Effect of Temperature, Salinity, and Pesticides on Oyster Hemocyte Activity

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ABSTRACT

Unexplained oyster deaths may relate to changes in salinity, temperature, and pesticides contaminating the seawater environment. The oyster's defense response to foreign material (i.e. bacteria, pesticides) is dependent on hemocytes (oyster blood cells). This research investigates the effects of temperature, salinity, and pesticides on oyster hemocyte function. Flow cytometry was used for monitoring *in vivo* hemocyte mortality and phagocytosis of *Escherichia coli* and *Vibrio vulnificus* in oysters acclimated at either 4°C, 25° C, or 37°C in 1^0/oo, 15^0/oo or 32^0/oo salinity at days 0, 3, 5, and 7; and in oysters challenged *in vivo* for 7 days with a mixture of pesticides, Imidacloprid, Permethrin, and Fipronil. Regardless of temperature, % hemocyte phagocytosis was 2-fold higher in 15 °/oo than in the 1 or 32 °/oo salinity groups. Pesticides *in vivo* and *in vitro* decreased ($p< 0.01$) oyster hemocyte phagocytosis of *E. coli* and pesticides *in vitro* increased ($p< 0.01$) hemocyte mortality. These results indicate that changes in salinity or exposure to pesticides in estuaries can impair hemocyte function and increase susceptibility to bacterial infection and contaminants consumed, thus increasing the mortality of oysters.

KEYWORDS: estuary, oyster, pesticide, salinity, temperature, hemocyte, *Escherichia coli, Vibrio vulnificus*

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1. INTRODUCTION

Oyster production represents an important global economic resource (USDA 2006). Periodic increases in oyster deaths have become a major problem globally, severely limiting oyster production. The causes of the sudden increase in oyster deaths, however, is unknown (Glude, 1974, Koganezawa, 1974, Goulletquer et al. 1998, Cheney, et al., 2000). Oysters are "sessile" or attached organisms; therefore, they are continuously exposed to the physiochemical modifications of their seawater environment. The circulatory systems of oysters are open, exposing the blood (hemolymph) and circulating cells (hemocytes) and tissues to variations in temperature, salinity, and pollutants, such as pesticides in the seawater (Shumway, 1977). Oysters, like other bivalve mollusks, are both osmo- and thermo-conformers, therefore, the hemolymph readily acquires the salinity and temperature of its seawater environment (Shumway, 1977).

The oyster's estuary is subjected to large variations in both temperature and salinity (Turner 2006, Pomeroy et al. 2000). Temperature is elevated (27^{o}C-33^{o}C) during the summer months and salinity fluctuates (2-32 ^{o}/oo) with tidal cycles, rainfall, and drainage from adjacent terrestrial sites such as farmland and lawns (Turner, 2006, Pomeroy et al. 2000).

The estuary is also subjected to pollutant loads. Among pollutants, pesticide contamination of shellfish has become more common in estuarine areas over the past several decades due, in part, to chemical run-off from farmland (EPA 2006). Pesticides are introduced into rivers when rainfall occurs and can enter marine areas, particularly estuarine and coastal zones (EPA 2006). These pollutants may have major ecological consequences and could endanger shellfish growth, reproduction, or survival (Banerjee et al. 1996). In the estuaries in the Apalachicola Bay, Florida, where 95% of Florida's oysters are harvested, three common pesticides that have been reported to contaminate water are Imidacloprid, Permethrin and Fipronil (EPA 2006).

Pathogenic organisms including bacteria, viruses, and parasites contaminate the oysters' estuaries (EPA 2006). Infectious agents, such as *Escherichia coli* and *Vibrio vulnificus*, have been previously correlated with low salinities and high temperatures (Cochran and Paul, 1998). Since the oyster does not possess a thymus, spleen, or bone marrow; the primary defense mechanism against foreign organisms such as bacteria involves circulating hemocytes, which are the invertebrate blood cells required for the immune response (Cheng, 1975). Hemocytes constitute the main line of defense against "non-self" material such as bacteria and pollutants and are involved in phagocytosis, pinocytosis, encapsulation, and wound healing (Cheng, 1996, Fournier et al. 2000). Granulocytes are one of the two types of hemocytes and have been previously shown to be involved in phagocytosis and pinocytosis of foreign material. They are thought to

contain hydrolytic enzymes and possibly produce reactive oxygen compounds that play a key role in killing and degrading invading pathogenic organisms (Pipe 1992, Cheng 1996).

Hemocytes have been used as immune capacity indicators in many bivalve species (Gelder and Moore 1986), even though some previous studies concluded that the factors that control oyster hemocyte phagocytosis and mortality remain unclear and have not been definitively characterized (Hegaret et al. 2003; Gagnaire et al. 2006; Pruzzo et al. 2005). Previous studies provided evidence that unidentified non-cellular components of hemolymph (serum) are also essential for the immune function of hemocytes (Leclerc 1996); however, little is known about whether changes in temperature, salinity, or exposure to pesticides could alter these protective serum factors.

