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Effect of Hypoxia on the Respiratory Burst and Associated Bactericidal Activity in the Pronephritic Cells of the Mummichog, Fundulus heteroclitus

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EFFECT OF HYPOXIA ON THE RESPIRATORY BURST AND ASSOCIATED BACTERICIDAL ACTIVITY IN THE PRONEPHRITIC CELLS OF THE MUMMICHOG, *FUNDULUS HETEROCLITUS*

by

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ABSTRACT

Estuaries provide habitat and spawning grounds for numerous species of plants, birds, fish and shellfish. Levels of dissolved oxygen, carbon dioxide and pH in estuarine waters vary with diurnal, tidal and seasonal cycles. Anthropogenic factors may further exacerbate these variations in water quality. The studies presented here tested the hypothesis that hypoxia, including hypercapnia and acidosis, suppresses antibacterial defenses provided by phagocytic cells. The respiratory burst of phagocytic cells was stimulated with zymosan and *Vibrio parahaemolyticus* to produce Reactive Oxygen Species (ROS) which are potent antibacterial agents. The respiratory burst is oxygen dependent and therefore may be inhibited by hypoxic conditions. Hypoxic conditions within physiologically relevant conditions (Po_2 = 15 Torr, Pco_2 = 8.0 Torr, pH = 7.0) significantly suppressed ROS production measured by the luminol-enhanced chemiluminescence assay. Hypoxic conditions also significantly suppressed superoxide production, measured by the reduction of NBT, when stimulated with both zymosan and *V. parahaemolyticus.* Furthermore, while ROS mediated bactericidal activity was not significantly suppressed under hypoxia at the 1:1 phagocyte:bacterium ratio, there was a significant suppression when the bacterial challenge was increased to 1:10. It can be concluded from these results that hypoxia plays a critical role in maintaining antibacterial defense mechanisms of the mummichog, *F. heteroclitus* against the opportunistic marine pathogen *V. parahaemolyticus.*

INTRODUCTION

Estuaries and tidal marshes are extremely important to the preservation of natural resources and the economic welfare of many communities worldwide. In fact, estuaries are among the most productive environments on earth, creating more organic matter each year than comparably sized areas of forest, grassland, or agricultural land (National Estuarine Research Reserve System, 1998). Estuaries provide habitat for 75% of the commercial fish catch and 85 to 90% of the recreational fish catch in the United States. The nation's economy benefits from the 28 million jobs connected with estuaries and the 111 billion dollars generated annually from the fishing industry alone. In addition to the 50% of the country's population living along the coast, an additional 180 million tourists visit estuaries and coastal waters on a yearly basis. Tourism and seafood consumption contribute an additional 38 billion dollars a year to the United States economy and this figure is expected to continue to rise (U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Ocean Service, October, 1990, National Estuarine Research Reserve System, 1998). Estuaries are highly productive, provide fisheries habitat for multiple life stages of numerous species and play an important role in filtering out sediment and nutrients in the water draining from the upland. Furthermore, estuaries act as natural buffers between the land and the sea, thus providing flood control (National Oceanic and Atmospheric Administration, National Ocean Service, October, 1990; National Estuarine Research Reserve System, 1998). In short, estuaries provide us

with numerous resources, benefits, and services. Many of these have economic value; others have a purely aesthetic value. Estuaries are an irreplaceable natural resource that must be managed carefully for the mutual benefit of all that enjoy and depend on them.

Although estuaries have long been valued for providing fish and shellfish for human consumption, it was not until the late 1960's with the onset of concern regarding the eutrophication of coastal waters that scientists began to pay close attention to the estuarine ecosystem and how human activities effect these ecosystems (Diaz and Rosenburg, 1995; Nixon, 1995; Burnett, 1997). In the eutrophication process, there is an increase in the rate of supply of organic matter to an ecosystem. This is thought to be caused in part by non-point source pollutants in runoff from industrial plants, recreational facilities and agricultural areas (Diaz and Rosenberg, 1995; Nixon, 1995; Cochran and Burnett, 1996; Burnett, 1997; Summers et al., 1996; Taft et al., 1980). This increase in organic matter results from the deposition of nitrogen and phosphorous, two main ingredients in fertilizers, into coastal waters leading to the enrichment of the organic content in the water (Nixon, 1995). This non-point source runoff can also lead to hypoxia, or low dissolved oxygen levels, a characteristic associated with and enhanced by eutrophication (Diaz and Rosenburg, 1995; Nixon, 1995; Summers et al., 1996; Burnett, 1997).

As a result of this concern regarding eutrophication and estuarine health, particular attention has been given to identifying the causes and effects of hypoxia, or low levels of dissolved oxygen, over the past few decades (Diaz and Rosenburg, 1995; Summers et al., 1996; Burnett, 1997). Increased amounts of organic matter and phytoplankton blooms have been associated with decreased bottom water oxygen

concentrations (Taft et al., 1980; Diaz and Rosenberg, 1995). The increased organic matter provides an abundant energy source for bacteria and benthic organisms, thereby increasing the biological oxygen demand (Diaz and Rosenberg, 1995). This concern is of particular interest in South Carolina where agricultural development is extensive and specifically between the areas of Savannah, Georgia, and Beaufort, South Carolina, which had the greatest population growth in the United States in 1996 and 1997 (Personal Communication, United States Army Corps of Engineers, 1999).

In addition to the anthropogenic causes of hypoxia, low dissolved oxygen levels occur naturally. Oxygen levels fluctuate both seasonally and diurnally (Truchot and Duhamel-Jouve, 1980; Diaz and Rosenberg, 1995; Summers et al., 1996; Burnett, 1997). Water quality data collected from the ACE Basin by the National Estuarine Research Reserve System in South Carolina show dissolved oxygen levels ranging from 2 Torr (0.1 mg/ml) to 383 Torr (23.9 mg/ml) with pH also fluctuating between 5.3 and 8.4 (water quality data provided by Dr. Elizabeth Wenner, Department of Natural Resources, Charleston, South Carolina). During the summer in Charleston Harbor, water P_{O2} ranges from 9 to 170 Torr, P_{CO2} from 0.3 to 12 Torr and pH from 6.48 to 7.64 (Cochran and Burnett, 1996). In a similar study of water quality along the Cooper River in Charleston, South Carolina, dissolved oxygen levels ranged from a low of 10% air saturation to a high of 150% air saturation accompanied by pH fluctuations between 6.7 and 8.3 (Holland et al., 1994).

As hypoxic conditions set in, the respiratory responses of organisms living in an estuary adapt to the low oxygen levels through various behavioral and physiological processes (Pihl et al., 1991; Breitburg, 1992; Diaz and Rosenberg, 1995; Burnett, 1997).

Behavioral responses include the migration of mobile species such as juvenile spot, *Leiostomus xanthurus,* and the penaeid shrimp to areas with adequate dissolved oxygen (Pihl et al., 1991; Breitburg, 1992; Cochran and Burnett, 1996; Burnett, 1997). Other species such as the grass shrimp *Paleomonetes pugio* and various species of crabs will jump out of the water to take advantage of the oxygen rich air (Herreid, 1980; Burnett, 1997). However, the ability to move to a well-oxygenated environment is not always an option. Both sessile organisms and mobile organisms trying to avoid the dangers of predation found outside the safe environments of estuaries and salt marshes must stay within the hypoxic environment (Burnett, 1997). For those organisms that remain in the hypoxic environment, various adaptations have been documented including aquatic surface respiration used by members of the fish genus *Fundulus* and other Cyprinodonts (Kramer and Mehegan, 1981). These fishes have dorsally located mouths that allow them to remove the abundant amount of oxygen from the surface layer of water (Kramer and Mehegen, 1981). However, during severe hypoxia, these adaptations are not sufficient for maintaining adequate oxygen levels within the organism and certain metabolic pathways dependent on a specific level of dissolved oxygen, may be affected by the decreased oxygen level (Jensen et al., 1993; Cochran and Burnett, 1996). For example, an increase in anaerobic processes evident by increased lactate levels in *Fundulus heteroclitus* appears to play an important role in maintaining metabolic processes while these fish are subjected to extreme hypoxic conditions of 10 Torr (Cochran and Burnett, 1996).

Environmental hypoxia is usually accompanied by hypercapnia or increased carbon dioxide levels and the accompanying acidosis or decreased pH (Cochran and

Burnett, 1996; Burnett, 1997). Hypercapnia and acidosis are often not considered when investigating the causes and effects of hypoxia, however, they were taken into consideration in the present study. Throughout this work, the term hypercapnic hypoxia will be used to describe the combination of low oxygen, high carbon dioxide and a decreased pH. The term hypoxia will be used to describe low dissolved oxygen levels only.

Levels of Po_2, Pco_2 and pH fluctuate diurnally due to interactions between respiration and photosynthesis. The consumption of oxygen by an organism results in the release of carbon dioxide. During the day, aquatic plants fix this carbon dioxide and release oxygen back into the water as a product of photosynthesis (Cochran and Burnett, 1996; Burnett, 1997). The respiration of estuarine organisms and the photosynthesis of aquatic plants and algae result in an equilibrium between oxygen consumption and carbon dioxide production during the day, favoring well-oxygenated water with low carbon dioxide pressures (Breitburg, 1990; Cochran and Burnett, 1996; Burnett, 1997). However, photosynthetic activity declines sharply at night, resulting in a depleted oxygen supply and a decreased pH caused by the accumulating levels of carbon dioxide (Truchot and Duhamel-Jouve, 1980; Breitburg, 1990; Burnett, 1997). Decreased oxygen levels are also exaggerated by seasonal factors such as increased temperature during the summer months leading to a reduction in oxygen solubility in the water column (Taft et al., 1980). Studies on the Chesapeake Bay, the largest estuary in the United States, suggest that in addition to increased water temperature, which decreases oxygen solubility, several other factors play a role in creating a hypoxic environment. First, as the temperature increases in the spring months, increases in respiration and primary productivity occur. As a result,

oxygen is depleted from the surface layer of water. This adds to an already stressed situation of oxygen depletion in the bottom water. As the spring rains increase the amount of fresh water in estuaries, salinity decreases in the surface layer of water, increasing the vertical salinity gradient. As the salinity gradient increases, oxygen transfer from the surface layer of water to deepwater decreases. Organisms respiring in the deepwater utilize the available oxygen, however, because oxygen is not being replenished, a depleted oxygen level results (Taft et al., 1980). This low ambient Po_2 results in even lower Po_2 within the blood of organisms living in the water. Similarly, high ambient Pco_2 results in a raised Pco_2 level in the blood of these organisms. These fluctuations may affect certain processes carried out by the cells within an organism, which require specific levels of Po_2, Pco_2 and pH (Holeton and Randall, 1966; Hughes, 1973; Burnett and Cochran, 1996). In an attempt to sustain high levels of oxygen consumption, many organisms have mechanisms that increase the extraction of oxygen from the water. For example, some organisms may increase the amount of respiratory pigments in response to chronic hypoxia (Holeton, 1972). Inevitably, in cases of extreme hypercapnic hypoxia, a critical point will be reached where compensation is not possible and the internal environment of an organism will begin to reflect its outside environment. Levels of blood P_{O2} and P_{CO2} of the rainbow trout, *Salmo gairdneri,* show fluctuations coinciding with diurnal fluctuations of water P_{O2} (Garey and Rahn, 1970). At the relatively high water P_{O2} of 225 Torr, tissue P_{O2} was 42.6 Torr. At the low water P_{O2} of 58 Torr, tissue P_{O2} in the same animals was 31.4 Torr. The lowest level of tissue P_{O2}, 25.5 Torr, came four hours after the minimal water P_{O2} suggesting a four hour lag period for the effects of hypoxia to be maximized within the organism (Garey and Rahn, 1970).

In a study of similar design in rainbow trout, Holeton and Randall (1966) showed that at an environmental P_{O2} of 120 Torr, the P_{O2} in the dorsal aorta was approximately 82 Torr. As the environmental P_{O2} declined, the P_{O2} in the dorsal aorta also declined to less than 5 Torr.

Effects of hypoxia on estuarine organisms are well documented and widespread. They include increased disease susceptibility, changes in biochemical processes and mortality (Hughes, 1973; Garlo et al. 1979; Glass et al., 1990; Winn and Knott 1992; Cochran and Burnett, 1996). Extreme hypoxia has been identified as the cause of extensive mortality of marine life (Diaz and Rosenburg, 1995). Vast marine animal mortality off the coast of New Jersey and in Little Egg Inlet has been documented as being caused by severe hypoxic conditions with DO levels of 2.0 mg/ml or less (Garlo et al., 1979). In the Savannah River estuary, in Savannah, Georgia, amphipods (*Gammarus tigrinus*) and bivalves (*Mercenaria mercinaria* and *Mulinia lateralis*) showed high levels of mortality after a three hour exposure to hypoxic water with oxygen saturation levels between 12 and 18% (Winn and Knott, 1992). Current research in our laboratory by Mikulski et al. (1998) has demonstrated that hypercapnic hypoxia conditions of 4% O_2 (30 Torr), 2% CO_2 and pH of 6.8-7.0, significantly decreases survival of the shrimp *Penaeus vannamei* and *Palaemonetes pugio* when challenged by injection with the bacterium *Vibrio parahaemolyticus.*

Sublethal effects of hypoxia on fish and shellfish are increasingly recognized and are now used as tools to monitor the overall health of an estuary. The Environmental Monitoring and Assessment Program (EMAP) in the Carolinas found increased infection of silver perch with parasites under low dissolved oxygen conditions (Landsberg et al.,

1998). EMAP studies conducted during 1991-1993 on estuaries in the mid-Atlantic states documented a high frequency of tumors and lesions on the skin of demersal species of fish. These lesions were often associated with bacterial or parasitic infection, malformations of the eye, gill abnormalities, and skeletal curvatures. These physical abnormalities have been attributed to poor water quality including low dissolved oxygen and contaminated sediments (Hargis, 1988; Hargis, 1989; Bunch and Bejerano, 1997). These bottom-feeding fish had gross pathologies four to ten times more often than other kinds of fish. Additionally, resistance to infection with various *Streptococcus* species was decreased in both Tilapia hybrids, *Oreochromis niloticus,* and carp, *Cyprinus carpio,* under hypoxic conditions (Bunch and Bejerano, 1997). Furthermore, another species of fish, Yellowtail, *Seriola quinqueradiata,* challenged with *Enterococcus seriolicida,* had increased mortality from disease under low dissolved oxygen levels (Fukuda et al., 1997).

These data demonstrating a link between hypoxia and infectious disease suggest that an oxygen dependent mechanism of immune defense such as the respiratory burst should be particularly sensitive to hypercapnic hypoxia. The respiratory burst generates a battery of reactive oxygen species (ROS) with bactericidal activity. The cells responsible for carrying out the respiratory burst, in response to a foreign antigen are found in both vertebrates and invertebrates and consist of phagocytes, cells that are capable of engulfing and destroying bacteria and other foreign targets. While identification of lymphoid cells in invertebrates and fish remains controversial due to interspecies differences, it is evident that the macrophages and granulocytes constitute the major phagocytic cell types in vertebrate species including fish (Hightower et al., 1984; Ainsworth, 1992). These cells are a critical factor in maintaining anti-bacterial defense

protection (Gabig and Baboir, 1981; Hightower et al., 1984; Chung and Secombes, 1988; Ainsworth, 1992; Secombes and Fletcher, 1992).

As shown by Boyd and Burnett (1998), phagocytic cells isolated from the hemolymph of *Crassostrea virginica* exposed to hypercapnic hypoxia have decreased respiratory burst activity. Additionally, in the shrimp *Penaeus stylirostris,* severe hypoxia of 1.0 mg/ml resulted in a significant decrease in total hemocyte counts and a decrease in the ROS superoxide anion production when stimulated with zymosan (Moullac et al., 1998).

Stimulation of the respiratory burst, the defense mechanism of interest here, requires adherence of the foreign particle, or antigen, to the phagocyte membrane (Chung and Secombes, 1988; Secombes and Fletcher, 1992; Roitt et al., 1996). In vertebrates, this interaction can be mediated by the binding of Fc receptors located on the phagocyte membrane with the constant region of the antibody attached to the antigen (Secombes and Fletcher, 1992; Roitt et al., 1996). Alternatively, complement proteins located in the serum can also mediate the recognition and binding of an antigen. The activated complement protein C3b, which adheres to bacteria, can be recognized by complement receptors (CR1) located on the phagocyte. Simultaneous binding of the Fc receptor with the antibody, and C3b with CR1 has been shown to have a synergistic effect on phagocytosis. Additional interaction between the antigen and the phagocyte can occur through the mannan-binding protein on the bacteria and the C1qR receptor on the phagocyte or through interactions between lectins and oligosaccharides on both the bacteria and the phagocyte. All of these interactions induce cell-signaling events that trigger an increased rate of phagocytosis, fusion of lysosomes with phagosomes and

increased bactericidal activity (Gabig and Baboir, 1981; Secombes and Fletcher, 1992; Roitt et al., 1996; Janeway and Travers, 1997).

