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Copper toxicity in the spiny dogfish (*Squalus acanthias*): Urea loss contributes to the osmoregulatory disturbance

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Abstract

Previous research showed that the spiny dogfish, *Squalus acanthias*, is much more sensitive to silver exposure than typical marine teleosts. The aim of the present study was to investigate if spiny dogfish were equally sensitive to copper exposure and whether the toxic mechanisms were the same. We exposed cannulated and non-cannulated spiny dogfish to measured concentrations of Cu (nominally 0, 500, 1000 and 1500 $\mu g\,L^{-1}$ Cu) for 72–96 h. All Cu exposures induced acidosis and lactate accumulation of either a temporary (500 $\mu g\,L^{-1}$) or more persistent nature (1000 and 1500 $\mu g\,L^{-1}$). At the two highest Cu concentrations, gill Na^+/K^+-ATPase activities were reduced by 45% (1000 $\mu g\,L^{-1}$) and 62% (1500 $\mu g\,L^{-1}$), and plasma Na^+ and Cl^- concentrations increased by approximately 50 mM each. At the same time urea excretion doubled and plasma urea dropped by ~100 mM. Together with plasma urea, plasma TMAO levels dropped proportionally, indicating that the general impermeability of the gills was compromised. Overall plasma osmolarity did not change.

Cu accumulation was limited with significant increases in plasma Cu and elevated gill and kidney Cu burdens at 1000 and 1500 $\mu g\,L^{-1}$. We conclude that Cu, like Ag, exerts toxic effect on Na^+/K^+-ATPase activities in the shark similar to those of teleosts, but there is an additional toxic action on elasmobranch urea retention capacities. With a 96 h LC_{50} in the 800–1000 $\mu g\,L^{-1}$ range, overall sensitivity of spiny dogfish for Cu is, in contrast with its sensitivity to Ag, only slightly lower than in typical marine teleosts.

Keywords: Elasmobranch; Silver; Copper; Na^+/K^+-ATPase; Urea; Ammonia

1. Introduction

In freshwater environments, the toxic action of copper has been related to the disturbance of osmoregulation (Laurén and McDonald, 1985, 1987a,b; Wood, 2001; Grosell et al., 2002) and waterborne silver and copper show very similar toxic actions, inhibiting Na^+/K^+-ATPase (Wood, 2001; Grosell et al., 2002). Both Ag and Cu can act as Na analogues and competitors in gill transport systems, and out-compete Na, thereby blocking transport systems (Wood, 2001; Grosell and Wood, 2002). At elevated concentrations they induce respiratory stress as well (De Boeck et al., 1995, 2001, 2006). In freshwater teleosts, the main target is the gill, while in marine teleosts which drink seawater; intestinal effects also play an important role (Hogstrand et al., 1999; Grosell et al., 1999, 2004a,b; Grosell and Wood, 2001). It is clear that also marine teleosts suffer from osmoregulatory disturbances under copper exposure, albeit at much higher concentrations (Stagg and Shuttleworth, 1982a,b; Steele, 1983a,b; Wilson and Taylor, 1993; Larsen et al., 1997; Grosell et al., 1999). A recent review summarises the role of developmental stage, size and salinity in Cu toxicity to estuarine and marine organisms (Grosell et al., 2007). From this, it is

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clear that much less is known about the effect of metals on elasmobranchs.

It has long been known that osmoregulation in elasmobranch fish is based on high urea levels in the blood and tissues which makes them slightly hyperosmotic compared to the surrounding seawater (Smith, 1931a,b; Hazon et al., 2003 for recent review). This strategy distinguishes them from marine teleosts that are hyposmotic compared to the seawater and thus constantly lose water to the environment. Therefore, in contrast to marine teleosts, elasmobranchs do not have to drink the external medium to compensate for any water loss, though very slight drinking has been reported (Webb and Wood, 2000; Hazon et al., 2003). However, they do have to maintain their urea levels against a huge diffusion gradient; typical urea levels in elasmobranchs are close to 350 mM while urea is virtually absent in seawater. Especially at the gills, a large surface with short diffusion distances designed for rapid diffusion of respiratory gases, the risk of continuously losing urea is high. Despite the fact that elasmobranchs have very tight and relatively impermeable gill epithelia with high cholesterol levels and a basolateral Na-coupled urea back-transporter that helps to retain urea (Part et al., 1998; Fines et al., 2001; Hill et al., 2004; reviewed by Walsh and Smith, 2001), diffusional urea loss occurs at about the same rate as urea is synthesised (Carrier and Evans, 1972; Wood et al., 1995). Plasma sodium and chloride levels are regulated at levels below those in seawater but are generally higher than to those in marine teleosts. However, in contrast to marine teleosts, Na and Cl gained by diffusion are not primarily excreted at the gills but by an additional excretory organ, the rectal gland (Shuttleworth, 1988). The rectal gland shows very high levels of Na^+/K^+-ATPase activity and excretes a fluid which is isosmotic to the plasma, but contains very low urea levels and is almost entirely composed of Na and Cl (Burger and Hess, 1960; Wood et al., 2007).

