The effects of silver on intestinal ion and acid-base regulation in the marine teleost fish, *Parophrys vetulus*

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Abstract

Exposure to elevated silver (as $AgNO_3$) concentrations (6–9 μM) in seawater was associated with comparably high silver concentrations in the intestinal fluids of the lemon sole (*Parophrys vetulus*), and a tendency for reduced drinking rate. The effects of silver on intestinal ion and acid-base regulation were studied using in situ perfusion of the intestine. Intestinal net Cl^- uptake was reduced from 0.4 to 0.1 and intestinal net Na^+ uptake from 0.2 to 0 mmol kg^{-1} h^{-1} during silver exposure (9 μM). At the same time, intestinal HCO_3^- net efflux was reduced from 0.2 to 0.1 mmol kg^{-1} h^{-1}. Both intestinal Na^+ and Cl^- uptake and Cl^-/HCO_3^- exchange are thus sensitive to silver, but to different extents. None of the observed effects were reversible during 24 h of recovery. Intestinal water transport was highly variable in vivo in the perfused preparation, and no significant effect of silver exposure was observed. However, in vitro intestine preparations exhibited reduction of intestinal net water flux from 4 to 1 μl cm^{-2} h^{-1} during silver exposure together with reduced unidirectional Cl^- influx. Reduced water intake and transepithelial water transport in silver-exposed fish resulted in moderate hemoconcentration evident from higher hematocrit values, but not in increased plasma ion levels. The latter could reflect a compensatory response via increased branchial Na^+/K^+-ATPase levels, observed in silver-exposed fish, indicative of increased branchial ion transport capacity. Impairment of intestinal ion and water transport as a result of silver intake via drinking could be an important part of the fatal cascade of physiological effects observed in marine fish during acute silver exposure. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Silver toxicity; Intestinal; Ion transport; Acid-base regulation; Marine teleost

1. Introduction

Silver is released to the environment by natural erosion, mining and photographic processing, and is discharged in variable amounts in the effluent of sewage treatment plants [12]. Waterborne silver toxicity has received increasing attention over the last decade (reviewed by Hogstrand and Wood [5] and Wood et al. [23]). Silver, when present in the form of free Ag^+, is potentially one of the most toxic metals to freshwater fish, but complexation by naturally occurring ligands (e.g. chloride, dissolved organic matter, sulfide) greatly attenuates toxicity in most natural waters. Silver toxicity to marine fish is not well described. While the toxic mechanisms for Ag^+ toxicity to freshwater fish are relatively well understood [10,19,22], only a few studies address the physiology of silver toxicity to marine fish [4,14,18]. Because of the high Cl^- concentration in seawater, Ag^+ is virtually non-existent in the marine environment and all silver exists in the form of various silver chloride complexes. Acute silver toxicity is much lower (requiring higher silver concentration) to marine fish than it is to freshwater fish — a difference of several orders of magnitude [5,14], but information about the toxic mechanism(s) of action in marine fish is still required for the establishment of sensible environmental regulations.

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While the site of silver uptake, and also the key target for Ag^+ toxicity in freshwater fish, is the gill (inhibition of active Na^+ and Cl^- uptake) [10], recent evidence suggests that the gastro-intestinal tract rather than the gill could be a more important route of silver uptake in marine fish [2,4,18]. Marine fish rely on drinking to compensate for the osmotic loss of water to their hyperosmotic environment [16], and intake of silver-contaminated water thus poses the potential for silver uptake by the gastro-intestinal tract. Recently, Grosell and Hansen [2] showed that during 72 h of silver (as $AgNO_3$) exposure, the gills of freshwater eels accounted for 60–80% of the whole body burden, while the gills of seawater eels accounted for less than 5%. The gastro-intestinal tract of the seawater adapted eels, however, accounted for as much as 20–30% of the whole body burden compared to only a few percent in freshwater eels. It thus appears that the gastro-intestinal tract could be an important route of silver uptake in marine fish during waterborne exposure.

In marine fish, water is absorbed from the gastro-intestinal tract by active transport of Na^+ and Cl^- into the body [16]. In addition, there is a substantial HCO_3^- secretion and excretion via the intestinal tract, processes that have been suggested to play a role in acid-base regulation [20]. The main transport enzyme responsible for transepithelial Na^+ and Cl^- transport is the Na/K-ATPase [13]. The branchial Na/K-ATPase activity of freshwater fish is highly sensitive to Ag^+ [10], and inhibition of the Na/K-ATPase activity is believed to be the key target for Ag^+ toxicity in freshwater fish. Ag^+ also interferes with acid-base regulation in freshwater teleost fish [19,22] and inhibits carbonic anhydrase in the gills [10], an enzyme which plays a key role in acid-base regulation. Since marine fish accumulate silver in the intestinal tract [2], similar inhibitions of the intestinal Na/K-ATPase activity and/or carbonic anhydrase activity could impair absorption of water across the intestinal epithelium leading to dehydration, or could impair acid-base regulation.

The aim of the present investigation on the lemon sole (*Parophrys vetulus*) was to describe the possible effects of silver intake via drinking on intestinal ion and water transport. To pursue this aim we conducted a series of in vivo experiments to first establish that water-borne silver exposure did in fact lead to silver intake via drinking. Subsequently, the effects of silver on intestinal ion and acid-base regulation were investigated using an in situ perfusion of the entire intestine on the intact fish, a modification of the technique of Wilson et al. [20]. Finally an in vitro approach using sacs made from freshly isolated segments of intestine was applied to describe any effect on intestinal water absorption. All presented experiments were conducted at a nominal silver concentration of 1000 μg l^{-1} equal to 9.1 μM. Pilot experiments revealed no significant

effects of 2.3 and 4.6 μM. This is in agreement with the study of Hogstrand and Wood [4] on another marine flatfish, the starry flounder (*Platichthys stellatus*), which showed negligible effects on physiology at 2.3 μM, but definite impact and partial mortality over 6 days at 9.1 μM. The total silver level exerting a comparable toxic effect in freshwater would be only 0.05–0.6 μM [5]. To put these values in environmental perspective, the US EPA (1980) acute criteria for the protection of marine aquatic life is 2.3 μg l^{-1}, or 0.02 μM.

2. Materials and methods

Lemon sole (*P. vetulus*) were obtained by otter trawls in the vicinity of Bamfield, BC, Canada in the summer of 1997 and held in 400-l fiberglass tanks with a flow-through (5 l min^{-1}) of aerated Bamfield marine station seawater (14°C, salinity = 30 ppt) 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. All exposures were performed using $AgNO_3$ (Fisher, analytical grade.)

