SERODIAGNOSIS IN PENAEID SHRIMP

Glenn L. Lawrence

University Undergraduate Fellow, 1988-1989

Texas A&M University

Department of Veterinary Anatomy

APPROVED

Fellows Advisor ______________________

Honors Director ______________________

ABSTRACT

Serodiagnostic techniques were studied using Penaeid shrimp to develop methods of evaluating the health status of shrimp. Three serodiagnostic techniques were evaluated: total hemocyte count, differential hemocyte count, and serum biochemistry. Ultrastructural of the circulating hemocytes was also compared. The serodiagnostic techniques were then tested in a Vibrio immunization challenge study.

ACKNOWLEDGEMENTS

I would like to thank Dr. Sis for his guidance and support throughout this project. I would also express my gratitude to Antony Chinnah for his advice and encouragement.

LIST OF ILLUSTRATIONS

TABLE OF CONTENTS

Introduction

Shrimp is the most valuable of the U.S. fisheries with most of the commercial shrimp coming from the Gulf of Mexico. Yet in 1980, the U.S. imports of shrimp was twice as much as its domestic production and accounts for almost ten percent of the national trade deficit. In addition, domestic commercial harvests are already at a maximum sustainable yield, with the U.S. demand for shrimp on the rise(Lawrence, et al., 1981). These are the events which have provided the setting for interest in shrimp mariculture to grow.

Current annual shrimp production in the U.S. through shrimp mariculture is approximately 1000 metric tons and is supplied by twenty shrimp farms (Rosenberry, 1989). The number of farms is expected to increase but in order for shrimp mariculture to remain successful, adequate control of disease must be maintained. One of the more important disease problems facing commercial shrimp farming is Vibriosis.

Vibrio sp. are opportunistic gram negative bacteria, part of the normal microflora of both naturally occurring and farm-raised shrimp. Conditions which can lead to production of the disease include overcrowding, stress of handling or capture, changes in the temperature and salinity of the water, organic contamination of the water, and cuticular wounds (Johnson, 1978; Lewis, 1981; Lightner and Lewis 1975).

Vibriosis has been suggested as a main cause of death in juvenile shrimp. Vibriosis produces disease usually resulting in 10-50 percent mortality, but which can reach as high as 100 percent (Lightner and Lewis, 1975). One species, Vibrio parahaemolyticus, is also a cause of food poisoning in man and has been found in white shrimp along the Texas Gulf coast.

Manifestation of Vibriosis is identified by opaqueness of the abdominal musculature in early development and in the intestinal tract in the later stages. The edges of the legs and carapace may also become blackened or lightened. The swimming of the diseased shrimp also becomes altered with the shrimp showing frantic swimming motions. Often the shrimp swim on their side and lay upright on the bottom close to the final stages of the disease and death (Lightner and Lewis, 1975; Shigueno, 1975).

Additional methods of assessing the health status of shrimp prior to impending death are needed. One possible alternative is using serodiagnostic techniques. Serodiagnosis is the analysis of body serum to diagnose disease and is routinely used in both human and veterinary medicine. For this project, I have investigated and developed three different serodiagnostic techniques: total hemocyte counts, differential hemocyte counts, serum biochemistry, and ultrastructure. The techniques were then used in a preliminary Vibrio immunization challenge study to test their efficacy.

REVIEW OF LITERATURE

Haeckel (1857) described the hemocytes of the crayfish Astacas fluvaitilis as amoeboid cells with or without cytoplasmic granules and became the first investigator to describe crustacean hemocytes (Sis et al., 1989). Since that time, hemolymph from many other species has been examined with the result of many different classifications of hemocytes arising.

Classification

Using light microscopy and Giemsa-stained hemocytes, Cornick and Stewart (1976) classified the hemocytes of the lobster Homarus americanus into four categories: prohyalocyte, hyalocyte, eosinophilic granulocyte, and chromophobic granulocyte. By differential counts, they found the hyalocyte in the greatest number(64.2%), followed by chromophobic granulocytes(21.9%), eosinophilic granulocytes(12.2%), and prohyalocytes(1.8%). They also used phase-contrast microscopy and Normarski interference-contrast microscopy. The criteria they used for distinguishing the hemocytes were: the size and refractile nature of the granules in the cytoplasm, the ratio of the size of the cytoplasm to that of the nucleus, and the staining characteristics with Giemsa stain. However, only two types of hemocytes were discernable under phase-contrast: granulocytes, described as being highly granulated and refractile, and hyalocytes, refractile and having sparse granules. With Normarski interference, Cornick and Stewart (1978) were able to identify a non-refractile granulocyte, prohyalocyte, and hyalocyte. They observed that the refractile hemocyte seen under Normarski interference corresponded to the chromophobic or eosinophilic Giemsa-stained granulocytes seen with light microscopy (Cornick et al., 1978).

Mix and Sparks (1980) used three different classes to group Dungeness crab, Cancer magister, hemocytes. These included hyalinocytes, intermediate granulocytes, and eosinophilic granulocytes. Their formalin-fixed hemocytes were stained with a modification of Wright's stain. They based differentiation of the cells on only two characteristics: the presence or lack of granules and the staining characteristics of the granules. Mix and Sparks believed that further characteristics for identification such as cell size, granule size, nuclear size, nuclear to cytoplasmic ratio, and staining of the cytoplasm and nucleus were not necessary because of "the great variation inherent in a dynamic cell population." Hyalinocytes had no granules present while the intermediate granulocytes contained large and small granules staining basophilic, or both basophilic and eosinophilic. The relative proportions of the cells were found to be hyalinocytes 16.3%, intermediate granulocytes 66%, and eosinophilic granulocytes 17.8% (Mix et al., 1980a).

The bivalve hemocytes, Mya arenaria, were classified by Seiler and Morse (1988) into two separate populations on the basis of ultrastructure. Cells were distinguished based on the number of granules present and called either agranulocytes or granulocytes. In addition to the number of granules, the agranulocyte had a large nucleus with distinctly clumped chromatin. It also contained electron-lucent vesicles, glycogen particles, and prominent organelles. The granulocyte had a lobulated nucleus with clumped chromatin. It also had no prominent organelles like the agranulocyte (Seiler and Morse, 1988).

