Effects of increased pCO_2 levels on the nematocyst densities in the symbiotic sea anemone *Anthopleura elegantissima*

Jack C Koch[1,2]

Marine Invertebrate Zoology Summer A 2014

[1]Friday Harbor Laboratories, University of Washington, WA 98250
[2]Department of Biology, University of North Carolina at Wilmington, Wilmington, NC 28407

Contact Information:
Jack C Koch
Biology Department
University of North Carolina at Wilmington
601 South College Road
Wilmington, NC 28407
jck5644@uncw.edu

Keywords: Anthopleura elegantissima, Aggregating Anemone, Green Aggregating Anemone, ocean acidification, nematocyst, nematocyst density, symbiosis, pCO_2

ABSTRACT

The temperate sea anemone *Anthopleura elegantissima* participates in a facultative symbiosis with two genera of unicellular photosynthetic algae. Under stressful conditions such as increased levels of pCO_2, anemones expel their algal symbionts causing the anemones to rely more heavily upon heterotrophic feeding. A heavier reliance on heterotrophic feeding could be accomplished by increasing the density of nematocysts. *Anthopleura elegantissima* individuals were collected from the Friday Harbor Laboratories shoreline (48° 32.7646 N, 123° 00.5932 W), Washington in June 2014, and the number of nematocyst per μg of protein was measured to determine if increasing oceanic pCO_2 levels will affect the density of nematocysts. The number of nematocyst per μg of protein was measured again after 7 days in higher (2200 μatm) or ambient (700 μatm) pCO_2 conditions. There was no difference in the density of nematocysts between individuals kept in different pCO_2 levels.

INTRODUCTION

The symbiosis between cnidarians and unicellular phototrophic algae can be a facultative or obligate relationship. The cnidarian-algal symbiosis is found worldwide and constitutes an important raw material trade between host and symbiont. For anemones, this symbiosis is considered endosymbiotic because the alga is associated with the gastrodermal tissue of the host. Symbiosis is metabolically advantageous for the host and the symbiont. Although the symbiont only receives waste products from the host, the symbionts use these waste products as resources for photosynthesis, the means by which the alga produces food for itself.

A model organism for studying the mechanisms, ecology, and disruptive factors of the cnidarian-algal symbiosis is the temperate sea anemone, *Anthopleura elegantissima* (Brandt, 1835). *Anthopleura elegantissima* is found on rocky and sandy shores on the Pacific coast of North America from Alaska (Hand 1955) to North Central California (Zamer 1986). Individuals that live in the high intertidal experience up to 18 hours of aerial exposure daily while individuals that live in the low intertidal experience complete immersion for days at a time (Zamer 1986). *Anthopleura elegantissima* shows biochemical and behavioral adaptations, which allows for extreme tolerance to high temperatures, high levels of irradiance, and large fluctuations of salinity in the intertidal zone. *Anthopleura elegantissima* maintains levels of critical enzymes in direct proportion to those levels observed in the symbiotic algae and contains UV absorbing pigments (Dykens 1984, Shick and Dykens 1984). In addition, *A. elegantissima* is able to contract during times of exposure, hold water in the gastrovascular cavity for evaporative cooling, and collect shells for cover. Shell covering also reduces the irradiance intensity experienced by the symbionts (Dykens and Shick 1984), whose photosystems can be damaged by intense light. Under such stressful conditions, symbionts may be evicted from the host tissue in a process known as bleaching.

Bleaching events are caused by factors such as changes in temperature, light intensity, food availability, or chemical composition of the water, like pCO_2 levels. When the symbiotic relationship is compromised the host must compensate for the loss of materials that the symbiont was once providing. The increasing occurrence of bleaching events across many taxa that participate in a symbiotic relationship has led to an

increased effort to understand the effects of environmental variability on the disruption of cnidarian-algal symbioses (Bates *et al.* 2010).

Anthopleura elegantissima has two methods of obtaining nutrients used in daily bodily process. The first is as a recipient of materials derived from symbiotic autotrophy and the second is as a passive suspension feeder.

