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ORIGINAL PAPER

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The mechanisms and costs of physiological and toxicological acclimation to waterborne silver in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract Juvenile rainbow trout were exposed to 0, 0.1, 1, 3, and 5 μg/l silver (Ag, as $AgNO_3$) for 23 days. Specific growth rate, cumulative food consumption, food-conversion efficiency, and critical swimming speed (U_{crit}) were significantly reduced during 5 μg/l Ag exposure, demonstrating a physiological cost of silver acclimation. Only the 5 μg/l Ag treatment had significant cumulative mortality (5.2%). Fish were most susceptible to silver on days 5 and 15. Exposure to 5 μg/l Ag significantly lowered plasma Na^+ and Cl^- on days 5 and 10, but plasma ion concentration recovered thereafter. Unidirectional Na^+ uptake and gill Na/K-ATPase activity were significantly inhibited by 3 and 5 μg/l Ag exposure. Na^+ uptake was inhibited by 3 μg/l Ag at day 5 alone, whereas the effects at the highest Ag exposure persisted until day 15. Gill Na/K-ATPase was inhibited on day 5 in both the 3 and 5 μg/l Ag treatments but increased to approx. 1.5 times of control levels by day 23. Only the 3 and 5 μg/l Ag treatments produced toxicological acclimation (at least twofold elevations in 168-h LC_{50} values in fish subsampled on days 15 and 23). We conclude that physiological acclimation results from compensatory changes in Na^+ transport at the gills, and that these changes may eventually lead to toxicological acclimation.

Keywords Rainbow trout · Silver acclimation · Unidirectional influx · Na^+/K^+-ATPase · Metabolic costs

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Abbreviations *AAS* Atomic absorption spectrophotometry · *FCE* Food conversion efficiency · LC_{50} Concentration resulting in 50% mortality · *SGR* Specific growth rate · U_{crit} Critical swimming speed

Introduction

It is well established that the free ionic silver species, Ag^+, is extremely toxic to freshwater fish, with estimates of concentrations resulting in 50% mortality (LC_{50}) after 96 h ranging between 5 and 65 μg/l Ag (Coleman and Cearley 1974; Davies et al. 1978; Hogstrand et al. 1996). Recent studies have shown that acute silver toxicity in juvenile rainbow trout is predicted well by the ionic Ag^+ concentration of water (Bury et al. 1999a; Galvez and Wood 1997; Hogstrand et al. 1996; McGeer and Wood 1998; Wood et al. 1996). The mechanism of acute silver toxicity in freshwater fish involves a blockade of gill Na^+ and Cl^- transport (Hogstrand and Wood 1998; Wood et al. 1999). Ionic Ag^+ acts by severely inhibiting Na/K-ATPase transporters on the basolateral surface of the gill epithelium (Bury et al. 1999b; Ferguson et al. 1996; McGeer and Wood 1998; Morgan et al.1997), inhibiting active Na^+ uptake, and severely reducing plasma Na^+ and Cl^- concentrations (Webb and Wood 1998; Wood et al. 1996).

In contrast, little is known about the physiological responses of freshwater fish to chronic low-level silver exposure. Studies suggest that growth rates are reduced in fish and mortality increased during long-term exposure to 0.09–0.17 μg/l Ag (Davies et al. 1978). Early life-stage tests on trout showed that 0.5 μg/l Ag significantly elevated fish mortality after 21 days of exposure (Nebeker et al. 1983). Similar to acute silver toxicity, the physiological basis for chronic toxicity may be in part related to impairment of Na^+ and Cl^- balance (Galvez et al. 1998), resulting in reductions in food-conversion efficiency (FCE) and impaired growth. However, the effects of silver on ion balance are short-lived, as plasma Na^+ and Cl^- typically return to control concentrations

during extended Ag exposure. This recovery of ion balance, despite the continued presence of silver, is characteristic of adaptive responses (physiological acclimation) seen with other metals during chronic exposure (McDonald and Wood 1993).

Acclimation to surface-acting metals is triggered by physiological and/or morphological disturbances at the gill epithelium, referred to as the "shock phase" (McDonald and Wood 1993). In order to compensate for the effects of the initial "shock phase" (e.g., ion loss) specific biochemical and physiological alterations at the gills can be implemented. If the initial physiological disturbance is large enough, this damage-repair process may result in increased tolerance of higher metal concentrations (toxicological acclimation) as assessed with acute toxicity tests. Although physiological acclimation to Ag is known to occur, nothing is known of the mechanism involved (Galvez et al. 1998), or whether toxicological acclimation occurs. The present study addresses these issues.

Juvenile rainbow trout were exposed for 23 days to 0.1, 1, 3, or 5 μg/l Ag (added as $AgNO_3$) and mortality, growth, appetite, and FCE monitored. Critical swimming speed tests (see Wilson and Wood 1992) were used to provide additional information on the physiological costs, if any, of silver acclimation. Toxicological acclimation was evaluated using either 96-h or 168-h toxicity tests. The effects of silver on ion regulation in trout were evaluated throughout the exposures using various physiological parameters, which included plasma Na^+ and Cl^- concentrations, whole-body Na^+ uptake rates, and gill Na/K-ATPase activities.

Materials and methods

Experiment 1

Fish holding conditions

Approximately 1500 juvenile rainbow trout were purchased from Humber Springs Trout Hatchery (Orangeville, Ont., Canada). All fish were initially mixed in one 450-l tank and then randomly transferred to three separate 450-l holding tanks. Each holding tank contained approximately 500 fish. Holding tanks were supplied with approx. 2,400 ml/min of aerated, dechlorinated Hamilton tap water (from Lake Ontario). The measured water chemistry included: $[Na^+] = 0.6$; $[Cl^-] = 0.7$; $[Ca^{2+}] = 1.0$; $[HCO_3^-] = 1.9$ mM; titratable alkalinity to pH 4.0 = 1.9 mM; total hardness as $CaCO_3 = 120$ ppm; pH 8.0 and natural organic matter at 1.3 mg/l measured as dissolved organic carbon; temperature = 14.5–15.5°C. Photoperiod was kept at 14 h light:10 h dark. Fish were fed to satiation once daily with dry trout pellets (Martin Feed Mills, Elmira, Ont., Canada).

