

White bass *Morone chrysops* is less susceptible than its hybrid to experimental infection with *Flavobacterium columnare*

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ABSTRACT: Hybrid striped bass (HSB) and white bass (WB) were evaluated for their susceptibility to *Flavobacterium columnare*, the causative agent of columnaris disease, in 3 fundamental studies. In the first experiment, we determined whether columnaris disease could be developed by experimental challenge in HSB. This challenge consisted of 3 levels of *F. columnare* (10, 30, and 60 ml volumes) determined to be 2.25×10^7 , 6.75×10^7 , and 1.35×10^8 CFU ml⁻¹, respectively. Each treatment group exhibited significantly different survival rates: 0, 3.3, and 13.3% in the 60, 30, and 10 ml groups, respectively. In Expt 2, using the 30 ml dose, both HSB and WB had a 0% survival rate, with WB taking significantly longer to reach 100% mortality. In Expt 3, using the 10 ml dose, no HSB survived, whereas 33% of WB survived ($p < 0.0001$). Compared to controls, HSB treated with 10 ml showed extensive gill damage at 24 h, which could have contributed to the higher mortality observed in HSB; in contrast, WB gills showed noticeably less damage. From these series of experiments, it is clear that HSB are more sensitive to *F. columnare*, having lower survival and more extensive histological damage compared to WB following challenge.

KEY WORDS: Columnaris disease · Hybrid striped bass · Dose response · Differential survival

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INTRODUCTION

Columnaris disease caused by *Flavobacterium columnare* is an important disease that adversely affects fish species worldwide, including those of the genus *Morone*. *F. columnare* is a Gram-negative rod that is ubiquitous in the freshwater aquatic environment, and is widely accepted as being present in most, if not all freshwater aquaculture settings, where it typically acts as a secondary pathogen, causing significant losses in the presence of predisposing stressors. However, *F. columnare* can also act as a primary pathogen and initiate disease in the absence of predisposing stressors (Plumb 1999).

Hybrid striped bass (HSB), a cross between white bass (WB) *Morone chrysops* female × striped bass

M. saxatilis male, is similar in appearance to both of the parental species. Due to heterosis, it possesses traits such as aggressive feeding behavior and tolerance to a wide range of environmental conditions that make it highly suitable for aquaculture (Bishop 1968, Myers & Kohler 2000), more so than either of its parental species (Bosworth et al. 1997). To develop effective selective breeding programs, it is essential to have baseline genetic information for commercially important traits such as growth and disease resistance.

A large portion of casualties during a production cycle are attributed to disease caused by an array of viral, bacterial, and parasitic pathogens of known and unknown etiologies. Despite the importance of columnaris disease, limited research has been con-

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ducted on its effects in *Morone* spp. The sensitivity of this hybrid has not been defined, nor has a method or model been developed to study the disease in bass. As a result, little is known regarding the basic pathobiology of infected HSB, which has led to a lack of information on potential treatments and their effectiveness, while markers of genetic resistance and selection have also not been identified.

For these reasons, we initiated a series of fundamental experiments to investigate the susceptibility to experimental *Flavobacterium columnare* challenges of HSB versus the parental WB, the effects of various doses on survival, and the histological consequences of *F. columnare* to each species.

MATERIALS AND METHODS

HSB and WB were spawned at the Harry K. Dupree Stuttgart National Aquaculture Research Center (HKD-SNARC), Stuttgart, Arkansas (USA), in April 2012. After approximately 120 d, fish were placed in 400 l tanks and acclimated for 2 wk prior to the initiation of experiments. Fish were then moved to 16 tanks (18 l) containing 10 l of water at a density of 10 fish tank⁻¹. Aeration was provided to each tank with submerged air stones. The tanks received filtered well water at a rate of approximately 4 water exchanges per day or about 30 ml min⁻¹ from an 'ultra-low-flow system' (Mitchell & Farmer 2010). Water temperatures ranged from 26.2 to 27.5°C. Temperature and dissolved oxygen were measured daily with a YSI Pro20. The mean dissolved oxygen concentration was 5.6 ± 1.2 mg l⁻¹. Total ammonia nitrogen (TAN) concentrations were determined in each tank (mean 1.2 ± 0.4 mg l⁻¹; range 0.6–1.8 mg l⁻¹) with a Hach DR/4000V spectrophotometer using the Nessler Method 8038. An Orion 720A pH meter (Fisher Scientific) was used to measure pH (7.5 to 8.2) at the beginning of the study. Standard titration methods (APHA et al. 2005) were used to measure total alkalinity (216 mg l⁻¹) and total hardness (114 mg l⁻¹).

