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Determination of Macrophage Chemiluminescent Response in Fundulus heteroclitus as a Function of Pollution and Temperature Stress

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DETERMINATION OF MACROPHAGE CHEMILUMINESCENT RESPONSE IN *FUNDULUS HETEROCLITUS* AS A FUNCTION OF POLLUTION AND TEMPERATURE STRESS

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Master of Arts

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by

Karen Kelly Reay

1990

To my parents and grandparents, who taught me
to love and respect the environment

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

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Master of Arts

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ABSTRACT

The Southern Branch of the Elizabeth River in Virginia has been shown to be a highly contaminated estuarine area. Correlations between environmental stress on a fish population and an altered immune response in comparison to non-stressed fish were tested using the chemiluminescent (CL) assay on *Fundulus heteroclitus* macrophages. Three experiments were performed to test kidney macrophage CL activity in two populations of *Fundulus heteroclitus*.

Eight trials in the field experiment, testing the CL response in Elizabeth River and York River populations, demonstrated that CL activity was significantly higher in Elizabeth River fish when water temperatures were similar. CL counts per minute for the Elizabeth River ranged from 0.82 to 7.98 and from 0.27 to 3.20 for the York River group. Mean peak macrophage counts per minute for the Elizabeth River population was 3.72 and mean peak counts per minute for the York River population was 1.20. Results of the York and Elizabeth River field CL assays were compared using the Mann-Whitney non-parametric ranked test for two populations. Significant differences were found between the two populations at an alpha level of 0.01. Macrophages may be stimulated from constant exposure to a stressful environment or certain chemicals may have stimulatory properties on CL activity.

An uptake and clearance study was performed to test for the effect of native versus non-native river water after a three week acclimation period. Results from this experiment showed that CL activity is reversible if water temperature changes. After three weeks of acclimation, York River fish began to exhibit CL activity found in Elizabeth River field populations and the Elizabeth River fish exhibited CL activity similar to York River field populations. Mean peak macrophage counts per minute for the Elizabeth River population held in York River water was 1.25 while mean peak counts per minute for York River fish held in Elizabeth River water was 5.28. In comparing the results of this experiment with the results of the field experiment, a reversal of trends can be demonstrated using the Mann-Whitney rank test. In the York River groups, a significant difference was found at an alpha level of 0.05. The results of the clearance group and Elizabeth River field results shows a significant difference at an alpha level of 0.10. A stimulatory agent or an increased stress factor in the Elizabeth River water may cause a modulation in macrophage activity.

A temperature experiment was performed to test the effect of stress water temperatures at the upper and lower stress levels. Two runs were made using three *in vivo* temperature regimes of 7, 15, and 28 °C. Six tanks of fish at each temperature regime were acclimated for three weeks prior to CL testing. Results show that statistically significant differences were found only in the York River population between the 15 and 28 °C temperature regimes. Temperature stress did not significantly affect the results of the CL assay in the Elizabeth River population. However, the results from both temperature runs demonstrate that the Elizabeth River activity is higher than the York River within each temperature regime. Highest activity occurred at 15 °C for both the York and Elizabeth River populations. CL activity may have been altered by the effects of temperature on the enzymatic activity or permeability of the cell. In addition, toxicity of certain chemicals or their uptake may be influenced by water temperature as a synergistic effect.

Major conclusions for this study are as follows: First, differences between the York and Elizabeth River populations of *Fundulus* exist when water temperatures are constant. Second, a reversal in macrophage activity is possible if fish are exposed to different environmental conditions. Third, optimum water temperature in this experiment for macrophage activity in *Fundulus heteroclitus* is 15 °C.

I. GENERAL INTRODUCTION AND STUDY OBJECTIVES

It was once thought that pollutants from industrial areas could be discharged in the nearby waterways, diluted and easily forgotten with no detrimental effects on the aquatic environment or the organisms within it. It is now known that this is not so. The result of unregulated or inadequately treated discharge is the decline of once thriving estuarine resources. This can be seen in degraded water quality and decreased abundance levels of marine organisms. Scientists have also noted an increase of morphological changes such as fin erosion, cataracts, and cancerous lesions occurring in existing fish populations within polluted regions.

It is hypothesized that poor fish health may be related to deterioration in the physical and chemical quality of the marine environment. Toxicity tests have identified the lethal dosage levels for a variety of chemicals. However, combinations of chemicals in the water column and sediment layers make it difficult to identify interactive effects and pinpoint which pollutants cause mortality. Although chemical concentrations in the field may not be high enough to directly kill an organism, sublethal effects may significantly affect a population through secondary infections or genetic mutations.

One hypothesis is that fish inhabiting a polluted estuarine environment reflect degraded environmental conditions through altered activity of the immune system. Such alterations may include changes in the cellular immune system such as the phagocytic activity of macrophages. Exposure to sublethal concentrations of toxicants has been shown to alter macrophage phagocytic activity in several fish species. This alteration is thought to stress the immune system and increase susceptibility to disease (Ziskowski and Murchelano, 1975; Overstreet and Howse, 1977; Sindermann, 1979; Weeks and Warinner, 1984; Wolke et al., 1985; Fries, 1986; Weeks et al., 1986). For example, studies performed with cells from the pronephros of striped bass, *Morone saxatilis*

(Elsasser et al., 1986), and rainbow trout, *Salmo gairdneri* (Stave et al., 1984), have shown varied chemiluminescent responses to bacterial and chemical stimuli after exposure to heavy metals. Evidence suggests that while low-level effects of toxicants may not be readily exhibited by external symptoms, small amounts of chemicals may increase susceptibility to disease and parasitic infections in exposed populations (Ziskowski and Murchelano, 1975; Wolke et al., 1985).

Stress may be defined as the modification of physiological processes where long-term survival is threatened unless conditions change (Heath, 1987). It can also be described as an environmental stimulus which, by exceeding a threshold value, disturbs normal function (Bayne, 1985). An organism may tolerate stress by altering its physiological or biochemical state. For this reason, species inhabiting polluted water serve as indicators to detect important interactions among chemicals and other environmental factors that traditional water analysis techniques may not fully detect (Heath, 1987). Since the macrophage/cellular immune system plays a fundamental role in the inhibition of cancer and disease, results from immunoassays may provide an early indication of whether a fish population is stressed before external damage or tumor development is observed.

It is hypothesized that alterations in the cellular immune system exist in fish that reside in the Elizabeth River due to a pollution-stressed environment. Gross abnormalities such as cataracts and skin lesions have been noted in fish taken from the Elizabeth River (Hargis et al., 1984; Huggett et al., 1986; Hargis et al., 1989). For these reasons, test fish for this study were obtained from the Southern Branch of the Elizabeth River, Virginia. This study was designed to detect modulations in the cellular immune system before overt toxic responses were evident.

1.1 Fish Immunology

The structure and function of fish immune systems is associated with phylogeny

(Sharma, 1981). Jawless (class Agnatha) and cartilaginous (class Chondrichthyes) fish such as hagfish and sharks, respectively, have a relatively simple immune system as compared to the more advanced bony fish groups (superorder Teleostei). Many invertebrates and some less advanced vertebrates have essentially a non specific cell mediated immune response. Slightly more "advanced" forms begin to demonstrate cell specificity and immunological memory (Albrecht, 1988). The immune systems of teleost fish begin to approach the complexity and efficiency of higher vertebrates (Sharma, 1981).

Fish, as a group, lack bone marrow and distinct regional lymph nodes (McCumber et al., 1982) which are critical regions of mammalian immune systems. The teleost kidney or pronephros area, performs the functions normally performed by the bone marrow and lymph nodes of mammals (Smith et al., 1970; Ellis and de Sousa, 1974). The pronephros of teleosts resembles mammalian lymph nodes by containing a phagocytic reticulum associated with lymphocytes and antibody producing cells (Chiller et al., 1969).

Teleosts have a humoral and cellular immune system but maintain a limited humoral response where only the IgM or natural antibody immunoglobulin class is present (Jurd, 1985). A type of cell mediated (T-cell) immune response exists in fish where they exhibit delayed hypersensitivity and reject foreign tissue grafts (Sharma, 1981). In cases where specific humoral defense mechanisms are inhibited, such as in extremely cold water, the phagocytic activity within the cellular defense system exhibits elevated activity (McCumber et al., 1982). Sharma (1981) suggested that the cellular immune system was a more reliable indicator of changes in fish immune systems since antibodies constitute less than 10 percent of the total serum proteins in fish.

In 1882, Metchnikoff was the first to propose "The Theory of Phagocytes" as a primary element in host resistence and the increased ability of phagocytic cells to destroy or digest parasitic organisms (Herbert et al., 1971). He also identified macrophages as the primary element in host resistance to bacterial and parasitic infections (Albrecht, 1979). In the cellular system of teleosts, macrophages are an important part of the total

immune system as well (Ellis, 1977; McKinney et al., 1977). In addition to phagocytic activity, macrophages function to activate T-helper cells, recognize antibody and/or complement associated with foreign material (Playfair, 1979), and secrete products that are associated with the inflammatory process (Unanune and Allen, 1987). Macrophages are produced in the pronephros of the kidney and spleen (Rijkers, 1981; MacArthur and Fletcher, 1985). In many fish species, these organs may perform multiple functions (Sharma, 1981), however, the excretion function of the pronephros is completely lost in adult fish and the organ performs mainly hemopoietic (blood forming) and lymphoid cell development functions.

Activated or stimulated macrophages exhibit elevated cytotoxic properties in relation to unactivated cells (Herbert et al., 1971). They seek out foreign material, including disease-causing agents such as microorganisms, and engulf them by phagocytic or "cell-eating" processes. Once activated, macrophages can ingest particles more rapidly, have higher quantities of enzymes to assist phagosomes and have greater oxidative activity, suggesting they have enhanced hydrogen peroxide formation (Stossel, 1974).

Macrophages are possibly stimulated after exposure to immunologic adjuvants or lymphokines. Endotoxin, the lipopolysaccharide component of gram-negative bacterial cell walls, has also been shown to induce an increased stimulation of the phagocyte system (Stuart, 1970). Many macrophage activating agents such as viruses, endotoxin, or lymphocyte activation products, all induce interferon. Interferon is an antiviral protein which is produced in response to infection by a virus or other chemical agent that prevents reproduction of the virus and induces resistance to other viruses (Barnhart, 1986). Therefore, it has been suggested that interferon, acting via cyclic nucleotides, is the common pathway for non specific macrophage activation (Albrecht, 1988).

The physical process of phagocytosis is similar in most species and begins with attachment of a foreign particle to the macrophage membrane. It is sometimes preceded *in vivo* by a chemically guided movement, or chemotaxis, toward its target (MacArthur and Fletcher, 1985). Extensions of the membrane or pseudopods, are formed and

subsequently surround the foreign particle. Gradually, the particle is engulfed when the opposing extensions meet and the pseudopodia fuse. Ultimately, the particle is enveloped within the cytoplasm of the cell and may be surrounded by a vacuole, or phagolysosome (McKinney et al., 1977; Albrecht, 1979)(Figure 1.1). Stimulated macrophages often show increased spreading and a greater degree of surface irregularity. Whereas non-stimulated and immature cells, perhaps in the process of differentiation, tend to have smoother surfaces (Albrecht, 1979).

During the ingestion and digestion process, various enzymes such as lysozymes and peroxidases are produced. Specific oxygen metabolites are used in killing internalized cells (MacArthur and Fletcher, 1985) as well as melanin which is used to aid the peroxide and peroxidase system in killing bacterial cells (Van Woert and Palmer, 1969; Ellis et al., 1976; Weeks et al., 1986). Several oxygen dependent killing mechanisms are postulated to occur during phagocytosis; these include: 1) the peroxide-halide-myeloperoxidase mechanism, and 2) a mechanism using highly reactive oxidizing radicals (Gabig and Babior, 1981).

In order to protect their own cells from destruction, macrophages must possess a system for neutralizing hydrogen peroxide and other cytotoxic agents. Macrophages defend themselves against self destruction by using enzymatic antioxidant systems such as: 1) superoxide dismutase, which converts O^{2-} to O_2 and H_2O_2, 2) glutathione reductase and peroxidase, to reduce H_2O_2 to H_2O, and 3) catalase which converts H_2O_2 to O_2 and H_2O (Gabig and Babior, 1981). After oxidization by hydrogen peroxide, reduced glutathione is regenerated by the biochemical reactions of the hexose monophosphate shunt. The peroxide produced during disturbances of the macrophage increases the activity of this shunt (Stossel, 1974).

