SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 17, No. 4, pp. 579–588, 1998
© 1998 SETAC
Printed in the USA
0730-7268/98 $6.00 + .00

PHYSIOLOGICAL ANALYSIS OF THE STRESS RESPONSE ASSOCIATED WITH ACUTE SILVER NITRATE EXPOSURE IN FRESHWATER RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

NATHAN A. WEBB* and CHRIS M. WOOD
Department of Biology, McMaster University, 1280 Main St. W, Hamilton, Ontario L8S 4K1, Canada

(*Received* 15 *May* 1997; *Accepted* 23 *October* 1997)

Abstract—Rainbow trout were exposed to $AgNO_3$ (9.2 μg/L total Ag, of which 35% was as the free ion, Ag^+) for a 6-d period in dechlorinated Hamilton tapwater. Our findings suggest that the primary toxic mechanism of Ag is an interruption of ionoregulation at the gills, stopping active Na^+ and Cl^- uptake without increasing passive efflux, thereby causing net ion loss. There is no recovery of influxes over 6 d, whereas effluxes are gradually reduced below control levels, and ion balance remains negative. The resulting fall in plasma [Na^+] and [Cl^-] leads to a decrease in plasma volume and hemoconcentration, but the red blood cells do not swell. A substantial metabolic acidosis with partial respiratory compensation occurs in the blood, due to a net uptake of acidic equivalents from the environmental water. This uptake greatly exceeds the measured acid load in the extracellular fluid, suggesting that acidosis also occurs in the intracellular compartment, which in turn explains the continual loss of K^+ to the water in the absence of any change in plasma [K^+]. Plasma ammonia, glucose, and cortisol rise. As there is no reduction but rather a progressive rise in ammonia excretion, the increase in plasma ammonia is due to elevated metabolic production rather than inhibited excretion. The cause is probably the stress-induced mobilization of cortisol. This increased plasma ammonia, in conjunction with hyperventilation, helps to counteract metabolic acidosis. However, because the fish is unable to counteract the loss of plasma ions, death eventually results from a severe ionoregulatory disturbance.

Keywords—Silver Toxicity Ionoregulation Rainbow trout

INTRODUCTION

While various forms of complexed Ag are relatively benign, free ionic Ag^+, as liberated from dissolved $AgNO_3$, is extremely toxic to freshwater fish, with 96 h 50% lethal concentrations (LC50s) in the range of 6.5 to 65 μg/L total Ag [1–4]. Recent data [5,6] suggest that these toxic effects of ionic Ag^+ are similar to those of both copper [7–12] and acid exposure (see Wood [13,14] and Reid [15] for reviews of the extensive low-pH literature). Both Cu^{2+} and H^+ cause net ion losses (J_{net}) from the fish by inhibition of active branchial Na^+ and Cl^- influxes (J_{in}) and increases in diffusive Na^+ and Cl^- effluxes (J_{out}). In addition, net K^+ losses have also been reported. Decreased uptake appears to be caused by the inhibition of gill basolateral Na^+/K^+-ATPase in the case of Cu^{2+}, whereas in the case of H^+, the decrease is due to the inhibition of apical ion-exchange processes. For both toxicants, increased efflux appears to be due mainly to a rise in paracellular leakage, probably by displacement of Ca^{2+} from the junctional complexes. The resulting drop in plasma ions causes internal fluid shifts that decrease plasma volume, exacerbating the hemoconcentration caused by red blood cell swelling and splenic contraction. Plasma glucose and cortisol levels rise, indicative of a typical stress response. Plasma ammonia levels also rise, but it is unclear whether this is due to an inhibition of branchial ammonia excretion that has been reported in several studies and/or due to the metabolic effects of cortisol mobilization (“proteolysis” [16]). Plasma acidoses of diverse origins, including a disturbance of acid–base exchange at the gills, have also been seen in some studies.

* To whom correspondence may be addressed (webbna@mcmail.cis.mcmaster.ca).

In the two investigations to date on the physiology of Ag^+ toxicity [5,6], both on adult rainbow trout (*Oncorhynchus mykiss*), elements of similarity to the effects of Cu^{2+} and/or H^+ include blockade of active Na^+ and Cl^- influxes, profound inhibition of gill Na^+/K^+-ATPase activity, large declines in plasma Na^+ and Cl^- levels, associated decreases in plasma volume, splenic contraction, hemoconcentration, elevated plasma glucose, and plasma acidosis. Elements of difference include an absence of stimulated diffusive effluxes at the gills and an absence of red cell swelling. Elements that have not yet been examined include K^+ fluxes, plasma cortisol levels, plasma ammonia levels, branchial ammonia fluxes, and branchial acid–base exchanges.

The present study was designed as a comprehensive examination of the stress response to Ag^+ in adult rainbow trout under the same exposure conditions as those used by Wood et al. [5] and Morgan et al. [6]. Fish were exposed to total Ag = 9.2 μg/L as $AgNO_3$ (approximately 1/3 as Ag^+, close to the 168 h LC50 [3,4]) for 6 d in moderately hard freshwater. The first specific objective was to confirm key responses of the previous studies, including effects on Na^+ and Cl^- exchanges at the gills, plasma ionic and acid–base status, and the nature of hemoconcentration. The second was to document responses, or lack thereof, in the elements listed above that were not examined in the two previous studies. Inasmuch as the occurrence of profound ionoregulatory disturbance and a complex plasma acidosis were confirmed, and elevations of both plasma ammonia and cortisol were documented for the first time, the final objective was to understand the mechanisms behind the acid–base and ammonia responses. In particular, because both ammonia excretion and acid–base exchange are

known to be intimately linked to ion-exchange processes at the gills [14], we tested whether the observed responses were due to disturbances of ammonia excretion and acid–base exchange with the environment.

MATERIALS AND METHODS

Experimental animals

Adult rainbow trout (*O. mykiss,* 200–400 g) were obtained from Humber Valley Trout Farm (Orangeville, ON, Canada) and held for at least 2 weeks prior to experimentation in flowing, aerated, dechlorinated Hamilton tapwater (Ca^{2+} = 1.0, Mg^{2+} = 0.2, Na^+ = 0.5, Cl^- = 0.7, K^+ = 0.05, in mM; titratable alkalinity to pH 4.0 = 1.9; total hardness = 140 ppm as $CaCO_3$ equivalents; pH = 8.0 ± 0.2; 15 ± 1°C). They were fed a ration of 1% body weight of commercial trout pellets three times a week.

To standardize metabolic conditions, all fish were starved for 7 d prior to experimentation. On the seventh day, the fish were either fitted with indwelling dorsal aortic catheters [5] while under MS-222 anesthesia (group A) or were left intact (groups B and C). The catheters consisted of PE50 tubing filled with Cortland saline heparinized at 50 IU/ml with sodium heparin (Sigma, Oakville, ON, Canada). All fish were then weighed and transferred to individual darkened plexiglas chambers (volume = 4 L [17]) served with 800 ml/min of flowthrough water, for 48 h of recovery. The chambers were individually aerated and could be closed to allow measurements of ammonia excretion and ionic and acid–base exchanges with the environment. By slowing the flow, ammonia excretion could also be continually monitored from measurements of differences in concentration between inflow and outflow (group B). The three groups of fish underwent similar exposure protocols with group A fish being used for blood parameter analysis over 6 d of exposure, group B fish being used to monitor the fine time course of ammonia excretion during the first 12 h of exposure, and group C being used for whole body ionic, acid–base, and ammonia fluxes over the 6-d period. Tests were performed on control and experimental fish simultaneously to yield a total N of 8 control (240.6 ± 14.4 g) and 14 experimental (216.3 ± 8.8 g) in group A, 5 control (228.7 ± 16.9 g) and 6 experimental (216.9 ± 6.6 g) in group B, and 13 control (380.8 ± 35.2 g) and 13 experimental (344.6 ± 29.5 g) in group C.

