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Physiology of acute silver toxicity in the starry flounder (*Platichthys stellatus*) in seawater

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Abstract Physiological effects of exposure to silver ($AgCl_n^{n-1}$; 250 μg Ag l^{-1} or 1000 μg Ag l^{-1}) in seawater fish were investigated using adult starry flounders. While all fish survived up to 10 days in 250 μg Ag l^{-1}, flounders started to die after day 4 in 1000 μg l^{-1}. Dose-dependent increases in plasma and hepatic silver concentrations showed that silver was available for uptake. There were minimal negative effects on hematological parameters, acid-base status, and blood gases. Plasma ammonia showed a pronounced (three- to four-fold), but transient increase in flounders exposed to either 250 μg Ag l^{-1} or 1000 μg Ag l^{-1}. Whole body ammonia and acid equivalent efflux measurements indicated that ammonia retention was due to a combination of stimulated production and inhibited excretion. In the 1000-μg Ag l^{-1} group there was a similar transient increase in plasma [magnesium], which was restored by day 4. In contrast, plasma chloride and sodium levels increased gradually towards the point when fish began to die. At 250 μg Ag l^{-1}, the Na^+/K^+-ATPase activity of the intestine was unaffected but there was a two-fold increase in branchial Na^+/K^+-ATPase activity. The latter effect was interpreted as compensation for an elevated chloride and sodium load. The increases in plasma chloride and sodium concentrations were accompanied by a marked suppression of drinking, thereby indicating that acute silver toxicity was likely caused by a combination of elevated electrolyte concentrations and dehydration.

Key words Ag · Osmoregulation · Ionoregulation · Marine · Fish

Abbreviations αCO_2 solubility coefficient for CO_2 · αO_2 solubility coefficient for O_2 · C_{aCO_2} total arterial plasma CO_2 content · C_{aO_2} total arterial blood O_2 content · *Hb* hemoglobin concentration · *^{3}H-PEG-4000* ^{3}H-polyethylene glycol 4000 · *Ht* hematocrit · J_{out} efflux · *MCHC* mean corpuscular hemoglobin concentration · *MS-222* tricaine-methyl-sulfonate · *Na^+/K^+-ATPase* sodium, potassium adenosine triphosphatase · P_{aO_2} arterial blood O_2 tension · pH_a arterial blood pH · P_{iO_2} inspired O_2 tension · T_{amm} total ammonia

Introduction

Predictions of the effects and impacts that chemicals may have on animals in different environments are very difficult to make without understanding the mechanisms by which the chemicals act to produce their toxic effects. There is now considerable knowledge of how many metals affect freshwater fish mechanistically, but the database is still shallow when it comes to marine species (Hogstrand and Wood 1996, 1998; Olsson et al. 1998; Wood et al. 1999). This is especially true for silver, for which there are very few published toxicological data dealing with marine fish species. Silver is of toxicological and environmental interest in the marine environment because of its potential discharge into waters from sewage treatment plants. These silver discharges originate from a variety of natural and anthropogenic sources, the latter including photographic processing effluents (Eisler 1997; Purcell and Peters 1998).

In the few marine teleost species studied to date, 96-h LC50 values for waterborne silver range from 183 μg l^{-1}

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to 1,200 μg l^{-1} (or 1.7 μM to 11.1 μM; Shaw et al. 1997, 1998; see also review by Hogstrand and Wood 1998). The mechanisms of silver toxicity to marine fish are largely unexplored. Some sublethal physiological effects, such as reduced activities of several liver enzymes (Jackim et al. 1970; Calabrese et al. 1977) and reduced oxygen consumption by gill tissue (Thurberg and Collier 1977), have been noted. However, it is not known if these aberrations have consequences for fitness or survival of the animal.

In contrast to the little investigated physiology of silver toxicity in marine fish, acute effects of waterborne silver to freshwater fish are relatively well characterized (see reviews by Hogstrand and Wood 1998; and Wood et al. 1999). In freshwater, like many other metals (e.g., copper, zinc, cadmium, aluminium), silver exerts its acute toxicity at the gills (Wood et al. 1996). Here the free Ag^+ ion is the toxic species of concern and it very specifically targets and blocks the inward transport of Na^+ and Cl^- across the gill epithelium (Morgan et al. 1997). The key molecular mechanism of action seems to be a potent inhibition of the Na^+/K^+-ATPase, which is located basolaterally in the gill epithelium and serves as the engine for both Na^+ and Cl^- transport (Ferguson et al. 1996; Morgan et al. 1997). The uptake of Na^+ and Cl^- by the gills is essential for maintenance of osmotic balance in the very hypoosmotic freshwater environment. Thus, blockage of Na^+ and Cl^- influx leads to a progressive net loss of plasma osmolytes, which in turn sets up a complex sequence of events that finally causes the fish to die from cardiovascular collapse (Wood et al. 1996; Webb and Wood 1998).

There are compelling reasons to expect that the etiology of acute silver toxicity to fish should be different in seawater than in freshwater. First, while freshwater fish are hyperosmotic relative to their dilute freshwater environment, the ion concentrations in plasma of marine teleost fish are much lower than that of seawater. Thus, if this hypo-osmoregulation is disrupted in a marine teleost it would likely lead to a net gain of ions as opposed to the net loss observed in freshwater teleost fish. Second, osmoregulation is fundamentally different in marine and freshwater fish in that marine fish drink seawater to prevent dehydration whereas freshwater fish, which need to avoid water gain, drink very little. The toxicokinetic consequence of this difference is that the intestine is a possible uptake route of waterborne silver to marine teleosts but most likely not to freshwater fish. Third, the free Ag^+ ion, which is the toxic silver species to freshwater fish (Hogstrand and Wood 1998; Wood et al. 1999), is virtually non-existent in seawater (Ferguson and Hogstrand 1998). Instead, the speciation of silver in seawater under aerobic conditions is dominated by silver chloride complexes ($AgCl^0_{(aq)}$, $AgCl_2^-$, $AgCl_3^{2-}$, and $AgCl_4^{3-}$) and possibly silver thiol complexes (Adams and Kramer 1998; Ferguson and Hogstrand 1998).

The aim for the present study was to elucidate the key mechanisms that lead to acute toxicity of waterborne silver in marine teleosts. As model species, we used the starry flounder (*Platichthys stellatus*). This flatfish species has a well-known physiology, is easily maintained, and tolerates well the type of experimental procedures employed (Wood et al. 1977, 1979a, b; Milligan and Wood 1987; Wood and Milligan 1987). The primary approach was to fit each fish with a chronic indwelling arterial catheter for repetitive measurements of blood chemistry and hematological variables during acute exposure to waterborne silver. The same procedure has been used in previous studies to delineate the physiological effects of exposure to silver (Janes et al. 1996; Wood et al. 1996; Webb and Wood 1998), copper (Wilson and Taylor 1993a, b; Pilgaard et al. 1994), zinc (Spry and Wood 1984, 1985), aluminium (Goss and Wood 1988; Wood et al. 1988a, b; Playle et al. 1989), and low pH (McDonald et al. 1980; McDonald and Wood 1981; McDonald 1983). The results from the catheterization experiment prompted various additional experiments to clarify specific aspects of the physiological responses. These experiments involved measurements of Na^+/K^+-ATPase activities, drinking rate, and excretion rates of total ammonia, urea, and acid equivalents.

Materials and methods

Animals

Starry flounders (*P. stellatus*; 799 ± 61 g mean ± SEM, $n = 32$, range: 300–1413 g) were collected by slow bottom trawl in Barkley Sound off the west coast of Vancouver Island, BC, Canada. Prior to experimentation, the fish were housed in 400-l fiberglass tanks with a sandy bottom substrate, without feeding (typically 7 days). Each tank was supplied with a continuous flow of aerated natural non-recirculating seawater with a salinity of 30–32‰ and a temperature of 11–13 °C.

Experiment 1: cannulation

Fish were fitted with indwelling arterial catheters (PE50 tubing), inserted into the caudal artery. The surgical procedure has been detailed by Wood et al. (1979) and followed the general method originally developed by Watters and Smith (1973). Each catheters was filled with heparinized Cortland saline (50 IU ml^{-1}; Wolf 1963) adjusted for seawater teleosts by raising the Na^+ concentration to 160 mM by addition of NaCl. After surgery, the fish were placed in individual 10-l plastic tubs, supplied with a continuous flow (350 ml min^{-1}) of well-aerated seawater with the same temperature (11–13 °C) and salinity (30–32‰) as before and allowed to recover for 48–60 h. Resting values for many physiological parameters are never attained when starry flounders are held in bare tubs, for the fish lack sandy substrate in which to bury (Wood et al. 1979a, b). Sand could not be used in the present study as it supports microorganism growth that removes silver from the solution and influences acid equivalent and ammonia flux determinations. Instead, a black plastic mesh was used to cover the fish inside the tub; Milligan and Wood (1987) demonstrated that this approach yields resting data comparable to those obtained from starry flounders allowed to bury in sand.

The exposure system consisted of a head tank, which delivered a constant flow (3.0 l min^{-1}) of aerated seawater to a 20-l, vigorously aerated, mixing chamber. The mixing chamber was equipped with an overflow drain to provide a constant gravity-driven flow through eight hoses fitted at the same level around the bottom of

the mixing chamber (also known as the "Octopus"). The water flow received by each individual fish was kept at 350 ml min^{-1} as before. Silver nitrate ($AgNO_3$), dissolved in distilled water and acidified with 0.05% HNO_3, was dispensed from a stock solution into the mixing chamber by a peristaltic pump at a rate of 1.0 ml min^{-1}. The silver stock solutions were kept in a darkened bottle and renewed every 48 h. The acid addition did not affect the pH of the mixed water down to one decimal place. Concentrations of the stock solutions were set so that the nominal concentrations of Ag in the tubs were 250 μg l^{-1} (2.3 μM) for one group of eight fish and 1000 μg l^{-1} (9.3 μM) for a second group. A third group of eight fish served as a sham-treated control group and were sampled identically but not exposed to silver. Measured silver concentrations in the water were 276.6 ± 3.8 μg l^{-1} for the "250-μg Ag l^{-1}" group (mean ± SEM, n = 10, range: 255–294 μg l^{-1}) and 1098 ± 97 μg l^{-1} for the "1000-μg Ag l^{-1}" group (mean ± SEM, n = 16, range: 863–1317 μg l^{-1}). These concentrations of silver were below the threshold for cerargyrite ($AgCl_{(s)}$) formation at the salinity present (Ferguson and Hogstrand 1998). Water silver concentrations in the sham-treated group were below the detection limit for the method used (0.2 μg l^{-1}). The sham control and the group exposed to 250 μg Ag l^{-1} were run simultaneously, whereas the 1000-μg Ag l^{-1} group was exposed in a separate subsequent run. The start of the exposure was carried out in a staggered fashion (30 min between fish) to allow for exact timing of all fish at each sampling point. At time 0 for each fish, the plumbing to the fish's tub was switched to the outflow of the mixing chamber, and the tub was spiked with the $AgNO_3$ stock solution to instantaneously reach the set exposure concentration.

