

Effects of Dissolved Copper on Select Hematological, Biochemical, and Immunological Parameters of Wild Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract. Rainbow trout (*Oncorhynchus mykiss*) were sampled from a creek in the western Sierra Nevada, Plumas County, CA, that receives run-off from a 40-ha copper (Cu) tailings pile. Reference sites included a site upstream of the Cu input and another site located on a nearby creek. Hepatic Cu concentrations were significantly elevated in trout from sites with elevated dissolved Cu concentrations compared with concentrations in trout from reference sites. Trout at the Cu-contaminated sites also exhibited decreased hematocrit (Hct), leukocrit (Lct), and percentage of lymphocytes in blood compared to trout from reference sites. The percentage of monocytes in blood and respiratory burst activity were affected by gender and age, respectively. Condition factor, percentage of neutrophils in blood, muscle glycogen and protein, and plasma acetylcholinesterase were not affected by dissolved Cu concentration or gender. Age also did not appear to be a factor. The data from this study support the use of immune system parameters to assess alterations in salmonids experiencing prolonged exposure to low-level Cu contamination and illustrate the variability in physiological responses of wild fish caused by demographic features. Overall, of the parameters measured, Hct, Lct, and percentage of lymphocytes in blood appeared to offer robust measures for assessing effects of metals on wild fish and did not appear affected by select demographic features.

Mining, industry, and agricultural activities can cause elevated metal concentrations in waterways that may result in deleterious effects on aquatic biota even at sublethal concentrations (Sorensen 1991). However, observable physiological impacts on biota may be masked by variability in responses caused by natural factors, such as temporal variation, interactions among organisms, demographic characteristics of organisms, and

physical characteristics of the system. Because of the inherent variability of natural systems, determining the appropriate parameters to measure to identify physiological alterations associated with metal pollution is problematic. While parameters such as metallothionein and tissue metal concentrations indicate whether fish have been exposed to metals (Hamer 1986; Laurén and McDonald 1987), other measurements are necessary to determine whether physiological functions have been compromised by the exposure. Parameters selected to identify compromised physiological responses are most useful if they measure alterations that can be linked to survival and reproduction of the individual and can be measured in natural populations with sufficient precision to differentiate between alterations due to metals and those caused by responses to natural variables.

The literature contains numerous examples of adverse effects of metals on biochemical and immunological parameters in fish exposed during laboratory studies. These assays often require sensitive techniques and instrumentation that are not readily available or amenable to conditions in the field. Thus, in evaluating measures of effect, it is logical to identify those laboratory assays that are sufficiently robust to be useful under field conditions. However, application of these measures under actual field conditions has received comparatively little attention. This study evaluated a number of assays in rainbow trout (*Oncorhynchus mykiss*) sampled from a drainage that received elevated copper (Cu) concentrations as a result of mine wastes and compared results to those from reference sites. The primary objectives were to determine if long-term exposure to elevated Cu concentrations altered physiological responses in wild rainbow trout and to validate assays used primarily in the laboratory for use in field applications. Parameters selected for investigation were immune system and hematologic state and function (hematocrit [Hct], leukocrit [Lct], differential leukocyte counts, respiratory burst), energy reserves (muscle protein, muscle lipid, muscle glycogen), and acetylcholinesterase (AChE) activity. Hepatic Cu concentrations were monitored as a measure of exposure. Not only do these parameters indicate changes in physiological systems, and potentially the health of the individual, but past laboratory and field studies have shown that metals can alter immune system functions (Dick and Dixon

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1985; Hutchinson and Manning 1996; Nussey *et al.* 1995), muscle biochemistry (Larsson *et al.* 1985), AChE levels (Verma *et al.* 1981), and Hct (Christensen *et al.* 1972; McKim *et al.* 1970). Many of these parameters were found to be altered during previous laboratory exposures of rainbow trout to Cu in waters that simulated the water quality (dissolved oxygen, pH, hardness, Cu concentration) of the streams sampled in this field study (Dethloff and Bailey 1998; Dethloff *et al.* 1999a, 1999b). Moreover, based on the results of the laboratory studies, the parameters selected were considered to have the greatest likelihood of detecting effects of dissolved Cu on wild trout.

Materials and Methods

Study Site

Little Grizzly Creek is located on the western side of the Sierra Nevada mountains (elevation 1,800 m) in Plumas County, California. The creek flows northwest from Lake Davis past the Walker Mine, a Cu mine in active operation from 1904 to 1941 (Sheehan 1980) and sealed in 1987 (Deanovic *et al.* 1999). One of its tributaries, Dolly Creek, is a small, first-order stream that originally received effluent directly from the Walker Mine. Although the mine seal has substantially reduced the Cu load from the mine, Dolly Creek still flows through a 40-ha tailings pile before converging with Little Grizzly Creek (Deanovic *et al.* 1999). Although the acutely toxic Cu concentrations previously documented in Dolly Creek and along a stretch of Little Grizzly Creek (Sheehan 1980) no longer exist, elevated Cu concentrations still occur in Little Grizzly Creek below its confluence with Dolly Creek and chronic toxicity is still reported (Bastin 1992; Deanovic *et al.* 1999). Concentrations of other metals are not considered to be of concern in this stream (California Water Quality Control Board, unpublished data).

Sites on Little Grizzly Creek that were sampled in this study have been used by previous researchers (Bastin 1992; Deanovic *et al.* 1999; Sheehan 1980), and the site locations have been retained to allow others to use the data for comparative purposes. Reference sites were located at site 1 on Little Grizzly Creek, approximately 4 km upstream of the confluence with Dolly Creek, and in Red Clover Creek, a stream approximately 10 km northeast of Little Grizzly Creek that is not in the Lake Davis watershed. Sites 4 and 6 on Little Grizzly Creek were located downstream of the confluence by 2 and 9 km, respectively.

