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RESEARCH ARTICLE

# Investigating the Sea Urchin Immune System: Implications for Disease Resistance and Aging

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Aging affects immune function, increasing an organism's vulnerability to infectious and neoplastic diseases, however efforts in medical research have focused on adaptive immunity, leaving the role of innate immunity largely open for investigation. Echinoderms are an excellent model for studying innate immunity as invertebrates lacking adaptive immunity and as deuterosomes being more closely related to humans than any other invertebrate group. A survey of the immune gene repertoire encoded by the sea urchin genome reveals enormous and unprecedented complexity and it has been hypothesized that the robust innate immune system may play a role in both the longevity and resistance to disease that these animals exhibit. To begin to test this hypothesis, the objectives of this study were to investigate the complexity and function of the sea urchin innate immune system with respect to age. Coelomocytes are the immune cells, found in coelomic fluid that are highly variably in size, shape, and function. There are currently four main accepted groups; macrophages, red and white spherules, and vibratile cells. The composition of coelomocytes from the sea urchin *Lytechinus variegatus* was investigated using microscopy and flow cytometry and coelomocyte function was evaluated by the level of reactive oxygen species (ROS) detected using 2',7'-Dichlorofluorescin diacetate. The results showed a high level of variability in morphology and concentration of coelomocytes. A comparison of total cells, red cells, and colorless cells showed that smaller urchins had generally higher cell concentrations than large urchins and higher variability in the proportion of red spherule cells. Reactive oxygen species were detected in the coelomocytes of small and large urchins with the larger urchins showing a trend to produce more ROS. This suggests that immune function is maintained (and perhaps enhanced) with age in sea urchins. The trend of higher ROS production in older urchins is particularly interesting considering they tend to have lower coelomocyte concentrations and may have important implications for the maintenance of innate immunity with age. Given the close genetic relationship between sea urchins and humans, investigating the mechanisms by which immune function is maintained with age could possibly have larger implications for improving human health.

## INTRODUCTION

The complexity of the genetic repertoire of sea urchins associated with the immune system is unprecedented in previous studies of invertebrates and vertebrates alike (Hibino et al. 2006). Urchins, like other invertebrates do not have an adaptive immune system, but rather rely fully on innate immunity for protection from pathogens (DeVeale et al. 2004). In vertebrates, and particularly in reference to the human immune system, the adaptive immune system has been studied extensively with little consideration for the role of innate immunity in disease resistance with age (Gomez et al. 2005). By focusing studies on invertebrates, complications due to the complexity of adaptive and innate immunity interactions can be avoided as they only have innate immune functions (DeVeale et al. 2004). Sea urchins are ideal among invertebrates for relating immunity studies to practical medical uses in humans because echinoderms are more closely related to vertebrates than any other relevant invertebrate model (Rast et al. 2006).

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In sea urchins, immunity is regulated by various cell types within the coelomic fluid called coelomocytes that are present on the order of millions (Hibino et al. 2006). There are four main types of coelomocytes described to date, macrophage-like phagocytes, white and red spherule cells (granulocytes), and vibratile cells (Hibino et al. 2006). Macrophages are large cells that play a major role in clotting and phagocytosis upon invasion of the host by non-specific pathogens (Matranga et al. 2006). The granular red and white spherule cells produce chemicals with antibacterial properties and can be found in different proportions based on the state of health the urchin is in, and what kind of immune pressures it has been under (Matranga et al. 2006). The function of vibratile cells is not very well understood but they may play some role in clotting, wound healing, and other immune functions, and they are very distinctive because of their characteristic rapid movement in a helicoidal pattern (Hibino et al. 2006; Matranga et al. 2006). A major goal of this study is to observe the presence of these cells within urchins of different sizes to get an idea of variation in coelomocyte proportions among similar individuals within a population as well as differences between young and old individuals within a population.

The cells of the innate immune system in vertebrates and invertebrates generate reactive oxygen species (ROS) by many intracellular mechanisms (DeVeale et al. 2004). This is a particularly important defense mechanism utilized by multicellular organisms, as innate immune cells provide the first

line of defense against non-specific pathogens, independently of adaptive immunity recognition (Gomez et al. 2005). Natural Killer cells are macrophages found in vertebrates that are attributed with the functions of phagocytosis of foreign pathogens followed by production of toxic chemicals such as ROS and nitric oxide (NO) to kill the pathogen (Gomez et al. 2005). In vertebrates it has been found that there is a decrease with age in natural production of the superoxide anion ($O_2^-$), and other ROS, indicating a distinct decrease in innate immunity function (Gomez et al. 2005).

