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**THE FUNCTION OF THE STRESS HORMONE
CORTISOL IN DISEASE SUSCEPTIBILITY OF
CHANNEL CATFISH, *Ictalurus punctatus*.**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Todd David Sink
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DEDICATION

This dissertation is dedicated to my parents, Steve Sink and Sheila Sink, who always encouraged me to pursue my interests and dreams, and my wife, Casey Sink, for her support, encouragement, and patient understanding, and to my daughter, Kylie Sink, who graced us with her debut appearance during my research.

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ABSTRACT

The purpose of these studies was to examine the roles that the stress hormone cortisol plays in disease susceptibility of channel catfish, *Ictalurus punctatus*, to enteric septicemia of catfish (ESC). ESC is a serious disease of the channel catfish industry with annual losses in the millions. Few treatment options such as antibiotics and vaccines are available, but efficacy of treatments has proven to be limited. Deeper investigations into the physiological causes of disease in fish that accompany commercial production are needed to promote understanding of disease treatment and prevention.

Disease challenges using *Edwardsiella ictaluri*, the pathogenic bacterium that causes ESC, were used to examine the effects of stress and the associated hormone cortisol on disease susceptibility of channel catfish. Channel catfish were subjected to confinement stress of various lengths of time by being placed into net baskets. Blood was collected from fish for the analysis of blood plasma cortisol concentrations. Stressed fish were then subjected to cultures of *E. ictaluri* along with nonstressed control fish. In some experiments, fish received additional treatments such as hormone injections to help explore the effects of stress on disease susceptibility. Fish were then monitored for 21 days for mortality.

Results demonstrated that cortisol was highly correlated with increases in disease susceptibility to ESC and that cortisol was an accurate predictor of increased risk of mortality in both small and large channel catfish fingerlings. A combination of both stress and bacterial pathogen concentration affected disease susceptibility of channel catfish, although stress and the concurrent physiologically high concentrations of cortisol were more highly correlated with disease susceptibility than bacterial pathogen load.

The synthetic hormone, dexamethasone (Dex), is capable of suppressing cortisol concentrations to baseline/control concentrations, and adrenocorticotrophic hormone (ACTH) injections can create cortisol concentration stress responses similar to confinement stress. Although Dex is capable of suppressing cortisol, its use as a stress preventative, especially for the reduction of disease susceptibility, is unfounded based on this study. Evaluation of the effect Dex and ACTH have on blood plasma mineral concentrations revealed that there were no differences in concentrations of sodium, potassium, and chloride in any treatment.

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NOMENCLATURE

°C	degrees Celsius
cc	cubic centimeter of volume
cm	centimeter
d	days
g	grams
h	hours
ha	hectare
IU/kg	international units per kilogram
L	liter
L/min	liters per minute
Log CFU/L	log of colony forming units per liter
mg	milligram
mg/cc	milligrams per cubic centimeter of volume
mg/L	milligrams per liter
mg/kg	milligrams per kilogram
min	minutes
mL	milliliters
mm	millimeters
ng/mL	nanograms per milliliter
nm	nanometer
pH	concentration of hydrogen ions in solution
ppm	parts per million
µg	microgram
µL	microliter

Abbreviations

ACTH	adrenocorticotrophic hormone
API	analytical profile index
API 20E	analytical profile index test strip number 20E
APHIS	Animal and Plant Health Inspection Service
ANOVA	analysis of variance
ARS	Agriculture Research Service
ASM	American Society of Microbiologists
BHI	brain and heart infusion microbiology media
BKD	bacterial kidney disease
CFU	colony forming units
CI	confidence interval
Dex	dexamethasone
<i>E. ictaluri</i>	<i>Edwardsiella ictaluri</i>
ESC	enteric septicemia of catfish
FDA	Food and Drug Administration
LD ₃₀	lethal dose 30 percent

LD-50	lethal dose 50 percent
MS-222	tricaine methano-sulfonate
N	population size
P	probability
<i>R. salmoninarum</i>	<i>Renibacterium salmoninarum</i>
SD	standard deviation
SE	standard error
U.S.	United States
USDA	United States Department of Agriculture

Chapter One

Introduction

1. Stress in Aquaculture

1.1 The stress response in fish

Any event or stimulus that causes a fish to physiologically or mentally (neurological recognition of a stress stimulus) change from a state of physiological balance or homeostasis is known as a stressor. It is the fish's response to such stimuli that is termed the stress response. Selye (1950) originated the idea that a consistent pattern in stress response occurs in mammals. Selye concluded that this response occurred in three stages, the alarm reaction, the stage of resistance, and the stage of physical exhaustion. It is reasonable to assume that this idea is applicable to fish, although we expect to find some differences between a homeothermic mammal and a poikilothermic fish.

The study of the stress response in fish is still rather incomplete when compared to mammals. Physiological data on the stress response of fish are fragmented. It is not possible to determine whether there are several different stress responses in fish, or rather variations of the same general response. Only a few fish stress responses seem related to Selye's mammalian stress model, while the significance of other physiological changes is unclear (Smith 1982). With this knowledge in mind scientists often make comparisons between the stress response of fish and mammals. However, of the three stages of Selye's model, fish only seem to exhibit the alarm reaction, although a valid case may be made for the exhaustion stage.

The resistance stage is not the same as in mammals, where the organism stops or reverses symptoms of the alarm reaction such as tissue catabolism, hypoglycemia, gastrointestinal erosions, and discharge of hormones from the adrenal gland (interrenal gland) (Selye 1950). In fish, we find that these events continue to occur, as long as the stressor is present, until the fish adapts, or succumbs to physical exhaustion. The term “adapt” is really not suitable, as adaptation implies a permanent modification based on changing stimuli that is often inheritable. Fish tend to acclimate, which is a similar physiological change to a stimulus. Acclimation can be overcome when the stressor becomes chronic, can be lost when the stressor is absent, necessary when the same stressor reappears after a prolonged absence, and is not inheritable. The exhaustion stage is also not the same, as fish often succumb to complications such as prolonged ion loss or imbalance, rather than physical loss of energy as in mammals.

Aquaculture production often requires handling, grading, netting, and confinement of fish as part of production practices. These procedures have long been known to be stressful to the fish involved (Mazeaud et al. 1977). Physiological stress and physical injury (also stress inducing) are the two primary contributing factors of fish disease and mortality in aquaculture (Rottmann et al. 1992).

1.2 Cortisol and the hypothalamo-pituitary-interrenal axis

Studies of stress have extensively centered on increases in blood cortisol concentrations as an indicator of the magnitude of the stress response. Cortisol is the major corticosteroid (glucocorticoid) produced by the interrenal gland in teleost fish

(Hazon and Balment 1998). The glucocorticoid cortisol is a product of the hypothalamo-pituitary-interrenal axis which operates as a negative feedback loop.

The negative feedback loop begins in the hypothalamus where corticotropin-releasing hormone (CRH) is secreted by the hypothalamic neuron's axon into the hypothalamic-hypophyseal portal circulation. CRH stimulates the secretion of adrenocorticotrophic hormone (ACTH) by the corticotropes in the adenohypophysis (as reviewed by Eiler 2004). ACTH enters the blood stream and circulates through the fish. In teleosts, adrenocortical or cortical tissue along with chromaffin tissue, forms a diffuse organ at the head or anterior kidney. This organ is situated around the posterior cardinal veins and their branches, allowing hormonal reception from the blood stream (Hazon and Balment 1998). This diffuse organ, the interrenal gland, conducts many of the biological processes that the adrenal gland conducts in mammals.

From the blood stream, ACTH binds receptors in the cortical tissue of the interrenal gland and stimulates the production of cortisol from progesterone (Hazon and Balment 1998). Cortisol continues to be produced by the interrenal gland as long as ACTH levels in the blood stream continue to rise. Eventually, the receptors become saturated and cortisol production peaks. Cortisol production is decreased by the negative feedback loop. Cortisol circulates in the blood saturating cortisol receptors on target cells. Excess cortisol in the blood stream inhibits both hypothalamic CRH secretions as well as adenohypophyseal ACTH secretions (as reviewed by Eiler 2004).

The effects of cortisol in fish are still debated. Cortisol has been found to be necessary for the induction of metabolic changes necessary for ion and water regulation. Cortisol is also known to increase renal sodium retention and reduce glomerular filtration

rates (Matty 1985). Cortisol in fish is also hyperglycemic and is reported to stimulate glycolysis and gluconeogenesis from proteins and lipids. Cortisol also activates several enzymes needed for intermediary metabolism in the liver (Hazon and Balment 1998).

Some evidence exists that cortisol plays a role in immune suppression of fish, especially in stressed fish. Maule and Schreck (1990) 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. This reduces the number of leukocytes available in the blood to combat infections such as septicemia. Handling and transport stress in channel catfish causes a decrease of lymphocytes and an increase of circulating neutrophils in the blood (Ainsworth et al. 1991). Weyts et al. (1997) determined that cortisol directly inhibits proliferation of peripheral blood lymphocytes, and induces apoptosis of lymphocytes in carp, *Cyprinus carpio*. Many of these authors are still uncertain if cortisol plays a primary role in immune suppression, or whether it plays a secondary role.

2. Bacterial Disease in Catfish Aquaculture

2.1 Common bacterial and protozoan diseases

Some bacteria in channel catfish production are considered opportunistic pathogens. These opportunistic bacteria are usually present in aquatic systems, but usually do not cause problems. However, in some situations with the correct conditions, they can cause disease in fish. Common examples of opportunistic pathogens, which can cause disease and death of catfish include *Aeromonas* sp., *Pseudomonas* sp., and

Flexibacter sp. Some bacteria are considered obligate pathogens, which means they are likely to be the sole cause of disease rather than a secondary problem (Francis-Floyd and Reed 1995). Examples of bacteria that fall into this category are *Edwardsiella ictaluri*, *Edwardsiella tarda*, and the protozoan *Ichthyophthirius multifiliis* (Ich).

Aeromonas and *Pseudomonas* cause very similar diseases. *Aeromonas* causes motile aeromonad septicemia characterized by large ulcers on the side of the fish, distended abdomen, complete erosion of the caudal peduncle and exophthalmia (Camus et al. 1998). *Flexibacter columnaris* causes a disease simply called columnaris. It is usually an external problem that begins by loss of color along the back of the fish. As the disease progresses, large white ulcers develop as well as yellow to brown lesions on the gills (Durborow et al. 1998). Mixed infections with *Aeromonas* and *Pseudomonas* are common in advanced cases. Poor management practices like rough or excessive handling and poor water quality are typically to blame for outbreaks of these bacteria.

The obligate pathogens, such as *E. tarda*, are often found in organically polluted waters. *E. tarda* causes a disease known as edwardsiellosis and is problematic of several species of fish as well as causing infections in reptiles, birds, mammals, and in some cases humans. *I. multifiliis* is capable of killing a large number of fish in a short period of time. Fish infected with Ich exhibit white spots on their body, and for this reason the infection is called white spot disease. Ich invades the gills and in some instances the only signs of infection are dead fish (Durborow et al. 1998). *E. ictaluri* causes more disease losses per year than all of the pathogens previously mentioned.

2.2 *Enteric septicemia of catfish*

Enteric septicemia of catfish (ESC) caused by the bacterium *E. 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 (USDA 2003). Producers often refer to ESC as "hole-in-the-head syndrome" as it frequently causes ulcers in the middle of the skull of fingerlings, just above the eyes.

E. ictaluri is a gram negative bacterium that accounts for 30% of all disease cases submitted to fish disease diagnostic laboratories in the southeastern United States. ESC was first recognized as a new infectious bacterial disease in 1976 from the examination of diseased catfish specimens from Georgia and Alabama by the disease diagnostic lab at Auburn University. The channel catfish is by far the most susceptible species to ESC, although the brown bullhead, white catfish, and walking catfish are also susceptible. Blue catfish have shown natural resistance to ESC. Other species such as rainbow trout and blue tilapia have been experimentally infected, although no natural disease outbreaks have occurred (Hawke et al. 1998).

Clinical signs of ESC infection include tail chasing or erratic circular swimming, red and white ulcers, raised red or white pimples, petechial hemorrhages, and a hole in the cranial cavity between the eyes. ESC is usually diagnosed by cultivation of bacteria isolated from the internal organs and brain cavity. An analytical profile index for enteric bacteria (API 20E) is typically used for identification of the bacteria, although infection and monitoring of healthy catfish is also suitable. Prevention of ESC is difficult due to its

wide distribution throughout the channel catfish industry (Hawke et al. 1998). The USDA's Agriculture Research Service developed the first approved ESC vaccine approved for use in channel catfish in 1999 and it was marketed in 2000 by Intervet, Inc. (ARS 1999). Although a vaccine was commercially available, only 18.1 % of all fry were vaccinated in 2002 (USDA 2003). Treatment of ESC outbreaks typically involves the use of medicated feeds.

2.3 *Disease treatments*

Drugs approved by the Food and Drug Administration for the treatment of disease in food fish are very limited. Treatment of ESC is restricted to only one antibiotic, ormetoprim combined with sulfadimethoxine (Romet-30® by Alpharma Inc) (USDA 2003). Extra-labeling by veterinarians (FDA 2001) does occur for the catfish approved antibiotic oxytetracycline HCL (Terramycin® by Pfizer, Inc.) (FDA 2003) although oxytetracycline is not very effective for ESC treatment.

Chapter Two

Linking Stress to the Increased Susceptibility of Channel Catfish to Enteric Septicemia Using Cortisol

This chapter is a lightly revised version of a paper by the same name published in the *Journal of Aquatic Animal Health* in 2004 by Todd D. Sink and Richard J. Strange:

Sink, T.D., and R.J. Strange. 2004. Linking Stress to the Increased Susceptibility of Channel Catfish to Enteric Septicemia Using Cortisol. *Journal of Aquatic Animal Health* 16(2): 93–98.

Any use of “we” or “our” in this chapter refer to my co-author, Richard Strange, and I. My primary contributions to this paper were: (1) literature review of the topic, (2) research and building of the experimental system, (3) obtaining and most of the culturing of bacteria for the challenges, (4) design of the experimental procedure, (5) acquiring, acclimating and maintaining the fish, (6) conducting most of the challenge experiment, (7) all of the mortality monitoring and data collection, (8) most of the blood plasma cortisol analyses, (9) most of the statistical analyses, and (10) most of the writing.

3. Introduction

Channel catfish *Ictalurus punctatus* is the major aquaculture crop in the southern United States. As commercial production has increased, so too have the losses to disease. Enteric septicemia of catfish (ESC), caused by the bacterium *Edwardsiella ictaluri*, is considered by the United States Department of Agriculture (USDA) to be the most infectious disease of commercially raised channel catfish. Enteric septicemia of catfish was present on 56% of catfish farms in the U.S. during 1996-1997, and on 84% of farms with production of 60 ha or greater (USDA 1997). Although channel catfish are susceptible to several diseases, ESC has become the focus of disease study in recent years.

The treatment of ESC is limited to two USDA approved antibiotics for catfish: ormetoprim and sulfadimethoxine under the brand name Romet, and oxytetracycline under the brand name Terramycin. Several isolates of *E. ictaluri* have already become resistant to these antibiotics, thus hindering treatment (Johnson 1991). This has led to the use of disease challenges using live or dead bacteria for vaccination purposes. These attempts at vaccination have not produced consistent results, and typically vaccinated fish also died (Thune 1997).

The limited success of vaccine development and lack of antibiotic treatment has led to the examination of other possibilities of ESC prevention. Examination of stress and its effects on disease susceptibility suggests a potential method of prevention. Fish subjected to stress are more susceptible to ESC than are nonstressed fish (81% versus 55% mortality) and longer exposure time to *E. ictaluri* after stress resulted in increased mortality (Wise et al. 1993).

The study by Wise et al. (1993) incorporated stress, but did not include a physiological indicator of level of stress. It is known that channel catfish stress response varies from individual to individual based on factors such as light exposure, density, and water quality. Stress reactions and cortisol levels can vary to a great extent dependent on temperature (Strange 1980). Rapid deterioration of water quality during mechanical failure or changes in pond conditions can lead to increases in cortisol levels due to stress (Tomasso et al. 1981). Tomasso and his colleagues determined that cortisol levels increase in response to stress associated with depleted oxygen levels. Stressors other than water quality that might increase susceptibility to ESC include stress incurred during stock out operations, grading, or pond transfer.

The objectives of this study were: (1) to document differences in cortisol levels between different lengths of acute confinement stress, (2) to document differences in mortality with associated stress levels following an *E. ictaluri* challenge, and (3) to examine prediction methods using cortisol level as an indicator of percent mortality. These studies are intended to help commercial catfish farmers reduce mortality by increasing our understanding of the relationship between the degree of stress and mortality due to *E. ictaluri*.

