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Flow Cytometric Analysis of Crayfish Hemocytes.

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Flow Cytometric Analysis of Crayfish Hemocytes

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by

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ABSTRACT

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Crayfish exhibit innate immune responses via hemocytes and their products. There are 3 hemocyte populations: hyaline cells, granular cells, and semigranular cells. Hemocytes from laboratory housed, untreated crayfish (normal crayfish) have been quantified on the basis of cell type, cell size, and cell granularity using Flow Cytometry. These data present the first overall picture of normal hemocytes from Red Swamp Crayfish with regard to cell type, cell size, and cell granularity and will serve as a baseline for all future studies in our lab. Experiments using crayfish injected with *Pseudomonas aeruginosa , Staphylococcus aureus,* or crayfish saline alone showed significant and consistent changes in cell type in cells from crayfish injected with bacteria with a decrease in hyaline cells and an increase in granular cells. This effect was greater in crayfish injected with Gram - bacteria. In addition, crayfish injected with *Pseudomonas aeruginosa showed* a significant difference in Granular cell size with a shift to larger cells and a significant decrease in granularity in the Granular cell population. Cells from crayfish treated with *Staphylococcus aureus* did not show these changes.

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CHAPTER 1

INTRODUCTION

Population Ecology

Freshwater crayfish are members of the superfamily Astacoidea. They encompass the largest population of freshwater crustaceans in North America, where there are 386 species and subspecies of crayfish. *Pacifastacus sp.* dominates California, Oregon, Washington, and Idaho, while *Procambarus sp.*is highly concentrated in the Southeast. They begin and end their life in littoral and riffle areas, but their lifecycle is centered primarily in deeper waters. Crayfish are aggressive which aides them in procuring both shelter and food. In addition to being aggressive, they are adaptive to a variety of environments from freshwater to saline. To understand these adaptations one must recognize that water continually diffuses into the hemolymph via the gills in freshwater crayfish. Osmosis and excretion take place in the antennal green glands, composed of an end sac that is complexly folded, an elaborate pathway leading to a nephridal canal followed by the bladder, and an excretory pore at the first segment of the second antennae. The green glands are located in front of the esophagus and manage water balance and prevent the loss of salts caused by diffusion. In order to maintain the balance between water and salts, crayfish produce hypotonic urine, lower their membrane permeability, and will undergo sodium uptake in their gills. Crayfish are able to obtain nutrition on every trophic level feeding as herbivores and carnivores as well as scavengers and detritivores (21).

Immunity

Crayfish only display an innate immune response that is characterized by no specificity in response and no long-term memory as well as the absence of cells involved in the adaptive immune system, such as the B-cells and the T-cells. The innate immune system of the crayfish is elaborate including phagocytosis, the phenoloxidase cascade, antibacterial peptides, clotting mechanisms, and

encapsulation (6). First, phagocytosis begins when a particle attaches to the plasma membrane of a phagocytic cell. Attachment triggers the engulfment of the particle forming a phagosome that eventually fuses particle with a lysososme forming a phagolysosome. The process is continued with the digestion of the particle within the phagolysosome via defensins, lysozymes, or digestive enzymes. Two proteins associated with phagocytosis have been isolated in the freshwater crayfish, *Pacifastacus leniusculus,* peroxinectin and masquerade (mas)- like protein. Peroxinectin is released from the hemocytes via exocytosis and functions as both a peroxidase upon microbial invasion and in cell adhesion. As a peroxidase, peroxinectin catalyzes the production of antimicrobial substances that may aide in killing pathogens. When functioning as a cell adhesion protein it can stimulate phagocytosis, degranulation, and capsule formation. The masquerade (mas)- like protein recognizes Gram negative bacteria and serves as an opsonin following cell wall attachment and proteolytic enzyme processing. Mas-like protein is located in hemocytes and consists of 2 subunits, one 134 kDa and the other 129 kDa, in heterodimeric form. These 2 subunits bind to LPS found in Gram negative bacteria, glucan, and microbes. Following microbial activation the mas-like protein undergoes proteolytic enzymatic processing (6, 10). The phenoloxidase cascade is initiated upon activation by either LPS, Peptidoglygan, or Beta-1,3-glucan. Activation is followed by entrance into a serine proteinase cascade. During the cascade inactive prophenoloxidase activating enzyme is converted to active prophenolxidase activating enzyme causing prophenoloxidase to be converted to phenoloxidase. The process is concluded with the oxidation of phenols and quinones generating melanin, which functions in melanization. This process involves the degranulation of large granular cells and complements other cellular defense processes ranging from cell adhesion and opsonization to encapsulation and nodulation as well as hemocyte aggregation (6, 11, 13). Liu and colleagues looked at the genes associated with the production of prophenoloxidase and pacifastin, a prophenoloxidase inhibitor, as well as crayfish response to *Aeromonas hydrophila,* a highly virulent bacterium found in freshwater crayfish. Depletion of

prophenoloxidase, produced a decrease in phagocytosis, phenoloxidase activity, and nodule formation, and an increase in bacterial proliferation and crayfish mortality. However deletion of pacifastin yielded a decrease in bacterial growth and crayfish mortality as well as an increase in phagocytosis, phenoloxidase activity, and nodule formation. In addition, they suggest that the synthesis of peroxinectin shares many steps with the production of prophenoloxidase. Therefore, increased production of prophenoloxidase yields a higher production of peroxinectin resulting in increased cell adhesion and phagocytic activity (11). Antibacterial peptides serve an integral role in crayfish immunity. Two antibacterial peptides have been isolated in the freshwater crayfish, *Pacifastacus leniusculus,* Astacidin 1 and the Crustin Family. These are thought to act on both Gram negative and Gram positive bacteria, although the functional mechanism is unknown (7). Clotting mechanisms prevent hemoymph loss and microbial entrance and migration through the open circulatory system, which is composed of the heart, arteries, and sinuses, but no veins. The heart is located in the large mid-dorsal pericardial sinus (21). In the open circulatory system the clotting process takes place via cross-linkage of a lipoprotein composed of 1721 amino acids that is directly cross-linked by transglutaminase. Finally, crayfish exhibit an encapsulation response, a process restricted to invertebrates. Encapsulation occurs when the bacterial load is too high or the infectious agent is too large for the phagocytic response of individual hemocytes. This results in the overlapping of many layers of hemocytes around the invader and ultimately its death, but the killing mechanism is unknown. However, several mechanisms have been proposed such as asphyxiation, free radicals, and antibacterial peptides (6).

Hemocytes

Nonspecific immunity exhibited in crayfish is accomplished by hemocytes and their products. Three types of hemocytes have been identified in the freshwater crayfish. They are hyaline cells, semigranular cells, and granular cells. It is thought that each type possesses a unique structure and serves specific functions in the crayfish immune response. The hyaline cells are the smallest of the

hemocytes with very few to no granules. Both light and transmission electron microscopy indicate they are spherical with free polyribosomes and rough endoplasmic reticulum. These cells are thought to function in phagocytosis. The semigranular hemocytes are elongated and oval shaped and possess both a large nucleus and numerous elliptical electron dense granules. In addition, light and transmission electron microscopy indicate they possess well established rough endoplasmic reticulum, Gogli apparatus, and mitochondria. Their function is not fully known; however, they apparently function in phagocytosis by producing opsonins and hyaline cell activators. The granular cells are circular to spindle shaped, and light and transmission electron microscopy has revealed that they have large electron dense granules that are structure-less and membrane-bound. Granular cells are thought to function both in inflammation and in melanization. However, the mechanism involved in the inflammatory response is unknown. In melanization the granules in these cells appear to function as storage chambers for the molecules involved in the phenoloxidase cascade (3).

Flow Cytometry

Flow cytometry is a powerful tool used in multiparametric analysis of individual cells within heterogeneous populations. It has been used in many ways including observation of cell surface markers via immunophenotyping, evaluation of cellular chromosomes through ploidy analysis, green fluorescent protein (GFP) gene expression analysis (17), and cell counting. Various components, each with unique functions, go into the operation of flow cytometry. First, the fluidic system presents the sample at the interrogation point and removes waste. Second, the laser beam serves as the source for both scatter and fluorescence. Third, the optics gather the light and direct it. Fourth, detectors then receive the light from the optics. Finally, the electronics and the computer system receive the signals from the detectors and convert them to digital data (5). A recent study used flow cytometry to observe and measure calcium uptake and homeostasis in crayfish during their molting cycles by looking at individual intermoult crayfish. Wheatly and colleagues used flow cytometry because it can be used to

analyze subcellular particles such as liposomes, endosomes, platelets, lymphocytes, and membrane vesicles by using light scatter and fluorescence transmission by individual cells as they quickly pass in front of the optics. Furthermore, it can be carried out with a minimal amount of material with the use of 90° angle light scatter. This study looked at calcium pumps in crayfish because they are vital membrane proteins consisting of 1000 amino acid residues consisting of 3 cytoplasmic domains. The first domain serves as an ATP binding site, the second as a phosphorylation site, and the third as a transduction domain. Wheatly and colleagues harvested basolateral membrane vesicles of the hepatopancreas from individual crayfish. Then, the vesicles were treated with the fluorescent dye Fluo-3, an excellent chelator with a high calcium affinity. The treated vesicles were incubated with polyclonal antifluorescein antibodies to remove excess Fluo-3 from the vesicles exterior and other debris. The vesicles were centrifuged; the pellet was resuspended in extravesicular medium and inserted into a flow cytometer. Calcium uptake and loss was monitored by comparing the response in fluorescence upon the addition of either ATP and calcium or ATP. Fluorescence was then measured by using the logarithmic fluorescence scale versus events, that correspond to each cell. Wheatly and colleagues observed a 6 fold increase in fluorescent labeled cells in the presence of both calcium and ATP but no change could be observed in the presence of either calcium or ATP (23). In addition, hemocyte production and maturation can be monitored by analyzing the BrdU expression in crayfish stem cells. Crayfish hemocytes are produced in the hematopoetic tissue, and this tissue is located on the dorsal side of the stomach. However, up to this point hematopoetic regulatory mechanisms in invertebrates, particularly crayfish, have been unknown and no successful method in harvesting and processing hematopoetic tissue in vitro existed. Söderhäll and colleagues chose to perform in vivo analysis by challenging crayfish with crayfish saline or laminarin, a β1, 3-glucan, which elicits an acute hemocyte deficiency in crayfish. This is similar to the response elicited by microbial challenge. They harvested the hemocytes and ran them through a flow cytometer. Total hemocyte count was assessed every 6 hours

postinjection and bleeding for 24 hours. After 6 hours the total hemocyte count dropped, but the total hemocyte count recovered over the 24-hour period. To determine if the recovery was due to an increase in proliferation of circulating hemocytes or the production and maturation of new hemocytes, crayfish were injected with BrdU, which stains the hemocytes. The hemocytes were harvested and run through the flow cytometer. After BrdU injection, hemopoietic tissue cells in the crayfish displayed an increased incorporation of the BrdU, whereas the circulating hemocytes did not. To determine if the circulating hemocytes were produced in the hemopoietic tissue or the hemolymph, crayfish were injected with BrdU 24 hours prior to treatment with laminarin or simultaneously with laminarin. Four hours postinjection, the total hemocyte count for crayfish treated with laminarin 24 hours after BrdU injection significantly increased. The animals that had been simultaneously treated with BrdU and laminarin did not show an increase in total hemocyte count. The authors concluded that most newly circulating hemocytes are not synthesized in the hemolymph, and laminarin causes the maturation of the hemopoietic stem cells in the hemopoietic tissue and release into the hemolymph (18). Flow cytometry can be used to display the effects of both LPS and its components. Cárdenas and colleagues used flow cytometry to show real time cellular responses to microbial challenge. They harvested the hemolymph and isolated the hemocytes by centrifugation. The hemocytes were resuspended in crayfish saline with HEPES. These hemocytes were then incubated with LPS, LPSdex, or LPS Rc. LPSdex is detoxified while LPS Rc is deficient in the O-antigen and has an incomplete core structure consisting of glucose and heptose linked to lipid A by a 2-keto-3-deoxy-D-mannooctonate. After 10 and 20 minute intervals aliquots of the incubated hemocytes were taken and analyzed by flow cytometry. This study used forward angle light scatter to measure cell size and cell viability was assessed by Calcein-AM and EthD-1 to measure live and dead cells respectively. This study revealed that when exposed to LPS crayfish hemocyte populations decrease both in size and viability. However, when exposed to LPS without the Lipid A moiety, hemocyte populations decreased only in size not viability. In addition LPS

challenge produced a gradual decline in cell size, but apoptosis took place only toward the end of the time point measured. The authors concluded that the Lipid A moiety is cytotoxic while the polyssacharide portion is regulatory, and crayfish respond innately to microbial infection and LPS is not mearly cytoxic (1). Earlier Cárdenas and colleagues measured the effects of zymosan A on crayfish hemocytes. Cell size was analyzed using forward and side angle light scatter, and cell viability was measured using calcein-AM and ethidium homodimer 1-by-2 color fluorescence staining. They observed a reduction in cell size and viability in crayfish hemocytes exposed to zymosan A. The addition of diethyldithiocarbamic acid, a phenoloxidase inhibitor, slowed cell size reduction and delayed cell death. Further addition of a trypsin inhibitor also delayed both cell size reduction and cell death. Cárdenas and colleagues concluded that the prophenoloxidase cascade serves an integral role in generating these responses (2). Flow cytometry has also been used to count hemocytes in crayfish that are free and captive. S. Taylor and collegues looked at hemocyte populations isolated from crayfish, *Paranephrops planifrons*, from 3 lakes located in New Zealand as well as captive crayfish. S. Taylor and colleagues isolated hemocytes from crayfish at each of the 3 lakes and from the captive crayfish. Then they analyzed them by flow cytometry. S. Taylor and colleagues observed crayfish held in captivity for 14 days. After 14 days crayfish exhibited a 63 percent decrease in overall hemocyte count, whereas the free crayfish exhibited no reduction (20). Flow cytometry has been used in studies into the immunity of shrimps, prawns, and crabs. One study revealed three hemocyte populations in shrimp: hyaline cells, granular cells, and semigranular cells through flow cytometry. Through PCR analysis white spot syndrome could be detected in shrimp hemocytes. However, electron microscopy revealed that only the granular and semigranular cells actually took up the virus (22). In addition, another study completed an in vivo analysis on shrimp infected with LPS or p43, a mitogenic protein developed from *Candida albicans* that serves as an immunosuppressive, and a combination of both. T. Segueira and colleagues injected shrimp with LPS, p43, or LPS and p43, all of which were suspended in shrimp salt solution. Five

days later the hemolymph was extracted, and the hemocytes were isolated and analyzed by flow cytometry. This study revealed a dramatic increase in hemocyte proliferation in response to LPS, p43, as well as LPS and p43 when compared to the control animals that were injected with shrimp salt solution (15). An in vitro analysis of the hemolymph of prawns displayed 3 types of hemocyte populations via flow cytometry. Lee and colleagues then harvested hemolyph and exposed it to heat killed *E.coli* that was stained with propidium iodide. They observed active engulfment of the bacteria by the hemocytes. This was confirmed by transmission electron microscopy (10). In addition, a study confirmed that hemocytes that were first incubated with antibody 6C1-1F followed by further incubation with Alexa 488-conjugated goat anti-mouse IgG, a fluorescence- labeled secondary antibody, displayed the highest affinity to the antigens present on the surface of the hemocytes of the horseshoe crab (12).

Focus of the Study

This study uses forward angle light scatter to measure cell size and 90° angle light scatter to measure cell granularity. These 2 single measurements done on crayfish hemolymph harvested directly into anticoagulant buffer allows us to separate the crayfish hemocytes into 3 populations, hyaline, granular, and semigranular. Each of these populations can then be assayed for cell size and cell granularity as well as the entire hemocyte population. The cell type assay uses a 2-parameter dot plot with the forward angle light scatter versus 90° angle light scatter and in each coordinate individual dots represent individual cells (Figure 1).

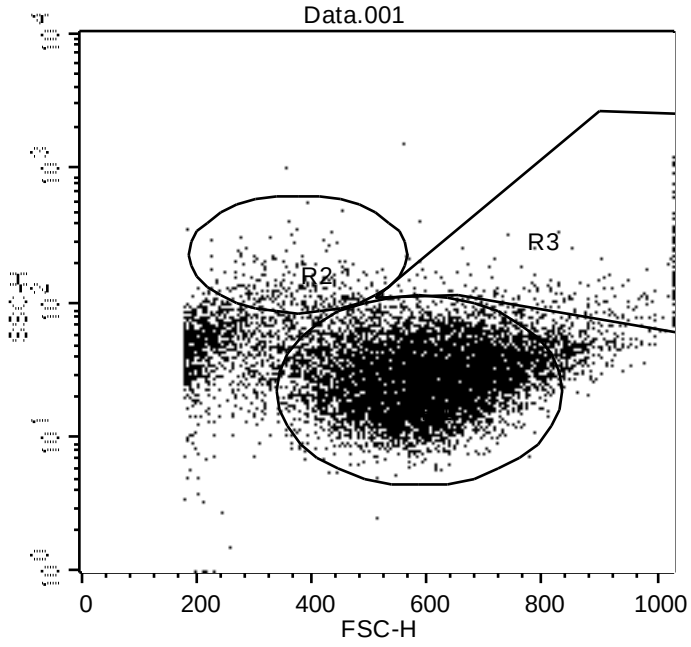


Figure 1. Flow cytometry of crayfish hemocytes in crayfish anticoagulant buffer. Dot plot of forward angle light scatter versus 90° angle light scatter parameters showing 3 individual hemocyte populations: R1, Hyaline; R2, Granular; R3, Semigranular.

The cell size assay uses a single parameter histogram with forward angle light scatter on the x-axis and counts (number of cells) on the y-axis (Figure 2).

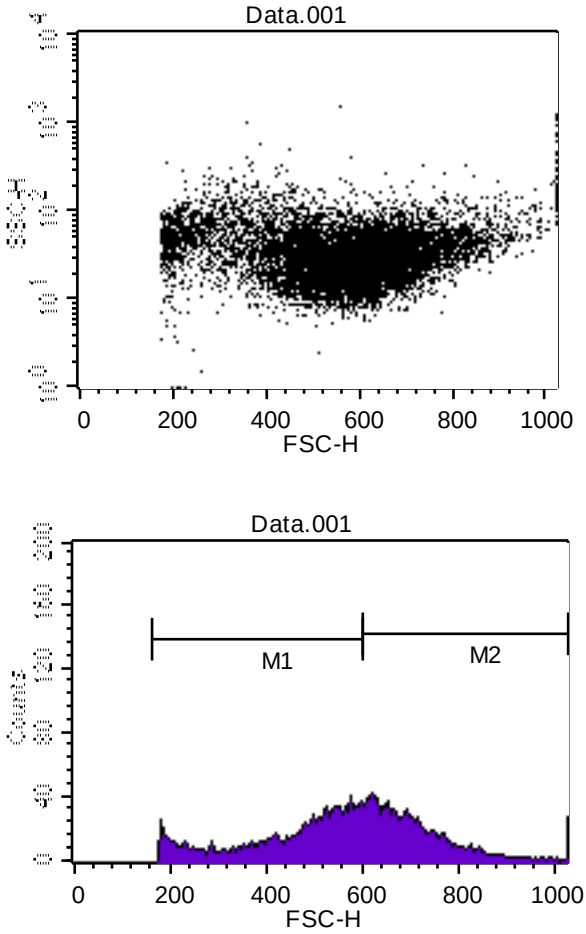


Figure 2. Flow cytometry of crayfish hemocytes in crayfish anticoagulant buffer. Dot plot of forward angle light scatter versus 90° angle light scatter parameters showing overall cell populations. One parameter histogram using the forward angle light scatter parameter to display the regions indicated in the analyses of cell size distribution. M1 and M2 represent cells of smaller and larger size respectively.

Measurement of cell granularity uses a single parameter histogram with 90° angle light scatter on the x-axis and counts (number of cells) on the y-axis (Figure 3).

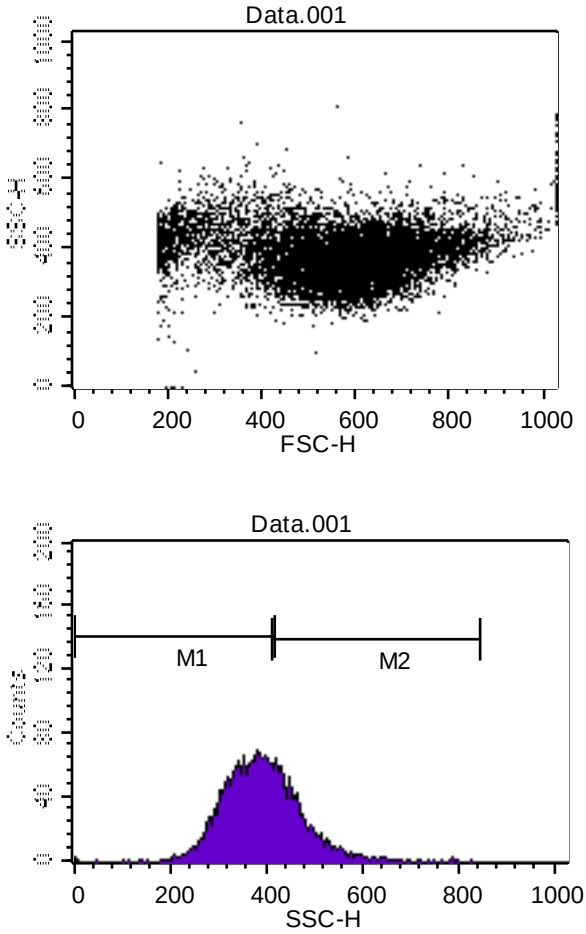


Figure 3. Flow cytometry of crayfish hemocytes in crayfish anticoagulant buffer. Dot plot of forward angle light scatter versus 90° angle light scatter parameters showing overall cell populations. One parameter histogram using the 90° angle light scatter parameter to display the regions indicated in the analyses of cell granularity distribution. M1 and M2 represent cells of low and high granularity respectively.

Normal Untreated Crayfish Hemocytes

Our work with normal untreated crayfish focuses on 3 areas:

1. Cell Type

   The percentages of hemocytes represented by the hyaline, granular, and semigranular populations will be measured.

2. Cell Size

   Cell size within each of the populations and for the entire hemocyte population will be determined.

3. Cell Granularity

   Cell Granularity within the granular and semigranular populations will be measured.

The variability of these measurements within the normal hemocyte populations will be used to help us describe which parameter might be the most valuable to use in the second most important aspect of our study, using flow cytometry to evaluate the status of the immune system after exposure to stressors. In addition, no systematic evaluation of these parameters has been done for normal untreated crayfish, and these measurements will provide new information regarding the status of the hemocyte populations in normal crayfish.

Control and Treated Crayfish Hemocytes

A major goal of this study is to find parameters that consistently change when crayfish are exposed to stress. The stressors used in our study are exposure to *Pseudomonas aeruginsoum*, a Gram negative bacterium, and *Staphylococcus aureus*, a Gram positive bacterium. From previous work in our lab it is known that exposure to Gram negative bacteria enhances the phagocytic activity of crayfish hemocytes as measured in an in-vitro phagocytic assay of sheep erythrocytes (Figure 4).

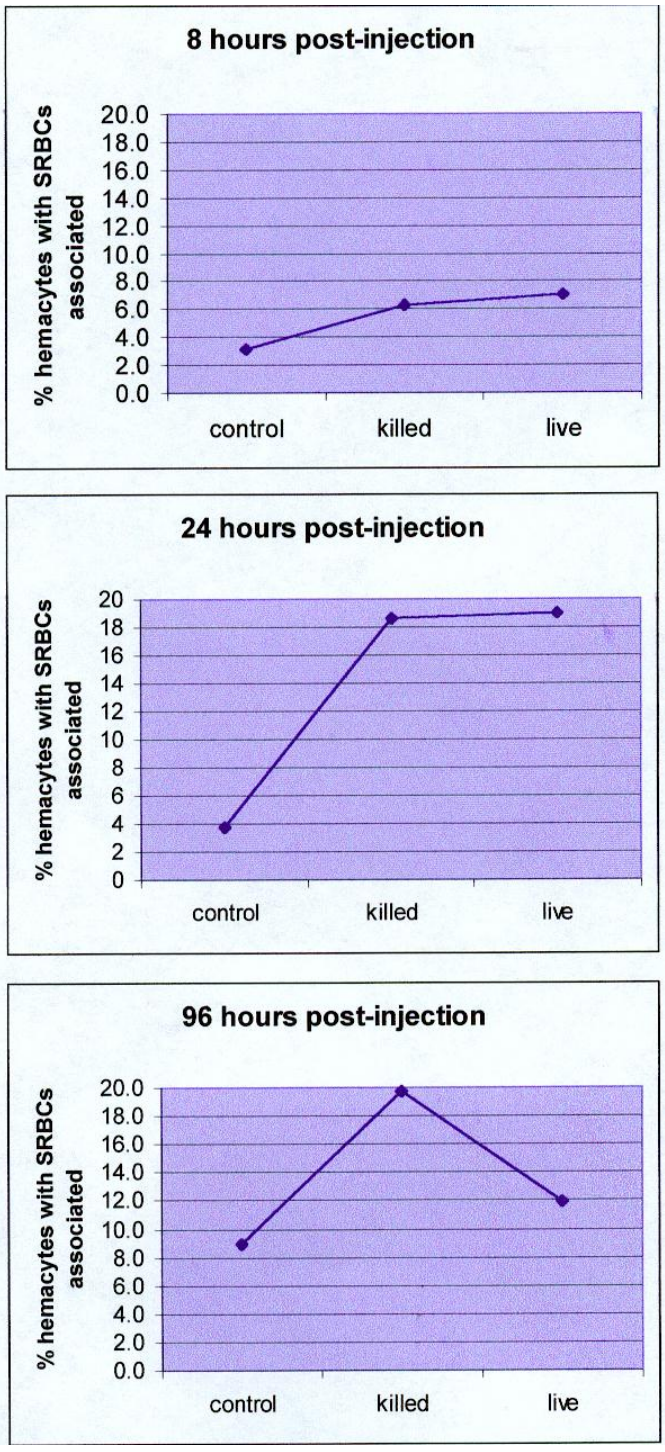


Figure 4. Phagocytic Assay Involving Hemocytes and Associated Sheep Erythrocytes 8, 24, and 96 Hours Postinjection with Crayfish Saline, Live and Killed Bacteria (4).

