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Primary Research Paper

Physiological and behavioral effects of zinc and temperature on coho salmon (*Oncorhynchus kisutch*)

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Abstract

Pacific salmon species including the U.S. federally endangered coho salmon (*Oncorhynchus kisutch*) and the U.S. federally threatened steelhead trout (*Oncorhynchus mykiss*) have declined at an alarming rate in the last 40 years. Two of the main causes for the decline in coastal coho populations include increases in temperature and contaminant loads in coastal watersheds. Zinc, in particular, is one of the most common contaminants in aquatic systems. Using an experimental mesocosm design, we examined physiological, biochemical, and behavioral responses of coho salmon to excess dietary zinc and increased temperatures, with the ultimate goal of relating results to wild populations of coho salmon and steelhead in the Navarro River, California. Fish were obtained from a hatchery and divided into four treatments: low water temperature-no dietary zinc, high temperature-no zinc, low temperature-zinc, and high temperature-zinc. Each treatment had four replicate tanks. Zinc concentrations in liver increased during exposure to a high zinc diet. Iron concentrations in liver increased during simultaneous exposure to high zinc diet and increased temperature, and growth was reduced in this experimental treatment. Expression of hsp-70 was not significantly different between treatments, but showed decreasing trends with high dietary zinc and high temperature. Feeding rate increased with exposure to a high zinc diet. Comparison with steelhead trout samples from the Navarro River, California, showed levels of zinc, iron, and hsp-70 greater than those found in the experimental Coho salmon. All comparisons between the hatchery coho salmon and wild steelhead should be viewed with caution due to the differences between species, the laboratory and natural environment, and the genetic differences between wild and hatchery fish.

Introduction

Pacific salmon species including the U.S. federally endangered coho salmon (*Oncorhynchus kisutch*) have declined at an alarming rate in the last 40 years (Brown & Moyle, 1991). During this time, 40% of California streams have lost their historic coho runs; 25% of the streams have intermittent runs of very few fish and only a few streams still retain self-sustaining runs of this once abundant salmon species (Brown & Moyle, 1991). Coho populations along the coast of California are currently perhaps at 6% of the levels seen in the 1890s (Brown et al., 1994). The main causes for the decline in coastal coho populations include alteration of stream systems and water quality from

factors such as logging and urbanization, periodic floods and drought, hatchery operations, disease, over harvest, and climate change (Brown et al., 1994). Many of these can lead to reduced stream flows and higher water temperatures, and the delivery of contaminants to the stream system.

Zinc is one of the most common contaminants in aquatic systems and is associated with urban runoff, soil erosion, industrial discharges, pharmaceuticals, and pesticides (Krenkel et al., 1975, Irwin, 1997). In some locations up to 50% of the zinc delivered to stream systems comes from highway runoff (Krenkel et al., 1975). Zinc is easily bioaccumulated in stream invertebrates – an important food source for juvenile salmonids while rearing in freshwater systems. Recent studies demonstrate that fish fed diets contaminated with zinc exhibited reduced survival, growth, and increased incidence of disease (Farag et al., 1994, Balasubramanian et al., 1995).

Water temperatures in most Northern California coastal watersheds are higher than historic levels, mostly due to the degradation of riparian vegetation and therefore reduced shading (Brown & Moyle, 1991). Coldwater, anadromous fishes in particular, may be subjected to sublethal heat stress due to large temperature fluctuations in shallow or unshaded streams (Wedemeyer, 1973). Studies of the acute effects of extreme temperatures (upper thermal tolerance limit) of salmonids have been numerous, but the consequences of sublethal temperature stress (thermal additions) have received less attention (Wedemeyer, 1973). One of the most common features of the sublethal response to temperature stress and exposure to xenobiotic compounds such as heavy metals is the increased cellular production of heat-shock or stress proteins (hsps) (Iwama et al., 1998, 1999). Hsps play a vital role in maintenance of protein integrity, preventing premature folding and aggregation of proteins (Iwama et al., 1998, 1999). Under normal conditions, cells produce relatively small amounts of hsps. However, the level of hsp production increases and regular protein synthesis is repressed during environmental conditions that result in thermal or proteotoxic stress to an organism (Sanders, 1993). Genes encoding hsps are grouped into four gene families, hsp-83, hsp-70, hsp-60 and the small hsps. The hsp70 family is the most highly conserved of the hsps and is found widely across species (Feige et al., 1996). Increased levels of hsp-70 protect cells from the deleterious effects of elevated temperatures or other stressors (Sanders, 1993).

