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Response of Striped Bass to Immunological Stimulation and Challenge

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Marine and Atmospheric Science

Stony Brook University

December 2009

Stony Brook University

The Graduate School

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Abstract of the Thesis

Response of Striped Bass (*Morone saxatilis*) to Immunological Stimulation and Challenge

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2009

Striped Bass (*Morone saxatilis*) is an economically and ecologically significant finfish species on the US Atlantic coast. Striped bass were studied here to characterize their responses to inflammatory stimuli and challenge with Mycobacteria infection. Inflammatory responses were rapidly stimulated with lipopolysaccharide (LPS) and Freud's complete adjuvant (FCA) within 6 hrs and maintained in most cases for over 48 hrs, similar to other teleosts and mammals. Striped bass inflammatory responses quickly increased following infection with *Mycobacterium marinum*, but they were down regulated in spite of bacterial counts remaining high. The quick up-regulation and then down-regulation of inflammatory cytokines was not much different than what was observed when just inoculating with LPS or FCA. The number of monocytes and neutrophils were initially increased at 1 day post infection. This confirms in striped bass the importance of these cell types in initial inflammation and bacterial infection in striped bass. Despite significant changes in leukocyte populations in the blood and inflammatory gene expression in the kidney, little changed in phagocytic index of splenic leukocytes. Finally, the effects of polyunsaturated fatty acid (PUFA) inclusion in fish diets were investigated on the innate immune system and associated host resistance to Mycobacteria infection. Fish fed high PUFA diets were characterized by higher expression of IL-1β and Nramp and enhanced granuloma formation, while fish fed low PUFA diets were characterized by higher expression of TNF-α with greater lesion formation. These observations suggest that a high PUFA diet is more beneficial to a successful immune response against *M. marinum* than a low PUFA diet. Although changes in PUFA content affected the host responses to Mycobacteria infection, the disease was cleared by all infected fish. Therefore, this suggests that more drastic changes to diet would be required for an effect to be realized in wild populations. Strain differences in host and Mycobacteria or pre-existing stress condition in striped bass may affect their immune response and the outcomes of infection.

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Acknowledgements

First and foremost I would like to thank my advisor, Dr. Mark D. Fast for his encouragement and confidence in my abilities. His assistance and review were essential to the completion of this dissertation. I would also like to thank members of my committee, Dr. Bassem Allam and Dr. Michael Frisk for their advises, and lab members Dr. Emmanuelle Pales Espinosa, Ms. Sujata Pawagi, Soren Dahl, Jesse Hornstein, Qianqian Liu, Mickael Perrigault, Mark Sokolowski, Sarah Winnicki. And finally, I want to thank all of my friends, especially Dr. Xing Jing and Jin Gao, who were there every time I needed them.

Title: Response of Striped Bass (*Morone saxatilis*) to Immunological Stimulation and Challenge

Chapter 1: Introduction

Striped Bass (*Morone saxatilis*) is an economically and ecologically significant finfish species on the US Atlantic coast. Kirkley et al. (2000) found that more than half of the total US landings of striped bass occur in the Chesapeake Bay states, Maryland and Virginia, where over 200 million dollars of revenue is generated each year, but they also play an important role in the ecosystem by controlling the population of smaller sized prey such as gizzard shad (Fourt et al. 2002).

Striped Bass are anadromous fish that spend most of their time at sea and run up through brackish or fresh water to spawn (Collette & Klein-MacPhee 2002). The Chesapeake Bay, Massachusetts Bay, Hudson River and Delaware River are the primary spawning and breeding areas for striped bass. Over the past decade, striped bass have been seriously impacted by endemic mycobacteriosis in the Chesapeake Bay (Cardinal 2001; Heckert et al. 2001; Overton et al. 2003; Rhodes et al. 2001, 2003, 2004, 2005), however, the major pathogens involved in this disease have also been described in Delaware Bay (Ottinger et al. 2006), Roanoke River (Overton et al. 2006) and recently in Long Island Sound and the New York Bight (Stine et al. 2009).

Mycobacteriosis is caused by several species of Mycobacterium which are widespread

in aquatic environments. *Mycobacterium marinum, M. chelonae, M. fortuitum* (Gauthier & Rhodes 2009), *M. chesapeaki* (Heckert et al. 2001), *M. shottsii and M. pseudoshotsii* (Rhodes et al. 2001, 2003, 2004, 2005) have been identified from infected striped bass. Infection of multiple mycobacteria species can also co-occur, and both *M. shottsii and M. pseudoshotsii* have been found in all four spawning areas (Rhodes et al. 2004; Stine et al. 2009).

Mycobacteriosis is characterized by granuloma formation which is a common vertebrate defense response against a mycobacterial infection. The granulomas can be found in any internal organ and are formed by activated macrophages including epithelioid and multinucleated giant cells, surrounded by T and B lymphocytes. This is followed by fibroblast and collagen aggregation around the macrophage core (Birkness et al. 2007). Macrophages generally phagocytose invading microorganisms and destroy them through the release of reactive-oxygen and –nitrogen species into the phagolysosome (Adam & Hamilton 1984; Nathan & Hibbs 1991). However, in the case of mycobacteria infection in mammals, the pathogen's ability to prevent lysosomal fusion with the phagosome prevents its destruction. When macrophages are initially unable to destroy bacteria they can become hyperactivated through increased production of inflammatory cytokines such as tumour necrosis factor (TNF)-α and interferon (IFN)-γ, while this is capable of enhancing the macrophages ability to kill microorganisms, in the case of mycobacteria some still survive. This survival induces the hyperactivated macrophages to fuse together and form a granuloma to wall off the bacteria from the rest of the host tissue. Beyond granuloma formation, the molecular mechanisms of pathogenesis and host response to mycobacteria in

striped bass, however, remain unexplained.

Inflammation is the initial response to trauma in all vertebrates and important in initiating an immune response to bacterial infection, which not only prevents the spread of bacteria but also promotes macrophage and neutrophil chemotaxis. Cytokines are secreted by phagocytic cells in response to immune and inflammatory stimuli and contribute to formation and maintenance of granulomas (Cooper et al. 1993; Ehlers et al. 2000). During mycobacterium challenge, host production of cytokines is essential to control mycobacterial proliferation, resulting in granuloma formation. However, this is a double-edged sword. Extremely excessive inflammation can result in damage to host tissues, contributing to death. Fortunately, inflammation is regulated by some pro- and anti-inflammatory cytokines to avoid damage to host tissues during normal immune responses. Thus, cytokine signaling plays an important role in the immuno-regulation, and consequently, the result of mycobacterial infection.

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine which is produced by activated monocytes, macrophages and dendritic cells during mycobacterial infection (Dahl et al. 1996; Gerosa et al. 1999). In particular a number of studies have reported that IL-1β is associated with directing granuloma formation and control of mycobacterial infection (Law et al. 1996; Juffermans et al. 2000; Yamada et al. 2000). IL-1 α and β double-knockout mice have shown poor granuloma formation, and enhanced growth of mycobacteria following infection (Juffermans et al. 2000). Although IL-1β is well described in mammals, little is known about the role of IL-1β in fish mycobacteriosis.

Tumor necrosis factor α (TNF-α) is directly involved in acute inflammatory response

to mycobacterial infection (Birkness et al. 2007) as well as development and persistence of the granulomas (Kindler et al. 1989; Rook 1994; Orme et al. 1999). A number of studies have found that TNF-α is activated early on in infection and leads to production of IL-6 and IL-8 (Goldsby et al. 2000). Inactivated TNF-α has caused granuloma disruption with bacterial growth (Jacobs et al. 2007), while excess TNF-α has induced hyperinflammation with subsequent necrosis (Bekker et al. 2000; Ehlers et al. 2001).

In contrast to IL-1β and TNF-α, transforming growth factor β (TGF-β) is an anti-inflammatory cytokine, and leads to macrophage deactivation and suppressive immuno-regulation. A number of studies have shown that excessive TGF-β inhibits production of Th1 cytokines including IFN-γ, TNF-α, TNF-β and IL-2 (Derynck 1994), and it prevents macrophage activation and macrophage respiratory burst (Ruscetti & Palladino 1991). In mammals, overexpression of TGF-β leads to the development of pathology, resulting from poor control of mycobacterial infection (McCartney-Francis & Wahl 1994). On the other hand, TGF-β also has pro-inflammatory effects such as activation of macrophage and neutrophil chemotaxis, IgA production, and secretion of some cytokines (McCartney-Francis & Wahl 1994). Harms et al. (2003) measured TGF-β mRNA expression in splenic mononuclear cells of mycobacteria-challenged striped bass, and found that TGF-β expression was significantly lower than uninfected control. They suggest that decreased TGF-β is associated with excessive inflammation and leads to organ damage in striped bass.

The natural resistance-associated macrophage protein (Nramp) is produced by phagocytic cells and two forms of Nramp, Nramp1 and Nramp2, exist in mammals. Nramp1 plays an important role in regulation of macrophage activation and antimicrobial

activity in response to infectious, inflammatory, or cytokine stimuli (Govoni et al. 1997; Blackwell et al. 1999, 2000). For example, Nramp1 gene was induced in a mouse macrophage cell line in response to IFN-γ and lipopolysaccharide (LPS), leading to a strong synergy for induction of inducible nitric oxide synthase (iNOS) (Govoni et al. 1997). Nramp2 plays a key role in the metabolism of transferrin-bound iron as a bivalent cation (Fe^{2+}, Zn^{2+} and Mn^{2+}) transporter (Fleming et al 1997; Govoni & Gros 1998). The protein transports free Fe^{2+} across the endosomal membrane into the cytoplasm. Similar to Nramp1, Nramp2 can be involved in host defense. The expression of Nramp 2 was stimulated by treatment with IFN-γ and LPS, increasing ^{59}Fe uptake from ^{59}Fe–nitrilotriacetic acid. The authors suggest that enhanced Nramp2 expression may be the result of exposure of macrophages to bacterial products and cytokines, which result in Fe deprivation within the endosomal compartment and thus inhibits bacterial growth (Wardrop & Richardson 2000). In striped bass, Nramp was strongly elevated at 4 h post treatment with LPS, heat-killed or live mycobacteria, and continued at 24 h in peritoneal exudate cells (Burge et al. 2004a). Furthermore, infection with *M. marinum* in peritoneal exudate cells induced up-regulation within 24 h, which lasted 15 days (Burge et al. 2004b). These data indicate that striped bass Nramp is regulated by intracellular infection and it may play an important role in resistance to mycobacterial infection.

Immune responses in vertebrates (and invertebrates) are highly integrated with and affected by other physiological processes, such that alterations to physiological homeostasis can have significant effects on an animal's immune response. Stress, is an abiotic or biotic challenge that pushes homeostatic processes beyond their ability to control routine

physiology. Stress can be experienced through changes in temperature, salinity, oxygen, crowding and numerous other factors in the aquatic environment. When stress is experienced over the long-term (chronic), animals will begin to experience secondary and tertiary effects that can negatively affect growth, reproduction and resistance to disease. In the case of fish, handling, hypoxia and cortisol implantation (i.e. stress hormone injection to mimick physiological induction due to stress), among other things, have been shown to be immunosuppressive (Pickering & Pottinger 1985; Choi et al. 2007; Fast et al. 2008).

Diet deficiency is another possible stressor in fish, and may be related to the reduced capacity of fish to resist infectious disease. This may occur through a reduced energy budget preventing significant responses from occurring and/or from malnourishment, in which improper/inefficient responses may occur. Jones et al. (2008) found that the expression of IL-8 was significantly lower in pink salmon on reduced ration within 7 days after exposure to *Lepeophtheirus salmonis*. These authors suggest that the transient reduction in IL-8 expression in head kidney-liver pools from pink salmon was the result of a limited energy reserve caused by reduced ration. In mycobacterium-infected striped bass, reducing diet (0.15 % bw/d) led to a severe, systemic infection, high bacterial loading ($>10^8$ CFU) and poor granuloma formation, suggesting decline of immune function (Jacobs et al. 2009). In all cases, data suggest that nutrition stress may have a negative effect on host immune responses.

Polyunsaturated fatty acids (PUFA) are an important component of a fish diet. Several studies have shown the effects of dietary PUFA on immune responses to bacterial infection.

Gil (2002) described that n-3 PUFA mediates the production of some inflammatory cytokines and inhibits the expression of a number of genes involved in leukocyte adhesion, blood coagulation and fibrinolysis. In mice, fish oil containing high PUFA enhanced production of IL-10 to suppress the lethal pro-inflammatory cytokines in response to LPS challenge (Sadeghi et al. 1999). Likewise, the survival of guinea-pigs following LPS challenge was significantly increased by fish oil feeding (Mascioli et al. 1988, 1989). These results indicate that PUFA may be deeply associated with immune defenses.

Despite the economic and ecological importance of striped bass and the extensive immunological work on a close relative, European sea bass (*Dicentrarchus labrax*), knowledge of striped bass immune regulation remains limited. Therefore, this project was conducted to:

1) Identify and characterize inflammatory/immune genes in striped bass following immune stimulation
2) Characterize immune regulation in striped bass infected with *Mycobacterium marinum*.
3) Characterize immune regulation and effects of diet changes in striped bass infected with *Mycobacterium marinum*.

Chapter 2: Striped Bass (*Morone saxatilis*) Response to Immunostimulation

Introduction

Many agents, known as immunogens, are capable of stimulating the immune system. One of the best studied is the T-independent antigen, lipopolysaccharide (LPS). LPS is a major component of the outer membrane and the endotoxin of gram-negative bacteria. LPS is recognized by monocytes and macrophages, which activates transcription factors including NF-kB/Rel proteins, AP-1 proteins, and NF-IL-6 by phosphorylation. The activation of transcription factors causes rapid induction of inflammatory mediators, such as cytokines and chemokines (Guha & Mackman 2001). Several studies have shown that LPS stimulation induces the production of cytokines in mammals (Davidson et al. 1998; Whyte 2007). Highly toxic in mammals leading to endotoxic shock, LPS administration in fish does not exhibit the same toxic effects. However, LPS does cause induction of inflammatory mediators in lower vertebrates and this has been described in carp (Saeij et al. 2003), Japanese flounder (Hirono et al. 2000), sea bass (Scapigliati et al. 2001) and trout (Zou et al. 1999; Brubacher et al. 2000; MacKenzie et al. 2003), showing that inflammatory cytokines such as IL-1β and TNF-α mRNA were greatly induced by LPS. Activation of pro-inflammatory cytokines by LPS also leads to the modulation of Nramp (Govoni et al. 1995; Overath et al. 1999; Chen et al. 2002) and TGF-β gene expression (Toossi et al. 1996).

Another commonly used immunostimulant in vertebrates is Freud's complete adjuvant

(FCA). FCA is made up of mineral oil including inactivated or dry mycobacterium, and it has been widely used as an adjuvant and non-specific inducer of the immune system. On the other hand, FCA can cause side effects such as inflammation, granuloma formation and pigmentation (Mutoloki et al. 2004; Koppang et al. 2005). Several studies have shown that rat IL-1β was significantly elevated by injection with FCA (Safieh-Garabedian et al. 1995; Li et al. 2005). Similarly, injection with FCA enhanced the expression of IL-1β gene in rabbits, with a maximum at around 24 h post injection (Sagara et al. 1990) and FCA injected BALB/c mice produced IL-1β and TNF-α gene expression in splenocytes (Victoratos et al. 1997).

According to these earlier studies on higher vertebrates, immunostimulants such as LPS and FCA induce production of pro-inflammatory cytokines, which is important in innate immune response to bacterial infection. However, we still know little about expression patterns of cytokines in response to immunological stimulation in fish. Victoratos et al. (1997) studied effects of adjuvants on cytokine production in response to thymus-dependent antigen. They found that cytokines were basically induced by adjuvants, but efficiency was various among adjuvants. Therefore, this study was conducted to understand cytokine expression pattern in response to LPS or FCA stimulation in striped bass over the short-term.

Materials and Methods

Experimental fish and maintenance

Study I

Age-0 Juvenile striped bass (3.44 ± 1.68 g, 70.6 ± 10.3 mm) sampled from Hudson river were immediately returned to the Flax Pond laboratory and allowed to acclimate for 4 weeks prior to any manipulation. During this time they were observed for any abnormal behaviour and a subsample of fish (n=15) were euthanized with MS-222 (250 mg/L) upon arrival and checked for infectious pathogens (wet mounts, histology, etc.). Fish were separated into 2 tanks (30-35 fish each). Striped bass were maintained on heated well water (20 ^{o}C +/- 1 ^{o}C, pH 7.5 at 18 ppt +/- 3 ppt) and 14:10 hr, light/dark photoperiod. This temperature and salinity is within the physiological optimal range for striped bass. Oxygen levels were maintained at 8-10 mg/L throughout the study. Fish were fed a combination of frozen brine/mysid shrimp and commercial fish pellets on all days except for 24 h prior to manipulation. After acclimation, 10 striped bass were euthanized (5 from each tank) using MS-222 (250 mg/L) and liver, spleen and head kidneys removed to set a baseline level of immune gene expression. The remaining fish were anaesthetized (MS-222 100 mg/L) and received an intraperitoneal injection (250 ul) of PBS (n=15), lipopolysaccharide (LPS) in PBS (3 mg/kg) (n=15), or Freud's complete adjuvant (FCA) (n=15) to simulate an immunological challenge. After injection, fish were recovered in a separate aerated bath before placement into one of three separate tanks (one for each treatment). Seven fish from

each group (control and treated) were euthanized using MS-222 at 250 mg/L at 6 and 24 h post injection. Kidney, spleen and liver tissue were removed and pooled for molecular analysis. Time points and tissues were chosen based on previous work on inflammatory gene expression in salmonids (Fast et al. 2007).

Study II

A 2nd Short-term study was similar to study I but striped bass were sampled over a longer time frame (by 48 hour post injection) and individual tissues (kidney, spleen and liver) were collected for molecular analysis. Larger age-0 juvenile striped bass were used for this study (20.6 ± 5.9 g, 129.2 ± 10.9 mm). After acclimation, fish were anaesthetized (same as previous) and received an intraperitoneal injection (250 ul) of PBS (n=20) or FCA (n=20) to simulate an immunological challenge. After injection, fish were euthanized using MS-222 at 250 mg/L and sampled at 24 and 48 h post injection.

