Immune function in the sea cucumber *Parastichopus californicus* during viscera atrophy and regeneration

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A Thesis Proposal

Submitted to

Walla Walla University

In partial fulfillment of

Requirements for the degree of

Master of Science

May 2014

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Introduction

Importance of Echinoderm Immune System

Echinoderms face threats from a wide variety of disease-causing agents including bacteria, fungi, cyanophyta, and protozoans such as flagellata, sarcodina, sporozoa, cilliata, and protophytans (Jangoux 1987). Despite such a wide variety of threats, echinoderms appear to have only an innate and simple immune system (Smith *et al.* 2010). This system mostly relies on coelomocytes, echinoderm immune cells, which are free moving through the coelomic fluid and many of the body tissues. There is no evidence of any kind of adaptive or secondary immunity in echinoderms (Smith *et al.* 2010, Yui & Bayne 1983).

Many echinoderm immune factors have homologues in the vertebrate immune system (Gross *et al.* 1998). Because these factors have similar roles and are likely to represent a common line of descent between the groups, echinoderms are a simpler model system for studying some immune responses, such as phagocytosis. Comparative studies of allograft rejection and self-recognition in sea stars and sea cucumbers have brought about a better understanding of these phenomena in humans. (Gross *et al.* 1998).

Coelomocytes Origins

There are at least six different categories of coelomocytes in echinoderms, with some or all being found in different species. These categories include lymphocytes, amoebocyte, spherule cells, vibratile cells, crystal cells, and haemocyte cells (Chia & Xing 1996). Two hypotheses have been formulated for coelomocyte generation. One hypothesis is that they are self-propagating cells, with one or more of the different coelomocyte populations serving as stem cells for the other populations. The other hypothesis is that these cells arise from a particular

organ. In sea cucumbers this organ could be the haemal ring or vessels, respiratory trees, or the peritoneum (Chia & Xing 1996, Ramirez-Gomez & Garcia-Arraras 2010).

Coelomocyte Types

These six coelomocyte categories can be further subdivided, and in fact might not all be independent. Some of these cells may have different immune functions as they mature, and may possibly switch between types (Ramirez-Gomez & Garcia-Arraras 2010, Chia & Xing 1996, Coteur *et al.* 2002). ***Amoebocytes*** use phagocytosis to remove foreign matter from the echinoderm, and they are the majority of cells that form large aggregations known as "brown bodies". In sea stars, at least three different sizes of amoebocytes may have different roles in immune response. However, it is unknown whether these different-sized cells have different origins or if they are the same type of cell at different stages of maturity (Coteur *et al.* 2002). ***Spherule cells*** (also called spherulocytes) currently are divided into two groups based on color—red spherule cells and colorless spherule cells (also sometimes called morula cells). These cells are filled with large spherule-shaped structures and have antibacterial activity (Li *et al.* 2010). ***Lymphocytes***, also known as progenitor cells, are small cells that have some phagocytic ability. These cells might be precursors of many of the other coelomocytes, hence the name progenitor cells (Chia & Xing 1996, Ramirez-Gomez & Garcia-Arraras 2010). ***Hemocytes*** are spheroid or biconvex cells of widely varying size. They contain hemoglobin and are responsible for oxygen transport. These cells are analogous to erythrocytes in vertebrates (Chia & Xing 1996). ***Vibratile cells*** have a flagellum and might be part of the echinoderm clotting reaction. They also might be responsible for coelomic fluid movement by the movement of the flagellum (Chia & Xing 1996, Ramirez-Gomez & Garcia-Arraras 2010). Finally, ***crystal cells*** are only found in Holothurians. They are thought to be part of the osmoregulatory system, but little is known about them because

they are extremely fragile and difficult to preserve. They have not been directly linked to immune function (Ramirez-Gomez & Garcia-Arraras 2010, Hetzel 1963, Dybas and Fankboner 1986).

