THE STRESS PROTEIN RESPONSE OF

Pimephales promelas

TO COPPER

THESIS

Presented to the Graduate Council of the

University of North Texas in Partial

Fulfillment of the Requirements

For the Degree of

Masters of Science

By

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Denton, Texas

May 1992

Covington, Sean M., <u>The Stress Protein Response of</u> Pimephales promelas <u>to Copper</u>. Master of Science (Biology), May 1992, 40 pp., 5 tables, 9 illustrations, reference list, 48 titles.

Organisms synthesize stress proteins in response to a variety of stressors. The 68/70-kDa proteins (synonymous to the 72/73-kDa proteins) have shown to be the most promising stress proteins, and have been proposed as a biomarker of general organismal stress. The 68/70-kDa proteins were used in an antigen/antibody based approach to determine the duration of the stress protein response of *Pimephales promelas* following an acute exposure to copper sulphate.

Elevated levels of the 70- and 68-kDa proteins were detected, following introduction of copper sulphate. Levels of both proteins remained high, even up to 10 days post exposure. Variability in control levels made it difficult to statistically detect differences at all of the sampling periods. Recovery of the fish was not observed via quantitative immunoblot techniques.

ACKNOWLEDGMENTS

I would like to recognize the following persons for their contributions in the culmination of this project, to each, I offer much appreciation and thanks:

Dr. Ken Dickson, my major professor, for creating a flexible, applied program which was conducive to learning, making friends, and accomplishing goals. His support and encouragement in the framework of this program helped me to broaden my thinking and be more openminded.

Dr. Tom Waller, served on my committee and gave me the benefit of his experience in data handling and statistics.

Dr. Earl Zimmerman, served on my committee, and gave me the opportunity to work with him on the project.

My wife, Julie, and my parents, who for the past few years supported and encouraged me to pursue my education and career goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Environmental stress is an inescapable component of the life of fish, compounded by the effects of adverse environmental conditions, including pollutants and land or water project developments within an area (Wedemeyer and McLeay,1981). These perturbations can severely damage populations and whole communities. During the past two decades, water quality biologists have searched for sensitive indicators of sublethal effects of contaminants on fish in an attempt to understand the mode of action of toxicants and to develop a basis for corrective action in cleaning up water bodies before the health of aquatic organisms is seriously threatened (Passino,1984). Standard approaches of measuring fish health following exposure to environmental pollution focus primarily on short-term studies of whole animal responses, such as mortality, gross abnormalities, altered condition indices, and behavioral changes (Neff,1985). These methods have been employed somewhat successfully as indicators of species, population, and community health and have been the best criteria to date for evaluating general stress (Jenkins and Sanders,1986). However they have several limitations , particularly sensitivity and time constraints.

1

The concept of biomarkers was introduced as a possible alternative to the conventional methods. Biomarkers refer to the measurement of selected endpoints in individual organisms, typically physiological or biochemical responses, that serve as sensitive indicators of exposure to contaminants and/or sublethal stress (Baker,1988). In essence, the organisms used in conventional methods are the same as those used in biomarker studies, however, the measurement endpoints are different. Biochemical indicators of environmental stress are comparatively cheaper and faster, more sensitive, less variable, more highly conserved, and often easier to measure than stress indices commonly examined at the organismic level such as inhibition of growth, changes in rate of development and reduced reproductive potential (Sanders,1990).

Esch and Hazen (1978), defined stress as "the effect of any environmental alteration or force that extends homeostatic or stabilizing processes beyond their normal limits, at any level of biological organization". In this context, organismal stress and organismal health are synonymous in response to environmental perturbations. Biotic responses to environmental perturbations begin at the molecular/cellular level and then extend to tissues and organs whose responses occur before changes in populations and ecosystems (Bouk,1984). Cellular responses, therefore, have the potential for providing exposure and effects

information prior to more traditional endpoints, such as mortality and altered condition indices. There is the expectation that the study of biochemical responses will provide reliable, sensitive biomarkers for exposure and toxicity in free living organisms (Di Giulio, et.al.,1989).

Recently, a biochemical technique that measures a cellular defense mechanism, termed the heat shock or stress protein response, has been proposed as a biomarker of environmental perturbations. The stress protein response (SPR) was first observed in *Drosophila* exposed to temperature stress (Ashburner and Bonner,1979). Since its discovery, the stress protein response has been observed in a variety of other organisms such as mammals, fish, plants, algae, and bacteria (Adams and Rinne,1982), thus lending support to the hypothesis that the SPR is a highly conserved and ubiquitous response.

Induction of stress proteins, via increased translation and or transcription of stress protein genes, is a cellular response to metabolic stress. Increased stress protein gene expression in response to environmental stress is brought about by heat, chemical reagents, starvation, anoxia, and wounding (Adams and Rinne,1982), among others. When an organism is subjected to a stressor, normal protein synthesis is suspended, and preferential synthesis of a suite of stress proteins begins (Sanders,1990; Schlesinger,1986). Several stress proteins have been

Overview of Stress Proteins
General Functions During Cellular Stress

Normal protein synthesis	STRESS →	Inhibition of normal protein synthesis
Decreased Sp levels, increased synthesis of normal proteins		Rapid synthesis of a suite of proteins
RECOVERY	Cellular repair and protection	
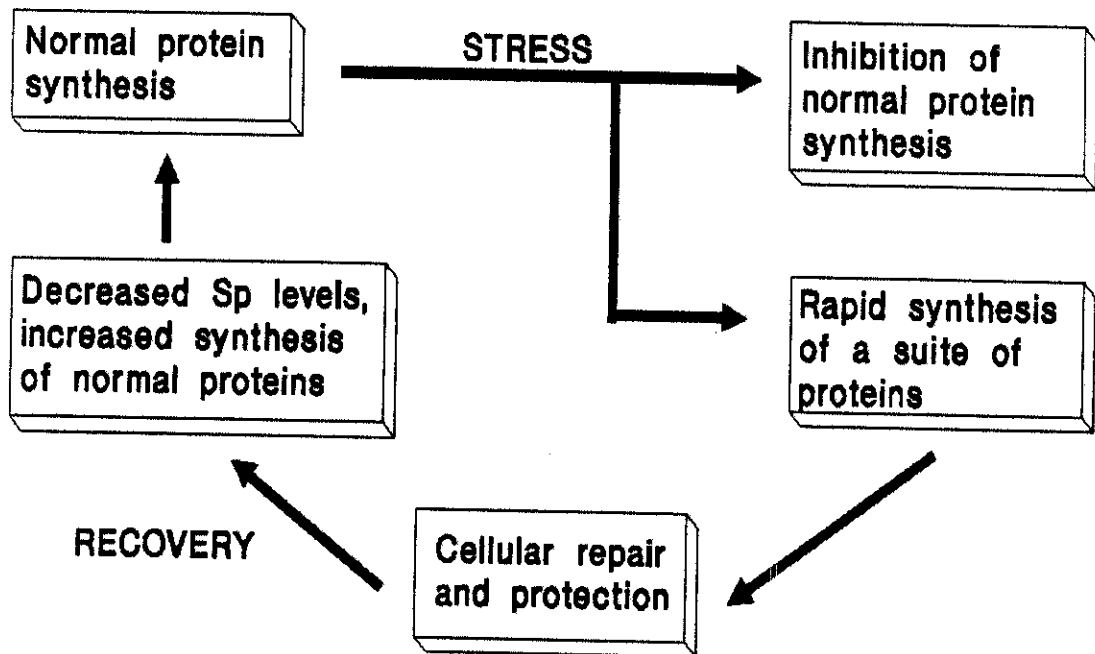
Figure 1. A flow diagram showing the events which occur during cellular stress with respect to the stress protein response.