Previous studies have failed to identify how the changes in water temperature and salinity may affect the ability of the hemocytes to protect the oyster from invading organisms or to determine whether the presence of pollutants may also negatively affect the oysters' immune response. It is proposed that a cause of oyster mortality outbreaks may be related to increased susceptibility to bacterial infection due to increased temperature or changes in salinity during the summer months coupled with exposure to pollutants such as pesticides. This study was specifically designed to determine whether changes in temperature, salinity, and exposure to pesticides may negatively affect the viability of hemocytes and normal immune function as assessed by hemocyte phagocytosis of bacteria. The specific species of oyster of interest to this investigation was the economically important eastern oyster, *Crassostrea virginica,* for which there is limited information regarding how environmental changes influence hemocyte activity (Hegaret et al. 2003).

The study hypothesis was that *in vivo* oyster hemocyte mortality and phagocytic activity against bacteria could be negatively affected by increases in temperature, salinity, and pesticides; and futher that normal hemocyte activity is dependent on the non-cellular serum component of hemolymph that may be influenced by pesticides. The specific objectives of the study were as follows:

1.Determine by flow cytometry whether *in vivo* exposure to different temperatures (4^{o}C, 25^{o}C, or 37^{o}C), salinities (1, 15, or 32 ^{o}/oo), and a mixture of three commonly used pesticides (Imidacloprid, Permethrin, and Fipronil) affect the mortality of oyster hemocytes or the hemocyte phagocytic activity against *E. coli* and *V. vulnificus*

2. Determine whether the serum component of the oysters' hemolymph is required to prevent hemocyte mortality and for normal phagocytosis of *E. coli* and *V. vulnificus*

3. Determine whether the *in vitro* exposure of pesticides affected oyster hemocyte mortality and normal phagocytosis of *E. coli* and *V. vulnificus*

2. METHODS

A. Overview and Rationale for Experimental Design

The Eastern oyster, *Crassostrea virginica,* was collected from approved shellfish harvesting waters from Apalachicola Bay, Apalachicola, Florida. Flow cytometry was used to evaluate the effects of different temperatures, salinities, and hemolymph serum on hemocyte mortality and phagocytosis of common pathogenic bacteria (*E. coli* and *V. vulnificus)* at timed intervals in acclimatized oysters. The specific salinity treatments (1, 15, 32 ^{o}/oo) were selected because they are representative of the average salinity (15 ^{o}/oo); the hypo- and hyper-salinities (1 and 32 ^{o}/oo, respectively) represent the range of salinities that may result from fluctuations in tides and rainfall in the normal aquatic environment of *C. virginica* (Berrigan et al. 1991).

The three seasonal water temperatures that the *C. virginica* species would be exposed to are on average 4^{o}C, 25^{o}C, 37^{o}C (EPA 2006). The three pesticides were chosen (Imidacloprid, Permethrin and Fipronil), since they are commonly used in the area of Apalachicola Bay, Florida, and the concentration utilized (0.7 mg/L) represents the average concentration that may occur in a Florida estuary (EPA 2006). The time periods (0, 3, 5, 7 days) were selected because the time required for the oyster to acclimate to different temperatures and salinities has previously been estimated to be up to 3-5 days (Shumway 1977). Flow cytometry was used as an evaluation technique because it is one of the most efficient methods for detecting differences in light scatter as a result of specific chemicals entering cells.

B. Experimental Design for Objective 1

a. Temperature Treatments. Oysters were acclimated at 4 ^{o}C, 25 ^{o}C, or 32^{o}C respectively, in one of nine tanks (225 L) of recirculating artificial seawater. The temperatures of the tanks (4^{o}C, 25^{o}C, or 37^{o}C) were controlled by placing them in one of three large temperature-controlled incubators.

b. Salinity Treatments. Three tanks at each temperature were adjusted to a different salinity (1, 15 or 32 ^{o}/oo) by using Instant Ocean Aquarium Sea Salt® (Aquarium Systems, Inc.).

c. Pesticide Treatments. Oysters were exposed to seawater with a mixture of pesticides (imidacloprid, Permethrin, and Fipronil), which were each present at a concentration of 0.7 mg/L of seawater.

d. Time Period for Treatments. Oysters (n=5) were removed from each of the nine tanks for hemocyte measurements on days 0, 3, 5, and 7.

e. Hemolymph/Hemocyte Collection. A hole was notched in each oyster using a drill. Hemolymph was then withdrawn from the pericardial cavity and posterior adductor muscle sinus of five oysters using a 10 mL.

syringe equipped with needle (0.9x25mm). Hemolymph samples were pooled, centrifuged for 10 minutes at 100 x g, and resuspended in the serum fraction and used for flow cell cytometry analysis and microscopy observation. Hemolymph samples were pooled to reduce inter-individual variation and to provide sufficient hemocytes for experiments.