2.1. In vivo experiments: drinking rate

Drinking rate was determined in non-exposed fish and in fish exposed to silver (9.1 μmol Ag l^{-1}) in Bamfield Marine Station seawater (14°C) for 96 h (static, 12-h renewal) prior to and during the measurement period by the use of 5 μCi [^{3}H]polyethylene glycol-4000 (PEG-4000) l^{-1} (specific activity: 2050 μCi g^{-1}; NEN-Dupont). Control (10) and silver-exposed fish (7) with a mean weight of 80 g (range 39–108 g) and 79 g (range 41–105 g), respectively, were transferred to 8-l PVC containers containing seawater of the appropriate composition at least 8 h prior to the experiments. The containers were kept covered to minimize stress. Most of the water was carefully removed and renewed by siphoning immediately prior to the 6 h of PEG-4000 exposure. Reference water samples (5 ml) were taken in triplicate at 0.25, 3 and 6 h for radioactivity measurements. At the end of the PEG-4000 exposure (6 h), the fish were killed by an overdose of MS-222 (0.2 g l^{-1}) from a neutralized stock solution and were carefully removed from the exposure tanks and rinsed briefly in seawater without PEG-4000. From each fish, a blood sample was obtained by caudal puncture and plasma was obtained by centrifugation at $10\,000 \times g$ for 2 min. Subsequently, rectal fluid was sampled with a syringe fitted with a short length of polyethylene tubing (PE160). One side of the buccal cavity was removed to gain access to the gastro-intestinal tract (GIT). The GIT was then clamped at the start of the esophagus and immediately anterior to the anus to prevent loss of contents, carefully removed and

weighed. The entire GIT was homogenized in 4 vol. of 10% perchloric acid. An aliquot of the homogenate was centrifuged ($500 \times g$ for 5 min). From each fish, aliquots of 0.5, 1.0 and 1.5 ml of the clear-protein free supernatant, 50 µl plasma and 50 µl of rectal fluid were prepared for determination of β-radioactivity as described below. Drinking rate was calculated by relating the counts from the GIT to the counts from the reference water samples, the weight of the individual fish and the PEG-4000 exposure time (cf. Ref. [20]).

2.2. In vivo experiments: water-borne exposure

A total of five fish (mean weight 298 g, range 58–790 g) were exposed to silver (6.72 ± 0.42 µM, $n = 23$) for 96 h (static, 12-h renewal), while nine non-exposed fish (mean weight 214 g, range: 74–910 g) served as controls for branchial and intestinal Na/K-ATPase activity.

To evaluate whether intake of silver-contaminated seawater by drinking resulted in elevated silver concentrations in the gastro-intestinal fluids, the fish were killed by an overdose of neutralized MS-222 (0.2 g l^{-1}), samples of rectal fluid were obtained as described above and the body cavity was exposed by removing the upper side of the buccal-opercular apparatus. The GIT was then ligated in situ at the pyloric sphincter, posterior to the last pyloric caecae, at the beginning of the posterior part of the intestine (where the gut wall becomes more transparent) and immediately anterior to the anus. The tract was cut at the esophagus and anus and removed from the body cavity. The three areas isolated by the above ligatures (anterior, mid and posterior intestine) were then opened with a longitudinal incision and the fluids were sampled by a Pasteur pipette.

Gill filaments, and segments of the anterior, mid and posterior part of the intestine were obtained by dissection, and plasma was obtained as described above.

The gill filaments and the intestinal segments from the silver-exposed and four of the control fish were frozen immediately in liquid nitrogen, and stored at $-70°C$ for later analysis of Na/K-ATPase activity. Samples of plasma and GIT fluids as well as water samples (acidified with 1% HNO_3^-) were stored at $-20°C$ for later analysis of [Ag] and ionic composition. Samples of GIT fluids from the non-exposed control fish were stored at $-20°C$ for later analysis of ionic composition.

2.3. In situ perfusion of the intestine

Having established that silver was taken in via drinking, leading to high concentrations of silver in the gastro-intestinal fluids, it was considered pertinent to examine potential effects of silver on intestinal ion and acid-base regulation. A total of 13 fish, mean weight 406 g (range 206–825 g) were prepared for in situ perfusion of the intestine. Methods were modified from those of Wilson et al. [20]. Fish were anaesthetised in a 100 mg 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 ml^{-1}) Cortland saline [21] adjusted to 160 mM Na with NaCl for seawater. The catheter was held in place by a sleeve of PE160 secured with two sutures. Once the caudal artery catheter was in place, a single incision was then made, posterior from the pectoral fin to just above the anal opening, exposing the gastro-intestinal tract. Three ligatures were placed under the pyloric end of the stomach, and a small slit was made in the stomach wall anterior to the pyloric sphincter. An inflow catheter (a length of PE160 tubing flared at the tip) was inserted through this slit, past the pyloric sphincter and tied in place with two ligatures leaving the tip of the catheter in the most anterior part of the intestine. One end of a similar but shorter length (10 cm) of PE160 tubing was tied into the esophageal end of the stomach with the last ligature. The latter allowed for external drainage of imbibed seawater, as the stomach was effectively tied off. A wide bore (i.d. ~ 4 mm) outflow catheter was inserted through the anal opening and secured with two ligatures just immediately anterior to the anus. The outflow catheter consisted of a length of modified urethral catheter tubing (size 12 French, elastic rubber; Davol) fitted with a 40-mm sleeve of silicone tubing flared at the end inserted into the fish. The three catheters were anchored externally, the wound was powdered with oxytetracycline and tightly closed by silk ligatures. During all the above surgery great care was taken not to damage the sub-intestinal vein, and following surgery the intestine was carefully flushed with at least 20 ml of gut saline (see below) through the inflow catheter to displace any intestinal fluid and solids. Once revived from the anaesthetic, fish were placed in individual 8-l PVC containers covered with a screen, supplied with flow-through of aerated seawater at a rate of ≥ 200 ml min^{-1}. Infusion of 'gut saline' at a rate of ~ 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 like this overnight prior to experiments.

Each experiment consisted of six consecutive periods of 12 h each. For the silver-exposed fish ($n = 8$), the first two 12-h periods served as internal control. During the following two 12-h periods the 'gut saline' (see below) contained 9.1 µM silver (nominal concentration) and finally the fish were infused with the silver free saline

during the last two 12-h 'recovery' periods. The control fish ($n = 5$) were infused with control saline throughout the six 12-h consecutive periods The control saline used for the gut perfusion consisted of the following (in mM); 99 NaCl, 62.5 MgSO$_4$, 17.5 MgCl$_2$, 5 KCl, 2.5 CaCl$_2$, 1 NaHCO$_3$. This saline was made on the basis of the ionic composition of the GIT fluids (Table 1). For HCO$_3^-$, however, only 1 mM was used in the gut saline in contrast to the 23–36 mM found in the intestine. This was done for practical reasons. Higher HCO$_3^-$ concentrations in the saline caused precipitation of CaCO$_3$ in the peristaltic pump tubing, the inflow catheters and in the intestine leading to blockage of the outflow catheters. During silver exposure, silver was added as AgNO$_3$.