Anthopleura elegantissima is facultatively symbiotic with two genera of unicellular photosynthetic algae, green algae (zoochorellae) and dinoflagellates (zooxanthellae) (Bates *et al.* 2010). Individuals may not host either of the symbionts (aposymbiotic), one or the other of the symbionts, or both of the symbionts. Algal symbionts inhabit the gastrodermal tissue of the anemones. The algae are taken into the gastrodermal cavity, engulfed in a vacuole of anemone tissue, and then transferred into the gastrodermal tissue safe from digestion. Once in the gastrodermal tissue the symbionts perform photosynthesis providing the host with sugars that are produced in the reaction. In exchange, the host provides the symbionts with waste products; materials that are used in the photosynthetic reaction. There are many potential physical and chemical factors that can influence the cnidarian-algal symbiosis. Some factors may strengthen the relationship while others may cause bleaching.

Anthopleura elegantissima is a passive suspension feeder that uses tentacles armed with nematocysts to capture prey (Figure 1). Nematocysts are nonliving secreted organelles derived from multi-potent stem cells (Kass-Simon and Scappaticci Jr. 2002). There are many different types of nematocysts that have been described, each falling into a category based on use. For example, basotrichs are used to inject toxins into a prey. A harpoon-like projection is ejected from basotrich-type nematocysts (Figure 2) when an

appropriate stimulus contacts a tentacle. Other types of nematocysts are used for attachment during locomotion, defense against predators, and entangling passing prey.

There are many potential positive and negative influences on the symbiotic relationship found between cnidarians and algae. Increased dissolved carbon dioxide levels will cause symbiotic cnidarians to bleach (Hajime Kayanne 2005) causing these organisms to rely more heavily upon heterotrophy to receive the same amount of food resources as received during symbiosis. Increased nematocyst density in anemones may be expected if they allow animals to compensate for lost nutrients after a bleaching event. The purpose of this study was to test whether nematocyst densities in *A. elegantissima* changed when animals were exposed to increased pCO_2 levels.

MATERIALS AND METHODS

Field Site Description and Animal Collection

This study was conducted at the Friday Harbor Laboratories (FHL) on San Juan Island, Washington, USA (Figure 3). The local shores are rocky and provide habitat space for many anemones. Animals were collected from the harbor shore because of the ease of access and abundance of the study organism, *Anthopleura elegantissima*. Sixteen randomly chosen medium to large *A. elegantissima* (26 mm – 47 mm diameter) were collected during low tide (-0.39 m) at 13:15 on 28 June 2014 from the FHL shoreline (48° 32.7646 N, 123° 00.5932 W [Figure 3]). All *A. elegantissima* were collected in the mid-tidal level zone within a 5 m radius of one another.

Animal Care and Preparation

All *A. elegantissima* were placed in running seawater tables and allowed to acclimate overnight. Twenty-six 15 mm x 100 mm petri dishes were numbered. The petri

dish top (PT) and bottom (PB) were measured with digital calipers (PB = 92.9 mm and PT = 89.1 mm). All petri dishes were soaked in freshwater overnight to remove any chemicals used in the production process and then allowed to soak in seawater for a day to further condition them. *Anthopleura elegantissima* were placed on damp petri dishes outside of the sea table with no water for up to six hours. Once attached, the anemones were placed in walled flow-through containers in the sea water tables to prevent escape and predation by other organisms. The anemones were fed once daily with ~2 mL of crushed Tetra Brine Shrimp Treat/seawater mixture. At the start of the experiment, individual anemones were photographed (Figure 4).

Preparation of Experimental Chambers

Experimental chambers (Figure 5) consisted of gardening starter trays. A 1.27 cm hole was cut in the left top corner of the tray and a 1.27 cm PVC 90° elbow was fitted into the hole. A bead of marine aquarium silicon was applied between the PVC and the starter tray to create a seal and then a push-to-connect pipe adapter was attached to the outflow of the PVC elbow to allow for a piece of flexible tubing to be attached for drainage. 2 mm hard plastic mesh was cut into strips and setup into 8 compartments in each tray so that individual anemones could be followed. The outflow of a larger scale ocean acidification project in the Ocean Acidification Lab (OAL) at FHL provided water for the experimental chambers. A flexible piece of hosing was attached to the outflow on the experimental chambers and then drained to the OAL drainage system. Prior to starting the experiment, the experimental chambers were allowed to condition overnight to remove any production chemicals.