f. Flow Cytometry Analysis of Hemocyte Mortality. Hemolymph samples were monitored within two hours of collection for hemocyte mortality by a modification of the method of Hegaret et al. (2003) using an EPICS XL 4, Beckman Coulter Flow Cytometer. Mortality was quantified using 200 µL of the oyster hemocyte suspension. Hemocytes were incubated in the dark for 30 minutes at room temperature with 10 µL;of propidium iodide (1.0 mg/L) (Interchim). Propidium iodide binds to double-stranded DNA and fluoresces at wavelengths above 630 nm; it enters and stains nonviable cells but cannot cross the membrane of viable cells, thereby making the dead hemocytes more fluorescent in the flow cytometer light detector (Hegaret et al. 2003). For each sample, 10,000 hemocytes were counted. Results were depicted as cell cytograms indicating cell size (FSC value) and cell complexity (SSC value) and the fluorescence channel(s) corresponding to the marker used. Hemocyte mortality was measured using red fluorescence and was expressed as the percentage of dead hemocytes relative to the total number of hemocytes.

g. Bacteria Preparation. Both *E. coli* and *V. vulnificus* were cultured separately in heart infusion (HI) broth at 37^{o}C for 24 hours. The culture broths were then centrifuged for 10 minutes at 500 x g. The bacterial pellets were washed two times with sterile phosphate buffer, pH 7.2. The bacterial phosphate buffer mixture was adjusted by adding phosphate buffer until an optical density of 0.69 at 540 µm wavelength light was achieved. This is equivalent to 1 x10^{6} bacterial cells per mL.

h. Flow Cytometry Analysis of Hemocyte Phagocytosis of Bacteria. Phagocytosis was measured using the two following methods: (1) oyster hemocyte ingestion of fluorosphere carboxylate-modified beads (1 µm) (Interchim) in which a 200 µL aliquot of cell suspension was incubated for 60 minutes at 25^{o}C with 10 µL of a 1/10th dilution of fluorescent beads coated with heat-killed *E. coli* or *V.vulnificus;* and (2) stained *E. coli* or *V. vulnifucus* with propidium iodide (1.0 mg/L, (Interchim) in which phagocytosis was expressed as the percentage of hemocytes that had ingested the fluorescent bacterial coated beads or the propidium iodide fluorescent *E .coli* or *V. vulnificus*.

C. Experimental Design for Objective 2

Hemolymph from oysters acclimated at 25^{o}C in 15 ^{o}/oo salinity for 3 days was collected and centrifuged for 10 minutes at 500 x g. The resulting cell-free serum was removed and artificial seawater

(ASW) (15 ^{0}/oo) was added back to the untreated hemocytes and evaluated by flow cytometry for cell mortality and phagocytosis activity against *E. coli* and *V. vulnificus* then compared to that observed when hemocytes were suspended in normal hemolymph or 15 ^{o}/oo artificial seawater after 2 and 4 hours incubation at 25^{o}C.

D. Experimental Design for Objective 3

Hemolymph from oysters acclimated at 25^{o}C in 15 ^{o}/oo salinity for 3 days was collected and centrifuged for 10 minutes at 500 x g. The resulting cell-free serum was collected and seawater with 0.7 mg/L of individual pesticides added and mixed. The resulting pesticide-treated serum was added back to the untreated hemocytes and evaluated by flow cytometry for cell mortality and phagocytosis activity against *E. coli* and *V. vulnificus* and compared to that observed when hemocytes were suspended in normal hemolymph or 15 ^{o}/oo artificial seawater after 2 and 4 hours incubation at 25^{o}C.

E. Microscopy Observation

Light microscopy was used to determine the uniformity of the oyster hemocyte preparations and to determine whether there were osmotic or temperature effects on the morphology of the hemocytes (Ford et al. 1994). Hemocytes were placed on a microscope glass slide with cover slip and observed using a Nikon light microscope equipped with a camera.

F. Statistical Analysis

The means and standard deviations were determined for hemocyte mortality at 4^{o}C, 25^{o}C, or 37^{o}C at 1, 15, or 32 ^{o}/oo salinity with and without pesticide exposure and hemocyte phagocytic activity against *E. coli* and *V. vulnificus* in oysters acclimated at 4^{o}C, 25^{o}C, or 37 ^{o}C in 1, 15, or 32 ^{o}/oo salinity on days 0, 3, 5, and 7 in the presence and absence of the pesticide mixture. Analysis of variance (ANOVA) was used to test the null hypothesis that the hemocyte mortality and phagocytosis of *E. coli* and *V. vulnlificus* at 4^{o}C, 25^{o}C, and 37 ^{o}C at 1, 15 and 32 ^{o}/oo salinity on days 0, 3, 5, and 7 with and without pesticide exposure would be the same and whether interactions between temperature, salinity, and pesticide exposure existed. ANOVA was also used to test for significant differences between means of phagocytosis of *E. coli* and *V. vulnificus* by hemocytes resuspended in normal hemolymph, positively-treated hemolymph and 15 ^{o}/oo artificial seawater after 2 and 4 hours incubation at 25^{o}C. The level of significance selected was (α) = 0.01, which

means that there was only a 0.01 chance that the observed differences in means were due to chance and a 99.99% probability that the differences were due to the temperature, salinity, and hemolymph treatments.