RNA extraction

RNA was extracted from fish tissues using TRIzol® LS Reagent (Invitrogen) according to the manufacturer's instructions. Kidney, spleen and liver tissue were immediately added to TRIzol® LS Reagent (0.75 ml, Invitrogen) and macerated manually with a micro-tube pestle. After 5 min incubation at room temperature, chloroform (0.2 ml, Sigma-Aldrich) was added to the homogenates and mixed vigorously. After incubation at room temperature for 15 min, the samples were centrifuged at 11500 x g for 15 min at 4 °C. Following

centrifugation, aqueous phase was transferred to a clean tube and mixed with isopropyl alcohol to precipitate the RNA from the aqueous phase. Samples were incubated at room temperature for 10 min and centrifuged at 11500 x *g* at 4 °C for 10 min. The RNA pellet obtained was washed with 0.75 ml of 75 % ethanol, air dried for 10 min and dissolved in 40 µl molecular biology grade water (MBGW - Sigma-Aldrich) before storage at −80 °C. Total RNA concentration was measured using the NanoDrop-1000 Spectrophotometer (v3.2.1). The mRNA extracted was then reverse transcribed to obtain cDNA using Superscript III reverse transcriptase and RNAse OUT (Invitrogen) following the manufacturer's protocol. The reverse transcription reaction was performed in a 20 µl mixture containing 1.5 µg of total mRNA, 2 µl of RT enzyme mix (dNTPs, MgCl, random hexamers and oligo dTs) and 10 µl of RT reaction mix.

Sequence generation and primer development

Quantitative real-time PCR (qPCR) was performed on a subset of four genes including important cytokine genes (IL-1β, TNF-α and TGF-β) and the natural resistance-associated macrophage protein gene (Nramp), which plays a major role in resistance to mycobacterial infections. Elongation factor-1α (EF-1α) gene was used as the internal control/housekeeping gene. Degenerate primers of IL-1β and TNF-α genes which have not yet been identified in striped bass, were designed through multiple nucleotide alignments of these genes to find conserved sequence domains from several teleost species using the Bioedit software package (www.mbio.ncsu.edu/BioEdit/bioedit.html). Table 2.1 shows the nucleotide sequence of oligonucleotide primers used for amplification of partial IL-1β

(*Danio rerio*: NM_212844, *Cyprinus carpio*: AJ245635, *Oncorhynchus mykiss*: AJ223954, *Dicentrarchus labrax*: AJ311925, *Diplodus puntazzo*: AJ459238, *Latris lineata*: FJ532282, *Lateolabrax japonicus*: AY383480) and TNF-α cDNAs (*Salmo salar*: NM_001123617, *Takifugu rubripes*: NM_001037985, *Danio rerio*: AB183467, *Oplegnathus fasciatus*: FJ623187, *Epinephelus awoara*: AY667275, *Lateolabrax japonicus*: AY376595, *Dicentrarchus labrax*: DQ070246). The gene-specific primers for qRT-PCR of IL-1β and TNF-α gene were designed after determined the nucleotide sequence of each partial gene using the direct DNA sequencing of PCR products with degenerate primers. Gene specific primers targeting the amplification of TGF-β (AF140363) and Nramp (AY008746) genes were designed based on the published sequences of the genes found in NCBI (http://www.ncbi.nlm.nih.gov/) using AlleleID version 3.0 (Premier Biosoft, Palo Alto, CA). All primers used in these studies are listed in Table 2.1.

Quantitative real-time PCR

A quantitative real-time PCR assay was performed using an Eppendorf Mastercycler ep realplex 2.0 and SYBR green kits (Invitrogen). The SYBR green mastermix kit was used as recommended by the manufacturer. Primers used were identified above.SYBR green (25 ul) was added to template cDNA (2 ul), ROX (1 ul), MBGW water (20 ul) and 10 μM of forward and reverse primers (2 ul) giving a total volume of 50 ul prior to dividing into separate wells for duplication of readings. The cycling conditions were as follows: denaturation for 10 min at 95 ^{o}C, followed by 40 cycles of 15 s at 95 ^{o}C and 30 s at 58 ^{o}C and finally temperature increasing from 60 ^{o}C to 95 ^{o}C for the melting curve step. I

calculated ΔCT (ΔCT = CT of the target gene - housekeeping gene) for each fish, which means expression of the target gene relative to the reference gene. Finally, I quantified fold change in expression relative to time 0 using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

Statistical analysis

Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). All values shown are means of individuals for each sampling time ± SEM. Statistical significance of gene expression differences was assessed on expression changes relative to elongation factor-1α (EF-1α) gene expression and non-injected control fish (time 0) using one-way ANOVA followed by Tukey test.

Results

Study I

Elongation factor-1α (EF-1α) was chosen as the house-keeping for this study and showed no significant change in its expression, regardless of any stimulation to the animal (Fig. 2.1). At 6 hour post injection (hpi), IL-1β gene was significantly higher in LPS injected fish when compared to PBS injected fish. FCA injected fish also showed significantly higher expression of IL-1β gene at 6 hpi. The increased expression was greatly decreased at 24 hpi which was comparable to those of uninjected (time 0) fish (Fig. 2.2). On the other hand, expression of IL-1β gene in PBS injected fish remained constant

between 6 h and 24 h post injection, but higher than T0 levels.

Expression of the TNF-α gene significantly increased in fish injected with LPS when compared to PBS injected fish at 6 hpi and then decreased at 24 hpi (Fig. 2.3). The expression levels in fish injected with FCA did not differ significantly from the levels in PBS injected fish at 6 hpi, although FCA stimulation showed slightly higher expression. PBS injected fish showed increased expression of TNF-α at 24 hpi.

Stimulation with LPS or FCA caused a significant elevation of Nramp gene when compared to PBS injected fish at 6 hpi, thereafter resulted in depressed expression at 24 hpi (Fig. 2.4). Similar to IL-1β and TNF-α, PBS injected fish showed higher expression at 24 hpi when compared to LPS or FCA injected fish.

LPS or FCA injected fish showed significant increase in TGF-β expression at 6 hpi when compared to PBS injected control, and declined to a level comparable to that seen in uninjfected fish at 24 hpi (Fig. 2.5). On the other hand, TGF-β gene expression was increased in PBS injected fish at 24 hpi, same as above.

Study II

In the second inoculation trial, larger striped bass were used, than in study I, and carried out longer to see if the inflammatory stimuli lasted longer than 24 hr. Individual tissues were investigated to determine differences in cytokine expression within the major hematopoetic organs. In kidney samples, the expression of IL-1β gene was up-regulated in FCA injected fish at 24 and 48 hpi when compared to T0 level, however, it did not significantly differ from PBS injected fish (Fig. 2.6a). Whereas TNF-α expression was

observed to be significantly lower in FCA injected fish at 24 hpi but significantly higher at 48 hpi when compared to PBS injected controls (Figure 2.6b). Nramp gene expression was higher in FCA injected fish at 24 hpi when compared to PBS injected control, but was not significantly different (Fig. 2.6c). The level of kidney Nramp expression in FCA injected fish dropped significantly at 48 hpi, while PBS injected controls remained constant at 48 hpi (significantly > FCA at this time point). Opposite to the expression profile seen in TNF-α, expression of TGF-β gene was significantly higher in FCA stimulated fish kidneys at 24 hpi, but significantly lower at 48 hpi, when compared to PBS injected controls (Fig. 2.6d). All genes studied at 24 hpi and 48 hpi were stimulated by either PBS or FCA when compared to T0 level.

Similar to kidney, in spleen, FCA stimulation was not effective in significantly inducing the expression of IL-1β and TNF-α gene at 24 hpi and 48 hpi when compared to PBS injection. The expression levels in PBS injected fish were significantly higher than those in FCA injected fish at 48 hpi (Fig. 2.7a, 2.7b). The expression of Nramp gene was significantly up-regulated by FCA stimulation when compared to PBS injected control at 24 hpi and 48 hpi (Fig. 2.7c). The expression peaked at 24 hpi and declined at 48 hpi in FCA injected fish. Similar to results in the kidney and in contrast to TNF-α gene, FCA injected fish expressed higher levels of the TGF-β gene at 24 hpi. The increased expression was reduced at 48 hpi, but still significantly higher when compared to PBS injected fish (Fig. 2.7d). Not surprisingly, there was a negative correlation between TGF-β gene expression and TNF-α gene expression observed in kidney (97.5 %, $p = 0.013$) and spleen (79.1 %, $p = 0.111$).

As exemplified by Figure 2.8A, expression of IL-1β was significantly lower, as were all immune genes studied, in the liver when compared to spleen and kidney samples. In liver, IL-1β expression was significantly higher in PBS injected fish than FCA injected fish at 24 hpi, and this was the same as in the spleen (Fig. 2.8B). TNF-α expression was slightly higher in PBS injected fish than FCA injected fish and this was also the same as in the spleen. On the other hand, Nramp expression was higher at 24 hpi in FCA injected fish than PBS injected fish, but did not significantly differ. Similar expression was found in kidney. TGF-β expression in FCA injected fish did not significantly differ from PBS injected fish. This did not follow the result seen in spleen and kidney, where FCA injected fish resulted in significantly higher expression in TGF-β when compared to PBS infected fish.

Discussion

Elongation factor 1 α, was chosen as a housekeeping gene for the current work, based on its common use as a gene for normalization, due to its lack of change under the stimuli tested (Olsvik et al. 2005; Fast et al. 2007). Our work confirmed the lack of change in spleen, liver and kidney tissues of striped bass, following PBS, FCA and LPS injection over the short-term.

The process of inoculation, while it did not stimulate changes in EF-1α, did show changes in inflammatory gene regulation as evidenced by increased cytokine expression in the PBS injected controls. As PBS itself contains no antigenic or immunogenic properties, the process of injection and the trauma caused at the injection site are the most likely

reasons for increased expression of IL-1β in pooled (6 and 24 hpi as compared to time 0, study I) and individual tissues (24 and 48 hpi, study II), TNFα, Nramp and TGFβ showing increased expression at 24 hpi in pooled and 24 and 48 hpi in individual tissues. Furthermore, fish used in these trials had mean weights of 3 and 20 g, respectively. The injection procedure itself, as well as, the injection of 100 µl of fluid in small fish can be expected to have significant physiological effects on blood/plasma volume and tissues, like those studied here that have some blood filtering capacity.

IL-1β gene in striped bass juveniles was rapidly and highly induced by treatment with either LPS or FCA. Within the spleen and kidney tissue, specifically, elevated expression was maintained for up to 48 hrs. This result was supported by previous studies. In mammals, IL-1β was quickly produced in response to LPS (Stylianou et al. 1998; Weining et al. 1998). In yellowfin sea bream (*Acanthopagrus latus*), IL-1β gene was highly induced by LPS injection with maximal level at 4 h (Jiang et al. 2008). In Nile tilapia (*Oreochromis niloticus*), IL-1β gene was elevated within 24 h after LPS injection (Lee et al. 2006). In carp, phagocytes expressed IL-1β at 2 h after stimulation with PMA and LPS (Engelsma et al. 2001). Likewise, FCA increased IL-1β gene expression at 6 hpi. Similar results were observed in rats (Li et al. 2005; Puehler et al. 2006).

Earlier studies have shown that TNF-α was inducible following injection with LPS (Taffet et al. 1989; Goldfeld et al. 1990). In rainbow trout, injection with LPS resulted in up-regulation of TNF-α gene in cultured leucocytes and macrophages (Laing et al. 2001). Likewise, striped bass TNF-α gene was elevated at 6 hpi in response to LPS or FCA injection (Fig. 2.3). These data suggest that stimulation with LPS or FCA is capable of

inducing pro-inflammatory genes within 6 h and being maintained up to 48 hr in hematopoetic tissue (kidney and spleen) in striped bass.

Nramp plays an important role in macrophage-mediated innate immunity and it is thought to contribute to resistance to infection by mycobacteria in mammals. Mammals have two forms of Nramp, Nramp1 and Nramp2. According to phylogenetic analysis of fish (Dorschner et al. 1999; Saeij et al. 1999; Chen et al. 2002; Donovan et al. 2002; Sibthorpe 2002; Burge et al. 2004b), fish Nramp is more closely related to mammals Nramp2. Striped bass Nramp gene also revealed high sequence similarity with mammals Nramp2 (Burge et al. 2004b). Thus, similar to mammals Nramp2, Nramp may transport metabolic divalent cations and play an important role in disease resistance to mycobacteria infection in striped bass. In the pooled tissues, striped bass induced Nramp expression in response to LPS or FCA at 6 hpi. At 24 hpi, the elevation was greatly reduced in LPS injected fish when compared to FCA infected fish, but still higher than T0 level. In kidney and spleen at 48 hpi, FCA injected fish resulted in down-regulation of Nramp following up-regulation at 24 hpi. This result corresponds to the finding of Burge et al. (2004a). They confirmed that striped bass Nramp gene was greatly increased at 4 h post treatment with LPS, heat-killed or live mycobacteria. Although the elevation by LPS was reduced at 24 h, Nramp expression by LPS or mycobacteria treatment remained high at 24 h when compared to untreated controls (Burge et al. 2004a). These data indicate that Nramp is rapidly modulated in the early stage of infection. Here, Nramp was also down-regulated by 24 hr under LPS/FCA stimulation in tissue pools (significantly lower than PBS) and by 48 hr in spleen and kidney samples from fish injected with FCA.

TGF-β is an important cytokine as a regulator of inflammatory response and it suppresses the production of pro-inflammatory cytokines such as TNF-α. When looking at pooled tissues, TGF-β was up-regulated by LPS or FCA injection at 6 hpi, and down-regulated at 24 hpi, showing no major differences with TNF-α. Interestingly, there was clearly a negative correlation between TNF-α gene and TGF-β gene in kidney (97.5 %, p = 0,013) and a negative correlation, though not significant, in the spleen as well (79.1 %, p = 0.111), in study II. This would agree with the suggested role in mammals of TNF-α as an essential cytokine to activate inflammatory response whereas TGF-β is an anti-inflammatory cytokine to control inflammation.

Similar patterns of gene expression were seen between kidney and spleen. On the other hand, fish injected with FCA showed low levels of IL-1β expression in liver when compared to kidney and spleen (Fig. 2.8A). It indicates that kidney and spleen are major tissues involved in inflammation, as compared to the liver, the inclusion of which in the pooled samples may have diluted the gene responses. Several studies have also shown the tissue specific expression in cytokines. In carp, IL-1β expression was observed to be highest in the head kidney and spleen (Engelsma et al. 2001). Likewise, Kono et al. (2002) confirmed that IL-1β in carp was strongly expressed in the immune lymphoid organs including head kidney and spleen, whereas it was poorly expressed in the liver and pituitary gland.

In some cases, expression data in study I did not show similar relationships in study II. For instance, there was no negative correlation between TNF-α and TGF-β gene expression in pooled tissues, and Nramp expression was also not maintained in FCA injected fish at

significantly higher levels than PBS injected fish at 24 hr, in pooled tissues as compared to all individual tissues (study II). The disagreement may be caused by difference in age. Juvenile striped bass at five different ages (4 – 19 months) showed different magnitude, duration, and time to peak level in humoral immune responses compared to controls, indicating that younger fish (< 6months old) showed significantly lower antibody levels than juveniles of 9 – 19 months (Hrubec et al. 2004). Fish in the current investigation were all y-o-y of less than 9 months of age, and those used in the first study were only an average of 3 g. Expression of innate immune genes are also known to change over the first few months of development in several teleost species (Douglas et al. 2001; Seppola et al. 2009).

The following work confirms the inducibility of striped bass immune responses with LPS and FCA, similar to other teleost species. However, as mentioned above, PBS injection significantly stimulated the inflammatory genes studied here as well. Unlike LPS and FCA stimulation, fish showed a slower response to PBS injection. Therefore, the rapid response due to LPS and FCA is most likely due to immunogen inclusion, whereas the increase in PBS injected fish may be explained through the stress involved in injection and handling. Similar results were observed in Fast et al. (2007), in which expression in IL-1β, TNF-α and IL-8 was higher in the saline injected chum salmon (<10 g) at 24 hpi when compared to expression at 6hpi. Furthermore, although striped bass were allowed to acclimate to lab conditions, they were wild stock, as compared to studies in which experimental animals are obtained from hatcheries, where some degree of domestication may have occurred. Finally, New York strains of striped bass have previously been described to be more susceptible to stress and development of subsequent bacterial

infections as compared to Maryland and Florida strains (Jacobs et al. 1999). Despite, stimulation due to injection (PBS), striped bass inflammatory responses were shown to be more rapidly induced due to common immunogens.

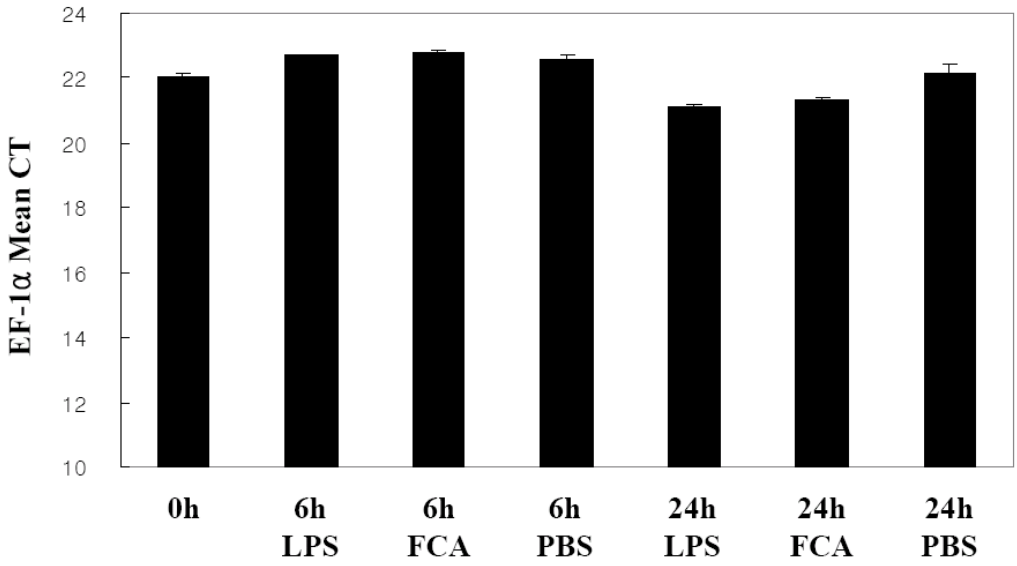


Fig. 2.1. Mean (±SEM) threshold cycle (CT) of elongation factor-1α (EF-1α) in striped bass (n=10 at time 0; n=7 at 6 h; n=8 at 24 h) prior to injection and at 6 h and 24 h post intraperitoneal injection with PBS, lipopolysaccharid (LPS) or freud's complete adjuvant (FCA). Mean CT indicates the cycle number at which the amount of amplified EF-1α reaches a fixed threshold.

