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PHYSIOLOGICAL RESPONSES TO ACUTE SILVER EXPOSURE IN THE FRESHWATER CRAYFISH (*CAMBARUS DIOGENES DIOGENES*)—A MODEL INVERTEBRATE?

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Abstract—Adult crayfish (*Cambarus diogenes diogenes*) exposed to 8.41 ± 0.17 μg silver/L (19.4% as Ag^+) in moderately hard freshwater under flow-through conditions for 96 h exhibited ionoregulatory disturbance, elevated metabolic ammonia (T_{amm}) production and substantial silver accumulation in the gills, hemolymph, and hepatopancreas. The ionoregulatory disturbance included both a generally reduced unidirectional Na^+ influx and an increased unidirectional Na^+ efflux, leading to a substantial net loss of Na^+ from the silver-exposed crayfish. The Na^+ uptake in silver-exposed crayfish differed overall from controls, while the increased Na^+ efflux recovered to control values 48 h into the 96 h of exposure. The general inhibition of Na^+ uptake could be explained by a reduced sodium/potassium-adenosine triphosphatase (Na/K-ATPase) activity in terminally obtained gill samples from the silver-exposed crayfish. The silver-induced effect on Na^+ uptake and loss translated to reduced hemolymph Na^+ concentrations but not significantly reduced hemolymph Cl^- concentrations. Hemolymph T_{amm} and T_{amm} efflux both increased in silver-exposed crayfish, indicating an increased metabolic T_{amm} production. The present study demonstrates that the toxic mechanism of waterborne silver exposure in freshwater crayfish resembles that of freshwater teleost fish. The crayfish might therefore be a useful model system for extending current environmental regulatory strategies, currently based on teleost fish, to invertebrates.

Keywords—Crayfish Silver toxicity Osmoregulatory disturbance Silver accumulation Unidirectional Na^+ flux

INTRODUCTION

Silver is toxic to aquatic organisms when present as ionic silver (Ag^+) [1–3]. In freshwater fish, toxicity occurs because silver specifically inhibits the activity of sodium/potassium-adenosine triphosphatase (Na/K-ATPase), thereby blocking Na^+ and Cl^- uptake and causing death from ionoregulatory failure [4–6]. However, natural ligands such as Cl^- and dissolved organic matter greatly reduce the toxicity of silver by the formation of silver complexes [4,6–12].

These important modifying factors for impact of silver in the environment have been incorporated into the biotic ligand model (BLM) with the goal to relate gill silver binding [7] to acute silver toxicity in freshwater fish. However, the BLM uses gill binding as a surrogate for toxicity and is calibrated to toxicity data directly rather than to gill silver binding data. In contrast to copper, where a correlation appears to exist between rapid gill copper binding and subsequent acute toxicity [13,14], silver accumulation on the gill and silver-induced toxicity do not appear to be directly correlated [15]. Nevertheless, McGeer et al. [16] used detailed knowledge about the physiological disturbance induced by silver exposure in freshwater fish to overcome this problem and designed a physiologically based BLM that successfully predicts acute silver toxicity using branchial Na/K-ATPase inhibition as an endpoint for silver toxicity.

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The success of these modeling approaches relied completely on a detailed knowledge of the physiological mechanisms of silver toxicity in rainbow trout [15,17]. In addition, most of the information regarding the ameliorating effects on silver toxicity by natural ligands, such as Cl^- and dissolved organic matter, arise from studies on rainbow trout or other teleost fish species [4,6–12,18].

The BLM is currently calibrated to protect crustacean invertebrates, which are much more acutely sensitive to silver than fish [19], based on toxicity data for *Daphnia* sp. [20]. This extrapolation from teleost fish to invertebrates relies on the assumption that the acute toxic mechanisms of waterborne silver exposure in the sensitive crustaceans are the same as those in the less sensitive teleost fish—inhibition of Na^+ (and Cl^-) uptake by inhibition of the branchial Na/K-ATPase.

