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Effects of an acute silver challenge on survival, silver distribution and ionoregulation within developing rainbow trout eggs (*Oncorhynchus mykiss*)

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Abstract

Rainbow trout eggs were acutely challenged with silver (as $AgNO_3$) at different stages of development from fertilization through to hatch in moderately hard water (120 mg $CaCO_3$ l^{-1}, 0.70 mM (25 mg l^{-1}) Cl^-, 1.3 mg l^{-1} DOC, 12.3 ± 0.1°C) at measured total silver concentrations of 0.11 ± 0.004, 1.55 ± 0.15, and 14.15 ± 1.52 μg l^{-1}. Four separate acute challenges were conducted, each consisting of 5 days exposure to the respective silver concentration, followed by 4 days recovery after transfer to silver-free water (series 1, 1–10 days post-fertilization; series 2, 8–17 days post-fertilization; series 3, 16–25 days post-fertilization; series 4, 23-32 days post-fertilization). Mortality was not significantly different from control during exposure to 0.11, 1.55, and 14.15 μg l^{-1} total silver in series 2, 3 and 4 (mortality for series 1 data could not be calculated for technical reasons). In the four days of recovery following silver exposure, however, there was significant mortality at 14.15 μg l^{-1} total silver reaching 100, 31 and 72% in series 2, 3 and 4, respectively, indicating eggs are more sensitive in the period of $8-17$ and 23–32 days post-fertilization at this temperature. Mortality following silver exposure was associated with ionoregulatory impairment in series 3 and 4, where up to 60% of whole egg [Na^+] and [Cl^-] was lost relative to controls at 14.15 μg l^{-1} total silver. Significant but smaller reductions in egg [Na^+] and/or [Cl^-] were also observed at 0.11 and 1.55 μg l^{-1} total silver. The greatest accumulation of silver in whole eggs and chorions occurred in series 4, reaching concentrations of 0.53 μg g^{-1} (eggs) and 15.5 μg g^{-1} (chorions) in the 14.15 μg l^{-1} treatment. The accumulation of silver in the whole eggs and chorions of the 0.11 μg l^{-1} treatment was not different from controls throughout embryonic development. Of the total silver content, only a small proportion of silver was found in the embryos (1–17%), indicating that the chorion is a protective barrier during acute silver exposure.

Keywords: Chorion; Delayed mortality; Ionoregulation; Rainbow trout eggs; Silver accumulation; Silver toxicity; Na^+; Cl^-

1. Introduction

While a major source of silver in surface waters is natural leaching, anthropogenic activities, such

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as mining, manufacture of silverware and jewellery, and photographic manufacturing and processing, can elevate silver levels in aquatic environments (Purcell and Peters, 1998). Silver complexes found in freshwater include silver chloride, silver sulfide, silver DOM (dissolved organic matter), and silver thiosulfate (Hogstrand and Wood, 1998; Purcell and Peters, 1998; Wood et al., 1999). The free Ag^+ ion is present only in minute amounts, as most silver is bound to these various ligands. In most laboratory toxicity studies, however, silver nitrate ($AgNO_3$) is used which is very soluble in fresh water and dissociates to yield high amounts of Ag^+, the most toxic form of silver to fish (Wood, 1999), thereby representing a 'worst case' scenario. For example, silver thiosulfate complexes are less toxic than Ag^+ (from $AgNO_3$) by as much as four orders of magnitude in fathead minnows (LeBlanc et al., 1984) and rainbow trout (Hogstrand et al., 1996).

In juvenile and adult fish, silver presented as $AgNO_3$ is extremely toxic, resulting in 96 h LC_{50} values between 6.5 and 65 μg l^{-1} total silver (Nebeker et al., 1983; LeBlanc et al., 1984; Hogstrand et al., 1996; Galvez and Wood, 1997). The primary mechanism of acute Ag^+ toxicity in juvenile and adult rainbow trout is ionoregulatory failure that arises from the binding of Ag^+ to specific sites on the gills (Janes and Playle, 1995), thereby impairing or abolishing branchial Na^+, K^+-ATPase activity (Morgan et al., 1997). The consequent non-competitive inhibition of Na^+ and Cl^- uptake (Morgan et al., 1997; Webb and Wood, 1998; McGeer and Wood, 1998; Bury et al., 1999) leads to a reduction in blood plasma ion levels resulting in a contraction of blood volume culminating in circulatory failure and death (Wood et al., 1996; Hogstrand and Wood, 1998). However, nothing is known of the mechanism of acute silver toxicity in developing embryos, often the most sensitive life stage to environmental contaminants (von Westernhagen, 1988). During exposure to other heavy metals, mortality often increases in developing fish embryos at specific developmental stages, called 'critical windows' (see Weis and Weis, 1989, for a review). For example, with cadmium exposure, separate peaks of egg mortality occurred during gastrulation and axiation, during the development of the vitelline circulation, and shortly before hatch in Atlantic salmon and rainbow trout (Rombough and Garside, 1982; Shazili and Pascoe, 1986). Nothing is known in this regard about the acute toxicity of silver in developing embryos.

The developing egg consists of an embryo supplied with a yolk, which is suspended in perivitelline fluid, all of which is encapsulated by a protective chorion. The chorion is acellular, containing negatively charged groups of glycoproteins which bind cations. Rombough (1985) observed variable metal toxicity in steelhead trout embryos depending upon the binding characteristics of the different metals. Mercury, copper and silver ions, all of which have a high affinity for sulfhydryl groups and often act as Na^+ antagonists, bound tightly to the chorion. When the chorion was removed, the embryos became less resistant to a static exposure of $AgNO_3$ (at a concentration greater than 50 μg l^{-1}; Rombough, 1985), implicating the chorion as a protective barrier. In contrast, zinc, cadmium, and lead ions, all of which are considered Ca^{2+} antagonists with lower sulfhydryl affinities, bound less effectively to the chorion, and penetrated the egg more rapidly (Rombough, 1985). During chronic exposure to silver, the chorion binds about 85% of the total egg silver content, and embryonic levels are very low (Guadagnolo et al., 2000), however, nothing is known of the relative partitioning of silver (or other metals) between the embryo, chorion and yolk during acute metal exposure.

The present study was conducted to investigate the effect of acute $AgNO_3$ challenge on mortality and whole egg Na^+ and Cl^- levels. The study also investigated silver accumulation and distribution between the chorion, embryo and yolk, at weekly intervals from fertilization through to hatch in developing rainbow trout acutely exposed to $AgNO_3$. Brauner and Wood (unpublished data) have demonstrated that there is pronounced delayed mortality associated with acute (48 h) silver exposure. Thus another objective was to further characterize this phenomena and determine whether there is an ionoregulatory basis for the delayed mortality.

In the present study, eggs were acutely challenged with a nominal concentration of 0, 0.1, 1.0 and 10 μg l^{-1} total silver (as $AgNO_3$). A concentration of 0.1 μg l^{-1} was chosen because this is the current maximum allowable concentration of silver in fresh water in Canada (CCME, 1995) representing both an acute and chronic guideline. In the United States, the Environmental Protection Agency (EPA) sets Ambient Water Quality Criteria (AWQC) for aquatic life protection based on water hardness (in mg l^{-1} of $CaCO_3$ equivalents) to protect only against acute toxicity of silver (US EPA, 1980). In the relatively hard water (120 mg l^{-1} $CaCO_3$) of Lake Ontario the acute US EPA AWQC would be 4.7 μg l^{-1} total silver. Thus, the silver concentrations used in this study were chosen because of their regulatory relevance. A parallel study has examined the effect of chronic $AgNO_3$ exposure to these same early life stages (Guadagnolo et al., 2000).