Tentacle Collection

After each individual was photographed, fine scissors were used to remove three tentacles from each anemone. Tentacles were collected from opposing sides of the anemones oral disk. The tentacles were removed because the tentacles contain large numbers of basotrichs and spirocysts (both used in food capture). Basotrichs and spirocysts are easy to visual and count using a differential interference contrast microscope. Clipping tentacles is a non-lethal and repeatable procedure. Clipped tentacles were placed into labeled micro centrifuge tubes and then placed into a standard freezer for further analysis.

Nematocyst Preparation and Counting

All solutions were kept on ice during use. 0.5 mL of filtered seawater was pipetted into a 5 mL Teflon tissue homogenizer. The homogenizer was attached to a power handheld drill for more efficient and thorough homogenizing. All three tentacles from an individual were defrosted. The water in each micro centrifuge tube was carefully removed using a glass Pasteur pipette. The tentacle itself was then removed and placed into the homogenizer. The tentacles were homogenized until no visible pieces of tissue were remaining. The resulting homogenate was poured into a labeled micro centrifuge tube and kept on ice. An additional 0.5 mL of filtered seawater was added to the homogenizer tube and used to clean the homogenizer. This dilute homogenate was added to the micro centrifuge tube to bring the total volume in the tube to 1 mL.

The homogenate was vortexed for five seconds. 2 μL of the homogenate was pipetted onto a flat microscope slide and covered with an 18 mm x 18 mm No. 1 cover slip. Nematocysts were counted on a Nikon Eclipse E600 differential interference contrast microscope. Most of the nematocysts were counted under the 10x power with the

condenser set to DIC L. To improve accuracy and ease of counting a red filter (G-2E/C TRITC) was used to enhance the contrast of the nematocysts. When a clump of nematocysts was encountered the power was increased to 20x and the clump was carefully counted to the best of the counters ability. Three subsamples of homogenate were counted per individual before experimental treatment and after experimental treatment.

Experimental Trials

Of the sixteen test anemones, eight were randomly picked out of the sea table and placed, one per compartment, in the high pCO_2 treatment chamber (28 ppt). The remaining eight anemones were placed in the low pCO_2 treatment chamber (28 ppt). These anemones were fed once daily with ~2 mL of crushed Tetra Brine Shrimp Treat/seawater mixture. The experimental period was seven days. The average pH and temperature of the high pCO_2 treatment chamber was 7.35 and 12°C, respectively. The high pCO_2 level was 2200 μatm (average taken from two measurements during experimental period). The average pH and temperature of the low pCO_2 treatment chamber was 7.81 and 12°C, respectively. The low pCO_2 level was 700 μatm (average taken from two measurements during experimental period).

Protein Assay

The protein assay was performed using a Sigma Diagnostics Protein Assay Kit (Procedure No. P 5656). Protein samples were originally frozen in filtered seawater. These samples were defrosted, spun down in a centrifuge (Beckman Microfuge E) at high speed for 20 minutes. The supernatant was decanted and the pellet was re-suspended in 1 mL of reverse osmosis water. A calibration curve (Figure 6) was created, using bovine

serum albumin (BSA) as a standard, following Procedure No. P 5656. Protein content in the anemone samples was beyond the calibration curve so a more concentrated known protein sample was made (600 μg, 800 μg, and 1600 μg) to extend the calibration curve and eliminate the need to extrapolate the calibration curve. Protein content was measured using a Hach DR 5000 spectrophotometer.

Statistical Analysis

Nematocysts were counted in 2 μL aloquots of homogenized tentacle material. The three nematocyst counts per individual (one of each tentacle) were averaged (Table 1.). The number of nematocysts per 1 mL of homogenized tissue was calculated by multiplying the average of the 2 μL counts by 500 (2 μL x 500 μL = 1 mL). The tentacle protein values were obtained by fitting a power curve regression to the protein assay calibration curve (BSA as standard) and plotting the recorded absorbance values on the curve. By dividing the nematocyst counts by the protein associated with each individual the average number of nematocysts per μg of protein was calculated.

