

Sensitivity of the spiny dogfish (*Squalus acanthias*) to waterborne silver exposure

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Abstract

The physiological effects of waterborne silver exposure (added as AgNO₃) on spiny dogfish, *Squalus acanthias*, were evaluated at 30, 200 and 685 µg silver per l in 30‰ seawater. These concentrations cover the toxic range observed for freshwater teleosts, where silver is extremely toxic, to seawater teleosts which tolerate higher silver concentrations. However, these levels are considerably higher than those that occur in the normal environment. At 685 µg l⁻¹, dogfish died within 24 h. Causes of death were respiratory as well as osmoregulatory failure. Arterial P_aO₂ rapidly declined below 20 Torr, and blood acidosis (both respiratory and metabolic) occurred. Urea excretion increased dramatically and plasma urea dropped from 340 to 225 mM. There were pronounced increases in plasma Na⁺, Cl⁻, and Mg²⁺, indicative of ionoregulatory failure due to increased diffusive permeability as well as inhibited NaCl excretion. At 200 µg l⁻¹, fish died between 24 and 72 h of silver exposure. The same physiological events occurred with a small time delay. At 30 µg l⁻¹, effects were much less severe, although slight mortality (12.5%) still occurred. Respiratory alkalosis occurred, together with moderate elevations in plasma Na⁺ and Cl⁻ levels. Silver accumulated to the highest concentrations on gills, with only low levels in the intestine, in accord with the virtual absence of drinking. Na⁺/K⁺-ATP-ase activities of gill and rectal gland tissue were impaired at the highest silver concentration. Normal gill function was impaired due to swelling and fusion of lamellae, lamellar aneurism and lifting of the lamellar epithelium. Our results clearly indicate that this elasmobranch is much more sensitive (about 10-fold) to silver than marine teleosts, with silver's toxic action exerted on the gill rather than on the intestine, in contrast to the latter. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Although only limited information concerning silver toxicity in the marine environment is available, it is clear that marine teleosts are a lot more tolerant to silver than freshwater teleosts. The 96h LC50 values for freshwater teleost fish are situ-

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ated in the 5–70 $\mu\text{g l}^{-1}$ range, whereas the values for seawater teleost fish vary between 183 and 1200 $\mu\text{g l}^{-1}$ (Shaw et al., 1997, 1998; Ferguson and Hogstrand, 1998; Hogstrand and Wood, 1998). In freshwater rainbow trout, acute toxicity seems to be caused only by ionic Ag^+ and the main mechanism of toxicity is the disruption of branchial ion transport by inhibition of the Na^+/K^+ -ATP-ase activity, which results in death from the consequences of ionoregulatory failure (Wood et al., 1996, 1999; Morgan et al., 1997; Hogstrand and Wood, 1998; McGeer and Wood, 1998). One of the principal causes of the reduced toxicity in seawater is presumed to be the high chloride level, such that ionic Ag^+ is virtually absent, and even AgCl^0 levels do not exceed a few percent of the total (Webb and Wood, 2000). Instead, anionic Ag chlorides (AgCl_n^-) and possibly organothiol-bound Ag complexes dominate Ag speciation in marine surface waters (Wood et al., 1999). Unlike freshwater fish, marine teleosts actively drink the medium. Surprisingly, recent evidence suggests that silver effects on the gills of seawater fish are minimal, and that most of the toxic effects can be explained by actions on the gastrointestinal tract (Grosell et al., 1999; Hogstrand et al., 1999; Wood et al., 1999). Water uptake in the intestine is impaired both by inhibition of drinking, and by blocking of ion and water transport across the intestinal epithelium; the fish appear to die from dehydration (Grosell et al., 1999; Hogstrand et al., 1999). Net loss of water across the gills continues while replacement from intestinal absorption is blocked.

However, virtually nothing is known about the effects of silver on marine elasmobranchs. Since these animals (sharks, skates and rays) have a very different osmoregulatory strategy from marine teleosts (Shuttleworth, 1988; Evans, 1993), differences in toxic mechanism and sensitivity might well occur. The dominant site of extrarenal secretion of excess NaCl is the rectal gland, which opens into the posterior intestine just proximal to the cloaca (Evans, 1993). Since the elasmobranchs are slightly hyperosmotic compared with the seawater, they have no need for drinking to absorb water, and the intestine plays little role in osmoregulation. This hyperosmotic situation is

maintained by high levels of two organic solutes, urea and trimethylamine oxide (TMAO) in their plasma and cells. The urea concentration of elasmobranch blood would be fatal to most other vertebrates because of the destabilising effect of urea on most proteins, including enzymes. These fish are able to tolerate this uremia because TMAO is able to counteract these effects when present at approximately 50% of the concentration of urea (Yancey and Somero, 1980). Gill water permeability is high but electrolyte permeability is low. Elasmobranchs, therefore, achieve a net influx of water, and since plasma Na^+ and Cl^- levels are below those of seawater, also receive a small net influx of NaCl. However, branchial ion flux rates are normally much lower than in marine teleosts; the quantitative contribution of the elasmobranch gill to ion regulation is at best modest, with some evidence that a portion of an excess NaCl load can be excreted across mitochondria-rich cells in the gill epithelium (Shuttleworth, 1988; Evans, 1993). More importantly, the elasmobranch gill appears to be involved in acid–base regulation through the operation of coupled Na^+ /'acid' and Cl^- /'base' exchanges as in the gills of freshwater teleosts (Payan and Maetz, 1973; Bentley et al., 1976; Evans, 1982; Claiborne and Evans, 1992). Therefore, this unique osmoregulatory strategy means that in some respects marine elasmobranchs more closely resemble freshwater teleosts than seawater teleosts.

The limited data available so far on the uptake of silver by marine elasmobranchs are strikingly different from those on marine teleosts. Elasmobranchs may accumulate up to 20 times more total Ag in their livers than do teleosts during extended periods of exposure to sublethal concentrations of silver in seawater. This was first noticed by Pentreath (1977) in a study on the thornback ray (*Raja clavata*) in comparison to the plaice (*Pleuronectes platessa*), and confirmed in a recent study by Webb and Wood (2000) on the spiny dogfish (*Squalus acanthias*) and the long-nose skate (*Raja rhina*). In the latter investigation, both fish accumulated much more total Ag in their gills, but virtually none in the intestines, in comparison to several teleost species at the same silver concentrations.

The aim of the present study was to explore the effects of waterborne silver exposure on the physiology of the spiny dogfish at three different silver concentrations. We wanted to assess if damage to osmoregulatory processes is the main cause of death as in freshwater and seawater teleosts, or if other processes are involved. In this study, using cannulated spiny dogfish, we followed osmoregulatory and respiratory disturbances over time, and assessed silver accumulation as well as possible disturbances of some metabolic processes. Sharks were exposed to three different Ag concentrations (30, 200, 685 $\mu\text{g l}^{-1}$) added as silver nitrate, covering the toxic range observed for freshwater to seawater teleosts. Effects on survival rate, blood gasses and pH, plasma ions and metabolites, ammonia, urea and acid/base fluxes, tissue silver accumulation, and Na^+/K^+ -ATP-ase activity were followed. Also drinking rates were measured to determine if stress-induced drinking would occur.

