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BIOLOGICALLY INCORPORATED DIETARY SILVER HAS NO IONOREGULATORY EFFECTS IN AMERICAN RED CRAYFISH (*PROCAMBARUS CLARKII*)

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Abstract—Two silver-contaminated diets were prepared by exposing juvenile rainbow trout for 8 d to waterborne silver thiosulfate as Ag at either 0.1 μg/L (low-Ag diet) or 80 mg/L (high-Ag diet). The level of total Ag accumulated in whole low-Ag fish was below the detection limit of analysis. Whole high-Ag fish accumulated Ag at 21.3 nmol/g. The livers of the low- and high-Ag fish accumulated Ag at 0.43 nmol/g and 1.01 μmol/g, respectively. The Ag-contaminated fish were then fed whole to adult crayfish in an 80-d dietary study to determine the effects of long-term trophic accumulation of Ag. In a second experiment, the livers of the high-Ag trout were fed to juvenile crayfish for either one or five weeks. Accumulation of Ag was demonstrated in both adult and juvenile crayfish. Silver accumulation in juvenile crayfish peaked at approximately 650 nmol/g at three weeks, after which Ag depuration occurred. In adult crayfish that consumed the high-Ag diet, the hepatopancreas accumulated more than 90% of assimilated Ag, rising 1,000-fold over control animals to approximately 740 nmol/g at 80 d. Crayfish that consumed the low-Ag diet had small, statistically insignificant elevations of Ag in some tissues. Dietary Ag had no effect on juvenile crayfish growth or adult mortality. Disturbances in osmoregulation, which are normally associated with acute waterborne Ag exposure, were not detected. Dietary Ag also had no effect on hemolymph concentrations of Na^+, Cl^-, Ca^{2+}, Mg^{2+}, or Cu; did not affect the concentration kinetics of Na^+ or Cl^- influx; and had no effect on the activity of gill Na^+/K^+-dependent adenosine triphosphatase. Hemolymph concentrations of glucose and lactate were similarly unaffected, indicating an absence of stress-related metabolic disturbance. However, a disproportionately low number of ecdysis events occurred among crayfish that consumed the high-Ag diet.

Keywords—Trophic transfer Silver *Procambarus clarkii* Ionoregulation Dietary toxicity

INTRODUCTION

Recent research has provided many insights into the toxicity of Ag to aquatic organisms [1,2]. Acute waterborne Ag toxicity typically is expressed as osmoregulatory impairment consequent to inhibition of Na^+ and Cl^- influx at the gills [3–6]. In trout and crayfish, the primary mechanism for this toxicity appears to be through inhibition of Na^+/K^+-dependent adenosine triphosphatase (Na^+/K^+-ATPase) on the basolateral membrane of the gill epithelium [3,4,7,8]. However, in rainbow trout, only the free Ag^+ ion is toxic in an acute sense [3,6,8,9]. When Ag is present in other waterborne forms (e.g., $Ag(S_2O_3)_x$, AgCl, and Ag_2S), where strong anionic ligands compete with the gill for Ag binding, Ag is not acutely toxic but can still accumulate within body tissues to very high levels in trout [9–11]. The physiological effects of long-term accumulation of Ag remain unclear.

Design of an experimental model that examines long-term accumulation of Ag requires consideration of the route of exposure. Silver discharged from sewage treatment plants as a consequence of industrial input generally is released as silver sulfides or is bound to colloidal and particulate matter [12]. Silver that is bound to particulate matter may be ingested by filter feeders and benthic organisms [13–15] or may be absorbed by aquatic algae and plants, as evidenced by substantial elevations in tissue Ag in plants and animals living within contaminated waters [16]. In this manner, Ag may be remobilized and made available for transfer to higher trophic levels [1].

For this reason, a dietary exposure is a realistic experimental model for examining the biological effects of bioaccumulated Ag. For this experiment, we chose a trophic model of exposure in which the food source was biologically contaminated with Ag [10]. Metallic contaminants that are biologically incorporated into prey food items may be more readily assimilated by predators than contaminants in artificially contaminated diets [17]. For example, rainbow trout (*Oncorhynchus mykiss*) assimilate Cd more efficiently from Cd-contaminated live amphipods than from a Cd-contaminated artificial diet [17]. The authors proposed that Cd absorbed by the amphipods became bound to sulfyhydryl groups on proteins or bound to metallothioneins, which are readily absorbed in the trout intestine.

In this study we used an approach similar to that of Galvez et al. [10]. Rainbow trout were exposed to waterborne silver thiosulfate so as to incorporate high internal levels of Ag. These contaminated trout were subsequently used as a food source in a dietary study with the American red crayfish (*Procambarus clarkii*). Silver sulfide may appear a more logical choice for producing contaminated food because this is the predominant species discharged from sewage treatment plants [12], but the use of silver thiosulfate is defensible on both ecological and experimental grounds. First, most of photographic facilities in the United States discharge Ag to munic-

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ipal sewers as silver thiosulfate complexes. These forms account for up to 44% of the total anthropogenic discharge to the aquatic environment [12]. Silver that is not fully reduced to silver sulfide or that escapes reactor treatment may enter the environment as silver thiosulfate, a form that is readily taken up by aquatic organisms. Second, from an experimental perspective, trout can be exposed to silver thiosulfate at high concentrations without succumbing to acute (osmoregulatory) toxicity [9–11] and will thereby accumulate substantial quantities of Ag [11]. In contrast, exposure to high concentrations of silver sulfide does not result in substantial accumulation of Ag in rainbow trout [18].

