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Final Report

Investigations of Mycobacteriosis in Chesapeake Bay Striped Bass *Morone saxatilis*: Large-Scale Field Survey and Molecular Diagnostics

Introduction

This is a large temporal- and spatial-scale survey of mycobacterial disease in Chesapeake Bay striped bass using the VIMS ChesMMAP trawl survey as a sampling platform. Striped bass were collected at 80-90 randomized stations throughout the Bay five times per year beginning in 2003 and continuing through November of 2006. Pending further support, the ChesMMAP mycobacteria survey will continue through 2007. A proposal requesting continued funding of this project has been submitted to the RFAB.

Beginning in 1997, striped bass exhibiting poor body condition and ulcerative skin lesions were observed in Virginia and Maryland waters of Chesapeake Bay (see Fig. 1). Histopathology revealed granulomatous inflammation associated with acid-fast bacteria, consistent with infection by *Mycobacterium* spp. (Vogelbein et al. 1999). Subsequent surveys of mycobacteriosis in Chesapeake Bay striped bass have demonstrated high visceral and dermal disease prevalence in Bay waters (Cardinal 2001, Overton et al. 2003). Mycobacteriosis has previously been described in wild and cultured striped bass from the US Pacific Coast, with prevalences reaching 68% and 80%, respectively (Sakanari et al. 1983, Hedrick et al. 1987). Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) are also known to be susceptible from spontaneous infections observed in aquaculture and via experimental exposures (Wolf & Smith 1999, Bowser et al. 2004).

Mycobacteriosis is a subacute to chronic disease common in wild and captive fishes worldwide. Mortality is not typically associated with mycobacteriosis in wild finfish populations, however, this may be attributed to the difficulties in observing long-term mortalities from a chronic disease in the field. High mortality is commonly observed in aquaculture (Nigrelli & Vogel 1963, Hedrick et al. 1987, Bruno et al. 1998). *Mycobacterium marinum, M. fortuitum* and *M. chelonae* are the most frequently cultured isolates from diseased fishes, although several other species have been reported (Lansdell et al. 1993, Herbst et al. 2001, Rhodes et al. 2004). In most cases, disease is visceral, with spleen, liver and kidney being the primary target organs. Granulomatous inflammation, often with extensive tissue destruction, is characteristic, although more poorly organized inflammatory responses are observed, typically in association with high bacterial loads (Kent et al. 2004). External clinical signs include scale loss, skin ulceration, emaciation, exophthalmia, pigmentation changes and spinal defects (Nigrelli & Vogel 1963, Snieszko 1978, Wolke & Stroud 1978, Bruno et al. 1998).

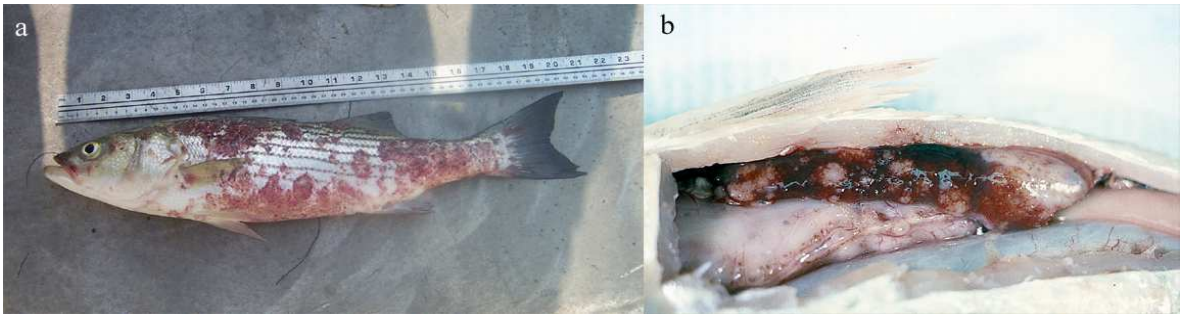


Fig. 1: Gross clinical signs of mycobacteriosis in Chesapeake Bay striped bass. a) Severe ulcerative dermatitis. Note shallow, hemorrhagic and hyper-pigmented (dorsal) ulcers. b) Multi-focal gray nodules (arrows) within the spleen.

Characterization and identification of agent(s) associated with the epizootic of mycobacteriosis in Chesapeake Bay striped bass are ongoing. A number of isolates have been cultured, including the new species, *M. shottsii* and *M. pseudoshottsii,* the pathogen *M. marinum,* and species typically considered to be harmless saprophytes (e.g., *M. gordonae*) (Rhodes et al. 2001, Rhodes et al. 2003, Rhodes et al. 2004, Rhodes et al. 2005). Other isolates resemble but do not exactly match known mycobacterial species, and are probably new to science (Rhodes et al. 2004) . *Mycobacterium shottsii* and *M. pseudoshottsii* are the most common isolates recovered from diseased striped bass, and co-infections with multiple mycobacterial species occur. Both *M. shottsii* and *M. pseudoshottsii* are closely related to *M. marinum* and *M. ulcerans*, important pathogens of fishes and humans, respectively.

Recently, researchers have reported a decline in striped bass tagging returns, suggesting that natural mortality of striped bass stocks in Chesapeake Bay has increased in recent years (Kahn 2004). The causes for this apparent increase in natural mortality are unknown, but the high observed prevalence of mycobacteriosis in Chesapeake Bay striped bass, as well as the decreased condition factor observed in many heavily infected fish, suggests that this disease may be at least partly responsible. Our research group at VIMS has an ongoing program, funded by Sea Grant, RFAB, and NOAA Chesapeake Bay Office, designed to determine whether or not diseased fish are experiencing increased mortality in Chesapeake Bay. In addition to the possibility that mycobacteriosis may be causing increased mortality among striped bass in Chesapeake Bay, skin lesions of mycobacterial origin have been reported with increasing frequency in these fish since 1997. Lesions can be highly disfiguring, to the point that anglers frequently do not wish to handle affected fish. Although negative impacts of skin disease on the recreational fishery cannot easily be measured, the product quality of this popular fishery is clearly being affected.

Prior surveys of mycobacteriosis in Chesapeake Bay striped bass have been greatly limited in terms of spatial and temporal coverage. Additionally, surveys relying on pound nets or hook-and-line sampling methodologies are subject to serious collection biases which may lead to erroneous conclusions. Therefore, extension of the findings of previous surveys to interpretation of the epizootiology of mycobacteriosis on a Bay-wide scale is problematic. Since March 2003, we have collaborated with the Chesapeake Bay

Multispecies Monitoring and Assessment Program (ChesMMAP) through the Fisheries Department at VIMS (http://www.fisheries.vims.edu/chesmmap/). This federal- and state-funded program is a large-scale trawl survey which samples adult fish five times per year over the entire Bay, ranging from the Bay mouth to Poole's Island. Sampling is performed in twenty-minute tows at 80 stations per cruise, with a stratified-random design that covers all depths of the Bay. Data collection on fish includes basic morphometrics (length, weight), otolith-determined age, and gut content analysis. In addition, comprehensive environmental data are collected at each station. The ChesMMAP survey is intended to develop data on the distribution and feeding habits of finfish species in Chesapeake Bay, and to apply these data to development of comprehensive multispecies management models. By virtue of its design and scale, this survey also provides an ideal sampling platform for studying disease in wild fishes. Consequently, we have been using the ChesMMAP platform to survey the presence and severity of internal mycobacteriosis, as well as presence of skin lesions.