Immune System Role in Regeneration

Current studies of echinoderm regeneration abilities have somewhat eclipsed interest in their immune system. However, evidence from them and other organisms supports the hypothesis that regenerative abilities may be controlled or at least supported by the immune cells. For example, in *Xenopus laevis* inflammatory factors are essential in limb regeneration, and extreme changes in the immune system occur during the metamorphosis from tadpole to adult (King *et al.* 2012 and Flajnik *et al.* 1987). Mammals have much lower regenerative abilities than either echinoderms or amphibians. However, a cellular link between the immune system and regenerative abilities has been demonstrated in them as well (King *et al.* 2012).

Echinoderms have the ability to regenerate all body parts (Tsonis 2000) and there is evidence that coelomocytes are heavily involved in regeneration. In *Holothuria glaberrima*, these cells congregate in the intestinal connective tissue after traumatic evisceration and release vesicles into the extracellular space. These vesicles are hypothesized to contain material to help rebuild the extracellular matrix (Garcia-Arraras *et al.* 2006).

Seasonal Atrophy in *Parastichopus californicus*

Parastichopus californicus undergoes a seasonal cycle of atrophy and regeneration of its visceral organs. The viscera is intact from late spring to early summer, with atrophy occurring mainly in the fall—typically beginning around late September with the cessation of eating and reduced movement. Two to four weeks after the atrophy process is completed, the visceral will regenerate, starting with the gut tube (Fankboner & Cameron 1985). The functions of this cycle

are unknown. Some hypotheses for this phenomenon include osmoregulation, seasonal food availability, or possibly immune response to gut bacterial load (Fankboner 2002).

Parastichopus californicus Coelomocytes

P. californicus has four of the six coelomocyte types: amoebocytes, spherule cells, lymphocytes, and crystal cells (Hetzel 1963). They also develop the characteristic "brown bodies" or large aggregations of coelomocytes (particularly amoebocytes) that engulf foreign material and help to remove it from the coelomic fluid. Very few studies have been done on the immune system of *P. californicus.* Dybas and Fankboner (1985) showed that this cucumber was able to effectively clear bacterial infections in less than 24 h, but they did not study how the population of coelomocytes changed during an immune challenge. Similarly, very little attention has been paid to how the coelomocyte population within the coelomic fluid changes over the atrophy/regeneration cycle.

Research Question:

- Is the annual cycle of visceral atrophy and regeneration of a marine species associated with changes in its immune system and its ability to respond to an immune challenge?

Research Objectives:

- To determine if the number and types of coelomocytes in coelomic fluid of *P. californicus* fluctuate during the annual atrophy/regeneration cycle.
- To determine if the response to an immune challenge within *P. californicus* varies with the atrophy state of the gut.

Methods

Timeline:

This study will be conducted during three seasons over approximately eight months.

1) July-August 2014 (Summer). *P. californicus* has all visceral organs and is at maximum body mass and feeding/movement rates.
2) November-December 2014 (Early Winter). The internal organs are fully atrophied, and animals have low body mass and very low feeding/movement rates.
3) January-February 2015 (Late Winter). Visceral organs are regenerating, and animal have high metabolic rates while feeding/movement rates remain very low.

Location & Animal Care:

Parastichopus californicus will be collected from depths between 10 and 20 m by SCUBA off of Sares Head near Rosario Beach Marine Laboratory, Anacortes, WA, USA (48°25’22”N 122°40’21”W) during the summer, early winter, and late winter periods. Adult animals (in situ length > 30 cm) will be used. Coelomic fluid (CF) for baseline studies will be sampled as soon as possible after collection and immune challenge studies will be done over the subsequent 10 days.

Coelomocyte Determination:

In each season, a 1 ml sample of CF will be taken from ten *P. californicus* as soon as possible after collection to determine the number and types of coelomocytes present in unstimulated coelomic fluid. To prevent contamination, the insertion site will be washed with sterilized artificial sea water before a sterile hypodermic needle is inserted. The CF will immediately be placed on ice.

P. californicus has at least four types of coelomocytes—amoebocytes, lymphocytes, spherical cells, and crystal cells (Dybas and Fankboner 1986). Each of these cell types will be identified in fresh mounts using a light microscope. All of the cells in three 20 µL aliquots of CF will be counted using a hemocytometer and the percentage of each cell type will be determined. These animals will be further used for cellular immune challenge (see below).