The main objective of the present study was to investigate whether the toxic mechanism of acute silver exposure in crustaceans is similar to that of freshwater teleost fish. We chose the freshwater crayfish as an experimental model since its size (unlike *Daphnia*) is well suited for mechanistic studies of silver toxicity and its basic osmoregulatory physiology is well described [21–27]. The endpoints employed in this investigation to assess silver-induced physiological disturbance included unidirectional Na^+ flux rates, total ammonia (T_{amm}) efflux rates, hemolymph Na^+, Cl^- and T_{amm} concentrations, and branchial Na/K-ATPase activity, all of which have been reported to be affected by acute silver exposure [5,28]. In addition, silver concentrations in gill and other tissues were analyzed at the end of the silver exposure.

MATERIALS AND METHODS

Experimental animals

Freshwater crayfish, *Cambarus diogenes diogenes* (wt range = 19.9–38.6 g) were obtained from Boreal Laboratory (St. Catharines, ON, Canada). A total of 46 crayfish were held in a 400-L fiberglass tank, supplied with a minimum of 2.5 L/min of dechlorinated, aerated Hamilton city tap water (ON, Canada) ($[Na^+]$ 0.6 mM; $[Cl^-]$ 0.7 mM; $[Ca^{2+}]$ 1.0 mM; $[HCO_3^-]$ 1.9 mM; DOC 1.3 mg/L; pH 7.9–8.2). The holding tank was supplied with a number of short lengths of polyvinylchloride (PVC) tubing of appropriate diameter for shelter to reduce aggression. The crayfish were allowed to acclimate to these laboratory conditions for at least 7 d prior to experimentation. The temperature was 9 ± 1°C throughout acclimation and all subsequent procedures. The crayfish were fed dry trout pellets (Martin's Feed Mills, ON, Canada) three times a week.

Experimental design

Two experiments were performed in this study. In the first experiment, two groups of eight crayfish were transferred from the holding tank to individual aerated PVC chambers (vol ~300 ml) for subsequent measurements of unidirectional Na^+ flux rates and total ammonia (T_{amm}) efflux rates. The crayfish from one group (mean wt 27.3 g, range 23.8–33.0 g) received dechlorinated, aerated Hamilton city tap water (200 ml/min) and served as a controls. The second group (mean weight 27.4 g, range 22.9–38.6 g) was exposed to silver via a flow-through system. A mixing chamber received a constant flow of dechlorinated, aerated city water (ionic composition as described previously) from a head tank, and a concentrated silver (as $AgNO_3$) stock solution (shielded from light) was added at a constant rate from a Mariotte bottle (SMS, Tuscan, AZ, USA). The silver concentration in the stock solution was adjusted to yield a nominal silver concentration of 10 μg (91 nmol)/L. This concentration was chosen to allow for a direct comparison with previous studies of silver toxicity on rainbow trout under identical conditions [4]. Water silver concentrations were measured by atomic absorption (Varian AA-1275 with GTA-9 atomizer, Varian, Mulgrave, Australia).

In the second experiment, four crayfish were placed in each of four aerated 5-L PVC containers for subsequent serial sampling of hemolymph. Two containers received silver-free tap water (300 ml/min), and these crayfish served as controls (mean weight 24.6 g, range 19.9–31.3 g). The other two containers received silver-contaminated tap water (300 ml/min) from the mixing chamber mentioned previously, and these eight crayfish (mean weight 26.5 g, range 24.4–28.1 g) were thus exposed to a silver concentration identical to those of the silver-exposed crayfish in individual containers. All crayfish were starved for 48 h prior to the start of the test and were allowed to acclimate to the holding chambers for 24 h prior to experimentation.

