Journal of Animal and Veterinary Advances 5 (3) : 208-214, 2006

Linking Stress to Increased Mortality of Channel Catfish at Varying Concentrations of *Edwardsiella ictaluri*

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Abstract: This study examined the effects of stress and pathogen concentration on ESC susceptibility in juvenile catfish. Three stress/disease challenges were conducted at concentrations of 1.4, 0.7 and 0.4 Log colony forming units/L of *Edwardsiella ictaluri*. Twenty-five fingerling catfish per tank were confined to plastic nets for 30 or 60 min, while unstressed fish remained free swimming. Blood samples were taken for cortisol analysis and the pathogen was administered at the conclusion of the stress period. Mortalities were monitored for 21 d. Cortisol concentrations differed ($p<0.05$) among stressor treatments in the high challenge. Additionally, increases in mortality occurred in conjunction with increases in cortisol (unstressed: 22.5±4.2, 30 min: 47.5±5.2, 60 min: 81.7±6.8%). The medium concentration challenge exhibited similar results for cortisol concentrations. Mortality rates were similar to the high dose challenge (unstressed: 7.5±4.2, 30 min: 60.8±13.9, 60 min: 94.2±5.8%). While cortisol concentrations were similar for the low dose challenge, mortality rates were lower (unstressed: 4.2±3.8, 30 min: 18.3±8.2, 60 min: 27.5±5.2%) but still significantly different from each other. This study demonstrates (1) both confinement stress and bacterial pathogen concentration affect disease susceptibility of channel catfish and (2) stress and the concurrent physiologically high concentrations of cortisol are more highly correlated with disease susceptibility than bacterial pathogen load.

Key words: Stress, *Edwardsiella ictaluri*, disease, cortisol, challenge

INTRODUCTION

Channel catfish, Ictalurus punctatus (Rafinesque, 1818), production is the largest aquaculture industry of the United States[1]. Commercial catfish production is up 4% while acres of water in production are down 3% [2]. As production intensifies and fish become more concentrated, diseases become more prevalent. Enteric Septicemia of Catfish (ESC) caused by the bacterium *Edwardsiella ictaluri*, is a serious infectious disease of commercially raised channel catfish. A recent United States Department of Agriculture (USDA) survey, Catfish 2003, found that 52.9% of all fry/fingerling operations experienced losses due to ESC for the previous two years. ESC accounted for the largest fry/fingerling losses at 24.8% of total disease fatalities[3]. While other diseases affect the channel catfish industry, the excessive occurrence of ESC at commercial facilities has caused this disease to become the focus of scientific disease research in channel catfish.

ESC treatment is limited to two USDA approved antibiotics for fish: Ormetoprim combined with sulfadimethoxine (Romet-30® by Alpharma Inc.) and oxytetracycline HCL (Terramycin® by Pfizer, Inc.)[4]. Low cost effectiveness of antibiotics and limited treatment options are a hindrance for the treatment of ESC outbreaks. Total catfish antibiotic use is estimated at 126,000 to 252,000 pounds annually for the treatment of ESC. Even with substantial drug use, losses in catfish production remain as high as 60 percent, with diseases accounting for by far the largest portion[5]. Several isolates of *E. ictaluri* have already become resistant to approved antibiotics[6,7]. The efficacy of current vaccinations has proved to be inconsistent and the use of vaccines has produced mortality in channel catfish[8].

The increasing resistance of *E. ictaluri* strains to approved antibiotics and limited success of vaccines has prompted research on other ESC prevention methods. Assessment of stress effects on disease susceptibility indicates a potential method of disease reduction. Channel catfish exposed to stress events are more susceptible to ESC than nonstressed fish (81% opposed to 55% mortality). Also, increased exposure time to *E. ictaluri* after stress resulted in increased mortality[9]. Ciembor *et al.*,[10] confirmed these results with stressed fish exposed to an *E. ictaluri* bath, exhibiting greater

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infection rates compared to intraperitoneal injected fish and unstressed control groups (52.7, 46.6 and 15.7%, respectively).

