Differences in mortality, growth, lysozyme, and Toll-like receptor gene expression among genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*

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Abstract Survivorship to ESC (enteric septicemia of catfish) varies among and within strains of commercially raised catfish, however the immunological basis for differences in susceptibility is not well-understood. We assessed the effect of pathogen challenge with *Edwardsiella ictaluri* on five genetic groups of catfish by measuring both phenotypic response (mortality, pathogen levels, specific growth rate), and three measures of immune response, including lysozyme activity and mRNA expression of two toll-like receptors (TLR3 and TLR5). Both mortality and pathogen loads, in addition to non-specific immune response, consistently ranged from the least susceptible Blue catfish (24%, $3.4 \times 10^2 \pm 9.3 \times 10^1$ cell-equivalents/mg, 13.2 ± 3.2 U/mL tissue, respectively) to the most susceptible 103 channel catfish (65%, $1.1 \times 10^4 \pm 6.4 \times 10^3$ cell-equivalents/mg tissue, 67.3 ± 28.7 U/mL, respectively). Similarly, specific growth rate was reduced in exposed fish, compared to non-exposed controls, only in the most susceptible genetic groups ($P = 0.0051$). Trends in mRNA expression levels were apparent in each tissue type for both genes. In kidney, differences were evident in expression of both TLR3 and TLR5 mRNA between strains early and late in challenge ($P < 0.01$). TLR5 mRNA showed significant downregulation in all strains on days 1 and 4 ($P = 0.0001$). In spleen, all strains had elevated levels of TLR3 ($P = 0.0050$) and TLR5 mRNA ($P < 0.0001$) only 1 day post-exposure. In stomach, only one strain (103 × RR) showed upregulation ($P = 0.0063$) throughout challenge. The relationship of phenotypic (mortality and growth) and immune responses measured here, suggests that variation in susceptibility to ESC is a function of differences in innate immune response. Understanding these differences will be crucial for enhancing the immune system through selective breeding and in developing disease management protocols for channel catfish.
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Introduction

Variation in susceptibility to disease may be based on the effectiveness of the innate and/or adaptive immune responses. Enteric septicemia of catfish (ESC), caused by the Gram-negative bacterium *Edwardsiella ictaluri* [1], is the most prevalent disease affecting commercial catfish production, resulting in up to 60% of total losses in a given year [2]. There is a large degree of variation in levels of resistance to ESC infection among catfish species, strains, and families [3–7], and the repeatability of response is high [7], suggesting that there is a genetic component to ESC resistance. This observation is further supported by a recent study that demonstrated that two generations of selection with a multi-trait selection index resulted in 10% reduction in channel catfish mortality following experimental challenge with *E. ictaluri* [7].

Direct comparison between resistant and susceptible families (full-sib groups) of channel catfish showed that pathogen loads were suppressed in fish from resistant families, whereas pathogen loads in fish from susceptible families were elevated [8,9]. In these same families, lysozyme activity became elevated 24 h earlier in resistant families [8]. Other measures of immune response have shown that macrophage aggregation increased in kidney and spleen of resistant families of channel catfish as compared to susceptible families, however, *E. ictaluri* can reproduce within macrophages [4,10]. These studies suggest that innate immunity plays a significant role in resistance to ESC.

The toll-like receptor (TLR) gene family is strongly associated with both the innate immune and adaptive immune systems. Toll-like receptors are pattern recognition receptors (PRRs) that recognize specific regions of sequences that are highly conserved among various microbial and viral pathogens. These sequences are referred to as pathogen associated molecular patterns (PAMPs) [11,12]. In mammals, TLR3 recognizes double stranded (ds) RNA from viral sources [13] and TLR5 recognizes flagellin from Gram-negative and Gram-positive bacteria [14,15]. Numerous TLRs have been described in various teleosts, *Danio rerio* [16], *Fugu rubripes* [17], *Ictalurus punctatus* [18,19], *Carrassius auratus* [20], *Paralichthys olivaceus* [21], and *Oncorhynchus mykiss* [22–24]. Most have high homology to their mammalian counterparts. However, the majority of these studies characterize the TLRs and there is little data regarding the functional role of toll-like receptors in teleosts. In channel catfish, expression levels of toll-like receptor (TLR) mRNA, specifically TLR3 and TLR5 mRNA, are upregulated in the presence of *E. ictaluri* [18,25].