Na^+ and T_{amm} flux experiment

Fluxes were measured for individual crayfish in the first experiment. The water flow to the individual flux chambers was terminated, and ^{22}Na (10 μCi/L, Amersham, Oakville, ON, Canada; specific activity of 303 Ci g/L Na^+) was added to the water of each flux chamber. After a 15-min equilibration period, two 5-ml samples of water were obtained from each flux chamber; one was designated for $^{22}Na^+$ radioactivity and total Na^+ measurements, and one was frozen immediately in liquid nitrogen for later analysis of T_{amm}. After 2 h, an additional two water samples were collected as described previously, and the water flow to each flux chamber was reestablished. For the silver-exposed crayfish, the water contained 8.41 ± 0.17 μg silver/L throughout the duration of the experiment. This procedure was repeated at 12, 24, 48, and 96 h of silver exposure and at the same times for control crayfish.

Unidirectional Na^+ influx rates were calculated from the disappearance of ^{22}Na radioactivity from the water in the flux chamber during the 2-h flux period, the mean specific activity of the ^{22}Na in the water, the chamber volume, the animal's body weight, and the elapsed time as outlined in detail in Grosell et al. [29]. The specific activity of plasma Na^+ never exceeded 5% of the corresponding specific activity in the water, and corrections for ^{22}Na efflux were thus not necessary (see Grosell et al. [29] for further details). The net Na^+ flux was calculated from the change in total Na^+ concentration in the water over the 2-h flux period, the flux chamber volume, the weight of the crayfish, and the time elapsed. The unidirectional Na^+ efflux was calculated by difference, using the corresponding equation for each individual set of measurements.

After the 96-h exposure to silver, a terminal hemolymph sample was obtained via a syringe fitted with a G26 needle inserted between the carapace of the thorax and the first tail segment. An aliquot of the hemolymph was frozen immediately in liquid nitrogen and stored at −70°C for later analysis of T_{amm}, and the remaining hemolymph was stored at −20°C for later analysis of Na^+, Cl^-, and silver concentration. After a period of time on ice sufficient to render the crayfish completely nonresponsive, the gills of thoracic appendages 2 to 7 were excised (the gills of thoracic appendages 1 and 8 are greatly reduced or absent). Gills from the left side of the animal were stored for later analysis of silver concentration, while gills from the right side were freeze clamped in liquid N_2 and stored at −70°C for later analysis of Na/K-ATPase activity. In addition, the antennal glands (left for silver analysis, right for Na/K-ATPase activity measurement), the hepatopancreas, a subsample of the tail muscle, and the thoracic carapace were obtained for later analysis of silver concentration.

Serial sampling of hemolymph experiment

In the second experiment, hemolymph was obtained from each individual crayfish prior to and at 24, 48, and 96 h of silver exposure and at parallel times for the controls. In addition, after the 96-h sampling, terminal tissue samples for silver analysis were taken as described previously. This second experiment was conducted because the serial percentage hemolymph sampling procedure might have affected the animals in the first experiment and thus might have interfered with the previously described flux measurements conducted with them.

Analytical techniques

Hemolymph ammonia was measured using an enzymatic assay (Sigma Chemical, St. Louis, MO, USA; Kit 171) modified for microtiter plate use. Ammonia concentrations in water samples were measured by the colorimetric method of Ivancic and Degobbis [30], hemolymph Cl^- concentrations were determined using the colorimetric assay of Zall et al. [31], and Na concentrations in the hemolymph and the water from the unidirectional flux study were analyzed using a Varian AA-

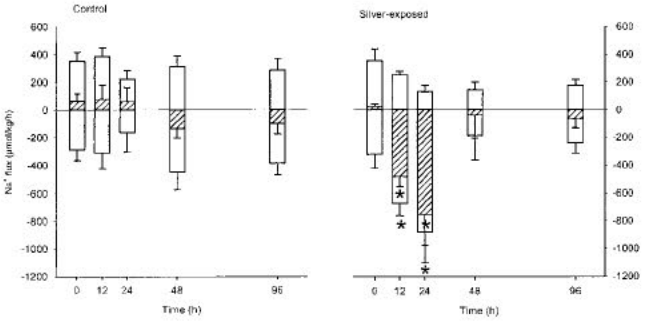

Fig. 1. Unidirectional Na^+ influx (open bars, positive values), efflux (open bars, negative values), and net flux (hatched bars) (μmol/kg/h) in control crayfish (left panel, $n = 8$) and silver-exposed (8.41 μg silver/L) crayfish (right panel, $n = 8$ for 0, 12, 24, and 48 h, $n = 7$ for 96 h). Means ± standard error of the mean (SEM). The asterisk indicates statistically significant difference from corresponding control at $p < 0.05$.

