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ORIGINAL PAPER

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Branchial versus intestinal silver toxicity and uptake in the marine teleost *Parophrys vetulus*

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Abstract Exposure to elevated waterborne silver as $AgNO_3$ (4.07 μM = 448 μg l^{-1}) in seawater resulted in osmoregulatory disturbance in the lemon sole (*Parophrys vetulus*). The main effects were increased plasma Na^+ and Cl^- concentrations which translated into increased plasma osmolality. Plasma Mg^{2+} levels were also slightly increased after 96 h exposure. Using radioisotopic flux measurements, a 50% reduction in branchial unidirectional Na^+ extrusion was observed after 48 h silver exposure. By applying an intestinal perfusion approach, we were able to separate and thus quantify the intestinal contribution to the observed silver-induced physiological disturbance and internal silver accumulation. This analysis revealed that the intestinal contribution to silver-induced ionoregulatory toxicity was as high as 50–60%. In marked contrast, internal silver accumulation (in liver and kidney) was found to be derived exclusively from uptake across the gills. Drinking of silver-contaminated seawater resulted in substantial silver accumulation in the intestinal tissue (but apparently not silver uptake across the intestine), which probably explains the intestinal contribution to silver-induced physiological disturbance.

Keywords Marine teleost · Silver toxicity · Silver uptake · Intestinal toxic response · Branchial toxic response

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Introduction

While the mechanisms of silver toxicity in freshwater teleost fish have been well documented over the past decade (see Hogstrand and Wood 1998; Wood et al. 1999 for reviews), much less is known about the exact toxic mechanisms of silver in marine teleost fish. In freshwater, the site of toxicity is the gill where silver enters primarily through the apical Na^+ channel (Bury and Wood 1999) and targets the basolateral Na/K-ATPase (Morgan et al. 1997; Webb and Wood 1998; Grosell et al. 1999a). Silver inhibits the maximal activity of this enzyme by binding competitively to the Mg^{2+} binding site (Ferguson et al. 1996). The main consequence of this interaction is a marked reduction in Na^+ uptake in vivo leading to a net Na^+ loss and subsequent ionoregulatory and cardiovascular failure (Morgan et al. 1997; Webb and Wood 1998; Grosell et al. 1999a).

It has been documented that the primary response to silver exposure in marine teleosts is osmoregulatory disturbance (Hogstrand et al. 1999) as it is in freshwater teleosts, but the mechanisms remain to be established. Relatively high intestinal silver levels in seawater-adapted eels, compared to freshwater-adapted eels exposed to the same silver concentrations (Grosell and Hansen 1997), revealed that the intestine could be a target organ for silver toxicity in marine teleost fish.

Osmoregulation in marine teleosts involves drinking to compensate for the diffusive loss of water to the surrounding hyperosmotic environment (Smith 1930; Shehadeh and Gordon 1969). The imbibed seawater is diluted in the oesophagus by extracellular fluid before entering the stomach and the intestine. From the intestine, an isotonic salt solution is taken up across the intestinal epithelium via active transport of Na^+ and Cl^- driven mainly by the intestinal Na/K-ATPase activity (see Loretz 1995 for a review). The salt gained by the fish (both from the intestinal uptake and passive branchial influx) is extruded across the gills with energy provided via the branchial Na/K-ATPase activity (McCormick

1995). Assuming that the Na/K-ATPase is the target for silver toxicity in marine teleosts as it is in freshwater teleosts, two possible sites for silver toxicity are thus the intestinal Na^+, Cl^- and water uptake and the branchial Na^+ and Cl^- extrusion.

We have recently shown that the drinking of silver-contaminated seawater leads to considerable silver concentrations in the intestinal fluids. Using in situ perfusion techniques and in vitro preparations we have documented that silver impairs both Na^+, Cl^- and water uptake as well as HCO_3^- secretion by this epithelium (Grosell et al. 1999b). This suggests that Na/K-ATPase could be the target for silver toxicity in the intestinal epithelium of marine teleosts as it is in the branchial epithelium of freshwater teleosts.

The inhibition of intestinal HCO_3^- secretion by silver previously observed (Grosell et al. 1999b) could be related to inhibition of carbonic anhydrase activity in this epithelium. Inhibition of carbonic anhydrase by silver exposure has been documented in freshwater teleost fish gills (Morgan et al. 1997). The function of the considerable amount of intestinal HCO_3^- secretion in marine teleosts is not known but it is clear that it is not involved in acid-base regulation (Wilson 1999). It has been proposed (by Wilson 1999) that one consequence of the high levels of HCO_3^- in the intestinal fluids (Walsh et al. 1991; Wilson, et al. 1996; Grosell et al. 2001) is the formation of Ca^{2+}- and Mg^{2+}-carbonate precipitate, thereby reducing the osmolality of the intestinal fluids and aiding water absorption by the intestinal epithelium.

We have recently proposed a model in which active Cl^- uptake is coupled to active HCO_3^- secretion by the intestine (Grosell et al. 2001). This means that silver exposure, in addition to potential inhibition of Na^+- and Cl^--driven water transport by inhibition of the Na/K-ATPase, could reduce Cl^- and water transport indirectly coupled to the carbonic anhydrase activity and thus HCO_3^- secretion.

In addition to the intestine, the gills are exposed when a marine teleost is faced with elevated ambient silver concentrations. Hogstrand et al. (1999) and Grosell et al. (1999b) reported increased branchial Na/K-ATPase in both starry flounder and lemon sole after acute high level silver exposure.

The aims of the present study were to quantify the relative importance of the branchial versus the intestinal toxic response to acute silver exposure and to assess the relative importance of the two routes of uptake. To pursue these aims, we applied an in vivo approach where we employed intestinal perfusion techniques similar to those of Wilson et al. (1996), Grosell et al. (1999b) and Grosell and Jensen (2000). During silver exposure, we compared the physiological disturbance, in fish which had their intestine perfused and thus did not drink the silver-contaminated water, to that in fish which were allowed to drink the silver-contaminated seawater. These two groups thus provided fish which were exposed to silver only at the gills and fish which were exposed to silver both at the gills and at the intestine. The physiological disturbance was evaluated based on comparison with a non-exposed control group. Furthermore, a sham-treated control group (non-exposed but with the intestine perfused) was included in the present study, because the involved surgery is elaborate and can induce some ion-regulatory and acid-base balance disturbances (Grosell and Jensen 2000).