identified, however, the most extensive work has involved the 72/73-kDa proteins. These proteins consists of a constitutive form (73 Kda) which is present in appreciable levels in unstressed cells, and an inducible form (72 Kda) which is usually present only in stressed cells (Welch,1990). The 73-kDa protein is mobile in the cell, serving as a "chaperon" for newly synthesized secretory and

organellular proteins and facilitates their translocation across a membrane (Sanders,1990; Welch,1990; Schlesinger, 1990, 1986; Lindquist,1986). During stress, there is a marked increase in the levels of the constitutive and inducible forms. The inducible form, 72-kDa, is speculated to resolubilize denatured pre-ribosomal complexes and help restore nucleolar function during recovery from stress (Sanders,1990; Welch,1990; Schlesinger, 1990, 1986; Lindquist,1986). Kinetics of induction and recovery of the stress response suggest differences which are specific to the type of environmental insult and severity of the stress (Sanders,1990), however, it is estimated that in most organisms, the 70-kDa protein has a half life of four days (Lindquist,1986). Consequently, the highly conserved nature of these proteins makes them good indicators of general stress. It is becoming clear that stress proteins have important functions in the cell under normal conditions and take on dual protective and repair roles under conditions of stress (Dyer,1991).

As previously mentioned, there are a host of other stress proteins ranging in molecular weights as low as 7 kDa to 100 kDa. Certain of these proteins are relevant to this research and deserve discussion, including species-specific low molecular weight (LMW) proteins, heme oxygenase, and metallothionen. The LMW proteins in the range of 20 to 30 kDa are the least conserved of the stress proteins and tend

to be species specific (Sanders,1990). These LMW proteins have been shown to be indicative of oxidizing agents, arsenite and heavy metal exposure (Dyer,1991; Shibahara et al.,1987; Keyse and Tyrrell,1989), indicating the potential for these proteins to be used as markers of stressor specific exposure. Heme oxygenase, a 32-kDa protein, has shown to be the most prominent stress protein induced by heavy metals upon *in vitro* exposure (Caltabiano et al.,1986; Shelton et al.,1986; Keyse and Tyrrell,1989). Heme oxygenase is an enzyme essential for heme catabolism that cleaves heme to form biliverdin which is subsequently reduced to bilirubin (Caltabiano et al.,1988; Keyse and Tyrrell,1989). Metallothionen ($\geq$ 10 kDa) has also been considered as a potential biomarker of metal stress. This metal binding ligand appears to be part of a cellular compartmentalization/sequestration system which evolved to regulate the uptake and tissue distribution of essential trace metals such as transition elements Zn and Cu (Viarengo, et al.,1985)

Recent research using the fathead minnow showed that the SPR is rapid, occurring within hours of stressor exposure, the SPR is sensitive to sublethal exposure and can be correlated to toxicant type, concentration, and percent mortality, and furthermore, certain stress proteins indicated the chemical class or mode of action of specific stressors (Dyer,1991). The ability of the organisms to

produce these proteins does not insure survivability, however it does contribute to the organism's ability to tolerate initial and subsequent stress episodes. Finally, as an assessment of fish health prior to, during, and after exposure, the stress protein response could provide the aquatic resource manager with a beneficial tool for evaluating fish health.

Although the literature reports certain stress proteins which are more indicative of metal stress, availability of antibodies for these proteins restricted this research to the use of 72/73-kDa antibodies. The 72/73-kDa stress proteins are general indicators of stress. Thus, using quantitative immunoblotting techniques with monoclonal antibodies specific for the 72/73 kDa proteins, and the stress protein response of the fathead minnow, *Pimephales promelas*, which was characterized by Dyer (1991), I attempted to accomplish the following objectives: 1) determine the duration of the stress protein response, *in vivo*, upon exposure to an acute dose of copper; 2) determine the degree of metal accumulation in gill and whole body tissues of the fathead minnow; 3) determine if a relationship exists between stress protein levels and metal accumulation in the gill tissues and whole body of the fish.

Copper, which occurs in natural waters primarily as the divalent cupric ion in free and complex forms, is a minor nutrient for both plants and animals at low concentrations

but is toxic to aquatic life at concentrations slightly higher (U.S.EPA,1980). Cuprous copper is unstable in aerated water over the pH range of most natural waters (6-8) and will oxidize to the cupric state (Garrels and Christ,1965). At pH 6, the cupric ion (Cu^{2+}) is dominant while at pH 6-9.3 the copper carbonate complexes ($CuCO_{3\ aq}$) dominate (Stumm and Morgan,1970). The major role of copper in biological systems is to stabilize sulphur radicals (Moore and Ramamoorthy,1984). Acute toxicity of copper to fresh water fish depends largely on water hardness and the copper species present (U.S.EPA,1980). The 96 hour LC50's for fathead minnows ranged from 0.023 to 1.450 mg/L, and as water hardness increased the toxicity of copper decreased (U.S.EPA,1980). Divalent copper ion and its hydroxy complexes are believed to be the toxic chemical species to fishes (Chakoumakos,1977).

The modes of action of copper are numerous, including precipitation of gill secretions (Tsai,1979), impairment of haemopoetic tissue in gill filaments resulting in loss of oxidative activity, possible acetylcholinesterase inhibition (Nemcsok and Hughes,1988) and disruption of osmoregulatory function via inhibiting the activity of gill Na^+ , K^+, ATPase enzyme (Lauren and McDonald,1986,1987; Lorz and McPherson,1976).

Because the gills are intimately associated with ionic regulation, it is predictable that heavy metals will

influence aspects of osmotic and ionic regulation in fish (Eddy,1981) resulting in a stress response. Most fishes are capable of accumulating metals via the gastrointestinal system and from water via various membrane surfaces, particularly the gill (U.S. EPA,1978). Eisler and Garden (1973) found that dead top minnows (*Fundulus heteroclitus*) accumulate copper more readily than living individuals. Fish exposed to copper show decreased feeding activity, reduced growth, and accumulation of copper in body tissues (Buckley, et.al.,1982). It has been reported that a good correlation exists between copper accumulation and development of chronic symptoms in fishes (U.S. EPA,1978). However, copper may exert a detrimental effect on fish only if tissue levels are significantly higher than background, and the degree of damage by copper is related to the body burden attained (Dixon and Sprague,1981). It has been suggested therefore, that fish accumulate significant copper only at levels of exposure above the threshold of sublethal injury (Brungs, et.al.,1973; McKim and Benoit,1974; Soble and Cooper,1976).

MATERIALS AND METHODS

Test Organisms

Fathead minnows, *Pimephales promelas*, were obtained from the University of North Texas stock culture. Seven days

prior to the test, fish were acclimated at 22°C, 16l:8d photoperiod, and maintained on a diet of frozen brine shrimp. In order to accommodate the large number of fish needed for the test, ninety day and older fish were used.

Experimental Design

The test was a static renewal design with aeration using dechlorinated Denton City tap water. Eighty-four, five gallon tanks were utilized, with three treatment replicates and three control replicates per time period of sampling. Ten to twelve fish were placed into each tank and subsequently dosed from a copper stock solution (1000 ppm) of $CuSo_4-5H_2O$. Tanks were dosed with a nominal concentration of 140 ug/l at 24 hour intervals up to 96 hours. Every 24 hours 80-90% of the water was renewed to a volume of 15 liters. Fish were fed frozen brine shrimp daily to satiation prior to renewal. Tanks were sampled every 24 hours which included: sacrificing fish via decapitation, wrapping in foil, and freezing at -80°C until dissection. Gill tissue was excised, minced, pooled and subdivided for body burden and stress protein analysis. Figure 2 shows a flow diagram of the experimental design followed for the experiment.

