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Effects of water hardness on the physiological responses to chronic waterborne silver exposure in early life stages of rainbow trout (*Oncorhynchus mykiss*)

T.P. Morgan [a,*], C.M. Guadagnolo [a], M. Grosell [b], C.M. Wood [a]

[a] *Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ont., Canada L8S 4K1*
[b] *RSMAS, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA*

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Abstract

Early life stages of rainbow trout were exposed to 0, 0.1 and 1 μg/L Ag (as $AgNO_3$) in very soft water (2 mg/L $CaCO_3$), moderately hard water (150 mg/L $CaCO_3$) and hard water (400 mg/L $CaCO_3$) of low dissolved organic carbon concentration (0.5 mg C/L) from fertilization to swim-up (64 days) under flow-through conditions, and monitored for whole embryo/larval silver accumulation, Na^+ and Cl^- concentrations, Na^+ uptake and Na^+K^+-ATPase activity. The objective of the study was to investigate potential protective effects of water hardness on the physiological responses to chronic silver exposure. In the absence of silver, there was little effect of hardness on the ionoregulatory parameters studied, though higher hardness did improve survival post-hatch. At all three water hardness levels, whole embryo/larval Na^+ uptake was low and relatively constant prior to 50% hatch, but dramatically increased following 50% hatch, whereas Na^+K^+-ATPase activity steadily increased over development. Whole embryo/larval Na^+ and Cl^- concentrations were low and constant prior to 50% hatch, but following 50% hatch Na^+ concentration increased, while Cl^- concentration decreased. Following 50% hatch, exposure to 0.1 and 1 μg/L Ag resulted in a decrease in whole embryo/larval Na^+ concentration, Cl^- concentration, Na^+ uptake and Na^+K^+-ATPase activity, indicating that the mechanism of chronic silver toxicity involves an ionoregulatory disturbance, and is similar to the mechanism of acute silver toxicity. An increase in water hardness reduced or eliminated the effect of silver on these parameters while enhancing survival, suggesting that the nature of the protective effect of hardness involves effects on the ionoregulatory disturbance associated with silver exposure. An increase in water hardness did not fully protect against the accumulation of silver associated with silver exposure. These results suggest that it may be possible to model chronic silver toxicity using a biotic ligand type model, and that a physiologically based model may be more appropriate because Na^+K^+-ATPase activity or Na^+ uptake is an endpoint for prediction rather than whole embryo or larval silver accumulation.
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Keywords: Egg; Larvae; Calcium; Na^+ uptake; Na^+K^+-ATPase activity; Na^+; Cl^-; Silver accumulation; Biotic Ligand Model

* Corresponding author. Tel.: +1 905 525 9140x27257; fax: +1 905 522 6066.
E-mail address: slewdog@sympatico.ca (T.P. Morgan).

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1. Introduction

Acutely, and when present in its ionic form, silver (Ag^+) is highly toxic to freshwater rainbow trout with 96-h LC50 values ranging from 6.5 to 13 μg/L (Davies et al., 1978; Nebeker et al., 1983; Hogstrand et al., 1996). Acute toxicity is strongly influenced by water chemistry. For example, Bury et al. (1999a) demonstrated a 4.3-fold increase and a 4.1-fold increase in the 96-h LC50 values for silver when the water concentrations of the anionic ligands chloride (Cl^-) and dissolved organic carbon (DOC) were increased from 50 to 1500 μM and 0.3 to 5.8 mg C/L, respectively. Elevating water hardness by increasing the calcium concentration from 50 to 1500 μM (approximately 5 to 150 mg/L as $CaCO_3$), had a small, but insignificant effect on acute silver toxicity (1.5-fold increase in the 96-h LC50 value). However, Davies et al. (1978) were able to demonstrate a 2-fold increase in the 96-h LC50 value when water hardness was increased from 26 to 350 mg/L as $CaCO_3$, while Karen et al. (1999) reported a 2.4-fold increase in the 96-h LC50 value when hardness was increased from 30 to 60 mg/L as $CaCO_3$. Still, the protective effect of water hardness on silver toxicity is much less than that of anionic ligands.

The primary mechanism of acute silver toxicity is a non-competitive inhibition of active Na^+ and Cl^- uptake at the gills, consequent to an inhibition of gill Na^+K^+-ATPase and carbonic anhydrase activity by Ag^+ (Morgan et al., 1997, 2004). This results in reductions in plasma Na^+ and Cl^- levels, and the fall in plasma ions eventually leads to circulatory collapse and death of the fish (Wood et al., 1996; Hogstrand and Wood, 1998). The protective effect of water chemistry on acute silver toxicity arises through effects on these physiological responses to silver exposure. For example, an increase in the water Cl^- and DOC concentrations from 50 to 600 μM and 0.31 to 5 mg C/L, respectively, reduced the degree of inhibition of whole body Na^+ uptake and gill Na^+K^+-ATPase activity caused by silver exposure (Bury et al., 1999b). Not surprising, given the relatively modest protective effect of water hardness on acute silver toxicity, increasing the water Ca^{2+} concentration from 50 to 1500 μM had no effect on the degree of inhibition of Na^+ uptake or gill Na^+K^+-ATPase activity induced by silver exposure (Bury et al., 1999b).

Chronically, Ag^+ is also toxic to rainbow trout. Exposure of rainbow trout embryos and larvae to 1 or 10 μg/L Ag from fertilization to hatch or swim-up was associated with elevated mortality (Guadagnolo et al., 2001; Brauner and Wood, 2002a,b; Morgan et al., 2005). However, the protective effect of anionic ligands on chronic silver toxicity is much less than the protective effect on acute silver toxicity. For example, 12 mg C/L DOC decreased the mortality of rainbow trout exposed to 10 μg/L total silver from fertilization to swim-up only up to hatch, but not following hatch (Brauner and Wood, 2002b), while an increase in water Cl^- from 30 to 3000 μM offered no protection in terms of mortality to trout exposed to either 0.1 or 1 μg/L Ag over the same time frame (Brauner et al., 2003). However, the protection afforded by water hardness during chronic silver exposure appears to be at least as effective as during acute exposure, if not more so. For example, elevating water hardness from 2 mg/L to 150 or 400 mg/L as $CaCO_3$ was significantly protective against the mortality following hatch in rainbow trout embryos and larvae exposed to 1 μg/L Ag from fertilization to swim-up (Morgan et al., 2005).

The mechanism of chronic silver toxicity appears to be similar to the mechanism of acute silver toxicity, involving an ionoregulatory disturbance (Guadagnolo et al., 2001; Brauner and Wood, 2002a,b). Interestingly, the protection afforded by DOC on chronic silver toxicity does not appear to involve effects on the physiological responses to silver (i.e. decreased whole body Na^+ uptake, Na^+K^+-ATPase activity and Na^+ concentration), with the exception of whole body Cl^- concentration. The degree of decrease in whole body Cl^- concentration was reduced in embryos and larvae exposed to 10 μg/L Ag in the presence of 12 mg C/L DOC (Brauner et al., 2003). The nature of the protective effect of water hardness on chronic silver toxicity is not known.

The objective of the present study was to investigate the effects of water hardness on the physiological responses to chronic silver exposure in rainbow trout early life stages to determine the nature of the protective effect of hardness on chronic toxicity. Early life stages of rainbow trout were exposed to nominal concentrations of 0, 0.1 and 1 μg/L total silver in water of three different hardness levels [very soft water (2 mg/L as $CaCO_3$), moderately hard water (150 mg/L) and hard water (400 mg/L), respectively] from fertil-

ization to swim-up and monitored for physiological responses, including whole embryo/larval silver accumulation, Na^+ concentration, Cl^- concentration, Na^+uptake and Na^+K^+-ATPase activity. The silver concentrations of 0.1 and 1 μg/L were chosen because the former represents the current Canadian Water Quality Guideline for silver (CCME, 1999), and the latter is the lowest concentration of silver previously reported to have significant effects on early life stages of rainbow trout (Brauner and Wood, 2002a). The water hardness levels of 2, 150 and 400 mg/L $CaCO_3$ were chosen because they are representative of the range of water hardnesses found in Canadian surface waters (Environment Canada, 1977). Understanding the effects of water hardness on the physiological responses to chronic silver exposure is important due to recent interest in the development of a chronic Biotic Ligand Model (BLM) for prediction of chronic silver toxicity.

2. Material and methods

2.1. Experimental animals

Approximately 15,000 freshly fertilized rainbow trout eggs were obtained from Rainbow Springs Trout Hatchery (Thamesford, ON, Canada) and were maintained for at most 3 h in a 10 L light-shielded container, which received flowing dechlorinated Hamilton city (ON, Canada) tap water (approximate ionic composition in mM: 0.6 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}], 0.05 [K^+]; 120 mg/L as $CaCO_3$ hardness; 1.3 mg C/L DOC; pH 7.5) while being transferred to the experimental exposure system (described below). The water temperature was 11.1 ± 0.1 °C during transfer to the exposure containers and for the duration of the experiment.

