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[Article]

Pathogen Levels, Lysozyme, and Cortisol Response in Channel Catfish with Susceptibility Differences to *Edwardsiella ictaluri*

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Abstract.—Differences in bacterial loads, clearance rates, nonspecific immune response, and cortisol response were measured for three family groups of channel catfish *Ictalurus punctatus* susceptible to enteric septicemia of catfish (ESC) and three ESC-resistant families. Resistant families had lower mortality during challenge ($P < 0.0001$). Bacterial loads on day 5 postexposure were higher ($P < 0.01$) in susceptible families than resistant families for blood, kidney, and spleen. Blood, kidney, and spleen bacterial levels were highly correlated. Pathogen clearance was evident by day 12 in fish from the susceptible families and measurable only in kidney and spleen from resistant families on day 20. Lysozyme activity increased on day 1 ($P = 0.0074$) in resistant fish and on day 2 in susceptible fish ($P < 0.0001$). An acute stress response was evident for both resistant (21.4 ± 1.7 ng/mL [mean ± SD]) and susceptible families (30.0 ± 1.6 ng/mL). Plasma cortisol levels were elevated in susceptible fish throughout challenge, except for day 12, but in resistant fish they recovered to near basal levels immediately after an acute response on day 2. There was no correlation between cortisol and lysozyme levels. Differences in lysozyme activity and pathogen levels suggest that the nonspecific immune response was effective during the early stages of infection in resistant fish.

Channel catfish *Ictalurus punctatus* are highly susceptible to enteric septicemia of catfish (ESC), which is caused by the bacterium *Edwardsiella ictaluri* (Hawke 1979). This is the most prevalent disease affecting farm-raised channel catfish in the United States and is responsible for up to 50% of total losses to catfish farmers each year, costing up to US$60 million in financial losses (USDA 1997). The pathology of ESC is clearly defined (Shotts et al. 1986; Newton et al. 1989; Baldwin & Newton 1993); however, the mechanisms driving innate resistance are not well understood. Current management tools for ESC include vaccination of fry (Shoemaker and Klesius 1997; Wise et al. 2000), application of medicated feed after infection is evident, and withdrawal of feed during the peak season for ESC outbreaks (fall and spring; Hawke et al. 1998). None of these management strategies are completely effective when used alone, and all have associated costs.

Selective breeding for increased ESC resistance would provide major economic benefit to commercial catfish producers by enabling farmers to maintain fingerlings on feed throughout late summer and fall when ESC outbreaks occur. The effectiveness of current disease management tools, such as the use of vaccination and medicated feed may be enhanced in a resistant strain of fish. Phenotypic variation for resistance is evident among commercially available catfish strains (Dunham and Smitherman 1987; Wolters et al. 1996). Among USDA103 channel catfish, family-level susceptibility to experimental ESC challenge is consistent across multiple challenges in a given year (W.R.W., unpublished data), suggesting that there is a genetic basis for differences in ESC re-

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sistance. To capitalize on this variation, selective breeding efforts are currently underway for genetic improvement of ESC resistance, as well as growth and fillet and carcass yield in USDA103 channel catfish.

Mortality data alone may be less than ideal for selection criteria for genetic improvement of disease resistance (Gjedrem 1983; Beacham and Evelyn 1992). Although selection based on phenotypic response to pathogen challenge is important and has had success (Chevassus and Dorson 1990; Cipriano et al. 2002), a better understanding of host response and immune function will provide information that may improve efficiency of selection for increased disease resistance. Use of resistant and susceptible lines or families of fish in experimental challenges provides the means for direct comparisons of potentially different host response mechanisms.