One of the major difficulties in understanding mycobacteriosis in Chesapeake Bay striped bass is that it does not appear to be a simple case of one bacterial species causing the disease. Multiple *Mycobacterium* spp. have been cultured from both diseased and healthy Chesapeake Bay striped bass, either individually or in combination (Rhodes et al. 2004). This strongly suggests that more than one species of mycobacteria is involved in production of disease. The limited number of fish from which cultures have been obtained, however, has not led to definitive understanding of which species cause the disease, and which species are responsible for disease on a Bay-wide scale. Detection and species identification of mycobacteria in a large number of fish over the entire area of the Bay is necessary in order to shed light on these questions. Because of the prohibitive time requirements of standard culture and the logistical difficulties of aseptic sampling on a large scale, accomplishment of these goals requires use of rapid molecular diagnostic tools. With previous support from the VMRC/RFAB, we have developed nested Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (nPCR/RFLP) assays capable of detecting and differentiating species of *Mycobacterium* from striped bass tissues. These techniques were used to analyze splenic tissue from striped bass collected in 2005 and 2006.

Methods: Field Collections and Histological Analyses

FIELD COLLECTIONS

All striped bass were collected from the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP). During 2003-2005, a total of 15 ChesMMAP cruises were conducted (March, May, July, September, and November annually) and approximately 80 to 90 sites were sampled each cruise within the mainstem of Chesapeake Bay. Sampling is ongoing for the 2006 season, and the final November cruise is underway at the time of this report. Sampling locations were chosen according to a stratified random design, with strata based on water depth (3 – 9 m, 9 – 15 m, and >15 m) within five 30 latitudinal minute regions of the bay. The number of locations sampled in each stratum of each region was randomly selected in proportion to the area

of that stratum. At each sampling location, a 13.7 m 4-seam balloon otter trawl (15.2 cm stretch mesh in the wings and body and 7.6 cm stretch mesh in the cod end) was towed for 20 min at approximately 6.5 km h^{-1}. Each catch was sorted and individual lengths (fork length, FL) of striped bass were recorded. If distinct size-classes of striped bass were evident in the catch, lengths measurements were coded according to a coarse size categorization (e.g., small, medium, large). A subsample of each size-class (if multiple were present) was further processed for total weight (nearest 0.01 kg) and sex determination. Otoliths were removed for age determination and portions of spleen were excised and placed in either Z-fix (Anatech, Battle Creek) for histopathology or 95% ethanol for molecular analyses.

LABORATORY PROCEDURES

The right sagittal otolith was used to determine the age of striped bass collected by the ChesMMAP survey. A thin transverse section was cut through the nucleus of the otolith and the resulting section was mounted on a glass slide (Secor et al. 1995). Annuli were counted by viewing the slide under a dissecting microscope using transmitted light (500X magnification). Three readers aged all specimens once and a randomly selected subsample of 200 specimens a second time. Two-dimensional tests of symmetry between and within readers were not significant (χ^2 tests, all p-values > 0.25) (Hoenig et al. 1995).

Year-class ages were assigned based on the conventional January 1 birthdate. Annulus formation occurs between May and June in striped bass (Bobko et al. 2003). The assigned age of a given specimen collected during the July, September, and November surveys was therefore equivalent to the number of annuli present on its otolith. For striped bass collected in March and May, the assigned age was equal to the number of annuli present when the most recently formed annulus occurred either along or near the edge of the structure (i.e., annulus had already formed that year), and was one plus the number of annuli present when there was a relatively large translucent zone between this annulus and the edge of the otolith section (i.e., annulus had yet to form that year).

Spleens were fixed for at least 72 hours and then divided transversely into six approximately equal portions, removing an additional 1-2 mm section between portions. Portions were then processed for routine paraffin histology (Prophet et al. 1994). If all six portions for an individual spleen would not fit in one cassette, portions were randomized and distributed to multiple cassettes. Sections were cut at 5 µm and stained with hematoxylin and eosin (HE). One section of up to six spleen portions and a 50µm step section were included on each slide. Tissue sections were examined on an Olympus AX-70 light microscope for the presence of granulomas. A granuloma was defined as any lesion containing epithelioid macrophages (Cotran et al. 1999). Granulomas containing helminth parasites were not counted. Granulomas with multiple necrotic core regions but confluent epithelioid cell layers were counted as single lesions. HE-stained sections were examined for granulomas until a) 12 sections (six sections + six 50µm step sections) per fish were determined to be negative, or b) granulomas were found. Original sections were always counted before step sections. In the case of b), granulomas were counted in all sections on that slide, excluding step sections, unless granulomas were

found in step sections and not in original sections. Splenic area measurements were taken by photographing sections at 6.3X magnification on an Olympus SZX-ZB9 dissecting microscope equipped with a Nikon DXM1200 digital camera. Splenic area (mm^2) was calibrated and measured with MetaMorph software (Universal Imaging, Downingtown, PA). Area measurements were taken on all sections examined for granulomas, excluding fish with no granulomas. Severity index (SI) was calculated as [$\log_{10}$ (granulomas/mm^2)+1], and severity category (S) was assigned as follows: **0-** SI=0; **1-** $0<SI\leq0.1$; **2-** $0.1<SI\leq0.5$; **3-** $SI>0.5$. In a small number of fish, SI could not be calculated due to tissue destruction by severe confluent granulomatous disease or large cystic lesions. These fish were assigned to S=3.

Results

- Note: Major results/findings are summarized in bulleted statements located at the end of each section.

SAMPLES COLLECTED

For the sampling period from 2003-2005, a total of 1,575 striped bass were collected by the ChesMMAP survey and examined histologically for the presence of mycobacterial granulomas. Of these, 963 were male, 568 were female, and sex was not determined in 44 fish. Ages ranged from 0 (young of the year) to 16. Fish between ages 1 and 3 were best represented in the sample. In 2006, an additional 363 fish were collected, and we anticipate an additional 200+ fish will be collected during the upcoming November cruise. In order to eliminate observer bias, otolith samples from fish collected during 2006 will not be analyzed until after the November cruise. Because age has a large effect on observed prevalence (see 2003-2005 results below) histological analyses for striped bass collected during 2006 will not be presented in this report. Publication of the findings of this study, however, will include full analysis of all fish collected from 2003-2006, including age-based analyses.