One of the more complex classification plans was designed by Blewett and Eble (1979) for the freshwater prawn Macrobrachium rosenbergii. They found the nuclear morphology of the hemocytes to be similar, with all the nuclei

having prominent chromatin masses against the nuclear envelope. Blewett and Eble found the distinguishing characteristic to be the number, size, and shape of the granules. The granules were classified into four types and the cells were placed into three different populations based on the total numbers and relative composition of the different types of granules. The most densely granulated cell reported was cytotype II with a mean of 233 granules per cell.(Blewett and Eble, 1979)

Hemocytes of marine penaeid shrimp were examined by Tsing et al. (1989) and differentiated on the basis of ultrastructural features into three classes: undifferentiated hemocyte, small granule hemocyte, and large granule hemocyte. An additional class was seen with phase-contrast microscopy and labeled as hemocytes after natural lysis. By electron microscopy, the undifferentiated hemocytes appeared elongated, and having occasional small granules and dispersed nuclear chromatin. The small granule hemocyte was more irregular in shape and had more granules in the cytoplasm. It also contained many free ribosomes, well-developed rough endoplasmic reticulum, and a smaller horseshoe-shaped nucleus. Dense chromatin was located adjacent to the nuclear membrane. The large granule hemocyte had a ovoid shape with a oval nucleus. It also had heterochromatin close to the nuclear membrane and many cytoplasmic granules. However, it had a slightly developed rough endoplasmic reticulum and few free ribosomes. Using phase-contrast microscopy, the cells were distinguished by the size of the inclusions found and the amount of refringency the cell had. Because of the size and number of granules in the large granule hemocyte, it was the most refringent. The lysed hemocyte appeared the least refringent and had only a small band of cytoplasm encircling the nucleus (Tsing et al., 1989).

Serum Biochemistry

Serum biochemistry parameter measurements were carried out by Sandnes et al. (1988) in the Atlantic salmon, Salmo salar. Serum enzyme activities were determined for alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. Total protein, albumin, creatinine, triglycerides, and cholesterol determinations were also performed. They showed reproducability for these values using a Technicon RA-1000 random access analyzer (Sandnes et al., 1988).

Hose et al. (1984) also measured hemolymph values in Penaeus californiensis infected with a fungus. Measurements were made for alkaline phosphatase, serum glutamic oxaloacetic transaminase, glucose, ammonia, and total protein (Hose et al, 1984.).

Alkaline Phosphatase

Alkaline phosphatase includes a group of enzymes having broad substrates and which catalyze the hydrolysis of monophosphate esters. Isoenzymes of alkaline phosphatase occur in every tissue with high activities in the bone, intestine, kidney, leukocytes, liver, and placenta. Alkaline phosphatase is found bound to intracellular microsomal membranes. Little is known about its intracellular function and there is some speculation that only recently synthesized alkaline phosphatase is leaves cells with normal membrane permeability. Its half-life in the serum varies between species (Duncan and Prasse, 1986).

Serum alkaline phosphatase is considered to be a sensitive measure of cholestasis, which causes increased liver induction and serum activity of alkaline phosphatase. Intrahepatic cholestasis and blockage of individual bile ducts outside the liver both are responsible for a progressive increase

in alkaline phosphatase. Increases in serum alkaline phosphatase can also be caused by drugs and corticosteroid-stimulated induction.

Bilirubin

Bilirubin is a yellow pigment which is formed by the breakdown of hemoglobin and is excreted in the bile. Increased amounts of serum bilirubin, like alkaline phosphatase, may indicate cholestasis. More acute increases in bilirubin may be associated with major internal hemorrhage. Necrosis of the liver and acute and chronic disease of the liver are responsible for hyperbilirubinemia (Duncan and Prasse, 1986).

Aspartate Aminotransferase

Aspartate aminotransferase, also called glutamic oxaloacetic transaminase, is an enzyme found in virtually all cells. It exists as two or more isoenzymes located in the cytosol and mitochondria of cells and catalyzes the reaction involving aspartate biosynthesis from carbohydrate intermediates or the reverse, as shown in the reaction: glutamate + oxaloacetate <------> aspartate + alpha ketoglutarate .

The highest activities for this enzyme are found in the liver and muscle. Serum measurements of aspartate aminotransferase may indicate leakage of cytosol from muscle and hepatic cells into the serum due to disease of these organs characterized by necrosis or degeneration. The half-life of the enzyme in the serum varies between species (Duncan and Prasse, 1986).

Total Serum Proteins

Measurement of serum proteins includes many different proteins with varied functions. Individual serum proteins serve as antibodies, coagulation

factors, enzymes, and transport substances. As a group, they are important in acid base balance and colloidal osmotic pressure (Duncan and Prasse, 1986).

Increases in serum protein may be due to dehydration if the fluid loss is severe. Inflammation or neoplastic disease causes an increase in the fibrinogen protein. Tissue injury and inflammation result in increases in the alpha globulin while beta globulin increases have occurred with hepatic disease. An increase in both beta and gamma globulin is seen during a strong immune response. Increased gamma globulin production is due to a response to antigens. Chronic inflammatory diseases, immune mediated diseases, and a few lymphoid neoplasms have been seen with polyclonal gammopathies (Duncan and Prasse, 1986).

Decreased concentrations of serum proteins are caused by inadequate production possibly due to malnutrition, low absorption, chronic liver disease, and neoplasia. Loss of production is another cause of decreased serum concentration.

Total Serum Cholesterol

The main site of cholesterol synthesis occurs in the liver and is an important structural component of membranes and precursor for other molecules. High levels of LDL and VLDL lipoproteins are responsible for high measurements of serum cholesterol. These lipoproteins may increase as a result of endocrine, hepatic disease, renal disease, or occasionally because of a lipid metabolism disorder. Increased serum cholesterol also may indicate diabetes mellitus, pancreatitis, or hyperadrenocorticism and serum concentrations are inversely proportional to thyroid hormone activity (Benjamin, 1978).

Blood Urea Nitrogen

Urea is synthesized from ammonia resulting from primarily from protein catabolism. Increased blood urea nitrogen may indicate prerenal renal and postrenal disease. Prerenal azotemia may be due to increased protein catabolism caused by necrosis, starvation, chronic exercise, infection, fever, and corticosteroids. Reduced renal blood flow and decreased glomerular filtration pressure are other indications of prerenal disease. Postrenal disease may be seen with urinary tract obstruction and perforation. Decreased blood urea nitrogen may mean hepatic insufficiency, low protein ration , malabsorption, or anabolic steroids (Duncan and Prasse, 1986).