In a two-family comparison of USDA103 channel catfish, ESC-resistant fish (as determined with prior challenges of the same full-sib groups) carried lower circulating bacterial loads than susceptible fish throughout the challenge period, although an increase in circulating bacterial concentrations occurred early in the challenge for both families (Bilodeau et al. 2003a). The lower bacterial loads measured in resistant fish is suggestive that innate immunity may be important in ESC resistance. During *E. ictaluri* challenge of USDA103 strain catfish, macrophage aggregation increased in resistant families in comparison to susceptible families (Camp et al. 2000). An early, nonspecific host response also exists in other teleosts when challenged with bacterial pathogens (Balfry et al. 2001; Villamil et al. 2003). Cell-mediated immunity (CMI), an adaptive response, has also been suggested to contribute to protection against ESC (Antonio and Hedrick 1994; Klesius and Sealey 1995; Shoemaker and Klesius 1997; Shoemaker et al. 1997; Thune et al. 1999). In direct comparison of ESC-resistant and susceptible strains of channel catfish, resistant strains carried lower antibody titers after experimental challenge, suggesting that this measure of adaptive immunity may have a reduced role in resistance (Wolters and Johnson 1994); however, reduced antibody titer may be the result of a limited response to lower bacterial loads. Despite this information, the specific mechanisms driving resistance to ESC remain unknown.

Factors such as stress may also influence disease resistance. Evidence in rainbow trout *Oncorhynchus mykiss* supports the role of an acute stress response in enhancing both cellular and humoral components of innate immunity (Demers and Bayne 1997). Cortisol is a common measure of stress in fish and is related to disease resistance in that an increase in cortisol levels is associated with peak bacterial loads in resistant fish (Bilodeau et al. 2003a). On the contrary, chronic stress results in a prolonged elevation of circulating cortisol and immunosuppression (Schreck 1996). A genetic predisposition in the degree of the stress response to pathogen exposure might play an important role in disease susceptibility.

In this paper we examine the relationship between pathogen loads, clearance, immune response, and stress response. This research expands on our previous study (Bilodeau et al. 2003a) by increasing the number of families examined, testing two immunologically important peripheral tissues (kidney and spleen), and assessing nonspecific immune response by measuring changes in lysozyme activity.

Methods

Experimental design and sample collection.— Resistance level was determined by prescreening 100 full-sib groups of USDA103 strain channel catfish from the USDA, Agricultural Research Service, Catfish Genetics Research Unit in Stoneville, Mississippi, by challenging them with virulent *E. ictaluri* and measuring mortality for 21 d. All fish were challenged with 10 mL of 10^9 colony-forming units (CFU)/mL virulent *E. ictaluri* culture with a 30-min static immersion challenge (Wolters and Johnson 1994). The prescreening challenge was replicated twice.

Six families (three ESC resistant and three ESC susceptible) from the 100 original families were selected based on mortality data from the prescreening challenges (Table 1). All fish were reared in the hatchery and were naïve to *E. ictaluri*. Juvenile fish (mean = 21.8 g, SE = 3.6) were stocked in 75-L aquaria according to the following regime: 10 fish/aquarium, 3 aquaria/full-sib group per day of sampling. For each full-sib group, a total of 17 aquaria (16 exposed to *E. ictaluri* and 1 nonexposed control) and 170 fish were used. Fish were acclimated for 7 d before challenge and were sampled just before challenge (control) and at 1, 2, 5, 12, and 20 d postchallenge. Aquaria were supplied with well water (26°C; pH, 8.6; total ammonia nitrogen, <1.5 ppm; nitrite nitrogen, 0 mg/mL) at a continuous flow rate of 8 L/min. All fish (except for controls) were challenged as described above and then harvested from a tank on each sample day. Of the nonexposed controls, five fish from

TABLE 1.—Mortality data for six families of channel catfish with different susceptibilities to enteric septicemia of catfish (ESC). Prior susceptibility is the mean (±SE) mortality percentage that was determined in two previous immersion challenges of 100 channel catfish families (USDA103 strain) with virulent *Edwardsiella ictaluri*, the causative bacterium in ESC. Mortality data is from the present study of six USDA103 channel catfish families challenged with *E. ictaluri*. Type mortality is the mean (±SE) of the three families in each type (resistant or susceptible).