Figure 2 presents prevalence (presence) of disease with respect to age and sex of striped bass from 2003-2005. While disease prevalence is similar in male and female fish until approximately age 6, a major contrast is observed between sexes in fish age 7 and older. There are several possible explanations for this finding. The possibility that male fish are inherently more susceptible to disease is unlikely, based on the similarity in prevalence observed between sexes in age ≤6 fish. Disease (and therefore infection) is observed in age 1 fish, which can be presumed not to have left Chesapeake Bay for coastal migration (Mansueti 1961). Therefore, both exposure and factors necessary for disease expression are found within Bay waters. Striped bass are sexually dimorphic both with respect to size and migratory behavior, with female fish typically beginning to leave the Bay at age 2+, while male fish do not migrate to an appreciable extent until at least age 4 (Kohlenstein 1981, Dorazio et al. 1994). Thus, male fish as a whole spend more time in Bay waters, and are thus presumably exposed to risk factors for disease for a longer period of time than females. The fact that apparent prevalence for both male and female fish increased until age 6, however, coupled with the lack of knowledge about

what disease risk factors may exist outside the Bay, makes this argument suspect, and much better information regarding the residence time and distribution of male and female striped bass in Chesapeake Bay is necessary to further evaluate it. Further, this argument would enjoy greater support if prevalence among female fish continued to increase with age, but at a lower rate than males. The fact that apparent prevalence decreased in age 7+ females relative to younger females suggests that additional mechanisms are at work. The observed decrease may be due to mortality of diseased females, regression/healing of disease (e.g. through lack of exposure to risk factors), or differential migration of diseased fish. It is generally assumed that mycobacteriosis in fishes, while chronic, is ultimately fatal, and regression of disease in laboratory exposure studies has not, to our knowledge, been observed.

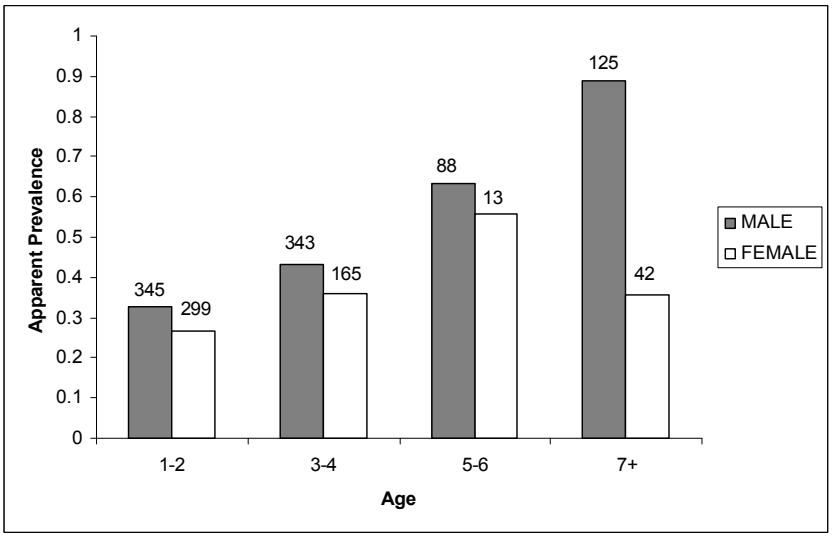


Fig. 2: Apparent prevalence of mycobacteriosis for male (gray bars) and female (clear bars) striped bass; 2003-2005 pooled data. Sample sizes are presented above bars.

Figure 3 shows apparent prevalence of mycobacterial granulomas in striped bass collected between 2003-2005 with respect to cruise (season). Cruises 1-5 were performed in March/April, May, July, September, and October/November, respectively. Apparent prevalence did not appear to vary by cruise for female fish, whereas male fish demonstrated a trend toward increasing apparent prevalence during warmer, summer months, and decreased prevalence during spring and winter.

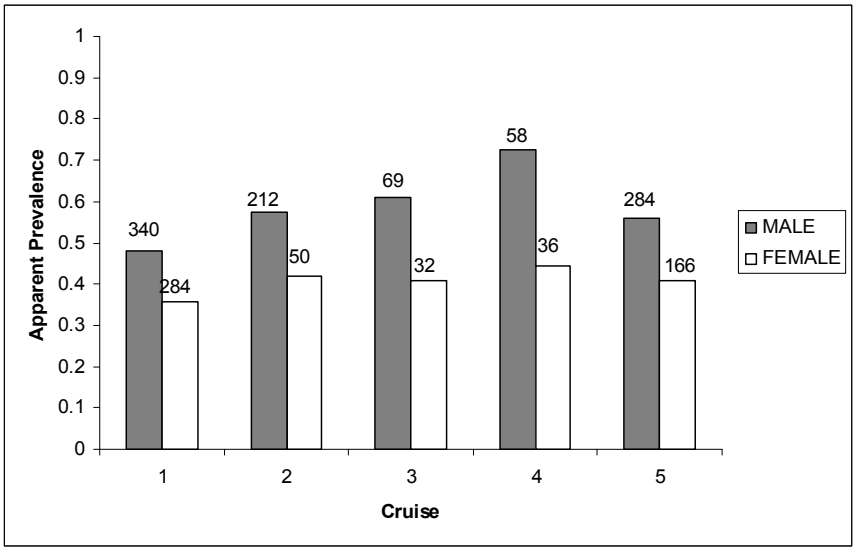


Fig. 3 Apparent prevalence of mycobacteriosis vs. cruise (season) for male (gray bars) and female (clear bars) striped bass. Pooled data 2003-2005. Sample sizes are presented above bars.

Figure 4a shows apparent prevalence of mycobacterial granulomas in striped bass versus region in which fish were collected.

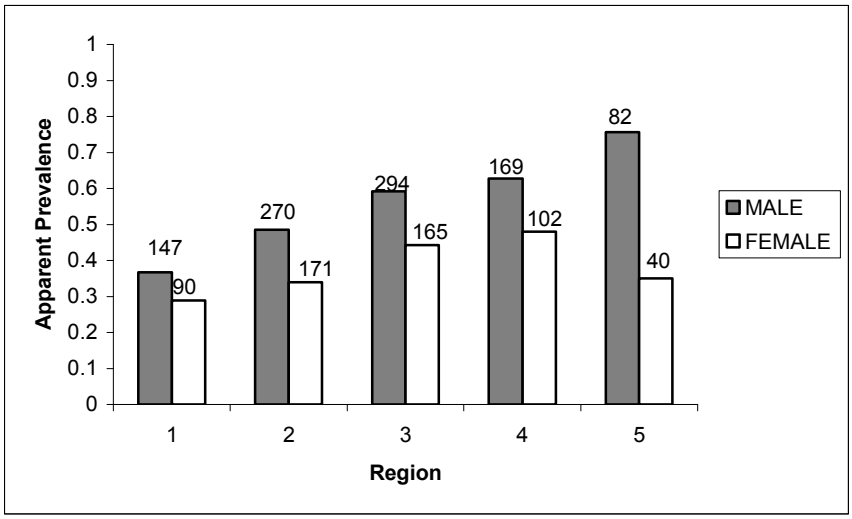


Fig. 4a Apparent prevalence of mycobacteriosis vs. region (1=northern Bay, 2=Southern Bay, see Fig. 3b) for male (gray bars) and female (clear bars) striped bass. Pooled data 2003-2005. Sample sizes are presented above bars.

