

The Effects of Copper on Blood and Biochemical Parameters of Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract. Metals are released into aquatic systems from many sources, often at sublethal concentrations. The effects of sublethal concentrations of metals on fish are not entirely understood. The objective of this study was to determine the hematological and biochemical effects of a range of copper concentrations (6.4, 16.0, 26.9 $\mu\text{g Cu/L}$) on rainbow trout (*Oncorhynchus mykiss*) over a prolonged period of time. Trout were exposed to copper, and, at intervals of 3, 7, 14, and 21 days, selected parameters were evaluated. Hemoglobin, hematocrit, plasma glucose, and plasma cortisol levels were elevated in trout exposed to 26.9 $\mu\text{g Cu/L}$ at day 3 and then returned to levels comparable to control fish. Plasma protein and lactate levels were not significantly altered in trout from any copper treatment. Hepatic copper concentration and hepatic metallothionein mRNA expression were consistently elevated in trout exposed to 26.9 $\mu\text{g Cu/L}$. Both of these parameters stabilized by day 3, with only hepatic copper concentration showing a further increase at day 21. Hepatic copper concentration and hepatic metallothionein mRNA expression appear to be robust indicators of copper exposure. Most blood-based parameters evaluated appear to be associated with a transitory, nonspecific stress response. The return of elevated hematological and biochemical parameters to control levels after 3 days and the stabilization of hepatic metallothionein mRNA expression and copper concentration over a similar time period suggested acclimation to dissolved copper at 26.9 $\mu\text{g/L}$. Further analysis of the data on blood-based parameters indicated that certain parameters (hemoglobin, hematocrit, plasma glucose, plasma cortisol) may be useful in field monitoring.

to regulatory measures, wastewater releases containing acutely toxic concentrations of metals have decreased (US EPA 1986). However, sublethal concentrations may still affect fish (Baatrup 1991; Hogstrand *et al.* 1996). For example, biochemical and hematological responses are known to be altered by metal exposure. Previous studies have shown that certain metals can either increase or decrease hemoglobin, hematocrit, plasma protein, plasma osmolality, cortisol, glucose, and blood enzymes depending on the metal type, species of fish, water quality, and length of exposure (McKim *et al.* 1970; Christensen *et al.* 1972; O'Neill 1981; Cyriac *et al.* 1989; Munoz *et al.* 1991). Many of these parameters respond rapidly following exposure to sublethal concentrations of metals as part of a nonspecific stress response. The response is transient if the animal can compensate for the stressor or if the stressor is removed (Thomas 1990).

This study focused on copper, a metal frequently found in freshwater systems that receive mining effluent (Roch and McCarter 1984b; Finlayson and Wilson 1989). Sublethal responses to copper were examined in rainbow trout (*Oncorhynchus mykiss*), a native species found in Northern California waterways polluted with copper (Wilson *et al.* 1981; Bastin 1992). In this study, the rainbow trout were exposed to copper concentrations that were environmentally relevant; copper concentrations within the range used in this study can be regularly measured in some Northern California waterways (Finlayson and Wilson 1989; Bastin 1992). Data were gathered on parameters associated with a nonspecific stress response (hemoglobin, hematocrit, plasma glucose, lactate, protein, and cortisol) to assess the sensitivity of these parameters to a range of copper concentrations. In addition, two biochemical indicators known to be affected by copper exposure, hepatic metallothionein and hepatic copper concentration (Hamer 1986; Laurén and McDonald 1987; McCarter and Roch 1983), were measured to evaluate any correlation between the two biochemical indicators and any correlation between hepatic copper concentration and parameters associated with the nonspecific stress response. Correlations between hepatic copper concentration and parameters associated with the nonspecific stress response were of interest because they would link an indicator of metal exposure to indicators of metal effects. Hepatic metallothionein measurements (as mRNA expression) were also made to determine if the dissolved copper concentrations used were capable of inducing increased production of metal-

Metals occur in effluents derived from urban, industrial, and mining sources (Mance 1987). As a result, numerous aquatic systems may receive elevated concentrations of these trace elements, which, in turn, may impact indigenous fish species (Nriagu 1988; Smith *et al.* 1990; McCormick *et al.* 1994). Due

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lothionein mRNA. The exposed fish were subsampled over a 3-week period to evaluate the response curves associated with the nonspecific stress parameters and the hepatic parameters. Selected assays were evaluated for potential use in a field monitoring program; *e.g.*, were they sensitive measures of effects, were the responses robust or transient indicators of exposure, and were the responses uniform or variable.

Materials and Methods

Experimental Setup and Acclimation

Yearling Shasta-strain rainbow trout (*O. mykiss*; average weight 148 g) were obtained from the California Department of Fish and Game, American River Trout Hatchery (Rancho Cordova, CA). Fish were transported in an aerated, fiberglass transport tank in which they were treated with 75 mg/L formalin for 1 to 2 h in an effort to eliminate external parasites. Diagnostic checks of randomly chosen trout 4 days after treatment found parasite loads to be either nonexistent or extremely light (one trematode/mount).