MATERIALS AND METHODS

Adult crayfish (mean wt 20 g) were purchased from Boreal Laboratories (St. Catherines, ON, Canada). Male and female crayfish were segregated and distributed among three tanks. Females were placed in two plastic tanks, each with a bottom area of 0.64 m^2. Males were placed in a third plastic tank with a bottom area of 0.28 m^2. The water depth of each tank was maintained at 10 cm; volume was 64 L for the large tanks with females and 28 L for the smaller tank with males. Each tank was supplied with aerated dechlorinated Hamilton (ON, Canada) tap water at a rate of 0.3 L/min with water exiting through a perforated central standpipe. Dechlorination was achieved by filtration through a bed of activated carbon. Hamilton tap water is sourced from Lake Ontario. The composition of this water has fluctuated by no more than 10% in 26 years of records kept by our laboratory. The composition of the water was $[Na^+]$ = 0.6 mM, $[Cl^-]$ = 0.7 mM, $[Ca^{2+}]$ = 1.0 mM, $[HCO_3^-]$ = 1.9 mM, $[Mg^{2+}]$ = 0.2 mM, $[SO_4^{2-}]$ = 0.25 mM, $[K^+]$ = 0.05 mM, $[NH_3] < 0.005$ mM, pH 7.8 to 8.0, dissolved organic carbon = 3 mg/L, and hardness 140 mg/L (as $CaCO_3$). Water temperature was maintained at 20 ± 2°C and a light: dark photoperiod of 16:8 h was established with dim lighting. To reduce aggression, all tanks were furnished with shelters in the form of short lengths (15 cm) of intact polyvinyl chloride (PVC) tube. Enough tubes were provided to allow all animals to find shelter. Food in the form of sole fillets was provided every 2 to 3 d. The quantity of each meal was roughly equivalent to 5% of total crayfish body weight. This occasionally was supplemented with trout pellets in addition to the sole. The animals were maintained in this manner for seven weeks. Some cannibalism occurred during this acclimation period.

Preparation of the experimental diet

Juvenile rainbow trout (1 g) were obtained from Humber Springs Hatchery (Orangeville, ON, Canada) and distributed among three 450-L tanks supplied with dechlorinated Hamilton tap water at a rate of 2.5 L/min. The water was aerated vigorously with air from a compressed air supply in our animal care facility. The animals were allowed to acclimate for 2 d before being exposed to waterborne Ag (see below). At the start of waterborne exposures, water flow to each tank was withdrawn, and each tank was assigned one of the following treatments: control treatment—trout were exposed to dechlorinated Hamilton tap water; low-Ag treatment—trout were exposed to Ag (as silver thiosulfate) at 0.1 μg/L; and high-Ag treatment—trout were exposed to Ag (as silver thiosulfate) at 80 mg/L. The low-Ag treatment concentration was selected because it represents the Canadian Water Quality Guideline [19], whereas the high-Ag treatment level was selected because exposure in this range results in substantial incorporation of Ag into trout within 7 d [9–11]. Silver thiosulfate ($Ag[S_2O_3]_x$) was prepared by reacting AgCl (Alfa Aesar, Ward Hill, MA, USA) with $Na_2S_2O_3$ (1:4 on a molar basis). The exposure regimes were maintained for 8 d with 55% exposure-water replacement every 24 h. All fish were then snap-frozen in liquid nitrogen.

Feeding trials

The experiment used three treatments, with four replicate 31-L polyethylene tanks (70 × 38 × 12 cm) per treatment. Treatments consisted of control (trout having no exposure to Ag), low-Ag diet (trout exposed to silver thiosulfate at an Ag concentration of 0.1 μg/L), and high-Ag diet (trout exposed to silver thiosulfate at an Ag concentration of 80 mg/L). The water depth of each tank was maintained at 8 cm (22 L); each tank was supplied with aerated dechlorinated tap water at a rate of 0.3 L/min with water exiting at the opposite end of the tank. Eight crayfish (six females and two males) were put into each tank. The water temperature was constant (20 ± 2°C) and a light:dark photoperiod of 16:8 h was established with dim lighting. To reduce aggression, all tanks were furnished with eight PVC shelters (described previously). Every 2 to 3 d, the crayfish were individually offered 5% of their body weight with the previously prepared frozen fish (i.e., one whole 1-g fish per crayfish). Any fish that remained uneaten after 6 h was removed and weighed.

The males were initially segregated from the females by using a perforated plastic barrier within each exposure tank. This was done to make sure that no mating occurred for the first four weeks of the feeding study, thereby ensuring bioaccumulation of Ag before mating. Mating occurred immediately upon removal of the barriers.

Twenty days after the experiment started, three crayfish were sampled for each treatment group for Ag analysis of some tissues. After 80 d, the remaining animals were sacrificed. Before being sacrificed, crayfish were anesthetized by cooling them to 1°C on ice.