2. Material and methods

Spiny dogfish (*S. acanthias*) were caught by either long lines or otter trawls in the vicinity of Bamfield, BC, Canada in the summer of 1997 and subsequently kept in a large concrete indoor tank (200 000 l) served with running aerated Bamfield Marine Station seawater (14°C, salinity 30‰). Dogfish caught by long lines were kept 1 month before the experiments began, and only fish with minor injuries were selected. Fish caught by otter trawls were kept at least 1 week before experiments began. Fish were fed twice a week with a mixture of marine teleosts (mainly sardines and flatfish).

2.1. Silver exposure

Adult dogfish with an average weight of 1706 g (1010–3020 g) were caught from the tank, and immediately anaesthetised in a 100 mg l^{-1} neutralised MS-222 seawater solution for surgery. Once anaesthetised, fish were placed on a wooden V-trough and gills were constantly irrigated with the anaesthetic throughout surgery. On the left

side of the body, approximately 5-cm anterior to the caudal fin, a small incision was made down to the vertebrae to expose the cartilaginous haemal canal, which was punctured with a #22 needle. Through this hole, the dogfish was provided with a cannula in the caudal artery (PE50 polyethylene tubing, filled with heparinised (50 i.u. ml^{-1}) dogfish saline (recipe as in Wood et al., 1994, but with urea level reduced to 325 mM), which was held in place by a sleeve of PE160 secured with two sutures to the skin. The wound was powdered with oxytetracycline to avoid infection and tightly closed with silk ligatures. Fish were then allowed to recover from surgery overnight in covered wooden fish boxes, which were coated with polyurethane. The boxes were 105 cm in length, 16.5 cm in width and 25 cm in height, and contained 32 l Bamfield Marine Station seawater (14°C, 30‰) with a flow-through of 1 l min^{-1} and perimeter aeration over the complete length of the box.

Four series were performed, using nominal total silver concentrations of 0, 35, 200, and 700 $\mu\text{g l}^{-1}$. *N* numbers of successfully cannulated dogfish were 4, 6, 8 and 4, respectively. Silver exposure of the shark began by adding these nominal concentrations as AgNO_3 from a stock solution (Fisher, analytical grade) to the fish box. During the control and exposure period, flow-through was switched off and the system was static (with aeration maintained) to allow measurement of acid–base exchange and N-waste (urea, ammonia) efflux to the water. Fish boxes were flushed every 12 h to renew the seawater, and subsequently spiked again with the appropriate amount of silver from the AgNO_3 stock solution. Flushing of the boxes consisted of three consecutive series of lowering of the water level halfway so that the dogfish remained submerged at all times, then filling back to 32 l. Water samples for determination of silver, ammonia, urea and excreted base content were taken at the beginning and end of each 12-h period of the exposure. For the four series of nominal total silver concentrations of 0, 35, 200, and 700 $\mu\text{g l}^{-1}$, actual measured silver concentrations in the water of the exposed fish were 0 (i.e. below detection limit of 0.1 $\mu\text{g l}^{-1}$), $30 \pm 1 \mu\text{g l}^{-1}$ ($N = 75$); $200 \pm 5 \mu\text{g l}^{-1}$ ($N = 60$)

and $685 \pm 30 \mu\text{g l}^{-1}$ ($N = 25$), respectively (mean \pm S.E.M., $N =$ number of measurements). The decline in silver concentration between the start and end of the 12-h periods did not exceed 14% in the highest silver concentrations, and was less than 4% in the lower exposure concentrations.

In each series, blood samples (1 ml) were taken 12 h before exposure (control) and after 12, 24, 48, 72 and 120 h of exposure, or until the sharks had died. At each time, the volume of the blood was replaced with non-heparinised dogfish saline. At the end of each exposure, surviving fish were quickly sacrificed by an overdose of neutralised MS-222. Tissues were taken and rinsed in dogfish saline, immediately frozen in liquid nitrogen, and then stored at -80°C for later determination of silver content (gill, muscle, intestine, liver, bile, kidney, rectal gland) and Na^+/K^+ -ATP-ase activity (gill, intestine, rectal gland). For the two highest silver concentrations, where no shark survived the experiment, six extra non-cannulated dogfish per concentration were exposed in the same manner (mean weight, 1173 ± 105 g). For the highest silver concentration ($685 \mu\text{g l}^{-1}$) this experiment was stopped after 12 h of exposure, for the group exposed to $200 \mu\text{g l}^{-1}$ after 36 h of exposure, time points at which the dogfish started to die in the experiment on the cannulated dogfish. Fish were killed and sampled as described above. For these two exposure groups, as well as for the control group, one gill arch was rinsed and fixed in Bouin's solution for determination of gill morphology. Gill filaments were embedded in paraffin and cut at $6 \mu\text{m}$. Microscopic slides were coloured with the classic hemalun-eosin stain for light microscopy.

2.2. Analytical procedures

Blood samples were immediately analysed for arterial oxygen pressure (P_{aO_2}), pH_{a} , hematocrit and hemoglobin content. The remainder of the blood sample was immediately centrifuged (5 min at $10\,000 \times g$), and subsamples were taken for determination of the levels of Cl^- , ammonia, urea, glucose, lactate, Ag, and cations in the plasma. Plasma $[\text{Cl}^-]$ was analysed using coulometric titration (Radiometer CMT-10), while sub-

samples for the other measurements were frozen for later determination. Blood samples were analysed for P_{aO_2} using a micro-oxygen electrode (Radiometer E5046). pH was measured with a micro-capillary pH electrode (Radiometer G279/G2 plus E5021) coupled to a PHM71 meter. Both electrodes were kept at 14°C by a water jacket perfused with Bamfield Marine Station seawater. Hematocrit was determined by centrifuging three stoppered and heparinised capillaries at $2000 \times g$ for 2 min, and measuring the percent of red blood cells. These capillaries were subsequently kept at 4°C for determination of total plasma CO_2 and plasma proteins after the sampling period (maximum one and a half hours after first sampling). Total plasma CO_2 was determined using a Corning total CO_2 analyser (CMT965, Corning Medical and Scientific). P_{aCO_2} was calculated using the solubility of carbon dioxide (α_{CO_2}) and the apparent pK (pK_{app}) for dogfish plasma according to Boutilier et al. (1984),

$$P_{\text{aCO}_2} = \frac{C_{\text{CO}_2}}{\alpha_{\text{CO}_2}(10^{\text{pH} - \text{pK}_{\text{app}}} + 1)}$$

with C_{CO_2} being total plasma CO_2 . Plasma HCO_3^- content was calculated as the difference between total plasma CO_2 and $\alpha_{\text{CO}_2} \cdot P_{\text{aCO}_2}$.

Plasma proteins were determined using a Goldberg refractometer (American Optical TS meter), with correction for the higher ionic strength of dogfish plasma, using elasmobranch saline as a reference. For determination of hemoglobin concentration, $20 \mu\text{l}$ of blood was added to a vial containing 5 ml of Drabkin's reagent (Sigma kit 527-A) and assayed spectrophotometrically as cyanmethemoglobin at 600 nm, with human hemoglobin as a reference (Sigma Standard 527-30). Gill, intestine and rectal gland were homogenised in and diluted with ice-cold homogenisation buffer (0.25 mol l^{-1} sucrose, 6 mmol l^{-1} EDTA) until protein levels were in the range between 0.1 and 0.5 mg ml^{-1} (Bradford reagent for protein determination: Sigma kit B6976). Na^+/K^+ -ATP-ase activity was evaluated in the homogenates as K^+ -dependent, ouabain sensitive ATP hydrolysis, using the method of Holliday (1985).