A repeated measures ANOVA was used to test whether nematocyst density per μg of protein differed among anemones exposed to different levels of pCO_2. The factors examined were individual (plate #1, plate #2, plate #3, etc.), time (before values and after values), treatment (high pCO_2 and ambient pCO_2), and nematocyst density per μg of protein. The statistical software JMP10 was used to run the ANOVA analysis. The model effects used were time, treatment, treatment cross time, and treatment nested within individuals with a random attribution.

RESULTS

Effect of pCO_2 on Nematocyst Density

There was no statistically significant difference ($p > 0.05$) in the density of nematocysts per μg of tentacle protein in relation to increased pCO_2 levels (Table 2.). There is a high amount of variance between the mean before values of nematocysts per μg of tentacle protein in both treatment groups. The control pCO_2 treatment group had an individual with the maximum nematocysts per μg of tentacle protein while the high pCO_2 treatment group had an individual with the minimum nematocysts per μg of tentacle protein. One of the individuals from the high pCO_2 treatment group was discarded from the statistical analysis because ¾ of the protein content was spilled before a protein reading could be taken. All anemones in the high pCO_2 treatment experienced a positive change in the density of nematocysts per μg of tentacle protein. Two of the anemones in the control pCO_2 treatment experienced a positive change and in the density of nematocysts per μg of tentacle protein. The other two individuals in the control pCO_2 treatment experienced a negative change and in the density of nematocysts per μg of tentacle protein.

DISCUSSION

Heterotrophic feeding in symbiotic cnidarians is directly affected by the productivity and presence of symbiotic algae (Anthony and Fabricius 2000). The efficiency of heterotrophic feeding is related to nematocyst density and nematocyst density is related to symbiotic state (Hiebert and Bingham 2012). The goal of this study was to determine if high pCO_2, which induces bleaching, affected the density of nematocysts in the tentacles of the temperate sea anemone *A. elegantissima*. When an anemone is symbiotic the need to capture passing food is less than if that same anemone is aposymbiotic. To find the happy medium between energy spent producing

nematocysts, food captured, and nutrients obtained from symbiotic algae, the anemone has a lower density of nematocysts per unit protein. When a bleaching event occurs, the need for food from heterotrophy increases drastically as ~65% of the anemones daily carbon is contributed by the algal symbionts (Muscatine and McCloskey 1981).

Hiebert and Bingham (2012) suggest that aposymbiotic individuals may have nematocyst densities that are similar in density but not in size and adhesive power to symbiotic individuals. By producing larger and stickier nematocysts an anemone may be able to save energy and catch larger food particles that would provide higher amounts of nutrient relative to energy put into capturing the food (Hierbert and Bingham 2012). Future research should examine food size capture capability for differing symbiotic states of temperate sea anemones.

There are additional tradeoffs between heterotrophy and symbiotic autotrophy that must be considered. If nematocyst densities are too high, there could be a decrease in the amount of photosynthetically active radiation (PAR) reaching the photosystems of the symbionts. Alternatively, if nematocyst densities are too low, there could be too much PAR reaching the photosystems of the symbionts causing damage. This damage shuts down the photosynthetic pathway, which provides nutrients for the sea anemone. Future research should examine the photo-protective properties of different types of nematocysts found in symbiotic cnidarians. If photo-protective, nematocyst density could play a key role in determining symbiont density as well. This also raises the question of the amount of energy to produce different types of nematocysts and how the distribution of different types of nematocysts changes with changing symbiotic state.

The results from this study did not match the expected results that were proposed at the beginning of the study. This deviation from the expected results may be explained by several ideas. Error in the experiment is most likely the largest contribution to findings that differing from the expected. Sources of potential error are in the protein assay and the counting of the nematocysts. If this study was performed again, tentacle clipping measurements would be taken to account for differing amounts of animal tissue. This difference in amount of animal tissue could affect the calculation of the density of nematocysts. Additionally, counting symbionts before and after exposing the sea anemones to differing pCO_2 levels would allow for confirmation that the symbiotic state of the anemones is actually changing and that a change in nematocyst density is expected.