In a second test, we used juvenile crayfish to evaluate the effect of dietary Ag on growth. In two trials, 3rd-instar juvenile crayfish were offered livers from high-Ag fish for one week and five weeks, respectively. The liver was selected because this organ represents the main internal site of Ag accumulation during silver thiosulfate exposure [2,9,11]. Each trial used four replicate 250-ml uncapped plastic tubs with 1-mm nylon-mesh bottoms suspended within the same tanks previously used for adult animals. The environmental conditions were the same as those used for the previous dietary study with adults. In trial 1, 20 juvenile crayfish were placed in each tub. In trial 2, 30 juvenile crayfish were placed in each tub. The crayfish were allowed to feed only on either the livers of control trout, or high-Ag trout for 7 d (trial 1) or 35 d (trial 2). The food consumption rate was 10 to 20% body weight per day. The high-Ag fish livers typically contained Ag at 1,010 nmol/g (wet wt). Growth (measured as wet mass) and Ag accumulation were measured daily (n = 9, trial 1) or weekly (n = 12, trial 2) by subsampling of the animals.

Physiology—Na^+ and Cl^- kinetic flux measurements and calculations

The following experiment was performed to test whether the long-term accumulation of Ag had an effect on Na^+ or Cl^- uptake in adult crayfish. After 70 d of exposure, seven adult crayfish from the control treatment and seven adult crayfish from the high-Ag treatment were used to measure the con-

centration-dependent kinetics of Na^+ and Cl^- influx. Flux measurements for each ion were carried out independently on sequential days with the same animals. The protocol was based on that of Shaw [20,21]. The crayfish were transferred to individual polyethylene beakers. The beakers contained 90 ml (i.e., ~4× crayfish mass + 10 ml) of formulated water. The solute composition of the water was 1.05 mM $CaCO_3$ and 0.15 mM $MgCO_3$. This composition approximated the hardness and acid–base characteristics of the holding water [22]. Sodium chloride levels were manipulated experimentally. Each beaker was individually aerated, and a plastic grate in each beaker was used to keep the crayfish fully submerged.

The experiment was started after a 15-min acclimation period. Aliquots of a 100 mM NaCl solution containing 11.7 kBq/ml of ^{22}Na (NEN, Boston, MA, USA) or ^{36}Cl (ICN, Irvine, CA, USA) were added at the beginning of five sequential flux intervals. The concentration of NaCl was sequentially increased from 0.1 mM to 2.0 mM over the five flux intervals. The duration of each flux interval was between 30 and 60 min. Each flux interval began 5 min after the addition of NaCl to allow the solutions to mix. Water samples (2 × 5 ml) were taken at the start and end of each flux interval for analysis of total Na or Cl. Additional water samples (2 × 5 ml) were also taken at the start and end of each flux interval for gamma (^{22}Na) or beta (^{36}Cl) spectroscopy. Formulated water (20 ml) was then added to each beaker to restore the original volume before the next flux interval.

Influx of Na^+ or Cl^- was determined by measuring the loss of radioactive isotope from the surrounding water. The isotopes are presumed to be taken up by the crayfish, because preliminary tests demonstrated that no loss of radioisotope occurred to the sides of the container or the surface of the animal. The protocol only measures influx of Na^+ and Cl^- because the duration of each flux interval is too short for any substantial efflux to occur [22]. The ^{22}Na radioactivity was measured with a MINAXI γ Auto-gamma 5000 series (Canberra-Packard, Meridian, CT, USA). The ^{36}Cl radioactivity was measured with a LKB Wallac 1217 Rackbeta liquid scintillation counter (Helsinki, Finland). Total water sodium was measured by atomic absorption spectroscopy (see below) by following the manufacturer's recommended protocol. Total water chloride was determined by a colorimetric assay described by Zall et al. [23]. The unidirectional fluxes of Na^+ (J_{in}^{NA}) and Cl^- (J_{in}^{Cl}) were calculated with the formula

$$J_{in}^{ion} = \frac{(cpm_i - cpm_f)V_{ext}}{\frac{1}{2}\left(\frac{cpm_i}{[ion]_i} + \frac{cpm_f}{[ion]_f}\right)W_t}$$

where cpm_i and cpm_f are the radioactivity (counts/min/ml) of the initial and final water samples; V_{ext} is the flux volume (ml); $[ion]_i$ and $[ion]_f$ are the initial and final concentrations of Na^+ or Cl^- (nM), respectively, in the flux water; W is the weight of individual crayfish (g); and t is the time (h) of the flux period. Ion influx data were plotted against the external ion concentration data. Lineweaver–Burk plots were generated to derive the maximal influx rate (J_{max}) and the binding affinity index (K_m) [22].

Physiology—Ion and metabolite analysis and calculations

After sacrifice, tissue samples were taken as follows. Hepatopancreas (whole), hemolymph, gill, antennal gland, and white muscle were removed, weighed, and stored at −20°C for analysis for Ag, Na, Cl, Ca, Mg, and Cu. The remaining carcasses were also stored at −20°C for later Ag analysis. Exuviae shed by crayfish during the course of the study were dried and assayed for Ag content as well. Tissue samples were prepared for ion analysis by adding five times the tissue volume of 1 N HNO_3 (trace metal grade; Merck, Darmstadt, Germany). The samples were digested overnight at 70°C, vortexed, and centrifuged. The supernatant was diluted for Ag and Cu analysis by graphite furnace atomic absorption spectroscopy (Varian AA-1275 with GTA-9 atomizer, Palo Alto, CA, USA) by following the manufacturer's recommended protocol with a multielement standard (Inorganic Ventures, Lakewood, NJ, USA). The digestates also were analyzed for Na, Ca, and Mg by flame atomic absorption spectroscopy (Varian AA-1275). Tissue weights (to the nearest 0.1 mg) were recorded for each tissue that was assayed for Ag. The tissue weights and Ag burden data for each tissue and the carcass were used to calculate the proportional allocation of Ag burden in the hepatopancreas.