Net intestinal transport of water was calculated by relating the difference between the volume of fluid infused via the inflow catheter and the volume of fluid collected via the outflow catheter to the time of infusion and the weight of the individual fish. Volumes were measured by weighing the inflow and outflow reservoirs at the start and the end of each of the six 12-h periods. Net intestinal transport of Cl$^-$, HCO$_3^-$, Na$^+$, Ca^{2+}, and Mg^{2+} was calculated from the difference between the product of the respective concentrations and the corresponding rates of the inflow and outflow of the gut saline, again related to infusion time and fish weight.

At the end of the second 12-h control period (i.e. 24 h), the end of the second 12-h silver exposure period (i.e. 48 h) and at the end of the 24-h recovery period (i.e. 72 h), a 600-μl blood sample was drawn from the caudal artery catheter into a heparinized syringe. The blood samples were replaced with an equal amount of saline. Blood pH was measured immediately and hematocrit was determined. Plasma was obtained by centrifugation and analysed for total CO$_2$ and a subsample was stored for later analysis of electrolytes.

2.4. In vitro experiments

Intestinal water transport in vivo was highly variable and therefore an in vitro approach was applied to test whether Ag influenced intestinal water transport. Preparations were made from a total of 13 fish, mean weight 165 g (range 108–280 g). Fish were killed by an overdose of neutralized MS-222 as above, and an inflow catheter (PE60 tubing flared at the tip) was inserted at the anterior end of the intestine (as described above), and the intestines were carefully removed by dissection. This catheter was used to flush the intestine with the gut saline to be used in the following experiments. The anterior, mid and posterior parts of the intestine were then separated. Each was fitted with a filling catheter (PE160) at the anterior end and closed at the posterior end by two silk ligatures. Subsequently, these 'gut bags' were filled with gut saline and transferred to glass vials containing 18 ml Cortland saline aerated with a mix of 99.7% O$_2$ and 0.3% CO$_2$. The resulting pH was 7.73 and the resulting total [CO$_2$] was 5.0 mM in the Cortland saline. The glass vials were placed in a water bath maintaining a constant temperature at 14°C. During the experiments, 15 min after transferring the gut bags to the glass vials, the gut saline was replaced with ^{36}Cl-labelled (NEN-Dupont; 0.32 μCi ml^{-1}) silver-free saline (controls, $n = 7$) or silver-containing saline ($n = 6$) (nominal silver concentration of 9.1 μM). Samples (0.5 ml) of the internal gut saline were obtained via the catheters which were then plugged, the gut bags were carefully blotted dry, weighed and again placed in the glass vial. Samples (0.5 ml) of the external Cortland saline were taken at 0, 1, 2 and 3 h, after which the gut bags were blotted dry and reweighed. Finally, a 0.5-ml sample of the internal gut saline was collected from the catheter of each prepara-

Table 1

pH and ionic composition of fluid from the gastro-intestinal tract in non-exposed control *P. vetulus*[a]

Part of GIT	[Cl$^-$]	[Na$^+$]	[K$^+$]	[Ca^{2+}]	[Mg^{2+}]	HCO$_3^-$ equiv.	pH
External seawater	489	420	89	92	48	2.3	7.900
Anterior intestine	135.8 ± 6.9 (5)	68.6 ± 12.4 (5)	7.6 ± 1.5 (3)	4.0 ± 0.4 (4)	97.0 ± 17.0 (4)	22.7 ± 5.3 (8)	7.906 ± 0.149 (8)
Mid intestine	128.9 ± 8.1 (7)	66.5 ± 10.9 (6)	10.5 ± 3.9 (4)	3.9 ± 0.5 (5)	123.0 ± 8.0 (5)	30.1 ± 4.6 (8)	8.264 ± 0.047 (8)*
Posterior intestine	124.0 ± 11.5 (5)	56.5 ± 17.1 (5)	17.4 ± 6.2 (4)	2.5 ± 0.2 (4)*,**2	165.0 ± 5.0 (4)*	36.0 ± 5.3 (8)	8.383 ± 0.042 (8)*
Rectal	103.0 ± 8.7 (6)*	41.8 ± 19.3 (6)	7.1 ± 2.8 (5)	1.8 ± 0.3 (5)*,**	117.0 ± 26.0 (5)	41.9 ± 7.9 (6)*	8.437 ± 0.122 (6)*

[a] Values from anterior, mid, posterior and rectal fluids were compared in an ANOVA followed by Duncan's test.

* Denotes statistically significant difference from the corresponding value in the fluid obtained from the anterior part of the intestine. Mean ± S.E.M. (n).

** Denotes statistically significant difference from the corresponding value in the fluid obtained from the mid part of the intestine. Mean ± S.E.M. (n).
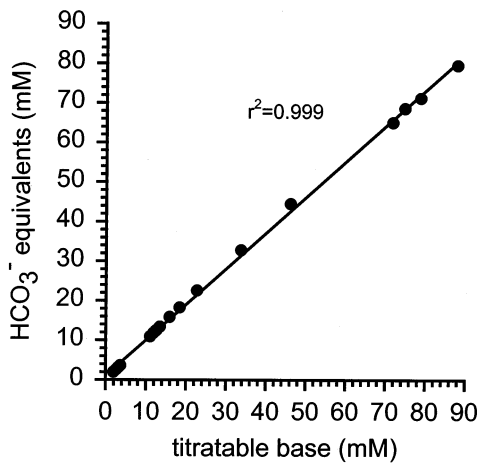
Fig. 1. Correlation between titratable base concentration and calculated HCO_3^- equivalent concentration in 19 randomly selected gut saline samples from the in vitro studies (see text for additional details). Slope of linear regression $= 1.073 \pm 0.034$ ($n = 19$), $r^2 = 0.99$.

tion. The internal exposed surface area of each gut bag was measured using graph paper. Net water transport was calculated from the difference in total mass over the experimental period, with the surface area of each gut bag and time taken into account. The pH and [total CO_2] were measured in the gut saline samples obtained at the start and the end of the experiment. The concentration of HCO_3^- equivalents was calculated from pH and [total CO_2] as described below. The net HCO_3^- equivalent flux was calculated from the increase in HCO_3^- equivalent concentration, gut bag surface area and time. Total [Cl^-] and β-radioactivity from ^{36}Cl was measured in the external Cortland saline samples and the initial and terminal samples of intestinal saline. Unidirectional mucosal to serosal Cl^- fluxes for the three, 1-h periods were calculated by relating appearance of ^{36}Cl radioactivity in the external Cortland saline to the specific ^{36}Cl radioactivity of the internal gut saline (time weighted mean of initial and terminal specific activity), and by taking the corresponding gut bag surface area and time into account.

2.5. Analytical techniques

^3H radioactivity in the water samples, plasma, rectal fluids and homogenized GIT samples from the drinking rate experiments, and ^{36}Cl radioactivity in the samples from the in vitro experiments were determined using liquid scintillation counting on an LKB Rackbeta 1017 counter. Quench correction was performed by the sample channels method and checked by internal standardisation. All samples were made up to a total volume of 5 ml by addition of seawater, to which was added 10 ml ACS fluor (Amersham).