1275 flame atomic absorption spectrophotometer (AAS) with methods as documented by the manufacturer.

The Na/K-ATPase activity in gill and antennal gland samples was analyzed by the method of McCormick [32] with modifications according to Wheatly and Henry [33]. Tissue samples were prepared for analysis of silver concentration by adding approximately five times the volume of 1 N HNO_3 (trace metal grade, Merck, Darmstadt, Germany). The samples were then digested overnight at 75°C, vortexed, and centrifuged. The supernatant together with water and hemolymph samples were diluted appropriately prior to silver analysis on a graphite furnace atomic absorption spectrophotometer (Varian AA-1275 with GTA-9 atomizer) using a 10-μl injection volume, N_2 gas, and standard operating conditions as documented by the manufacturer.

The ^{22}Na radioactivity in water samples from the unidirectional Na^+ flux study was determined using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra-Packard, Mississauga, ON, Canada).

Data presentation and statistical evaluation

All values presented in Figures 1 to 5 are expressed as means ± 1 standard error of the mean (SEM). Unidirectional Na^+ flux rates, hemolymph Na^+, Cl^-, and ammonia during silver exposure were compared to control values by a two-factor analysis of variance (ANOVA) with exposure condition and time of exposure as main variables. The ANOVAs were followed by comparisons of silver-exposed and corresponding control values using a two-tailed t test with Bonferroni multisample comparison correction. Silver concentrations and Na/K-ATPase activity values from silver-exposed and control crayfish were compared using a two-tailed t test with Bonferroni multisample comparison correction. In all cases, $p < 0.05$ was applied. For the presentation of silver concentrations in various tissues of silver-exposed and control crayfish, values from the individual and group-exposed crayfish were pooled because they did not differ significantly.

RESULTS

The crayfish were exposed to a measured silver concentration of 8.41 ± 0.17 μg (76 ± 2 nmol) silver/L ($n = 12$). Using the geochemical speciation program MINEQL (4.06 for Windows, Hallowell, ME, USA) and a Ag-DOC log K of 9.0 [7], the calculated silver speciation in the experimental water was 17.7% Ag^+, 53.5% AgDOC, 26.7% AgCl(aq), and 2.1% $AgCl_2$.

The effects of silver on Na^+ flux

Unidirectional Na^+ flux rates were influenced by silver exposure. The control crayfish exhibited an approximately constant Na^+ influx during the 96 h of experimentation that equaled the Na^+ efflux rate, resulting in a net Na^+ flux not significantly different from 0. Thus, the control crayfish were in Na^+ balance throughout the experiment (Fig. 1). In contrast, the silver-exposed crayfish exhibited a gradual decline in Na^+ influx to a minimum of less than 50% of the corresponding control value after 24 h of exposure (ANOVA, $p < 0.05$). The mean Na^+ influx rates tended to remained lower, although not statistically significant than the corresponding control values (Fig. 1).

Unidirectional Na^+ efflux increased at 12 h and more than doubled at 24 h in silver-exposed crayfish, after which it recovered to control values at 48 and 96 h of exposure.

Together, the reduction in Na^+ influx and the increase in Na^+ efflux in the silver-exposed crayfish led to a substantial net Na^+ loss at both 12 and 24 h of silver exposure. Interestingly, a net Na^+ balance was regained by 48 h, mainly by the reduction in Na^+ efflux (Fig. 1).

