ABSTRACT

Stress Response Associated with Hatchery Conditions in
Developing Chum Salmon (*Oncorhynchus keta)*

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To assess the impact of hatchery techniques on the stress response of Chum Salmon, developing fish were subjected to treatments of formalin, low-medium-high densities, thermal shock, and mechanical distress. Mean cortisol levels for formalin treated eggs immediately increased ~350% and remained elevated for 60 minutes until returning to control treatment levels. Mean cortisol levels differed significantly between formalin treatment and control at all density levels. Both mortality rate and mean cortisol differed significantly between low, medium, and high densities. Shock and pick, as well as transport of salmon fry, was mimicked with mechanical distress and cortisol levels did not increase until mechanical distress continued for 90 minutes whereby cortisol concentrations increased ~295% above control levels. These results should help identify stress responses during early life stages of chum salmon as well as identify potential sources of anthropogenic stress associated with hatcheries.

Stress Response Associated with Hatchery Conditions in Developing Chum Salmon *(Oncorhynchus keta)*

by

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A Thesis

Approved by the Department of Biology

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Robert D. Doyle, Ph.D., Chairperson

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of
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CHAPTER ONE

Introduction

In 2007, commercial hatcheries raised and released more than 1.5 billion salmon into Alaskan streams, lakes and near shore marine habitats to enhance fisheries (White, 2008). Chum salmon (*Oncorhynchus keta)* are typically released after approximately 8 months of incubation, whereas coho *(Oncorhynchus kisutch)* and chinook salmon *(Oncorhynchus tshawytscha)* are retained for up to two years before being released. Hatcheries and the rearing process, however, can be stressful to developing fish, exposing them to unnatural levels of trauma during early stages of development (Moran et al. 1997).

While most investigators have used developmental abnormalities to evaluate the effects of stress on captive fish, few studies have assessed fish health in a hatchery directly using physiological indicators such as cortisol. Cortisol plays a critical role in regulating both acute and chronic responses to stress (Norris, 1997). The pathway for cortisol release begins in the hypothalamic-pituitary-interrenal (HPI) axis with the release of corticotropin-releasing hormone (CRH) chiefly from the hypothalamus in the brain, which stimulates the corticotrophic cells of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) (Bonga, 1997). Circulating ACTH, in turn, stimulates the interrenal cells embedded in the head kidney to synthesize and release corticosteroids into circulation for distribution to target tissues (Bonga, 1997). During an acute stress response episode in teleosts, cortisol prevents amino acids from being used for protein synthesis, and promotes conversion of amino acids to glucose via

gluconeogenesis (Donaldson, 1981). It also causes chromaffin cells to increase the release of catecholamines which aids in modulating cardiovascular and respiratory functions (Reid et al. 1998). Vijayan et al. (1996) subjected tilapia *(Oreochromis mossambicus)* to acute confinement stress for 24 hours, and reported higher plasma free amino acid concentrations, increased phosphoenolpyruvate carboxykinase (PEPCK), increased plasma glucose, and elevated cortisol which suggested amino acid mobilization for gluconeogenesis.

If a stressor becomes chronic, generally greater than five days in teleosts (Ruane *et al.* 2002), cortisol continues inhibiting protein synthesis and promoting gluconeogenesis (Norris, 1997). Additionally, it prevents utilization of glucose by skeletal muscle and stimulates lipolysis by nonneural tissues (Norris, 1997). All of these actions serve to protect glucose dependent tissue such as the brain and heart (Norris, 1997). Cortisol also helps suppress the inflammatory response during times of stress to prevent tissue damage (Mommsen et al. 1999). However, exposure to acute or chronic environmental stress during early development in fish can produce abnormal phenotypes and developmental asymmetry (Moran et al. 1997). Increased cortisol levels due to stress causes an inhibition in protein synthesis which can cause a disruption in the musculature development of juvenile salmon during a critical time of growth. The increase in cortisol levels above a control baseline has been used as a diagnostic tool to indicate stress in teleosts (Mazur and Iwama, 1993; Davis and Parker, 1986). Mechanical trauma (Strong et al. 1986), resource limitation (Payan et al. 2004), density effects (Casselman, 1990; Ruane, 2002), and temperature stress (Johansson 1966) are all known to impact development and survival in fish. The hatchery environment can increase these factors,

so hatchery-reared fish can be more asymmetrical than their wild counterparts (Moran et al. 1997). The relationship between stress and developmental variability described in these studies, however, did not appear causative. Developmental variability associated with hatchery systems can also be caused by the interaction between genotype and environment (Leary et al. 1983), rather than environmental trauma. Understanding the causes of abnormal development can affect survival and therefore the effectiveness of hatchery-based enhancement programs at augmenting fisheries and protecting wild stock. An understanding of population-level stress is critical for augmentation of wild populations for fisheries and aquaculture. From an ecological as well as a management perspective, factors affecting broodfish quality can be reflected in the number and quality of their progeny. A decrease in reproductive fitness causes ecological implications for wild stock, and monetary implications for an aquaculture facility.

Cortisol is a corticosteroid and is synthesized from cholesterol in the adrenal cortical cells of the chromaffin tissue in the head kidney of teleostean fish (Norris, 1997). Biosynthesis of cortisol in fish is similar to that of mammals. The side-chain-hydrolysis of cholesterol produces pregnenolone, ultimately producing cortisol after a series of enzymatic conversions (Mommsen et al. 1999). It can then be secreted and transported to wherever needed.

Corticosteroids fall into two subcategories, glucocorticoids and mineralocorticoids, depending on the physiological role of the steroid (Norris, 1997). Generally, glucocorticoid function affects metabolism and growth while mineralcorticoid function affects movement of ions and water (McCormick et al. 2008). Whereas cortisol functions as a glucocorticoid in mammals, it performs a dual role in teleosts by

functioning as both a glucocorticoid and mineralocorticoid (Norris, 1997). Elevated levels of cortisol in teleosts can confirm that a biological response to stress has occurred and provide a potential causative link between abnormal physical expression and trauma. Most importantly, because the response of this physiological indicator to stress is relatively instantaneous, cortisol can potentially be used to identify problematic exposure before irreparable harm is done to developmental pathways, whereas the physical consequences of exposure are often delayed, making it difficult to identify and eliminate the potential causes.

Cortisol circulates via binding to plasma proteins. Depending on the physiological process needing regulation, cortisol travels to specific target tissues where a corticosteroid receptor (CR) has been expressed (Mommsen et al. 1999; Milla et al. 2009). Teleosts have two GR receptors and one mineralocorticoid receptor (MR), all with high affinity for cortisol (McCormick et al. 2008; Stolte et al. 2006). Since cortisol binds with both types of receptors in fish, it regulates many diverse functions including a trigger between life stages, a permissive agent, osmoregulation of sodium, and perhaps most importantly, regulation of the stress response.

Development in teleosts is often accompanied by an increase in cortisol concentration. Most teleosts undergo three developmental phases, larva, juvenile, and adult (Szisch et al. 2004). De Jesus et al. (1991) found that cortisol decreased in chum salmon eggs as the maternal cortisol was depleted, increased toward the end of yolk absorption after hatching, and peaked when the fry emerged from the gravel bed. Emergence from the gravel bed is also when fry develop the ability to osmoregulate so they can migrate downstream, and marks the transition from larva to juvenile. Elevated

cortisol levels upon hatching were also discovered in chinook salmon (Feist and Schreck, 2002). There appears to be a positive relationship between egg size and cortisol levels in teleosts sampled to date. Moreover, teleosts which lay large eggs (~6-7mm) had elevated cortisol upon hatching, whereas teleosts with smaller eggs (~0.8-3.0mm) tended to hatch with immature organs and no elevated cortisol levels were detected (Wada, 2008). While plasma cortisol levels vary widely during the reproductive cycle, especially among different species of teleosts, an increase in levels has been detected in both pre-spawning and spawning of several species of fish (Westring et al. 2008). Increases in cortisol were measured in female rainbow trout (*Oncorhynchus mykiss)*, brown trout *(Salmo trutta)*, goldfish *(Carassius auratus)*, and common carp *(Cyprinus carpio)* during ovulation. In male teleosts, cortisol levels increase earlier in the reproductive cycle with lower overall levels than females (Milla et al. 2009).