The experimental results may be explained by the fact that *A. elegantissima* is a coastal and intertidal species. The west coast of North America experiences periods of upwelling, which brings cold, nutrient-rich waters to the surface. In addition to high levels of nutrients, this water also contains high levels of pCO_2 (Feely *et al*. 2008). These periods of upwelling can last for a few weeks and could put bleaching stresses on symbiotic organisms. There is no evidence of large numbers of bleaching events that happen in conjunction with these upwelling events. If future research found that there is no association between bleaching events and upwelling event then if would be possible to hypothesize that symbiotic cnidarians living in coastal areas that experience periods of upwelling have adapted to high spikes in the pCO_2.

In addition to the possibility that coastal symbiotic cnidarians are adapted to high levels of pCO_2, intertidal symbiotic cnidarians also may have evolved an adaptive advantage. During low tide, anemones will hold water in their gastrovascular cavities and

allow it to evaporate to keep them cooler than the surrounding air. During this time there is no flushing of water in the gastrovascular cavity so, there is a build up of respiration waste products including pCO_2. Future research should examine the levels of pCO_2 in the gastrovascular cavity of an intertidal symbiotic cnidarian to see if the levels of pCO_2 reach beaching levels during different tidal regimes. Comparing the effects of increasing pCO_2 levels on nematocysts and symbiont densities in a deep-water symbiotic cnidarian versus a similar coastal symbiotic cnidarian may also provide insight into adaptive advantages of symbiotic cnidarians.

FIGURES AND TABLES

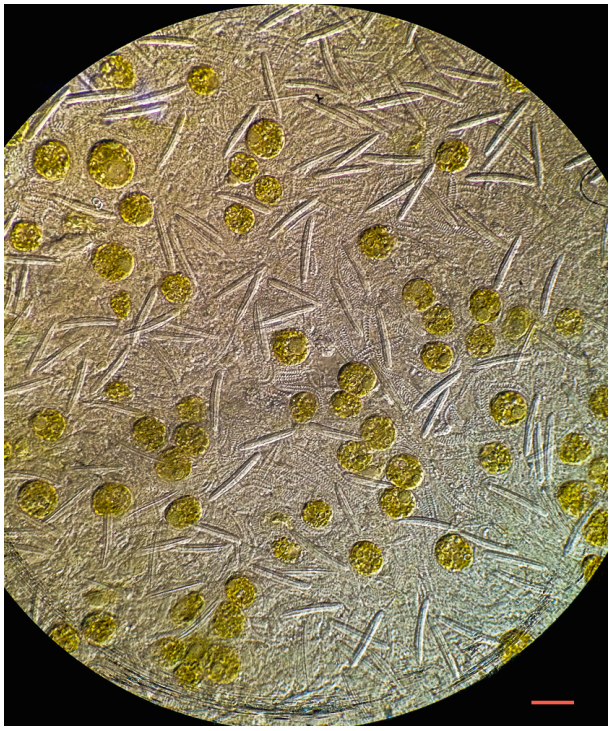

Figure 1. *Anthopluera elegantissima* tentacle squash at 40x power oil immersion under differential interference contrast microscope. Basotrichs are cylinder-like nematocysts, spirocysts are spiral-like nematocysts, and zooxanthellae are golden-brown circles. Bar is 10 microns.