While the effects of temperature and contaminants on fish have been studied as individual stressors (Hodson & Sprague, 1975), there is relatively little information about the combined effects of such stressors on growth and fitness of Pacific salmon. The combined effects of multiple stressors on organisms can be complex. For example, while zinc has been found to be more acutely toxic to fish at higher temperatures than at lower temperatures, water temperature can also reduce the toxicity of zinc (Hodson & Sprague, 1975, Donker et al., 1998) by increasing its metabolic elimination or detoxification rate, and by changing the physiological state of the animal (e.g. by induction of protective enzymes such as heat shock proteins) (Donker et al., 1998). Sublethal stress caused by exposure to elevated water temperature and high concentrations of zinc may also modify behavior (Hogstrand & Wood, 1996, Shumway, 1999). Metals have been shown to reduce aggression at sublethal concentrations (Henry & Atchison, 1986, Atchison et al., 1987). Toxicants can also affect swimming performance and compromise the ability to escape predators, impair predator detection abilities, and increase conspicuousness due to erratic behavior or hyperactivity (Mesa, 1994, Weis et al., 1999).

The objective of this study was to examine the physiological, biochemical, and behavioral responses of coho salmon to excess dietary zinc and increased temperatures, and to determine if the combination of stressors acts synergistically.

Materials and methods

Experimental conditions

Juvenile coho salmon (approximately two to three months old) were obtained from the Cascade Fish Hatchery in Cascade Locks, Oregon, and transported in chilled (10 °C) water to the University of California, Davis, CA. Fish were acclimated at 10 °C for 7 days and then randomly separated into 16 tanks (40 l each), 6 fish per tank at 10 °C. Temperature in eight tanks was increased by 1 °C per

day to a final temperature of 15 °C. Fish were acclimated to final experimental temperatures for 14 days. During the course of the experiments, ambient air temperatures reached 43 °C, contributing to intermittent breakdown of the water-cooling system. As a result, temperatures in the 10° tanks averaged between 8.6° and 11.5° during the experiment. Temperatures in the 15° tanks averaged between 13.8° and 16.2° prior to the experiment and between 14.6° and 15.9° during the experiment.

During acclimatization, all fish were fed 2.5% of their body weight twice per day with Biodiet Oregon 1-mm pellet size. Prior to introducing the experimental diet, all fish were weighed to the nearest 0.5 g and measured to the nearest millimeter. Fish were not individually marked, and all growth measurements were based on averages for each replicate tank.

On day 1 of the experimental phase, the diet in eight tanks (4 at 10 °C, 4 at 15 °C) was changed to a zinc-enhanced diet. The zinc-enhanced diet was prepared by mixing distilled water and $ZnCl_2$ with 1-mm pellet size Biodiet Oregon. The mixture was then freeze-dried, ground, and passed through a 1 mm mesh sieve. The final zinc concentration was 1900 ppm as analyzed by the California Veterinary Diagnostic Laboratory (CVDLS) at the University of California at Davis. During the exposures, fish were fed 2.5% of their body weight twice per day of either Biodiet Oregon or zinc-enhanced Biodiet Oregon. Fish were exposed for 21 days to one of four combinations of diet and temperature, high zinc/high temperature (15 °C), control/high temperature, high zinc/low temperature (10 °C), and control/low temperature. End points included length, weight, tissue metal accumulation, heat shock protein expression, aggression, and feeding frequency.

