ABSTRACT

Title of Document: MYCOBACTERIOSIS IN CHESAPEAKE BAY STRIPED BASS *MORONE SAXATILIS*

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Striped bass, *Morone saxatilis,* is an economically and ecologically important species in the Chesapeake Bay and along the East coast of the United States. In 1997 an epizootic of mycobacterial infections was discovered in the Chesapeake Bay stock and subsequent reports indicated that up to three-fourths of subpopulations of striped bass in the Bay were infected, primarily older fish. This study investigated regional and age class differences in mycobacterial infections among younger striped bass in the Chesapeake Bay, and identified putative risk factors for infection. Approximately 2,000 0+ to 3+ age striped bass, a limited number of spawning stock, and bycatch species were evaluated for microbiology, histopathology and parasitology. Mycobacterial isolates were grouped according to gas chromatography fatty-acid methyl-ester profiles and multi-locus sequencing. Twenty-nine groups of mycobacteria were discerned including *M. scrofulaceum*, *M. septicum*, *M. interjectum*, *M. triplex/M. montefiorense*, *M. szulgai*, *M. moriokaense*, *M. duvalii*, *M.*

avium, *M. terrae*, *M. pseudoshottsii*/*M. marinum* and *M. shottsii*, and several putative new species. The majority of mycobacteria groups observed had host overlap. Data revealed that prevalence of mycobacterial infection increased with age, up to 59%. Location of capture was associated with higher infection prevalence in fish sampled from the Pocomoke River compared with fish sampled from the Upper Bay (1+), the Choptank River (1+) and the Potomac River (0+, 1+). The presence of copepods, isopods, acanthacephalans, nematodes and trichodinid ciliates was associated with an increased prevalence odds ratio (POR) for mycobacterial infection, while the presence of bacteria other than mycobacteria was associated with a decreased POR for 0+ fish. Gender was not a risk factor for mycobacterial infection, however, gonads from some mature fish were infected. In addition, mycobacterial infections were observed in 12 other Chesapeake Bay fishes, including Atlantic menhaden, *Brevoortia tyrannus*, an important prey species. Mycobacterial infections in Chesapeake Bay fish appear to be more complex than the one pathogen-one host scenario. Further, vertical and food-borne transmission cannot be ruled out. Future research requires an holistic approach including evaluation of multiple host species in association with water quality and other environmental parameters.

MYCOBACTERIOSIS IN CHESAPEAKE BAY STRIPED BASS *MORONE SAXATILIS*

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my mom who taught me to laugh and that a positive outlook is the key to life. I'm also acknowledging that she tried to convince me that her master's sub-sub-basement laboratory where she played with pig pituitaries was worse than my master's basement laboratory where I played with fish livers.

Mary Susan Bee Stine, M.D.

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Chapter 1: Introduction

1.1 Problem statement and hypotheses

Striped bass *Morone saxatilis* are an important species both economically and ecologically in the Chesapeake Bay region as well as on the East Coast of the United States. However, striped bass landing decreased so dramatically during the 1970s and 1980s that a moratorium on fishing the stock was instituted in 1984. Through management that included restocking efforts, the stock numbers rebounded to the extent that fishing was marginally opened in 1990 and the species was declared recovered in 1995. However in 1997, a mycobacterial epizootic was discovered in Chesapeake Bay striped bass and reports from data collected between 1997-2001 indicated as many as 76% of Chesapeake Bay striped bass were infected (Heckert et al. 2001, Rhodes et al. 2001, Rhodes et al. 2004). Public concern was raised because of the unsightly nature of this disease in fish as well as possible transmission of piscine mycobacterial infections to humans. Three new species of mycobacteria isolated from striped bass were identified in this epizootic: *M shottsii*, '*M. chesapeaki'* and *M. pseudoshottsii* (Heckert et al. 2001, Rhodes et al. 2001, Rhodes et al. 2003, Rhodes et al. 2005). Thus, with prevalence data abounding, and amidst species identification efforts, an epidemiologic study was needed to begin to understand the infections in the wild stock. Therefore, the current striped bass health study was designed to investigate regional differences in mycobacterial infections among younger striped bass in the Chesapeake Bay, elucidate the differences among age classes, and identify putative risk factors.

To that end, in Chapter 1, I begin with a review of mycobacteria in general. I cover those mycobacteria which infect fish, specifically *M. marinum*, concentrating on wild stocks but including aquaculture information because more is known in that arena, and finish with the potential role environmental mycobacteria play in human health.

In Chapter 2, I describe two methods, one phenotypic and the other genotypic, of environmental mycobacterial speciation as applied to isolates from Maryland fish. Gas chromatography and a multi-locus sequencing technique are applied to over 40 isolates and results are compared.

In Chapter 3, I review the mycobacteria isolated from Chesapeake Bay striped bass and other species. A gas chromatography technique of tracking isolates is introduced. This method is developed in more detail in Chapter 4.

In Chapter 4, I present prevalence data on parasites of striped bass encountered during this health assessment survey of Chesapeake Bay striped bass, 2003-2005. Included are plates designed to help the parasitology student quickly identify parasitic organisms grossly and *en section*.

In Chapter 5, I investigate the prevalence of mycobacterial infection and disease in young-of-the-year to 3 year old Chesapeake Bay striped bass, 2003-2005. The associations with putative risk factors are explored, and I finish with a discussion of the relevance of findings within the context of striped bass health within the Chesapeake Bay.

H_0: The Chesapeake Bay stock of striped bass is not infected with *Mycobacterium* spp.

H_0: No differences exist in the prevalence of infection between age classes of striped bass in the Chesapeake Bay (alpha=0.05).

H_0: No differences exist in the prevalence of infection among young-of-the-year taken from 5 collection sites in the mid and upper regions of the Chesapeake Bay (alpha=0.05).

H_0: The presence of external lesions, internal granulomas, bacteria other than mycobacteria and parasites on and in Chesapeake Bay striped bass are not related to the presence of mycobacteria within individual fish.

Chapter 6 is a discussion of problem areas within this dissertation, further discussion on results, and suggested areas for continued study.

1.2 Introduction to Mycobacterium spp.

Mycobacteria are widely distributed in nature. Most live in soil and/or water and are thus termed 'environmental' mycobacteria (EM), however, they are opportunistic pathogens and can infect vertebrate and invertebrates alike. A few mycobacterial species prefer various warm-blooded animals and humans; *Mycobacterium tuberculosis* and *M. leprae* cause tuberculosis and leprosy, respectively, in humans, while *M. avium* subsp. *paratuberculosis* causes Johne's disease in ruminants. Aquatic mycobacteria are environmental mycobacteria that are, in turn, members of the group also collectively called nontuberculous mycobacteria. Previously, all nontuberculous mycobacteria were termed 'atypical' because they did not exhibit typical characteristics of *M. tuberculosis*.

Mycobacteria are slightly curved or straight bacilli (Metchock et al. 1999). They are classified as gram positive even though they exhibit notable variability in staining

(Metchock et al. 1999). Mycobacteria have high lipid content waxy cell walls that create their characteristically acid fast qualities, yet not all colonies or bacilli stain positive at all times. This variable staining is possibly dependant on the type of stain used, strain characteristics, or phase of growth (Gauthier et al. 2003). Mycobacteria are aerobic, non-spore-forming, and non-motile (Metchock et al. 1999). Colonies can be smooth to rough, and colors range from yellow, buff, orange and pink to non-pigmented. Some pigmented species are photochromogens, which require light to form pigment, while others are scotochromogens, which can produce pigment in either the presence or absence of light.

The characteristic slow growth of mycobacteria can be explained by two qualities. First, mycobacteria only have one or two copies of the *16S rDNA* gene, which slows down replication, but also allow mutations to more readily incorporate into the genome (Primm et al. 2004). Second, mycobacteria expend more energy to produce longer-chain mycolic acids for their impermeable wax-rich cell wall than faster growing bacteria expend for their cell walls (Primm et al. 2004). While this does limit transport of nutrients, the impermeable wall also affords protection from antimicrobial entities and general stress (Primm et al. 2004). Another advantage of the cell wall is hydrophobicity, which allows EM to easily affix to particulates (Hall-Stoodley and Lappin-Scott 1998, Primm et al. 2004). Slow growth of mycobacteria may be viewed as a drawback, but it is also an advantage as slowly growing bacteria are more resistant to starvation and are harder to kill (Falkinham et al 2004).

As previously mentioned, EM are widely distributed in nature, and potential reservoirs include soils, water, biofilms, amoebae, and ciliates (Falkinham et al. 2004, Primm et al. 2004). EM are recovered from fresh, brackish and sea water sources, and

can inhabit a wide range of niches with varying temperatures, oxygen levels, acidities and salinities (Falkinham et al. 2004). EM can metabolize a variety of complex hydrocarbons, which further enhances their colonization potential (Primm et al. 2004). EM can quickly form biofilms, which may afford protection in hostile environments (Hall-Stoodley and Lappin-Scott 1998, Hall-Stoodley et al. 2004). EM can also survive, and even replicate faster, within amoebae vacuoles, which further expands their repertoire of ecological niches (Adekambi et al. 2006, Thomas and McDonnell 2007).

While the exact ecological niches and reservoirs for all the EM are not known, EM can infect a wide range of animals, including fish and humans. Fish infections with EM can produce a disease termed mycobacteriosis, yielding granulomas in internal organs, external lesions, and can potentially cause death.

Human *M. marinum* infections usually cause granulomatous skin lesions termed 'fish fancier's finger,' 'fish tank granuloma,' 'mariner's TB' and 'swimming pool granuloma' (Philpott et al. 1963, Littlejohn and Dixon 1984, Vincenzi et al. 1992, Dobos et al. 1999). Contact with infected water or infected animals (usually fish) is a risk factor, especially for *M. marinum* infections (Zeligman 1972, Aubry et al. 2002). However, EM can also aerosolize and cause pulmonary infections (Wendt et al. 1980). Disseminated infections may occur in immunodeficient patients (Tchornobay et al. 1992, Streit et al. 2006). Therefore, mycobacterial zoonoses are a potential problem with anyone coming in contact with contaminated water, including watermen, anglers, recreational boaters, swimmers, and both hobby and professional aquarists.

Along with public health concerns, environmental mycobacteria have the potential to cause financial damage in the form of commercial and recreational fishery

losses and lost recreational use of waterways, including the Chesapeake Bay. Therefore, more needs to be known about the ecology of environmental mycobacterial species including; host specificity, epidemiology of disease, their mode of transmission in piscine vectors, and natural reservoirs.

1.3 Mycobacteria in Fish

1.3.1 Introduction

A group of French scientists originally described piscine mycobacteria in 1897 (Bataillon et al. 1897, cited in Chinabut 1999) and Von Betegh described the first report of mycobacteriosis in a marine fish in 1910 (cited in Chinabut 1999). *Mycobacterium marinum* was first discovered and isolated as a salt-water fish pathogen in 1926 when 'tubercles', with acid-fast bacilli inside, were found in several organs of dead fish in the Philadelphia aquarium (Aronson 1926, Zeligman 1972). *M. marinum* (then termed *M. platypoecilus*) was also determined as the cause of a 'tuberculin outbreak' in Mexican platyfish in 1942 (Zeligman 1972). Several species names have appeared in the literature for *M. marinum*, including *M. platypoecilus* and *M. balnei*, but *M. marinum* has historical precedence (Kullavanijaya et al. 1993, Ang *et al.* 2000).

Multiple *Mycobacterium* spp. infect fish (Table 1-1), including three species or strains recently isolated from wild stocks of striped bass in the Chesapeake Bay, '*M. chesapeaki', M. shottsii* and *M. pseudoshottsii*. The two newly-named isolates, '*M. chesapeaki'* and *M. shottsii*, differ in response to several biochemical tests, including growth at 37°C, niacin production, pyrazinamidase activity (7d) and 16S rDNA gene sequence (Rhodes et al. 2003). *M. pseudoshottsii* differs from '*M. chesapeaki'* and *M.*

shottsii by photochromogenicity, growth at 37°C and antibiotic resistance profiles (Rhodes et al. 2005). Pathogenicity of *Mycobacterium* spp. appears to differ. For example, *M. marinum* produced more severe pathology in experimentally infected striped bass than *M. shottsii* or *M. gordonae* (Gauthier et al. 2003).

As of 1963, mycobacterial species were documented to infect over 150 species of fish and numerous other aquatic species (Nigrelli and Vogel 1963). The list of susceptible species has grown in recent years, and it is possible that all piscine species are potential hosts (Noga 2000). Affected fish include individuals from wild stocks as well as aquacultured and aquarium species (Nigrelli and Vogel 1963). Wild stocks may have high infection rates (Table 1-2), including wild fish associated with cage farms in the Gulf of Eliat, Red Sea (Diamant et al. 2000). The authors postulated that highly susceptible cage-farmed aquacultured species, such as sea bass (*Dicentrarchus labrax*) and red drum (*Sciaenops ocellatus*), become infected and act as a reservoir of bacterial infection for wild fish which pass through cages and interact with infected captive fish. Wild rabbitfish (*Siganus rivulatus*) associated with these cage farms had a 50% prevalence of mycobacterial infections whereas rabbitfish at a local reef 10 km away were not infected (Diamant et al. 2000).

Infection prevalence increases with age (Ashburner 1977; Abernethy and Lund 1978; MacKenzie 1988; Gauthier et al. 2006). This increase may be due to the slower growth rate of mycobacteria, the prolonged disease progression, the more chances to have contact with infected sources, or other unknown factors.

Until recently, there were few reports of mycobacterial infections in wild striped bass. Tubercular lesions containing acid-fast bacteria in necrotic centers were first

reported in striped bass from the San Joaquin and Sacramento rivers, San Francisco Bay and Coos Bay in California (Sakanari et al. 1983). These tubercles were most prevalent in livers, but were also found in spleen, anterior kidney, posterior kidney and occasionally in mesentery. The bacterium was not cultured but, presumably *en section,* did not have the filamentous, branching morphology of the other acid-fast bacterial fish pathogen *Nocardia* spp. The striped bass did not appear to be clinically affected by the infection. More recently, mycobacterial infections have been reported in wild striped bass in the Chesapeake Bay (Heckert et al. 2001, Rhodes et al. 2001, Rhodes et al. 2004). Up to seventy-six percent of striped bass collected from Virginia and Maryland waters of the mid to lower Chesapeake Bay from 1997 to 2001 were infected with mycobacteria (Rhodes et al. 2004). Diagnosis was made by culture, and recovered isolates belonged to three non-pigmented groups, two scotochromogenic groups and three photochromogenic groups. However fish in the resident Chesapeake Bay stock, under three years of age (n=16), were grouped together and had a relatively low prevalence (19%). Conversely, of the striped bass coastal stock that migrate annually into the Bay, 90% of striped bass 3-4.9 years-old (n=119) were infected, 80% of 5-6.9 year-olds (n=25) were infected, and 47% of fish over 7 years-old (n=34) were infected. Recently, striped bass in the Delaware Bay, also in the mid-Atlantic region with the Chesapeake Bay, have been found to be infected with mycobacteria (Ottinger et al. 2007). Prevalence ranged from 7-53% dependent on sampling day, and while the majority of isolates recovered were not speciated, 9.1×10^7 cfu/g tissue of *M. chelonae* was isolated from one fish, and 1.5×10^3 cfu/g tissue of a *M. shottsii*-like mycobacteria was recovered from another.

Although mycobacterial infections can cause severe ailments in individual wild fish, infections of wild fish stocks generally seem to be relatively mild compared to the more severe cases that are found in cultured or aquarium fish. Disease in cultured fish is potentially attributed to high stocking densities and decreased water quality (Chinabut 1999). Stress associated with intensive culture practices and handling is known to reduce immune competence in several fish species, and has been proposed as a cause of high mycobacterial prevalence and incidence (Schreck 1996, Smith 1996, Antonio et al. 2000). In one case, fifty percent of cultured yearling striped bass died over a 6 month period; *M. marinum* was isolated from 80% of the remaining yearlings (Hedrick et al. 1987). Another form of stress, accidental higher temperatures of culture (16ºC versus the optimal 9-12ºC), and repeated handling were believed to have increased infection rates in delta smelt *Hypomesus transpacificus* (Antonio et al. 2000).

1.3.2 Clinical manifestations of mycobacteriosis in fish

Mycobacteriosis can be a chronic wasting disease in fish. Fish may appear emaciated, have reddish skin lesions with skin and/or scale loss, ulcers, hemorrhage, exophthalmia, fin rot, skeletal deformities, and their colors made fade or darken (Baker and Hagan 1942, Van Duijn 1981, Plumb 1994, Austin and Austin 1999, Chinabut 1999, Sanders and Swaim 2001). Infected fish may be sluggish, exhibit equilibrium disturbances, and/or go off feed (Van Duijn 1981, Plumb 1994, Swanson et al. 1996, Chinabut 1999, Conroy and Conroy 1999, Wolf and Smith 1999, Sanders and Swaim 2000).

Mycobacteriosis can cause white to grayish nodules called granulomas in internal organs, primarily the spleen, head kidney, liver and heart, and these organs may become

enlarged (Van Duijn 1981, Gomez et al. 1993, Plumb 1994). Colorni et al. (1998) suggested that because fish lack lymph nodes, the spleen initially traps mycobacteria, and only when the spleen is overwhelmed does the infection spread systemically to form granulomas in other organs. Other observations include adhesions within the peritoneal cavity, smaller gonads and decreased length of adult wild salmonids, as well as spawning abnormalities (Wood 1974, MacKenzie 1988). Spawning inhibition was also observed in delta smelt (Antonio et al. 2000).

Histologically, granulomas consist of epitheliod macrophage cells surrounded by connective tissue from a chronic inflammatory response, with or without central necrosis (Gomez et al. 1993, Noga 2000). Chinabut (1999) proposed that mycobacterial infections can be classified as subacute or chronic. The subacute form is characterized by granulomas with a central caseous necrotic area of reticuloendothelial cells and macrophages interspersed with acid-fast bacteria. A chronic infection may produce hard or soft granulomas (Chinabut 1999, Phanwichien et al. 1999). Soft granulomas have a central area of caseous necrosis possibly containing nuclear debris surrounded by spindle-shaped epitheliod cells. These are encircled by highly eosinophilic, flattened, epitheliod cells that are, in turn, encapsulated with fine fibrous connective tissue (Chinabut 1999). Hard granulomas have central epitheliod cells with a fibrous connective tissue capsule (Chinabut 1999). Calcification of granulomas may occur in more chronic infections (Majeed et al. 1981, Van Duijn 1981, Chinabut 1999).

Acid-fast bacteria may or may not be present in these granulomas, and it has been postulated that they are potentially seen only in new granulomas (Van Duijn 1981, Anderson et al. 1987, Plumb 1994). Conversely, Gauthier et al. (2003) suggests that with

M. marinum infections, acid-fast bacteria may only be seen in later developing bacillary granulomas and proposed a histologic progression of granuloma formation in experimentally infected striped bass (*Morone saxatilis*). Briefly, epitheliod cells surround a central core containing eosinophilic cellular debris (epitheliod granuloma) then spindle-shaped cells form between the epitheliod cells and the necrotic core (spindle-cell granuloma). Few or no acid-fast bacilli are seen within the core in the first two stages, but in the third stage (bacillary granuloma) numerous acid-fast bacilli are seen in the core and spindle layers. In the fourth stage (recrudescent lesion), the lesion lacks organization and acid-fast bacilli may or may not be present.

It is important to note that whether or not granulomas may be histologically similar across fish species, different host species may have different susceptibilities and rates of granuloma formation based on unknown factors. For example, striped bass had considerably more granulomas than hybrid tilapia when experimentally infected under identical conditions (Wolf and Smith 1999).

1.3.3 Transmission

Much debate has ensued over the routes of transmission of mycobacterial infections in fish. As discussed earlier, EM are ubiquitous in nature, and can live in a wide range of ecological niches (Primm et al. 2004). Reservoirs of EM include water, soils, biofilms and potentially amoeba (Primm et al. 2004, Hall-Stoodley and Stoodley 2005, Adekambi et al. 2006). It is also generally accepted that mycobacteria are shed into surrounding water from various sources, including skin ulcers or lesions, feces, or dead carcasses (Clark and Shepard 1963, Van Duijn 1981, Hedrick et al. 1987, Plumb 1994, Belas et al. 1995, Antonio et al. 2000, Noga 2000). Nevertheless, the question lies

in how the bacteria invade an individual fish to cause infection. Whereas stress is believed to lower immunity and increase the chance of infection, the bacteria still require an initial 'in' (Schreck 1996, Smith 1996, Antonio et al. 2000).

Several authors have provided reports of ingestion as a route of exposure. In fact, mortality and morbidity ended when contaminated feed was avoided in several salmon hatcheries during the 1950's in the northwestern United States and in cultured snakehead (*Channa striatus*) from Thailand (Ross 1970, Wood 1974, Chinabut et al. 1990). Other sources of infection through ingestion may include infected invertebrate prey items including arthropods, snails, shrimp or prawns (Michelson 1961, Beerwerth et al. 1979, Brock et al. 1986, Lightner and Redman 1986, Mohney et al. 1998, Chinabut 1999). More recently, Harriff et al. (2007) suggest ingested mycobacteria infect fish by passing from the intestinal lumen through the gastrointestinal wall and into the viscera. Additionally, passage of ingested *M. peregrinum* through the amoeba *Acanthamoeba castellani* before intubation enhanced mycobacterial growth in fish tissues, thus indicating a potential natural reservoir for some environmental mycobacteria (Harriff et al. 2007).

Mycobacteria are also believed to be able to enter through pre-existing lesions that create a hole in protective skin or gill barriers (Frerichs 1993, Chinabut 1999). Subtle, early skin lesions containing acid-fast bacilli, believed to be pathognomonic, have been observed in Chesapeake Bay striped bass (Vogelbein et al. 2006). However whether the mycobacteria creating these lesions stem from internal organs or if external mycobacteria are the potential cause remains unknown.

Vertical transmission has been proposed in some cases where horizontal infection has been dismissed or the route of exposure is unclear (Nigrelli and Vogel 1963, Ashburner 1977, Chinabut et al. 1994, Hedrick et al. 1987). Conroy (1966) demonstrated a trans-ovarian route of infection in Mexican platyfish (*Platypoecilus maculuatus*) when acid-fast bacteria were found within embryonic tissues. Ashburner (1977) suggested mycobacteria might infect salmon eggs through the micropyle since mycobacteria were isolated from the peritoneal fluid that comes in contact with eggs. In addition, Conroy and Conroy (1999) isolated mycobacteria from the unborn embryos of infected gravid guppies (*Lebistes reticulates*). Conversely, Ross and Johnson (1962) were unable to demonstrate vertical transmission in salmon but did not rule out its possibility.

1.3.4 Diagnosis

Historically, some researchers have relied on the presence of acid-fast bacilli in histologic sections to diagnose mycobacteriosis (Van Duijn 1981, Stoskopf 1993). However, the various methods of acid-fast staining seemingly give variable results, possibly dependant on the phase of bacterial growth. Herbst et al. (2001) suggested that the commonly applied, unmodified Ziehl-Neelsen stain can give false negatives while Gauthier et al. (2003) found a pre-treatment for 24 hours of 10% periodic acid (HIO_4) followed by Ziehl-Neelsen, as proposed by Nyka and O'Neill (1970), to be a more sensitive staining method for bacteria within early-stage granulomas.

Whatever the case, the presence of acid-fast organisms or internal granulomas is not diagnostic of only mycobacteriosis. The list of differential diagnoses must include several granuloma-forming organisms, including a second genus of acid-fast bacteria, *Nocardia*. With granulomas, differential diagnoses include flavobacteria (which cause

pseudotuberculosis (Majeed et al. 1981)), *Ichthyophonus hoferi*, ulcerative mycosis, and migrating helminthes and protozoa including microsporidians and myxosporidians (Dulin 1979, Anderson et al. 1987, Plumb 1994, Noga 2000). Anderson et al. (1987) cited ways to distinguish some of these organisms through histology. The characteristic epitheliod cells around the granulomas in mycobacteriosis are usually absent in infections of flavobacteria, nocardia, and myxospridia. *Nocardia* granulomas, while possibly found in association with acid-fast bacteria, also do not have the mycobacterial distinctive layers. *Nocardia* may appear filamentous, while mycobacteria most likely will not (Baya, personal communication). Finally, migrating helminth granulomas are not associated with acid-fast bacteria, are unusually large and irregularly shaped, and have a distinctive thick tissue capsule (Anderson et al. 1987). Ulcerative mycosis occurs primarily in the musculature and within the granulomatous tissue (Noga 2000). The fungal hyphae associated with this disease can be visualized histologically in tissues stained with Gomori's methenamine silver stain (GMS) (Noga 2000).

Other methods of detection include molecular, immunohistochemical and serology techniques. Polymerase chain reaction (PCR) techniques have been used to detect 16S rDNA gene fragments in tissues (Whipps et al. 2003). Immunohistochemical methods can detect mycobacterial antigens in section and circulating antibodies have been used to diagnose previous exposure (Colorni et al. 1998, Gomez 2001, Lescenko et al. 2003).

Culturing the actual mycobacterial organisms is often considered the definitive way to diagnose an infection as long as contamination of specimens is prevented. However, speciation by traditional biochemical characterization can be costly and time

consuming. Cook et al. (2003) performed a cost analysis of laboratory techniques to identify commercial DNA probe-negative, difficult to identify non-tuberculous mycobacteria, based on year 2000 costs. Cost per sample using conventional biochemical methods ranged from CAD$80 (Canadian dollars) to CAD$174 whereas 16S rDNA sequencing cost CAD$48 per sample. Additionally, species were identified within 1 to 2 days using sequencing compared with 2 to 6 weeks using conventional methods. Therefore, recent developments in molecular biological techniques may afford a more cost effective and timely diagnosis.

Once it is established that the infection exists, speciation of the causative mycobacteria can be difficult. The 16S rDNA has been used widely for species identification (Talaat et al. 1997, Astrofsky et al. 2000, Hecket et al. 2001, Rhodes et al. 2001, Whipps et al. 2003, Kaattari et al. 2005). However *16S rDNA* is highly conserved, and as little as 5 nucleotide changes in the entire *16S rDNA* gene, with accompanying phenotypic changes, may be enough to name a new species (Turenne et al. 2001). Compounding the situation is that some mycobacteria, primarily the rapid growers, may contain more than one *16S rDNA* allele and consequently intraspecies variability is introduced (Turenne et al. 2001). Therefore, as the intraspecies variability and the interspecies conservation within *16S rDNA* cannot necessarily distinguish among closely related mycobacteria, researchers are turning to other genes with more sequence variability in an attempt to find species specific regions for differentiation and identification, specifically in closely related complexes such as the tuberculosis clade (Turenne et al. 2001, Devulder et al. 2005, Kaattari et al. 2006). These genes include 65-kDa heat shock protein (*hsp65*), RNA polymerase B subunit (*rpoB*), exported repeated

protein (*erp*) , superoxide dismutase (*sod*), heat shock protein J (*dnaJ*), chromosomal replication initiation protein (*dnaA*), DNA repair protein gene *recA* and 16S-23S internal transcribed spacer (ITS) insertion sequences (Turenne et al. 2001, Devulder et al. 2005, Kaattari et al. 2006, Mukai 2006).

Additional molecular techniques for mycobacterial speciation include restriction enzyme analysis and probe analysis. PCR restriction enzyme profiles for *Apa*I and *Ban*I have been developed for speciation of three commonly found fish mycobacteria, *M. marinum*, *M. fortuitum* and *M. chelonae* (Talaat et al. 1997). Commercially available probes have been developed for mycobacteria of human clinical concern, including some EM (Mijs et al. 2002, Tortoli et al. 2003).

Characterizing the species-specific fatty-acids in the mycobacterial cell wall through fatty-acid methyl ester (FAME) analysis using gas chromatography can also be used for mycobacterial speciation (Teng et al. 1997, Muller et al. 1998, Ozbek and Aktas 2003). A library is currently being developed for fish-specific mycobacterial isolates (A. Baya, personal communication). A computer aided high-performance liquid chromatography technique has also been developed for mycobacterial speciation, and was found more cost efficient than either commercially available probes or biochemical characterization (Kellogg et al. 2001). Nevertheless, at present, PCR-based techniques seem to be considered by some to be the gold standard (Chemlal and Portaels 2003).

1.3.5 Prevention and Treatment

Mycobacterial infections are persistent, primarily because of the long generation times, as well as waxy cell walls that are impervious to many chemical treatments and antibiotics (Stoskopf 1993). In fact, environmental mycobacteria have been known to

survive 2 years without a host in the environment, however the amount of proliferation was unreported (Reichenbache-Klinke 1972, Noga 2000). Therefore, preventive measures are the ideal way to combat mycobacterial infections (Belas et al. 1995, Antonio et al. 2000, Noga 2000). Measures to control spread through a facility include 1) preventing overcrowding to contribute to good water quality and reduced stress, 2) removing affected fish by culling infected populations, 3) thoroughly disinfecting aquaria, 4) quarantining newly acquired fish, and 5) instituting chlorine foot baths (200 mg/L available chlorine) for husbandry personnel (Van Duijn 1981, Hedrick et al. 1987, Kawakami and Kusuda 1990, Stoskopf 1993, Plumb 1994, Belas et al. 1995, Antonio et al. 2000, Noga 2000, Jacobs et al. 2004). Additionally, disinfecting nets and hands, and changing gloves when switching from tank to tank contribute to proper disease control (Jacobs et al. 2004). Further, Astrofsky et al. (2000) detailed a successful method of system disinfection that used bleach at a concentration of 1/4 cup of Clorox per gallon of water (800 ppm) for three days followed by running the system with freshwater for an additional three days.

In addition to physical control of infections, although no bacterins are currently available, engineered measures may be useful in the relatively near future (Stoskopf 1993). Chen et al. (1996) showed elevated nonspecific and specific immune responses in rainbow trout, *Oncorhynchus mykiss*, following an intraperitoneal injection of a mixture of mycobacterial extracellular products and Freund's complete adjuvant (Chen et al. 1996). The non-specific immune responses of phagocytic activity and lysozyme activities were elevated post-injection as well as the specific immune response of serum antibody titers (Chen et al. 1996). Subsequently, a recombinant vaccine that expressed

M. bovis BCG 85A antigen was developed for potential use in fish (Pasnik et al. 2003). While it produced significant specific humoral and cell-mediated immune responses in juvenile striped bass, the vaccine was not protective in fish challenged with *M. marinum* 70 days post-inoculation (Pasnik et al. 2003). Pasnik and Smith (2005) subsequently constructed a DNA vaccine from the *M. marinum* Ag85A gene. Intra-muscular injection was found to be more protective than intra-peritoneal and the degree of protection was dose-dependant. The authors suggest that both humoral and cellular responses were stimulated, however they failed to demonstrate increased phagocytic or respiratory burst functions in macrophages. This study demonstrated that 90% of hybrid striped bass vaccinated by 50 µg i.m. injection survived twice as long (36 days) as control fish when challenged with $8x10^5$ cfu *M. marinum*/g body weight at 90 days post-inoculation. However, long-term vaccine efficacy was unknown at this point. A subsequent study showed this vaccine provided significant but short lived protection against *M. marinum* in hybrid striped bass (Pasnik and Smith 2006). While control fish showed mortalities, elevated splenic bacterial counts and higher granuloma formation at 14 days post-challenge, vaccinated fish eventually showed similar results at 28 days post-challenge (Pasnik and Smith 2006). Since *M. marinum* is one of the more prevalent and devastating mycobacterial species in aquaculture, vaccine development efforts for fish have concentrated on this species.

Once infections occur two options remain for fish in a captive setting; infected fish can either be destroyed or treated. Euthanizing infected fish is the preferred response, followed by burning or, historically, by burying carcasses with quicklime, and

thoroughly disinfecting tanks and tank paraphernalia (Dulin 1979, Leibovitz 1980, Plumb 1994, Austin and Austin 1999).

The second option is to attempt treatment, and is usually only used with financially or emotionally high value fish. Antibiotics have been used with varying clinical success as antibiotic resistance is common and differs among *Mycobacterium* species and strains (Van Duijn 1981, Decostere et al. 2004). Penicillins and anti-tuberculin drugs have been used and include cyclosporine, doxycycline, minocycline, ethambutol, ethionamide, streptomycin, sulfanomides, and tetracycline (Van Duijn 1981, Hedrick et al. 1987, Stoskopf 1993, Plumb 1994, Noga 2000, Decostere et al. 2004). Rifampicin, streptomycin and erythromycin were shown to control, but not eradicate, mycobacterial infections in yellowtail, *Seriola quinqueradiata*, as was streptomycin and garlic extract in sea bass, *Dicentrarchus labrax* (Kawakami and Kusuda 1990, Colorni et al. 1998). Note that food fish in the United States should not be treated as there are currently no approved FDA drugs for mycobacterial control in fish (Jacobs et al. 2004). Additionally, treating the water with ozone, ultra-violet light or chloramines (either B or T) have been suggested as treatments (Van Duijn 1981, Plumb 1994, Decostere et al. 2004). In fact, Miyamoto et al. (2000) found UV light over 45 mW/cm^2 to control *Mycobacterium avium*, a ubiquitous environmental species. The treatment option should be tailored to the mycobacterial species present though, as organisms likely have differing susceptibilities.

The natural progression of infection can depend on a fish species' environment and natural immunity to the bacteria, although all fish species are believed to be susceptible hosts (Stoskopf 1993). Overcrowding in culture facilities can compound

infections and end in high mortality rates, whereas wild fish populations may live with minor background infections (Plumb 1994). In the end, however, lesion resolution may occur, as recrudescent lesions have been found in experimentally infected striped bass *Morone saxatilis* (Gauthier et al. 2003). Though many of the measures described in this section are not feasible for wild fish, improving water quality and reducing stress (i.e., assuring adequate biomass of nutritionally rich prey items) may be possible intervention strategies for wildlife managers.

1.4 Environmental mycobacteria in humans

Environmental mycobacteria represent potential zoonotic diseases and have been identified as emerging water-borne pathogens (Dobos et al. 1999; Sharma et al. 2003). Infections can occur from exposure during swimming, boating or fishing in natural waters or by handling infected wild, aquacultured or aquarium animals. In fact, human infections have been associated with contact with salt- and fresh-water fish, shrimp and oysters (Kullavanijaya et al. 1993, Lehane and Rawlin 2000).

Epidemiological data suggests that up to 80% of *M. marinum* infections are associated with a history of local trauma such as a puncture wound, scrape or bite (Zeligman 1972, Feldman et al. 1974, Bhatty et al. 2000, Casal and Casal 2001). Specifically, *M. marinum* infections associated with traumas have been reported from the Chesapeake Bay region (Zeligman 1972, Hoyt et al. 1989). Luckily, infections are usually limited to extremities because the optimal growth temperature of *M. marinum*, and most environmental mycobacteria, is lower than core body temperature (Weitzul et al. 2000). Through passive laboratory-based surveillance, reported human *M. marinum*

cases increased in the United States in the 1990's, with an estimated 198 cases per year from 1993 to 1996 (Dobos et al. 1999). The southeast, as a region, reported the most cases, while Maryland, Florida and Virginia reported the most cases from individual states (Dobos et al. 1999). Additionally, Maryland and Virginia counties bordering the Chesapeake Bay reported 275 cases of *M. marinum* infection from 1995-2005 while only 4 cases of infection were reported from counties not bordering the Bay (Panek and Bobo 2006). The authors caution that while no information was available on where the infections were contracted, the potential of the Chesapeake Bay as a reservoir for human infections is highlighted.