The effect of silver on hemolymph Na^+ and Cl^- concentrations

In accord with the previously described effects on Na^+ flux rates, hemolymph Na^+ concentrations were significantly reduced after 24 h of silver exposure (Fig. 2A). The hemolymph Na^+ concentration remained low after 48 h of exposure, but this value was not significantly different from the control value, which exhibited a slight decline over the first 48 h of experimentation, presumably because of the serial sampling procedure. At 96 h, the serially sampled crayfish showed some recovery; however, this appeared to be due partly to a slightly reduced control hemolymph Na^+ concentration (Fig. 2A). Crayfish not disturbed by serial sampling percentage exhibited significantly reduced hemolymph Na^+ concentrations in the terminal samples taken after 96 h of exposure (Fig. 2A).

No statistically significant differences were observed between hemolymph Cl^- concentrations in silver-exposed and control crayfish from either of the two experimental series (Fig. 2B).

The effect of silver on T_{amm} efflux and hemolymph T_{amm} concentrations

The T_{amm} efflux gradually decreased in the control crayfish over the 96 h of experimentation. In contrast, T_{amm} efflux during silver exposure gradually increased to approximately three times higher than the corresponding control value at 96 h (Fig. 3A). The hemolymph T_{amm} concentration remained more or less constant in the control animals but was significantly elevated in silver-exposed crayfish at 24 h (Fig. 3B). Consistent with T_{amm} efflux rates (Fig. 3A), the hemolymph T_{amm} concentration was approximately two times higher than the corresponding control value at 96 h of exposure (Fig. 3B).

The effect of silver on branchial and antennal gland Na/K-ATPase activity

In the control animals, distal thoracic appendages exhibited lower branchial Na/K-ATPase activity than the corresponding frontal segments (Fig. 4). In agreement with the unidirectional

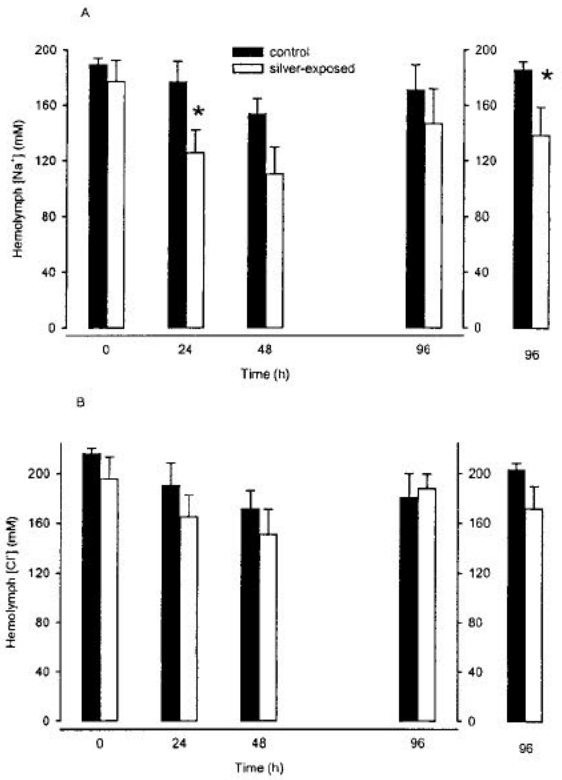

Fig. 2. (**A**) Hemolymph Na^+ concentration and (**B**) hemolymph Cl^- concentration in serially sampled control crayfish (black bars, $n = 8$ for 0–48 h and $n = 7$ for 96 h) and silver-exposed (8.41 μg silver/L) crayfish (white bars, $n = 8$, 7, 6, and 3 for 0, 24, 48, and 96 h, respectively) after 0, 24, 48, and 96 h of experimentation as well as terminally sampled control and silver-exposed crayfish after 96 h of experimentation (far right bars, $n = 8$ and 7 for control and silver-exposed crayfish, respectively). Means ± standard error of the mean (SEM). The asterisk indicates statistically significant difference from corresponding control value at $p < 0.05$.

