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Physiological Responses of Juvenile Rainbow Trout to Chronic Low Level Exposures of Waterborne Silver

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ABSTRACT. The physiological effects of chronic exposure to $AgNO_3$ in moderately hard freshwater were investigated in juvenile rainbow trout (*Oncorhynchus mykiss Walbaum*). Two separate 28-day exposures were performed at silver concentrations of 0.5 and 2.0 μg/L in flowing Hamilton dechlorinated tap water. Exposure to 0.5 μg/L Ag resulted in a slight increase (~14.9%) in food consumption, whereas growth rates remained unaltered. Both plasma Na^+ and Cl^- levels were significantly decreased by 11.8% and 9.3%, respectively at day 16 of the exposure. Hepatic Ag concentrations were elevated approximately 4-fold in 0.5 μg/L Ag-exposed fish. However, no significant increases in liver metallothionein (MT) concentrations were noted. No mortalities were observed during this 28-day exposure. In comparison, chronic exposure to 2.0 μg/L Ag resulted in a 28.8% decrease in food consumption and a 43.0% reduction in growth rate. Plasma [Na^+] was decreased by 18.3%, whereas plasma [Cl^-] was reduced by 12.2% at day 7. At both concentrations of silver, plasma ion concentrations appeared to recover thereafter. Silver accumulated steadily in the liver up until day 15 when concentrations were 39.7 μg/g wet weight (285-fold increase) above control levels. In addition, MT levels were increased by 81.2% at day 7. Silver exposure at 2.0 μg/L resulted in approximately 15.0% mortality over the 28-day period. comp biochem physiol 119C;2:131–137, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Accumulation, chronic, feeding, growth, ion regulation, metallothionein, rainbow trout, silver nitrate

INTRODUCTION

It has been recently suggested that the primary mechanism of acute Ag toxicity in fish is related to a severe ionoregulatory disturbance at the gills (15,22). Adult rainbow trout (*Oncorhynchus mykiss*) exposed to 10 μg/L Ag (as $AgNO_3$) showed immediate reductions of over 50% in branchial Na^+ and Cl^- influx, while complete inhibition (~99%) was produced after only 8 hr. The net result was a progressive loss of Na^+ and Cl^- from blood plasma. The subsequent decrease in plasma osmolality was partially compensated for with shifts of plasma and interstitial fluids to the surrounding tissues (22). Death was postulated to occur due to cardiovascular collapse resulting from systemic plasma volume loss as has been reported in cases of acidity (10,12,13).

Despite the dramatic physiological effects seen in adult rainbow trout at 10 μg/L Ag (as $AgNO_3$), exposure to silver concentrations as high as 30,000 μg/L (in the same water quality) failed to produce any significant effects to fish when presented as silver thiosulphate ($Ag(S_2O_3)_n$) (23), demonstrating the importance of silver speciation on toxicity (8). Photoprocessing effluent, the primary source of silver released into the environment, is almost entirely composed of silver thiosulphate. The two major complexes found in this include mono- and di-thiosulphate, with log K values of 8.8 and 13.7 respectively (14,19). During photographic processing (fixation), most of the free Ag^+ becomes strongly complexed with thiosulphate (20); consequently, even though total Ag concentrations at these contaminated point sources may reach levels as high as 35 μg/L Ag (9), Ag^+ concentrations will only range between 0.0001 and 0.2 μg/L Ag^+ (2).

In this experiment fish were exposed to either 0.5 or 2.0 μg/L Ag (as $AgNO_3$) over a 28-day period. Silver speciation modeling using a geochemical equilibrium program (19) was used to predict nominal Ag^+ concentrations for each of these treatments. A total [Ag] of 0.5 μg/L (in Hamilton dechlorinated tap water) was calculated to yield approximately 0.2 μg/L of free Ag^+, whereas a total [Ag] of 2.0 μg/L was expected to give 0.6 μg/L Ag^+. The 2.0 μg/L Ag exposure was performed despite yielding a high Ag^+ so as to compare results obtained here with previous studies (15).