Ammonia in water samples was determined using the salicylate-hypochlorite method (Verdouw et al., 1978), and in plasma samples using the Sigma enzymatic kit 171-A. Urea was analysed with the diacetyl monoxime method (Price and Harrison, 1987). Plasma lactate was determined using the Sigma 826 diagnostic kit adapted for microtiterplate readings, while plasma glucose was determined using the Sigma 16-10 diagnostic kit. Water samples were analysed for base content by titration, using Gilmont micro-burettes filled with 0.02 N HCl. Water samples (10 ml) were continuously bubbled with CO₂ free air, titrated down to pH 4.2 in order to remove all HCO₃⁻ and CO₃²⁻ as gaseous CO₂, allowed to equilibrate (15 min) and then carefully titrated further down to a pH 4.00. 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). Base content was calculated by the amount of acid necessary to reach pH 4.00. Branchial net fluxes (J_{net}) over any interval were calculated as,

$$J_{\text{net}} = \frac{(C_i - C_f)V}{Wt}$$

where C_i and C_f refer to initial and final concentrations (in $\mu\text{equiv ml}^{-1}$); V is the volume of the system (ml); W is the body mass (kg) and t is the elapsed time (h). Thus net losses by the animal are negative, net gains are positive. By reversing the C_i and C_f , the net titratable acid flux was calculated from the titratable base concentrations. The sum of the titratable acid and ammonia fluxes gives the net flux of acidic equivalents (net H⁺ flux) (Wood, 1988).

Total silver concentrations were determined in plasma diluted with 1% HNO₃ (Fisher, trace metal grade) using graphite furnace atomic absorption spectroscopy (Varian AA-1275 with GTA atomiser) with a 10 μl injection volume, N₂ gas, and standard operating conditions as documented by the manufacturer. Tissue samples were dissolved in 2 ml of 70% HNO₃ (w/w, Fisher, trace metal grade) plus 0.75 ml of 10% H₂O₂ (w/w, Fisher, trace metal grade) at 120°C until total digestion occurred, acid was allowed to

evaporate and the samples were rediluted to the appropriate volume with 1% HNO₃. Seawater samples were acidified by adding 100 μl of 10% HNO₃ to 900 μl of seawater. The Ag analysis was calibrated against standards of known [Ag] in the appropriate media (seawater, dogfish saline, tissue) since matrix components, principally related to osmolarity, interfered with Ag light absorption. Cations in plasma were analysed, after appropriate dilution in 1% HNO₃ by standard atomic absorption spectroscopy (Varian 1275), again using standard operating conditions.

2.3. Drinking experiment

Drinking rate of spiny dogfish was determined in a static system, following a similar protocol to that of Wilson et al. (1996), for six non-exposed (weight range, 242–283 g; mean weight, 257 g) and six silver-exposed juvenile sharks (weight range, 235–340 g; mean weight, 269 g). The fish were held in two separate 150 l PVC tanks filled with 100 l of Bamfield Marine Station seawater (14°C, salinity 30‰). Exposure to 100 $\mu\text{g l}^{-1}$ of silver, added as AgNO₃ (Fisher, analytical grade) in one of the tanks, began 14 h before the start of the drinking measurement. The other tank served as the non-exposed group. At the beginning of the experiment, water of both groups was renewed, spiked with 5 $\mu\text{Ci l}^{-1}$ [³H]polyethylene glycol-4000 (PEG-4000, specific activity, 2050 $\mu\text{Ci g}^{-1}$, NEN-Dupont) and the silver concentration was again brought to 100 $\mu\text{g l}^{-1}$ for the silver-exposed group. Reference water samples (5 ml) were taken in triplicate at the beginning and end of the experiment. Measured silver concentration was $95.2 \pm 5.8 \mu\text{g l}^{-1}$. The PVC tanks were kept covered during the experiments to minimise stress. After 6 h of exposure, the experiment was ended and fish were carefully removed from the tanks and sacrificed by a quick blow on the head. They were briefly rinsed in seawater without PEG-4000, a blood sample was obtained by caudal puncture and plasma was obtained by centrifugation at $10\,000 \times g$ for 2 min (to check for PEG-absorption), and rectal fluid was collected with a 1 ml syringe (to check if any imbibed PEG had passed out of the anus). The gastro-intestinal tract (GIT)

was clamped at the start of the oesophagus and immediately anterior to the cloaca to prevent loss of content, carefully removed and weighed. The entire GIT was homogenised in 4 volumes of 10% of perchloric acid. An aliquot of the homogenate was centrifuged ($5000 \times g$ for 5 min). From each fish, β radioactivity was determined in multiple aliquots of 0.5–1.5 ml of the clear-protein free supernatant, 50 μ l of the plasma and 50 μ l of the rectal fluid using liquid scintillation counting on an LKB Rackbeta 1017 counter. 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). Quench correction was performed by the sample channels method and checked by internal standardisation. 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.

2.4. Statistics

All values are mean values \pm S.E.M. Water, blood and plasma values were analysed for significant differences ($P < 0.05$) using a paired *t*-test for the group exposed to the highest Ag level where only the control sample and the first experimental sample were completed before death occurred, or a repeated measures analysis of variance (ANOVA) for the other exposure groups. Values for tissue silver and Na^+/K^+ -ATP-ase were analysed using an ordinary ANOVA. In both cases the ANOVA was followed by the Dunnett comparison post test if significant differences were found (GraphPad In-stat 3.01, GraphPad Software Inc.), comparing each group to its own pre-exposure control value.

3. Results

For the all parameters shown in the Figs. 1, 2, 4, 5 and 7, data for the control group (not exposed to silver) were stable throughout the experiment except from a minor increase in plasma Cl^- concentration after 24 h ($P < 0.05$). Control data are, therefore, shown as dotted lines in these

figures while mean values (\pm S.E.M., $N = 24$) of all measurements on the four control fish are displayed in Table 1.

Mortality of the dogfish at the two highest Ag concentrations was substantial (Fig. 1). At $685 \mu\text{g l}^{-1}$ Ag, all dogfish died within 24 h after the exposure started. At $200 \mu\text{g l}^{-1}$ Ag, all dogfish died between 36 and 72 h, while mortality after the 120 h of exposure to $30 \mu\text{g l}^{-1}$ Ag was 12.5%. During the exposure to the two highest silver concentrations, a substantial decline in arterial blood pH was observed, from 7.86–7.92 to 7.25–7.31 in the $685 \mu\text{g l}^{-1}$ Ag and the $200 \mu\text{g l}^{-1}$ Ag exposure group, respectively (Fig. 2b). The $30 \mu\text{g l}^{-1}$ Ag exposure group showed an increase in arterial blood pH rather than a decrease, with levels reaching up to 8.17 after 48 h of Ag exposure, with a later return to pre-exposure values. The drop in pH_a in the two highest exposure groups was coupled to a decrease in arterial oxygen pressure ($P_a\text{O}_2$), which dropped below 20 Torr within the first 12 h of exposure to $685 \mu\text{g l}^{-1}$ of silver (Fig. 2a). The group exposed to $200 \mu\text{g l}^{-1}$ Ag showed the same decrease in $P_a\text{O}_2$ levels, but only after 24–48 h of exposure. In the lowest exposure group ($30 \mu\text{g l}^{-1}$ Ag) and the control group this decline was not observed, and a small increase rather than a decrease was observed after 48 h of exposure to the low Ag levels. Calculated $P_a\text{CO}_2$ increased significantly for the