Because phagocytosis is an extremely important defense mechanism in crayfish, we decided to use this model as our stressor. Three crayfish were injected with *Pseudomonas aeruginosa* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline, 3 with *Staphylococcus aureus* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline, and 3 crayfish were injected with crayfish saline alone to serve as the control. Twenty-four hours postinjection hemocytes were harvested and analyzed by flow cytometry. Following this, 3 more crayfish were injected with *Pseudomonas aeruginosa* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline, and 3 more crayfish were injected with crayfish saline alone to serve as the control. Twenty-four hours postinjection hemocytes were harvested and analyzed by flow cytometry. Finally, 3 additional crayfish were injected with *Pseudomonas aeruginosa* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline, and 3 additional crayfish were injected with crayfish saline alone to serve as the control. Twenty-four hours postinjection hemocytes were also harvested and analyzed by flow cytometry.

If upon completion of these assays we find parameters that are significantly and consistently different in treated versus control crayfish, then we are one step closer to using flow cytometry as a screening test for the stress on the crayfish immune system in both the laboratory and natural habitats. Crayfish have successfully occupied a wide range of habitats. Of all freshwater crustacea, crayfish are the largest and have been present the longest in North American freshwater ecosystems from lotic to lentic environments. Crayfish process a wide variety of organic matter in these systems. By serving as decomposers they breakdown detritus and change it biochemically. Then they release this material back into the environment. This contributes extensively to the flow of energy throughout aquatic systems. In addition crayfish, can alter their position in freshwater ecosystems. As mentioned before, crayfish serve as herbivores, carnivores, and scavengers depending on food allocation. For example, as herbivores crayfish can selectively feed on a narrow range of plant material. They do this with very little energy expenditure, and this can significantly reduce species richness in the environment. In

addition, as carnivores crayfish will gravitate toward prey that will require the least energy investment. This is observed as crayfish feed more on snails with thin shells than those with thick shells. Through this they are able to maintain their large population densities. By feeding on nearly all trophic levels in aquatic ecosystems crayfish manipulate a wide range of both interspecific and intraspecific interactions. Thus crayfish can serve as an indicator subject for the health of both lotic and lentic systems (21) because stressors detected in crayfish could indicate stressors in the ecosystem.

CHAPTER 2

MATERIALS AND METHODS

Animal Protocol (4)

This study used red swamp crayfish, *Procambarus clarki,* (Lot Number: WF-14-2502) purchased from Wauburn Laboratories (102 West Main Street #A Schriever, LA 70395) through Carolina Biological Supply Company (2700 York Road, Burlington, NC 27215). They were kept in fresh water aerated aquariums held at 25°C. and fed twice weekly (Tuesday and Thursday) with 6 pellets (~1.42 g) of Kaytee Timothy Complete Guinea Pig Food. (Kaytee Products: 521 Clay Street, P.O. Box 230, Chilton, WI 53014).

Reagent Preparation

Crayfish Anticoagulant Buffer Solution (19)

A buffer 100.0ml buffer solution of 0.82g of NaCl was used. This solution consisted of 1.80g glucose, 0.88g trisodium citrate, 0.50g citric acid, 0.37g EDTA, and 70.0 ml of double distilled water. This was mixed via a stir bar in a 600.0ml beaker, and double distilled water was again used to both raise the volume to 100.0ml. This was followed by adjusting the pH to 4.60 and filter sterilization via a 0.2 µm filter pore and storage at 4°C.

Crayfish Saline Solution (19)

A 500ml saline solution was used. This consisted of 5.84g NaCl, 201.4mg KCl, 555.0mg $CaCl_2$, 264.4mg $MgCl_2 + 6H_2O$, 84.0mg $NaHCO_3$, and 450.0ml double distilled H_2O. This was mixed in a 600.0ml beaker with a stir bar until thoroughly dissolved and double distilled H_2O was then used to raise the solution volume to 500.0ml. This was followed by adjusting the pH to 6.75, autoclaving, and storage at 4°C.

Nutrient Agar (4)

A 100.0ml solution was used. This consisted of 2.3g nutrient agar dissolved in 100.0ml double distilled H_2O. This was mixed in a 250.0ml volumetric flask, autoclaved for sterilization, and cooled in a water bath at 50°C.

Nutrient Broth

A 100.0ml solution was used. This consisted of 0.3g beef extract powder and 0.5g bacto peptone dissolved in 100.0ml double distilled H_2O. This was mixed in a 250.0ml Erlenmeyer flask and autoclaved for sterilization.

Bacterial Cultures

Pseudomonas aeruginosa

A *Pseudomonas aeruginosa* stock culture strain (Lot Number 10145) purchased from American Type Culture Collection (P.O. Box 1549 Manassas, VA 20108) was used in this study. This bacterium had been cultured on a nutrient agar slant before being transferred to nutrient broth for incubation at37°C for 12 hours. Following this an innoculum of this bacterium was transferred to nutrient broth and aerated in a shaker for 24 hours at 37°C. Then colonies were isolated by streaking the bacterial culture onto plates, and incubated for 24 hours at 37°C. Once isolated the purity of these colonies was determined via Gram staining. The pure *Pseudomonas aeruginosa* colonies were then be incubated in nutrient broth for 24 hours at 37°C (4).

Staphylococcus aureus (4)

A *Staphlococcus aureus* stock culture strain (Lot Number 25923) purchased from American Type Culture Collection (P.O. Box 1549 Manassas, VA 20108) was used in this study. This strain was inoculated into sterile nutrient broth for 12 hours, incubated at 37°C on a shaker, and used in the experiments.

Immunization Protocol (4)

Pseudomonas aeruginosa stock culture was used to immunize the crayfish. These bacterial cells were incubated and centrifuged for 20 minutes at 10°C at 1200 rpm. Following this, the pellet was placed in 1ml of crayfish saline and counted via hemocytometer and diluted to one million cells per ml concentration. The following vaccination procedures were accomplished using 10^6 bacterial cells/ml as crayfish have exhibited tolerance to this concentration in previous studies in the lab. Then, 3 crayfish were injected with 1ml of the live bacterial vaccine, and 3 crayfish are injected with 1ml of the crayfish saline solution to serve as a control.

Pseudomonas aeruginosa and Staphlococcus aureus stock cultures were used to immunize the crayfish. These bacterial cells were incubated and centrifuged for 20 minutes at 10°C at 1200 rpm. Following this, the pellet was placed in 1ml of crayfish saline and counted via hemocytometer and diluted to a concentration of 10^6 bacterial cells/ml. The following vaccination procedures were accomplished using 10^6 bacterial cells/ml as crayfish have exhibited tolerance to this concentration in previous studies in the lab. Then, 3 crayfish were injected with 1ml of the *Pseudomonas aeruginosa* vaccine, 3 crayfish were injected with 1ml of the *Staphlococcus aureus* vaccine, and 3 crayfish were injected with 1ml of the crayfish saline solution to serve as a control.

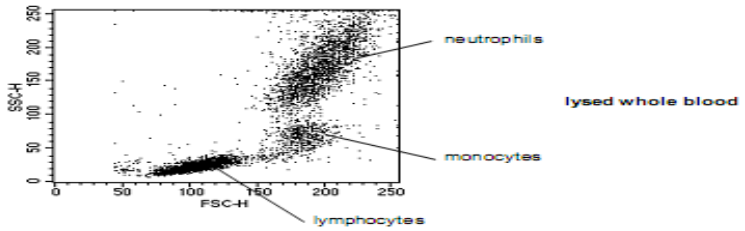
Obtaining and Preparing Hemocytes (4)

Crayfish were held in separate aquariums based upon their course of treatment (Normal Untreated, *Pseudomonas aeruginosa*, *Staphlococcus aureus,* or control), and each crayfish was moved to an ice bath for 30-45 minutes in order to slow its metabolism and allow for proper handling. Then 1.0mL of their hemolymph was isolated via abdominal hemocoel with a 22 gauge sterile needle. The hemolymph from each group was collected with a 3cc sterile syringe loaded with 0.5mL of crayfish anticoagulant buffer.

Flow Cytometry

Background

Flow cytometry was used to detect cell size and granularity. This was accomplished using the FACS Calibur flow cytometer. Cell size was measured via forward angle light scatter, and granularity measured via 90° light scatter. The following scatter plot is an example of human whole lysed blood that has had large phagocytic cells such as monocytes and neutrophils isolated:



(5).

The higher forward angle light scatter value corresponds to a larger cell size, and the higher the 90° light scatter value corresponds to a cell with more granularity.

This study focused on the 3 types of previously isolated hemocytes, hyaline cells, granular cells, and semigranular cells (3). Hyaline cells are the smallest with very little to no granularity that function in phagocytosis. The granular hemocytes, the largest of the hemocytes, function in melanization and are granulated, and these granules serve as storage chambers for molecules involved in the prophenoloxidase cascade in the melanization process. Semigranular hemocytes are larger than the hyaline cells and have dense granularity. These hemocytes also function in phagocytosis (6).

Process

Hemocytes were isolated as in the above mentioned procedure. Following this the hemolymph was then emptied into 2 mL vials (sterile) and placed on ice. The hemocytes were then analyzed by placing the hemolymph into the FACSCalibur tubes and allowing the sample to run through the flow cytometer at 10,000 cells per cycle. This was followed by measurements of cell type, cell size, and cell granularity. Cell type was determined by looking at dot plots with forward angle light scatter on the x-axis and 90° angle light scatter on the y-axis and drawing bit maps around the isolated populations. These bit maps corresponded to the cell size and cell granularity levels observed in each population. Cell size was assayed by looking at a histogram dot plot with forward angle light scatter on the x-axis and counts (the number of cells) on the y-axis. This was then followed by dividing the histogram dot plot in half, the first half corresponding cells in the smaller size range and the second half corresponding to cells in the larger size range. Cell granularity was determined by looking at a histogram dot plot with 90° angle light scatter on the x-axis and counts (the number of cells) on the y-axis. This was then followed by dividing the histogram dot plot in half, the first half corresponding to cells of low granularity and the second half corresponding to cells of high granularity.

Statistical Analysis

In order to compare means calculated from the control samples to means calculated from the experimental samples, all statistical analysis was completed using the Student's t-test with a 0.95 confidence interval, and a pvalue ≤ 0.05 was seen as significant.

CHAPTER 3

RESULTS

Normal Untreated Crayfish

Cell Type

In 20 normal untreated crayfish average percentages of the 3 individual hemocyte populations were 89.7 (± 0.87; Range 82.8-94.4) for the hyaline cell population, 6.6 (± 0.66; Range 2.49-12.0) for the granular cell population, and 3.7 (± 0.40; Range 1.54-8.46) for the semigranular cell population (Figure 5 & Table 1).

Cell Size

When looking at overall cell size, 85.5 percent (± 3.17) of the normal crayfish cells were in the smaller cell range whereas 15.5 percent (± 3.17) were in the larger cell range (Figure 6A). Analysis of hyaline cells revealed, 62.9 percent (± 2.93) of the cells were in the smaller cell range and 13.1 percent (±2.93) of the cells were in the larger cell range (Figure 6B). Looking at the granular cells revealed 51.2 percent (± 2.58) of the cells were in the smaller cell range and 48.7 percent (± 2.58) of the cells were in the larger cell range (Figure 6C). Analysis of the semigranular cells showed 63.1 percent (± 4.13) of the cells were in the smaller cell range and 36.8 percent (± 4.13) of the cells were in the larger cell range (Figure 6D).

Cell Granularity

When overall cell granularity was measured, 76.8 percent (±2.78) of the normal crayfish cells were in the low granularity range, whereas 23.2 percent (± 2.78) of the cells were in the high granularity range (Figure 7A). Looking further at the granular cells showed 77.5 percent (± 2.09) of the cells were in the low granularity range and 22.5 percent (±2.09) of the cells were in the high granularity range (Figure 7B). When analyzing the semigranular cells 85.2 percent (±2.79) of the cells were in the low granularity range and 14.8 percent (± 2.79) of the cells were in the high granularity range (Figure 7C).

Table 1. Percentage of Individual Hyaline, Granular, and Semigranular Hemocyte Populations from Normal Untreated Crayfish with Mean Percentages, Standard Error, and Ranges.

Sample	Hyaline Cells	Granular Cells	Semigranular Cells
1	93.6	4.84	1.6
2	82.8	8.76	8.46
3	85.1	9.82	5.12
4	94.4	4.1	1.54
5	94.2	3.09	2.48
6	86.3	10.4	3.3
7	88.7	7.7	3.6
8	92.5	4.75	2.75
9	91	7.24	1.7
10	89.4	8.29	2.34
11	93.9	2.49	3.61
12	90.6	3.07	6.32
13	85.4	10.7	3.88
14	91.4	4.93	3.71
15	90.5	6.82	2.71
16	84.4	9.58	5.96
17	92.4	5.61	1.98
18	91.1	3.94	4.95
19	93.8	3.22	2.92
20	83.2	12	4.83
Mean	89.7	6.57	3.69
S.E.M.	±0.866	±0.660	±0.402
Range	82.8-94.4	2.49-12.0	1.54-8.46

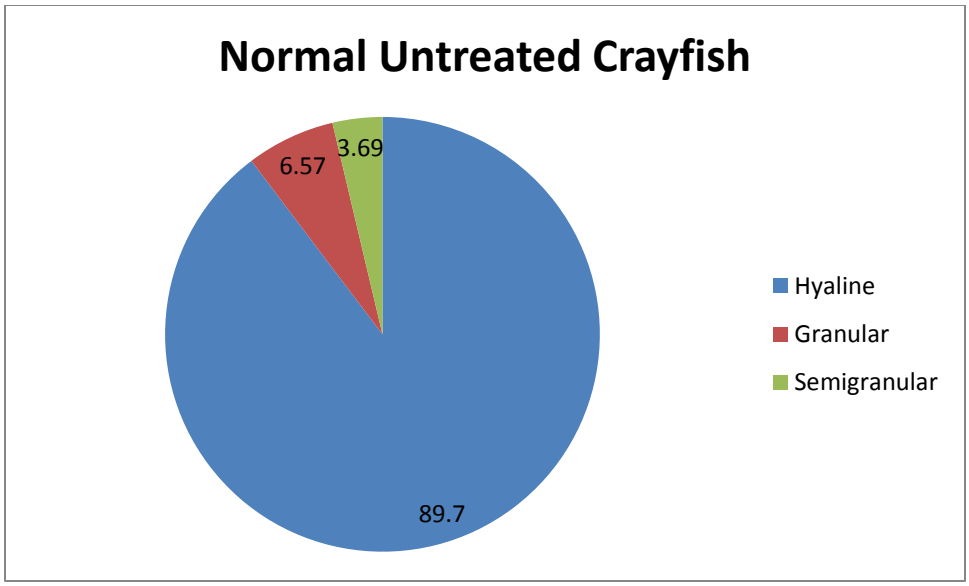


Figure 5. Overall Distribution of Hemocyte Populations for Normal Untreated Crayfish. Chart sections represent mean percent hemocytes. n = 20.

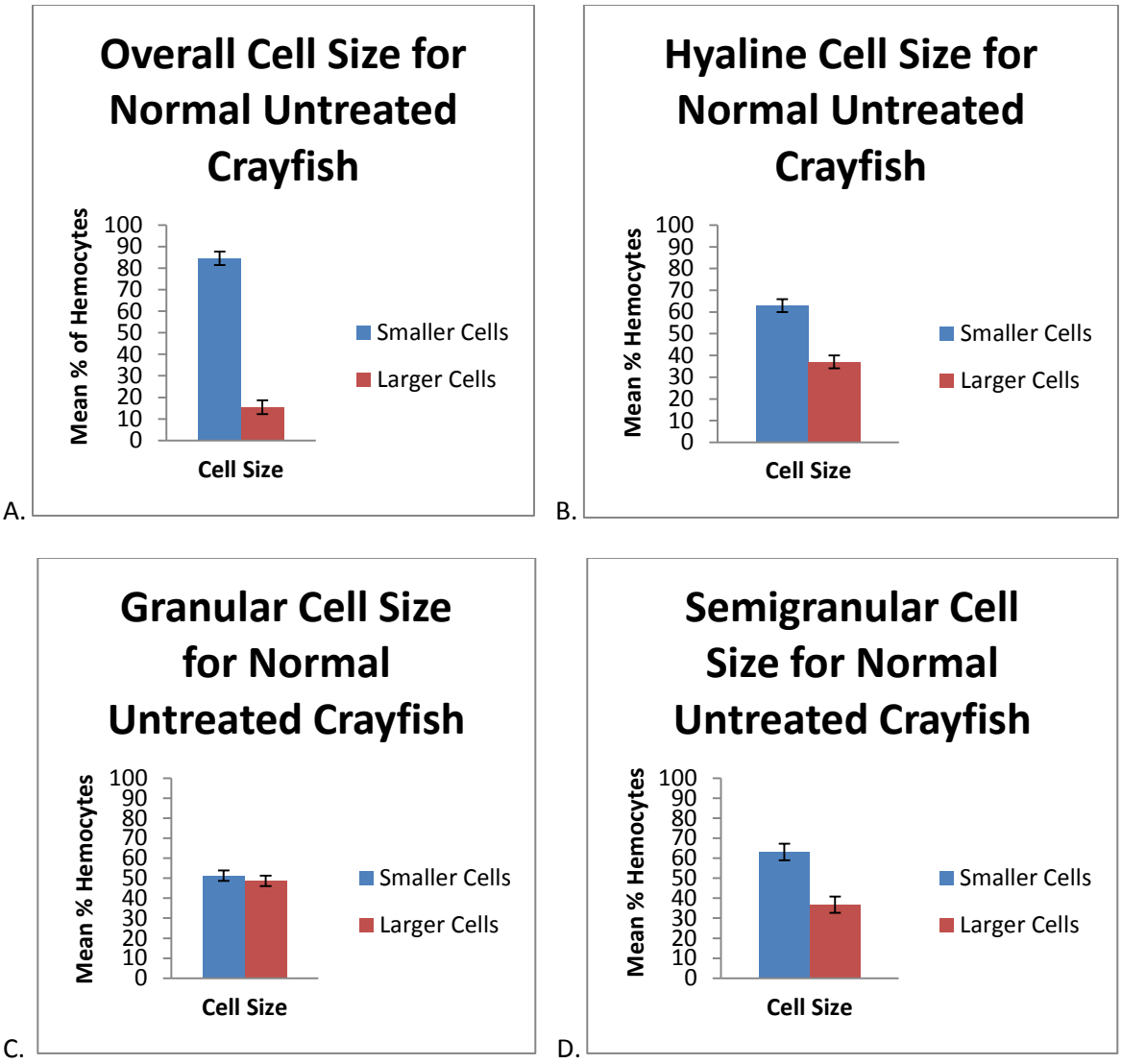
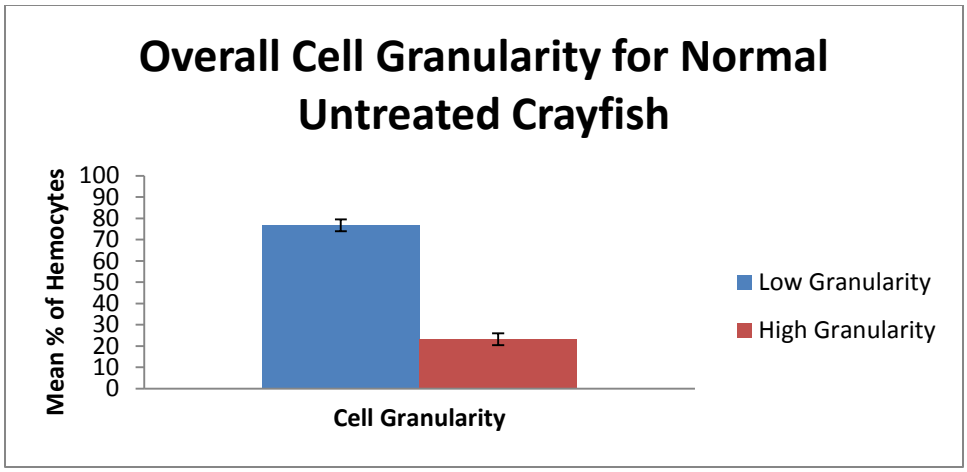


Figure 6. Cell Size Distribution for Overall Cells (A), Hyaline Cells (B), Granular Cells (C), and Semigranular Cells (D) for Normal Untreated Crayfish. n = 20.

A.



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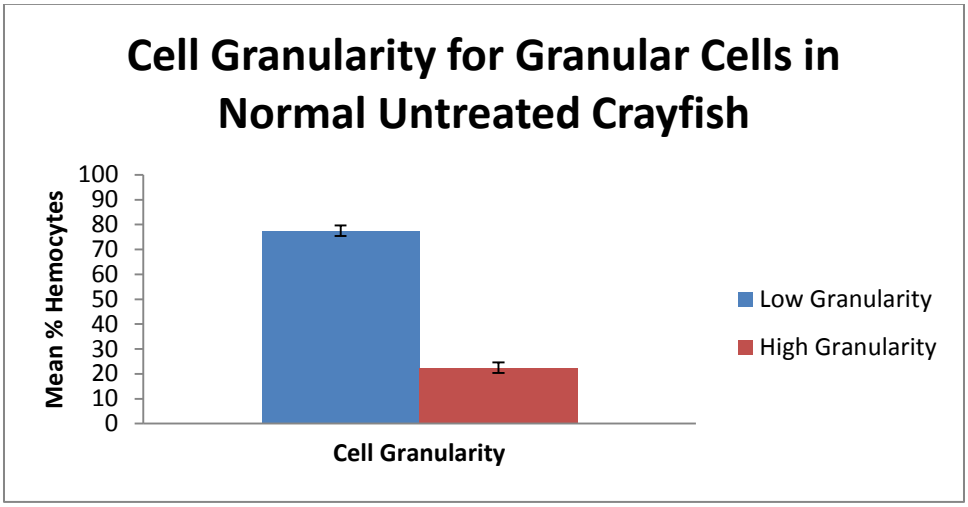


C.

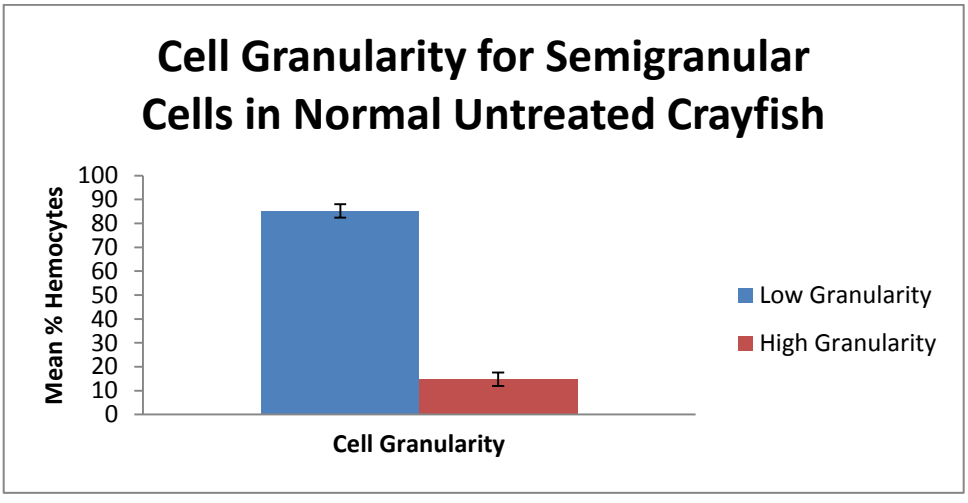


Figure 7. Cell Granularity for Overall Cells (A), Granular Cells (B), and Semigranular Cells (C) for Normal Untreated Crayfish. n = 20.

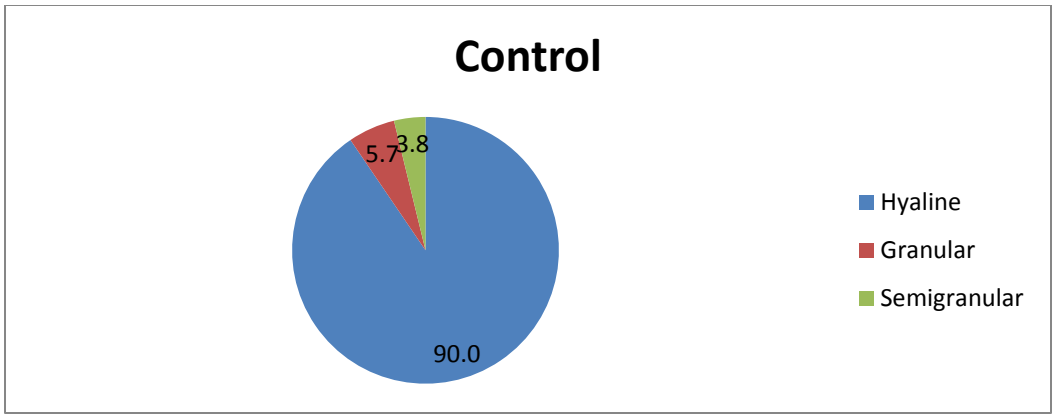
Effect of Treatment with Gram Negative Bacteria versus Gram Positive Bacteria on Hemocyte Distribution

Experiment 1 was designed to measure the effects of injection with Gram negative or Gram positive bacteria on hemocyte cell type, cell size, and cell granularity. Three crayfish were injected with *Pseudomonas aeruginosa* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline, 3 with *Staphylococcus aureus* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline, and 3 crayfish were injected with crayfish saline alone to serve as the control. Twent- four hours postinjection hemocytes were harvested and analyzed by flow cytometry.