A 700-μl blood sample was drawn from each fish via the catheter immediately prior to exposure, and then at 12, 24, 48, 96, and 144 h. Blood was sampled anaerobically into ice-cold gas-tight glass syringes (Hamilton). A water sample was also drawn from in front of the fish's mouth for measurement of inspired O_2 tension (P_{IO_2}), which was found to remain above 124 torr throughout the experiment. The arterial blood samples were split into two portions. One was analyzed immediately for partial pressure of O_2 (P_{aO_2}), total blood O_2 content (C_{aO_2}), total true plasma CO_2 content (C_{aCO_2}), pH (pH_a), hematocrit (Ht), and hemoglobin (Hb). The other portion was centrifuged at 10,000 g for 2 min. The plasma was immediately decanted, analyzed for refractive index (plasma protein), and then quickly frozen in liquid N_2 for later analysis of plasma levels of inorganic ions (Na^+, Cl^-, and Mg^{2+}), total Ag, glucose, lactate, cortisol, and total ammonia (T_{amm}). The sampled blood was replaced by infusion of the same volume of modified Cortland saline (Wolf 1963) mixed with re-suspended red blood cells not used for analyses. After the final sample on day 6, the fish were deeply anaesthetized in tricaine-methyl-sulfonate (MS-222; 1 g l^{-1}, neutralized with NaOH) and immediately perfused with heparinized Cortland saline (200 IU ml^{-1}) via the heart. Gills, pyloric intestine (10-cm section of intestine immediately posterior to the stomach), liver, and kidney were dissected out. The intestine was flushed with 50 ml Cortland saline to remove residual silver-containing seawater, opened and blotted dry, and then frozen in liquid N_2. Similarly, the gills were rinsed in Cortland saline, blotted dry, and frozen in liquid N_2. The other tissues were frozen directly in liquid N_2. Tissue samples were stored at −80 °C for subsequent determination of total silver concentration and Na^+/K^+-ATPase activity (intestine and gills) or silver concentration only (liver and kidney). Na^+/K^+-ATPase activity was not analyzed on fish from the 1000-μg Ag l^{-1} group because these particular samples were lost during shipping between institutions.

Experiment 2: ammonia, urea, and acid excretion

Effects of silver on nitrogenous waste and acidic equivalent excretion were investigated in a separate experiment. Starry flounders (n = 5) were placed in individual 10-l plastic tubs filled with 30–32‰ seawater at 12 °C and equipped with an air line and a plastic mesh to minimize visual stress as described above for the cannulation experiment. However, there was no flow of seawater through the tubs in this experiment, and an external water bath was used to maintain temperature. The water in the tubs was replaced every 8 h with either plain natural seawater (pre-exposure) or with seawater supplemented with $AgNO_3$ from a stock solution to give a nominal exposure concentration of 250 μg Ag l^{-1} (measured concentration: 293 ± 29 μg l^{-1}, mean ± SEM, n = 12, range: 224–357 μg l^{-1}). The exposure water was prepared fresh every 16 h and kept aerated. Water was changed by gently decanting most of the water in the tubs and replacing it with new water. This procedure likely imposed little stress to the animals because total ammonia excretion rates did not increase during four consecutive 8-h flux periods prior to the silver exposure. Water samples (5 ml) were taken immediately prior to and after each water change for measurements of T_{amm}, urea, and titratable alkalinity, from which acidic equivalents were calculated (see below). Titrations for titratable alkalinity in water samples were carried out immediately after sampling. Water samples for ammonia and urea analysis were stored at 4 °C and analyzed within 24 h following sampling. Exposure to Ag was continued for 144 h during which time twelve 8-h fluxes were carried out. After the last flux period on day 6, the five starry flounders were kept exposed to silver at the same level as before and on the 10th exposure day used in the drinking rate experiment described below.

Experiment 3: drinking rate

Drinking rate was measured in five starry flounders after exposure to 250 μg Ag l^{-1} for a total of 10 days (see Experiment 2) and in five unexposed individuals. Fish were kept in the same plastic tubs as before with either 250 μg Ag l^{-1} or with no silver added to the water. The volume was set to 6 l. Following the methods of Wilson et al. (1996), a non-absorbed tracer, ^{3}H-polyethylene glycol 4000 (^{3}H-PEG-4000, specific activity: 2050 μCi g^{-1}; NEN-Dupont), was introduced to the water (5.0 μCi l^{-1}) and the fish were allowed to drink the medium during the following 6–8 h (exact time recorded). This time period is considerably shorter than the passage time through the gastrointestinal tract, a fact confirmed by counting rectal fluid samples in the present study. Plasma counts confirmed that the ^{3}H-PEG-4000 was not absorbed. Water samples were taken throughout the exposure to monitor ^{3}H-PEG-4000 concentration, which in fact remained constant. At the end of the incubation period, the fish were euthanized by an overdose of neutralized MS222 (1 g l^{-1}). Plasma and rectal fluid samples were taken for analysis of silver, chloride, and ^{3}H-PEG-4000 radioactivity. The gastrointestinal tract was then ligated off at the oesophagus and the rectum, followed by removal of the whole gastrointestinal tract, which was homogenized in 5 volumes of 8% $HClO_3$. Further processing of the gastrointestinal homogenate, scintillation counting, quench correction, and calculation of drinking rate were performed exactly as described by Wilson et al. (1996).

Analytical methods

Most blood parameters were analyzed by methods detailed previously by Wood et al. (1988a) and Playle et al. (1989). pH_a was determined at the experimental temperature using a capillary electrode assembly (Radiometer E-5021 A) connected to a Radiometer PHM72 blood gas analyzer. P_{aO_2} was measured with an O_2 electrode (Radiometer E-5046) connected to the PHM72 analyzer. C_{aCO_2} was determined with a Corning 965 total CO_2 analyzer or by the method of Cameron (1971). These two methods were cross-validated. C_{aO_2} in the control and 250-μg Ag l^{-1} groups was determined by the method of Tucker (1967) whereas the same data were obtained with an Oxicon blood O_2 analyzer (Cameron Instruments) for the 1000-μg l^{-1} group. Again, the two methods were cross-validated. Plasma protein was determined from the refractive index of the plasma, using a refractometer (Wood et al. 1988b). Hemoglobin was measured colorimetrically by the cyanomethemoglobin method (Sigma diagnostic kit no.525-A). Plasma glucose,

lactate, and ammonia were determined enzymatically using standard kits (HK 16-UV, 826-UV, and 171-UV, Sigma). Ammonia and urea in seawater were analyzed by the standard colorimetric methods described by Ivancic and Deggobis (1984) and Price and Harrison (1987) respectively. Titratable alkalinity in seawater was determined by titration to pH 4.0, as described by McDonald and Wood (1981). Plasma cortisol levels were determined by radioimmunoassay using a commercial kit (^{125}I RIA kit, ICN Biomedical) with appropriate dilutions of standards to match the protein levels in flounder blood plasma. An automated chloride titrator (CMT10 Radiometer) was used to analyze Cl^- concentrations. Plasma Na^+ and total Mg levels were determined by air-acetylene flame atomic absorption spectroscopy (Varian 1275 or Instrumentation Laboratory model S-11). Silver analyses of blood plasma and tissues were performed as in Hogstrand et al. (1996). In brief, proteins and lipids were digested by evaporating the samples in a mixture of HNO_3 (trace metal grade) and H_2O_2 (analytical grade) under heat. The samples were reconstituted in 0.5% HNO_3 and analyzed by graphite furnace atomic absorption spectroscopy (Varian 1275 or Varian SpectrAA-20). Water samples were acidified with HNO_3 to give a final concentration of 0.5% and analyzed for silver with graphite furnace atomic absorption spectroscopy (Varian 1275). Na^+/K^+-ATPase was assayed in homogenates of gill epithelia and intestinal mucosa as K^+-dependent, oubain-sensitive ATP hydrolysis (Holliday 1985).

Calculations

P_{aCO_2} and plasma [HCO_3^-] were calculated from measured values of pH_a and C_{aCO_2} using the Henderson-Hasselbach equation as detailed in Playle et al. (1989). Values for pK' and the solubility coefficient for CO_2 (αCO_2) at the experimental temperature and pH were taken from Boutilier et al. (1984). Physically dissolved O_2 content in the arterial blood was calculated as the product of P_{aO_2} and the measured O_2 solubility coefficient (αO_2) in the blood plasma of *P. stellatus* (1.91 μmol O_2 l^{-1} $torr^{-1}$ at 12 °C; Wood et al. 1979a). To adjust for differences in Hb and physically dissolved O_2 concentrations between fish, Hb-bound O_2 content per unit Hb ([O_2]/[Hb]) was calculated as:

$$[O_2]/[Hb] = \frac{C_{aO_2} - P_{aO_2} \times \alpha O_2}{[Hb]} \qquad \text{(Wood and Milligan 1987)}$$

Mean corpuscular hemoglobin concentration (MCHC) was calculated as the ratio of Hb to Ht values, Hb (g 100 ml^{-1})/Ht (ml 100 ml^{-1}) = MCHC (g Hb ml RBC^{-1}). The inverse ratio between plasma protein concentration at each time point and the pre-exposure value ("C") taken before the start of the exposure was used as an index of relative blood volume (Wood et al. 1988b).