4. Methods

4.1 Fish

Fingerling channel catfish (Figure 2.1) were obtained from commercial aquaculture ponds at Greenwater Fish Farm (Gibson County, Tennessee). Two size classes of fish were obtained; small fingerlings 63-98 mm (mean 82.1 mm, SD 6.3 mm) and large fingerlings 114-168 mm (mean 147.3 mm, SD 13.5 mm). These fish were hatched from eggs of broodstock maintained within the same facility and stocked into commercial grow-out ponds with no previous outbreaks of *E. ictaluri*. These fish exhibited no clinical signs of infection. Additionally, plate isolates of brain-heart infusion (BHI) agar from a subsample (13 fish) failed to generate any colonies of *E. ictaluri* after incubation at 30°C for 72 h. A subsequent subsample (10 fish) failed to generate any colonies of *E. ictaluri* in BHI broth after incubation at 30°C for 48 h.



Figure 2.1: Channel catfish fingerlings.

The above figure depicts large fingerling channel catfish used in the large fingerling challenge (range: 114-168 mm, mean: 147.3 mm, SD: 13.5 mm). The fingerlings were found to be disease free, with no previous ESC infections.

4.2 *Edwardsiella ictaluri*

A test strain isolate of *E. ictaluri* (Figure 2.2) was obtained from a natural outbreak in a commercial catfish operation (Auburn University Fish Health Laboratory). An additional isolate (S97-773) from a natural disease outbreak was obtained from the Thad Cochran National Warmwater Aquaculture Center. The identities of these isolates were verified by biochemical isolation tests described by Hawke et al. (1998) and produced API 20E analytical profile index codes of 4004000.

To test virulence, the isolates were propagated with BHI broth in a shaking bath at 27°C for 48 h. Fifteen fish (70-85 mm) were exposed to 5.6 Log colony-forming units per

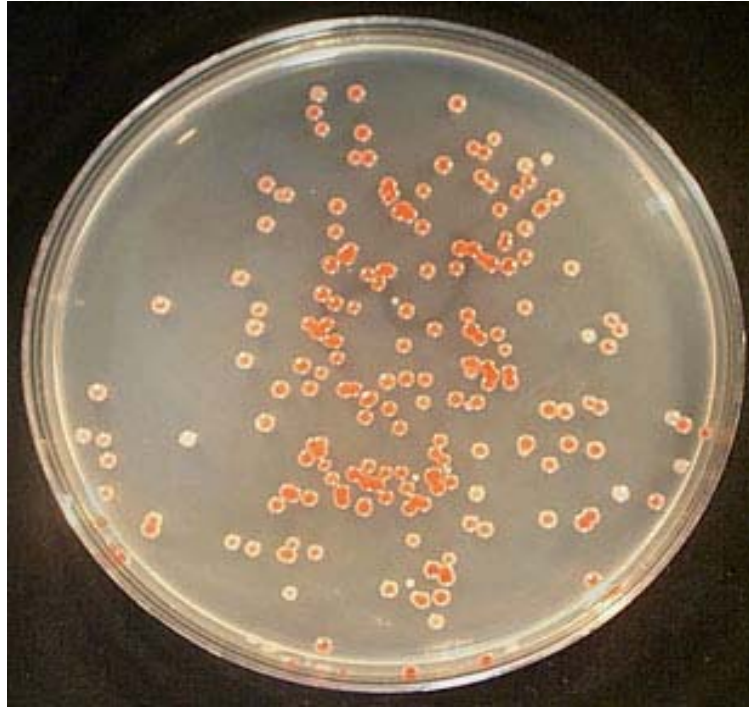


Figure 2.2: *Edwardsiella ictaluri* on BHI agar plate.

The above photo shows *Edwardsiella ictaluri* colonies cultured on a brain and heart infusion plate. Colonies were grown for enumeration and identification of bacteria. The colonies in the photo have been stained red with iodine for visual purposes. Colonies typically appear small white and punctuate.

L (Log CFU/L) in 85-L tanks for each isolate. The exposed fish were reared for 9 d with most (>90%) exhibiting one or more clinical signs of ESC infection. *E. ictaluri* was reisolated from brain tissue and the posterior kidney for the test strain of *E. ictaluri*, but was not reisolated from the S97-773 strain. 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 use in the challenges.

4.3 *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 through charcoal and total chlorine content was less than 0.003 ppm. Water temperatures were maintained between 24 and 26°C. An air stone was used in each aquarium for supplemental aeration keeping dissolved oxygen levels near saturation. Water quality (Appendix A) was monitored continuously for dissolved oxygen (always > 6.5 mg/L), temperature (always $25 \pm 2^\circ\text{C}$), and total chlorine (< 0.003 ppm). Water quality was tested weekly for pH (7.0-7.3), total ammonia (< 1.0 mg/L), and nitrite concentration (as nitrite-nitrogen < 0.01 mg/L). Lighting was automatically controlled by timer with a 16 h light / 8 h dark cycle. The small fingerlings were fed a Zeigler Bros, Inc. slow-sinking finfish starter (50% protein, 15% fat) at a rate of 3% of body weight daily. Large fingerlings were fed Zeigler Bros, Inc. sinking finfish silver (38% protein, 12% fat) at a rate of 2% of body weight daily.

4.4 *LD-30 infection challenge*

A lethal dose 30 % (LD_{30}) was performed to determine the appropriate concentration of *E. ictaluri* for the disease challenges. Two Erlenmeyer flasks containing 500 mL of BHI broth were inoculated with a thawed aliquot of *E. ictaluri* isolate. The flasks were cultured with agitation at 28°C for 36 h. Triplicate plate counts were made from each flask to determine *E. ictaluri* concentration. Spectrophotometer readings were taken for each flask at 650 and 700 nm. *E. ictaluri* concentrations of 1×10^6 CFU yielded

spectrophotometer readings of 0.388 for 650 nm and 0.343 for 700 nm. These values were used for reproducibility of *E. ictaluri* concentrations in subsequent challenges.

Six 85-L tanks were stocked with 20 naïve fingerlings (70-85 mm) each. The fingerlings were allowed to acclimate to the tanks for 14 d. One tank was dosed for each of six concentrations of *E. ictaluri* (0.2, 0.4, 0.7, 1.4, 2.8, 5.6 Log CFU/L). Water flow was discontinued for 24 h with supplemental aeration remaining constant. Water flow was restored and the bacteria were flushed after the 24 h period. Mortality in each tank was monitored for 21 d.

4.5 *Method of stress inducement*

A nonpathogenic stress challenge was conducted to determine appropriate levels of stress for the disease challenge as indicated by cortisol concentrations. Twenty-five fingerlings (70–85 mm) were stocked into each of 12 tanks, where they were acclimated for 14 d. Stress treatments were applied to three tanks for each of the four treatments (0, 30, 60, and 90 min). Five fish were removed from the tank and blood samples were drawn for cortisol analysis at the conclusion of each treatment. Stress was induced in 25 fish from each tank by confinement in baskets (12.5 x 15 x 10 cm) constructed of rigid vinyl netting. Times of stress (0, 30, and 60 min) for the small and large fingerling challenges depended upon on treatment group.

4.6 *Small fingerling disease challenge*

Twenty-five small fingerling catfish were stocked into each of 18 tanks. The fingerlings were acclimated for 14 d. Stress treatments (0, 30, 60 min) were applied to two tanks each in three banks. Five fish were removed from each tank and blood samples

were drawn for cortisol analysis at the conclusion of each treatment. The remaining fish 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 of *E. ictaluri* (20 mL of inoculated BHI @ 1×10^6 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.

4.7 *Large fingerling disease challenge*

A subsequent disease challenge was conducted with large fingerlings (mean 147.3 mm, SD 13.5 mm). The challenge was conducted in the same manner as above duplicating the disease challenge of the small fingerlings with larger fish.

4.8 *Blood plasma cortisol collection and assays*

Fish were anesthetized in 100 mg tricaine methanesulfonate/L (MS-222) upon removal from basket or tank. The caudal peduncle was severed and blood was collected from the caudal vasculature using 370 μ L heparinized Caraway tubes. Tubes were blown into 1.5-mL centrifuge tubes and the plasma was separated from the red blood cells. The plasma was removed and placed into 1.5-mL cryotubes and frozen until cortisol assays were performed.

Plasma was later thawed and used in conjunction with Coat-A-Count® solid-phase ^{125} I-cortisol radioimmunoassay (Appendix B). The competitive assay was used for quantitative measure of the cortisol (hydrocortisone, compound F) in the blood plasma. The assay was modified from 25 μ L to 10 μ L of plasma and calibrators in order to accommodate the small blood volumes received from the fish. All samples and

calibrators were done in duplicate. Samples with %CV greater than 10 were rerun through the assay using the same kit. Internal controls of high and low cortisol concentration plasma from pooled channel catfish, trout, and pigs were run with the plasma from each challenge. The difference in the controls between assays was negligible (< 3-7 ng/mL).

4.9 *Statistical analyses*

Differences in cortisol and mortality among fish due to exposure time to the bacterial challenge were tested using analysis of variance (ANOVA) and Tukey's post hoc tests (SPSS 11.0 2000). 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.

5. Results

5.1 *LD³⁰ infection challenge*

Mortality rates for bacterial doses were recorded (Table 2.1). A mortality rate of 30% provides a suitable rate of infection while still allowing a large enough increase in mortality to allow differences between treatments to be detectable. The *E. ictaluri* concentration of 1.4 Log CFU/L produced the objective mortality of 30%. Therefore, 1.4 Log CFU/L was used for all ensuing challenges.

5.2 *Stress Challenge*

Cortisol levels (Table 2.2) from the 0, 30, and 60min treatments were significantly different ($P < 0.05$) and no mortality was observed due to stress after seven

Table 2.1: Mortality rates from bacterial exposure during the LD₃₀ test.

Bacterial Concentration (Log CFU/L)	Mortality (Individuals)	Percent Mortality
0.2	1	5
0.4	3	15
0.7	5	25
1.4	6	30
2.8	15	75
5.6	18	90

Mortalities for channel catfish during a lethal dose 30% test were recorded as individuals per treatment and expressed as % of total for treatment tank. Bacterial levels are reported as colony forming units per L.

Table 2.2: Cortisol concentrations for timed treatments of the stress challenge.

Treatment (min)	Cortisol Concentration (ng/mL)		
	Minimum	Maximum	Mean $\pm$ SE
0	1.4	8.1	4.42 $\pm$ 1.32 x
30	24.3	57.3	36.38 $\pm$ 5.86 y
60	56.6	91.5	71.88 $\pm$ 6.77 z
90	61.9	90.4	73.98 $\pm$ 5.77 z

Cortisol concentrations of channel catfish as determined by timed treatments during non-bacterial stress challenge. Means with different letters are significantly ($P < 0.05$) different.

d. These increments allow for detection of differences between stress levels when a pathogen is added. Therefore, these treatment levels were used for the subsequent disease challenges. Because the results for the 90 min treatment were not significantly ($P > 0.05$) different from those for the 60 min treatment, we did not use the longer treatment in the challenges.

5.3 *Small fingerling disease challenge*

Both cortisol and mortality levels (Table 2.3) (Appendix C) differed significantly (one-way ANOVA, $P < 0.05$) between treatments. A strong relationship between cortisol concentration (Appendix D) and percent mortality (Appendix E) was found, with a Pearson correlation coefficient of 0.861 ($P < 0.01$, two-tailed test). A simple linear regression of cortisol concentration on mortality rate was conducted (Figure 2.3). The R^2 value showed that 74% of mortality differences could be explained by cortisol concentration ($P = 0.001$). The regression line for small fingerlings was 13.5 % mortality + 0.51 * cortisol ng/mL (95% confidence intervals, 0.09–26.91 for the intercept and 0.35–0.67 for the slope).

5.4 *Large fingerling disease challenge*

Statistical methods for the large fish disease challenge were as above for the small fish challenge. Mortality levels (Table 2.3) (Appendix F, G) differed ($P < 0.05$) among the stress treatments. Cortisol concentrations (Table 2.3) (Appendix F, H) were significantly different ($P < 0.05$) between the 0 and 30 min treatments but not ($P = 0.61$) between the 30 and 60 min treatments. The strong relationship between cortisol concentration and

Table 2.3: Mortality rates and cortisol concentrations for channel catfish.

Treatment (min)	Small Fingerling Challenge		Large Fingerling Challenge	
	Cortisol (ng/mL)	Percent Mortality	Cortisol (ng/mL)	Percent Mortality
0	23.63 ± 2.46	22.5 ± 1.7	31.44 ± 7.31 z	25.8 ± 3.0
30	77.70 ± 7.82	47.5 ± 2.1	103.14 ± 8.47 y	62.5 ± 4.2
60	115.37 ± 10.88	81.7 ± 2.8	109.64 ± 10.16 y	100 ± 0.0

Means ± SEs of mortality rates and cortisol concentrations for channel catfish by treatment time for small (63–98-mm) and large (114–168-mm) fingerling challenges. Means with different lowercase letters are significantly ($P < 0.05$) different.

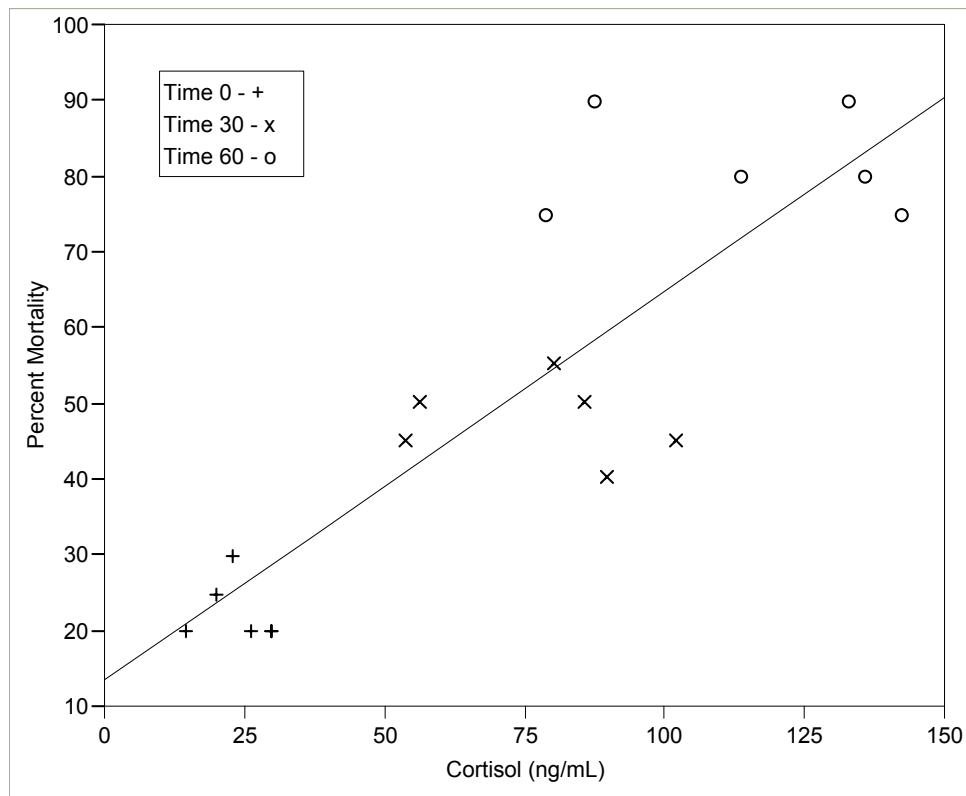


Figure 2.3: Small channel catfish fingerling regression.

Regression of channel catfish mortality rate on cortisol concentration for small fingerling ESC disease challenge. Mortality rate is expressed as percent mortality per tank. Cortisol is expressed as mean cortisol (ng/mL) per tank.

percent mortality was indicated by a Pearson correlation coefficient of 0.787 ($P < 0.01$, two-tailed test). A simple linear regression of percent mortality on cortisol (Figure 2.4) had an R^2 fit of 0.620, indicating that the correlation of percent mortality with cortisol concentration was significant ($P = 0.001$). The regression line for large fingerlings is $13.7\% \text{ mortality} + 0.60 * \text{cortisol ng/mL}$ (95% confidence intervals, 29.13 to 36.24 for the intercept and 0.35 to 0.85 for the slope).

6. Discussion

Few studies have examined the relationship between biological indicators of stress and increases in mortality rates. Because channel catfish fingerlings are often exposed to *E. ictaluri* in combination with various acute and chronic stressors, we assumed that a biological indicator of stress, such as cortisol, might be used to predict increases in infection rates during stress. Our results indicated that cortisol might be used as a predictor of mortality rates.

In response to non-specific stressors, cortisol is the major corticosteroid released from the interrenal gland into the blood stream of teleost fish in both fresh and saltwater. Cortisol has been shown to suppress immune system function, decrease growth rates, and inhibit reproduction in fish (Evans 1997). Cortisol has long been used as a biological indicator of level of stress. Our study demonstrated that cortisol might be used as an indicator of increased infection due to its ability to suppress the immune system.

