Regeneration and Regrowth of *Mycale* sp.*, Halochondira panicea* and *Phakellia* sp. Sponges in the Pacific Northwest

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Abstract

Sponges (phylum Porifera) fall short of being considered true animals, Eumetazoas, because of their unique tissue organization. This organization is questionability multicellular because of their cell-to-cell nutrient transfer and lack of epithelialization. Sponge cells have, however, been shown to have cell to cell communication and recognition within an individual. To assess the degree of a sponge's ability to act as a coherent metazoan organism by recognizing and repairing damage to a specific area, three morphologically different demosponge sponges of the species *Halochondria panicea*, *Phakellia* sp. and *Mycale* sp. were wounded and monitored. Both growth and regeneration were evaluated by observing surface area of growth per unit time with regards to overall percent area change and oscula formation respectively in the field over an 18-day period by time series photographs and analyzed with ImageJ software. The individuals of the *Phakellia* sp. could not display results due to environmental disturbance. The intertidal encrusting sponges, *Mycale sp*., *Halochondria panicea*, both showed signs of overall growth in the wounded region without a statistical difference between the two species (P=0.877). The new outside growth rate of the entire sponge opposed to healing growth rate of the wounded region in both sponge species also proved to be statistically insignificant (*Halochondria panacea* P=0.114 and *Mycale* sp. P=0.343). Regeneration by oscula formation was observed in both encrusting species after day two for *Mycale* sp. and by day 10 in *Halochondria panicea*. These results further show that growth and regeneration in a specific wounded region may be an adaptation to remain a specific shape for the entire sponge individual as well as to maintain survival in a competitive environment such as the intertidal.

Introduction

The phylum Porifera contains organisms which are defined by their unique tissue organization. Sponge 'tissues' are composed of single cells or syncytia that function as units to form oscula, the main space through which water and nutrients flow out (Hooper and Van Soest, 2002). Sponges lack epithelia or true gut. For this reason they are considered to be metazoans but not eumetazoans. The question has been raised whether sponges should be considered a single organism or colony of cells working together (Simpson, 1984). Sponges in the class Demospongiae typically consist of at least seven cell types: choanocytes, sclerocytes, archeocytes, porocytes, oocytes, pinacocytes, and lophocytes. Choanocytes, porocytes, lophocytes and pinacocytes help to shape the sponge tissue. Sclerocytes are involved in spicule formation. Archeocytes

perform a variety of functions in the sponge including reproduction, aiding in digestion and waste disposal. Being totipotent the archeocytes have the ability to turn into more specialized cell types (Hooper and Van Soest, 2002). Further, sponges have the ability to regenerate after the individual has been damaged (Ayling, 1983).

Sponges are sessile and vulnerable to disturbances such as predation (Dayton, 1971), disease (Webster, 2007), or wave surge (Ayling, 1981). This may cause damage to the sponge. Their success relies heavily on their ability to regrow and regenerate after damage has been done and sponge material is removed (Chadwick and Loya, 1990). Some sponges have the ability in the most extreme case to regenerate and grow even when dissociated down to the cellular level (Hildemann Johnson and Jokiel, 1979).

Cellular recognition was shown in dissociated sponge cells when two individuals of different species were placed together (Van de Vyver, 1975). The sponges would aggregate into separate colonies rather than combine into one sponge. This phenomenon has also been observed in two individual sponges of the same species (Hildemann Johnson and Jokel, 1979). Grafting experiments have shown demosponge to have non-self recognition when grafted with another sponge individual (Smith and Hildemann, 1986). These kinds of experiments have served to show self-recognition in sponges. Sponges maintain the ability to recognize self from nonself via cell to cell communication (Van de Vyver, 1975). Growth rates in damaged demosponge were found by Ayling (1983) to be faster than growth rates in undamaged sponges, implying that sponges indeed have the ability to recognize damage.

Regrowth is seen by change in the area or volume of the damaged sponge; regeneration is seen by osculum formation in the damaged sponge (Duckworth, 2003). In this experiment the growth of the three sponge species was expected to be different due to their morphological variation. In addition, the wound site was expected to grow at a faster rate than that of the undamaged edge of the sponge. Three different species were observed to measure differences in each species' ability to regrow and regenerate. *Halochondria panicea* is a thin encrusting sponge with nipple-like oscula usually no more than a centimeter tall. It is typically green, yellow, or purple in color and may be found along the Pacific Coast. *Mycale* sp. is a thick encrusting yellow sponge with many perforations and more integrated oscula. It may be found in temperate waters (Hooper and Van Soest, 2002). *Phakellia* sp. is a leafy cone shaped sponge, which is typically found in the subtidal (Hooper and Van Soest, 2002). Individuals from each species were damaged and observed. Sponges were noted on their ability to regenerate by observing osculum formation and regrow by noting changes in wound and sponge area since both regrowth and regeneration are considered separate processes.

