ABSTRACT

Choi, Kyoungju. Modulation of Immune Function Parameters in Fish Caused by Sudden Environmental Changes (Under the direction of Mac Law and Gregory Cope)

Three related projects were conducted using multiple tiered bioassays to determine the effects of acute changes in salinity, low oxygen saturation (hypoxia), or triamcinolone on immune function in teleost (bony) fish using the well characterized tilapia fish model.

We hypothesized that sudden changes in the aquatic environment, specifically salinity and dissolved oxygen, would compromise immune function in teleost fish leading to alterations of cytokine expression, namely increase in TGF-ß and decrease in IL-1ß; suppression of phagocytosis; and adverse changes in plasma chemistry and hematology. Further, we hypothesized that these immune system changes would be similar to those caused by administration of triamcinolone. Our overall hypothesis was that the total immunocompromise caused by rapid environmental changes leads to increased susceptibility to diseases such as epizootic ulcerative syndrome (EUS).

To model the rapid environmental changes often experienced by fish in North Carolina estuaries, tilapia (*Oreochromis niloticus*) were exposed under controlled laboratory conditions to either acute salinity changes, acute drop in dissolved oxygen, or intraperitoneal injection of triamcinolone (positive control). Tiered bioassays of increasing specificity for immune function were performed on tissue samples: Tier I, histopathology; Tier II, hematology, plasma biochemistry, and plasma cortisol; Tier III, phagocytosis of peripheral

blood leukocytes (PBL), and mRNA expression of transforming growth factor-ß (TGF-ß) & interleukin-1ß (IL-1ß) splenic mononuclear cells using real time PCR.

While histopathology was a useful tool for assessing overall health of the test fish, no remarkable lesions were found in that could be attributed to any of the three stressors. Triamcinolone administration (i.p., 10mg/kg) for 3 days induced lymphopenia, neutrophilia with overall leukopenia, and monocytosis. Hematocrit and circulatory red blood cell count did not change with triamcinolone administration nor with acute decrease in salinity. Increase in salinity (5-20 ppt) had no effects on any of the aforementioned parameters. Hypoxia induced lymphopenia and neutrophilia without change in monocytes and additionally inhibited values of PO_2, SO_2, total protein, and lipase in exposed fish. Decrease in salinity (20-5 ppt) did not affect plasma cortisol levels, nor did triamcinolone administration or hypoxia treatment. Plasma protein level was not changed in response to decrease in salinity or triamcinolone administration. Triamcinolone administration suppressed phagocytosis of peripheral blood leukocytes (PBL) without a significant change in phagocytic index (P.I). Decrease in salinity elevated phagocytosis without change in P.I. in exposed tilapia. Increase in TGF-β transcription and decrease in IL-1β transcription with in vitro LPS stimulation were identified after triamcinolone administration. Acute decrease in salinity upregulated TGF-β transcription, whereas IL-1β transcription without in vitro LPS stimulation was not detectable in exposed fish. During hypoxia it was observed that changes in phagocytosis and TGF-β transcription were correlated negatively and that changes in IL-1β transcription and phagocytosis correlated positively.

Taken together, these controlled laboratory experiments suggest that rapid changes in the aquatic environment can lead to increased disease susceptibility via compromise of the

fish immune system. In these studies, this immunocompromise was especially evident in the modulation of the inflammatory mediators TGF-ß and IL-1ß, as well as in shifts in blood leukocyte distribution. It is likely that such perturbations of teleost immune system parameters have much wider implications, including in North Carolina estuaries.

Modulation of Immune Function Parameters in fish Caused by Sudden Environmental Changes

by

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BIOGRAPHY

Kyoungju Choi was born in Seoul, South Korea on January 23, 1970. After graduating from the In-Wha High School in Inchun in 1988, she began studies at Seoul Woman's University in Seoul. She earned a Bachelor of Science Degree in Food Science in 1991. After graduation she worked as a teaching assistant at the Research Institute of Food Development in Kon Kuk University, and then she earned a Master of Science Degree with emphasis in Food Fermentation and Chemistry in 1995 at Kon Kuk University. During the Masters program she also worked as a sanitation worker at Hongsung Food Co. in Korea. After graduation, she worked as a researcher at the Korean Institute of Food Hygiene in Seoul. On June 15, 1996 she was married to Hyun Joo and moved to the United States. She then began a doctoral program in 1999 in the Department of Environmental and Molecular Toxicology at North Carolina State University under the direction of Dr. Mac Law.

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APPENDIX 1.

Introduction and Literature Review

Vast fish kills and fish disease outbreaks in the estuaries of North Carolina have been attracting public and media attention since the mid-1980s (Borsuk 2004; Dykstra 2000; NCDENR 1997; NCDNER-DWQ 2000). In particular, major tributaries of the Albemarle-Pamlico estuarine system, including the Neuse and Pamlico Rivers, have been impacted by these events (Miller et al. 1990; NOAA 1996). Studies concerned with these events focused on the effects of a variety of interacting environmental stressors that induce protective alterations in physiology, histopathology, and immune function in the host, thereby reducing susceptibility to infectious disease and eventual death (Adams et al. 1989; Borsuk 2004). These adverse aquatic conditions include i) increased nutrient load from agricultural runoff and anthropogenic compounds like pesticides and organic compounds; ii) adverse environmental conditions such as hypoxia , fluctuations of temperature, salinity and sediment influx; and iii) toxic algal blooms (*Pfiesteria* sp.), parasitic infections, fungal/oomycete infections including *Aphanomyces* sp., and other diseases (Adams et al. 1989; Anderson and Zeeman 1995; Bly et al. 1997; Dykstra 2000). Adverse environmental conditions continue to challenge the capability of fish to maintain homeostasis (Bly et al. 1997; Wedemeyer et al. 1984). When this capability is exceeded, there is adverse modulation of hormonal status, immune function, and reproduction, and elevated susceptibility to infectious diseases (Adams et al. 1989; Wendelaar-Bonga 1997).

The hypothalamus-pituitary-chromaffin cell axis (equivalent to the hypothalamus-pituitary-adrenal medullary axis in mammals) and hypothalamus-pituitary-interrenal axis (equivalent to the hypothalamus-pituitary-adrenocortical axis in mammals) mediate the

response to stressors in fish (Wendelaar-Bonga 1997). These neuroendocrine axes are also involved with immune function (Harris and Bird 2000). Glucocorticoids and catecholamines are hormones secreted in primary-adaptive responses to stressors that are involved in energy mobilization, osmoregulation, and synthesis of metallothionins (Sapolsky et al. 2000). The primary corticosteroid expressed in teleost interrenal tissue is cortisol, as opposed to the dominant mammalian corticosteroids, cortisone and corticosterone (Kime 1987). Glucocorticoids (GCs) are generally considered to be immunosuppressive and anti-inflammatory. Immune functions affected by GCs are: i) inhibition of synthesis, release and potency of cytokines such as IL-1, IL-2 and TNF-α, as well as other inflammatory mediators such as histamine and nitric oxide (NO); ii) suppression of eicosanoid synthesis through decrease in cyclooxygenase 2 (COX-2); iii) alteration of antigen presentation and expression of major histocompatibility complex (MHC) II protein; iv) alteration of lymphocyte proliferation; v) shift from Th1 to Th2 cell responses and elevation of anti-inflammatory cytokines such as transforming growth factor (TGF-β); and vi) induction of redistribution of peripheral blood cells including CD4 cells, macrophages, monocytes and eosinophils, but not neutrophils (Brinkmann and Kristofic 1995; Brown et al. 1982; Darlington et al. 1990; Masferrer et al. 1992; Oursler et al. 1993; Radomski et al. 1990; Ramírez et al. 1996; Sapolsky et al. 2000; Wiegers and Reul 1998; Wu et al. 1991). These immune function effects are accomplished via glucocorticoid receptors (Sapolsky et al. 2000; Wiegers and Reul 1998). Thus, GC receptor activation may result in morphological changes in tissues involved in immune function, including atrophy of the thymus and other lymphoid tissues (Sapolsky et al. 2000; Wiegers and Reul 1998). However, GCs can also act as permissive agents and

have immunostimulatory functions such as T cell mitogenesis in adrenalectomized rats and proliferation of splenic lymphocytes (mediated by anti-T cell antigen and mineralocorticoid receptors) (Wiegers et al. 1993; Wiegers et al. 1995; Wiegers et al. 1994). It is also reported that altered values of GCs are often associated with elevated susceptibility to infectious diseases (Harris and Bird 2000). Therefore GCs can function permissively at the basal level during normal diurnal variation as well as immunosuppressively in response to stressors (Munck and Náray-Fejes-Tóth 1992; Munck and Náray-Fejes-Tóth 1994).

Many studies have focused on the effects of cortisol administration in fish immune function (Fox and Heald 1981; Kay and Czop 1994; Narnaware and Baker 1996; Wa-Yu Law et al. 2001; Narnaware et al. 1994). Law et al. reported that tilapia (*Oreochromis niloticus* x *O. aureus*) and common carp (*Cyprinus carpio*) treated with cortisol and dexamethasone suppressed phagocytosis both time- and dose-dependently (Wa-Yu Law et al. 2001). *In vivo* dexamethasone administration decreased the phagocytic index of splenic and pronephric macrophages of rainbow trout, whereas *in vitro* cortisol did not affect the value of pronephric macrophages (Narnaware et al. 1994). Yet, at basal concentrations, cortisol elevated phagocytosis in humans and bovines (Fox and Heald 1981; Kay and Czop 1994), and stress-induced suppression of phagocytic capability in trout was counteracted by cortisol injection (Narnaware and Baker 1996).

Seasonal and diurnal low dissolved oxygen (hypoxia) or lack of oxygen (anoxia) have been commonly reported in estuarine and coastal marine waters (Harper et al. 1981; Turner and Allen 1972). Agricultural run-off, vertical stratification, increased temperature, and salinity influx can worsen these naturally occurring conditions (Malone

1991; Paerl et al. 2001; Turner et al. 1987). Most fish species will avoid waters with low oxygen, whereas some species such as the sand goby (*Poecilia latipinna*) have air breathing ability (Petersen and Petersen 1990). In mammals, hypoxia-inducible factor 1 (HIF-1), a ubiquitous DNA-binding protein, regulates many genes induced by hypoxia including α- and β- subunits of the heterodimeric transcription factor, generally degraded in normoxic conditions (Salceda and Caro 1997; Wang and Semenza 1993). Hypoxia-activated HIF-1 signals redox or phosphorylation events activating transcription of hypoxia-inducible genes such as oxygen-elevating vascular endothelial factor (VEGF) (Arany et al. 1996). It has been reported that fish, like mammals, have HIF-1 and that its sequence similarity with human is 61 % (Soitamo et al. 2001). Genes induced by hypoxia in mammals are involved in i) locomotion and contraction such as skeletal α and β-actin and protein translation; ii) amino acid metabolism such as glutamine synthase; iii) ATP metabolism including glucose-6-phospatase and cytochrome c oxidase; and iv) antigrowth, for example insulin-like growth factor binding protein (Wu 2002). In fish, hypoxia activates genes associated with protein synthesis, locomotion, anaerobic ATP production, gluconeogenesis and cell growth suppression (Gracey et al. 2001). The expression and translation of these genes may lead to biochemical and physiological responses that maintain oxygen levels, conserve energy, and promote anaerobic metabolism (Wu 2002). These adjustments may negatively affect the growth and feeding habits of many fish and marine organisms (Petersen and Pihl 1995) as well as reproduction and development (Zhou 2000). Indeed in Carp (*Cyprinus carpio*), chronic hypoxia interferes with the reproductive system, resulting in endocrine disruption (Wu et al. 2003). As a result, hypoxia may permanently modify certain species resulting in

organisms of new tolerances and dominations in benthic communities, leading to an overall reduction of fish biomass (Wu 2002). Reperfusion[1] following hypoxia may impair tissues more than hypoxia alone due to excessive generation of free radicals, excessive cytosolic ca^{+} and altered release of cytokines such as TGF-β and other pro-inflammatory components (Chen 2003; Jordan et al. 1999; Yellon and Baxter 1999). In addition, exposure to immunotoxic agents like heavy metals, polycyclic aromatic hydrocarbons (PAH), polychlorobiphenyl (PCB) and volatile organic chemicals (VOCs) (Zelikoff et al. 2000) during hypoxia has been reported to modulate innate and adaptive immune responses in fish (Boleza et al. 2001; Bunch and Bejerano 1997; Cecchini and Saroglia 2002; Cuesta et al. 2003; Ortuno et al. 2002; Woo 2003).

Salinity is influenced by wind-driven tides, precipitation and water flow and is often stratified in estuaries, becoming less concentrated as one moves toward the water's surface (NCDNER-DWQ 2000). Salinity influences levels of dissolved oxygen, represented by normal or high dissolved oxygen level at the surface and low or absent dissolved oxygen near the bottom (NCDNER-DWQ 2000). Studies concerned with effects of salinity changes on immune function focus on host resistance to infectious disease, endocrinology, cellular or humoral immune responses, and histopathological characteristics (Chang and Plumb 1996; Farghaly et al. 1973; Fernandes and Rantin 1994; M.C.J.Verdegem et al. 1997; M.S. Sawants et al. 2001; Marc et al. 1995; Morgan

[1] The term,"reperfusion" is used here to denote restoration of oxygen to the tissues and "reperfusion injury," the consequences thereof; namely, free radicals and their potential damaging effects on cells/tissues. It is acknowledged, however, that reperfusion is not technically synonymous with re-oxygenation, since in the case of fish swimming in hypoxic waters, there is no apparent constriction of blood vessels (ischemia) followed by restoration of blood flow. Blockage of blood supply (to a coronary artery, for example) robs tissues of not only oxygen but also nutrients such as glucose.

et al. 1997; Narnaware et al. 2000; T. Yada et al. 2001; Woo and K. C 1995; Zeltoun et al. 1974).

Hyperosmotic salinity-adapted tilapia followed by challenge with *Streptococcus* isolates show significantly higher mortality than those kept in fresh water (Chang and Plumb 1996) and brackish water-adapted hybrid red tilapia often develop fin and tail lesions (M.C.J.Verdegem et al. 1997). In hybrid red tilapia, high salinity alters hematological measurements including leukocrit values, immature lymphocytes, and granulocytes without change in hematocrit values (M.C.J.Verdegem et al. 1997). Rainbow trout adapted to iso-osmotic salinity (12 ppt) show an increase in plasma lysozyme activity without change in plasma immunoglobulin M (IgM) levels (T. Yada et al. 2001). However, estuary-origin tilapia adapted to seawater show no significant differences in plasma cortisol or lysozyme activity with decreased plasma levels of prolactin (Iwama et al. 1997). Upon adaptation to changing salinity, fish attempt to adjust osmolarity by altering gill Na^+-K^+-ATPase activity, accompanied by hypertrophy of chloride cells, modification of plasma hormone levels including growth hormone, prolactin and cortisol, and increase in oxygen consumption (Borski et al. 1994; Hwang et al. 1989; Iger et al. 1995; McCormick 1995; Morgan et al. 1997; Sargent et al. 1980). Thus, adaptation depends on the duration and nature of the change in salinity (Iwama et al. 1997; Morgan et al. 1997).

In the present study, a laboratory fish model, fresh water-adapted euryhaline tilapia (*Oreochromis niloticus*), was used to investigate the effects of acute salinity stress, low dissolved oxygen, or triamcinolone on physiological and histopathological characteristics and on immune function parameters. The tilapia is a proven species for

mechanistic studies attempting to model exposure of fish to adverse environmental conditions. Osmoregulation and hypoxia studies are possible since tilapia exhibit high tolerance to low dissolved oxygen concentrations and are euryhaline in nature (tolerant up to 120 ppt) (Stickney 1986). They also exhibit high resistance to stress and infections, and thrive in captivity (Evans 1984; Foskett et al. 1983; Foskett et al. 1981; M.C.J.Verdegem et al. 1997).

The study of fish immunology gives a unique perspective into the evolution of defense mechanisms and disease resolution in vertebrates (Scapigliati et al. 2001) The fish model would be useful in environmental toxicology investigations or substitutive biomedical research (Stolen and Fletcher 1994), but unfortunately is limited due to the lack of available species-specific experimental reagents (Harms et al. 2000a). However, the recent development of advanced techniques including cloning, sequencing and RTqcPCR allow for easier immunological measurements in fish, including cytokine expression (Harms et al. 2000a; Harms et al. 2000b; Holland et al. 2003; Jun Zou et al. 2000).

Cytokines are any immunity-mediating proteins secreted by immune cells. Immune functions regulated by cytokines include i) the clonal expansion of lymphocytes, ii) innate immune responses, and iii) effector actions of most immune cells (Charles A. Janeway et al. 2001). Cytokine expression analysis allow us to perceive the immunologic state of fish (Harms et al. 2000a). Recent studies have demonstrated the relationship between stress-induced immunosuppression and altered cytokine production (Ottaviani et al. 1997; Saperstein et al. 1992).

TGF-β. The transforming growth factor-β (TGF-β) superfamily comprises over twenty bone morphogenetic proteins (BMPs), four activins/inhibins and three TGF-β proteins (Attisano and Wrana 2002). Only five isoforms (TGF-β_1--TGF-β_5) were found to be immunoregulatory (Ruscetti and Palladino 1991). Three isoforms of TGF-β (TGF-β_1, TGF-β_2 and TGF-β_3) are found in mammals; TGF-β_2 is found in rainbow trout and carp; TGF-β_4 in chickens and TGF-β_5 in *Xenopus lavis* (Harms et al. 2000a). TGF-βs mediate signal transduction through both SMAD-associated pathways and non-SMAD-associated pathways comprised of Rho family guanosine triphosphatate (GTPase), mitogen-activated protein (MAP) kinase, TGF-β-activated kinase-1 (TAK-1), and protein kinase B (PKB; Ark). The MAP kinase pathway, in particular, mediates neuroprotective effects of TGF-β_1 (Dhandapani and Brann 2003). TGF-βs are expressed by most immune effector cells including $CD4^+$ and $CD8^+$ cells, B cells, large granular lymphocytes (LGL), lymphocyte activated killer (LAK) cells and macrophages (Ruscetti and Palladino 1991). TGF-β is considered to be primarily immunosuppressive, but can also be immunostimulatory. *In vitro*, TGF-β suppresses human immune function mediators including lymphocyte antigens-DR, Fc receptor expression, cytokine production including IFN-γ, TNF-α, IL-1β, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), proliferation of T/B cell and thymocyte, IgG/IgM production, IL-2 receptor p55 expression, cytotoxic T cell generation/function, LAK/NK cell activation/function, cytotoxic macrophages, macrophage H_2O_2 production, neutrophil adhesion to endothelium, and hematopoiesis (Ruscetti and Palladino 1991). Alternatively, immunostimulatory functions *in vitro* include IgA and cytokine production (THF-α, IL-1α, IL-1β, TGF-α/β, IL-6, contra IL-1), macrophage and neutrophil

chemotaxis, and granulopoiesis (Ruscetti and Palladino 1991). TGF-β functions are complex. For example, TGF-β increases the steady state level of mRNA synthesis of IL-1α/β and TNF-α in monocytes, whereas it downregulates LPS-induced expression of IL-1α/β, TNF-α but not by 12-myristate-12-acetate (PMA) induction (Chantry et al. 1989; Espevik et al. 1987). *In vivo,* immunoregulatory functions of TGF-β, as *in vitro,* include downregulation of TNF-α, CSF and NK activity, Concanavalin A responses of T cells, *E.coli*-induced septic shock, allograft rejection in heart and skin and hematopoiesis, and upregulation of granulopoiesis and contra-IL-1 production (Ruscetti and Palladino 1991). In addition, TGF-β influences cell proliferation and differentiation, extracellular matrix regulation, wound healing, angiogenesis, apoptosis, production of cytokines and lipid metabolism (Harms 2000; Zhang and Phan 1999; Zuckerman et al. 2001).