Other environmental mycobacteria also occur in various water sources, including tap water, and can cause nosocomial infections in immunocompromised patients (Metchock et al. 1999, Gebo et al. 2002). Higher chlorine levels have all but erased swimming pool infections in recent years, but it is possible drinking water chlorination has selected resistant mycobacterial strains (Lehane and Rawlin 2000; Primm et al. 2004).

Purified protein derivative (PPD) skin tests, routinely used in the United States to test for past *M. tuberculosis* exposure, can become positive following *M. marinum* infection (Mollohan and Romer 1961, Lewis et al. 2003). These tests may help diagnose environmental mycobacterial infections but cannot distinguish *M. marinum* infections from *M. tuberculosis*, thus further tests are required to identify the actual mycobacterial species causing the response (Feldman et al. (1974).

Treatment can be long and expensive, so prevention is the best way to approach environmental mycobacterial infections. One should minimize contact with presumably

infected water or organisms. Gloves are recommended when cleaning fish tanks or handling fish, especially those from populations with a known infection (Jacobs et al. 2004). Cuts and scrapes should be thoroughly scrubbed with soap and water after contact with purportedly infected water or fish, especially if the trauma was caused during such contact (Jacobs et al. 2004). Immunocompromised individuals are especially at risk for contracting infections, and should therefore exercise extreme caution when handling potentially infective material or specimens.

Mycobacterium species found in fish, other than *M. marinum,* can be pathogenic to humans and include, but are not limited to, *M. gordonae*, *M. fortuitum*, *M. chelonae*, and *M. szulgai*. These species are ubiquitous in the environment, and epidemiological data investigating route of exposure may not be listed in case studies and whether these cases stem from contact with fish or water is unclear. Regardless, the point remains that these species are found in fish and therefore represent possible zoonoses.

Mycobacteria can be aerosolized, when a filter is being replaced on an aquarium, or from bubbles bursting at the water-air interface (Wendt et al. 1980; Meunier et al. 2003). Since environmental mycobacteria have the ability to cause pulmonary diseases resembling tuberculosis, this aerosolization associated with water sources is another potential mode for transmission (Jarikre 1991, Swetter et al. 1993, Pozniak and Bull 1999, Smith et al. 2001).

1.5 Conclusion

Mycobacterial infections are generally insidious in nature, but can cause unsightly ulcers and death in fish, and represent possible zoonoses. Mycobacterial infections can

cause losses to commercial and recreational fisheries both by population decline if fish are killed by the disease and by public refusal of ulcerated fish as well as, possibly, fish in apparently excellent condition. For example, public perception of a high infection rate of *Pfiesteria*-like dinoflagellates in 1997 caused a public outcry that damaged fisheries associated with the Chesapeake Bay. An estimated $43 million was lost in commercial seafood industry sales and $4.3 million was lost in recreational fishing revenue (Lipton 1998). Interestingly, Lipton (1998) further postulated that public education could have helped to limit these losses. Therefore, more needs to be known about mycobacterial infections in wild stocks of fish to prevent public overreaction. This study will provide important insight into the nature of mycobacterial infections within the Chesapeake Bay stock of striped bass, and will provide a base for further research into these troubling infections. The following chapters include investigations into the epidemiology of infections in Chesapeake striped bass, the specific mycobacteria infecting striped bass as well as other Chesapeake fishes, and development of a method of mycobacterial speciation based on gas chromatography

1.6 Tables

Table 1-1. *Mycobacterium* spp. reported to infect fish.

Species	**Reference(s)**
M. abscessus	Lansdell et al. 1993, Teska et al. 1997
M. chelonae	Lansdell et al. 1993, Bruno et al. 1998, Beran et al. 2006
M. 'chesapeaki'	Heckert et al. 2001
M. flavescens	Beran et al. 2006
M. fortuitum	Nigrelli and Vogel 1963, Lansdell et al. 1993, Beran et al. 2006
M. gordonae	Lescenko et al. 2003, Beran et al. 2006
M. interjectum-like	Rhodes et al. 2004
M. marinum	Lansdell et al. 1993, Lescenko et al. 2003
M. montefiorense	Levi et al. 2003
M. neoaurum	Backman et al. 1990,
M. poriferae	Tortoli et al. 1996
M. pseudoshottsii	Rhodes et al. 2005
M. scrofulaceum	Lansdell et al. 1993, Rhodes et al. 2004
M. shottsii	Rhodes et al. 2003
M. simiae	Lansdell et al. 1993
M. smegmatis	Talaat et al. 1999 (experimental)
M. szulgai-like	Rhodes et al. 2004

M. terrae	Beran et al. 2006
M. triplex-like	Rhodes et al. 2004
*M. ulcerans**	Portaels et al. 2001

* Identification was based on the PCR detection of IS2404, which has since also been discovered in *M. marinum*, *M. shottsii* and *M. pseudoshottsii* (Rhodes et al. 2005).

Table 1-2. Wild fish infected with mycobacteria, presented chronologically.

Species	Location	Population affected	Reference
Mountain whitefish (*Prosopium williamsoni*)	Washington State	8%	Abernethy and Lund 1978
Striped bass (*Morone saxatilis*)	California	25-68%	Sakanari et al. 1983
	Oregon	46%	
Coho salmon (*Oncorhynchus kisutsch*)	Pacific-caught juveniles	1.4%	Arakawa and Fryer 1984
Chinook salmon (*Oncorhynchus tschawytscha*)	Pacific-caught juveniles	4%	Arakawa and Fryer 1984
Mountain whitefish (*Prosopium williamsoni*)	Montana	36-49%	Peterson 1986
North-east mackerel (*Scomber scombrus*)	Portugal to North Sea	Increased with age from <10-100%	MacKenzie 1988
Yellow perch (*Perca flavescens*)	Alberta	-	Daoust et al. 1989
Rabbitfish (*Siganus rivulatus*)	Israel	21-50%	Diamant et al. 2000
Silver mullet (*Mugil curema*)	Venezuela	10% (n=20)	Perez et al. 2001

Striped bass (*Morone saxatilis*)	Chesapeake Bay	76%	Rhodes et al. 2004
Atlantic menhaden (Brevoortia tyrannus)	Chesapeake Bay	18% (n=287)	Kane et al. 2007
Blueback herring (*Alosa aestivalis*)	Chesapeake Bay	12 (n=17)	Kane et al. 2007
Summer flounder (*Paralichthyes dentatus*)	Chesapeake Bay	12% (n=26)	Kane et al. 2007
Striped killifish (*Fundulus majalis*)	Chesapeake Bay	100% (n=1)	Kane et al. 2007
Mummichog (*F. heteroclitus*)	Chesapeake Bay	33% (n=3)	Kane et al. 2007
Largemouth bass (*Micropterus salmoides*)	Chesapeake Bay	100% (n=1)	Kane et al. 2007
Weakfish (*Cynoscion regalis*)	Chesapeake Bay	50% (n=2)	Kane et al. 2007
Spot (*Leiostomus xanthurus*)	Chesapeake Bay	7% (n=27)	Kane et al. 2007
White perch *(Morone americana*)	Chesapeake Bay	20% (n=87)	Kane et al. 2007
Striped bass (*Morone saxatilis*)	Delaware Bay	7-53% (n=80)	Ottinger et al. 2007

Chapter 2: Gas Chromatographic Clustering and Multi-Locus Sequencing of Mycobacteria Isolated from Fish

2.1 Abstract

Mycobacterial species are notoriously hard to differentiate, and biochemical testing can be time consuming. Therefore, the Microbial Identification System (MIS: MIDI Inc., Newark, DE) was investigated as a faster alternative to biochemical testing for the speciation of mycobacteria isolated from fish. *16S rDNA*, *hsp* and *rpoB* of 44 mycobacterial isolates were sequenced as part of a multi-locus sequencing technique that was used to validate the differentiation of isolates included in this study. Few isolates were speciated by the database included in the MIS software. Approximately half (5/9) of the putative species specific clusters produced by the MIS software were supported by the sequencing data. Therefore, along with sequencing more isolates to determine within cluster relatedness, manipulation of the clustering guidelines is suggested to fine-tune the MIS software for routine identification of environmental mycobacteria isolated from fish.

2.2 Introduction

Traditional biochemical testing for classification of mycobacteria is both costly and time consuming, and often fails to provide adequate species-level identification. For bacterial isolates other than mycobacteria, capillary gas chromatography (GC) has proven to be a useful speciation tool (Whittaker et al. 2003). The method exploits the varying ignition temperatures of fatty acid methyl esters (FAMEs) derived from the fatty acids in

bacterial cell walls. As each species of bacteria has a unique composition of cell wall fatty acids, resulting chromatograms can be compared with chromatograms for known species, thus speciating field-derived isolates. The Sherlock® Microbial Identification System (MIS; MIDI Inc, Newark, DE) is an automated method of comparing unknown chromatograms with known chromatograms stored in a digital database.

The current MIS database, however, contains mostly fast growing mycobacteria derived from human and veterinary isolates, with a total of 31 entries for a genus believed to have over 120 species (Tortoli 2006). Therefore, a large majority of environmental mycobacteria are not in the database, specifically those infecting fish in Maryland (Brondz 2002). Our laboratory has isolated a large number of environmental mycobacteria and requires a revised method of GC/MIS to allow for altered growth regimes, including countertop incubation, and includes those mycobacterial species most commonly found infecting wild, aquacultured and aquarium fish species in Maryland.

Use of the MIS gas chromatography method for mycobacterial speciation has not been widely accepted, although successful identification of clinical mycobacterial samples have been reported using MIS with or without few additional biochemical tests (Teng et al. 1997; Ozbek and Aktas 2003). Other authors, however, state that traditional capillary gas chromatography, using preparative methods releasing fatty acid chains with less than 20 carbons (Teng et al. 1997), does not differentiate mycobacterial species sufficiently for speciation, and that a modified method of sample preparation is required to release the mycolic acid cleavage products with chain lengths of 22 to 26 carbons (Muller et al. 1998).

Molecular tools, such as sequencing the *16S rDNA* gene, have also been applied to mycobacterial speciation. Recent reports, however, indicate that genetic speciation of mycobacteria by sequencing the *16S rDNA* gene fails to provide sufficient differentiation as *16S rDNA* is a highly conserved region of the genome (Devulder et al. 2005; Kaattari et al. 2006). For example, *M. marinum* and *M. ulcerans 16S rDNA* are practically identical, with variation of only two nucleotides at the 3' end (Stinear et al. 2000). Sequence analyses of other genes, including *rpoB* and *hsp* have also proven useful, but not consistently definitive, in mycobacterial speciation (Kim et al. 1999; Ringuet et al. 1999; Kim et al. 2005; Lee et al. 2003; Cheunoy et al. 2005). Therefore, a multi-locus sequencing approach has been suggested as a more distinctive method to identify mycobacteria (Devulder et al. 2005; Kaattari et al. 2006). As such, *16S rDNA*, *hsp* and *rpoB* were sequenced in this study to: 1) identify the mycobacterial species isolated from Maryland fish and 2) investigate the ability of multi-locus sequencing to validate capillary gas chromatography as a rapid method for fish mycobacterial identification.

2.3 Materials and Methods

2.3.1 Isolates

Isolates were obtained from the collection maintained at the Fish Health Laboratory. This collection is comprised of mycobacteria isolated from fish diagnostic cases 1999-2006, as well as two ATCC isolates. Isolates were sub-cultured on Middlebrook 7H10 Agar supplemented with OADC (Difco, Detroit MI) and incubated at room temperature, approximately 22°C.

2.3.2 Capillary Gas Chromatography

Over 900 isolates from the collection were run through the gas chromatograph. Sufficient amount of growth occurred (approx. 40mg of live wet cells), between 1 week and 3 months. Isolates were harvested and processed for gas chromatography using a modified method (MIDI 2002A). Isolates were boiled for 30 minutes after vortexing with 3 mL saponification reagent (sodium hydroxide, methanol, and deionized distilled water) to lyse cells and remove fatty acids from cellular lipids. Then, 2 mL of methylation reagent (6 N hydrochloric acid and methanol) was added, the tube vortexed, and placed in an 80ºF water bath for 10 minutes to form the methyl esters of the fatty acids (FAMEs). Following, 1.25 mL of extraction solvent (hexane and methyl tert-butyl ether) was added to the tube as well as 5 drops of saturated salt solution (12.5M sodium chloride in distilled deionized water) and vortexed to transfer the FAMEs to the organic phase from the aqueous phase. The bottom aqueous phase was then removed by pipette. Finally, 3 mL of base wash (sodium hydroxide and deionized distilled water) and 10 drops (~250 µL) of salt solution were added to the remaining top layer and vortexed to perform an aqueous wash of the organic extract. As the top layer should be clear, further clearing of the top layer was occasionally required for mycobacterial isolates. In such cases, more salt solution was added, the tube vortexed and then allowed to sit for up to ten minutes before collection. The sample was then frozen and subsequently visually inspected for good phase separation, i.e. no aqueous layer remained.

Samples were run on a Hewlett-Packard 6890 Series GC System with a Hewlett-Packard 7683 Series Injector attached to a computer with proprietary Sherlock® Microbial Identification System software (MIS: version 3.80, MIDI Inc., Newark, DE).

Standards were included in each run to calibrate peaks and assure column integrity (MIDI 2002A). The MIS output includes a similarity index (SI: poor match=0, 1.000=exact match) that ranks the similarity of unknown isolate chromatograms to the MIS library definition of mean chromatograms for known species included in the MIS database. A match with a SI ≥0.500 with greater than 0.100 to the next highest SI is regarded as an acceptable library comparison (MIDI 2002A). Essentially, the higher the SI, the better the match of the unknown isolate is with a known species.

Note, reproducibility of the gas chromatography method was tested with known isolates obtained from the ATCC. Isolates were duplicate plated, incubated per the protocol, and run through the MIS-GC system. Some isolates were run in replicate from simultaneous cultures, others were chromatographed sequentially with days to weeks between runs.

2.3.3 Dendogram

A dendogram of mycobacterial isolates was produced using the MIS software. The software produced a cluster analysis with relative Euclidian Distances (EDs) in multi-dimensional space between and among isolates using an un-weighted pair matching method (MIDI proprietary method). Essentially, the dendogram of isolate EDs supported visualization of phenotypically similar clusters, based on fatty acid composition (MIDI 2002B). Clusters were considered phenotypically related if isolates were ≤10 EDs (MIDI 2002A).

2.3.4 Multi-locus Sequencing

One or two isolates were selected from each major cluster in the dendogram, except the cluster containing *M. shottsii* and *M. chesapeaki*, from which 10 isolates were selected. The larger number of isolates assayed from this cluster, compared with other dendogram clusters, was associated with our interest in further discerning these two species, and that *M. shottsii* has been implicated as the cause of a highly publicized epizootic in Chesapeake Bay striped bass (*Morone saxatilis*). The majority of isolates selected for this part of the study were from wild Chesapeake Bay species, mostly striped bass, with additional isolates from white perch (*Morone americana)* and Atlantic menhaden (*Brevoortia tyrannus),* as well as two ATCC isolates, *M. shottsii* and *M. chesapeaki*.

Mycobacterial DNA was prepared from colonies of a subset of the isolates used in the gas chromatography. Prepman Ultra Sample Preparations Reagent (Applied Biosystems, Foster City, CA) was used, following the manufacturer's protocols. Briefly, 100µL of reagent was pipetted into a 2mL screw cap tube with a loop of bacteria. The tube was vortexed for 20 seconds and put on a heat block at 100˚C for 10 minutes. The tube was then centrifuged at 13,000rpms for 3 minutes, and the supernatant was removed and used as template for PCR after a 1:10 dilution in TE Buffer.

For the initial PCR reaction, the FailSafe™ PCR System (EpiCentre Biotechnologies, Madison, WI) was used according to manufacturer's protocols. Each reaction consisted of 9.25uL deionized water, 1uL each of 2-10uM primers (Table 2), 12.5uL Premix, 0.25uL enzyme and 1uL template DNA. PCR reaction conditions were as follows: 4 min at 98˚C followed by 25 cycles of 30 sec at 98˚C, 1 min at 61˚C and 70

sec at 72˚C. The initial PCR reaction conditions for the three genes, *16S rDNA*, *hsp* and *rpoB*, were identical.

PCR products were visualized on a 1% agarose gel stained with ethidium bromide, then cleaned up with a Qiaquick PCR Purification Kit (Quiagen Inc., Valencia, CA) following manufacturer's protocols.

The sequencing reaction was performed using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Foster City, CA). 2uL of purified PCR product was added to 1uL Big Dye, 1uL of the Big Dye Buffer, and 1uL of 2uM Primer. PCR conditions were as follows: 1 min 94˚C initial denaturation step followed by 25 cycles of 30 sec 96˚C, 5 sec 50˚C and 4 min 60˚C. Both forward and reverse strands were sequenced for higher confidence in sequence fidelity.

2.3.5 BLAST Sequences

Nucleotide sequences for each gene were BLASTed against the National Center for Biotechnology Information (NCBI) nucleotide collection (nr/nt) database with BLASTn to determine similarity with known species. Similarity was identified based on high query coverage (≥97%) and maximum identity percent.

2.3.6 Neighbor Joining Trees

Neighbor joining trees for the isolates were created for each gene using MEGA 3.1 (Kumar et al. 2004). The three gene sequences were also concatenated together, to create a supergene, from which a neighbor joining tree was also created. The arbitrary order of the genes within the supergene was *hsp*, *rpoB* followed by *16S rDNA*.

2.3.7 Validation of GC by MLST

The locations of each isolate in relation to other isolates were compared among neighbor joining trees for the three genes, for the supergene tree, and the gas chromatography dendogram.

2.4 Results

2.4.1 Capillary Gas Chromatography

Of the 44 isolates, 18 did not have matches with known species. Of the remaining 26 isolates with matches, only 1 had a similarity index >0.500. Isolate SIs are presented in Table 2-2.

2.4.2 Reproducibility

Results from the isolates run in tandem indicated good reproducibility of chromatograms. 92% (12/13) of ATCC isolates provided excellent matches with their respective duplicate. i.e. ≤ 5ED (data not shown). The one outlier duplicate set (clustered at 10ED) was duplicate plated a second time, and produced excellent matches both with themselves but also with one of the previous runs (data not shown).

2.4.3 Dendogram

The dendogram of the isolates used for genetic characterization (Figure 2-1) was based on the relationships found when over 900 isolates from Maryland fish were used, but was abbreviated for space considerations. According to MIDI (2002A) for non-mycobacterial isolates, isolates that are approximately 10 ED from each other are considered the same species. The current study still used ≤10 ED in spite of the relaxed growth parameters. The clusters which warranted the 10 ED nomenclature were isolates 3 and 6, isolates 9 and 10, 30 and 31, 7 and 14, 20 and 24, 40 and 41, 11 and 26, 1 and 19, and the cluster of 44 through 36. Therefore all other isolates were considered separate species, based on gas chromatographic FAME analyses. Since gas chromatography is based on phenotype, unlike genetic relationship trees, assumptions about phylogeny or genetic relationships between isolates cannot be made further than the 10 ED distance.

2.4.4 Multi-locus Sequencing

Portions of *16S rDNA* (810bp), *hsp* (840bp) and *rpoB* (266bp) were sequenced for 44 isolates (data not shown). Successful sequencing, however, did not occur for *16S rDNA* from isolate 5, *hsp* from isolates 10 or 17, nor *rpoB* from isolate 5, even after repeated attempts. This missing data prevented supergene construction for these three isolates.

2.4.5 BLAST of *16S rDNA*, *hsp* and *rpoB*

BLAST search results were sufficient to suggest species identification for some isolates, but not in others (Table 2-3). For example, sequencing data suggested that isolate 4 was *M. pseudoshottsii*, isolate 13 was *M. marinum*, and isolate 27 was *M. interjectum*. Additionally, isolates 21, 22, 25, 29, 32, 33, 34, 35, 36, 43 and 44 were *M. shottsii*. Interestingly, while all these *M. shottsii* isolates were identical in *rpoB*, they varied in their *16S rDNA* sequences, as evidenced by those matches ranging from 99-100% with M175, the type isolate of *M. shottsii*. It is interesting to note that several isolates had multiple 99% matches in the 16S rDNA, which is most likely the most sequenced gene of the three, but also the most conserved.

2.4.6 Neighbor Joining Trees

Genetic relatedness between the isolates for the three genes and the supergene are presented as neighbor joining trees (Figures 2-5). In most instances, isolates clustered similarly in the three individual gene trees. For example, isolates 1 and 19 were always in a group together, as were 26, 40 and 41 as well as 31 and 42. One notable exception is the case of isolates 27, 28 and 37, where 28 and 37 grouped together consistently, however 27 grouped with 28 and 37 in *16S rDNA*, but not in *hsp* or *rpoB*. The former relationships were conserved in the supergene, as 1 and 19 grouped together, 26, 40 and 41 grouped together, and 31 and 42 grouped together. Isolates 21, 22, 25, 29, 32, 33, 34, 35, 36, 43, and 44 consistently grouped together, and additionally with isolates 4 and 13.

2.4.7 Validation

Genetic evidence, based on neighbor joining trees, including the supergene tree, supported the clustering of 5 out of the 9 clusters identified as related within 10ED by GC FAME analyses (Table 2-4). Isolates 3 and 6 clustered at 10 ED based on gas chromatography, and grouped together in neighbor joining trees with all three genes and the supergene. However, the genetic differences in *hsp* and *rpoB* were large (Figures 3 and 4), which indicated GC was not good at differentiation between these two related isolates. Isolates 40 and 41 grouped at 10 ED, and genetic evidence supported this grouping as they clustered in all three genes. Interestingly, isolate 26 also grouped with 40 and 41 in the genetic analysis yet was 17ED away in the dendogram, which indicated closely related isolates can have considerably different fatty acid cell wall make-ups (Figure 2-1). Of the remaining 4 GC clusters not supported by genetic analysis, one (isolates 9and 10) included missing data, and the remaining three were clustered by GC right at the cut-off of 10ED (Table 2-4).

2.4.8 The contentious *M. shottsii* versus 'M. chesapeakii' debate

Isolate 33 (the type isolate for *M. chesapeakii*) was genetically identical to the ATCC type isolate *M. shottsii* (isolate 21) in both *16S rDNA* and *rpoB*. Isolate 33 differed from *M. shottsii* in *hsp* by 3 base pair mutations and 2 deletions (data not shown). Interestingly, isolate 33 was also genetically identical to isolate 25 (the type isolate of *M. chesapeakii* after it had been passaged through a striped bass) in both *16S* rDNA and *rpoB*, but differed in *hsp* by 5 base pair mutations. Therefore, the type isolates

of *M. chesapeakii* (33 and 25) were identical to the type isolate of *M. shottsii* (isolate 21) in *16S rDNA* and *rpoB*. All three differed in *hsp*.

2.5 Discussion

The purpose of the current study was to speciate the mycobacteria isolated from Maryland fish, to study the GC method of speciation currently used in our lab, and to compare phenotypic GC results with genotypic multi-locus sequencing.

In some cases the MIDI database appeared sufficient to speciate mycobacteria in the current study based on GC FAME analyses. For example, isolate 3 had a SI of 0.305 with *M. gordonae* (Table 2-1). The sequencing data supported this speciation as the *16S rDNA* had a 99% similarity and *rpoB* a 100% similarity with NCBI sequences for *M. gordonae* (Table 3). However the current MIDI database needs to be expanded in order to encompass the isolates found in Maryland fish as evidenced by the number of 'no matches' in the ID by GC column in the isolates selected for sequencing (Table 2-1). One potential reason for this lack of identification by MIS-GC was a discrepancy in the incubation parameters for the isolates. The existing MIS database uses 30-35˚C incubation in 5-10%CO_2 until adequate growth occurs (MIDI 2002A). Countertop incubation (~22˚C) was used in the current study as it is a more environmentally relevant temperature for fish pathogens, and it was consistently used, so results would be comparable with all isolates from our laboratory (Buller 2004). Isolation plates in the current study were also wrapped in parafilm, not incubated with CO_2. A second potential reason for the inadequacy of MIS-GC to identify fish isolates was that they were not included in the MIS database. Efforts to increase the usefulness of MIS-GC for our

laboratory purposes include creating species library definitions within the MIDI software to account for our laboratory's altered growing conditions and to include isolates our lab routinely finds. A library definition is created by averaging 15-20 chromatograms to account for the variability seen within the same species (J. Abell, personal communication). Therefore, isolate groups from Maryland fish can be tracked as the definitions will be included in the GC output with SI indices afforded to definitions already included in the software. Additionally, this tracking system inherent to the GC method of speciation will quickly alert the researcher if new mycobacteria are found in fish, thus assisting in the identification of potentially new and emerging pathogens. The potential of this latter point is immense, as a probe technique of speciation can either miss emerging pathogens or incorrectly identify isolates as known species.

Previously, gas chromatography successfully speciated common clinical mycobacterial isolates (Mosca et al. 2007). The authors were able to differentiate among *M. tuberculosis*, *M. gordonae*,*M. xenopi*, *M. kansasii*, *M. fortuitum*, and *M. avium-intracellular* as validated by biochemical testing. Additionally, Teng et al. (1997) used gas chromatography with enzyme profiles, namely API-ZYMs, to identify clinical and ATCC isolates. This method could be useful with some of our isolates, as the alkaline phosphatase microtubule in the API-ZYM could differentiate between *M. fortuitum* and *M. chelonae*, two species of clinical interest in the fish world. Although our laboratory has not investigated API-ZYMs with our isolates, this report highlights to potential of using two methods for clearer species designations. For example, FAME analyses placed isolates 11 and 26 in the same cluster, however genetic analyses identified them most likely as different species (Table 2-4). Colony morphology varied between these isolates:

11 was a relatively faster growing dry isolate whereas 26 was a relatively slower growing creamy isolate (Table 2-1). Therefore, a combination of the FAME analysis and general growth characteristics are potentially sufficient to differentiate these two species.

The MIS-GC method, as it stands now, is not sufficient for mycobacterial speciation, however, shows immense promise with further modifications. It is important to note that the 10ED cut-off was an initial attempt at species designation. Perhaps using fewer EDs in certain cases would be advisable, such as in the case of isolates 3 and 6 where they were 5 ED apart, yet genetic data suggest they were separate species (Table 2-4). Comparably, 9.9ED was sufficient for *M. shottsii* as genetic evidence indicated all isolates included in that group matched either 99% or 100% with *M. shottsii* in *16S rDNA* and *rpoB*. Clearly more research is required to elucidate these relationships. Suggested avenues include dropping the cut-off ED value lower and sequencing more isolates to determine the genetic relatedness within clusters, not only among clusters. Further complicating the picture, it is important to note that isolates related at a distance farther than 10ED did not automatically imply the isolates were separate species (MIDI 2002B). Therefore, separate dendogram clusters could in fact be the same species. Finally, while MIDI has since come out with the Sherlock® Mycobacteria Identification System, it employs a different technology, high performance liquid chromatography, and the database still only includes 40 mycobacteria (MIDI website 2007).

The multi-locus sequencing technique was used in this study to name the isolates clustered in the dendogram. However as the project proceeded, it became clear that the MLST would fall short of its intended purpose. MLST was able to speciate some isolates in the current study, e.g. isolate 3 was *M. gordonae* and isolate 4 was *M. pseudoshottsii*,

however it failed to assign species names to the majority of the isolates (Table 2-3). The potential exists for several of these isolates to represent novel species, not just species whose genes have not been submitted to NCBI. Therefore, further efforts on these isolates should include biochemical testing, and DNA-DNA hybridization, which along with the genes already sequenced, will allow for the designation of putative new species (Devulder et al. 2005). All isolate sequences will be submitted to the NCBI database to assist the scientific community's further inquest into mycobacteria.

It is important to note that the MLST was also initially intended to validate MIS-GC. However, MIS-GC is a phenotypic method of identification and is only suggested to discriminate species, whereas MLST is a genotypic method and can investigate taxonomic relationships. As such, MLST can only validate GC within the confines of species designations. As stated above, it appears as though the 10ED cut-off value used in the current study was not sufficient for species designations in all cases, and further research is needed to elucidate the relationships proposed by MIS-GC clustering. Additionally, while not included in this study, the taxonomic relationships revealed by MLST will be investigated further, including the potential existence of recombination, in a separate study.

The three genes sequenced in this study were chosen for specific reasons. All three genes have been sequenced previously in mycobacteria and so would allow comparison with previous findings (Devulder et al. 2005; Kaattari et al.2006). 16S rDNA is a highly conserved region of the genome, and has been highlighted as the gold standard for differentiating bacterial isolates (Devulder et al. 2005). According to the *16S rDNA* neighbor joining tree in the current study, isolates 27, 28 and 37 were very similar, and

may even be considered the same species (Figure 2-2). However, *hsp* and *rpoB* neighbor joining trees placed isolate 27 far away from isolates 28 and 37, indicating isolate 27 was a separate species from 28 and 37 (Figures 2-3 and 2-4). These data support the growing evidence that *16S rDNA* is not the most discriminatory gene for speciation (Devulder et al. 2005: Kaattari et al. 2006*)*. *hsp* has also been used to differentiate non-tubercular mycobacteria because it contains divergent sequences (Kim et al. 2005). *hsp* appeared to be the most divergent of the genes sequenced in the current study (Figure 2-3). *rpoB* contains polymorphic regions used previously to differentiate non-tubercular mycobacteria (Kim et al. 1999; Lee et al. 2003). Additionally, the region of *rpoB* chosen for this study contains the region believed to be related to rifampicin resistance in *M. tuberculosis* (Kim et al. 1999). Therefore, further studies can investigate genotypically the resistance of environmental fish isolates to rifampicin.

DNA sequencing of three genes indicated that the type isolate of *M. chesapeakii* (isolate 33) was most likely *M. shottsii*. The sequences were identical in two genes, but differed in *hsp*. However, there are biochemical differences which indicate *M. chesapeakii* may be a subspecies of *M. shottsii* (Rhodes et al. 2003). Although *M. chesapeakii* has historical precedent in the literature, *M. shottsii* was the name used first in the *International Journal of Systemic and Evolutionary Microbiology*, the authority in naming microorganisms (VanDamme 2003).

Isolate 33 is the type isolate of *M. chesapeakii* (*M. shottsii*) which has been sub-cultured repeatedly since 1997 on Middlebrook 7H10 agar. This isolate was passaged through 100g striped bass and re-isolated from the spleen. Isolate 25 was this new isolate. These two isolates were identical in *16S* rDNA and *rpoB*, however differed by 5

bp in *hsp*, a molecular chaperone that assists protein folding in times of stress (Feder 1999). These data indicate a mutation may have occurred, which may have been caused by passage through the fish. This hypothesis requires further investigation, however it is reasonable to assume that the environmental pressures put on the microorganism are different when growing on an agar plate, a stable environment, versus in a fish, a dynamic environment. It is interesting to note that these isolates failed to cluster together in the GC dendogram, but after one subculture on a Middlebrook agar plate, the fish pass isolate (isolate 25) reverted back to clustering with isolate 33 (data not shown). Further investigation is required, however these data are suggestive of a mutation in the genetic code that responds to environmental conditions, and may be associated with a phenotypic change in the fatty acid composition of the cell wall. Interestingly, mycobacteria isolated from striped bass from the Delaware Bay, DE, USA, have been biochemically characterized as *M. shottsii*-like, however share similarity with *M. chesapeakii* in two tests used to differentiate between *M. shottsii* and M. *chesapeaki*, namely optimum growth at 28-30°C and niacin negative (Heckert et al. 2001; Rhodes et al. 2003; Ottinger et al. 2007). These results, combined with data from the current study, indicate this cluster or species of mycobacteria may reveal high plasticity both phenotypically and genetically as a result of environmental conditions.

The group of *M. marinum*, *M. shottsii* and *M. pseudoshottsii* were clustered closely together in the neighbor-joining trees and are all considered members of the *M. tuberculosis* clade (Kaattari et al. 2006). The BLAST searches indicated their close relationship as 16S sequences were 99% homologous in the region studied. However, closely related mycobacterial species may differ by only several base pairs, if any, in the

16S rDNA and it has been suggested that clear phenotypic differences even with identical sequences is sufficient to identify separate species (Tortoli 2003). Therefore the 5-15 bp difference suggested for other genera does not hold for mycobacterial species (Tortoli 2003). Conversely, some well defined species vary in 16S sequences by up to 7 bp (Tortoli 2003). Therefore, the homology seen among *M. marinum*, *M. shottsii* and *M. pseudoshottsii* in the 16S rDNA is not surprising and more loci are required to differentiate among these species. Further, a plasmid encoding mycolactone, a toxin, was originally thought to only exist in *M. ulcerans*, however recent evidence indicates it is more globally distributed (Ranger et al 2006). Strains of *M. pseudoshottsii* from the Chesapeake Bay and *M. marinum* isolated from fish in the Red and Mediterranean Seas possess a variant of mycolactone, however *M. marinum* isolates from humans in the same geographic region lack mycolactone (Ranger et al. 2006). Therefore, it has been suggested that mycolactone producing mycobacteria arose from a single species, and using subspecies instead of species nomenclature to differentiate strains would be more appropriate (Yip et al. 2007).

This study investigated the ability of two methods, MIS-GC and a multi-locus sequencing technique (MLST), to speciate mycobacteria isolated from fish in Maryland. Neither method was able to assign species names to all isolates, nor for that matter, the majority of isolates. The incubation parameters used in this study did not allow for a defensible comparison with the MIS database, nor did the database appear to have the library definitions required to name the isolates encountered in this study. The downfall of MLST appeared to be the incompleteness of the NCBI database for these isolates, whether because the isolates are new species or these isolates represent existing species

without sequencing data coverage for the genome regions used in the current study. In spite of this lack of species naming, with further inquest, both methods can provide useful contributions. MIS-GC currently under development in our laboratory is aimed at creating library definitions which will allow the tracking of clinical isolates, whether a species name is assigned or not. Additionally, MLST sequences can be used to look at the genotypic relatedness of isolates encountered in this study, and will help elucidate relationships created by GC dendograms.

2.6 Acknowledgements

I would like to thank Mamuka Kotetishvili for his guidance and supervision throughout the design and execution of the molecular aspects of this project. I would also like to thank Ana Baya for guidance and enlightening conversation in the realm of gas chromatography. Additionally, I would like to ackowledge the assiatance of Lisa Sadzewicz for visually assembling and inspecting genetic sequences. This research was supported by grants from the Centers for Disease Control, the National Oceanic and Atmospheric Administration, and the Maryland Agriculture Experiment Station.

2.7 Tables and Figures

Table 2-1. Primers used for amplification of *16S*, *hsp*, and *rpoB* target regions in multi-locus sequencing of mycobacteria isolated from fish.

Primer name	Sequence	Reference
myco16S-1	5' - ACA CAT GCA AGT CGA ACG GAA AGG - 3'	de novo
myco16S-2	5' - TGC GGG ACT TAA CCC AAC ATC TCA - 3'	de novo
Mycohsp-1	5' - GAT CCG GAG GAA TCA CTT CGC AAT - 3'	de novo
Mycohsp-2	5' - TCG TCC TTG GTG ATG ACG ACC TT - 3'	de novo
mycorpoB-1 MF	5' - CGA CCA CTT CGG CAA CCG - 3'	Kim et al. 1999
mycorpoB-2 MR	5' - TCG ATC GGG CAC ATC CGG- 3'	Kim et al. 1999

Table 2-2. Mycobacterial isolates selected from gas chromatography dendogram for multi-locus sequencing. DTC= days to collection, which occurred when there was enough growth to collect ~40mg of live wet weight cells. Note that while this is not directly comparable to growth time as described in species descriptions, it does give a relative measure of how quickly isolates grow. W=wild, C=aquacultured, and LA=wild caught but lab acclimated.