Water quality data were collected at all sites on July 10, 14, 17, 21, 24, and 28, 1997. Temperature, pH, total dissolved solids, conductivity, and dissolved oxygen were measured in-stream. Water samples from each site were taken on June 21, 1997, and on the dates listed above for analysis of dissolved Cu, hardness, and alkalinity. Bottles were stored on ice for transport and at 4°C until analysis (within 48 h). A portion of each water sample (100 ml) was filtered through a 0.45- μ m nylon filter (Micron Separations, Westboro, MA), then acidified with 0.25 ml 7 N trace-metal-grade nitric acid. Dissolved Cu concentrations were determined with a Varian 400 Zeeman graphite furnace atomic absorption spectrometer. Hardness and alkalinity were measured by titrimetric methods (APHA 1989). Water quality values are presented in Table 1.

Trout Sampling

Rainbow trout were collected from Red Clover Creek on July 15, site 4 on July 22, site 6 on July 25, and site 1 on July 29, 1997. Sampling was conducted in the summer as streams are Wadeable, spawning is completed, and immune responses are expected to be at or near their

highest annual levels. The reference sites were sampled first and last to bracket the sampling period. Trout were collected by electrofishing. A stretch of stream of approximately 500 m was sampled over a 2.5-h period. This period was established due to time constraints imposed by travel and laboratory protocols requiring viable cells. Between 11 and 13 fish were collected at each site. Fish were netted and immediately anesthetized in a solution of buffered MS222 (200 mg/L; Sigma Chemical, St. Louis, MO). They were quickly transported to the processing table where they were measured, weighed, and bled from the caudal vessel using cooled, heparinized vacutainers (McKim *et al.* 1970) that were placed back on ice. Trout were terminally anesthetized. After expiration, livers and dorsal epaxial muscle tissue samples were removed, frozen in liquid nitrogen, and stored on dry ice. Head kidneys were removed and placed in 5-ml tubes with 2 ml of Eagle's minimum essential media (ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 15 mM HEPES (Gibco BRL, Gaithersburg, MD), 2 mM L-glutamine (Gibco BRL), 10 units/ml heparin, and 50 μ g/ml gentamicin sulfate (Sigma). Head kidneys remained on ice until processing. After examination of the gonads, fish were designated as "immature," "mature female," or "mature male." An area above the lateral line was cleaned, and a scraping of scales was taken for annuli counts (Devries and Frie 1996). A Fulton-type condition factor (CF) was calculated for each fish (Anderson and Neumann 1996).

Blood Processing

Hct and Lct values were determined by filling microcapillary tubes with whole blood, centrifuging them, and reading the packed cell percentages. Tubes were then scored and broken above the packed cells. The plasma was removed into a cryovial and stored on dry ice. Whole blood was also used to make blood smears for differential leukocyte counts. All procedures were performed within 3 h of blood collection. Upon return to the laboratory, smears were fixed and stained using a Wright-Giemsa modified stain (Sigma). Differential leukocyte counts were made by identifying 200 leukocytes on each slide (Dick and Dixon 1985). Neutrophils, lymphocytes, and monocytes were differentiated based on previous descriptions (Zinkl *et al.* 1991a). Relative percentages of neutrophils, lymphocytes, and monocytes present in blood were calculated by multiplying the counted value by the Lct value (A. P. Farrell, personal communication). To determine absolute values, these percentages would be multiplied by total leukocytes per volume blood. Since this count could not be done in the field, the percent volume of leukocytes (Lct) was used as a correction factor to determine relative percentages of each leukocyte type in blood.

Samples of whole blood were chilled until they were centrifuged. A portion of the plasma was diluted to 20% in phenol red-free RPMI 1640 (Gibco BRL) supplemented with 50 μ g/ml gentamicin sulfate and heat-inactivated. This plasma was used in the respiratory burst assay.

Respiratory Burst Assay

Head kidneys were brought to the laboratory and processed as previously described (Dethloff and Bailey 1998). Briefly, head kidneys were homogenized through mesh screens and cells were counted using a Bright-Line hemacytometer. A total of 8×10^6 cells from each fish were washed, resuspended in 1.6 ml of phenol red-free RPMI 1640 supplemented with gentamicin sulfate and autologous plasma, and plated out at 200 μ l/well in 96-well microtiter plates (Falcon, Franklin Lakes, NJ). Macrophages were allowed to adhere, and nonadherent cells were washed off. Detection of superoxide anions by ferricytochrome c reduction was based on a standard procedure (Pick 1986).

Table 1. Water quality data from Red Clover Creek and Little Grizzly Creek for June and July 1997

Sampling Site	Dissolved Oxygen (mg/L)	Temperature (°C)	pH	Total Dissolved Solids (mg/L)	Conductivity (µS)	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)	Dissolved [Cu] (µg/L)
RCC	8.4 ± 0.8	18.0 ± 1.0	8.09 ± 0.22	82.1 ± 2.2	164.3 ± 4.9	69 ± 3	82 ± 3	0.56 ± 1.03
Site 1, LGC	8.4 ± 0.7	19.9 ± 2.6	7.27 ± 0.24	52.4 ± 3.4	104.6 ± 6.4	44 ± 7	56 ± 7	1.71 ± 2.10
Site 4, LGC	9.0 ± 1.1	17.5 ± 0.5	7.82 ± 0.15	70.8 ± 2.7	141.0 ± 5.3	55 ± 4	64 ± 6	10.06 ± 2.08
Site 6, LGC	8.8 ± 0.7	17.3 ± 0.6	7.92 ± 0.09	70.0 ± 2.4	139.0 ± 6.7	59 ± 8	66 ± 3	3.82 ± 1.58

Values are presented as mean ± SD. Method detection limit for dissolved Cu = 1.0 µg/L.