Cellular Immune Challenge:

For each of the three seasons, *P. californicus* will be monitored to determine if the cellular response to an immune change within the CF varies with viscera state. After taking the CF sample for coelomocyte counts (see above), each of the ten sea cucumbers in each season will be injected with 0.2 ml of 6 $x10^8$ cells/ml of citrated sheep erythrocytes (SRBC) based on procedures developed by Canicatti (1989), Up to ten control sea cucumbers will be injected with the same volume of sterile artificial sea water (Canicatti 1989). Samples of coelomic fluid will be taken once a day for the next ten days and coelomocyte counts will be done as described above. After ten days, each sea cucumber will be dissected to confirm viscera state.

Immune Enzyme Activities:

Immune enzyme activities will be measured in ten *P. californicus* from each of the three seasons. Animals will have aliquots of coelomic fluid removed using a hypodermic needle and immediately frozen.

Tests will be done on thawed cell-free coelomic fluid and also on resuspended coelomocytes (Klanian 2013, Zang et al. 2012). Coelomocytes will be removed from the fluid by centrifugation. After resuspension in PBS, coelomocytes will be lysed using sonification (22 kHz and 0°C for 25s, then centrifugation at 4000xg at 4°C for 10 min; Klanian 2013).

Acid Phosphatase and Alkaline Phosphatase activity will be measured using fluorescence assay kits (Sigma, MAK087 & APF) following the protocol provided with the kit. Similarly, Superoxide Dismutase (SOD) and Total Antioxidant Capacity (TAC) will be measured using spectrophotometric kits (Sigma, 19160 & C S0790).

Phenoloxidase will be measured following methods adapted from Smith and Soderhall (1991) by Zhang *et. al* (2010). The activity of phenoloxidase will be measured spectrophotometrically at 490 nm. For both the cell-free and coelomocytes samples, 50 µl of sample will be incubated at 25°C for 10 minutes with an equal amount of 0.1% trypsin in cacodylate buffer (CAC). Then 100 µl of $_{L}$-3, 4 dihydroxyphenylalanine (L-DOPA, 0.3% in CAC buffer) will be added. Immediately samples will be mixed and then the optical density will be measured.

Total protein concentration will be measured using a Bio-Rad DC Protein Assay kit (Bio-Rad, 500-0122).

Statistical Analysis:

Data will be analyzed using ANOVA with the appropriate post hoc test.

Results

Coelomocyte Determination

The mean number of total free living coelomocytes in the CF is likely to highest during the summer (Figure 1). I expect that the mean number of amoebocytes to be approximately equal during the late winter and summer with the lowest mean in the early winter (Figure 2). The mean spherule cell population will be at its lowest during the late winter, with higher means in both summer and early winter (Figure 3).

Cellular Immune Challenge

I expect mean number of amoebocytes to change seasonally, with the lowest mean during the early winter. During the immune challenge with SRBCs, the phagocytic amoebocytes will exhibit the largest magnitude change when compared with the other coelomocyte populations. The immune challenge should elicit the smallest magnitude change in amoebocytes during the early winter (Figure 2).

Immune Enzyme Activities

For all immunoenzyme activities measured, I expect the activities in cell-free CF and in the coelomocytes to be highest during the early winter. Additionally, the late winter samples will most likely have higher overall immunoenzyme activities than the summer samples (Figure 4).

Discussion

The number of coelomocytes that are free living in the CF varies depending on whether the animal has complete viscera or regenerating and whether is responding to an immune challenge. During the summer, *P. californicus* is less likely to be responding to a coelomic bacterial infection or to be undergoing regeneration. Thus, I expect to see the highest mean number of coelomocytes during this time. During both the early and late winter periods, certain coelomocyte populations are not likely to be free in the CF, thus reducing the mean number of total coelomocytes at those times (Figure 1).