In a previous study, examining the effects of metals on an elasmobranch, silver appeared to be 10 times more toxic to Pacific spiny dogfish than to similarly sized marine teleosts and in fact, sensitivity approached that of freshwater teleosts (De Boeck et al., 2001). This sensitivity coincides with high Ag accumulation rates in gill and other tissues (Webb and Wood, 2000; De Boeck et al., 2001). As in teleosts, toxicity appeared to be related to osmoregulatory disturbance; however, in this elasmobranch, failure of the urea retention mechanism played an important role in the osmoregulatory disturbance (De Boeck et al., 2001). Grosell et al. (2003) compared sublethal Cu toxicity between an elasmobranch, *Raja erinacea*, and a marine teleost, the sculpin *Myoxocephalus octodecemspinosus* at Cu concentrations $\leq 110\,\mu g\,L^{-1}$. At these Cu levels, they did not observe any reduction in Na^+/K^+-ATPase activity or any other indication of osmoregulatory disturbance. Cu accumulation in the gills was about 15 times higher in the skate compared to the sculpin, but no further Cu accumulation was observed in any of the other tissues.

Despite the fact that normal background levels for Cu in the marine environment are low (between 0.04 and 0.127 $\mu g\,L^{-1}$ in the open ocean, but as high as 7 $\mu g\,L^{-1}$ in estuaries, Baeyens, 1998) the differences in response between marine teleosts and elasmobranchs are intriguing. Because of the apparent similarity in toxic mechanisms between Ag and Cu, we wanted to further elucidate the effects of Cu exposure in elasmobranchs. Therefore, the goals of the present study were to determine which Cu levels are detrimental to elasmobranchs and to compare the effects to those previously seen under Ag exposure (De Boeck et al., 2001). For this purpose, we exposed spiny dogfish, *Squalus acanthias*, to increasingly higher Cu levels and measured parameters related to osmoregulation, respiration and Cu accumulation.

2. Materials and methods

Spiny dogfish (*S. acanthias*) were caught by angling in the vicinity of Bamfield, British Columbia, Canada in the summer of 2005 and subsequently kept in a large concrete indoor tank (151,000 L) served with running aerated Bamfield Marine Station seawater (14 °C, salinity 30‰). Dogfish were kept at least 1 week before experiments began. Fish were fed twice a week with commercially purchased frozen hake (*Merluccius productus*, a marine teleost) which were first thawed and deheaded. In August 2005, two series of fish were exposed to four different Cu concentrations (nominal values: 0, 500, 1000 and 1500 $\mu g\,Cu\,L^{-1}$). In the first series, fish were fitted with a cannula in the caudal artery and kept in separate wooden boxes (details see below). In the second series, fish were free-swimming in large fibreglass tanks and were sampled for blood with a syringe by caudal puncture after a brief, partial sedation (see details below).

2.1. Copper exposed cannulated fish

Sixteen adult dogfish with an average weight of 2.25 ± 0.15 kg were caught from the tank, and immediately anaesthetised in a 100 $mg\,L^{-1}$ MS-222 seawater solution neutralised with $NaHCO_3$ for surgery. Once anaesthetised, fish were placed on a wooden V-trough and gills were constantly irrigated with the anaesthetic throughout surgery. A PE50 polyethylene cannula was fitted into the caudal artery as described by De Boeck et al., 2001. The cannula was filled with heparinised (50 IU mL^{-1}) dogfish saline (recipe as in Wood et al., 1994, but with urea level reduced to 325 mM). Fish were then allowed to recover from surgery overnight in covered wooden fish boxes. 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 exposure series were performed on four dogfish each, using nominal total copper concentrations of 0, 500, 1000, and 1500 $\mu g\,L^{-1}$. Copper exposure of the shark began by adding these nominal concentrations as $CuCl_2 \cdot 2H_2O$ 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 urea and 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 copper from the $CuCl_2$ stock solution. Flushing of the boxes consisted of three

consecutive series of lowering the water level by two thirds so that the dogfish remained submerged at all times, then filling back to 32 L. Water samples for determination of copper, ammonia and urea content were taken at the beginning and end of each 12 h period of the exposure. Actual measured copper concentrations in the water of control fish were below the detection limit of 1 $\mu g\,L^{-1}$, and water copper levels for the exposed fish were $444.5 \pm 11.5\ \mu g\,L^{-1}$ ($N = 38$), $852.6 \pm 41.0\ \mu g\,L^{-1}$ ($N = 24$) and $1442.0 \pm 29.3\ \mu g\,L^{-1}$ ($N = 24$), respectively (mean ± S.E.M., N = number of measurements).

In each series, blood samples (1 mL) were taken 12 h before exposure (control) and after 12, 24, 48 and 72 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 point of death, or at the end of the exposure, tissues were taken and rinsed in dogfish saline, immediately frozen in liquid nitrogen, and then stored at −80 °C for later determination of copper content (gill, muscle, intestine, liver, bile, kidney, rectal gland). Samples for Na^+/K^+-ATPase activity (gill, rectal gland) were only taken from the surviving fish, and one fish at 1500 $\mu g\,L^{-1}$ that died during blood sampling and was dissected immediately thereafter. Surviving fish were quickly killed by an overdose of neutralised MS-222 at the end of each exposure.