Fish with significant increases in cortisol level displayed significant increases in mortality when compared to fish not subjected to stress. Increases in cortisol levels were

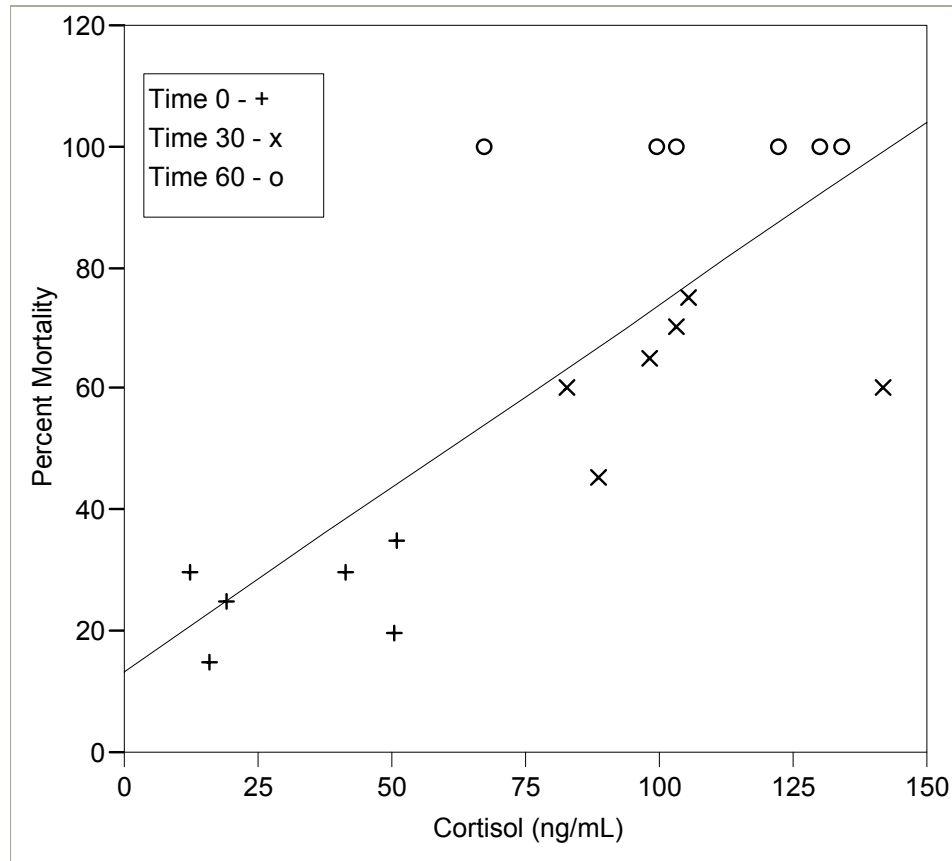


Figure 2.4: Large channel catfish fingerling regression.

Regression of channel catfish mortality rate on cortisol concentration for large fingerling disease challenge. Mortality rate is expressed as percent mortality per replicate per stress treatment. Cortisol is expressed as mean cortisol (ng/mL) per tank per stress treatment.

found to be proportional to increases in stress levels in the small fingerling challenge. For the large fingerling challenge, the stress groups displayed significant increases in cortisol levels when compared to the nonstressed fish, but the cortisol levels were not significantly different among the stress groups (mean $\pm$ SE: 103.14 ± 8.47 , and 109.64 ± 10.16 ng/mL). This was likely due to the large fish becoming more stressed when placed in the same sized confinement cages as the small fish (mean $\pm$ SE for 30 and 60 min small fish: 77.70 ± 7.82 , and 115.37 ± 10.88 ng/mL, mean $\pm$ SE for 30 and 60 min large fish: 103.14 ± 8.47 , and 109.64 ± 10.16 ng/mL). Mortality rates most likely differed due to the prolonged stress effects of the time 60 min fingerlings. Since they were subjected to stress for a longer period than the time 30 min fish, cortisol concentrations were elevated for longer periods. This would mean an increased or lengthened period of immunosuppression by cortisol.

The cortisol levels were highly correlated with the tightly grouped mortality rates for each of the six replicates in the small fish challenge. The large fish also exhibited a strong correlation between cortisol levels and mortality levels. Because these mortality rates were so tightly grouped at an associated cortisol level for each size of fish, it appears that cortisol may be used to predict mortality levels.

The small fingerling regression model for cortisol by mortality exhibits a definite linear trend with a moderate R^2 of 0.741. We observed that the cortisol model for small fingerlings was capable of estimating mortality rates in commercial catfish culture with error less than 6.3%. The large fingerling challenge resulted in a lower R^2 of 0.620. This lowered R^2 was partially due to the percent mortality reaching the upper limit of 100% in all replicates and skewing the data. Results from the large fish challenge would still

accurately estimate mortality rates for large fingerlings, although it exhibited a larger possible prediction error of $\pm 10.7\%$. As shown by the R^2 , cortisol cannot explain all of the variation in catfish fingerling mortality, but it can explain a large enough portion to be viewed as an adequate predictor of mortality.

As previous studies have shown, different factors affect stress and associated cortisol levels. We know from Strange (1980) that cortisol levels increase significantly more in fish acclimated at 20 °C or 30 °C when compared to cortisol levels of fish acclimated at 10 °C. Strange's study showed little differences in the stress responses of channel catfish between 20 °C and 30 °C, with cortisol levels for the two temperatures remaining quite similar (Strange 1980). It is also known that *E. ictaluri* has a typical infectious temperature range referred to as the "ESC window" of 20 – 28 °C (Hawke et al. 1998). Since fluctuations in cortisol concentrations throughout the infectious temperature window remain similar, it is not necessary to account for changes in temperature when estimating mortality based on cortisol concentration.

When water quality conditions become degraded from improper management practices and not mechanical failure, conditions typically worsen at a slower rate than confinement or transfer stress and may be chronic rather than acute. While conditions in the study by Tomasso et al. (1981) may be considered both chronic and acute, fish often become conditioned to cyclical, repeated, or constant stressors such as oxygen depletion during the diurnal cycle. This acclimation to stress levels results in a reduction of cortisol release during these stress events and a return to pre-stressed cortisol levels (Evans 1997). Acclimation to chronic stressors reduces the risk of overestimation of mortality when using cortisol concentrations during an acute stress event. While our study focused on an

acute stress event such as transport, future studies may be necessary to examine the effects of chronic stressors on cortisol levels and subsequent mortality rates.

It has been reported that acute stressors significantly increase the percent mortality of channel catfish fingerlings to ESC (Wise et al. 1993). This study did not incorporate a biological indicator of stress. It is important to have an indicator of stress in order to standardize the response of fish to a stress event. It is important to consider that a stress response not only varies from individual to individual, but also between stocks and strains by region. Cortisol may be used as an indicator for a specific population of fish on a given farm.

Increases in cortisol are associated with decreases in leukocytes in the blood and spleen of juvenile coho salmon after stress treatments (Maule et al. 1990). This reduction in leukocytes reduces the effectiveness of the immune system to defend against infection. This may make it possible to correlate cortisol level with level of decreased immune function. Mesa et al. (2000) examined the physiological responses of juvenile spring chinook salmon *Oncorhynchus tshawytscha* infected with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD). The cortisol levels of infected fish were compared with levels of noninfected fish. Also, infected fish were exposed to a series of acute stressors and cortisol concentrations were compared to those of infected fish that had not received the stressors (Mesa et al. 2000).

Cortisol level and degree of infection were increased in the stressed groups when compared to the nonstressed groups. Cortisol levels increased after infection with *R. salmoninarum* and exposure acute stressors. Infected fish exhibited continuously elevated levels of cortisol, but increases in mortality did not occur when infected fish were

exposed to additional stressors (Mesa et al. 2000). This study examined the relationship between stress, increases in infection, and cortisol concentration of previously infected fish. It did not examine the link of a stress event to the start of infection. Our study showed that cortisol could indicate that the stress event itself, led to increased infection rates and subsequent mortality.

Our study proposes to help commercial catfish farmers regulate mortality by limiting exposure of stressed fish to *E. ictaluri*. In the presence of the pathogen *E. ictaluri*, monitoring the cortisol level of fish during or immediately following an acute stress event such as transport, grading, or stock out will allow fish farmers and scientists to accurately estimate mortality rates. This will allow farmers to make two management decisions: (1) limit amount of stress to acceptable mortality levels of production, or (2) make a decision of intensity of antibiotic treatment that will be needed to lower the predicted mortality levels.

This study could have applications in the majority of fisheries industries in the United States. All aquacultural systems produce stress events at some stage during production. Likewise, many industries experience one or more naturally occurring bacterial diseases that can have devastating economic impacts on a regional scale if not industry wide. Subsequent studies on the effects of stress on increased infection or mortality using a biological indicator such as cortisol may lead to a decrease of disease outbreaks and decreased economic losses.

Based on results from this study, future challenges will be conducted with different concentrations of bacterium to develop regression charts that may be used as predictors of disease susceptibility when fish are stressed. These charts are intended to

enable farmers to predict mortality rates from stress events based on the amount of *E. ictaluri* present in their ponds. Farmers may be able to sample their water prior to stocking or grading to determine the actual level of *E. ictaluri* present, and then consult the corresponding chart for that concentration to predict mortality. Other studies may include the use of antibiotics (Romet or Terramycin) fed as prophylactic and post-stress treatments, in order to evaluate the effectiveness of medicating fish under stress. This study may allow farmers to evaluate the effectiveness as well as the economics of medicating fish in the treatment of ESC.

Chapter Three

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

This chapter is a lightly revised version of a paper by the same name in review for publication in the journal *Aquaculture Research* in 2004 by Todd D. Sink, Richard J. Strange, Henry G. Kattesh, and Alan G. Mathew:

Sink, T.D., R.J. Strange, H.G. Kattesh, and A.G. Mathew. 2004. Linking Stress to Increased Mortality of Channel Catfish at Varying Concentrations of *Edwardsiella ictaluri*. *Aquaculture Research*. *In review*.

Any use of “we” or “our” in this chapter refer to my co-authors Richard Strange, Henry Kattesh, Alan Mathew and I. My primary contributions to this paper were (1) literature review of the topic, (2) research design, and construction of the experimental system, (3) obtaining and culturing bacteria for the challenges, (4) design of the experimental procedure, (5) acquiring, acclimating, and maintaining the fish, (6) conducting most of the challenge experiment, (7) most of the mortality monitoring and data collection, (8) all of the blood plasma cortisol analyses, (9) all of the statistical analyses, and (10) most of the writing.

7. Introduction

Channel catfish, *Ictalurus punctatus* (Rafinesque 1818), production is the largest aquaculture industry of the United States (USDA 1998). 2002 commercial catfish production is up 4% while acres of water in production are down 3% compared to 2001(USDA 2002). 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 (USDA 2003). 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.) (FDA 2003). 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 %, with diseases accounting for by far the largest portion (USDA 1997). Several isolates of *E. ictaluri* have already become resistant to approved antibiotics (Cooper et al. 1993; Starliper et al. 1993). The efficacy of current vaccinations has proved to be inconsistent and the use of vaccines has produced mortality in channel catfish (Thune 1997).

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 (Wise et al. 1993). Ciembor et al. (1995) confirmed these results with stressed fish exposed to an *E. ictaluri* bath, exhibiting greater infection rates compared to intraperitoneal injected fish and unstressed control groups (52.7%, 46.6%, and 15.7%, respectively).

Wise et al. (1993) 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 (Strange 1980; Tomasso et al. 1981; Davis et al. 1984). 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 (Sink and Strange 2004).

Several studies have demonstrated a link between increased infection rates of ESC and stress (Wise et al. 1993; Ciembor et al. 1995; Sink et al. 2004). However, these studies typically use inflated levels of *E. ictaluri* for infection. Wise et al. (1993) address the shortcomings of inflated bacterial concentrations suggesting that they do not adequately reflect natural routes of infection. Studies often use LD₃₀ or LD₅₀ infection levels to insure separation of treatment groups (Wise et al. 1993; Thune et al. 1999; Sink et al. 2004).

The main objective of this study was to examine the effects of stress on catfish mortality when *E. ictaluri* concentrations were reduced from experimental LD₃₀ 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 conditions.

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 (Pickering and Duston 1983; Maule et al. 1989; Sink et al. 2004). Although the means of immune suppression by cortisol in fish is not fully understood, it has been thoroughly documented in mammals (Hadley 1996). Weyts et al. (1999) refuted cortisol's role as a direct immune-suppressant in fish, but increased production of cortisol during stress was associated with decreased immune system function.

8. Methods

8.1 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, SE 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.

8.2 *Edwardsiella 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. (1998) 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) 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.

8.3 *Experimental system*

The experimental system (Figure 3.1) contained 24 independently maintained 85-L tanks with four banks of six tanks. Each tank was operated in a flow-through mode with filtered de-chlorinated city water supplied at 2.2 L/min. The water was filtered with activated carbon and total chlorine content was less than 0.003 ppm. 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), temperature (25 ± 2°C), and total chlorine (<0.003 mg/L). Water quality was tested weekly for pH (7.0-7.3), and total ammonia (<1.0 mg/L). Lighting was automatically controlled by timer with a 16 h light / 8 h dark cycle. The



Figure 3.1: Experimental system.

The above photo shows the experimental system used in all challenges. The system was operated as a flow through, with water capacities of the tank being changed as necessary by changing the height of the standpipe.

fingerlings were fed Zeigler slow-sinking finfish starter (50% protein, 15% fat) at a rate of 3% of body weight daily.

8.4 *Stress challenge*

A non-pathogenic stress challenge was conducted to determine appropriate levels of stress as indicated by cortisol for the disease challenge. The effect of stress on mortality was also examined. Fingerlings (n=25, 70-85 mm) were stocked into each of 12 tanks and were acclimated for 14 d. Stress treatments were applied to three tanks for each of the four treatments (0, 30, 60, 90 min). Five fish were removed from the tank and

blood samples were drawn for cortisol analysis at the conclusion of each treatment. The remaining twenty fish in each tank were then monitored for 7 d for mortality.

8.5 *Edwardsiella ictaluri* lethal dosage determination

A lethal dose 30 % (LD₃₀) was performed to determine the appropriate concentration of *E. ictaluri* for the experimental disease challenges (Sink et al. 2004). Mortality in each tank was monitored for 21 d. The determined LD₃₀ was the starting point for the high dose challenge in this study.

8.6 *Stress inducement and blood collection*

Stress was induced in 25 fish from each tank by confinement in baskets (12.5 x 15 x 10-cm) constructed of rigid vinyl netting (Figure 3.2). Stress treatments included confining fish in the nets for 30 or 60 min, 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 5 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 methanesulfonate/L (MS-222) upon removal from basket or tank prior to bleeding. All five fish were bled in less than five minutes after removal from the tank. Blood collection, storage, and radioimmunoassays were conducted as in Sink and Strange (2004).

8.7 *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



Figure 3.2: Confinement stress of channel catfish.

The above photo depicts channel catfish undergoing confinement stress treatment. The fish were confined in baskets (12.5 x 15 x 10-cm) constructed of rigid vinyl netting for 30 or 60 min treatments.

applied to two 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 (Figure 3.3) with 1.4 Log CFU/L of *E. ictaluri* (20 mL of inoculated BHI @ 1×10^6 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.



Figure 3.3: *Edwardsiella ictaluri* challenge dose regimens.

The above photo depicts prepared tank doses of *Edwardsiella ictaluri*. Tanks were dosed with 1.4 Log CFU/L (20 mL of inoculated BHI @ 1×10^6 CFU), 0.7 Log CFU/L (10 mL of inoculated BHI @ 1×10^6), or 0.4 Log CFU/L (5 mL of inoculated BHI @ 1×10^6) for the high, medium, and low dose challenges, respectively.

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 of *E. ictaluri* (10 mL of inoculated BHI @ 1×10^6) and a low dose consisting of a challenge with 0.4 Log CFU/L of *E. ictaluri* (5 mL of inoculated BHI @ 1×10^6).

8.8 Statistical analyses

Differences in cortisol and mortality among stress levels were tested using ANOVA and Tukey's post hoc tests (SPSS 2000). 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.

9. Results

9.1 *Stress challenge*

Cortisol levels from time 0, 30, 60, and 90 min treatments were significantly different ($P < 0.05$) and were presented in chapter II. These increments allow for detection of differences between stress levels when a pathogen is added. Therefore, these treatment levels were used for the subsequent disease challenges. Time 90 min treatment was not significantly different ($P > 0.05$) from time 60 min treatment and was not used in the challenges. No mortalities occurred in any tank at any stress increment.

9.2 *High dose disease challenge*

A one-way ANOVA was used to compare differences among mortalities for the net confinement stress levels. Mortality levels (Appendix I) differed ($P < 0.05$) among the net confinement stress levels (Table 3.1). A one-way ANOVA was also used to compare differences among cortisol concentrations (Appendix I) for the net confinement stress levels. Cortisol concentrations were different ($P < 0.05$) among all three treatment groups and between net confinement stress levels (Table 3.1).

A Pearson correlation analysis was performed to determine if a relationship existed 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

Table 3.1: Mortality rates and cortisol concentrations for channel catfish in high, medium, and low bacterial concentration challenges.

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

Percent mortality of channel catfish 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, 20 mL @ 1.0×10^6), medium (0.7 Log CFU/L, 10 mL @ 1.0×10^6), and low (0.4 Log CFU/L, 5 mL @ 1.0×10^6) bacterial dosage. Three net confinement stress levels were used for each challenge: 0 min or non-stressed, 30 min, net confinement or 60 min net confinement.

level indicated trends in mortality rate (Figure 3.4). 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 $13.5 \% \text{ mortality} + 0.51 * \text{cortisol ng/mL}$ (95% confidence intervals of 0.09 to 26.91 for intercept and 0.35 to 0.67 for slope).