The role of the gill in the toxic response to silver exposure was evaluated directly both by unidirectional ^{22}Na efflux measurements and by terminal Na/K-ATPase assays, while the intestinal response was evaluated more indirectly by comparison of blood plasma electrolyte and acid-base status between the above-mentioned groups.

The present study was conducted at a silver concentration of 4.07 μM (448 ± 21 $\mu g \cdot l^{-1}$) which is considerably higher than the US EPA acute criteria for the protection of marine aquatic life (0.02 μM or 2.3 $\mu g \cdot l^{-1}$ total silver). The high concentration used in the present study was chosen to ensure significant physiological disturbance arising from the silver exposure within the time frame limited by the applied surgical and experimental procedures.

Materials and methods

Experimental fish

Lemon sole (*P. vetulus*) (weight range 115–552 g) were obtained by otter trawls in the vicinity of Bamfield, British Columbia, Canada in the summer and held in 400-l fibreglass tanks with a flow-through (> 5 $l \cdot min^{-1}$) of aerated Bamfield marine station seawater (14 °C, salinity = 30‰) for at least 5 days prior to experiments. The bottom of the holding tanks were covered by fine sand. The fish were not fed during holding.

Experimental groups

A total of four groups of fish were anaesthetized in a 100-$mg \cdot l^{-1}$ solution of MS-222 in seawater for surgery. Once anaesthetized, fish were placed on a plexiglass plate where the gills were constantly irrigated with aerated MS-222 solution, as above, throughout surgery. For repetitive blood sampling, each fish was fitted with a caudal artery cannula, a length of polyethylene tubing (PE50) filled with heparinized (50 $iu \cdot ml^{-1}$) Cortland saline (Wolf 1963) adjusted to 160 mM Na with NaCl for seawater. The catheter was held in place by a sleeve of PE160 secured with two sutures.

In addition to the caudal artery catheter, one group was prepared for intestinal perfusion as described by Grosell et al. (1999b) and Grosell and Jensen (2000). In brief, this preparation allowed for perfusion of the entire intestine and for in-situ recordings of the intestinal transport processes. The fish in this experimental group ($n = 6$) were exposed to silver in the water but had their intestine perfused with a silver-free saline. This approach thus eliminated any direct effect of silver on the intestinal contribution to whole body ion and acid-base regulation while the gills were exposed. A second group of fish ($n = 7$) were allowed to drink the silver-containing seawater and were thus exposed to silver both at the gills and at the intestine. The fish from these two experimental groups ($n = 27$) were exposed to 4.07 ± 0.21 μM silver (448 ± 21 $\mu g \cdot l^{-1}$).

Fish from a third group ($n = 5$) were prepared for intestinal perfusion with the same silver-free saline as above but were not exposed to silver in the water. This group served as a "sham control" for the silver-exposed, perfused group (described above) and

was necessary because the applied surgical procedure itself can cause physiological disturbance (Grosell and Jensen 2000). The two silver-exposed groups and the sham-operated control group were compared to a fourth experimental group of non-exposed and non-perfused control fish ($n = 7$).

Once revived from the anaesthetic, fish from the four experimental groups were placed in individual 8-l PVC containers partly covered with a wooden lid and supplied with flow-through aerated seawater at a rate of ≥200 ml·min^{-1}. Infusion of "gut saline" at a rate of approximately 15 ml·kg^{-1}·h^{-1} was started immediately from an inflow reservoir via a peristaltic pump, and the outflow perfusate was collected into covered glass beakers (using a siphon of at least 5 cm). Fish were left to recover overnight prior to experiments. The gut saline composition has been reported elsewhere (Grosell et al. 1999b). With exception of the HCO_3^- concentration, it resembles the measured composition of intestinal fluids from lemon sole.

Experimental design

The experiment consisted of four successive 24-h periods. A 500-µl blood sample was obtained via the caudal artery catheter prior to and after 24, 48, 72 and 96 h silver exposure. The two control groups were treated as the silver-exposed groups, except for the silver exposure. The silver exposure conditions were flow-through in Bamfield marine station seawater (14 °C, salinity 30‰). A head-tank ensured a constant water pressure and thus constant water flow to a mixing chamber. Silver was added as silver nitrate to the water in the vigorously aerated mixing chamber from a concentrated stock solution via a Mariotte bottle to achieve a final concentration of 4.07 µM. From the mixing chamber, water was fed to each of the individual tubs by gravity.

To evaluate the effect of silver in the gill tissue and to test the hypothesis of branchial compensation for silver-induced effects on the intestinal epithelium (Grosell et al. 1999b; Hogstrand et al. 1999), Na^+ efflux rates were determined in each individual fish from all four experimental groups. The efflux measurements were conducted prior to silver exposure and after 24, 48, 72 and 96 h exposure by injecting radio-labelled ^{22}Na (100 µCi·kg^{-1} Amersham, specific activity; 303 Ci·g^{-1} Na^+) in Cortland saline (1 µl·g^{-1}) through the caudal artery catheter. This was done shortly after completion of blood sampling (described below). Pilot experiments revealed that a period of 1 h after injection was sufficient to ensure equilibrium between injected ^{22}Na and extracellular "cold Na". Water flow was terminated 1 h after ^{22}Na injection and an initial water sample (5 ml) was obtained from each container. In addition, a 100-µl blood sample was drawn from the caudal artery catheter (after thorough rinsing of the catheter) to determine specific ^{22}Na activity of the extracellular fluid. This water and blood sampling procedure was repeated after 1 h and 2 h, after which water flow was re-established. See below for details regarding determination of ^{22}Na radioactivity and Na^+ flux rate calculations.

After the blood sampling was completed and the Na^+ flux experiment was terminated at 96 h, each fish was killed by an overdose of MS-222 and samples were taken as follows. Gill filaments and the anterior, mid and posterior segments of the intestine were sampled immediately, wrapped in aluminium foil and frozen in liquid nitrogen. The kidney, spleen, liver and white muscle were sampled and stored at –20 °C.