Ferricytochrome c (2 mg/ml; Sigma) and phorbol myristate acetate (1 µg/ml; Sigma) in 100 µl phenol red-free RPMI 1640 were added to triplicate wells for each trout to stimulate superoxide anion production. The above solution with the addition of superoxide dismutase (300 units/ml; Sigma) was added to triplicate wells for each trout for blanking. The two remaining wells received crystal violet lysis buffer (Chung and Secombes 1988). Plates were incubated, then read on a multiscan plate reader (BioTek Instruments, Laguna Hills, CA). Nuclei from the crystal violet wells were counted, and the average was calculated. Results are expressed as nmols superoxide anion produced/1 × 10⁵ cells.

Muscle Biochemistry

Muscle samples were stored at -80°C before and between analyses. For the measurement of muscle total protein approximately 30 mg of tissue were removed, ground into fine powder under liquid nitrogen, and homogenized in 1 ml of TES buffer (20 mM TES in 250 mM sucrose). Protein concentration was determined by the Lowry (Folin-Ciocalteu) protein determination method (Lowry *et al.* 1951). Bovine serum albumin was used as the protein standard.

Muscle glycogen was measured using a modification of a previously described method (Hassid and Abraham 1957). Approximately 100 mg of tissue was ground into a fine powder under liquid nitrogen. Samples from fish that did not contain at least 100 mg of tissue were not analyzed. The powder was boiled with periodic vortexing in 1 ml of 30% KOH for 10–15 min. Tubes were cooled to room temperature and 500 µl of saturated sodium sulfate were added followed by 2 ml of 95% ethanol. Tubes were vortexed, returned to the hot water bath until boiling began, cooled to room temperature, and centrifuged. Supernatants were discarded by inversion. Pellets were resuspended in 2 ml H₂O followed by 2 ml of 95% ethanol. Tubes were again heated until the contents began to boil, centrifuged, and inverted. Pellets were resuspended in 2 ml H₂O. From this solution, 500 µl were removed and combined with 750 µl acetate buffer (pH 4.8) and 20 µl amyloglucosidase (Boehringer-Mannheim, Indianapolis, IN) which was incubated for 2 h at 37°C. The reaction breaking glycogen down into free glucose was stopped by adding 25 µl of 70% perchloric acid. Samples were stored at -20°C. Within 3 days, extracts were neutralized with 80 µl of 3M potassium carbonate and centrifuged. Supernatants (500 µl) were removed and assayed for glucose concentration on a YSI 2700 Select Biochemistry Analyzer.

For lipid measurement, samples were placed on aluminum boats in a drying oven for 24 h. Samples and boats were then placed in cheesecloth bags, tagged, weighed, and subsequently extracted with ether using a modified protocol (Garrett and Hinman 1969). After lipid extraction, the bags and their contents were placed in a drying oven for 24–48 h and reweighed.

Plasma AChE

Plasma samples were stored at -80°C until analysis. A standard method (Ellman *et al.* 1961) with modifications (Donovan and Zinkl 1994) was used to determine AChE activity. Activities were determined in 2.9 ml of 0.05 M tris buffer (Sigma), pH 7.4, with 2.5 × 10⁻⁴ M DTNB (Sigma). Plasma (20 µl) was added to the DTNB solution and preincubated at 25°C for 1–2 min. Then 100 µl of 0.156 MATCI (Sigma) were added, and samples were read on the Beckman Instruments DU-68 spectrophotometer at 25°C for 3 min. For calculation of the AChE activities, the molar absorbance coefficient constant of 1.33 × 10⁴ M⁻¹cm was used (Ellman *et al.* 1961). Activity was measured as mU/ml (Zink *et al.* 1987a). Samples that did not contain 20 µl of plasma were not analyzed.

Liver Metal Analysis

Livers were stored at -80°C prior to Cu analysis. For analysis, a subsample of liver ranging between 0.25 and 1.0 g was mixed and subsequently digested in 70% nitric acid. Digested samples were assayed for metal content using flame atomic absorption spectrophotometry on a Varian SpectraAA-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) was used to verify methods and estimate recovery efficiency; recovery efficiencies were 94.7% and 96.8%, respectively.

Statistical Analyses

Percentage data were arcsin transformed. Log transformations were performed on the muscle glycogen and hepatic Cu concentration data to correct for nonhomogeneity of error variances detected by Bartlett's test (Neter *et al.* 1990). Differences among sites were investigated first for weight and CF responses to determine whether dissolved Cu (represented by the "site" term) affected weight and CF and, therefore, might affect other responses indirectly through these two parameters. An analysis of variance (ANOVA) was conducted with site and gender as main effects and included their interaction. If the interaction was found to be insignificant, it was removed from the model (Neter *et al.* 1990). Significant main effects were examined further by contrasts among factor levels (Sokal and Rohlf 1995). Significant interaction effects were analyzed by comparing the factor levels of one term with those of the other term using ANOVA (Neter *et al.* 1990). In the case of a significant main effect of site, the two reference sites were first compared by a t-test (Sokal and Rohlf 1995). If these two sites were not significantly different, they were combined and compared to each of the two sites receiving elevated Cu concentrations. An ANOVA with site, age, and their first-order interaction was performed using a

subset of the data [age groups 1 (0+–1+) and 2 (2+) at Red Clover Creek, site 1, and site 4] due to the incomplete representation of age groups at all sites. Results of this ANOVA are presented when significant results were found.