Lipopolysaccharides (LPS) are large molecules found on the outer membrane of Gram-negative bacteria, which the immune system uses to identify the bacteria as a foreign pathogen (Smith et al. 1995). LPS elicits an immune response in mammals and many other organisms including sea urchins and is often used to study immune responses in various organisms (Smith et al. 1995). In addition to a natural decrease in the production of ROS related to immune function, (LPS)-stimulated ROS and NO are also lower in macrophages of aged vertebrates in immune studies (Gomez et al. 2005). It has also been reported that macrophages from the wounds of aged mice exhibit diminished phagocytic activity (Gomez et al. 2005). Additionally, production of some cytokines decreases with age while others show increased production compared to young mice, indicating that ageing in vertebrates has effects on different stages of intracellular signaling pathways (Gomez et al. 2005). Macrophages and white granulocytes have been reported in many invertebrates to produce ROS by similar chemical processes as they are produced in Natural Killer cells of vertebrates (Beck et al. 2001). Although invertebrates also utilize ROS to target pathogens, it is possible that ageing affects this process on different levels due to complicated changes in signaling pathways throughout the life cycle.

To better understand this we will look at differences in the presence of ROS in coelomocytes from urchins of different ages to get an idea of how urchins respond to natural conditions at different ages. It is possible that urchins experience a decline in innate immunity as vertebrates do, however a similar or more acute response in older urchins relative to young urchins may indicate an important difference in the ageing of the immune system that would maintain disease resistance with age. If an effective way to induce an immune response in coelomocytes of young and old urchins is established the immune responses could be compared to infer whether urchins respond less effectively with age as many vertebrates do, or if their innate immunity is consistent despite age. In this study I explored the use of lipopolysaccaride (LPS) to elicit an immune response in sea urchin coelomocytes.

In reference to human medicine, vertebrates (particularly mice) have been studied extensively when studying the immune system and processes of new drugs. Being more similar to humans, comparable vertebrates are logical models for medical studies, however sea urchins exhibit many interesting qualities such as a complex innate immune system and an absence of reported cases of cancer (Bodnar 2009). As echinoderms are more closely related to humans than other invertebrates, studying their immune systems could potentially be very beneficial to medical advances.

## MATERIALS AND METHODS

### Collecting Urchins and Coelomic Fluid

*Lytechinus variegatus* were collected from Mangrove Bay, Bermuda and stored in tanks with a constant flow of fresh seawater. The urchins were placed in a clean tank 24 hours prior to each experiment. Once coelomic fluid was removed urchins were placed in a separate tank so that no urchin provided coelomic fluid more than once. Coelomic fluid was removed using a syringe fitted with an 18 guage needle and mixed with an equal volume of the coelomic fluid media, calcium and magnesium free seawater with EDTA (CMFSW-E) containing 460 mM NaCl, 10 mM KCl, 7 mM $Na_2SO_4$, 2.4 mM $NaHCO_3$, 30 mM EDTA at pH 7.4. Additionally the diameter of each urchin's test was measured to determine the approximate age by comparison with growth curves established for *L. variegatus* (Beddington and McClinktock 2000; Moore et al. 1963). The cells were then centrifuged for five minutes at 6000rpm and resuspended in CMFSW-E. The concentration of cells was then calculated using a hemocytometer. Cells were counted as separate groups, red cells, white cells, and vibratile cells. Red cells are easily identified by their distinct color, and only red granulocytes have this color. Vibratile cells were identified by their rapid spinning movement, while white granulocytes and macrophages were both counted at white cells. Although it might be possible to distinguish the cells using differential density centrifugation, we pelleted all coelomocytes at one speed, which did not allow separaton of the different white cell populations. The vibratile cells were counted as a separate group, however all vibratile cells were counted as white cells during data analysis because the EDTA added to the cells causes them to stop or slow movement (Matranga et al. 2006). Counts of vibratile cells in coelomic fluid treated with EDTA are probably not accurate due to the effects of EDTA, so for simplicity the category "white cells" refers to macrophages, white granulocytes, and vibratile cells. The total cell counts were used to determine how much coelomic fluid was needed before each of the following experiments was performed.

### Flow Cytometry and Microscopy

Flow cytometry (FCM) and microscopy were used to characterize the different types of coelomocytes and their proportions within in the coelomic fluid. The cells were resuspended in 4mL of CMFSW-E. A fluorescent dye that binds DNA (SYTO13, from Molecular Probes Inc.) was used to treat 2mL of this sample, making the cells easier to find on the slides. The sample was filtered through a 100 µm nylon mesh filter to remove aggregates of cells (>100µm) that would clog the nozzle

of the cytometer. Four populations were determined based on size and two slides containing 5000 cells each were made for each population. The best slide (usually whichever had the least or no air bubbles) from each population was observed using an Olympus Provis AX70 fluorescence microscope at 10x and 40x magnification. Images were photographed with a Toshiba IK-TU40A camera, so that the populations could be analyzed by size, shape, and appearance of the cells using Image Pro Plus software, version 4.0 (Media Cybernetics). Flow cytometry was performed using an Influx Benchtop Sorter from Cytopeia with 488nm excitation.