Experimental protocol

At the start of the exposure (T = 0), $AgNO_3$ was added to a well-mixed head tank by a peristaltic pump, from a light shielded stock bottle (20.0 μg/ml Ag as $AgNO_3$, BDH) to give a nominal total Ag concentration in the inflow water of 10 μg/L. At time 0, each flux box was spiked with the stock solution to bring the water immediately to the exposure level. Water samples were taken from the fish boxes throughout the experiment to verify the exposure concentration for each fish. Water leaving the fish boxes exited to waste; there was no recirculation.

Group A

For both control and experimental fish, blood samples (0.8 ml) were taken via the dorsal aortic catheters prior to the exposure (control or ''C'' sample) and again at 4, 24, 48, 96, and 144 h during the exposure. Blood was drawn anaerobically into ice-cold gas-tight Hamilton syringes for analysis of arterial blood pH (pH_a), total plasma CO_2 content (Ca_{CO_2}), hematocrit, hemoglobin, and plasma levels of protein, total ammonia, glucose, cortisol, Na^+, Cl^-, K^+, and total Ag. This volume was replaced by reinfusion of nonheparinized Cortland saline plus the resuspended red blood cells not used in analysis. After the final blood sample on day 6, the fish were immediately sacrificed by an overdose of MS-222 (1 g/L, neutralized with NaOH; Syndel Labs, Vancouver, BC, Canada). Gill, liver, and white muscle samples were dissected, frozen in liquid N_2, and stored at −80°C for future analysis.

Group B

Ammonia excretion rates were monitored hourly on a flowthrough basis prior to and throughout the first 12 h of exposure for both control and experimental fish. The water inflow to all boxes was decreased to 200 ml/min. Water samples (10 ml) from both inflow and outflow were taken and analyzed for pH and total ammonia concentration. The goal of these fine timescale measurements was to test whether there was any initial inhibition of ammonia excretion.

Group C

Measurements of ionic exchange, acid–base exchange, and ammonia excretion in both control and experimental fish were carried out over 3-h periods prior to the start of exposure (''C'' sample) and also starting at 4, 24, 48, 96, and 144 h. For each measurement period, the water flow was stopped and the volume of the box was set to exactly 4 L. At this time, 4 μCi of ^{22}Na (as NaCl; NEN-Dupont, Boston, MA, USA) and 5 μCi of ^{36}Cl (as NaCl; ICN Radiochemicals, Irvine, CA, USA) were added to the water and allowed to mix for 5 min. Vigorous aeration was used to ensure thorough mixing and to maintain the partial pressure of O_2 at >85% of air-saturation levels. A 50-ml water sample was taken at this time and again at the end of 3 h. The boxes were then reopened to a flowthrough system. Each water sample was analyzed for gamma (^{22}Na only) and total radioactivity (^{22}Na plus ^{36}Cl), pH, and total water levels of Na^+, Cl^-, K^+, Ag, ammonia, and titratable alkalinity. After the final sample period (day 6) the fish were immediately sacrificed by an overdose of MS 222 (1 g/L), and a terminal blood sample was taken by caudal puncture to monitor internal ^{22}Na and ^{36}Cl, and plasma Na^+, and Cl^- levels. Gill, liver, and white muscle samples were dissected, frozen in liquid N_2, and stored at −80°C for future analysis.

ANALYTICAL METHODS

Blood and tissue parameters

Arterial blood pH was determined at the experimental temperature by a Radiometer microelectrode system (E-5021) connected to a Radiometer PHM72 meter. Hemoglobin was measured colorimetrically using the cyanmethemoglobin method and Sigma reagents. Total carbon dioxide concentration (Ca_{CO_2}) in the plasma was determined with a Corning 965 CO_2 analyzer. Plasma glucose and ammonia were determined enzymatically using Sigma kits (HK 16-UV and 171-UV, respectively). Cortisol levels were determined by a radioimmunoassay kit from Immunocorp (^{125}I RIA kit, ICN Biomedical, Irvine, CA, USA). Cl^- levels were measured with a Radiometer CMT10 chloridometer, while Na^+ and K^+ concentrations were determined by atomic absorption spectrophotometry (AAS, Varian 1275). All silver levels were determined as detailed by Hogstrand et al. [3] using graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer).

Water parameters

Water pH was measured with Radiometer electrodes (GK 2401C) connected to Radiometer PHM84 meters. Water P_{O_2} was monitored with a Radiometer O_2 electrode (E-5046) connected to the PHM72 meter. Ammonia levels were determined using the colorimetric assay of Verdouw et al. [18], which employs the reaction of ammonia with salicylate and hypochlorite. Water Cl^- was measured colorimetrically by the liberation of thiocyanate from mercuric thiocyanate to form mercuric chloride [19]. Na^+ and K^+ levels were determined by atomic absorption spectrophotometry (Varian 1275). Water silver levels were determined by graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer), again as described by Hogstrand et al. [3]. Titratable alkalinity was determined as described by McDonald and Wood [20] by titrating continually aerated 10-ml water samples to pH 4.00 with 0.02 N HCl using Gilmont digital microburettes and Radiometer electrodes (GK 2401C) attached to Radiometer PHM82 meters.

^{36}Cl is a pure β-emitter, while ^{22}Na is a mixed γ- and β-emitter. Dual-labeled 5-ml water samples (and 100-μl terminal plasma samples) were prepared in duplicate. ^{22}Na radioactivity alone was measured by γ-counting in a well counter (Canberra-Packard Minaxi Auto-Gamma 5000) and ^{22}Na plus ^{36}Cl radioactivity by scintillation counting (LKB Rackbeta 1217). ^{36}Cl radioactivity was obtained by subtraction after accounting for differences in efficiency of ^{22}Na counting by the two instruments, as described by Wood [21].

Calculations

Group A. Acid–base calculations employed the Henderson–Hasselbalch equation, as detailed in Playle et al. [22], to determine Pa_{CO_2} and plasma $[HCO_3^-]$ from measured values of pH_a and Ca_{CO_2}. CO_2 solubility (αCO_2) and pK′ values at experimental temperatures for trout plasma were taken from Boutilier et al. [23]. The metabolic acid load (ΔH_m^+) in the blood plasma was calculated from changes in plasma HCO_3^- concentrations and pH_a using the formula of McDonald et al. [24]. In this equation, the whole blood buffer capacity (β) for true plasma was estimated from the measured Hb concentration and the regression relationship determined for rainbow trout blood by Wood et al. [25]. Mean cell Hb concentration (MCHC) was calculated as the ratio of Hb to hematocrit. The inverse ratio of the plasma protein concentration at day 6 to the initial (time ''C'') value was used as an index in the change of plasma volume [5,24]. Ag speciation in the exposure water was calculated using the determined water chemistry and the aquatic geochemical equilibrium program MINEQL+ [26].

Partial pressures of ammonia (P_{NH_3}) in the plasma and water were determined from the measured total ammonia concentrations (T_{Amm}) and pH by the equations detailed in Wright and Wood [27], using NH_3 solubility (αNH_3) and pK′ values for trout plasma and freshwater at the experimental temperature from Boutilier et al. [23].

Group B. Ammonia excretion rates during the first 12 h of exposure were calculated by the Fick principle from measurements of body weight, water flow rate, and inflowing and outflowing concentrations, as detailed by Booth et al. [28].