All other physiological responses were examined by ANOVA with site, gender, weight, and CF as main effects and included first-order interactions. Inclusion of these main effects tested for the effects of dissolved Cu along with effects of demographic and morphological factors. Interactions and significant effects were treated as above. Age was evaluated as stated above. Data on Hct could not be transformed to equalize error variances among sites. Therefore, Hct data were analyzed as above using a weighted ANOVA (Neter *et al.* 1990). For all analyses, differences were considered significant at $p \leq 0.05$. All statistical operations were run using JMP 3.2.1 for Macintosh (SAS Institute, Cary, NC).

Results

Demographics

Twelve trout were collected from site 1, 11 from Red Clover Creek and from site 4, and 13 from site 6. The genders and ages of these fish are presented in Table 2.

Morphological Parameters

In the analysis of weight, site and gender were significant (Table 3). When age was analyzed, it was also significant ($p = 0.001$). Weights were significantly different only between the two reference sites, site 1 and Red Clover Creek ($p = 0.024$), with Red Clover Creek having heavier fish. Therefore, dissolved Cu concentrations did not affect weight differences, and analyses of the indirect effects of Cu on other physiological parameters through alterations in weight were not required. Site 1 had more young (age group 1), immature fish than Red Clover Creek. Contrasts on gender revealed that mature males were significantly heavier than immature fish ($p < 0.001$) and mature females ($p = 0.005$). Although the difference was not significant, mature females were, on average, heavier than immature fish (57.9 g versus 38.9 g). Further supporting a demographic explanation, a t-test found that age group 2 was significantly heavier than age group 1 ($p = 0.001$).

In the ANOVA for CF, no interactions and no main effects were significant (Table 3). Therefore, dissolved Cu did not appear to affect CF, and analysis of the indirect effects of dissolved Cu on other physiological parameters through alterations in CF was not performed.

Hepatic Cu Concentration

Site had a significant effect on hepatic Cu (Figure 1; Table 3). Reference sites were combined. Contrasts between the combined reference sites and site 4 ($p = 0.002$) and site 6 ($p = 0.008$) were significant. Hepatic Cu concentrations were elevated at sites 4 and 6, indicating exposure to and uptake of Cu. Hepatic Cu concentrations were similar at the two sites even though site 4 had a higher mean dissolved Cu concentration than site 6.

Table 2. Demographic data for rainbow trout collected from Red Clover Creek and Little Grizzly Creek

Sampling Site	# of Males (Ages)	# of Females (Ages)	# of Immatures (Ages)
RCC	2 (2+)	3 (2+)	6 (0+,1+,2+)
Site 1, LGC	1 (2+)	3 (2+)	8 (0+,1+)
Site 4, LGC	1 (4+)	3 (2+,3+)	7 (1+,2+)
Site 6, LGC	4 (2+,3+)	6 (2+,3+)	3 (2+)

Hct

For Hct, the only significant effect was site (Figure 2; Table 3). As site 1 and Red Clover Creek were almost significantly different ($p = 0.06$), the reference sites were not combined. Contrasts were significant between site 1 and site 4 ($p < 0.001$), site 1 and site 6 ($p < 0.001$), Red Clover Creek and site 4 ($p = 0.009$), and Red Clover Creek and site 6 ($p = 0.006$). Hct values were higher at reference sites than at the sites with elevated dissolved Cu concentrations. Though site 4 had a higher mean dissolved Cu concentration than site 6, both had similar mean Hct values.

Lct and Differential Counts

In the analysis of Lct, site had a significant effect (Figure 3; Table 3). The two reference sites were combined. Contrasts between the combined reference sites and site 4 ($p < 0.001$) and site 6 ($p = 0.005$) were significant. Mean Lct values at sites 4 and 6 were depressed in a concentration-dependent manner.

For the percentage of lymphocytes in blood, site was significant (Table 3). Site 1 and Red Clover Creek were combined and contrasted with sites 4 ($p < 0.001$) and 6 ($p = 0.05$). The percentages of lymphocytes were depressed at sites 4 and 6 in a concentration-dependent manner.

Site, gender, site \times gender, and site \times CF were significant in the analysis of percentage of monocytes in blood (Table 3). The reference sites were combined. The contrast between the two reference sites and site 6 was significant ($p = 0.006$). Site 6 had a significantly higher value, while site 4, with a higher mean dissolved Cu concentration, was not significantly different from the reference sites. This suggested that the fish at site 6 might differ from other sites in a way unrelated to Cu exposure.