3. RESULTS

A. Objective 1

Oyster *in vivo* hemocyte mortality was significantly ($p< 0.01$) increased for oysters acclimated in 1 ^{o}/oo and 32 ^{o}/oo salinity without pesticide exposure when compared to hemocyte cell mortality in oysters acclimated in 15 ^{o}/oo without pesticide exposure. Hemocyte mortality was significantly ($p< 0.01$) increased for the oysters exposed to the mixture of pesticides when compared to oysters that were not exposed to the mixture of pesticides regardless of salinity or temperature treatment. The data illustrated in Figure 1 indicate that exposure to either low (1 ^{o}/oo) or high (32 ^{o}/oo) salinity significantly ($p<0.01$) increased the mortality of oyster hemocytes relative to water with salinity equal to15 ^{o}/oo. The negative effect of either hypo- (1 ^{o}/oo) or hyper- (32 ^{o}/oo) salinity on hemocyte viability was independent of the water temperatures evaluated in this study. Regardless of temperature, the mortality of hemocytes exposed to 15 ^{o}/oo salinity was on average 10% compared to either 40% or 30% for the 1 and 32 %o salinities, respectively (Figure 1). No significant differences ($p>0.01$) were detected between acclimatization days. No significant ($p>0.01$) interactions between temperature, salinity, and incubation times were detected.

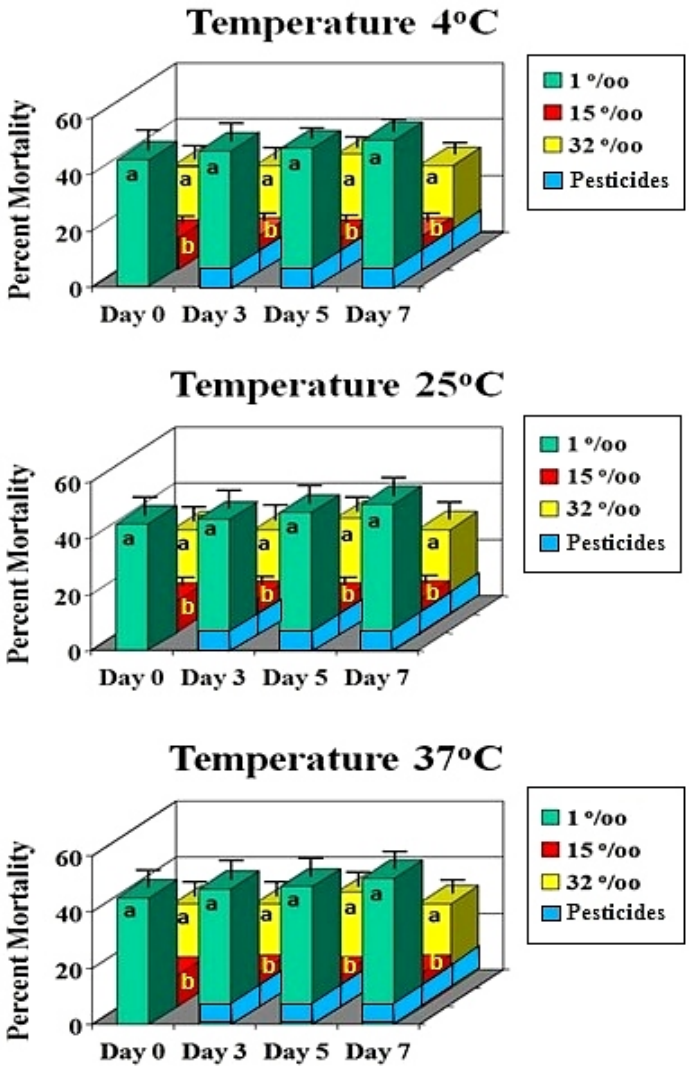

Figure 1. *In vivo* percent oyster hemocyte mortality at 4°C, 25°C, 37°C at 1, 15, and 32 °/oo salinity at days 0, 3, 5 and 7 with and without pesticide exposure. Values are means of 3 replicates ± SD. Different letters indicate significant differences ($p < 0.01$) between salinity groups.

Oyster *in vivo* hemocyte phagocytois of *E. coli* and *V. vulnificus* monitored by flow cytometry was significantly lower ($p < 0.01$) in oysters acclimated in 1 and 32 °/oo salinity compared to hemocyte phagocytosis in oysters acclimated in 15 °/oo salinity (**Figure 2**). The effect of salinity on hemocyte phagocytosis was independent of the water temperature. Regardless of temperature, the percent hemocyte phagocytosis for the 15 °/oo was approximately 2-fold higher than in the 1 and 32 °/oo salinity groups (**Figure 2**). In addition, the percentage of phagocytosis of *E. coli* by oyster hemocytes was markedly higher than the phagocytosis of *V. vulnificus* regardless of the temperature or salinity. The percent hemocyte

phagocytosis of *V. vulnificus* was only a fraction of 1% regardless of treatments in contrast to a 59% maximum hemocyte phagocytosis of *E. coli*. No significant differences ($p>0.01$) were detected between acclimatization days and no significant ($p>0.01$) treatment interactions were detected.

