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Amebocyte Diameter and Density after Partial Exsanguinations in Limulus Polyhemus Hemolymph

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ABSTRACT

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William Weiner, Ph.D.

The presence of endotoxins, a potentially life threatening bacterial contamination in medical devices and supplements, is determined by a product know as Limulus Amebocyte Lysate (LAL). The creation of LAL is known to raise the mortality rates of the crabs that are also seeing population declines due to environmental pressures and fishing industries. Because of the decline in the number of crabs, a method for the production of LAL, outside of the bleeding animals, is necessary. This research worked to quantify systematically the cell size, shape, and concentration of the amebocytes.

After baseline data were collected, 40-50% of the hemolymph from six different animals was removed, and the recovery of the weight, amebocyte density, and diameter was regularly determined. Follow-up data showed the weight decreasing immediately after the large hemolymph removals, and recovery to baseline occurring within two weeks. Around day 8 after the large hemolymph removals, both the amebocyte density and diameter decrease. Between days 18 and 20 the density and diameter returned to normal, implying that new amebocytes can be seen in the hemolymph of a horseshoe crab about two weeks after a large hemolymph removal and the new cells will have a smaller diameter than the more mature amebocytes in the hemolymph.

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1 BACKGROUND

1.1 Introduction

The presence of endotoxins, a potentially life threatening bacterial contamination in medical devices and medication, is determined by the Limulus Amebocyte Lysate (LAL). LAL is created by collecting hemolymph from the American Horseshoe Crab where the sole cell, the amebocyte, is separated and purified. This lysate is highly sensitive and incredibly important with respect to the health industry, but the bleeding procedure is known to raise the mortality rates of the crabs up to 10%.[1] The populations of these very important animals are declining because of environmental pressures and fishing industries, aside from the LAL industry. This population decline suggests that the need for an alternative production method is vital to the survival of the horseshoe crab. This research worked to answer a number of questions hindering the progress of finding an alternative LAL production method such as: the recovery of hemolymph volume after large hemolymph removals, amebocyte density, and amebocyte diameter in the hemolymph under normal conditions and straining conditions modeling amebocyte production in the gill lamellae where amebocytes are grown *in-vitro*.

1.2 Endotoxins and the Human Reaction

Endotoxins are the lipid-A portion of the lipopolysaccharides from the membrane of Gram-negative bacteria[1]. This ubiquitous endotoxin is only harmful to humans when coming into contact with the blood stream through drugs, medical devices, or rupturing

of the intestinal tract. When endotoxins are recognized by the human immune system, the hypothalamic system responds by raising the set point inducing fever[2]. This fever response becomes more detrimental to the human than the endotoxins because the temperature necessary to neutralize endotoxins is higher than the temperature of protein denaturing.

The immune response to endotoxins, commonly known as endotoxemia or septic shock, can be characterized as "a state of inadequate tissue perfusion induced by microbial products and characterized by low blood pressure and biochemical signs of oxygen [deficiency]"[2]. Common symptoms of endotoxemia are fever with a raise in temperature of 1-2° C, myalgia, headache, nausea, chills, increased heart rate, and lowering of blood pressure[2]. As endotoxemia progresses, it can also cultivate symptoms of "reduced oxygen to vital organs, reduced nutrient transport capability to vital organs, generalized inflammatory response to vasodilatation, intravascular coagulation, renal failure, adult respiratory distress syndrome", and ultimately death[2]. Recorded cases of endotoxemia reach 500,000 cases per year, with 35% being fatal[2]. Endotoxemia can be prevented by ensuring endotoxins do not come into contact with the human bloodstream. The established mode of this prevention is using LAL to test for sterile medical instruments and medication.

1.3 Amebocytes and the Immune System of Horseshoe Crabs

The LAL industry was founded through the research of Frederick Bang and Jack Levin who studied a 300 million year old species, the American Horseshoe Crab, which lives in an aquatic environment along the Atlantic Coast[3]. Levin and Bang's research

focused on the innate immunity of the horseshoe crab based on their hemolymph. The hemolymph of the horseshoe crab consists of one main amebocyte: the granular amebocyte. These amebocytes generate in the gills of the horseshoe crab and are ovoid in structure, non-adhesive to the surroundings, and are ~15-20 μm in diameter[4].

Since Gram-negative bacteria are ubiquitous to the horseshoe crab's aquatic environment, endotoxins from the surrounding environment come in contact with the hemolymph whenever the horseshoe crab's shell or soft tissue is broken [3]. The crab's only defense against endotoxins involves the degranulation of the amebocyte. This lysate binds to the endotoxins, thereby inactivating and immobilizing the endotoxins through clotting mechanisms[5]. The coagulation acts as positive feedback encouraging other amebocytes to undergo degranulation forming a clot that neutralizes the endotoxins and rebuilds the barrier between the outside environment and the hemolymph.

1.4 Current Endotoxin Testing Using Limulus Amebocyte Lysate

Currently, the method for endotoxin testing is LAL, which is a serum made from the hemolymph of the American Horseshoe Crab[6]. Harvesting the hemolymph of the American Horseshoe Crab and commercial production of LAL has become a $15,000 per quart industry; where one quart of LAL requires the bleeding of 15-20 animals[7]. The procedure for bleeding crabs has changed very little since being outlined in Levin and Bang's original methods[3]. After the animals are bled the hemolymph and amebocytes are separated creating the lysate. The lysate is then freeze dried for storage or used immediately for endotoxin testing.

LAL testing is performed by mixing equal parts of the sample and LAL in a test tube which is incubated at 37° C for one hour[8]. After incubation, the test tube is completely inverted. The solution will clot in the presence of endotoxins, but will remain liquid if no endotoxins are present. This method provides sensitivity levels up to 3 picograms of endotoxins and is known as the gel clot method[8].

1.5 Limulus Amebocyte Lysate Challenges

The American Horseshoe Crab population experienced a steady decline over the last three decades due to many factors such as the LAL industry, depletion of their environment, natural predators, and being harvested for bait and fertilizer. In a five year period, the bait industry alone decreased the horseshoe crab population from 1.2 million to around 100,000, which is detrimental to their industry and the crabs[3]. The depletion of the horseshoe crab population not only affects the LAL industry but also harms the Red Knot, which rely on crab eggs as their sole food source during migration[3]. Throughout history, the presence of Red Knots did not threaten the horseshoe crab population, but other factors have caused the amount of crab eggs the birds are eating to be debilitating to the population.

The necessity and expense of producing LAL has gradually increased over the years causing another pressure on the animals and the product. Over an eight year period the number of crabs bled for the LAL industry went from 127,000 to 240,000[8]. As the industry continues to grow, the amount of hemolymph necessary increases. Which implies that the number of animals bled also increases, putting a strain on the animals

especially if they were previously bled or are injured? Bleeding protocols have been placed to protect the animals, but in 2006 over 300,000 crabs were harvested and over 4,600 were rejected although a significantly larger percent of the population was unfit to be bled[9]. Because of the importance of the industry to the medical field, the population of American Horseshoe Crabs and the prosperous LAL industry need to be protected.

1.6 Previous Research Attempts

Because of the decline in population accompanied by the demand for horseshoe crabs, alternate testing for endotoxins or forms of producing LAL has become important. Thus far, no other aquatic or land animal is well suited for endotoxin testing; suggesting, alternative methods for LAL production should be attempted. Research was attempted to synthesize LAL from one of the three proteins that neutralize endotoxins; this synthetic peptide is more expensive and less sensitive than LAL making this artificial option unrealistic[8]. Another research project attempted was "The *In Vitro* Cultivation of Limulus Amebocytes" which utilized various medias trying to create a protocol for long term maintenance of hemolymph[5]. Three month cultures of hemolymph with frequent media changes were completed[5]. Culturing of amebocytes via a nutrient mist bioreactor was attempted, but abandoned because of the difficulty of having an endotoxin free environment[10]. The "*In Vitro* Production of Amebocytes from Tachypleus Gigas in Leibovitz Culture Medium", patent outlines a procedure used on the Indian Horseshoe Crab which cultures the gill flaps for up to 90 days, at time of publication[11]. The successful cultures via the Indian Horseshoe Crab have allowed for a means to culture the

gills and harvest amebocytes in the American Horseshoe Crab, which would allow for producing LAL without the frequent harvesting of crabs.

Since 2006 two students from Rose-Hulman Institute of Technology have performed original research and published theses regarding the American Horseshoe Crab. Amber Brannan generated a detailed protocol for creating an endotoxin free environment and successfully produced cultures of the gill lamellae for ten weeks[12]. Kirk Thompson built off of Amber Brannan's work and developed a protocol for staining amebocytes to test for the presence of amebocytes in the cultures of gill lamellae. His research provided photographs and descriptions that suggested the presence of amebocytes in cultures 22 days old[13]. Brannan and Thompson's research both suggested that amebocytes were present in the cultures but neither were able to confirm them. The primary objective of this research is to identify characteristics of amebocytes present in hemolymph for comparison to amebocytes grown in *in-vitro* cultures. To create a profile of the amebocytes, the following three criteria were taken into account: changes in hemolymph volume following large hemolymph removals, average amebocyte density, and average amebocyte diameter.

2 RESEARCH OBJECTIVES

2.1 Establishment of Baseline Data

The horseshoe crabs' baseline information is obtained for comparison to animals under various conditions and stresses. The literature suggests the average sized animal has amebocytes averaging 15-20 μm, but the literature does not provide any information on the possible amebocyte density in an average animal[4]. These baseline data give an accurate representation of the weight, amebocyte density, and amebocyte diameter of each animal under normal conditions.

2.2 Large Hemolymph Removals

After the collection and subsequent profile development of amebocytes for each test animal, a series of large blood removals can be performed. The literature suggests that each animal can be safely bled 50 +/- 13 % of their total hemolymph volume[14]. By removing a significant amount of hemolymph it can be determined how these bleeds affect the amebocyte density and size, as well as how long it takes for each parameter to return to its baseline levels.

2.2.1 Weight, Amebocyte Density, and Amebocyte Diameter Recovery

Current literature suggests several methods for estimating the volume of hemolymph in each animal. One of these possible methods uses the wet weight of the animal to estimate the hemolymph volume. By monitoring the weights of the animals

before and after large hemolymph removals the volume of hemolymph in the animal can be estimated, giving a mode for monitoring the hemolymph recovery of the animal.

While monitoring the recovery of the animal's hemolymph volume, the amebocyte density can be calculated by taking small amounts of hemolymph at designated intervals providing insight into changes in amebocyte density.

The small hemolymph removals also allow for profiles of the amebocyte diameter to be made. The amebocyte profiles in relation to the change in amebocyte density will provide information on when new amebocytes are being added to the blood, as well as how the size of these amebocytes compare to their average size. By monitoring all three criteria simultaneously a better understanding of how the amebocytes behave in the hemolymph will be established which provides a reference with which to compare amebocytes cultured *in-vitro*.

3 METHODOLOGY

Both investigators, Amber and Kirk, worked on a protocol for gill lamellae culturing and were confronted by the issue of how to confirm the growth of new amebocytes. This challenge was the motivation for this research which delves into the amebocytes naturally present in the *Limulus Polyhemus* hemolymph. *The Effect of Hemolymph Extraction Volume and Handling Stress on Horseshoe Crab Mortality* by Lenka Hurton provided useful insight into determining the total hemolymph volume in each animal. *The American Horseshoe Crab* by Jack Levin also provided information in the form of a possible mode for slowing degranulation of amebocytes. Through the guidance of these and other works, I was able to establish a conceptual basis and an understanding of the complexity of the research. The remainder of this section outlines the reasoning and actions necessary for completion of the research.