In contrasting the percentage of monocytes in blood between genders, a difference between mature males and immature fish approached significance ($p = 0.054$). The mean value was higher in mature males. Given the significant site \times gender term, comparisons were made among sites within genders. This analysis was only conducted for immature fish and mature females because sites 1 and 4 each had one mature male. Site was significant for both immature fish and mature females. In contrasts for immature fish, site 1 and Red Clover Creek were combined and found to be significantly different from site 6 ($p = 0.012$). The mean response at site 6 was elevated in immature fish. The greater number of mature males at site 6 and the elevated percentage of monocytes in blood in immature fish at this site compared to reference values were likely the cause of

Table 3. Final statistical models and results for each parameter

Factors	F-Ratio	prob > F
A. Weight		
Site	4.8	0.006
Gender	18.6	< 0.0001
B. Condition factor		
Site	1.1	0.35
Gender	0.5	0.59
C. Hepatic copper		
Site	7.0	0.0008
Gender	0.3	0.74
CF	0.5	0.5
Weight	1.1	0.3
D. Hematocrit		
Site	12.7	< 0.0001
Gender	1.0	0.39
CF	2.3	0.14
Weight	1.6	0.21
E. Leukocrit		
Site	10.1	< 0.0001
Gender	1.0	0.38
CF	0.3	0.60
Weight	1.2	0.28
F. % lymphocytes		
Site	9.7	0.0001
Gender	1.8	0.17
CF	0.3	0.57
Weight	1.3	0.26
G. % monocytes		
Site	4.7	0.01
Gender	4.0	0.03
CF	3.6	0.07
Weight	0.0	0.99
Site × gender	3.6	0.01
Site × CF	4.8	0.01
H. % neutrophils		
Site	0.3	0.85
Gender	1.3	0.29
CF	0.0	0.89
Weight	0.0	0.89
I. Respiratory burst		
Site	2.7	0.06
Gender	0.0	0.97
CF	0.6	0.44
Weight	0.2	0.64
J. Muscle protein		
Site	8.3	0.0002
Gender	0.7	0.51
CF	0.3	0.56
Weight	0.0	0.85
K. Muscle glycogen		
Site	0.2	0.9
Gender	1.1	0.36
CF	3.8	0.06
Weight	0.1	0.76
L. Plasma acetylcholinesterase		
Site	0.4	0.76
Gender	0.3	0.78
CF	0.0	0.99
Weight	0.0	0.97

the significant difference between site 6 and the reference sites. In contrasts for mature females, site 1 and Red Clover Creek were combined and found to be significantly different from site

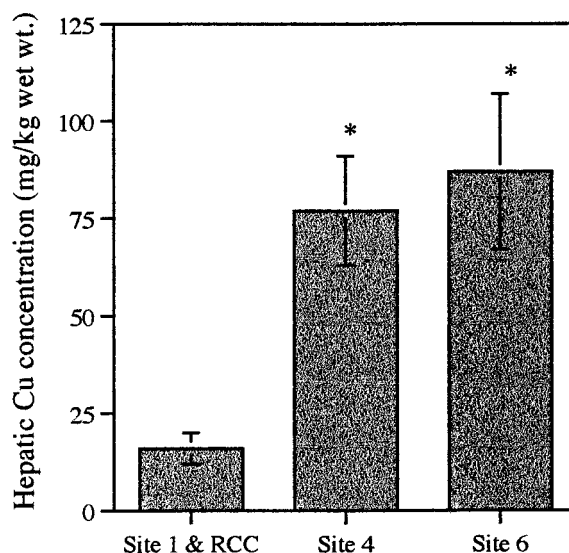


Fig. 1. Hepatic Cu concentrations in rainbow trout at two reference sites (site 1 and Red Clover Creek combined) and at two sites with elevated levels of dissolved copper (sites 4 and 6). Values are presented as mean ± SE. Significant differences from reference sites are indicated by an asterisk

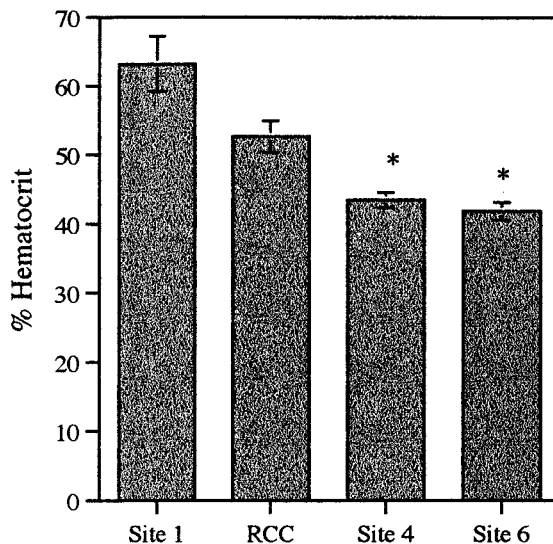


Fig. 2. Hematocrit values of rainbow trout at two reference sites (site 1 and Red Clover Creek) and at two sites with elevated levels of dissolved copper (sites 4 and 6). Values are presented as mean ± SE. Significant differences from reference sites are indicated by an asterisk

4 ($p = 0.042$). The mean response at site 4 was depressed in mature females. Although values for mature males were not analyzed, the profile plot of responses at each site was similar to that of mature females. Therefore, mature fish at site 4 showed a pattern of depressed monocyte numbers. For the site × CF term, a similar relationship occurred at all sites except Red Clover Creek, suggesting that dissolved Cu concentration did not cause the effect.

In analyzing the percentage of neutrophils in blood, no factors were significant (Table 3).