Cell Type

Crayfish treated with crayfish saline exhibited a mean percentage of 90.0 (± 0.80; Range 89.4-92.0) for the hyaline cell population, 5.7 (± 0.72; Range 4.31-6.60) for the granular population, and 3.8 (± 0.23; Range3.5-4.27) for the semigranular population (Figure 8A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed a mean percentage of 79.0 (± 1.1; Range 77.3-81.1) for the hyaline cell population, 17.0 (± 0.77; Range 15.8-18.4) for the granular population, and 3.6 (± 0.37; Range 3.08-4.29) for the semigranular population (Figure 8B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline presented with a mean percentage of 84.0 (±0.74; Range 82.4-84.9) for the hyaline cell population, 12.0 (±0.68; 11.4-13.6) for the granular cell population, and 3.9 (± 0.14; Range 3.64-4.13) for the semigranular population (Figure 8C).

A.



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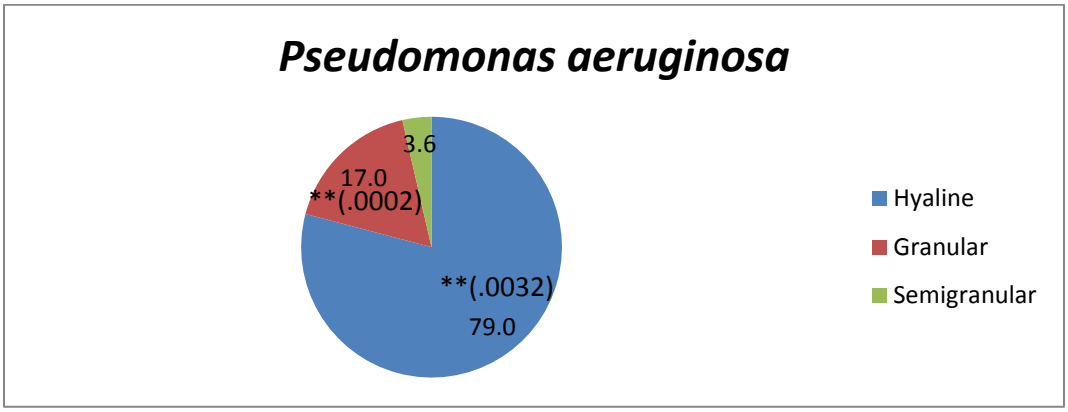

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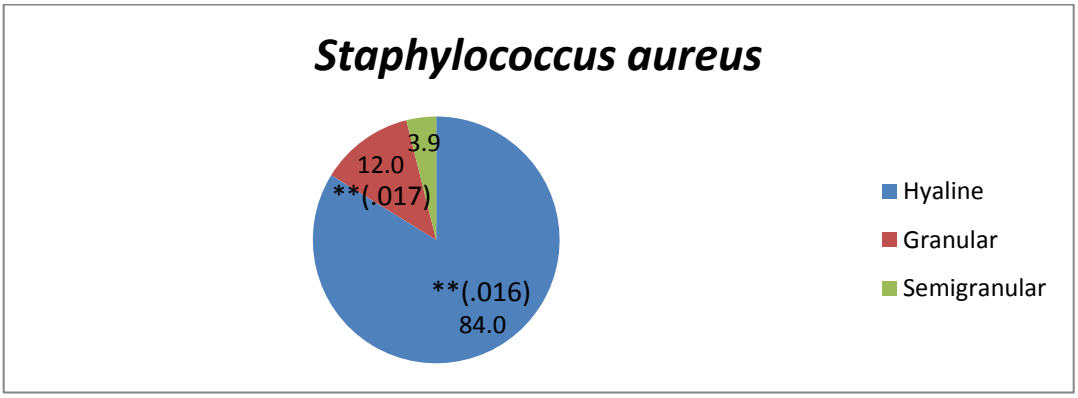
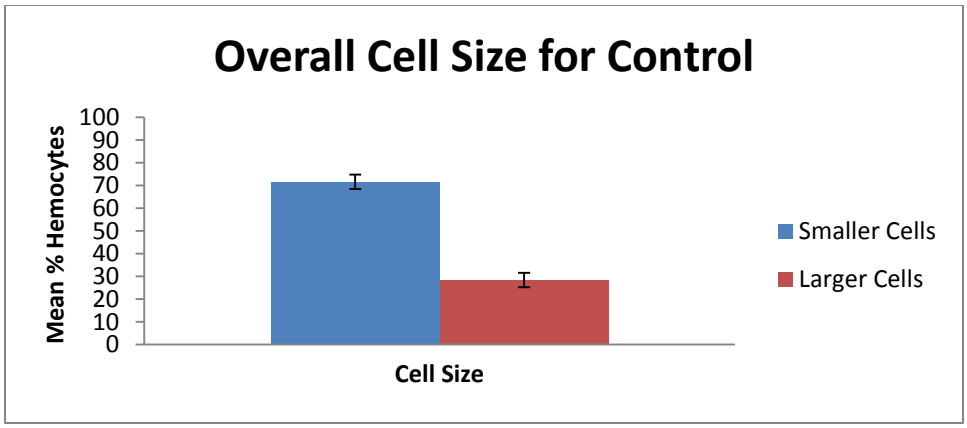


Figure 8. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C*) Staphylococcus aureus* on Distribution of Hemocyte Populations 24 Hours Postinjection. Chart sections represent mean percent hemocytes. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

Cell Size

When analyzing overall cell size, crayfish treated with crayfish saline 72.0 percent (± 3.2) of the cells were in the smaller cell range and 28.0 percent (± 3.2) of the cells were in the larger cell range (Figure 9A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 64.0 percent (± 4.1) of the cells in the smaller cell range and 36.0 percent (±4.1) of the cells in the larger cell range (Figure 9B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline presented with 71.0 percent (± 9.3) of the cells in the smaller cell range and 29.0 percent (± 9.3) of the cells in the larger cell range (Figure 9C).

A.



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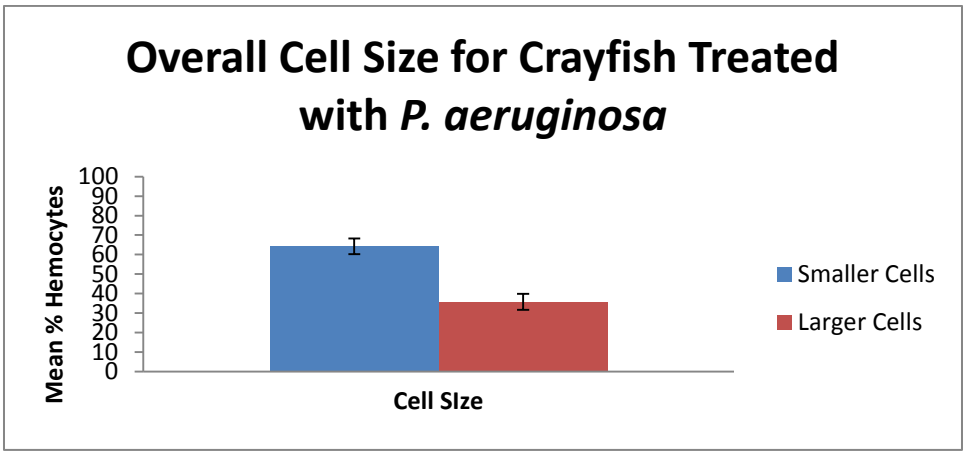

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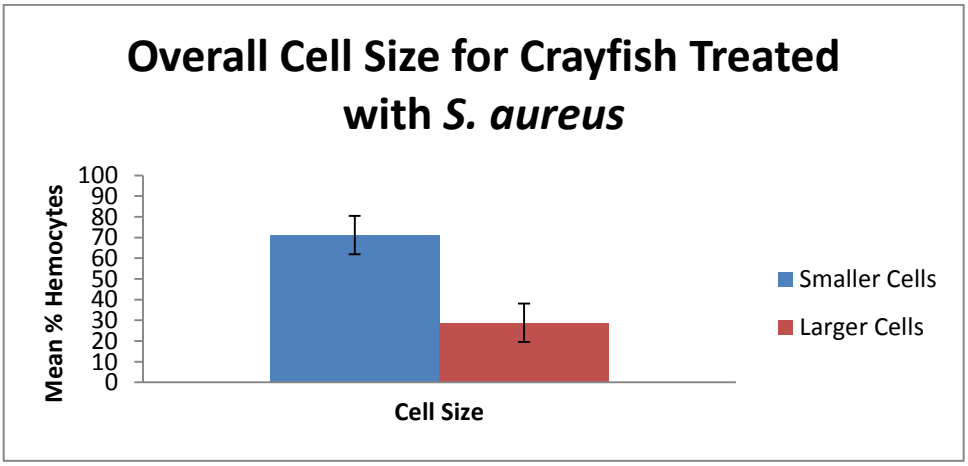
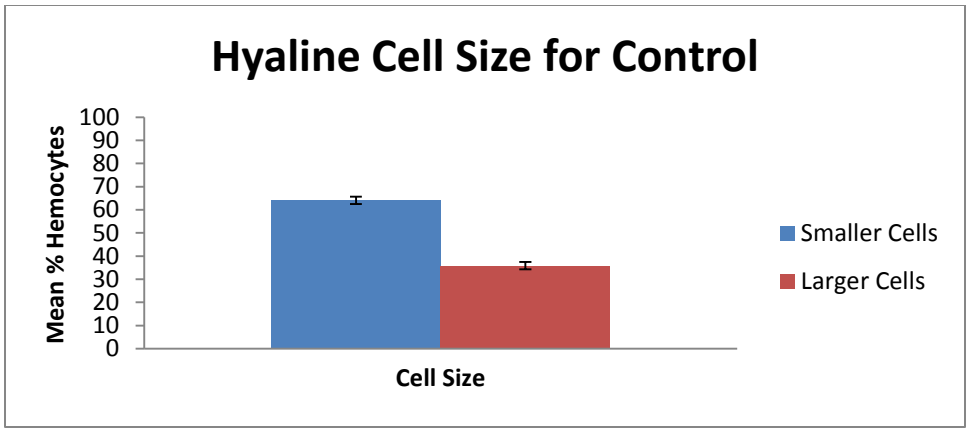


Figure 9. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Overall Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

In evaluating cell size in the hyaline cell population, crayfish treated with crayfish saline exhibited 64.0 percent (± 1.6) of the cells in the smaller cell range and 36.0 percent (± 1.6) of the cells in the larger cell range (Figure 10A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 57.0 percent (± 5.1) of the cells in the smaller cell range and 44.0 percent (±5.1) of the cells in the larger cell range (Figure 10B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline presented with 66.0 percent (± 9.7) of the cells in the smaller cell range and 34.0 percent (±9.7) of the cells in the larger cell range (Figure 10C).

A.



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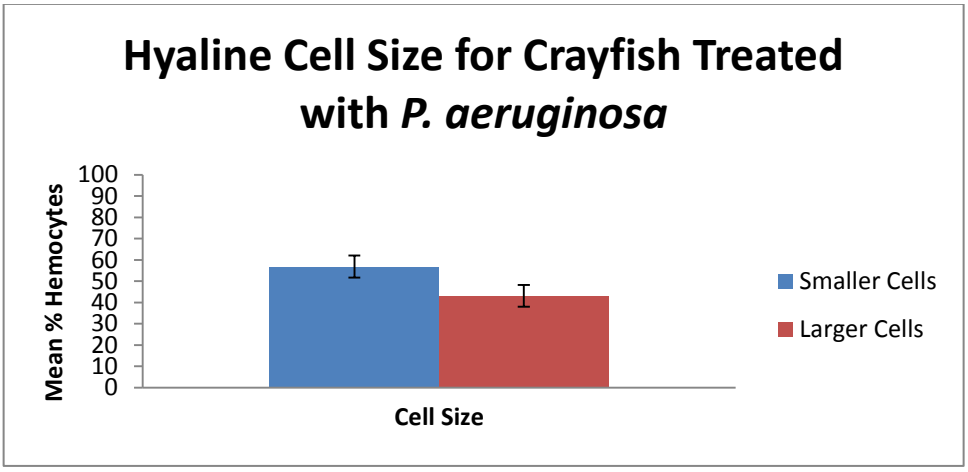

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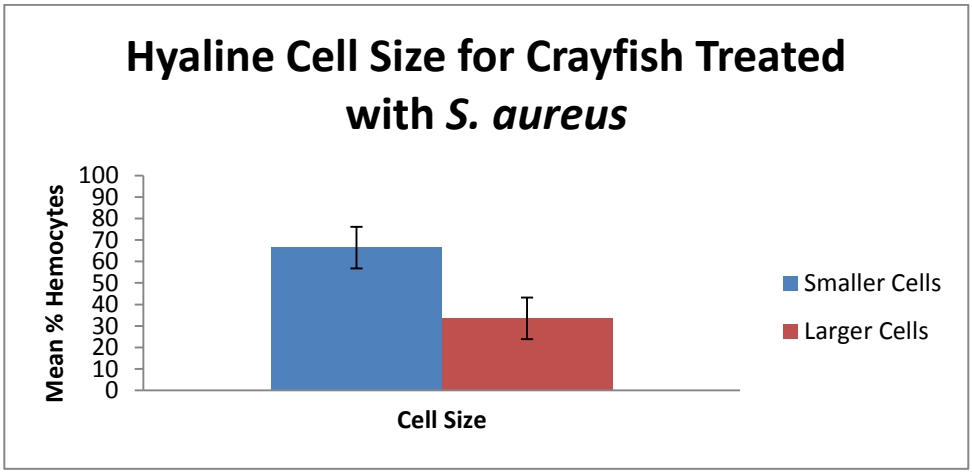
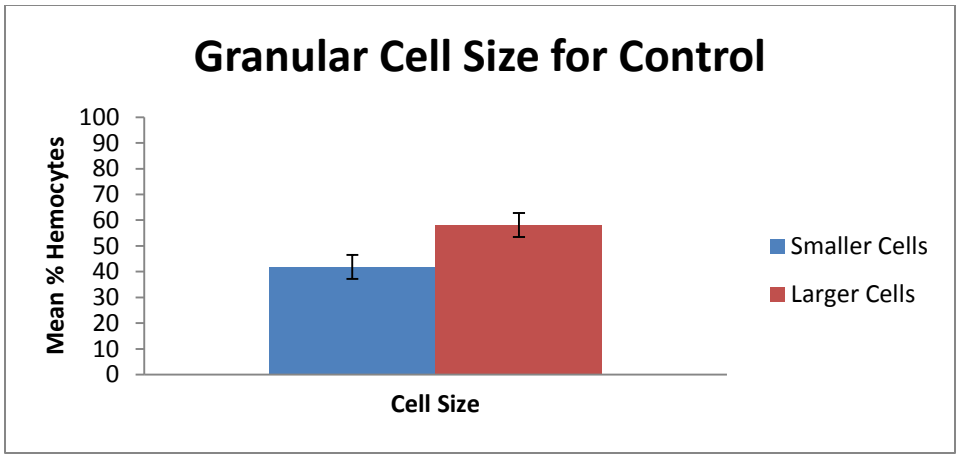


Figure 10. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Hyaline Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

In assaying cell size in the granular cell population, crayfish treated with crayfish saline displayed 42.0 percent (±4.7) of the cells in the smaller cell range and 58.0 percent (± 4.7) of the cells in the larger cell range (Figure 11A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 50.0 percent (± 4.9) of the cells in the smaller cell range and 50.0 (± 4.9) of the cells in the larger cell range (Figure 11B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline exhibited 44.0 percent (± 14.0) of the cells in the smaller cell range and 56.0 percent (± 14.0) of the cells in the larger cell range (Figure 11C).

A.



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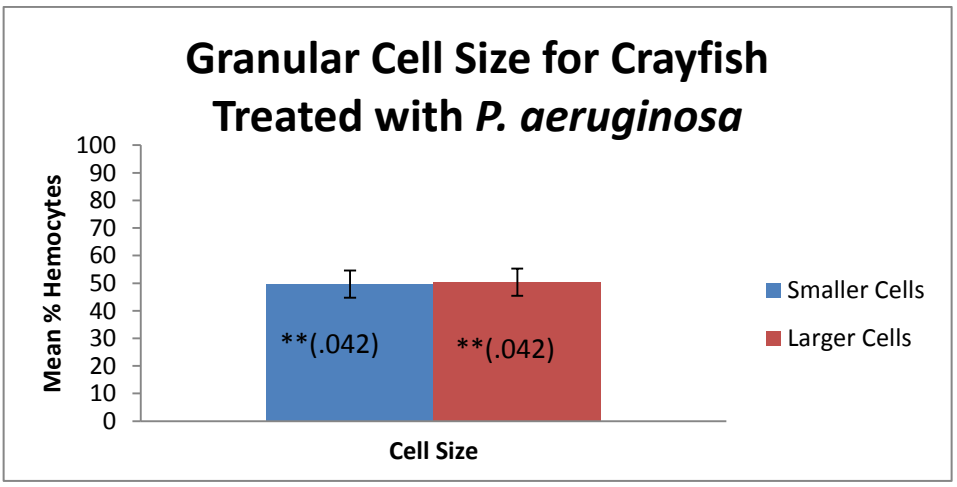

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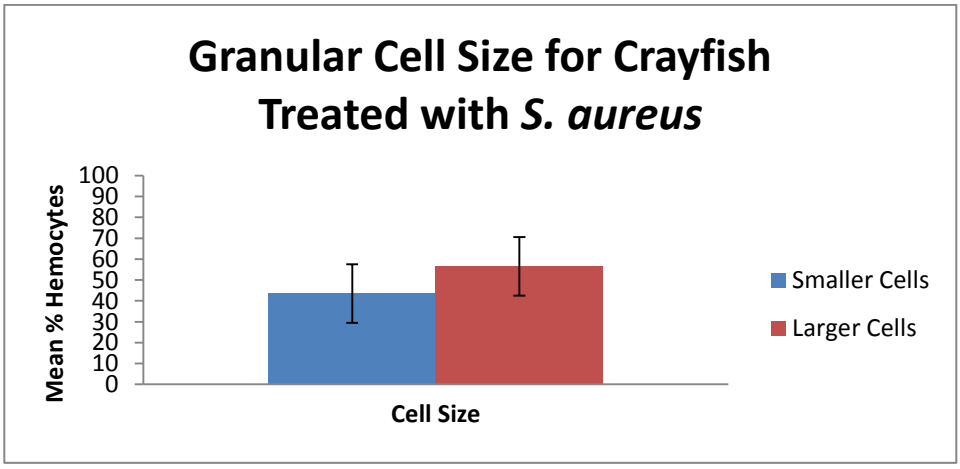
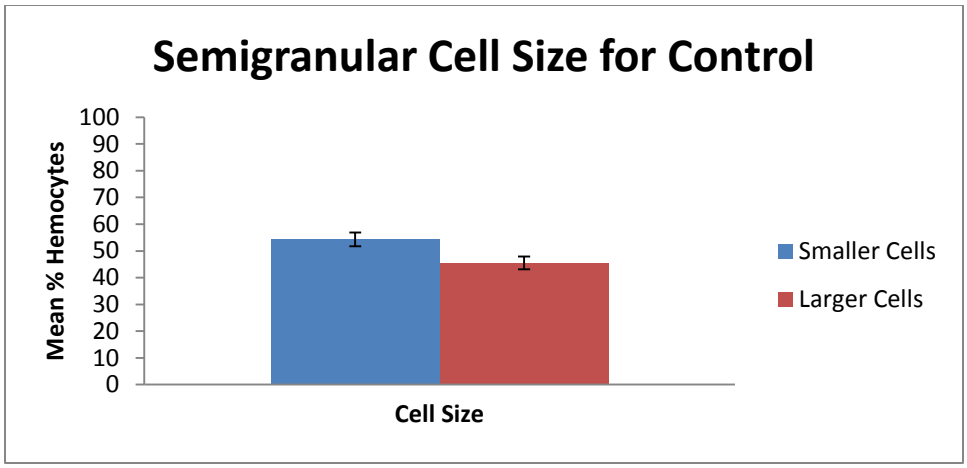


Figure 11. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Granular Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

When observing cell size in the semigranular cell population, crayfish treated with crayfish saline exhibited 54.0 percent (±2.6) of the cells in the smaller cell range and 46.0 percent (± 2.4) of the cells in the larger cell range (Figure 12A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 75.0 percent (± 1.1) of the cells in the smaller cell range and 25.0 percent (± 1.1) of the cells in the larger cell range (Figure 12B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline displayed 59.0 percent (±1.2) of the cells in the smaller cell range and 41.0 percent (± 1.2) of the cells in the larger cell range (Figure 12C).

A.



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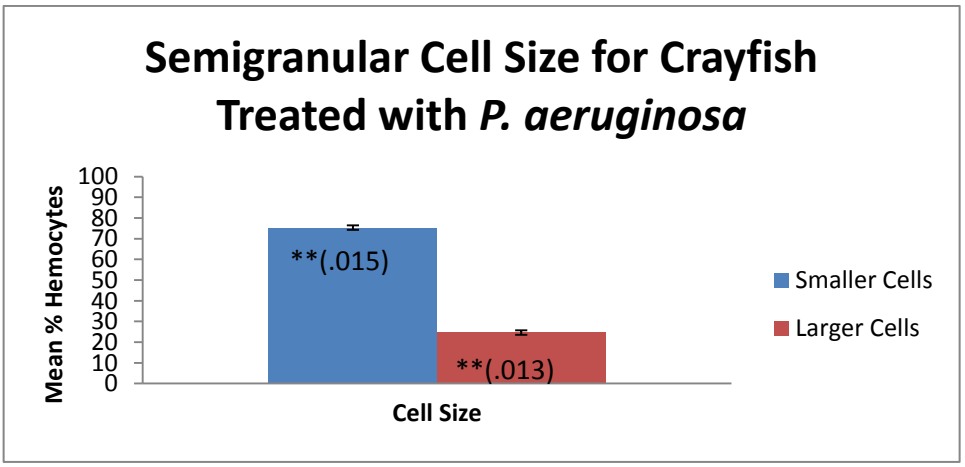

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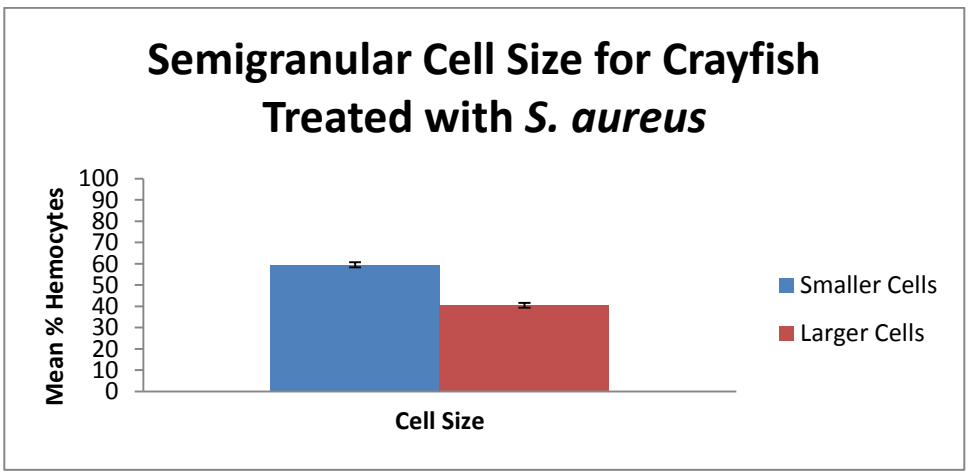
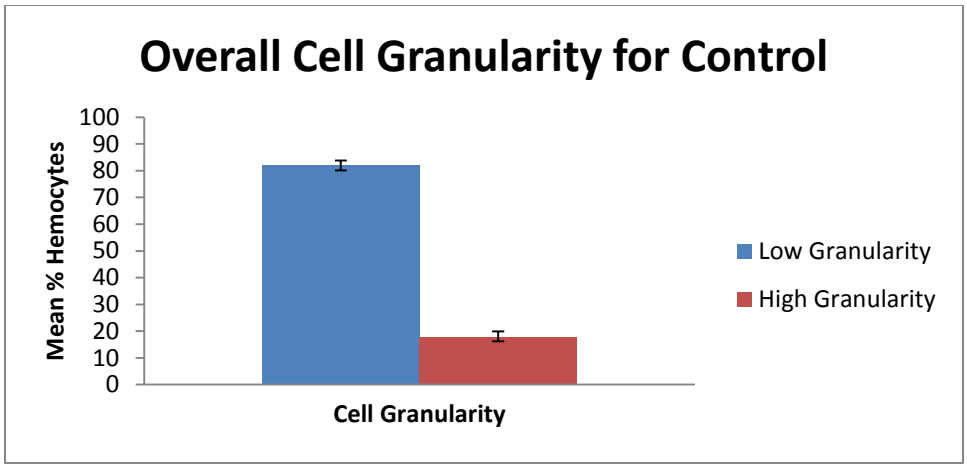


Figure 12. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Semigranular Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

Cell Granularity

When looking at overall cell granularity, crayfish treated with crayfish saline displayed 82.0 percent (±1.8) of the cells in the low granularity range and 18.0 percent (± 1.8) of the cells in the high granularity range (Figure 13A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 68.0 percent (±1.1) of the cells in the low granularity range and 32.0 percent (± 1.1) of the cells in the high granularity range (Figure 13B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline exhibited 40.0 percent (± 11.0) of the cells in the low granularity range and 60.0 percent (± 11.0) of the cells in the high granularity range (Figure 13C).

