

## Mycobacterial Infection in Laboratory-Maintained Atlantic Menhaden

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**Abstract.**—Mycobacteriosis can be a chronic wasting disease found in many species of fish examined from wild-caught, cultured, and aquarium-reared populations. An Atlantic menhaden *Brevoortia tyrannus* with an open ulcer from a wild-caught, laboratory-maintained population was sampled for microbiology. *Mycobacterium* spp. was recovered from the ulcer and *M. fortuitum* complex was recovered from the spleen of this fish. Subsequently, 20 additional fish were subsampled from this population to determine the prevalence of the infection. Bacteriology samples were taken from the brain, liver, and kidney. Spleens were homogenized and plated on media enriched for mycobacterial growth, full necropsies were performed, and samples were taken for histology. Bacteriology results showed 100% of the fish in the subsample were infected with mycobacteria. Three species of mycobacteria were isolated from the spleen tissues: *M. marinum*, *M. fortuitum* complex, and *M. goodii*. Histology results revealed that granulomas, characteristic of mycobacteriosis, were most prevalent in livers but were also found in spleens, posterior kidneys, and hearts. This report adds Atlantic menhaden to the list of species susceptible to mycobacteria.

Mycobacteriosis can be a chronic wasting disease found in many species of fish examined from wild-caught, cultured, and aquarium-reared pop-

ulations (Nigrelli and Vogel 1963; Chinabut 1999). This disease is caused by a variety of bacteria in the genus *Mycobacterium* spp. It can infect over 80% of the individuals in a population and is associated with large-scale mortalities and considerable economic losses (Chinabut 1999; Noga 2000a). Clinical observations of mycobacteriosis include the presence of internal granulomas, particularly in the spleen, kidney, and liver (Austin and Austin 1999; Chinabut 1999; Noga 2000a; Rhodes et al. 2004). Additional signs may include emaciation, external reddish lesions, fin rot, granulomas in all organs, internal adhesions, as well as behavioral alterations including equilibrium disturbances.

Different theories have been proposed regarding the mode of transmission, including vertical and horizontal transmission (e.g., gill or skin epithelial exposure or ingestion of infected prey; Conroy 1966; Chinabut et al. 1994; Chinabut 1999; Antonio et al. 2000; Noga 2000a). Treatment for the disease is challenging and often expensive (Plumb 1994) and different fish species probably have different levels of sensitivity. However, some procedures have proved worthwhile to help control spread throughout a facility or when expensive aquarium populations are at risk. These procedures include culling infected populations, disinfecting

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infected aquaria, and quarantining newly acquired fish (Noga 2000a; Jacobs et al. 2004).

Bataillon et al. (1897) originally described piscine mycobacteria in the late 19th century. The first report of mycobacteriosis in a marine fish was noted by Von Betegh in 1910 (described by Chinabut 1999). Aronson (1926) documented the first isolation of *M. marinum* from a fish infection at the Philadelphia Aquarium. Since then, over 160 fresh- and saltwater fish species have been reported to be susceptible to mycobacterial infection, but it is not unreasonable that all fish species may be potential hosts (Nigrelli and Vogel 1963; Chinabut 1999). Although Atlantic menhaden *Brevoortia tyrannus* are not included on this list of potential hosts, acid-fast bacilli have been previously observed in this species (Noga et al. 1989).

### Methods

This report describes a mycobacterial infection in a wild-caught, laboratory-maintained Atlantic menhaden population. Seventy-four young-of-the-year Atlantic menhaden were collected from the Choptank and Nanticoke rivers of the Chesapeake Bay, Maryland, in the fall of 2001. The fish were transported to the laboratory and held in three 450-L round fiberglass tanks that were part of a 2,000-L recirculating system. Adequate water quality was maintained through the use of physical and biological filtration as well as 20% weekly system water changes. Holding conditions consisted of a pH of 7.4–7.6, a salinity of 5–7‰, a water temperature of 22–25°C, and a 16:8 light:dark photoperiod. Fish were fed trout grower diet (40% protein mash; Zeigler Bros, Inc., Gardners, Pennsylvania) daily. After 10 months, fish were euthanatized for histology and bacteriology.