Group C. Detailed rationales for net and unidirectional flux calculations are given by Wood [21]. In brief, net flux rates of ammonia and ions were calculated from changes in water concentrations over the 3-h period of box closure:

$$J_{net} = \frac{([initial] - [final]) \times V}{W \times t} \quad (1)$$

where [initial] and [final] are the initial and final concentrations in the water respectively (μmol/L), V is the volume of the water in the flux chamber (L), W is the weight of the fish (kg), and t is the duration of the flux period (h). Thus net losses from the fish have a negative sign, and net gains a positive sign. By reversing the [initial] and [final] terms, the net titratable acid flux was calculated from the titratable alkalinity measurements. The sum of titratable acid and ammonia fluxes, signs considered, yields the net flux of acidic equivalents. As pointed out by McDonald and Wood [20], this method does not distinguish between ammonia movements in the NH_3 versus NH_4^+ forms nor between the net excretion of acidic equivalents and the net uptake of basic equivalents, or vice versa. Fortunately, this does not matter in terms of the net acid–base balance of the fish, which is still faithfully represented.

Unidirectional influx rates for sodium and chloride were determined by monitoring the disappearance of radiotracers from the external water into the fish:

$$J_{in} = \frac{(cpm_i - cpm_f) \times V}{MSA \times W \times t} \quad (2)$$

where cpm_i and cpm_f are the initial and final concentrations of counts per minute (cpm/L), respectively, MSA is the mean specific activity of the test water during the flux period (cpm/μmol), and the other variables are as above.

Unidirectional efflux rates were determined by subtraction:

$$J_{out} = J_{net} - J_{in} \quad (3)$$

By convention, unidirectional influx rates and net uptake rates by the fish have a positive sign, while unidirectional efflux rates and net loss rates have a negative sign.

Repeated radioisotope flux determinations may lead to internal accumulation and associated ''backflux'' of radiotracers that would require a correction of the calculated J_{in} values. This becomes important when the internal SA of the isotope is ≥10% of the external specific activity [21]. However, as the final SA measured from terminal plasma samples for all fish was ≤6% of the external SA on day 6 in the present study, correction was not required.

Data have been expressed as means ± SEM. Differences between control and experimental treatment means at the same time were analyzed by the Student's unpaired two-tailed t test. Comparisons of changes over time within treatments were assessed with Student's two-tailed paired t test using the Bonferroni procedure to adjust the t value for multiple comparisons. Significant differences ($p < 0.05$) between treatments are indicated by asterisks (*) while significant differences within treatments are indicated by a plus sign (+).

RESULTS

Water chemistry and survival

The mean total water Ag concentration during the exposure was 9.2 ± 0.2 μg/L. Ag speciation calculated by MINEQL+ showed similar species distribution as in Wood et al. [5] for Hamilton tapwater, indicating that 35% of total Ag was in the free ionic form (Ag^+), with the rest being bound up in various forms of silver chloride (AgCl [aq] = 60%, $AgCl_2^-$ = 5%). The lack of any $AgNO_3$ remaining in bound form is directly

Table 1. Tissue Ag burdens of rainbow trout exposed to 9.2 μg/L Ag (as $AgNO_3$) for 6 d[a]

Total Ag (μg/kg wet wt.)	Control	$AgNO_3$
Gill	164 ± 10	1,305 ± 202[b]
Liver	7,450 ± 352	26,150 ± 7,240[b]
White muscle	5,420 ± 1,280	6,320 ± 1,050

[a] Values are means ± 1 SEM ($n = 8$).
[b] $p < 0.05$.

due to the dissociation constant of NO_3^- being extremely high in freshwater [2,26,31].

Of the fish exposed for 6 d (groups A and C), three experimental fish died after 4 d and two fish after the fifth day (i.e., 5/27). Only one of the control fish died (i.e., 1/21) immediately before the sixth day of sampling. Data from all individuals that died were included in calculations as they did not bias any of the trends seen. None of the short-term exposed fish (group B) died.

Tissue silver burden

Gill silver levels were found to be approximately 1,300 μg/kg (or about 12 μmol/kg), 8 times those of control fish by the end of 6 d exposure. Silver levels in liver samples from exposed fish were much higher (~26,000 μg/kg), but only 3.5 times those of control fish. White muscle concentrations were intermediate (6,000 μg/kg), and there was no difference seen between the mean values for control and exposed fish (Table 1).

Group A—blood chemistry

Plasma total Ag concentration in exposed fish increased continually throughout the exposure (Fig. 1A). This increase was significant by day 1 and reached about 7 μmol/L (750 μg/L), again nearly eight times the pre-exposure levels by day 6. The Ag levels in the plasma of control fish remained less than 1 μmol/L and did not change during the treatment (Fig. 1A).

Plasma K^+ levels remained unchanged throughout the exposure (Table 2), while both Na^+ and Cl^- levels dropped by 16% over the 6 d (Fig. 1B and C). Ion concentrations in control fish remained unchanged throughout the experiment.

The hematocrit values for both exposed and control fish did not differ significantly from pre-exposure levels (Fig. 2A). However, Ag exposure prevented the decrease in Hb seen in control fish that resulted from repetitive blood sampling (Fig. 2B). MCHC did not change significantly (Table 2). A significant increase in plasma protein levels to nearly twice the control value was seen by the end of the exposure (Fig. 2C). This corresponded to a marked 40% drop in relative plasma volume by the sixth day (Table 2).

Acid–base measurements demonstrated progressive, highly significant decreases in both Pa_{CO_2} (Fig. 3A) and plasma $[HCO_3^-]$ (Fig. 3B). By day 6, both parameters had fallen to about 50% of pre-exposure values (Fig. 3A and B). Blood pH_a in these fish dropped by 0.2 units after 6 d of Ag exposure, with the drop becoming significant by the fourth day (Fig. 3C). The decreases in $[HCO_3^-]$ and pH_a was associated with an increase in the metabolic acid load (ΔH_m^+) of the plasma, significant by day 4 (Fig. 3D). By this time, the Ag-exposed trout exhibited an increased depth of breathing indicative of hyperventilation.

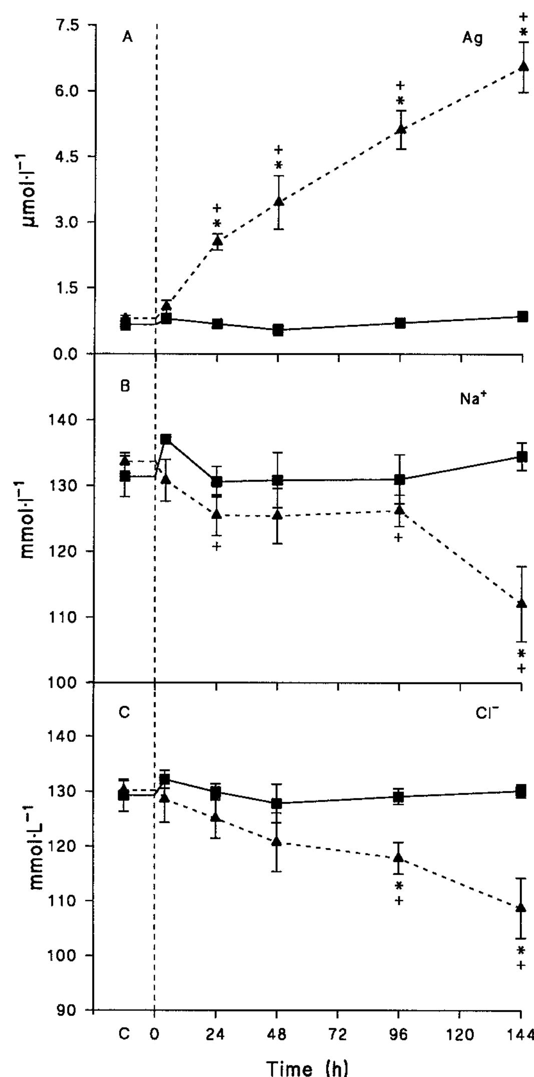

Fig. 1. The effects of 6 d exposure to 9.2 μg/L Ag (as $AgNO_3$) on (**A**) plasma total Ag levels, (**B**) plasma Na^+, and (**C**) plasma Cl^- in both control (■, $n = 8$) and Ag-exposed fish (▲, $n = 14$). Data points are means ± 1 SE. Asterisks indicate a significant difference ($p < 0.05$) between groups, while + indicates a significant difference from pre-exposure (day C) values.