A.



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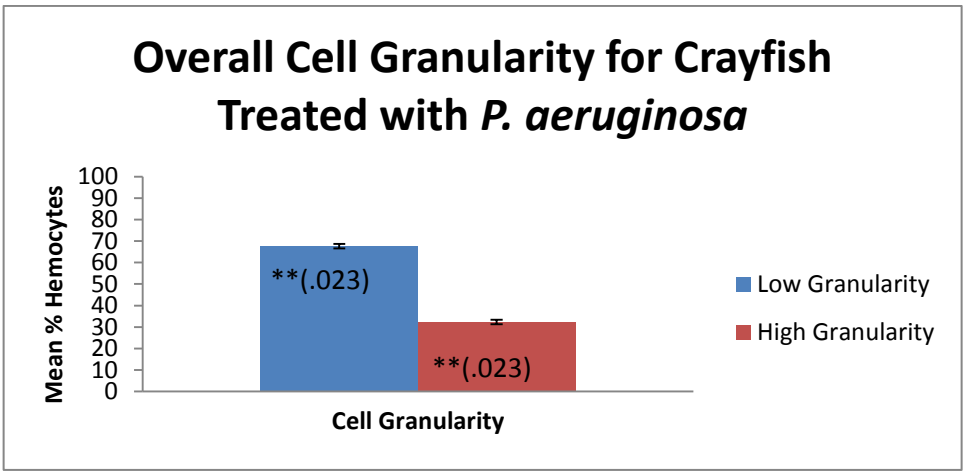

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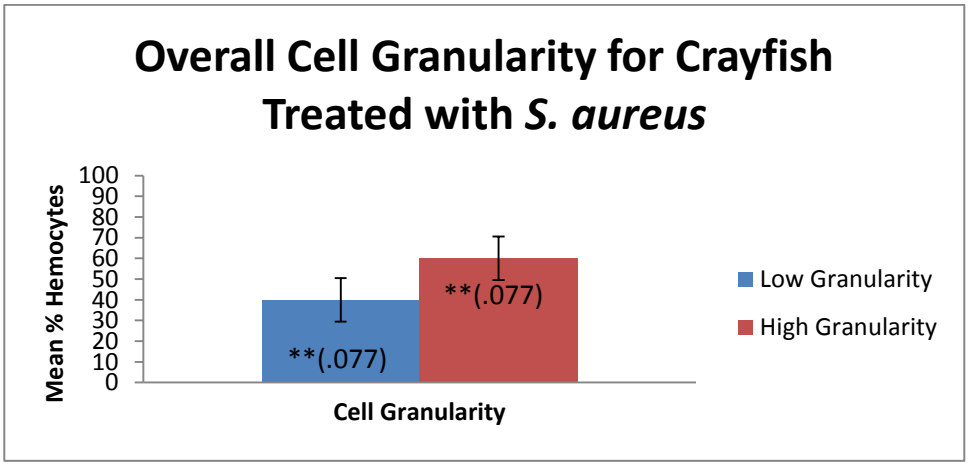
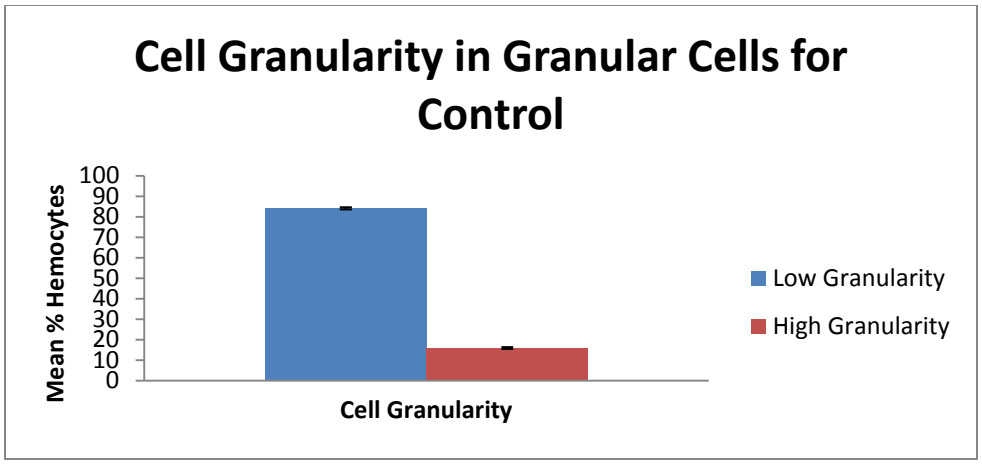


Figure 13. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Overall Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

In analyzing cell granularity for the granular cell population, crayfish treated with crayfish saline exhibited 84.0 percent (± 0.51) of the cells in the low granularity range and 16.0 percent (± 0.51) of the cells in the high granularity range (Figure 14A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 91.0 percent (± 0.90) of the cells in the low granularity range and 8.8 percent (± 0.90) of the cells in the high granularity range (Figure 14B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline presented with 91.0 percent (±1.8) of the cells in the low granularity range and 9.0 percent (± 1.8) of the cells in the high granularity range (Figure 14C).

A.



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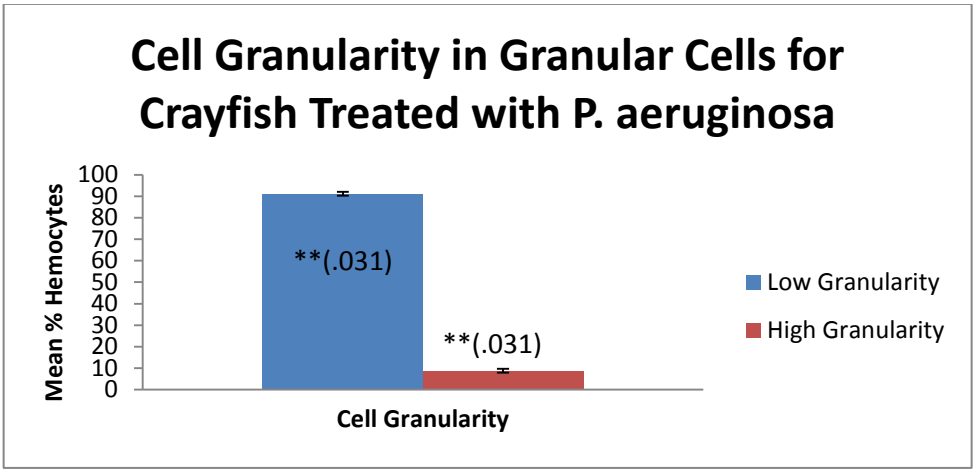

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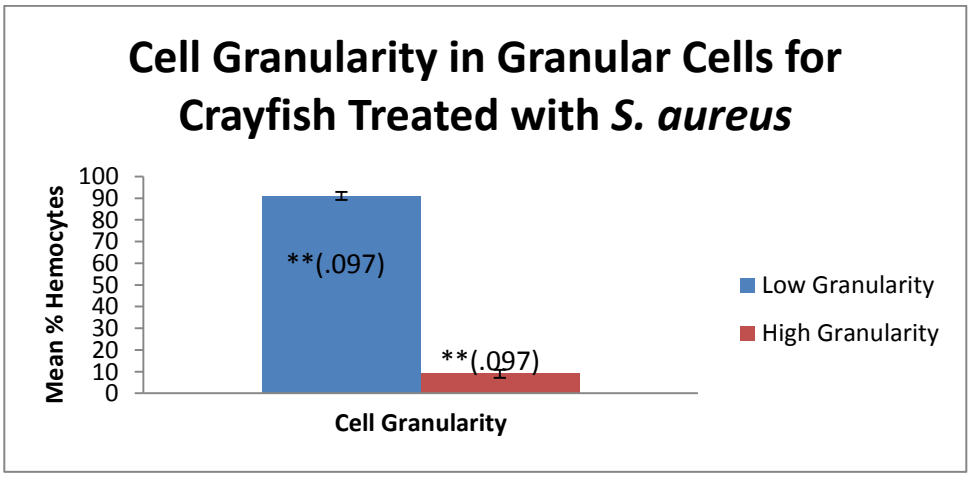
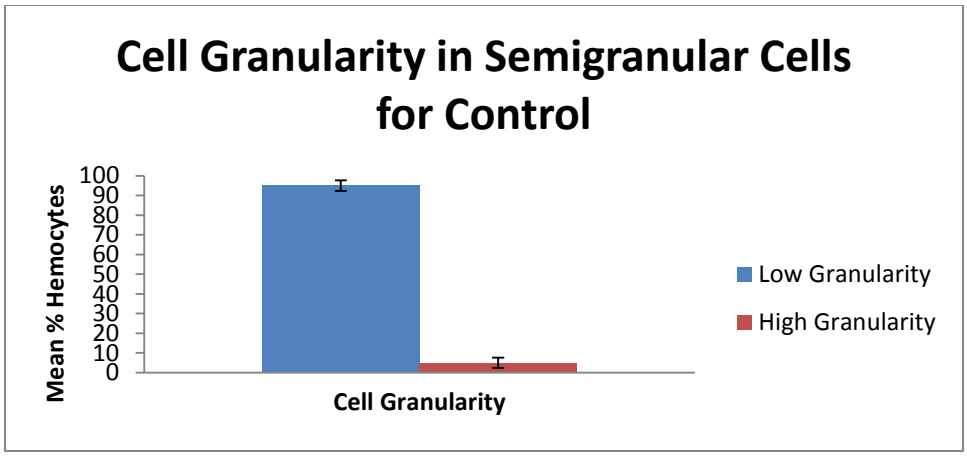


Figure 14. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Granular Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

When evaluating cell granularity for the semigranular cell population, crayfish treated crayfish saline displayed 95.0 percent (± 2.6) of the cells in the low granularity range and 5.0 percent (± 2.6) of the cells in the high granularity range (Figure 15A). Crayfish treated with *Pseudomonas aeruginosa* suspended with crayfish saline exhibited 99.0 percent (±0.70) of the cells in the low granularity range and 1.4 percent (± 0.69) of the cells in the high granularity range (Figure 15B). Crayfish treated with *Staphylococcus aureus* suspended in crayfish saline presented with 95.0 percent (±2.2) of the cells in the low granularity range and 4.9 percent (± 2.3) of the cells in the high granularity range (Figure 15C).

A.



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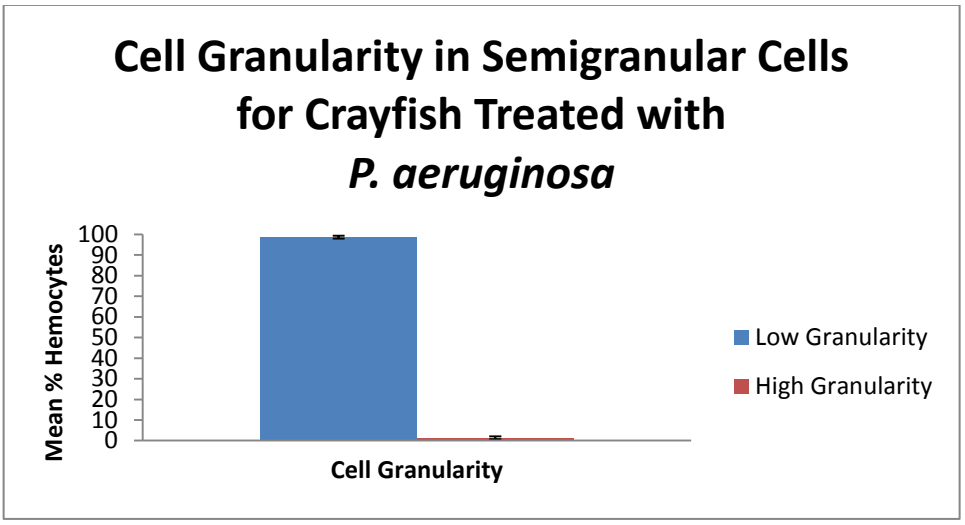

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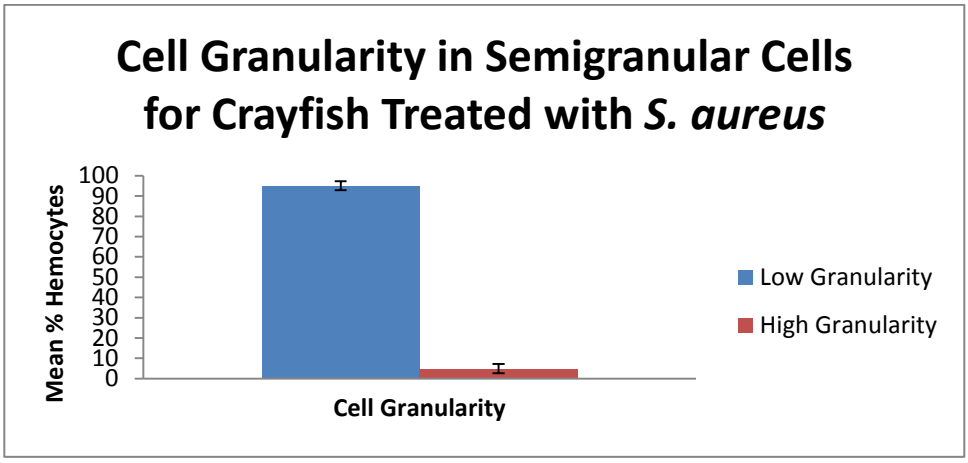


Figure 15. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* on Semigranular Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing each treatment to (A) control using t-test. n = 3. **(pvalue).

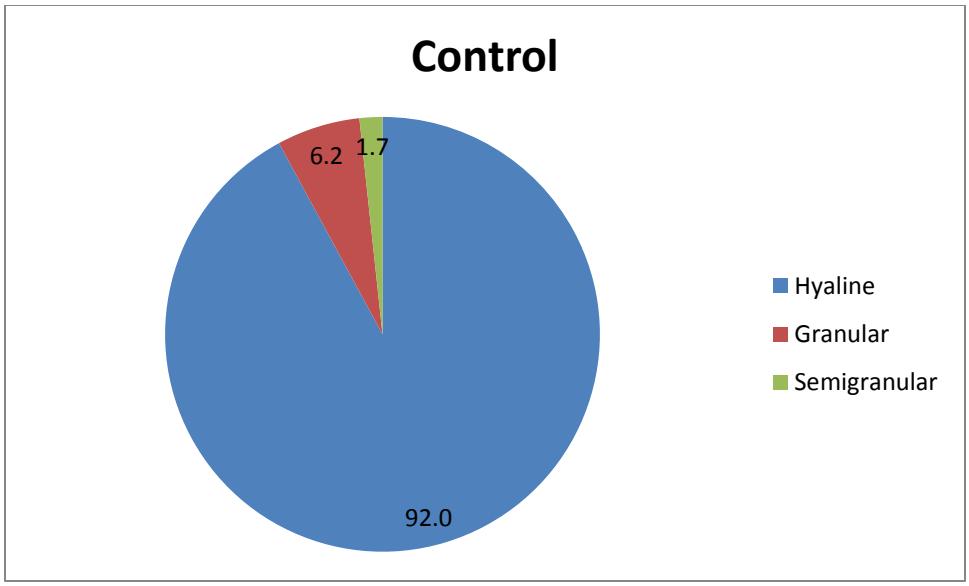
Effect of Treatment with Gram Negative Bacteria on Hemocyte Distribution

Experiment 2 was designed to measure the effects of injection with Gram negative bacteria on hemocyte cell type, cell size, and cell granularity. Three crayfish were injected with *Pseudomonas aeruginosa* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline and 3 crayfish were injected with crayfish saline alone to serve as the control. Twenty-four hours postinjection hemocytes were harvested and analyzed by flow cytometry.

Cell Type

Crayfish treated with crayfish saline exhibited a mean percentage of 92.0 (± 1.3; Range 89.6-93.6) for the hyaline cell population, 6.2 (± 1.3; Range 4.18-8.76) for the granular population, and 1.7 (± 0.33; Range 1.14-2.27) for the semigranular population (Figure 16A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed a mean percentage of 81.0 (± 0.82; Range 79.7-82.2) for the hyaline cell population, 14.0 (± 1.1; Range 12.2-15.6) for the granular population, and 5.2 (± 0.27; Range 4.75-5.68) for the semigranular population (Figure 16B).

A.



B.

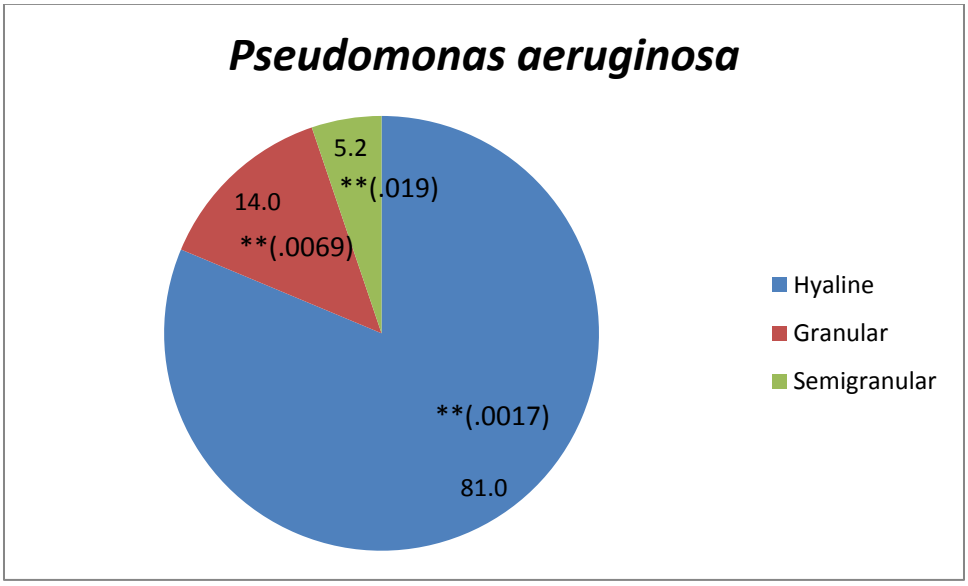
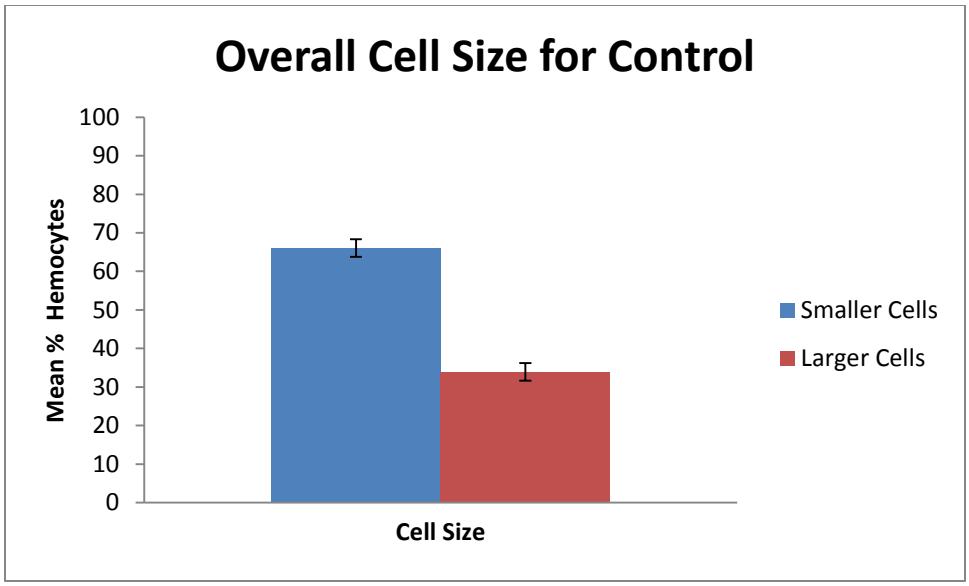


Figure 16. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Distribution of Hemocyte Populations 24 Hours Postinjection. Chart sections represent mean percent hemocytes. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

Cell Size

When assaying overall cell size, crayfish treated with crayfish saline exhibited 66.0 percent (± 2.3) of the cells in the smaller cell range and 34.0 percent (± 2.3) of the cells in the larger cell range (Figure 17A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 73.0 percent (± 8.0) of the cells in the smaller cell range and 27.0 percent (± 8.0) of the cells in the larger cell range (Figure 17B).

A.



B.

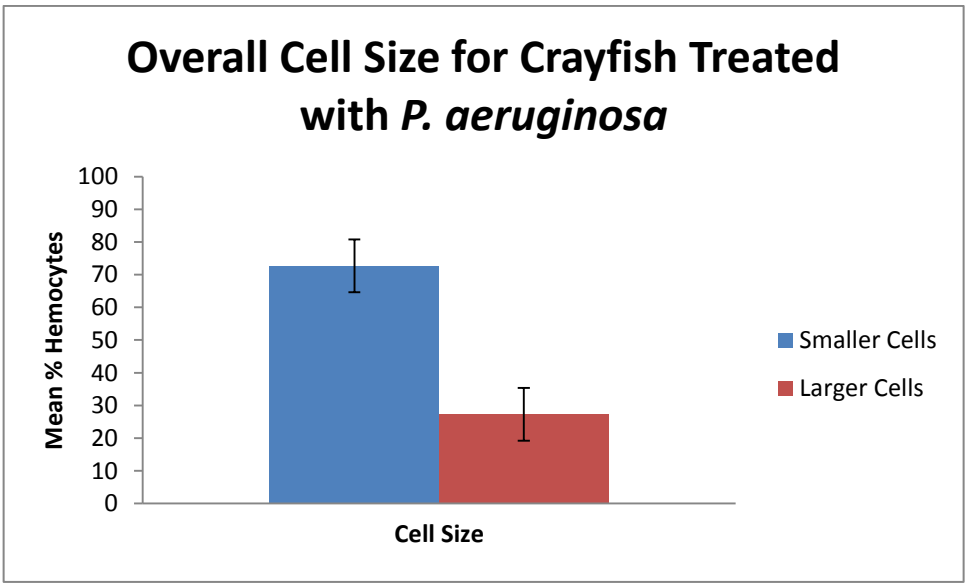
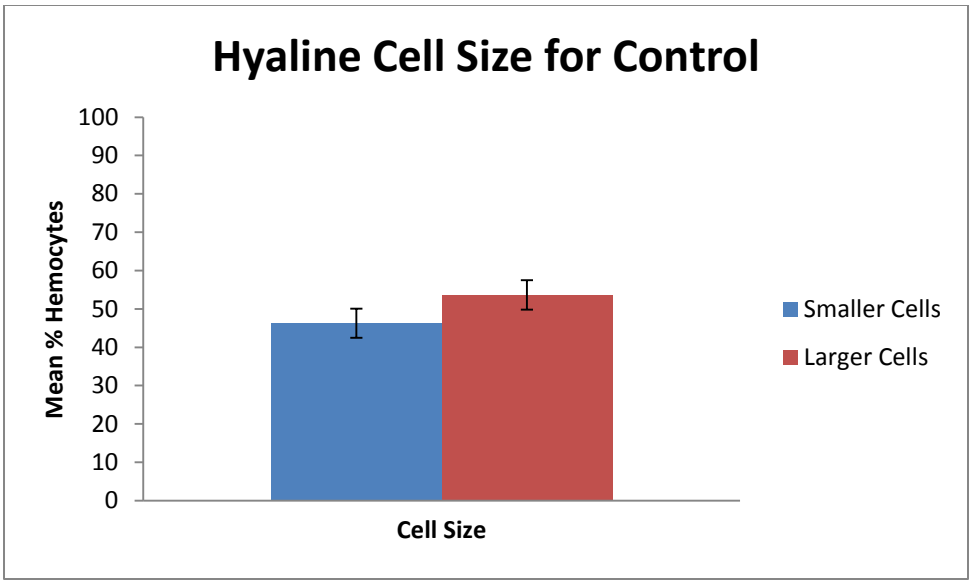


Figure 17. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Overall Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

In observing cell size in the hyaline cell population, crayfish treated with crayfish saline presented with 46.0 percent (±3.8) of the cells in the smaller cell range and 54.0 percent (± 3.8) of the cells in the larger cell range (Figure 18A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 49.0 percent (± 10.0) of the cells in the smaller cell range and 51.0 percent (± 10.0) of the cells in the larger cell range (Figure 18B).

A.



B.