### Detecting ROS using Fluorometry and FCM

Twelve urchins were used to test for natural ROS levels and ROS following immune stimulation with LPS. When detecting natural ROS levels, six urchins were small (<50mm) and six were large (>60mm), to compare results between urchins of distinctly different ages. I removed 1.5mL of coelomic fluid from each urchin and washed and resuspended the coelomocytes in CMFSW-E. After counting the cells on a hemocytometer, two samples of 500,000 cells were diluted into a volume of 0.5mL with CMFSW-E in 1.5mL micro tubes. One of these samples received a treatment of 50µM (1.25µg) DCFH-DA (2',7'-Dichlorofluorescin diacetate) (SIGMA Chemical Company) while the other sample received the same volume of ethanol which is the solvent for DCFH-DA. The samples were incubated for 15 minutes and then washed with 1mL of CMFSW-E. The samples were resuspended in 500µL and 100µL was removed from each sample and transferred to pre-labeled 1.5mL micro tubes. The remaining 400µL samples were analyzed by FCM for overall fluorescent levels and fluorescent levels within different populations of cells. Fluorescence was detected between the wavelengths of 495nm-521nm, a commonly used dye FITC fluoresces in this range, as well as many others including DCFH-DA. 100µL of 2x Cell Lysis buffer (Oxiselect Intracellular ROS Assay Kit, Cell Biolabs, Inc.) was added to each remaining 100µL sample for five minutes. The samples were centrifuged at 6000rpm for two minutes and then transferred to special micro fluorometer tubes for use in an Invitrogen Qubit fluorometer. The overall fluorescence for each sample was recorded under the settings for double strand DNA high sensitivity, and the units were replaced with relative fluorescent units. Control and DCFH-DA treated samples were graphed as averages and paired t-tests were used to compare overall control and experimental samples and within predetermined populations. Unpaired t-tests were used to compare fluorescent levels in large and small urchin groups.

### Determining ROS levels in urchins with LPS and $H_2O_2$ treatments

A sample size of five urchins was used to determine ROS levels in urchins with no treatment, LPS, and $H_2O_2$ treatments. I removed 3mL of coelomic fluid from each urchin and divided each sample into four microtubes containing approximately 500,000 cells, for each treatment, control (EtOH only), DCFH-DA only, LPS and DCFH-DA, and $H_2O_2$ and DCFH-DA. All control samples received a treatment of 1.25µL EtOH and all other samples received 1.25µL of DCFH-DA. The stock LPS solution of 1mg/mL was added to each sample to give a final concentration of 1 µg/ml 15 minutes after the DCFH-DA was added. The LPS used was 055-B5 *E.coli.* To the $H_2O_2$ samples, 5µL of a 1M solution were added to all samples to give a final concentration of 1 mM 15 minutes after the DCFH-DA had been added. Multiple preliminary experiments were performed to determine the best parameters to apply the LPS treatment. Different types of LPS from *E.coli* as well as marine bacteria were used, different concentrations of LPS and incubation times were used and the dye and LPS were added in different sequences to determine which method caused the most immune stimulation. The same procedure following DCFH-DA treatments as detailed above was used for these samples omitting the step where the samples are separated for the flow cytometer. All samples were resuspended in 100µL of CMFSW-E and 100µL of 2x cell lysis buffer before being analyzed by the Invitrogen Qubit fluorometer.

## RESULTS

### Coelomocyte grouping based on FCM and microscopy

For all parts of the experiments utilizing the flow cytometer, coelomocytes were divided into the four populations based on cell size shown in figure 1. Population 2 P2 contained numerous round cells that were about 10-20 µm in size and very granular (figure 2 P2a), however the population was dominated mostly by larger clumps of cells that may not have been separated when passing through the flow cytometer. Population 1 (P1, figure 1) largely contains the more uniform group of round granular cells (figure 2 P1a & 2 P1b). Cells range from 10-20µm with larger cells being more amorphous in shape and found in clumps, although fewer cell clumps were found in population 1 than population 2.

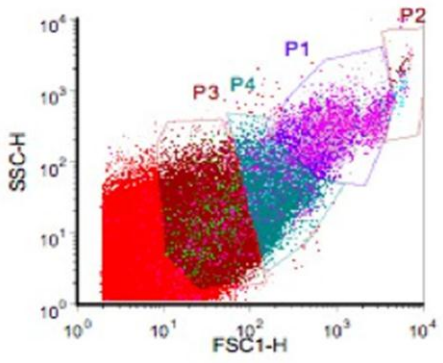


**Figure 1.** Flow cytometry dot plots showing forward scatter (FSC1-H) versus side scatter (SSC-H) for *L. variegatus* coelomocytes. The four assigned population divisions are indicated by P1, P2, P3 and P4.

Population 4 contained numerous red granular cells (figure 2 P4a) as well as some clear cells of similar size, shape, and granularity to the red cells (figure 2 P4b). Population 3 contained cells ranging from about 4-10µm. Smaller cells tended to be more rounded or oblong while larger cells varied more in shape (figure 2 P3a &2 P3b).