Wise *et al.*,[9] incorporated stress with disease challenges, but a physiological indicator of level of stress was not used. Channel catfish stress response varies depending upon environmental and production factors such as density and water quality. Physiological changes such as increases in cortisol found in channel catfish exposed to a variety of stressors have been extensively documented[11-13]. Stress induced by netting or seining during stock out, grading, or pond transfer increases susceptibility to ESC. Increased catfish mortality rates due to ESC caused by handling and confinement stress are correlated to increases in cortisol concentrations[14].

Several studies have demonstrated the link between increased infection rates of ESC and stress[9,10,14]. However, these studies typically use inflated levels of *E. ictaluri* for infection. Wise *et al.*,[9] address the shortcomings of inflated bacterial concentrations suggesting that they do not adequately reflect natural routes of infection. Studies often use LD-30 or LD-50 vinfection levels to insure separation of treatment groups [9,14,15].

The main objective of this study was to examine the effects of stress on catfish mortality when *E. ictaluri* concentrations were reduced from experimental LD-30 levels to concentrations that more accurately reflect natural levels of bacteria in culture ponds. No accurate data currently exist as to the actual concentrations of *E. ictaluri* in ponds during infectious outbreaks of ESC. While higher concentrations of bacteria may be necessary for testing procedures in laboratories, this study should help demonstrate the relationship between amount of stress and infection susceptibility in culture practices.

The second objective of this study was to examine cortisol as an indicator of stress for examining increases in mortality due to ESC. Releases of cortisol after stress events are associated with immune system suppression that can lower disease resistance[14,16,17]. Although the means of immune suppression by cortisol in fish is not fully understood, it has been thoroughly documented in mammals[18]. Weyts *et al.*,[19] refute cortisol's role as a direct immune-suppressant in fish, but increased production of cortisol during stress is associated with decreased immune system function.

MATERIALS AND METHODS

Fish: Fingerling channel catfish were obtained from commercial aquaculture ponds at Greenwater Fish Farm (Gibson County, Tennessee). Fingerlings ranged from 62-105 mm (mean 85.3 mm, SD 6.9 mm) in length. The fingerlings were hatched on-site from eggs of broodstock maintained at the facility and stocked into commercial grow-out ponds with no previous outbreaks of *E. ictaluri*. The fingerlings exhibited no clinical signs of infection. Plate isolates of Brain-Heart Infusion (BHI) agar from a subsample (8 fish) did not produce any colonies of *E. ictaluri* after incubation at 30°C for 72 h. Additional subsamples (8 fish) failed to generate any colonies of *E. ictaluri* in BHI broth after incubation at 30°C for 48 h.

***E. ictaluri*:** A test strain isolate of *E. ictaluri* was obtained from a natural outbreak in a commercial catfish operation (Auburn University Fish Health Laboratory). The identity of this isolate was verified by biochemical isolation tests described by Hawke *et al.*,[20] and produced API 20E analytical profile index codes of 4004000. To test virulence, the isolate was propagated with BHI broth in a shaking bath at 27°C for 48 h. Ten fish (73-91 mm) were exposed to 5.6 Log colony-forming units per L (Log CFU L^{-1}) in 85-L tanks for each isolate. The exposed fish were reared for 14 d with all exhibiting one or more clinical signs of ESC infection. *E. ictaluri* was reisolated from the posterior kidney for the test strain of *E. ictaluri*. The identity of the test strain isolate was verified using the API 20E. The BHI broth containing the isolate was then frozen in 20 aliquots of 1 mL for the challenges. These aliquots of isolate were cultured for use in the challenges.

Experimental system: The experimental system contained 24 independently maintained 85-L tanks with four banks of six tanks. Each tank was operated in a flow-through mode with filtered city water supplied at 2.2 L/min. The water was filtered with activated carbon and total chlorine content was less than 0.03 mg L^{-1}. Water temperatures were maintained between 24 and 27°C. An air stone was used in each aquarium for supplemental aeration to maintain dissolved oxygen levels. Water quality was monitored for dissolved oxygen (>6 mg L^{-1}), temperature (25±2°C) and total chlorine (<0.03 mg L^{-1}). Water quality was tested weekly for pH (7.0-7.3) and total ammonia (<1.0 mg L^{-1}). Lighting was automatically controlled by timer with a 16 h light /8 h dark cycle. The fingerlings were fed Zeigler slow-sinking finfish starter (50% protein, 15% fat) at a rate of 3% of body weight daily.