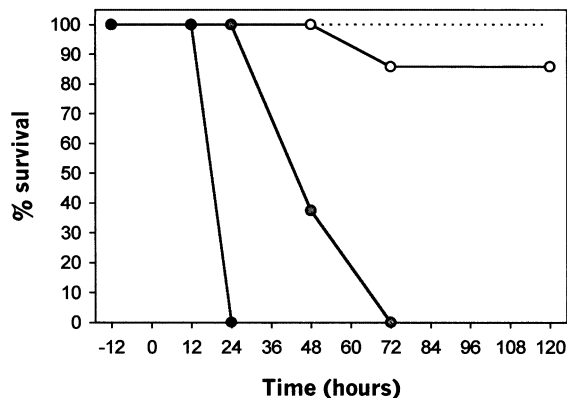


Fig. 1. Survival rate (% survival) of spiny dogfish exposed to different Ag concentrations, dotted line, $0 \mu\text{g l}^{-1}$; white circles, $30 \mu\text{g l}^{-1}$; grey circles, $200 \mu\text{g l}^{-1}$; black circles, $685 \mu\text{g l}^{-1}$.

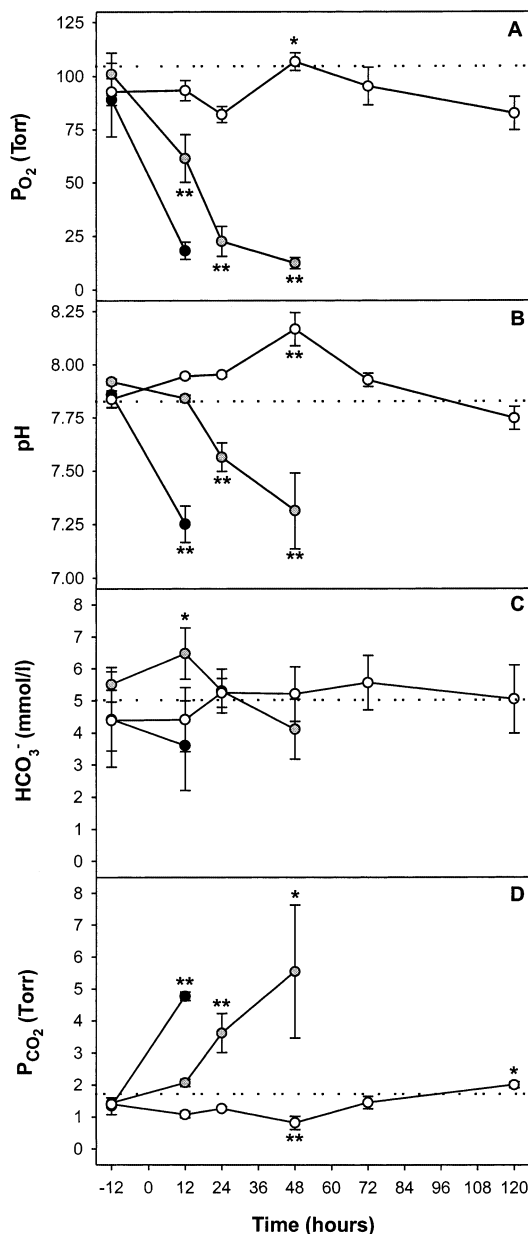


Fig. 2. Arterial blood P_aO_2 , (A), pH_a (B) HCO_3^- (C) levels and P_aCO_2 of spiny dogfish exposed to different Ag concentrations: white circles: $30 \mu g.L^{-1}$; grey circles: $200 \mu g.L^{-1}$; black circles: $685 \mu g.L^{-1}$ (Mean values \pm SEM, significant differences relative to the pre-exposure value at $-12h$ are indicated: *, $P < 0.05$; **, $P < 0.01$, mean data for the control group (non-exposed) are indicated by the dotted line).

two highest exposure groups, while in the lowest exposure group, a small decrease was observed

after 48 h of exposure (Fig. 2d), i.e. reciprocal changes to those in P_aO_2 (Fig. 2a). Despite these pronounced changes in pH and P_aCO_2 , HCO_3^- concentrations in arterial blood remained reasonably stable, except for a small increase in the intermediate exposure group at 12 h (Fig. 2c).

Due to the repeated blood sampling, hematocrit values decreased significantly in the control and the lowest exposure, but in the two highest exposure groups hematocrit values increased rather than decreased (Fig. 3). No significant changes were observed in any treatment group in blood hemoglobin (from 3.7 ± 0.2 ($N = 22$) to 3.3 ± 0.4 mM ($N = 16$) at the last sampling period overall) or plasma protein content (from 3.0 ± 0.1 ($N = 22$) to 2.7 ± 0.2 g 100 ml^{-1} ($N = 16$) at the last sampling period overall).

Plasma glucose decreased in all the Ag exposure groups (Fig. 4a), but in the lowest exposure group glucose concentrations rebounded, and returned to normal levels after 72 h of Ag exposure. Lactate levels showed a steep increase in the two highest Ag exposure groups (Fig. 4b), but remained low and constant in the lowest exposure and control group. Whereas plasma ammonia levels remained stable around 0.15 ± 0.06 mM (re-

Table 1

Control values (mean values \pm S.E.M., $N = 24$ measurements on four fish) for the different parameters represented as dotted lines in the graphs

| | Mean value | S.E.M. | Unit |
|-----------|------------|------------|-------------------------|
| PH_a | 7.83 | ± 0.02 | |
| P_aO_2 | 104.7 | ± 2.7 | Torr |
| P_aCO_2 | 1.71 | ± 0.07 | Torr |
| HCO_3^- | 5.02 | ± 0.11 | mM |
| Ht | 13.92 | ± 0.43 | % |
| Hb | 3.72 | ± 0.20 | mM |
| Cl^- | 244 | ± 1.31 | mM |
| Na^+ | 246.7 | ± 3.8 | mM |
| K^+ | 3.17 | ± 0.17 | mM |
| Mg^{2+} | 1.60 | ± 0.09 | mM |
| Ca^{2+} | 3.13 | ± 0.05 | mM |
| Lactate | 0.10 | ± 0.04 | mM |
| Glucose | 6.38 | ± 0.46 | mM |
| Ammonium | 0.15 | ± 0.06 | mM |
| Urea | 380.8 | ± 5.3 | mM |
| Protein | 3.16 | ± 0.09 | g 100 ml^{-1} |

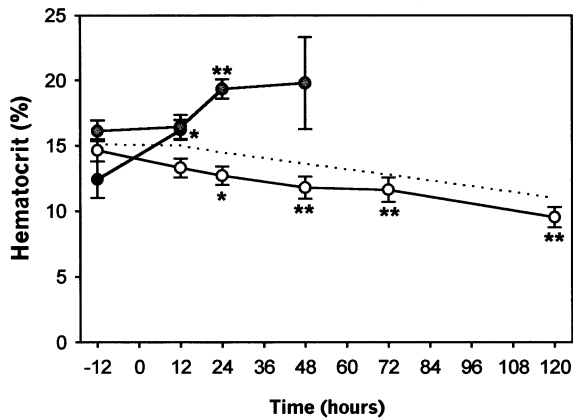


Fig. 3. Arterial blood hematocrit levels of spiny dogfish exposed to different Ag concentrations, white circles, $30 \mu\text{g l}^{-1}$; grey circles, $200 \mu\text{g l}^{-1}$; black circles, $685 \mu\text{g l}^{-1}$ (mean values \pm S.E.M.; significant differences relative to the pre-exposure value at $t = -12$ h are indicated, *, $P < 0.05$; **, $P < 0.01$). Mean data for the control group (non-exposed) are indicated by the dotted line, and were significantly decreased compared with $t = -12$ h at $t = 120$ h ($P < 0.01$).