Water Chemistry

The renewal water was tested daily for pH, temperature,

EXPERIMENTAL DESIGN

EQUAL CONTROL AND TREATMENT TANKS (REPLICATES N=3)
TIME SERIES SAMPLING EVERY 24 HOURS

GILL TISSUE EXCISED
POOLED AND SEPARATED

RENEWAL WATER
CHEMISTRY

TANK WATER
SAMPLED

SP ANALYSIS
SDS PAGE
WESTERN BLOT
ANTIBODY PROBE
VISUAL DETECTION

BODY BURDEN ANALYSIS
TISSUE DIGESTION
COPPER RESIDUE

GRAPHITE FURNACE ANALYSIS
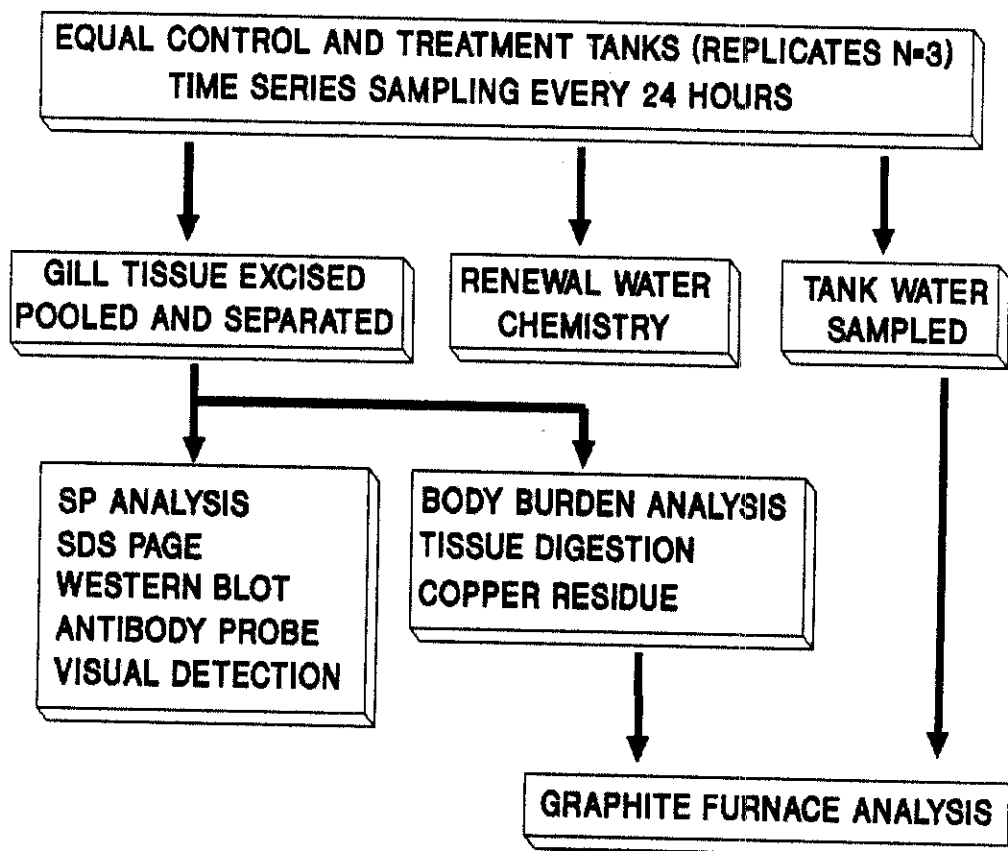
Figure 2. A flow chart depicting the stepwise procedure used in the study. Chart also briefly displays steps in determining the stress protein response.

alkalinity, hardness, conductivity, and dissolved oxygen. Test aquaria water was monitored daily for pH, temperature, and dissolved oxygen. Water samples were taken daily from treated and control tanks, acidified to pH 2 with AR select ultra purity grade Nitric acid (70%), and stored for later

analysis. Total copper in the tanks was determined using a Perkin-Elmer Atomic Adsorption Spectrophotometer with HGA-400 Graphite Furnace attachment (EPA method 220.6).

Body Burden Analysis

Tissues were dried for 24 hours at 103°C, weighed and digested with heat using AR select ultra purity grade Nitric Acid (70%). Further digestion was done with AR hydrogen Peroxide (30%) with heat. Digestate was brought up to volume with Milli-Q water. Analysis for total copper utilized a Perkin-Elmer Atomic Absorption Spectrophotometer with HGA-400 Graphite Furnace.

Sample Preparation and SDS-PAGE

Sample preparation and electrophoresis was performed according to the methods described by Dyer (1991). Half of the pooled gill tissue was used in the analysis, which contained gill from survivors of the test. Tissues for stress protein analysis were minced, homogenized and sonicated in 150 ul of tris buffer (150 uM Tris-HCL pH 7.8, 1 uM phenylmethylsulfonylfluoride). Homogenates were centrifuged at 10,000 x g, 4°C, for 30 minutes. The supernatents were then collected and protein concentrations determined using the method of Bradford (1976).

Proteins were separated on 12.5% polyacrylamide gels using the discontinuous buffer system described by Laemmli

(1970). Samples containing 100 ug of protein were diluted to 50, 25, 12.5, 6.125, 3.06, 1.53, and .765 ug of total protein. Prestained low molecular weight markers were used to determine protein location on the gels. Samples were loaded onto gels and run for 2-3 hours, at 230 mV at 4°C.

Gels which were used for the time series profile were stained in Commassie R-250 for 45 minutes, and then destained in 7% acetic acid and 8% methanol for 2-3 hours. Gels were subsequently stored in 5% acetic acid and photographed.

Quantitative Immunoblots

Following SDS-PAGE, proteins were transferred to nitrocellulose using a transfer buffer containing 1% SDS, 0.192 M glycine, tris and 20% methanol (v/v), pH 8.3. Transfers were performed in a Western Blot apparatus at 25 mV for 15 h, at 4°C. The nitrocellulose was blocked for 1h in tris buffered saline (TBS) with 3% gelatin. A monoclonal antibody (Ab) raised against HSP 72/73 from HeLa cells was then used to probe the nitrocellulose at a 1:1200 dilution. Antibody incubation was performed at room temperature for 2 hours. Immunoblots were then rinsed with TBS containing 0.5% Tween-20 (TTBS) following incubation. A second incubation included the use of a goat anti-mouse alkaline phosphatase (GAM-AP) conjugate antibody for 2 hours. Immunoblots were washed again with TTBS and TBS. Color

development of the blots was initiated with 5-bromo 4-chloro 3-indoyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride. Immunoblots were incubated for 30-45 minutes at room temperature to visualize protein-AB complex. The minimum stress protein level (MSPL) was determined from the blots as the least amount of protein required for visualization. Verification of bands was performed using the LKB 2202 Ultroscan Laser Densitometer with Gelscan(LKB) software on an Apple IIe computer via the methods of Maciewicz and Knight (1988).

Data Analysis

Differences in MSPL from treatment and recovery fish were tested for significance using nonparametric analysis of variance. Comparisons of MSPL in treated vs. control fish at each time interval was determined using nonparametric analysis of variance and Duncan, Dunn's and Student Neuman Keuls multiple range tests. The Spearman rank correlation method was used to determine if a relationship existed between copper accumulation and stress protein levels. Significance for all statistical tests was at $\alpha = 0.05$.

