

Temperature, hypoxia, and mycobacteriosis: effects on adult striped bass *Morone saxatilis* metabolic performance

Dominique Lapointe^{1,4,*}, Wolfgang K. Vogelbein¹, Mary C. Fabrizio¹,
David T. Gauthier², Richard W. Brill³

¹Virginia Institute of Marine Science, College of William & Mary, PO Box 1346, Gloucester Point, Virginia 23062, USA

²Department of Biological Sciences, Old Dominion University, Norfolk, Virginia 23529, USA

³Ecosystem Processes Division, Northeast Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Atlantic Highlands, New Jersey 07732, USA

⁴Present address: Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada

ABSTRACT: Mycobacteriosis, a chronic bacterial disease of fishes, is prevalent in adult striped bass from Chesapeake Bay (USA). Although environmental factors may play a role in disease expression, the interaction between the disease and environmental stress remains unexplored. We therefore examined the individual and interactive effects of elevated temperature, hypoxia, and mycobacteriosis on the metabolism of wild-caught adult striped bass from Chesapeake Bay using respirometry. Because the spleen is the primary target organ of mycobacteriosis in striped bass, we hypothesized that the disease interferes with the ability of fish to increase their hematocrit in the face of increasing oxygen demands. We determined standard metabolic rate (SMR), maximum metabolic rate under normoxia (MMR_N), critical oxygen saturation (S_{crit}), and MMR under hypoxia ($3\text{ mg O}_2\text{ l}^{-1}$; MMR_H) for healthy and visibly diseased fish (i.e. exhibiting skin lesions indicative of mycobacteriosis). Measurements were taken at a temperature within the preferred thermal range (20°C) and at an elevated temperature (28°C) considered stressful to striped bass. In addition, we calculated aerobic scope ($AS_N = MMR_N - SMR$, $AS_H = MMR_H - SMR$) and factorial scope ($FS_N = MMR_N\text{ SMR}^{-1}$, $FS_H = MMR_H\text{ SMR}^{-1}$). SMR increased with increasing temperature, and hypoxia reduced MMR, AS, and FS. Mycobacteriosis alone did not affect either MMR_N or MMR_H . However, elevated temperature affected the ability of diseased striped bass to tolerate hypoxia (S_{crit}). Overall, our data indicate that striped bass performance under hypoxia is impaired, and that elevated water temperatures, hypoxia, and severe mycobacteriosis together reduce aerobic scope more than any of these stressors acting alone. We conclude that the scope for activity of diseased striped bass in warm hypoxic waters is significantly compromised.

KEY WORDS: Oxygen · Critical · Aerobic scope · Disease · Hematocrit · Spleen

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INTRODUCTION

The striped bass *Morone saxatilis* is an ecologically and economically important finfish inhabiting much of the US Atlantic coast. In the mid-1980s, extremely low production prompted implementation of coast-

wide restrictions on both commercial and recreational fisheries for this species (Richards & Rago 1999), but since the mid-1990s, striped bass abundance along the Atlantic coast has rebounded and fishing restrictions have been eased (Hartman & Margraf 2003). Although the abundance of Chesa-

peake Bay striped bass is currently considered high, concerns have developed with respect to the health of the Atlantic coastal migratory stock. Striped bass exhibiting poor body condition and clinical signs of mycobacteriosis (including severe skin ulceration in some fish) were first reported from Virginia waters in 1997 (Vogelbein et al. 1998). Concomitant with the first reports of this disease, significant increases in natural mortality for Chesapeake Bay striped bass were reported (Jiang et al. 2007). The disease is caused by acid-fast bacteria belonging to the genus *Mycobacterium*. In aquaculture situations, outbreaks of mycobacteriosis are associated with stressful environmental conditions and are invariably lethal (Nigrelli & Vogel 1963, Backman et al. 1990, Bruno et al. 1998, Chinabut 1999). In Chesapeake Bay striped bass, the disease is characterized by granulomatous inflammation with the presence of acid-fast bacteria, predominantly in the spleen and kidney. However, severe skin ulceration is also commonly observed (Vogelbein et al. 1999, Cardinal 2001). The prevalence of mycobacteriosis in Chesapeake Bay striped bass is alarmingly high, exceeding 90% by the time fish are 4 to 5 yr of age (Overton et al. 2003, Gauthier et al. 2008). A recent study provided the first evidence of mortality associated with mycobacteriosis in Chesapeake Bay striped bass (Gauthier et al. 2008). However, the magnitude of this disease-associated mortality, the role of environmental stressors in disease expression, and the specific physiological impacts of the disease remain largely unexplored.

Aside from the etiologic agents, the underlying causes of mycobacteriosis in striped bass are currently not known. Two predominant hypotheses proposing underlying environmental stressors as modulators of disease expression have recently been forwarded. The first hypothesis suggests that disease emergence may be linked to food limitation, as poor nutrition of laboratory-held fish resulted in severe systemic disease, high bacterial loads, and rapid progression to mortality in experimentally infected striped bass (Jacobs et al. 2009). A second hypothesis posits that increasing eutrophication of Chesapeake Bay and the resulting expansion of hypoxic (<4 mg O₂ l⁻¹) bottom waters is forcing striped bass to inhabit waters outside their normal thermal range (Kemp et al. 2005, Martino & Secor 2009). The recent increase in hypoxic events has had significant impacts on the productivity of Chesapeake Bay (Price et al. 1985, Adams et al. 2003, Breitburg et al. 2003, Kemp et al. 2005, Diaz & Breitburg 2009). Adult striped bass prefer temperatures <25°C, and in freshwater reservoirs, they have been demonstrated to use deeper,

cooler waters as summer thermal refugia (Coutant 1985). It is possible that striped bass in Chesapeake Bay experience a 'temperature–oxygen squeeze,' whereby hypoxic bottom water forces them to inhabit more oxygenated but warmer, more stressful surface waters during the summer, predisposing them to this infectious disease. Nonetheless, this 'temperature–oxygen squeeze' hypothesis has yet to be linked to the physiological requirements of wild striped bass.

Physiological responses of finfishes to both elevated temperatures and hypoxia have been well documented (e.g. Pörtner & Lanning 2009). Temperature controls and limits metabolic rates in fishes, and aerobic scope (AS) expressed as a function of temperature typically takes the form of a bell-shaped curve (Fry 1947, Claireaux & Lagardère 1999, Claireaux et al. 2000, Farrell 2009). AS represents the metabolic confines within which all energy-requiring work (e.g. gonadal and somatic growth, activity) must be undertaken, and is typically defined as the difference between maximum metabolic rate (MMR) and standard metabolic rate (SMR) (Fry 1971, Claireaux & Lefrançois 2007). Factorial scope (FS) is defined as the ratio of MMR to SMR (Schurmann & Steffensen 1997). SMR can be estimated from the lowest metabolic rate of a fish in a post-absorptive state, when movements are restricted and the fish is protected from outside stimuli (Fry 1971, Brett 1972). MMR is often reached during recuperation from exhaustive exercise (Bushnell et al. 1994, Reidy et al. 1995, Svendsen et al. 2012) and can be assessed by measuring oxygen consumption after a chase protocol (Black 1958). Moreover, oxygen consumption during activity or following exhaustive exercise decreases with reductions in ambient oxygen (Fry 1947, Basu 1959, Claireaux & Lagardère 1999, Claireaux et al. 2000, Dutil et al. 2007). The relationship between dissolved oxygen and AS in European sea bass showed that at a fixed temperature, AS decreases with decreasing ambient water oxygen content (Claireaux & Lagardère 1999). Critical oxygen saturation (S_{crit}) is commonly used as a measure of hypoxia tolerance in fishes (Chapman et al. 2002, Mandic et al. 2009, 2013). S_{crit} is defined as the ambient oxygen level at which the SMR can no longer be sustained. Thus S_{crit} is thought to reflect the ability of a fish to extract oxygen from the environment (Fry 1947, Schurmann & Steffensen 1997, Speers-Roesch et al. 2012).