Total silver concentrations was determined in GIT fluids, gut saline, plasma, and water samples by graphite furnace atomic absorption spectroscopy (Varian AA-1275 with GTA-9 atomiser) using a 10-μl injection volume, N_2 gas, and standard operating conditions as documented by the manufacturer. The Ag analysis was calibrated against standards of known [Ag] in the appropriate saline since plasma and GIT osmolarity were found to interfere with Ag light absorbance.

Na/K-ATPase activity in homogenates of gill and intestinal tissue were determined by the method of Holliday [6] as K^+-dependent, ouabain-sensitive, ATP hydrolysis.

Cations in plasma, GIT fluids and gut salines were analysed by standard atomic absorption spectrophotometry (Varian 1275) again using standard operating conditions. [Cl^-] was analysed using either coulometric titration (Radiometer CMT-10) or a colorimetric assay; the two methods were cross-validated.

The pH of plasma and GIT fluids were measured using a micro-capillary pH electrode (Radiometer G279/G2) coupled with a PHM71 meter. The total base content of gut saline samples and GIT fluid samples were analysed using a double titration procedure (cf. Ref. [3]) as modified by Wilson et al. [20]. This was carried out on 50-μl samples diluted in 10 ml of 40 mM NaCl in order to stabilize the pH electrodes. The sample was continuously bubbled with CO_2-free air. The continuously aerated sample was titrated to $< pH$ 4 with 0.02 N HCl in order to remove all HCO_3^- and CO_3^{2-} as gaseous CO_2, and then titrated back to the starting pH using 0.02 N NaOH. Acid and base were added using digital micro-burettes (Gilmont). The difference in number of moles of HCl and NaOH required to return to the starting pH is then equivalent to the number of moles of HCO_3^- equivalents in the original sample. For these titrations, glass combination pH electrodes were used (Radiometer GK2401C or Cole-Palmer combination electrodes, in conjunction with Radiometer PHM82 or Fisher 119 meters). Samples of saline from the in vitro experiments were analysed for pH as described above, and for total CO_2 using a Corning total CO_2 analyser (CMT965, Corning Medical and Scientific). The concentration of HCO_3^- equivalents was calculated according to the Henderson-Hasselbalch equation as $[HCO_3^-] + 2[CO_3^{2-}]$ using values for CO_2 solubility and pK^I and pK^{II} in one-third seawater at the appropriate temperature from Walton Smith [17]. To compare the two methods of [HCO_3^- equivalent] determination, a total of 19 samples were measured using both techniques. This comparison revealed a very good correlation between titratable base and calculated [HCO_3^- equivalent] (Fig. 1; linear regression, slope 1.073 ± 0.034, $r^2 = 0.99$). For simplicity, hereafter in the text, [HCO_3^- equivalent] is referred to as [HCO_3^-], recognising that the HCO_3^- component represents on average 95% of the total [HCO_3^- equivalent].

2.6. Statistical evaluation

All values are expressed as means ± S.E.M. Significant differences between data obtained during silver exposure and the corresponding data obtained from the
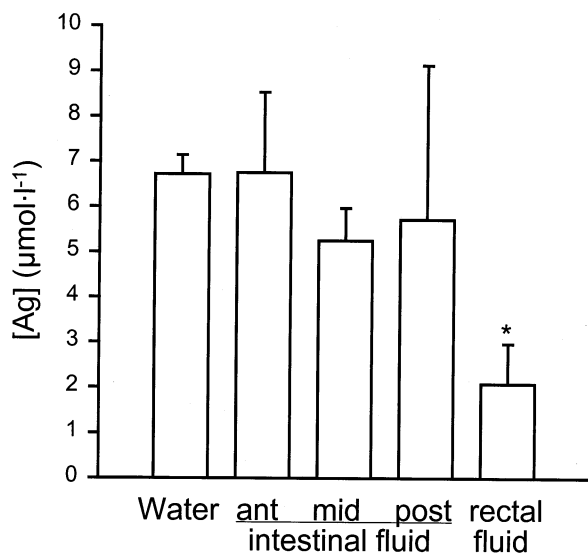

Fig. 2. [Ag] (μM) in ambient water ($n = 23$), and in fluid from the anterior ($n = 3$), mid ($n = 4$) and posterior ($n = 3$) intestine and in rectal fluids ($n = 4$) from the lemon sole after 96 h of silver exposure. Means ± S.E.M. The silver concentration in the rectal fluid was significantly lower than the silver concentration in the water and in the fluid obtained from the anterior part of the intestine, indicated by '*' ($P < 0.05$).
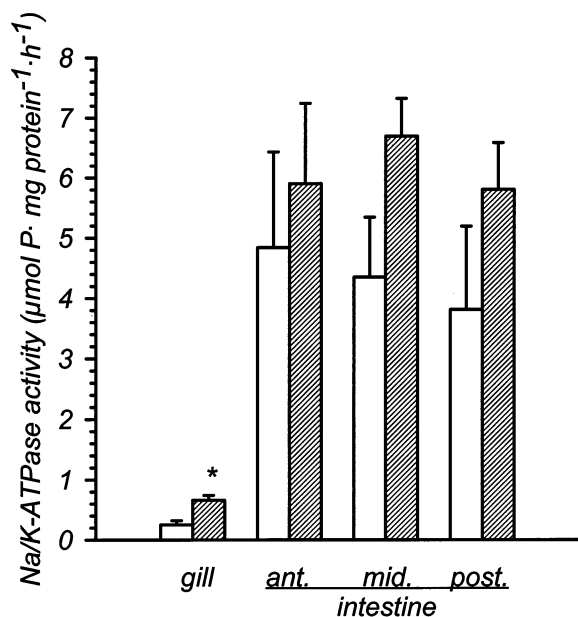
Fig. 3. Na/K-ATPase activity in the gills, the anterior, mid and posterior part of the intestine of control fish (white bars, $n = 4$) and fish exposed for 96 h to waterborne silver (black bars, $n = 5$) in lemon sole. Means ± S.E.M. '*' indicates statistically significant difference from the control ($P < 0.05$).

same experimental fish during the initial control period were evaluated using paired Student's t-test (two-tailed). Differences between the silver-exposed experimental fish and their corresponding parallel controls were evaluated using unpaired Student's t-test (two-tailed). Significant differences from the internal control (paired t-test) are indicated by an 'a' and significant differences from the parallel control (unpaired t-test) are indicated by a 'b' throughout. In all cases, groups were considered significantly different at $P < 0.05$. Values of ionic composition and pH in fluids obtained from the gastro-intestinal tract were compared by an ANOVA followed by a Duncan's multi-sample comparison (see Table 1 for further details).