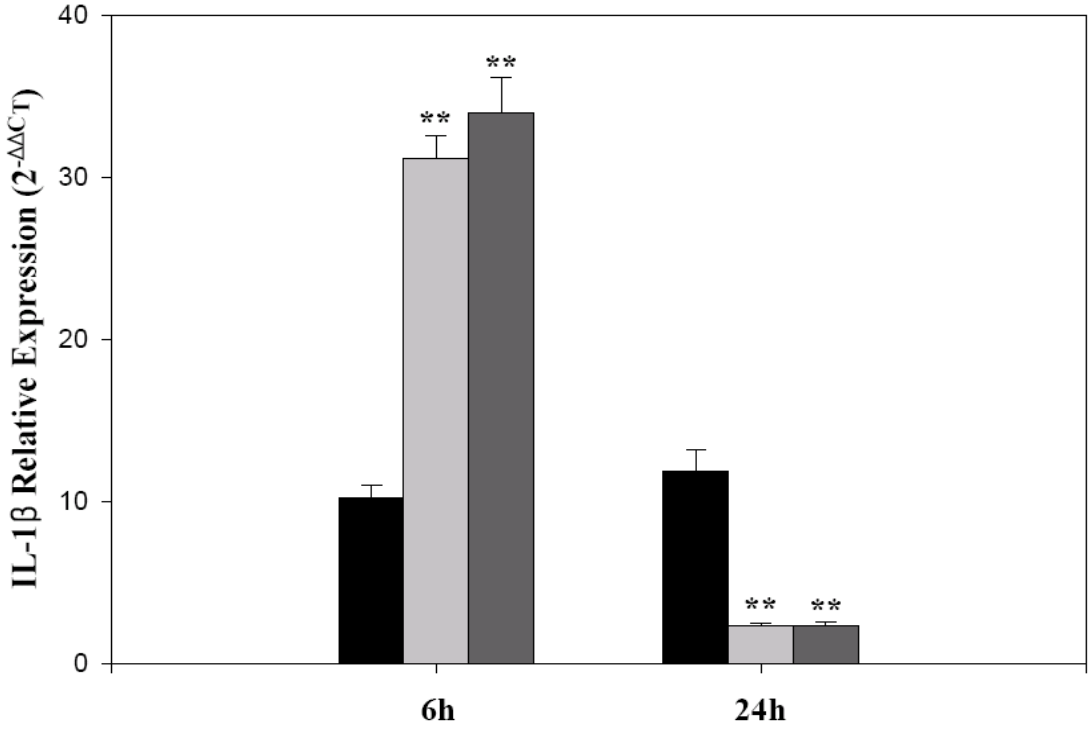


Fig. 2.2. Mean (±SEM) expression of interleukin-1β gene (IL-1β), relative to elongation factor-1α (EF-1α) in striped bass (3.44 ± 1.68 g, 70.60 ± 10.31mm) at 6 h and 24 h post intraperitoneal injection with PBS (■), lipopolysaccharid (■) or freud's complete adjuvant (■). Time 0 represents the 1X expression of the target gene normalized to EF-1α. Significant differences from PBS injected controls on that time (** $p < 0.001$).

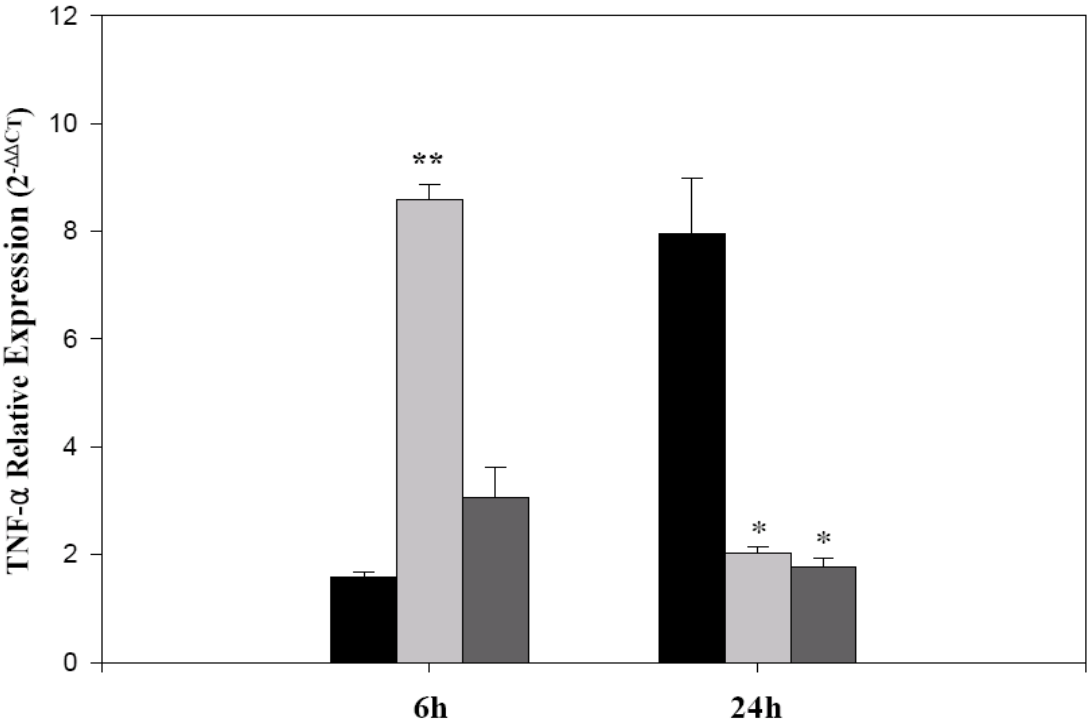


Fig. 2.3. Mean (±SEM) expression of tumor necrosis factor α gene (TNF-α), relative to elongation factor-1α (EF-1α) in striped bass (3.44 ± 1.68 g, 70.60 ± 10.31mm) at 6 h and 24 h post intraperitoneal injection with PBS (■), lipopolysaccharid (■) or freud's complete adjuvant (■). Time 0 represents the 1X expression of the target gene normalized to EF-1α. Significant differences from PBS injected controls on that time (** $p < 0.001$, * $p < 0.05$).

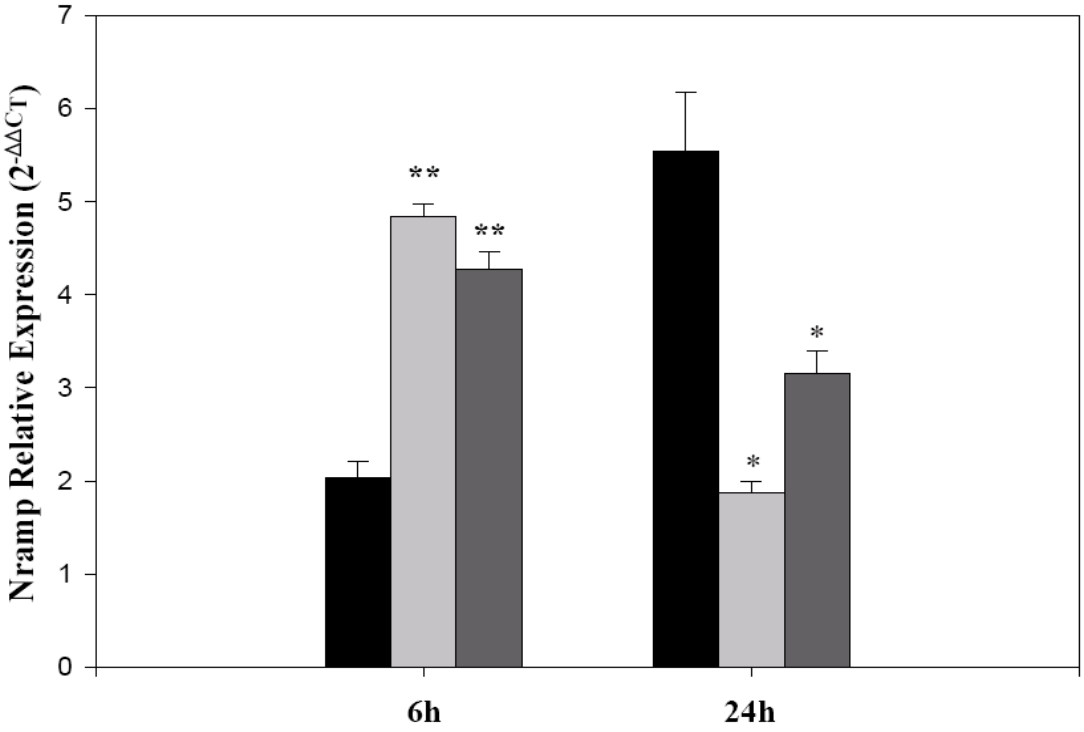


Fig. 2.4. Mean (±SEM) expression of natural resistance associated macrophage protein gene (Nramp), relative to elongation factor-1α (EF-1α) in striped bass (3.44 ± 1.68 g, 70.60 ± 10.31mm) at 6 h and 24 h post intraperitoneal injection with PBS (■), lipopolysaccharid (■) or freud's complete adjuvant (■). Time 0 represents the 1X expression of the target gene normalized to EF-1α. Significant differences from PBS injected controls on that time (** $p < 0.001$, * $p < 0.05$).

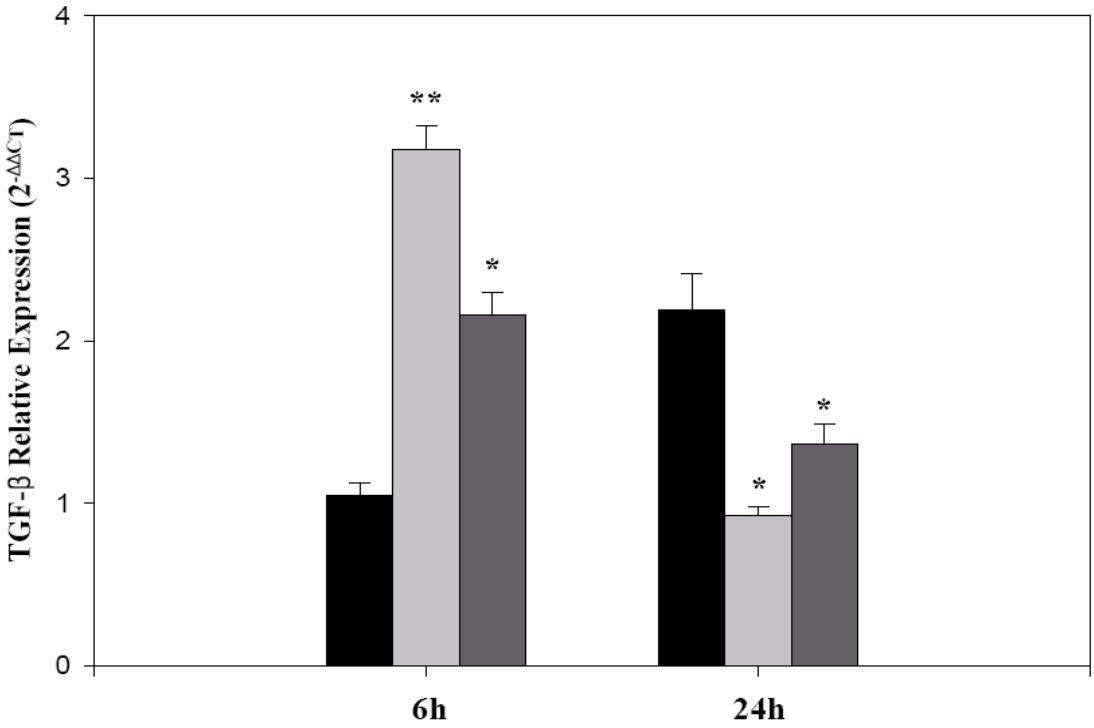


Fig. 2.5. Mean (±SEM) expression of transforming growth factor β gene (TGF-β), relative to elongation factor-1α (EF-1α) in striped bass (3.44 ± 1.68 g, 70.60 ± 10.31mm) at 6 h and 24 h post intraperitoneal injection with PBS (■), lipopolysaccharid (■) or freud's complete adjuvant (■). Time 0 represents the 1X expression of the target gene normalized to EF-1α. Significant differences from PBS injected controls on that time (** $p < 0.001$, * $p < 0.05$).

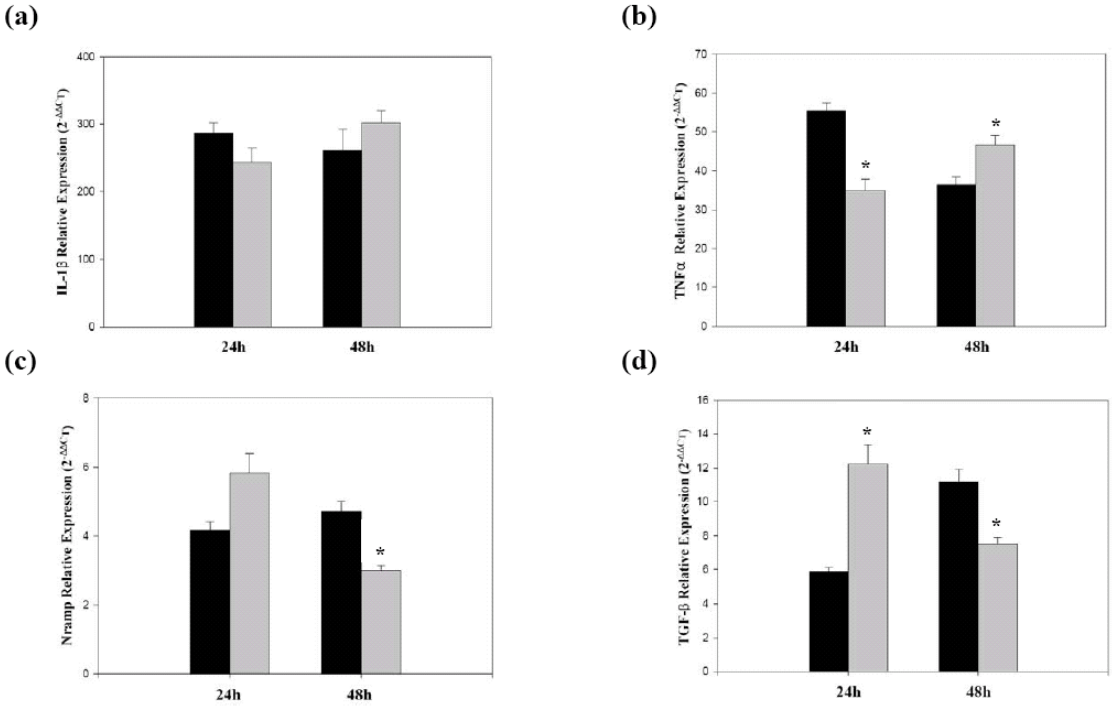


Fig. 2.6. Mean (±SEM) expression of immune-related genes relative to elongation factor-1α (EF1α) in striped bass kidney (20.62 ± 5.9 g, 129.22 ± 10.97 mm), 24 h and 48 h post intraperitoneal injection with PBS (■) or freud's complete adjuvant (■). (a) interleukin-1β; (b) tumor necrosis factor α; (c) natural resistance associated macrophage protein; and (d) transforming growth factor β. Time 0 represents the 1X expression of the target gene normalized to EF-1α. Significant differences from PBS injected controls on that time (* $p < 0.05$).

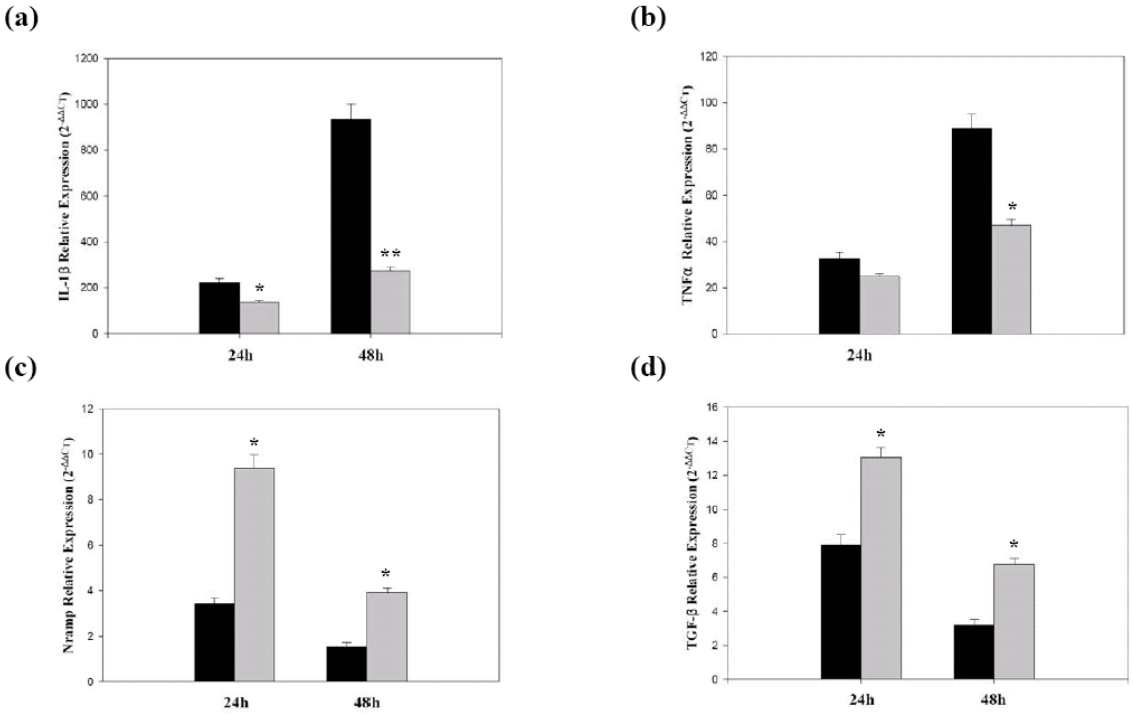
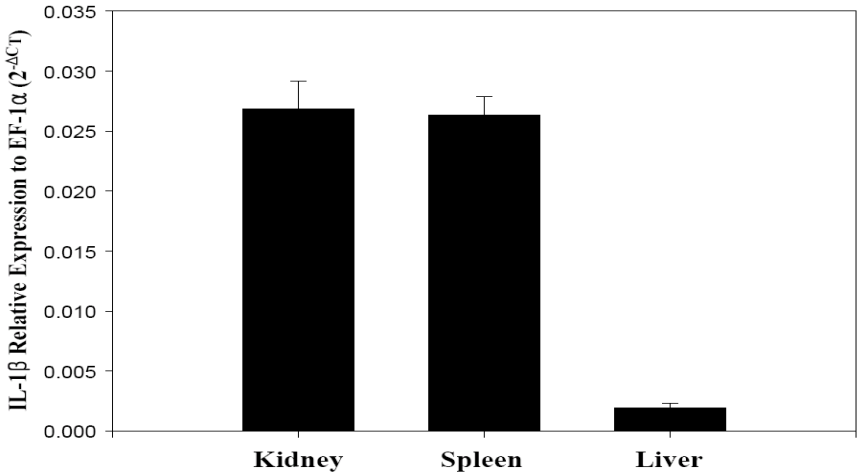


Fig. 2.7. Mean (±SEM) expression of immune-related genes relative to elongation factor-1α (EF1α) in striped bass spleen (20.62 ± 5.9 g, 129.22 ± 10.97 mm), 24 h and 48 h post intraperitoneal injection with PBS (■) or freud's complete adjuvant (■). (a) interleukin-1β; (b) tumor necrosis factor α; (c) natural resistance associated macrophage protein; and (d) transforming growth factor β. Time 0 represents the 1X expression of the target gene normalized to EF-1α. Significant differences from PBS injected controls on that time (** $p < 0.001$, * $p < 0.05$).