Twenty-one fish were aseptically necropsied and observed for gross external and internal lesions. Portions of the spleen, liver, anterior kidney, posterior kidney, heart, brain, gill, gut, gonad, eye, muscle, and external lesions were preserved in 10% neutral buffered formalin and processed for routine histology. Slides were stained with hematoxylin and eosin following the Kinyoun method for the detection of acid-fast bacteria or with Gomori methenamine silver (GMS) stain for the detection of fungal hyphae (Profet et al. 1992). Prepared slides were evaluated for general pathology and the presence of granulomas. Bacteriology samples of the liver, anterior kidney, and brain were plated on 1% tripticase soy agar and inoculated in 1% tripticase soy broth. Resulting bacterial colonies were isolated for pure culture

and identified using routine biochemical characterizations or API strips. Spleens were aseptically removed, stomached with 2 mL B-phosphate-buffered saline for 2 min, and 0.5 mL of the homogenate was plated on Middlebrook 7H10 agar supplemented with Bacto Middlebrook oleic acid–albumin–dextrose–catalase supplement (OADC). Plates were incubated at room temperature (22°C) and were checked for growth every week for 8 weeks. Types and numbers of mycobacterial colonies were tallied for each plate after 8 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, were processed for fatty acid methyl ester (FAME) analysis. 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). Fatty acid profiles of the prepared samples were generated on a gas chromatograph (Hewlett-Packard Company, Palo Alto, California) and analyzed with MIDI software (Sasser 1990; MIDI 2002).

### Results

One fish submitted for diagnosis presented with a large, well-circumscribed ulcer (Figure 1A). Histologically, the ulcer was associated with a focal loss of epidermis and dermis, penetrating through the chromatophore layer of the dermis and into the underlying musculature (Figure 1B). The ulcer contained areas of mild to moderate focal necrosis with minimal infiltration of mononuclear inflammatory cells, fragmentation of myocytes, and several granulomas. The granulomas were composed of focal aggregates of epithelioid macrophages, many of which contained phagocytosed acid-fast bacteria and often had necrotic centers (Figure 1C). Fungal hyphae were not evident based on sections stained with GMS. There was some re-epithelialization as evidenced by 1- to 2-cell layers of flattened, squamous cells on peripheral areas of the ulcer. Numerous acid-fast bacteria not contained within granulomas were found in spleen and posterior kidney interstitium (Figure 1D). The peritoneal mesentery also contained acid-fast bacteria.

Of the 20 remaining Atlantic menhaden, 1 individual had marked adhesions in the peritoneal cavity. Granulomas were grossly visible in the spleens of 8 individuals. Histologically, granulomas were most common in the liver (75% prevalence). They were also found in the spleen (40%),