Sampling procedures and analytical techniques

The 500-µl blood samples were drawn into heparinized, gas-tight Hamilton syringes. The sampled volume was replaced with a non-heparinized Cortland saline (as described above) and the catheter was subsequently filled with 200 µl heparinized Cortland saline (100 IU·ml^{-1}) to prevent clotting. Blood pH was measured immediately using a micro-capillary pH electrode (Radiometer G279/G2) coupled with a PHM71 acid-base analyser and haematocrit was determined by centrifugation at 5000 *g* for 5 min. Plasma was obtained by centrifugation (10,000 *g* for 2 min) and analysed for total CO_2 using a Corning total CO_2 analyser (CMT965, Corning Medical and Scientific), and a sub-sample was stored for later analysis of electrolytes as described below.

The plasma PCO_2 and concentration of HCO_3^- equivalents were calculated from pH and total CO_2 according to the Henderson-Hasselbalch equation using values for CO_2 solubility and for pK at the appropriate temperature from Boutilier et al. (1986). The validity of this approach has previously been documented (Grosell et al. 1999b).

Na/K-ATPase enzyme activity was measured in samples of gill filaments and in samples of anterior, mid and posterior intestine by the method of McCormic (1993) modified for microtitre plate reading. Muscle water content was determined by weighing a sample of muscle tissue before and after 24 h drying at 60 °C.

Sub-samples of all sampled tissues were prepared for analysis of silver concentration by adding 5 volumes of 1 N HNO_3 (trace metal grade, Merck chemicals). The samples were left overnight at 75 °C, vortexed and centrifuged. The supernatant and water 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 were set as documented by the manufacturer. The silver analysis was calibrated against standards of known silver concentrations in the appropriate matrix since osmolality of tissue digests, plasma, and water samples were found to differentially interfere with the silver signal.

Cations in plasma samples were analysed by standard atomic absorption spectrophotometry (Varian 1275) again using standard operating conditions. Chloride concentration was analysed using coulometric titration (Radiometer CMT-10). The osmolarity of plasma was measured using a Wescor 5100C vapor pressure osmometer.

Radioactivity of water and plasma samples (50 µl) obtained during the ^{22}Na-flux experiments were determined by liquid scintillation counting at constant quench on a LKB Rackbeta 1017 counter. Prior to counting, all samples were made up to a total volume of 5 ml by addition of seawater, to which 10 ml ACS fluor (Amersham) was added.

Na^+ efflux rates were calculated by relating the appearance of radioactivity in the water to the mean specific activity of ^{22}Na in the plasma over the duration of the flux period, the fish weight, the volume of the exposure containers and the flux time elapsed. Specific activity of the plasma was calculated from total Na^+ concentrations and ^{22}Na radioactivity measurements. In most cases, the calculations were based on the two 1-h flux periods combined.

Data presentation and statistical evaluation

All values are expressed as means ± SEM (n). Significant differences from control fish, from sham-operated fish and between perfused silver-exposed and non-perfused silver exposed fish were evaluated using an unpaired students t-test (two tailed). In all cases a limit for significance of $P < 0.05$ was applied. For the presentation of silver concentrations in various tissues, the control group and the perfused control group were pooled and presented as one value since they, as expected, showed no differences in silver concentration.

Results

Plasma electrolytes

The control fish exhibited constant levels of Na^+, Cl^-, Mg^{2+}, Ca^{2+}, HCO_3^- and osmolality throughout the 96-h experimentation period (Fig. 1A–E and Table 1). Silver exposure resulted in a gradual increase in the plasma concentration of Na^+, Cl^- and the plasma osmolality to values significantly higher than the corresponding control values after 48–72 h and onwards. Silver exposure

