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Influence of nutritional state on the progression and severity of mycobacteriosis in striped bass *Morone saxatilis*

John M. Jacobs[1,*], Matt R. Rhodes[1], Ana Baya[2], Renate Reimschuessel[3], Howard Townsend[4], Reginal M. Harrell[5]

[1]Center for Coastal Environmental Health and Biomolecular Research (CCEHBR)/Cooperative Oxford Lab, NOAA National Ocean Service (NOS), Oxford, Maryland 21654, USA
[2]Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland 20742, USA
[3]Center for Veterinary Medicine, US Food and Drug Administration (USFDA), Laurel, Maryland 20708, USA
[4]Chesapeake Bay Office, NOAA National Marine Fisheries Service (NMFS), Oxford, Maryland 21654, USA
[5]Department of Environmental Science and Technology, University of Maryland, College Park, Maryland 20742, USA

ABSTRACT: Challenge studies with *Mycobacterium marinum* clearly demonstrate that a poor diet affects the progression and severity of mycobacteriosis in striped bass *Morone saxatilis*. Fish (n = 512 total, wt = 65 ± 15 g) were inoculated intraperitoneally with 10^4 colony-forming units (CFU) g^{-1} body weigth (BW) or a physiological saline solution (controls) and evaluated for 8 mo. Inoculated fish fed a low-ration diet (0.15% BW d^{-1}) developed a severe, systemic infection characterized by a high bacterial load (>10^8 CFU g^{-1} spleen) and poor granuloma formation, which commonly progressed to mortality by 6 wk. In contrast, inoculated fish fed an adequate ration diet (1% BW d^{-1}) developed classic granulomatous inflammation of reduced severity and total body energy similar to that found in uninoculated controls ($p > 0.05$). After 4 wk, fish fed adequate rations maintained an equilibrium state throughout the study period, even though 10^6 CFU g^{-1} spleen mycobacteria were consistently cultured. In a second study, reactivation of an acute inflammatory state was demonstrated by placing previously infected fish on reducing diets (0.073% BW d^{-1}). In both studies, the energetic demand of this disease was only appreciable when associated with active, severe, inflammatory states. To our knowledge, this study is the first to demonstrate the interaction of diet and mycobacteriosis in fish.

KEY WORDS: Mycobacteriosis · Nutrition · Disease · Stress · Striped bass

INTRODUCTION

In terms of economically and socially important diseases of avian, mammalian, and piscine species, few are of more significance worldwide than those associated with the genus *Mycobacterium*. Johne's disease, caused by *M. avium* subsp. *paratuberculosis*, is estimated to cause over US $1.5 billion in losses annually to the US cattle industry (Stabel 1998). Chronic infections from the *M. avium* complex have been well documented in a wide range of species from horses to poultry (Thorel et al. 1997). Piscine species are equally vulnerable, with tremendous losses related to mycobacteriosis in aquaculture (Colorni et al. 1998) as well as documented epizootics in wild populations (Sakanari et al. 1983, MacKenzie 1988, Rhodes et al. 2001, Gauthier & Rhodes 2009, Jacobs et al. 2009). Of increasing concern is the ability of many of these nontuberculosis or environmental species to cause disease in humans, especially in individuals who are immunocompromised (Dobos et al. 1999).

Currently an epizootic of mycobacteriosis is affecting striped bass *Morone saxatilis* in the Chesapeake Bay (Heckert et al. 2001, Rhodes et al. 2001, 2004, Overton

*Email: john.jacobs@noaa.gov

et al. 2003, Kaattari et al. 2005, Ottinger & Jacobs 2006). Of great interest is the large number of mycobacterial species that have been isolated from affected fish (Rhodes et al. 2004, Stine et al. 2009), including 2 new isolates named *M. shottsii* (Rhodes et al. 2001) and *M. pseudoshottsii* (Rhodes et al. 2005). While *M. shottsii* is the predominant isolate from wild-collected striped bass (Rhodes et al. 2004), only *M. marinum* has demonstrated an ability to cause the same pathology when experimentally infected (Gauthier et al. 2003). Co-occurring in the population is a high incidence of external lesions and reports of emaciated fish (Overton et al. 2003, Rhodes et al. 2004, Maryland Department of Natural Resources [MDNR] unpubl. data). Recent work suggests that the probability of survival of infected fish may be reduced to 69% of uninfected cohorts (Gauthier et al. 2008).

Dietary changes have been documented in striped bass, suggesting a shift from historically preferred pelagic sources to benthic sources (Hartman & Brandt 1995, Griffin & Margraf 2003, Overton et al. 2003, Pruell et al. 2003, Walter et al. 2003). Concurrently, populations of principal prey items for age 2+ striped bass Atlantic menhaden *Brevoortia tyrannus*, bay anchovy *Anchoa mitchilli*, and spot *Leiostomas xanthurus* have declined (Uphoff 2003, MDNR unpubl. data). In addition, decreased growth of age 3+ fish (Overton et al. 2003, Warner et al. 2005) increased variability in weight at length (Uphoff 2003, Warner et al. 2005), and low concentrations of tissue lipids (Jacobs 2007) have also been reported. Temporal association of these findings in combination with historically high population abundance (ASMFC 2005) led to hypotheses linking food limitation to disease state (Hartman & Margraf 2003, Uphoff 2003). However, the relationship between diet and mycobacteriosis is poorly understood in striped bass and difficult to determine from field observations because prior history of the animal is unknown.

The issue of nutrition and mycobacterial disease is the classic 'chicken or egg' question. Wasting has been associated with infection in humans (Macallan 1999, Paton & Ng 2006), ruminants (Harris & Barletta 2001), and fish (MacKenzie 1988, Inglis et al. 1993) among others. Once called 'consumption,' the mechanisms behind the loss of body mass associated with tuberculosis are poorly understood but may reflect decreased appetite, altered metabolism, and demands of the inflammatory and immune response (Schwenk et al. 2004). Johne's disease caused by *Mycobacterium avium* subsp. *paratuberculosis* commonly results in wasting in many ruminants and reduced milk production in dairy cattle (Harris & Barletta 2001). Here infection results in severe gastroenteritis, diarrhea, and loss of body condition, which may be associated with tissue damage altering the efficiency of post-absorptive processes (Harris & Barletta 2001). As with mammals, reduced condition has been reported in many cases of freshwater tropical and marine aquaria fishes in association with mycobacteriosis (Inglis et al. 1993, Chinabut 1999, Conroy & Conroy 1999). However, in most cases these reports are from moribund fish in the final stages of disease progression, when emaciation is a common clinical sign of many bacterial diseases (Inglis et al. 1993).

In a large-scale investigation of mycobacteriosis in Atlantic mackerel *Scomber scombrus* (n = 9470), MacKenzie (1988) noted increased prevalence and severity with age and corresponding declines in length and condition. However, these differences were minor and rarely significant ($p < 0.05$). Similar results are reported for striped bass in Chesapeake Bay, where declines in condition are noted in more severe cases of disease, especially in combination with external lesions (Cardinal 2001, Overton et al. 2003, Ottinger & Jacobs 2006). Depending perhaps on the study location and methodology, the reduction in condition associated with mycobacteriosis may be significant (Ottinger 2006), minor (Gauthier et al. 2006), or insignificant (Overton et al. 2003, Jacobs 2007).

