ABSTRACT

Title of Dissertation: MYCOBACTERIOSIS IN CHESAPEAKE BAY STRIPED BASS (MORONE SAXATILIS): THE INTERACTION OF NUTRITION AND DISEASE.

John M. Jacobs, Doctor of Philosophy, 2007

Dissertation directed by: Professor Reginal M. Harrell and Associate Professor Ana Baya

A series of field and laboratory studies were conducted from 1998 - 2005 to examine the relationship between nutritional status and mycobacteriosis in Chesapeake Bay striped bass (*Morone saxatilis*). A review of archived tissue blocks of wild striped bass collected from 1975 – 1995 revealed that this is not a new disease of the species. Application of genus specific PCR and multi-gene sequencing from 1984-1985 samples identified the species as one of the tuberculosis clade common in the current epizootic in Chesapeake Bay. Closest identity was found with the recently described *M. pseudoshottsii.* These findings, 13 years before the initial isolations from Chesapeake Bay fish, suggest that other factors may be relevant in enhancing an endemic disease to the current elevated prevalence (> 50% in age 3+ fish). Field surveys and feeding trials were conducted from 1998-1999 to determine the overall nutritional condition of striped bass and the association with disease state. Proximate composition revealed elevated moisture (~ 80%) and low storage lipids (< 0.5% ww), characteristic of a poorly nourished population. Wild collected fish (age 3 and 4) were not significantly different in chemical composition, weight at length, or mesenteric body fat than fish

experimentally deprived of food for two months ($p > 0.05$). These findings were not consistent with data collected in 1990-1991, or with experimentally fed fish used as benchmarks for this study. Mycobacteriosis explained little of the variance in chemical composition ($p > 0.2$); however elevated moisture and low lipid concentration were associated with fish with ulcerative lesions ($p < 0.05$). This suggests that age 3 and 4 striped bass were in poor nutritional health in 1998-1999, which may be independent from the disease process. Based on these findings, challenge studies were performed to address the hypothesis that disease progression and severity may be altered by nutritional status of the host. Intraperitoneal inoculation of 10^4 colony forming units (CFU) *M. marinum* resulted in high mortality, elevated bacterial density, and poor granuloma formation in low ration (0.15% bw/d) groups while adequately fed fish (1% bw/d) followed a normal course of granulomatous inflammation with low associated mortality to a steady, equilibrium state. Further, we demonstrated that an active inflammatory state could be reactivated in fish through reductions in total diet. The energetic demand of mycobacteriosis, determined by tissue chemical composition, was insignificant in comparison to sham inoculated controls in adequately fed fish ($p > 0.05$). Declines in total body energy were only apparent during active, inflammatory stages of disease. Overall, these findings suggest that: 1) mycobacteriosis is not a new disease of Chesapeake Bay striped bass, 2) the disease has little energetic demand in the normal, chronic progression, and 3) poor nutritional health can greatly enhance the progression and severity, and reactivation of disease. The implications of this research are that management strategies focused on enhancing the nutritional state of striped bass could potentially alter the disease dynamics in Chesapeake Bay.

MYCOBACTERIOSIS IN CHESAPEAKE BAY STRIPED BASS (MORONE SAXATILIS): THE INTERACTION OF NUTRITION AND DISEASE

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DEDICATION

In memory of my grandmother, Elizabeth McIntosh

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Chapter 1 – INTRODUCTION

An epizootic of mycobacteriosis is affecting striped bass (*Morone saxatilis*) in Chesapeake Bay (Heckert et al. 2001, Rhodes et al. 2001, Overton et al. 2003, Rhodes et al. 2004, Ottinger and Jacobs 2006). Conditions leading to the onset or continuation of this chronic, progressive disease have been the subject of much debate among the scientific and lay communities alike. The Atlantic Coastal population of striped bass has undergone considerable changes in the past two decades, increasing by an order of magnitude in a ten year period (ASMFC 2005). It is evident that corresponding declines in prey availability and changes in the diet of striped bass have occurred (Hartman and Margraf 2003, Overton 2003, Pruell et al. 2003, Uphoff 2003, Walters et al. 2003). In addition, novel pathogens and multiple species of *Mycobacterium* have been isolated further complicating the picture as the relative contribution and virulence for many is largely unknown (Heckert et al. 2001, Rhodes et al. 2001, Rhodes et al. 2004). Finally, the Chesapeake Bay is a eutrophic, temperate estuary characterized by high summer water temperatures, elevated nutrient loading and primary productivity, and extensive "dead zones" or areas of hypoxic waters (Kemp et al. 2005). Thus, multiple stressors are present and may be impacting host susceptibility and disease progression. These stressors may act through a common mechanism of host immunosupression, or divergent mechanisms relating to pathogen abundance or virulence. Of the plausible factors relevant to this epizootic, changes in host nutritional competence merits investigation and is the focus of this dissertation. In this introductory chapter, I review the status of the Atlantic Coast striped bass population and changes in prey availability and consumption. Further, parallels are drawn with the influence of nutritional state in human tuberculosis

to what is known in fish. Finally, hypotheses are presented concerning the potential for a relationship between nutritional competency and disease in striped bass.

A Restored Population

The striped bass is the largest member of the family Moronidae, and can attain sizes of up to 57 Kg and 200 cm in length (Seltzer et al. 1980). From the Roanoke River, NC to southern Maine, striped bass undergo extensive coastal seasonal migrations (north in summer, south in spring and fall) with females most likely returning to natal river systems each spring to spawn (Chapman 1990). South of North Carolina, and north of Maine, populations are largely riverine with little to no migration (Freeman 1977, Boreman and Lewis 1987). In addition, extensive stocking of the species on the Pacific coast and in a variety of freshwater reservoirs from the 1800s on has led to new self-sustaining populations (Seltzer et al 1980).

The striped bass is a prized recreational species in most all of its range, with commercial importance varying state by state. In the mid-Atlantic region, over 12 million pounds of fish were captured by recreational anglers (total catch, including released) in 2001 with almost 2 million pounds harvested commercially (National Marine Fisheries Service, Fisheries Statistics and Economics Division, personal communication). The current level of angler success is due to intensive management efforts after a serious population decline in the late 1970s and early 1980s (Richards and Rago 1999).

By the mid 1970s, declines in commercial harvest and spawning stock biomass were evident preceding a number of management actions aimed at limiting harvest and further investigating causes for decline (Figure 1-1). In 1979, the Emergency Striped

Bass Study Program was passed as an amendment to the Anadromous Fish Act (VERSAR 1990). This research effort was quickly followed by the establishment of an inter-juristictional fisheries management plan by the Atlantic States Marine Fisheries Commission (ASMFC) with recommendations adopted by most member states by 1984 (Richards and Rago 1999). Also in 1984, Congress passed the Atlantic Striped Bass Conservation Act which essentially empowered ASMFC by allowing for the imposition of a Federal moratorium in States that were found out of compliance. In 1985, Maryland voluntarily declared a moratorium on striped bass harvest and sales, followed by Virginia in 1989. Recruitment over-fishing and habitat issues were identified as likely causes. Efforts from the mid 1980s through the early 1990s concentrated on rebuilding the stock structure through management regulations supplemented with intensive stocking efforts. During this time period increases in spawning stock biomass and juvenile production led to vast improvements in the age structure of the population (VERSAR 1990, Richards and Rago 1999). By 1990, stocks had sufficiently recovered to allow for limited fisheries in both states. By 1994, the Atlantic coast striped bass population had reached historic levels of abundance (Figure 1-2). At the same time, reports of ulcerative lesions on striped bass began to surface (Eric May, University of Maryland Eastern Shore (UMES), personal communication).

Disease in Chesapeake Bay striped bass is not a recent phenomenon, but may have increased in regularity and severity in recent years. In the spring and summer of 1988, an outbreak of *Streptococcus* sp. affecting striped bass, weakfish (*Cynoscion regalis*) and bluefish (*Pomatomus saltatrix*) occured Bay-wide (Baya et al. 1990). Sporadic reports of dead striped bass were received by the Maryland Department of

Natural Resources (MDNR) Fish Health Program between 1988 and 1994 with bacteria isolated being primarily gram-negative enterics (Eric May, UMES, personnel communication). In this case, rafts of dead fish were seen at varying locations from the Bay Bridge-Tunnel to Kent Island (upper Maryland portion of Chesapeake Bay).

In the spring of 1994 an isolated disease outbreak occurred in the Potomac River and at the mouth of the Wicomico River, which involved over 50 fish. This outbreak was diagnosed as being the result of *Edwardsiella tarda* (Baya et al. 1997). In 1995 a second outbreak in the Potomac was seen. In this case, affected fish yielded isolates of *Aeromonas* sp. and *Pseudomonas* sp. Both cases were initially reported by watermen based on the observation of a high prevalence of external lesions in pound net captured fish. Clinically, the lesions appeared as multiple red sores, which often coalesced to cover large areas of the epidermis.

Samples taken from fish exhibiting external lesions from 1994 - 1997 revealed an assortment of pathogens including: *E. tarda, A. hydrophila, Escherichia coli, P. putrifacians*, and several species of *Vibrio (*Ana Baya, unpublished data). The prevalence of these lesions often exceeded 25%, and had a strong seasonal trend of elevation in the early summer, declining by late fall (MDNR, Striped Bass Tagging Program, and Beth Rodgers, MDNR, personal communication). Infectivity trials conducted with *E. tarda* revealed pathogenicity at 10^5 cells (LD_{50}), however external ulcers were not formed (Baya et al. 1997). Similar results were obtained previously by Herman and Bullock (1986) with age-0 hatchery-raised striped bass.

In 1997, mycobacterium was isolated from striped bass in Maryland and Virginia waters (Heckert et al. 2001, Rhodes et al. 2001). Over the past decade, numerous efforts

by state, federal, and academic institutions have served to define the distribution and prevalence of this disease, as well as the major mycobacterial pathogens involved (Rhodes et al. 2004, Ottinger and Jacobs 2006). However, few efforts have directly examined the role of environmental stressors and disease state.

The biomass of Atlantic coast striped bass increased by an order of magnitude in the ten year period from 1984-1994 (ASMFC 2005). While touted as a true management success story (Richards and Rago 1999), the implications of this rapid increase in a single, higher trophic order predator to the ecosystem as a whole may not have been thoroughly considered. The concept of density dependence in fish populations has most often been addressed in larval and juvenile stages in terms of mortality (Shepard and Cushing 1990). In general, at these sensitive life history stages, competition for food, habitat, or other limiting needs will result in higher mortality within a cohort to maintain equilibrium within the system. Thus the concept of density dependence is one of self regulation to maintain balance at the population level (Krebs 1985). Density dependence in adult fish is thought to be a rare occurrence (Shepard and Cushing 1990). However, it can become an issue when food limitation differentially affects year classes based on ontogenetic shifts in the prey consumed or size selectivity (Shutter 1990, Jonsson et al. 1998). Infectious disease general exhibits density dependent transmission as well, so the *per capita* risk of infection is greater at higher population density (Wilson et al. 2002). Thus, a population, or segment of a population nearing or exceeding its carrying capacity may be expected to experience density dependent regulation expressed in ways such as increased disease incidence and food limitation. These conditions may not be independent.

A Case for Food Limitation

Striped bass are opportunistic feeders and forage at various trophic levels throughout their life. Feeding generally ensues at 5 days post hatch (dph), with larvae targeting *Cyclops* nauplii and copepodites, gradually moving onto Mysids by 30 dph. By 100 dph, fish sources are targeted (Seltzer et al. 1980). Juvenile striped bass are non-selective, feeding on insect larvae, polychaetes, larval fish, mysids, and amphipods. (Setlzer-Hamilton and Hall 1991). Age-1 striped bass undergo an ontogenetic shift in foraging from primarily invertebrate sources to fish (Hartman and Brant 1995). However, the timing of this shift may be spatially influenced by prey availability (Overton 2003). Age 2+ fish historically have preferentially fed on soft-rayed fish such as Atlantic menhaden (*Brevoortia tyrannus*) and Norfolk spot (*Leiostomus xanthurus*) (Hartman and Brant, 1995, Griffin and Margraf 2003). However, in recent years, the relative abundance and contribution of prey items in the diet of adult striped bass has changed.

Since 1958, the MDNR has conducted annual seine surveys which serve as an index of relative prey abundance and recruitment for several species in Chesapeake Bay. While abundance has been variable, sustained reductions in juvenile Atlantic menhaden, bay anchovy *(Anchoa mitchilli)*, and spot have persisted since the mid 1990s (Figure 1-3).

Griffin and Margraf (2003) analyzed historic stomach content data from striped bass collected from 1955-1959 using an index of relative importance (IRI). Small striped bass (< 600 mm TL, age-5 and under) relied predominately on Bay anchovy (IRI = 67%), while the diet of larger striped bass (> 600 mm TL) was dominated by Atlantic menhaden (IRI = 93%). In the late 1990s and early 2000s, Overton (2003) found a comparative

reduction in Atlantic menhaden consumption, and increased feeding on bay anchovy and blue crab. The impact of this shift in dietary sources was greatest in fish age 3+ (Figure 1-4). Consequently, growth was reduced in age 4+ fish, a process suggested to occur because of lower energy density of invertebrate sources (Overton 2003). This shift in diet to benthic sources was also corroborated by Pruell et al. (2003) who examined stable carbon isotope ratios in 3-5 year old fish from 1982-1997 and Walter et al. (2003) who found increased prevalence of benthic fishes in stomach content of age 4+ striped bass suggesting an overall dietary shift with the decline of preferred pelagic sources. The impact of these findings is supported by monitoring data of the MDNR who show reduction in length at age of 3-5 year old striped bass (Warner et al. 2005) (Figure 1-5).

Hartman and Margraf (2003) suggest that seasonal food shortages have been limiting striped bass potential growth since at least 1993. This concept of predator-prey imbalance was recently addressed by Uphoff (2003) who found menhaden populations are currently incapable of supporting striped bass production. In addition, in one of the only long-term data sets available where weight and length were taken, Uphoff (2003) found increased variability in weight at length in the later part of the 1990s. An updated version of this data continues to support Uphoff's analysis (Table 1-1), however high intra-annual variability exists, and analysis of covariance of slopes, or of Fulton's condition factor are inconclusive (unpublished data). However, the weight of evidence concerning predator-prey imbalance, shifts in forage, and decline in growth combined with the current disease status of striped bass is suggestive of density dependent forces.

The Interaction of Mycobacterial Diseases and Nutrition

The issue of nutritional status and mycobacterial disease is complex. Wasting is a recognized manifestation of tuberculosis (TB) in humans (Macallan 1999, Paton and Ng 2006), however, the associated pathology remains poorly understood (Schwenk et al. 2004). The cause is most likely a combination of decreased appetite and energy loss due to altered metabolism and demands of the inflammatory and immune response. Wasting associated with TB typically results in the loss of whole-body fat mass and lean tissue mass in relatively equal proportions (Paton and Ng 2006).

In clinical trials, TB infection does not alter protein flux (Patton et al. 2003), but may change the protein anabolic response to food ingestion (Macallan et al. 1998). Schwenk et al. (2004) noted that patients recovering from TB, human immunodeficancy virus (HIV), and bacterial septis tended to gain fat mass in greater proportion than lean tissue mass. However, the ratio of protein energy to total energy continued to decline after six months of treatment reflecting a lack of protein accretion despite positive total energy balance. These findings are consistent with those of Macallan et al. (1998) where TB patients failed to channel food protein into endogenous protein synthesis, a process termed "anabolic block". It is suggested that this failure to efficiently use exogenous protein sources is due to impairment of the use of amino acids for protein synthesis by pro-inflammatory cytokines (Macallan 1999).

Wasting is also a trademark of infection with non-tuberculosis mycobacterium in other mammals. Most notably, Johne's disease, caused by *Mycobacterium. avium subsp. paratuberculosis,* commonly results in wasting in most ruminants, and reduction of milk production in dairy cattle. In ruminants, infection results in severe gastroenteritis and

associated diarrhea and loss of body condition. Wasting may be associated with tissue damage altering the efficiency of post-absorptive processes (Harris and Barletta 2001).

As with mammals, reduction in condition has been reported in many cases of freshwater tropical and marine aquaria fishes in association with mycobacteriosis (Inglis et al. 1993, Chinabut 1999, Conroy and Conroy, 1999). However, in most cases these reports are from moribund fish in the final stages of disease progression. In a large scale investigation of mycobacteriosis in Atlantic mackerel (*Scomber scrombus*) (n=9470), MacKenzie (1980) noted increased prevalence and severity with age, and corresponding declines in length and condition. However, these differences were inconsistent, minor and rarely significant ($p < 0.05$) in comparisons of fish of the same sex, age and region.

While Mycobacteria are associated with wasting, susceptibility and progression of the disease can be directly influenced by nutritional status in humans further complicating the issue. The ability of inadequate nutrition to adversely alter the progression and outcome of TB is well known (Chandra 1996). Homeless individuals and alcoholics are at higher risk of pulmonary tuberculosis than other groups (Takano et al. 1999). Over eight million new cases of tuberculosis are reported annually leading to an estimated two million deaths worldwide (Wieland et al. 2005). The primary risk factor is immunosupression, which is known to be greatly influenced by both food quantity, and specific macro-and micro-nutrient deficiencies (Chandra 1996, Wieland et al. 2005). While the literature on this subject is dominated by clinical trials and with murine and other higher-order vertebrate models, there are many parallels that exist with fish immune function.

Examinations of the effect of overall reduction in energy intake on disease resistance in fish have demonstrated a range of responses, which may be species specific. Lim and Klesius (2003) recently demonstrated reduced mortality, anemia, and elevated macrophage chemotaxis in well-fed channel catfish (*Ictalurus punctatus*) challenged with *Edwardsiella ictaluri*, the pathogen responsible for enteric septicemia (ESC). In contrast, the immune system of chinook salmon (*Oncorhynchus tshawytscha*) may be more robust with respect to ration. Only minor changes were noted in immune function by Alcorn et al. (2003) with increasing ration, which suggests that composition of the diet may be more important than available energy.

Much work in clinical medicine has focused on the association of protein calorie malnutrition (PCM) and reduced immune function. Chan et al. (1996) demonstrated that mice receiving a reduced protein diet (2%) rapidly succumbed to TB accompanied by a reduced expression of interferon γ, tumor necrosis factor ά, and nitric oxide synthase in the lungs. These cytokines, as well as interleukin -1, interleukin -4, and transforming growth factor β are critical to the production of nitrous oxides and reactive nitrogen intermediates, which is the principal mechanism by which phagocytes kill (Plouffe et al. 2005). The mycobacteriocidal process is dependent on the amino acid L-arginine as a substrate for cytokine production. Remarkably, fulminant TB characterized by poorly formed granulomas and elevated bacterial density could be reversed in mice by increasing protein levels to match those of the controls (20%).

Dia and McMurray (1998) obtained similar results in protein malnourished guinea pigs (*Cavia porcellus*) and *in vivo* challenges of harvested spleen macrophages. Low protein intake reduced the production of interferon γ, tumor necrosis factor ά, and tumor

necrosis factor β; essentially altering the cytokine profile to favor macrophage deactivation. Non specific responses, such as the mobilization of inflammatory cells, phagocytosis, intracellular killing, neutrophil mobility, and production of macrophage cytokines may also be reduced under conditions of inappropriate or insufficient food sources (Dia and McMurray 1998).

Lipids have also been shown to influence immune function with respect to mycobacterial pathogenesis. Of particular interest to this dissertation are the essential fatty acids alpha linolenic acid (omega-3), and linoleic acid (omega-6) due to their high concentration in Atlantic menhaden. Paul et al. (1997) challenged guinea pigs with *M. tuberculosis* previously held on diets containing elevated concentrations of linoleic, linolenic, or saturated fatty acids. Supplementing diet with linolenic fatty acids resulted in significantly higher splenic bacterial loads, and enhanced severity of infection in comparison to all other diets. The immunosuppressive influence of unsaturated fatty acids is supported by *in vitro* work of Huges et al. (1996) who demonstrated their capability to alter immune cell membrane profiles. In fish, Sheldon and Blazer (1991) found high levels of n-3 fatty acids to enhance bactericidal activity to *Edwardsiella tarda* in channel catfish, but did not enhance overall phagocytic activity. Low levels of n-3 fatty acids, and density associated stress have been correlated with increased macrophage aggregates (melano-macrophage centers) in splenic tissue of gilthead seabream (Montero et al. 1999). Thus the relationship between fatty acids and immune function is not completely understood, and may not be consistent in fish and mammals.

Micro-nutrients are those required in minimal amounts, but their absence in diet can be equally influential on immune function. In particular, their role in oxidant

mediated innate immune response has received considerable attention in clinical medicine (see review by Erickson et al. 2000). In beige mice, reductions in dietary calcium are protective against *M. avium* subsp. *paratuberculosis* (Stabel et al. 1996). However, a corresponding increase in vitamin D reverses the calcium effect (Stable et al. 1998). In fish, most effort has been directed towards the ability of ascorbic acid (vitamine C) and vitamin E to enhance macrophage activity and function (Blazer 1991).

Much of this discussion of the influence of nutrition on immune function refers to mammalian systems. While our understanding of fish immunology continues to grow, it is clear there are many homologies to higher-order vertebrate immune systems (Plouffe et al. 2005). Fish possess both specific and non-specific immune response capabilities, although the former is somewhat slower and less refined than in mammalian systems. Of particular interest in immune response to mycobacterial infection is the capacity of the innate immune system. In higher-order vertebrates, the immune response to TB exposure is highly dependent on T-lymphocytes and their associated cytokines, which are particularly sensitive to nutritional insult (Dia and McMurray 1998). Fish possess a full complement of cytokines similar in function to mammalian systems (Plouffe et al. 2005).

In response to microbial pathogens, phagocytosis is associated with increased oxygen consumption by specific immune cells, a phenomenon known as "respiratory burst." This process is associated with the production of reactive oxygen intermediates, is known to occur in fish (Secombes et al. 1992), and demonstrated to be inducible by mixed cytokine preparations (Neumann and Belosovic 1996). Reactive nitrogen intermediates are controlled by the enzyme nitric oxide synthase, which in turn is regulated by a host of cytokines. Nitrous oxide synthase is inducible in fish, responds to

bacterial challenge, and is inactivated by antagonists to L-arganine as demonstrated in mammalian systems (Plouffe et al. 2005, Chan et al. 1996). To date, only transforming growth factor β has been investigated in response to mycobacterial infection in fish, with results suggesting a lack of involvement in the control of bacterial proliferation (Harms et al. 2003).

The model that is evolving from the medical literature is one of a cat and mouse game between host immune function and mycobacterial replication (Chandra 1996). Once engulfed by macrophages, bacteria may replicate freely within the cell. This triggers a cascade of cytokine-mediated events leading to the formation of granulomas in an attempt to limit the spread of disease and focus efforts to destroy the pathogen. In immunocompetent hosts, the acute phase of disease often gives way to either a latent or chronic state where bacteria are often readily culturable and visible within granulomas (Flynn and Chan 2001). Recent work suggests that there is a dynamic equilibrium between host immune function and mature granulomas (Bouley et al. 2001), in contrast to theories of bacteria persisting in a resting state. Their findings suggest that bacterial killing within the granuloma is balanced by pockets of freely replicating cells, sometimes within the same macrophage. Exactly how some mycobacteria evade the attempts of the host immune system is unclear, but the implications are that a reservoir is maintained within the host for potentially a lifetime. It is estimated that 1/3 of the worlds population is infected with tuberculosis (Flynn and Chan 2001). Similarly, over 50% of age 3+ striped bass are infected with related fish pathogens in Chesapeake Bay (Ottinger and Jacobs 2006). Whether through disruption of cytokine profiles and subsequent macrophage activation (Chan et al. 1996, Dia and McMurray 1998) or mechanisms yet to

be determined, it is clear that nutritional insult can disrupt this equilibrium in favor of the pathogen.

Hypothesis Relating Nutritional State to Mycobacterial Disease in Striped Bass

In the preceding pages, I have expressed in detail changes that have resulted in association with a restored Atlantic coast migratory stock of striped bass, and the potential of nutritional deficiency of various forms to influence immune function and disease expression. In my discussion, I suggest that 1) mycobacterial disease is associated with ecosystem imbalance, 2) this imbalance is reflected in changing predator-prey dynamics and nutritional competency of striped bass; and 3) poor nutrition is capable of negatively impacting disease state. Causative relationships in disease are often difficult to discern due to the complex relationship between host, pathogen, and the environment (Sindermann 1970). New pathogens or changes in virulence can impact hosts directly. It is the premise of this dissertation that environmental stressors negatively impact disease state.

In Chapter 2, I review the state of knowledge regarding mycobacteriosis in marine fish. The review extends from early reports of "piscine tuberculosis" in cod (*Gadus morhua*) (Aronson 1926) through published works available by June 2006. Much additional work is ongoing in the Chesapeake region, which is highlighted in Ottinger and Jacobs (2006). Chapter 2 concludes with acknowledgement of the complexity of disease in wild populations and a call for increased collaboration among traditional fisheries ecologists, human epidemiologists, and fish-health professionals to examine disease in a holistic, ecosystem-based framework.

In Chapter 3, I address the specific hypothesis that mycobacterial disease in Chesapeake Bay striped bass is not a new occurrence. This is an important concept in the framework of host, pathogen, and environment. New diseases affecting a population may result in epizootics solely due to lack of host defense against an introduced pathogen. It is the premise of this thesis that environmental stressors have influenced a disease that was always present. Through archival review and isolation and sequence analysis of mycobacterial DNA from formalin-fixed tissue blocks, I explore the occurrence and mycobacterial species present from 1970 to present.

H_0: The current epizootic is the first indication of mycobacteriosis in Chesapeake Bay striped bass based on available tissue archives.

Chapter 4 relates findings of laboratory and field efforts in defining the nutritional status of fall (September – November) collected striped bass, and the association of status with disease state. I apply proximate composition to wild collected fish to provide a detailed assessment of nutritional health.

H_O: Chemical composition of fall collected Chesapeake Bay striped bass is reflective of poor nutritional state.

H_1: Chemical composition and condition indices of striped bass with histologically detectable mycobacteriosis do not differ from those without.

In Chapter 5, I expand on the methods employed in the previous chapter to develop a cost-effective means of collecting whole-body energy data while allowing for full health assessment of the same organism. Developing this method is essential for energetic comparisons used in the following chapter.

H_O: Proximate composition of specific fish tissues do not correlate with whole body composition.

In Chapter 6, I use controlled laboratory studies to examine questions derived from field studies concerning the energetic demand of mycobacteriosis in striped bass. Here I use *M. marinum* as a model to describe the influence of ration on disease progression and host energetics.

H_0: The progression and severity of mycobacteriosis associated with *M. marinum* is independent of nutritional state.

Finally, Chapter 7 summarizes my findings pertaining to the issue of nutritional status and striped bass mycobacteriosis.

Table 1-1. Log length-weight regressions of fall collected adult striped bass in Maryland's Chesapeake Bay from 1990 -2003. Data originally presented in Uphoff (2003) and updated with data courtesy of the Maryland Department of Natural Resources.

		Slope		Intercept		
Year	N	Mean	SE	Mean	SE	R2
1990	41	3.19	0.09	-15.1	0.56	0.97
1991	1595	2.98	0.02	-18.3	0.14	0.92
1992	1362	3.10	0.03	-19.0	0.02	0.87
1993	1690	3.32	0.20	-20.6	0.13	0.95
1997	667	2.84	0.04	-17.5	0.26	0.87
1998	929	3.30	0.04	-20.5	0.24	0.89
1999	427	3.17	0.05	-19.6	0.32	0.90
2000	768	3.23	0.05	-20.0	0.28	0.87
2001	302	2.34	0.05	-14.4	0.28	0.90
2002	678	2.79	0.05	-17.2	0.28	0.85
2003	1374	2.65	0.02	-16.4	0.12	0.94

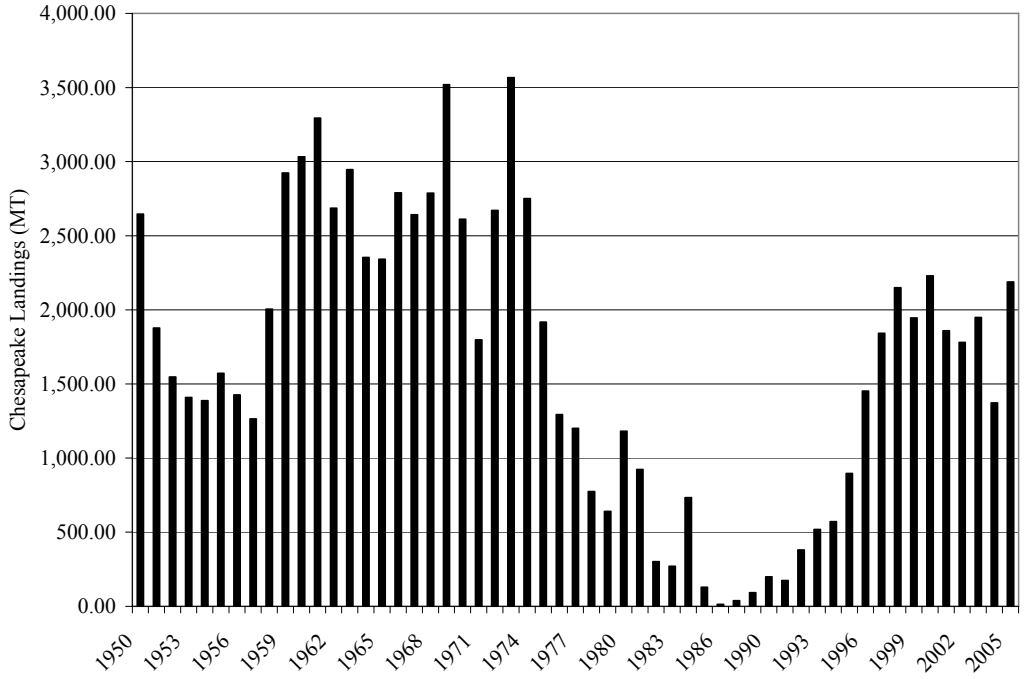

Figure 1-1. Total landings of Chesapeake Bay striped bass in metric tons from 1950-2005. Moratoria were declared in MD from 1985-1990 and in Virginia from 1989-1990. Data courtesy of the National Marine Fisheries Service, Fisheries Statistics.

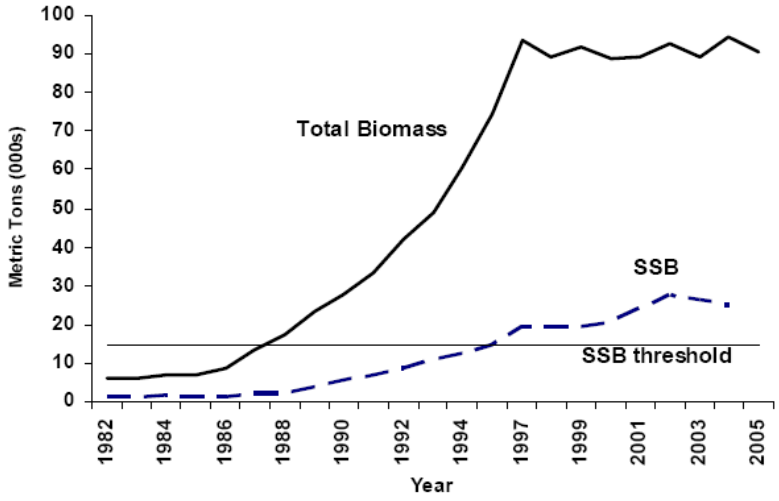

Figure 1-2. Total Atlantic coast striped bass biomass (metric tons) and spawning stock biomass (SSB) based on the virtual population analysis (VPA) of the ASMFC 2004 stock assessment (ASMFC 2005).

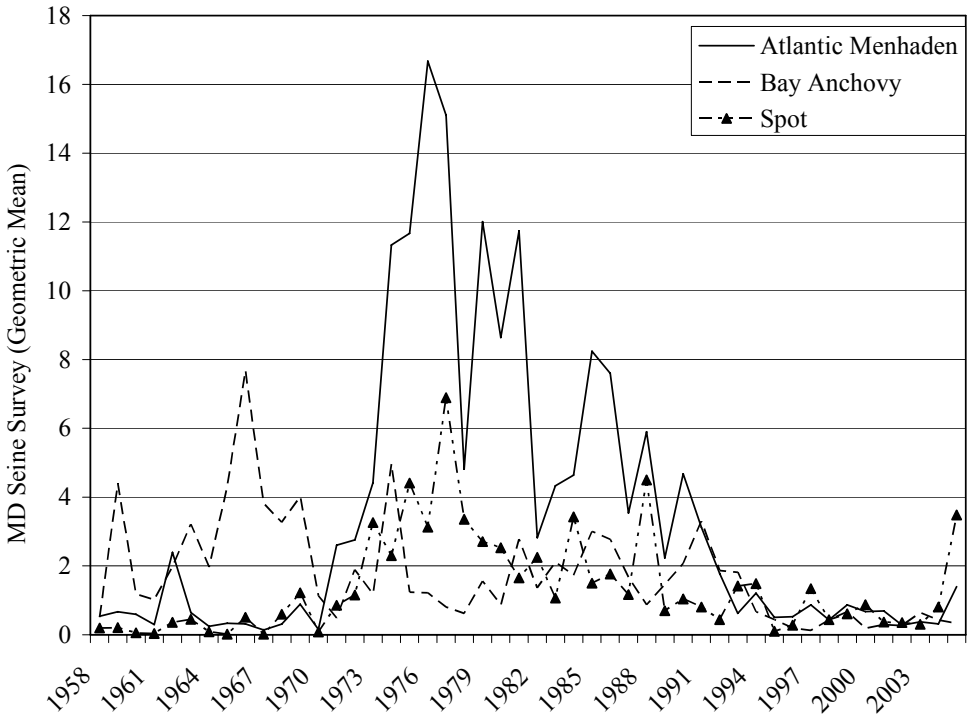

Figure 1-3. Geometric mean catch per seine haul of Atlantic menhaden, bay anchovy, and spot in the Maryland Department of Natural Resources juvenile seine survey. Data courtesy of the Maryland Department of Natural Resources.

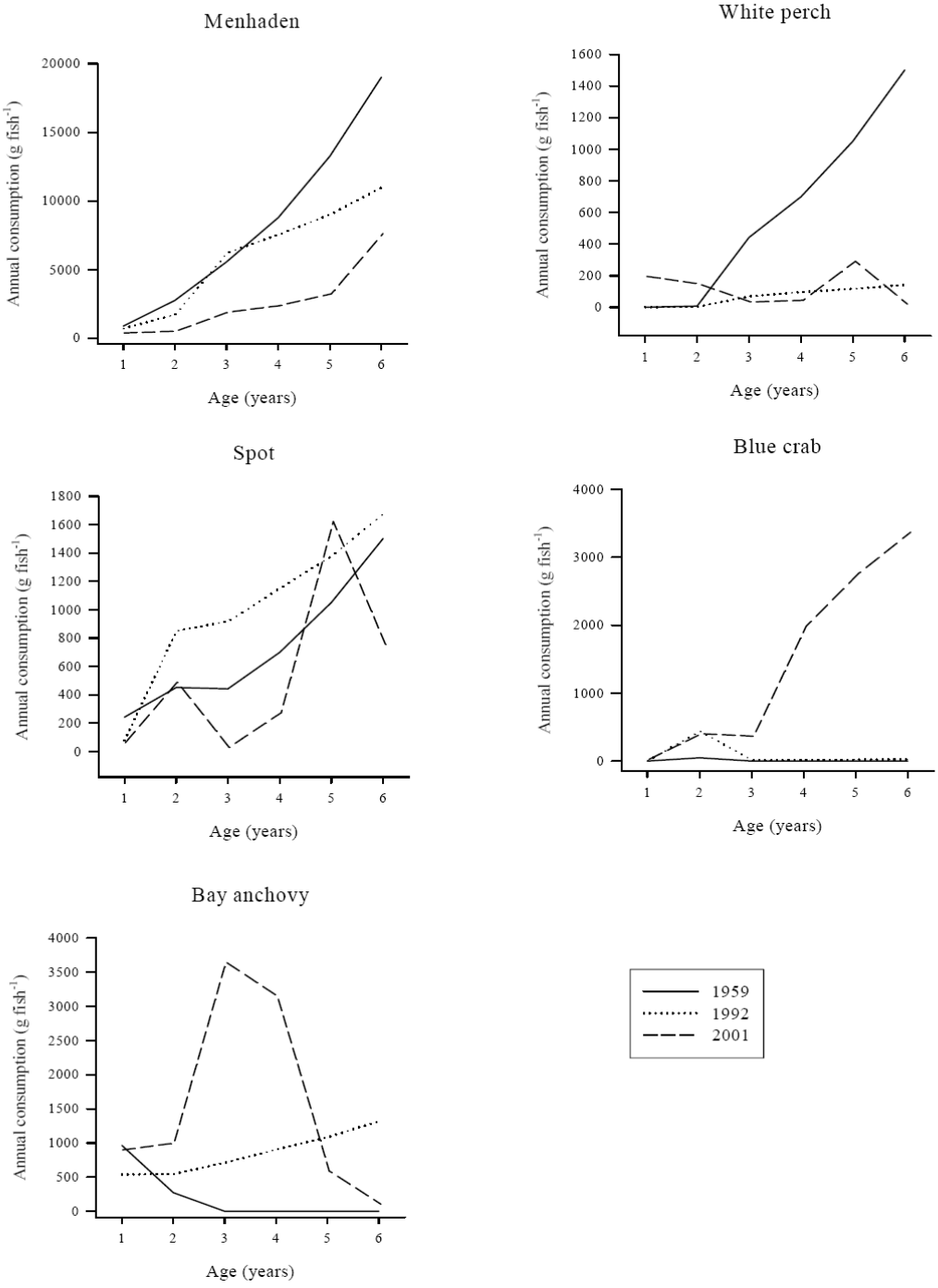

Figure 1-4. Change in Chesapeake Bay striped bass consumption of prey by age from 1959, 1992, and 2001. Figure courtesy of Overton (2003) with permission.