Trout were randomly sorted into 24 cylindrical, high-density polyethylene tanks (54.5 L volume) at the UC Davis Institute of Ecology; six trout were placed in each tank. The tanks were provided with chilled groundwater passed through deionizing columns and remixed with chilled groundwater to obtain a nominal hardness of 50 mg/L as CaCO₃. This hardness value was chosen because it is similar to the hardness measured in Northern California waterways that receive copper-laden effluent and are populated by rainbow trout (Wilson *et al.* 1981; Bastin 1992). The flow rate to each tank was 0.2 L/min. Flows were measured twice daily. Fish were acclimated in the tanks for 7 days before initiation of the exposure study. During acclimation and exposure, all fish were fed at 1% body weight once per day with Silver Cup 3/32" trout feed (Nelson and Sons, Murray, UT). Tanks were checked twice daily for mortalities; fish that had lost equilibrium were considered moribund and removed. During acclimation, total dissolved solids (TDS) and temperature were measured daily for all 7 days, pH and dissolved oxygen (DO) were measured daily over the final 4 days, and ammonia levels were measured twice. After the acclimation period, each tank was impartially thinned to five trout.

Copper Exposure

Nominal copper concentrations were 0, 5, 15, and 30 µg/L. Each of the four treatments was composed of six replicate tanks. The three copper stock solutions (5, 15, and 30 µg Cu/L) were prepared from copper sulfate (99.9% purity; Aldrich Chemical, Milwaukee, WI), acidified with trace metal grade nitric acid to pH 2.6, and aerated continuously. The stock solutions were delivered to the treatment tanks by peristaltic pumps; stock solution flow rates were measured daily. Feeding, mortality checks, and measurements of flows to the tanks continued as during acclimation. The exposure duration was 21 days. The study was conducted during May and June 1996, and a natural photoperiod was maintained throughout the study.

Temperature, DO, and TDS were monitored daily in all tanks; pH was monitored on alternating days in either all tanks or six randomly chosen tanks. Measurements were taken with the Checkmate 90 modular testing system (Corning, Corning, NY). Hardness was measured four times per week in a subset of tanks by titrimetric methods; alkalinity was measured by titrimetric methods on three occasions: before initiation of the copper exposure, on day 12 and on day 20 (APHA 1989). Ammonia was measured two to three times per week in six random tanks using the Nessler method (Kit# FF-2; Hach, Loveland, CO). The measured values for water quality parameters during the exposure period were similar to those measured during

acclimation (presented as mean ± SD: alkalinity, 45 ± 2 mg/L as CaCO₃; ammonia, 0.003 ± 0.001 mg/L as toxic NH₃; dissolved oxygen, 8.9 ± 0.7 mg/L; hardness, 44 ± 5 mg/L as CaCO₃; pH, 7.42 ± 0.07; temperature, 11.7 ± 0.4°C; total dissolved solids, 52.5 ± 4.6 mg/L).

On days 1, 4, 11, and 18, water samples from each tank were taken in 1-L high-density polyethylene bottles (Superfund-analyzed; IChem, Newcastle, DE) for dissolved copper analysis. A portion of each water sample (100 ml) was filtered through a 0.45-µm nylon filter (Micron Separations, Westboro, MA), then acidified with 0.25 ml 7 N trace metal grade nitric acid in a 125-ml high-density polyethylene bottle (Superfund-analyzed; IChem). Samples were analyzed in a Perkin-Elmer Model 5100 Zeeman-corrected atomic absorption spectrophotometer.

Sampling

Trout were fasted for a 24-h period before sampling. One fish was removed from each tank on days 3, 7, 14, and 21, anesthetized in a solution of buffered MS222 (150 mg/L; Sigma Chemical, St. Louis, MO), weighed, and bled from the caudal vessel using 3-ml heparinized vacuum containers cooled on ice before use (McKim *et al.* 1970). Blood samples were placed on ice. Trout were terminally anesthetized. The liver was removed, frozen in liquid nitrogen, and placed on dry ice.

Blood Processing

Whole blood was removed from each vacuum container for the following: (1) hemoglobin was measured in duplicate using the cyanmethemoglobin method (Sigma, Kit #525-A); (2) one microcapillary tube was filled and centrifuged for hematocrit determination. These procedures were performed within 2 h of blood collection. Hemoglobin tubes were kept on ice and read within 3 h of addition of whole blood.

Blood was centrifuged at 500 *g* for 10 min at 15°C. Plasma was subsequently aliquoted into three tubes for determination of plasma glucose, lactate, cortisol, and protein levels. Tubes were stored at -20°C until analyzed. Two days after sampling, glucose and lactate levels were measured simultaneously using a YSI 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Plasma protein was quantified in duplicate by the Lowry (Folin-Ciocalteu) protein determination method (Lowry *et al.* 1951) 3 days after sampling. Cortisol was analyzed within 6 weeks by enzyme immunoassay (Munro and Stabenfeldt 1985).

Liver Copper Analysis

Livers were stored at -80°C prior to copper and metallothionein mRNA analyses. For copper analyses, a subsample of liver ranging between 0.25 and 1.0 g was mixed and digested in 10 ml of 70% nitric acid in a microwave digester (CEM Corporation, Mathews, NC). Digested samples were assayed for copper content using flame atomic absorption spectrophotometry on a Varian SpectrAA-20. Standard curves were established using Spex WP-9 Quality Control Standard (Spex Industries, Edison, NJ). National Institute of Standards and Technology freeze-dried oyster tissue (NIST 1566A) and spiked laboratory control material were used to verify methods and estimate recovery efficiency; recovery efficiencies were 104 and 108%, respectively.