Changes in TLR mRNA expression in response to *E. ictaluri* exposure have been examined in strains of channel catfish with similar levels of susceptibility [26] and in comparisons of channel and blue catfish which are known to have substantial differences in their challenge response [25]. These TLRs show promise for having an association with susceptibility to ESC and survival. In order to generate a thorough test of association of TLR3 and TLR5 with ESC susceptibility, in the present study we chose five genetic groups of catfish that represented a range in susceptibilities to ESC from the highly susceptible USDA103 channel catfish to blue catfish with low susceptibility [3]. We measured changes in growth and immune response of these genetic groups of catfish during experimental challenge with virulent *E. ictaluri*. Specific tests were of mortality, pathogen loads, specific growth rate, lysozyme activity, and mRNA expression of two toll-like receptors, TLR3 and TLR5, in catfish during experimental *E. ictaluri* challenge. Measurement of these factors in groups of fish with a range of susceptibilities should provide the framework for identification of genes/characteristics with strong association to susceptibility to *E. ictaluri*. This will ultimately aid in the search for suitable genetic markers for the improvement of resistance to ESC in channel catfish.

Materials and methods

Growth and experimental challenge

Fingerling fish from multiple families from five genetic groups were tested. Three genetic groups were channel catfish (USDA103(103), 103 × Red River (103 × RR), Red River (RR)), and the remaining two were blue catfish (*Ictalurus furcatus*) and USDA103 channel × blue catfish hybrids (103 × blue). All fish were spawned and grown in the USDA-ARS Catfish Genetics Research Unit in Stoneville, MS, and were naïve to *E. ictaluri*. Fish were stocked randomly into a total of 165 75-L aquaria according to the following regime: 30 fish per aquarium; 3 aquaria per genetic group per treatment (control or exposed) per sampling timepoint (see below). Aquaria were supplied with 26 °C well water. All fish were weighed prior to stocking. Fish were acclimated for 7 days and then challenged with 4 mL of 10^9 cfu/mL virulent *E. ictaluri* (S97-773; with a 30 min static challenge) [5]. Virulence was confirmed by passing the bacteria through channel catfish, resulting in acute ESC, after which *E. ictaluri* cells were isolated and grown in culture for the present study, as was previously described [18]. Fish were sampled just prior to challenge (15 aquaria), and then 2 h, 1, 4, 8, and 14 days post-challenge (150 aquaria). All remaining fish were maintained in aquaria until 21 days post-challenge. At this point, all remaining fish were weighed and specific growth rate was calculated. Throughout acclimation and during the trial, fish were fed a diet at 3% of their body weight.

The number of *E. ictaluri* cells/mL of culture was enumerated using standard plate count methods on tryptic soy agar plates supplemented with 5% sheep's blood [27]. An aliquot of 50 μL of culture used in the challenge was plated on BHI agar plates and incubated for 48 h at 26 °C.

Sample collection

Anterior kidney, spleen, gut, and whole blood (100 μL treated with sodium heparin) were collected for five fish from three replicate aquaria per genetic group per treatment (control or exposed) at the following times: 2 h, 1, 4, 8 and 14 days post-exposure. Fish were anesthetized in a 100 mg/L solution of tricaine methanesulfonate (MS-222) for collection of blood samples and then euthanized in a 300 mg/L solution of MS-222 before collection

of anterior kidney, spleen, and stomach. All samples were flash frozen in liquid nitrogen and then stored at −80 °C until DNA or RNA extraction. Plasma was collected from the 100 μL of heparinized blood following centrifugation, and stored at −80 °C until lysozyme analyses were conducted.

Pathogen detection and quantification

Bacterial genomic DNA was extracted from 20−50 mg of kidney tissue using a proteinase K digestion and ethanol precipitation as previously reported [8]. All samples were eluted in 50 μL dH₂O. An *E. ictaluri*-specific target sequence was then amplified using a validated real-time PCR assay that enabled direct quantification of bacterial DNA/cell-equivalents [11]. All real-time PCR reactions were carried out on a Bio-Rad iQ iCycler™ and were run in triplicate. Although this assay has been validated against standard plate counts of viable *E. ictaluri*, small amounts of target DNA fragments from dead bacteria may also be included in the quantification. Hence, the data are expressed in cell-equivalents.