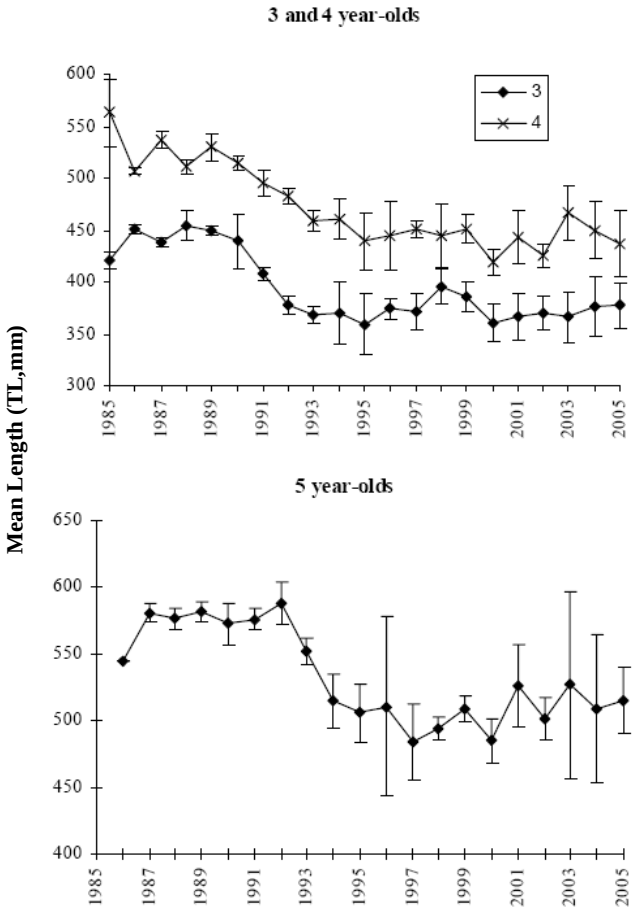

Figure 1-5. Reduction in length at age of 3-5 year old spring collected striped bass from the upper reaches of Maryland's Chesapeake Bay. Figure from Warner et al. (2005).

CHAPTER 2 – A REVIEW OF MYCOBACTERIOSIS IN MARINE FISHES

ABSTRACT

Mycobacteriosis is a serious and often lethal disease of fishes, affecting a wide range of species globally both in culture and wild settings. Caused by several species of the genus *Mycobacterium,* the disease has received considerable attention in recent years due to the discovery of new species in piscine hosts, epizootics in wild fisheries, and the ability of a few species to infect humans. The impact of this disease in aquaculture and the aquaria trade has been well reported and there is currently no widely accepted cure other than depopulation and facility disinfection. However, the impact on wild fisheries is poorly understood and may relate to species-specific interactions (host-pathogen) and possibly environmental stressors. In this review, much of what is known about mycobacteriosis in marine fish is summarized with particular attention to an epizootic in striped bass (*Morone saxatilis*) in Chesapeake Bay, USA.

INTRODUCTION

Mycobacteriosis, once known as "piscine tuberculosis," is a chronic progressive disease caused by several species of the genus *Mycobacterium*. Mycobacterial species are capable of causing serious and costly diseases in most vertebrates including humans (most notably tuberculosis, leprosy, and Buruli ulcer), livestock (bovine tuberculosis), and fish. While commonly reported in aquaculture and the aquaria trade (Colorni et al. 1998, Noga 2000), reports in wild fishes have been infrequent with the exception of recent years (Decostere et al. 2004).

In this review, we discuss the specific pathogens responsible for mycobacteriosis in marine fish, and what is known about the distribution, transmission, and host response to these species. While mycobacteriosis is equally problematic in freshwater fish (Stoskopf 1993, Noga 2000), for the purpose of brevity, they are largely excluded from this review.

The Species

Non-tuberculosis mycobacteria (NTM) designate all species other than those in the *M. tuberculosis* complex and *M. leprae*. In general, most NTM are aerobic, acid-fast, gram-positive, non-spore forming, non-motile, and free-living saprophytes in soil and water (Falkinham et al. 1980, Frerichs 1993, Iivanainen et al. 1993). Currently, there are over 90 accepted species of NTM (Devulder et al. 2005). Until recently, only *M. marinum, M. fortuitum, M. chelonae, and M. abscessus* (elevated from *M. chelonae* subsp. *abscessus*, Kusunoki and Ezaki 1992) were recognized as fish pathogens (Frerichs 1993, Chemlal and Portaels 2003). *M. marinum* is commonly reported from a wide range of saltwater species, while *M. chelonae* is documented principally in Pacific salmonids (Decostere et al. 2004). *M. chelonae* has also been isolated from wild salmon in Oregon (Arakawa and Fryer 1984), and farmed Atlantic salmon (*Salmo salar*) in marine environments (Bruno et al. 1998, Brocklebank et al. 2003). *M. abscessus* and *M. fortuitum* are isolated less frequently in marine fish, but have been reported from silver mullet (*Mugil curema*) in hyper-saline lagoons in Venezuela (Perez et al. 2001). Excellent reviews concerning disease in fishes caused by *M. marinum, M. fortuitum,* and *M. chelonae* have been published by Frerichs (1993) and Chinabut (1999).

In recent years, novel species, strains, and isolates have been recovered from wild marine fishes world-wide (Table 2-1). In the Chesapeake Bay, three novel species have been proposed from an ongoing epizootic in striped bass, *M. shottsii* (ATCC# 700981) (Rhodes et al. 2001, 2003), *M. psuedoshottsii* (ATCC# BAA-883) (Rhodes et al. 2005) and 'M. chesapeaki' (Heckert et al. 2001). Growth and biochemical characteristics are very similar for the nonpigmented *M. shottsii* and photochromogenic *M. psuedoshottsii,* however, differ from 'M. chesapeaki' in their inability to grow at 37°C, and several biochemical reactions (Heckert et al. 2001, Rhodes et al. 2005). These isolates are reported to share a cladic relationship with each other phylogenetically, as well as with species of clinical significance including *M. tuberculosis*, *M. marinum*, *M. ulcerans*, and *M. bovis* (Rhodes et al. 2005). However, complete molecular characterization of 'M. chesapeaki' has not been completed. *M. shottsii* is the predominate isolate from surveys conducted on wild striped bass by the Virginia Institute of Marine Science (VIMS), where species phenotypically resembling *M. interjectum, M. marinum, M. scrofulaceum, M. szulgai,* and *M. triplex* have also been isolated (Kaattari et al. 2002, Rhodes et al. 2004). In addition, several more isolates from this epizootic in Chesapeake Bay are currently being characterized (unpublished data).

A new species of mycobacteria related to *M. triplex*, *M. montefiorense,* was determined to be a cause of granulomatous lesions in wild and captive moray eels (*Gymnothorax* spp.) (Herbst et al. 2001, Levi et al. 2003). Mycobacteriosis is also common in rockfishes (*Sebastes* spp.)in the Pacific northwest (Kent et al. 2001). Culture efforts have not been successful; however, Whipps et al. (2003) sequenced rDNA directly from tissue. The mycobacteria from rockfish had the highest sequence identity with *M.*

montefiorense. Multiple granulomas containing acid-fast bacilli were observed in commercially-caught Pacific ocean perch (*S. alutus)*, yellowtail rockfish (*S. flavidus)* and yellowmouth rockfish (*S. reedi)*(Kent et al. 2001).

The list of recognized mycobacterial isolates and species continues to grow, in large part reflecting the advancement and acceptance of molecular characterization of species (Turenne et al. 2001, Devulder et al. 2005). However, much work remains in defining the relative roles of these isolates and the significance of strain variation. Ucko et al. (2002) and Sechi (2002) have demonstrated strain variability among *M. marinum* isolates obtained from different geographic regions of Europe. This approach will allow for future epidemiological evaluation of pathogen sources, modes of introduction, and distribution of great importance for the protection of both piscine and human health.

Pathology

Over 167 species of fish have been documented to be susceptible to mycobacteriosis (Nigrelli and Vogel 1963 , Chinabut 1999) spanning cultured populations and wild fisheries globally. The primary pathology associated with mycobacteriosis is that of classic granulomatous inflammation (Roberts 2001). Microscopically, granulomas are characterized by a central area of necrosis surrounded by macrophages, epithellioid cells, and fibrous connective tissue (Talaat et al. 1997, Diamant et al. 2000). Granulomas are primarily found in the spleen, liver, and kidney during earlier stages of the disease, but may spread to all organs in more advanced cases. Advanced infection with the disease has been suggested to be terminal (Sakanari et al.

1983, Hedrick et al. 1987, Heckert et al. 2001), however this assumption may be highly dependent on the mycobacterial species and piscine host.

Controlled challenge studies have served to elucidate the progression of mycobacteriosis in several fish species, and offer insight into the relative pathogenicity of various mycobacterial isolates and host-specific differences. Timur and Roberts (1977) outlined the progression of granulomatous inflammation in plaice (*Pleuronectes platesa*). In general, the response initiates with the aggregation of macrophages in an inflammatory focus, with gradual shift towards epithellioid cell morphology. Giant cells may or may not be present in early stages of infection. Gauthier et al. (2003) compared granuloma formation in striped bass (*Morone saxatilis*) infected with *M. marinum*, *M. shottsii*, and *M. gordonae*. Whereas *M. marinum* caused a severe granulomatous reaction, *M. shottsii* and *M. gordonae* only rarely produced granulomas. Wolf and Smith (1999) found striped bass (*Morone saxatilis*) presented with more severe clinical and microscopic disease manifestations than did hybrid tilapia (*Oreochromis* spp.) when challenged with high concentrations of *M. marinum*. Thus individual variation in susceptibility and host-pathogen interactions limit the ability to generalize the specific impact of mycobacteriosis.

Transmission

Transmission of the disease is not well established in fish, although ingestion (Nigrelli and Vogel 1963), transovarian transmission in viparous fishes (Conroy 1966, Stoskopf 1993) and direct transmission from water contact have been suggested (Frerichs 1993). High incidence of infection in cultured Pacific salmon was linked directly to

feeding of infected fish carcasses, which was remedied by ceasing the practice in favor of pasteurized fish meal (Parisot and Wood 1960). Transovarian transmission was investigated by Ross and Johnson (1962) in chinook salmon (*Oncorhynchus tshawytscha*), but definitive results were not obtained leading the authors to conclude that this route is probably of little importance to fisheries management. Evidence for this route of transmission comes from Conroy (1966) who demonstrated this may be a potential source in the viparous Mexican platyfish (*Platypoecilus maculates*). I have found no similar, or recent references for strictly marine fish regarding this mode of transmission. High density of fish in an intensive culture system was suggested as a contributing factor to an outbreak of *M. marinum* in juvenile striped bass, leading to increased opportunity for transmission through the water column, fecal products, or cannibalism (Hedrick et al. 1987).

Water has received considerable attention as a source of human infection with NTM, with many of the same species infecting fish (Nichols et al. 2004) (Table 2-1). Water-born transmission has been demonstrated in hybrid striped bass (*Morone chrysops X M. saxatilis*). Li and Gatlin (2005) used *in situ* exposure to contaminated culture systems in their investigation of prebiotics. Infection with *M. marinum* was established in 16 weeks. Dos Santos et al. (2002) postulated that water was the source of mycobacterial infections in farmed turbot (*Scophthalmus maximus*) after culturing *M. marinum* from inlet water. An interesting observation of possible sources for *M. marinum* in the Red Sea was noted by Diamant et al. (2000) where the authors describe high prevalence of infection in cage-cultured rabbitfish (*Siganus rivulatus*) (50%) which may have been transmitted to or from the wild population. Between 21 and 42% of wild

rabbitfish sampled regionally were affected by the same strain of mycobacteria discovered in samples of caged fish.

Other possibilities are equally plausible, including entrance through dermal wounds, or through vector organisms. As intracellular pathogens, survival in alternate hosts is likely. Indeed, *M. avium* has been shown to be phagocytized, survive and replicate within protozoa and amoeba (Strahl et al. 2001). A close relative to many of the fish pathogens, *M. ulcerans*, may be transmitted through aquatic insects as intermediate hosts (Marsollier et al. 2002). It is likely that several modes of transmission exist, and are case, host, and mycobacterial species specific. Nevertheless, understanding the mechanisms could severely aid in delineating environmental reservoirs and limiting the impact of this disease in aquaculture.

Diagnosis

The techniques for diagnosing mycobacteriosis in fish are continually evolving, but clinical signs and gross pathology may give an initial indication of infection with mycobacterial species. Clinical signs of mycobacteriosis are not specific to the disease and can vary in occurrence and severity. Signs may include dermal lesions, pigmentary changes, emaciation, stunted growth, exophthalmia, and slowed swimming (Noga et al. 1990, Colorni et al. 1998, Wolf and Smith 1999, Rhodes et al. 2001, Swanson et al. 2002). In many cases, no external signs are revealed. Gross examination of affected organs may reveal grayish to white granulomatous nodules, typically apparent in the spleen, liver, and head kidney. However, inconsistent clinical signs, and the non-specific

nature of granulomatous inflammations (i.e., other bacteria, fungus, and parasites can cause a similar response), often make diagnosis by gross observations inaccurate.

Historically, researchers have relied on histology and culture to identify mycobacteria. Detection of granulomas in tissue sections containing acid-fast rods after Ziehl-Neelsen staining is helpful in diagnosis because host response and the severity of infection can be visualized (Figure 2-1). However, the mycobacteria cannot always be detected in section, depending perhaps on species, abundance, and/or the growth phase of the pathogen (Nyka and O'Neill 1970, Gauthier et al. 2003). Culture continues to be an important method for diagnosis of a mycobacterial infection. Tissue samples are homogenized with buffer and plated on appropriate media, such as Middlebrook 7H10 or Lowenstein-Jensen agar, to encourage *Mycobacterium* spp. growth. Several *Mycobacterium* spp. are slow growers necessitating the maintenance of plates for two-three months before being considered negative. The higher temperatures frequently used for human clinical isolates can inhibit the growth of fish mycobacteria. Once growth occurs and a bacterial isolate is found to be acid-fast, the colony can be subjected to further tests for speciation. Standard biochemical characterization can be costly and time consuming because of the slow growing nature of many mycobacterial species.

Methods currently under development include genetic techniques, high-performance liquid chromatography (HPLC), and gas chromatography for fatty-acid methyl-ester (FAME) analysis. FAME analysis identifies isolates to species based on the composition of fatty acids in the cell wall of bacteria (Tønjum et al. 1998). Since cell wall composition is conserved within species, dendograms can be produced to group isolates as well as measure the relatedness among groups. Once the groupings are

formed, subsequent isolates can be readily identified when they fall within a specific group. Similarly, HPLC uses the unique profile of mycolic acids in cell walls and is a well-accepted method for the speciation of mycobacteria (Butler et al. 1992, Tortoli et al. 1996).

Alternatively, many efforts are currently directed towards genetic techniques to detect and identify mycobacteria from culture and in biological material. DNA probes and gene sequencing have been extensively evaluated for species of clinical importance in the human medical field (Turenne et al. 2001, Chemlal and Portaels 2003), allowing for rapid identification to the species level in many cases. Efforts to distinguish the more common fish isolates have focused on the PCR amplification of the 16S ribosomal gene (Heckert et al. 2001, Herbst et al. 2001, Chemlal and Portaels 2003, Whipps et al. 2003), the 65-kDa heat shock protein gene (*hsp65*) (Kim et al. 2005, Ucko and Colorni 2005), the RNA polymerase B subunit gene (*rpoB*) (Kim et al. 1999, Devulder et al. 2005) and the exported repeated protein gene (*erp*) (de-Mendoca-Lima 2001) among others. Techniques such as restriction enzyme analysis (Talaat et al. 1997, Ucko et al. 2002) or hybridization (Puttinaowarat et al. 2002) have also been employed with varying success. Direct sequencing from tissues can be useful when *in vitro* culture fails (Knibb et al. 1993, Astrofsky et al. 2000, Whipps et al. 2003). However, many of the fish isolates share high homology in these genes requiring the use of multiple gene targets (Devulder et al. 2005). In her review, Kaattari et al. (2006) recommends the use of a minimum of two genes when using sequencing for differentiation of the *M. tuberculosis* clade. The continued development of rapid and sensitive techniques capable of identification to the

species level is essential for furthering our understanding of the relative roles of various mycobacterial pathogens, sources, and future epidemiological approaches.

Human Health Concerns

In addition to concern for fish health, infection of humans with NTM has been well documented and can reach epidemic proportions (Adams et al. 1970, Stoskopf 1993). NTM are an increasing concern, particularly in developing countries and individuals stricken with AIDS and other forms of immunodeficiencies. Buruli ulcer, a skin condition caused by *M. ulcerans,* has been linked to severe deformities in exposed children, including functional disability of limbs and atrophy, with incidents steadily increasing (Dobos et al. 1999). Eddyani et al. (2004) suggest that fish may act as a reservoir for *M. ulcerans* through the ingestion of infected plankton and insects in an area of Ghana where Buruli ulcer is endemic. *Mycobacterium marinum* is the most common piscine-related NTM to infect humans, resulting in local granulomatous inflammation, usually at the extremities such as hands and fingers. However, a few strains of *M. marinum* are capable of growth at 37°C and systemic infections in humans have been reported (Parent et al. 1995). Human infection rarely occurs from person to person contact, but more often from contaminated water sources (including pet fish tanks), inhalation, or direct ingestion (Schulze-Robbecke and Buchholtz 1992, Aubry et al. 2002). In Anne Arundel County, Maryland, 41 cases were reported from 1988-1994, all related to exposure to Chesapeake Bay waters (Joe and Hall 1995). Previously, nine cases of *M. marinum* infection had been found in a community on Virginia's Eastern Shore of the Chesapeake Bay, and all but one had a direct connection to a wound

occurring in or around saltwater (Hoyt et al. 1989). *M. chelonae-abscessus group*, *M. fortuitum group*, *M. szulgai*, and *M. gordonae* are also human pathogens and therefore represent possible zoonoses, primarily for immunosuppressed patients (Swetter et al. 1993, Gebo et al. 2002, Abalain-Colloc et al. 2003, Pozniak and Bull 1999) (Table 2-1).

Impact on Foodfish and Marine Ornamental Culture

Pathogenic NTM are most commonly reported in aquaculture and ornamental fish. Winsor (1946) had documented 36 edible marine fish, including wild and aquarium species that were 'tubercular.' Reported cases in aquaculture identify stress due to overcrowding, poor water quality, or contaminated food sources as factors predisposing infection (Hawke 2000). Commercial sea bass (*Dicentrarchus labrax*) production in Israel has been severely hampered by *M. marinum* infection in both inland and net pen operations (Colorni et al. 1998). Mycobacterial infections have also been found in farmed turbot (*Scophthalmus maximus*) in Portugal (dos Santos et al. 2002), captive delta smelt (*Hypomesus transpacificus*) in California (Antonio et al. 2000), cultured yellowtail (*Seriola quinqueradiata*) in Japan (Kusuda and Kawai 1998), juvenile tautog (*Tautog onitis*) held for nutritional studies in Connecticut (Pitchford et al. 2000), commercially-reared summer flounder (*Paralichthyus dentatus*) (Hughes et al. 2002), and in anadramous salmonids held in freshwater hatcheries (Ashburner 1977, Bruno et al. 1998).

Marine ornamental fish are also subjected to intensive culture conditions and are thus susceptible to mycobacterial infections. Giavenni et al. (1980) documented a severe case of *M. marinum* infection in 97 marine tropical fish representing 17 genera. Although only 5-10% mortality occurred, the employee who cared for the fish developed

nodular skin lesions on his hands, from which mycobacteria were isolated. Efforts to control mycobacteriosis in aquaculture through culture practices or feed supplements have met with mixed success (Conroy and Conroy 1999, Astrofsky et al. 2000, Jacobs et. al 2005, Li and Gatlin 2006). There are currently no FDA approved drugs for the treatment of mycobacteriosis leaving depopulation and facility disinfection as the only option. Mycobacteriosis is clearly a serious and costly issue in the culture and propagation of many fish species and future advancements in treatment options are warranted.

Impact on Wild Fisheries

The first description of naturally occurring tubercular lesions in a marine fish was provided by Alexander (1913) and Johnstone (1913) in reference to a single cod (*Gadus morhua*) landed in England. Excellent reviews of the early history of mycobacteriosis and presumptive cases through mid-1980s are provided by Conroy (1970) and Chinabut (1999). In perhaps the largest examination in geographic scale and age classes, MacKenzie (1988) reported presumptive mycobacteriosis (not culture confirmed) in northeast Atlantic mackerel based on observations of grayish-white nodules in the spleen. From age 0 to 3, prevalence of the condition increased from 10% to almost 90%, suggesting early onset of disease. Severity, however, increased slowly and linearly with age over the 19 year classes reported, suggesting a chronic condition of low pathogenicity. While no external pathology was apparent, decreased condition factor was noted in affected individuals. In the early 1980s, Sakanari et al. (1983) described a high prevalence of tubercular lesions in Pacific coast striped bass. While the species was not

identified, mycobacteria was suggested to be the causative agent. Unfortunately, no further information on population level effects or fate exists from this case. However, Lansdell et al. (1993) isolated *M. marinum* from Pacific wild-captured striped bass several years later. Low background rates of infection in ocean-caught juvenile coho salmon (*Oncorhynchus kisutsch*) and chinook salmon (*O. tshawytscha*) have also been found (Arakawa and Fryer 1984).

The Chesapeake Story

Of current interest is an epizootic of mycobacteriosis in Chesapeake Bay striped bass first detected in 1997 (Heckert et al. 2001, Rhodes et al. 2001). Multiple survey efforts have been conducted to examine the prevalence and distribution of mycobacteriosis in both Maryland and Virginia waters. Estimates range from ~50% using standard histological methods, and up to 75% with molecular techniques (Kaattari et al. 2002, Overton et al. 2003, Kaattari et al. 2005). It should be noted that these estimates are generalities, as prevalence is highly dependent on age classes sampled. While age of onset has not been fully established, Kaattari et al. (2005) found infection prevalence based on culture to increase with age to approximately age 5, with incidence subsequently declining through age 6+. Similar results were obtained by Cardinal (2001) and Overton et al. (2003) relying primarily on histopathlogy. Little evidence of differences in spatial distribution of infection have been reported to date (Cardinal 2001, Kaattari et. al 2002), however, sex and seasonal changes may be apparent. Cardinal (2001) noted a higher prevalence in males than females, as well as increased prevalence in the fall in a single river system. Of note in the Chesapeake epizootic is an elevated

prevalence of ulcerative skin lesions (Rhodes et al. 2001, Overton et al. 2003). While the majority of infected fish do not show external signs of disease, it has been suggested that they may be more prevalent in later stages of mycobacterial infection (Kaattari et al. 2005). Mycobacterial infections in Chesapeake Bay are most commonly associated with a newly described species, *M. shottsii*, however, polymycobacterial infections are common and at least seven other isolates have been obtained (Rhodes et al. 2004, Heckert et al. 2001). Of interest is the low level of *M. marinum* cultured (>5%) (Rhodes et al. 2004).

Of particular interest in Chesapeake Bay are the potential population level effects. It is largely held that infection with certain species of *Mycobacterium*, such as *M. marinum*, will eventually progress to a state where host survival is unlikely. However, the slow progression of the disease may take years to reach this endpoint, allowing for multiple spawning opportunities, and obscuring population level impacts. Challenge studies with the primary Bay isolate (*M. shottsii*) have shown a low degree of pathogenicity (Gauthier et al. 2005) Much work remains to clarify population level impacts and the relative roles of the various Bay isolates.

Many questions remain regarding the current epizootic in Chesapeake Bay striped bass, which has persisted now for nearly a decade (Hedrick et al. 2001). Epizootics of disease commonly reflect complex relationships between the host, pathogen and the environment (Sindermann 1970). As such, several hypotheses have surfaced questioning the impact of environmental stressors and ecosystem imbalance. Principally, these relate to issues of water quality and anthropogenic influences and density-dependent factors perhaps leading to trophic shifts in prey consumption and subsequent declines in

nutritional health. The Chesapeake is a highly eutrophic temperate estuary, subject to elevated summer temperatures and large areas of hypoxia (Kemp et al. 2005). These factors may serve as direct stressors, favor conditions for mycobacterial growth, or limit optimal habitat for striped bass. In addition, a shift in striped bass foraging has been reported resulting in a movement towards feeding at benthic oriented, lower trophic levels (Hartman and Brant 1995, Overton et al. 2000, Griffin and Margraf, 2003). Determining the impact of environmental stressors on disease state is inherently difficult owing to the complexities of the interactions that may occur. Whether disease is a reflection of ecosystem change or an independent process remains to be seen.

Summary

Mycobacteriosis is a chronic, progressive fish disease most likely capable of affecting all fish species. While the study of *Mycobacterium* spp. affecting fish is progressing, there remains much work to be completed in delineating sources, modes of transmission, and the impact of environmental conditions on the concentration of pathogenic species and host susceptibility. Much of this work is currently hampered by the lack of rapid identification tools at the species level for these slow growing pathogens.

For both cultured and wild marine fish management, mycobacteriosis represents a serious challenge. In culture, depopulation and facility disinfection is the most commonly adapted strategy, as there is currently no approved drug for the treatment of this disease. However, disinfection is not always successful due in large part to the resistance of many species of mycobacteria to common disinfectants (Jacobs et al. 2004).

In wild fisheries, management is faced with both the potential of transmission to humans from infected fish, as well as the difficult task of determining population level impacts, which may vary with species of host and bacteria as well as environmental factors. Understanding this disease and its implications from an ecosystem perspective will require strong interaction of fish health professionals with traditional fisheries ecologists and epidemiologists.

ACKNOWLEDGEMENTS

This chapter benefited from the efforts of Cindy Stine, Dr. Ana Baya, and Dr. Mike Kent. I appreciate the critical review provided by Dr. Wolfgang Vogelbein.

Table 2-1. Species of *Mycobacterium* reported from marine fish. In most cases, speciation is based on phenotypic characteristics. C= common, I = infrequent, Y = yes and U = unknown.

Species	**Frequency**	**Transmission to Humans[A]**	**Reference**
M. chelonae	C	Y	Ross 1960
M. fortuitum	C	Y	Perez et al. 2001
M. marinum[B]	C	Y	Aronson, 1926
M. abscessus	I	Y	Perez et al. 2001
'M. chesapeaki'	I	U	Heckert et al., 2001
M. interjectum	I	Y	Rhodes et al. 2004
M. montefiorense	I	U	Herbst et al., 2001
M. psuedoshottsii	I	U	Rhodes et al. 2005
M. scrofulaceum	I	Y	Lansdell et al., 1993
M. shottsii	I	U	Rhodes et al., 2001
M. szulgai	I	Y	Rhodes et al. 2004
M. simiae	I	U	Landsdell et al. 1993

[*] *M. ulcerans* has also been detected in fish tissue, but not directly isolated (Eddyani et al. 2004)

[A] Nichols et al., 2004

[B] Endemic strains described from European isolates (Ucko et al. 2002)

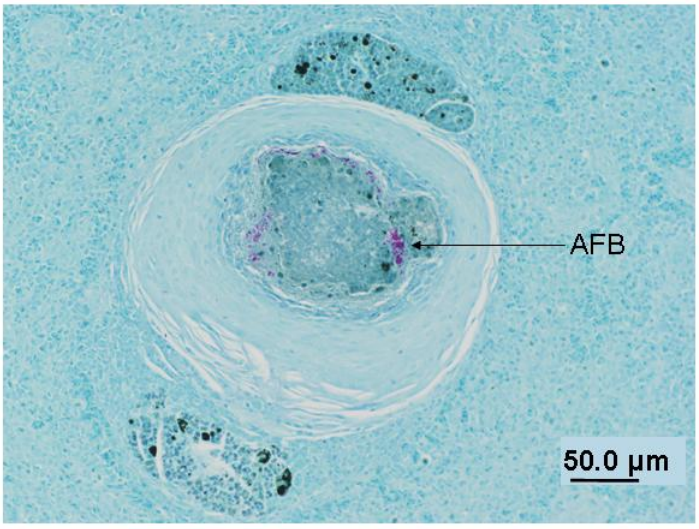

Figure 2-1. Typical mature granuloma in spleen section of striped bass. Bacteria are readily visible in granuloma core (AFB). Ziehl-Neelsen stain, 40x

Chapter 3- AN ARCHIVAL REVIEW OF MYCOBACTERIOSIS IN CHESAPEAKE BAY STRIPED BASS FROM 1975 – 1995.

ABSTRACT

Archived tissue samples of wild-collected striped bass (*Morone saxatilis*) were examined for the historical presence of mycobacteriosis. Of 57 cases available from 1975 – 1990, two fish were found positive for the disease. PCR amplification and sequencing of the internal transcribed spacer region (ITS) of the 16-23s rRNA gene, the exported repeated protein gene (*erp*), and insertional sequences *IS2404* and *IS2606* place both within the *M. tuberculosis* clade with closest resemblance to the recently described fish pathogen *M. psuedoshottsii*. The prevalence of granulomas containing acid-fast bacteria in this collection was greater in 1990 – 1995 (7 of 32) than in all previous periods. Our data confirms that mycobacteriosis is not a new disease of Chesapeake Bay striped bass.

INTRODUCTION

Mycobacteriosis is a chronic progressive bacterial disease of fish caused by several species of the genus *Mycobacterium*. While common and problematic in aquaculture (Stopskopf 1993, Colorni et al. 1998), observation of mycobacteriosis in wild marine fish has been less frequent. In 1997, mycobacteria was cultured from Chesapeake Bay striped bass (*Morone saxatilis*) exhibiting external ulcerative lesions and internal granulomatous inflammation (Heckert et al. 2001, Rhodes et al. 2001). Over the past 10 years, several research efforts have demonstrated that disease prevalence increases with age, and a high proportion (>50%) of mature fish are affected (Overton et al. 2003,

Rhodes et al. 2004, Kaattari et al. 2005, Ottinger and Jacobs 2006). Further complicating the matter is the isolation of several species of *Mycobacterium* including three novel members of the genus; *M. shotttsii* (Rhodes et al. 2003), *M. psuedoshottsii* (Rhodes et al. 2005), and 'M. chesapeaki' (Heckert et al. 2001), as well as others phenotypically resembling *M. marinum*, *M. interjectum*, *M. scrofulaceum*, *M. szulgai* and *M. triplex* (Rhodes et al. 2004). While *M. marinum* is known to cause severe and often fatal infections in fish (Colorni et al. 1998, Wolf and Smith 1999), it is rarely cultured from affected striped bass in Chesapeake Bay (Rhodes et al. 2004, Baya et al., unpublished data). *M. shottsii* and *M. pseudoshottsii* are the primary isolates (Rhodes et al. 2004). In challenge studies, the former has not been shown to consistently cause the same pathology as noted in the wild (Gauthier et al. 2003), while the relative roles of many of the other isolates have yet to be evaluated. The roles of environmental stressors such as elevated water temperature and lack of forage are only beginning to be addressed in combination with these pathogens (Ottinger and Jacobs 2006).

Only with the recent threat posed by mycobacteriosis in Chesapeake Bay have health surveys been routinely conducted for the striped bass with statistically valid designs. Thus the timing of the actual onset of the disease is questionable. Timing is important because many of the hypotheses surrounding the high prevalence of disease in striped bass are centered on density dependent issues with a population at historically high abundance and management strategies to enhance stock production following the decline in the Atlantic Coastal population in the late 1970's – early 1980's. (Richards and Rago 1999, Hartman and Margraf 2003). Mycobacteriosis in striped bass was reported from the Pacific coast in the early 1980's (Sakanari et al. 1983) and in Atlantic

mackerel (MacKenzie 1988) based on histological evidence. Thus, species of mycobacteria capable of causing pathological change were regionally present (Atlantic Ocean) and have been shown to affect wild striped bass in different systems. Knowledge of whether this disease, and these particular species, were present in striped bass before the current epizootic would lend powerful information to refining efforts to understand the source and associated factors germane to this issue.

While intensive health surveys for striped bass did not exist until recently, the National Oceanic and Atmospheric Association - Maryland Department of Natural Resources Oxford Laboratory (COL) has investigated fish and shellfish disease issues in Chesapeake Bay since 1965 and maintain an archived library of historic tissue blocks. In this paper, we describe an effort to review these tissues with the specific intent to determine if mycobacteriosis was apparent in Chesapeake Bay before 1997. In addition, we apply molecular tools to candidate blocks offering a level of sensitivity and discrimination not possible in earlier investigations (Crumlish et al. 2007).

METHODS

Tissue Samples

Since 1965, COL has maintained archived, paraffin embedded tissues in a climate-controlled facility. Unique accession numbers are given to each tissue to allow for subsequent retrieval. In 2002, we initiated a process to organize earlier submissions (1965-1987) electronically and query the data set for wild collected striped bass. Archived laboratory notebooks, institutional knowledge, and correspondence with previous employees were used to discern individual investigators accession numbers and

provide clues for candidate submissions. Later years (1988 - present), had been maintained in a searchable database easing the process. Only confirmed, age 1+ wild collected striped bass were included in this review. While large scale coastal surveys were routinely conducted by COL, no such surveys were done in Chesapeake Bay for finfish during this time period. Thus the majority of collected tissues from 1965 – 1988 are from "Service Samples", or those brought to the laboratory by other researchers or concerned anglers and watermen for expert opinion on disease status. Necropsies were routinely performed and tissues preserved predominantly in formalin-based fixatives. After 1988, MDNR biologists routinely conducted studies with wild striped bass for various reasons, while also charged with responding to fish kill events and reports of abnormal conditions in fish. Tissue samples from these cases were predominantly preserved in Bouin's fixative. A total of 89 cases of wild-collected striped bass were available for examination.

Histopathology

In some cases, replicate tissue sections were found in excellent condition previously mounted on slides negating the need to re-section archived blocks. For the majority of early fish, replicate 5 μm sections were taken from archived tissue blocks and stained with hemotoxylin and eosin for initial screening. All available sections were examined with light microscopy for the presence of granulomas, inflammatory foci, or necrotic lesions characteristic of mycobacterial infection. Only obvious verminous granulomas were excluded from the initial screening. All tissues containing the above pathologies were stained with Ziehl-Neelsen for visualization of acid-fast bacteria

performed at 60x. Only those containing clearly visible acid-fast, non-branching rods were considered histopathology positive (AFB+). Otherwise, samples were categorized as negative.

Molecular Detection and Identification

Molecular characterization of acid-fast bacteria in paraffin embedded tissues was limited to cases before 1998. All cases available after 1998 were preserved in Bouin's fixative which is known to degrade RNA and DNA, a process that may become more significant with time in storage (Bonin et al. 2005). Regardless, several attempts were made to amplify DNA from these tissues with no success.

Briefly, microtomes were dismantled, sterilized with 70% ETOH, and blades autoclaved before sectioning. After removal of several sections from the blocks surface, 3 serial 5 μm sections were cut, specific tissue sections segregated when possible, captured on a sterile wooden applicator, and transferred directly to sterile 1.5 ml micro-centrifuge tubes. Sections were deparaffinized with xylene and DNA extracted with Qiagen's DNeasy kit according to manufacturers instructions for paraffin-embedded tissues (Qiagen Inc. Valencia, CA 91355, USA) with modification. Three ethanol (100%) rinses were performed after the use of xylene for paraffin removal, followed by complete evaporation in a vacuum centrifuge for 15 min. Elution from spin filters was performed twice to a total volume of 70 μl. Paraffin-embedded splenic tissue from a single fish challenged with *M. marinum* was used as an extraction control. Samples were screened for the presence of mycobacteria using a real-time PCR assay targeting a genus specific region of the ribosomal 16-23S intergenic transcribed spacer (ITS) region

(Bruijenstein van Coppenraet 2004). While the ITS is variable enough to distinguish fast and slow growing mycobacteria, and many species, most of the tuberculosis clade commonly infecting fish in Chesapeake Bay are highly homologous necessitating the use of multiple primer sets for subsequent sequencing (Kaattari et al. 2006). Sequence analysis of the exported repeated protein (*erp*) gene and the insertional sequences *IS2404* and *IS2606* were used to further distinguish members of the tuberculosis clade (Stinear et al. 1999, Kaattari et al. 2006, Rhodes et al. 2005, de Mendonça-Lima et al. 2001). All primers and probes used in this study are given in Table 3-1. Reaction conditions were similar to those of the reference listed in Table 3-1. Positive samples were either gel purified and extracted using a Qiagen QIAquick Gel extraction kit (Qiagen Inc. Valencia, CA 91355, USA) or directly purified from PCR product using Agencourt's AMPure kit (Agencourt Bioscience Corporation, Beverly, MA, 01915 USA). Sequencing was performed at the University of Maryland's, Center of Marine Biotechnology's BioAnalytical Services Laboratory. All sequencing was performed on an ABI 3130 XL Genetic Analyzer using the dye terminator method. Sequences were aligned to our own databases and those of Genbank using the freeware BioEdit (Hall 1999).