There is equal evidence in clinical medicine suggesting malnutrition is a major risk factor for tuberculosis and can alter the progression and severity of the disease (Chandra 1996, Wieland et al. 2005). Much work in this area has focused on the association of protein calorie malnutrition (PCM) and reduced immune function. Chan et al. (1996) demonstrated that mice receiving a reduced protein diet (2%) rapidly succumbed to tuberculosis accompanied by a reduced expression of interferon γ, tumor necrosis factor ά, and nitric oxide synthase in the lungs. These cytokines, as well as interleukin-1 (IL1), interleukin-4 (IL4), and transforming growth factor β are critical to the production of nitrous oxides and other reactive nitrogen intermediates, which are used by phagocytes to kill pathogens (Plouffe et al. 2005). Remarkably, fulminant tuberculosis characterized by poorly formed granulomas and elevated bacterial load was reversible in mice by increasing protein intake to match that of the controls (20%) (Chan et al. 1996). Other researchers (Dai & McMurray 1998) obtained similar results through *Mycobacterium tuberculosis* challenge of splenocytes harvested from protein-malnourished guinea pigs *Cavia porcellus*. Low protein intake reduced the production of interferon, tumor necrosis factor ά, and tumor necrosis factor β; essentially altering the cytokine profile to favor macrophage deactivation. Non-specific responses, such as the mobilization of inflammatory cells, phagocytosis, intracellular killing, neutrophil mobility, and production of macrophage cytokines, may also be reduced under conditions of

inappropriate or insufficient food sources (Dai & McMurray 1998). To our knowledge, similar studies looking at nutritional effects on mycobacterial disease challenge have not been conducted with fish.

MATERIALS AND METHODS

Fish and fish husbandry. Wild-collected, Choptank River strain striped bass were spawned and reared (Harrell et al. 1990) at the Horn Point Aquaculture Facility (University of Maryland, Center for Environmental Science, Horn Point Laboratory, Cambridge, MD, USA) for use in this study. To verify the absence of pre-existing conditions, a representative sample of juveniles (n = 60) (Ossiander & Wedemeyer 1973) were transported live to the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) and evaluated using the methods described below. Three fish cultured positive for *Mycobacterium marinum* from this cohort, all from a single tank. Fish from the remaining tanks examined were gradually transported in small batches (80 to 100) to the Cooperative Oxford Lab (COL) to allow for fish and system acclimation. A total of 512 fish (wt = 65 ± 15 g) were randomly stocked into 16 circular tanks (568 l, 32 fish per tank) and allowed to acclimate for 1 mo from the final stocking. The experiment was conducted in 8 identical experimental units, each comprised of 2 tanks serviced by a common bio-filter (Red Sea). Each unit was equipped with a UV sterilizer (Red Sea) and automatic pH controller (Automated Aquarium Systems). Four units were used for uninfected fish, and 4 units were used for infected fish, located in a separate treatment room. The 2 areas were physically partitioned with separate air-handling systems designed to meet or exceed all requirements for working with class II agents (USDHHS 1999). Experimental conditions were: photoperiod 12:12 fluorescent, pH 8.2, salinity 10, temperature 21°C. Nutrient water quality (NH_4, NO_2, NO_3) remained in a healthy range for the species (Harrell et al. 1990) through weekly monitoring of all systems and water exchange (10% vol wk^{-1}).

***Mycobacterium marinum* isolate and inoculation.** *M. marinum* isolate FL03-23 was obtained from wild Chesapeake Bay Atlantic menhaden *Brevoortia tyrannus* in 2003 and maintained in pure culture at the VMRCVM. This isolate was passed through 6 striped bass (wt = 30 g) once and re-isolated from spleen homogenates before being used in the experiments. Approximately 0.2 g of each spleen was homogenized in 2 ml of Butterfield's phosphate-buffered saline (BPBS), and directly plated on Middlebrook 7H10 agar supplemented with oleic acid, albumin, dextrose, catalase (OADC) enrichment and 0.5% glycerol (Difco) and allowed to incubate at room temperature. Cells were harvested from a single plate during exponential growth and inoculum prepared (via Gauthier et al. 2003) with slight modification. Briefly, on the morning of inoculation, cells were removed, suspended in BPBS, spun at 12000 × *g* for 15 min, and supernatant removed. Immediately following, the pellet was resuspended in BPBS, vortexed for 2 min with approximately 25% v/v 50 µm glass beads to disrupt clumping. Finally, the suspension was filtered through Whatman No. 1 filter paper. Bacterial concentration was estimated by turbidity measured at 590 nm against a BPBS blank and adjusted to 0.05 nm or approximately 10^7 colony-forming units (CFUs). A dilution of this suspension was prepared in sufficient quantity to inoculate all fish (n = 256) at roughly 10^4 CFU g^{-1} body weight (BW). Replicate serial dilutions of inoculum were subsequently spread plated to verify dose. All fish were removed from their tanks, anesthetized in FINQUEL MS-222 (Argent Chemical Laboratories), weighed, and measured before inoculation. Fish were inoculated intraperitoneally with 100 µl of either diluted *M. marinum* suspension (infected) or sterile BPBS (control). Final dose of *M. marinum* was 6636 ± 1691 (SD) cells g^{-1} BW.

Experimental design and rations. Two experiments were performed in succession to evaluate the effect of ration on (1) disease progression and severity, and (2) reactivation of disease. A 4 mm pelleted diet comprised of 45% crude protein, 12% fat, and 4% fiber was used in all experiments (Melick Aquafeed). Fish were fed a high- (1% BW) or a low-ration diet (0.15% BW Expt 1, or 0.073% BW Expt 2). In Expt 1, a factorial design was used consisting of replicate high-ration controls (HRC), low-ration controls (LRC), and high- and low-ration infected (HRM and LRM, respectively). Fish were placed on either the high or low ration 1 mo before intraperitoneal (IP) inoculation. Rations were adjusted monthly based on mean weight of experimental group. Two fish per tank (8 per group) were sampled at Time 0, and 3 per tank (12 per group) at 4 and 8 wk post-inoculation. At the conclusion of the short-term (Week 8) study (Expt 1), all remaining low-ration fish were removed and euthanized. HRM and HRC fish were then each randomly divided into 2 different ration groups for a longer-term (Weeks 8 to 32) study (Expt 2). Half of the original high-ration fish were assigned the 1% BW d^{-1} diet (HRC, HRM), and half of the fish were assigned to a reducing diet of 0.073% BW d^{-1} (LRC, LRM). Twelve fish per group were sampled at 16, 24, and 32 wk post-inoculation.

Gross pathology and hematology. At each sampling period, all fish were visually examined and abnormalities measured, photographed, and described. Weight and length were taken immediately after taking 1 ml of

whole blood with a 25 g syringe from the blood vessels at the caudal peduncle. Blood was transferred to capillary tubes for determination of packed cell volume (PCV) and leucocrit (white blood cell, WBC) volume by direct measurement using an ocular micrometer. Capillary tubes were immediately spun at 17900 × *g* for 5 min after sample collection and plasma removed to measure total protein by refractometer. Gross pathology was noted during necropsy.

***Mycobacterium marinum* re-isolation and enumeration.** At each sampling period, sections of spleen were removed aseptically and stored at –20°C in sterile whirlpacks. Subsequently, spleens were weighed and stomached in 2.0 m BPBS. Homogenates were diluted in duplicate 1:100 and 1:1000, or 1:1000 and 1:10 000, and 200 µl replicate plated on Middlebrook 7H10 agar with OADC enrichment and 5% glycerol. Plates were incubated at room temperature and colonies counted manually at 3 and 6 wk.