The interactive effects of hypoxia, temperature, and infectious disease remain largely unexplored in finfishes. Our objective, therefore, was to quantify the individual and combined influences of temperature and hypoxia on the metabolic performance of

healthy and diseased wild adult striped bass from Chesapeake Bay. In teleosts, the spleen functions as a reservoir for red blood cells which can be ejected into the circulation to increase blood oxygen carrying capacity during periods of hypoxia and elevated metabolic oxygen demands (Yamamoto et al. 1980, 1983, Yamamoto 1987, Yamamoto & Itazawa 1989, Gallagher & Farrell 1998). Because the spleen is the primary target organ of mycobacteriosis in striped bass, we suggest that the disease compromises splenic function by interfering with the ability of fish to increase their hematocrit in the face of increasing oxygen demands. We thus hypothesized that mycobacteriosis exacerbates the negative consequences of hypoxia and elevated temperatures on metabolic performance, and that the deleterious effects of temperature, hypoxia, and mycobacteriosis are additive. We further contend that skin ulceration interferes with hydromineral balance, thereby increasing the osmoregulatory burden (Kieser et al. 1991, Briand et al. 2012). Hence, we hypothesized that severe dermal mycobacteriosis results in an increase in SMR as well as an increase in the activity of sodium–potassium adenosine triphosphatase ($\text{Na}^+\text{-K}^+\text{-ATPase}$). In order to test these hypotheses, we determined SMR, MMR under normoxia (MMR_N), S_{crit} , and MMR under a fixed level of hypoxia (MMR_H) at normal (20°C) and elevated (28°C) temperatures, in both healthy and diseased fish. We also calculated AS (AS_N , AS_H) and FS (FS_N , FS_H). To assess the ability of the spleen to serve as an erythrocyte reservoir, we determined hematocrit and hemoglobin concentration. Finally, we measured gill and intestinal $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity.

MATERIALS AND METHODS

Animal care and experimental protocols were approved by the College of William & Mary Institutional Animal Care and Use Committee (protocol no. IACUC-2010-11-02-6990-rwbril), and complied with all applicable laws of the USA.

Fish collection and maintenance

Adult striped bass were captured by commercial pound net at the mouth of the Rappahannock River (December 2010 sample) and the Great Wicomico River (Spring 2011 and Fall 2011 samples); both rivers are tributaries to Chesapeake Bay. Fish of varying dermal disease severity (see below) were

collected and transported live in a 1000 l aerated tank to the Virginia Institute of Marine Science (Gloucester Point, VA) where they were tagged with individually numbered T-bar anchor tags. Fish were maintained in a filtered recirculating system of 3 circular 2500 l fiberglass tanks (mean \pm SD; $21.4 \pm 2.3^\circ\text{C}$, 18.1 ± 2.3 psu) supplied with a constant input of filtered and UV-sterilized York River water (dissolved oxygen, $\text{DO} > 80\%$). An artificial lighting regime matched the natural photoperiod cycle (revised monthly). Once acclimated to laboratory conditions, striped bass were fed ad libitum 3 times wk^{-1} with commercially prepared food (Zeigler Brothers), blue crabs *Callinectes sapidus*, or goldfish *Carassius auratus*. Fish were acclimated for a minimum of 3 wk prior to the beginning of the experiments.

Experimental design

We determined MMR_N , SMR, S_{crit} , and MMR_H using an intermittent-flow respirometry system (Schurmann & Steffensen 1997, Horodysky et al. 2011, Roche et al. 2013) at 20 and 28°C . Two independent single respirometry chambers were run simultaneously. Each cylindrical respirometry chamber (24 cm diameter and 75 cm long, 34 l volume, Loligo Systems) was constructed of plexiglass. Oxygen levels in the respirometer were measured with a galvanic oxygen sensor (Cellox 325) and accompanying meter (Oxi 1970i, both from WTW) in one chamber, and with a fluorescence oxygen sensor (Fibox 3 mini sensor, PreSens) in the other. Both oxygen sensors were mounted in a flow-through cell inserted in the water recirculation tubing. The digital outputs from the oxygen meters were recorded with computers running custom-designed software developed in DasyLab 9.02 (National Instruments, www.ni.com). Oxygen saturation (%) values were converted to oxygen content ($\text{mg O}_2 \text{ l}^{-1}$) using standard equations (Richards 1965) within the DasyLab software routines. The respirometers were submerged in separate temperature-controlled water basins bubbled with air (for normoxic conditions) or nitrogen (for hypoxic conditions). The flow of nitrogen was controlled by oxygen meters with galvanic oxygen sensors (OX10000, Loligo Systems) and solenoid valves to maintain fixed levels of oxygen saturation. The water surface of each basin was covered with a plastic sheet to minimize air–water exchange of gases, and an insulated cover was placed on top of the plastic sheet to minimize external stimuli.

The oxygen levels in the respirometers were recorded at either 1 or 5 s intervals (from the fluorescence or galvanic oxygen sensors systems, respectively). The 10 min cycle required for a metabolic rate measurement consisted of a 5 min flush, followed by a 2 min equilibration interval, then 5 min of data recording for calculation of metabolic rate (MO_2). At the conclusion of the data recording interval, the DasyLab software executed a call to an Excel macro routine which calculated the rate of change of O_2 content with time ($\Delta[O_2] t^{-1}$) based on a linear regression of the recorded oxygen levels against elapsed time (t). The Excel macro routine then calculated MO_2 as:

$$MO_2 = (\Delta[O_2] t^{-1}) \times V \times W^{-1} \quad (1)$$

where V = respirometer volume (l) corrected for fish volume and W = weight of the fish (kg). To account for variations in MO_2 due to size differences among the fish, MO_2 estimates were adjusted to a standard body weight of 1 kg using a weight exponent of 0.82 (Edwards et al. 1972):

$$X_s = \left(\frac{1}{W}\right)^{0.82} \times X_m \quad (2)$$

where X_s is the standardized MO_2 value, W is the weight of the fish (kg), and X_m is the measured MO_2 value. Background oxygen consumption ($\Delta[O_2] t^{-1}$) was measured and subtracted from $\Delta[O_2] t^{-1}$ measured when fish were in the respirometer. Fish were fasted for 48 h prior to their introduction into the respirometer. Fasting period and time intervals between sequential determination of MMR_N , SMR , S_{crit} , and MMR_H were established based on preliminary experiments and ensured minimal impact of a given measurement on the others.

Maximum metabolic rate under normoxia

To measure MMR_N , individuals were transferred into a 900 l circular tank (chase tank) and then gently prodded to produce short periods of burst swimming until exhaustion (i.e. until fish showed no response to being handled and removed from the water; Black 1958, Lapointe et al. 2006). Fish were then immediately transferred to the respirometer where MO_2 was measured for a minimum of 48 h, or until the MO_2 displayed minimal to no variation. We defined MMR_N as the highest MO_2 measured during the first 12 h based on results from preliminary experiments.

Standard metabolic rate

SMR was estimated using the average of the 10 lowest routine MO_2 values observed during the last 24 h of the 48 h period during which the fish were in the respirometer (Schurmann & Steffensen 1997, Claireaux et al. 2000, Lapointe et al. 2006). Under these conditions, the rate of oxygen consumption is influenced only by spontaneous activity because fish movements are restricted and external stimuli are minimal (Fry 1971).