Once antigen is recognized, an increase in oxygen consumption and the assembly and activation of the membrane bound enzyme NADPH oxidase occurs (Chung and Secombes, 1988). Oxygen consumption increases upon stimulation with Phorbol 12-Myristate 13-Acetate (PMA) from 2 nmol $O_2/10^7$ macrophages to approximately 58 nmol $O_2/10^7$ macrophages (Secombes and Fletcher, 1992). NADPH oxidase is a membrane bound enzyme consisting of low potential cytochrome b and a flavoprotein, thus forming an electron transport chain using NADPH as the reducer. NADPH is derived from the conversion of glucose-6-phosphate to ribulose-5-phosphate, otherwise known as the hexosemonophosphate shunt. Therefore, the respiratory burst is also dependent on a source of glucose (Chung and Secombes, 1988; Secombes and Fletcher, 1992). These two events signal the initiation of the respiratory burst, resulting in the production of ROS, which are microbicidal agents in both vertebrates and invertebrates (Chung and Secombes, 1988; Gabig and Baboir, 1981; Secombes and Fletcher, 1992; Sharp and Secombes, 1993). As oxygen is consumed, it is reduced to the superoxide anion through the one electron reduction catalyzed by NADPH oxidase. Singlet oxygen is also produced spontaneously and as it returns to a stable configuration, it emits light, a form of chemiluminescence.

$$2O_2 + NADPH \text{----------} > O_2^- + NADP^+ + H^+$$

The superoxide generated is then dismutated spontaneously or catalyzed by superoxide

dismutase (SOD) into hydrogen peroxide.

$$2H^{+} + O_2^{-} \text{----------} > O_2 + H_2O_2$$

Hydrogen peroxide may act on the bacteria directly or be further converted to water and oxygen through the activity of the enzymes catalase and glutathione peroxidase. Hydrogen peroxide may also combine with superoxide in the presence of iron (Fe) to generate the highly reactive hydroxyl radical through the Haber-Weiss/Fenton reaction. The conversion to water and oxygen acts as a protective mechanism to the cell itself (Chung and Secombes, 1988; Secombes and Fletcher, 1992; Sharp and Secombes, 1993).

Suppression of ROS production may increase the susceptibility of the organism to infection with bacteria, viruses and fungi. For example, humans diagnosed with Chronic Granulomatous Disease (CGD) are diagnosed with frequent and severe bacterial infections. The cells of these individuals are unable to undergo a respiratory burst and therefore, are unable to produce ROS (Gabig and Baboir, 1981; Roitt et al., 1996). Also, *Fundulus heteroclitus* phagocytic cells exposed to pentachlorophenol (PCP), a known inhibitor of phagocytosis and ROS production, have decreased bactericidal activity against the fish bacterial pathogen *Listonella anguillarum* (Roszell and Anderson, 1997). Furthermore, the addition of trifluoroperazine (TFP), a drug commonly used to treat psychiatric disorders, however; also used by Secombes (1993) as an NADPH oxidase inhibitor, to cultures containing phagocytes and bacteria, has demonstrated increased survival of *Aeromonas salmonicida* in the macrophages of the rainbow trout, *Oncorhynchus mykiss* (Secombes and Fletcher, 1992; Sharp and Secombes, 1993). The

addition of another inhibitor of NADPH oxidase, diphenyleneiodonium (DPI), to cultured striped bass (*Morone saxatilis)* macrophages inhibited superoxide production causing a 50% decrease in the ability to kill *Bacillus megaterium* and a 100% decrease in bactericidal activity against *Pseudomonas fluorescens* (Bramble and Anderson, 1999).

Many studies of protective immunity against bacterial pathogens in fish and shellfish employ bacteria from the family Vibrionicae, which causes Vibriosis. Vibriosis is characterized in fish by severe hemorrhaging, skin lesions, necrosis of the liver, spleen and kidney and in the past has been referred to as "red pest," "red boil" or "red plague" (Bullock, 1987; Thune et al., 1993). Vibriosis can lead to the death of both marine organisms and humans that have consumed an animal infected with *Vibrio*. Furthermore, not only is Vibriosis a concern in the wild, it is also a major cause of mortality in mariculture operations (Bullock, 1987). Routes of infection in fish include invasion of the skin, bypassing the protective mucus membrane or through the intestinal tract during consumption of contaminated food. Several species of *Vibrio* have been associated with disease and mortality in fish and shellfish including *V. anguillarum, V. ordalii, V. carchariae, V. damsela, V. vulnificus, V. salmonicida, V. algynoliticus* and *V. parahaemolyticus* (Bullock, 1997).

Bacteria from the family Vibrionacae comprise approximately 10 to 50% of marine heterotrophic bacteria cultured from coastal seawater samples. Diseases caused by these bacteria have been documented since the early 1500's (Bullock, 1987; Thune et al., 1993). Of these species, *V. parahaemolyticus*, a known opportunistic pathogen, has been found in coastal waters along the East Coast of the United States and Gulf of Mexico (DePaola et al., 1990). In July of 1998, an outbreak of *V. parahaemolyticus* was

documented in the Gulf of Mexico, infecting shellfish and sending hundreds of people to hospitals in Texas and Florida. *V. parahaemolyticus* may infect reef silversides, *Menidia menidia,* by invading abrasions made by dinoflagellates and by contaminating food which is consumed by the fish (Rand and Wiles, 1988). Once inside a host, bacteria proliferate and damage the host tissue through several important mechanisms. First, virulent strains have been shown to be resistant to the bactericidal activity of fresh serum. Secondly, *Vibrio anguillarum* and other *Vibrio* species contain a plasmid that enables them to sequester iron from the host, which is necessary for the bacteria's metabolism. This may decrease the ability of cells to become activated as a result of decreased iron levels. Lastly, it has been shown that the majority of *Vibrio* species produce exotoxins such as hemolysins and proteases which act directly on host tissue (Bullock, 1987; Thune et al., 1993). Because of the Vibrionicae's predominance in the estuarine and mariculture communities, there is increased pressure to understand the mechanisms by which fish and shellfish protect against infection and disease caused by these pathogens.

Phagocytes and their oxygen radical products provide a major first line of defense against invading pathogens. Hypoxia and hypercapnic hypoxia suppress bacterial disease resistance *in vivo* as well as ROS production *in vitro* (Landsberg et al., 1998; Mikulski et al., 1998; Moullac et al., 1998; Boyd and Burnett; 1998). The studies presented here test the hypothesis that suppression of ROS production by hypercapnic hypoxia would directly decrease the bactericidal activity of the phagocytic cells. The abundant estuarine teleost *Fundulus heteroclitus,* otherwise known as the mummichog or the killifish, was selected as the test animal for these experiments. *F. heteroclitus* have been widely used in toxicological, immunological, pharmacological and tumorogenic studies. They are

geographically distributed along the eastern Atlantic coast from Canada to Florida. Because of their availability, adaptability to laboratory conditions and sensitivity to immunological and toxicological testing, they are often used as a test model for estuarine organisms.

In order to test this hypothesis, experiments were designed to quantify ROS production and ROS mediated bactericidal activity of *F. heteroclitus* phagocytes against *V. parahaemolyticus* under conditions simulating *in vivo* levels of oxygen, carbon dioxide and pH in fish exposed to atmospheric, normoxic and hypoxic conditions in an estuary. ROS production in response to zymosan, a commonly used stimulant of the respiratory burst, was quantified using the luminol enhanced chemiluminescence procedure described by Austin and Paynter in 1995. This assay has been widely used to measure oxygen radical production by phagocytic cells from both vertebrates and invertebrates (Stave et al., 1984; Stave et al., 1985; Kelly-Reay and Weeks-Perkins, 1994; Austin and Paynter, 1995; Lambert and Nicolas, 1998). The chemiluminescence assay could not be used to measure ROS production in response to *V. parahaemolyticus* due to the high chemiluminescent response of the bacteria alone. As an alternative approach, the production of the superoxide anion; the first ROS produced during the respiratory burst, was measured using the nitroblue tetrazolium reduction method described Pick et al. (1981) and modified by Secombes (1990). Bacterial killing was quantified with a bactericidal assay originally described by Sharp and Secombes (1993). This assay has been used to monitor suppression of bactericidal activity due to a wide variety of causes such as stress and contaminant exposure (Graham et al., 1988; Kelly-Reay and Weeks-Perkins, 1994).

The results of these experiments will demonstrate if inhibition of ROS production by hypercapnic hypoxia will lead to a decrease in the ability of phagocytic cells to kill the opportunistic pathogen *V. parahaemolyticus.*

MATERIALS AND METHODS

***Animal Maintenance*:**

Fundulus heteroclitus were collected from local tidal creeks on James Island in Charleston, South Carolina, and examined for any obvious signs of infection or disease. Healthy fish were transported to the Medical University of South Carolina's animal holding facility at the Fort Johnson Marine Biology complex in Charleston, South Carolina. Fish were held in laboratory aquaria for no less than fourteen days prior to use in experiments. Aquaria were equipped with recirculating filtration systems maintained at 21-24°C, pH of 8.0-8.2 and 25-30% salinity with biweekly monitoring of ammonia/nitrite/nitrate levels. Fish were fed a standard diet of Marine Tetra Flakes twice daily.

Opsonization of bacteria was required for the assays performed in this research. *Sciaenops ocellatus* (red drum) serum, from animals caught in the Wando River in 1996 was used. Serum was heat inactivated at 56°C for 30 minutes, aliquotted and placed in a -20°C freezer until use.

***Macrophage Isolation*:**

F. heteroclitus were anesthetized with 0.15g/L of 3-amino benzoic acid ethyl ester (MS-222) (Sigma Chemical Company, St. Louis, MO) and decapitated. The anterior head kidney was removed and washed in an antibiotic incubating medium (AIM)

composed of 24.35 ml L-15 (Life Technologies, Rockville, Maryland) containing 0.21% NaCl and supplemented with 150 µl Penicillin/Streptomycin, 150 µl gentamicin, 250 µl fungizone, (Sigma) for thirty minutes to remove possible bacterial contamination caused by the dissection. Anterior head kidney tissue was then washed in Fundulus Media (FM)(L-15, 0.21% NaCl, 5mM HEPES) and dissociated by pestle grinding. Cells were placed in a 50 ml centrifuge tube and washed by addition of FM to a total of 10 ml FM followed by centrifugation for 10 minutes at 258 x g. The supernatant was removed and the cells were resuspended to the desired density in FM.

***Differential Cell Counts*:**

Fifty microlitres of 1.0×10^6 cells in FM for a total of 50,000 cells were placed on a microscope slide and air-dried. The slide was then stained with Diff-Quik differential stain (Gibco, Grand Island, NY). For each measurement one hundred cells were counted in one field and categorized as macrophages, granulocytes, red blood cells, or other based on staining characteristics defined in the Diff-Quik directions for use.

***Bacteria Preparation*:**

A known pathogenic strain of *V. parahaemolyticus* (90-69B3) was provided by Dr. Donald Lightner and Ms. Leone Mahoney of the University of Arizona. Identification of this strain was confirmed using NE API bacterial identification strips supplied by bioMerieux (St. Louis, MO). Upon arrival, the bacteria were grown for 24 hours on Tryptic Soy Agar (TSA)(Gibco, Grand Island, NY) containing 2.5% NaCl. Aliquots of 0.5 ml were stored in freezing media (TSA, 2.5% NaCl, 20% glycerol) at -

80°C. For use in assays, bacteria were thawed and grown on TSA 2.5% NaCl plates for 24 hours. Bacterial densities were quantified by measuring optical density in Tryptic Soy Broth (TSB)(Gibco, Grand Island, NY) 2.5% NaCl at 540nm. An optical density (OD) of 0.1 is equal to a bacterial density of 1.0×10^8cells/ml as determined by counting colony forming units grown on double layer plates consisting of TCBS and Marine Agar (Gibco, Grand Island, NY). The assays performed in this research required the use of different amounts of bacteria. Serial dilutions were done from the stock of 1.0×10^8 bacteria/ml. To obtain a higher bacterial concentration than 1.0×10^8 bacteria/ml, an OD of 0.1 was obtained in 50 ml of TSB 2.5% NaCl. This suspension was then centrifuged for 30 minutes at 3632 x g. The supernatant was removed and the bacteria were resuspended to the desired density.

Demonstration of Phagocytic Activity by Macrophages and Granulocytes

To determine intracellular killing of the bacteria, it was first necessary to demonstrate phagocytic activity of the macrophages and granulocytes against *V. parahaemolyticus.* Phagocytosis was monitored using a procedure adapted from Kiel (1998). Cells were resuspended in FM to a final concentration of 1.0×10^6 cells/ml. Two hundred microlitres of this suspension was added to the wells of a sixteen well chamber slide (Fisher Scientific, Springfield, IL) for a total of 2.0×10^5 cells/well. The slide was then incubated for seven hours at 25°C under atmospheric conditions. At this point, the medium was removed and replaced with 100 µl of fresh FM. Approximately 50,000 cells were lost when the medium was removed leaving a total of 1.5×10^5 cells/well. This was determined by counting the number of cells present in the removed media over twelve

trials. Thirty microlitres of heat inactivated red drum serum was then added to all the wells with gentle mixing and allowed to settle for ten minutes. Fifty microlitres of a 3.0 x 10^6 bacteria/ml suspension was added to duplicate wells to give a 1:1 phagocyte:bacterium ratio. Fifty microlitres of a 3.0 x 10^7 bacteria/ml suspension was added to duplicate wells to give a 1:10 phagocyte:bacterium ratio. Fifty microlitres of a 3.0 x 10^8 bacteria/ml suspension was added to duplicate wells to give a 1:100 phagocyte:bacterium ratio. Fifty microlitres of a 3.0 x10^7 zymosan/ml solution was added to duplicate wells for a 1:100 phagocyte:zymosan ratio. The slides were then incubated at 25°C under atmospheric conditions, removing media from duplicate wells at fifteen minute intervals over a 105-minute period to determine when peak phagocytosis occurred. Once all wells were empty, the chamber section of the slide was removed and the slide was air dried. Once dry, cells were stained with Diff-Quik differential stain and phagocytosis was quantified under a 100X oil immersion lens on a Zeiss microscope by counting the number of cells with ingested bacteria in a field of 100 cells.

Obtaining Hypoxic, Hypercapnic and Acidic Conditions

The remaining assays were performed under atmospheric, normoxic and hypoxic conditions to determine if these factors had an effect on the respiratory burst and associated bactericidal activity. Atmospheric conditions were chosen to allow for comparison of these results to numerous assays in the literature performed under atmospheric conditions. Normoxic and hypoxic exposure was used to demonstrate the respiratory burst activity of the cells under conditions they would experience in a fish that was exposed to normoxic or hypoxic conditions in an estuary. Fundulus media was

gassed to appropriate pressures of nitrogen, oxygen and carbon dioxide using Wosthoff gas mixing pumps. All media were gassed for three hours the day before running the assay and kept at 2-8°C overnight. To obtain atmospheric conditions, the medium was gassed using 21.0% oxygen, 0.1% carbon dioxide and 79.0% nitrogen. To obtain normoxic conditions, the medium was gassed with 6% oxygen, 0.5% carbon dioxide and 94.0% nitrogen. To obtain hypoxic conditions mimicking hypercapnic hypoxia, the medium was gassed with 2.0% oxygen, 1.0% carbon dioxide and 97.0% nitrogen. The final pH for the atmospheric and normoxic conditions was 7.6 while the final pH for the hypoxic conditions was 7.0. During the course of this research, buffer curves were determined to establish the starting pH of the FM necessary to obtain the final pH of 7.6 for atmospheric and normoxic conditions and 7.0 for hypoxic conditions after gassing and to determine the amount of HEPES (Sigma, St. Louis, MO) required to buffer the media.

Physiologically Relevant Conditions

The functional assays described below were performed under the physiologically relevant conditions outlined here. Physiology literature refers to the partial pressure of these gasses in Torr, however, most water quality data is presented in mg/L. Therefore, for the present studies, dissolved oxygen values are given in both Torr and mg/L. These values are based on previous research demonstrating that the physiological levels of these gasses are approximately one half of the environmental level (Garey and Rahn, 1970). For example, a moderate level of environmental hypoxia at slightly less than 30 Torr would result in a dissolved oxygen level within the organism of approximately 15 Torr.

Test Condition	Po_2	Pco_2	pH
Atmospheric	155 Torr / 8.43 mg/L	0.76 Torr	7.6
Normoxic	45 Torr / 2.45 mg/L	3.8 Torr	7.6
Hypoxic	15 Torr / 0.82 mg/L	8.0 Torr	7.0

:Reactive Oxygen Species Production From Zymosan Stimulation Measured By Luminol Enhanced Chemiluminescence

Production of ROS by phagocytes stimulated with zymosan was quantified using luminol-enhanced chemiluminescence. Luminol (Sigma, St. Louis, MO) was prepared according to the method of Scott and Klesius (1981) and stored in 1.0 ml aliquots at -20°C for no more than five days prior to use. Zymosan (Sigma, St. Loius, MO) was prepared according to the method of Austin and Paynter (1995) and stored in 1.0 ml aliquots at -70°C until use. For use in this assay, zymosan was thawed and centrifuged for three minutes at 3635 x g. The supernatant was removed and the pellet resuspended to the same concentration in Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, Grand Island, NY) with 0.21% NaCl. Anterior head kidney cells were isolated (as previously described) and resuspended to 5.5 x 10^5cells/ml in FM. One milliliter of this cell suspension was added to each 10 ml glass scintillation vial (Fisher Scientific, Springfield, IL) and incubated at 25°C under atmospheric conditions for seven hours. At this point, the media was replaced with fresh FM, removing approximately 50,000 nonadherent cells. The cells were then incubated overnight at 25°C under atmospheric conditions.