3. Results

3.1. In vivo experiments

Drinking rates in silver-exposed fish were 0.90 ± 0.36 ml kg^{-1} h^{-1} (7), compared to 1.73 ± 0.38 (10) ml kg^{-1} h^{-1} in the controls. The difference was just below statistical significance ($P < 0.06$) reflecting considerable variation, especially in the silver-exposed fish. Intake of seawater containing silver resulted in relatively high silver concentrations in the gastro-intestinal (GIT) fluids. The silver concentration in the fluid obtained from the anterior part of the intestine was almost identical to the ambient silver concentration (6.75 and 6.72 μmol l^{-1}, respectively). However, there was a tendency for reduction in the concentration of silver in the fluids obtained from the mid and posterior part of the intestine. In the rectal fluids the silver concentration was significantly reduced to 2.08 μmol l^{-1} (Fig. 2) ($P < 0.05$). Na/K-ATPase activity in all parts of the intestine was not significantly affected by in vivo silver-exposure (Fig. 3). Branchial Na/K-ATPase activity was on the other hand significantly higher in the silver-exposed fish compared to the controls ($P < 0.05$) (Fig. 3).

3.2. Ionic composition of GIT fluids

The ionic composition of GIT fluids obtained from the non-exposed control fish is given in Table 1. The [Cl$^-$], [Na$^+$] and [Ca^{2+}] decreased in concentration from 136, 69 and 4 mM in the anterior part of the intestine to 103, 42 and 2 mM, respectively, in the rectal fluids. [K$^+$] and [Mg^{2+}] showed a slight increase in concentration from the anterior to the posterior part of the intestine, but concentrations were decreased in the rectal fluids to levels similar to those of the anterior part of the intestine. The [HCO$_3^-$] concentration was increased from 23 mM in the anterior part of the intestine to 37 mM in the rectal fluids. For statistical details, see Table 1.
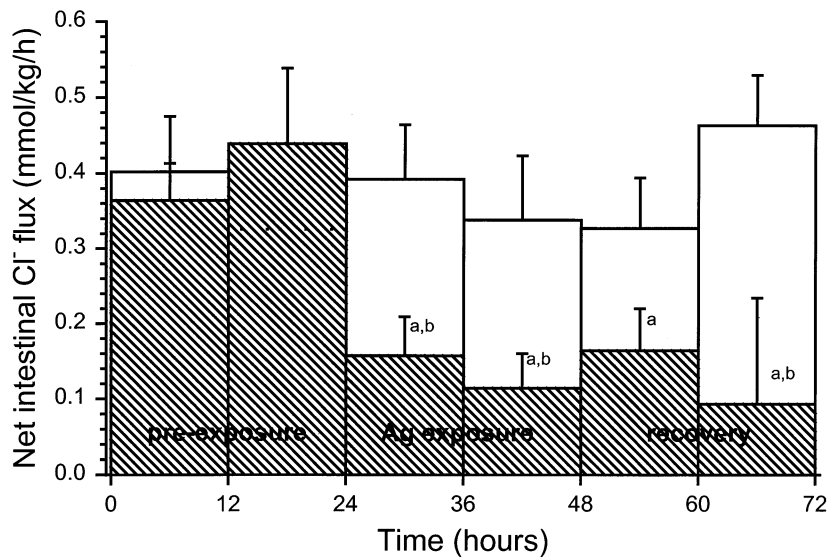
Fig. 4. Net intestinal Cl$^-$ flux (mmol kg^{-1} h^{-1}) in control (white bars, $n = 5$) and silver-exposed (hatched bars, $n = 8$) lemon sole during in situ perfusion: pre-exposure, intestinal silver exposure and recovery. Means ± S.E.M. 'a' denotes statistically significant difference from internal (initial) pre-exposure value ($P < 0.05$) and 'b' denotes statistically significant difference from parallel control values ($P < 0.05$).
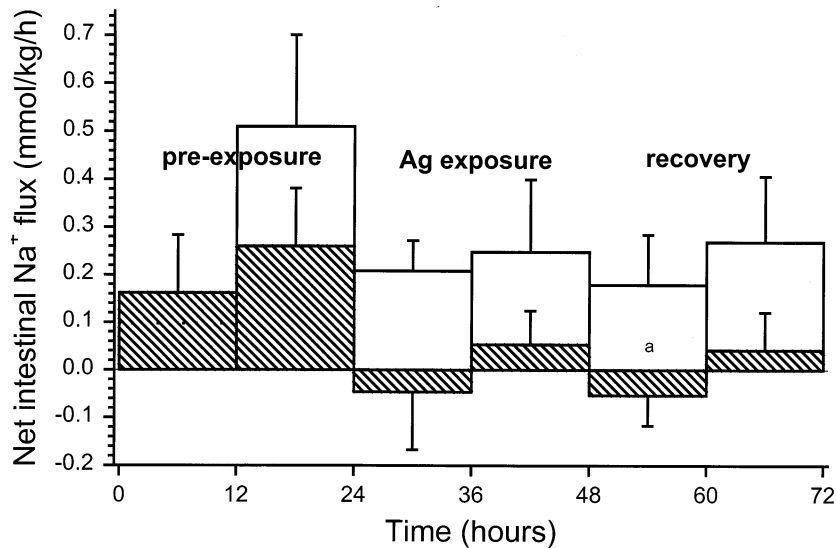

Fig. 5. Net intestinal Na$^+$ flux (mmol kg^{-1} h^{-1}) in control (white bars, $n = 5$) and silver-exposed (hatched bars, $n = 8$) lemon sole during in situ perfusion: pre-exposure, intestinal silver exposure and recovery. Means ± S.E.M. Overall, silver exposure significantly reduced Na$^+$ transport ($P < 0.05$). 'a' denotes statistically significant difference from internal (initial) pre-exposure value ($P < 0.05$) and 'b' denotes statistically significant difference from parallel control values ($P < 0.05$).

3.3. In situ perfusion of the intestine

The unexposed control fish showed constant net fluxes of Cl$^-$, Na$^+$ and HCO$_3^-$ over the entire 72 h of ~ 0.4, 0.2 and -0.2 mmol kg^{-1} h^{-1} (i.e. net efflux of HCO$_3^-$), respectively (Figs. 4–6). Net Cl$^-$ uptake was reduced to ~ 0.1 mmol kg^{-1} h^{-1} during the first 12 h of intestinal exposure to 9.1 μM silver and remained constant during the silver exposure and the following recovery period (Fig. 4). Overall, intestinal net Na$^+$ uptake was completely inhibited by silver exposure and

as for Cl$^-$ there was no recovery during the 24 h after termination of the silver exposure (Fig. 5). The inhibition of Na$^+$ uptake was statistically significant both on an overall basis and during the first 12-h recovery period. Net intestinal HCO$_3^-$ flux was gradually reduced during silver exposure to around -0.1 mmol kg^{-1} h^{-1} and remained at that level during the post-exposure recovery period (Fig. 6). The control fish showed no net transport of either Mg^{2+} or Ca^{2+} during the 72 h of exposure and the intestinal silver exposure had no effect on the net transport of either

ion (data not shown). Despite the marked effects on both Cl^- and Na^+ uptake, silver exposure did not influence intestinal water absorption, which stayed unchanged at ~ 2 ml kg^{-1} h^{-1} in both controls and silver-exposed fish during the 72 h (data not shown). Water absorption rates from both control and silver-exposed fish were highly variable, presumably due to the periodic contractions of the intestine leading to a somewhat pulsatile water outflow.