Critical oxygen saturation

Following the estimation of SMR , S_{crit} was determined by decreasing the oxygen content in the water basin and hence, respirometer, in a stepwise fashion (Schurmann & Steffensen 1997). MO_2 was monitored at 9 levels of oxygen saturation (90, 80, 70, 60, 50, 40, 30, 20, and 10%), or until the fish was not able to maintain SMR as evidenced by a drastic decrease in MO_2 . At each O_2 level, 2 to 4 MO_2 measurements were performed, each of which lasted approximately 10 min. On average, S_{crit} trials were completed within 4 h. S_{crit} was determined using a 2-segment piecewise linear regression of MO_2 on the DO content of the water, with the first segment forced through the origin (Schurmann & Steffensen 1997, Capossela et al. 2012), using Sigmaplot 11 (Systat Software). After reaching S_{crit} , the oxygen content of the water basin and respirometer was restored to full saturation and the fish were left undisturbed until the next day, when they were transferred into the chase tank and allowed to recover for 24 h.

Maximum metabolic rate under hypoxic conditions

Following the 24 h recovery period, striped bass were subjected to a second chase protocol. Once exhausted, fish were transferred into the respirometer, and the oxygen content was decreased to 3 mg $O_2 l^{-1}$. The targeted O_2 level was reached in 36 ± 9 and 18 ± 5 min (mean \pm 1 SD) at 20°C and 28°C, respectively. The MO_2 was recorded from the time of transfer into the respirometer until MO_2 stabilized or for a maximum of 3 h. The highest MO_2 recorded during that 3 h period corresponded to MMR_H . The oxygen content of the water was restored to full saturation and the fish were left undisturbed for at least 1 h before transfer to the chase tank. On the following day, they were fed, and the day after feeding, the

temperature in the chase tank was increased to 28°C (over a 15 to 18 h period). Fish were then subjected to the protocols described above, at the increased temperature.

Fish were euthanized after completion of the experimental protocol via immersion in a tricaine methanesulfonate (MS-222) solution (400 mg l⁻¹), measured (fork length, cm), weighed (kg), and aseptically necropsied. Spleen, skin (if pigmented foci or ulcers were present), gill, gonad, and liver tissue samples were taken and placed in Z-fix fixative (Anatech) for histological processing. The spleen was divided into 6 pieces of equal size prior to fixation. Gill and intestinal tissue samples were collected for measurement of Na⁺-K⁺-ATPase activity and immediately placed in a -80°C freezer.

Determination of disease status and severity

Fixed tissues were processed by routine methods for paraffin histology (Prophet et al. 1992). Briefly, some tissues (e.g. gill) were decalcified, washed for 3 h in running tap water, dehydrated in ethanol, cleared in xylene, and infiltrated and embedded in paraffin wax. The 6 pieces of spleen were embedded in 2 paraffin blocks to facilitate simultaneous sectioning at 6 levels within the spleen. Tissues were sectioned at 5 µm on a rotary microtome and stained with hematoxylin and eosin (HE). All sections were examined on an Olympus AX-70 light microscope for the presence of granulomas. Quantification of mycobacterial disease severity was based on counting granulomas in the 6 sections of spleen obtained from each of the fish. Splenic lesions comprised mainly of epithelioid cells with or without a necrotic core were considered to be mycobacterial granulomas (Cotran et al. 1999) and were counted in all 6 splenic sections to obtain a total granuloma count. Granulomas containing helminth parasites were excluded from the counts. Digital image files of spleen sections examined for granulomas were then acquired using a scanner. Total area (mm²) of all 6 splenic sections was measured using MetaMorph software (Universal Imaging). A disease severity index (SI) was then calculated as $\log_{10}([\text{total count of granulomas in 6 splenic sections} \times \text{total splenic sectional area}^{-1}] + 1)$ (Latour et al. 2012). Based on the number of granulomas mm⁻² of spleen, fish were then assigned to 1 of 3 mycobacterial visceral disease severity categories: (1) 'Healthy' if $SI \leq 0.1$ ($n = 16$ individuals); (2) 'Moderate' if $0.1 < SI \leq 0.5$ ($n = 6$); and (3) 'Heavy' if $SI > 0.5$ ($n = 10$).

Dermal disease status was determined by visual examination of both sides of the fish. Lesions were classified as either 'ulcer,' for areas in which scales and epidermis were completely eroded, or 'pigmented focus,' a small (<2 mm) pigmented (pale tan to brown) focal lesion. We have examined both of these types of lesions extensively using histological methods and have found that they are consistently associated with granulomatous inflammation and acid-fast bacteria (W. K. Vogelbein et al. unpubl.). Fish with no skin lesions were classified as 'Healthy.' Fish with <50 pigmented foci and no ulcer >2 cm² were considered 'Moderate,' and fish with >50 pigmented foci and/or ulcers >2 cm² were classified as 'Heavy.' We have used this classification system extensively for tag-recapture studies of striped bass in the Rappahannock River, VA (Sadler et al. 2012). Dermal disease status was used to establish the groups for Na⁺-K⁺-ATPase analyses.

Na⁺-K⁺-ATPase activity

Na⁺-K⁺-ATPase activity in gill and intestinal tissue was used to estimate osmoregulatory costs across the 3 dermal disease groups (Gibbs & Somero 1990, Kirschner 1995), as this enzyme plays an important role in active ion transport in fishes (Jampol & Epstein 1970). Frozen gill and intestinal tissue samples were thawed on ice and individually homogenized (using a Tissuemizer Model DT-1810, Tekmar) in 30 volumes of ice-cold buffer (pH 7.3; 0.05 M imidazole, 0.02 M sodium EDTA; 0.3 M sucrose, 0.1% sodium deoxycholate). The homogenate was centrifuged at 16 000 × *g* (30 s), and the supernatant was removed and stored on ice.

The assay procedure used to determine Na⁺-K⁺-ATPase activity was modified from that described by Kültz & Somero (1995). The assay is based on the coupling of ATPase activity to the conversion of NADH to NAD measured spectrophotometrically at 340 nm. The assay buffer contained 30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM MgCl₂·6H₂O, and 0.4 mM KCN, with pH adjusted to 7.4. The reaction mixture was prepared fresh daily using the assay buffer and contained 1 mM Na₂ATP, 0.2 mM Na₂NADH, 2 mM PEP, 3 IU ml⁻¹ pyruvate kinase, and 2 IU ml⁻¹ lactate dehydrogenase. The assay volume included 1 ml of reaction mixture, 100 µl of distilled water (replaced with 100 µl of 11.25 mM ouabain for controls), and 20 µl of supernatant combined in a spectrophotometer cuvette. All assays were run in duplicate and performed at 24°C using a

temperature-controlled spectrophotometer (Shimadzu Bio-Spec 1601, Shimadzu Scientific Instruments). Na⁺-K⁺-ATPase activity was determined as the difference between total ATPase activity and that measured in controls. Total protein content was measured using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Activities were expressed in IU (mmol of substrate converted to product min⁻¹) mg⁻¹ of protein and IU g⁻¹ of wet tissue weight.

Hematocrit and blood hemoglobin concentration

Following the first bout of exhaustive exercise at 20°C, blood samples were taken via direct puncture of the caudal vein to determine hematocrit and hemoglobin concentration. Microhematocrit tubes were filled with whole blood and centrifuged at 10 000 × *g* (10 min). Whole blood hemoglobin concentration (mg ml⁻¹) was measured using Drabkin's reagent (Product code D5941; Sigma-Aldrich).