Liver Metallothionein mRNA Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Isolation of RNA from Livers: Total RNA was extracted from approximately 75 mg of frozen fish liver using TRI reagent (Molecular

Research Center, Cincinnati, OH). The final RNA pellet was dissolved in 40 µl of diethyl pyrocarbonate (DEPC)-treated water and the concentration was measured using a Hitachi U-2000 spectrophotometer. Agarose gel electrophoresis was used to check the integrity of the RNA and the density of the band corresponding to the 18S subunit was measured by image analysis (NIH Image 1.5.2).

Synthesis of cDNA and PCR: RT-PCR was carried out using the 1st Strand cDNA Synthesis Kit and PCR Master kit (Boehringer Mannheim, Indianapolis, IN) as previously described (Schlenk *et al.* 1997). The Universal RACE-T primer (5'-CCGAA TTCTC GAGAT CGATT TTTT TTTT TT-3') for the RT reaction as well as the 5'-(5'-ATGGA TCCNT GCGAA TG-3') and 3'-adaptor primer (5'-CCGAA TTCTC GAGAT CGA-3') were designed from previous studies (Chan 1994) and obtained from National Biosciences (Plymouth, MN). Primers were homologous with nucleotide sequences encoding metallothionein from rainbow trout and the observed product was the appropriate size based on comparison to published cDNAs of rainbow trout (Zafarullah *et al.* 1988). For the RT reaction, 1.0 µg of RNA was used in a total reaction volume of 50 µl containing 1 × reaction buffer, 5 mM MgCl₂, 1 mM dNTP mix, primer 1 µM, 10 units of RNase inhibitor, 0.8 µl of AMV reverse transcriptase, and 10 ng of RACE-T primer. The reaction mixture was incubated at 42°C for 1 h and then diluted with 150 µl water to a total volume of 200 µl. PCR was carried out on a 2-µl aliquot of the single-stranded cDNA solution resulting from RT. The PCR master mix contained 25 units of *Taq* DNA polymerase in 20 mM Tris-HCl, 100 mM KCl, 3 mM MgCl₂, 0.01% Brij 35 (v/v), dNTP mix (dATP, dCTP, dGTP, dTTP) each 0.4 mM, pH 8.3 (20°C) in a final volume of 0.1 ml. A set of specific primers for metallothionein expression was used as mentioned above. The PCR was performed using 25 cycles comprised of three segments of 94°C for 1 min; 50°C for 2 min; and 72°C for 3 min and then a final extension at 70°C for 5 min. Amplification of a 800-bp fragment of β-actin was used to normalize quantities of the amplified transcript. The primers for actin were 5'-ACTCACATCTGCTGGAAGGT-3' and 5'-TCACCAACTGGGATGACATG-3'. Studies carried out with coinubation of β-actin primers and metallothionein primers led to reduced metallothionein signals (Schlenk *et al.* 1997). Consequently, separate amplification reactions were performed with equal volumes of single-stranded cDNA from the RT reaction. Each PCR product was resolved on 1.0% agarose gels, photographed, and analyzed by image analysis as above.

Statistical Analyses

Data were transformed when necessary to correct for nonhomogeneity of variance detected by Bartlett's test. Log-transformations were performed on the plasma cortisol, lactate, and glucose data and on the data for hepatic metallothionein mRNA and copper concentrations (Neter *et al.* 1990). Since replicates (tanks) were resampled, repeated measures analyses were run on each parameter using a contrast and sum response design (Neter *et al.* 1990; Paine 1996). Because mortality depleted a tank before the final sampling date or there was insufficient tissue sampled on a specific date, data from certain tanks were not available at each sampling time. Predicted values were calculated for those replicates using SAS 6.09 for open VMS (SAS Institute, Cary, NC). Consequently, repeated measures analyses were run with six replicates at 0 and 5 µg Cu/L, five replicates at 15 µg Cu/L, and four replicates at 30 µg Cu/L, unless otherwise noted. Means calculated with predicted values were within 7% of means calculated with raw data, with the exception of cortisol, for which the means were within 13%. All bar graphs, however, are presented as means, standard errors, and replicate numbers of raw data.

The repeated measures analysis produced two terms of interest: (1) a concentration term, which indicated whether, over the entire study period, a parameter was significantly altered among the treatments; and (2) an interaction term, which indicated whether a significant but

transient alteration occurred in a given parameter among the treatments. If the repeated measures analysis resulted in a significant concentration term ($p \leq 0.05$), follow-up one-way ANOVAs in conjunction with Dunnett's test were used to distinguish fish responses in the copper concentrations that differed from fish responses in the control over the course of the exposure period (Dunnett 1955; Neter *et al.* 1990). Significant interaction terms (concentration × time) were analyzed by one-way ANOVAs and Dunnett's test performed on contrasts among times (Paine 1996). Results of these tests identified transient responses elicited in copper treatments that differed from the control response. Differences were considered significant at $p \leq 0.05$.

Survival data from the highest concentration were compared to the control using a Chi-square test for independence (Steel and Torrie 1960). This procedure utilized a conservative approach in that all fish removed from the population during the different sampling periods were assumed to have survived until the end of the exposure period.