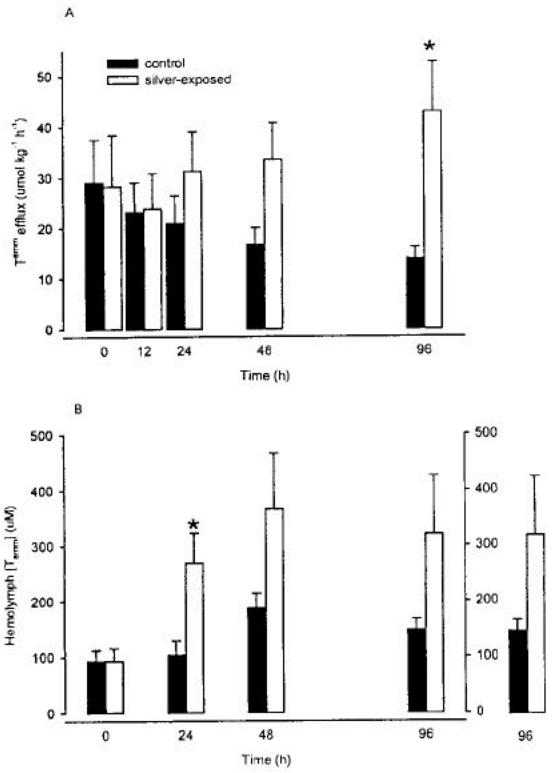

Fig. 3. (**A**) The T_{amm} efflux (μmol/kg/h) in control crayfish (black bars, $n = 8$) and silver-exposed (8.41 μg silver/L) crayfish (white bars, $n = 8$ for 0, 12, 24, and 48 h, $n = 7$ for 96 h) and (**B**) hemolymph T_{amm} concentration (μM) in serially sampled control and silver-exposed crayfish (details as in Fig. 2). Means ± standard error of the mean (SEM). The asterisk indicates statistically significant difference from corresponding control value at $p < 0.05$.

Na^+ flux measurements (see the previous discussion), 96 h of silver exposure generally reduced branchial Na/K-ATPase activity by 30 to 40% relative to controls. In agreement with the lack of silver accumulation in the antennal glands (see the following discussion), silver exposure had no effect on antennal gland Na/K-ATPase activity. The antennal gland Na/K-ATPase activity was more than twofold higher than the highest branchial Na/K-ATPase activity recorded in the frontal thoracic segment (Fig. 4).

The effects of silver on tissue silver concentrations

Silver exposure caused substantial branchial silver accumulation in all thoracic appendages (Fig. 5A). In addition, both hemolymph and hepatopancreas silver concentrations were greatly increased at 96 h. In contrast, the antennal glands, tail muscle, and carapace did significantly accumulate silver after 96 h of exposure (Fig. 5B).

Mortality

Of the nonserially sampled silver-exposed crayfish, only one out of eight died during the 96 h of silver exposure. No mortality was observed in the corresponding control group. The experimental group subjected to a combination of silver exposure and serial hemolymph sampling exhibited the highest mortality, with five of eight crayfish dying during the 96 h of silver exposure. In the corresponding control group, one of the eight crayfish died, presumably from effects of the serial sampling.

DISCUSSION

Mechanisms of silver toxicity in the freshwater crayfish

Silver exposure reduced Na^+ uptake by approximately 50% within 24 h, and Na^+ uptake remained low throughout the remaining 72 h of exposure. This corresponded well with terminal samples of gills from all thoracic appendages of silver-