Histopathology. Samples of spleen, anterior and posterior kidney, mesenteries, liver, intestine, stomach, gills, heart, and gonad were preserved in 10% neutral-buffered formalin for routine paraffin embedding and microtome sectioning. All slides were stained with hemotoxylin and eosin (H&E) initially for routine histopathology (Luna 1968). Re-cuts of organs where inflammatory foci or granulomas were detected from H&E were stained with Ziehl-Neelsen for the detection of acid-fast bacteria. Severity of inflammation was categorized for each organ relative to all fish, as previously described by Talaat et al. (1998). A scale of 0 to 5 was used, with 0 being normal, 1 minimal, 2 mild, 3 moderate, 4 marked, and 5 severe or complete loss of organ architecture.

Proximate composition/energetics. At the completion of necropsy, each carcass was individually wrapped in freezer paper, vacuum sealed in commercial freezer bags, and stored at –80°C for later analysis. The carcass was thawed at room temperature and fillets removed. Abdominal wall tissue or belly flap (BF) was removed from the fillet by following the line marked by termination of rib bones and initiation of peritoneum of body wall. This region has been demonstrated to provide precise estimates of total body proximate components in this size striped bass (Jacobs et al. 2008). BF was weighed to the nearest 0.01 g wet wt and homogenized in a Retsch mixer mill (Retsch). Proximate composition followed standard Association of Official Analytical Chemists (AOAC) methods with single samples per tissue due to the amount of material available (AOAC 2005). Briefly, BF were dried at 90°C overnight, neutral lipids extracted over 8 h via Golfich apparatus with petroleum ether. Crude protein was determined by the Kjeldahl nitrogen method with a 6.2 conversion factor, and ash by muffle furnace overnight at 550°C. Total energy was calculated using the conversions for lipid and protein derived by Brett & Groves (1979): 8.7 kcal g^{-1} lipid and 5.7 kcal g^{-1} protein.

Statistical analyses. For most variables, general linear models (ANOVA) (PROC GLM; SAS 1990) were used to examine overall effects of ration, treatment, time, and interactions. Least-squares means were examined where appropriate with Tukey-Kramer adjustment (LS means; SAS 1990). The data were analyzed separately for the short- and long-term studies, with HRM and HRC at Week 8 used as starting data for the long-term study. Bacterial counts were log transformed before analysis and data and confidence intervals back transformed for reporting. Analysis of covariance (ANCOVA) (Proc GLM; SAS 1990) was also used to test equality of slopes and treatment effects of resulting energetic growth over time within ration. A novel approach was employed for analysis of survival because of the need to sacrifice experimental subjects before they could reach the study endpoint. The Cox proportional hazards model (Cox 1972) was applied to calculate the relative risk associated with predictor variables. Data were analyzed using PROC PHREG (SAS 1990) to model days until death as a function of each combination of ration and treatment.

RESULTS

Gross pathology

In the short-term Expt 1, we examined the influence of ration on the progression and severity of disease. Clinical signs were not apparent 4 wk after inoculation, and both HRM and LRM fish appeared grossly similar internally. At necropsy, the visceral mass in both groups appeared hardened, fused, and was occasionally adhered to the body wall. Nodular red foci were readily apparent throughout the visceral fat, mesenteries, and body wall. Spleens were generally enlarged and friable as occasionally were head kidneys. Between 4 and 6 wk, extensive mortality occurred in LRM fish with characteristically greater severity of ascites, visceral fusion, and abundance of red foci on mesenteries. Moribund fish typically moved to the bottom of the tank, became dark, lethargic, and did not feed. By Week 8, these conditions generally subsided in HRM fish but remained intense in remaining LRM fish. Necropsy revealed nodular lesions in spleens, head kidney, and occasionally liver in both groups. However, ascites was more common in LRM survivors. Rarely, ascites presented as a gelatinous mass encompassing the entire visceral cavity. More often, varying amounts of clear to yellowish liquid were noted.

In the long-term Expt 2, previously infected fish placed on a limiting diet (0.073% BW d^{-1}) had minimal influence on clinical presentation or gross pathology. No difference was noted between HRM or LRM at Week 16, with both groups showing further reduction in the abundance of red foci and splenic enlargement. By Week 24, some LRM fish had enlarged spleens, a fused abdominal organ mass, and, occasionally, ascites. The majority of fish had no visible stores of mesenteric body fat. Between Weeks 26 and 32, this group had increased mortality compared to the HRM group. Behavior and clinical signs were identical to that described during Expt 1. Gross necropsy finding in LRM survivors at 26 to 32 wk varied from similar appearance to HRM fish internally, to hardened and fused viscera with intense red foci and ascites reminiscent of the LRM fish at 8 wk. In general, gross pathological changes of the low ration infected groups in Expts 1 and 2 were identical, presenting with advanced ascites, fused viscera and red foci than high-ration counterparts in each experiment.

Histopathology

In the first short-term Expt 1, granulomatous inflammation in HRM fish followed a classical progression from loosely organized inflammatory cells and early granulomas to distinct, well-formed nodular lesions as described previously (Colorni et al. 1998, Gauthier et al. 2003). Bacteria were visible at all progressive stages by Ziehl-Neelsen staining. By Week 8, granulomas generally had a well-developed fibrous capsule and were rarely found outside of spleen, anterior kidney, and mesenteric tissue. In contrast, an active, systemic infection associated with intense inflammation in many organs generally persisted throughout the initial study period in LRM fish. This was characterized by a high prevalence of fused, poorly developed granulomas with large, pale necrotic cores (Fig. 1). The central pale eosinophilic necrotic material was surrounded by vacuolated macrophages, and the cells comprising the fibrous capsules had a thicker, epitheliod morphology versus the flat fibroblastic appearance of those in HRM fish. Acid-fast staining generally revealed a greater concentration of bacteria in LRM than HRM splenic granulomas. Mortalities in the LRM group were generally characterized by systemic infection with intense peritonitis. Severity of lesions was significantly greater in spleen, head kidney, liver, posterior kidney, and heart in LRM fish than in HRM fish at 4 and 8 wk ($p < 0.05$). Severity of the peritoneal inflammatory response was equal among rations at 4 wk, becoming significantly greater in LRM fish by Week 8 ($p < 0.05$) (Fig. 2).

In the long-term Expt 2, HRM fish histopathology remained relatively stable from Weeks 8 to 32, characterized by well-formed granulomas surrounded by normal tissue. Cores of many granulomas had condensed, and visible bacteria within granulomas were greatly reduced after 8 wk. In Week 24, minor renewed inflammation was noted in a few HRM fish but was not associated with disintegrating granulomas. In contrast, renewed inflammation predominantly in the spleen was readily visible in LRM fish by Week 24, increasing in severity by Week 32 (Fig. 1). Lesion severity and inflammation increased in all organs in LRM fish from Week 24 until Week 32, resembling lesions noted in the LRM of Expt 1 at 8 wk (Fig. 2).

Bacteriology

Inoculation of 10^4 CFU g^{-1} BW resulted in a differential response in bacterial replication among rations and over time. In Expt 1, bacterial density in spleens of LRM fish and mortalities was over 3 orders of magnitude greater than HRM fish at Week 4 ($p < 0.0001$). By Week 8, bacterial density increased in HRM fish from ~10^4 to ~10^6 CFU g^{-1} spleen. Bacterial density similarly increased in LRM fish (~10^6 to ~10^8 CFU g^{-1} spleen) remaining significantly higher than HRM fish ($p = 0.0016$). Mortalities at 4 and 6 wk had statistically similar bacterial densities to LRM fish ($p > 0.05$) (Fig. 3). Overall, ration explained 64% of the model variation ($p < 0.0001$, $F = 38.56$, df = 2,77) with time accounting for 23% ($p = 0.0004$, $F = 13.75$, df = 1,77), and the interaction accounting for the remainder ($p = 0.0005$, $F = 8.51$, df = 2,77).