In this study, potential ionoregulatory disturbances were assessed by analysis of plasma Na^+ and Cl^-. In addition, the

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performance of fish, as measured by growth and feeding, was evaluated. Finally, hepatic Ag and metallothionein content were measured. Metallothionein is a protein believed to be involved in the regulation and detoxification of trace metals. Induction of this protein may additionally serve as a useful indicator of bioavailable Ag. The aims of the present study were to expose fish to Ag^+ concentrations near the upper range of environmental relevance (0.2 μg/L Ag^+) and to assess the physiological effects of such an exposure in rainbow trout.

MATERIALS AND METHODS

Animals

Juvenile rainbow trout (*O. mykiss*, 3.01 ± 0.27) were purchased from Rainbow Springs Hatchery (Thamesford, Ontario). The entire stock was initially held in a 400-L tank supplied with dechlorinated, Hamilton city tap water ($[Na^+]$ = 0.6 mM; $[Cl^-]$ = 0.7 mM; $[Ca^{2+}]$ = 1.0 mM; $[Mg^{2+}]$ = 0.2 mM; $[K^+]$ = 0.05 mM; titratable alkalinity to pH 4.0 = 1.9 mM, total hardness of approximately 140 ppm as $CaCO_3$; $[HCO_3^-]$ = 1.9 mM; pH = 8.0) at a flow rate of 2000 mL/min. Water was vigorously aerated to maintain the dissolved oxygen concentration near saturation. Fish were kept at ambient conditions, with water temperatures ranging from 15.5°C to 17.5°C. Fish were hand-fed dry trout pellets (Ziegler Brothers Inc., Gardners, PA) to satiation daily.

Experimental Protocol

The study involved two separate 28-day flow-through exposures at nominal total silver concentrations of 0.5 and 2.0 μg/L as $AgNO_3$ (Fisher Scientific, Toronto, ON). Measured Ag concentrations were 0.7 ± 0.2 and 1.7 ± 0.4 μg/L, respectively. In conjunction with each Ag exposure a simultaneous control (no Ag added) was also tested. For the 0.5 μg/L Ag exposure, 450 fish were randomly selected from the original stock and divided into two equal groups of 225. For the 2.0 μg/L exposure, 300 fish were randomly selected from the original stock and divided into two equal groups of 150. In all cases, fish were placed in 400-L tanks, each supplied with 1000 mL/min of dechlorinated tap water (see above). Fish were allowed to acclimate to these conditions for at least one week before the start of the experiment.

$AgNO_3$ stock solutions were made up in light-shielded plastic carboys with Ag concentrations 1000-fold greater than the desired amount in the exposure water. Concentrated HNO_3 acid was added to the stock solution to a pH of 4.0 to help minimize absorption of silver to the container. Silver from the stock solutions was added to their respective tanks at a rate of 1.0 mL/min using peristaltic pumps. Adequate mixing of Ag stock (1.0 mL/min) and water (1000 mL/min) at their points of addition into the tanks was achieved by vigorous aeration. Exposure tanks were initially spiked with an appropriate amount of $AgNO_3$ stock to immediately achieve nominal silver concentrations of either 0.5 or 2.0 μg/L.