Cortisol plays a vital role in the osmoregulation of sodium balance in teleosts (McCormick, 2001). Regardless of the salinity of their external environment, a teleost must maintain its plasma osmotic concentration to about 1/3 that of sea water (McCormick, 2001). If salinity approaches fresh (0ppt) a fish will compensate for the ion loss by producing large amounts of dilute urine and taking up ions across the gills (McCormick, 2001). If the water is saline (30ppt), a fish will counteract the gain in ions by "drinking" seawater, absorbing water and salts across the gut, and secreting excess monovalent ions across the gills and divalent ions through the kidney (McCormick, 2001). Cortisol has been shown to stimulate the differentiation of the seawater chloride cell to promote salinity tolerance (McCormick, 2001). There is also some evidence to suggest that cortisol is important in maintaining the sodium pump in both seawater and

freshwater chloride cells which would give it a dual osmoregulatory function (McCormick, 2001). Several physiological parameters, including cortisol levels were studied in tilapia by Kammerer et al (2010). They acclimated tilapia from fresh water to 25% seawater over five days, and observed an increase in cortisol after three hours of exposure, a peaking of cortisol at three days. Cortisol level was significantly different from the control by five days. Plasma osmolality (Na^+, K^+, Cl^-) was significantly different from the control at eight hours and peaked at 24 hours (Kammerer et al. 2010). They concluded that those previous results combined with significantly different plasma osmolality after eight hours in the new study, indicated a rapid turnover event in the re-differentiation of chloride cells from freshwater type to seawater type following the rise in cortisol at three hours (Kammerer et al. 2010). Recent evidence indicates cortisol is responsible in regulating ion and water movement across the intestinal epithelium of freshwater fish (Sakamoto and McCormick, 2005).

During this study, I examined the effects of hatchery-induced stress on larval fish physiology and development using whole body cortisol levels and physical responses (length, weight, mortality rate) to measure immediate and long-term responses and consequences to stress exposure. Because knowledge regarding the effects of stress on health can help to develop production tactics that lessen the impact of a stressor, this project will provide information that will allow managers to evaluate and formulate effective production strategies which will provide hatcheries with ways to minimize stress and maximize the production of healthy, viable fish.

I surmise there will be a positive correlation between cortisol levels and stress exposure, as well as with alterations in morphology. H_{o1} = no differences in mean

cortisol levels among treatments. H_{o2} = no differences in mean cortisol levels among densities. H_{o3} = no differences in mean morphological indices among treatments. H_{o4}=no differences in mean morphological indices among densities

CHAPTER TWO

Methods and Materials

Fertilized chum salmon eggs were acquired from Macaulay Hatchery in Juneau, Alaska during September, 2010. Eggs from different parents within the same stock were randomly mixed to minimize potential genetic influences. Once eggs reached the eyed stage, a subsample was collected and shipped overnight to the Laboratory of Ecological and Adaptational Physiology (LEAP) at Baylor University where they were allowed to acclimate to their new environment for approximately 5 days. Eggs incubated at LEAP were housed in an Aquatic Habitat® six-shelf system at temperatures mimicking that of Macaulay Hatchery (2 - 8ºC). Water for the shelf system was filtered by reverse osmosis, treated with UV light, and flowed in a 160L per day (6.71L/hr) semi-open design through a carbon filter to ensure oxygenation and waste removal. Water was chilled using a two-stage, flow-through, inline chiller (Aqua Logic DS5 as well as a coil chiller (Aqua Logic AE3D). Cortisol level, percent mortality, weight, and length were compared within and among treatments and density levels for all samples (Figure 1).

Eggs were reared under the following conditions, control, formalin exposure (1ppt), thermal shock, and low, medium, or high density. Each condition was designed to replicate stressors that typically occur within a commercial hatchery. All samples were flash frozen in liquid nitrogen and stored in a freezer at -80ºC for further analysis. Each stressor had two replicates. Fish from similar treatment tanks were transferred to replace sampled fish and maintain density in all treatments.

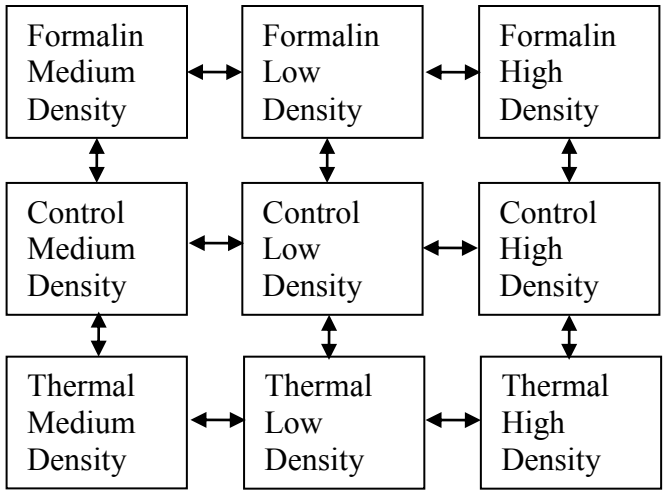

Figure 1: Experimental Design

Control: A group of fish was raised at a low density of 525 eggs/L, under minimal stress to serve as a reference population. Aliquots sampled consisted of three eggs to five fish were collected daily through hatching and weekly or biweekly thereafter.

Densities: The control group of 525 eggs/L served as the low density treatment. A medium density of 1050 eggs/L was used to represent artificial incubation conditions at Macaulay Hatchery. Based on hatchery recommendation, a treatment of 1600 eggs/L represented a "high" population density. Aliquots were sampled daily through hatching and weekly or biweekly thereafter.

Acute mechanical trauma: Eyed-eggs are typically exposed to vibrational stress as they are sorted and counted during "shock and pick" procedures at commercial hatcheries. Mechanical trauma is also experienced as fish are moved to their release sites. Vibrational trauma was mimicked using a plate shaker placed beneath aquaria for 360 minutes at 180 vibrations per minute.

Algal control: Formalin (37% formaldehyde), which is routinely used to control algal outbreaks, was administered (1ppt) to the formalin exposed tanks until hatching. Water circulation was such that all eggs had equal exposure to formalin which remained in each 1L tank for an estimated 15 minutes after injection. Formalin was absorbed by carbon filtration and removed from the system as the water recirculated. To produce a time series of cortisol levels, formalin was also administered to a group of low density eggs and samples collected immediately, every 15 minutes for 3 hours, every 3 hours through 24 hours, and a sample at 36 hours.

Acute Thermal Shock: During otolith marking experiments, eggs and fish were subjected to thermal trauma in which water temperature was raised to 10^{o}C for 12 hours, then lowered back to ambient (6^{o}C) for another 12 hours for a total time of 36 hours. This marking was done as hatching ended. Aliquots were immediately collected after each change in temperature.

Cortisol Extraction and Quantification

Cortisol was extracted using a whole-body extraction method, utilizing methods modified from Feist et al. (1990) and further developed by Sink et al. (2006). During pre-hatch, three eggs were used to determine cortisol levels, and during post-hatch, five fish were used.

The modified extraction procedure is as follows: Eggs or fish were cut into small pieces, 2ml of Phosphate Buffered Saline (PBS) was added to the sample, placed in a stomacher (Seward Stomacher80) for six minutes, and transferred to a 50ml beaker. The stomacher bag was rinsed with 1ml PBS to remove any remaining sample, and 100ug of vegetable oil (Great Value 100% pure vegetable oil) per gram of sample was added to the

beaker along with 5ml of diethyl ether. Beaker contents were transferred to a 15ml centrifuge tube, and the beaker was rinsed with an additional 2ml of diethyl ether with contents transferred to the same centrifuge tube. The centrifuge tube was vortexed for 30-60 seconds until contents were well-mixed. The sample was centrifuged for ten minutes at 3000rpm, and frozen upright for two hours at -20ºC. The unfrozen portion of the extract was decanted into a new 15ml centrifuge tube and dried under a gentle stream of nitrogen for 2 hours or until all liquid was gone. The remaining extract was frozen at -20ºC for further analysis.

