Winter 2013

Observing the Location and Orientation of Nematocysts Through Aeolidiella Stephaniae

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OBSERVING THE LOCATION AND ORIENTATION OF NEMATOCYSTS THROUGH *AEOLIDIELLA STEPHANIAE*

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Animal & Veterinary Sciences; Pre-Veterinary Concentration)

The Honors College

University of Maine

December 2013

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Abstract

The anemone species *Aiptasia* is a nuisance pest that can quickly out-compete many species in a coral reef aquarium. The aeolid nudibranch *Berghia verrucicornis*, now officially known as *Aeolidiella stephanieae*, consumes only anemones of the *Aiptasia* species, a feature that has increased its popularity among aquarium overseers everywhere. Not much information exists on the digestive process of these aeolids, but what exists notes that *A. stephaniae* seem to house parts of the anemone it consumes in the cerata on its back, a practice commonly seen in other aeolids that feed on cnidarian species. By observing the location of nematocysts in *A. stephaniae* at different intervals, we may be able to determine the path that nematocysts take on their route to the cerata. To observe this trend, we tested three different live stains to determine its potential as a viable stain. The anemones were bathed in the selected stain and fed to the nudibranchs. We found that using a 6.8×10^{-5} M solution of Aniline Blue was the best for viewing. The *A. stephaniae* were collected at different times after feeding and fixed so that they might be viewed by light microscopy. Data based on the orientation and location of nematocysts was collected and analyzed.

Dedication

To my parents, John and Martha, my twin brother, Justin, and all my remaining family who have always been a great source of love and support. Life would be a lot duller without you all.

Acknowledgements

I would like to thank Dr. Ian Bricknell and Erin Switzer for their guidance in designing the experiment and obtaining the necessary components of the experiment. I would also like to thank Patricia McNamara for her assistance in caring for the *Aeolidiella stephaniae* and the *Aiptasia*. I am grateful for Dr. Ian Bricknell, Erin Switzer, and Dr. Martin Stokes for their review of either the drafts or portions of this manuscript. I would like to recognize Dawna Beane of the U. Maine Animal Health Lab for her help in acquiring stains and sectioning the specimens. I would like to thank Neil Greenberg of the Aquatic Research Center for his help in building the system the nudibranch system, as well as in the acquisition of the *A. stephaniae*.

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Introduction

The anemone *Aiptasia pallida* is a species that grows well in tank conditions under heavy lighting. Normal tank conditions cause the anemone to flourish, and it forces other species out of their environment during its growth if it is not regulated. Originally, manual control of the anemone was the only form of regulation. It was later found that some nudibranch organisms, particularly the *Aeolidiella stephaniae* (formerly known as *Berghia verrucicornis*) could be used as a natural control of *Aiptasia*. (Reefs.org, 2012).

Aiptasia is a sea anemone that is a scourge of modern saltwater tank systems. *Aiptasia* can be autotrophic organisms, which means they are able to use the energy from light nutrients to fuel their biological processes. The bright fluorescent lighting in tanks provides overly sufficient light, which allows the *Aiptasia* to grow at exponential rates (Kempf and Brittsan, 1996). The rapid growth can be extremely detrimental to the ecosystem in which the *Aiptasia* exists. The anemone can latch onto stationary organisms and prevent organisms in the ecosystem from proper feeding and reduce the intake of nutrients by those organisms, negatively affecting their growth. The asexual reproduction of *Aiptasia* is another characteristic in its favor. *Aiptasia* easily reproduce through pedal reproduction, a form of asexual reproduction in which the organism loses small regenerative portions of itself as it moves (Kempf and Brittsan, 1996).

Cnidarians are characterized by multi-use stinging cells called cnidocytes. There are varying types of cnidocytes, differing in style and firing mechanism. Nematocysts are found in all of these stinging structures. These cells can be used offensively to hunt and capture prey, as well as defensively to defend the cnidarian against potential predators. (Greenwood, 2009). Nematocysts are made up of a barb connected to a thread-like filament contained within the cell, as seen in figure 1. Once triggered, a flap called the operculum opens, allowing the barb and filament to exit the cell.

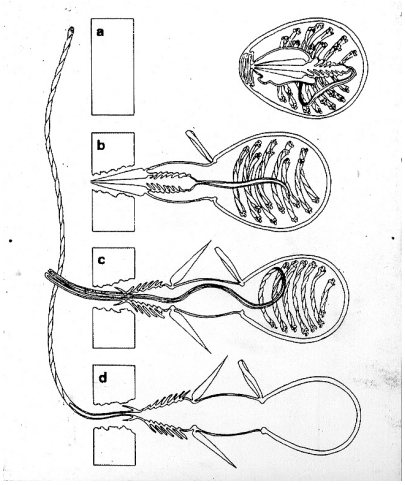

Figure 1. Diagram of unfired nematocyst. Note the trigger, or cilium, that can cause the firing mechanism. The barb can be seen surrounded by the coil of filament. When fired, the barb will exit the nematocyst and inject the filament into the predator. (From Moffett, 1996)

In *Aiptasia*, as well as in other organisms that use nematocysts for defensive purposes, the nematocyst containing cells are imbedded in the epidermis between other epidermal cells (figure 2).