RESULTS

Fathead minnows were exposed to a sublethal copper concentration for 96 hours and allowed to recover up to 240

hours. The time course of the experiment was 336 hours (14 days). Gill tissue was used for SP analysis to determine the effect of copper on the stress protein response. Renewal and test aquaria water was sampled every 24 hours to insure homogenous conditions.

Temperature, dissolved oxygen, and pH were measured every 24 hours and had the following characteristics: 18.5 - 21 °C, 6.0 - 6.6 mg/L O_2, 7.0 - 8.5 pH, respectively. Table I shows the water quality parameters which were measured during the experiment.

Table I. Mean (S.D.) characteristics of the renewal water. Measurements were made prior to each renewal.

Parameter		Mean	S.D.	n
Alkalinity	(mg/L as $CaCo_3$)	99.3	6.7	14
Hardness	(mg/L as $CaCo_3$)	113.4	13.6	14
Temperature	(°C)	22.2	0.93	14
Dissolved oxygen	(mg/L O_2)	3.6	0.91	14
Conductivity	(umHos)	307.1	9.9	14
pH range		6.9 - 7.3		14

Mean (+/- S.D.) total copper concentrations in treated tanks for times 24, 48, 72, and 96 hours as measured by graphite furnace, were 156.39 (12.40), 134.58 (28.49),
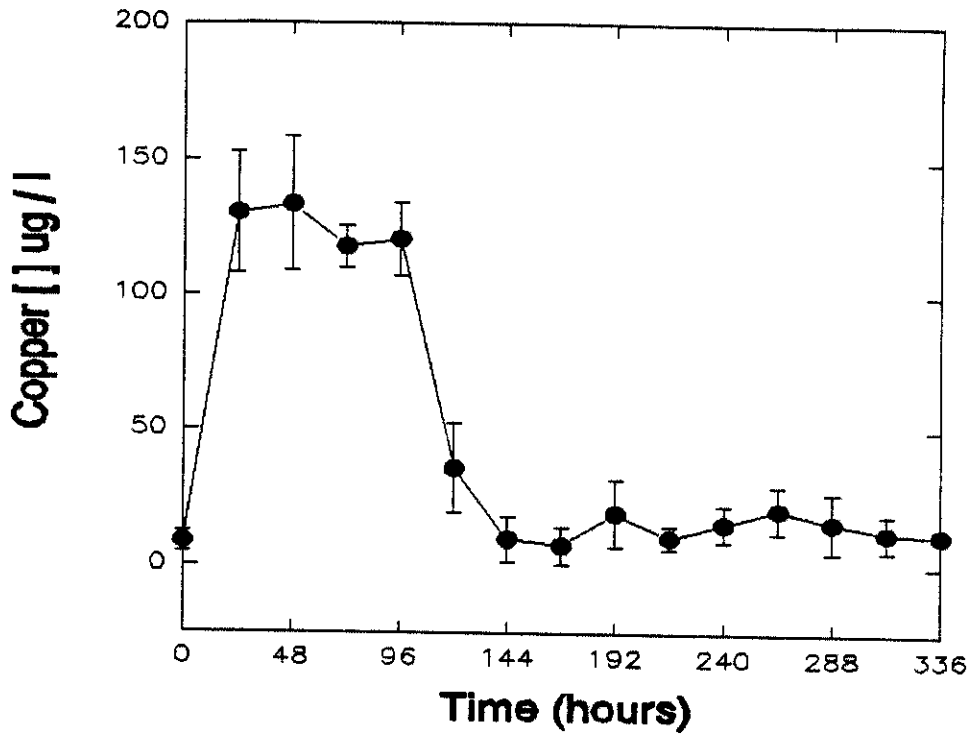
Figure 3. Mean (+/- S.D.) copper concentrations (ug/l) as measured by graphite furnace for each time period.

129.01 (6.95), and 137.29 (10.04) ug/l, respectively. Total copper concentrations returned to within 0 hour background levels by 144 hours. Figure 3 shows the measured levels.

Body burden analysis of gill tissue was not reported due to a contamination of the samples which resulted in abnormally high copper concentrations in control, treated and blank samples. Body burden analysis of whole fish (less gills) showed no significant accumulation of copper over control levels (ANOVA $p >> .05$ at each time). Gill tissue was expected to be the primary site of copper accumulation,
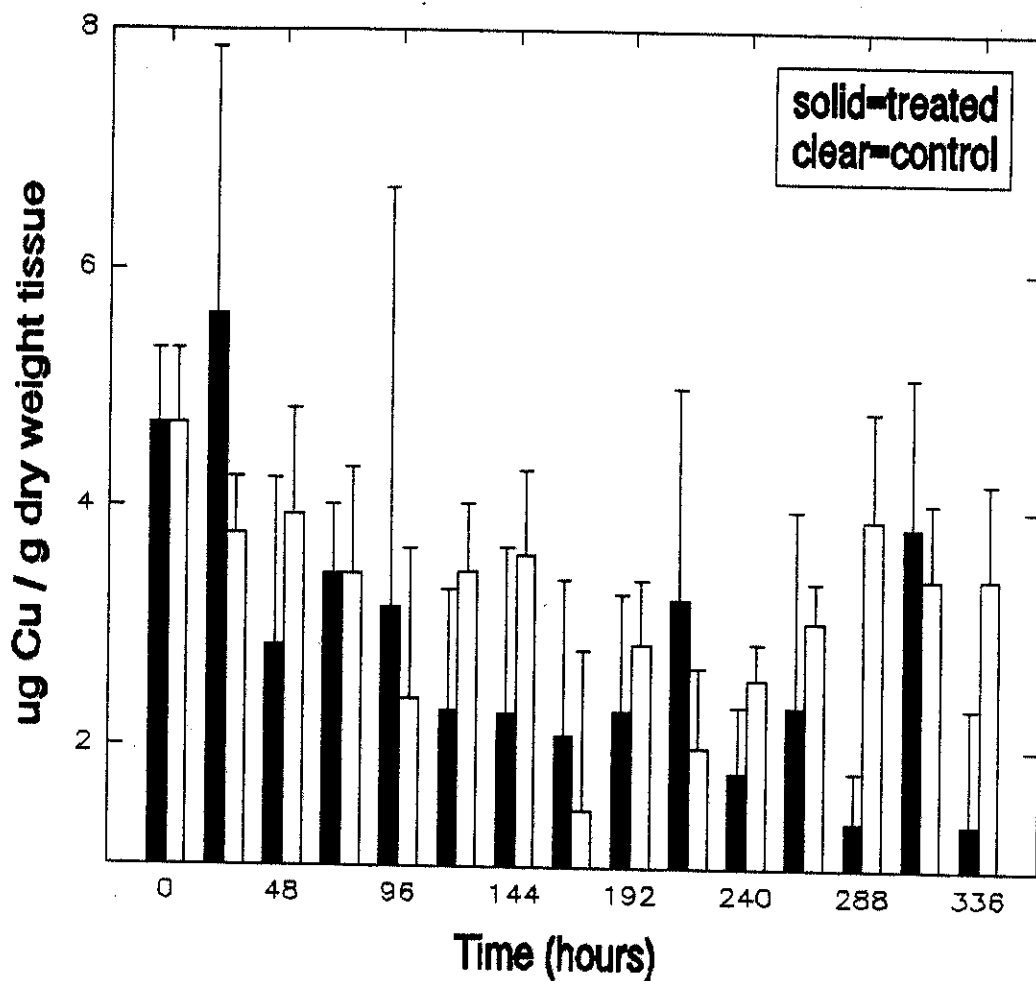
Figure 4. Body burden analysis of whole body control and treated fish. Bars represent the mean total copper concentration in ug/l (+ S.D.) for each time period (n=3).

and liver tissue the secondary site. Whole body analysis served to effectively dilute any accumulation which might have occurred in the liver due to the mass of liver to remaining body tissues. Figure 4 shows the mean total copper levels (ug copper/gram dry weight tissue) found in treated and control fish.