Prior to running this assay, the media was once again removed and replaced with 675 µl of medium previously gassed at the appropriate combination of O_2, CO_2, and N_2. Two hundred microlitres of heat inactivated red drum serum was then added to each of the vials and the vials were capped with rubber stoppers. Each vial was gassed for three minutes under the specified conditions (atmospheric, normoxic, hypoxic). Twenty-five microlitres of luminol was then added to each vial in a darkened room, as luminol is light sensitive, using a 25 µl Hamilton syringe. Background chemiluminescence was measured for 20 minutes on a Wallace 1414 liquid scintillation counter set for single photon monitoring at 10-second intervals. Once background chemiluminescence had stabilized, 100 µl of the zymosan suspension was added to the vials and chemiluminescence was measured for 100 minutes . Total chemiluminescence was calculated as area under the curve post-zymosan exposure where the last background count prior to zymosan exposure was used as the baseline for the curve.

Reactive Oxygen Species Production In Response To Vibrio parahaemolyticus Exposure Measured By Nitroblue Tetrazolium

When *V. parahaemolyticus* was used to stimulate ROS production in the chemiluminescence assay described above, the bacteria alone generated high background luminescence that obscured any response by the phagocytic cells themselves. As an alternative method, the production of intracellular superoxide anion was measured using the nitroblue tetrazolium assay described by Secombes et al. (1990) with slight modifications similar to that of Pick (1981). Superoxide is the first product of the ROS cascade and is therefore an indicator of respiratory burst activation. Phagocytic cells

were isolated as previously described and resuspended to a final concentration of 1.0 x 10^6 cells/ml in FM containing 2mM $CaCl_2$ (Sigma, St. Louis, MO). Two hundred microlitres of this cell suspension was placed in each well of a 96 well flat bottom plate (Fisher Scientific, Springfield, IL) for a final concentration of 2.0 x 10^5 cells/well. The cells were then incubated at 25°C under atmospheric conditions for seven hours. At this point, the medium was replaced with 150 µl of fresh FM supplemented with 2mM $CaCl_2$, removing approximately 50,000 non-adherent cells, leaving 1.5 x 10^5 cells/well. The cells were then incubated overnight at 25°C under atmospheric conditions. Prior to running the assay, the medium was again replaced with 150 µl of FM gassed to atmospheric, normoxic or hypoxic conditions, containing 2mM $CaCl_2$. Fifty microlitres of heat inactivated red drum serum was then added to all of the wells. After ten minutes, 25 µl of NBT (Sigma, St. Loius, MO) from a stock of 3.0 mg/ml made in Phenol Red Free L-15 (Gibco, Grand Island, NY) was added to each well for the measurement of intracellular superoxide production. After ten minutes, 25 µl of the stimulant, either zymosan or bacteria, was added to the wells. For stimulation with zymosan, a 1.5 ml Eppendorf tube of stock zymosan at 50 mg/ml was thawed. From this, approximately 100 µl was added to 2.5 ml of PBS 0.21% NaCl to achieve a concentration of 6.0 x 10^8 zymosan/ml. Zymosan density was verified by counting on hemacytometer. This gave a final 1:100 phagocyte: zymosan ratio in the test wells. For stimulation with bacteria, a bacterial density of 1.0 x 10^8 cells/ml in Tryptic Soy Broth (TSB) (Gibco, Grand Island, New York) was obtained as described above. Serial dilutions were performed as follows. For the 1:1 phagocyte:bacterium ratio, 60 µl of 1.0 x 10^8 cells/ml was pipetted into 940 µl of TSB 2.5%NaCl. This gave a bacterial concentration of 6.0 x 10^6cells/ml. The addition

of 25 µl of this concentration to each well gave a final concentration of 1.5×10^5 bacteria/well. For the 1:10 phagocyte:bacterium ratio, 600 µl of 1.0×10^8 cells/ml was pipetted into 400 µl of TSB 2.5%NaCl. This gave a bacterial concentration of 6.0×10^7cells/ml. The addition of 25 µl of this concentration to each well gave a final concentration of 1.5×10^6 bacteria/well. For the 1:100 phagocyte:bacterium ratio, an OD of 0.1 in 50 ml's of TSB 2.5% NaCl was obtained on the spectrophotometer at 540nm. This bacterial density was centrifuged for 30 minutes at 4500 RPM. The supernatant was then removed and the pellet resuspended in 8.3 ml of TSB 2.5% NaCl. This gave a 6.0×10^8 bacteria/ml concentration. Twenty-five microliters of this concentration to each well gave a final bacterial concentration of 1.5×10^7 bacteria/well.

After addition of the stimulant, the contents of the wells were gently mixed with a pipet and the plates were centrifuged for ten minutes at 258 x g to bring the zymosan and bacteria into contact with the phagocytic cells attached to the bottom of the plate. The plates were then placed under the specific gassed environments for sixty minutes. Upon removal from the gassed environment the plates were centrifuged at 300 x g for three minutes. Media was removed and 120 µl of 2M KOH and 140 µl of DMSO (Sigma, St. Louis, MO) were added to dissolve the reduced NBT. Optical densities were read on a Multiskan spectrophotometer at 620 nm. Microscopy revealed formazan filled cells. To correct for NBT reduction caused by the phagocytes, optical densities of wells containing zymosan or bacteria alone were subtracted from the wells with phagocytes. Additionally, as a positive control, 30 µg of superoxide dismutase (SOD, Sigma, St. Louis, MO) was added to some of the wells in each plate. SOD catalyzes the reaction in which the superoxide anion is dismutated to hydrogen peroxide. NBT can also be

reduced by unstimulated cells through the action of the diaphorase enzyme Thus, the amount of reduction that was performed by the superoxide anion was determined by the addition of SOD to the cell culture.

***Bactericidal Activity Associated With Reactive Oxygen Species Production*:**

Once ROS production by the phagocytes was confirmed, association of this production with bactericidal activity against *V. parahaemolyticus* was investigated. The killing assay described by Sharp and Secombes (1993) was performed with slight modifications. Phagocytic cells were isolated as previously described and resuspended in FM to a concentration of 1.0×10^6 cells/ml. Two hundred microlitres of this suspension was then added to the wells of a 96 well flat bottom plate in triplicates yielding a total of 2.0×10^5 cells/well. The cells were incubated at 25°C under atmospheric conditions for seven hours. The medium was then replaced with 150 µl of fresh FM, removing approximately 50,000 non-adherent cells and adherent cells were incubated overnight at 25°C under atmospheric conditions. Prior to running the assay, the FM was again replaced with 150 µl FM gassed to physiologically relevant levels of oxygen and carbon dioxide. Next, 10 µl of 5 mM trifluoroperazine (TFP)(Sigma, St. Louis, MO) was added to specified wells and mixed with gentle pipetting. TFP, a drug which has been used as an NADPH oxidase inhibitor (Secombes, 1993) was used in this assay for the determination of the amount of killing that the respiratory burst was responsible for. After ten minutes, 40 µl of heat inactivated red drum serum was added to all of the wells and mixed with gentle pipetting. Ten minutes later the *V. parahaemolyticus* was added at the following concentrations. For the 1:1 phagocyte:bacterium ratio, 50 µl of a 3.0×10^6

bacteria/ml suspension was added to the wells. For the 1:10 phagocyte:bacterium ratio, 50 µl of a 3.0 x 10^7 bacteria/ml suspension was added to the wells. For the 1:100 phagocyte:bacterium ratio, 50 ml of TSB 2.5% NaCl inoculated with bacteria to give an OD of 0.1 at 540 nm, was centrifuged for 30 minutes at 3632 x g. The supernatant was removed and the pellet was resuspended in 3.3 ml of TSB 2.5% NaCl. This gave a 3.0 x 10^8 bacteria/ml concentration of which 50 µl/well would give a 1.5 x 10^7 bacteria/well or a 1:100 phagocyte:bacterium ratio. Plates were spun for ten minutes at 258 x g to bring the bacteria into contact with the then placed under the specific gassed environments for 180 minutes. At this point, the medium was removed and the phagocytic cells were lysed with 0.1% Tween (Sigma, St. Loius, MO). After ten minutes, 100 µl of TSB 2.5% NaCl was added to each well to support surviving bacterial growth and the plate was incubated in room conditions for nine hours. Then, 10 µl of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) in distilled water was added to each well and placed on a shaker at medium speed for fifteen minutes. MTT is chemically reduced to a blue/purple formazan in proportion to the number of viable bacteria and therefore was used to quantitate surviving bacteria in this assay. One hundred microliters of 10% SDS in 0.01N HCl was added to the wells to dissolve the formazan crystals and the plate was shaken for 30 minutes. Optical density was then determined on a Multiskan spectrophotometer with a 561 nm filter.

Bactericidal Activity As A Function Of Oxygen Only

This assay was performed identically to the bactericidal assay under hypoxic, hypercapnic and acidic conditions with slight modifications in the environmental

conditions. To test the effect of oxygen alone, the levels of CO_2 and pH were maintained at 0.1% and 7.6 respectively. The oxygen level was decreased from atmospheric conditions (21.0%) to hypoxic conditions (2.0%).

Bacterial Growth Curves

To determine if bacterial growth was enhanced under hypoxic conditions, *V. parahaemolyticus* was grown for twelve hours under conditions simulating those in the functional assays. Stock cultures of *V. parahaemolyticus* were diluted to 0.1 OD_{540nm} in TSB 2.5% NaCl as previously described. For growth curves under atmospheric, normoxic and hypoxic conditions, 45 ml of TSB 2.5% NaCl in 100 ml glass bottles was gassed under 21.0% oxygen and 0.1% CO_2 for atmospheric conditions, 6% O_2 0.5% CO_2 for normoxic conditions and 2.0% O_2 and 1.0% CO_2 for hypoxic conditions. The bottles were placed on a shaker and held at a constant temperature of 25°C and gassed for one hour prior to the addition of the bacteria. Five hundred microlitres of 1.0 x 10^8 bacteria/ml was added to 4500 :l TSB 2.5% NaCl for a total of 5 ml. This suspension was then added to the 100 ml bottles to begin the growth curve. Optical densities were read at 540nm every hour for the first five hours. At this point, the bacteria began to grow and OD_{540nm} was determined every 45 minutes.

For growth curves under acidic conditions, TSB 2.5% NaCL was brought to a pH of 7.6 or 7.0, the pHs of the media under atmospheric and hypoxic conditions. Bacteria were added the same as for the oxygen growth curves and OD_{540nm} was measured as a function of time.

Cell Viability

Viability of phagocytic cells after isolation was measured using trypan blue exclusion. Trypan blue is a dye commonly used to measure cell viability based on its exclusion from viable cell membranes. To determine cell viability in the presence of TFP, these cells were incubated for a period of three hours in the presence of TFP. The cells were then removed from the well using gentle scraping and viability was tested using trypan blue exclusion. To test the viability of the cells under hypoxic conditions, the chemiluminescence assay was run as described below. Cells were exposed to hypoxic conditions for a period of 3 hours and then placed in atmospheric conditions. The chemiluminescent response of these cells was then compared to the response of cells held under atmospheric conditions only. Cell viability after exposure to hypoxic conditions was also tested using trypan blue exclusion.

Statistical Analysis

For each treatment in the NBT and bactericidal assay, samples were run in triplicate. Results were expressed as mean +/- standard error. To determine if hypoxia significantly affected the processes being evaluated, statistical analysis was performed using the statistical program JMP IN, SAS Institute Inc. For data that met the assumptions of normality, a one way paired t-test was performed comparing data from normoxic and hypoxic responses. A p value of < 0.05 was considered significant. For data that did not meet the assumptions of normality, the nonparametric Wilcoxen rank-sum test was performed. A p value of < 0.05 was considered significant. Statistical

analysis was not performed on the atmospheric-normoxic relationship. The data presented from this relationship are expressed as percentages, normalized to the atmospheric response.

RESULTS

Characteristics of Isolated Anterior Head Kidney Cells

Anterior head kidney cells isolated from mummichogs consisted of macrophages, granulocytes, red blood cells and other cells (undifferentiated cells, lymphocytes, thrombocytes) based on Diff-Quik differential staining. For the functional assays used in this research, head kidney cells were allowed to adhere to 16 well chamber slides or 96 well plates for seven hours at which point non-adherent cells were washed away. Cell counts performed on the media showed an average of approximately 50,000 cells or 25% of the total of 200,000 cells were lost during the washing.

Differential cell counts on the initial cell population (prior to washing) yielded on average 45% macrophages, 28% granulocytes, 23% red blood cells and 4% other (lymphocytes, thrombocytes and undifferentiated cells) Differential cell counts from individual fish are given in Table 1.The adherent cell population consisted on average of 70% macrophages, 28% granulocytes, 2% red blood cells and <1% other (Table 1).

Peak phagocytosis of zymosan and the 1:10 phagocyte:bacterium ratio occurred at 45 minutes in contrast to 75 minutes for the 1:1 phagocyte:bacterium ratio (Figure 1). Additionally, percent phagocytosis was greater for zymosan than either bacterial concentration. The macrophage was the predominant phagocytic cell with the granulocyte showing minimal phagocytic behavior (Table 2). The percent of phagocytic activity varied from fish to fish, evident by the wide range of values (Table 2).

Zymosan-Stimulated Reactive Oxygen Species Production Measured by Luminol Enhanced Chemiluminescence

Once the preliminary assays confirmed that head kidney adherent cells could phagocytize zymosan and *V. parahaemolyticus,* immune function of these phagocytic cells could be assessed. First experiments tested ROS production in mummichog phagocytic cells using the luminol-enhanced chemiluminescence assay. Responses to zymosan stimulation were compared under normoxic and hypercapnic hypoxic conditions. Hypercapnic hypoxia significantly suppressed ROS production (Wilcoxen rank-sum test, $p = 0.01$, Figure 2). ROS production by cells under hypercapnic hypoxic conditions was 24.3% of ROS production under normoxic conditions.

Individual responses of the ten fish varied considerably, particularly under atmospheric and normoxic conditions. All fish demonstrated at least a 58% decrease in ROS production under hypercapnic hypoxic conditions (Figure 3).

Zymosan and Vibrio parahaemolyticus Stimulated Superoxide Anion Production Measured by Nitroblue Tetrazolium Reduction

The inherent chemiluminescence exhibited by *V. parahaemolyticus* was so great that it was not possible to determine the amount of ROS production by the phagocytic cells in response to bacterial stimulation using the luminol-enhanced chemiluminescence assay. The NBT reduction assay, which measures intracellular production of the superoxide anion, was employed as an alternative means of measuring ROS production . NBT reduction stimulated by zymosan or *V. parahaemolyticus* was significantly suppressed under hypercapnic hypoxia based on a one way paired t-test ($p = 0.01$ for

zymosan, p = 0.03 for 1:1 phagocyte:bacterium ratio and p = 0.009 for 1:10 phagocyte:bacterium ratio, Figure 4). Hypercapnic hypoxia suppressed superoxide production by 58.6%, 75% and 47.4% when stimulated by zymosan, 1:1 or 1:10 phagocyte:bacterium ratio respectively.

Bactericidal Activity Time Course

The ability of head kidney cells to kill bacteria *in vitro* was assessed using a bactericidal assay optimized for *F. heteroclitus*. Bactericidal activity was suppressed by the addition of TFP, indicating that substantial killing activity was largely dependent on the respiratory burst (Secombes, 1993). Higher optical densities of wells with TFP treated cells suggest that phagocytosis is occurring, despite inhibition of ROS production in these cells (Figure 5).

A time course was performed under atmospheric conditions to determine the time of peak bactericidal activity (Figure 5). Measurements made at 90, 180 and 300 minutes showed bacterial killing was optimal at 180 minutes for both phagocyte:bacterium ratios. Therefore, subsequent assays were conducted for three hours.

Bactericidal Activity Against Vibrio parahaemolyticus

The chemiluminescence and NBT reduction assays showed that hypercapnic hypoxia decreases ROS production in response to both zymosan and *V. parahaemolyticus* (Figures 2 and 4). To demonstrate that this suppression might be associated with increased disease susceptibility, the bactericidal activity of the phagocytes was evaluated under conditions of hypercapnic hypoxia. Bactericidal activity of mummichog

phagocytic cells against *V. parahaemolyticus* at a 1:1 phagocyte:bacterium ratio was not significantly suppressed under hypercapnic hypoxia ($p = 0.299$, Figure 6). However, when the effector:target ratio was changed to 1 phagocyte for 10 bacteria, bactericidal activity was significantly suppressed under hypercapnic hypoxic conditions (Wilcoxen rank sum test, $p = 0.0191$, Figure 7). Bactericidal activity under hypercapnic hypoxic conditions was suppressed by 23.3% at the 1:1 ratio and 72% at the 1:10 ratio.