Experimental design

For each experiment, treatments each consisted of 3 replicates for survival data, with an additional tank for each treatment reserved for tissue collection/sampling (i.e. no fish were removed from survival tanks for sampling). Each study had a total duration of 10 d. Expt 1 (Dose Response, HSB mean

weight ± SD = 14.6 ± 3.1 g;) consisted of 4 treatments: (1) control (0 ml) without bacterial challenge, (2) Dose 1: 10 ml bacterial suspension added to each replicate tank, (3) Dose 2: 30 ml bacterial suspension added to each replicate tank, and (4) Dose 3: 60 ml bacterial suspension added to each replicate tank. Expt 2 (30 ml, WB versus HSB) consisted of 4 treatments (WB 15.4 ± 2.5 g; HSB 16.8 ± 2.0 g): (1) WB control (0 ml) without bacterial challenge, (2) HSB control (0 ml) without bacterial challenge, (3) WB with 30 ml bacterial suspension added to each replicate treatment tank, and (4) HSB with 30 ml bacterial suspension added to each replicate treatment tank. Expt 3 (10 ml, WB versus HSB) consisted of 4 treatments (WB 17.3 ± 3.3 g; HSB 19.6 ± 4.3 g): (1) WB control (0 ml) without bacterial challenge, (2) HSB control (0 ml) without bacterial challenge, (3) WB with 10 ml bacterial suspension added to each replicate treatment tank, and (4) HSB with 10 ml bacterial suspension added to each replicate treatment tank. The experiments were initiated in sequence, with Expt 1 completed first, followed by Expts 2 and 3.

Flavobacterium columnare challenge

Fish were experimentally challenged with *F. columnare* isolate LSU-066-04 (genomovar II) obtained from Dr. J. Hawke (Louisiana State University), an isolate originating from an outbreak in largemouth bass which has previously been used to initiate columnaris disease in channel catfish *Ictalurus punctatus* in our challenge system (Beck et al. 2014). The isolate was retrieved from storage at -80°C and streaked on Ordal's media (Anacker & Ordal 1959); after 48 h, the isolate was dislodged from the agar using a sterile cotton swab and inoculated into 5 ml of *F. columnare* growth medium (FCGM; Farmer 2004). This suspension was incubated at 28°C for 48 h and then used to inoculate 1 l of FCGM. The inoculated 1 l broth was incubated overnight at 28°C in an orbital shaker incubator set at 200 rpm. When the bacterial growth reached an absorbance 0.70 at 550 nm (approximately 2.0 × 10¹⁰ bacteria ml⁻¹), the flask was removed and placed on a stir plate at room temperature. A 10 ml sample was removed from the broth for serial dilution and CFU data. The remaining inoculated broth was added to tanks receiving bacterial challenge according to the prescribed treatment dose (i.e. 10, 30, or 60 ml); the control tanks received 60 ml of sterile media.

Fish were observed twice daily for clinical signs associated with columnaris disease. Individuals unable to maintain neutral buoyancy were considered moribund and were removed for sampling. Fish were not fed during the exposure or during the first day after challenge, but were offered food on Day 2 and throughout the rest of the study. Animal care and experimental protocols were approved by the HKD-SNARC Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

Bacteriology and histology

Dead and moribund fish were removed from the tanks daily, and samples were taken from the caudal fin, gills, and liver. The samples were cultured on selective cytophaga agar containing $5 \mu\text{g ml}^{-1}$ neomycin sulfate and $200 \text{ units ml}^{-1}$ polymyxin B. This selective medium has previously been shown to be effective in inhibiting all bacterial species tested except *Flavobacterium* spp. and *Streptococcus* spp. (Hawke & Thune 1992). If present, a maximum of 3 moribund or dead fish were sampled from each tank per day. Cultures were incubated at 28°C for 48 h and then scored as being positive or negative for growth based on colony morphology (i.e. flat, yellow colonies with a rhizoid morphology).