sults not shown), plasma urea levels declined quickly in the two highest exposure groups, with decreases reaching 35–40% prior to death (Fig. 4c). This was reflected in the urea excretion rates, which increased to about $2000 \mu\text{mol kg}^{-1} \text{h}^{-1}$, 8-fold their original values (Fig. 5). In marked contrast, ammonia fluxes remained low and constant ($< 40 \mu\text{mol kg}^{-1} \text{h}^{-1}$; Fig. 6). No significant differences were found in the net acid–base exchange with the water (Fig. 6), but where control levels remained around $1.21 \pm 13.34 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ($N = 24$ measurements on the four control fish), a net H^+ influx from the water seemed to occur at the two highest Ag exposures.

Dogfish did drink very slightly at a rate of $0.133 \pm 0.011 \text{ ml kg}^{-1} \text{h}^{-1}$ at a silver concentration of $95 \mu\text{g l}^{-1}$. This drinking rate was not stress-induced since drinking rates in the Bamfield Marine Station seawater (salinity 30‰) for the control group were similar at $0.163 \pm 0.017 \text{ ml kg}^{-1} \text{h}^{-1}$. No absorption of PEG-4000 into the bloodstream or passage into the rectal fluid was detected over the 6-h measurement period.

Plasma ion levels increased due to silver exposure (Fig. 7). Cl^- concentrations were elevated in all three exposure groups and Na^+ concentrations

in the two highest exposure groups by about 20–30%. Mg^{2+} concentrations showed an extremely marked elevation ($\sim 500\%$), but only in the two highest exposure groups. For plasma K^+ and Ca^{2+} , the rise was only significant after 48 h of exposure to $200 \mu\text{g l}^{-1}$ Ag.

Despite the short exposure period (all fish died within 24 h of exposure) total Ag concentrations

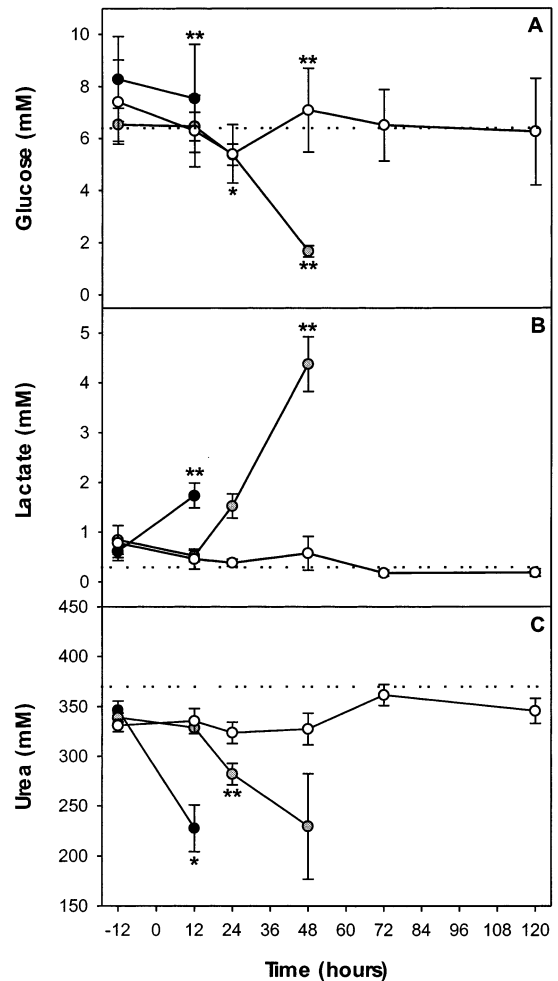


Fig. 4. Plasma glucose (A), lactate (B) and urea (C) levels of spiny dogfish exposed to different Ag concentrations: white circles: $30 \mu\text{g.L}^{-1}$; grey circles: $200 \mu\text{g.L}^{-1}$; black circles: $685 \mu\text{g.L}^{-1}$ (Mean values \pm SEM, significant differences relative to the pre-exposure value at -12 h are indicated: *, $p < 0.05$; **, $p < 0.01$, mean data for the control group (non-exposed) are indicated by the dotted line).

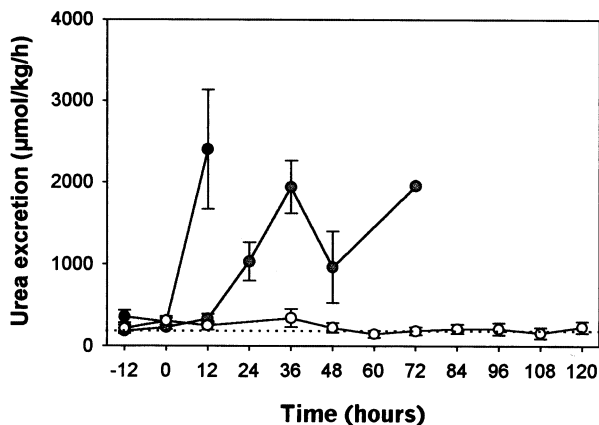


Fig. 5. Urea excretion of spiny dogfish exposed to different Ag concentrations, white circles, $30 \mu\text{g l}^{-1}$; grey circles, $200 \mu\text{g l}^{-1}$; black circles, $685 \mu\text{g l}^{-1}$ (mean values \pm S.E.M.; mean data for the control group (non-exposed) are indicated by the dotted line).

increased significantly in all tissues except muscle tissue when fish were exposed to $685 \mu\text{g l}^{-1}$ Ag (Table 2). In all exposures, the gills accumulated more Ag than any of the other tissues sampled. Note, however, the significant endogenous Ag concentrations in some of the tissues of the non-exposed control fish, most notably the gills themselves, the kidney, and the bile. Notably, intestine and kidney exhibited no increase at lower silver exposure levels, despite the slight drinking of the medium which was detected. At an exposure concentration of $200 \mu\text{g l}^{-1}$ Ag, silver accumulated in gill, rectal gland and liver tissue while at the lowest silver concentration the accumulation occurred in gill and liver tissue only. Na^+/K^+ -ATPase activity was impaired (by 20–25%) in gill and rectal gland at the highest Ag exposure concentration only (Fig. 8).

When studying gill morphology (Fig. 9), effects observed in the groups exposed to 685 and $200 \mu\text{g l}^{-1}$ Ag were substantial — swelling of epithelial cells, fusion of lamellae, lamellar aneurism, and lifting of the lamellar epithelium away from pillar cells were observed.