Rates of ammonia and urea excretion (J_{out}) into the external water were calculated from changes in concentrations of these substances in the water multiplied by the volume of the flux chamber and factored by time and mass. Titratable acidity flux was calculated in an analogous fashion from changes in titratable alkalinity over the same time period, with the signs reversed. Net acidic equivalent flux was calculated as the sum of the ammonia and titratable acidity flux data, signs considered, as outlined by McDonald and Wood (1981). Drinking rate was calculated from the total ^{3}H-PEG-4000 radioactivity in the gastrointestinal tract divided by the 'concentration' of ^{3}H radioactivity in the external water (cpm ml^{-1}), the drinking time, and the mass of the fish.

Data have been expressed as means ±1 SEM (n), where n = number of fish. Within each experimental treatment, differences were assessed by Student's two-tailed paired t-test with each fish serving as its own control, comparing data to the initial pre-exposure value ("C"). The Bonferroni procedure was employed to adjust the t-value for such multiple comparisons. Differences between treatment groups and the unexposed control, at the same sampling time, were analyzed by means of Student's two-tailed unpaired t-test, again using the Bonferroni procedure. Significant differences between values at $P < 0.05$ are denoted by asterisks (*).

Results

Cannulation experiment

There were a few significant differences amongst the pre-exposure (control) means of the three experimental groups (e.g., higher cortisol, higher plasma [Ag], and lower plasma [Na^+] and [Cl^-] in the 1000-μg l^{-1} group; higher hematocrit, [Hb], and plasma [protein] in the 250 μg Ag l^{-1} group; higher plasma [T_{amm}] in the sham-treated control group). These differences were probably a consequence of the variability in wild-caught fish. However, most variables were initially uniform and typical of those seen in resting, unstressed starry flounder (Wood et al. 1979a, b; Milligan and Wood 1987; Wood and Milligan 1987) and showed negligible changes throughout the experiment in the sham-treated control group. Nevertheless, the focus of our analysis is upon the significance of changes *within* individual treatment groups (i.e., changes over time compared with the respective pre-exposure value), so the relatively small differences in pre-exposure values between groups were not a compromising factor.

Most physiological parameters remained stable in starry flounders exposed to the lower silver concentration (250 μg l^{-1}), but the 1000-μg l^{-1} exposure was in the lethal range. Three out of eight fish exposed to 1000 μg Ag l^{-1} died between the 96-h (day 4) and 144-h (day 6) sampling points. To indicate that the blood chemistry data of the day 6 sampling are based on surviving fish, and therefore biased, the lines between the means on day 4 (n = 8) and day 6 (n = 5) of the 1000-μg l^{-1} group in Figs. 1–7 have been drawn dotted. The increased stress in fish within this group was reflected in a highly variable and rapidly increasing plasma glucose concentration (Fig. 1A) as well as by an increase in plasma cortisol level on day 6 (Table 1). In the control and 250-μg Ag l^{-1} groups, there were slight but only temporary drops in plasma glucose concentrations that were significant on days 1–4 for the sham-treated control and on day 1 for the group exposed to 250 μg Ag l^{-1}. In the sham control and the 250 μg Ag l^{-1} group, there were temporary significant elevations in plasma [cortisol] at 48 h and 12 h, respectively, but both these groups were back at pre-exposure levels at the 96 h and 144 h samplings (Table 1).

The only dramatic change occurring in the 250-μg Ag l^{-1} group was a large but transient increase in plasma T_{amm} level that was evident already at 12 h, peaked at 3.4-times the pre-exposure level on day 2, and was over on day 4 (Fig. 1B). A very similar effect was observed in the 1000-μg Ag l^{-1} group. The plasma ammonia response was, in fact, almost identical at the two Ag concentrations tested. In spite of the apparent lack of dose-dependency, the rise in plasma ammonia was likely to be a result of silver exposure because plasma ammonia levels in the unexposed sham controls gradually declined over time. In the group exposed to 250 μg

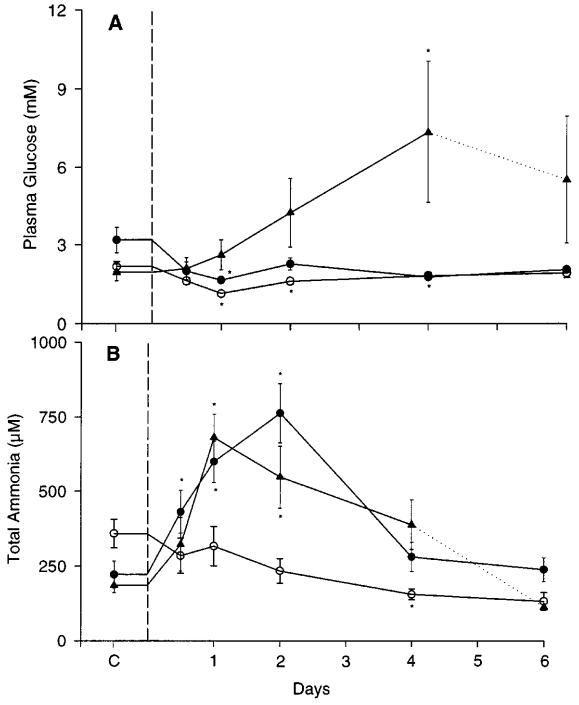

Fig. 1 Plasma glucose (**A**) and ammonia (**B**) concentrations in starry flounder during exposure to 250 μg l^{-1} (*filled circles*) or 1000 μg l^{-1} (*filled triangles*) of silver ($AgCl_n^{n-1}$) for 6 days or during sham-treatment of unexposed individuals (*open circles*). Each point, except for day 6 in the 1000-μg Ag l^{-1} group, represents the mean of eight fish. *Error bars* show SEM. The *dotted line* between the values on days 4 and 6 in the 1000-μg Ag l^{-1} group reflects the fact that three fish died between these sampling points (i.e. $n = 5$ for 1000 μg Ag l^{-1} on day 6). Asterisks indicate where means were significantly different ($P < 0.05$) from the pre-exposure value ("C") in the respective treatment

Ag l^{-1}, the increase in plasma ammonia corresponded reasonably well with the observed changes in plasma cortisol (Table 1), thus, possibly linking the increase in plasma ammonia to cortisol-induced proteolysis. However, in neither of the other groups (control and 1000 μg Ag l^{-1}) was there any discernible correlation between patterns of cortisol and ammonia variations in the plasma.

Table 1 Plasma cortisol concentrations in starry flounder during exposure to silver as $AgCl_n^{n-1}$ (250 μg Ag l^{-1} or 1000 μg Ag l^{-1}) for 6 days in 30–32‰ seawater. Values are expressed as mean ± SEM ($n = 5$–8)

Time (h)	Plasma cortisol (μg dl^{-1})		
	Control	250 μg l^{-1}	1000 μg l^{-1}
C	13.5 ± 3.1	14.2 ± 4.2	25.9 ± 5.1
12	25.0 ± 4.1	32.8 ± 5.1*	35.3 ± 5.3
24	20.1 ± 2.8	37.2 ± 10.9	29.4 ± 7.5
48	35.8 ± 4.3*	25.6 ± 9.7	32.9 ± 5.0
96	13.0 ± 2.5	14.7 ± 5.9	32.5 ± 5.4
144	12.5 ± 2.0	14.4 ± 6.1	54.6 ± 3.3*

* Significant difference from the pre-exposure value (*C*) of the respective group ($P < 0.05$)

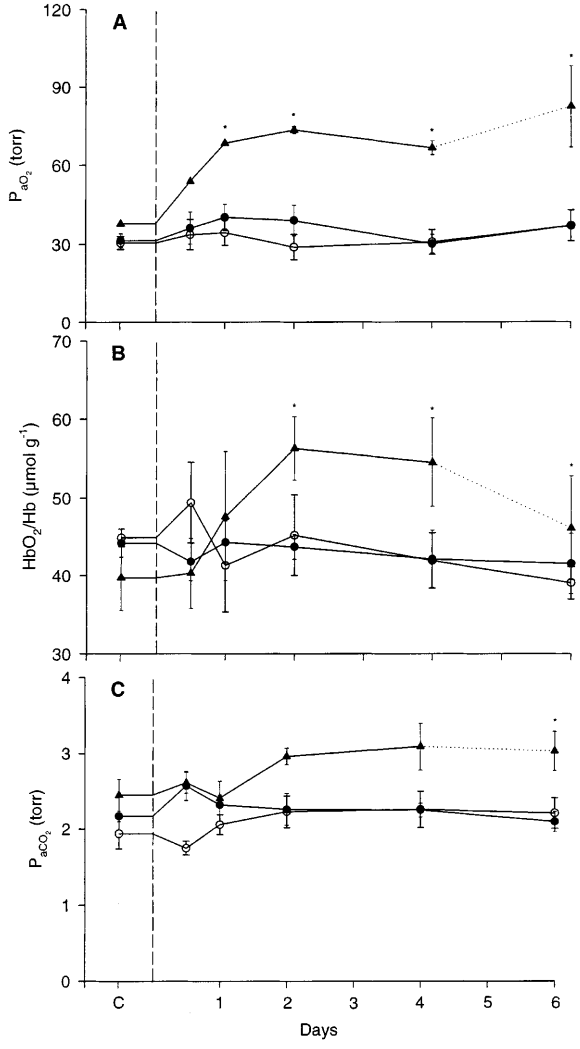

Fig. 2 Oxygen tension (P_{aO_2}) (**A**), hemoglobin-bound oxygen content per unit hemoglobin (HbO_2/Hb) (**B**), and carbon dioxide tension (P_{aCO_2}) (**C**) in arterial blood of starry flounder during exposure to 250 μg l^{-1} (*filled circles*) or 1000 μg l^{-1} (*filled triangles*) of silver ($AgCl_n^{n-1}$) for 6 days or during sham-treatment of unexposed individuals (*open circles*). Other details as in Fig. 1