Lysozyme analysis

Plasma lysozyme activity was determined using the EnzChek® lysozyme assay kit (E22013; Molecular Probes, Eugene, OR). Briefly, 25 mL of plasma was diluted with 25 mL of reaction buffer (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5) and incubated with 50 mL of fluorescein-labeled *Micrococcus lysodeikticus* (50 μg/mL) for 30 min at 37 °C. The fluorescence was measured in a fluorescence microplate reader using excitation/emission wavelengths of 485/535 nm. Background fluorescence, determined for a no-enzyme control, was subtracted from each value. Lysozyme activity of the experimental samples was calculated from a standard curve prepared with lysozyme from chicken egg white.

RNA extraction, cDNA synthesis, and expression

Total RNA was extracted from all tissues following instructions included with the MagMAX™-96 Total RNA Isolation Kit (Ambion, Inc, Austin, TX). RNA was quantified on a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Rockland, DE) and 1 μg of total RNA was used for cDNA synthesis. cDNA was synthesized using the iScript kit (BioRad Laboratories) as per the manufacturer's instructions on the Eppendorf MasterCycler. The cDNA was then quantified by UV absorption on the NanoDrop spectrophotometer and all samples were diluted to 200 ng/μL. A total of 400 ng per sample of cDNA was used in expression analyses.

Expression of target mRNA was measured with real-time PCR on the BioRad iQ™ iCycler. All real-time PCR amplifications were performed in triplicate. Each amplification reaction mixture (12.5 μL) contained 400 ng of cDNA; 1× iQ™ Supermix (BioRad Laboratories); 10 nM (TLR3) or 20 nM (TLR5, α-tubulin) of each primer; and 20 nM dual-labeled probe. Primer and probe sequences were designed with Beacon Designer 2.0 (Premier BioSoft, Inc.) software and have been previously reported [26]. The amplification

profile was: 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification products were quantified by comparison of experimental C_t (threshold cycle, defined as the PCR cycle where an increase in fluorescence first occurred) levels with those of the standard curve.

Data analysis

Specific growth rates (SGR) were calculated ((ln(WT) −ln(wt)/ $(T) \times 100$), where WT and wt are initial and final weights, respectively, and T is time in days). Bacterial levels (cell-equivalents), lysozyme levels, and SGR for each genetic group were log-transformed and subjected to analysis of variance (ANOVA) to determine differences between genetic groups and days (MIXED procedure: SAS v9.0, SAS Institute, Inc., Cary, NC). P-values were calculated for comparisons by day (pdiff) when the MIXED results were significant. Pearson's correlation coefficient was used to determine the relationship between lysozyme levels and cumulative mortality.

All expression data were measured in copy number and normalization was calculated as the ratio of the expression level of the gene of interest/housekeeping gene. The housekeeping gene used was α-tubulin and there was no genetic group or time effect on α-tubulin mRNA expression. Normalized expression data passed Levene's test of homogeneity and were subjected to analysis of variance to determine differences between genetic groups and days as above. Fold induction of each gene was calculated as the normalized copy number for exposed fish/normalized copy number for control fish at each timepoint.

Results

Mortality and pathogen levels

Mortalities began on day 5 post-challenge and continued until day 19 (103, 103 × RR), day 20 (RR), and day 21 (103 × Blue, Blue). Cumulative mortality differed among genetic groups ($P < 0.0001$). The 103 group had the highest mortality ($65.0 \pm 4.0\%$) and Blue had the lowest mortality ($24.3 \pm 2.4\%$; Fig. 1). All dead fish showed clinical signs of ESC infection, including blistering of the skin, lack of appetite, internal hemorrhaging, discoloration of the liver, and an enlarged spleen [28].

Pathogen loads, as determined by PCR quantification of bacterial cell-equivalents, varied among genetic groups throughout the challenge (Fig. 2). However, no significant differences were found ($P > 0.05$). By day 14, pathogen loads diminished to below 10^3 bacterial cell-equivalents/ mg of sample in all genetic groups. Only 103 × RR nearly cleared the bacterial cells by day 14. Pathogen loads in Blue remained consistent from days 1 to 14.