Creatinine

Creatinine primarily is formed from the conversion of creatine in the muscle. Creatine stores energy in the form of phosphocreatine. Increased serum creatinine gives similar information concerning renal disease as blood urea nitrogen except it is not affected by protein diet, catabolism, or exercise. Decreased creatinine values have no significance (Benjamin, 1978).

Glucose

It is common to see abnormal serum glucose concentrations and glucose metabolism caused by many diseases. Increased concentrations in the blood may indicate diabetes mellitus, pancreatitis, excitement, exercise, hyperadrenocorticism, chronic hepatic disease, hyperthyroidism, and recent high-carbohydrate meal. Hypoglycemia may be due to gram negative septicemia, neoplasms, malabsorption, starvation, hyperinsulinism, glycogen storage disease, hypoadrenocorticism hypothyroidism, and hepatic insufficiency (Duncan and Prasse, 1986).

Phosphorus

Phosphorus is present in almost all cells. It is a component of phospholipids, which are important in lipid transport and metabolism and cell membrane structure. Phosphorus is also vital in energy metabolism as a component of adenosine monophosphate(AMP), adenosine diphosphate(ADP), adenosine triphosphate(ATP), and of creatinine phosphate. As phosphate, this mineral is incorporated into RNA and DNA, which are needed for protein synthesis, and several enzyme systems (Church and Pond, 1982).

Increased serum phosphorus is a possible indication of renal insufficiency, vitamin D intoxication, hemolysis of blood sample, hypoparathyroidism, and a high-phosphorus and low-calcium ration. Decreased phosphorus concentrations in the serum may be caused rickets, osteomalacia, primary hyperparathyroidism, a low-phosphorus ration with a high calcium:phosphorus ratio, and hyperinsulinism (Duncan and Prasse, 1986).

Calcium

Calcium controls the excitability of nerve and muscle and is required for blood coagulation. Primary hyperparathyroidism, vitamin D intoxication, and neoplasms of the bone are suspected when serum calcium levels are elevated. With low levels of serum calcium, hypoparathyroidism, rickets, pancreatitis, renal insufficiency, tetany, hypoproteinemia, and malabsorption are indicated (Church and Pond, 1982).

MATERIALS AND METHODS

The animal used in this project to develop serodiagnostic techniques was Penaeus vannamei. The shrimp were obtained from the South Texas coast. Shrimp used for this project were brought to Texas A&M University and housed in artificial tanks at the College of Veterinary Medicine Aquatic Animal Laboratory. Shrimp were maintained at 27 degrees centigrade and 28 ppt salinity.

Collection of Hemolymph

Hemolymph was collected from the shrimp by inserting a 1 cc syringe with 25 gauge needle at the base of the third periopod or walking leg and withdrawing .2-.3 cc hemolymph. If an anticoagulant was used, the inside of the syringe was coated with the anticoagulant before removing hemolymph from the shrimp.

Total Count

Preparation of hemolymph for total hemocyte counts was performed following the method described by Fontaine (1978). Crustacean anticoagulant (Ford, 1988) was used in collecting the hemolymph. After dilution, the hemolymph was counted using a hemocytometer. Hemocytes in nine square millimeters were counted. Ten percent was added to the number counted and multiplied by the dilution factor as given by the formula: $10(n + 1/10n)$ = hemocytes/ul.

Differential Count

Hemolymph was collected with crustacean anticoagulant. After drawing the hemolymph into the syringe, the plunger was pulled back and the syringe inverted several times to ensure thorough mixing of the hemolymph with the anticoagulant. The hemolymph was then ejected onto a precleaned glass slide and spread with the tip of the needle. Spreading the hemolymph in this manner was found to be more beneficial in preserving the integrity of the hemocytes than streaking the hemolymph with another slide. After air-drying, the hemolymph smears were stained using a Diff-Quik stain set. If the smears were not stained within 15 minutes following air-drying, they were fixed in absolute methanol before storage.

Hemolymph smears were observed and counted under oil immersion using a 100x objective. A total of 100 hemocytes were counted on each slide to represent the relative proportion of each cell population.

Serum Biochemistry

Two different methods were used to prepare the serum for biochemical analysis.

(1) Anticoagulant method:

Hemolymph was collected into crustacean anticoagulant coating the syringe. Hemolymph was then placed in a 1.5 ml eppendorf tube and spun at 5000 rpm for 10 min. Cells formed a pellet at the bottom and the serum was then removed with a pipette and stored frozen.

(2) Heat inactivation:

Hemolymph was collected without anticoagulant and placed into a 1.5 ml

eppendorf tube. The hemolymph was processed by heat inactivation at 60 degrees C. for 8 minutes, placed in a freezer overnight, thawed at 40 degrees C. and centrifuged at 4500 rpm for 30 minutes. Serum above the resulting pellet was then removed and stored frozen.

After collecting the serum, it was then analyzed for cholesterol, alkaline phosphatase, bilirubin, blood urea nitrogen, calcium, creatinine, glucose, phosphorus, total protein, and serum glutamic oxaloacetic transaminase content using a Kodak Ektachem automated analyzer. The disadvantage in using the crustacean anticoagulant is that it contains EDTA. EDTA chelates divalent cations and will interfere with the calcium test. It also interferes with the creatinine test.

Electron Microscopy

Hemolymph pellets were collected using the above procedure for collecting serum. After the serum was removed, 3% fresh glutaraldehyde in .2M cacodylate buffer was added to the hemolymph pellet. Pellets were then fixed with Karnovsky's fixative and washed with 0.1M cacodylate buffer. Post fixation was done using 1% OsO_4 and washed with 0.1M cacodylate buffer. Pellets were washed with water, dehydrated, and embedded in 100% Epon-Araldite 502.

Vibrio Challenge Study

The serodiagnostic techniques were tested in a Vibrio immunization challenge study. Penaeus vannamei shrimp were used for this experiment and were immunized and raised in Panama. The adult vaccinated shrimp were then

sent to Texas A&M University for the challenge. In Panama, five to eight day old post larvae were vaccinated with two doses of Vibrio bacterin. Group A received 200 ug/L and group B received 1000 ug/L. A third group received no vaccine and served as a control.

The immunized and control shrimp were challenged with five different levels of Vibrio bacteria. The shrimp were injected in the third abdominal segment with either 4×10^5, 2×10^5, 8×10^4, 4×10^4, or 2×10^4 organisms. Shrimp were maintained in the same manner during the experiment. At the end of 48 hours, hemolymph samples were collected from each immunization group and used for total counts, differential counts, serum biochemistry, and electron microscopy.