9.3 *Medium dose disease challenge*

Mortality levels (Appendix J) differed ($P < 0.05$) among the net confinement stress levels (Table 3.1). A one-way ANOVA was also used to compare differences among cortisol levels for the net confinement stress levels. Cortisol levels (Appendix J) were also different ($P < 0.05$) among the net confinement stress levels (Table 3.1).

A Pearson correlation analysis was performed to determine if a relationship existed between cortisol level 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 (Figure 3.4). 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 $5.6 \% \text{ mortality} + 0.64 * \text{cortisol ng/mL}$ (95% confidence intervals of -8.43 to 19.69 for intercept and 0.49 to 0.79 for slope).

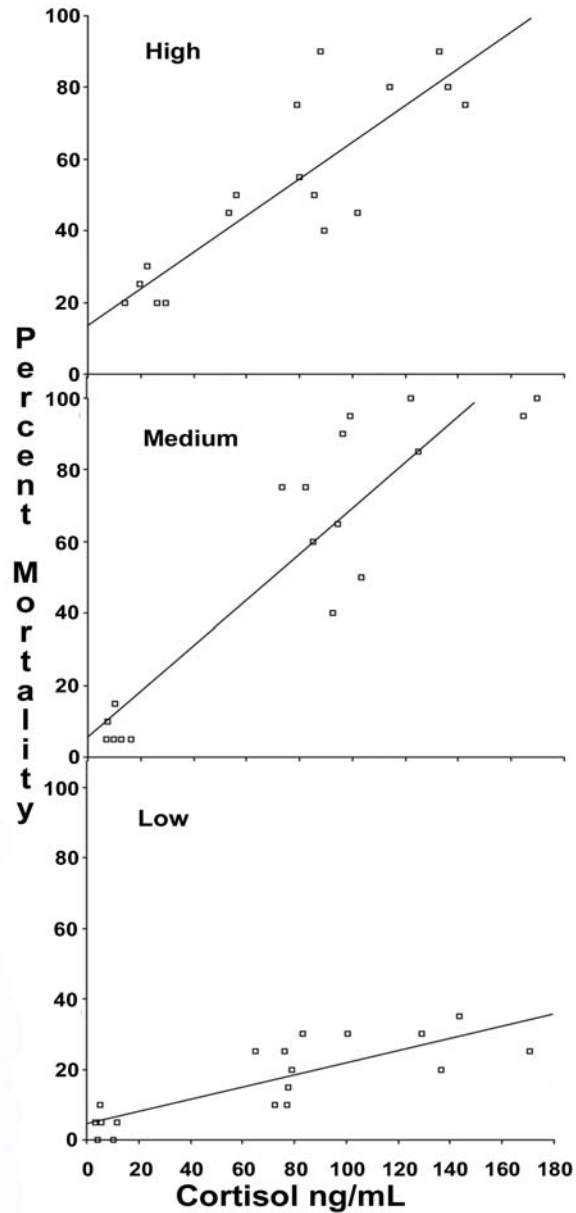


Figure 3.4: Regression of mortalities rates and cortisol for channel catfish in the high, medium, and low disease challenges.

Regression of mortality rate of channel catfish (n=450 for each challenge) 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 tank. Cortisol is expressed as mean cortisol (ng/mL) per tank. The regression line for the high concentration challenge is $13.5 \% \text{ mortality} + 0.51 * \text{cortisol ng/mL}$. The medium concentration challenge regression line is $5.6 \% \text{ mortality} + 0.64 * \text{cortisol ng/mL}$. The low concentration challenge regression line is $4.6 \% \text{ mortality} + 0.17 * \text{cortisol ng/mL}$.

9.4 Low dose disease challenge

Mortality levels (Appendix K) differed ($P < 0.05$) among the net confinement stress levels (Table 3.1). A one-way ANOVA was also used to compare differences among cortisol levels for the net confinement stress levels. Cortisol concentrations (Appendix K) were also different ($P < 0.05$) among the controls and net confinement stress levels (Table 3.1).

A Pearson correlation analysis was performed to determine if a relationship existed 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 (Figure 3.4). 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 $4.6 \% \text{ mortality} + 0.17 * \text{cortisol ng/mL}$ (95% confidence intervals of -1.01 to 10.27 for intercept and 0.11 to 0.24 for slope).

10. 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 (Wise et al. 1993; Ciembor et al. 1995). However, it is not known if these experiments accurately reflect what are 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 (Ainsworth et al. 1991), decrease growth rates, and inhibit the reproduction of fish (Pickering 1992). 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 demonstrated 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 rate for the non-stressed group for the high concentration was similar to the time 30 treatment for the low concentration dose. This indicated that both stress and bacterial concentration played 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 on fish mortality due to disease. This is likely attributed to greater cortisol concentrations in the medium dose group. The increased cortisol concentration confirmed that the fish were acutely stressed and the Pearson correlation coefficient confirmed 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 continued to show that increases in stress, as indicated by cortisol concentration, led 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 indicated that cortisol concentrations were linked to mortality rates at various concentrations of bacteria. The regression models for cortisol by mortality exhibited definite linear trends at every concentration. Cortisol concentrations appear 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 confirmed that the stress hormone cortisol was highly correlated with increased susceptibility of channel catfish to ESC infection and mortality. The

regression models indicated 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 (1990) asserted 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. This however could mean a reduction of the number of leukocytes in the blood available for fighting septicemia. Alford et al. (1994) found that apoptosis of leukocytes was lower in stressed channel catfish than in non-stressed fish. Alford's study indicated a direct effect of stress on individual leukocyte cells, causing them to remain active for a longer period of time, rather than cell lysis. However, Weyts et al. (1997) determined that cortisol produced during handling stress inhibited proliferation of peripheral blood leukocytes and caused induced apoptosis of activated leukocytes. Handling and transport stress causes a decrease of lymphocytes and an increase of circulating neutrophils in the blood (Ainsworth et al. 1991). These researchers also indicated that cortisol alone does not cause 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 mortality of channel catfish to ESC. This seems to support the claim of Ainsworth and his coworkers (1991) 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 (1990) 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.

Chapter Four

Changes in Susceptibility of Channel Catfish, *Ictalurus punctatus*, to Enteric Septicemia of Catfish by Hormonal Alteration of the Hypothalamo-Pituitary- Interrenal Axis

This chapter is a lightly revised version of a paper by the same name in review for publication in the *Journal of Aquatic Animal Health* in 2004 by Todd D. Sink, Richard J. Strange, Hugo Eiler, and Kellie Fecteau:

Sink, T.D., R.J. Strange, H. Eiler, and K. Fecteau. 2004. Changes in Susceptibility of Channel Catfish, *Ictalurus punctatus*, to Enteric Septicemia of Catfish by Hormonal Alteration of the Hypothalamo-Pituitary- Interrenal Axis. *Journal of Aquatic Animal Health*. *In review*.

Any use of “we” or “our” in this chapter refer to my co-authors Richard Strange, Hugo Eiler, Kellie Fecteau and I. My primary contributions to this paper were (1) literature review of the topic, (2) research and building of the experimental system, (3) obtaining and culturing bacteria for the challenges, (4) most of the design of the experimental procedure, (5) acquiring, acclimating and maintaining the fish, (6) conducting most of the challenge experiment, (7) most of the mortality monitoring and data collection, (8) all of the blood plasma cortisol analysis, (9) all of the statistical analysis, and (10) most of the writing.

11. Introduction

Channel catfish, *Ictalurus punctatus* Rafinesque, farming is the largest aquaculture industry in the United States with production reaching 317 million food size fish and 1.3 billion fingerlings as of July 1, 2004 (USDA 2004). Channel catfish are more susceptible to infections when they are subjected to stressful conditions such as handling, improper nutrition, poor water quality, and close confinement (USDA 2003). Channel catfish have been shown to be more susceptible to enteric septicemia of catfish (ESC) when subjected to confinement stress (Wise et al. 1993; Sink and Strange 2004).

ESC, caused by the bacteria *Edwardsiella ictaluri*, is the most serious disease of commercially reared channel catfish. The United States Department of Agriculture (USDA) in conjunction with the Animal and Plant Health Inspection Service (APHIS) recently published a fact sheet of compiled survey materials, Catfish 2003. This survey found that 57 % of large fingerling operations experienced losses to ESC in the previous 2 years. ESC was the leading cause of all fry and fingerling loss at 27.3 % (USDA 2003).

Treatment of ESC is limited with only one antibiotic, ormetoprim combined with sulfadimethoxine (Romet-30® by Alpharma Inc.), approved for ESC treatment of channel catfish (USDA 2003). Extra-labeling by veterinarians (FDA 2001) does occur for the catfish approved antibiotic oxytetracycline HCL (Terramycin® by Pfizer, Inc.) (FDA 2003).

The American Society of Microbiologists claim antibiotic use in disease treatment is becoming a concern due to increasing antibiotic resistance of bacteria and environmental contamination of antibiotics (ASM 1994). Isolates of *E. ictaluri* have been exhibiting antibiotic resistance to Romet-30® since the early 1990's (Cooper et al. 1993; Starliper et al. 1993).

The ASM also states that aquaculture treatment practices are a large focal point due to non-standardized and unregulated treatment practices (ASM 1994). Total antibiotic use for the treatment of ESC is estimated at 126,000 to 252,000 pounds annually (Benbrook 2002). Yet with substantial drug use, losses in catfish production remain as high as 60 %, with diseases accounting for by far the largest portion (USDA 1997).

New treatment methods and preventions are currently being sought due to the current shortcomings of antibiotic use. The efficacy of previous vaccinations for ESC has proved to be inconsistent and the use of vaccines has produced mortality in channel catfish (Thune 1997). The first approved ESC vaccine for use in channel catfish was developed by the USDA's Agriculture Research Service in 1999 and marketed in 2000 by Intervet, Inc. (ARS 1999). The current vaccine still has shortcomings due to a limited window of age that it can be administered (7-31 d after hatching). The vaccine also contains live bacteria, so it presents storage, shipping and shelf life problems, in addition to creating an exposure source to catfish stocks already in commercial facilities. Although a vaccine was commercially available, only 18.1 % of all fry were vaccinated in 2002 (USDA 2003).

Physiological evidence indicates that another option in ESC prevention is the limitation of stress, or possibly reducing the physiological effects of stress. Wise et al. (1993) found channel catfish exposed to stress events are more susceptible to ESC than nonstressed fish (81% and 55% mortality, respectively). These researchers also indicated increased exposure time to *E. ictaluri* after stress resulted in increased mortality rates. Ciembor et al. (1995) confirmed that stressed fish were more susceptible to infection by ESC. Unstressed control groups exhibited lower infection rates than stressed fish exposed to an *E. ictaluri* bath and intraperitoneal injected fish (15.7%, 52.7%, and 46.6%, respectively).

Sink and Strange (2004) confirmed the earlier results of Wise et al. (1993) and Ciembor et al.'s (1995), reporting mortality rates (mean) of 22.5% for nonstressed and 47.5 %, and 81.7 % for 30 and 60 min stressed fish, respectively, but linked infection

rates to the hormone cortisol, a physiological indicator of stress in fish. As stress increased, blood cortisol and infection rates increased proportionally when fish were exposed to *E. ictaluri* baths post confinement stress. Cortisol increases have long been associated with stress events in channel catfish (Strange 1980; Tomasso et. al.1981). It is unclear whether cortisol directly causes immune suppression, or whether it plays a secondary role.

Apoptosis of leukocytes was found to be lower in confinement stressed channel catfish than in non-stressed fish (Alford et al.1994). However, Weyts et al. (1997) determined that cortisol directly inhibits proliferation of peripheral blood lymphocytes, and induces apoptosis of lymphocytes in carp, *Cyprinus carpio*. Alford's study could indicate a direct effect of stress on individual leukocyte cells, causing them to remain active for a longer period of time. Another study found that handling and transport stress produces a decrease of lymphocytes and an increase of circulating neutrophils in the blood (Ainsworth et al. 1991). These studies indicated cortisol alone does not cause suppression of phagocyte function but that elevated physiological concentrations of cortisol can initiate phagocyte suppression.

Cortisol concentrations have been manipulated in fish to create simulated stress responses by either feeding or injecting cortisol, dexamethasone (Dex), or adrenocorticotrophic hormone (ACTH) (Pottinger and Carrick 2001). Pickering and Duston (1983) fed cortisol to brown trout, *Salmo trutta*, in order to evaluate susceptibility to *Saprolegnia* infection. Although they fed cortisol in order to evaluate disease susceptibility, only blood cortisol concentrations would have been elevated.

The use of ACTH would both increase blood ACTH and cortisol concentrations as the use of Dex would decrease blood cortisol concentrations. It is also appropriate to note that the half-life of Dex is much greater than that of cortisol. The bioactivity of Dex is greater than 48 h, whereas cortisol bioactivity is less than 12 h. The hypothalamic-pituitary-interrenal axis needs to be thoroughly manipulated hormonally in order to begin to examine the effects that stress hormones may play on disease susceptibility. Physiological rationale in this research is that inhibition of the renal axis by the synthetic glucocorticoid dexamethasone will inhibit secretion of corticotropin releasing hormone, adrenocorticotrophic hormone, and cortisol and possibly may prevent some of the negative effects of stress response on disease susceptibility.

This study had three main objectives: 1) to examine how manipulation of the hypothalamic-pituitary-interrenal axis using Dex and ACTH changes disease susceptibility of channel catfish to ESC, 2) to evaluate Dex as a possible way to reduce the negative effects of stress during confinement and transport, and 3) to determine the effects of the synthetic hormones dexamethasone and Cortrosyn® (ACTH) (Amphastar Pharmaceuticals, Inc) on blood plasma cortisol concentrations of stressed and unstressed channel catfish fingerlings in addition to evaluate the effect Dex and ACTH have on blood plasma mineral concentrations in channel catfish.

12. Methods

12.1 Fish

Fingerling channel catfish, *Ictalurus punctatus*, (N=1000) were obtained from commercial aquaculture ponds at Greenwater Fish Farm (Gibson County, Tennessee).

Fingerlings (N=450) were hand graded for weight, with weights ranging from 86-111 g (mean 98.2 g, SD 5.3 g). The fingerlings were hatched on-site at Greenwater Fish Farm from eggs of broodstock maintained at the facility and stocked into commercial grow-out ponds with no previous outbreaks of *E. ictaluri*. The fingerlings did not exhibit clinical signs of infection (unusual swimming, hemorrhaging, pustules, lesions, hole in head syndrome) and appeared healthy upon inspection. A sample swipe from the abdominal cavity, liver, and brain cavity of seven fish was plated on brain-heart infusion (BHI) agar. Samples from all seven fish failed to produce any colonies of *E. ictaluri* after incubation at 30°C for 72 h. Additional samples (10 fish) failed to generate any *E. ictaluri* cells in BHI broth after incubation at 30°C for 48 h. All samples were verified using analytical profile index 20E (API 20E).

12.2 *Edwardsiella ictaluri*

An *E. ictaluri* unknown strain isolate was obtained from a naturally occurring outbreak at a commercial catfish operation (Auburn University Fish Health Laboratory). The identity of this isolate as *E. ictaluri* was verified by biochemical isolation tests described by Hawke et al. (1998) and produced API 20E analytical profile index codes of 4004000. This isolate was taken from frozen aliquots used in previous experiments by Sink and Strange (2004) and Sink et al. (*In review*).

To assure virulence, one aliquot of the isolate was propagated in BHI broth in a shaking bath at 27°C for 48 h. Ten fish (97-105 g) were exposed by immersion bath to 7.1 Log colony-forming units per L (Log CFU/L) in 70-L tanks. The exposed fish were reared for 10 d with all exhibiting one or more clinical signs of ESC infection (red

pustules, hemorrhaging, and ulcers were the most common). The posterior kidney of five fish was swabbed and inoculated into BHI broth tubes for reisolation of *E. ictaluri*. The identity of the isolate was verified using the API 20E. Seven of the 10 inoculated tubes produced API 20E codes of 4004000. The BHI broth containing the isolate was then frozen in six aliquots of 1 mL for future verification and use in other challenges. The one remaining aliquot was used to inoculate two, 500-mL samples of BHI broth for use in the challenge. The 500-mL BHI samples were then cultured at 30°C for 48 h. Spectrophotometer readings were taken in association with plate counts to allow comparison with future challenges.

12.3 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 0.002 mg/L. Water temperature was maintained at $27 \pm 2^\circ\text{C}$. An air stone was used in each aquarium for supplemental aeration to maintain dissolved oxygen levels. Water quality was monitored daily for dissolved oxygen (>6 mg/L), temperature ($27 \pm 2^\circ\text{C}$), and total chlorine (<0.002 mg/L). Water quality was tested weekly for pH (7.0-7.3), and total ammonia (<1.0 mg/L). Lighting was automatically controlled by timer with a 16 h light / 8 h dark cycle. The fingerlings were fed Zeigler floating finfish bronze (35% protein, 5% fat) 3mm pellets at a rate of 2% of body weight daily.