For each experiment, fish were euthanized 24 h after bacterial challenge, and gill samples were collected and immediately fixed in 10% neutral buffered formalin solution. After 24 to 48 h of fixation, gills were briefly rinsed with water, transferred to 70% isopropyl alcohol, and stored until routine paraffin embedding with a Leica TP1020 tissue processor (Straus et al. 2012). Tissues were sectioned with a Leica RM2135 microtome to $5\text{--}6 \mu\text{m}$, mounted on slides, and stained with hematoxylin and eosin. Gills were evaluated for pathological changes as compared to unexposed fish, and comparative pathology between HSB and WB was assessed.

Statistical analysis

Survival data were analyzed with SigmaPlot 11 using Kaplan-Meier log rank survival analysis, and all pairwise multiple comparisons used the Holm-Sidak method with Type III adjusted p-values. Each experiment was analyzed separately, with tank as the fixed effect and replicate as the random effect. Treatment effects were considered significant at $p \leq 0.05$.

RESULTS

Expt 1: Dose response of HSB to *Flavobacterium columnare* challenge

Percent survival was calculated by dividing the number of mortalities by the total number of fish in the tank. In addition, the rate of mortality as a function of time was calculated to generate Kaplan-Meier survival curves. The bacterial challenge concentrations (in CFU ml^{-1}) were determined post hoc to be as follows: (1) Dose 1 (10 ml): 2.25×10^7 ; (2) Dose 2 (30 ml): 6.75×10^7 ; and (3) Dose 3 (60 ml): 1.35×10^8 . At 24 h after bacterial challenge, no mortalities had occurred. Mortalities began in the 60 ml challenge-group on Day 2 and in the 30 and 10 ml groups on Day 3. In the control group (0 ml), survival was not 100%, as 1 fish died on Day 2 and 2 died on Day 3, most likely due to socio-behavioral effects (i.e. chasing, bullying); we found no obvious signs of disease, and the final survival rate of control fish was 83.3%. All mortalities ceased on Day 6, and fish were observed for an additional 4 d. Kaplan-Meier survival curves depicting the probability of survival as functions of time are represented in Fig. 1. The probability of survival was analyzed with a log rank survival curve, and we determined the survival of each of the treatment groups to be significantly different

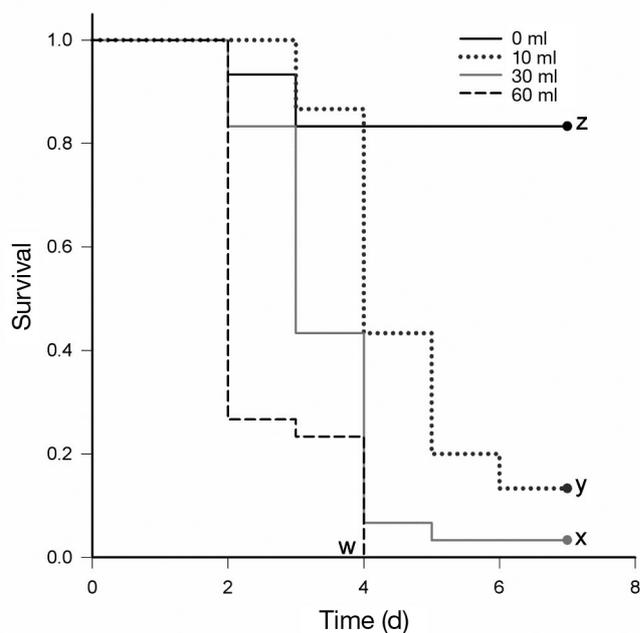


Fig. 1. *Morone chrysops* × *M. saxatilis*. Kaplan-Meier log rank survival analysis of hybrid striped bass challenged with varying doses of *Flavobacterium columnare* in an ultra-low-flow challenge system. Survival curves not sharing the same letter indicate a significant difference ($p \leq 0.05$)

from the unchallenged (0 ml) group ($p < 0.05$); moreover, survival differed between the treatment groups ($p < 0.05$).