There was little evidence for any direct impairment of gas exchange in silver-exposed fish. P_{O_2} was unchanged in the group exposed to 250 μg Ag l^{-1} and actually increased in the 1000-μg l^{-1} group (Fig. 2A). This resulted in a rise in the amount of O_2 bound to hemoglobin (Fig. 2B) in spite of a mild decrease in pH_a (Table 2) as well as an increase in the concentration of O_2 carried in physical solution (from 0.07 ± 0.01 mM before the exposure to 0.16 ± 0.03 mM at day 6, $P < 0.05$, data not shown). P_{aCO_2} showed a small elevation which was statistically significant only in the 1000-μg l^{-1} group on day 6 (Fig. 2C). This increase was associated with a reduced pH_a and was not accompanied by a simultaneous elevation in $[HCO_3^-]$, which usually is the case if CO_2 transfer across the gills is compromised (Table 2). Furthermore, total CO_2 was actually not increased on day 6 although it was at an earlier point of the exposure

Table 2 Arterial pH (pH_a) and bicarbonate concentration (HCO_3^-) in starry flounder during exposure to silver as $AgCl_n^{n-1}$ (250 μg l^{-1} or 1000 μg l^{-1}) for 6 days. Values are expressed as mean ± SEM (n = 5–8)

Time (h)	pH_a			HCO_3^- (mM)		
	Control	250 μg l^{-1}	1000 μg l^{-1}	Control	250 μg l^{-1}	1000 μg l^{-1}
C	7.905 ± 0.023	7.874 ± 0.015	7.853 ± 0.019	5.88 ± 0.47	6.20 ± 0.37	6.74 ± 0.58
12	7.917 ± 0.019	7.818 ± 0.019	7.882 ± 0.032	5.53 ± 0.32	6.30 ± 0.36	7.68 ± 0.27
24	7.894 ± 0.018	7.874 ± 0.026	7.923 ± 0.024	6.12 ± 0.31	6.51 ± 0.15	7.83 ± 0.34
48	7.824 ± 0.019*	7.880 ± 0.022	7.889 ± 0.011	5.49 ± 0.41*	6.37 ± 0.37	8.98 ± 0.42*
96	7.849 ± 0.013	7.852 ± 0.007	7.820 ± 0.024	5.99 ± 0.24	6.04 ± 0.26	7.90 ± 0.76
144	7.835 ± 0.022*	7.847 ± 0.016	7.689 ± 0.021*	5.58 ± 0.29	5.52 ± 0.23	5.61 ± 0.59

* Significant difference from the pre-exposure value (C) of the respective group ($P < 0.05$)

(day 2; data not shown) a time when HCO_3^- was also elevated in this treatment group. The reduced pH_a in the 1000-μg Ag l^{-1} group on day 6 occurred concurrently with a small but sudden increase in plasma lactate concentration from 0.23 ± 0.01 mM (mean ± 1 SEM, n = 8) on day 4 to 0.77 ± 0.02 mM (mean ± 1 SEM, n = 5, different $P < 0.05$ from pre-exposure value) on day 6. There were no other changes in lactate, which remained below 0.3 mM in the other two groups throughout the experiment (data not shown). Thus, in the fish of the 1000-μg Ag l^{-1} group, still surviving on day 6, there was a mild mixed metabolic and respiratory acidosis combined with a slight lactacidosis occurring in the presence of hyperoxemic blood. Thus, the acid-base profile indicates that overall there were only slight disturbances in acid-base regulation.

Table 3 summarizes the hematological measurements taken throughout the 6-day cannulation experiment. There were statistically significant changes (relative to the respective pre-exposure values) in several of the hematological parameters analyzed, but the alterations were not very large and in most cases they were only temporary. The only persistent changes were found in the 250-μg Ag l^{-1} group in which there was a progressive drop in Ht and a steadily increasing MCHC. Notably, this group started with higher Hb, Ht, and plasma [protein], and lower MCHC than the other two groups. The increase in MCHC was likely a compensation for the drop in Ht, which in turn was caused by the repetitive blood sampling. Presumably, new red blood cells with higher Hb were mobilized into the circulation. Decreases in Ht were also evident in the control and in the fish exposed to 1000 μg Ag l^{-1}, but in these two groups the Ht recovered by the final sampling on day 6. Similarly, the reduction in Hb occurring in the 1000-μg Ag l^{-1} group on days 2 and 4, recovered by day 6, simply reflected the loss of red blood cells due to sampling. Plasma protein, which can be used as an indicator of changes in blood volume (plasma protein is inversely related to plasma volume; Wood et al. 1988b, 1996) was decreased in the 250-μg Ag l^{-1} group during the 1st 4 days of exposure. Thus, there were indications of a temporary increase in blood volume of up to 25%. Plasma protein was also reduced in the 1000-μg Ag l^{-1} group, but only on day 4 (~20% increase in blood volume).

The most striking effects of silver exposure were disturbances in plasma electrolyte concentrations (Fig. 3). Plasma levels of chloride and sodium rose gradually in fish exposed to 1000 μg Ag l^{-1} towards the point where fish began to die (day 4; Fig. 3A, B). The concentration of plasma chloride (Fig. 3A) appeared to be more strongly affected than that of sodium (Fig. 3B). In one fish in the 1000-μg Ag l^{-1} treatment, sampled right at the point of death on day 5, plasma [Cl^-] and [Na^+] were 245 mM and 224 mM, respectively, in comparison to the pre-exposure values of 149 mM (Cl^-) and

Table 3 Hematological parameters in starry flounder during exposure to silver as $AgCl_n^{n-1}$ (250 μg l^{-1} or 1000 μg l^{-1}) for 6 days. Values are expressed as mean ± SEM (n = 5–8). (*MCHC* mean corpuscular hemoglobin concentration, *RBC* red blood cell)

		Pre-exposure	12 h	24 h	48 h	96 h	144 h
Hemoglobin (g 100 ml^{-1})	Control	4.36 ± 0.23	3.92 ± 0.32	4.17 ± 0.38	3.76 ± 0.40	4.08 ± 0.21	4.44 ± 0.28
	250 μg l^{-1}	5.36 ± 0.34	4.83 ± 0.19	4.74 ± 0.23	4.55 ± 0.17	4.77 ± 0.39	4.42 ± 0.42
	1,000 μg l^{-1}	4.53 ± 0.70	4.21 ± 0.66	4.14 ± 0.75	3.95 ± 0.60*	3.75 ± 0.69*	4.63 ± 0.17
Hematocrit (%)	Control	17.3 ± 0.7	15.0 ± 0.9	14.5 ± 0.8*	14.9 ± 0.9	14.2 ± 0.8*	17.3 ± 1.1
	250 μg l^{-1}	23.3 ± 1.7	21.9 ± 1.3	20.8 ± 0.9*	16.3 ± 1.2*	16.2 ± 1.4*	14.9 ± 1.2*
	1,000 μg l^{-1}	15.9 ± 1.5	14.9 ± 1.6	14.1 ± 1.8	13.7 ± 1.3*	13.2 ± 1.9*	15.5 ± 1.5
MCHC (g Hb ml RBC^{-1})	Control	0.273 ± 0.013	0.266 ± 0.022	0.288 ± 0.023	0.253 ± 0.022	0.296 ± 0.023	0.256 ± 0.004
	250 μg l^{-1}	0.237 ± 0.020	0.224 ± 0.013	0.236 ± 0.018	0.288 ± 0.017*	0.305 ± 0.032*	0.307 ± 0.035*
	1,000 μg l^{-1}	0.279 ± 0.021	0.269 ± 0.020	0.277 ± 0.021	0.277 ± 0.020	0.267 ± 0.021	0.300 ± 0.029
Plasma protein (g 100 ml^{-1})	Control	2.78 ± 0.16	2.53 ± 0.24	2.52 ± 0.22	2.49 ± 0.26	2.40 ± 0.30	2.47 ± 0.29
	250 μg l^{-1}	3.45 ± 0.18	2.97 ± 0.16*	2.97 ± 0.15*	2.75 ± 0.20*	2.81 ± 0.30*	2.92 ± 0.33
	1,000 μg l^{-1}	2.6 ± 0.21	2.61 ± 0.22	2.57 ± 0.25	2.57 ± 0.25	2.18 ± 0.22*	2.78 ± 0.43

* Significant difference from the pre-exposure value (C) of the respective group ($P < 0.05$)

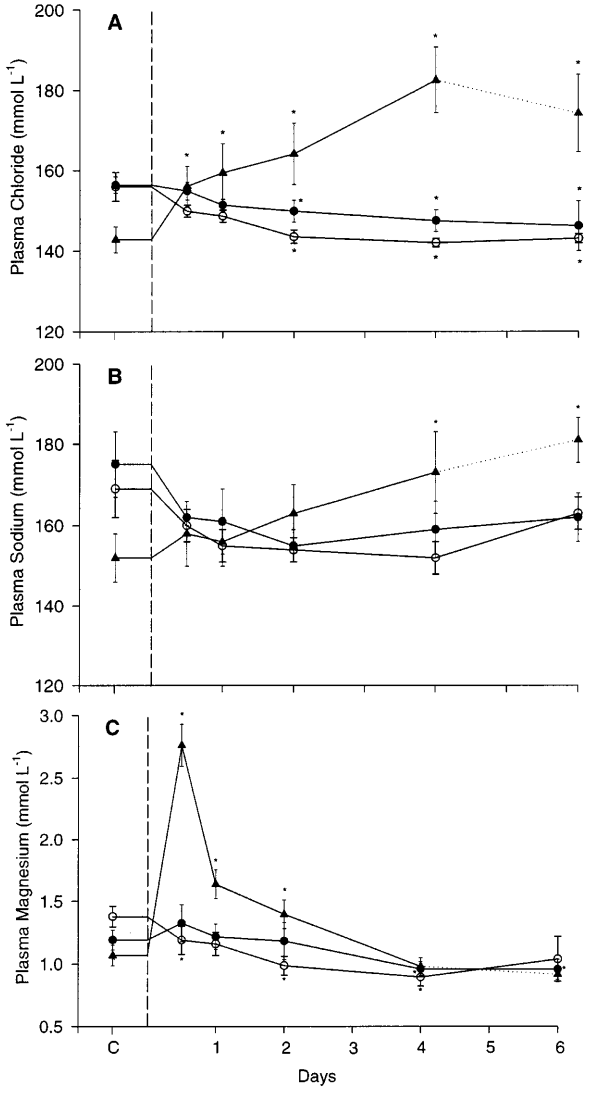

Fig. 3 Plasma chloride (**A**), sodium (**B**), and magnesium (**C**) concentrations in arterial blood plasma of starry flounder during exposure to 250 μg l^{-1} (*filled circles*) or 1000 μg l^{-1} (*filled triangles*) of silver ($AgCl_n^{n-1}$) for 6 days or during sham-treatment of unexposed individuals (*open circles*). Other details as in Fig. 1