Methods

Study site and experimental design

Three different sponge species from the class Demospongiae were chosen to compare growth rates and regeneration in different morphologies. The first site located at Eagle Cove on the southern part of San Juan Island, Washington, consisted of a rocky intertidal environment. Five individuals of the species *Halichondria panicea* were chosen

at this location. The Friday Harbor Laboratory dock intertidal served as the second site harboring a total of eight sponge individuals. Four *Phakellia* sp. individuals (originally obtained from a subtidal region in the harbor) were hung from a rope off the dock and four *Mycale* sp. sponges were found growing on tires bordering the dock. Individuals at both sites were chosen at random in locations accessible to observe daily and obtain consistent pictures from. All the sponges were large enough to have at least an entire 2x2 cm^2 section removed. Because the experiment remained fully in the field, undamaged controls were observed near each sponge throughout the experiment to note predation, disease and wave surge effects.

Tissue removal

Each sponge was wounded with a sharp dissecting scalpel to ensure as much initial precision in the field and done so as follows: In the most central part of the sponge a 2x2 cm^2 section was removed. Tissue removal larger or smaller than the intended dimensions was also noted. The wound went through to the substrate for the encrusting (*Halichondria panicea*) sponges, through the entire tissue of the *Phakellia* sp. sponges and approximately one centimeter deep for all *Mycale* sp. individuals unless substrate was met beforehand. Majority of the encrusting sponge basopinacoderm was removed via forceps and any other remnants noted with pictures. The wound tissue served as a sample to be taken back in collection vials back to the lab for identifying the sponge species.

Monitoring/observations

The experiment ran from 28 June 2012 to 16 July 2012. To examine the wound recovery and regeneration of the different sponge species observations were made right before, immediately after, two, six, 10 and 18 days after wounding. Observations consisted of photographing the individual at the same angle with a ruler to provide a scale to ensure precision and note for later data analysis using ImageJ software (ImageJ 1.43u). Using this method a full body photograph before and after wounding plus a close up of the wound were taken. Additional monitoring included recording wound dimensions to the closest one millimeter, overall sponge health, wound edge healing (noted by smoothing or rounding out of surface wound edges), near vegetation, organisms, substrate, the controls health, and potential predators.

Osculum development measured overtime indicated the amount of regeneration a sponge individual has undergone. Oscula on all three species were identified as being fewer in number and a notably larger canal than the smaller incurrent pores. By the time series photographs and during each observation new oscula formation was recorded for each individual.

Data analysis

In the lab, each sponge time series photograph was processed using ImageJ software to accurately calculate the surface area of each wound over time. Through this the surface areas of growth per day were recorded to calculate mean growth of both *Mycale* sp. and *H. panicea* by day 18 and compared with a traditional t-test using the statistical sigma pi program, due to a normal distribution. Overall percent growth of the wound area from the initial wounding until the last experiment observation was

calculated using the overall change in area of the wounded region. Both the percent growth for each individual and overall mean for each species was calculated.

$$\% \text{ growth} = 1 - A_t/A_0 \times 100$$

Where A_0 and A_t are the area of the wounded regions on day 0 (immediately after injury) and day t, respectively.

The total final surface area in cm^2 at day 18 of each sponge individual was calculated by subtracting the remaining wound area from the whole sponge area.

$$TA = A_s - A_w$$

Where A_s is total area of sponge and A_w being remaining wound area.
The rates, G1: new outside growth and G2: new "healing" growth, were recorded in cm^2/t. The G1 and G2 values were compared to each other within each sponge species using the Mann-Whitney U Test under the assumptions that the difference between the samples is not normally distributed and they were chosen at random