A)



B)

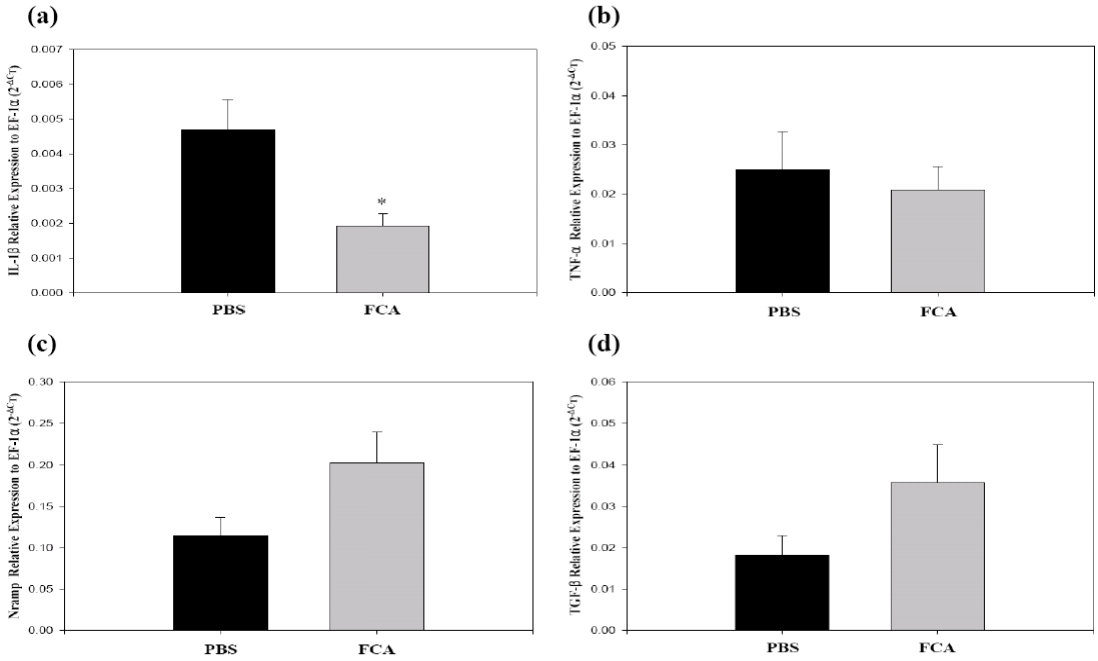


Fig. 2.8. A) Mean (±SEM) expression of IL-1β gene relative to EF-1α in kidney, spleen and liver at 24 h post intraperitoneal injection with FCA. B) Mean (±SEM) expression of immune-related genes relative to elongation factor-1α (EF1α) in liver, 24 h post intraperitoneal injection with PBS (■) or FCA (■). a) interleukin-1β; (b) tumor necrosis factor α; (c) natural resistance associated macrophage protein; and (d) transforming growth factor β. Expression of the target gene was normalized to EF-1α (ΔCT method). Significant differences from PBS injected controls on that time (* $p < 0.05$).

Table 2.1. Sequences of oligonucleotide primers used for sequencing and real-time PCR

Primers name	5'-3' sequence	Length (bp)
IL-1β-F	CYGTGRCTCTGDRCATCAAGG	146
IL-1β-R	GAAGAGVAATCGYRCCATGTCGCT	
TNF-α-F	TACTTHGTCTACWSCCAGGCGTCGTTC	238
TNF-α-R	GCTGRAACACBGCDCCHAGATARATGG	
MsIL-1β-RT-F	CAGACTGGCTTTGTCCACTG	77
MsIL-1β-RT-R	AGTCCTGCTGATTTGATCTACC	
MsTNF-α-RT-F	AACGATGGTGAAGAGGAAAG	80
MsTNF-α-RT-R	CCTATGGAGTCTGAGTAGCG	
MsNramp-RT-F	TATTGTGATGTGCGTGCAGC	85
MsNramp-RT-R	AGGCTCTGAGGATCAAGCTG	
MsTGF-β-RT-F	ATGGTTAAGAAAAAGCGCATTGAA	80
MsTGF-β-RT-R	TCCGGCTCAGGCTCTTTG	
EF-1α-F	CTTGACGGACACGTTCTTGA	151
EF-1α-R	GTGGAGACCGGTGTCCTGAA	

Chapter 3: Short-term Infection of Young-of-the-Year Striped Bass (*Morone saxatilis*) with *Mycobacterium marinum*

Introduction

Mycobacterium marinum is an important fish pathogen that was first identified by Aronson in 1926. It causes chronic and systemic disease with granulomatous lesions, similar to human tuberculosis. It can affect all organs, but granulomas are mainly observed in spleen, kidney and liver during early stages of the disease (Jacobs et al. 2009). *M. marinum* has been used to study tuberculosis due to relatively fast growth (~ 4 h generation time) and genetic similarity to *Mycobacterium tuberculosis* (Tonjum et al. 1998; Cosma et al. 2003). Several aquatic animal models have been used for this reason in the past to study immune responses to infection with *M. marinum,* including goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), leopard frog (*Rana pipiens*) and medaka (*Oryzias latipes*) (Ramakrishnan et al. 1997; Talaat et al. 1998; Decostere et al. 2004; Broussard & Ennis 2007).

Striped bass is naturally susceptible to tuberculosis caused by *Mycobacterium* spp. (Gauthier & Rhodes 2009). Recently, several surveys have shown that striped bass exhibit high prevalence (> 50 %) of mycobacteriosis in the Chesapeake Bay (Vogelbein et al. 1999; Cardinal 2001; Overton et al. 2003). Furthermore, prevalence of Mycobacteriosis in striped bass varies with sex and age. Gauthier & Rhodes (2009) have reported that prevalence increases with age through age 5, and male fish show higher prevalence than female fish among older fish. Due to the chronic nature of mycobacteriosis in striped bass, mortality

caused by mycobacteriosis is unclear in wild populations, however, commonly observed in aquaculture (Nigrelli & Vogel 1963; Hedrick et al. 1987; Bruno et al. 1998). Although recently, Gauthier et al. (2008) have proposed a model whereby endemic mycobacteriosis is contributing significantly to striped bass mortality in the Chesapeake Bay region.

Striped bass experimentally infected with *M. marinum* show large granulomas and progressive inflammation as early as 2 weeks post injection in liver, kidney and the visceral cavity (Gauthier et al. 2004). To date, little is known about immune response to chronic infection with *M. marinum* in young striped bass, compared to adult striped bass, and nothing exists in terms of the inflammatory mechanisms involved. In this study, I describe short-term immune responses to infection with *M. marinum* of young-of-the-year striped bass.

Materials and Methods

Experimental fish and maintenance

Striped bass, Choptank River, MD strain, obtained for this study were spawned and reared at the Horn Point Aquaculture Facility (University of Maryland, Center for Environmental Science, Horn Point Laboratory, Cambridge, MD 21613) from wild-collected fish. Fish were transported to the Cooperative Oxford Lab (COL) to allow for fish and system acclimation. A total of 100 fish (wt = 50 +/- 5 g) were randomly stocked into 16 - 568 L circular tanks (12-13 fish/tank) and allowed to acclimate for one month. Experimental conditions were set as: photoperiod 12:12 fluorescent, pH 8.2, salinity 10 ppt,

temperature 21° C. Water quality remained in a healthy range for the species (Harrell et al. 1990) through weekly monitoring of all systems and water exchange (10 % volume/wk).

M. marinum Isolate and Inoculation

The *Mycobacterium marinum* isolate and inoculation procedures used, were identical to those employed by Jacobs et al. (2009). Briefly, *M. marinum* isolate FL03-23 was obtained from wild Chesapeake Bay striped bass in 2003 and maintained in pure culture at the Virginia-Maryland Regional College of Veterinary Medicine. This isolate was passed through six 30g striped bass once before the initiation of the experiments and re-isolated from spleen homogenates. Approximately 0.2 g of each spleen were homogenized in 2 mL of Butterfield's Phosphate Buffered Saline (BPBS), and plated directly on Middlebrook 7H10 agar with OADC enrichment and 0.5 % glycerol, and allowed to incubate at room temperature. Cells were harvested from a single plate during exponential growth and the inoculum prepared using a modification of Gauthier et al. (2003). Briefly, on the morning of inoculation, cells were removed, suspended in BPBS, spun at 12,000 X *g* for 15 min, and supernatant collected. Immediately following, the pellet was re-suspended in BPBS, vortexed for 2 min with approximately 25 % v/v 50 µm glass beads to disrupt clumping. Finally, the suspension was filtered through Whatman No. 1 paper. Bacterial concentration was estimated by turbidity (590 nm) against a BPBS blank, and adjusted to approximately 10^7 colony forming units (CFUs). A dilution of this suspension was prepared in sufficient quantity to inoculate all fish at roughly 10^4 CFU/g body weight. Replicate serial dilutions of inoculum were subsequently spread plated to verify dose. All fish were removed from

their tanks, anesthetized in MS-222, weighed and measured before inoculation. Fish were inoculated intraperitoneally with 100 µl of either diluted *M. marinum* suspension (Treatment) or sterile BPBS (Control).

Tissue sampling

Fish were sacrificed prior to infection (time 0) and at 1, 3, 7 and 14 days post infection. Head kidney was collected for RNA analysis, spleen was taken for bacterial counts and phagocytosis assay (FITC-yeast) and blood was extracted for leukocyte counts at each sampling point.

Bacterial count

Portions of spleen were frozen immediately at -20 ˚C following extraction from the fish. Prior to plating, each spleen portion was weighed and homogenized inside of a whirl pack using a pestle. Spleen slurries were further homogenized in 2 ml of phosphate buffer solution by placing the whirl packs inside a stomacher for 2 min. 200 µl of spleen homogenate were plated onto Middlebrook agar plates without dilution and allowed to grow at ambient temperature for 2-4 weeks before counting the number of colony forming units. Each spleen sample was plated in duplicate.

Molecular Analysis

RNA extraction, reverse transcription reaction and real-time PCR followed the same protocols from Chapter 2.

Differential leukocyte population determination

Blood smear slides were made from each of 5 fish (control and treatment) at each sampling time. Smears were allowed to dry at room temperature for 1-2 hr prior to fixation and staining using a Wright's Giemsa staining method. 200 leukocytes were counted per slide to determine the differential leukocyte percentages using a Nikon ECLIPSE E200 light microscope at 1000 x magnification.

Phagocytosis Assay

The phagocytosis assay was completed by collaborators at COL and the University of Maryland Eastern Shore, Lonnie Gonsalves. Following retrieval, each spleen portion was placed immediately in cRPMI (RPMI solution with phenol red and L-glutamine supplemented with 10% fetal bovine serum, 1% EDTA, and 100nU penicillin/streptomycin) and held on ice. Leukocytes were isolated on the same day of extraction by teasing the spleen portions through a 100 µl nylon mesh strainer. Following centrifugation at 400 g at 4˚ C for 5 minutes, the cell pellet was resuspended in 5 ml cRPMI. Macrophages were isolated by layering the cell suspension over a 40% / 51% Percoll gradient (Polonio et al. 2000). Cell counts and cell viability were calculated using a hemocytometer and trypan blue staining. Each cell suspension was adjusted to 1 X 10^7 cells/ml.

The phagocytosis assay was performed using a 96-well plate format. 100 µl of each cell suspension (1x 10^6 cells) were plated in triplicate on 96-well tissue culture plates and incubated for several hours. 100 µl of fluorescein isothiocyanate (FITC) labeled yeast was added to each well along with an additional 25 µl of cRPMI. The plates were incubated for

2 hours at 27˚ C and 5 % CO_2. Following incubation, 25 µl of ice cold 0.4 % trypan blue were added to each well in order to quench extracellular fluorescence. Absorbance was then read at 495 nm. In order to quantify the amount of yeast engulfed, standard curves using FITC-yeast were developed during each assay run (R^2 >0.94). Absorbance readings caused by extracellular FITC-yeast not quenched by trypan blue was calculated by comparing values generated from wells with FITC-yeast to wells with FITC-yeast + trypan blue. The percent difference in these values was subtracted from the total absorbance measured in each sample well. Linear regression was used to interpret the final concentrations of yeast engulfed during the incubation period. Each phagocytosis assay was performed 24 hours post-sacrifice of the fish.

Statistical analysis

Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). All values shown are means of individuals for each sampling time ± SEM. Statistical significance of gene expression differences was assessed on expression changes relative to gene expression of saline injected control using one-way ANOVA followed by Tukey test. A value of $p < 0.05$ was considered significant.

Results

Striped bass were injected with 10^4 CFU of *M. marinum* and bacterial count was assessed in the spleen over 14 days. Bacterial counts in the treatment group began to increase at 3 dpi and continued to increase by 14 dpi, while no bacteria were observed in control through the experiment (Fig. 3.1).

Expression of IL-1β did not differ between the control and treatment group at 1dpi. At 3 and 7 dpi, however, expression in head kidneys of infected fish was significantly higher when compared to control fish (Fig. 3.2). After IL-1β peaked at 7 dpi, it dramatically dropped by 14 dpi, with significantly lower expression when compared to head kidney expression in control fish. Expression of TNF-α, similar to IL-1β, was significantly higher in head kidneys of infected fish when compared to control at 3 and 7 dpi before returning to baseline levels at 14 dpi (Fig. 3.3). However, the level of expression in infected fish head kidneys at 3 dpi was similar to that seen at T0 (uninjected control). In contrast to IL-1β and TNF-α, Nramp and TGF-β expression were rapidly up-regulated at 1 dpi, however, the expression was not significantly higher, when compared to controls (Fig. 3.4, 3.5). Nramp and TGF-β both showed significantly elevated expression in infected fish head kidneys at 3 dpi and 7 dpi, when compared to controls. The increased expression of Nramp and TGF-β in infected fish, similar to IL-1β and TNF-α, was ameliorated by 14 dpi.

Leukocyte populations in the blood also exhibited changes over the course of the experiment. In *M. marinum* injected fish, the number of monocytes and neutrophils were initially increased at 1 dpi (Fig. 3.6a). The percentage of leukocytes made up by monocytes

peaked at 1dpi, while neutrophils peaked at 3 dpi. At 7 dpi, the increased proportion of these cells in the blood disappeared. In PBS injected controls, a small increase of neutrophils was observed at 3 dpi however, no obvious changes in cell distribution were found on other days (Fig. 3.6b).

Despite significant changes in leukocyte populations in the blood and inflammatory gene expression in the kidney, little changed in phagocytic index of splenic leukocytes of striped bass (Fig. 3.7). Phagocytic ability in infected fish initially increased at 1dpi, and continued to increase by 14 dpi, however, there was no significant difference between treatment and control groups.

Discussion

Infection with *M. marinum* results in acute or chronic disease, depending on the dose of inoculum. Injection of 10^8 and 10^9 CFU /g body weight have been shown to induce acute disease, whereas injection of 10^2 and 10^7 CFU /g body weight have caused chronic disease in goldfish (wt= 30 g) (Talaat et al. 1998). Injection of 10^4 CFU /g body weight showed chronic granulomatous inflammation with low mortality in previous work on sea bass (Colorni et al. 1998) and striped bass (Gauthier et al. 2003; Jacobs et al. 2009). Due to the slow growth rate and chronic nature of *M. marinum* disease, previous studies have studied immune responses over a long-term period, when granulomas were obviously found in host organs. However, little is known about early immune response to mycobacterial infection, prior to granuloma formation. Here, striped bass were injected intraperitoneally with a dose

of about 10^4 CFU of *M. marinum* to produce chronic disease and investigated immune response over 2 weeks to study early immune response to chronic mycobacteriosis.

Infection with *M. marinum* caused significant and rapid up-regulation of inflammatory genes in striped bass head kidneys. IL-1β and TNF-α were up-regulated at 3 and 7 dpi, but then returned to normal levels by 14 dpi. The enhanced production of cytokines may be a direct response to the increase of systemic bacteria (i.e. bacterial counts in spleen). It is likely that the migration of monocytes to infected tissues (1 and 3 dpi) was initiated by chemotaxis and the observed changes in gene expression in the kidney may be a measure of increased neutrophil and monocyte populations in the blood stream and later on extravasated into these tissues.