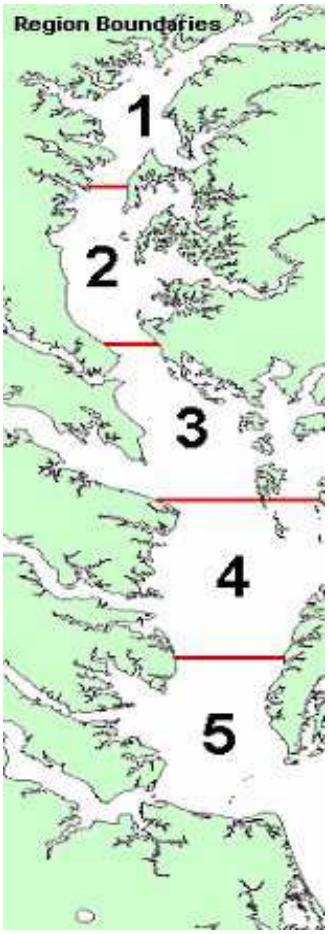


Fig. 4b: Regional boundaries of Chesapeake Bay used in analysis of prevalence data

Regional prevalence data is somewhat difficult to interpret, as the subject of study, the striped bass, is a highly migratory animal. Therefore, a fish inhabiting region 1 during cruise 1 may be found in region 5 later in the year. On a purely descriptive level, apparent prevalence appeared to increase in both male and female fish with decreasing latitude, although prevalence appeared to decrease in female fish in the southernmost reaches of the Bay.

In Figures 1-3, we see a number of variables (e.g. age, sex, cruise, and region) plotted individually against an outcome (i.e. disease prevalence). In order to analyze the data properly, we must be cautious of the phenomenon of *confounding*. Confounding occurs when variables of interest are associated with the outcome, and at the same time with other variables. To illustrate this concept, consider a case in which disease prevalence is found to be higher in one region than another. This increased prevalence may be due to an actual regional effect, or it may be due to the fact that there were more older fish (which have higher disease prevalence) in that region. In this case, region and age are confounders, as

either or both may be directly related to the outcome (disease), but age may additionally be directly related to region.

Fortunately, there are statistical methods that allow us to control for confounding and simultaneously examine the effect of multiple variables on an outcome. One such method is known as logistic regression. Logistic regression examines multiple variables simultaneously, and assigns an "odds ratio" to each variable that is corrected for the other variables. This corrected odds ratio indicates whether a particular exposure (variable) is significantly positively or negatively related to risk for disease. An odds ratio (abbreviated OR) greater than one indicates that the variable is a positive risk factor for disease, whereas an odds ratio of less than one indicates that a variable is negatively related to disease, or in some cases, protective against disease. An odds ratio that is not significantly different from one indicates no relationship between disease and the variable of interest. Logistic regression analysis of data from 2003-2005 is presented in Table 1. An important point to note here is that prior to generating the data in Table 1, it was determined that year of collection (2003, 2004, 2005), did not have a significant relationship to disease when corrected for other variables (age, sex, cruise, region). This finding is important for two reasons. First, because year was not a significant risk factor, this allowed us to pool data from 2003-2005, which increased sample sizes and precision. Second, this finding may be directly interpreted as indicating no change in overall disease status of striped bass in Chesapeake Bay from 2003-2005. This is the first indication of long-term dynamics of this disease in the Bay, and at least partially answers the question of whether disease is getting worse or better overall in the striped bass population.

From Table 1, we can see that, even when corrected for cruise and region, there was a highly significant increased risk of disease for male fish with increasing age. In fact, older male fish (age 7+) were over 22 times as likely as younger fish (age 1-2) to have splenic disease. Female fish also showed increased risk of disease with age, however, as was seen in the raw apparent prevalence data (Fig. 2), risk of disease was only slightly elevated in age 7+ fish relative to age 1-2. In both male and female fish, risk for disease appeared decreased for fish collected during cruise 1 (March/April), and male fish also showed this reduced risk during cruise 2 (May). This may reflect increased expression of disease during summer months, and/or mortality of heavily diseased fish during periods of increased water temperature. A significantly reduced risk for disease was observed in region 1 (northern Bay) for male fish, and a barely significant increase in risk was observed for female fish in region 3 (mid-Bay). As stated previously, interpretation of these results is difficult due to the highly migratory nature of striped bass.

Sex		Parameter	OR	95%CI	*p*
Male	Age	7+	**22.088**	**42.327-11.526**	**<0.001**
		5-6	**6.485**	**11.316-3.716**	**<0.001**
		3-4	**4.347**	**6.23-3.033**	**<0.001**
		1-2	Reference		
	Cruise	1	*0.346*	*0.52-0.231*	*<0.001*
		2	*0.404*	*0.645-0.254*	*<0.001*
		3	0.612	1.207-0.31	0.156
		4	1.059	2.151-0.521	0.875
		5	Reference		
	Region	1	*0.314*	*0.623-.158*	*0.001*
		2	0.692	1.288-0.371	0.245
		3	0.978	1.83-0.522	0.944
		4	0.973	1.894-0.5	0.936
		5	Reference		
Female	Age	7+	**2.304**	**4.723-1.124**	**0.023**
		5-6	**42.913**	**349.195-5.274**	**<0.001**
		3-4	**4.687**	**7.52-2.922**	**<0.001**
		1-2	Reference		
	Cruise	1	*0.447*	*0.756-0.265*	*0.003*
		2	0.714	1.539-0.331	0.39
		3	0.922	2.258-0.376	0.858
		4	1.067	2.457-0.464	0.878
		5	Reference		
	Region	1	1.093	2.75-0.434	0.85
		2	1.414	3.245-0.616	0.414
		3	**2.326**	**5.378-1.006**	**0.048**
		4	1.775	4.241-0.743	0.196
		5	Reference		

Table 1: Results of logistic regression analysis of prevalence data from 2003-2005. Analyses are stratified (separated) by sex. Logistic regression requires one subcategory for each variable (e.g. 1-2 for Age) to serve as a reference category. Therefore, the ORs presented for other subcategories are interpreted relative to the reference. For example, male fish of age 3-4 have an odds ratio of 4.347 relative to control. This indicates the risk of an age 3-4 fish having splenic disease is 4.35 times that of an age 1-2 fish. 95% CI is the 95% confidence interval for the calculated OR. This is essentially the error associated with the measurement, and indicates the range in which we can be 95% certain that the "true" OR lies. If this range does not include one, this indicates that the variable is significantly related to the outcome (disease). In this table, ORs significantly above 1 (positive risk factor for disease) are presented in bold type, and ORs significantly below 1 (negative risk factor for disease are presented in italics.