Results

Encrusting sponge species, *H. panicea* and *Mycale* sp., both showed almost total regrowth by day 18 of the experiment, however both failed to display complete regrowth of the wounded areas (Fig. 1). *Mycale* sp. initially showed a higher percentage of regrowth, however, by day 18 both sponges were fairly equivalent, 76.1% percent growth in *H. panicea* and 83.0% in *Mycale* sp. (Table 1). Both *H. panicea* and *Mycale* sp. initially reconstructed the surface pinacoderm by smoothing out and rounding the wounded edges, however, subsequent regrowth and regeneration began to differ. The inner, yellow colored tissue, distinguishable from the thicker, green surface, of the thin, encrusting sponge, *H. panicea*, grew in from the wound edges onto the substrate. This thin layer of tissue never demonstrated thickening of the sponge tissue until oscula formation. By day 18 *H. panicea* appeared to of filled in approximately one third of the sponge wound. Because the individual sponges were thicker, the wounds of *Mycale* sp. were surrounded by live tissue. Growth and regeneration was observed from all surfaces of the wound, and created a gradual encroachment of new tissue. By day 18 majority of the *Mycale* sp. individuals appeared to of filled in over 50 percent of the wound depression area. *H. panicea* had a mean growth into the wound of 3.421 cm2 and *Mycale* sp. a mean growth of 3.297 cm2 from day 1 to day 18 , but there was no significant statistical difference (T=0.162, n = 4, P=0.877).

To determine if there was preferential growth, the overall sponge growth rate was compared to the wound growth rate (Table 2). *H. panicea* had a new outside growth rate (G1) median of 2.444 cm2/day and new "healing" growth rate (G2) median of 0.186 cm2/day, and there was no significant difference between the two (T=24.00, n=4, P (exact)=0.114). *Mycale* sp. had a median G1 of 0.357 cm2/day and median a G2

of 0.189 cm2/day. The difference between the two was not significant (T=22.00, n=4, P (exact)=0.343).

As a measure of regeneration, new oscula formation was observed with differing trends between the two species (Table 3). One *H. panicea* individual formed one new osculum by 10 days and by the end of 18 days 75 percent of the H. panicea individuals formed oscula. In contrast, *Mycale* sp. had one individual form one new osculum by day 2 and all individuals account for new oscula formation by day 10.

Discussion

The two morphologically encrusting sponge species, *Halochondria panicea* and *Mycale* sp., both showed amazing regrowth capability in the experimental time of 18 days. *Phakellia* sp. individuals of shaped morphology never displayed the ability to regrow or regenerated and failed to be analysed in data because of degraded health over the course of the experiment caused by too many unknown variables. The *Phakellia* sp. were kept in the intertidal, a foreign environment in comparison to their native environment, the subtidal. Differing conditions of the two environments like wave surge, nutrition, and predation could all of been contributing factors to their degraded health and influenced the ability for a sponge individual to regrow and regenerate. *H. panicea* and *Mycale* sp. did not however display regrowth of the entire wounded area. This helped to obtain more precise growth rates measured by surface area of growth per day (cm^2 / day). It could be assumed that the sponges were still in the process of growing and full regrowth potential was just about met, giving an accurate overall growth rate for most individuals. To observe more complete regrowth an experimental design with a longer period of monitoring would be advised.

Initial percentage of growth in each wounded area were higher in *Mycale* sp., however, percentage of regrowth became equivalent between the two species by day 18. The initial growth difference could be attributed to both the morphology of the sponge individual and its spatial location environmentally e.g. substrate and the presence of remaining sponge tissue. Both sponge species began to repair and regrow by reconstructing their surface pinacoderm. *H. panicea* is a thin encrusting sponge usually no more than a centimeter tall (Hooper and Van Soest, 2002). By growing very closely to the rock substrate, post wounding left the bare area clear, exposing the substrate with only small remains of *H. panicea* tissue left over. *H. panicea* could only grow in from the wound edges, regrowing its entire basopinacoderm first before further thickening of tissue and oscula formation may be possible. This initially slower percentage of growth and appearance of only one third total regrowth of the wound area could be credited to the more intensive preliminary growth demands and a wounded region with limited surface area to grow from. The thick encrusting morphology with many perforations of the *Mycale* sp. individuals could be the explanation for initial high percentages of growth in the wounded region. The thicker morphology also influenced the result of each individual having a wound area completely surrounded by live tissue. Therefore the *Mycale* sp. individuals had more overall surface area for potential regrowth.