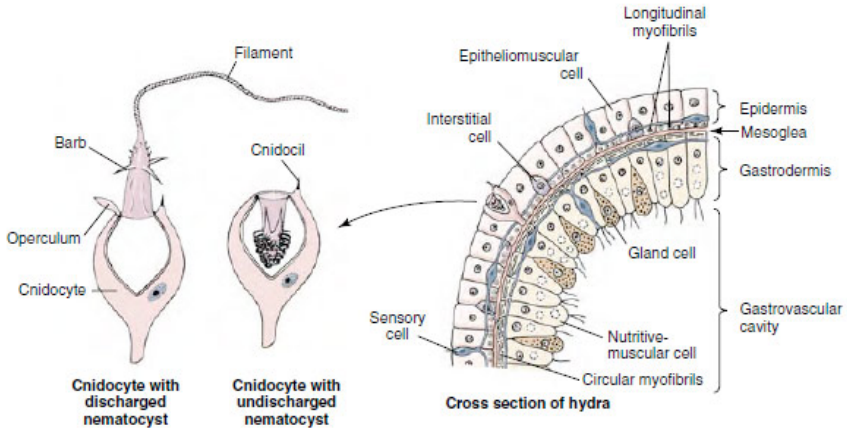

Figure 2. Placement of cnidocytes in epidermis layer. The orientation of the cnidocyte allows the nematocyst to be fired out from the epidermis of the cnidarian. (From Phylum Cnidaria, n.d.)

A cilium, called a cnidocil, that can be a trigger for the firing mechanism is located on the outside of the cell, so the cells position in such a way that the barb, if triggered, will fire out of the cell towards the potential threat, rather than into the cnidarian. When an organism brushes against the cilium, it triggers the firing of the barb located inside the nematocyst. The firing mechanism is most likely influenced by a sudden change in the osmotic pressure of the cnidocyte caused an influx of calcium. The pressure within the cell rises, eventually leading to the expulsion of the barb from the nematocyst. The barb punctures the offending organism, and the thread is released through the barb. This process can be seen in figure 1. A toxin is then pumped through multiple spines on the thread into the affected organism. This toxin causes paralysis, as well the stinging sensation that is associated with the creatures that possess them. (Ruppert and Barnes, 1996).

Aiptasia is naturally consumed by some nudibranchs, which can help control the growth of the anemone. Nudibranchs feed mainly on a specific genus of cnidarians (Kempf and Brittsan, 1996). The *Spurilla neapolitana,* a nudibranch found in the Mediterranean, West Atlantic, and East Pacific, feeds on as many as 37 species of sea anemones as a part of the organism's diet (Schlesinger, Goldshmid, Hadfield, Kramarsky-Winter, and Loya, 2009). Doekpe, Herrmann, and Schuett (2011) noted that nudibranchs possess the ability to incorporate unfired nematocysts in the cerata, which are dorsal structures that protrude from the backs of nudibranchs. Kempf (1991) demonstrated that ingested pieces of *Aiptasia* pass through the digestive system and into the cerata. Once there, the *Aiptasia* are engulfed by nutrient processing cells. Kempf did not find any signs of digestion in the cerata, which could suggest that the cnidosacs in the cerata only act as storage, not digestive entities.

It is possible that mucus and other structures in the organism behave as protective barriers against nematocysts. Studies on the movement of nematocysts throughout various nudibranch species observed that nematocysts are stored in cells of the ingestor organism, called cnidosacs, through foreign organellar retention (Schlesinger *et al*., 2009). Another study tracked the movement of the nematocysts throughout the digestive structures of *Cratena peregrina*, a nudibranch related to *A. stephaniae*, finding the nematocysts to be stored in the cerata of the nudibranch before being excreted (Martin, 2003).

Like many nudibranchs, *A. stephaniae* has cerata along its dorsal side that change color after the organism has fed (Carroll and Kempf, 1990).

It is hypothesized is that the nematocysts would pass through a portion of the digestive system and align in the epidermis of the cerata similarly to nematocyst alignment in *Aiptasia*. The null hypothesis is that the nematocysts will be evenly distributed among the tissue of the cerata, as well as oriented so that the firing mechanism will expel the barb towards the outside of the cerata. The purpose of this experiment is to determine if the nematocysts pass through the digestive system unaffected, and if so, whether they orient themselves in the epidermis in the same manner they are known to be oriented in the original host.

Materials and Methods

Aiptasia Care

The *Aiptasia* anemones were removed from a tank containing a sump of a larger aquarium system. Clumps of green algae were placed into a large, plastic container filled with water from the sump tank, where the algae was separated into smaller sections. The anemones were removed from the algae with the aid of forceps and a dissecting microscope and placed into a tank, with the dimensions 20”x11”x12”, equipped with a filter, heater, and two sections of plastic egg crate grids. The *Aiptasia* anemones were kept in a tank separate from the other

organisms. Salinity, pH, temperature, ammonia, nitrite and nitrate levels were measured everyday. Salinity was kept at approximately 34% ± 2%. Temperature was kept at about 23°C ± 3°C. Ammonia levels were kept at <0.1 ppm, nitrite at <0.2 ppm, and nitrate at <0.2 ppm (Holmes-Farley, 2008). A water change of a fourth was performed if any of the listed water parameters were found to be outside the acceptable range. Anemones were fed small, frozen mysis shrimp dropped directly into the arms of the anemones every other day. Visible waste on the floor of the tank was siphoned out on days when the *Aiptasia* were not fed.

Three vital dyes (Aniline blue, Tryphan blue, Bismarck brown) were tested on the anemones. One gram (g) of the diluted or powered dye mixed with 20 milliliters (mL) of filtered seawater formed each dye. Three anemones were removed from the tank and placed into a small container with enough water to cover the top of the anemones. Anemones were bathed in the stain for 30 seconds, one dye per anemone. The effect of the dye within the anemones was observed. Dyes were also injected into the anemones. The dyes were compared based on the ability of the dye to be retained in the *Aiptasia*.