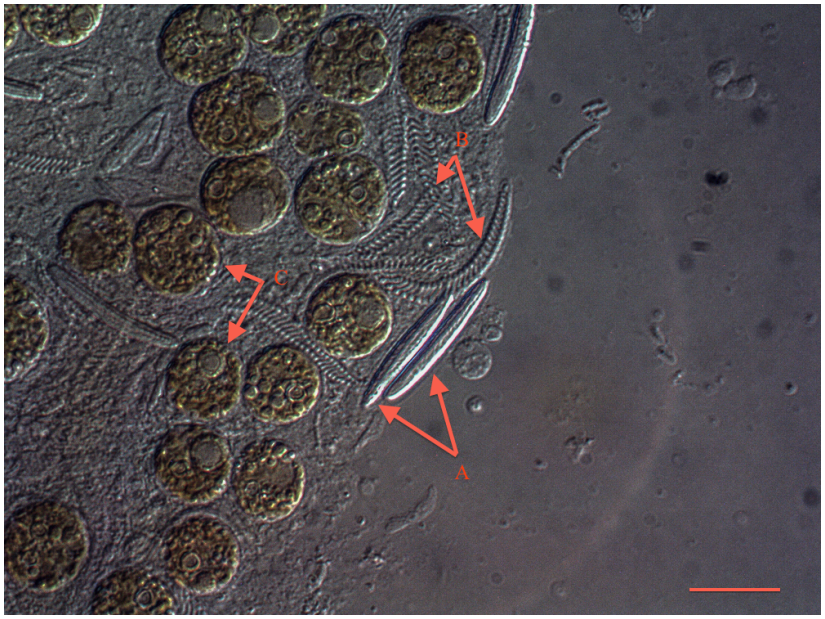

Figure 2. *Anthopluera elegantissima* nematocyst count clump at 100x power oil immersion under differential interference contrast micropscope (A are basotrichs [6 pictured], B are spirocysts [13 pictured], and C are zooxanthellae [21 pictured]). Scale bar is 10 microns.

Figure 3. A. Map of San Juan Island. B. Map of Friday Harbor. C. Map of Friday Harbor Laboratories (FHL). Red pins indicates collection site on the shore of FHL.

Figure 4. Individual anemone photographs. The petri dish top (PT) [pictured on the right] or bottom (PB) [pictured on the left] is the scale bar (PB = 92.9mm and PT = 89.1mm).

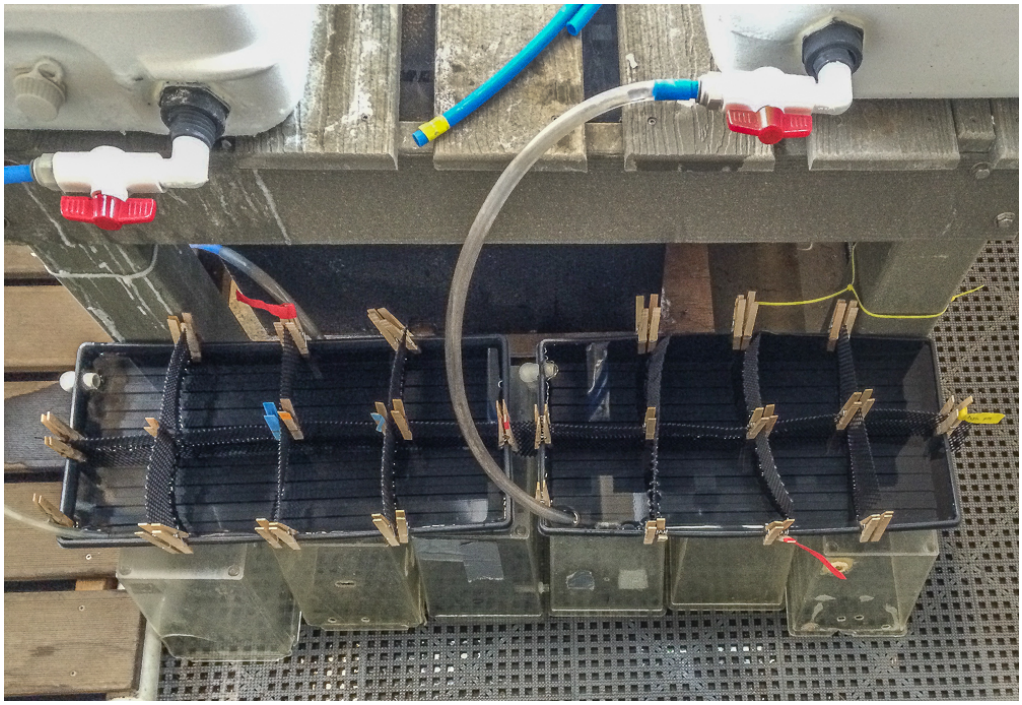

Figure 5. Experimental chambers setup in the ocean acidification lab prior start of experiment.