3.1 Tank and Water Maintenance

After ordering and the arrival of ten horseshoe crabs from the Marine Biological Laboratory (Woods Hole, MA) all packing material was removed from the surface of the animals, and they were placed in a 75 gallon salt water sand bottom tank. The water in the tank was kept at a specific gravity between 1.020 and 1.024, by testing it weekly with a hydrometer, and 21-27 °C. An aerator, filter, and fecal matter remover were also present to provide sufficient oxygen and maintain a clean environment. Three gallons of deionized water were added about once a week or as the water levels decreased and Instant Ocean® was added when the salinity dropped below the ideal range. Every 10-12 weeks ten gallons of water was changed and the salinity was corrected as necessary.

3.2 Feeding the Animals

After monitoring the weight of the animals and consulting with Shannon Tieken regarding proper animal care procedures, the animals were placed on a two week feeding cycle; this schedule minimized disturbances to the data collection process. Frozen seafood was defrosted in deionized water and fed to the animals outside of the tank in order to preserve the cleanliness of the water. One at a time the animals were removed from the tank, laid on their backs, and a piece of food was placed in their mouth. If the animals finished the piece of food they were given another. After giving them sufficient time to eat they were returned to the tank.

3.3 Distinguishing Animals from One Another

When ordering horseshoe crabs, the males are shipped more often because of their smaller size compared to the females. Since all of the animals were of similar size, a mechanism for identifying each animal was necessary. A commonly used technique calls for boring holes using a small bit drill into the carapace of the animals. Each of the ten animals were assigned a specific number of holes in a unique pattern or area to insure the animals could be distinguished from one another. The holes were drilled in areas of the animal's carapace that would not cause pain or infection.

Figure 1. Schematic of a horseshoe crab; red dots correspond to locations where holes can be drilled to differentiate the animals.

3.4 Endotoxin Removal and Sterile Techniques

An endotoxin free environment is essential to work with hemolymph and subsequent amebocytes from the *Limulus polyhemus*. The sensitivity of the amebocytes causes them to begin clotting as soon as they come in contact with a non-sterile surface or the air, which is contaminated with endotoxins. Amber Brannan provided a set of protocols for rendering an endotoxin free environment which were slightly adapted for this research[12].

3.4.1 Endotoxin Removal from Storage Vials

Amber Brannan originated this method through the aid of a Sigma E-TOXATE kit for the preparation of her metal and glass equipment. After careful assessment of all equipment necessary it was determined that the majority could be purchased in individually wrapped sterile containers, with the exception of glass storages vials. A large quantity of 8 mL and a few 100 mL glass vials would be used to store collected blood and pre-made solutions. These vials were expensive to purchase separately, so Amber Brannan's procedure was used for the sterilization of glass. A 1% alkaline detergent solution was made using Alconox Liqui-Nox and deionized water in a 2-gallon polypropylene lidded bucket. All lids and glass vials were added to this bucket and soaked for a minimum of 24 hours. Wearing nitrile gloves, each vial was removed from the soak solution and rinsed with deionized water. The lids were then removed because the rest of the procedure would damage their rubber lining. The vials were then transferred to sheets of aluminum foil and heated in a hot air oven at 175°C for 10 minutes. Upon removal from the oven they were wrapped in aluminum foil and placed in

an autoclave at 121°C for 1 hour. Once the autoclave cycle was complete they were immediately transported back to the hot air oven for 3 hours. Upon completion of the process the vials were left to cool in the aluminum foil before the lids were tightly screwed on for storage in cardboard boxes.

3.4.2 Treatment of Non-sterile Equipment

Before the data collection process began, it became clear that the blood could not be collected and processed in an endotoxin free environment, but the amount of contamination to the hemolymph needed to be minimized. The hemacytometer used in the data collection process was cleaned before and after every use with deionized water and Kimwipes to eliminate as much contamination as possible. The time between hemolymph removal and data collection was less than three minutes which allowed for sufficient time to photograph the amebocytes before endotoxins induced degranulation.

3.5 Hemolymph Removal Apparatus

A piece of particle board was cut to a width of sixty-one centimeters; the animals were folded in half along their hinge and placed on the board with their eyes facing upward. From here a rubber bungee cord was attached to either side of the board, holding the animals in place. The size of the board and the bungee cord

Figure 2. Particle board and rubber strap used to hold the animals in place during bleeding.

assured the animals were secure enough to restrict their movement but not so secure as to injure the animals. This allowed for the investigator to draw the hemolymph without the assistance of another person holding the animal in place.

3.6 Pre-Hemolymph Removal Animal Preparation

Pre-hemolymph removal protocols were established to estimate the volume of hemolymph removed and ensure animal health. Prior to bleeding, each animal was weighed in a bucket on a scale. The animal's wet weight was recorded. To ensure water on their bodies was not significantly affecting their weight they were allowed to drip until all excess water was removed. These weights were recorded for hemolymph volume estimates; which provided insight into each animal's health, eating habits, and hemolymph recovery after large hemolymph removals. Animal weights ranged from five hundred to eight hundred grams. Hemolymph removal utilized a syringe puncturing the soft tissue of the hinge, much like blood removal from a human, which means a puncture remains after the syringe is removed. For the safety of the animals, the soft tissue and surrounding shell was cleaned with Kimwipes and a 95% alcohol blend. This procedure minimized puncture site infections promoting better overall health of the animals.

3.7 Hemolymph Removal

For the safety of the animals, sterile techniques and equipment were necessary. These techniques also assured the best results for hemolymph collection. Since there was little literature providing insight into a hemolymph removal protocol, a series of test

hemolymph removals was performed. The following protocols minimized the amount of damage done to the animal's soft tissue without compromising the hemolymph samples.

3.7.1 Large Hemolymph Removals

The literature suggested the following equation for estimating the total hemolymph volume in an animal of a specified wet weight:

$V = 257.2 * w - 5.693$ where V = volume (ml) w = weight (kg)[14].

Once the total hemolymph volume was determined, the appropriate percentage to be removed was calculated. In the case of the large bleeds, an 18 gauge needle was used without a syringe. The needle was inserted through the soft tissue of the animal into the tubular heart where the beating of the animal's heart caused the hemolymph to flow freely into a collection vial. This method provided less stress on the animal by eliminating the vacuum pressure which is associated with using a syringe. After hemolymph removal the animals were weighed again along with the container of hemolymph to estimate the volume of hemolymph removed. The animals were then immediately returned to their tank for recovery.

Figure 3. Horseshoe crab folded at the hinge exposing an area of soft tissue which leads to the tubular heart.

3.7.2 Small Hemolymph Removals

The small hemolymph removals consisted of removing 1 mL of hemolymph originally, but after initial testing the removal of 0.5 mL of hemolymph proved to be sufficient for analysis purposes. The 0.5 mL of hemolymph removal amounts to between

0.25 and 0.5% of the animal's total hemolymph volume. Similar to the large hemolymph removals, the animals were pierced with a needle through their soft tissue into their tubular heart and were returned to the tank immediately after hemolymph removal. The small hemolymph removals differed by using a 26 gauge needle which created a smaller puncture hole while still allowing for the amebocytes to be collected. The small collections also utilized a syringe which allowed for a precise volume of hemolymph to be collected. Originally when 1 mL of hemolymph was being collected, it was separated into two sterile glass vials, one in which an equal amount of the anti-clotting solution was present. These bleeds were continued for ninety days to monitor the change in density and diameter of the amebocytes.

Table 1. Over the duration of the bleeding series, the number of bleeds per week was decreased to minimize the number of puncture wounds on the animal while allowing for sufficient data collections.

Week	Number of bleeds per week
1	13
2	5
3-9	3
9-12	2

3.8 Photographic Documentation of Amebocytes

Since the amebocytes present in hemolymph samples are susceptible to degranulation in a short amount of time, it was necessary to find a way to collect data before the amebocytes degranulated. The DinoCapture 2.0 system was used, which consisted of a camera inserted into the eyepiece of a standard microscope connected to a computer. The camera allowed for pictures to be taken of the amebocytes on glass slides or on hemacytometers for later analysis.

Immediately after hemolymph removals, the animals were returned to their tank and the vial or syringe was taken to the microscope for analysis. Here a hemacytometer

was filled on both sides via the syringe or a sterile micropipette. The hemacytometer was placed on the microscope stage and pictures were captured at optical magnifications of 5X, 10X, and 40X with the eye piece adding 10X magnification. Each set of pictures captured at least fifty amebocytes for adequate sample size and both sides of the hemacytometer provided a self check if a data set showed an anomaly. A series of time lapse photographs and videos were taken to observe the process of degranulation.

3.9 Data Collection

Photographs were used to estimate the amebocyte density per milliliter of hemolymph as well as to observe the amebocyte diameter before and after large hemolymph collections. The videos and time lapse photography were taken when the protocol was initially being developed at one or five minute intervals for thirty minutes to two hours to observe the rate and process of degranulation. These time lapse data provided information about how long hemolymph could be exposed to endotoxins before degranulation of amebocytes occurred appreciably.

3.9.1 Amebocyte Density per Milliliter of Hemolymph

The hemacytometers used were comprised of nine square 1.0 mm^2 boxes; within the center one mm box was twenty five 0.4 mm^2 boxes. Theses 0.4 mm^2 boxes were further subdivided into sixteen 0.0025 mm^2 boxes. The depth of fluid held by hemacytometer was 0.1 mm. When using the 10X magnification on the microscope, six of the 0.4 mm^2 were visible and all amebocytes within this area could be counted. A random number generator was used to select six of the 0.4 mm^2 boxes in which all

amebocytes were counted. Amebocyte densities were calculated using the following equation:

$$\frac{Amebocytes}{ml} = \#of\ cells\ counted\ \times (25 \times 10 \times 1000)$$

Cell counts are generally performed by counting all cells in one 1.0 mm^2 box, this number is then multiplied by 10 to find the number of cells/mm^3 and then by 1000 to find cells per ml. In this case only six of the 0.4 mm^2 boxes were counted; therefore, the counted amebocytes were not only subjected to the normal protocol but also multiplied by twenty-five since there are twenty-five 0.4 mm^2 boxes within a 1.0 mm^2 box. Since six boxes were counted and subjected to the same method for determining amebocytes/ml the average was taken and used as the value for that sample.

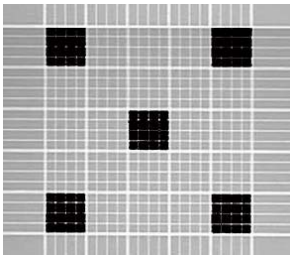

Figure 4. Hemacytometer showing the 0.4 mm^2 boxes, five which are blackened, with 16 smaller boxes within each.

3.9.2 Amebocyte Diameter Profiles

After further exploration into the DinoCapture 2.0 program a measurement feature was found; this feature allowed for three point circles to be made around fifty amebocytes and then the program would calculate the diameter. DinoCapture 2.0 utilized an input of the magnification along with the measurement of an already known area, which in this case was the outline of a box from the hemacytometer, to convert the measurement to actual diameter. This data was then transferred directly to Microsoft Excel where further calculations were performed and compared to other data collections.