Intestinal silver exposure did not cause dramatic effects on plasma electrolytes (Table 2). Hematocrit levels decreased with blood sampling in the control fish, but in the silver-exposed fish, an identical sampling protocol did not result in reduced hematocrit (at least not to the same extent; Table 2).

3.4. In vitro experiment

In the isolated gut bags, net water transport in control fish was similar in the anterior, mid and posterior part of the intestine at ~ 4 μl cm^{-2} h^{-1}. In contrast to the in situ perfusion experiments, silver exposure greatly reduced water transport in the in vitro experiments to 1 μl cm^{-2} h^{-1} (Fig. 7). The reduction was significant both on an overall basis and in the mid intestine alone. Unidirectional Cl^- influx in isolated gut bags measured by ^{36}Cl appearance in the external medium was ~ 1.0, 1.4 and 1.9 nmol cm^{-2} h^{-1} during the 1st hour in the non-exposed controls in the anterior, mid and posterior part of the intestine, respectively (Fig. 8). Cl^- influx in the anterior part of the intestine
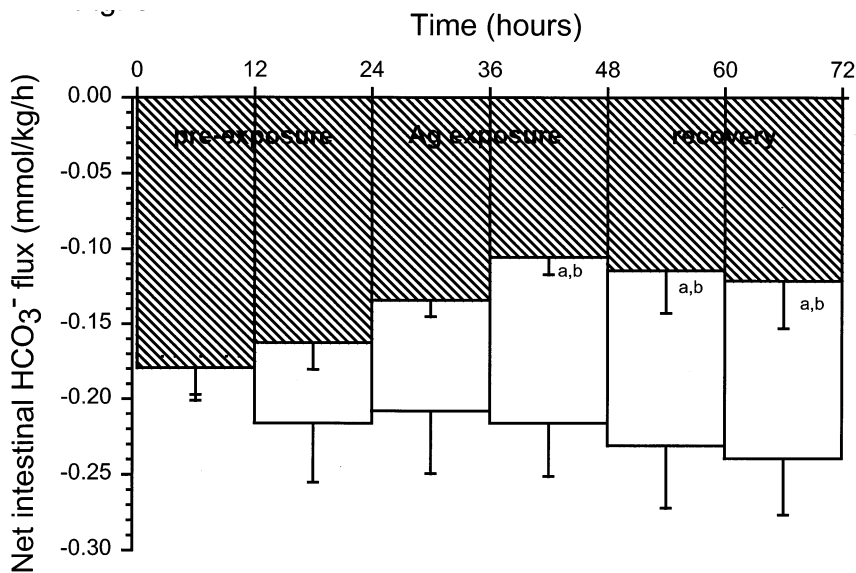

Fig. 6. Net intestinal HCO_3^- flux (mmol kg^{-1} h^{-1}) in control (white bars, $n = 5$) and silver-exposed (hatched bars, $n = 8$) lemon sole during in situ perfusion: pre-exposure, intestinal silver exposure and recovery. Means $\pm$ S.E.M. 'a' denotes statistically significant difference from internal (initial) pre-exposure value ($P < 0.05$) and 'b' denotes statistically significant difference from parallel control values ($P < 0.05$).

Table 2
Hematocrit (%), blood pH, plasma total CO_2 (mM), plasma protein (g/100 ml) and plasma ionic composition (mM) from control and silver-exposed *P. vetulus*[a]

	Time (h)	$[Cl^-]$	$[Na^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$	$[CO_2]$	pH	Hematocrit	Plasma protein
Controls	24	138.0 ± 6.3	152.4 ± 2.0	1.4 ± 0.1	1.3 ± 0.1	4.23 ± 0.19	7.816 ± 0.019	12.4 ± 1.0	2.77 ± 0.13
	48	131.1 ± 2.2	152.4 ± 1.5	1.4 ± 0.1	1.5 ± 0.3	4.20 ± 0.27	7.808 ± 0.039	$10.4 \pm 1.0*$	2.74 ± 0.31
	72	131.5 ± 2.4	150.2 ± 2.1	1.3 ± 0.1	1.3 ± 0.2	4.00 ± 0.25	7.834 ± 0.014	$8.7 \pm 1.5*$	$2.22 \pm 0.19*$
Experimental									
Pre-exposed	24	143.6 ± 3.4	151.0 ± 1.6	$1.7 \pm 0.18**$	1.6 ± 0.3	4.41 ± 0.28	7.786 ± 0.029	11.1 ± 1.1	3.17 ± 0.19
Silver-exposed	48	$139.9 \pm 3.0**$	152.6 ± 1.6	1.5 ± 0.1	1.8 ± 0.6	4.35 ± 0.51	7.874 ± 0.026	11.3 ± 1.2	3.22 ± 0.17
Recovery	72	144.0 ± 6.5	150.3 ± 1.5	$1.6 \pm 0.1**$	1.3 ± 0.2	4.26 ± 0.40	7.866 ± 0.025	$10.5 \pm 0.6*$	$2.90 \pm 0.19*$

[a] Mean $\pm$ S.E.M., $n = 5$ and 8 for controls and silver-exposed, respectively.
* Denotes statistical significant difference from internal initial pre-exposure value at 24 h (paired *t*-test).
** Denotes statistical significant difference from the values at the corresponding time in the control series (unpaired *t*-test) $P < 0.05$.
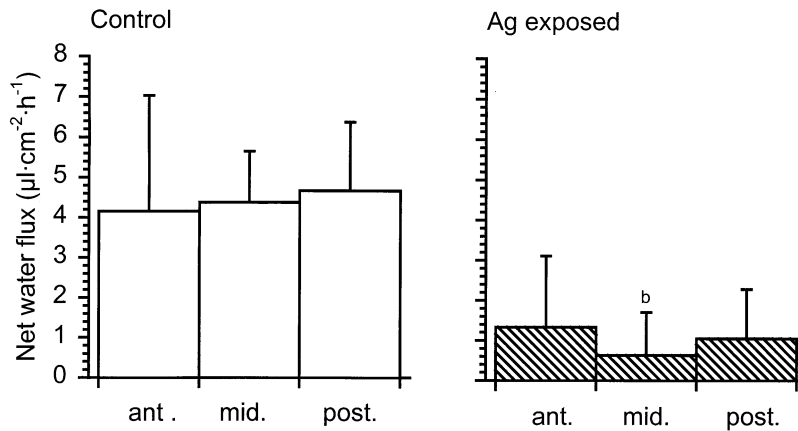
Fig. 7. Water transport ($\mu l\ cm^{-2}\ h^{-1}$) in freshly isolated control (white bars, $n = 7$) and silver-exposed (hatched bars, $n = 6$) segments ('gut bags') of the anterior, mid and posterior part of the intestine from lemon sole. Means $\pm$ S.E.M. Overall, silver exposure significantly decreased water transport ($P < 0.05$, unpaired t-test). 'b' denotes statistically significant difference from control ($P < 0.05$).

remained constant over the entire experimental period, however, in both the mid and posterior part of the intestine Cl^- fluxes were reduced over time. During the 3rd hour, the Cl^- flux was $\sim 60\%$ of the initial value in both the mid and posterior part of the intestine, a difference which was statistically significant in the posterior segment. For all three parts of the intestine, silver exposure inhibited Cl^- influx by at least 50% in the 1st hour of exposure. Generally, in contrast to the controls, Cl^- influx remained constant over the 3 h in all three parts of the intestine during silver exposure.