Measurements of phagocytosis and the byproducts of the associated biochemical reactions are important in demonstrating the actions of the cellular immune system. Allen et al. (1972) found that cells phagocytosing bacteria exhibit chemiluminescence (CL), or an emission of photons, in a dose-dependent manner. This assay has been used by several researchers as a tool to study the phagocytic activity of fish cells (Scott and

Klesius, 1981; Stave et al., 1984; Weeks et al., 1986) as well as numerous studies on mammalian systems including humans (Allen et al., 1972). The chemiluminescent assay was used in this study to demonstrate the phagocytic activity of macrophages obtained from *Fundulus heteroclitus.*

1.2 *Fundulus heteroclitus*

Fundulus heteroclitus or the mummichog, was chosen as the test species for several reasons. First, these fish normally feed on algae, invertebrates, and detritus found along marsh edges and the bottom (Valiela, 1984; Abraham, 1985), where sediments are likely to contain pollutants such as the creosote residues found in the Elizabeth River (Bieri et al., 1982). Second, Hightower et al. (1984) noted that *F. heteroclitus* are hardy, yet sensitive to chemical carcinogens. Carcinogen tests that use rats or mice are relatively expensive and may take up to two years to complete. In comparison, fish models are faster and less expensive (Couch and Hargis, 1984). Third, *F. heteroclitus* are nonmigratory resulting in a limited home range (Hoff, 1985). This is a very important characteristic in a test species since the health of the fish should reflect environmental conditions within its home range (Bigelow and Schroeder, 1953). Fourth, *Fundulus* spp. are available nearly year-round in the marsh areas and can be trapped rather than seined or trawled in a net, thus reducing the risk of injury during capture. In addition, *F. heteroclitus*, due to their small size, are easily handled and maintained. This species of fish was also found to feed in captivity unlike some other testable fish species, such as hogchoker (*Trinectes maculatus*)(Robinson, Personal Communication).

For the selection of a standard test fish, Adelman and Smith (1976) listed several necessary criteria. Fish must: 1.) have a constant response to a broad range of toxicants tested under similar conditions, 2.) be available all year, 3.) be available in large quantities, 4.) be easy to handle and easy to transport, 5.) have a complete life cycle within 1 year or less, 6.) have easily distinguished sexes, and 7.) have chronic testing results in 3-4 months. *Fundulus heteroclitus* meets these requirements, and thus

qualifies as a favorable aquatic species to test.

1.3 Objectives

Various questions were investigated regarding the health of the *Fundulus heteroclitus* population in the Elizabeth River. Differences in macrophage activity between the York and Elizabeth River populations, the effect of depuration opportunity for Elizabeth River fish and uptake of chemical pollutants from Elizabeth River water in York River fish, and the effect of water temperature on the cellular immune system were investigated.

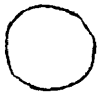
The purpose of this study can be stated as three major objectives:

1. Can a functional assay be developed to determine if *Fundulus heteroclitus* have been stressed by environmental pollutants?

2. Does long-term or chronic exposure to heavily polluted water modulate or alter the cellular immune macrophage response in *Fundulus heteroclitus?*

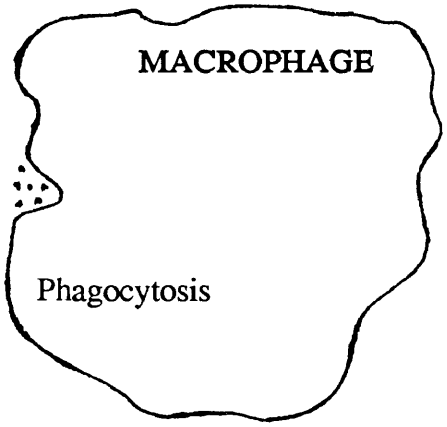
3. Can *in vivo* environmental temperature stress in *Fundulus heteroclitus* compound pollution effects with regard to changes in macrophage activity?

Figure 1.1: Interactions of macrophages and macrophage products (taken from Albrecht, 1979).

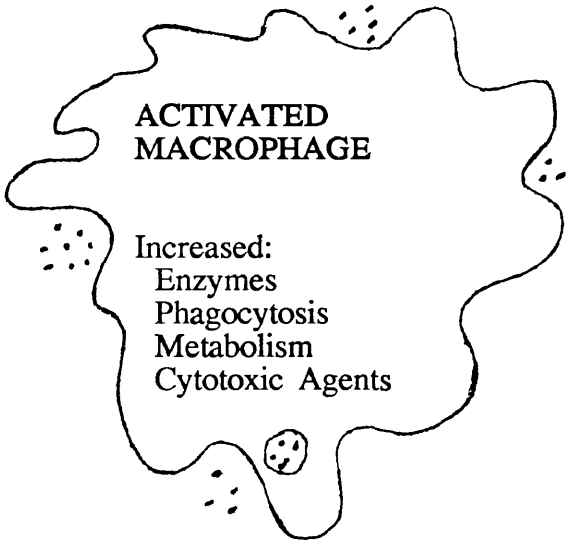


MONOCYTE

Location
Peripheral Blood



Location
Kidney
Spleen
Peritoneal Cavity
Peripheral Blood
Gills



Location
Sites of Inflammation

Characteristics
Viracidal Microbiological Macrophage
Suppressor Macrophage
Tumoricidal Macrophage
Delayed Hypersensitivity Responses

II STUDY SITE DESCRIPTION

2.1 Elizabeth River

The Elizabeth River, located in Southeastern Virginia, is a major tributary of the James River which empties into the James River at Hampton Roads. The Elizabeth River is divided into a main branch with 4 major tributaries: the Lafayette River, and the Eastern, Western, and Southern branches. The Elizabeth River serves as the drainage basin for approximately 777 km^2 of highly urbanized and industrialized land (Cerco and Kuo, 1981). There is very low topographic relief within this drainage basin, approximately 6.1 m, resulting in low net water movement to flush out pollutants (Neilson and Sturm, 1978; Cerco and Kuo, 1981).

The Elizabeth River has been a populated and industrialized area with extensive civilian and military shipbuilding activity, industry and shipping commerce for nearly 300 years. It has been associated with manufacturing and military activity due to its strategic location and protected port (Hargis et al., 1984). Most of the pollutants deposited in this area remain close to deposition sites and usually adhere to the sediment. Researchers conducting studies on the water column and sediments of the Elizabeth River found concentrations of 10 ug g^{-1} of benzo(a)pyrene in the top 2 centimeters of sediment cores (Lu, 1982). A geochemical study conducted by Bieri et al. (1982) indicated that the Southern Branch (Station 217), where creosote biproducts (PAHs) once were discarded, was one of the most polluted areas of the Elizabeth River (Table 2.1).

The study site was located in the lower segment of the Southern Branch, approximately 16 miles south of the city of Norfolk, Virginia at coordinates of latitude 36° 47' 30'' and longitide 76° 17' 30'' (Figure 2.1). Tidal range varies from 0.9 meters near the headwaters at Great Bridge, Virginia to 0.8 meters at the mouth in Hampton

Roads, Virginia (U.S. Army, 1976). The current at the collection site was moderate with additional wave action due to tugs, barges and recreational boats using the Intercoastal Waterway. Salinity and water temperature during the time of collection averaged 21.5 ppt and 25 °C, respectively (Table 2.2). Dissolved oxygen level was measured at 5.4 mg l^{-1}. The substrate at the collection area was composed of sand, brick fragments and an area with rip-rap rocks for erosion purposes. The site was adjacent to the Tenneco Oil Company pipeline loading dock and a small fringing marsh.

2.2 York River

The York River estuary is formed by the joining of the Mattaponi and Pamunky Rivers at West Point, Virginia. It is approximately 32 miles long and has an average width of 1.6 miles with the narrowest part at Yorktown. The river is relatively shallow and ranges in depth from 1 to 20 feet (Galtsoff et al., 1938). Timberneck Creek, a tributary of the York River, near White Marsh, Virginia, located at latitude 37° 18' 30'' and longitude 76° 31' 30'', served as a comparable control collection site. For clarity to the reader, the control site will be referred to as the York River. Sediment at the collection site was composed of mud and silt and a fringing *Spartina alterniflora* marsh lined the banks along both sides of the creek and residential dock. Salinity and temperature during the time of collection averaged 21.2 ppt and 25 °C, respectively. Dissolved oxygen levels averaged 8.05 mg l^{-1}.

Table 2.1 Chemicals found in surficial sediments from the Southern Branch of the Elizabeth River, Virginia and their potential effects on aquatic organisms. (Chemical list from O'Connor and Huggett, 1988 and Hargis et al., 1984)

Chemical/Agent	Effect	Reference
Benzothiophene 2-Methylnaphthalene 1-Methylnaphthalene Biphenyl Fluorene Dibenzothiophene Phenanthrene	Decreased growth - mud crabs	Laughlin and Neff, 1980
Anthracene	Destabilization of lysosomal membranes	Moore et al., 1978, 1980
Fluoranthracene Pyrene Benzo[a]fluorene Benzo[b]fluorene Benz[a]anthracene Chrysene Benzofluoranthrene Benzo[e]pyrene	Tumors - bottom fish	Black et al., 1980
Benzo[a]pyrene	Liver tumor - rainbow trout	National Cancer Inst. Monograph, 1984
	Oocyte Abnormalities - flathead sole	Malins, 1982
Indenopyrene Perylene Benzo[g,h,i]perylene Indeno[1,2,3-cd]pyrene		
Tributyltin	Alteration of macrophage activity	Rice and Weeks, 1989
General polluted sediments	Fin erosion - hogchokers, toadfish	Bender et al., 1988 Murchelano and Ziskowski, 1976
	Cataracts - spot, croaker, weakfish	Bender et al., 1988
	Neoplasms - brown bullhead	Black et al., 1985
	Decreased immune response - flatfish	Mearns and Sherwood, 1976
	Decreased macrophage activity - spot and hogchokers	Weeks and Warinner, 1984
Heavy Metals - Cu, Al	Decreased macrophage CL	Elsasser et al., 1986
Cd	Increased macrophage CL	Elsasser et al., 1986

Figure 2.1: Chesapeake Bay region showing York and Elizabeth River study site locations.