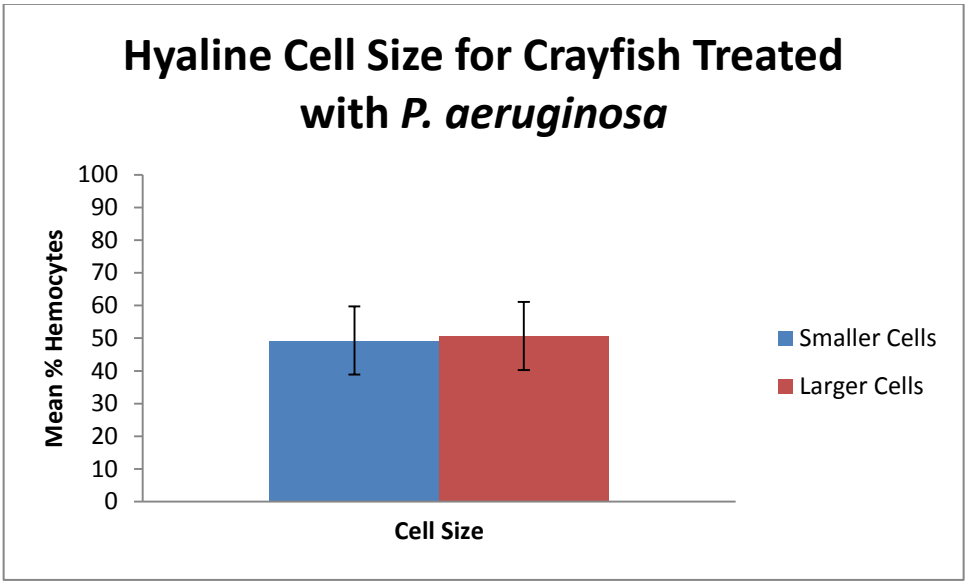
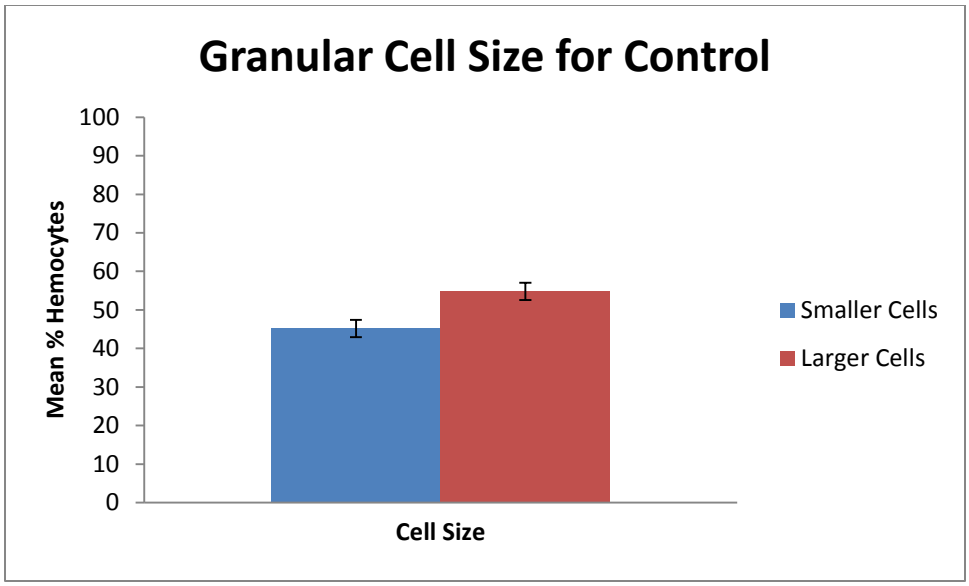


Figure 18. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Hyaline Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

In analyzing cell size in the granular cell population, crayfish treated with crayfish saline displayed 45.0 percent (± 2.2) of the cells in the smaller cell range and 55.0 percent (± 2.2) of the cells in the larger cell range (Figure 19A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 62.0 percent (±5.8) of the cells in the smaller cell range and 38.0 percent (± 5.8) of the cells in the larger cell range (Figure 19B).

A.



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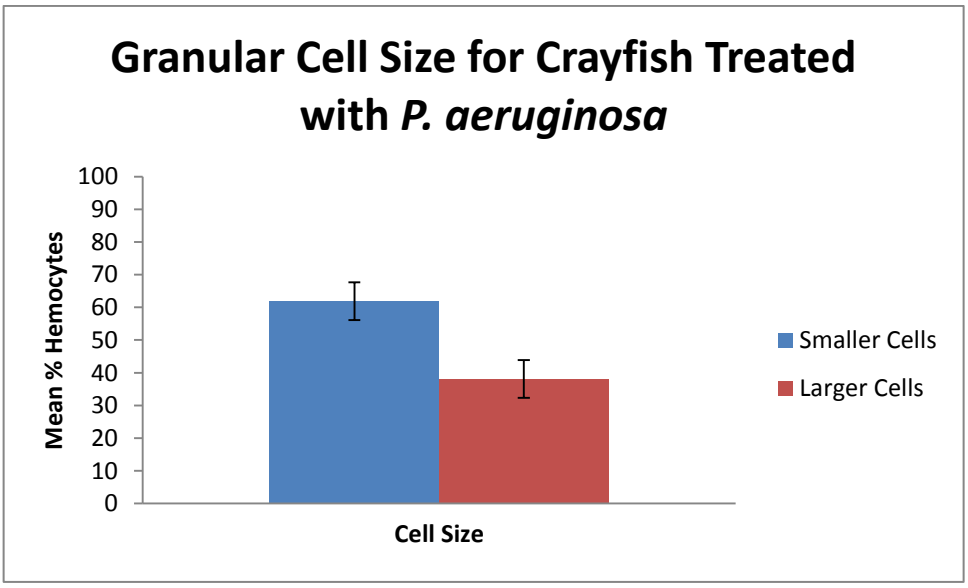
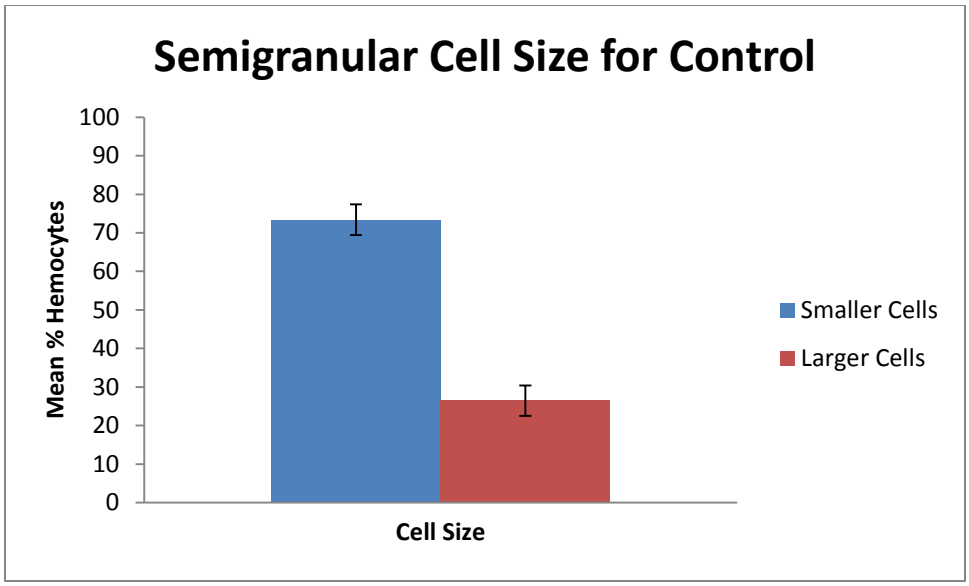


Figure 19. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Granular Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

When evaluating cell size in the semigranular cell population, crayfish treated with crayfish saline exhibited 73.0 percent (±3.4) of the cells in the smaller cell range and 26.0 percent (± 3.4) of the cells in the larger cell range (Figure 20A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 69.0 percent (± 3.5) of the cells in the smaller cell range and 30.0 percent (±3.8) of the cells in the larger cell range (Figure 20B).

A.



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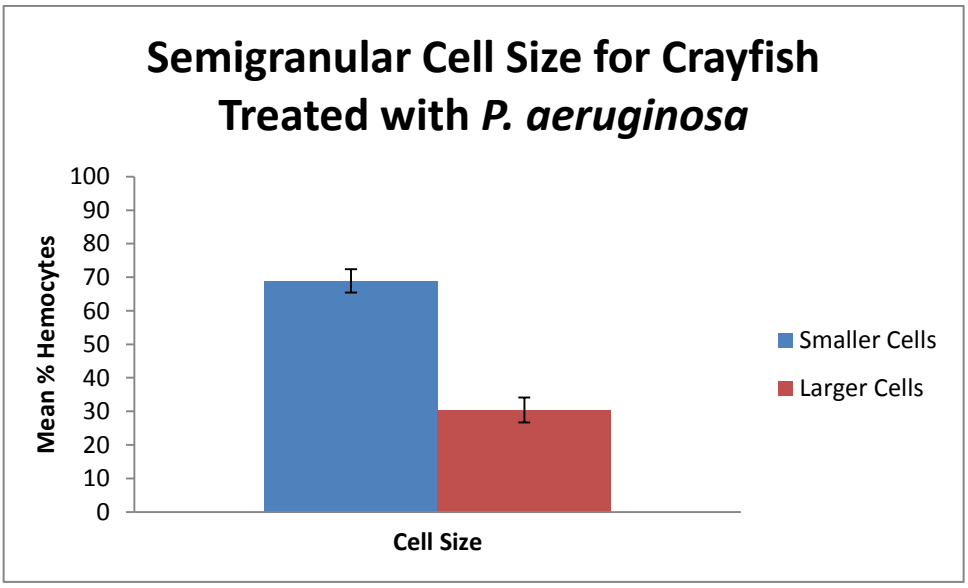
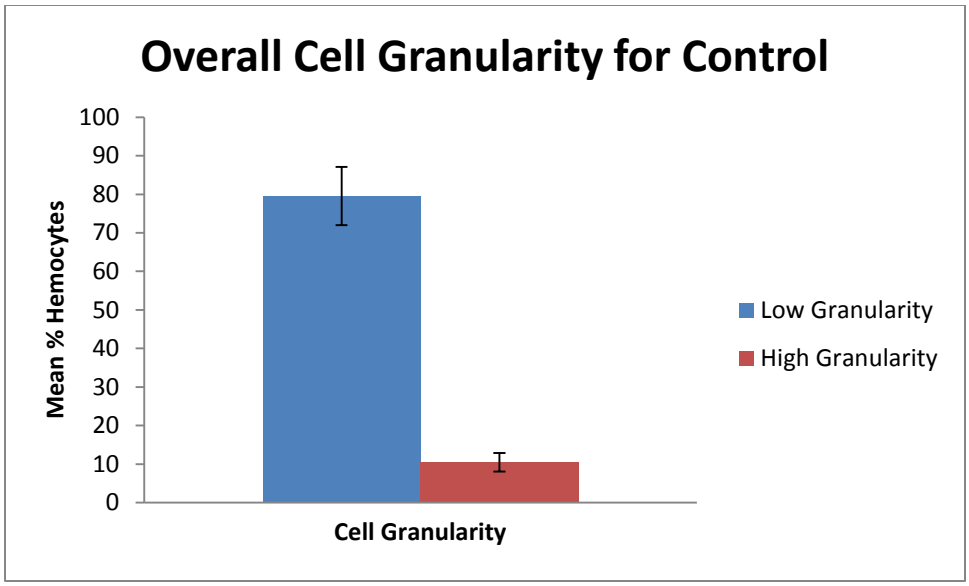


Figure 20. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Semigranular Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

Cell Granularity

When evaluating overall cell granularity, crayfish treated with crayfish saline exhibited 80.0 percent (± 7.6) of the cells in the low granularity range and 10.0 percent (± 2.4) of the cells in the high granularity range (Figure 21A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 70.0 percent (±6.1) of the cells in the low granularity range and 30.0 percent (±6.1) of the cells in the high granularity range (Figure 21B).

A.



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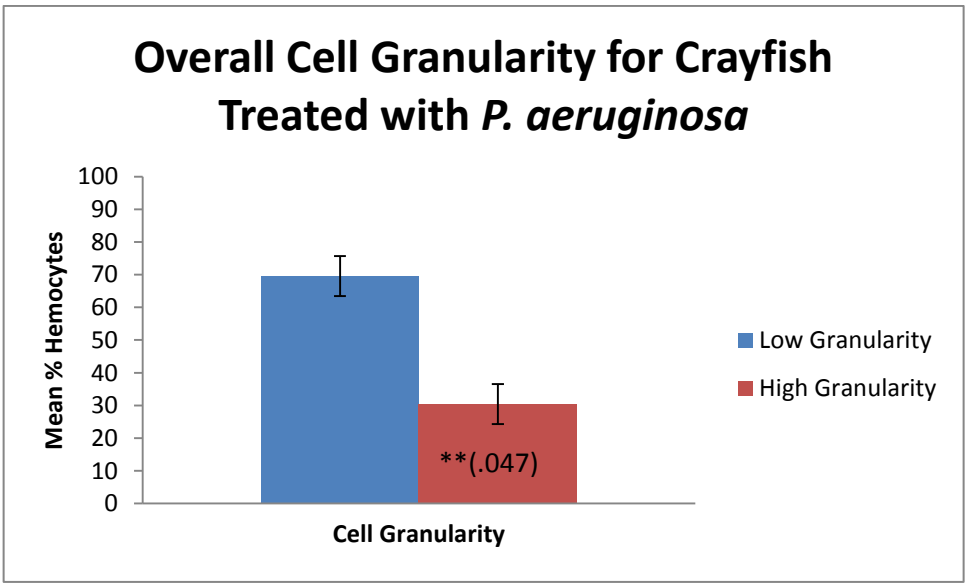
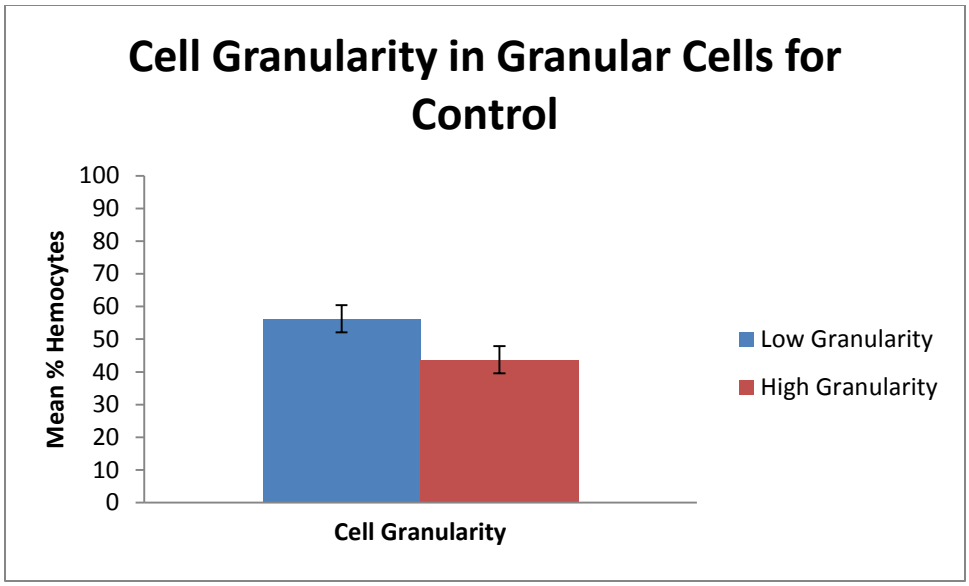


Figure 21. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Overall Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

In looking at cell granularity for the granular cell population, crayfish treated with crayfish saline displayed 56.0 percent (±4.2) of the cells in the low granularity range and 43.0 percent (±4.2) of the cells in the high granularity range (Figure 22A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 65.0 percent (±6.9) of the cells in the low granularity range and 35.0 percent (±6.9) of the cells in the high granularity range (Figure 22B).

A.



B.

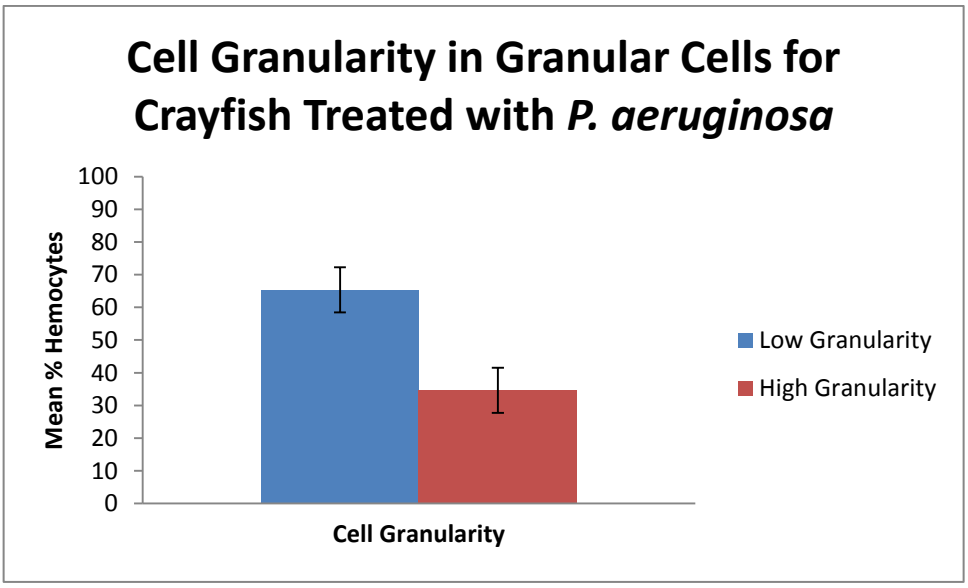
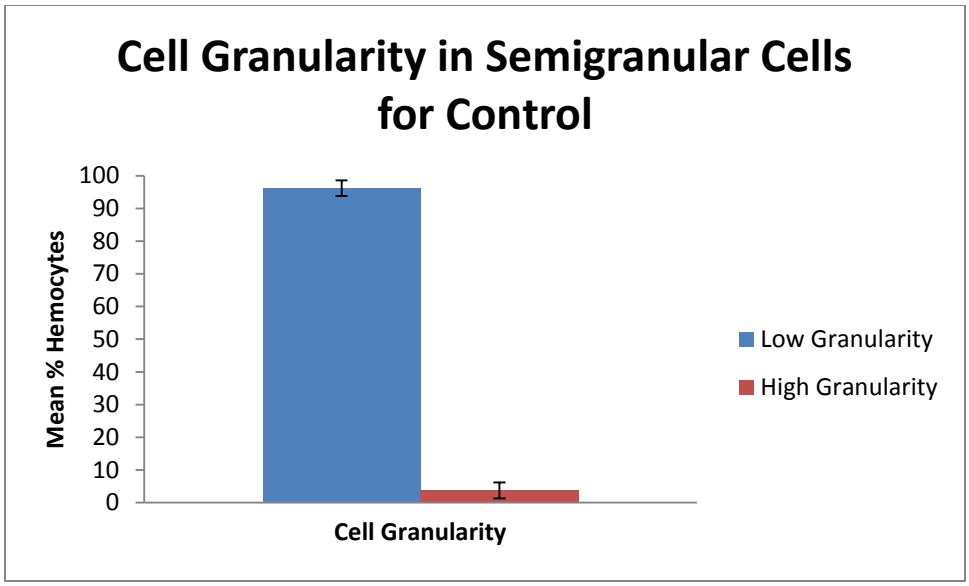


Figure 22. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Granular Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

When analyzing cell granularity for the semigranular cell population, crayfish treated with crayfish saline presented with 96.0 percent (±2.4) of the cells in the low granularity range and 3.7 percent (±2.4) of the cells in the high granularity range (Figure 23A). Crayfish treated with *Pseudomonas aeruginosa* suspended with crayfish saline displayed 96.0 percent (±1.9) of the cells in the low granularity range and 3.5 percent (±1.9) of the cells in the high granularity range (Figure 23B).

A.



B.

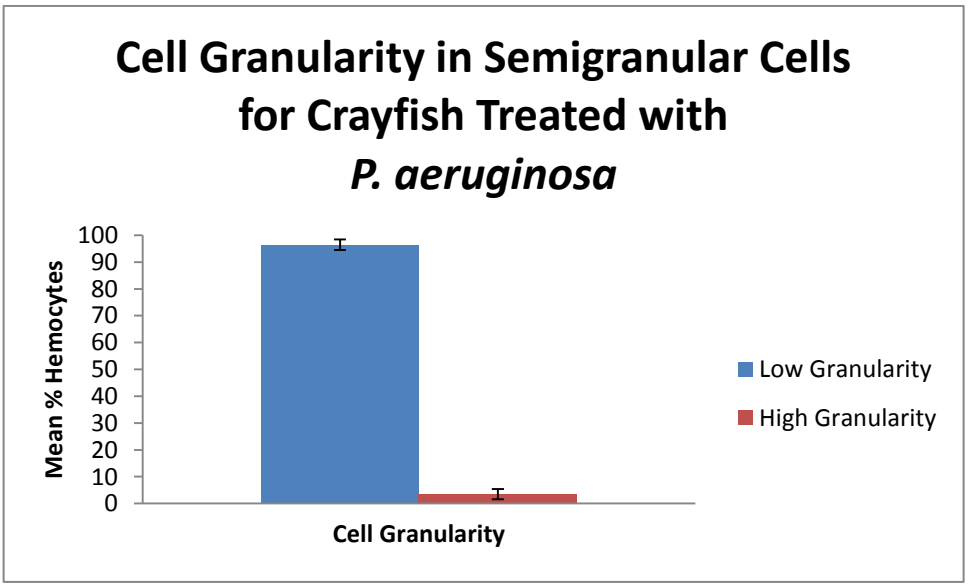


Figure 23. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Semigranular Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

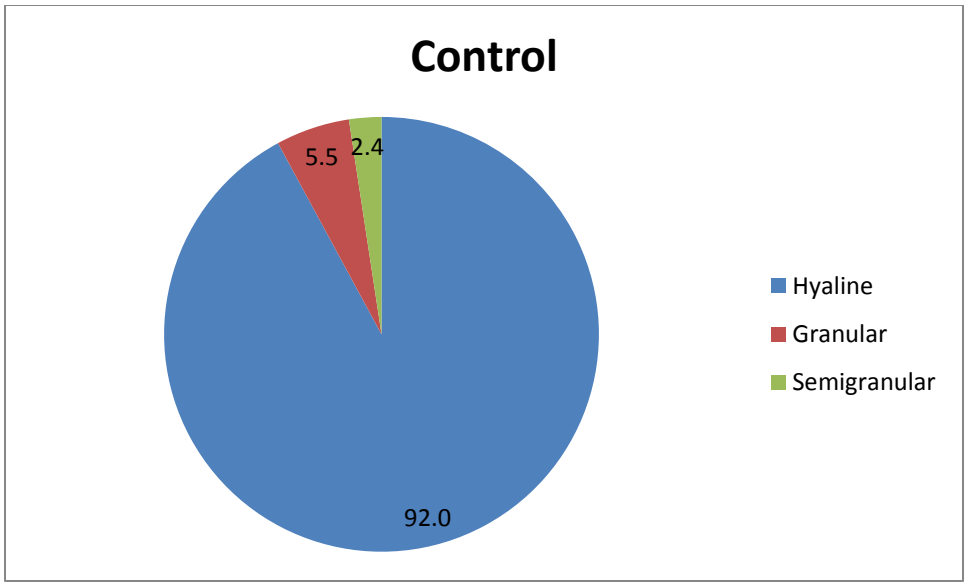
Effect of Treatment with Gram Negative Bacteria on Hemocyte Distribution

Experiment 3 was designed to measure the effects of injection with Gram negative bacteria on hemocyte cell type, cell size and cell granularity. Three crayfish were injected with *Pseudomonas aeruginosa* (Concentration: 1 x 10^6 bacterial cells/mL) suspended in crayfish saline and 3 crayfish were injected with crayfish saline alone to serve as the control. Twenty-four hours postinjection hemocytes were harvested and analyzed by flow cytometry.

Cell Type

Crayfish treated with crayfish saline exhibited a mean percentage of 92.0(±1.0; Range 90.1-93.6) for the hyaline cell population, 5.5 (±1.0; Range 4.15-7.56) for the granular population, and 2.4 (±0.15; Range 2.24-2.73) for the semigranular population (Figure 24A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed a mean percentage of 76.0 (±0.94; Range 74.2-77.4) for the hyaline cell population, 22.0 (±0.99; Range 20.3-23.4) for the granular population, and 2.1 (±0.26; Range 1.58-2.44) for the semigranular population (Figure 24B).

A.



B.

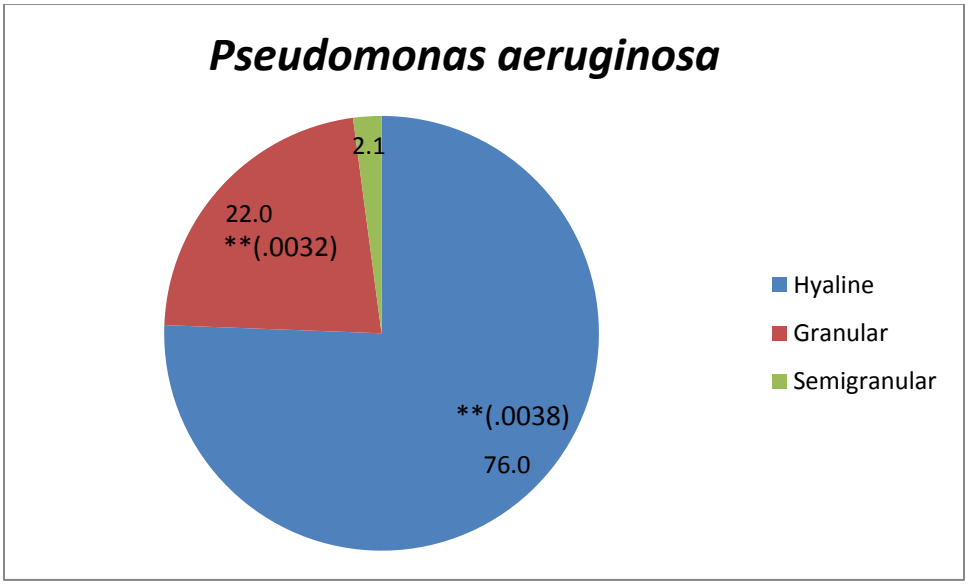
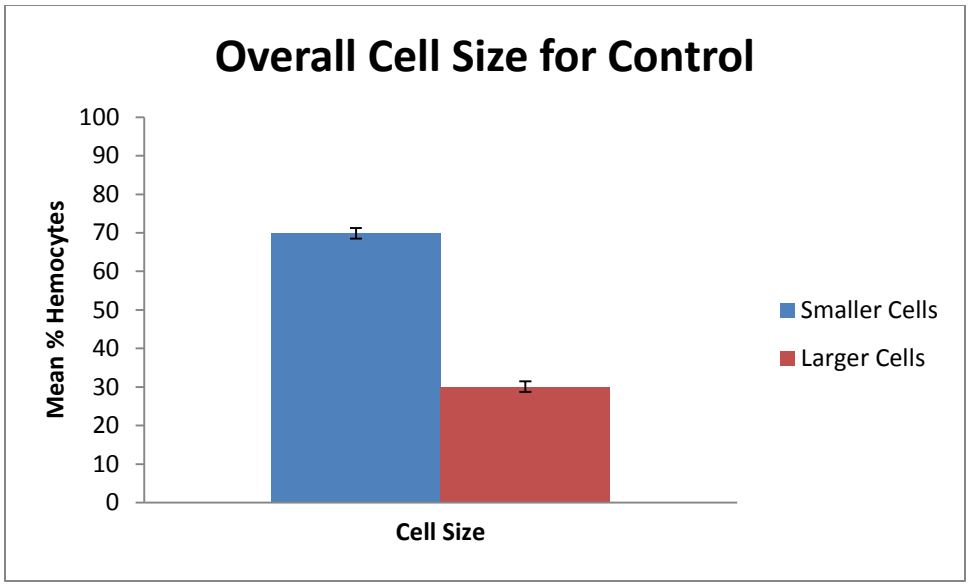


Figure 24. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Distribution of Hemocyte Populations 24 Hours Postinjection. Chart sections represent mean percent hemocytes. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

Cell Size

When observing overall cell size, crayfish treated with crayfish saline exhibited 70.0 percent (±1.4) of the cells in the smaller cell range and 30.0 percent (±1.4) of the cells in the larger cell range (Figure 25A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 87.0 percent (±2.6) of the cells in the smaller cell range and 13.0 percent (±2.6) of the cells in the larger cell range (Figure 25B).