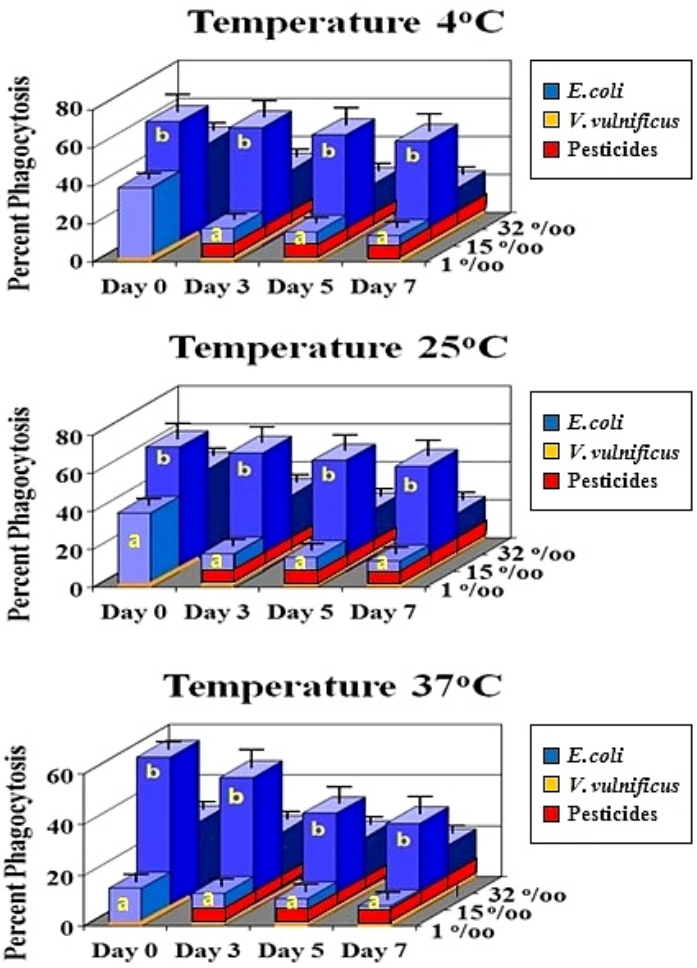

Figure 2. *In vivo* percent oyster hemocyte phagocytosis of *E. coli* ( ) , *V. vulnificus* ( ) measured by flow cytometry at 4, 25, 37 ^{0}C at 1, 15, and 32 ^{0}/oo salinity at Days 0, 3, 5 and 7. With ( ) and without pesticide exposure. Values are means of 3 replicates ± SD. Different letters indicate significant differences ($p< 0.01$) between salinity groups for *E. coli*. For *V. vulnificus* no differences were detected between the same salinity group**s.**

B. Objectives 2 and 3

After incubation in either pesticide-treated cell-free serum or 15 ^{o}/oo artificial seawater (ASW), hemocyte mortality was significantly increased ($p< 0.01$) when compared to hemocytes incubated in normal

hemolymph serum (Figure 3). The hemocyte percent mortality was approximately 5-fold higher regardless of incubation time (2 or 4 hours) in hemocytes incubated in either ASW or pesticide-treated serum compared to hemocytes incubated in normal hemolymph serum (Figure 3).

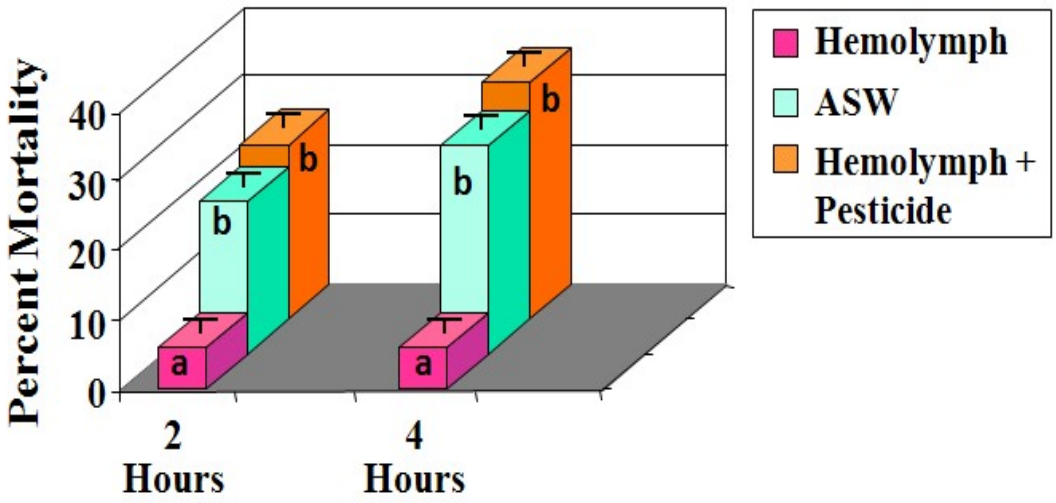

Figure 3. Percent hemocyte mortality in hemocytes incubated for either 2 or 4 hours in either hemolymph, artificial seawater (ASW), or pesticide-treated hemolymph. Values are means of 3 replicates. Different letters indicate significant differences ($p<0.01$).