### Determining ROS levels following LPS and $H_2O_2$ treatments

2',7'-Dichlorofluorescin diacetate (DCFH-DA) was used to measure the levels of ROS in coelomocytes. DCFH-DA readily crosses cell membranes and is deacetylated by cellular esterases into the non-fluorescent DCFH which is rapidly oxidized in the presence of ROS to the highly fluorescent compound DCF. Results showing relative differences in fluorescence indicating ROS levels of coelomocytes with different treatments can be seen in figure 3. The control samples had the least fluorescence, indicating that the cells have some natural fluorescence however the fluorescence levels increase significantly with the treatment of DCFH-DA (paired t-test, p=0.0073, figure 3). There was no significant change in ROS from samples treated with DCFH-DA only, to the cells that were treated with LPS and DCFH-DA (paired t-test, p=0.43, figure 3), however there was a significant change from DCFH-DA only samples to those treated with $H_2O_2$ and DCFH-DA (paired t-test, p=0.036, figure3)

### ROS levels in small and large urchins

Both fluorometry (figure 4B) and FCM (figure 4A) were used to analyze ROS levels in small and large urchin samples. Both methods demonstrated that DCFH-DA treated samples had significantly higher fluorescence than untreated controls in small urchins [(paired t-test, p=0.015, figure 4B), (paired t-test, p=5.51E-4, figure 4A)]. In large urchins the FCM data showed that DCFH-DA treated samples were significantly higher than control samples (paired t-test, p=5.0E-4, figure 4A), while the difference in fluorescence between controls and DCFH-DA treated groups measured by fluorometry was nearly significant (paired t-test, p=0.077, figure 4B). There was no significant difference between DCFH-DA treated samples in small and large urchins for the FCM data (unpaired t-test, p=0.18, figure 4A), or fluorometry (unpaired t-test, p=0.61, figure 4B), although in both cases the group of large urchins demonstrated higher fluorescence (figure 4). This experiment was repeated twice with different urchins and yielded similar results in both fluorometry and FCM data for both trials.

When the fluorescence detected by FCM was broken down into the amount of fluorescence for each population, populations 1 and 2 showed the highest fluorescence levels in both large and small urchins treated with DCFH-DA (figure 5), while populations 3 and 4 have considerably lower fluorescence measured in the range 495nm-521nm. For all small urchins the difference between control and DCFH-DA treated groups was significant (paired t-test, pop. 1 p=2.2E-3, pop. 2 p=3.03E-3, pop. 3 p=1.89E-3, pop. 4 p=9.76E-4, figure 5). This is also the case for all control and DCFH-DA groups of large urchins (paired t-test, pop1. p=4.34E-6, pop. 2 p=4.53E-4, pop. 3 p=8.65E-4, pop. 4 p=1.54E-4, figure 5), however there is no significant difference between small and large DCFH-DA treated groups in population 1 (unpaired t-test, p=0.079, figure 5), population 2 (unpaired t-test, p=0.41, figure 5), population 3 (unpaired t-test, p=0.10, figure 5), and population 4 (unpaired t-test, p=0.076, figure 5).

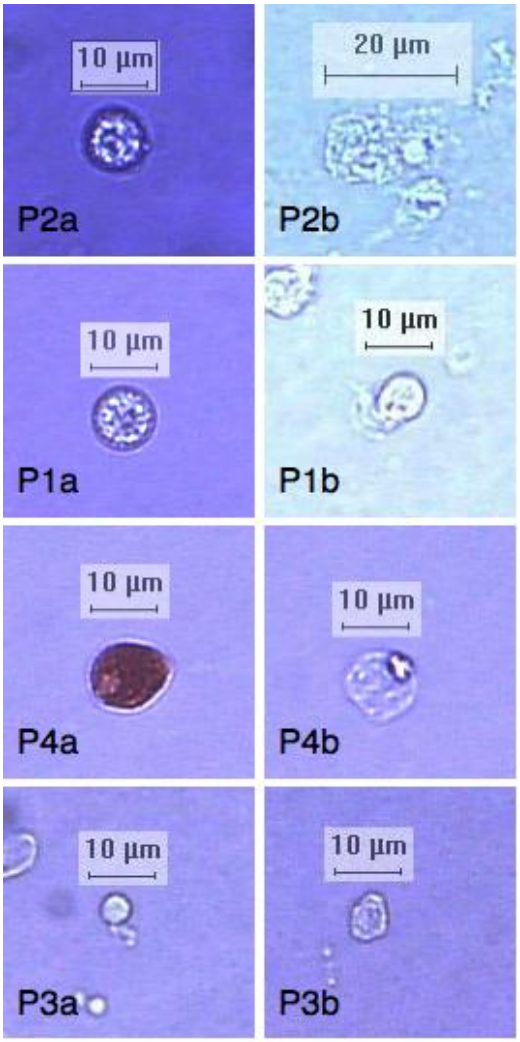


**Figure 2.** Representative coelomocytes of *L. variegatus* found in populations P1 through P4 defined by the cytometry analysis.

The P-values comparing large and small urchin samples within each population are fairly low, in most cases nearly significant (except population 2), and in all cases the large urchin samples demonstrated higher fluorescence. These results were generated from two separate experiments and showed similar trends in data when broken down by population.