4. Discussion

In freshwater environments, the toxic action of silver is primarily caused by the Ag^+ ion (Terhaar et al., 1972; Leblanc et al., 1984; Hogstrand et al., 1996) and the addition of Cl^- , with formation of AgCl and AgCl_2^- as a consequence, decreases the toxicity of AgNO_3 , at least in the rainbow trout (Hogstrand et al., 1996; Galvez and Wood, 1997; McGeer and Wood, 1998). In the marine environ-

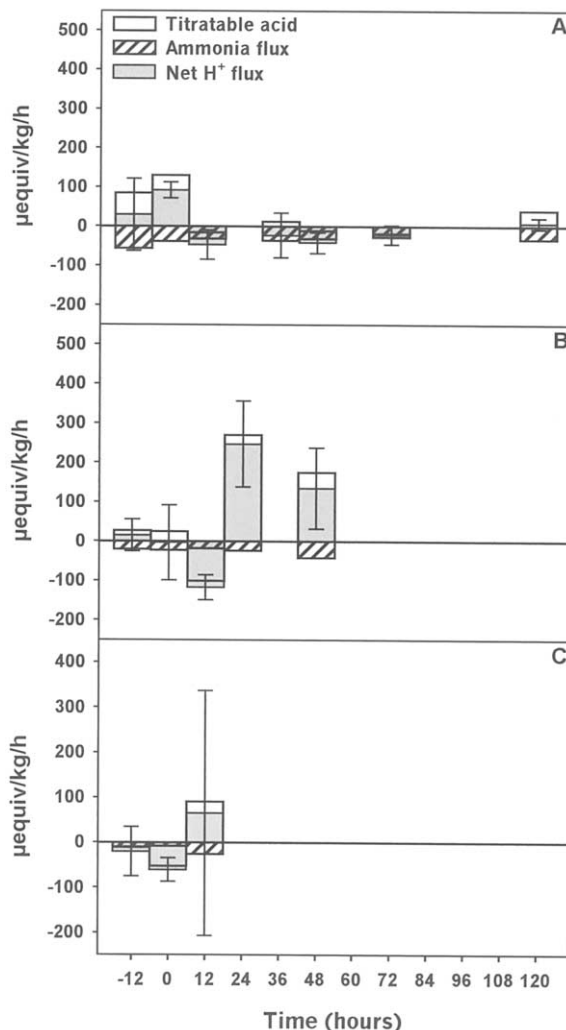


Fig. 6. Acid–base balances of spiny dogfish exposed to different Ag concentrations, (A) 30 ; (B) 200 ; (C) $685 \mu\text{g l}^{-1}$ (mean values for titratable acid and ammonia flux, mean values \pm S.E.M. for net H^+ flux).

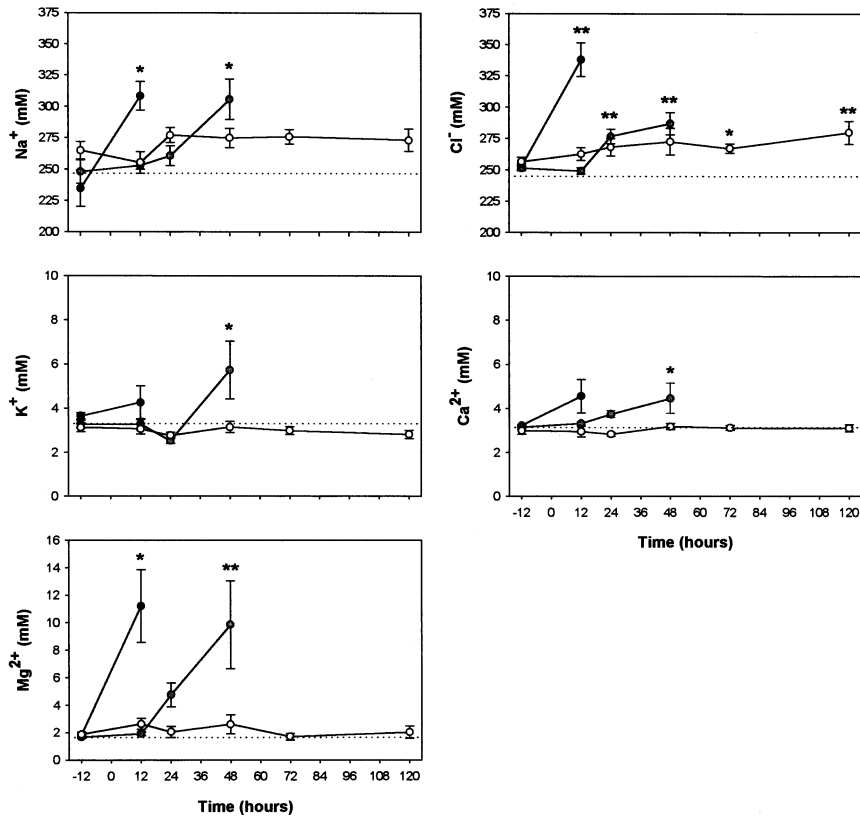


Fig. 7. Plasma ion levels of spiny dogfish exposed to different Ag concentrations, white circles, $30 \mu\text{g l}^{-1}$; grey circles, $200 \mu\text{g l}^{-1}$; black circles, $685 \mu\text{g l}^{-1}$ (mean values \pm S.E.M.; significant differences relative to the pre-exposure value at -12 h are indicated, *, $P < 0.05$; **, $P < 0.01$; mean data for the control group (non-exposed) are indicated by the dotted line).

ment ionic Ag^+ is virtually absent due to the abundance of chloride, resulting in mainly anionic silver chlorides (Wood et al., 1999; Webb and Wood, 2000). Therefore, toxic actions in the marine environment are expected to be a lot lower than in freshwater. Indeed, in contrast to findings in freshwater fish, exposure of a marine teleost fish (the starry flounder, *Platichthys stellatus*) to sublethal concentrations up to $250 \mu\text{g l}^{-1}$ did not disturb Na^+ and Cl^- plasma concentrations (Hogstrand et al., 1999). At $1000 \mu\text{g l}^{-1}$ Ag, plasma Na^+ and Cl^- did rise, indicating ionoregulatory disruption, but these teleosts survived at least 96 h.

However, in the present study using elasmobranch dogfish, a whole series of deleterious toxic effects leading to death within 36–72 h were

revealed at silver concentrations of only $200 \mu\text{g l}^{-1}$, and at $685 \mu\text{g l}^{-1}$, all dogfish died within 24 h with massive internal disturbances. These effects occurred despite the fact that these marine sharks were mainly exposed to anionic Ag chlorides (approximately 60% as AgCl_2^- , 20% as AgCl_3^{2-} , and 20% as AgCl_4^{3-} with less than 2% as AgCl^0 , by MINQEL + geochemical speciation modelling; Schecher and McAvoy, 1991). It is obvious that spiny dogfish are much more sensitive to silver intoxication than are marine teleosts. In the literature, 96h LC50 values for adult marine teleost fish in full strength seawater (30–35‰) vary between $664 \mu\text{g l}^{-1}$ for tidepool sculpins (*Oligocottus maculosus*, Shaw et al., 1998), $800 \mu\text{g l}^{-1}$ for English sole (*Parophrys vetulus*, Dinnel et al., 1983) to $1170 \mu\text{g l}^{-1}$ for sheepshead minnow (*Cyprinodon*