Table 2. Plasma K^+ and hematological parameters of rainbow trout exposed to 9.2 μg/L Ag (as $AgNO_3$) for 6 d[a]

	Control	$AgNO_3$
Plasma K^+ (mmol/L)	3.418 ± 0.509 (6)	3.900 ± 0.227 (9)
MCHC (g Hb/ml RBC)	0.464 ± 0.083 (6)	0.475 ± 0.063 (14)
Relative plasma volume (%)	89.0 ± 3.4 (6)	62.6 ± 2.9[b] (14)

[a] Values are means ± 1 SEM (n).
[b] $p < 0.05$.

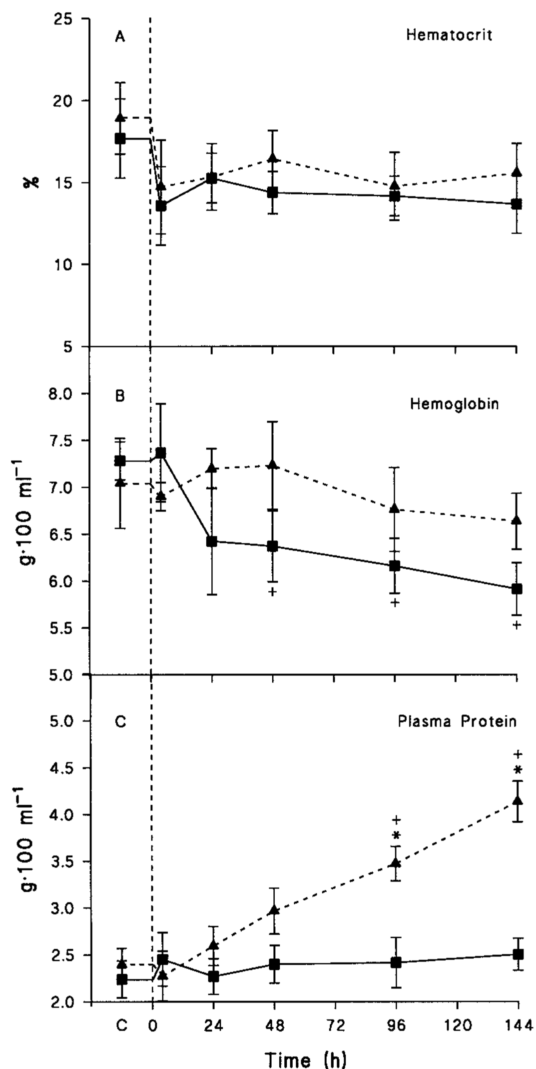

Fig. 2. The effects of 6 d exposure to 9.2 μg/L Ag (as $AgNO_3$) on (**A**) hematocrit, (**B**) hemoglobin, and plasma protein (**C**) levels in rainbow trout. Other details as indicated in legend to Figure 1.

Total ammonia (T_{Amm}, Fig. 4A) and P_{NH_3} (data not shown) levels in the blood plasma increased in Ag-exposed fish by almost 150% and 75% respectively, by day 6, compared to control fish. T_{Amm} in control fish dropped at 4 h, perhaps an effect of blood sampling, but thereafter recovered to pre-exposure levels. Ag-exposed fish showed a progressive increase in plasma glucose and cortisol levels (150–230%; Fig. 4B and C) that were significantly different from controls by day 4. Cortisol levels did not change in control fish, but plasma glucose dropped below pre-exposure levels at 4 h and remained slightly depressed for the remainder of the experiment.

Changes in the partial pressure gradient for ammonia between blood and water (ΔP_{NH_3}), thought to be the major driving force for ammonia excretion (see Discussion), are summarized in Table 3. ΔP_{NH_3} increased significantly by day 2 in Ag-exposed trout, whereas it fell significantly over this same period in control fish. By day 6, the gradient in the exposed fish had increased by about 60%, whereas it had returned to pre-exposure levels in the control group.

Group B—initial ammonia excretion rates

Ammonia flux rates for both control and exposed fish were essentially the same over the first 12 h of exposure, although the exposed fish, on average, had a slightly higher (but nonsignificant) excretion rate compared to controls (Fig. 5A). These measurements demonstrated that the progressive build-up of plasma T_{Amm}, seen as early as 4 h (Fig. 4A), was not due to an inhibition of ammonia excretion to the environment.

Group C—ammonia, acid–base, and ionic flux rates

Measurements of ammonia fluxes over the longer term (Fig. 5B) demonstrated a dramatic increase in ammonia excretion that became significant at 24 h and reached almost threefold control levels by day 6, in concert with the build-up of T_{Amm} in the blood plasma (Fig. 4A) and the increase in ΔP_{NH_3} (Table 3). Ammonia excretion in the control fish remained constant over the 6 d of the exposure (Fig. 5B).

These ammonia excretion rates are shown in relation to the net acid–base exchange of the fish with the environment (Fig. 6). In control fish, there were no significant changes in the net uptake of titratable acidity or the net excretion of ammonia over the 6 d of the experiment (Fig. 6A). The arithmetic sum of the two components remained positive and constant, indicating a continuing uptake of acidic equivalents (or loss of basic equivalents) over this period. However, in Ag-exposed trout, a significant rise in the net uptake of titratable acidity from the environment occurred concomitant with the rise in ammonia excretion (Fig. 6B). This increase in titratable acidity flux exceeded that in ammonia flux, so the net uptake of acidic equivalents increased significantly to approximately twice the control levels by day 6 (Fig. 6B). Thus, the internal acidosis seen in the blood plasma of Ag-exposed fish (Fig. 3) was associated with an increased net uptake of acidic equivalents or loss of basic equivalents by the fish.

The movements of major ions (Na^+, Cl^-, and K^+) between the fish and the environmental water were markedly affected by Ag exposure. Despite the constancy of plasma K^+ concentration (Table 2), K^+ balance changed from a net uptake rate to a net loss rate during the first 4–7 h of Ag exposure, and remained negative at about the same level (−25 μmol/kg/h) for the duration of the experiment (Fig. 7).

In addition to net flux rates (J_{net}), unidirectional influx (J_{in}) and efflux (J_{out}) components were measured for Na^+ and Cl^- balance (Fig. 8). Prior to exposure, J_{net} values were positive, as J_{in} exceeded J_{out} for both Na^+ and Cl^-. Throughout the experiment, the control fish remained close to zero balance for both ions, with J_{in} and J_{out} values approximately equal. However, following only 4 h of Ag exposure, J_{in} values for Na^+ and Cl^- decreased by 88% (Fig. 8B) and 64% (Fig. 8D), respectively, and were completely abolished after 48 h. As a result, J_{net} became highly negative for both ions (−100 to −300 μmol/kg/h) over this period. These negative balances were largely due to the inhibitions of the active influx (J_{in}) components. Although J_{out} values also appeared to increase (significant only for Cl^- at 4–7 h; Fig. 8D), a similar phenomenon occurred in the controls (Fig. 8A and C). The absolute J_{out} values in the Ag-exposed trout were not significantly different from those of the control fish at this time. As time progressed, the net loss rates of Na^+ and Cl^- from the fish decreased due entirely to decreases of the J_{out} components that eventually reached values less than half the control levels. There was no significant recovery of influx (J_{in}) for either ion during the exposure (Fig. 8B and D).