12.4 Hormone level determination

Hormone dosage was calculated based on an average fish weight of 100 g. Hormones were dissolved in sterile physiological saline to a desired concentration. Fish received intramuscular injections of Dex 20 μ g/fish (approximately 200 mg/kg) (Figure 4.1) and Cortrosyn® (Amphastar Pharmaceuticals, Inc.) (cosyntropin a synthetic ACTH) 5.0 μ g/fish (approximately 50 μ g/kg) in 0.5 cc saline.

Selection of dosage was arbitrary, taking into consideration that suppression of cortisol secretion (inhibition of ACTH secretion) by Dex is usually attained with dosages of Dex between 10-100 μ g/kg (intravenously) in dogs and 40-100 μ g/kg (intravenously) in horses (Appendix L). Dosage of Dex for therapy of different conditions in different species often varies between 0.5 to 6.0 mg/kg. Dose of synthetic ACTH used for adrenal stimulation varies between 1.0 and 20 μ g/kg (intravenously) depending upon specie (Plumb 2002). In summary, dosages of Dex and ACTH injected fish were comparable with diagnostic dosages used in mammals. To our knowledge, optimization of dose-response and time-response relationships for Dex and ACTH are not available in channel catfish.

In order to test these dosages, six fish (100-108 g) were given doses of Dex or ACTH (3 each) by intramuscular injection. Fish were quickly anaesthetized previous to injection using 100 mg/L of MS-222. Fish were then returned to their tank to recover immediately after the injection was administered. Fish receiving Dex were bled (under anesthetic 150 mg/L MS-222) by vivisection of the tail at the caudal peduncle for cortisol analysis at time 0, time 180, and time 240 min after receiving the injection. The time 0 min fish had a cortisol concentration of 18 ng/mL, similar control levels for channel



Figure 4.1: Measuring dexamethasone doses for injection in channel catfish.

The above photo shows doses of dexamethasone being measured from stock solution. Hormones were injected using 1 mL tuberculin syringes and were administered intramuscularly.

catfish in Sink and Strange (2004). Time 180 (3 ng/mL) and time 240 min (5 ng/mL) fish exhibited suppressed cortisol concentrations. Twenty ug/100g of Dex was deemed sufficient to suppress cortisol response for use in the main challenge. Fish receiving ACTH were anaesthetized and bled in the same manner. Bleeding times for ACTH fish were times 0, 60, and 120 min. Once again, cortisol concentrations of the time 0 min fish (22 ng/mL) were similar to the controls (23 ng/mL) in Sink and Strange (2004). Time 60 (77 ng/mL) and time 120 (81 ng/mL) min fish were similar to the time 30 min (77 ng/mL) confinement stress fish in Sink and Strange (2004). The cortisol concentrations for the ACTH injections were elevated enough to be comparable to an elevated stress level, so they were considered sufficient for use in the main challenge.

12.5 Hormonal disease challenge

Twenty-five graded (86-111 g, mean 98.2 g, SD 5.3 g) fingerling catfish (total N = 450) were stocked into each of 18 experimental tanks. The fish were allowed to acclimate for 14 d. Six treatments were applied to three tanks each for replication.

12.5.1 Treatment I – control/baseline

The objective of this treatment was to establish a baseline for cortisol and mortality of unstressed and non-injected fish for control comparisons of other treatments. Twenty-five fish were removed from the tank and placed in 100 mg/L MS-222. All fish achieved stage IV anesthesia (total loss of muscle tone and equilibrium; slow but regular opercular rate; loss of spinal reflexes) in less than 3 min. Once the fish achieved stage IV anesthesia, each was injected with 0.3 cc sterile saline (Figure 4.2) intramuscularly to simulate hormone injection and placed back in the tank to recover. All fish were injected in less than 5 min. Once all fish were replaced in the tank, five fish were randomly selected and placed in 150 mg/L MS-222. Once the fish achieved stage V anesthesia (total loss of reactivity; opercular movements slow and irregular; heart rate very slow; loss of all reflexes), they were bled using 370 μ L heparinized Caraway tubes by vivisection of the tail at the caudal peduncle for analysis of cortisol. Water flow to the tank was then shut off with aeration remaining constant. The remaining 20 fish per tank were exposed to 1.4 Log colony forming units/L (Log CFU/L) of *E. ictaluri* (20 mL of inoculated BHI @ 1×10^6 CFU) verified by spectrophotometer readings of 0.390 for 650 nm and 0.346 for 700 nm. Water flow was restored allowing the bacteria to flush from



Figure 4.2: Hormonal injections of channel catfish previous to the disease challenge.

The above photo illustrates the intramuscular injection of channel catfish with dexamethasone, adrenocorticotrophic hormone, or saline dependant on treatment.

the system at the end of the 8 h exposure period. Fish were then monitored for mortalities daily for 21 d.

12.5.2 Treatment II – confinement stress 30 min

The objective if this treatment was to establish mortality and cortisol concentrations of a stress event similar to handling or transport stress for comparison with hormonally treated fish. Twenty-five fish were anesthetized and injected with saline as in treatment I. The fish were placed back into the tanks in confinement baskets (12.5 x 15 x 10-cm) constructed of rigid vinyl netting. The baskets caused the fish to be restricted in movement and to be in contact with other fish as well as the basket at all times. Five



Figure 4.3: Bleeding of channel catfish during the disease challenge.

The above photo illustrates the method of bleeding channel catfish during disease challenges. The fish were anesthetized in 150 mg/L of MS-222 until they achieved stage V anesthesia. The tail was then severed at the caudal peduncle and blood was collected using heparinized caraway tubes from the caudal artery.

fish were then removed from each tank for bleeding (Figure 4.3) and cortisol analysis as in treatment I. The remaining 20 fish per tank were released into the tanks at the conclusion of the 30 min confinement stress period. Water flow and bacteria administration was completed as in the previous treatment. The fish were monitored for mortalities for 21 d.

12.5.3 Treatment III – dexamethasone injection

The purpose of this treatment was to evaluate Dex's cortisol suppression capabilities and evaluate its effect on mortality in channel catfish exposed to ESC.

Twenty-five fish were anesthetized as in the previous treatments and injected intramuscularly with 20 µg of Dex. All fish were then placed back into the tanks. Five fish from each tank were randomly removed for bleeding and cortisol analysis after 180 min, allowing the Dex time to suppress cortisol concentrations. Water flow manipulation and bacteria administration was then completed as in previous treatments. Mortalities were monitored for 21 d.

12.5.4 Treatment IV – adrenocorticotrophic hormone injection

The objective of this treatment was to examine if cortisol concentrations brought about by ACTH injection were similar to those caused by stress events and if elevated cortisol concentrations caused by ACTH injection would produce similar mortality rates to stress treatments. Twenty-five fish were anesthetized as in the previous treatments and injected intramuscularly with 5 µg of ACTH. All fish were then placed back into the tanks. Five fish from each tank were randomly removed for bleeding and cortisol analysis after 60 min, allowing the ACTH time to stimulate cortisol production. Water flow manipulation and bacteria administration was then completed as in previous treatments. Mortalities were monitored for 21 d.

12.5.5 Treatment V – dexamethasone injection and confinement stress 30 min

The objective of this treatment was similar to treatment III, but was used to examine whether stress stimuli can overcome Dex suppression of cortisol and what the effect on mortality would be. Twenty-five fish were anesthetized as in the previous treatments and injected intramuscularly with 20 µg of Dex. All fish were then placed

back into the tanks. The 25 fish in each tank were netted and placed in confinement baskets (12.5 x 15 x 10-cm) at time 150 min. At the conclusion of the confinement stress (time = 180 min), the fish were released into the tank and 5 fish from each tank were removed and bled for cortisol analysis as in previous treatments. Water flow manipulation and bacteria administration was then completed as in previous treatments. Mortalities were monitored for 21 d.

12.5.6 Treatment VI – adrenocorticotrophic hormone injection and confinement stress 30 min

The objective of this treatment was to determine what the combined effects of stress and ACTH injection were on cortisol concentration and mortality. Twenty-five fish were anesthetized as in the previous treatments and injected intramuscularly with 5 µg of ACTH. All fish were then placed back into the tanks. The 25 fish in each tank were netted and placed in confinement baskets (12.5 x 15 x 10-cm) at time 30 min. At the conclusion of the confinement stress (time = 60 min), the fish were released into the tank and five fish from each tank were removed and bled for cortisol analysis as in previous treatments. Water flow manipulation and bacteria administration was then completed as in previous treatments. Mortalities were monitored for 21 d.

12.6 Cortisol analysis

The 370 µL heparinized Caraway tubes containing collected blood were blown into 1.5-mL centrifuge tubes (Figure 4.4). The plasma was separated from the red blood cells by centrifugation. The plasma was removed by disposable pipettes and placed into



Figure 4.4: Blood collection and separation.

The above photo depicts blood collection and setup for separation and storage of plasma from channel catfish in the disease challenges.

1.5-mL cryotubes and frozen until cortisol assays were performed. Plasma was later thawed at room temperature for 30 min and used in conjunction with Coat-A-Count® solid-phase ^{125}I -cortisol radioimmunoassay. The competitive assay was used for quantitative measure of cortisol (hydrocortisone, compound F) in the blood plasma. The assay was modified from 25 μL to 10 μL of plasma and calibrators in order to accommodate the small blood volumes received from the fish. Validation of the assay was done with known plasma pools from previous experiments done with 25 μL plasma volumes randomly conducted during the assay procedure. All samples and calibrators were done in duplicate.

12.7 Mineral analysis

Blood plasma mineral analysis was conducted by the Clinical Pathology Laboratory, College of Veterinary Medicine, The University of Tennessee. The technique used was based on the use of ion selective electrodes and a Hitachi 911 chemistry analyzer. Plasma from fish for each treatment was measured for sodium, potassium, and chloride concentrations.

12.8 Statistical analyses

Differences in cortisol concentrations, mortality rates, and plasma mineral concentrations among treatments were tested using one way analysis of variance (ANOVA) and Tukey's post hoc tests (SPSS 11.0 2000). Differences were considered significant at $P < 0.05$. Pearson correlation was used to measure the relationship between cortisol level and mortality.

13. Results

13.1 Effects of hormones and stress on blood plasma cortisol concentration

A one-way ANOVA of cortisol (Appendix M) for the six treatments showed that cortisol concentrations for treatments were different ($P < 0.05$). Tukey's HSD post hoc test demonstrated three treatment groupings that were not different from each other. Control/Baseline cortisol concentrations were not different from Dex and Dex stress 30 min treatments ($P = 0.973$, and 0.971 , respectively). Stress 30 min cortisol concentrations did not differ from ACTH concentrations ($P = 0.468$), and ACTH treated cortisol concentrations were not different from ACTH stress 30 min treatments ($P = 0.129$).

However, stress 30 min cortisol concentrations were different ($P = 0.001$) from ACTH stress 30 min. Treatments were divided into three separate groups from lowest cortisol concentration to highest: 1 – control/baseline, Dex and Dex stress 30 min, 2 – stress 30 min and ACTH, and 3 – ACTH, and ACTH stress 30 min. Mean cortisol concentrations for each replicate in each treatment may be found in Table 4.1.

13.2 Effect of hormones and stress on mortality

A one-way ANOVA of percent mortality (Appendix N) for channel catfish in the six treatments showed that treatments were different. A Tukey's HSD post hoc test revealed many overlapping treatment groups (Table 4.2). The control/baseline treatment mortalities were different ($P < 0.05$, mean $\pm$ SE: $8.3 \% \pm 1.7$) than the other five treatments (mortality - Table 4.3). Dex ($78.3 \% \pm 11.7$) was not different from ACTH treatments ($66.7 \% \pm 6.0$). ACTH treated mortalities were also similar to ACTH stress 30 min ($51.7\% \pm 1.7$). ACTH stress 30 min mortalities could not be differentiated from Dex stress 30 min ($40.0 \% \pm 0.0$) and stress 30 min ($36.7 \% \pm 1.7$). Only the control/baseline treatment mortalities could be distinguished as a separate treatment group, with all other treatment means exhibiting overlap with at least two other treatments.

13.3 Effects of treatments on plasma concentrations of sodium, potassium and chloride

One-way ANOVAs of sodium ($P = 0.251$), potassium ($P = 0.457$), and chloride ($P = 0.390$) revealed that all three were not statistically different for any treatment. Sodium had a range of 15.0 mEq/L (max:148, min:133 mEq/L) and a mean of 139.9 ± 1.3 mEq/L for all treatments combined. Potassium's range was 2.4 mEq/L (max: 6.2, min: 3.8

Table 4.1: Mean cortisol concentrations by treatment for channel catfish in hormonal disease challenge.

Mean ($\pm$ SE) Cortisol Concentrations (ng/mL)						
Treatment						
Tank	Control/ Baseline	Dex	Dex Stress 30 min	Stress 30 min	ACTH	ACTH Stress 30 min
1	10.3 $\pm$ 1.6	9.0 $\pm$ 3.5	13.5 $\pm$ 5.0	82.0 $\pm$ 21.0	81.7 $\pm$ 15.3	60.5 $\pm$ 6.8
2	13.8 $\pm$ 1.6	7.2 $\pm$ 0.9	16.9 $\pm$ 2.5	85.9 $\pm$ 10.8	71.5 $\pm$ 9.7	64.9 $\pm$ 4.0
3	13.2 $\pm$ 1.5	6.0 $\pm$ 0.8	22.1 $\pm$ 4.1	98.2 $\pm$ 11.0	77.4 $\pm$ 9.1	55.7 $\pm$ 7.9

Mean cortisol concentrations (ng/mL) of channel catfish for each replicate of hormonal treatment from the disease challenge.

Table 4.2: Treatment groupings for mortality of channel catfish in hormonal disease challenge.

Treatment	Mean $\pm$ SE	Group
Control	8.3 % $\pm$ 1.7	A
Dexamethasone	78.3 % $\pm$ 11.7	B
Adrenocorticotropic	66.7 % $\pm$ 6.0	B C
ACTH Stress 30 min	51.7 % $\pm$ 1.7	B C D
Dex Stress 30 min	40.0 % $\pm$ 0.0	C D
Stress 30 min	36.7 % $\pm$ 1.7	C D

Statistically overlapping treatment groups for percent mortality means ($\pm$ SE) of channel catfish in hormonal disease challenge. ABCD means with no common letter differ (Tukey $P < 0.05$).

Table 4.3: Mortality rates for channel catfish in each replicate of six hormonal treatments.

Percent Mortalities by Treatment						
Treatment						
Tank	Control/ Baseline	Dexamethasone	Dexamethasone Stress 30 min	Stress 30 min	ACTH	ACTH Stress 30 min
1	5	100	75	35	40	50
2	10	60	55	40	40	50
3	10	75	70	35	40	55

Percent mortality rates for six hormonal treatments for channel catfish in each replicate of ESC disease challenge.

mEq/L), and a mean of 5.3 ± 0.2 mEq/L. Chloride possessed a range of 11.0 mEq/L (max: 114, min: 103 mEq/L), and a mean of 109.4 ± 1.2 mEq/L for all six treatments.

14. Discussion

Channel catfish are often exposed to various physical and environmental stressors during production that often occur in the presence of one or more pathogens. Stress often facilitates disease outbreaks in commercial fish rearing operations. These stressors increase the susceptibility of channel catfish fingerlings to ESC in experimental situations (Wise et al. 1993). Disease outbreak intensity, frequency, and duration can be reduced by minimizing stress (Lewis and Shelton 2003). This study examined the hormonal manipulation of the hypothalamo-pituitary-interrenal axis, or stress axis, in an attempt to evaluate changes in the stress response and disease susceptibility of channel catfish to ESC.

The first objective was to examine how manipulation of the hypothalamo-pituitary-interrenal axis using Dex and ACTH changes disease susceptibility of channel catfish to ESC. The results were unexpected as cortisol concentrations are assumed to be correlated with immune suppression in fish. Mortalities for the baseline/control treatments (Table 3) were low and comparable to control mortalities in Sink and Strange (2004). Slightly elevated mortalities in the control/baseline treatment would not have been surprising due to the fact that their skin was punctured by injection previous to exposure creating a new route of infection.

The lack of differentiation in mortality between other treatments (Table 4.2) could not be explained by cortisol concentrations as we expected those with lower

concentrations to be less susceptible to ESC. However, we determined that Dex exhibited the greatest mortality ($78.3 \% \pm 11.7$) with the lowest mean cortisol concentration (7.4 ± 1.2 ng/mL). Dex was not different from ACTH ($66.7 \% \pm 6.0$) and ACTH stress 30 min ($51.7 \% \pm 1.7$) which were expected to exhibit the greatest mortality.

Mineral imbalance or depletion due to hormonal overdose was thought to be a possible culprit in the unexplained mortality. This led us to evaluate the effect Dex and ACTH have on blood plasma mineral concentrations in channel catfish. However, the results from the mineral analysis revealed that there were no differences in blood plasma concentrations of sodium, potassium and chloride in any treatment. This does not mean that a mineral imbalance was not occurring at the cellular level, but this was not the focus of this study. Mineral analysis at the cellular level should be considered in future studies.