Fish from the 0.5 μg/L Ag exposure and its simultaneous control were sampled on days 0, 1, 7, 16, 23, and 28, whereas fish from the 2.0 μg/L Ag exposure (and control) were sampled on days 0, 7, 15, 20 and 28. The day 0 sampling period was performed prior to spiking the tank with Ag. Ten fish per treatment were analysed for a variety of physiological parameters at each sampling period. Individually sampled fish were randomly selected and immediately sacrificed with a cephalic blow. Blood samples were withdrawn via caudal puncture using a 100 μL Hamilton syringe pre-rinsed with 50 i.u./mL ammonium heparin (Sigma, St. Louis, MO), and placed in small plastic centrifuge tubes (Eppendorf). Whole blood was spun for two minutes to obtain plasma. Samples were subsequently frozen in liquid N_2 and stored at −70°C until analyzed for total plasma Na^+ and Cl^- concentrations. Plasma Na^+ was measured using atomic absorption spectroscopy, whereas plasma Cl^- concentrations were determined using the mercuric thiocyanate colorimetric assay (24). Livers were excised, blotted dry, weighed in plastic centrifuge tubes, frozen in liquid N_2, then stored at −70°C for analysis of total Ag and metallothionein.

Feeding and Growth

Fish were fed to satiation once daily at 17:00. A special feeding protocol was implemented to accurately assess satiety in fish (21). In short, food was carefully sprinkled into each tank allowing it to float on the water surface. Small quantities (0.5 g-1.0g) from a pre-weighed bag were administered at one minute intervals. Attention was given to ensure that all food added was completely consumed before more food was given. Cessation of appetite was arbitrarily set as the point at which floating food was still present after two consecutive minutes. Appetite was expressed as the average cumulative amount of food consumed (in grams) during the exposure per fish. Fish weights obtained at each sampling period (n = 30) were used as an index of fish growth over the course of the experiment. Growth was expressed as average weight per fish.

Analysis of Silver in Water

Water samples were taken daily from each tank and immediately acidified with 0.5% (v/v) of trace metal grade 70% HNO_3 (J.T. Baker; Toronto, ON) for analysis of total Ag. Samples were analysed using atomic absorption spectroscopy (Varian AA 1275, Mississauga, ON) equipped with a graphite furnace atomizer (Varian GTA-95). The graphite furnace was programmed with the following temperature

ramping profile: ambient temperature to 75°C over 5 s, 75°C to 90°C over 12 s, 90°C to 120°C over 30 s, with atomization occurring at 2000°C. Between samples, the graphite tube was flushed with pre-purified N_2 gas to eliminate contamination and memory effects. An automated sample injector was used to dispense 10 μL samples, giving a detection limit of 0.25 μg/L Ag.

Analysis of Ag and Metallothionein (MT) in Livers

Livers (12–160 mg) were individually homogenized in 1.00 mL of 50 mM Tris-HCl, pH 8.0 (Sigma, St. Louis, MI), at 0°C using an ice-cold glass-teflon homogenizer (Thomas Scientific). Five hundred μL aliquots of liver homogenate were stored in individual, acid-washed test tubes for total Ag analysis. Homogenates were digested in 2.5 mL of concentrated HNO_3 for 2 hours at 120°C. Samples were allowed to cool to room temperature and 375 μL of 30% H_2O_2 (Fisher) were added to each test tube. Tubes were then slowly heated to 120°C and each of the homogenates was evaporated to dryness. Five mL of 0.5% HNO_3 were added to each tube, and subsequently analysed by graphite furnace AAS.

The remaining ice-cold homogenate was immediately centrifuged at 16,000 ***g*** for 20 min at 4°C. The supernatant (approximately 200 μL) from each sample was collected in centrifuge tubes, frozen in liquid N_2, and stored at −70°C until analysis. The MT assay used a double antibody radioimmunoassay as described by Hogstrand and Haux (7). This included rabbit antiserum raised against perch (*Perca fluviatilis*) as the first antibody, ^{125}I-labelled rainbow trout MT as tracer, and goat anti-rabbit IgG as the second antibody.

Statistical Analysis

Mean values of both Ag-exposed groups were statistically compared to their simultaneous controls using Student's two-tailed *t*-test ($p < 0.05$)* or ($p < 0.01$)**.