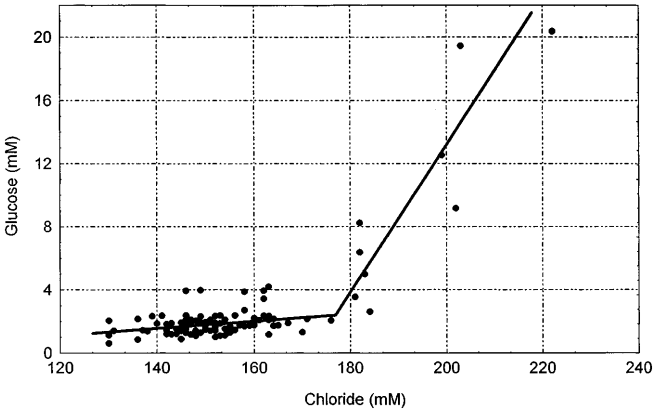

Fig. 4 Relationship between concentrations of glucose and chloride in arterial blood plasma as analyzed by piecewise regression. All measured plasma glucose concentrations at all sampling points were plotted against the corresponding plasma chloride concentrations. The relationship can be described by two lines with a breakpoint at 177 mM of chloride ($r = 0.88$)

172 mM (Na^+) from the same animal. In this experiment, there were no increases in either chloride or sodium levels in the plasma of fish exposed to the lower concentration of silver. In fact, plasma chloride and sodium levels decreased slightly during the experiment in both the 250-μg l^{-1} group and in the unexposed sham control group.

Notably, the plasma magnesium concentration did not increase (Fig. 3C) in concert with the progressive rise in plasma chloride and sodium levels in the 1000-μg Ag l^{-1} group. To the contrary, during the first 12 h of exposure, the magnesium level of the plasma increased nearly three-fold, but recovered and was back to pre-exposure levels on day 4 and day 6, the times when plasma chloride and sodium concentrations exhibited greatest elevations. As with chloride and sodium, the plasma magnesium concentrations in fish of the 250-μg Ag l^{-1} group and the sham control declined slowly during the experiment. The decreases in plasma chloride, sodium, and magnesium in the sham control and 250-μg Ag l^{-1} groups were likely due to a gradual acclimation to the experimental conditions as the experiment progressed. These two groups were tested simultaneously whereas the 1000-μg Ag l^{-1} exposure was conducted in a subsequent run and it can be noted that the pre-exposure values ("C") of electrolytes were higher in the two former groups than in the 1000-μg l^{-1} group.

In an attempt to relate ionoregulatory dysfunction to acute toxicity, we investigated the possible influence of plasma chloride on a stress parameter, namely the plasma glucose concentration. Figure 4 shows all plasma glucose measurements in all fish at all time points plotted against the corresponding plasma chloride concentrations. The plot was analyzed by piecewise linear regression (STATISTICA version 4.0) and it was found that 78% of the variation ($r = 0.88$) can be described by two straight lines, with a breakpoint at 177 mM [Cl^-], beyond which the glucose concentration rises drastically.

Although the measured disturbances of blood composition were small in the 250-μg Ag l^{-1} group, silver did accumulate in all silver-exposed fish. Plasma silver concentrations increased markedly in both exposure groups during the course of the experiment (Fig. 5A). Accumulation of silver in plasma appears to be dose-dependent although this is not fully clear because the fish of the 1000-μg Ag l^{-1} group started out with higher plasma silver concentration than the two other groups. Furthermore, the mortalities in the 1000-μg Ag l^{-1} group were likely to have eliminated the fish with highest silver accretion. Tissues analyzed for silver content on day 6 showed that the putative organs for silver uptake, gill and intestine, both accumulated high concentrations of silver (Fig. 5B). Although the absolute concentrations of silver in intestinal and branchial tissues were very

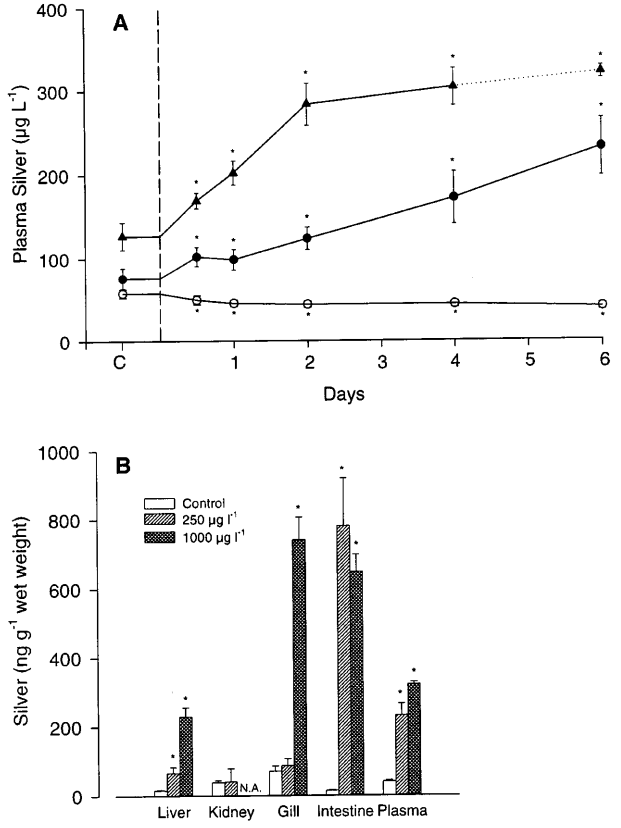

Fig. 5 **A** Silver concentrations in blood plasma of starry flounder during exposure to 250 µg l^{-1} (*filled circles*) or 1000 µg l^{-1} (*filled triangles*) of silver ($AgCl_n^{n-1}$) for 6 days or during sham-treatment of unexposed individuals (*open circles*). Other details as in Fig. 1. **B** Silver concentrations in tissues after 6 days of exposure to sham treatment (*open bars*, $n = 8$), 250 µg Ag l^{-1} (*hatched bars*, $n = 8$) or 1000 µg Ag l^{-1} (*crosshatched bars*, $n = 5$). *Bars* represent the mean and error bars SEM. All silver levels are given on wet-weight basis. Significant differences ($P < 0.05$) from the respective value of the sham-treated control group are indicated by asterisks

similar in fish of the 1000-µg Ag l^{-1} group, the increase in silver burden over the unexposed sham control (on day 6) was 50-fold in the intestine versus 10-fold in the gills (Fig. 5B). Furthermore, while the intestinal silver concentration of the 250-µg Ag l^{-1} group was as high as that of the 1000-µg Ag l^{-1} group, there was no significant increase in gill silver level of the 250-µg Ag l^{-1} group compared to the sham control. The silver concentration in liver tissue increased with increasing exposure concentration. Together with the highly elevated plasma silver levels (Fig. 5A), the hepatic silver content shows that substantial quantities of silver entered the starry flounders and accumulated in the animals in a dose-related manner. There was no increase in kidney silver concentration of fish exposed to silver at 250 µg l^{-1}. Kidneys from the 1000-µg Ag l^{-1} group were not analyzed for silver content.

In addition to the tissue residues of silver, gills and intestines from the 250-µg Ag l^{-1} group were assayed for Na^+/K^+-ATPase activities. The activity of this sodium transporting enzyme was twice as high in gill tissue of fish from the 250-µg Ag l^{-1} group as in gill tissue of the unexposed sham control (Table 4). Intestinal Na^+/K^+-ATPase activities were not statistically different between these two groups.

Table 4 Activity of sodium, potassium adenosine triphosphatase (Na^+/K^+-ATPase) in gill and intestine of starry flounder exposed to 250 µg Ag l^{-1} (as $AgCl_n^{n-1}$) for 6 days. Values are expressed as mean ± SEM ($n = 5$–8)

	Na^+/K^+-ATPase (pmol P_i mg^{-1} protein h^{-1})	
	Control	250 µg Ag l^{-1}
Gill	58.5 ± 17.6	118.7 ± 16.3*
Intestine	57.1 ± 8.5	70.9 ± 6.4

* Significantly different value from the unexposed control at the same time point

Ammonia, urea, and acid excretion

In the cannulation experiment, it was found that silver exposure caused a large, but transient, increase in plasma ammonia concentrations during exposure to silver at both 250 µg Ag l^{-1} and 1000 µg Ag l^{-1} (Fig. 1B). Therefore, in a separate experiment, the excretion of ammonia (NH_4^+ and NH_3) was measured during silver

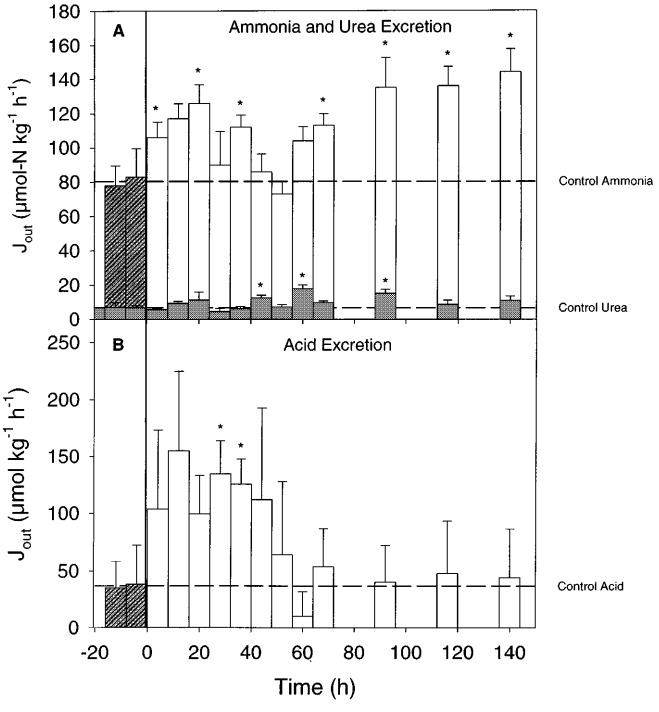

Fig. 6 Excretion rates (J_{out}) for **A** ammonia (*open bars*) and urea (*shaded bars*; expressed as N-units) and **B** acid equivalents to the water in starry flounders before (*hatched bars*) and during exposure to 250 µg Ag l^{-1} for 6 days. The *solid vertical line* shows the time point at which the exposure started ($t = 0$). The *dashed horizontal lines* indicate the average values for the two 8-h pre-exposure flux periods. *Bars* show the mean value at each flux period and *error bars* the SE ($n = 5$). Asterisks indicate means significantly different ($P < 0.05$) from those obtained before the exposure