Isolate	SIs from Gas Chromatography	DTC	Morphology	W/C	Host Species
1	0.352 *M. nonchromogencium*	30	pink creamy	C	tilapia
2	No match	12	orange creamy	W	striped bass
3	0.305 *M. gordonae*	30	orange yellow creamy	W	striped bass
4	0.409 *M. marinum*	33	yellow dry	W	striped bass
5	0.210 *M. chelonae*	6	white creamy	W	striped bass
6	0.136 *M. gordonae*	41	orange yellow, light halo	W	menhaden
7	0.444 *M. gordonae*	30	orange creamy	W	striped bass
8	0.146 *M. fortuitum*	74	white dry	W	mummichog

9					
	0.226 *M. smegmatis*	12	white dry	W	striped bass
10					
	0.060 *M. smegmatis*	34	white dry	C	Tilapia
11					
	No match	26	orange yellow dry	W	Menhaden
12					
	No match	90	yellow opaque creamy	W	striped bass
13					
	0.174 *M. marinum*	30	yellow creamy	LA	Menhaden
14					
	0.094 *M. gordonae*	30	orange creamy	C	hybrid striped bass
15					
	0.130 *M. scrofulaceum*	26	pale yellow creamy	W	white perch
16					
	0.091 *M. scrofulaceum*	11	orange watery	W	Menhaden
17					
	No match	34	bright yellow dry	W	Menhaden
18					
	No match	11	orangish yellow moist	W	striped bass
19					
	0.531 *M. nonchromogencium*	21	white creamy	C	Tilapia
20					
	0.091 *M. scrofulaceum*	14	orange creamy glistening	W	striped bass
21					
	0.141 *M. marinum*	55	white dry	-	ATCC 700981, *M. shottsii*
22					
	No match	47	white dry	W	striped bass
23					
	No match	40	yellow creamy	W	striped bass

24	0.062 *M. scrofulaceum*	67	yellow creamy	W	striped bass
25	No match	120	white dry	C	striped bass
26	0.105 *M. scrofulaceum*	61	yellow creamy	W	striped bass
27	0.153 *M. chelonae*	74	yellow translucent creamy	W	Alewife
28	No match	35	yellow creamy	W	striped bass
29	No match	64	white dry	W	striped bass
30	0.254 *M. genavense*	76	White creamy	W	striped bass
31	0.087 *M. smegmatis*	90	white creamy	W	white perch
32	No match	74	white very dry	W	striped bass
33	0.108 *M. marinum*	54	white dry	-	ATCC BAA-422, *M. chesapeakii*
34	0.099 *M. marinum*	64	white dry	W	striped bass
35	No match	70	white dry	W	striped bass
36	No match	65	white dry	W	striped bass
37	No match	58	yellow creamy	W	striped bass
38	No match	77	yellow creamy	W	striped bass

39	No match	34	yellow orange	W	Menhaden
40	0.165 *M. scrofulaceum*	68	white creamy	W	striped bass
41	0.116 *M. scrofulaceum*	96	orange creamy	W	striped bass
42	0.157 *M. smegmatis*	35	white translucent creamy	W	striped bass
43	No match	74	white dry	W	striped bass
44	No match	64	white dry	W	striped bass

Table 2-3. Basic Local Alignment Search Tool (BLAST) results against the National Center for Biotechnology Information (NCBI) database for *Mycobacterium* spp. isolated from fish, with maximum identity percent given. Only the matches with the highest identity percent were included, except when the match was with an unknown species (i.e. isolates 8, 11, 12 and 17). M175 is the type isolate for *M. shottsii* (Rhodes et al. 2003). All results are *Mycobacterium* spp. Coverage of query was no less than 97% in all cases.

Isolate	16S	hsp	rpoB
1	arupense 99% nonchromogencium 99%	gilvum 93%	arupense 99%
2	ratisbonense 98% phocaicum 98% farcinogenes 98% senegalense 98% mucogenicum 98% fortuitum 98% conceptionae 98% aubagnense 98% houstonense 98%	gilvum 94% mucogenicum 94% vanbaalenii 94%	chlorophenolicum 98%
3	gordonae 99%	marinum 94%	gordonae 100%
4	marinum 99% pseudoshottsii 99% ulcerans 99% liflandii 99% M175 99% asiaticum 99%	marinum 99%	pseudoshottsii 100%
5	x	185-409 (from fish) 98%	x
6	gordonae 98% asiaticum 98%	marinum 94% ulcerans 94%	gordonae 94% asiaticum 94%
7	gordonae 99%	marinum 95% ulcerans 95%	gordonae 95%
8	septicum 99% peregrinum 99% fortuitum 99%	smegmatis 96%	Nepal-2 100% farcinogenes 97% senegalense97%

9	fortuitum 99%	smegmatis 96%	fortuitum 97%
10	brisbanense 97% fallax 97% rhodesiae 97% tokaience 97%	x	porcinum 93% vanbaalenii 93% fortuitum 93%
11	moriokaense 98% barrassii 98% pulveris 98% elephantis 98% phlei 98% goodii 98%	smegmatis 94%	czh-42 (degrade PAH) 97% aichiense 97%
12	intracellulare 99% columbiae 99% chimaera 99% avium 99%	marinum 94% avium 94%	03-19 seoulense 95% interjectum 93% conspicuum 93%
13	ulcerans 99% marinum 99% M175 99% liflandii 99%	marinum 99% ulcerans 99	marinum 100%
14	saskatchewanense 98% interjectum 98%	avium 94%	asiaticum 93%
15	columbiae 99% intracellulare 99% chimaera 99% avium 99%	avium 97% paratuberculosis 97%	chimaera 95% intracellulare 95%
16	poriferae 99%	graecum DL049' 97%	phlei 98% vanbaalenii 98% poriferae 98% austroafricanum 98%
17	moriokaense 98% barrassii 98% elephantis 98% phlei 98%	x	JS19b1 (degrades PAH) 98% rhodesiae 97% aichiense 97%
18	frederiksbergense 99% sacrum 99%	smegmatis 93% phocaicum 93% mucogenicum 93%	peregrinum 97% frederiksbergense 97%
19	arupense 99% nonchromogenicum 99%	gilvum 92% phocaicum 92% mucogenicum 92%	arupense 99%
20	aurum 98%	gilvum 95%	aurum 100%

21	M175 100%	marinum 99% ulcerans 99%	shottsii 100%
22	M175 99% ulcerans 99% marinum 99% liflandii 99% pseudoshottsii 99% asiaticum 99%	marinum 98% ulcerans 98%	shottsii 100%
23	peregrinum 98% tokaiense 98% murale 98%	vanbaalenii 93%	barrassii 95%
24	parascrofulaceum 100%	avium 94% marinum 94% paratuberculosis 94%	parascrofulaceum 96% scrofulaceum 96%
25	M175 100%	marinum 99% ulcerans 99%	shottsii 100%
26	simiae 99% lentiflavum 99% palustre 99%	marinum 94% ulcerans 94% avium 94% paratuberculosis 94%	03-19 seoulense 95% saskatchewanense 95% intermedium 95%
27	interjectum 100%	intracellulare 95% avium 95% chimaera 95%	interjectum 99%
28	interjectum 99% intermedium 99% saskatchewanense 99%	marinum 95% ulcerans 95%	parmense 94%
29	M175 100%	marinum 99% ulcerans 99%	shottsii 100%
30	montefiorense 99% florentinum 99% triplex 99% sherrisii 99% kubicae 99%	marinum 95% ulcerans 95%	lentiflavum 96% triplex 96%
31	montefiorense 99% sherrisii 99% florentinum 99% triplex 99% heidelbergense 99% simiae 99%	avium 95% paratuberculosis 95% marinum 95% ulcerans 95%	montefiorense 97%
32	M175 99% ulcerans 99% marinum 99%	marinum 98%	shottsii 100%

	liflandii 99%		
33	M175 100%	marinum 98% ulcerans 98%	shottsii 100%
34	M175 99% ulcerans 99% marinum 99% liflandii 99% pseudoshottsii 99% asiaticum 99%	marinum 99% ulcerans 99%	shottsii 100%
35	M175 99% ulcerans 99% marinum 99% liflandii 99% pseudoshottsii 99% asiaticum 99% lacus 99%	marinum 99% ulcerans 99%	shottsii 100%
36	M175 99% ulcerans 99% marinum 99% liflandii 99% pseudoshottsii 99% asiaticum 99% lacus 99%	marinum 99% ulcerans 99%	shottsii 100%
37	interjectum 99% intermedium 99% saskatchewanense 99%	marinum 94% ulcerans 94% avium 94%	parmense 94%
38	conspicuum 99%	marinum 95%	conspicuum 97%
39	moriokaense 98% barrassii 98% elephantis 98% pulveris 98$ phlei 98% goodii 98% smegmatis 98% flavescens 98% marinum 98%	smegmatis 94%	JS19b1 (degrades PAH) 97% aichiense 97% rhodesiae 97% sphagni 97%
40	simiae 99% lentiflavum 99% palustre 99% heidelbergense 99% parascrofulaceum 99% parmense 99% sherrisii 99% kubicae 99% montefiorense 99%	marinum 95% ulcerans 95% intracellulare 95%	03-19 seoulense 94%

41	simiae 99% lentiflavum 99% palustre 99% heidelbergense 99% parascrofulaceum 99%	marinum 95%	03-19 seoulense 95%
42	montefiorense 99% sherrisii 99% florentinum 99% triplex 99% heidelbergense 99% simiae 99% parmense 99% kubicae 99% tilburgii 99%	paratuberculosis 95% avium 95% marinum 95%	montefiorense 96% lentiflavum 96%
43	M175 100%	marinum 99% ulcerans 99%	shottsii 100%
44	M175 99% ulcerans 99% marinum 99% liflandii 99% pseudoshottsii 99% asiaticum 99%	marinum 99% ulcerans 99%	shottsii 100%

Table 2-4. Mycobacteria isolates clustered by GC (within 10ED). GDA= Growth days apart, i.e. number of days separating days til collection (DTC) of isolates from Table 2-1. Note that GDA is based on DTC, not when growth first appeared. Gene columns note whether the isolates clustered genetically. However grouping genetically did not necessarily imply same species as this table does not show how closely the isolates are grouped.

Clusters	**GC ED**	**GDA**	**Similar colony morphology*?***	***16S rDNA***	***hsp***	***rpoB***	**Supergene**
3, 6	5	11	Yes	Yes, with 7	Yes	Yes	Yes
9, 10	9	22	Yes	No	Missing data	No	Missing data
30, 31	10	14	Yes	Yes, with 42	No	Yes, with 42	Yes, with 42
7, 14	10	0	Yes	No	No	No	No
20, 24	10	53	orange vs. yellow	No	No	No	No

40, 41	10	28	white vs. orange	Yes, with 26	Yes, with 26	Yes, with 26	Yes, with 26
11, 26	10	34	dry vs. creamy	No	No	No	No
1, 19	10	9	pink vs. white	Yes	Yes	Yes	Yes
44, 29, 21, 33, 34, 43, 35, 32, 22, 36	9.9	27	Yes	Yes	Yes, with 13	Yes	Yes, with 13

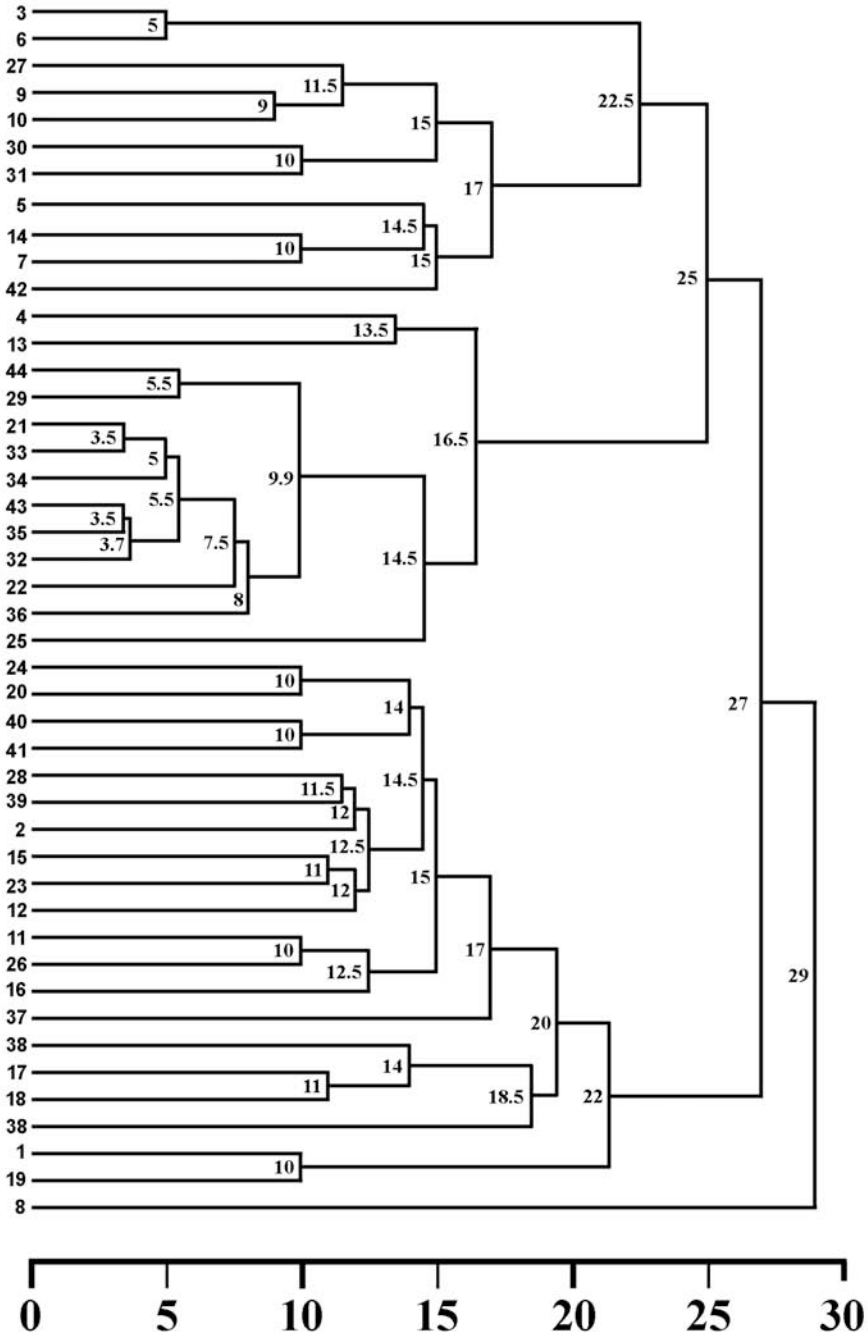

Figure 2-1. Abbreviated dendogram of phenotypic relatedness of mycobacteria isolated from fish. Isolate numbers are on the y-axis. Numbers at intersections are distance from zero. Scale along x-axis is in Euclidean Distance (ED).

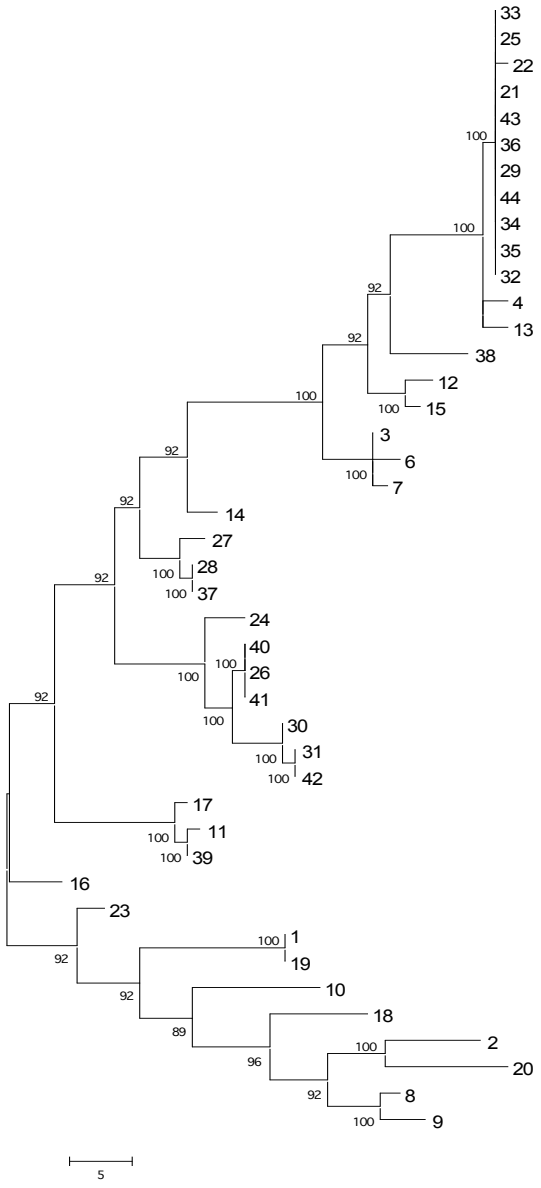

Figure 2-2. Consensus maximum parsimony tree for *16S rDNA* of mycobacteria isolated from fish. Bootstrap proportions for 100 iterations are indicated at nodes. Bar represents maximum parsimony changes.

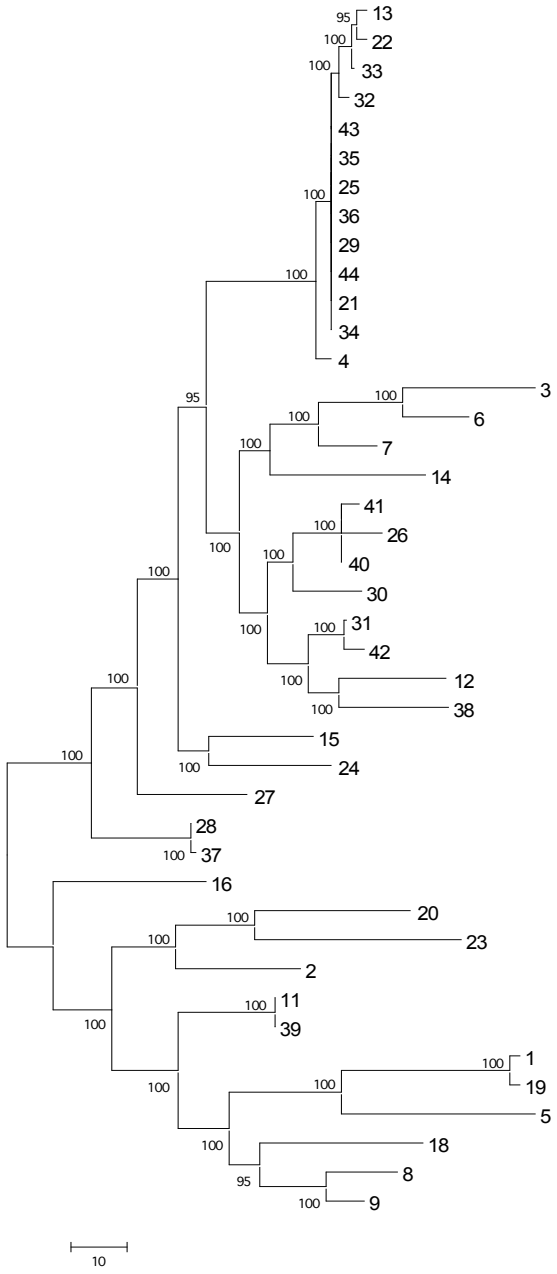

Figure 2-3. Consensus maximum parsimony tree for *hsp* of mycobacteria isolated from fish. Bootstrap proportions for 100 iterations are indicated at nodes. Bar represents maximum parsimony changes.

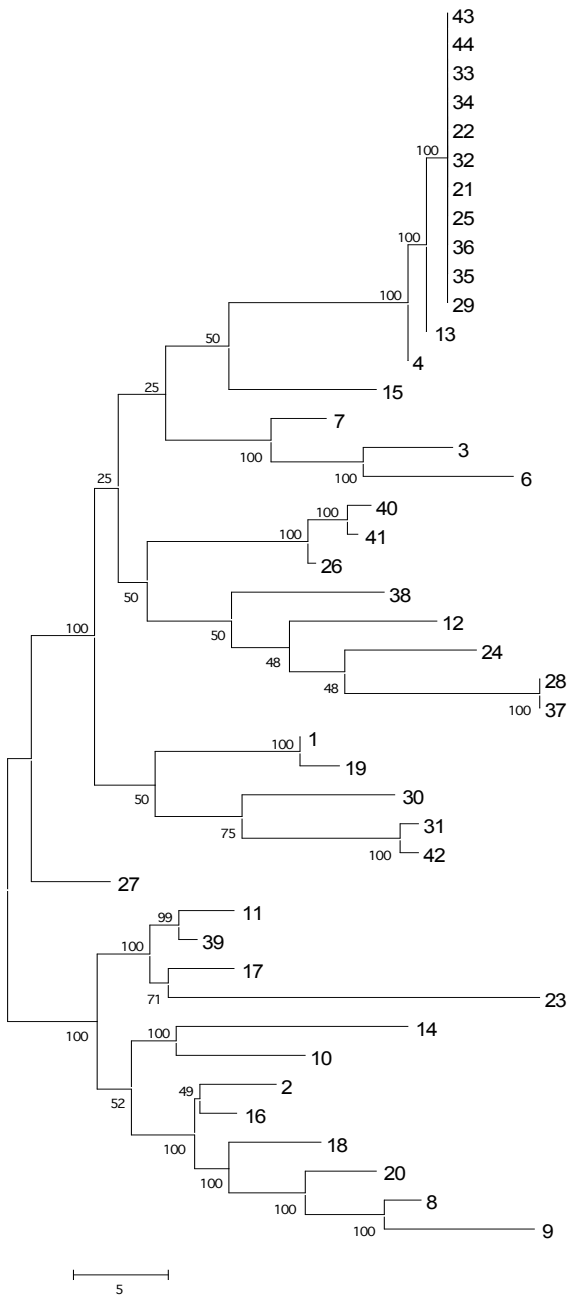

Figure 2-4. Consensus maximum parsimony tree for *rpoB* of mycobacteria isolated from fish. Bootstrap proportions for 100 iterations are indicated at nodes. Bar represents maximum parsimony changes.

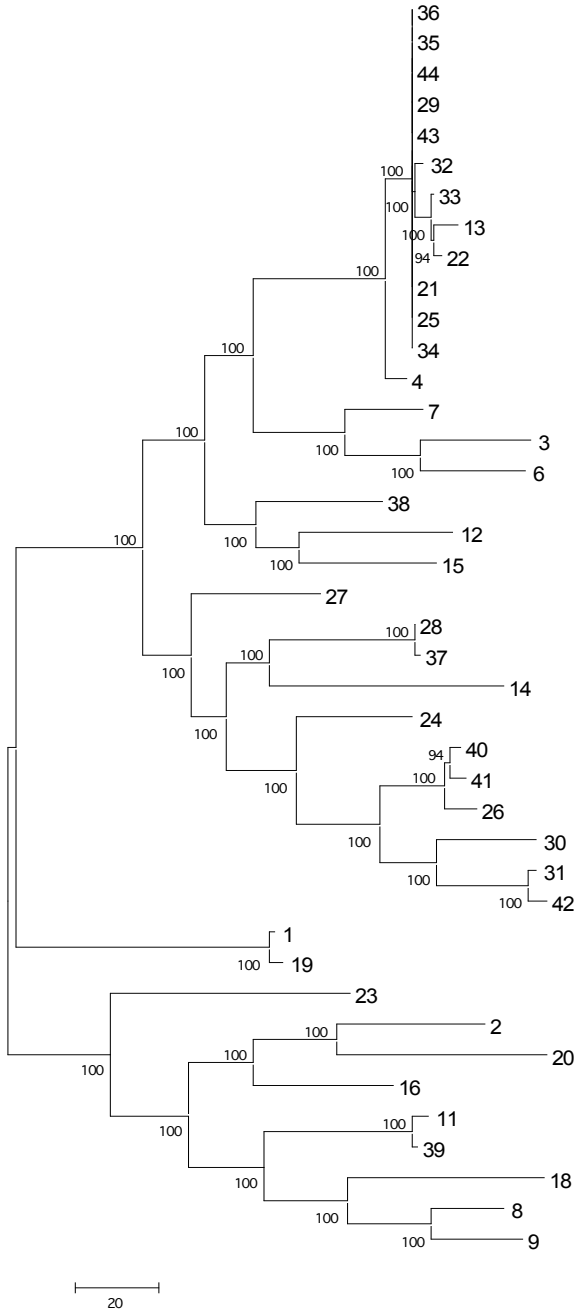

Figure 2-5. Consensus maximum parsimony tree for supergene of mycobacteria isolated from fish. Bootstrap proportions for 100 iterations are indicated at nodes. Bar represents maximum parsimony changes.

Chapter 3: Mycobacteria Isolated from Chesapeake Bay Fishes

3.1 Abstract

Mycobacteria were isolated from wild fishes of the Chesapeake Bay collected from the Upper Bay, the Choptank River, the Middle Bay (Maryland portion), the Chicamicomico River, the Pocomoke River and the Potomac River in 2003-2005. Mycobacterial isolates were recovered from striped bass (*Morone saxatilis*), Atlantic menhaden (*Brevoortia tyrannus*), white perch (*Morone americana*), summer flounder (*Paralichthys dentatus*), spot (*Leiostomus xanthurus*), largemouth bass (*Micropterus salmoides*), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio carpio*), spotted seatrout (*Cynoscion nebulosus*), killifish (*Fundulus* sp.), blueback herring (*Alosa aestivalis*), American gizzard shad (*Dorosoma cepedianum*), and American silver perch (*Bairdiella chrysoura*). Twenty-nine well-defined groups resulted from gas chromatography dendogram clustering of isolates. The majority of groups included more than one host species and more than one site of collection. However, 4 groups contained only striped bass isolates, three of which were similar to *M. shottsii*. Therefore, multiple Chesapeake Bay fish species are infected with multiple mycobacterial isolates, of which few appear to be host or location specific.

3.2 Introduction

Mycobacteria are ubiquitous, insidious, and thought to potentially infect all fish species. Until recently, however, most reports of mycobacterial disease in fish have been from aquarium and cultured stocks. Recent evidence indicates mycobacteria are found in wild fish as well as captive stocks, and more than six mycobacterial species are causative agents (Chinabut 1999, Levi et al. 2003, Rhodes et al. 2004). A recent epizootic of mycobacteriosis in Chesapeake Bay striped bass has received much attention because of ulcerated fish (Heckert et al. 2001, Rhodes et al. 2001). Subsequent inquiry has resulted in the discovery and description of two new species, *Mycobacterium shottsii* and *M. pseudoshottsii* (Rhodes et al. 2003; Rhodes et al. 2005). Efforts are currently underway to determine the population level effects of mycobacteriosis on striped bass stocks in the Chesapeake Bay (Vogelbein et al. 2006).

Mycobacterial infections have also been observed in Chesapeake fish other than striped bass (Stine et al. 2005; Kane et al. 2007). In order to further explore the extent of mycobacterial infections in Chesapeake Bay fishes, this study investigated the potential of mycobacteria as a group to infect multiple fish species in the Bay, and to determine if individual mycobacteria species had more than one piscine host species. Note, the isolates included in this study are only from wild Chesapeake Bay fish from 2003-2006 while the MLST Chapter 2 in this dissertation includes all isolates recovered from fish submitted to the Fish Diagnostics Laboratory, Maryland Department of Agriculture, College Park, MD from 1999-2005.

3.3 Methods

3.3.1 Isolates

Chesapeake Bay fishes were collected by hook and line, seine net, cast net and bank trap from the Upper Bay, the Choptank River, the Middle Bay, the Chicamacomico River, the Pocomoke River and the Potomac River. Internal organs were aseptically collected. Approximately 50 mg of each organ was stomached with 2 mL Butterfield phosphate buffered solution and 200 uL were plated on Middlebrook agar supplemented with OADC (Difco, Detroit, MI). Plates were incubated at room temperature, in order to more realistically mimic the environment of mycobacteria in wild fish (Buller 2004). Plates were checked every week for 12 weeks. Acid-fast colonies were sub-cultured on Middlebrook agar and, when sufficient amount of growth had occurred (1 to 3 months of culture yielded approx. 40mg of live wet cells), were collected for gas chromatography. Seventeen ATCC reference isolates were also included in this study (Table 3-1).

3.3.2 Capillary Gas Chromatography

Isolates were harvested and processed for gas chromatography using a modified method (MIDI 2002B). Isolates were boiled for 30 minutes after vortexing with saponification reagent (sodium hydroxide, methanol, and deionized distilled water) to lyse cells and remove fatty acids from cellular lipids. Then, 2 mL of methylation reagent (hydrochloric acid and methanol) was added, the tube vortexed, and placed in an 80ºF water bath for 10 minutes to form the methyl esters of the fatty acids (FAMEs). Following, 1.25 mL of extraction solvent (hexane and methyl tert-butyl ether) was added to the tube as well as 5 drops of saturated salt solution (sodium chloride in distilled

deionized water) and vortexed to transfer the FAMEs to the organic phase from the aqueous phase. The bottom aqueous phase was then removed by pipette. Finally, 3 mL of base wash (sodium hydroxide and deionized distilled water) and 10 drops (~250 μL) of salt solution were added to the remaining top layer and vortexed to perform an aqueous wash of the organic extract. As the top layer should be clear, further clearing of the top layer was occasionally required for mycobacterial isolates. In such cases, more salt solution was added, the tube vortexed and then allowed to sit for up to ten minutes before collection. The top, organic layer was then collected for analysis and put in a gas chromatography sample vial. The sample was then frozen and subsequently visually inspected for good phase separation, i.e. no aqueous layer remained.Samples were analyzed on a Hewlett-Packard 6890 Series GC system with a Hewlett-Packard 7683 series injector attached to a computer with proprietary Sherlock Microbial Identification System software (MIS; MIDI Inc., Newark, DE). Reference samples were included in each run to calibrate peaks and assure column function (MIDI 2002B).

3.3.3 Dendogram

A dendogram of the mycobacterial isolates was produced using the MIS software. Essentially, the cluster analysis produced a relational diagram, based on Euclidian Distances (EDs), in multi-dimensional space between and among isolates based on fatty acid compositions of the bacterial cell walls (MIDI 2002A). Clusters of isolates were then visualized based on the dendogram and classified as such if isolates were related under 12 ED.

3.4 Results

Mycobacterial isolates were recovered from striped bass (*Morone saxatilis*), Atlantic menhaden (*Brevoortia tyrannus*), white perch (*Morone americana*), summer flounder (*Paralichthys dentatus*), spot (*Leiostomus xanthurus*), largemouth bass (*Micropterus salmoides*), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio carpio*), spotted seatrout (*Cynoscion nebulosus*), killifish (*Fundulus* sp.), blueback herring (*Alosa aestivalis*), American gizzard shad (*Dorosoma cepedianum*), and American silver perch (*Bairdiella chrysoura*) from the Upper Bay, the Choptank River, the Middle Bay, the Chicamacomico River, the Pocomoke River and the Potomac River (Table 3-2).

Over 600 isolates were collected and analyzed by gas chromatography. Approximately 550 isolates produced chromatograms of sufficient resolution for analysis in the dendogram. The resulting dendogram produced 29 well defined groups (Table 3-2). The groups were not all-inclusive for all mycobacteria isolates found in Chesapeake Bay fish, i.e., isolates that did not fall within well defined groups were not described individually. For example, one mycobacterial isolate that was recovered from blueback herring was not included in this analysis or Table 23- because it did not fall within a well defined group.

The majority of groups included isolates from more than one host species (Table 3-2). However Groups 13, 22, 23, and 24 only included striped bass isolates. Additionally, all groups, except Group 13, included isolates from more than one Bay location (Table 3-2). Group 13 isolates were recovered only from striped bass collected from the Potomac River, however no other fish species were collected from this location.

Twelve groups contained the majority of isolates found in the most intensively sampled host species, striped bass (number of isolates=247), Atlantic menhaden (n=110) and white perch (n=57; Table 3-3). It is interesting to note that some groups overlaped considerably among hosts. For example, Groups 10 and 17 were predominant isolates found in striped bass, Atlantic menhaden and white perch (Table 3-3). Conversely, some mycobacteria groups appeared to almost be host specific, i.e. Group 27 was found commonly in Atlantic menhaden but not commonly in striped bass and white perch. Additionally, Group 7 was isolated commonly in striped bass but rarely in white perch and not at all in Atlantic menhaden. The most obvious case of host specificity was Group 22, *M. shottsii*, where all 79 isolates were recovered from striped bass (Table 3-3 and 3-4) but not other host species. Ages of striped bass infected with *M. shottsii* ranged from 1+ to 6+, but primarily from fish 4+ and older (Table 3-5).

The inclusion of ATCC isolates in this study allowed eleven of the groups to be named (Table 3-2). Note that two ATCC isolates, *M. triplex* and *M. montefiorense* both fell within Group 5, and *M. marinum* and *M. pseudoshottsii*, both fell within Group 20. Four ATCC isolates, *M. chelonae*, *M. fortuitum*, *M. gordonae* and *M. peregrinum*, failed to fall within well defined groups.

Sequencing of 16S, hsp65 and rpoB genes (data not shown; see MLST chapter) and GenBank BLAST searches provided the names assigned to several groups. Group 1 included an isolate sequenced as *M. gordonae* (100% homology in rpoB and 99% in 16S) and Group 4 included an isolate sequenced as *M. interjectum* (100% in 16S, 99% in rpoB). Group 12 contained 2 sequenced isolates, one as *M. aurum* (100% in rpoB and 98% in 16S) and one as *M. parascrofulaceum* (100% in 16S and 96% in rpoB). Group

20 included an isolate sequenced as *M. pseudoshottsii* (100% in rpoB), Group 21 included an isolate sequenced as *M. marinum* (100% in rpoB), and Group 22 included 9 isolates sequenced as *M. shottsii* (100% or 99% in 16S and/or rpoB).

3.5 Discussion

Previous studies have shown that multiple Chesapeake fishes are infected with mycobacteria (Kane et al. 2007). This study provides evidence that multiple mycobacterial species are involved in those infections, which corroborates previous findings in striped bass (Rhodes et al. 2004). In general, these mycobacterial isolates were not river specific nor host specific. However certain isolates were found more predominantly in some host species (Table 3-3).

Striped bass and Atlantic menhaden, historically a striped bass food source, share some mycobacterial pathogens, i.e., Groups 10 and 17, thus indicating that Chesapeake Bay striped bass are potentially infected by eating an infected diet (Table 3-3). This is well regarded as a potential route of transmission in other systems, and was initially shown in salmonids in the Pacific Northwest (Ross and Johnson 1962). However, it is also possible that these mycobacteria are common in the environment, and therefore infective by other routes of transmission to a large range of host species. Clearly, further research is required to elucidate the methods of transmission for mycobacterial infections in wild hosts, and it is possible that routes differ for mycobacterial species as well as host species.