Although hypercapnic hypoxia did not significantly suppress bactericidal activity at the 1:1 phagocyte:bacterium ratio, four fish had at least a 40% decrease in bactericidal activity under hypercapnic hypoxic conditions (Figure 8). Additionally, at the 1:10 phagocyte:bacterium ratio, seven fish had at least a 40% decrease in bactericidal activity under hypercapnic hypoxic conditions (Figure 9).

Bactericidal Activity Against Vibrio parahaemolyticus As A Function Of Oxygen Only

While the respiratory burst is oxygen dependent, it was not clear that the levels of oxygen used in these studies was limiting ROS production *in vitro*. Therefore, tests were designed to evaluate if low oxygen levels alone would suppress ROS-mediated bactericidal activity. Bactericidal activity of mummichog phagocytic cells against *V. parahaemolyticus* at a ratio of 1:1 and 1:10 was not significantly suppressed under hypoxia (Wilcoxen rank-sum test, $p=0.3013$ for the 1:1 phagocyte:bacterium ratio, $p = 0.0527$ for the 1:10 phagocyte:bacterium ratio) (Figures 10 and 11). Bactericidal activity under hypoxia was 77.7% of the atmospheric response at the 1:1 ratio phagocyte:bacterium ratio and 73% at the 1:10 ratio.

Cell Survival And Bacterial Growth Curves

Results of the ROS and bactericidal assays under hypercapnic hypoxia may be explained by reduced viability of effector cells or enhancement of bacterial cell growth. To exclude these possibilities, cell viability and bacterial growth was measured under hypercapnic hypoxic conditions. Cell viability was greater than 98% based on trypan blue exclusion after isolation and after a three hour incubation in the presence of TFP. Cells exposed to hypercapnic hypoxia for three hours had greater than 96% viability based on trypan blue exclusion, and once returned to normoxic conditions, demonstrated the same level of chemiluminescence as cells that had only been exposed to normoxic conditions.

V. parahaemolyticus grown under the conditions of low dissolved oxygen and high carbon dioxide used in the functional assays of this research, did not exhibit enhanced growth when compared to the growth under normoxic or atmospheric conditions (Figure 12). Additionally, conditions with a low pH mimicking those seen in the functional assays did not enhance the growth of *V. parahaemolyticus* (Figure 13).

Comparison Between Responses Under Atmospheric And Normoxic Conditions

Many studies on immune function of marine animals do not consider physiologically relevant conditions comprised of dissolved oxygen, carbon dioxide and pH. To determine whether responses under atmospheric conditions differ from the same responses under physiologically relevant conditions,. the chemiluminescent, NBT and bactericidal assays were also performed under atmospheric conditions. A comparison of responses under atmospheric and normoxic conditions clearly shows that these two

conditions do not result in the same response (Figure 14). ROS production measured by luminol-enhanced chemiluminescence under normoxic conditions was 57.9% of the production under atmospheric conditions. NBT reduction in response to stimulation with zymosan and the 1:10 phagocyte:bacterium ratio was approximately the same however at the 1:1 concentration, NBT reduction under normoxic conditions was 194% of the reduction under atmospheric conditions. Bactericidal activity under normoxic conditions at the 1:1 phagocyte:bacterium ratio was only 79% of the killing that occurred under atmospheric conditions. At the 1:10 ratio bactericidal activity under normoxia was 96% of the response under atmospheric conditions.

DISCUSSION

Hypercapnic hypoxia, caused by both natural and anthropogenic factors, exists in oceans, lakes, rivers and, most importantly for this research, in estuaries. The extreme fluctuations of dissolved oxygen, carbon dioxide and pH occurring in estuaries pose a significant stress on the organisms that depend on these areas for habitat and nursery grounds. This poor water quality is tightly correlated with poor health of aquatic organisms (Anderson et al., 1981; Miller and Tripp, 1982; Stave et al., 1984; Diaz and Rosenberg, 1995; Lenihan and Peterson, 1998; Moullac et al., 1998). While other environmental stresses such as temperature fluctuations, exposure to toxins and high particulate matter also appear to affect the health of organisms, low levels of dissolved oxygen most often co-occur with poor animal health such as bacterial infection (Clean Water Act Amendments, 1972; Miller and Tripp, 1982). For example, the ubiquitous, opportunistic pathogens *Pseudomonas fluorescens, Aeromonas hydrophila* and *Vibrio anguillarum* generally do not cause disease in fish unless the water quality declines (Rodsaether et al., 1977; Miller and Tripp, 1982). Although there is circumstantial evidence of an interaction between hypoxia and disease in aquatic organisms, there is little direct evidence supporting the action of hypercapnic hypoxia on a specific mechanism of disease resistance. In this research, I have elucidated one mechanism of disease resistance that is suppressed by hypercapnic hypoxia, and have shown that this suppression leads to decreased bactericidal activity in the mummichog, *Fundulus*

heteroclitus. These results suggest that the innate immune response of *F. heteroclitus* is suppressed when it is exposed to environmental hypercapnic hypoxia.

The anterior head kidney is composed largely of phagocytic cells including macrophages and granulocytes (Table 1). This result agrees with other studies in which phagocytic cells are by far the most abundant in the anterior head kidney of two species of mummichogs, *Fundulus heteroclitus* and *Fundulus grandis* (Hightower et al., 1984; Roszell and Rice, 1998). In contrast, the pronephros of the East African killifish, *Nothobranchius guentheri,* contains primarily lymphocytes and histiocytes with neutrophils and eosinophils appearing at a later age (Hightower et al., 1984).

Phagocytic cells including macrophages and granulocytes in vertebrates and hemocytes in invertebrates, play a primary role in protecting an organism from infection and disease caused by invading pathogens (Gabig and Babior, 1981; Chung and Secombes, 1988; Secombes and Fletcher, 1992; Ainsworth, 1992; Roitt et al., 1996; Roszell and Rice, 1998). The present study demonstrates that adherent macrophages and granulocytes from anterior head kidney of *F. heteroclitus* phagocytose efficiently both zymosan and *V. parahaemolyticus.* Phagocytosis of both zymosan and *V. parahaemolyticus* was evident as early as 15 minutes after exposure and continued through 105 minutes (Figure 1). Phagocytosis peaked at 45 minutes for zymosan and the higher concentration of bacteria (1:10) and 75 minutes for the lower concentration of bacteria (1:1)(Figure 1). These results are similar to those of other studies in which investigators have found that peak phagocytosis occurs from 30 minutes to 1 hour for zymosan and heat killed *Vibrio anguillarum* and from 1 to 2 hours for polystyrene latex

microspheres (Hightower et al., 1984; Roszell and Anderson, 1994; Roszell and Rice, 1998)

The dominant phagocytic cell type was the macrophage with an average of 46% of this cell population ingesting one or more particles of zymosan or bacteria (Table 2). This result is similar to those of other studies on *F. grandis* and *F. heteroclitus* in which the macrophage was also the predominant phagocytic cell type (Hightower et al., 1984; Roszell and Rice, 1998).

Phagocytosis of an antigen, such as zymosan and *V. parahaemolyticus,* activates the respiratory burst, the major antibacterial defense mechanism evaluated in this research, leading to the production of ROS (Gabig and Babior, 1981; Chung and Secombes, 1988; Secombes and Fletcher, 1992; Ainsworth, 1992; Roitt et al., 1996; Roszell and Rice, 1998). ROS are produced in both vertebrate and invertebrate phagocytic cells and are responsible for damaging the invading microorganism through protein degradation, enzyme inactivation and DNA damage (Chung and Secombes, 1988; Roitt et al., 1996). The studies performed as part of this thesis research demonstrated that phagocytic cells of *F. heteroclitus* produce ROS in response to stimulation with zymosan and that this process is significantly suppressed under hypercapnic hypoxia (Figure 2). Viability of these cells under hypoxic conditions was greater than 96%, supporting the conclusion that this decrease is a result of inhibited respiratory burst activity and not cell death. Similarly, other investigators found that hypoxia does not have an effect on viability of oyster hemocytes or their rate of phagocytosis (Alvarez et al., 1989), however hypoxia does reduce total hemocyte counts in the shrimp, *Penaeus stylirostris* (Moullac et al., 1998).

This study demonstrated that hypercapnic hypoxia suppresses the production of ROS measured by the luminol-enhanced chemiluminescence assay (Figure 2). There was a great deal of variation in the chemiluminescent responses among individual fish (Figure 3). High variation in the chemiluminescent response has been documented in wild species of fish including spot and hogchoker (Warriner et al., 1988). This high variation, particularly under atmospheric conditions and to a lesser degree normoxia, may be related to the activation state of the macrophage, the health of the fish or its history of pathogen exposure (Secombes, 1990). Mummichogs exposed to polynuclear aromatic hydrocarbons (PAH) in Virginia's Elizabeth River, displayed a significantly higher chemiluminescent response than fish taken from pristine waters (Kelly-Reay and Weeks-Perkins, 1994). Additionally, Miller and Tripp (1982) showed that immunocytoadherance is significantly decreased in fish maintained in the laboratory for four weeks. Although this study did not evaluate the effect of captivity on the chemiluminescent response, it cannot be ruled out as a possibility. In the present study, animals exhibiting signs of stress or disease evident by physical examination were removed from aquaria and were not used for this research. However, some fish may have been stressed or infected but displayed no observable signs associated with stress or disease. If this were the case, the chemiluminescent responses may have been affected through suppression or enhancement.

Measurements of ROS production by chemiluminescence commonly employ zymosan to stimulate the respiratory burst. Many fewer studies have employed live bacteria as a stimulant. Due to the inherently high chemiluminescence of *V. parahaemolyticus,* I was unable to determine the response attributable to phagocyte ROS

production when stimulated with *V. parahaemolyticus*. As an alternative approach, evaluation of ROS production using the NBT assay, a simple, colorimetric, objective technique, which quantifies production of superoxide, the first ROS produced during the respiratory burst was substituted (Pick et al., 1981; Rook et al., 1985; Secombes, 1990; Roszell and Rice, 1998). The results from the NBT assay support the results from the zymosan stimulated chemiluminescence assay. Phagocytic cells exposed to hypercapnic hypoxia and stimulated with either zymosan or *V. parahaemolyticus* produced significantly less superoxide than cells exposed to normoxic conditions (Figure 4). Although simple and objective, there is some concern expressed in the literature regarding the accuracy of the NBT assay (Pick et al., 1981; Rook et al., 1985). Unstimulated macrophages display some ability to reduce NBT through the action of the intracellular diaphorase enzyme, although this reduction occurs much more rapidly in stimulated cells (DeChatelet et al., 1974; Secombes, 1990). To account for this reduction, exogenous superoxide dismutase (SOD), the enzyme responsible for the dismutation of superoxide to hydrogen peroxide was added to the mixture to demonstrate the specificity of the reaction for NBT reduction by superoxide. The superoxide anion was responsible for 98% of the NBT reduced under normoxic conditions and 70% under hypoxic conditions. By comparison, Secombes (1990) and Pick (1981) linked only 50% of NBT reduction to the presence of the superoxide anion. However, they speculated that SOD might not be able to gain access to cellular areas where superoxide was being produced. If true, then the amount of NBT reduction due to superoxide may have been underestimated in the assays reported here.

Another concern encountered with this assay but not addressed in the literature is the reduction of NBT by *V. parahaemolyticus*. In this study, it was possible to determine NBT reduction by phagocytic cells by subtracting the amount of NBT reduced by the bacteria alone from the amount reduced by both phagocytes and bacteria. However, there may be complex interactions occurring between the bacteria and the phagocyte that have not yet been determined. A large number of prokaryotic and eukaryotic organisms have been assayed for SOD production and only three have been identified as non-producers of SOD (Archibald and Duong, 1986). For example, pathogenic bacteria from the genus *Neisseria* and the species *Shigella flexneri* have been characterized as producers of SOD (Archibald and Duong, 1986; Franzon et al., 1990). In these organisms, SOD becomes activated when they shift from an anaerobic environment to an aerobic environment (Franzon et al., 1990). *V. parahaemolyticus* has also been identified as an SOD producer. Once *V. parahaemolyticus* is ingested by a phagocyte, SOD production may be stimulated or increased above constitutively expressed levels, due to the increase in oxygen seen in the phagocyte. This raises a few questions. First, do the controls in the present study (NBT reduction by *V. parahaemolyticus* in the absence of phagocytic cells) accurately portray the amount of NBT reduced by the bacteria in the presence of phagocytes? Secondly, is SOD produced by the bacteria sensitive to hypercapnic hypoxic conditions? If so, would this increase susceptibility to phagocytic ROS production under hypercapnic hypoxia?

The data from both the chemiluminescence assay and the NBT assay demonstrate that hypercapnic hypoxia significantly reduces ROS production in phagocytic cells. The significance of this suppression in immune function could only be evaluated by

measuring the sensitivity of ROS mediated bactericidal activity to hypercapnic hypoxia. There is evidence that suppression of ROS production by exposure to pollutants or drugs which act as metabolic inhibitors, can lead to decreased bactericidal activity (Sharp and Secombes, 1993; Roszell and Anderson, 1997). To date however, it appears that the direct effects of hypercapnic hypoxia on ROS-mediated bactericidal activity have not been examined.

The use of trifluoroperazine (TFP) to inhibit the respiratory burst provided a means of measuring the amount of bacterial killing due to this bactericidal pathway. The high optical densities for the TFP treated cells indicated a strong ROS-mediated bactericidal pathway in these phagocytes (Figures 6 and 7). TFP has been used frequently in bactericidal assays to inhibit NADPH oxidase activity (Secombes, 1990; Graham et al., 1988; Sharp and Secombes, 1993). More recent studies on bactericidal activity are using an alternative NADPH oxidase inhibitor, diphenyleneiodonium (DPI) (Bramble and Anderson, 1999). Results in the present study show that bactericidal activity of phagocytic cells against *V. parahaemolyticus* under hypercapnic hypoxia is not significantly suppressed when phagocytic cells are challenged at a 1:1 ratio of phagocytes to bacteria (Figure 6). However, when the challenge is increased to 1:10, hypercapnic hypoxia causes a significant inhibition of bactericidal activity (Figure 7). This result indicates that when phagocytes are presented with relatively low numbers of a potential pathogen, the capacity for ROS production exceeds the cells' need for antibacterial defense. Below this threshold level of bacterial infection, phagocytic cells are capable of providing protection under moderate levels of stress. When this threshold level is exceeded, the cell requires all of its energy and resources to generate ROS for

antibacterial defense and becomes more sensitive to environmental stress. Bactericidal activity of individual animals varied such that the challenge dose of bacteria resulting in sensitivity to hypercapnic hypoxia also varied. The response from individual fish at the 1:1 phagocyte:bacterium ratio shows that although there was not a significant difference at this ratio, 4 of the 10 fish (#2, 3, 4 and 10, Figure 8) did demonstrate decreased bactericidal activity under hypercapnic hypoxia. Similarly, the majority of the fish at the 1:10 phagocyte:bacterium ratio demonstrated decreased bactericidal activity under hypercapnic hypoxia conditions. However, the degree of killing varied among fish (Figure 9).

These data show that the respiratory burst is inhibited by hypercapnic hypoxia and that the reduced level of ROS production suppresses anti-bactericidal capacity of phagocytic cells (Figures 2, 4 and 7). ROS production is oxygen dependent and therefore it was of interest to determining if the suppressive effects associated with hypercapnic hypoxia were due to oxygen alone or a combination of low oxygen, high carbon dioxide and low pH. As the results show, bactericidal activity at both the 1:1 and 1:10 phagocyte:bacterium ratios was not significantly suppressed under conditions of low oxygen only as determined by the non-parametric Wilcoxen rank-sum test, although the p-value approached significance at the 1:10 phagocyte:bacterium ratio (Figures 10 and 11). However, in contrast to these results, Boyd and Burnett, (1998) demonstrated that low pH and low oxygen levels independently suppress ROS production by oyster hemocytes. Carbon dioxide pressure, independent of pH, had no effect (Boyd and Burnett, 1998). Furthermore, the effects of low oxygen and low pH were additive. If mummichog phagocytes behave the same way as oyster hemocytes, then both low

oxygen and low pH may account for the hypercapnic hypoxia-induced reduction in bactericidal activity in the present study.