Source of Reference Cultures, DNA, and Sequences

Mycobacterial species and strains used in this study were obtained either as pure cultures or DNA preparations from the Virginia-Maryland Regional College of Veterinary Medicine (VA-MDRCVM) or the USGS National Fish Health Laboratory (USGS-NFHL). All other sequences used in the analysis were obtained directly from GenBank, or entered manually from referenced literature. DNA was extracted from

isolates for sequencing from known cultures in all cases by simple boiling of cell suspensions in TE buffer (Afghani and Stutman 1996). *M. marinum* isolated from wild Chesapeake Bay striped bass (VAMDRCVM FL03-23) was used as the inoculum to generate control tissue blocks. Sources and strains used in this study are given in Table 3-2.

Sequence Analysis

Chromatograms from all sequences were visually inspected before consensus based assembly of forward and reverse complement strands (CAP Contig Assembly Program, Huang 1992). Sequences were aligned using Clustal W (Thompson et al. 1994) and homology calculated for pairwise comparisons (Hall 1999). Phylogenetic trees were assembled using Neigbour-joining and maximum likelihood methods with DNADIST version 3.5c (Felsenstein 1993).

RESULTS

1975-1980

The first cases of striped bass submitted through COL were found in 1975, ten years after the establishment of the laboratory. Of the 21 cases available, only three fish with non-verminous granulomas were apparent (Table 3-3). New sections of these tissues stained with Ziehl-Neelsen revealed no acid-fast bacteria. PCR amplification of the ITS was not successful for these tissues. No other information was available for these cases other than location and date of collection.

1980-1985

During the period of stock decline, moratoria, and rebuilding, 16 cases were available for analysis. Little information other than the location of collection was available for these cases. During 1984-1985, two adult striped bass were found with severe mycobacteriosis (Figure 3-1). Fish 84-42 was captured in the Tred Avon River, a tributary of the Choptank River, MD, in October of 1984. It was an adult fish based on tissue size, but no other information was available. Granulomatous inflammation was apparent in liver, spleen, posterior kidney, and heart with acid-fast bacteria present in all tissues. The extent of tissue damage varied with organ. Poorly developed, large, and often coalescing granulomas accompanied almost complete fibrosis of the spleen. Eosinophillic granular cells and lymphocytic infiltrate was common within the fibrous meshwork. High concentrations of bacteria were present within many granuloma cores, however, individual bacteria were also readily discernable within macrophages outside of granulomatous lesions. Liver sections contained multiple granulomas in various stages of development, largely surrounded by normal hepatic tissue. Mature granulomas were most often condensed with cores containing ceroid pigments and necrotic debris surrounded by a thin fibrous capsule. Pericarditis was evident on ventricle tissues of the heart with granulomas present both within this inflammatory layer and the ventricle and atrium. While granulomas were less numerous, acid fast bacteria were visible within cores. Anterior kidney sections contained numerous granulomas in various stages of development accompanied by extensive renal tubular necrosis.

Fish 85-4 was collected in January of 1985 from the Choptank River, MD based on tissue submission logs. Again, an adult specimen based on tissue size, but no other information was available. Splenic granulomas varied considerably in size and organization ranging from small (10-12 μm diameter) and well organized, to large (~200 μm diameter) and poorly contained as described previously. However, fibrosis of the organ was not apparent, and most tissue was normal suggesting reduced severity of infection. Acid-fast bacteria were readily visible in both types of granulomas, however bacteria in the latter was found throughout the confines of the granuloma and not restricted to pockets within the core. Anterior kidney, muscle tissue, and intestine were also examined and appeared normal.

Sequence analysis of the ITS gene from bacterial DNA isolated from spleens of both specimens showed high homology with the fish pathogenic clade reported in Chesapeake Bay striped bass (Figure 3-2). Only one substitution was noted for *M. ulcerans* and for *M. shottsii* at differing loci among this group. PCR amplification of *IS2404* and *IS2606* were weakly positive for both archived tissues and strongly positive for *M. psuedoshottsii* and *M. ulcerans* (Figure 3-3). *M. marinum* and *M. shottsii* did not produce bands. Sequence analysis of a 500 bp region of the *IS2404* showed high homology between both isolates (99.5%) and with *M. psuedoshottsi* (99%) and less with published sequences for *M. ulcerans* (AF003002) (97%) and *M. marinum* (EF164897) (87.0%). Sequences of a 330 bp segment of *IS2606* were identical between *M. pseudoshottsii* and our isolates, with all differing from *M. ulcerans* by only one substitution (99% homology). Products from primers ERP1F and ERP1R showed 100%

homology of DNA from archived tissues with *M. psuedoshottsii* and *M. ulcerans*, and 98.8 % with *M. marinum* and *M. shottsii.* New primers were designed to amplifly the adjacent region of the the *erp* gene reported by Rhodes et al. (2005) (Table 3-2). 100% homology was found with *M. psuedoshottsii* and 84-42, with *M. ulcerans*, *M. marinum*, and *M. shotttsii* having a minimum of 30 additional nucleotides in this central region of the *erp* gene (Table 3-4). Amplification of this region for 85-4 was unsuccessful. In summary, mycobacterial DNA retrieved from 84-42 and 85-4 are highly homologous to each other and *M. psuedoshottsii* for all genes examined (> 99%).

1986-1990

A total of 20 cases were available from 1988 – 1990 with two fish found with NVG. However, both cases failed to yield acid-fast bacteria, and PCR was not performed due to extensive time in Bouin's fixative. This time period represented a shift in focus of the laboratory away from fish from 1986 - 1988. In 1988 MDNR joined the laboratory and renewed investigations into fish health. Granulomatous inflammation accompanied by acid-fast bacteria were apparent in wild striped bass held in net pens and flow through tanks at the laboratory during this time period (authors data, not presented), but have been excluded from this review since the impact of artificial environment cannot be separated.

1991-1995

During the period of peak stock abundance, 32 cases were available. Of these, 20 contained NVG and seven contained acid-fast bacteria (AFB). Of those containing AFB, six of the cases occurred from 1993-1995. Predominantly, these lesions were observed in

splenic tissue. However, in some cases other organs were involved, and direct relationships of granulomatous inflammation and external lesions were observed (Figure 3-1, plate D). Again, no attempts were made to amplify DNA from these tissues as all were preserved in Bouin's fixative.

DISCUSSION

Mycobacteriosis was apparent in archived tissue of wild-collected striped bass at least 12 years before the initial reports in Chesapeake Bay striped bass (Heckert et al. 2001, Rhodes et al. 2001). Given other reports during the same time period, the presumed ubiquitous distribution of mycobacterium in the environment, and the known susceptibility of striped bass (Wolf and Smith 1999) these findings should not be surprising. In the early 1980s, Sakanari et al. (1983) described a high prevalence of tubercular lesions in Pacific coast striped bass. While the species was not identified, mycobacteria was suggested to be the causative agent based on histopathology. MacKenzie (1988) reported presumptive mycobacteriosis (not culture confirmed) in Northeast Atlantic mackerel based on observations of grayish-white nodules in the spleen. Thus reports of presumptive mycobacteriosis in wild fishes of the same species, and others in the same environment as the coastal migratory stock coincide with our initial findings in Chesapeake Bay.

While the occurrence of granulomatous inflammation increased in later years in our retrospective analysis, we cannot speak to prevalence of disease during any time period reported. Fish examined during this review were collected in an opportunistic manner from concerned anglers or watermen, or from small-scale investigations of

reports of unhealthy fish. Thus sampling may be biased, and comparisons should not be made to outside of this collection.

In 1988, mortalities of bluefish (*Pomatomus saltatrix*), striped bass, and sea trout (*Cynoscion regalis*) were reported throughout the Bay and associated with hemorrhagic lesions. Culture efforts routinely isolated *Streptococcus sp.* (Baya et al. 1990). From 1993 – 1995, reports of external lesions on striped bass began to surface with increasing frequency. Many of the archival cases during this time period were in response to such reports from concerned watermen and anglers and represent the initiation of extensive efforts within the states of Maryland and Virginia to identify causative agents. Efforts to culture causative organisms during this time period resulted in numerous species being isolated, however they were primarily gram-negative enterics (MDNR, Unpublished Data).

The presence of non-verminous granulomas did not go unnoticed, and early investigators suspected the involvement of Actinomycetes organisms (such as *Norcardia, Corynebacterium, Mycobacterium*) (MDNR, unpublished case reports). *Carnobacterium sp.* was isolated in 1993, however the pathology associated with this pathogen and *Corynebacterium aquaticum* differ from the observed granulomatous inflammation, (Toranzo et al. 1993, Baya et al 1992). In the spring of 1994 an isolated lesion event occurred in the Potomac River at the mouth of the Wicomico River, MD which involved over 50 fish. This outbreak was diagnosed as being the result of *Edwardsiella tarda,* however, subsequent challenge studies failed to yield similar lesions as those seen in wild collected fish (Baya et al. 1997). In 1995 a second outbreak occurred in the Potomac River. In this case affected fish yielded isolates of *Aeromonas* sp. and *Pseudomonas* sp.

Continued efforts in 1996 and 1997 resulted in the culture of *Mycobacterium sp.* (Hedrick et al. 2001, Rhodes et al. 2001).

One hypothesis concerning the current epizootic infers that intensive stocking efforts during the mid 1980's to early 1990's may represent a source of mycobacterial pathogens. Our initial finding in 1984 pre-dates the extensive stocking efforts coordinated by the Atlantic States Marine Fisheries Commission (Upton 1993). From 1985 – 1992 over 6.5 million phase II and III juveniles were released into Chesapeake Bay (Rulifson and Laney 1999). However, striped bass strains have been readily stocked around the country since the late 1800's. More specifically, both Roanoke River, NC strain and Chesapeake strain striped bass were stocked in Virginia Rivers as early as 1975 (Rulifson and Laney 1999), and similar small scale stockings occurred in Maryland as early as 1960 (Steve Early, Maryland Departement of Natural Resources, personal communication). In addition, nearly 1.2 million fry were released in 1981 by the State of Maryland (Tarnowski 1999). Thus the timeline established with our study yields little insight to the potential role of stocking, and it is unlikely that these linkages can be made with the paucity of data available.

Other hypotheses state that stressors (i.e., nutrition, poor water quality and habitat, elevated temperature) may have influence in combination with record high numbers of fish (Ottinger and Jacobs 2006). Indeed, the frequency of reports of external lesions increased in the early 1990s, a time when stocks reached historical highs. It should be noted that the State of Maryland declared moratoria on striped bass harvest from 1985-1991 which may have greatly influenced reporting as anglers and watermen were not targeting the species. We cannot infer prevalence from our data, however, the frequency

of archived samples containing acid fast bacteria was greater in the early 1990s than any other period while sample size remained consistent (Table 3-3). The majority of these samples (6/7) were from 1993 to present.

Retrospective analysis of tissues using current molecular techniques offers an important epidemiological tool. The sensitivity and specificity of PCR in particular allow for rapid identification of pathogens not possible in earlier investigations. However, the process of fixation and subsequent embedding of tissues is not ideal for recovery of RNA or DNA. Bouin's fixative, used for all fish in this study after 1987, is particularly problematic. Bouin's is composed of picric acid, formalin, and acetic acid. This acidic environment can rapidly degrade DNA, a process that can continue for years after fixation (Bonin et al. 2005). Our experience, and that of other investigators, suggests that extracting quality DNA from tissues preserved in this manner is extremely difficult (Greer et al. 1991). Bonin et al. (2005) had limited success in amplifying fragments less than 200 bp, although, DNA restoration was generally required and no mention is given to the age of tissues examined.

For the positive samples preserved in 10% neutral buffered formalin, we amplified mycobacterial DNA and applied multiple gene sequencing to attempt identification to species level. The ITS region of the 16-23S rRNA gene has successfully been used for the differentiation of fish mycobacterial isolates before (Levi et al. 2003, Kent et al. 2004), but is also conserved enough for the development of genus level assays of use in screening unknown samples (Bruijnesteijn van Coppenraet 2003). Within the fish pathogens of the *M. tuberculosis* clade, the ITS fragment does not have sufficient variability to separate *M. marinum*, *M. shottsii*, *M. psuedoshottsii* and *M. ulcerans*

(Figure 3-2). These closely related fish pathogens are difficult to separate owing to their high homology for many gene targets, especially the most common 16S rRNA gene (Rhodes et al. 2005).

The exported repeated protein (*erp*) gene codes for an extracellular protein thought to be genus specific to mycobacteria (de Mendoça-Lima 2001). Recent work suggests that it is a virulence determinant in both *M. marinum* and *M. tuberculosis* allowing for bacterial replication within host macrophages (Cosma et al. 2006). The primer set ERP 1F and ERP 1R produced strong amplicons from most species, however, was highly homologous among those sequenced with only a two nucleotide difference separating our isolates and *M. psuedoshottsii* from *M. marinum*, *M. ulcerans*, and *M. shottsii*. An adjoining fragment of the central region of the *erp* gene was shown by Rhodes et al. (2005) to be capable of distinguishing *M. marinum*, *M. shotttsii*, and *M. psuedoshottsii*. While sequence analysis of this region clearly demonstrates the presence of *M. psuedoshottsii* (Table 3-4) in case 84-42, we were unable to obtain product from 85-4. With most genes examined, 85-4 provided less template for sequencing than 84-42, suggesting lower concentration of the pathogen in this tissue.

The insertional sequences (IS) IS2404 and IS2606 were previously thought to be specific for *M. ulcerans* (Stinear et al. 1999). However, recent evidence suggests that they may be associated with many members of the *M. tuberculosis* clade (Rhodes et al. 2005, Ranger et al. 2006). Insertional sequences are mobile genetic elements that are available in high copy numbers within *M. ulcerans*, and are of interest in their potential to serve as a marker for mycolactone producing mycobacteria (Ranger et al. 2006). Few sequences for either IS have been published, however our results suggest that they are

fairly well conserved. We were not able to amplify IS2404 or IS2606 from *M. shottsii* or *M. marinum.* Strain variability in the detection of these elements has been reported and success may be somewhat dependent on the use of high fidelity polymerase (Rhodes et al. 2005, Ranger et al. 2006).

In conclusion, we have found evidence that mycobacterial infections in Chesapeake Bay striped bass are not a recent phenomenon. Members of the same group of pathogens involved in the current epizootic were present in striped bass since at least 1984. The DNA isolates obtained most closely resemble *M. psuedoshottsii*, however, further taxonomic evaluation is ongoing to validate this finding for 85-4 and to evaluate additional archived samples from other species and geographic regions. Archived tissues are an invaluable source for examining the epidemiology of disease and establishing timelines relevant to other factors influencing a population.

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Table 3-1. Primer sets used for the determination of mycobacterial species present in tissue blocks and source.

Primer	Sequence (5' to 3')	Reference
ITS F	GGGGTGTGGTGTTGAG	Bruijnesteijn et al. 2004
ITS R	CTCCCACGTCCTTCATC	
ERP 1 F	GTGCCGAACCGACGCCGACG	de Mendoça-Lima et al. 2001
ERP 1 R	GGCACCGGCGGCAGGTTGATCCCG	
ERP OX F	TTGTCCTCGTTCGGGATCAATCTG	This study
ERP OX R	TCAACGCCGGATTGGTGAGT	
IS2404 F	AGCGACCCCAGTGGATTGGT	Stinear et al. 1999
IS2404 R	CGGTGATCAAGCGTTCACGA	
IS2606 F	GGCCTGGCGGATTGCTCAAGG	Stinear et al. 1999
IS2606 R	CGTAGATGTGGGCGAAATGG	

Table 3-2. Mycobacterium strains sequenced for comparison to DNA isolated from archived tissues.

Species	Strain
M. fortuitum complex	VAMDRCVM FL04-42-34 LB
M. gordonae	VAMDRCVM FL04 -25-2 LivA
M. interjectum	ATCC 51457
M. marinum	ATCC 927
M. mariokaensis	VAMDRCVM FL04-42-217A
M. psuedoshottsii	USGS L-1A *
M. scrofulaceum	ATCC 700734
M. shottsii	ATCC 700981
M. szulgai	ATCC 23069
M. triplex	ATCC 700071
M. ulcerans	ATCC 19423 *

* DNA preparations only, culture maintained by USGS
VAMDRCVM - Virginia-Maryland Regional College of Veterinary Medicine, College Park, MD
USGS - United States Geological Survey, National Fish Health Laboratory, Kearnysville, WV

Table 3-3. Wild collected, Chesapeake Bay striped bass available from 1975 – 1995 presented in five year intervals. NVG = non verminous granuloma, AFB+ = positive identification of non-branching, acid fast bacteria, and PCR + reflects successful amplification of the ITS.

Year	Cases	NVG	AFB+	PCR +
1975-1980	21	3	0	0
1981-1985	16	2	2	2
1986-1990	20	2	0	NA
1991-1995	32	20	7	NA

Table 3-4. Sequence alignment of the central region of the erp gene. *M. psuedoshottsii* and 84-42 are identical, and differ from *M. shotttsii*, *M. marinum*, and *M. ulcerans* by a minimum 30 base pair reduction.

```
                                10        20        30        40        50
                                .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
M. ulcerans (AF213153)          A T C T G C C T C C A G T G C C C A G C C T C A C C G G C G G T A C C G G C A C C G G C A T G A G C  50
M. marinum (CP000325)           A T C T G C C T C C A G T G C C C A G C C T C A C C G G C G G T A C C G G C A C C G G C A T G A G C  50
M. shottsii *                   A T C T G C C T C C A G T G C C C A G C C T C A C C G G A G G T A C C G G C A C C G G C A T G A G C  50
M. shottsii                     A T C T G C C T C C A G T G C C C A G C C T C A C C G G A G G T A C C G G C A C C G G C A T G A G C  50
M. pseudoshottsii *             A T C T G C C T C C A G T G C C C A G C C T C A C C G G C G G T A C C G G C A C C G G C A T G ~ ~ ~  47
M. psuedoshottsii               A T C T G C C T C C A G T G C C C A G C C T C A C C G G C G G T A C C G G C A C C G G C A T G ~ ~ ~  47
84-42                           A T C T G C C T C C A G T G C C C A G C C T C A C C G G C G G T A C C G G C A C C G G C A T G ~ ~ ~  47

                                60        70        80        90        100
                                .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
M. ulcerans (AF213153)          C C G G G G T T G A C C A G C C C G G G G T T G A C C A G C C C C G G C T T G A C C A G C C C G G G  10
M. marinum (CP000325)           C C G G G C C T G A C C A G C C C G G G G T T G A C C A G C C C G G G G T T G A C C A G C C C C G G  10
M. shottsii *                   C C A G G C C T G A C C ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ A G C C C G G G G T T G A C C A G C C C C G G  85
M. shottsii                     C C A G G C C T G A C C ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ A G C C C G G G G T T G A C C A G C C C C G G  85
M. pseudoshottsii *             ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ A G C C C G G G G T T G A C C A G C C C C G G  70
M. psuedoshottsii               ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ A G C C C G G G G T T G A C C A G C C C C G G  70
84-42                           ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ A G C C C G G G G T T G A C C A G C C C C G G  70

                                110       120       130       140       150
                                .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
M. ulcerans (AF213153)          T C T C A C C A G C C C A G G G C T G A C C A G C C C A G G T C T C A C C A G C C C A G G C C T G A  15
M. marinum (CP000325)           C T T G A C C A G C C C A G G G C T G A C C A G C C C G G G T C T C A C C A G C C C A G G C C T G A  15
M. shottsii *                   C T T G A C C A G C C C A G G G C T G A C C A G C C C G G G T C T C A C C A G C C C A G G C C T G A  13
M. shottsii                     C T T G A C C A G C C C A G G G C T G A C C A G C C C G G G T C T C A C C A G C C C A G G C C T G A  13
M. pseudoshottsii *             C T ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ T G A C C A G C C C G G G T C T C A C C A G C C C A G G C C T G A  10
M. psuedoshottsii               C T ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ T G A C C A G C C C G G G T C T C A C C A G C C C A G G C C T G A  10
84-42                           C T ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ T G A C C A G C C C G G G T C T C A C C A G C C C A G G C C T G A  10

                                160       170       180       190       200
                                .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
M. ulcerans (AF213153)          C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C C C T G C C G G G C  20
M. marinum (CP000325)           C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C C C T G C C G G G C  20
M. shottsii *                   C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C T C T G C C G G G C  18
M. shottsii                     C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C T C T G C C G G G C  18
M. pseudoshottsii *             C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C C C T G A C G G G C  15
M. psuedoshottsii               C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C C C T G A C G G G C  15
84-42                           C C A C C C C G G G T C T G A C G C C G G G A A T G C C G G G A G A C T T G G C C C T G A C G G G C  15

                                210       220       230       240       250
                                .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
M. ulcerans (AF213153)          A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  25
M. marinum (CP000325)           A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  25
M. shottsii *                   A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  23
M. shottsii                     A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  23
M. pseudoshottsii *             A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  20
M. psuedoshottsii               A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  20
84-42                           A C G A C G C T G C C C C C G A C A C C G G G C G C C G C C G T C A A C C C C G C A C T C A C C A A  20

                                260
                                .    |    .    |    .
M. ulcerans (AF213153)          T C C G G C G T T G A  261
M. marinum (CP000325)           T C C G G C G T T G A  261
M. shottsii *                   T C C G G C G T T G A  246
M. shottsii                     T C C G G C G T T G A  246
M. pseudoshottsii *             T C C G G C G T T G A  216
M. psuedoshottsii               T C C G G C G T T G A  216
84-42                           T C C G G C G T T G A  216
```

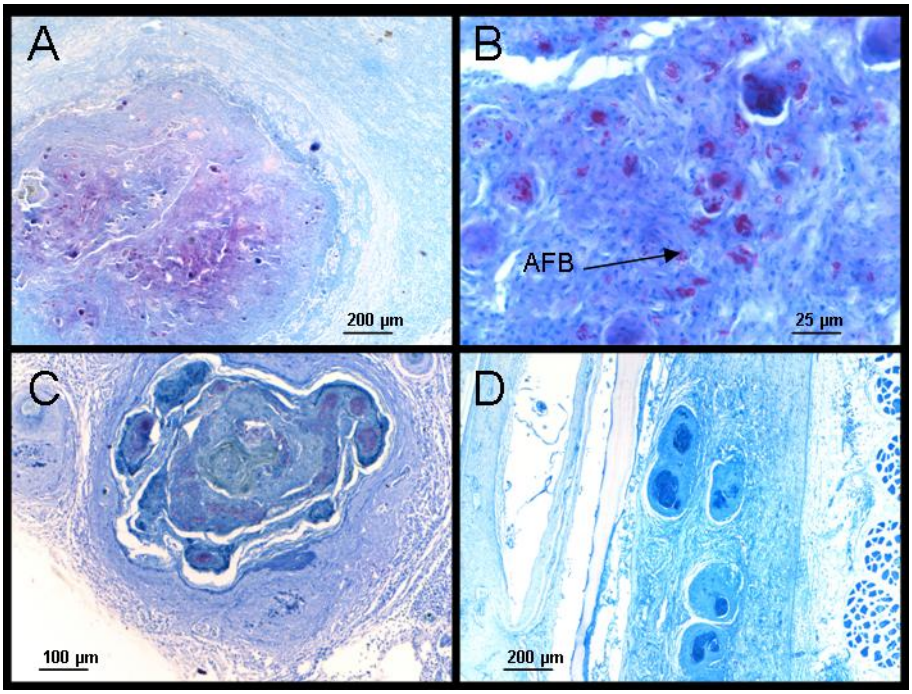

Figure 3-1. Photomicrographs of granulomatous inflammation from archival striped bass tissues. All tissues stained with Ziehl Neelsen. A = splenic granuloma 1985 (4x), B = acid fast bacteria (AFB) in granuloma core 1985 (40x), C = granuloma of poster kidney 1984 (10x), and D = dermal inflammation and granuloma 1993 (4x).

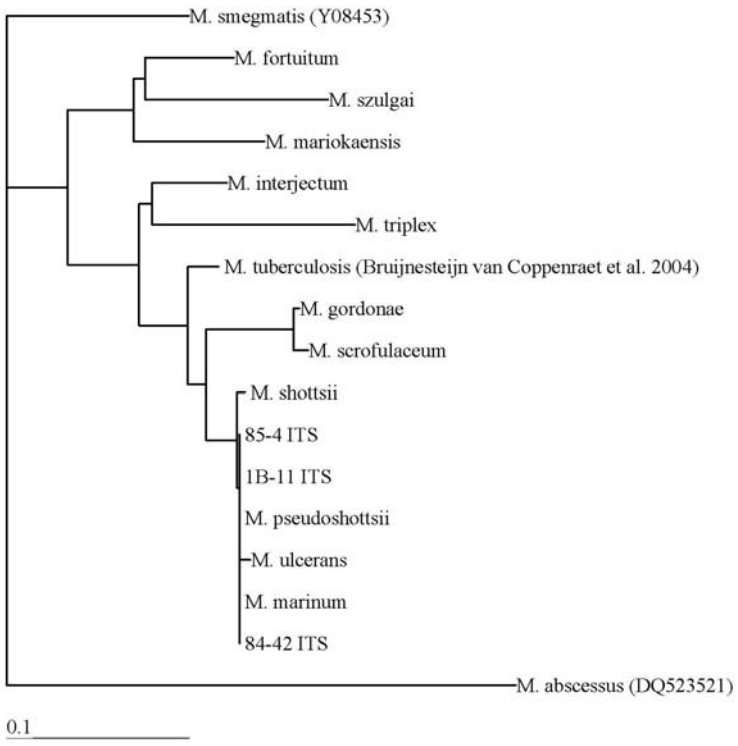

Figure 3-2. Neighbour-joining tree based on the intergenic transcribed spacer region of the 16-23S rRNA gene for mycobacteria. Bar length represents 0.1 substitutions per site.

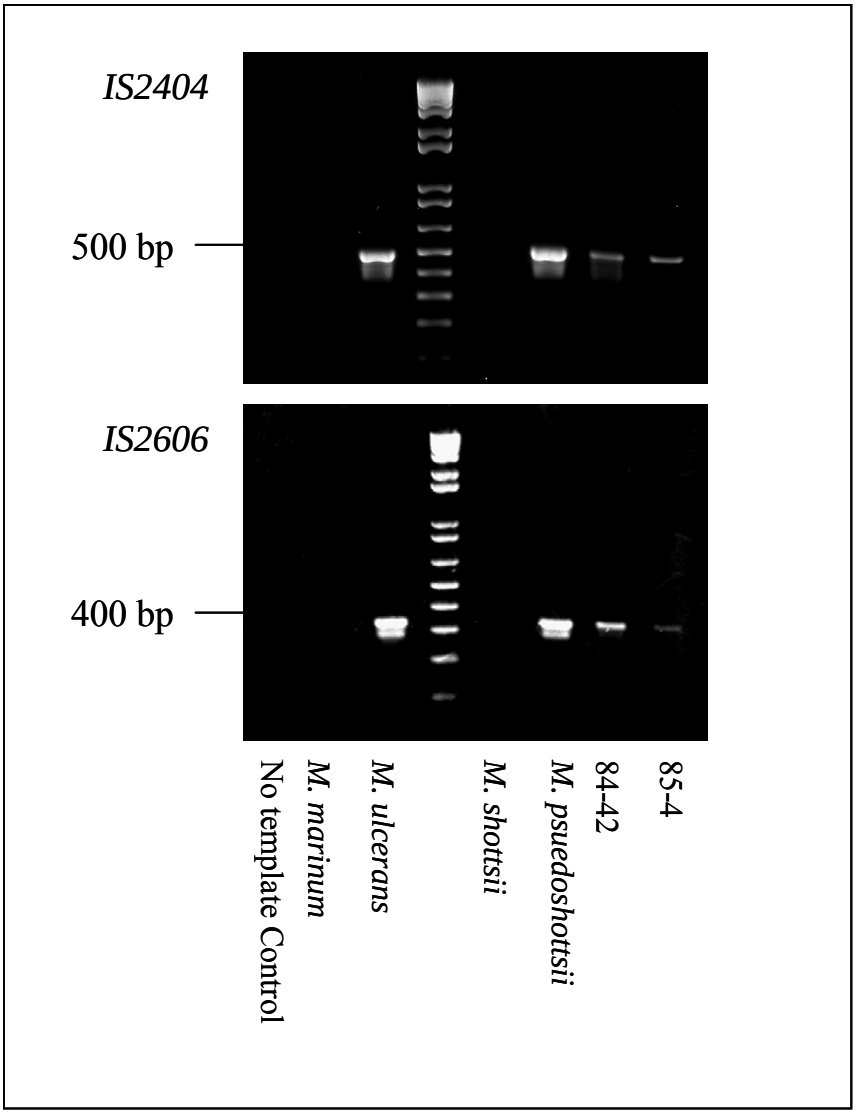

Figure 3-3. PCR amplification of insertional sequences IS2404 (492-bp product) and IS2606 (332-bp product) from selected mycobacteria and DNA extracted from paraffin embedded tissue blocks. IS2602 gel photo enhanced to show weak positive band of sample 85-4.

CHAPTER 4 - NUTRITIONAL STATUS OF CHESAPEAKE BAY STRIPED BASS (*Morone saxatilis*) AND RELATIONSHIP TO DISEASE STATE.

ABSTRACT

During 1998-1999, we used proximate composition to determine the nutritional status of fall collected striped bass, and associations of nutritional competency with mycobacteriosis and ulcerative conditions. To provide reference intervals for this method, food deprivation studies were conducted with 40 wild-caught striped bass (18 - 20°C, starved and fed treatments, 2 months). Results clearly demonstrate low weight at length, elevated moisture (~80%), and a lack of storage lipids (< 0.5 %) in the fall population compared to experimental fish and historic data (1990-1991). Wild-collected fish were not significantly different in chemical composition, weight at length, or mesenteric body fat than fish deprived of food for two months ($p > 0.05$). In addition, the presence of mycobacteriosis did not result in further reductions of lipid or moisture ($p > 0.05$). However, ulcerative conditions clearly impacted chemical profiles ($p < 0.05$). These findings suggest that fall collected Chesapeake Bay striped bass were in poor nutritional health, which may be independent of disease.

INTRODUCTION

Since at least 1997, mycobacteriosis has been highly prevalent in Chesapeake Bay striped bass (*Morone saxatilis*) with concurrent reports of emaciated fish and external, ulcerative lesions (Heckert et al. 2001, Rhodes et al. 2001, Overton et al. 2003). Mycobacteriosis is a chronic, progressive disease caused by several species of gram – positive bacteria of the genus *Mycobacterium*. Clinical signs of mycobacteriosis are not

specific to the disease and can vary in occurrence and severity. Signs may include dermal lesions, pigmentary changes, emaciation, stunted growth, exophthalmia, and slowed swimming; however, some fish show no external symptoms (Noga et al. 1990, Colorni et al. 1998, Wolf and Smith 1999, Rhodes et al. 2001, Swanson et al. 2002). The primary pathology is that of granulomatous inflammation and associated tissue damage particularly to visceral organs. While particularly problematic in aquaculture (Colorni et al. 1998, Hawke 2000), mycobacteriosis has occurred in wild marine fisheries including cod (*Gadus morhua*) (Alexander 1913) striped bass (Sakanari et al. 1983, Landsdell et al. 1993), Atlantic mackerel (*Scombur scombrus*) (MacKenzie 1988), and several species of Pacific salmon (*Oncorhynchus spp.*) (Arakawa and Fryer 1984).

Conditions leading to the onset or continuation of the current epizootic of mycobacteriosis affecting striped bass in the Chesapeake Bay have been the subject of much debate among the scientific and lay communities. One hypothesis suggests that striped bass are food limited, which in turn may be related to disease state (Hartman and Margraf 2003, Uphoff 2003). Following a period of marked decline in the mid 1970s, management actions successfully increased the Atlantic coastal population of striped bass 10-fold in the period from 1984-1994 (ASMFC 2005, Richards and Rago 1999). Uphoff (2003) suggests that predatory demand for Atlantic menhaden (*Brevoortia tyrannus*), an important forage species for striped bass, has exceeded their population abundance in Chesapeake Bay. While primary prey populations fluctuate annually, in the period from 1994 to present, Atlantic menhaden, bay anchovy (*Anchoa mitchilli*), and Norfolk spot (*Leiostomus xanthurus*) have all suffered poor recruitment (MDNR Juvenile Seine Survey, Unpublished data). This lack of forage base is supported by bioenergetics

modeling efforts of Overton (2003) which show a reduction in menhaden consumption, and lack of adequate forage after age 2. In addition, both length and weight at age have declined in 3-6 year old males collected during Maryland's winter gill net fishery (Walleret al. 1999, Waller and Warner 1999), and variance in weight at length has increased in the latter half of the 1990's (Uphoff 2003).

The issue of nutritional status and mycobacterial disease is complex. Wasting is a recognized manifestation of tuberculosis in humans (Paton and Ng 2006), however, the associated pathology remains poorly understood (Shwenk et al. 2004). The cause is most likely a combination of decreased appetite and energy loss due to altered metabolism and demands of the inflammatory and immune response. Emaciation and wasting has been associated with mycobacteriosis in cultured finfish (Inglis et al. 1993, Chinabut 1999, Conroy and Conroy, 1999), and there is evidence of decline in condition factor with more severe stages of disease in striped bass (Cardinal, 2001, Ottinger et al. 2006), particularly if external lesions are present (Overton et al. 2003). However, macronutrient malnourishment has been clearly demonstrated to alter the progression and severity of mycobacterial disease through disruption of immune function in higher order vertebrates (Dia and McMurray 1998, Chan et al. 1996), and the relationship of immune response and nutritional status in fish has been established (Blazer et al. 1991, Wise et al. 1993, Lim and Klesius 2002). Thus the relationship between nutritional status and disease state is a difficult question of causality. Are striped bass food limited leading to enhanced susceptibility or progression of the disease, or does mycobacteriosis alone lead to wasting?

Proximate analysis is a widely accepted standard for precise measurement of chemical components in a variety of food products and animals (AOAC 1999). In fish, the principal components normally include moisture, lipid, protein and ash and occasionally carbohydrate (Busacker et al. 1990). As a fish starves, it first uses glycogen deposits primarily from the liver. Once depleted, triglycerides are mobilized from muscle tissues and mesenteric lipid deposits. Lipids used are replaced with water in a linear fashion. As water and lipid weight do not differ substantially, changes in lipid energetics may go undetected in weight-based assessments. Finally, protein catabolism ensues in severe cases of starvation (Love 1980). While its application to wild studies has been limited by the extensive cost and time requirements, no other method provides the same detailed picture of physiological state.

We determined the chemical composition of muscular tissue of wild collected fish during the period of 1998-1999 and experimentally food deprived controls to evaluate: 1) the nutritional status of Chesapeake Bay striped bass, and 2) the association of nutritional status and disease state. In addition, several rapid observational indices for field application were evaluated against traditional laboratory assays.

METHODS

Sample Collection

In September-November 1998, and late August-November 1999, striped bass were collected from commercial pound nets and by hook and line with the assistance of the Maryland Department of Natural Resources (DNR) striped bass stock assessment project and commercial hook and line watermen. Sampling locations (Figure 1) were

stratified by Upper (Bay Bridge and North), Middle (Bay Bridge to Little Choptank River, MD) and Lower (Little Choptank, MD to Virginia Line) portions of Maryland's Chesapeake Bay. Each strata and gear combination was sampled twice per year, totaling 120 fish (3 strata, 2 gear types, 2 replicates, 10 fish per trip).

Necropsy and Sample Processing

Fish were immediately euthanized by severing the cervical spine, weighed (g), measured (total length, mm) and observations of gross pathology recorded. Fillets were removed from the left side of each fish, vacuum packed in commercial freezer bags, and placed on dry-ice for transportation to the laboratory. All samples were maintained at -80° C until analysis. Standard necropsies were performed on all fish, with gross observations recorded. A series of observational indices were conducted including presence of visual granulomas in the spleen, parasite burden, and quantity of mesenteric body fat. The latter was scaled from 0 (no fat present) to 3 (complete coverage of visceral organs) by committee as outlined by Goede and Barton (1980). Sections of gill, eye, brain, spleen, liver, anterior and posterior kidney, gonad, intestine, stomach, and heart were preserved in 10% neutral buffered formalin. All tissues were embedded and sectioned in accordance with routine paraffin histology. Initially, sections were stained with hemotoxylin and eosin and visualized by light microscopy. Subsequent sections were stained with Ziehl-Neelsen for the visualization of acid-fast bacteria when non-verminous granulomas were detected. Severity of infection was scaled using only the spleen as follows: 0 – no granuloma, 1 – focal lesion, 2 – 2-5 granulomas, and 3 – greater than 5 granulomas.

Experimental Study

A total of 62 striped bass were obtained from MDNR after being captured by hook and line, and retained in a net pen at the Sarbanes Cooperative Oxford Laboratory for two weeks. Fish were transported to the University of Maryland, Horn Point Laboratory and maintained in 500 gallon flow-through tanks. All fish were fed cut menhaden every other day for two weeks prior to the start of the experiment. Forty fish were randomly selected from the group, weighed (g), measured (TL), photographed, and tagged for individual identification. Mean starting weights and lengths were 1365 ± 487 g (SD) and 492 ± 56 mm (SD) respectively. Tagging was accomplished by inserting Passive Integrated Transponder (PIT) tags into the dorso-ventral musculature. Fish were randomly divided into four 500 gallon tanks (10 each) designated as either fed (control, 2 tanks) or starved (treatment, 2 tanks). Control fish were fed cut menhaden *ad libinum* 5 days per week, while treatment fish were not fed. Water temperature was set to mimic fall conditions and ranged from 16.9 - 23.9°C, with salinity ranging from 10.3 - 14.5 ppt. Temperature control was accomplished by mixing heated and ambient Choptank River water, thus it fluctuated some with the environment. The experiment was terminated after two months (11/16/99 - 1/15/00) and all fish were euthanized and sampled as outlined previously for field collections.