In addition, the phagocytic activity of the hemocytes toward *E. coli* and *V. vulnificus* was significantly ($p< 0.01$) decreased when compared to hemocytes incubated in normal hemolymph serum for 2 and 4 hours (Fig 4).

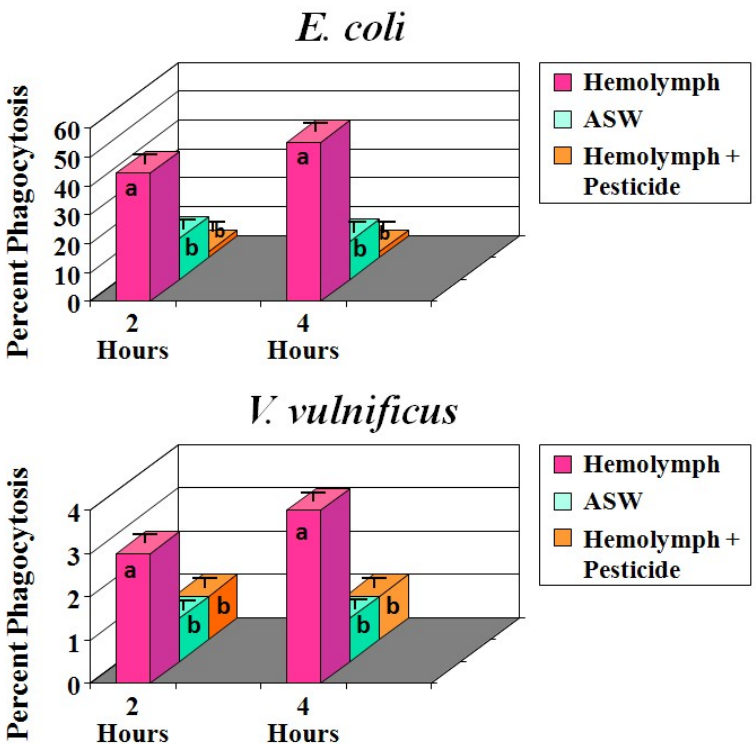

Figure 4. Percent *in vitro* phagocytosis of either *E. coli* or *V. vulnificus* when hemocytes were incubated in hemolymph, artificial seawater (ASW), or pesticide-treated hemolymph. Values are the means of 3 replicates. Different numbers indicate significant differences ($p<0.01$) between treatments. (Note different scale-higher % phagocytosis for *E. coli*).

C. Light Microscopy of Hemocytes

Hemocytes taken from the acclimated oysters were observed to be morphologically intact and normal by examination of photographs taken by light microscopy (Figure 5).

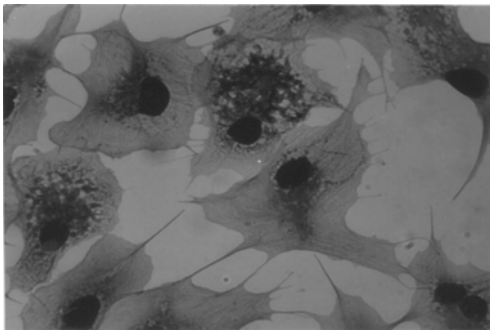

Figure 5. Light Microscopy of Oyster Hemocyte

4. DISCUSSION

The results of this study indicate that in contrast to our hypothesis, the viability and phagocytic activity of hemocytes did not change when the oysters were exposed to different water temperatures. The surprising and significant effect of hypo- and hyper-salinity on the oysters' defense mechanism, provides new evidence for potential negative effects of salinity changes on oyster production (Turner 2006).

The hypothesis that pesticides would have a negative effect on the oysters' immune system was supported by the *in vitro* data, which indicated that exposure of the oyster hemocytes to pesticides significantly increased hemocyte mortality and depressed hemocyte phagocytosis of both *E. coli* and *V. vulnificus.* In comparison to the *in vitro* exposure, *in vivo* exposure of oysters to pesticides resulted in a statistically significant lower rate of hemocyte mortality. *In vivo* exposure also depressed hemocyte phagocytosis of *E. coli* and *V. vulnificus*. The reasons for the differences between *in vitro* and *in vivo* hemocyte mortality when exposed to pesticides may possibly involve a biological transformation of the pesticides once these chemicals entered the oyster's digestive tract. It is known that many chemicals can be altered through digestive tract enzymes and the P-450 microsomal enzymes (Woodburn et al. 2003).