Silver exposures

Trout were maintained for 5 weeks under laboratory holding conditions prior to experimentation. In order to distinguish between treatments fish were assigned a unique marking with Alcian blue dye using a Panjet injector (Wright Health Group; Dundee, U.K.). During the marking procedure fish were lightly anesthetized with 0.1 g/l MS-222 buffered with 0.2 g/l sodium bicarbonate and allowed to recover in fresh water before being placed back in the holding tanks. All fish were marked 2 weeks before the start of the experimental exposures. At the start of the experiment concentrated silver stock was added to two of the holding tanks to immediately achieve nominal total Ag concentrations of 0.1 and 1 μg/l Ag. One treatment of fish was maintained at control conditions. Silver, as $AgNO_3$ (Sigma, St. Louis, Mo., USA), was continually added to holding tanks via a Marriott bottle metering system. Each silver stock was made at 6,000-fold the concentration of the respective exposure tank and acidified to 0.5% (v/v) with trace metal grade HNO_3 acid (Fisher, Nepean, Ont., Canada). Concentrated silver stocks were shielded from light and replenished every 3 days. Silver stocks were delivered at 0.5 ml/min to header tanks, and diluted with 3,000 ml/min dechlorinated Hamilton tap water. Each header tank was aerated with an air stone to adequately mix the silver before delivery to the experimental tanks. Silver-amended water was supplied to each experimental tank at 2,400 ml/min; the remainder of the water in the header tank was allowed to overflow to waste. Total Ag concentrations were measured daily. The measured Ag concentrations were control tank = below detection (0.05 μg/l); low silver exposure = 0.20 ± 0.02 μg/l ($n = 24$); high silver exposure = 1.04 ± 0.43 μg/l ($n = 24$).

Experiment 2

Fish holding conditions

Approximately 1,350 juvenile rainbow trout were purchased from Humber Springs Trout Hatchery and held under similar conditions as those of experiment 1. At the time of arrival, ambient water temperature was only 5°C. In order to maintain consistency between the studies water temperature was gradually increased to 15°C over a period of 2 weeks. Fish were held for 7 weeks before the start of the experiment.

Silver exposure

Silver was added to tanks in a similar fashion to experiment 1, except that nominal total Ag concentrations were 3 and 5 μg/l Ag. A simultaneous control treatment was also included in experiment 2. The measured Ag concentrations were: control tank = below detection (0.05 μg/l); low silver exposure = 3.02 ± 0.10 μg/l ($n = 24$); high silver exposure = 4.81 ± 0.28 μg/l ($n = 24$).

Experimental protocol

Food consumption, indices of growth, and mortality

Fish were hand-fed to satiation once a day at 17:00 hours using the same protocol as Galvez et al. (1998). The amount of food consumed daily was recorded and used to calculate cumulative food consumption per fish. Mean fish weights ± SE were calculated from the recorded weights of fish sampled for tissues, Na^+ uptake fluxes, and swimming tests on days 0, 5, 10, 15, and 23. Specific growth rates (SGR) in percentages per day were calculated for each treatment from the slope of the least-squares regression through the natural logarithm (ln) transformed weight versus time data (SPSS, version 8.0). The amount of food consumed at satiation was expressed as percentage of mean fish weight per day. Estimates of average fish weight were obtained using the SGR equations. FCE (in percentage) for each treatment is the ratio of mean SGR (percentage per day) and daily food consumed (percentage per day), multiplied by 100. Mortality was recorded daily, and dead fish were removed as soon as observed. To correct for the removal of fish by sampling, cumulative mortality was expressed as a percentage of the total number of fish in the tank at any time.

Tissue sampling

Fish were sampled on days 0, 5, 10, 15, and 23. The day 0 tissue sampling was performed prior to spiking the tanks with Ag. Ten

fish per treatment were analyzed for a variety of physiological parameters. Fish were randomly selected and immediately killed by a quick cephalic blow. Blood was taken by caudal puncture using 1-ml syringes prerinsed with ammonium heparin (50 IU/ml). Blood was centrifuged at 10,000 *g* for 2 min, and plasma was collected for analysis of total Na^+ and Cl^-. Plasma Na^+ was analyzed by flame atomic absorption spectrophotometry (AAS; Varian 1275), whereas plasma Cl^- was measured using the mercuric thiocyanate method (Zall et al. 1956), adapted for use on a microtiter plate reader. Entire gill baskets were excised from fish, rinsed in 18 M double-deionized water, blotted dry, and stored at –70°C until analyzed for Na/K-ATPase activity. Gill Na/K-ATPase analysis was performed using the UV detection method of McCormick (1993). Briefly, an enzymatically coupled reaction was employed in which each mole of ATP hydrolyzed by ATPase enzymes would result in conversion of NADH to NAD^+. Absorbance change due to NADH oxidation was measured at 340 nm at 15-s intervals over a 10-min period, or until the reaction substrates became depleted. Na/K-ATPase was calculated as the difference in ATP hydrolosis in the absence and presence of oubain (Sigma). Gill Na/K-ATPase activity was normalized against total gill protein, as measured by the Bradford (1976) method using a commercial dye reagent (Sigma).

Unidirectional sodium fluxes

Unidirectional sodium fluxes were measured using radioisotopic ^{22}Na (Mandel Scientific, Guelph, Ont., Canada) over a 4-h period on days 0, 5, 10, 15, and 23. For each treatment group, all fluxes (including day 0) were performed at the nominal Ag exposure concentrations (0, 0.1, or 1 µg/l Ag for experiment 1, or 0, 3, and 5 µg/l Ag for experiment 2). Ten fish per treatment were transferred from their experimental tanks to 11-l polyethylene containers containing 5 l aerated water at the appropriate Ag concentration. Fish were placed in chambers 1 h before the start of fluxes. At the start of each flux 4.5 µCi ^{22}Na (as NaCl in H_2O) was added to each chamber and allowed to equilibrate for 5 min. After equilibration and again 5 min before the end of the 4-h period 4×5 ml water samples were taken for analysis of total Na and Ag, and ^{22}Na radioactivity. Total Na and Ag were analyzed by flame AAS and graphite furnace AAS (Varian GTA-95), respectively, whereas ^{22}Na radioactivity was measured by γ-counting (Minaxi γ; Canberra-Packard, Meridan, Conn., USA). After the 4-h flux period fish were given an overdose (approx. 1 g/l) of MS-222 and rinsed for 1 min in a 7 mmol/l NaCl solution to displace superficially bound radioactivity from the fish. Fish were blotted dry, and their weights and lengths were recorded before being stored in scintillation vials for γ-counting. The unidirectional Na fluxes were calculated in nanomoles per gram per hour as:

$$\mathrm{Jin} = \frac{\mathrm{FishCPM}}{\mathrm{MSA} \cdot \mathrm{W} \cdot \mathrm{t}} \tag{1}$$

in which MSA is the mean specific activity of Na in the water, W is the weight of fish in grams, and t is time in hours. Note that the MSA can be calculated as:

$$MSA = \frac{\frac{\mathrm{CPM_i Na}}{[\mathrm{Na}]_\mathrm{i}} + \frac{rmCPM_\mathrm{f}\mathrm{Na}}{[\mathrm{Na}]_\mathrm{f}}}{2} \tag{2}$$

in which cpm_i Na and cpm_f Na are the radioactivity of the initial and final water samples in counts per minute per milliliter of ^{22}Na, and $[Na]_i$ and $[Na]_f$ are the total Na concentrations for the initial and final water samples, respectively, in nanomoles per milliliter.

Toxicity testing

Toxicological acclimation to silver (i.e., increased tolerance to acute silver exposure) was assessed using either 96-h or 168-h toxicity tests. To allow for direct comparison of silver sensitivity between treatments marked fish from all exposure groups in an experimental series were tested in the same container (i.e., one container per concentration).