3.10 Statistical Analysis

After the data collection and processing a series of statistical tests can be performed to better understand the trends being observed. Unstacked one-way ANOVA tests were used for comparing days of a hemolymph removal series to another and for the comparison between different animals. If statistically significant variation exists within or between animals, post-hoc testing was performed using Tukey grouping. This post-hoc test organizes the data in groups that are statistically similar. Similar Tukey groups are identical using a letter scheme, meaning that a series designated as “group A” are similar to one another but dissimilar to those in “group B”. Some data points may be in overlapping groups. When comparing day data points after the large hemolymph removal to the baseline data, one-sample t-tests with a hypothesis were used to test for statistically significant deviation from the baseline mean.

4 RESULTS

4.1 Analysis of Baseline Data

The baseline data collected over two weeks provided the mean weight, amebocyte density, and amebocyte diameter for the eight animals as summarized in Table 2 Four separate bleeds were conducted on different days and each bleed was compared to determine the sampling error. For each animal, the weight varied no more than two percent from the mean for any given day. Figure 5 shows the animal with the least variation between days and the animal with the most variation between days for both amebocyte density and diameter. Data for the remaining animals can be found in Appendix B. When comparing between animals, there is up to 60% variation for the density and up to 19% variation for the diameter (Table 7,12).

Table 2. Baseline data collected from eight animals over a two week period providing insight into the animals average amebocyte diameter, density, and hemolymph volume prior to treatments. Standard deviations are given in parenthesis.

Animal	**Weight(g)**	**Amebocyte density (amebocytes x 10^7/ml)**	**Amebocyte diameter(μm)**
1	524(7.2)	1.98(0.4)	11.02(1.54)
2	536(3.5)	2.34(0.6)	10.48(1.69)
3	539(4.2)	1.09(0.3)	9.55(1.84)
4	577(7.1)	1.71(0.5)	11.52(1.52)
5	635(6.9)	1.81(0.3)	11.68(1.81)
6	657(3.0)	1.51(0.5)	9.49(1.58)
7	676(7.1)	2.85(0.7)	9.85(1.77)
8	782(5.5)	2.25(0.4)	10.86(1.75)
Average	616(89.1)	1.93(0.7)	10.53(1.87)

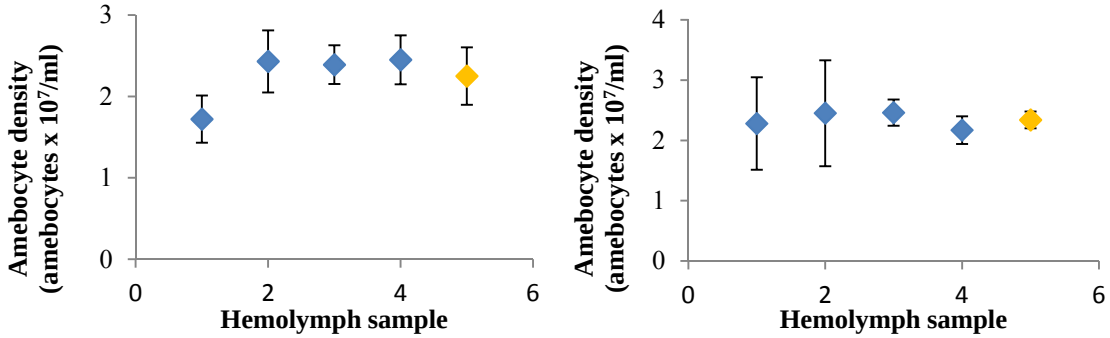

Figure 5. The yellow point indicates the mean generated from all data collected in the baseline series. (Left) The first bleed in the baseline data for Animal 8 has a significantly different ($p \leq 0.05$) density then the remaining three hemolymph draws. (Right) Animal 2's amebocyte density for all four days are statistically similar ($p = 0.59$) showing little variation between the four bleeds making up the baseline density value.

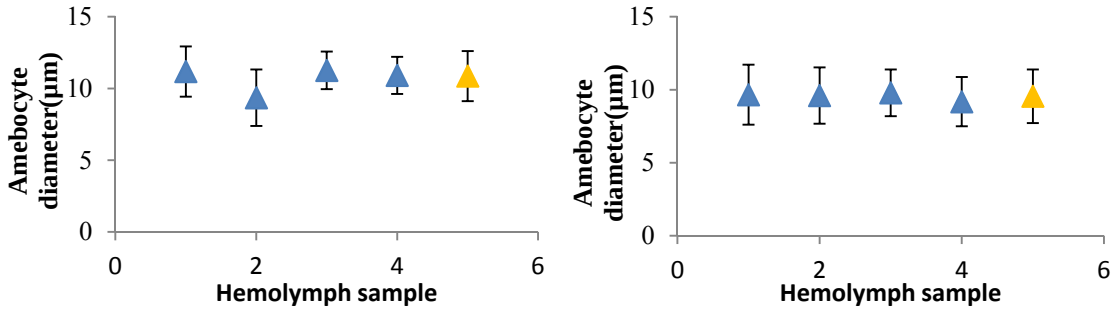

Figure 6. (Left) Animal 8's amebocyte diameter over the four bleeds in the baseline the second day was statistically different ($p \leq 0.05$) than the others. (Right) Animal 3's amebocyte diameters in four separate bleeds are statistically similar to one another ($p = 0.101$).

4.2 Large Hemolymph Removals

4.2.1 Weight

Immediately following the large bleeds, the animals were weighed along with their respective vials of collected hemolymph. The mass lost and the mass of the hemolymph removed are recorded in Table 3 which illustrates the variability between the two values. Additionally, animals were weighed every subsequent hemolymph removal to monitor the rate of hemolymph volume recovery. Figure 7 shows each animal's weight

decreased between two and nine percent of the animal's baseline weight after the initial bleed and recovered to within two percent of the baseline mean by eight days into the series. The weight of each animal after recovering stabilizes to within two percent of the baseline for the remainder of the experiment.

Table 3. The difference in wet weights for each animal before and after the large hemolymph removal approximately corresponds to the amount of hemolymph removed.

Animal	Weight Change (g)	Hemolymph Removed (g)	Hemolymph Removed(ml)
1	34	51	58
2	62	51	53
3	62	57	70
4	90	68	74
5	80	68	63
6	74	68	67

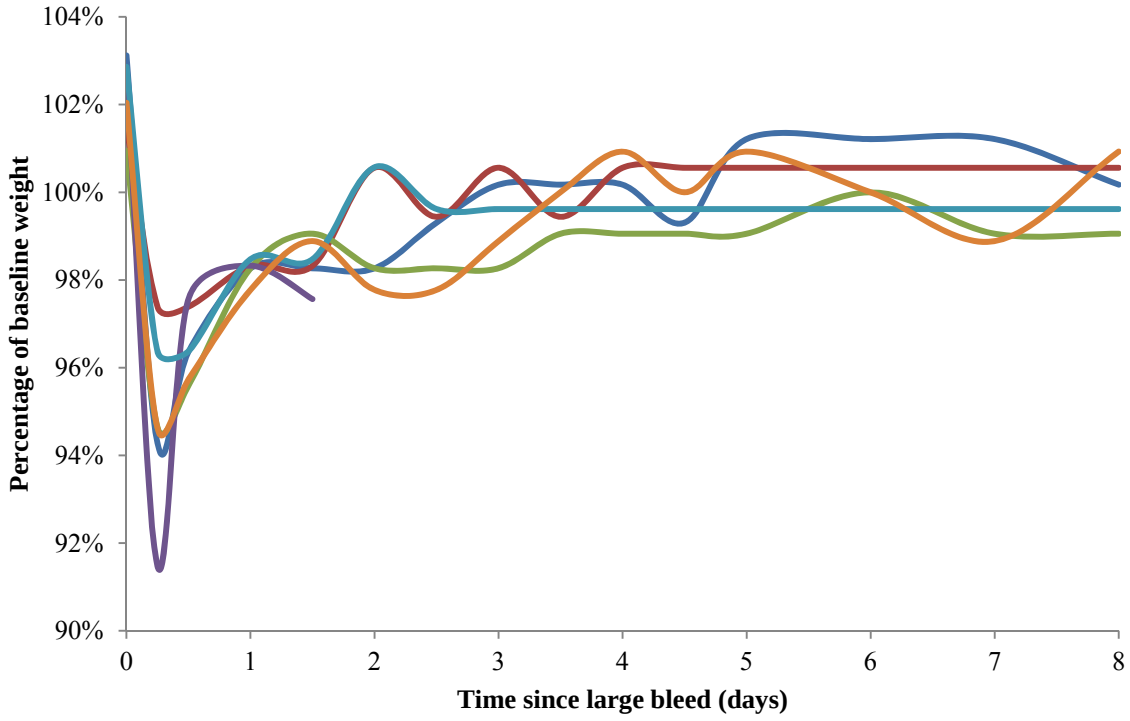

Figure 7. All six animals experienced a significant drop in their weight after the initial large bleed but saw an increase and return to withing five precent of their baseline weight within eight days.

4.2.2 Amebocyte Density

For each subsequent hemolymph draw the density was calculated showing a common pattern between five of the six animals. [The sixth animal died of unknown causes the day after the large hemolymph removal.] The pattern observed in the five animals was a significant decrease in density within the first week of the series with p-values ≤ 0.05. The density returned to the baseline mean within three weeks of the initial decrease with p-values ≥ 0.05. A second decrease was seen in all five animals, with unknown causation.

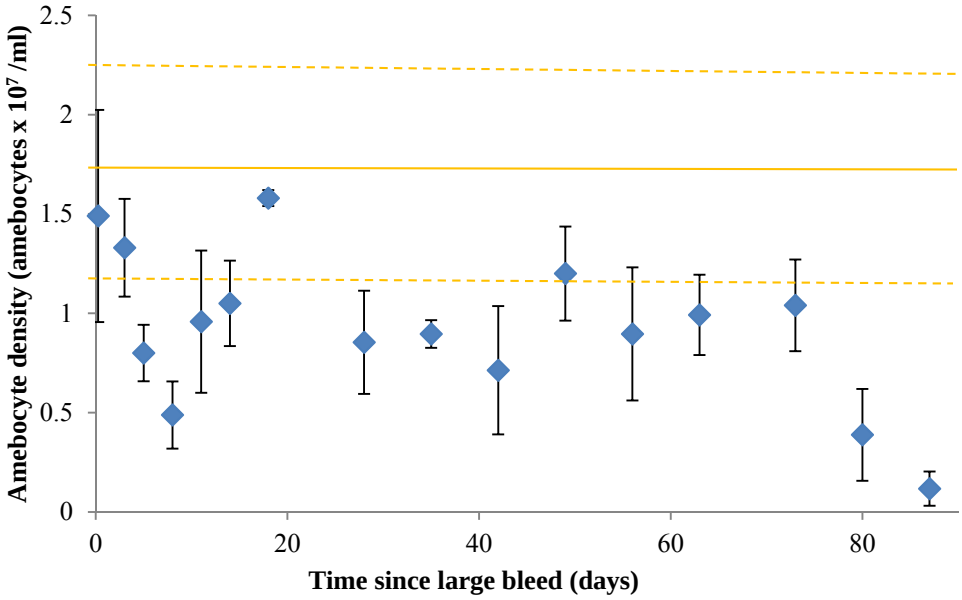

Figure 8. The mean and standard deviation of the amebocyte density data for Animal 4 suggests the density dropped below the baseline value at day three and returned to the mean baseline at day eighteen.