Summary: Logistic regression analyses of histological data 2003-2005

- Mycobacteriosis in Chesapeake Bay striped bass appeared to remain fairly constant in terms of apparent prevalence between 2003-2005. This suggests that the disease situation in the Bay is currently stable.

- Disease prevalence is dependent on both age and sex of striped bass. Therefore, any future studies must take these variables into account for the purposes of calculating spatial or temporal prevalence values.

- Risk of disease increased in both male and female fish with increasing age until around age 6, then dropped considerably in female fish. This could be due to death of diseased females, regression of disease, altered migration patterns, or a combination of these factors.

- Risk for disease appeared to be decreased during the early months of the year for both male and female fish.

During 2006, groundbreaking statistical methods for analyzing the type of prevalence data collected in this study became available. The theory behind these methods is complex, and will not be explained in detail here, but they enable estimation of population-level mortality in addition to risk factors for disease as described above (Heisey et al. 2006)

The new statistical approach employed for analyses of these data begins by modeling the "force of infection," or the rate at which disease-negative animals become disease-positive. Variables are then added to the model, in a similar manner to logistic regression. The real innovation in this technique is that disease-associated mortality may be added to the model as another variable. Multiple models containing different combinations of variables are applied to the cross-sectional apparent prevalence data, and the models with the best "fit" are assumed to best describe the observed data. In the case of the data generated by this study, models including a disease-associated mortality term improved the fit of the model to the data significantly, indicating that disease-associated mortality did play a role in structuring the observations. Once the "best-fit" model, including a mortality term, was determined, it was further possible to estimate the disease-associated mortality in the striped bass population. Disease-associated mortality was calculated to be 0.64. This means that relative to a non-diseased fish, a diseased fish had a 64% probability of surviving from year to year.

This work represents the first attempt to detect and quantify the level of disease-associated mortality in striped bass of Chesapeake Bay. Additional studies, funded by VMRC, SeaGrant, and NOAA, which use more direct tag-and-release methodologies, are currently being performed by our group. Together, these studies will provide answers to the very important question of whether mycobacteriosis is causing the death of striped bass in Chesapeake Bay.

- Statistical analysis of cross-sectional apparent prevalence data collected between 2003-2005 indicates that mycobacteriosis-associated mortality of striped bass is occurring on a population level in Chesapeake Bay.

- Year-to-year survival of diseased fish was estimated to be 64% that of non-diseased fish

Severely emaciated (skinny) striped bass have been observed with increasing frequency by fishers in recent years. These fish often have external ulcerative skin lesions consistent with mycobacteriosis, and it is thought that mycobacterial disease leads to wasting. To date, no studies have definitively examined the association between mycobacterial disease and fish condition. In order to explore the relationship between disease and condition of striped bass, we performed a regression analysis of eviscerated weight vs. length, comparing the slopes of regression for different disease severity categories. Analysis of covariance (ANCOVA) indicated no significant differences in the regression slopes for the different disease categories, indicating that disease did not have a significant impact on weight-at-length (condition). The p value of this analysis, however, was small ($p=0.073$), and barely missed significance at the $\alpha=0.05$ level. This indicates that a trend toward decreasing condition was observed in the most severely diseased fish. It should be noted here that these analyses only examine the association between disease and condition, and do not have the power to reveal a causal relationship. Controlled laboratory studies will be necessary to examine whether disease eventually leads to decreased condition, or if decreased condition predisposes fish to development of disease.

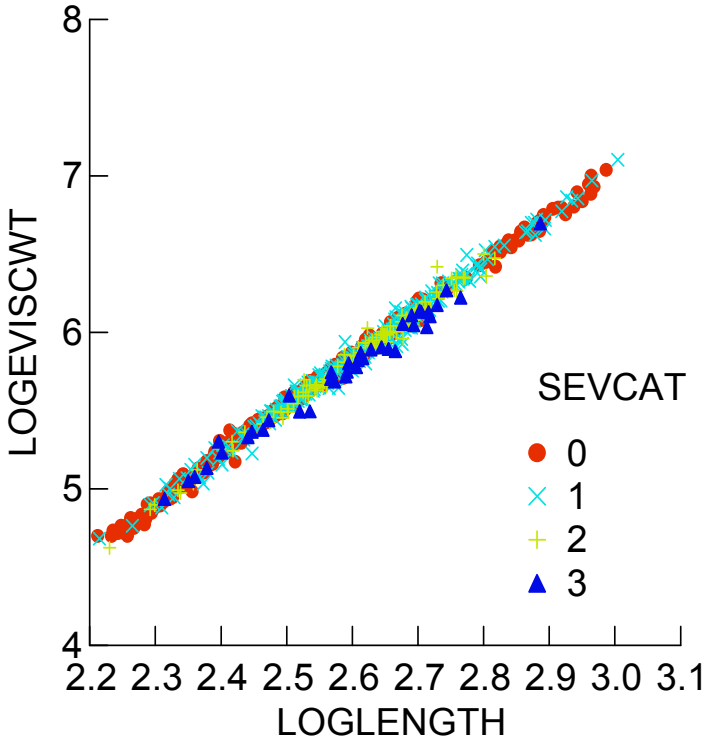


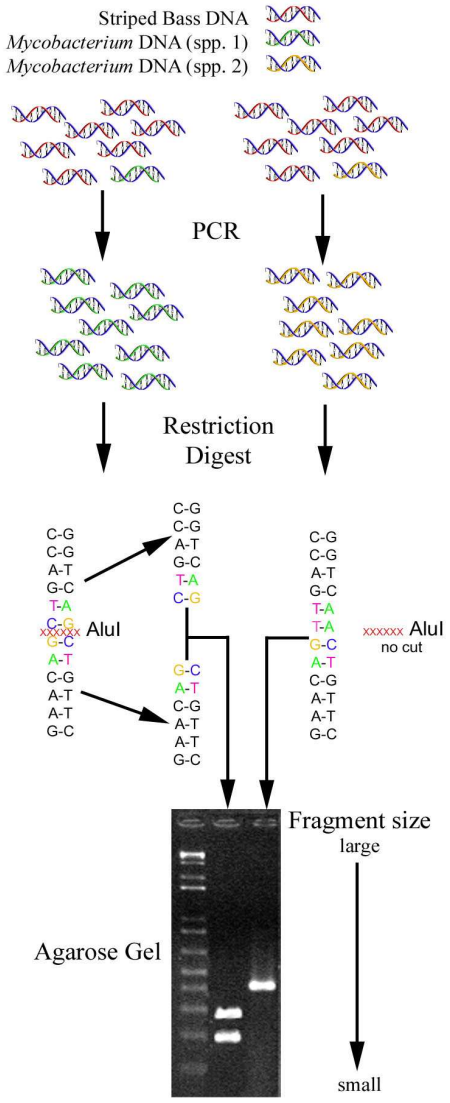
Fig. 5: Log eviscerated weight plotted vs. log fork length, and stratified by disease category (SEVCAT). SEVCAT 0 is no disease, 1 is light disease, 2 is moderate disease, and 3 is heavy disease (critera for categories described in section on histological methodology).