RESULTS

Results are reported and discussed for the data collected from the Vibrio immunization challenge study. Because of the limited amount of shrimp, the samples were grouped according to whether the shrimp had received immunization and to whether the shrimp had been challenged with live Vibrio. This was done to provide some comparison and to demonstrate the feasibility of serodiagnostic techniques in detecting differences between the treatment groups.

Total Hemocyte Count

Ten samples representing 10 different shrimp from the Vibrio challenge study were utilized for total hemocyte counts (table 1). Five samples were taken from the immunized and Vibrio challenged group, two from the immunized and noninfected, two from the control Vibrio challenged group, and one from the control noninfected group. The shrimp in the immunized infected group showed the widest range of results with one shrimp having a count of 3850 cells/ul and one shrimp having a count of 13200 cells/ul, which is higher than the count for the control noninfected shrimp. The results are also displayed graphically in figure 1.

Differential Hemocyte Count

The classification scheme I developed for circulating hemocytes was based on classifications of hemocytes by other authors and by the appearance of the hemocytes as I examined them. I based my classification system on three criteria: (1)presence of cytoplasmic granules, (2)color of cytoplasm

Comparison of Total Hemocyte Counts(hemocytes/ul)

Group	No. shrimp	Range	Mean
Nonimmunized, Noninfected	1	12007	12007
Nonimmunized, Infected	2	1793-1942	1867
Immunized, Noninfected	2	3289-7469	5379
Immunized, Infected	5	3850-13200	7252

Table 1 Total hemocyte count results from Vibrio challenge study.

and/or granules, and (3)appearance of nucleus. Based on this, I determined that there existed three readily distinguishable populations of circulating hemocytes: (1)granulocyte, (2)eosinophilic granulocyte, and (3)hyalinocyte (figure 2).

(1)Granulocyte:

The granulocyte is most distinguishable by the presence of blue to violet staining granules in the cytoplasm. It has the largest variance in size, often the largest of the circulating hemocytes, but occasionally approaching the size of a hyalinocyte. The nuclear chromatin in the larger granulocytes is generally more fine, but becoming more dense in the smaller granulocytes. Dark staining nucleoli also appear in the smaller granulocytes. In addition, the cytoplasm becomes increasingly filled with granules in the smaller version of the granulocyte.

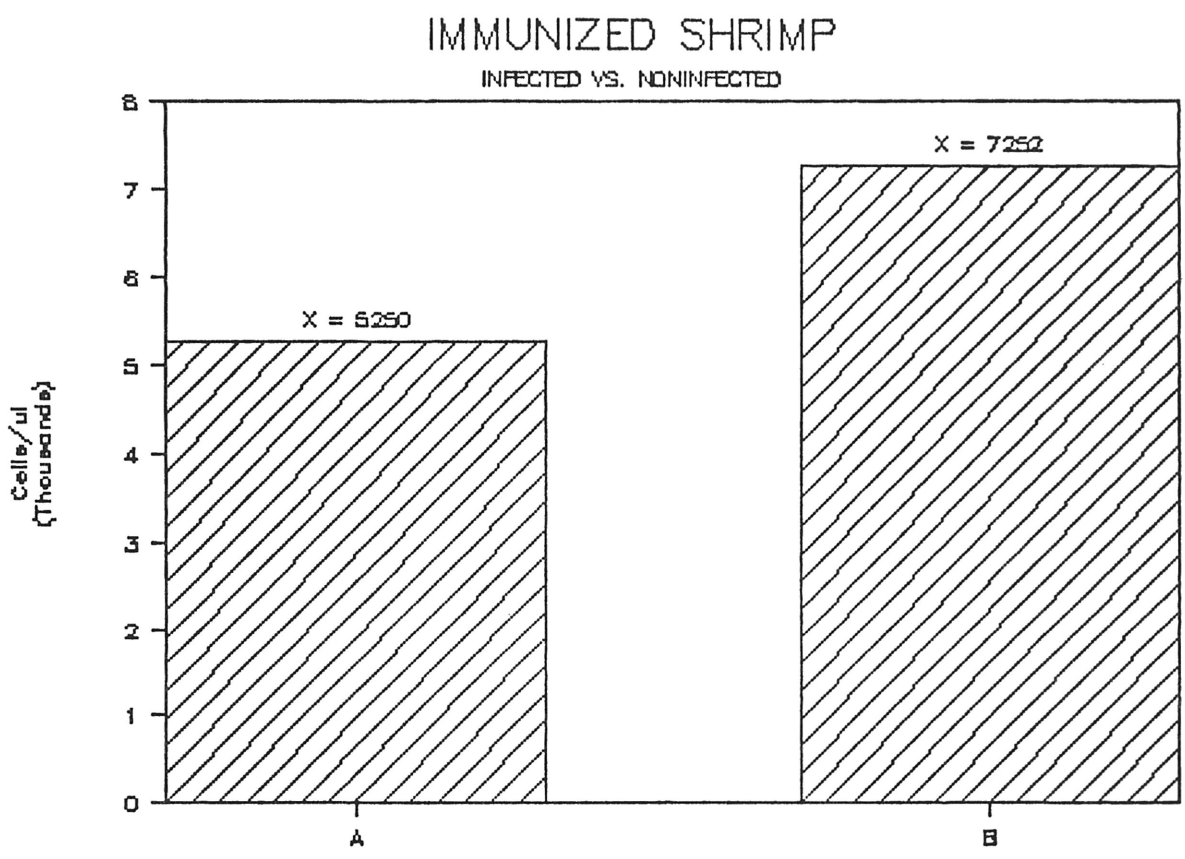
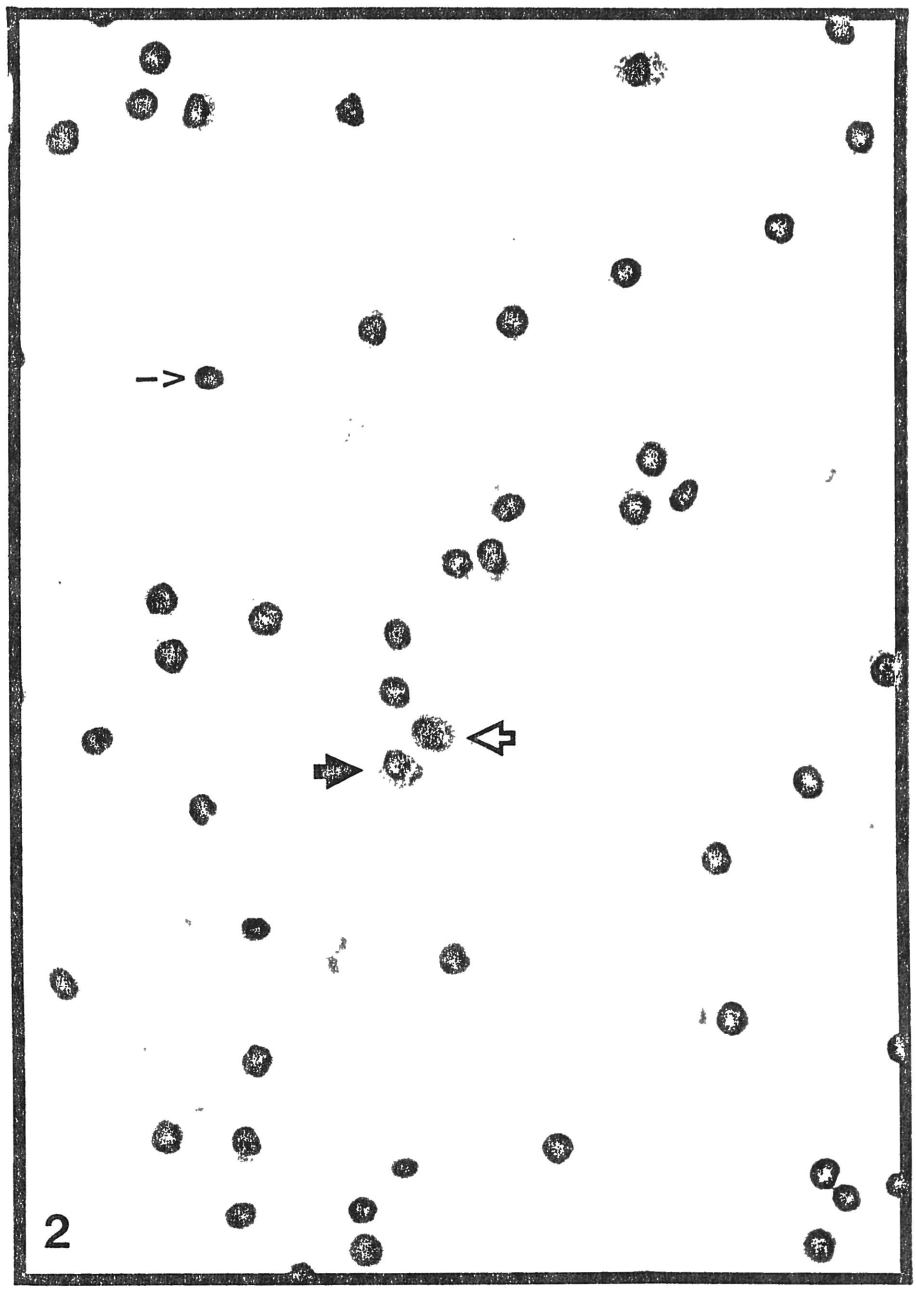