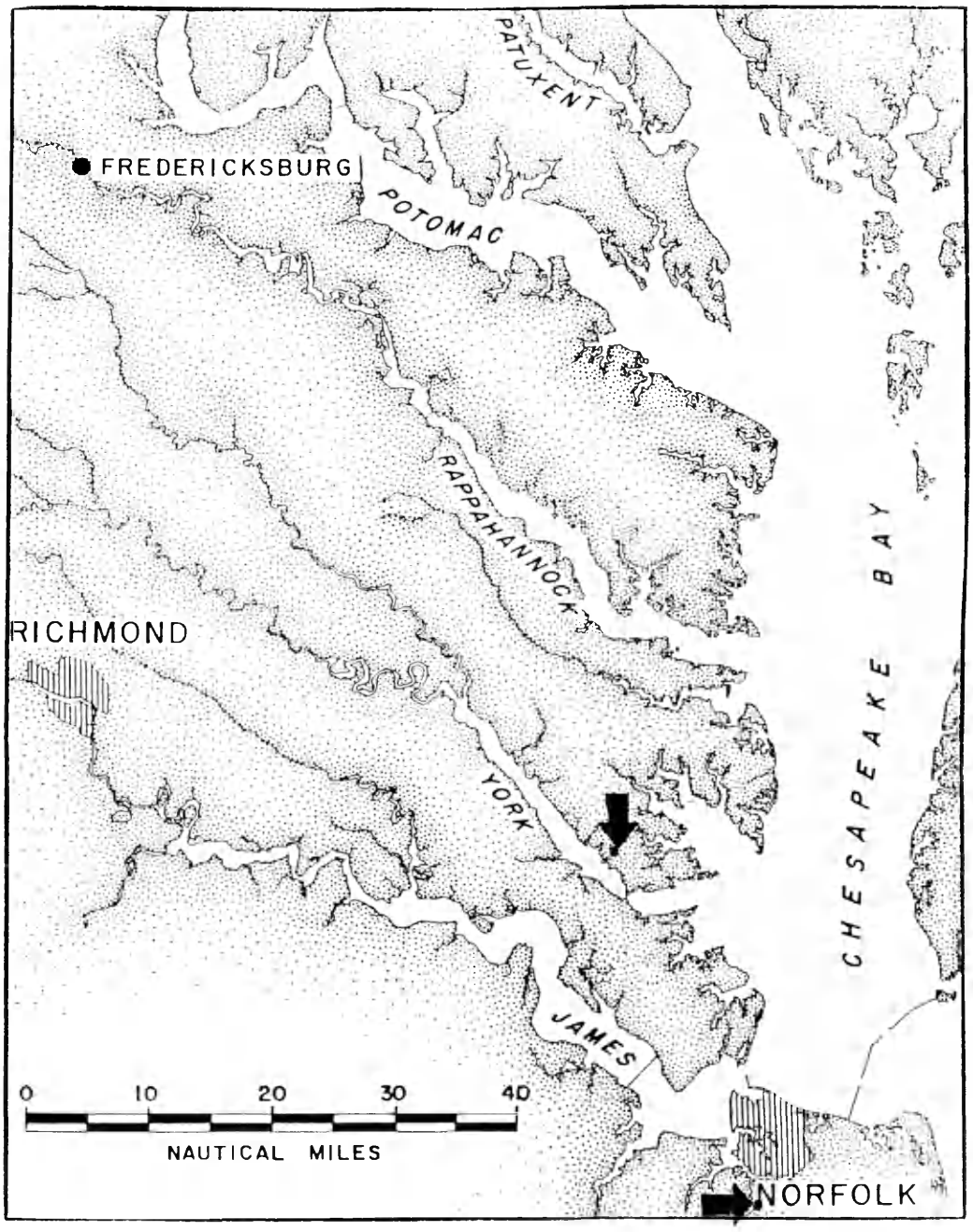

PATUXENT
FREDERICKSBURG
POTOMAC
RAPPAHANNOCK
RICHMOND
YORK
JAMES
CHESAPEAKE BAY
0 10 20 30 40
NAUTICAL MILES
NORFOLK

Table 2.2: Temperature, salinity and dissolved oxygen measured in the York and Elizabeth Rivers.

	Elizabeth River	York River
Water Temperature (°C)		
	28 (8/13/87)	29 (8/11/87)
	28 (8/24/87)	29 (8/24/87)
	27 (8/27/87)	28 (8/27/87)
	28 (8/31/87)	29 (8/31/87)
	27 (9/8/87)	28 (9/8/87)
	27 (9/11/87)	28 (9/11/87)
	27 (9/16/87)	28 (9/16/87)
	27 (10/6/87)	25 (10/6/87)
	22 (10/13/87)	15 (10/13/87)
	18 (4/25/88)	22 (4/25/88)
	23 (6/10/88)	24 (6/10/88)
	28 (7/22/88)	27 (7/22/88)
Salinity (ppt)		
	23 (8/13/87)	22 (8/11/87)
	22 (8/24/87)	21 (8/24/87)
	21 (8/27/87)	21 (8/27/87)
	21 (8/31/87)	20.5 (8/31/87)
	21 (9/11/87)	21 (9/11/87)
	21.5 (10/13/87)	20 (10/13/87)
	16 (4/25/88)	15 (4/25/88)
	15 (6/10/88)	16 (6/10/88)
	21 (7/22/88)	20 (7/22/88)
Dissolved O_2 (mg/l)		
	------	6.9 (8/11/87)
	5.4 (8/24/87)	9.2 (8/24/87)

III. CHEMILUMINESCENT ASSAY

3.1 General Overview

The chemiluminescent (CL) assay was first described by Allen et al. (1972) using human polymorphonuclear leucocytes and has been used to study phagocytosis in many fish species including channel catfish (*Ictalurus punctatus*)(Scott and Klesius, 1981), striped bass (*Morone saxatilis*) (Stave et al., 1984), rainbow trout (*Salmo gairdneri*) (Elsasser et al., 1986), spot (*Leistomus xanthurus*) and croaker (*Micropogon undulatus*)(Weeks and Warinner, 1984; Weeks et al., 1986). The CL assay measures the emission of photons that are produced during biochemical and cytophysical reactions during macrophage phagocytic activity (Herbert et al., 1971). A phagocytic "respiratory burst" occurs when there is a change in oxygen metabolism resulting in cell membrane depolarization (Gabig and Babior, 1981; Stave et al., 1984).

Superoxide radicals and hydrogen peroxide are used by macrophages to destroy foreign cells such as bacteria, yeast, and viruses (Gabig and Babior, 1981). During phagocytic activity, superoxide radicals become electronically excited and electrons are elevated to orbitals higher than ground state. This excess energy is dissipated by thermal decay, light emission and increased chemical reactivity (Rosen and Klebanoff, 1976; Welch, 1980). The light generated during the relaxation of electrons to the ground state can be detected and quantified using a liquid scintillation counter (LSC). The emission of light can be amplified using luminol, which upon oxidation, results in the production of an excited aminophthalate anion that produces light as it relaxes to ground state (DeChatelet and Shirley, 1982).

The following biochemical processes occur in macrophages during phagocytosis in response to a stimulating agent:

Stimulus

|

Membrane depolarization

|

O_2 production, change in cyclic

AMP levels, and lysosomal enzyme release

Measuring the CL response is considered a more sensitive method for detecting the biochemical reactions asssociated with phagocytosis than direct microscopic observation of phagocytosis or the phagocytosed materials (Stave et al., 1984). Correlations between CL response and bactericidal function have been shown to be greater than with procedures that directly quantify superoxide anion and hydrogen peroxide formation. The CL assay can be used to demonstrate the mechanism of phagocytosis, specifically the kinetics and peak phagocytosing ability of the cells.

3.2 Methods

Macrophages were obtained from the kidneys of *Fundulus heteroclitus* in sufficient numbers to be separated and used for *in vitro* studies. Fish were sacrificed during the morning of the experiment by severing the spinal cord directly behind the head. Kidneys were removed, rinsed in 5 ml of Teleost Buffered Saline (TBS) and placed in 7 ml complete Minimum Essential Medium (MEM). Complete MEM consisted of 500 mls of medium containing 0.15 g streptomycin, 0.15 g penicillin, 1.65 g glucose, and 0.1 ml heparin. Kidneys were pooled in order to obtain sufficient tissue for cell

separation. Following collection, kidneys were homogenized in a sterile tissue grinder and the supernate poured into a sterile test tube and refrigerated at 10 °C for one hour until Percoll gradients could be made for cell separation.

Isoosmotic Percoll (Phamacia brand) discontinuous density gradients, made from a colloidal suspension of silica, were layered in 25 ml centrifuge tubes for use in cell centrifugation. The cells settle through the gradient until they reach a point at which their specific gravity is equal to that of the medium, thus separating into bands of differing densities (Herbert et al., 1971). Percoll gradients were carefully layered by pipette with the highest density at the bottom. Densities of the four layers were 1.60, 1.50, 1.45, and 1.40 g ml^{-1}. Approximately 3 ml of cells, at a concentration of 2 to 3 x 10^6 cells ml^{-1}, was the fifth layer to be added to the gradient tubes. Gradients were spun in a Sorvall centrifuge, refrigerated to 4 °C, at 1800 rpm for 15 minutes. Preliminary tests indicated that the majority of *F. heteroclitus* macrophages were contained between gradient densities of 1.50 and 1.60 g ml^{-1} (Figure 3.1). Macrophages from other species of fish, such as spot (*Leistomus xanthurus*), flounder (*Paralichthyes dentatus*), and hogchoker (*Trinectes maculatus*) also exhibit similar density gradient separation in Isoosmotic Percoll (Weeks and Warinner, 1984). During separation, a small amount of undesirable material such as lymphocytes, artifacts, and some red blood cells may also be retained in the macrophage layer of the Percoll gradient.

The macrophage layer was carefully extracted, placed into sterile test tubes and rinsed with 6 ml of Hanks Buffered Saline Solution (HBSS). Cells were recentrifuged for five minutes and the supernate was discarded. The cellular pellet was resuspended in 2 ml of HBSS and refrigerated until CL tests were performed the following morning.

3.3 Reagent Preparation

Preparation, concentration, and volumes of chemicals and buffers used for CL experiments were adapted from Trush et al. (1978), Scott and Klesius (1981), and Stave

et al. (1984). Zymosan, a mixture of polysaccharides, proteins, and ash derived from the yeast cell wall fraction of *Saccharomyces cerevisiae*, was used to stimulate and activate macrophages (Herbert et al., 1971). Twenty milligrams of zymosan was added to two milliliters of teleost buffered saline (TBS) and mixed thoroughly. The mixture was boiled for 30 minutes in a water bath then centrifuged for 5 minutes at 600 rpm. The zymosan pellet was resuspended in 20 ml TBS and stored in three 50 ml centrifuge tubes until use with a maximum storage time of 2 weeks. The final concentration of zymosan was 1 mg ml^{-1} (Scott and Klesius, 1981; Trush et al., 1978).

Luminol (Aldrich), a light amplifier, was prepared using a potassium salt preparation (Trush et al., 1978). Stock Luminol solution consisted of 14 mg of Luminol (3-aminophthalhydrazide), 0.78 g KOH, and 0.62 g boric acid, diluted in 10 ml of distilled water. The final concentration of stock Luminol solution was 1.4 mg ml^{-1}. Working dilutions of Luminol were made with stock solution and HBSS and used within 3 hours of preparation. Stock HBSS (10X) consisted of 53.2 g NaCl, 2.6 g KCL, 6.6 ml glucose, 0.4 g KH_2PO_4, and 0.32 g Na_2HPO_4 dissolved in 100 ml of distilled water.

3.4 Chemiluminescence Methodology

Trypan blue stain was used to assess cell number and viability prior to the CL assays. A cell suspension of 0.1 ml was added to 0.9 ml trypan blue dye. Dye enters the cell only when the plasma membrane is severely disrupted; thus dead and dying cells stain blue (Herbert et al., 1971). Cell count was performed using a hemocytometer and a light microscope.

Chemiluminescent activity was determined using a modified method of Trush et al. (1978) and Warinner et al. (1988). A Beckman liquid scintillation counter (LSC), model LS-150 set in the out-of-coincidence mode for lower energy reactions, was used to measure the CL response. The out-of-coincidence mode allows the events from both photomultiplier tubes to be seen individually and summed (Trush et al., 1978). Gain

was set at 60 percent and discriminators were set from 0 to 1000 (open window). Although Scott and Klesius (1981) recommended a benchtop Pico-Lite Luminometer, preliminary testing with *Fundulus heteroclitus* indicated that the LSC could record the CL activity at a higher scale. In addition, the sample changer of the LSC can be precisely timed. This permits the use of a multiple vial system whereby a series of vials can be rotated such that control and experimental vials can be measured almost simultaneously. Macrophage cell concentrations were standardized to 1 x 10^5 cells ml^{-1} for the CL assay to maintain LSC readings within the appropriate range. The liquid scintillation counter, operated in the out-of-coincidence mode, can record a maximum of 900,000 cpm. Macrophage and luminol concentrations that generate CL responses exceeding this value were unusable. Samples were run in triplicate using aliquots of 0.5 ml macrophage cells (1 x 10^5 cells ml^{-1}), 0.5 ml luminol at 14 *u*g ml^{-1}, and 0.5 ml unopsonized zymosan at 1 mg ml^{-1} for each scintillation vial. Since exposure of luminol to fluorescent light may affect the results, luminol and zymosan were added to the vials in a dark room with a red safelight.