A. stephaniae Care

Fifteen adult *A. stephaniae* were bought from aquaculture stock supplier and placed into a tank. The nudibranchs were placed into containers that separated the organisms into groups (Figure 3). Salinity, pH, temperature, ammonia, nitrite and nitrate levels of the system were measured everyday. Salinity was kept at

approximately 33% ± 2%. Temperature was kept at about 18°C ± 2°C. Ammonia levels were kept at <0.1 ppm, nitrite at <0.2 ppm, and nitrate at <0.2 ppm (Holmes-Farley, 2008). A water change of half was performed if any of the listed water parameters were found to be outside the acceptable range. *Berghia* were fed *Aiptasia* every two days.

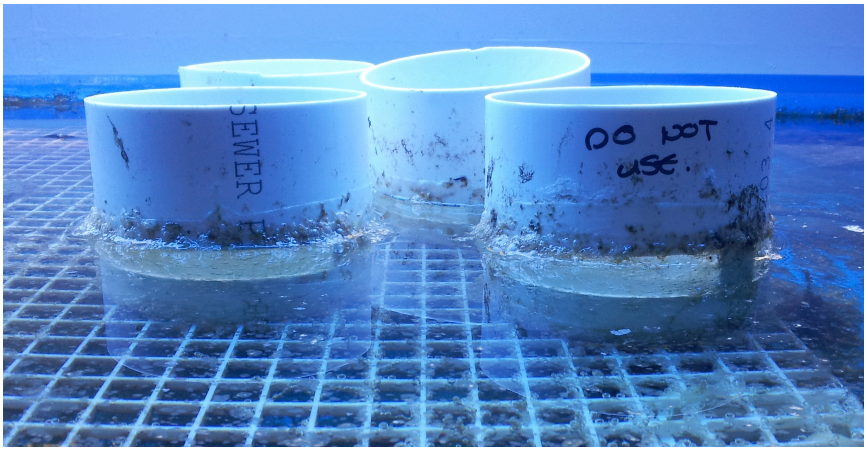

Figure 3. Set up of containers used to house *A. stephaniae*

Experimental Design

The *A. stephaniae* were starved for one day prior to the experiment day. Stained *Aiptasia* placed in each container allowed for observation to select a reasonable set of times to assign to the *A. stephaniae* containers. The *A. stephaniae* were removed at 1 hour (h), 1.5 h, 2 h, and 2.5 h. The samples were placed into a fixative of 10% formalin in filtered salt water and sent away for sectioning (Kempf, 1984). The sectioned samples were stained with a hematoxylin and eosin stain and mounted on slides, which were observed using a light microscope. Nematocysts were located, and notes were made of the distance and orientation of each in relation to the epidermis of the cerata. Five orientations in relation to the

epidermis were determined, and nematocysts were assigned a value between 0 and 4. These values are summarized in figure 4. A value of 4 features the barb assembly of the nematocyst facing outwards and perpendicular to the epidermis. A value of 3 features the barb assembly facing approximately 45 degrees from the position of value 4. A value of 2 features the barb assembly of the nematocyst facing parallel to the epidermis. A value of 1 features the barb assembly facing approximately 45 degrees from the position of value 2. A value of 0 features the barb assembly facing inwards and perpendicular to the epidermis. A Fisher's Exact Test and a chi-squared test were performed on the data to calculate for significance through the use of online programs (citations).

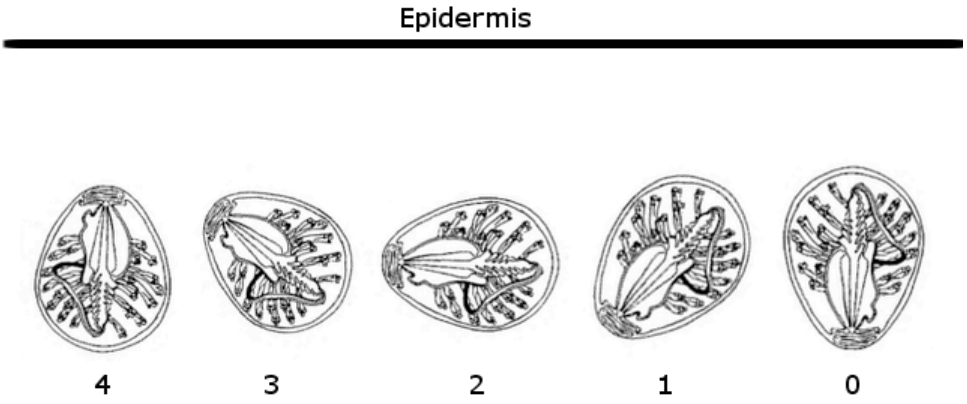

Figure 4. Orientation values in relation to nematocyst cerata epidermis. Note that the orientation for 3 and 1 can be to the right or the left. Figure obtained in part from Moffett, 1996.

Results

Staining Determination

The live stains that stained the *Aiptasia* anemones were Aniline blue, Tryphan blue, and Bismarck brown. All three of the stains that remained in the *Aiptasia* caused a noticeable change in the color of the cerata of *A. stephaniae*, although the intensity of each varied (Table 1). The visibility of each stain can be seen in figure 5. Aniline blue was found to be the most favorable choice for staining, while Bismarck brown was the least favorable choice for staining.