The second objective of this study was to evaluate Dex as a possible way to reduce the negative effects of stress during confinement and transport. Dex was effective at suppressing the primary stress hormone cortisol in fish, in both stressed (17.5 ± 2.3 ng/mL) and unstressed (7.4 ± 1.2 ng/mL) channel catfish fingerlings. Although Dex is capable of suppressing ACTH and cortisol, its use as a stress preventative, especially for the reduction of disease susceptibility is unfounded based on this study. Dex is a synthetic glucocorticoid that competes with naturally produced cortisol by binding receptors. It is possible that some or all of the receptors in fish could be occupied by Dex enforcing the stress response in the absence of cortisol.

The final objective was to determine the effects of the synthetic hormones Dex and ACTH on blood plasma cortisol concentrations of stressed and unstressed channel catfish fingerlings. Results from the study were consistent with those from the hormone

level determination pre-study. Control/baseline cortisol concentrations (mean $\pm$ SE: 12.4 ± 0.9 ng/mL) were low and comparable to controls in Sink and Strange (2004). Dex (7.4 ± 1.2 ng/mL) and Dex stress 30 min (17.5 ± 2.3 ng/mL) were also low and not different from the controls as expected. The Dex stress 30 min cortisol mean concentration was slightly higher than both the Dex and control/baseline concentrations, but Dex was still capable of cortisol suppression under induced stress conditions.

ACTH treated cortisol concentrations (76.9 ± 6.4 ng/mL) were expected to be similar to stress 30 min concentrations (88.7 ± 8.2 ng/mL) based upon results of the hormonal level determination study. Our assumption was proven to be correct by the cortisol concentration ANOVA. However, ACTH stress 30 min (60.3 ± 3.6) mean concentrations were lower than stress 30 min concentrations. This was not as expected as stress compiled with ACTH injections should theoretically push cortisol concentrations beyond what stress alone can produce. We however know that there must be a maximum rate of production for cortisol after which further stimulation has no effect, but we expect that cortisol concentrations of ACTH stress 30 min would equal those of the ACTH treated fish.

Two possible explanations are: 1) the fish placed in the confinement baskets struggled and moved constantly increasing metabolic rate. This could have lead to greater metabolic breakdown and faster use of the ACTH, causing faster recovery times for cortisol concentrations, 2) blood plasma cortisol profiles could have still been in transition, and sampling at time = 60 min was not the best stage to measure cortisol concentrations. Time response studies for the duration of each treatment are needed to remedy this possible problem. From this study, we can see that Dex is capable of

suppressing cortisol concentrations to baseline/control concentrations, and that ACTH injections can create cortisol concentration stress responses similar to confinement stress.

Mortality increased in all treatments above control/baseline rates. All treatments included stress components: dexamethasone, adrenocorticotrophic hormone, and/or confinement. The common denominator was increased glucocorticoid concentrations: either Dex or cortisol. The “lethal” effect of ACTH injection was probably mediated by cortisol in part, not due to ACTH itself. However the fact that mortality remained high when cortisol was low (but Dex was high) indicated that elevated glucocorticoid activity, rather than specific cortisol was a “lethal” factor in disease susceptibility of catfish.

What is so bad in glucocorticoids to increase susceptibility to enteric septicemia and become a “lethal” factor? There is not a final answer. The following effects of glucocorticoids should be considered. Dexamethasone is a potent anti-inflammatory steroid (inflammation is a defensive response) that may cause gastrointestinal complications including septicemia and endotoxic shock. It does block the acquisition and expression of cell-mediated immunity and inhibit the protective mechanism by the white blood cells in mammals.

New treatment and prevention methods must be devised as prevalence of disease continues to increase in aquaculture industries. Current unregulated use and overuse of the few antibiotics available for treatment (Benbrook 2002) must be alleviated by new technologies. Development of new vaccines, such as the ESC vaccine available from Intervet, Inc. (ARS 1999) must be complimented with reduction of pathogen exposure and reduction of stressors at the source of outbreaks. Further understanding of stress, cortisol, and their relationship to disease susceptibility is still needed. This study helps to

formulate an understanding of the underlying causes of infection and their relationship to stress in fish.

Chapter Five

General Conclusions

Section 15. Cortisol and Stress

We began these studies with one question in mind. Why should a typical aquaculture operation care about the known cortisol increases that occur in fish in conjunction with stress? From this general question we developed more concise questions such as: 1) Is cortisol an accurate biological indicator of stress in channel catfish? 2) Can cortisol be used as a predictor of mortality in channel catfish subjected to pathogens? 3) Is stress as determined by cortisol concentration more important than pathogen concentrations? 4) Does cortisol play a direct role in immune suppression of channel catfish, or is some other component of stress causing immune suppression? We used disease challenges in conjunction with stress to attempt to answer these as well as other, questions.

Previous studies by Strange (1980) and Tomasso et al. (1981), as well as our first challenge (Sink and Strange 2004) with small and large channel catfish fingerlings, confirmed that cortisol was an accurate biological indicator of stress in channel catfish. Cortisol was highly correlated with increases in time of confinement stress in all of the disease challenges presented in this dissertation. We assumed, based on our results in chapter II, that a biological indicator of stress, such as cortisol, might be used to predict increases in infection rates in channel catfish subjected to stress. My conclusions, based on the small, large, high, medium, and low disease challenges that cortisol might be used

as an indicator of increased infection due to its ability to suppress the immune system, were premature. This issue will be addressed more in the next section.

Based on the series of studies that we have conducted, we believe that cortisol may be capable of predicting mortality rates, but that these studies have not provided clear evidence that cortisol itself is an immune suppressant in channel catfish. Although cortisol is highly correlated with increases in stress, as well as increases in mortality, it may be an indirect correlation and does not point to a direct cause and effect relationship. Cortisol is an important tool for diagnostic use in stress studies, but further research is necessary in order to make a definitive statement that cortisol is an immune suppressant in fish.

Section 16. Stress, Cortisol, and Disease

Previous work by Wise et al. (1993) has already demonstrated that stress increases channel catfish mortality in the presence of a pathogen (*E. ictaluri*). Several other researchers such as Maule et al. (1989) and Mesa et al. (2000) have shown similar results in other species of fish. The studies presented in this dissertation included the biological indicator of stress, cortisol, which was not used by Wise et al. (1993). The use of cortisol was for indication and quantification of the amount of stress that channel catfish were undergoing during the studies.

The first two studies, presented in Chapters II and III, indicated that stress and cortisol were highly correlated with increases in infection. In Chapter III, evidence was reported that stress, as indicated by cortisol concentration was more important in increasing infection rates than concentration of the pathogen in the environment.

However, no direct evidence was provided as to the means of cortisol increasing infection. Rather it may be concluded that some other function of stress, or stress as a whole, causes increased susceptibility.

As addressed in the previous section, it appeared that cortisol may have been responsible for immune suppression and increased mortality based on the studies included in Chapters II and III. Cortisol's immune suppressive capabilities were also confirmed by the controls and natural stress treatments in the hormonal disease challenge presented in Chapter IV. Controls that were not subjected to stress maintained low concentrations of cortisol and low levels of mortality. Fish subjected to confinement stress exhibited the typical increases in cortisol concentration and corresponding increases in mortality similar to previous studies. Additional studies by Weyts et al. (1997, 1999) provided evidence that cortisol does produce immune suppressive effects, such as apoptosis of leukocytes, suppressed phagocyte recruitment, and redistribution of leukocytes (Maule and Schreck 1990). However, in the hormonal disease challenge, evidence that cortisol alone is not an immune suppressant was found.

The lack of differentiation in mortality among treatments could not be explained by cortisol concentrations, since we expected those with lower concentrations to be less susceptible to ESC. Instead, dexamethasone suppressed cortisol to one of the lowest concentrations in the challenge, yet had the greatest mortality rates. Hormones were administered in the hope of eliminating other factors of the stress response so that the interrenal loop and cortisol's immune function could be studied without other effects of stress.

If we eliminate all other factors of stress, and are able to examine the effects of cortisol alone on disease susceptibility, these facts would allow us to make the assumption that cortisol alone does or does not affect immune suppression. Perhaps the unexpected results that were obtained stemmed from unnatural routes of creating an “artificial” stress response. The synthetic hormones could have triggered unexpected and unaccounted for reactions. We do see that the two natural modes of stress, controls and stress 30 min, exhibited expected cortisol concentrations and mortality rates based on previous challenges. This again suggests that cortisol and mortality are correlated, although there was no direct cause and effect relationship that is established. From the results presented in the hormonal disease challenge, it appears that cortisol does play a role in immune suppression, although it is not directly responsible for immune suppression. Cortisol seems to play a secondary role, perhaps triggering other physiological processes that are the main contributors to immune suppression during stress in channel catfish.

Section 17. Summary

The main purpose of the research presented in this dissertation was to examine the function of the stress hormone cortisol in disease susceptibility of channel catfish, *Ictalurus punctatus*. These studies were conducted in an attempt to further scientific understanding of cortisol’s roles in stress and disease of channel catfish, as well as benefit fish farmers with possible ESC prevention methods.

Key objectives were: to 1) to document differences in cortisol levels between different lengths of acute confinement stress, 2) to document differences in mortality with

associated stress levels following an *E. ictaluri* challenge, 3) to examine if cortisol concentration is capable of predicting increases in mortality during disease challenges, 4) 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, 5) to examine how manipulation of the hypothalamo-pituitary-interrenal axis using Dex and ACTH changes disease susceptibility of channel catfish to ESC, 6) to evaluate Dex as a possible way to reduce the negative effects of stress during confinement and transport, and 7) to determine the effects of the synthetic hormones dexamethasone and adrenocorticotrophic hormone on blood plasma cortisol concentrations of stressed and unstressed channel catfish fingerlings, and in addition, to evaluate the effects Dex and ACTH have on blood plasma mineral concentrations in channel catfish.

Results from the challenge series indicated that cortisol had significant and defined increases as stress increases, as well as corresponding increases in mortality rates for channel catfish subjected to *E. ictaluri*. Chapter II results showed that cortisol was capable of predicting mortality rates based on stress in disease challenges using *E. ictaluri* to infect channel catfish. However, based on these results it was not justified to say that cortisol is a direct immune suppressant, or that cortisol was capable of predicting mortality in real world situations. There are entirely too many factors that contribute to stress, pathogen concentration, pathogen virulence, and individual or strain resistance of channel catfish, all of which effect ESC susceptibility, to be capable of making prediction tables.

Mortality rates in channel catfish subjected to *E. ictaluri* were determined by both amount of stress and pathogen concentration. However, stress was the most important factor in determining mortality rates in channel catfish. Reduction in pathogen concentration may decrease mortality rates, but stress in each pathogen concentration determines which fish are more susceptible to infection. Stress with the greatest stress level as determined by cortisol concentration were always the most susceptible to ESC.

Manipulation of the hypothalamo-pituitary-interrenal axis using Dex and ACTH changes disease susceptibility of channel catfish to ESC. Mortalities for the baseline/control treatments were low and comparable to control mortalities in Chapters II and III. The lack of differentiation in mortality between other treatments could not be explained by cortisol concentrations, as we expected those with lower concentrations to be less susceptible to ESC. However, Dex exhibited the greatest mortality with the lowest mean cortisol concentration. Dex was not different from ACTH and ACTH stress 30 min, both of which were expected to exhibit the greatest mortality. Mineral imbalance or depletion due to hormonal overdose was thought to be a possible culprit in the unexplained mortality. This led us to evaluate the effects Dex and ACTH had on blood plasma mineral concentrations in channel catfish. However, the results from the mineral analysis revealed that there were no differences in blood plasma concentrations of sodium, potassium, and chloride in any treatment.

Dex was effective at suppressing cortisol in both stressed and unstressed channel catfish fingerlings. Although Dex was capable of suppressing ACTH and cortisol, its use as a stress preventative, especially for the reduction of disease susceptibility, was unfounded based on the studies of this dissertation. Dex is a synthetic glucocorticoid that

competes with naturally produced cortisol by binding receptors. It is possible that some or all of the receptors in fish could be occupied by Dex enforcing the stress response in the absence of cortisol.

Finally, the effects of the hormones Dex and ACTH on blood plasma cortisol concentrations of stressed and unstressed channel catfish fingerlings were examined. Results were consistent with those from the hormone level determination pre-study. Control/baseline cortisol concentrations were low and comparable to controls in Chapter II. Dex and Dex stress 30 min were also low and not different from the controls as expected. The Dex stress 30 min cortisol mean concentration was slightly higher than both the Dex and control/baseline concentrations, but Dex was still capable of cortisol suppression under induced stress conditions.

ACTH treated cortisol concentrations were expected to be similar to stress 30 min concentrations based upon results of the hormonal level determination study. However, ACTH stress 30 min mean concentrations were lower than stress 30 min concentrations. This was not as expected as stress compiled with ACTH injections should increase cortisol concentrations to or beyond those of the stress 30 min treatment.

Conclusions based on these studies, were that cortisol is important as a biological indicator of stress. Also, stress and cortisol concentrations are highly correlated in channel catfish, producing definite interval increases. Cortisol concentrations and mortality rates due to ESC infection are also highly correlated. This, however, does not mean that cortisol directly caused immune suppression, but may play a secondary role in disease susceptibility of channel catfish. Stress was a very important and perhaps overlooked aspect of disease susceptibility in fish. Hormonal manipulation of the

hypothalamo-pituitary-interrenal axis in fish was experimentally viable and valuable as a research tool. Hormonal manipulation also indicated no basis for cortisol being a direct immune suppressant in channel catfish.

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APPENDICES

Appendix A

Water Quality Report for the Johnson Animal and Research Teaching Unit Aquaculture Lab, Summer 2004

Appendix A:

General Water Quality Parameters
Johnson Animal Research and Teaching Unit
Aquaculture Lab

Parameter	Average Level Detected
Temperature	27.1 °C
Chlorine	0.003 mg/L
Dissolved Oxygen	8.0 mg/L
Alkalinity	70 mg/L
pH	7.21
Total Hardness	101
Flouride	1 mg/L
Nitrate (as Total Nitrogen)	1.1 mg/L

Appendix B

Cortisol Coat-A-Count® Radioimmunoassay Procedure

Appendix B:

Cortisol Coat-A-Count® Radioimmunoassay Procedure

1. Retrieve Coat-A-Count® assay kit from refrigeration storage at 2-8°C.
2. Heat water bath to 37°C.
3. Remove plasma from freezer storage at -4°C and allow thawing at room temperature during the labeling process.
4. Collect four plain 12 x 75 mm polypropylene tubes and label as follows:
 - 1 TC (total count)
 - 2 TC
 - 3 NSB (non-specific binding)
 - 4 NSBPlace the tubes in order in a test tube rack.
5. Label the blue polypropylene antibody coated 12 x 75 mm tubes that come with the assay kit as follows:
 - 5 A (calibrator A)
 - 6 A
 - 7 B (calibrator B)
 - 8 B
 - 9 C (calibrator C)
 - 10 C
 - 11 D (calibrator D)
 - 12 D
 - 13 E (calibrator E)
 - 14 E
 - 15 F (calibrator F)
 - 16 F
 - 17 Hi (pooled control stressed catfish plasma)
 - 18 Hi
 - 19 Lo (pooled control non-stressed catfish plasma)
 - 20 Lo
 - 21 Pig (pig plasma control)
 - 22 Pig
 - 23 Sample 1
 - 24 Sample 1
 - 25 Sample 2
 - 26 Sample 2
 - 27 Etc. until all samples have been accounted for in duplicate.

N ₊₁	Hi (pooled control stressed catfish plasma)
N ₊₂	Hi
N ₊₃	Lo (pooled control non-stressed catfish plasma)
N ₊₄	Lo

6. As the blue tubes are labeled, place them in the test tube racks in order.
7. Pipette 10 μ L of calibrator A into the bottom of NSB and A tubes. Pipette 10 μ L of calibrators B, C, D, E, and F into corresponding labeled tubes. Pipette 10 μ L of Hi, Lo, and pig controls to corresponding labeled tubes. Finally, pipette 10 μ L of the samples to each of their corresponding duplicate tubes. Always pipette to the bottom of the tube.
8. Place the tubes under an approved radiation hood. Pour the radioactive ^{125}I solution into a beaker. Use a 1 mL repipet to add 1 mL of iodine solution to every prepared tube. No more than ten min should pass from the first to the last tube. If this cannot be accomplished, the assay should be broken into two or more parts with the addition of more Hi, Lo, and pig controls.
9. Vortex each tube and place back into test tube rack.
10. Place the tubes in the water bath and allow them to incubate for 45 min.
11. Return the racks containing the tubes to the hood and place all the tubes in order into foam decanting racks except for the TC tubes. The TC tubes are not to be decanted and should remain in the original rack. Flip the foam racks upside down into an appropriate radiation container to remove the iodine solution from the tubes. The tubes should then be struck against absorbent lab paper to remove any remaining moisture inside the tubes.
12. Once the tubes are well drained, replace the tubes in order in the original rack. Take the tubes to a gamma counter for analysis.
13. Place the tubes in numerical order starting with TC tubes in the gamma counter. Once the counter is done, remove the tubes, place them back in the original rack and replace under the hood in case a recount is necessary.
14. Evaluate the data. If it is acceptable, dispose of the tubes and radioactive material as directed by the University of Tennessee radiation safety committee guidelines.
15. If any samples have a %CV greater than 10, rerun the assay with those samples.