2.2. Copper exposed free swimming fish

In a second experiment, the last 15 dogfish of a suitable size (average weight of 0.786 ± 0.18 kg) were caught from the holding tank, and divided among three 1500 L fibreglass tanks. Two groups of six sharks each were exposed to the lower copper concentrations (nominal levels of 500 and 1000 $\mu g\,L^{-1}$), while one group of three sharks was exposed to the highest copper level (1500 $\mu g\,L^{-1}$). Due to the small number of dogfish available, we chose to have only a limited number of dogfish in the high exposure group, where mortality and distinct physiological effects were expected, thereby increasing the number of fish available for the lower exposure groups where more subtle physiological effects were more likely. For physiological parameters, pre-exposure levels of each dogfish served as controls.

Copper exposure of the shark began by adding the nominal concentrations as $CuCl_2{\cdot}2H_2O$ from a stock solution (Fisher, analytical grade) to the tanks. During the control and exposure period, flow-through was switched off and the system was static (with aeration maintained). Two thirds of the water in the tanks was renewed every 12 h, and subsequently spiked again with the appropriate amount of copper from the $CuCl_2$ stock solution. Water samples for determination of copper, ammonia and urea content were taken at the beginning and end of each 12 h period of the exposure, but ammonia and urea levels in the tanks remained too low to obtain trustworthy efflux rates. Actual measured copper concentrations in the water were below the detection limit of 1 $\mu g\,L^{-1}$ before exposure, and water copper levels during exposure were $542.8 \pm 6.6\ \mu g\,L^{-1}$ ($N = 26$), $1092.8 \pm 6.7\ \mu g\,L^{-1}$ ($N = 26$) and $1610.0 \pm 13.9\ \mu g\,L^{-1}$ ($N = 12$), respectively (mean ± S.E.M, N = number of measurements).

In each series, blood samples (0.5 mL) were taken 12 h before exposure (control) and after 12, 24, 48, 72 and 96 h of exposure, or until the sharks had died. At the point of death, or at the end of the exposure, tissues were taken and rinsed in dogfish saline, immediately frozen in liquid nitrogen, and then stored at −80 °C for later determination of copper content (gill, muscle, intestine, liver, bile, kidney, rectal gland). Samples for Na^+/K^+-ATPase activity (gill, rectal gland) were only taken from the surviving fish. Surviving fish were quickly killed by an overdose of neutralised MS-222 at the end of each exposure. Of the surviving fish, 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 μm. Microscopic slides were coloured with the classic hemalun–eosin stain for light microscopy.

2.3. Analytical procedures

Blood samples of cannulated fish were immediately analysed for arterial pH and haematocrit content. Arterial pH was measured with a micro-capillary pH electrode (Radiometer G279/G2 plus E5021) coupled to a PHM71 meter and kept at 14 °C by a water jacket perfused with Bamfield Marine Station seawater. The remainder of the blood sample was immediately centrifuged (5 min at 10,000 *g*), and plasma sub-samples were taken and kept at 5 °C for determination of the levels of total CO_2, Na^+, Cl^-, later that day, or frozen in liquid nitrogen and kept in the freezer (−80 °C) until later determination of ammonia, urea, glucose, lactate, protein and Cu. Plasma total CO_2 was analysed using a Cameron chamber (Cameron, 1971) kept at 14 °C by a water jacket perfused with Bamfield Marine Station seawater and connected to a PCO_2 electrode (Radiometer E5046) coupled to a PHM71 meter. 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): $P_{aCO_2} = C_{CO_2}/(\alpha_{CO_2}(10^{pH-pK_{app}} + 1))$ with C_{CO_2} being total plasma CO_2. Plasma HCO_3^- content was calculated as the difference between total plasma CO_2 and $\alpha_{CO_2} \cdot P_{aCO_2}$.

Plasma [Na^+] and [Cl^-] were analysed using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium). Plasma proteins were determined with the Bradford method (Bradford, 1976) using BSA as a reference (US Biochemical, Cleveland, OH, USA). Plasma ammonia, glucose and lactate were determined using enzymatic kits (E1112732, E0716251 and E0139084, respectively, R-Biopharm, Boehringer Mannheim, Darmstadt, Germany). Water and plasma urea were analysed with the diacetyl monoxime method (Price and Harrison, 1987) and ammonia in water samples was determined using the salicylate-hypochlorite method (Verdouw et al., 1978). Plasma TMAO levels were analysed by a modification of the method of Wekell and Barnett (1991) as described in Treberg and Driedzic (2006).

Total copper concentrations were determined using ICP-AES (Varian, Liberty Series II Ax, St-Katelijne-Waver, Belgium). Seawater samples were acidified to 1% using analytical grade 69% HNO_3 (Merck, Darmstadt, Germany). Plasma samples were diluted with 1% HNO_3 made with Milli-Q grade water before measurement (Millipore, Bedford, MA, USA). Tissues

were dried in a 60 °C drying oven for a minimum of 1 week, cooled in a desiccator, weighed, dissolved with 69% HNO_3 and 30% H_2O_2 (Merck, Darmstadt, Germany) and placed in a microwave oven until total digestion had occurred. They were then diluted with Milli-Q grade water (Millipore, Bedford, MA, USA). Standard curves were made by standard addition.