Family identity	Type	Prior susceptibility (%)	Mortality (%)	Type mortality (%)
183	Resistant	25 ± 6.4	18.3	
208	Resistant	16 ± 1.2	31.7	28.9 ± 5.5
232	Resistant	19 ± 2.9	36.7	
268	Susceptible	82 ± 9.2	63.3	
287	Susceptible	85 ± 8.7	46.7	69.4 ± 15.2
294	Susceptible	88 ± 6.9	98.3	

each family were harvested before challenge and another five were harvested on day 20 of the challenge and tested for the presence of *E. ictaluri*.

Head kidney, spleen, and whole blood (200 μL treated with sodium heparin) were collected from all fish in three replicate aquaria per family at 1, 2, 5, 12, and 20 d postexposure. Fish were removed from each tank, anesthetized with 0.6 mg/L metomidate hydrochloride (Small 2003) before blood collection and then overdosed in a 200-mg/L solution of MS-222 (tricaine methanesulfonate) before collection of head kidney and spleen. Metomidate hydrochloride blocks the handling-related release of cortisol into circulation, thus decreasing plasma cortisol variability due to sampling (Small 2003). Following centrifugation, plasma was collected from 100 μL of the heparinized blood and stored at −80°C until cortisol analyses were conducted.

Pathogen detection and quantification.—Genomic DNA was extracted from 100 μL of each blood sample via the High-Pure PCR Template Preparation Kit (Roche Applied Science); 5 μL of lysozyme (10 mg/mL) was added to each sample, which was then incubated at 37°C for 15 min before adding binding buffer. All samples were eluted in 100 μL prewarmed (70°C) elution buffer. Genomic DNA was extracted from kidney and spleen samples by overnight digestion with 50 μg/mL proteinase K at 55°C, followed by protein precipitation with 7.5 M NH_4OAc and DNA precipitation with isopropanol. An *E. ictaluri*-specific target sequence was then amplified via a validated real-time polymerase chain reaction (PCR) assay that enabled direct quantification of bacterial DNA per cell equivalent (Bilodeau et al. 2003b). All real-time PCR reactions were carried out on a Bio-Rad iQ iCycler (results expressed in cell-equivalent units).

The number of *E. ictaluri* cells per milliliter of blood sample was enumerated using standard plate count methods on tryptic soy agar plates supplemented with 5% sheep's blood (Wise and Terhune 2001). An aliquot of 50 μL of whole blood from each fish sampled was plated on brain–heart infusion agar plates and incubated for 48 h at 26°C.

Cortisol and lysozyme analyses.—Plasma cortisol concentrations were determined using a time-resolved fluoroimmunoassay (TR-FIA) kit (R060–101; PerkinElmer Life Sciences, Akron, Ohio) modified and validated for channel catfish (Small and Davis 2002). The TR-FIA satisfied strict criteria of precision (intra-assay CV, <7%) and reproducibility (interassay CV, <10%). Accuracy of the TR-FIA, calculated as the percent of exogenous cortisol recovered from spiked catfish plasma, averaged 99.5%. Assay sensitivity (minimum detection limit) in catfish plasma was 1.2 ng/mL, and the displacement curve for serially diluted channel catfish plasma paralleled the cortisol standard curve.

Plasma lysozyme activity was determined using the EnzChek lysozyme assay kit (E22013; Molecular Probes, Eugene, Oregon). 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 g/mL) for 30 min at 37°C. The fluorescence was measured in a fluorescence microplate reader using an excitation wavelength of 485 nm and emission wavelength 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.