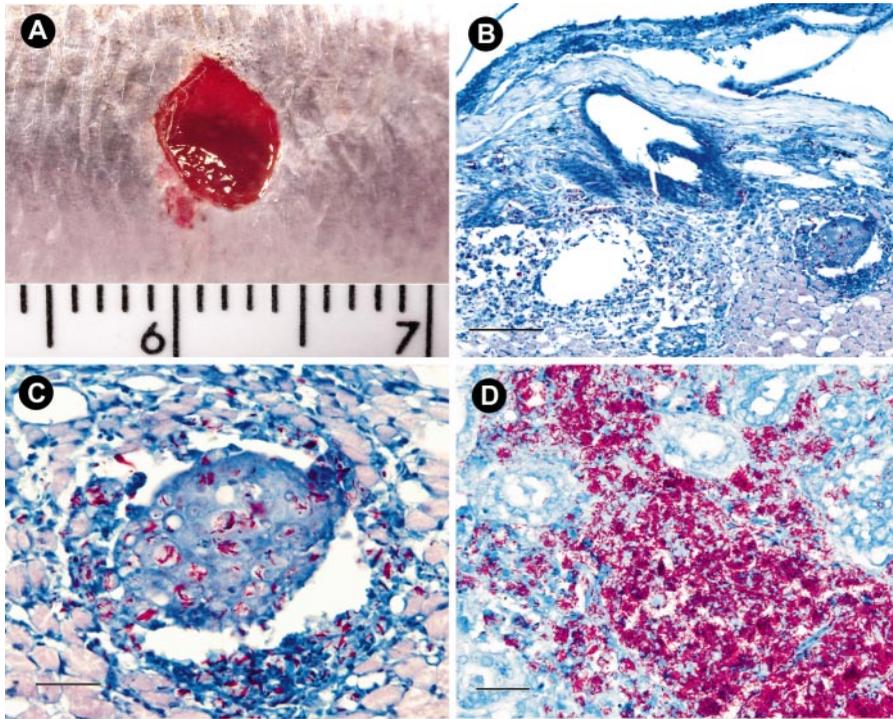


FIGURE 1.—Mycobacterial infection in a wild-caught, laboratory-maintained Atlantic menhaden. Panels are as follows: (A) photograph of a well-circumscribed, 5-mm  $\times$  6-mm lesion from the ulcerated fish located in the middle of the left flank halfway between the insertion of the pectoral fin and the peduncle (centimeter scale shown); (B) low-magnification micrograph showing the ulcer from the specimen in panel (A) (Kinyoun stain; scale bar = 100  $\mu$ m); (C) acid-fast bacteria (red-stained bacilli) within the same granuloma shown in panel (B) at lower magnification (Kinyoun stain; scale bar = 25  $\mu$ m); and (D) acid-fast bacteria within the interstitium of the posterior kidney; as shown, portions of the kidney were heavily loaded with bacteria (Kinyoun stain; scale bar = 25  $\mu$ m).

posterior kidney (24%), and the ventricle and bulbus arteriosus of the heart (25%). No granulomas were found in the brain, gills, gut, gonads, eye, or muscle. Numerous macrophage aggregates were observed in all splenic tissue samples.

*Kudoa*-like organisms were found in the musculature of 22% of the Atlantic menhaden. These myxosporean parasites did not appear invasive and were contained within cysts within the myomeres.

Bacteriology results from spleen cultures revealed four types of *Mycobacterium* spp. colonies with a 100% infection prevalence. The dominant colony type isolated was determined to be from the *M. fortuitum* complex. These were observed as white, round, rough or wrinkled, dry colonies. The FAME analysis gave a similarity index of 0.784 for *M. chelonae* and 0.625 for *M. fortuitum*; however, sequencing of the 16S rRNA (data not shown) shows closest relation to *M. septicum* or *M. peregrinum*. *M. marinum* closely followed *M. fortuitum* complex in dominance and were observed as

yellow, round, smooth, and moist colonies. The FAME analysis gave a similarity index of 0.475 for *M. marinum*. *M. gordonae* was third in dominance and presented as small, orange, smooth, and moist colonies. The FAME analysis gave a very low similarity index to *M. gordonae* (similarity index = 0.082). However, based on dendrogram clustering, this isolate appeared closely related to other isolates identified as *M. gordonae* through 16S rRNA sequencing. The fourth isolate was found only in the spleen of the ulcerated individual and was identified as *M. smegmatis* by FAME analysis (similarity index = 0.731).

The number of mycobacterial colonies per plate ranged from 1 to over 3,500. Spleens from 9 fish yielded pure culture of only one of the three mycobacterial species. *Vibrio hollisae* was also isolated from the kidney, liver, or brain of 8 fish. This corresponds to a 40% co-infection rate of *Mycobacterium* spp. and *V. hollisae*. *Photobacterium damsela* was isolated from the kidney, liver, or

TABLE 1.—Summary of bacteriology and histology observations for each Atlantic menhaden studied. Symbols are as follows: “+” denotes <100 colony-forming units (CFU); “++” denotes 101–1,000 CFUs; “+++” denotes >1,000 CFUs; “–” denotes not cultured; and “□” denotes no data. Granulomas were observed in spleen (S), liver (L), posterior kidney (K), heart (H), and the ulcer (U) of fish 21 other bacteria (*Photobacterium damsela* and *Vibrio hollisae*) were isolated from the liver, posterior kidney, or brain (B). *Kudoa*-organisms were observed only in muscle tissue.