Proximate Composition

Random sub-sampling of 33% of the 240 samples collected in 1998 and 1999 and from the tank studies were analyzed for proximate composition. Three samples were randomly chosen from each sampling day by use of a random number generator

(Microsoft Excel, 2004). Frozen fillets were skinned prior to homogenization in a commercial blender and a 50 gram sub-sample removed. Triplicate 5 gram samples were placed in desiccated aluminum pans and dried at 90° F for 15 h for determination of moisture content. The remainder was dried similarly to allow for sufficient sample for other components. Dried sample (2.5 grams) were then wrapped in Wheaton #1 filter paper in triplicate, and lipids were extracted with petroleum ether over 15 h via Goldfish apparatus (Labconco Corporation, Kansas City, MO). Protein was determined from dried, de-fatted samples using the Kejldahl nitrogen, acid digestion technique, and ash was determined by muffle furnace (500°F). All methods were in accordance with the Association of Official Analytical Chemists (1999).

Data Analysis

Data analysis was conducted within SAS v. 8.02.(SAS 1990). Contingency table analysis and Spearman's rank correlation (SRr) was used to determine relationships between categorical and ordinal variables (Proc Freq). Fisher's exact test (FE) was employed where expected cell frequency was less than 5. Otherwise, Chi square (χ^2) was used. Continuous data was analyzed using ANOVA (proc GLM) with subsequent least square means comparison (pdiff, Tukey-Kramer adjustment) unless otherwise noted. Analysis of covariance was used determining weight at length relationships (Proc GLM) with $\log_e$ transformed data. Proximate composition data was analyzed as concentrations of component per gram dry weight (lipid) or fat-free dry weight (protein and ash). In some cases, data is presented as wet weight percentage for ease of comparison to

published data. In instances where data is presented as pooled, year and regional effects were not significant ($p > 0.05$).

RESULTS

Characterization of Sample Sources: Prevalence of Mycobacteriosis and External Lesions (1998-1999)

Mycobacteriosis was apparent in 30.6% of all striped bass collected during 1998-1999. Prevalence was significantly associated with increased age (FE, $p = 0.026$) and fish length ($F=5.2$, 1,221 df, $p = 0.024$) in age 2 though 4+ fish examined (Table 4-1). Severity of infection as determined from splenic granuloma counts was also positively correlated with age. However, the strength of this relationship was weak ($SRr = 0.186$, $p = 0.0055$) and length explained only a marginal proportion of the total variance ($F = 2.04$, 1,221 df, $p = 0.11$). Spatially, mycobacterial infections declined slightly with distance from the head waters of Chesapeake Bay, (Upper Bay (33%), Middle (29%), Lower (27%)), however this association was not significant ($\chi^2 = 0.7881$, $p = 0.6743$). External lesions were noted on 11% of fish over the two year period, and increased significantly with age (FE, $p = 0.021$) (Table 4-1). Lesions were also significantly associated with histological detection of mycobacteriosis (FE, $p = 0.0012$). However, only 14 of 222 fish (6.3%) displayed both symptoms, whereas 61 (27.4%) had only one of these symptoms, so this association was not consistent. Fish collected during the study were predominantly male (85.3%) and associations with dermal lesions or disease state were not significant by sex ($p> 0.05$).

Experimental Food Deprivation: Establishment of Benchmarks

The purpose of the starvation study was to provide benchmarks for the wild-collected fish in terms of proximate composition, and weight-length relationships. The 58 day feeding trials provided clear separation of weight ($p = 0.0014$) and condition ($p<0.001$) in fish, while lengths remained similar ($p = 0.11$). Mean weight loss over the 58 day period in food deprived striped bass was -159.4 ± 54.6 g (SD) while fed fish experienced averaged gains of 593.3 ± 198.6 g (SD) during the same period. Tank effects and the interaction of tank and treatment were not significant ($p > 0.05$). Mesenteric body fat was largely absent from starved fish, but generally covered over 75% of the pyloric caecae in their fed counterparts (FE, $p <0.0001$). In addition, gonadal development was slightly depressed in starved fish. No serious external or internal abnormalities were noted among any of the fish from gross examination. No bacteria were cultured from brain, liver, or head kidney samples (Dr. Ana Baya, VA-MD Regional College of Veterinary Medicine, personal communication).

Nutritional Status of Wild Collected Striped Bass

Indicators of nutritional health in wild-collected fish during the years 1998 and 1999 were generally poor. Observations of mesenteric body fat consistently showed the vast majority of fish (mean = 76%) had no visible reserves. In comparison to experimentally fed and food deprived fish, wild-collected striped bass had statistically similar reserves as fish starved for two months (FE, $p = 0.30$), but different from well-fed fish (FE, $p < 0.0001$)(Table 4-2). This index of mesenteric body fat correlated strongly with measured tissue lipids confirming its utility as a rapid indicator when necropsy is

performed (Spearman's rank correlation, n=80 r = 0.73, $p < 0.0001$). The relationship is linear, with each progressive index level significantly different from the next in measured tissue lipid (F=50.5, 3,79 df, $p < 0.0001$; least square means $p < 0.03$) (Figure 4-2). Weight at length relationships showed a similar pattern as mesenteric body fat (Figure 4-3). Analysis of covariance (ANCOVA, F = 309.6, 7,260 df, $p < 0.0001$) suggests that the slopes of the logistic regressions were similar (F = 1.28, 3df, p = 0.28), however the treatments differed (F= 8.1, 3df, $p < 0.0001$) with the fish experimentally fed having greater weight at length than all the others (LS means comparison, $p < 0.0005$). No significant difference was noted in weight at length between fish starved for 2 months, and those collected in the wild from 1998-2001 (LS means, $p > 0.05$).

One third of all fish collected were randomly sampled for proximate composition. No significant difference in moisture (F= 2.13, 3,55 df, p = 0.1071), lipid (F=1.71, 3,55 df p = 0.18), protein (F = 1.24, 3,55 df, p= 0.30) or ash (F = 0.52, 3, 58 df, p= 0.67) was noted by year or region for wild-collected fish. Wild-collected fish had elevated moisture and low lipid content, similar to the experimentally starved group, but not characteristic of fed fish (LS means, $p < 0.05$) (Table 4-3). Protein and ash levels were not significantly different among any of the groups examined suggesting protein catabolism was not largely apparent ($p > 0.05$)(Table 4-4). However, the 90^{th} quartile (N = 6) represented a significant reduction in total protein from the population mean (18.2 ± 0.17 and 13.9 ± 0.56 respectively, F = 56.2, 1,68 df, $p < 0.0001$). In comparison to historical data, lipid values were generally lower and moisture higher than encountered in fall collected striped bass in 1990-1991 by Karahandian et al. (1995) (Table 4-3). However,

only summary data was available from this publication and direct comparison of lipid values is hampered by differences in extraction technique.

Association of Nutritional Status with Disease State

Moisture concentration was significantly elevated in fish with external lesions ($F = 3.97$, 1,65 df, $p = 0.050$), but was not explained by the presence of mycobacteriosis or the interaction of conditions ($p > 0.05$) (Table 4-5). Mean lipid concentration was also reduced in fish with external lesions (Y=1.03 ± 0.79 vs N=1.97 ± 0.39), however the proportion of variance explained by these lesions was not significant ($F = 1.22$, 1,65 df, $p = 0.27$). Mycobacterial infection had no influence on lipid concentration, nor did the interaction of lesions and disease (Table 4-5). The highest proportion of the variance in protein was again explained by exterior lesions (56%), but overall, both protein and ash were insignificant explanatory variables of disease state (Table 4-5). Of the six fish in the 90^{th} quartile for protein suggestive of catabolism, four had mycobacteriosis, three external lesions, and one neither malady.

DISCUSSION

Our results are indicative of an overall level of poor health in Chesapeake Bay striped bass during 1998 and 1999. Striped bass were characterized by an elevated prevalence of mycobacteriosis and external lesions, low tissue lipid reserves, elevated moisture, and weight at length and chemical profiles similar to experimentally food deprived fish. Further, nutritional condition was not explained by mycobacteriosis

suggesting that the chronic disease process does not exert a strong energetic demand above environmental determinants.

Overall, mycobacteriosis was present in 31% of striped bass, with prevalence increasing with age. Isolation and identification of mycobacteria was not performed, and only granulomas of appropriate epitheliod morphology containing clearly visible acid-fast, non-branching bacteria were considered positive. The use of histopathology represents a conservative estimate of infection, as acid-fast bacteria are not always visible within granulomas even though they may be readily cultured (Gauthier et al. 2003). The reasons for this are not completely understood, but may relate to growth phase of the pathogen (Nyka and O'Neill 1970).

The prevalence of external lesions on striped bass shows a strong seasonal component peaking in late summer-early fall (Maryland Department of Natural Resources, unpublished data). External lesions on fish are not uncommon, however the apparent increase in cutanaceous lesions in a number of species regionally has gained recognition (Levine et al. 1990). Lesion prevalence in this study was 11% and based solely on visual observation. In our data, there was a significant association of external lesions with mycobacteriosis (FE, $p = 0.0012$). This has been previously reported for Chesapeake Bay striped bass and is suggested to be indicative of advanced cases of mycobacterial infection (Rhodes et al. 2001). However, in our study only 14 of 222 fish (6.3%) displayed both symptoms, whereas 61 (27.4%) had only one of these symptoms, so this association was not consistent. The reduction in tissue moisture and lipid associated with fish with external lesions is consistent with the findings of Overton et al. (2003) who found significant reduction in condition factor. Ulcerative lesions represent a

disruption of the primary mucosal and dermal barrier in fish, and as such may provide a portal of entry for a variety of opportunistic pathogens (Levine et al. 1990). While enhanced immune response may elevate energetic demands, it is likely the osmotic challenges faced by this disruption of epithelium are equally costly.

Proximate composition is a sensitive measure of nutritional status applied widely for characterization of physiological state (Love 1980, Brown and Murphy 1991, Neff and Cargnelli 2004). Commonly, whole body homogenates are evaluated as lipids in particular can vary throughout the body (Love 1980, Einen et al. 1998, Jacobs et al. Submitted). However, the use of whole body preparations negates the opportunity to examine disease status of the fish. Because of this limitation, and to provide comparison to historical data (Karahadian et al. 1995) we used skinless fillets. Lipids in striped bass are predominantly stored in viscera as non-polar triglycerides (Love 1980, Brown and Murphy 1991). Muscle tissue is generally lower in lipid, but is relative to whole body concentrations in age 1 and 2 striped bass (Jacobs et al. Submitted). In our experimental study, we demonstrated that incorporation of lipids into muscle tissue and utilization is directly related to feeding in striped bass (Table 4-3), thus the use of muscle tissue is an appropriate surrogate for comparative studies.

Karahadian et al. (1995) collected striped bass from the Upper Bay, Choptank, and Potomac Rivers in 1990 and 1991 for comparative proximate composition. In general, her findings suggest improved condition in the fall (October, November) in comparison to the spring (April-May). Seasonal changes, as well as age, diet, migration, sex, and temperature can all influence body composition (Brett et al. 1969, Simpson et al. 1992, Neff and Cargnelli 2004). The fall represents a period where striped bass should be

feeding heavily in preparation for over wintering and outmigration (Uphoff 2003). Thus this time period should represent optimal energy gain. Data reported from Karahadian et al. (1995) is reflective of good nutritional status (Table 4-3), but differs greatly from fish collected in 1998 and 1999 in this study. Lipid levels reported as a percentage of wet weight are an order of magnitude greater than those of wild-collected fish in our study, however they were similar to our fed controls (Table 4-3). This comparison is hampered by methodological differences. Karahadian et al. (1995) employed chloroform-methanol extraction which removes both polar and non-polar lipids (Bligh and Dyer 1959). Polar lipids are predominantly represented by phospholipids, which serve as structural components of the cell wall. The relative proportion of these classes is variable, but a general approximation of 1:4 (polar to non-polar) may be appropriate for nutritionally stable fish (Jeong et al. 2000, Joe Soares, UMD(ret) personal communication). Applying this to Karahadian et al.'s (1995) mean lipid value brings the non-polar concentration to 2.5 %, or essentially identical to wild fish fed menhaden diets in our experimental study (2.6%).

While low lipid and elevated moisture was characteristic of wild-collected striped bass in this study, mean protein concentration was relatively stable. In general, protein changes little in fish except in instances of prolonged starvation where the more energetically efficient lipid stores are reduced. Thus protein catabolism represents an advanced stage of malnourishment (endogenous metabolism) (Love 1980). In our study only six fish, representing the lower 90^{th} quartile showed evidence of protein catabolism. Although the majority of these fish had one of the two disease states examined, the

lowest protein was encountered in a fish having neither external lesions or mycobacteriosis (10% ww).

This study has served to define the general status of Chesapeake Bay striped bass, but establishing causal relationships between nutritional health and disease state can not be accomplished from field evaluations. It is impossible to determine the direction the relationship without knowing the prior history of the animal. Our data questions the energetic demand of mycobacteriosis, however, these answers are more likely to come from controlled laboratory approaches.

The overall condition of striped bass in this survey raises many questions of relevance to the management of this species. Is food limitation solely driving condition, or do energetic demands of high summer temperatures and habitat limitation play a major role in the manner suggested by Coutant and Benson (1990)? Most likely they are interrelated which may, as Hartman and Margraf (2003) suggest, make it difficult to manage separately for predator and prey. Are stressful Bay conditions leading to increased or earlier age of outmigration? The unavailability of age 3+ could have substantial financial consequences for Chesapeake states. However, Haeseker et al. (1996) found that striped bass in the Albemarle Sound-Roanoke River remained in the system even though water temperature rose well above suitable limits. Dermal lesions and ectoparasites were extensive as well as reduced condition during summer months, but returned to a normal state for the remainder of the year. This last point is perhaps of most importance in that we do not know whether reduced condition persists into the winter, affects over-winter survival, or has any influence on subsequent spring spawning. These

are answerable questions, and warrant future efforts in spatially and temporally defining the overall health of striped bass in Chesapeake Bay.

ACKNOWLEDGEMENTS

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Table 4-1. Prevalence of mycobacteriosis (Afgrans), severity, and external lesions (Ext Les) in wild striped bass collected from 1988-1999.

Age	N	size	Afgrans[A]	Severity[B]	Ext Les[A]
2	18	260 - 356 mm	16.67%	2.00	0.00%
3	90	357 - 456 mm	22.22%	2.40	7.07%
4+	114	> 457 mm	37.72%	2.44	15.00%

A - Fishers Exact Test of association with age ($p < 0.05$)
B - Spearman's rank correlation ($r = 0.1857$, $p = 0.0055$)

Table 4-2. Index of mesenteric body fat from wild-collected striped bass (1988-1999) in comparison to experimentally fed and food deprived (Food Dep.) fish. Same letter denotes statistical similarity (Fishers Exact Test, $p > 0.05$)

BFI	1998[A]	1999[B]	Food Dep.[A,B]	Fed[C]
0	69%	83%	95%	0%
1	15%	9%	5%	6%
2	14%	4%	0%	18%
3	3%	3%	0%	75%

Table 4-3. Proximate composition of wild-collected striped bass and experimental controls. Mean data from publication is presented from Karahadian et al. (1995), but was not available for statistical analysis. * depicts significance from all others within component ($p > 0.05$), A – chloroform-methanol extraction.

	N	Moisture	Lipid	Protein	Ash
1998 Wild	35	80.67	0.35	17.54	1.25
1999 Wild	37	79.75	0.50	18.19	1.26
2000 Exp. Food Dep.	7	79.30	0.33	19.03	1.29
2000 Exp. Fed	6	74.44 *	2.59 *	22.92	1.41
Karahadian et al. (1995) 1990-1991 Fall Survey	57	77.15	3.14 [A]	21.37	1.33

Table 4-4. ANOVA results for overall comparison of compositional components among 1998 and 1999 wild collected striped bass and experimentally fed and food deprived controls.

	ndf	ddf	F	p
Moisture	3	81	16.71	<0.0001
Lipid	3	81	18.68	<0.0001
Protein	3	81	1.31	0.2782
Ash	3	81	2.07	0.1107

Table 4-5. Model results for mixed model anlaysis of variance of chemical components by disease state and least square means. Values presented for least square means are in concentrations (mg/g dry weight) and errors are standard errors of the mean.

	Moisture		Lipid		Protein		Ash	
	F	***p***	**F**	***p***	**F**	***p***	**F**	***p***
Afgran	0.17	0.68	0.01	0.90	0.04	0.84	0.89	0.35
Ext Les	3.97	0.05	1.22	0.27	0.93	0.34	0.03	0.86
Afgran*Ext les	0.03	0.88	1.43	0.24	0.70	0.40	0.03	0.87
				Least Square Means				
	Y	**N**	**Y**	**N**	**Y**	**N**	**Y**	**N**
Ext lesion	81.52	80.04	1.03	1.97	91.29	90.25	6.38	6.40
SE (+/-)	(0.684)	(0.316)	(0.788)	(0.386)	(0.996)	(0.454)	(0.267)	(0.123)
Afgran	80.94	80.63	1.55	1.45	90.88	90.66	6.54	6.27
SE (+/-)	(0.507)	(0.558)	(0.592)	(0.649)	(0.736)	(0.818)	(0.198)	(0.218)

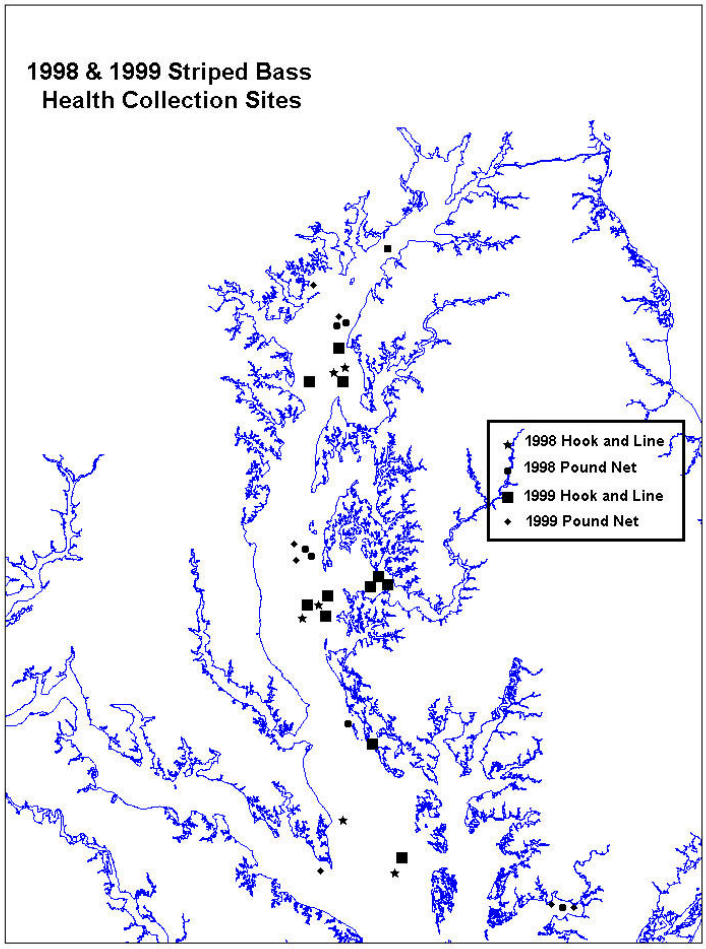

Figure 4-1. Sample collection locations for 1998-1999 MDNR Striped Bass Health Survey. Aggregates represent Upper, Middle, and Lower Chesapeake Bay stratification.

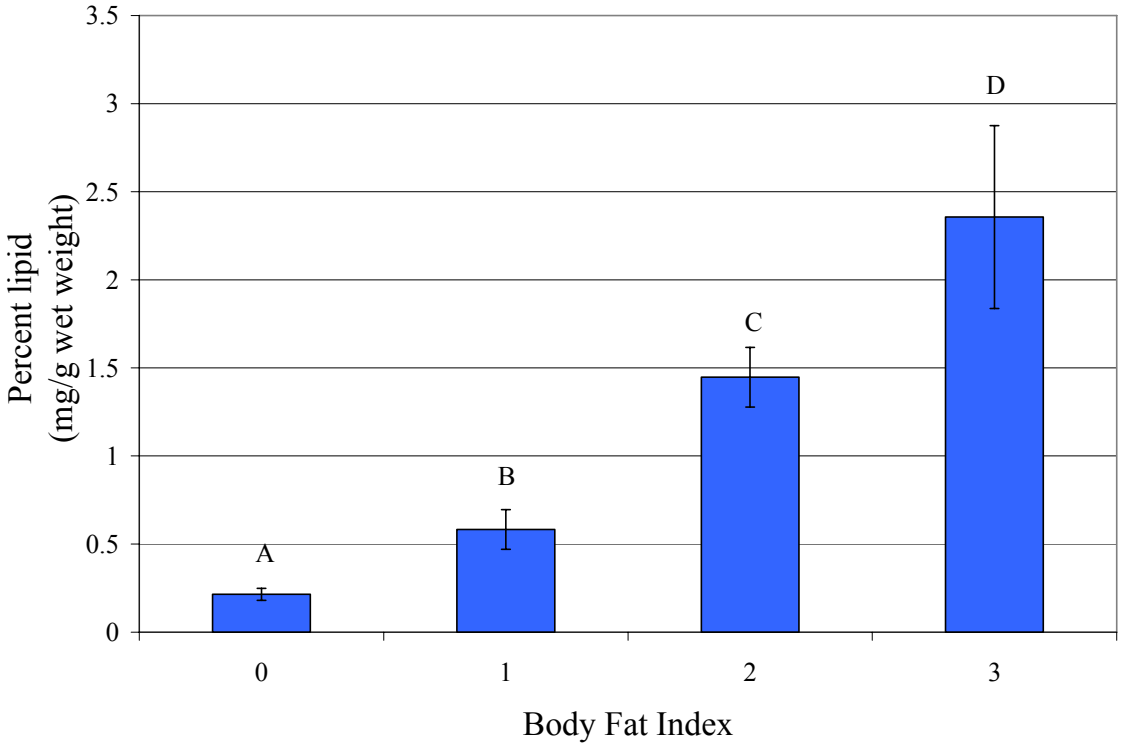

Figure 4-2. Relationship of mesenteric body fat index of Goede and Barton (1990) to measured fillet lipid. Same letter denotes lack of significance ($p > 0.05$).

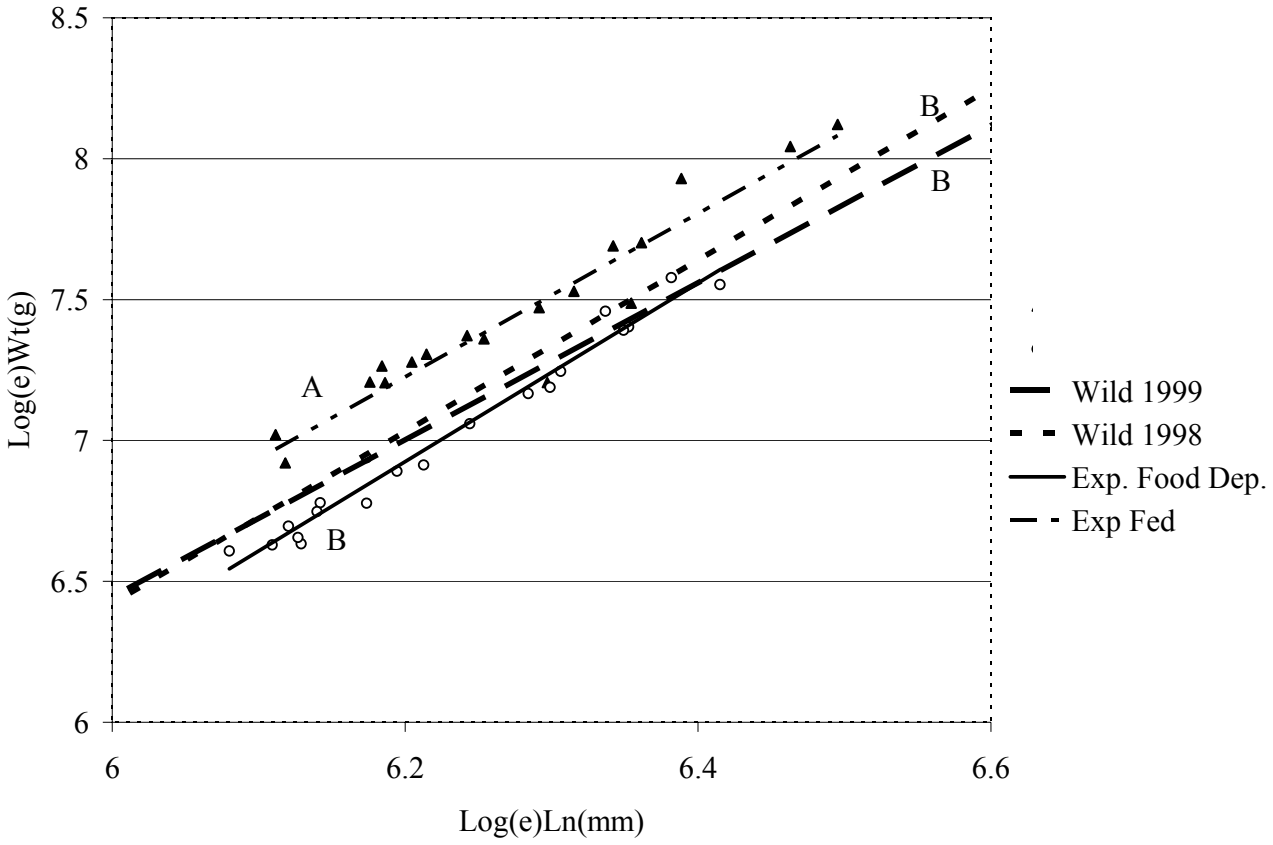

Figure 4-3. Log weight-length regressions for wild fish collected in 1998-1999 compared to experimentally fed and food deprived controls. Overall model was significant (ANCOVA ($F = 309.6$, 7,260 df, $p < 0.0001$) with slopes identical ($F = 1.28$, 3df, $p = 0.28$), and wild-collected fish similar in weight at length to those experimentally food deprived. Same letter denotes lack of significance for least square means comparisons ($p< 0.05$).

Chapter 5 – TISSUE SPECIFIC SAMPLING FOR THE ESTIMATION OF WHOLE-BODY PROXIMATE COMPOSISITION OF STRIPED BASS (MORONE SAXATILIS)

ABSTRACT

The need for precise estimates of chemical components of fish is common among the fields of aquaculture, fish health, and bioenergetics in fisheries management. Proximate composition is a widely used tool for obtaining this level of information, but is time consuming and requires homogenization of the entire fish limiting the ability to obtain additional information from the same organism. Exploratory chemical component analysis of differing body regions of age 1 and 2 striped bass (*Morone saxatilis*) suggests that the abdominal wall (belly flap) is capable of predicting whole-body proximate composition. Belly flaps showed strong linear relationships with total composition for lipid ($R^2 = 0.91$) and moisture ($R^2 = 0.82$), but were more variable with respect to protein ($R^2 = 0.22$) and ash ($R^2 = 0.26$). Equations derived from these linear relationships allow for precise prediction of total body energy, water, lipid, dry mass, fat-free dry mass and protein ($R^2 > 0.90$). In addition, lipid and moisture composition of the belly flap has good linear relationship with that of the fillet ($R^2 = 0.82$, 0.73 respectively). We conclude that sampling an otherwise unused portion of the fish can provide precise chemical compositional information without sacrificing product and can allow for additional information to be obtained from the same organism.

INTRODUCTION

Proximate analysis is a widely accepted standard for measurement of chemical components in a variety of food products and animals (AOAC 2005). In fish, the principal components normally include moisture, lipid, protein and ash and occasionally carbohydrate (Busacker et al. 1990). The relative proportions of these components allow for detailed assessment of the physiological or nutritional condition of fish (Love 1980), calculation of energy content (Eder and Lewis 2005), and determination of the quality of food product (Karahadian et al. 1995). Thus, the methodology for determining proximate composition shares wide importance to the fields of aquaculture, fish health, and fisheries management.

While offering precise measurements of compositional components, there are several issues with proximate analysis that have served to limit its application. These issues involve the extensive time required per sample, expense, and need to homogenize the entire organism when energetic data is needed (Walsberg 1988, Brown et al. 1993, Crossin and Hinch 2005). The basis for requiring whole-body homogenization for determination of proximate components stems largely from the variability of lipid distribution within fish. In teleosts, neutral lipids are primarily stored in the visceral mesenteries, and to a lesser extent in musculature (Love 1980). The use of these reserves during intervals of starvation may or may not be equally partitioned (Einen et al. 1998). However, if the concentration of components in a specific body region is directly related to whole-body composition, tissue-specific sampling could be used resulting in considerable savings in effort and equipment costs making this methodology more feasible for smaller laboratories. In addition, tissue specific sampling would allow for

other information to be obtained from the same fish (e.g., necropsy), which is currently not feasible where total body compositional data is required. Thus, the objective of this study was to determine if specific body regions of striped bass are predictive of total body composition.

METHODS

Pilot Study

A pilot study was conducted to examine the variability in chemical composition of various body components of striped bass (*Morone saxatilis*, Walbaum). Fish used in this effort were of mixed family Choptank River, MD strain spawned and reared at the University of Maryland Center for Environmental Science's Horn Point Laboratory via standard culture methods (Nicholson et al. 1990). Fish were transported to the NOAA Cooperative Oxford Lab (COL) and maintained in 1,893 L flow through-tanks receiving ambient Choptank River water (approx 10 ppt salinity). Eleven age-one striped bass were selected from the stock maintained at COL based on body shape to represent a range of nutritional states. All fish were removed, euthanized by severing the cervical spine, weighed (0.01g), measured (mm, TL), and wrapped tightly in freezer paper followed by vacuum packing individually in freezer bags. Fish were immediately stored at -80°C for later analysis. Prior to processing, fish were allowed to partially thaw at room temperature and sectioned into five components. Whole fillets were removed, skinned, and sectioned into three parts: belly flap (BF) by following the line marked by termination of rib bones and initiation of peritoneum of body wall, front fillet (FF) and rear fillet (RF) separated by extending a straight line from the posterior terminus of the

belly flap through the dorsal segment of the fillet (Figure 5-1). Liver (L) and viscera (V) were removed by cutting the esophagus at nearest anterior point possible, removing the entire visceral mass, and subsequently separating the entire liver. All tissues were processed as described below. Relative distribution of components in the pilot effort was compared by general linear models (PROC GLM) using the PDIFF option for determination of difference in least square mean values (alpha = 0.05) (SAS 2005).

Food Deprivation Experiment

On 31 May, 2005, one year old striped bass (24.18 ± 1.97 (SD) cm) were transported to the Delaware State University Aquaculture Research and Demonstration Facility in Dover, DE (DSU). A total of 168 striped bass were stocked into a 5,000-L recirculating aquaculture system. This system was comprised of four 1.2 m x 1.2 m round polypropylene culture tanks. Mechanical and biological filtration were provided by a propeller-washed bead filter (PBF-3, Aquaculture Systems Technologies, LLC, New Orleans, LA, USA) and water was further treated using ultraviolet sterilization (EU65P, Emperor Aquatics Inc., Pottstown, PA, USA). The bead filter was backwashed 3x per week to ensure optimal operation. Water was circulated through the system with a 0.75-hp centrifugal pump (MAG 1, Jacuzzi Brands, Inc., West Palm Beach, FL, USA).

Seawater for the system was obtained from the Indian River Inlet (Rehoboth Beach, DE, USA) and adjusted to 11 ± 0.1 ppt salinity using aerated well water. Throughout the 42-day experimental period, key water quality variables remained within acceptable ranges for striped bass (Harrell et al. 1990). Temperature (mean = 19.06 ± 0.06°C), dissolved oxygen (mean = 5.75 ± 0.12 mg/l), pH (mean = 6.47 ± 0.10) and

salinity (mean = 11 ± 0.10 ppt) were checked daily, while alkalinity (mean = 70 ± 7.1 mg/l), ammonia (mean = 1.19 ± 0.31 mg/l), nitrite (mean = 0.23 ± 0.10 mg/l) and nitrate (mean = 2.94 ± 0.60 mg/l) were checked approximately every other day.

Lighting was provided by six 60-watt incandescent bulbs and the photoperiod was maintained at 12 hours light:12 hours dark. To minimize the potential effects of changing day length, the photoperiod was held constant for the duration of the experiment. All fish were fed a 4 mm striped bass grower diet (Melick Aquafeed, Catawissa, PA 17820) 3x a week to satiation and allowed to acclimate for a period of two weeks before initiation of the experiment.

A food deprivation study was subsequently initiated at DSU for the purpose of establishing a range of nutritional states in similar sized fish. Of the four tanks, two were hand fed Melick 4 mm striped bass grower to satiation three times/week and two were given no food for a period of 42 days. On day 0, three fish per tank were removed, euthanized by severing the cervical spine, weighed (0.01g), measured (mm), and wrapped tightly in freezer paper followed by vacuum packing individually in freezer bags to establish baseline parameters. Six fish per tank (24 total) were removed on day 21 and 42 and treated in the same manner. Before all sampling periods, all treatments were not fed for five days to allow for gastric evacuation. All samples were transported on wet ice to the Oxford Laboratory and stored at -80°C for subsequent analysis.

Tissue Processing and Proximate Composition

Based on pilot study results, tissue sections were prepared by separating each fish into only whole fillet (F), belly flap (BF) and remains (R) using the same protocol as described above. Each component was weighed to the nearest 0.01 gram wet weight and homogenized in a Retsch mixer mill (Retsch Inc., Newtown, PA 18940). Proximate composition followed standard AOAC methods with single samples per tissue due to the amount of material available (AOAC 2005). Briefly, fish were dried at 90°C overnight, neutral lipids extracted over 8 h via Golfich apparatus with petroleum ether, protein determined via Kjeldahl nitrogen with 6.2 conversion factor, and ash by muffle furnace overnight at 550°C. All tissue sections were treated in this fashion with the exception of fillet and remains from the food deprivation trials which were frozen at -80°C following lipid extraction and subsequently pooled for protein and ash determination.

Data Treatment and Analysis

Moisture, neutral lipid, protein, and ash values were examined as concentrations in grams of component per gram of sample weight. Relative distribution of components in the pilot effort were compared by general linear models (PROC GLM) using the PDIFF option for determination of difference in least square mean values (alpha = 0.05) (SAS 2005). Whole fish concentrations from the food deprivation trial were determined by multiplying individual measured tissue concentrations by the total weight of that tissue (wet for moisture, dry for lipid, and fat free dry weight for protein and ash) to obtain total gram values for each component. These values summed and divided by whole fish mass values were used for whole fish concentration of proximate components. Linear

regression analysis (PROC REG) was used to examine relationships between tissue sections and whole fish concentrations in grams of component per gram tissue weight (SAS, 2005). Predicted values from these models were then used to calculate all proximate components and tested for their ability to predict whole body mass values. Gross energy was calculated using the conversion factors of Brett and Groves (1979): 8.7 kcal g^{-1} lipid, and 5.7 kcal g^{-1} protein. Slope comparisons were conducted via analysis of covariance (PROC GLM) (SAS 1990). Changes in tissue composition over time were also examined with general linear models (SAS 1990).

RESULTS

Component Distribution

The primary target of this exercise was to assay lipids, thus data collected for the pilot study was only moisture and lipid components. Percent lipid of the various tissues was more than two times higher in the viscera and belly flap than any other tissue examined (Table 5-1). Moisture concentrations followed the expected inverse relationship with lipids with viscera being significantly lower than all others ($p<0.05$). Sampling the front or rear fillet did not significantly influence lipid or moisture values ($p>0.05$). Viscera and liver were not included in subsequent studies.

Food Deprivation Study

The food deprivation study proved sufficient for establishing a range of lipid concentrations in similar sized fish. Minor, non-significant changes in composition and total energy were observed in fed fish after 21 days (Table 5-2). By day 42, a significant

increase in lipid was noted (+ 3.15 g/g ww, $p< 0.05$), accompanied by a minor increase in protein (+ 1.91 g/g ww), and a reduction in moisture (- 1.32 g/g ww) and ash (- 0.26 g/ g ww). Net energy gain (+ 1106 J/g ww) was significant in fed fish after 42 days. Conversely, food-deprived fish gained moisture (+ 86.4 mg/day) and used lipid reserves (-115 mg/day) in a relatively equal, and more linear fashion across sampling intervals (Table 5-2). Protein catabolism was not apparent after 21 days of food deprivation, but became significant after 42 days resulting in a net loss of 3.18 g/g ww ($p<0.05$). Ash values did not change significantly, increasing by 0.31 g/g ww over the course of the treatment. Total energy declined in food deprived fish by 1,562 J/g ww ($p<0.05$).

Relationships of moisture and lipid concentrations measured in fillets and belly flap were strongly related to whole fish concentrations, however variance from predicted lipid values was less in the belly flap ($R^2 = 0.91$) (Table 5-3). Slope comparisons for lipids in these components show equal distance from 1:1 relationships but are significantly different from each other (ANCOVA, $p = 0.0007$ interaction term). Concentrations of both lipid and moisture in the belly flap also showed a strong ability to predict those of the fillet (Table 5-3). For these reasons, and the fact that the belly flap is a normally discarded portion, ash and protein were only determined for belly flap and pooled remains. The belly flap proved to have a weaker ability to predict whole body protein than moisture and lipid components, with a high degree of variance ($R^2 = 0.22$) (Table 5-3). A significant relationship between belly flap ash and total fish ash was observed, but was poor ($R^2 = 0.26$) (Table 5-3).