Nramp has been shown to be associated with resistance to intracellular pathogens including *Mycobacterium* spp. (Vidal et al. 1993). Burge et al (2004b) have shown Nramp expression of Chesapeake Bay striped bass (200 g) in anterior kidney and peritoneal exudate (PE) cells, following mycobacterial exposure. In that work, striped bass received approximately 5000 CFU/ g of *M. marinum* or *M. shottsii* to initiate a chronic infection. In anterior kidney, no significant expression of Nramp was observed until 15 days after injection when compared to controls. At 15 days post injection, Nramp was greatly elevated in fish injected with *M. marinum*, but not seen in fish injected with *M. shottsii*. On the other hand, in PE cells, Nramp was significantly induced at 1 day after injection with *M. marinum* or *M. shottsii* when compared to controls. They explained that rapid expression of Nramp may be caused by higher proportion of macrophages in PE cells compared to anterior kidney, and late increases of Nramp expression in anterior kidney may be due to

trafficking of mycobacteria by PE cells. In this study, Nramp expression of Chesapeake Bay striped bass (50 +/- 5 g) was measured in head kidney over 14 days, following injection with *M. marinum*. Nramp was significantly up-regulated by 3 dpi, and declined to the control level at 14 dpi. In contrast to the previous study by Burge et al (2004b), quick expression of Nramp was observed in head kidney. The difference may be caused by the different doses and/or strains of *M. marinum,* or size of striped bass (200 g vs. 50 g in this study). Based on earlier work, early induction of Nramp would be expected because stimulation with LPS or FCA has shown rapid induction of Nramp in striped bass head kidney (Chapter 2). The early production of Nramp again may be produced by the increased number of monocytes/macrophages (1-3 dpi). Whereas, the increase in bacterial counts in the spleen, correspond to the down-regulation of Nramp by 7 dpi. Although these changes are occurring in separate tissues, both tissues filter blood quickly and may be showing related mechanisms, whereby Nramp down-regulation or even exhaustion, allows enhanced survival and replication of *M. marinum.*

TGF-β is a well known anti-inflammatory cytokine that inhibits production of pro-inflammatory cytokines as well as recruitment and activation of macrophages and respiratory burst activity in mammals (Jang et al. 1994, 1995; Toossi et al. 1996). However, the function of TGF-β in fish is not as well known. In this study, IL-1β and TNF-α reached maximum expression after TGF-β was down-regulated (7 dpi). While all three cytokines studied here, exhibited significantly elevated expression at 3 dpi, maximum expression of the pro-inflammatory genes (IL-1β and TNF-α) may have lagged due to crosstalk with TGF-β and its anti-inflammatory affects. Rapid production of TGF-β may be the result of

leukocyte differentiation, showing increase of monocytes at 1 dpi in response to mycobacterial infection. It has been reported that monocytes and dendritic cells induce production of TGF-β during tuberculosis (Toossi et al. 1995).

Nramp and TGF-β expression in the kidney were stimulated by 24 hpi in fish infected with *M. marinum*, similar to that shown in Chapter 2 following FCA injection. These fish also showed similar changes due to the injection procedure in PBS controls. However, this was not the case for IL-1β and TNF-α. Expression of these genes was not as significantly enhanced in the kidney of fish following *M. marinum* injection. The PBS injected controls also showed no changes in IL-1β and TNF-α expression in the kidney as compared to that shown in Chapter 2. As the injection volume was the same across studies, but the sizes of striped bass here were approximately 50 g (vs. 3 and 20 g in study I and II, respectively), inflammatory genes may have been specifically affected in the kidney of smaller fish (Chapter 2) due to a greater physiological stress of having to clear this volume of fluid. The fish in these studies were also of different strain, and were maintained at different salinity and pH. Chesapeake Bay strain of striped bass was used in this study, while NY strain of striped bass was used in Chapter 2. NY strain of striped bass have previously been described to be susceptible to stress and development of subsequent bacterial infections (Jacobs et al. 1999). Similarly, NY strain in Chapter 2 increased inflammatory/immune gene expression in response to PBS injection, which may reflect stress response involved in injection and handling.

Differential leukocyte count reflects immune response to a pathogenic agent at a given time. Maurício et al. (2009) have shown differential leukocyte populations and the

phagocytic activity in Nile tilapia (*Oreochromis niloticus*) in response to injection with *Enterococcus* sp. They suggested that the increased numbers of WBC and lymphocytes were followed by a decreased number of monocytes at 24 h post injection. On the other hand, no change in the neutrophil and monocyte counts were observed in Nile tilapia (*Oreochromis niloticus)*, following infection with *M. marinum* (Ranzani-Paiva et al. 2004). This indicates that differentiation in leukocyte population can vary with bacterial virulence. As pro-inflammatory mediators IL-1β and TNF-α were significantly elevated, in this study, it is no surprise that differential populations of leukocytes were also observed. However, although neutrophils are normally considered to be the 'first responders' to infection, it is surprising that the peak neutrophil percentages in the blood were observed at 3dpi, and monocytes peaked at 1 dpi. It is possible that neutrophils occur quickly within 24 h, considering the result of Chapter 2, where cytokine expression was quickly and highly induced by immunological stimulation by 6 h. Otherwise, initial elevation in monocytes may be inevitable in response to mycobacterial infection, in terms of its sensitivity to differ between pathogenic and non-pathogenic mycobacteria (Sahar et al. 2001).

Phagocytic cells play an important role in innate defense mechanism. They present foreign antigens to T-cells, which causes the initiation and direction of adaptive T-cell immunity as well as expression/secretion of cytokines that can regulate other phagocytic cells (van Crevel et al. 2002). Here, phagocytosis was not observed to change significantly between infected and control fish. Similarly, Dautremepuits et al. (2006) found percentage of phagocytosis and phagocytosis activity in brook trout (*Salvelinus fontinalis*) infected with *Aeromonas salmonicida* was also not significantly different from controls. They

suggested that it may be due to pathogen's ability to resist to ROS release by macrophages. In this study, no change in phagocytosis may be observed in striped bass infected with *M. marinum because M. marinum* is intracellular bacteria and is capable of preventing lysosomal fusion with the phagosome, avoiding phagocytosis as well as interaction with antibody, complement possibly other detrimental host responses.

In summary, leukocyte population fluctuated in the blood and cytokine expression was induced in the head kidney, early on, following infection with *M. marinum,* indicating rapid inflammation. On the other hand, bacterial cell counts did not peak until inflammation responses had decreased. As expected, *M. marinum* was able to survive and replicate regardless of phagocytic activity in striped bass. While inflammatory responses and Nramp expression, appear to be stimulated quickly under *M. marinum* challenge, they do not appear to differ much from that observed in other immunogen exposure (LPS/FCA). Since Nramp is known to affect intracellular survival and observed to be quickly down-regulated in this case, followed by bacterial density increases, a lengthier Nramp induction may be beneficial or even required in successful resolution of Mycobacterial infection in striped bass.

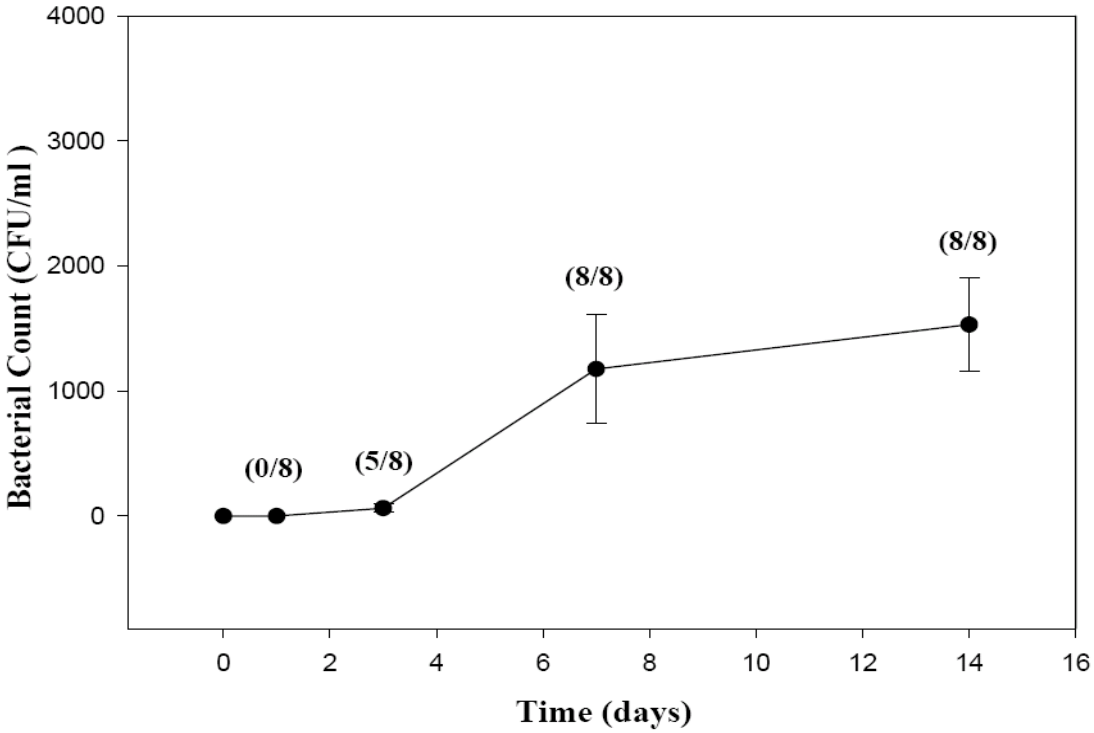


Fig. 3.1. Mean (±SEM) bacterial count in striped bass spleens over 14 days. Striped bass were injected with 10^4 CFU /g body weight of *M. marinum.* The numbers denote infected fish out of each group (8 fish/gp).

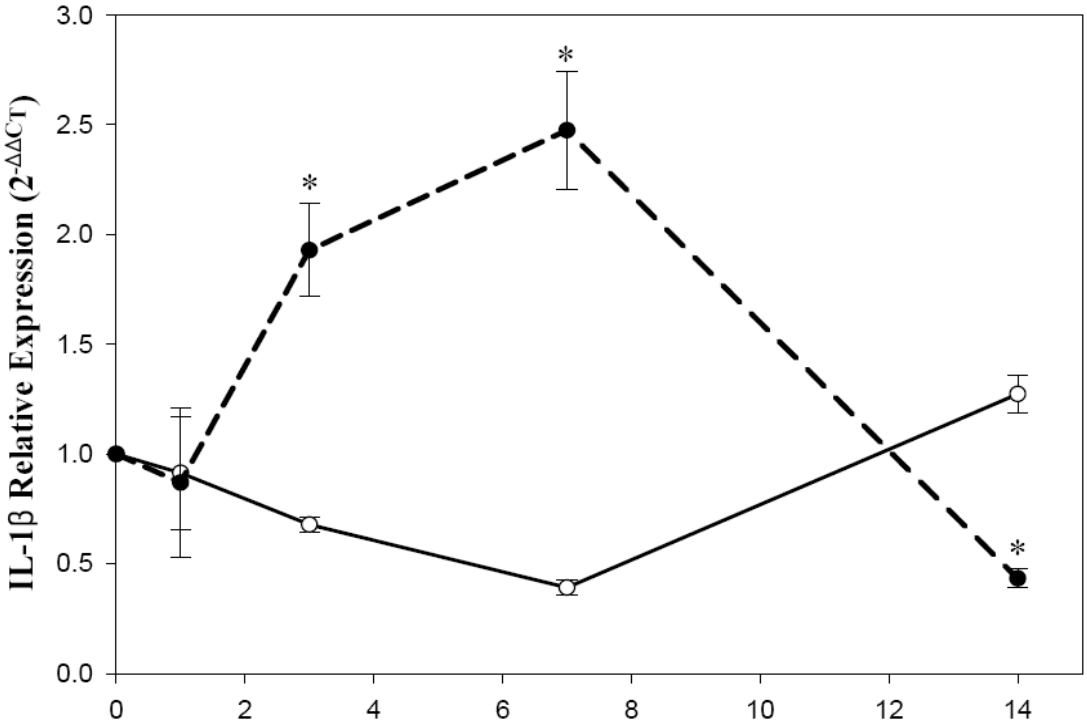


Fig. 3.2. Time course of mean (±SEM) expression of interleukin-1β gene (IL-1β), relative to elongation factor-1α (EF-1α) in striped bass following intraperitoneal injection with PBS (○) or *M. marinum* (●). * indicates significant higher expression from PBS injected controls on that time ($p < 0.05$).

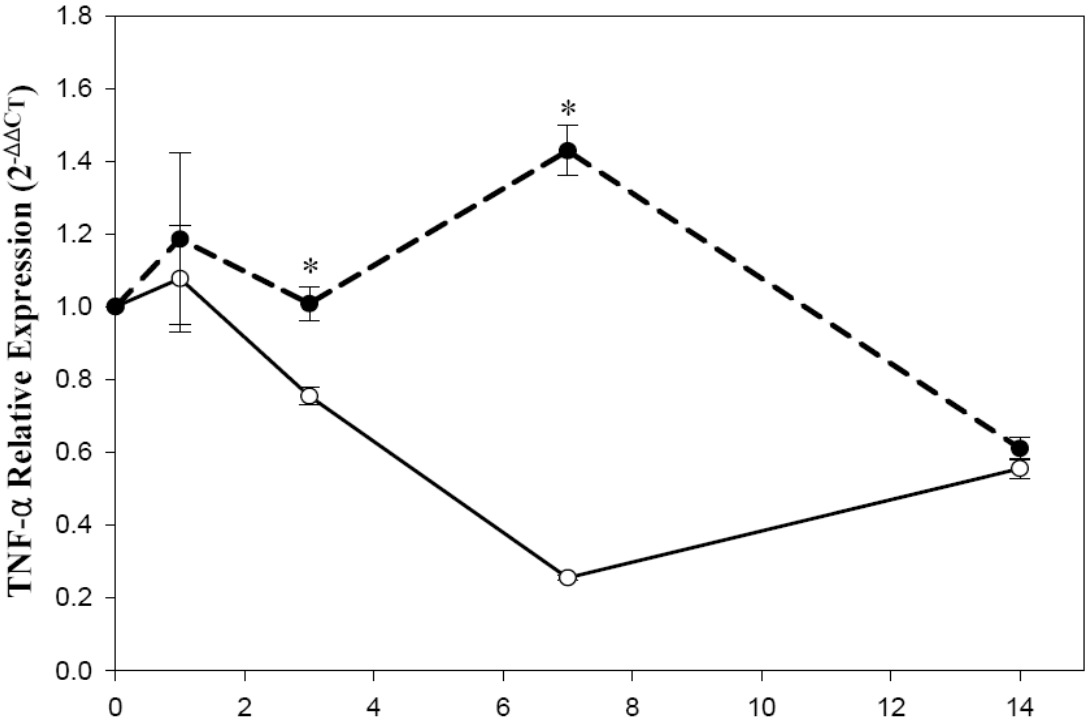


Fig. 3.3. Time course of mean (±SEM) expression of tumor necrosis factor α gene (TNF-α), relative to elongation factor-1α (EF-1α) in striped bass following intraperitoneal injection with PBS (○) or *M. marinum* (●). * indicates significant higher expression from PBS injected controls on that time ($p < 0.05$).

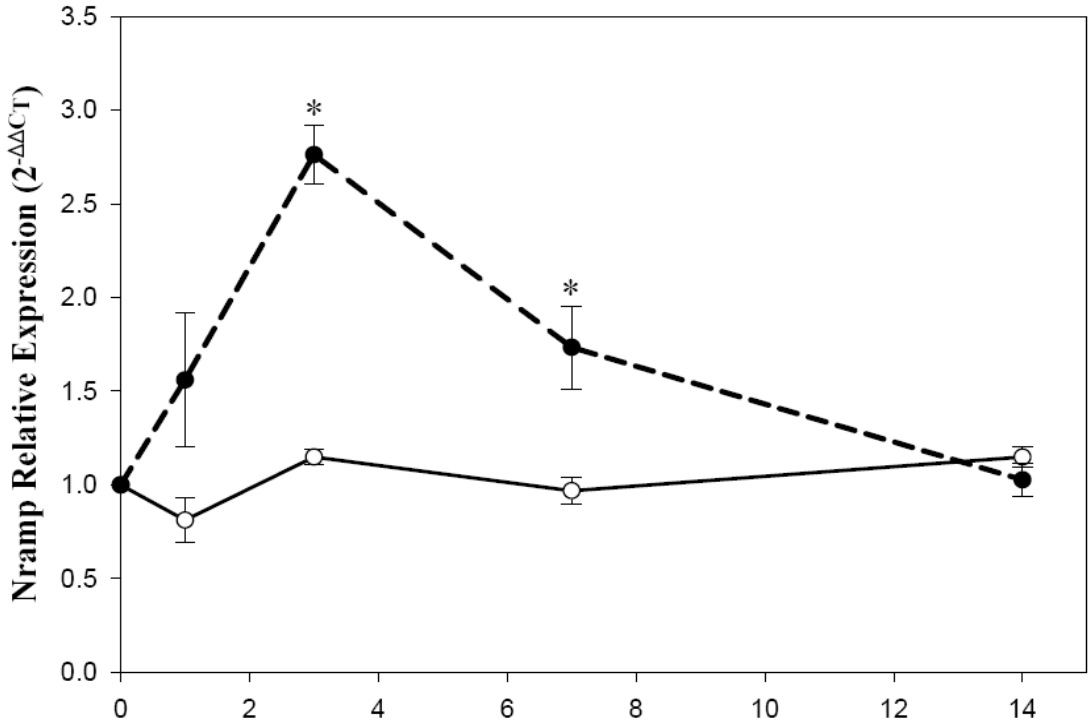


Fig. 3.4. Time course of mean (±SEM) expression of natural resistance associated macrophage protein gene (Nramp), relative to elongation factor-1α (EF-1α) in striped bass following intraperitoneal injection with PBS (○) or *M. marinum* (●). * indicates significant higher expression from PBS injected controls on that time ($p < 0.05$).