Fatheads exposed to copper exhibited rapid gill

ventilation, coughing, and erratic swimming followed by periods of lethargy. Feeding was greatly reduced in treatment tanks as opposed to non treatment tanks. Normal feeding activity more closely resembled control feeding by 192 hours of the test.

The use of quantitative immunoblots allowed the detection of two proteins, 68 and 70 kDa, which are synonymous to the 72/73-kDa proteins, respectively. Minimum stress protein levels (MSPL) were measured as the minimum number of micrograms of total protein required for detection. In effect, the specific binding of the monoclonal antibodies to the 72/73-kDa proteins was proportional to the amount of proteins present. Proteins were obtained from the pooled gill tissue of fathead minnows (n=12). Replicate means (n=3) were used for each analysis. In order to determine if the 68/70-kDa proteins were indicative of stress, a series of statistical tests were run independently of each other, on the MSPL recorded for each protein. Nonparametric analysis of the ranks of log10 transformed MSPL for SP70 revealed that the control means were not significantly different through time (ANOVA, p =.0580). However, analysis of MSPL for SP70 treated means were significantly different through time (ANOVA p =.0231). Student-Neuman-Keuls MRT showed the MSPL of treated means at time 0 was significantly different from MSPL at times 288 and 312 hours (p ≤ 0.05). Figure 5 shows the mean

Figure 5. Minimum stress protein levels for SP70 as measured via quantitative immunoblots. Levels are plotted as the mean reciprocal SP70 levels (n=3), plus the standard deviation of each mean.

reciprocal MSPL for SP70 in gill tissue of control and

treated fish. The untransformed values used to generate

Figure 5 are recorded in Table II. Relative assessment of

those levels showed that at approximately half of the time

intervals, control levels were actually higher than treated

levels. However, comparison of the ranks of log10

transformed MSPL for SP70 at each time interval showed

control and treated levels were not significantly different (Nonparametric ANOVA p >.05 at each time).

Analysis of the ranks of log10 transformed MSPL for SP68 revealed that the control means were not significantly different (ANOVA, p=.5996) through time, however, similarly transformed data for treated SP68 levels showed the mean ranks were significantly different (ANOVA, p =.0430).

Table II. Mean (S.D.) of the MSPL for SP70 from gill tissue as determined via quantitative immunoblots. Values are expressed as ug of total protein

Time	Mean control	S.D.	Mean treated	S.D.
0	12.5	0	12.5	0
24	2.04	0.8833	2.55	0.8833
48	1.78	1.16	5.44	6.21
72	3.57	2.33	2.55	0.8833
96	5.44	6.21	2.55	0.8833
120	2.55	0.8833	3.06	0
144	3.06	0	3.06	0
168	4.08	1.76	5.10	1.76
192	3.06	0	4.08	1.76
216	2.55	0.8833	3.06	0
240	4.08	1.76	3.06	0
264	2.04	0.8833	2.55	0.8833
288	4.08	1.76	2.04	0.8833
312	3.57	2.33	2.04	0.8833
336	5.10	1.76	3.06	0

Minimum stress protein levels for SP68 in treated fish at times 24 and 72 hours were significantly different from MSPL at 96, 216, 264, 312, and 336 hours (Duncans MRT, p≤ 0.05).
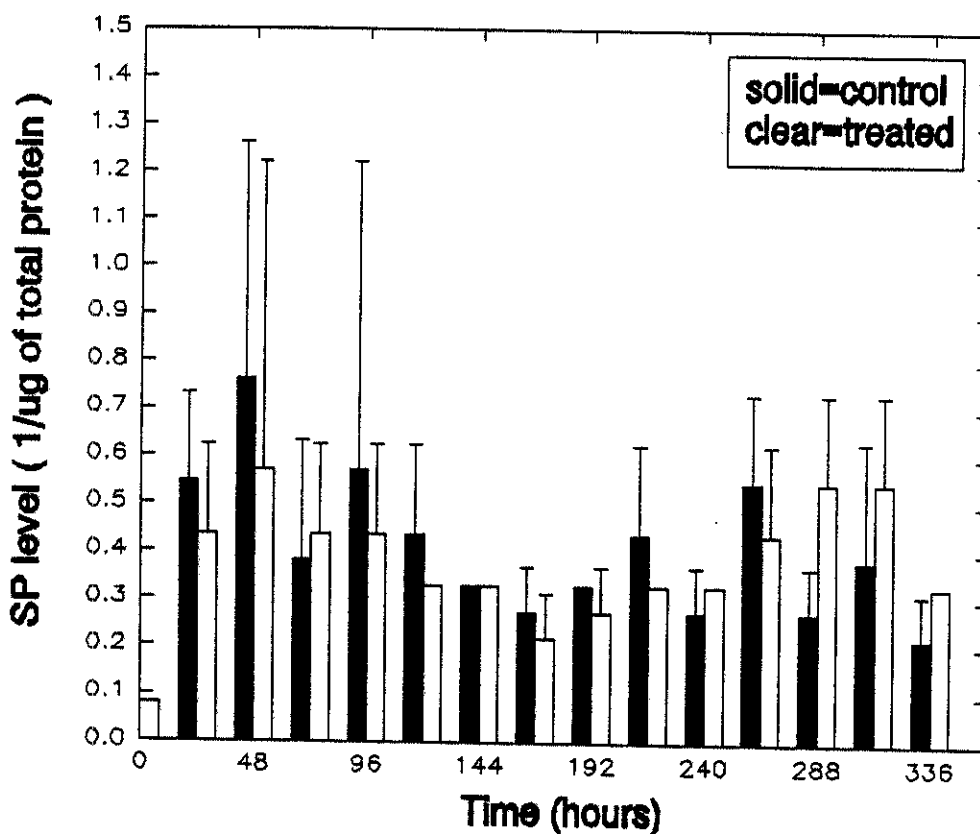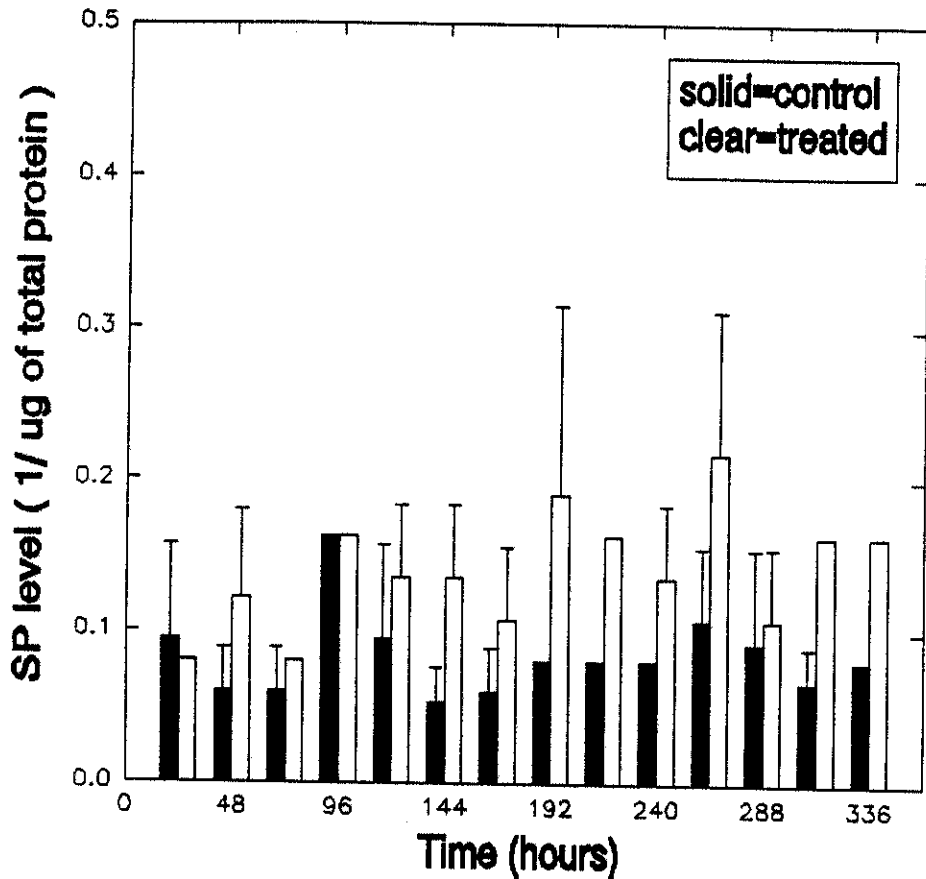
Figure 6. Minimum stress protein levels for SP68 as measured via quantitative immunoblots. Levels are plotted as the mean reciprocal SP68 levels (n=3), plus the standard deviation of each mean.