2.2. Experimental protocol

Rainbow trout were exposed in duplicate to three different water silver concentrations (nominal concentrations of 0, 0.1 and 1 μg/L total silver as $AgNO_3$) in water of three different hardness levels (very soft water, moderately hard water and hard water; nominal levels of 2, 150 and 400 mg/L as $CaCO_3$, respectively) from 3 h post-fertilization to swim-up (a period of 64 days) in a flow-through system. The first replicate exposure vessel contained 1000 eggs in 4 L of water and the second contained 500 eggs in 2 L of water. Whole embryo/larval silver accumulation, Na^+ and Cl^- concentrations, Na^+ uptake and Na^+K^+-ATPase activity were measured in eggs and larvae taken from the first replicate exposure container. Mortality, % hatch and % swim-up were measured in eggs and larvae from both replicates. (See Morgan et al., 2005 for a detailed analyses of these results. The Results section provides an overview of the mortality data for reference to the present physiological data). Exposure containers were covered and dark, and room lights were turned off during non-sampling periods because rainbow trout eggs are photosensitive early in development.

For the 2 mg/L $CaCO_3$ exposures, acidified stock solutions (0.5% HNO_3, trace metal grade, Fisher Scientific, Toronto, ON, Canada) of $AgNO_3$ in distilled, deionized water were made up in 1 L amber bottles at 1000 times the final desired concentration. For the 150 and 400 mg/L $CaCO_3$ exposures acidified stock solutions (0.5% HNO_3, trace metal grade, Fisher Scientific, Toronto, ON, Canada) of $AgNO_3$, calcium sulfate and magnesium sulfate in distilled, deionized water were made up in 4 L light-shielded glass flasks at 1000 times the final desired concentration. Calcium sulfate and magnesium sulfate were mixed in a 3 to 1 ratio to achieve the desired levels of water hardness of 150 mg/L $CaCO_3$ (1.125 mM Ca^{2+} + 0.375 mM Mg^{2+}), and 400 mg/L $CaCO_3$ (3 mM Ca^{2+} + 1 mM Mg^{2+}), respectively (Welsh et al., 2000). The stock solutions were constantly stirred on magnetic stirrers for the duration of the experiment due to the insolubility of calcium sulfate at such high concentrations. All stock solutions were delivered via peristaltic pump to 150 mL header tanks at a flow rate of 0.1 mL/min and were mixed with synthetic very soft water (approximate ionic composition in mM; 0.05 [Na^+], 0.02 [Cl^-], 0.02 [Ca^{2+}], non-detectable [Mg^{2+}], non-detectable [K^+]; 2 mg/L as $CaCO_3$ hardness; 0.5 mg C/L DOC; pH 7), generated by reverse osmosis of Hamilton city (ON, Canada) dechlorinated tap water. This resulted in 1000-fold dilutions of the stock solutions, yielding nine nominal exposure solutions of 0 μg/L Ag + 2 mg/L $CaCO_3$, 0 μg/L Ag + 150 mg/L $CaCO_3$, 0 μg/L Ag + 400 mg/L $CaCO_3$, 0.1 μg/L Ag + 2 mg/L $CaCO_3$, 0.1 μg/L Ag + 150 mg/L

$CaCO_3$, 0.1 μg/L Ag + 400 mg/L $CaCO_3$, 1 μg/L Ag + 2 mg/L $CaCO_3$, 1 μg/L Ag + 150 mg/L $CaCO_3$ and 1 μg/L Ag + 400 mg/L $CaCO_3$. The header tanks were vigorously aerated to facilitate mixing of the stock solutions with the soft water, and the overflow was directed to the exposure containers at a flow rate of 100 mL/min.

To facilitate measurement of the water silver concentrations of the 0.1 μg/L Ag treatment groups (note: 0.1 μg/L Ag is very close to the detection limit of 0.05 μg/L Ag of the graphite furnace atomic absorption spectrophotometer), 10 μCi (370 kBq) of ^{110m}Ag/L (RISOE Nuclear Research Reactor, Roskilde, Denmark) was added to the stock solution, enabling the silver concentration to be measured by radioisotopic dilution (see below).

Dead embryos or larvae were removed daily from the exposure chambers. The criterion for mortality was whiteness early in development, while later in development it was cessation of heartbeat. On days 8, 15, 19, 22, 28, 36, 43, 57 and 64 post-fertilization, 10 embryos (with yolk-sac attached) or larvae from each treatment were euthanized with a lethal dose of tricaine methanesulphonate (MS-222), rinsed once with distilled, deionized water, blotted dry, weighed and placed in bullet tubes for determination of whole embryo/larval silver, Na^+ and Cl^- concentrations (see below). An additional eight embryos or larvae were collected in the same manner, but not on day 8 or 15 post-fertilization, and were immediately frozen in liquid nitrogen for determination of whole embryo/larval Na^+K^+-ATPase activity (see below). Twenty embryos or larvae were also collected as above at each sampling time, except on day 8 post-fertilization, for determination of whole embryo/larval Na^+ uptake, as described below. Yolk sacs were not separated from the embryos prior to embryo analysis because these data may be used for chronic biotic ligand modeling purposes. If so, it is important to establish the relationship between whole body silver accumulation and chronic silver toxicity because whole body accumulation may be used as the endpoint to predict toxicity in a biotic ligand model, and the yolk sac can accumulate a significant portion of the whole body silver burden (Guadagnolo et al., 2001) which is likely accessible to the organism to cause toxicity.

Up to and including day 43 post-fertilization, embryos were sampled, whereas larvae were sampled on days 50, 57 and 64 post-fertilization. Because of mortality, we do not have data for larvae exposed to 0, 0.1 and 1 μg/L Ag in water of hardness 2 mg/L $CaCO_3$. For this reason, comparisons were made among all water hardness levels up to and including day 43 post-fertilization, while on all subsequent days comparisons were made among only the 150 and 400 mg/L $CaCO_3$ water hardnesses. Ideally, larvae should have been sampled on days 36 and 43 post-fertilization, because 50% hatch occurred on approximately day 30 post-fertilization (Morgan et al., 2005). However, once post-hatch larval mortality was taken into consideration, there were not enough larvae for sampling on days 36 and 43 post-fertilization, so embryos, but not larvae, were sampled on these days.

Water samples were taken once a week from each exposure chamber and immediately acidified to 0.5% (v/v) with HNO_3 (trace metal grade, Fisher Scientific, Toronto, ON, Canada) for determination of water total and dissolved (0.45 micron Acrodisc polyethersulfone syringe filters, Pall Gelman Laboratory, Ann Arbor, MI, USA) silver concentrations, and calcium and magnesium concentrations. Water and stock solution flow rates were checked every second day. The temperature of the exposure water was recorded daily, and remained between 11.0 and 11.2 °C.

2.3. Water analyses

Analysis of the water total and dissolved silver concentrations from the 1 μg/L Ag treatment groups was conducted by graphite furnace atomic absorption spectrophotometry (Varian 1275, GTA-95, Varian, Mississauga, ON, Canada). Analysis of the water total silver concentrations from the 0.1 μg/L Ag treatment groups was conducted by radioisotopic dilution. To do this, the total silver concentration of the stock solution was measured by graphite furnace atomic absorption spectrophotometry (Varian 1275, GTA-95, Varian, Mississauga, ON, Canada), while the radioactivity of ^{110m}Ag in the stock solution was measured by gamma counting (Hansen et al., 2002, MINAXI Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada). The specific activity of the stock solution was then calculated (cpm/μg Ag). The water total silver concentrations in the exposure containers were calculated by dividing the measured ^{110m}Ag

activity (cpm) of the exposure water by the measured specific activity of the stock solution (cpm/μg Ag).

The calcium and magnesium concentrations of the exposure water were determined by flame atomic absorption spectrophotometry (Varian 1275, Varian, Mississauga, ON, Canada).

Silver speciation in each exposure was calculated using the aquatic geochemical program MINEQL+ (Schecher and McAvoy, 1992) based on the known water chemistry (see above) and appropriate binding constants from Janes and Playle (1995).

2.4. Ag, Na^+ and Cl^- concentrations in embryos and larvae

All samples collected for analysis of whole embryo/larval Ag, Na^+ and Cl^- concentrations were digested in five times the sample mass of 1 N HNO_3 (trace metal grade, Fisher Scientific, Toronto, ON, Canada) for 24 h at approximately 60 °C. Digested samples were homogenized, spun down and then a portion of the supernatant was diluted with 1% HNO_3 (trace metal grade, Fisher Scientific, Toronto, ON, Canada) and analyzed for either silver via graphite furnace atomic absorption spectrophotometry (Varian 1275, GTA-95, Varian, Mississauga, ON, Canada), Na^+ by flame atomic absorption spectrophotometry (Varian 1275, Varian, Mississauga, ON, Canada) or Cl^- using the colorimetric mercuric thiocyanate method (Zall et al., 1956).