Expt 2: Survival of HSB and WB after challenge with 30 ml (Dose 2) of *Flavobacterium columnare*

Mortalities began in the HSB challenge-group on the morning after the challenge (Day 1) and in the WB challenge-group on the afternoon of Day 1 (i.e. Day 1.5). Survival of the non-treated controls was 100%. Both the HSB and the WB challenge-groups showed 0% survival, with all fish succumbing to the disease by Day 3. However, there was a significant difference in the amount of time it took the 2 groups to reach 100% mortality ($p = 0.00851$). All challenged HSB had expired by Day 2, whereas 5% of the WB survived to Day 3. Kaplan-Meier survival curves depicting the probability of survival as functions of time are represented in Fig. 2a. The probability of survival was analyzed with a log rank survival curve, and we determined the survival of each of the treatment groups to be significantly different ($p < 0.05$) from the controls.

Expt 3: Survival of HSB and WB challenged with 10 ml (Dose 1) of *Flavobacterium columnare*

Mortalities began in the HSB challenge-group on Day 2 and in the WB challenge-group on Day 3. Survival of the non-treated controls was 100%. The HSB challenge-group had 0% survival, with all fish succumbing to the disease by Day 4. In contrast, WB exhibited a significantly higher survival rate of 33% ($p < 0.0001$). Kaplan-Meier survival curves depicting the probability of survival as functions of time are represented in Fig. 2b. The probability of survival was analyzed with a log rank survival curve, and we determined the survival of each of the treatment groups to be significantly different ($p < 0.05$) from the controls.

Clinical signs and gross pathology

Moribund HSB and WB in challenge tanks displayed signs consistent with a *Flavobacterium columnare* infection. Fish were lethargic and exhibited little feeding activity. Macroscopic lesions were typical, with the skin of challenged fish initially showing discrete areas of depigmentation that over time