2. Materials and methods

2.1. Experimental animals

Experiments were performed on about 4000 freshly fertilized rainbow trout eggs obtained from Rainbow Springs Trout Farm, Ontario. They were maintained in flowing dechlorinated Hamilton tapwater (in mM: Na^+, 0.60; Cl^-, 0.70; Ca^{2+}, 1.0; Mg^{2+}, 0.2; K^+, 0.05, HCO_3^-, 1.9; DOC (dissolved organic carbon), 1.3 mg l^{-1}, alkalinity, 95 mg $CaCO_3$, hardness, 120 mg $CaCO_3 l^{-1}$, pH, 7.5) in a 10 l light-shielded chamber until transfer to the experimental set up as described below. A constant water temperature of 12.3 ± 0.1°C was maintained. Because eggs are photosensitive early in development, they were always kept in dark containers and the lights were off during non-sampling periods.

2.2. Exposure system

Over a period of 32 days, four acute challenges were performed at different developmental periods using eggs from the holding tank. Stock silver solutions of 0 μg l^{-1} (control), 100, 1000, and 10 000 μg l^{-1} were made using $AgNO_3$ (BDH) in 0.5% nitric acid (trace metal grade — Fisher Scientific) in deionized, distilled water and were kept in light shielded containers. These stock solutions were delivered at a flow rate of 0.14 ml min^{-1} by a peristaltic pump into 150 ml header tanks where they mixed with dechlorinated Hamilton tap water (from Lake Ontario) flowing at a rate of 140ml min^{-1}, thereby, yielding continuously flowing exposure concentrations of 0, 0.1, 1.0, and 10 μg l^{-1} total silver (nominal values). Header tanks were vigorously aerated to facilitate mixing and the overflow was directed to the egg exposure chambers. The plastic exposure chambers ($10 \times 8 \times 7.5$ cm) each containing 150–250 eggs (see Section 2.3 below), held water volumes of approximately 450 ml each and received a flow of approximately 140 ml min^{-1}. Recovery from silver exposure was accomplished by transferring surviving eggs to chambers supplied with silver-free dechlorinated Hamilton tap water.

Graphite furnace AAS was used to measure total silver levels in the water in the 1.0 and 10 μg l^{-1} treatments with a limit of detection of 0.05 μg l^{-1} silver. Control levels were below this detection limit. In the 0.1 μg l^{-1} treatment, 10 μCi of $^{110m}Ag\ l^{-1}$ (Amersham International, Coustaboef, France) was added to the 100 μg l^{-1} silver stock solution so that final total silver concentration in the exposure chamber could be measured by radioisotopic dilution as described below. Table 1 reports the mean measured silver concentration and calculated silver speciation in each experimental treatment. Calculation of total free silver ion, silver chloride (aqueous) and silver-DOC (dissolved organic carbon) in each experimental treatment (Table 1) was conducted using the geochemical modelling program MINEQL+ (Schecher and McAvoy, 1994) based on the known chemistry of Hamilton tap water (from Lake Ontario) and appropriate binding constants from Janes and Playle (1995).

2.3. Experimental procedure

Four separate acute challenge series were performed, each during different development periods over 32 days following fertilization (series 1,

1–10 days post-fertilization; series 2, 8–17 days post-fertilization; series 3, 16–25 days post-fertilization; series 4, 23–32 days post-fertilization). In each acute challenge, eggs (250 for series 1, 2 and 3; and 150 for series 4) were exposed for five days to nominal silver concentrations of 0.1, 1.0, and 10 μg l^{-1} total silver (as $AgNO_3$, see Table 1 for measured values), followed by four days of recovery in silver-free water. Eggs from each chamber were sampled on days 1, 3 and 5 of silver exposure and on days 2 and 4 of the recovery period. Prior to sampling, mortalities in each treatment were recorded and dead eggs were removed. Eggs that had turned white were assumed to be dead and when embryos were sufficiently developed, the criteria for death was cessation of heartbeat. In series 1, it was difficult to determine whether all opaque eggs had been fertilized and therefore mortality is not reported in series 1. Following sampling for series 1 all unfertilized eggs could easily be identified by lack of an embryonic axis and were removed from the experimental set-up and not included in the mortality calculations. Eggs that were sampled were carefully checked to ensure that they were alive in all series.

In all treatments, whole eggs ($n = 10$) and chorions (taken from 16 eggs) were removed at each sampling time in each acute challenge series. Embryos (8) were sampled during series 3 and 4. Silver levels were measured in all these samples and Na^+ and Cl^- concentrations were measured in whole eggs of series 3 and 4. When egg mortality was high (series 2 and 4) or eggs began to hatch (series 4) priority was given for collection of whole eggs for silver and ion determinations and consequently eggs could not be sampled for chorion silver concentrations during some recovery periods (Fig. 3).

For egg dissection a small, shallow puncture was made in the chorion with an 18 gauge needle. The chorion was then gently torn off using tweezers. After dechorionation, the embryo was separated from the yolk sac and collected for analysis.

All samples were rinsed with deionized, distilled water, blotted dry, weighed, and acidified with three times the sample weight of 1N nitric acid (trace metal grade, Fisher Scientific). All acidified samples (in 1.5 ml polyethylene centrifuge tubes) were digested in an oven over a 24-h period at approximately 60°C. All samples were homogenized using a micro-centrifuge sample pestle (teflon fluorocarbon resin, Scienceware, Fisher Scientific) at a speed of approximately 5 rps for about 10 s. The digested, homogenized samples were spun down and a portion of the supernatant appropriately diluted with deionized, distilled water for total silver analysis and measurement of Na^+ and Cl^- concentrations.

Water samples were taken every second day from chambers during silver exposure and immediately acidified to 0.5% (v/v) with trace metal grade nitric acid (Fisher Scientific) and kept in the dark for later total silver analysis. Water and silver stock flow rates were also measured every second day during exposure and the temperature was recorded daily.

2.4. Analysis

Analysis of total silver concentration of the water from the 1.0 and 10 μg l^{-1} treatments

Table 1
Measured water total silver concentration ([silver]; $n = 12$) during acute exposures and calculation of silver speciation based upon measured values using MINEQL+ (Schecher and McAvoy, 1994).

Nominal total [silver] (μg l^{-1})	Measured total [silver] (μg l^{-1})	Ag^+ (μg l^{-1})	$AgCl_{aq}$ (μg l^{-1})	Ag-DOC (μg l^{-1})
0.1	0.11 ± 0.004	0.0019	0.0025	0.10
1.0	1.55 ± 0.15	0.033	0.042	1.48
10	14.13 ± 1.45	2.63	3.49	8.01

and of diluted supernatant from digested samples was carried out using a graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer) with the following operating parameters: 75°C for 5 s, 90°C for 30 s, 120°C for 10 s, 120°C for 2 s (no gas flow), 2000°C for the next 3 s (no gas flow, absorption read), 2000°C for 1 s. Analysis of silver concentration in the water from the 0.1 μg l^{-1} total silver treatment was done by radioisotopic dilution. Atomic absorption spectrophotometry (Varian 1275) was used to measure the silver concentration of the stock solution and a gamma counter (Packard Instruments, Downers Grove, IL, USA) was used to measure radioactivity (total counts per minute; cpm) of ^{110m}Ag. The specific activity (SA) of the stock solution was calculated (cpm μg^{-1}) and the total silver concentration of the exposure chamber was calculated by dividing ^{110m}Ag activity (cpm) of the water by the SA of the stock solution.

Standards used to measure silver in whole eggs and different egg compartments were made by mixing known certified Fisher silver standard with digested rainbow trout tissue. This was done to correct for background and matrix effects.

Measurement of whole egg Na^+ concentration was performed by atomic absorption spectrophotometry (Varian 1275) and whole egg Cl^- levels were measured using the colorimetric mercuric thiocyanate method (Zall et al., 1956).

2.5. Statistics

Mean values for silver concentration in various compartments and whole egg ion concentrations were statistically compared between treatments on individual days, and between days within the exposure regime of individual treatments using one-way analysis of variance in Sigma Stat for Windows (version 1.0) package (Jandel Corporation). Mortality between treatments during silver exposure or during recovery was also compared using a one-way ANOVA. The Student–Newman–Keuls post-hoc test (Steel and Torrie, 1960) was used to isolate groups that differed significantly from one another with a level of significance for all analyses of $P < 0.05$.