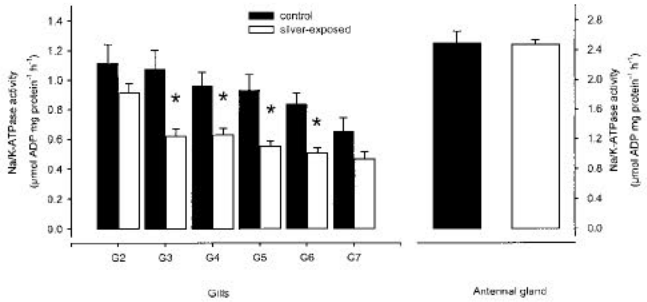

Fig. 4. Sodium/potassium-adenosine triphosphatase (Na/K-ATPase) activity in gills and antennal gland of control (black bars, $n = 8$) and silver-exposed (8.41 μg silver/L) crayfish (white bars, $n = 7$) after 96 h. G2 to G7 refer to podobranchs on the corresponding thoracic appendages; podobranchs on appendages 1 and 8 are greatly reduced or absent. Means ± standard error of the mean (SEM). The asterisk indicates statistically significant difference from corresponding control value at $p < 0.05$. Note different scale on the y-axis of the left and right panel.

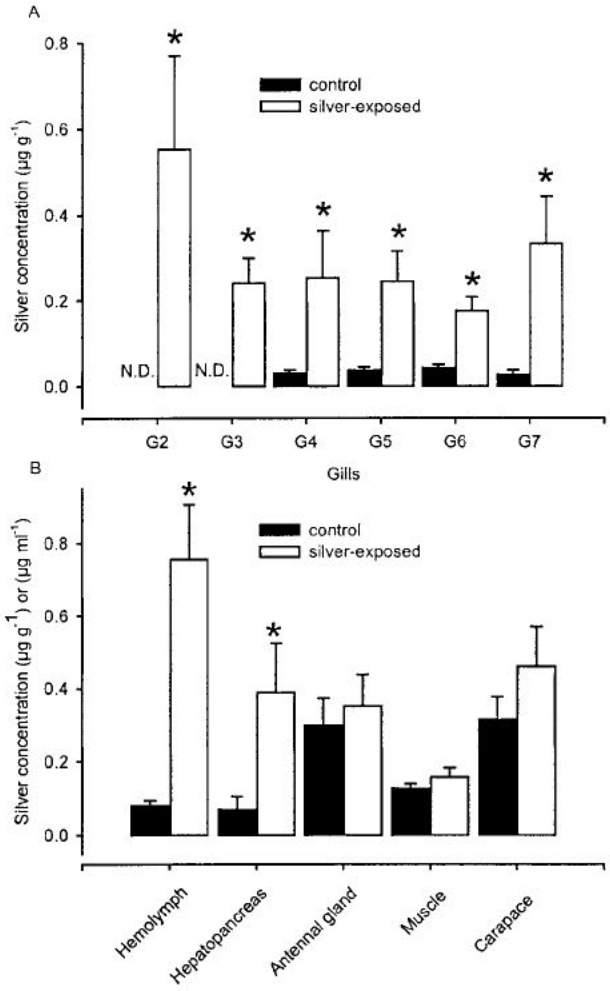

Fig. 5. Silver concentration in (A) gills and (B) in hemolymph, hepatopancreas, antennal gland, muscle, and carapace of control crayfish (black bars, $n = 15$) and silver-exposed (8.41 µg silver/L) crayfish (white bars, $n = 10$). G2 to G7 refer to podobranchs on the corresponding thoracic appendages; podobranchs on appendages 1 and 8 are greatly reduced or absent. ND = nondetectable. Means ± standard error of the mean (SEM). The asterisk indicates statistically significant difference from corresponding control value (unpaired t test) at $p < 0.05$.

exposed crayfish, which exhibited silver accumulation and associated reduction in Na/K-ATPase activity. These observations agree with the physiological effects of acute silver exposure in freshwater teleost fish [5,6,28,29] and thus support the hypothesis that the mechanism of silver toxicity is similar in freshwater crustaceans and teleosts. In addition to the reduced Na^+ uptake, silver-exposed crayfish had greatly elevated Na^+ efflux during the first 24 h. Although this effect was absent at 48 and 96 h of exposure, it contributed greatly to the overall net Na^+ loss during silver exposure and was probably an important factor in reducing hemolymph Na^+ concentrations. The elevated Na^+ efflux during silver exposure in crayfish differs from all reports of silver-induced osmoregulatory disturbance in freshwater teleosts, where Na^+ uptake but never Na^+ efflux is impacted by silver exposure. The hemolymph Cl^- concentrations were never significantly reduced by silver exposure.