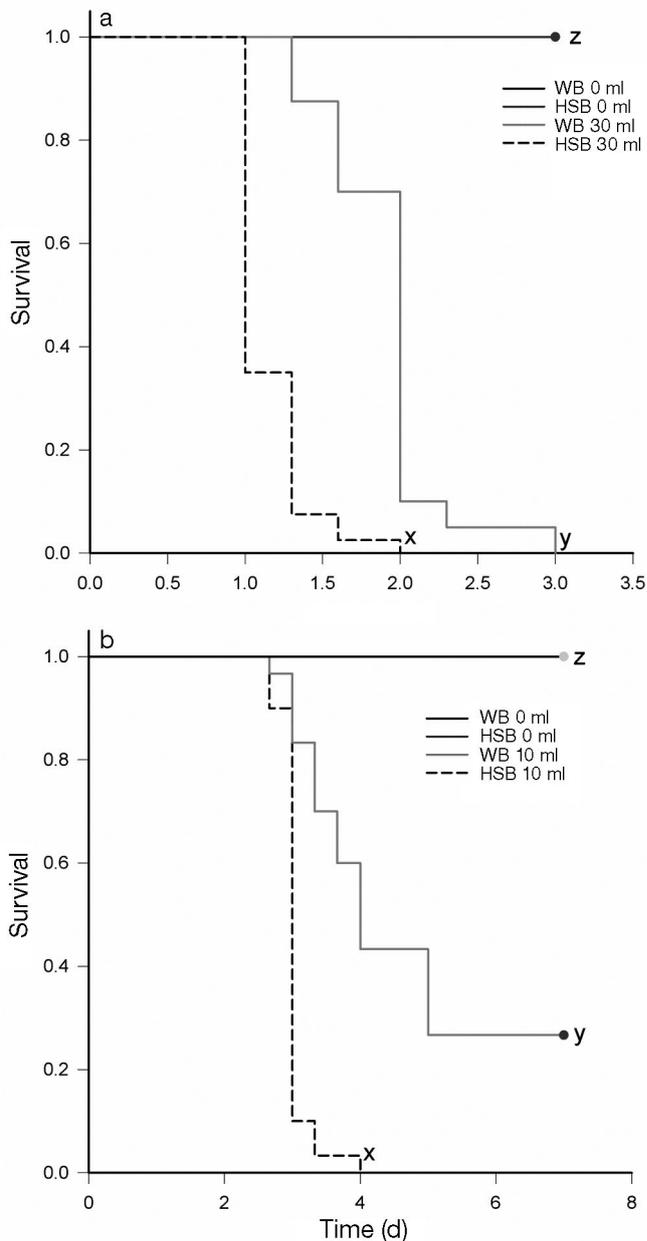


Fig. 2. *Morone chrysops* and *M. chrysops* × *M. saxatilis*. Kaplan-Meier log rank survival analysis of white bass (WB) and hybrid striped bass (HSB) subjected to a challenge with (a) 30 ml (6.75×10^7 CFU ml⁻¹) or (b) 10 ml (2.25×10^7 CFU ml⁻¹) of *Flavobacterium columnare* in an ultra-low-flow challenge system. Survival curves not sharing the same letter indicate a significant difference ($p \leq 0.05$)

became multifocal and diffuse, often encompassing most of the body as the infection progressed. The gills had multifocal to widespread necrotizing branchitis, which was more common and extensive in HSB (Fig. 3) in all studies as compared to WB. Severely frayed fins, especially the caudal fin, were common as the infection progressed. No internal



Fig. 3. *Morone chrysops* × *M. saxatilis*. Representative macroscopic image depicting gill damage in hybrid striped bass challenged with *Flavobacterium columnare* in an ultra-low-flow challenge system

grossly visible lesions were observed in any tissues examined for either species.

Histology (Expt 3)

HSB that were challenged with 10 ml of *Flavobacterium columnare* showed extensive gill damage at 24 h compared to unchallenged fish (gross pathology is depicted in Fig. 3; histopathology is shown in Fig. 4A,B). Lesions were characterized by severe necrosis and marked hyperplasia that resulted in the complete loss of the intralamellar space and fusion of adjacent lamellae. While not enumerated,

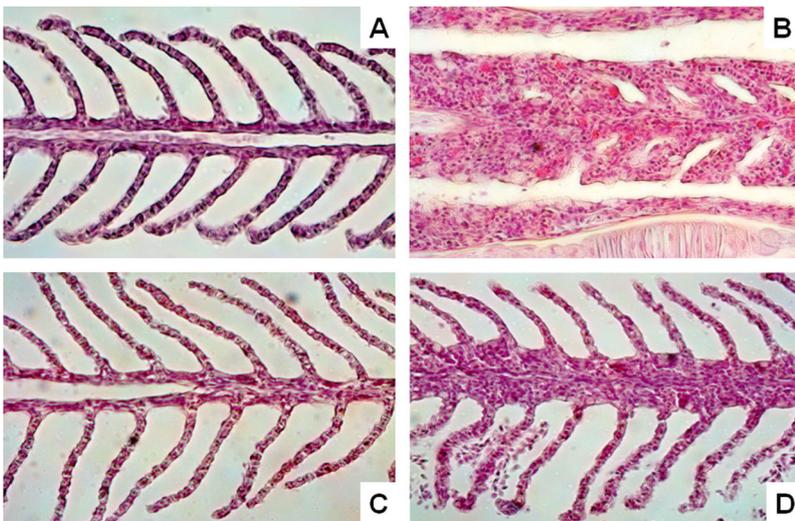


Fig. 4. *Morone chrysops* and *M. chrysops* × *M. saxatilis*. Representative photomicrographs of gills 24 h after experimental challenge with *Flavobacterium columnare*. (A) Unchallenged hybrid striped bass. (B) Challenged hybrid striped bass (10 ml, 2.25×10^7 CFU ml⁻¹); note the extensive degradation of branchial structure and lamellar fusion. (C) Unchallenged white bass. (D) Challenged white bass (10 ml, 2.25×10^7 CFU ml⁻¹). All images: stained with hematoxylin and eosin

eosinophilic granular cells increased in abundance compared to unchallenged HSB. The gills of WB challenged with 10 ml of *F. columnare* were not normal, but were altogether less severely damaged (Fig. 4C,D). The gill of challenged WB routinely showed evidence of mild hyperplasia which typically involved less than 25% of the intralamellar space. Gills of unchallenged fish had no detectable lesions. No parasites were observed in any gill section.