Figure 1 Graph of total hemocyte counts comparing immunized noninfected shrimp (A) with immunized infected shrimp (B).

Figure 2 Light microscopy picture of hemolymph smear showing an eosinophilic granulocyte (solid arrow), granulocyte (hollow arrow), and hyalinocyte (-->).


2

(2)Eosinophilic granulocyte:

Pink, acidic granules distinguish the eosinophilic granulocyte from the granulocyte. The size of the granules in the two granulated cells are approximately the same size. Other features of the eosinophilic granulocyte resemble those of the large granulocyte.

(3)Hyalinocyte:

The hyalinocyte is the smallest of the circulating cells and morphologically resembles a mammalian lymphocyte. The nucleus has a homogenous appearance, staining dark blue and appearing black in normal light levels. The nucleus was encircled by a thin rim of royal blue staining cytoplasm devoid of any granules.

In the challenge study, eight differential counts were made: 2 from the immunized infected group, three from the control infected group, and three from the control noninfected group (table 2). No differential counts were able to be done for the immunized noninfected group. A graphical representation of the results show an increasing number of hyalinocytes in infected shrimp (figure 3).

Serum Biochemistry

Hemolymph serum was collected both with and without the use of anticoagulant. Although more results were obtained with the serum obtained without anticoagulant, results for all of the tests were not realized. The results obtained for the samples collected by heat inactivation are also likely to represent a more true value than those results obtained from samples with anticoagulant in the serum. An average of the results for those tests in which values were obtained are presented in table 3.

Comparison of Differential Counts(%)

Group	No. shrimp	% Cell Type		
		Gran	Eosin	Hyal
Nonimmunized, Noninfected	3	91	3	5
Nonimmunized, Infected	3	69	1	27
Immunized, Noninfected	0	0	0	0
Immunized, Infected	2	81	5	15

Table 2 Differential count results from Vibrio challenge showing the percentage of granulocytes(Gran), eosinophilic granulocytes(Eosin), and hyalinocytes(Hyal).