3. Results

3.1. Experimental silver concentrations

The measured silver concentrations in the treatment water are shown in Table 1, however, nominal values will predominantly be referred to throughout the manuscript for clarity. Results of silver speciation calculations based on known chemistry (see Section 2) of Hamilton tap water (from Lake Ontario) are also summarized in Table 1. In this experimental setup, equilibration time between the silver stock solution and the water was short because water flow rate had to be kept relatively high to maintain a constant temperature. While interaction between silver and water chloride is fast, the interaction with DOC is considerably slower. For copper, Ma et al. (1999) have demonstrated that increasing the reaction time from 1 to 24 h between the metal and humic acid (at 5 mg l^{-1} DOC) reduces toxicity of the solution in bioassays by 50%. Thus, the speciation values reported in Table 1 may overestimate the proportion of silver as Ag-DOC and the conditions of the present study may result in greater toxicity than if the silver solutions were permitted to equilibrate for several hours.

3.2. Mortality

Due to the difficulty in assessing whether opaque eggs were truly dead or had never been fertilized at early stages of development, data for mortalities of series 1 (1–10 days post-fertilization) are not reported. At later stages this distinction was not a problem.

In series 2 (8–17 days post-fertilization), mortality did not differ significantly from controls at any silver concentration during exposure (Fig. 1A). During recovery, however, there was a significant increase in mortality relative to controls in eggs that had been exposed to 10 μg l^{-1} total silver. Cumulative mortality rose from 26% on day 5 of exposure to 78% on day 2 of recovery and 100% by day 3. Mortalities in the 0.1 and 1.0 μg l^{-1} treatments during recovery were not significantly different from controls.

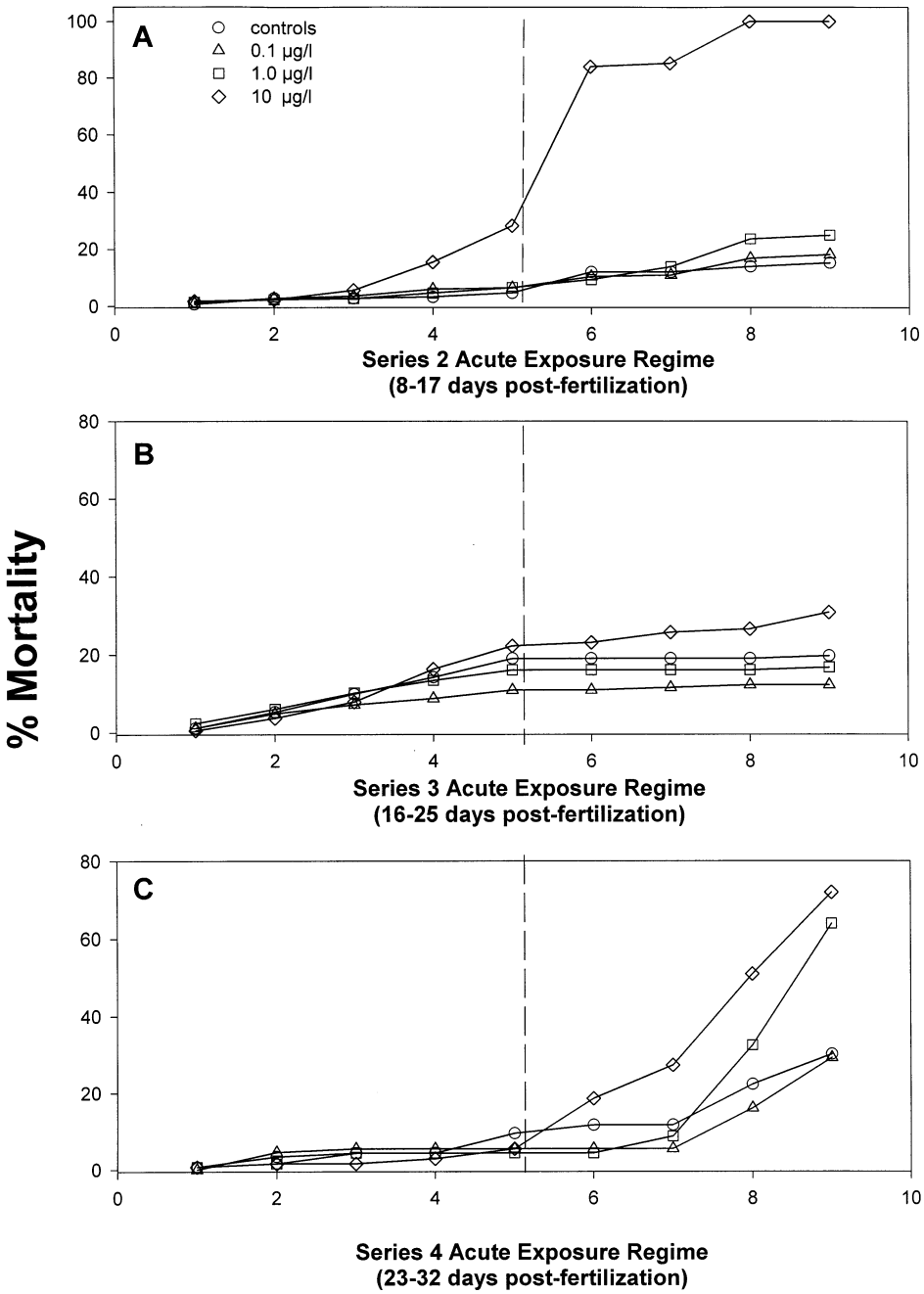

Fig. 1. Cumulative percent mortality in eggs of rainbow trout acutely challenged with 0 µg l^{-1} (open circles), 0.1 µg l^{-1} (open triangles), 1.0 µg l^{-1} (open squares), and 10 µg l^{-1} (open diamonds), total Ag (as $AgNO_3$) for 5 days at three different stages of development; (A) 8–17 days post-fertilization (series 2); (B) 16–25 days post-fertilization (series 3); (C) 23–32 days post-fertilization (series 4). A 4-day recovery period followed each 5-day exposure period the start of which is indicated by the vertical dashed line. In all the treatments, none had significantly greater mortality compared to controls during the 5-day exposure. However, in all series (2, 3, 4), mortality in the 10 µg l^{-1} treatment was significantly greater than controls ($P < 0.05$) during the 4-day recovery period.

In series 3 (16–25 days post-fertilization) mortality did not differ significantly from controls at any silver concentration during exposure (Fig. 1B). During recovery, mortality in the 10 μg l^{-1} treatment was again significantly greater than controls, reaching a maximum of 31% by day 4 of recovery.

In series 4 (23–32 days post-fertilization) mortality again did not differ significantly from controls at any silver concentration during silver exposure (Fig. 1C). Throughout the recovery period, mortality in the 10 μg l^{-1} treatment was significantly greater than controls and increased from 19 to 72% by the end of the recovery period. Mortality in the 0.1 and 1.0 μg l^{-1} treatments was not statistically different from controls, though in the 1.0 μg l^{-1} treatment it had reached 64% by the end of recovery.

3.3. Silver concentration in whole eggs

There were no significant differences in whole egg silver content between the 0.1 μg l^{-1} treatment group and controls in any of the series (1–4). In series 1 (1–10 days post-fertilization) eggs of the 10 μg l^{-1} treatment had significantly greater silver concentrations than controls throughout silver exposure and during recovery (Fig. 2A). In both the 1.0 and 10 μg l^{-1} treatment, whole egg silver concentration increased from day 3 to 5 of silver exposure and remained at that level until day 2 of recovery. By day 4 of recovery, whole egg silver concentration fell and was not significantly different from day 1 values in the 1.0 μg l^{-1} Ag exposed group.