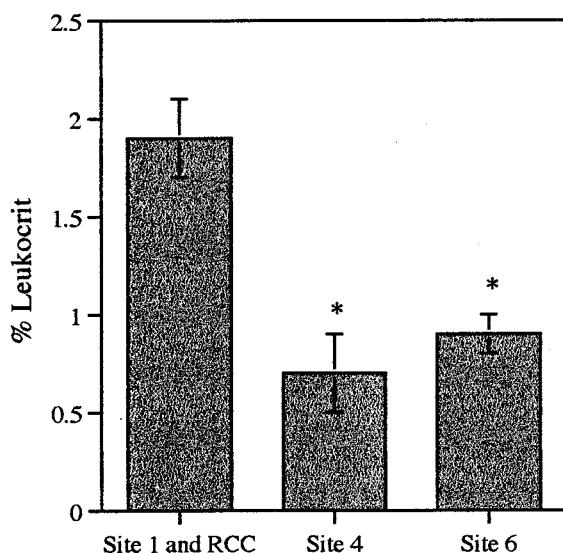


Fig. 3. Leukocrit values of rainbow trout at two reference sites (site 1 and Red Clover Creek combined) and at two sites with elevated levels of dissolved copper (sites 4 and 6). Values are presented as mean \pm SE. Significant differences from reference sites are indicated by an asterisk

Overall, elevated dissolved Cu concentrations at sites 4 and 6 appeared to depress the mean Lct and percentage of lymphocytes in blood in a concentration-dependent manner but did not affect the mean percentage of neutrophils in blood. Demographic and morphological factors did not complicate the results. Elevated Cu concentrations may have caused a depression in the percentage of monocytes in blood in mature fish at site 4, the site with high dissolved Cu.

Respiratory Burst

Differences in respiratory burst activity among sites approached significance (Table 3). The site effect was most likely due to differences between the responses of trout at sites 1 and 6. The mean response of trout at site 1 was almost twice that of trout at site 6. However, the response of trout at Red Clover Creek was only 10% higher than that at site 6. The mean response at site 4, at the upper end of the dissolved Cu gradient, was similar to that at site 1. This pattern suggested that dissolved Cu was not affecting respiratory burst activity. Because respiratory burst data for age group 1 were not available at site 4, age was not analyzed by ANOVA. Mean values (\pm SD) for all three age groups (group 1— 0.97 ± 0.48 ; group 2— 0.38 ± 0.07 ; group 3— 0.28 ± 0.11) suggested that age influenced differences among sites. The largest difference in respiratory burst activity was between site 1 (no individuals in age group 3) and site 6 (no individuals in age group 1).

Muscle Biochemistry

In the ANOVA for muscle protein concentration, site was significant (Table 3). Muscle protein values were significantly

different between Red Clover Creek and site 1 ($p = 0.007$), between Red Clover Creek and site 4 ($p = 0.008$), and between Red Clover Creek and site 6 ($p < 0.001$) with Red Clover Creek having fish with higher muscle protein. Dissolved Cu concentration did not appear to influence site differences.

For muscle glycogen concentration, no factors were significant, although CF approached significance (Table 3).

Data on muscle lipid content were collected but sample weights were found to have increased slightly or not changed after lipid extraction. Even varying the drying time did not decrease weights. To calculate lipid content, a loss in ether-extracted muscle weight had to occur. Therefore, lipid content was not calculated. These results were postulated to be due to the small sample weights used in this procedure (< 0.2 g) and precision problems with the balances.

Plasma AChE

No factors significantly affected plasma AChE (Table 3).

Discussion

Metals dissolving into the waters of Dolly Creek, primarily from a Cu tailings pile, result in elevated dissolved Cu concentrations in both Dolly Creek and a 19-km stretch of Little Grizzly Creek below its confluence with Dolly Creek (Deanovic *et al.* 1999). Although the dilution factor of Little Grizzly Creek reduces the Cu concentrations in this stretch relative to the concentrations found in Dolly Creek, a detectable Cu gradient still exists year-round in this section of Little Grizzly Creek. By measuring responses in various systems of rainbow trout along this gradient, we investigated whether there was evidence of chronic toxicity in Little Grizzly Creek and evaluated the ability of laboratory-tested assays to detect sublethal responses in rainbow trout chronically exposed to Cu in a natural environment.

Hepatic Cu Concentration

Hepatic Cu was chosen as a measure of exposure because the liver is a primary accumulation site for Cu during exposure to elevated concentrations in the water column (Sorensen 1991). This parameter was elevated at both sites with elevated dissolved Cu concentrations, indicating exposure. A clear concentration-dependent response was not seen. The range of data (represented by the standard error) at site 6 was expected given that this site was the most likely to be a mixture of resident fish and fish emigrating from upstream and downstream sites. Site 4 was the only site that had no fish with undetectable or single-digit hepatic Cu concentrations.

Demographic and morphological factors did not have an effect on hepatic Cu concentration, though it was believed they might have since they represented exposure duration and liver size. Movement along the Cu gradient by an individual or changes in Cu uptake and turnover with age or condition could explain why these factors had no effect, but investigation of the mechanism was beyond the scope of this study. Even with the

many potential confounding factors, the mean response of hepatic Cu concentration reflected dissolved Cu concentration and appeared to be a robust measure of exposure for this diverse population.

Other studies that examined wild populations of fish along a gradient of metal contamination have reported similar findings. The mean hepatic metal concentrations in rainbow trout in a series of metal-contaminated lakes roughly followed the gradient of dissolved metals; a large amount of variation was associated with the hepatic metal concentrations in the most-contaminated lakes (Roch *et al.* 1982). Also, hepatic levels of zinc in perch (*Perca fluviatilis*) living downstream of a brassworks reflected dissolved zinc concentrations (Hogstrand *et al.* 1989).

Morphological Parameters

Our study did not show significant differences in weights between sites with different concentrations of Cu. The significant difference in weights between the two reference sites was most likely the result of demographic differences between fish at the sites. CF also did not differ among sites.