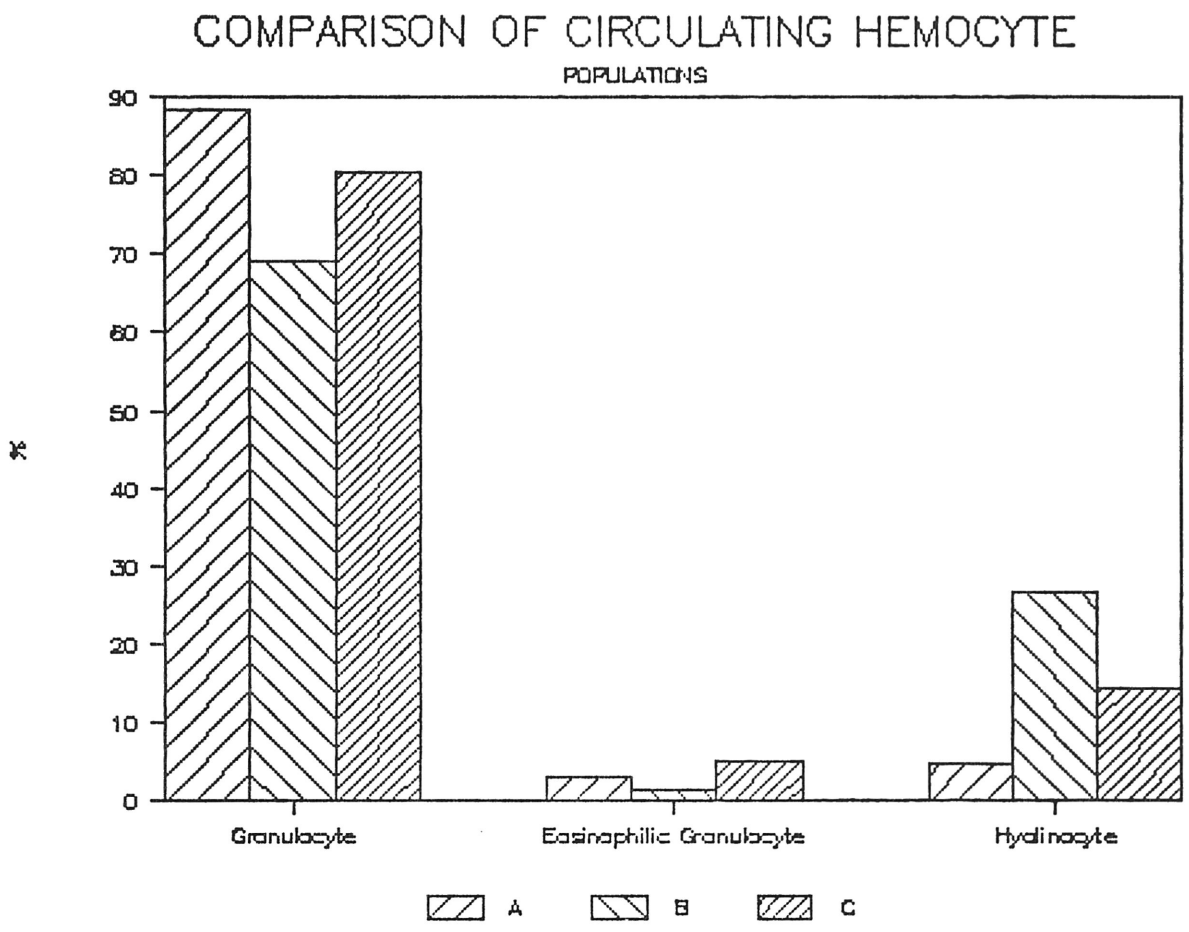


Figure 3 Graph of results from differential counts comparing the percentage of each cell population for nonimmunized noninfected shrimp (A), nonimmunized infected shrimp (B), and immunized infected shrimp (C).

Selected Serum Biochemistry Results	
Test	Result
Alkaline Phosphatase	33.0 U/L
BUN	6.22 mg/dl
Creatinine	0.31 mg/dl
Glucose	38.14 mg/dl
Phosphorus	2.03 mg/dl
Total Protein	6.94 g/dl
SGOT	206.75 U/L

Table 3 Mean values obtained for serum biochemistry tests from the Vibrio challenge study having measurable results.

Electron Microscopy

The pellets from the Vibrio challenge study to be used in electron microscopy were not processed in time for this report. They will be used to compare the ultrastructure of infected and noninfected hemocytes. However, results from electron microscopy work done on normal shrimp prior to the challenge study are presented for an ultrastructural comparison of the different hemocyte populations.

Four different transmission electron microscopy pictures are shown in figures 4-7. All of the hemocytes studied in electron microscopy shared several common features. All of the cells appeared round or oval in shape. The nucleus was centrally located and had dark staining material resembling heterochromatin adjacent to the inside of the nuclear membrane.

Figure 4 Hemocyte with small granules showing mitochondria (M), rough endoplasmic reticulum (RER), and small granules (SG).

Figure 5 Agranular hemocyte. Note also the lack of organelles in the cytoplasm.