A.



B.

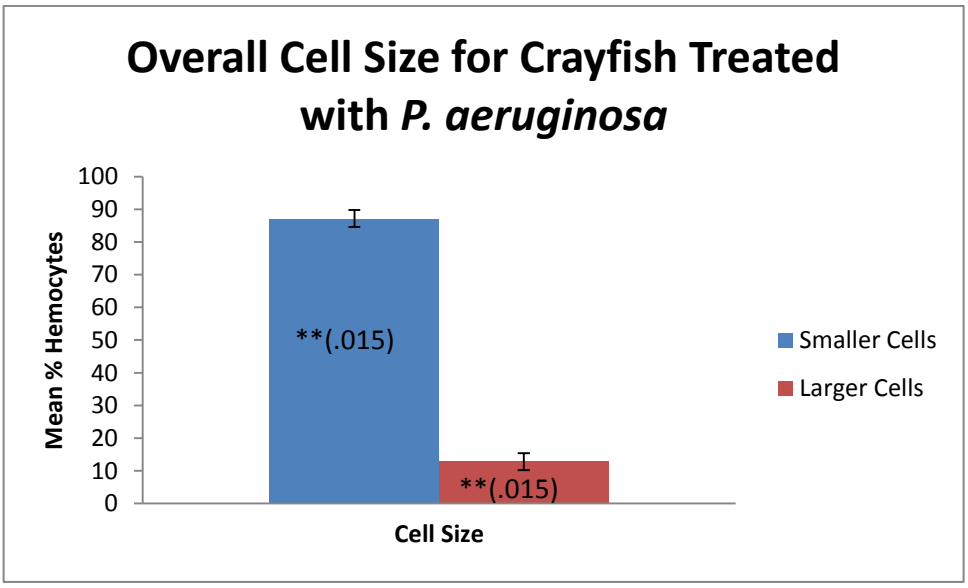
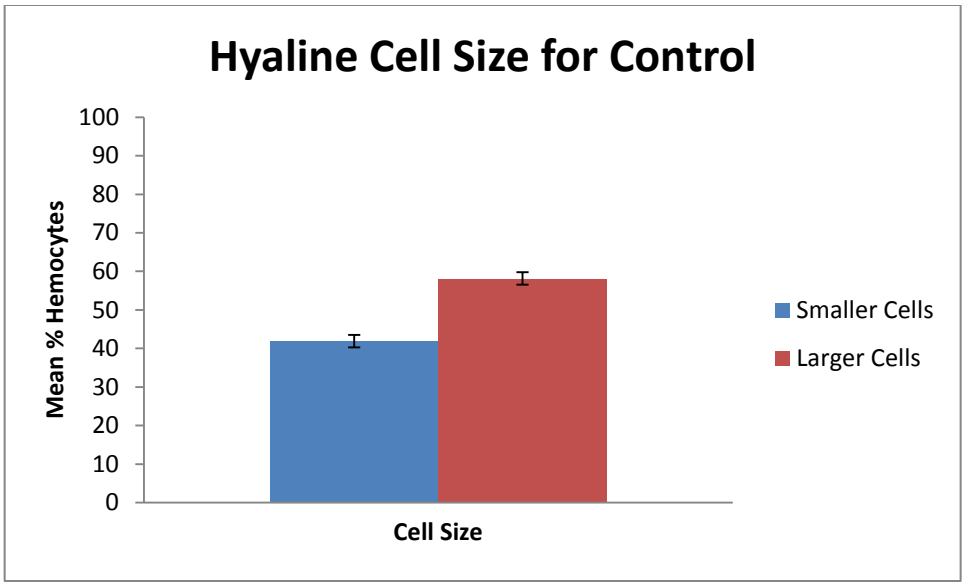


Figure 25. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Overall Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

In assaying cell size in the hyaline cell population, crayfish treated with crayfish saline presented with 42.0 percent (±1.6) of the cells in the smaller cell range and 58.0 percent (±1.6) of the cells in the larger cell range (Figure 26A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 61.0 percent (±3.0) of the cells in the smaller cell range and 39.0 percent (±3.0) of the cells in the larger cell range (Figure 26B).

A.



B.

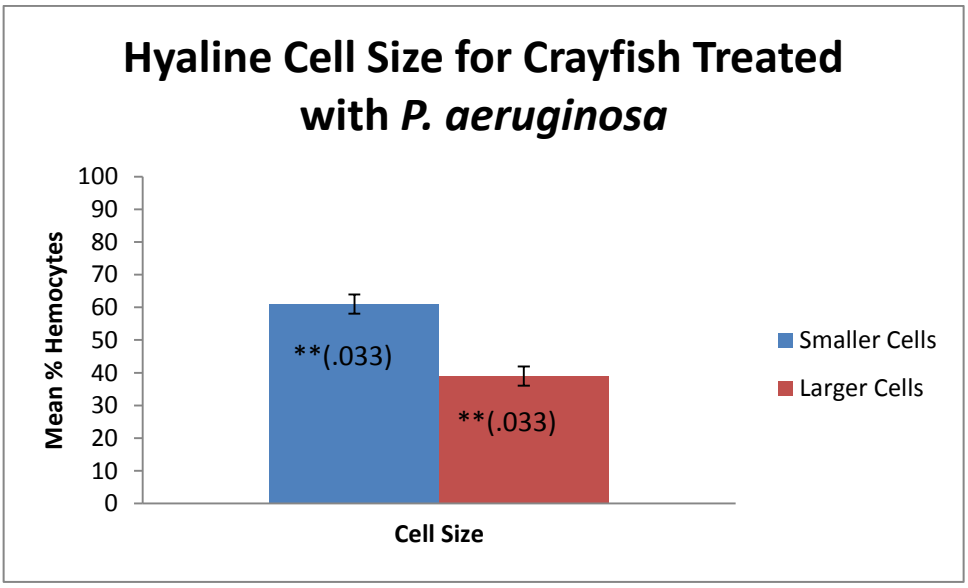
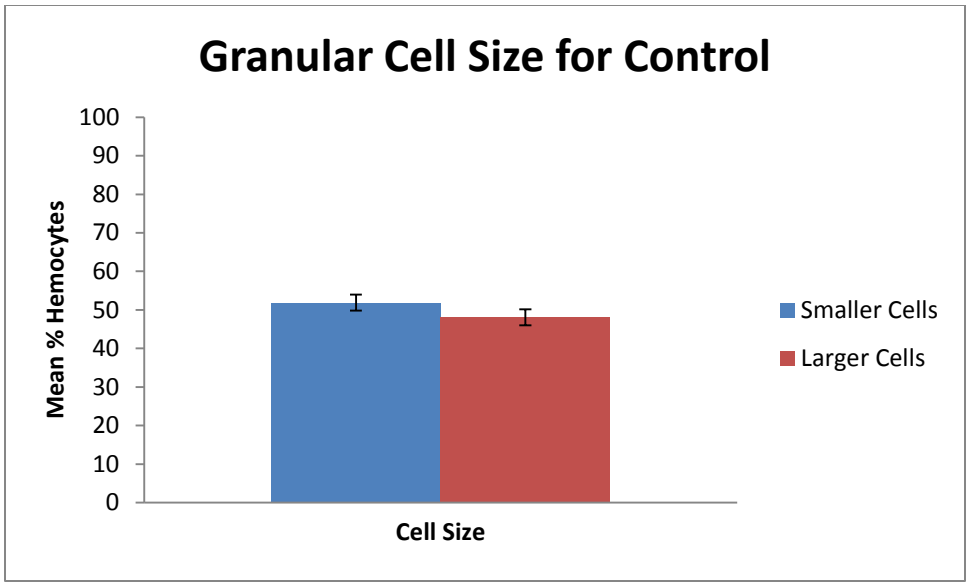


Figure 26. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Hyaline Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

In evaluating cell size in the granular cell population, crayfish treated with crayfish saline displayed 52.0 percent (±2.1) of the cells in the smaller cell range and 48.0 percent (±2.1) of the cells in the larger cell range (Figure 27A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 65.0 percent (±6.2 of the cells in the smaller cell range and 35.0 percent (±6.2) of the cells in the larger cell range (Figure 27B).

A.



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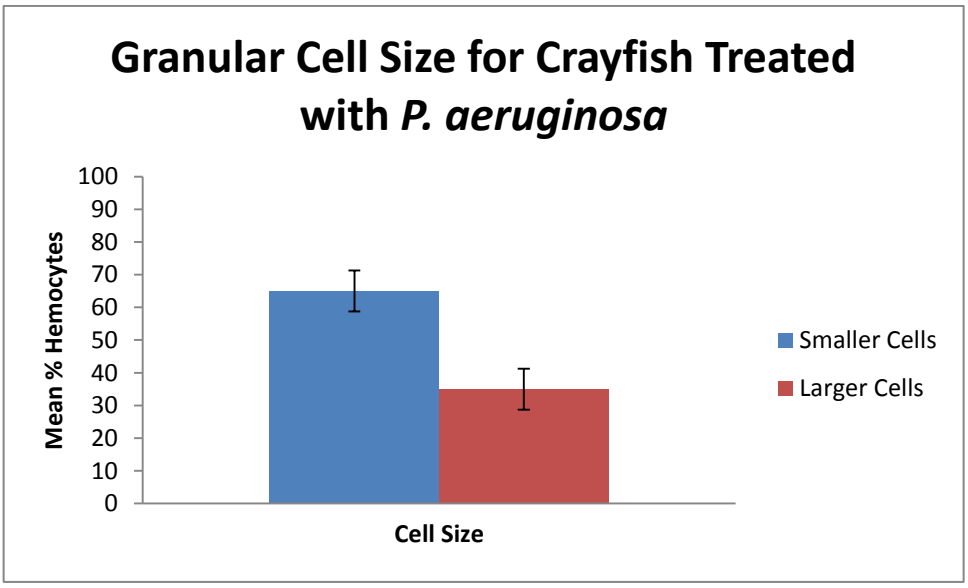
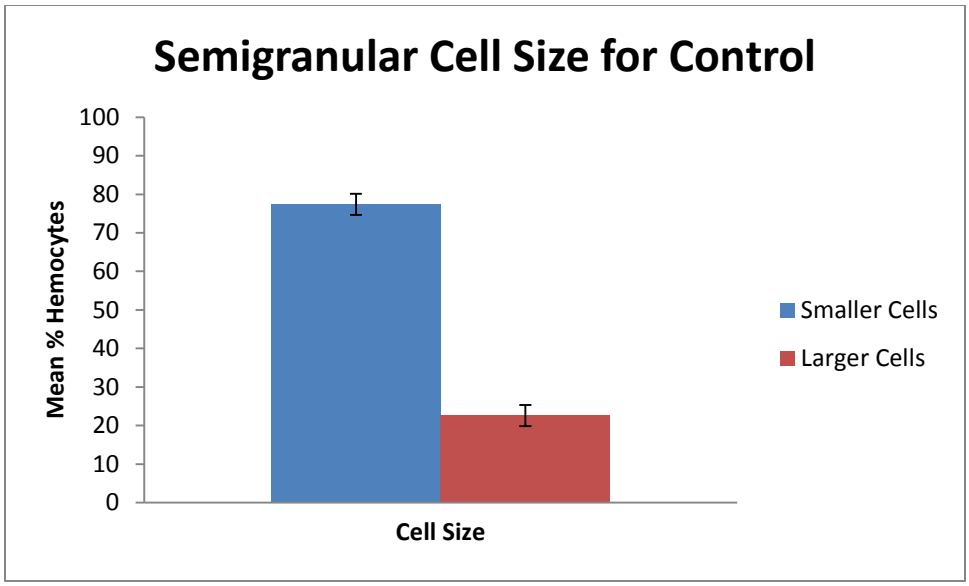


Figure 27. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Granular Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

When analyzing cell size in the semigranular cell population, crayfish treated with crayfish saline exhibited 77.0 percent (±2.8) of the cells in the smaller cell range and 23.0 percent (±2.8) of the cells in the larger cell range (Figure 28A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 64.0 percent (±3.9) of the cells in the smaller cell range and 36.0 percent (±3.9) of the cells in the larger cell range (Figure 28B).

A.



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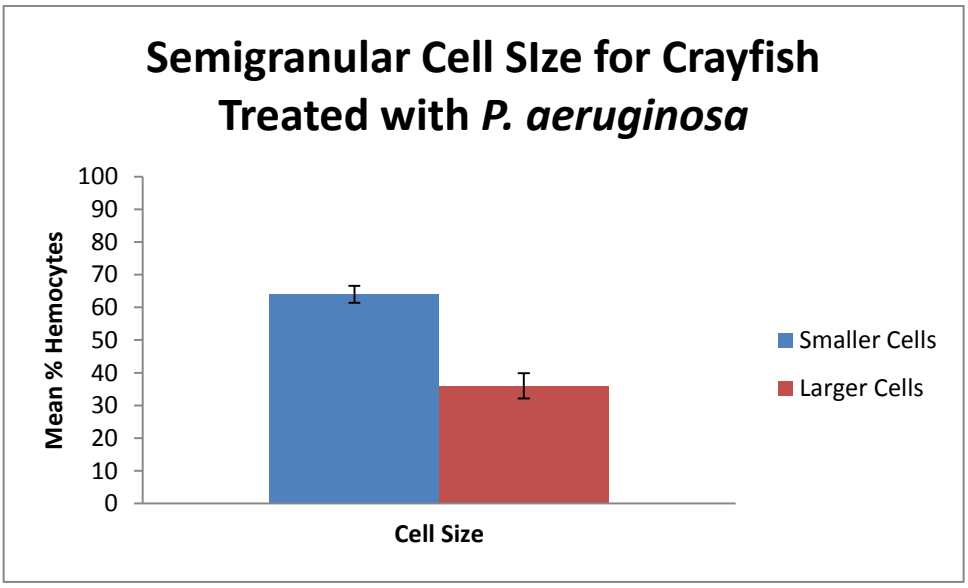
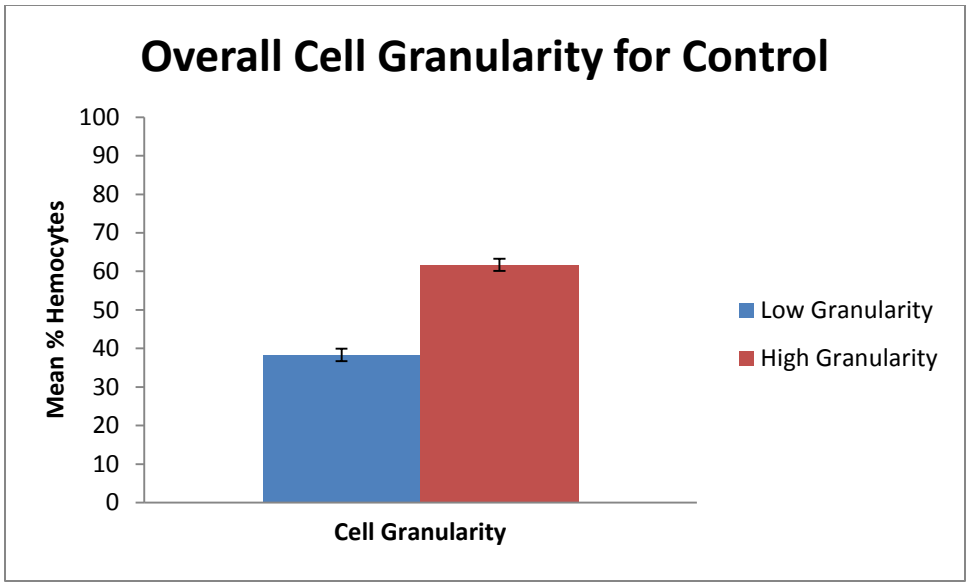


Figure 28. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Semigranular Cell Size 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

Cell Granularity

When assaying overall cell granularity, crayfish treated with crayfish saline exhibited 38.0 percent (±1.6) of the cells in the low granularity range and 62.0 percent (±1.6) of the cells in the high granularity range (Figure 29A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 41.0 percent (±18.0) of the cells in the low granularity range and 59.0 percent (±18.0) of the cells in the high granularity range (Figure 29B).

A.



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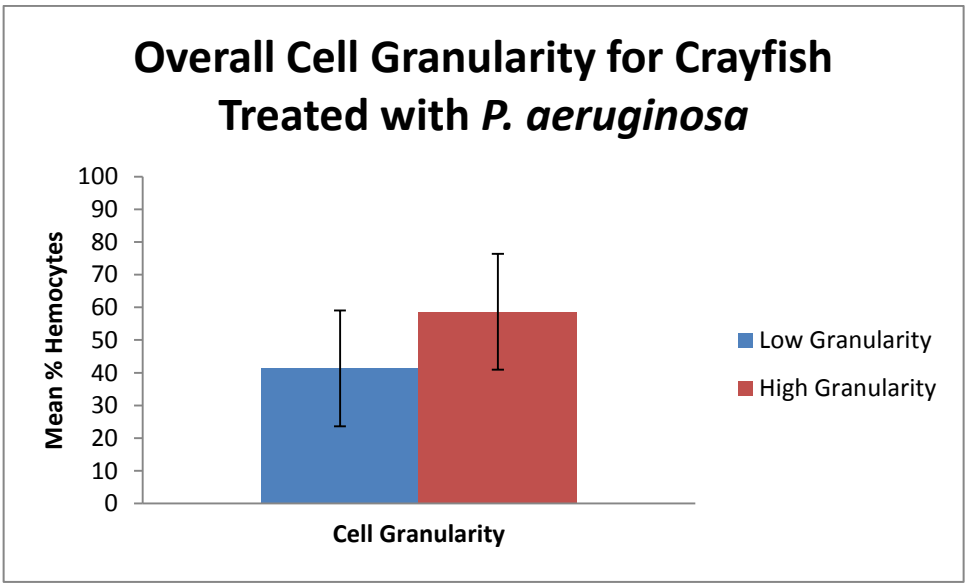
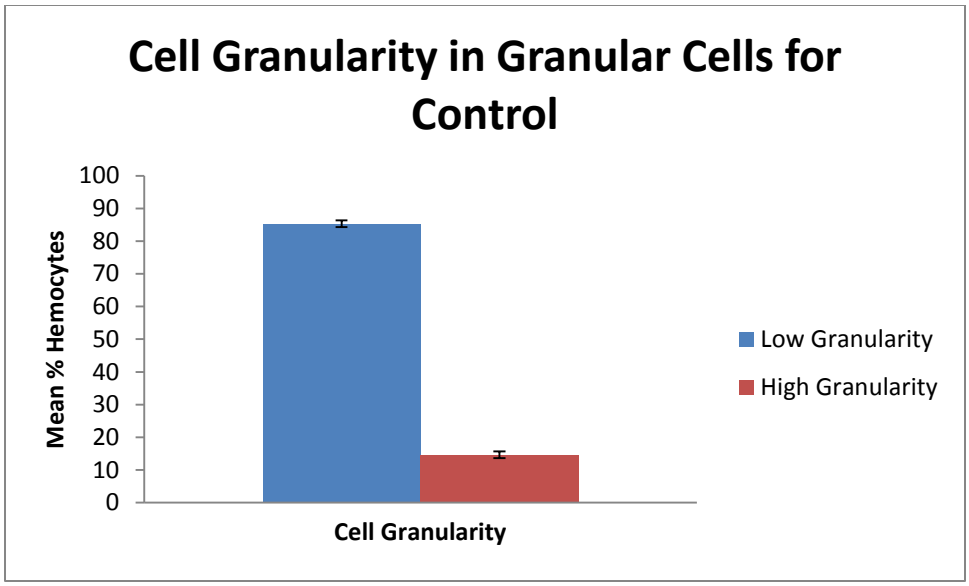


Figure 29. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Overall Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

In analyzing cell granularity for the granular cell population, crayfish treated with crayfish saline displayed 85.0 percent (±1.0) of the cells in the low granularity range and 15.0 percent (±1.0) of the cells in the high granularity range (Figure 30A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 87.0 percent (±2.1) of the cells in the low granularity range and 13.0 percent (±2.1) of the cells in the high granularity range (Figure 30B).

A.



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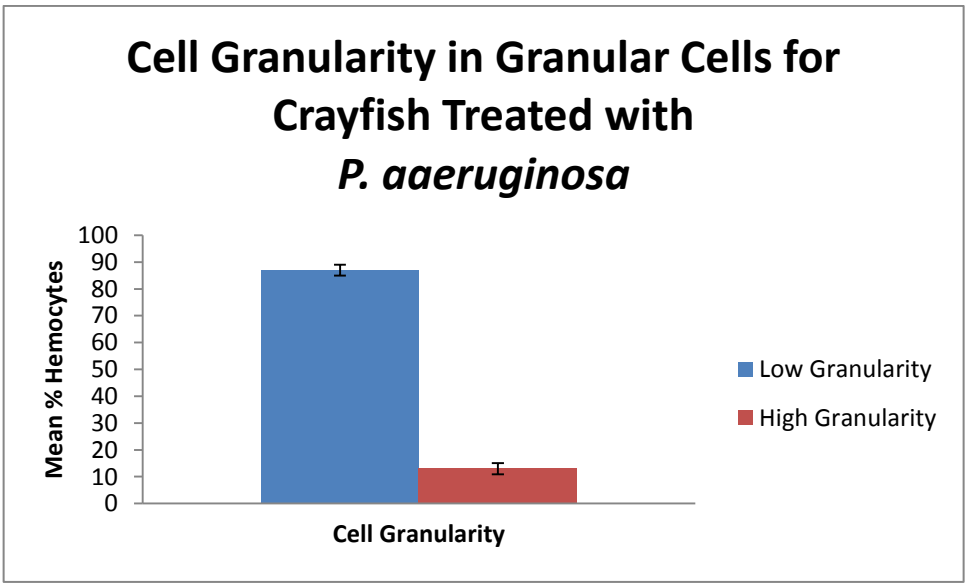
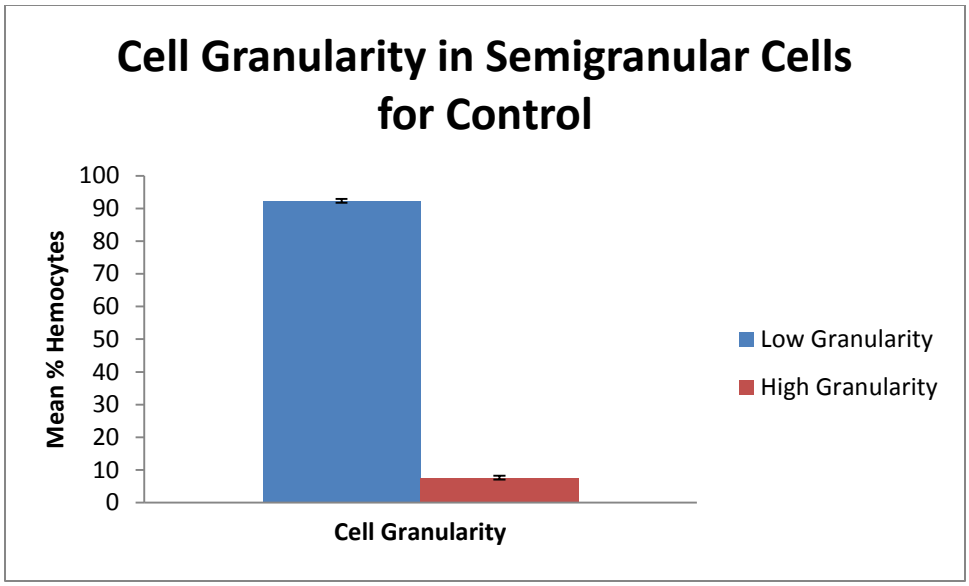


Figure 30. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Granular Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

When evaluating cell granularity for the semigranular cell population, crayfish treated crayfish saline presented with 92.0 percent (±0.57) of the cells in the low granularity range and 7.6 percent (±0.57) of the cells in the high granularity range (Figure 31A). Crayfish treated with *Pseudomonas aeruginosa* suspended with crayfish saline displayed 81.0 percent (±7.8) of the cells in the low granularity range and 19.0 percent (±7.8) of the cells in the high granularity range (Figure 31B).

A.



B.

