Figure 5B are organized in Table 2. The one-way ANOVA test found no significance between group means of crown diameters from different colonies (F=2.143).
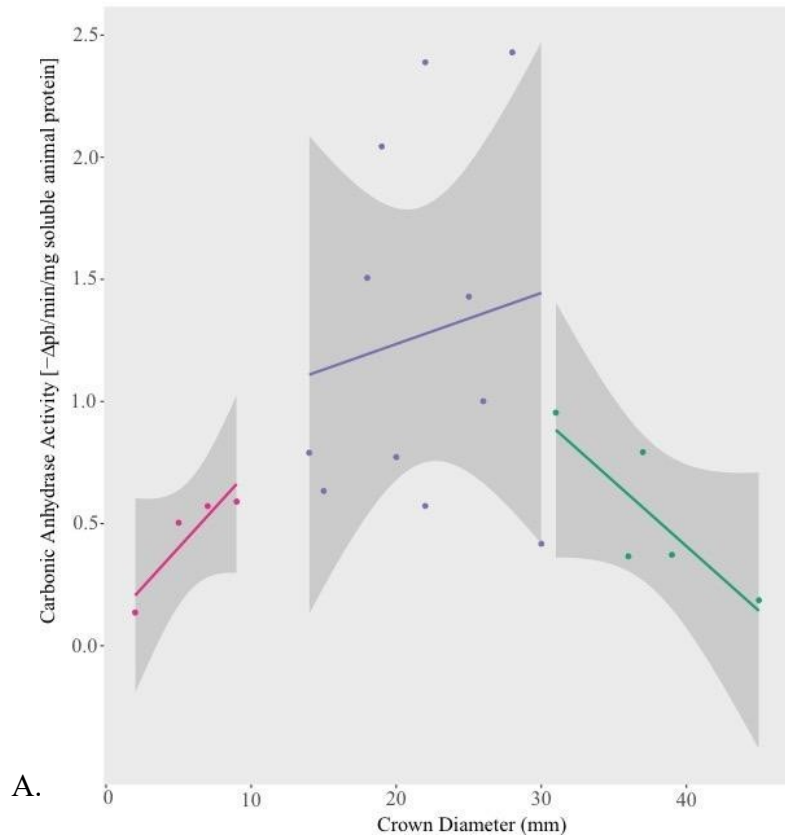
Figure 6. Multiple linear trends of carbonic anhydrase activity as a function of anemone size, as measured by crown diameter, organized by colony and group size. **A.** Linear trends of colony 1 organized by group size and **B** represents colony 2. The magenta, purple, and green data points respectfully represent the small group (1.0 – 15.0 mm), the medium group (15.1 – 30.0 mm), and the large group (30.1 – 45 mm) of anemones. The respective linear regression equations, R^2 values, and p-value can be found in Table 3.
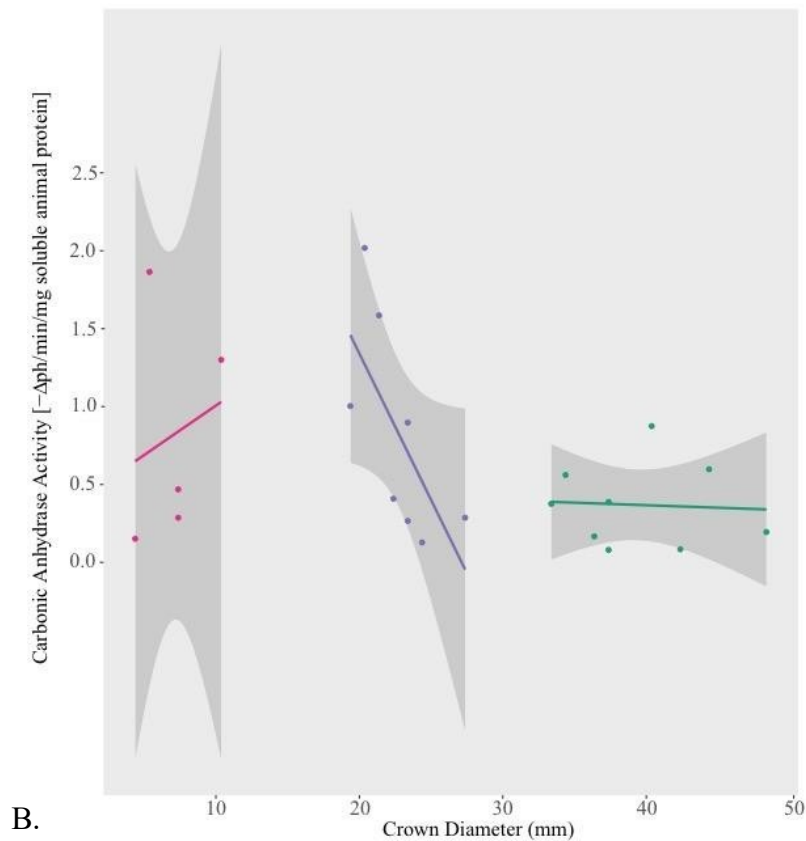
Table 1. Linear trends and statistical measures of carbonic anhydrase activity as a function of *A. elegantissima* size, as measured by column crown diameter.

Column Crown Diameter (mm)	Equation	R^2	Adjusted R^2	P-value
1.0 – 15.0	y = 0.07622x + 0.17822	0.0122106	-0.01657	0.3821
15.1 – 30.0	y = 0.04323x + 0.31643	0.0006548	-0.02813	0.4972
30.1 – 45.0	y = -0.02251x + 1.29498	0.0200223	0.06993	0.185
1.0 – 45.0	y = -0.010656x + 1.029536	2.20×10^{-4}	-0.009186	0.00097

Table 2. Linear and smooth trends and statistical measures of carbonic anhydrase activity as a function of *A. elegantissima* size, as measured by column crown diameter, and colony identity.

Colony	Equation	R^2	Adjusted R^2	P-value	Peak Value (mm)
		Linear			*Smooth*
1	y = 0.0004578x + 0.9126243	3.87×10^{-9}	-0.055	0.9737	23
2	y = 0.016339x + 1.053832	0.0198	0.0978	0.08534	14

Table 3. Linear trends and statistical measures of carbonic anhydrase activity as a function of *A. elegantissima* size, as measured by column crown diameter, organized by group size and colony identity.

Column Crown Diameter (mm)	Colony	Equation	R^2	Adjusted R^2	P-value
1.0 – 15.0	1	$y = 0.06522x + 0.07526$	0.70090	0.7555	0.08511
15.1 – 30.0	1	$y = 0.02091x + 0.81712$	0.0004567	-0.08737	0.668
30.1 – 45.0	1	$y = -0.05291x + 2.52387$	0.47596	0.05865	0.08155
1.0 – 15.0	2	$y = 0.06344x + 0.39550$	0.001539	-0.281	0.7495
15.1 – 30.0	2	$y = -0.18802x + 0.03116$	0.22109	0.3819	0.06046
30.1 – 45.0	2	$y = -0.003242 + 0.495728$	0.00001233	-0.1388	0.8796