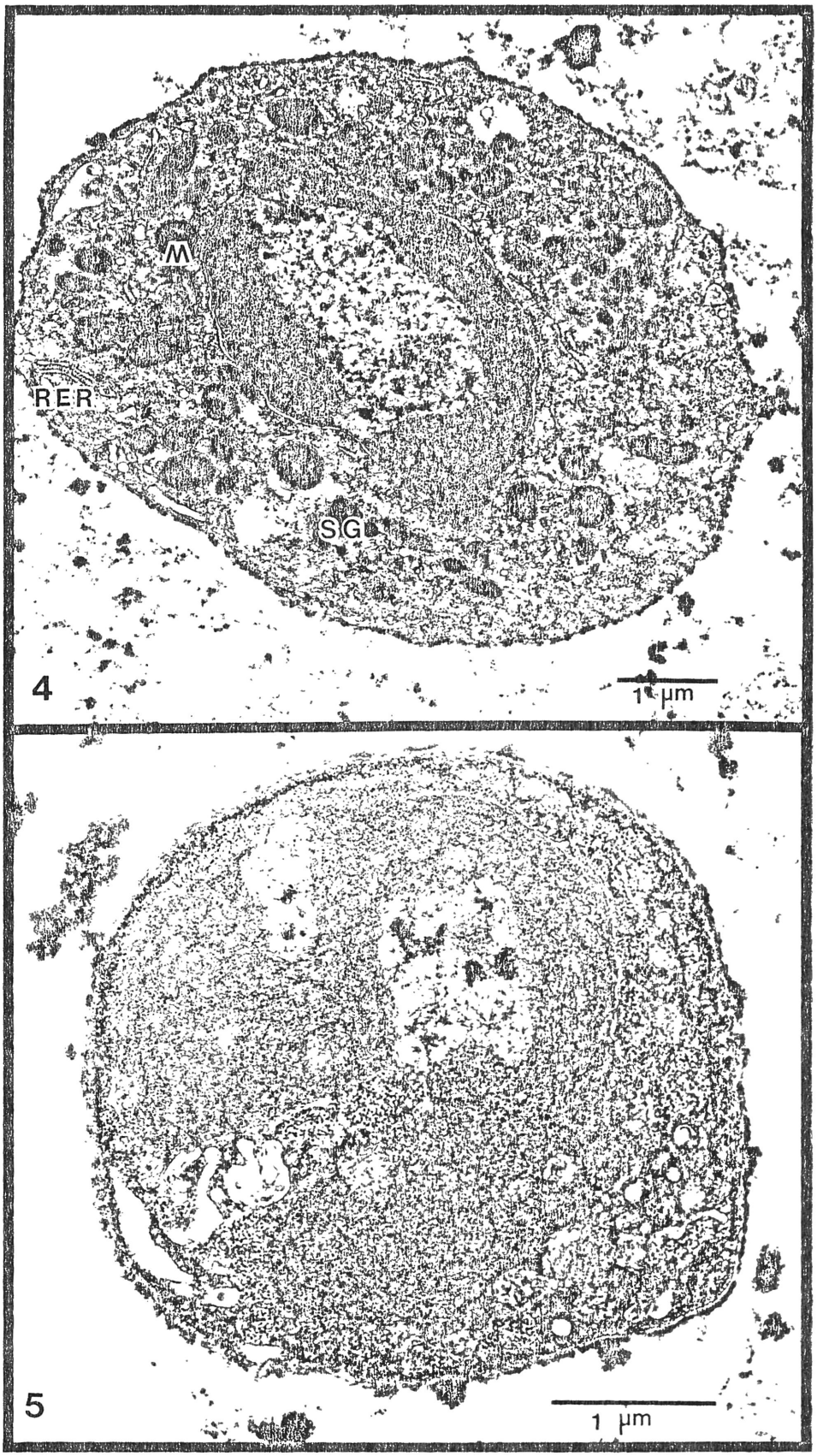

M
RER
SG
4
1 µm
5
1 µm

Figure 6 Hemocyte containing large granules. A centrally located nucleus (N) is also shown.

Figure 7 Hemocyte with large granules and prominent Golgi apparatus (--> arrow).

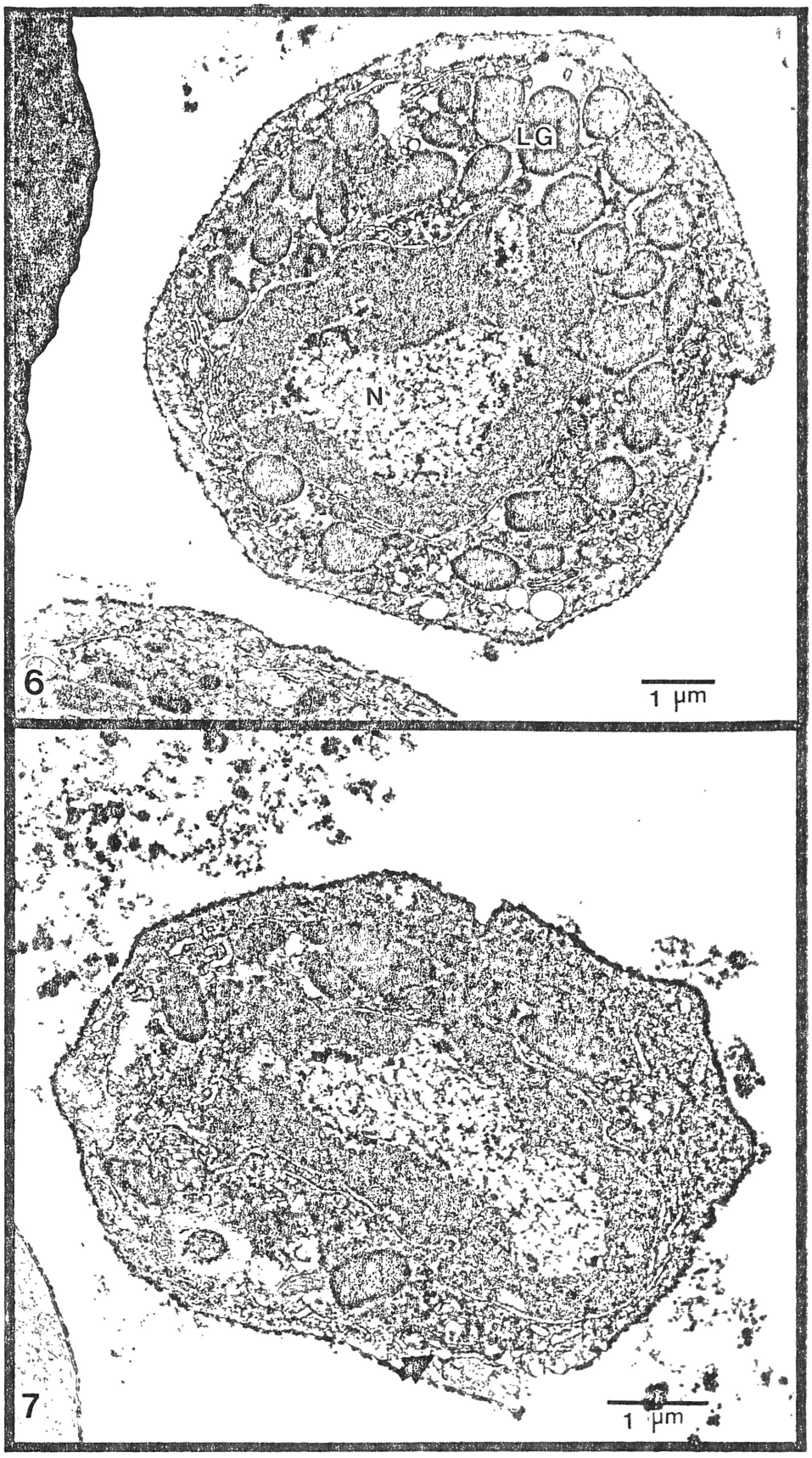

LG
N
6
1 µm
7
1 µm

Figure 4 displays a hemocyte with small granules. This small-granule cell probably corresponds to the granulocyte observed in light microscopy. This cell also shows noticeable rough endoplasmic reticulum and mitochondria.

A second type of cell observed was an agranulocytic cell (figure 5). This cell is likely corresponds to the hyalinocyte and also shows fewer organelles than hemocytes containing granules.

The third type of hemocyte was characterized by having large, prominent granules dominating the cytoplasm (figure 6 & 7). This large-granule hemocyte resembles the eosinophilic granulocyte seen in hemolymph smears(1989). Figure 7 also shows a noticeable Golgi apparatus. Several mitochondria and rough endoplasmic reticulum are also seen in both figures 6 and 7.

CONCLUSIONS

Serodiagnostic techniques are routinely used in mammalian, including human, diagnosis. Although the techniques investigated in this project were tested in a Vibrio challenge study, the main goal was to develope techniques for the use of serodiagnostics in shrimp disease. Not enough data was collected from the experiment to show results of any statistical significance, but enough to show that these techniques show promise for further use. The limited data also allows one to speculate about possible role of hemocytes during infection and the concomitant changes which might occur.