***E. ictaluri* lethal dosage determination:** A lethal dose 30 % (LD-30) was performed to determine the appropriate concentration of *E. ictaluri* for the experimental disease challenges[14]. Mortality in each tank was monitored for 21 d.

Table. 1: Percent mortality of channel catfish, *Ictalurus punctatus* (Rafinesque, 1818), due to enteric septicemia of catfish infection and cortisol levels for bacterial dosage groups. Three *Edwardsiella ictaluri* challenges (N=450 each) were used: high (1.4 Log CFU L^{-1}), medium (0.7 Log CFU L^{-1}) and low (0.4 Log CFU L^{-1}) bacterial dosage. Three net confinement stress levels were used for each challenge: 0 min or non-stressed, 30 min net confinement or 60min net confinement

Dose group	Bacterial concentration	Stress time (min)	Mortality %±SE	Cortsisol (ng/mL)±SE
		0	22.5±1.7	23.6±2.5
High	1.4 Log CFU L^{-1}	30	47.5±2.1	77.7±7.8
		60	81.7±2.8	115.4±10.9
		0	7.5±4.2	10.5±3.5
Medium	0.7 Log CFU L^{-1}	30	60.8±13.9	88.5±10.6
		60	94.2±5.8	129.5±31.5
		0	4.2±3.8	6.7±3.4
Low	0.4 Log CFU L^{-1}	30	18.3±8.2	80.5±10.0
		60	27.5±5.2	121.3±39.7

Stress inducement and blood collection: The stress model used in this study was based on previous work by our group[14]. A non-pathogenic stress challenge was previously conducted to determine appropriate levels of stress as indicated by cortisol for the disease challenge as reported in Sink *et al.*,[14]. Stress was induced in 25 fish from each tank by confinement in baskets (12.5x15x10-cm) constructed of rigid vinyl netting. Stress treatments included confining fish in the nets for 30 or 60 minutes, with Time 0 treatment, or non-stressed fish remaining free swimming in the tanks. Each treatment was assigned to six tanks per bank. Start times for stress were staggered by five min with actual treatment time beginning when the last fish was netted from the tank and placed into the net. Fish were anesthetized in 150 mg tricaine/L (MS-222) upon removal from basket or tank prior to bleeding. Blood collection, storage and radioimmunoassays were conducted as in Sink and Strange[14].

Challenge dose regimens: For the high disease dose, twenty-five fingerling catfish were stocked into each of 18 tanks (total N = 450). The fingerlings were acclimated for 14 d. Stress treatments were applied to six tanks per bank for each of the three stress treatments (0, 30 or 60 min of net confinement). Five fish were removed from each tank and blood samples were drawn for cortisol analysis at the conclusion of each treatment. The remaining fish (N=20 per tank) in the basket were immediately released and water flow was discontinued for 24 h with supplemental aeration remaining constant. Each tank was dosed with 1.4 Log CFU L^{-1} of *E. ictaluri* (20 mL of inoculated BHI @ $1x10^6$ CFU). Water flow was restored and the bacteria were flushed after the 24 h period. Mortality in each tank was monitored daily for 21 d.

Medium and low dose disease challenges (N=450 for each challenge) were conducted in the same manner as above with the medium dose consisting of a challenge with 0.7 Log CFU L^{-1} of *E. ictaluri* (10 mL of inoculated BHI @ $1x10^6$) and a low dose consisting of a challenge with 0.4 Log CFU L^{-1} of *E. ictaluri* (5 mL of inoculated BHI @ $1x 10^6$).