Table 4. Animal 4's mean amebocyte density decreases below the baseline on day three and the mean density returns to the baseline average at day eighteen with p-values ≤ 0.05 confirming a statistically significant change. N = 6 for each day.

Time (days)	Mean Density (amebocytes x 10^7/ml)	StDev	SE Mean	P
Baseline	1.71E+07	5.41E+06	9.02E+05	1.00
3	5.29E+06	3.03E+06	1.24E+06	0.00
5	8.00E+06	1.69E+06	6.89E+05	0.00
8	4.88E+06	3.58E+06	1.46E+06	0.00
11	9.58E+06	2.15E+06	8.80E+05	0.00
14	1.05E+07	4.01E+05	1.64E+05	0.00
18	1.58E+07	2.60E+06	1.06E+06	0.26

4.2.3 Amebocyte Diameter

The pattern seen in Animal 4's amebocyte profile (Figure 11) is representative of three of the other animals whose amebocyte profiles are included in Appendix B. Animal 4 shows a change in diameter distribution three days after the large hemolymph removal, with a wider range of diameters and a smaller mean (p-values ≤ 0.05). The next five samples also show a larger range in distribution and a smaller mean, but they begin an upward trend toward the baseline mean value. On day fourteen, the mean diameter returns to the mean baseline value with p-values ≥ 0.05.

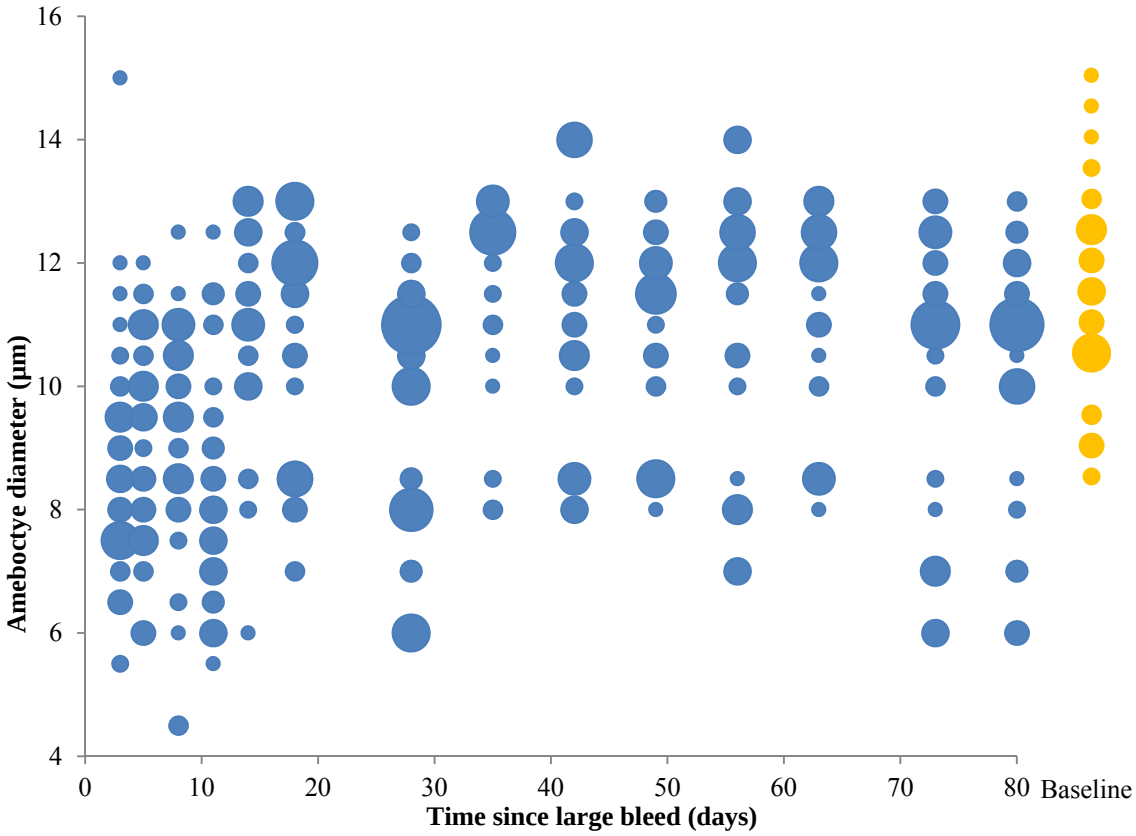

Figure 9. After the initial large bleed, Animal 4's mean amebocyte diameter decreases below the baseline mean at day three and returns to the baseline mean at day fourteen.

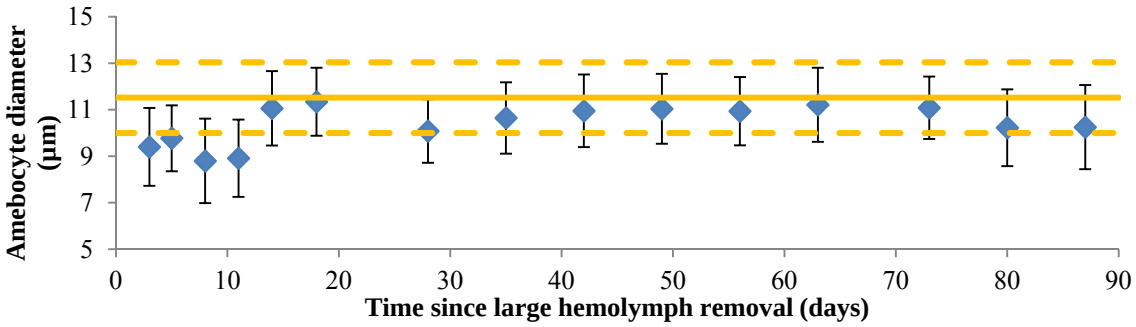

Figure 10. Animal 4's amebocyte diameter decreases below the baseline mean at day three followed by an increase back to the mean at day fourteen. The diameter remains statistically similar size to the baseline mean for the remainder of the experiment ($p \geq 0.05$).

Table 5. After the initial large bleed, Animal 4's mean amebocyte diameter decreases below the baseline mean at day three and returns to the baseline mean at day fourteen with corresponding $p \leq 0.05$ showing a statistically different mean diameter.

Time (days)	N	Mean Diameter (µm)	StDev	SE Mean	P
Baseline	300	11.52	1.52	0.09	1.00
3	100	9.40	1.68	0.17	0.00
5	51	9.77	1.42	0.20	0.00
8	51	8.80	1.82	0.25	0.00
11	51	8.91	1.66	0.23	0.00
14	51	11.06	1.60	0.22	0.05
18	51	11.34	1.46	0.21	0.39

4.3 Amebocyte Viability Time Frame

The time lapse photographs and videos of the amebocytes taken from multiple samples on separate days provided an idea of the rate of amebocyte degranulation. The photographs, which were taken between 45 seconds and 90 seconds after hemolymph removal, showed minimal amebocyte degranulation beginning immediately after initial viewing and being fully degranulated after about an hour. The videos showed the amebocytes moving through the hemolymph towards one another and creating large clots of degranulated amebocytes. These data suggest that as long as photographs are collected within three minutes after exposure to air, then no significant degranulation will have occurred yet.

5 DISCUSSION

5.1 Amebocyte Density and Size

The baseline analysis of amebocyte density and size showed statistically significant variability between certain days for all of the animals studied. The day in which the amebocyte density varied was not always the same day in which the amebocyte diameter differed. Because the variation in amebocyte density and size is not occurring on the same day, suggests some sampling error may be occurring or some uncontrolled mechanism within the animal may be present. If a sample began clotting or the amebocytes were not evenly distributed across the hemacytometer then the data will be skewed. Clotting may have been induced at different rates if the needles, syringes, and hemacytometers had differing amounts of endotoxins present. The variation in amebocyte densities within animals also may be attributed to the natural fluctuation in the life cycle of amebocytes in the hemolymph. Draws may have been taken on days where amebocytes were being released at higher quantities, skewing the density in the upper direction. The animals also may have experienced encounters in the tank that caused injury or stress, thereby decreasing the amebocyte density.

Since the change in amebocyte diameter did not occur on the same day as the density changes, the difference in diameters may be attributed to a number of different complications or unknown animal mechanisms. The computer program used to calculate diameter used discrete sizes, which explains the gaps in the amebocyte diameter data seen in Figure 9 (between eight and ten μm). Aside from the computer program, the amebocytes may not be evenly distributed by size in the hemolymph. For instance, the

smaller amebocytes may remain closer to the gill lamellae for longer amounts of time or the larger amebocytes may move slower causing them to congregate in certain areas. The hemolymph was drawn directly from the tubular heart in attempt to best capture a range of amebocytes in the circulating hemolymph. Although one day of the baseline data may have varied from the other three, the aggregate data provide a representation of amebocyte density and size. Because of the variation within and between the animal's future researchers should be cautious when interpreting the data from a single draw; instead taking multiple samples for one single day or looking at trends over time would be preferable given this significant variation.

5.2 Analysis of the Animals after Large Hemolymph Removals

5.2.1 Weight Recovery

After the initial large hemolymph removal, the weight of the animals dropped immediately. However, recovery was exhibited within hours, suggested by the increase in weight three and six hours after the large hemolymph removal. This immediate recovery likely occurred because the animal's sequestered water from the tank to replace the hemolymph volume removed. This water sequestration is common in many aquatic animals, allowing for the organisms to regulate their volume and ion concentrations. The animals all saw a complete recovery of their weight within two weeks. The normal feeding schedule was not disrupted by the large hemolymph removal because the animal's weights recovered prior to the scheduled feeding, and thus, feeding is not the

reason for the return to baseline weight. Throughout the remainder of the experiment the animal's weights fluctuated up to ten grams in either direction, most likely because of the animal's metabolism.

The difference between the weight of the animals before and after the large hemolymph removal was compared to the weight of the hemolymph removed. Although in each case the value in grams varied, the discrepancy can partially be explained by the presence of water on the animals. The equation used to estimate the hemolymph volume of the animals took into account their wet weight, but the process of weighing, bleeding, and reweighing each animal spanned ten to fifteen minutes. This time outside of the water was sufficient to allow the salt water to drip off the animals or evaporate, slightly altering its weight. For a more accurate reading of the change in weights, the animal could be left out of the water for ten minutes before the initial weighing. Although there was a difference in the weight of the hemolymph removed and the weight change in the animal, the hemolymph volume equation is still a reliable method for estimation. Also noteworthy, the grams and milliliters of hemolymph removed show a near one-to-one relationship explained by a significant portion of the hemolymph being water (which also has a one to one ratio between milliliters and grams). By weighing the animals and using the equation that relates animal weight to hemolymph volume, the hemolymph volume can be estimated. Thus, hemolymph recovery from water sequestration after large hemolymph removals can be monitored.