Extra hemolymph samples were stored at −70°C for subsequent analysis of lactate, glucose, and protein. Extra gill filaments were also stored at −70°C for analysis of Na^+/K^+-ATPase activity. For analysis of lactate and glucose, hemolymph samples were thawed and a 20-μl aliquot was deproteinized in 40 μl of 1 M $HClO_4$, then centrifuged. The supernatants were placed on ice just before analysis. Total protein was measured on whole hemolymph samples that had been thawed and placed on ice just before analysis. Lactate, glucose, and total protein were measured by using protocols outlined in Bergmeyer [24] with Sigma reagents (St. Louis, MO, USA). The Na^+/K^+-ATPase activity in gill tissue was analyzed by using the method outlined by McCormick [25] with modifications for crayfish tissues according to Wheatly and Henry [26]. Results were expressed as the amount of adenosine diphosphate formed per milligram of protein per hour.

Statistical analyses

An analysis of variance with a Tukey post hoc test was used to test for significant ($\alpha = 0.05$) differences among fish exposure treatments. Silver concentration data were transformed (natural log) to achieve normality. Analysis of variance with a Tukey post hoc test was used to test for significant ($\alpha = 0.05$) differences between the concentrations of accumulated Ag within the various tissues of control crayfish and crayfish exposed to either a low-Ag diet or a high-Ag diet. Only the 80-d data were tested because of the low sample size ($n = 3$) for the 20-d data. All data were normally distributed except for the carapace data (80 d). A Mann–Whitney nonparametric test was used to evaluate carapace data. Analysis of variance with a Tukey post hoc test was used to test for statistically significant ($\alpha = 0.05$) differences between the concentrations of accumulated Ag over sequential weeks of the five-week trial with juvenile crayfish. Logarithmic curves were fitted to Na^+ and Cl^- influx data by using logarithmic regression analysis in Microsoft Excel (Redmond, WA, USA). We used Student's t tests for unpaired data to test for differences ($\alpha = 0.05$) between mean values of J_{max} and K_m for each of the seven control-diet and seven high-Ag–diet crayfish in the Na^+ and Cl^- flux trials. A nonparametric chi-square test was used to evaluate mortality and ecdysis events between treatment groups.

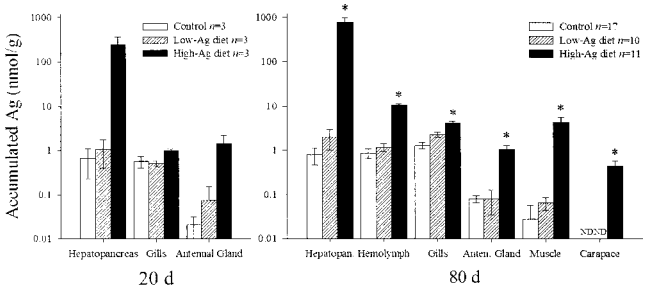

Fig. 1. Accumulation of Ag by adult crayfish after 20 d and 80 d of feeding, respectively. Error bars represent standard error. Asterisks denote data that differ significantly ($p < 0.05$) from control crayfish. ND = not detected. Detection limit = 0.005 nmol/g.

RESULTS

Diet preparation

The water concentration of Ag in the low-Ag tank (nominally 0.1 μg/L) could not be reliably monitored because it was below the detection limit of analysis (0.25 μg/L). The water concentration of Ag in the high-Ag tank (nominally 80 mg/L) remained consistently between 70 and 80 mg/L for the duration of the exposure. Fish in both treatments accumulated Ag; this was reflected in the livers of fish sampled after 8 d of exposure. The mean (± SE) concentrations of Ag in the livers of fish from the control ($n = 9$), low-Ag ($n=7$), and high-Ag ($n=8$) treatments were 0.146 ± 0.021 nmol/g, 0.43 ± 0.18 nmol/g, and 1,010 ± 137 nmol/g, respectively. The livers of both high-Ag and low-Ag fish had significantly ($p < 0.05$) higher levels of Ag than the livers of control fish. For whole fish, Ag concentrations were below the analytical detection limit (0.005 nmol/g) in control and low-Ag diet treatments, whereas the whole high-Ag–diet fish ($n = 18$) contained 21.3 ± 0.93 nmol/g.

Feeding observations

Observations showed that crayfish generally consumed the entire fish within a few minutes. On average, more than 80% of the total mass of fish was consumed. A feeding crayfish that failed to consume a whole fish preferentially consumed the contents of the body cavity, including the liver. Crayfish with larvae adherent to pleopods rarely ate. Juvenile crayfish consumed the entirety of the food offered within 1 to 2 h.