Cortisol extractions were assayed using a competitive immunoassay kit manufactured by Enzo Life Sciences (900-071). This kit was validated for use in fish by Sink *et al.* (2007). Each extraction was run in duplicate on a 96-well microtiter plate. The wells were precoated with goat antibody specific to mouse IgG. Mouse monoclonal antibody to cortisol and an alkaline phosphatase conjugate were used to bind the cortisol in the extract in a competitive manner. During incubation, samples with high antigen content result in unlabeled antigen being bound in greater amounts than conjugated antigen. After incubation, the excess reagents were washed away and a p-nitrophenyl phosphate chromogenic substrate added to develop color. Samples with high antigen concentration generated a lower optical density than those containing low antigen concentration. This resulted in an inverse relationship, which was easily calculated using a standard curve.

Morphology

Beginning 14 days post-hatching, five fish per density for each treatment were sampled every 8 to 10 days for a total of 52 days. These fish were weighed and measured

for morphological comparison. Each fish was weighed in a beaker containing 100ml of water to obtain an accurate wet weight. Body length was measured from head to the start of the tail fin using calipers.

Statistical Analyses

Cortisol levels, mortality rates, weight, and length within and among treatments, were analyzed with a Multivariate Analysis of Variance (MANOVA), a Randomized Complete Block Analysis (RCB), and a Tukey's Honestly Significant Difference (HSD). α was set at $P \leq 0.05$ for all comparisons. When needed, log-transformed cortisol was used to normalize the data for statistical analysis. Mass (g) and length (mm) comparisons were made using simple linear regression. Each stressor had two replicates and each assay was run in duplicate. Standardized cortisol serial dilutions were used to create a cortisol curve for each microtiter plate series.

CHAPTER THREE

Results

Cortisol Curve

When compared to the control, cortisol levels for the formalin treated eggs (1ppt) immediately increased after injection at time zero to 340pg/ml (mean = 323.84±18.16 pg/ml for formalin treated eggs; mean = 70.84±5.42 pg/ml in the control eggs) and remained elevated for 60 minutes until returning to control treatment levels (Figure 2).

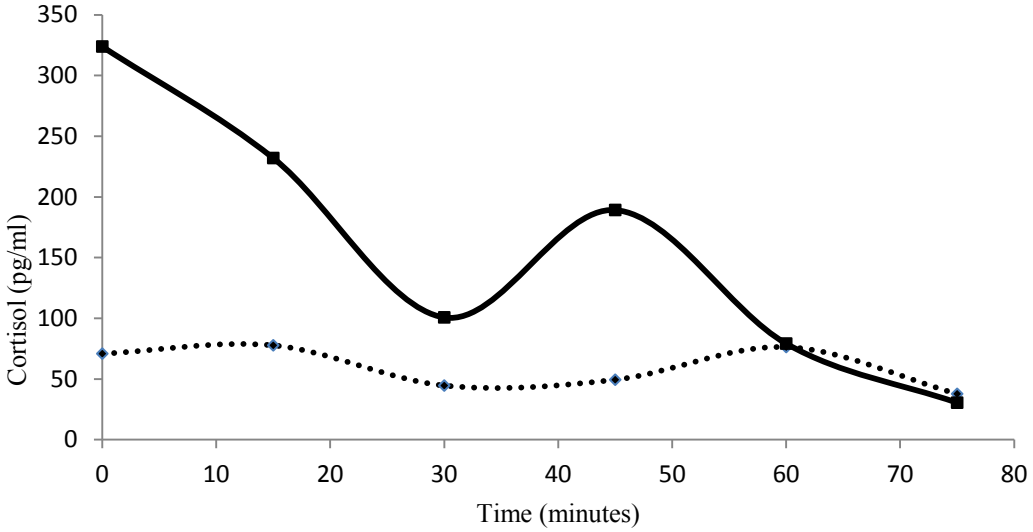

Figure 2. Cortisol concentrations in chum salmon eggs after administration of formalin (1ppt; solid line) compared against a control (dotted line). Formalin was administered at time zero.

Manipulative Study

Formalin and Thermal vs. Control

Overall mean cortisol levels differed significantly between the formalin treatment (mean = 1213.80±99.07 pg/ml) and control (mean = 789.86±83.69 pg/ml) at all density levels (low-medium-high). MANOVA; $P<0.05$; $F_{(treatment)} = F_{(2,2)} \sim 3.4554$, $P = 0.0333$. Mean cortisol levels did not differ significantly between the thermal treatment (mean = 934.38±98.14 pg/ml) and control. When comparing mean cortisol levels by treatment within the time periods of pre-hatch (sample days 1-15), hatching (sample days 16-22), and post-hatch (sample days 23-36), there was no significant difference. However, there was a significant difference between mean cortisol levels for overall pre-hatch (mean = 186.25±116.87 pg/ml) vs. hatching (mean = 1336.65±156.18), and for pre-hatch vs. post-hatch (1530.26±113.94). RCB; $P<0.05$; $F_{(treatment)} = F_{(2,2)} \sim 36.6836$, $P = <0.0001$ (Figures 3, 4, and 5).

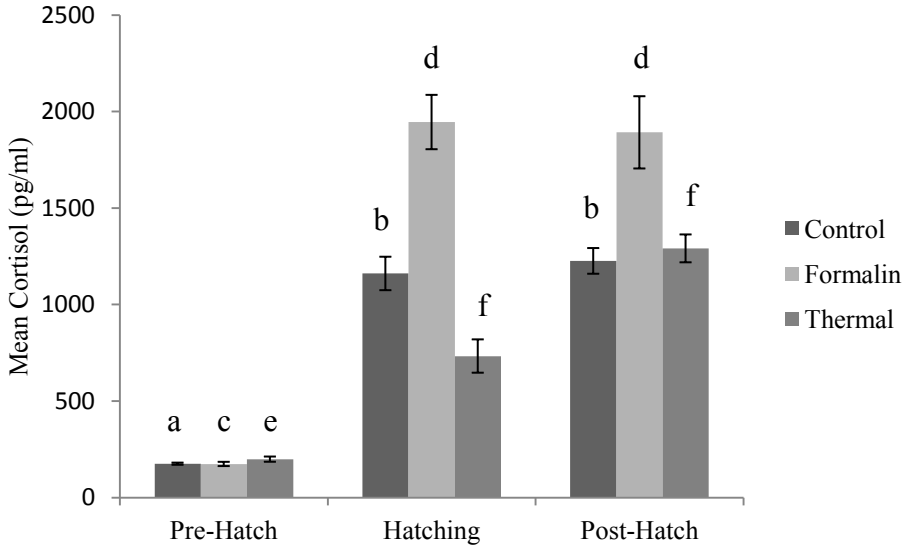

Figure 3. Mean cortisol levels of low density treatment for control, formalin (1ppt) and thermal treatments.

Low Density; Formalin and Thermal

Control pre-hatch had a mean of 174.96±5.56 pg/ml. Control hatch had a mean of 1160.60±86.63 pg/ml which is six times elevated over pre-hatch. Control post-hatch had a mean of 1125.61±66.58 pg/ml which is not significantly different from hatch. Formalin pre-hatch had a mean of 173.72±10.95 pg/ml. Formalin hatch had a mean of 1945.04±140.76 pg/ml which is ten times elevated over pre-hatch, is 40% higher than control, and is significantly different from pre-hatch. Formalin post-hatch had a mean of 1891.67±187.12 pg/ml which is 40% higher than control, and is not significantly different from hatch. Thermal pre-hatch had a mean of 198.58±13.45 pg/ml. Thermal hatch had a mean of 732.69±86.32 pg/ml which is almost three times elevated over pre-hatch, is 36% lower than control, and is significantly different from pre-hatch. Thermal post-hatch had a mean of 1290.53±72.24 pg/ml which is not significantly different from hatch.