The growth of neoplastic cells is associated with lack of TGF-β receptors on the cell surface and tumor-induced immunosuppression is mediated by TGF-β (Ruscetti and Palladino 1991; Wahl et al. 1991). In addition, TGF-β overexpression by HIV-infected monocytes may often be responsible for AIDS-related impairment of the central nervous system (Ruscetti and Palladino 1991; Wahl et al. 1991). In *Mycobacterium*-infected striped bass, TGF-β induces excessive granulomatous inflammation in internal organs, resulting in death of susceptible species (Harms et al. 2003). In mammals, upregulation of TGF-β expression is associated with kidney fibrosis (Basile et al. 1998), whereas TGF-β is protective against generation of superoxide anions and other inflammatory cytokines generated by hypoxia and reperfusion during cerebral ischemia (Lefer et al. 1993). Ottaviam et al. reports cytokines are involved with the endocrine axis leading to modulation of immune function (Ottaviani et al. 1997). Therefore, TGF-β can be anti- or

pro-inflammatory depending on its concentration, destination, and interaction with other proinflammatory compounds (McCartney-Francis and Wahl 1994).

IL-1β. Interleukin-I (IL-1) is a proinflammatory cytokine, often associated with increase in host resistance, that acts in concert with other proinflammatory cytokines including tumor necrosis factor (TNF) (Dinarello 1997; Engelsma et al. 2001; Rosenwasser 1998). It is also a potent enhancer of immune responses, as well as an inducer of acute phase responses and inflammation (Dinarello 1997). IL-1 also serves as a signal molecule between immune and internal systems such as the hypothalamus-pituitary-adrenal (HPA) axis in mammals (Dinarello 1997; Engelsma et al. 2001; Rosenwasser 1998). The IL-1 family is composed of four isoforms: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-18 (interferon-γ-inducing factor) (Christopher J. Secombes et al. 1999; Dinarello 1997; M.Y.Engelsma et al. 2001). IL-1α is produced as an active precursor and rarely found in the circulation or body fluids except in severe disease where IL-1α may be released from dying cells or by proteolysis after calpain-mediated cleavage (Christopher J. Secombes et al. 1999; Dinarello 1997; M.Y.Engelsma et al. 2001). IL-1β is produced as an inactive precursor and is only active upon cleavage by IL-1β converting enzyme (ICE). IL-1 receptor antagonist (IL-1Ra) and IL-1β are measured in clinical trials to assess disease severity due to loss of agonist activity (Christopher J. Secombes et al. 1999; Dinarello 1997; M.Y.Engelsma et al. 2001).

IL-1β mediates proliferation of lymphocytes (T and B cells) and thymocytes, phagocytosis and bactericidal activity, hepatic metastases of melanoma *in vivo*, development of fever and pain hypersensitivity, angiogenesis and invasion of tumor cells *in vivo*, and progressive death of oligodendrocytes in multiple sclerosis (Beauséjour

et al. 1998; Dinarello 1991; Takahashi et al. 2003; Vidal-Vanaclocha et al. 2000; Voronov et al. 2003; Youssef and P.Stashenko 2004). Not surprisingly, excessive production of IL-1β has been implicated in the pathogenesis of septic shock and inflammatory bowel diseases (Nemetz et al. 1999; Sharma and Mink 2004).

IL-1β transcription can be stimulated in different ways. *In vitro,* transcription without translation is stimulated in leukocytes without exogenous factors and is simply caused by adherence to glass or plastic. Stimulants such as LPS, microcystins, and cyanobacterium significantly upregulate IL-1β expression in lymphoid tissue models above basal levels. In human peripheral blood mononuclear cells, immature Il-1β transcript is stimulated by retinoic acid exposure. (Chen et al. 2004; Dinarello 1991; Jarrous and Kaempfer 1994; Scapigliati et al. 2001). However, corticosteroids inhibit macrophage IL-1β transcription by glucocorticoid receptor-mediated suppression of NF-κB activity. (Frieri 1999; McKay and Cidlowske 1999).

Studies focused on IL-1 mediation of biological activity in fish have been reported. Channel catfish and carp macrophages and neutrophils express biologically active IL-1-like molecules (Siegel et al. 1986; Verburg-van Kemenade et al. 1995) and the complete sequences are identified in rainbow trout, plaice, sea bass and carp (Engelsma et al. 2000; Scapigliati et al. 2001; Secombes et al. 1999; Zou et al. 1999).

Summary. Measurable adverse changes in an aquatic organism's immune function have been used as sensitive biomarkers of overall health status and host susceptibility to a variety of pollutants and adverse environmental factors, especially when present below overtly toxic levels (Zelikoff et al. 2000). In the studies described herein, to address the mechanisms of this putative immunosuppression, we used the tilapia (*Orechromis niloticus*) fish model under controlled laboratory conditions to investigate the effects of acute changes of salinity or dissolved oxygen, or administration of triamcinolone (a synthetic cortisol), on immune function and (indirectly) susceptibility to infectious or opportunistic pathogens. Immunological status was determined by examining changes in histopathology, hematology, plasma chemistry and plasma cortisol levels. In addition, determination of phagocytic activity and TGF-β and Il-1β transcript quantitation were correlated with hematological characteristics, plasma chemistry and histopathology.

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Biomarkers of Immunomodulation Induced by Triamcinolone in Tilapia (*Oreochromis niloticus*)

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Abstract

To determine the effects of the synthetic glucocorticoid, triamcinolone, on the immune system of teleost fish, tilapia (*Oreochromis niloticus*) were injected intraperitoneally with 10 mg/kg triamcinolone and a series of tiered bioassays were performed 3 days later. Tiered bioassays consisted of the following: Tier I histological evaluation, Tier II hematological characteristics and Tier III peripheral blood leukocyte phagocytosis and splenic mononuclear cell TGF-β and IL-1β mRNA. Histopathology of internal organs showed no lesions that were attributed to treatment. Lymphopenia and neutrophilia and elevated macrophages and decrease in white blood cells were observed in treated tilapia, whereas hematocrit and red blood cell count remained not significant. Phagocytic capability was suppressed without significant change of phagocytic index in treated tilapia. TGF-β transcription was elevated, whereas IL-1β transcription was inhibited after triamcinolone administration. Triamcinolone triggers overall downregulation of immune response in tilapia.

Introduction

Biomarkers of immunotoxicity in fish assessed under controlled laboratory conditions allow prediction of the adverse effects of contaminated aquatic environments and help to extrapolate to the health status of wild populations (Zelikoff et al. 2000). Such extrapolations are based on innate and acquired immune function including lymphocytes mitogenesis, phagocytosis, phagocyte respiratory burst and antibody production (Charles D. Rice et al. 1996; Espelid et al. 1996; Harms 2000; Pickering and Pottinger 1985; Wa-Yu Law et al. 2001; Yuwaraj K. Narnaware et al. 1994). In addition, recent studies concerned with cytokine expression have been reported based on techniques of cloning, sequencing and RT qcPCR (Harms et al. 2000a; Harms et al. 2000b; Holland et al. 2003; Jun Zou et al. 2000). Glucocorticoids are the primary hormone secreted in the primary response to stressors via the hypothalamus-pituitary-interregnal (HPI) axis (equivalent to the hypothalamus-pituitary-adrenal (HPA) axis) resulting in adaptation through energy mobilization, osmoregulation or synthesis of metallothionins. They are generally considered to be immunosuppressive through glucocorticoid receptors in terms of i) the inhibition of synthesis, release and potency of cytokines such as IL-1 and ii) the stimulation of TGF-β release that is a primarily anti-inflammatory cytokine leading to elevated susceptibility of fish to infectious diseases. Glucocorticoids may also cause permissive effects on the immune system through the mineralocorticoid receptor (Ellis 1981; Iger et al. 1995; Sapolsky et al. 2000).

Triamcinolone (Kenalog® Injection) is an intermediate acting, synthetic glucocorticoid associated with no cross-reactivity with mineralocorticoids. It is used for bronchial asthma, rheumatic disorders, systemically for adrenocortical insufficiency and

systemic lupus erythematosus and other diseases requiring anti-inflammatory and immunosuppressive effects (W. S. Messer Jr.). Tilapia are euryhaline fresh water fish exhibiting high tolerance to varying adverse environmental effects and infection. They are easy to handle and show hardiness in captivity (M.C.J.Verdegem et al. 1997). The present study used tiered bioassays to determine the effects of *in vivo* triamcinolone administration on immune function in tilapia (*Oreochromis niloticus*) including cytokine expression and compared with classical immunoassays and hematological characteristics and histopathology. We used this synthetic glucocorticoid under controlled laboratory conditions in order to establish benchmark levels of biomarkers for teleost immune function that could be useful in solving "real world" problems such as ulcerative mycosis.

Methods

Fish and experimental design. Tilapia (*Oreochromis niloticus* L.), weighing 55±7.9 g were transported from the NCSU aquaculture facility (LakeWheeler, NC) to the NCSU College of Veterinary Medicine. Fish were acclimated in fresh water for at least 1 week. Fish (n=6) were randomly placed in the tank and then experimental fish were injected with 10 mg triamcinolone acetonide/kg (Kenalog-10, Apothecon, Princeton, New Jersey; 10mg/ml suspension) intraperitoneally and placed back into the previous tank. This dose (230 nM) was based on the physiological dose (10nM to 100nM) and pharmacological dose (1μM to 100μM) of cortisol (Wa-Yu Law et al. 2001), as well as on the stressed concentration (62.5 nM to 0.675 μM) (Barcellos et al. 1999). In addition, in a recent study by Harms (Harms 2000), a dose of 10 mg/kg was used in hybrid striped bass. Three days after administration, fish (n=6) were euthanized with an overdose of 3-aminobenzoate ethyl ester methanesulphonate (MS-222, Sigma, St. Louise, MO). Blood samples were collected in lithium vacutainer tubes from the caudal vein and spleen removed aseptically was placed in sterile RPMI media added with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100μg/ml streptomycin, and 2mM EDTA. The buffy coat layer of blood diluted in phosphate-buffered solution (PBS) was placed on 55 % Percoll gradient (specific gravity 1.0697 g/mL) at 800 x g for 30 min. at 16 °C. Peripheral blood leukocytes (PBL) were adjusted to a cell density of 1 x 10^6/ml. The isolation of splenic leukocytes was prepared as described in splenic leukocytes from hybrid striped bass (Harms et al. 2000a). Finely minced spleen was forced through a wire mesh (50 meshes) and resuspended in sterile RPMI media. Then the cell suspension was placed on two-step percoll gradients (specific gravity 1.052 and 1.065 g/mL) at 400 x

g for 5 min. and then 800 x g for 25 min. and splenic leukocytes in interface were adjusted to a cell density of 1 x 10^7/mL. Cell viability was performed in 0.2 % trypan blue. All experiments were repeated two times.

Tier I: Histology. Samples of spleen, head kidney, gill, intestines and gonad were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin (HE) followed by the examination with light microscopy. A six-point scoring system for evaluation was performed as reported recently (Hurty et al. 2002a). The splenosomatic index was calculated by the following:

Splenosomatic index = (spleen weight (g)/body weight (g)) x 100.

Tier II: Hematological characteristics. Differential cell counts were performed on blood smears from each fish stained using Wright's stain according to manufacturer's instructions (Fisher, Middle Town, VA), then examined by light microscopy under oil immersion. One hundred leukocytes were counted and categorized into lymphocytes, monocytes/macrophages and neutrophils.

White Blood Cell (WBC) and Red Blood Cell (RBC) counts were performed using a manual counting method on a hemacytometer using Natt & Herricks staining method**.**

Tier IIIa: Phagocytosis Measurement In vitro phagocytic assay was prepared for phagocytic cells in blood from tilapia. Peripheral blood leukocytes (PBL) of tilapia were isolated as described above. PBL, 1x10^6/ml were cultured per well with opsonized-fluoresceinated (FITC) latex beads (diameter = 1.75μm) (the ratio of cell: beads, 1:30)

(Polysciences, Northampton, U.K.) in 5% CO_2/95% air in a humidified incubator for 5 hr at 26°C. At the end of incubation 1 ml of cold PBS was added to stop phagocytosis and centrifuged at 400 x g for 10 min. Pellets were resuspended in 100 µ of 1 % PBS with the addition of 50 µl 1 % triton X and 50 µl of Propidium Iodide (0.5 mg/ml) (Roche, Indianapolis) and analyzed by fluorescence microscope. At least 100 cells were counted and the percentages of phagocytic cells engulfing latex beads as phagocytic capability were examined. Phagocytic index (PI) was the average number of ingested latex beads per phagocytic cell and its calculation was the following:

PI = total number of ingested beads / total number of phagocytic cells.

IIIb. TGF-1β and IL-1β mRNA Expression.

Isolation of Total RNA and Reverse transcription. The assays of total RNA isolation and reverse transcription were followed by the procedure described in rainbow trout (Harms et al. 2000a). For the isolation of total RNA of splenic leukocytes one ml of Tri Reagent (Sigma, MO, USA) for each 5-10 x 10^6 cells was used according to manufacturer's instructions. RNA pellet was resuspended in sterile water (Sigma, MO, USA) at a concentration of 5 x 10^4 cell equivalents/µl. For RNA reverse transcription oligo dT_{15} (300-pmol, Promega) was added to 3 x 10^6 cell equivalents of total RNA in a 72 µl volume with heating at 94 °C for 5 min. and immediate chilling on ice. Then mixed solution of Reverse Transcription was placed in a final volume of 150 µl. This solution comprised 1 x buffer (50mM Tris-HCI, pH 8.3; 75mM KCl, 3mM $MgCl_2$), 0.4 mM each dNTP, 10 mM DTT, 0.8U/µl RNAsin and 2 U/µl reverse transcriptase (Superscript II RT, Gibco-BRL, Gaithersburg, MD, U.S.A.). Its cycle was at 45 °C for 60 min. with

immediate chilling on ice and dilution in 1:2 in sterile water (Sigma, MO, USA) and stored at –20 °C until use.

Primer Preparations. The assay of primer preparation was prepared as described in rainbow trout (Harms et al. 2000a). For TGF-β, initial primers (Con A and Con B2) from hybrid striped bass (Harms et al. 2000a) and tilapia-specific primers against TGF-β (TIF and TIR) were prepared based on initial primer-generated sequences. The similarity between the generated sequences and other known sequences was compared with BLAST and DNASTAR software.

β-actin was used as housekeeping gene in the quantitative real time PCR assay. β-actin primers (BacF1 and BacR1) were based on alignment with known- teleost sequences to amplify a 139 bp fragment verified by PCR product size. IL-1β initial primers (MUF4 and MUR3) from conserved region of known-teleost sequences and tilapia-specific primers against IL-1β (ABF1 and ABR1) were designed against initial primer-generated sequences to allow amplification of 115bp fragment for Real Time PCR (Table 1). For IL-1β expression it was required to incubate splenic mononuclear cells with 10 µl of LPS (1 mg/mL) (Sigma, St. Louise, MO) for 4h at 26°C.

IIIc. Quantitative Real Time PCR. Quantitative PCR analyses for TGF-β and IL-1β mRNA were performed. Reaction mixtures comprised of 1x PCR buffer (50m m Tris-HCl, pH8.3, 10 mM KCl, 5 mM $[NH_4]SO_4$ and 2 mM $MgCl_2$) (Roche, Mannheim, Germany), 0.2 mM PCR dNTP Mix (Roche, Mannheim, Germany), 5U/ul FastStart Taq DNA Polymerase (Roche, Mannheim, Germany), 1x SYBR Green (Sigma, MO, USA), 150 nM each primers and 5 µl cDNA in a final volume of 50 µl. The cycling conditions of Real Time PCR were 1 cycle of 95 °C 2min, 31 cycles of 95 °C 30 s denature, 57 °C

30s annealing and 72 °C 2 min extend and 1 x 72 °C 7 min final extension. Four 10 fold serial dilutions of plasmid DNA were used in standard curves for both targets and housekeeping gene. The quantification in the cytokine signal was normalized by β-actin signal.

Statistical Methods. The effects of triamcinolone on immune responses were analyzed by Student t-test and $p<0.05$ was accepted as a statistically significant level of difference between control and treated fish. Data were expressed as means ± standard error (SE).

Results

Histopathology. No mortality of tilapia was observed prior to and during the experiment. All the control samples with no lesions were scored as zero. Internal organs had no remarkable lesions attributable to treatment. In the spleen, pigmented macrophage aggregates (PMAs) were commonly found in both treated fish and controls. Control fish (n=4) and treated fish (n=5) had mild PMAs. Mild vacuolation (n=2) of the liver in control and mild to moderate vacuolation (n=5) along with no inflammation in treated fish were present. Head kidney, gill and intestine of triamcinolone-administrated tilapia were not remarkable. Most of the fish were female, and the reproductive organs were essentially normal.

Splenosomatic index was significantly lower in treated fish than controls after 3 days of administration (p=0.005) (Fig. 3).

Hematology and Plasma Chemistry. The total white blood cells (WBC) were significantly different compared to controls (p= 4.4E-0.6). The number of white blood cells was suppressed in treated fish (p=0.0002), whereas no significant changes were observed in red blood cells (RBC) (data not shown). After 3 days of administration the percentages of lymphocytes in peripheral blood of treated fish significantly decreased compared to control value (p=1.3E-07), whereas the percentages of neutrophils (p=0.001) and macrophages (p=0.0004) in treated fish significantly increased compared to control value (Fig.2).

Phagocytosis. Phagocytic capability was significantly different after 3 days of administration compared to control value (p=6E-5). Its value decreased in treated fish (Fig.4). The percentages of phagocytic frequency of ingested beads were modulated after

3 days of administration (Table 2). The frequency of phagocytic cells ingesting only one bead ($p<0.001$), two beads ($p=0.04$) and over three beads ($p=0.02$) was significantly depressed from control ($p<0.05$), whereas there was no differentiation of over three beads. However phagocytic index was not significant between control and treated fish ($p=0.32$).

Cytokines. TGF-β mRNA expression of splenic mononuclear cells was significantly different compared to control ($p=0.003$). Its level increased in treated fish (Fig. 5). Splenic mononuclear cell IL-1β transcription was significantly different from control level ($p=8.7E-05$). Its level significantly decreased in treated fish (Fig. 6).