HCO_3^- excretion in the isolated gut bags was ~ 0.4 nmol $cm^{-2}\ h^{-1}$ and showed no marked differences between the different parts of the intestine (Fig. 9). No effect of silver exposure on HCO_3^- excretion was observed over the 3 h.

4. Discussion

The observed drinking rate of 1.73 ± 0.38 ml kg^{-1} h^{-1} is within the range of drinking rates ($1–5$ ml kg^{-1} h^{-1}) observed in other marine teleost fish [1,11,15,20]. Silver exposure resulted in silver concentrations in GIT fluids comparable to those in the silver containing water (Fig. 2), and reduced drinking by $\sim 50\%$. Although this effect was not statistically significant ($P < 0.06$), due to one outlayer in the silver-exposed group, it is in agreement with the significant decrease in drinking rate associated with exposure to lower water-borne silver levels in two other marine teleosts, the starry flounder [4] and the tidepool sculpin [18]. To our knowledge there are no reports on effects of other metals or other xenobiotics on drinking rates in marine teleost fish. Drinking by marine teleosts compensates for osmotic loss of water to the hyperosmotic marine environment. Water is drawn from the intestinal lumen across the intestinal epithelium into the body by active

transport of Na^+ and Cl^-. Therefore any reduced drinking could lead to dehydration. The mechanisms of the effect is unknown. As discussed by Wood et al. [23], it could result from 'taste' aversion, mechanical distension or other toxic responses associated with the presence of silver in the intestine, or by interference with the hormonal control of drinking.
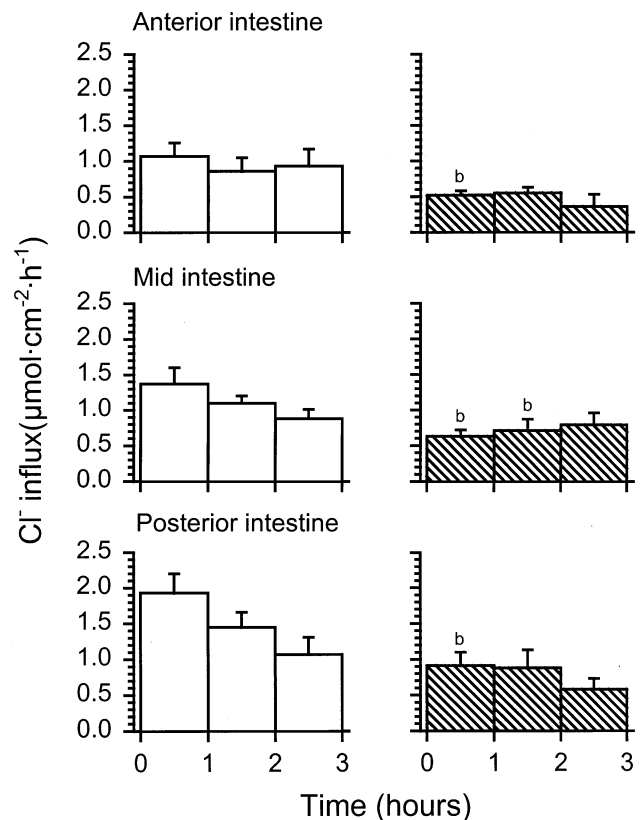

Fig. 8. Cl^- influx (nmol $cm^{-2}\ h^{-1}$) during 3 h in freshly isolated control (white bars, $n = 7$) and silver-exposed (black bars, $n = 6$) segments of the anterior, mid and posterior part of the intestine from lemon sole. Means $\pm$ S.E.M. 'b' denotes statistically significant difference from control ($P < 0.05$).
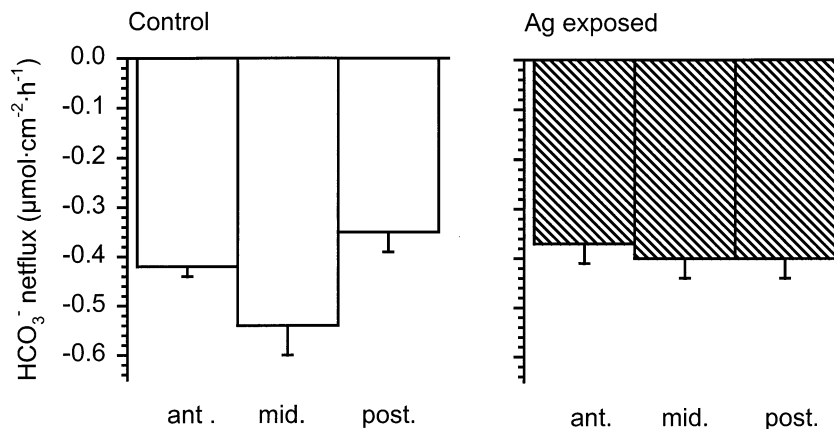
Fig. 9. HCO_3^- influx (mmol cm^{-2} h^{-1}) during 3 h in freshly isolated control (white bars, $n = 7$) and silver-exposed (black bars, $n = 6$) segments of the anterior, mid and posterior part of the intestine from lemon sole. Mean ± S.E.M. There were no significant differences.

Despite a reduced net intestinal influx of both Na$^+$ and Cl$^-$ during intestinal silver exposure in the in situ perfusion experiments (Figs. 4 and 5), there was no significant effect on intestinal water absorption. However, in the in vitro experiments, where more accurate measurements of water transport could be conducted, silver exposure clearly reduced net water transport across the intestinal epithelium (Fig. 7). One serious consequence of silver exposure to marine teleosts could thus be dehydration, since both water intake via drinking and transepithelial water transport in the intestine can be reduced during silver exposure.