In series 2 (days 8–17 post-fertilization) eggs in the 10 μg l^{-1} (days 3 and 5) and 1.0 μg l^{-1} (day 5) treatment had significantly greater whole egg silver concentrations than controls during silver exposure (Fig. 2B). In the 1.0 μg l^{-1} treatment, whole egg silver concentration decreased substantially by day 2 of recovery.

In series 3 (days 16–25 post-fertilization) eggs exposed to 10 μg l^{-1} total silver had significantly greater silver concentrations than controls throughout the exposure regime but levels tended to fluctuate (Fig. 2C). On day 2 of recovery, eggs that had been exposed to 1.0 μg l^{-1} silver possessed significantly greater silver concentrations than controls (Fig. 2C).

In series 4 (days 23-32 post-fertilization) eggs exposed to 10 μg l^{-1} total silver had significantly greater silver concentrations than controls throughout exposure and recovery (Fig. 2D). The silver concentration in the whole eggs of the 1.0 μg l^{-1} treatment was not significantly different from controls at any time during exposure and recovery.

3.4. Silver concentration in chorions

In general, silver concentrations per unit weight in chorions were much greater than in whole eggs in the 1.0 and 10 μg l^{-1} treatments at all developmental stages. Again, there were no significant differences between chorion silver concentration in the 0.1 μg l^{-1} treatment and controls in any of the series (1–4). In series 1 (1–10 days post-fertilization) total silver concentration in the chorions of the 10 μg l^{-1} treatment was significantly greater than that in control chorions throughout exposure (Fig. 3A), reaching a maximum value of 1.92 μg g^{-1} by day 5 of exposure. In the 1.0 μg l^{-1} treatment, chorion silver concentration was significantly different from control concentrations on days 3 and 5 of exposure and on day 2 of recovery.

In series 2 (days 8–17 post-fertilization), chorions from eggs in the 10 μg l^{-1} treatment had significantly greater silver concentrations than control chorions (Fig. 3B) at all times during exposure, reaching a maximum value of 5.3 μg g^{-1} by day 5. In the chorions of the 1.0 μg l^{-1} treatment, silver concentrations increased from day 1 to 5 of exposure and remained at about 1.0 μg g^{-1} throughout recovery, significantly greater than controls (Fig. 3B).

In series 3 (days 16–25 post-fertilization), chorions of the 10 μg l^{-1} treatment had significantly greater silver concentrations than controls throughout exposure (Fig. 3C). The chorion silver concentration in the 1.0 μg l^{-1} treatment increased significantly from day 1 to 5 of silver exposure (Fig. 3C).

In series 4 (days 23–32 post-fertilization) chorion silver concentration in the 10 μg l^{-1} treat-

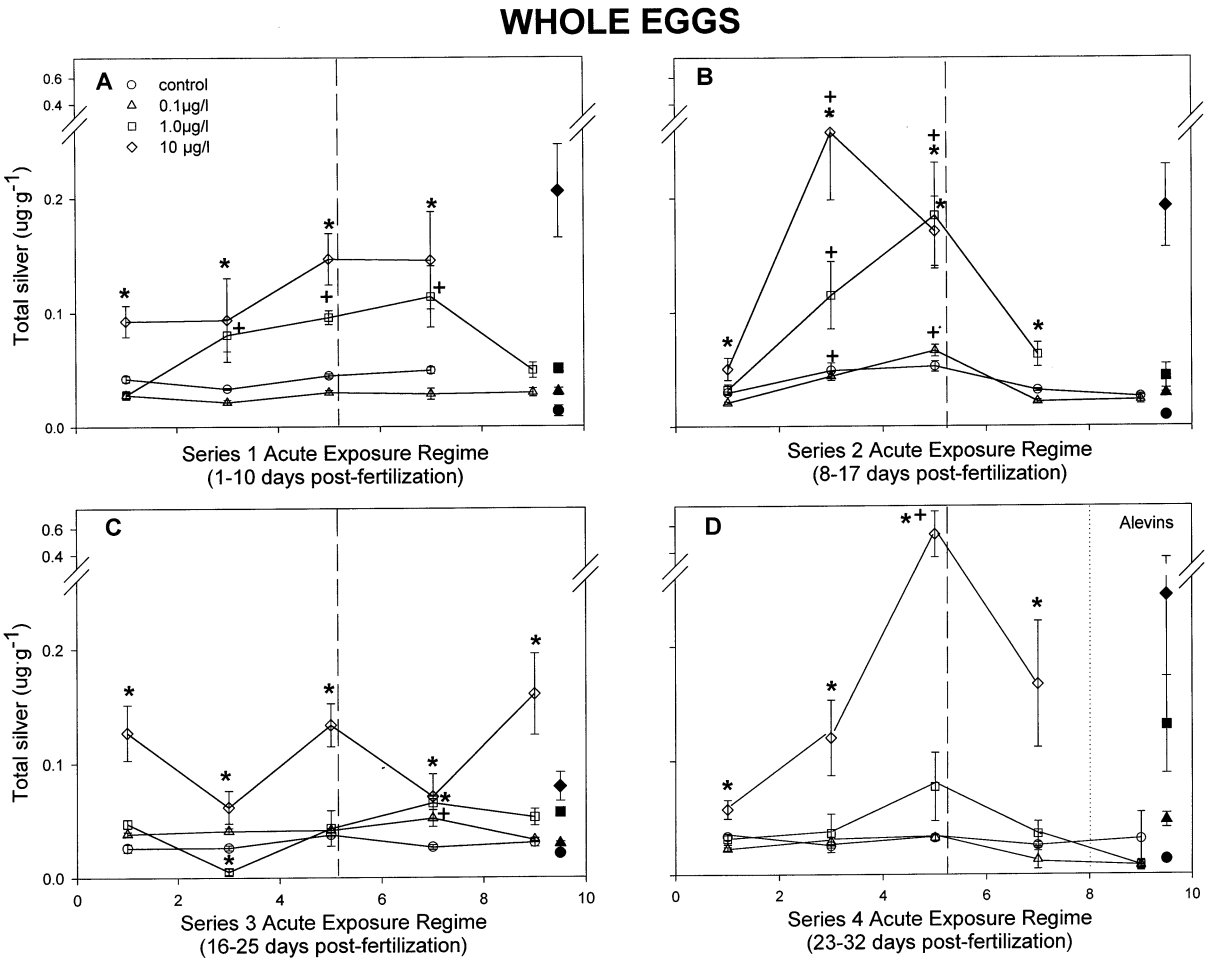

Fig. 2. Silver concentration in whole eggs acutely challenged with 0 µg l^{-1} (open circles), 0.1 µg l^{-1} (open triangles), 1.0 µg l^{-1} (open squares), and 10 µg l^{-1} (open diamonds) total Ag (as $AgNO_3$) for 5 days during four different developmental stages; (A) 1–10 days post-fertilization (series 1); (B) 8–17 days post-fertilization (series 2); (C) 16–25 days post-fertilization (series 3); (D) 23–32 days post-fertilization (series 4). The vertical dashed line indicates the start of the 4-day recovery period following silver exposure. Solid symbols represent data for eggs chronically exposed to the same silver concentrations (0 µg l^{-1}; closed circles, 0.1 µg l^{-1}; closed triangles, 1.0 µg l^{-1}; closed squares, 10 µg l^{-1}; closed diamonds) from fertilization to sampling time on day 11 (A), 15 (B), 23 (C) and 30 (D) post-fertilization (Guadagnolo et al., 2000). Values are mean ± SEM ($n = 8–10$); *, indicates significant difference from controls at respective time ($P < 0.05$); +, represents significant difference from day 1 values within each silver exposure group ($P < 0.05$).

ment were significantly greater than controls throughout exposure (Fig. 3D). These levels are much higher than those observed at other developmental stages (Fig. 3A, B and C). Silver concentration of the chorions in the 0.1 and 1.0 µg l^{-1} treatments were not significantly different than controls, however, there was a significant increase from day 1 values in both treatments. Silver concentration of chorions during recovery in surviving embryos could not be measured because most of the eggs had hatched by the end of the 5-day exposure period.