2.5. Na^+ uptake

Whole embryo/larval Na^+ uptake measurements were conducted in 60 mL Nalgene bottles. At each sample time 20 embryos/larvae were added to the bottles containing 20 mL of water (for measurements in embryos) or 40 mL of water (for measurements in larvae) to which the organisms had been exposed. ^{22}Na (7.2 μCi, 266.4 kBq) was then added to each bottle. The water was aerated over the course of the measurement. Ten minutes after the start of the measurement and again at the end of the measurement, 2-mL water samples were taken in duplicate for determination of water ^{22}Na radioactivity by gamma counting (MINAXI Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada) and Na^+ concentration by flame atomic absorption spectrophotometry (Varian AA-1275, Varian, Mississauga, ON, Canada). The Na^+ uptake measurements lasted a total of 6 h, however, because there is a large, non-specific binding of ^{22}Na to the embryos within the first hour of measurement, only the ^{22}Na accumulation between 2 and 6 h of the measurement was used to calculate uptake. At 2 and at 6 h, 10 embryos or larvae were collected as described above, except embryos and larvae were rinsed three times with 5 mM NaCl to remove surface bound radioisotope by displacement before the single rinse with distilled, deionized water and were placed in vials. Embryos/larvae were then measured for radioactivity by gamma counting (MINAXI Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada). Whole embryo/larval Na^+ uptake was calculated as follows:

$$\text{Whole embryo/larval Na}^+ \text{ uptake} = \frac{\text{cpm}_6 - \text{cpm}_2}{\text{SA} \times \text{wt} \times t}$$

where cpm_6 and cpm_2 are the total counts per minute in the embryos or larvae at 6 and 2 h, respectively, SA is the measured specific activity of the water, wt is the wet weight of the embryo or larvae (g), and t is the time of exposure (h). The measured average specific activity of the water was calculated as follows:

$$\text{SA} = \frac{[(\text{cpm}_i/[\text{Na}]_i) + (\text{cpm}_f/[\text{Na}]_f)]}{2}$$

where cpm_i represents the ^{22}Na cpm/mL in the water 10 min following the addition of ^{22}Na to the water, cpm_f represents the ^{22}Na cpm/mL in the water at the end of the measurement, and $[Na]_i$ and $[Na]_f$ represent the initial and final sodium concentrations of the water, respectively.

2.6. Na^+K^+-ATPse activity

Whole embryo/larval Na^+K^+-ATPase activity was measured according to the method of McCormick (1993), using a plate reader (MRX, Dynex Technologies, Chantilly, VA, USA). Activity was expressed as the concentration of ADP liberated per unit time and was standardized to protein content. The protein content was measured using Bradford reagent (Sigma, Oakville, ON, Canada) with bovine serum albumin as a standard (Sigma, Oakville, ON, Canada).

2.7. Statistics

Differences in mean values were compared between silver treatments within a given water hardness treatment on any given day, and between water hardness treatments within a given silver treatment on any given day using a one-way analysis of variance, followed by a Student-Newman Keuls post-hoc test. Because water hardness had significant effects on mean values measured in the absence of silver, significant differences between hardness treatments within a given silver treatment were not used to determine protection, but rather hardness was deemed protective if any significant effects of silver exposure at 2 mg/L $CaCO_3$ (up to and including day 43 post-fertilization) or 150 mg/L $CaCO_3$ (day 50–64 post-fertilization) were not observed at 150 or 400 mg/L $CaCO_3$. Values are mean ± 1 standard error of the mean (S.E.M.), and $N = 10$ for silver, Na^+ and Cl^- concentrations and Na^+ uptake and $N = 8$ for Na^+K^+-ATPase activity. The level of statistical significance employed was $P < 0.05$.

3. Results

3.1. Water chemistry

The measured water total silver concentrations were generally within 10% of the nominal water silver concentrations, averaging below detection (<0.05 μg/L), 0.10 ± 0.01 and 0.94 ± 0.14 μg/L Ag ($N = 19$–21) in the control, 0.1 and 1 μg/L Ag nominal treatments, respectively. In the 1 μg/L Ag exposures, measured filtered water silver concentrations averaged 75 ± 7% of the total silver concentrations, indicating that a substantial proportion of the silver was dissolved, and filtered concentrations did not differ between water hardness treatments (mean 0.69 ± 0.03 μg/L Ag, $N = 57$). We do not have measured filtered water silver concentrations for the 0.1 μg/L exposures. The measured water calcium concentrations and magnesium concentrations were generally within 30% of nominal concentrations. Mean calcium concentrations were 0.02 ± 0.02, 1.33 ± 0.15 and 2.76 ± 0.34 mM, while mean magnesium concentrations were below detection (<0.005 mM), 0.32 ± 0.02 and 0.73 ± 0.10 mM ($N = 19$–21) in the nominal 2, 150 and 400 mg/L $CaCO_3$ hardness treatments. For the remainder of the paper, we have referred to nominal concentrations because of the close agreement between nominal and measured values. Results of the silver speciation calculations indicate that both ionic silver and silver-DOM (dissolved organic matter) complexes were present in the exposure waters. At 0.1 μg/L Ag, the ionic (Ag^+) concentration was 0.006 ± 0.001 μg/L and the Ag-DOM complex concentration was 0.09 ± 0.01 μg/L, while at 1 μg/L Ag, the Ag^+ concentration was 0.09 ± 0.02 μg/L and the Ag-DOM complex concentration was 0.84 ± 0.10 μg/L ($N = 19$–21).

3.2. Mortality

Detailed mortality data are presented by Morgan et al. (2005). In brief, exposure to 0.1 and 1 μg/L Ag had no significant effects on mortality prior to 50% hatch in water of hardness 2 mg/L $CaCO_3$. However, following 50% hatch, exposure to 1 μg/L Ag, but not 0.1 μg/L Ag, significantly increased mortality relative to controls. Increasing water hardness was protective against the mortality associated with exposure to 1 μg/L Ag in water of hardness 2 mg/L $CaCO_3$. Mortality was significantly greater at 2 mg/L than at 150 or 400 mg/L $CaCO_3$.

Even in the absence of silver, water hardness was protective against mortality. Prior to 50% hatch, mortality was significantly greater at 2 mg/L, than at 150 or 400 mg/L $CaCO_3$, while following 50% hatch, mortality was significantly greater at 2 mg/L $CaCO_3$ than at 150 mg/L $CaCO_3$, although there was no difference in mortality between the 2 and 400 mg/L water hardnesses.

3.3. Silver accumulation

Prior to 50% hatch, exposure to 1 μg/L Ag, but not 0.1 μg/L Ag, in water of hardness 2 mg/L $CaCO_3$ significantly increased whole body silver accumulation relative to controls on days 15, 19 and 28 post-fertilization (Fig. 1A). In general, this effect of exposure to 1 μg/L Ag was also observed in water of hardness 150 and 400 mg/L $CaCO_3$ (Fig. 1B and C).

Following 50% hatch, exposure to 1 μg/L Ag significantly increased whole body silver accumulation relative to controls on day 43 post-fertilization in water of hardness 2 mg/L (Fig. 1A), and increasing water