5.2.2 Amebocyte Density Recovery

Assuming these aquatic animals take in water from their environment to compensate for the large hemolymph loss, a decrease in amebocyte density is expected when animal weight first begins to recover. The same trend in weight increase and amebocyte density decrease was seen in all five of the surviving animals. As shown in Figure 11, Animal 4's weight returned to normal the same time the amebocyte density began dropping, ultimately to forty percent of the baseline value. The decrease in amebocyte density is mostly likely due to the animal replacing its lost hemolymph with water; which explains both why the animal's weight recovered as well as why amebocyte density decreased. After the amebocyte density bottomed out at forty percent of the baseline value it increased over a two week period to a statistically similar amebocyte density within ten percent of the baseline value. This increase in amebocyte density may have been caused by the gill lamellae generating new amebocytes and releasing them in the hemolymph.

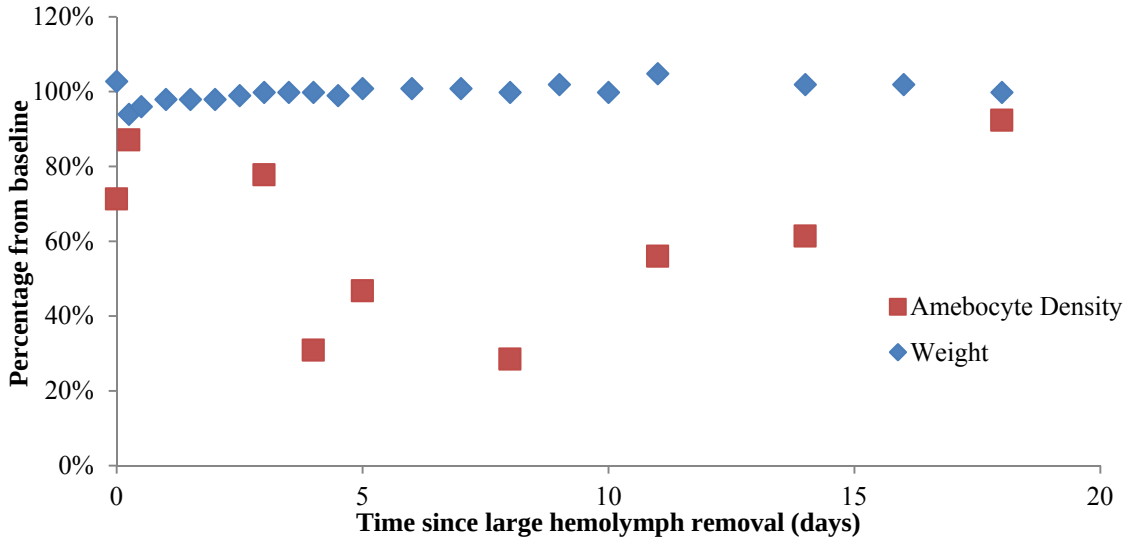

Figure 11. Percentage of animal weight and amebocyte density (from the baseline) following the large hemolymph removal shows the amebocyte density began to decrease in Animal 4 at the same time that its weight recovered.

The animals that died throughout the series showed a steady decrease in amebocyte density two weeks prior to death, which suggests that amebocyte density may provide a measure of the animals overall health. The cause of death for each animal that died during the series is unknown, especially since animals who were not subjected to large hemolymph removals or the follow up series also died. Their deaths suggest something other than the hemolymph removals was playing a factor. The large hemolymph removals may have played a role in lowering the immune system of the animals causing them to die. However, since the animals died at various times, there is no way of knowing their exact cause of death. This study suggests that after a large hemolymph removal the amebocyte density will recover to within ten percent of the baseline value within three weeks, suggesting gill lamellae being cultured should be monitored for amebocyte growth two to four weeks after culture initiation.

5.2.3 Amebocyte Diameter Recovery

The increase in amebocyte density observed and discussed above can be explained two ways: the hemolymph sans amebocytes was being removed from the animals or the gill lamellae were releasing new amebocytes into the hemolymph system. Evidence for the second reason is proposed when looking at the amebocyte profile over time, specifically the diameter. If the amebocyte density was increasing because of the production of new amebocytes, a larger proportion of smaller amebocytes should be present, which is consistent with what is observed. The literature suggests that new amebocytes are smaller than fully mature and degranulating amebocytes.[15, 16] Following

this decrease in diameter, the mean diameter returned to its baseline within a week, possibly suggesting the new amebocytes matured and grew in the hemolymph.

Large hemolymph removals caused a statistically significant drop in the amebocyte density, but amebocytes still remained in the hemolymph after 40-50% of the hemolymph was removed. These remaining amebocytes mask the change in mean diameter as new amebocytes are released. This challenge was compensated for by not only looking at the mean diameter, but also by observing the distribution of amebocyte diameters in each subsequent hemolymph draw. When the mean diameter shifted, the distribution of amebocyte diameters also changed from that seen in the baseline data. Figure 12 shows this trend on days three through eighteen, with a change in the distribution of diameters from the baseline correlating with the same time as the density changes. The change in amebocyte density, lowered mean diameter, and change in the distribution of the amebocyte diameters suggests noticeable amounts of new amebocytes were being released into the hemolymph. As the amebocyte density was decreasing the gill lamellae were releasing amebocytes into the hemolymph at the same rate as when the animals are healthy. Because of this release of amebocytes and the decreased density of mature amebocytes the diameter drops below the baseline mean as the density is decreasing. As the density began increasing the mean diameter decreased further, from ~9 μm to ~8 μm, also showing the production of new amebocytes. When the animal experiences a large decrease in amebocyte density, the gill lamellae may be up-regulating amebocyte production to compensate. Over the remainder of the experiment, the amebocyte profiles may have revealed a steady-state between the rate of amebocyte renewal and death; evidenced by the similar mean diameter and distribution. When

looking at gill lamellae cultures two to three weeks after initiation, new amebocytes will have an average diameter between six and ten µm. After initial confirmation of amebocyte production, the amebocytes will begin growing and maturing reaching an average size of ten to fifteen µm around day twenty for animals between 500-800 grams.

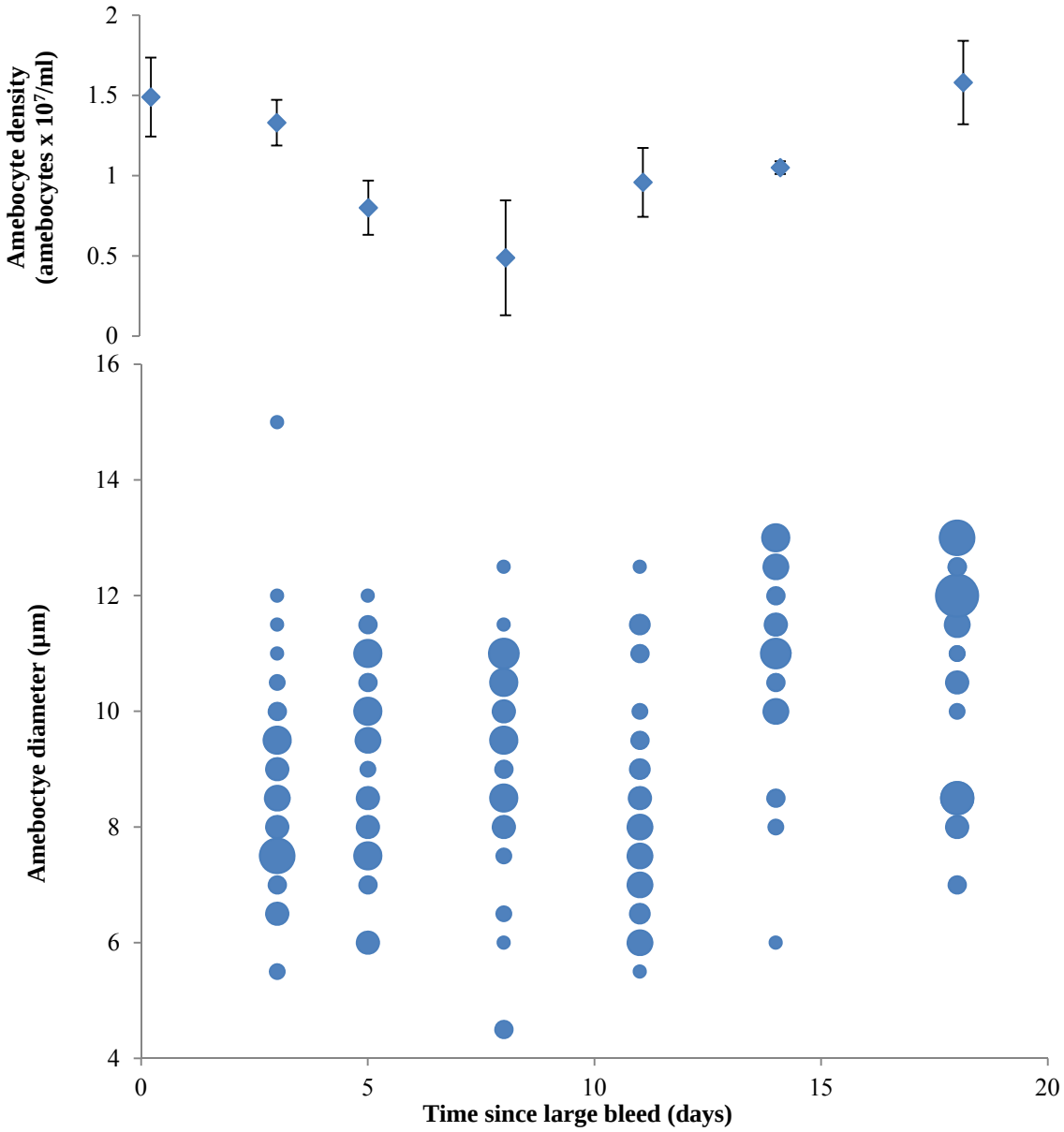

Figure 12. Animal 4's amebocyte profile, show a correlation between the recovery of amebocyte density and a shift in the discrete profiles toward smaller diameter amebocytes.

6 FUTURE RESEARCH

6.1 Replication of Current Methods

To further support the data from this research, using more replicates is necessary. Additionally, for this research, all but one animal was male, which may misrepresent the population as a whole. Because males were used in this study, there is no way of knowing if some variation between the sexes exists with respect to: amebocyte density, diameter, and regeneration rate. This study used matched sizing of smaller animals potentially limiting some variability in the data. Therefore, a larger range of animal size should be utilized since it may result in a different range of amebocyte diameters.

6.2 Sensitivity Testing

After providing a profile for the amebocyte production in the American Horseshoe Crab, subsequent quantification of amebocyte sensitivity would provide a means of comparing the sensitivity of cultured amebocytes to those harvested from the hemolymph. A protocol outlining the creation of endotoxin standards via dilutions of E. coli should be established. These endotoxin standards can then be used find the average clotting time for amebocytes in the presence of various endotoxin concentrations. Upon finding baseline sensitivities, a series of large bleeds should be performed and the hemolymph should be tested for changes in sensitivity as the amebocyte density decreases and is repopulated by new amebocytes. This understanding of amebocyte sensitivities will provide a means to test whether amebocytes grown *in-vitro* can be as or more effective at producing LAL than those harvested from the hemolymph.

6.3 Comparison to Amebocytes Grown *In-Vitro*

Upon successful completion of the two previous aims, a series of gill lamellae cultures can be grown and observed for amebocyte production. After successful growth, they can be tested for comparable sensitivity to amebocytes produced in a living animal. If the amebocytes from the *in-vitro* cultures are comparable in density per milliliter, diameter, and sensitivity to those in the hemolymph, they may be collected and used for the production of LAL. Upon confirmation of amebocytes usable for LAL production, then a method for upregulating the production of amebocytes can be attempted. In order for *in-vitro* cultures to replace current LAL production methods the *in-vitro* production of amebocytes must produce substantial enough quantities as compared to the levels currently produced by the LAL industry.