Silver accumulation in crayfish

Crayfish that consumed the low-Ag fish had a small, statistically insignificant increase in Ag accumulation over 80 d (Fig. 1). Crayfish that consumed the high-Ag fish accumulated substantial quantities of Ag in all tissues examined (hepatopancreas, gills, and antennal gland) after 20 d and continued to accumulate Ag up to 80 d (Fig. 1). The greatest amount of Ag accumulated in the hepatopancreas. The average concentrations of Ag in the hepatopancreas were 245 nmol/g and 784 nmol/g after 20 and 80 d, respectively. These levels of Ag represent 370 times and 1,000 times more Ag than in the hepatopancreas of crayfish that fed on control fish. Also, after 80 d of exposure, the hepatopancreas Ag burden was >90% of the body Ag burden in high-Ag–diet crayfish and 9% of the body Ag burden in crayfish that ate control fish. Of the tissues examined, the gills showed the lowest degree of Ag accumulation. After 20 and 80 d, the gills had accumulated, on average, 1.8 and 3.2 times more Ag than the gills of control crayfish, respectively (Fig. 1). No Ag was detected in the carapace of the crayfish on the control or low-Ag diet. Also, no Ag was detected in any of the exuviae shed by control or high-Ag animals.

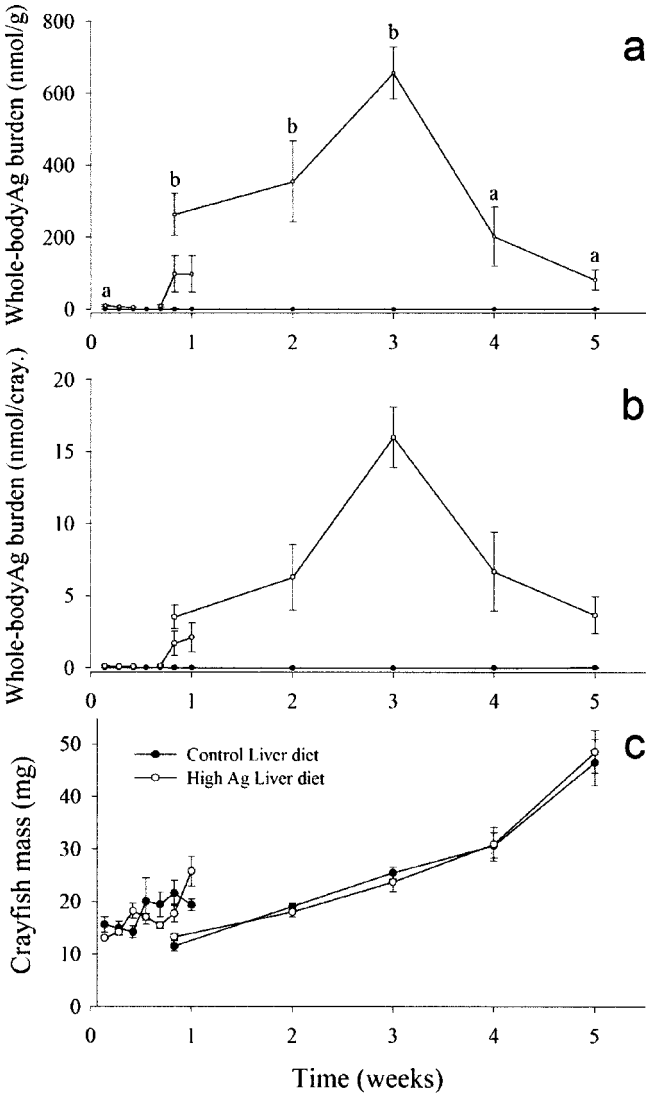

Fig. 2. Whole-body accumulation of Ag in juvenile crayfish in trials 1 and 2, expressed as (**a**) nmol/g and (**b**) nmol/crayfish. Silver levels in control animals remained below instrument detection limits for the duration of both trials. (**c**) Juvenile crayfish growth over the one-week and five-week trials. Each of the sample points was based on 9 animals (trial 1, one week), or 12 animals (trial 2, five weeks). Error bars represent standard error. Lowercase letters denote statistically significant ($p < 0.05$) groupings identified by analysis of variance with Tukey post hoc tests.

Juvenile crayfish that consumed livers from the high-Ag fish also accumulated Ag. The accumulation peaked at three weeks, before declining (Fig. 2a). The decline was not attributable to growth dilution, because the trend was similar even when the body burden of Ag was expressed as nanomoles per animal (Fig. 2b).

Mortality, growth, and reproduction

Mortality of adult crayfish was high among all treatment groups (Table 1): 48.1% of the crayfish on the control diet, 48.3% of crayfish on the low-Ag diet, and 60.0% of crayfish on the high-Ag diet. More than 50% of the mortality among

Table 1. Mortality, growth, ecdysis, and reproduction among crayfish consuming control, low-Ag, or high-Ag diets. Asterisks denote values that differ significantly ($p < 0.05$) from control crayfish

	Control diet	Low-Ag diet	High-Ag diet
Mean mass ± SE[a] at 0 d (g)	20.4 ± 0.62	20.4 ± 0.49	20.6 ± 0.52
Mean mass ± SE at 80 d (g)	20.2 ± 1.07	20.9 ± 0.90	21.5 ± 1.50
Mortality at 80 d (%)	48.1	48.3	60
Number of ecdysis events	13	16	5*
Number with young at 80 d	2	6	4

[a] SE = standard error.

all crayfish could be explained by cannibalism during the vulnerable postmolt stage when the animals were very soft. The number of deaths among crayfish fed on the high-Ag diet was not significantly different ($p = 0.188$; chi-square analysis) from the number of deaths among crayfish fed the control diet. During the trial, 13 control, 16 low-Ag–diet, and five high-Ag–diet crayfish molted. The low number of ecdysis events among crayfish (Table 1) on the high-Ag diet was statistically significant ($p \leq 0.001$; chi-square analysis). Of the animals surviving to 80 d, only two control, six low-Ag–diet, and four high-Ag–diet crayfish were carrying young (Table 1).