Predictive Capability

The predictive capability of using only belly flap to obtain whole fish proximate compositional data was evaluated by calculating all components using measured concentrations in the belly flap and the linear equations derived above and regression against measured whole body components in mass. Near 1:1 relationships were obtained for all components, with the exception of ash which showed a high degree of variation (Figure 5-2). In addition, predictions of whole body energy by using data derived from the belly flap proved to be very strong ($R^2 = 0.91$, Figure 5-3).

DISCUSSION

Compositional components in fish vary both within and among species and individual fish which has historically made whole-body homogenization necessary for proximate composition. However, we have shown that there are strong relationships between fillet and belly flap components and whole fish that provide a means for sub-sampling, greatly reducing time and effort while allowing for other data to be collected from the same individual organism. These relationships are particularly strong for lipid and moisture concentrations derived from sampling only the belly flap, a normally discarded portion of the fish.

Striped bass store lipids preferentially in the mesenteries surrounding the viscera. During periods of starvation, liver glycogen reserves are used first, followed by neutral lipids, and final breakdown of protein for energy. As lipids are utilized, they are replaced with water (Love 1980). During periods of high energy intake, lipid reserves can be visualized in the body cavity, and these observations may be scaled and used as a general indicator of nutritional status (Goede and Barton 1990). While the viscera would seem a

logical place to sample for lipids, logistically it requires extreme attention during removal and additional steps to recover lipids after moisture has been removed. The liver is a prominent storage location for glycogen, and maintains lipids at a relatively high proportion. While this may be a useful organ to sample for nutritional status, we have found liver lipid levels to vary greatly, limiting their prospects for estimating total body compositional data. Some of this may be due to the small size of the organs used in this study, which left little material after moisture was removed for lipid extraction. However, the liver is a very transient site for lipid and sugar storage, which may provide increased sensitivity for relative nutritional assessment.

Variance in proximate components along the length of a fillet has been reported in other species, being depleted in a cranial-caudal direction in salmonids (Einen et al. 1998). Anterior fillets in this study were slightly higher in moisture and lower in lipid than their adjacent posterior sections (Table 5-1), however this difference was not significant ($p<0.05$). Further evaluation of these regions is warranted, as preferential lipid utilization in one section may provide increased sensitivity in nutritional assessment.

Belly flaps and fillets both showed strong relationships with total body concentrations of lipid and moisture ($R^2>0.80$) (Table 5-3). We chose the belly flap for further evaluation because of its strong linear relationship with total body lipids ($R^2 = 0.91$), ease of collection, relative size, and lack of use for other applications. If these relationships hold for larger organisms than evaluated in this study, the need for expensive and specialized equipment for homogenization could be alleviated.

Protein and ash concentrations in the belly flap did not show strong relationships with whole fish measured concentrations in grams per gram fat free dry weight. The poor

fit with protein is most likely explained by the limited range of values obtained in these fish, and the general lack of variability normally encountered with protein (Shearer et al. 1994). Protein catabolism generally demarks a fairly severe period of food depravation, as it is energetically more costly to use than lipid (Love 1980). It is likely that the fish used in this experiment were not deprived of food long enough to necessitate use of body protein as no significant difference was noted between food deprived and fed groups ($p>0.05$). This finding is supported by the work of Einen et al. (1998) who reported no significant loss of protein in Atlantic salmon (*Salmo salar*) after 86 days of food deprivation. Even with poor fit, the equation derived does predict total body protein (g) extremely well (Figure 3). Since the measured values for protein showed strong stability regardless of treatment in our experiment, it is likely that this is due primarily to the influence of weight of the organism on the regression. In other words, larger fish will have higher total body protein due solely to mass when values are similar.

Ash concentrations provide a measurement of mineral weight per gram fat free dry mass free of all other components. As such, it is not surprising that skinless belly flap with few to little bone present does a poor job of predicting whole body ash. Ash concentrations were significantly (47%) higher in whole fish homogenates than belly flap ($p<0.0001$).

The limitations of proximate composition in terms of time, costs, and logistics have led many investigators to examine correlations with wet and dry weights and other meristic-based condition indices (Brown and Murphy 1991; Herbinger and Friars 1991; Hartman and Brandt 1995), and more recently electrical conductivity (Walsberg 1988; Lantry et al. 1999; Cox and Hartman 2005). While varying degrees of success have been

reported, these methods offer another potential means of obtaining energetic and compositional data that does not require euthanasia of the organism. However, all these approaches, including the one used in this study, require validation and model development for each new species across multiple age classes.

We conclude that tissue sub-sampling offers a cost effective and less labor intensive means for calculating proximate components while affording the opportunity to obtain additional information from the same fish. In our hands, this approach allows for the examination of changes in compositional components and energetics associated with disease. The application of this method is currently limited to age 1 and 2 striped bass, and should not be applied to other age classes or species until further validated. If proven for mature fish, this would eliminate the need for expensive commercial equipment for the homogenization of large striped bass, and potentially allow for sampling of commercially obtained fish without sacrificing product.

ACKNOWLEDGEMENTS

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Table5-1. Moisture and neutral lipid by body section of age 1 striped bass (99.01 g, ± 32.97 sd). Same letter denotes lack of significance within component ($p > 0.05$).

		Percent Moisture		Percent Lipid (dry wt)	
Sect	*n*	LS Mean	SE	LS Mean	SE
V	11	69.37	0.82 ^{A}	37.65	1.72 ^{A}
BF	11	72.41	0.82 ^{B}	20.29	1.72 ^{B}
L	11	72.31	0.82 ^{B}	9.71	1.72 ^{C}
RF	11	75.75	0.82 ^{C}	6.28	1.72 ^{C}
FF	11	76.75	0.82 ^{C}	5.42	1.72 ^{C}

Table 5-2. Least Square Means for whole body proximate components by time period and treatment. All data presented as percentage wet weight with the exception of energy.

Component	Time 0	Day 21		Day 42	
		Fed	Starved	Fed	Starved
Moisture	69.58	69.45	71.11 **	68.26	73.21 **
Lipid	9.56	9.96	7.56 **	12.71 **	4.73 **
Protein	21.87	22.09	20.61	23.78	18.69 **
Ash	3.04	2.87	3.16	2.78	3.35
Energy (J/gWW)	7677	7837	6805	8837 **	6115 **

** significant difference from time 0 ($p > 0.05$)

Table 5-3. Relationships between body part and whole body lipid and moisture (g/g). BF = Belly Flap, FL = Fillet, WF = Whole Fish.

Independent	Dependent	*n*	Slope (SE)	Intercept (SE)	*F* statistic	*p* value	R^2
BF Lipid	WF Lipid	52	0.65 (0.028)	0.05 (0.008)	524.94	0.0001	0.91
FL Lipid	WF Lipid	51	1.47 (0.098)	0.07 (0.011)	207.37	0.0001	0.81
BFMoist	WF Moist	52	0.62 (0.041)	0.26 (0.030)	223.21	0.0001	0.82
FL Moist	WF Moist	51	1.45 (0.089)	-0.41 (0.684)	265.96	0.0001	0.84
BF Protein	WF Protein	48	0.41 (0.111)	0.47 (0.09)	13.50	0.0006	0.22
BF Ash	WF Ash	47	0.59 (0.148)	0.08 (0.015)	16.04	0.0002	0.26
BF Lipid	FL Lipid	51	0.39 (0.026)	0.002 (0.007)	228.78	0.0001	0.82
BF Moist	FL Moist	50	0.37 (0.032)	0.50 (0.023)	131.64	0.0001	0.73

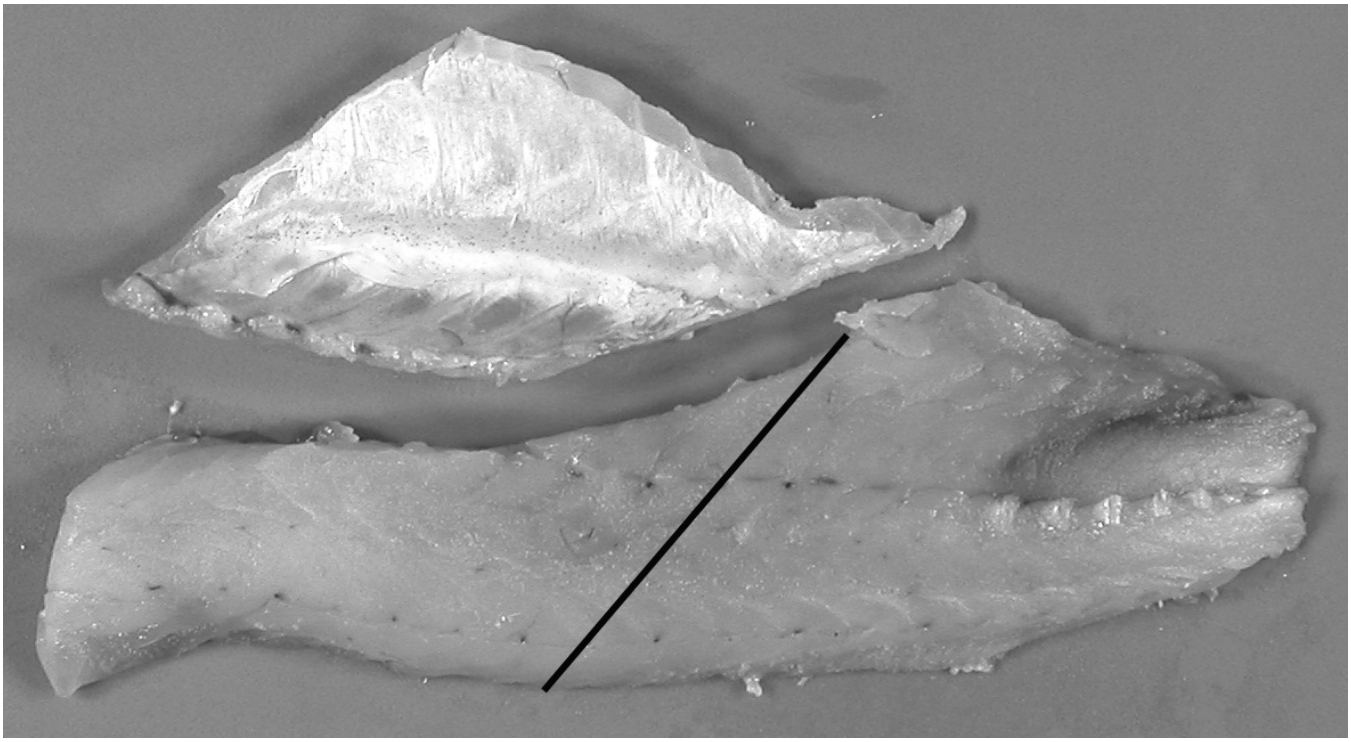

Figure 5-1. Striped bass fillet skinned and prepared for homogenization. Line represents separation of front (FF) and rear (RF) filet sections for pilot effort. Whole fillets were used for subsequent evaluation.

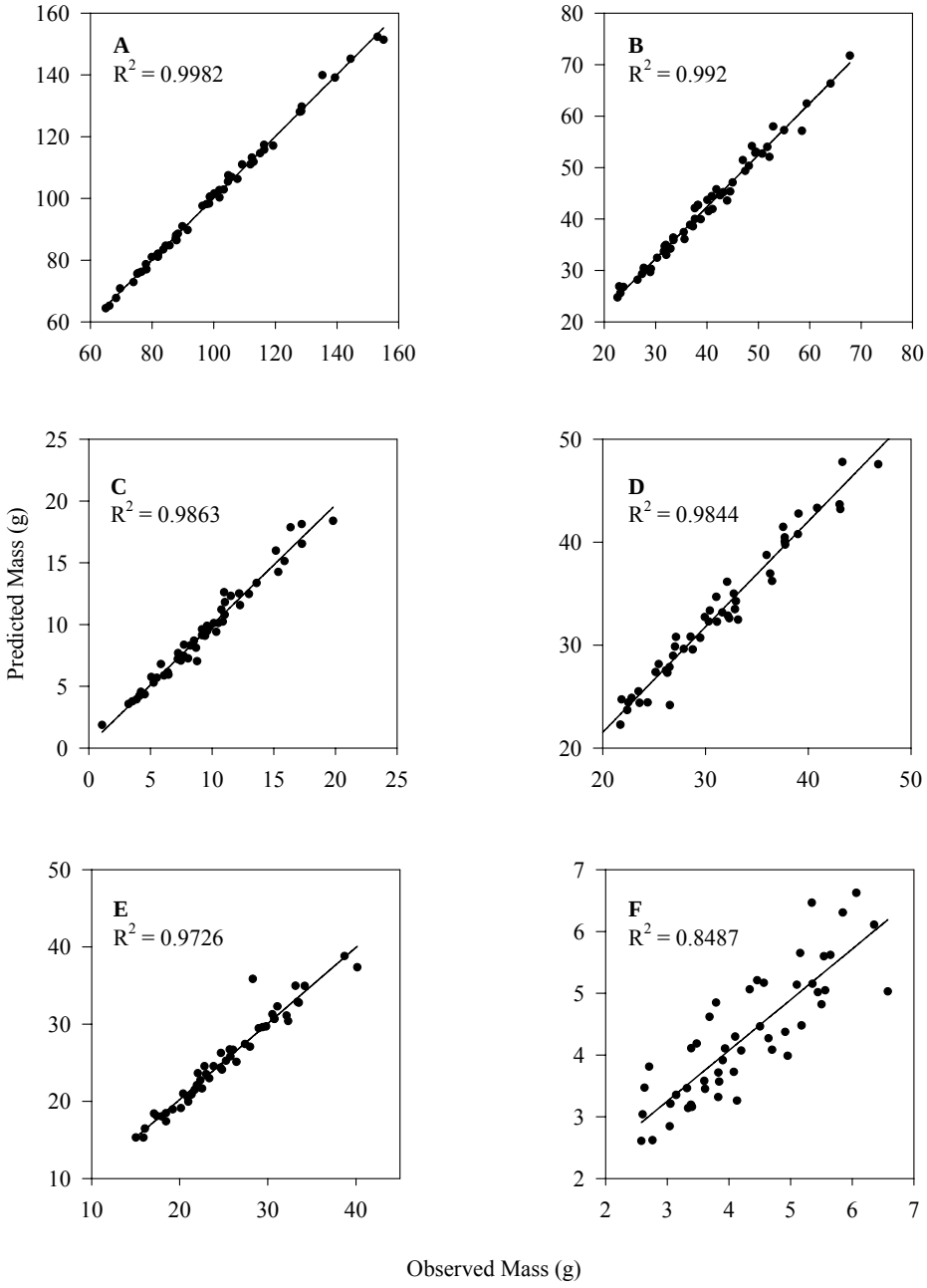

Figure 5-2. Regression analysis of predicted and observed proximate components based on using equations derived from the belly flap. A = total body water, B = dry mass, C = total body lipid, D = fat free mass, E = total body protein, F = total body ash.

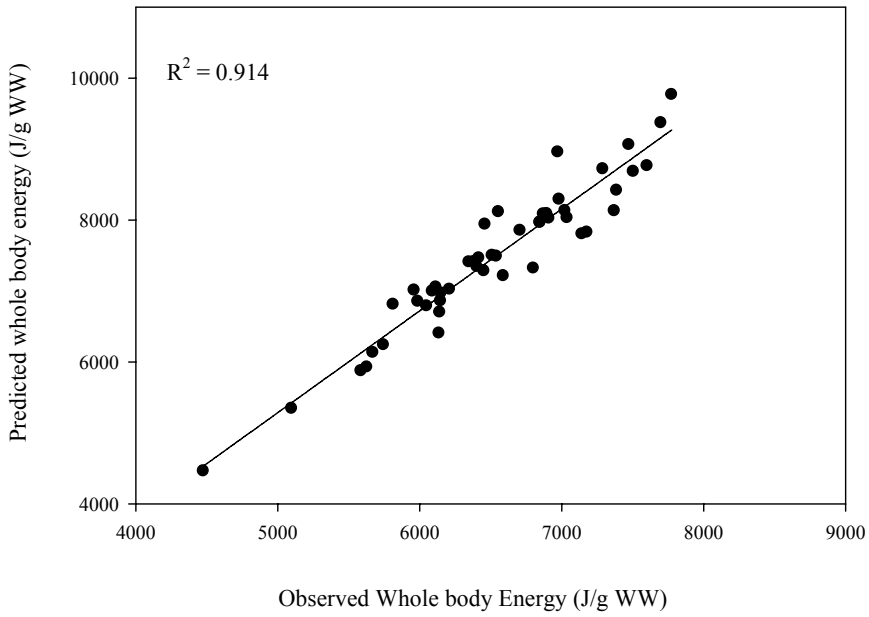

Figure 5-3. Regression analysis of predicted and observed gross energy based on equations derived from the belly flap.

Chapter 6 - THE INFLUENCE OF NUTRIONAL STATE ON THE PROGRESSION AND SEVERITY OF MYCOBACTERIOSIS IN STRIPED BASS (*MORONE SAXATILIS*)

ABSTRACT

Challenge studies with *Mycobacterium marinum* clearly demonstrate the capability of poor diet to influence the progression and severity of mycobacteriosis in striped bass (*Morone saxatilis*). Fish (n= 512 total, wt = 65 +/- 15g) were inoculated intraperitoneally with 10^4 CFU/g bw or a physiological saline solution (controls) and evaluated over an eight month period. Feeding a low ration diet (0.15% bw/d) resulted in a severe, systemic infection, characterized by high bacterial loading (>10^8 CFU), and poor granuloma formation, which commonly progressed to mortality by six weeks. In contrast, adequate ration (1% bw/d) resulted in classic granulomatous inflammation of reduced severity, and similar total body energy as found in un-inoculated controls ($p > 0.05$). After four weeks, fish fed adequate rations maintained an equilibrium state throughout the study period even though 10^6 CFU mycobacteria were consistently cultured. In a second study, reactivation of acute inflammatory state was demonstrated by placing previously infected fish on reducing diets (0.073% bw/d). In both studies, the energetic demand of this disease was only appreciable when associated with active, severe, inflammatory states. To our knowledge, this study is the first to demonstrate the interaction of diet and mycobacteriosis in fish.

INTRODUCTION

In terms of economically and socially important diseases of avian, mammalian, and piscine species, few are of more significance world-wide than those associated with the genus *Mycobacterium*. Johne's disease, caused by *M. paratuberculosis*, is estimated to cause over 1.5 billion in losses annually to the U.S. cattle industry (Stabel 1998). Chronic infections from the *M. avium* complex have been well documented in a wide range of species from horses to poultry (Thorel et al. 1997). Piscine species are equally vulnerable with tremendous losses related to mycobacteriosis in aquaculture (Colorni et al. 1998) as well as documented epizootics in wild populations (Sakanari et al. 1983, Mackenzie 1988, Rhodes et al. 2001). Of increasing concern, is the ability of many of these non-tuberculosis, or environmental isolates to cause disease in humans, especially in individuals who are immuno-compromised (Dobos et al. 1999).

Currently, an epizootic of mycobacteriosis is affecting striped bass (*Morone saxatilis*) in the Chesapeake Bay (Heckert et al. 2001, Rhodes et al. 2001, Overton et al. 2003, Rhodes et al. 2004, Kattari et al. 2005, Ottinger and Jacobs 2006). Of great interest is the number of species (10) that have been isolated from affected fish (Rhodes et al. 2004), including three new isolates named 'M. chesapeaki' (Heckert et al. 2001), *M. shottsii* (Rhodes et al. 2001), and *M. psuedoshottsi* (Rhodes et al. 2005). While *M. shottsii*, is the predominant isolate from wild-collected striped bass (Rhodes et al. 2004), only *M. marinum* has demonstrated ability to cause the same pathology (Gauthier et al. 2003). Co-occurring in the population is a high incidence of external lesions and reports of emaciated fish (Overton et al. 2003, Rhodes et al. 2004, MDNR Unpublished Data).

Changes in diet have been documented in striped bass suggesting a shift from historically preferred pelagic sources to benthic sources (Hartman and Brant 1995, Griffin and Margraf, 2003, Overton 2003, Pruell et al. 2003, Walter et al. 2003). At the same time, populations of principal prey items for age 2+ striped bass (Atlantic menhaden, bay anchovy, spot) have declined (MDNR, unpublished data, Uphoff 2003). In addition, a reduction in growth of age 3+ fish (Overton 2003, Warner et al. 2005), increased variability in weight at length (Uphoff 2003, Warner et al. 2005), and low concentrations of tissue lipids (Jacobs et al. 2004) have also been reported. The temporal association of these findings, in combination with historically high population abundance (ASMFC 2005), has led to hypotheses linking food limitation to disease state (Hartman and Margraf 2003, Uphoff 2003). However, the relationship between nutritional competency and mycobacteriosis is poorly understood in striped bass and extremely difficult to determine from field observations because prior history of the animal is unknown.

The issue of nutrition and mycobacterial disease is the classic "chicken and the egg" question. Wasting has been associated with infection in humans (Macallan 1999, Paton and Ng 2006), ruminants (Harris and Barletta 2001) and fish (MacKenzie 1988, Inglis et al. 1993) among others. Once called "consumption," the mechanisms behind the loss of body mass associated with tuberculosis are poorly understood, but may reflect decreased appetite, loss due to altered metabolism, and demands of the inflammatory and immune response (Schwenk et al. 2004). Johne's disease, caused by *M. avium subsp. paratuberculosis* commonly results in wasting in many ruminants, and reduction of milk production in dairy cattle (Harris and Barletta 2001). In this case, infection results in

severe gastroenteritis, diarrhea, and loss of body condition which may be associated with tissue damage altering the efficiency of post absorptive processes (Harris and Barletta 2001). As with mammals, reduction in condition has been reported in many cases of freshwater tropical and marine aquaria fishes in association with Mycobacteriosis (Inglis et al. 1993, Chinabut 1999, Conroy and Conroy, 1999). However, in most cases these reports are from moribund fish in the final stages of disease progression, and emaciation is a common clinical sign of many bacterial diseases in moribund fish (Inglis et al. 1993). In a large scale investigation of mycobacteriosis in Atlantic mackerel (*Scomber scrombus,* n=9470), MacKenzie (1980) noted increased prevalence and severity with age, and corresponding declines in length and condition. However, these differences were minor and rarely significant ($p < 0.05$). Similar results are reported for Chesapeake Bay, where declines in condition are noted in more severe cases of disease, especially in combination with external lesions (Cardinal 2001, Overton et al. 2003, Ottinger 2006). Depending perhaps on the study location and methodology, the reduction in condition associated solely with mycobacteriosis may be significant (Ottinger 2006), minor (Gauthier et al. 2006), or insignificant (Overton et al. 2003, Jacobs et al. 2004).

There is equal evidence in clinical medicine suggesting malnutrition is a major risk factor for tuberculosis, and can alter the progression and severity of disease (Chandra 1996, Wieland et al. 2005). Much work in clinical medicine has focused on the association of protein calorie malnutrition (PCM) and reduced immune function. Chan et al. (1996) demonstrated that mice receiving a reduced protein diet (2%) rapidly succumbed to tuberculosis accompanied by a reduced expression of interferon γ, tumor necrosis factor ά, and nitric oxide synthase in the lungs. These cytokines, as well as

Interleukin -1, interleukin -4, and transforming growth factor β are critical to the production nitrous oxides and reactive nitrogen intermediates, which is a principal mechanism by which phagocytes kill (Plouffe et al. 2005). Remarkably, fulminant tuberculosis characterized by poorly-formed granulomas and elevated bacterial density was reversible in mice by increasing protein levels to match those of the controls (20%). Dia and McMurray (1998) obtained similar results in protein malnourished guinea pigs (*Cavia porcellus*) and *in vivo* challenges of harvested spleen macrophages. Low protein intake reduced the production of interferon, tumor necrosis factor ά, and tumor necrosis factor β; essentially altering the cytokine profile to favor macrophage deactivation. Non-specific responses, such as the mobilization of inflammatory cells, phagocytosis, intracellular killing, neutrophil mobility, and production of macrophage cytokines may also be reduced under conditions of inappropriate or insufficient food sources (Dia and McMurray 1998). To our knowledge, similar challenge studies have not been conducted with fish.

METHODS

Fish and Fish Husbandry

Fish spawned and reared (Harrell et al. 1990) at the Horn Point Aquaculture Facility (University of Maryland, Center for Estuarine and Environmental Science, Horn Point Laboratory, Cambridge, MD 21613) from wild-collected, Choptank River strain, striped bass were used in this study. To verify the absence of pre-existing conditions, a representative sample of juveniles (n=60, Ossiander and Wedemeyer 1973) were transported live to the Virginia-Maryland Regional College of Veterinary Medicine

(VMRCVM) and evaluated using the methods described below. Three fish were found to be culture positive for *M. marinum* from this effort, all being held in a single tank. Fish from the remaining tanks examined were gradually transported in small batches (80-100) to the NOAA/NOS Cooperative Oxford Lab (COL) to allow for fish and system acclimation. A total of 512 fish (wt = 65 +/- 15g) were randomly stocked into 16 - 568 L circular tanks (32 fish/tank) and allowed to acclimate for one month from the last stocking. The COL experimental systems consist of eight identical experimental units, each comprised of two tanks serviced by a common bio-filter. Each system is equipped with a UV sterilizer and automatic pH controller that remained on for the duration of the study. Four systems are located in one half of the facility designated as the control side, with the remainder located in the treatment room. The two sides are physically partitioned with separate air handling systems designed to meet or exceed all requirements for working with class II agents (U.S. Dept. Health and Human Services, 1999). Other experimental conditions were set as follows: 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

M. marinum isolate FL03-23 was obtained from wild Chesapeake Bay striped bass in 2003 and maintained in pure culture at the VMRCVM. This isolate was passed through six 30g striped bass once prior to 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 directly plated on Middlebrook 7H10 agar with OADC enrichment and 0.5% glycerol (Difco, Detroit, MI) and allowed to incubate at room temperature. Cells were harvested from a single plate during exponential growth and inoculum prepared via Gauthier et al. (2003) with slight modification. Briefly, on the morning of inoculation, cells were removed and suspended in BPBS, spun at 12,000 X g for 15 minutes, and supernatant removed. Immediately following, the pellet was re-suspended in BPBS, vortexed for 2 minutes with approximately 25% v/v 50 um glass beads to disrupt clumping. Finally, the suspension was filtered through Whatman No. 1 paper. Bacterial concentration was estimated by turbidity measured at 590 nm against a BPBS blank, and adjusted to 0.05 nm or approximately 10^7 CFU. 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 Finquel (MS-222, Argent Chemical), weighed, and measured prior to inoculation. Fish were inoculated intraperitoneally with 100 µl of either diluted *M. marinum* suspension (Treatment) or sterile BPBS (Control). Final dose of *M. marinum* was calculated to be 6636 +/- 1691 (SD) cells per gram body weight.

Experimental Design and Rations

Two experiments were performed in succession to evaluate 1) the influence of ration on disease progression and severity, and 2) reactivation of disease associated with food limitation. A 4 mm pelleted diet comprised of 45% crude protein, 12% fat, and 4% fiber was used in all experiments (Melick Aquafeed, Catawissa, PA, 17820). In the first

study, fish were placed on one of two rations one month prior to intraperitoneal inoculation. A factorial design was used consisting of replicate high ration controls (HRC, 1% body weight/d), low ration controls (LRC, 0.15% body weight/d), and high and low ration treatments (HRM and LRM respectively). Rations were adjusted monthly based on mean weight of treatment group. Two fish per tank (8 per treatment) were sampled at time 0, and 3 per tank (12 per treatment) at 4 and 8 weeks post inoculation. At the conclusion of the short term study (week 8), all remaining low ration fish were removed and euthanized. HRM and HRC fish were subsequently randomly assigned within bacterial treatment across all tanks and maintained under the above listed conditions with the exception of ration. HRM and HRC fish remained on the 1% bw/day diet, while LRC and LRM fish received a reducing diet of 0.073% bw/day. Twelve fish per treatment were sampled at 16, 24, and 32 weeks post inoculation.

Gross Pathology and Hematology

At each sampling period, all fish were visually examined and abnormalities measured, photographed, and described. Weight and length were taken immediately following the withdrawal of 1ml of whole blood with a 25 g syringe from the dorsal aorta. Blood was transferred to capillary tubes for determination of hematocrit (RBC) and leucocrit (WBC) volume. Capillary tubes were immediately spun at 13,000 rpm for 5 min after sample collection, with plasma removed for determination of total protein by refractometer. Gross pathology was noted during necropsy.

Re-isolation and Enumeration of *M. marinum*

At each sampling period, sections of spleen were removed aseptically and stored at -20 °C in sterile whirlpacks. Subsequently, spleens were weighed, and stomached in 2.0 ml BPBS. Homogenates were diluted in duplicate 1:100 and 1:1000, or 1:1000 and 1:10000 and 200 ul replicate plated on Middlebrook 7H10 agar with OADC enrichment and 5% glycerol. Plates were incubated at room temperature and colonies counted manually at three and six weeks.

Histopathology

Samples of spleen, anterior and posterior kidney, mesenteries, liver, intestine, stomach, gills, heart, and gonad were preserved in 10% neutral-buffered formalin for routine paraffin embedding and microtome sectioning. All slides were stained with hemotoxylin and eosin initially for routine histopathology. Re-cuts of organs where inflammatory focus or granulomas were detected from H&E were stained with Ziehl-Neelsen for the detection of acid-fast bacteria. Severity of inflammation was categorized for each organ relative to all fish as previously described by Talaat et al. (1998). A scale of 0-5 was used with 0 being normal, 1 minimal, 2 mild, 3 moderate, 4 marked and 5 being severe, or complete loss of organ architecture.

Proximate Composition/Energetics

At the completion of necropsy, each fish was individually wrapped in freezer paper, vacuum sealed in commercial freezer bags, stored at -80°C for later analysis. Whole fish were allowed to thaw at room temperature and fillets removed. Abdominal wall tissue or belly flap (BF) was removed from the fillet by following the line marked by

termination of rib bones and initiation of peritoneum of body wall. This region has been demonstrated to provide precise estimates of total body proximate components in this size striped bass (Jacobs et al., submitted, Chapter 5). BF was weighed to the nearest 0.01 gram wet weight and homogenized in a Retsch mixer mill (Retsch Inc., Newtown, PA 18940). Proximate composition followed standard AOAC methods with single samples per tissue due to the amount of material available (AOAC, 2005). Briefly, fish were dried at 90°C overnight, neutral lipids extracted over eight hours via Golfich apparatus with petroleum ether. Crude protein was determined by the Kjeldahl nitrogen method with a 6.2 conversion factor, and ash by muffle furnace overnight at 550°C. Total energy was calculated using the conversions for lipid and protein derived by Brett and Groves (1979): 8.7 Kcal g-1 lipid and 5.7 kcal g-1 protein.

Statistical Analyses

For most variables, general linear models (ANOVA) (PROC GLM, SAS 1990) were used to examine overall effects of ration, treatment, time, and interactions. Least square means were examined where appropriate with Tukey/Kramer adjustment (LSmeans, SAS 1990). The data was analyzed separately for the short and long-term studies, with HRM and HRC at week eight used as starting data for the long-term study. Bacterial counts were log transformed prior to analysis and data and confidence intervals back-transformed for reporting. Analysis of Covariance (Proc GLM, SAS 1990) was also used to test equality of slopes and treatment effects of resulting energetic growth over time within ration. A novel approach was employed for analysis of survival because of the need to sacrifice experimental subjects before they could reach the study endpoint.

The Cox proportional hazards model (Cox 1972) was applied to calculate the relative risk associated with predictor variables. Data was censored and analyzed using PROC PHREG (SAS 1990) to model days until death as a function of each combination of ration and treatment.

RESULTS

Gross Pathology

In the initial experiment, we examined the influence of ration on the progression and severity of disease. Four weeks after inoculation, clinical signs were not apparent and both high (HRM) and low ration (LRM) fish appeared grossly similar internally. The visceral mass in both groups appeared hardened, fused, and was occasionally adhered to the body wall. Nodular red foci were readily apparent throughout the visceral fat, mesenteries and body wall. Spleens were generally enlarged and friable as occasionally were head kidneys. Between four and six weeks, extensive mortality occurred in LRM fish with characteristically greater severity of ascites, visceral fusion, and abundance of red foci on mesenteries. Moribund fish typically moved to the bottom of the tank, became dark in color, lethargic, and did not feed. By week eight, these conditions generally subsided in HRM fish, but remained intense in remaining LRM fish. Nodular lesions were apparent in spleens, head kidney, and occasionally liver in both groups. However, acites was more common at week eight in LRM survivors. Rarely, ascites presented as a gelatinous mass encompassing the entire visceral cavity. More often, varying amounts of clear to yellowish liquid were noted.

Placing the previously infected fish receiving adequate rations on a limiting diet (0.073% bw/d) had little initial influence on clinical presentation or gross pathology. No difference was noted between treatments at week 16 with both groups showing further reduction in the abundance of red foci and splenic enlargement. By week 24 some LRM fish showed fusion of organs, enlarged spleens and occasionally ascites. The majority of fish had no visible stores of mesenteric body fat. This was shortly followed by enhanced mortality that continued to week 32. Behavior and clinical signs were identical to that described during the short-term study. LRM survivors at this point ranged from similar appearance to HRM fish internally, to hardened and fused viscera with intense red foci and ascites reminiscent of mortalities and LRM fish at 8 weeks. In general, internal observations in the short-term and long-term studies were identical with low-ration groups presenting more severely than high-ration counterparts.

Histopathology

Granulomatous inflammation in HRM fish followed a classical progression from loosely organized inflammatory cells and early granulomas to distinct, well formed nodular lesions as described previously (Colorni et al. 1998, Gauthier et al. 2003). Bacteria were visible at all progressive stages by Ziehl-Neelsen staining. By week eight, granulomas were generally well developed and rarely found outside of spleen, anterior kidney, and mesenteric tissue. In contrast, an active, systemic, inflammatory state generally persisted throughout the initial study period in LRM fish characterized by a high prevalence of fused, poorly-developed granulomas with enlarged, pale necrotic cores (Figures 6-1 and 6-2). The central pale eosinophilic necrotic material was

surrounded by vacuolated macrophages and the cells comprising the fibrous capsules had a thicker, epitheliod morphology, versus the flat fibroblastic appearance of those in HRM fish. Acid fast staining generally revealed a greater concentration of bacteria in LRM than HRM splenic granulomas. Mortalities were generally characterized by systemic infection with intense peritonitis. Severity of infection was significantly greater in spleen, head kidney, liver, posterior kidney, and heart in LRM fish than in HRM fish at four and eight weeks ($p < 0.05$). Severity of the peritoneal response was equal among rations at four weeks, becoming significantly greater in LRM fish by week 8 ($p < 0.05$) (Figure 6-3).

In the long-term study, HRM fish remained relatively stable from weeks 8-32 characterized by well-formed granulomas surrounded by normal tissue. Cores of many granulomas had condensed, and visible bacteria within granulomas were greatly reduced after eight weeks. In week 24, minor renewed inflammation was noted in a few HRM fish, but was not associated with degrading granulomas. Renewed inflammation predominantly in the spleen was readily visible in LRM fish by week 24, increasing in severity by week 32 (Figure 6-4). Severity increased in all organs over time in LRM fish, equaling that noted in the short term study by week 32 (Figure 6-3).

Bacteriology

Inoculation of 10^4 CFU/g bw resulted in a differential response in bacterial replication among rations and over time. Bacterial density in spleens of LRM fish and mortalities was over 3 orders of magnitude greater than HRM fish at week four ($p < 0.0001$). By week eight, bacterial density increased in HRM fish from ~ 10^4 to ~ 10^6 CFU/g spleen. Bacterial density similarly increased in LRM fish (~10^6 to ~10^8 CFU/g

spleen) remaining significantly higher than HRM fish ($p = 0.0016$). Mortalities at four and six weeks had statistically similar bacterial densities to LRM fish ($p > 0.05$) (Figure 6-5). Overall, ration explained 64% of the model variation ($p < 0.0001$, $F = 38.56$, 2/77 df) with time accounting for 23% ($p = 0.0004$, $F = 13.75$, 1/77 df), and the interaction accounting for the remainder ($p = 0.0005$, $F = 8.51$, 2/77 df).

In the long-term study, bacterial density increased from week 8-16 in both LRM and HRM fish from 5×10^6 to approximately 9×10^6 CFU/g spleen. From 24 – 32 weeks, LRM bacterial density continued to increase resulting in a ten fold difference between ration groups by week 32 (Figure 6-6). Overall, ration explained 67% of the total variability ($p = 0.02$, $F = 5.43$, 1/79 df), while time accounted for the remainder ($p = 0.05$, $F = 2.73$, 3/79 df) and the interaction being non-significant ($p > 0.5$) A single moribund fish between weeks 8-16 had a bacterial density of 4×10^8, approximately twice that of LRM fish at that time period. Mortalities by week 32 were highly variable with respect to bacterial density with mean density similar to LRM fish (Figure 6-6). Mortalities were not included in the analysis due to lack of representation over time.

Bacterial species other than *Mycobacterium* were isolated from 30.5% of moribund fish from either the liver or kidney. Most common were *Vibrio damsella* and *Vibrio vulnificus*, with all other species being isolated from only a single fish (Table 6-1). Of the nine control mortalities during the short term study, four were found in a moribund state allowing for culture. Of these, three were culture positive for *M. marinum* at densities similar to their inoculated counterparts ($10^6 – 10^8$ cfu/g spleen wt), all belonging to the low ration group. In the long-term study, the single control mortality was also culture positive containing 2×10^8 cfu/g spleen wt.