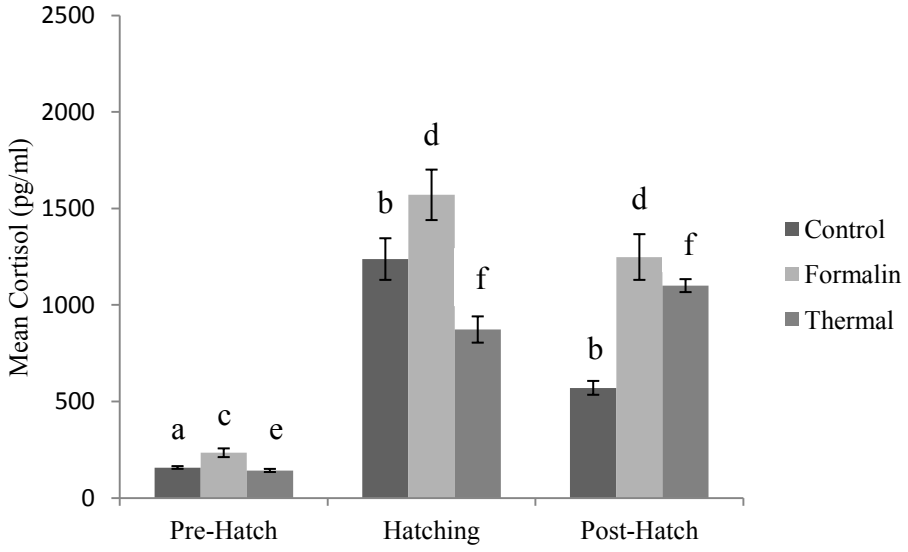

Figure 4. Mean cortisol levels of medium density treatment for control, formalin (1ppt) and thermal treatments.

Medium Density; Formalin and Thermal

Control pre-hatch had a mean of 157.45±7.06 pg/ml. Control hatch had a mean of 1237.24±107.85 pg/ml which is seven times elevated over pre-hatch and is significantly different. Control post-hatch had a mean of 570.00±35.90 pg/ml which is not significantly different from hatch. Formalin pre-hatch had a mean of 233.82±22.59 pg/ml. Formalin hatch had a mean of 1570.04±130.69 pg/ml which is six times elevated over pre-hatch, is 21% higher than control, and is significantly different from pre-hatch. Formalin post-hatch had a mean of 1247.78±118.41 pg/ml which is 54% higher than control, and is not significantly different from hatch. Thermal pre-hatch had a mean of 142.09±8.03 pg/ml. Thermal hatch had a mean of 872.29±68.05 pg/ml which is five times elevated over pre-hatch, is 29% lower than control, and is significantly different from pre-hatch. Thermal post-hatch had a mean of 1099.66±33.59 pg/ml which is 48% higher than control, and is not significantly different from hatch.

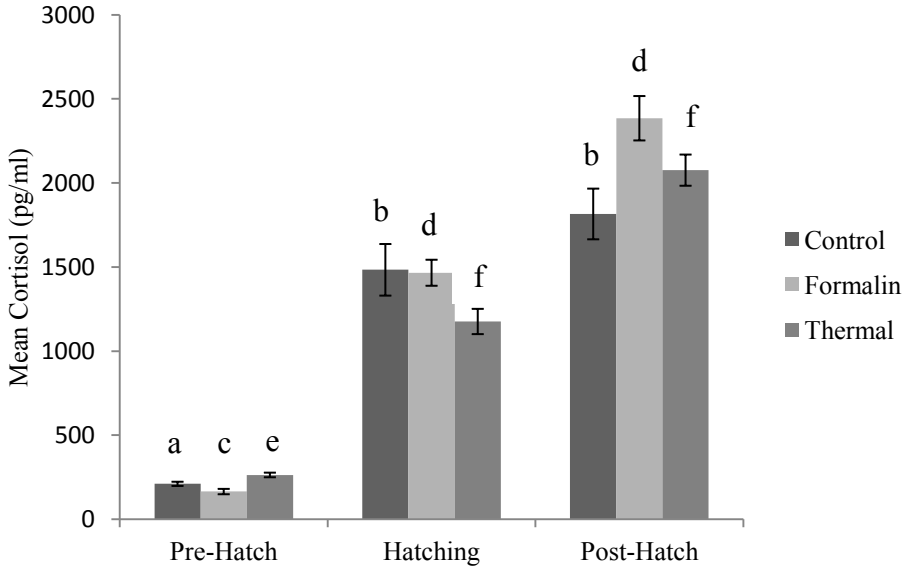

Figure 5. Mean cortisol levels of high density treatment for control, formalin (1ppt) and thermal treatments.

High Density; Formalin and Thermal

Control pre-hatch had a mean of 210.95±12.55 pg/ml. Control hatch had a mean of 1483.62±153.37 pg/ml which is six times elevated over pre-hatch and is significantly different. Control post-hatch had a mean of 1815.71±150.71 pg/ml which is not significantly different from hatch. Formalin pre-hatch had a mean of 165.11±15.95 pg/ml. Formalin hatch had a mean of 1466.19±77.36 pg/ml which is eight times elevated over pre-hatch, and is significantly different from pre-hatch. Formalin post-hatch had a mean of 2384.89±132.00 pg/ml which is 23% higher than control, and is not significantly different from hatch. Thermal pre-hatch had a mean of 263.92±13.68 pg/ml. Thermal hatch had a mean of 1176.37±75.06 pg/ml which is three times elevated over pre-hatch, is 20% lower than control, and is significantly different from pre-hatch. Thermal post-hatch had a mean of 2076.18±92.63 pg/ml which is 12% higher than control, and is not significantly different from hatch.

Density Effects

When density effects are examined within a treatment rather than among treatments, mean cortisol level for high density (mean = 1244.66±90.21 pg/ml) differed significantly from medium density (mean = 702.77±95.22 pg/ml MANOVA; $P<0.05$; $F_{(density)} = F_{(2,2)} \sim 9.3373$, $P = 0.0001$). Mean cortisol levels also differed significantly between high (mean = 1244.66±129.49 pg/ml) and medium (mean = 702.77±132.27 pg/ml) densities between pre-hatch, post-hatch and hatching. (RCB; $P<0.05$; $F_{(density)} = F_{(4,4)} \sim 1.9769$, $P = 0.0987$).

Morphology

Beginning 14 days post-hatching, five fish were sampled every eight to ten days for a total of seven sample periods covering 52 days. Mass (g) and length (mm) did not differ significantly, except for low density length where control was significantly lower than thermal and formalin treatments. (ANOVA; $P < 0.05$) (Figures 6 – 11). High density thermal treatment had a linear regression between cortisol (pg/g of tissue) and mass (g), $R^2 = 0.985$ (Figure 12).

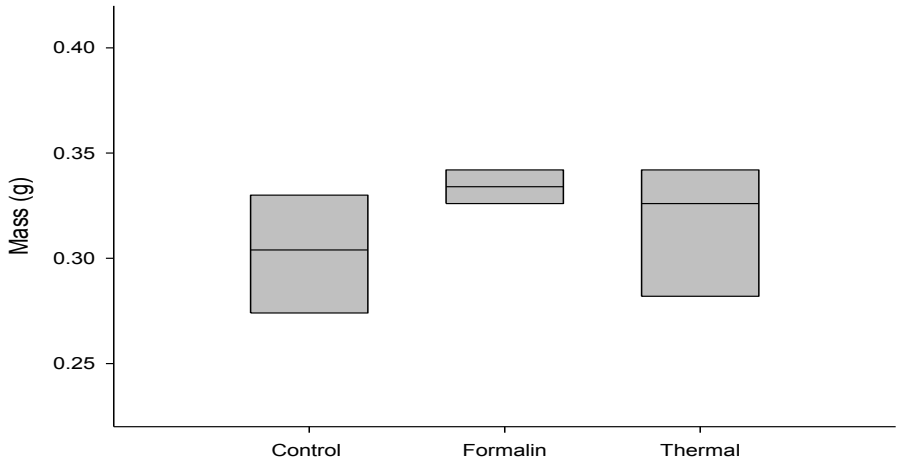

Figure 6. Mass (g) of fish in low density whose eggs were formalin exposed (1ppt), and eggs exposed to a thermal treatment, compared to a control.