The studies presented here demonstrate that adequate levels of oxygen, carbon dioxide and pH, play a critical role in maintaining antibacterial defense mechanisms of the mummichog, *F. heteroclitus.* The chemiluminescence assay has shown decreased ROS production under hypercapnic hypoxic conditions in response to zymosan exposure (Figure 2). Superoxide production stimulated by *V. parahaemolyticus* was also significantly inhibited under hypercapnic hypoxia, demonstrated by decreased reduction of NBT (Figure 4). Furthermore, bactericidal activity against *V. parahaemolyticus* was suppressed by hypercapnic hypoxia (Figure 7). The increase in pathogenicity under hypoxic conditions was not a result of increased bacterial growth under these conditions (Figure 9 and 10). Although the respiratory burst process is oxygen dependent, hypoxia alone did not significantly suppress bactericidal activity (Figures 10 and 11). There appears to be an interaction between hypoxia, hypercapnia and low pH that act concomitantly in suppressing disease resistant mechanisms. Although not evaluated here, it is likely that pH plays a large role in suppression of these responses (Boyd and Burnett, 1998). Furthermore, although there is a great deal of variation in oxygen affinity among various oxidases, the cytochrome oxidases including NADPH oxidase, have a particularly high affinity for O_2 (de Groot and Littauer, 1989). As a result, impairment of enzyme activity may occur at an oxygen level of 5 Torr, however, significant inhibition usually requires an extracellular Po_2 of 2 Torr (de Groot and Littauer, 1989). Therefore, it is unlikely that low oxygen is substrate limiting for NADPH oxidase activation.

Several factors should be considered when applying the results of these studies to evaluate disease susceptibility in whole organisms. First, pathogenic organisms may protect themselves against cellular defenses by taking specific action against the phagocyte. For example, several species of *Vibrio* including *V. anguillarum,* elicit a very weak chemiluminescent response measured as ROS production, or do not elicit a response at all (Lambert and Nicolas, 1998). This suggests that the pathogenicity of these bacteria may be enhanced by not stimulating the production of ROS from the phagocyte. Similarly, pre-exposure of bivalve hemocytes (*Pecten maximus* and *Crassostrea gigas),* to numerous strains of *Vibrio*, decreases the zymosan stimulated chemiluminescence response at a later time (Lambert and Nicolas, 1997). As previously discussed, a large number of bacteria including *V. parahaemolyticus* produce SOD and catalase. Catalase, also produced by phagocytic cells, is the enzyme responsible for the conversion of hydrogen peroxide to water and oxygen (Chung and Secombes, 1988). Therefore, catalase and SOD positive microorganisms such as *Vibrio parahaemolyticus,* may be further protected against ROS produced by the phagocytic cells. Cells which are challenged by catalase and SOD-positive pathogens, may need to produce excess ROS, and consequently be more sensitive to the effects of hypercapnic hypoxia.

It is of interest to note that while several studies have confirmed the importance of ROS-mediated bactericidal activity in teleost fish (Chung and Secombes, 1988; Graham et al., 1988; Sharp and Secombes, 1993; Bramble and Anderson, 1999), a recent study provides evidence that ROS are not involved in the bactericidal activity of hemocytes from the Eastern oyster, *Crassostrea virginica* (Bramble and Anderson, 1999). NADPH oxidase inhibition with DPI resulted in a significant decrease in ROS production by

oyster hemocytes. Despite this reduction in ROS production, there was no decrease in bactericidal activity against *Pseudomonas fluorescens* and only a 4% reduction in bactericidal activity against *Bacillus megaterium.* Bramble and Anderson (1999) that the overall low chemiluminescent response exhibited by bacteria stimulated oyster hemocytes may not be sufficient to counteract the antioxidant activity of the bacteria. However, these investigators were able to correlate decreased ROS production in the striped bass, *Morone saxatilis,* with decreased bactericidal activity against both *P. fluorescens* and *B. megaterium.*

Future direction of this research should include investigation into reactive nitrogen species. Activation of nitric oxide synthase results in the production of nitric oxide, which has also been shown to be toxic to bacteria and tumor cells (Roitt et al., 1996). DPI, the inhibitor used in the previously described hemocyte study, has been shown to inhibit nitric oxide synthase (NOS), an enzyme associated with the production of reactive nitrogen intermediates (RNI) (Bramble and Anderson, 1999). Consequently, the possibility that RNI or products of RNI and ROS such as peroxinitrate and not ROS are playing the primary role in bactericidal activity cannot be ruled out in the hemocyte study.

A final point of discussion concerns the results of the chemiluminescence, NBT and bactericidal assays under atmospheric conditions. Phagocytic cells of the mummichog are not exposed to atmospheric conditions *in vivo*. *In vitro* exposure of these cells in this research created an artificial environment, one in which there is no "normal" response. Therefore, statistical analysis was not performed on these data. However, it is important to point out that the responses under normoxic and atmospheric

conditions were not the same; the normoxic response was almost always lower than the atmospheric response (Figure 14). This is meaningful when reviewing the large body of literature on the immunology and physiology of fish. By far, the majority of the experiments have been performed under atmospheric conditions. The results presented here show that physiologically relevant responses are not identical to the responses under atmospheric conditions. Therefore, future experiments should be performed under conditions reflecting the physiological state of the organism.

Hypercapnic hypoxia in estuaries, caused by both natural variation and anthropogenic sources, are an increasing concern worldwide. A healthy estuarine ecosystem requires sufficient amounts of dissolved oxygen, carbon dioxide and a specific pH range to support the abundant life that exists there. The Clean Water Act Amendments of 1972 state a criterion level of 5 mg/l of dissolved oxygen for a body of water to be considered healthy and unimpacted. A concentration of approximately 2 mg/l is thought to be extremely stressful to most estuarine organisms. This criterion was set largely in response to mortality associated with poor water quality and does not consider carbon dioxide and pH levels. The results presented here demonstrate that when dissolved oxygen levels are maintained internally at 0.86 mg/l (15 Torr), carbon dioxide at 8.0 Torr and a pH of 7.0, a level which reflects an environmental condition of moderate hypercapnic hypoxia, estuarine organisms may be subject to increased risk of infection with opportunistic pathogens. These sub-lethal effects may occur at even higher dissolved oxygen levels, necessitating further investigation into identifying the critical dissolved oxygen level at which immune function is compromised. This should ultimately lead to re-examination of water quality standards, specifically in impacted

areas where development and discharge of material into the water column can be regulated.

CONCLUSION

The present study has demonstrated that environmental hypoxia, including low dissolved oxygen, high carbon dioxide and a decreased pH, suppresses at least one component of immune function in the mummichog, *Fundulus heteroclitus*. ROS mediated bactericidal activity of phagocytes under physiologically relevant conditions provides evidence to support the hypothesis that hypercapnic hypoxia decreases disease resistance to pathogenic infections in aquatic organisms. This research thus provides a stepping stone for further investigation into the interaction between pathogen and immune cell and other possible bactericidal pathways found in phagocytic cells in addition to the effects of environmental stressors. Most importantly however, these studies have provided yet another piece of information to use in support of maintaining healthy estuarine communities and recognizing the impact that human activity can have on these estuaries.

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Table 1. Differential cell counts of anterior head kidney cells from individual mummichogs. Cells were stained with Diff-Quik and evaluated using light microscopy under a 100X oil immersion lens. Values represent cell counts from a field of 100 cells. Before adherence n=3. After adherence n=3.

Differential Cell Counts of Anterior Head Kidney Cells from Individual Mummichogs

Cell Type	% Before Adherence	% After Adherence
Macrophage	**Fish 1: 51%** **Fish 2: 42%** **Fish 3: 43%**	**Fish 1: 76%** **Fish 2: 64%** **Fish 3: 70%**
Granulocyte	**Fish 1: 26%** **Fish 2: 27%** **Fish 3: 31%**	**Fish 1: 22%** **Fish 2: 32%** **Fish 3: 29%**
Red Blood Cell	**Fish 1: 20%** **Fish 2: 24%** **Fish 3: 24%**	**Fish 1: 1%** **Fish 2: 4%** **Fish 3: 1%**
Other (Lymphocytes, undifferentiated cells)	**Fish 1: 3%** **Fish 2: 7%** **Fish 3: 2%**	**Fish 1: 1%** **Fish 2: 0%** **Fish 3: 0%**

Table 2. Percent of adherent cell types demonstrating phagocytosis of *Vibrio parahaemolyticus* after a 45-minute incubation under atmospheric conditions. Cells were stained with Diff-Quik and evaluated using light microscopy under a 100X oil immersion lens. Values represent the range of percent phagocytosis from 3 mummichogs. Counts were performed on a field of 100 cells.

Percent of Adherent Cell Types Demonstrating Phagocytosis of *Vibrio parahaemolyticus*

Cell Type	% Phagocytic
Macrophage	**Fish 1: 74%** **Fish 2: 54%** **Fish 3: 6%**
Granulocyte	**Fish 1: 11%** **Fish 2: 31%** **Fish 3: 0%**
Red Blood Cell	**Fish 1: 0%** **Fish 2: 0%** **Fish 3: 0%**
Other (Lymphocytes, undifferentiated cells)	**Fish 1: 0%** **Fish 2: 0%** **Fish 3: 0%**

Table 3. Summary of results from the chemiluminescence, NBT and bactericidal assays performed under hypercapnic hypoxic conditions. Statistical test performed along with p values are given. Significant difference from normoxic conditions is determined by a p value of less than 0.05. Gray cells indicate assay not performed with the specific stimulus.

Summary of Results From the Chemiluminescence, NBT and Bactericidal Assays Performed Under Hypercapnic Hypoxic Conditions.

Stimulant	**CL**	**NBT**	**Bactericidal**	**Bactericidal** (as a function of O2)
Zymosan	Wicoxen rank Sum p = 0.01	One way paired t–test p= 0.01		
Vibrio Parahaemolyticus (1:1)		One way paired t–test p= 0.03	Wicoxen rank Sum p = 0.299	Wicoxen rank Sum p = 0.3013
Vibrio parahaemolyticus (1:10)		One way paired t–test p= 0.009	Wicoxen rank Sum p = 0.0191	Wicoxen rank Sum p = 0.0527

Figure 1. Phagocytosis of zymosan and *Vibrio parahaemolyticus* by mummichog phagocytic cells. Maximum phagocytosis of zymosan and bacteria at the 1:10 ratio occurred at 45 minutes. Maximum phagocytosis of bacteria at the 1:1 ratio occurred at 75 minutes. Values represent the average of three fish.

Time Course For Phagocytosis Of Zymosan And *Vibrio parahaemolyticus* By Mummichog Phagocytic Cells

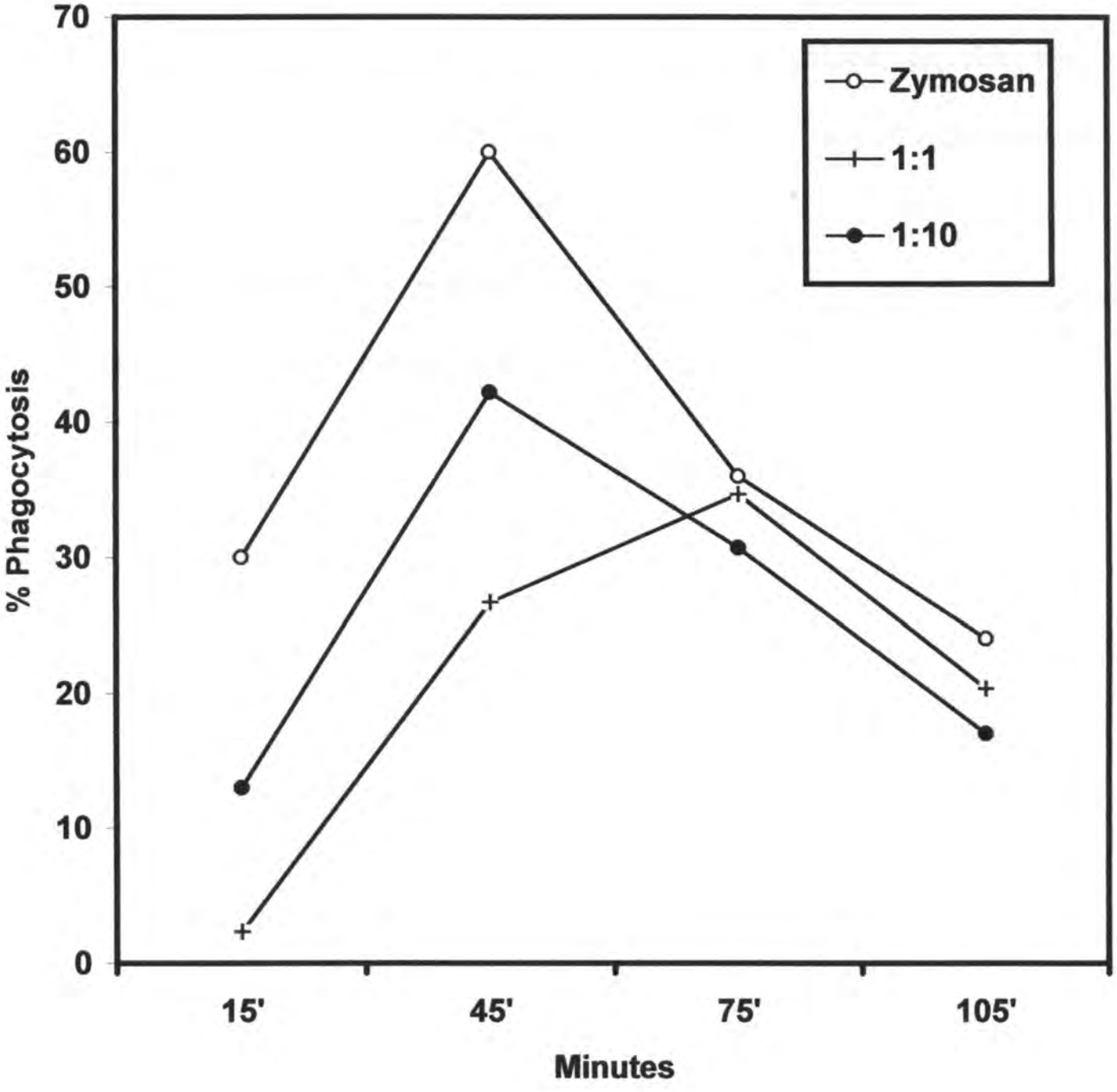

Figure 2. Production of ROS by mummichog phagocytic cells measured as chemiluminescence in response to stimulation with zymosan and held under conditions simulating a normoxic and hypoxic environment. Background chemiluminescence due to luminol alone was measured for 20 minutes. Once background chemiluminescence had stabilized, 100 µl of the zymosan suspension was added to the vials and chemiluminescence was measured for 100 minutes. Total ROS produced was measured as area under the curve starting with the addition of zymosan. ROS production was significantly suppressed under hypoxic conditions (Wilcoxen rank-sum test, $p=0.01$, $n=10$). Values are mean +/- standard error.

Reactive Oxygen Species Production By Mummichog Phagocytic Cells In Response To Zymosan Exposure

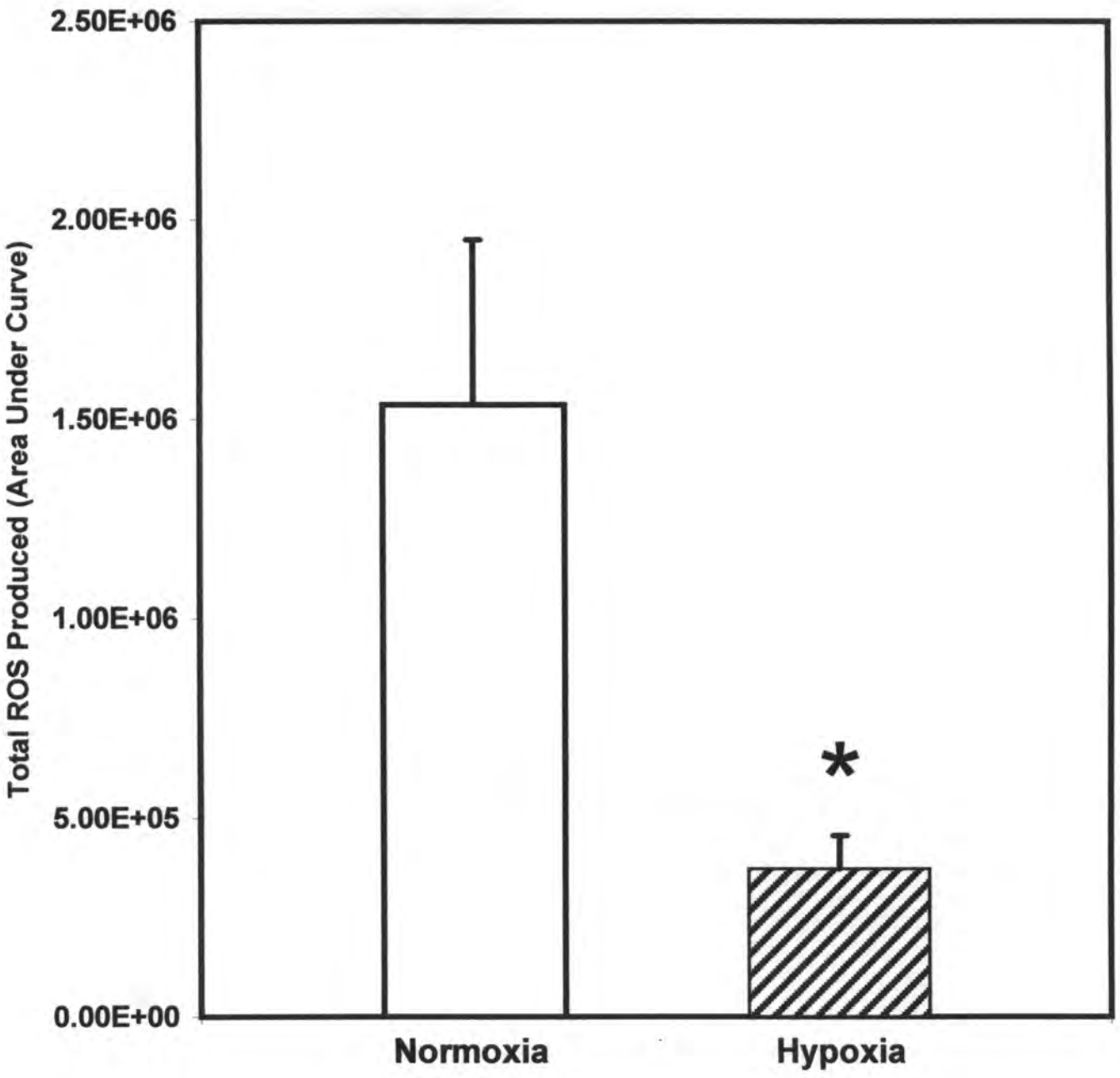

Figure 3. ROS production measured as chemiluminescence by mummichog phagocytic cells in response to zymosan stimulation and held under atmospheric, normoxic and hypoxic conditions. Procedure described in Figure 2 applies to this figure as well. Chart represents individual responses of 10 animals. All fish demonstrated at least a 58% decrease in ROS production under hypoxic conditions.