Adult crayfish did not grow much over the 80-d duration of the feeding trial regardless of the dietary treatment (Table 1). Juvenile crayfish grew substantially and growth was not affected by dietary Ag (Fig. 2c). Comparison of growth in treated versus control juvenile crayfish in trial 1 revealed a relatively high degree of growth fluctuation. This high degree of fluctuation was not observed in the five-week trial.

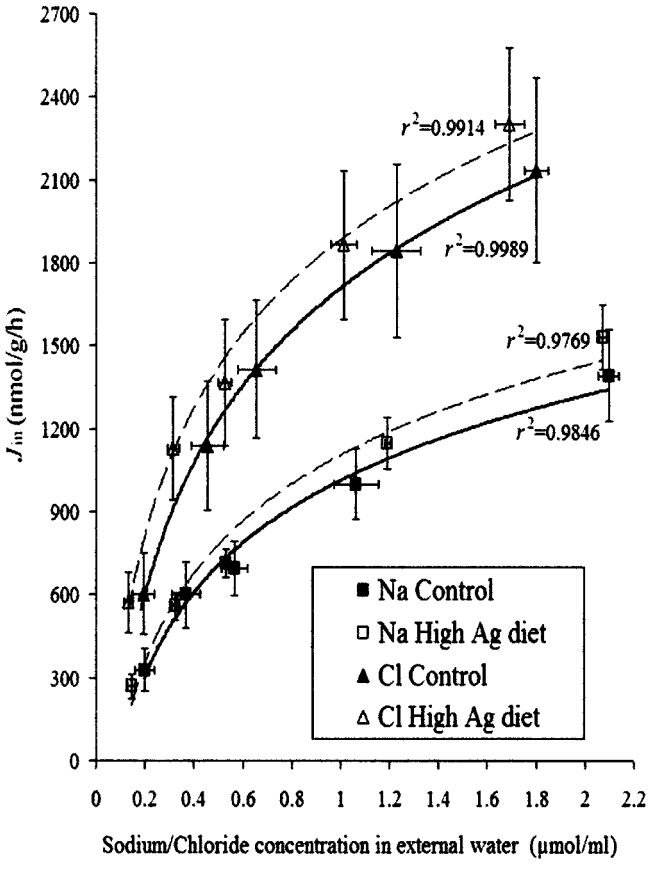

Fig. 3. Kinetics of sodium influx (J_{in}^{Na}) and chloride influx (J_{in}^{Cl}) as a function of external sodium or chloride concentration for crayfish that had been fed either a control diet (solid line) or a high-Ag diet (broken line) for 70 d. Logarithmic curves were fitted that closely approximate mean estimates of K_m^{Na}/K_m^{Cl} and $J_{max}^{Na}/J_{max}^{Cl}$. Mean J_{in} values have been plotted against mean sodium and chloride concentrations. Error bars represent standard error.

Physiology

Consumption of a high-Ag diet by adult crayfish for 70 d did not affect Na^+ or Cl^- influx (Fig. 3 and Table 2). Also, adult crayfish exposed for 80 d did not differ in respect to hemolymph concentrations of Na, Cl, Mg, Ca, Cu (Fig. 4), glucose, lactate (Fig. 5a), or total protein. The diets also did not affect the activity of gill Na^+/K^+-ATPase (Fig. 5b).

DISCUSSION

The trophic transfer protocol

Silver usually enters environmental waters through publicly owned sewage treatment works [12,27]. Concentrations of waterborne Ag in the environment are almost always exceedingly low because Ag has a high affinity for colloidal and particulate matter [12,27]. However, many marine and freshwater algal species can bioconcentrate Ag (and other metals) to levels much higher than in the surrounding waters [1]. Even though bioconcentration and biomagnification factors for ingested Ag are generally low [1,28,29], ingestion of particulate Ag may be the predominant route of Ag accumulation by filter-feeding invertebrates, benthic detritivores [29,30], and pelagic fish [31]. Crayfish, as opportunistic detritivores [32], will consume material derived from either plant or animal tissue and are likely to consume and accumulate Ag associated with other organisms and organic matter within their environment.

The concentration of silver thiosulfate in the high-Ag fish exposure was greater than can typically be found in the environment. However, the levels of total Ag accumulated by whole juvenile trout (21.3 nmol/g) after 8 d of exposure to silver thiosulfate were within the range of concentrations measured in field sampled fish, benthic invertebrates, and algae. A review of field data by Ratte [1] indicated Ag concentrations in freshwater periphyton up to 33.4 nmol/g, and Ag concen-

Table 2. Michaelis–Menten kinetic parameters for Na^+ and Cl^- influx in *Procambarus clarkii* that consumed either a control diet ($n = 7$) or high-Ag diet ($n = 7$) for 70 d. No statistically significant differences ($p < 0.05$) were found for comparisons of J_{max} and K_m between treatments[a]

Treatment	J_{max} ± SE (nmol/g/h)	K_m ± SE (µM)
Control (Na^+ influx)	2,182 ± 189	1,270 ± 206
High-Ag diet (Na^+ influx)	2,199 ± 211	1,095 ± 275
Control (Cl^- influx)	2,922 ± 487	835 ± 156
High-Ag diet (Cl^- influx)	2,756 ± 371	591 ± 125

[a] J_{max} = maximal influx rate; K_m = binding affinity index; SE = standard error.