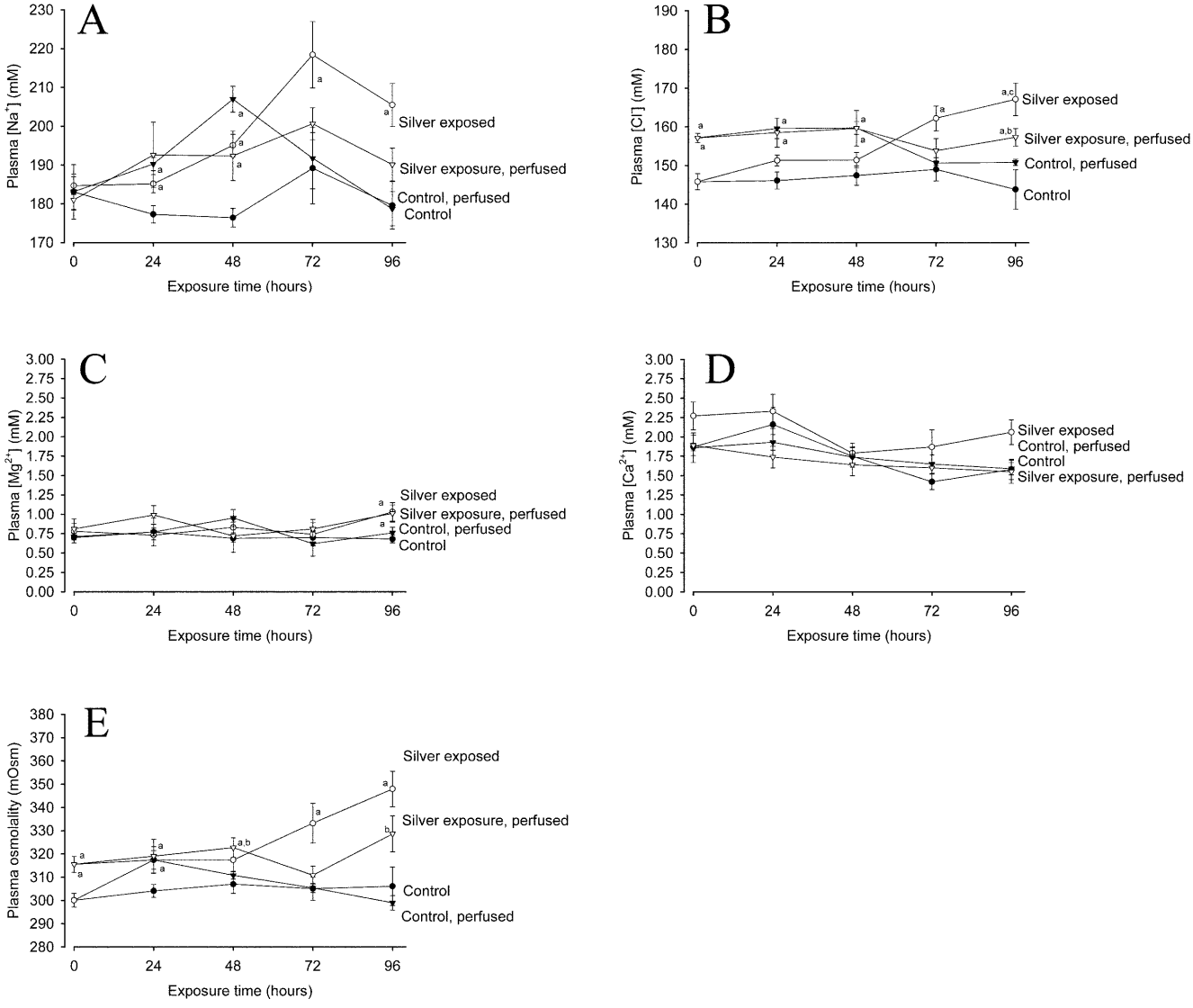

Fig. 1 **A** Na^+, **B** Cl^-, **C** Mg^{2+} and **D** Ca^{2+} concentrations (all in mM) and **E** osmolality (in mosmol) in plasma samples obtained via caudal artery catheters from lemon sole during 96 h experimentation. Control fish are shown with *filled circles* ($n=7$), silver-exposed fish are shown with *open circles* ($n=7$), perfused control fish are shown with *filled triangles* ($n=5$) and perfused silver-exposed fish are shown with *open triangles* ($n=6$). Means ± SEM. *a*, statistically significant difference from control fish at the same time; *b*, statistically significant difference from the "sham control" (perfused, non-exposed); *c*, statistically significant difference from perfused, silver-exposed fish (two-tailed students *t*-test, $P<0.05$)

did not affect plasma Ca^{2+} concentrations but resulted in a slight yet significant increase in plasma Mg^{2+} concentrations after 96 h exposure (Fig. 1A–E). Similarly, HCO_3^- was elevated in plasma samples from the silver-exposed fish after 96 h (Table 1).

Perfusion of the intestine during the recovery period following extensive surgery did not affect the plasma concentration of any of the measured cations at the pre-exposure control sampling time, 0 h (Fig. 1A, C, D). Plasma Na^+ concentrations in the perfused non-exposed fish increased over the first 48 h but then gradually recovered to control values after 96 h experimentation. The Cl^- concentration and the plasma osmolality in the two perfused groups, however, were slightly (yet significantly) elevated compared to the non-perfused fish at the start of the experimentation period. Comparing the control and the perfused control group, the fish recovered from this apparent effect of perfusion and/or surgery after 48 h and 72 h, for Cl^- and osmolality, respectively (Fig. 1B, E). The plasma HCO_3^- concentrations tended to remain lower in the perfused groups than in the non-perfused groups, an effect which was statistically significant after 24 h and 72 h experimentation (Table 1).

Blood pH, PCO_2 and haematocrit

Blood pH was not affected by silver exposure. At 96 h, the non-perfused, silver-exposed fish exhibited a parallel rise in HCO_3^- and PCO_2 indicating respiratory acidosis. In concordance with the observed reduction in plasma

Table 1 Blood pH, plasma [HCO_3^-], PCO_2 (torr) and haematocrit of lemon sole during 96 h experimentation. $n = 7$, 7, 5 and 6 for the controls, the silver-exposed (4.07 M silver), the perfused controls and the perfused, silver-exposed fish (4.07 M silver) respectively. Means ± SEM

Treatment		0 h (control)	24 h	48 h	72	96 h
Control	pH	7.869 ± 0.061	7.764 ± 0.132	7.967 ± 0.042	7.970 ± 0.015	7.934 ± 0.027
Silver exposed		7.776 ± 0.056	7.896 ± 0.018	7.991 ± 0.030	7.872 ± 0.068	7.816 ± 0.103
Control perfused		7.978 ± 0.018	7.807 ± 0.025	7.763 ± 0.038[a]	7.790 ± 0.029[a]	7.770 ± 0.015[a]
Silver exposed, perfused		7.793 ± 0.053	7.834 ± 0.031	7.791 ± 0.085[a]	7.782 ± 0.057[a]	7.775 ± 0.108
Control	HCO_3^- (mM)	4.52 ± 0.26	4.58 ± 0.33	4.15 ± 0.36	4.93 ± 0.25	3.84 ± 0.67
Silver exposed		4.10 ± 0.49	3.98 ± 0.52	5.03 ± 0.70	3.96 ± 0.75	5.81 ± 0.34
Control perfused		4.00 ± 0.52	2.94 ± 0.25[a]	4.17 ± 0.34	3.33 ± 0.30[a]	2.60 ± 0.21
Silver exposed, perfused		4.24 ± 0.19	3.24 ± 0.23[a]	3.53 ± 0.47	3.15 ± 0.46[a]	2.38 ± 0.85
Control	PCO_2 (torr)	2.83 ± 0.73	3.16 ± 0.90	1.63 ± 0.53	1.58 ± 0.11	1.56 ± 0.30
Silver exposed		3.62 ± 0.85	1.81 ± 0.29	1.49 ± 0.31	1.62 ± 0.61	5.12 ± 1.90a
Control perfused		1.23 ± 0.16	2.07 ± 0.28	3.58 ± 0.37[a]	2.62 ± 0.48[a]	2.11 ± 0.08
Silver exposed, perfused		3.60 ± 0.79	2.01 ± 0.23	2.79 ± 0.55	2.49 ± 0.41[a]	1.79 ± 0.45
Control	Haematocrit (%)	11.8 ± 0.9	9.7 ± 1.0	9.2 ± 0.9	7.4 ± 1.2	7.9 ± 1.2
Silver exposed		12.1 ± 0.6	9.6 ± 0.6	9.4 ± 0.8	8.6 ± 0.9	8.4 ± 1.3
Control perfused		14.1 ± 1.1	12.4 ± 1.3	10.5 ± 1.2	10.0 ± 0.9	8.9 ± 0.7
Silver exposed, perfused		12.4 ± 1.0	12.6 ± 1.4	9.5 ± 1.8	10.4 ± 2.9	7.2 ± 1.4