DISCUSSION

Infection with *Flavobacterium columnare* is a problematic disease that is global in distribution and plagues numerous species of freshwater wild, cultured, and ornamental fish species including but not limited to carp, channel catfish, goldfish, tilapia, salmonids, and eel (Declercq et al. 2013). In effect, it is widely held that no wild or farmed freshwater fish, including ornamental fish in aquaria, are completely resistant to columnaris disease (Plumb & Hanson 2011).

To our knowledge, this is the first report in which columnaris disease susceptibility has been assessed in WB, and only limited information on columnaris disease is available for HSB. Darwish et al. (2012) evaluated the efficacy of different therapeutants in HSB exposed to a mixed infection of *Aeromonas hydrophila* and *F. columnare* during both natural and experimental infections. In a study to evaluate the immunocompetency of HSB fed test diets with varying levels of vitamin E, Trushenski & Kohler (2008) reported an incidental *F. columnare* outbreak in test fish, which was presumably a contaminant in a stock culture of *Streptococcus iniae*, the intended pathogen to be tested. In the current series of experiments, while neither species was completely resistant to columnaris disease, the results showed a significant difference in susceptibility between WB and its hybrid under the experimental challenge conditions.

The notion that a hybrid species may not exhibit heterosis towards disease resistance is not new. Indeed, there is mixed evidence on superior disease resistance in hybrid species of fish, and heterosis for survival has not shown large effects (Fjalestad et al.

1993). Goldberg et al. (2005) found a 14 % reduction in performance in hybrid largemouth bass infected with largemouth bass virus relative to non-hybrid largemouth bass. Cai et al. (2004) concluded that hybrid tilapia had higher resistance to *Aeromonas sobria* than its parent species. Wolters et al. (1996) found that, following an experimental bath immersion, hybrid catfish (*Ictalurus punctatus* female \times *I. furcatus* male) had lower resistance to *Edwardsiella ictaluri* than blue catfish but higher than channel catfish. However, hybrid catfish were significantly more resistant to columnaris disease than either parent species when experimentally challenged with a highly virulent isolate (Arias et al. 2012). Clearly, a precise understanding of the heritability of immune traits is lacking, and future studies should focus on identifying markers that may drive immunity in farmed fish species.

As expected, HSB showed a dose-dependent decrease in survival. In the 30 ml challenge, 100 % mortality was observed in both species, but WB showed a more delayed time to death. Previously, juvenile striped bass were shown to be highly susceptible to a columnaris disease challenge, with 100 % of fish dying over a 7 d period after challenge (Macfarlane et al. 1986). Interestingly, Macfarlane et al. (1986) used a lower dose (5×10^6 cells ml⁻¹) of bacteria and exposed fish to bacteria for only 2 min before a complete water change. In comparison, in our study, the 10 ml challenged (2.25×10^7 cells ml⁻¹) WB exhibited a survival rate of 33 %, while all HSB had succumbed to the disease by Day 4. It is important to note that isolates of *Flavobacterium columnare* are known to differ widely in terms of their virulence; therefore, it is difficult to compare rates of mortality between previous studies and our work. Nevertheless, future studies should compare the susceptibility of striped bass in parallel with WB and HSB using the same isolate and size-matched individuals.