Discussion

Histopathology. Histopathology helps to identify effects of stressors at the individual animal level based on specific cell types, tissues and affected organs and these changes also represent cumulative effects of biochemical and physiological changes and often actual injury to organisms (Myers and Fournie 2002). The administration of triamcinolone (i.p.) did not cause demonstrable lesions in internal organs including spleen, head kidney, liver, gill, intestine and gonad. Spleen, a lymphoid tissue, was found to have pigmented macrophage aggregates (PMAs), but its level was not significantly different in treated vs. controls. Studies concerned with histopathological changes of spleen in response to varying stressors were reported (Ekman 2003; Wolke 1992). Wolke observed that increased density of PMAs often is associated with natural conditions such as aging, starvation and bacteria and parasite infection (Wolke 1992). Enlarged spleen and kidney with necrosis and hemorrhages, edema and congestion were observed among salmonid species infected with acute *F. psychrophilum* (Ekman 2003). In the present study, mild vacuolation of hepatocytes with no inflammation was observed in the liver. The degree of hepatocyte vacuolation is an indicator of the relative amounts of hepatic glycogen and/or fat present in the liver (Vandenberg et al. 1998). Other organs like gill, head kidney, intestine and gonad were considered to be normal in control and treated fish. In contrast, histopathological changes of gills induced by stressors including toxic chemicals, bacterial infection and cortisol injection are necrosis, lamellar fusion, hypertrophy and hyperplasia, excess mucus secretion and epithelial lifting of the outer layer of the lamellar epithelium (Smith 2000), and increased mitosis, apoptosis and

excessive leukocyte infiltration (Iger et al. 1995). Lesions seen in head kidney include atrophy of the interrenal tissue in juvenile coho salmon (McLeay 1973).

Splenosomatic index, along with histopathology data, allows interpretation of the general health status in affected animals under varying environmental stressors (Adams 1993). In this study, excessive decrease in splenosomatic index was observed, which was consistent with decreased index induced by handling and the fish anesthetic, clove oil (Tort et al. 2002). Additionally, chronic exposure to toxic thiocyanate caused a decreased splenosomatic index in rainbow trout (Lanno and Dixon 1996). This result can be explained by the release of immature red blood cells from the spleen (Tort et al. 2002). However some stressors such as crowding result in no change in splenosomatic index of rainbow trout (Fox et al. 1997).

Hematology. In this study, triamcinolone administration (i.p.) was associated with a decreased number of white blood cells and lymphopenia, neutrophilia and elevated macrophages along with constant red blood cells in peripheral blood, consistent with other studies (McLeay 1973; Pickering and Pottinger 1985). *In vivo* administration of cortisol and dexamethasone cause a decrease in small lymphocytes along with increase in neutrophils and large lymphocytes of juvenile Coho Salmon, whereas circulating immature erythrocytes remains constant (McLeay 1973). Cortisol administration shows altered circulating lymphocytes, erythrocytes and thrombocytes in brown trout without change in neutrophils (Pickering and Pottinger 1985). Those results can be explained by the redistribution of circulating lymphocytes or increased susceptibility to stress-hormone leading to cell lysis by the stress-induced hormone, cortisol (Espelid et al. 1996). This

occurs via altered adhesion molecules between blood cells and various tissue stromata (Sapolsky et al. 2000).

Phagocytosis. The present study demonstrates that triamcinolone depresses phagocytosis; percentage of phagocytic frequency in terms of the number of ingested beads was decreased. These results are supported by a decrease in phagocytic activity of splenic and pronephros macrophages of trout subjected to stress including noradrenaline and saline injection (Narnaware and Baker 1996). Law et al reported that cortisol administration (100 pM to 10 μM) downregulates phagocytosis of tilapia (*Oreochromis niloticus* x *O. aureus*) and common carp (*Cyprinus carpio*) time and dose-dependently. Additionally, tilapia are more susceptible to cortisol (Wa-Yu Law et al. 2001). *In vivo* dexamethasone administration alters phagocytic index of splenic and pronephric macrophages of rainbow trout, associated with significant decreased phagocytosis of pronephric macrophage, whereas *in vitro* cortisol did not affect phagocytic index of pronephric macrophages (Yuwaraj K. Narnaware et al. 1994). In contrast, low concentration of low dose of cortisol upregulates the phagocytosis of human and bovine (Fox and Heald 1981; Kay and Czop 1994). Additionally, no effects of cortisol on splenic and pronephric phagocytosis were found in rainbow trout (Yuwaraj K. Narnaware et al. 1994).

In this study cortisol did not affect phagocytic index of treated fish, whereas it did alter phagocytic capability of same treated group significantly. This may be explained by the calculation methods of phagocytes because phagocytes ingesting over three beads were considered to be in the same category without detailed sorting in terms of beads numbers. This result is explained by involvement of glucocorticoid receptor on

peripheral blood leukocytes (Weyts et al. 1998) and the sensitivity of tilapia to cortisol (Wa-Yu Law et al. 2001).

Cytokines. Cytokines, soluble proteins or growth factors, are known to play important roles in regulating and maintaining immune responses under stress and stress-induced hormones (Frieri 1999). Cytokine production and release may be inhibited or stimulated by glucocorticoids (GCs) (Batuman et al. 1995; Wilckens 1995).
The present study shows triamcinolone injection elevated the production of splenic mononuclear cell TGF-β mRNA, which is supported by increase in TGF-β expression induced by GCs in human T and glial cells (Batuman et al. 1995). In contrast, triamcinolone-treated hybrid striped bass had decreased TGF-β production by splenic and pronephric mononuclear cells (Harms 2000). Loss of immunomodulatory capability of TGF- β in *Mycobacterium*-infected striped bass caused profound granulomatous inflammation, associated with high mortalities in this susceptible species (Harms et al. 2003).

In the present study, the production of splenic mononuclear cells IL-1β mRNA was suppressed by triamcinolone administration. This result is similar with Wilckens's report that glucocorticoids mediate inhibition of IL-1β synthesis in human monocytes, whereas IFN-γ counteracts this inhibition (Wilckens 1995). In addition, *in vitro* cortisol prevents IL-1β transcription of head kidney leukocytes of rainbow trout (Jun Zou et al. 2000). This can be explained by the presence of cortisol receptors on fish leukocytes (Weyts et al. 1999). It depends on the types of GCs, dose used, specific tissue examined and its bioavailability whether glucocorticoids (GCs) act as immuno-suppressive or as immuno-stimulants with regard to cytokine suppression or release (Wilckens 1995).

Additionally, chronic stress or cortisol administration are found to impair the hypothalamus-pituitary-interrenal (HPI) axis via a decrease in specific binding sites, resulting in protection from over-reactions in responses to additional stressors (Pottinger 1990).

Conclusions

This study demonstrates that triamcinolone disrupts the overall immune response in tilapia (*Oreochromis niloticus*). This was supported by the changes in the affected biomarkers including the primary defense mechanism, phagocytosis, and production of the cytokines, TGF-β and IL-1β. The altered immune responses were consistent with the changes in hematology. This experiment indicated that triamcinolone suppressed overall immune responses and caused leukopenia, lymphopenia, neutrophilia and monocytosis in circulation. Therefore the biomarkers in this experiment were beneficial to establish benchmarks for immunocompromise of teleost fish. These biomarkers may help us better understand the mechanisms of diseases found in the environment, such as ulcerative mycosis.

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Figures

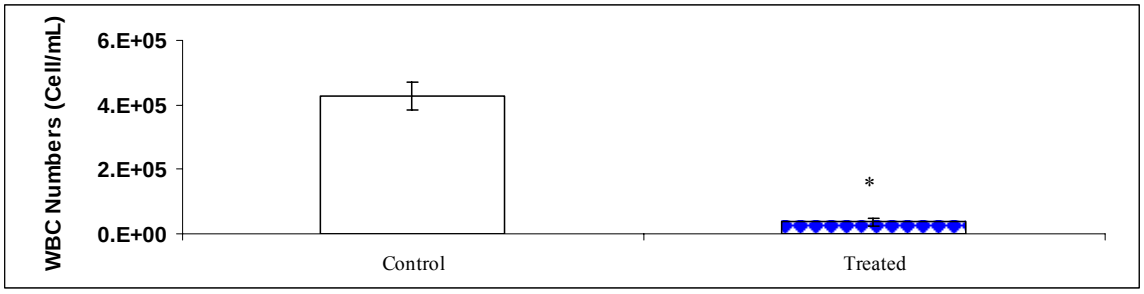

Fig. 1. The effect of triamcinolone on white blood cells counts. Tilapia (n=6) were administered triamcinolone intraperitoneally (10mg/kg body weight). White blood cells were counted 3 days after administration. * represents significant difference in means between control and treated tilapia (p=0.00016). Error bars represent standard error (SE).

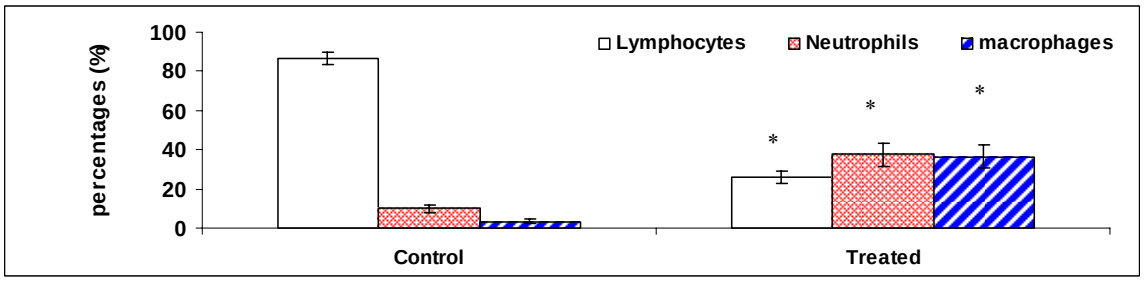

Fig. 2. The effect of triamcinolone on lymphocytes, neutrophils, and macrophages counts. Tilapia (n=6) were administered with triamcinolone (10mg/kg body weight). Each blood cell type from blood smear was measured 3 days after administration. * represents significant difference in means between control and treated tilapia (n=6) ($p<0.005$). Error bars represent standard error (SE).

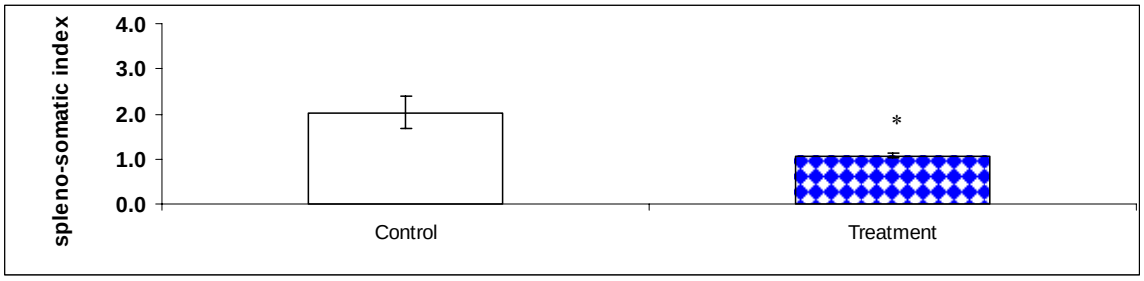

Fig. 3. The effect of triamcinolone on spleno-somatic index. Tilapia (n=6) were administered with triamcinolone (10mg/kg body weight) intraperitoneally. Aseptically removed spleen was weighed and divided by body weight of each fish. Its value was measured 3 days after administration. * represents significant difference in means between control and treated tilapia (p=0.01). Error bars represent standard error (SE).

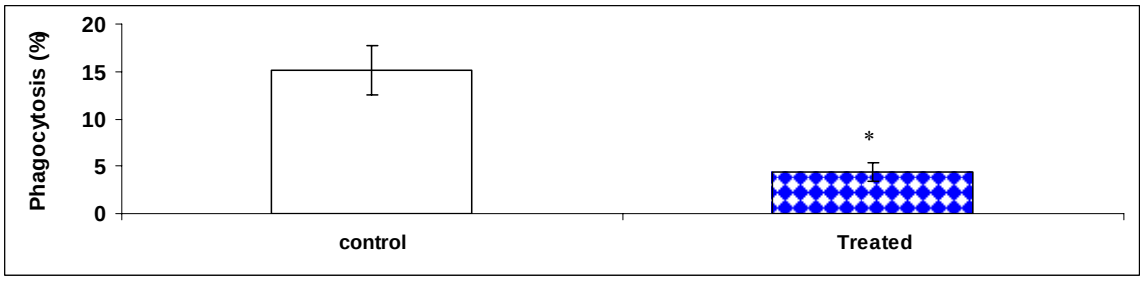

Fig. 4. . The effect of triamcinolone on phagocytosis of peripheral blood leukocytes (PBL). Tilapia (n=6) were administered with triamcinolone (10 mg/kg body weight). Phagocytosis was measured 3 days after administration. * represents significant difference in means between control and triamcinolone-treated tilapia (p=0.0001). Error bars represent standard error (SE).

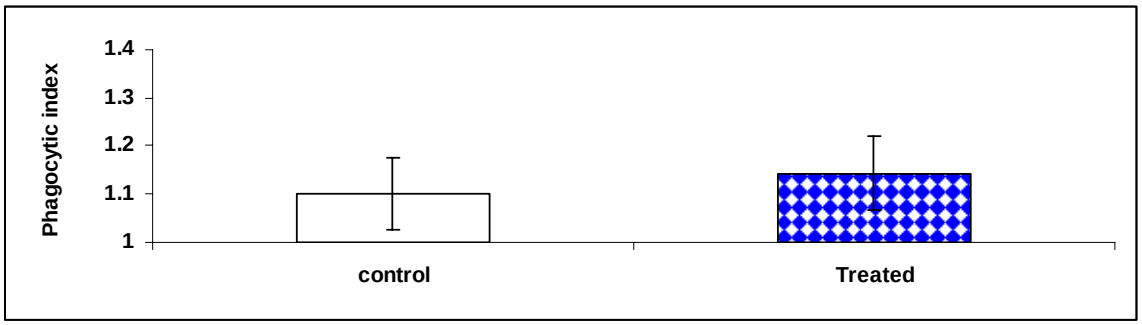

Fig. 5. . The effect of triamcinolone on phagocytic index (PI) of peripheral blood leukocytes (PBL). Tilapia (n=6) were administered with triamcinolone (10 mg/kg body weight). Phagocytic index was measured 3 days after administration. Error bars represent standard error (SE).

Fig. 6. The effect of triamcinolone on splenic mononuclear cells TGF-β mRNA expression. Tilapia (n=6) were administered with triamcinolone (10 mg/kg body weight) intraperitoneally. TGF-β transcription was measured 3 days after administration. * represents significant difference in means between control and treated tilapia (p=7E-06). Error bars represent standard error (SE).

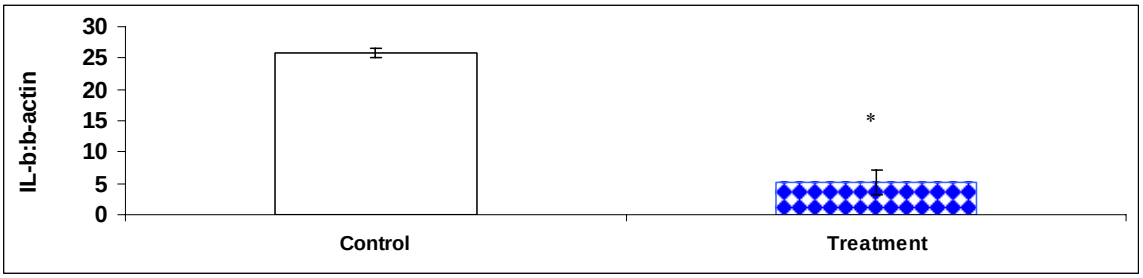

Fig. 7. The effect of triamcinolone on splenic mononuclear cells IL-1β mRNA expression. Tilapia (n=6) were administered with triamcinolone (10 mg/kg body weight) intraperitoneally. IL-1β transcription was measured 3 days after administration. * represents significant difference in means between control and treated tilapia (p=8.7E-05). Error bars represent standard error (SE).

Tables

Table 1. Sequences of primers used to investigate each cytokine expression including splenic mononuclear cells TGF-β and IL-1β mRNA in this study.

Target	primers	Sequences (5'-3')	Target species	PCR product size (bp)
TGF-β	Con A	GACCTGGGATGGAAGTGGAT (20 mer)*	Rainbow trout	300
	Con B2	CAGCTGCTCCACCTTGTGTTG (21 mer)		
	TIF	TATATCTGGGATGCCGAAAACA (22 mer)$	Tilapia	114
	TIR	TGGCAGTGGCTCTAGTGTCTGT (22 mer)		
IL-1β	MUF4	GAGTGTGGTCAACCTCATCAT (21 mer) $	Tilapia	324
	MUR3	GCTGTGCTGATGTACCAGTTG (21mer)		
	ABF1	TGCACTGTCACTGACAGCCAA (21 mer) $	Tilapia	113
	ABR1	ATGTTCAGGTGCACTATGCGG (21 mer)		
b-actin	BacF1	TGGCATCACACCTTCTATAACGA (23 mer) $	Tilapia	139
	BacR1	TGGCAGGAGTGTTGAAGGTCT (21mer)		

* was used from (Choi et al. 2004; Harms et al. 2000a) and $ were chosen from (Choi et al. 2004)

Table 2. Percentage of phagocytic frequency in terms of engulfed beads of tilapia (n=6) 3 days after intraperitoneal administration of triamcinolone (10 mg/kg). One hundred peripheral blood leukocytes were counted from each fish. * means significant difference between control and treatment group ($p<0.05$). Values are means ±SE.

The numbers of engulfed beads	P value	Phagocytic frequency (%)	
		Control	Treated
Only one	0.001	12.4±2.1	4.2±0.9
two beads	0.04	1.4±0.6	0.3±0.2
over three beads	0.02	0.8±0.3	0.1±0.1

Modulation of Immune Function Parameters in Tilapia (*Oreochromis niloticus* L.) Induced by Acute Changes in Salinity

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Abstract

Adverse environmental conditions are considered to be potential factors in fish mortality events and in fish morbidity. In this study, the effects of acute salinity changes on the immune response of fish were investigated. Two different salinity exposures were performed: Exp. I increase in salinity from 0 ppt to 5 ppt, 10 ppt and 20 ppt; Exp. II, decrease in salinity from 20 ppt to 15 ppt, 10 ppt and 5 ppt. Samples of head kidney, gill, gonad, intestine and liver were examined for histopathology and spleen was removed for cytokine expression. Peripheral blood leukocytes (PBL) were used for phagocytosis. No remarkable lesions were found by histopathology that could be attributed to salinity changes. In decreased salinity exposures lymphopenia, neutrophilia and monocytosis were observed in peripheral blood without modification of hematocrit, plasma protein, or plasma cortisol levels. Phagocytosis was increased in response to decrease in salinity from 20 ppt to 15 ppt, 10 ppt and 5 ppt, whereas phagocytic index was not significantly altered. TGF-β transcription continued to increase during the acute decrease in salinity from 20 ppt to 15 ppt, 10 ppt and 5 ppt. However its value at 5 ppt was less than those in 15 ppt and 10 ppt. In increased salinity exposure IL-1β transcription was not significantly different, whereas it was not detectable in the decreased salinity experiment. Acute salinity changes appear to trigger an overall upregulation of immune response of tilapia associated with the alteration of physiological conditions.