2.4. Statistics

All values are mean values ± S.E.M. Water, blood and plasma values were analysed for significant differences ($P < 0.05$) using a repeated measures ANOVA. Values for tissue Cu and Na^+/K^+-ATPase were analysed using an ordinary ANOVA. In both cases the ANOVA was followed by the Dunnett's comparison post-test if significant differences were found (GraphPad Instat 3.01, GraphPad Software Inc.), comparing experimental values in each group to its own pre-exposure control value.

3. Results

Fig. 1 shows the survival of all dogfish, cannulated and free-swimming, during the first 72 h of exposure. Free-swimming fish were exposed up to 96 h, but no further mortalities occurred after 72 h of exposure. No mortality was observed in controls and in the group exposed to 500 μg L^{-1} Cu. About 50% of the dogfish in the 1000 μg L^{-1} group died over the 72 h interval, without a difference between the cannulated or free-swimming fish. At 1500 μg L^{-1}, none of the free-swimming fish survived and only two of the cannulated dogfish survived up the final sampling (72 h), of which one died during the sampling procedure. Since 50% of the animals died at 1000 μg L^{-1}, an estimated LC_{50} at 96 h would be slightly below this value, likely in the 800–1000 μg L^{-1} Cu range. Cu levels in the plasma were elevated at the two highest exposure levels, but accumulation in the tissues was surprisingly low (Table 1). The only significant accumulation occurred in gill and kidney of the 1000 and 1500 μg L^{-1} groups.

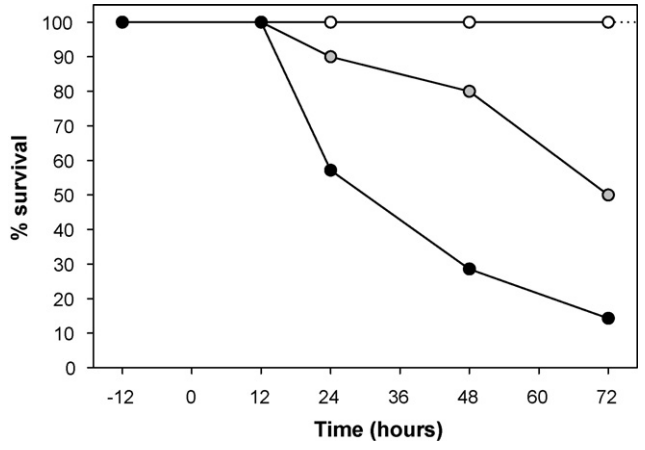

Fig. 1. Survival of spiny dogfish exposed to copper levels of 0 μg L^{-1} (dotted line), 500 μg L^{-1} (open circles), 1000 μg L^{-1} (grey circles) and 1500 μg L^{-1} (black circles) (at the start of the exposure $N = 4$ at 0 μg L^{-1}, $N = 10$ at 500 μg L^{-1} and 1000 μg L^{-1}, and $N = 7$ at 1500 μg L^{-1}).

Table 1
Tissue copper concentrations (μg g^{-1} dry weight or μg L^{-1} for plasma) of spiny dogfish exposed to copper levels of 0, 500, 1000 and 1500 μg L^{-1} ($N = 4$–8 at 0 μg L^{-1}, $N = 10$ at 500 μg L^{-1} and 1000 μg L^{-1}, and $N = 7$ at 1500 μg L^{-1})

	0	500	1000	1500
Gill	3.2 ± 0.5	21.6 ± 1.5	41.0 ± 6.1*	142.4 ± 26.2*
Plasma	8.6 ± 0.3	13.3 ± 1.7	20.9 ± 1.7*	20.0 ± 2.7*
Liver	4.3 ± 1.0	6.5 ± 0.8	8.5 ± 1.9	7.6 ± 1.4
Bile	18.1 ± 1.5	13.5 ± 2.1	14.6 ± 5.59	10.1 ± 4.5
Kidney	5.7 ± 0.8	8.5 ± 0.7	26.4 ± 6.5*	82.7 ± 30.9*
Intestine	4.5 ± 0.2	4.7 ± 1.4	4.6 ± 0.4	4.6 ± 0.7
Rectal gland	17.1 ± 0.5	23.7 ± 2.9	22.8 ± 1.6	24.6 ± 2.2
Muscle	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.6 ± 0.1

Time of exposure varies according to survival (see Fig. 1). Means ± S.E.M., significant differences ($P < 0.05$) from control group indicated with asterisk.

Due to practical constraints blood haematocrit, pH and total CO_2 were only measured in the cannulated dogfish. Except for haematocrit values, all blood and plasma parameters were stable over time in the control group and are therefore shown as a dashed and dotted line in Figs. 2–5 representing the overall average value with the S.E.M. indicated as dotted lines ($N = 20$). For the parameters measured in both the cannulated and the free-swimming fish, only glucose levels differed significantly between the two groups with consistently higher glucose levels in the cannulated group, but without any obvious trends over time (4.7 ± 0.2 mM compared to 3.9 ± 0.4 mM on average, respectively). Therefore, all other data available for both groups of fish were pooled.