DISCUSSION

The present study was successful in its first objective, that of confirming key responses to ionic Ag^+ exposure in rainbow

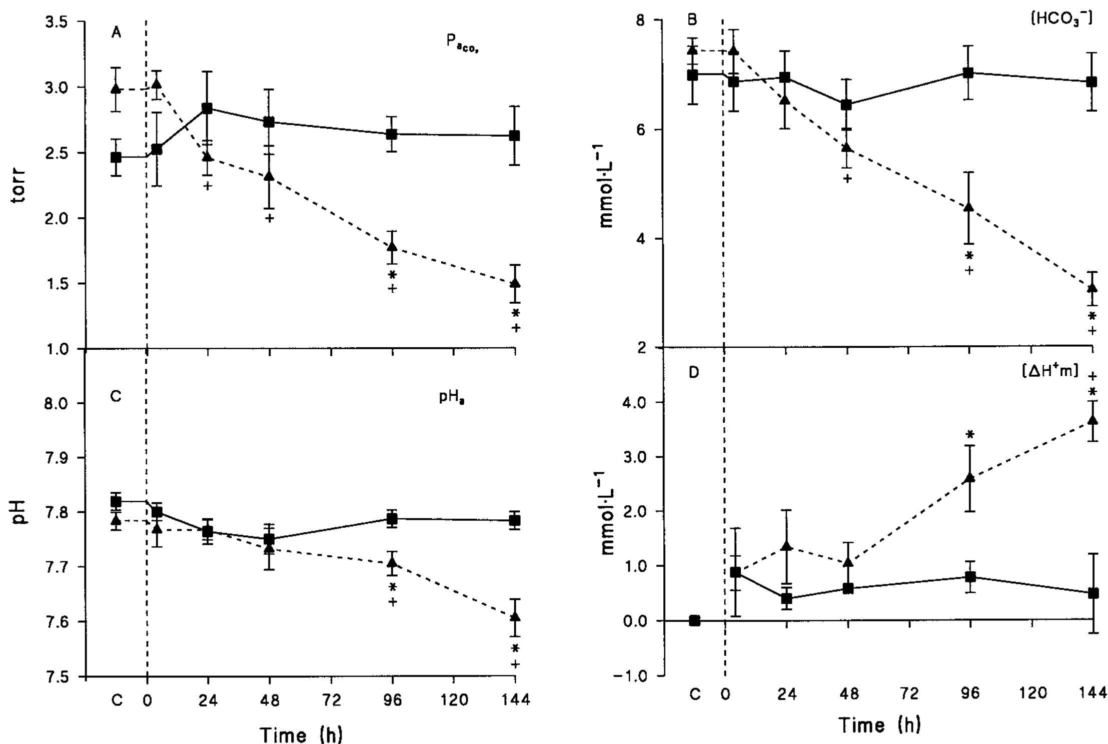

Fig. 3. The effects of 6 d exposure to 9.2 μg/L Ag (as $AgNO_3$) on (**A**) partial pressure of CO_2 in the blood (P_{CO_2}), (**B**) plasma [HCO_3^-], (**C**) blood pH, and (**D**) metabolic acid load (ΔH^+_m) in rainbow trout. Other details as indicated in legend to Figure 1.

trout. Thus, with only minor quantitative differences, the observed decreases in plasma Na^+ and Cl^- levels, associated increases in plasma protein and Hb (the latter relative to sampling-induced decreases in control fish), decreases in plasma volume, and complex acidosis in the blood plasma (see below) were very similar to those reported by Wood et al. [5,29] for adult rainbow trout similarly exposed to 10 μg/L total Ag (as $AgNO_3$) for 6 d in moderately hard Hamilton tapwater. The results also confirm that the red cells do not swell (i.e., unchanged MCHC), which suggests that Ag in the plasma may in some way block the β-adrenergically stimulated Na^+/H^+ exchange on the erythrocyte membrane, which is thought to be responsible for this response during low pH or Cu^{2+} exposure (see Introduction). Plasma [Ag] rose steadily over the 6 d, rather than levelling off after 2–4 d as reported by Wood et al., and by day 6 were twofold higher than the levels seen in the previous study [5,29]. However, gill and liver Ag concentrations were very comparable in the two investigations. Notably in white muscle, a tissue that has not previously been examined, there was a high background level of Ag in control samples but no further Ag accumulation during the exposure. In this regard, muscle appears very similar to the kidney tissue monitored by Wood et al. [5]. These studies therefore confirm that nonexposed fish have considerable background levels of Ag in tissues that they have presumably accumulated from trace levels in the water or the diet during their lifetimes.

Our study also confirms that the Na^+ and Cl^- losses are due to a rapidly developing inhibition of active Na^+ and Cl^- uptake processes (J_{in}) at the gills and are not due to a stimulation of diffusive effluxes (J_{out}), in agreement with the recent report of Morgan et al. [6]. Assuming that measured declines (~20 mmol/kg) in plasma Na^+ and Cl^- levels occurred throughout an internal distribution space of about 270 ml/kg (as determined by Wood [21]—i.e., largely extracellular fluid), they would amount to about −5,400 μmol/kg, approximately 1/3 of the measured net loss rates to the water over 6 d (~ −100 μmol/kg/h × 144 h = −14,400 μmol/kg). Considering that the flux measurements represent only isolated "snapshots" in time versus an integrated plasma measurement, agreement is not unreasonable. As for Cu^{2+} [9,30], the influx blockade is undoubtedly related to the potent inhibition of branchial Na^+/K^+-ATPase caused by Ag^+ [6]. However, the absence of efflux stimulation (which is normally thought to be due to Ca^{2+} displacement from the gill [14]) is a clear difference from the actions of both Cu^{2+} [7,8] and low pH [14] but is in accord with the very limited protective effect of water [Ca^{2+}] against Ag^+ toxicity [4] and the very weak affinity of the Ag^+ binding ligands on gills for Ca^{2+} [31]. These observations all suggest that Ag^+ does not attack the Ca^{2+} binding sites that stabilize paracellular permeability. The later reductions in unidirectional Na^+ and Cl^- effluxes to less than 50% of control levels are much larger than can be explained by the drop in the gradient alone (i.e., falling plasma [Na^+] and [Cl^-]). As with acid stress, hormonal factors such as prolactin mobilization are probably also involved [15].

The present observations confirm that the blood acid–base response to Ag exposure comprises a dominant metabolic acidosis (ΔH^+_m accumulation leading to HCO_3^- reduction) and partially compensating respiratory alkalosis (Pa_{CO_2} reduction, also leading to HCO_3^- reduction). An obvious hyperventilation is the probable cause of the latter, without which the drop in pH_a would be more severe. Earlier, Wood et al. [5] demonstrated all of these same responses and also reported that there was no appearance of lactate in the blood, thereby ruling out anaerobic metabolism as the cause of the metabolic acidosis.