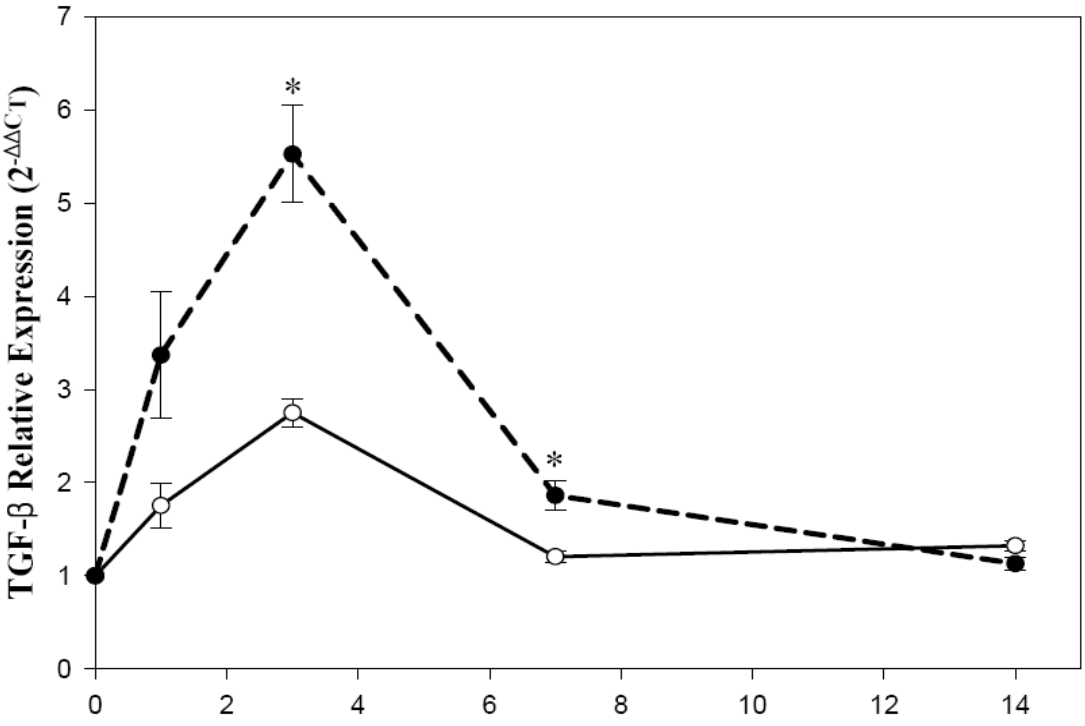


Fig. 3.5. Time course of mean (±SEM) expression of transforming growth factor β gene (TGF-β), relative to elongation factor-1α (EF-1α) in striped bass following intraperitoneal injection with PBS (○) or *M. marinum* (●). * indicates significant higher expression from PBS injected controls on that time ($p < 0.05$).

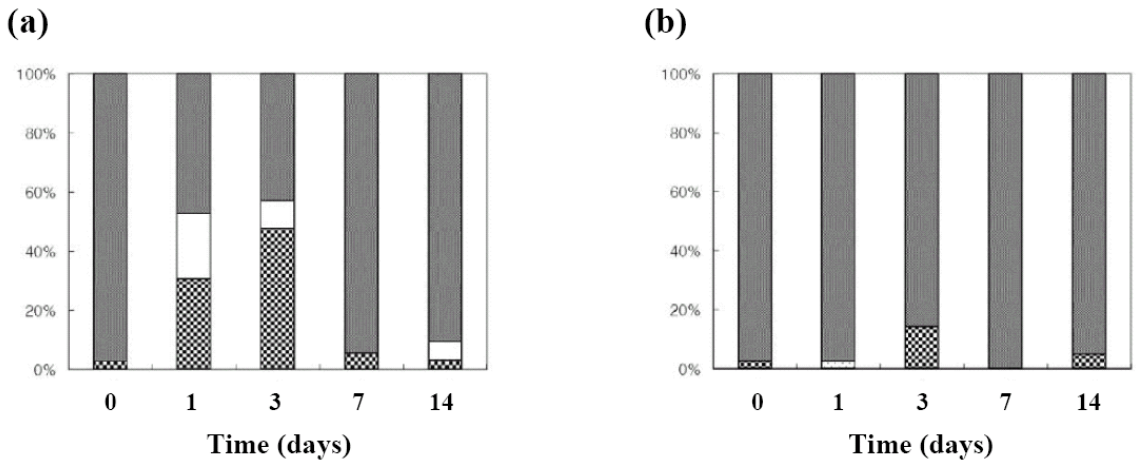


Fig. 3.6. Percentage of differential leukocyte population in response to infection with *M. marinum* (a) or with PBS (b). Lymphocyte (▩), Monocyte (□) and Neutrophil (▩).

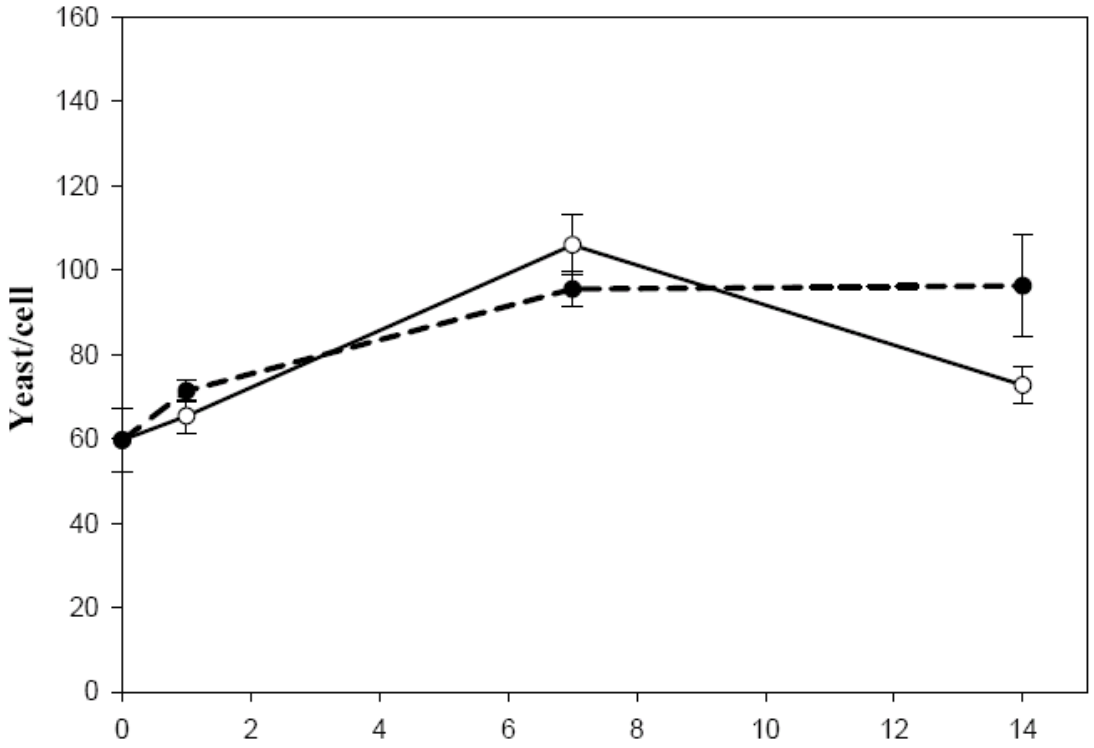


Fig. 3.7. Mean (±SEM) phagocytic activity of FITC-labelled yeast by striped bass spleen leukocytes in treatment with *M. marinum* (solid circles) or PBS (open circles).

Chapter 4: Long-term Infection of Young-of-the-Year Striped Bass (*Morone saxatilis*) with *Mycobacterium marinum*: The effects of a low PUFA diet

Introduction

Mycobacterium spp. infections have been increasing in Chesapeake Bay striped bass, since it was first described in 1997 (Vogelbein et al. 1998; Heckert et al. 2001; Rhodes et al. 2001; Rhodes et al. 2004). Although the impact of mycobacteriosis on the striped bass population is unclear, Jiang et al. (2007) suggested that natural mortality of striped bass increased with increasing mycobacteriosis. According to previous surveys, the prevalence of disease reached more than 50 % in striped bass from Chesapeake Bay. (Vogelbein et al. 1998; Cardinal 2001; Overton et al. 2003; Rhodes et al. 2004; Gauthier & Rhodes 2009). It has been suggested that the unusually high prevalence in Chesapeake Bay striped bass may be the result of anthropogenic stressors and nutritional stress (Coutant 1985; Price et al. 1985; Jacobs et al. 2009).

Mycobacteriosis results in chronic and systemic infection in wild fish, leading to emaciation and deaths in the infected fish population over a period of months to years. The disease is characterized by granuloma formation. Talaat et al. (1998) have shown mycobacterial pathogenesis in goldfish (30 g). Fish inoculated by 10^2 to 10^7 CFU *M. marinum* led to progressive, systemic granuloma formation but survived until sacrifice (56 days). Zebrafish also show similarly large granulomas and progressive inflammation in the

kidney, liver, and visceral cavity as early as 4 weeks post infection with 10^4 CFU *M. marinum*, and the inflammation was activated by 8 weeks. Striped bass have also been shown to develop similar granulomas over a period of 4-5 weeks in response to *M. marinum* infection (Gauthier et al. 2004).

Previous work has demonstrated beneficial effects of dietary unsaturated fatty acids on the vertebrate immune system (Stulnig 2003). However, based on the essential fatty acid (EFA) limitation hypothesis, animals cannot synthesize polyunsaturated fatty acids (PUFA). In addition, there is low efficiency conversion from short chain omega3-polyunsaturated fatty acids (omega3-PUFA) to highly unsaturated fatty acid (HUFA), that is a subset of PUFA molecules with 20 or more carbon atoms (Olsen 1999). Thus, polyunsaturated fatty acids (PUFA) are an important component of diets. Montero et al. (1998) has shown dietary effect on the alternative complement pathway activity in gilthead seabream. Fish fed deficient a-tocopherol and n-3 HUFA diets resulted in a chronic elevation in plasma cortisol (i.e. stress hormone), suggesting that both a-tocopherol and n-3 HUFA nutritional deficiencies is involved in fish complement activity and stress. Ortuño et al. (2002) have shown depressed complement activity after stress as a probable consequence of the high circulating levels of cortisol. In particular, PUFAs can affect host immune responses and resistance to infection with *M. tuberculosis*. Guinea pigs fed a (n-3) PUFA showed pronounced progression of disease and higher bacterial counts in the spleen in response to *M. tuberculosis* infection (McFarland et al. 2008). To date, the relationship between nutrition and disease is poorly understood in fish, compared with mammals. Thus, in this study, I studied the effects of changing PUFA diets on the innate immune system and

associated host resistance to mycobacterial infection.

Materials and Methods

Fish maintenance

Striped bass, Choptank River strain, MD, obtained for this study were spawned and reared at the Horn Point Aquaculture Facility (University of Maryland, Center for Environmental Science, Horn Point Laboratory, Cambridge, MD 21613) from wild-collected fish. Fish were transported to the Cooperative Oxford Lab (COL) to allow for fish and system acclimation. A total of 216 fish (wt = 182 +/- 37 g) were randomly stocked into 16 - 568 L circular tanks (14-15 fish/tank) and allowed to acclimate for one month. These fish were then fed either a high or low PUFA diet for 10 weeks prior to inoculation. The COL experimental system was similarly constructed to that of (Jacobs et al. 2009). Briefly, the system consisted of eight identical experimental units, each comprised of two tanks serviced by a common bio-filter. Each dietary group was randomly assigned four experimental units per diet and two of these units per group were inoculated with *M. marinum*. Each experimental unit was equipped with a UV sterilizer and an automatic pH controller. Four units were located in one half of the facility designated as controls. The remaining four units were located in the treatment room. The two sides of the facility were physically partitioned with separate air handling systems designed to meet or exceed all requirements for working with class II agents (USDHHS 1999). Fish were maintained as described in Chapter 3. Water levels were decreased approx. 15 % per month to account for

changes in biomass to maintain constant biomass per tank over the course of the study. Two types of diets, low or high polyunsaturated fatty acid (PUFA) were used in this study. The high PUFA diet was formulated to contain 30.1 % PUFA and the low PUFA diet was formulated to contain 8.75 % PUFA (Ziegler Bros. Inc.).

Sample Collection

Striped bass were infected intraperitoneally with a dose of 10^4 CFU/g body weight of *M. marinum*. Control fish received sterile PBS. Fish (8 fish/gp) were sacrificed prior to injection and at 2, 4 and 8 wk post injection (wpi) for bacteriological, molecular and histological sampling. Similar to Chapter 3, the head kidney was sampled for gene studies and spleens were taken for bacterial counts. All visceral tissues were also taken for histological analysis.

Molecular Analysis

Striped bass head kidneys were collected at 0, 2, 4 and 8 weeks post injection (wpi) to investigate diet effects on inflammatory responses of striped bass during *M. marinum* challenge. mRNA expressions of IL-1β, TNF-α, Nramp and TGF-β were measured using the quantitative real-time RT-PCR assays developed in Chapters 2 and 3.

Histological Analysis

Tissues for histological analysis were fixed in 10 % buffered formalin immediately after sampling (for approximately 48 hrs) from fish. Tissues were dehydrated, paraffin

embedded and 7 um sections prepared on slides. Slides were then stained using hematoxylin and eosin stain by the COL diagnostic service (Prophet et al. 1992). Histological sections were used for index of lesion and granuloma formation in the tissues including kidney, spleen, liver and heart. Index was scored for presence of absence of lesion or granuloma, respectively (0 = absence, 1 = presence). Lesion index = total organ affected per fish / number of fish. Granuloma index = total organ with granulomas per fish / number of fish.

Statistical Analysis

Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). All values shown are means of individuals for each sampling time ± SEM. Statistical significance of gene expression differences was assessed on expression changes relative to gene expression of PBS injected control using one-way ANOVA followed by Tukey test. Interaction between diet change and infection was statistically evaluated by two-way ANOVA followed by Holm-Sidak method. A value of $p < 0.05$ was considered significant.

Results

Fish from 2 systems/diet were injected with *M. marinum* and portions of spleen were cultured to obtain mycobacterial cell counts over time. As shown Fig. 4.1, abundance of *M. marinum* peaked at 2 wpi, when most of the fish were infected with *M. marinum*, but bacterial count was higher in fish fed a low PUFA diet than fish fed a high PUFA diet. At 4

and 8 wpi, bacterial infection disappeared in fish fed a low PUFA diet as no bacteria were cultured from these fish. Bacterial were also cleared in fish fed a high PUFA diet although mean bacterial counts were approximately 95 CFU's /ml and 140 CFU's /ml at 4 and 8 wpi, respectively, this was the result of 1 fish on each sampling day containing 735 of CFU's/ml and 1060 CFU's/ml, respectively. Furthermore, although gene expression and histological analyses were not done, fish were maintained to 16 and 32 wpi, during this study and no mycobacteria were found following the 8 wpi time point.

The expression of genes studied was variable in infected fish head kidneys over time. Regardless of diet change, IL-1β gene was significantly higher in infected fish head kidneys at 2 wpi and 4 wpi when compared to control, but a significant interaction between diet and mycobacteria infection was also observed at 2 wpi (Fig. 4.2). The increase in IL-1β gene at 2 wpi was observed to decrease by 8 wpi.

In contrast to IL-1β gene, the expression of TNF-α gene was significantly higher in infected fish head kidneys fed a low PUFA diet at 2 wpi and 4 wpi when compared to control fish head kidneys (Fig. 4.3). There was also a significant interaction between diet and mycobacterial infection at 2 and 4 wpi. At 4 wpi, TNF-α expression in infected fish was reduced and stayed low in all fish at 8 wpi.

Similar to IL-1β, the expression of Nramp was highly elevated in the head kidneys of fish infected with *M. marinum* and a significant interaction between diet and mycobacterial infection was observed at 2 wpi (Fig. 4.4). The expression levels of Nramp also declined at 4 wpi. In the treatment group, fish fed a high PUFA diet showed higher expression of Nramp than fish fed a low PUFA diet at 2 wpi.

Similar to the other genes studied the expression of TGF-β gene was stimulated in response to infection with *M. marinum* at 2 wpi (Fig. 4.5). TGF-β gene was significantly higher in the head kidneys of fish fed a high PUFA diet when compared to kidneys of fish fed a low PUFA diet and again a significant interaction was observed between diet and mycobacteria infection at 2 wpi. Again, by 8 wpi, treated and control fish, regardless of diet, had similar TGF-β expression levels in the head kidney.

Here, lesion index is a proxy for tissue trauma and pathology associated with bacterial infection. As was seen in the bacterial counts, some controls were observed to have tissue lesions, but infected fish had higher lesion indices throughout the study (Fig. 4.6). Lesion index showed a peak at 2 wpi, when lesion index was higher in fish fed a low PUFA diet than fish fed a high PUFA diet similar to the expression level of the TNF-α gene and bacterial count, and then decreased by 8 wpi.

As with bacterial counts and the lesion index, some controls would be expected to have a small number of granulomas in their tissues, as such, time 0 was used as a baseline for the granuloma index. Using the pre-inoculation granuloma index as a baseline, only infected fish at 2 and 4 wpi exhibited above background numbers of granulomas (Fig. 4.7). In addition, fish fed a high PUFA diet showed a higher maximum of granuloma index at 2 wpi (4 of 8 fish), when compared to fish fed a low PUFA diet (2 of 8 fish), whereas there were no differences at any other time point (2 of 8 fish). Interestingly, granulomas were eliminated over time, and none were observed by 8 wpi.

Discussion

In this study, striped bass were injected intraperitoneally with a dose of 10^4 CFU/ g of *M. marinum* to produce chronic disease, similar to Chapter 3 (Colorni et al. 1998; Talaat et al. 1998; Gauthier et al. 2003; Jacobs et al. 2009). The infection was accompanied with lesion and granuloma formation in most visceral organs (kidney, spleen, liver and heart) without gross pathology or morbidity at 2 wpi. Unexpectedly, however, bacterial infection and granuloma formation was cleared within 8 weeks, as shown by the decreased bacterial abundance, as well as lesion and granuloma indices. These results indicate that *M. marinum* infection did not successfully replicate or survive in the host macrophages past 2 wpi. This is in stark contrast with the finding of Jacobs et al. (2009), where following the same inoculation procedure and starting bacterial density, bacterial density in striped bass spleens increased over an 8 week period. Similarly, Talaat et al. (1998) also found a positive relationship between time and replication of *M. marinum* colony formation in goldfish to 56 days following inoculation of 10^7 or less CFU. In addition, these authors found granulomas forming in infected animals up to 16 weeks after initial inoculation. This is likely due to difference in *M. marinum* strain or fish size. The FL03-23 strain of *M. marinum* isolated from a wild Chesapeake Bay striped bass was used in this study, whereas the F-110 strain of *M. marinum* obtained from *Cichlids* sp. fish was used by Talaat et al. (1998). The difference in strain may affect bacterial virulence, however, the *M. marinum* isolate and inoculation procedures used, were identical to those used by Jacobs et al. (2009), but size difference in striped bass was observed between this study (wt = 182 +/- 37 g) and Jacobs

et al. (2009) (wt = 65 +/- 15g). Fish size may have effect on an ability of host to fight off mycobacterial infection. Previous work in striped bass has shown differences in humoral responses related to size/age in juvenile fish (Hrubec et al. 2004).