Chemical analysis of liver samples

Portions of fish livers were composited by tank and sent to the CVDLS at the University of California at Davis for analysis. Livers were analyzed according to methods described by Martin et al. Briefly, livers were analyzed using an ICP analytical procedure for nine metals. Samples were prepared by nitric acid/hydrochloric acid digestion. Based on a one gram sample size, this screen quantitates for Fe >0.2 ppm, Mn >0.04 ppm, Cu >0.1 ppm, Zn >0.1 ppm, Cd >0.3 ppm, and Mo >0.4 ppm, while semi-quantitative results were obtained for Pb >1 ppm, and Hg >1 ppm. On day 21, fish were removed from the tanks individually, weighed, measured, and sacrificed. Fish were placed in liquid nitrogen for immediate freezing. Condition factor was determined by the equation: C.F. = body weight (g)/length^3 (cm). All fish were stored in a −80° freezer until dissection for heavy metal and hsps analysis. Fish were dissected and gill, muscle, and 1/2; liver were removed for analysis of hsp induction.

Hsp-70 analysis

Hsp-70 proteins were analyzed using Western blotting techniques following methods described by Werner et al. (2001). Muscle and gill tissues were separated into two fractions, a supernatant or soluble fraction and a pellet (5% sodium dodecyl sulfate – SDS) fraction containing membranes and subcellular organelles. For liver tissue only the soluble fraction was analyzed. Briefly, samples were homogenized in a hypotonic solution containing 66 mM Tris–HCl (pH 7.5), 0.1% Nonidet, 10 mM EDTA, 10 mM DTT and protease inhibitors, i.e. 10 mM benzamidine, 5 μM pepstatin, 0.001% aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged, supernatants were collected then pellets resuspended in homogenization buffer containing 5% SDS, vortexed for 10 s, centrifuged and sample buffer added. Total protein concentration in each fraction was determined using the Biorad DC Protein Assay based on Lowry et al., (1951).

Subsamples of equal total protein content (50 μg) were separated by SDS-PAGE on 10% polyacrylamide gels with 5% stacking gels (Blattler et al., 1972) using the buffer system described by Laemmli (1970). Hsp-70 antigen was applied to one lane per gel to serve as an internal standard for blotting efficiency. Proteins were separated then electroblotted onto Immobilon-P membrane at constant voltage (40 V) over night. Membranes were blocked with 5% skim milk in 20 mM tris buffer and 0.4 M NaCl (pH 7.5) with 0.05% Tween-20 for 30 min. A monoclonal antibody for hsp70 (1:500; Affinity Bioreagents, MA3-001) was used as probe. Blots were incubated for 1 h 30 min with primary antibody, then washed three times for 30 min in tris-buffered saline solution containing 0.05% Tween-20. Alkaline phosphatase-conjugated

goat-anti-rat IgG (1:30000; Sigma) was used to detect hsp-70 probes. Bound antibody was visualized by a chemiluminescent substrate (CDP-Star; Tropix, Bedford, MA), and protein bands were quantified by densitometry (Biorad GS710).

Behavioral endpoints

Each fish was observed for a total of 5 h over the course of the 21-day experiment (each tank was observed for 30 min every other day). Fish were monitored for aggression measured as strikes/minute against conspecifics, and for feeding activity measured as strikes/minutes at food pellets.

Statistical analysis

To reduce handling stress, fish were not individually marked. Instead, all differences in behavior, growth and condition factor were based on averages of individuals in each tank. Data were analyzed using a 2-way Analysis of Variance with temperature and zinc as the main treatment effects. In tests of the effects of diet and temperature on post-trial condition factor and aggression (strikes/min), pretrial condition factor and post-trial condition factor, respectively, were used as covariates. Because multiple tests were performed, we used a Bonferroni correction to adjust the Type I error rate to the 0.05 level across all statistical tests performed. The Bonferroni correction is simply the 0.05 error rate divided by the number of tests performed. It is generally acknowledged that the Bonferroni correction is conservative in that it makes statistical significance more difficult to demonstrate (Perneger, 1998). Removing the effect of the covariates significantly reduced the possibility that differences in post-trial condition factor could be due to pre-trial condition or that changes in aggression could result from differences in body size of the fish. For correlated response variables like behavioral measures or fish size, condition factor, MANOVAs were performed.