RESULTS

Feeding, Growth and Mortality

Cumulative food eaten per fish was monitored during each of the 28-day silver exposures and simultaneous controls. Fish exposed to 0.5 μg/L Ag had a significant increase ($p < 0.05$) in their average cumulative food consumption (per fish) compared to controls. Average values for cumulative food consumption were 4.27 and 3.72 g per fish respectively, representing a 14.9% elevation (Fig. 1A). Growth rates were not significantly different between controls (150.0 mg/fish/day) and fish exposed to 0.5 μg/L Ag (167.5 mg/fish/day) (Fig. 1B). In comparison, exposure to 2.0 μg/L Ag significantly decreased ($p < 0.01$) average cumulative food consumption by 28.8% (Fig. 2A). Average growth

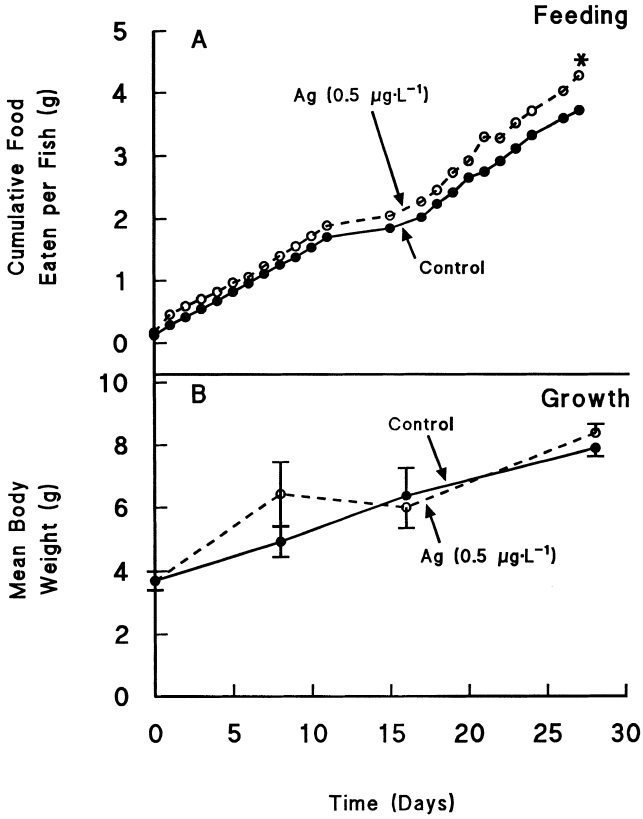

FIG. 1. (A) Food consumption and (B) growth rates in juvenile rainbow trout during a 28-day exposure to 0.5 μg/L Ag as $AgNO_3$. Fish were fed to satiation once daily. Food consumption represented as cumulative food consumed per fish. Mean body weights were measured ± 1 SEM (N = 30) at each sampling period. The effects of Ag exposure were compared with a simultaneous control. Vertical bars represent one SEM. An * represents a statistically significant difference from the control at $p < 0.05$.

rates calculated over the 28 day exposure were reduced by 43.0% (Fig. 2B). No fish mortalities were reported in either of the controls or in the 0.5 μg/L Ag-exposed group throughout the 28- day period. In comparison, exposure to 2.0 μg/L Ag resulted in a mortality rate of 15% over 28 days. Approximately 85% of these mortalities were evenly distributed between days 4 and 20 of the treatment.

Plasma Ion Levels

Plasma Na^+ concentrations were reduced ($p < 0.05$) by 11.8% at day 16 and 15.8% at day 28 of the 0.5 μg/L Ag exposure (Fig. 3). Similarly, a decrease in plasma Cl^- levels at day 16 (lowered by 9.3%; $p < 0.05$) was also seen in these Ag-exposed fish. Following day 16, plasma Cl^- concentrations were not significantly different from those of control fish. Reductions in plasma Na^+ and Cl^- were most severe during treatment to 2.0 μg/L Ag. Plasma Na^+ concentra-