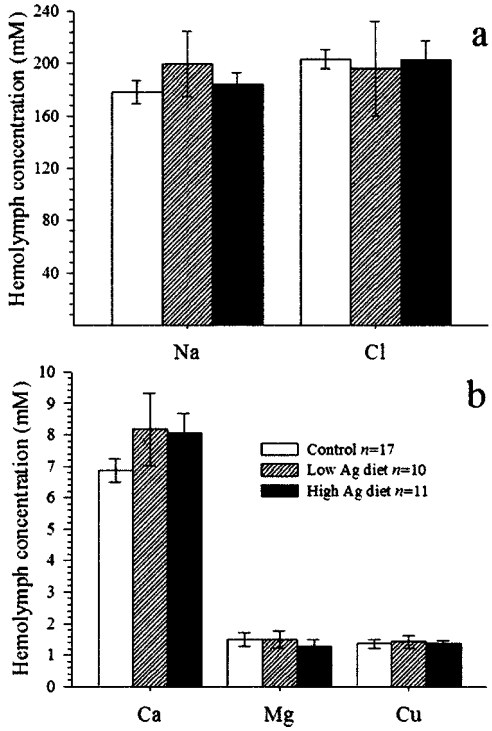

Fig. 4. (**a**) Concentrations of Na and Cl in crayfish hemolymph. (**b**) Concentrations of Ca, Mg, and Cu in crayfish hemolymph. Error bars represent standard error.

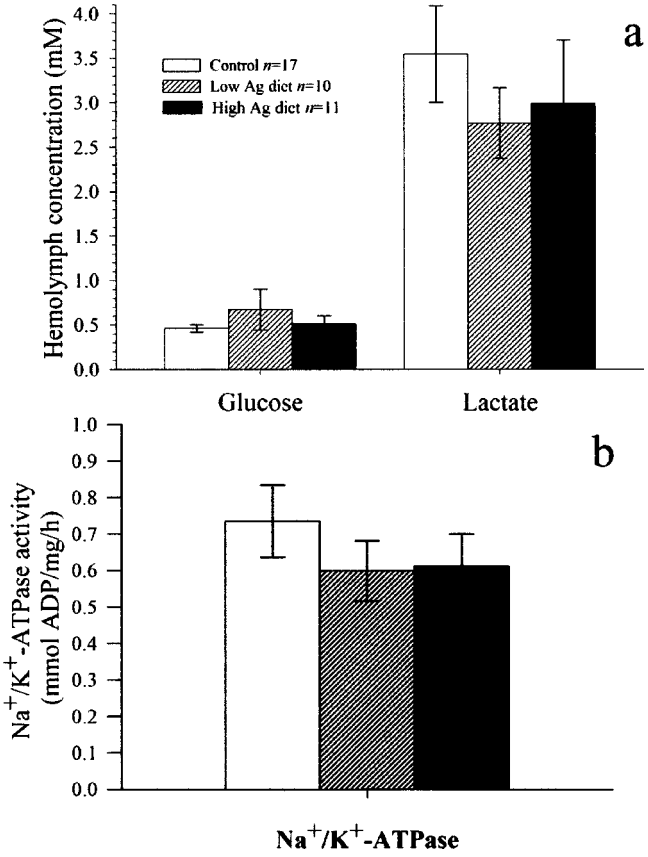

Fig. 5. (**a**) Concentrations of glucose and lactate in crayfish hemolymph. (**b**) Activity of sodium/potassium-dependent adenosine triphosphatase (Na^+/K^+-ATPase) in the first and second podobranchs (combined) of gills from crayfish that consumed control, low-Ag, or high-Ag diets (expressed as amount of adenosine diphosphate [ADP] formed per milligram of protein). Error bars represent standard error.

trations in fish from contaminated sites ranged from 4.2 nmol/g (*Lepomis microlophus*) to 25.3 nmol/g (*Campostoma anomalum*). Furthermore, freshwater mesocosm studies indicated that algae, crustaceans, mollusks, and fish all have the potential to accumulate Ag far in excess of the concentrations reached by trout in this study [1].

Silver accumulation

All tissues examined accumulated Ag in response to a high-Ag diet, but the hepatopancreas accumulated the bulk of the Ag. The estimated rate of Ag consumption by adult crayfish in this study was 0.35 to 0.54 nmol/g/d. This rate is similar to that of the juvenile rainbow trout used by Galvez et al. [10], 0.32 to 0.57 nmol/g/d. In the study by Galvez et al. [10], fish livers accumulated approximately 185 nmol/g over 126 d. In contrast, the crayfish in our study accumulated more than 742 nmol/g in the hepatopancreas over 80 d. This finding indicates that crayfish have a large capacity for Ag bioconcentration. A similar disparity in bioconcentration factors was found for marine snow crabs (*Chionoecetes opilio*) versus American plaice (*Hippoglossoides platessoides*), with the crustacean having greater accumulations of ^{110m}Ag (consumed as $AgNO_3$) as a consequence of higher assimilation efficiency and longer biological half-life [33].