Zero hour could not be included in this analysis, because no observable levels of SP68 were detected. Similar comparisons of the MSPL for SP68 at each time interval showed control and treated levels were significantly different at the following intervals: 144 hours, 216 hours, 312 hours, and 336 hours (Nonparametric ANOVA p=.05, .0001, .0056, and .0001 respectively). Interestingly, all of these

time periods were in the recovery phase of the experiment.

Differences between control and treated MSPL at these time

periods however, does not indicate that the fish were

recovering.   Relative assessment of the mean reciprocal

Table III. Means (S.D.) of the MSPL for SP68 from gill tissue
as determined via quantitative immunoblots.   Values are
expressed as ug of total protein.

Time	Mean control	S.D.	Mean treated	S.D.
0	0	0	0	0
24	14.54	9.60	12.5	0
48	18.75	8.83	9.31	4.5
72	18.75	8.83	12.5	0
96	6.12	0	6.12	0
120	14.54	9.60	8.25	3.68
144	20.83	7.21	8.25	3.68
168	18.75	8.83	10.37	3.68
192	12.5	0	7.22	4.81
216	12.5	0	6.125	0
240	12.5	0	8.25	3.68
264	10.37	3.68	5.10	1.76
288	14.54	9.60	10.37	3.68
312	16.66	7.21	6.12	0
336	12.5	0	6.12	0

MSPL for SP68 shows that the treated levels were

consistently detected at levels higher than those of the

controls (Fig. 6).   In other words, SP68 levels were

detected at lower dilutions of equal micrograms of total

protein in treated fish as opposed to control fish.   The

values used to generate Figure 6 are recorded in Table III.

Relationship of SP68 Levels
Time of exposure and Cu concentration
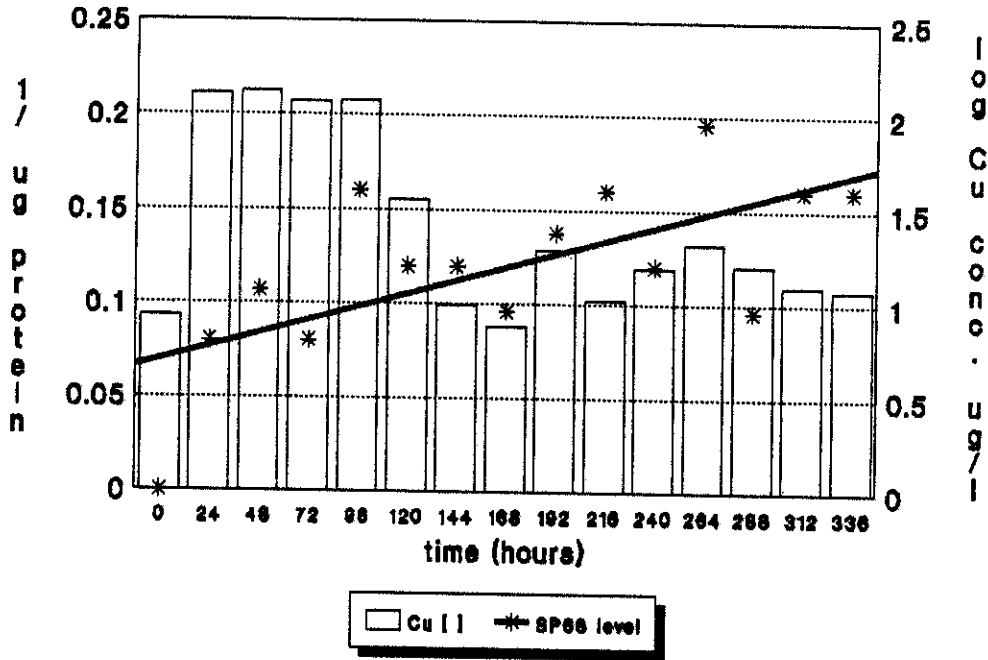
Figure 7. A diagram of mean copper concentrations (ug/l) in treatment tanks overlaid with the reciprocal plot of the mean MSPL for SP68. Note, this is not a regression, only a trend of the observed levels.

Figure 7 shows the trends observed for SP68 levels and total copper concentration in the water at the times specified. Relative assessment of Figure 7 shows that as copper concentration declined after 96 hours of dosing, the MSPL observed for SP68 continually increased. Furthermore, this overlay shows a mirrored effect of copper concentration and SP68 levels. Increased copper levels after 168 hours

Relationship of SP68 Levels
Time of exposure and total mortality
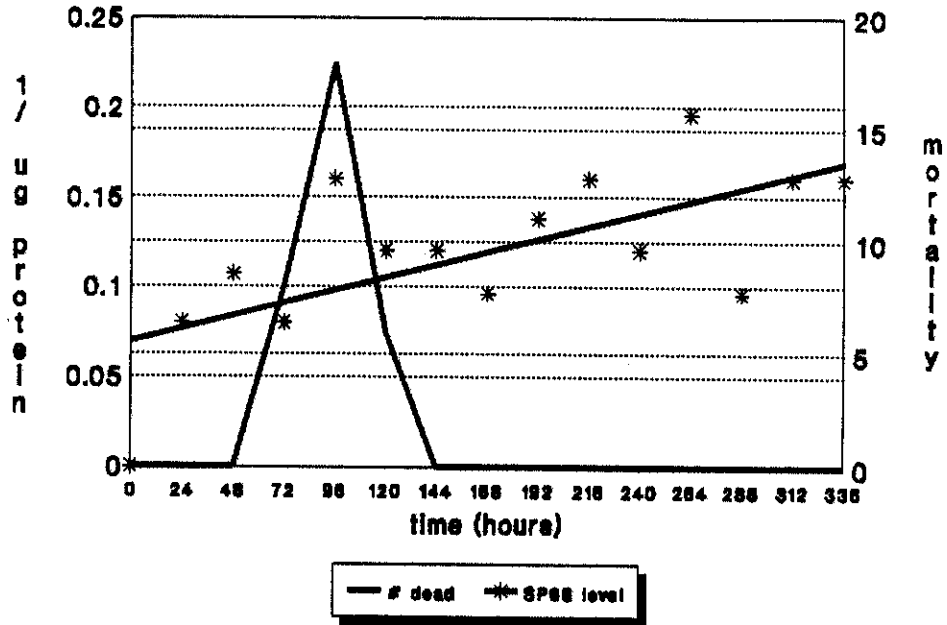
Figure 8. A diagram of the mean MSPL for SP68 plotted as the reciprocal value as in Figure 7. Overlay shows raw mortality counts obtained from all treatment tanks at each sampling time.

may be due to the excretion of copper by the fish.