Two completely new, and likely interrelated, findings of the present study are that stimulated uptake of acidic equivalents (or loss of basic equivalents) and steady net loss of K^+

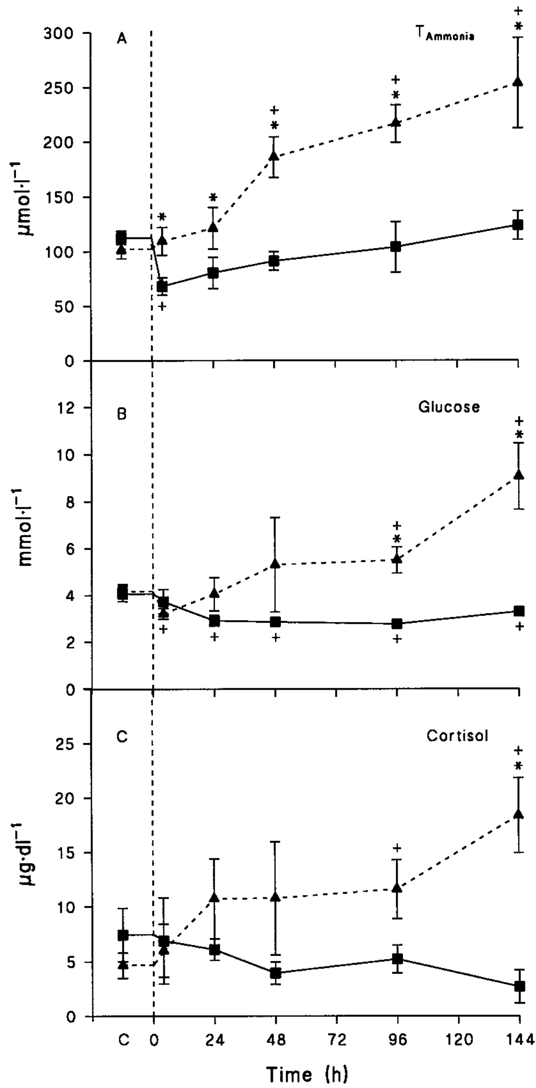

Fig. 4. The effects of 6 d exposure to 9.2 μg/L Ag (as $AgNO_3$) on (**A**) plasma ammonia, (**B**) glucose, and (**C**) cortisol levels in rainbow trout. Other details as indicated in legend to Figure 1.

to the water both occur during Ag exposure. The former is the probable cause of the marked build-up of metabolic acid (ΔH^+_m) in the blood. Applying the same sort of calculations as for Na^+ and Cl^- losses (above), the net uptake of acidic equivalents from the water over 6 d, relative to controls, was about 12,000 μmol/kg, or at least 10-fold the measured accumulation

Table 3. Plasma to water ammonia gradients in control and fish exposed to 9.2 μg/L Ag (as $AgNO_3$) for 6 d[a]

Hour	ΔP_{NH_3} (μTorr)	
	Control	Exposed
C	33.17 ± 1.83	27.64 ± 3.62
4	16.69 ± 1.57[b]	23.88 ± 5.86
24	18.34 ± 2.18[b]	25.46 ± 4.67
48	21.44 ± 1.10[b]	40.67 ± 2.38[b,c]
96	26.44 ± 4.87	41.20 ± 7.70
144	35.18 ± 3.61	45.23 ± 4.56[b]

[a] Values are means ± 1 SEM ($n = 8$).
[b] $p < 0.05$ from pre-exposure value of same group.
[c] $p < 0.05$ between groups.

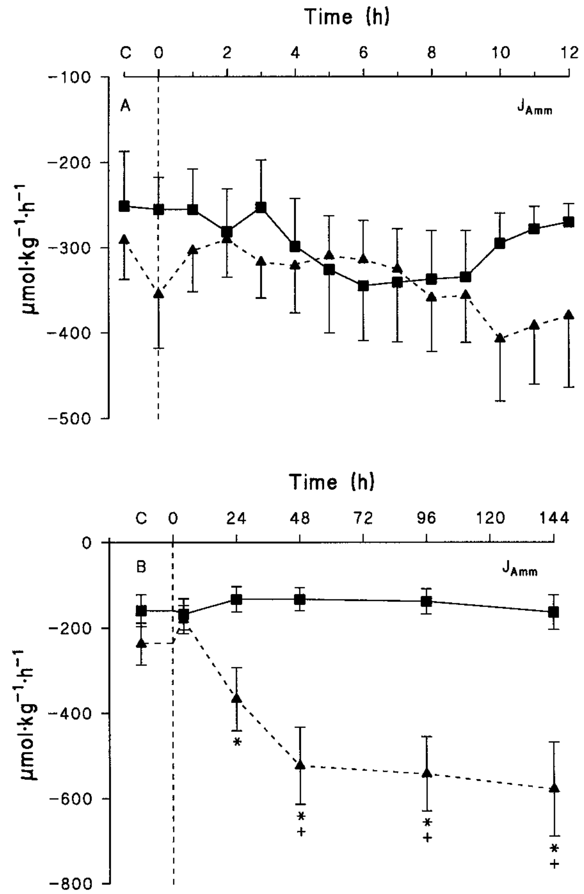

Fig. 5. Ammonia excretion rates for both control (■, $n = 5$) and rainbow trout exposed to 9.2 μg/L $AgNO_3$ (▲, $n = 6$) during the first 12 h of exposure (**A**) and throughout 6 d of exposure (**B**) $n = 13$ for both control and Ag-exposed fish. Other details as indicated in legend to Figure 1.

of ΔH^+_m in the extracellular compartment. This discrepancy is too large to be an artifact and strongly suggests that a considerable portion of the net acid uptake penetrated the intracellular compartment that has about a 10-fold greater ability to tolerate ΔH^+_m loading because of its 5-fold larger buffer capacity and 2-fold larger compartmental size [25,32]. The major intracellular compartment lies in the white muscle (>50% of body mass). Strong corroborative evidence for this intracellular acid loading was the steady loss of K^+ to the water (∼−5,000 μmol/kg relative to controls over 6 d) in the absence of any change in plasma [K^+]. Exchange of H^+ for K^+ is the classic mechanism by which metabolic acid enters muscle, accompanied by a net excretion of K^+ to prevent hyperkalemia from occurring in the blood. Exactly the same phenomenon of acid uptake from the water in excess of the extracellular load and K^+ loss to the environment has been seen during exposure of trout to low pH in the same water quality [13,20,24]. We are unaware of any acid flux data for Cu^{2+}-exposed fish, but a similar loss of K^+ has been reported [7].

The mechanism responsible for this disturbance of acid–base exchange with the environment is unknown. One possibility is that it is a direct consequence of the observed disruption of Na^+ and Cl^- uptake at the gills, which are thought to involve Na^+/"acid" and Cl^-/"base" exchange processes [14]. Another possibility, which may be synergistic, is an inhibition of branchial carbonic anhydrase, the enzyme that provides the acidic (H^+) and basic (HCO_3^-) counterions through

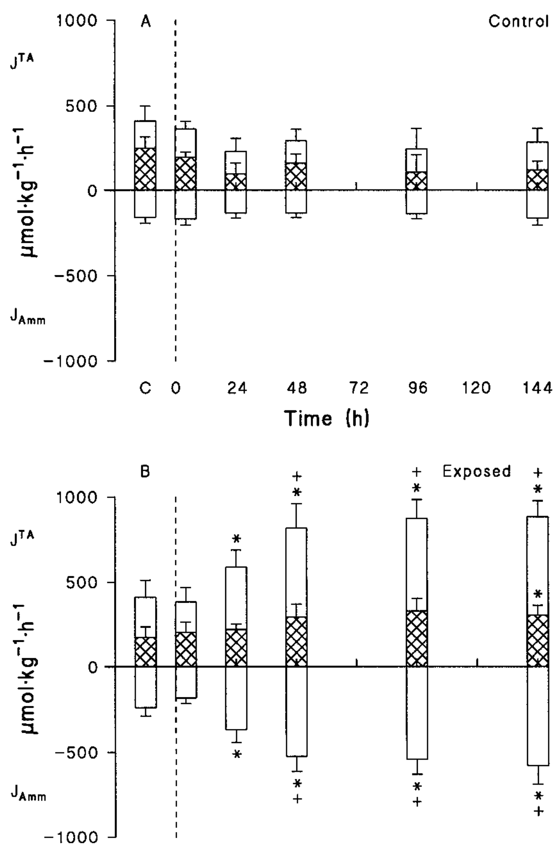

Fig. 6. The movement of acid and base equivalents across the gills for control (**A**, $n = 13$) and Ag-exposed (**B**, $n = 13$) rainbow trout. Open bars above the x-axis represents titratable acidity (J^{TA}); open bars below the axis represent the ammonia excretion (J_{Amm}), with the hatched bars representing the net acid flux at the gills. Other details as indicated in legend to Figure 1.

the catalysis of CO_2 hydration. Indeed, Morgan et al. [6] demonstrated that in fish exposed to 10 μg/L Ag for 48 h in vivo, there was a 30% reduction of carbonic anhydrase activity in the gills, as well as when added in vitro. Ag has also been reported to inhibit erythrocytic carbonic anhydrase [33].