7 CONCLUSION

The results from this study show that the amebocyte density and diameter range from 1-3 x 10^7 amebocytes per ml and from nine to twelve μm in diameter for animals in the weight range of 500-800 grams. When forty to fifty percent of the hemolymph is removed, hemolymph volumes recover within twelve days to within two percent of the baseline weight. After twenty-four days the density recovers to within five percent of the baseline value. The amebocyte diameter recovers to within one percent of the baseline value after thirty days following the large hemolymph removal.

LIST OF REFRENCES

[1] Novitsky, Thomas J. "Biomedical Applications of Limulus Amebocyte Lysate." *Biology and Conservation of Horseshoe Crabs*. Ed. John T. Tanacredi, Mark L. Botton, and David R. Smith. New York: Springer, 2009. 315-29. Print.

[2] Williams, Kevin L. "Fever and the Host Response." *Endotoxins: Pyrogens, LAL Testing and Depyrogenation*. Ed. Kevin L. Williams. New York: Informa Healthcare, 2007. Print.

[3] Sargent, William. *Crab Wars: a Tale of Horseshoe Crabs, Bioterrorism, and Human Health*. Hanover: University of New England, 2002. Print.

[4] Armstrong, Peter B. *The American Horseshoe Crab*. Ed. Carl N. Shuster, Robert B. Barlow, and H. Jane. Brockmann. Cambridge, MA: Harvard UP, 2003. 288-309. Print.

[5] "Medical Uses." *The Horseshoe Crab: Natural History, Anatomy, Conservation and Current Research*. Web. 02 Jan. 2011. <http://www.horseshoecrab.org/med/med.html>.

[6] Swan, Benjic Lynn. "A Unique Medict Product (LAL) from the Horseshoe Crab and Monitoring the Delaware Bay Horseshoe Crab Population." *Limulus in the Limelight: a Species 350 Million Years in the Making and in Peril?* Ed. John T. Tanacredi. New York: Kluwer Academic/Plenum, 2001. Print.

[7] "Horseshoe Crab LAL Limulus Amoebocyte Lysate Research." *College of Earth, Ocean, and Environment | The College of Earth, Ocean, and Environment*. Web. 02 Jan. 2011. <http://www.ceoe.udel.edu/horseshoecrab/Research/lal.html>.

[8] Levin, Jack, Donald Hochstein, and Thomas J. Novitsky. *The American Horseshoe Crab*. Ed. Carl N. Shuster, Robert B. Barlow, and H. Jane. Brockmann. Cambridge, MA: Harvard UP, 2003. 309-40. Print.

[9] Hurton, Lenka V., Jim M. Berkson, and Stephen A. Smith. "Selection Of A Standard Culture Medium For Primary Culture Of Limulus Polyphemus Amebocytes." *In Vitro Amebocyteular & Developmental Biology - Animal* 41.10 (2005): 325-29. Print.

[10] Friberg, Jill A., Pamela J. Weathers, and Daniel G. Gibson. "Culture of Amebocytes in a Nutrient Mist Bioreactor." *In Vitro Amebocyteular & Developmental Biology - Animal* 28.3 (1992): 215-17. Print.

[11] Bhonde, Ramesh Ramchandra, and Anil Chatterji. In Vitro Production of Amebocytes From Tachylpleus Gigas in Leibovitz Culture Medium. Council of Scientific and Industrial Research, assignee. Patent 6790659. 28 Mar. 2002. Print.

[12] Brannan, Amber G. *In-Vitro Generation of Amebocytes from the American Horseshoe Crab, Limulus Polyphemus*. Thesis. Rose-Hulman Institute of Technology, 2006. Print.

[13] Thompson, Kirk M. *In-Vitro Generation of Amebocytes from the American Horseshoe Crab, Limulus Polyphemus*. Thesis. Rose-Hulman Institute of Technology, 2010. Print. [3]"Limulus and Endotoxin - Fever." *Marine Biological Laboratory*. Web. 02 Jan. 2011. <http://www.mbl.edu/marine_org/images/animals/Limulus/blood/fever.html>.

[14] Hurton, Lenka, Jem Berkson, and Stephen Smith. "The Effect of Hemolymph Extraction Volume and Handling Stress on Horseshoe Crab Mortality." *Biology and Conservation of Horseshoe Crabs*. Ed. John T. Tanacredi, Mark L. Botton, and David R. Smith. New York: Springer, 2009. 331-46. Print.

[15] Armstrong, Peter A. "Adhesion and Spreading of Limulus Blood Cells on Artifical Surfaces." *Journal of Cell Science* 44 (1980): 243-62. Print.

[16] Coursey, Yvonne, Barbara M. McGee, Nina Ahmad, Nancy Steimel, and Mary Kimble. "Amebocyte Production Begins at Stage 18 During Embryogenesis in Limulus Polyphemus, the American Horsheshoe Crab." *Biological Bulletin* 204 (2003): 21-27. Print.

APPENDIX

A Additional Methods

A.1 Preparation of Solutions

The preparation of an anti-clotting agent was attempted to prolong the storage period of the hemolymph sample. Through study of the literature and industry standards it was suggested that the presence N-ethylmaleimide would aid in preventing amebocyte degranulation. After purchasing the N-ethylmaleimide it was mixed with deionized water and sodium chloride to achieve a solution with .125% N-ethylmaleimide and 3% sodium chloride. Hydrochloric acid and additional sodium chloride was used when necessary to make the solution have a pH of 7.4, equivalent to that of the hemolymph. The solution was made in 100 mL quantities and stored in a previously sterilized Wheaton bottle. Initial testing showed an allotment of up to an hour of expanded storage life. As time passed, the solution lost effectiveness; even when the solution was made again, there was little to no difference in clotting speeds with and without the solution. Importantly, use of the anti-clotting chemical was discontinued because the chemical caused the amebocytes to swell, skewing the amebocyte profiles.

A.3 Alternate Methods for Estimating Amebocyte Diameter Profiles

Since there was no industry standard for determining the average amebocyte diameter in each animal, three separate methods were attempted. The first method

utilized a technique suggested by Dr. Ella Ingram which required the use of photographs taken under 400X magnification. Enough pictures to view fifty amebocytes were printed, and individual amebocytes were cut out using a scalpel. Each amebocyte was then measured and weighed. A series of conversion factors were used to check this method: the ratio of amebocyte weight to a known dimension of weighed paper, a ratio between a known size square and the amebocyte measurements, and a direct conversion using the magnifications were all used. Although this is a well established method other proved to be less time consuming.

The second method utilized the Paint program on a laptop and the 100X magnified pictures. The pictures were then magnified to 800% and a grid was added to the pictures. For fifty amebocytes the diameter was measured by counting the number of grid boxes from one side of the amebocyte to the other. The diameter was corrected for by using a ratio of the known and measured size of a box from the hemacytometer. This method, although tedious, produced consistent results with the methods used in this study.

B Additional Animal Data

This appendices is a collection of all tables and figures for the additional animals used in the experiment but not directly discussed in the results or discussion. Tables from the statistical analysis of the baseline information for intra and interanimal comparisons are also contained here.

B.1 Amebocyte Density Figures and Statistical Tables

Table 6. Variablity was seen between days of the baseline bleed series suggesting sampling error, unknown variables, or uncontrollable variables occurred during the small bleeds.

Animal	Date	N	Mean (amebocytes x 10^7 /ml)	StDev.	F	P	Tukey Grouping
1		48	1.98	0.45	4.07	0.012	
	8-2	12	1.99	0.33			A, B
	8-4	12	2.26	0.27			A
	8-9	12	1.68	0.58			B
	8-11	12	1.97	0.39			A, B
2		48	2.34	0.60	0.65	0.59	
	8-3	12	2.28	0.77			A
	8-5	12	2.45	0.88			A
	8-10	12	2.46	0.22			A
	8-12	12	2.17	0.23			A
3		48	1.09	0.50	19.13	0.000	
	8-3	12	1.38	0.22			A
	8-5	12	0.93	0.13			B
	8-10	12	0.92	0.16			B
	8-12	12	1.14	0.16			C
4		36	1.71	0.54	33.57	0.000	
	8-2	12	1.40	0.29			A
	8-4						
	8-9	12	2.33	0.43			B
	8-11	12	1.41	0.19			A
5		48	1.81	0.30	11.66	0.000	
	8-2	12	1.50	0.27			A
	8-4	12	1.85	0.30			B
	8-9	12	1.81	0.15			B
	8-11	12	2.05	0.16			B
6		48	1.51	0.54	8.05	0.000	
	8-3	12	1.13	0.45			A
	8-5	12	2.01	0.73			B
	8-10	12	1.44	0.22			A
	8-12	12	1.44	0.15			A
7		42	2.85	0.73	7.18	0.001	
	8-3	12	3.15	0.46			A,B
	8-5	12	2.34	0.17			C
	8-10	6	3.60	0.33			A
	8-12	12	2.68	0.99			B,C
8		48	2.25	0.35	15.96	0.000	
	8-2	12	1.72	0.29			A
	8-4	12	2.43	0.38			B
	8-9	12	2.39	0.24			B
	8-11	12	2.45	0.30			B

Table 7. Comparing the baseline amebocyte density for eight animals shows significant variability between the animals.

Animal	N	Mean (amebocytes x 10^7 /ml)	Std	F	P	Tukey Group
1	48	1.98	0.45			C,D
2	36	2.34	0.60			B
3	48	1.09	0.25			F
4	48	1.71	0.54			D,E
5	48	1.81	0.30			D,E
6	48	1.51	0.54			E
7	48	2.85	0.73			A
8	42	2.25	0.43			B,C
	366	1.93	0.71	54.18	0.000	

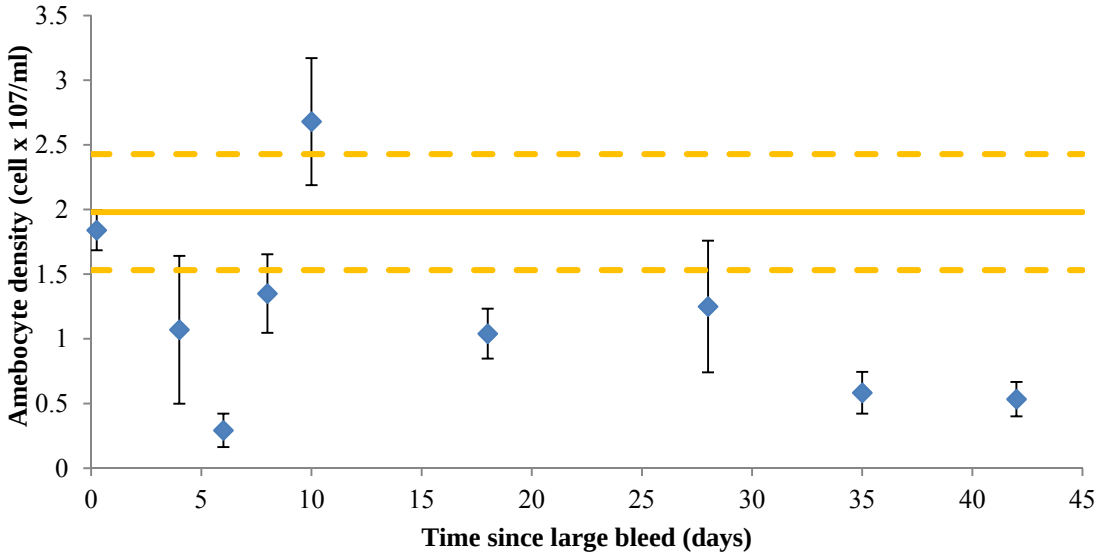

Figure 13. Animal 1 showed an amebocyte density decrease at day four followed by a dramatic increase on day ten.