[a]Statistically significant difference from control fish at the same time (two-tailed students *t*-test, $P < 0.05$)

HCO_3^- concentrations and increased PCO_2, it appeared that the surgery and/or the perfusion caused a statistically significant acidosis around 48–72 h experimentation (Table 1).

Haematocrit levels (Hct) were similar amongst all groups throughout the 96 h but showed a gradual decline over time with progressive blood sampling (Table 1).

Muscle water content

Water content in white muscle tissue samples obtained at the end of the 96 h exposure was 80.19 ± 0.46% (control fish), 79.05 ± 0.76% (silver-exposed fish), 81.40 ± 0.91% (perfused, non-exposed fish) and 79.95 ± 0.28% (perfused, exposed fish). While none of these values were statistically significant, it is worth noting that the mean muscle water content of the silver-exposed fish was the lowest followed by the silver-exposed, perfused group.

Unidirectional Na^+ efflux and Na/K-ATPase activity

Control Na^+ efflux rates averaged around 20 mmol·kg^{-1}·h^{-1} (Fig. 2). Silver exposure gradually decreased this Na^+ efflux rate to approximately 50% after 48-h exposure after which it remained relatively constant. The perfused fish exhibited lower control Na^+ efflux rates than the non-perfused fish. While the Na^+ efflux rates gradually recovered to control levels in the perfused non-exposed fish, they remained low and decreased slightly in the silver-exposed perfused fish. These observations were paralleled by the Na/K-ATPase enzyme activity in gill tissue obtained from the very same fish at the end of the 96-h experiment (Table 2). While silver exposure did not reduce branchial Na/K-ATPase activity in a statistically significant manner, the mean values of silver-exposed fish were approximately 60% of the corresponding control values. Plotting individual values of branchial Na^+ efflux rates as a function of corresponding values of branchial Na/K-ATPase activity revealed no significant correlation (Fig. 3A). Individual Na^+ efflux rates (Fig. 3B) however, were inversely correlated to branchial silver concentrations. Na/K-ATPase activity levels did not correlate with the corresponding branchial silver concentrations (data not shown).

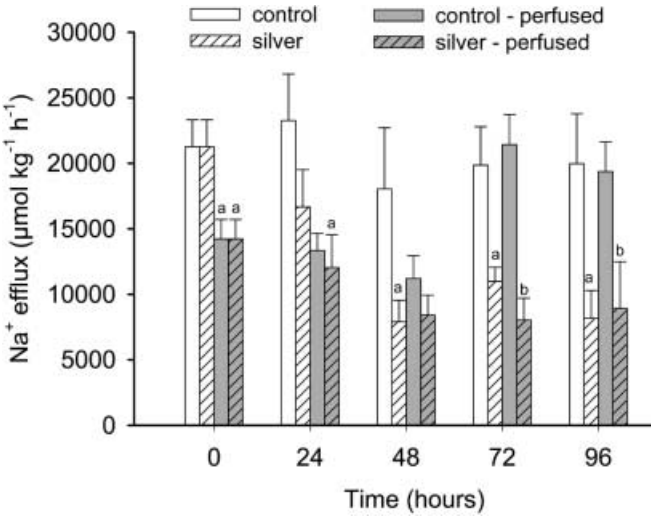

Fig. 2 Unidirectional Na^+ efflux (µmol·kg^{-1}·h^{-1}) in lemon sole during 96 h experimentation. Control fish are shown with *open bars* ($n = 7$), silver exposed fish are shown with *hatched open bars* ($n = 7$), perfused control fish are shown with *filled bars* ($n = 5$) and perfused silver-exposed fish are shown with *hatched filled bars* ($n = 6$). Means ± SEM. *a*, statistically significant difference from control fish at the same time; *b*, significant difference from sham control (two-tailed students *t*-test, $P < 0.05$)

Table 2 Na/K-ATPase activity (mol ADP·g protein^{-1}·h^{-1}) in gills, and anterior, mid and posterior intestine after 96 h experimentation. Means ± SEM. No statistically significant differences were observed (two-tailed students *t*-test, $P < 0.05$). $n = 7$, 7, 5 and 6 for the controls, the silver exposed, the perfused controls and the perfused, silver exposed fish respectively

Treatment	Gill	Anterior intestine	Mid intestine	Posterior intestine
Control	13.51 ± 4.17	46.32 ± 6.57	37.05 ± 3.72	32.99 ± 7.35
Silver exposed	8.98 ± 4.35	53.90 ± 7.85	43.99 ± 5.80	32.37 ± 10.63
Control perfused	14.70 ± 1.23	62.30 ± 9.88	48.69 ± 4.49	43.52 ± 6.63
Silver exposed, perfused	8.37 ± 2.70	53.47 ± 9.30	45.63 ± 8.33	33.83 ± 13.07