Reactive Oxygen Species Production By Mummichog Phagocytic Cells In Response To Zymosan Exposure (Response Of Individual Fish)

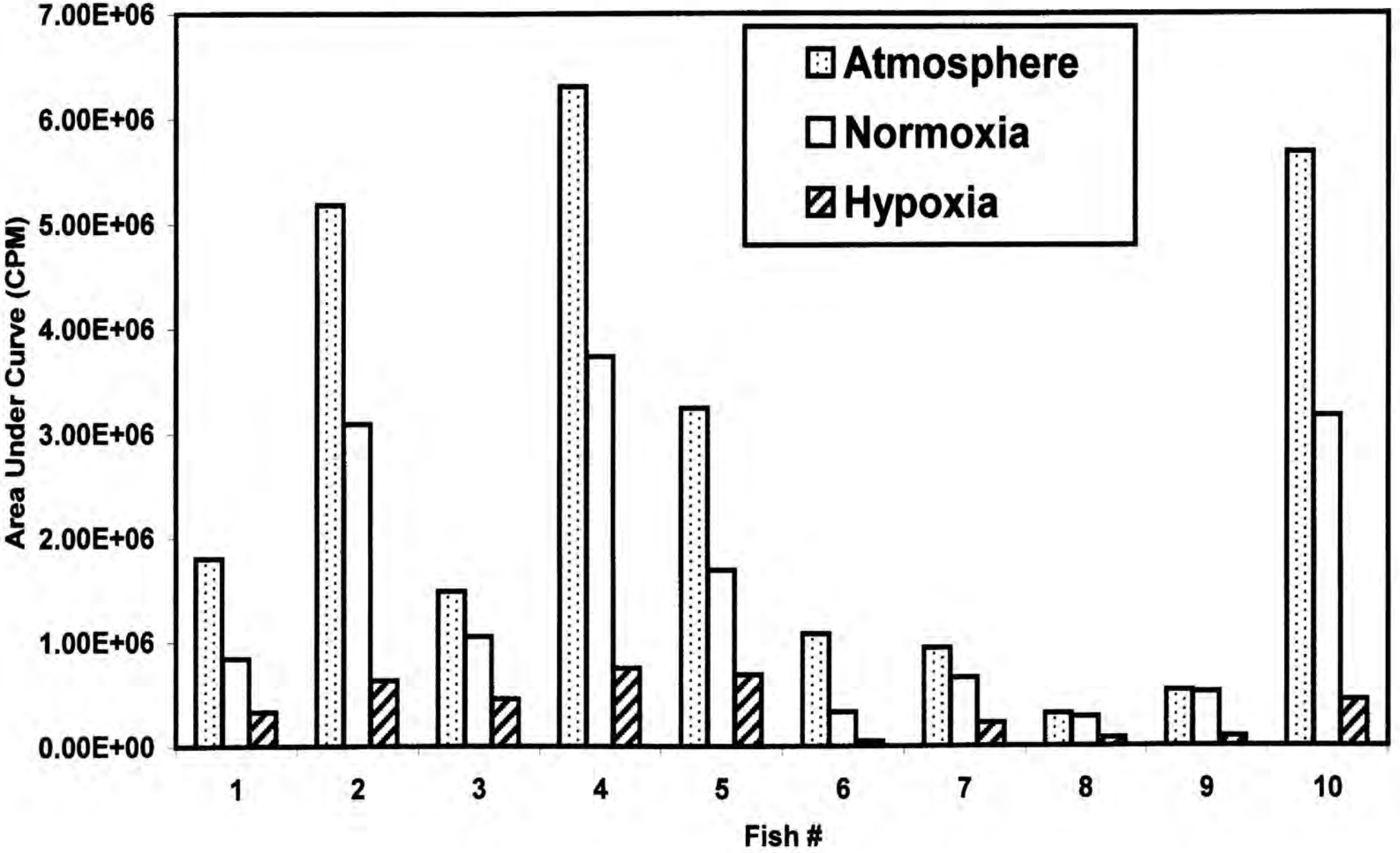

Figure 4. Reduction of NBT by mummichog phagocytic cells in response to stimulation with zymosan or *Vibrio parahaemolyticus*. Phagocytic cells were incubated with zymosan or *V. parahaemolyticus* for one hour under normoxic or hypoxic conditions. Reduced NBT in the form of formazan was then solubilized and the OD was read at 630nm. NBT reduced by stimulant alone was subtracted from NBT reduction by phagocytes and stimulant. Reduction of NBT due to zymosan stimulation is significantly suppressed under hypoxic conditions based on a one way paired t-test ($p=0.002$) ($n=5$). NBT reduction induced by stimulation with *Vibrio parahaemolyticus* is significantly suppressed under hypoxic conditions at both a 1:1 and 1:10 phagocyte:bacterium ratio based on a paired t-test ($p=0.03$, 1 to 1) ($p=0.049$, 1 to 10)($n=5$). Values represent +/- standard error.

NBT Reduction By The Superoxide Anion Produced When Phagocytic Cells Are Stimulated With Zymosan or *Vibrio parahaemolyticus*.

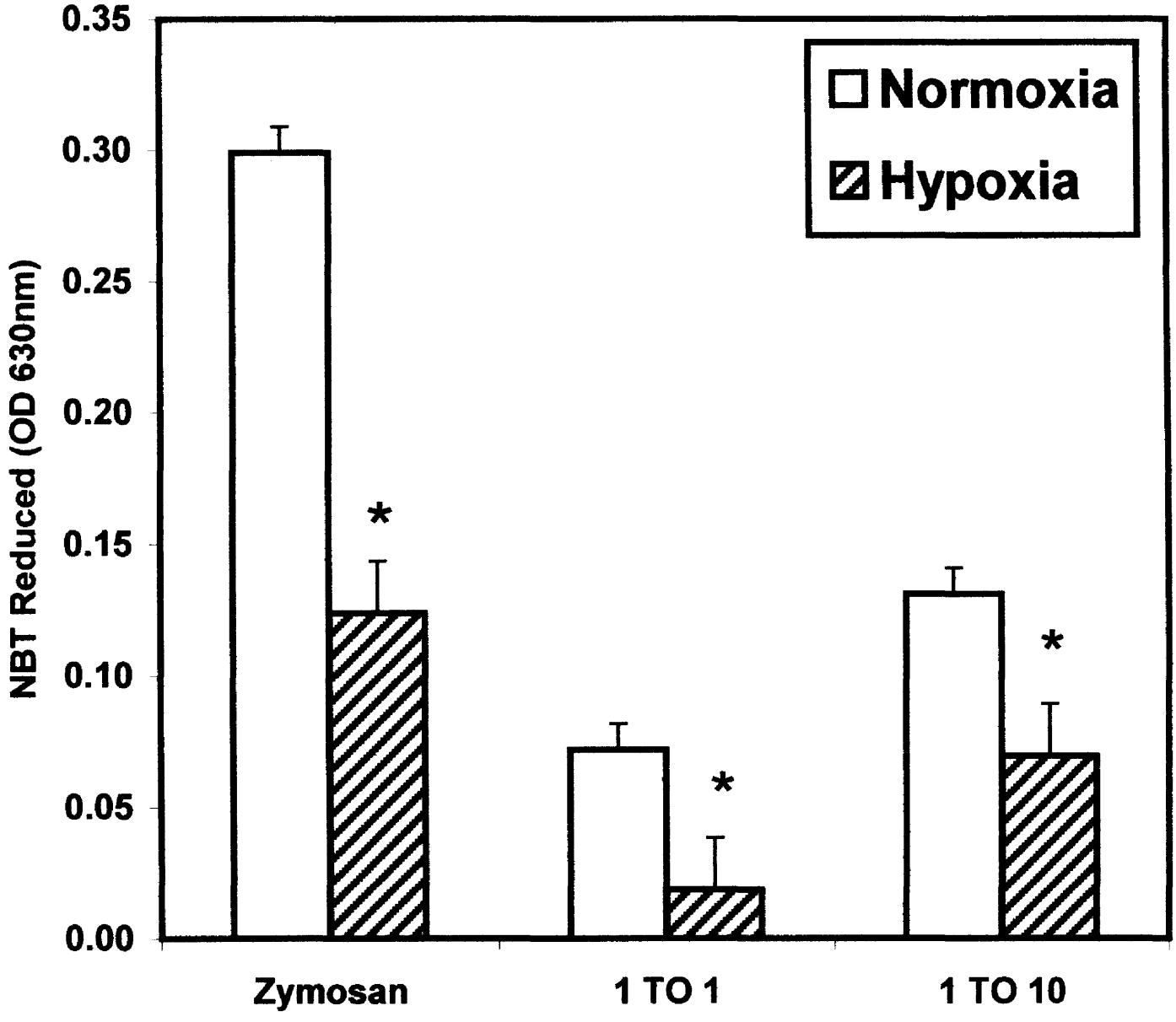

Figure 5. Time course of bactericidal activity by mummichog phagocytic cells at phagocyte:bacterium ratios of 1:1 and 1:10 in the presence and absence of 50 μM. Phagocytes and bacteria were incubated for a five hour period during which cells were lysed at specific time points and surviving bacteria grown for 9 hours. Bacterial survival was quantified by the addition of MTT and measurement of the OD at 561nm. Maximum bacterial killing occurred at 180 minutes for both ratios. Values represent the average of three animals.

Time Course For Bactericidal Activity Of Mummichog Phagocytic Cells Against *Vibrio parahaemolyticus*

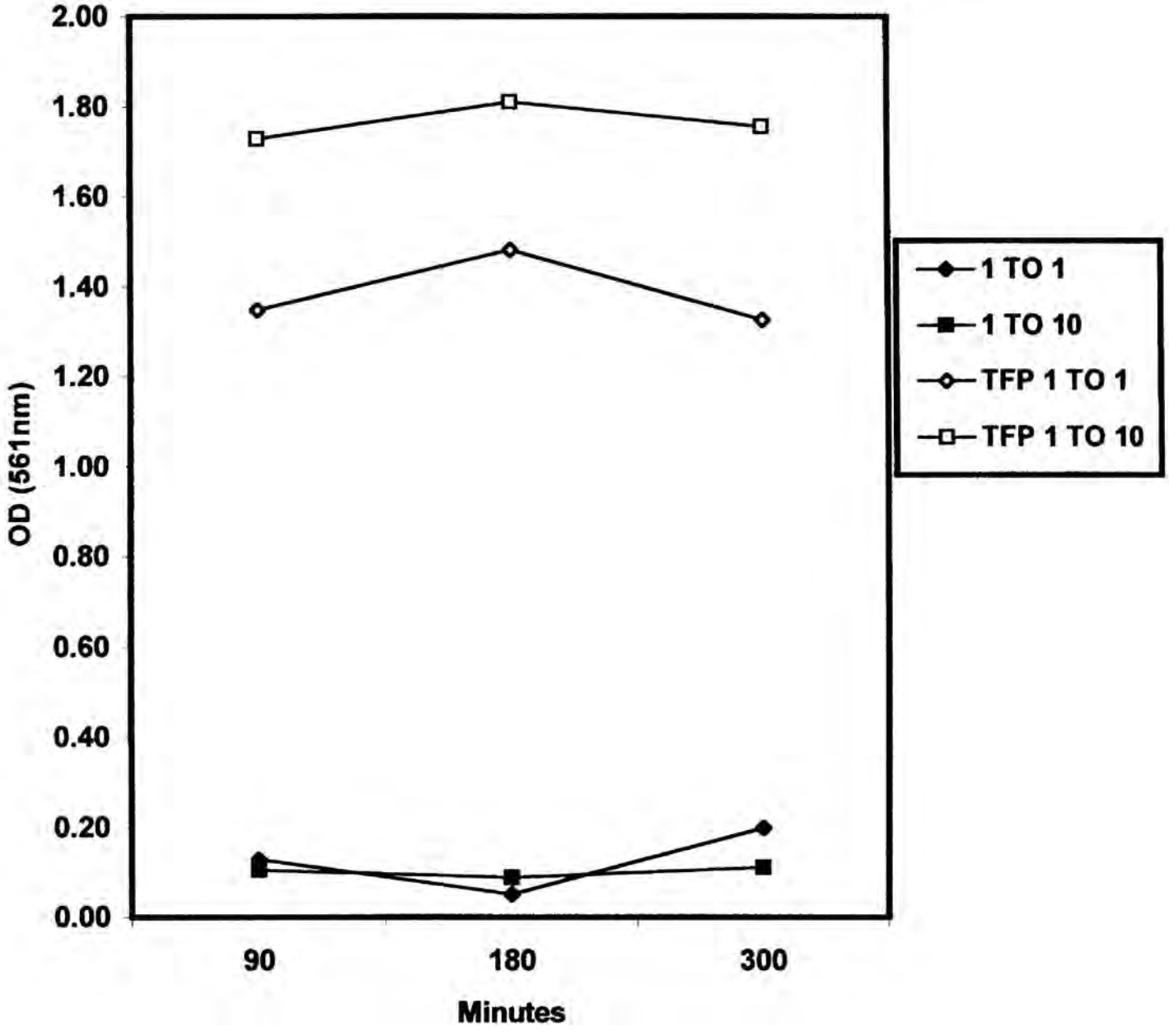

Figure 6. Mean bactericidal activity of mummichog phagocytic cells at a 1:1 phagocyte:bacterium. Phagocytes were incubated with bacteria for 3 hours at which point media was removed and phagocytes lysed, releasing surviving bacteria. Surviving bacteria was quantitated after a 9-hour growth period by the addition of MTT and measurement of the OD at 561nm. Values have been normalized to the TFP response and expressed as percent killing. (TFP has 0% killing due to NADPH oxidase inhibition.) There is no significant difference in bactericidal activity between the normoxic and hypoxic groups (Wilcoxen rank-sum test, $p=0.2899$, $n=10$). Values represent +/- standard error.