3.5. Silver distribution between egg compartments

Fig. 4 summarizes the calculated total silver content (in μg) in the embryos and the remainder of the egg (predominantly chorion and yolk) in eggs exposed to silver in series 3. Values for the combined chorion and yolk compartment were calculated by subtracting the measured embryonic silver content from the measured whole egg silver content. On days 3 and 5 of exposure and day 4 of recovery, only 2–4% of the total egg silver was found in the embryos in the 10 μg l^{-1} treatment, while the remainder was bound by the chorion and the yolk (Fig. 4). In the 0.1 and 1.0 μg l^{-1} treatment, the embryo contained between 4 and 17% of the total silver.

3.6. Whole egg Na^+ and Cl^- concentration

Eggs acutely exposed to silver in series 3 and 4 were analyzed for whole egg Na^+ and Cl^- concentrations, which are reported in Figs. 5 and 6. The Na^+ concentration of eggs in series 2 did not differ between treatments and are not reported.

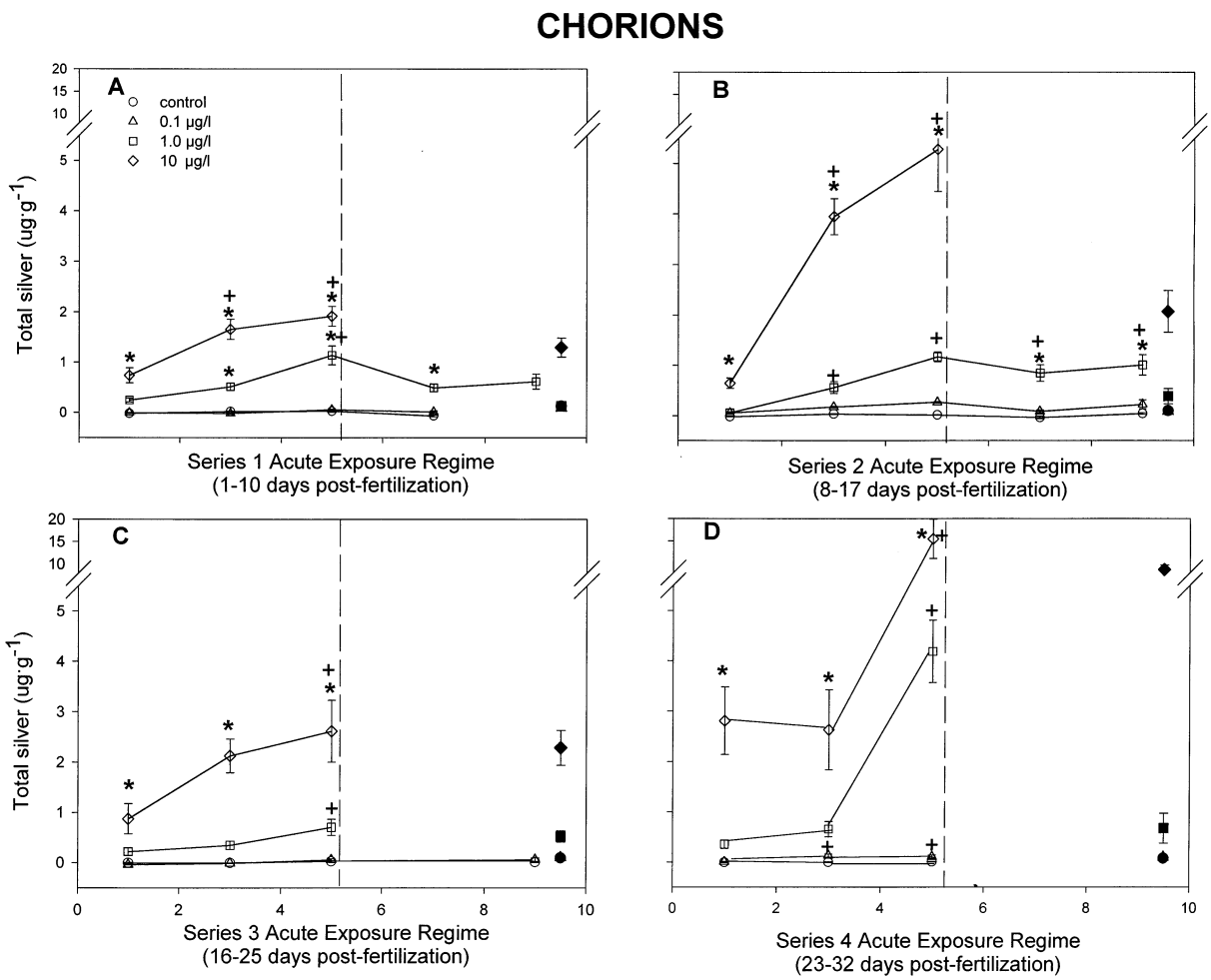

Fig. 3. Silver concentration in chorions of eggs acutely challenged with 0 μg l^{-1} (open circles), 0.1 μg l^{-1} (open triangles), 1.0 μg l^{-1} (open squares), and 10 μg l^{-1} (open diamonds) total Ag (as $AgNO_3$) for 5 days and then following 4 days of recovery. Four different developmental stages were examined; (A) 1–10 days post-fertilization (series 1); (B) 8–17 days post-fertilization (series 2); (C) 16–25 days post-fertilization (series 3); (D) 23–32 days post-fertilization (series 4) ($n = 8$). See legend of Fig. 2 for further details.