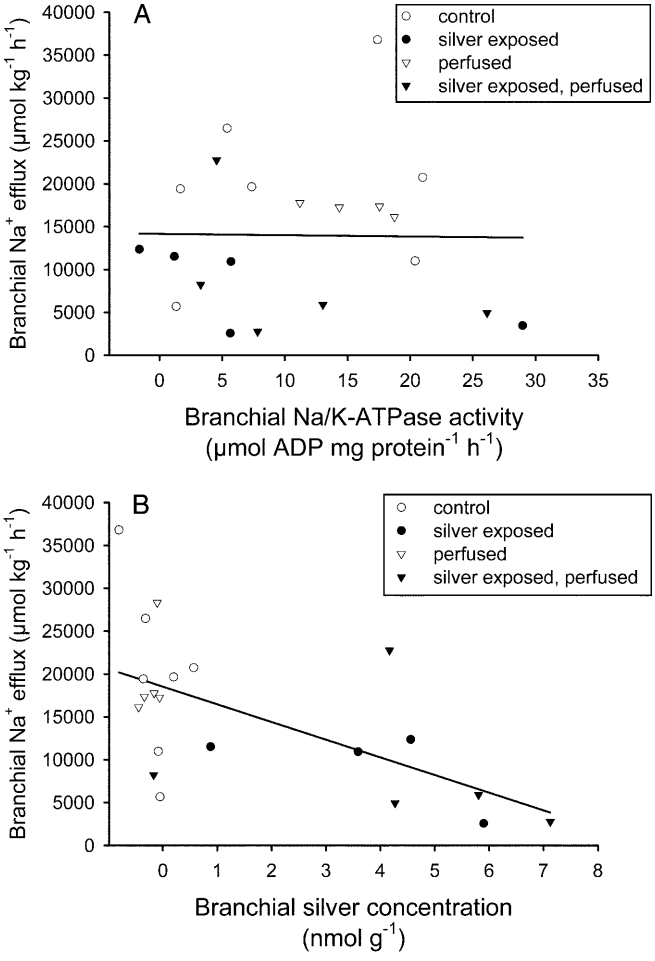

Fig. 3 **A** Plot of individual rates of branchial Na^+ efflux as a function of individual values of branchial Na/K-ATPase levels. **B** Plot of individual rates of branchial Na^+ efflux as a function of individual concentrations of branchial silver. *Open symbols* represent non-exposed fish, *filled symbols* represent silver-exposed fish, *circles* represent non-perfused fish and *triangles* represent perfused fish. There was no significant correlation between Na^+ efflux rates and Na/K-ATPase activity. Branchial Na^+ efflux rates were inversely correlated to the branchial silver concentrations by the following equation: Na^+ efflux rate = 18526.5–18.7523×(silver concentration), slope significantly different from 0 ($P < 0.05$), $R^2 = 35.78\%$

Tissue silver levels

Background silver levels in all investigated tissue samples from non-exposed fish were very low (< 1 nmol·g^{-1}; Fig. 4). In all tissues but the white muscle, silver exposure caused a marked increase in tissue silver levels to the range of 2–10 nmol·g^{-1}. In the gill, liver and kidney (from both the non-perfused and perfused fish) silver exposure resulted in similar levels of silver. In contrast, the perfused fish accumulated much less silver (approximately 5%) in all intestinal segments than the corresponding non-perfused fish. The low silver accumulation in these perfused fish is, however, still statistically significantly higher than in the non-exposed control fish. Note also that silver accumulation was about 1.5-times higher in the posterior intestine than in the anterior and mid intestine (Fig. 4).

Discussion

Physiological effects of silver exposure

A comparison, first, of the non-perfused control fish and the fish exposed to 4.1 µM (= 448 µg·l^{-1}) silver reveals osmoregulatory disturbance since plasma Cl^- and Na^+ concentrations and plasma osmolality all gradually increased in the silver-exposed fish over the 96 h exposure. This is in agreement with observations from the starry flounder exposed to 9.1 µM silver, presented by Hogstrand et al. (1999).

Blood pH did not change significantly during silver exposure but mean values tended to be lower in silver-exposed fish compared to control fish. Plasma HCO_3^- concentration was slightly elevated after 96 h exposure. This in combination with the increased PCO_2 indicates respiratory acidosis after 96 h silver exposure. These observations are in general agreement with reports from Hogstrand and co-workers (1999), who showed a similar acidosis after exposure to 9.1 µM silver but no effect of exposure to 2.3 µM. Such an acidosis could be caused by inhibition of carbonic anhydrase as previously reported for silver-exposed freshwater rainbow trout (Morgan et al. 1997).

Silver exposure did not reduce intestinal Na/K-ATPase activity in any of the three segments investigated. In contrast, the mean values of activity in the anterior and mid segment of the intestine tended to be higher than in the corresponding controls. These observations, even though not statistically significant, are in perfect agreement with previous observations from the same species exposed to a similar silver concentration (Grosell

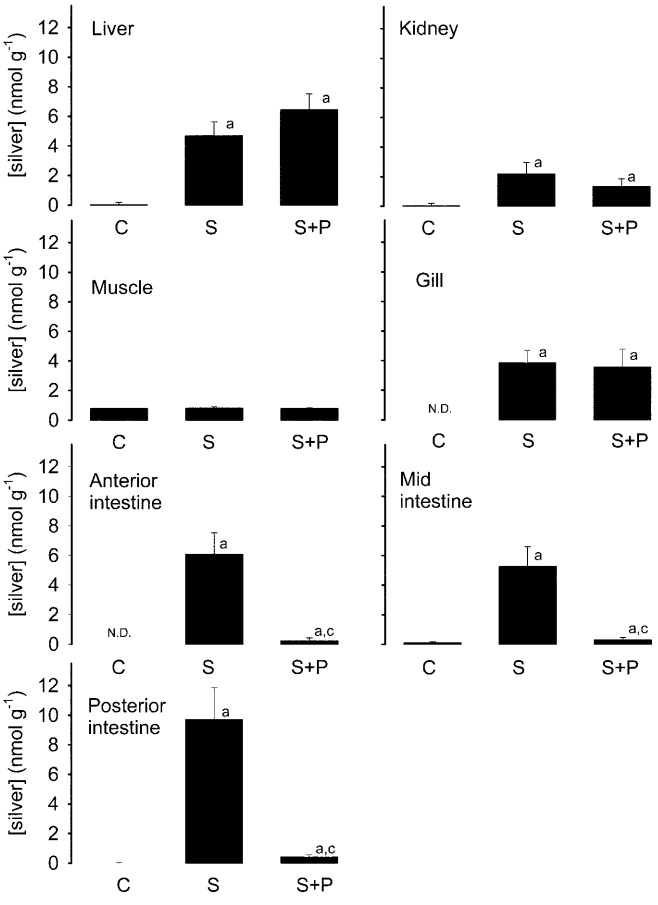

Fig. 4 Silver concentration in liver, kidney, muscle, gill, anterior intestine, mid intestine and posterior intestine obtained from lemon sole after 96 h of experimentation. *Bars labelled* "*C*" show the control and perfused control groups (pooled), "*S*" shows the silver exposed fish and "*S*+*P*" shows the perfused, silver exposed fish. Means ± SEM. *a*, statistically significant difference from pooled control fish; *c*, statistically significant difference between silver exposed and perfused silver exposed (two-tailed students *t*-test, $P < 0.05$)