As suggested by Rouleau et al. [33], the higher capacity for bioconcentration of Ag in some crustaceans might be related to the similarity between Ag and Cu, and the important role that Cu plays in crustacean respiration. Copper and Ag are both group I-b transition metals that are chemically and toxicologically similar [2], and the respiratory pigment in crustacean hemolymph (hemocyanin) uses Cu to transport oxygen in much the same way as hemoglobin transports oxygen in vertebrates. Silver may have the capacity to displace Cu from the hemocyanin complex in much the same way that Ag competes for the Mg^{2+} binding site in the Na^+/K^+-ATPase complex [2,34]. However, we found no difference in hemolymph [Cu] for control versus high-Ag diet crayfish, even though hemolymph [Ag] was substantially elevated.

The increase, then subsequent loss of Ag in juvenile crayfish (Fig. 2), is intriguing. It is possible that Ag was being eliminated with the exuviae as the animals grew. However, the shed exuviae of these animals were not collected for analysis and this possibility is not verified. The lack of Ag in the shed exuviae of the adult animals, together with the presence of Ag in the carapace of sacrificed crayfish, strongly suggests that Ag deposited within the exoskeleton is extracted and excreted before ecdysis. Such mechanisms are used for the partial recovery of Ca from the exoskeleton in the premolt stage with approximately 40% of whole-body calcium excreted into the surrounding medium [35]. Because ecdysis occurs more frequently during early growth phases, it is possible that juveniles can excrete their Ag burden more efficiently than can adults.

Physiology, growth, and reproduction

In trout, acute toxicity of free Ag^+ is manifested as osmoregulatory impairment consequent to inhibition of Na^+ and Cl^- influx at the gills [3–6]. Mechanistically, this inhibition appears to be caused by noncompetitive inhibition of Na^+/K^+-ATPase bound to the basolateral surface of the gill epithelium [3,8,36]. In crayfish (*Cambarus diogenes diogenes*), the mechanism of acute waterborne Ag^+ toxicity appears to be similar

and results in impairment of Na balance, accompanied by gill Na^+/K^+-ATPase inhibition [7]. In addition, crayfish exposed to waterborne Ag^+ [7] had significant accumulations of Ag within gill tissues (~12 times the [Ag] as control gill tissue). In the present study, accumulations of Ag at the gills of crayfish that consumed the high-Ag diet were only 3.2 times greater than accumulations of Ag of control animals, despite the high body burdens of Ag. This finding indicates little retrograde movement of Ag into the gill tissue after high accumulations of Ag from the diet. Also, the Ag within the gills is likely bound within a biological molecule such as metallothionein, or persists as an Ag–thiosulfate complex that is less toxic than free Ag^+ [9]. The absence of an accumulation of toxic ionic Ag in the gills is therefore consistent with the observed absence of osmoregulatory effects. In trout exposed to waterborne Ag^+, noncompetitive inhibition of Na^+/K^+-ATPase reduces the maximal rate of Na^+ influx (J_{max}) [4]. In contrast, in daphnids exposed to waterborne Ag^+, the inhibition of Na^+/K^+-ATPase was competitive and resulted in a decrease in the affinity of binding sites for Na^+ (i.e., an increase in K_m) [37]. Neither J_{max} nor K_m was affected by dietary Ag exposure in this study.

Hemolymph glucose and lactate concentrations were not significantly affected by trophic Ag accumulation, although lactate levels were somewhat high [38]. In general, these results are consistent with those obtained in rainbow trout [10], and argue against any alterations in metabolism as a consequence of dietary exposure to Ag. However, increased metabolic costs as a consequence of dietary Ag may be masked by background noise associated with the health of our animals, behavioral compensation [39], or some other metabolic trade-off. For example, it is conceivable that the low number of ecdysis events among crayfish that consumed the High-Ag diet reflects a metabolic trade-off.

The low number of ecdysis events among crayfish that consumed the high-Ag diet (Table 1) is notable, but remains unexplained. Retardation of ecdysis also has been described in juvenile and adult estuarine crabs after waterborne exposure to Cd [40,41].

We were unable to make any definitive observations on reproductive indices in this study because of the high mortality and low numbers of animals that produced young (Table 1). We can state that some of the animals exposed to high-Ag diets produced young during the course of the study, and that a similar number of control crayfish and crayfish that consumed the low-Ag diet also produced young. No conspicuous differences were found in clutch size or viability among the treatments. Reproductive impairment has been demonstrated in cladocerans and copepods that consumed Ag-contaminated diets [42]. Deleterious effects on reproduction, if any, would likely be seen only if the period of dietary exposure and Ag accumulation coincided with ovary maturation. In the study presented here, Ag exposure only commenced after ova development.

CONCLUSIONS

The crayfish in this study had high levels of trophic assimilation of Ag, with most of the Ag deposited within the hepatopancreas. The gills did not accumulate substantial amounts of Ag, and osmoregulation in the gill did not appear to be impaired. The results of this study are therefore in agreement with those of Galvez et al. [10], who reported no osmoregulatory effects of dietary Ag in rainbow trout. This study highlights the importance of exposure route in toxicity studies with Ag and other metals. The osmoregulatory toxicity of Ag in aquatic organisms is likely exclusively associated with exposure of the gills to waterborne Ag^+. Dietary exposure of trophically incorporated Ag or other nonessential metals may allow for greater accumulation without deleterious effects. Indeed, metals presented to a consumer in a form that is potentially nonbioreactive, as was the case in this study (i.e., complexed with biological molecules), may simply pass into the compartmentalized and biologically detoxified body burden.

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