Introduction

Massive fish kills and algal blooms, often associated with epizootic ulcerative syndrome (EUS), have been increasingly observed in estuaries of North Carolina (NCDNER-DWQ 2000). Studies concerned with these events considered a variety of interacting environmental conditions as potential factors (Law and Levine 2000; Law 2001; NCDNER-DWQ 2000). The capability of fish to maintain homeostasis is challenged continuously with adverse environmental conditions such as anthropogenic compounds, salinity fluctuations, hypoxia and sediment influx (Bly et al. 1997). Fish subjected to those stressors separately or in concert respond in various ways to maintain homeostasis associated with modulation of hormonal status and immune function, impairment of reproduction, and susceptibility to infectious diseases (Adams et al. 1989; Wendelaar-Bonga 1997). Salinity varies seasonally and its stratification with more concentrated salinity near the bottom of the water column is often influenced by wind driven tide, precipitation, and water flow; this is often accompanied by dissolved oxygen layering (NCDNER-DWQ 2000). Recent reports concerned with changes in salinity demonstrated its effects on survival, hatching rate, endocrinology, immune responses, and histopathological characteristics (Chang and Plumb 1996; Farghaly et al. 1973; Fernandes and Rantin 1994; M.C.J.Verdegem et al. 1997; M.S. Sawants et al. 2001; Marc et al. 1995; Morgan et al. 1997; Narnaware et al. 2000; T. Yada et al. 2001; Woo and K. C 1995; Zeltoun et al. 1974). Tilapia adapted to hyper-osmotic salinity and challenged with *Streptococcus* isolates showed significantly higher mortality than those at "zero" salinity (Chang and Plumb 1996). Verdegem et al. reported that high salinity induces the modulation of hematological characteristics with a constant hematocrit value and lesion development on the fin area of hybrid red tilapia adapted to brackish water (M.C.J.Verdegem et al. 1997). Rainbow trout adapted to iso-osmotic salinity

shows increases in plasma lysozyme activity and no change of plasma immunoglobulin M (IgM) (T. Yada et al. 2001), whereas tilapia caught in the estuary followed by adaptation to seawater had no significant differences of plasma cortisol and lysozyme activity but decrease in plasma level of prolactin (Iwama et al. 1997). Tilapia transferred from fresh water to sea water (25 ppt) had significant increases in plasma osmolarity, plasma level of [Na^+] and [Cl^-], cortisol and growth hormone, whereas those held in freshwater and isosmotic salinity show no change among these values (Morgan et al. 1997). In this study a laboratory fish model, fresh water-adapted euryhaline tilapia (*Oreochromis niloticus*), was used to investigate the effects of acute salinity stress on physiological, histological and immune responses. Tiered bioassays of increasing specificity for the immune system were performed with this fish model. Tilapia have proven to be an excellent model to study the mechanisms of acute salinity changes based on their high tolerance to varying ranges of salinity and high resistance to stress and infection (M.C.J.Verdegem et al. 1997).

Methods

Experimental animals. Laboratory-reared Tilapia (Oreochromis niloticus L.), 14.5 to 19 cm fork length and weighing 110 – 150 g were transported from the NCSU aquaculture facility to a 190 L fresh water tank for at least one week and fed ad libitum commercial foods (Ziegler Brothers, Gardners, PA). The feeding was suspended 24h prior to the experiments and 50 % of the tank water was replaced on alternate days. Water conditions were 26 °C, pH 7.0 ± 0.5, ammonia 0.25 mg/L, nitrate less than 20 mg/L, nitrite less than 3 mg/L, 16h light:8h dark photoperiod, and tanks were constantly aerated.

Experiment I: Increase in salinity

After the initial acclimation twelve fish were placed randomly into two tanks. The salinity of the test water was gradually increased to 5 ppt. And further increasing rate of salinity was set at 5 ppt per day until each final level of 10 ppt, 15 ppt and then 20 ppt based on Al-Amoudi's study that immediate transfer from fresh water to 18 ppt salinity caused mortality of tilapia (Al-Amoudi 1987). The control group was continuously kept in fresh water.

Experiment II: Decrease in salinity

Fish in fresh water were acclimated to 20 ppt gradually for one week, then salinity of the test water was gradually reduced to 15 ppt. Further reducing rate of salinity was set at 5 ppt per day until each level of 10 ppt and 5 ppt was reached. The control group was continuously kept in 20 ppt.

Saline water was prepared with synthetic salt (Marine Enterprise International Inc., Baltimore, MD). At each interval, three fish from each group were processed after overdose of 3-aminobenzoate ethyl ester methanesulphonate (MS-222, Sigma, St. Louis, MO) (150 ppm). Time intervals were 2 days at each salinity exposure and all experiments were repeated three

times. Blood samples were collected from the caudal vein in heparinized syringes (22G) and stored at room temperature until use. Spleen was removed aseptically and placed in sterile RPMI media supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100µg/ml streptomycin, and 2mM EDTA. The blood was diluted 1:1 in 1% Phosphate buffered solution (PBS) at the room temperature and centrifuged at 45 x g for 5 min. The buffy coat layer was resuspended in 1% PBS and placed on 55 % Percoll gradient (specific gravity 1.0697 g/mL) at 800 x g for 30 min. at 16 °C. Peripheral blood leukocytes (PBL) were adjusted to a cell density of 1 x 10^6/ml. The isolation of splenic leukocytes was prepared as described in splenic leukocytes from hybrid striped bass (Harms et al. 2000a). Finely minced spleen was forced through a wire mesh (50 meshes) and resuspended in sterile RPMI media. The cell suspension was placed on two-step percoll gradients (specific gravity 1.052 and 1.065 g/mL) at 400 x g for 5 min. and then 800 x g for 25 min. and splenic leukocytes in interface were adjusted to a cell density of 1 x 10^7/mL. Cell viability was performed in 0.2 % trypan blue.

Tier I: Histological Evaluation

Samples of gill, liver, head kidney, intestine and gonad were routinely processed, embedded in paraffin, and sectioned at 5 microns. Sections were mounted on glass slides, stained with HE, and examined by light microscopy. A six-point grading scheme was used for evaluation; "0"no remarkable microscopic abnormalities, "1" very mild changes with minimal inflammatory cells, "2" mild changes, "3" moderate changes, "4" moderately severe changes and "5" severe changes (Hurty et al. 2002a). The splenosomatic index was calculated as follows:

Splenosomatic index = (spleen weight (g)/body weight (g)) x 100.

Tier II: Hematology

Differential cell counts were performed on blood smears from each fish using Wright's stain according to manufacturer's instructions (Fisher, Middle Town, VA), then examined by light microscopy (OPELCO, VA, USA) under oil immersion. One hundred leukocytes were counted and categorized into lymphocytes, monocytes/macrophages and neutrophils. The assay of hematocrit values was a modification of procedure described by Wedemeyer et al. (Wedemeyer et al. 1983). Hematocrit was measured by microcapillary method at 6000 x g for 5 min. Its value was determined by index. The concentration of plasma protein in each sample was determined using a clinical refractometer (American optical Co., Buffalo, NY) according to the manufacturer's instructions. Whole blood was centrifuged at 3,000 x g for 10 min. and plasma was harvested then kept at –80 °C until the analysis. Plasma cortisol was analyzed by the Clinical Pharmacology Laboratory at the North Carolina State University College of Veterinary Medicine (NCSU-CVM) with the IMMULITE chemilumicent immunoassay (Diagnostic Products Co., Los Angeles, CA).

Tier III: Immune Function Parameters

1. Phagocytosis Measurement

Peripheral blood leukocytes (PBL) of tilapia were isolated as described above. PBL ($1x10^6$/ml) were cultured per well with opsonized-fluoresceinated (FITC) latex beads (diameter = 1.75μm) (the ratio of cell: beads, 1:30) (Polysciences, Northampton, U.K.) in 5% CO_2/95% air in a humidified incubator for 5 hr at 26°C. At the end of incubation 1 ml of PBS was added to stop phagocytosis and centrifuged at 400 x g for 10 min. Pellets were resuspended in 100 μ of 1 % of PBS with the addition of 50 μl of 1 % triton X and 50 μl of Propidium Iodide (0.5 mg/ml) (Roche, Indianapolis) and analyzed by fluorescence microscope. At least 100 cells were counted

and the percentages of phagocytic cells engulfing latex beads as phagocytic capability were examined. Phagocytic index (PI) was defined as the average number of ingested latex beads per a phagocytic cell. Therefore, the total number of ingested beads was divided by the total number of phagocytic cells.

2. TGF-1β and IL-1β mRNA Expression.

Isolation of Total RNA and Reverse transcription

The assays of total RNA isolation and reverse transcription were the modification of procedure described from rainbow trout (Harms et al. 2000a). Total RNA of splenic leukocytes was isolated with one mL of Tri Reagent (Sigma, MO, USA) for each 5-10 x 10^6 cells according to manufacturer's instructions. RNA pellet was resuspended in sterile water (Sigma, MO, USA) at a concentration of 5 x 10^4 cell equivalents/μl. For RNA reverse transcription total RNA (3 x 10^6 cell equivalents) was mixed with 300-pmol oligo dT_{15} (Promega) in a 72 μl volume followed by heating at 94 °C for 5 min. and chilling on ice immediately. Then 78 μl RT mixtures were added to a final volume of 150 μl. RT mix comprised 1 x buffer (50mM Tris-HCI, pH 8.3; 75mM KCl, 3mM $MgCl_2$), 0.4 mM each dNTP, 10 mM DTT, 0.8U/μl RNAsin and 2 U/μl reverse transcriptase (Superscript II RT, Gibco-BRL, Gaithersburg, MD, U.S.A.). Transcription cycle was one cycle at 45 °C for 60 min. followed by chilling on ice immediately and diluted in 1:2 in sterile water (Sigma, MO, USA).

Primer Preparations

Initial primers (Con A1: GACCTGGGATGGAAGTGGAT (20 mer) and Con B2: CAGCTGCTCCACCTTGTGTTG (21 mer)) against hybrid striped bass TGF-β were used (Harms et al. 2000a). PCR product was purified with gel-extraction kit (Qiagen, Valencia, CA)

according to manufacturer's method. Purified PCR products were automated-sequenced by NC-CH Automated DNA Sequencing Facility (Chapel Hill, NC) with an ABI 377 Automated Sequencer (Applied Biosystems, Foster City, CA). After alignment with other known sequences, tilapia-specific primers against TGF-β were made (TIF: TATATCTGGGATGCCGAAAACA (22 mer) and TIR: TGGCAGTGGCTCTAGTGTCTGT (22 mer)). β-actin was used as a housekeeping gene in the quantitative real time PCR assay. β-actin primers were made against known teleost β-actin sequences (Table 1) to amplify a 139 bp fragment (BacF1: TGGCATCACACCTTCTATAACGA (23 mer) and BacR1: TGGCAGGAGTGTTGAAGGTCT (21mer)). IL-1β initial primers were designed against conserved region of known sequences of teleost in the GenBank Databases (Table 1) (MUF4: GAGTGTGGTCAACCTCATCAT (21 mer) and MUR3: GCTGTGCTGATGTACCAGTTG (21mer)). Based on initial primers-generated sequences tilapia-specific primers against IL-1β were designed to allow amplification of 115bp fragment for Real Time PCR (ABF1: TGCACTGTCACTGACAGCCAA (21 mer) and ABR1: ATGTTCAGGTGCACTATGCGG (21 mer)).

Four 10-fold serial dilutions of plasmid DNA were prepared for standard curves of both targets and housekeeping gene.

3. Quantitative Real Time PCR

Quantitative PCR analysis for both TGF-β and IL-1β mRNA was performed as previously described for the triamcinolone experiment. Relative quantitation in the cytokine signal was normalized by β-actin signal.

Statistical Methods

The effects of acute salinity were analyzed by the SAS analysis of variance (ANOVA) procedure (SAS 2003). When ANOVA is significant the multiple comparisons were performed with Least Significance Difference (LSD) and Tukey Test. $P<0.05$ was accepted as a statistically significant level of difference.

Results

Histopathology. No mortality of tilapia was observed prior to or during the experiments. Two types of lesions were present in the head kidney; pigmented macrophage aggregates (PMAs) and granulomas. During the both increased and decreased salinity experiment pigmented macrophage aggregates (PMAs) were often observed in the head kidney of tilapia with and without treatment. Very mild to moderate density of PMAs was present. In the increased salinity treatment one fish had mild granulomas and in decreased salinity treatment two fish had mild granulomas. In the gill in response to increased salinity change two fish had mild to moderate lamellar aneurysms and three fish had mild to moderately severe lamellar thickness (Fig 1). In the decreased salinity experiment, there was moderate lamellar atrophy (n=3) in the gills of exposed tilapia. In addition lamellar aneurysm-like lesions were commonly found in tilapia with and without decrease in salinity change. In the liver, one fish had mild lymphocyte aggregates in the increased salinity treatment and two had mild lymphocyte aggregates and congestion in the decreased salinity treatment. Intestines and gonads had no remarkable changes. Tilapia in both treatments were all male except two female.
No changes in splenosomatic index were observed in response to increased salinity or decreased salinity ($p>0.05$).

Hematology. In the increased salinity experiment there were no significant changes in levels of lymphocytes, macrophages and neutrophils. In the decreased salinity experiment the percentages of lymphocytes in peripheral blood continued to decrease compared to control level ($p<0.0001$), whereas its value remained the same as the initial control value at 5 ppt (Fig.2). Its value within the treated group continued to increase linearly over salinity changes ($p=0.001$). The percentages of neutrophils increased at 10 ppt and 15 ppt compared to control levels

($p<0.0001$), whereas their values were the same as control values at 5 ppt salinity (Fig.4). Within treated groups, the percentage of neutrophils at 10 ppt and 15 ppt was higher than those at 5 ppt salinity ($p=0.0038$). The percentage of monocytes was significantly increased at 15 ppt salinity compared to controls ($p=0.0034$) (Fig.3). Their value within treated fish seemed excessively increased at 15 ppt ($p=0.0006$). The values of hematocrit (Hct), plasma protein and splenosomatic index were not significantly different compared to controls in either experiment.

Phagocytosis. Phagocytic capability of peripheral blood leukocytes (PBL) was not significantly different in the increased salinity treatment (data not shown). However, in the decreased salinity experiment its value was significantly different at 15 ppt, 10 ppt and 5 ppt compared to controls ($p<0.0001$) (Fig.5). Phagocytosis increased over the salinity range from 20 ppt to 15 ppt, 10 ppt and 5 ppt and especially its value was increased the most at 10 ppt salinity ($p<0.03$). The percentages of cells with varying ingested beads were modulated during decreased salinity treatment (Table 1). The cells with only one bead were significantly different at 10 ppt ($p<0.05$) and those with two beads were also significant at 10 and 5 ppt ($p<0.05$) compared to control levels, whereas those with over three beads were not significantly different. Phagocytic Index of both salinity changes remained constant between treated and control fish ($p>0.05$).

Cytokine Expression. TGF-β mRNA expression of splenic mononuclear cells was not significantly different during the increased salinity experiment. However, in the decrease in salinity treatment its level was significantly different at 15 ppt, 10 ppt and 5 ppt compared to controls ($p<0.0001$) (Fig.6). TGF-β transcription continued to increase at 15 ppt, 10 ppt and 5 ppt ($p<0.01$). IL-1β mRNA expression of splenic mononuclear cells was not significantly different in the increased salinity experiment. However, during the decrease in salinity change

splenic mononuclear cell IL-1β transcription was constitutively expressed in the control group, whereas its expression was not detectable in the treated group.

When compared with phagocytosis, TGF-β transcription was not correlated ($p>0.05$).

Discussion

Histopathology. Histopathology can be a valuable tool for detecting cellular/tissue modifications from prior or ongoing exposure to multiple toxic agents and gives an indication of overall health status of the animals at risk. The present study showed no gross or microscopic lesions in fish exposed to rapid salinity changes that would be consistent with ulcerative mycosis (UM) as reported in Atlantic menhaden and other fish species (Dykstra et al. 1986). Severe skin ulcers in fish associated with oomycetes have been found most commonly in areas of low salinity (2-10 ppt) in North Carolina estuaries (Levine et al. 1990). In the current study, internal organs likewise had no remarkable lesions attributed to salinity changes. Increased pigmented macrophage aggregates (PMAs) density can be induced by natural conditions such as aging, starvation and bacteria and parasitic protozoal infections (Wolke 1992). In head kidney, pigmented macrophage aggregates (PMAs) in both control and exposed tilapia were commonly found, whereas it was not remarkable compared to control. Granulomas in liver and head kidney of exposed fish were observed with no associated degenerative changes. But it was not likely correlated with the exposure of acute salinity changes. In the gills, lamellar aneurysms associated with no fibrosis were found in control and exposed fish during acute salinity change although a number of morphological changes of fish in response to decrease in salinity change were difficult to define in terms of classical lamellar aneurysms. Lamellar aneurysm is breakdown of vascular integrity via rupture of the pillar cells encountering thrombosis and fibrosis (Brand et al. 2001). This histopathological change was observed in some teleost fish including sea water-adapted pink salmon fry exposed to crude oil (Brand et al. 2001) and rainbow trout subjected to acute aflatoxicosis (Wales 1970). This result seems likely to be induced by incidental damage although it needs more investigation to characterize this change.

Gonads and intestine were considered normal in control and exposed fish in both increase and decrease in salinity experiments. Most fish were males.

Splenosomatic index adds quantitative results to histopathology data leading to prediction of general health in affected animals under varying environmental stressors (Adams 1993). In this study, acute changes of salinity did not cause significant changes in splenosomatic index ($p>0.05$). This result is consistent with the study that no changes of splenosomatic index were observed in rainbow trout subjected to crowding (Fox et al. 1997). However, chronic exposure of toxic thiocyanate caused decreased splenosomatic index in rainbow trout (Lanno and Dixon 1996).

Hematology. In this study increase in salinity from 0 ppt to 20 ppt induced no changes of hematologic characteristics, in contrast with the abundance of immature lymphocyte and granulocytes of hybrid red tilapia kept at 19 ppt for 6 weeks (Farghaly et al. 1973; M.C.J.Verdegem et al. 1997).

In decrease in salinity, relative lymphopenia was observed from 20 ppt to 15 ppt and 10 ppt, neutrophilia from 20 ppt to 15 ppt and 10 ppt and monocytosis from 20 ppt to 15 ppt, in contrast with Morgan et al study that sudden drop of salinity causes no changes of leukocyte numbers in dolphin (*Coryphaena hippurus*) (John D. Morgan et al. 1996). These results suggest that salinity drop from 20 ppt to 15 ppt and 10 ppt allows changes in hematological characteristics of exposed fish. Under stress, blood leukocytes in different organs of dab are found to be redistributed (Pulsford et al. 1995). The result of no significant changes in hematocrit and plasma protein over both salinity changes in this study agreed with the constant levels of hematocrit and plasma protein of coral reef fish (*Pomacanthus imperator*) throughout the salinity range from 30 ppt to 7 ppt (Woo and K. C 1995). Tilapia over the salinity range from 0 ppt to 19 ppt had no significant

changes in hematocrit value (M.C.J.Verdegem et al. 1997), whereas rainbow trout from 10 ppt to 20 ppt had increase in its value (Zeltoun et al. 1974). It has been postulated in other studies that stress, regardless of its nature, influences hematologic characteristics like leukopenia, lymphopenia and neutrophilia directly or indirectly (Ainsworth et al. 1991; Ellsaesser and L.W.Clem 1986; Pickering and Pottinger 1985).