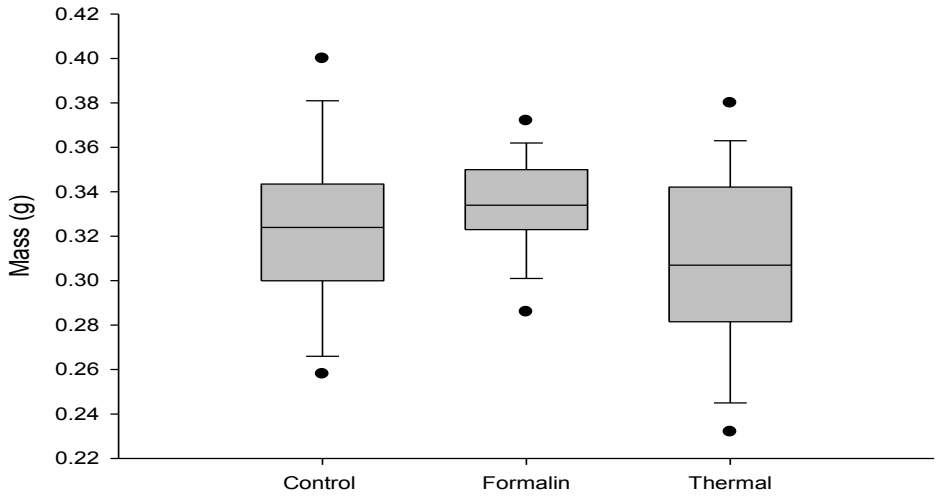

Figure 7. Mass (g) of fish in medium density whose eggs were formalin exposed (1ppt), and eggs exposed to a thermal treatment compared to a control.

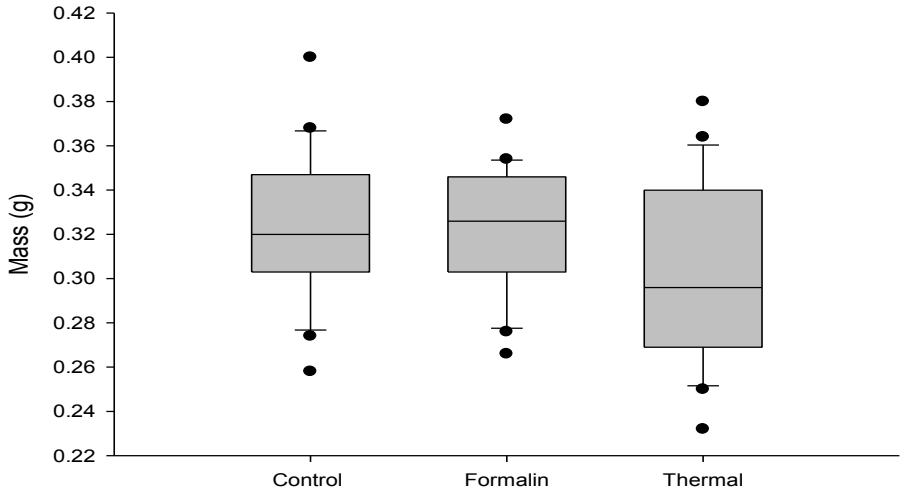

Figure 8. Mass (g) of fish in high density whose eggs were formalin exposed (1ppt), and eggs exposed to a thermal treatment compared to a control.

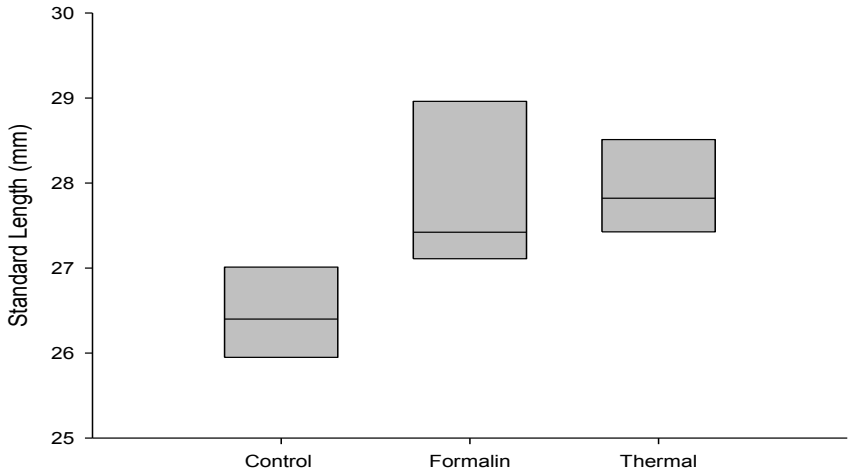

Figure 9. Standard length (mm) of fish in low density whose eggs were formalin exposed (1ppt), and eggs exposed to a thermal treatment compared to a control.

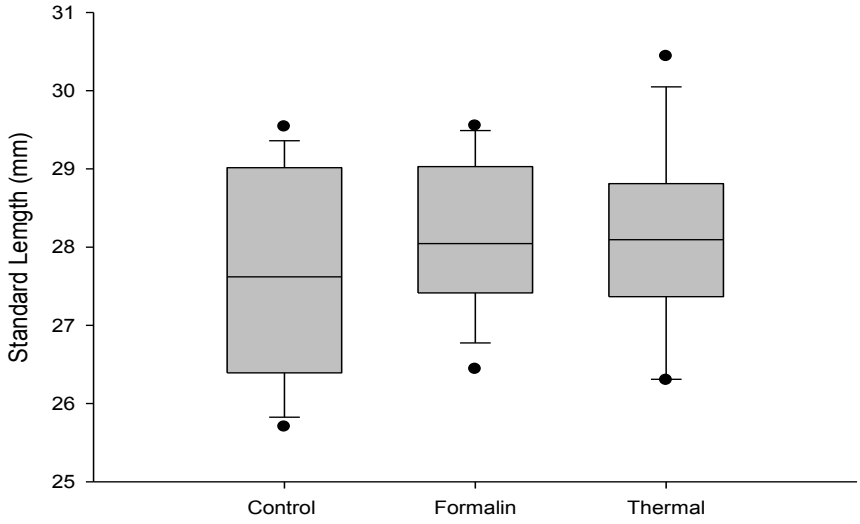

Figure 10. Standard length (mm) of fish in medium density whose eggs were formalin exposed (1ppt), and eggs exposed to a thermal treatment compared to a control.

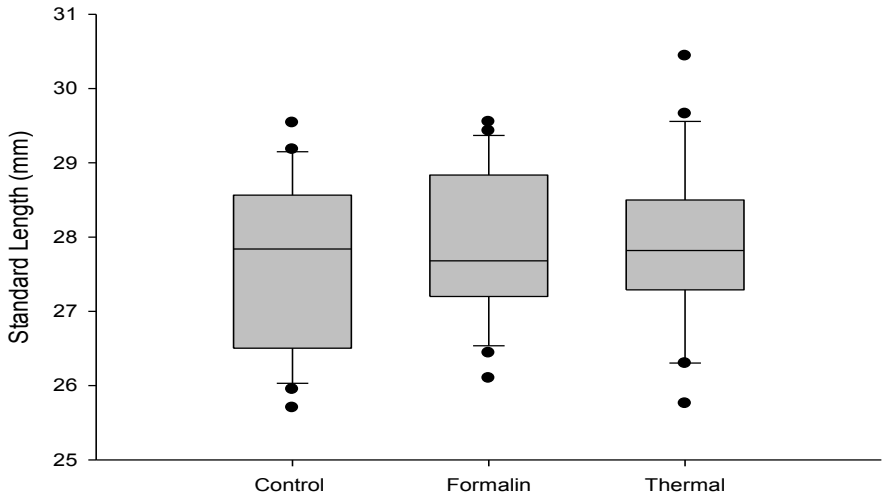

Figure 11. Standard length (mm) of fish in high density whose eggs were formalin exposed (1ppt), and eggs exposed to a thermal treatment compared to a control.