The mean growth area of the wounded region from day one to 18 showed no significant statistical difference with *H. panicea* having a mean growth into the wound of 3.421 cm^2 and *Mycale* sp. a mean growth of 3.297 cm^2 (T=0.162, n = 4, P=0.877). This result falsified the expectation that *H. panicea* and *Mycale* sp. sponges would overall differ in regrowth of the wound area. Where the method of regrowth varied, both sponges equivalently performed the ability and displayed both self recognition of the damaged area and the perhaps the necessity to redeem its initial morphology. Further research will need to be made because no valid conclusion that morphology influences regrowth can be made considering these results.

Growth rates in damaged sponges were found to be faster than growth rates in undamaged sponges (Alying, 1983). To further examine this, preferential growth was compared within individuals of the same species. Preferential growth of each sponge individual was tested by comparing the growth rate at the specific wound site to the growth rate of the undamaged outside edge. *H. panicea* showed no significant difference between the two rates with new outside growth rate (G1) median of 2.444 cm^2/day and new "healing" growth rate (G2) median of 0.186 cm^2/day (T=24.00, n=4, P (exact)=0.114). *Mycale* sp. had a median G1 of 0.357 cm^2/day and median a G2 of 0.189 cm^2/day. The difference between the two also was not significant therefore negating the expectation that wound growth rate would be greater than unwounded edge growth rate within the same individual (T=22.00, n=4, P (exact)=0.343). While the need for regrowth in the wounded region proves important due to prior results, the priority of wound regrowth is not greater than the need to grow and simply maintain its space in its environment. This may be due to the fact that all sponges were left in the field for the duration of the experiment and therefore vulnerable to disturbances of the field. At the Eagle Cove site, competition by other sponge individuals of different species along with predation of sea stars and crabs were recorded. The Friday Harbor dock field site appeared to have greater wave surge and competition due to other organism such as tunicates, bryozoans, and barnacles. In the variable environment of the field, each sponge individual needs to maximize its own chances of survival and maintain its spatial niche. Regrowth and regeneration appear to be one way of doing just this.

The impressive ability to regrow and recover from damage in a remarkably short period of time (18 days) was further supported by a sponge's regenerative ability, which both species displayed. Regeneration, in the most extreme of cases, has been viewed after sponge cells were totally dissociated (Smith and Hildemann, 1986). However regeneration holds its greatest ecological importance where damaged areas of sponges in their natural environment are commonly regenerated. The Regeneration capability was measured by new oscula formation in the wounded area. Regeneration is key for the sponges ability to recover in a functional manner; oscula prove to be a functional part of sponge morphology (Hooper and Van Soest, 2002). Trends varied between the two different species. One *H. panicea* individual formed one new osculum by 10 days post wounding and 75 percent of *H. panicea* individuals formed new oscula by day 18. *Mycale* sp., in contrast, displayed its regenerative capabilities earlier with one individual forming new osculum two days after wounding and all individuals accounted for new oscula formation by day 10. Observationally, osculum was formed initially by a thin

translucent layer of tissue, membrane like, followed by new pinacoderm formation for support in the *Mycale* sp. individuals. *H. panicea* displayed oscula formation only after the new basopinacoderm was reconstructed along with other supporting pinacoderm tissues. These trends could be attributed to once again greater wound surface area already existing for the *Mycale* sp. individuals in addition to a need to reform a basopinacoderm on the substrate prior to oscula formation for the *H. panicea* individuals.

For the two encrusting sponge species *Halichondria panicea* and *Mycale* sp, however, varying morphologically, did not display significant difference in their overall regrowth in a wounded area. The growth rates within one individual of the wounded area and unwounded edge also appeared to be statistically insignificant, showing no preference to growth. Oscula formation as a measurement of regeneration was noted in both species by the end of the experiment however the trends over the duration of the 18 day experiment differed. Both sponges displayed the remarkable ability to regrow and regenerate in the wound areas however could not support a difference in the species of varying morphology. Future research will be needed to define whether individuality is of a single sponge cell or an entire sponge colony. More overall sponge individuals as a sample size, a longer experimental interval of time in addition to a larger amount of sponge species with contrasting morphologies would be advised for future research.