Our results are in reasonable agreement with a number of studies investigating the effects of metals on weight and CF. Reduced weights were noted in juvenile brown trout (*Salmo trutta*) exposed to a metal mixture over 5 weeks, but a similar inhibition was not seen in juvenile rainbow trout (Marr *et al.* 1995). At the end of a 60-day exposure, weight and CF were not significantly different between rainbow trout fry in control treatments and those exposed to a waterborne mixture of metals in conjunction with dietary exposure to a single metal (Mount *et al.* 1994). A field study of white suckers (*Catostomus commersoni*) inhabiting a chain of metal-contaminated lakes found that weight differences among the lakes were not correlated with the gradient of metal contamination, though a gradual shift in CF occurred with white suckers from the least-contaminated and reference lakes having the highest CF (Miller *et al.* 1992). Finally, free-ranging brown trout exposed to a mixture of metals in water and sediments did not have significantly different weights or lengths at sites with different levels of contamination (Frag *et al.* 1995).

Hct

Rainbow trout at sites receiving Cu effluent had depressed Hct values compared to those of individuals from reference sites. Age and gender have previously been noted as factors that affect hematologic parameters (McCarthy *et al.* 1975), but they did not have a significant effect on Hct in this study, leaving dissolved Cu concentration as the only significant factor. Hct values at site 1 appear somewhat high, but the mean value is within the normal range for a demographically mixed population of rainbow trout (McCarthy *et al.* 1975). Also, although the sampling method could elevate Hct, such an alteration should be seen at all sites so that values can still be compared. The results of this study indicate that prolonged exposure to Cu can depress red blood cell volume, and potentially oxygen-carrying capacity (Houston 1990), in wild rainbow trout. Short-

term laboratory studies (≤ 30 days) have suggested that acclimatization processes act during exposure to sublethal concentrations of metals to return Hct to pre-exposure levels after initial alteration (Christensen *et al.* 1972; Dethloff *et al.* 1999b; McKim *et al.* 1970). This process was not evident in this field population experiencing extended exposure. Our results agree instead with those of a 337-day study of brook trout responses to Cu concentrations between 5 and 20 $\mu\text{g/L}$ (McKim *et al.* 1970). In that study, exposed brook trout exhibited depressed Hct.

Immune System Parameters

One of the goals of this study was to determine whether certain immune parameters altered in rainbow trout during previous laboratory exposures (Dethloff and Bailey 1998; Dethloff *et al.* 1999a) were altered in a wild population of rainbow trout exposed to Cu where factors such as water quality, gender, and size could also influence measured physiological parameters. Lct, which has been advocated as a tool for monitoring the condition of wild fish (Adams *et al.* 1993), was also included in the study due to its straightforward interpretation and ease of measure. The insignificant effects of all main factors on the percentage of neutrophils in blood was surprising, considering that this parameter displayed a consistent response to metal exposure in laboratory studies (Dethloff and Bailey 1998; Dethloff *et al.* 1999a).

While Lct and the percentage of lymphocytes in the blood exhibited concentration-dependent responses to dissolved Cu concentrations in the field, the percentage of monocytes in blood was influenced by gender. Respiratory burst activity may have differed between sites due to differences in the representation of the three age groups. Because an equitable number of each age group and each gender was not available from each site (due to nonselective collection and 1–2 fish per site having insufficient tissue or blood), it was difficult to determine whether these demographic factors were complicating a response to dissolved Cu. For the respiratory burst, older animals (age group 3) showed lower activity relative to younger animals (age group 1). Since all age groups were not represented equally at each site, sites that had a higher proportion of older animals (*i.e.*, site 6) were more likely to have lower mean respiratory burst activity. For the percentage of monocytes in blood, different response patterns were seen for the different genders at the different sites (significant site \times gender interaction) with the two mature groups showing similar trends of depression at site 4 whereas the immature fish had elevated values at site 6. However, the mature age groups were only represented by three individuals at site 4, making it difficult to draw any firm conclusions about Cu effects from this small sample.

In past laboratory studies on rainbow trout exposed to Cu in waters simulating Sierra Nevada creeks, certain immunoassays (respiratory burst and differential leukocyte counts) were identified as indicators of chronic physiological alterations (Dethloff and Bailey 1998; Dethloff *et al.* 1999a). Laboratory studies conducted by other researchers have also demonstrated the sensitivity of respiratory burst activity in fish undergoing metal exposure (Hutchinson and Manning 1996; Zelikoff *et al.* 1995)

as well as alterations in differential leukocyte counts. In general, the relative proportion of lymphocytes decreased (Dick and Dixon 1985; Murad and Houston 1988), the relative proportion of neutrophils increased (Dick and Dixon 1985; Murad and Houston 1988; Nussey *et al.* 1995) and the relative proportion of monocytes either increased or decreased (Murad and Houston 1988; Nussey *et al.* 1995) with metal exposure.

The results for this study suggested that Lct and the percentage of lymphocytes in blood were appropriate measurements to detect immune system alterations in rainbow trout exposed to Cu regardless of gender and age. To better determine the potential usefulness of respiratory burst activity and the percentage of monocytes in blood for assessing alterations in the immune system of wild trout, future work requires a protocol specifying equitable sampling of genders and ages.

Muscle Energy Reserves

Glycogen, protein, and lipid content of muscle tissue were measured to estimate the effects of Cu on the energy reserves of rainbow trout, although, unfortunately, the lipid results were not usable. Glycogen and proteins, along with lipids, are considered important energy sources for fish (Chellappa *et al.* 1995) that can be altered by exposure to contaminants. Decreased visceral lipid and liver glycogen levels and also higher liver protein have occurred after exposure of fishes to organic pollutants (Adams *et al.* 1992; Vignier *et al.* 1992). Muscle glycogen content was depleted in rainbow trout exposed to cadmium for 30 weeks with this effect persisting after 57 weeks of recovery (Larsson *et al.* 1985). White sucker from lakes containing elevated levels of Cu and zinc exhibited decreased muscle lipid stores, although liver glycogen and lipid stores were not affected by collection site (Munkittrick and Dixon 1988).