In experiment 1, 96-h toxicity tests were performed starting on days 0, 5, 10, 15, and 23. For experiment 2, it was necessary to extend the exposure period to 168- h in order to observe incipient lethal limits for control treatments. Consequently 168-h tests could only be performed starting on days 5, 15, and 23, to avoid overlap. For each toxicity test five Ag concentrations plus a simultaneous control were used. At the start of the tests 60 fish were removed from each exposure tank and randomly distributed into the six test containers. $AgNO_3$ stocks were delivered at 0.5 ml/min using Marriott bottles. Ag stocks were diluted with approx. 500 ml/min Hamilton dechlorinated water and mixed by gentle aeration prior to being gravity fed to 19-l covered containers on a flow-through basis. The nominal Ag concentrations used for the lethality tests included: 10, 18, 32, 56, and either 5.6 or 100 µg/l (nominal values). Water samples were taken daily, acidified to 0.5% (v/v) with HNO_3 acid, and analyzed for total Ag by graphite furnace AAS. Mortalities were monitored during the course of the 96 or 168 h. Fish were not fed during acute toxicity testing. Cessation of opercular movement and lack of response to gentle prodding were the criteria for death. Dead fish were removed as soon as observed and their treatment and time of death recorded. The 96-h and 168-h LC_{50} values ±SE were calculated by log probit analysis using measured total aqueous Ag concentrations (SPSS, version 8.0).

Critical swimming speed

In experiment 1 swimming tests were performed on days 0, 5, 10, 15, and 23 of exposures, whereas for experiment 2 swimming tests were conducted only on days 5, 10, and 15. Fish were tested at their nominal Ag concentrations. An additional series of swimming tests was conducted on day 6 (experiment 2 only) using the same fish as on day 5 except with no Ag present in the tunnel. Swimming tests were performed in a modified 150-l Beamish-style swimming tunnel. Water flow rate was calibrated prior to use with a Kent Miniflow Type 265 flowmeter. Fish (ten per treatment) were placed in the swim tunnel and allowed to adjust to their surroundings for 45 min at a velocity of 10 cm/s. Swimming tests involved increasing the water velocity by 7.5 cm/s every 40 min until the fish exhausted and would no longer swim when reintroduced into the current. At the completion of the test fish were blotted dry, weighed, and measured for total length. Fish tested on day 5 of experiment 2 were lightly anesthetized (0.1 g/l MS-222, buffered with 0.2 g/l $NaHCO_3$) and were recorded for their weights and lengths. These fish were compartmentalized in their respective holding tanks and tested again on day 6, with no Ag present. Critical swimming speed was calculated as follows (Brett 1964):

$$\mathrm{Ucrit}(\mathrm{cm} \cdot \mathrm{s}^{-1}) = \mathrm{V_f} + \left[\left(\frac{\mathrm{T}}{\mathrm{t}}\right) \times \mathrm{dV}\right] \tag{3}$$

in which V_f is the final velocity the fish was able to swim for the complete time (t) of 40 min, T is the time the fish was able to swim at the velocity causing exhaustion, and dV is the velocity increment (cm/s). U_{crit} was normalized to body lengths per second by dividing U_{crit} (in centimeters per second) by the total length of the fish (in centimeters).

Statistics

Data are expressed as mean ±standard error (N). SGR values for silver treatments were statistically compared to simultaneous controls using an unpaired, two-tailed *t* test. The α level was modified using Bonferroni's correction to allow for multiple comparisons. All other data were tested using a one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons. All data sets were tested for normality and homogeneity of variances prior to performing a one-way analysis of variance. A *P* value of 0.05 was considered statistically significant throughout.

Results

Effects of silver exposure

A cumulative mortality of 5.2% was obtained following exposure for 23 days to 5 μg/l Ag. Fish were most susceptible to the silver exposure between days 5 and 15, when mortality rates averaged 0.58% per day. In comparison, less than 1.6% cumulative mortality occurred in the other silver treatments after 23 days. Control mortalities were 0.6% and 0.2% during experiments 1 and 2, respectively.

Cumulative food consumption per fish was 23% lower in the 5 μg/l Ag treatment than it was in the control (Fig. 1). These fish were significantly smaller than were controls on days 10 and 23, a difference that reached 22.2% by the latter day (Fig. 2). The SGR values tended to be slightly higher in experiment 1 than in experiment 2 (Table 1). Exposure to 5 μg/l Ag significantly inhibited SGR by 70%. There were no other significant differences. Daily ration consumed (when expressed as percentage body weight per day) was not significantly different between treatments, whereas FCE in the 5 μg/l Ag treatment was 58% lower relative to controls due to the significant reduction in SGR in this group (Table 1).

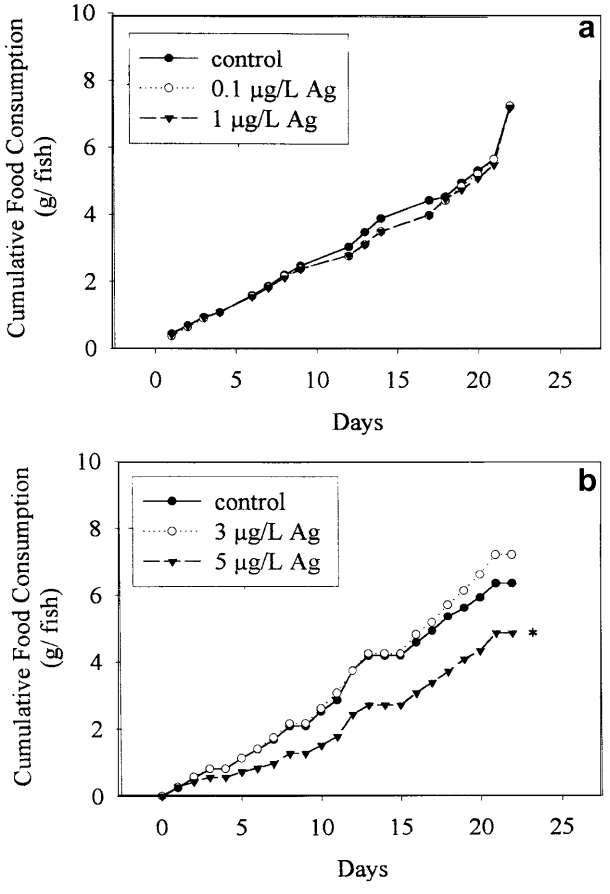

Fig. 1. Cumulative food consumption in juvenile rainbow trout during 23-day exposures to 0, 0.1, and 1 μg/l Ag (**A**) and 0, 3, and 5 μg/l Ag (**B**). Fish were fed to satiation once daily. Data expressed as the average amount of food consumed cumulatively per fish. $*P < 0.05$ vs. controls