Bactericidal Activity of Phagocytic Cells Against *Vibrio parahaemolyticus* at a 1:1 Phagocyte:Bacteria Ratio

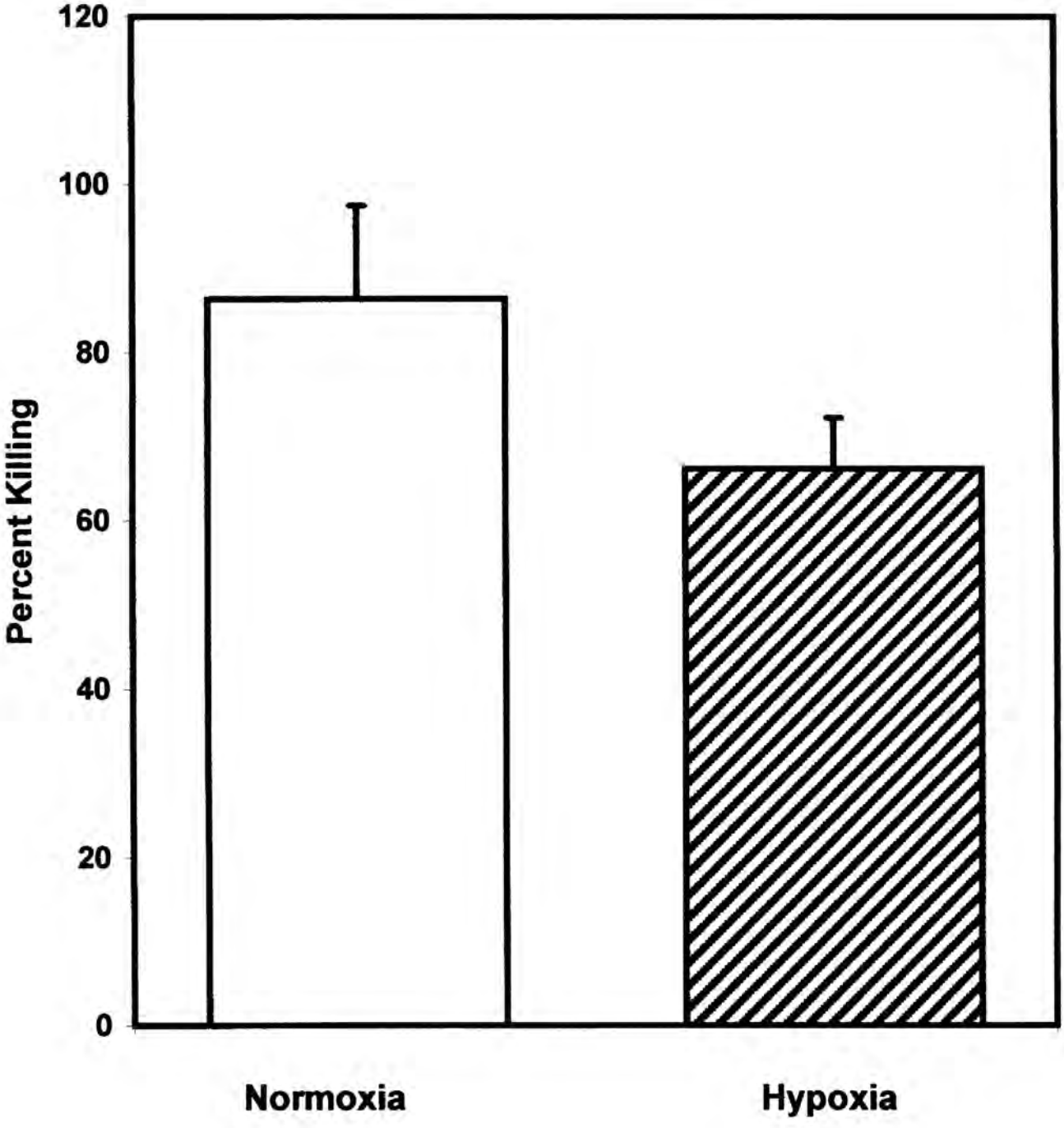

Figure 7. Bactericidal activity of mummichog phagocytic cells at a 1:10 phagocyte:bacterium ratio. Same procedure described in Figure 6 was performed here. Values have been normalized to the TFP response and presented as percent killing (TFP has 0% killing due to NADPH oxidase inhibition.) There is a significant difference in bactericidal activity between the normoxic and hypoxic groups based on the Wilcoxen rank-sum test ($p=0.019$)($n=10$). Values represent +/- standard error.

Bactericidal Activity of Phagocytic Cells Against *Vibrio parahaemolyticus* at a 1:10 Phagocyte:Bacteria Ratio

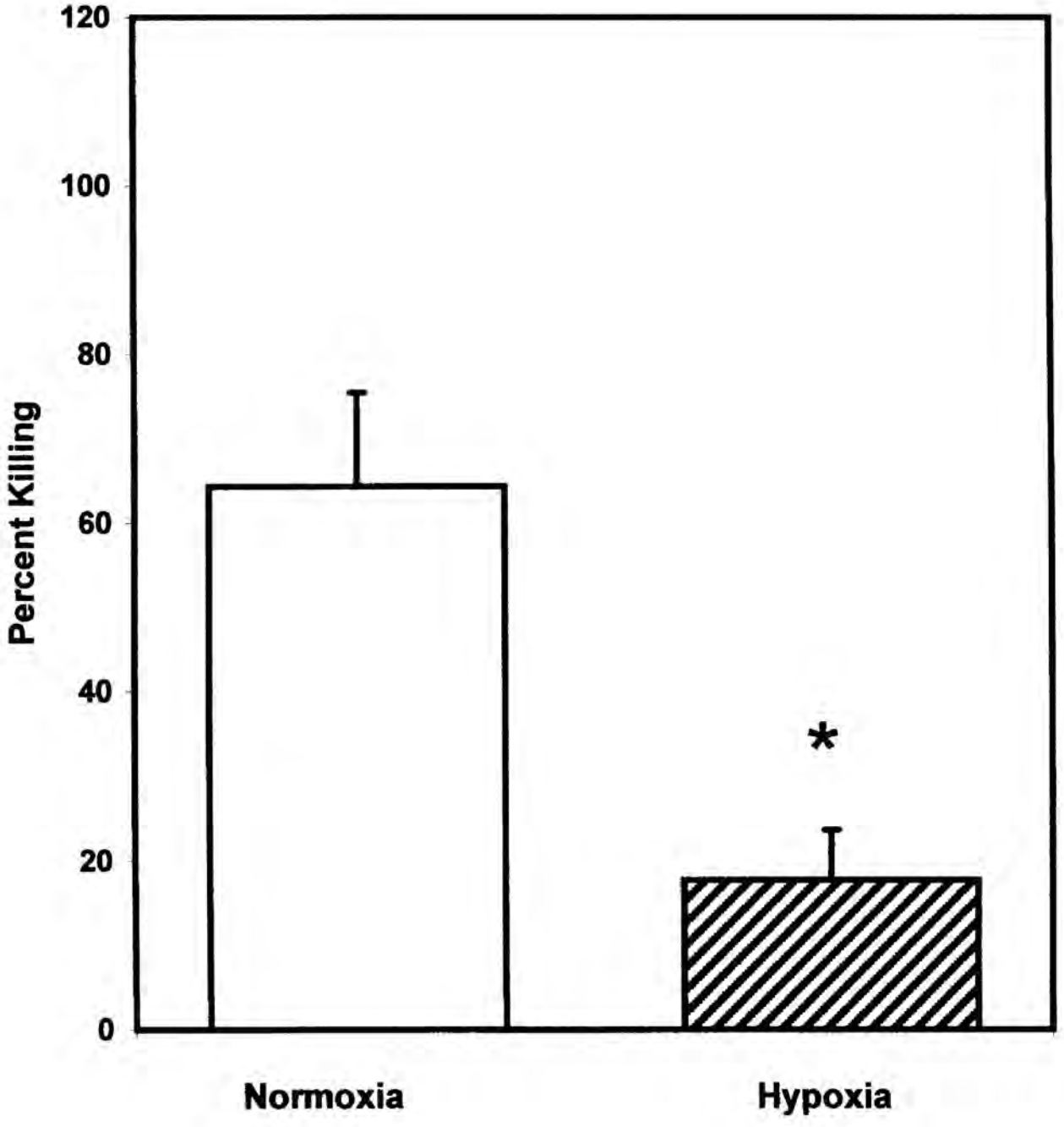

Figure 8. Individual bactericidal activity of mummichog phagocytic cells at a 1:1 phagocyte:bacterium ratio. Same procedure described in Figure 6 was performed here. The atmospheric and hypoxic values have been normalized to the TFP response and presented as percent killing. Wells containing TFP have 0% killing due to NADPH oxidase inhibition.

Bactericidal Activity After a 180' Incubation of Phagocytes and *Vibrio parahaemolyticus* at a 1 to 1 Ratio(Response Of Individual Fish)

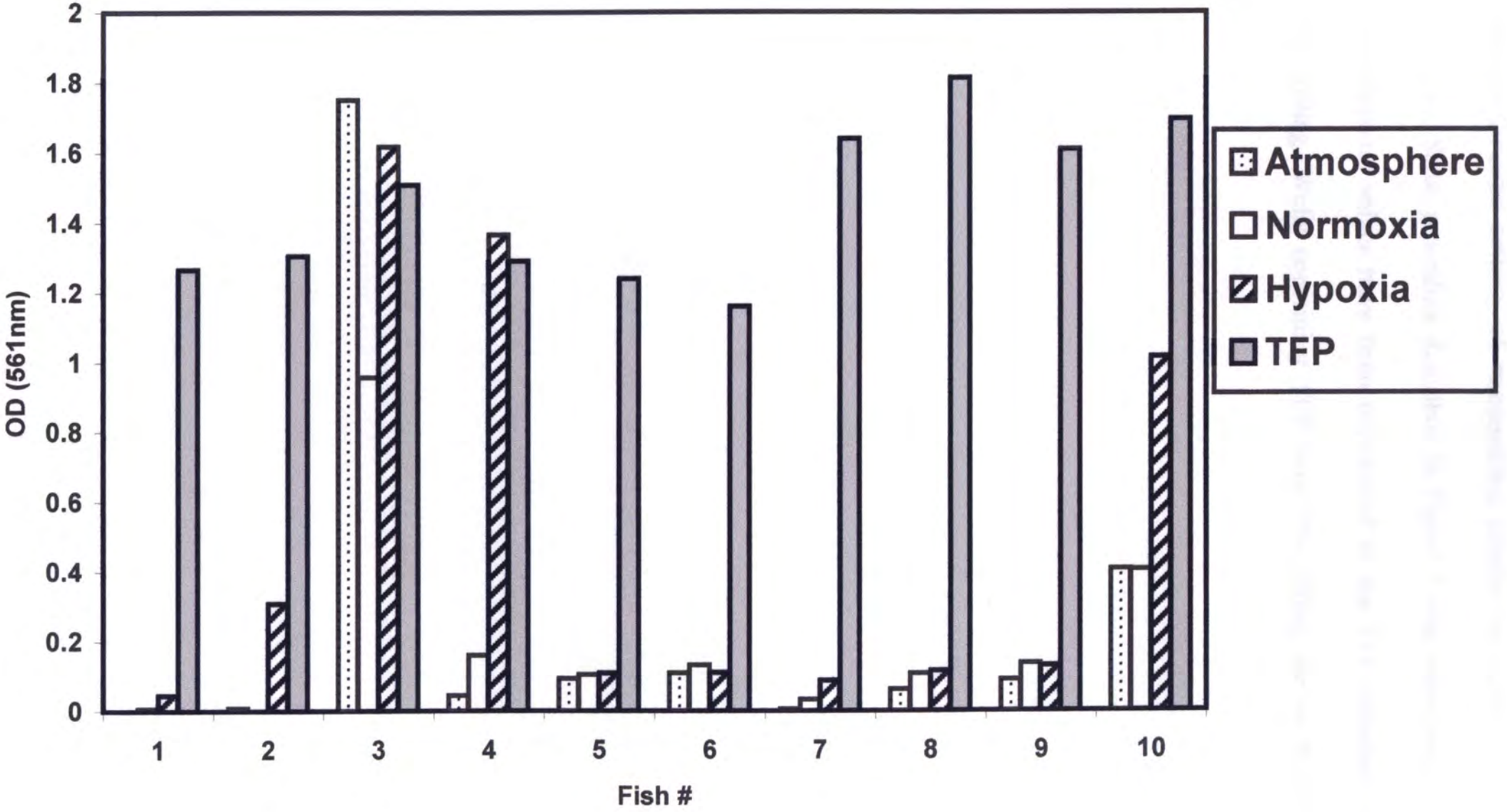

Figure 9. Individual bactericidal activity of mummichog phagocytic cells at a 1:10 phagocyte:bacterium ratio. Same procedure described in Figure 6 was performed here. The atmospheric and hypoxic values have been normalized to the TFP response and presented as percent killing. Wells containing TFP have 0% killing due to NADPH oxidase inhibition.

Bactericidal Activity After a 180' Incubation of Phagocytes and *Vibrio parahaemolyticus* at a 1 to 10 Ratio (Response Of Individual Fish)

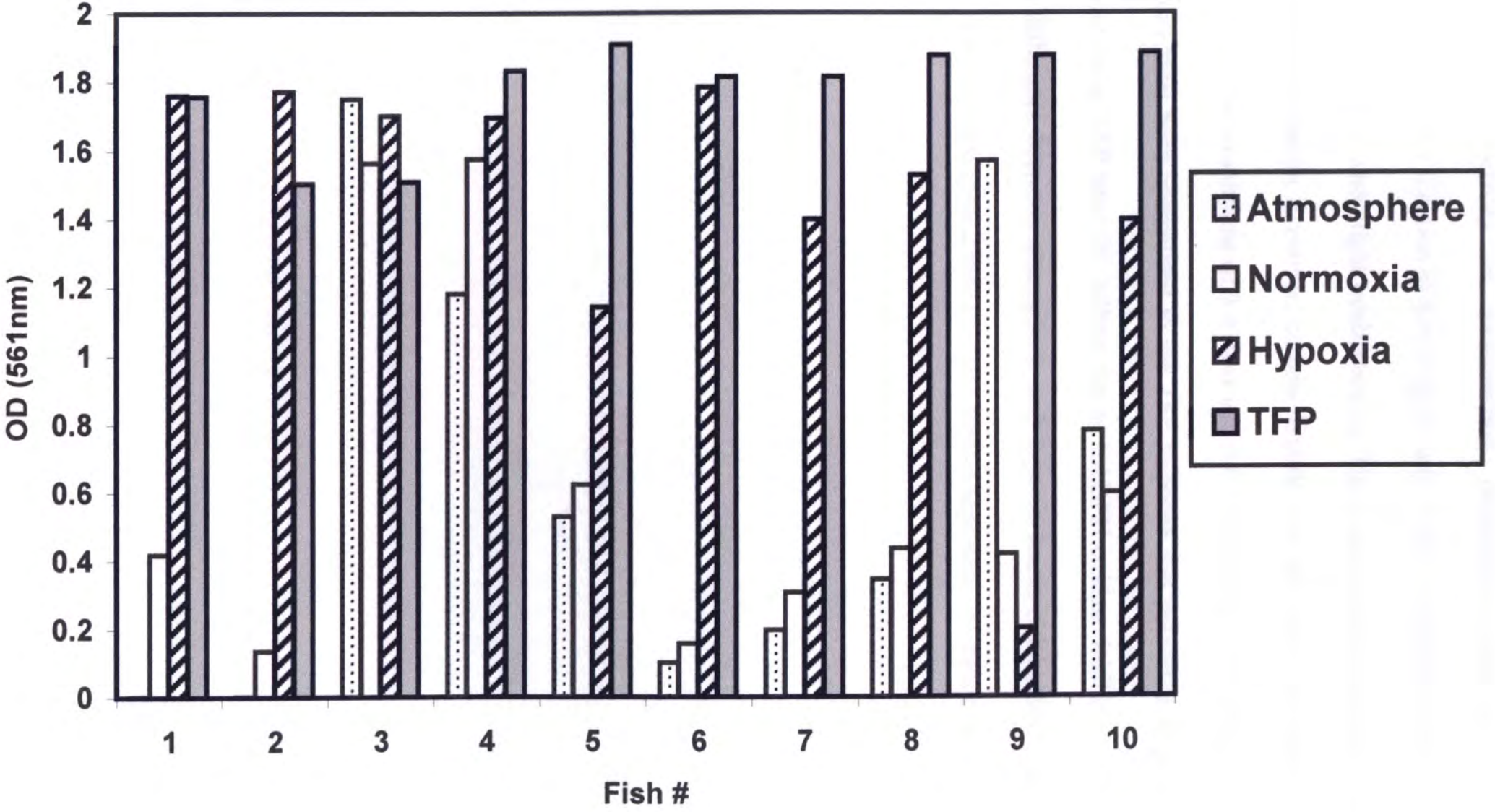

Figure 10. Bactericidal activity of mummichog phagocytic cells at a 1:1 phagocyte:bacterium ratio as a function of low oxygen only. Same procedure described in Figure 6 was performed here with slight modifications. The oxygen levels was decreased to 15 Torr to simulate hypoxia, however, carbon dioxide and pH were maintained at levels simulating normoxic conditions of 3.8 Torr and 7.6 respectively. The atmospheric and hypoxic values have been normalized to the TFP response and presented as percent killing. Wells containing TFP have 0% killing due to NADPH oxidase inhibition. There is no significant difference between atmospheric and hypoxic bactericidal activity based on the Wilcoxen rank-sum test ($p=0.2594$) ($n=10$). Values represent +/- standard error.