The present observations on ammonia metabolism and cortisol mobilization are also entirely new. Ammonia excretion (J_{Amm}), more than 90% of which occurs across the gills [14],

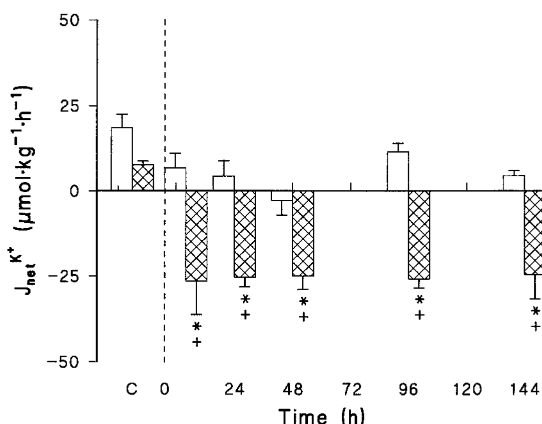

Fig. 7. Net flux rates of potassium across the gills in control (open bars, $n = 8$) and Ag-exposed fish (hatched bars, $n = 8$) during 6 d in 9.2 μg/L Ag (as $AgNO_3$). Other details as indicated in legend to Figure 1.

was monitored throughout the exposure and no inhibition was seen at any time. J_{Amm} increased, in association with an increase in ΔP_{NH_3}, the driving gradient for NH_3 diffusion across the gills. Thus, the situation appears to be different to that for low pH and Cu^{2+} exposure, where ammonia retention has been attributed to an initial reduction of J_{Amm} at the gills due to an inhibition of linked Na^+ uptake and NH_4^+ efflux [12,14]. The whole question of whether Na^+ influx is really coupled to NH_4^+ efflux at the gills or rather to H^+ efflux (with NH_3 diffusing passively) remains controversial [34] and beyond the scope of this Discussion. The key point here is that the increased plasma T_{Amm} observed during Ag exposure was due to an increased metabolic production, not retention due to a blockade of J_{Amm}.

The substantial mobilization of cortisol reported here for the first time had been earlier predicted on the basis of observed glucose mobilization [5]. These two parameters are standard "stress responses" in fish and usually occur simultaneously [35]. Cortisol has well-known proteolytic and gluconeogenic effects [16]. The increased rate of ammonia production was therefore likely caused by the action of cortisol in stimulating protein catabolism, subsequent amino acid oxidation and gluconeogesis, and resulting ammoniogenesis. Some longer-term studies with both low pH [36] and Cu^{2+} [37] have suggested a similar scenario.

The levels of plasma T_{Amm} accumulating during Ag exposure are well below those known to be acutely damaging in trout [34], so it is unlikely that ammonia build-up directly contributes to toxicity. Indeed, from an acid–base standpoint, because ammonia is produced metabolically as NH_3 but exists almost entirely as NH_4^+ at physiological pH, its action in buffering H^+ ions may be helpful in reducing the extent of acidosis. This may be particularly important in alleviating intracellular acidosis. Tissue T_{Amm} levels in fish are usually far greater than plasma T_{Amm}. However, recent studies on Cu^{2+} [12] have suggested that internal ammonia retention, especially in white muscle, may have an important sublethal action to reduce swimming performance in fish. In future studies it would be useful to measure exercise performance, muscle intracellular pH_i, and muscle T_{Amm} in Ag-exposed trout.

This study was designed to elucidate the acute toxic mechanism of silver in teleosts. Due to the level and type of Ag used (10 μg/L Ag as $AgNO_3$, 35% as Ag^+), the degree of toxicity seen here is severe compared to a natural environment. Ag levels in nature tend to be much lower, with concentrations being no more than a few percent of the levels used here [38]. However, the parameters measured here (plasma Na and Cl, and ion flux rates) can serve as nonspecific indicators of sublethal stress in fish. This nonspecificity is due to the similarity of the toxic action of Ag^+ to that of acid (H^+) and other heavy metals (Cu^{2+}), as discussed earlier (see Introduction).

Clearly, the next step is to examine the mechanism of toxicity of these three toxicants at the cellular and molecular levels in greater detail and also to evaluate whether these sublethal stress effects result in impaired growth, reproduction, or long-term survival.

Acknowledgement—This study was supported by grants from Kodak Canada and the NSERC Industrially Oriented Research Program to C.M. Wood. We thank Fernando Galvez for valuable input and liaison and Joe Gorsuch, Ken Robillard, Daland Juberg, and Tom Bober of Kodak for their helpful comments.

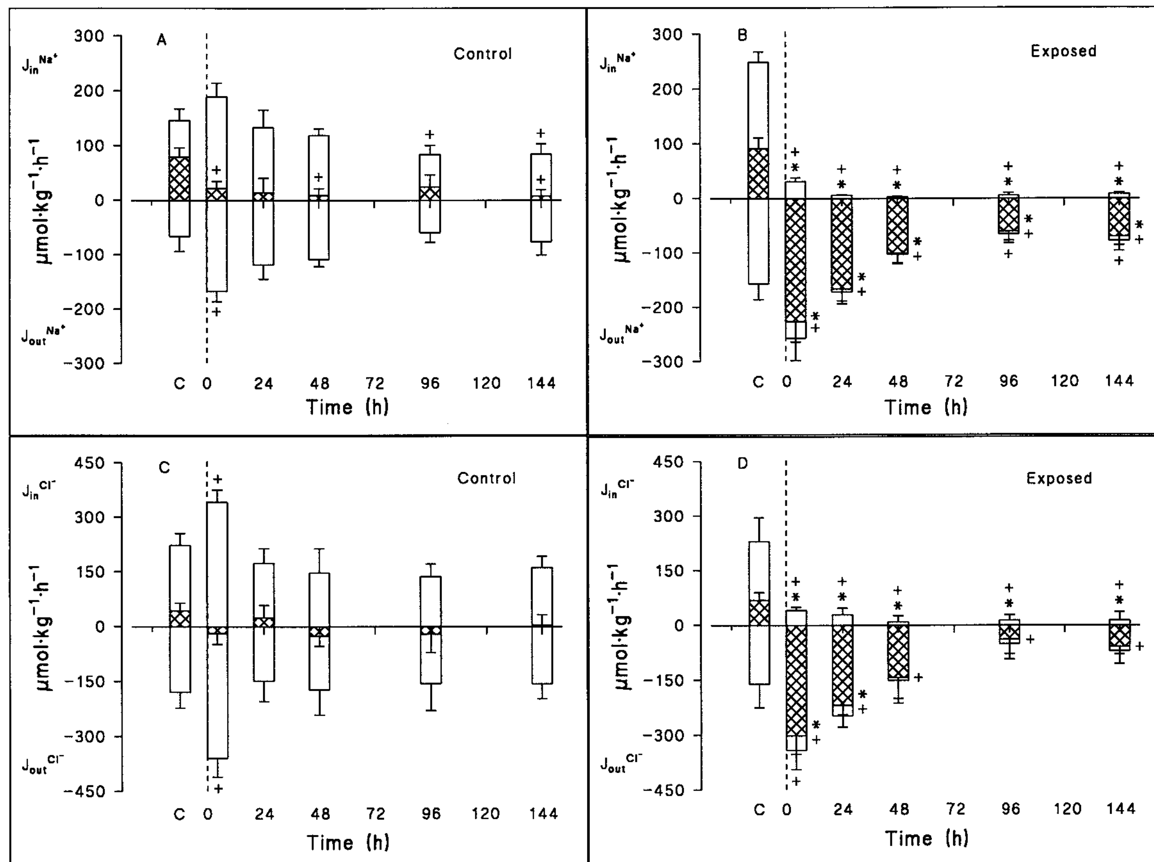

Fig. 8. Unidirectional Na^+ (**A**: control fish, n = 13; **B**: Ag-exposed fish, n = 13) and Cl^- (**C**: control fish, n = 13; **D**: Ag-exposed fish, n = 13) flux rates during 6 d of exposure to 9.2 μg/L Ag (as $AgNO_3$). Open bars above the axis indicate influx, open bars below the axis indicate efflux, and hatched bars indicate the net ion flux. Other details as indicated in legend to Figure 1.