A standard kinetic curve for macrophage chemiluminescent activity is represented in Figure 3.2. The ideal complete kinetic cycle should form a bell shaped curve with macrophages responding to the stimulant at an increasing rate until the cells become exhausted and the CL rate drops. To determine if varying dilutions of Luminol had a proportionate effect on the CL response of macrophages, a test was performed using a graded series of concentrations. The four stock Luminol dilution factors tested were: 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. The results of CL activity on York River *Fundulus* macrophages, un-opsonized zymosan (1 mg ml^{-1}), and varied Luminol concentrations are presented in Figure 3.3. The lowest dilution (1:1000) gave the highest CL reading of 3.37 cpm (SE = 1.77; N = 4). Likewise, the highest dilution gave the lowest CL reading of 0.92 cpm (SE = 0.07; N = 4) . The 1:10,000 dilution was chosen for the CL experiments due to its ability to enhance peak values without overextending the upper limits of the LSC counter.

Blank vials were prepared by combining 0.5 ml (1 x 10^5 cells ml^{-1}) macrophages, 0.5 ml (14 *u*g ml^{-1}) luminol, and 0.5 ml HBSS (instead of zymosan). Blank vials were placed with each triplicate set and counted for a series kinetic cycle. Vials were counted in 12 second intervals during the duration of the kinetic cycle. The baseline response was defined as CL counts obtained in the absence of any stimulus (ie, blank vials). The baseline response was subtracted from experimental counts and activity was recorded as counts per minute per cell. Each series triplicate was averaged to obtain a single mean value. Statistical analysis was performed on the average peak value of each trial for each river.

Figure 3.1: Percoll density gradients depicting location of macrophage layer for *Fundulus heteroclitus*.

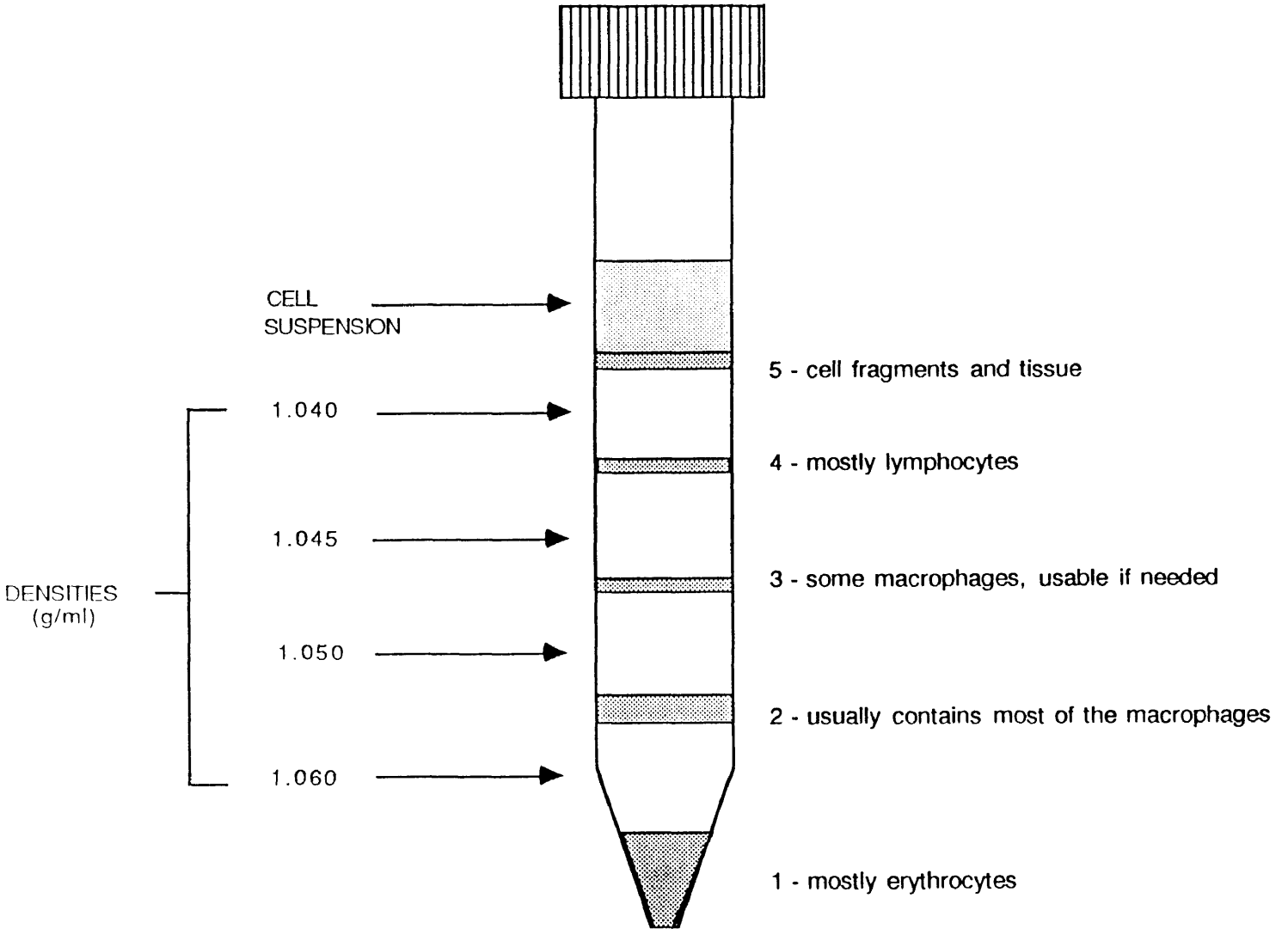
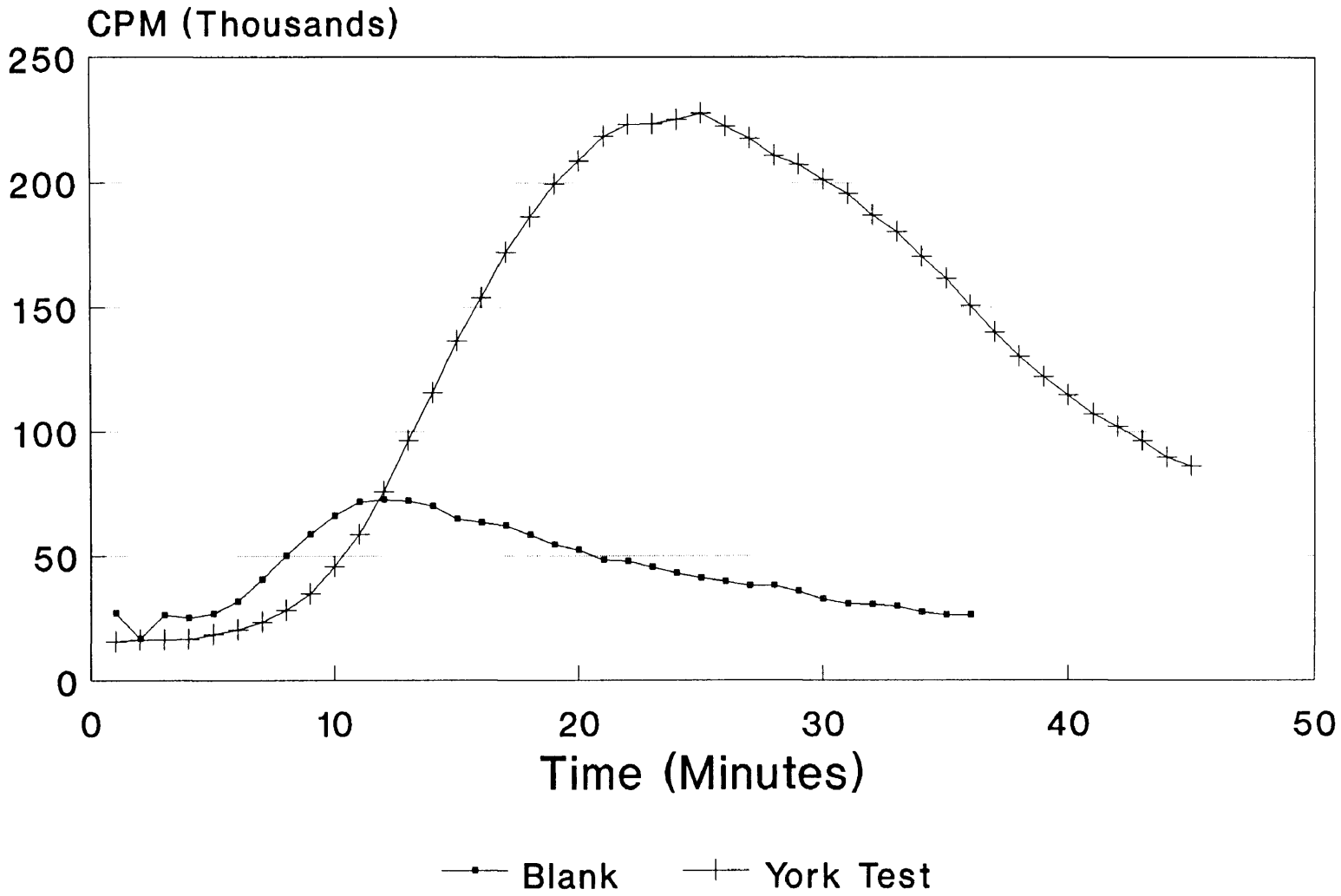
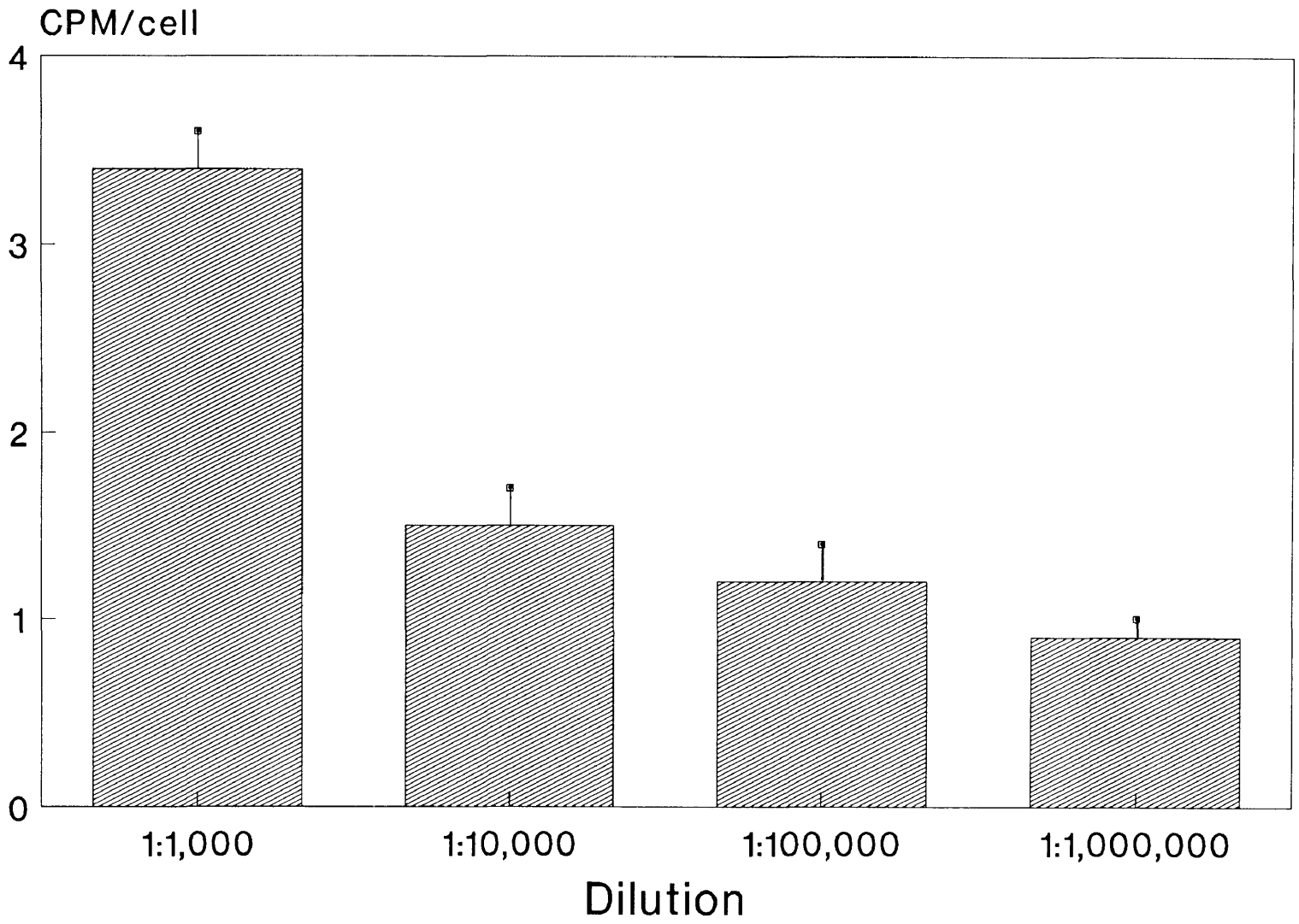

CELL
SUSPENSION
1.040
1.045
1.050
1.060
DENSITIES
(g/ml)
5 - cell fragments and tissue
4 - mostly lymphocytes
3 - some macrophages, usable if needed
2 - usually contains most of the macrophages
1 - mostly erythrocytes

Figure 3.2: Representation of a standard kinetic curve of the chemiluminescent response for *Fundulus heteroclitus*.