In Expt 2, bacterial density increased from Weeks 8 to 16 in both LRM and HRM fish, from 5×10^6 to approximately 9×10^6 CFU g^{-1} spleen. From Weeks 24 to 32, LRM bacterial density continued to increase, resulting in a 10-fold difference between ration groups by Week 32 (Fig. 3). Overall, ration explained 67% of the total variability ($p = 0.02$, $F = 5.43$, df = 1,79), while time accounted for the remainder ($p = 0.05$, $F = 2.73$, df = 3,79) and the interaction being non-significant ($p > 0.5$) A single moribund fish (LRM) between Weeks 8 to 16 had a bacterial density of 4×10^8 CFU g^{-1} spleen, approximately twice that of LRM fish at that time period. Moribund fish in Expt 2 were highly variable with respect to bacterial density by Week 32, with mean density similar to LRM fish (Fig. 3). These fish were not included in the analysis due to lack of representation over time.

Bacterial species other than *Mycobacterium* were isolated from either liver or kidney in 30.5% of moribund fish. Most common were *Vibrio damsela* and *V. vulnificus*, with all other species being isolated from

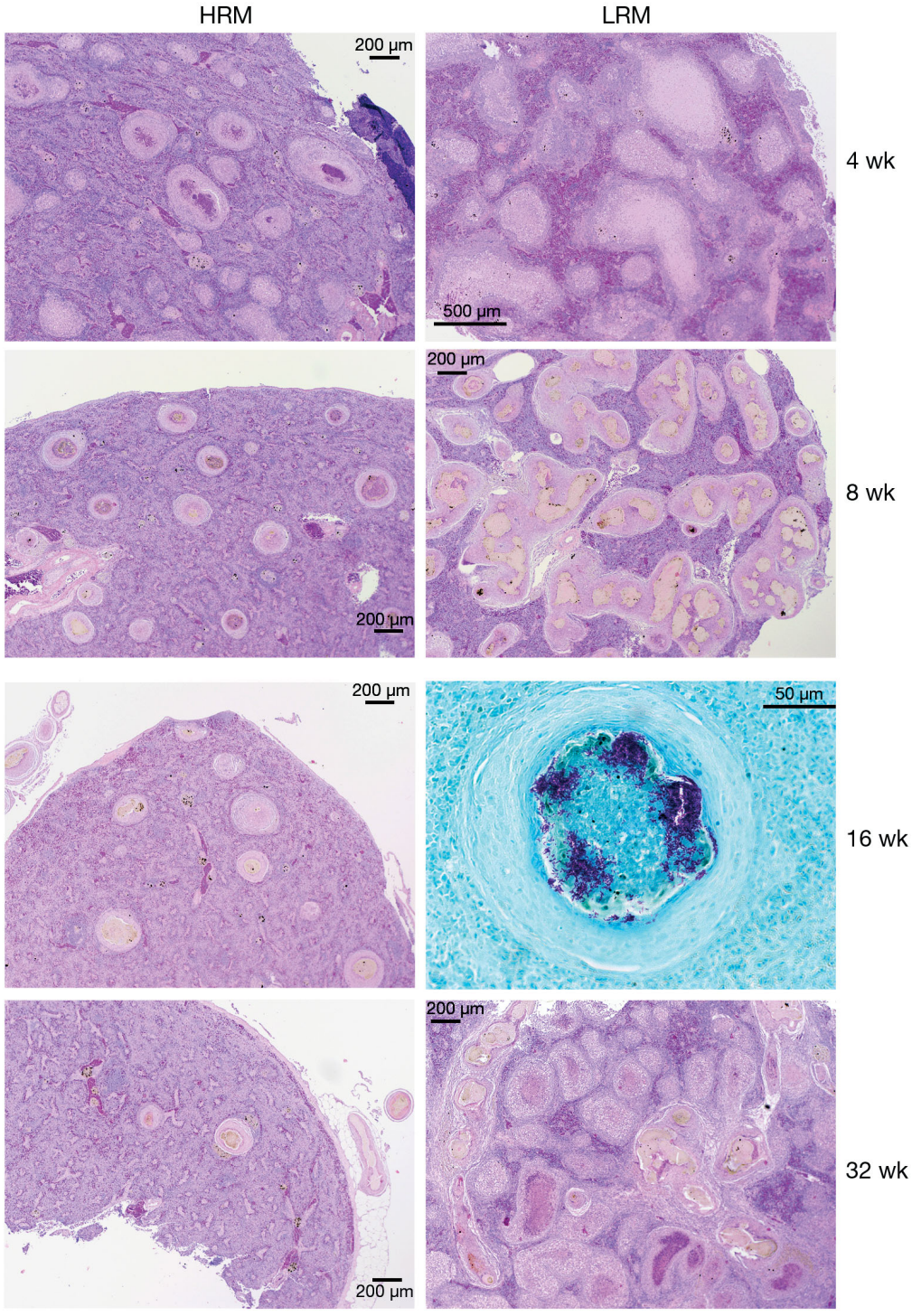

Fig. 1. *Mycobacterium marinum* infecting *Morone saxatilis*. Progression of inflammatory response in striped bass inoculated with *M. marinum* by diet and time. All sections are splenic tissue stained with hemotoxylin and eosin (H&E) or Ziehl-Neelsen. High-ration fish progressed through stages of classic granulomatous inflammation resulting in well-encapsulated granulomas by 8 wk surrounded by largely normal tissue. This condition remained throughout the course of the short-term and long-term studies. In contrast, low-ration fish developed poorly formed, vacuolated, early granulomas, without fibrous capsules at 4 wk, progressing to fused granulomas with pale eosinophillic cores by Week 8 and complete mortality by Week 10. In the long-term study previously infected high-ration fish inoculated with *M. marinum* (HRM) placed on a reducing diet (low-ration fish inoculated with *M. marinum*; LRM) largely maintained well-formed granulomas with high bacterial density by Week 16. However, inflammation was reactivated in LRM fish with poorly formed and fused granulomas dominated by vacuolated macrophages by Week 32 (LRM)