Due to sampling, haematocrit levels dropped in the control fish from 17.9 ± 0.5% at the start to 14.6 ± 0.8% after 24 h ($P < 0.05$) and 12.7 ± 1.1% at 72 h ($P < 0.01$) (data not shown). This effect disappeared in all exposed fish, suggesting that haemo-concentration occurred which counteracted the effects of sampling. Arterial pH decreased significantly at 12 h for the 500 μg L^{-1} group (Fig. 2). This effect was short-lived and complete recovery was observed within 24 h of exposure. For the two highest exposure groups the pH drop was significant after 24 h of exposure and plasma pH stayed around 7.50 for the remaining exposure period without any sign of recovery. The acidosis was not respiratory in nature, since no effects were observed in bicarbonate or P_{CO_2} levels at this time (Fig. 2). P_{CO_2} levels of surviving dogfish at the higher exposure concentrations were increased only at 72 h. For the 500 μg L^{-1} group, the reduction in pH coincided with a peak in lactate accumulation (Fig. 2) followed by a comparable recovery within 24 h. In the two highest exposure groups, there was a tendency for a much slower lactate accumulation again corresponding with the acidosis, but the effect was not significant.

We observed no change in either ammonia excretion or plasma ammonia levels at any of the exposure levels, but urea excretion increased significantly during copper exposure at 1000 and 1500 μg L^{-1} (Fig. 3). Urea efflux increased significantly between 12 and 24 h of exposure for the two highest copper concentrations from 443.5 ± 23.8 to 808.1 ± 68.2 μmol/(kg h) (average for the two exposure groups). If this efflux rate persisted over the remaining time, which at least for the highest exposure groups seems to be the case, dogfish would lose about

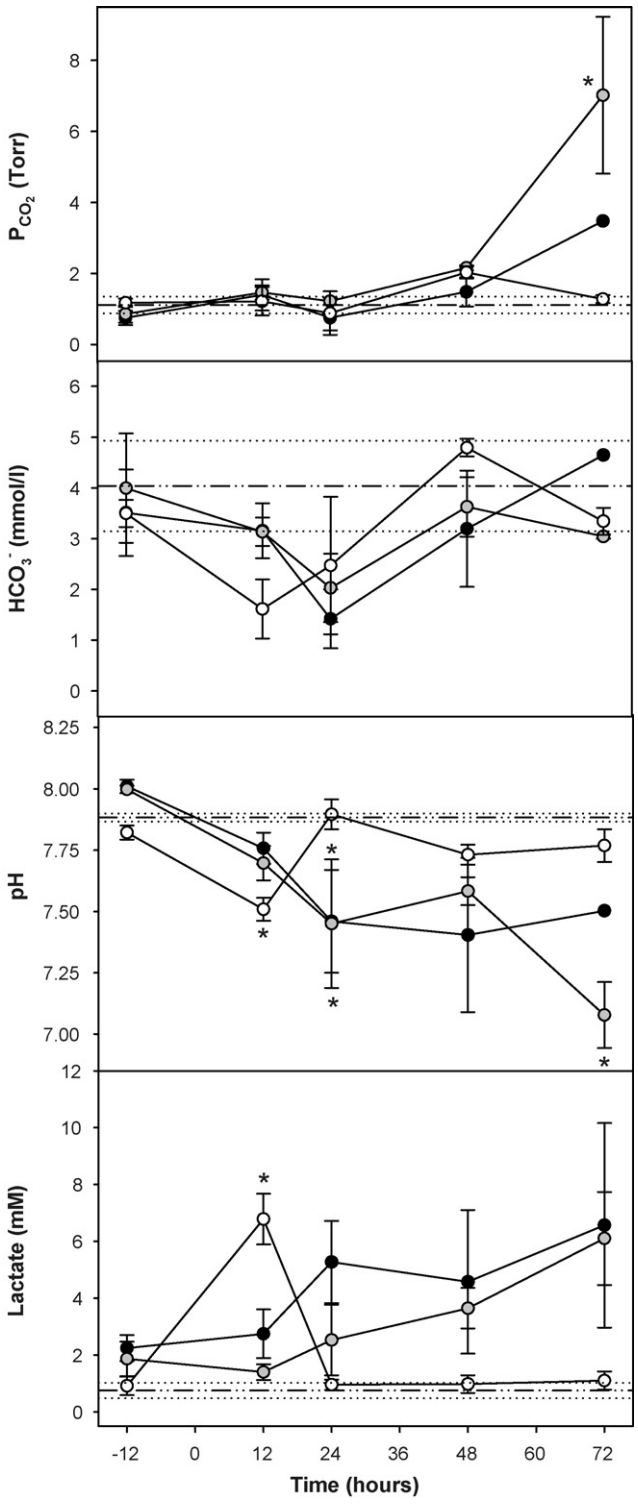

Fig. 2. Arterial P_{CO_2}, HCO_3^-, pH and plasma lactate levels of spiny dogfish exposed to copper levels of 0 μg L^{-1} (dashed-dotted line), 500 μg L^{-1} (open circles), 1000 μg L^{-1} (grey circles) and 1500 μg L^{-1} (black circles). Means ± S.E.M., significant differences ($P < 0.05$) from own pre-exposure values indicated with asterisk.