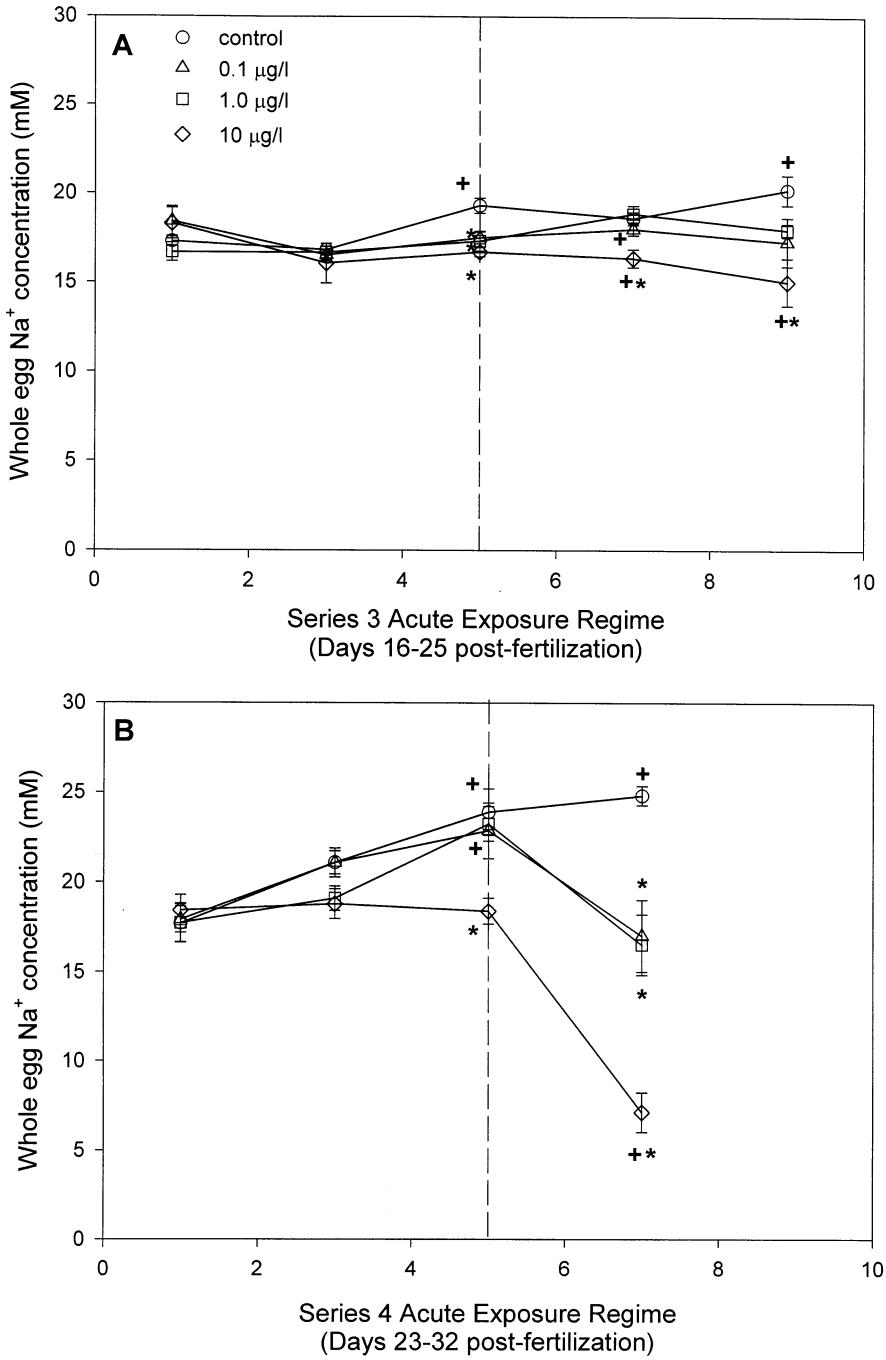

Fig. 5. Whole egg Na^+ concentration (mM) in eggs acutely challenged with 0 μg l^{-1} (open circles), 0.1 μg l^{-1} (open triangles), 1.0 μg l^{-1} (open squares), and 10 μg l^{-1} (open diamonds) total Ag (as $AgNO_3$) for 5 days and then following 4 days of recovery. Two different developmental stages were examined; (A) 16–25 days post-fertilization (series 3); (B) 23–32 days post-fertilization (series 4) ($n = 8$). See legend of Fig. 2 for further details.

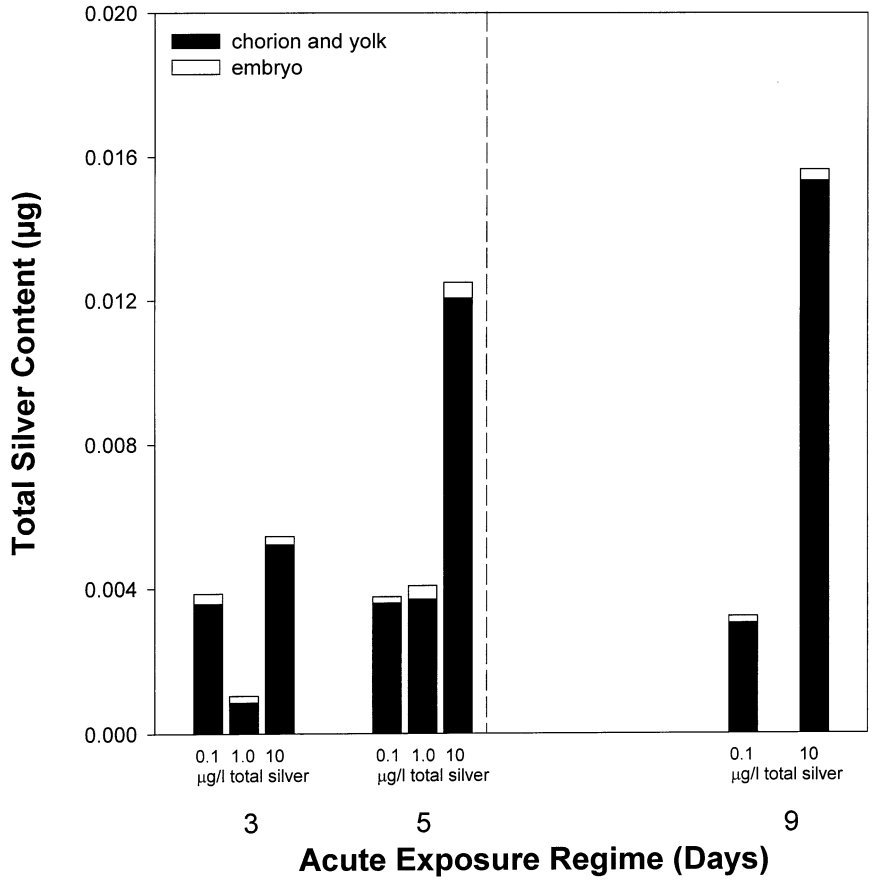

Fig. 4. Comparison of the total amount of silver in the embryo and the chorion + yolk of eggs acutely challenged with 0.1, 1.0 and 10 µg l^{-1} total silver (as $AgNO_3$) in series 3. The vertical dashed line indicates the start of the 4-day recovery period following silver exposure. Values presented for embryos were measured and those for the chorion + yolk were calculated from the difference in whole egg and embryo silver contents.

In series 3 (days 17–25 post-fertilization), there was a significant reduction in whole egg Na^+ concentration in all treatments (0.1, 1.0 and 10 µg l^{-1} total silver) relative to controls following 5 days of silver exposure (Fig. 5A) while no significant differences were observed at this time for whole egg Cl^- concentration (Fig. 6A). On days 2 and 4 of recovery from silver exposure, only eggs exposed to 10 µg l^{-1} total silver had a significantly lower Na^+ concentration than controls and no differences were observed for Cl^-. In control eggs, Na^+ concentration increased from 17.3 to 20.2 mM from day 1 to day 9 of the exposure regime, while in the 10 µg l^{-1} treatment group it decreased from 18.3 to 15.1 mM over the same duration (Fig. 5A).

In series 4 (days 24–32 post-fertilization),10 µg l^{-1} total silver was the only treatment that significantly reduced whole egg Na^+ concentration relative to controls following 5 days of silver exposure (Fig. 5B) while no differences were observed in whole egg Cl^- concentration over this duration (Fig. 6B). Following 2 days of recovery, however, there was a significant reduction in both whole egg Na^+ and Cl^- concentrations relative to controls in all treatments (0.1, 1.0 and 10 µg l^{-1} total silver). On day 1 of the exposure regime, whole egg Na^+ concentration was about 18 mM in all

treatments which increased to 24.8 mM by day 7 in controls. In contrast, Na^+ concentration did not change significantly at 0.1 and 1.0 μg l^{-1} and decreased greatly to 7.1 mM in the 10 μg l^{-1} treatment. Thus, following 2-days of recovery, whole egg Na^+ concentration was reduced relative to controls by 31, 33 and 71% in the 0.1, 1.0 and 10 μg l^{-1} treatments, respectively.