Data analysis.—Bacterial levels (cell equivalents) for each family were log-transformed and subjected to analysis of variance (ANOVA) to determine differences between families (GLM procedure: SAS 2001. Clearance rate of the pathogen

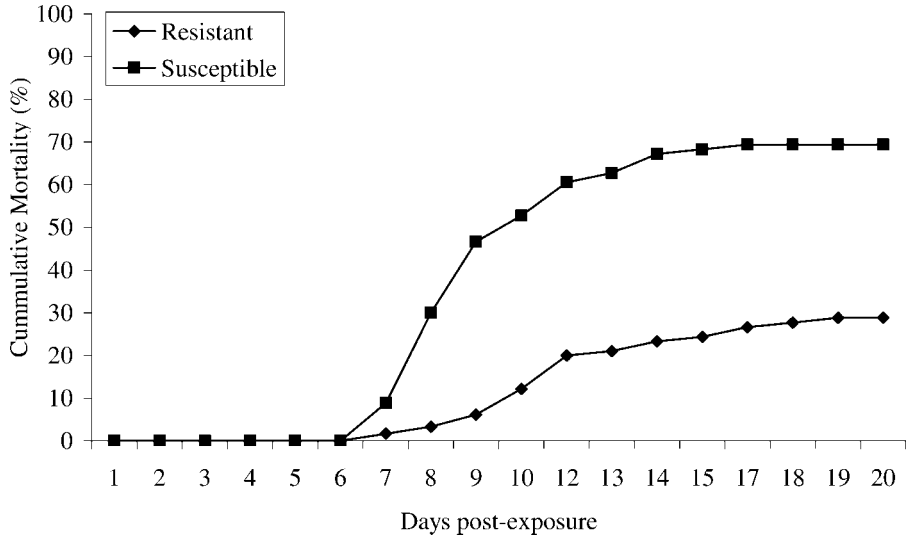

FIGURE 1.—Cummulative mortality following exposure to virulent *Edwardsiella ictaluri*, the causative bacterium in enteric septicemia of catfish (ESC), of ESC-resistant and ESC-susceptible channel catfish families. Resistance level was determined by prescreening 100 full-sib groups by challenge with *E. ictaluri* and measuring mortality for 21 d. Based on mortality levels in the prescreening trials, three resistant and three susceptible full-sib groups were chosen and challenged with *E. ictaluri* and then monitored for 20 d.

was measured as the change in pathogen loads (cell equivalents) over time and was assessed by performing a linear regression and comparison of slopes for days 5 through 21 postexposure for each family (GLM procedure of SAS; Armitage 1980). Plasma cortisol concentrations and lysozyme activity for each family were subjected to ANOVA to determine differences between families (GLM procedure of SAS). Correlations between mortality, bacterial levels, and cortisol levels were determined using the CORR procedure of SAS.

Results and Discussion

Mortalities first occurred on day 7 postchallenge and continued for approximately 12 d (Figure 1). The mortality (cumulative mortality and days to first mortality) observed in this study was consistent with previous studies of channel catfish challenged with *E. ictaluri* (Wolters and Johnson 1994; Wise et al. 1997; Bilodeau et al. 2003a). The differences in mortality rates between susceptible and resistant families were consistent with differences in two previous *E. ictaluri* challenges (Table 1) that included the same families tested here and with other families from the same generation (Bilodeau et al. 2003a). There was some change in mortality levels in our present study compared with the levels in the prescreening trials (Table 1, prior susceptibility). This variation may be due to differences among individuals within each family. However differences in mortality levels between the resistant and susceptible families remain clear (Table 1; Figure 1).

Statistical analyses showed no tank effect for pathogen levels in blood ($P = 0.2654$), kidney ($P = 0.6212$), or spleen ($P = 0.5523$), so tank and family data were pooled by resistance level. Resistant and susceptible families differed in their response to *E. ictaluri* challenge in blood ($P = 0.0038$), kidney ($P = 0.0008$), and spleen ($P = 0.005$; Figure 2). Bacterial levels (both bacterial DNA and viable bacterial cells) on day 5 were lower in resistant families ($P < 0.001$) than susceptible families ($P < 0.0001$; Figures 2, 3). There was a high positive correlation between bacterial DNA levels in the blood and bacterial plate counts, as well as DNA levels in spleen and kidney ($P < 0.0001$ for all correlations). The difference in bacterial loads between resistant and susceptible fish indicates that fish from the resistant families were able to limit the systemic infection in early stages of the challenge. Bilodeau et al. (2003a) demonstrated that pathogen loads in a resistant channel catfish family were suppressed early in a challenge with virulent *E. ictaluri*. A similar suppression of pathogen loads via innate immunity was indicated for coho salmon *O. kisutch* with innate resistance to the bacterial pathogen *Vibrio anguillarum* (Bal-