Appendix C

Mean Cortisol Concentrations and Percent Mortalities by Treatment for the Small Fingerling Disease Challenge

Appendix C:

Means by Treatment for Small Fish Challenge

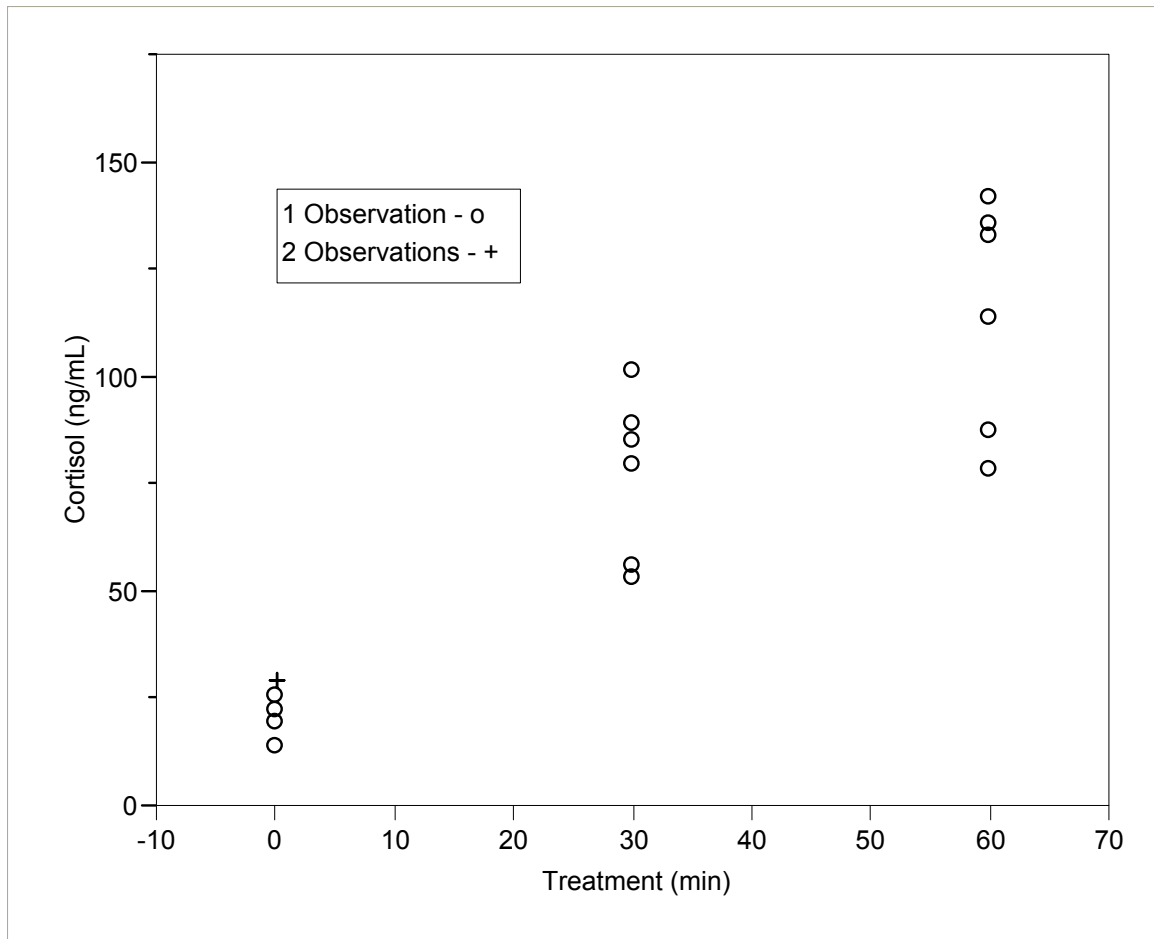
Treatment	Percent Mortality	Cortisol Concentration (ng/mL)
Control 1	20	14.20
Control 2	30	22.60
Control 3	20	29.60
Control 4	20	26.00
Control 5	20	29.60
Control 6	25	19.80
Stress 30 min 1	45	53.40
Stress 30 min 2	50	56.20
Stress 30 min 3	50	85.40
Stress 30 min 4	55	80.00
Stress 30 min 5	45	101.80
Stress 30 min 6	40	89.40
Stress 60 min 1	90	87.80
Stress 60 min 2	75	79.00
Stress 60 min 3	90	133.00
Stress 60 min 4	80	114.00
Stress 60 min 5	80	136.00
Stress 60 min 6	75	142.40

Appendix D

Scatter Plot of Cortisol by Treatment for Small Fingerling Challenge

Appendix D:

Scatter Plot of mean cortisol concentrations (ng/mL) for small fingerling channel catfish in the disease challenge. Each treatment contains six replicates.

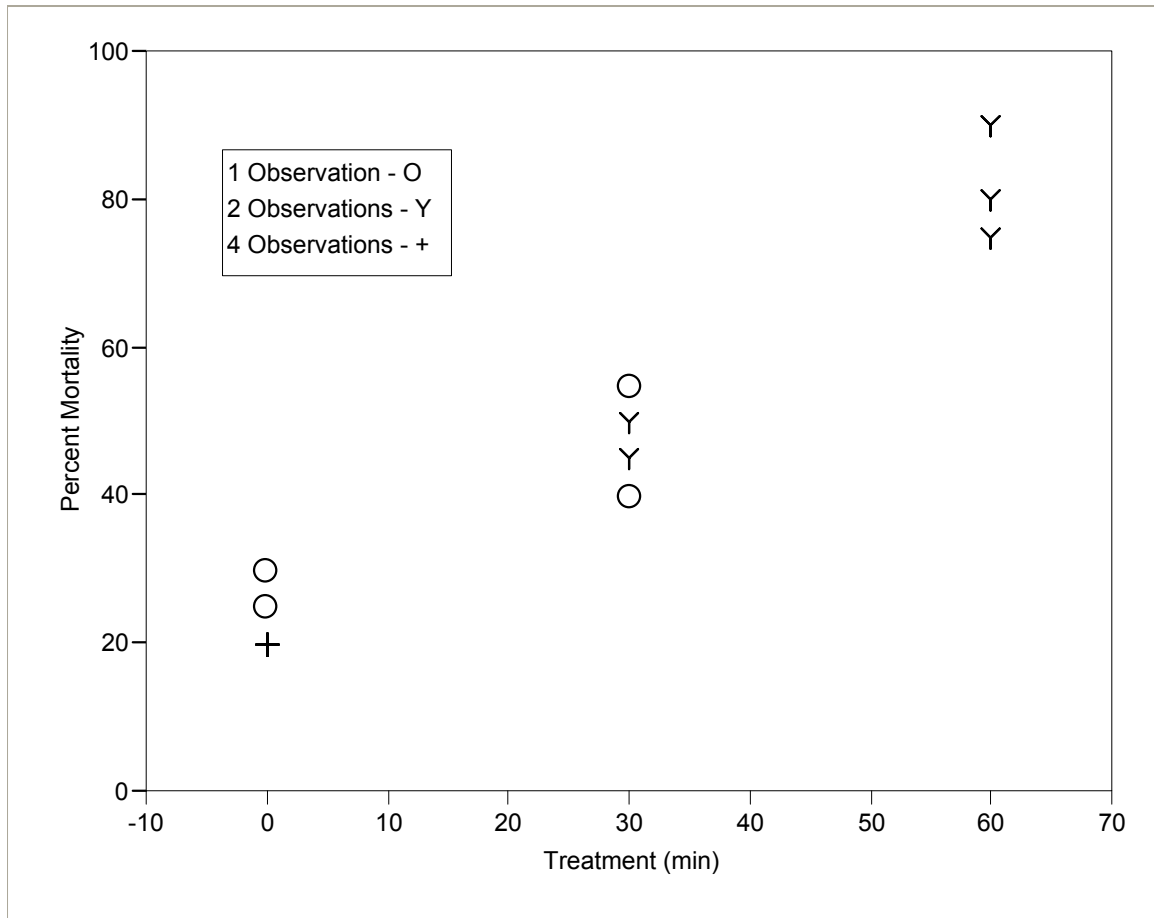


Appendix E

Scatter Plot of Percent Mortality by Treatment for Small Fingerling Challenge

Appendix E:

Scatter Plot of percent mortality for small fingerling channel catfish in the disease challenge. Each treatment contains six replicates. Several replicates are overlain.



Appendix F

Mean Cortisol Concentrations and Percent Mortalities by Treatment for the Large Fingerling Disease Challenge

Appendix F:

Means by Treatment for Large Fish Challenge

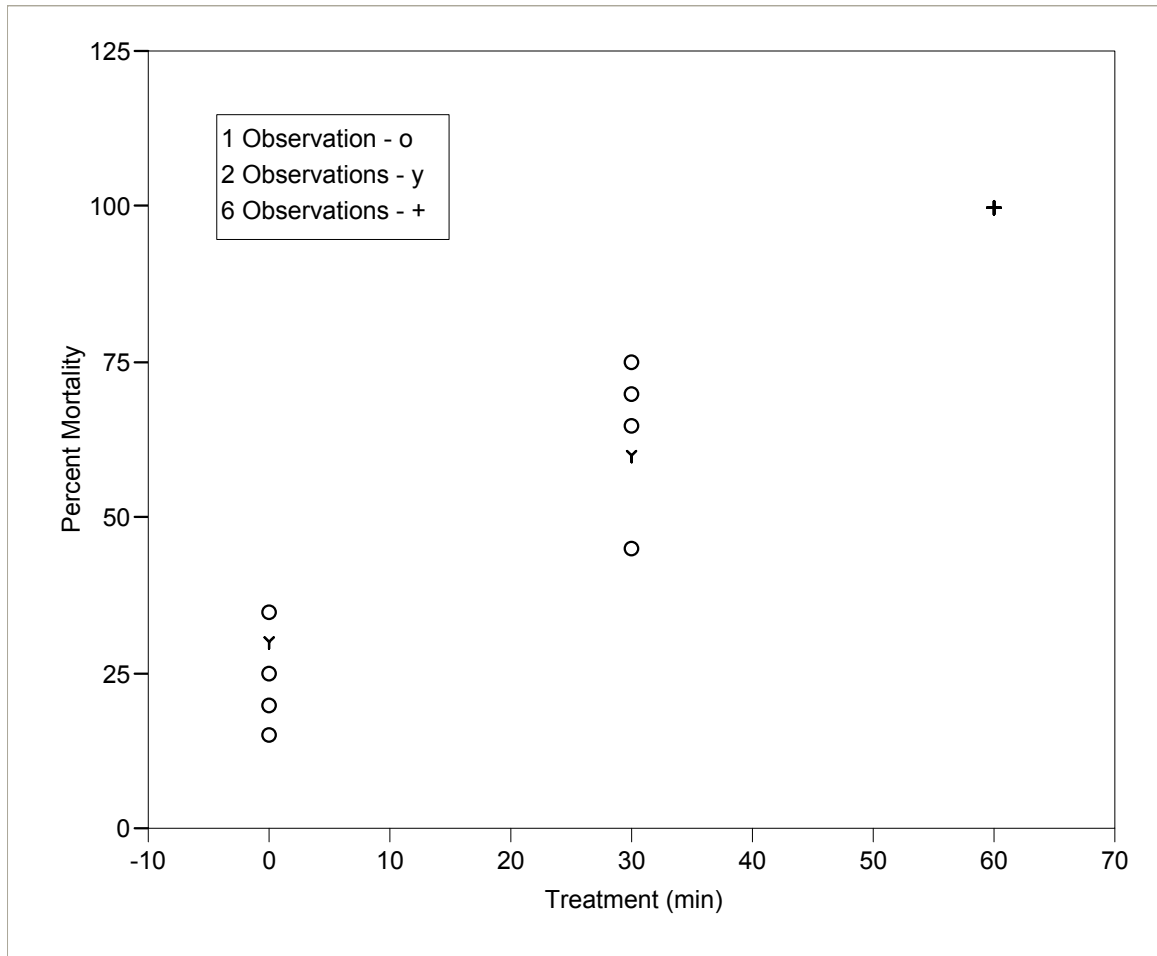
Treatment	Percent Mortality	Cortisol Concentration (ng/mL)
Control 1	30	12.20
Control 2	35	50.64
Control 3	15	15.46
Control 4	30	41.22
Control 5	20	50.26
Control 6	25	18.86
Stress 30 min 1	70	102.78
Stress 30 min 2	60	141.68
Stress 30 min 3	45	88.62
Stress 30 min 4	60	82.50
Stress 30 min 5	65	98.18
Stress 30 min 6	75	105.06
Stress 60 min 1	100	122.48
Stress 60 min 2	100	99.70
Stress 60 min 3	100	130.00
Stress 60 min 4	100	67.60
Stress 60 min 5	100	103.56
Stress 60 min 6	100	134.48

Appendix G

Scatter Plot of Percent Mortality by Treatment for Large Fingerling Challenge

Appendix G:

Scatter Plot of percent mortality for large fingerling channel catfish in the disease challenge. Each treatment contains six replicates. Several replicates are overlain (all time 60 min replicates are overlain).

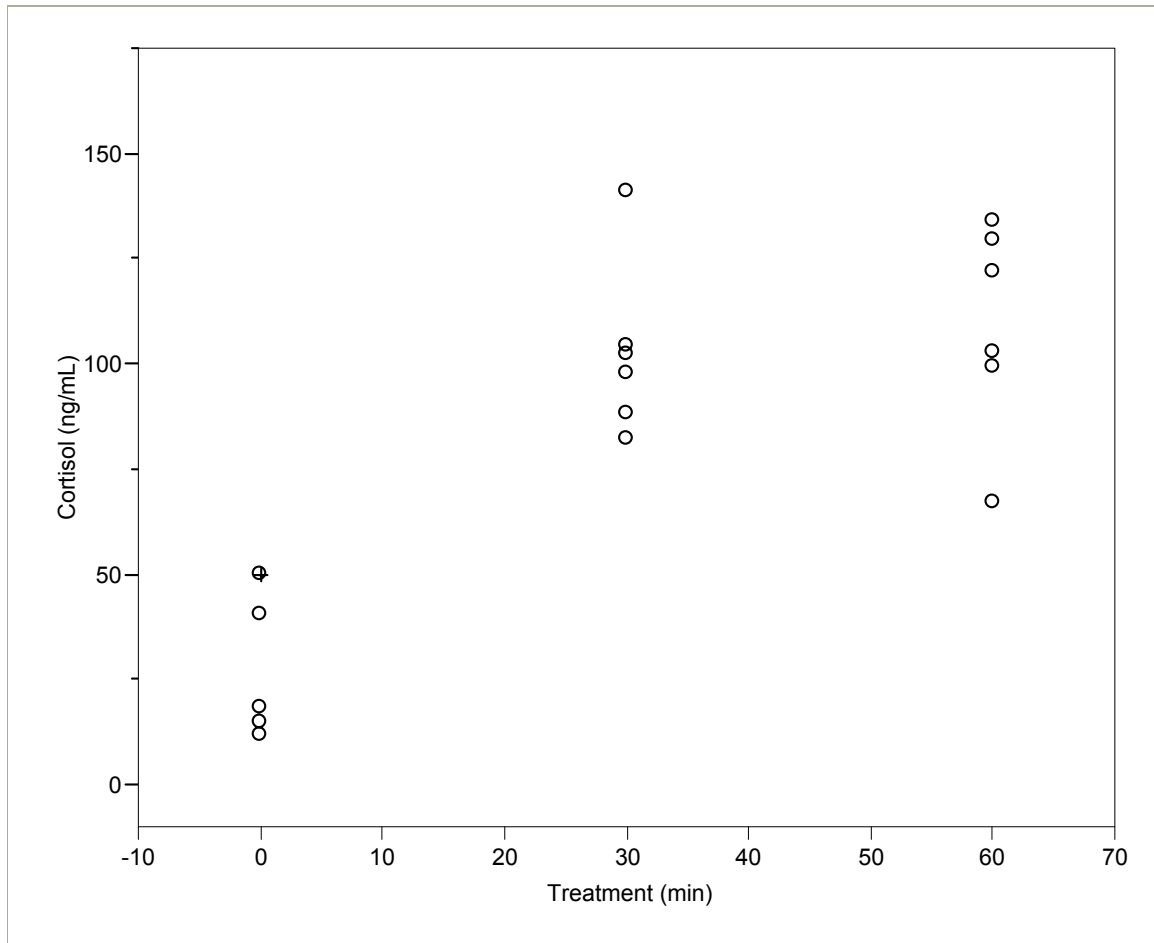


Appendix H

Scatter Plot of Cortisol by Treatment for Large Fingerling Challenge

Appendix H:

Scatter Plot of mean cortisol concentrations (ng/mL) for large fingerling channel catfish in the disease challenge. Each treatment contains six replicates.