Minimum suggested sample sizes necessary to detect significant differences in parameters were calculated from data collected on hemoglobin, hematocrit, plasma glucose, and plasma cortisol at 3 days of exposure using the least significant number calculation. Predicted values were not used in this analysis. The value for the standard deviation used to calculate the minimum sample size was the average of the highest and lowest standard deviations of the four treatments at 3 days of exposure. All statistical operations were run using JMP 3.1.5 for Macintosh (SAS Institute), except as noted above.

Results

Copper Concentrations

Measured concentrations of copper closely approximated nominal values (Table 1). The coefficients of variation for each of the concentrations were between 9 and 22%, indicating that the concentrations were relatively consistent throughout the exposure period.

Weight and Survival

Average wet weights of the fish sampled during the study ranged between 129 and 200 g, irrespective of exposure duration or concentration, suggesting that none of the copper concentrations affected this parameter. Conversely, survival appeared adversely affected in the two highest concentrations. The majority of the fish that died in the 16.0 and 26.9 µg Cu/L groups did so within the first 48 h of exposure (four fish and 10 fish, respectively). A total of 11 fish died in the 26.9 µg Cu/L concentration (37% mortality), compared with a total of four fish in the control (13% mortality); these values were significantly different. A total of two fish died at 6.4 µg Cu/L (6.5% mortality) and seven fish at 16.0 µg Cu/L (23% mortality). The cumulative mortality for each treatment is presented in Figure 1.

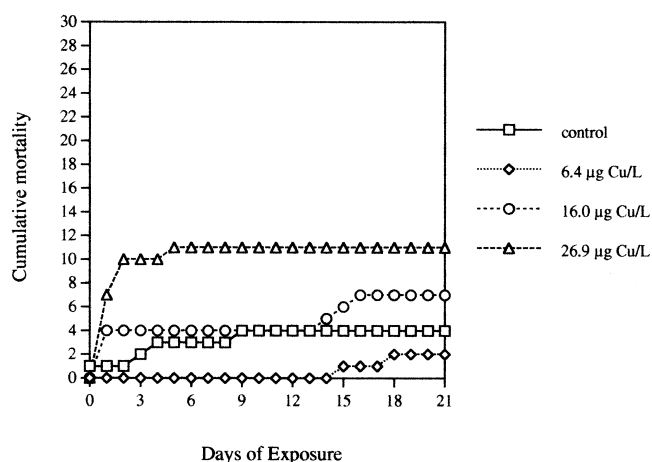
Hepatic Copper Concentration

The relationship between hepatic copper concentrations and dissolved copper concentrations over the course of the study is shown in Figure 2a. A significant concentration effect was found; fish exposed to 26.9 µg Cu/L had significantly greater liver copper levels than control fish over the course of the

Table 1. Dissolved copper concentrations (in $\mu\text{g Cu/L}$)^a

Nominal	5	15	30
Measured	6.4 ± 1.4	16.0 ± 2.0	26.9 ± 2.5
Range	2.3–8.1	13.0–21.0	23.3–31.4
n	24	21	18

^a Measured values are presented as mean \pm standard deviation. For the control tanks, 19 measurements were taken; measured concentrations ranged from $\leq 1.2 \mu\text{g Cu/L}$ (n = 5) to $1.8 \mu\text{g Cu/L}$ (n = 1). Method detection limit = $1.2 \mu\text{g Cu/L}$

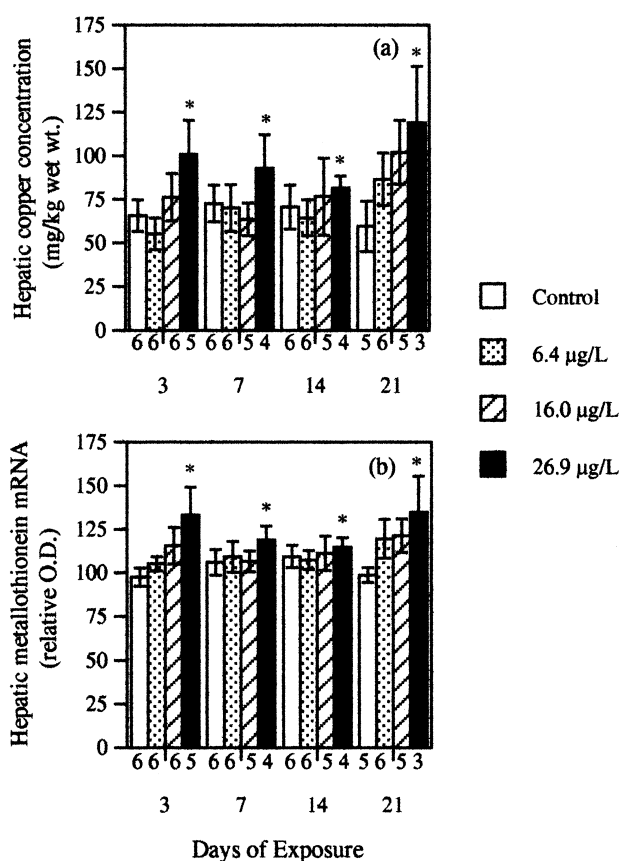
**Fig. 1.** Cumulative mortality in each treatment over the 21-day exposure period

exposure. Hepatic copper concentrations for fish at 6.4 and 16.0 $\mu\text{g Cu/L}$ showed no consistent elevation above control, and a classic dose-response relationship was not apparent until 21 days of exposure. At that time, trout exposed to 6.4, 16.0, and 26.9 $\mu\text{g Cu/L}$ contained hepatic copper concentrations that were elevated to 145%, 171%, and 199%, respectively, of the control concentration (control = 100%). No interaction effect was noted.