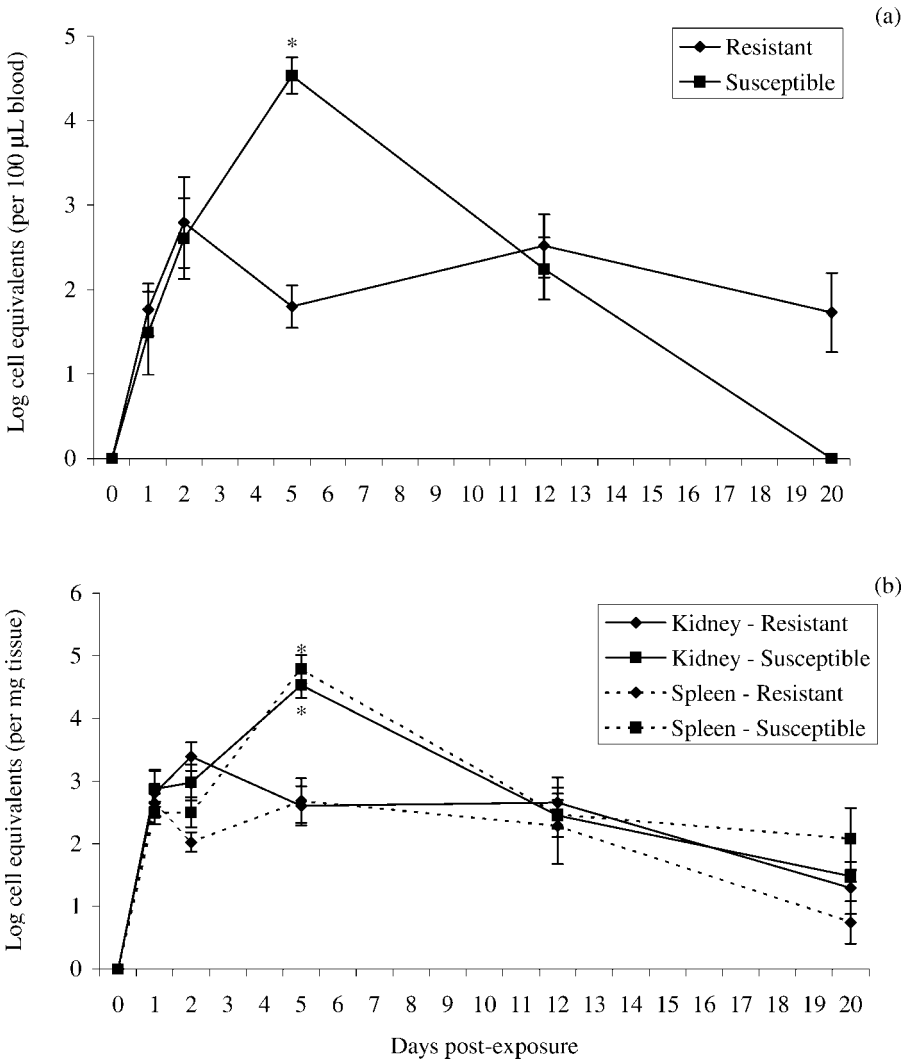

FIGURE 2.—Means (±SEs) of log-transformed bacterial cell-equivalents in channel catfish tissues after exposure to virulent *Edwardsiella ictaluri*, the causative bacterium in enteric septicemia of catfish (ESC). Panel **(a)** shows bacterial cell equivalents in 100-µL whole blood samples, panel **(b)** bacterial cell equivalents per milligram of anterior kidney and spleen tissue. A total of 170 fish per ESC-resistant and ESC-susceptible family were challenged with virulent *E. ictaluri* and sampled over 20 d. Bacterial loads were measured through direct quantification of bacterial DNA with real-time polymerase chain reactions. Asterisks represent significant differences ($P < 0.05$) between resistant and susceptible families on a given day.