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Table 1. Osculum formation in the wound area in both *Haichondria panicea* and *Mycale* sp. at day two, six, 10, and 18 after wounds were made. All *Mycale* sp. showed initial osculum formation before *H. panicea* however between day 10 and 18 three of the four *H. panicea* showed osculum formation.

	day 2	day 6	day 10	day 18
Halichondria panicea				
sample 1	0	0	0	0
sample 2	0	0	0	8
sample 3	0	0	0	2
sample 4	0	0	3	0
Mycale sp.				
sample 1	0	1	3	0
sample 2	0	0	1	0
sample 3	1	3	0	0
sample 4	0	1	1	2

Table 2. Percentage growth in wound area of *Halichondria panicea* and *Mycale* sp. at day two, six, 10, and 18 after wounding. Using a t-test the amount of growth in both sponges was shown to be not statistically different over an 18 day period (T=0.162, n = 4, P=0.877).

	day 2	day 6	day 10	day 18
Halichondria panicea				
sample 1	15.6%	25.9%	35.7%	71.0%
sample 2	21.9%	24.3%	84.4%	99.1%
sample 3	6.9%	14.1%	27.6%	62.0%
sample 4	34.6%	58.5%	59.9%	72.8%
mean	19.8%	30.7%	51.9%	76.2%
Mycale sp.				
sample 1	14.9%	26.6%	64.6%	97.8%
sample 2	58.5%	65.9%	76.0%	77.9%
sample 3	54.5%	80.7%	82.4%	82.4%
sample 4	32.2%	46.2%	48.1%	73.8%
mean	40.0%	54.9%	67.8%	83.0%

Table 3. Growth rate of the undamaged edge of the sponge (G1) and growth rate of the wound area (G2) after 18 days in both *Halichondria panicea* and *Mycale* sp. G1 and G2 within a species were shown to be statistically not different using a Mann Whitney U test (T=24.00, n=4, P (exact)=0.114 in *H. panicea*) and (T=22.00, n=4, P (exact)=0.343 in *Mycale* sp.).

	Total area	G1 (cm2/d)	G2 (cm2/d)
Halichondria panicea			
sample 1	251.061	5.666	0.128
sample 2	207.931	3.037	0.245
sample 3	112.659	1.851	0.126
sample 4	121.639	0.222	0.262
mean	173.323	2.694	0.190
Mycale sp.			
sample 1	34.230	0.414	0.187
sample 2	31.834	0.082	0.127
sample 3	136.666	0.300	0.228
sample 4	161.536	2.911	0.191
mean	91.067	0.927	0.183

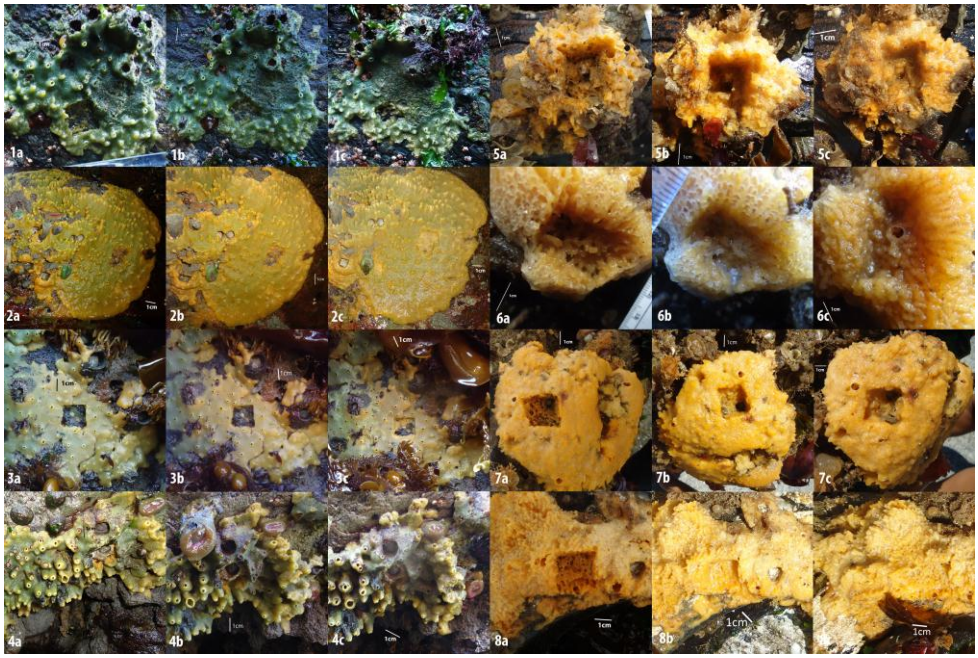

Figure 1. Time series photographs of *Halichondria panicea* (1-4) and *Mycale* sp. (5-8). Sponge when initially wounded is indicated with an "a." Sponge at day 6 is indicated with a "b." Sponge at day 18 is indicated with a "c."