Cytokine production (Jacobs et al. 2007; van Crevel et al. 2002) and granuloma formation (Talaat et al. 1998; Bouley et al. 2001; Gauthier et al. 2003; Gauthier & Rhodes 2009) are important components in the host immune response to mycobacterial infection. This study specifically investigated the expression of IL-1β, TNF-α, Nramp and TGF-β genes over an 8 week infection period in striped bass. Similarly, the expression of IL-1β, TNF-α, Nramp and TGF-β genes were all stimulated by mycobacterial infection at 2 wpi, and they showed trends toward decreased expression between 2 weeks and 8 weeks. Since bacterial densities dropped off after 2 weeks, it is reasonable to conclude that the infection and subsequent inflammatory stimuli were ameliorated at 4 and 8 weeks.

Unlike IL-1β, Nramp and TGF-β, TNF-α was significantly up-regulated in fish fed low PUFA at 2 wpi (Fig. 4.3). This may be an indication of the difficulty in granuloma formation at a low PUFA diet. As TNF-α is directly involved in development and maintenance of granulomas, and an increased or over-expression of this gene may be required in animals under dietary stress to form normal granulomas (Kindler et al. 1989; Friedland et al. 1992; Rook 1994; Orme et al.1999). Furthermore, although lesion index was higher in fish fed the low PUFA diet, they exhibited equal or lower granuloma formation in visceral tissues and higher bacterial count in spleen.

Expression of IL-1β, Nramp and TGF-β were all much lower in this study as compared to expression observed in Chapter 2 and 3. TNF-α expression, however was

similar across studies in fish of different strain and size. As mentioned previously, fish used in Chapter 4 were approximately 182 g, as compared to <50 g in Chapter 2 and 50 g in Chapter 3. Again, as size/age are known to dramatically affect striped bass immune development, as well as other vertebrates, especially within the first year of life, this is not surprising (Douglas et al. 2001; Hrubec et al. 2004; Seppola et al. 2009). This variability high lights the need for further work in strain and size effects on striped bass immune responses.

A number of studies have shown that PUFAs play important roles in regulation of inflammatory genes (Weaver et al. 2009). PUFA can result in either increase in IL-1β, IL-2, IL-6 and TNFα (Endres et al. 1989, 1993; Meydani et al. 1991, 1993; Caughey et al. 1996) or decrease in IL-1β and TNFα (Caughey et al. 1996). In this study, infection with *M. marinum* caused up-regulation in inflammatory genes at 2 wpi, with a significant interaction between diet and mycobacterial infection. Fish fed a low PUFA diet showed higher expression of TNF-α gene and lesion index, as well as bacterial densities were higher in fish fed a low PUFA diet when compared to fish fed a high PUFA diet. On the other hand, fish fed a high PUFA diet exhibited higher expression of IL-1β, Nramp and TGF-β gene with granuloma formation, but bacterial densities were lower than fish fed a low PUFA diet (Table 4.1). These data indicate that high PUFA diet resulted in positive host response to *M. marinum* infection, suggesting that a high PUFA diet is more beneficial to successful immune response against *M. marinum* than a low PUFA diet, possibly by increasing cytokine production and granuloma formation.

Previous studies have shown the effect of food quantity or quality on the progression

and severity of disease in fish. Following infection with *M. marinum*, striped bass fed low rations resulted in a severe, active systemic infection, characterized by high bacterial loading, which commonly progressed to mortality, whereas fish fed proper rations controlled infection with classic granulomatous inflammation (Jacobs et al. 2009). Jones et al. (2008) have described that proinflammatory gene expression was modulated by reduced rations in juvenile pink salmon (*Oncorhynchus gorbuscha*), in response to *Lepeophtheirus salmon*, where IL-8 was down-regulated at 7 days after exposure, but IL-1β and TNF-α were increased by 14 d after exposure in association with reduced rations among exposed salmon. They suggested that reduced body energy caused by low ration may have negative effect on cytokine expression, leading to high susceptibility to infectious diseases. Similar results were observed in Chan et al. (1996) who showed the relationship of protein calorie malnutrition and tuberculosis in mice. Mycobacteria-infected mice fed protein-deficient diets (2 %) reduced expression of IFN-γ, TNF-α, and iNOS in the lungs with increased bacillary burden, and died within 90 days by infection with 10^4 CFUs of *M. tuberculosis*. On the other hand, mice receiving a full protein (20 %) survived under tuberculosis. These results described that protein malnutrition may have a negative effect on cell-mediated immune responses, leading to increase in bacterial burden and poor granuloma formation. It must be noted, that although changes in PUFA inclusion within the diet affected the host responses to mycobacteria infection, they did not affect the overall outcome of infection, as the disease was cleared by all infected fish.

This work highlights the importance of inflammatory regulation in the formation of lesions and granulomas under mycobacteria infection. Furthermore, it shows a significant

interaction with diet. As this has also been shown in other studies, using similar or different pathogens and hosts, it speaks to the importance of proper diets in immunocompetence.

It has been reported that Atlantic menhaden, a key forage species, has experienced significant declines in the Chesapeake Bay (Uphoff 2003), while striped bass have recovered to near historic highs (Field 1997). This imbalance may result in malnutrition that may affect susceptibility of hosts to infectious disease. On the other hand, striped bass may consume different forage fish, instead of Atlantic menhaden (Hartman & Brandt 1995; Griffin & Margraf 2003; Overton et al. 2003; Pruell et al. 2003; Walter & Austin 2003). Dietary shifts can lead to deficiencies in polyunsaturated fatty acids (PUFA) by altering the nutritional content of the diet and possibly affect immune function and overall progression of disease, without the contribution of PUFAs to regulate the expression of signal transduction genes and pro-inflammatory cytokines (Weaver et al. 2009). These factors may all contribute to immunodeficiencies in Chesapeake Bay striped bass populations and the endemic mycobacteriosis problem there, but will require further investigation.

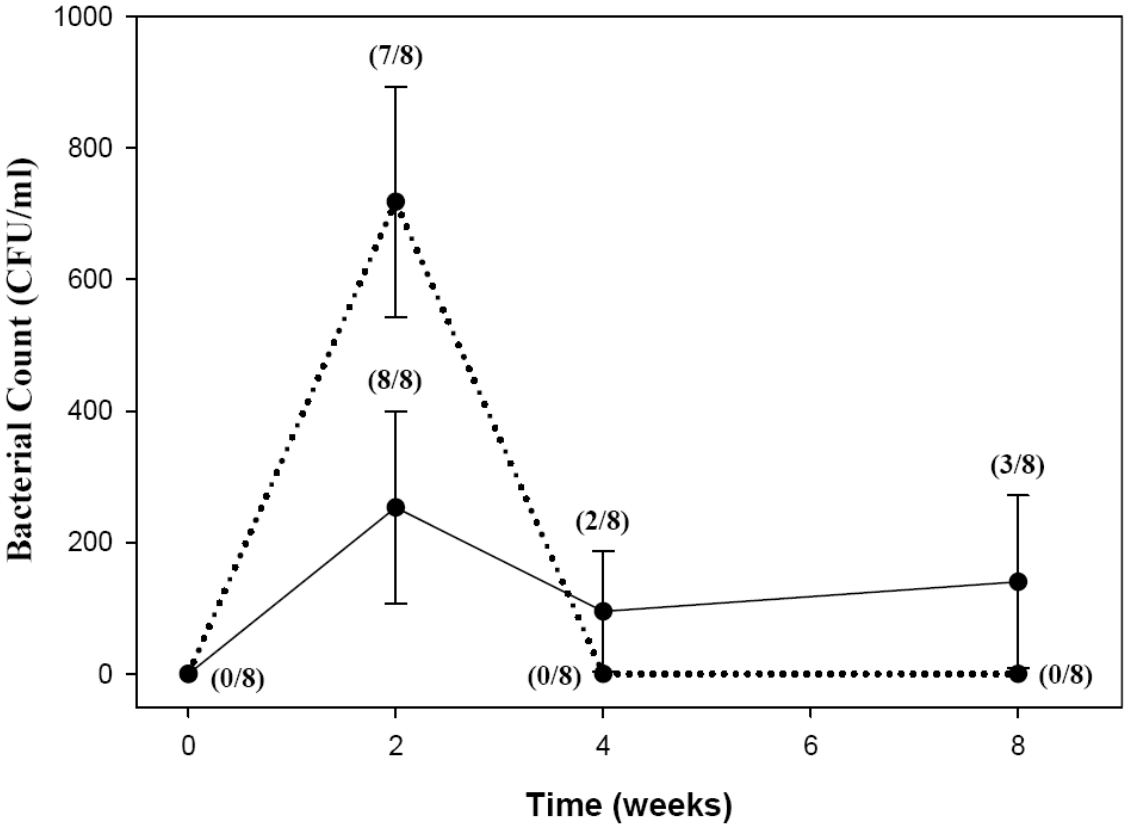


Fig. 4.1. Mean (±SEM) bacterial count in striped bass spleens of experimental fish over 8 weeks post injection with 10^4 CFU /g fish *M. marinum.* The numbers denote infected fish of each group (8 fish/gp). Dotted line indicates fish fed a low PUFA diet and solid line indicates fish fed a high PUFA diet.

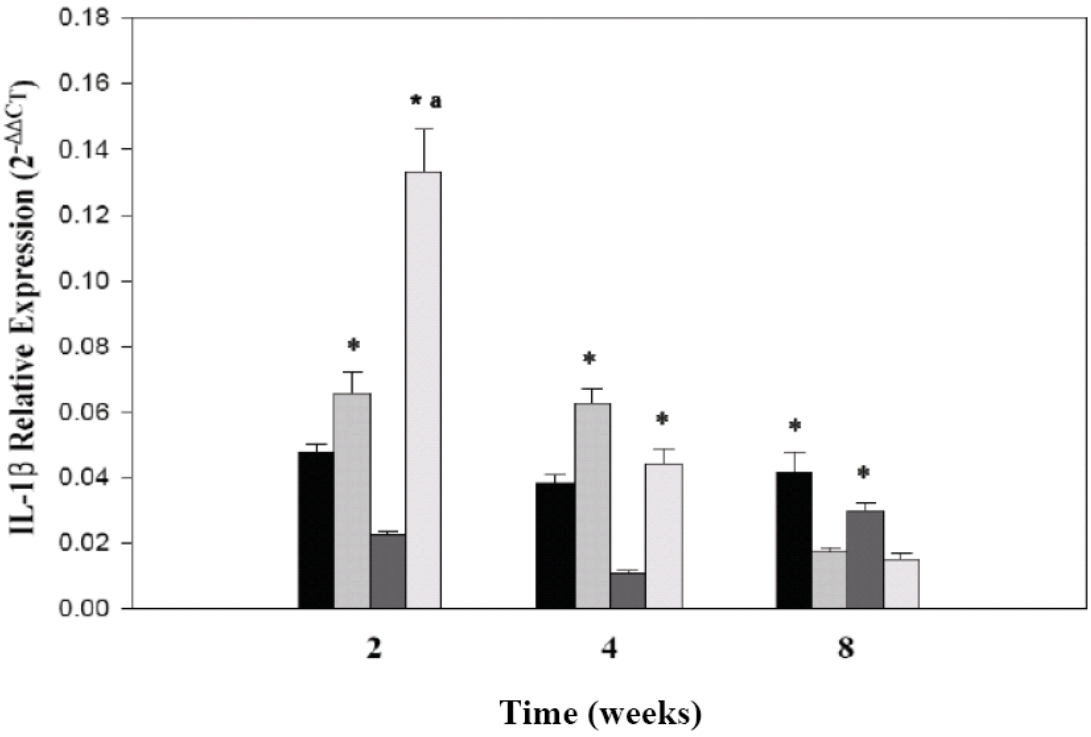


Fig. 4.2. Mean (±SEM) expression of interleukin-1 β gene, relative to elongation factor-1α (EF1α) of striped bass (8 SB/gp) fed either low PUFA or high PUFA for 10 weeks and sampled at 0, 2, 4 and 8 weeks post injection with *Mycobacteria marinum* (Treatment) or sterile PBS (Control). * indicates significant differences between control and treatment group on that time ($p < 0.05$). [a] indicates significant interaction between diet change and mycobacterial infection on that time ($p < 0.05$). Bars represent low PUFA control (■), low PUFA treatment (■), high PUFA control (■) and high PUFA treatment (■).

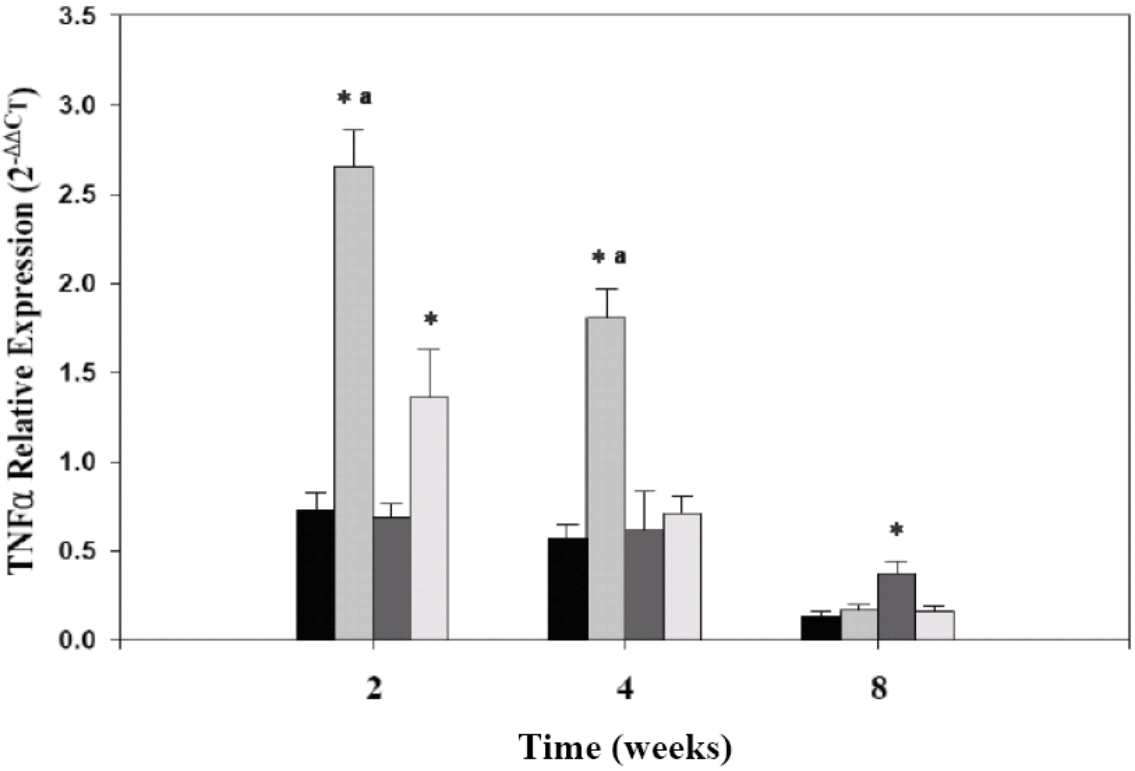


Fig. 4.3. Mean (±SEM) expression of tumor necrosis factor α gene, relative to elongation factor-1α (EF1α) of striped bass (8 SB/gp) fed either low PUFA or high PUFA for 10 weeks and sampled at 0, 2, 4 and 8 weeks post injection with *Mycobacteria marinum* (Treatment) or sterile PBS (Control). * indicates significant differences between control and treatment group on that time ($p < 0.05$). [a] indicates significant interaction between diet change and mycobacterial infection on that time ($p < 0.05$). Bars represent low PUFA control (■), low PUFA treatment (■), high PUFA control (■) and high PUFA treatment (■).

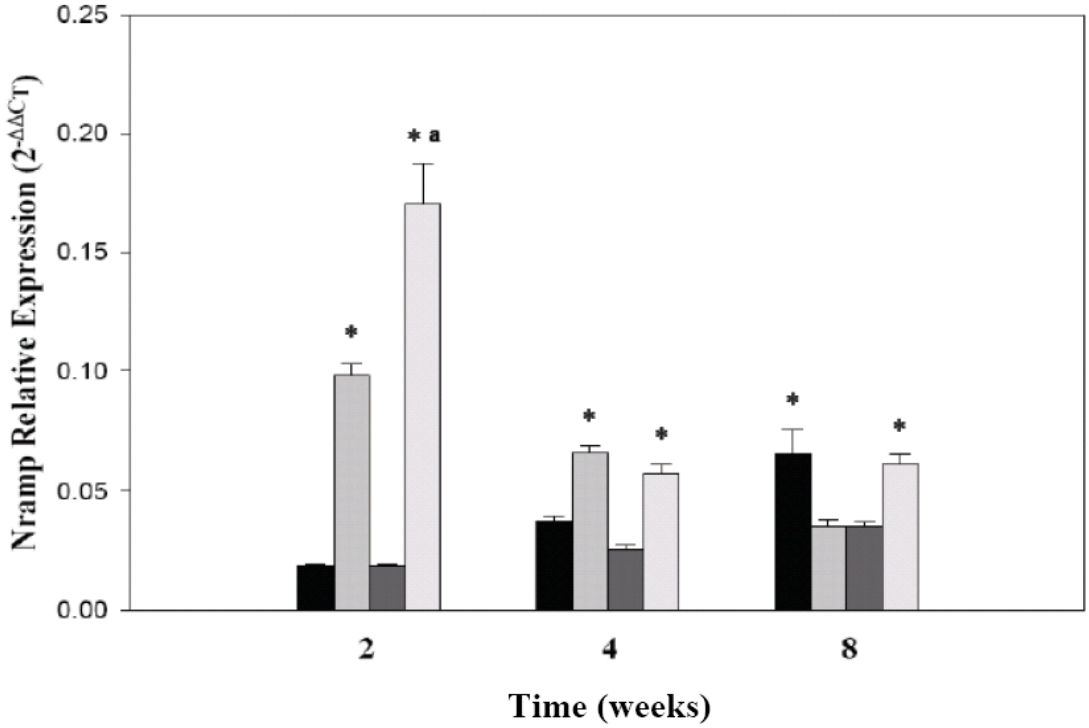


Fig. 4.4. Mean (±SEM) expression of natural resistance associated macrophage protein gene, relative to elongation factor-1α (EF1α) of striped bass (8 SB/gp) fed either low PUFA or high PUFA for 10 weeks and sampled at 0, 2, 4 and 8 weeks post injection with *Mycobacteria marinum* (Treatment) or sterile PBS (Control). * indicates significant differences between control and treatment group on that time ($p < 0.05$). [a] indicates significant interaction between diet change and mycobacterial infection on that time ($p < 0.05$). Bars represent low PUFA control (■), low PUFA treatment (■), high PUFA control (■) and high PUFA treatment (■).