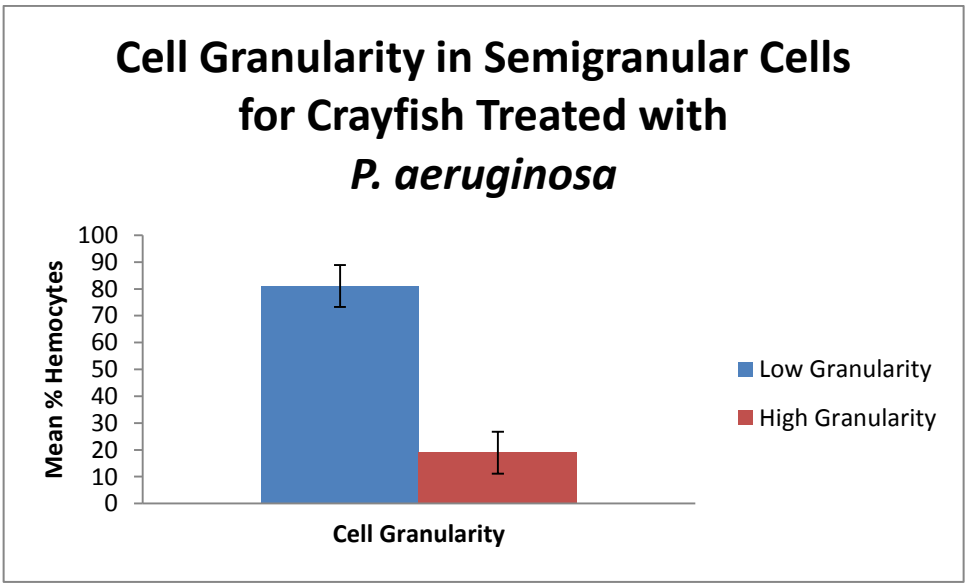


Figure 31. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Semigranular Cell Granularity 24 Hours Postinjection. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 3. **(pvalue).

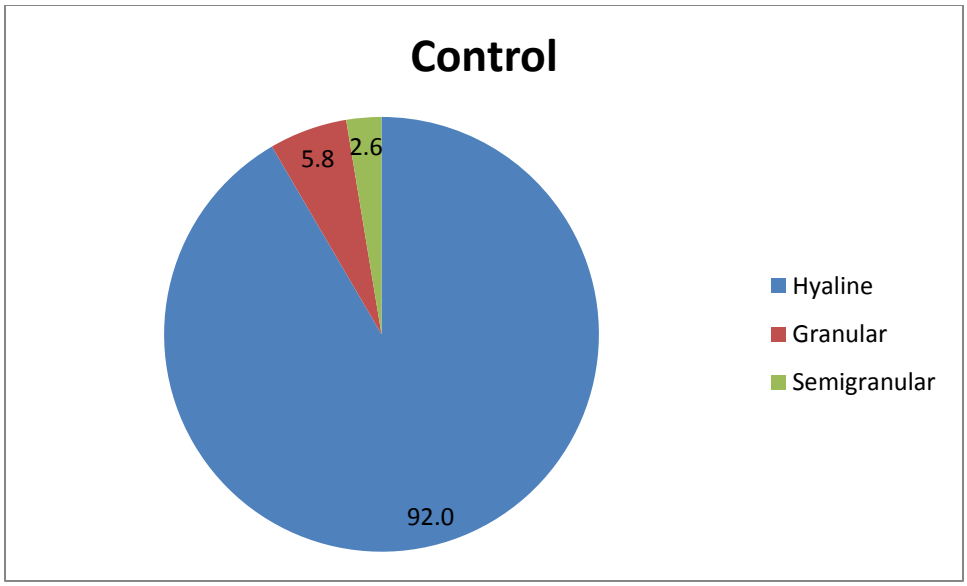
Combined Experimental Results

Data pertaining to the effects of Gram negative bacteria, *Pseudomonas aeruginosa,* were pooled from the previous experiments. Overall hemocyte distribution was compared to the control group. Further comparisons of overall cell size as well as cell size within each population were completed. Finally, overall cell granularity as well as cell granularity within each population was compared to the control group.

Cell Type

Crayfish treated with crayfish saline exhibited a mean percentage of 92.0 (±0.59; Range 89.4-93.6) for the hyaline cell population, 5.8 (±0.54; Range 4.15-8.76) for the granular population, and 2.6 (±0.34; Range 1.14-4.27) for the semigranular population (Figure 32A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed a mean percentage of 79.0 (±0.96; Range 74.2-82.2) for the hyaline cell population, 18.0 (±1.36; Range 12.2-23.4) for the granular population, and 3.6 (±0.48; Range 1.58-5.68) for the semigranular population (Figure 32B).

A.



B.

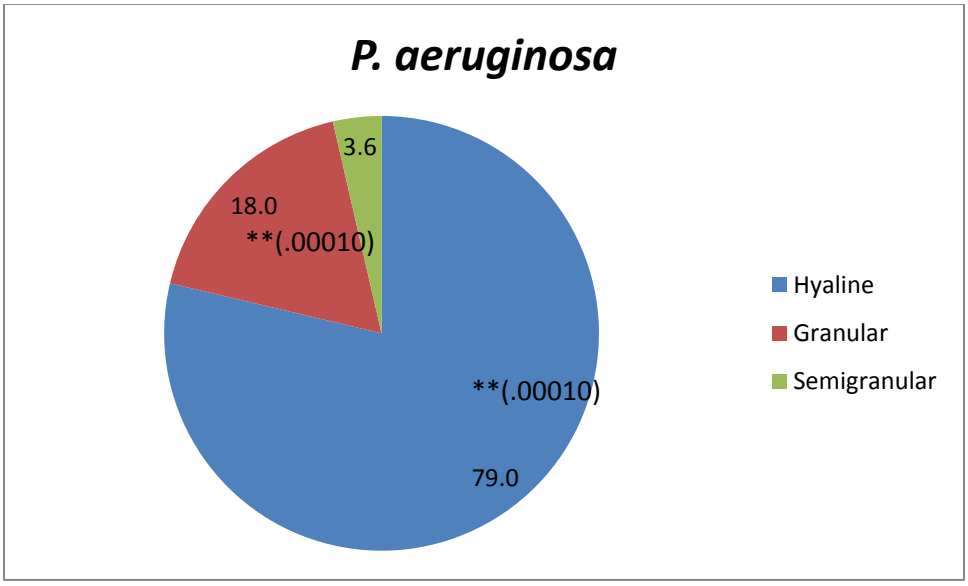
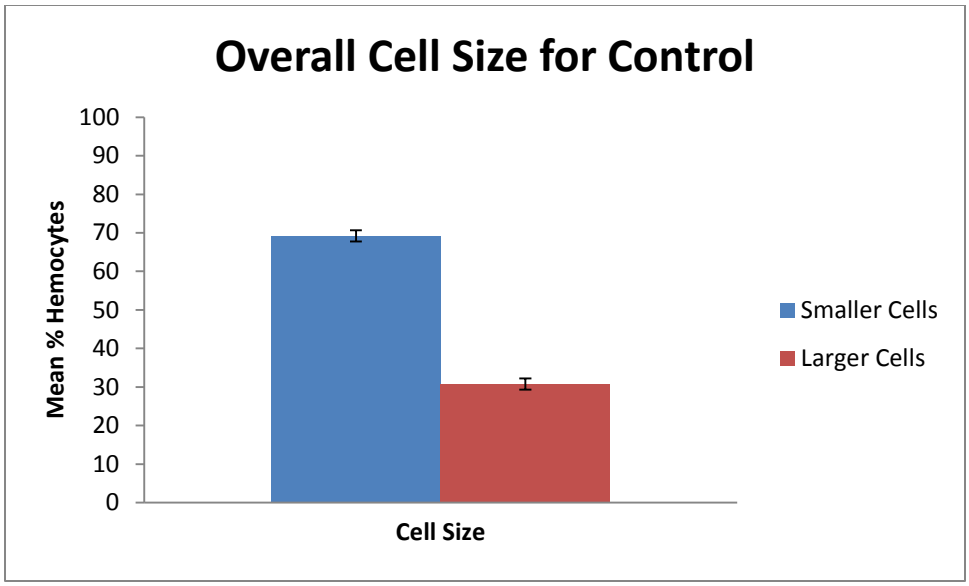


Figure 32. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Distribution of Hemocyte Populations 24 Hours Postinjection. Chart sections represent mean percent hemocytes. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

Cell Size

When observing overall cell size, crayfish treated with crayfish saline displayed 69.0 percent (±1.4) of cells in the smaller cell range and 31.0 percent (±1.4) of the cells in the larger cell range (Figure 33A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 75.0 percent (±4.3) of the cells in the smaller cell range and 25.0 percent (±4.3) of the cells in the larger cell range (Figure 33B).

A.



B.

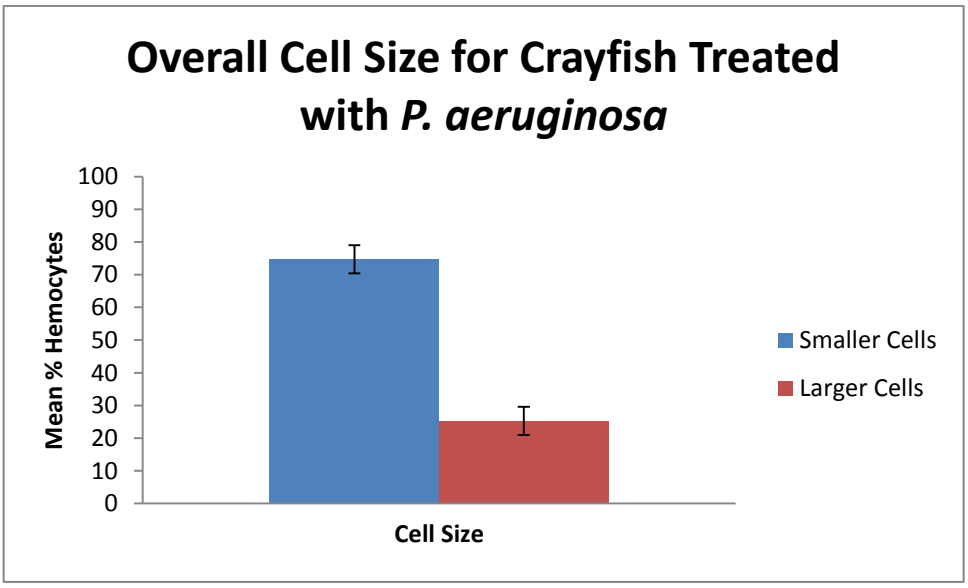
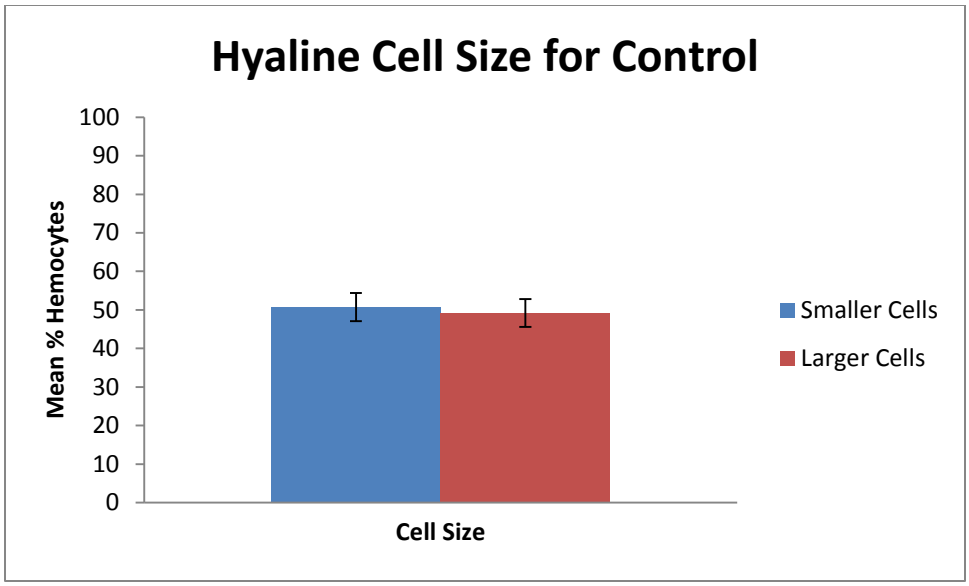


Figure 33. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Overall Cell Size 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

In assaying cell size in the hyaline cell population, crayfish treated with crayfish saline displayed 51.0 percent (±3.6) of the cells in the smaller cell range and 49.0 percent (±3.6) of the cells in the larger cell range (Figure 34A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 56.0 percent (±3.9) of the cells in the smaller cell range and 44.0 (±3.9) of the cells in the larger cell range (Figure 34B).

A.



B.

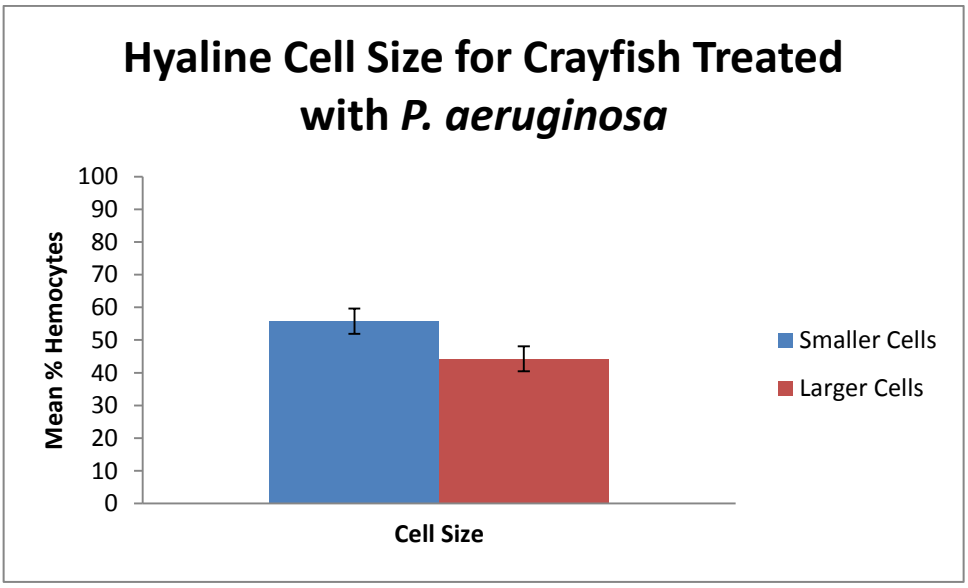
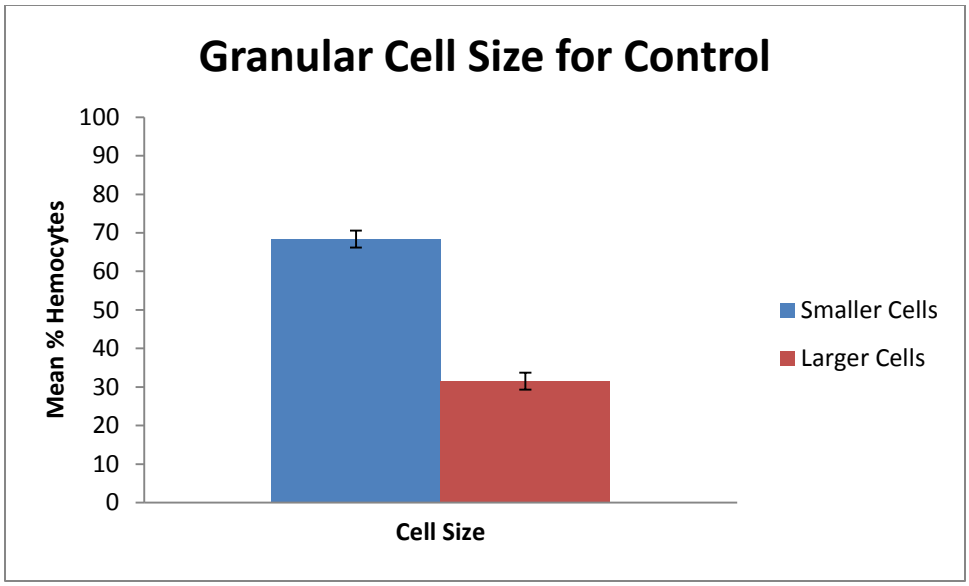


Figure 34. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Hyaline Cell Size 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

In evaluating cell size in the granular cell population, crayfish treated with crayfish saline exhibited 46.0 percent (±2.2) of the cells in the smaller cell range and 54.0 percent (±2.2) of the cells in the larger cell range (Figure 35A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 59.0 percent (±3.7) of the cells in the smaller cell range and 41.0 percent (±3.7) of the cells in the larger cell range (Figure 35B).

A.



B.

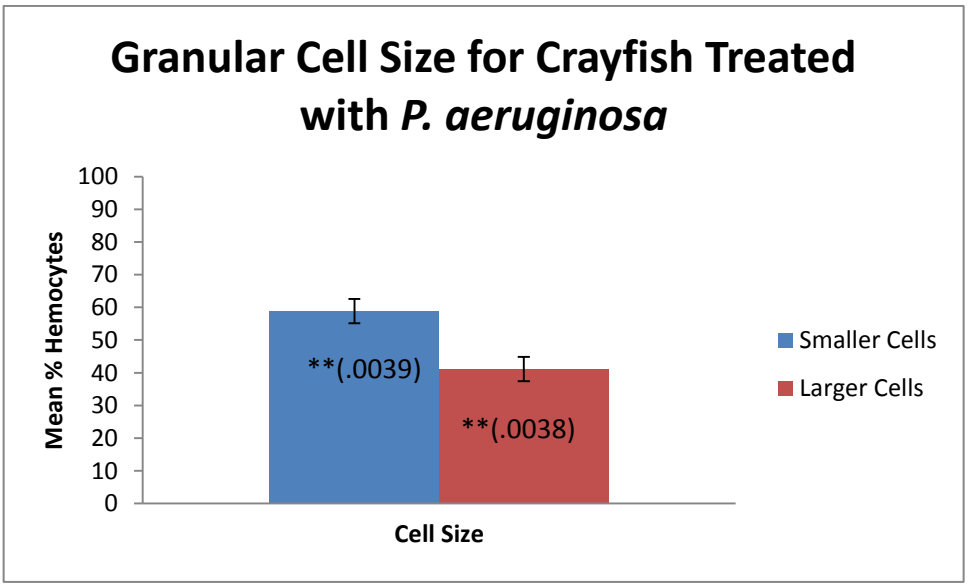
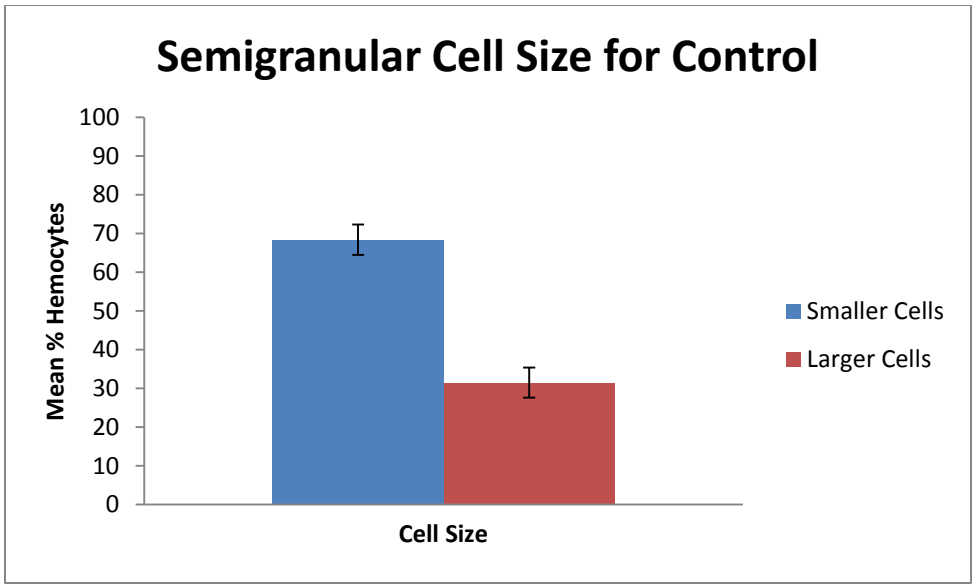


Figure 35. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Granular Cell Size 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

When analyzing cell size in the semigranular cell population, crayfish treated with crayfish saline presented with 68.0 percent (±3.9) of the cells in the smaller cell range and 32.0 percent (±3.9) of the cells in the larger cell range (Figure 36A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline exhibited 69.0 percent (±2.2) of the cells in the smaller cell range and 30.0 percent (±2.3) of the cells in the larger cell range (Figure 36B).

A.



B.

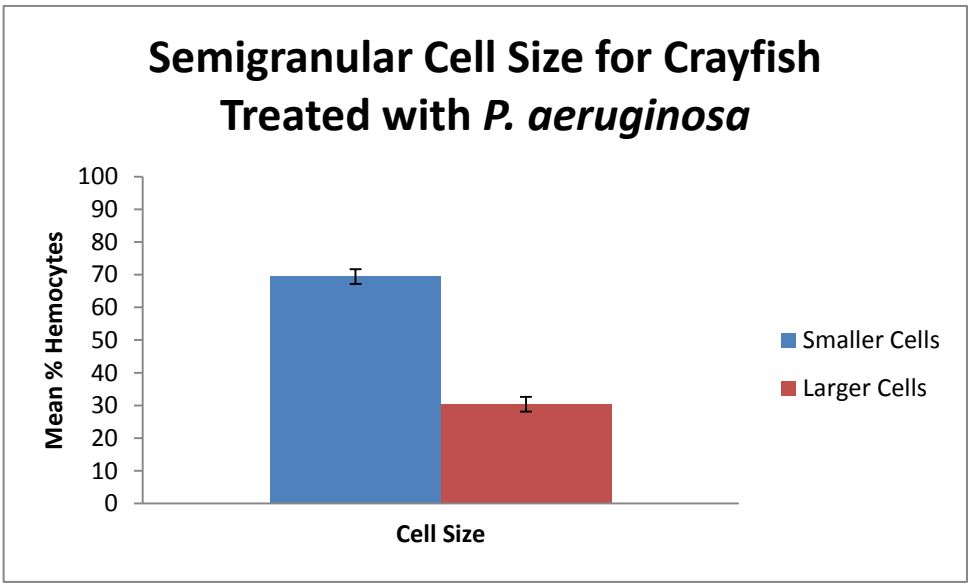
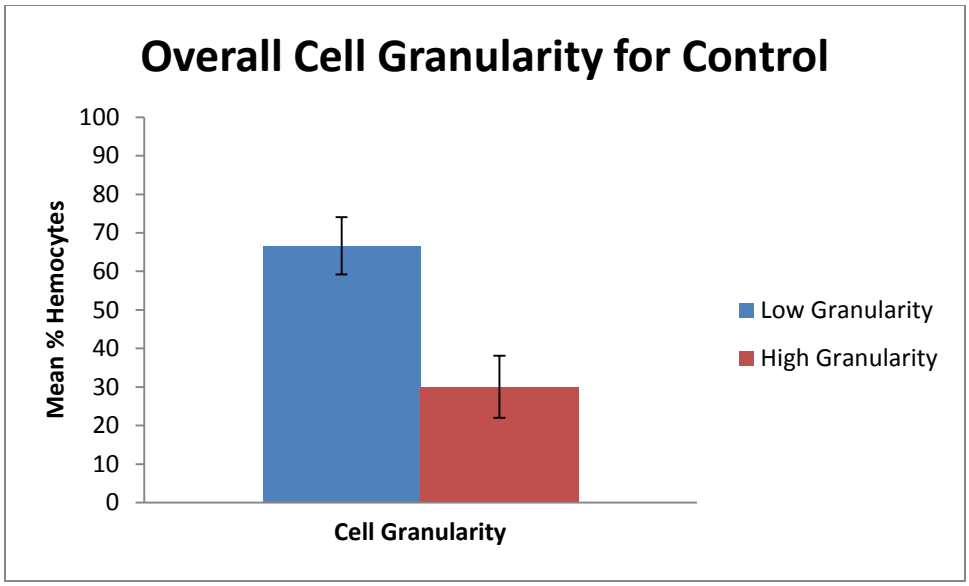


Figure 36. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Semigranular Cell Size 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

Cell Granularity

When evaluating overall cell granularity, crayfish treated with crayfish saline displayed 67.0 percent (±7.4) of the cells in the low granularity range and 30.0 percent (±8.0) of the cells in the high granularity range (Figure 37A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 60.0 percent (±7.1) of the cells in the low granularity range and 40.0 percent (±7.1) of the cells in the high granularity range (Figure 37B).

A.



B.