et al. 1999b) and from the starry flounder (Hogstrand et al. 1999). This lack of reduction (or trend to a slight increase) in Na/K-ATPase activity in intestinal tissue is surprising considering the substantial silver accumulation in this tissue both in the present study and in the study by Hogstand et al. (1999).

We have previously shown that silver exposure leads to substantial silver concentration in the intestinal fluids of the lemon sole. In this same species, applying a radioisotope approach we have also shown reduced intestinal Cl^- uptake across isolated segments and by using in situ perfusion of the intestine we found a reduced net intestinal transport of Na^+ and Cl^- in fish exposed to silver (Grosell et al. 1999b). Cl^- uptake across the intestinal epithelium is (at least partly) linked to Na^+ uptake and thus the activity of the Na/K-ATPase in the basolateral membrane of the epithelial cells (see Loretz 1995 for a review). It thus seems contradictory that there was no effect of silver exposure on measured maximal Na/K-ATPase activity in the intestinal epithelium, but reduced Cl^- uptake in vitro, reduced Na^+ and Cl^- uptake during in-situ perfusion of the intestine and obvious effects of silver on the gastro-intestinal contribution to whole body ion regulation in vivo (see below).

One possible explanation for these contradictory observations could be related to the enzymatic assay. The assay procedure is designed to provide optimal conditions for function of this particular enzyme and involves substantial dilution of the intestinal cells in silver-free homogenization and assay buffers (McCormic 1993). This dilution together with the homogenization may alter any interaction between the enzyme and silver in the intact intestinal tissues, thereby masking the effect of silver on the Na/K-ATPase activity in vivo.

Another possible explanation, at least for reduced Cl^- uptake, could be inhibition of the cytosolic carbonic anhydrase. Carbonic anhydrase has previously been shown to be susceptible to silver exposure at least in freshwater fish (Morgan et al. 1997). We have recently proposed a model for active HCO_3^- secretion and Cl^- uptake via the Cl^-/HCO_3^- exchanger in the intestinal epithelium of marine teleosts (Grosell et al. 2001). This system relies on a basolateral proton pump and cytosolic carbonic anhydrase. Inhibition of this cytosolic carbonic anhydrase would impair both intestinal HCO_3^- secretion and Cl^- uptake. In support of this hypothesis are previously reported findings showing that the total inhibition of Cl^- transport by the intestinal epithelium exceeds the inhibition of Na^+ transport but matches the sum of inhibition of Na^+ and HCO_3^- transport (Grosell et al. 1999b). While carbonic anhydrase inhibition could be part of the explanation for reduced intestinal Cl^- transport in marine teleosts, it can, however, not explain reduced Na^+ transport.

Even though branchial Na/K-ATPase activities have been reported in studies of marine teleosts exposed to silver (Hogstrand et al. 1999; Grosell et al. 1999b) and copper (Stagg and Shuttleworth 1982) and in the elasmobranch spiny dogfish exposed to silver (De Boeck et al. 2000), this is to our knowledge the first report of branchial Na^+ efflux during metal exposure in marine fish. Control levels of branchial Na^+ efflux are two orders of magnitude higher than branchial Na^+ uptake in most freshwater teleosts, but are in close agreement with values previously reported for other marine flatfish (e.g. Maetz 1969; Wood and Randall 1971). Silver exposure to 4.1 μM resulted in substantial branchial silver accumulation and gradually reduced branchial Na^+ efflux over the 1st 48 h to approximately 50% of the control values after which Na^+ efflux remained constant.

The observed decrease in Na^+ efflux did not correlate with the branchial Na/K-ATPase activity (Fig. 3A). The tendency to lower mean values of Na/K-ATPase activity after silver exposure (Table 2) is in contrast to our previous study (Grosell et al. 1999b) and to the study of Hogstand et al. (1999) both showing increased branchial Na/K-ATPase activity after high level acute silver exposure. A more recent study, however, showed both

increased and decreased branchial Na/K-ATPase activity after lower level chronic silver exposure depending on species, exposure time and silver concentration (Webb et al. 2000). The reasons for the above differences are unknown. One strength of the present study is the branchial Na^+ efflux measurement and the terminal Na/K-ATPase activity measurements in the same animals. While the rates of Na^+ efflux correlated well with the branchial silver concentrations, there was no correlation between branchial Na/K-ATPase activity and branchial silver concentration. These observations suggest that the branchial Na^+ efflux rather than the branchial Na/K-ATPase could be a more suited endpoint for assessing silver toxicity in marine fish.

Branchial versus intestinal contribution to silver accumulation and toxicity

Evaluation of the experimental procedure

The perfusion clearly influenced the experimental animals. This was most pronounced in plasma HCO_3^- concentrations and blood pH. Also PCO_2 and the major plasma ions appeared to be affected at least initially, the latter also resulting in changes in plasma osmolality. However, in contrast to the disturbance in blood acid-base status which progressively increased throughout the experimental period despite recovery of PCO_2, the plasma ions and osmolality in non-exposed perfused fish recovered to control values at 72 h and 96 h. This perfusion technique has previously been shown to influence both blood acid-base status and plasma electrolyte levels (Grosell and Jensen 2000) underscoring the need for a "sham-control" group.