- No clear relationship was found between disease severity and fish condition. A trend was observed, however, of decreasing weight-at-length for severely diseased fish.

Methods: Molecular analyses

DNA was extracted from ethanol-fixed tissue with a commercially available kit (DNeasy, Qiagen). Mycobacterial DNA was amplified from extracted total DNA samples using nested PCR (nPCR) with Restriction Fragment Length Polymorphism assay (RFLP). An explanatory diagram of the nPCR/RFLP techniques is given in Fig. 6.

Fig. 6: Diagram of nPCR/RFLP technique. Mycobacterial DNA is first specifically amplified from mixture of extracted striped bass and mycobacterial DNA (spleen sample). Amplified mycobacterial DNA is then subjected to digestion with specific restriction enzymes (AluI in this example). Restriction enzymes cut DNA at very specific sequences, in this example at TCGA. Because species 1 has this sequence and species 2 does not (species 2 sequence is T*T*GA), AluI cuts DNA from species 1 into two fragments, while DNA from species 2 remains intact. When the digested DNA is run on an agarose gel, which separates DNA fragments by size, one large fragment is seen for species 2, while two smaller fragments are seen for species 1.



Briefly, amplification of mycobacterial DNA was accomplished in two PCR reactions. In the first, large subunit ribosomal DNA (LSU) was amplified by primers specific to *Mycobacterium* spp. A small aliquot of this reaction, enriched in *Mycobacterium* specific sequences, was further amplified with an additional set of primers that amplified a segment of DNA contained within that amplified by the first reaction (Fig. 7).

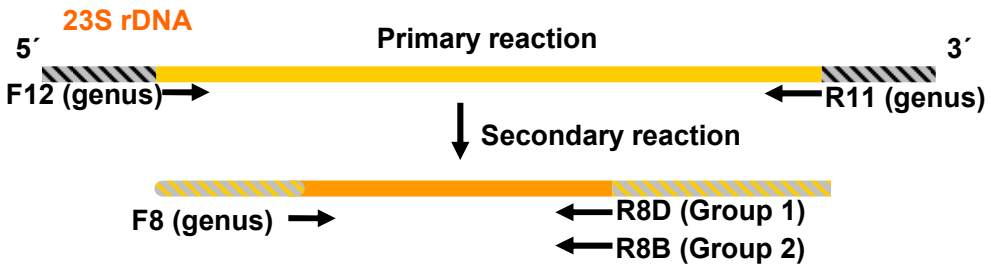


Fig. 7: Schematic of nPCR reaction. In the primary reaction, genus-specific primers are used to amplify a small fragment of the 23S rDNA gene (yellow). A portion of this reaction, now enriched in the genus-specific 23S fragment, is placed in a secondary reaction, which uses internal sub-genus specific primers. After the secondary reaction, the sample is enriched in sub-genus specific DNA fragments (orange), which can be detected on an agarose gel and digested for RFLP analysis.

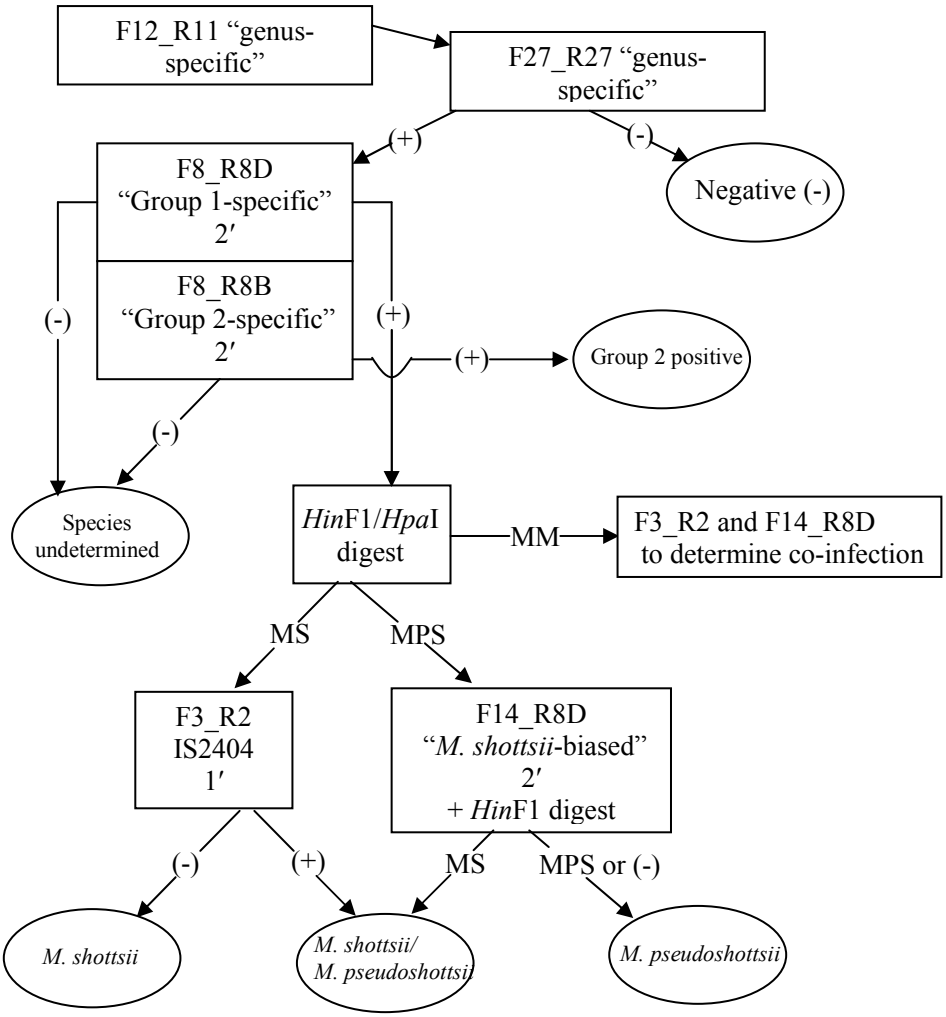


Fig. 8: Flow chart diagramming order of reactions for nPCR-RFLP testing.