### Variation in coelomocytes of *L variegatus*

Of the 45 urchins used in various stages of these experiments, the diameter of the test was measured in 37 urchins. Figure 6 shows the wide variation in white to red cell ratios for each of the 37 urchins that were measured. The bars are shown in order by the size of the urchin, and variability appears higher in smaller urchins, which have the highest and lowest proportions of red cells (1.01%-14.07%) while larger urchins have a somewhat more consistent percentage of red cells (figure 6). The group called "white cells" in this chart represent all macrophages, granulocytes, and vibratile cells as many of them could not be identified due to variation in size and shape.

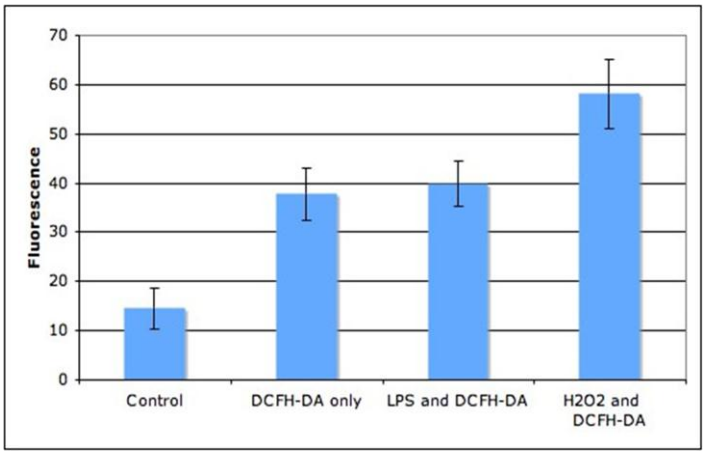


**Figure 3.** Average fluorescence with standard error bars, in *L. variegatus* coelomocyte samples from five intermediate size urchins with four different treatments. Fluorescence measured in relative fluorescent units attained using a Qubit Fluorometer.

Figure 7A shows the mean concentration (cells/mL) of cells in the coelomic fluid of all 37 measured urchins. Mean concentrations of red, white, and total cells are given for each size category, and urchins are broken down into groups of every 10mm from 30-69mm. The group of urchins 30-39mm in diameter had significantly higher total cell concentration than urchins in the groups, 40-49mm (unpaired t-test, p=0.023, figure 7A), 50-59mm (unpaired t-test, p=0.014, figure 7A), and 60-69mm (unpaired t-test, p=5.59E-4, figure 7A). The differences in white cell concentrations also follow this trend while red cell concentrations tend to be more consistent. Figure 7B shows a subset of the total urchins measured, including only the 24 urchins used in the two ROS experiments. The difference in mean total cell concentrations between small and large groups is nearly significant (unpaired t-test, p=0.13, figure 7B), as well as for mean concentration of white cells (unpaired t-test, p=0.14, figure 7B) while red cells' mean concentrations tend to be more similar (unpaired t-test, p=0.59, figure 7B). As in figure 7A, the smaller urchins consistently have higher cell concentrations than large urchins.

## DISCUSSION

Flow cytometry as a method for separating different types of coelomocytes of *L. variegatus* has proved to be difficult mainly because there is no clear separation between the populations on the FCM plot. Generally in echinoderms macrophages range from 3-20µm, spherules from 8-20µm, and vibratiles from 6-20µm (Ramirez-Gomez and Garcia-Arraras, 2010). These size ranges may be smaller for particular species but significant overlap is still present.

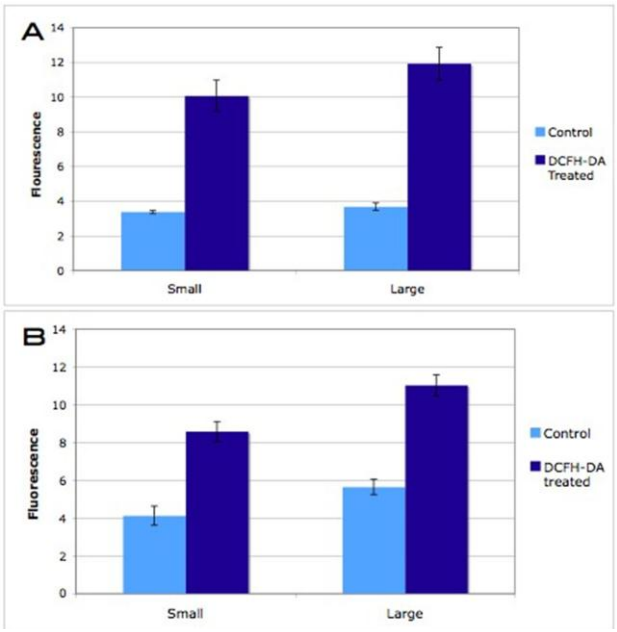


**Figure 4.** Fluorescence of coelomocytes from small and large *L. variegatus* following treatment with DCFH-DA which indicates overall ROS levels. **Panel** A: shows average fluorescence with standard error bars, in coelomocytes analyzed by flow cytometry. Panel B: shows the average fluorescence in relative fluorescent units with standard error bars attained using a Qubit Fluorometer.