Results

Pre-experiment conditions

After random assignment of fish to treatments, fish did not differ in condition factor. However, there was a statistically significant difference in body mass and length between temperature treatments (Table 1) with the fish at 15 °C being heavier ($F = 8.54$, $p = 0.012$) and longer ($F = 15.6$, $p = 0.002$). The fish in the 15 °C/high zinc treatment were slightly heavier (~13%) than fish in the two 10 °C treatments. Fish in the 15 °C/control diet treatment were almost 1 g heavier and 8 mm longer (~12%) than fish in both 10 °C treatments. In the analyses of post-treatment results, pre-treatment length was used as a covariate. However, the covariate was not significant in the majority of the analyses and is not reported unless it is significant.

Growth and condition factor

Growth, measured as increased length, differed slightly between treatments. Fish not exposed to zinc grew approximately 15% longer during the course of the experiment (17% at 10 °C, 14% at 15 °C), while fish on high-zinc diets experienced a lower growth rate of 6% (8% at 10 °C, 5% at 15 °C). The two-way ANOVA indicated that the difference due to zinc was significant ($F = 23.8$, $p < 0.001$) The effect of temperature on these results was not significant ($F = 1.46$, $p = 0.250$), and there was no interaction between temperature and zinc ($F = 0.031$, $p = 0.863$). There was a significant difference in growth of body mass due to temperature with fish at the lower temperatures growing heavier than at higher temperatures ($F = 8.9$, $p = 0.011$). The change in condition factor between pre-treatment and post-treatment was positive (increase in condition factor) in all treatments. The increase in condition factor was significantly less in the high zinc treatments ($F = 18.93$, $p = 0.000$) than in fish fed control diets OK. Pretrial condition factor used as a covariate was not significant, indicating that the change in condition factor was not a function of the condition of the fish prior to the initiation of the experiment. There was also an effect of temperature on change in condition factor ($F = 6.02$, $p = 0.03$) with lower temperatures exhibiting a greater increase in condition factor. Change in length, change in weight, and post-test condition factor were combined into a single response variable in a two-way MANOVA. There were significant differences between temperatures (Wilkes

Table 1. Effects of temperature and zinc treatments on physiological and behavioral parameters in coho salmon (Oncorhynchus kisutch)

Parameter	Treatment			
	10 °C – no zinc	10 °C – zinc	15 °C – no zinc	15 °C – zinc
Pretreatment length (mm)	60.1	60.9	64.5	65.0
Post-treatment length (mm)	70.3	65.7	73.5	68.1
Growth (mm)	10.2	4.7	9.0	3.1
Pretreatment mass (g)	4.6	4.6	5.5	5.2
Post-treatment mass (g)	14.2	14.2	14.3	14.1
Growth (g)	9.6	9.5	8.8	9.0
Pretreatment condition factor	0.021	0.020	0.021	0.019
Post-treatment condition factor	0.041	0.050	0.036	0.045
Zinc in liver (ppm)	20.0	21.5	19.5	23.0
Fe in liver (ppm)	51.0	66.3	85.8	120.0
Aggression (Strikes/minute)	3.6	1.3	2.5	1.3
Feeding (Strikes/minute)	9.5	15.2	11.2	18.0
Hsp-70 (muscle)	8.41 (1.26) 17.16 (3.48)	9.00 (1.76) 14.74 (2.86)	6.9 (2.02) 12.55 (3.35)	7.9 (1.12) 14.58 (4.44)
Hsp-70 (liver)	17.38 (2.76)	15.44 (3.21)	16.67 (3.93)	12.66 (3.55)
Hsp-70 (gill)	8.1 (3.41)	6.96 (2.95)	5.82 (2.23)	4.25 (3.42)

$\lambda = 0.264$, $F = 9.26$, $p = 0.003$), zinc treatment (Wilkes $\lambda = 0.286$, $F = 8.32$, $p = 0.005$), but no interaction (Wilkes $\lambda = 0.951$, $F = 0.17$, $p = 0.91$).