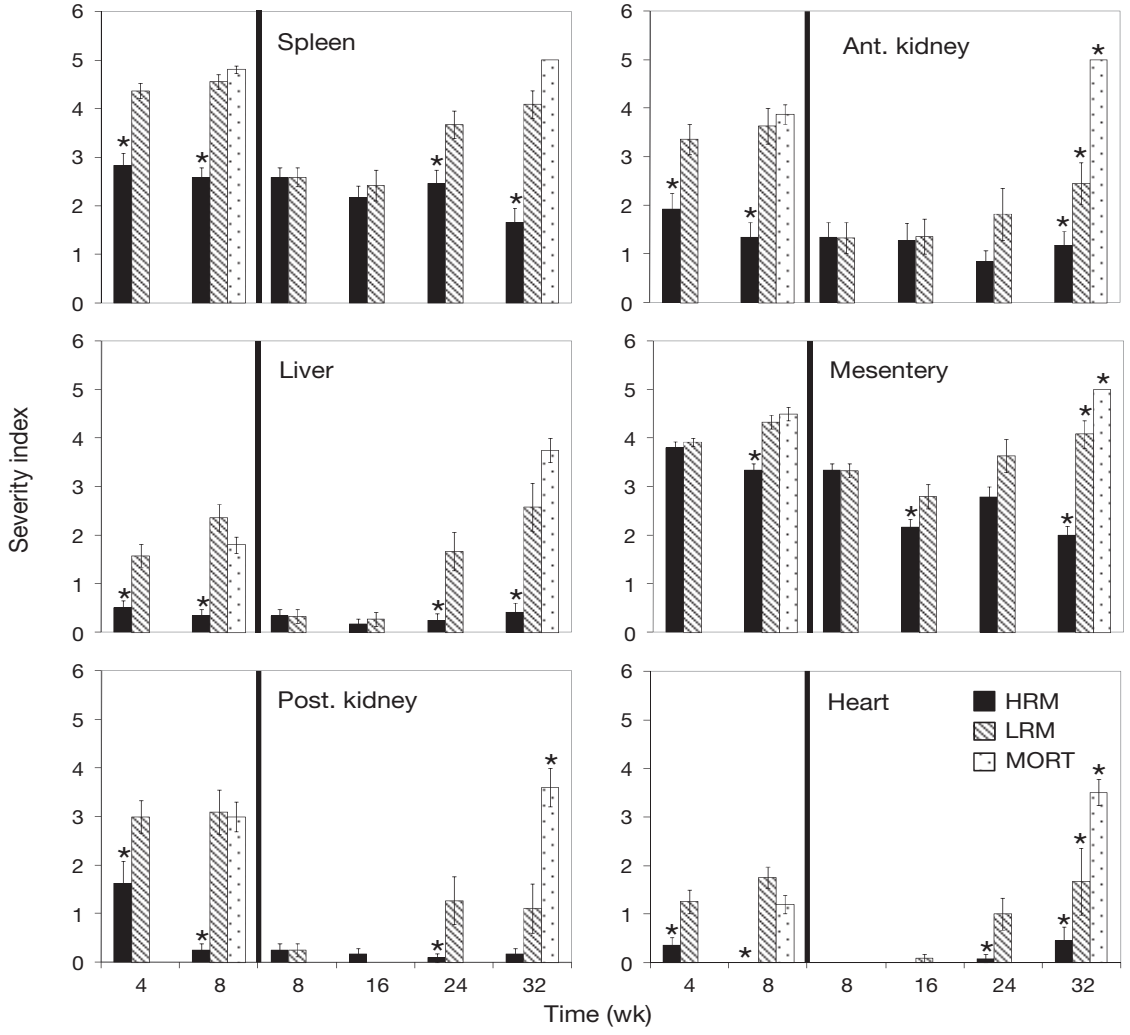

Fig. 2. *Mycobacterium marinum* infecting *Morone saxatilis*. Severity of pathology associated with mycobacteriosis by organ for short- and long-term studies combined. Dark vertical line divides short- from long-term experiment. Severity index is scaled from 0 to 5, with 0 being normal tissue and 5 being complete change of tissue architecture. *Significantly different from all others within time ($\alpha = 0.05$). HRM, LRM: high- and low-ration inoculated fish, respectively; MORT: low-ration mortalities

only a single fish (Table 1). Of the 9 control mortalities during the short-term Expt 1, 4 were found in a moribund state allowing for culture. Of these, 3 were culture-positive for *M. marinum* at densities similar to their inoculated counterparts (10^6 to 10^8 CFU g^{-1} spleen), all belonging to the low-ration group. In the long-term Expt 2, the single control mortality was also culture-positive, containing 2×10^8 CFU g^{-1} spleen of *M. marinum*.

Hematology/plasma chemistry

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

In the long-term Expt 2, leukocrit was highly variable both within and among treatments, with no consistent trends (Fig. 4). Treatment, ration, and time explained only 7% of the total variation ($p = 0.0293$, $F = 2.56$, df = 5,167), with only time explaining a significant proportion of the model variance ($p = 0.064$, $F =$

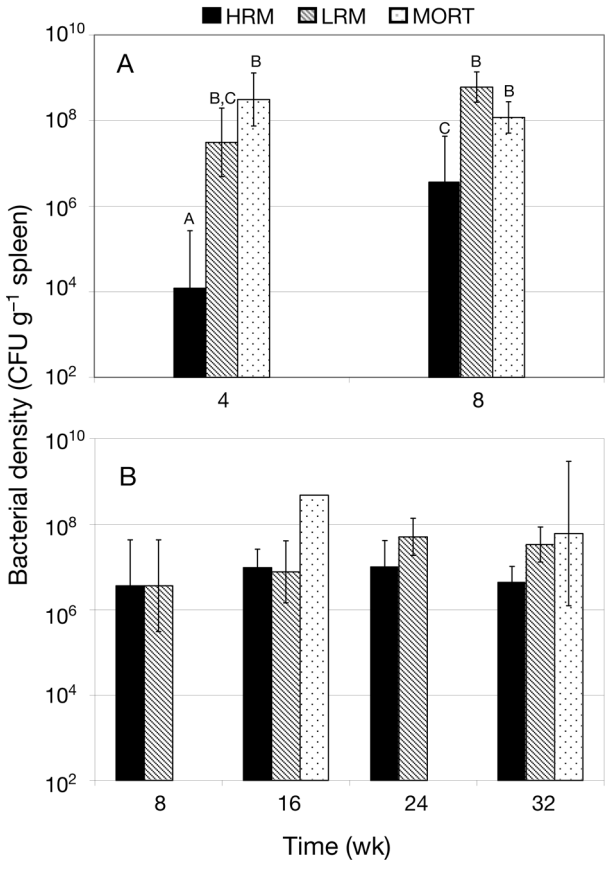

Fig. 3. *Mycobacterium marinum* infecting *Morone saxatilis.* Bacterial density in striped bass spleens of experimental fish over time for (A) the short-term and (B) long-term studies. Error bars are 95% confidence intervals. Same letter denotes lack of significance (α = 0.05). HRM, LRM: high- and low-ration inoculated fish, respectively; MORT: low-ration mortalities

4.24, df = 3,167). Red cell volume declined in all treatments over time, but more substantially in low-ration fish (Fig. 4). Overall, a model containing ration, treatment, time, ration × treatment, and ration × time explained 76% of the total variation ($p < 0.0001$, $F = 64.39$, df = 8,167) with 62% of the model variance accounted for by ration alone ($p < 0.0001$, $F = 39.30$, df = 1,167). Treatment effects did not explain a significant proportion of the model variance ($p = 0.66$, $F = 0.02$, df = 1,167).

Plasma protein levels increased in high-ration fish and declined in low-ration fish over time in the long-term study (Fig. 4). A full model explained 83% of the total variation ($p < 0.0001$, $F = 64.25$, df = 11,148), with ration alone explaining the vast majority of model variance (75%; $p < 0.0001$, $F = 530.47$, df = 1,148) followed by time (21%; $p < 0.0001$, $F = 50.11$, df = 3,148). Individual differences in treatment means at any given time did not account for significant proportions of the model variance for any of the blood parameters investigated ($p > 0.05$).

Table 1. *Morone saxatilis.* Percentage of non-mycobacterial isolates from liver (L) and head kidney (K) of all moribund striped bass. Both treatments and short- and long-term studies are combined

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

Survival

Mycobacterium marinum inoculation severely reduced survival in LRM fish in Expt 1. Only 25% of LRM fish survived by Week 8 compared to 97% of HRM fish or either control (92% LRC, 100% HRC) (Fig. 5). Mortality in LRM fish peaked between Weeks 4 and 6 post-inoculation. The survival model with diet and infection explained survival times better than a null model (likelihood ratio test; $\chi^2 = 241.7$, $p < 0.0001$). The mortality risks (hazard ratio) for each treatment group are given in Table 2. Subjects in the HRC group were at no appreciable risk of increased mortality. Subjects in the HRM and LRC groups were at a similar slight risk level, statistically significantly greater than zero ($p < 0.001$) but probably insignificant biologically. The LRM group was at a high risk level, being 37× more likely to die than the other groups.