Calculated parameters and statistical analysis

Fulton's condition factor was calculated as $K = \text{mass (g)} \times 100 \times \text{length}^{-3}$ (cm). AS_N was calculated as the difference between MMR_N and SMR (Fry 1971), whereas FS_N was calculated by dividing MMR_N by SMR (Schurmann & Steffensen 1997). To determine AS and FS under a fixed level of hypoxia (AS_H and FS_H), MMR_N was replaced by MMR_H.

Mean morphological parameters, hematocrit, blood hemoglobin levels, and Na⁺-K⁺-ATPase activities among groups were compared using ANOVAs with a *posteriori* Tukey-Kramer tests in SigmaPlot 11.0 (Systat Software). All tests used the 5% significance level, and the normality and homogeneity of variance criteria were verified using the Shapiro-Wilk and Levene Median tests, respectively.

To evaluate the effect of temperature and disease status on SMR and S_{crit}, and the effect of temperature, disease status, and oxygen level on MMR, AS, and FS, we used a multivariate repeated-measures model implemented with the MIXED procedure in SAS 9.3 (SAS Institute) (Seco et al. 2007, Withers & Cooper 2011). Responses (i.e. SMR, S_{crit}, MMR, AS, or FS) were not independent and were thus modeled using a repeated measures approach (Seco et al. 2007). SMR and S_{crit} were modeled as a function of the disease status (Healthy, Moderate, or Heavy), temperature (20 or 28°C), and the interaction of status and temperature; MMR, AS, and FS were simi-

larly modeled, but this model included the effect of oxygen condition (normoxia or hypoxia), and all 2- and 3-way interactions between the main effects. Individual fish were treated as the subject of these analyses; furthermore, because disease state could not be assigned to each fish in a randomly sequential order, the proper treatment of such data required nesting individual fish within disease state (see e.g. Littell et al. 2006).

We modeled the heterogeneity in responses among fish assigned to the 3 disease states using the group option in the MIXED procedure in SAS and specified the Kenward-Roger method for calculating the degrees of freedom (Kenward & Roger 1997). Several covariance structures were fit to the data using restricted maximum likelihood estimation, and Akaike's Information Criterion adjusted for small sample sizes (AICc) was used to identify the model with the appropriate random structure (Littell et al. 2006). The model for SMR and S_{crit} was best fit using a double-banded unstructured covariance structure, whereas the model for MMR, AS, and FS was best fit using a banded main diagonal unstructured covariance structure; these are highly flexible but complex structures that account for the unequal correlations and variances among observed measures, in contrast to the simple (but unrealistic) compound symmetric structure typically assumed by repeated measures models (Littell et al. 2006). Finally, *a priori* contrasts of least-squares means were obtained using the LSM estimate statement in the MIXED procedure and used to make inferences about the effects of temperature, hypoxia, and disease state on the 5 measured responses.

RESULTS

Mean fish weight did not differ among groups, whereas mean length and condition factor did (Table 1). Striped bass in the Moderate and Heavy groups had a lower condition factor compared to Healthy fish (Table 1). Mean values of post-exercise hematocrit and blood hemoglobin levels did not significantly differ among groups, and gill and intestinal Na⁺-K⁺-ATPase activities were not significantly higher in diseased fish (Table 1).

The mean S_{crit} for the Healthy, Moderate, and Heavy groups at 20°C was 25 ± 2, 24 ± 6, and 30 ± 6%, respectively, and at 28°C, it was 35 ± 2, 34 ± 5, and 43 ± 5%, respectively (Fig. 1a). S_{crit} was higher under elevated water temperature compared to values observed at 20°C, across all groups (Fig. 1a).

Table 1. *Morone saxatilis*. Morphometric characteristics, blood parameters, and sodium-potassium adenosine triphosphatase ($\text{Na}^+\text{-K}^+\text{-ATPase}$) activities of striped bass from 3 groups (Healthy, Moderate, and Heavy) determined based on the visceral disease severity index; for $\text{Na}^+\text{-K}^+\text{-ATPase}$, groups were determined based on the dermal disease status (see 'Materials and methods'). Data are shown as means \pm 95% CI. Different letters indicate a significant difference ($p < 0.05$) between groups based on a simple ANOVA. Absence of letters indicates that all groups were statistically similar. n: number of individuals; M: male; F: female; U: unknown; FL: fork length; K: Fulton's condition factor; Ht: hematocrit; Hb: hemoglobin concentration

Group	n (M/ F/ U)	FL (mm)	Mass (kg)	K	Ht		Hb		$\text{Na}^+\text{-K}^+\text{-ATPase}$ activity		
					n	(%)	n	(mg ml^{-1})	n	Gill	Intestine
Healthy	9/ 4/ 3	464 \pm 17 ^a	1.3 \pm 0.1	1.24 \pm 0.04 ^a	4	33 \pm 3	3	152 \pm 10	4	5.2 \pm 0.9	3.6 \pm 0.3
Moderate	5/ 0/ 1	489 \pm 27 ^{ab}	1.3 \pm 0.3	1.1 \pm 0.1 ^b	5	32 \pm 6	5	144 \pm 13	8	5.3 \pm 0.8	3.8 \pm 1.0
Heavy	9/ 1/ 0	510 \pm 27 ^b	1.4 \pm 0.2	1.00 \pm 0.06 ^b	5	26 \pm 5	6	125 \pm 25	13	4.3 \pm 0.8	2.8 \pm 0.8

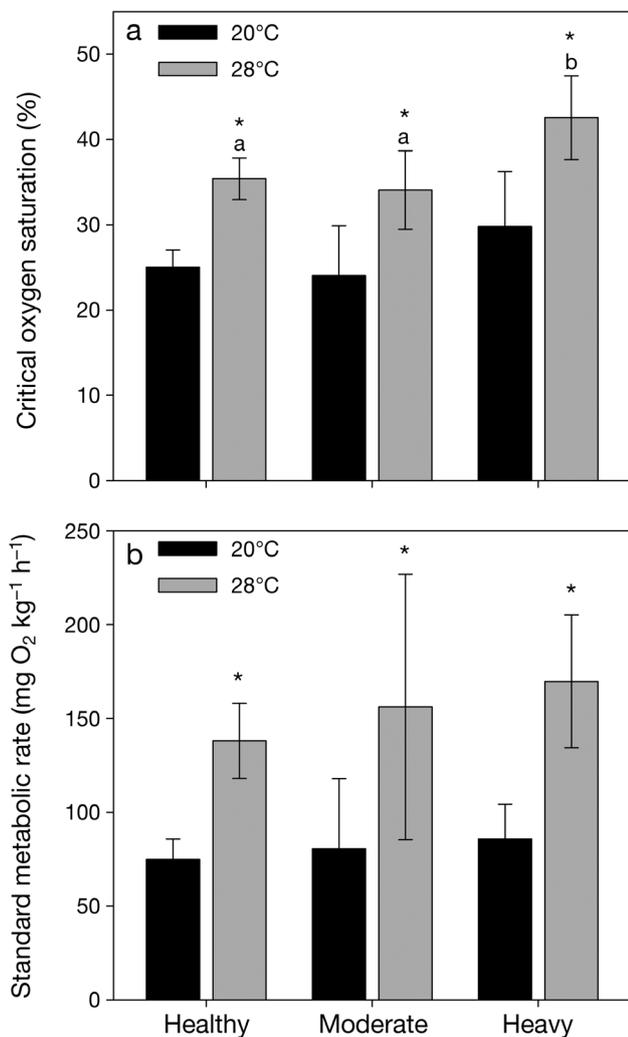


Fig. 1. *Morone saxatilis*. (a) Critical oxygen saturation and (b) standard metabolic rate measured at 20 and 28°C for 3 groups of striped bass classified according to their disease severity. Data are model estimates of the mean \pm 95% CI. Stars (*) indicate a significant effect of temperature on the measured responses for a given group. Different letters indicate a significant difference between groups at a given temperature. Absence of letters indicates no significant differences

However, S_{crit} at either temperature was 1.2-fold higher in Heavy animals compared to fish in the Healthy and Moderate groups, although this difference was significant only at 28°C (Fig. 1a).