Hematology/Plasma Chemistry

In the short-term study, the proportion of circulating white blood cells was significantly reduced by low ration ($p = 0.005$, $F=8.09$, 1/125 df) and mycobacterial treatment ($p = 0.001$, $F = 11.18$, 1/125 df). Leukopenia was apparent in both HRM and LRM fish four weeks after IP injection, but returned to normal in HRM fish by week eight while the condition persisted in LRM fish (Figure 6-7). Red cell volume was reduced mainly by ration, which explained 65% of the total model variance ($p <0.0001$, $F = 218.34$, 1/125 df) (Figure 6-7). Protein levels generally increased in high ration fish and declined in low ration fish over time, with a greater reduction noted from weeks 4-8 than 0-4 in low ration fish (Figure 6-8). Ration alone accounted for 83% of the model variance ($p < 0.0001$, $F = 347.49$, 1/105 df).

In the long term study, leukocrit was highly variable both within and among treatments with no consistent trends (Figure 7). Treatment, ration, and time explained only 7% of the total variation ($p = 0.0293$, $F = 2.56$, 5/167 df), with only time explaining a significant proportion of the model variance ($p = 0.064$, $F = 4.24$, 3/167 df). Red blood cell volume declined in all treatments over time, but more substantially in low ration fish (Figure 6-8). Overall, a model containing ration, treatment, time, ration x treatment, and ration x time explained 76% of the total variation ($p < 0.0001$, $F = 64.39$, 8/167 df) with 62% of the model variance accounted for by ration alone ($p < 0.0001$, $F = 39.30$, 1/167 df). Treatment effects did not explain a significant proportion of the model variance ($p = 0.66$, $F = 0.02$, 1/167 df).

Plasma protein levels increased in high ration fish and declined in low ration fish over time in the long-term study (Figure 6-9). A full model explained 83% of the total

variation ($p < 0.0001$, $F = 64.25$, 11/148 df), with ration alone explaining the vast majority of model variance (75%, $p < 0.0001$, $F = 530.47$, 1/148 df) followed by time (21%, $p < 0.0001$, $F = 50.11$, 3/148 df). Individual differences in treatment means at any given time did not account for significant proportions of the model variance for any of the blood parameters investigated ($p > 0.05$).

Survival

Inoculation of *M. marinum* severely reduced survival in LRM fish. Only 25% of LRM fish survived by week eight compared to 97% of HRM fish or either control (92% LRC, 100% HRC)(Figure 6-10). Mortality in LRM fish peaked between weeks four and six post inoculation. The survival model with diet and infection explained survival times better than a null model (likelihood ratio test, $\chi^2=241.7$, $p<0.0001$). The mortality risks for each treatment group are given in Table 6-2. Subjects in the HRC group were at no appreciable risk of increased mortality. Subjects in the HRM and LRC groups were at a similar slight risk level, statistically significantly greater than zero, but probably insignificant biologically ($p < 0.001$). The LRM group was at a high risk level, being 37 times more likely to die than the other groups.

Survival in the long-term study followed a similar, although protracted trend with 96% percent survival in all groups with the exception of LRM. Between weeks 13-28, a single mortality was noted in LRM fish. By week 32, 44% of the starting population had died (Figure 6-11). The model with diet and infection explained survival times better than a null model (likelihood ratio test, $\chi^2=20$ $p=0.0002$). The mortality risks for each treatment group are given in Table 6-3. Subjects in the HRC, HRM and LRC groups

were at a similar slight risk level. The LRM group was again at a high risk level, being 14 times more likely to perish than the other groups.

Energetics

Rations used in the initial study proved to have the desired effect of strong, linear growth in HRC fish and weak, positive growth in LRC fish during the first eight weeks. Growth in terms of total body energy (Kcal) was reduced slightly in comparison to controls in both LRM (- 5.04 Kcal) and HRM (-20.73 Kcal) fish four weeks following inoculation. By week eight, growth rate had increased in HRM fish becoming more comparable to HRC fish (1.83 and 2.10 Kcal/day respectively) with no significant difference in total body energy ($p > 0.05$). Conversely, growth rate declined in LRM fish by week eight (-0.61 Kcal/day) while LRC continued positive growth (0.22 Kcal/day) with total body energy at this time period being significantly greater in LRC fish ($p > 0.05$) (Figure 6-12). The overall energetic impact of active infection with *M. marinum* was estimated to be nearly identical for high and low ration fish costing 0.52 Kcal/day and 0.51 Kcal/day respectively.

For the long term study, the reduction ration (0.073% BW/day) again had the desired effect of gradually reducing total body energy in both LRM and LRC fish. While the reduction was significant over time ($p <0.0001$, $F= 41.7$, 1/61 df), slopes were identical ($p= 0.53$, $F=.40$, 1/61 df) as were treatment means ($p = 0.18$, $F = 1.90$, 1/61 df) suggesting little energetic demand of mycobacteriosis during this time. Separation was only apparent at the final sampling period with LRM fish having lower total body energy, although not significant (ANOVA, $p = 0.336$, $F = 1.0$, 1/14 df). A strong negative

correlation was found between of severity of splenic infection and both lipid ($r = -0.755$, $p < 0.0001$,N= 24) and protein concentration ($r = -0.65$, $p < 0.0001$, N = 24). Significant growth occurred over time in both HRC and HRM fish ($p < 0.001$, $F = .09$, 1/60 df) and both maintained identical slopes ($p = 0.99$, $F = 0.0$, df = 1/60) and total energy at time ($p = 0.77$, $F = 0.09$, 1/60 df) (Figure 6-13).

DISCUSSION

To our knowledge, this is the first effort that has directly demonstrated the influence of food quantity on the progression and severity of mycobacteriosis in fish. Low ration resulted in a severe, active systemic infection, characterized by high bacterial loading, which commonly progressed to mortality. In contrast, classic granulomatous inflammation leading to a persistent but controlled infection, was characteristic of properly nourished fish. In a second study, reactivation of acute inflammatory state was demonstrated by placing fish with contained infections on reducing diets (0.073% bw/d). In both studies, the energetic demand of this disease was only appreciable when associated with active, severe, inflammatory states.

The progression of mycobacteriosis associated with *M. marinum* has been previously described in goldfish (*Carassius auratus*) (Talaat et al. 1998), sea bass (*Dicentrarchus labrx*) (Colorni et al. 1998), hybrid tilapia (*Orechromis spp*.), and striped bass (Wolf and Smith 1999, Gauthier et al. 2003) among others. In experimental mycobacteriosis, dose administered is a critical consideration in the interpretation of results. Talaat et al. (1998) found median survival time of four, and 10 days after administering doses of 10^9 and 10^8 respectively to 30g goldfish, while fish survived to the

end of the 56 day study with doses of 10^7 or less. Minimum dose for producing pathology within eight weeks was determined to be 600 CFU per fish. Wolf and Smith (1999) found that a dose of 10^6 CFU/g body weight resulted in severe inflammation and 50% mortality by eight days in striped bass, while the same dose resulted in complete survival and less severe of a response in tilapia. In sea bass, 10^4 CFU/g body weight resulted in classic granulomatous inflammation, intensified at 4-6 weeks post inoculation, with low mortality and evidence of lesion regression by 26 weeks (Colorni et al. 1998). Similarly, Gauthier et al. (2003) found 10^4 cfu resulted in a persistent, chronic disease state with low associated mortality over a 45 week period in striped bass. 10^4 CFU/g body weight was administered in this study, as it has been demonstrated to be a biologically relevant dose, resulting in a measurable pathology, with low associated mortality in the absence of other stressors.

Strain variation in *M. marinum* isolates is only beginning to be appreciated (Ucko et. al 2002, van de Sar et al. 2004), but is an important consideration in experimental studies. van der Sar et al. (2004) demonstrated a marked difference in survival and disease progression in zebrafish (*Danio rerio*) challenged with several fish and clinical isolates. Most clinical isolates caused an acute disease characterized by uncontrolled proliferation of the pathogen and complete mortality within 16 days, while the fish isolates generally resulted in classic granulomatous inflammation characteristic of piscine mycobacteriosis. In addition, the two fish isolates examined in their study caused moderate differences in granuloma number, size and morphology, with one isolate also associated with external ulcerations. The strain of *M. marinum* used in this study was isolated from a wild Chesapeake Bay striped bass and has not been characterized beyond

species. This strain proved to be highly appropriate as it caused a demonstrable and measurable pathology in both ration groups allowing for direct examination of ration effects. Clearly, the issue of strain variability demands further attention in epidemiological approaches aimed at understanding mortality and the genesis of ulcerative lesions.

The rations used in this study were based on the work of Cox and Coutant (1981) who demonstrated basal requirements for age 1 striped bass, and the extensive experience of the authors with cultured striped bass growth dynamics (Harrell et al. 1990, Jacobs et al. 1999). The low-ration diet in the short-term study was designed to minimally exceed basal requirements, while the high ration diet designed for adequate, linear growth. The performance of the controls fed both diets clearly demonstrate that these objectives were fully realized (Figure 6-12).

The use of a reducing diet in the long-term study was necessary to bring fish into a poor nutritional state in a reasonable time frame. Fish are well adapted physiologically to undergo long periods of complete starvation (Love 1980). At 21° C, striped bass can maintain visceral, non-polar lipid reserves for several months in the complete absence of food (Jacobs et al. submitted). As a fish starves, it first uses glycogen deposits primarily from the liver. Once depleted, triglycerides are mobilized from muscle tissues and mesenteric lipid deposits. Lipids used are replaced with water in a linear fashion. Finally, protein catabolism ensues in severe cases of starvation (Love 1980). Coarsely, renewed inflammation was associated with depletion of lipid reserves. Severity of infection was strongly correlated with decreasing lipid and protein concentrations ($p < 0.0001$, $r's > -0.65$, $N=24$) but not in their adequately fed cohorts. Because of the time

between sampling (2 months), a definitive chemical profile associated with renewed inflammation could not be determined the data. More detailed approaches are certainly warranted to examine this issue.

A minor proportion of experimental fish were previously infected with *M. marinum* based on positive culture of three out of 60 fish in our initial screening. As fish possess acquired immunity, it is possible that previous exposure to mycobacterium led to a hastened immune response over that capable by truly naïve fish (Plouffe et al. 2005). Thus prior exposure to mycobacteria may have served as a form of vaccination. This principle is the basis for the tuberculosis vaccine BCG, where the less virulent *Mycobacterium bovis* is used to prime the immune system for subsequent protection against *M. tuberculosis*. However, this process is suggested to be effective only 50% of the time (Colditz et al. 1994). Efforts to develop a DNA vaccine for fish mycobacteriosis have demonstrated only marginal efficacy as well. Pasnik and Smith (2006) challenged juvenile hybrid striped bass with *M. marinum* after vaccination. While significant protection was noted after 14 days, by 28 days mortality, bacterial counts, and splenic granulomas were similar to unvaccinated controls. Thus vaccination could aid in rapid antigen recognition and containment, but may not influence long term survival of the bacteria or intracellular killing.

There is no evidence that previous exposure had any influence on our results, and in fact, allowed for limited information on the impact of diet in naturally-infected fish with a second strain of *M. marinum.* In both experiments, the majority of the few control mortalities in the low ration group that were removed in time for processing were identical to inoculated counterparts in terms of bacterial density, and severity of the

active inflammatory state. This, in conjunction with the long-term study, provide further evidence that IP inoculation is an appropriate dosing strategy. The only major difference in pathogenesis between naturally infected controls and those inoculated was the elevated prevalence of granulomatous inflammation in the mesenteric tissue of the later; undoubtedly an artifact of concentration of pathogens in the body cavity. Others have used water exposure as a route of infection for the study of mycobacteriosis, which may provide a more natural route of infection, but inherently enhances variability in the degree and severity of infection, and the concentration of pathogen in exposure waters (Li and Gatlin 2005).

The use of proportional hazard analysis to describe mortality associated with infectious disease has received considerable attention in the medical literature, but has only been used sparingly in fish health investigations (Dale et al. 1997, Park and Reno 2003, Ogut and Reno 2004, Becker et al. 2006). Bebak-Williams et al. (2002) applied survival analysis to examine the influence of stocking density and pathogen concentration on survival of rainbow trout (*Onchorhynchus mykiss*) experimentally challenged with IPN virus. The authors used the Kaplan-Meier model to examine changes in risk over time associated with IPN outbreaks. This model differs from the Cox model (Cox 1972) in that it is fully parametric and thus requires larger sample sizes than possible in our evaluation. The advantage of using these models in studies of infectious disease is that they allow for removal of organisms from the study over time, provide relative estimates of risk of mortality, and allow for the evaluation of survival distribution over time.

In the current epizootic of mycobacteriosis in wild Chesapeake Bay striped bass, reduction in condition measures have been reported in association with external lesions

and mycobacteriosis (Overton et al 2003, Ottinger et al. 2006). The disease itself has also been referred to informally as a "chronic wasting disease" implying that reduction in fitness is caused by the bacteria itself. It is impossible to discern causal relationships between disease state and nutritional health from field evaluations because they are endpoint observations, and thus the prior history of the animal is unknown. In our experiments, a measurable reduction in total body energy was only apparent during active, acute inflammatory states. This state occurred regardless of ration for four weeks post IP administration of 10^4 cfu *M. marinum* , but was resolved in HRM fish by week eight while LRM fish continued to decline energetically. In the long term study, the artifact of high dose IP inoculation was removed by using previously infected fish. Although splenic bacterial density averaged over 10^6 cfu/g, total body energy remained identical to sham inoculated controls in adequately fed fish over the course of the study interval. Fish fed sub-optimal diets also maintained similar body energy through the initial four month period regardless of bacterial treatment. Only during the final two months, with the re-emergence of active, acute inflammation preceding elevated mortality in LRM fish did body energy decline. Thus our data suggests that the energetic demand of mycobacteriosis in striped bass as caused by *M. marinum* is negligible in chronic states where adequate energy reserves are present.

Remarkably similar results to this study were previously obtained by Chan et al. (1996) in their examination of the relationship of protein calorie malnutrition and tuberculosis. Using a mouse model, those fed low protein diets (2%) rapidly succumbed to tuberculosis within two months accompanied by a reduced expression of interferon γ, tumor necrosis factor ά, and nitric oxide synthase in the lungs. Those receiving a high

protein diet (20%) survived through the end of the study. Of great interest is the authors demonstration that the fate and course of infection could be reversed by re-administering the high protein diet.

The model that is evolving from the medical literature is one of a "cat and mouse" game between host immune function and mycobacterial replication (Chandra 1996). Once engulfed by macrophages, bacteria may replicate freely within the cell. This triggers a cascade of cytokine mediated events leading to the formation of the granuloma in attempt to limit the spread of disease and focus efforts to destroy the pathogen. In immunocompetent hosts, the acute phase of disease often gives way to either a latent or chronic state where bacteria are often readily culturable and visible within granulomas (Flynn and Chan 2001). Recent work suggests that there is a dynamic equilibrium between host immune function and mature granulomas (Bouley et al. 2001), in contrast to theories of bacteria persisting in a resting state. Bouley et al.'s findings suggest that bacterial killing within the granuloma is balanced by pockets of freely replicating cells, sometimes within the same phagosome. Exactly how some mycobacteria evade the attempts of the host immune system is still unclear, but the implications are that a reservoir is maintained within the host for potentially a lifetime. It is estimated that 1/3 of the worlds population is infected with tuberculosis (Flynn and Chan 2001), and over 50% of striped bass greater than age 3 with related fish pathogens in Chesapeake Bay (Ottinger and Jacobs 2006). Whether through disruption of cytokine profiles and subsequent macrophage activation (Chan et al. 1996, Dia and McMurray 1998) or mechanisms yet to be determined, it is clear that nutritional insult can disrupt this equilibrium in favor of the pathogen.

Causative relationships in the study of disease epizootics are often difficult to discern because of complex interactions between the host, pathogen, and the environment (Sindermann 1970). Stressors such as high density or crowding, poor water quality, or elevated temperature could also play a role in the dynamics of this disease (Hawke 2000). It is plausible that a combination of environmental stressors, combined with a susceptible host, and the numerous mycobacterial pathogens whoes virulence have yet be thoroughly explored will ultimately illuminate mechanisms driving this epizootic. However, the importance of the potential role of food limitation and/or changes in dietary quality should not be understated. Effective mulit-species management of predator and prey offers one of the only potential mechanisms for adressing this disease in a relatively short time frame. With the clarity of our results concerning nutritional stress, future efforts should proceed with the examination of dietary quality in combination with other stressors (i.e., temperature; low dissolved oxygen), and the multiple species of *Mycobacterium* isolated in Chesapeake Bay.

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Table 6-1. Percentage of non-mycobacterial isolates by organ and species from sampled mortalities. Both short and long term studies are combined.

Species	**L**	**K**
Vibrio damsela	10.14%	8.70%
Vibrio vulnificus	5.80%	11.59%
Micrococcus sp.	1.45%	1.45%
Pseudomonas florescens	1.45%	0.00%
Vibrio pelagius bv II	0.00%	1.45%
Listonella anguillarum	0.00%	1.45%

Table 6-2. Hazard ratios and 95% confidence intervals for the short term portion of the study. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

Effects	χ^2	**p**	**Hazard ratio**	**95% CL**
HRC	0.0010	0.975	0.000	
HRM	44.1100	<.0001	0.020	0.006 – 0.063
LRC	61.5955	<.0001	0.062	0.031 – 0.125
LRM	134.1408	<.0001	37.169	20.150 – 68.530

Table 6-3. Hazard ratios and 95% confidence intervals for the long term portion of the study. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

Effects	χ^2	**p**	**Hazard ratio**	**95% CL**
HRC	6.3062	0.0120	0.072	0.009 – 0.561
HRM	6.1905	0.0128	0.073	0.009 – 0.574
LRC	6.3715	0.0116	0.071	0.009 – 0.553
LRM	15.9267	<.0001	13.909	3.818 – 50.672

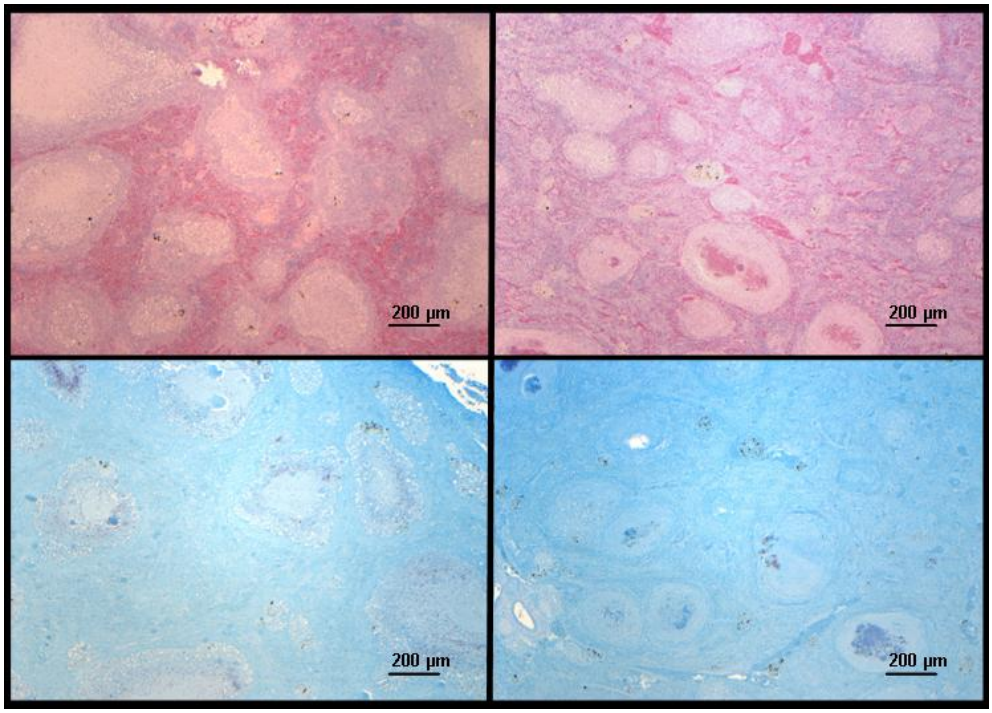

Figure 6-1. Inflammation in splenic tissue at 4 weeks post inoculation. A and B are LRM fish stained with H&E (A) and ZN (B) showing poorly formed, vacuolated, early granulomas. C and D are HRM fish at 4 weeks showing reduced severity and later stage granulomas. All magnifications are 4X.

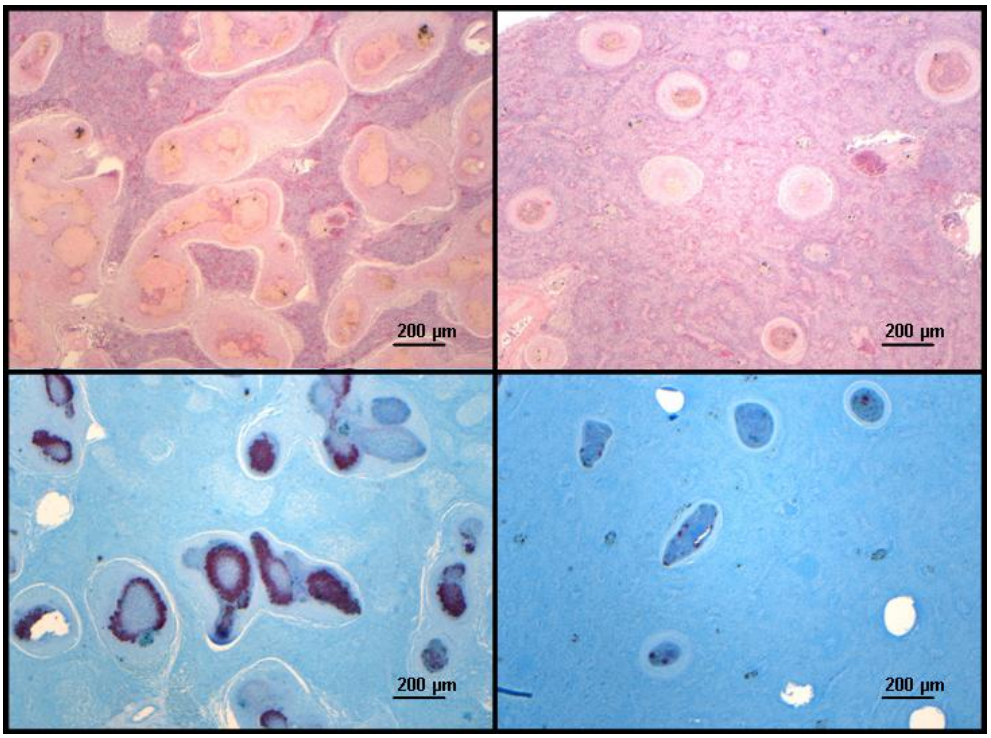

Figure 6-2. Inflammatory lesions at 8 weeks post inoculation. A and B are H&E and ZN stains respectively from LRM fish while C and D are the same stains from HRM fish. Note fused, poorly formed granulomas (A) with highly concentrated bacteria (stained red, B) in LRM fish in comparison to well formed granulomas surrounded by mostly normal tissue (C) with reduced bacterial concentrations (B). All magnifications are 4X.

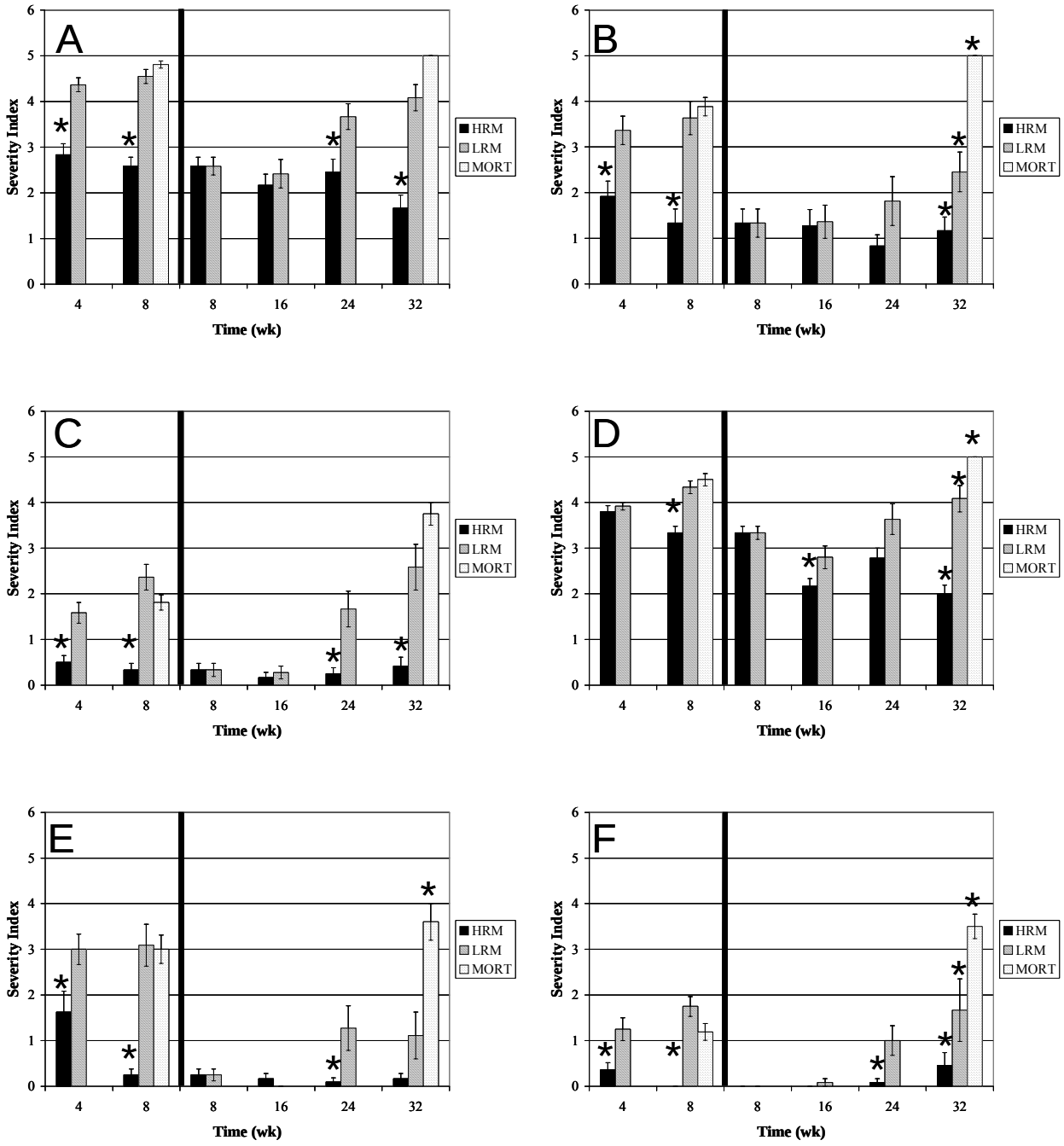

Figure 6-3. Severity of pathology associated with mycobacteriosis by organ for short and long-term studies combined. Dark vertical line divides short from long-term experiments. Severity index is scaled from 0-5 with 0 being normal tissue and 5 being complete change of tissue architecture. A = Spleen, B = Anterior Kidney, C = Liver, D =

Mesenteric tissue, E = Posterior Kidney, and F = Heart. * = significantly different from all others within time ($\alpha = 0.05$)

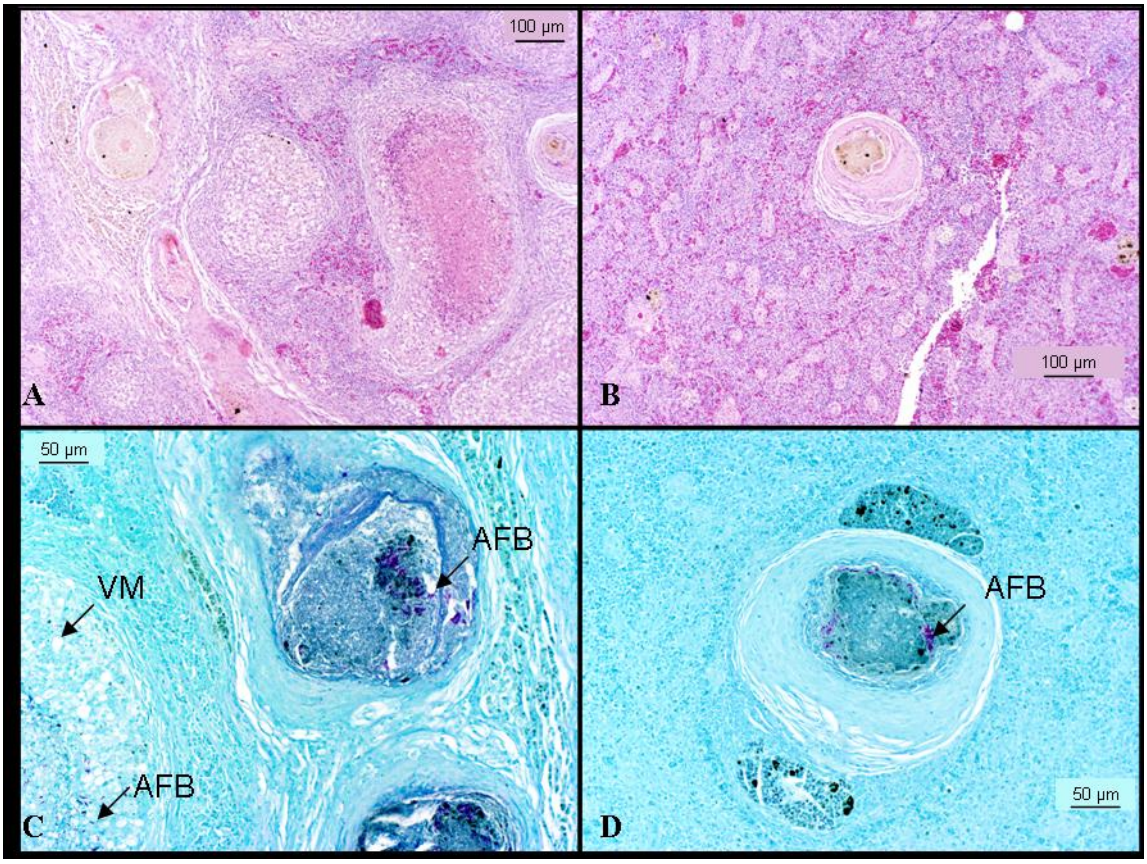

Figure 6-4. Inflammatory lesions at 32 weeks post inoculation for the long-term study. A and C are H&E and ZN stains respectively from LRM fish while B and D are the same stains from HRM fish. Note renewed inflammation in LRM fish with inflammatory foci dominated by vacuolated macrophages (VM) and individual bacteria (AFB) readily visible (panel C). In contrast, HRM fish largely maintained a steady state throughout the long-term study characterized by well formed, mature granulomas containing varying concentrations of acid fast bacteria (AFB) (Panel D).

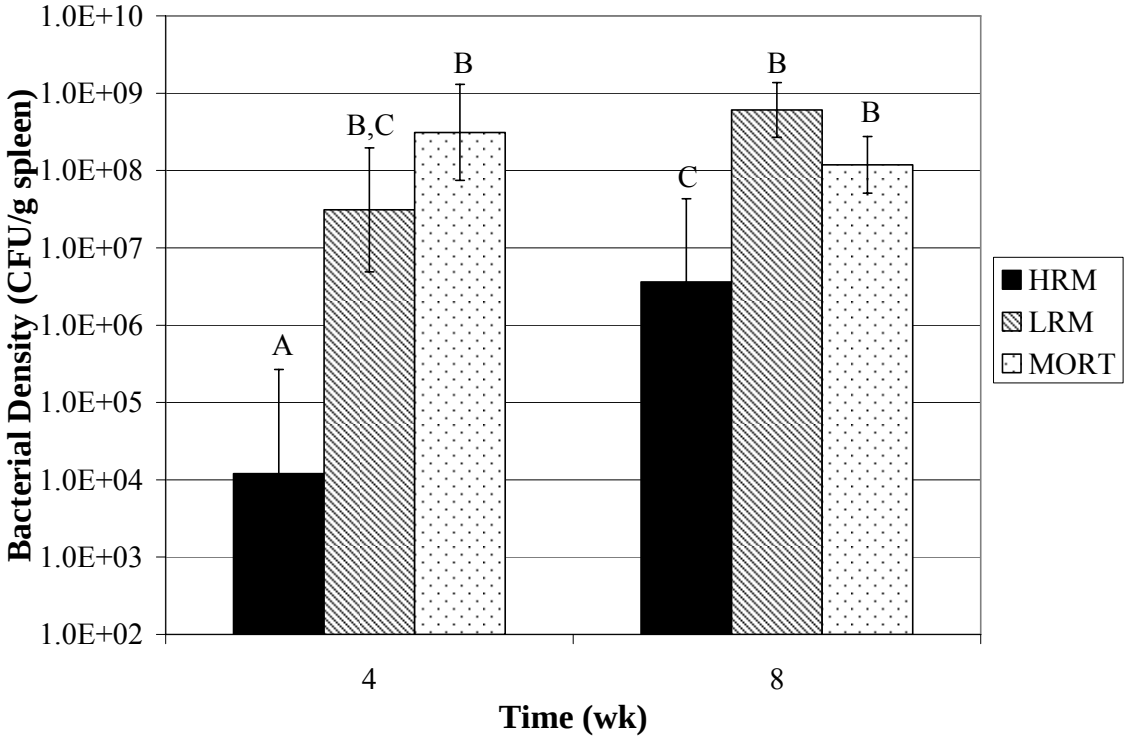

Figure 6-5. Bacterial density in spleens of experimental fish over time for the short-term study. Error bars are 95% confidence intervals. Same letter denotes lack of significance ($\alpha = 0.05$). HRM, LRM = high and low ration inoculated fish respectively. MORT = low ration mortalities.

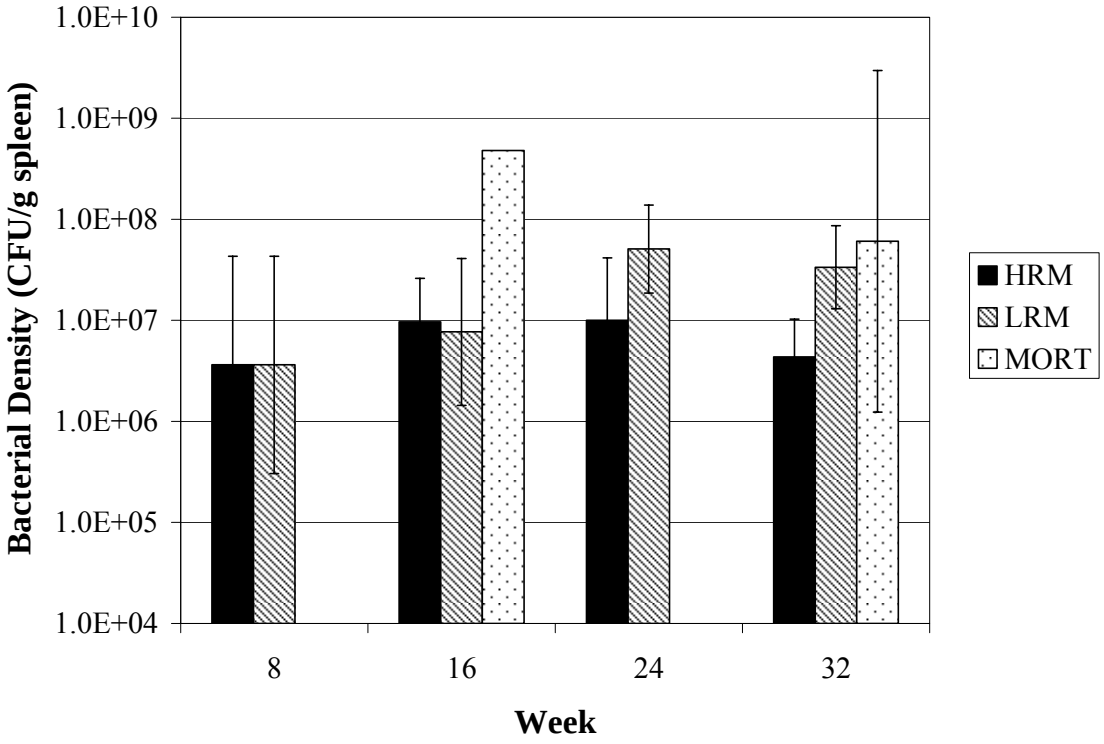

Figure 6-6. Bacterial density in spleens of experimental fish over time for the long term study. Mortality at 16 weeks represents a single fish. Error bars are 95% confidence intervals. HRM, LRM = high and low ration inoculated fish respectively. MORT = low ration mortalities.