Survival in the long-term Expt 2 followed a similar, although protracted, trend with 96% percent survival in all groups with the exception of LRM. Between Weeks 13 and 28, a single mortality was noted in LRM fish. By Week 32, 44% of the starting population had died (Fig. 5). The model with diet and infection explained survival times better than a null model (likelihood ratio test; $\chi^2 = 20$, $p = 0.0002$). The mortality risks for each treatment group are given in Table 3. Subjects in the HRC, HRM, and LRC groups were at a similar slight risk level. The LRM group was again at a high-risk level, being 14× more likely to perish than the other groups.

Energetics

Rations used in the initial study proved to have the predicted effect of strong, linear growth in HRC fish

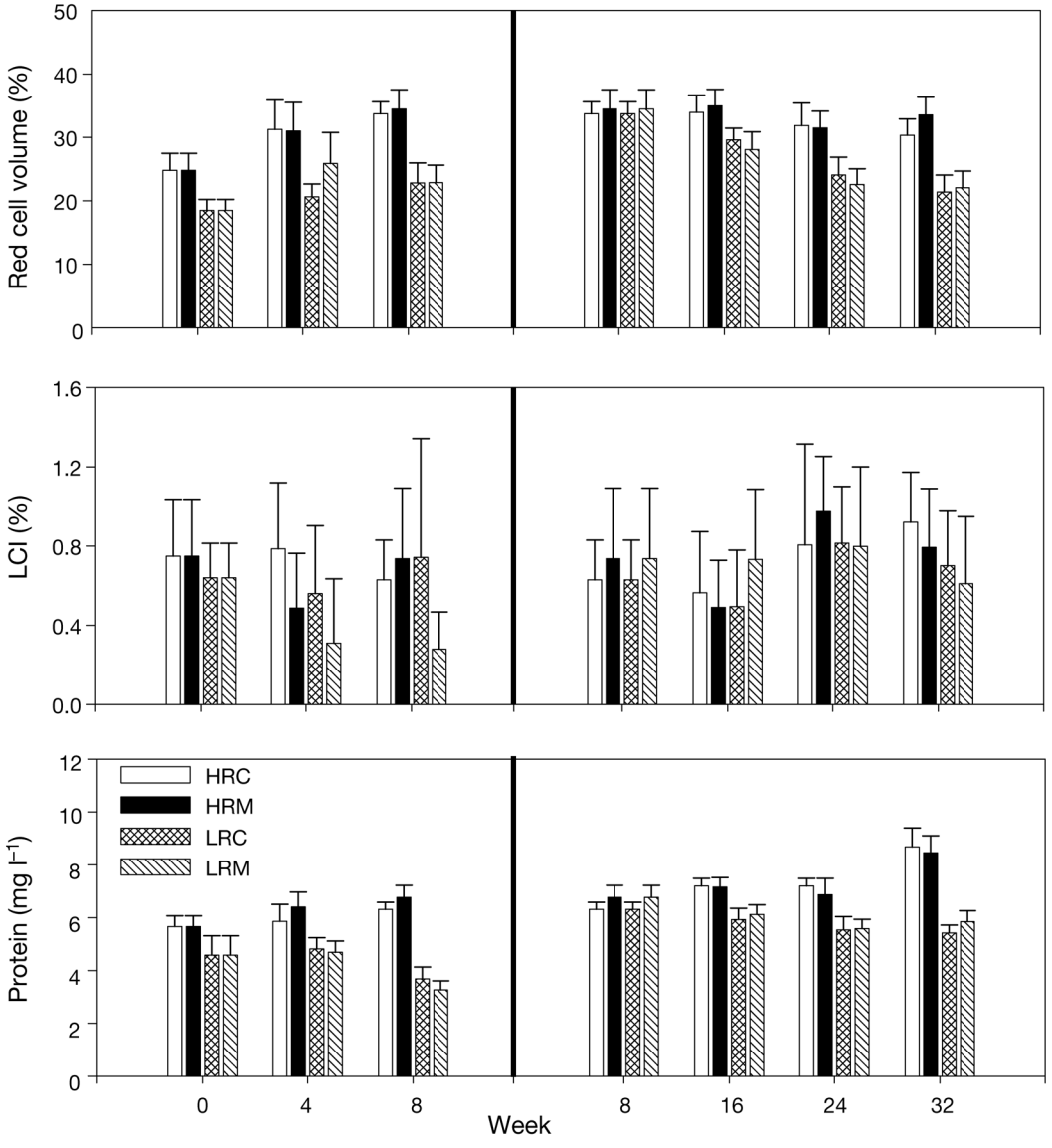

Fig. 4. *Morone saxatilis.* Changes in striped bass red and white cell volume and plasma protein by treatment over time (LCI: leucocrit index). Vertical bar separates short- and long-term evaluations. Error bars: +SD. HRC: high-ration control; LRC: low-ration control; HRM: high-ration, inoculated with *Mycobacterium marinum*; LRM: low-ration, inoculated with *M. marinum*

and weak, positive growth in LRC fish during the first 8 wk. Growth in terms of total body energy (kcal) was reduced slightly in comparison to controls in both LRM (–5.04 kcal) and HRM (–20.73 kcal) fish 4 wk following inoculation. By Week 8, growth rate had increased in HRM fish, becoming more comparable to HRC fish (1.83 and 2.10 kcal d^{-1}, respectively) with no significant difference in total body energy ($p > 0.05$). Conversely, growth rate declined in LRM fish by Week 8 after inoculation, 12 wk after the start of the low-ration feed (–0.61 kcal d^{-1}) while LRC continued positive growth (0.22 kcal d^{-1}), with total body energy at this time period being significantly greater in LRC fish ($p > 0.05$) (Fig. 6). The overall energetic impact of active infection with *Mycobacterium marinum* was estimated to be nearly identical for high- and low-ration fish, costing 0.52 and 0.51 kcal d^{-1}, respectively.

In the long-term Expt 2, the reduction ration (0.073 % BW d^{-1}) had the predicted effect of gradually reducing total body energy in both LRM and LRC fish. These fish had been on the high-ration diet for 12 wk prior to being placed on the low-ration diet. While the reduction was significant over time ($p < 0.0001$, $F = 41.7$, df = 1, 61), slopes were identical ($p = 0.53$, $F = 0.40$, df = 1, 61) as were treatment means ($p = 0.18$, $F = 1.90$, df = 1, 61), suggesting little energetic demand of mycobacteriosis