fry et al. 2001) and for rainbow trout (Fevolden et al. 1992). Together, these results suggest that an early response by the nonspecific immune system may limit the severity of systemic infection in resistant lines of fish. This is supported by change in lysozyme activity measured in our present study.

Among fish from the resistant families, plasma lysozyme activity was elevated ($P < 0.0001$) throughout the challenge compared with basal levels; however, the timing of activation differed (Figure 4). The increase in lysozyme activity in susceptible fish ($P < 0.0001$) was delayed by 24 h (until day 2), after which levels became higher than in resistant fish ($P = 0.0001$). On day 2, when lysozyme activity increased in susceptible fish, pathogen loads in the same fish had already increased ($P = 0.0301$). During the challenge, lysozyme activity was higher ($P = 0.0074$) in resistant families 1 d postexposure and higher ($P =$

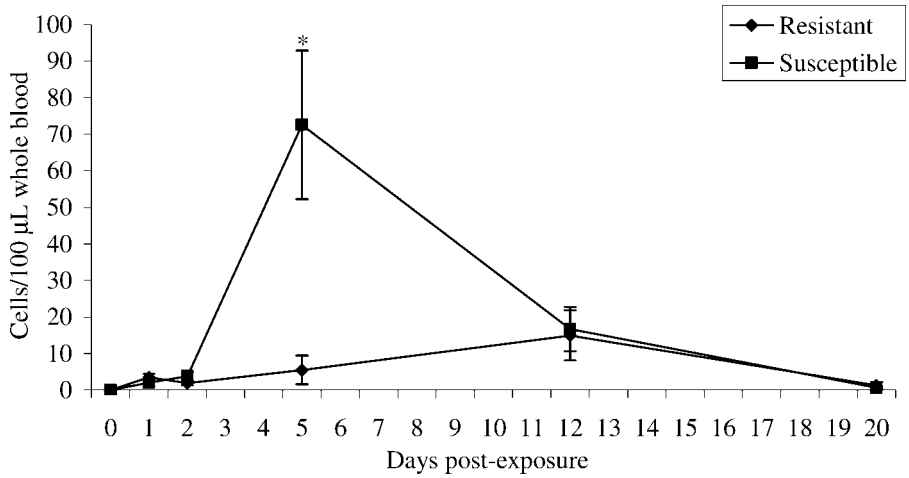

FIGURE 3.—Means (±SEs) of bacterial cell counts in whole blood of channel catfish after exposure to virulent *Edwardsiella ictaluri*, the causative bacterium in enteric septicemia of catfish (ESC). A total of 170 fish per ESC-resistant and ESC-susceptible family were challenged with *E. ictaluri* and sampled over 20 d. Bacterial cell counts were enumerated with standard plate counts. Asterisks represent significant differences ($P < 0.05$) between resistant and susceptible families on a given day.

0.0001) in susceptible families 5 d postexposure. The timing of peak lysozyme activity in susceptible fish was coincident with peak pathogen levels in all tissues ($P < 0.05$). The 24-h delay and coincident increase in pathogen levels in susceptible fish suggests that the timing of the nonspecific immune response measured in resistant families contributed to limiting infection levels.