Striped bass and white perch, two closely related species, appeared to share some pathogen specificity, i.e., Groups 12 and 15 (Table 3-3). Whether this occurs because of similarities in genetic code and immune function, ecological niche, behaviors, or other

similarities between these two fish species, however, is unknown. Findings from this study agreed with those of Rhodes et al. (2004), in that *M. shottsii* (Groups 22, 23, and 24) was the most common mycobacteria isolated from striped bass. However, the current study also suggests that *M. shottsii* is host specific. As with mycobacterial species that appeared to prefer *Morone* hosts, i.e., striped bass and white perch, why *M. shottsii* prefered striped bass is unknown.

Rhodes et al. (2004) described 8 groups of mycobacteria isolated from striped bass and named them according to phenotypic characterization. The current study named groups according to FAME ED relationships between isolates, and in relation to ATCC reference isolates. The synthesis of the group names is provided in Table 3-4. Whereas all Rhodes et al. (2004) groups, except H, were found in the current study, additional groups of mycobacteria were also discerned (Table 3-2). The former study used a decontamination process when samples were not aseptically collected, and plated samples on a variety of media whereas the current study relied on aseptic technique (plates with contamination were removed from the current study) and plated samples only on Middlebrook media. Additionally, the former study collected samples from 1997 to 2001 in the southern Chesapeake Bay and tributaries (i.e. the Potomac River to the mouth), whereas the current study sampled from 2003 to 2006 and focused on the Maryland portion of the Chesapeake Bay and its tributaries (i.e., the Potomac and Pocomoke Rivers northward). A notable difference also occurred in the age structure of the striped bass sampled; 92% (178/194) of the striped bass in the former study were 4 years or older, whereas the current study concentrated on younger fish (only 9% sampled (180/2078) were 4 years or older).

Group 20 included both the *M. pseudoshottsii* and the *M. marinum* ATCC isolates. These species are closely related in the same clade (Kaattari et al. 2006), and it is therefore not surprising the composition of the fatty acids within their respective cell walls is similar. This close relationship of FAMEs is not problematic for distinguishing these two species since their morphology in culture is distinctly different. While *M. pseudoshottsii* appears as pale golden yellow dry colonies, *M. marinum* appears as bright yellow creamy colonies (personal observations, C. Stine and A. Baya).

A few groups may represent additional isolates of already named groups. For example, Group 3 is believed to be *M. interjectum,* or a closely related species, because of its close ED relationship with Group 4, although with different pigmentation. Group 3 produced white creamy colonies whereas Group 4 produced yellow creamy colonies. Additionally, both Groups 8A and 8B are believed to be *M. szulgai* because of their close relationship at 10.5 ED, but 8A has dry colonies whereas 8B has creamy colonies. Further, Group 21 is thought to be *M. marinum* (along with a portion of Group 20 as described in the previous paragraph). This name results from good correlation to *M. marinum* in the MIDI software library. Incidentally, Group 21 was recovered from hosts in high colony forming units (CFUs) whereas Group 20 generally had low CFUs (data not shown). It is possible that strains of the same species, in this case *M. marinum*, may have differences in the fatty acid composition of their cell walls which would account for their being placed in separate groups. Alternatively, as *M. marinum* and *M. pseudoshottsii* were in the same clade, the *M. marinum* isolates, found within Group 20 with *M. pseudoshottsii,* could potentially be intermediate strains between the two species

(Kaattari et al. 2006). Clearly the relationships among these isolates and between these two groups require further study.

Several putative new species were discerned during the course of this study, although more groups remain unnamed than named. Additional ATCC isolates have been ordered and will be run through the gas chromatograph to assist in further group naming. However, isolates from several groups will inevitably need to be sequenced to assist in naming. Additionally, whereas isolates within groups have similar morphology, growth characteristics and cell wall composition, sequencing regions of multiple isolates within each group will further strengthen the group designations. This proposed sequencing was beyond the scope of the current project.

Mycobacteriosis can cause mortality in aquaculture, however the effect on wild stocks is unknown. Studies are currently underway to study the population level effects of mycobacterial infections on striped bass, specifically, whether or not infections kill fish (Vogelbein et al. 2006). Further, PCR evidence exists that mycobacteria, consistent with *M. pseudoshottsii*, have been present in Chesapeake Bay striped bass since 1984 (Jacobs 2007). Previous efforts to find mycobacteria in other host species have been unsuccessful although minimal effort was initially put forth (Rhodes et al. 2004). With the understanding that more host species are now known to be affected, further questions are raised about the effects of mycobacterial infections on other wild species, such as Atlantic menhaden or white perch, and whether other species served as a source of infection for striped bass.

With decreased immune functions of humans resulting from auto-immune diseases and drug therapies, environmental mycobacteria pose health threats to those

people coming in contact with sources of infection (Dobos et al. 1999, Panek and Bobo 2006). The threats resulting from piscine mycobacterial infections described in the current study are not clear, although there are reports of human infections from Chesapeake Bay contact (Zeligman 1972; Hoyt et al. 1989; Joe and Hall 1995). Therefore caution is warranted when handling fish from the Chesapeake Bay, and hand washing and/or wearing gloves is suggested (Jacobs et al. 2004, Panek and Bobo 2006)

This study reports that multiple mycobacterial species infect multiple piscine host species. Infected wild hosts included striped bass, Atlantic menhaden, white perch, summer flounder, spot, largemouth bass, channel catfish, common carp, spotted seatrout, killifish, blueback herring, American gizzard shad, and American silver perch. Twenty-nine groups of mycobacteria were discerned, of which eleven were speciated as *M. scrofulaceum*, *M. septicum*, *M. interjectum*, *M. triplex/M. montefiorense*, *M. szulgai*, *M. moriokaense*, *M. duvalii*, *M. avium*, *M. terrae*, *M. pseudoshottsii*/*M. marinum* and *M. shottsii*. Several putative new species were found during the course of this study and require further description.

3.6 Acknowledgements

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3.7 Tables

Table 3-1. American Type Culture Collection (ATCC) isolates included in fatty acid methyl ester and dendogram analysis.

Isolate	Accession Number
Mycobacterium avium	25291
Mycobacterium chelonae	19235
Mycobacterium duvalii	43910
Mycobacterium fortuitum	9820
Mycobacterium gordonae	14470
Mycobacterium interjectum	51457
Mycobacterium marinum	927
Mycobacterium montefiorense	BAA-256
Mycobacterium moriokaense	43059
Mycobacterium peregrinum	700686
Mycobacterium pseudoshottsii	BAA-883
Mycobacterium scrofulaceum	700734
Mycobacterium septicum	700731

Mycobacterium shottsii	700981
Mycobacterium szulgai	23069
Mycobacterium terrae	15755
Mycobacterium triplex	700071

Table 3-2. Dendogram groupings, based on fatty acid methyl ester analysis, of mycobacteria isolated from Chesapeake Bay fishes. Morphology includes color (W=white, Y=yellow, O=orange and P=pink) and texture. DTC=days to collection for gas chromatography (1= <15 days, 2=2 weeks to 2 months, and 3= >2 months). Note that while DTC is not identical to days to growth in species descriptions, it is a measure of comparable growth rates.

Group	# isolates	Host Species										River						Morphology	DTC	ATCC matches
		striped bass	Atlantic menhaden	white perch	summer flounder	spot	largemouth bass	channel catfish	carp	sea trout	mummichog/ killifish	gizzard shad	silver perch	Upper Bay	Choptank	Middle Bay	Chicamacomico	Pocomoke	Potomac			
1	11	9	2											7	1	1		2		O-Y creamy	2	*M. scrofulaceum*
2	2	1					1							1			1			W moist	1	X
3	11	7		4										1	1	1		8		W creamy	3	*M. septicum*
4	3	1	2												1		1	1		Y creamy	3	*M. interjectum*
5	20	15	1	4										1	2			17		W Y creamy	2, 3	*M. triplex* *M. montefiorense*
6	7	4	3											1	3		1	1	1	W P O creamy	1, 2, 3	X
7	9	8		1														6	3	O-Y creamy	3	X
8A	20	2	18												2		17		1	Y dry	2	X
8B	7	2	4	1											5			2		Y creamy	2	*M. szulgai*
9	3	2						1							1			2		W Y creamy	2	X

10	42	16	15	5	1		1	1		1			1	4		16	17	4	Y creamy	1, 2, 3	X
11	13	2	7	1	1	1			1				1	6			6		Y P W creamy	1, 2, 3	*M. moriokaense*
12	26	16	3	6										2		3	14	6	Y creamy	2	*M. duvalii*
13	3	3																3	W P creamy	3	X
14	5	3		1	1									2			3		Y creamy	3	*M. avium*
15	24	12	1	9		1				1			1			1	21	1	Y creamy	3	X
16	3	1		1							1				1		2		Y creamy	3	X
17	74	21	30	20		1	2						4	12		31	28	2	O-Y creamy	2	*M. terrae*
18	6	2	4											6					O creamy	1	X
19	4		3			1								4			1		W dry	2	X
20	16	13		2								1	2	4		1	7	2	Y creamy pale Y dry	2	*M. marinum* *M. pseudoshottsii*
21	7	5		1									2	1			1	3	Y creamy	2	X
22	79	79											20	17	5		7	30	W dry	3	*M. shottsii*
23	12	12											3	4	3			2	W dry	3	X
24	3	3												2				1	W dry	3	X
25	4	2	2											1		1	2		W Y creamy	2	X
26	8	2	6											1		6		1	W Y moist/dry	2	X
27	15	2	12	1									1	4		8	2		Y creamy/dry	2	X
28	4	2	1		1											1	1	2	W creamy	3	X

Table 3-3. Predominant dendogram mycobacterial groups isolated from striped bass *Morone saxatilis*, Atlantic menhaden *Brevoortia tyrannus* and white perch *Morone americana*. '+' indicates a predominate isolate, 'u' indicates that group of isolates is uncommon in that host, and '-' indicates that type of isolate was not found in that host. A group of isolates was considered predominant if more than 5% of the individuals of the infected host species were infected with that isolate group.

Group	Striped bass	Atlantic menhaden	White perch
5	+	u	+
8 A	u	+	u
8 B	u	+	u
10	+	+	+
11	u	+	u
12	+	u	+
15	+	u	+
17	+	+	+
20	+	-	u
22	+	-	-
23	+	-	-
26	u	+	-

27 u + u

Table 3-4. Comparison of the current study mycobacterial groupings with those groupings found by Rhodes et al. 2004. The BLAST matches are from MLST chapter in this dissertation.

Study Group	ATCC match	BLAST matches	Rhodes et al. 2004 group
1	*M. scrofulaceum*	*M. gordonae* (100% rpoB, 99% 16S)	D
4	*M. interjectum*	*M. interjectum* (100% 16S, 99% rpoB)	C
5	*M. triplex*		B
8B	*M. szulgai*		E
12		*M. aurum* (100% rpoB, 98% 16S)	
		M. parascrofulaceum (100% 16S, 96% rpoB)	
20	*M. pseudoshottsii*	*M. pseudoshottsii* (100% rpoB)	F
20	*M. marinum*		G?

21		*M. marinum* (100% rpoB)	G?
22	*M. shottsii*	*M. shottsii* (100% 16S, 100% rpoB)	A
unknown	unknown		H

Table 3-5. Prevalence of *M. shottsii* infected Chesapeake Bay striped bass *Morone saxatilis* by host age. Number positive/sample size in parentheses.

Age	Percent *M. shottsii* positive
0+	0
1+	1 (2/163)
2+	3 (5/192)
3+	11 (13/116)
4+	34 (61/177)
5+	44 (19/43)
6+	100 (1/1)

Chapter 4: Pictorial atlas and ecology of the parasitic fauna of striped bass *Morone saxatilis* (Percichthyidae) from the Chesapeake Bay, USA

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4.2 Abstract

The parasitic fauna of 0+ to 4+ Chesapeake Bay striped bass was investigated from 2003-2006. Striped bass were collected from the Upper Bay, the Choptank River, the Nanticoke River, the Pocomoke River and the Potomac River. Thirteen parasite taxa were observed: a bodonid (flagellate), two solitary sessile peritrichs (ciliates), *Trichodina* sp. (ciliates), larval digenetic trematodes, larval cestodes, *Philometra rubra* (nematodes), larval nematodes, *Pomphorhynchus* sp. (acanthocephalan), *Argulus* sp., *Ergasilus* sp., *Livoneca ovalis* (crustaceans), and 1 leech. Crustacean parasites (*Ergasilus* sp. and *Livoneca* sp.) or parasites with crustacean intermediate hosts (*P. rubra* and *Pomphorhynchus* sp.) were found in higher prevalence in the Pocomoke River than other sites. Further, prevalence of *P. rubra*, *Pomphorhynchus* sp. and *Ergasilus* sp. were lower in 0+ striped bass than the older fish collected in this study. Samples collected in 2004 were significantly higher for sessile peritrichs and trichodinids than those collected in 2003. Therefore, as various factors influence the presence and prevalence of parasites, generalizations about the parasitic load of a population of fish are not appropriate. Each parasite should be treated individually as well as investigations into the relationship of the parasite to the fish host and the environment.

4.3 Introduction

The Chesapeake Bay is the largest and most productive estuary in the United States, and is the spawning ground for most of the Atlantic striped bass *Morone saxatilis* (ASMTC: Atlantic States Marine Fisheries Commission 2003). This fish is a top predator, and a species of great ecological, commercial, and recreational importance. Following a decline in the population in the 1980's, a moratorium was imposed by the

state of Maryland in 1985 – 1989, and the species has recently been placed under federal legislative protection (Bush 2007).

Although considerable attention has been focused on the spawning stock biomass and certain diseases of the Chesapeake Bay striped bass namely mycobacteriosis, ulcerative dermatitis syndrome, and nutritional deficiencies (ASMFC 2003), there have been no recent reports on their parasites. Knowledge of parasites affecting Chesapeake Bay striped bass is important not only for parasitologists, but also because of the potential role of parasites, especially acanthocephalans, in transmission of mycobacterial infections (Dove, 2005). Mycobacterial infections have recently gained much attention in the popular media and in the scientific literature because of their zoonotic potential and as causative agents for emaciation and skin lesions in fish (Heckert et al. 2001; Rhodes et al., 2001, 2004).

The most comprehensive study of parasites of Chesapeake Bay striped bass was published in 1976 by Paperna and Zwerner (1976). Subsequent studies focused on host parasite relationships and identification of specific parasites (Paperna and Zwerner 1976b; Zachary and Paperna 1977; Paperna and Zwerner 1982). The comprehensive 1976 study concentrated on identification of whole parasites, with histology samples being taken only when pathology was recognized, thus some parasites, especially larval stages of some metazoans, would probably not have been seen. Therefore, a new investigation is needed, which incorporates identification of parasites in tissue sections. Furthermore, documentation of the current parasite community is needed, because the ecology of the Chesapeake Bay has undergone dramatic changes in the 30 years since the 1976 study (Kemp et al. 2005). Of particular concern is the increased population pressure, with the number of Bay area residents having increased from 11.7 million in 1970 to 16.6 million in 2005, an increase of 42% in just 35 years (www.chesapeakebay.net), and the

introduction of non-native fish species, potentially harboring new parasites. It is known that invasive species, including parasites, can pose threats to local ecosystems (Ruiz et al. 1999, Torchin et al. 2002, Prenter et al., 2004).

We recently conducted a four-year study of parasites from striped bass collected in Maryland portions of the Chesapeake Bay in order to investigate the relationships between parasite infection and other aspects of the health of the striped bass, especially mycobacterial infections. The present publication documents the parasite fauna from 2003 - 2006, and provides a pictorial atlas with gross and histological images that will be helpful in providing taxonomic identification for other investigators. This paper supplements the publications of Bruno et al. (2006) and Dykova and Lom (2007) which focus on protozoans and myxozoans. The possible relationships between parasites and mycobacterial infections in Chesapeake Bay striped bass are considered in a subsequent publication.

4.4 Methods

4.4.1 Sampling sites

Striped bass were collected from five sites in the Chesapeake Bay: the Choptank, Nanticoke, Pocomoke, and Potomac rivers, and the Upper Bay (Figure 4-1, Table 4-1). These sites were chosen as they were also used for collection of striped bass during a recent survey of mycobacterial infections (Chapter 5), and because they have historically been used by Maryland Department of Natural Resources in striped bass stock assessment.

4.4.2 Fish collection

Our aim was to document parasitism by host age, collection site and year, for age 0+ to 4+ striped bass from 5 sites in the Chesapeake Bay between 2003 and 2006. Due to limitations in sampling resources and erratic collection of fish older than 1+, we were unable to collect all age classes from all sites in all years.

Fish were collected in summer and fall seasons of 2003-2006 by beach seine, cast net, pound net, or hook and line. Age 0+ fish were usually collected in July and August, and older fish were collected August through October; Choptank fish were collected first followed by the Upper Bay, the Potomac and the Nanticoke. However, in the Pocomoke, 0+ and older fish were collected simultaneously from July to August. Upon capture, fish were transported live, with oxygen support, to the laboratory in coolers or circular holding tanks. Time from collection to parasite evaluation was typically under 6 hours. Age of striped bass was determined by length-at-age data and confirmed by scale analysis.

4.4.3 Examination of fish and recording ecological parasitology data

We focused our attention on the skin and gills since these were the organs most commonly infected with parasites according to Paperna and Zwerner 1976a. We also studied the distal gut because of a report that acanthocephalans may provide a conduit for mycobacteria to travel from the digestive tract to the coelom (Dove 2005).

Grossly visible ectoparasites were noted during necropsy. Skin scrapes and gill biopsies were taken for the first 10 fish on each sampling day. Skin was scraped from anterior to posterior adjacent to the first dorsal fin. Gill biopsies were taken from the middle to ventral, distal edge of the 1st gill arch. The first gill arch on the left side of the fish was examined for noting density of copepods. The peritoneal cavity was examined

for grossly visible endoparasites. The distal gut was longitudinally opened and the lumen was grossly examined for acanthacephalans.

Presence/absence was recorded for all parasites for calculation of prevalence. Density and intensity of infection was recorded semi-quantitatively for copepods and acanthacephalans respectively, on a scale of 1-3 where 1 = light infection, 2 = moderate infection, and 3 = heavy infection. Ecological terminology follows Bush et al. (1997).

4.4.4 Identification of parasites in fresh preparations

Parasites were identified by standard keys to the lowest taxonomic level possible (Lom and Dykova 1992; Hoffman 1999). Skin smears were air dried and stained using Klein's dry silver impregnation method for visualization of denticle morphology of trichodinids (Lom and Dykova 1992).

Speciation of some parasites lay beyond the scope of the present study, for example speciation of *Ichthyobodo,* a bodonid, requires sequencing of the small subunit rDNA (Callahan et al. 2005), and speciation of sessile peritrichs requires impregnation by Klein's method or with protargol (silver protein) to visualize the somatic and oral ciliature, and nuclei (Lom & Dykova 1992).

4.4.5 Identification of parasites in tissue section

Further observations were made by examination of parasites in the spleen, liver and anterior kidney sample, tissues which were routinely taken for histology because of their involvement in mycobacterial infections (the focus of a larger umbrella study as noted in the introduction). We also routinely sampled gills from 0+ fish in 2003. Occasional incidental samples were also available for brain, gastro-intestinal tract, muscle and gonad. Tissues were preserved in 10% neutral buffered formalin and

processed for routine histology (Profet et al., 1992). Sections were cut 7µm thick and stained with hematoxylin and eosin. Parasites were identified *en section* by observing features of taxonomic significance, as outlined in Lom and Dykova (1992), Gardiner and Poynton (1999), Bruno et al. (2006) and Dykova and Lom (2007).

Larval digenean trematodes, cestodes and some nematodes were not observed grossly, but only *en section*.

To investigate the possibility of parasites with traumatic sites of attachment as vectors for mycobacterial infections, a sub-set of sections of gill heavily infested with copepods and sections of gut heavily infested with acanthacephalans were stained with aqueous acid-fast.

4.4.6 Statistics

Fisher's exact test (SAS 9.1, SAS Institute Inc., Cary, NC or EpiInfo 6.04d, Centers for Disease Control, USA and World Health Organization, Geneva, Switzerland) was used to investigate differences in prevalence between host age, collection sites, and collection years. For determination of statistical significance in prevalence, we used data sets with a sample size of $n \geq 15$. This allowed us to make comparisons for seven parasites: the bodonid, sessile peritrichs, trichodinids, *Philometra* sp., *Pomphorhynchus* sp., *Ergasilus* sp., and *Livoneca* sp. (Table 4-2). Differences were considered significant if $p < 0.05$.

4.4.7 Deposition of reference specimens

Preserved whole parasites, and histological sections stained with hematoxylin and eosin will be deposited in the US National Parasite Collection, USDA, BARC East, Building 1180, Beltsville MD 20705-2350, USA (E-mail: ehoberg@anri.barc.usda.gov).

4.5 Results

From the 1,438 striped bass examined, 13 parasite taxa were observed: a bodonid (flagellate), two solitary sessile peritrichs (ciliates), *Trichodina* sp. (ciliates), larval digenetic trematodes, larval cestodes, *Philometra rubra* (nematodes), larval nematodes, *Pomphorhynchus* sp. (acanthocephalan), *Argulus* sp., *Ergasilus* sp., *Livoneca ovalis* (crustaceans), and 1 leech.

The parasites are described below and illustrated in Figs. 2 – 7. Prevalence data is given for 7 parasites in Table 4-2: the bodonid, sessile peritrichs, *Trichodina* sp., *P. rubra*, *Pomphorynchus* sp., *Ergasilus* sp. and *Livoneca ovalis*.

4.5.1 Protozoans

Bodonid

A bodonid, more common on the gills than on the skin, was found on all ages of fish, at all sites, and in each year (Table 4-2). Prevalence, which ranged from 0 – 90%, was generally lower than that of the other ectoparasitic protozoans, and there were many occasions on which the parasite was not found.

There were significant differences in prevalence between sites: in the most complete data set, 0+ fish in 2004, prevalence in the Nanticoke (0%) was significantly lower than at the Upper Bay (50%), the Pocomoke (42%), and the Potomac (29%); but not significantly different from the Choptank (20%). In 0+ fish in 2003, prevalence of the bodonid was not significantly different between the Upper Bay, Potomac and Choptank (the same as in 2004). However in 4+ fish in 2006, the only other comparison possible

for these three sites, there was a significant difference, with prevalence in the Potomac (26%) being significantly higher than in the Upper Bay (3%) and Choptank (3%).

There were no significant differences in prevalence of infection in 0+ fish between 2003 and 2004 at the three sites we compared, Upper Bay, Potomac and Choptank.

Sessile peritrichs

Sessile peritrichs (Figure 4-2) believed to be *Ambiphyra* spp. and/or *Riboscyphidia* spp., and *Apiosoma* spp. were found on all host age groups, at all collection sites and in all years sampled (Table 4-2). Prevalence ranged from 0 – 100%. Sessile peritrichs were more prevalent on the gills than on the skin (data not shown).

There were significant differences in prevalence between sites: in the most complete data set, 0+ fish in 2004, prevalence in the Upper Bay (30%) was significantly lower than in the Potomac (86%), the Nanticoke (75%), and the Choptank (65%); but not significantly different from the Pocomoke (42%). Prevalence in the Pocomoke was also significantly lower than in the Potomac and Nanticoke. In 2003 in 0+, prevalence in the Potomac (41%) was significantly higher than the Upper Bay (0%), but not different from the Choptank (18%).

There were significant differences in prevalence of infection on 0+ fish between 2003 and 2004, being higher in the latter, at each of the three sites we compared, Upper Bay (0% versus 30%), Potomac (41% versus 86%) and Choptank (18% versus 65%).

***Trichodina* spp.**

Trichodinids (Figure 4-2), more common on the gills than on the skin, were found on all ages of fish, at all sites, and in each year (Table 4-2). Prevalence ranged from 0 –

100%. Preliminary staining not presented here indicates multiple trichodinid species were present.

There were some significant differences in prevalence between sites: in the most complete data set, 0+ fish in 2004, although prevalence was high (≥ 70%) at all sites, the Upper Bay (70%) was significantly lower than the Choptank and Potomac (100% for each). In 0+ fish in 2003, there were again significant differences between these three sites, with both the Upper Bay (30%) and the Choptank (50%) being significantly lower than the Potomac (86%).

There were differences in prevalence of infection on 0+ fish in 2003 and 2004, being higher in the latter at each of the three sites we compared, this difference being significant for the Choptank (50% versus 100%) and the Upper Bay (30% versus 70%).

4.5.2 Metazoans

Digenetic trematodes

Digenetic trematode larvae were observed histologically in the liver, anterior kidney, posterior kidney, spleen, distal gut, gill and brain tissues (Figure 4-3).

Cestodes

Larval cestodes were found rarely in the liver and the intestinal lumen (Figure 4-4). A Cyclophyllidian adult cestode was also observed in the intestinal lumen

***Philometra* sp.**

Nematodes consistent with *Philometra rubra* (Figure 4-5) were observed in the abdominal cavity in three forms: 1) red and actively writhing, 2) dead, black or 3) dead, in encapsulated plaques frequently occurring at the posterior end of the abdominal cavity

attached to mesentery or the dorsal aspect of the gut. All three forms could occur singly or in combinations in individual hosts. When infected, fish usually hosted more than one nematode.

Philometra sp. was found in all age groups, at all sites, and in each year (Table 4-2). Although only a minority of the 0+ fish were infected (prevalence was usually less than 10%), most fish 1+ and older were infected (prevalence of 32 – 93%). The most complete data set, 0+ v1+ v2+ v3+ in the Choptank in 2005, showed the prevalence in 0+ fish was significantly lower than that in each subsequent age class, and that there were no significant differences in prevalence between 1+, 2+ and 3+ fish. While the other less complete data sets supported the significantly lower prevalence in 0+ fish compared with older fish, there were significant differences in prevalence among the 1+, 2+ and 3+ fish, although no consistent patterns were seen.

There were significant differences in prevalence of *Philometra* sp. between sites. In the complete data set, 0+ fish in 2004, prevalence in the Pocomoke (63%) was significantly higher than in each of the other four sites; the only difference among the other four sites was that the prevalence in the Choptank (3%) was significantly lower than that in the Nanticoke (14%). In the data set for 0+ fish in 2003, although data for the Pocomoke was not available, the data again showed only one significant difference between the remaining four sites, (prevalence in the Potomac (2%) was significantly lower than in the Choptank (11%)). In the other less complete data sets for in 1+, 2+ and 3+ fish 2005, the significantly lower prevalances were: 1+ fish in the Upper Bay, in 2+ fish in the Pocomoke, and in 3+ fish in the Upper Bay.

There were no significant differences in prevalence of *Philometra* in 0+ fish between 2003 and 2004 in three of four sites (the Nanticoke river was the exception), nor in 0+, 1+ or 2+ fish between 2004 and 2005 in the Choptank river.

Adult nematodes were also found *en section* in the liver, spleen, and rarely in the kidney. Occasionally, nematodes were observed with bacilli-like projections exterior to the cuticle (Figure 4-4E). Aphasmid nematode larvae were rarely observed in gut sections (Figure 4-4D-F). In one instance, nematode larvae were observed migrating along the granulomatous tissue associated with an acanthacephalan proboscis (Figure 4-5G).

***Pomphorhynchus* sp.**

Acanthacephalans, consistent with *Pomphorhynchus* sp. (Hoffman 1999), were found attached to the luminal surface of the distal gut, with their heads often observed as firm, 1-3 mm nodular protuberances on the opposing serosal surface (Figure 4-6). Multiple acanthacephalans were commonly found in a host, when present.

Pomphorhynchus sp. was found in all age groups, at all sites and in all years (Table 4-2). Prevalence was typically lower in 0+ fish that in older year classes, with fish at some sites not becoming infected in their first year, and the prevalence increased and fluctuated among the age classes 1+ to 4+. The most complete data set, 0+ v1+ v2+ v3+ in the Choptank in 2005, showed that the only significant difference in prevalence with age was between the 0+ fish which were uninfected, and the 1+ fish in which prevalence was 33%. In the less complete data sets, there were some significant differences in prevalence among the 1+, 2+ and 3+ fish, but no consistent patterns were seen.

There were some marked differences between sites. Of particular note was the fact that 0+ fish from the Choptank, Potomac and Upper Bay sites were not infected, in contrast to 0+ fish from the Nanticoke and Pocomoke rivers; these site differences were statistically significant in the complete data set for 0+ fish in 2004, i.e. prevalence in the Nanticoke (20%) and Pocomoke (58%) were significantly higher than in the Choptank,

Potomac and Upper Bay. At age 1+ and older, fish at all sites were infected, with prevalence being highest in the Pocomoke, and in most cases statistically significantly higher.

There were no significant differences in prevalence of acanthacephalans between years in the possible comparisons, Choptank 2+ fish (2004-2005) and Upper Bay 2+ (2004-2005).

Acanthacephalans were found *en section* both within the lumen, and transecting the intestinal wall (Figure 4-5G). No acid-fast bacilli were observed in intestinal walls in association with heavy infections of acanthacephalans.

***Argulus* sp.**

Argulus sp. (Figure 4-7I) was found solely on the skin in this study, often on the peduncle and/or on the caudal fin. Observations of these branchiurans were occasionally associated with skin lesions. More than one argulid was commonly found per host when present. *Argulus* sp. was not a common finding, and was only observed in 1+, 2+ and 3+ striped bass collected from the Potomac River, and in 1+ and 3+ fish collected from the Upper Bay.

***Ergasilus* sp.**

Copepods were found primarily clustered closer to the anterior edge of the first gill arch, close to the insertion of gill lamellae into the raker (Figure 4-7A-D). In severe infections (ranking of 3), however, copepods were found on the distal edge of the first gill arch as well as on the second and third arches.

Copepods were found in all age groups, at all sites and all years sampled (Table 4-2). Prevalence was lower in 0+ fish compared with other age groups: in the most

complete data set, prevalence of copepods in Choptank 0+ (2004, 0%; 2005, 4%) was lower than 1+ (87%), 2+ (100%) and 3+ (100%) in 2005, and lower than 1+ (84%) and 2+ (63%) in 2004.

Site differences were also found in prevalence of copepods. In the most complete data set, 0+ in 2004, Pocomoke fish (37%) had a higher prevalence than all other sites (Choptank (0%), Nanticoke (4%), Potomac (3%) and Upper Bay (14%)).

Only one yearly difference was found, Choptank 2+ in 2005 (100%) had a higher prevalence than Choptank 2+ in 2004 (63%).

No acid-fast bacilli were observed in gill tissue in association with heavy copepod infections.

Livoneca ovalis

Isopods were observed in the gill chambers, typically attached to skin overlying the cleithrum and/or clavicle (Figure 4-7E-H). Only rarely did a host have more than one isopod attached. Isopods were consistent with *Livoneca ovalis*.

Isopods were found in all host age groups, at all collection sites and in all years sampled (Table 4-2). Age generally did not affect prevalence of isopods. In the most complete data sets, no differences were found in the Choptank in 2004, 0+, v1+, v2+, nor in 2005, 0+, v1+, v2+, v3+. The one statistically significant difference was the 1+ (63%) had a higher prevalence than the 2+ (31%) in the Pocomoke in 2005.

Site differences were found relating to the Pocomoke River. In 2004, 0+ in the Pocomoke (26%) had a higher prevalence than the 0+ in the Upper Bay (0%). Additionally, in 1+ and 2+ in 2005, prevalence in the Pocomoke was higher than in both the Choptank (1+, 0% versus 63%; 2+, 0% versus 31%)and in the Upper Bay (1+, 0% versus 63%; 2+,0% versus 31%).

There were differences in prevalence of infection on 0+ fish in 2003, 2004, and 2005, with 2004 being higher than the other years. This difference was significant for the Choptank (4% and 4% versus 23%) and the Nanticoke (0% versus 17%).

Leeches

Occasionally, leeches were observed on striped bass, most commonly on the 1st or 2nd dorsal fin. They were found on 2+ and 3+ striped bass from the Potomac River and on 1+ fish from the Pocomoke River.

4.6 Discussion

4.6.1 Overview

In this 2003 – 2006 study, we studied the parasites of striped bass, ages 0+ to 3+, from five sites in the Chesapeake Bay. A total of 13 taxa were recorded, at least 5 of which appear to be new host/parasite/geographic recordings when compared to the last study of striped bass parasites by Paperna and Zwerner (1976). Of particular note is the *Eustrongylides* sp., because of its putative zoonotic potential (Guerin et al. 1982). Additionally, we observed two apparently novel presentations of parasites, i) the nematode *en section* with apparent bacteria attached to the epidermis (Figure 4-4E), and ii) nematode larvae migrating through the intestinal wall along the granulomatous tissue created by an acanthacephalan proboscis (Figure 4-5G). In relation to metazoan parasites, 0+ striped bass appeared have lower prevalence than older fish collected from the same sites. Additionally, the prevalence of metazoan parasites that were either crustaceans or used a crustacean intermediate host was higher in the Pocomoke River than other sites. These topics will be discussed further in more detail. It is important to

note here that it is not appropriate to make generalizations about all parasites of striped bass in general. Each parasite should be treated as its own entity and analyzed as such, specifically because parasites employ different life cycle strategies.

4.6.2 New host-parasite-geographic records

The current study observed parasites not previously found by Paperna and Zwerner (1976), and vice versa (Table 4-3). The current study's findings of *Ambiphyra* sp., larval digenes in the gills, a cyclophyllidian adult cestode in the intestinal lumen, and *Eustrongylides* sp. in the gonad appear to be new host records when compared with Paperna and Zwerner (1976), however *Ambiphyra* sp. has been reported previously in striped bass (Hoffman 1999). Further, aphasmid nematode larvae were found in the current study, and while the exact identity is unknown, no aphasmid nematodes were found in the former study. Several explanations for the observed disparity in parasitic assemblages between these two studies are possible - i) the parasitic assemblages may have changed over 30 years, ii) the studies were performed on striped bass collected from different parts of the Chesapeake Bay, or iii) the sampling strategies used in the two studies differed. It is likely that the differences observed between the two studies are a function of a combination of the reasons mentioned.

First, it is possible that the parasitic assemblages of striped bass have changed in the 30 years separating the studies, from Paperna and Zwerner's study in 1972-1973 to the current study in 2003-2006. In the light of the pressures placed on the Chesapeake Bay in the past 30 years, which includes an increase in nutrient enrichment which in turn can alter the communities of fish and invertebrates through realigning trophic structures, it is reasonable to assume that the parasitic assemblages of striped bass have, in fact, been

altered (Kemp et al 2005). However the absence in the current study of parasites found previously seem to suggest the effect of changing Chesapeake Bay ecology may be more of a decrease in species diversity than an alteration of species composition. It is important to note that definitive statements on alterations of parasitic assemblages should be based on studies including longer time frames as yearly variations may influence results (see below).

Second, the striped bass were collected from different sites in the Chesapeake Bay in Paperna and Zwerner (1976) and the present study. The previous study sampled primarily from the lower, or Virginia, portion of the Chesapeake Bay. The present current study sampled primarily from the northern, or Maryland, portion of the Chesapeake Bay. Only the Potomac River was sampled in both studies. Further, as the ages of fish sampled in both studies were comparable, ranging from 0+ to 3+, it is important to note that even within these age groups, some individuals 1+, 2+ and 3+ may be migratory, and thus the location disparity between the two studies may not be as significant in the older fish (Kohlenstein 1981). Finally, mycobacterial infection prevalence appears to vary with age only in the 0+ and 1+ fish (Chapter 5), therefore this data suggests site of capture for the striped bass host may only affect prevalence distribution of parasites in 0+ and 1+ fish as well.