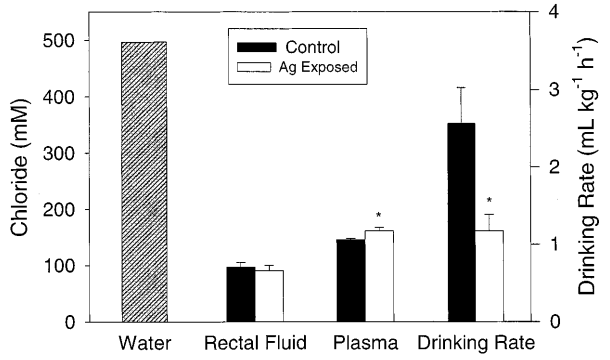

Fig. 7 Chloride concentrations in rectal fluid and blood plasma ($[Cl^-]$ in 32‰ seawater included for comparison), and drinking rate in starry flounder after exposure to 250 μg Ag l^{-1} (*open bars*) for 10 days or in starry flounder kept under identical conditions (*black bars*). *Bars* show the mean and *error bars* the SEM ($n = 5$). Asterisks indicate values statistically different ($P < 0.05$) from the control

exposure (250 μg Ag l^{-1}) to elucidate whether the peak in plasma ammonia could be attributed to an increased ammonia production (reflected in increased excretion) or if silver blocked ammonia excretion. Ammonia excretion increased during the first 24-h of exposure (Fig. 6A), reflecting the increased plasma ammonia levels noted in the cannulation experiment (Fig. 1B). During the following flux periods from 24 h to 56 h, ammonia excretion fell back to pre-exposure levels, followed by a gradual second increase that continued through the remainder of the experiment (Fig. 6A). Net acidic equivalent excretion, which may be partially coupled to ammonia excretion (Wood 1993), was markedly elevated during the initial 16 h of exposure followed by a recovery towards (and even below) pre-exposure rates (Fig. 6B). This recovery largely paralleled the temporary recovery in ammonia efflux, but in contrast to the ammonia excretion, acid excretion stayed at pre-exposure rates from 58 h into the exposure onwards.

Urea-N excretion, expressed as N-equivalents (Fig. 6A), was about 10% of ammonia-N excretion. Urea-N excretion exhibited a tendency for slight increase later in the period of silver exposure, significant at several flux periods between 40 h and 100 h.

Drinking rate

Drinking rate in starry flounders exposed to 250 μg Ag l^{-1} for 10 days was reduced by 54% compared to control fish (Fig. 7). In the same silver-exposed fish, a slight but highly significant ($P < 0.009$) increase in plasma chloride concentration was observed. The plasma chloride concentration of rectal fluid collected from the distal portion of the intestine was not affected by silver exposure at this level (Fig. 7). The measured silver concentration in the same rectal fluid of the silver-exposed starry flounders was 3.87 ± 0.86 μg l^{-1}, which is only 1.5% of the silver concentration in the imbibed seawater. This indicates that much of the silver imbibed is either absorbed into the animal or retained in the wall of the digestive tract. However, an alternative explanation is that the decrease in chloride concentration in the gut caused silver to precipitate out of solution as an insoluble, mono-chloro $AgCl_{(s)}$ (cerargyrite) complex.

Discussion

While most of the measured blood parameters were minimally affected by silver exposure, the effects on plasma ammonia and inorganic ion concentrations were substantial. Of these, only plasma sodium and chloride concentrations showed a progressive increase towards the point of death and correlated strongly with stress, as assessed by plasma glucose concentrations. Thus, silver seems to be an ionoregulatory toxicant to marine teleost fish. In this respect, the key toxic effect appears to be analogous to that in freshwater fish (Wood et al. 1996), but the mechanisms involved seem to be very different. While silver exposure causes strong and, at higher concentrations, even fatal inhibition of the branchial Na^+/K^+-ATPase in freshwater fish (Morgan et al. 1997; Wood et al. 1999), we found an *increase* in the Na^+/K^+-ATPase activity in starry flounders that were exposed to the sub-lethal concentration of silver (250 μg l^{-1}). This increased Na^+/K^+-ATPase activity could be interpreted as a compensation for a dysfunctional regulation of chloride and sodium (i.e. increased influx or decreased efflux) levels and an attempt to eliminate these ions by up-regulation of active branchial excretion. Although the chloride and sodium concentrations of the plasma did not increase during 6 days of exposure to 250 μg Ag l^{-1}, the same exposure level resulted in a significantly increased plasma chloride concentration in the experiment that was extended to 10 days. The mechanism behind this disturbed ionoregulation is less certain. The documented inhibition of drinking could have reduced water uptake resulting in a net loss of water. However, since water is taken up from the intestine by solute-linked (chloride and sodium) transport it is not obvious how a reduced water uptake would be linked to increases in plasma chloride and sodium levels. An as yet unexplored possibility is that silver interfered with ion excretion across the gills, e.g., blockage of the apical Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) chloride channel. Such a mechanism might explain the observation that silver had a greater effect on chloride than on sodium in the plasma. However, the fact that there was no detectable accumulation of silver in the gill tissue from fish exposed to 250 μg Ag l^{-1}, while the Na^+/K^+-ATPase activity in the same fish was doubled, seems to argue against a direct effect of silver on the gills at this level of exposure. In future studies, this problem may be addressed directly by exposing gills and gut separately to Ag and by measuring chloride and sodium effluxes across the gills.

The fact that plasma magnesium concentrations recovered to control levels during the period when plasma

chloride and sodium concentrations continued to increase is an important piece of evidence suggesting that silver did *not* just cause a general increase in the permeability of the gills for ions. The relative gradient of magnesium between seawater and blood plasma (approximately 50:1) is much greater than the relative gradients of chloride and sodium between seawater and plasma (approximately 3:1). Any generalized increase in permeability caused by silver exposure would be expected to have a much greater effect on plasma magnesium levels than on plasma chloride and sodium concentrations. Indeed, a general stress-induced dilation of the gills in response to the shock of exposure to 1000 μg Ag l^{-1} is the likely explanation for the initial "spike" observed in plasma [magnesium] at 12 h, which occurred well before plasma chloride and sodium levels became critical. The observation of declining or stable plasma magnesium levels later in the exposure indicate that specific effects on ion and water economy, as might be caused by impaired ion excretion and inhibited water absorption, were the likely causes of elevated plasma chloride and sodium concentration, not a general increase in gill permeability.

Actual water uptake from the gastrointestinal tract, and body water content were not measured in the present study so we cannot conclusively tie the reduced drinking rate to silver toxicity. However, the effect of silver on drinking has very recently been confirmed using a different species, the English sole (*Parophrys vetulus*; M. Grosell, G. De Boeck, O.E. Johannsson, and C.M. Wood, personal observation). Furthermore, the same investigators found strong evidence that silver is blocking both salt (chloride and sodium) and water uptake across the intestinal epithelium, the former providing the transepithelial osmotic gradient which drives the latter. In combination, all these pieces of information point towards the intestine as a major site for toxicity during exposure of marine fish to waterborne silver presented as $AgCl_n^{n-1}$. Interference with ion excretion through the gills may also contribute to silver toxicity, but as yet there is no direct evidence for such an effect.

Copper is chemically related to silver, and the mechanism of copper toxicity to freshwater fish is similar to that of silver. Just like silver, copper is a strong inhibitor of branchial sodium and chloride influx, by inhibition of Na^+/K^+-ATPase (Stagg and Shuttleworth 1982a, b; Laurén and McDonald 1985; Li et al. 1996). However, unlike silver, copper also stimulates large diffusive effluxes of sodium and chloride across the branchial epithelium of freshwater fish (Laurén and McDonald 1985). Copper and silver seem to act very similarly on marine fish as well. For both metals, the acutely lethal effect appears to be increases in plasma chloride and sodium concentrations along with a pronounced, but not lethal, hyperammoniaemia (Stagg and Shuttleworth 1982b; Wilson and Taylor 1993a; present study).

There are two major reasons why we do not believe that the elevation in plasma ammonia played a major role in the etiology of silver toxicity to starry flounders. First, although the plasma ammonia level in silver-exposed fish rose by a maximum 240% of the pre-exposure values, the peak was subsiding well before the onset of mortality. Second, the plasma ammonia concentration was not higher in the group exposed to the lethal 1000 μg Ag l^{-1} than in the group exposed to the sub-lethal 250-μg Ag l^{-1}. Thus, the response was not dose-dependent in this range of water silver concentrations although it might have been at concentrations lower than the ones tested. While certainly not the direct cause of death in the starry flounder, the effect on ammonia is physiologically interesting and may be toxicologically significant. In a recent study, Shaw et al. (1998) showed that addition of silver to the water induced mortality in the marine tidepool sculpin (*Oligocottus maculosus*) at water ammonia concentrations that otherwise caused no acute toxicity. Conversely, addition of ammonia to the water decreased both LC50 and LT50 values for silver. The physiological basis for these toxicological interactions is that ammonia excretion in fish is at least partly a passive process that is driven by the concentration gradient between ammonia in plasma and water (Wood 1993). An increase in the water ammonia concentration, therefore, impedes ammonia excretion, leading to accumulation of ammonia in the blood. As shown in the present study for the marine starry flounder, and by Webb and Wood (1998) for freshwater rainbow trout, waterborne silver also causes ammonia to accumulate in the fish, but by a different mechanism, involving a stimulation of metabolic ammonia production rate (see below). Thus, together ammonia and silver in the water could boost the plasma ammonia concentration to toxic levels even if each of the toxicants is present at levels that are individually sub-lethal. Furthermore, a sub-lethal elevation of plasma ammonia can have detrimental consequences for fish as elevated plasma ammonia has been shown to reduce swimming performance and cause early onset of fatigue (Beaumont et al. 1995).