Table 1

Summary of live stain results; concentration and visibility

Live Stain Name	Molecular weight	Concentration	Visibility in *Aiptasia*	Visibility in *A. stephaniae* cerata
Tryphan Blue	0.4% m/v solution	5.2×10^{-5}	High	Medium
Bismarck Brown	419.32 g/mol	1.2×10^{-4}	Medium	Medium
Aniline Blue	737.76 g/mol	6.8×10^{-5}	High	High

Figure 5. Comparison of staining effect on *A. stephaniae*. a. *Aiptasia* anemone stained with Tryphan blue. b. *Aiptasia* anemone stained with Aniline blue. c. Unstained *Aiptasia* anemone. d. *Aiptasia* anemone stained with Bismarck brown.

A. stephaniae Observation

An initial observation of feeding of the *A. stephaniae* suggested a rate of passage of approximately 3.5 hours or less from consumption to retention in the cerata. Figure 6 represents the difference in color of *A. stephaniae* fed stained and unstained *Aiptasia*.

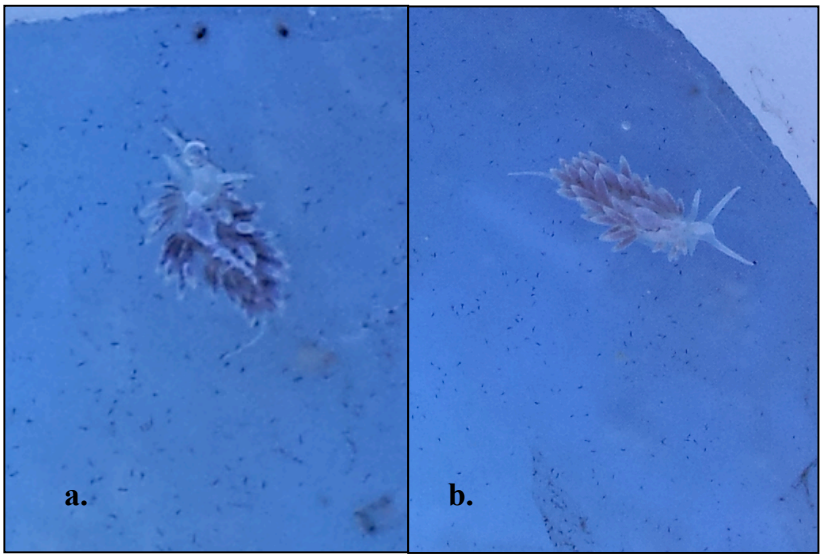

Figure 6. Physical difference of appearance stained and unstained *A. stephaniae*. (a) *A. stephaniae* that ingested Aniline blue stained *Aiptasia*. (b) *A. stephaniae* that did not ingest stained *Aiptasia*

A. stephaniae staining

The following pictures are of intact nematocysts located in the cerata of an *A. stephaniae* organism. The intact nematocysts appear as a bright pink and approximately 20 micrometers long. The ridges along the outer border of the nematocyst are the coiling of the barb. Nematocysts were observed within 25 µm of the epidermis, as well as at distances farther than 125 µm. Figures 7 and 9 depict nematocysts at 10x magnification. Figures 8 and 10 show the previous mentioned nematocysts at 40x magnification, so that the position of the nematocysts can be seen in context to their position within the lumen of the cerata.

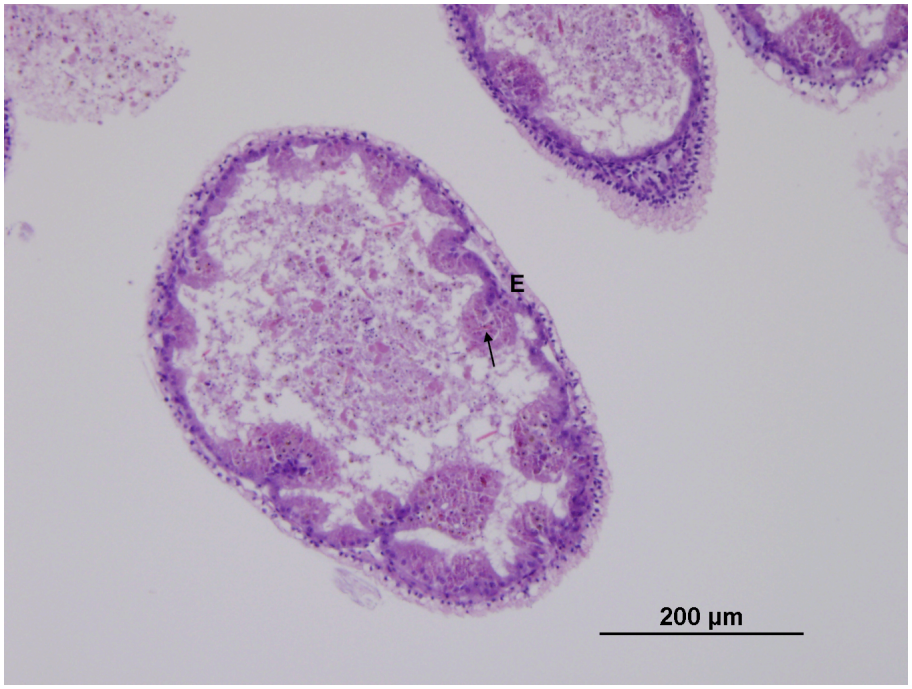

Figure 7. Intact nematocyst in relation to the portion of cerata. The 'E' labels the epidermis. The arrow indicates the location of the nematocyst.