Assessment of the same SP68 trend and mortality in Figure 8

shows that mortality peaked at the final dosing period of

the test, 96 hours. This observation corresponds to SP68

levels for that time period in which 96 hour MSPL were

significantly higher than levels at 24 and 72 hours. In

terms of percent mortality, at 72 hours (n=432 fish)

mortality equaled 1.8 %, while at 96 hours (n=396 fish)

mortality equaled 4.5 %. Thus, at relatively low percent

mortalities, MSPL of SP68 were able to detect signs of

stress in fish acutely exposed to copper during, and post

exposure over non exposed zero hour fish. In addition, the

stress protein response was rapidly induced and the levels

of the 68-kDa protein continued to increase with time, even

after 240 hours of recovery to an acute exposure.

Table IV.   Means, probabilities, and minimum significant
differences of the ranks of log10 transformed MSPL for SP70 as
determined by nonparametric ANOVA (alpha = 0.05).

Time	Mean control	Mean treated	Probability	MSD
24	23.50	35.00	.5185	45.18
48	20.17	45.00	.4234	77.45
72	45.33	35.00	.6650	61.50
96	45.00	35.00	.7297	74.94
120	35.00	46.50	.3739	34.94
144	46.50	46.50	0	0
168	56.83	67.17	.5185	40.59
192	46.50	56.83	.3799	28.70
216	35.00	46.50	.3739	31.92
240	56.83	46.50	.3739	28.70
264	23.50	35.00	.5185	45.18
288	56.83	23.50	.0973	42.94
312	45.33	23.50	.3799	61.50
336	67.17	46.50	.1161	28.70

Assessment of the minimum significant differences at

each time indicates that actual difference necessary to

achieve a significant difference between control and treated

Table V. Means, probabilities, and minimum significant differences of the ranks of log10 transformed MSPL for SP68 as determined by nonparametric ANOVA (alpha = 0.05). Values marked with an asterisk are significantly different.

Time	Mean control	Mean treated	Probability	MSD
24	46.50	50.00	.8358	43.95
48	61.00	33.75	.2994	84.43
72	61.00	50.00	.2722	26.09
96	17.50	17.50	0	0
120	46.50	28.33	.3672	53.28
144	64.67	28.33	.05	36.32 *
168	61.00	39.17	.2708	51.53
192	50.00	23.00	.2390	58.62
216	50.00	17.50	.0001	0 **
240	50.00	28.33	.2191	44.51
264	39.17	12.17	.0890	33.52
288	46.50	39.17	.7217	53.25
312	57.33	17.50	.0056	20.37 *
336	50.00	17.50	.0001	0 **

levels for SP70 would be on the order of one to two dilution series. This is evident in Table IV, which shows the transformed values obtained from the ANOVA. Significant differences detected for SP68 between control and treated fish show that 144 hours is an anomaly, and control levels dictated that a difference would occur (Table V). The Kruskal-Wallis test reinforces this, as it detected 216, 312, and 336 hours to be significantly different when controls were compared to treated levels. Figure 9 illustrates the minimum significant differences of control and treated values for SP70 and SP68. The minimum

Minimum significant differences
stress protein 70 and 68
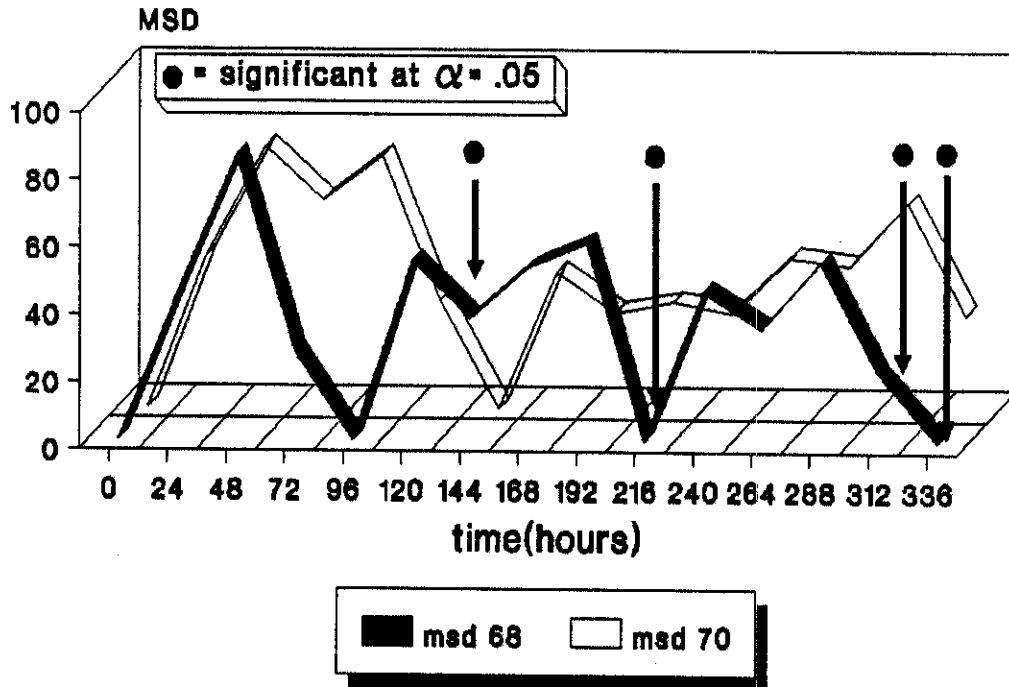
Figure 9. Minimum significant differences obtained from the ANOVA to better illustrate the magnitude of change necessary to achieve statistical significance. Values are graphed as the ranks of log10 transformed MSPL for both proteins.

significant differences for SP68 levels between control and treated fish reveal that differences which were detected were dependent on the increased levels of SP68 through time and the changing control levels of the 68-kDa protein. Although the control levels for SP68 were not significantly different through time, the variability of the response directly influenced the determination of treatment effects.

While more statistical differences were not detected for the SP68 the ecological significance of the observed trend may be important in terms of fish health and water quality. The final objective of the experiment was to determine if there was a relationship between accumulation of copper and the stress protein response. Spearman rank correlation of MSPL for SP68 showed no significant correlation of measured body burden in whole fish to the response of the 68-kDa protein ($p \gg .05$). Regression analysis of SP68 levels vs. time, and SP68 levels vs. copper concentration yielded significant effects, however, only 10 % of the variation in SP68 levels was accounted for by the model, hence a low coefficient of determination. Data from immunoblots illustrate a threshold, on/off effect, which are not conducive to regression analysis (Dyer,1991).