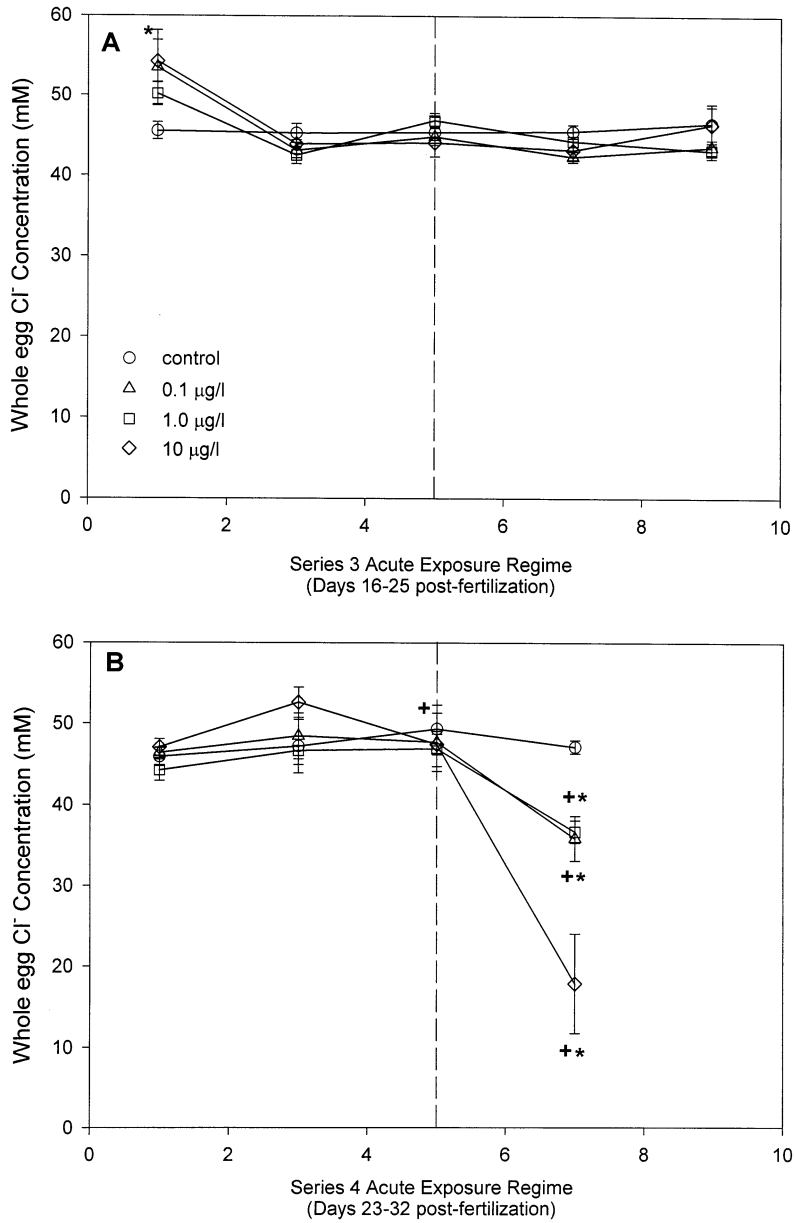

Fig. 6. Whole egg Cl^- concentration (mM) in eggs acutely challenged with 0 μg l^{-1} (open circles), 0.1 μg l^{-1} (open triangles), 1.0 μg l^{-1} (open squares), and 10 μg l^{-1} (open diamonds) total Ag (as $AgNO_3$) for 5 days and then following 4 days of recovery. Two different developmental stages were examined; (A) 16–25 days post-fertilization (series 3); (B) 23–32 days post-fertilization (series 4) ($n = 8$). See legend of Fig. 2 for further details.

Whole egg Cl^- concentration was about 46 mM in all treatments on day 1 of the exposure regime. This value did not change in controls but decreased to 35.9, 36.7 and 17.9 mM in the 0.1, 1.0 and 10 μg l^{-1} treatment groups, respectively by day 2 of recovery (Fig. 6B). Thus, relative to controls, whole egg Cl^- concentration decreased by 22, 17 and 61%, respectively (Fig. 6B).

4. Discussion

Acute challenge with silver (as $AgNO_3$) at 0.1, 1.0 or 10 μg l^{-1} total silver, in moderately hard water did not result in significant mortality relative to controls during the 5-day exposure. However, significant mortality (reaching 100%) was observed during the 4-day recovery period in silver-free water in eggs that had previously been exposed to the 10 μg l^{-1} treatment. This delayed mortality was associated with a 60–70% loss of whole egg [Na^+] and [Cl^-] in series 4, just prior to hatch. Although no significant delayed mortality was observed following exposure to 0.1 and 1.0 μg l^{-1} total silver in series 4, there was a significant reduction in whole egg [Na^+] and [Cl^-] at these concentrations relative to controls, indicating an ionoregulatory disruption at these low silver concentrations. The majority of silver was contained within the chorion and yolk with only 1–3% found in the embryo following exposure to 10 μg l^{-1} total silver in series 3 and 4, indicating that the chorion plays a protective role against silver accumulation during an acute silver challenge.

4.1. Mortality

During a 5-day acute challenge to 0.1, 1.0, and 10 μg l^{-1} total silver (as $AgNO_3$), there was no significant increase in mortalities relative to controls at any period of development. However, during a 4-day recovery period following 10 μg l^{-1} total silver exposure, eggs experienced significant mortality relative to controls in series 2, 3, and 4 (days 8–17, 16–25, and 23–32 post-fertilization; Fig. 1). This delayed mortality must be partly associated with handling stress as control eggs experienced a cumulative mortality of up to 30% over the 9-day exposure regime (series 4). At all developmental stages, delayed mortality was far greater in the 10 μg l^{-1} total silver treatments than in controls. In series 4, there was pronounced delayed mortality at 1.0 μg l^{-1} total silver by days 3 and 4 of recovery, reaching 64% (49% when the control mortality are taken into account according to Sprague, 1969). However, when mortality over the entire recovery period for this treatment was examined, there were no statistically significant differences from controls. While a synergistic effect between silver exposure and handling stress probably occurred, these data indicate that just prior to hatch, eggs may become more sensitive to an acute silver challenge, an effect that is only seen when delayed mortality is taken into account.

Sensitivity to silver exposure varied with developmental stages as indicated by delayed mortality rates of greater than 70% in series 2 and 4 in the 10 μg l^{-1} treatment, while it was much lower (31%) in series 3. Thus, under our exposure conditions rainbow trout appear to be more sensitive to an acute silver challenge 1 to 2 weeks following fertilization (series 2) and just prior to hatch (series 4). In the only other study conducted to examine changes in sensitivity to acute heavy metal challenge, rainbow trout eggs were most sensitive to Cd, Cu, and Zn during development of the embryonic axis (Shazili and Pascoe, 1986), approximately the same developmental stage as series 2 eggs in the present study. Sensitivity to Cd and Zn then decreased with development and increased again (for Zn only) just before hatch (Shazili and Pascoe, 1986). Increased sensitivity near hatch has also been observed in eggs of Atlantic salmon chronically exposed to cadmium (Rombough and Garside, 1982). The increased sensitivity to silver near hatch is associated with an increased egg silver burden likely related to increased active ion uptake from the water at this stage as described below.

4.2. Whole egg ion concentrations

In juvenile and adult fish, mortality following acute silver exposure arises from circulatory col-

lapse following ion regulatory disruption (see Section 1). Plasma ion levels decrease by up to 35% prior to death (Wood et al., 1996). Although plasma ion levels in embryos acutely exposed to silver in this study could not be measured, whole egg [Na^+] and [Cl^-] decreased by 70 and 60% respectively, during recovery from exposure to 10 μg l^{-1} total silver in series 4 (Figs. 5 and 6). At the lower silver concentrations of 0.1 and 1.0 μg l^{-1} total silver in series 4, there was a significant reduction in whole egg [Na^+] and [Cl^-] of approximately 30 and 20%, respectively. Thus, it appears that the mechanism of acute toxicity in developing embryos also involves ion regulatory disruption.

In embryos chronically exposed to 10 μg l^{-1} total silver from fertilization under identical conditions (Guadagnolo et al., 2000), whole egg [Na^+] was significantly reduced relative to controls just prior to hatch. Interestingly, whole egg [Na^+] in the control group increased by 70% but that in silver exposure eggs remained constant (Guadagnolo et al., 2000) due to elimination of active Na^+ uptake (Brauner and Wood, unpublished). In contrast to the chronic study (Guadagnolo et al., 2000), there was a net loss of whole egg [Na^+] in silver exposed eggs in the present study. This may have arisen from a stimulation of efflux in addition to an inhibition of influx. Unidirectional flux studies would be needed to verify this. It is not clear whether the net loss in whole egg [Na^+] and [Cl^-] observed in this study is solely a result of the acute silver challenge or due to a combined effect of exposure and handling. This question clearly warrants further investigation.