Third, the sampling techniques differed between the studies. Previously, Paperna and Zwerner (1976) only took tissue samples when pathological changes were observed. In contrast, in the current study, tissue samples of spleen, kidney and liver were routinely taken and thus several parasites were seen *en section* that were not observed grossly, i.e. *Eustrongylides* sp., the aphasmid nematodes, and the digenetic trematodes. However the current study did not routinely sample some tissues, such as brain, heart, gill, gonads, intestinal lumen, mesenteries and muscle, some of which harbor parasites as shown by

Paperna and Zwerner (1976), i.e. digenetic trematodes. It is important to note that several digenetic trematodes were found in the current study, and few overlapped with those found in the previous study, and vice versa. This disparity underscores the importance of routinely taking comprehensive histology samples for a full parasitic study. Further, the majority of parasites found by Paperna and Zwerner (1976) and not in the current study (Table 4-3) were at low prevalence, and could have been missed by the current study sampling methods.

4.6.3 Potential zoonoses

Larval migration of *Eustrongylides* sp. has been found previously to occur in humans after ingestion of parasitized fish hosts (Guerin et al. 1982). Symptoms included extensive abdominal cramping and required surgery in two of three patients for removal of the nematodes (Guerin et al. 1982). Therefore, while it is not common, in our knowledge, to eat the raw gonads of striped bass from the Chesapeake Bay, the occurrence of *Eustrongylides* sp. in striped bass gonads should be noted as a potential zoonoses.

4.6.4 Novel presentations of parasites

Through the course of this study, we have found an unusual presentation of nematodes *en section* (Figures 4-4E). These adult nematode had an arrangement of basophilic entities external to their acellular eosinophilic cuticle. These bodies did not appear to be spinous projections, such as on the genera *Goezia*, as they did not extend into the cuticle and their staining qualities differed from the cuticle. Therefore, we propose these bodies are symbiotic bacteria. Further research is required into this

possibility, including the use of special stains such as acid-fast, gram or azure eosin (Pat Wilcox, personal communication). Additionally, pictures will be sent to a nematode specialist (Dr. Moravec) for further characterizations. However, there is precedent for symbiotic bacteria to attach externally to nematodes, including marine species (Musat et al. 2007).

The second novel presentation of a parasite encountered in this study was the occurrence of larval nematodes migrating along the granulomatous tissue created by an acanthacephalan proboscis (Figure 4-5G). While it is known that larval nematodes migrate through the intestinal wall to enter the peritoneal cavity and develop into adults, this is possibly the first histologic proof of such an occurrence (Williams and Jones 1994).

4.6.5 Ecological parasitology

Statistical differences were found among sites, host age and years of collection. The prevalence of metazoan parasites was generally higher in the Pocomoke River. Prevalence of metazoan parasites was also generally lower in 0+ fish than 1+, 2+ and 3+ fish. Yearly differences were significant for sessile peritrichs and trichodinids as 2004 prevalence was higher than 2003 in comparable age classes and site of host capture.

The prevalence of Acanthacephalans, nematodes, copepods and isopods was higher in the Pocomoke River than in other sites sampled. Each of these parasites is either a crustacean, or uses a crustacean in its life cycle. Copepods and isopods are crustaceans, acanthacephalans use copepods, ostracods, amphipods and isopods as first intermediate hosts, and nematodes use free-living copepods as intermediate hosts (Hoffman 1999). Therefore it appears as though crustaceans in general thrive in the

Pocomoke River, however the reason for this is unclear. For example, the Pocomoke River may have increased levels of prey items available. Alternatively, although not investigated in this study, copper, a minor nutrient, is potentially toxic to crustaceans at elevated levels (Pinho 2007). Therefore, the Pocomoke River may have lower levels of copper than the other sites in this study. Further studies investigating the increased prevalence of crustaceans in the Pocomoke River is warranted.

The prevalence of nematodes, acanthacephalans and copepods was lower in 0+ fish than the older fish, 1+, 2+ and 3+, in this study. These data are consistent with the previous study on Chesapeake Bay striped bass performed by Paperna and Zwerner (1976) as lower prevalence of nematodes, acanthacephalans and *Ergasilus* sp. was found on 0+ striped bass than on 1+, 2+ and 3+ fish. However age dependent prevalence of these three parasites differed with the previous study. For nematodes, Paperna and Zwerner (1976) found higher prevalence in 0+ fish (39%) than the current study when all sites and years were combined (8%; 67/804). Prevalence in 1+ fish was identical (64%) between the two studies, but whereas prevalence increased in 2+ (77%) and 3+ (100%) fish in Paperna and Zwerner (1976), prevalence remained relatively constant in the current study in the 2+ (62%; 120/193) and 3+ (53%; 73/138) fish. For acanthacephalans, prevalence in the 0+ fish was similar (9% (37/413) in the current study versus 14% in the previous study), as prevalence was in 1+ and 2+ fish (range 21-28% in the current study versus the range of 25-29% in the previous). Prevalence in the 3+ fish in the current study (39%; 44/114) was lower than the prevalence in the previous study (67%). For *Ergasilus* sp., 0+ striped bass prevalence was similar (12% in the current study, 19% in the former study). Prevalence on older fish was also similar. The range of prevalence was 89-94% in the current study and 80-100% in Paperna and Zwerner

(1976). Finally, as the previous study did not find isopods on 0+ fish, the current study found 6% (52/804) to be infected.

The significant yearly differences found in the sessile peritrichs and the trichodinids were similar as prevalence was higher in 2004 compared with 2003. These yearly variations could be accounted for by differences in either yearly rainfall or in temperatures, for example. In the Washington, D.C. area, 2003 was an exceptionally wet and cold year, as rainfall exceeded 20 inches from normal and annual temperatures were the coolest since records began in 1967 (Horvitz 2003). In contrast, 2004 was an average year in terms of rainfall and annual temperatures (Horvitz and Magnus 2004). Sessile peritrichs and trichodinids replicate by binary fission, the rate of which may be increased by elevated temperatures, which may explain why the warmer year, 2004 had elevated prevalence for these two parasites (Rohde 2005). In contrast, the increased rainfall observed in 2003 would increase the amount of suspended organic solids (SOS) in the water from runoff. Peritrichs and trichodinids feed off SOS, suspended bacteria and cell debris, so increased prevalence of these protozoa in the year with higher rainfall, 2003, would be expected (Hoffman 1999, Rohde 2005). However, as this was not the case, rainfall is not likely an explanation for the yearly variation observed in these two protozoan ectoparasites. It is important to note that these yearly differences reflect the ability of yearly variation to affect prevalence data and therefore several years of data should be used to determine long term trends in prevalence, or possibly presence/absence as well.

It is critical to mention that comments on the general parasitic load of a fish population are not appropriate. Some parasites have direct life cycles, such as copepods, where larvae and adults are found on the same host, whereas other parasites require multiple hosts as is the case for the aptly named digenetic trematodes (Hoffman 1999).

Therefore, the factors influencing direct life cycle parasites may be different than the factors influencing another parasite's intermediate host. Further, external parasites, such as protozoan parasites of the skins and gills, may be subjected to environmental conditions not encountered by internal parasites such as acanthacephalans or nematodes. Therefore, as various factors influence the presence and prevalence of parasites, it is not appropriate to make generalizations about the parasitic load of a population of fish, but to treat each parasite individually and identify the relationship of the parasite to the fish host and the environment.

4.6.6 Comments on taxonomy

During the current study, several limitations and problems were encountered when trying to determine the lowest taxonomic level for some parasites. Limited time precluded identification of some taxa of ectoparasitic ciliate to genus and species since special stains were not used. For example, use of a silver protein stain on the sessile peritrichs would highlight the oral and somatic ciliature, and elucidate the shape of both the macro- and micronuclei (Lom and Dykova 1992, Lynn and Small 2000). For trichodinids, mobile peritrichs, a silver nitrate stain is needed to show the details of the adhesive disc, and a Fuelgen's stain will highlight the location of the micronucleus in relation to the micronucleus, both features are needed for determination of species (Lom and Dykova 1992, Lynn and Small 2000).

Second, there appears to be a lack of adequate comprehensive keys. Many keys include only representative species, or are published only for specific regions or range of hosts (Margolis and Kabata 1988, Margolis and Kabata 1989, Hoffman 1999). Further, some keys only go to the genus level such as those found in Hoffman (1999) or Lynn and

Small (2000). It is generally required to find individual species descriptions within the literature to ultimately identify to the species level. Authors are systematically approaching this lack of keys in certain genera, such as Moravec for *Philometra* spp. (Moravec 2004, Moravec et al. 2004, Moravec and Ali 2005). This systematic approach would be helpful in taxa such as protozoans of the world not just one geographic region, copepodid crustaceans, and digenetic trematodes. Additionally, some parasites in this study were identified only *en section*. While higher order taxonomy identification may be possible, and occasionally identification to genus is possible, species identification is difficult in this situation as features of significance may be missing from a particular cut. As such, the photomicrographs presented in this study were chosen specifically to illustrate the majority of features of taxonomic significance required for identification.

Third, some species descriptions in the literature may be incorrect. For example, an updated key on *Philometra* spp. found within the host body cavity (Moravec et al., 2004) claims that the adult females of the nematode *P. rubra* are 10mm long whereas the original description from Leidy (1856) states that they are 4.5 inches long (approximately 11.5 cm) (Morevec et al. 2004). This discrepancy may purely be a typographical error, since 100 mm – not 10 mm – would be the metric length on the order of 4.5 inches.

Fourth, there exists the potential for intermediate individuals to be collected, or presentation of individuals that do not fit a description exactly. For example, following the key provided by Hoffman (1999), the isopods collected during this study fit the description of *Livoneca ovalis*, except for the length of the uropods. The key claims the uropods (balancing fins) of *Livoneca ovalis* are much longer than the terminal shield, whereas in the specimens collected in the current study, the uropods reach only to the end of the terminal shield (Hoffman 1999).

In light of the discussion above, these problems with taxonomy highlight the crucial importance of depositing reference material in established repositories for potential comparisons with future specimens.

4.6.7 Critique and recommendations

While the purpose of this study was to identify potential risk factors for mycobacterial infections in individual striped bass, and this aim was accomplished in all the age groups sampled (Chapter 5), this chapter describes those potential parasitic risk factors. Additionally, this project has produced a pictorial atlas of parasites, both grossly observable and *en section*, which will be useful as a reference for future studies. However generalized statements regarding the ecological aspects of striped bass parasites were unable to be made from these data as sample sizes were small. Further, as the study encompassed only three years, it is unknown whether the differences in species composition with Paperna and Zwerner's work (1976) is real or natural fluctuation. Future studies will be able to address such drawbacks with the following recommendations.

Some alterations in study methodology, such as taking quantitative density and intensity data, taking larger data sets, and performing parasitology on the entirety of the samples instead of a subset, would permit stronger comparative investigations of the ecological aspects. For example, ecological parasitology recommends taking semi-quantitative or quantitative density data, and quantitative intensity data (Bush et al. 1997), rather than the more simplistic and less accurate ranking, as used for the parasites in the present study. Larger data sets would additionally increase the robustness of the data, as sample sizes ≤ 40 have been shown to under- or over-estimate prevalence

(Marques and Cabral 2007). However it is important to note that the initial intent of this study was to determine parasite infection in relation to mycobacterial infection on individual fish, not to generate ecological prevalence data. Furthermore, given that such comprehensive studies usually operate within financial and temporal constraints, a strategy of sampling fewer age classes would allow more fish within each age class to be sampled, and thus strengthen the data sets for comparative purposes. Additionally, future studies should perform skin scrapes and gill biopsies on all the fish sampled, not only a sub-set, to increase the sample size.

4.6.8 New illustrations of parasites in tissue sections

Previous identification guides of parasites *en section* have been presented by Bruno et al. (2006) for fish, and Gardiner and Poynton (2006) have compiled veterinary parasites. Both highlight features of taxonomic significance, but stress it may be difficult to identify parasites in histologic section because sections may lack the required features. Therefore, the presentation of the photomicrographs in the current study were selected from a vast compilation of many sections, and chosen to highlight the features available *en section*. This compilation is intended to assist studies in striped bass health in that future studies may lack all the features required for identification, but may appear similar to the sections presented here and thus make identification possible.

4.6.9 Parasitic association with mycobacteria

One purpose of this study was to investigate whether parasites with traumatic sites of attachment potentially provide a portal for environmental mycobacteria to enter into the otherwise sterile internal host environment. Therefore, histologic sections of heavily

infected fish were acid-fast stained to attempt visualization of mycobacteria in association with parasites. Additionally, prevalence data was taken to identify 'hot spots' of parasitic infection to determine putative associations with 'hot spots' of mycobacterial infection. Sections of intestine heavily infected with acanthacephalan and of gills heavily infected with copepod were acid-fast stained, yet no acid-fast bacilli (AFB) were seen in relation to these parasites, either within the parasites or in the granulomatous tissue surrounding the point of attachment. These findings were contrary to Dove's (2005) anecdotal result for one bluefish, in which AFB+ were found both in the host granulomatous response as well as in the space between parasite and host (Dove 2005). During the study period, the Pocomoke River had a significantly higher prevalence of mycobacterial infections than the Potomac River in 0+ fish and versus the Upper Bay, the Choptank River and the Potomac River in 1+ fish. The Pocomoke River was also identified as a location with increased prevalence of parasites including a crustacean stage in their lifestyle. Therefore it appears as though there is an overlap of high prevalence of parasites and mycobacterial infections in the Pocomoke River, but it is important to note that the 'hot spots' identified in this paper only show geographical co-occurrence with mycobacterial infections. To find an association of parasites and mycobacterial infections, the association must be investigated within individual fish, not geographical regions (Chapter 5).

4.6.10 Conclusion

The purpose of this study was to i) compare the current assemblage with that found by Paperna and Zwerner (1976) 30 years ago, ii) derive spatially relevant data on parasitic loads of striped bass for a larger umbrella study investigating mycobacterial

infections in striped bass, and iii) provide a pictorial atlas of the parasitic fauna of Chesapeake Bay striped bass. The parasitic assemblage of striped bass has changed in the past 30 years, however it is unknown whether this is a real alteration or an artifact of study design. Parasites have been associated with mycobacterial infections (Chapter 5) and should therefore be included in further investigations into mycobacterial disease processes and natural reservoirs. Finally, this project has produced a visually rich overview of parasites commonly encountered in Chesapeake striped bass and will be an important reference tool for future studies into the health of this ecologically and economically important species.

4.7 Acknowledgements

This chapter is a draft of a manuscript and as such, co-authorship is listed in the title page. Dr. S. Poynton is acknowledged for her expertise, and key role and assistance in parasite identification, data analysis, and drafting of the results and figure captions. The authors would like to thank František Moravec of the Institute of Parasitology, Academy of Sciences of the Czech Republic for discussions on *Philometra* spp., Jim Salierno, Jaime Gonzales, Maddy Sigrist, Mohammed Ali, Eddy Johnson, Jessica Yen, and Jordyn Wolfand of the Aquatic Pathobiology Center (UMD) for assistance with field collections and processing, and the Sarbanes Cooperative Oxford Laboratory for histology services. This research was supported by grants from the Centers for Disease Control, the National Oceanic and Atmospheric Administration, and the Maryland Agriculture Experiment Station.

4.8 Tables and Figures

Table 4-1. Global Positioning System (GPS) coordinates of Chesapeake Bay sites sampled for striped bass *Morone saxatilis*.

		0+	1+ to 3+
Upper Bay		39° 22.5'N 75° 59.0'W to 39° 23.5'N 76° 02.4'W	Centered around 39° 04.1'N 76° 17.4'W
Choptank	Tred Avon	38° 41.7'N 76° 10.3'W	Centered around 38° 39'N
	Hambrooks	38° 35.2'N 76° 04.9'W	76° 17.0'W
	Horn Point	38° 35.3'N 76° 07.5'W	
Nanticoke	Roaring Point	38° 15.9'N 75° 55.1'W	None collected
	Ragged Point	38° 17.5'N 75° 54.4'W	
Pocomoke	Williams Point	37° 57.2'N 75° 39.3'W	Same as 0+
	East Creek	37° 58.8'N 75° 44.7'W	
Potomac	Tall Timbers	38° 10.2' N 76° 32.9'W	Centered around 38° 02.5'N
	Blake Creek	38° 12.3' N 76° 34.8'W	76° 20.0'W

Table 4-2. Prevalence of parasites in Chesapeake Bay striped bass *Morone saxatilis* by host age and site and year of capture. Sample size in parentheses.

	Upper Bay				Choptank				Nanticoke		Pocomoke		Potomac			
Age	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2004**	**2005**	**2003**	**2004**	**2005**	**2006**
Bodonid (flagellate)																
0+	50 (20)	50 (20)	-	-	27 (22)	20 (20)	88 (8)	-	83 (12)	0 (20)	42 (19)	80 (5)	36 (22)	29 (21)	-	-
1+	-	0 (3)	0 (6)	-	20 (10)	0 (3)	0 (8)	-	-	-	8 (12)	50 (22)	0 (2)	0 (7)	0 (2)	-
2+	0 (12)	0 (6)	0 (9)	-	10 (10)	25 (8)	0 (4)	-	-	-	0 (6)	90 (10)	0 (7)	33 (3)	0 (10)	-
3+	0 (7)	0 (1)	0 (11)	25 (8)	0 (3)	-	6 (17)	67 (3)	-	-	50 (2)	50 (6)	0 (4)	-	0 (7)	-
4+	-	-	0 (1)	3 (34)	-	-	0 (1)	3 (39)	-	-	0 (1)	-	-	-	0 (1)	26 (23)
Sessile Peritrichs (ciliates)																
0+	0 (20)	30 (20)	-	-	18 (22)	65 (20)	100 (8)	-	83 (12)	75 (20)	42 (19)	80 (5)	41 (22)	86 (21)	-	-
1+	-	33 (3)	67 (6)	-	60 (10)	0 (3)	63 (8)	-	-	-	75 (12)	50 (22)	0 (2)	29 (7)	100 (2)	-
2+	42 (12)	17 (6)	11 (9)	-	40 (10)	25 (8)	100 (4)	-	-	-	50 (6)	90 (10)	43 (7)	67 (3)	70 (10)	-
3+	29 (7)	0 (1)	27 (11)	13 (8)	67 (3)	-	59 (17)	0 (3)	-	-	50 (2)	50 (6)	25 (4)	-	29 (7)	-
4+	-	-	0 (1)	15 (34)	-	-	0 (1)	10 (39)	-	-	100 (1)	-	-	-	0 (1)	26 (23)

Table 4-2 cont. Prevalence of parasites in Chesapeake Bay striped bass *Morone saxatilis* by host age and site and year of capture. Sample size in parentheses.

	Upper Bay				Choptank				Nanticoke		Pocomoke		Potomac			
Age	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2004**	**2005**	**2003**	**2004**	**2005**	**2006**
Trichodina spp. (ciliates)																
0+	30 (20)	70 (20)	-	-	50 (22)	100 (20)	100 (8)	-	83 (12)	95 (20)	79 (19)	100 (5)	86 (22)	100 (21)	-	-
1+	-	100 (3)	17 (6)	-	60 (10)	100 (3)	100 (8)	-	-	-	83 (12)	45 (22)	0 (2)	43 (7)	100 (2)	-
2+	33 (12)	100 (6)	11 (9)	-	30 (10)	88 (8)	75 (4)	-	-	-	67 (6)	60 (10)	43 (7)	100 (3)	60 (10)	-
3+	43 (7)	100 (1)	9 (11)	13 (8)	33 (3)	-	41 (17)	33 (3)	-	-	50 (2)	50 (6)	75 (4)	-	29 (7)	-
4+	-	-	100 (1)	18 (34)	-	-	100 (1)	0 (39)	-	-	0 (1)	-	-	-	100 (1)	22 (23)
Philometra rubra (nematodes)																
0+	7 (120)	5 (58)	-	-	11 (121)	3 (60)	11 (27)	-	5 (88)	14 (120)	63 (19)	0 (9)	2 (122)	5 (60)	-	-
1+	-	36 (11)	40 (25)	-	80 (10)	64 (25)	87 (15)	-	-	-	58 (12)	78 (27)	0 (2)	74 (19)	70 (10)	-
2+	54 (13)	39 (18)	71 (24)	-	70 (10)	81 (16)	57 (23)	-	-	-	67 (6)	42 (26)	43 (7)	71 (14)	78 (36)	-
3+	29 (7)	0 (1)	32 (37)	0 (9)	100 (3)	-	63 (24)	75 (4)	-	-	50 (2)	76 (17)	25 (4)	-	77 (30)	-
4+	-	-	50 (2)	50 (34)	-	-	100 (1)	93 (58)	-	-	100 (1)	0 (1)	-	-	100 (2)	74 (58)

Table 4-2 cont. Prevalence of parasites in Chesapeake Bay striped bass *Morone saxatilis* by host age and site and year of capture. Sample size in parentheses.

	Upper Bay				Choptank				Nanticoke		Pocomoke		Potomac			
Age	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2004**	**2005**	**2003**	**2004**	**2005**	**2006**
Pomphorhynchus sp. (acanthacephalans)																
0+	-	0 (58)	-	-	-	0 (120)	0 (27)	-	-	20 (120)	58 (19)	22 (9)	-	0 (60)	-	-
1+	-	27 (11)	4 (25)	-	-	12 (25)	33 (15)	-	-	-	83 (12)	63 (27)	-	5 (19)	10 (10)	-
2+	-	22 (18)	17 (24)	-	-	6 (16)	9 (23)	-	-	-	67 (6)	31 (26)	-	21 (14)	22 (36)	-
3+	-	0 (1)	41 (27)	100 (9)	-	-	13 (24)	25 (4)	-	-	50 (2)	76 (17)	-	-	20 (30)	-
4+	-	-	50 (2)	59 (34)	-	-	0 (1)	55 (58)	-	-	0 (1)	100 (1)	-	-	0 (2)	21 (58)
Ergasilus spp. (crustaceans)																
0+	24 (120)	14 (58)	-	-	10 (121)	0 (20)	4 (27)	-	0 (12)	4 (120)	37 (19)	33 (9)	14 (22)	3 (60)	-	-
1+	-	100 (11)	100 (25)	-	60 (10)	84 (25)	87 (15)	-	-	-	75 (12)	94 (33)	100 (2)	89 (19)	90 (10)	-
2+	100 (13)	100 (18)	96 (24)	-	60 (10)	63 (16)	100 (23)	-	-	-	83 (6)	96 (26)	86 (7)	86 (14)	100 (36)	-
3+	100 (7)	100 (1)	100 (27)	100 (8)	33 (3)	-	100 (24)	100 (4)	-	-	50 (2)	100 (17)	50 (4)	-	90 (30)	-
4+	-	-	100 (2)	100 (34)	-	-	100 (1)	90 (58)	-	-	0 (1)	100 (1)	-	-	50 (2)	91 (58)

Table 4-2 cont. Prevalence of parasites in Chesapeake Bay striped bass *Morone saxatilis* by host age and site and year of capture. Sample size in parentheses.

	Upper Bay				Choptank				Nanticoke		Pocomoke		Potomac			
Age	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2005**	**2006**	**2003**	**2004**	**2004**	**2005**	**2003**	**2004**	**2005**	**2006**
Livoneca ovalis (crustaceans)																
0+	0 (120)	0 (58)	-	-	4 (121)	23 (60)	4 (27)	-	0 (88)	17 (120)	26 (19)	0 (9)	2 (122)	10 (60)	-	-
1+	-	18 (11)	0 (25)	-	0 (10)	8 (25)	0 (15)	-	-	-	25 (12)	63 (27)	0 (2)	11 (19)	20 (10)	-
2+	8 (13)	0 (18)	0 (24)	-	0 (10)	0 (16)	0 (23)	-	-	-	17 (6)	31 (26)	0 (7)	0 (14)	3 (36)	-
3+	0 (7)	0 (1)	0 (27)	0 (8)	0 (3)	-	8 (24)	0 (4)	-	-	0 (2)	7 (14)	0 (4)	-	3 (30)	-
4+	-	-	0 (2)	0 (34)	-	-	0 (1)	0 (58)	-	-	0 (1)	0 (1)	-	-	0 (2)	2 (58)

Table 4-3. Comparison of parasitic taxa of Chesapeake Bay striped bass *Morone saxatilis*.

	Presence		
Parasite	**Paperna and Zwerner (1976)**	**Current Study**	**Comments**
Protozoa			
Trichodina davisi	+	?	The current study found the same genus, but species is unknown.
Trichodina sp.	-	+	Found on gills and skin
Trichodinella sp.	+	-	
Epistylus sp.	+	-	
Glossatella sp.	+	?	Genus renamed as *Apiosoma*. Further characterization required of current peritrichs to determine similarity.
Solitary sessile peritrich type 1	-	+	Possible *Apiosoma* sp. found on gills

Schyphidia sp.	+	-	Genus renamed as *Riboscyphidia*
Ambiphyra sp.	-	+	Found on gills
Colponema sp.	+	?	The current study has a bodonid flagellate, however genus is unknown.
Bodonid	-	+	Found on skin and gills, however free-living, not attached
Trichophyra sp.	+	-	
Myxosoma morone	+	-	Not sought in current study (cartilage and bones)
Kudoa cerebralis	+	-	Not sought in current study (meninges of brain and spinal cord)
Nosema sp.	+	-	
Oodinium sp.	+	-	

Metazoa			
Monogenea			
Gyrodactylus sp.	+	-	
Ancyrocephalinae	+	-	
Microcotyle macrura	+	-	
Digenea			
Leptocreadium setiferoides	+	-	Not sought in current study (intestinal lumen)
Leptocreadium areolatum	+	-	Not sought in current study (intestinal lumen)
Stephanostmum tenue	+	-	Not sought in current study (intestinal lumen)
Immature opecoelids	+	-	Not sought in current study (intestinal lumen)

Digenea sp. 1	+	-	Not sought in current study (intestinal lumen)
Digenea sp. 2	+	-	Not sought in current study (intestinal lumen)
Metacercariae			
Neascus sp.	+	-	
Diplostomulum sp.	+	-	
Ascocotylid type sp.	+	+	Found in spleen, bulbous arteriosis, liver and brain
Clinostomum marginatum	+	-	
Metacercariae mystery	-	+	Larval digene found in gills
Cestodes			

Proteocephalid larvae type A	+	?	Descriptions not in previous paper to determine similarity with current study
Proteocephalid larvae type B	+	?	Descriptions not in previous paper to determine similarity with current study
Scolex pleuronectis	+	-	
Trypanorhynchid pleuroceroid	+	-	
Cyclophyllidian adult	-	+	Found in intestinal lumen
Nematoda			
Philometra rubra	+	+*	Found in peritoneal cavity
Cuculanus sp.	+	-	Not sought in current study (intestinal lumen)
Spinithectus sp. (larvae)	+	-	
Goezia sp. (larvae)	+	-	
Eustrongylides sp. (adult/larvae)	-	+#	Found in gonad

Aphasmid nematodes (larvae)	-	+	Found within intestinal lamellar folds. Some individuals with putative symbiotic bacteria on cuticle
Phasmid nematodes (larvae)	-	+	Encapsulated in somatic musculature
Nematode type 1 (female adult)	-	+	Found in bulbous arteriosis
Nematode type 2 (adult/larvae?)	-	+	Found in lumen of anterior kidney vessel
Acanthocephala			
Pomphorhynchus rocci (adult)	+	+*	Found in intestinal lumen with proboscis inserted through intestinal wall
P. rocci (larvae)	+	-	
Crustacea			
Ergasilus labracis	+	?^	The current study found same genus
Ergasilus lizae	+	?^	The current study found same genus

Ergasilus sp.	-	+	Found on gills
Argulus bicolor	+	?^	
Argulus sp.	-	+	Found on skin
Caligus sp.	+	-	
Livoneca ovalis	+	+	Found within gill chamber
Aegathoa oculuata	+	?	Genus no longer recognized, was used for many Cymothoid juveniles
Hirudinea			
Myzobdella lugubris	+	?	Current study had a leech, however genus and species unknown
Mollusca			
Glochidia	+	-	

*Identity to species level difficult because original species descriptions were incomplete or confusing. Therefore, since parasites were found within the same host, in the same location within the host, we assumed same species.

Zoonotic

^ Morphologically the current study's individuals did not agree with species descriptions

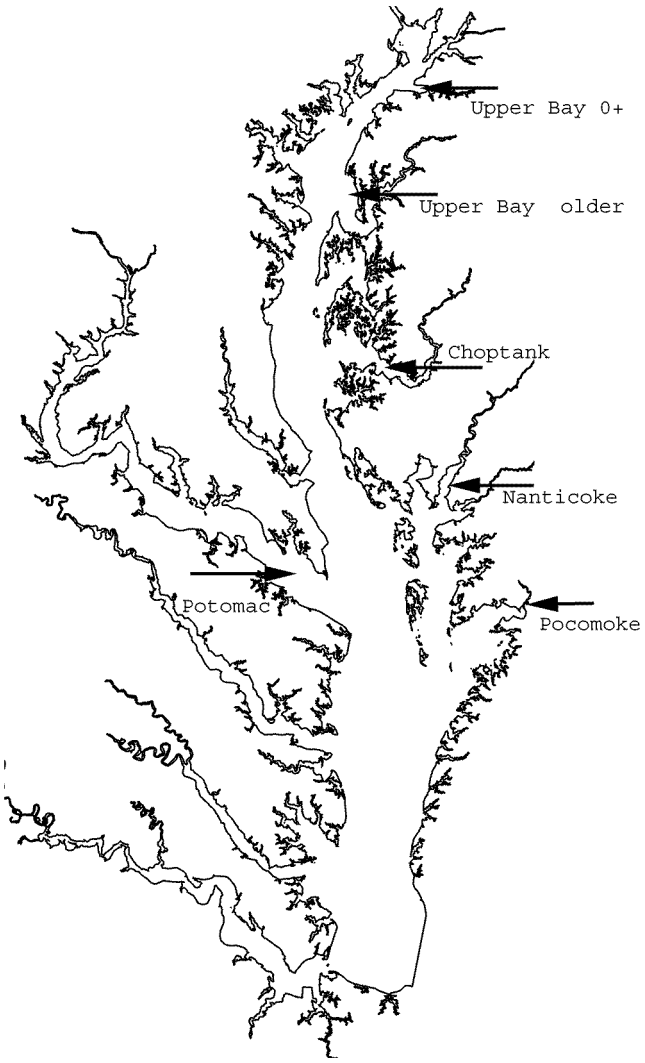

Figure 4-1. Map of the Chesapeake Bay with sites sampled for striped bass *Morone saxatilis* indicated by arrows. Line drawing provided by Maryland Sea Grant.

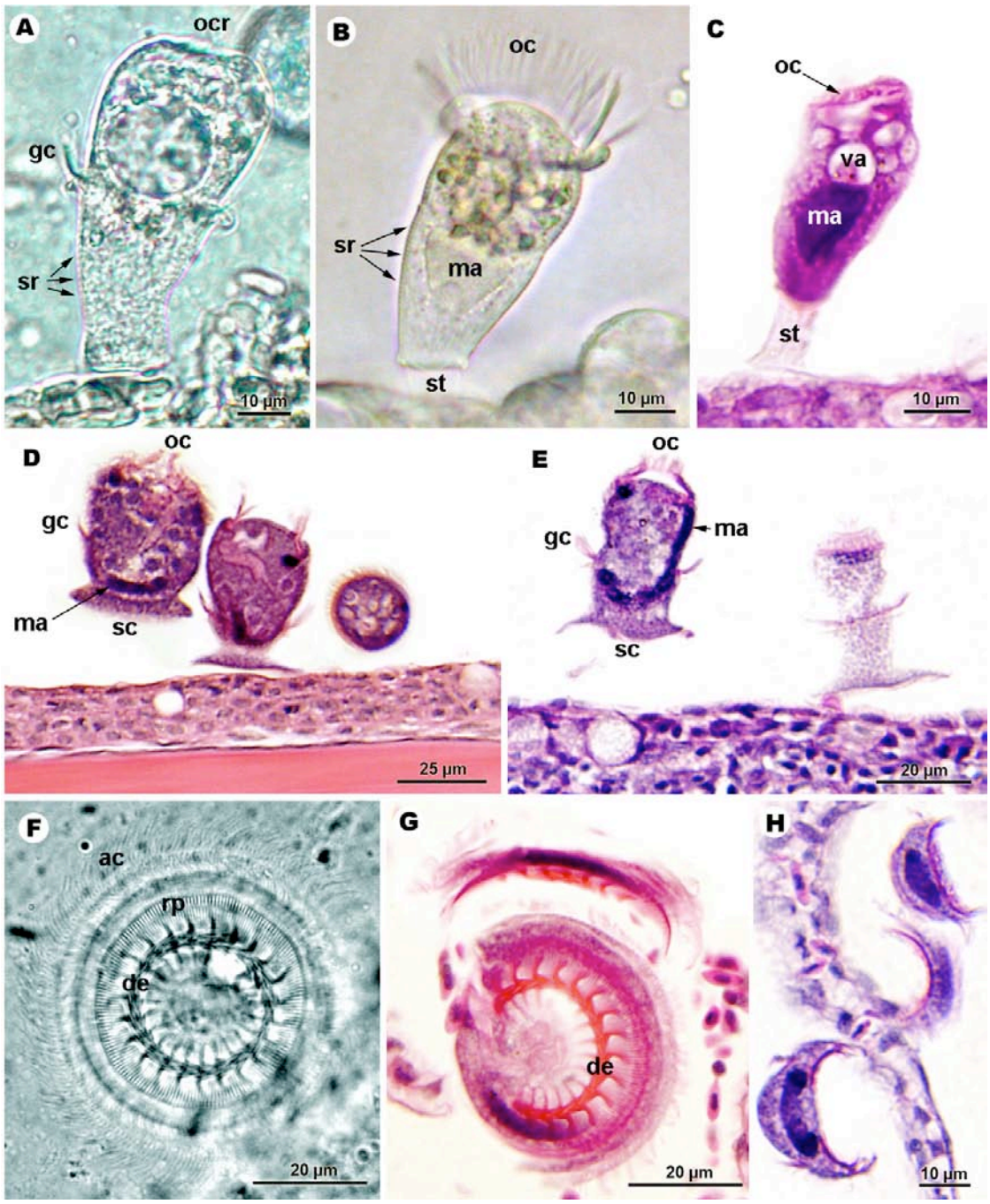

Figure 4-2. Peritrich ciliates from striped bass, *Morone saxatilis,* from the Chesapeake Bay. (A) Sessile peritrich, fresh mount. The oral cilia are retracted in this individual (ocr), and the girdle of cilia (gc) and transverse striations (sr) are easily recognizable. Note conical body shape. (B) Stalked sessile peritrich, fresh mount. Note the oral cilia

(oc) directed away from the host surface, the large conical macronucleus (ma), the non-contractile stalk (st) that attached the zooid to the host, and transverse striations (sr) of the surface; compare with (f). (C) Stalked sessile peritrichs similar to (B) in histological section of skin. Note the oral cilia (oc) directed away from the host surface (the oral cilia are retracted and less easy to see in this individual – compare with (B)), the large conical macronucleus (ma), vacuoles (va) in the cytoplasm, and the non-contractile stalk (st) that attached the zooid to the host. (D, E) Sessile peritrichs in histological section of skin, four organisms in longitudinal section, one in transverse section. Note the oral cilia (oc) directed away from the host surface, girdle of cilia (gc), broad scopula for attachment to the host (sc), and profiles of the long, ribbon-like macronucleus (ma). Note differences in body shape, barrel-like in (D) and more cylindrical in (E). (F) *Trichodina* sp., fresh mount showing adoral surface. Note girdle of adoral cilia (ac), and the characteristic adhesive disc with interlocking denticles (de), surrounded by radial pins (rp). (G, H) *Trichodina* sp, in histological section of gills. Note the concave adhesive disc with refractile denticles (de). All histological sections are stained with hematoxylin and eosin.