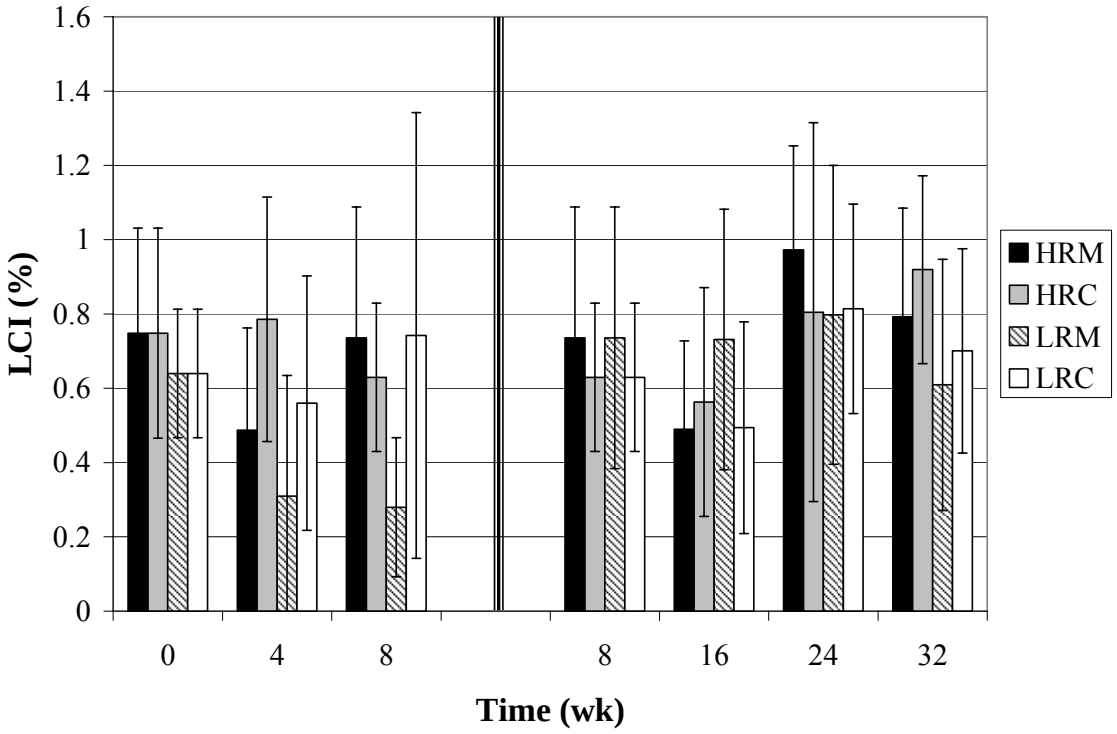

Figure 6-7. Changes in white cell volume by treatment over time (LCI= leucocrit index). Vertical bar separates short and long-term evaluations. Error bars are SD. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

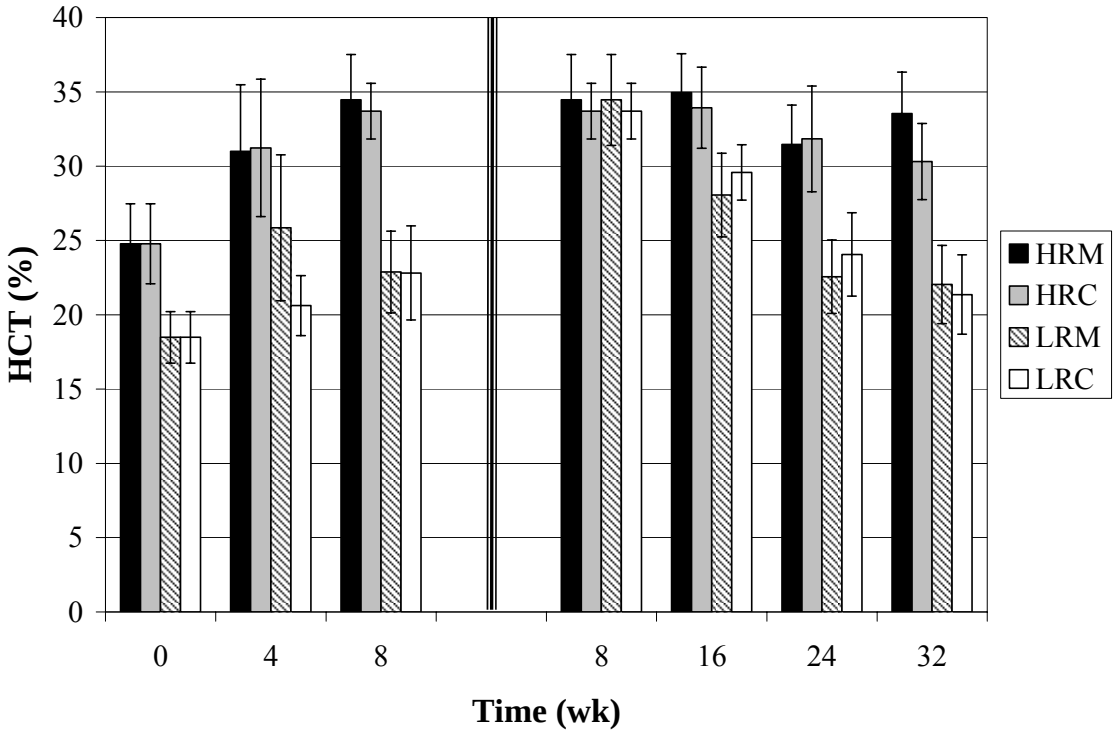

Figure 6- 8. Percentage red cell volume by treatment and time (HCT = hematocrit). Vertical bar separates short and long term studies. Error bars are SD. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

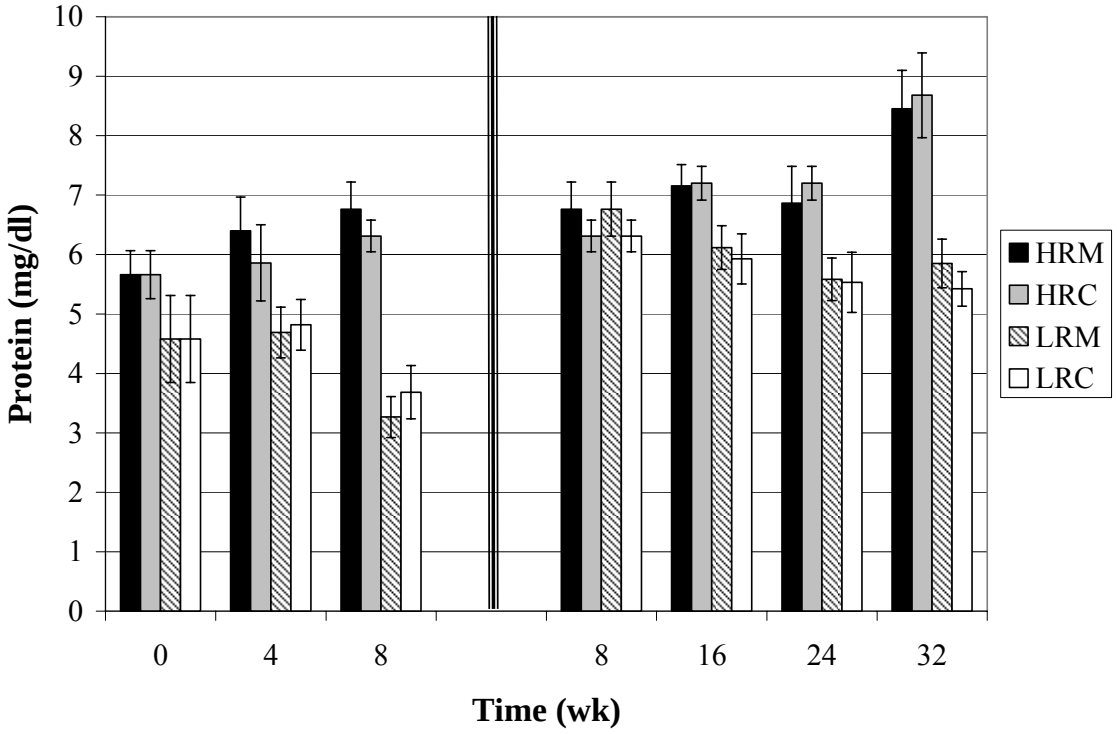

Figure 6- 9. Plasma protein by treatment and time. Vertical bar separates short and long term studies. Error bars are SD. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

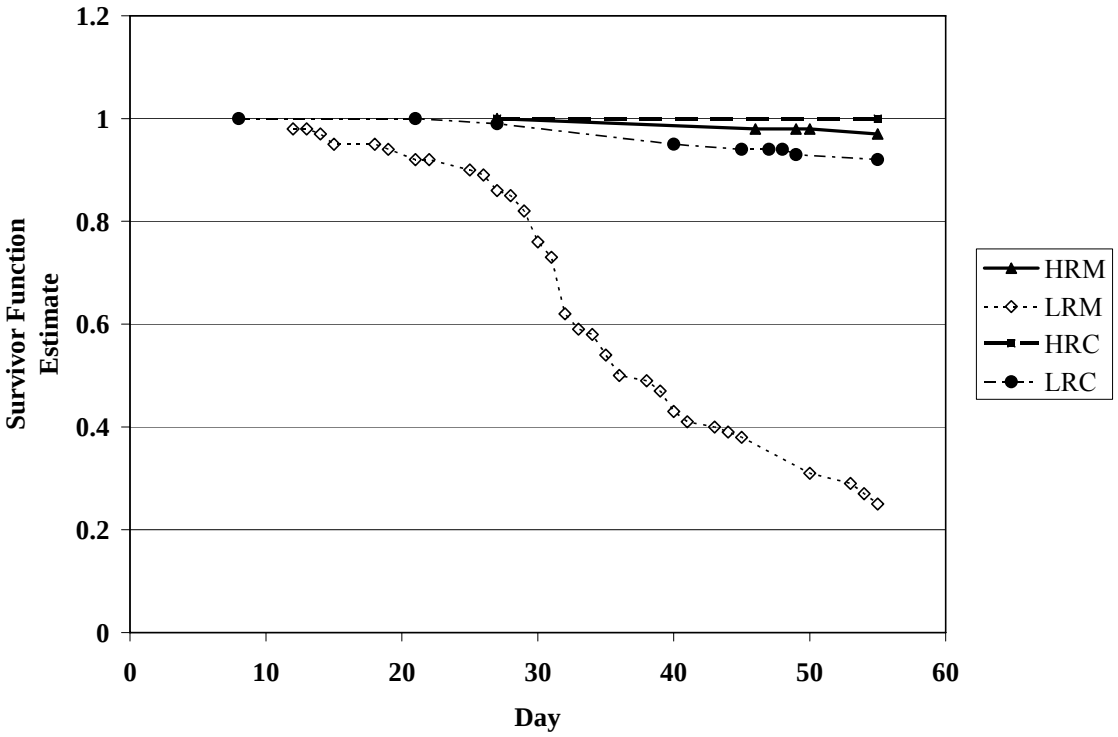

Figure 6-10. Maximum likelihood estimates of survival by bacterial treatment and ration. Short term study was terminated after week 8 due to lack of LRM survivors. First data point for each treatment marks first mortality or sampling interval if point is equal to 1. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

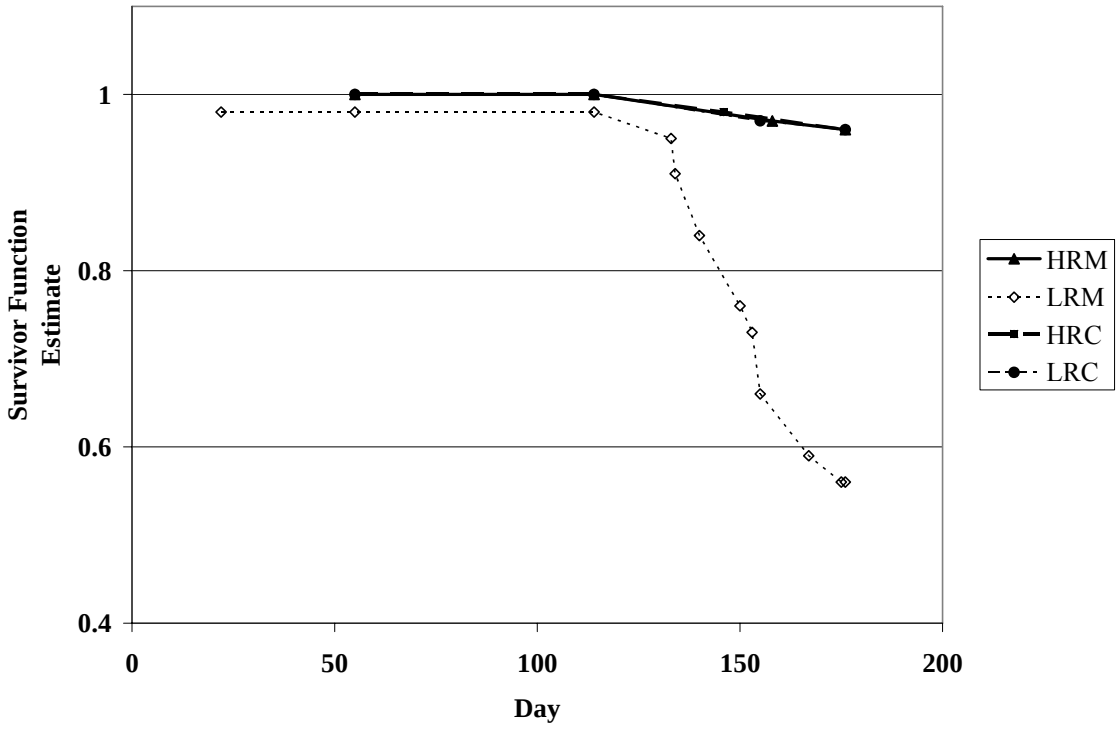

Figure 6-11. Maximum likelihood estimates of survival for long term study. First data point for each treatment marks first mortality or sampling interval if point is equal to 1. HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

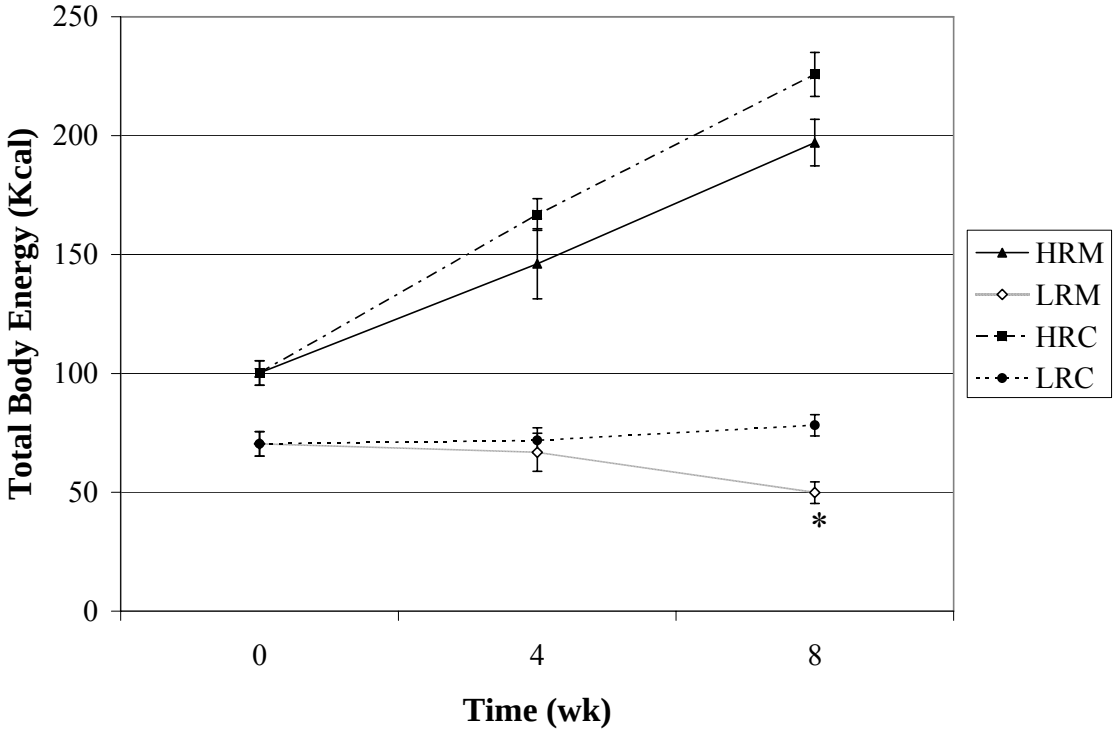

Figure 6-12. Estimated total body energy by treatment and ration over time through week 8. * denotes value is significantly different from controls at specified time interval (α = 0.05). HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

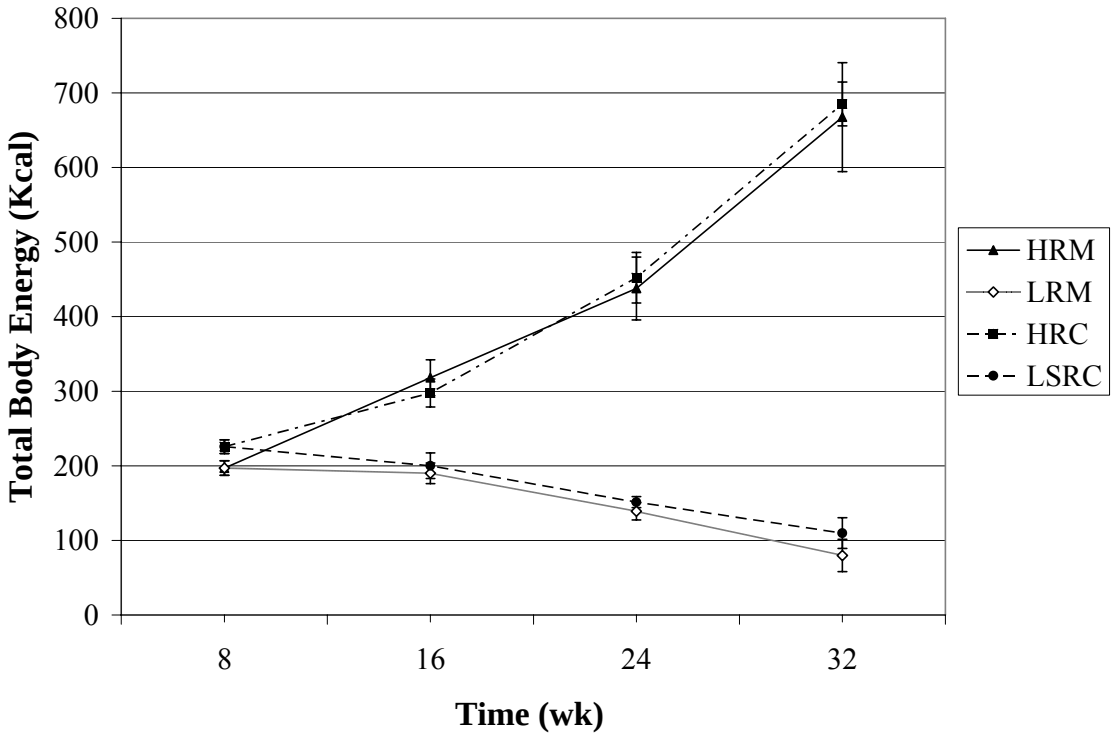

Figure 6-13. Change in total body energy over time in long term study. No significant difference was noted at any time period between treatment and controls ($p > 0.05$). HRC= high ration control, LRC = low ration control, HRM = high ration, inoculated with *M. marinum*, and LRM = low ration, inoculated with *M. marinum*.

Chapter 7 - SUMMARY

The findings of this dissertation emphasize the complexity of the relationship between nutritional health and mycobacterial disease. In humans and fish, wasting has been associated with infection (Macallan 1999, Paton and Ng 2006, MacKenzie 1988, Inglis et al. 1993). However, there is equal evidence in clinical medicine that malnutrition is a major risk factor for tuberculosis (Chandra 1996, Wieland et al. 2005), and can alter the progression and severity of disease (Chan et al. 1996, Dia and McMurray 1998). The major findings of this dissertation are: 1) Mycobacteriosis is not a new disease of striped bass and few examples exist in the historic record prior to the current epizootic; 2) chemical composition of wild-collected striped bass is reflective of poor nutritional health, a condition not explained by the presence of mycobacteriosis; 3) reduction in food quantity can severely impact the progression and severity of disease, and 4) the energetic demand of mycobacteriosis is negligible in the normal chronic course of infection.

Mycobacteriosis has been affecting Chesapeake Bay striped bass since at least 1984 (Chapter 3). However, the number of positive cases from 1975 – 1990 was less than those from 1990-1995. This is important in that it places the disease in the population prior to the current epizootic suggesting that other factors may be relevant. The use of multiple gene sequencing shows that the DNA isolated from the earliest fish is most closely related to a newly described species, *M. psuedoshottsii*.

The chemical composition of three and four year old wild-collected striped bass from 1998 – 1999 was demonstrated to be remarkably similar to experimentally food

deprived fish and significantly different than fish fed a menhaden diet (Chapter 4). Moisture and lipid concentrations were not consistent with similar size fish collected during in the same locations and seasons in 1990 and 1991 (Karahadian et al. 1995). This suggests an overall reduction in nutritional health that may have implications for susceptibility and disease progression in striped bass. Advanced stages of malnourishment represented by protein catabolism was apparent in less than 10% of sampled fish. These individuals tended to have either mycobacteriosis or ulcerative conditions, but rarely both. Overall, no significant difference was found between fish with mycobacteriosis and those without ($p > 0.05$). However, the presence of external lesions was associated with reduced lipid and elevated moisture. These findings may be indicative of dietary shifts (Overton 2003) or habitat issues such as elevated summer temperatures and minimal optimal habitat operating as an energetic stress (Coutant and Benson 1990). In either case, a causal relationship between environmental stressors and disease susceptibility, progression, and severity cannot be determined from field evaluations as prior history of the organism is unknown.

The use of proximate composition to determine nutritional state is preferable because it allows for an overall assessment of physiological condition. However, the high cost and time requirements impose limits on sample size. In addition, to obtain whole body energetic estimates, the entire organism must be sacrificed which limits the ability to obtain other information. Exploratory chemical component analysis of differing body regions of age 1 and 2 striped bass (*Morone saxatilis*) suggests that the abdominal wall (belly flap) is capable of predicting whole body proximate composition (Chapter 5). Belly flaps showed strong linear relationships with total composition for lipid ($R^2 = 0.91$)

and moisture ($R^2 = 0.82$), but were more variable with respect to protein ($R^2 = 0.22$) and ash ($R^2 = 0.26$). Equations derived from these linear relationships allow for precise prediction of total body energy, water, lipid, dry mass, fat-free dry mass and protein (R^2's > 0.90). These methods are important in the study of disease as they allow for precise, whole organism energetic data to be obtained without sacrificing the ability to fully diagnose causative organisms and pathological change.

Challenge studies with *M. marinum* clearly demonstrate the capability of poor diet to influence the progression and severity of mycobacteriosis in striped bass. Low ration diet (0.15% bw/d) resulted in a severe, chronic, systemic infection, characterized by high bacterial loading, which commonly progressed to mortality. In contrast, adequate ration (1% bw/d) resulted in classic granulomatous inflammation of reduced severity primarily associated with mesenteric and haematopoetic tissues, substantially reduced bacterial replication, and similar total body energy as found in un-inoculated controls. These fish maintained a steady state or latency throughout the study period even though high levels of mycobacteria were consistently cultured. The negative impact of low ration on the progression of mycobacteriosis was demonstrated to occur both directly as a result of acute exposure to the pathogen, and through renewed inflammation in previously infected fish. In addition, the energetic demand of this disease is only appreciable when associated with active, severe, inflammatory states. This data is consistent with findings in murine and guinea pig models with human tuberculosis (Chan et al. 1996, Dia and McMuray 1998), suggesting periods of latency or controlled equillibrium in immunocompetent organisms. However, stressors leading to immunosupression such as poor nutrition can cause reactivation of this disease.

The implications of this dissertation are that inadequate food supply for the Chesapeake Bay striped bass population can influence the progression and severity of this disease. Susceptibility was not examined, however striped bass and many other fish are carriers of mycobacterium at young ages (Stine et al. 2006, Matsche et al. 2006) when food supply may not be limiting (Overton 2003). The transition from carrier state to disease or from latency to active infection may be influenced by environmental stressors. It is plausible that this scenario of an equilibrium state between host and pathogen is routinely disrupted in Chesapeake Bay, perhaps seasonally. The potential exists for nutritional stressors to disproportionately affect age 3+ striped bass leading to reactivation and the elevated severity of disease noted in older fish. This dissertation clearly demonstrates that poor nutritional health can be a mechanism for this disruption, but other stressors such as poor water quality, elevated summer temperature, and lack of suitable habitat cannot be ruled out. It is equally possible that a combination of these issues serve as stressors in Chesapeake Bay and are not mutually exclusive.

From a management perspective, disease in wild populations represents a significant challenge. Population level impacts with a chronic disease are difficult to discern because mortality may not be grossly apparent in the form of rafts of floating fish as seen with acute cases. In addition, the slow progression may mask changes in recruitment as infected fish may be afforded multiple opportunities to spawn. It is apparent that changes in the striped bass population abundance (ASMFC 2005) have coincided with a shift in diet, particularly affecting age 3+ fish (Overton 2003, Uphoff 2003), reductions in growth of age 3+ fish (Overton 2003, Warner et al. 2005), increased variability in weight at length (Warner et al. 2003, Uphoff et al. 2003), and a high

prevalence of mycobacteriosis (Cardinal et al. 2001, Overton et al. 2003, Rhodes et al. 2004, Ottinger and Jacobs 2006). Thus the current capability of Chesapeake Bay to adequately support older age classes (> 3) is questionable. It was the intent of this dissertation to explore this relationship between nutritional health and disease state as it offers one of the few management possibilities for impacting stock health. It is clear that mycobacteriosis is an integral component of the ecology of Chesapeake Bay striped bass and that managing this species in isolation cannot account for important linkages within the ecosystem. While difficult in application, this case offers a prime opportunity for integration of disease into ecosystem based fisheries management approaches.

LITERATURE CITED

Abalain-Colloc, M.L., D. Guillerm, M. Saläun, S. Gouriou, V. Vincent, and B. Picard. 2003. *Mycobacterium szulgai* isolated from a patient, a tropical fish and aquarium water. European Journal of Clinical Microbiology and Infectious Diseases 22:768-769.

Afghani, B., and H.R. Stutman. 1996. Polymerase chain reaction for diagnosis of *M. tuberculosis*: Comparison of simple boiling and a conventional method for DNA extraction. Clinical and Molecular Medicine 57: 14-18.

Adams, R.E., J.S. Remington, J. Steinberg, and J.S. Seibert. 1970. Tropical fish aquariums. A source of *Mycobacterium marinum* infections resembling sporotrichosis. Journal of the American Medical Association 211:457-461.

Alcorn, S.W., R.J. Pascho, A.L. Murray, and K.D. Shearer. 2003. Effects of ration level on immune functions in chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture, 217:529-545.

Alexander, D.M. 1913. A review of piscine tubercle, with a description of an acid fast bacillus found in the cod. Proceedings of the Transactions of the Liverpool Biological Society 27:219-226.

Antonio, D.B., C. Swanson, J.J. Cech Jr., R.C. Mager, S. Doroshov, and R.P. Hedrick. 2000. Prevalence of *Mycobacterium* in wild and captive delta smelt. California Fish and Game 86:233-243.

AOAC (Association of Official Analytical Chemists). 1999. Official Methods of Analysis of the Association of Official Analytical Chemists. Association of Official Analytical Chemists, Washington, DC.

AOAC (Association of Official Analytical Chemists). 2005. Official methods of analysis, 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD, USA

Arakawa, C.K. and J.L. Fryer. 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infectious for salmonid fish. Helgoländer eeresuntersuchungen 37:329-342.

Aronson, J.D. 1926. Spontaneous tuberculosis in salt water fish. Journal of Infectious Diseases 39:315-320.

Ashburner, L.D. 1977. Mycobacteriosis in hatchery-confined chinook salmon (*Oncorhynchus tshawytscha* Walbaum) in Australia. Journal of Fish Biology 10:523-528.

Astrofsky, K.M., M.D. Schrenzel, R.A. Bullis, R.M. Smolowitz, and J.G. Fox. 2000 Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. Comparative Medicine 50:666-72.

Atlantic States Marine Fisheries Commission (ASMFC). 2005. 2005 stock assessment report for Atlantic striped bass: catch-at-age based VPA and tag release/recovery based survival estimation. Report by the striped bass technical committee. ASMFC, Washington DC, USA 129 pp.

Aubry, A., O. Chosidow, E. Caumes, J. Robert, and E. Cambau. 2002. Sixty-three cases of *Mycobacterium marinum* infection: clinical features, treatment, and antibiotic susceptibility of causative isolates. Archives of Internal Medicine 162:1746-1752.

Baya, A.M., B. Lupiani, I. Bandin, F.M. Hetrick, A. Figueras, A. Carnahan, E.M. May, and A.E. Toranzo. 1992. Phenotypic and pathobiological properties of *Corynebacterium aquaticum* isolated from diseased striped bass. Diseases of Aquatic Organisms 14:115-126.

Baya, A.M., B. Lupiani, F.M. Hetrick, B.S. Roberson, R. Lukacovic, E. May, and C. Poukish. 1990. Association of Streptococcus sp. with fish mortalities in the Chesapeake Bay and its tributaries. Journal of Fish Diseases 13:215-253.

Baya, A.M., J.L. Romalde, D.E. Green, R.B. Navarro, J. Evans, E.B. May, and A.E. Toranzo. 1997. Edwardsiellosis in wild striped bass from the Chesapeake Bay. Journal of Wildlife Diseases 33(3):517-525.

Bebak-Williams J., P.E. McAllister, G. Smith, and R. 2002. Effect of fish density and number of infectious fish on the survival of rainbow trout fry, *Oncorhynchus mykiss* (Walbaum), during epidemics of infectious pancreatic necrosis. Journal of Fish Disease 25:715-726

Becker J.A., D.J. Speare, and I.R. Dohoo. 2006. Interaction of water temperature and challenge model on xenoma development rates for *Loma salmonae* (Microspora) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Disease 29:139-145.

Blazer, V.S. 1991. Piscine Macrophage Function and Nutritional Influences: a review. Journal of Aquatic Animal Health 3:77-86.

Bligh, E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37(8): 911-917.

Bonin, S., F. Petrera, J. Rosai, and G. Stanta. 2005. DNA and RNA obtained from Bouin's fixed tissues. Journal of Clinical Pathology 58:313-316.

Boreman, J. and Lewis, R.R. 1987. Atlantic coastal migration of striped bass. Pages 331-339 *in* M.J. Dadswell, R.J. Klauda, C.M. Moffitt, R.L. Saunders, R.A. Fulifson and J.E. Cooper, editors. Common strategies of anadromous and catadromous fishes. Proceedings of an international symposium held in Boston, Massacusetts, USA, March 9-13, 1986. American Fisheries Society Symposium Seriers, vol 1.

Bouley, D.M., N. Ghori, K.L. Mercer, S. Falkow, and L. Ramakrishnan. 2001. Dynamic nature of host-pathogen interactions in *Mycobacterium marinum* granulomas. 69(12): 7820-7831.

Brett, J.R. and T.D.D. Groves. 1979. Physiological energetics. Pages 279-351 *in* W.S. Hoar and D.J. Randall, editors. Fish physiology, Vol. VIII. Academic Press, New York.

Brett, J.R., J.E. Shelbourn, and C.T. Shoop. 1969. Growth rate and body composition of fingerling sockeye salmon, Oncorhynchus nerka, in relation to temperature and ration size. Journal of the Fisheries Research Board of Canada 26:2363-2394.

Brocklebank, J., S. Raverty, and J. Robinson. 2003. Mycobacteriosis in Atlantic salmon farmed in British Columbia. Canadian Veterinary Journal 44(6):486-489.

Brown, M.L., D.M. Gatlin, and B.R. Murphy. 1993. Non-destructive measurement of sunshine bass, *Morone chrysops* (Rafinesque) x *Morone saxatilis* (Walbaum), body composition using electrical conductivity. Aquaculture and Fisheries Management 24: 585-592.

Brown, M.L. and B.R. Murphy. 1991. Relationship of relative weight to proximate composition of juvenile striped bass and hybrid striped bass. Transactions of the American Fisheries Society 120: 509-518.

Bruijnesteijn van Coppenraet, E.S., J.A. Lindeboom, J.M Prins, M.F. Peeters, E.C.J. Claas, and E.J. Kuijper. 2004. Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadentis in children. Journal of Clinical Microbiology 42(6):2644-2650.

Bruno, D.W., J. Griffiths, C.G. Mitchell, B.P. Wood, Z.J. Fletcher, F.A. Drobniewski, and T.S. Hastings. 1998. Pathology attributed to *Mycobacterium chelonae* infection among salmon farmed and laboratory infected Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 33:101-109.

Busacker, G.P., I.A. Adelman, and E.M. Goolish. 1990. Growth. Pages 363-382 in C.B. Shreck and P.B. Moyle, editors. Methods for fish biology. American Fisheries Society, Bethesda, Maryland.

Butler, W.R., L. Thibert, and J.O. Kilburn. 1992. Identification of *Mycobacterium avium* complex strains and some similar species by high-performance liquid chromatography. Journal of Clinical Microbiolgy 30 (10):2698-2704.

Cardinal, J.L. 2001. Mycobacteriosis in striped bass *Morone saxatilis*, from Virginia waters of the Chesapeake Bay. MS Thesis, School of Marine Science, College of William and Mary, Williamsburg, VA.

Chan, J., Y. Tian, K.E. Tanaka, M.S. Tsang, K. Yu, P. Salgame, D. Carroll, Y. Kress, R. Teitelbaum, and B.R. Bloom. 1996. Effects of protein calorie malnutrition on tuberculosis in mice. Proceedings of the Natural Academy of Sciences of the United States of America 93(25):14857-14861

Chandra, R.K. 1996. Nutrition, immunity and infection: From basic knowledge of dietary manipulation of immune responses to practical application of ameliorating suffering and improving survival. Proceedings of the National Academy of Science 93:14304-14307.

Chapman, R.W. 1990. Mitochondrial DNA analysis of striped bass populations in Chesapeake Bay. Copeia 1990:335-336.

Chemlal, K. and F. Portaels. 2003. Molecular diagnosis of nontuberculous mycobacteria. Current Opinion in Infectious Diseases 16:77-83.

Chinabut, S. 1999. Mycobacteriosis and nocardiosis. Pages 319-340 *In* P.T.K. Woo and D.W. Bruno, editors, Fish Diseases and Disorders, Vol 3: Viral, Bacterial and Fungal Infections. CAB International, New York, NY.

Colditz, G.A., T.F. Brewer, C.S. Berkey, M.E. Wilson, E. Durdick, H.V. Fineberg, and F. Mosteller. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. Journal of the American Medical Association 271(9):698-702.

Colorni, A., R. Avtalion, W. Knibb., E. Berger, B. Colorni, and B. Timan. 1998. Histopathology of sea bass *(Dicentrarchus labrax*) experimentally infected with *Mycobacterium marinum* and treated with streptomycin and garlic *(Allium sativum*) extract. Aquaculture 160:1-17.

Conroy, D.A. 1970. Piscine tuberculosis in the sea water environment. Pages 273-278 *In* S.F. Snieszko, editor, A Symposium on Diseases of Fishes and Shellfishes, Special Publication No. 5. American Fisheries Society, Washington, D.C

Conroy, D.A. 1966. Observaciones sobre casos espontaneos de la tuberculosis ictica. Microbiologia espanola 19 :93-113.

Conroy, G. and D.A. Conroy. 1999. Acid-fast bacterial infection and its control in guppies (*Lebistes reticulates*) reared on an ornamental fish farm in Venezuela. Veterinary Record 144:177-178.

Cosma, C.L., K. Klein, R. Kim, D. Beery, and L. Ramakrishnan. 2006. *Mycobacterium marinum Erp* is a virulence determinant required for cell wall integrity and intracellular survival. Infection and Immunity 74(6):3125-3133.

Coutant, C.C., and D.L. Benson. 1990. Summer habitat suitability for striped bass in Chesapeake Bay: reflections on a population decline. Transactions of the American Fisheries Society 119:757-778.

Cox, D. R. 1972. Regression Models and Life Tables. Journal of the Royal Statistical Society, Series B, 20, 187 - 220.

Cox D.K. and C.C. Coutant 1981. Growth dynamics of juvenile striped bass as functions of temperature and ration. Transactions of the American Fisheries Society 110:226–238

Cox, M.K. and K.J. Hartman. 2005. Nonlethal estimation of proximate composition in fish. Canadian Journal of Fisheries and Aquatic Science 62: 269-275.

Crossin, G.T. and S.G. Hinch 2005. A nonlethal, rapid method for assessing the somatic energy content of migrating adult pacific salmon. Transactions of the American Fisheries Society 134:184-191.

Crumlish, M. A.M. Diab, S. George, and H.W. Ferguson. 2007. Detection of the bacterium *Flavobacterium psychrophilum* from a natural infection in rainbow trout, *Onchorhynchus mykiss* (Walbaum), using formalin-fixed, wax-embedded fish tissues. Journal of Fish Diseases 30:37-41.

Dale O.B., S.K. Gutenberger, and J.S. Rohovec. 1997. Estimation of variation of virulence of *Renibacterium salmoninarum* by survival analysis of experimental infection of salmonid fish. Journal of Fish Disease 20:177-183

Decostere, A., K. Hermans, and F. Haesebrouck. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Veterinary Microbiology 99(3-4):159-166.

Devulder, G., M. Perouse de Montclos, and J.P. Flandrois. 2005. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. International Journal of Systematic and Evolutionary Microbiology 55:293-302.

Dia, G., and D.N. McMurray. 1998. Altered cytokine production and impaired antimycobacterial immunity in protein -malnourished guinea pigs. Infection and Immunity, 66(8):3562-3568.

Diamant, A., A. Banet, M. Ucko, A. Colrni, W. Knibb, and H. Kvitt. 2000. Mycobacteriosis in wild rabbitfish *Siganus rivulatus* associated with cage farming in Gulf of Eilat, Red Sea. Diseases of Aquatic Organisms 39:211-219.

Dobos, K.M., F.D. Quinn, D.A. Ashford, C.R. Horsburgh, and C.H. King. 1999. Emergence of a unique group of necrotizing mycobacterial diseases. Emerging Infectious Diseases 5(3):367-378.

dos Santos, N.M.S., A. do Vale, M.J. Sousa and M.T. Silva. 2002. Mycobacterial infection in farmed turbot *Scophthalmus maximus*. Diseases of Aquatic Organisms 52:87-91.