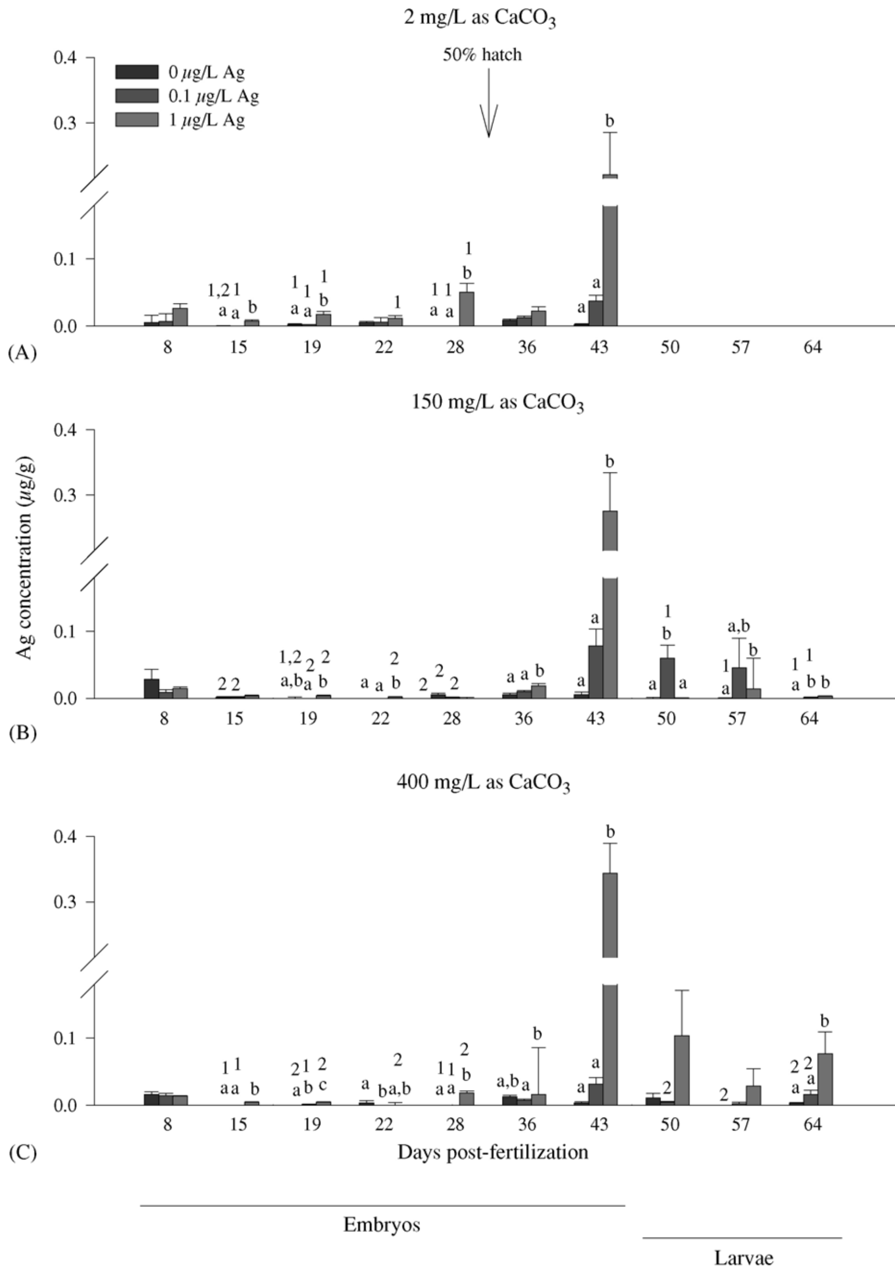

Fig. 1. Whole body silver accumulation in rainbow trout during flow-through exposure to 0 (black bars), 0.1 (light gray bars) and 1 (dark gray bars) μg/L total Ag (as $AgNO_3$, nominal values) from fertilization to swim-up in water of hardness (A) 2 mg/L as $CaCO_3$; (B) 150 mg/L as $CaCO_3$ and (C) 400 mg/L as $CaCO_3$. Data for days 8, 15, 19, 22, 28, 36 and 43 are for embryos, while data for days 50, 57 and 64 are for larvae. There are no data for days 50, 57 and 64 in the 2 mg/L $CaCO_3$ treatment group due to mortality. The arrow indicates 50% hatch. Values are mean ±1 S.E.M. ($N = 10$). Letters that differ for a given day indicate statistically significant differences among silver treatments at the given water hardness, while numbers that differ for a given day indicate statistically significant differences among hardness treatments at the given silver concentration (one-way analysis of variance followed by a Student-Newman Keuls post-hoc test, $P < 0.05$).

hardness to 150 or 400 mg/L $CaCO_3$ did not prevent this accumulation (Fig. 1B and C).

In larvae sampled from water of hardness 150 mg/L $CaCO_3$ following 50% hatch, exposure to 0.1 μg/L Ag significantly increased whole body silver accumulation relative to controls on days 50 and 64 post-fertilization, while exposure to 1 μg/L Ag increased whole body silver accumulation on days 57 and 64 post-fertilization (Fig. 1B). An increase in water hardness to 400 mg/L $CaCO_3$ prevented the accumulation of silver associated with exposure to 0.1 μg/L Ag on days 50 and 64 post-fertilization, but did not prevent the accumulation associated with exposure to 1 μg/L Ag on day 64 post-fertilization (Fig. 1B and C).

3.4. Na^+ concentration

In the absence of silver, at all three water hardness levels, whole embryo Na^+ concentration was low and relatively constant prior to 50% hatch at 15.06 ± 1.84, 11.01 ± 1.80 and 12.19 ± 0.69 mmol/kg wet weight in water of hardness 2, 150 and 400 mg/L $CaCO_3$, respectively (Fig. 2). Following 50% hatch, there was a steady increase in Na^+ concentration with development until the concentration leveled off at about 30 mmol/kg on day 50 and onwards, at least at 150 and 400 mg/L $CaCO_3$.

Prior to hatch, exposure to 0.1 and 1 μg/L Ag significantly decreased whole body Na^+ concentration relative to controls on days 15 and 22 post-fertilization in water of hardness 2 mg/L $CaCO_3$ (Fig. 2A). This effect of silver exposure was not observed in water of hardness 150 or 400 mg/L $CaCO_3$ (Fig. 2B and C). Interestingly, although there was no effect of 1 μg/L Ag on whole body Na^+ concentration on day 19 post-fertilization in water of hardness 2 mg/L $CaCO_3$ (Fig. 2A), there was a decrease in Na^+ concentration at this silver concentration relative to controls in the two higher hardnesses (Fig. 2B and C).

Following 50% hatch, exposure to 0.1 and 1 μg/L Ag significantly decreased whole body Na^+ concentration relative to controls on day 43 post-fertilization in water of hardness 2 mg/L $CaCO_3$, while exposure to 1 μg/L Ag (only) significantly decreased Na^+ concentration relative to controls on day 36 post-fertilization (Fig. 2A). Increasing water hardness to 150 mg/L, but not to 400 mg/L $CaCO_3$, eliminated the effect of 0.1 μg/L Ag on whole body Na^+ concentration on day 43 post-fertilization (Fig. 2B and C). An increase in water hardness also eliminated the effect of 1 μg/L Ag on Na^+ concentration, although an increase to 400 mg/L $CaCO_3$ was required (Fig. 2A–C).

In larvae sampled from water of hardness 150 mg/L $CaCO_3$ following hatch, exposure to 0.1 μg/L Ag significantly decreased whole body Na^+ concentration relative to controls on day 50 post-fertilization, while exposure to 1 μg/L Ag significantly decreased the concentration on days 57 and 64 post-fertilization (Fig. 2B). These effects of silver exposure were also observed in water of hardness 400 mg/L $CaCO_3$, as well as, a decrease in Na^+ concentration relative to controls on day 50 post-fertilization, although the latter was not observed at 150 mg/L $CaCO_3$ (Fig. 2A–C).

3.5. Cl^- concentration

In the absence of silver, Cl^- concentration was constant prior to hatch at 38.37 ± 2.52, 36.56 ± 1.72 and 38.85 ± 2.02 mmol/kg wet weight in water of hardness 2, 150 and 400 mg/L $CaCO_3$, respectively (Fig. 3). Following 50% hatch, the Cl^- concentration remained at approximately these levels, but by day 50 post-fertilization, the Cl^- concentration had decreased relative to day 43 values to about 27 mmol/kg in both of the higher hardness waters, and remained at these lower values until the end of the experiment.

Prior to hatch, exposure to silver had no significant effects on whole embryo chloride concentration in water of hardness 2 mg/L $CaCO_3$ (Fig. 3A). Interestingly, exposure to 1 μg/L Ag at hardness 400 mg/L $CaCO_3$ resulted in a decrease in Cl^- concentration on day 28 post-fertilization, although there was no effect of this silver concentration in water of hardness 2 or 150 mg/L $CaCO_3$. (Fig. 3A–C).

Following 50% hatch, exposure to 1 μg/L Ag significantly decreased whole body Cl^- concentration on days 36 and 43 post-fertilization in water of hardness 2 mg/L $CaCO_3$ (Fig. 3A). An increase in water hardness to 150 mg/L $CaCO_3$ eliminated this effect of silver on day 36 post-fertilization (Fig. 3B), but an increase in water hardness to 400 mg/L $CaCO_3$ was required to eliminate the effect of silver exposure on day 43 post-fertilization (Fig. 3C).