Figure 1 shows the FCM plot for coelomocytes, which had a similar distribution for all FCM analyses. Since there is very little previous work on coelomocytes using FCM, we tried gating different populations and then observing them using microscopy to determine what types of cells were present in that population. The gates shown in figure 1 were determined after multiple FCM and microscopy analyses. Each time this was done, the cells observed by microscopy showed that our gates were not exclusive to a particular type of cell, and that there was a great deal of overlap.

The gates used here can be used as a basis for future FCM work on coelomocytes of *L. variegatus* however different

gates (possibly narrower) could be used to isolate specific cell types. Population 2 represents the largest cells (10-20 µm) as well as many aggregates of cells. Upon exposure to the environment echinoderm phagocytes are known to undergo a petaloid-filopodial transition in which the cytoplasm transforms from flat petal shapes surrounding the nucleus to thin spikes surrounding the nucleus (Matranga et al. 2006). This transition facilitates the cell-cell interactions that lead to clot formation (Smith et al. 2010). Phagocytes have been reported to make up anywhere from 60-85% of coelomocytes in echinoderms (Matranga et al. 2006), with great variability among species but consistently acting as major mediators of cellular immunity in nearly all echinoderms (Smith et al, 2010). EDTA was added to the cells as an anti-coagulant, which may account for the fact that the petaloid-filipoidal transition was not observed, however a certain amount of clotting was unpreventable, as shown by the observation of many clumps of cells. In general the size of cells in population 2 are indicative of their location on the FCM plot, with clumps of cells being about 20µm in length and individual cells approximately10-20 µm (figure 2 P2a). Round granular cells in this group probably represent white spherule cells, which get their name from the rounded shape due to the application of EDTA, although they appear more amorphous in shape in their natural state, as they are motile cells that move by changes in cell shape (Matranga et al. 2006).

Population 1 is a group containing the second largest cells and has a similar composition to population 2, including clumps of coelomocytes although fewer were present. The population is dominated by more rounded white spherules (figure 2 P1a) and other smaller less distinct round cells less than 10µm in size (figure 2 P1b).

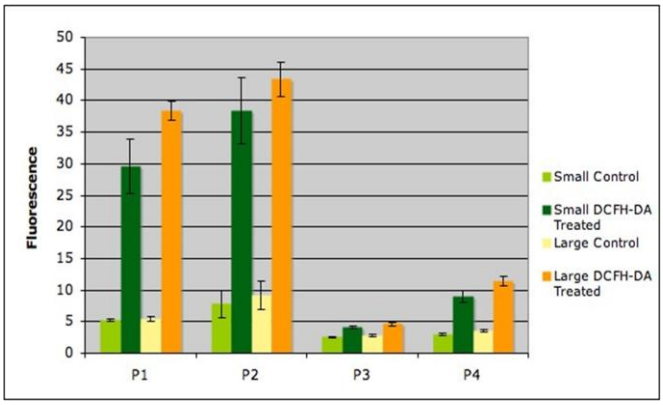


**Figure 5.** Average fluorescence with standard error bars for control and DCFH-DA treated coelomocytes divided into populations P1 through P4 defined by flow cytometry analysis.

This shows that although populations 1 and 2 are mostly different size cells, there is a significant amount of overlap so neither shows a distinct separation between two groups of cells. The variability in the size and composition of cell clumps contributes significantly to this variability. Population 4 was a group with a lot of variation, so many narrow gates were used within this population for observation by microscopy to try and classify different groups. Both red (figure 2 P4a) and white spherules (figure 2 P4b) were observed, however this is the only population containing red spherules, which are considerably less prevalent than their colorless counterparts. Only individual cells were found in this population, which were generally smaller than the cells and aggregates of cells found in populations 1 and 2.

Population 3 contained the smallest colorless cells of varying shapes, which cannot be identified as any specific coelomocyte type (figure 2 P3a & 2 P3b). Overall very few cells were observed with flagellum, and so very few vibratile cells were distinctly identified. General observations can be made about these widely varying populations but they do show somewhat distinct size differences, and in the future these gates can be used as a reference for flow cytometry to divide coelomocytes into more distinct populations. Coelomocytes have been traditionally difficult to classify as definite cell surface markers have not been characterized, and so have been identified mainly by morphological observations (Ramirez-Gomez and Garcia-Arraras. 2010), which are very variable due to different treatment cells and observational methods used in different studies (Matranga et al.2006). Some antibodies have been used to positively identify spherulocytes by cell surface markers, and this method may prove more efficient in the future for identifying cell types (Ramirez-Gomez and Garcia-Arraras.2010).

The main purpose of the results displayed in figure 3 is to show that the coelomocytes are producing ROS detected by DCFH-DA being converted to the fluorescent DCF, and that $H_2O_2$ increases the fluorescent response of DCFH-DA. The control sample shows that despite some natural fluorescence within the cells, adding DCFH-DA and treatments of $H_2O_2$ indicate additional fluorescent activity due to the presence of ROS. Numerous experiments were performed testing different parameters of the LPS treatment to induce an immune response in the coelomocytes, however none showed a significant difference in ROS levels than samples treated with DCFH-DA alone.