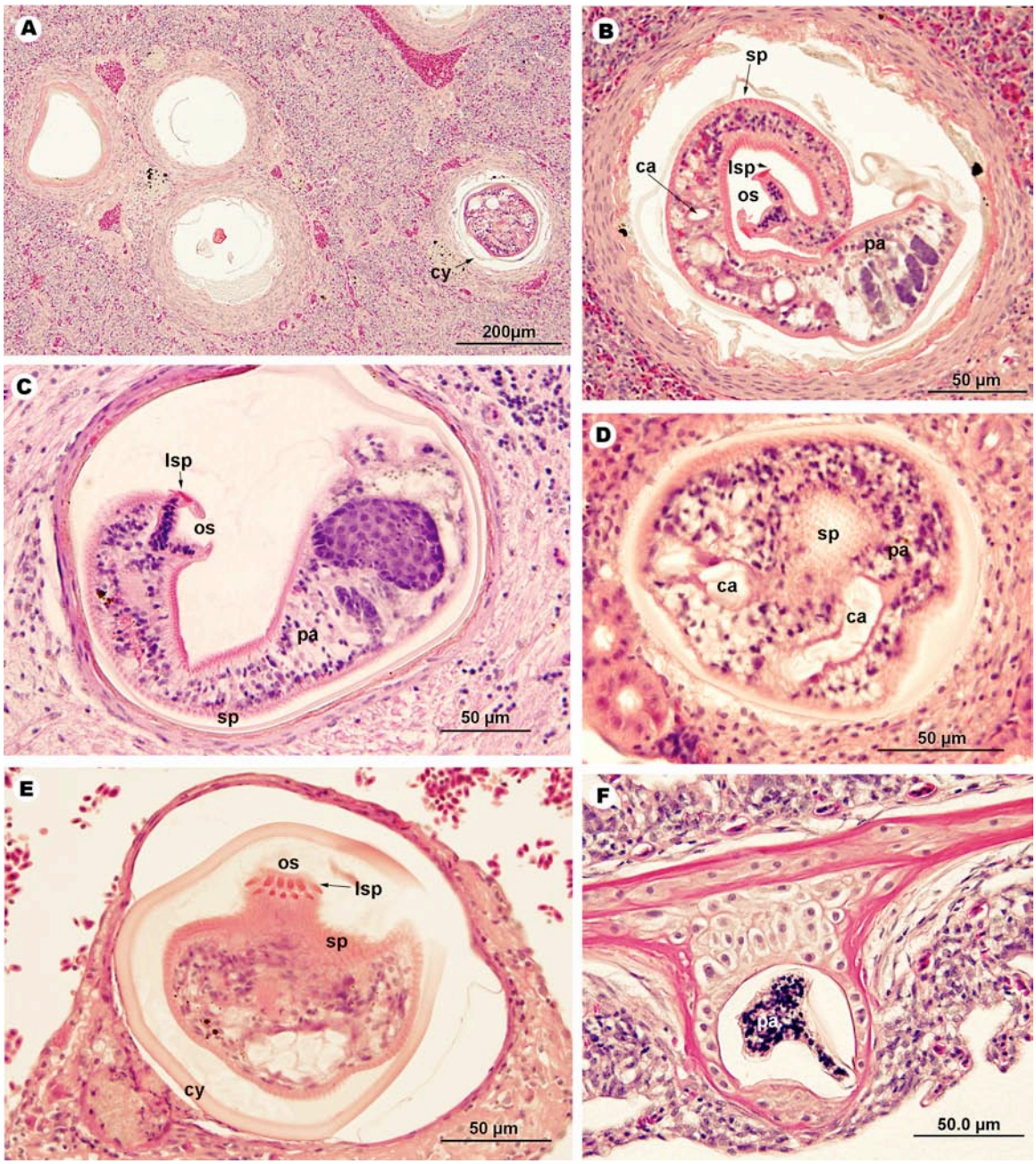

Figure 4-3. Metacercaria (encysted larval trematodes) from striped bass, *Morone saxatilis,* from the Chesapeake Bay. (A) Spleen with encysted metacercaria (right), and similar lesions with parasites missing from the section (left and center). The parasite is enclosed in a cyst (cy). (B) Longitudinal section of metacercaria in the spleen. The parasite is distinguished by a spinous tegument, with spines (sp) being more prominent

anteriorly than posteriorly, there are also large spines (lsp) around the oral sucker (os). Parenchyma (pa) fills the body cavity, and there are intestinal caecae (ca). (C) Longitudinal section of metacercaria in the brain. The parasite is distinguished by a spinous tegument, with spines (sp) being more prominent anteriorly than posteriorly, there are also large spines (lsp) around the oral sucker (os). Parenchyma (pa) fills the body cavity. (D) Grazing section of metacercaria in posterior kidney. Note the spinous tegument (sp), parenchyma (pa) filling the body cavity, and paired intestinal caecae (ca). (E) Longitudinal section through anterior end of metacercaria encysted in the bulbus arteriosis. Note the thick cyst (cy), the spinous tegument (sp), and the large spines (lsp) around the oral sucker (os). (F) Metacercaria in the gill. Note the dense parenchyma (pa) that fills the body cavity. All histological sections are stained with hematoxylin and eosin.

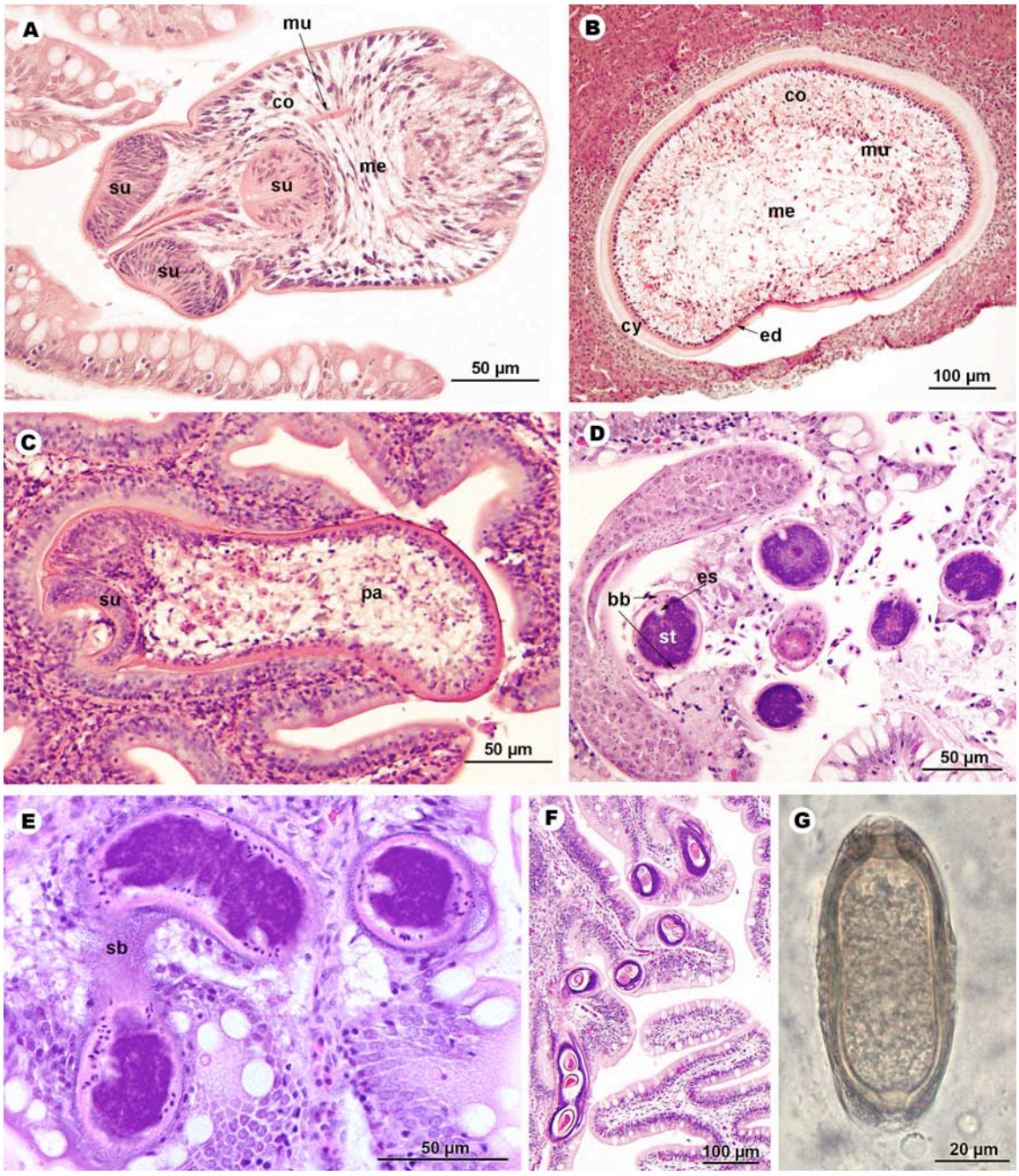

Figure 4-4. Cestodes and aphasmid nematodes from striped bass, *Morone saxatilis,* from the Chesapeake Bay. (A) Scolex (holdfast organ) of cyclophyllidian cestode in lumen of intestine. Cyclophyllidians are characterized by an acetabulate scolex with four suckers (su), three of which can be seen in this section, and circular muscles (mu) that divide the

parenchyma into cortical (co) and medullary regions (me). (B) Plerocercoid cestode larva encysted in the liver. At the periphery are the dense, basophilic, epidermal cells (ed), and circular muscles (mu) dividing the parenchyma into a more dense cortical region (co) and a less dense medullary region (me). The parasite lacks a digestive tract. A cyst (cy) is closely apposed to the surface of the parasite. (C) Anterior end of a platyhelminth in the intestine. The sucker (su), parenchyma (pa) and basophilic cells at the periphery, coupled with the absence of a digestive tract, are consistent with a cestode; however firm identification is difficult with the limited number of taxonomically important features present in this section. (D) Five transverse sections of anterior ends, and one longitudinal section (right), of aphasmid nematodes within the intestinal tissue. Note the characteristic hypodermal bacillary bands (bb), and the stichosome (row of esophageal gland cells or stichocytes) (st) surrounding the esophagus (es). (E) Aphasmid nematodes within the folds of the intestine. Note the symbiotic basophilic rod-shaped bacteria (sb) covering the surface of the cuticle. (F) Nematodes within the intestinal tissue. Note the bipolar plugged eggs, characteristic of aphasmid nematodes. (G) Fresh preparation of a bipolar, plugged, unembryonated, nematode egg. Egg morphology is consistent with the aphasmid group. All histological sections are stained with hematoxylin and eosin.

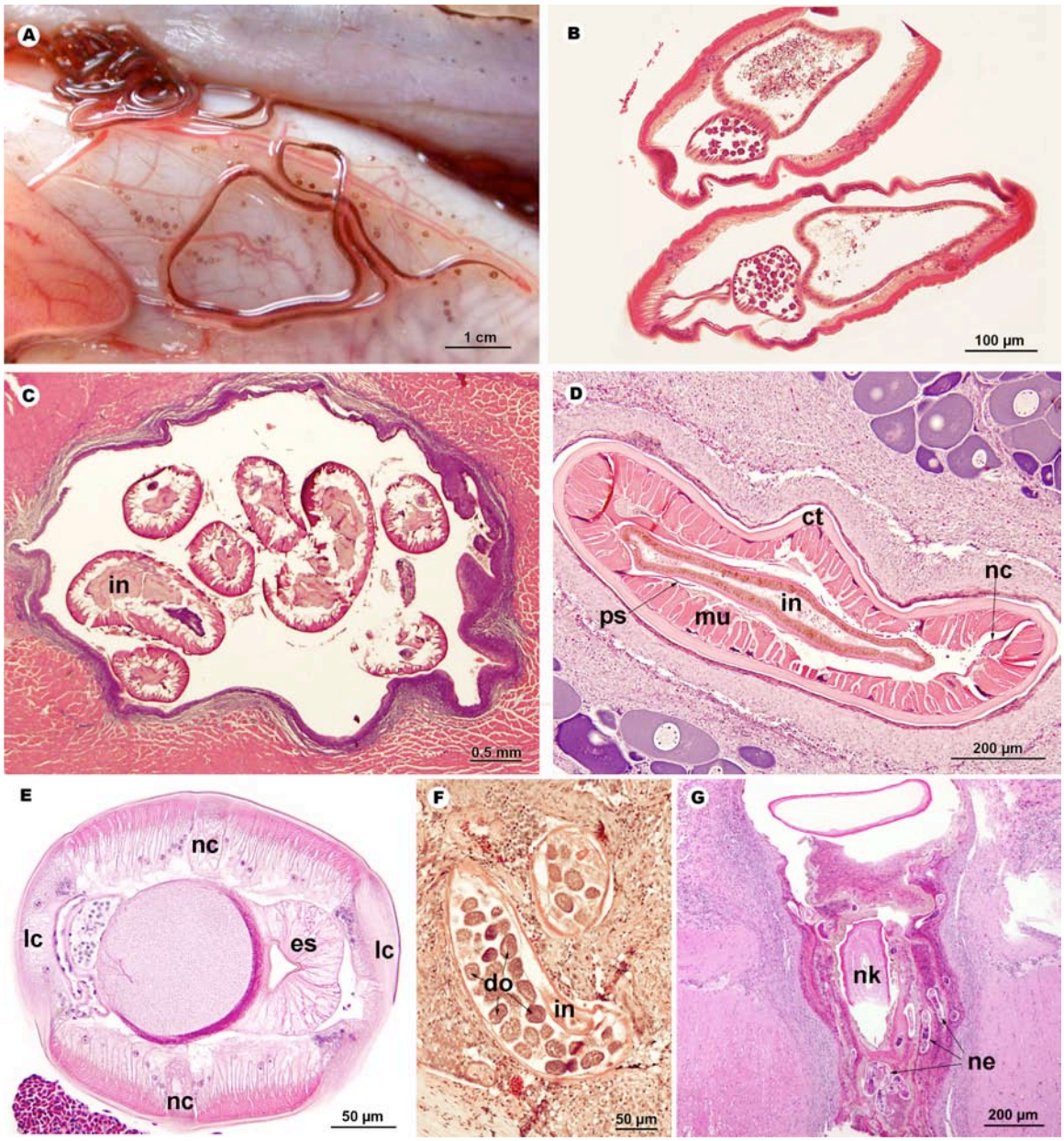

Figure 4- 5. Nematodes from striped bass, *Morone saxatilis,* from the Chesapeake Bay. (A) Adult *Philometra* sp. (phasmid) in the body cavity. Note the characteristic red color of these nematodes. (B) Oblique sections through two nematodes from the body cavity. (C) Transverse section of eight nematodes encapsulated in somatic musculature. The intestine (in) is large relative to the diameter of the nematode. (D) Transverse section of *Eustrongylides* sp. nematode encapsulated in the ovary. Note the cuticle (ct) is very thick,

the musculature (mu) is distinctly Eustrongylid, and the intestine (in) is large relative to the diameter of the worm. Nerve chords (nc) and pseudomembranes (ps) are present. Note the inflammatory infiltrate among the ova. (E) Transverse section of nematode from lumen of vessel in anterior kidney. The lateral chords (lc) are low and broad, ventral and dorsal nerve chords (nc) protrude, the esophagus (es) is lined with cuticle and has a tri-radiate lumen. (F) Transverse section (top) and oblique section (bottom) through adult female nematodes in bulbous arteriosis of heart. The intestine (in) is small relative to the diameter of the nematode, developing ova are distinct (do). (G) Numerous nematodes (ne) associated with the neck (nk) of the presoma of an acanthacephalan penetrating from the lumen to the serosal surface of the intestinal wall. All histological sections are stained with hematoxylin and eosin.

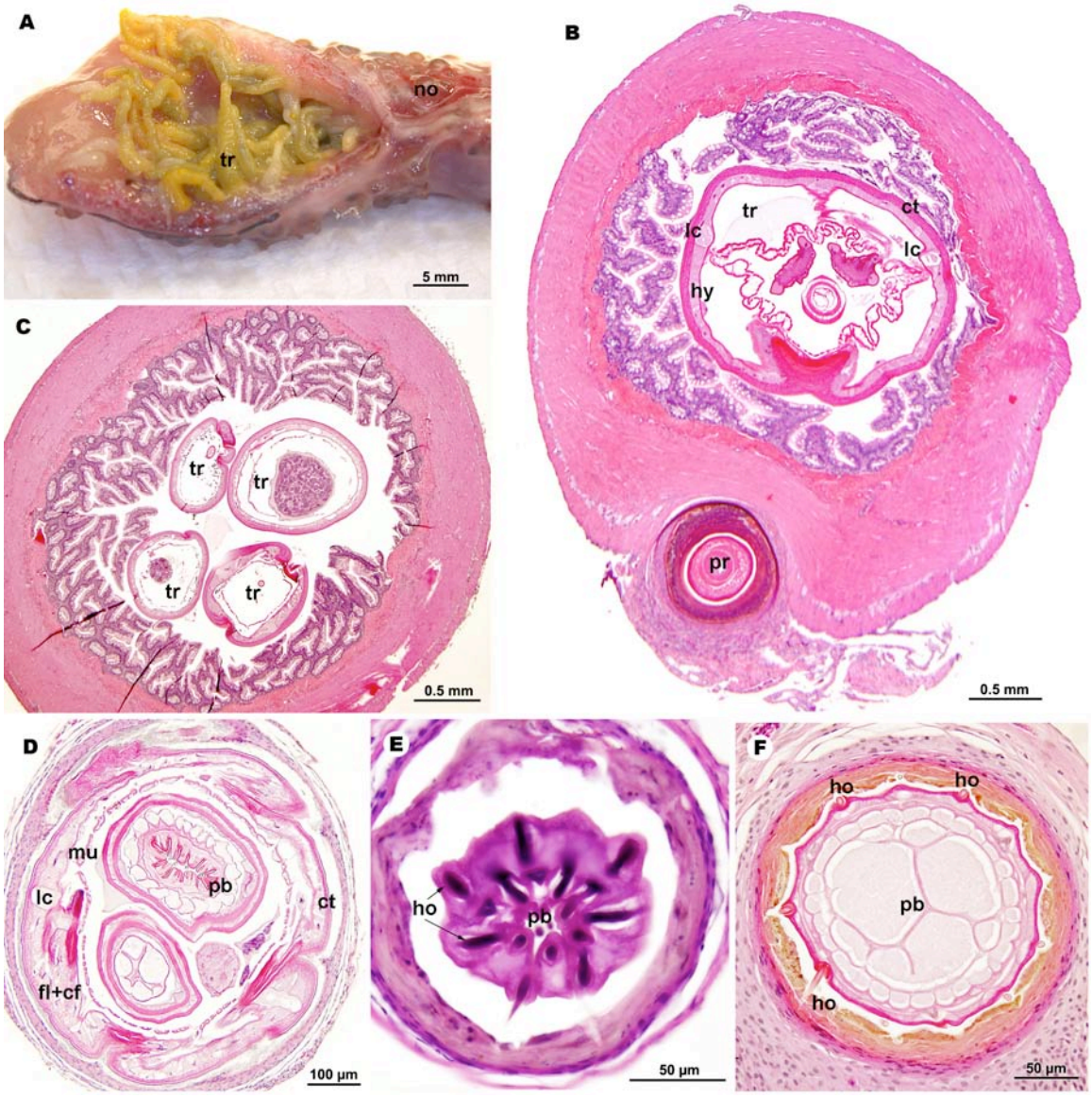

Figure 4-6. The acanthocephalan *Pomphorhynchus* sp. from the posterior intestine of striped bass, *Morone saxatilis,* from the Chesapeake Bay. (A) Gross view of opened posterior intestine showing heavy infection. The trunk (tr) (metasoma) of the worm hangs in the lumen, the neck penetrates through the wall of the intestine, and the presoma comprising the inflated bulb and the proboscis, encapsulated by host tissue, is visible as a bulb/nodule (no) on the serosal surface. (B) Transverse section of intestine with section

of trunk (tr) in the lumen, and section of presoma (pr) encapsulated by host tissue on the serosal surface. The worm has a cuticle (ct), a thick hypodermal layer (hy) comprising a felted layer under the cuticle and a layer of cross fibers containing lacunar channels (lc), and a thin layer of muscles lining the pseodocoelom; there is no digestive tract. (C) Transverse section of intestine with sections of four trunks (tr) in the lumen. (D) Transverse section of presoma encapsulated by host tissue on the serosal surface. In this section the proboscis (pb), with inverted hooks, is surrounded by the bulbus in which the characteristic layers can clearly be seen: cuticle (ct), felted layer and cross fibers (fl+cf), lacunar channels (lc) and muscles (mu). (E, F) Transverse section of probosces (pb) encapsulated by host tissue on the serosal surface of the intestine. In (E) the proboscis is partially retracted, as evident by the hooks (ho) pointing inwards, in (F) this section of a proboscis is fully everted, as evident by all the hooks (ho) pointing outwards. There are 12 hooks per cross-section. All histological sections are stained with hematoxylin and eosin.

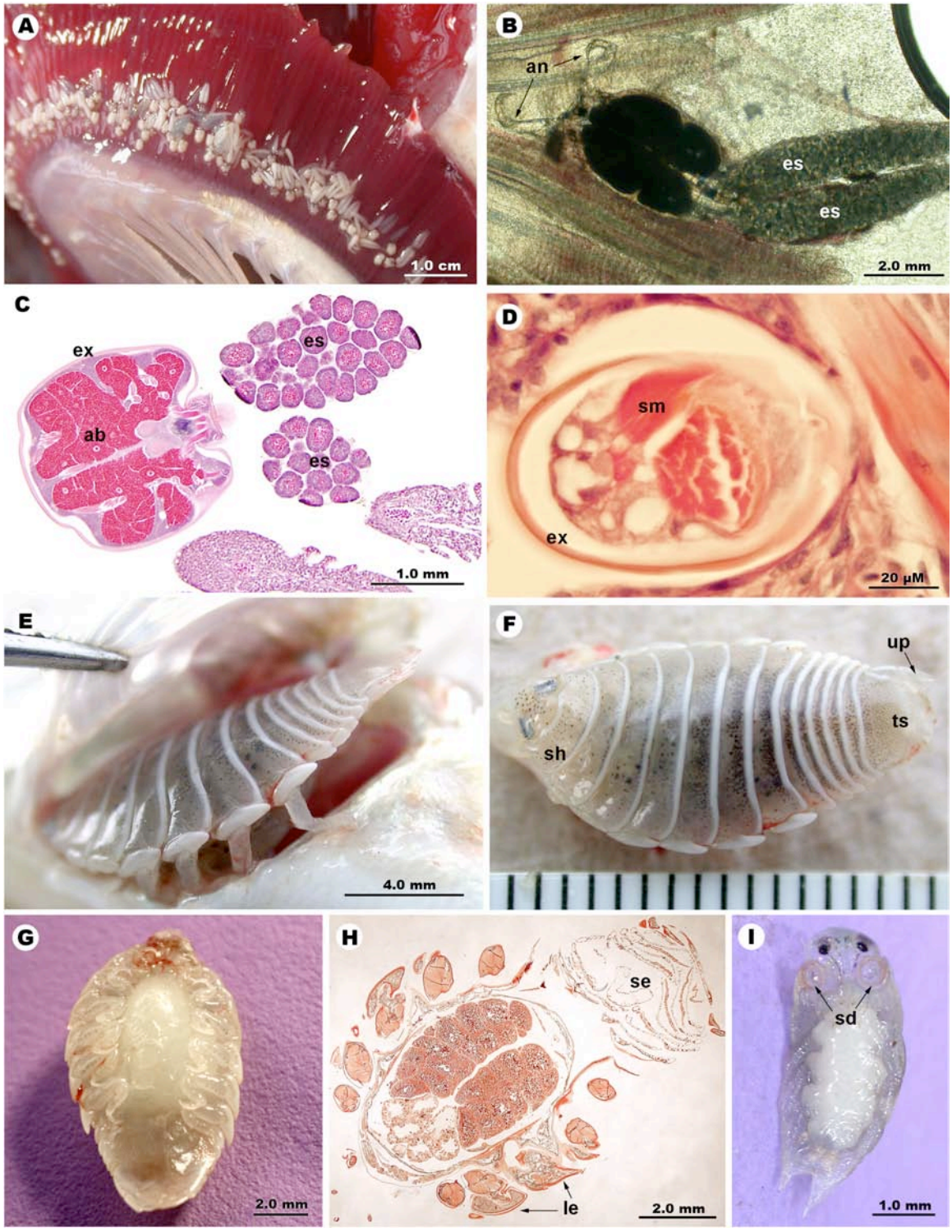

Figure 4-7. Ectoparasitic crustaceans from striped bass, *Morone saxatilis,* from the Chesapeake Bay. (A) Heavy infection of the copepod *Ergasilus* sp. on the proximal

region of the gills. (B) Adult female *Ergasilus* sp., note the second antennae (an) are modified into claw-like structures for attachment and there are paired egg sacs (es). (C) Adult female *Ergasilus* sp., in histological section, longitudinal section. Note the refractile chitinous exoskeleton (ex) of the abdomen (ab), and the paired egg sacs (es). (D) Adult female *Ergasilus* sp. in histological section, transverse section of modified second antennae embedded in the gill. The refractile chitinous exoskeleton (ex) can be seen, along with striated muscle (sm). (E) The isopod *Livoneca* sp. in the gill chamber, lateral view. (F) *Livoneca* sp., dorsal view. Note in this species, the shoulders (sh) do not surround the head, and at the posterior, the uropods (up) are longer than the terminal shield (ts). Ruler is in mm. (G) *Livoneca* sp., ventral view. (H) *Livoneca* sp. in histological section, longitudinal section. Note the segmented body (se) and the paired, jointed legs (le). (I) The branchiuran *Argulus* sp. Ventral view. Note the maxillules which serve as sucking discs (sd). All histological sections are stained with hematoxylin and eosin.

Chapter 5: Mycobacterial infections in resident Chesapeake Bay striped bass *Morone saxatilis*

5.1 Abstract

Striped bass, aged 0+ to 3+, were collected from the Upper Bay, the Choptank River, the Nanticoke River, the Pocomoke River and the Potomac River in 2003-2005 to investigate the risk factors associated with mycobacterial infections. In addition, gonads were sampled from spawning stock in the spring seasons of 2003 and 2004 to determine the possibility of vertical transfer of mycobacterial infections. Gonads of spawning stock were positive for mycobacterial species, but no differences were found between rivers or genders. Further, acid-fast bacilli were observed within granulomas in gonads and aseptically cultured from gonads. Three multivariate models of mycobacterial infection were fit to the data in order to account for putative variations in migratory behavior at different ages. 0+ striped bass remain within their natal river, and location of capture other than the Pocomoke River (POR=0.06 for Potomac River), presence of grossly observable parasites (POR=8.86) and presence of bacteria other than mycobacteria (POR=0.27) were associated with prevalence of mycobacterial infections. 1+ fish remain close to their natal river, and only location of capture other than the Pocomoke River (POR ranged from 0.02 to 0.36) was associated with prevalence of infection. 2+ and 3+ striped bass generally migrate within the Chesapeake Bay and presence of acanthacephalans in the intestine (POR=1.74) and presence of moderate to severe trichodinid infections (POR=3.15) on the skin and gills were associated with prevalence of mycobacterial infection. The existence of multiple risk factors, and the variability

introduced into the models by age-dependant life history factors, indicate mycobacterial infections in Chesapeake Bay striped bass may be a result of a series of complex processes.

5.2 Introduction

Striped bass, *Morone saxatilis,* with ulcerative lesions were first observed in the Chesapeake Bay in 1997. It wasn't until 2001, however, that published reports described the causative agent of these diseased fish as mycobacteria (Heckert et al. 2001, Rhodes et al. 2001). Multiple *Mycobacterium* spp., including three newly described strains, *M. shottsii*, *M. pseudoshottsii* and *M. chesapeaki*, were isolated from the visceral organs of lesioned fish, and mycobacteria have subsequently been reported to infect over 50% of the Chesapeake Bay striped bass population (Heckert et al. 2001, Rhodes et al. 2001, Rhodes et al. 2003, Rhodes et al. 2004, Rhodes et al. 2005). While the current epizootic of mycobacterial infections resulting in lesioned fish began in 1997, recent evidence indicates that mycobacteria have been present in Chesapeake Bay striped bass since the mid 1980's, and sequencing data speciated the causative agent as *M. pseudoshottsii* (Jacobs 2007). Published reports further suggest that these infections in striped bass are not confined to the Chesapeake Bay. For example, 7-54% of striped bass from Delaware Bay were positive for mycobacteria by either culture or histology, and 18% of striped bass sampled from the Roanoke River, NC, were positive for acid-fast bacilli by histology (Overton et al. 2006, Ottinger et al. 2007). Further, striped bass are not the only affected host species in the Chesapeake Bay as multiple fish species, including Atlantic menhaden, *Brevoortia tyrannus,* and white perch, *Morone Americana*, have been infected

with mycobacteria (Kane et al. 2007). Therefore, the extent of the current mycobacterial epizootic appears to be wider than previously thought, and Chesapeake Bay striped bass may only be the tip of the proverbial iceberg.

Previous reports of mycobacterial infections have concentrated primarily on prevalence and the *Mycobacterium* spp. associated with infections in older striped bass. However less is known about natural reservoirs and the progression and epidemiology of mycobacteriosis in this population. Studies are underway, however, to investigate very early skin lesions believed to be pathognomonic of the disease (Vogelbein et al. 2006). These early lesions are focal pigmented "spots," underneath which are granulomas that may contain acid-fast bacilli as viewed *en section*. Additionally, a tag-recapture study is currently underway to suggest the fate of these lesioned fish, aimed at investigating the progression of the lesions on individual fish and whether lesioned fish are recaptured at the same rate as non-lesioned fish (Vogelbein et al. 2006). While the fate of lesioned/infected fish is unknown, evidence based on the presence of acid-fast bacilli within granulomas *en section*, suggests gender has no effect on prevalence at younger ages (data presented in this chapter), but males 4+ and older seem to have a higher prevalence than females of the same age (Gauthier et al. 2006).

With the current lack of knowledge of the epidemiology of mycobacterial infection in Chesapeake Bay striped bass, the current study was designed to target the younger, typically resident fish, i.e., 0+ to 3+ fish to 1) identify potential risk factors including parasitism or infection with other bacteria, 2) determine age at infection and suggest age-based prevalence trends and 3) identify spatial distribution of infections.

5.3 Methods

5.3.1 Sampling sites and fish collections

Striped bass were collected from five sites in the Chesapeake Bay: the Upper Bay and the Choptank, Nanticoke, Pocomoke and Potomac rivers (Figure 5-1; Table 5-1). These sites were chosen since they have been historically used by Maryland Department of Natural Resources (MD DNR) in stock assessment, they represent mid- and upper Bay nursery grounds, and sampling could be facilitated though direct access or with assistance from MD DNR.

Collections were carried out during Summer and Fall seasons of 2003-2005 using beach seine, cast net, pound net, and hook and line. In general, 0+ fish were collected in July and August while older fish were collected August through October. Choptank River collections occurred first and were followed by collections from the Upper Bay, the Potomac and the Nanticoke rivers. Collections from the Pocomoke River were the exception where 0+ and older fish were collected simultaneously anywhere from July to August. The purpose was to provide a snapshot of each age class per river per year.

After collection fish were immediately transported live to the laboratory in oxygenated coolers or circular holding tanks. Time from collection to processing was under 8 hours and typically less than 4 hours. Survival to the time of processing was typically 100%. Fish were euthanized with an overdose of buffered tricaine methanesulfonate (MS222, Sigma-Aldrich, St. Louis, MO). Morphometric data was collected for length and weight, and fish were observed for external lesions and parasites. Parasitology data collection is described below. Age of striped bass was determined by length-at-age data and a subset was confirmed by scale analysis.

Gamete samples were taken from mature striped bass from the Choptank and Nanticoke rivers during the spring spawning run in April of 2003 and 2004. Thirty males and 30 females were collected by electro-fishing and were anaesthetized with MS222. The vent was sterilized with 70% ethyl alcohol. Milt was expressed directly into sterile collection tubes. Eggs were collected by inserting a modified sterile 5mL serological pipette into the vent. Samples were transported to the laboratory on ice. Sampled fish were returned to river water, observed until anesthesia wore off, and seen to swim away.

In 2006, 3+, 4+ and 5+ fish were collected by hook and line. Visceral organs were taken aseptically and processed for virology as described below.

5.3.2 Bacteriology

Bacteriology samples of liver, anterior kidney and brain were taken aseptically, and were plated on 1% Tripticase Soy Agar and inoculated in 1% Tripticase Soy Broth (Difco Laboratories, Detroit, MI). Isolates were identified using routine biochemical procedures (specifically gram stain, oxidase, catalase, fermentation O/F, and 0/129 sensitivity), 20E and 20NE API strips (Biomerieux, Marcy l'Etoile, France) and the Microbial Identification System associated with gas chromatography (MIDI Inc., Newark DE; Sasser 1990).

Spleens were aseptically removed, stomached with 2mL Butterfield's Phosphate-Buffered Dilution Water (B-PBS; Anonymous 2002) for two minutes, and 0.5mL of the homogenate was spread plated on Middlebrook 7H10 agar supplemented with Bacto Middlebrook OADC (Difco Laboratories, Detroit, MI). Plates were checked for growth every week for 12 weeks. Colonies with mycobacterial morphology were stained for

Ziehl–Neelsen acid-fastness. Characteristic acid-fast positive colonies were subsequently isolated on Middlebrook agar and when sufficient growth had occurred, fatty acid methyl ester (FAME) profiles were determined from colony growths by gas chromatography for identification. Briefly, colony samples were saponified for 30 min, methylated for 10 min, and then subjected to an acid wash and a base wash (MIDI 2002). FAME profiles of the prepared samples were generated on a gas chromatograph (Hewlett-Packard Company, Palo Alto, CA) and analyzed with MIDI software (Sasser 1990). Types and numbers of mycobacterial colonies were counted on each plate at 12 weeks. Kidney, liver and gonad samples were also routinely taken in 2005 and processed for mycobacteria isolation.

5.3.3 Histology

Tissues routinely taken for histology included the spleen, liver and anterior kidney. Gills were also routinely taken in 2003 for the young-of-the-year. Sample parasites and tissues were preserved in 10% neutral buffered formalin or 70% ethanol, and processed for routine histology (Profet et al. 1992). Sections were cut 7µm thick and stained with hematoxylin and eosin (H&E) or with aqueous acid-fast (Della Speranza and Fail 2000).