This lack of a clear effect of silver on hemolymph Cl^- concentrations differs from that observed in rainbow trout, which exhibit reduced plasma Cl^- concentrations during silver exposure, but it is in agreement with observations of nonaffected plasma Cl^- levels in the European eel [4,5, vs 29]. Silver exposure probably does not alter plasma Cl^- concentration (and branchial Cl^- uptake rates) in the European eel because European freshwater eels rely not on active uptake of Cl^- across the gills but rather on Cl^- uptake through the diet (J.C. Rankin, unpublished data). In contrast, freshwater crayfish have considerable branchial Cl^- uptake [24,26] like most freshwater teleost fish. It is clear that uptakes of Na^+ and Cl^- are not strictly coupled but can occur independently in crayfish [22,23]. Thus, a differential response of Na^+ and Cl^- transport during silver exposure is plausible. Such a differential response has been reported for intestinal ion transport in marine teleosts, in which the Cl^- transport pathway is considerably less sensitive to silver exposure than the Na^+ transport pathway [34]. The lack of an effect on hemolymph Cl^- concentration, despite reduced hemolymph Na^+ concentration in these silver-exposed crayfish, implies that the crayfish may suffer from metabolic acidosis during silver exposure. While this remains to be confirmed, it would agree with observations from silver-exposed rainbow trout, which exhibit metabolic acidosis during silver exposure [28].

Hemolymph T_{amm} was increased in silver-exposed crayfish, but since this was related not to a decrease in T_{amm} efflux but rather to a comparable increase in T_{amm} efflux rates, the rise in hemolymph T_{amm} must have been related to an elevated metabolic production rather than to an inhibited excretion. These observations are in agreement with those from silver-exposed rainbow trout [28].

Silver accumulation in the freshwater crayfish

A marked silver accumulation in the gills during silver exposure explains the inhibited Na/K-ATPase activity and the reduced Na^+ uptake rate, in agreement with reports from teleost fish [5]. Lack of silver accumulation in the antennal glands (the functional equivalent of the freshwater teleost kidney) may explain why the relatively high levels of Na/K-ATPase activity in this osmoregulatory organ were not affected by silver exposure. Elevated silver concentrations in the hemolymph and hepatopancreas (the functional equivalent of the teleost liver) and lack of silver accumulation in other investigated tissues demonstrate that the uptake and internal distribution pattern of silver is very similar in freshwater crayfish and teleost fish [4,8,28]. In both types of organisms, the gill appears to be the major (if not only) site of uptake and toxicity during waterborne exposure. Silver is subsequently transported via the hemolymph/plasma to a highly specific accumulation in the hepatopancreas/liver. Because this general uptake and distribution pattern is similar to that of copper in trout [35], silver might act as a substrate for various copper transporters [36].

Is the crayfish a suitable model invertebrate?

In conclusion, the freshwater crayfish used in the present study exhibits silver uptake and accumulation patterns similar to that of the few freshwater teleost fish in which the physiology of silver toxicity has been investigated. In addition, it appears that the main toxic action of silver in this freshwater crustacean is similar to that reported for teleost fish. One clear exception, however, is the elevated Na^+ efflux observed during the first 24 h of silver exposure. Based on the similarities between silver-induced physiological disturbances in freshwater crayfish and teleost fish, it appears that much of the current understanding of silver toxicity and how it is modulated

by environmental factors (arising from studies on fish) may apply also to invertebrates.

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