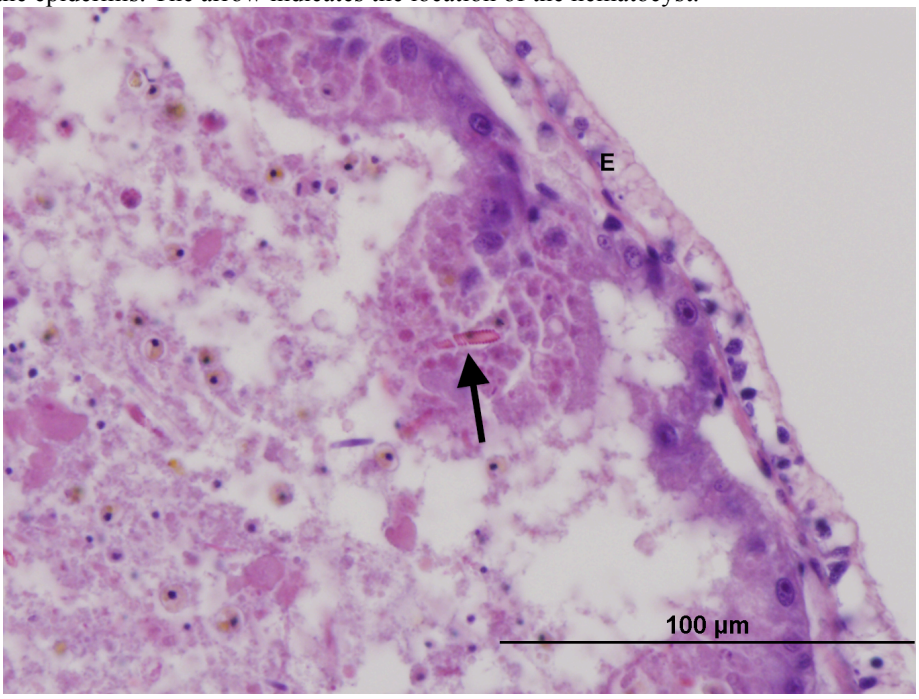

Figure 8. Intact nematocysts with visible coiling. The 'E' labels the epidermis. The arrow indicates the location of the nematocyst. Note the classic coiled appearance resulting from the contracted barb assembly.

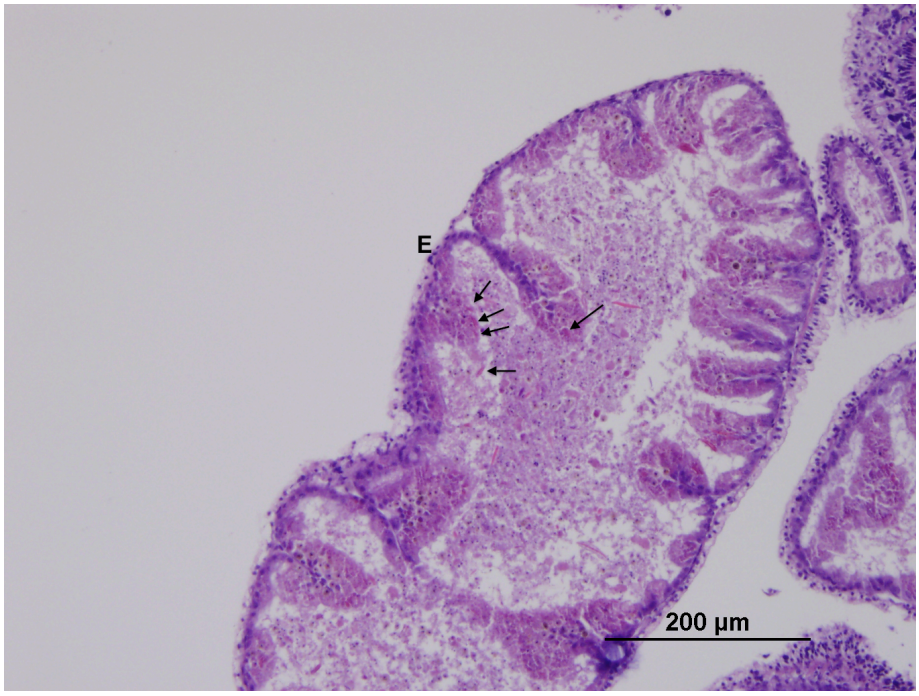

Figure 9. Distribution of nematocysts. The ‘E’ labels the epidermis. The arrows indicate the location of the nematocysts.

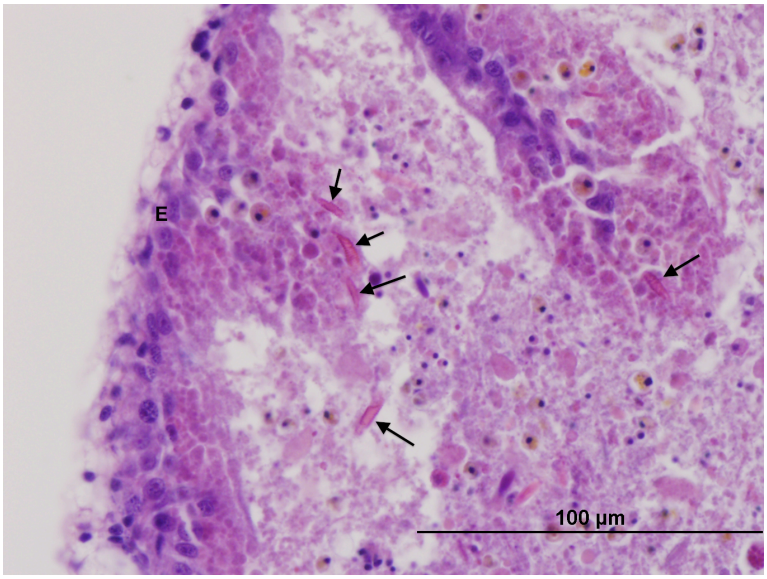

Figure 10. Distribution of nematocysts. Two nematocysts are within 25 of the epidermis. Two nematocysts are with 100 of the epidermis. One nematocyst is located with 125 of the epidermis. The ‘E’ labels the epidermis. The arrows indicate the location of the nematocysts.