Figure 8 shows the order of operations for the nPCR-RFLP assay used in this study. All samples were amplified in a primary reaction using the genus-specific primers F12 and R11 (all primers except for F3 and R2 describe below are specific for large ribosomal subunit DNA [LSU]). Subsamples of the primary reaction were then re-amplified with the genus-specific primers F27 and R27 in a secondary reaction. Negative F27/R27 results indicated mycobacterial DNA was not present in the sample, and the sample was classified as negative. When a sample tested positive by F27/R27, additional aliquots of the primary reaction were re-amplified with primers F8 and R8D (specific to *M. pseudoshottsii*, *M. shottsii*, and *M. marinum*; Group 1) and separately with primers F8 and R8B (specific to "*M. triplex*-like" mycobacteria; Group 2). Samples showing bands with F8/R8D primers were then digested with restriction enzymes *Hin*F1 and *Hpa*I, and the resulting banding pattern analyzed. Samples showing a banding pattern consistent with *M. pseudoshottsii* were further analyzed with primers F14 and R8D, which preferentially amplify *M. shottsii* in the presence of *M. pseudoshottsii* DNA (see below). Positive samples were digested with *Hin*F1. No reaction or a single band was indicative of a monoinfection with *M. pseudoshottsii*, whereas a double band was indicative of co-infection with *M. shottsii.* Samples showing restriction patterns consistent with *M. shottsii* after the F8/R8D reaction were re-amplified from genomic DNA (primary reaction) with primers F3/R2, which are specific to the insertion sequence IS2404, which is unique to *M. pseudoshottsii*. Samples not showing amplification with IS2404-specific primers were classified as having *M. shottsii* monoinfection, whereas IS2404-positive samples were classified as co-infected. Samples showing restriction digest patterns consistent with *M. marinum* were re-tested with both IS2404-specific primers, and primers F14 and R8D to reveal co-infections with *M. pseudoshottsii* or *M. shottsii*.

Figure 9 shows examples of the end result of nPCR/RFLP reactions detecting "Group 1" mycobacteria in the spleens of striped bass. Samples positive for *M. shottsii* (4 bands), *M. pseudoshottsii* (2 bands), and *M. marinum* (3 bands) are demonstrated.

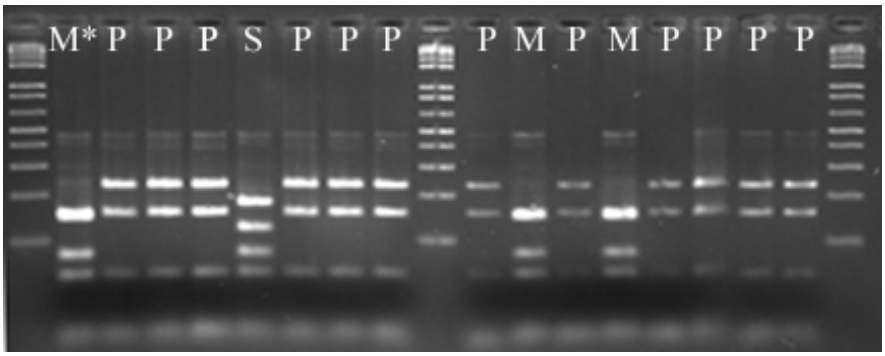


Fig. 9: RFLP of positive Group 1 nPCR products from striped bass spleens. Lanes 1, 10, and 19 are size standards. Lane 2 is a *M. marinum* (marked "M*") standard for comparison. Lanes marked with "P" indicate an RFLP pattern consistent with *M. pseudoshottsii*, "S" indicates *M. shottsii*, and "M" indicates *M. marinum*.

One of the complicating factors of mycobacterial infection in striped bass is the observation of co-infections of *M. shottsii*, *M. pseudoshottsii*, and/or *M. marinum*. Due to a phenomenon called primer competition, when DNA from more than one of these highly similar species is present, only that species will be detected by nPCR/RFLP. Two methods were used to get around this problem. First, we know from existing literature that *M. pseudoshottsii* possesses IS2404, which is a unique DNA sequence known as an insertion sequence (Rhodes et al. 2005). Neither *M. shottsii* or *M. marinum* possesses this insertion sequence, so a PCR reaction specific to IS2404 was developed to detect *M. pseudoshottsii* in co-infections where it would have otherwise been masked by the presence of *M. shottsii* and/or *M. marinum*. Unfortunately, no unique gene targets are yet known for *M. shottsii*, so a different approach was necessary. Primers were designed that amplified both *M. shottsii* and *M. pseudoshottsii*, but were highly "biased" toward amplifying *M. shottsii* (Fig. 10). Using these methods, it was possible to detect *M. shottsii* DNA even when larger amounts of *M. pseudoshottsii* DNA were present in the same sample. For the purposes of this study, no methodologies were developed to detect *M. marinum* in the presence of larger quantities of *M. pseudoshottsii* and/or *M. shottsii*. Previous culture-based work has demonstrated *M. marinum* to be present in a small percentage of Chesapeake Bay striped bass (Rhodes et al. 2004), so we do not believe this to be a major shortcoming. Further refinement of these diagnostic techniques, however, will focus on the capability to resolve co-infections with any two, or all three of these species. Recently, using a technique called genomic subtractive hybridization, we have been successful in identifying DNA sequences putatively specific to *M. shottsii.* Testing of these gene targets for their usefulness in diagnostic assays is ongoing.

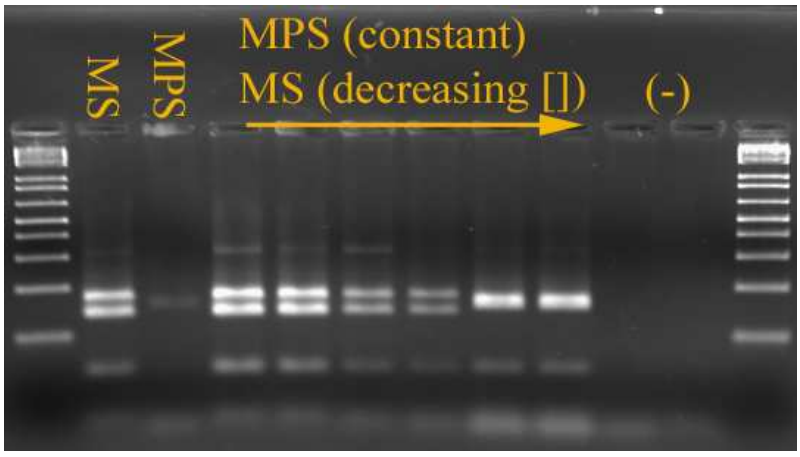


Fig. 10: nPCR/RFLP of samples containing DNA from both *M. shottsii* and *M. pseudoshottsii*. Samples were amplified with primers F14 and R8D, then digested with the restriction enzyme *Hin*F1. Lanes 1 and 12 are size markers. Lanes 2 (MS) and 3 (MPS) are samples containing DNA from only *M. shottsii* or *M. pseudoshottsii*, respectively. Lanes 4-9 are reactions containing constant amounts of *M. pseudoshottsii* DNA and decreasing amounts of *M. shottsii* DNA. A double band is characteristic of *M. shottsii*, whereas a single band is characteristic of *M. pseudoshottsii*. Lane 7, the last lane in which a double band is present, represents a 5000-fold lower concentration of *M. shottsii* DNA than *M. pseudoshottsii* DNA. Lanes 10 and 11 are negative controls.