Zn and Fe

No pretreatment measurements of zinc or iron in liver were available due to the limited number of fish. Therefore fish fed control diets, i.e. not exposed to zinc were used as the control for concentration measurements of zinc and iron in the liver. Zinc in liver increased significantly ($F = 5.00$, $p = 0.045$) with exposure to a high-zinc diet. Fe in the liver increased with exposure to both zinc and high temperature. The increase due to zinc in the diet was significant ($F = 5.63$, $p = 0.035$), and the increase due to temperature was highly significant ($F = 17.98$, $p = 0.001$). No temperature–zinc interaction was present in either analysis. Also, no factor such as length, body mass, or condition factor was significant as a covariate, indicating that there was no size dependence in zinc or iron processing.

Differences in hsp-70 levels were not statistically significant, but several trends could be observed. Overall, expression of hsp-70 in both muscle and liver supernatant extractions was higher in the 10 °C treatments than in the 15 °C treatments. Expression of hsp-70 in liver was lower than controls in the zinc-exposed fish.

Behavior

Exposure to high-zinc diet decreased aggression as measured by the number of strikes per minute at other fish in the tank ($F = 11.65$, $p = 0.005$). Condition factor used as a covariate was not significant indicating that larger fish were not more aggressive. Feeding rate increased with exposure to zinc ($F = 15.41$, $p = 0.002$).

Discussion

One of the primary results of this 21-day study was the lack of a significant interaction between temperature and zinc. Statistical analyses demonstrated that there was no interaction for any of the experimental endpoints measured indicating that there are neither synergistic nor antagonistic effects between exposure to a diet containing 1900 ppm zinc and a moderately elevated temperature (15 °C).

Increased dietary zinc reduced incidents of intraspecific aggressive behaviors, but caused a significant increase in feeding rate, (Table 2). Environmental stressors have been shown to impair predator avoidance (Weis et al., 1999) and would potentially affect competitive behaviors in general. Less time spent defending resources would allow for additional time spent feeding. However, even with increased feeding rates, growth of fish from this treatment was lower than that of control fish. This indicates that there was a significant diversion of energy due to uptake of zinc, and that fish may have tried to compensate for this energetic demand by increasing their food intake. It is known that there is a large energetic cost associated with the induction of stress-responsive and detoxifying enzymes (Barton & Schreck, 1987). Zinc can induce metal-specific detoxifying enzymes such as metallothionein as well as components of the cellular response to oxidative stress.

Exposure to elevated temperature (15 °C) also reduced growth rate, a common result in studies of this type. Increasing temperature results generally in an increase in metabolic rate and, consequently, in energy demand. If food is supplied at a greater rate, growth rate can in fact increase. However, we kept the feeding rate constant at 2.5% body mass and therefore likely placed the fish in an energetic deficit resulting in a reduced growth rate at higher temperature.

It is generally believed that juvenile salmonids cannot tolerate temperatures greater than 23–26 °C, and the preferred temperature of juvenile coho salmon is about 10–12 °C (Konecki et al., 1995). We chose treatment temperatures of 10 °C (control) and 15 °C (experimental thermal stress) in order to investigate response to continuous thermal stress. We were surprised to see a decreased rather than increased expression of heat shock proteins in livers of fish in increased temperature and zinc treatment groups (1900 ppm zinc/15 °C). The same trend was seen in gill tissues of these fish. Although a decreased expression of hsps in apparently stressed organisms has been observed in other studies (Werner & Hinton, 1999, Kohler, 1999, Kohler, 2001, Viant et al., 2004, Werner, 2004) we still lack the mechanistic understanding for this phenomenon. Viant et al. (2004) suggest that steelhead trout are able to adapt to a constant 5 °C elevation in temperature, from 15 to 20 °C, within ca. 3 weeks. Induction of hsps may also depend more upon the relative increase in environmental temperature than upon the absolute temperature experienced by these fish (Fader et al.,

Table 2. Results of two-way ANOVA analyses on the physiological and behavioral parameters. All values are p values. Bonferroni correction factors for the number of tests resulted in $p = 0.0036$ as the alpha value that allowed an experiment-wide type I error rate of $p = 0.05$. All significant results at the $p = 0.0036$ level are in bold