Phagocytosis. Phagocytosis plays an important role to defend against invasive pathogens, and is associated with increase or decrease in its ability after non-specific or specific disturbances (Michel and Hollebecq 1999). In this study, it is apparent that acute salinity changes stimulated phagocytic capability, without alteration of its value in the increased salinity experiment. Additionally phagocyte numbers engulfing one and two beads were increased during decrease in salinity exposure although its value ingesting over three beads was not significantly different (Table 2). These results are in contrast with the report by Narnaware et al. that macrophage phagocytosis of black sea bream is not affected over salinity changes, whereas its value is stimulated during adaptation to iso-osmotic salinity (Narnaware et al. 2000). Marc et al reported that the phagocytic activity of pronephric leukocytes of brown trout adapted to seawater is significantly increased (Marc et al. 1995). It has been reported that mild to severe infection with vibriosis stimulates phagocytic activity and phagocytic index of pronephric and splenic macrophages of sea bream (Deane et al. 2001). In this study, phagocytic index of decreased salinity water-held fish was not changed, whereas phagocytosis of the same fish was significantly different. This is explained by the phagocyte counting method, because phagocytes ingesting over three beads were placed into the same category without differentiation according to ingested beads number. Additionally, Atlantic salmon infected with *Neoparamoeba* sp. followed by being kept in fresh water showed no changes in phagocytic index but augmented

phagocytosis (Gross et al. 2004). Based on relative increase in monocytes and neutrophils considered to be effective phagocytes, it is speculated that decrease in salinity stimulated the phagocytic ability through other inflammatory agents such as TGF-β, which may be protective against the stressors.

Cytokines. There are number of studies that suggest cytokines are involved in stress-induced immunosuppression (Ottaviani et al. 1997; Saperstein et al. 1992). TGF-β and platelet-derived growth factor (PDGF) are involved to intervene the release of biogenic amines via the axis of corticotrophin hormone (CRH)-adernocorticotropin (ACTH)-biogenic amines, associated with modulation of immune responses (Ottaviani et al. 1997). Saperstein et al also reported that IL-1β alters innate immune function of rats in response to electric shock stress (Saperstein et al. 1992). TGF-β, primarily immunosuppressive, depends on its concentration, differentiated target cells and correlation with other proinflammatory compounds to play the role of anti- or pro-inflammatory agent (McCartney-Francis and Wahl 1994). Increase in salinity did not affect cytokine expression, whereas in response to decrease in salinity change 20 ppt to 15 ppt, 10 ppt and 5 ppt, splenic mononuclear cell TGF-β transcription was stimulated. In mammals, upregulation of TGF-β expression is involved in the malfunctioning kidney with fibrosis (Basile et al. 1998) although it protects from generation of superoxide anions and other inflammatory cytokines under hypoxia and reperfusion during cerebral ischemia (Lefer et al. 1993). Inability to induce immunomodulatory TGF-β in *Mycobacterium*-infected striped bass is associated with granulomatous inflammation leading to the death of this susceptible species (Harms et al. 2003). The correlation between innate immune function, peripheral blood leukocytes phagocytosis and TGF-β mRNA expression under the decreased salinity change was not significant, in contrast with studies concerned with the relationship between innate immune function and cytokine

expression (Harms 2000). Tilapia in response to hypoxia and reperfusion show a negative relationship between phagocytosis and TGF- IL-1β transcription and positive one between phagocytosis and IL-1β transcription, and hybrid striped bass administrated with triamcinolone also had a negative relationship between bactericidal activity and TGF-β transcription (Harms 2000).

Under increased salinity changes IL-1β mRNA was expressed constitutively with and without treatment, whereas under decreased salinity its expression was found in only the controls. In addition, increase in salinity did not affect splenic mononuclear cells IL-1β transcription ($p>0.05$). Scapigliati et al. reported that IL-1β transcription of different organs was observed without LPS stimulation due to leukocytes adherence to plastic culture dishes, whereas *in vivo* and *in vitro* LPS stimulation enormously upregulated its expression in lymphoid tissues (Scapigliati et al. 2001). Decreased salinity change somehow seems to affect leukocyte's ability, resulting in no expression of IL-1β mRNA, whereas it is not clear how this ability reacts with IL-1β transcription in teleost fish. In future studies, stimulation with LPS or bacteria can be used to investigate IL-1β transcription.

Although acute increases in salinity did not appear to have significant effects on immune responses of tilapia, the results indicate the possible complexity of the interrelationships of salinity changes and host responses. It is reported that drops in salinity (into the 2 – 10 ppt range) may be correlated with ulcerative mycosis due to *Aphanomyces* sp. (Dykstra et al. 1986; Levine et al. 1990). Ulcerative mycosis in fish may demand predisposing conditions in the host such as systemic or mucosal immune dysfunction (Law 2001). Therefore, with this result it is speculated that changes in host immune responses induced by acute salinity changes may play a role in ulcerative mycosis indirectly. However, future studies still need to investigate the effects

of salinity combined with other environmental stressors, which may act synergistically or antagonistically leading to more susceptibility to or protection from infectious diseases. Additionally, this study suggests that alteration of immune responses depends on the nature and degree of stressors and interspecies variation.

Acknowledgements

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Figures

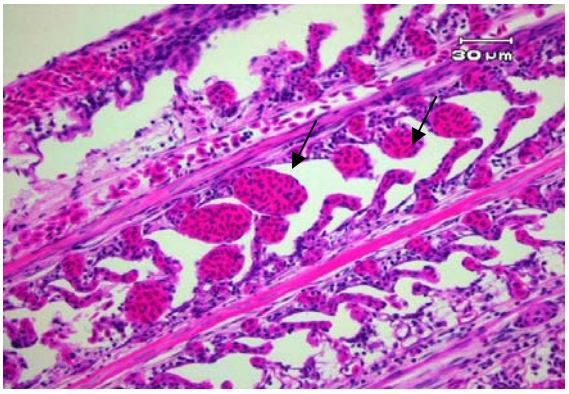

Fig. 1. Lamellar aneurysms in the gills of tilapia in response to acute changes in increased salinity (10 ppt). Arrows point to example lamellar aneurysms.

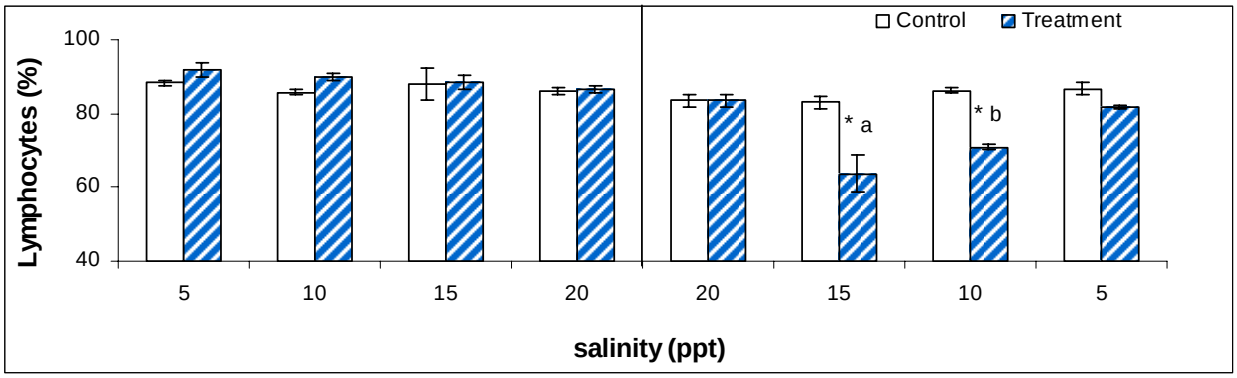

Fig. 2. The effects of acute salinity change on lymphocytes of tilapia (n=3). The perpendicular line divides Exp. I, increased salinity treatment and Exp. II, decreased salinity treatment. * represents significant difference in means between control and exposed values ($p<0.0001$). The lowercase a and b denote significant difference in lymphocyte levels within treated group ($p=0.001$). Error bars represent standard error (SE).

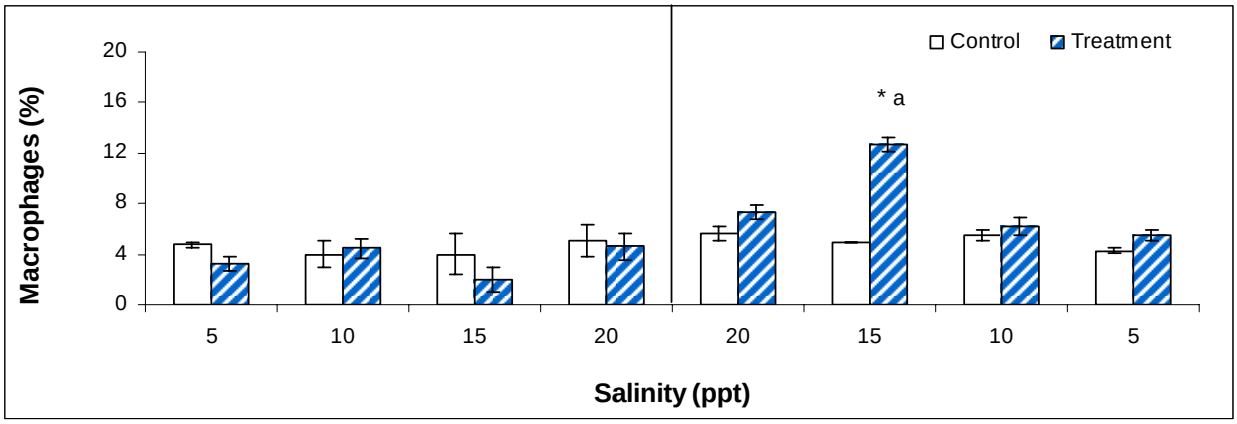

Fig. 3. The effects of acute salinity on macrophages of tilapia (n=3). The perpendicular line divides Exp. I, increased salinity treatment and Exp. II, decreased salinity treatment.

* represents significant difference in means between control and exposed values (p=0.0034).

The lowercase a denotes significant difference in macrophage levels within treated groups (p=0.0006). Error bars represent standard error (SE).

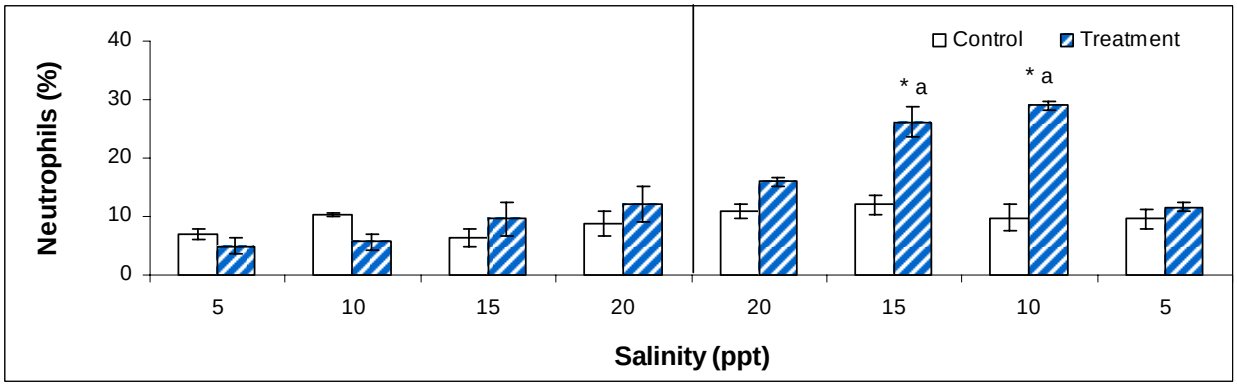

Fig. 4. The effects of acute salinity on neutrophils of tilapia (n=3). The perpendicular line divides Exp. I, increased salinity treatment and Exp. II, decreased salinity treatment.

* represents significant difference in means between control and exposed values (p<0.0001).

The lowercase a denotes significant difference in neutrophil level within treated groups (p=0.0038). Error bars represent standard error (SE).

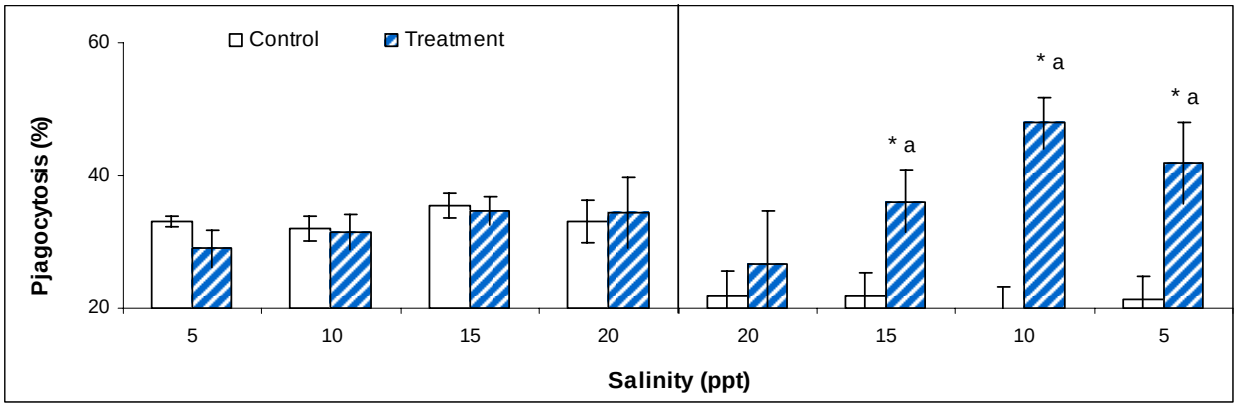

Fig. 5. The effects of acute salinity on phagocytosis of tilapia (n=3). The perpendicular line divides Exp. I, increased salinity treatment and Exp. II, decreased salinity treatment. * represents significant difference in means between control and exposed values ($p<0.0001$). The lowercase a denotes significant difference in phagocytosis level compared to its value at 5 ppt in decreased salinity exp. ($p<0.03$). Error bars represent standard error (SE).

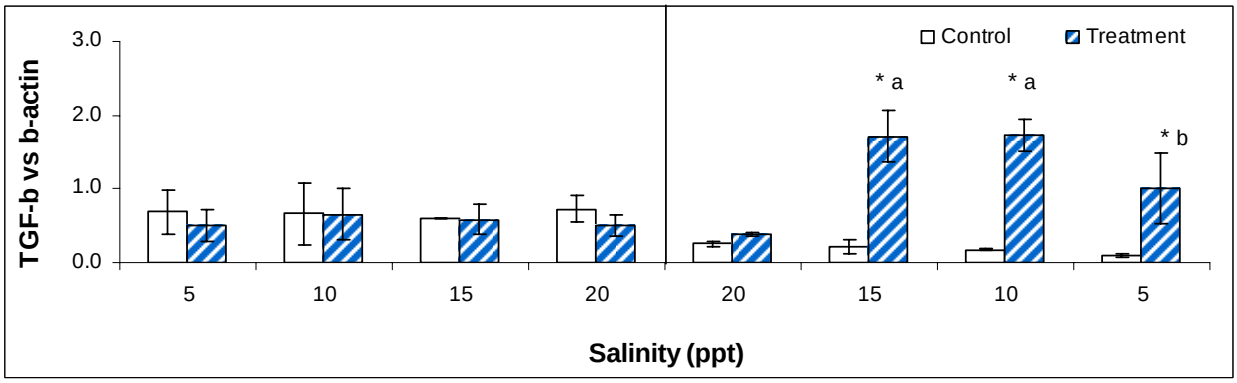

Fig. 6. The effects of acute salinity changes on TGF-β mRNA expression of tilapia (n=3). The perpendicular line divides Exp. I, increased salinity treatment and Exp. II, decreased salinity treatment. * represents significant difference in means between control and exposed values ($p<0.0001$). The lower case a and b denote significant difference in TGF-β expression level ($p<0.03$). Error bars represent standard error (SE).

Tables

Table 1. Genbank identification number of known teleost fish cytokines used to prepare the primers for real time PCR.

Cytokine	Common Name	Genbank Identification number
β-actin	zebra fish	(GenBank, AA566386)
	Red Seabream	(GenBank, AB036756)
	Rainbow trout	(GenBank, AF254414)
	Grass Carp	(GenBank, M25013)
	Common Carp	(GenBank, M24113)
	Gold fish	(GenBank, AB039726)
	Fugo	(GenBank, U38849)
IL-1β	Sea bream	(GenBank AJ277166)
	flounder	(GenBank, AB070835)
	Sea bass	(GenBank AJ269472)

Table 2. Percentage of phagocytic cells in terms of engulfed bead numbers by peripheral blood leukocytes (PBL) of Tilapia (n=3) subjected to decreased salinity. * means significant difference between control and treatment group ($p<0.0001$). Values are means ±SE.

Phagocytic cells	Salinity (ppt)							
	20		15		10		5	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
PBL w/ one bead	13.8±1.7	18.5±6.0	16±2.5	15.8±1.7	11.5±3.1	27.5±1.9*	13.3±1.7	27±2.4
PBL w/ two beads	4.5±0.3	5±1.6	3.8±1.1	5.3±1.6	3±1.1	8.75±0.7*	3.5±0.3	8.25±1.3*

Modulation of Immune Function Parameters in Tilapia (*Oreochromis niloticus*) Induced by Hypoxia-Reperfusion

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Abstract

Inadequate dissolved oxygen in the aquatic environment is a well established cause of fish morbidity and mortality. The specific effects of hypoxia on the immune system of fish, however, are not well established. In this study, the effects of hypoxia as a source of immunocompromise in fish were investigated. Using a precision apparatus developed in our laboratory for hypoxia exposures, a series of assays of increasing specificity for immune function were performed on acutely hypoxia stressed fish: Tier I, histopathology; Tier II, hematology, plasma chemistry, and cortisol concentration; and Tier III, phagocytic index and expression of the cytokines TGF-β and IL-1β. Tilapia (*Oreochromis niloticus*) were exposed to 7% oxygen saturation for 96h followed by reperfusion for 84h. Sampling intervals were 48h and 96h during the hypoxia and 12h and 84h during the reperfusion. Samples of head kidney, gill, intestine, heart and liver were examined for histopathology and spleen was extracted for cytokine expression. Peripheral blood leukocytes (PBL) were used for measuring phagocytosis, while whole blood was examined for hematology plasma biochemistry, blood gases and cortisol levels. Histopathology, while the least specific for immune function, showed no remarkable microscopic abnormalities in lymphoid or other tissues. Lymphopenia and neutrophilia were observed in peripheral blood but with no significant changes in monocytes. There were no significant changes in plasma chemistry and blood gases except for total protein, lipase, partial pressure oxygen (PO_2) and oxygen saturation (SO_2). Total protein, PO_2, and SO_2 were decreased in response to hypoxia and reperfusion. Plasma lipase decreased in response to hypoxia but not to reperfusion. Phagocytic capability and phagocytic index decreased during hypoxia and reperfusion, whereas its values were recovered by 84h reperfusion. TGF-β transcription continued to

increase during the exposure with the most excessive production at 12h reperfusion, whereas IL-1β transcription decreased in response to hypoxia and reperfusion.

In this study, hypoxia appeared to trigger overall downregulation of the immune response in the test fish. This would suggest a possible factor in the pathogenesis of disease outbreaks in fish such as ulcerative mycosis, in which repeated bouts of environmentally induced hypoxia may lead to lesion events and individual mortalities rather than massive fish kills.

Introduction

It is axiomatic that lack of oxygen causes injury and death of cells and tissues and, thus, whole organisms. Fish are especially vulnerable to hypoxia since, compared to air, water contains only small amounts of oxygen available for respiration (Boyd 1990). While many fish kills have been attributed to low dissolved oxygen (DO), it is the *sublethal* effects of hypoxia that are less well understood. Relatively little is known regarding the specific effects of hypoxia on immune function in fish and, by extension, its role in disease pathogenesis.