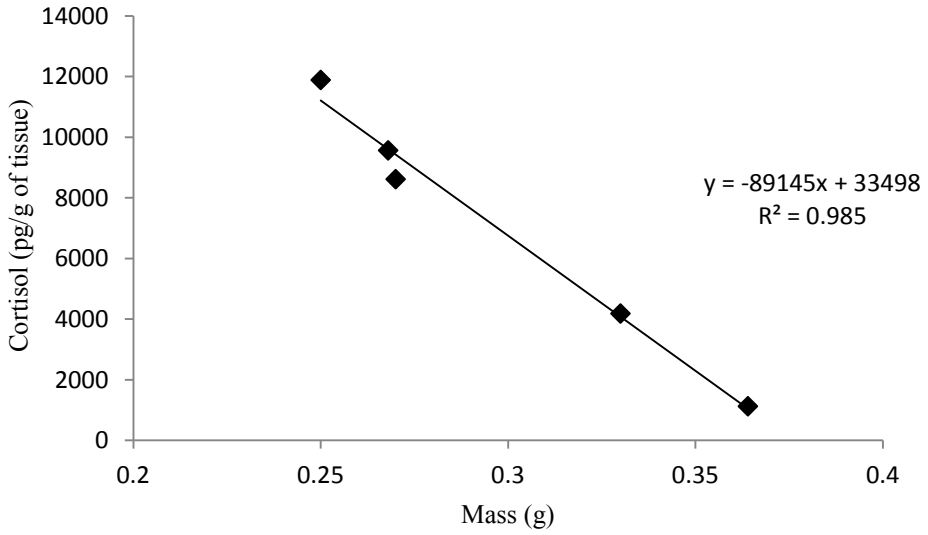

Figure 12. Linear regression of cortisol (pg/g of tissue) in high density thermal treatment; Samples were collected approximately every 10 days from 22 days post hatching through 52 days post hatching.

Mortality, Density Effects

Mortality rates varied significantly between low (mean = 0.066±0.0076), medium (mean = 0.046±0.0076), and high (mean = 0.033±0.0076) densities. (RCB; $P<0.05$;

$F_{(density)} = F_{(2,2)} \sim 4.8313$, $P = 0.0096$). However, mortality rates did not vary significantly between treatments. Mortality peaked during time period from ~50% hatched through 100% hatched (Figure 13).

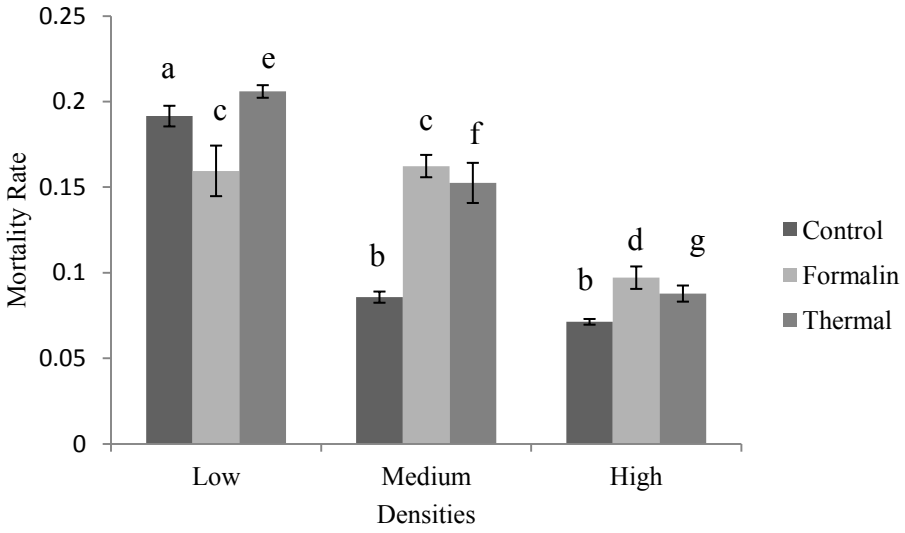

Figure 13. Mean mortality rate for the time period of ~50% through 100% of hatching completed for low, medium, and high densities for control, formalin (1ppt), and thermal treatments.

Mechanical Distress

Shock and pick, as well as transport of salmon fry, was mimicked with mechanical distress and cortisol levels measured to assess stress response. Interestingly, cortisol levels did not increase until mechanical distress continued for 90 minutes whereby cortisol concentrations increased ~295% above control levels (Figure 14).

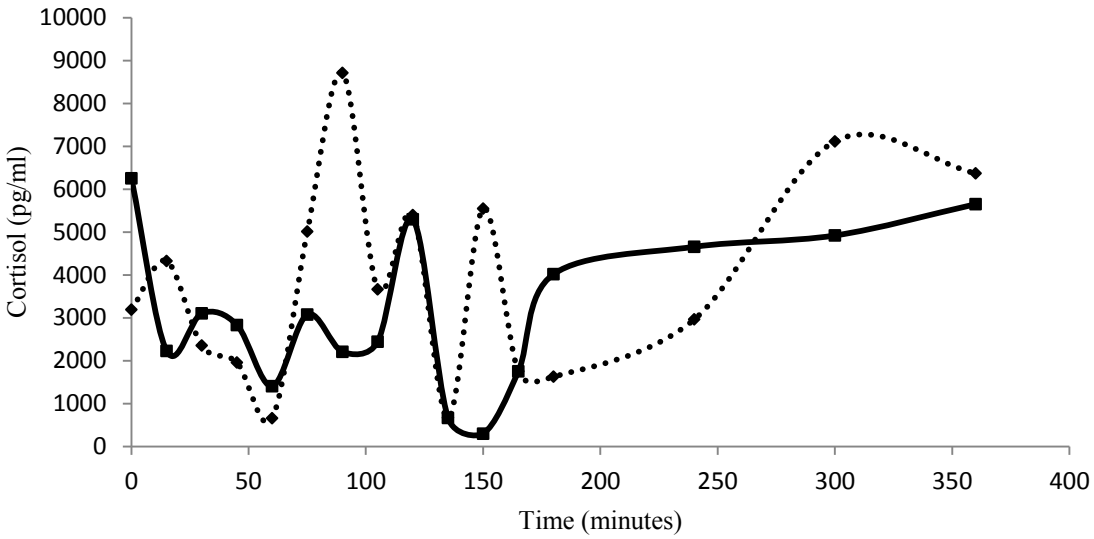

Figure 14. Cortisol concentration over time in control (solid line) compared to mechanical distress (dotted line).

CHAPTER FOUR

Discussion

In this study, chum salmon eggs treated with formalin exhibited an immediate increase in mean cortisol levels indicating an acute stress response (Figure 2). Also, the increase in cortisol appeared to decrease mass in developing salmon, especially in the high densities. High densities had the highest cortisol levels, but the lowest body mass. The thermal high density in particular had a direct negative linear relationship between cortisol level and body mass (Figure 12). Surprisingly, the high density also had the lowest mortality rates (Figure 13).

Formalin

One of the concerns with formalin use is that it removes oxygen from the water at the rate of 5mg/l of formalin per 1/mg/l of dissolved oxygen. The Food and Drug Administration permits application of formalin in concentrations of 1000mg/l to 2000mg/l for 15 minutes on eggs to aid in control of fungus in hatcheries (Francis-Floyd 1996). Formalin cannot be applied to fish as it will cause death (Stoskopf, 1993). Mean cortisol levels differed significantly between formalin exposed eggs and control eggs at every density level with the formalin treated eggs having a higher mean cortisol level. When compared to control during hatching and post-hatch, formalin treated eggs had a 35% (hatching) and 40% (post-hatch) higher mean cortisol level at the low density, a 21% (hatching) and 54% (post-hatch) higher mean cortisol level at medium density, and a 24% (post-hatch) higher level at high density (Figures 3–5). Interestingly, the mean

cortisol levels were almost the same as control at high density hatching (Figure 5). With the exception of low density, formalin treated eggs had a higher mortality rate during peak hatching time (~50% - 100% hatched) with the formalin medium density (0.16) exhibiting a rate double that of control (0.08) (Figure 13). The same toxic properties that make formalin effective as a treatment for ectoparasites may be causing a physiological response leading to an acute stress response in the eggs. Further research would need to be done to clarify the exact physiological response responsible for the stress, but hypoxia due to a reduction in dissolved oxygen in the water is a likely cause. Smith and Piper (1972) found that rainbow trout *(Salmo gairdneri)* exposed to formalin for one hour had higher hematocrit values and more immature red blood cells which suggested a physiological response to hypoxia. During the acute stress response following short term formalin exposure, cortisol causes an increase in catecholamine release from the chromaffin tissue (Reid et al. 1998). Increased heart rate, blood pressure, and blood glucose levels due to catecholamines can cause physiological damage in developing embryos. The subsequent release of cortisol can stop or slow protein synthesis, which can also harm embryo development. While it is apparent that formalin is causing stress which elevates cortisol levels above that of control, this effect is least pronounced in the high densities. This may be due to individual eggs receiving less of the overall formalin as the same amount of formalin was applied to each tank regardless of the egg stocking density, however further study is needed to support this hypothesis.