The mean SMR for the Healthy, Moderate, and Heavy groups at 20°C was 75 ± 11 , 80 ± 37 , and $86 \pm 18 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively, and at 28°C, it was 138 ± 20 , 156 ± 71 , and $170 \pm 35 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively (Fig. 1b). As expected, SMR significantly increased with increasing temperature (Fig. 1b), but we measured no significant effect of mycobacteriosis on SMR at either 20 or 28°C (Fig. 1b).

The mean MMR_N for the Healthy, Moderate, and Heavy disease groups at 20°C was 284 ± 40 , 336 ± 89 , and $283 \pm 66 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively, and at 28°C, it was 384 ± 69 , 413 ± 153 , and $362 \pm 105 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively (Fig. 2a). The mean MMR_H for the Healthy, Moderate, and Heavy groups at 20°C was 198 ± 42 , 198 ± 67 , and $225 \pm 101 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively, and at 28°C, it was 278 ± 55 , 265 ± 82 , and $228 \pm 64 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively (Fig. 2a). Time to reach MMR_N was $3.2 \pm 3.2 \text{ h}$, whereas MMR_H was reached in $1.6 \pm 1.0 \text{ h}$. Except for the Heavy group at 20°C and the Moderate group at 28°C, MMR_H was significantly lower than MMR_N , regardless of temperature (Fig. 2a). Mean MMR_N and MMR_H were higher after transfer to 28°C, but this increase was significant only for the Healthy group (Fig. 2a). Surprisingly, MMR_N and MMR_H did not differ between disease groups, under any set of conditions.

AS_H was significantly lower than AS_N at both temperatures, except for the Heavy group at 20°C (Fig. 2b). The mean AS_N for the Healthy, Moderate, and Heavy groups at 20°C was 210 ± 34 , 255 ± 69 , and $197 \pm 50 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively, and at 28°C, it was 246 ± 55 , 257 ± 115 , and $198 \pm 75 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively (Fig. 2b). The mean AS_H for the Healthy, Moderate, and Heavy groups at 20°C was 123 ± 33 , 118 ± 31 , and $140 \pm 87 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, respectively, and at 28°C, it was 140 ± 43 , 109 ± 44 ,

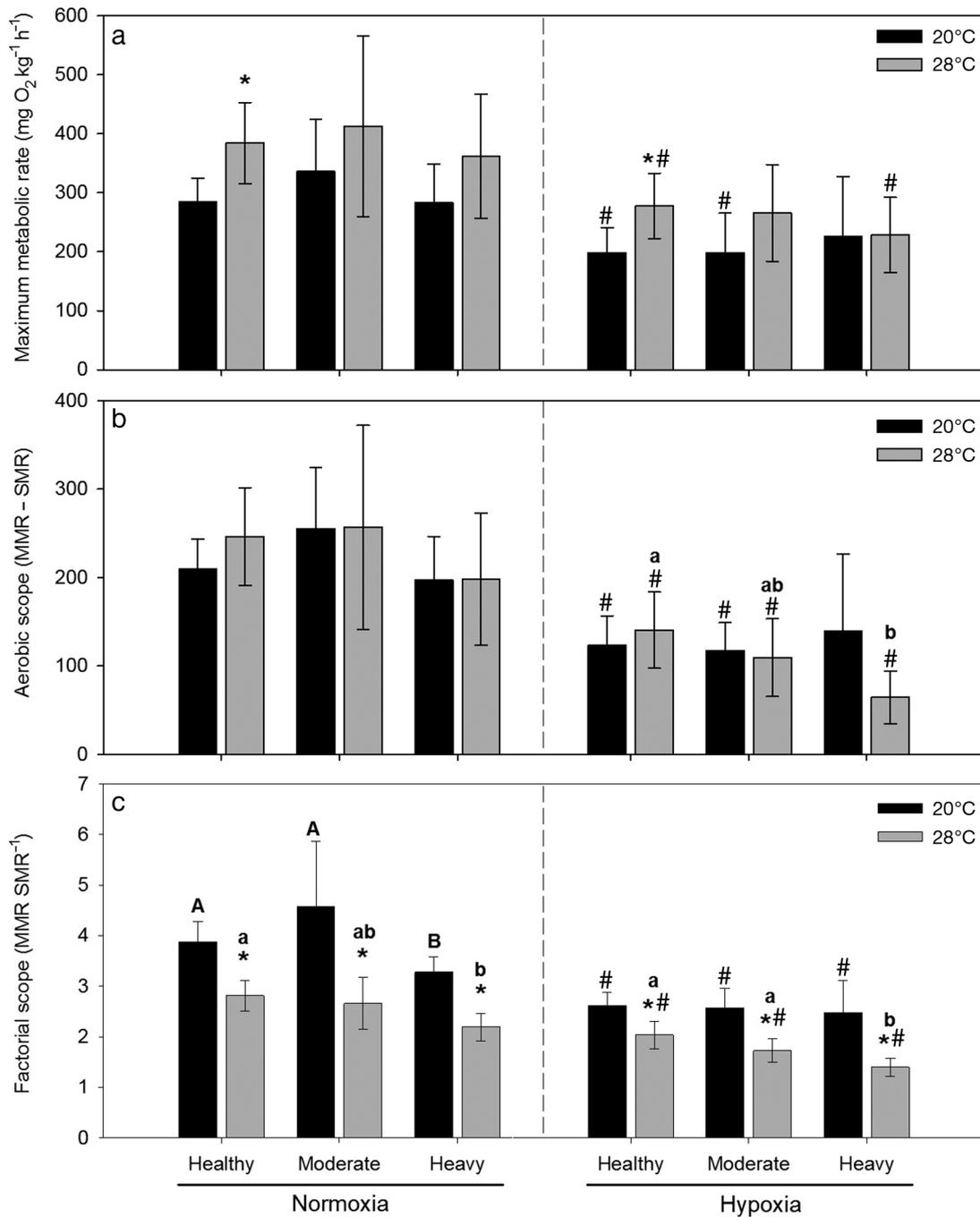


Fig. 2. *Morone saxatilis*. (a) Maximum metabolic rate (MMR), (b) aerobic scope, and (c) factorial scope measured at 20 and 28°C, under normoxic and hypoxic conditions, for 3 groups of striped bass classified according to their disease severity. Data are shown as model estimates of the mean \pm 95% CI. Stars indicate a significant effect of temperature on the measured responses for a given group and oxygen level. Different letters indicate a significant difference between groups at a given temperature. Absence of letters indicates no significant differences. The hash symbols (#) indicate a significant effect of oxygen level on the measured responses for a given group and at a given temperature. SMR: standard metabolic rate

and 64 ± 30 mg O₂ kg⁻¹ h⁻¹, respectively (Fig. 2b). We measured no significant influence of temperature on either AS_N or AS_H. Interestingly, AS_H of Heavy fish under elevated temperature was significantly lower

than AS_H of the Healthy group under the same conditions (Fig. 2b).