During the last two decades, U.S. mid-Atlantic estuaries have been plagued by large scale fish kills, often accompanied by outbreaks of ulcerative skin lesions (Dykstra 2000; NCDENR 1997; NCDNER-DWQ 2000). Recent studies have demonstrated the potential roles of microbial pathogens in these epizootics, including Oomycetes (fungi) such as *Aphanomyces invadens* and blooms of harmful algae such as *Pfiesteria* spp. (Dykstra 2000; Noga 2000; Noga et al. 1996). It has become apparent that the pathogenesis of these lesion outbreaks is multi-factorial, and both geographically and seasonally variable. Since the role of sudden changes in water quality parameters in disease pathogenesis is less clear, the objective of the present study was to determine the effects of hypoxia and reperfusion on the immune system of fish.

Adverse water quality in terms of anthropogenic activity or adverse environmental conditions such as hypoxia , temperature changes and sediment influx may induce compromise of the immune system, leading to less resistance to pathogen invasion (Adams et al. 1989; Anderson and Zeeman 1995; Bly et al. 1997). Seasonal and diurnal hypoxia or anoxia have been commonly found in estuarine and coastal marine waters (Harper et al.

1981; Turner and Allen 1972) associated with mass mortality of benthic organisms and fish over large marine coastal areas (Wu 2002) . Low dissolved oxygen has become worse due to loads of agricultural run-off, vertical stratification, temperature and salinity influx (Malone 1991; Paerl et al. 2001; Turner et al. 1987). A number of agents are reported to cause immunotoxicity in fish, including heavy metals, pesticides, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCBs), and volatile organic chemicals (VOC) (Zelikoff et al. 2000). Like these agents, hypoxia also has been shown to modulate the innate and adaptive immune responses in fish (Boleza et al. 2001; Bunch and Bejerano 1997; Cecchini and Saroglia 2002; Cuesta et al. 2003; Ortuno et al. 2002; Woo 2003). Bunch and Bejerano reported high mortality induced by streptococcal infection was found in Tilapia exposed to hypoxic conditions (Bunch and Bejerano 1997). Cecchini et al. demonstrated antibody responses against human-γ-globulin in sea bass held at hypoxia was produced less than those kept at hyperoxia (Cecchini and Saroglia 2002). Air exposure-induced hypoxia reduced respiratory burst of seabream (Ortuno et al. 2002) and additionally inhibited head-kidney natural cytotoxic cell activity of gilthead seabream (Cuesta et al. 2003)

In response to acute hypoxia, systemic responses are triggered such as increased ventilation and cardiac output followed by tissue/cell anaerobic metabolism, whereas for chronic exposure molecular changes occur in terms of up-regulated gene expression to increase oxygen transport capacity and capillary blood supply (Michiels 2004). In both mammals and fish hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor, is up-regulated in response to hypoxia that regulates hypoxia inducible genes involved in erythropoiesis, glycolysis, and angiogenesis (Wu 2002). Chronic hypoxia also interferes with the reproductive system, resulting in endocrine disruption of carp (*Cyprinus carpio*)

(Wu et al. 2003). Hypoxia followed by reperfusion can damage tissue more than hypoxia alone due to overload of free radical generation, excess cytosolic Ca^{+}, modulation of cytokines such as TGF-β activity, and other pro-inflammatory components (Chen 2003; Jordan et al. 1999; Yellon and Baxter 1999).

Transforming growth factor-β (TGF-β) is an immunoregulatory, primarily suppressive, cytokine that also influences cell proliferation and differentiation, extracellular matrix regulation, wound healing, angiogenesis, apoptosis, production of cytokines, and lipid metabolism (Harms 2000; Zhang and Phan 1999; Zuckerman et al. 2001). There is some evidence that TGF-β_1 is involved in hypoxia-reperfusion injury in mammals (Basile et al. 1998; Dhandapani and Brann 2003). In general, upregulation of TGF-β expression is associated with fibrosis and atrophy of renal function in hypoxia-reperfusion injury (Basile et al. 1998) , whereas it contributes to neuroprotective factor in cerebral ischemia (Dhandapani and Brann 2003). IL-1 is a polypeptide proinflammatory cytokine that is a potent inducer of acute phase responses and inflammation, and a signaling molecule between the immune system and other body systems such as the hypothalamus-pituitary-adrenal (HPA)-axis in mammals (Engelsma et al. 2001; Rosenwasser 1998). IL-1-like factor is found in carp (*Cyprinus carpio*) and its bioactivity, along with induction of IL-1β transcript by stimulation with LPS or challenge with bacteria, is similar to that in mammals (Christopher J. Secombes et al. 1999).

To address the mechanisms of this putative immunosuppression, a laboratory fish model was developed which has enabled us to investigate the effects of acute hypoxia on responses of fish which may affect the susceptibility of the host to infectious diseases. Tilapia are a well known freshwater teleost fish and have been used in numerous laboratory

studies due to their hardiness in captivity and high resistance to varying environmental stressors (M.C.J.Verdegem et al. 1997). A series of bioassays, grouped in three tiers of increasing specificity for immune function, were performed on hypoxia stressed Tilapia (*Oreochromis niloticus*): Tier I, histopathology; Tier II, hematology, plasma chemistry, and serum cortisol; and Tier III, phagocytic index and expression of the cytokines TGF-β and IL-1β.

Methods

Fish

Tilapia (*Oreochromis niloticus* L.), weighing 235±30 g were transported from NCSU aquaculture facility (LakeWheeler, NC) to NCSU College of Veterinary Medicine. Fish were kept at 756L freshwater tank for 1 week, exposed to 16h light: 8h dark photoperiod and constantly aerated. The fish were fed ad libitum commercial foods (Ziegler Brothers, Gardners, PA) and feeding was suspended 24h before the experiment and restarted feeding after restoring the normoxic level and 50 % of the tank water was replaced on alternate days. Water conditions were 25 ± 0.5°C, pH 7.0 ± 0.5, ammonia 0.25 mg/L, nitrate less than 20 mg/L and nitrite less than 2 mg/L.

In the preliminary study lethal concentration (LCs) of DO were estimated between 3 % and 80% and LC_{30} was measured at the 3% DO level. However, 7% oxygen level without the mortality was chosen for acute hypoxia experiment.

Fish were exposed to 7% dissolved oxygen level (0.578 mg/L) for 96h followed by 84 h of reperfusion of normoxic condition that was maintained by N_2 gas bubbling into the tank. Oxygen electrodes were placed into the treatment tank and maintain oxygen level through opening or closing N_2 gas valve. The oxygen tension in tank was controlled uniformly by submersible pump and surface water of treatment tank was covered with vinyl sheet to prevent fish from gasping the air from surface during the exposure. Control groups were kept at the normoxic condition. Time intervals were 48h and 96h of hypoxia and 12h and 84h of reperfusion. Five fish from each group were collected at each time point and anaesthetized with 3-aminobenzoate ethyl ester methanesulphonate (MS-222, Sigma, St. Louise, MO) at a final concentration of 150 parts per million (ppm). Blood samples were

collected from caudal vein to heparinized syringe (22G) to drain a blood, thus depleting the spleen of interfering erythrocytes during the in vitro culture and stored at room temp. until use. Spleen was removed aseptically and placed in complete RPMI supplemented with 10% heat – inactivated fetal bovine serum, 100 U/ml penicillin, 100µg/ml streptomycin, and 2mM EDTA. The blood was diluted 1:1 in 1% Phosphate buffered solution (PBS) at the room Temp. and centrifuged at 45 x g for 5 min. The buffy coat layer was harvested, resuspended in 1% PBS and placed on 55 % Percoll gradient (specific gravity 1.0697 g/mL) at 800 x g for 30 min. at 16 °C. Peripheral blood leukocytes (PBL) band was harvested, washed 3 times in complete RPMI 1640, counted and adjusted to a cell density of 1 x 10^6/ml. The isolation of splenic leukocytes was prepared as described in splenic leukocytes from hybrid striped bass by C. A. Harms (2000). Spleen was minced finely, forced through a wire mesh (50 meshes) and resuspended in complete RPMI. The cell suspension was placed on two-step percoll gradients (specific gravity 1.052 and 1.065 g/mL) at 400 x g for 5 min. and then 800 x g for 25 min. and splenic leukocytes in interface were harvested, counted and adjusted to a cell density of 1 x 10^7/mL. Cell viability was performed in 0.2 % trypan blue.

Tier I: Histopathology

Samples of gill, intestine, heart, liver and head kidney were routinely processed, embedded in paraffin, sectioned at 5 microns and examined by light microscopy. The following six-points grading was used for evaluation. Grade 0 as no remarkable microscopic abnormalities, Grade 1 as very mild changes with minimal inflammatory cells, Grade 2 as mild changes, Grade 3 as moderate changes, Grade 4 as moderately severe changes and Grade 5 as severe changes (Hurty et al. 2002b).

Tier II:

1. Plasma Chemistry

The blood samples described above were analyzed with Hitachi 717 chemical analyzer (Roche Diagnostics, Indianapolis, IN) by Antech laboratory at Antech Diagnostics (Cary, NC). Tested items were glucose, urea nitrogen, creatinine, cholesterol, triglyceride, lipase total protein, alkaline phosphatase, alanine aminotransperase (ALT), aspartate aminotransperase (AST), calcium (Ca), phosphorus (P), chloride (Cl), magnesium (Mg). 100 µl of whole blood was also analyzed with iSTAT system equipped with cartridge (EG+7) (Sensor Device INC, Waukesha, WI) according to manufacturer's instructions. These include sodium (Na), potassium (K), ionized Ca (iCa), hematocrit (Hct), pH, partial pressure of carbon dioxide (PCO_2), partial pressure of oxygen (PO_2), bicarbonate (HCO_3), oxygen saturation (SO_2) and hemoglobin (Hb).

2. Differential Cell Count

The blood smears from each fish were stained using Wright's stain according to manufacturer's instructions (Fisher, Middle Town, VA 22645) and examined by light microscopy (OPELCO, Virginia, USA) under oil immersion. One hundred leukocytes were counted and categorized into lymphocytes, monocytes/macrophages and neutrophils.

3. Cortisol Measurement

Whole blood was centrifuged at 10,000 x g for 10 min. and plasma was harvested then kept at –80 °C until the analysis. Plasma cortisol was analyzed by the Clinical Pharmacology Laboratory at the North Carolina State University College of Veterinary Medicine (NCSU-

CVM) measured with the IMMULITE chemiluminescent immunoassay (Diagnostic Products Co., Los Angeles, CA, USA).

Tier III:

1. Phagocytosis Measurement

The in vitro phagocytic assay was a modification of that described for phagocytic cells in blood from rainbow trout (A. Thuvander, L. Norrgren and C. Fossum, 1987). Peripheral blood leukocytes (PBL) of Tilapia were isolated as described above. The peripheral blood leukocytes (PBL) suspensions ($1x10^6$/ml) were cultured per well in 6-well flat bottom tissue cultures with fluoresceinated (FITC) latex beads (diameter = 1.75μm) (Polysciences, Northampton, U.K.) in 5% CO_2/95% air in a humidified incubator for 5 hr at 26°C. To opsonize the beads the latex beads were incubated with newborn calf serum for 30 min. at 37 °C and washed with cold PBS two times and stored at 4 °C until the use. Before the use opsonized beads were washed twice in phosphate buffered saline (PBS) and sonicated for 15min. to resuspend aggregated beads. The cell to bead ratio was approximately 1:30. At the end of incubation 1 ml of PBS were added in order to stop phagocytosis and centrifuged at 400 x g for 10 min. Pellets were resuspended in 100 μ of 1 % of PBS. Then 50 μl of 1 % triton X and 50 μl of Propidium Iodide (0.5 mg/ml) (Roche, Indianapolis) were added and analyzed by fluorescence microscope. At least 100 cells were counted and the percentages of phagocytic cells engulfing latex beads as phagocytic capability (PC) were estimated. Phagocytic index (PI) was defined as the average number of ingested latex beads per a phagocytic cell. Therefore, the total number of ingested beads was divided by the total number of phagocytic cells.

2. Expression of Cytokines: TGF- β and IL-1 β

Isolation of Total RNA and Reverse Transcription

The assay of total RNA isolation was a modification of procedure described by C.A.Harms (Harms 2000). One mL of Tri Reagent (Sigma, MO, USA) for each 5-10 x 10^6 cells was added to splenic leukocyte pellet and total RNA was isolated according to manufacturer's instructions. RNA pellet was resuspended in sterile DNase RNase free water (Sigma, MO, USA) at a concentration of 5 x 10^4 cell equivalents/µl. 300-pmol oligo dT_{15} (Promega) for reverse transcription was added to Total RNA (3 x 10^6 cell equivalents) in a 72 µl volume and heated 94 °C for 5 min. and chilled on ice immediately. Then 78 µl of additional RT mixture was mixed in a final volume of 150 µl: 1 x buffer (50mM Tris-HCI, pH 8.3; 75mM KCl, 3mM $MgCl_2$), 0.4 mM each dNTP, 10 mM DTT, 0.8U/µl RNAsin and 2 U/µl reverse transcriptase (Superscript II RT, Gibco-BRL, Gaithersburg, MD, U.S.A.). Sample cDNA was transcribed at 45 °C for 60 min. and chilled on ice and diluted in 1:2 in sterile DNase RNase free water (Sigma, MO, USA) and then stored at –20 °C until the analysis.

TGF- β Primers of Tilapia

Initial primer (Con A1 and Con B2) against hybrid striped bass TGF-β was used for initial PCR (Harms 2000). The cycling protocol was one cycle of 94 °C 1min, 31 cycles of 94 °C 30 s denature, 57 °C 1.5 min annealing and 72 °C 2 min extend and one cycle of 72 °C 7 min final extension. PCR reaction was conducted using the thermal controller (MJ Research, INC., Miami, Florida). PCR product was visualized on 1.5 % agarose gel containing ethidium bromide (0.5 mg/ml) and purified using gel-extraction kit (Qiagen, Valencia, CA) according to manufacturer's method. DNA amplified by PCR was submitted to NC-CH

Automated DNA Sequencing Facility (Chapel Hill, NC) and automated sequenced using an ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences generated were analyzed for similarity with other known sequences using BLAST and DNASTAR software. Further primers for real time PCR (TIF and TIR) was made based on sequence generated above.

Primers for beta-actin were generated as housekeeping gene in the quantitative real time PCR assay. Beta-actin primers (BacF1 and BacR1) are based on alignment of zebra fish (GenBank, AA566386), Red Sea bream (GenBank, AB036756), Rainbow trout (GenBank, AF254414), Grass Carp (GenBank, M25013), Common Carp (GenBank, M24113), Gold fish (GenBank, AB039726) and Fugo (GenBank, U38849) to amplify a 139 bp fragment verified by PCR product size.

The sequences of PCR primer pairs are shown in Table 1.

3. IL-1β Cloning and Sequencing

Initial primers (MUF4 and MUR3) were designed against conserved region from known teleost fish IL-1β sequences in the GenBank Databases. Initial primers are based on alignment of Sea bream (GenBank AJ277166), flounder (GenBank, AB070835) and Sea bass (GenBank AJ269472). These primers were used for PCR and sequenced as above. PCR was performed with cDNA from a range of tissues (spleen, gill and liver) of flounder, tilapia and rainbow trout whose mononuclear cells were isolated followed by in vitro administration with 10 µl of LPS (1 mg/mL) (Sigma, St. Louise, MO) for 4h at 26°C. In vitro stimulation was required to study IL-1b expression since no expression showed in unstimulated fish (Jun Zou, 1999). Further primers (ABF1 and ABR1) were designed against initial primer-generated sequences to allow amplification of 115bp fragment for Real Time PCR. Obtained

product (115 bp) was cloned using TA cloning kit (Invitrogen, Carlsbad, CA) followed by manufacturer's method and an accurate colony was confirmed by PCR. Plasmid DNA was quantified by VersaFluorometer (Bio-rad, Hercules, CA) using Fluorescent DNA quantitation kit (Bio-rad, Hercules, CA) according to manufacturer's method. For each reaction of real time PCR using four 10 fold serial dilutions of plasmid DNA made standard curves for both targets and housekeeping gene.

4. Real Time Quantitative PCR Analysis

Real time quantitative PCR analysis of TGF-β and IL-1β was performed using iCycler and software (Bio-rad, Hercules, CA). PCR reaction mixtures were as described: 1x PCR buffer (50m m Tris-HCl, pH8.3, 10 mM KCl, 5 mM $[NH_4\}SO_4$ and 2 mM $MgCl_2$) (Roche, Mannheim, Germany), 0.2 mM PCR dNTP Mix (Roche, Mannheim, Germany), 5U/ul FastStart Taq DNA Polymerase (Roche, Mannheim, Germany), 1x SYBR Green (Sigma, MO, USA), 150 nM each primers and 5 µl cDNA in a final volume of 50 µl. The cycling protocol was one cycle of 95 °C 2min, 31 cycles of 95 °C 30 s denature, 57 °C 30 s annealing and 72 °C 2 min extend and one cycle of 72 °C 7 min final extension. Relative quantitation of the cytokine signal was performed by normalized to β-Actin signal.

Statistical Methods

The results of the hypoxia effect on teleost immunity were analyzed by the SAS analysis of variance (ANOVA) procedure (SAS. 2003). Multiple Comparison of mean response was performed by Least Significant Difference (LSD) and Tukey test with the MEANS/GLM procedures of SAS. $P<0.05$ was accepted as a statistically significant level of difference.

Results

Histopathology

Complete postmortem examination of each fish in this study, including necropsy and histopathology, revealed no remarkable lesions attributable to hypoxia exposure. Nor were there any remarkable lesions suggesting prior disease conditions or confounding lesions that might mask changes in the spleen, hematopoietic tissue, or other tissues related to the immune system.

Several relatively common, predominantly incidental lesions were found in the head kidney, gills, and liver of both control and treated fish. The head kidney of nearly all fish contained small numbers of pigmented macrophage aggregates, while two treated fish had a few small granulomas. The gills were generally within normal limits; however, two treated fish had scattered, mixed protozoal parasites along with mild thickening of the secondary lamellae. These areas were not associated with any remarkable inflammation in the gills. The livers of four treated and one control fish had very mild to moderate perivascular inflammation and one fish had several small granulomas.

Hematology

The percentages of lymphocytes in peripheral blood decreased during 46h and 96h hypoxia and 12h reperfusion compared to hypoxia ($p=0.0001$) whereas it returned to the initial control value at 84h reperfusion (Table 2). The percentages of neutrophils increased during 46h and 96h hypoxia and 12h reperfusion, whereas its value returned to the level of control at 84h

reperfusion. However, monocyte/macrophage was not significantly changed during hypoxia and reperfusion (Table 2). The value of Hct and Hb did not change during hypoxia and reperfusion.

Plasma Chemistry, Blood Gases and Cortisol

There were no significant differences in plasma chemistry, blood gases and cortisol between control and exposed fish during hypoxia and reperfusion (Appendix II) except for PO_2, SO_2, total protein and lipase (Table 3). PO_2 and SO_2 values significantly decreased compared to control during hypoxia and reperfusion ($p<0.001$). Total protein in plasma was significantly lower at 96h hypoxia and 12h reperfusion compared to control and its value returned to control level at 84h reperfusion ($p<0.001$). At 48h hypoxia total protein was observed to be the lowest during hypoxia and reperfusion ($p<0.05$). Plasma lipase decreased significantly compared to control during hypoxia but not reperfusion ($p<0.001$). However, its value was not significantly different between hypoxia and reperfusion exposure.