Growth rate

Overall, SGR over the 21 day challenge differed between exposed and non-exposed fish ($P = 0.0051$). Unexposed control fish grew faster than exposed fish for the three
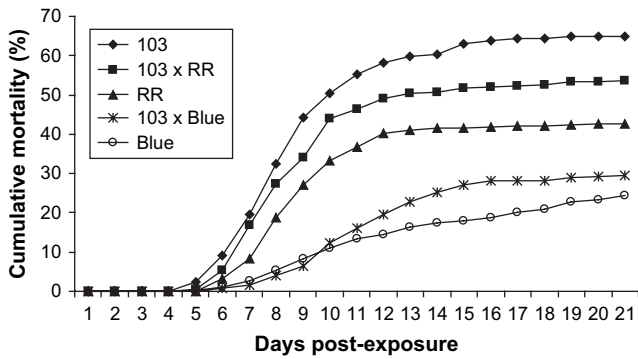
Figure 1 Cumulative mortality of five genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*. Catfish were challenged by 30 min immersion in an *E. ictaluri* bath (1.2 × 10^9 cfu/mL). Data are presented as means. Genetic groups differed in cumulative mortality ($P < 0.0001$).

channel catfish groups, but there was no difference in growth rates of control and exposed groups of blue and 103 × blue (Fig. 3).

Lysozyme activity

Lysozyme activity was elevated from 4 days through to 14 days post-challenge (Fig. 4). Genetic group differences were evident on days 4, 8, and 14 ($P < 0.05$). On day 4 post-challenge, both 103 and 103 × RR lysozyme activity was higher ($P < 0.05$) than that of the other genetic groups. A more specific gradation was found on day 8, with genetic group 103 having the highest lysozyme levels ($P = 0.0141$), 103 × RR and RR having moderate levels ($P < 0.05$), and 103 × Blue and Blue having the lowest lysozyme activity ($P < 0.05$). Lysozyme activity across the challenge period was correlated with cumulative mortality levels for all genetic groups ($P = 0.0236$). The correlation between pathogen levels and lysozyme activity throughout the challenge period was positive, but weak ($P = 0.0610$).
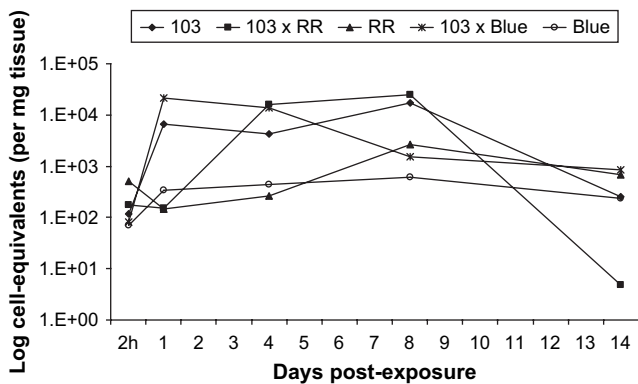
Figure 2 Pathogen loads in kidney of five genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*. Kidney samples were taken at 2 h, 1, 4, 8 and 14 days post-exposure. Data are presented as means. Differences between genetic groups were non-significant ($P > 0.05$).
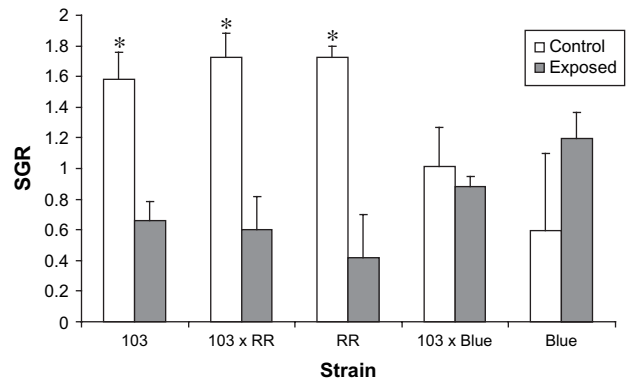

Figure 3 Specific growth rate (SGR) of five genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*. Weights of five fish per aquarium were taken before and 21 days after challenge for both non-exposed (control) and exposed fish. Data are presented as means ± SEM. The asterisk (*) represents a significant difference ($P < 0.05$) between exposed and control fish per genetic group.

Expression of TLR3 and TLR5 mRNA after experimental challenge

The standard curve for each gene was generated from two replicates of serial dilutions of each recombinant plasmid. PCR efficiencies (as calculated by the iCycler software (BioRad Laboratories) for each gene were as follows: TLR3 94.6%; TLR5 91.8%; α-tubulin 84.6%. Normalized expression levels of both TLR3 and TLR5 mRNA were elevated during challenge for all genetic groups, regardless of susceptibility levels.