During the degeneration process, *P. californicus* will have a higher level of bacteria in the coelom (Dybas and Fankboner 1986). Because of this, I expect to see the lowest mean numbers of amoebocytes during this season. In clearing the coelom of bacteria, much of the amoebocyte population will be sequestered in brown bodies and removed from the coelom, thus reducing the number of amoebocytes sampled. This previous stress on their immune system will likely prevent a large magnitude immune response when SRBCs are injected into the coelom to elicit

and artificial immune responce (Figure 2). Also, because I expect the immune system to have been previously activated during the early winter, the overall immunoenzyme activities at this time should be higher than what is observed during summer or late winter (Figure 4).

It has been demonstrated in other holothurian species that spherule cells play a role in the regeneration of tissue and these cells will migrate into the regenerating tissues during this process (Garcia-Arraras *et al.* 2006). During the late winter when *P. californicus* is undergoing the regeneration of its viscera, I expect to see a lower mean number of spherule cells in the CF because of this migration (Figure 3).

Overall, during each season *P. californicus* should exhibit a characteristic immune response that depends on both the cycle of viscera atrophy and on recent immune system stressors. I expect that the trends observed during the previously described experiments will be comparable to the results from similar experiments that have been conducted in other holothurians.

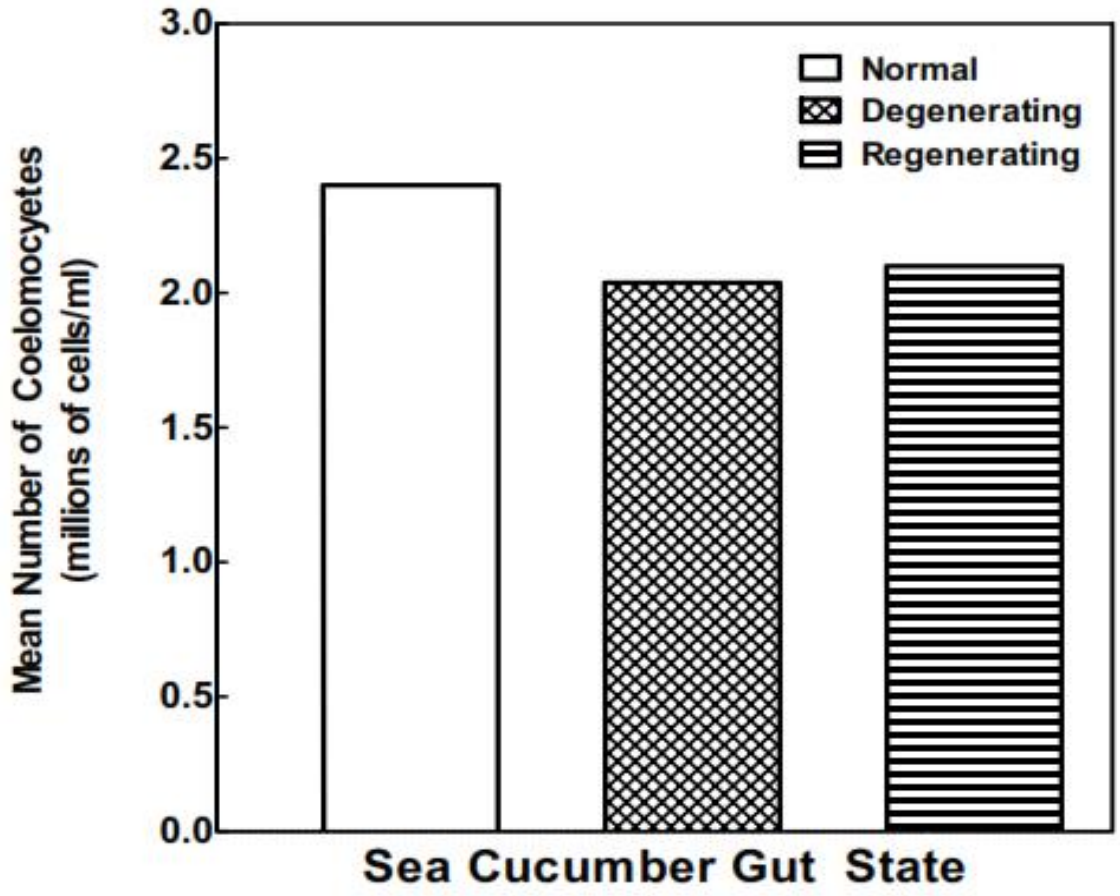

Figure 1. Expected mean number of coelomocytes found in coelomic fluid taken from *Parastichopus californicus* at three points during the yearly regeneration cycle. Samples taken from animals with three different viscera states: normal (summer), degenerating (early winter), and regenerating (late winter). n=10