Phagocytosis

Phagocytic capability is significantly different during hypoxia and reperfusion compared to control except 86h reperfusion ($p<0.0001$) (Fig.1). Its value continuously decreased at 46h and 96h and 12 h hypoxia but returned to initial control value at 84h reperfusion ($p<0.004$). The percentages of cells with varying ingested beads were modulated during hypoxia and reperfusion (Table 4). The cells with only one bead and with over three beads were significantly different from control except 86h reperfusion ($p<0.0001$) and its value continuously decreased at 48h and 96h hypoxia and 12h reperfusion and returned to control

level at 86h reperfusion ($p<0.007$,and $p<0.05$). The cells with only two beads were significantly different from control at 96h hypoxia and 12h reperfusion ($p<0.0001$). Its value decreased most significantly at 96h hypoxia and returned to control value at 84h reperfusion ($p<0.04$).

Phagocytic index (PI) was significantly different from control value except at 86h reperfusion ($p<0.0001$) (Fig.2). Its level continuously decreased during hypoxia and reperfusion, whereas it recovered its control level at 84h reperfusion ($p<0.02$).

Cytokine Expression

TGF-β mRNA expression of splenic mononuclear cells was significantly different during hypoxia and reperfusion compared to control ($p<0.0001$). Its level constantly increased during 48h and 96h hypoxia and 12h reperfusion, whereas it returned to control level at 84h reperfusion ($p<0.03$). Splenic mononuclear cell IL-1β transcription was significantly different from control at 96h hypoxia and 12h reperfusion ($p=0.006$). Its level significantly decreased the most at 12h reperfusion, whereas it recovered to its control level at 84h reperfusion ($p<0.002$) (Fig. 4).

Each cytokine expression was correlated with phagocytosis in this study (Fig.5). When its regression was conducted between phagocytosis and TGF-β it was negatively correlated ($y= -0.085x+1.02$, $r^2=0.39$, $p=0.0017$). However, IL-1β and phagocytosis as expected were positively correlated ($y= 4.08x+1.32$, $r^2= 0.989$, $p=0.005$).

Discussion

Taken together, the results of this study are consistent with the hypothesis that environmental hypoxia triggers an overall downregulation of the immune response in fish. To evaluate immune function in the test fish, we used a series of assays of increasing specificity for the immune system which allowed us to "cast a broader net" in order to detect changes in overall health, while also taking a narrow focus on specific immune function parameters. The broadest tier for evaluation was histopathology. While it did not detect any remarkable lesions that could be attributed to acute changes in DO, histopathology showed that the test fish had no predisposing adverse health conditions and that lymphoid tissues were morphologically normal. While a relatively blunt instrument for evaluation of immune function, histopathology can provide a wealth of information at the tissue/cellular levels for comparatively little added cost. Especially in situations where the fish species being studied is not laboratory reared, systemic evaluation of major organ systems of both treated fish and controls is important.

The second tier in this study comprised hematology, plasma chemistry, blood gases and cortisol. Relative lymphopenia and neutrophilia were observed in fish subjected to hypoxia and reperfusion ($p<0.0001$), whereas no significant change of monocyte percentage was found (Table 2). At 84h reperfusion those values returned to the initial control level. These results show affected tilapia were recovered from hypoxia in terms of normal oxygen level and its duration. These results are consistent with other studies that stress, regardless of nature of stress, and cortisol directly or indirectly allow the characteristic changes in hematologic profiles like leukopenia, lymphopenia and neutrophilia (Ainsworth et al. 1991;

Ellsaesser and L.W.Clem 1986; Pickering and Pottinger 1985). Also Jordan et. al. found that neutrophils are activated by cytokines and pro-inflammatory mediators induced by ischemia-reperfusion (Jordan et al. 1999). Therefore it is speculated that this change in hematology seems likely to be stereotypical responses to stressors rather than unique effect of hypoxia or reperfusion.

The result of no significant change in hematocrit in this study is consistent with Ellsaesser's report that the hematocrits and actual erythrocyte numbers did not change under stress such as transportation and handling (Ellsaesser and L.W.Clem 1986). However, in contrast sudden increase in hematocrit value of rainbow trout was found during short term hypoxia resulting in elevated urine flow rate and swelling erythrocytes (Thomas and Hughes 1982). With these results it is suspected that hypoxia and reperfusion alter the characteristics of fish hematology, whereas it is not clear that it is through direct cytolytic change or indirect redistribution into other lymphoid tissues.

Plasma cortisol and glucose are considered as primary and secondary responses to stress and used to measure severity and duration of stress (Henrique et al. 1998; Mazeaud et al. 1977). In this study no significant change was found in plasma cortisol and glucose concentration during hypoxia and reperfusion. It is consistent with acute exposure of hypoxia resulting in no changes of plasma glucose and cortisol concentrations in seabream, *Sparus aurata* (Henrique et al. 1998). This is probably due to the interval of blood collection since cortisol level fluctuates diurnally and is maintained by feedback system (Henrique et al. 1998). In contrast to these results there is evidence that hypoxic stress causes increased plasma cortisol and glucose (Evans et al. 2004; Ishibashi et al. 2002; Leach and Taylor 1980; Palti et al.

1999). Acute hypoxia (1mg/L for 24h) induced increased glucose in Nile tilapia (*Oreochromis niloticus*) (Evans et al. 2004) and short term reperfusion caused increased glucose in two tilapia species (*Oreochromis aureus* and *O. mossambicus*) (Palti et al. 1999). In response to 6h hypoxia (3mmHg) Nile tilapia shows increase in glucose, cortisol and hematocrit levels, whereas hemoglobin value was not significantly changed (Ishibashi et al. 2002).

When exposed to hypoxia and reperfusion the values of PO_2 and SO_2 decreased compared to control (Table 3). It is reported that partial pressure of oxygen (PO_2) and oxygen saturation (SO_2) were decreasing in response to hypoxia in tilapia (Fernandes and Rantin 1994) and in rainbow trout (Fernandes and Rantin 1994; Thomas and Hughes 1982).
The pH value was not significantly different from control level during hypoxia and reperfusion, along with no significant difference of PCO_2 and bicarbonate value (data not shown). This result is converse to a study in which at the early onset of hypoxia respiratory alkalosis occurred in rainbow trout due to decrease in partial pressure of carbon dioxide (PCO_2), followed by continuous decrease in bicarbonate (HCO_3^-) and carbonate (CO_3^{-2}) concentration and increased lactate concentration (Lykkeboe and Weber 1978; Thomas and Hughes 1982).
Kakizawa et al demonstrate that at the beginning of acute hypoxia (9.3 kPa) there were no changes of blood pH and bicarbonates of rainbow trout, whereas severe hypoxia (6.1 kPa) caused decreased pH and bicarbonate (Kakizawa et al. 1997).

Low total protein concentration in response to hypoxia and reperfusion was observed compared to control values except at 86h reperfusion (Table 3). Its value decreased most at 48h hypoxia, and returned to the control value at 86h reperfusion. This is contrast to the result of the significant increase in total plasma protein of striped bass exposed to hypoxia (Lebelo et al. 2001). Higher levels of serum protein, globulin and IgM are considered to be related with effective innate immune responses in fish (Wiegertjes et al. 1996), whereas low level of plasma protein is associated with diseased fish (Medemeyer et al. 1984).
These results may occur through decrease in protein synthesis or increased protein catabolism in order to reduce energy utilization induced by low oxygen stress (hypoxia or anoxia) (Lutz and Nilsson 1997; Mazeaud et al. 1977).

Impaired lipid metabolism, including low cholesterol, has been correlated with reduced host resistance to pathogen invasion (Maita et al. 1998). In this study plasma lipase in tilapia continued to decrease in response to hypoxia compared to control, without changing during reperfusion, whereas there were no changes of cholesterol and triglyceride concentrations. This is consistent with a study that acute hypoxia induced no change of plasma total cholesterol of tilapia (*Oreochromis aureus* and *O. mossambicus*) (Palti et al. 1999). In contrast, hypoxia induced significant increases in plasma cholesterol, triglycerides and nonesterified fatty acids in a murine model (Raff et al. 2000).

Plasma electrolytes (Na^+, Cl^-, Ca, iCa, K, P and Mg) were not found to differ significantly in response to hypoxia and reperfusion. These findings are consistent with a study in which acute hypoxia (9.3KPa) did not affect plasma levels of Na^+, Cl^-, iCa, and K, whereas a

significant decrease in plasma K was observed in response to severe hypoxia (6.1kPa) in rainbow trout (Kakizawa et al. 1997).

Plasma chemistry values seem to be depends on severity and duration of hypoxia and reperfusion.

Phagocytosis is usually considered to be a primary defense against invasive pathogens, associated with increased or decreased activity after non-specific or specific stimulation (Michel and Hollebecq 1999). The common types of phagocytic cells in fish, as in mammals, are the granulocytes and mononuclear phagocytes (Secombes and Fletcher 1992). In response to acute hypoxia and reperfusion tilapia showed a significantly lower phagocytic capability compared to control values ($p<0.0001$). This suggests that acute hypoxia and reperfusion influenced phagocytosis adversely, whereas normoxia allowed recovery to original values at 84h reperfusion (Figs.1, 2). The percentage of phagocytic cells according to ingested bead numbers also decreased during hypoxia and reperfusion periods (Table 4). These results indicate that hypoxia and reperfusion impaired phagocytosis and suggest that affected fish may be more susceptible to opportunistic infections.

This is consistent with altered phagocytic activity of macrophage in Oyster Toadfish collected from PAH-polluted Elizabeth River, Virginia in terms of PAH gradient (Seeley and Weeks-Perkins 1991)

Based on no significant change in percentage of circulating monocytes, it is speculated that acute hypoxia detrimentally influenced their function, leading to alteration of host resistance to invasive or opportunistic pathogens. However neutrophilia in affected fish was not

consistent with decreased phagocytosis. It is speculated that hypoxia likely influences neutrophil bioactivity through other anti-inflammatory responses such as modulation of cytokine expression, whereas neutrophil count was increased in circulation.

Immunological responses are dependent on the actions of various agents, their interactions with each other or with immuno competent cells as well as with other endogenous factors such as cytokines (Harris and Bird 2000). In this study it is demonstrated that cytokine transcription is altered in tilapia under conditions of hypoxia and reperfusion.

During hypoxia and reperfusion TGF-β mRNA expression of splenic mononuclear cells was significantly increased, whereas its level returned to baseline under normoxic conditions (84h). The most elevated TGF-β transcription was found at 12 h reperfusion. Either anti- or pro- inflammatory ability of TGF-β depends on its concentration, differentiated target cells and interaction with other inflammatory mediators (McCartney-Francis and Wahl 1994) When compared with IL-1β there was no significant correlation between them (data not shown). However TGF-β expression was negatively correlated with phagocytosis by peripheral blood leukocytes (PBL). Based on known TGF-β immunosuppressive effects on macrophage activation this negative correlation is expected. This is also consistent with the reverse relationship between anterior kidney mononuclear cell TGF-β mRNA and bactericidal activity in triamcinolone as immunomodulator-treated hybrid striped bass (Harms 2000). In mammals, upregulation of TGF-β expression is observed generally in the kidney, which is associated with fibrosis and inhibition of renal function (Basile et al. 1998), whereas it also serves as neuroprotector during cerebral ischemia via inhibited generation of

superoxide anions and other inflammatory cytokines under hypoxia and reperfusion (Lefer et al. 1993). In response to low oxygen level TGF-β_3 inhibited human trophoblast differentiation through hypoxia-inducible factor-1 (Caniggia et al. 2000).
Reduced TGF-β transcription in *Mycobacterium*-infected striped bass was associated with excessive granulomatous inflammation in internal organs (Harms et al. 2003).
However the relationship between hypoxia-reperfusion injury and TGF-β is not well established in fish.

Downregulation of splenic mononuclear cells IL-1β transcription was observed during hypoxia and reperfusion. Especially during early reperfusion (12h) IL-1β mRNA expression was decreased in contrast with the Vannay et al study that reported IL-1β mRNA expression in rat kidney was unaffected during the post-ischemia (Vannay et al. 2004). However this downregulated IL-1β expression was not consistent with the neutrophilia observed under hypoxia and reperfusion (Table 2) based on the its ability to induce accumulation of inflammatory cells (Rosenwasser 1998). This also did not agree with the rapid transitory increase in IL-1β detected in *Bacillus anthracis* lethal toxin-induced hypoxia in the mouse model, which results in increase in mortality of infected mice (Moayeri et al. 2003). But it was consistent with reduced phagocytic activity of peripheral blood leukocyte in exposed tilapia, which was expected based on knowledge of IL-1β proinflammatory activity in mammals (Dinarello 1991). Youssef et al reported that IL-1β enhances leukocyte phagocytosis and bacteriocidal activity resulting in prevention of disseminating orofacial infection and sepsis (Youssef and P.Stashenko 2004). In vitro Microcystins cyanobacterium toxin in freshwater, a known immunomodulator, inhibited IL-1β mRNA expression dose-

dependently in a mouse model (Chen et al. 2004). There is some evidence that may correlate IL-1β expression with low oxygen/hypoxia (Brenchley 2000; Testa et al. 1999; Vannay et al. 2004). Testa et al demonstrated ischemia induced IL-1β expression in hypoperfused skeletal muscle of patient with peripheral arterial disease (Testa et al. 1999). Brenchley et al reported that hypoxia-induced vascular endothelial growth factor (VEGF) may regulate IL-1β and TGF-β gene expression (Brenchley 2000). Additionally the positive correlation between IL-1β and phagocytosis makes it possible to predict that IL-1β transcription is attributable to amplitude of phagocytosis based on its ability to augment toll-like-receptor 2 (TLR2) production to play a role in bacterial phagocytosis (Sakai et al. 2004).

In this study, tiered approaches including classic cellular immunoassays and cytokine production improved our understanding of the health status and mechanisms of hypoxic changes in affected animals. The altered cytokine expression and inhibited phagocytosis accompanied by changes in hematology and plasma chemistry suggest that hypoxia and reperfusion induce considerable physiological stress in fish. This may imbalance Th1/Th2 homeostasis leading to inhibition of Th1 responses and reduced resistance to opportunistic or invasive pathogens. In further studies, the combined effects of hypoxia and other environmental stressors should be investigated using these tiered assays in order to better understand the health status of aquatic habitats impacted by multiple stressors.

Acknowledgements

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Figures

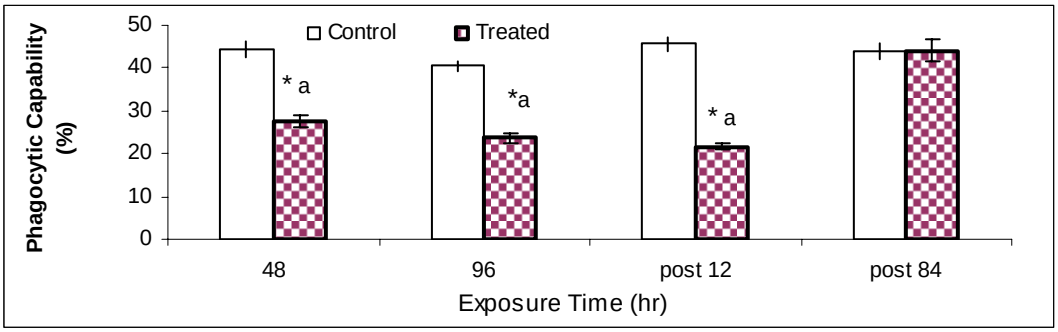

Fig. 1. The effects of hypoxia followed by reperfusion on phagocytic capability in peripheral blood leukocytes from control and hypoxia-exposed tilapia. * represents significant difference in means between control and exposed values ($p<0.0001$).The lowercase a denotes significant difference in phagocytic capability levels compared to 84h reperfusion ($p<0.004$). Error bars represent standard error (SE).

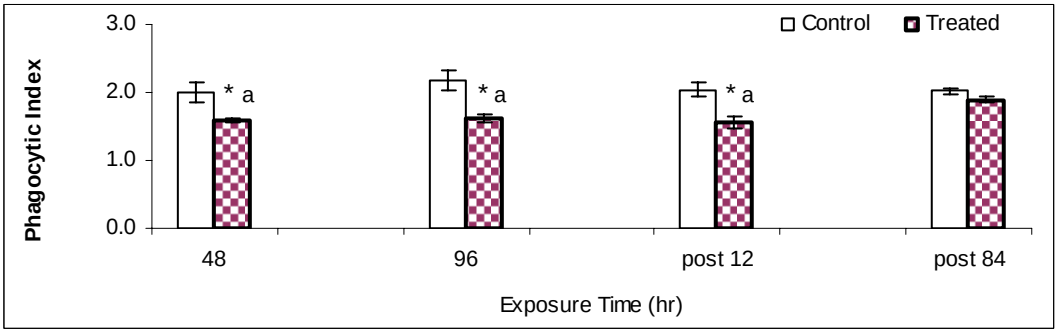

Fig. 2. Effects of hypoxia followed by reperfusion on phagocytic index in peripheral blood leukocytes (PBL) from control and hypoxia-exposed tilapia. * represents significant difference in means between control and exposed values ($p<0.0001$).The lowercase a denotes significant difference in phagocytic index compared to its value at 84h reperfusion ($p<0.01$). Error bars represent standard error (SE).

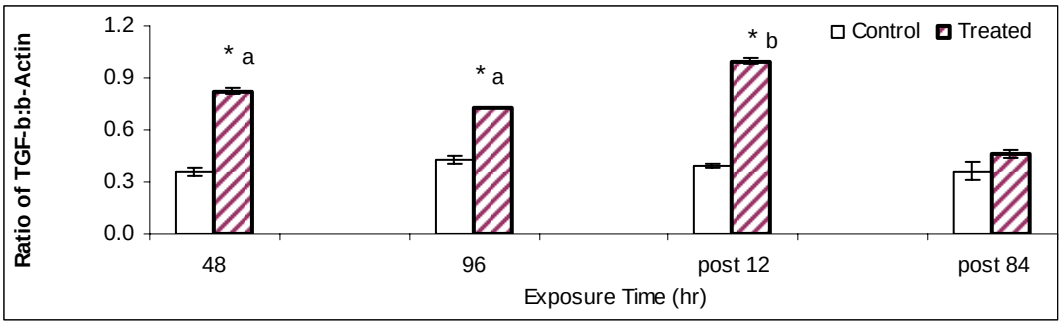

Fig 3. TGF-β mRNA expression of tilapia (n=5) splenic mononuclear cells subjected to 96h hypoxia followed by 84h reperfusion. . * represents significant difference in means between control and exposed values ($p<0.0001$). The lowercase a and b denote significant difference from 84h reperfusion and also significantly different between at 96h hypoxia and 12h reperfusion ($p<0.03$). Error bars represent standard error (SE).

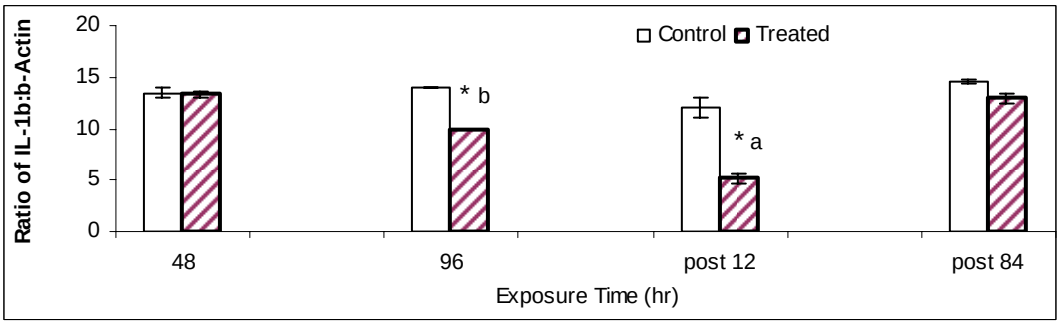

Fig. 4. IL-1β mRNA expression of tilapia (n=5) splenic mononuclear cells subjected to 96h hypoxia followed by 84h reperfusion. * represents significant difference in means between control and exposed values ($p=0.006$). The lowercase a and b denote significant difference in IL-1β transcription from 86h reperfusion and also significantly different between at 48h hypoxia and at 12h reperfusion ($p<0.002$). Error bars represent standard error (SE).