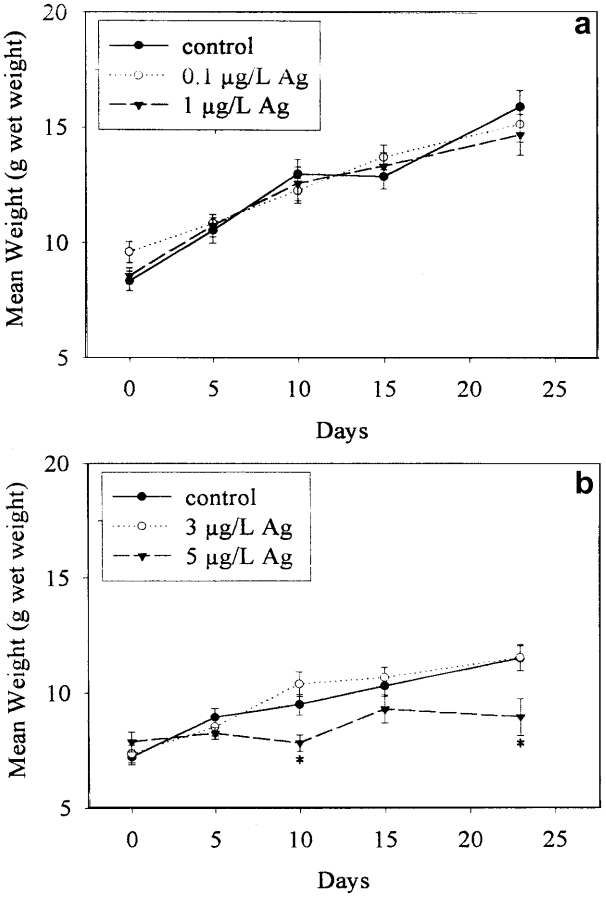

Fig. 2. Mean body wet weight of juvenile rainbow trout on days 0, 5, 10, 15, and 23 for fish exposed to 0, 0.1, and 1 μg/l Ag (**A**) and 0, 3, and 5 μg/l Ag (**B**). Values are expressed as means ±SE ($n = 30$; except for days 0 and 23 of **B**, for which $n = 20$). $*P < 0.05$ vs. controls

Lethal challenges with silver

Fish subjected to either 0.1 or 1 μg/l Ag for 23 days did not develop increased tolerance to acutely lethal challenges of silver (Fig. 3A). The 96-h LC_{50} values for the control fish (days 5, 10, 15, 23) ranged between 7.6 ± 0.6 and 15.1 ± 2.8 μg/l Ag. The 168-h LC_{50} values for control fish (days 5, 15, and 23) ranged between 9.9 ± 1.6 and 22.4 ± 7.6 μg/l Ag (Fig. 3B). Fish that had been chronically exposed to 5 μg/l Ag appeared to be more tolerant to acute silver exposure on days 15 and 23 (at least twofold higher 168 h LC_{50}). Unfortunately, accurate estimates of LC_{50} could not be obtained for this treatment because fewer than 50% of the fish died at the highest Ag concentrations (10% mortality at 40.4 μg/l Ag on day 15 and 30% mortality at 53.6 μg/l Ag on day 23). The LC_{50} value of fish exposed to 3 μg/l

Table 1. Specific growth rate (*SGR*), ration of food consumed daily, and food-conversion efficiency (*FCE*) of rainbow trout exposed to 0, 0.1, and 1 μg/l Ag in experiment 1, and 0, 3 and 5 μg/l Ag in experiment 2. SGR is the slope ±SE for the least-squares regression through the ln-transformed wet weight versus time data. Ration is the mean ±SE. FCE is SGR/ration

	SGR (% per day)	Ration (% per day)	FCE (%)
Control 1	2.65±0.45	3.38±0.49	78.4
0.1 μg/l Ag	2.04±0.18	4.05±0.69	50.4
1 μg/l Ag	2.29±0.44	3.54±0.58	64.7
Control 2	1.86±0.30	4.02±0.36	46.3
3 μg/l Ag	1.99±0.41	4.39±0.24	45.3
5 μg/l Ag	0.66±0.33*	3.37±0.40	19.6

*$P<0.05$ vs. control. There were no significant differences in ration within each experiment

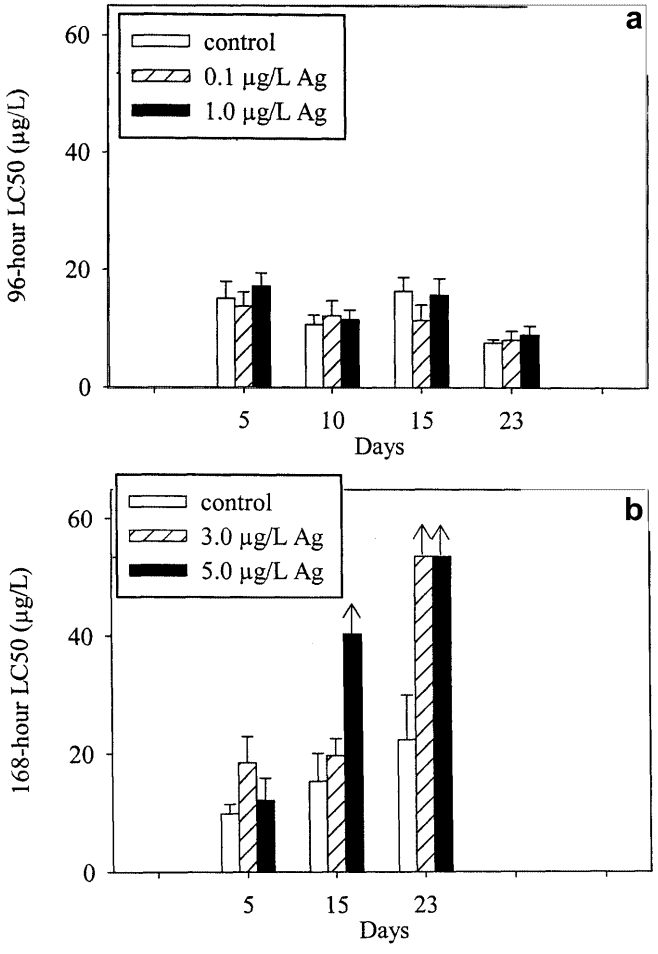

Fig. 3. **A** 96-h LC_{50} values of juvenile rainbow trout exposed to 0, 0.1 and 1 μg/l Ag for 5, 10, 15, and 23 days. **B** 168-h LC_{50} values of juvenile rainbow trout exposed to 0, 3 and 5 μg/l Ag for 5, 15, and 23 days. On the latter 2 days LC_{50} values were greater than the values *with arrows*, but absolute values could not be calculated due to mortalities of 50% or less. Values are expressed as LC_{50} value ±SE derived from log probit analysis of toxicity curves ($n=60$)

Ag also appeared to be slightly higher than the controls on day 23. However, a true estimate of LC_{50} could not be obtained for this treatment since only 50% mortality was produced at the highest silver concentration.