Eddyani, M., D. Ofori-Adfei, G. Teugels, D. De Weirdt, D. Boakye, W.M. Meyers, F. Portaels. 2004. Potential role for fish in transmission of *Mycobacterium ulcerans* disease (Buruli ulcer): an environmental study. Applied and Environmental Microbiology 70(9):5679-5681.

Eder, E.B. and M.N. Lewis. 2005. Proximate composition and energetic value of demersal and pelagic prey species from the SW Atlantic Ocean. Marine Ecology Progress Series 291: 43-52.

Einen, O., B. Waagan, and M.S. Thomassen. 1998. Starvation prior to slaughter in Atlantic salmon (*Salmo salar*): I. Effects on weight loss, body shape, slaughter- and fillet-yield, proximate and fatty acid composition. Aquaculture 166:85-104.

Erickson, K.L., E.A. Medina, and N.E. Hubbard. 2000. Micronutrients and innate immunity. The Journal of Infectious Diseases 182(Suppl 1):S5-10.

Falkinham, J.O., B.C. Parker, and H. Gruft. 1980. Epidemiology of infection by nontuberculosis Mycobacteria. American Review of Respiratory Diseases 121:931-938.

Felenstein, J. 1993. DNADIST V. 3.5c: A program to compute distance matrix from nucleotide sequences. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Flynn, J.L. and J. Chan. 2001. Tuberculosis: Latency and Reactivation. Infection and Immunity. 69(7): 4195- 4201.

Freeman, B.L. 1977. Notes on striped bass migrations. Underwater Naturalist 10(4): 13-19.

Frerichs, G.N. 1993. Mycobacteriosis: Norcardiosis. Pages 219-234 *In* V. Inglis, R.J. Roberts, and N.R. Bromage, editors, Bacterial Diseases of Fish. Halsted Press, New York.

Gauthier D.T., R.Latour, and W. Vogelbein. 2006. Epizootiology of mycobacteriosis in Chesapeake Bay striped bass (*Morone saxatilis*): Large-scale field survey. In: Ottinger C.A., and J.M. Jacobs (eds) USGS/NOAA Workshop on Mycobacteriosis in Striped Bass. USGS Scientific Investigations Report 2006-5214/ NOAA NOS NCCOS Technical Memo 41.

Gauthier, D.T., M.W. Rhodes, W.K. Vogelbein, H. Kator, and C.A. Ottinger. 2003. Experimental mycobacteriosis in striped bass *Morone saxatilis*. Diseases of Aquatic Organisms 54:105-117.

Gebo, K.A., A. Srinivasan, T.M. Perl, T. Ross, A. Groth, and W.G. Merz. 2002. Pseudo-outbreak of *Mycobacterium fortuitum* on a human immunodeficiency virus ward: transient respiratory tract colonization from a contaminated ice machine. Clinical Infectious Diseases 35:32-38.

Giavenni,R., M. Finazzi, G. Poli, and E. Grimaldi. 1980. Tuberculosis in marine tropical fishes in an aquarium. Journal of Wildlife Disease 16(2):161-168.

Goede, R.W., and B.A. Barton. 1990. Organismic indices and an autopsy-based assessment as indicators of health and condition of fish. Pages 93-108 *in* Adams, A.M. (ed), Biological Indicators of Stress in Fish. American Fisheries Society Symposium 8, Bethesda, MD.

Greer, C.E., S.L. Peterson, N.B. Kiviat, and M.M. Manos. 1991. PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. American Journal of Clinical Pathology 96(1):144-145.

Griffin J.C., and F.J. Margraf. 2005. The diet of Chesapeake Bay striped bass in the late 1950's. Fisheries Management and Ecology 10:323-328.

Haeseker, S.L., J.T. Carmichael, and J.E. Hightower. 1996. Summer distribution and condition of striped bass within Albemarle Sound, North Carolina. Transactions of the American Fisheries Society 125:690-704.

Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98.

Harms, C.A., K.E. Howard, J.C. Wolf, S.A. Smith, and S. Kennedy Stoskopf. 2003. Transforming growth factor-β response to mycobacterial infection in striped bass *Morone saxatilis* and hybrid tilapia *Oreochromis* spp. Veterinary Immunology and Immunopathology 95:155-163.

Harrell R.M., J.H. Kirby, and R.V. Minton. 1990. Culture and Propogation of Striped Bass and their Hybrids. Striped Bass Committee, Southern Division of the American Fisheries Society, Bethesda, MD.

Harris, N.B. and R.G. Barletta. 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clinical Microbiology Reviews 14(3):489-512.

Hartman K.J. and S.B. Brandt. 1995. Trophic resource partitioning, diets and growth of sympatric estuarine predators. Transactions of the American Fisheries Society 124:520-527.

Hartman, K.J. and F.J. Margraf. 2003. US Atlantic coast striped bass population: Issues with a recovered population. Fisheries Management and Ecology 10:309-312.

Hawke, J.P. 2000. Bacterial disease agents. Pages 87-88 *In* R. R. Stickney, editor, Encyclopedia of Aquaculture. John Wiley and Sons, New York.

Heckert, R.A., S. Elankumaran, A. Milani, and A. Baya. 2001. Detection of a new *Mycobacterium* species in wild striped bass in the Chesapeake Bay. Journal of Clinical Microbiology 2(39):710-715.

Hedrick, R.P., T. McDowell, and J. Groff. 1987. Mycobacteriosis in cultured striped bass from California. Journal of Wildlife Disease 23(3):391-395.

Herbiner, C.M. and G.W. Friars. 1991. Correlation between condition factor and total lipid content in Atlantic salmon, *Salmo salar* L., parr. Aquaculture and Fisheries Management 22, 527-529.

Herbst, L.H., S.F. Costa, L.M. Weiss, L.K. Johnson, J Bartell, R. Davis, M. Walsh, and M. Levi. 2001. Granulomatous skin lesions in moray eels caused by a novel *Mycobacterium* species related to *Mycobacterium triplex.* Infection and Immunity 69(7):4639-4646.

Herman, R.L., and G.L. Bullock. 1986. Pathology caused by the bacterium *Edwardsiella tarda* in striped bass. Transactions of the American Fisheries Society 115(2):232-235.

Hoyt, R.E., J.E. Bryant, S.F. Glessner, F.C. Littleton Jr., R.W. Sawyer, R.J. Newman, D.B. Nichols, A.P. Franco Jr., and N.R. Tingle Jr. 1989. *M. marinum* infections in a Chesapeake Bay community. Virginia Medical Quarterly 116:467-470.

Huang, X. 1992. A contig assemble program based on sensitive detection of fragment overlaps. Genomics 14(1):18-25.

Hughes, D.A., A.C. Pinder, Z. Piper, I.T. Johnson, and E.K. Lund. 1996. Fish oil supplementation inhibits the expression of major histocompatibility complex class II molecules and adhesion molecules on human monocytes. American Journal of Clinical Nutrition 63:267-272.

Hughes, K.P., R.B. Duncan, and S.A. Smith. 2002. Renomegaly associated with a mycobacterial infection in summer flounder *Paralichthys dentatus*. Fish Pathology 37(2):83-86.

Iivanainen, E.K., P.J. Martikainen, P.K. Vaananen, and M.L. Katila. 1993. Environmental factors affecting the occurrence of mycobacteria in brook waters. Applied and Environmental Microbiology 59(2):398-404.

Inglis, V., R. J. Roberts, and N.R. Bromage. 1993. Bacterial Diseases of Fish. Halsted Press, New York. Pages 219-233.

Jacobs J.M., M. Rhodes. Martin, D. McIntosh, and R.M. Harrell (Submitted). Tissue specific sampling for the estimation of proximate compositional components in striped bass *Morone saxatilis*. Transaction of the American Fisheries Society X:XX

Jacobs, J.M., A. Baya, and A. Lazur. 2004. Prevention and disinfection of *Mycobacterium* sp. in aquaculture. Publ. No. UM-SG-SGER-2004-01, Maryland Sea Grant Extension, Univ. of Maryland, College Park. 4 pp.

Jacobs J.M., S. Lindell, W. Van Heukelem, E.M. Hallerman, and R.M. Harrell. 1999. Strain evaluation of striped bass (*Morone saxatilis*) under controlled conditions. Aquaculture 173 (1-4):171-177.

Jacobs J.M., H.L. Rogers, W.F. Van Heukelem, B. Coakley, C. Gieseker, M. Matsche, and R.M. Harrell. 2004. Nutritional health of Chesapeake Bay striped bass *Morone saxatilis* in relation to disease. Proceedings of the 60th Annual Northeast Fish & Wildlife Conference, Ocean City, MD. p. 21.

Jeong, B., S. Moon, W. Jeong, and T. Ohshima. 2000. Lipid classes and fatty acid compositions of wild and cultured sweet smelt Plecoglossus altivelis muscles and eggs in Korea. Fisheries Science 66:716-724.

Joe, L. and E. Hall. 1995. *Mycobacterium marinum* disease in Anne Arundel County: 1995 update. Maryland Medical Journal 44(12):1043-1046.

Johnstone, J. 1913. Diseased conditions of fishes. Report of the Lancashire Sea Fish Laboratory 21:20-25.

Jonsson, N., B. Jonsson, and L.P. Hansen. 1998. The relative role of density-dependent and density-independent survival in the life cycle of Atlantic salmon *Salmo salar*. Journal of Animal Ecology 67:751-762.

Kaattari, I.M., M.W. Rhodes, S.L. Kaattari, and E.B. Shotts. 2006. The evolving story of *Mycobacterium tuberculosis* clade members detected in fish. Journal of fish diseases 29:509-520.

Kaattari, I., M. Rhodes, and H. Kator. 2002. Mycobacteriosis in striped bass of the Chesapeake Bay: expansion of studies emphasizing cultural and rapid molecular diagnostic methods to evaluate disease prevalence. Final report to the Virginia Marine Resources Commission, November 2002. VMRC, Newport News, VA.

Kaattari, I.M., M. Rhodes, H. Kator, and S.L. Kaattari. 2005. Comparative analysis of mycobacterial infections in wild striped bass *Morone saxatilis* from Chesapeake Bay. Diseases of Aquatic Organisms 67: 125-132.

Karahadian, C., K.P. Fowler, and D.H. Cox. 1995. Comparison of chemical composition of striped bass (*Morone saxatilis*) from three Chesapeake Bay tributaries with those of two aquaculture hybrid striped bass types. Food Chemistry 54(4): 409-418.

Kemp, W.M., W.R. Boynton, J.E. Adolf, D.F. Boesch, W.C. Boicourt, G. Brush, J.C. Cornwell, T.R. Fisher, P.M. Glibert, J.D. Hagy, L.W. Harding, E.D. Houde, D.G. Kimmel, W.D. Miller, R.I.E. Newell, M.R. Roman, E.M. Smith, and J.C. Stevenson. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. Marine Ecology Progress Series 303:1-29.

Kent, M.L., V. Watral, S.C. Dawe, P. Reno, J.R. Heidel, and S. M. Jones. 2001. *Ichthyophonus* and *Mycobacterium*-like bacterial infections in commercially-important rockfishes (*Sebastes* spp.) in the eastern North Pacific Ocean. Journal of Fish Disease 24:427-431.

Kent, M.L., C.M. Whipps, J.L. Matthews, D. Florio, V. Watral, J.K. Bishop-Stewart, M. Poort, and L. Bermudez. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comparative Biochemistry and Physiology, Part C 138: 383-390.

Kim, B.J., S.H. Lee, M.A. Lyu, S.J. Kim, G.H. Bai, S.J. Kim, G.T. Chae, E.C. Kim, C.Y. Cha, and Y.H. Kook. 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). Journal of Clinical Microbiology 37:1714-1720.

Kim, H., S.H. Kim, T.S. Shim, M.N. Kim, G.H. Bai, S.J. Kim, G.T. Chae, E.C. Kim, C.Y. Chae, Y.H. Kook, and B.J. Kim. 2005. Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene (*hsp65*). International Journal of Systematics and Evolutionary Microbiology 55:1649-1656.

Knibb, W., A. Colorni, M. Ankaoua, D. Lindell, A. Diamant, and H. Gordin. 1993. Detection and identification of a pathogenic marine *Mycobacterium* from the European seabass *Dicentrarchus labrax* using polymerase chain reaction and direct sequencing of 16S rDNA sequences. Molecular Marine Biology and Biotechnology 2, 225-32.

Krebs, CJ. 1985. Ecology: The Eperimental Analysis of Distribution and Abundance. Third Edition. Harper and Row, New York.

Lantry, B.F., D.J. Stewart, P.S. Rand, and E.L. Mills. 1999. Evaluation of total-body electrical conductivity to estimate whole-body water content of yellow perch, *Perca flavescens*, and alewife, *Alosa pseudoharengus*. Fisheries Bulletin 97: 71-79.

Levi, M.H., J. Bartell, L. Gandolfo, S. C. Smole, S. F. Costa, L.M. Weiss, L. K. Johnson,, G. Osterhout, and L. H. Herbst. 2003. Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic *Mycobacterium* from moray eels that is related to *Mycobacterium triplex*. Journal of Clinical Microbiology 41:2147-2152.

Levine, J.F., J.H. Hawkins, M.J. Dykstra, E.J. Noga, D.W. Moye, and R.S. Cone. Species distribution of ulcerative lesions on finfish in the Tar-Pamlico river estuary, North Carolina. Diseases of Aquatic Organisms 8:1-5.

Li, P. and D.M. Gatlin. 2005. Evaluation of the prebiotic GroBiotic® - A and brewers yeast dietary supplements for sub-adult hybrid striped bass (*Morone chrysops* x *M. saxatilis*) challenged in situ with *Mycobacterium marinum*. Aquaculture 248:197-205.

Lim, C. and P.H. Klesius. 2003. Influence of feed deprivation on hematology, macrophage chemotaxis, and resistance to *Edwardsiella ictaluri* challenge of channel catfish. Journal of Aquatic Animal Health, 15(1):13-20.

Love, R.M. 1980. The chemical biology of fishes, Volume 1. Academic Press, New York

Macallan, D.C. 1999. Malnutrition and tuberculosis. Diagnostic Microbiology of Infectious Disease 34:153-157.

Macallan, D.C, M.A. McNurlan, A.V. Kurpad, G. De Souza, P.S. Shetty, A.G. Calder, and G.E. Griffin. 1998. Whole body protein metabolism in human tuberculosis and undernutrition: evidence for anabolic block in tuberculosis. Clinical Science 94:321-331.

MacKenzie, K. 1988. Presumptive mycobacteriosis in North-east Atlantic mackerel, *Scomber scombrus* L. Journal of Fish Biology 32:263-275.

Marsollier, L., R. Robert, J. Aubry, J.P. Saint Andre, H. Kouakou, P. Legras, A.L. Manceau, C. Mahaza, and B. Carbonnelle. 2002. Aquatic insects as a vector for *Mycobacterium ulcerans*. Applied and Environmental Microbiology 68:4623-4628.

Matsche, M., L. Pieper, C.B. Stine, A.S. Kane, A.M. Baya, K. Rosemary, and J.M. Jacobs. 2006. Survey of gametes and juvenile striped bass (*Morone saxatilis*) for mycobacteriosis from the Chesapeake Bay: Sampling methods, external lesions, and histopathology. *In* Ottinger and Jacobs (eds). USGS/NOAA Workshop on Mycobacteriosis in Striped Bass, May 7-10 2006, Annapolis, MD. NOAA Technical Memorandum NOS NCCOS 41.

de Mendonça-Lima, L., M. Picardeau, C. Raynaud, J. Rauzier, Y.O. Goguet de la Salmoniere, L. Barker, F. Bigi, A. Cataldi, B. Gicquel, and J.M. Reyrat. 2001. *Erp*, an extracellular protein family specific to mycobacteria. Microbiology 147:2315-2320.

Montero, D., V.S. Blazer, J. Socorro, M.S. Izquierdo, and L. Tort. 1999. Dietary and culture influences on macrophage aggregate parameters in gilthead seabream *(Sparus aurata)* juveniles. Aquaculture, 179:523-534.

Neff, B.D. and L.M. Cargnelli 2004. Relationships between condition factors, parasite load, and paternity in bluegill sunfish, *Lepomis macrochirus*. Environmental Biology of Fishes 71:297-304.

Neumann, N.F., and M. Belosevic. 1996. Deactivation of primed respiratory burst response of goldfish macrophages by leukocyte-derived macrophage activating factor(s). Developmental and Comparative Immunology 20:427 – 439.

Nichols, G., T. Ford, J. Bartram, A. Dufour, and F. Portaels. 2004. Introduction. Pages 1-14 In S. Pedley, J. Bartman, G. Rees, A. Dufour, and J. Cotruvo, editors, Pathogenic mycobacteria in water: A guide to public health consequences, monitoring, and management. World Health Organization, IWA publishing, London, UK.

Nicholson, L.C., L.C. Woods, and J.G. Woiwode. 1990. Intensive culture techniques for striped bass and its hybrids. Pages 141-157 *in* R.M. Harrell, J.H . Kirby, and R.V. Minton, editors. Culture and propagation of striped bass and its hybrids. American Fisheries Society, Bethesda, Maryland.

Nigrelli, R.F. and H. Vogel. 1963. Spontaneous tuberculosis in fishes and other cold-blooded vertebrates with specieal reference to *Mycobacterium fortuitum* Cruz from fish and human lesions. Zoologica 48:131-143.

Noga, E.J. 2000. Fish Disease: Diagnosis and Treatment. Iowa State Univ. Press. Ames, Iowa.

Noga, E.J., J.F. Wright, and L. Pasarell. 1990. Some unusual features of mycobacteriosis in the cichlid fish *Oreochromis mossambicus*. Journal of Comparative Pathology 102:335-344.

Nyka, W. and E.F. O'Neill. 1970. A new approach to the study of non-acid fast mycobacteria. Annals of the New York Academy of Sciences 174:862-871.

Ogut H. and P.Reno. 2004. Prevalence of furnunculosis in Chinook salmon depends on density of the host exposed by cohabitation. North American Journal of Aquaculture 66:191-197.

Ossiander F.J. and G. Wedemeyer. 1973. Computer programs for sample sizes required to determine disease incidence in fish populations. Journal of the Fisheries Research Board of Canada 30(9):1383-1384

Ottinger, C.A. 2006. Mycobacterial infections in striped bass (*Morone saxatilis*) from upper and lower Chesapeake Bay: 2002 and 2003 pound net studies. In: Ottinger C.A. and Jacobs J.M. (eds) USGS/NOAA Workshop on Mycobacteriosis in Striped Bass. USGS Scientific Investigations Report 2006-5214/ NOAA NOS NCCOS Technical Memo 41.

Ottinger, C. A. and J.M. Jacobs. 2006. USGS/NOAA Mycobacteriosis in Striped Bass Workshop. USGS Scientific Investigations Report Series #206-5214: NOAA NOS Technical Memo Series #41.

Overton, A.S. 2003. Striped bass predator-prey interactions in Chesapeake Bay and along the Atlantic Coast. Doctoral Dissertation. University of Maryland Eastern Shore, Princess Anne, MD.

Overton A.S., F.J. Margraf, J.C. Griffin, and E.B. May. 2000. Changes in feeding habits of striped bass in the Chesapeake Bay: A health assessment. Final Report 2034. Maryland Department of Natural Resources, Annapolis, MD.

Overton, A.S., F.J. Margraf, C.A. Weedon, L.H. Pieper, and E.B May. 2003 The prevalence of mycobacterial infections in striped bass in Chesapeake Bay. Fisheries Management and Ecology 10(5):301-308.

Paton, N.I. and Y. Ng. 2006. Body composition in patients with wasting associated with tuburculosis. Nutrition 22(3):245-251.

Parent, L.J., M.M. Salam, P.C. Appelaum and J.H. Dosset. 1995. Disseminated *Mycobacterium marinum* infection and bacteremia in a child with severe combined immunodeficiency. Clinical Infectious Disease 21(5):1325-1327.

Park, K.C., and P.W. Reno. 2003. The effect of *in vitro* passage of infectious pancreatic necrosis virus (IPNV) on virulence and sensitivity of the virus to rainbow trout serum. Journal of Aquatic Animal Health 15:128-135.

Parisot, T.J. and E.M. Wood. 1960. A comparative study of the causative agent of a mycobacterial disease of salmonid fishes. II. A description of the histopathology of the disease in chinook salmon (*Oncorhynchus tshawytscha*) and a comparison to the strain characteristics of the fish disease with leprosy and human tuberculosis. American Review of Respiratory Diseases 82:212-222.

Pasnik, D.J. and S.A. Smith. 2006. Immune and histopathology responses of DNA-vaccinated striped bass *Morone saxatilis* x *M. chrysops* after acute *Mycobacterium marinum* infection. Diseases of Aquatic Organisms 73(1):33-41.

Paton, N.I. and Y. Ng. 2006. Body composition in patients with wasting associated with tuburculosis. Nutrition 22(3):245-251.

Paton, N.I., Y. Ng, C.B. Chee, C. Persaud, and A. Jackson. 2003. Effects of tuberculosis and HIV infection on the whole-body protein metabolism during feeding, measured by the [^{15}N]glycine method[1-3]. The American Journal of Clinical Nutrition 78:319-325.

Paul, K.P., M. Leichsenring, M. Pfisterer, E. Mayatepek, D. Wagner, M. Domann, H.G. Sonntag, and H.J. Bremer. 1997. Influence of n- and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. Metabolism 46(6):619-624.

Perez, A.T., D.A. Conroy, and L. Quinones. 2001. Presence of acid-fast bacteria in wild and cultured silver mullets (*Mugil curema* VAL., 1863). Interciencia 26(6): 252-256.

Pitchford, S., R. Robohm, S. MacLean, and L. Ramseyer. 2000. Observations on mycobacteriosis in the tautog (*Tautog onitis*). Journal of Shellfish Research 19:579.

Plouffe, D.A., P.C. Hanington, J.G. Walsh, E.C. Wilson, and M. Belosevic. 2005. Comparison of select innate immune mechanisms of fish and mammals. Xenotransplantation 12:266-277.

Pozniak A. and T. Bull. 1999. Recently recognized mycobacteria of clinical significance. Journal of Infection 38:157-161.

Pruell, R.J., B.K. Taplin, and K. Cicchelli. 2003. Stable isotope ratios in archived striped bass scales suggest changes in trophic structure. Fisheries Management and Ecology 10:329-336.

Puttinaowarat, S., K.D.Thompson, A. Kolk, and A. Adams. 2002. Identification of *Mycobacterium* spp. isolated from snakehead, *Channa striata* (Fowler), and Siamese fighting fish, *Betta splendens* (Regan), using polymerase chain reaction-reverse cross blot hybridization (PCR-RCBH). Journal of Fish Diseases 25(4):235-243.

Ranger, B.S. E.A. Mahrous, L. Mosi, S. Adusumilli, R.E. Leee, A. Colorni, M. Rhodes, and P.L. Small. 2006. Globally distriuted mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. Infection and Immunity 74(11): 6037-6045.

Rhodes, M.W., H. Kator, I. Kaattari, D. Gauthier, W. Vogelbein and C.A. Ottinger. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Diseases of Aquatic Organisms 61:41-51.

Rhodes, M.W., H. Kator, S. Kotob, P. van Berkum, I. Kaattari, W. Vogelbein, M.M. Floyd, W.R. Butler, F.D. Quinn, C. Ottinger, and E. Shotts. 2001. A unique *Mycobacterium* species isolated from an epizootic of striped bass (*Morone saxatilis*). Emerging Infectious Diseases 7(5):896-899.

Rhodes, M.W., H. Kator, S. Kotob, P. van Berkum, I. Kaattari, W. Vogelbein, F. Quinn, M.M. Floyd, W.R. Butler, and C. Ottinger. 2003. *Mycobacterium shottsii* sp. nov., a slowly growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). International Journal of Systematic and Evolutionary Microbiology 53:421-424.

Rhodes, M.W., H. Kator, A. McNabb, C. Deshayes, J. Reyrat, B.A. Brown-Elliott, R. Wallace, K. Trott, J.M. Parker, B. Lifland, G. Osterhout, I. Kaattari, K. Reece, W. Vogelbein, and C.A. Ottinger. 2005. *Mycobacterium pseudoshottsii* sp. nov., a slowly growing chromogenic species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). International Journal of Systematic and Evolutionary Microbiology 55(3):1139-1147.

Richards, R.A. and P.J. Rago. 1999. A case history of effective fishery management: Chesapeake Bay striped bass. North American Journal of Fisheries Management 19:356-375.

Roberts, R.J. 2001. The bacteriology of teleosts. Pages 297-331 *In* R.J. Roberts, editor, Fish Pathology. W.B. Saunders, New York.

Ross, A.J. 1960. *Mycobacterium salmoniphilum* sp. nov. from salmonid fishes. American Review of Respiratory Diseases 81:241-250.

Ross, A.J., and H.E. Johnson. 1962. Studies of transmission of mycobacterial infections in chinook salmon. Progressive Fish Culturist 24:147-149.

Rulifson, R., and R.W. Laney. 1999. Striped bass stocking programs in the united states: Ecological and resource management issues. Canadian Stock Assessment Secretariat Research Document 99/007.

Sakanari, J.A., C.A. Reilly, and M. Moser. 1983. Tubercular lesions in Pacific Coast populations of striped bass. Transactions of the American Fisheries Society 112:565-566.

SAS Institute Inc. 1990. SAS Procedures Guide: Reference. Version 6, First Edition. SAS Institute, Inc., Cary, NC, USA

Schulze-Robbecke, R. and K. Buchholtz. 1992. Heat susceptibility of aquatic mycobacteria. Applied and Environmental Microbiology 58(6):1869-1873.

Schwenk, A., L. Hodgson, A.Wright, L.C. Ward, C.F.J. Rayner, S. Grubnic, G.e. Griffin, and D.C. Macallan. 2004. Nutrient partitioning during treatment of tuberculosis: gain in body fat mass but not in protein mass. American Journal of Clinical Nutrition 79:1006-1012.

Sechi, L.A., Colorni, A., Dupre, I., Molicotti, P., Fadda, G., and S. Zanetti. 2002. Strain variation in Mediterranean and Red Sea *Mycobacterium marinum* isolates. Microbiologica 25:351-356.

Secombes, C.J., A.R. Cross, G.J. Sharp, and R. Garcia1992. NADPH oxidase-like activity in rainbow trout Oncorhynchus mykiss (Walbaum) macrophages. Developmental and Comparative Immunology 16:405 – 413

Seltzer, E.M., W.R. Boynton, K.V. Wood, H.H. Zion, L. Lubbers, N.K. Mountford, P. Frere, L. Tucker, and J.A. Mihursky. 1980. Synopsis of Biological Data on Striped Bass, *Morone saxatilis* (Wlabaum). NOAA Technical Report NMFS Circular 433, FAO synopsis No. 121.

Seltzer-Hamilton, E.M., W.R. Boynton, J.A. Mihursky, T.T. Plogar, and K.V. Wood. 1981. Spatial and temporal distribution of striped bass eggs, larvae, and juveniles in the Potomac Estuary. Transactions of the American Fisheries Society 110:121-136

Seltzer-Hamilton, E.M., and L. Hall. 1991. Striped Bass. *In*; S.L. Funderburk, J.A. Mihursky, S.J. Jordan, and D. Riley (Eds). Habitat Requirements for Chesapeake Bay Living Resources. 2nd Edition. Chesapeake Bay Program, Annapolis, MD.

Shearer, K.D., T. Asgard, G. Andorsdottir, and G.H. Aas. 1994. Whole body elemental and proximate composition of Atlantic salmon (*Salmo salar*) during the life cycle. Journal of Fish Biology 44: 785-797.

Sheldon, W.M., and V.S. Blazer. 1991. Influence of dietary lipid and temperature on bactericidal activity of channel catfish macrophages. Journal of Aquatic Animal Health, 3:87-93.

Shepherd, J.G., and D.H. Cushing. 1990. Regulation in fish populations: myth or mirage? Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 330:151-164.

Shuter, B.J. 1990. Population-level indicators of stress. In: Adams, S.M. (ed), Biological indicators of stress in fish. Pp. 145-166. American Fisheries Society, Bethesda, MD.

Simpson, A.L. 1992. differences in body size and lipid reserves between maturing and nonmaturing Atlantic salmon parr, *Salmo salar* L. Canadian Journal of Zoology 70:1737-1742.

Sindermann, C.J. 1970. Principle diseases of marine fish and shellfish. Academic Press, New York and London.

Stabel JR. 1998. Johne's disease: a hidden threat. Journal of Dairy Science 81(1) 283-288

Stabel, J.R., J.P. Goff, and M.R. Ackermann. 1998. Dietary calcium modulates *Mycobacterium paratuberculosis* infection in beige mice. Veterinary Immunology and Immunopathology 66:377-390.

Stabel, J.R., J.P. Goff, D.L. Whipple, M.R. Ackermann, and T.A. Reinhardt. 1996. Low calcium diet and 1,25-dihydroxyvitamin D3 infusion modulate immune responses during *Mycobacterium paratuberculosis* infection in beige mice. Veterinary Immunology and Immunopathology 50:127-143.

Stine, C.B., A.S. Kane, M. Matsche, L. Pieper, K. Rosemary, J.M. Jacobs, J. Abel, C. Driscoll, and A.M. Baya. 2006. Microbiology of gametes and age 0-3 striped bass (*Morone saxatilis*). *In* Ottinger and Jacobs (eds). USGS/NOAA Workshop on Mycobacteriosis in Striped Bass, May 7-10 2006, Annapolis, MD. NOAA Technical Memorandum NOS NCCOS 41.

Stinear, T., B.C. Cross, J.K. Davies, L. Marino, R.M. Robins-Browne, F. Oppedisano, A. Sievers, and P.D.R. Johnson. 1999. Identification and characterization of IS2404 and IS2606: Two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. Journal of Clinical Microbiology 37(4);1018-1023.

Stoskopf, M.K. 1993. Bacterial diseases of freshwater tropical fishes. Pages 559-563 *In* S.K. Stoskopf, editor. Fish Medicine. W.B. Saunders Company, Philadelphia.

Strahl, E.D., G.E. Gillaspy, and J.O. Falkinham III. (2001). Fluorescent acid-fast microscopy for measuring phagocytosis of *Mycobacterium avium, Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* by *Tetrahymena pyriformis* and their intracellular growth. Applied and Environmental Microbiology 67:4432-4439.

Swanson, C., D.V. Baxa, P.S. Young, J.J. Chech, and R.P. Hedrick. 2002. Reduced swimming performance in delta smelt infected with *Mycobacterium* spp. Journal of Fish Biology 61:1012-1020.

Swetter S.M., S.E. Kindel, and B.R. Smoller. 1993. Cutaneous nodules of *Mycobacterium chelonae* in an immunosuppressed patient with preexisting pulmonary colonization. Journal of the American Academy of Dermatology 28(2):352-355.

Takano, T., K. Nakamura, S. Takeuchi, and M. Watanabe. 1999. Disease patterns of the homeless in Tokyo. Journal of Urban Health, 76(1):73-84.

Talaat, A.M., R. Reimschuessel, and M. Trucksis. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. Veterinary Microbiology 58(2-4):229-237.

Talaat A.M., R. Reimschuessel, S.S. Wasserman, and M. Trucksis.1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. Infection and Immunity 66(6): 2938-2942.

Tarnowski, M. 1999. A historical background for striped bass landings in Maryland. Maryland Department of Natural Resources Fisheries Service, http://mddnr.chesapeakebay.net/mdcomfish/stripedbass/sbfactrev.htm

Thompson, J.D., D.G. Higgings, and T.J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22(22):4673-4680.

Thorel MF, Huchzermeyer H, Weiss R, Fontaine JJ (1997) *Mycobacterium avium* Infections in animals. Literature review. Veterinary Research 28(5):439-447

Timur, G. and R.J. Roberts. 1977. The experimental pathogenesis of focal tuberculosis in the and plaice (*Pleuronectes platessa*). Journal of Comparative Pathology 87:83-87.

Toranzo, A.E., B. Novoa, A.M. Baya, F.M. Hetrick, J.L. Barja, and A. Figueras. 1993. Histopathology of rainbow trout, *Oncorhynchus mykiss* (Walbaum) and striped bass, *Morone saxatilis* (Walbaum) experimentally infected with *Carnobacterium piscicola.* Journal of Fish Diseases 16(3): 261-267.

Tortoli, E., A. Bartonloni, E. Bozzetta, C. Burrini, C. Lacchini, A. Mantella, B. Penati, M.T. Simonette, and C. Ghittino. 1996. Identification of the newly described *Mycobacterium poriferae* from tuberculous lesions of the snakehead fish (*Channa striatus*). Comparative Immunology Microbiology and Infectious Diseases 19(1):22-29.

Tønjum T, DB Welty, E Jantzen and PL Small. 1998. Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: Mapping of their relationships to M. tuberculosis by fatty acid profile analysis, DNA-DNA Hybridization, and 16S rRNA gene sequence analysis. Journal of Clinical Microbiology 36(4): 918-925.

Turenne, C.Y., L. Tschetter, J. Wolfe, and A. Kabani. 2001. Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. Journal of Clinical Microbiology 39:3637-3648.

Ucko, M., and A. Colorni. 2005. *Mycobacterium marinum* infections in fish and humans in Israel. Journal of Clinical Microbiology 43:892-895.

Ucko, M., A. Colorni, H. Kvitt, A. Diamant, A. Zlotkin, and W.R. Knibb. 2002. Strain variation in *Mycobacterium marinum* fish isolates. Applied and Environmental Microbiology 68:5281-5287.

Uphoff, J.H. 2003. Predator-prey analysis of striped bass and Atlantic menhaden in upper Chesapeake Bay. Fisheries Management and Ecology 10:313-322.

Upton, H.F. 1993. Atlantic States Marine Fisheries Commission striped bass stocking summary 1985-1992. Striped Bass Stocking Technical Advisory Committee, ASMFC, Washington, D.C.

U.S. Department of Health and Human Services. 1999. Biosafety in Microbiological and Biomedical Laboratories. 3rdEdition. U.S. GPO, Washington, D.C., USA.

Van der Sar, AM, AM Abdallah, M Sparrius, E Reinders, CMJE Vandenbroucke-Grauls, and W. Bitter. 2004. *Mycobacterium marinum* strains can be divided into two distinct types based on genetic diversity and virulence. Infection and Immunity 72(11):6306-6312.

VERSAR 1990. Atlantic States Marine Fisheries Commission Interstate Fisheries Management Plan for the Striped Bass of the Atlantic Coast from Maine to North Carolina. Versar, Inc. April 1990.

Waller L.M. and L.Warner. 1999. Job No. 2A: Characterization of striped bass spawning stocks in Maryland. *Investigation of Striped Bass in Chesapeake Bay*. U.S. Fish and Wildlife Service Federal Aid Project F-42-R-12, MDDNR, Annapolis, MD, USA, pp 37-73.

Waller L.M., L. Warner, and E. Durell. 1999. Job No. 1: Fall and winter stock assessment and commercial fishery monitoring. *Investigation of Striped Bass in Chesapeake Bay*. U.S. Fish and Wildlife Service Federal Aid Project F-42-R-12, MDDNR, Annapolis, MD, USA, pp 5-36.

Walter, J.F., A.S. Overton, K.H. Ferry, and M.E. Mather. 2003. Atlantic coast feeding habits of striped bass: a synthesis supporting a coast-wide understanding of trophic biology. Fisheries Management and Ecology 10:349-360.

Walsberg, G.E. 1988. Evaluation of a nondestructive method for determining fat stores in small birds and mammals. Physiological Zoology 61(2):153-159.

Warner L., C. Weedon, and B. A. Versak. 2005. Project 2: Characterization of striped bass spawning stocks in Maryland. Chesapeake Bay Finfish / Habitat Investigations 2006, U.S. Fish and Wildlife Service Performance Report F-61-R-1, Maryland Department of Natural Resources, Annapolis.

Whipps, C.M., V.G. Watral, and M.L. Kent. 2003. Characterization of a *Mycobacterium* sp. in rockfish, *Sebastes alutus* (Gilbert) and *Sebastes reedi* (Westrheim & Tsuyuki), using rDNA sequences. Journal of Fish Diseases 26(4):241-245.

Wieland, C.W., S. Florquin, E.D. Chan, J.C. Leemans, S. Weijer, A. Verbon, G. Fantuzzi, and T. van der Poll. 2005. Pulmonary *Mycobacterium tuberculosis* infection in leptin-deficient *ob/ob* mice. International Immunology 17(11):1399-1408.

Wilson, K., M.B. Thomas, S. Blanford, M. Doggett, S.J. Simpson, and S.L. Moore. 2002. Coping with crowds: density-dependent disease resistance in desert locusts. Proceedings of the Natural Academy of Sciences 99(8):5471-5475.

Winsor H. 1946. Cold-blooded tuberculosis from the Fairmount Park Aquarium, Philadelphia. Proceedings of the Pennsylvania Academy of Science 20:43-46.

Wise, D.J., J.R. Tomasso, T.E. Schwedler, V.S. Blazer, and D.M. Gatlin III. 1993. Effect of vitamin E on the immune response of channel catfish to Edwardsiella ictalui. Journal of Aquatic Animal Healty 5:183-188.

Wolf, J.C. and S.A. Smith. 1999. Comparative severity of experimentally induced mycobacteriosis in striped bass *Morone saxatilis* and hybrid tilapia *Oreochromis* spp. Diseases of Aquatic Organisms 38:191-200.