Hematological Parameters

Hemoglobin was elevated in trout exposed to 26.9 $\mu\text{g Cu/L}$ at 3 days of exposure with a return to near control levels by 14 days of exposure (Figure 3a). This pattern resulted in a significant interaction term. The elevation in the mean hemoglobin value in fish at 26.9 $\mu\text{g Cu/L}$ was significant in comparison to the control at 3 days. Hemoglobin levels in these fish began to decrease by day 7; the elevation was transient. An apparent dose-response pattern was seen at 3 days of exposure when mean hemoglobin gradually increased with copper concentration. Hemoglobin in trout exposed to 6.4 $\mu\text{g Cu/L}$ was 117% of the control value, hemoglobin in trout exposed to 16.0 $\mu\text{g Cu/L}$ was 120% of the control value, and hemoglobin in trout exposed to 26.9 $\mu\text{g Cu/L}$ was 141% of the control value. The concentration term was insignificant.

Elevated hematocrit levels were seen in fish exposed to 26.9 $\mu\text{g Cu/L}$ only at 3 days (Figure 3b). The interaction term was significant. Hematocrit followed the same pattern as hemoglobin with trout exposed to 26.9 $\mu\text{g Cu/L}$ having a significantly

**Fig. 2.** Effect of dissolved copper on (a) hepatic copper concentrations and (b) hepatic metallothionein mRNA expression of rainbow trout. Values are mean \pm SE. The number below each bar is the number of replicates. An asterisk indicates that a consistent, significant alteration from the control value was detected over all time points

different response trend from control trout due to an initial elevation of hematocrit (142% of the control) which declined by day 7. Hematocrit levels in trout exposed to either 6.4 $\mu\text{g Cu/L}$ or 16.0 $\mu\text{g Cu/L}$ were not significantly different from the control value on any sampling day. The concentration term was not significant.

The concentration term and the interaction term were not significant for plasma protein.

Biochemical Parameters

Plasma glucose levels appeared markedly elevated at 16.0 and 26.9 $\mu\text{g Cu/L}$ at 3 days of exposure, but not at any other time period (Figure 4a). The interaction term was significant while the concentration term was not. Fish exposed to 26.9 $\mu\text{g Cu/L}$ experienced a significant, transient elevation of mean plasma glucose at 3 days compared to the control. This elevation in trout exposed to 26.9 $\mu\text{g Cu/L}$ was 254% of the control value on day 3 and fell to 151% on day 7; by day 21 it approximated control levels. While fish exposed to 16.0 $\mu\text{g Cu/L}$ experienced an initial elevation to 239% of the control glucose level, the difference from the control was not significant.

Fish exposed to 26.9 $\mu\text{g Cu/L}$ responded with a rapid increase in plasma lactate at 3 days (191% of control) followed by a drop

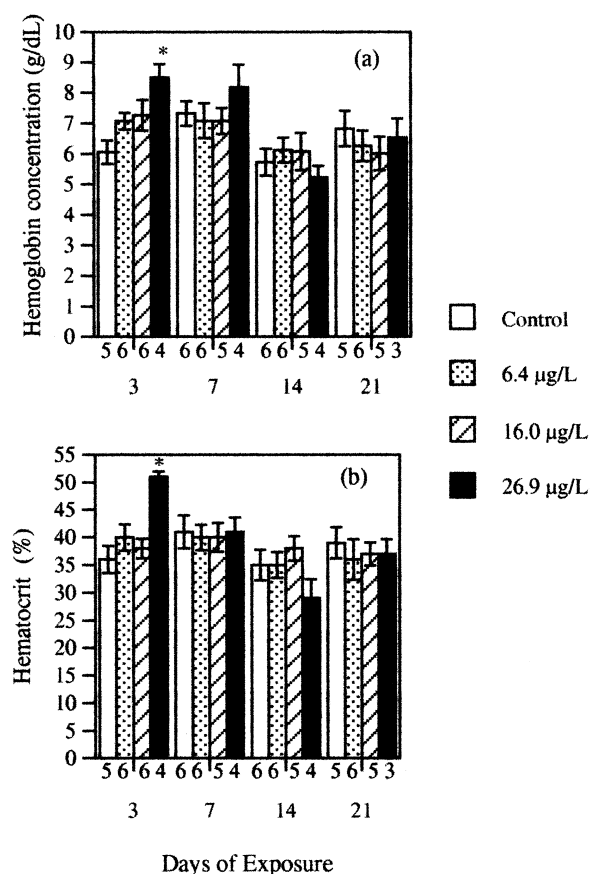


Fig. 3. Effect of dissolved copper on (a) hemoglobin concentration and (b) hematocrit in rainbow trout. Values are mean \pm SE. The number below each bar is the number of replicates. An asterisk denotes a significant, transitory alteration from the control value

to control levels. However, the interaction term did not indicate that the response was significantly different from the control response. The concentration term was significant, but Dunnett's test found no copper treatment to be significantly different from the control.