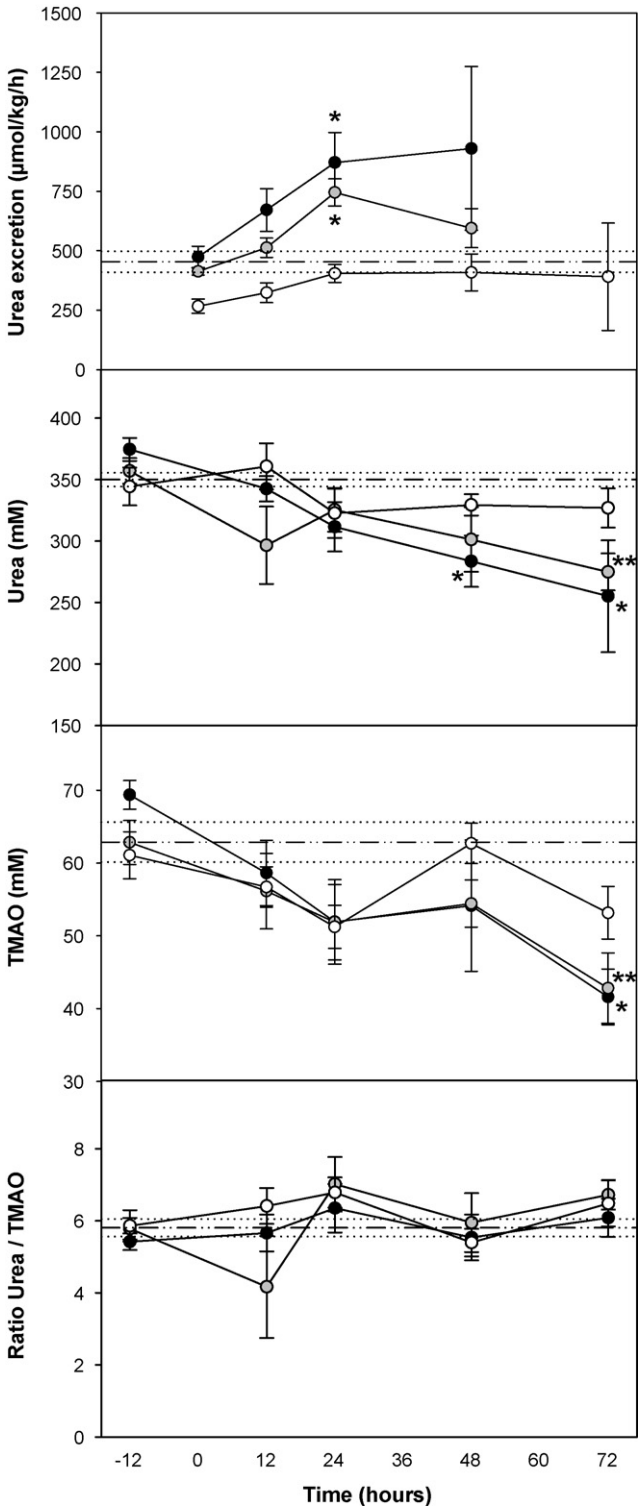

Fig. 3. Urea excretion, plasma urea and plasma TMAO as well as plasma urea/TMAO ratios of spiny dogfish exposed to copper levels of 0 μg L^{-1} (dashed-dotted line), 500 μg L^{-1} (open circles), 1000 μg L^{-1} (grey circles) and 1500 μg L^{-1} (black circles). Means ± S.E.M., significant differences ($P < 0.05$) from own pre-exposure values indicated with asterisk.

21.9 ± 3.4 mmol per kg in excess of the control efflux rate for the last 60 h of the exposure. This urea efflux induced a reduction in plasma urea levels which was significant at 48 and 72 h for the 1500 μg L^{-1} group and at 72 h for the 1000 μg L^{-1} group. Plasma urea had dropped by about 80–100 mmol L at 72 h of exposure. This value largely exceeds the value of 21.9 mmol per kg, indicating that the observed urea loss mainly occurred from the plasma at this stage and as a consequence tissues would be