Granulomas observed in H&E sections for each internal organ were categorized as 1) 'bacterial', 2) 'parasitic' or 3) 'other' when the etiology was unclear. Acid-fast stained sections of granulomas were further characterized as 1) acid-fast bacillary, 2) having non-specific staining or 3) acid-fast negative. Each type of granuloma for each organ was ranked on a relative, qualitative scale of 1-3 where "1" indicated few, small

granulomas; “2” indicated a moderate amount of granulomas of typically a moderate or larger size; and “3” indicated the most numerous and/or largest granulomas.

5.3.4 Parasitology

Grossly visible ectoparasites were noted during necropsy. Skin scrapes and gill biopsies were taken for the first 10 fish on each sampling day. Skin was scraped from anterior to posterior, just ventral to the first dorsal fin. Gill biopsies were taken from the middle to ventral distal edge of the 1st gill arch. The peritoneal cavity was examined for grossly visible endoparasites. The distal gut was opened and the lumen grossly examined for acanthacephalans, since these were known to be the most common intestinal helminth.

Semi-quantitative density of infection was recorded for bodonids, sessile peritrichs, trichodinids, copepods and acanthacephalans, on a scale of 1-3 where 1 represented a mild infection and 3 represented a severe infection. Presence/absence data was recorded for all other parasites. Ecological terminology follows Bush et al. 1997; however the term ‘location’ in this paper reflects the geographical area from which the striped bass host was collected.

5.3.5 Virology

Spleen, liver and kidney tissues were additionally taken from 82 striped bass, aged 3+, 4+ and 5+, from the Choptank and Potomac rivers and Herring Bay in 2006. Tissues were homogenized in Minimal Essential Media (Sigma-Aldrich, St. Louis, MO) with 1mg/L of gentamycin sulfate (Sigma-Aldrich, St. Louis, MO). Fifty microliters of

homogenate was used to inoculate Epithelioma Papulosum Cyprini (EPC) and Chinook Salmon Embryo (CHSE) cell lines in 48 well cell culture plates. Four replicates of each cell line were inoculated. The first pass was observed for cytopathic effect (CPE) for 7-10 days as was the second blind pass.

5.3.6 Case definition

In epidemiologic analyses, a fish was designated positive if a mycobacterial strain was isolated from the internal organs (i.e., culture positive) or if acid fast bacilli (AFB+) were found within granulomas in the internal organs, primarily the spleen, kidney or liver. Therefore, these analyses determine the epidemiology of exposure to mycobacterial species, regardless of state of infection. Note that 'infected' fish in this study included both colonized fish without grossly visible signs of disease as well as animals with frank pathology.

5.3.7 Potential risk factors

Variables examined in this study included age at collection, river of collection, gender, grossly visible signs of disease, presence of bacteria other than mycobacteria, and presence of parasites.

Age of striped bass was determined by length-at-age data (Maryland Department of Natural Resources) and a subset was confirmed by scale analysis. 1+ fish were initially used as the reference for analysis of age effects in the older fish.

Sites of collection included the Upper Bay, the Choptank River, the Nanticoke River, the Pocomoke River and the Potomac River. Only 0+ fish were collected from the

Nanticoke River, so analyses on older fish excluded this site. For univariate analyses, the Upper bay was used as the reference. In multivariate analyses the Pocomoke River was used as the reference site.

Gender was determined by either visual inspection of gonads at necropsy, or characterization of gonads in histology. Gender analyses were only performed on older fish since small sample size of known genders in the 0+ fish precluded including this variable in 0+ analyses. Male was used as the reference gender.

Grossly visible signs of disease included external skin lesions and/or granulomas on internal organs grossly visible at necropsy. The absence of disease signs was used as the reference in analyses.

Presence of bacteria other than mycobacteria was determined by sampling the brain, liver and anterior kidney as described above. Outcome was restricted to presence/absence as species of bacteria were lumped for this analysis.

Skin, gills, peritoneum and distal gut were observed for parasites as described above. Presence/absence data was used for risk factor evaluation where absence was used as the reference, except for gill ciliates (trichodinids and sessile peritrichs). In this case, analyses of ciliates compared "high rank" (moderate to severe, rank of 2 or 3 during evaluation at necropsy) with the reference "low rank" (absent or mild, ranking of 0 or 1 during evaluation at necropsy).

5.3.8 Analytical methods

Prevalence of mycobacterial infection, with 95% confidence intervals, was calculated for age groups and sites of collection. Univariate logistic regression (SAS

9.3.1, SAS Institute, Cary, NC) was used to calculate unadjusted prevalence odds ratios (POR), confidence intervals and Wald statistic p-values for potential risk factors. Associations with $p<0.25$ were further examined for effects in a multivariate model (Hosmer and Lemeshow 2000, King 2002). Each variable was tested with each other variable and the inclusion of each variable in the final model was determined by Wald statistics and the Hosmer and Lemeshow Goodness-of-fit test. The best subsets method was used to identify factors that were important contributors to the model in various combinations (Hosmer and Lemeshow, 2000). Variables were then individually introduced and excluded from base models to identify the best potential models based on Wald statistics, changes in coefficients of other variables, and the Hosmer and Lemeshow Goodness-of-fit test (Rothman and Greenland, 1998). The final model was selected based on statistical significance of variables, model fit and potential biological relevance. Note, 'signs of disease' was excluded from the multivariate model because this variable was considered an effect of disease, not a predictor. Estimations of sensitivity and specificity for culture and histology methods were performed on TAGS software (Pouillot et al. 2002.)

5.4 Results

5.4.1 Gametes

Gametes sampled in 2003-2004 were infected with mycobacteria, *Pseudomonas fluorescens*, *Micrococcus* spp., and an unidentified yeast (Table 5-2). River differences were significant for the yeast in 2003 ($p<0.01$) and the *Micrococcus* spp. in 2004 ($p<0.05$). No gender differences were found with any of the putative pathogens. Neither

the yeast, *P. flourescens,* nor the *Micrococcus* spp. were identified as a risk factor for mycobacterial infection by statistical tests for homogeneity.

5.4.2 General bacteriology

A wide variety of bacteria were isolated from striped bass, and the most commonly encountered are presented in Table 5-3. Species presented in Table 5-3 reached the cut-off value of 10% prevalence in at least one sub-sample of striped bass. The full data set is presented in Appendix A. The range of 0+ fish positive for bacteria, *other than mycobacteria,* was 12-90%, the range was 0-68% for 1+ fish, 0-65% for 2+ fish and 0-44% for 3+ fish (Table 5-3). Some bacterial species were found in both the older and younger fish, such as *Vibrio vulnificus*, *V. fluvialis* and *Photobacterium damsela*, however younger fish were infected with a wider variety of species of bacteria (n=28; Appendix A) than the older fish (n=19; Appendix A). For example, *Aeromonas hydrophila* was commonly found in the 0+ fish, but was only isolated from Pocomoke 2+ in 2004. In addition, *V. cholera* non-01, *Pleisomonas shigelloides* and *Stenotrophomonas maltophilia* were only isolated from 0+ fish.

5.4.3 AFB+ granulomas *en section*

The AFB+ granulomas presented over a range of organization, from well defined distinct layers and margins, indicating contained infections, to not well encapsulated amorphous lesions, suggestive of active infections (Figures 5-2A and 5-2B). When present, the central core would consist of cellular debris, liquefaction, rarely caseous necrosis, or a combination of the three. Occasionally, melanomacrophages were seen

within granuloma cores. The core was surrounded by a spindle-cell layer, from 2 to 10 cell layers thick, sometimes with an acellular hyaline layer separating this layer from the core. A layer of epitheliod cells surrounded the spindle-cell layer and ranged in thickness from 2 cell layers thick to almost 30 cells thick in the less well defined margin granulomas. AFB+ were typically seen within the core, but also occasionally in the spindle-cell layers and rarely in the epitheliod cell layers. Eosinophilic coarse granulocytes (ECGs) were commonly seen surrounding the granuloma and within the epitheliod layer. Macrophage aggregates (MA) were commonly found on the periphery of granulomas, occasionally with ECGs streaming between the two bodies, however no AFB+ were associated with MAs.

AFB+ granulomas were found in the spleen, anterior kidney, liver, mesentery, heart, muscle and gonad. Granuloma morphology was similar (see above) across all organs, except the muscle and gonad. Grossly visible skin ulcers were taken for histology, and the muscle granulomas were situated proximal to the ulcerated skin tissue. These granulomas were well defined, and while similar in morphology to those described above, were encased within large regions of connective tissue (Figure 5-2D). AFB+ were also visualized external to granulomas within this connective matrix. The one AFB+ granuloma observed in the gonads, in the testes of a 2 year old from the Pocomoke River, retained the central cellular debris with areas of caseous necrosis. An epitheliod layer lacking well defined outer margins surrounded the central core as the spindle-cell layer was absent. Inflammatory infiltrate, including ECGs surrounded the epitheliod layer (Figure 5-2C).

5.4.4 Non-AFB+ granulomas *en section*

Granulomas observed other than AFB+ granulomas were termed 'parasitic' or 'other'. Parasitic granulomas consisted of a thin epitheliod layer around the visible parasite. ECGs were occasionally seen in association with these lesions. No parasitic granulomas were AFB+ but non-specific staining was common in these granulomas. 'Other' granulomas included all granulomas not defined as bacillary or parasitic, and ranged in presentation from empty shells to well-defined granulomas lacking bacilli in H&E section, let alone AFB+ in aqueous acid-fast stained sections. Non-specific staining was also common in 'other' granulomas.

5.4.5 Concordance

Culture samples for both spleen and kidney were taken from a total of 284 fish. A total of 227 fish were either positive or negative by both organs, which resulted in a total concordance of 80% (227/284). Total discordance, where only one of the two organs sampled was positive, was 20% (57/284). However these statistics were driven by a large number of negative fish (n=208). Therefore these statistics were also calculated including only positive fish (n=76). Positive fish concordance was 25% (19/76) and positive fish discordance was 75% (57/76). Within the positive fish discordance statistic where only one organ was positive, spleen (49%; 28/57) and kidney (51%; 29/57) were almost equally likely to designate a fish as positive. Note, one fish, while negative by culture in both spleen and kidney, was positive in the liver. Interestingly, one fish was positive by gonad in culture, however it was also positive in both spleen and kidney.

Spleen and kidney samples were present in histology for a total of 1,826 fish. Total concordance of spleen and kidney was 88% (1612/1,827) and total discordance was 12% (215/1,827). Similar to the culture data, these statistics were driven by a large number of negative fish (n=1,459), so positive fish statistics were also calculated. Positive fish concordance for spleen and kidney was 64% (215/337) and positive fish discordance was 36% (122/337). Within the positive fish discordance statistic where only one organ was positive, spleen (84%; 180/215) was more likely than kidney (16%; 35/215) to designate a fish as positive. Note, 31 fish were negative by both spleen and kidney, but had AFB+ within granulomas in other tissues, such as the liver (15/31) which was routinely sampled for histology, but also the mesenteries (16/31) which was not.

5.4.6 Comparison of histology and microbiology

Both histology and microbiology data was available for 644 fish. Concordance, when AFB+ were found within granulomas by histology and at least one *Mycobacterium* sp. was cultured from internal organs of the same fish, was 69% (446/644) and discordance was 31% (198/644). Within the positive fish discordance statistic where a fish was positive by only one detection method, histology (62%; 122/198) was more likely than culture (38%; 76/198) to designate a fish as positive. The estimations of sensitivity and specificity for the two methods of diagnosis used in this study are presented in Table 5-4.

5.4.7 Epidemiology of infection

The age pattern of mycobacterial infection differed among rivers (Figure 5-3). Therefore, a generalized statement regarding this variable could not be made as logistic regression analysis had a low goodness-of-fit p-value. Three age patterns emerged from these data. The first age pattern of mycobacteria positive fish was observed in Potomac River and the Upper Bay fish. Prevalence in these 0+ fish was very low, with a 6-fold and 11-fold increase in the 1+ fish from the two river systems, respectively, and a leveling out at 40% in the 2+ and 3+ fish. The second age pattern, observed in Choptank River fish, retained a very low prevalence in the 0+ fish, but leveled out around 40% in the 1+ and older fish. The third age pattern, observed in the Pocomoke River, had a comparatively high prevalence in 0+ fish, and leveled out in the 1+ and older fish at around 55% instead of around 35-50% as in the other rivers. In essence, more Pocomoke River fish were infected at an earlier age, i.e., 0+, with Choptank River fish following with higher numbers infected at 1+. The Potomac River and Upper Bay fish retained low prevalence in these earlier ages with higher prevalence occurring with the 2+ and 3+ fish. Note, the Nanticoke River was excluded from age pattern analysis as only 0+ fish were collected from this location.

No consistent cohort or year effects were found, therefore data was lumped. Goodness-of-Fit decreased when these factors were included in regression analysis, so they were excluded from further analyses.

Univariate modeling, however, found age and location to be significant, as well as age by location interaction (Table 5-5). Therefore, analyses were split into three age

groups to account for this interaction. The final models assessed were i) 0+ fish, ii) 1+ fish, and iii) 2+ and 3+ fish.

In univariate analyses for 0+ fish, possessing any grossly visible sign of disease and presence of acanthacephalans, nematodes, any gut associated parasites, copepods, or grossly visible gill parasites, i.e., copepods and/or isopods, resulted in an increased prevalence odds ratio (Table 5-6). These parameters along with location and presence of other bacteria (6/542=1% were co-infected with a mycobacteria and another species of bacteria), isopods or high sessile peritrichs were included in multivariate modeling, as selection was based on $p<0.25$ in univariate analyses. In the final multivariate model, location other than Pocomoke River and presence of other bacteria resulted in lower prevalence odds ratios (Table 5-7). The presence of any grossly observable parasite, i.e., acanthacephalans, nematodes, isopods or copepods, resulted in an increased prevalence odds ratio (Table 5-7). No interactions were found to be significant.

Univariate analyses for older fish possessing a grossly visible sign of disease, or having an acanthacephalan infection resulted in an increased prevalence odds ratio (Table 5-8). Location, presence of other bacteria (47/469=10% were co-infected with a mycobacteria and another species of bacteria), presence of any gut associated parasites, i.e., acanthacephalans and/or nematodes, and presence of a high trichodinid infection were included in multivariate modeling, as selection was based on $p<0.25$ in univariate analyses. In the final model for 1+ fish, location other than the Pocomoke River resulted in lower prevalence odds ratios (Table 5-9). In the final model for 2+ and 3+ fish, presence of acanthacephalans and high trichodinid infections resulted in increased prevalence of mycobacterial infections (Table 5-10).

5.4.8 Virology

All samples screened for CPE were negative both on the first pass as well as the blind pass.

5.5 Discussion

5.5.1 Definitions and diagnosis of infections

As this study proceeded, it became evident that definitions would be a key component of any discussion on mycobacteriosis. Researchers have primarily used three methods of detecting mycobacteria within tissues: by histology, culture and molecular techniques. By convention in histology, acid-fast bacilli must be seen, primarily within granulomas for the fish to be considered positive for mycobacteria. Culturing *Mycobacterium* sp. from tissue also renders a fish positive. Additionally, PCR techniques can be performed on tissues to detect mycobacterial genomic DNA (Kaattari et al. 2005). On the surface it appears as though the purpose of each technique is to identify an individual fish either positive or negative for mycobacteriosis. However in reality, each technique is looking for, and thus diagnosing, different states of infection within the fish.

First, finding acid-fast bacilli within granulomas indicates that a fish has been exposed to mycobacteria and has mounted an immune response to the insult. The mycobacterial cell wall integrity is retained, however whether the bacteria are viable or non-viable, and whether the infection is active or not is unknown. In essence, histology

confirms prior exposure to mycobacteria. Second, culturing mycobacteria from tissues indicates viable bacteria within the tissues, and the degree of intensity may suggest whether the infection is active or not. The state of the host immune response is unknown. Culture confirms the fish is a carrier of viable mycobacteria. Third, detecting mycobacterial DNA in fish tissues fails to confirm viability of the bacteria, fails to suggest immune status of the host, and fails to indicate the status of the infection. Molecular techniques indicate prior exposure to mycobacteria, but unlike histology, further assumptions about cell integrity are unknown.

In light of the previous discussion, culturing mycobacteria from tissues appears to provide the most information about the status of the bacteria, however data on potential previous exposure is lacking. Therefore, as the concordance of histology and culture was only 69%, this study used both culture and histology to identify individual fish as either positive for negative for mycobacteria. Further, this study highlights the importance of sampling both the spleen and the kidney when determining mycobacterial infections, as shown not only in total concordance, but more so in positive fish concordance.

5.5.2 Gametes

The gametes sampled in this study were found to harbor not only mycobacteria, but also other putative pathogens. The sampling method used for the spawning stock was as sterile as possible for a non-destructive field technique (NDT), however there still existed the possibility for contamination. Data obtained from the destructive technique (DT) used during sterile necropsy supported the results that gametes can be infected with mycobacteria. In fact, the ovaries of one 5+ female from the Pocomoke River were

positive for *M. shottsii* (data not shown) and AFB+ granulomas were found in gonadal tissue (Figure 5-2C). Although mycobacteria prevalence was generally low (≤11%) using NDT, it is possible that the discrepancy in prevalence with the DT could be explained for reasons other than contamination in the NDT. The NDT was sampling an older portion of the stock, the spawning stock, and included individuals estimated to be up to 17 years old based on length at age, compared with the resident striped bass in the rest of this study, the majority of which were 3+ or younger with only stray 4+ and 5+ individuals taken. Additionally, spawning stock are coastal migrants whereas the younger fish are resident to the Chesapeake Bay (Kohlenstein 1981) so habitat use may be a factor as well. Previous studies have indicated gonadal tissue, fluids or ova, can be infected with mycobacteria and suggest disease can be transmitted to the F_1 generation (Chinabut 2003).

5.5.3 Epidemiology

Analyses produced three models of infection based on age at capture. During initial analyses, age and location interactions were significant. Therefore partitions were made to follow the biology of the fish. 0+ fish are believed to be non-migratory and stay within their natal river, 1+ fish remain close to their natal river, and 2+ and 3+ fish migrate within the Chesapeake Bay (Kohlenstein 1981). Therefore, final analyses included three models, one for 0+ fish, the second for 1+ fish and the third for 2+ and 3+ fish.

The final model for 0+ fish included location, presence of grossly visible parasites, and presence of bacteria other than mycobacteria (Table 5-7). The Pocomoke

River, the reference river in these analyses, was identified as an area of higher mycobacterial prevalence. Reasons for this elevated prevalence are unclear, but this river effect may represent factors not included in this study including flow dynamics or water quality parameters such as temperature, dissolved oxygen levels, salinity or pH (Kirschner et al. 1992, Falkinham et al. 2004). Elevated levels of mycobacteria have been associated with high humic and fulvic acids, high soluble zinc, and low dissolved oxygen (Kirschner et al. 1992). Therefore, water quality parameters including those listed above should be included in further investigations into location effects on mycobacterial infections in non-migratory fish.

The grossly visible parasites included in this model create continual breaks in the barrier between the sterile internal environment from the non-sterile external environment (in this case, while internal to the fish, the gut lumen was considered external to the sterile internal organs). Therefore, it appears as though 0+ can be infected with mycobacteria either through the gills (Chinabut 2003) or through the intestinal tract, as has been suggested by Harriff et al. (2007). Interestingly, a model including grossly visible parasites other than nematodes also produced a good fit. Larval nematodes are thought to infect fish through the intestinal tract, migrating across the gut wall into the peritoneum to develop into adults (Williams and Jones 1994), but do not produce a continual break in the barrier between sterile internal and non-sterile external environments. However, the results were the same including or excluding nematodes from the final model as variable p-values retained significance and prevalence odds ratios differed only slightly (data not shown).

The presence of bacteria other than mycobacteria appeared to have a protective effect on prevalence of mycobacterial infection in 0+ fish (Table 5-7). Several explanations for this result are possible. First, infection with other bacteria may have, in effect, primed the immune system to combat pathogens, or vice versa. Increased macrophage activity against one bacterial infection may prevent colonization of another bacterial species. Therefore, the mycobacteria infecting these individuals had either been killed or reduced to numbers too low to likely derive a culturable isolate. A second possibility was that individuals cannot survive with a dual infection, mycobacteria and other bacteria, and therefore die and are excluded from our samples. A corollary to this theory is the potential for 0+ fish infected with other bacteria to die, whether or not they have also been infected with mycobacteria. Therefore, while a mycobacterial infection alone would not cause death, the 0+ fish infected with other bacteria would not live long enough to acquire a secondary mycobacterial infection. Generally, the majority of fish larvae do not survive their first year of life. Although bacterial infection may partly explain this reduced survival rate of young-of-the-year individuals, predation may be a more likely source of mortality which may or may not be related to infected fish having a greater likelihood of being caught.

Location of collection was identified as a risk factor for mycobacterial infections in 1+ fish (Table 5-9). Presence of other bacteria was also included in the model to retain continuity with the 0+ fish and because the relationship appeared biologically relevant although not statistically significant ($p=0.07$). Therefore, presence of other bacteria may be a risk factor in 1+ fish, but the study sampling failed to identify it. The presence of any grossly visible parasite, a risk factor in 0+ fish, was tested as a variable in the final

model, however it failed to be significant (p=0.85). Therefore, location was the driving factor in mycobacterial infections in 1+ fish, potentially for the reasons listed above for 0+ fish. These findings support previous evidence for 1+ striped bass to maintain location fidelity, as opposed to migrating around the Bay (Kohlenstein 1981).

The presence of acanthacephalans and the presence of a moderate to severe infection with trichodinids were identified as risk factors for 2+ and 3+ striped bass (Table 5-10). Acanthacephalans insert a thorny anchor into the gut wall, and may provide a conduit for mycobacteria to enter the sterile visceral environment, as was proposed for the 0+ fish above. Previously, mycobacteria have only been anecdotally associated with acanthacephalans in bluefish (Dove 2005), however further investigation may prove useful. Conversely, acanthacephalan presence was not found to be associated with mycobacterial infections in Delaware Bay striped bass (Ottinger et al. 2007). However, the Delaware Bay study found only a small number of mycobacteria positive fish (17.5%, 14/80) and this small sample size may have prevented finding this association at the level found in the current study (POR=1.74, Table 5-10). Additionally, the infection dynamics in older Delaware Bay stock (total length approximately 600mm, probably 5+ or older) may be different from those of the younger Chesapeake Bay resident individuals. It is important to note that in the current study, as the presence of acanthacephalans was associated with mycobacterial infections in 2+ and 3+ fish, this association was also tested in the 1+ fish model and was found there to be insignificant (p=0.58).

Trichodinids also create breaks in the epithelial barrier of the gills and/or skin by the action of their adhesive disc (Rohde 2005). Protozoa, specifically ciliates and

amoebae, are known to phagocytose mycobacterial species. In fact, mycobacteria are known to survive for extended periods of time within ciliates and amoebae, and it has been suggested that intracellular existence may confer increased virulence to mycobacteria (Cirillo et al. 1997, Strahl et al. 2001, Harriff et al. 2007). Therefore, the potential exists for trichodinid species to, in essence, concentrate mycobacteria by the act of phagocytosis, and release viable bacteria at the specific point where a break in the epithelial barrier exists. However the appearance of trichodinids as a risk factor in older fish, and not younger fish, is interesting. As was suggested in Chapter 4, multiple trichodinid species were found on Chesapeake Bay striped bass. It is possible that trichodinid species composition on older fish differs from that found on younger fish, and the pathogenicity, i.e. the physical damage caused to gill and skin epithelium of these different species may be variable as well. Perhaps, as with the river effects in the 0+ fish, the presence of moderate to severe trichodinid infections may not be the effect of interest itself, but rather a marker for some other factor not investigated. Interestingly, the presence of moderate to severe infection trichodinids was also investigated in 1+ fish, but small samples sizes of mycobacteria positive fish precluded full analyses. Sample sizes of 1+ fish in the Pocomoke River were, however, sufficient to perform analysis, and while not significant, trichodinids were associated with higher prevalence of mycobacterial infections (POR=1.35 [0.69, 2.70]) as was found with the 2+ and 3+ fish. Finally, it is reasonable that the association with location fell out of the model of older fish, as older fish are believed to migrate around the Bay, and therefore an association with location of capture would be lacking.

It is important to note that, while not included in modeling, grossly visible signs of disease, including external lesions and internal granulomas, were associated with mycobacterial infections in all age groups (Tables 5-6 and 5-8).

5.5.4 General bacteriology

Yearly variation in samples was apparent in i) species of bacteria isolated and ii) prevalence of fish positive. For example, the Upper Bay 0+ fish consistently had a high prevalence of positive fish (85% in 2003 and 70% in 2004; Table 3). However species of bacteria varied as *A. hydrophila* was the dominant isolate (75% positive) in 2003 while in 2004, *Pl. shigelloides* was the dominant isolate (47%). Prevalence of positive fish also varied, i.e., in the Choptank 0+ fish, 82% were positive in 2003, but only 15% were positive in 2004 (Table 5-3). Further, all sub-sampled groups of 0+ were positive for bacteria (prevalence ranged from 12-90%). Older fish groups, however, had lower prevalence ranges of positive fish (0-68% for 1+; 0-65% for 2+; 0-44% for 3+). This may indicate a decreasing trend of fish positive for bacteria other than mycobacteria with increasing age.

Vibrio spp. have previously been associated with morbidity and mortality events in Chesapeake Bay striped bass (A. Baya personal communication). Ten named *Vibrio* species were isolated from striped bass in the current study, along with additional unnamed species (Table 5-3; Appendix A). *V. vulnificus*, *V. fluvialis* and *V. parahaemolyticus* were the most commonly isolated *Vibrio* spp. Of note is that *V. cholera* non-01, a human pathogen which can cause gastroenteritis, was isolated from 35% (21/60) 0+ fish from the Choptank in 2003, as well as an individual 0+ fish from the

Potomac River in 2003 (Table 3; Lupiani et al. 1993, Kaper et al. 1995). Buller (2004) has suggested that *V. cholera* non-01 is normal flora of striped bass, but may prove to be an opportunistic pathogen.

Interestingly, some pathogens other than *Vibrio* spp. previously associated with morbidity and mortality in Chesapeake Bay striped bass were either not isolated or observed at low prevalence (A. Baya, personal communication; Baya et al. 1990, Baya et al. 1997). No isolates of *Streptococcus* spp. nor *Edwardsiella tarda* were found in the current study. *Carnobacterium piscicola* was isolated from 8% and 20% of 2005 Potomac 1+ and 2+ striped bass, respectively, and *Listonella anguillarum* was isolated from 4% of Choptank 2004 1+ striped bass (Appendix A).

A. hydrophila and *P. damsela* are considered both fish and human pathogens, causing gastroenteritis and wound infections, respectively, in humans (Buller 2004). *Pl. shigelloides* causes primarily intestinal disease in humans and is believed to be an opportunistic pathogen of fish (Gilligan et al. 2003, Buller 2004, Stock 2004). *St. maltophilia* pathogenicity to fish is unclear, however it has been known to produce nosocomial infections in humans (Gilligan et al. 2003, Buller 2004).

5.5.5 Final thoughts

Based on multivariate modeling, the results from this study indicate that the presence of mycobacterial infections in resident Chesapeake Bay striped bass are dependant on age, location, the presence of other bacteria in internal organs, and the presence of parasites. Univariate models indicated that during the study period, prevalence increased with age, from 0+ to 1+, and from 1+ to 2+ and 3+. Additionally

during the study period, 0+ striped bass from the Pocomoke River had an elevated prevalence of infection compared to 0+ striped bass from the Potomac River and marginally same-aged fish from the Upper Bay. Similarly, in 1+ fish, the Pocomoke River had an elevated prevalence of infection compared to the Upper Bay, the Choptank River and the Potomac River. Further, the presence of other bacteria was inversely related to mycobacterial infections in 0+ and 1+ striped bass. Finally, the presence of any grossly observable parasite resulted in an increased prevalence odds ratio in 0+ fish, while the presence of acanthacephalans and elevated trichodinid levels were associated with increased prevalence odds ratios in 2+ and 3+ fish. The existence of multiple risk factors, and the variability introduced into the models by age-dependant life history factors, indicate mycobacterial infections in Chesapeake Bay striped bass may not follow one route of infection and disease process, but instead are a result of a series of complex processes.

5.6 Acknowledgements

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Oceanic and Atmospheric Administration, and the Maryland Agriculture Experiment Station.

5.7 Tables and Figures

Table 5-1. Global Positioning System (GPS) coordinates of Chesapeake Bay sites sampled for striped bass *Morone saxatilis*.

Site		0+	1+ to 3+
Upper Bay		39° 22.5'N 75° 59.0'W	Centered around 39° 04.1'N
		to	76° 17.4'W
		39° 23.5'N 76° 02.4'W	
Choptank	Tred Avon	38° 41.7'N 76° 10.3'W	Centered around 38° 39'N
	Hambrooks	38° 35.2'N 76° 04.9'W	76° 17.0'W
	Horn Point	38° 35.3'N 76° 07.5'W	
Nanticoke	Roaring Point	38° 15.9'N 75° 55.1'W	None collected
	Ragged Point	38° 17.5'N 75° 54.4'W	

Pocomoke	Williams Point	37° 57.2’N 75° 39.3’W	Same as 0+
	East Creek	37° 58.8’N 75° 44.7’W	
Potomac	Tall Timbers	38° 10.2' N 76° 32.9'W	Centered around 38° 02.5'N
	Blake Creek	38° 12.3' N 76° 34.8'W	76° 20.0'W

Table 5-2. Sample prevalence of microorganisms colonizing striped bass *Morone saxatilis* gametes (genders combined), 2003-2004. Sample sizes in parentheses.

	2003			2004	
	***Mycobacterium* spp.**	**Yeast**	***Pseudomonas fluorescens***	***Mycobacterium* spp.**	***Micrococcus* spp.**
Choptank	3 (65)	22 (65)	8 (65)	2 (52)	8 (52)
Nanticoke	11 (62)	5 (62)	2 (62)	5 (60)	0 (60)

Table 5-3. Prevalence of select bacteria isolated from the brain, liver and kidney of Chesapeake Bay striped bass, 0+ to 3+. See Appendix A for complete data set.

Age	River	Year	n	% positive	*V. vulnificus*	*V. fluvialis*	*V. parahaemolyticus*	*A. hydrophila*	*Ph. damsela*	*Ps. flourescens*	*Sh. putrifaciens*	*V. cholera non-01*	*Vibrio spp.*	*Pl. shigelloides*	*St. maltophilia*
0+	Upper Bay	2003	60	85	5			75		3	5			20	2
		2004	60	70	2	12		13		3				47	
		2005	0												
	Choptank	2003	60	82	47	7		2	20			35		2	15
		2004	60	15	10										
		2005	28	57	50			4		4	4				
	Nanticoke	2003	60	90	60	13		12	23		2			18	
		2004	59	12					12						
		2005	0												
	Pocomoke	2003	0												
		2004	19	53	21	5	5		5	5	11		5		
		2005	11	73	18	9	9	9	18		9				
	Potomac	2003	60	68	7	8	2	2	28	2		2	32		
		2004	60	33	7	2				18	2				10
		2005	0												

Table 5-3 cont. Prevalence of select bacteria isolated from the brain, liver and kidney of Chesapeake Bay striped bass, 0+ to 3+. See Appendix A for complete data set.

Age	River	Year	n	% positive	*V. vulnificus*	*V. fluvialis*	*V. parahaemolyticus*	*A. hydrophila*	*Ph. damsela*	*Ps. flourescens*	*Sh. putrifaciens*	*V. cholera non-01*	*Vibrio spp.*	*Pl. shigelloides*	*St. maltophilia*
1+	Upper Bay	2003	0												
		2004	11	9	9										
		2005	25	68	36	40			8	4	4				
	Choptank	2003	10	0											
		2004	24	50	4	8	8			25	21				
		2005	15	13						7					
	Nanticoke	2003	0												
		2004	0												
		2005	0												
	Pocomoke	2003	0												
		2004	12	17	8				8						
		2005	33	42		9	6		21		6				
	Potomac	2003	2	0											
		2004	19	26	5					5	16				
		2005	10	30							10				

Table 5-3 cont. Prevalence of select bacteria isolated from the brain, liver and kidney of Chesapeake Bay striped bass, 0+ to 3+. See Appendix A for complete data set.

Age	River	Year	n	% positive	*V. vulnificus*	*V. fluvialis*	*V. parahaemolyticus*	*A. hydrophila*	*Ph. damsela*	*Ps. flourescens*	*Sh. putrifaciens*	*V. cholera non-01*	*Vibrio spp.*	*Pl. shigelloides*	*St. maltophilia*
2+	Upper Bay	2003	13	0											
		2004	18	22	6				11		6				
		2005	24	50	38	8			13						
	Choptank	2003	10	0											
		2004	19	16		5	5			11					
		2005	23	65	43	4			30						
	Nanticoke	2003	0												
		2004	0												
		2005	0												
	Pocomoke	2003	0												
		2004	6	17	17			17							
		2005	26	31	4		12		4		4				
	Potomac	2003	6	0											
		2004	14	14							7				
		2005	36	39	11	3	8				3				

Table 5-3 cont. Prevalence of select bacteria isolated from the brain, liver and kidney of Chesapeake Bay striped bass, 0+ to 3+. See Appendix A for complete data set.

Age	River	Year	n	% positive	*V. vulnificus*	*V. fluvialis*	*V. parahaemolyticus*	*A. hydrophila*	*Ph. damsela*	*Ps. flourescens*	*Sh. putrifaciens*	*V. cholera non-01*	*Vibrio spp.*	*Pl. shigelloides*	*St. maltophilia*
3+	Upper Bay	2003	7	0											
		2004	1	0											
		2005	27	44	33	7			4						
	Choptank	2003	0												
		2004	0												
		2005	24	4	4										
	Nanticoke	2003	0												
		2004	0												
		2005	0												
	Pocomoke	2003	0												
		2004	2	0											
		2005	17	0											
	Potomac	2003	5	0											
		2004	0												
		2005	30	40	7		20		13						

Table 5-4. Sensitivity and specificity of histology and culture for diagnosis of mycobacterial colonized Chesapeake Bay striped bass *Morone saxatilis*.

	Sensitivity	**Specificity**
Histology	0.1824	0.7667
Culture	0.3439	0.9080

Table 5-5. Risk factors for piscine mycobacterial infection in Chesapeake Bay striped bass with univariate logistic regression analysis. Analyses are on total mycobacteria prevalence, including culture and histology positive fish.

	Myco Positives		Myco Negatives		Prevalence Odds Ratio	95% confidence interval	Wald test p value
Risk factor	**No.**	**%**	**No.**	**%**			
Age							
0+	17	8.6	954	76.8	0.04	0.02, 0.08	<0.01
1+	48	24.2	113	9.1	1.00	Reference	
2+	80	40.4	112	9.0	1.68	1.08, 2.62	0.02
3+	53	26.8	63	5.1	1.98	1.20, 3.26	<0.01

Site							
Upper	42	21.2	322	25.6	1.00	Reference	
Bay							
	51	25.8	343	27.2	1.14	0.74, 1.76	0.56
Choptank							
	0	0.0	208	16.5			
Nanticoke							
	64	32.3	62	4.9	7.92	4.92, 12.73	<0.01
Pocomoke							
Potomac	41	20.7	324	25.7	0.97	0.61, 1.53	0.9

Table 5-6. Risk factors for piscine mycobacterial infection in 0+ striped bass with univariate logistic regression analysis. Analyses are on total mycobacteria prevalence, including culture and histology positive fish. For trichodinids and sessile peritrichs, 'low' indicates no or mild infection and 'high' indicates moderate to severe infection.