The repeated measures analysis for plasma cortisol was run with only three replicates at 26.9 µg Cu/L. Means calculated with predicted values of the fourth replicate exceeded means calculated from the raw data by >400%; therefore, this replicate was not used in further analyses. The concentration term for plasma cortisol was not significant (Figure 4b). The interaction term approached significance ($p = 0.088$). The response trend in plasma cortisol of rainbow trout exposed to 26.9 µg Cu/L was not significantly different than the response trend of the control, though the raw data showed a transient elevation in cortisol to over ten times the control level at 3 days. The lack of statistical significance in the interaction term was likely due to the small sample size and large variability associated with this parameter. After this initial elevation, cortisol levels in trout exposed to 26.9 µg Cu/L declined to 711%, 376%, and 171% of control values at day 7, 14, and 21, respectively.

Analysis of hepatic metallothionein mRNA data resulted in a significant concentration term; fish exposed to 26.9 µg Cu/L had significantly greater mRNA expression than control fish over the study period (Figure 2b). Hepatic metallothionein mRNA

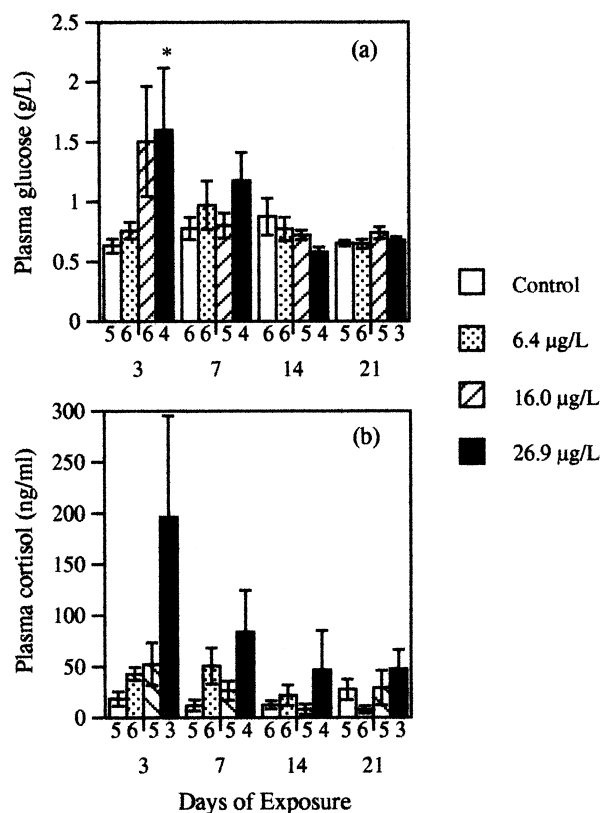


Fig. 4. Effect of dissolved copper on (a) plasma glucose concentrations and (b) plasma cortisol concentrations of rainbow trout. Values are mean \pm SE. The number below each bar is the number of replicates. An asterisk denotes a significant, transitory alteration from the control

was not consistently elevated above control levels for fish at 6.4 and 16.0 µg Cu/L. A classic dose-response relationship was apparent at 3 days of exposure. At 7 and 14 days, all treatments produced similar responses before a dose-response relationship was again noted at 21 days of exposure. The interaction term was not significant. In addition, measured metallothionein mRNA in trout at 26.9 µg Cu/L did not change markedly between 3 and 21 days of exposure, suggesting that this protein was fully induced by 3 days of exposure. To more fully understand the relationship between copper exposure and metallothionein mRNA expression, metallothionein mRNA expression was plotted against hepatic copper concentration using all of the sampling points obtained in the study. There was a strong, positive relationship between hepatic copper concentration and the level of metallothionein mRNA present ($R^2 = 0.83$). Thus, while there may have been appreciable variation in hepatic copper uptake in individual fish experiencing similar dissolved copper concentrations, the metallothionein concentration was related to the actual amount of copper present in the liver.

Relationship of Other Parameters to Hepatic Copper Concentration

The other parameters (plasma cortisol, plasma glucose, plasma lactate, hemoglobin, hematocrit, plasma protein) were similarly evaluated with respect to their relationship with hepatic copper concentration. None of the parameters appeared to be related

to hepatic copper concentration since the R^2 values ranged between 0.000 and 0.025.

Discussion

Survival

Mortality occurred rapidly following the onset of exposure in the 16.0 and 26.9 $\mu\text{g Cu/L}$ concentrations. Mortality was, in general, limited to the first 48 h of exposure, presumably due to acclimation through the process of increasing metallothionein production to bind copper (Dixon and Sprague 1981; Benson and Birge 1985; Laurén and McDonald 1987). A small increase was seen in mortality for fish at 6.4 and 16.0 $\mu\text{g Cu/L}$ between 14 and 21 days (Figure 1). This trend raises a question about whether the continuous exposure to these relatively low levels of copper coupled with the lack of consistent metallothionein elevation and copper sequestration in the livers of fish at these concentrations could result in delayed mortality; a study with a longer exposure period would be needed to address this.