In larvae sampled from water of hardness 150 mg/L $CaCO_3$ following hatch, only exposure to 0.1 μg/L Ag significantly decreased whole body Cl^- concentration,

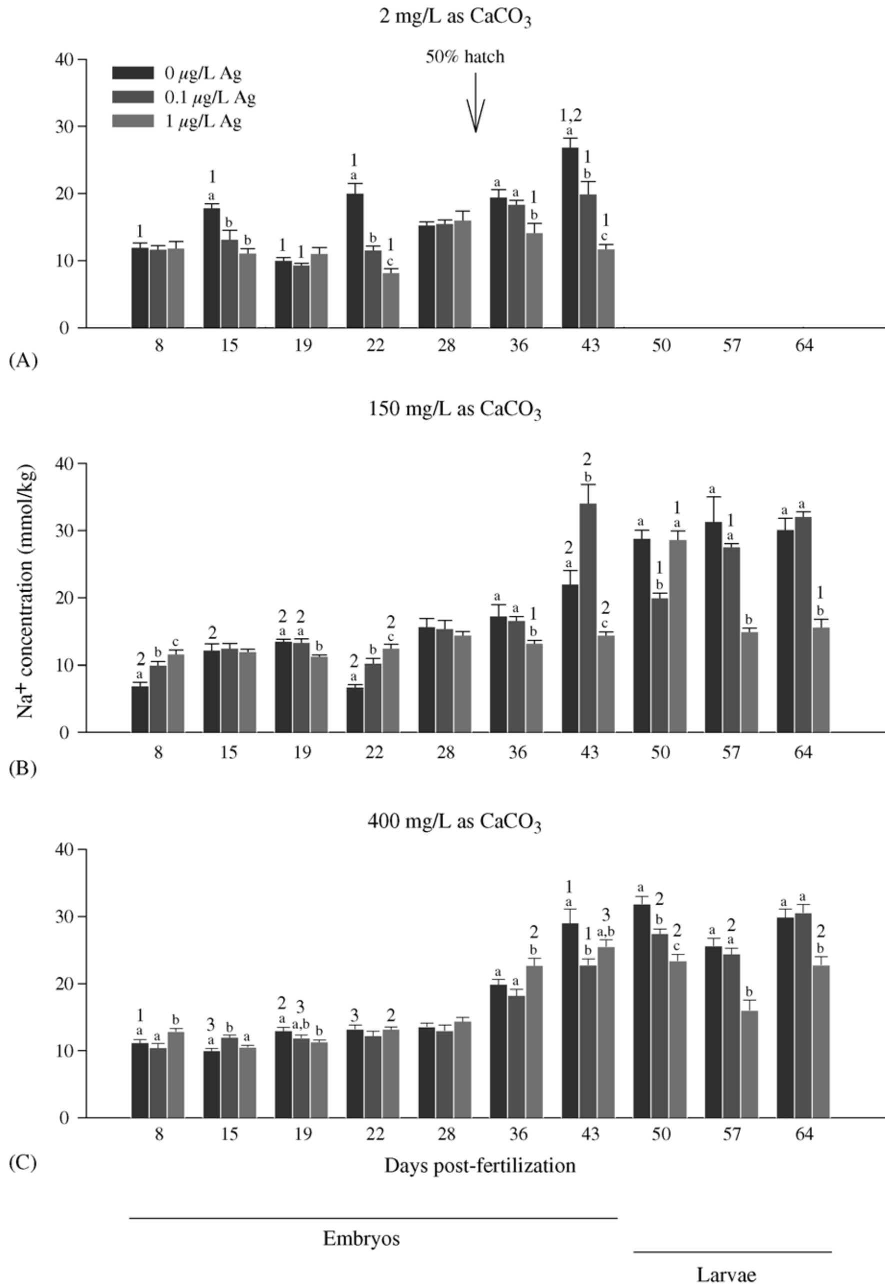

Fig. 2. Whole body Na^+ concentration in rainbow trout during flow-through exposure to 0 (black bars), 0.1 (dark gray bars) and 1 (light gray bars) µg/L total Ag (as $AgNO_3$, nominal values) from fertilization to swim-up in water of hardness (A) 2 mg/L as $CaCO_3$; (B) 150 mg/L as $CaCO_3$ and (C) 400 mg/L as $CaCO_3$. See legend of Fig. 1 for further details.

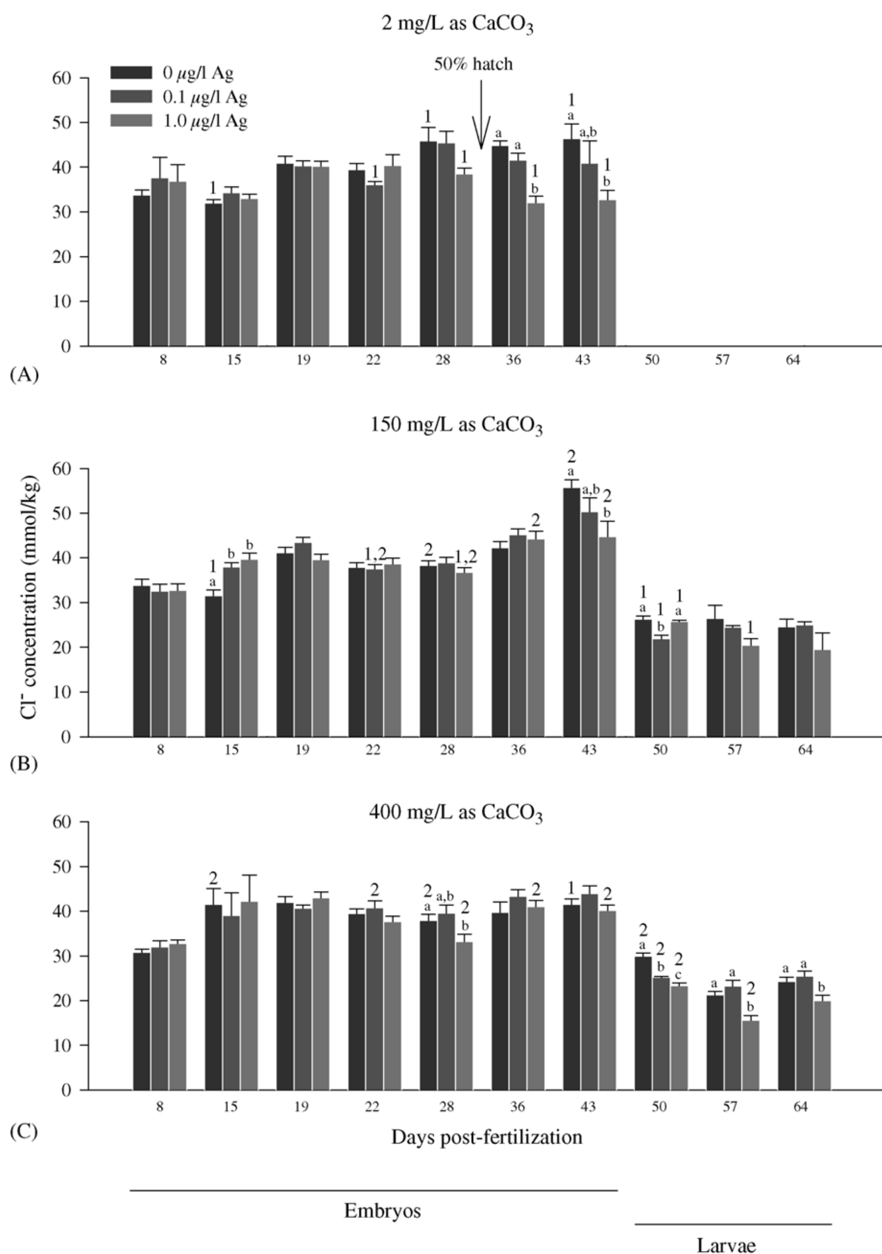

Fig. 3. Whole body Cl^- concentration in rainbow trout during flow-through exposure to 0 (black bars), 0.1 (dark gray bars) and 1 (light gray bars) μg/L total Ag (as $AgNO_3$, nominal values) from fertilization to swim-up in water of hardness (A) 2 mg/L as $CaCO_3$; (B) 150 mg/L as $CaCO_3$ and (C) 400 mg/L as $CaCO_3$. See legend of Fig. 1 for further details.

and only on day 50 post-fertilization (Fig. 3B). An increase in water hardness to 400 mg/L $CaCO_3$ had no effect on this decrease in Cl^- concentration and, in fact, was accompanied by a significant decrease in Cl^- concentration on days 50, 57 and 64 post-fertilization in larvae exposed to 1 μg/L Ag (Fig. 3C), although there was no effect of exposure to this silver concentration in water of hardness 150 mg/L $CaCO_3$ (Fig. 3B).