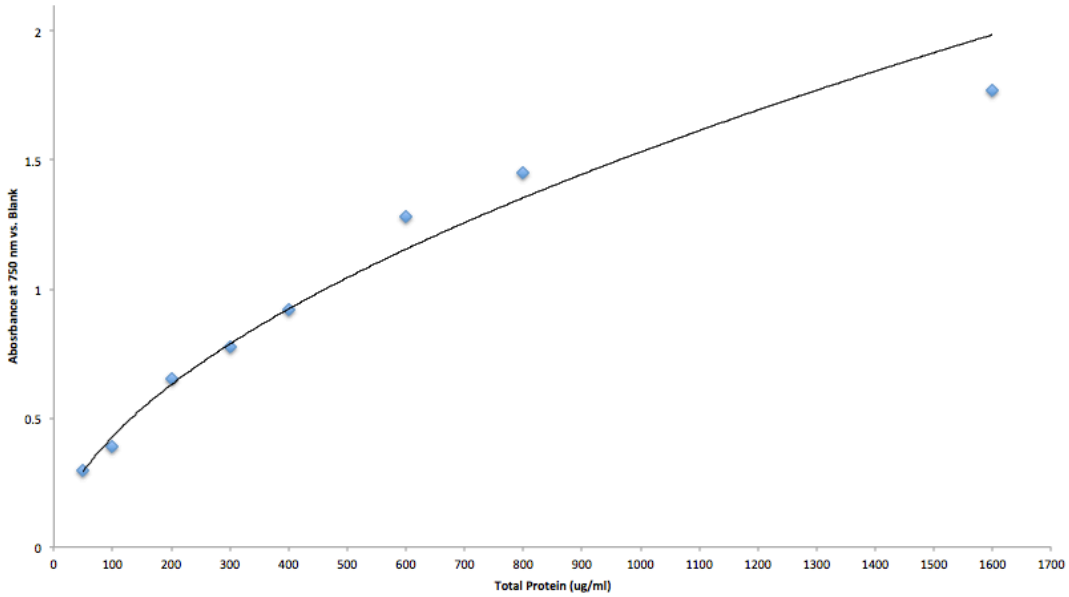

Figure 6. Calibration curve of absorbance values for protein standard (P7656) solutions vs. their protein concentrations using Sigma Protein Assay Kit No. P5656. Bovine serum albumin (BSA) was used as the standard. Blue squares are protein standard absorbance readings at 750 nm. Power fit regression curve ($y = 0.0336x^{0.553}$ and R2 = 0.98583).

Table 1. Mean start and end data for nematocyst counts, protein content, and nematocysts per protein content.

Sample Size	Data	Value	Units
	average start nematocysts control	547500.00	nematocysts per 1 mL
	average start nematocysts high pCO2	595750.00	nematocysts per 1 mL
n = 4	average start protein control	590.77	ug protein per 1 mL
n = 3	average start protein high CO2	736.99	ug protein per 1 mL
n = 4	average start nematocyst/ug protein control	1185.64	nematocysts per ug protein
n = 3	average start nematocyst/ug protein high pCO2	957.26	nematocysts per ug protein
	average end nematocysts contol	665958.33	nematocysts per 1 mL
	average end nematocysts high pCO2	781083.33	nematocysts per 1 mL
n = 4	average end protein control	900.97	ug protein per 1 mL
n = 3	average end protein high CO2	747.53	ug protein per 1 mL
n = 4	average end nematocyst/ug protein control	526.94	nematocysts per ug protein
n = 3	average end nematocyst/ug protein high pCO2	777.12	nematocysts per ug protein

Table 2. Summary of Repeated Measures ANOVA results for the effects of increased pCO_2 levels on nematocyst density in the tentacles of the temperate symbtiotic sea anemone, *Anthopleura elegantissima*.

Fixed Effects Test		
Source	**d.f.**	**Prob > F**
Time	1	0.9537
Treatment * Time	1	0.2376
Treatment	1	0.2506

ACKNOWLEDGEMENTS

I would like to thank the instructors of the Summer A FHL Marine Invertebrate Zoology course for their assistance in the development and statistics of this study and editing of this paper. I would like to thank Rebecca Guenther and the Ocean Acidification Lab at FHL for allowing me to use their facilities. I would like to thank Lisbeth Francis for her help in the development of the methods of this study. Thank you FHL for their financial support to attend this course.

PROCEDURES

Sigma Diagnostics Protein Assay Kit (Procedure No. P 5656) - http://legacy.library.ucsf.edu/tid/ese93a99/pdf

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