The inferior performance of the HSB relative to WB as shown in these experiments, as well as the seemingly high susceptibility of striped bass to columnaris disease as observed by Macfarlane et al. (1986), could be explained by differences in how each species responds to stress. A significant body of literature has recorded elevated plasma cortisol concentrations during a variety of viral, bacterial, fungal, and parasitic infections (see Table 1 in Ellis et al. 2007 for a good overview). Additionally, Fevolden et al. (1992) found that 2 lines of rainbow trout *Oncorhynchus mykiss* selected for either a high- or low-stress response had significantly different survival rates following experimental challenges with 2 bac-

terial pathogens. Following exposure to an acute low-water stress event, WB had the lowest physiological stress response and the fastest return to basal levels, while striped bass had the highest response and the slowest return to basal levels (Davis & McEntire 2009). Interestingly, HSB exhibited a response that was intermediate to both parental species. While the present experiments did not directly measure classical stress indices, it stands to reason that the superior stress response and recovery mechanism of WB, relative to HSB as demonstrated by Davis & McEntire (2009), could lead to improved survival following an experimental challenge with a bacterial pathogen. Based on the conclusions of Malham et al. (2003), it is also possible that the significantly higher and/or earlier mortality in HSB relative to WB observed in the present series of experiments could be due to inherent differences in the mechanism and timing with which each species redirects bioenergetic resources towards adaptive physiological functions such as respiration, oxygen uptake, and glycolysis to cope with this massive physiological insult, which could result in downregulation of immune functions. Detailed studies are needed on the metabolic changes that occur during bacterial infections and a better understanding of the mechanisms involved in the regulation of their immune function for each species.

Between Expts 1 and 3, the mean weight of both species increased in mass by 10 to 15 %. In channel catfish, susceptibility to columnaris disease decreases as fish increase in size/age (Shoemaker et al. 2008, Plumb & Hanson 2011). While not directly tested in the present study, the differences in survival between the initial trial and the last trial may be due in some part to this increase in the size of the host fish.

Due to the heightened mortality kinetics (>60 % mortality at 24 h) in HSB in the 30 ml dose, we chose to focus our efforts on characterizing the pathology in the 10 ml study as it would better approximate a disease outbreak that we would encounter at our facility. One of the most unexpected and striking aspects of this study was the marked grossly visible and microscopic branchial lesions observed in HSB gill, while gill involvement was noticeably reduced in WB. This level of pathologic change in HSB could have greatly contributed to both the accelerated and overall higher mortality observed. The differences in gill condition could be related to differences in the ability of *Flavobacterium columnare* to adhere to the gill. A clear correlation between *F. columnare* virulence and adherence to the gill epithelium has previously been demonstrated (Decostere 2002). Differences in the ad-

hesion of *F. columnare* to gills was noted in a study comparing zebrafish versus channel catfish (Olivares-Fuster et al. 2011). Successful bacterial adhesion enables the pathogen to withstand cleansing mechanisms operating on the surfaces of the gill, such as the resistance to water flow, mucus secretion, and the constant cellular turnover of the respiratory epithelium (Decostere et al. 1999a,b). Moreover, in comparison with the aqueous environment, the gill surface may serve as a nutrient-rich environment, offering growth advantages to the pathogen (Decostere et al. 1999a,b). In this line of reasoning, we recently identified a putative host receptor utilized by *F. columnare* for adhesion to the gill, a rhamnose-binding lectin (RBL) whose expression was dramatically upregulated early after infection (Sun et al. 2012). A more comprehensive study of RBL activity revealed that higher expression levels were strongly linked to increased columnaris susceptibility and that saturation of the receptor with its putative ligands (L-rhamnose or D-galactose) resulted in significantly decreased columnaris mortality (Beck et al. 2012). Studies are ongoing to examine the kinetics and magnitude of bacterial adhesion to ectopic mucosal surfaces in WB versus HSB.

The present study was designed as an initial investigation into the comparative susceptibility of HSB and WB to columnaris disease and not as an exhaustive analysis of comparative pathology or the comparative stress response. Nevertheless, the present findings are important for a number of reasons. First, these results heighten our understanding of columnaris disease susceptibility in Moronidae, for which little information existed previously. These findings will also serve as a foundation for future studies to elucidate the physiological or molecular underpinnings governing the differential survival between these 2 species. We contend that these 2 species could serve as valuable models of columnaris disease pathogenesis and be utilized to bridge knowledge gaps on the mechanisms contributing to columnaris disease susceptibility/resistance.

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