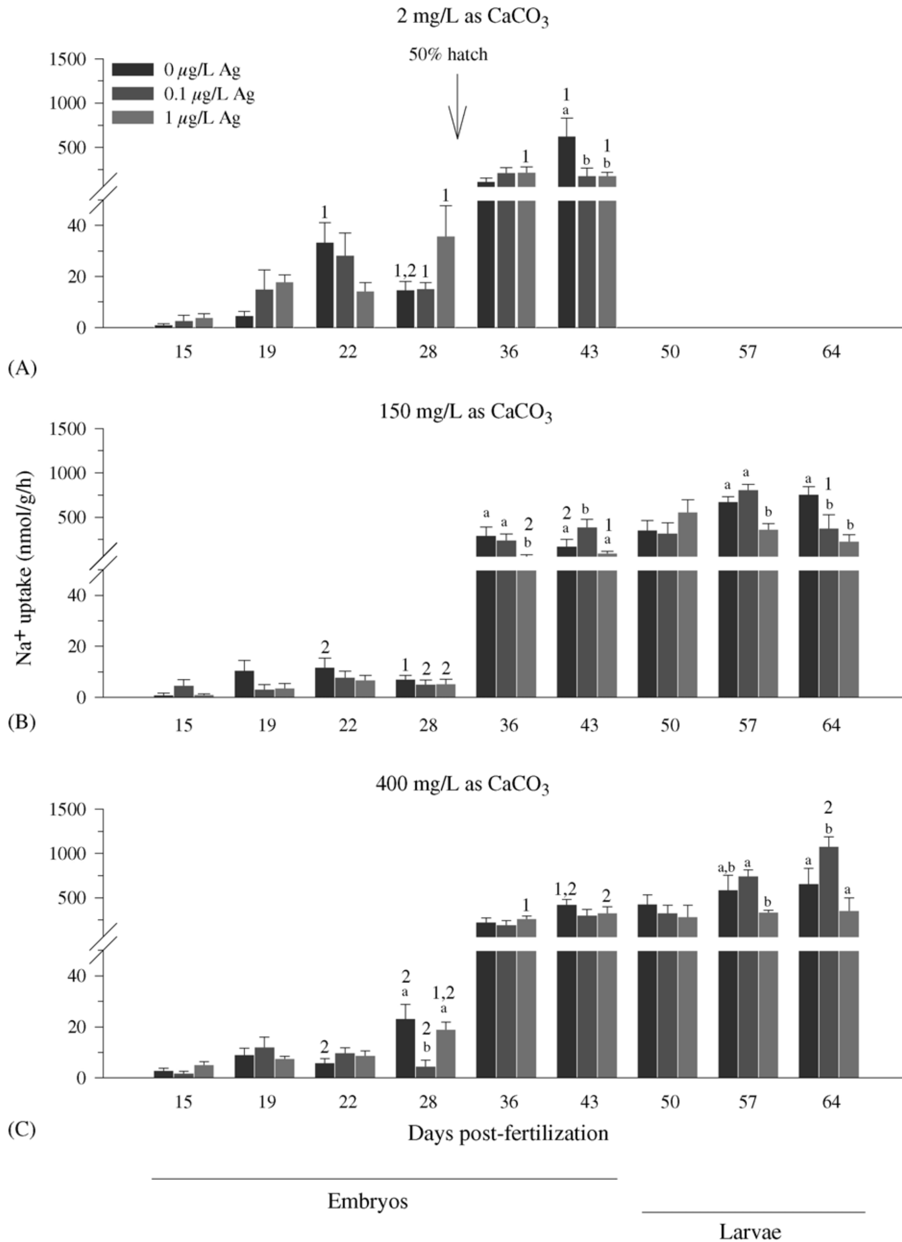

Fig. 4. Whole body Na^+ uptake in rainbow trout during flow-through exposure to 0 (black bars), 0.1 (dark gray bars) and 1 (light gray bars) μg/L total Ag (as $AgNO_3$, nominal values) from fertilization to swim-up in water of hardness (A) 2 mg/L as $CaCO_3$; (B) 150 mg/L as $CaCO_3$ and (C) 400 mg/L as $CaCO_3$. Data for days 15, 19, 22, 28, 36 and 43 are for embryos, while data for days 50, 57 and 64 are for larvae. See legend of Fig. 1 for further details.

3.6. Na^+ uptake

Prior to hatch and in the absence of silver, whole embryo Na^+ uptake was low and relatively constant at all water hardness levels at 13.46 ± 7.27, 7.61 ± 2.44 and 4.15 ± 1.26 nmol/g/h in water of hardness 2, 150 and 400 mg/L $CaCO_3$, respectively (Fig. 4). Following 50% hatch, there was a dramatic increase in Na^+ uptake, and uptake continued to increase with development, reaching 626 ± 202 nmol/g/h on day 43 post-fertilization in water of 2 mg/L $CaCO_3$, and 760 ± 85 and 661 ± 169 nmol/g/h on day 64 post fertilization in water of hardness 150 and 400 mg/L $CaCO_3$, respectively.

Exposure to silver had no significant effect on whole body Na^+ uptake prior to hatch in water of hardness 2 mg/L $CaCO_3$ (Fig. 4A), although an inhibition was observed on day 28 post-fertilization during exposure to 0.1 μg/L Ag in 400 mg/L $CaCO_3$ hardness (Fig. 4C).

Following 50% hatch, exposure to both 0.1 and 1 μg/L Ag significantly inhibited Na^+ uptake on day 43 post-fertilization in water of hardness 2 mg/L $CaCO_3$ (Fig. 4A). There was no inhibitory effect of silver on this day in water of hardness 150 or 400 mg/L $CaCO_3$ (Fig. 4B and C). There was no effect of silver on day 36 post-fertilization in water of hardness 2 mg/L $CaCO_3$ (Fig. 4A), but at 150 mg/L $CaCO_3$ exposure to 1 μg/l silver significantly inhibited Na^+ uptake on this day (Fig. 4B). This effect was eliminated when water hardness was further increased to 400 mg/L $CaCO_3$ (Fig. 4C).

In larvae sampled from water of hardness 150 mg/L $CaCO_3$ following hatch, exposure to 0.1 μg/L Ag inhibited whole body Na^+ uptake relative to controls on day 64 post-fertilization, and exposure to 1 μg/L Ag inhibited uptake on days 57 and 64 post-fertilization (Fig. 4B). There was no significant effect of silver exposure on these days in water of hardness 400 mg/L $CaCO_3$ (Fig. 4C).

3.7. Na^+K^+-ATPase activity

Na^+K^+-ATPase activity was low during exposure to 0 μg/L Ag, but increased steadily over the course of development, reaching 0.36 ± 0.07 μmol ADP/mg protein/h on day 43 post-fertilization in water of hardness 2 mg/L $CaCO_3$, and 4.89 ± 0.81 and 3.82 ± 0.63 μmol ADP/mg protein/h on day 64 post-fertilization in water of hardness 150 and 400 mg/L $CaCO_3$, respectively (Fig. 3).

Prior to 50% hatch, there was no significant effect of silver on Na^+K^+-ATPase activity in water of hardness 2 mg/L $CaCO_3$, except for an increase in activity during exposure to 1 μg/L Ag on day 22 post-fertilization (Fig. 5A). In water of hardness 150 or 400 mg/L $CaCO_3$ there was no significant effect of exposure to 1 μg/L Ag on this day (Fig. 5B and C).

Following 50% hatch, there was no significant effect of silver on Na^+K^+-ATPase activity in water of hardness 2 mg/L $CaCO_3$ (Fig. 5A), although at a water hardness of 400 mg/L $CaCO_3$, exposure to 0.1 μg/L Ag inhibited activity on days 36 and 43 post-fertilization (Fig. 5C).

In larvae exposed in water of hardness 150 mg/L $CaCO_3$, exposure to 1 μg/L Ag significantly inhibited Na^+K^+-ATPase activity on days 50 and 64 post-fertilization (Fig. 5B). This effect of exposure to 1 μg/L Ag was not observed in water of hardness 400 mg/L $CaCO_3$ (Fig. 5C).

4. Discussion

4.1. Ionoregulation and development

In the absence of silver at all three water hardness levels, Na^+ uptake was a constant low value prior to 50% hatch (Fig. 4), as has been demonstrated in an earlier study on rainbow trout at 120–140 mg/L $CaCO_3$ water hardness (Brauner and Wood, 2002a). Low Na^+ uptake has also been demonstrated in Atlantic salmon embryos at this time (Rudy and Potts, 1969; Eddy and Talbot, 1985). Following 50% hatch, there was a dramatic increase in Na^+ uptake, and uptake continued to increase until day 64 post-fertilization (Fig. 4).