600000 cells/ml

Figure 3.3: Macrophage chemiluminescent response for *Fundulus heteroclitus* as a function of luminol concentration.


CPM/cell
4
3
2
1
0
1:1,000
1:10,000
1:100,000
1:1,000,000
Dilution

IV. FIELD EXPERIMENT

4.1 Introduction

Research on the effects of pollution on the cellular immune system has generally focused on the *in vitro* application of chemicals to cells or the accute LC_{50} levels of pollutants. This chapter investigates the effect of *in vivo* exposure of environmental pollutants using a field study of *Fundulus heteroclitus.*

Numerous studies conducted on the water, sediment and aquatic organisms in the Elizabeth River have indicated high concentrations of environmental pollutants in comparision to surrounding rivers (VSWCB 1983, 1984). *Fundulus heteroclitus* living in these severely degraded waters could be affected by these conditions and consequently show an altered cellular immune response to stimulation. This chapter discusses an attempt to determine if *Fundulus heteroclitus* taken from the Elizabeth River exhibit an altered macrophage chemiluminescent response as compared to those from the York River which are not exposed to high levels of chemical pollution.

4.2 Methods

Methods used for the field experiment are given in sections 3.2 to 3.4. Water temperature and salinity were measured at each test site at the time of collection (Table 2.2). Fish were transported in aerated coolers and immediately placed in 10 gallon glass aquaria equipped with charcoal filtration and airstone systems upon return to the laboratory. Each fish population was maintained in its original river water and acclimated for 48 hours prior to experimentation. Fish were randomly chosen from each tank, sacrificed and their kidneys were extracted. Fish lengths and weight measurements were determined and are given in Table 4.1.

4.3 Results

Average body weight and length of *Fundulus heteroclitus* collected from the York River were 8.1 g and 8.3 cm, respectively. Fish collected from the Southern Branch of the Elizabeth River averaged 9.3 g in body weight and 8.7 cm in total length. All fish for the field experiments were collected between August 1987 and October 1987.

Water temperature in the York River ranged from 15 to 29 °C with an average temperature of 25.3 °C. In the Elizabeth River, water temperature ranged from 22 to 28 °C with an average temperature of 26.3 °C. The water temperature was measured at trap collection sites located near the shallow edge of the rivers where temperatures were usually higher during the summer months. Fish were subjected to this higher temperature while feeding at the river edges. Salinity of the York River and Elizabeth River averaged 19.1 and 19.0 ppt, respectively. The phagocytic activity of the two populations of *Fundulus* in the test and control areas was based on the average peak response of triplicate trials minus the blank count. Maximum peak CL response was higher in all trials of the Elizabeth River population where water temperatures were similar. Average peak CL response of the field experiment results is shown in Figure 4.1.

On October 13, 1987, water temperature in the York River was considerably lower than in the Elizabeth River (YR = 15 °C, ER = 22 °C) (Table 2.2). While using the cells from these fish, macrophage CL results were inverted as compared to other field responses in the temperature range between 27 and 29 °C. The CL tests were re-run and similar results were obtained. Because of the rapid drop in York River water temperature and the considerable temperature differences in the two rivers, the CL results from fish collected on October 13, 1987 were excluded from statistical analysis in this part of the experiment. A comparison of field results and water temperature is presented in Figure 4.2. The modulation of CL activity initiated the investigation of

temperature as a significant factor in influencing chemiluminescent activity. The results of the temperature experiment follow in Chapter 6, Section 3.

Results indicate a higher peak chemiluminescent activity in Elizabeth River *F. heteroclitus* macrophages (Mean = 3.72 cpm, SE = 0.77, N= 8) compared to peak chemiluminescent activity in York River *F. heteroclitus* macrophages (Mean = 1.20 cpm, SE = 0.31, N=8). Results were statistically compared using the nonparametric ranked sum Mann-Whitney Test for two groups (Zar, 1984). Test results indicate a significant difference at an alpha level of 0.01, N = 8, between York and Elizabeth River *F. heteroclitus* CL activity (U' = 58, $U_{0.01\ (2)\ 8,8}$ = 57).

4.4 Discussion

There are several lines of evidence that may explain the observed pattern of an elevated chemiluminescent response in pollutant-exposed fish. First, macrophages may be stimulated from constant activity to counteract the effects of pollution. In Adams and Hamilton (1984), authors cite that lymphokines present in fish immune systems due to irritation or exposure to polluted waters, may induce activation of normal tissue macrophages. This suggests that macrophages of *Fundulus* from the Elizabeth River may be rendered fully active due to the effect of lymphokines.

The chemicals in the water column or sediment of the Elizabeth River may themselves have a stimulatory effect on macrophages. Rice (1989) found that toadfish (*Opsanus tau*) macrophages exposed *in vitro* to 50 ug l^{-1} of tributyltin (TBT) had an increased macrophage CL response. This stimulatory response decreased to baseline levels when cells were exposed to TBT concentrations of 500 ug l^{-1}. Rice and Weeks (1989) hypothesize that the increased CL activity was the result of calcium influx to the macrophage and the resultant formation of reactive oxygen intermediates. In another study, Larson et al. (1988) reported *in vivo* exposure to low levels of naphthalene and dieldrin initially stimulated and increased the CL response of hemocytes isolated from

exposed oysters. In addition, stimulatory effects from the interaction of metals on enzymes in *Fundulus* was reported by Heath (1987). However, this stimulatory property was eventually detrimental to the organism over time or if exposed to higher concentrations. It is possible that stimulatory levels of PAH, TBT or other chemicals present in the Elizabeth River system were absorbed or assimilated to cause the increased CL response of *Fundulus heteroclitus* in the field study.

A second possible explanation is that macrophages of *Fundulus heteroclitus* may be genetically more responsive or preadapted to pollution levels. This statement implies that Elizabeth River fish are the survivors of a harsh environment and thus hardier than those sampled from the York River. Weis et al. (1981) found an increased tolerance of *F. heteroclitus* embryos to methylmercury in a polluted river versus a nonpolluted river. It was suggested that the polygynous mating system of this species of fish may provide a mechanism for rapid gene frequency change and development of a preadapted tolerance to pollution.

In summary, these conclusions suggest that Elizabeth River *Fundulus heteroclitus* macrophages were possibly stimulated by constant exposure to various chemical pollutants in the water column or sediment, or are genetically predisposed to the effects of the chemicals. It is difficult to pinpoint one chemical in the Elizabeth River system that may cause the alteration, however, synergistic effects or the combination of carcinogenic or mutagenic chemicals, may also be affecting the CL response seen in these experiments.

Figure 4.1: Average peak chemiluminescent values for the field experiment of 1987.

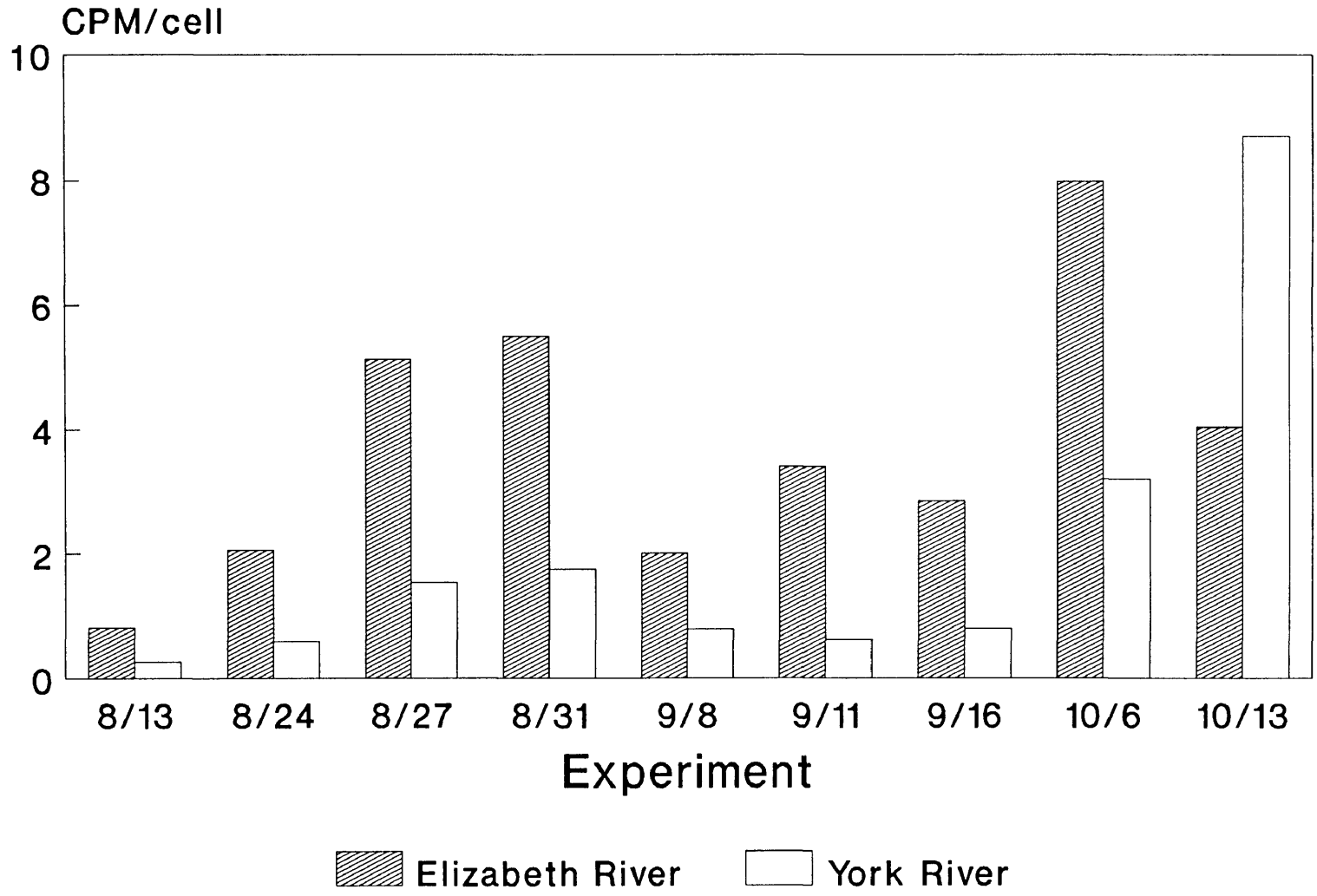
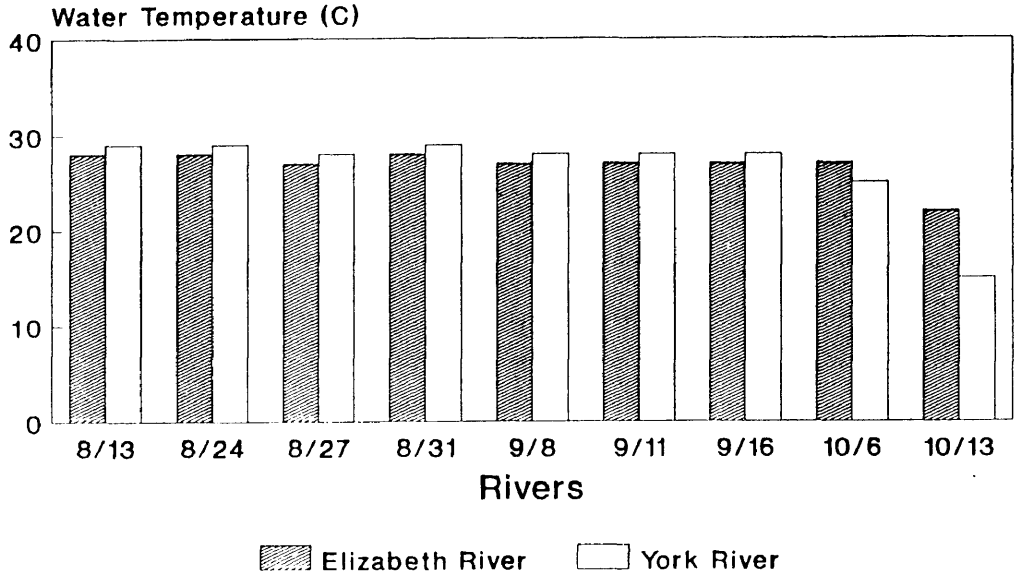

CPM/cell
10
8
6
4
2
0
8/13
8/24
8/27
8/31
9/8
9/11
9/16
10/6
10/13
Experiment
Elizabeth River
York River

Figure 4.2: Comparison of river water temperatures and 1987 field experiment.