The design of the study also provided the opportunity to observe whether there were differences in the ability of the oysters' hemocyte to phagocytize *E. coli* compared to *V. vulnificus*. The data clearly indicated that the oysters' hemocyte was much less capable of phagocytizing *V. vulnificus* compared to *E. coli.* One explanation for this is that there may be differences between how the hemocyte membrane receptor recognizes either *E. coli* or *V. vulnificus* before these bacteria are engulfed and phagocytized.

These findings indicate that salinity and the presence of pesticides may play a more important role in the oyster hemocyte viability and activity than previously thought. The current study alerts the scientific community to the negative effects that alterations in salinity have on the oysters' defense mechanism, which could be linked to observed oyster mortality outbreaks. The results of this study could also lead to a better understanding of the possible interactions between abiotic environmental factors (temperature, salinity, pesticides) and biotic factors associated with the immune system of oysters and the resulting effect on susceptibility to infections. The finding that the oysters' hemocyte is much less capable of phagocytizing *V. vulnificus* compared to *E. coli* provides an explanation for the previously observed *in vivo* natural decay of *E. coli* compared to *V. vulnificus* (Wright et al. 1996).

It was not possible to investigate all of the potential environmental factors that may have affected the oyster's hemocyte activity. It is recognized that the hemocyte response to the specific temperature, salinity,

and pesticide treatments may actually be somewhat different within the estuary where the oyster is also exposed to other variables including other pollutants, different amounts of dissolved oxygen, and changes in pH. Since estuaries in different regions of the country are exposed to different seasonal changes in temperature and salinity, the application of these data may be most relevant to the southeastern United States.

The fact that it was only possible to evaluate a limited number of environmental factors in these studies sets the stage for future investigations which are needed to characterize how other pollutants such as chemicals used in fertilizers and contaminants from industrial wastes may also impact the observed seasonal increase in oyster mortality. In addition, future studies are needed to identify and characterize the serum factors required for the oysters' immune response and to investigate the role of these factors in hemocyte viability. Future investigations are needed to determine the physiological basis of the observed differences in hemocyte phagocytosis of *E. coli* and *V. vulnificus*, as well as the mortality of these bacteria to oysters.

Since the study identified the fact that the oyster's immunity to common bacteria may be impaired when salinity is either too high or too low and pesticides are present, monitoring systems should be put in place to track changes in salinity and pesticide contamination of the estuaries. The reason this is important is that the bacterial content of the oysters harvested under these conditions are likely to be elevated, which may lead to the previously unexplained increase in oyster deaths. In addition, when the oyster's ability to kill invading bacteria is impaired due to changes in environmental conditions, the increased bacterial content may also pose a health risk to humans when the oysters are consumed raw (Potassman et al. 2002).

5. CONCLUSIONS

1. *In vivo* variations in salinity affects the ability of oyster hemocytes to resist foreign bacterial invasion.
2. The significant differences in the eastern oyster hemocyte phagocytic activity towards *E. coli* compared to *V. vulnificus* may be partially related to changes in salinity.
3. Non-cellular serum components are required for the hemocyte to phagocytize *E. coli* and *V. vulnificus*.
4. *In vitro* exposure of pesticides to the oyster hemocytes significantly increased oyster hemocyte mortality and depressed hemocyte phagocytosis of both *E. coli* and *V. vulnificus.*
5. In comparison to the *in vitro* exposure, *in vivo* exposure of oysters to pesticides resulted in a statistically significant lower rate of hemocyte mortality.

6. ABBREVIATIONS AND ACRONYMS

FCS: Forward light scatter

SSC: Side light scatter

ASW: Artificial Seawater

7. ACKNOWLEDGEMENTS

A. Credits

I would like to thank Mr. Donald Noel, biological scientist at the University of Florida, Department of Food Science and Human Nutrition, for his assistance and guidance in conducting these experiments. I also appreciate the fact that the University of Florida provided me with the opportunity to use research space in the Aquatics Food Lab in the Food Science and Human Nutrition Department. In addition, I would like to thank Mrs. Sandra Swindler, Oak Hall science teacher, for her assistance and guidance with completion of the required forms for conducting the research and presenting my findings at the regional, state, and international science fairs and at the Florida Junior Academy of Sciences meetings.

B. Author

I am a 17 year rising senior at Oak Hall High School in Gainesville, FL, and have been actively conducting research studies spanning a period of four years related to the effect of seawater conditions on bacterial contamination of oysters. I became interested in how water quality affects pathogenic bacteria in shellfish after reading about deaths due to consumption of raw oysters. My research has been conducted in laboratories at the University of Florida with live oysters collected from Florida estuaries with seawater conditions simulated in the laboratory. I was a Discovery Channel finalist and received top honors multiple times for my water-related research at the Florida Junior Academy of Sciences. I received top-place awards during this four year period for my research at the regional, state, and international science fairs.

8. REFERENCES

Banatvala, N, Hdady WG, Ray, B J., McFarland LM, Thompson S, Tauxe RV (1997). *Vibrio vulnificus* infection reporting on death certificates: the invisible impact of an often fatal infection. Epidemiology Infection. 118: 221-225.