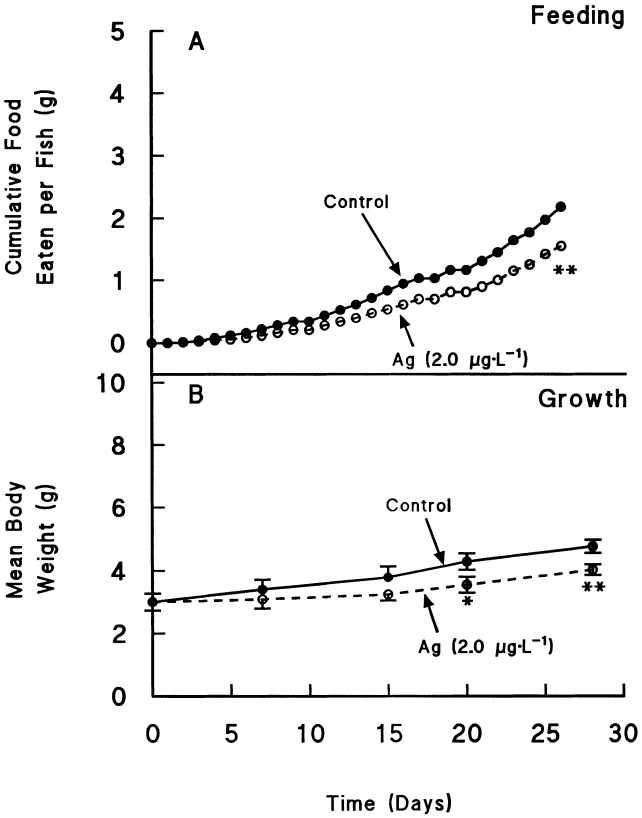

FIG. 2. (A) Food consumption and (B) growth rates in juvenile rainbow trout during a 28-day exposure to 2.0 μg/L Ag as $AgNO_3$. Fish were fed to satiation once daily. Food consumption represented as cumulative food consumed per fish. Mean body weights were measured ± 1 SEM (N = 30) for each sampling period. The effects of Ag exposure were compared with a simultaneous control. Vertical bars represent one SEM. An * represents a statistically significant difference from the control at $p < 0.05$, whereas ** represents a statistically significant difference from the control at $p < 0.01$.

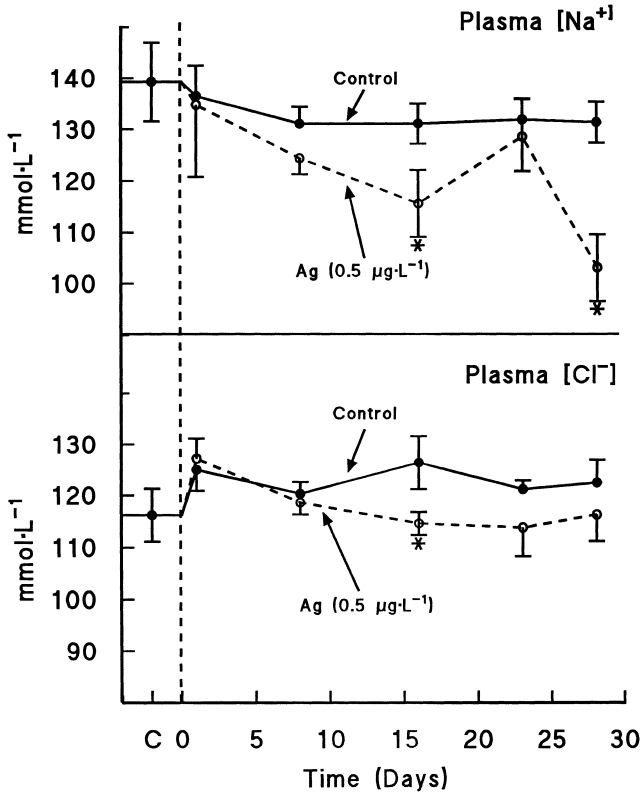

FIG. 3. Plasma Na^+ and Cl^- levels in juvenile rainbow trout, exposed for 28 days to 0.5 μg/L Ag as $AgNO_3$. Each data point represents mean value ± 1 SEM (N = 10). Ag-exposed fish are compared with their simultaneous control. Vertical bars represent 1 SEM. An * represents a statistically significant difference from the control at $p < 0.05$.