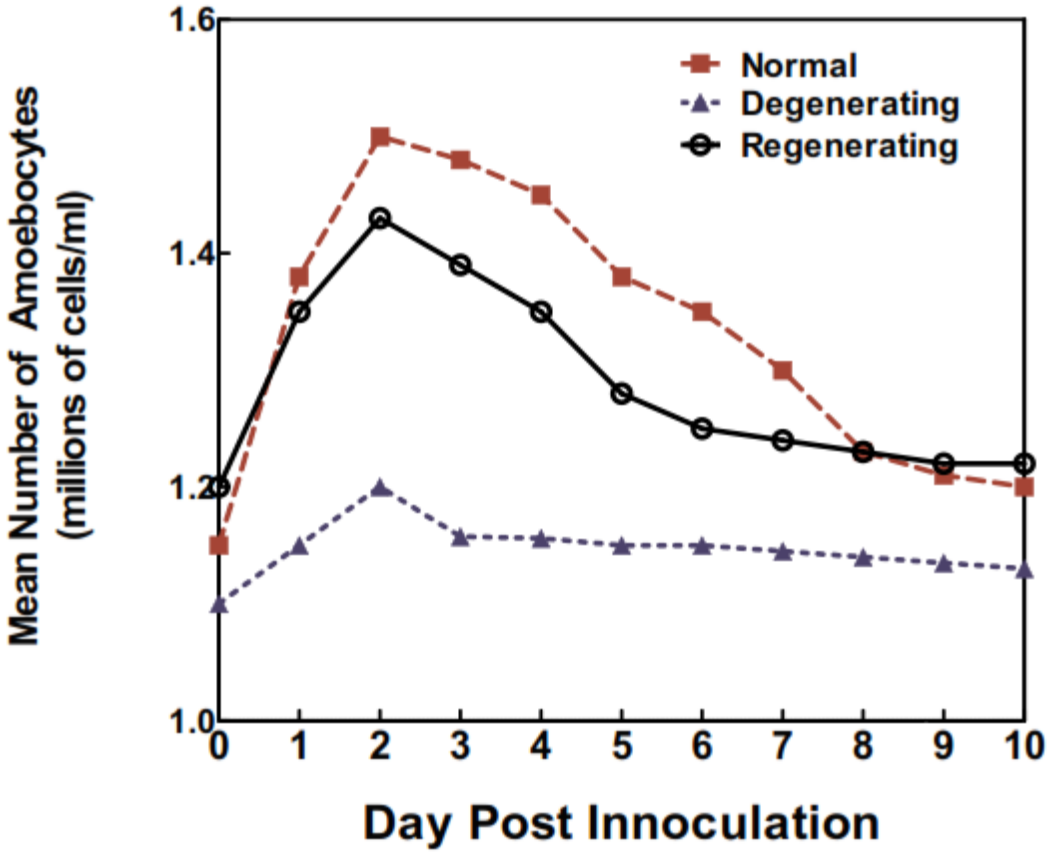

Figure 2. Expected mean number of amoebocytes in coelomic fluid from *Parastichopus californicus* each day post innoculation (PI) with formalized sheep erythrocytes at three different points in the yearly regeneration cycle. Samples taken from animals with three different vicera states: normal (summer), degenerating (early winter), and regenerating (late winter). n=10