Appendix I

Mean Mortality Rates and Cortisol Concentrations for High Dose Disease Challenge

Appendix I:

Means by Treatment for High Dose Challenge.

Treatment	Percent Mortality	Cortisol Concentration (ng/mL)
Control 1	20	14.2
Control 2	30	22.6
Control 3	20	29.6
Control 4	20	26.0
Control 5	20	29.6
Control 6	25	19.8
Stress 30 min 1	45	53.4
Stress 30 min 2	50	56.2
Stress 30 min 3	50	85.4
Stress 30 min 4	55	80.0
Stress 30 min 5	45	101.8
Stress 30 min 6	40	89.4
Stress 60 min 1	90	87.8
Stress 60 min 2	75	79.0
Stress 60 min 3	90	133.0
Stress 60 min 4	80	114.0
Stress 60 min 5	80	136.0
Stress 60 min 6	75	142.4

Appendix J

Mean Mortality Rates and Cortisol Concentrations for Medium Dose Disease Challenge

Appendix J:

Means by Treatment for Medium Dose Challenge.

Treatment	Percent Mortality	Cortisol Concentration (ng/mL)
Control 1	5	12.5
Control 2	5	9.6
Control 3	5	16.3
Control 4	15	10.1
Control 5	5	6.9
Control 6	10	7.5
Stress 30 min 1	75	85.3
Stress 30 min 2	75	92.4
Stress 30 min 3	65	73.2
Stress 30 min 4	90	82.1
Stress 30 min 5	70	94.6
Stress 30 min 6	85	103.3
Stress 60 min 1	90	99.2
Stress 60 min 2	100	122.0
Stress 60 min 3	100	124.8
Stress 60 min 4	95	96.3
Stress 60 min 5	100	164.8
Stress 60 min 6	100	169.9

Appendix K

Mean Mortality Rates and Cortisol Concentrations for Low Dose Disease Challenge

Appendix K:

Means by Treatment for Low Dose Challenge.

Treatment	Percent Mortality	Cortisol Concentration (ng/mL)
Control 1	5	3.4
Control 2	0	10.2
Control 3	5	5.6
Control 4	5	11.7
Control 5	10	5.2
Control 6	0	4.1
Stress 30 min 1	20	79.1
Stress 30 min 2	15	77.4
Stress 30 min 3	10	77.3
Stress 30 min 4	25	76.1
Stress 30 min 5	10	72.5
Stress 30 min 6	30	100.3
Stress 60 min 1	20	64.9
Stress 60 min 2	25	128.9
Stress 60 min 3	20	136.7
Stress 60 min 4	30	83.0
Stress 60 min 5	25	170.5
Stress 60 min 6	35	143.6

Appendix L
Hormone Pilot Studies

Appendix L:

Hormone Pilot Studies.

A pilot study was done for the purpose of establishing a steroidal hormone panel for channel catfish. The study was conducted as an individual case study for channel catfish specimens. The goal of the study was to establish known hormonal responses due to injections with various hormones.

Fish

Channel catfish, *Ictalurus punctatus*, were obtained from commercial aquaculture ponds at Greenwater Fish Farm (Gibson County, Tennessee) and Fort Loudon Reservoir (Knox County, Tennessee). Weights ranged from 115-250 g. The catfish did not exhibit signs of infection and appeared healthy upon inspection. Twenty –two fish were used in the hormone panel pilot study. Physical characteristics of these fish may be found in the table on the following page. All of the channel catfish were tagged for identification as shown in the photos on following pages and given a corresponding number for the study. Fish were placed in the experimental system and allowed to acclimate for 14 d post tagging.

Fish Number	ID Number	Treatment	Weight (grams)	Length (mm)
1	688	ACTH no stress	115	250
2	692	ACTH no stress	136	136
3	851	ACTH no stress	187	187
4	856	ACTH no stress	206	255
5	684	ACTH no stress	167	258
6	690	Stress 30	244	263
7	852	Stress 30	177	257
8	853	Stress 30	201	255
9	854	Stress 30	137	231
10	857	Stress 30	155	240
11	691	Dex Stress 30	190	256
12	695	Dex Stress 30	230	265
13	697	Dex Stress 30	153	233
14	698	Dex Stress 30	218	270
15	855	Dex Stress 30	157	243
16	687	Dex ACTH	175	240
17	689	Dex ACTH	156	241
18	694	Dex ACTH	250	280
19	696	Dex ACTH	136	225
20	699	Dex ACTH	153	243
21	859	Dex TRH	149	249
22	860	Dex TRH	163	258
23	NA	NA	NA	NA
24	861	TRH	222	269
25	862	TRH	169	254
26	863	TRH	161	257
		Average	176.28	244.6



Photos of channel catfish identification tags.

Experimental system

The experimental system contained six independently maintained 1050-L (280 gallon) tanks. Each tank was operated in a flow-through mode with filtered city water supplied at 3.4 L/min. The water was filtered with activated carbon and total chlorine content was less than 0.002 mg/L. Water temperature was maintained at $27 \pm 2^\circ\text{C}$. Water quality was monitored daily for dissolved oxygen (>6 mg/L), temperature ($27 \pm 2^\circ\text{C}$), and total chlorine (<0.002 mg/L). Water quality was tested weekly for pH (7.0-7.3), and total ammonia (<1.0 mg/L). Lighting was automatically controlled by timer with a 16 h light / 8 h dark cycle. The fingerlings were fed Zeigler floating finfish bronze (35% protein, 5% fat) 5mm pellets at a rate of 2% of body weight daily.

Hormone level determination

Hormones were dissolved in sterile physiological saline to a desired concentration. Fish received intramuscular injections of Dex approximately 200 mg/kg and ACTH 5.0 μg /fish approximately 50 μg /kg in 0.5 cc saline. Dosage was determined based on suppression of cortisol secretion (inhibition of ACTH secretion) by Dex is usually attained with Dex doses between 10-100 μg /kg (intravenously) in dogs and 40-100 μg /kg (intravenously) in horses. Dosage of Dex for therapy of different conditions in different species often varies between 0.5 to 6.0 mg/kg. Dose of synthetic ACTH used for adrenal stimulation varies between 1.0 and 20 μg /kg (intravenously) depending on species (Plumb 2002). In summary, dosages of Dex and ACTH injected fish were compatible with diagnostic dosages used in mammals.

Treatment I – control/baseline

The objective of this treatment was to establish a baseline for hormones of unstressed for control comparisons of other treatments. Fish 1-5 were removed from the tank and placed in 100 mg/L MS-222. All fish achieved stage IV anesthesia (total loss of muscle tone and equilibrium; slow but regular opercular rate; loss of spinal reflexes) in less than 3 min. Once the fish achieved stage IV anesthesia, each was injected with 0.5 cc sterile saline intramuscularly to simulate hormone injection. All fish were injected in less than 5 min. Fish were then placed in 150 mg/L MS-222. Once the fish achieved stage V anesthesia (total loss of reactivity; opercular movements slow and irregular; heart rate very slow; loss of all reflexes), they were bled using 3 cc syringes with 18 gauge 1 1/2" needles for analysis of cortisol, progesterone, 17-OH progesterone, androstenedione, testosterone, and estradiol. The syringes were used to draw blood from the caudal vein at the caudal peduncle. The fish were then returned to the tank to recover. The fish were then anesthetized and bled in the same manner at time 60 min.



Photos of a channel catfish being bled.

Treatment II – confinement stress 30 min

Fish 6-10 were anesthetized and bled treatment I. The fish were placed back into the tanks in confinement baskets constructed of rigid vinyl netting. The baskets caused the fish to be restricted in movement and to be in contact with other fish as well as the basket at all times. The fish were then removed from the basket for bleeding at time 30 min. The fish were then returned to the tank to recover. The fish were then anesthetized and bled at time 90 min.



Photo of hormones being administered to a channel catfish.

Treatment III – Dexamethasone injection

Fish 11-15 were anesthetized and bled as in the previous treatments and injected intramuscularly with 200 mg/kg of Dex. The fish were then placed back into the tank. Fish were again anesthetized and bled at time 180 and 210 min post injection.

Treatment IV – Combined dexamethasone adrenocorticotrophic hormone injection

Fish 16-20 were anesthetized and bled as in the previous treatments and injected intramuscularly with 200 mg/kg of Dex. All fish were then placed back into the tanks. Fish were again anesthetized and bled at time 180 then injected with 50 μ g/kg of ACTH before being placed back in the tanks. The fish were then anesthetized and bled at time 240 min.

Treatment V – Combined dexamethasone thyrotropin releasing hormone (TRH) injection

Fish 21-22 were anesthetized and bled as in the previous treatments and injected intramuscularly with 200 mg/kg of Dex. Fish 23 was in this treatment but was not used due to a visible infection on the day of the study. All fish were then placed back into the tank. The fish were then bled at 180 min and injected with TRH 1 μ g/kg. The fish were returned to the tanks and bled again at 210 min.

Treatment VI – Thyrotropin releasing hormone (TRH) injection

Fish 24-26 were anesthetized and bled as in the previous treatments and injected intramuscularly with TRH 1 μ g/kg. All fish were then placed back into the tanks. The fish were then bled at time 30 min.

Steroid analysis

The steroid panels were ran by the University of Tennessee, Clinical Pathology Lab.

Cortisol

Fish #	Cortisol (ng/mL)						
	Time (min)						
	0	30	60	90	180	210	240
1	20.52		24.23				
2	16.57		37.94				
3	22.41		57.46				
4	18.18		46.66				
5	38.90		46.51				
6	7.67	41.40		16.32			
7	7.71	50.22		12.89			
8	13.91	50.22		26.90			
9	6.64	58.69		60.98			
10	18.70	44.41		41.30			
11	20.96				15.03	34.18	
12	3.21				5.22	12.47	
13	6.47				3.13	13.31	
14	7.76				4.76	20.12	
15	11.20				4.19	15.71	
16	40.66				5.68		48.08
17	11.56				3.38		23.89
18	29.58				7.16		40.42
19	7.03				7.15		63.15
20	10.18				16.90		24.08
21	14.18				4.65	9.23	
22	44.61				17.54	21.43	
24	15.48	41.59					
25	27.68	33.98					
26	17.20	49.90					

Progesterone

Fish #	Progesterone (ng/mL)						
	Time (min)						
	0	30	60	90	180	210	240
1	0.13		0.14				
2	0.12		0.27				
3	0.26		0.33				
4	0.07		0.29				
5	0.22		0.63				
6	0.03	0.06					
7	0.09	0.22					
8	0.09	0.16					
9	0.03			0.17			
10	0.09	0.21		0.16			
11	0.14					0.13	
12	0.11				0.16	0.24	
13	0.07				0.10		
14	0.07				0.05	0.12	
15	0.20				0.22	0.62	
16	0.19				0.18		0.59
17	0.28				0.12		0.62
18					0.09		0.41
19	0.11				0.25		0.49
20	0.13				0.18		0.25
21	0.15				0.17	0.12	
22	0.15				0.32	0.39	
24		0.16					
25	0.16	0.26					
26	0.17	0.30					

17-OH Progesterone

Fish #	17-OH Progesterone (ng/mL)						
	Time (min)						
	0	30	60	90	180	210	240
1	0.14		0.33				
2	0.11		0.43				
3	0.16		0.31				
4	0.09		0.29				
5	0.29		0.45				
6	0.14	0.18		0.09			
7	0.13	0.37		0.11			
8	0.14	0.41		0.18			
9	0.07	0.24		0.15			
10	0.12	0.27		0.13			
11	0.14				0.19	0.18	
12	0.11				0.16	0.22	
13	0.23				0.10	0.20	
14	0.23				0.08	0.32	
15	0.32				0.12	0.40	
16	0.32				0.17		0.39
17	0.23				0.17		0.22
18	0.28				0.16		0.19
19	0.11				0.14		0.45
20	0.13				0.24		0.20
21	0.28				0.19	0.17	
22	0.21				0.41	0.44	
24	0.12	0.26					
25	0.16	0.28					
26	0.16	0.45					

Androstenedione

Fish #	Androstenedione (ng/mL)						
	Time (min)						
	0	30	60	90	180	210	240
1	1.4		3.3				
2	0.9		1.3				
3	1.7		2.4				
4	1.2		3.9				
5	2.5		2.0				
6	1.2	3.8		1.4			
7	2.2	2.6		1.3			
8	2.0	2.9		1.5			
9	0.7	2.0		1.0			
10	10.2	3.4		2.2			
11	1.0				1.4	2.4	
12	6.1				2.7	2.1	
13	2.3				3.1	2.3	
14	2.9				1.2	1.5	
15	3.1				2.2	1.4	
16	2.6				0.9		1.4
17	1.3				1.6		1.2
18	1.3				1.8		1.5
19	2.2				1.2		2.2
20	6.1				1.6		1.3
21	0.6				1.1	1.0	
22	0.6				1.0	1.2	
24		2.7					
25	0.8	1.2					
26	2.2	3.5					

Testosterone

Fish #	Testosterone (ng/mL)						
	Time (min)						
	0	30	60	90	180	210	240
1	0.03		0.04				
2	0.04		0.04				
3	0.10		0.06				
4	0.04		0.04				
5	0.03		0.05				
6	0.04	0.04					
7	0.04	0.04		0.05			
8	0.04	0.03		0.06			
9	0.05	0.04		0.05			
10	0.17	0.15		0.09			
11	0.05				0.06	0.05	
12	0.32				0.11	0.10	
13	0.04				0.04	0.07	
14	0.06				0.07	0.04	
15	0.05				0.05	0.08	
16	0.10				0.04		0.06
17	0.10				0.05		0.09
18	0.10				0.04		0.06
19	0.12				0.05		0.06
20	0.28				0.05		0.05
21	0.04				0.03	0.03	
22	0.18				0.05	0.06	
24		0.03					
25	0.04	0.06					
26	0.06	0.12					

Estradiol

Fish #	Estradiol (ng/mL)						
	Time (min)						
	0	30	60	90	180	210	240
1	90.67		77.13				
2	171.82		102.46				
3	283.40		211.13				
4	94.49		165.12				
5	70.37		47.22				
6	55.81	62.67		34.80			
7	64.06	63.50		40.94			
8	81.35	65.36		44.34			
9	79.30	65.72		40.10			
10	74.35	79.08		41.03			
11	60.05				22.57	27.37	
12	72.07				65.56	49.38	
13	75.09				50.05	34.99	
14	154.10				124.72	101.16	
15	77.41				64.14	48.83	
16	253.14				139.51		98.60
17	56.43				50.88		33.89
18	65.03				61.63		39.89
19	234.22				96.11		60.69
20	290.90				125.16		75.01
21	41.10				43.26	25.36	
22	198.15				113.12	67.90	
24	68.03	54.06					
25	49.55	50.33					
26	95.04	81.23					

Appendix M

Cortisol Concentrations by Treatment for the Hormonal Disease Challenge

Appendix M:

Cortisol Concentrations by Treatment for the Hormonal Disease Challenge
Cortisol is listed in ng/mL.

Treatment					
Control	Dex	ACTH	Stress 30	Dex Stress	ACTH Stress
13.2	5.8	78.3	69.9	2.7	72.40
6.2	5.3	117.3	164.4	13.9	52.00
7.8	9.8	55.4	56.5	22.1	39.30
9.4	21.9	115.7	69.5	2.0	62.10
14.8	2.0	41.9	49.7	27.0	76.60
17.9	7.2	41.6	78.2	8.5	69.10
15.6	6.4	68.3	125.0	18.0	60.10
8.8	4.8	62.8	90.8	20.4	54.70
11.2	10.1	90.4	73.6	14.6	62.40
15.4	7.6	94.6	61.9	22.9	78.10
17.0	2.9	81.7	97.1	14.6	30.70
13.1	6.7	72.3	102.5	23.9	56.10
13.7	7.5	64.9	136.2	11.9	48.80
14.1	5.8	110.3	84.9	25.3	65.20
7.9	7.0	57.7	70.5	34.6	77.50

Appendix N

Mean Percent Mortality by Treatment for the Hormonal Disease Challenge

Appendix N:

Mean Percent Mortality by Treatment for the Hormonal Disease Challenge.

Treatment	Replicate		
	1	2	3
Control	5	10	10
Dex	100	60	75
Dex Stress	75	55	70
Stress	35	40	35
ACTH	40	40	40
ACTH Stress	50	50	55

Vita

Todd David Sink was born in Lafayette, Indiana on December 16, 1977. He graduated from Connersville High School in Fayette county Indiana in May of 1996. He then entered Purdue University in West Lafayette, Indiana in August of 1996. He graduated from Purdue University in December of 2000 with a B.S. degree in Fisheries. He completed two specializations as an undergraduate, one in fisheries management and the other in fish physiology and nutrition. He then entered the graduate program in Natural Resources at the University of Tennessee, Knoxville in January 2001 to begin his doctoral studies. While attending the University of Tennessee he worked as a graduate research assistant in the Department of Forestry, Wildlife, and Fisheries. He also worked as the fisheries lab manager, overseeing the daily function and activities of the fisheries lab.