tions were decreased by 18.3% at day 7, however, levels were recovered to control values by the next sampling period (day 15) (Fig. 4). The average plasma Cl^- concentration was significantly reduced by 12.2% on day 7 and by 10.7% at day 15; complete recovery of plasma Cl^- levels was seen by day 20 (Fig. 4).

Silver Accumulation and Metallothionein (MT) Levels

Fish exposed to 0.5 μg/L Ag exhibited moderate accumulation of Ag in their livers only after day 23. Levels of hepatic Ag in this group were approximately four times greater than in control fish (Fig. 5). Despite the accumulation of Ag in these Ag-exposed fish, no significant induction of hepatic MT was observed (Fig. 5). In comparison, exposure to 2.0 μg/L Ag resulted in a 130-fold elevation of liver Ag content after only 7 days (Fig. 6). Silver accumulation was greatest at day 15 when levels were increased approximately 285-fold in the Ag-exposed group compared to control fish. Hepatic MT concentrations in the 2.0 μg/L Ag- exposed fish were increased by 81.2% at day 7, but elevations were not statistically significant thereafter (Fig. 6).

DISCUSSION

Effects of Ag on Plasma Sodium and Chloride Levels

Exposure of juvenile rainbow trout to low levels of Ag elicited an ionoregulatory disturbance qualitatively similar although quantitatively smaller than that seen at acutely lethal Ag concentrations (22). At 0.5 μg/L Ag, 10% reductions in plasma Na^+ and Cl^- concentration were produced by day 16 (Fig. 3), whereas plasma Na^+ losses as high as 18% were seen following 7 days of 2.0 μg/L Ag exposure (Fig. 4). Previous studies have concluded that plasma Na^+ losses over 30% were lethal to rainbow trout (11). Similar results were seen following 6 days of exposure to 10 μg/L Ag (as $AgNO_3$) (21). Fish mortalities commenced when plasma ion concentrations were decreased by 30%. In this study, mortalities were only reported at 2.0 μg/L Ag. The majority of these deaths occurred between days 7 and 20 when plasma Na^+ and Cl^- losses were most pronounced. Furthermore, it appears that amelioration of Ag toxicity is