Effects of silver on ion regulation

Silver exposure up to 3 μg/l produced no significant differences in plasma Na^+ compared with controls over 23 days apart from a small reduction in plasma Na^+ concentration in the 0.1 μg/l Ag treatment at day 15 (Fig. 4A). Nonetheless, plasma Na^+ concentrations in the 1 μg/l and 3 μg/l Ag treatments tended to decrease on days 5 or 10, with recovery thereafter. Exposure to 5 μg/l Ag had the most marked influence on plasma Na^+ concentration (Fig. 4B). Plasma Na^+ concentrations were 23% and 18.3% lower than found in controls ($P<0.05$) on days 5 and 10, respectively. This ionoregulatory disturbance was transient in nature, and plasma Na^+ returned to control concentrations by day 15.

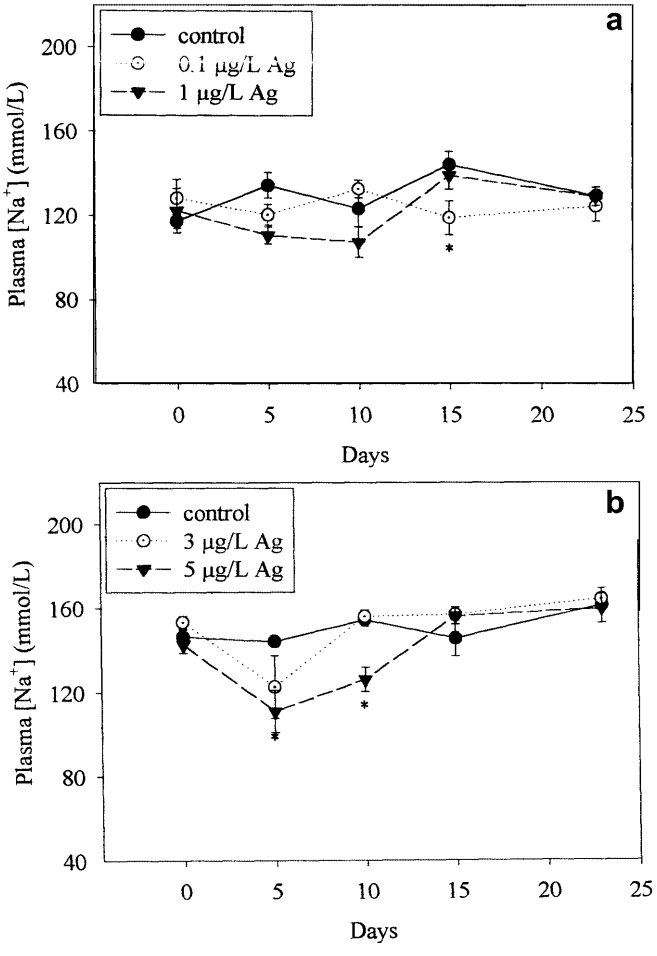

Fig. 4. Plasma Na^+ concentrations in juvenile rainbow trout exposed for 0, 5, 10, 15, and 23 days to 0, 0.1, or 1 μg/l Ag (**A**) and 0, 3, or 5 μg/l Ag (**B**). Values are expressed as means ±SE ($n=10$). *$P<0.05$ vs. controls

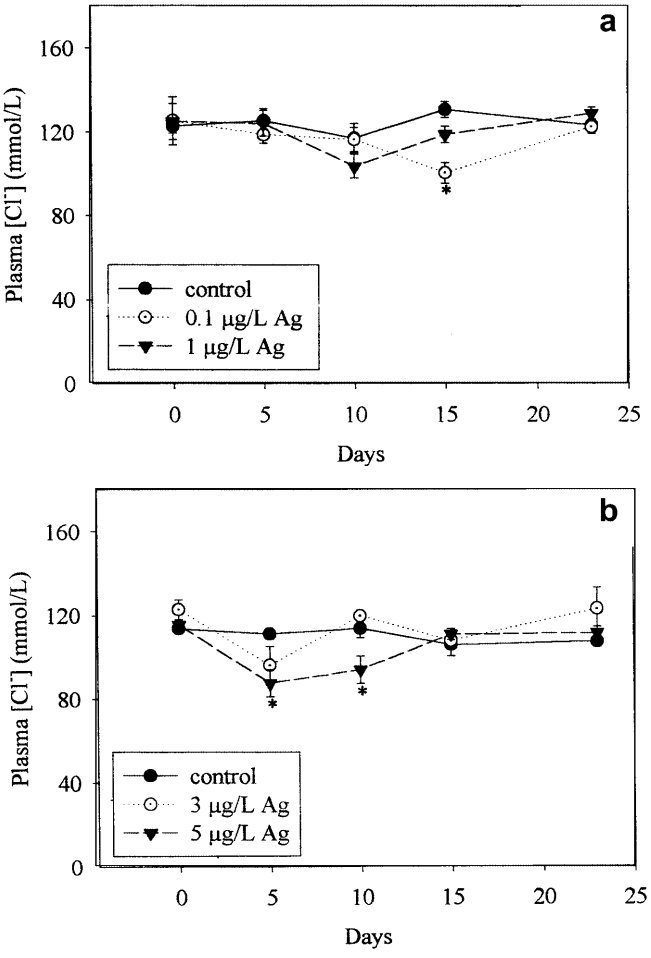

Fig. 5. Plasma Cl^- concentrations in juvenile rainbow trout exposed for 0, 5, 10, 15, and 23 days to 0, 0.1, or 1 µg/l Ag (**A**) and 0, 3, or 5 µg/l Ag (**B**). Values are expressed as means ± SE ($n = 10$). *$P < 0.05$ vs. controls

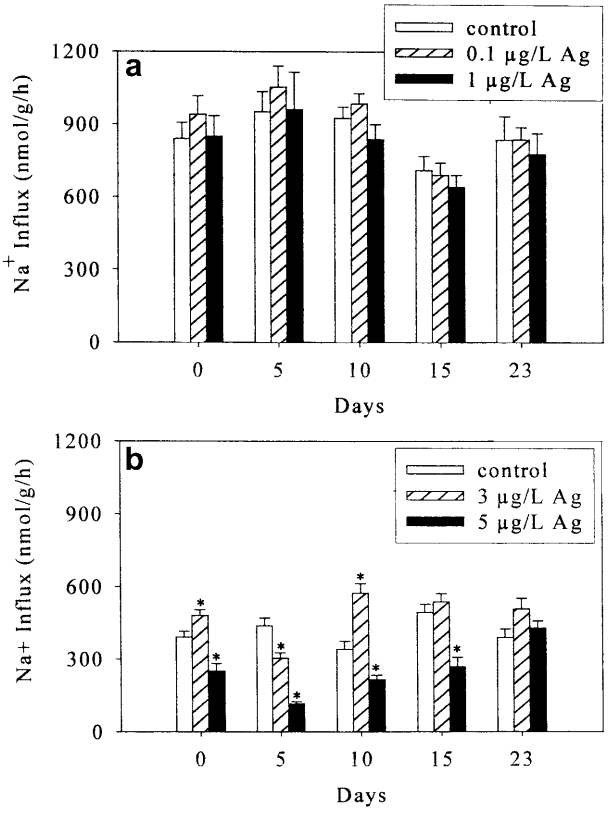

Fig. 6. Na^+ influx rates in juvenile rainbow trout following exposure for 0, 5, 10, 15, and 23 days to 0, 0.1, or 1 µg/l Ag (**A**) and 0, 3, or 5 µg/l Ag (**B**). Day 0 measurements were performed at nominal Ag concentrations given above. Values are expressed as means ± SE ($n = 10$). *$P < 0.05$ vs. controls

Silver exposure had similar transient effects on plasma Cl^- concentrations (Fig. 5). The mean plasma Cl^- concentration was significantly reduced only in fish exposed to 0.1 µg/l (on day 15) and 5 µg/l Ag (on days 5 and 10). The most dramatic effects on plasma Cl^- were noted in the 5 µg/l Ag treatment, with a 21% reduction by day 5 and a 17% decrease in concentration by day 10 ($P < 0.05$).