Table 8. Animal 1's Amebocyte density dropped below average three days after the large hemolymph removal and increased to above average on day ten.

Time (days)	N	Mean Density (amebocytes x 10^7 /ml)	StDev	SE Mean	P
Baseline	48	1.98E+07	4.49E+06	6.48E+05	1.00
4	6	1.07E+07	5.72E+06	2.33E+06	0.01
6	6	2.92E+06	1.29E+06	5.27E+05	0.00
8	6	1.35E+07	3.04E+06	1.24E+06	0.00
10	6	2.68E+07	4.91E+06	2.00E+06	0.02

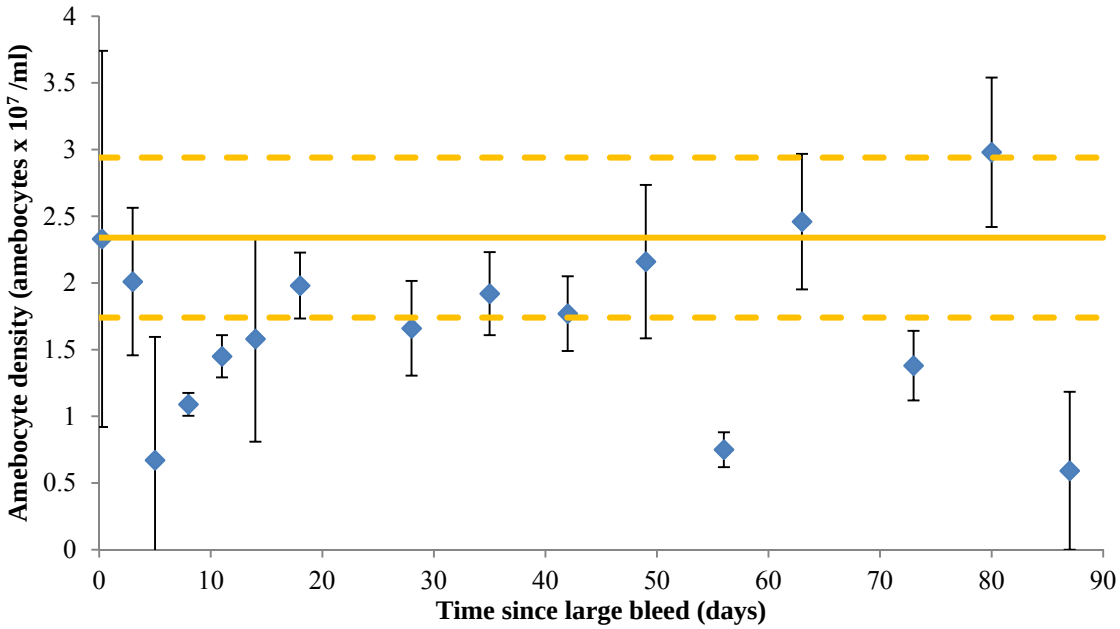

Figure 14. Animal 2's amebocyte density decreased below the baseline mean at day five and recovered by day eighteen.

Table 9. Animal 2's amebocyte density statistically drops below the baseline mean after day three and returns to baseline mean by day fourteen after the large hemolymph removal.

Time (days)	N	Mean Density (amebocytes x 10^7 /ml)	StDev	SE Mean	P
Baseline	48	2.34E+07	5.97E+06	8.62E+05	1.00
3	6	2.01E+07	5.53E+06	2.26E+06	0.21
5	6	6.71E+06	9.24E+06	3.77E+06	0.01
8	6	1.09E+07	8.61E+05	3.52E+05	0.00
11	6	1.45E+07	1.58E+06	6.44E+05	0.00
14	5	1.58E+07	7.70E+06	3.44E+06	0.09

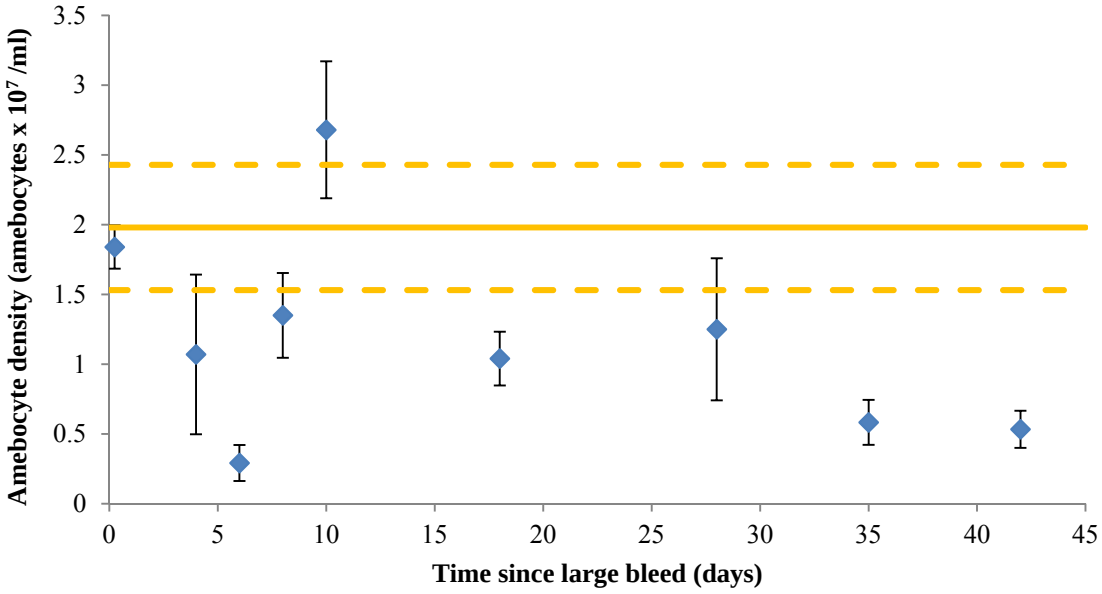

Figure 15. Animal 3's sees an initial decrease in amebocyte density on day three followed by an return to the baseline mean at by eight.

Table 10. The one-sample t-test with the baseline mean as the hypothesis shows the amebocyte density dropped below the baseline mean at day three and recovered by day eight.

Time (days)	N	Mean Density (amebocytes x 10^7 /ml)	StDev	SE Mean	P
Baseline	48	1.09E+07	2.50E+06	3.61E+05	1.00
3	6	3.29E+06	7.65E+05	3.12E+05	0.00
5	6	6.17E+06	5.40E+05	2.20E+05	0.00
8	6	9.96E+06	1.31E+06	5.34E+05	0.13

B.2 Amebocyte Diameter Figures and Statistical Tables

Table 11. A one-way unstacked ANOVA showed variation between the four bleeds that made up the baseline data suggesting some mechanism in the animal or from the bleed causing variability.

Animal	Date	N	Mean Diameter (µm)	StDev.	F	P	Tukey Grouping
1		400	11.02	1.54	6.18	0.000	
	8-2	100	11.18	1.36			A
	8-4	100	11.38	1.52			A
	8-9	100	11.05	1.44			A
	8-11	100	10.48	1.72			B
2		400	10.48	1.69	5.54	0.001	
	8-3	100	10.99	2.04			A
	8-5	100	10.51	1.59			A,B
	8-10	100	10.55	1.36			A,B
	8-12	100	9.95	1.57			B
3		400	9.55	1.84	2.09	0.101	
	8-3	100	9.66	2.06			A
	8-5	100	9.60	1.93			A
	8-10	100	9.79	1.60			A
	8-12	100	9.18	1.69			A
4		300	11.52	1.52	3.89	0.009	
	8-2	100	11.15	1.57			A
	8-4	50	11.95	1.92			B
	8-9	100	11.54	1.32			A,B
	8-11	50	11.79	1.18			A,B
5		400	11.68	1.81	7.47	0.000	
	8-2	100	11.42	1.82			A
	8-4	100	12.39	1.65			B
	8-9	100	11.35	2.01			A
	8-11	100	11.57	1.56			A
6		400	9.49	1.58	12.61	0.000	
	8-3	100	9.00	1.65			A
	8-5	100	9.24	1.62			A
	8-10	100	9.46	1.29			A
	8-12	100	10.25	1.50			B
7		350	9.85	1.77	6.23	0.000	
	8-3	100	10.16	2.10			A
	8-5	100	9.23	1.92			A
	8-10	50	10.23	1.09			B
	8-12	100	9.97	1.36			A
8		350	10.86	1.75	17.43	0.000	
	8-2	100	11.18	1.97			A
	8-4	50	9.35	2.06			B
	8-9	100	11.26	1.31			A
	8-11	100	10.91	1.30			A

Table 12. The eight animals, used in the baseline series, showed variation between one another for the average amebocyte diameter.

Animal	N	Mean Diameter (µm)	Std	F	P	Tukey Group
1	400	11.02	1.54			B
2	400	10.48	1.69			C
3	400	9.55	1.84			D
4	300	11.52	1.52			A
5	400	11.68	1.81			A
6	400	9.49	1.58			D
7	350	9.85	1.77			D
8	350	10.86	1.75			B
	3000	10.53	1.87	96.35	0.000	

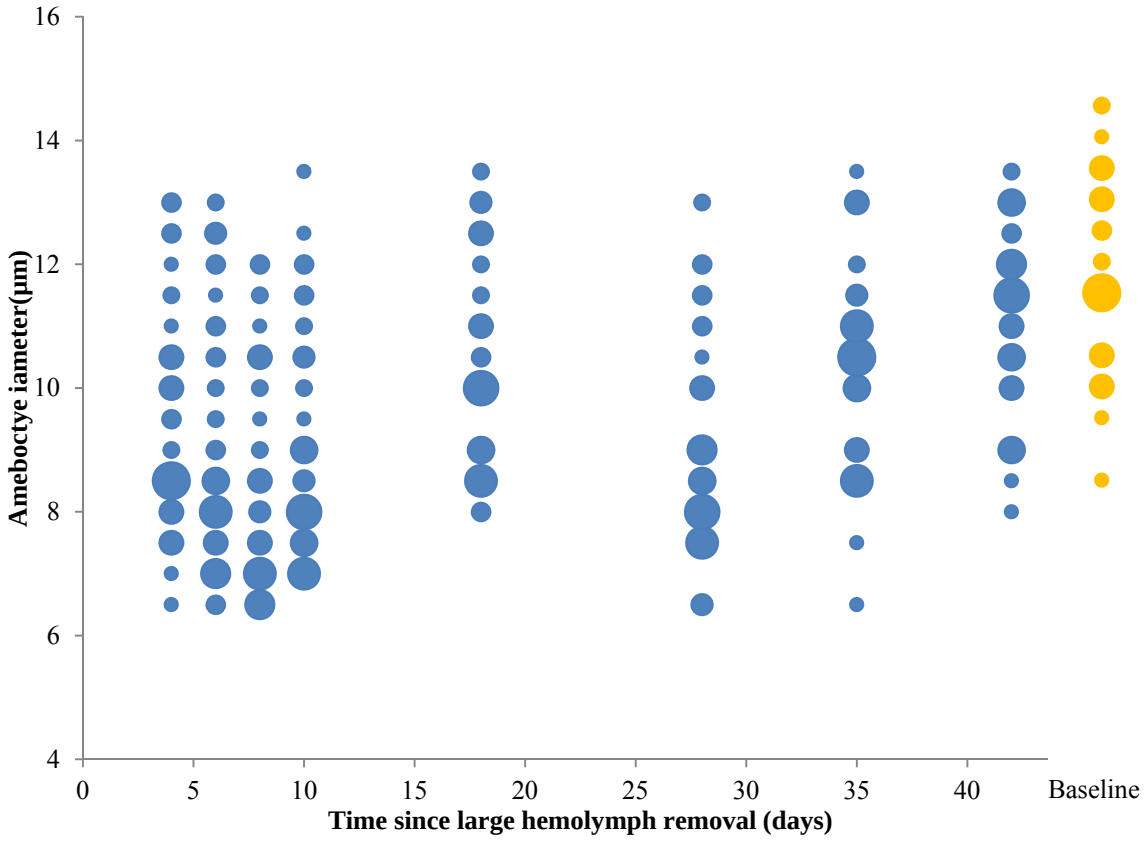

Figure 16. Animal 1's amebocyte diameter decrease below the baseline mean day three after the large hemolymph removal and although the distribution of the cells changed the diameter never recovered.