Elevated pathogen levels probably contribute to increased mortality, and ability to remove a pathogen from tissues (i.e., pathogen clearance) affects survivorship. Although there was no evidence (*P*

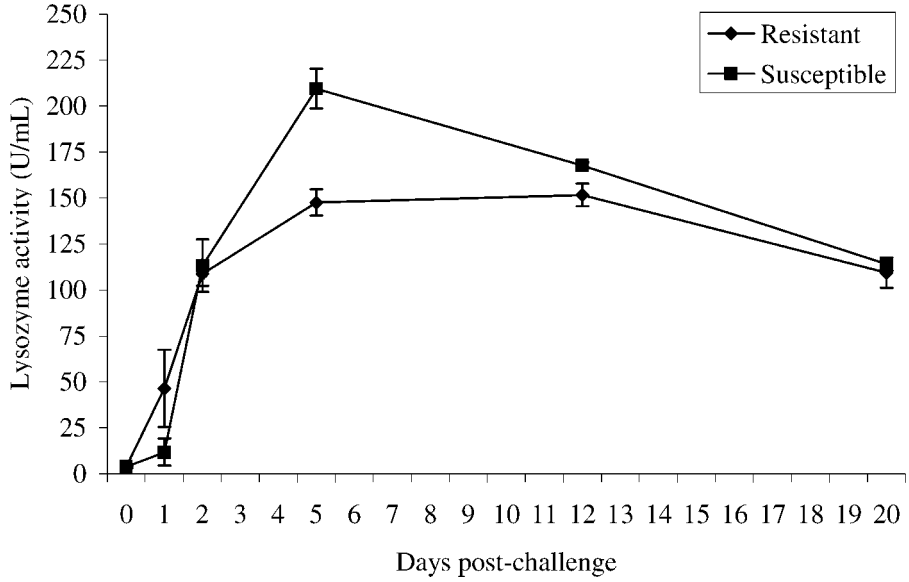

FIGURE 4.—Means (±SEs) of plasma lysozyme activity during challenge with *Edwardsiella ictaluri*, the causative bacterium in enteric septicemia of catfish (ESC), for ESC-resistant and ESC-susceptible families of channel catfish. Lysozyme activity was elevated throughout challenge ($P < 0.0001$) for resistant families and after day 2 ($P < 0.0001$) in susceptible families. Asterisks represent significant differences ($P < 0.05$) between resistant and susceptible families on a given day.

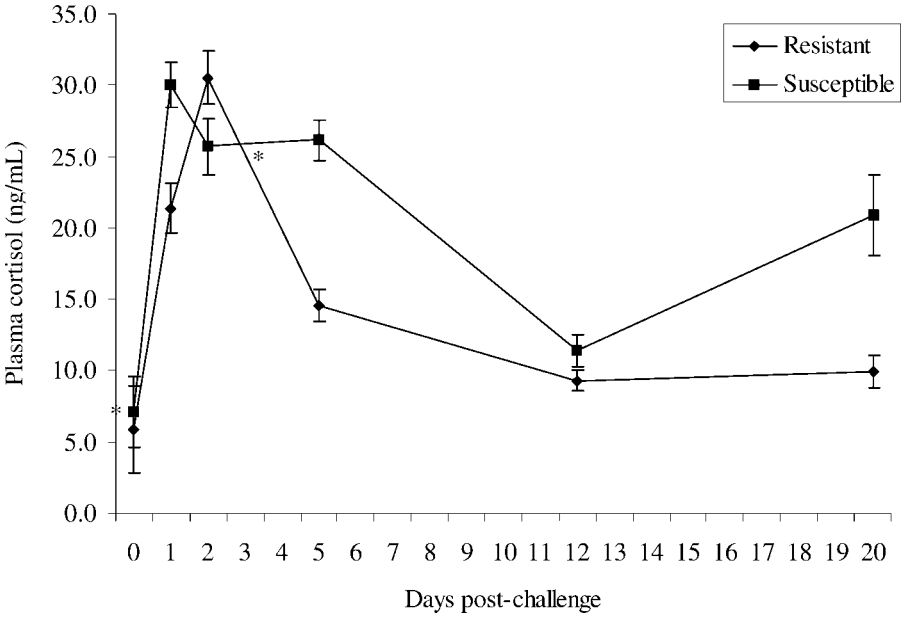

FIGURE 5.—Means (±SEs) of plasma cortisol levels during challenge with *Edwardsiella ictaluri*, the causative bacterium in enteric septicemia of catfish (ESC), for ESC-resistant and ESC-susceptible families of channel catfish. Resistant and susceptible families differed in their cortisol response both overall ($P < 0.0034$) and by day ($P < 0.0001$); however, the interaction of the two effects (type and time) was not significant ($P > 0.05$).