The reason for the blood acid-base disturbance is probably partly related to the composition of the "gut saline". The applied saline is composed to best mimic the composition of the intestinal fluids of these fish (Grosell et al. 1999b), however, for practical reasons, the HCO_3^- concentration was considerably lower than the corresponding concentration in the intestinal fluids (1 mM as opposed to 30–40 mM). Higher HCO_3^- concentration in the gut saline causes problems due to precipitation of carbonate salts eventually clotting the perfusion catheters. The conclusions regarding branchial and intestinal contribution to silver uptake and toxicity below are not affected by the obvious disturbance to blood acid-base status since they concern ion-regulatory disturbance and silver accumulation.

The disturbance of plasma electrolytes arising from the perfusion technique prevailed until 72 h experimentation at which time the non-exposed perfused fish exhibited plasma electrolyte and osmolality values very similar to the control fish. Apparently, the 24-h recovery period after surgery in the present study was not sufficient to ensure stable and normal plasma electrolyte levels. Consequently, the following discussion and conclusions of the relative contribution of the gills and the intestine to silver-induced disturbance was based on the 72-h and 96-h data.

Branchial versus intestinal contribution to silver accumulation

Silver accumulation in the gill was not affected by intestinal perfusion. This demonstrates that branchial silver accumulation, as could be expected, arises from direct uptake from the water into/onto the gill. More importantly, silver accumulation in the liver and kidney was also not affected by intestinal perfusion. The liver and the kidney are the major internal silver-accumulating organs and are thus good indicators of whole-body silver uptake (Webb and Wood 1998). The lack of effect of intestinal perfusion demonstrates that the branchial route dominates for whole body silver uptake. In fact there was no evidence for any intestinal contribution at all to whole body silver uptake and accumulation.

Intake of silver via drinking did, however, result in highly elevated silver concentrations in the intestinal tissues. Perfusion of the intestine almost completely abolished intestinal silver accumulation demonstrating that silver accumulated in the intestine was derived mainly from the intestinal lumen. The complete lack of intestinal contribution to whole body silver uptake, despite the highly elevated intestinal silver concentrations in the non-perfused silver-exposed fish demonstrates that the basolateral membrane of the intestinal epithelium is the rate-limiting step in intestinal silver uptake. The present conclusion is therefore very different from earlier suggestions that the intestine might provide the dominant route for silver uptake in marine teleosts (Hogstrand et al. 1999; Webb et al. 2000). However, these suggestions were based simply on measurements of net burdens of silver in gill and gut tissue; no quantitative partitioning on internalization was attempted, in contrast to the present study.

The present findings are in agreement with observations of copper accumulation in dietary exposed freshwater rainbow trout showing very high copper concentrations in the intestinal tissue but only slightly elevated internal copper concentrations (Kamunde et al. 2000).

This observation of a strongly dominant branchial route of silver uptake is in marked contrast to the sites of silver toxicity discussed below.

Branchial versus intestinal contribution to silver toxicity

The major effect of silver exposure in the lemon sole was disturbance of Na^+ and Cl^- balance. This effect translated into a parallel disturbance of plasma osmolality. Considering only the 96-h values (for reasons outlined above) of the three above parameters, it appears that at least 50% of the silver-induced disturbance occurs at or in the intestinal transport processes. This conclusion is

based on 50% or less reduced ion-regulatory disturbance in the intestinally perfused, silver-exposed fish compared to the corresponding non-perfused fish.

An additional effect of silver exposure was the slight disturbance of plasma Mg^{2+} levels. Unlike the disturbance of plasma Na^+ and Cl^- levels, this effect on plasma Mg^{2+} was not affected by intestinal perfusion, demonstrating that it was strictly of branchial origin. It likely reflected an elevation of gill permeability caused by water-borne silver.

While the intestine appears to be a very important site of silver toxicity in this marine teleost it is also evident that branchial ion extrusion and general permeability can be affected by acute silver exposure. While the present experimental protocol was suited to estimate the relative importance of the intestine to silver toxicity, it disregards the potential inhibition of drinking which has been reported from teleosts during silver exposure (Grosell et al. 1999b; Hogstrand et al. 1999; Webb et al. 2000). In the non-perfused fish, the physiological disturbance includes any reduced drinking which might have occurred in response to silver exposure. Such a response is completely bypassed in the intestinally perfused fish where saline is pumped through the intestine at a constant rate. Reduced drinking could well result in dehydration and thus increased plasma ion levels as seen in the present study. By perfusing the intestine in the present study, we protected against the direct effect of silver on the intestinal epithelium as intended, but also eliminated any effect of silver-induced reduction of drinking. Thus, if anything, we have underestimated the gastro-intestinal contribution to the ionoregulatory toxicity caused by water-borne silver because we supplied the intestine with excess gut fluid in a situation where water intake might have been reduced.

In summary, we can state that the direct contribution of the intestine to silver-induced ion-regulatory disturbance was at least 50%, but the gastro-intestinal contribution (including a drinking rate response) may well have been higher leaving the branchial contribution lower than simply the difference between total and intestinal silver effects. These conclusions are similar to findings of approximately 60–70% intestinal contribution to NO_2 uptake and effects in the European flounder (*Platicthys flesus*; Grosell and Jensen 2000). Furthermore, ongoing studies suggest that copper toxicity in the marine gulf toadfish (*Opsanus beta*) may also have a considerable intestinal component (M. Grosell, M.D. McDonald, C.M. Wood and P.J. Walsh, unpublished observations).

In conclusion, it appears that silver exerts its toxic effect at two different sites, the intestine and the gill, in marine teleost fish. The major effect is disturbance of Na^+ and Cl^- balance. Disregarding possible effects of silver exposure on drinking rate, it seems that the two sites were of comparable importance to whole body ion-regulatory disturbance. This is in marked contrast to the relative importance of these two routes of silver uptake and accumulation. Branchial silver uptake was by far the dominant contribution to internal silver accumulation. However, during acute exposure of this nature, it is ionoregulatory disturbance, not internal silver accumulation, that kills the fish (Hogstrand et al. 1999). During chronic low-level silver exposures, internal silver accumulation appears to be subject to physiological regulation (Webb et al. 2000). In future work, it will be of interest to determine whether this physiological regulation of silver accumulation occurs at the gills or elsewhere, and whether the bio-accumulation itself causes any toxicity.

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