Sodium influx rates of control fish ranged from 710 ± 58 to 920 ± 47 nmol/g/h for experiment 1 (Fig. 6A) and from 340 ± 33 to 490 ± 35 nmol/g/h for experiment 2 (Fig. 6B). The higher influx rates in series 1 were likely associated with the lower concentrations of plasma Na^+ in these fish (Fig. 4), but the reason for this difference is unknown. Silver exposure at 0.1 and 1 µg/l did not significantly affect sodium uptake rates in trout (Fig. 6A). In contrast, Na^+ uptake was significantly inhibited on days 0 (in the presence of Ag), 5, 10, and 15 of the 5 µg/l Ag treatment (Fig. 6B). The most pronounced effect of Ag exposure on Na^+ uptake was seen on day 5 when its rate was decreased by 74% from the control value. By day 23 Na^+ uptake rates in the 5 µg/l Ag group returned to control rates. Exposure to 3 µg/l Ag first slightly stimulated and then inhibited Na^+ uptake by 30% compared to control values at day 5. Na^+ uptake rates in the 3 µg/l Ag treatment increased thereafter to rates either similar to or significantly higher than control rates.

Mean Na/K-ATPase activity in crude gill homogenates of nonexposed control trout ranged between (in µmol P/mg/h): 1.50 ± 0.10 and 2.50 ± 0.16 for experiment 1 and 1.06 ± 0.10 and 1.40 ± 0.20 for experiment 2 (Fig. 7). Na/K-ATPase activities were significantly lower in the 0.1 µg/l and 1 µg/l Ag treatments relative to controls on day 0 or 15 (Fig. 7A), whereas there was a significant increase in the rates of Na/K-ATPase in fish in the 0.1 µg/l Ag treatment on day 10. However, the Na/K-ATPase activities of gills sampled in exposure 1 varied considerably, and there were no discernable trends in the low level Ag exposure. In comparison, the Na/K-ATPase activities of control fish from experiment 2 were relatively constant. Exposure to 3 and 5 µg/l Ag resulted in a significant inhibition of the enzyme on day 5, followed by a recovery to control concentrations by day 10 (Fig. 7B). On days 15 and 23 Na/K-ATPase activities of both silver treatments were significantly elevated above controls.

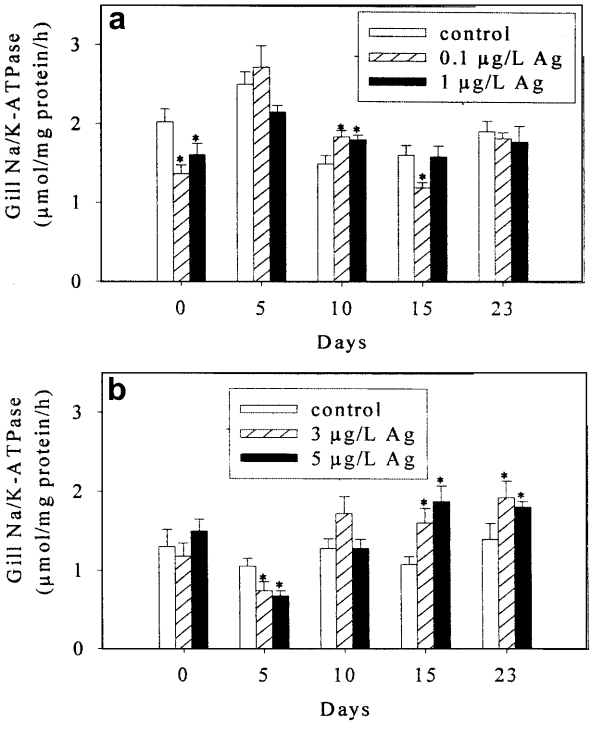

Fig. 7. Na/K-ATPase activity in gill filaments of juvenile rainbow trout following exposure for 0, 5, 10, 15, and 23 days to 0, 0.1, or 1 µg/l Ag (**A**) and 0, 3, or 5 µg/l Ag (**B**). Values are expressed as means ±SE. ($n=10$). *$P<0.05$ vs. controls

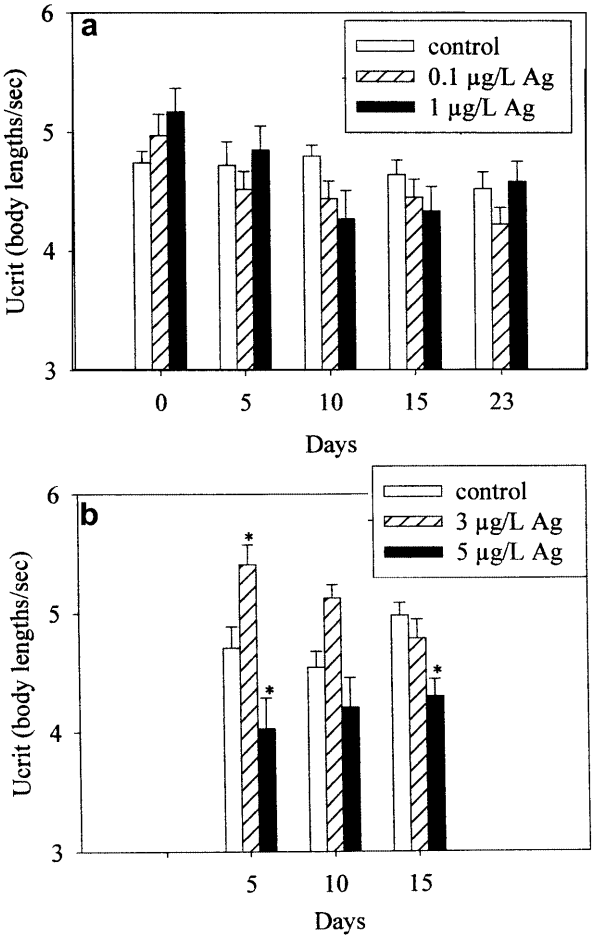

Fig. 8. Critical swimming speeds (U_{crit}) of juvenile rainbow trout after exposure for 0, 5, 10, 15, and 23 days to 0, 0.1, or 1 µg/l Ag (**A**) or for 5, 10, and 15 days for fish exposed to 0, 3, or 5 µg/l Ag (**B**). Fish were tested in the presence of Ag at nominal concentrations. Values are expressed as means ±SE ($n=10$). *$P<0.05$ vs. controls