During these acute studies of intestinal silver exposure, only minor effects were observed on blood parameters (Table 2). This virtual lack of effect on plasma electrolytes is somewhat unexpected in the light of the impaired intestinal net ion and water transport (Figs. 4–9), but branchial compensation could be one explanation. In the in situ perfusion experiments, the gills were not exposed to silver externally. However, after 96 h of exposing both the gills and GIT to silver in the in vivo experiment, branchial Na/K-ATPase activity was actually increased compared to the controls as also reported by Hogstrand and Wood [5]. This is in contrast to the response seen in freshwater teleosts exposed to Ag$^+$, where branchial Na$^+$ and Cl$^-$ influxes are greatly impaired, caused by inhibition of the branchial Na/K-ATPase activity. This impairment leads to mortality [10,19,22]. The increased branchial Na/K-ATPase activity found in the present study indicates increased branchial electrolyte excretion capacity, and could thus be an adaptive response by fish facing elevated plasma salt levels due to reduced GIT water intake and absorption during silver exposure. One sign of hemoconcentration caused by dehydration can be seen in the hematocrit levels (Table 2). Mean hematocrit fell in the control fish by 30% from blood sampling during the experiments. In the silver-exposed fish,

however, mean hematocrit actually increased slightly during the silver exposure period showing evidence of hemoconcentration.

In the current model of ion transport in teleost fish intestine presented by Loretz [9], transepithelial transport depends on the Na/K-ATPase on the basolateral membrane. This enzyme generates appropriate electrochemical gradients for coupled and channel mediated ion flows. Na$^+$ that enters the cell is extruded to the serosal solution by this enzyme and Cl$^-$ follows passively down its electrochemical gradient across the basolateral membrane. Along with the Na/K-ATPase, the suggested location of the Cl$^-$/HCO$_3^-$ exchanger is on the basolateral membrane exchanging Cl$^-$ from the cell with HCO$_3^-$ from the serosal solution (blood side). HCO$_3^-$ is then believed to be transported from the intestinal epithelial cells to the lumen by diffusion across the apical membrane. Transport of Cl$^-$ and Na$^+$ across the apical membrane occurs at both the Na$^+$, K$^+$, 2Cl$^-$ and the Na$^+$, Cl$^-$ cotransporters.

In the control series, intestinal net flux of all investigated ions as well as blood parameters (except for hematocrit) remained constant throughout the duration of the experiment. This indicates that the fish were not seriously stressed by the applied surgical and experimental procedure. The composition of the gut saline was made to closely mimic the overall composition of the fluid found in the intestine (Table 1). In the unexposed control fish, net intestinal influxes of Cl$^-$ and Na$^+$ were 0.4 and 0.2 mmol kg^{-1} h^{-1}, respectively, and the net intestinal HCO$_3^-$ efflux was 0.2 mmol kg^{-1} h^{-1} (Figs. 4–6). It thus appears that 0.2 mmol Cl$^-$ kg^{-1} h^{-1}, or 50% of the total Cl$^-$ transport, could be accounted for by Na$^+$-coupled Cl$^-$ transport, while the remaining 50% of the total Cl$^-$ transport could be contributed by the Cl$^-$/HCO$_3^-$ exchanger.

Both Na$^+$ and HCO$_3^-$ intestinal transport were inhibited by silver exposure. While net Na$^+$ transport

was totally abolished, HCO_3^- excretion was reduced by $\sim 50\%$. This indicates that the Na^+ transport system (Na/K-ATPase) and the Cl^-/HCO_3^- exchanger are both sensitive to silver exposure, but to different extents. During silver exposure, no Cl^- transport can thus be coupled to Na^+ transport and the total Cl^- transport of ~ 0.1 mmol kg^{-1} h^{-1} is quantitatively well matched by the remaining HCO_3^- excretion after 50% inhibition. The resulting overall inhibition of Cl^- transport was 75%, corresponding to the inhibition of water absorption seen in vitro (Fig. 7).

In the in vitro experiments, silver exposure again resulted in inhibition of Cl^- transport (Fig. 8). The overall inhibition was only up to 50% and thus lower than the 75% inhibition observed in the in situ perfusion experiments. In contrast to the in situ experiments, the in vitro experiments showed no effect of silver exposure on HCO_3^- flux over the 3 h. The lack of effect of silver exposure on the HCO_3^- efflux could be due to the short experimental period in the in vitro experiments (3 h compared to 2×12 h in the perfusion experiments). This could indicate that it takes longer for silver-induced effects to develop on HCO_3^- efflux than on Na^+ influx. Thus, in agreement with the in situ perfusion experiments in live animals, overall Cl^- transport seems to be more sensitive to silver exposure than HCO_3^- excretion in the in vitro experiments.

In both the mid and posterior part of the intestine, Cl^- uptake was reduced over time in the controls. This could indicate some deterioration of the isolated intestinal segments during the 3-h duration of the experiment. However, the total Cl^- concentration in the gut saline was reduced during the experiments due to a net mucosal to serosal Cl^- transport. Thus Cl^- transport at the end of the experiment was against a higher chemical gradient than at the start of the experiment. The Cl^- transport in the 3rd hour of the experiments was similar in all three parts of the intestine, but the mid and posterior parts started out at higher levels than the anterior part of the intestine. It therefore appears that part of the Cl^- transport was sensitive to the preparation and experimental procedure, or to increased mucosal to serosal [Cl] gradient, and furthermore that this part of the Cl^- transport is located predominantly in the mid and posterior part of the intestine. Such a spatial differentiation between components of intestinal Cl^- transport clearly deserves more attention.

The apparent lack of effect on intestinal Na/K-ATPase activity observed in fish exposed to silver-containing seawater seems in contrast to the total inhibition of Na^+ transport in the in situ perfused fish. A likely explanation is that the intestinal 'silver dose' is much higher in the in situ perfused fish than it is in fish which are drinking silver containing seawater. The silver concentration of the seawater and the gut saline were similar, but the infused fish received 15 ml kg^{-1} h^{-1}

gut saline whereas the non-infused, but silver-exposed, fish drank only 0.9 ml kg^{-1} h^{-1} of seawater.

The conclusions that can be drawn from the present findings and those of Hogstrand and Wood [4] and Webb [19], are that waterborne silver exposure, albeit at levels far above environmental significance, can lead to reduced drinking in marine teleost fish. Although silver is present as various silver chloride complexes rather than the free Ag^+ (the toxic species in freshwater), it can still exert toxic effects when present in very high concentrations. Despite the reduced drinking rate, silver exposure leads to relatively high silver concentrations in the gastro-intestinal fluids. The presence of silver in intestinal fluids can potentially lead to impairment of ion and water transport as well as disturbed acid-base regulation in marine teleost fish. Further studies are required to assess the relative importance of silver-induced effects at the gills and in the intestine to the overall cascade of physiological effects seen during exposure to high waterborne silver concentrations.

Intestinal metal toxicity could also be an important factor for marine fish drinking seawater contaminated with other metals. Recently Cd has been shown to impair Cl^- transport in intestinal epithelium from the European eel (*Anguilla anguilla*) in vitro and to impair intestinal carbonic anhydrase and $Na^+/K+$-ATPase activity in vivo [7,8].

Acknowledgements

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