Changes in TLR3 mRNA expression levels during challenge were evident (Fig. 5). Levels of TLR3 mRNA in kidney
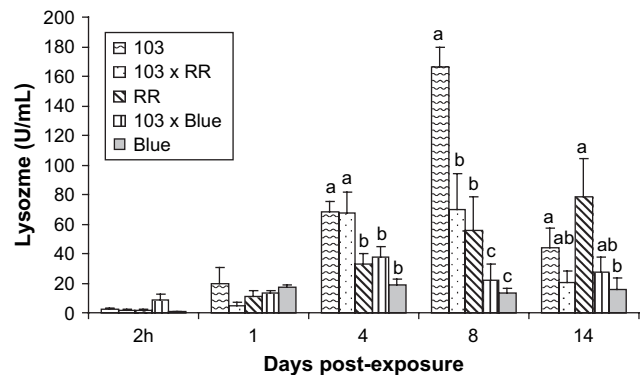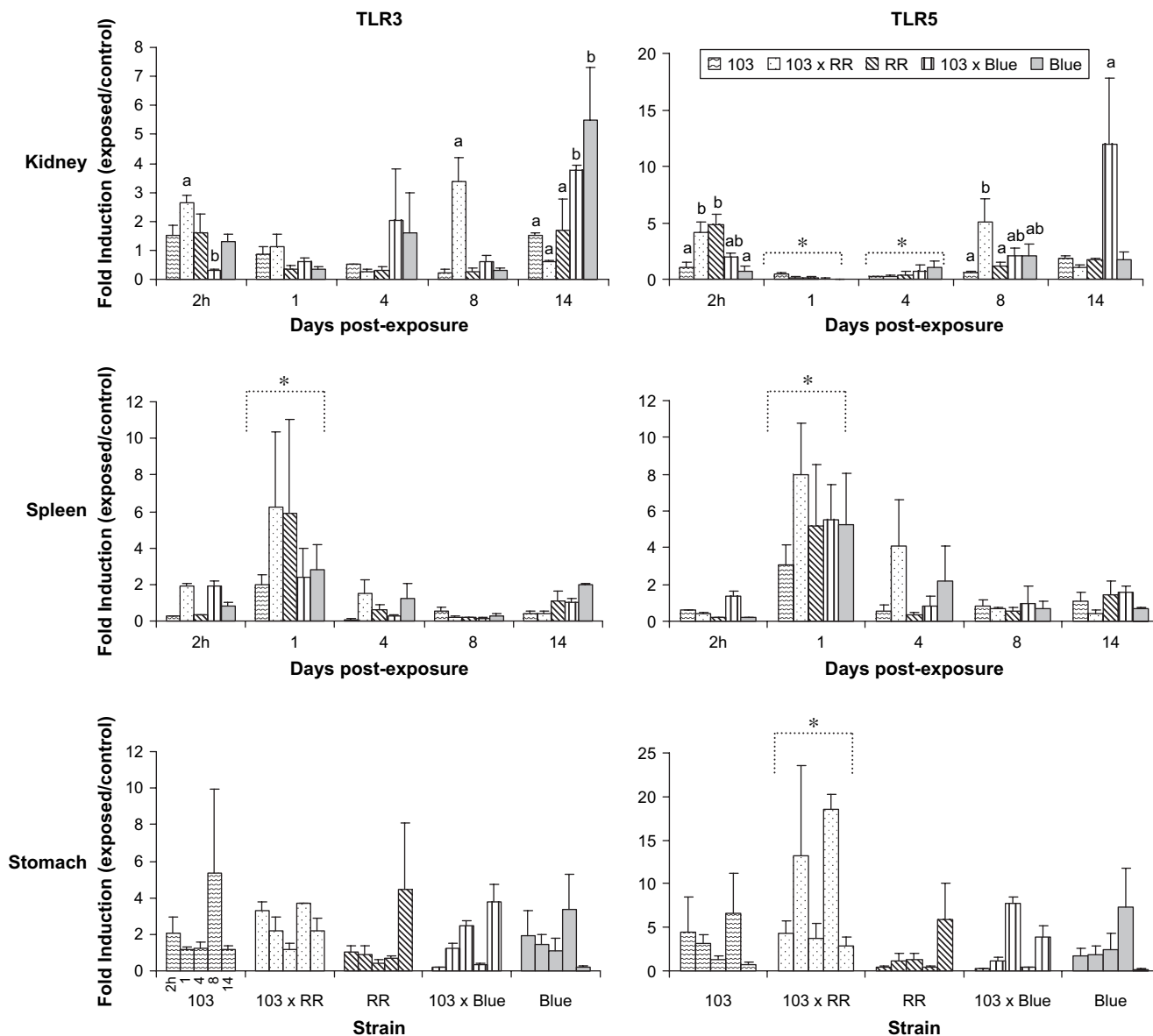