Critical swimming speed

A critical swimming speed test was used to assess possible physiological costs associated with chronic exposure and acclimation to silver. Exposure to 0.1 and 1 µg/l Ag produced no significant effects on critical swimming speed relative to simultaneous controls (Fig. 8A). In comparison, U_{crit} was raised by 15% on day 5 at 3 µg/l, and lowered by 14% on days 5 and 15 ($P<0.05$) in the 5 µg/l Ag group. Fish tested on day 5 at their respective nominal Ag concentrations were subjected to an additional swimming trial on day 6 in Ag-free water. No significant differences were seen in the swim performances of fish from each treatment (control, 3 and 5 µg/l) between days 5 and 6 (Table 2). Nonetheless, the significant differences on day 5 in U_{crit} values of the 3 µg/l and 5 µg/l Ag treatments relative to controls, were no longer present on day 6 (when tested without Ag), although the general trends appeared to be similar.

Table 2. Critical swimming speeds (U_{crit}; body lengths/s) of juvenile rainbow trout on days 5 and 6 of exposure to 0, 3, or 5 µg/l Ag. On day 5 the swimming test was performed in the presence of Ag and on day 6 in the absence of Ag. The same groups of ten fish per treatment were used during both testing periods; values are expressed as means ±SE ($n=10$)

	Control	3 µg/l Ag	5 µg/l Ag
Day 5 (Ag present)	4.71 ± 0.18	5.41 ± 0.18*	4.03 ± 0.26*
Day 6 (no Ag present)	4.68 ± 0.23	5.14 ± 0.11	4.21 ± 0.24

*$P<0.05$ vs. controls. No significant differences were noted between treatments on day 6 or between days 5 and 6 within treatments

Discussion

Environmental relevance of experiment

Silver concentrations of 0.1, 1, 3, and 5 µg/l Ag were chosen in this study based on their environmental relevance and regulatory importance. The lowest silver concentration tested (0.1 µg/l Ag) represents the upper range (0.01–0.10 µg/l Ag) of silver concentrations recently monitored using "clean" techniques for surface water near United States urban centers (Shafer et al. 1998). Moreover, a chronic guideline close to 0.1 µg/l Ag has been proposed or implemented in Europe (RIVM 1999), Australia (NWQMS 1999), and Canada (CCME 1999) and has been adopted as the water quality objec-

tive in the Canadian provinces of Manitoba and Ontario (CCME 1999). The 1 and 3 μg/l Ag treatments were tested to address the hardness-based criterion recently promulgated in British Columbia (BC MOELP 1995). This guideline stipulates that at hardness exceeding 100 mg/l, as $CaCO_3$ (i.e., comparable to the Lake Ontario water used in the present study; 120 mg/l), a 30-day average (chronic) limit of 1.5 μg/l Ag, and a criterion maximum concentration (acute) of 3 μg/l Ag is enforceable. Finally, the 5 μg/l Ag treatment tests the effects of Ag at the hardness-based US EPA Ambient Water Quality Acute Criterion for total recoverable silver in Lake Ontario water (i.e., approx. 5.5 μg/l Ag at 120 mg/l as $CaCO_3$; US EPA 1980).

Physiological acclimation

Exposure to 5 μg/l Ag produced approximately equimolar reductions in both plasma Na^+ and Cl^-, with ion losses of approx. 22% and 18%, respectively on days 5 and 10 (Figs. 4, 5). Nonetheless, impairment in ion balance was short-lived, and plasma Na^+ and Cl^- returned to control concentrations by day 15. The ionoregulatory disturbance produced in the 3 μg/l Ag treatment was less pronounced than seen in the 5 μg/l Ag treatment, although it followed the same general trend. These findings are consistent with the study of Galvez et al. (1998), which demonstrated physiological acclimation in response to 28-day exposures at 0.5 and 2 μg/l Ag. Fish experienced reductions in plasma concentrations of approx. 18% for Na^+ and 12% for Cl^- by day 7 of exposure to 2 μg/l Ag (Galvez et al.1998). In comparison, only moderate changes in plasma Na^+ and Cl^- were observed during exposure to either 0.1 or 1.0 μg/l Ag. Furthermore, these effects were difficult to ascertain due to the high variability of the controls.

Toxicological acclimation

The present study is the first to demonstrate an increased tolerance to a lethal Ag exposure resulting from preexposure to the metal. Toxicological acclimation was elicited at a threshold of about 13% of the control LC_{50} value (i.e., at 3 μg/l Ag). Toxicological acclimation was not produced below 3 μg/l Ag, despite the fact physiological acclimation had occurred. The threshold required to elicit toxicological acclimation in terms of total Ag (as a percentage of the LC_{50} value) is consistent with that seen for other metals. In general, threshold concentrations ranged from 8% to 18% of control LC_{50} values in Cu (Dixon and Sprague 1980), Zn (Alsop et al. 1999; Bradley and Sprague 1985), and Cd (Hollis et al. 1999). Acclimation to Cu is especially relevant to the discussion here because Cu and Ag share similar mechanisms of acute toxicity in freshwater fish. Both metals impair branchial Na^+ uptake and reduce plasma Na^+ concentration due to inhibition of gill Na/K-ATPase activity (Laurén and McDonald 1987a, 1987b; Morgan et al. 1997).

Fish tolerance to silver was increased at least twofold following exposure to either 3 or 5 μg/l Ag for 23 days. Since only 50% and 10% mortality rates, respectively, were obtained at the highest total Ag concentrations (i.e., 40.4–53.6 μg/l), it was impossible to calculate exact LC_{50} values for these groups. However, if we assume that the LC_{50} was greater than the highest total Ag concentration used, we can at least suggest that toxicological acclimation had occurred in these treatments. In addition, the relative magnitude of the increase in LC_{50} appears to be in line with values reported for Cu (Dixon and Sprague 1980) and Zn (Alsop et al.1999), but about fivefold less than was reported for Cd (Hollis et al.1999). Since cumulative mortality did not exceed 5.2% in any pretreatment during 23 days, the acclimation responses seen here likely represent real compensatory changes, rather than preselection for tolerant fish.