Most evidence to date suggests that chloride cells are the principal cells involved in branchial chloride uptake in freshwater fish, and uptake across the apical membrane occurs via a Cl^-/HCO_3^- exchanger, while uptake across the basolateral membrane likely occurs via a Cl^- selective channel (Perry, 1997). A current model for branchial Na^+ uptake suggests that apical uptake occurs via a proton pump-coupled Na^+ channel, such that proton pumping in the chloride cell creates an electrochemical gradient that drives Na^+

uptake through epithelial Na^+ channels on adjacent, but electrically coupled, pavement cells (Perry and Fryer, 1997), while basolateral uptake occurs via the Na^+K^+-ATPase (Perry, 1997). Extrabranchial chloride cells in the skin are believed to be the sites of ionoregulation in early developmental stages of teleosts until chloride cells in the gills become functional (Rombough, 2004). If we assume that extrabranchial chloride cells

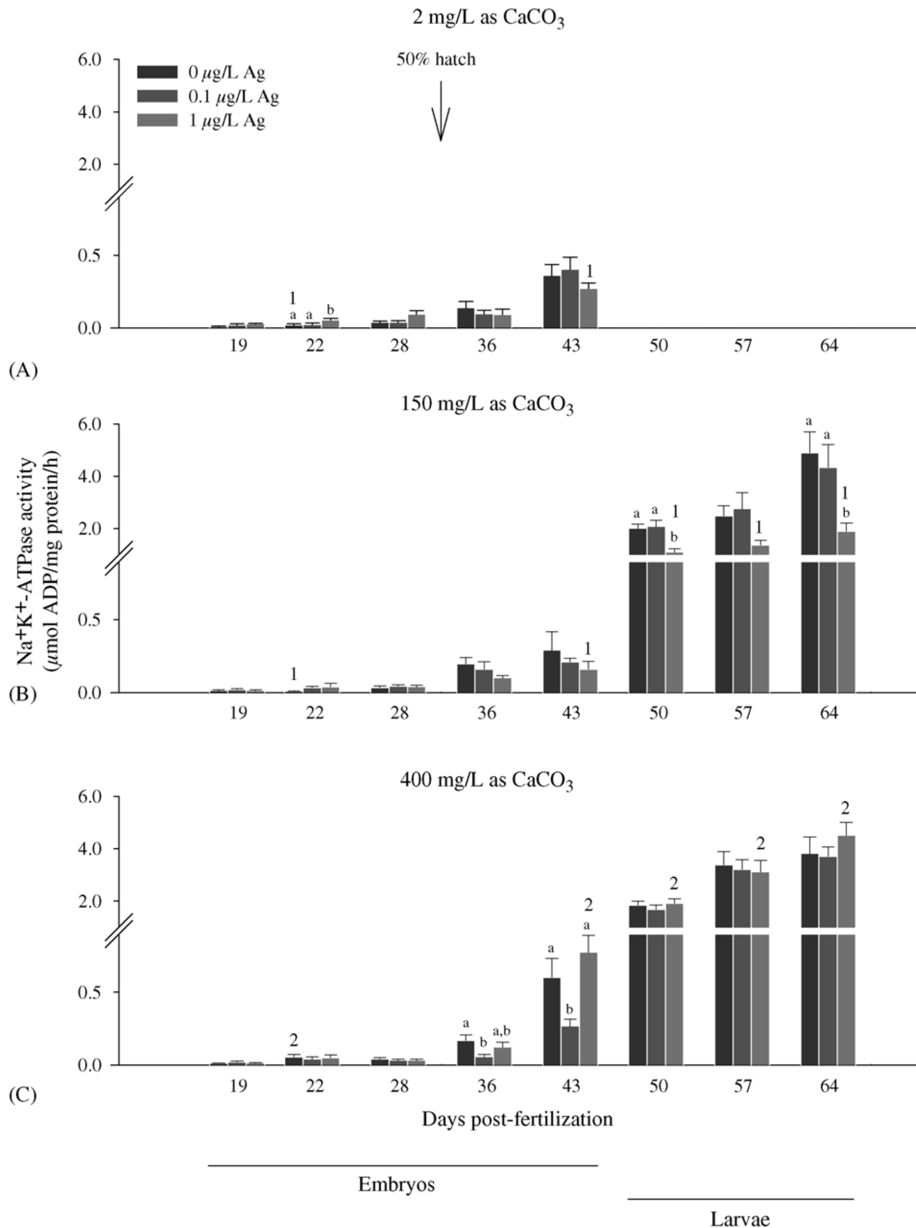

Fig. 5. Whole body Na^+K^+-ATPase activity in rainbow trout during flow-through exposure to 0 (black bars), 0.1 (dark gray bars) and 1 (light gray bars) μg/L total Ag (as $AgNO_3$, nominal values) from fertilization to swim-up in water of hardness (A) 2 mg/L as $CaCO_3$; (B) 150 mg/L as $CaCO_3$ and (C) 400 mg/L as $CaCO_3$. Data for days 19, 22, 28, 36 and 43 are for embryos, while data for days 50, 57 and 64 are for larvae. Values are mean ± S.E.M. ($N = 8$). See legend of Fig. 1 for further details.

function similarly to branchial chloride cells, and that chloride cell number and ion uptake are correlated in the early developmental stages of rainbow trout because uptake of ions occurs either directly or indirectly through this gill cell type, the constant Na^+ uptake prior to hatch correlates with a constant number of chloride cells in the fish at this time, while the dramatic increase in uptake following hatch correlates with an increase in the number of chloride cells at this time (Rombough, 1999), an observation also made by Brauner and Wood (2002a). Not surprisingly, the time course of whole embryo/larval Na^+ concentration paralleled the time course of whole embryo/larval Na^+ uptake (Figs. 2 and 4). Surprisingly, however, the time course of Na^+ uptake did not parallel the time course of Na^+K^+-ATPase activity, where activity progressively increased over the course of development (Figs. 4 and 5). Brauner and Wood (2002a) attributed the lack of correlation, which was similarly noted in their study, to two possibilities. First, the chorion may act as a barrier to ion uptake, masking any relationship between uptake and activity prior to hatch. Second, in early life stages, Na^+K^+-ATPase may be associated with processes other than ion uptake, which could explain the lack of correlation following hatch.

4.2. Water hardness and development

Exposure of embryos and larvae in water of hardness 2 mg/L $CaCO_3$ was associated with mortality, even in the absence of silver (Morgan et al., 2005, see also the Results section of the present study). In fact, mortality was significantly greater at 2 mg/L than at 150 mg/L and 400 mg/L, or 150 mg/L only, prior to hatch and following hatch, respectively. Brown and Lynam (1981) noted that 10 mg/L Ca^{2+} was required for survival of freshly fertilized brown trout (*Salmo trutta* L.) eggs in water of low pH, while 1 mg/L Ca^{2+} was required for survival of larvae. The required concentrations of Ca^{2+} noted in the study of Brown and Lynam (1981) are higher than the concentration of Ca^{2+} in our soft water exposure of 0.80 mg/L (0.02 mM), suggesting that inadequate water Ca^{2+} was responsible for the mortality we observed.

The presence of Ca^{2+} in the water decreases Na^+ and Cl^- effluxes in the gills of adult goldfish and brown trout through its effects on the permeability of the gills to these ions (Hunn, 1985). As discussed above, in early developmental stages of teleosts, extra-branchial chloride cells in the skin are believed to be the sites of ionoregulation until chloride cells in the gills become functional (Rombough, 2004). Whether Ca^{2+} has the same effects on permeability in the skin and gills during early development is unknown, but if so, low Ca^{2+} could increase the permeability of the skin and gills to Na^+ and Cl^-, increasing their respective effluxes and potentially, mortality. However, there were not large differences in whole embryo/larval Na^+ concentrations, Cl^- concentrations or Na^+ uptake rates among the soft water, moderately hard water and hard water treatments (Figs. 2–4), suggesting that Na^+ and Cl^- effluxes had not been increased during soft water (and hence, low Ca^{2+}) exposure as a result of changes in skin and gill permeability to these ions. Alternatively, low Ca^{2+} could compromise the process of egg hardening, leading to elevated mortality. During this process the enzyme transglutaminase, using Ca^{2+} as a cofactor, catalyzes the formation of γ-glutamyl-ε-lysine isopeptide cross-links between proteins within the chorion which strengthens it, and provides a protective barrier for the embryos from physical, chemical and biological stresses (Yamagami et al., 1992). A third explanation is that low water Ca^{2+} compromises the process of skeletal calcification, elevating mortality. Reader et al. (1988) noted that skeletal Ca^{2+} deposition was impaired at a low water Ca^{2+} concentration (1 mg/L), although survival was not affected.