Results: Molecular analyses

Table 2 shows nPCR/RFLP results from 764 striped bass splenic samples collected during 2005 and 2006. PCR-negative samples were negative for splenic granulomas in 64.4% (319/495) of cases; an additional 34.6% (171/495) displayed mild disease; and 1% (5/495) displayed moderate disease. No (0/21) severely diseased samples tested negative by PCR. PCR-positive samples were negative by histology in 21.9% (59/269) of cases. Samples displaying moderate- or severe disease were always positive for *M. pseudoshottsii*, *M. shottsii*, or both. *M. marinum* was detected in 2.6% (7/269) of PCR-positive samples, with co-infection of *M. pseudoshottsii* or *M. shottsii* detected in 3 of these cases. In 51 of 269 PCR-positives, species could not be determined because of non- or weak amplification with either Group 1 or Group 2 primers. No (0/269) samples showed amplification with Group 2 primers. *M. pseudoshottsii* was present, whether alone or in combination with *M. shottsii* or *M. marinum*, in 79.8% (174/218) of cases where species was determined by RFLP. *M. shottsii* was present, whether alone or in combination, in 30.3% (66/218) of these cases, and *M. marinum* was present alone or in combination in 3.2% (7/217) of these cases.

	HISTOLOGY				
nPCR-RFLP	NEGATIVE	MILD	MODERATE	SEVERE	Total
NEGATIVE	319	171	5	0	495
?SPP	24	23	4	0	51
MPS	30	78	25	14	147
MS	3	18	13	5	39
MPS/MS	2	16	5	2	25
MM	0	4	0	0	4
MM/MPS	0	1	0	0	1
MM/MS	0	1	0	0	1
MPS/MS/MM	0	0	1	0	1
	378	312	53	21	764

Table 2: Results of nPCR/RFLP analysis of fish collected during 2005-2006. Columns show numbers of fish in each disease category as classified by histology. Rows show PCR results: Negative=no bands present; ?spp=positive genus-level PCR reaction, but species undetermined; MPS=*M. pseudoshottsii*; MS=*M. shottsii*; MPS/MS=*M. pseudoshottsii*/*M. shottsii* co-infection; MM=*M. marinum*; other co-infection combinations use abbreviations defined above.

The data presented above clearly demonstrate the association of *M. pseudoshottsii* and *M. shottsii* with diseased fish, and strongly indicates that *M. pseudoshottsii* may be playing a more important role than *M. shottsii* in production of disease in Chesapeake Bay striped bass. In agreement with previous studies, *M. marinum* was found in a small minority of infected fish (Rhodes et al. 2004). Surprisingly, and in disagreement with previous studies, no samples tested positive for Group 2 (*M. triplex*-like) mycobacteria. The dominance of *M. pseudoshottsii* over *M. shottsii* is also surprising in light of previous studies, and indicates that more attention should be paid to the former in future studies examining mycobacterial disease in striped bass.

The moderate degree of disagreement between PCR and histological methods observed in these data is inconvenient, but not unexpected. The first important consideration in this comparison is the fact that the two methods measure fundamentally different things. Whereas PCR measures the presence of mycobacterial DNA, histology measures the presence of disease (host reaction to mycobacterial infection). Therefore, instances in which PCR is positive and histology is negative may be viewed as cryptic infections without detectable host response. The reverse situation, in which PCR is negative and histology is positive, may be interpreted as a host reaction in the absence of mycobacterial DNA, whether by complete degradation of the eliciting mycobacterial agent, or granulomas caused by a non-mycobacterial agent. The next important factor that complicates direct comparison between PCR and histology is one of sampling. Mycobacteria in the spleen are likely to be contained within granulomas, rather than evenly distributed, and since different portions of the spleen are taken for PCR and histological analyses, one piece of tissue may contain mycobacteria while another may not. This is likely only a significant concern in spleens with light infection/disease, as reflected in the increasingly good agreement between PCR and histology with increasing disease severity.

- Molecular techniques were developed and successfully applied to detection and species determination of *Mycobacterium shottsii*, *M. pseudoshottsii*, and *M. marinum* in tissues of striped bass.

- *M. shottsii*, *M. pseudoshottsii*, and/or *M. marinum* were found in a majority of spleen samples also showing granulomatous disease by histological methods. These mycobacteria were also detected in spleen samples showing no disease.

- In contrast to previous studies, *M. pseudoshottsii* was detected in the majority of positive samples, followed by *M. shottsii*. *M. marinum* was found in a minority of PCR-positive samples.

- In contrast to previous studies, mycobacteria other than *M. shottsii*, *M. pseudoshottsii*, and *M. marinum* were not detected in striped bass spleen tissue by molecular techniques.

- Co-infections of *M. pseudoshottsii*, *M. shottsii*, and/or *M. marinum* were detected in some samples.

- Samples in which PCR was negative, but granulomas were demonstrated histologically, were typically lightly diseased, and inconsistency between techniques likely represented sampling artifact.

Project Summary

The results of this study represent major advancements in our understanding of mycobacteriosis in Chesapeake Bay striped bass. Apparent prevalence data from fish collected between 2003-2005 clearly demonstrate the relationship between disease and age and sex of striped bass. Temporal (cruise/season) and spatial (region) factors were also examined as risk factors for disease. Year of collection did not appear to have a significant effect on disease prevalence when corrected for other variables. This is one indication that the disease situation in Chesapeake Bay is currently stable, or changing over long time periods. The addition of fish collected during 2006 upon completion of otolith-based ageing will further enhance this data set and enable us to further understand long-term disease dynamics.

In addition to examination of individual risk factors for disease, new statistical methodologies were employed to detect disease-specific mortality in Chesapeake Bay striped bass. The question of whether mycobacteriosis kills striped bass is a crucial one, both to managers and fishers. This study has provided the first indication that mycobacteriosis is, in fact, causing mortality on a population level within Chesapeake Bay. The findings of this study will be used in conjunction with ongoing tag-and-release research over the next several years to arrive at an accurate and well-supported answer to the question of mycobacteriosis-related mortality in striped bass.

A major goal of this study was to apply newly developed molecular tools toward the detection and species determination of *Mycobacterium* in striped bass tissues. To this end, we were successful in detecting *M. pseudoshottsii*, *M. shottsii*, and *M. marinum* in both diseased and non-diseased striped bass spleen tissue. In contrast to previous studies, *M. pseudoshottsii* appeared to be the most commonly detected species, followed by *M. shottsii* and *M. marinum.* Also in contrast to previous studies, other "*M. triplex*-like" mycobacteria were not detected in striped bass spleens. The reasons for the differences between these two studies are under investigation, but it seems clear that future research efforts regarding mycobacteriosis in Chesapeake Bay striped bass should concentrate on *M. pseudoshottsii* and *M. shottsii*. The successful application of molecular tools in this study represents a major advance in our capability to study disease in striped bass, as these tools allow rapid and sensitive detection of mycobacteria in striped bass tissues, without the considerable expense and labor associated with traditional culture-based techniques.

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