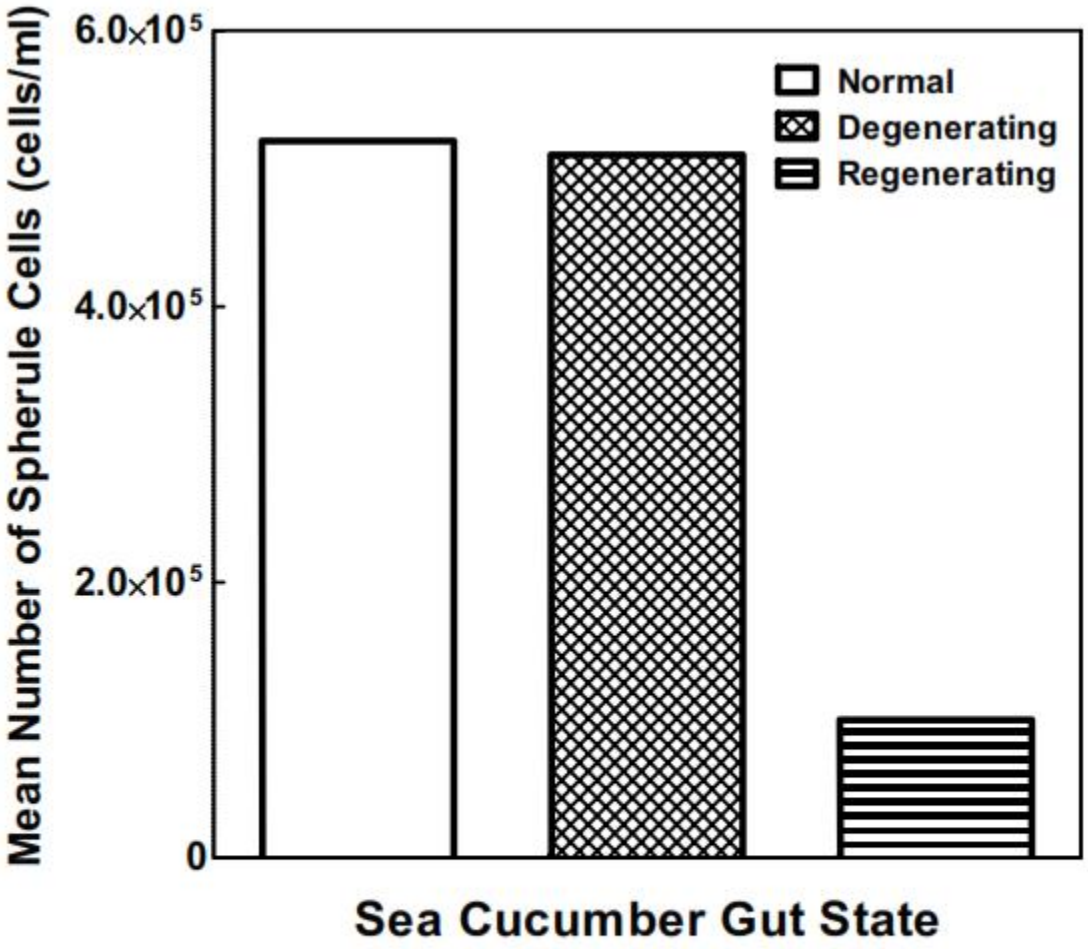

Figure 3. Expected mean number of circulating spherule cells in coelomic fluid from *Parastichopus californicus* at three points during the yearly regeneration cycle. Samples taken from animals with three different viscera states: normal (summer), degenerating (early winter), and regenerating (late winter). n=10