Figure 4 Lysozyme activity in five genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*. Blood samples were taken at 2 h, 1, 4, 8 and 14 days post-exposure. Plasma was separated and tested for lysozyme activity. Data are presented as means ± SEM. Differing letters above the data bars represent significant differences ($P < 0.05$) within each time-point. Differences among genetic groups were evident 4, 8, and 14 days post exposure.

were elevated on day 14 post-challenge ($P = 0.0004$), when Blue levels of TLR3 mRNA were higher than the three channel catfish strains ($P < 0.0001$; Fig. 5). Group 103 × RR also showed elevated levels of TLR3 mRNA in kidney, but at 2 h and 8 days post-challenge ($P < 0.01$). In spleen all genetic groups had elevated levels of TLR3 1 day post-exposure

($P = 0.0050$). No differences among genetic groups or over time for TLR3 in stomach samples were detected.

Upregulation of TLR5 mRNA was evident in all tissues of the five genetic groups (Fig. 5). In kidney, differences were observed among genetic groups ($P = 0.0171$) and across time ($P = 0.0001$). Downregulation in all genetic groups was

Figure 5 Expression levels (fold induction compared to non-exposed at each timepoint) of TLR3 and TLR5 in five genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*. Kidney, spleen, and stomach samples were taken 2 h, 1, 4, 8 and 14 days post-exposure for RNA extraction. TLR3 and TLR5 mRNA expression was measured with real-time PCR to determine differences in upregulation, both between genetic groups and between non-exposed fish and exposed fish 2 h, 1, 4, 8 and 14 days post-exposure. Data are presented as means ± SEM. Differing letters above the data bars represent significant differences ($P < 0.05$). The asterisk (*) represents significant differentiation ($P < 0.05$) at a timepoint or of a strain over all timepoints. Samples along the *x*-axis are grouped by timepoint in kidney and spleen and by strain/genetic group in stomach to more clearly show trends in significance. In kidney, expression of TLR3 differed between genetic groups early and late in challenge ($P = 0.0011$). TLR5 differences were evident among genetic groups throughout challenge ($P = 0.0008$) with significant downregulation occurring in all genetic groups on day 1 and 4 ($P = 0.0001$). In spleen, all genetic groups had elevated levels of TLR3 ($P = 0.0050$) and TLR5 ($P < 0.0001$) 1 day post-exposure. In stomach, only one genetic group (103 × RR) showed upregulation ($P = 0.0063$) throughout challenge.

evident at both 1 day and 4 days post-exposure ($P < 0.05$). Conversely, TLR5 mRNA levels were elevated and genetic group differences were detected at all remaining timepoints ($P < 0.05$). In spleen, TLR5 mRNA was upregulated 1 day post-exposure ($P < 0.0001$). In stomach, only 103 × RR showed upregulation of TLR5 mRNA ($P = 0.0063$).

Discussion

Prior knowledge of ESC-susceptibility levels of various catfish strains allowed us to select strains/genetic groups for this study to increase the likelihood of finding relationships between ESC susceptibility and factors associated with growth and immunity. The overall levels of cumulative mortality and the differences in mortality observed among genetic groups were consistent with previous studies [3,7]. Also, as expected, the crosses had intermediate mortality rates as compared to the parental genetic groups. Pathogen levels were highly variable both among and within groups. However, infection kinetics of 103 catfish was similar to previous studies, but peaked at lower levels [8]. The low pathogen levels and lack of clearance (except in 103 × RR) suggest chronic infection and may have a different effect on immune response than an acute infection. This is also the first time that pathogen levels in the remaining genetic groups have been measured with the genetic pathogen detection assay.