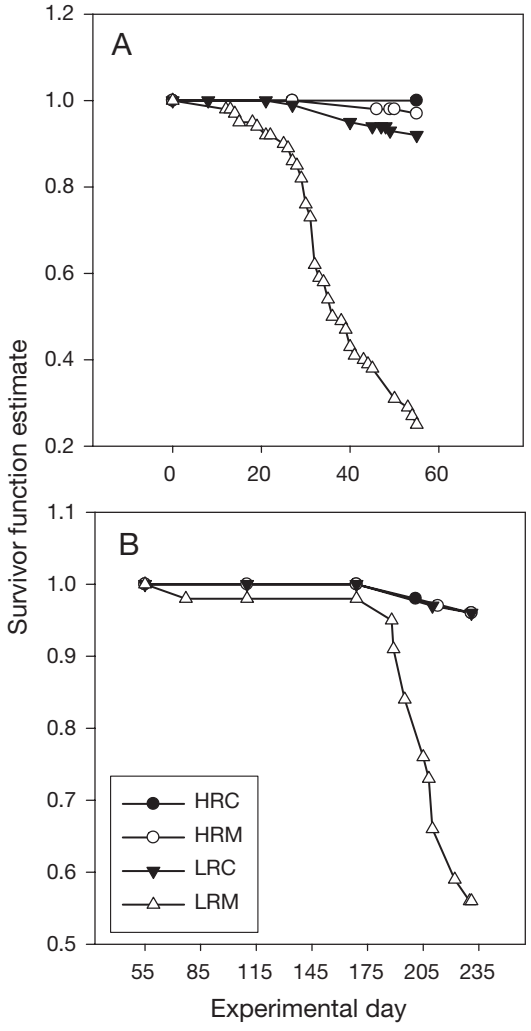

Fig. 5. *Morone saxatilis*. Maximum likelihood estimates of survival by bacterial treatment and ration for (A) the short-term and (B) long-term studies. Short-term study was terminated after Week 8 due to lack of LRM survivors. First data point for each treatment marks first mortality or sampling interval if point is equal to 1. See Fig. 4 for abbreviations

during this time. Separation was only apparent at the final sampling period, with LRM fish having lower total body energy, although not significant (ANOVA; p = 0.336, $F = 1.0$, df = 1,14). A strong negative correlation was found between severity of splenic infection and both lipid ($r = -0.755$, $p < 0.0001$, $n = 24$) and protein concentration ($r = -0.65$, $p < 0.0001$, $n = 24$). Significant growth occurred over time in both HRC and HRM fish ($p < 0.001$, $F = 0.09$, df = 1,60), and both maintained identical slopes ($p = 0.99$, $F = 0.0$, df = 1,60) and total energy at time ($p = 0.77$, $F = 0.09$, df = 1,60) (Fig. 6).

DISCUSSION

To our knowledge, this is the first effort that has directly demonstrated the influence of food quantity on the progression and severity of mycobacteriosis in fish. Inoculated fish fed low rations developed a severe, active systemic infection, characterized by high bacterial loads, ending in death within 4 to 6 wk. In contrast, classic granulomatous inflammation leading to a persistent but controlled infection was characteristic of inoculated, yet properly nourished, fish. In the second study, reactivation of acute inflammatory state was induced by placing fish with contained infections on reducing diets (0.073% BW d^{-1}). In both studies, the energetic demand of this disease was only appreciable when associated with active, severe inflammatory states.

The progression of mycobacteriosis associated with *Mycobacterium marinum* has been previously described in goldfish *Carassius auratus* (Talaat et al. 1998), sea bass *Dicentrarchus labrax* (Colorni et al. 1998), hybrid tilapia *Orechromis* spp., and striped bass *Morone saxatilis* (Wolf & Smith 1999, Gauthier et al. 2003) among others. In experimental mycobacteriosis, dose administered is a critical consideration in the interpretation of results. Talaat et al. (1998) found median survival time of 4 and 10 d after administering doses of 10^9 and 10^8, respectively to goldfish weighing 30 g, while fish survived to the end of the study period of 56 d with doses of 10^7 or less. Minimum dose for producing pathology within 8 wk was determined to be 600 CFU per fish. Wolf & Smith (1999) found that a dose of 10^6 CFU g^{-1} BW resulted in severe inflammation and 50% mortality by 8 d in striped bass, while the same dose resulted in complete survival and less severe of a response in tilapia. In sea bass, 10^4 CFU g^{-1} BW resulted in classic granulomatous inflammation, intensified at 4 to 6 wk post-inoculation, with low mortality and evidence of lesion regression by 26 wk (Colorni et al. 1998). Similarly, Gauthier et al. (2003) found 10^4 CFU resulted in a persistent, chronic disease state with low associated mortality over a period of 45 wk in striped bass. An inoculation of 10^4 CFU g^{-1} BW was

Table 2. *Morone saxatilis.* Hazard ratios and 95% confidence intervals for the short-term portion of the challenge study. HRC: high-ration control; LRC: low-ration control; HRM: high-ration inoculated with *Mycobacterium marinum*; LRM: low-ration inoculated with *M. marinum*

Effect	χ^2	p	Hazard ratio	95% confidence limit
HRC	0.0010	0.975	0.000	
HRM	44.1100	<0.0001	0.020	0.006–0.063
LRC	61.5955	<0.0001	0.062	0.031–0.125
LRM	134.1408	<0.0001	37.169	20.150–68.530

Table 3. *Morone saxatilis.* Hazard ratios and 95% confidence intervals for the long-term portion of the challenge study. See Table 2 for abbreviations

Effect	χ^2	p	Hazard ratio	95% confidence limit
HRC	6.3062	0.0120	0.072	0.009–0.561
HRM	6.1905	0.0128	0.073	0.009–0.574
LRC	6.3715	0.0116	0.071	0.009–0.553
LRM	15.9267	<0.0001	13.909	3.818–50.672

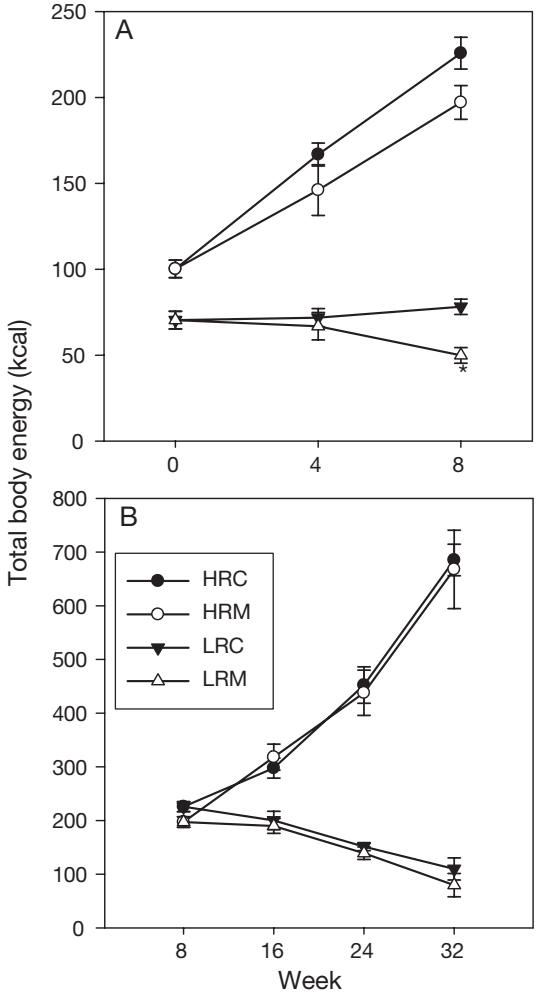

Fig. 6. *Morone saxatilis.* Estimated total-body energy by treatment and ration over time for the (A) short-term and (B) long-term studies. *Significantly different from controls at specified time interval (α = 0.05). See Fig. 4 for abbreviations

administered in this study, as it has been demonstrated to be a biologically relevant dose, resulting in a measurable pathology, with low associated mortality in the absence of other stressors.

Strain variation in *Mycobacterium marinum* isolates is only beginning to be appreciated (Ucko et al. 2002, van der Sar et al. 2004) but is an important consideration in experimental studies. van der Sar et al. (2004) demonstrated a marked difference in survival and disease progression in zebrafish *Danio rerio* challenged with several fish and clinical isolates. Most clinical isolates caused an acute disease characterized by uncontrolled proliferation of the pathogen and complete mortality within 16 d, while the fish isolates generally resulted in classic granulomatous inflammation characteristic of piscine mycobacteriosis. In addition, the 2 fish isolates examined in their study caused moderate differences in granuloma number, size, and morphology, with one isolate also associated with external ulcerations. The strain of *M. marinum* used in this study was isolated from a wild Chesapeake Bay fish and has not been characterized beyond species. This strain proved to be highly appropriate as it caused a demonstrable and measurable pathology in both ration groups allowing for direct examination of ration effects. Clearly, the issue of strain variability demands further attention in epidemiological approaches aimed at understanding mortality and the genesis of ulcerative lesions.