Comparison of Water Temperature
York and Elizabeth Rivers 1987



Field Collection 1987

Average Peak CL Response
Field Experiment 1987

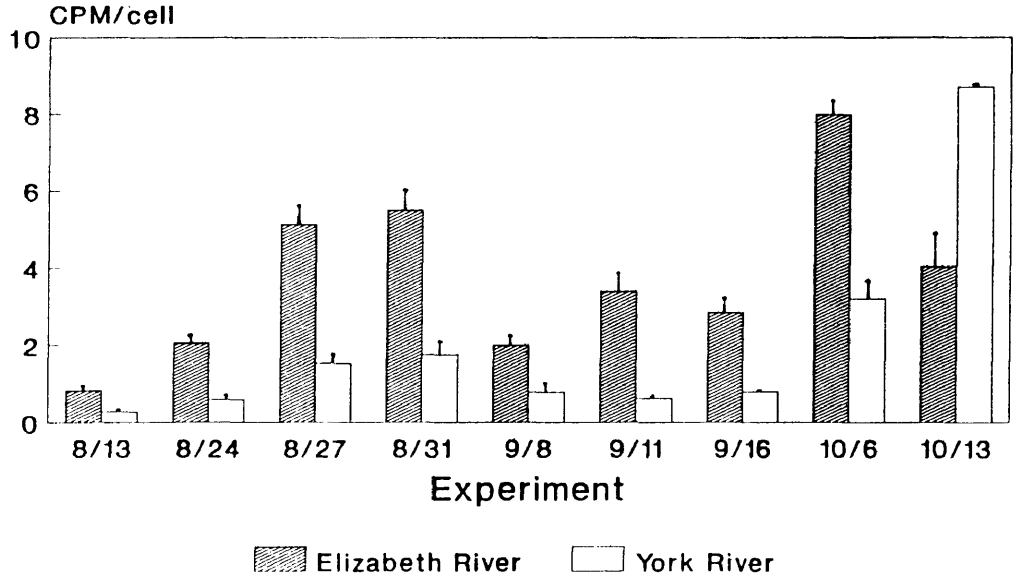


Table 4.1: Average total length and body weight of *Fundulus heteroclitus* used in the field experiment.

Elizabeth River

Experiment	BodyWeight (g)	Total Length (cm)	N
1	10.0	8.8	8
2	10.2	8.7	9
3	8.8	8.3	8
4	8.7	8.3	8
5	10.8	9.0	8
6	8.0	7.8	8
7	8.0	8.0	8
8	9.8	9.0	8

York River

Experiment	Body Weight (g)	Total Length (cm)	N
1	7.6	8.0	10
2	8.1	8.3	9
3	5.9	7.3	8
4	5.8	7.2	8
5	9.0	8.5	9
6	6.0	7.3	9
7	5.9	7.3	9
8	7.7	8.1	10

V. UPTAKE AND CLEARANCE STUDY

5.1 General Introduction

Many factors influence the uptake, metabolism and clearance of chemicals in marine organisms. Such biotic factors include growth, lifestage, gill ventilation, age, size and sex of the organism (Murty, 1986). Environmental or physiochemical parameters such as temperature, salinity, pH, photoperiod, nutrients, suspended sediments and dissolved oxygen/gases have also been shown to influence interactions between organisms and the chemical pollutants (Stuart, 1970; MacArthur and Fletcher, 1985). Spacie et al. (1983) has shown that fish can directly take up polyaromatic hydrocarbons or PAHs (naphthalene, alkylated naphthalenes, anthracene, and BAP) from the water column. Aquatic organisms often have higher levels of PAH in fatty body tissue than levels found in the water column, but equal to or less than those of bottom sediments (Neff and Anderson, 1981). Time required to achieve maximum chemical uptake and subsequent retention by fish is directly related to compound persistence in the water (Murty, 1986).

Murty (1986) reported that elimination of residues in body tissue after transfer to noncontaminated water is usually biphasic, with an initial rapid phase followed by a slower, later phase. In addition, larger fish may require a longer period of time to eliminate certain accumulated chemicals. Upon transfer to clean water, complete elimination of pesticides required about 30 to 50 days in rainbow trout (*Salmo gairdneri*) but only 1 week in spot (*Leiostomus xanthurus*)(Murty, 1986). Data on the rate of a 50 percent reduction of DDT in fish have varied from 10 to 30 days (Edgren, 1979).

Temperature has also been shown to influence the rate of uptake or clearance of pollutants within fish species. Significantly higher concentrations of chlorinated hydrocarbons were found in fish from a 15 °C aquarium than in the fish from a 5 °C

aquarium (Murty, 1986). An increase from 5 to 15 °C resulted in doubling the uptake of DDT and PCBs which was attributed to the subsequent doubling of the metabolic rate. A direct relationship between metabolic rate and accumulation was also suggested by Edgren (1979). However, Bender et al. (1988) found that the rate of uptake of pollutants in Elizabeth River sediments was not affected by temperature in oysters or clams.

5.2. Methods

Fundulus heteroclitus were collected from the York and Elizabeth Rivers as described in Methods (Chapter 4) . Water from both rivers was placed into six separate 10 gallon aquaria at ambient room temperature (22 °C). Fish that were collected from the York River were placed into three tanks containing Elizabeth River water. Fish collected from the Elizabeth River were placed into three tanks containing York River water. Tanks were filtered using a charcoal box filtration system which was cleaned weekly. Fish were fed the commercial fish food Tetramin ad libitum. After a 21 day acclimation period, the fish were sacrificed. Macrophage extraction was performed as described in Methods (Chapter 4).

5.3. Results of Uptake and Clearance Study

This was an accessory experiment to determine if uptake and clearance could alter the macrophage CL activity. Results of this experiment were clearly inverted from field results, as seen in Figure 5.1. Average peak macrophage CL activity for the York River field test was 1.20 cpm (SE = 0.31, N = 8). Mean peak CL activity in York River fish held in Elizabeth River water (uptake group) was 5.28 cpm (SE = 1.05, N = 3). In fish from the Elizabeth River field test, the average peak macrophage CL activity was

3.72 cpm (SE = 0.77, N = 8) while the average peak CL activity for Elizabeth River fish held in York River water (clearance group) was 1.25 cpm (SE = 0.12, N = 3).

Statistical analysis using the nonparametric rank sum Mann-Whitney test shows that CL activity of Elizabeth River fish held in York River water significantly differed ($P < 0.10$) from York River fish held in Elizabeth River water ($U_{0.10(1)3,3} = 8$, $U = 9$). In comparing the results of the field test with the uptake and clearance test, Elizabeth River clearance results were significantly different ($P < 0.10$) from Elizabeth River field samples ($U_{0.10(2)3,8} = 21$, $U = 22$). York River uptake results also differed significantly ($P < 0.05$) from York River field samples ($U_{0.05(2)3,8} = 22$, $U = 24$).

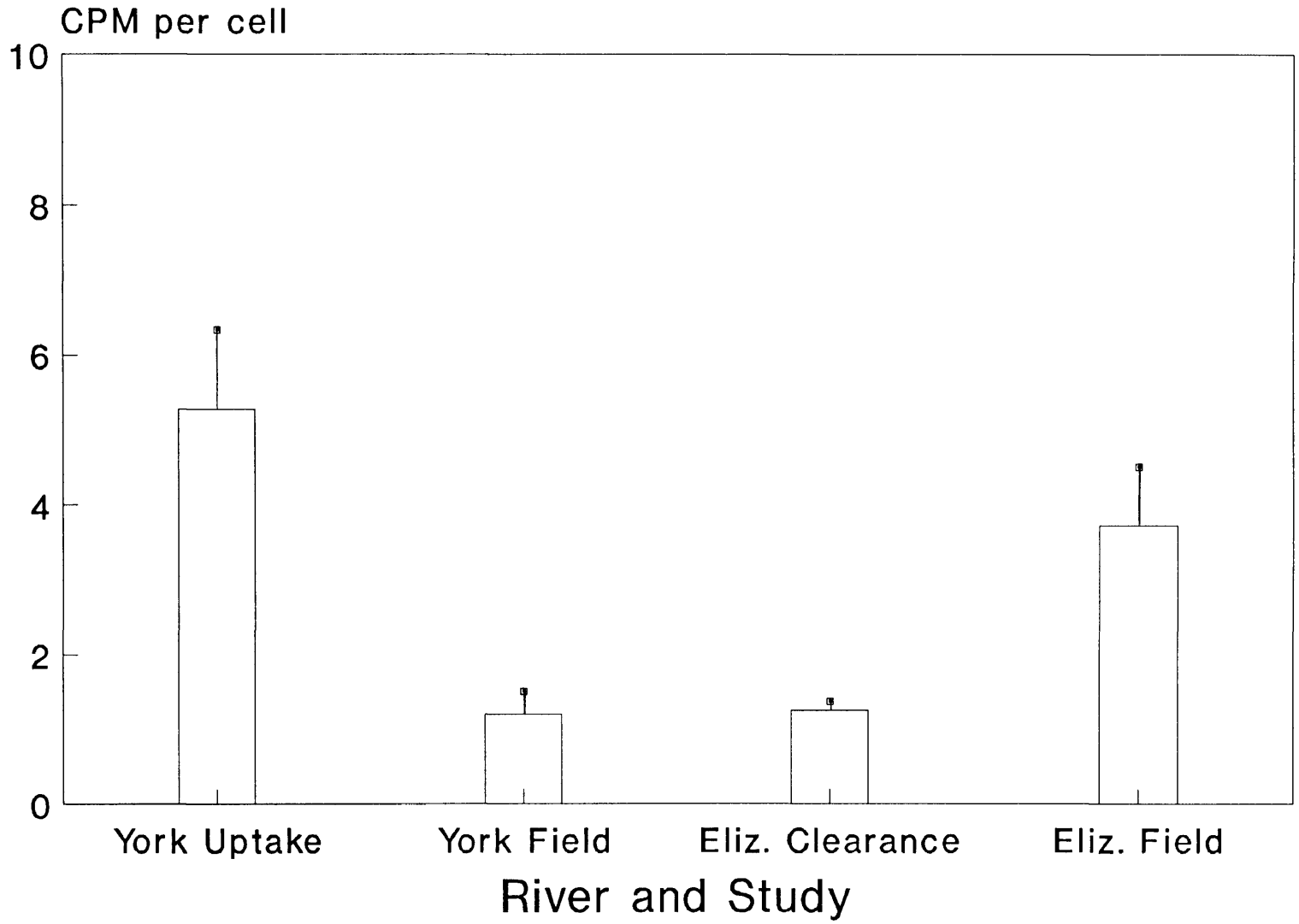
5.4 Discussion

Modulation of the macrophage activity of *Fundulus* from the two river systems in this experiment may be due to exposure to or absence of pollutants existing in the water column of the tanks. Weeks et al. (1986) held Elizabeth River spot (*Leiostomus xanthurus*) and hogchokers (*Trinectes maculatus*) in clean water for three weeks. After that time, macrophage chemotactic activity was equivalent to that of control fish. This suggests that the effects caused by pollution may be reversible after transfer to a cleaner water source or through reduction of pollutant levels in the original water column (Weeks et al., 1986). Likewise, York River macrophages began to exhibit the CL response characteristic of those from the Elizabeth River, possibly due to exposure to chemicals found in the Elizabeth River tank water.