Using LPS from different types of bacteria had no apparent change on ROS levels. We determined that out of numerous concentrations, 1 µg/µL had the most consistent affect. Using this concentration, numerous experiments were performed changing the amount of time both LPS and DCFH-DA were applied to the samples to maximize immune response. DCFH-DA was applied before and after LPS, as well as after LPS was applied to cells and then washed. It is possible that despite these trials the LPS was still interacting with the DCFH-DA in some way to prevent it from detecting the ROS levels, however it is more likely that that coelomocytes are simply not being stimulated by the LPS for an unknown reason. Many previous studies injected the LPS directly into the urchins and tested specific gene expression (Smith et al.1995 ; Ramirez-Gomez and

Garcia-Arraras.2010). LPS has been shown to activate the immune system in other ways, for example inducing production of the protein profilin, which modifies the cytoskeleton allowing phagocytosis and encapsulation (Ramirez-Gomez and Garcia-Arraras.2010) while ROS levels have not been specifically tested in this way before.

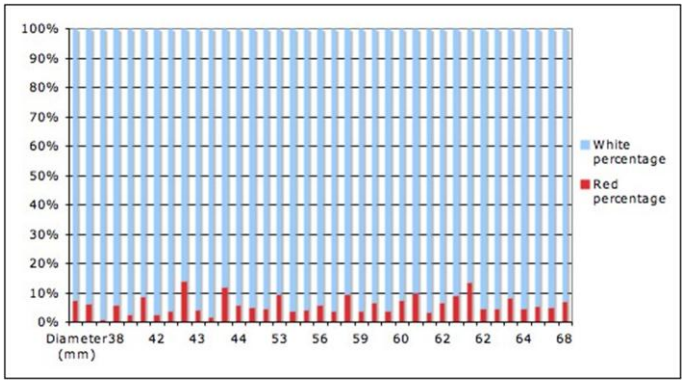


**Figure 6.** Percentage of white to red coelomocytes in 37 individuals of *L. variegatus* shown in order by diameter of test in mm. The white percentage represents all macrophages, clear granulocytes, and vibratile cells. The red percentage is made up solely of red granulocytes.

One study done by Coteur et al in 2005 used the peroxidase, luminal-enhanced chemiluminescence (PLCL) method, which is similarly effective in measuring ROS levels as our methods, to observe ROS levels in starfish amebocytes that were stimulated by LPS under different environmental conditions (Coteur et al. 2005). ROS production following LPS stimulation increased greatly at 6°C and less while even 1-2°C raise in temperature greatly decreased ROS production (Coteur et al. 2005). It has been suggested that this is due to higher dissolved oxygen content in colder waters, making oxygen more available for ROS production (Coteur et al. 2005). Heat shock proteins (Hsp70) that are overexpressed in increased and decreased water temperatures, have antioxidant properties that may protect the amebocytes and allow for higher production of ROS without damage to the cells (Coteur et al.2005). Although the water temperatures in Bermuda are fairly variable they never reach 6°C. It may not be practical to keep urchins endemic to Bermuda in such low temperatures, but storing coelomocytes at a colder temperature while they are being treated with LPS is an interesting parameter that could be done to further this study in attempt to induce an immune response. Increased salinity resulted in decreased ROS production while stress, gender, and paratisitism has no effect (Coteur et al. 2005), and all of these factors as well as others must be taken into account when testing ROS levels in animals that come directly from their natural environment.

Similar to the results shown in figure 3, figures 4 and 5 show that DCFH-DA treated samples had considerably higher fluorescence than untreated control samples. The majority of the fluorescence produced comes from populations 1 and 2 indicating that these cells are the most active coelomocytes in ROS production (figure 5). Because of the complications in dividing these populations we cannot definitively say that a specific cell type is producing most of the ROS however it is clear that these groups are dominated by white spherule cells and macrophages, which have been previously identified as very active immune cells (Smith et al. 2010; Coteur et al. 2005). Macrophages are likely to be the main effectors of the clotting observed in populations 1 and 2 and probably contribute to the high fluorescence seen in these two populations.