> 0.05) of clearance for resistant families in blood pathogen levels throughout the trial, some pathogen clearance did occur in the kidney ($P = 0.0046$) and spleen ($P = 0.0077$) on day 20 (Figure 2). Conversely, susceptible families exhibited pathogen clearance in all tissues by day 20 ($P < 0.0001$). The pronounced clearance from blood in susceptible fish may be an artifact of measuring pathogen levels only in surviving fish. Fish that survived the acute infection either had some innate resistance, or were able to clear an active infection from the blood. Residual bacterial loads in the kidney and spleen suggest that *E. ictaluri* cells remain in tissues and are probably harbored in macrophages, either as a carrier state or as cells that will be eventually eliminated. *Edwardsiella ictaluri* is known to survive and even multiply in macrophages of susceptible fish (Ciembor et al. 1995). Further study into duration of infection, even chronic infection, is needed.

Plasma cortisol levels were elevated ($P < 0.0001$) in both resistant and susceptible families; however, fish from the susceptible families had higher cortisol levels than fish from the resistant families ($P = 0.0034$; Figure 5). The cortisol response to pathogen exposure observed in this study was rapid among both susceptible and resistant families. The degree of the cortisol response suggests the fish were stressed by the presence of the pathogen. Plasma cortisol levels for channel catfish associated with moderate acute stress typically range from 30 to 60 ng/mL (Small and Davis 2002; Small 2004). After the initial increase, cortisol levels remained elevated throughout the challenge, except for day 12 in susceptible fish, but decreased immediately to near basal levels in resistant fish.

Although both chronic and acute stress in fish can result in immunosuppression and increased disease susceptibility (Schreck 1996), there is evidence that over the short term cortisol plays a positive role in innate immune response and disease resistance (Maule et al. 1989; Demers and Bayne 1997; Bilodeau et al. 2003a). However, there was no correlation ($P > 0.05$) between cortisol and either blood pathogen levels or lysozyme activity. This does not support an association of cortisol levels with innate immune response in channel catfish. The delay in cortisol response until day 2 in resistant fish followed by immediate recovery suggests that reduced pathogen loads may limit stress and associated immunosuppression. Environmental stress increases mortality in channel catfish exposed to the bacterium *Aeromonas hydrophila* (Walter and Plumb 1980), and crowding stress increases mortality in channel cat-

fish exposed to *E. ictaluri* (Wise et al. 1993). Administration of exogenous cortisol to channel catfish appears to have no effect on susceptibility to channel catfish virus and a dose-dependant effect on susceptibility to *Ichthyopthirius multifiliis* (Davis et al. 2003).

This study demonstrates two patterns of disease kinetics in resistant and susceptible families of channel catfish. Both innate and adaptive immunity probably contribute to these patterns. An early host-response mechanism, such as innate immunity, may be delayed and less effective in susceptible families and lead to increased levels of infection and increased stress, as indicated by cortisol levels. Although there were no differences between families at early stages of the challenge, blood-borne pathogen loads among resistant families remained constant after day 1 of challenge. In resistant families the nonspecific immune response, as indicated by lysozyme activity, was probably effective during early stages of infection, thereby reducing pathogen loads that subsequently prevented high levels of mortality at later stages of infection. To better assess such a mechanism, future research should include the measurement of pathogen loads and innate immune response throughout the first 5 d of challenge.

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