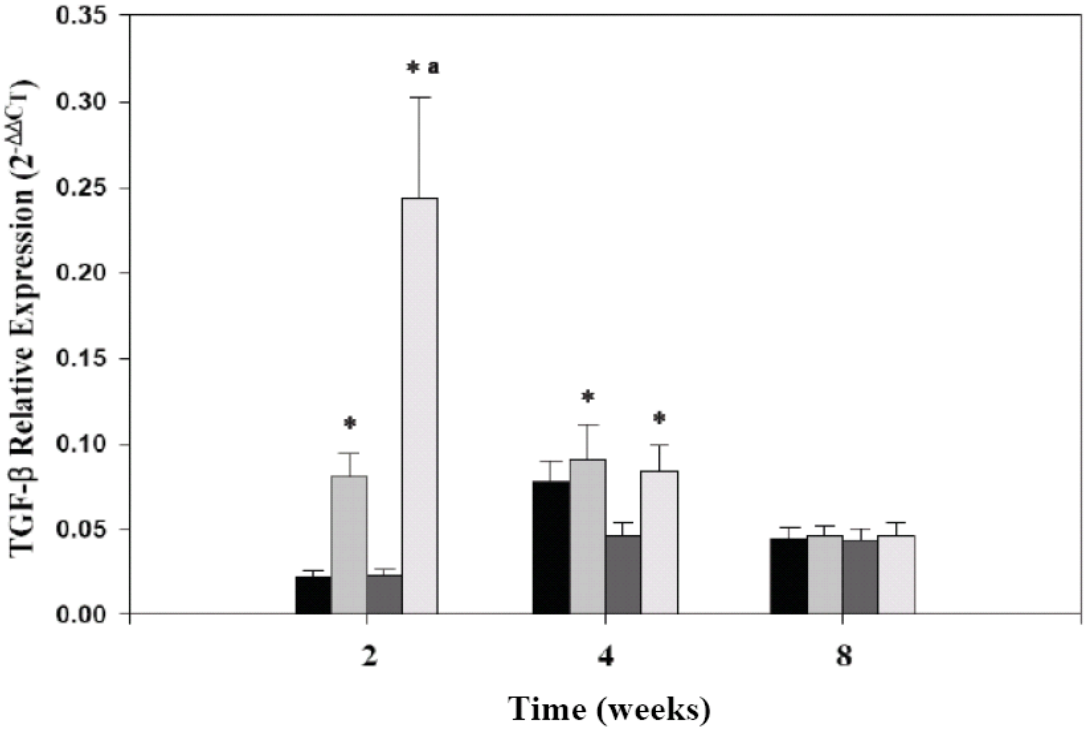


Fig. 4.5. Mean (±SEM) expression of transforming growth factor β gene, relative to elongation factor-1α (EF1α) of striped bass (8 SB/gp) fed either low PUFA or high PUFA for 10 weeks and sampled at 0, 2, 4 and 8 weeks post injection with *Mycobacteria marinum* (Treatment) or sterile PBS (Control). * indicates significant differences between control and treatment group on that time ($p < 0.05$). [a] indicates significant interaction between diet change and mycobacterial infection on that time ($p < 0.05$). Bars represent low PUFA control (■), low PUFA treatment (■), high PUFA control (■) and high PUFA treatment (■).

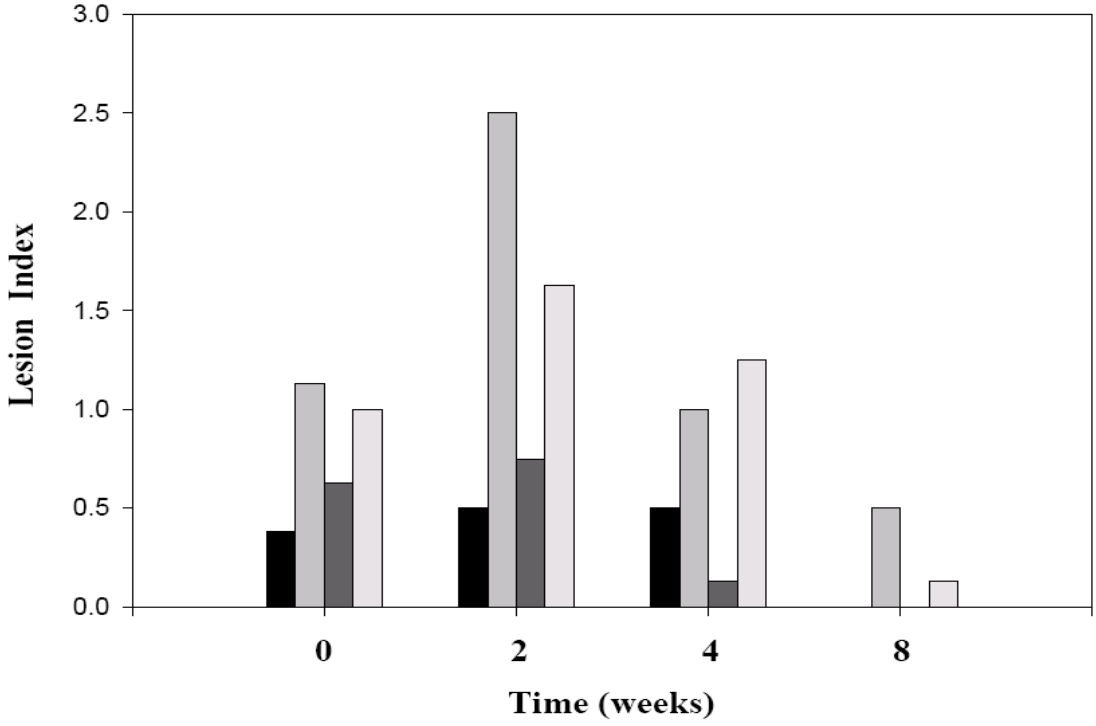
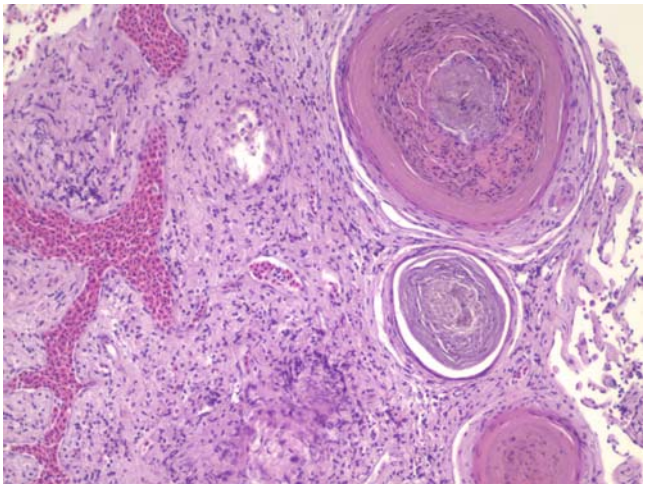


Fig. 4.6. Lesion index in the tissues including kidney, spleen, liver and heart. Index is scored for presence of absence of lesion. Lesion index = total organ affected per fish/ number of fish. Bars represent low PUFA control (■), low PUFA treatment (■), high PUFA control (■) and high PUFA treatment (■).

A)



B)

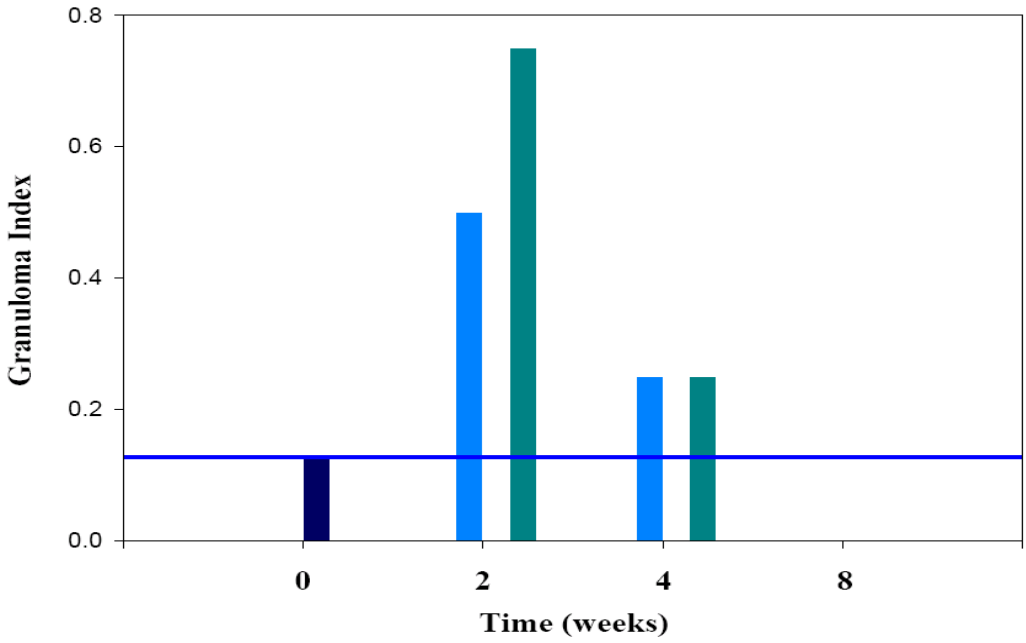


Fig. 4.7. A) H+E stain of liver granuloma in striped bass (200X magnification). B) Granuloma index in the tissues. Index was scored for presence of absence of granuloma (0 = absence, 1 = presence). Granulomas index = total organ with granulomas per fish/number of fish. Bars represent low PUFA treatment (■), high PUFA control (■) and high PUFA treatment (■).

Table 4.1. Comparison a low PUFA diet with a high PUFA diet at 2 weeks following *M. marinum* injection. ++ indicates higher production than + .

	Low PUFA Diet	**High PUFA Diet**
Bacterial Count	+ +	+
IL-1β **Nramp** **TGF-β**	+	+ +
TNF-α	+ +	+
Lesion Formation	+ +	+
Granuloma Formation	+	+ +

Chapter 5: Summary and Future Research

Striped bass (*Morone saxatilis*) is an economically and ecologically significant finfish species, and detection of mycobacterial infections in striped bass have increased in the Chesapeake Bay, Delaware Bay, Roanoke River, and recently in Long Island Sound and the New York Bight. The disease results in chronic, progressive granulomas, which is an important defense response against a mycobacterial infection. Cytokine production attribute to formation and maintenance of granulomas (Cooper et al. 1993; Ehlers et al. 2000). The current study indicated that Age-0 striped bass are capable of strong cytokine induction in response to immunological stimulation within a very short period of time. Likewise, juvenile striped bass are capable of controlling replication of *M. marinum* and clearing infection in less than a month. Strong inflammatory cytokine expression and leucocyte differentiation were associated with the response to infection. This suggests that cell mediated immune responses play an important role in resistance to mycobacterial infection very early on. It will be important for future research to identify the role of T- and B-lymphocytes in mycobacteria-related granuloma formation in striped bass (possibly through flow cytometry), as well as the signaling cascades involved and potentially driving T_{h1} vs. T_{h2} immune responses.

Data also indicate that high PUFA diets are more beneficial to a successful immune response against *M. marinum* than a low PUFA diet, by increasing cytokine production and granuloma formation. Although changes in PUFA content affected the host responses to mycobacteria infection, they did not affect the overall outcome of infection, as the disease

was cleared by all infected fish. Therefore, this suggests that more drastic changes to diet would be required for an effect to be realized in wild populations. As mentioned above, declines in Atlantic menhaden may cause reduction of PUFA intake in striped bass. Furthermore, low intake of PUFA diet may weaken the immune system, and it may lead to severe mycobacteriosis in striped bass. Therefore, further study should be done to identify how menhaden inclusion or other prey items in the diet can contribute to PUFA intake and how this affects mycobacterial infection.

Disease impacts on striped bass populations have not yet been established due to chronic nature of mycobacteriosis. Recently, however Gauthier et al. (2008) estimated mycobacteriosis-associated mortality in wild striped bass from Chesapeake Bay using an epidemiological model, where age and sex variants were significant factors. Prevalence increased with age through age 5 and male fish revealed higher prevalence than female fish. Environmental factors such as temperature, dissolved oxygen levels, salinity or pH as well as nutritional stress may be involved in severity of disease (Coutant 1985; Price et al. 1985; Falkinham et al. 2004; Jacobs et al. 2009). Parasite infection may also be associated with prevalence of mycobacteriosis in terms of the effect on health of the striped bass and transmission of mycobacterial infections. *Philometra* sp. (Nematodes), *Pomphorhynchus* sp. (Acanthacephalans) or *Argulus* sp. etc, are commonly found in Chesapeake Bay and other populations of striped bass. With regard to *Pomphorhynchus* sp. infections in Chesapeake Bay striped bass, Stine (2008) found it to be a significant factor contributing to the prevalence of mycobacterial infections. As *Pomphorhynchus* sp. are observed to perforate the intestinal wall with their proboscis (Pers. Obs.), they may provide an entry point for

mycobacteria infection.

Enhanced exposure due to age, is an obvious reason for why older/larger fish have higher prevalences, however, generally age 0 fish show lower prevalence even when compared to age 1-3 fish (Stine 2008). The above factors involved in mycobacteriosis may have different effects on striped bass populations, depending on habitat usage by particular age groups. Age-1 striped bass used in long-term infection experiment exhibited resistance to mycobacterial infection as bacteria for the most part were cleared in less than 8 weeks, in comparison to other studies using younger fish. As the immune system matures, especially specific immunity after the first year, we can expect to observe greater resistance to pathogens as compared to y-o-y, all things being equal. Whereas age-variant disease-associated mortality in striped bass may be the sum of exposure incidence, endoparasitic co-infection, mating and nutritional stress, etc.

This investigation enhanced our knowledge of striped bass immunology and lower vertebrate responses to mycobacteria infection. Investigation of immune responses and their interaction with stress in striped bass, may be useful in predicting disease outbreaks in relation to increasing anthropogenic stressors. Further work needs to be done to elucidate the relationship of host strain to mycobacteria infection, as well, since only Chesapeake Bay strains show the high prevalence and severity of disease in the wild.

Polymorphisms in Nramp, TNF-α or IL-1β have been shown to negatively affect resistance to tuberculosis (TB). Several studies have shown that deletions and/or point mutations of the Nramp1 gene were associated with susceptibility to TB (Bellamy et al. 1998; Gao et al. 2000; Ryu et al. 2000). Deficiency in TNF-α receptor 1 or the TNF-α gene

showed high susceptibility to TB infection and resulted in death (Flynn et al. 1995; Marino et al. 1997). Furthermore, KO mice without IL-1 α/β or TNF-α were susceptible to mycobacteria with poor granuloma formation (Senaldi et al. 1996; Bean et al. 1999; Kaneko et al. 1999; Juffermans et al. 2000; Roach et al. 2001). An interesting area of future research would be to look at variants in these genes both at the genomic and transcriptomic level in these different strains of striped bass. Potential differences in susceptibility across striped bass strains or stocks are even more important now than ever. With increases in ocean temperature continuing and anthropogenic stressors in estuarine environments not showing any signs of decline in the Northeast, the potential for the right set of circumstances to cause epizootics of mycobacteria infection in other stocks of striped bass remains.

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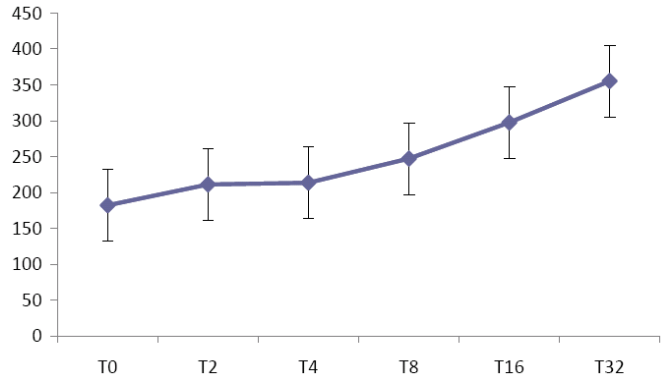
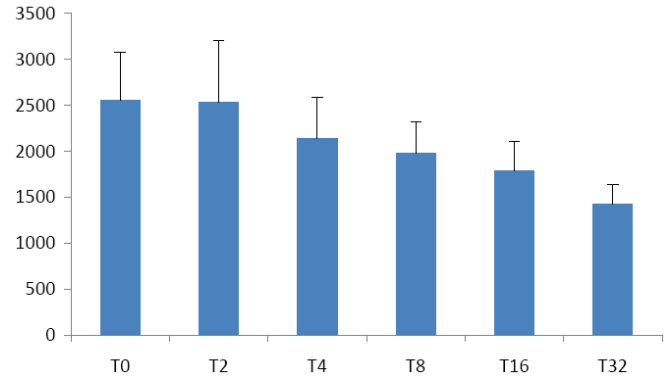
Appendix

a)



b)



Appendix 1. a) The average size (+/- SD) and b) the average biomass per tank (+/-) over 32 weeks for the diet study (Chapter 4).