Bactericidal Activity Of Phagocytic Cells Against *Vibrio parahaemolyticus* at a 1:1 Phagocyte:Bacteria Ratio As A Function Of Oxygen Only

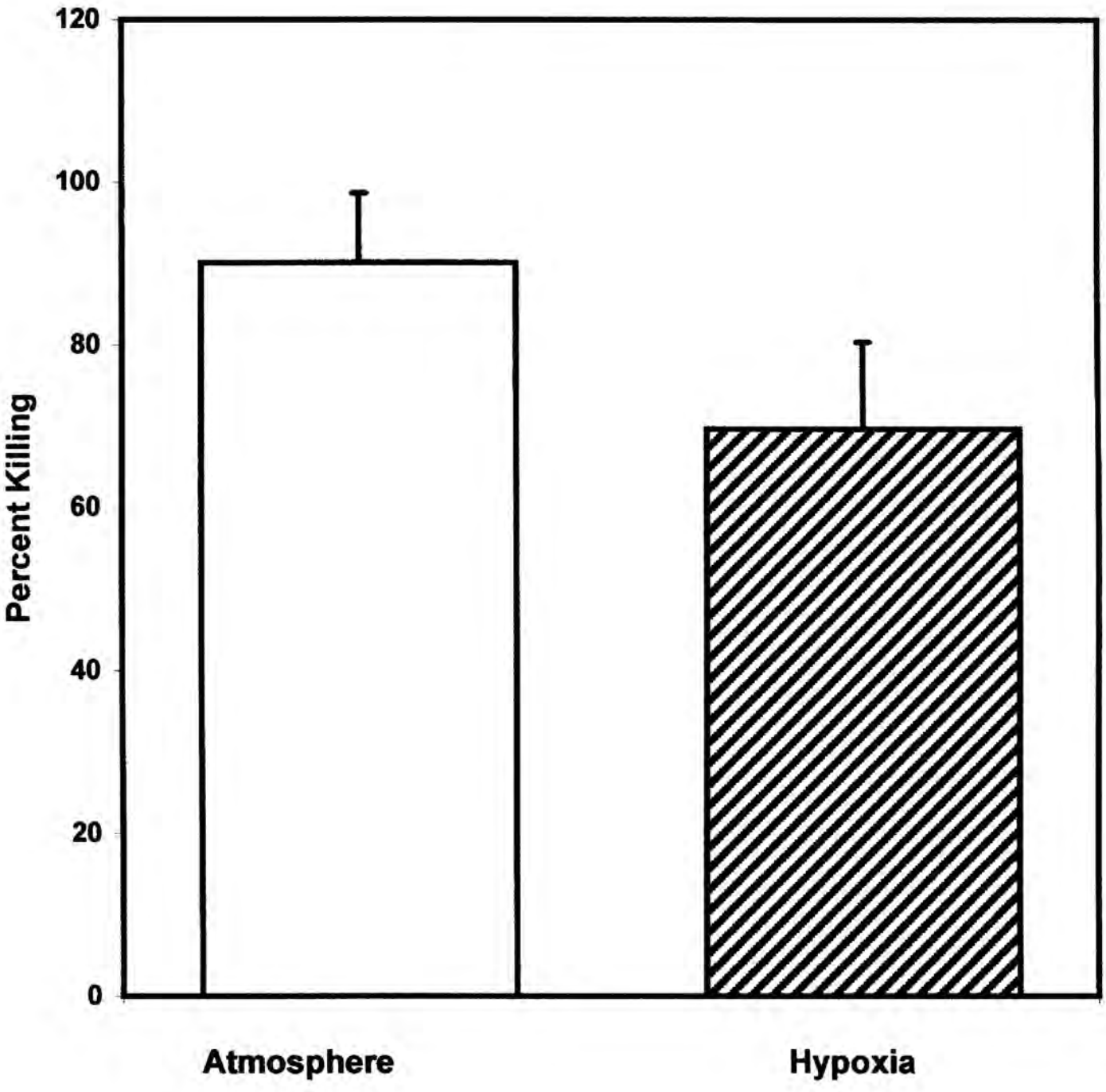

Figure 11. Bactericidal activity of mummichog phagocytic cells at a 1:10 phagocyte:bacterium ratio as a function of oxygen only. Same procedure described in Figure 10 was followed here. The atmospheric and hypoxic values have been normalized to the TFP response and presented as percent killing. (Wells containing TFP have 0% killing due to NADPH oxidase inhibition. There was no significant difference between atmospheric and normoxic bactericidal activity based on the Wilcoxen rank-sum test ($p=0.0527$) ($n=10$). Values represent +/- standard error.

Bactericidal Activity Of Phagocytic Cells Against *Vibrio parahaemolyticus* at a 1:10 Phagocyte:Bacteria Ratio As A Function Of Oxygen Only

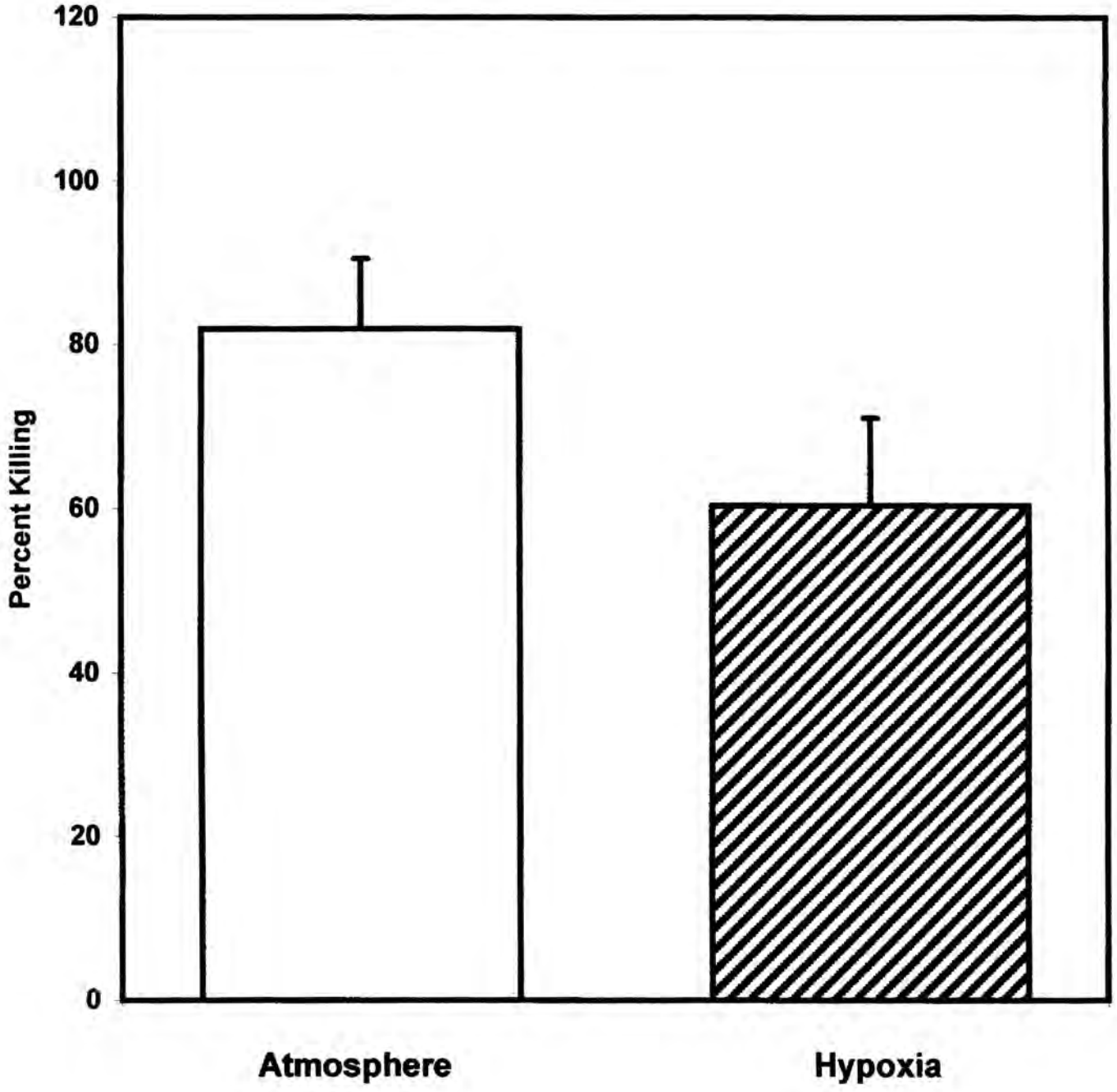

Figure 12. Growth curve of *Vibrio parahaemolyticus* over a 12 hour period. Tryptic Soy Broth 2.5% NaCl was inoculated with *V. parahaemolyticus* and placed under conditions simulating an atmospheric, normoxic or hypoxic environment with respect to levels of dissolved oxygen and carbon dioxide only. Optical densities were read at specified time intervals. Hypoxic conditions employed in the chemiluminescence, NBT reduction and bactericidal assays, do not enhance growth of *Vibrio parahaemolyticus*.

Growth of *Vibrio parahaemolyticus* As A Function of Oxygen and Carbon Dioxide

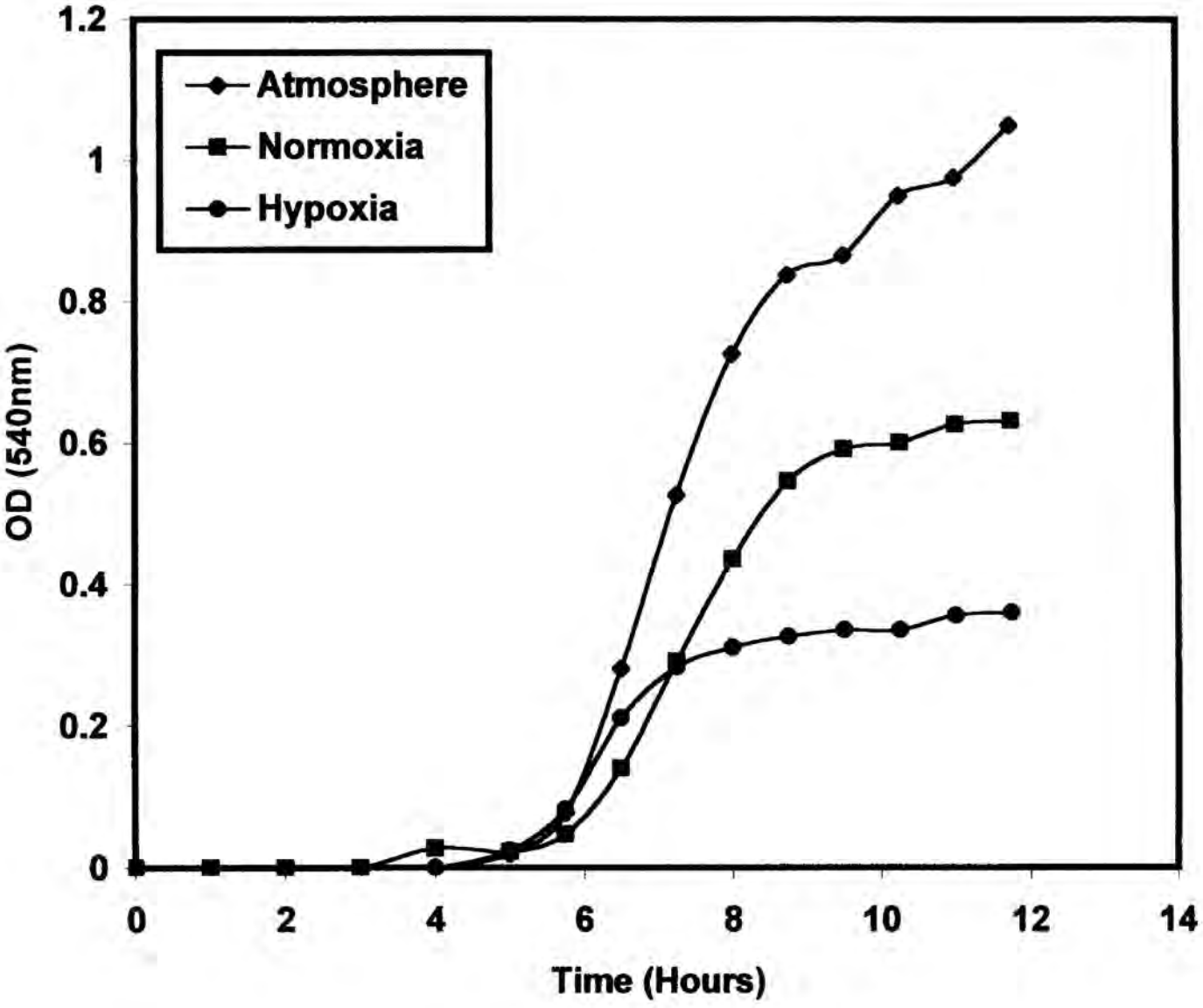

Figure 13. Growth curve of *Vibrio parahaemolyticus* over a 12 hour period as a function of pH. Tryptic Soy Broth 2.5% NaCl was inoculated with *V. parahaemolyticus* and placed under conditions simulating an atmospheric, normoxic or hypoxic environment with respect to levels of pH only. A pH of 7.6 was used to measure growth under atmospheric and normoxic conditions and a pH of 7.0 was used to simulate a hypoxic environment. The pH's of 7.0 and 7.6 used in this research do not effect the growth of the bacteria.

Growth of *Vibrio parahaemolyticus* Under a pH of 7.0 and 7.6

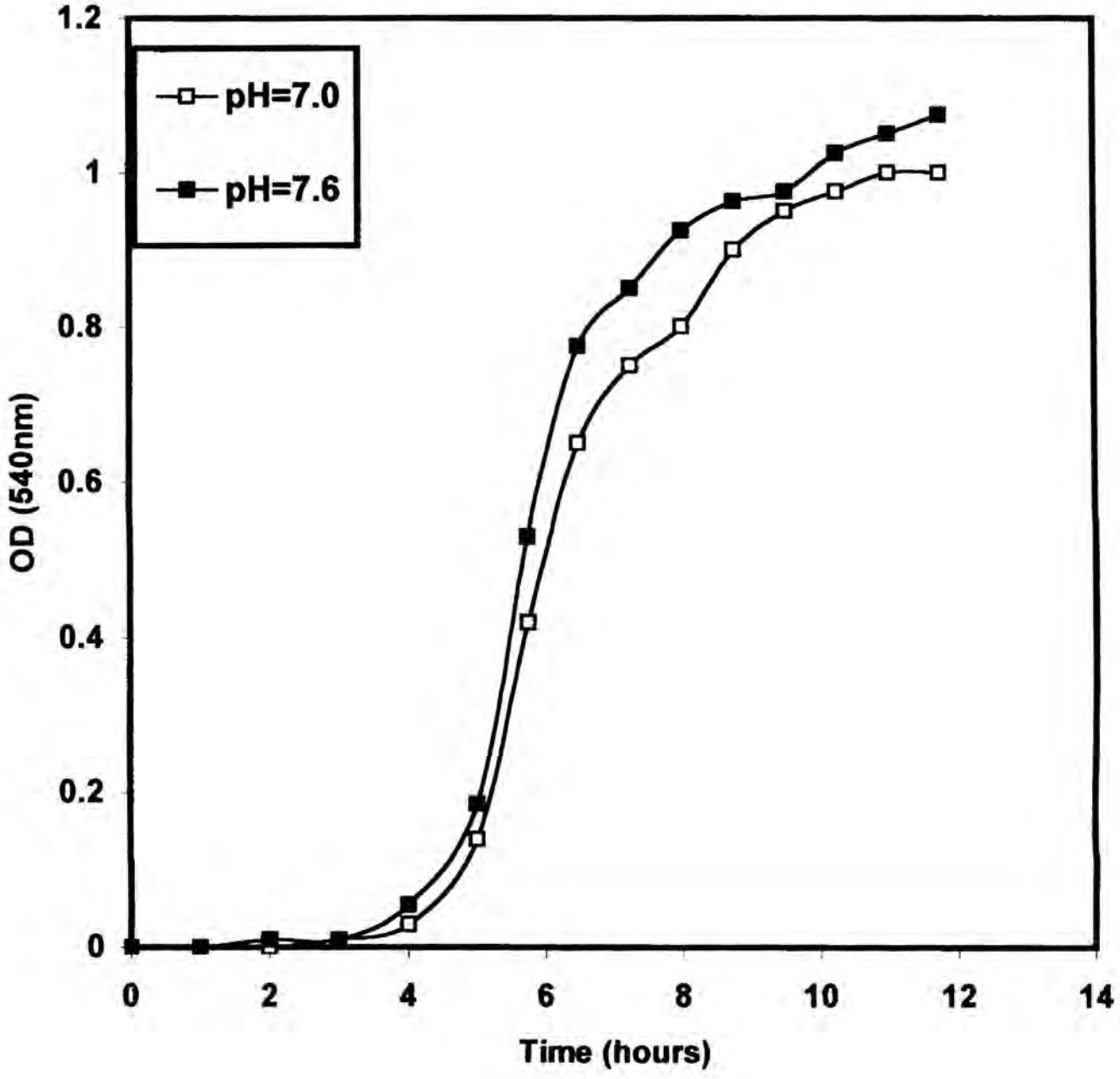

Figure 14. Comparison between atmospheric and normoxic responses for the chemiluminescent, NBT and bactericidal assays. The chemiluminescent response under atmospheric conditions is slightly higher than the response under normoxic conditions. Similarly, the bactericidal response at the 1:1 phagocyte:bacterium ratio is increased under atmospheric conditions. The opposite effect is seen in the NBT reduction assay at the 1:1 phagocyte:bacterium ration. Here, the response under atmospheric conditions is lower than the response under normoxic conditions. These results suggest that responses under atmospheric conditions most commonly employed in these assays do not accurately represent the response under physiologically relevant conditions.

Comparison Of Responses Under Atmospheric And Normoxic Conditions