Table 2

Ag concentrations ($\mu\text{g kg}^{-1}$ dry tissue or $\mu\text{g l}^{-1}$ for plasma and bile) in the different tissues at the time of death (685, 200 and 30 $\mu\text{g l}^{-1}$) or the end of the experiment (30 and 0 $\mu\text{g l}^{-1}$) of spiny dogfish exposed to different Ag concentrations^a

| | 0 $\mu\text{g l}^{-1}$ Ag | 30 $\mu\text{g l}^{-1}$ Ag | 200 $\mu\text{g l}^{-1}$ Ag | 685 $\mu\text{g l}^{-1}$ Ag |
|--------------|---------------------------|----------------------------|-----------------------------|-----------------------------|
| Gill | 11.8 \pm 2.7 | 52.6 \pm 15.9* | 1107.9 \pm 186.6** | 3628.7 \pm 582.1** |
| Plasma | 0.1 \pm 1.9 | 0.0 \pm 1.1 | 1.7 \pm 1.6 | 176.2 \pm 66.6** |
| Kidney | 39.4 \pm 1.7 | 29.2 \pm 18.4 | 54.6 \pm 29.0 | 257.1 \pm 65.7** |
| Rectal gland | 2.4 \pm 4.4 | 9.4 \pm 7.2 | 40.0 \pm 6.6** | 314.7 \pm 79.1** |
| Intestine | 14.6 \pm 12.7 | 10.2 \pm 16.9 | 91.0 \pm 41.3 | 282.4 \pm 83.2** |
| Liver | 2.9 \pm 4.8 | 33.6 \pm 15.2* | 26.2 \pm 9.0* | 62.0 \pm 17.8** |
| Bile | 68.5 \pm 14.3 | 70.9 \pm 15.7 | 97.0 \pm 5.4 | 124.6 \pm 15.7* |
| Muscle | 8.7 \pm 4.4 | 4.4 \pm 3.3 | 0.2 \pm 8.4 | 24.8 \pm 9.4 |

^a Mean values \pm S.E.M.; significant differences are indicated, *, $P < 0.05$; **, $P < 0.01$.

variegatus, Environmental Protection Agency, 1980). Calculating a 96h LC50 for spiny dogfish from our (limited) set of data according to the graphical method (Environmental Protection Agency, 1991), we obtained an Ag concentration of approximately 100 $\mu\text{g l}^{-1}$, thus 7–12 times lower than for adult marine teleosts.

Earlier work investigating the toxicity of zinc and copper to the Mediterranean dogfish (*Scyliorhinus canicula*) did not show such a high sensitivity for heavy metal exposure, in fact, LC50 values were in the same range or higher than LC50 values for marine teleosts exposed to these metals (Crespo and Balash, 1980; Torres et al., 1987). However, the exceptionally high silver accumulation observed in elasmobranch fish compared with teleost fish under the same exposure conditions, supports the idea that a different mechanism is at work here. During long-term low-level exposures to silver the elasmobranch thornback ray (*R. clavata*) has been seen to accumulate substantially more silver than the plaice (*P. platessa*), particularly in the liver (Pentreath, 1977). Webb and Wood (2000) also found that spiny dogfish (*S. acanthias*) and long-nose skate (*R. rhina*) accumulated much more total Ag in their gills, in comparison to several teleost species at the same silver concentrations during long-term sublethal exposures. The fact that these dogfish seem to accumulate silver so easily, might explain the relatively high kidney silver content in control fish, accumulated from the low environmental concentrations during their life time.

To put the present Ag bioaccumulation data for acutely exposed dogfish in perspective, the gill concentration accumulated in 12–24 h exposure to 685 $\mu\text{g l}^{-1}$ was approximately 5-fold greater than that accumulated in starry flounder exposed to 1000 $\mu\text{g l}^{-1}$ Ag for 4–6 days (Hogstrand et al., 1999). Interestingly, liver concentrations were similar in the two species after these exposures. However, it must be remembered that liver mass per unit body weight is up to ten-fold larger in elasmobranchs than in teleosts, so the absolute liver uptake rate was comparably elevated in the sharks.

In contrast to some early anecdotal reports of suffocation at very high silver levels (Cooper and

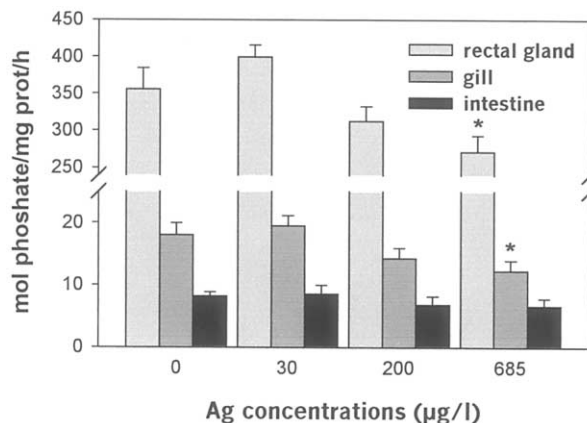


Fig. 8. Na^+/K^+ -ATP-ase activity in rectal gland, gill, and intestine of spiny dogfish exposed to different Ag concentrations (mean values \pm S.E.M.; significant differences relative to the 0 $\mu\text{g l}^{-1}$ treatment are indicated, *, $P < 0.05$).

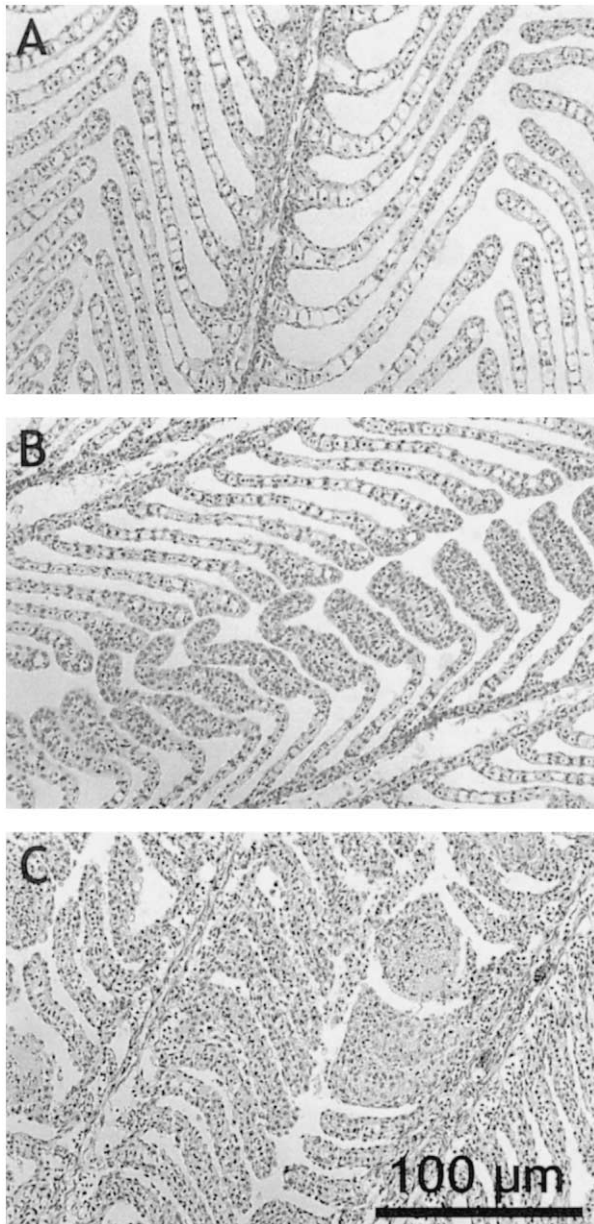


Fig. 9. Gills of spiny dogfish exposed to different Ag concentrations, (A) 0; (B) 200; (C) 685 $\mu\text{g l}^{-1}$. Swelling of epithelial cells, fusion of lamellae, lamellar aneurism, and lifting of lamellar epithelium away from pillar cells can be observed in the dogfish gills exposed to Ag.