Variable	Model Terms				
	Model	Covariate	Diet	Temp	Diet × Temp (Interaction Term)
Pre-trial condition factor	0.124	N/A	N/A	N/A	N/A
Growth (length/mm)	**0.003**	N/A	**0.000**	0.250	0.863
Growth (weight/g)	0.066	N/A	0.782	0.011	0.532
Zinc in liver	0.168	N/A	0.045	0.663	0.389
Iron in liver	**0.003**	N/A	0.035	**0.001**	0.381
Hsp-70 Muscle supernatant	0.036	N/A	0.102	0.008	0.680
Hsp-70 Muscle pellet	**0.000**	N/A	0.753	0.234	0.016
Hsp-70 Gill supernatant	0.182	N/A	0.947	0.110	0.227
Hsp-70 Gill pellet	0.772	N/A	0.351	0.729	0.493
Hsp-70 Liver supernatant upper band	0.580	N/A	0.425	0.453	0.327
Hsp-70 Liver supernatant lower band	0.009	N/A	0.004	0.072	0.259
Aggression (strikes/min)	0.023	N/A	0.005	0.305	0.349
Feeding (strikes/min)	0.011	N/A	**0.002**	0.192	0.736

1994). It has also been hypothesized that a lack of energy, potentially stemming from increased metabolic demands related to stressors, leads to a reduction in cellular protein expression (Sanders et al., 1991, Werner & Hinton, 1999). It has also been suggested that the basis of the decrease in stress protein levels may lie in the pharmacological kinetics of the hsp70 level in response to increasing intensity of stressors; the induction of hsp70 follows an optimum curve with an optimum stress response (Kohler et al., 2001). Vijayan et al. (1998) demonstrated a surpassing of the stress response optimum and declining hsp70 levels following exposure to increasing concentrations of chemical stressors. While increasing temperature alone has been shown to induce an optimum hsp70 response, in theory, high temperature is potentially capable of reducing contaminant-induced hsp70 levels when they occur together (Kohler et al., 2001).

Increasing concentrations of iron in the liver, as measured in fish exposed to dietary zinc and elevated temperature, are considered to be an indicator of liver damage (Skibba & Gwartney, 1997, Mori & Hirayama, 2000) in mammals. Mammalian hepatotoxicity in response to hyperthermia may be the result of oxidative stress from superoxide generation, and ferritin released from the liver appears to play a central role in hyperthermic toxicity (Martin et al., 1998). Whether this same phenomenon is occurring in fish and by what mechanism is unknown, but livers did accumulate iron in response to increasing temperature and increasing zinc indicating that hepatotoxicity was occurring.

As populations of coho salmon in many Pacific North coast watersheds are federally listed, field experimentation and investigation of this species is nearly impossible. Coho salmon used in this experiment were obtained from hatchery stock considered genetically different from wild coho salmon. Since differences in tolerance to environmental factors appear to have a genetic basis, results may not be directly transferable to field situations (Weis et al., 1999). Additionally, methods used to incorporate metals into fish diets can influence the degree of toxicity caused by metals (Farag et al., 1994). When metals were added surficially to commercial diets, the toxicity to trout was less than when zinc occurred naturally in the diet. Thus, although the concentrations of metals in diets may be similar, toxicological effects of those diets can differ (Farag et al., 1994).

Species with similar life history strategies and habitat requirements such as steelhead may be studied in the field to gain insights into the complex interactions and subsequent population consequences of environmental stressors. In the Navarro River, for example, we have examined steelhead in an attempt to gain insight into the decline of the coho salmon populations in that watershed. Preliminary investigations have shown levels of zinc in steelhead livers averaging 32.8 ppm, approximately 1.5 times the levels found in livers of our experimental coho. Levels of iron in livers taken from Navarro steelhead averaged 114 ppm, while values from our experimental coho averaged 66 ppm (10° + Zn), 51 ppm (10°), 120 ppm (15° + Zn), and 86 ppm (15°). Hsp levels in a small sample of Navarro steelhead averaged 9.6, while levels in our experimental coho averaged approximately 8.05 (muscle supernatant) and 7.05 (muscle pellet). These values demonstrate strong responses to environmental stressors present in the Navarro River. Clearly, however, further research is necessary in order to begin to separate the multiple factors involved in declines of fish species and populations.

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