Table 13. The amebocyte diameter of Animal 1 significantly decreased after the large hemolymph removal after three weeks the diameter began recovering but never reestablished the same diameter mean as the baseline.

Time (days)	N	Mean Diameter (µm)	StDev	SE Mean	P
Baseline	309	11.19	1.46	0.08	1.00
4	51	9.25	1.65	0.23	0.00
6	51	9.06	1.78	0.25	0.00
8	51	8.46	1.65	0.23	0.00
10	51	9.76	1.64	0.23	0.00
18	51	10.05	1.55	0.22	0.00
28	51	9.63	1.38	0.19	0.00
35	51	10.45	1.60	0.23	0.00
42	51	10.12	1.40	0.20	0.00

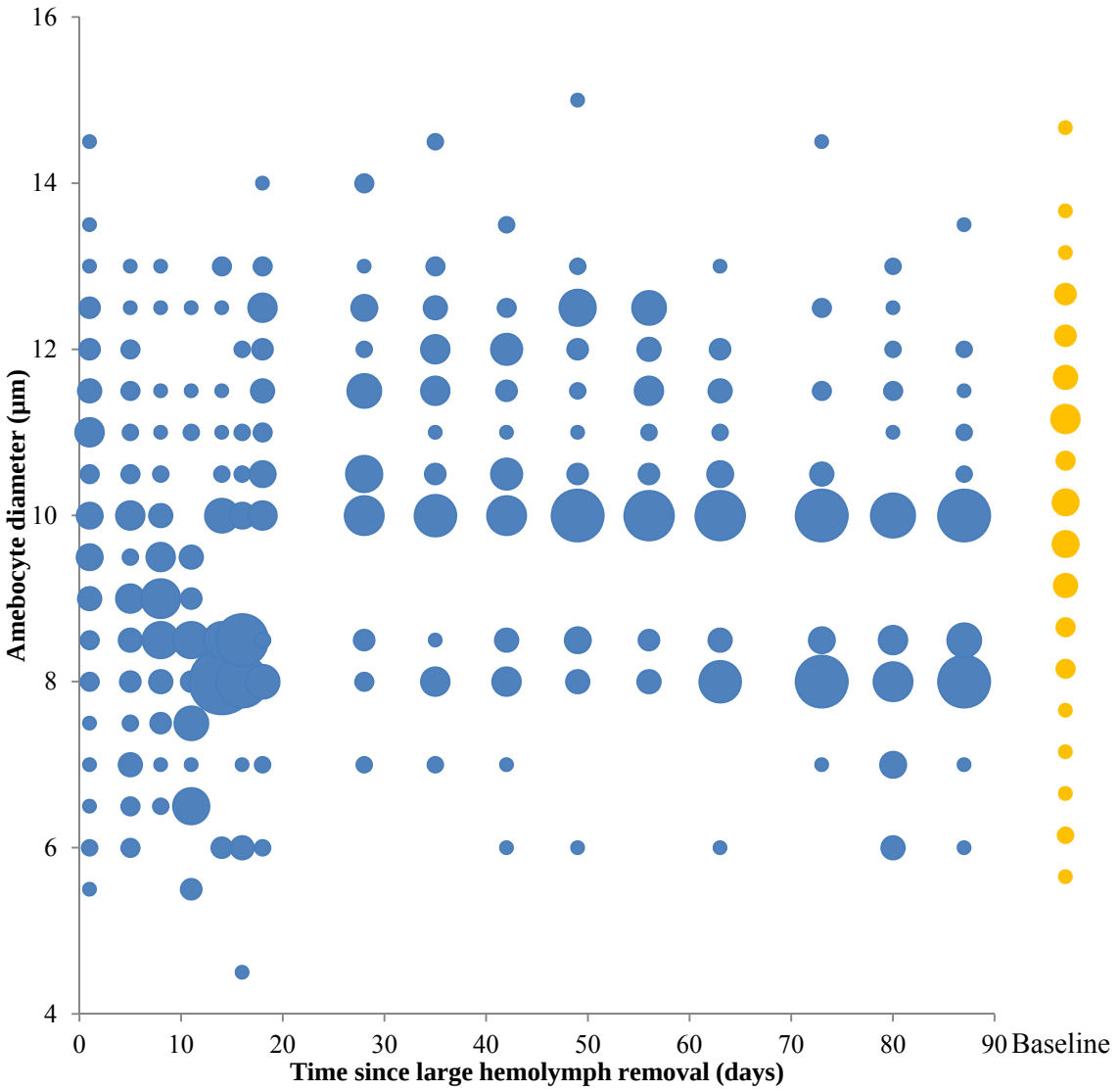

Figure 17. Animal 2's baseline diameter decreases and changes distribution compared to the baseline on day three and returns to the baseline mean by day eighteen.

Table 14. Animal 2 shows a decrease in mean amebocyte diameter from day three after the large hemolymph removal to day eighteen.

Time (days)	N	Mean Diameter (µm)	StDev	SE Mean	P
Baseline	400	10.48	1.69	0.08	0.97
3	51	9.38	1.83	0.26	0.00
5	51	9.26	1.31	0.18	0.00
8	51	8.25	1.55	0.22	0.00
11	51	9.04	1.62	0.23	0.00
14	51	8.78	1.52	0.21	0.00
18	51	10.54	2.03	0.29	0.85

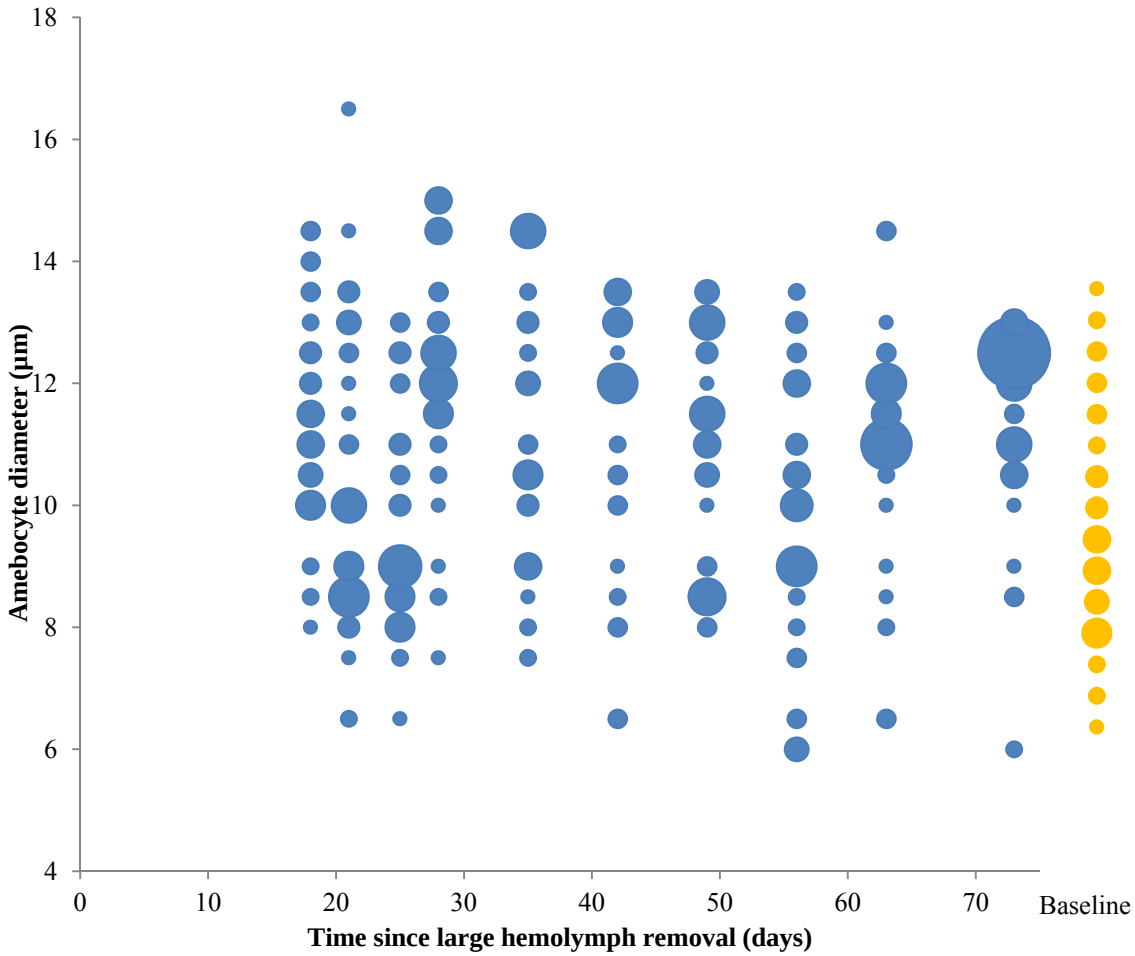

Figure 18. Animal 3's distribution and average diameter varied from the baseline values on day twenty-eight returning by day forty-two.

Table 15. Animal 3's mean amebocyte diameter varied from the baseline value on day eighteen after the large hemolymph removal. There was a second change in diameter beginning day twenty-eight and returning the baseline value by day forty-two.

Time (days)	N	Mean Diameter (µm)	StDev	SE Mean	P
Baseline	400	9.55	1.84	0.09	0.96
18	51	10.14	1.82	0.26	0.02
21	51	9.68	2.19	0.31	0.67
25	51	9.39	1.62	0.23	0.48
28	51	11.14	1.94	0.27	0.00
35	51	10.95	1.73	0.24	0.00
42	51	9.88	1.93	0.27	0.24
49	51	10.11	1.57	0.22	0.01
56	51	9.53	1.67	0.23	0.92
63	51	9.98	1.70	0.24	0.08
73	51	10.01	1.52	0.21	0.04