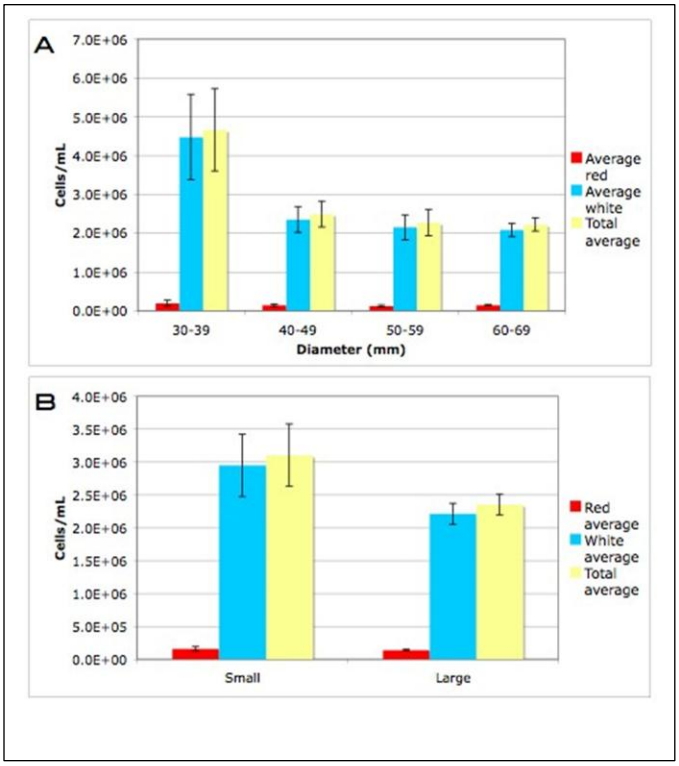


**Figure 7.** Concentration of coelomocytes in different urchin size categories. Panel A: shows average concentration of cells with standard error bars, of red, white, and total cells within the coelomic fluid of all 37 urchins sampled and measured, organized by size categories of every 10mm. Panel B: shows a subset of these urchins containing the 12 small urchins (<50mm) and 12 large urchins (>60mm) used in the two trials of the ROS experiments.

The macrophage's main role is phagocytosis and clotting, but also plays a significant role in cytotoxity and ROS production, while spherule cells participate in inflammatory responses, wound healing, and antibacterial activity particularly ROS production (Ramirez-Gomez and Garcia-Arraras 2010; Coteur et al. 2005). Although the population divisions used

cannot tell us exactly how much ROS is produced by particular cell types, it is clear that the high ROS production in populations 1 and 2 can be attributed to macrophage and spherule cells. Populations 3 and 4 have considerably less ROS present than the first two populations however population 4 has more than 3, which is logical based on figure 4 showing white and red spherules, both active cell types in the immune system. Population 3 on the other hand only had very small cells that are probably not capable of producing high levels of ROS.

When comparing the proportions of different cell types a fair amount of variability was found among the urchins sampled (figure 6). We were only able to compare proportions of white to red cells rather than the various cell types, however the variability in this proportion can tell us that the urchins collected were in varying immune states at the time of sampling, which is expected of urchins that are taken directly from the wild despite being collected from the same location. The proportion of red spherules in echinoderms has been identified as an accurate biosensor as they have been seen to increase from 5-40% in polluted waters or when the organism is subjected to injury or bacterial infection (Smith et al. 2010). While white spherules are mainly potent in cytolytic activity, augmented by presence of phagocytes, red spherules are more active, migrating towards bacteria or the edge of wounds or infections initiating the encapsulation response and degranulating in the presence of bacteria (Smith et al.2010). An increase in these antibacterial cells indicates a recent bacterial infection, wound, or contamination (Smith at al.2010).

Our data show that smaller urchins tended to have higher variability in the proportion of red cells, representing individuals with the lowest and the highest proportions of red spherules (figure 6), while larger urchins tend to have more consistent proportions. This could indicate that the small urchins are responding more strongly to bacterial infection, which would be especially beneficial if the younger urchins are more susceptible harm from infection. Only three urchins under 40mm were measured so to improve this comparison more small urchins could be measured and coelomocytes counted.

The total concentration of cells and concentrations of red and white cells were compared in figure 7 showing some interesting results especially in relation to the trends seen in ROS levels of different size urchins. Figure 7A shows that the smallest urchins (30-39mm) had the overall highest cell concentrations, while all other groups had approximately the same total cell concentration. To further analyze this I looked specifically at the urchins used in the ROS experiments, comparing their cell concentrations in figure 7B. The ranges of sizes that are considered small and large in this chart are different (<40mm for small, >50mm for large), but they show the same trend of smaller urchins having higher cells concentrations than larger urchins. This trend is seen in both figures 7A and 7B for total cell concentrations and white cell concentrations while red cells appear to be more consistent throughout. Because small urchins have less volume of coelomic fluid, the higher concentration of cells is an interesting observation implying that perhaps all of the urchins have approximately the same amount of coelomocytes, and as they grow in size they do not increase the number of coelomocytes proportionally to the amount of coelomic fluid.

The trend of larger urchins having higher ROS levels is consistent throughout all results shown and is contrary to the decreased ROS production as a result of age seen in humans and other model animals. This suggests that immune function is maintained (and perhaps enhanced) with age in sea urchins. The trend of smaller urchins having a higher coelomocyte cell concentrations is interesting and merits for further investigation before a conclusion can be drawn. Investigation into these trends has potential to lead to interesting conclusions about the functioning of the urchin immune system at different ages, especially if an effective method is developed for stimulating the immune system using LPS or something similar. This study provides a basis to begin research into the urchin immune system that may possibly have larger implications into human health, in particular for enhancing disease resistance with age.

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