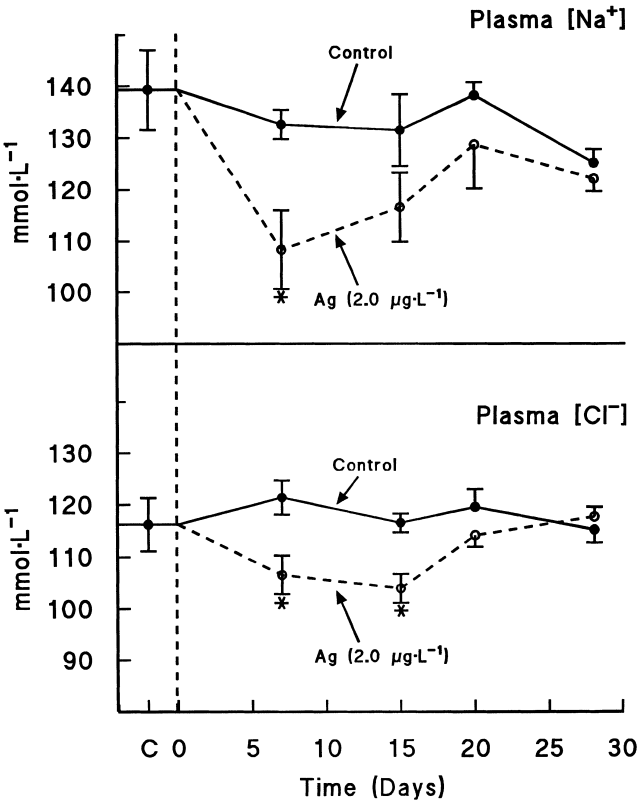

FIG. 4. Plasma Na^+ and Cl^- levels in juvenile rainbow trout, exposed for 28 days to 2.0 μg/L Ag as $AgNO_3$. Remaining details as outlined in legend for Fig. 3.

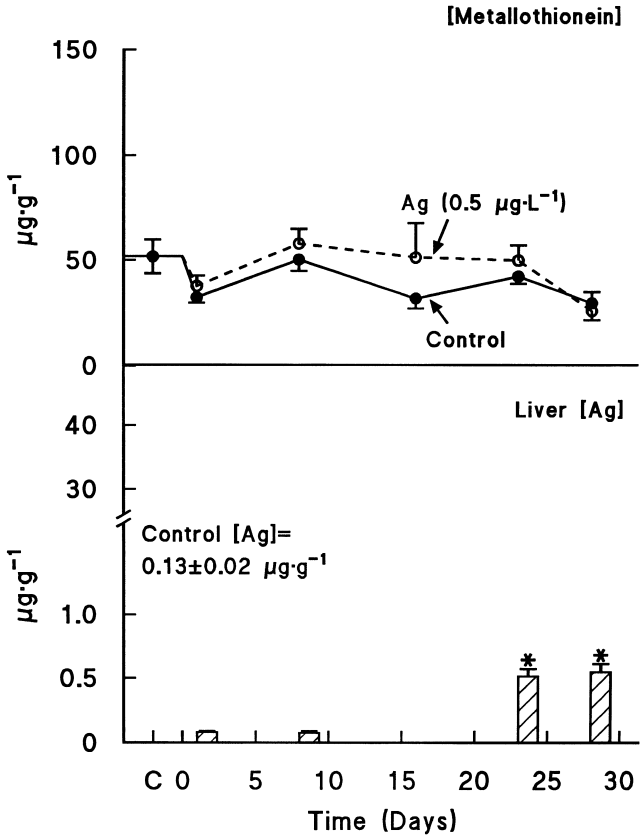

FIG. 5. Metallothionein and silver concentrations in livers of juvenile rainbow trout, exposed for 28 days to 0.5 μg/L Ag as $AgNO_3$. Each data point represents mean value ± 1 SEM (N = 10). An * represents a statistically significant difference from the control at $p < 0.05$.

correlated well with the recovery of Na^+ and Cl^- concentrations back to control levels (as seen by day 28 in the 2.0 μg/L Ag exposure). It is unclear whether this recovery in electrolyte homeostasis represents a true acclimatory response which would involve fish acquiring an improved resistance to subsequent lethal challenges of waterborne silver (12). For example, rainbow trout which have experienced an ionoregulatory disturbance in response to low pH exposure were shown to recover back to their original ionic status. Despite this recovery, these fish were no better able to survive future low pH exposures when compared to pre-exposed fish.

Growth and Food Consumption

In this study, growth rates were reduced in the 2.0 μg/L Ag-exposed group (Fig. 2b), but not in fish exposed to 0.5 μg/L Ag (Fig. 1b). This is consistent with other studies suggesting that growth is impaired during waterborne silver exposure (1,3). Early life stage tests performed on steelhead trout (over 21 days) showed decreased survivorship at 1.1 μg/L Ag, and significant alterations in growth rates at concentrations as low as 0.1 μg/L Ag (16). Additionally, a persistent decrease in the growth rates of oysters has been observed following exposure to 2.0 μg/L Ag (18). Additionally, food consumption was significantly elevated in the 0.5 μg/L Ag exposure (Fig. 1a). It is possible that fish consume more food to increase their dietary uptake of osmolytes in order to compensate for loss of ions due to branchial impairment. For instance, trout exposed to low pH (4.4–5.2) and given a ration of 2% of their body weight per day, were able to alleviate the resulting ionoregulatory disturbance. However, if trout were starved during low pH treatment, plasma Cl^- and whole body Na^+ levels remained low (17).