Johnson(1980) reports that, "most investigators who have used light microscopy believe that circulating hemocytes form a developmental series, beginning with a hyaline or semihyaline cell and ending with a mature, fully granulated cell." Although in crabs the hyalinocyte might be the largest cell, this is not the case in my investigation. Hyalinocytes had a high nuclear to cytoplasmic ratio typical of mammalian immature cells, but the hyalinocytes also were the smallest of the circulating cells and uncharacteristic of mammalian precursor cells.

Seiler and Morse (1988) also suggested a possible role for bivalve hemocytes in removing pollutants. In their study, they found a statistically higher percentage of granulated hemocytes from a polluted area than a nonpolluted area. They proposed that the hemocytes provided a protective function and that clams exposed to a higher level of pollutants over a lifetime would need additional granules in the hemocytes to remove the pollutant particles (Seiler and Morse, 1988).

Mix and Sparks(1980), also showed a shift in the percentage of cell populations present in infected and noninfected tanner crabs. They showed an

increase in the relative number of granulocytes and a decrease in the percentage of hyalinocytes during infection. Results revealed a positive relationship between the severity of infection and an increase in the number of circulating eosinophilic granulocytes. An eosinophilic granulocyte mobilization in response to infection was cited as one possible explanation for the increase in the numbers of eosinophilic granulocytes (Mix and Sparks, 1980).

In studying fungal infections in the California brown shrimp, Hose et al. (1984) found significant changes in some hemolymph parameters of shrimp with advanced fungal infections. These shrimp with advanced infections were found to be hypoproteinemic and had lower numbers of circulating hemocytes. The finding of reduced numbers of circulating hemocytes corresponds well with the results of the challenge experiment in this project, since some of the shrimp at the end of the 48 hour challenge also experiment exhibited gross signs of severe Vibrio infection and had reduced numbers of hemocytes.

Although these findings suggest possible roles for circulating hemocytes, other experiments can be done to add support to existing ideas. For example, acid phosphatase is found in the lysosomes of phagocytic mammalian leukocytes. Acid phosphatase stains of infected shrimp hemolymph might indicate cells having more phagocytic activity. In addition, I feel that the question of an immune response in crustaceans might be better understood with the use of immunofluorescence. This can be accomplished by isolating shrimp immunoglobulins. By then developing antibodies against these shrimp immunoglobulins and labeling the antibodies with a fluorescent material, an immunofluorescent study can be done to determine which hemocytes are responsible for the production of immunoglobulins.

This preliminary data will be used to establish the feasibility of selected biochemical values in the serodiagnosis of shrimp diseases. Serodiagnosis shows good potential to be effective in assessing the health status in crustaceans.

LITERATURE CITED

Benitez, M. 1985. *Biochemical Parameters on the Nutritional Diet of the Marmoset with a Manufactured Food Source.* Thesis, Texas A&M University Library.

Benjamin, M. M. 1978. *Outline of Veterinary Clinical Pathology.* Iowa State University Press, Ames, Iowa.

Blewett, C. and Eble, A. F. 1979. Cytology and Cytochemistry of Hemocytes from the Freshwater Prawn *Macrobrachium rosenbergii.*

Church, D. C. and Pond, W. G. 1982. *Basic Animal Nutrition and Feeding.* J. Wiley and Sons, New York, New York.

Cornick, J. W. and Stewart, J. E. 1978. Lobster (*Homarus americanus*) Hemocytes: Classification, Differential Counts, and Associated Agglutinin Activity. *Journal of Invertebrate Pathology*, 31, 194-203.

Duncan, J. R. and Prasse, K. W. 1986. *Veterinary Laboratory Medicine: Clinical Pathology*, Iowa State University Press, Ames, Iowa.

Fontaine, C. T. 1978. *The Circulating Hemocytes of White Shrimp, Penaeus setiferus: Origin, Abundance, Morphology, and Function.* Thesis, Texas A&M University Library.

Ford, L. 1988. Personal communication, Louisiana State University.

Hose, J. E., Lightner, D. V., Redman, R. M., and Danald, D. A. 1984. Observations on the pathogenesis of the imperfect fungus, *Fusarium solani*, in the California Brown Shrimp, *Penaeus californiensis*, *Journal of Invertebrate Pathology*, 44, 292-303.

Johnson, P. T. 1980. *Histology of the Blue Crab, Calinictes sapidas: A Model for the Decapoda.* Praeger Publishers, New York, New York.

Johnson, S. K. 1978. *Handbook of Shrimp Diseases.* TAMU-S6-603.

Lawrence, A. L., Chamberlain, G. W., and Hutchins, D. L. 1981. Shrimp Mariculture: An Overview. Texas A&M University Sea Grant College Program.

Lightner, D. V. and Lewis, D. H. 1975. A Septicemic Bacterial Disease Syndrome of Penaeid Shrimp. *Marine Fisheries Review*, 37, 25-28.

Mix, M. C. and Sparks, A. K. 1980a. Hemocyte Classification and Differential Counts in the Dungeness Crab, *Cancer magister.* *Journal of Invertebrate Pathology*, 35, 134-143.

Mix, M. C. and Sparks, A. K. 1980b. Tanner crab *Chionocetes bairdi* Rathbun haemocyte classification and an evaluation of using differential counts to measure infection with a fungal disease. *Journal of Fish Diseases*, 3, 285-293.

Rosenberry, B. 1989. *World Shrimp Farming.* Aquaculture Digest, San Diego, California.

Sandnes K., Lie O. and Waagbo, R. 1986. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon (*Salmo salar*). *Journal of Fish Biology*, 32, 129-136.

Seiler, G. R. and Morse, M. P. 1988. Kidney and hemocytes of *Mya arenaria* (Bivalvia): Normal and pollution-related ultrastructural morphologies. *Journal of Invertebrate Pathology*, 52, 201-214.

Shigueno, K. 1975. *Shrimp culture in Japan*. Association of International Technical Promotion, Tokyo, Japan. Ch. 4:112-117.

Sis, R. F., Lewis, D. H., and Caceci, T. 1989. Morphology and Classification of the Hemocytes of a Penaeid Shrimp (*Penaeus vannamei*). (publication pending).

Tsing, A., Arcier, J., and Brehelin, M. 1989. Hemocytes of Penaeid and Palaemonid Shrimps: Morphology, Cytochemistry, and Hemograms. *Journal of Invertebrate Pathology*, 53, 64-77.