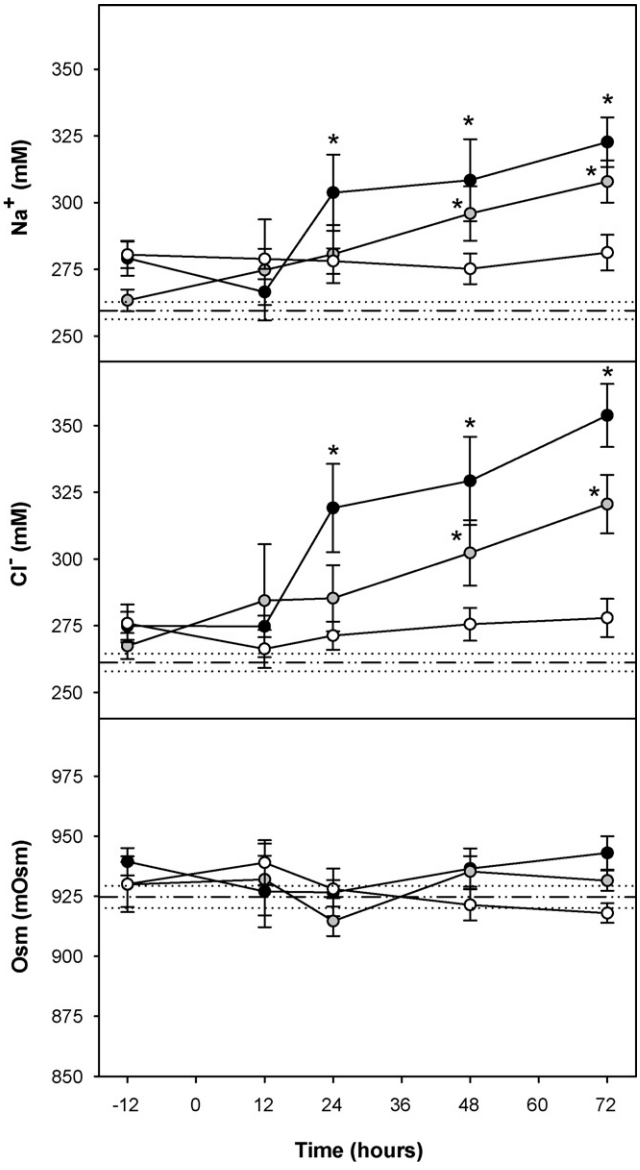

Fig. 4. Plasma sodium and chloride levels and plasma osmolarity of spiny dogfish exposed to copper levels of 0 μg L^{-1} (dashed-dotted line), 500 μg L^{-1} (open circles), 1000 μg L^{-1} (grey circles) and 1500 μg L^{-1} (black circles). Means ± S.E.M., significant differences ($P < 0.05$) from own pre-exposure values indicated with asterisk.

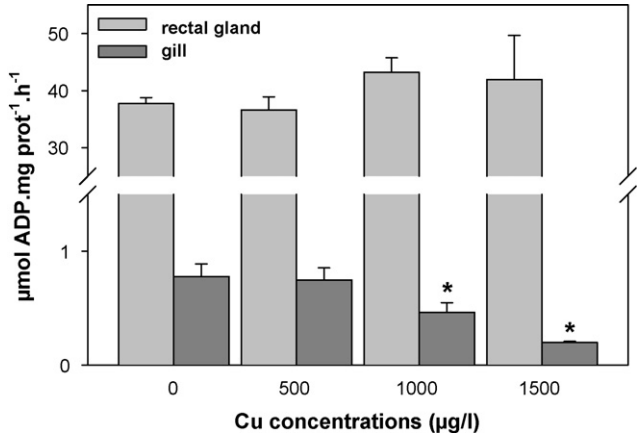

Fig. 5. Na^+/K^+-ATPase activity in gill and rectal gland of spiny dogfish exposed to copper levels of 0, 500, 1000 and 1500 μg L^{-1} ($N = 4$ at 0 μg L^{-1}, $N = 10$ at 500 μg L^{-1} and 1000 μg L^{-1}, and $N = 7$ at 1500 μg L^{-1}). Means ± S.E.M., significant differences ($P < 0.05$) from control group indicated with asterisk.

less affected. Plasma TMAO levels dropped in a proportional way to plasma urea levels, leaving the plasma urea/TMAO ratio unaffected, at a value of approximately 6.0 (Fig. 3).

Despite the drop in plasma urea and TMAO, plasma osmolality did not differ between groups and remained stable over time (Fig. 4). Also plasma protein did not change (overall average 14.4 ± 0.4 mg mL^{-1}). However, we observed an increase in plasma ions (Fig. 4) with significantly elevated levels of Na^+ and Cl^- starting from 24 h onwards in the highest exposure group, and from 48 h onwards in the 1000 μg L^{-1} group. No changes were observed in control animals or in the lowest exposure group. In the surviving fish of the two highest exposure groups, Na^+/K^+-ATPase activities were significantly reduced at the end of the exposure by 40 and 75%, respectively (Fig. 5).

4. Discussion

It is clear from the observed effects that the spiny dogfish can tolerate fairly high amounts of waterborne Cu. Our 96 h LC_{50} is estimated to be in the 800–1000 μg L^{-1} range, clearly higher than the 96 h LC_{50} value for Ag in these fish which is estimated to be above 30 μg L^{-1} but substantially lower than 200 μg L^{-1} where 100% mortality occurred within 72 h of exposure (De Boeck et al., 2001). However, a comparison with the scarce other LC_{50} data on Cu in marine fish still indicates that the spiny dogfish is relatively sensitive to metal exposure. The 48 h LC_{50} for Cu on Mediterranean dogfish, *Scyliorhinus canicula*, was reported to be 4000 μg L^{-1}, probably leaving the 96 h LC_{50} to be above 2000 μg L^{-1} (Torres et al., 1987). Marine teleosts are even less sensitive, with reported 96 h LC_{50} values of 2400 μg L^{-1} for sea catfish (*Arius felis*), of 1140 μg L^{-1} for sheepshead (*Archosargus probatocephalus*), of 5660 μg L^{-1} for Atlantic croacker (*Micropogan undulatus*) and of 2750 μg L^{-1} for pinfish (*Lagadon rhomboides*) (Steele, 1983a,b). Toadfish (*Opsanus beta*) are even more resistant with the 96 h LC_{50} between 21,600 and 36,100 μg L^{-1} (Grosell et al., 2004a). Younger life stages can be considerably more sensitive, with a 96 h LC_{50} of 294 μg L^{-1} for killifish (*Fundulus heteroclitus*) larvae (Grosell et al., 2007).