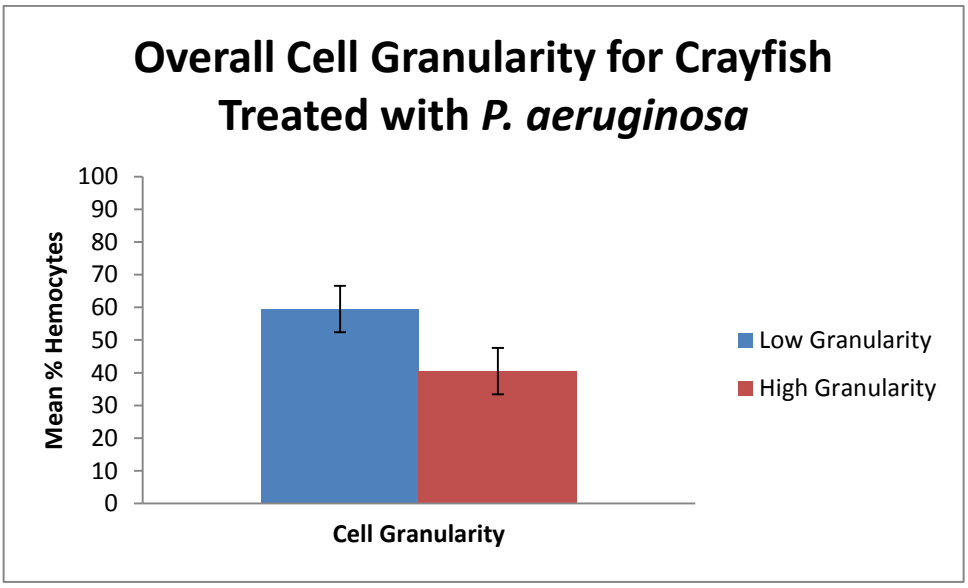
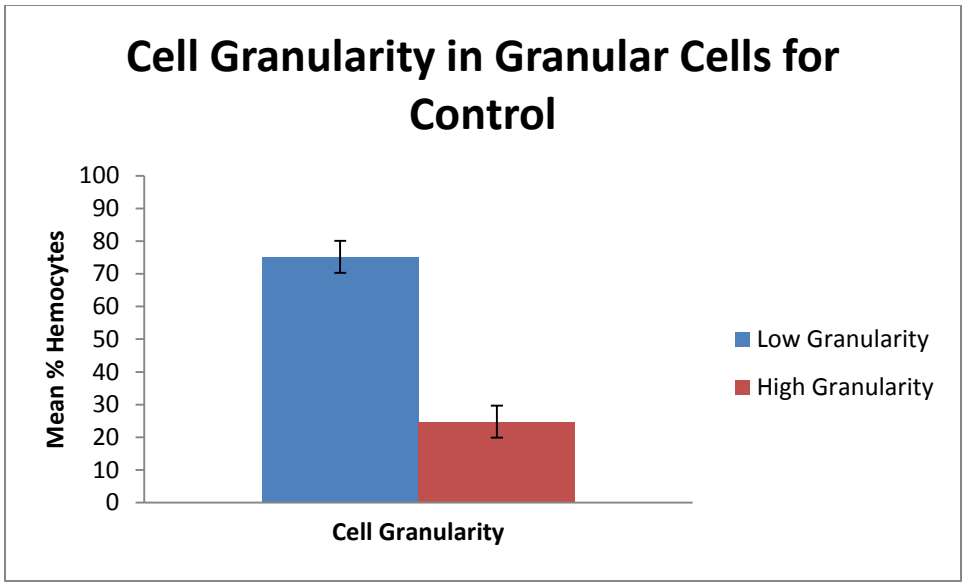


Figure 37. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Overall Cell Granularity 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

In analyzing cell granularity for the granular cell population, crayfish treated with crayfish saline exhibited 75.0 percent (±4.9) of the cells in the low granularity range and 25.0 percent (±4.9) of the cells in the high granularity range (Figure 38A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline displayed 81.0 percent (±4.5) of the cells in the low granularity range and 19.0 percent (±4.5) of the cells in the high granularity range (Figure 38B).

A.



B.

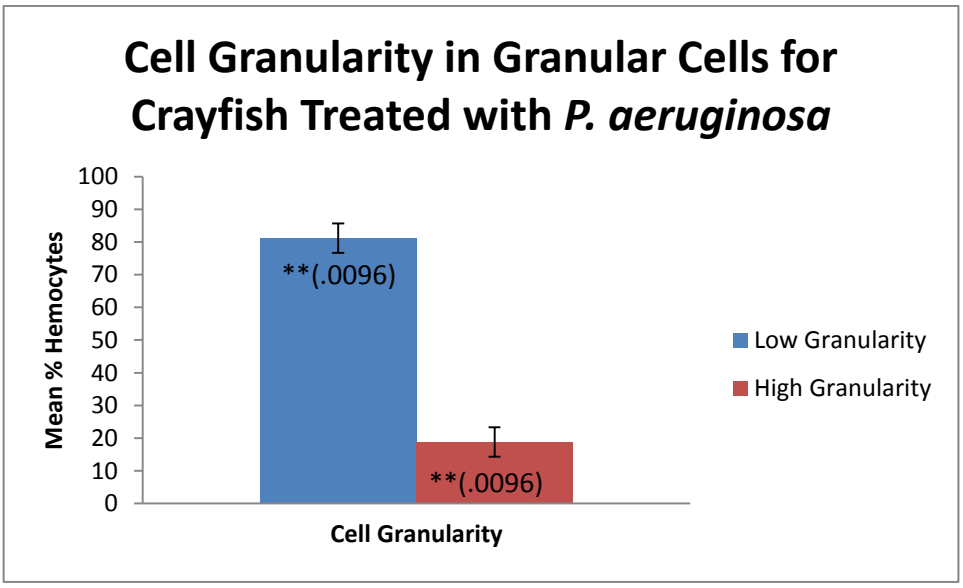
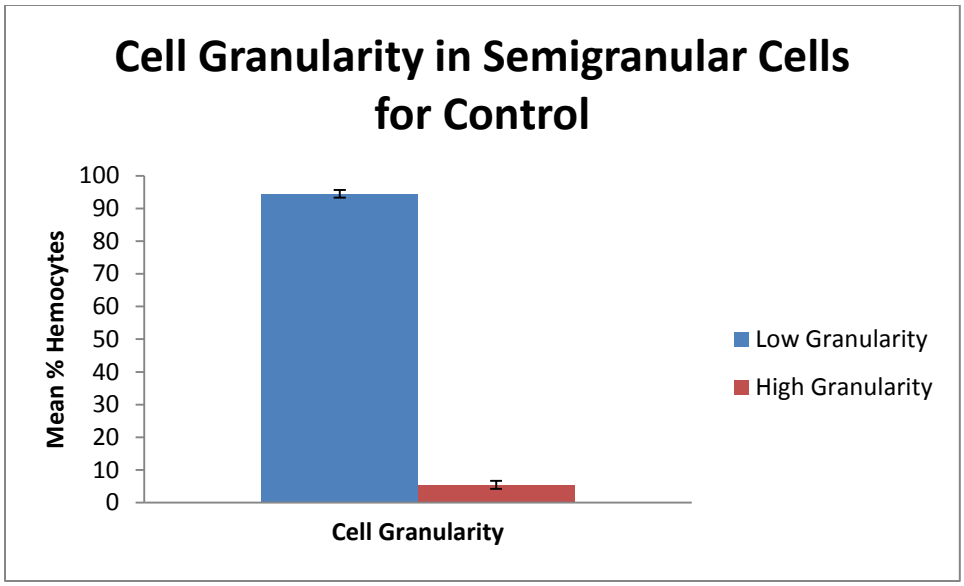


Figure 38. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Granular Cell Granularity 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

When observing cell granularity for semigranular cell population, crayfish treated with crayfish saline exhibited 94.0 percent (±1.2) of the cells in the low granularity range 4.4 percent (±1.2) of the cells in the high granularity range (Figure 39A). Crayfish treated with *Pseudomonas aeruginosa* suspended in crayfish saline presented with 92.0 percent (±3.6) of the cells in the low granularity range and 7.9 percent (±3.6) of the cells in the high granularity range (Figure 39B).

A.



B.

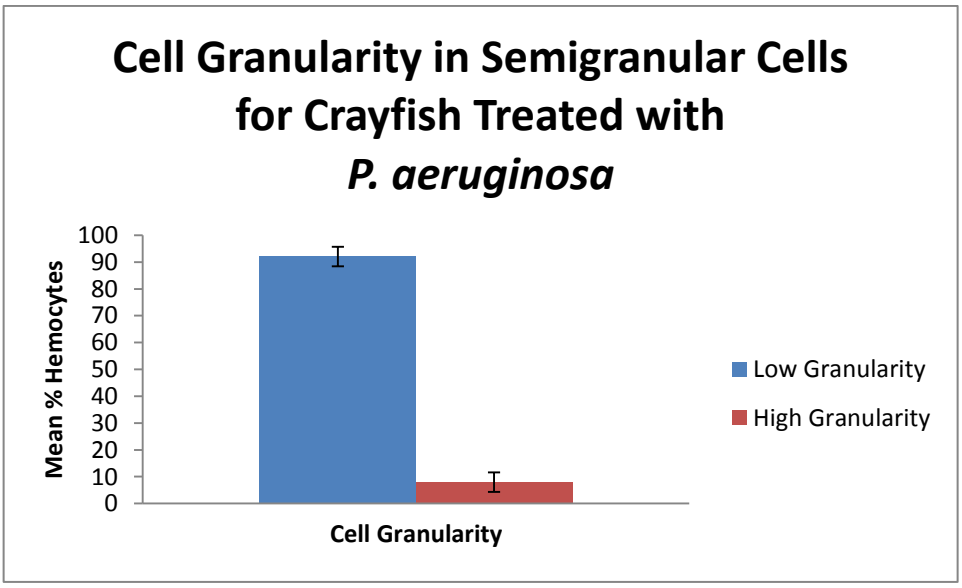


Figure 39. Effect of Injection of Crayfish with (B) *Pseudomonas aeruginosa* on Semigranular Cell Granularity 24 Hours Postinjection. Combination of 3 experiments. Significant difference (**≤ .05) shown by comparing treatment to (A) control using t-test. n = 9. **(pvalue).

CHAPTER 4

DISCUSSION

Freshwater crayfish only display innate immunity. It comprises many processes ranging from phagocytosis and encapsulation to antibacterial peptides and clotting mechanisms as well as the prophenyloxidase cascade (6). All of these processes are mediated by hemocytes and their products. There are 3 distinct types of hemocytes in freshwater crayfish, the hyaline cells, the granular cells, and the semigranular cells (3).

In the first set of experiments normal hemocyte populations in 20 normal untreated crayfish were assayed. Three parameters were analyzed, cell type, cell size within all cells and the different cell populations, and cell granularity within all the cells and the different cell populations. This study produced the first picture of the hemocyte population in normal Red Swamp Crayfish with regards to hemocyte type, overall hemocyte size, size within each hemocyte population, overall hemocyte granularity, and granularity within the granular and semigranular populations. First, cell type was measured by using both forward angle light scatter and 90° angle light scatter to separate the individual hemocyte populations. For normal untreated crayfish, average percentages of the individual hemocyte populations were 89.7 (±0.87) for the hyaline cell population, 6.6 (±0.66) for the granular cell population, and 3.7(±0.40) for the semigranular cell population (Figure 5). These findings were in contrast to some values reported in the literature. Washington Cárdenas and colleagues observed *Procambarus zonangulus* and reported average percentages for individual hemocyte populations. Their results indicated a 77.1 average percentage for the hyaline cells, a 12.6 average percentage for granular cells, and a 2.3 average percentage for the semigranular cells. However, these percentages add up to only 92 percent, leaving an 8 percent disparity (2). When looking at *Procambarus clarkii,* our flow cytometry data appeared to be more consistent with data reported under microscopy analysis.

Washington Cárdenas and colleagues reported an average percentage of 88.7 for the hyaline cells, an average percentage of 9.2 for the granular cells, and an average percentage of 2.2 for the semigranular cells for *Procambarus zonangulus* (2). The variability in the hyaline cell population exhibited a range of 82.8 to 94.4, the granular cell population, 2.49 to 12.0, and the semigranular cell population, 1.54 to 8.46 (Table 1). These findings were different from some reported in the literature where the hemocyte populations were assessed via miscroscopy. H. Lanz and colleagues reported a range of 75 to 77 percent for the hyaline cell population for *Procambarus clarkii*, and K. Söderhäll and V.J. Smith reported a range of 15 to 16 percent for the granular population in *Astacus astacus* (8 , 19). H. Lanz and colleagues also reported a range of 8 to 9 percent for the semigranular cells for *Procambarus clarkii* (8). Our findings using flow cytometry allowed for greater accuracy and more objectivity than microscopy because more cells can be analyzed and results are garnered faster with more precision. Furthermore, flow cytometry may possess a greater potential in cell typing than Percoll gradient separation, especially for our application, because hemolymph could be analyzed from individual animals and not pooled from a set of animals. This provided more accuracy into overall cell characteristics for each animal because more cells could be assayed. Second, cell size within all cells and the different cell populations was observed by using forward angle light scatter. When looking at overall cell size in normal untreated animals, 85.5 percent (±3.17) of the cells were in the smaller cell range, whereas 15.5 percent (±3.17) of the cells were in the larger cell range (Figure 6A). Analysis of hyaline cells revealed that 62.9 percent (±2.93) were in the smaller cell range, whereas 13.1 percent (±2.93) of the cells were in the larger cell range (Figure 6B). This finding was indicative of observations in previous studies where hyaline cells were shown to be the smallest of the hemocytes (2, 3). Looking at the granular cells showed that 51.3 percent (±2.58) of the cells were in the smaller cell range, but 48.7 percent (±2.58) of the cells were in the larger cell range (Figure 6C). Analysis of the semigranular cells showed that 63.1 percent (±4.13) of the cells were in the smaller cell range, whereas 36.8 (±4.13) of the cells were in the larger cell range (Figure 6D). Finally, cell

granularity within all cells and the different cell populations was analyzed via 90° angle scatter analysis. When granularity was assessed in normal untreated crayfish 76.8 percent (±2.78) of the cells were in the low granularity range, whereas 23.2 percent (±2.78) of the cells were in the high granularity range (Figure 7A). Looking further at the granular cells showed that 77.5 percent (±2.09) of the cells were in the low granularity range, but 22.5 percent (±2.09) of the cells were in the high granularity range (Figure 7B). When looking at the semigranular cells 85.2 percent (±2.79) of the cells were in the low granularity range, whereas 14.8 percent (±2.79) of the cells were in the high granularity range (Figure 7C). The granular cells demonstrated a greater level of high granularity than the semigranular cells, a finding that could represent an increased granular density in the granular and a lower granular density in the semigranular cells (3).

This study also measured the effect on crayfish hemocytes produced by exposure to Gram positive bacteria, *Staphylococcus aureus*, and Gram negative bacteria, *Pseudomonas aeruginosa*, after 24 hours via flow cytometry. In this study 3 parameters were analyzed, cell type, cell size, and cell granularity. First, cell type was assessed by using both forward angle light scatter and 90° angle light scatter to separate the individual hemocyte populations. Then, cell size within all cells and the different cell populations was observed by using forward angle light scatter. Finally, cell granularity within all cells and the different cell populations was detected via 90° angle scatter analysis. In doing this study it was hypothesized that cell type, cell size, and cell granularity may be significantly affected by exposure to both *Staphylococcus aureus*, a Gram positive bacterium, and *Pseudomonas aeruginosa,* a Gram negative bacterium.

Cell Type

Forward angle light scatter and 90° angle light scatter were used to separate and analyze the 3 types of hemocytes identified in freshwater crayfish (3, 5). When freshwater crayfish*, Procambarus clarkii*, were exposed to *Pseudomonas aeruginosa*, there was a significant decrease in the percentage of the hyaline cell population (Figures 8B, 16B, 24B; 32B) with respect to the control animals (Figures 8A, 16A, 24A; 32A). In addition crayfish exposed to *Staphylococcus aureus* exhibited a significant decrease in the percentage of hyaline cells (Figure 8C) with respect to the control crayfish (Figure 8A). However, treatment with *Pseudomonas aeruginosa* elicited a more significant response in the hyaline cell population (Figure 8B; pvalue, 0.0032) than treatment with *Staphylococcus aureus* (Figure 8C; pvalue, 0.016). Exposure to *Pseudomonas aeruginosa* also revealed a significant increase in the percentages of the granular but not the semigranular populations (Figures 8B, 24B; 32B) with respect to the control animals (Figures 8A, 24A; 32A). Treatment with *Staphylococcus aureus* also revealed a significant increase in quantity for the granular cells (Figure 8C) but not the semigranular cells (Figure 8C) when compared to the controls (Figure 8A). These findings (Figure 8C; pvalue, 0.017) were not as significant as those exhibited by *Pseudomonas aeruginosa* (Figure 8B; pvalue, 0.0002). These responses could be elicited for several reasons. First, treatment with these 2 bacteria could stimulate death of the hyaline cells or a higher production of granular cells by the crayfish immune system. Second, higher percentages in the granular population could indicate production of higher levels of phenoloxidase which complements various defensive processes ranging from phagocytosis to encapsulation and nodulation (3, 6). Finally, the greater significance demonstrated by exposure to *Pseudomonas aeruginosa* versus *Staphylococcus aureus* could be due to the presence of LPS in the *Pseudomonas aeruginosa*. LPS, an endotoxin found in Gram negative bacteria, could cause cell lysis in the hyaline cell population and the granular cell population (2).

Cell Size

Both forward angle light scatter and 90° angle light scatter isolated individual hemocyte populations. However, forward angle light scatter was used in evaluating cell size. This was accomplished by analyzing forward angle light scatter versus counts, and each count corresponded to each cell (5). In the combined results of 3 experiments freshwater crayfish, *Procambarus clarkii,* exposed to *Pseudomonas aeruginosa* exhibited a significant difference in granular cell size with a shift toward larger cells (Figure 35B) when compared to animals treated with crayfish saline (Figure 35A). In a single experiment freshwater crayfish exposed to *Staphylococcus aureus* generated no significant differences in cell size (Figure 11C) when compared to animals treated with crayfish saline (Figure 11A). These results could be due to several mitigating factors. Initially, administration of *Pseudomonas aeruginosa* in freshwater crayfish, *Procambarus clarkii*, could cause an increase in the production of the granular cells. Then, heightened production of the granular cells could signal a higher production of phenoloxidase, which stimulates melanization, a process that augments the different immune procedures such as phagocytosis, nodulation, and encapsulation (3 ,6). Finally, the significant responses exhibited by *Pseudomonas aeruginosa* over *Staphylococcus aureus* could be indicative of the effect of LPS found in Gram negative bacteria. LPS could cause cell lysis in the hyaline cell population as well as the granular and semigranular cell populations (2).

Cell Granularity

Forward angle light scatter was used to quantify changes in cell size within hemocyte populations, but 90° angle light scatter was used in analyzing cell granularity. This was done by plotting cell granularity versus counts, and each count corresponded to a cell (5). In the combined results of 3 experiments , freshwater crayfish, *Procambarus clarkii,* exposed to *Pseudomonas aeruginosa* showed a significant difference with regard to reduced granularity for the granular hemocytes (Figure 38B) when

compared to animals treated with crayfish saline (Figure 38A). In addition, a single experiment freshwater crayfish exposed to *Staphylococcus aureus* exhibited no significant differences in granularity (Figures 13C, 14C; 15C) when compared to animals treated with crayfish saline (Figures 13A, 14A; 15A). The significant findings in animals treated with *Pseudomonas aeruginosa* versus *Staphylococcus aureus* could be due to the presence of LPS found in *Pseudomonas aeruginosa*. LPS could generate 2 responses. First, LPS, an endotoxin found in Gram negative bacteria, *Pseudomonas aeruginosa*, could cause degranulation in the granular cells (2). Second, LPS could stimulate the production of phenoloxidase for melanization, a process that causes degranulation of granular cells (3,6).

This study showed significant changes in hemocyte populations as well as cell size and granularity as a result of exposure to Gram negative bacteria, *Pseudomonas aeruginosa*. Freshwater crayfish, *Procambarus clarkii* , also showed significant changes in hemocyte populations when exposed to Gram positive bacteria, *Staphylococcus aureus.* The tests performed in this study represented bacterial stress on the crayfish immune system. Further studies into the effects of bacterial stress on crayfish immunity could possibly be used to monitor the health of freshwater habitats. This could be done by repeating these current experiments with native crayfish in various freshwater environments. Normal baselines would have to be established for native crayfish population and procedures for collection of hemolymph for native populations developed before this approach could be tested. In addition, future studies into the effects of other xenobiotics on the innate immune system in red swamp crayfish (our laboratory model) need to be completed. Looking at the effects of xenobiotics could be accomplished by exposing red swamp crayfish to both herbicides and pesticides and analyzing their effects on hemocyte population distribution, cell size with all the cells and within the different cell populations, and cell granularity in all the cells and within the different hemocyte populations. Furthermore, future investigations into the reasons, ranging from cell death and cell proliferation to cell degranulation and conversion of one cell type to another, behind the results that have been observed

need to be determined. Finally, more experiments of the effects of Gram positive bacteria on red swamp crayfish hemocytes need to be completed.

Understanding the immune system in freshwater crayfish could be important in looking at the overall health of freshwater ecosystems. Crayfish have invaded all trophic levels in freshwater habitats (21). Therefore, they could serve as a valuable indicator species in almost any fresh water environment.

Crayfish are also good model systems for innate immune responses. The innate immune system is very important in human health. Studies in innate immunity will give rise to more knowledge about how the adaptive immune and the innate immune response work together to protect us from pathogens in our environment.

BIBLIOGRAPHY

1. Cárdenas, Washington, John R. Dankert; Jill A. Jenkins. (2004). Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides. *Fish and Shellfish Immunology*.17,3,223-233.
2. Cárdenas, Washington, Jill A Jenkins; John R. Dankert. (2000). A flow flow cytometric approach to the study of crustacean cellular immunity. *J. invertebrate Pathology.* 76, 112-9.
3. Giuliani, Piero Giulio, Manuel Bierti, Simonetta Lorenzon, Silvia Battistella; Enrico Antonio Ferrero. (2006). Ultrastructual and functional characterization of circulating hemocytes from the freshwater crayfish *Astacus leptodactylus* : Cell types and their role after in vivo artificial non-self challenge. *Micon.* 38,1, 49-57.
4. Goins, Kimberly R. (2003). Host Defense Mechanisms in the Crayfish: The Effect of Injection with Live or Killed Bacteria. Thesis East Tennessee State University.
5. *Introduction to Flow Cytometry: A Learning Guide*. April. (2000). BD Biosciences
6. Jiravanichpaisal, Pikul, Bok Luel Lee; Kenneth Söderhäll. (2006). Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. *Immunobiology.* 211, 4, 213-36.
7. Jiravanichpaisal, Pikul, So Young Lee, Young-A Kim, Tove Andren; Irene Söderhäll. (2007). Antibacterial peptides in hemocytes and hemopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: Characterization and expression pattern. *Developmental & Comparative Immunology.* 31,5,441-55.
8. Lanz, H., V. Tsutsumi; H. Arèchiga. (1993). Morphological and Biochemical Characterization of *Procambarus clarkii* Blood Cells. *Dev. Comp. Immunol*. 17,389-97.
9. Lee, So Young; Kenneth Söderhäll. (2001). Characterization of a Pattern Recognition Protein, a Masquerade-Like Protein, in the Freshwater Crayfish *Pacifastacus leniusculus. The Journal of Immunology.* 166,7319-26.
10. Lee, YK., BS Soh; JH Wu.(2001).Quantitative analysis of phagocytic activity of hemocytes in the prawn, *Penaeus merguiensis*, by flow cytometric analysis. *Cytometry*. 43, 1, 82-5.
11. Liu, Haipeng, Pikul Jiravanichpaisal, Lage Cerenius, Bik Luel Lee, Irene Söderhäll; Kenneth Söderhäll. (2007). Phenoloxidase Is an Important Component of the Defense against *Aeromonas hydrophila* Infection in a Crustacean, *Pacifastacus leniusculus. J. Biol. Chem.* 282,46,33593-98.
12. Osaki, T., N Okino, F Tokunaga, S Iwanaga; S. Kawabata.(2002). Proline-rich cell surface antigens of horseshoe crab hemocytes are substrates for protein cross-linking with a clotting protein coagulin. *Journal of Biological Chemistry*.277,42,40084-90.
13. Park, Ji Won, Byung-Rok Je, Shunfu Piao, Seiichi Inamura, Yukari Fujimoto, Koichi Fukase, Shoichi Kusumoto, Kenneth Söderhäll, Nam-Chul Ha; Bok Luel Lee. (2006). A Synthetic Peptidoglycan Fragment as a Competitive Inhibitor of the Melanization Cascade. *J. Biol Chem*. 281,12,7747-55.

14. Perry, Kristi. (2002). The Optimization of the Technique for Studying the Phagocytosis and Attachment of Sheep Erythrocytes by Crayfish Hemocytes *in vitro*. Honors Thesis East Tennessee State University.
15. Sequeira, T., D. Tavares; M. Arala-Chaves.(1996).Evidence for circulating hemocyte proliferation in the shrimp, *Penaeus japonicas.* *Developmental and Comparative Immunology.* 20, 2, 97-104.
16. Smith, V.J.; K. Söderhäll. (1983). Induction of degranulation and lysis of hemocytes in the freshwater *Astacus astacus* by components of the prophenoloxidase activating system *in vitro*. *Cell Tissue Res*. 233,295-303.
17. Sobolski, Mark R., Jason Oaks; William P. Halford. (2005). Green Fluorescent protein is a qualitative reporter of gene expression in individual eukaryotic cells. *The FASEB Journal.* 19,440-42.
18. Söderhäll, Irene, Eakaphun Bangyeekhun, Susan Mayo; Kenneth Söderhäll. (2003). Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of *Pacifastacus leniusculus*. *Developmental & Comparative Immunology*. 27, 8, 661-72.
19. Söderhäll, K.; V.J. Smith. (1983).Separation of the haemocyte populations of *Carcinus maenas* and other marine decopods, and prophenoloxidase distribution. *Developmental & Comparative Immunology.* F,229-39.
20. Taylor, S., MJ Landman; M. Ling.(2009).Flow Cytometric Characterization of freshwater crayfish hemocytes for the examination of physiological status in wild and captive animals. *J. Aquatic Animal Health*. 3, 195-203.
21. Thorp, James H.; Alan P. Covich, Ed. (1991). Ecology and Classification of North American Freshwater Invertebrates. 823-58.
22. Wang, YT., W. Liu, JN Sean, CS Lam, JH Xiang, V Korzh; J Kwang.(2002). White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. *Diseases of Aquatic Organisms*.52, 3, 249-59.
23. Wheatly, Michele G., Zhiping Zhang, Jennifer R. Weil, James V. Rogers; La'Tonia M. Stiner. (2001). Novel Subcellular and Molecular Tools to Study Ca^{2}+ Transport Mechanisms During the Elusive Moulting Stages of Crustaceans: Flow Cytometry and Polyclonal Antibodies. *J. Experimental Biology*. 204; 959-66.

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