REFERENCES

1. Nebeker AV, McAuliffe CK, Mshar R, Stevens DG. 1983. Toxicity of silver to steelhead and rainbow trout, fathead minnows and *Daphnia magna*. *Environ Toxicol Chem* 2:95–104.
2. LeBlanc GA, Mastone JD, Paradice AP, Wilson BF. 1984. The influence of speciation on the toxicity of silver to fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 3:37–46.
3. Hogstrand C, Galvez F, Wood CM. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environ Toxicol Chem* 15:1102–1108.
4. Galvez F, Wood CM. 1997. The relative importance of water hardness (Ca) and chloride levels in modifying the acute toxicity of silver to rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 16:2363–2368.
5. Wood CM, Hogstrand C, Galvez F, Munger RS. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. The effects of ionic Ag^+. *Aquat Toxicol* 35:93–109.
6. Morgan IJ, Henry RP, Wood CM. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. *Aquat Toxicol* 38:145–163.
7. Laurén DJ, McDonald DG. 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. *J Comp Physiol B* 155:635–644.
8. Laurén DJ, McDonald DG. 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* 43:1488–1496.
9. Laurén DJ, McDonald DG. 1987. Acclimation to copper by rainbow trout, *Salmo gairdneri*: Biochemistry. *Can J Fish Aquat Sci* 44:105–111.
10. Wilson RW, Taylor EW. 1993. The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. *J Comp Physiol B* 163:38–47.
11. Pilgaard L, Malte H, Jensen FB. 1994. Physiological effects and tissue accumulation of copper in freshwater rainbow trout (*Oncorhynchus mykiss*) under normoxic and hypoxic conditions. *Aquat Toxicol* 29:197–212.
12. Taylor EW, Beaumont MW, Butler PJ, Mair J, Mujallid MSI. 1996. Lethal and sublethal effects of copper upon fish: A role for ammonia toxicity. In Taylor EW, ed, *Toxicology of Aquatic Pollution: Physiological, Cellular and Molecular Approaches*, Cambridge University Press, London, UK, pp 85–113.
13. Wood CM. 1989. The physiological problems of fish in acid waters. In Morris R, Taylor EW, Brown DJA, Brown JA, eds, *Acid Toxicity and Aquatic Animals*, Cambridge University Press, London, UK, pp 125–152.
14. Wood CM. 1992. Flux measurements as indices of H^+ and metal effects on freshwater fish. *Aquat Toxicol* 22:239–264.
15. Reid SD. 1995. Adaptation to and effects of acid water on the fish gill. In Hochachka PW, Mommsen TP, eds, *Biochemistry and Molecular Biology of Fishes*, Vol 5. Elsevier, New York, NY, USA, pp 213–227.
16. van der Boon J, van den Thillart GEEJM, Addink ADF. 1991. The effects of cortisol administration on intermediary metabolism in teleost fish. *Comp Biochem Physiol A* 100:47–53.
17. McDonald DG, Rogano MS. 1986. Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion-poor water. *Physiol Zool* 59:318–331.
18. Verdouw H, Echteld CJA, Dekkers EMJ. 1978. Ammonia determination based on indophenol formation with sodium salicylate. *Water Res* 12:399–402.
19. Zall DM, Fisher D, Garner MQ. 1956. Photometric determination of chlorides in water. *Anal Chem* 28:1665–1678.
20. McDonald DG, Wood CM. 1981. Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. *J Exp Biol* 93:101–118.

21. Wood CM. 1988. Acid–base and ionic exchanges at gills and kidney after exhaustive exercise in the rainbow trout. *J Exp Biol* 136:461–481.
22. Playle RC, Goss GG, Wood CM. 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminum exposures in soft water to two calcium concentrations. *Can J Zool* 67:314–324.
23. Boutilier RG, Heming TA, Iwama GK. 1984. Physicochemical parameters for use in fish respiratory physiology. In Hoar WS, Randall DJ, eds, *Fish Physiology,* Vol 10A—*Gills.* Academic, New York, NY, USA, pp 403–430.
24. McDonald DG, Hobe H, Wood CM. 1980. The influence of calcium on the physiological responses of the rainbow trout, *Salmo gairdneri,* to low environment pH. *J Exp Biol* 88:109–131.
25. Wood CM, McDonald DG, McMahon BR. 1982. The influence of experimental anaemia on blood acid–base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). *J Exp Biol* 96:221–237.
26. Schecher WD, McAvoy DC. 1991. MINEQL$^+$: A software environment for chemical equilibrium modeling. *Comput Environ Urban Systems* 16:65–76.
27. Wright PA, Wood CM. 1985. An analysis of branchial ammonia excretion in the freshwater rainbow trout: Effects of environmental pH change and sodium uptake blockade. *J Exp Biol* 114:329–353.
28. Booth CE, McDonald DG, Simons BP, Wood CM. 1988. Effects of aluminum and low pH on net ion fluxes and ion balance in the brook trout (*Salvelinus fontinalis*). *Can J Fish Aquat Sci* 45:1563–1574.
29. Wood CM, Hogstrand C, Galvez F, Munger RS. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 2. The effects of silver thiosulfate. *Aquat Toxicol* 35:111–125.
30. Li J, Lock RAC, Klaren PHM, Swarts HGP, Schuurmans Stekhoven FMAH, Wendelaar Bonga SE, Flik G. 1996. Kinetics of Cu^{2+} inhibition of Na^+/K^+-ATPase. *Toxicol Lett* 87:31–38.
31. Janes N, Playle RC. 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 14:1847–1858.
32. Milligan CL, Wood CM. 1986. Intracellular and extracellular acid–base status and H^+ exchange with the environment after exhaustive exercise in the rainbow trout. *J Exp Biol* 123:93–121.
33. Christensen GM, Tucker JH. 1976. Effects of selected water toxicants on the *in vitro* activity of fish carbonic anhydrase. *Chem-Biol Interact* 13:181–192.
34. Wilson RW, Wright PM, Munger S, Wood CM. 1994. Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: Lack of evidence for Na^+/NH_4^+ exchange. *J Exp Biol* 191:37–58.
35. Thomas P. 1990. Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. In Adams SM, ed, *Biological Indicators of Stress in Fish,* Symposium 8. American Fisheries Society, Bethesda, Maryland, USA, pp 9–28.
36. Audet C, Wood CM. 1993. Branchial morphological and endocrine responses of rainbow trout (*Oncorhynchus mykiss*) to a long-term sublethal acid exposure in which acclimation did not occur. *Can J Fish Aquat Sci* 50:198–209.
37. De Boeck G, De Smet H, Blust R. 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cypinus carpio. Aquat Toxicol* 32:127–141.
38. Wen LS, Santschi PH, Gill GA, Peternostro CL, Lehman RL. 1997. Colloidal and particulate silver in rivers and estuarine waters of Texas. *Environ Sci Technol* 31:723–731.