These alterations to metabolic activities have been attributed to both indirect and direct effects of xenobiotics on fish. It has been suggested that metal contamination can cause nutritional deficiencies in certain fish by contaminating sediments and adversely affecting the organisms on which the fish feed (Munkittrick and Dixon 1988). Direct effects occur through a secondary stress response characterized by several metabolic alterations and can result in depletion of tissue reserves of glycogen, protein, and lipid (Larsson *et al.* 1985; Neff 1985). The current study investigated the possibility that tissue reserves had been depleted though it did not investigate the possible mechanisms. No depletion or increase was seen in rainbow trout occupying Cu-contaminated reaches of Little Grizzly Creek. Information on lipid stores is needed, but it appears that Cu contamination did not cause alterations that affected muscle energy stores.

Plasma AChE

Alterations in AChE activity have been associated with altered reproduction, behavior, and motor abilities in fish. While AChE inhibition and its effects have been extensively studied as an indicator of environmental contamination primarily by organophosphate and carbamate pesticides (Zinkl *et al.* 1991b),

few studies have investigated the impacts of prolonged *in vivo* exposure to metals. Plasma AChE concentrations in wild rainbow trout from this study showed no effect of dissolved Cu concentration. Although brain AChE activity has been found to increase with size in rainbow trout (Zinkl *et al.* 1991b), weight and age had no notable effect on plasma AChE activity in this study. Mean values of plasma AChE calculated for wild rainbow trout were within the range found for hatchery rainbow trout (Zinkl *et al.* 1987b; Dethloff, unpublished data). Thus, dissolved Cu concentrations at this field site did not appear to be having any adverse effect on AChE activity in plasma.

In other studies on metals, stinging catfish (*Saccobranchus fossilis*) exposed to Cu and common carp exposed to sublethal concentrations of mercury and zinc showed significant decreases in AChE activity in several organs (Suresh *et al.* 1992; Verma *et al.* 1981). Rainbow trout exposed to sublethal Cu and zinc concentrations for 21 days experienced elevated brain AChE activity caused by dissolved Cu through day 14 (Dethloff *et al.* 1999a). A consideration when comparing these studies to the current study is that AChE alterations were measured in organ tissue rather than plasma. Plasma was chosen as the medium for analysis in this study due to logistical considerations of working in the field, although plasma is considered less ideal than brain tissue due to greater variability among individuals (Donovan and Zinkl 1994; Zinkl *et al.* 1991b).

Summary

Hematological and immunological changes are among the first detectable responses of fish to environmental stressors (Mayer *et al.* 1992; Weeks *et al.* 1992), such as metals. These parameters respond even after short-term exposure. Exposed fish can compensate for the stressor, with hematological and immunological parameters returning to pre-stress levels. If fish cannot successfully compensate for the stressor's effects, an altered physiological state may be reached in which the organisms continue to function. In extreme cases, the acclimation response will be exhausted with subsequent impacts on fitness (Mayer *et al.* 1992).

In Little Grizzly Creek, Cu exposure was indicated by significantly elevated hepatic Cu concentrations at sites characterized by elevated concentrations of dissolved Cu. Lct, percentage of lymphocytes in blood, and Hct were significantly reduced in fish from these sites, reflecting hematological and immunological effects. These findings suggested that the trout exposed to Cu for a prolonged period were either still compensating for the stressor or had made physiological adjustments that resulted in a physiological state different than that observed in reference trout.

In contrast to Hct, Lct, and percentage of lymphocytes in blood, parameters indicating energy reserves and condition (muscle protein and glycogen, CF, weight) did not differ between the reference and Cu-impacted sites. A possible explanation for this is that trout were, at the time of sampling, equilibrated and the individuals were no longer expending energy to return the affected systems to a normal range. Another consideration in explaining these findings is that food is relatively abundant in the summer months, allowing trout at

these sites to replace energy lost to compensatory physiological measures. Collections during periods of limited food would provide information regarding Cu impacts on energy reserves and condition during less optimal times. Also, inclusion of muscle lipid and liver glycogen (this study's initial protocol did not allow liver subsampling), both primary stores for energy mobilization, would allow a more thorough assessment of energy reserves.

Though responses associated with a higher level of organization (energetic output, growth) were not altered, the comparative differences between the blood-based parameters of exposed and reference fish suggested that the status of the Cu-exposed trout was not optimal and the capacity of the trout to withstand additional stressors was potentially compromised. For example, lowered Lct and percentage of lymphocytes may have increased susceptibility of the Cu-exposed trout to a pathogen challenge. These parameters therefore provide useful insights into subtle, potentially deleterious effects of certain stressors on the physiological condition of wild trout. Our data suggest that measurements of Lct, percentage of lymphocytes in blood, and Hct provide statistically powerful data and should be part of a suite of parameters used to monitor the condition of wild salmonids exposed to sublethal Cu concentrations. Inclusion of other immune system parameters (respiratory burst activity, percentage of monocytes in blood) may also strengthen the ability of monitoring programs to detect sublethal effects and protect healthy salmonid populations. However, these two parameters appear to be affected by demographic factors and require additional work to separate demographic influences from possible impacts of sublethal metal exposure.

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