Elevation of plasma ammonia seems to be a general effect of waterborne silver because it occurs in both freshwater and seawater fish (Webb and Wood 1998; present study). In the freshwater rainbow trout, the accumulation of ammonia in the plasma was paralleled by increases in ammonia excretion rate and plasma cortisol concentration. A likely explanation was that cortisol induced proteolysis with increases in production and excretion of ammonia as direct and secondary effects, respectively (van der Boon et al. 1991). The results from the present study indicate a more complex scenario in marine fish. In the starry flounder, the plasma ammonia concentration did not correlate with either plasma cortisol or plasma glucose concentrations, making the connection between cortisol and ammonia accumulation less obvious. Also, the measured efflux of ammonia did not entirely reflect the changes in plasma ammonia build-up, again suggesting that a more rapid ammonia synthesis rate was not the sole contributor to the observed peak in plasma ammonia. Instead, our results

hint that there may be a combination of stimulated synthesis and impaired excretion of ammonia as well as a slightly increased reliance on urea excretion as a mechanism for removal of N-waste. The increase in ammonia excretion during the first 24 h of exposure corresponds to the rise in plasma ammonia, suggesting an increase in ammonia production. However, while plasma ammonia levels remained high for at least another 24 h, the ammonia excretion quickly returned to pre-exposure values. These results are indicative of an inhibited ammonia excretion occurring at this stage of the exposure. Then, after 2 days of silver treatment, while the plasma ammonia levels were declining, there was a second increase in ammonia excretion and a small rise in urea excretion. The elevated ammonia excretion persisted through to the end of the experiment. The ammonia excretion rates in the later part of the exposure were 35–44% higher than the pre-exposure values and were probably involved in the restoration of plasma ammonia homeostasis.

Although mechanisms of ammonia excretion in fish remain a matter of controversy, there is a general consensus that the gill is the major site for ammonia efflux and that both NH_3 and NH_4^+ are eliminated across the gills (Wood 1993). Ammonia is produced metabolically as NH_3, which means that branchial efflux of NH_4^+ under most conditions has the effect of *net* acidic equivalent (H^+) excretion (Wood 1993). In this context it is interesting to note that while the first peak in ammonia excretion rate was paralleled by a large increase in H^+ excretion, the second increase in ammonia excretion occurred without a concomitant rise in H^+ excretion. In fact, H^+ excretion stayed at or below pre-exposure levels after day 2 of the exposure. A possible interpretation of these data, and also the observed peak in plasma ammonia, is that silver might have blocked the part of ammonia excretion that is tied to H^+ excretion (i.e. functional Na^+/NH_4^+ exchange and/or unmediated NH_4^+ diffusion) forcing a subsequent switch to alternative acid-base neutral modes of ammonia excretion (i.e. unmediated NH_3 diffusion and/or H^+/NH_4^+ exchange). A number of alternative interpretations are entirely possible, but a partial blockade of ammonia excretion with a delayed up-regulation of an alternative pathway would explain the transient increase in plasma ammonia concentration of silver-exposed starry flounders.

Summarizing data from a number of experiments in a variety of water chemistries, Hogstrand and Wood (1998) concluded that the accumulation of silver in internal organs of fish is three to four orders of magnitude slower (per unit gradient) from seawater (silver as $AgCl_n^{n-1}$) than freshwater (silver as Ag^+ or $AgCl^0_{(aq)}$). Nevertheless, the results from the present study clearly show that silver is able to enter marine fish. However, in contrast to freshwater fish in which silver very rapidly passes across the gills and is distributed to internal organs (Mayer et al. 1997), extremely high levels of the silver in the starry flounders were found in (or on) the intestinal tissue. Indeed, at the lower silver exposure level, 250 μg Ag l^{-1}, the gill tissue did not show any increased silver burden whereas the silver level in the intestine was dramatically elevated. These results would indicate that at lower concentrations of silver in the water, the intestine might be the primary route of silver uptake. There is an additional piece of rather surprising evidence that points towards an important role of the intestine in silver uptake. In starry flounders, exposed to 250 μg Ag l^{-1}, the silver concentration of the fluid in the rectal portion of the intestine was no more than 3.9 μg l^{-1}. Add to this that the volume of the rectal fluid is only about 15% of the imbibed water (Wilson et al. 1996) and it can be concluded that over 99% of the ingested silver did not leave the fish (at least not in dissolved form)! Whether all this extracted silver was actually absorbed, bound to mucus, or precipitated as cerargyrite ($AgCl_{(s)}$) in the intestinal lumen remains to be investigated. The key point here is that the intestine is extremely effective in retaining silver from the ingested water. Taken together with the high silver content of the intestinal tissue, this information implicates the intestine as the a probable major route of silver uptake in starry flounder.

Baseline values of measured parameters of blood composition corresponded well to those previously reported for starry flounder (Wood et al. 1977, 1979a, b). P_{aO_2} in control fish and the 250-μg Ag l^{-1} group stayed at the low resting level of 30–40 torr, which is typical of values for arterial blood in truly resting, non-stressed starry flounder (Wood et al. 1979a, b). The more than two-fold increase in P_{aO_2} of fish exposed to 1000 μg Ag l^{-1} and associated increase in both plasma and Hb-mediated O_2-transport shows that gas exchange across the gill epithelium was not a problem even in dying fish. The same conclusion was made for rainbow trout exposed to a lethal concentration of silver in freshwater (Wood et al. 1996; Webb and Wood 1998) or to copper in seawater (Wilson and Taylor 1993a). Elevated P_{aO_2} is a hallmark of stress disturbance in starry flounder (Wood et al. 1979a, b; Milligan and Wood 1987). Although not analyzed, the likely reason for the increase in P_{aO_2} was mobilization of catecholamines, which facilitate blood flow through the gills and may cause hyperventilation (Perry and Wood 1989). We did observe hyperventilation in fish of the 1000-μg Ag l^{-1} group, which surely contributed to the observed hyperoxemia. In spite of the increased blood oxygenation there was a small temporary increase on day 2 in total carbon dioxide concentration, and P_{aCO_2} was significantly elevated at the final sampling on day 6. The explanation for this slight CO_2 retention is unclear because the phenomenon normally occurs concomitantly with hypoxemia. Since oxygenation of the blood was not only adequate, but actually increased, there must be some other reason for the selective impediment of carbon dioxide excretion. One possible explanation could be inhibition of the zinc-dependent enzyme carbonic anhydrase, which is known to be sensitive to silver (Christensen and Tucker 1976; Morgan et al. 1997).

Most carbon dioxide (90–95%) in the blood is transported as HCO_3^- and converted to CO_2 at the gills by erythrocytic carbonic anhydrase (see Perry and McDonald 1993). Thus, branchial excretion of carbon dioxide is dependent upon the function of carbonic anhydrase. Reduction of erythrocytic carbonic anhydrase activity by experimental anaemia in starry flounder caused exactly the same phenomenon as observed here, CO_2 retention in the face of elevated P_{aO_2} (Wood et al. 1979b). Ultimately, measurements of carbonic anhydrase activity in red blood cells of starry flounders would tell whether or not the observed hypercapnia was caused by carbonic anhydrase inhibition.

In the present study we have provided novel data on the physiology of acute silver toxicity to a marine fish species. Silver has relatively low toxicity in seawater compared to environmentally relevant levels. In the present study, the threshold for acute effects was between 250 μg l^{-1} and 1000 μg l^{-1}. The US Environmental Protection Agency (EPA) acute criterion for silver in seawater is 2.3 μg l^{-1}, and measured concentrations in open oceans are in the range of 0.1–0.2 ng l^{-1} and in estuaries, 0.3–2.0 ng l^{-1} (Schafer 1995). Thus, the lower concentration of silver used in the present study was five orders of magnitude higher than levels that can be expected to be encountered in marine environments. Although many details remain to be sorted out, it seems very likely that osmo- and ionoregulatory dysfunctions are the major causes of acute silver toxicity to the starry flounder in seawater. Silver inhibits drinking and apparently obstructs the mechanism for water absorption from the intestinal lumen (M. Grosell, G. De Boeck, O.E. Johannsson, and C.M. Wood, personal observation). In addition to the proposed dehydration effect, inhibition of chloride and sodium efflux might explain the accumulation of these osmolytes in blood plasma, but there is currently no direct evidence to this regard. The key toxic effect of silver to seawater fish is analogous, but mechanistically different from the situation in freshwater where silver causes acute toxicity by inhibiting chloride and sodium uptake by the gills (Wood et al. 1996; Morgan et al. 1997). It should also be noted that it takes much greater concentrations of silver to elicit toxic ionoregulatory malfunction in seawater than in freshwater and this undoubtedly reflects the very different speciation of silver in seawater, where the high Cl^- concentration renders the free Ag^+ ion unavailable. In addition to the osmo- and ionoregulatory problems, silver causes a large increase in plasma total ammonia accumulation. Data from the present study would suggest that there are two mechanisms contributing to this ammonia build-up, one of them being stress-induced proteolysis and the other blockade of the portion of ammonia excretion that is coupled to H^+ efflux. Ammonia did not kill the starry flounders in the present study. However, because ammonia accumulation seems to occur at lower concentrations than those required to upset osmo- and ionoregulation, this effect might be of chronic importance, especially in ammonia-sensitive species.