The following graphs represent the orientation and distance in relation to the epidermis of all observed nematocysts within the four sample *A. stephaniae*. The graph in Figure 11 groups the data in five clusters, with each cluster representing an orientation value (0-4). Within each cluster are three separate entries for the distances from the epidermis. The graph in Figure 12 groups the data in 3 clusters. Each cluster represents a range from the epidermis. Within each cluster are five separate entries representing the orientation of the nematocysts. The distances were split into smaller segments, such that the nematocysts were counted for every 25 μm from the epidermis. This data represents the frequency distribution of the nematocysts (figures 13 and 14). The chosen statistical test for significance, the Fisher's Exact Test displayed a p value of 5.9×10^{-5} (figure 15).

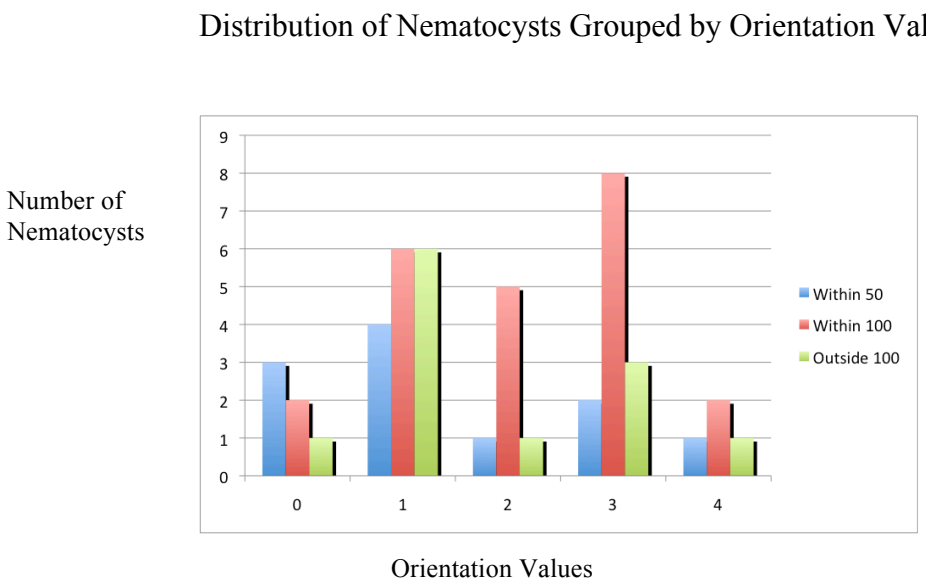

Figure 11. Distribution of Nematocysts grouped by orientation value. Each orientation value has three separate entries for the three distance ranges from the epidermis.

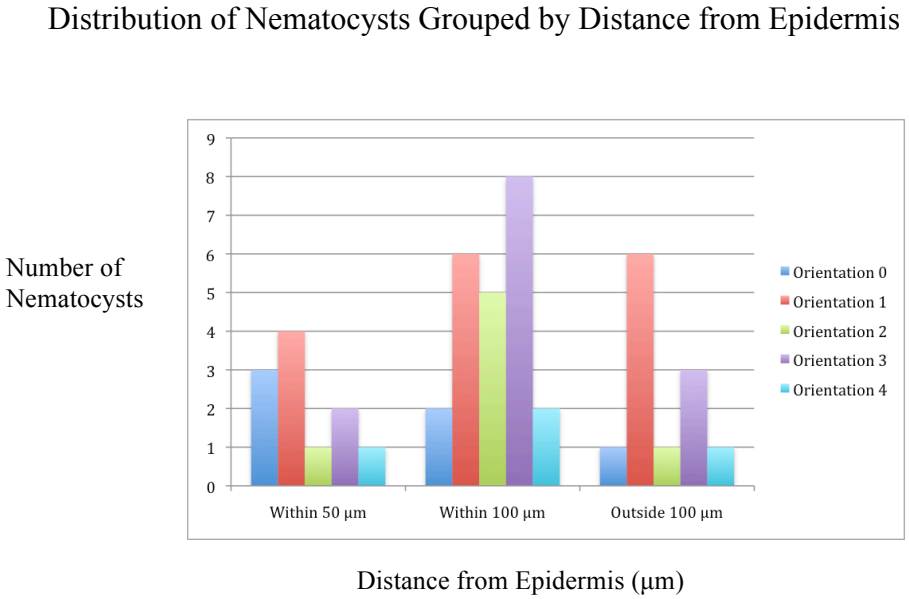

Figure 12. Distribution of nematocysts, grouped by distance from epidermis. Each distance has five separate entries for the assigned orientation values.