Ag Accumulation and MT Induction

Hepatic Ag accumulation was at least 40 times greater in the 2.0 μg/L exposure, compared to the 0.5 μg/L Ag-exposed group. Ag levels in the liver, following 8 days of exposure to 2.0 μg/L Ag were similar to those seen in other studies (4,6). Previous studies have shown that liver tissue is able to accumulate extremely high concentrations of Ag (>300 μg/g) during waterborne silver thiosulphate exposure. Despite this large increase in hepatic Ag no apparent deleterious effects to these exposed fish were observed (22). Recently however, hepatic Ag accumulated from a Ag-

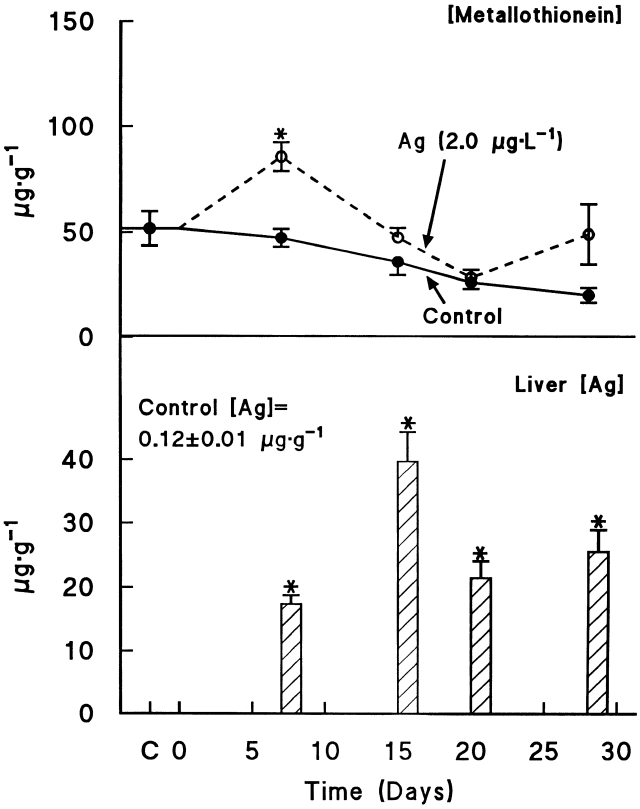

FIG. 6. Metallothionein and silver concentrations in livers of juvenile rainbow trout, exposed for 28 days to 2.0 μg/L Ag as $AgNO_3$. Each data point represents mean value ± 1 SEM (N = 10). An * represents a statistically significant difference from the control at $p < 0.05$.

contaminated diet was shown to result in a significant decrease to cytosolic copper concentrations in the liver (5). It seems feasible that as Ag accumulates in the liver, it begins to strip copper away from low-affinity ligands and metallothionein. This could result in either a loss of copper from hepatic tissue, or simply a redistribution of this metal to other subcellular compartments.

The long-term Ag exposures performed in this study suggest that branchial Na^+ and Cl^- uptake is a sensitive marker of Ag exposure. The fact that 15% of the 2.0 μg/L exposed group died despite plasma Na^+ reductions of only 18% (compared to 30% reductions at higher Ag concentrations (22)) may suggest that some fish in the population are more sensitive than others to chronic Ag exposure. The reduced ionoregulatory disturbance and lack of mortality observed in the 0.5 μg/L Ag treatment may indicate that a no-observed-effect limit is being reached. Future work should attempt to better characterize this dose-response relationship.

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