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References

Adams NWH, Kramer JR (1998) Reactivity of Ag^+ ion with thiol ligands in the presence of iron sulfide. Environ Toxicol Chem 17: 625–629

Beaumont MW, Butler PJ, Taylor EW (1995) Plasma ammonia concentrations in brown trout (*Salmo trutta*) exposed to acidic water and sublethal copper concentrations and its relationship to decreased swimming performance. J Exp Biol 198: 2213–2220

Boon I van der, Thillart GEEJM van der, Addinte ADF (1991) The effects of cortisol administration on intermediary metabolism in teleost fish. Comp Biochem Physiol A 100: 47–53

Boutilier RG, Heming TA, Iwama GK (1984) Physico-chemical parameters for use in fish respiratory physiology. In: Hoar WS, Randall DJ (eds) Fish physiology. Academic Press, New York, pp 403–430

Calabrese A, Thurberg FP, Gould E (1977) Effects of cadmium, mercury, and silver on marine animals. Mar Fish Rev 39: 5–11

Cameron JN (1971) Rapid method for determination of total carbon dioxide in small blood samples. J Appl Physiol 31: 632–634

Christensen GM, Tucker JH (1976) Effects of selected water toxicants on the in vitro activity of fish carbonic anhydrase. Chem-Biol Interact 13: 181–192

Eisler R (1997) Silver hazards to fish, wildlife and invertebrates: a synoptic review. US Fish Wildl Serv Biol Rep 32, Washington DC

Ferguson EA, Leach DA, Hogstrand C (1996) Metallothionein protects against silver blockage of the Na^+/K^+-ATPase. In: Andren AW, Bober TW (eds) Proceedings of the 4th International Conference on the Transport, Fate, and Effects of Silver in the Environment, University of Wisconsin, Madison, WI, pp 191–200

Ferguson EA, Hogstrand C (1998) Acute silver toxicity to seawater acclimated rainbow trout: influence of salinity on toxicity and silver speciation. Environ Toxicol Chem 17: 589–593

Goss GG, Wood CM (1988) The effects of acid and acid/aluminum exposure in circulating plasma cortisol levels and other blood parameters in the rainbow trout, *Salmo gairdneri*. J Fish Biol 32: 63–76

Hogstrand C, Wood CM (1996) The physiology and toxicology of zinc in fish. In: Taylor EW (ed) Toxicology of aquatic pollution. Cambridge University Press, Cambridge, pp 61–84

Hogstrand C, Wood CM (1998) Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: implications for water quality criteria. Environ Toxicol Chem 17: 547–561

Hogstrand C, Galvez F, Wood CM (1996) Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. Environ Toxicol Chem 15: 1102–1108

Holliday CW (1985) Salinity-induced changes in Na,K-ATPase activity in the mud fiddler crab, *Uca pugnax*. J Exp Zool 233: 199–208

Ivancic I, Deggobis D (1984) An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. Water Res 18: 1143–1147

Jackim E, Hamlin JM, Sonis S (1970) Effects of metal poisoning on five liver enzymes in the killifish (*Fundulus heteroclitus*). J Fish Res Board Can 27: 383–390

Janes N, Richards J, Playle RC (1996) Influence of temperature and thiosulphate on active and passive uptake of silver by rainbow trout. In: Andren AW, Bober TW (eds) Proceedings of the 4th International Conference on the Transport, Fate and Effects of Silver in the Environment. University of Wisconsin, Madison, pp 231–235

Laurén DJ, McDonald DG (1985) Effects of copper on branchial ionoregulation in the freshwater rainbow trout, *Salmo gairdneri* Richardson. J Comp Physiol B 155: 635–644

Li J, Lock RA, Klaren PH, Swarts HG, Schuurmans SF, Wendelaar BS, Flik G (1996) Kinetics of Cu^{2+} inhibition of Na^+/K^+-ATPase. Toxicol Lett 87: 31–38

Mayer GD, Galvez F, Wood CM, Hogstrand C (1997) Uptake, accumulation and elimination of silver in juvenile rainbow trout. In: Andren AW, Bober TW (eds) Proceedings of the 5th International Conference on the Transport, Fate, and Effects of Silver in the Environment. University of Wisconsin, Madison, WI, 229–263

McDonald DG (1983) The interaction of calcium and low pH on the physiological responses of the rainbow trout, *Salmo gairdneri* I. Branchial and renal net ion and H^+ fluxes. J Exp Biol 102: 123–140

McDonald DG, Wood CM (1981) Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. J Exp Biol 93: 101–118

McDonald DG, Hobe H, Wood CM (1980) The influence of calcium on the physiological responses of the rainbow trout, *Salmo gairdneri*, to low environmental pH. J Exp Biol 88: 109–131

Milligan CL, Wood CM (1987) Effects of strenuous activity on intracellular and extracellular acid-base status and H^+ exchange with the environment in the inactive, benthic starry flounder, *Platichthys stellatus*. Physiol Zool 60: 37–53

Morgan IJ, Henry RP, Wood CM (1997) The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncothunchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. Aquat Toxicol 38: 145–163

Olsson P-E, Kling P, Hogstrand C (1998) Mechanisms of heavy metal accumulation and toxicity in fish. In: Langston WJ, Bebianno MJ (eds) Metal metabolism in aquatic environments. Chapman and Hall, London, pp 321–350

Perry SF, McDonald DG (1993) Gas exchange. In: Evans DH (ed) The physiology of fishes. CRC, Boca Raton, pp 251–278

Perry SF, Wood CM (1989) Control and coordination of gas transfer in fishes. Can J Zool 67: 2961–2970

Pilgaard LH, Malte H, Jensen FB (1994) Physiological effects and tissue accumulation of copper in freshwater rainbow trout (*Oncorhynchus mykiss*) under normoxic and hypoxic conditions. Aquat Toxicol 29: 197–212

Playle RC, Goss GG, Wood CM (1989) Physiological disturbances in rainbow trout (*Oncorhynchus mykiss*) during acid and aluminum exposures in soft water of two calcium concentrations. Can J Zool 63: 314–324

Price NM, Harrison PJ (1987) Comparison of methods for the analysis of urea in seawater. Mar Biol 94: 307–313

Purcell TW, Peters JJ (1998) Sources of silver in the environment. Environ Toxicol Chem 17: 539–546

Schafer MM (1995) Sampling and analytical techniques for silver in natural waters. In: Andren AW, Bober TW (eds) Proceedings of the 3rd International Conference on Transport, Fate and Effects of Silver in the Environment. University of Wisconsin, Madison, Wis. pp 99–112

Shaw JR, Hogstrand C, Price DJ, Birge WJ (1997) Acute and chronic toxicity to silver in marine fish. In: Andren AW, Bober TW (eds) Proceedings of the 5th International Conference on the Transport, Fate and Effects of Silver in the Environment. University of Wisconsin, Madison, WI, pp 317–324

Shaw JR, Wood CM, Birge WJ, Hogstrand C (1998) Toxicity of silver to the marine teleost the tidepool sculpin (*Oligocottus maculosus*): effects of salinity and ammonia. Environ Toxicol Chem 17: 594–600

Spry DJ, Wood CM (1984) Acid-base, plasma ion, and blood gas changes in rainbow trout during short term zinc exposure. J Comp Physiol B 154: 149–158

Spry DJ, Wood CM (1985) Ion flux rates, acid-base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. Can J Fish Aquat Sci 45: 2206–2215

Stagg RM, Shuttleworth TJ (1982a) The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (*Platichthys flesus* L.). J Comp Physiol 149: 83–90

Stagg RM, Shuttleworth TJ (1982b) The accumulation of copper in *Platichtys flesus* L. and its effects on plasma electrolyte concentrations. J Fish Biol 20: 491–500

Thurberg FP, Collier RS (1977) Respiratory response of cunners to silver. Mar Pollut Bull 8: 40–41

Tucker VA (1967) Method for oxygen content and dissociation curves on microliter blood samples. J Appl Physiol 23: 410–414

Watters KW Jr, Smith LS (1973) Respiratory dynamics of the starry flounder *Platichtys stellatus* in response to low oxygen and high temperatures. Mar Biol 19: 133–148

Webb NA, Wood CM (1998) Physiological analysis of the stress response associated with acute silver exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 17: 579–588

Wilson RW, Taylor EW (1993a) Differential responses to copper in rainbow trout (*Oncorhynchus mykiss*) acclimated to sea water and brackish water. J Comp Physiol B 163: 239–246

Wilson RW, Taylor EW (1993b) The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J Comp Physiol B 163: 38–47

Wilson RW, Gilmour KM, Henry RP, Wood CM (1996) Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid-base balance? J Exp Biol 199: 2331–2343

Wolf K (1963) Physiological salines for freshwater teleosts. Prog Fish Cult 25: 135–140

Wood CM (1993) Ammonia and urea metabolism. In: Evans DH (ed) The physiology of fishes. CRC, Boca Raton, pp 379–426

Wood CM, Milligan CL (1987) Adrenergic analysis of extracellular and intracellular lactate and H^+ dynamics after strenuous excercise in the starry flounder. Physiol Zool 60: 69–81

Wood CM, McMahon BR, McDonald DG (1977) An analysis of changes in blood pH following exhausting activity in the starry flounder, *Platichthys stellatus*. J Exp Biol 69: 173–185

Wood CM, McMahon BR, McDonald DG (1979a) Respiratory gas exchange in the resting starry flounder, *Platichthys stellatus*: a comparison with other teleosts. J Exp Biol 78: 167–179

Wood CM, McMahon BR, McDonald DG (1979b) Respiratory, ventilatory, and cardiovascular responses to experimental anaemia in the starry flounder, *Platichthys stellatus*. J Exp Biol 82: 139–162

Wood CM, Playle RC, Simons GG, Goss GG, McDonald DG (1988a) Blood gases, acid-base status, ions, and hematology in adult brook trout (*Salvelinus fontinalis*) under acid/aluminum exposure. Can J Fish Aquat Sci 45: 1586–1596

Wood CM, Simons GG, Mount DR, Bergman HL (1988b) Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 2. Blood parameters by cannulation. Can J Fish Aquat Sci 45: 1597–1605

Wood CM, Hogstrand C, Galvez F, Munger RS (1996) The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. The effects of ionic Ag^+. Aquat Toxicol 35: 93–109

Wood CM, Playle RC, Hogstrand C (1999) Physiology and modelling of the mechanisms of silver uptake and toxicity in fish. Environ Toxicol Chem 18: 71–83

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