FS_H was significantly lower than FS_N at both temperatures (Fig. 2c). In addition, both FS_N and FS_H

were reduced at elevated temperature (Fig. 2c). The mean FS_N for Healthy, Moderate, and Heavy groups at 20°C was 3.9 ± 0.4 , 4.8 ± 1.3 , and 3.3 ± 0.3 mg O₂ kg⁻¹ h⁻¹, respectively, and at 28°C, it was 2.8 ± 0.3 , 2.7 ± 0.5 , and 2.2 ± 0.3 mg O₂ kg⁻¹ h⁻¹, respectively (Fig. 2c). The mean FS_H for the Healthy, Moderate, and Heavy groups at 20°C was 2.6 ± 0.3 , 2.6 ± 0.4 , and 2.5 ± 0.6 mg O₂ kg⁻¹ h⁻¹, respectively, and at 28°C, it was 2.0 ± 0.3 , 1.7 ± 0.2 , and 1.4 ± 0.2 mg O₂ kg⁻¹ h⁻¹, respectively (Fig. 2c). Striped bass in the Heavy group had a significantly lower FS_N than fish in the Healthy group, regardless of temperature. Finally, FS_H of Heavy animals was significantly lower than that of Healthy striped bass at elevated temperature. Assuming that the value obtained for FS_N within the Healthy group in normoxia at 20°C represents the value for healthy adult striped bass under preferred temperature and oxygen conditions, hypoxia, temperature, and severe mycobacteriosis occurring alone reduced the FS by 1.5-, 1.4-, and 1.2-fold, respectively. When the stressors of hypoxia and elevated temperature were combined, however, FS was reduced by 2.8-fold (Fig. 2c).

DISCUSSION

To the best of our knowledge, this is the first study to report the interactive effects of temperature, oxygen levels, and infectious disease in finfishes. Our results indicate that when 3 stressors are present together (elevated water temperature, hypoxia, and mycobacteriosis), they act synergistically to reduce AS more than any single stressor acting alone (Fig. 2b). The AS was not influenced by temperature alone but was affected by hypoxia ($AS_N > AS_H$), except for the Heavy group at 20°C. SMR in striped bass did, however, increase with increasing water temperature. This has been shown to be true for other species, as demonstrated in numerous studies (e.g. Fry 1947, Beamish 1964, Kruger & Brocksen 1978, Schurmann & Steffensen 1997, Claireaux & Lagardère 1999, Claireaux et al. 2000, Sylvestre et al. 2007). Disease alone did not influence AS_N . However, AS_H at 28°C was significantly lower in Heavy fish compared to the Healthy group. Under the influence of all 3 stressors (28°C, hypoxia, severe disease), the AS was reduced to approximately one-third of that of healthy fish held at 20°C under normoxia.

AS in fishes has long been hypothesized to increase with temperature until an optimal temperature is reached, and then decline with further increases in temperature, taking the form of a bell-shaped curve

(Claireaux & Lagardère 1999, Claireaux et al. 2000, Pörtner & Farrell 2008, Farrell 2009, Pörtner 2010). Alternatively, Clark et al. (2013) hypothesized that AS continues to increase beyond the optimal temperature, until close to the upper critical temperature. In both instances, the temperature dependence of AS is driven by the failure of maximum metabolic rate to continue increasing with temperature, while SMR exponentially increases until temperature approaches a lethal level (Fry 1947, Fry & Hart 1948, Farrell 2009). Thus, following the first hypothesis, the absence of a significant difference between AS measured at 20 and 28°C in adult striped bass may indicate that these 2 temperatures are respectively below and above the preferred or optimal temperature for adult striped bass. However, following the second hypothesis, our results for AS may suggest that 28°C is close to their upper critical temperature, and that mycobacteriosis narrows their thermal window. Alternatively, the absence of a significant influence of temperature on AS might be caused by the high inter-individual variability observed in this study. In contrast to temperature, hypoxia significantly reduced AS. For striped bass in the Healthy group, AS_H was 1.70- and 1.75-fold lower than AS_N measured at 20 and 28°C, respectively. This reduction in AS under hypoxia was similar to that reported for European sea bass (Claireaux & Lagardère 1999), common sole (Lefrançois & Claireaux 2003), and Atlantic cod (Claireaux et al. 2000, Dutil et al. 2007).

Temperature, hypoxia, and mycobacteriosis had a synergistic effect on FS, similar to the observed effects of these factors on AS of striped bass (Fig. 2c). FS appeared to be a more sensitive indicator of physiological impairment because temperature alone elicited a significant effect. Both FS_N and FS_H were significantly lower at 28°C than at 20°C, regardless of disease severity. Additionally, FS_H was significantly lower than FS_N , regardless of water temperature and/or disease severity. Disease state also significantly influenced FS. FS_N was significantly lower in fish from the Heavy group than Healthy and Moderate animals, except for Moderate at 28°C. Likewise, FS_H was significantly lower in fish from the Heavy group compared to the Healthy and Moderate groups, but in this case only at 28°C. Under the influence of all 3 stressors (28°C, hypoxia, severe disease), like AS, FS was reduced to approximately one-third of that of Healthy fish held at 20°C under normoxia.

Because of the large differences in SMR and MMR among fish species (e.g. Brill & Bushnell 2001, Korsmeyer & Dewar 2001), we argue that FS is a more informative measure for inter-species comparisons, especially when considering the effects of tem-

perature. In line with our results, a reduction in FS with increasing water temperature was previously reported for Atlantic cod measured at an acclimation temperature of 7°C and following an acute transfer to 11°C (Sylvestre et al. 2007). However, in Atlantic cod acclimated to different water temperatures (acclimation period ranged from 3 wk to several months), FS did not vary with temperature (Schurmann & Stefensen 1997, Claireaux et al. 2000). In contrast, in European sea bass acclimated for 10 to 15 d to different temperatures, FS increased with increasing acclimation temperature (Claireaux & Lagardère 1999). Similar to our findings, a reduction in FS under hypoxia has been reported for European sea bass (Claireaux & Lagardère 1999) and Atlantic cod (Claireaux et al. 2000). To our knowledge, this is the first report of a significant reduction in FS associated with an infectious disease.

The teleost spleen is a blood-filtering organ that functions to trap and degrade foreign matter and effete erythrocytes. Additionally it is a secondary lymphoid organ involved in hematopoiesis and initiation of specific immunity (Fänge & Nilsson 1985, Takashima & Hibiya 1995, Genten et al. 2009). It also serves as an erythrocyte reservoir that can, by a process termed splenic contraction, release large numbers of erythrocytes into circulation during times of elevated oxygen demand, including that associated with exposure to hypoxia (Yamamoto et al. 1980, 1983, 1985, Wells & Weber 1990, Pearson & Stevens 1991, Lecklin & Nikinmaa 1998, Lai et al. 2006).