Thermal Shock

Mean cortisol levels of the thermal shock treatment did not differ significantly from that of the control (Figures 3-5). Thermal treatment mean cortisol levels stayed

near or even under that of control until after hatching. During post-hatch, mean cortisol levels for the thermal treatment were elevated above control, particularly in the medium density where a 48% increase over control was observed (Figure 4). This effect is most likely due to the thermal shock taking place near the end of hatching and the beginning of post-hatch. Mortality rate also did not differ significantly between thermal and control, however the thermal mortality rate (0.15) was almost double that of control (0.08) at the medium density level (Figure 13) which is also where the cortisol levels were the most elevated over control. Further study is needed to determine why the thermally shocked fish did so poorly in the medium density. Thermal shock has been linked to errors in branchiostegal ray development (Campbell, 2003) and abnormal otolith development (Gauldi, 1986, Oxman et al. 2004). Since fish were sacrificed upon absorption of the yolk sac, further research is needed to determine if the thermally shocked fish suffered developmental abnormalities upon maturation.

Hatching Effect

Elevated mean cortisol levels were observed in control, formalin, and thermal treatments in all stocking densities during hatching (Figures 3-5). Control had a mean cortisol level six to seven times greater during hatching than during pre-hatch, so it is apparent that the hatching process is itself causing an acute stress response. Feist and Schreck (2002) found low cortisol levels during pre-hatch, and a nearly two-fold increase in cortisol levels at hatching in chinook salmon. Cortisol levels for the formalin treatment during hatching were above that of control except for the high density, so formalin had an additive stress effect over that of the hatching process itself. Thermal

treatment cortisol levels stayed at or below that of control during hatch, but as stated earlier, thermal shock did not take place until the end of hatching.

Density Effects

Within formalin, thermal, and control treatments, medium and high densities differed significantly. With the exception of the formalin treatment during the hatching time period, the high density for all treatments had a significantly higher mean cortisol level. This was not surprising as higher densities have less oxygen, higher pressure, and less room to hatch which can lead to a chronic stress response. Ruane et al (2002) found an increase in transient plasma cortisol levels, plasma glucose levels, free fatty acids, and lactate in common carp, *(Cyprinus carpio)* when stocking densities were increased. They also discovered that the fish stressed from crowding in the higher stocking density were more susceptible to an additional acute stressor which resulted in an increase in oxygen radical production (Ruane et al. 2002). Contrary to what one would initially expect, the high density treatments had the lowest mortality rate and the low densities had the highest mortality rates (Figure 16). One possible explanation is that the lower densities had a higher exposure to formalin since there were fewer eggs in the same amount of formalin treated water as the other densities. The hatching process also causes stress (Feist and Schreck 2002) which further exacerbates the stress response associated with the higher densities. Interestingly though, the low density did not differ significantly from either the medium or high densities. One possible explanation is that the water flow in the low density tanks moved the eggs around with more force than in the medium and high density tanks. This buffeting of the eggs could cause stress and increased cortisol levels. In the wild, chum salmon eggs, and hatched alevin would be buried beneath the gravel

river bed following fertilization by a male. This burial would still allow water flow, but with minimal buffeting of the eggs and alevin.

Morphology

With the exception of low density control length, mass and length did not differ significantly between treatments (Figures 6-11). However, high densities had a lower mean body mass (g) than the low and medium densities. Unusually, the thermal high density treatment had a negative linear correlation between mean cortisol level per gram of tissue and mass of the fish (Figure 12) which is opposite of what one would expect in an unstressed fish. However, during either an acute or chronic stress response such as would result from formalin exposure, thermal shock, or high stocking density, cortisol inhibits protein synthesis in order to promote gluconeogenesis (Donaldson 1981). Additionally, cortisol prevents the skeletal muscles from using the glucose in order to protect vital glucose-dependent tissue like the heart and brain. This protein synthesis inhibition may be one cause of the negative linear correlation between mass (g) of the fish and cortisol level per gram of tissue in the thermal high density, and the lower mean mass (g) in the high densities in general. After hatching during the alevin stage, the fish are developing appropriate swimming musculature in preparation for the swim downstream during the fry stage (Stoskopf, 1993). Inhibition of protein synthesis during this critical time of growth could be devastating for developing fish.

One would expect the juvenile salmon to gain mass and length as they aged and developed. It is also expected that the cortisol levels would increase as cortisol acts as a permissive agent assisting the action of the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4). Szisch et al (2004) investigated the ontogeny of thyroid hormones and

cortisol in the gilthead sea bream *(Sparus aurata)*, and correlated the hormone concentrations with each phase of larval development. They found that cortisol levels paralleled T_3 and T_4 levels throughout the process and increased after the initial feeding (Szisch et al. 2004). First feeding usually occurs just after the development of a functional mouth stomach, gastrointestinal tract, and eyes. While there was an overall body increase after intial feeding, the second increase for hormones occurred with the development of fin rays and the flexion of the notochord which is the start of metamorphosis (Szisch et al. 2004). The final phase, melanophore and scale formation also showed parallel increases in cortisol and the thyroid hormones (Szisch et al. 2004). They concluded that cortisol, T_3, and T_4 were required to act in permissive roles among each other (Szisch et al. 2004). Geven et al. (2009) investigated the interaction between thyroid hormones and cortisol further using common carp. They found that the preoptic area and the renal tissues were sites of interaction between mediators of the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-interrenal (HPI) axis. *In vivo*, T_4 affected the CRH system in the preoptic area, whereas cortisol and ACTH stimulated the release of thyroid hormones from renal tissues (Geven et al. 2009). Further research is needed to determine if the morphological differences persist as the fish mature into adulthood.

Mechanical Distress

During the mechanical distress of salmon fry, cortisol levels did not increase until stress continued for 90 minutes (Figure 14). One theory is that while the salmon fry are adapted to some mechanical stress such as one would find in a typical stream, the

extended time of the mechanical distress such as one would encounter during transport caused them to exhibit an acute stress response.

Conclusion

While increased cortisol is generally good for fish survival, especially in terms of the primary acute stress response, elevated levels of cortisol during chronic stress disrupts many regulatory processes which in turn can cause a host of physiological problems for teleosts (Norris 1997). A chronic rise in plasma cortisol increases susceptibility to pathogens (Bonga 1997). Saxena et al. (2009) observed significantly higher T lymphocytes in common carp after exposure to the stress of heavy metals and concluded elevated cortisol acting as an immune suppressor was the primary cause. Recent studies have shown bidirectional communication between the teleostean HPI axis and its immune system (Holland et al. 2002). A study investigating the effect of increasing temperature on Atlantic cod *(Gadus morhua)* found that increased cortisol levels correlated with the expression of four immune-related genes (Perez-Casanova et al. 2008). The chronic stress of exhaustive exercise during spawning in Pacific salmon (genus *Oncorhynchus*) is accompanied by loss of pituitary control of the interrenal secretion of cortisol. Concurrently, there is a decrease in the ability to clear cortisol from the circulation. The net effect is that plasma levels of cortisol become elevated and the fish develop characteristics very similar to Cushing's Syndrome (Norris 1997; Carruth et al. 2002). Chronic stress during the reproductive cycle can affect time of ovulation, time of spawning, and egg size (Schreck et al. 2001). Tilapia who encountered stress during ovarian development delayed ovulation, whereas those who were stressed during late vitellogenesis (yolk formation) spawned immediately (Schreck et al. 2001). Rainbow

trout undergoing severe stress for nine months had both delayed ovulation and small egg size (Schreck et al. 2001). High cortisol levels of Atlantic salmon *(Salmo salar)* subjected to handling stress twice a day for 30 days resulted in a growth rate 50% lower than that of controls (McCormick et al. 1998). Understanding the mechanisms and roles of cortisol in teleostean fish has enabled scientists to use cortisol concentrations as a valuable tool for investigations to improve fish management and conservation strategies.