Fish number	Mycobacteria cultured in spleen				Granulomas	Other bacteria		<i>Kudoa</i> -like organisms
	<i>M. fortuitum</i> complex	<i>M. marinum</i>	<i>M. gordonae</i>	<i>M. smegmatis</i>		<i>P. damsela</i>	<i>V. hollisae</i>	
1	+	++	–	–	S L K			–
2	++	+	–	–	S L	L K		–
3	–	+++	–	–	S L			–
4	+	–	–	–				–
5	+	–	–	–	L K	L		–
6	+	+	–	–	L	L		+
7	+	–	++	–	L	L K		+
8	+	++	–	–			L	–
9	+	–	–	–	L	L K	L	–
10	□	□	□	–	S L H	L K		□
11	+	+	++	–				–
12	+++	+	–	–		L K	L K	–
13	+	–	–	–	K	L K	L K	–
14	+	–	–	–	L	L K B		□
15	–	–	+	–	L			–
16	++	++	–	–	S L H		L K B	–
17	++	–	–	–	S L K		L K	–
18	+++	+	+	–	S L H	K B	L B	–
19	+	–	–	–	L	K B	K B	+
20	+	+++	–	–	S L	L		+
21	–	–	–	+		U		–

brain of 12 fish. This corresponds to a 60% co-infection rate of *Mycobacterium* spp. and *P. damsela*. Both *V. hollisae* and *P. damsela* were isolated from 5 fish, which corresponds to a 25% co-infection rate of both bacterial species with *Mycobacterium* spp. Table 1 presents a summary of bacterial and histological findings from this study.

### Discussion

This study describes a case of mycobacteriosis in a wild-caught, laboratory-maintained Atlantic menhaden population. Histologically, granulomas were most common in the liver, followed by spleen, posterior kidney, and heart tissues. Observations of granulomas associated with mycobacteria in these tissues are consistent with previously published reports (Austin and Austin 1999; Noga 2000a; Rhodes et al. 2004).

We know that wild young-of-the-year Atlantic menhaden can carry mycobacteria based on microbiology and FAME analysis results from field-collected specimens (Kane et al. 2005). However, it is unclear whether the Atlantic menhaden in this study were infected with mycobacteria in the field, during transport, or during laboratory culture. However, this report demonstrates that Atlantic

menhaden are susceptible to mycobacterial infections and can now be documented as another susceptible fish species (Nigrelli and Vogel 1963; Chinabut 1999). The ecological significance of mycobacterial infections in Atlantic menhaden remains unclear; however, it may be notable since Atlantic menhaden are an important prey species, particularly in the Chesapeake Bay.

Myxosporean cysts of *Kudoa*-like organisms were observed within the musculature of the population sampled. These observations are consistent with previous reports of *Kudoa clupeiidae* spores commonly found in wild-caught Atlantic menhaden from the Pocomoke River, another Maryland eastern shore tributary of the Chesapeake Bay. However, unlike previous reports on Atlantic menhaden in the Pocomoke River (Reimschuessel et al. 2003), the *Kudoa*-like organisms found in this group of Atlantic menhaden did not appear to be associated with ulcerative lesions. Both *V. hollisae* and *P. damsela* have been known to affect humans (Love et al. 1981; Levine et al. 1993), and while *P. damsela* is a known fish pathogen, *V. hollisae* is suggested to be nonpathogenic for fish (Austin and Austin 1999; Buller 2004). The biological significance of finding these bacteria in Atlantic menhaden is unclear.

Since humans can also be infected with mycobacteria, mycobacteriosis poses a public health concern for fisheries workers and researchers, aquaria hobbyists, recreational anglers, and swimmers who may come into contact with infected fish or water (Zeligman 1972; Bhatti et al. 2000). Clinical observations of mycobacterial infection in humans typically involve painful or painless reddish nodules at the site of infection, usually adjacent to a cut or scrape and, rarely, sporotrichoid spread of nodules (Bhatti et al. 2000; Lewis et al. 2003).

Based on the zoonotic potential for the spread of mycobacteria from fish to humans and widespread observations of mycobacteria in different fish species from many different facilities, persons who routinely come in contact with fish have a higher risk of infection. Therefore, it may be wise for public display aquarists, researchers, and large-scale hobbyists to consider careful, periodic sampling of specimens and equipment to determine relative mycobacterial loading in different facilities. Clearly, prevention and appropriate, routine disinfection should be viewed as the primary means to control mycobacteria in culture facilities (Jacobs et al. 2004). Also needed are epidemiological studies that can help identify risk factors associated with loading of different mycobacterial species in different fish species under different environmental conditions.

This study substantiates Atlantic menhaden as a host species for mycobacteria. Additional observations of *Kudoa* spp., other bacteria, and an ulcerated individual underscore the multifactorial nature of bacterial diseases and lesion pathogenesis (Sindermann 1988; Dykstra and Kane 2000; Noga 2000b).

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