	Myco Positives		Myco Negatives		Prevalence Odds Ratio	95% confidence interval	Wald test p value
Risk factor	**No.**	**%**	**No.**	**%**			
Signs of Disease							
Negative	15	88.2	762	99.9	1.00	Reference	
Positive	2	11.8	1	0.1	101.60	8.73, 999.99	<0.01
Presence of other							

bacteria							
Negative	11	64.7	217	41.3	1.00	Reference	
Positive	6	35.3	308	58.7	0.38	0.14, 1.05	0.06
Presence of Parasite							
No acanthacephalans	10	58.8	916	96.0	1.00	Reference	
Acanthacephalan (AC)	7	41.2	38	4.0	16.90	6.1, 46.7	<0.01
No nematodes	10	58.8	891	93.4	1.00	Reference	
Nematodes (NEM)	7	41.2	63	6.6	9.90	3.65, 26.89	<0.01

No gut parasites	10	58.8	915	95.9	1.00	Reference	
Gut parasites (AC and NEM)	7	41.2	39	4.1	16.40	5.94, 45.44	<0.01
No isopods	15	88.2	904	94.8	1.00	Reference	
Isopods (ISO)	2	11.8	50	5.2	2.41	0.54, 10.83	0.25
No copepods	11	64.7	890	93.3	1.00	Reference	
Copepods (COPE)	6	35.3	64	6.7	7.59	2.72, 21.17	<0.01
No gill parasites	10	58.8	843	88.4	1.00	Reference	
Gill parasites (ISO and COPE)	7	41.2	111	11.6	5.32	1.98, 14.25	<0.01
Low trichodinids	7	53.9	103	57.9	1.00	Reference	

High trichodinids	6	46.1	75	42.1	1.18	0.38, 3.65	0.78
Low sessile peritrichs	8	61.5	146	82.0	1.00	Reference	
High sessile peritrichs	5	38.5	32	18.0	2.85	0.88, 9.29	0.08

Table 5-7. Multivariate model for mycobacterial infection in Chesapeake Bay 0+ striped bass *Morone saxatilis*.

Variable	Beta coefficient	Standard error	Prevalence Odds Ratio	95% confidence interval	Wald test *p* value
Location					
Reference Pocomoke					
Upper Bay	-1.85	0.97	0.16	0.02, 1.05	0.06
Choptank	-1.50	0.93	0.22	0.04, 1.37	0.11
Nanticoke	-14.56	257.5	<0.01	<0.01, >99.99	0.95
Potomac	-2.83	1.24	0.06	0.01, 0.68	0.02
Presence of any grossly observable parasite	2.18	0.83	8.86	1.75, 44.97	<0.01

Presence of other bacteria	-1.33	0.62	0.27	0.08, 0.89	0.03

Table 5-8. Risk factors for piscine mycobacterial infection in 1+, 2+ and 3+ striped bass with univariate logistic regression analysis. Analyses are on total mycobacteria prevalence, including culture and histology positive fish. For trichodinids and sessile peritrichs, 'low' indicates no or mild infection and 'high' indicates moderate to severe infection.

	Myco Positives		Myco Negatives		Prevalence Odds Ratio	95% confidence interval	Wald test p value
Risk factor	**No.**	**%**	**No.**	**%**			
Gender							
Female	76	42.0	100	34.7	1.00	Reference	
Male	69	38.1	93	32.3	0.98	0.63, 1.50	0.91
Unknown	36	19.9	95	33.0			
Signs of Disease							

Negative	146	80.7	270	93.8	1.00	Reference	
Positive	35	19.3	18	6.2	3.60	1.97, 6.57	<0.01
Presence of other bacteria							
Negative	134	74.0	195	67.7	1.00	Reference	
Positive	47	26.0	93	32.3	1.36	0.90, 2.06	0.15
Presence of Parasite							
No acanthacephalans	75	58.6	130	80.3	1.00	Reference	
Acanthacephalan	53	41.4	32	20.7	2.87	1.70, 4.84	<0.01

(AC)							
No nematodes	67	37.0	113	39.0	1.00	Reference	
Nematodes	114	63.0	177	61.0	1.10	0.75, 1.61	0.63
(NEM)							
No gut parasites	98	54.1	175	60.8	1.00	Reference	
Gut parasites	83	45.7	113	39.3	1.31	0.90, 1.91	0.16
(AC and NEM)							
No isopods	164	90.6	264	91.7	1.00	Reference	
Isopods (ISO)	17	9.4	24	8.3	1.14	0.60, 2.19	0.69
No copepods	16	8.8	25	8.7	1.00	Reference	
Copepods	165	91.2	263	91.3	0.98	0.51, 1.89	0.95
(COPE)							
No gill parasites	15	8.3	23	8.0	1.00	Reference	

Gill parasites (ISO and COPE)	166	91.7	265	92.0	0.96	0.49, 1.89	0.91
Low trichodinids	66	80.5	122	89.1	1.00	Reference	
High trichodinids	16	19.5	15	10.9	1.97	0.92, 4.24	0.08
Low sessile peritrichs	73	89.0	119	86.9	1.00	Reference	
High sessile peritrichs	9	11.0	18	13.1	0.82	0.35, 1.91	0.64

Table 5-9. Multivariate model for mycobacterial infection in Chesapeake Bay 1+ striped bass *Morone saxatilis*.

Variable	Beta coefficient	Standard error	Prevalence Odds Ratio	95% confidence interval	Wald test *p* value
Location					
Reference Pocomoke					
Upper Bay	-2.35	0.62	0.10	0.03, 0.32	<0.01
Choptank	-1.03	0.44	0.36	0.15, 0.84	0.02
Potomac	-3.85	1.07	0.02	<0.01, 0.17	<0.01
Presence of other bacteria	-0.77	0.43	0.46	0.20, 1.08	0.07

Table 5-10. Multivariate model for mycobacterial infection in Chesapeake Bay 2+ and 3+ striped bass *Morone saxatilis*. For trichodinids, high' indicates moderate to severe infection.

Variable	Beta coefficient	Standard error	Prevalence Odds Ratio	95% confidence interval	Wald test *p* value
Presence of Acanthacephalans	0.55	0.28	1.74	1.01, 2.99	<0.05
Presence of high Trichodinids	1.15	0.58	3.15	1.02, 9.73	<0.05

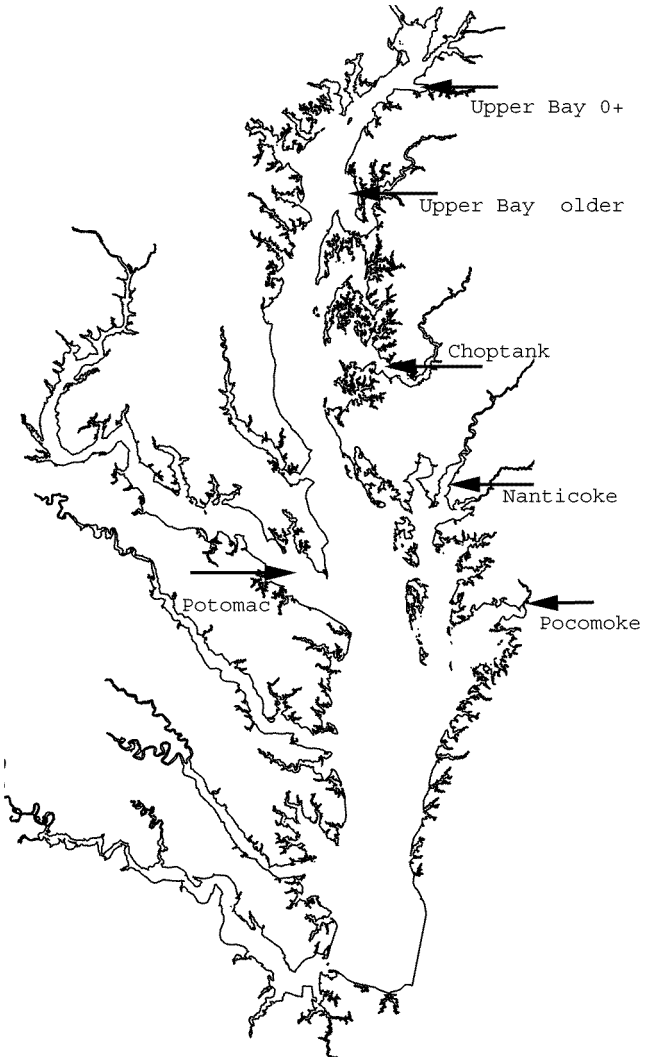

Figure 5-1. Map of the Chesapeake Bay with sites sampled for striped bass *Morone saxatilis* indicated by arrows. Line drawing provided by Maryland Sea Grant.

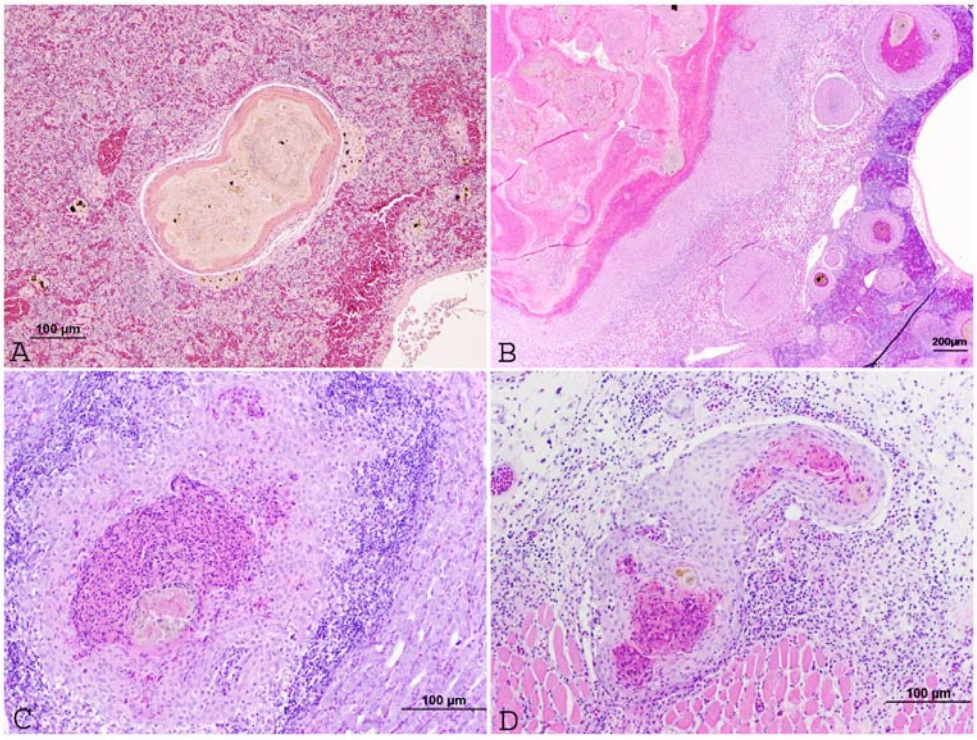

Figure 5-2. Acid fast positive bacilli granulomas from Chesapeake Bay striped bass. (A) Well-defined granuloma in the spleen. Note the central necrotic core surrounded with an eosinophilic spindle-cell layer. Slightly basophilic epitheliod cell layer is several layers thick, surrounding the spindle-cell layer. (B) Poorly defined large granuloma in the anterior kidney with ancillary granulomas surrounding the primary granuloma. The primary granuloma lacks the well-defined encapsulation visible in (A). (C) Granuloma in the gonad that lacks a spindle-cell layer and the epitheliod cell layer is poorly demarcated. Note inflammatory cells surrounding the epitheliod layer. (D) Granuloma in muscle, associated with skin epithelial lesion. All tissues stained with hematoxylin and eosin.

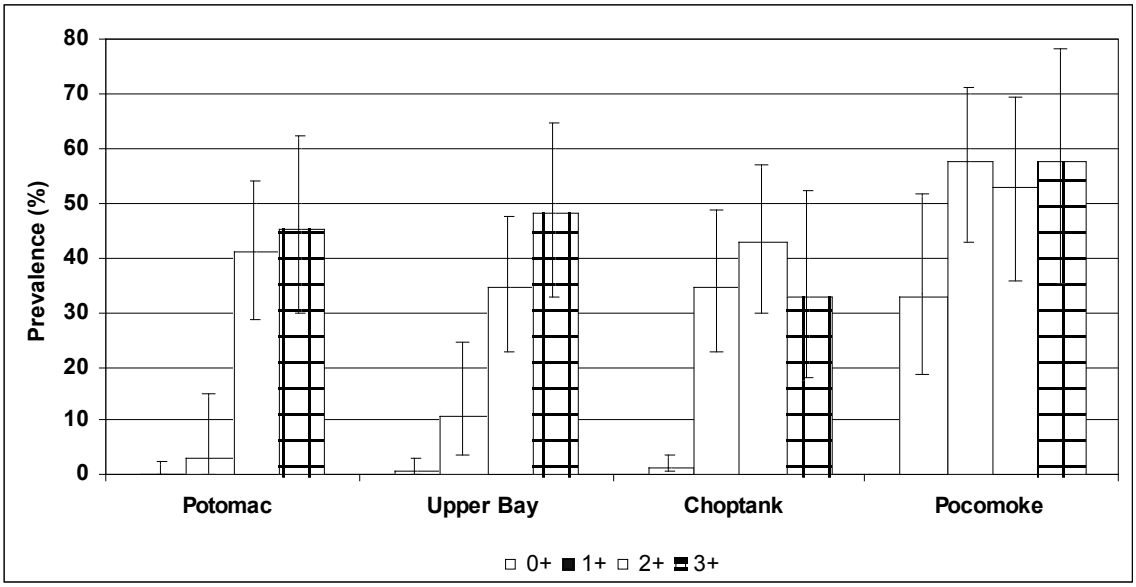

Figure 5-3. Prevalence (with 95% confidence intervals) of mycobacteria positive striped bass for four age classes from four collection sites, all years combined.

Chapter 6: Discussion

6.1 Overview

The research performed in the ten years since the discovery of *Mycobacterium* spp. as the causative agent of ulcerated striped bass in the Chesapeake Bay has raised as many questions as it has come up with answers. The disease process appears to be more complex than the one pathogen-one host scenario, and as such, future research into the process should take a more holistic approach including host species other than striped bass and investigating associations with environmental parameters. To focus attention on 'where to go from here,' this chapter will address challenges associated with this study, comment on the biological relevance of mycobacteriosis, and suggest areas for continued study.

6.2 Problems with data collection

Several methodological issues regarding data collection and specimen processing could have been altered to make this research stronger. However it is important to note that most of the factors to be discussed would require substantially greater cost. Nevertheless, what was accomplished in this study was done well, and within the existing (and not insignificant) available budget.

In the field, in order to synthesize environmental conditions to disease prevalence, it may have been advantageous to take more complete water quality

parameters, including presence of mycobacterial species in the water column. In addition, higher numbers of environmental mycobacteria have been associated specifically with high zinc, and humic and fulvic acids so including these factors in analyses would be beneficial (Kirschner et al. 1999). This may have been more relevant with younger fish that do not migrate, and these potential associations would be interesting to investigate specifically in the Pocomoke River, where prevalence was higher during the study period (Epidemiology Chapter 5).

Several actions, while increasing necropsy time, would allow for a more complete data set and fuller analyses. For example, the 0+ fish were extremely small, and thus, in general, culture data was collected on one set whereas histology data was collected on a separate set. Collecting both microbiology data and histopathology data from each fish would increase the power of analyses by increasing the small sample of mycobacteria positive fish. In addition, as it appears the level of trichodinid infection on the skin and gills may be associated with mycobacterial infection, while again significantly increasing the amount of necropsy time per fish, taking complete parasite data on all fish would make data analyses more complete. Further, instead of relying on scale confirmation of the length-at-age tables, aging fish by counting otolith rings would provide more reliable ages for individual fish.

Post-necropsy processing would also benefit from several changes and alterations. Again, however, this would increase the financial burden on the project. In histology, acid-fast slides were only stained and observed when a granuloma was present in the H&E slide. This would invariably miss those new active infections where the immune response has not yet been able to mount a response. In culture

processing, samples were plated on Middlebrook 7H10 agar, on which some mycobacterial species may be difficult to grow. For example, *M. avium* does not grow well on Middlebrook (author, A. Baya personal communication) and some isolates in the project, while acid-fast bacilli, failed to thrive on the Middlebrook media. In this case, fish were marked as mycobacteria positive, but since no FAME analyses were performed, speciation could not be attempted. Therefore, this highlights the potential of the project to have missed culturing some strains. Additionally, while incubating at room temperature is more environmentally relevant (Buller 2004), the decreased temperature may have prohibited the growth of some of the more fastidious strains. It is possible that a different composition of species/strains would be found if additional media or additional incubation temperatures were added to the project. However, the media and incubation temperature were chosen to facilitate the gas chromatography FAME analyses. Exacting growth regimes are required for the comparison of FAME chromatograms, and while primary isolation may have benefitted from additional media and incubation temperatures, secondary isolation would need to be on Middlebrook at room temperature for FAME.

6.3 Discussion

Whereas a wealth of research proves striped bass in the Chesapeake Bay are infected with mycobacterial species, the ramifications of this situation are less clear. The existence of mycobacteria-infected striped bass in the Chesapeake Bay is a problem, but in what ways? The questions raised by this scenario are complex and

diverse, and require a more thorough investigation into the disease process and its ecological context within the Chesapeake Bay.

The concept of infection and disease within the context of the age structure of the stock is an intriguing discussion. Evidence from this dissertation shows that 0+ fish can be infected, and infection prevalence increases from 0+ to 1+ and from 1+ to 2+ and 3+ striped bass (Chapter 5). Previous studies on older fish indicate that risk of disease (determined by histology) increased with age, and up to 90% of 3+ and 4+ striped bass are culture positive (Rhodes et al. 2004, Gauthier et al. 2006). Previous research further found per-year risk was higher in males (Gauthier et al. 2006). The current study found no association of infection with gender, however the previous study sampled 0-16 year old fish. The authors suggest the gender difference may be a function of males spending more time in the Bay than females, a factor with less weight in the current study as only a portion of the older age groups in the current study would be potential migrants to the ocean. Therefore, as discussed in Chapter 5, the prevalence of disease at age may be a factor of the ecological niche occupied. Additionally, as different age groups inhabit various environments, the mode(s) of transmission or natural reservoirs may vary as well. Further, individual mycobacterial species may reservoir separately than other species, and therefore the infection dynamics may be altered depending on the specific pathogen.

One of the more intriguing discussions regarding this epizootic pertains to why *M. shottsii* is found more commonly in older fish. Previous research found *M. shottsii* in more than 50% of infected fish, however, as discussed in Chapter 3, 92% of fish sampled were 4 years or older (Rhodes et al. 2004). Conversely, only 1%

(2/163) of 1+, 3% (5/192) of 2+ and 11% (13/116) of 3+ striped bass in this study were infected with *M. shottsii*. Eighty percent (81/101) of *M. shottsii* isolates in this study, however, were cultured from fish 4 years of age and older (Chapter 3). It is possible that the current sampling scheme missed the population of younger fish infected with *M. shottsii*. It is also possible that *M. shottsii* kills younger fish rapidly and therefore that population would be missing from the sampling. The latter theory is most likely not as realistic as it is important to note that *M. shottsii* is believed to be a species of low pathogenicity (Gauthier et al. 2003). More reasonably, older fish are more likely to come into contact with *M. shottsii* from an unknown reservoir, perhaps not even within the Chesapeake Bay, as other East Coast striped bass stocks are also infected with this mycobacterial species (Ottinger et al. 2007, author, A Baya, J Jacobs and A Overton, personal communication). Perhaps the distribution of *M. shottsii,* specifically, in Chesapeake Bay striped bass is related to the capture location within the Bay. The 1+ infected fish in this study were from the Pocomoke River, the 2+ from the Pocomoke, the Choptank and the Potomac rivers, and only in the 3+ fish was the Upper Bay included along with the Choptank and the Potomac rivers (data not shown). As the ages of infected fish increased, so did the latitude of their site of capture, which brings to mind putative association with distance from the mouth of the Bay. Whereas *M. shottsii* is not the only mycobacterial species involved, this discussion suggests some mycobacteria species may behave differently, or reservoir separately, than other pathogens, and should be analyzed within the context of the epizootic as a whole.

One set of questions raised by the findings in this dissertation regards the effects of disease on the individual fish, as well as on the stock as a whole. Researchers at the Virginia Institute of Marine Science (VIMS) have instituted a tag and release study to investigate whether infected fish die (Vogelbein et al. 2006), a factor important to managers attempting to set fishery regulations. However, as several of the mycobacterial species involved appear to have low pathogenicity, a rapid death for the majority of infected fish is not likely (Gauthier et al. 2003). Therefore, as mounting an immune response to these chronic infections inevitably diverts energy away from growth and reproduction, the potential exists for larger effects on striped bass stocks than purely the loss of individuals. It is unknown if reproductive potential is affected, and subsequently if recruitment to the stock and, in turn, the fishery, is altered.

The effects of a diseased stock also have economic consequences as striped bass support important commercial and recreational fisheries in the Chesapeake Bay. Commercial fishermen cannot sell overtly diseased fish. Additionally, the publicity associated with a diseased population may cause public refusal of that species, or seafood from the region in general, and cause considerable financial losses (Lipton 1998). Further, recreational fishers may plan trips in other regions, or avoid charter fishing in general.

Some of the more important questions that the mycobacterial epizootic raises focus on the health of the Chesapeake Bay as an ecosystem. While *M. shottsii* appears to be an important species involved in the current epizootic in older fish, there are certainly other mycobacterial species involved (Rhodes et al. 2004, Chapter

3). Additional hosts, also commercially important species including white perch, Atlantic menhaden, summer flounder and spot, have also been identified (Kane et al. 2007). Therefore, as the epizootic is larger than the one pathogen-one host scenario, and mycobacteria are commonly found in the aquatic environment, external factors potentially influence the current disease situation. It is important to note here that infection with mycobacteria does not necessarily imply disease state. Mycobacteriosis is a chronic disease, and as such, individuals may be infected, act as carriers, and/or mount granulomatous response, but lack the clinical disease symptoms such as emaciation or ulcerations. Therefore, it is possible that there exists a background of mycobacterial infections in wild fish which will cause disease and impair healthy functioning only after an insult. This decrease in immune function could be caused by any number of factors, such as decreased water quality including low dissolved oxygen, which occurs regularly in the main stem of the Chesapeake. The recent increase in the striped bass population in the 1990s and 2000s may have created crowding of preferred habitat, including the availability of preferred prey, and led to decreased nutrition, which in turn may exacerbate mycobacterial disease (Jacobs 2007). Further, as mycobacteria are known to metabolize a wide array of compounds not used by other microorganisms, e.g., polycyclic aromatic hydrocarbons, the increase of man-made compounds being introduced to the Bay ecosystem may be introducing new ecological niches in which mycobacteria can thrive (Falkinham 1996). It is possible that wild striped bass in the Chesapeake Bay can successfully live, potentially for years, with these infections, mount immune responses, and only when stressors render them incapable to further ward off even a

low pathogenic mycobacterial species does disease in the form of ulcers, or death, occur. Therefore, to more fully understand mycobacteriosis in the wild, analyzing the external stressors placed on the infected fish would contribute greatly to the investigations into the pathogens themselves. As mycobacterial infections are also found in other East coast striped bass stocks, namely Delaware Bay, DE and Roanoke River, NC, investigations should take into account the parameters and host-environment relationships similar to the Chesapeake Bay scenario, as well as those unique to the other ecosystems.

6.4 Future research

As the Chesapeake Bay striped bass mycobacterial epizootic appears unconfined to striped bass or the Chesapeake Bay, future research should continue into this complex and important disease process. As most previous research has concentrated on identifying the isolates obtained, and prevalence and distribution of infection, the potential for areas of continued research are immense.

As has been suggested in technical meetings, looking along the East coast for mycobacterial infections, not only in striped bass but also other species, would allow for a diverse array of environmental conditions to be investigated for effects on disease (Ottinger and Jacobs 2006). Additionally, monitoring other estuaries and potentially mapping disease spread may suggest preferred areas of disease, or lend insight to mode(s) of transmission. In addition, determining the history of disease presence in these other estuaries would be useful in analyzing disease spread. Jacobs (2007) undertook a retrospective analysis of striped bass tissue blocks from

Chesapeake Bay striped bass and determined that isolates molecularly speciated as *M. pseudoshottsii* appeared as early as 1984, 13 years before the current epizootic. Performing these studies on Delaware Bay and Roanoke River striped bass tissue blocks, if they exist, is warranted.

Continued development of better methods of speciation will allow faster results, analyses and conclusions. At present, however, concentration appears to be on molecular diagnostics. While rapid molecular methods are important, culturing the actual organism is necessary as well. For example, new species may be misidentified as existing species or missed entirely. Additionally, strains may have different virulence factors; e.g., *M. pseudoshottsii* possesses a plasmid encoding mycolactone, a virulence factor similar to that of *M. ulcerans* (Ranger et al. 2006). Further, the gas chromatography FAME method of speciation shows promise, but requires more strain sequencing for full validation, specifically sequencing multiple strains believed to be the same species. Finally, culturing the actual organism also allows for infectivity studies to be performed.

Understanding the current epizootic requires a working knowledge of the pathogenicity of mycobacterial species to hosts. *M. shottsii* has been shown to have low pathogenicity, yet appears to be of concern in older wild striped bass (Gauthier et al 2003, Rhodes et al. 2004). Conversely, *M. marinum* has high pathogenicity and is of great concern in aquaculture settings, yet is infrequently isolated from wild striped bass (Chinabut 1999, Gauthier et al. 2003, Rhodes et al. 2004). However it is possible that the discovered pathogenicity of isolates may support field observations in that low pathogenic strains result in chronic disease as opposed to large-scale die

offs. Further, nothing is known about the relative pathogenicity of the isolates cultured from other wild fish, including Atlantic menhaden or white perch, and the ramifications of infections to those stocks. A corollary to the interest of pathogenicity in piscine hosts is the potential of these mycobacteria to infect humans and the health threats they pose to populations encountering infected fish.

Elucidating specific host-pathogen relationships would be critical to determining modes of transmission and virulence factors. As mentioned previously, *M. shottsii* and *M. pseudoshottsii* appear to only infect striped bass. This may be a function of the susceptibility of the striped bass host, the mode of transmission as only striped bass come into contact with the natural reservoir for these particular species, or potentially previous research has just not discovered other existing hosts yet. In particular, *M. shottsii* and *M. pseudoshottsii* appear more often in older striped bass and potentially older individuals of other species occupying similar ecological niches have not yet been sampled. Conversely, striped bass appear particularly susceptible to this disease, which is one of the reasons why striped bass hybrids, which benefit from hybrid vigor, are a preferred aquaculture species (Harrell 1997, Wolf and Smith 1999). Nevertheless, if specific pathogens are host specific, particular virulence factors may be the cause. In this case, specific pathogens may have evolved to infect that particular host.

Evidence supports the existence of genetic recombination among *M. tuberculosis* strains and this occurrence warrants further investigations in environmental species (Liu et al. 2006, Lazzarini et al. 2007). As the various niches occupied by mycobacteria are pressured by environmental change, the effect of this

change on mycobacteria should be explored. Mycobacteria are known to persist in harsh environments, and there exists the potential for mycobacteria to interact with ciliates and amoebae, or to form biofilms in order to combat less hospitable conditions (Falkinham et al. 2004).

The natural reservoir(s) of environmental mycobacteria associated with the current epizootic are unknown. Environmental mycobacteria are found in soils and water; they may be free-floating in water, possibly attached to suspended sediments because of their hydrophobicity (Falkinham et al. 2004). Environmental mycobacteria are known to persist within amoebae, and this existence has been suggested as a training ground for mycobacteria, specifically for enhanced entry into macrophages and intracellular replication (Cirillo et al. 1997, Adekambi et al. 2006, Thomas and McDonnell 2007). Further, mycobacteria are found within biofilms, which are suggested to facilitate clonal expansion of virulent strains (Hall-Stoodley and Stoodley 2005). Therefore, a systematic investigation into the potential of mycobacteria involved in piscine mycobacteriosis to infect free-living amoebae, their presence in biofilms, and the putative increased virulence in such is warranted.

6.5 Final thoughts

The current epizootic of mycobacterial infections in Chesapeake Bay striped bass should be examined within an ecosystem context. Schrag and Weiner (1995) suggest that emerging infectious diseases typically occur because the ecology of the host, the pathogen or both has been altered. Further, Belden and Harris (2007) suggest that emerging infectious diseases in wildlife may be associated with climate

changes in those normal bacterial communities, and thus proportions and natural competitors of pathogens, are altered. Therefore, emerging infectious wildlife diseases are complex and thus an understanding of the disease process and emergence requires an ecosystem approach.

Appendix

Appendix A. Bacteria isolated from Chesapeake Bay striped bass, ages 0+ to 3+, during 2003-2005. Values given are percent positive.

Age	River	Year	n	% positive	*Vibrio vulnificus*	*V. fluvialis*	*V. alginolyticus*	*V. parahaemolyticus*	*V. splendidus*	*Aeromonas hydrophila*	*Photobacterium damsela*	*Pseudomonas flourescens*	*Ps. putida*	*Ps. stutzeri*	*Shewanella putrifaciens*	*Acinetobacter haemolyticus*	*V. anguillarum*	*V. cholera non 01*	*V.mimicus*	*V. ordalii*	*V. pelagius II*	*Vibrio spp.*	*A. sobria*	*Pleisomonas shigelloides*	*Bacillus cereus*	*Citrobacter freundii*	*Escherichia coli*	*Moraxella sp.*	*Stenotrophomonas maltophilia*	*Acin. junii*	*Acin. Calcoaceticus*	*Acin. Johnsonii*	*Ps. aeriginosa*	*Pseudomonas sp.*	*Listonella anguillarum*	*Brevundimonas vesicularis*	*Carnobacterium piscicola*	*Pasturella multocida*
0+	UB	03	60	85	5		2			75		3		2	5				2					20	2	2	2		2	2		2						
		04	60	70	2	12				13		3											7	47														
		05	0																																			
	Chop	03	60	82	47	7				2	20			2				35	3					2					15		2							
		04	60	15	10												7																					
		05	28	57	50					4		4			4																							
	Nan	03	60	90	60	13			8	12	23		7	2	2						2			18						7								
		04	59	12							12																											
		05	0																																			
	Poc	03	0																																			
		04	19	53	21	5		5			5	5			11							5						11										
		05	11	73	18	9	9	9		9	18				9																							
	Pot	03	60	68	7	8		2		2	28	2	5					2		3	2	32																
		04	60	33	7	2						18			2	5													10									
		05	0																																			

Age	River	Year	n	% positive	*Vibrio vulnificus*	*V. fluvialis*	*V. alginolyticus*	*V. parahaemolyticus*	*V. splendidus*	*Aeromonas hydrophila*	*Photobacterium damsela*	*Pseudomonas flourescens*	*Ps. putida*	*Ps. stutzeri*	*Shewanella putrifaciens*	*Acinetobacter haemolyticus*	*V. anguillarum*	*V. cholera non 01*	*V. mimicus*	*V. ordalii*	*V. pelagius 11*	*Vibrio spp.*	*A. sobria*	*Pleisomonas shigelloides*	*Bacillus cereus*	*Citrobacter freundii*	*Escherichia coli*	*Moraxella sp.*	*Stenotrophomonas maltophilia*	*Acin. junii*	*Acin. Calcoaceticus*	*Acin. Johnsonii*	*Ps. aeriginosa*	*Pseudomonas sp.*	*Listonella anguillarum*	*Brevundimonas vesicularis*	*Carnobacterium pisicola*	*Pasturella multocida*
1+	UB	03	0																																		
		04	11	9	9																																
		05	25	68	36	40					8	4			4																						
	Chop	03	10	0																																	
		04	24	50	4	8		8				25			21																			4	4		
		05	15	13								7																							7		
	Nan	03	0																																		
		04	0																																		
		05	0																																		
	Poc	03	0																																		
		04	12	17	8						8																										
		05	33	42		9		6			21			3	6																				3		
	Pot	03	2	0																																	
		04	19	26	5							5			16																						
		05	10	30											10																					20	

Age	River	Year	n	% positive	*Vibrio vulnificus*	*V. fluvialis*	*V. alginolyticus*	*V. parahaemolyticus*	*V. splendidus*	*Aeromonas hydrophila*	*Photobacterium damsela*	*Pseudomonas flourescens*	*Ps. putida*	*Ps. stutzeri*	*Shewanella putrifaciens*	*Acinetobacter haemolyticus*	*V. anguillarum*	*V. cholera non 01*	*V.mimicus*	*V. ordalii*	*V. pelagius II*	*Vibrio spp.*	*A. sobria*	*Pleisomonas shigelloides*	*Bacillus cereus*	*Citrobacter freundii*	*Escherichia coli*	*Moraxella sp.*	*Stenotrophomonas maltophilia*	*Acin. junii*	*Acin. Calcoaceticus*	*Acin. Johnsonii*	*Ps. aeriginosa*	*Pseudomonas sp.*	*Listonella anguillarum*	*Brevundimonas vesicularis*	*Carnobacterium piscicola*	*Pasturella multocida*
2+	UB	03	13	0																																	
		04	18	22	6						11				6																						
		05	24	50	38	8			4		13		4																								
	Chop	03	10	0																																	
		04	19	16		5		5				11																									
		05	23	65	43	4					30																										4
	Nan	03	0																																		
		04	0																																		
		05	0																																		
	Poc	03	0																																		
		04	6	17	17					17																											
		05	26	31	4		8	12			4				4	4																			4		
	Pot	03	6	0																																	
		04	14	14											7																		7				
		05	36	39	11	3		8							3																				8	8	

Age	River	Year	n	% positive	*Vibrio vulnificus*	*V. fluvialis*	*V. alginolyticus*	*V. parahaemolyticus*	*V. splendidus*	*Aeromonas hydrophila*	*Photobacterium damsela*	*Pseudomonas flourescens*	*Ps. putida*	*Ps. stutzeri*	*Shewanella putrifaciens*	*Acinetobacter haemolyticus*	*V. anguillarum*	*V. cholera non 01*	*V.mimicus*	*V. ordalii*	*V. pelagius II*	*Vibrio spp.*	*A. sobria*	*Pleisomonas shigelloides*	*Bacillus cereus*	*Citrobacter freundii*	*Escherichia coli*	*Moraxella sp.*	*Stenotrophomonas maltophilia*	*Acin. junii*	*Acin. Calcoaceticus*	*Acin. Johnsonii*	*Ps. aeriginosa*	*Pseudomonas sp.*	*Listonella anguillarum*	*Brevundimonas vesicularis*	*Carnobacterium piscicola*	*Pasturella multocida*
3+	UB	03	7	0																																	
		04	1	0																																	
		05	27	44	33	7					4																					4					
	Chop	03	0																																		
		04	0																																		
		05	24	4	4																																
	Nan	03	0																																		
		04	0																																		
		05	0																																		
	Poc	03	0																																		
		04	2	0																																	
		05	17	0																																	
	Pot	03	5	0																																	
		04	0																																		
		05	30	40	7			20			13																									3		

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