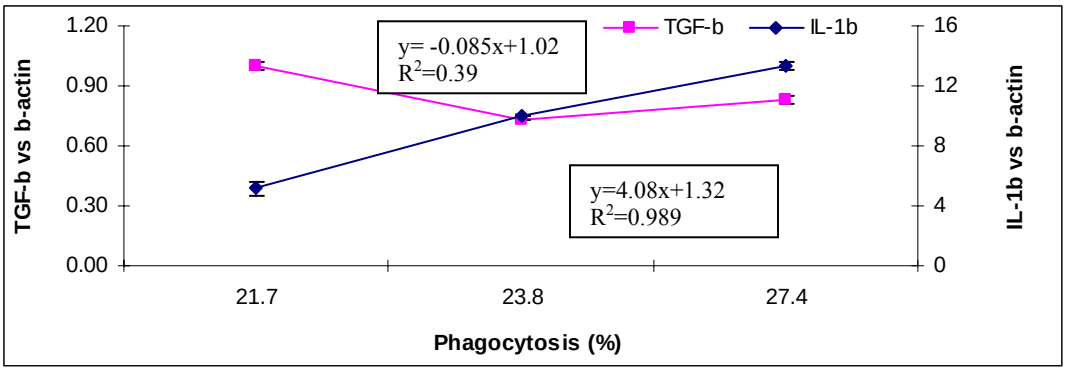

Fig. 5. Correlation between phagocytosis, IL-1β mRNA and TGF-β mRNA expression of tilapia subject to 96h hypoxia followed by 84h reperfusion. The plot of phagocytosis with IL-1β transcription shows positive correlation ($y=4.08x+1.32$ $R^2=0.989$, $p=0.005$) and with TGF-1β shows negative correlation ($y= -0.085x+1.02$ $R^2=0.39$, $p=0.0017$). Error bars represent standard error (SE).

Tables

Table 1. Sequences of primers used in this study.

Target	Primer	Sequences (5'-3')	PCR product size
TGF-β	Con A1	GACCTGGGATGGAAGTGGAT (20 mer)	300 bp
	Con B2	CAGCTGCTCCACCTTGTGTTG (21 mer)	
	TILF1	TATATCTGGGATGCCGAAAACA (22 mer)	114 bp
	TILR1	TGGCAGTGGCTCTAGTGTCTGT (22 mer)	
β-actin	BACF1	TGGCATCACACCTTCTATAACGA (23 mer)	139bp
	BACR1	TGGCAGGAGTGTTGAAGGTCT (21mer)	
IL-1β	MUF4	GAGTGTGGTCAACCTCATCAT (21 mer)	324 bp
	MUR3	GCTGTGCTGATGTACCAGTTG (21mer)	
	ABF1	TGCACTGTCACTGACAGCCAA (21 mer)	113 BP
	ABR1	ATGTTCAGGTGCACTATGCGG (21 mer)	

Table 2. Differential blood cell counts of tilapia (n=5) subjected to 96h hypoxia followed by 84h of reperfusion. * means significant difference between control and treatment group ($p<0.0001$). The lowercase a and b denote significant difference in lymphocyte ($p<0.0001$) and neutrophils counts ($p<0.03$). Error bars represent standard error. Values are means±SE and sample units are percentage (%).

Cell Types	**Exposure Time**				**Post Exposure Time**			
	48 h		96 h		12 h		84 h	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Lymphocytes	93.5 ±2.1	81.7±2.6 [a] *	93.1±1.8	82.4±2.5 [a] *	90.3±1.4	80.4±3.3 [a] *	92.3±1.7	93.0±1.9 [b]
Neutrophils	3.5±3.2	12.2±4.1 [a] *	2.7±3.0	14.2±4.9 [a] *	5.8±3.8	15.2±7.9 [a] *	3.6±1.9	3.5±2.8 [b]

Table 3. Plasma concentration of partial pressure of oxygen (PO_2, mmHg), oxygen saturation (SO_2, %), total protein (g/dL) and lipase (U/L) of tilapia (n=5) subjected to 96h hypoxia followed by 84h reperfusion. * means significant difference between control and treatment group ($p<0.001$). The lowercase a and b denote significant difference in total protein ($p<0.05$). Means followed by the same letter are not significantly different. Values are means ±SE.

	Exposure Time				**Post Exposure Time**			
	48 h		96 h		12 h		84 h	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
PO_2	11±2.4	5±0*	12±5.2	5±0*	8.4±1.5	5.2±0.2*	9.17±1.9	5±0*
SO_2	12.4±4.2	0*	13±10.06	2.2±1.4*	6±2.5	1.33±1.0*	10.25±2.6	0*
Total Protein	2.78±0.4	2.48±0.28^{a}	3.28±0.1	2.74±0.1^{ab}*	3.48±0.3	2.86±0.2^{ab}*	3.39±0.2	3.4±0.1^{b}
Lipase	51.8±8.6	29.3±13.4*	56.8±1.9	35.8±8.7*	42.8±5.3	32.3±5.0	43.1±6.8	32.6±6.5

Table 4. Percentage of phagocytic cells in terms of engulfed bead numbers of peripheral blood leukocytes (PBL) of Tilapia (n=5) subjected to 96h hypoxia followed by 84h reperfusion. * means significant difference between control and treatment group ($p<0.0001$). The lowercase a denotes significant difference in values of PBL with one ($p<0.007$) and over three beads ($p<0.05$) compared to its value at 84h reperfusion and a and b means significant difference in PBL with two beads ($p<0.04$). Means followed by the same letter are not significantly different. Values are means ±SE.

Phagocytic cells	**Exposure Time**				**Post Exposure Time**			
	48 h		96 h		12 h		84 h	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
PBL w/ one bead	24.6±1.8	16.9±1.4^{a}*	20.3±1.7	15.4±2.9^{a}*	22±1.8	12.2±1.5^{a}*	24.4±2.6	24.7±1.9
PBL w/ two beads	10.2±1.2	7.3±1.2^{ab}	10.5±2.2	4.3±1.3^{a}*	15.5±2.4	7.1±1.2^{ab}*	12.2±1.7	9.4±1.5^{b}
PBL w/ over three beads	9.7±1.8	3.2±0.7^{a}*	9.7±2.5	4.1±0.6^{a}*	9.1±1.1	2.4±1.6^{a}*	7.22±1.2	9.8±1.7

OVERALL SUMMARY

The present study has determined:

1. The synthetic cortisol, Triamcinolone, altered hematological characteristics, phagocytosis and cytokine production of tilapia (*Oreochromis niloticus*) without change in histological characteristics.
    - Lymphopenia and neutrophilia with overall leukopenia and elevated macrophage levels were noted.
    - Red blood cell count and hematocrit was not altered with treatment.
    - Phagocytic capability was suppressed without significant changes in phagocytic index.
    - TGF-β mRNA production was increased whereas IL-1β transcription was suppressed in splenic mononuclear cells.
    - Internal organs including spleen, head kidney, gill, intestine and gonad were considered to be intact without preceding or ongoing degenerative conditions.
2. Acute changes in salinity modulated immune responses and hematological characteristics without change in plasma cortisol level or histopathological characteristics.
    - Increase in salinity exposure did not alter health parameters, as measured above.
    - Decrease in salinity induced:

a. Lymphopenia, neutrophilia and elevated macrophage levels in the circulation.
b. No changes in hematocrit, plasma protein, or plasma cortisol level.
c. Phagocytosis was elevated in response to varying salinity range from 20 ppt to 5 ppt although phagocytic index was not affected in response to those salinity changes.
d. TGF-β transcription in splenic mononuclear cells increased with decreased salinity, with minimal transcriptional change seen at 5 ppt.
e. Splenic mononuclear cell IL-1β transcription was not detected with decrease in salinity, whereas its value was not significant in response to increase in salinity change.
f. Internal organs including gill, liver, head kidney, intestine and gonad were considered integral with no prior impaired conditions.

3. Hypoxia-induced immunosuppression was determined by quantifying TGF-β and IL-1β mRNA and phagocytosis then comparing results with characteristic observations of hematology, histopathology and plasma chemistry.
    - The altered cytokine expression, hematology and plasma chemistry returned to control values in response to 84h reperfusion.
    - Circulatory lymphopenia and neutrophilia, without changes in macrophage levels, were noted.
    - No modulation of plasma chemistry with the exception of suppressed PO_2, SO_2 and total protein in response to hypoxia and reperfusion and suppressed lipase in response to hypoxia alone.

- Phagocytosis and phagocytic index were inhibited in response to hypoxia and reperfusion.
- TGF-β transcription in splenic mononuclear cells increased in response to hypoxia and reperfusion where maximum upregulation was seen at reperfusion.
- IL-1β transcription in splenic mononuclear cells was suppressed under conditions of hypoxia and reperfusion.
- Internal organs including head kidney, gill, intestine, heart and liver were considered normal.

OVERALL CONCLUSIONS

In this study, potential mediators of fish mortality and morbidity were investigated using the tilapia fish model. A tilapia-specific bioassay was developed to quantitate TGF-β and IL-β transcription using real time PCR. Measurement of TGF-β and IL-1β transcriptional activity was utilized as a biomarker of immune function in response to triamcinolone administration, acute salinity change, and hypoxia. Changes in the transcriptional levels of the immunosuppressive cytokine TGF-β and the immunostimulatory cytokine IL-1β, as well as changed in phagocytosis, were consistent with changes in hematological characteristics, values of plasma enzymes and metabolic characteristics. With triamcinolone administration, a known immunomodulator, the immunological measurements agreed with the expected changes. However, decrease in salinity induced stimulation of immune responses that were not completely consistent with other parameters. The data suggest that hypoxia may be a contributing factor to large disease outbreaks (such as ulcerative mycosis), whose mechanisms are associated with suppressed immune function of the host. Additionally, decrease in salinity appears to act as an immunostimulatory agent in tilapia. In other words, these responses are species-specific and depend on the nature and duration of exposure to deleterious agents. Using established measurements of histopathological characteristics, as well as newly developed species-specific methods, such as measurement of TGF-β and IL-1β transcription, we may better understand the health status of populations at risk in waters increasingly impacted by environmental degradation.

APPENDIX 1

Nucleotide Sequence of Tilapia (*Orechromis niloticus*) IL-1β mRNA

Table 1. Nucleotide sequence of tilapia (*Oreochromis niloticus*) IL-1β mRNA and primers. Initial primers were designed against conserved region from known teleost fish IL-1β sequences in the GenBank Databases. Initial primers are based on alignment of Sea bream (GenBank AJ277166), flounder (GenBank, AB070835) and Sea bass (GenBank AJ269472). These primers were used for PCR and sequenced. PCR was performed with cDNA from a range of tissues (spleen, gill and liver) of flounder, tilapia and rainbow trout whose mononuclear cells were isolated followed by in vitro administration with 10 µl of LPS (1 mg/mL) (Sigma, St. Louise, MO) for 4h at 26°C. In vitro stimulation was required to study IL-1b expression since no expression showed in unstimulated fish (Jun Zou, 1999).

Primers used	Sequences (324bp)
forward primer (MUF4): GAGTGTGGTCAACCTCATCAT (21 mer) reverse primer (MUR3): GCTGTGCTGATGTACCAGTTG (21mer)	GGAA TTCC AGGA TGAA AGAC CTGC TTTC CATC ATGC TGGA GAGC ATTG TGGA ATGA GCAC AGTA TAAT AGAG AGGA CAGC TGGA CCAC CTCA GTTC ACCA GCAG GGAT GAGA TTGA GTGC ACTG TCAC TGAC AGCC AAAAGAGG AGCT TGGT TCTT CTCC AAAG CAGC ATGG AGCT CCAT GCAG TGAT GCTG CAGG GAGG CAGT GAAG ACCG CATA GTGC ACCT GAAC ATGT CGAC CTAC GCGC ACCC TACA CCCA TCGC TGAG ACCA GGCC TGTC GCTC TGGG CATC AAAG GCAC AAAC CTCT ATCT GTCT TGTC ACAA GGAT

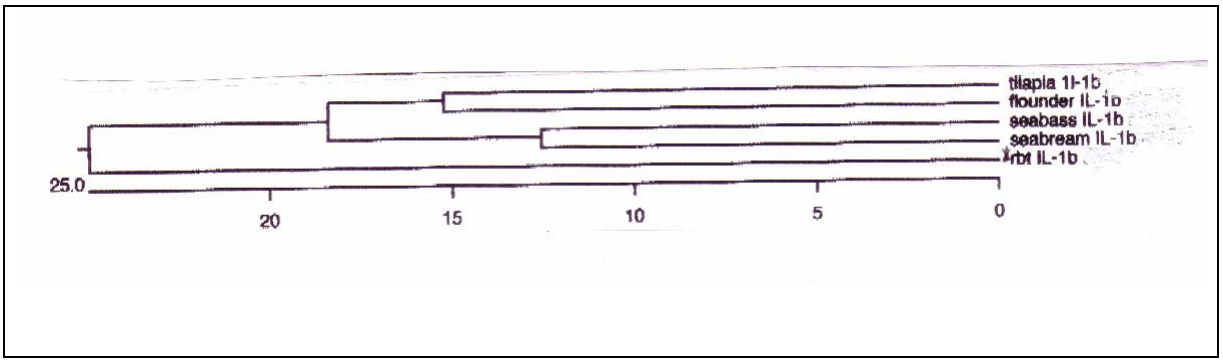

Fig. 1. Phylogenetic tree of IL-1β nucleotide in tilapia (*Oreochromis niloticus*), flounder (*Paralichthys olivaceus*), seabream (*Sparus aurata*), seabass(*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) using Clustal method in MEGALIGN (DNASTAR) with weighted residue weight table. *rbt represents rainbow trout.

APPENDIX 2

Plasma Chemistry Profile of Tilapia (*Oreochromis niloticus*) Subjected to Hypoxia and Reperfusion

Table 1. List of plasma biochemistry analyzed with Hitachi 717 chemical analyzer and iSTAT analyzer and plasma cortisol analyzed with IMMULITE chemiluminescent immunoassay. Tilapia (n=5) were exposed to 96h hypoxia followed by 84h reperfusion. * = data performed by iSTAT analyzer. Values are means ±SE.

	Exposure Time				**Post Exposure Time**			
	48 h		96 h		12 h		84 h	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Glucose (mg/dL)	35.9±7.5	41.7±4.5	34±2.4	39.9±3.5	38.3±3.9	23.4±2.1	40.6±8.6	34.9±3.6
Cortisol (10mg/mL)	2.5±0.8	2.4±0.4	3.0±0.9	1.2±0.1	3.0±0.9	5.4±2.1	3.8±2.5	5.3±1.5
Creatinine (mgldL)	0.6±0.1	0.3	0.2	0.5±0.1	0.2±0.1	0.2	0.4±0.1	0.6±0.1
Bilirubin (mgldL)	16.4±3.0	17.8±2.4	14.9±3.0	17.9±3.3	16.9±2.8	19.1±2.1	15.7±2.5	17.4±3.0
ALP (U/L)	23.7±3.6	18.4±2.1	22.3±2.4	23±2.9	25.1±1.4	23.7±2.0	26.3±2.9	20.5±3.2
AST (U/L)	34.7±10.9	28±8.1	23.5±5.8	24.3±6.1	36.9±10.8	40.1±24.4	48.5±9.1	41.3±7.3
Lipase (U/L)	45.6±9.6	29.3±13.3	45.9±8.7	35.8±8.7	42.8±5.3	32.3±5.0	37.6±7.9	32.6±6.5
Ca (mgldL)	9.2±1.2	8.8±0.8	10.8±0.3	10.6±0.7	10.8±0.4	9.6±0.5	10.7±0.4	12.6±1.6
iCa* (mmol/L)	1.8±0.02	1.7±0.02	1.8±0.03	1.8±0.02	1.8±0.03	1.8±0.04	1.8±0.02	1.8±0.1
Phosphorus (mg/dL)	12.9±2.4	9.9±1.2	13.8±1.7	15.1±1.0	13.5±1.4	14.2±2.1	17.7±1.4	17.5±0.8
Na (Meq/L)	201.8±27.1	163.4±15.5	183.4±2.8	188±2.2	185.8±2.2	182.4±2.9	180.8±2.4	162.9±28.9
Na* (mmol/L)	157.7±1.2	153.7±1.3	159.2±0.9	157.7±0.8	15.7±2.6	160±1.8	156.3±0.9	151.8±1.5
K (Meq/L)	3.8±0.1	4.0±0.2	3.9±0.2	3.9±0.1	3.7±0.2	3.8±0.1	3.5±0.1	3.65±0.1
K* (mmol/L)	3.9±0.9	3.1±0.4	3.0	3.4±0.4	2.8±0.3	3.1±0.1	3.1±0.1	3.0±0.1
Mg (Meq/L)	2.4±0.4	1.9±0.2	2.7±0.2	2.7±0.2	2.8±0.2	2.8±0.3	3.4±0.3	3.1±0.21
Cl (Meq/L)	129.2±4.1	118.8±12.6	140.5±1.9	139. 4±3.3	139.4±1.8	135.4±2.1	122.1±16.1	138±2.0
pH*	7.2	7.3	7.2	7.3±0.1	7.3	7.3±0.1	7.3	7.3
PCO_2* mmHg	16.2±0.9	18.7±3.6	18.1±0.8	15.8±1.5	16.5±0.7	13.9±0.9	16.2±0.7	14.97±0.9
HCO_3* Mmol/L	6.7±0.2	8.3±1	6.8±0.2	7±0.3	7.2±0.5	6.2±0.7	7.2±0.3	6.5±0.2

TCO_2* Mmol/L	7.3±0.2	8.8±1.1	7.2±0.2	7.7±0.2	7.6±0.4	6.7±0.8	7.7±0.4	7
Hct* %PCV	21.5±1.3	22.8±2.2	19.8±0.7	21.2±1.2	19.8±0.7	27±9.5	21.7±1.7	20±1.4
Hb* (g/dL)	7.3±0.6	8±0.8	6.6±0.2	7.2±0.5	6.6±0.2	9.2±3.3	7.2±0.7	6.8±0.5
Triglyceride (mg/dL)	48.4±9.3	43.7±6.6	40.9±4.3	41.3±11	65±8.8	44.3±6.1	65.5±7.1	81±6.3
CPK (U/L)	134±71.3	109.1±21.2	140.7±54.7	107.7±36.4	138.4±23.3	167.4±19	156±27.6	197±16.4
Cholesterol (mg/dL)	105.4±23.1	125.3±26.4	171.4±13.6	131.4±22.5	139.6±9.8	148.1±9.2	160.4±11.1	164.9±29.8

(PCO_2 = partial pressure carbon dioxcide, HCO_3 = bicarbonate, TCO_2 = total carbon dioxide, Hct = hematocrits, Hb = hemoglobin, AST = aspartate aminotransferase, ALT = alanine aminotransferase)