Hematology and Biochemistry

With the exception of hepatic metallothionein mRNA, significant responses in the hematological and biochemical parameters generally followed the pattern displayed by the mortality data—an initial elevation followed by a return to baseline values in the higher dissolved copper concentrations. This pattern is suggestive of acclimation to the toxicant. While it is difficult to directly compare our observed responses to those of other studies due to differences in factors that influence toxicity (*e.g.*, species, life history stage, water quality, exposure duration, copper concentration), there are similarities between our findings and those from other studies investigating copper toxicity. Continuous exposure to sublethal copper concentrations resulted in significantly elevated levels of both hemoglobin and hematocrit in Mozambique tilapia (*Oreochromis mossambicus*) at 72, 120, and 168 h (Cyriac *et al.* 1989) and in brown bullheads (*Ictalurus nebulosus*) at 6 and 30 days (Christensen *et al.* 1972). When exposed to copper for 6, 21, and 337 days, yearling brook trout (*Salvelinus fontinalis*) experienced an increase in hematocrit at day 6 only and an increase in hemoglobin at days 6 and 21 (McKim *et al.* 1970). The results of our study are consistent with these findings, particularly McKim *et al.* (1970), where transient responses were noted.

Protein (plasma, serum, or whole body) measurements from previous studies exhibited no consistent pattern of response; protein levels increased, decreased, or were unaffected by copper exposures of 48 h to 15 weeks' duration in brown trout (*Salmo trutta*), brown bullheads, rainbow trout, brook trout, and common carp (*Cyprinus carpio*) (McKim *et al.* 1970; Christensen *et al.* 1972; Lett *et al.* 1976; Beaumont *et al.* 1995). This study recorded no effect of copper on plasma protein. Plasma lactate levels in past studies have not shown significant alterations when measured in brown bullheads and brown trout exposed to copper (Christensen *et al.* 1972; Beaumont *et al.* 1995). Plasma lactate in rainbow trout was not significantly affected by exposure to copper in the current study. In contrast, plasma glucose levels have been found to increase with exposure to copper, often in a dose-response pattern, in brown

bullheads and rainbow trout (Christensen *et al.* 1972; Laurén and McDonald 1985; Nemcsók and Hughes 1988). Of the two studies that examined the glucose response to copper in rainbow trout, neither had an exposure period that exceeded 48 h (Laurén and McDonald 1985; Nemcsók and Hughes 1988), while the study using brown bullheads did not sample until day 6 when an elevated, but insignificant glucose response was recorded (Christensen *et al.* 1972). Therefore, a transient, significant elevation in plasma glucose levels could not be noted. This study, however, observed glucose levels with subsampling over short and long exposure periods, allowing observation of a transient, dose-response pattern.

Plasma cortisol levels measured in yearling sockeye salmon (*Oncorhynchus nerka*) and juvenile rainbow trout after varying durations of exposure to copper were elevated initially and then declined (Donaldson and Dye 1975; Munoz *et al.* 1991). Yearling coho salmon (*Oncorhynchus kisutch*) exposed to sublethal copper concentrations showed a short-term elevation of plasma cortisol in the first 24 h followed either by a return to control levels or an increase in cortisol concentration over the remainder of the 7-day exposure, depending on the copper concentration (Schreck and Lorz 1978). In the current study, rainbow trout experienced a transient elevation in cortisol with exposure to dissolved copper.

In general, the biochemical and hematological parameters measured in this study responded rapidly to the onset of exposure and were reduced thereafter. Such a response is consistent for parameters associated with a nonspecific stress response (Thomas 1990). This response was also similar to the pattern of mortalities that occurred rapidly following the onset of exposure and then remained stable over the remainder of the study. Thus, it would appear that the exposed trout responded rapidly to the increased copper concentrations with the survivors acclimating to the copper over the course of the exposure. Possible mechanisms of acclimation leading to these transient biochemical and hematological responses include metallothionein production, sequestration of copper, and branchial responses. Metallothionein production and sequestration of metal in response to exposure remove free metal ions, allowing affected physiological systems to recover. Metal exposure can also result in gill damage, which can affect blood-based parameters (Wilson and Taylor 1993; Pelgrom *et al.* 1995). However, gill functions have been found to recover after the initial insult if concentrations are sublethal (Laurén and McDonald 1987; review by McDonald and Wood 1993); blood-based parameters may then return to normal. The role of the gills in the acclimation of fish in this study was not documented; the roles of copper sequestration and metallothionein production are discussed below.

Hepatic Copper and Metallothionein

Hepatic copper concentrations in exposed rainbow trout at the highest dissolved copper concentration were consistently elevated, stabilizing between days 3 and 14 and increasing again at day 21. A similar result was found in juvenile rainbow trout exposed to 55 $\mu\text{g/L}$ copper; mean liver copper concentrations were relatively stable between 2 and 21 days with a larger increase between 21 and 28 days (Laurén and McDonald 1987). This result suggests that the increased hepatic copper concentrations seen at day 21 in the current study may have increased further if the exposure had continued. Further support of the

potential for hepatic copper concentrations to increase after 21 days of exposure is provided by a study of rainbow trout fry in which whole-body copper concentrations reached steady-state between 40 and 60 days of exposure to copper (Marr *et al.* 1996). The variability in the hepatic copper concentrations that were measured within each treatment in the current study suggests that the response of individual fish with respect to copper uptake and elimination varied considerably.