When comparing results of the uptake and clearance experiment with the field experiment, a complete reversal in trends of average peak macrophage CL values occurred (Figure 5.2). During the field experiment, Elizabeth River fish exhibited a higher overall macrophage CL activity as compared to York River fish. However, in the uptake and clearance study, the York River fish held in Elizabeth River water (uptake sample) exhibited a higher macrophage CL response than the Elizabeth River

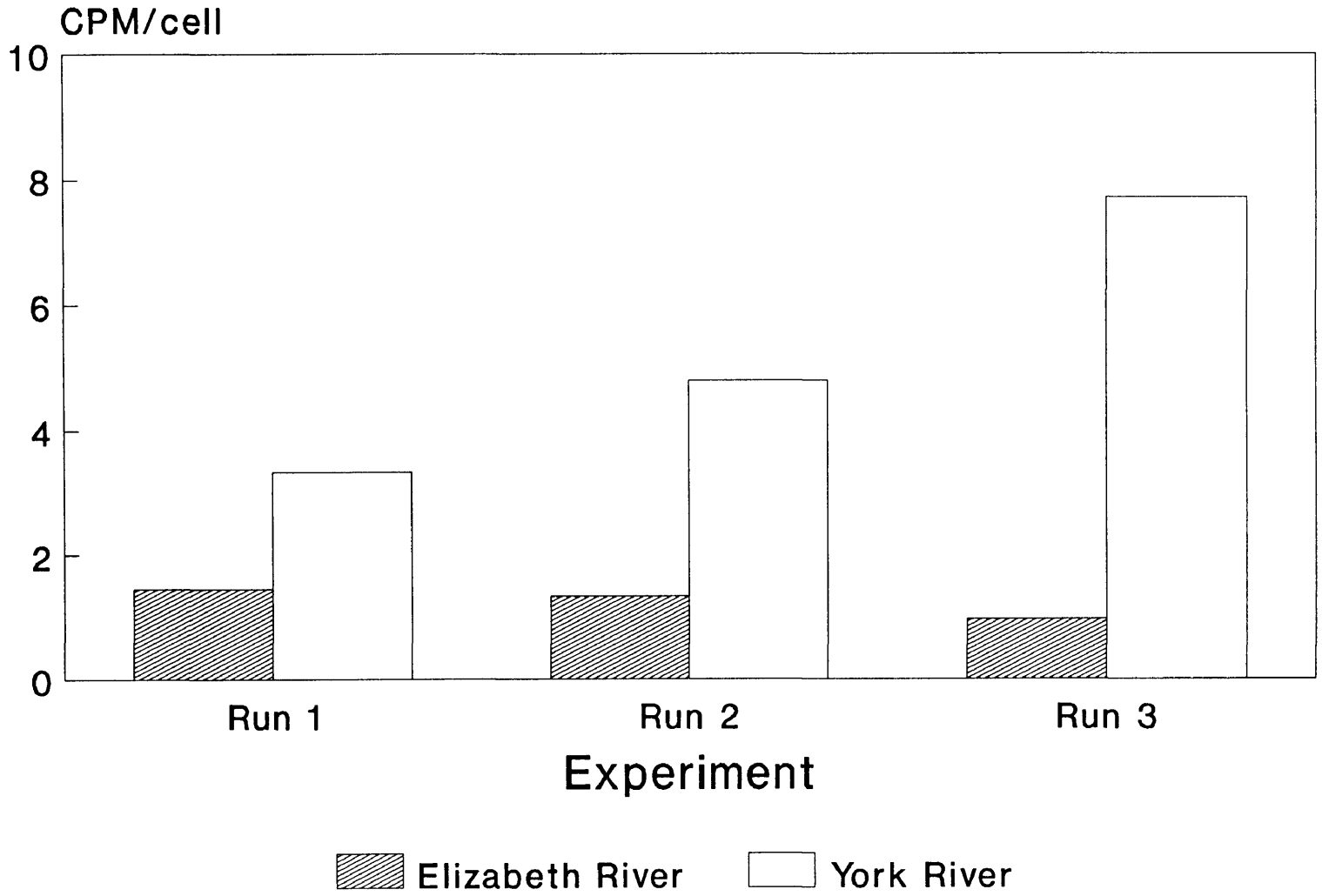
fish held in York River water (clearance sample). In other words, macrophages from control fish held in polluted water began to demonstrate the rate of CL activity found in fish from polluted water. In the same respect, polluted water fish held in clean water exhibited CL activity similar to fish originally found in control water. Although this segment of the study was an auxillary investigation, it can be postulated that environmental modification or a change in pollutant levels in aquatic environments may cause corresponding effects on macrophage CL activity. This study also weakens the theory of genetic hardiness of the Elizabeth River population.

Figure 5.1: Average peak chemiluminescent value comparisons for the uptake and clearance and field experiments.


CPM per cell
10
8
6
4
2
0
York Uptake
York Field
Eliz. Clearance
Eliz. Field
River and Study

+/- SE

Figure 5.2: Average peak chemiluminescent response in the uptake and clearance experiment.


CPM/cell
10
8
6
4
2
0
Run 1
Run 2
Run 3
Experiment
Elizabeth River
York River

VI. TEMPERATURE EXPERIMENT

6.1 Introduction

"Environmental temperature is probably one of the most significant external factors involved in the life processes of fish, affecting all aspects of cell metabolism and activity" (O'Neill, 1985). The suppressive effect of low environmental temperatures on the immune response was investigated for the first time by Ernst in 1890. Simkiss and Watkins (1988) found that the mussel, *Mytilus edulus* exhibited variations in intracellular metal concentration if exposed to oscillating temperatures under normal, clean conditions. Jimenez and Burtis (1988) found that mixed function oxidase activity in bluegill sunfish (*Lepomis macrochirus*) held at 4 and 26 °C was dependent on temperature and not on the amount of benzo[a]pyrene accumulated in the liver. This was not true for fish acclimated to 13 °C. Scott and Klesius (1981) found that during the spring and fall, as water temperatures pass through the 15 to 25 °C range, cultured channel catfish experience a high incidence of disease. These studies identified temperature as an important external factor, but the combined impact of temperature and pollution remains a challenge. In this study, temperature was investigated as a factor in CL activity since temperature was shown to affect the immune response in fish during the transition of seasons in the Field Experiment (Chapter 4).

6.2 Methods

Three temperature regimes were established at 7, 15, and 28 °C with 15 °C serving as a control. Fifteen °C corresponds to the average yearly temperature of the York

River (Anderson, personal communication). A total of eighteen aquaria, six at each temperature, were maintained. Three tanks from each temperature contained water from the control river (York River) and three contained water from the polluted site (Elizabeth River). Tanks were filtered and oxygenated through use of a charcoal box filter. The 7 and 15 °C tanks were set up in two separate cold rooms, whereas the 28 °C tanks were maintained in an ambient temperature room and were heated with standard aquarium heaters adjusted to 28 °C. Water temperature was monitored daily in the morning and evening and filters were cleaned weekly.

Fish were acclimated to the *in vivo* test temperatures for 21 days with a 12 hour light and 12 hour dark illumination cycle. Lumination was provided in all rooms by fluorescent bulbs and measured using a Li Cor Quantum/Radiometer/Photometer. Lumination was standardized for all tanks at each temperature and ranged from 6.5 to 7.0 microeinsteins M^{-2} sec^{-1} x 0.1 watts M^{-2} x 10 lux.

Fish held at 15 and 28 °C were fed ad libitum with Tetramin while those held at 7 °C were fed very little since metabolism rates were reduced and the unconsumed food fouled the tanks. Dissolved oxygen levels were measured in all tanks at the conclusion of the experiment to denote oxygen levels at the time of testing (Table 6.1). After a three week acclimation period, fish were sacrificed. Kidney macrophages were obtained and held *in vitro* at a constant temperature of 10 °C and tested using the CL assay as described in Chaper 4. Average total lengths and body weights are given for each river and tank temperature in Table 6.2

6.3 Results

Results of the CL response from York and Elizabeth River *Fundulus heteroclitus* populations, with respect to water temperature are shown in Figures 6.1 and 6.2. As in the field experiments, macrophages from Elizabeth River fish displayed elevated CL activity as compared to those from the York River. Fish acclimated to 15 °C had the

highest activity in both groups.

Nonparametric analysis of variance by ranks (Kruskal-Wallis Test) was utilized to determine if significant differences occurred in the macrophage CL response between temperature treatments for York and Elizabeth *F. heteroclitus* populations. Statistically significant differences were observed in York River populations for both experimental runs (Run 1, $H = 5.0$, $P < 0.05$; Run 2, $H = 6.0$, $0.05 < P < 0.10$). Significant differences were not observed in the Elizabeth River populations (Run 1, $H = 2.49$, $P > 0.10$; Run 2, $H = 0.27$, $P > 0.10$).

Since significant differences were found for runs 1 and 2 of the York River population, a non-parametric multiple comparison test was performed to determine between which of the temperature groups the difference occurred (Zar, 1984). A statistically significant difference ($P < 0.10$) was found in runs 1 and 2 between temperature groups 15 and 28 °C.

The nonparametric analysis by ranks (Mann-Whitney Test) was used to compare the two river populations exposed to experimental temperature regimes. Results from this test show that a significant difference ($P < 0.05$) existed between the two river groups. The Elizabeth River group had a higher overall peak CL response than the York River group (ER = 4.47 cpm, SE = 0.92, N = 3; YR = 1.81 cpm, SE = 1.55, N = 3). In addition, fish from both river groups acclimated *in vivo* to 15 °C showed higher overall peak CL activity as compared to the 7 or 28 °C acclimation group for both river systems (ER = 6.51 cpm, SE = 3.26; YR = 3.71 cpm, SE = 0.58).

6.4 Discussion

From the results presented in the laboratory experiment, one can state that it is important to know the effects of natural environmental variables on test organisms before postulating the consequences of pollution stress. It is interesting to note that although temperature stress did not significantly affect the Elizabeth River population, a

significant difference was found between the 15 and 28 °C groups in the York River. Fifteen °C acclimation was shown to increase CL activity in both the York and Elizabeth river tank groups. This finding may explain the sudden increase in CL activity of macrophages from York River fish collected on October 13, 1987 in the field study. The difference in reaction to temperature stress between York and Elizabeth *F. heteroclitus* may be caused by such factors as better stress adaptability or genetically selected hardiness to temperature stress in pollution-exposed fish. Robohm (1986) found similar reversed effects with pollution and temperature in striped bass (*Morone saxatilis*) but not in cunners (*Tautogolabrus adspersus*).

McCumber et al. (1982) found that between 6 and 12 °C, viral infections are extremely widespread in fish hatcheries. By elevating the temperature to 15 °C the situation appeared to be corrected even though this temperature does not affect viral growth *in vitro*. However, this theory is contradictory to the laboratory results of this study where peak CL activity of fish held at 7 °C was not significantly different from those held at 15 °C.

Phagocytic activity or the level of cellular immune response of macrophages was demonstrated by measuring the chemiluminescent response to a stimulant. A relatively high CL activity indicated that the macrophages were active and stimulated to phagocytize and destroy foreign invaders. Differences in macrophage CL activity between two population cell samples, or a modulatory effect, was demonstrated after exposure to pollution and various temperature levels. This study, as well as other studies, have shown that stress factors can act as either stimulatory or suppressive to the cellular immune system.

The CL activity of macrophages from the Elizabeth River indicates that this population responds differently than the York River population. The reversal of CL activity in the uptake and clearance study and differences in the temperature acclimated macrophage CL activity indicates that the populations are being affected by their environmental condition. This study suggests that these populations may be susceptible to fluctuations in their immune systems caused by the additional environmental stress of extreme water temperature.

Figure 6.1: Average peak chemiluminescent response comparison for run 1 in the temperature experiments for the York and Elizabeth Rivers.