Jolly, 1970), more recent work on silver indicates that it is not a respiratory toxicant at concentrations up to 96h LC50 levels (i.e. to well above the

range of environmental relevance) in either freshwater or seawater teleosts. Wood et al. (1996) reported increases in arterial $P_{\text{a}}\text{O}_2$ while $P_{\text{a}}\text{CO}_2$ gradually decreased during exposure (10 $\mu\text{g l}^{-1}$ Ag) of freshwater rainbow trout (*Oncorhynchus mykiss*). They attributed this effect to hyperventilation as a consequence of the observed metabolic acidosis. In the marine starry flounder (*P. stellatus*) exposed to 1000 $\mu\text{g l}^{-1}$ Ag, Hogstrand et al. (1999) reported a similar increase in $P_{\text{a}}\text{O}_2$ associated with hyperventilation. However, in the present study it is obvious that respiratory failure was one of the main causes of death at the two highest Ag concentrations (200 and 685 $\mu\text{g l}^{-1}$). Admittedly, these concentrations are far above the Ag levels that could occur in any natural environment. Maximum levels are generally reported in coastal and intertidal areas, and are in the 0.06–2.9 $\mu\text{g l}^{-1}$ range (Fowler and Nordberg, 1986; Eisler, 1996), but naturally occurring silver levels in the open ocean are usually below 0.0025 $\mu\text{g l}^{-1}$ (Eisler, 1996). The US Environmental Protection Agency (EPA) Ambient Water Quality Criterion for the protection of marine life is 2.3 $\mu\text{g l}^{-1}$ (Environmental Protection Agency, 1980). In the exposure to the lowest silver concentration, we observed similar effects as in freshwater trout, a transient increase in $P_{\text{a}}\text{O}_2$ and a decrease in $P_{\text{a}}\text{CO}_2$. It is not clear what triggered the temporary hyperventilation in these dogfish because there was no decrease in blood pH, and the acid–base exchange measurements showed no indications of an elevated H^+ influx in this treatment group. Perhaps simple irritation caused the fish to hyperventilate.

At the two highest silver concentrations, gill damage with swelling of epithelial cells, fusion of lamellae, lamellar aneurism, and lifting of lamellar epithelium most probably caused the dramatic respiratory failure. Increase in the diffusion distance and a disturbed blood flow through the lamellae can explain the restricted transport of blood gasses. The increased hematocrit (compared with control fish) indicating thickening of the blood, could add to the difficulties in blood gas transport. Likely as a consequence of the disturbance in gas exchange, anaerobic metabolism was initiated, causing rapid increases in plasma lactate

concentrations. The decrease in plasma glucose levels observed in these two exposure groups could be related to the substantial activation of the anaerobic metabolism, or it could be due to elevated losses across the gills accompanying massive gill damage, as for example, was seen for urea.

The decrease in arterial blood pH_a appears to be caused by several different processes. The interference with respiratory gas exchange and transport resulted in lactate mobilisation, and therefore, probably caused metabolic acidosis, which would tend to decrease plasma HCO_3^- concentration. However, an increase in $P_a\text{CO}_2$ from the disturbance of gas exchange was also obvious in the two highest exposure groups, which would tend to raise plasma HCO_3^- . Thus plasma HCO_3^- levels exhibited little change. The increase in $P_a\text{CO}_2$ represents a direct respiratory acidosis contributing to the drop in pH_a . But whereas the drop in pH_a should normally lead to fast H^+ extrusion across the gills (Heisler, 1993), the measured acid–base fluxes with the environment did not show such an effect. Acid extrusion in elasmobranch fish is normally coupled to Na^+ influx due to $\text{NH}_4^+/\text{Na}^+$ and H^+/Na^+ exchange (Payan and Maetz, 1973), so it is possible that silver exposure blocked this compensating process. Despite the massive Na^+ influx observed at the two highest silver concentrations, H^+ excretion did not occur, suggesting a large increase in diffusive permeability rather than an activation of these ion exchange mechanisms. Furthermore, we did not observe any NH_4^+ loading in the plasma or changes in the net ammonia flux to the water, which would suggest a compromised NH_4^+ excretion.

A further argument for the changes in diffusive capacity of the dogfish gills is the considerable loss of plasma urea and elevated urea fluxes to the environment at the two highest silver concentrations, which coincided with increases in all plasma ion levels. Dogfish gills are normally very impermeable to the high levels of urea that are present in the plasma and it is suggested that some ‘back-transport’ mechanism exists to minimise urea losses (Wood et al., 1995; Part et al., 1998), likely by a Na^+ -coupled urea transporter (Fines et al., 2001). The leaking of urea at such high rates

indicates that either the low permeability of the gills is seriously compromised, or that this ‘back-transport’ mechanism is blocked by the silver exposure. A consequence of the increased gill permeability would be a diffusion of the seawater ions into the plasma, normally a restricted process (Evans, 1979). The dramatic increases in all ion concentrations in the plasma, and especially in Mg^{2+} concentration for which the relative seawater-to-plasma gradient is greatest, indicate that this indeed occurred. At the $30 \mu\text{g l}^{-1}$ Ag exposure level, no decrease in plasma urea, or elevation of plasma Mg^{2+} was seen, while small increases in plasma Na^+ and Cl^- concentrations were observed. As in freshwater and seawater teleosts, the toxic action of silver seems to be directed most potently towards the transport of these two ions in particular, a fact that is supported by the inhibition of the Na^+/K^+ -ATP-ase inhibition in gills and rectal gland at the highest silver concentration. For marine fish, this results in a reduced elimination of these ions from the body, since the ions have to be excreted by active transporters to compensate for the passive diffusion into the animals.

For marine teleosts, the toxic action of silver is mainly directed towards the gastro-intestinal tract due to continuous drinking (Grosell and Hansen, 1997; Grosell et al., 1999; Hogstrand and Wood, 1998; Webb and Wood, 1998), but since drinking rates of elasmobranch fish are less than 10% of the drinking rates of marine teleosts, the importance of this uptake route is probably very limited. Drinking levels observed in this study are close to the values reported for *S. canicula* (Hazon et al., 1989, 1999), and as was to be expected from the low drinking rates, no effects were observed on the intestinal Na^+/K^+ -ATP-ase activity. Only at the highest exposure level was there any increase in intestinal Ag levels. The main iono-regulatory disturbances were thus situated at the gills, as for freshwater teleosts. Interestingly, the rectal gland also appears to have been impacted, as judged by a relative inhibition of Na^+/K^+ -ATP-ase activity at the highest silver concentration, comparable to that seen in the gills. This was probably due to the significant Ag burdens, which were acquired in the rectal gland by transport

through the bloodstream in only 12–24 h (note elevated plasma and rectal gland Ag concentrations in Table 2).

We conclude that adult spiny dogfish, *S. acanthias*, are about one order of magnitude more sensitive to silver toxicity than adult marine teleosts in full strength seawater. At high Ag concentrations causes of death appeared to be respiratory as well as osmoregulatory failure, the latter with disturbances in ion and urea regulation due to increases in diffusive permeability as well as disturbed ion transport. At lower Ag concentrations, physiological effects of silver toxicity were much less severe and only osmoregulatory toxicity was seen, probably due to specific effects on NaCl excretion mechanisms.

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