The mechanistic basis for silver acclimation

The present study is the first to demonstrate that the inhibitory action of Ag^+ on Na/K-ATPase activity in vivo is transient (Fig. 7). Although exposure to 3.0 and 5.0 μg/l Ag resulted in significant reductions in Na/K-ATPase activities, the enzyme activities eventually recovered and were in fact significantly increased above control levels on days 15 and 23 of Ag exposure. This is similar to the response reported during chronic sublethal exposure to Cu (Laurén and McDonald 1987a, 1987b). In comparison, plasma Na^+ (Fig. 4B) and unidirectional Na^+ influx (Fig. 6B) in fish acclimated to 5.0 μg/l Ag recovered only to control levels by day 15 and day 23, respectively. Fish acclimated to 3.0 μg/l Ag showed slightly different trends, with Na^+ influx significantly elevated by day 10 before stabilizing back to control levels (on days 15 and 23). Although the occurrence of physiological acclimation during exposure to the high concentrations of Ag (experiment 2) was quite apparent, these trends were much more difficult to discern in the low-level Ag exposures (experiment 1). The concentrations of plasma Na^+ and Cl^- and Na/K-ATPase activities in the control group varied considerably over time making it difficult to observe effects in the Ag-treated groups. Clearly, these ionoregulatory parameters are difficult to interpret at such low concentrations of Ag exposure.

It is unclear why little agreement exists between the recoveries of Na/K-ATPase activities, plasma Na^+ concentrations and unidirectional Na^+ influx, with the recovery of each parameter back to control levels requiring varying lengths of time. Recently Bury and Wood (1999) found evidence that silver, likely as Ag^+, is taken up across the apical membrane of gill epithelia via a Na^+ channel coupled to a V-type H^+-ATPase. Furthermore, silver taken up via this Na^+ transport mechanism was found to competitively inhibit Na^+ transport, at least at very high concentrations of Na^+.

Acclimation could potentially involve an alteration in the affinity of the Na^+ channel in order to favor Na^+ transport over Ag^+. Although this explanation is speculative, an analogous mechanism has been proposed for rainbow trout acclimated to low concentrations of zinc (Hogstrand et al. 1994, 1995). Zinc acclimation has been shown to reduce the affinity (increases the K_m) of specific Ca^{2+} channels that are also known to transport calcium and zinc across the apical membrane. Hogstrand et al. (1994, 1995) have suggested that increasing the K_m of Ca^{2+}/Zn^{2+} transport via this specific channel would significantly decrease zinc uptake into the cell, while having little effect on Ca^{2+} transport. It seems logical that preventing Ag^+ from entering the gill cells would help to protect Na/K-ATPase transporters. If not, any increase in Na/K-ATPase transporters for the purpose of alleviating the ionoregulatory impairment would be futile, since these transporters too would be susceptible to Ag^+. Morgan et al. (1997) have demonstrated that the inhibition of Na/K-ATPase by Ag^+ is quickly reversed once fish are placed in Ag-free water. A physiological acclimation response involving a reduction in the apical entry of Ag^+ (as previously discussed) would be analogous to transferring the fish to Ag-free water. Therefore it might be expected that Na/K-ATPase activity would recover once apical Ag^+ transport were reduced (i.e., initial changes to apical Na^+ transporter), whereas recovery in Na^+ influx would require further changes to the putative apical Na^+ channel for it to resume normal Na^+ transport (despite the continued presence of Ag^+). Future studies should address the exact cause of the increase in Na/K-ATPase activity and delayed recovery in Na^+ influx in Ag acclimated fish.

To this point, our discussion has revolved around the impact and changes of silver acclimation on branchial Na^+ influx mechanisms. Nonetheless, there is some evidence that reductions in Na^+ efflux may also contribute to the silver acclimation response. Morgan et al. (1997) demonstrated that Na^+ efflux was reduced during short-term silver exposure. They proposed that this response involved a progressive decrease in the diffusive gradient for Na^+ efflux as a result of the low osmolarity of the blood. As mentioned above, Na^+ influx rates in the 5 μg/l Ag treatment were reduced up until day 15 (Fig. 6) whereas plasma Na^+ concentration was reduced only to day 10 (Fig. 4). These data suggest that at least part of the recovery in plasma electrolyte balance was elicited by a reduction in Na^+ efflux. In addition to a reduction in the diffusive gradient, either a general reduction in the permeability of the gill epithelium or increased retention of Na^+ by the kidneys (as seen with Cu^-, e.g., Grosell et al. 1998) could explain the decrease in Na^+ efflux from fish.

The physiological costs of silver acclimation

This study incorporated a controlled feeding regime in order to decipher the effects and costs of Ag acclimation on maximal food consumption rate (appetite) and FCE. Although fish from all silver treatments underwent physiological and/or toxicological acclimation, only the 5 μg/l Ag group showed any physiological cost of silver acclimation. Reductions in SGR and FCE (Table 1), as well as increased mortality during acclimation were seen in the 5 μg/l Ag treatment alone. The reduction in overall food consumption in fish exposed to 5 μg/l Ag (Fig. 1), together with the lack of effect of this Ag treatment on ration (i.e., food consumption normalized to body weight), implies that the reduction in total food consumed is due to the decrease in the body mass of the group and not due to a change in appetite per se. This is contrary to the effects of sublethal exposure to Al (Wilson and Wood 1992; Wilson et al. 1996), in which appetite was suppressed to the extent that FCE appeared enhanced. SGR remained impaired throughout the entire 23-day period, in contrast to the lack of persistent SGR impairment seen during acclimation to other metals (Alsop et al. 1999; Dixon and Sprague 1980; Hollis et al. 1999; Wilson et al. 1996). It is generally believed that the greatest cost of metal acclimation is imposed during the first 7–15 days of exposure, during which time the acclimation response is typically being established (McDonald and Wood 1993). In the present study it appears that the greatest cost of silver acclimation may be associated with maintaining the response over time, rather than its initial development.

Reduction in the U_{crit} of fish acclimated to 5 μg/l Ag suggests that aerobic capacity in these fish was affected (Fig. 8). Reduction in U_{crit} appeared to be independent of the presence or absence of silver during testing (Table 2), suggesting that Ag acclimation represented a real physiological cost to these fish and was not simply an irritant. Critical swimming speed was significantly lowered on day 5 and thereafter recovered only slightly. A similar response has been noted for Al (Wilson and Wood 1992), Zn (Alsop et al. 1999), and Cu (at low pH) (Waiwood and Beamish 1978) and may represent an inescapable cost of metal acclimation. U_{crit} can be affected by a number of factors. Generally, factors that decrease the maximum rate of oxygen uptake are termed "limiting stressors," whereas factors that decrease aerobic scope by increasing metabolic costs are termed "loading stressors" (Brett 1964). Typically, the diagnosis employs oxygen uptake measurements at rest and at different swimming speeds. Nonetheless, the fact that U_{crit}, SGR, and FCE were affected throughout the whole course of the exposure suggests that silver acted as a loading stressor. At present there is no experimental evidence available to assess whether silver also acted as a limiting stressor. However, metals such as Al (Wilson et al. 1994) and Cu (Waiwood and Beamish 1978) are known to be both loading and limiting stressors. McDonald and Wood (1993) have proposed that acclimation to metals involves a damage/repair process, and that it occurs only at concentrations high enough to elicit damage to the gills. According to Mallatt (1985), necrosis of the gill epithelium, mucus hypersecretion,

and branchial epithelial cell hypertrophy are the most common alterations in gill morphology following metal exposure. These structural responses would certainly be expected to decrease oxygen transport across the gill epithelium and reduce the aerobic capacity in Ag-acclimated fish.

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