Statistical analysis: Differences in cortisol and mortality among stress levels were tested using paired-sample t-tests and ANOVA. Differences were considered significant at $p<0.05$. Pearson correlation was used to measure the relationship between cortisol level and mortality. A simple linear regression was performed to estimate mortality rate based on cortisol level for each concentration of bacteria.

RESULTS

High dose disease challenge: A one-way ANOVA was used to compare differences among mortalities for the net confinement stress levels. Mortality levels differed ($p<0.05$) among the net confinement stress levels (Table 1). A one-way ANOVA was also used to compare differences among cortisol levels for the net confinement stress levels. Cortisol levels were also different ($p<0.05$) among the net confinement stress levels (Table 1).

A Pearson correlation analysis was performed to determine if a relationship exists between cortisol level and percent mortality. A strong relationship was found between cortisol level and percent mortality, with a Pearson correlation coefficient of 0.861 ($p<0.01$ two-tailed test). Simple linear regression was used to determine if cortisol level indicates trends in mortality rate (Fig. 1). The R^2 analysis demonstrated that 74% of mortality differences could be explained by cortisol level ($p=0.001$). The regression line equation for the high dose was determined to be 13.5 ng mL^{-1} + 0.51 x cortisol ng mL^{-1} (95% confidence intervals of 0.09 to 26.91 for intercept and 0.35 to 0.67 for slope).

Medium dose disease challenge: Mortality levels differed ($p<0.05$) among the net confinement stress levels (Table 1). A one-way ANOVA was also used to compare differences among cortisol levels for the net confinement stress levels. Cortisol levels were also different ($p<0.05$) among the net confinement stress levels (Table 1).

A Pearson correlation analysis was performed to determine if arelationship exists between cortisol level