Expression of hepatic metallothionein mRNA had been induced and appeared to have stabilized by day 3 in trout exposed to the highest copper concentration. Maximum induction of hepatic metallothionein within a few days of initial exposure has been reported by other investigators studying rainbow trout responses to metals (Bradley *et al.* 1985; Anadu *et al.* 1989), although coho salmon exposed to copper did not achieve maximum metallothionein protein levels until 4 weeks after exposure (McCarter and Roch 1983). Assuming that hepatic metallothionein confers tolerance to copper exposure (McCarter and Roch 1983; Benson and Birge 1985; Bradley *et al.* 1985), hepatic metallothionein mRNA levels in the current study are consistent with the general lack of mortalities and stabilization of the short-term stress indicators at control levels in the 26.9 $\mu\text{g Cu/L}$ treatments after 3 days of exposure. Protection from copper-induced lethality by metallothionein has been hypothesized to explain species differences in toxicity (Marr *et al.* 1995). Along with the liver, other tissues may also be upgrading metallothionein protein transcription and thus contributing to the protection of rainbow trout from copper toxicity (Hogstrand and Haux 1991). Since significant hepatic metallothionein mRNA induction was only observed at 26.9 $\mu\text{g Cu/L}$, it suggests that the minimum dissolved metal concentration capable of increasing expression over a 21-day period in this exposure system was between 16.0 and 26.9 $\mu\text{g/L}$ (Hamer 1986). Increases could also have occurred at lower concentrations, but may have been obscured by variability in the individual response.

The strong correlation between hepatic copper concentration and metallothionein mRNA in rainbow trout supports the hypothesis that increased hepatic copper loads may be due to increased binding capacity of copper by a larger number of metallothionein binding sites following hepatic metallothionein mRNA induction and transcription. A positive relationship between liver metallothionein and liver copper levels has been previously noted for rainbow and brown trout exposed to metal mixtures (Roch and McCarter 1984a; Marr *et al.* 1995). Our correlation coefficient was higher than these previously calculated values, possibly due to the fact that metallothionein mRNA was measured in the current study as opposed to metallothionein protein by polarography (Roch and McCarter 1984a) or radioimmunoassay (Marr *et al.* 1995). Following copper binding, metallothionein protein can easily dimerize under aerobic conditions, leading to underestimated concentrations (Schlenk and Brouwer 1991). The lack of correlation between hepatic copper and the short-term stress responses is reasonable given that hepatic copper was elevated throughout the exposure period whereas parameters associated with the nonspecific stress response were not.

Utility of Parameters in Field Monitoring

The usefulness of different parameters for monitoring the physiological status of an organism depends on a variety of

factors, including how closely the parameter is coupled with the exposure or response of interest, the variability associated with the parameter, the variability in measuring the parameter, and whether the parameter is transient in nature or is a robust and long-lasting indicator of exposure. There appears to be a consensus that it is useful to monitor tissue metal concentrations and hepatic metallothionein as indicators of either acute or chronic metal exposure of wild fish (Roch and McCarter 1984b; Benson and Birge 1985; Olsson *et al.* 1989), but these parameters may not directly indicate if physiological damage has occurred in exposed organisms. Therefore, the utility of using other hematological and biochemical parameters as indicators of damage to wild fish due to chronic copper exposure remains of interest.

The hematological and biochemical parameters measured in this study generally demonstrated short-term responses at levels $\geq 16.0 \mu\text{g Cu/L}$, where some mortality of previously unexposed fish also occurred. Consequently, these parameters may not be useful for indicating damage from sublethal toxicity in field populations, but could have applications for monitoring short-term responses of fish to infrequent, episodic events. To aid in identifying such responses, we calculated minimum suggested sample sizes for measuring hemoglobin, hematocrit, glucose, and cortisol at sites where dissolved copper concentrations have temporarily reached $\geq 16.0 \mu\text{g Cu/L}$ and have exceeded water quality standards. Alterations in this set of parameters would suggest disruptions in a number of processes, including hematopoiesis, blood oxygen-carrying capacity and ion composition, and energy reserves. For an episodic event with copper concentrations similar to those seen in this study's highest concentration, at least eight fish would need to be sampled to determine differences from a reference level in all parameters. For an exposure to approximately 16.0 $\mu\text{g Cu/L}$, at least 13 trout should be compared to a like-numbered reference group. Any researchers using these parameters to assess the effects of episodic copper exposure must employ procedures that sufficiently minimize capture and handling stresses (Thomas 1990).

It should be noted that these sample sizes were derived from data on naive hatchery trout. Whether episodic events actually elicit a hematological or biochemical response in wild trout may depend on the water chemistry of the system they inhabit and any previous exposures that resulted in acclimation to metals (Dixon and Sprague 1981; McCarter and Roch 1983; Bradley *et al.* 1985; Laurén and McDonald 1987; Anadu *et al.* 1989). Acclimated animals would be less likely to respond to copper concentrations similar to those used in this study.

In conclusion, the blood-based biochemical and hematological parameters measured in this study responded to relatively low copper concentrations, but the responses were transient. Therefore, these parameters do not seem useful for monitoring the long-term effects of copper exposure on wild fish. However, they could be useful for monitoring trout in situations where episodic exposures to high, but sublethal copper concentrations occur. Because of their continued elevation during exposure, hepatic metallothionein and copper concentration can serve as robust indicators of exposure, although not necessarily of deleterious physiological effects of copper on trout.

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