Banerjee BC, Koner BC and Ray A (1996). Immunotoxicity of pesticides: perspectives and trends, Indian Journal Experimental Biology 34: 732-733.

Cheney, DP, MacDonald, BF and Elston, RA (2000). Summer mortality of Pacific oysters, *Crassostrea gigas* (Thunberg): Initial findings on multiple environmental stressors in Puget Sound, Washington, 1998. Journal of Shellfish Research. *19*: 353-359.

Cheng, TC (1975). Functional morphology and biochemistry of molluscan phagocytes. Annuals New York Academy of Sciences. *266*: 343-379.

Cheng, TC (1996). Hemocytes: Forms and Functions. **In**: The Eastern Oyster, *Crassostrea virginica.* (eds. VS Kennedy, RIE Newell and AF Eble), pp.299-333. Maryland Sea Grant, College Park, Maryland.

Cochran, PK and Paul, JH (1998). Seasonal abundance of lysogenic bacteria in a subtropical esturary. Applied and Environmental Microbiology 64: 2308-2312.

Environmental Protection Agency (2006). Challenges Facing Our Estuaries. Key Management Issues. http://www.epa.gov/owow/estuaries/about3.htm (date accessed June 21, 2007)

Fournier, M, Cyt, D, Blakley, B, Boermans, H and Brousseau V (2000). Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics. American Zoologist 40: 412-420.

Ford, SE, Ford, KA and Kanaley, SA (1994). Comparative cytometric and microscopic analyses of oyster hemocytes. Journal of Invertebrate Pathology 64: 114-122.

Gagnaire, B, Frouin, H, Moreau, K, Thomas-Guyon, H and Renault, T (2006). Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish and Shellfish Immunology 20: 536-547.

Gelder, SR and Moore, CA (1986). Cytochemical demonstration of several enzymes associated with processing of foreign material within hemocytes of *Mercenaria mercenaria*. Transactions of the American Microscopical Society 105: 51-58.

Glude, JB (1974). A summary report of Pacific Coast oyster mortality investigations 1965-1972. **In**: Proc of the Third U.S.-Japan Meeting on Aquaculture at Tokyo. pp 28. Tokyo Press, Tokyo, Japan

Goulletquer P, Soletchnik P, Le Moine O, Razet D, Geairon P, Faury N, and Tailade,S (1998). Summer mortality of the Pacific cupped oyster *Crassostrea gigas* in the Bay of Marennes-Oleron (France). ICES Mariculture Committee CM, Copenhagen.

Hegaret H, Wikfors G H, Soudant, P (2003). Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation. Journal Experimental Marine Biology and Ecology 293: 249-265.

Koganezawa, A (1974). Present status of studies on the mass mortality of cultured oysters in Japan and its prevention. **In**: Proceedings of the Third U.S.-Japan Meeting on Aquaculture at Tokyo, pp 29-34. Tokyo Press, Tokyo Japan.

Leclerc M (1996). Humoral factors in marine invertebrates. **In**: Invertebrate Immunology. (eds. B Rinkevich, and WEB Muller), pp. 1-10. Springer Verlag, Berlin, Germany

Pipe, RK (1992). Generation of reactive oxygen metabolites by the hemocytes of the mussel *Mytilus edulis*. Developmental and Comparative Immunology. *16*: 111-122.

Pomeroy, LR, Sheldon JE, Sheldon Jr, WM, Blanton JO, Amft J, Peters F (2000). Seasonal changes in microbial processes in estuarine and continental shelf waters of the south-eastern U.S.A. Estuarine, Coastal and Shelf Science 51: 415-428.

Potassman, I, Pax, A, and Odeh, M (2002). Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. Clinical Infectious Disease 35: 921-928.

Pruzzo, C, Gallo G, Canesi L (2005). Persistence of vibrio in marine bivalves: the role of interactions with haemolymph components. Environmental Microbiology 7: 761-772.

United States Department of Agriculture (2006). Census of Aquaculture (2005), Vol. 3, Part 2, National Agricultural Statistics Service, USDA, Washington, D.C. 1-114.

Shumway, SE (1977). Effect of salinity fluctuation on the osmotic pressure and Na+, Ca2+, and Mg2+ ions in the hemolymph of mollusks. Marine Biology 41: 153-177.

Turner R.E. 2006. Will lowering estuarine salinity increase Gulf of Mexico oyster landings? Esturaries and Coasts 29: 345-352.

Woodburn KB, Hansen SC, Roth GA, and Strauss K (2003). The bioconcentration and metabolism of chlorpyrifos by the eastern oyster, *Crassostrea virginica*. Environmental Toxicology and Chemistry 22: 2766-284.

Wright AC, Hill, RT, Johnson, JA, Roghman, MC, Colwell RR, Morris, JG, Jr (1996). Distribution of *V. vulnificus* in the Chesapeake Bay. Applied and Environmental Microbiology 62: 717-724.