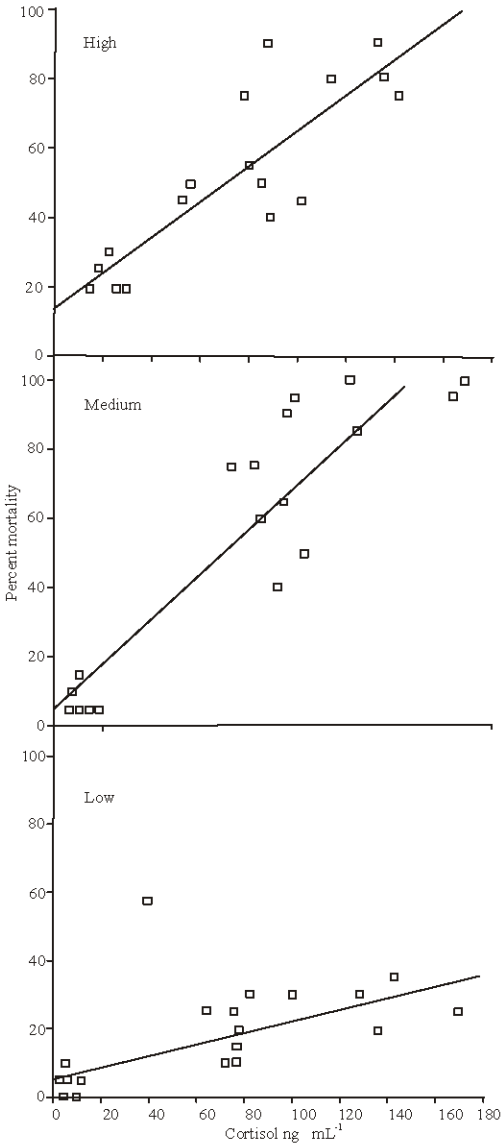

Fig. 1: Regression of mortality rate of channel catfish, *Ictalurus punctatus* (Rafinesque, 1818), (N=450 times three trials) due to enteric septicemia of catfish on cortisol level for high, medium and low bacterial concentration disease challenge. Mortality rate is expressed as percent mortality per replicate (N=6) per net confinement stress level (N=3). Cortisol is expressed as mean cortisol (ng/mL) per tank per stress treatment. For the high concentration challenge R^2 = .74, (P=0.001). Regression line: 13.5 ng/mL + 0.51 x cortisol ng/mL (95% confidence intervals of 0.09 to 26.91 for intercept and 0.35 to 0.67 for slope). For the medium concentration challenge R^2 = .83, ($p<0.001$). Regression line: 5.6 ng mL^{-1} + 0.64 x cortisol ng mL^{-1} (95% confidence intervals of –8.43 to 19.69 for intercept and 0.49 to 0.79 for slope). For the low concentration challenge R^2 = .67, ($p<0.001$). Regression line: 4.6 ng mL^{-1} + 0.17 x cortisol ng/mL (95% confidence intervals of –1.01 to 10.27 for intercept and 0.11 to 0.24 for slope)

and percent mortality. A strong relationship between cortisol level and percent mortality was found, as indicated by a Pearson correlation coefficient of 0.912 ($p<0.01$ two-tailed test). Simple linear regression was conducted to determine if cortisol level indicates trends in mortality rate (Fig. 1). The R^2 analysis confirmed 83% of mortality differences could be explained by cortisol level ($p<0.001$). The regression line equation for the medium dose was determined to be 5.6 ng mL^{-1} + 0.64 x cortisol ng mL^{-1} (95% confidence intervals of –8.43 to 19.69 for intercept and 0.49 to 0.79 for slope).

Low dose disease challenge: Mortality levels differed ($p<0.05$) among the net confinement stress levels (Table 1). A one-way ANOVA was also used to compare differences among cortisol levels for the net confinement stress levels. Cortisol levels were also different ($p<0.05$) among the net confinement stress levels (Table 1).

A Pearson correlation analysis was performed to determine if a relationship exists between cortisol level and percent mortality. A strong relationship was found, with a Pearson correlation coefficient of 0.817 ($p<0.01$ two-tailed test). Simple linear regression was conducted to determine if cortisol level indicates trends in mortality rate (Fig. 1). The R^2 analysis showed that 67% of mortality differences could be explained by cortisol level ($p<0.001$). The regression line equation for the medium dose was determined to be 4.6 ng/mL + 0.17 x cortisol ng mL^{-1} (95% confidence intervals of –1.01 to 10.27 for intercept and 0.11 to 0.24 for slope).

DISCUSSION

Channel catfish fingerlings are often exposed to acute stressors in the presence of *E. ictaluri* during production. Acute stressors increase the mortality of channel catfish fingerlings to ESC in experimental situations[9,10]. However, it is not known if these experiments accurately reflect what is natural bacterial concentrations occurring in pond production. With no reliable data on the concentrations of *E. ictaluri* present in ponds during outbreaks of ESC, it is not known if stressors or bacterial concentration play a greater role in mortality of channel catfish.

Additionally, high physiological cortisol concentrations during stress responses suppress the

immune system function by suppressing neutrophil phagocytic function[21], decrease growth rates and inhibit the reproduction of fish[22]. This information has led us to suspect that the degree of stress may play a greater role in ESC infection than actual concentrations of *E. ictaluri* present. Such information may be important to producers as production practices intensify and fish are grown at greater densities, which may increase stresses experienced by the fish. This study demonstrates that *E. ictaluri* concentrations are not always the main factor in catfish susceptibility to ESC.

Stressed fish at all concentrations of *E. ictaluri* demonstrated significant increases in cortisol concentration and significant increases in mortality, when compared to fish not subjected to stress. Mortality rates of non-stressed fish remained lower than stressed groups, although the non-stressed group for the high concentration was similar to the Time 30 treatment for the low concentration dose. This indicates that both stress and bacterial concentration play key roles in susceptibility to ESC. Mortality levels increased from the high dose group to the medium dose group when bacterial levels were lowered.

This increase in mortality at a lower bacterial concentration may indicate that stress has a greater impact than bacterial concentrations. This is likely attributed to greater cortisol concentrations in the medium dose group. The increased cortisol concentration confirms that the fish were more stressed and the Pearson correlation coefficient confirms a greater correlation with mortality in the medium dose group compared to high dose group. A lowered concentration of *E. ictaluri* did not reduce mortality rates.