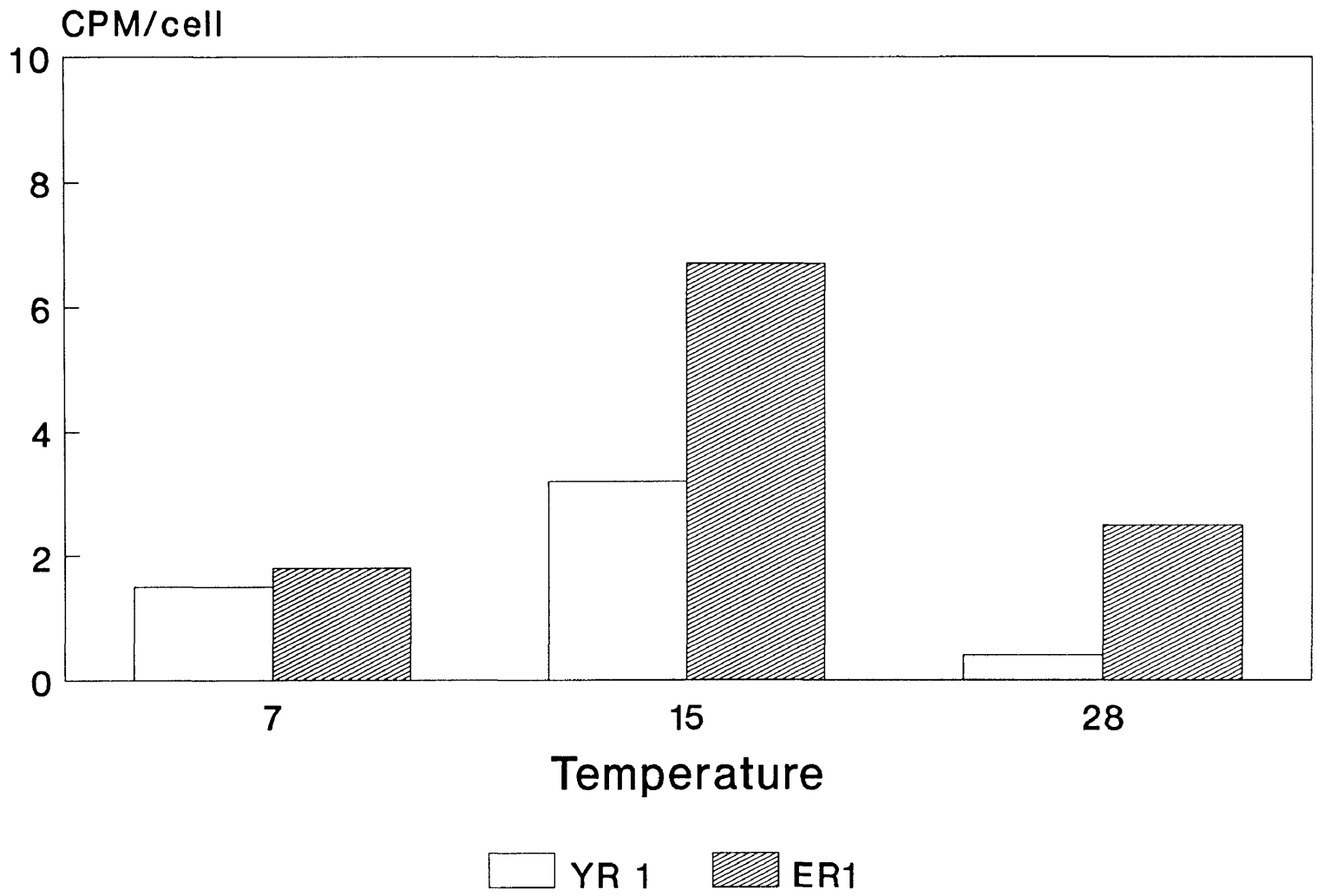

CPM/cell
10
8
6
4
2
0
7
15
28
Temperature
YR 1
ER1

Figure 6.2: Average peak chemiluminescent response comparison for run 2 in the temperature experiments for the York and Elizabeth Rivers.

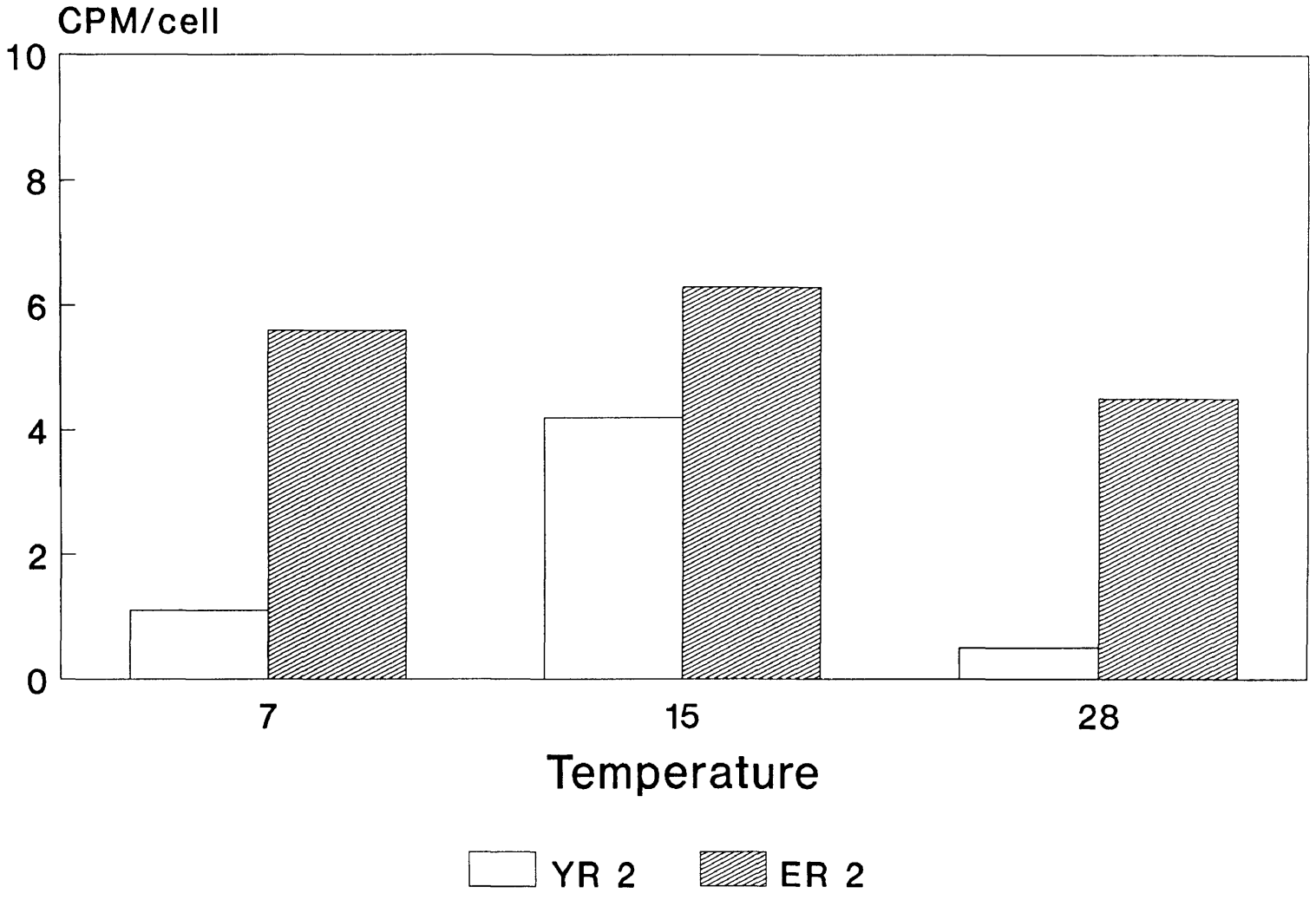

CPM/cell
10
8
6
4
2
0
7
15
28
Temperature
YR 2
ER 2

Table 6.1: Dissolved oxygen reading in the aquaria after three weeks of temperature acclimation.

Elizabeth River

Temp.	Dissolved Oxygen (mg/l)			X
7 °C	8.3	8.6	8.8	8.6
15 °C	6.0	7.1	7.2	6.8
28 °C	4.7	6.0	4.7	5.1

York River

Temp.	Dissolved Oxygen (mg/l)			X
7 °C	8.9	9.2	9.2	9.1
15 °C	7.3	7.4	4.0	6.2
28 °C	5.6	5.5	6.2	5.8

Table 6.2: Average total length and body weight of *Fundulus heteroclitus* used in the temperature experiments.

Elizabeth River

Temperature	Total Length (cm)	Body Weight (g)
7 °C	8.3	8.4
	8.9	9.6
	8.5	8.7
15 °C	8.6	9.8
	7.8	7.2
	9.3	12.4
28 °C	8.6	8.6
	9.0	9.9
	8.7	9.1

York River

Temperature	Total Length (cm)	Body Weight (g)
7 °C	9.1	11.5
	8.3	8.2
	8.1	6.9
15 °C	8.1	7.2
	8.5	8.3
	8.2	8.3
28 °C	8.4	7.6
	7.9	7.0
	8.2	8.1

VII. SUMMARY AND CONCLUSIONS

The objective of the temperature experiment was to relate temperature variations found in the field to the more controlled conditions in the laboratory. This was accomplished by acclimating two populations of *Fundulus heteroclitus* to similar *in vivo* temperatures of 7, 15, and 28 °C and examining the effects of the additional physical stress on the cellular immune system. Combined environmental stress factors, interaction of predator/prey, and foraging activities are significantly reduced in the laboratory, thus emphasizing effect of the induced stressor.

Although the results of this study are limited and circumstantial, it would appear that environmental contaminants may cause stress-related effects on the cellular immune system. Additional experiments with *Fundulus heteroclitus* will be necessary before an exact cause and effect can be pinpointed. Conclusions drawn from this study are limited by two considerations. First, variations in cellular responses to pollutants exist between fish species (Weeks and Warinner, 1984); therefore, application of the techniques used for *Fundulus heteroclitus* may not be feasible for other species. Second, there is an absence of baseline information about the test organisms and their habitats prior to pollution. Determination of such a baseline figure with seasonal and yearly time variations is difficult since biochemical and physiological changes can sometimes be larger than the effects caused by pollution.

Macrophages may be stimulated by two separate mechanisms. First, stimulation may result from a polluted environment where the cells are always prepared to fight infection or experience direct chemical stimulation from the pollutant. Rice (1989) found that particular *in vitro* levels of tributyltin caused increased CL activity. Secondly, macrophage stimulation may also be associated with optimal temperature conditions for cellular immune activity (15 °C). This indicates that since environmental

temperature does influence the cellular immune activity, consideration must be made regarding the environmental fluctuations that occur while sampling over a transitional season such as spring or fall.

Literature states that pollution-exposed fish have suppressed immune systems, and therefore a decreased CL activity (Ziskowski and Murchelano, 1975; Overstreet and Howse, 1977; Sindermann, 1979; Weeks and Warinner, 1984; Wolke et al., 1985; Fries, 1986; Weeks et al., 1986). This was not the case with macrophages from *Fundulus heteroclitus* obtained from the Elizabeth River. The question then remains, why is CL actvity elevated at the "optimal" temperature but also elevated at the non-optimal habitat conditions? Does a high CL activity mean the fish are healthy and exhibit a better response to challenge, or does high activity indicate a system that is stressed? Results from from these experiments indicate that Elizabeth River fish live in a stressful environment and thus compensate with an elevated CL response. However, the CL assay measures only a small fragment of the total immune response. Further study is needed using additional assays and environmental conditions to obtain a complete background on the cellular immune system of Elizabeth River fish. It is important to understand what constitutes the normal condition in the phagocytic system and how the system is activated before conclusions can be made about the health of the overall *Fundulus* population.

The major conclusions reached were:

1. A difference between the two populations of *Fundulus heteroclitus* exists when *in vivo* temperatures are constant.

2. A reversal in macrophage activity is possible if fish are exposed to different environmental conditions.

3. Optimum water temperature for macrophage chemiluminescent activity in *Fundulus heteroclitus* in this experiment was 15 °C.

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