The analysis of data from this project supports the conclusion that the measurement of cortisol concentrations is a valuable tool for investigators to improve fish management and conservation strategies by understanding the factors in a hatchery causing fish stress. My hypothesis that there would be a positive correlation between cortisol levels and stress exposure, as well as alterations in morphology was supported. This knowledge can help to develop production tactics that lessen the impact of a stressor, thereby lowering fish mortality and improving overall fitness. Scientists and hatchery managers can use the information to evaluate and formulate effective production strategies that will maximize the production of healthy, viable fish, and ultimately a more efficient enhancement program.

BIBLIOGRAPHY

Barcellos LJG, Marqueze A, Trapp M, Quevedo RM, Ferreira D (2010) The effects of fasting on cortisol, blood glucose, and liver and muscle glycogen in adult jundia *Rhamdia quelen*. Aquaculture 300:231-236.

Bonga SEW (1997) The stress response in fish. Physiol Rev 77.3:591-625.

Campbell WB (2003) Assessing developmental errors in branchiostegal rays as indicators of chronic stress in two species of Pacific salmon. Can. Jour. Zool. 81(11):1876-1884.

Carruth LL, Jones RE, Norris DO (2002) Cortisol and Pacific salmon: a new look at the role of stress hormones in olfaction and home-stream migration. Integr Comp Biol 42:574-581.

De Jesus EG, Hirano T, Inui Y (1991) Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. Gen Comp Endocr 82.3:369-376.

Davis KB, Parker NC (1986) Plasma corticosteroid stress response of fourteen species of warm water fish to transportation. Trans. Am. Fish. Sot. 115, 495-499.

Donaldson EM (1981) The pituitary-interrenal axis as an indicator of stress in fish. In: Picering, A.D. (Ed.), Stress and Fish. Academic Press, London, pp. 1 l-47.

Feist G, Schreck CB (2002) Ontogeny of the stress response in chinook salmon, *Oncorhynchus Tshawytscha*. Fish Physiol Biochem 25:31-40.

Francis-Floyd R (1996) Use of formalin to control fish parasites (VM-77). Gainesville: University of Florida Institute of Food and Agricultural Sciences. Retrieved February 5, 2011, from http://edis.ifas.ufl.edu/vm061.

Gauldie RW (1986) Vaterite otoliths from Chinook salmon (*Oncorhynchus tshawytscha*). New Zealand Jour. of Mar. and Fresh. Res. 20: 209-217.

Geven EJW, Flik G, Klaren PHM (2009) Central and peripheral integration of interregnal and thyroid axes signals in common carp (*Cyprinus carpio* L.). J Endocrinol 200:117-123.

Holland JW, Pottinger TG, Secombes CJ (2002) Recombinant interleukin-1β activates the hypothalamic–pituitary–interrenal axis in rainbow trout, *Oncorhynchus mykiss*. J Endocrinol 175:261-267.

Kammerer BD, Cech JJ Jr., Kultz D (2010) Rapid changes in plasma cortisol, osmoality, and respiration in response to salinity stress in tilapia (*Oreochromis mossambicus*). Comp Biochem Phys A 157:260-265.

Li M, Bureau DP, King WA, Leatherland JF (2010) The actions of *in ovo* cortisol on egg fertility, embryo development and the expression of growth-related genes in rainbow trout embryos, and the growth performance of juveniles. Mol Reprod Dev 77:922-931.

McCormick SD, Hansen LP, Quinn TP, Saunders RL (1998) Movement, migration, and smolting of Atlantic salmon (*Salmo salar*). Can J Fish Aquat Sci 55:77-92.

McCormick SD (2001) Endocrine control of osmoregulation in teleost fish. Amer Zool 41:781-794.

McCormick SD, Regish A, O'Dea MF, Shrimpton JM (2008) Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone, and aldosterone on osmoregulation, gill Na^+, K^+-ATPase activity and isoform mRNA levels in Atlantic salmon. Gen Comp Endocr 157:35-40.

Mazur CF, Iwama GK, (1993) Handling and crowding stress reduces number of plaque-forming cells in Atlantic salmon. J. Aquat. Anim. Health 5, 98-101.

Milla S, Wang N, Mandiki SNM, Kestemont P (2009) Corticosteroids: Friends or foes of teleost fish reproduction? Comp Biochem Phys A 153:242-251.

Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fisher 9:211-268.

Monette MY, Bjornsson BT, McCormick SD (2008) Effects of short-term acid and aluminum exposure on the parr-smolt transformation in Atlantic salmon (*Salmo salar*): Disruption of seawater tolerance and endocrine status. Gen Comp Endocr 158:122-130.

Moran P, JI Izquierdo, AM Pendas, and Garcia-Vazquez E (1997) Fluctuating asymmetry and isozyme variation in Atlantic salmon: Relation to age of wild and hatchery fish. Trans. Am. Fish. Soc. 126:194-199.

Norris DO (1997) Vertebrate Endocrinology. San Diego, California.

Oxman DS, Hagen P, Smoker W, and Gharrett AJ (2005) Genetic and Environmental Effects on Otolith Formation in Pink Salmon (*Oncorhynchus gorbuscha*) from Auke Creek, Alaska. American Fisheries Society 135th Annual Meeting. Anchorage, AK. pp. 211.

Perez-Casanova JC, Rise ML, Dixon B *et al* (2008) The immune and stress response of Atlantic cod to long-term increases in water temperature. Fish Shellfish Immun 24:600-609.

Reid SG, Bernier N J & Perry S F (1998) The adrenergic stress response in fish: control of catecholamine storage and release. Comp Bio Phys Part C, 120: 1-27.

Ruane NM, Carballo EC & Komen J (2002) Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpi.* Aqua Res, 33: 777-784.

Sakamoto T, McCormick SD (2005) Prolactin and growth hormone in fish osmoregulation. Gen Comp Endocr 147.1:24-30.

Saxena MP, Kaur P, Saxena HM, Kaur K (2009) Humoral and cellular immune responses to *Pasteurella multocida* in fish exposed to heavy metal polluted water. Inter J Vet Med 6:1.

Schreck CB, Contreras-Sanchez W, Fitzpatrick MS (2001) Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197:3-24.

Sink TD, Kumaran S, and Lochmann RT (2007) Development of a whole-body cortisol extraction procedure for determination of stress in golden shiners, (*Notemigonus crysoleucas)*. Fish Physiol Biochem 33:189-193.

Smith CE, and Piper RG (1972) Pathological effects in formalin-treated rainbow trout (*Salmo gairdneri*). J Fish Res Board Can 29:(3) 328-329.

Stolte EH, Van Kemenade BMLV, Savelkoul HFJ, Flik G (2006) Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. J Endocrinol 190:17-28.

Stolte EH, de Mazon AF, Leon-Koosterziel KM, *et al* (2008) Corticosteroid receptors involved in stress regulation in common carp, (*Cyprinus carpio)*. J Endocrinol 198:403-417.

Stoskopf MK (1993) Fish Medicine. Philadelphia, PA.

Szisch V, Papandroulakis N, Fanouraki E, Pavlidis M (2005) Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. Gen Comp Endocr 142:186-192.

Vijayan MM, Pereira C, Grau EG, Iwama GK (1996) Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp Biochem Phys C 116:89-95.

Wada H (2008) Glucocorticoids: Mediators of vertebrate ontogenetic transitions. Gen Comp Endocr 156:441-453.

Westring CG, Ando H, Kitahashi T *et al* (2008) Seasonal changes in CRF-I and urotensin I transcript levels in masu salmon: correlation with cortisol secretion during spawning. Gen Comp Endocr 155:126-140.

White B (2008) Alaska salmon enhancement program 2007 annual report, Alaska Department of Fish and Game, Fishery Management Report No. 08-03, Anchorage