Mycobacteriosis damages and in some instances completely destroys significant portions of the splenic parenchyma, which is largely replaced by granulomatous inflammatory tissues (Fig. 3), likely compromising splenic function of striped bass. This may effectively reduce or completely eliminate the spleen's ability to serve as an erythrocyte reservoir. We therefore expected hematocrit and blood hemoglobin concentration in diseased animals to be significantly below that of non-diseased fish during periods of high oxygen demand (i.e. after exercise). Although post-exercise hematocrit and blood hemoglobin concentration tended to be lower in Heavy animals compared to the Healthy group (Table 1), we found no significant differences in these parameters among disease states. This may explain why we likewise observed no effect of disease state alone on either MMR_N or MMR_H . Thus, in adult striped bass, compromised splenic function associated with mycobacteriosis may not translate into a significantly reduced blood oxygen carrying capacity, although this was not directly tested in the present study. Our

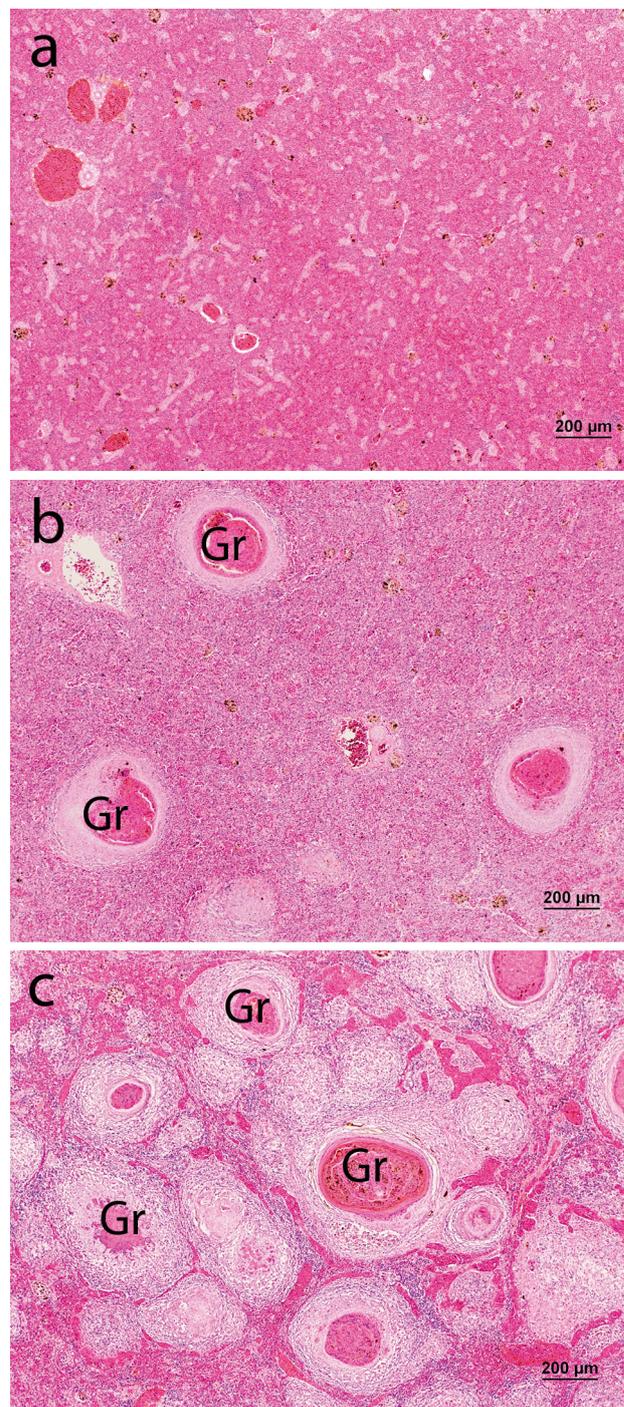


Fig. 3. *Morone saxatilis*. Splenic mycobacteriosis in Chesapeake Bay striped bass. (a) Healthy spleen. (b) Moderately affected spleen (Gr: mycobacterial granulomas). (c) Severe splenic mycobacteriosis (Gr: mycobacterial granulomas)

results suggest the presence of compensatory mechanisms that enable diseased fish to maintain near optimal circulating erythrocyte concentrations (hematocrit) and hemoglobin values, thereby maintain-

ing blood oxygen carrying capacity in the face of compromised splenic function. Given the chronic nature of mycobacteriosis, with severe disease developing slowly over extended time periods (months to years), it is probable that these blood parameters are maintained by elevated levels of hematopoiesis within the head kidney.

The skin of fishes presents an effective protective barrier to the aqueous environment. Given that mycobacteriosis causes severe skin ulceration in striped bass, we hypothesized that osmoregulatory burden would increase with increasing dermal disease severity. This should manifest as an increase in SMR and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in the gill and intestine of Heavy fish (in this case based on dermal rather than splenic disease severity). Surprisingly, dermal disease severity had no effect on any of these parameters (Fig. 1b, Table 1). Histological examination of the dermal lesions provides a possible explanation. Among tissue samples displaying the range of dermal disease, most but not all lesions had become re-epithelialized (Fig. 4). This was true for both small incipient pigmented foci (Fig. 4a) and many expansive ulcers (Fig. 4b). Most of these skin lesions exhibited a prominent re-growth of the epidermis, although in some large ulcers this was not the case and the entire epidermal layer and scales had been lost (Fig. 4c), exposing the underlying dermis and the prominent granulomatous inflammatory lesions to the environment. It is plausible that in many instances, regrowth of epidermis minimizes any increase in osmoregulatory burden associated with ulceration and may explain why we did not see evidence of increased SMR and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in gill and intestine of these fish.

Measurements of S_{crit} suggest that severe mycobacteriosis exacerbates the influence of increased water temperature on hypoxia tolerance. Although S_{crit} was not different among the 3 disease groups at 20°C, it was significantly higher in Heavy fish at 28°C. In addition, the relative difference between the Heavy group and the other 2 groups was the same (1.2 fold) regardless of water temperature, suggesting that severe mycobacteriosis alone reduces hypoxia tolerance in striped bass.

Hypoxia tolerance in fishes, as indicated by S_{crit} , varies considerably. Adult striped bass from Chesapeake Bay have a relatively high S_{crit} value in comparison with other species held at their preferred temperature (Table 2), suggesting that striped bass are more vulnerable to hypoxia. Furthermore, elevated water temperature in this study increased the S_{crit} of striped bass, impacting their ability to cope