4.3. Silver concentrations

The ability of eggs to depurate silver to some degree upon transfer to silver-free water was indicated in series 2, 3 and 4 (Fig. 2). In the 10 μg l^{-1} treatment of series 2 (day 8 to 17 post-fertilization) whole egg silver concentration decreased from day 3 to 5, while chorionic silver concentration increased. These data indicate that the eggs may have been able to depurate silver from the inside of the egg back to the chorion, though the mechanism of this is unknown. Regardless of silver depuration, mortality rates after one day of recovery increased to almost 80%. In series 3, the reduced degree of delayed mortality in the 10 μg l^{-1} treatment during recovery may have been due to the lower egg silver burden and the egg's ability to depurate silver. Guadagnolo et al. (2000) also noted an apparent depuration of silver during chronic silver exposure at this developmental stage where eggs continuously exposed to silver from fertilization to hatch exhibited a reduction in whole egg silver concentration from days 17 to 25 post-fertilization.

Both whole eggs and chorions of the 10 μg l^{-1} treatment of series 4, accumulated significant amounts of silver from day 3 to 5 of exposure; whole eggs then lost significant amounts of silver from day 5 of exposure to day 2 of recovery. This rapid uptake and depuration of silver by the eggs may have been related to increased Na^+ exchange during this developmental period. Previous studies have demonstrated that there is an increased active uptake of Na^+ by the embryo starting at the eyed stage of development (Rudy and Potts, 1969; Eddy and Talbot, 1985) which increases exponentially through to hatch (Brauner and Wood, unpublished data). Bury and Wood (1999) demonstrated that both Na^+ and Ag^+ uptake at the gills of juvenile rainbow trout were reduced by specific blockers of Na^+ channels (phenamil) and a specific blocker of the H^+-ATPase (bafilomycin A_1). These data indicate that the uptake of Ag^+ occurs across the apical membrane of the fish gill via a Na^+ channel coupled to an H^+-ATPase, and therefore in direct competition with Na^+ (Bury and Wood, 1999). By analogy, Ag^+ uptake in eggs may be related to Na^+ exchange rates of embryos resulting in the 3 to 6-fold higher egg silver burden seen on day 5 of series 4 in the 10 μg l^{-1} treatment relative to the other series.

Interestingly, at each developmental stage there was not a large difference in whole egg silver burden following 5-days acute exposure or following a much longer continuous exposure to

silver under an identical experimental setup from fertilization (Guadagnolo et al., 2000). In some cases, egg silver burden was even greater following a 5-day acute challenge than following continuous exposure from fertilization for a much longer duration. For example, in series 1 and 2, eggs exposed to 1.0 μg l^{-1} for 5 days (Fig. 2) had two- and fivefold greater egg silver burdens than those chronically exposed to 1.0 μg l^{-1} for 11 and 15 days, respectively (c.f. Fig. 3 of Guadagnolo et al., 2000). The same trend was observed for the 10 μg l^{-1} treatment in series 3 and 4. The silver concentration in the chorions was qualitatively similar (c.f. Fig. 3 of Guadagnolo et al., 2000). These data indicate that it may be the developmental stage of the embryo rather than exposure duration that is most important in determining silver uptake into the egg. Another explanation is that chronic exposure to silver results in some form of acclimation, reducing the rate of silver uptake relative to that of a shorter acute challenge duration, although this remains to be tested experimentally.

4.4. Ag content in different egg compartments and the role of the chorion

The embryo contained only a small percentage of the whole egg silver content (1–17%), the remainder being contained in the chorion and yolk (Fig. 4). In the eggs of the 1.0 and 10 μg l^{-1} treatments in series 3 and 4, the majority of the total egg silver content was bound by the chorion. Similarly, in eggs chronically exposed to 1.0 and 10 μg l^{-1} total silver as $AgNO_3$ under a similar experimental setup, the percent silver bound by the chorion was about 85% (Guadagnolo et al., 2000). Thus, the chorion binds a large proportion of the egg silver burden during both chronic and acute exposure.

The chorion is known to be a protective barrier for the embryo from environmental stress (Rombough, 1988). In the present study, chorions accumulated the highest silver concentration compared to other egg compartments, especially in the eggs of the 10 and 1.0 μg l^{-1} treatments. Previous studies have demonstrated that in rainbow trout eggs exposed to cadmium, most of the metal was found associated with the chorion (Beattie and Pascoe, 1978; Rombough and Garside, 1982). Similarly, mercury and copper are also tightly bound by the chorion (Rombough, 1985).

The mechanism for metal transport across the chorion is unknown. However, Rombough (1985) reported that cations, such as metals, probably bind selectively to anionic glutamic acid sites on the chorion. Brivio et al. (1991) demonstrated that the rainbow trout chorion possesses about 650–700 μg total protein, which corresponds to 13–14% of the chorion. Although about 80% of the chorion protein consists of 'asparagine linked' glycoproteins (Brivio et al., 1991), there are significant amounts of cysteine (1–10%; Reiger et al., 1978; Sugiyama et al., 1996) that possess sulfhydryl groups with a very high binding affinity for silver (Bell and Kramer, 1999). Thus, most of the silver ions likely enter the egg by initially binding to these sulfhydryl groups, as well as other anionic sites.

The silver concentration of chorions following 5 days of acute exposure to 1.0 and 10 μg l^{-1} total silver in series 4 was almost 10-fold that in the other series, indicating that the composition of the chorion may be changing at this stage of development. Similar observations were made during chronic exposure to silver (Guadagnolo et al., 2000). These changes may be related to the presence of newly exposed sulfhydryl groups of previously cross-linked cysteine residues, brought about by protease activity of the hatching enzymes.

4.5. Implications for water quality criterion for silver

Under the current national guideline for freshwater life protection in Canada, total silver concentrations of up to 0.1 μg l^{-1} are acceptable (CCME, 1995). Cumulative mortality of eggs acutely exposed to 0.11 and 1.55 μg l^{-1} total silver (as $AgNO_3$) treatments for 5 days followed by 4-days recovery were not significantly different from controls throughout embryonic development. The general lack of significant silver uptake in whole eggs and different egg compartments was

correlated with low mortality rates. Therefore, based upon mortality data, the current Canadian water quality criterion is sufficient in preventing mortality due to toxic effects of silver during the embryonic development stage of rainbow trout in dechlorinated tap water that comes from Lake Ontario. In series 4, however, there was a significant effect of 0.11 and 1.55 μg l^{-1} total silver on whole egg Na^+ and Cl^- concentrations during recovery from silver exposure (Fig. 5). Although a combined effect between handling and silver exposure cannot be ruled out, these data indicate that these low silver concentrations may result in sub-lethal effects.

In the US, the Environmental Protection Agency (EPA) guidelines are based on water hardness (in mg l^{-1} of $CaCO_3$, US EPA, 1980). Using hardness measurements of Hamilton water, the acceptable acute silver criterion would be approximately 4.7 μg l^{-1} which represents a value that should not be exceeded for more than 1 h every 3 years on average (US EPA, 1980). According to the data presented, the minimum concentration of silver that caused statistically significant mortality was between 1.55 and 14.15 μg l^{-1} following recovery from the acute silver challenge. However, on day 4 of recovery from 5 days of exposure to 1.55 μg l^{-1} total silver in series 4, 49% mortality (accounting for control mortality according to Sprague, 1969) was observed. During chronic silver exposure in a parallel study on the same population of eggs an effect of silver exposure was also observed at 13.5 but not 1.2 μg l^{-1} total silver (Guadagnolo et al., 2000).

The present experiment with $AgNO_3$ represents a 'worst case' scenario; it is unlikely that levels of free Ag^+ in natural waters would ever reach those calculated in Table 1 due to the presence of other high affinity ligands such as DOC and reactive sulfides. Further, as discussed, some effects may have been due to the additional handling that is part of this experimental protocol. Nevertheless, the results, particularly taken with the observation of sub-lethal ionoregulatory effects, provide impetus for continued investigation into a possible US EPA chronic criterion for silver that is representative of realistic field conditions.

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