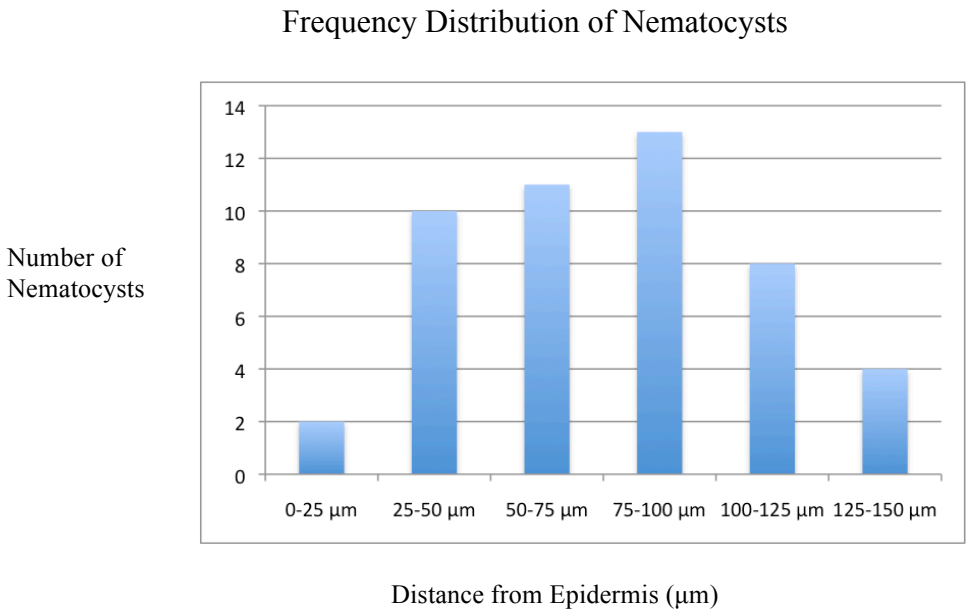

Figure 13. Frequency of nematocysts, grouped by distance observed from epidermis.

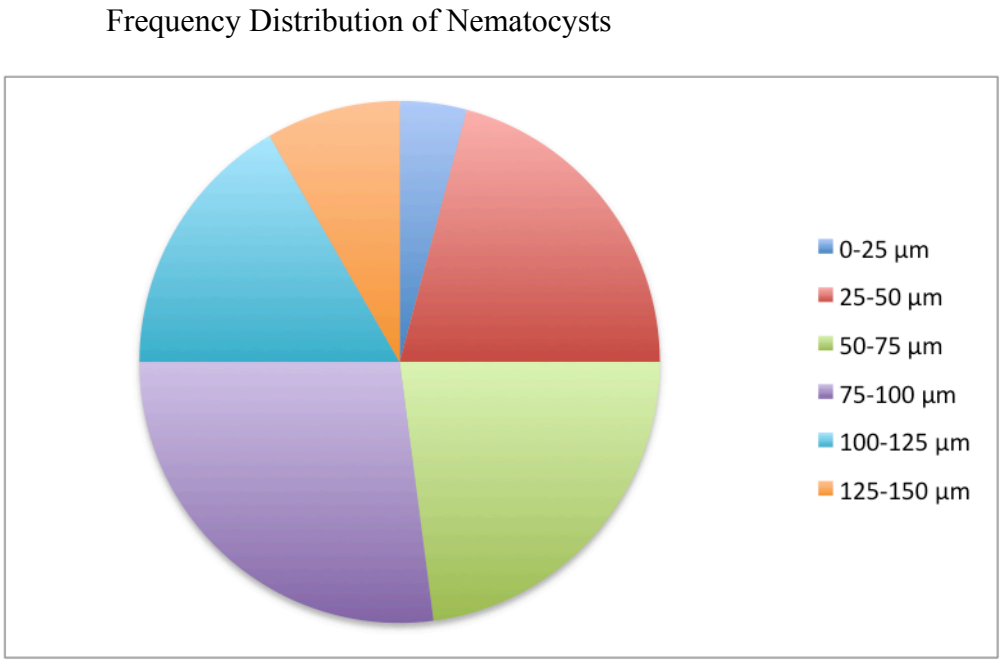

Figure 14. Frequency distribution of nematocysts, grouped by distance from epidermis.

r × *c* Exact Contingency Table: Results

The results of an exact contingency table test performed at 20:54 on 11-DEC-2013

data: contingency table

	A	B	C	D	E	
1	3	4	1	2	1	11
2	2	6	5	8	2	23
3	1	6	1	3	1	12
	6	16	7	13	4	46

expected: contingency table

	A	B	C	D	E
1	1.43	3.83	1.67	3.11	0.957
2	3.00	8.00	3.50	6.50	2.00
3	1.57	4.17	1.83	3.39	1.04

The given table has probability 5.9E-05
The sum of the probabilities of "unusual" tables, $p = 0.767$

Figure 15. Results of a Fisher's Exact Test. (From Kirkman, 1996).

	Gp 1	Gp 2	Gp 3	Gp 4	Gp 5	Gp 6	Gp 7	Gp 8	Gp 9	Gp 10	
Cond. 1:	6	16	7	13	4						46
Cond. 2:											0
Cond. 3:											0
Cond. 4:											0
Cond. 5:											0
Cond. 6:											0
Cond. 7:											0
Cond. 8:											0
Cond. 9:											0
Cond. 10:											0
	6	16	7	13	4	0	0	0	0	0	46

Output:

Calculate | Reset all

Chi-square: 11.174
degrees of freedom: 4
p-value: 0.02467655
Yates' chi-square: 9.005
Yates' *p*-value: 0.06097463

Status: Status okay

Figure 16. Results of a Chi Squared Test. The result of the chi-squared test was a p-value of approximately 0.02 (From Preacher, 2001).