DISCUSSION

Stress in fish has long been a topic of great interest and research. Behavioral, physiological and biochemical studies have searched for a definitive indicator to target stress responses to anthropogenic perturbations. The stress protein response is the latest of a series examined in an attempt to link cellular stress to physiologic, organismal, and population responses in environmental studies. However, the stress protein response is still most widely

studied *in vitro*, and there is little information in the technical literature on whole organism responses. Furthermore, most of the available literature examines the rate of synthesis and transcription/translation patterns of the response. These techniques (i.e. radiolabel incorporation), however, are not appropriate for long term studies. Sanders (1990) found that under continuous exposure to moderate stress conditions, the translational patterns in *Mytilus* were transient and the translational activity reverted to patterns similar to those found in controls.

My study focused on using the accumulation of stress proteins as part of the total protein pool to reflect the degree of stress. Quantitative immunoblots used in the study, therefore, expressed the titers of stress proteins, not the synthesis rates. The immunological methodology was found to be several fold more sensitive than radiolabel incorporation used to look at transcription/translation patterns (Dyer,1991).

The objectives of this research were to determine the duration of the stress protein response to copper exposure and attempt to make relationships to metal accumulation. Gill tissue of fathead minnows exhibited a stress response to acute exposure of copper sulphate. The response was observed up to 240 hours post treatment. Stress protein 70 levels used as an indicator of stress showed no significant

differences between gill tissue of control and treated fish. This corresponds to Dyer's (1991) findings which showed that sodium chromate concentrations had no significant effect on the levels of SP70 in both gill and muscle tissue of control vs. treated fathead minnows using similar techniques. However, MSPL's for SP70 in treated fish were significantly different for non treated zero hour levels and post treatment levels after exposure to copper. The MSPL for SP70 peaked at 288 and 312 hours. In effect, this contradicts previous statements about the low variability of the stress protein response. It was obvious that MSPL's for SP70 in both treated and control fish were greater than zero hour levels, however the variability of the response in control fish made it difficult to detect statistical differences in the levels. In some cases, the MSPL for SP70 in control fish was measured at levels greater than those for treated fish. Misra et.al. (1989) reported that in salmon fish cell lines, synthesis of the 70-kDa protein was relatively high in the absence of stress. Thus, cells apparently titrate the amount of the 72/73-kDa protein produced as a function of both severity of stress treatment and the preexisting levels of the two proteins in the cell prior to the stress event (Mizzen and Welch,1988). As a result of this regulation, it is quite likely that under chronic stress conditions, elevated stress proteins persist in time by balancing the rate of synthesis against the rate

of degradation (Sanders,1990). In addition, Lindquist (1986) found that in a number of different organism: 1) continuous exposure to a contaminant which resulted in moderate stress elicited a transient response; 2) continuous exposure to a contaminant which resulted in a more severe stress resulted in a sustained response. Other studies have reported that synthesis levels of the 72/73-kDa protein returned to control levels within hours of stressor exposure (Mizzen and Welch,1988; Sanders,1990; Caltabiano et.al.,1986; and Misra,1988), however, synthesis rates of the stress proteins were not examined in this study.

By contrast, the inducible 68-kDa stress protein in gill tissue, showed a stress response at time periods during treatment and recovery. Nonparametric ANOVA of MSPL for SP68 showed that levels in treated fish were significantly different over time (p=.0430). Dunn's MRT revealed that SP68 levels in treated fish at times 24 and 72 hours were significantly lower than at times 96, 216, 264, 312, 336 hours. The levels of the 68-kDa protein detected at 96 hours corresponded well to the culmination of the dosing period, and the period of highest mortality. However, the response of the 68-kDa protein peaked at 168 hours post treatment at a mean concentration of 5.10 ug of total protein. Relatively, this is not much lower than 6.12 ug of total protein observed at 96 hours. Comparison of MSPL for SP68 in treated and control fish showed the levels were

significantly different at times 144, 216, 312, and 336 hours (Nonparametric ANOVA, p = .05, .0001, .0056, and .0001 respectively). Once the response was initiated, it did not return to zero hour levels in control or treated fish. In treated fish the response of the 68-kDa protein showed a relative increase with time once the initial stress was induced. Dyer (1991) reported that the 68-kDa protein accumulated and was significantly correlated to Cr concentration and percent mortality, furthermore, the accumulation of the 68 kDa protein was five fold more sensitive than 96-hour mortality.

The whole organism response to a stressor measured by quantitative immunoblots is sensitive, yet variable, which is reflected by treatment and control levels of the 70 and 68-kDa protein. The duration of the response could not be determined since MSPL for SP68 and SP70 never returned to zero hour levels for control or treated fish. In effect, recovery was not observed during the course of this experiment. Mizzen and Welch (1988) found that of the stress proteins induced by a variety of stressors, the 72/73-kDa synthesis was the most responsive to stress severity and also the first to be repressed during recovery.

Factors which could have caused elevated levels of SP68 and SP70 in control fish include: frequent handling, and introduction of water with a low dissolved oxygen concentration, both of which occurred during renewal. The

dissolved oxygen was measured in the tanks, ranging from 6.0
- 6.6 mg/l O_2 , however renewal water had a mean D.O. of 3.6
mg/l O_2. Test aquaria were aerated, but the short exposure
period to low dissolve oxygen concentrations in the renewal
water could have caused elevated stress protein levels in
controls. The renewal process also could have influenced
the stress protein levels observed in controls. Most
likely, a combination of these events was responsible for
the elevated levels of SP68 and SP70 observed in control
fish. Acute forms of handling stress, such as those
associated with the routine hatchery procedures of grading,
transportation and artificial stripping, almost invariably
result in marked physiological stress responses with an
elevation of plasma catecholamines and corticosteroids
(Pickering,1981). Furthermore, it has been found that the
70-kDa protein is associated with transformed and
untransformed steroid receptors in human cells (Sanchez,
et.al.,1990). It is possible that stress protein levels can
be either directly or indirectly influenced by increased
hormones, or the association of stress proteins and steroid
receptors. However, literature to support this claim was
not found. Long holding periods of *Mytilus*, while not
subjected to a stressor, apparently showed elevated stress
protein levels (Sanders, personal communication). This may
suggest that exposure regime, collection techniques and
routine handling of organisms may greatly increase the

background levels of the stress protein response.

CONCLUSIONS

The objectives of this research were: 1) determine the duration of the stress protein response, *in vivo*, upon exposure to an acute sublethal dose of copper; 2) determine the degree of metal accumulation in gill and whole body tissues of the fathead minnow; 3) determine if a relationship exists between stress protein levels and metal accumulation in the gill tissues and whole body of the fish.

In summary, the stress protein response of fathead minnows exposed to copper sulphate was detected in excess of 10 days post exposure. Quantitative immunoblots provided a reliable and sensitive method for assessing stress protein levels. The exposure regime of this study made it possible to examine acute and chronic effects of the exposure regime, and copper stress on the stress protein response. The highly inducible 68-kDa protein was more indicative of stress than the 70-kDa protein. Recovery in copper exposed fish was not observed via the stress protein response. Furthermore, the response of the 68- and 70-kDa protein in copper exposed fish provided insufficient results to determine treatment effects. However, both proteins reflected that the fish were undergoing stress. A relationship of stress protein levels to body burden was not

established, furthermore, metal accumulation was not observed in whole fish. Metal toxicity may have been reflected better by other stress proteins, which would have defined a stressor specific response, but the availability of antibodies was the limiting factor.

Future research using the stress protein response in environmental studies should examine the use of quantitative immunoblots and stressor specific proteins to pinpoint toxicant exposure. *In vivo* studies would be more beneficial as a diagnostic tool to determine stress from external factors and physiological interactions. Exposure regimes should also be examined to determine if stress is induced via the methodology chosen. This information coupled with standard aquatic toxicological methods may prove to be effective as an early warning to adverse conditions affecting fish health.

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