The low dose group did exhibit decreased mortality rates when compared to the high and medium dose groups, but percent mortality continued to increase with stress and cortisol concentration. This may again indicate that stress and bacterial concentration both play key roles in ESC susceptibility. However the low dose group continues to show that increases in stress as indicated by cortisol level lead to increased mortality due to ESC even at decreased bacterial concentrations. Groups exhibiting greater stress and cortisol concentrations within the same challenge dose treatment exhibited greater mortality in all three trials These results denote that stress is a larger factor in susceptibility of channel catfish to ESC than concentration of *E. ictaluri* in the water.

Increases in cortisol concentrations were found to be proportional to increases in stress in all challenges. Cortisol concentrations were highly correlated with the tightly grouped mortality rates for each of the six replicates in each dose challenge. This indicates that cortisol concentrations are linked to mortality rates at various concentrations of bacteria. The regression models for cortisol by mortality exhibited definite linear trends at every concentration. Cortisol appears to be a clear clinical indicator of increased risk to ESC in fingerling catfish. The regression models support this claim in channel catfish of this size for each concentration.

This study also confirms that the stress hormone cortisol is highly correlated with increased susceptibility of channel catfish to ESC infection and mortality. The regression models indicate that greater than 67% of catfish mortality may be explained by cortisol for all three concentrations of bacteria. The models for high and medium dose can explain 74 and 83% of the mortality rate, respectively based on cortisol alone. This does not mean that cortisol acts alone in increased susceptibility.

The mechanism of cortisol's immune suppression in fish is still unknown at this time. Authors debate whether cortisol itself plays a role in immune suppression or if it plays a secondary role. Maule and Schreck[23] assert that increased cortisol concentrations in juvenile coho salmon cause a redistribution of leukocytes to the thymus and anterior kidney, resulting in a reduction of leukocytes in the blood and spleen. Alford *et al.*,[24] found that apoptosis of leukocytes was lower in stressed channel catfish than in non-stressed fish. The above studies indicate a direct effect of stress on individual leukocyte cells, causing them to remain active for a longer period of time, rather than cell lysis or redistribution. Handling and transport stress causes a decrease of lymphocytes and an increase of circulating neutrophils in the blood [21]. These researchers also report that cortisol alone does not cause immune suppression of phagocyte function but that high physiological concentrations of cortisol can initiate phagocyte suppression.

As these authors have shown, the role that cortisol plays in immune suppression of fishes has not yet been clearly defined. This study does show that an increase in cortisol concentration to physiologically high levels is directly correlated to an increase in susceptibility of channel catfish to ESC. This seems to confirm the claim of Ainsworth and his coworkers that high concentration of cortisol can lead to immune suppression through initiation of phagocyte suppression. It is important to note, however, that we did not measure phagocyte activity in this study. Maule and Schreck[23] noted decreases of leukocytes in the blood of coho salmon after acute stress or cortisol treatments. These findings are noteworthy in the correlation of cortisol with increased disease susceptibility to ESC, a bacterial disease of the blood. This decrease of leukocytes in the blood stream may give *E. ictaluri* an increased opportunity to increase in numbers and colonize.

This study has demonstrated that (1) a combination of confinement stress and bacterial pathogen concentration both affect disease susceptibility of channel catfish to ESC and (2) stress and the concurrent physiologically high concentrations of cortisol are more highly correlated with disease susceptibility than bacterial pathogen load. In this study, we have only examined one species of fish, the channel catfish and one type of stressor. It is important to note that not all species of fish have the same response to stress and disease challenges. Cortisol may not act alone in immune suppression, or it may simply initiate the process of immune suppression. Further understanding of stress and cortisol is still needed. Additionally, investigations that focus on the pathways of stress hormones in combination with disease challenges may shed light on stress and its effects on disease susceptibility.

ACKNOWLEDGEMENTS

This study was supported by the Southern Regional Aquaculture Center, the Department of Forestry, Wildlife and Fisheries, University of Tennessee and the Department of Animal Science, University of Tennessee. Thank you to Dan and Jenny Fagin for supplying channel catfish for research.

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