Discussion

Staining Determination

Two tests considering the effectiveness of a list of live stains were designed. The stains were selected for testing based on what parts of the cell were stained. The first test was a determinant of whether the stain would be taken up in the *Aiptasia*. The stain was given by two methods: bathing and injection. The stains that remained within the *Aiptasia* were moved to the next test. The dyed *Aiptasia* were fed to the *A. stephaniae*, which were observed a day later. Since it is known that the cerata of *A. stephaniae* will turn a dark color after feeding, the theory was that the stain from the consumed *Aiptasia* would be visible once it had reached the cerata. The stain that was most visible was selected as the dye to be used.

Originally, there was a list of six complied live stains (Aniline blue, Tryphan blue, Janice Green, Bismarck brown, Carmine, and Neutral red) that would be tested. Of the original list, three of these were ruled out before testing on *Aiptasia*. Janice green was dropped from the list because it was not available from the source the stains were acquired from. Carmine was ruled out for two reasons, the first of which was the lack of a molecular weight for the actual compound I had. The MSDA sheet that accompanied the dye did not have a listed molecular weight, and multiple sources listed different molecular weight for the dye, so I decided to discount it as a possibility.

Aniline Blue was determined to be the most appropriate dye to use due to the strength of its color in the *Aiptasia* and *A. stephaniae*. Though all three live stains were visible in *Aiptasia* and anemones, the color that Tryphan blue was highly visible in the *Aiptasia*, but provided a weaker visible color in the cerata. Bismarck brown produced a highly visible color, but the color was too similar to the natural color of the *Aiptasia*, so it was given a rating of medium visibility in the *Aiptasia*. When the stained anemone was given to *A. stephaniae*, the color did affect the dorsal section of the nudibranch. However, the color of a nudibranch having fed on Bismarck brown stained *Aiptasia* was not significantly different from the color of *A. stephaniae* having fed on unstained *Aiptasia*. This lack of difference in color indicated that the nematocysts might not have stood out much from the normal interior structures of *A. stephaniae.* For this reason, Bismarck brown was rejected as an optimal choice. Aniline blue produced a highly visible color in the *Aiptasia* and the cerata.

A. stephaniae Observation

The dyed *Aiptasia* was fed to the *A. stephaniae*, and the *A. stephaniae* were observed at one-hour time intervals over the course of a day to ensure that the time frame used to take samples taken from the *A. stephaniae* could be tailored to fit the rate of passage through the *A. stephaniae*. It would not have made sense to carry out the experiments over time intervals of six hours if the nematocysts take only two hours to reach the cerata.

A. stephaniae Staining

The nematocysts were only observed in the lumen of the cerata, mostly with 25-100 micrometers of the epidermis of the cerata. The nematocysts were expected to be found evenly throughout the cerata, and yet the data actually displayed a pattern similar to a bell curve. Many of the nematocysts were found at between 25 and 100 μm from the epidermis. This could indicate a preference for this range of distances from the epidermis. The p-value from the fisher's exact test, selected because of the small sample size, was extremely low at 0.000059. This low value indicates that the data input into the fisher's exact test was not significantly different, which allows the null hypothesis that the nematocysts would be evenly distributed through the cerata to be rejected.

It was expected that the nematocysts would orient themselves with in the epidermis so that the nematocysts could be fired outwards. As the data reflects, this was not the case, as observed in the four sample *A. stephaniae*. Had the nematocysts in the *A. stephaniae* oriented as in cnidarians, it would be expected that all of the nematocysts would have been observed with orientation values of 4. This was not what was observed. The nematocysts were found in multiple orientation values with most of the nematocysts laying in the orientation of values 1, 2, and 3. This could indicate that the orientation of the nematocysts is random, which is supported by the result of the chi-squared test. The result of the p-value was approximately 0.02, and since the threshold for significance for this

experiment was 0.05, it can be concluded that the data is not significantly different, as the p-value was lower than 0.05.

The sample size was fairly small, so that could be a contributing factor to our results, but using the two conclusions from the results of the fisher's exact test and the chi-squared test, the null hypothesis that the nematocysts are evenly distributed and oriented in one direction can be rejected. It can be inferred that the nematocysts may not act in *A. stephaniae* as in *Aiptasia* and other cnidarians. Most likely the nematocysts act as a deterrent to potential predators in a slightly different way. The nematocyst may function by positioning so that the predator receives a mouth full of nematocysts if the cerata are bitten off rather than firing when the predator brushes against the outside of the cerata of the *A. stephaniae*.

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Author Biography

Siobhan Bolinger was born to John and Martha Bolinger on the first of January, 1991 in England. Her twin brother, Justin, followed soon after. After moving to Florida shortly after her birth, the family decided to settle in Gorham, ME, where Siobhan received her elementary and secondary education. After graduating from Gorham High School in 2009 magna cum laude, Siobhan went on to attend the University of Maine. Along with juggling studies, Siobhan found time to participate in 3 student organizations. She also found time to participate in the UMADCOWS program twice, milking the Holsteins at J.F. Witter Teaching and Research Center twice a week for a semester. Siobhan has enjoyed her time in the Honors College for the wonderful opportunities and challenges it provided her. She hopes to eventually continue her education to become a veterinarian for large animals, but she is excited to see where life will take her after graduation.