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

The use of a reducing diet in the long-term study was necessary to bring fish into a poor nutritional state in a reasonable time frame. Fish are well-adapted physiologically to undergo long periods of complete starvation (Love 1980). At 21°C, striped bass can maintain visceral, non-polar lipid reserves for several months in the complete absence of food (Jacobs et al. 2008). As a fish starves, it first uses glycogen deposits primarily from the liver. Once depleted, triglycerides are mobilized from muscle tissues and mesenteric lipid deposits. Lipids used are replaced with water in a linear fashion. Finally, protein catabolism ensues in severe cases of starvation (Love 1980). Coarsely, renewed inflammation was associated with depletion of lipid reserves. Severity of infection was strongly correlated with decreasing lipid and protein concentrations ($p < 0.0001$, $r > -0.65$, $n = 24$) but not in their adequately fed cohorts. Because of the time between sampling (2 mo), a definitive chemical profile associated with renewed inflammation could not be determined.

More detailed approaches are certainly warranted to examine this issue.

A minor proportion of experimental fish were previously infected with *Mycobacterium marinum* based on positive culture of 3 out of 60 fish in our initial screening. As fish possess acquired immunity, it is possible that previous exposure to mycobacterium led to a hastened immune response over that capable by truly naïve fish (Plouffe et al. 2005). However, there is no evidence that previous exposure had any influence on our results and, in fact, allowed for limited information on the impact of diet in naturally infected fish with a second strain of *M. marinum.* In both experiments, the majority of the few control mortalities in the low-ration group that were removed in time to culture were identical to inoculated counterparts in terms of bacterial density and severity of the active inflammatory state. These results, in conjunction with the long-term study, provide further evidence that IP inoculation is an appropriate dosing strategy. The only major difference in pathology between naturally infected controls and those inoculated was the high prevalence of granulomas and inflammation in the mesenteric tissue of the latter, undoubtedly an artifact due to injecting the pathogen into the body cavity. Others have used water exposure as a route of infection for the study of mycobacteriosis, which may provide a more natural route of infection but inherently enhances variability in the degree and severity of infection and the concentration of pathogen in exposure waters (Li & Gatlin 2005).

The use of proportional hazard analysis to describe mortality associated with infectious disease has received considerable attention in the medical and statistical literature (e.g. Turnbull & Mitchell 1984) but has only been used sparingly in fish health investigations (Dale et al. 1997, Park & Reno 2003, Ogut & Reno 2004, Becker et al. 2006). Bebak-Williams et al. (2002) applied survival analysis to examine the influence of stocking density and pathogen concentration on survival of rainbow trout *Oncorhynchus mykiss* experimentally challenged with infectious pancreatic necrosis (IPN) virus. The authors used the Kaplan-Meier model to examine changes in risk over time associated with IPN outbreaks. This model differs from the Cox model (Cox 1972) in that it is fully parametric and thus requires larger sample sizes than possible in our evaluation. The advantage of using survival analysis models in studies of infectious disease is that they allow for removal of organisms from the study over time (i.e. destructive sampling), provide relative estimates of risk of mortality, and allow for the evaluation of survival distribution over time.

In the current epizootic of mycobacteriosis in wild Chesapeake Bay striped bass, reduced condition can occur in association with external lesions and mycobacteriosis (Overton et al. 2003, Ottinger 2006). The disease itself has also been referred to informally as a 'chronic wasting disease,' implying that reduction in condition is caused by the bacteria themselves. It is impossible to discern causal relationships between disease state and nutritional health from field evaluations because they are endpoint observations, and thus the prior history of the animal is unknown. In our experiments, a measurable reduction in total body energy was only apparent during active, acute inflammatory states. This state occurred regardless of ration for 4 wk post IP administration of 10^4 CFU g^{-1} BW of *Mycobacterium marinum*, but was resolved in HRM fish by Week 8, while LRM fish continued to decline energetically. In the long-term study, the artifact of high-dose IP inoculation was removed by using previously infected fish. Although splenic bacterial density averaged over 10^6 CFU g^{-1} spleen, total body energy remained identical to sham-inoculated controls in adequately fed fish over the course of the study interval. Fish fed suboptimal diets also maintained similar body energy through the initial 4 mo period, regardless of bacterial treatment. Only during the final 2 mo with the re-emergence of active, acute inflammation preceding elevated mortality in LRM fish did body energy decline. Thus our data suggest that the energetic demand of mycobacteriosis in striped bass as caused by *M. marinum* is negligible in chronic states where adequate energy reserves are present.

Remarkably similar results to this study were previously obtained by Chan et al. (1996) in their examination of the relationship of PCM and tuberculosis. Using a mouse model, those fed low-protein diets (2%) rapidly succumbed to tuberculosis within 2 mo, accompanied by a reduced expression of interferon γ, tumor necrosis factor ά, and nitric oxide synthase in the lungs. Those receiving a high-protein diet (20%) survived through the end of the study. Of great interest is the demonstration that the fate and course of infection could be reversed by re-administering the high-protein diet.

The model that is evolving from the medical literature is one of a cat-and-mouse game between host immune function and mycobacterial replication (Chandra 1996). Once engulfed by macrophages, bacteria may replicate freely within the cell. This triggers a cascade of cytokine-mediated events leading to the formation of the granuloma in attempt to limit the spread of disease and focus efforts to destroy the pathogen. In immunocompetent hosts, the acute phase of disease often gives way to either a latent or chronic state where bacteria are often readily culturable and visible within granulomas (Flynn & Chan 2001). Recent work suggests that there is a dynamic equilibrium between host immune function and mature granulomas (Bouley

et al. 2001), in contrast to theories of bacteria persisting in a resting state. Some findings (Bouley et al. 2001) suggest bacterial killing within the granuloma is balanced by pockets of freely replicating cells, sometimes within the same phagosome. Exactly how some mycobacteria evade the attempts of the host immune system is still unclear, but the implications are that a reservoir is maintained within the host for potentially a lifetime. It is estimated that ⅓ of the world's population is infected with tuberculosis (Flynn & Chan 2001), as are 50% of striped bass greater than age 3 with related fish pathogens in Chesapeake Bay (Ottinger & Jacobs 2006). Whether through disruption of cytokine profiles and subsequent macrophage activation (Chan et al. 1996, Dai & McMurray 1998) or mechanisms yet to be determined, it is clear that nutritional insult can disrupt this equilibrium in favor of the pathogen.

Causal relationships in the study of disease epizootics are difficult to discern because of complex interactions between the host, pathogen, and the environment (Sindermann 1970). Stressors such as high density or crowding, poor water quality, or elevated temperature could easily play a role in the dynamics of this disease (Hawke 2000). It is plausible that a combination of environmental stressors, combined with a susceptible host and the numerous mycobacterial pathogens whose virulence have yet to be thoroughly explored may be driving this epizootic. However, the importance of the potential role of food limitation and/or changes in energetic state should not be understated. Effective multi-species management of predator and prey offers one of the few potential intervention strategies for addressing this disease in a relatively short time frame. Future efforts should proceed with the examination of dietary quality in combination with other stressors (i.e. temperature, low dissolved oxygen) and the multiple species of *Mycobacterium* isolated in Chesapeake Bay.

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