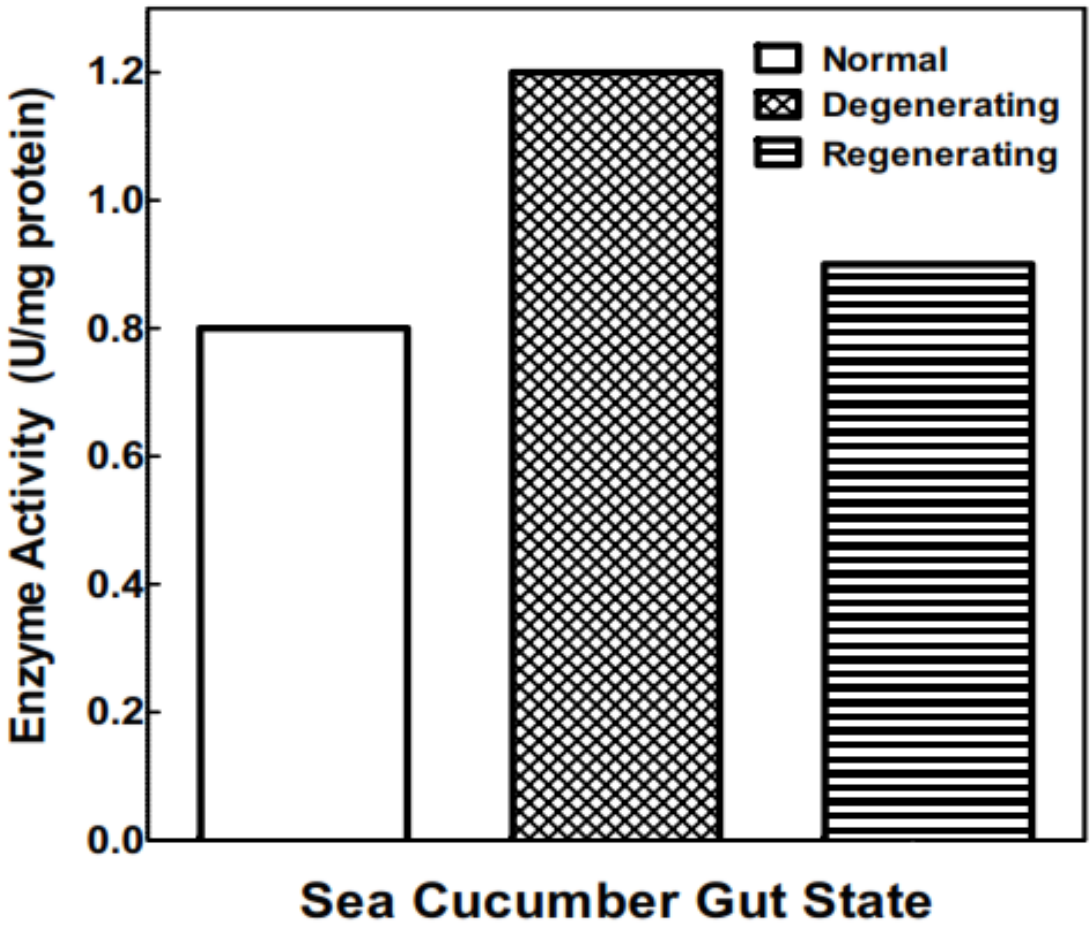

Figure 4. Expected mean activity of immunoenzymes in coelomic fluid sampled from *Parastichopus californicus* at three points during the yearly regeneration cycle. Samples taken from animals with three different viscera states: normal (summer), degenerating (early winter), and regenerating (late winter). n=10

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Appendix

Proposed Budget

Product	Cost	Justification
Sigma Total Antioxidant Capacity Kit (CS0790)	$461.50	Identify the presence and activity of antioxidants in CF samples
Sigma Acid Phosphatase Activity Fluorometric Assay Kit (MAK087)	$332.00	Identify the presence and activity of acid phosphatase in CF samples
Sigma Alkaline Phosphatase Detection Kit, Fluorescence (APF)	$584.00	Identify the presence and activity of alkaline phosphatase in CF samples
Sigma Superoxide Dismutase Assay Kit (19160)	$342.50	Identify the presence and activity of superoxide dismutase in CF samples
Bio-Rad Protein Assay Kit (500-0001EDU)	$137.00	Measure protein concentration in samples in order to quantify immune enzyme activity
Chemicals for Phenoloxidase Test	$128.70	Identify the presence and activity of phenoloxidase in CF samples
Sheep Erythrocytes (Citrated) 50 mL	$40.95	Elicitor for immune challenge
Liquid Nitrogen Fills	$180.00	Three fills/$60 apiece
SCUBA tank refills	$108.00	$6 per refill, two divers with 3 dives each season to collect organisms
Transportation to Rosario Beach Marine Lab from Walla Walla, WA	$206.40	Two round trips (480 miles) at $0.43/mile
Total Expenses:	$2,520.55	