Although we did not measure feed intake specifically, reduced feeding activity was more prominent in those groups with higher susceptibility. This was expected because a common indicator of ESC in the pond environment is reduction in feeding activity. Concomitant with low feeding activity is reduction in growth rate. Specific growth rate decreased during challenge in exposed fish, as compared to non-exposed controls, only in the most susceptible genetic groups. Similarly, a positive relationship between growth and disease resistance exists in juvenile Atlantic Halibut, *Hippoglossus hippoglossus* [29], and chinook salmon, *O. tshawytscha*, following challenge with vibriosis and furunculosis [30]. In contrast, the relationship between growth and survival from bacterial kidney disease (caused by *Renibacterium salmoninarum*) varies by population in coho salmon, *O. kisutch* [9].

Immune response (innate or adaptive) is a major factor affecting survival after pathogen exposure. Lysozyme activity is a parameter of the non-specific immune response. The variation in lysozyme activity, shown here among genetic groups, coupled with the positive correlation of lysozyme activity with cumulative mortality rates and pathogen loads suggest that changes in lysozyme activity are acting in response to disease state. The involvement of lysozyme in suppressing pathogen loads was suggested by Bilodeau et al. [8] in a comparison between families (full-sib groups) of USDA103 catfish with differing levels of susceptibility to ESC, where lysozyme levels increased 24 h earlier in families with low susceptibility to ESC. In the present study lysozyme activity increased in all genetic groups early in the challenge, however only in the most susceptible strains of channel catfish did lysozyme levels decrease towards the end of the challenge. In the strains with the lowest susceptibilities, lysozyme activity either continually increased or

increased and then stabilized during the challenge. This observation suggests that elevated lysozyme activity may contribute to an increase in survival during ESC challenge.

Toll-like receptors are associated with a targeted immune response in mammals and teleost fish. In each of the five genetic groups of catfish tested, all showed upregulation of both TLR3 and TLR5 mRNA at some point during the challenge with *E. ictaluri*. In human and mouse models, the TLR3 ligand is dsRNA (i.e., a viral signal) [13]. It has been shown that in catfish, TLR3 mRNA responds to the presence of the Gram-negative bacterium, *E. ictaluri* [25,26,31,32]. Those data are further supported by the current study, where upregulation of TLR3 mRNA was evident. TLR5 mRNA expression in all tissues was affected by challenge as well. While the specific ligand has not been demonstrated in catfish, it has been repeatedly shown that TLR5 mRNA is upregulated in the presence of *E. ictaluri* [26,31,32].

Consistent patterns of expression were evident for both TLR genes in each tissue, although patterns varied between tissues. In kidney, differences among genetic groups over time were evident. The downregulation that was measured for TLR5 mRNA in all groups was bracketed by differences among groups in upregulated TLR5 mRNA expression early and late in the challenge period. This contrasts with what has been reported in previous studies involving channel catfish and ESC that showed upregulation of TLR5 mRNA during early-mid challenge [26,31−33]. Downregulation of TLR5 mRNA in kidney during challenge may be indicative of a response to chronic infection. Downregulation of TLR5 mRNA during chronic infection has been demonstrated in humans and mice [34,35]. In both cases, low levels of bacterial infection triggered downregulation of TLR5 mRNA during mid-stages of disease progression.

The response of TLR3 and TLR5 mRNA expression in both spleen and stomach showed a different pattern from kidney. Perhaps the function of these genes in the innate immune response in the spleen is conserved across genetic groups.

In contrast, upregulation of TLR5 mRNA in stomach was limited to 103 × RR. The only group to show any indication of pathogen clearance (as indicated by pathogen levels) was also 103 × RR. Toll-like receptors may be involved in both innate and adaptive response mechanisms (see review in [36]) and pathogen clearance is understood to be an adaptive response. The data reported here then suggest that TLR5 may have some involvement in invoking pathogen clearance in the 103 × RR.

Both the phenotypic (mortality and growth) and immune responses measured suggest that large-scale (strain/species level) variation in susceptibility to ESC is related to differences in innate immune response. Understanding these differences will be crucial for enhancing the immune system through selective breeding and in developing disease management protocols for channel catfish. While no direct relationship between susceptibility to ESC and TLR3 and TLR5 mRNA expression was evident, a pattern of response to chronic or low-levels of infection was suggested. Perhaps a clearer relationship may emerge if infection levels were to reach acute levels. Nonetheless, this information may be useful when testing for fine-scale associations of TLR function and ESC susceptibility and the family level in a search for markers suitable for genetic improvement of disease resistance in catfish.

Acknowledgements

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