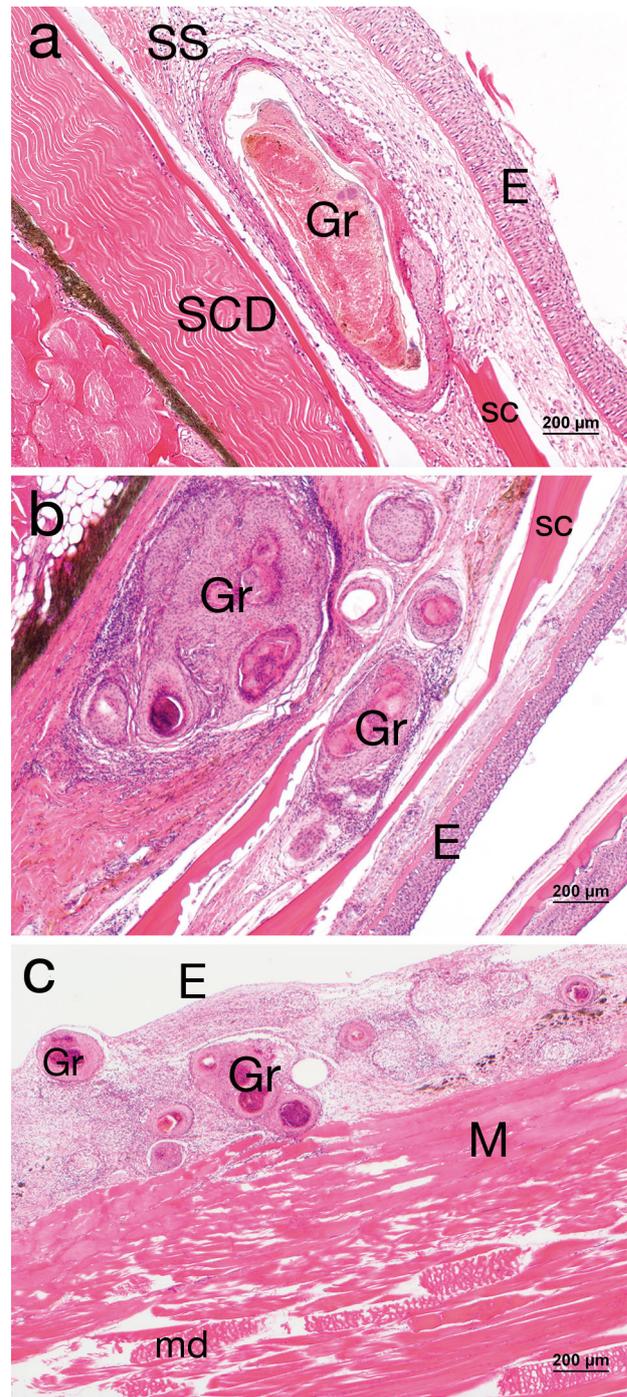


Fig. 4. *Morone saxatilis*. Dermal mycobacteriosis in Chesapeake Bay striped bass. (a) Re-epithelialization (E) of small pigmented focus. Gr: granuloma; SS: stratum spongiosum of dermis; SCD: stratum compactum of dermis; sc: scale. (b) Re-epithelialization (E) of large ulcer. Gr: granuloma; sc: scale. (c) Large ulcer exhibiting widespread loss of epidermis and scales (E). Gr: granuloma; M: muscle; md: myodegeneration

Table 2. Critical oxygen saturation (S_{crit}) and critical oxygen content (C_{crit}) of striped bass *Morone saxatilis* acclimated to 20°C and following an acute increase to 28°C, in comparison with values previously measured for other fish species. Data are shown as means \pm 95% CI. n: number of individuals. Species are ranked based on their vulnerability to hypoxia at a normal temperature, from the less tolerant to the most tolerant (descending S_{crit})

Species	Temperature (°C)	n	S_{crit} (%)	C_{crit} (mg l ⁻¹)	Source
<i>Pangasianodon hypophthalmus</i>	27	13	37 \pm 6	3.0	Lefevre et al. (2011)
<i>Cyprinodon variegatus</i>	20	8–18	36.5–39.6	3.3–2.8	Haney & Nordlie (1997)
<i>Astronotus ocellatus</i>	28	10	30 \pm 4	2.3	Scott et al. (2008)
<i>Paralichthys dentatus</i>	22		27.2 \pm 1.7	2.0 \pm 0.1	Capossela et al. (2012)
	30		39.0 \pm 1.9	2.4 \pm 0.1	Capossela et al. (2012)
<i>Morone saxatilis</i>	20	16	25.0 \pm 2.0	2.0 \pm 0.1	This study
	28	16	35.4 \pm 2.4	2.5 \pm 0.2	This study
<i>Etheostoma</i> spp. (6 species)	10	47	34.5	3.9	Ultsch et al. (1978)
	20	61	23.1	2.1	Ultsch et al. (1978)
<i>Dicentrarchus labrax</i>	10–25	64–80	18–29	~2	Claireaux & Lagardère (1999)
<i>Scophthalmus maximus</i>	17	7	19	1.5	Maxime et al. (2000)
<i>Oreochromis niloticus</i>	22	7	18.0 \pm 1.5	1.6	Speers-Roesch et al. (2010)
<i>Gadus morhua</i>	2–10	27	15–20	1.4–2.2	Claireaux et al. (2000)
	5	10	16.5 \pm 2.4	1.7	Schurmann & Steffensen (1997)
	10	10	23.2 \pm 5.3	2.1	Schurmann & Steffensen (1997)
	15	11	30.3 \pm 5.9	2.5	Schurmann & Steffensen (1997)
<i>Silurus meridionalis</i> Chen	25	10	16.4	1.31	Zhang et al. (2010)
<i>Oncorhynchus mykiss</i>	10	–	13.5	1.2	Svendsen et al. (2012)
<i>Oreochromis niloticus</i>	20	5	12 \pm 2	1.1	Fernandes & Rantin (1989)
	25	6	12 \pm 3	1.0	Fernandes & Rantin (1989)
	30	5	19 \pm 3	1.4	Fernandes & Rantin (1989)
	35	5	19.4 \pm 0.4	1.4	Fernandes & Rantin (1989)
<i>Carassius auratus</i>	5–35	10–20	26–16	3.3–1.1	Fry & Hart (1948)
	20	20	11.5	1.0	Fry & Hart (1948)

with oxygen deprivation. Critical oxygen saturation was 1.4-fold higher at 28°C compared to 20°C, indicating that a fish's capacity to sustain its oxygen consumption rate under hypoxic conditions is significantly reduced at elevated water temperatures. This was expected, as similar results have been observed in other fishes (e.g. Fry & Hart 1948, Fernandes & Rantin 1989, Schurmann & Steffensen 1997).

The absence of a significant increase in MMR_N and MMR_H with increasing temperature in Moderate and Heavy striped bass suggests that mycobacteriosis shifted the relationship between maximum metabolic rate and temperature compared to Healthy fish. This was particularly evident for MMR_H measured in the Heavy group. Therefore, mycobacteriosis appears to reduce maximum rates of oxygen delivery by the cardio-respiratory system at elevated water temperatures under hypoxic conditions. In exercising goldfish *Carassius auratus*, maximum metabolic rate ceased to increase with temperature above an optimal temperature (Fry 1947, Fry & Hart 1948). Moreover, modeling the relationship between active metabolic rate (AMR), temperature, and oxygen content in European sea bass showed that AMR increases

with temperature to a maximum and then decreases as temperature further increases, whereas at a given temperature, AMR decreases with decreasing ambient water oxygen content (Claireaux & Lagardère 1999). As for the effect of hypoxia, MMR_H was generally significantly lower than MMR_N regardless of disease state. This is consistent with other studies where oxygen consumption during activity, or following exhaustive exercise, was found to decrease in concert with reductions in ambient oxygen (Fry 1947, Basu 1959, Claireaux & Lagardère 1999, Claireaux et al. 2000, Dutil et al. 2007).

In conclusion, given that the scope for activity (both AS and FS) of diseased striped bass in warm hypoxic waters is greatly compromised, diseased fish under similar conditions are likely to fatigue more rapidly, be restricted in their ability to elude predators or secure prey, have lower growth rates, and exhibit reduced reproductive performance and immune function. Because of the high prevalence and severity of mycobacteriosis in adult striped bass from Chesapeake Bay, and our demonstration of the disease's impact on their physiological capacities (especially scope for activity under elevated temperature

and hypoxia), any attempts to quantify the impacts of environmental factors on striped bass must include healthy and diseased fish. Our results therefore suggest that the increasing occurrence and severity of summer hypoxic events in Chesapeake Bay may have negative impacts on striped bass populations. Indeed, there are documented increases in natural mortality rates associated with mycobacteriosis (Jiang et al. 2007, Gauthier et al. 2008), and a recent modeling analysis of adult striped bass from Chesapeake Bay showed that growth in disease-positive fish was compromised (Latour et al. 2012). Hence, it is likely that the differences in condition factor we measured among the 3 groups at the end of the trials reflected the differences among individuals before they were captured. We specifically argue that anthropogenic impacts on environmental quality, possible changes in forage abundance, the recent decline in striped bass condition, and the emergence of mycobacteriosis are completely interlinked processes. Therefore, we contend that summer temperature–oxygen conditions increase the prevalence and severity of mycobacteriosis, and that in turn, the disease reduces the tolerance of striped bass to elevated water temperature and hypoxia. This situation creates multiple positive feedback loops, possibly resulting in the observed high prevalence of disease, reduced growth rates, and elevated rates of natural mortality.

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