4.3. Physiological responses to chronic silver exposure

Exposure of rainbow trout embryos and larvae to 0.1 or 1 μg/L Ag in water of hardness 2 mg/L $CaCO_3$ had no significant effects on mortality prior to 50% hatch (Morgan et al., 2005, Results section of the present paper). However, following 50% hatch, exposure to 1 μg/L Ag, but not 0.1 μg/L Ag, in water of hardness 2 mg/L $CaCO_3$ significantly increased mortality relative to controls. The present study demonstrates that an ionoregulatory disturbance was associated with the mortality at 1 μg/L Ag. Following 50% hatch, exposure to 1 μg/L Ag significantly decreased whole embryo/larval Na^+ concentration, Cl^- concentration, Na^+ uptake and Na^+K^+-ATPase activity (Figs. 2–5). Prior to 50% hatch, when exposure to 1 μg/L Ag had no significant effects on mortality (Morgan et al., 2005,

Results section of the present study), there were no significant effects of 1 μg/L Ag on whole embryo/larval Cl^- concentration, Na^+ uptake or Na^+K^+-ATPase activity (Figs. 3–5), although Na^+ concentration was decreased on days 15, 19 and 22 post-fertilization (Fig. 2). Thus, the mechanism of chronic silver toxicity in early life stages of rainbow trout involves an ionoregulatory disturbance, as has been concluded in a number of other studies investigating chronic silver toxicity in rainbow trout early life stages (Guadagnolo et al., 2001; Brauner and Wood, 2002a, 2002b; Brauner et al., 2003). A decrease in Na^+ concentration prior to hatch in the absence of effects on whole embryo/larval Na^+ uptake or Na^+K^+-ATPase activity has been demonstrated before in rainbow trout early life stages, but at a higher silver concentration (10 μg/L Ag, Brauner and Wood, 2002b). The mechanism responsible for the decrease prior to hatch is unknown, but may involve increased Na^+ loss by the embryo, although there are not large effects on Na^+ efflux during acute silver exposure in adult rainbow trout (Morgan et al., 1997; Webb and Wood, 1998).

Ionoregulatory disruption, and consequently mortality, may increase following 50% hatch with exposure to 1 μg/L Ag due to the loss of the chorion at hatch. The chorion surrounds the embryo and is thought to protect the embryo from the toxic effects of silver by impeding the movement of silver into the embryo (Rombough, 1985; Guadagnolo et al., 2001). As such, loss of the chorion at hatch may result in greater silver accumulation in larvae, greater ionoregulatory disruption and greater mortality following hatch. In the present study, we measured only whole embryo or whole larval silver accumulation (Fig. 1), and to determine if chorion loss plays any role in chronic silver toxicity, we would need to determine whole embryo silver accumulation following hatch in embryos with their chorions removed, and in whole larvae at the same time point. If following hatch silver accumulation was greater in larvae, then loss of the chorion may play an important role.

Although exposure to 0.1 μg/L Ag has no significant effects on mortality following hatch (Morgan et al., 2005, Results section of the present study), exposure significantly reduced whole embryo/larval Na^+ concentration, Cl^- concentration and Na^+ uptake (Figs. 2–4). The consequences of these sublethal ionoregulatory effects on the health of the fish are unknown, but could involve delayed effects on survival and growth, although time to 50% hatch and time to 50% swim-up were not affected by exposure to this silver concentration (Morgan et al., 2005).

4.4. Water hardness and physiological responses to chronic silver exposure

Increasing water hardness is protective against the mortality following 50% hatch associated with exposure to 1 μg/L Ag in water of hardness 2 mg/L $CaCO_3$ (Morgan et al., 2005, Results section of the present study), and the present study demonstrates that the mechanism appears to involve a reduction in the ionoregulatory disturbance associated with silver exposure. Specifically, an increase in water hardness to 150 and 400 mg/L as $CaCO_3$ eliminated the inhibitions of whole body Na^+ uptake and Na^+K^+-ATPase activity demonstrated during exposure to 1 μg/L Ag in embryos in water of hardness 2 mg/L $CaCO_3$ and in larvae in water of hardness 150 mg/L $CaCO_3$, respectively (Figs. 4 and 5).

Surprisingly, the full protection afforded to whole embryo/larval Na^+ uptake and Na^+K^+-ATPase activity did not translate into full protection against the decrease in whole embryo/larval Na^+ concentration. Increasing water hardness to 150 or 400 mg/L from 2 mg/L $CaCO_3$ eliminated the effect of 1 μg/L Ag on whole embryo Na^+ concentration on days 36 and 43 post-fertilization, but increasing water hardness to 400 mg/L $CaCO_3$ from 150 mg/L $CaCO_3$ had no effect on the decrease in whole larval Na^+ concentration on days 57 and 64 post-fertilization (Fig. 2). It is possible that if the experiment had proceeded longer, increasing water hardness would have revealed a greater protective effect on whole embryo/larval Na^+ concentration. However, this does not explain why an increase in water hardness was fully protective against the decrease in whole embryo and whole larval Cl^- concentration associated with exposure to 1 μg/L Ag in water of hardness 2 and 150 mg/L $CaCO_3$, respectively (Fig. 3).

In general, increasing water hardness was also protective against the ionoregulatory disruption following hatch associated with exposure to 0.1 μg/L Ag. However, it is interesting to note that at some sample times there was no significant effect of silver exposure in water of hardness 2 mg/L $CaCO_3$, but at 150 or 400 mg/L as $CaCO_3$ exposure to silver was associated with decreases in whole embryo/larval

Na^+ concentration, Cl^- concentration, Na^+ uptake and Na^+K^+-ATPase activity relative to controls. The reason for these decreases with silver exposure in water of higher hardness is unknown, but the decreases in Na^+ uptake and Na^+K^+-ATPase activity may be secondary, a response to decreased Na^+ efflux across the gills due to the stabilizing effect of Ca^{2+} on the permeability of the gill epithelium (Hunn, 1985).

4.5. Implications for environmental regulations

In a previous study, we concluded that the current Canadian Water Quality Guideline of 0.1 μg/L Ag, which represents both an acute and chronic guideline, is more than sufficient in preventing mortality in early life stages of rainbow trout during chronic silver exposure over 64 days because exposure to 0.1 μg/L Ag had no significant effects on mortality (Morgan et al., 2005). However, in the present study the guideline does not appear to be sufficient in preventing ionoregulatory disruption because exposure to 0.1 μg/L Ag had significant effects on whole body Na^+ concentration, Cl^- concentration, Na^+ uptake and Na^+K^+-ATPase activity (Figs. 2–5). This suggests the guideline may not be fully protective against chronic silver toxicity because sublethal effects on ionoregulation are observed at this concentration in rainbow trout early life stages. However, it is important to note that in the present study, silver nitrate was used in all exposures, which dissociates readily to yield ionic silver (Ag^+), the most toxic form of silver (Hogstrand et al., 1996) but which is found in very low concentrations in the natural environment (Kramer et al., 2002). Furthermore, exposures were conducted in water with low DOC and sulfide concentrations, two water quality constituents which greatly ameliorate silver toxicity (Wood et al., 1999).

At present, water chemistry (other than hardness) is not taken into account when generating acute or chronic water quality regulations for silver, although the importance of considering water chemistry has been recognized for some time (Hogstrand et al., 1996; Hogstrand and Wood, 1998). The goal of incorporating water chemistry into acute guidelines has led to the development of two acute silver Biotic Ligand Models, which predict acute silver toxicity as a function of water chemistry. The toxicological Biotic Ligand Model of Paquin et al. (1999) predicts toxicity from assumed gill silver accumulation, while the physiological Biotic Ligand Model of McGeer et al. (2000) uses gill Na^+K^+-ATPase inhibition.

A Biotic Ligand Model for prediction of chronic silver toxicity does not, at present, exist. In an effort to develop a chronic model, several studies have investigated the mechanism of chronic silver toxicity together with the protective effects of various water chemistry parameters on chronic toxicity, including dissolved organic matter (Brauner and Wood, 2002b), chloride (Brauner et al., 2003) and hardness (Morgan et al., 2005). Together with the present study, these studies demonstrate that the mechanism of chronic silver toxicity is similar to the mechanism of acute silver toxicity, involving an ionoregulatory disturbance. Furthermore, these studies demonstrate protective, albeit modest, effects of water chemistry on mortality, with DOC and Cl^- being somewhat less protective against chronic toxicity than against acute toxicity (see Introduction), while hardness appears to be equally or even more protective (Bury et al., 1999a; Karen et al., 1999). These studies also suggest that the nature of the protective effect involves reductions in the ionoregulatory disturbance associated with silver exposure. Thus, it appears possible to model chronic silver toxicity using the same framework used in the two types of acute models described above. However, it is important to note that an increase in water hardness did not fully protect against whole embryo/larval silver accumulation (Fig. 1), but was protective against the ionoregulatory disturbance (Figs. 2–5) and ensuing mortality (Morgan et al., 2005). With this in mind, a physiologically based biotic ligand model, similar to that of McGeer et al. (2000), may be more appropriate to model chronic silver toxicity because silver accumulation is not an endpoint for prediction of mortality, but rather Na^+K^+-ATPase inhibition is. Because Na^+ and sulfide are two additional water chemistry parameters that provide protection against acute silver toxicity (Bury et al., 1999b; Mann et al., 2004), their effects on chronic silver toxicity need to be investigated in the future to develop a chronic model.

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