At 500 μg L^{-1} effects were non-existent or very modest. We observed only a transient drop in plasma pH matching an increase in plasma lactate at 12 h of exposure. Despite the fact that there were no signs of osmoregulatory disturbance, haemoconcentration occurred. Since plasma protein levels remained stable and plasma osmolarity was not disturbed, this probably indicates a release of extra red blood cells from the spleen, which could be either stress-induced or caused by the transient respiratory disturbance indicated by lactate production. Morphological examination of the gill lamellae (Fig. 6) indicates that gill damage is substantial at all three Cu concentrations, with swelling of epithelial cells and lifting of lamellar epithelium at 500 μg L^{-1} and some additional fusion of lamellae and severe lamellar aneurysm in the dogfish gills exposed to the two highest Cu

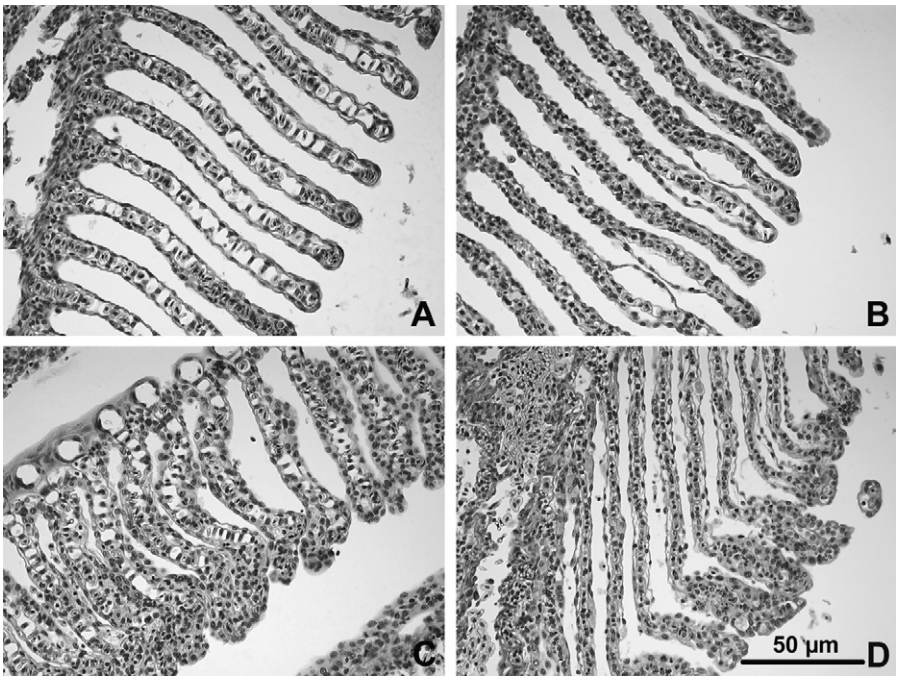

Fig. 6. Gills of spiny dogfish exposed to different Ag concentrations: (A) 0 $\mu g\,L^{-1}$; (B) 500 $\mu g\,L^{-1}$; (C) 1000 $\mu g\,L^{-1}$; (D) 1500 $\mu g\,L^{-1}$. At 500 $\mu g\,L^{-1}$ some swelling of epithelial cells and lifting of lamellar epithelium can be observed, this is supplemented with fusion of lamellae and severe lamellar aneurysm in the dogfish gills exposed to the two highest Cu concentrations.

concentrations. These features result in increased diffusion distances even at 500 $\mu g\,L^{-1}$ and a reduction in respiratory surface at the highest Cu exposure levels.

At the high Cu exposure levels, ionoregulation was clearly disturbed with reduced Na^+/K^+-ATPase activities at the gills and increased levels of Na^+ and Cl^- in the plasma. Thus it seems that at least part of the toxic effects are comparable to those seen in freshwater and marine teleosts (Stagg and Shuttleworth, 1982a,b; Steele, 1983a,b; Laurén and McDonald, 1985, 1987a,b; Wilson and Taylor, 1993; Larsen et al., 1997; Grosell et al., 1999, 2002, 2007; Wood, 2001), and to Ag exposure in elasmobranchs (De Boeck et al., 2001). In contrast to the earlier Ag exposure studies (De Boeck et al., 2001), the rectal gland did not seem to be affected. We observed no inhibition of rectal gland Na^+/K^+-ATPase activities, in accordance with the lack of Cu accumulation in this tissue. Since Na^+ and Cl^- levels were disturbed while rectal gland functioning was not inhibited, our results suggest that the gills do play a role in ionoregulation contrary to earlier indications (Shuttleworth, 1988). However, this consideration must be taken with care since gill membranes probably became leaky (see below). The interactions between gills and rectal gland ionoregulatory capacities, and the factors influencing this interaction, certainly warrant further research under non-toxic (and non-leaky) circumstances. Despite the increase in plasma Na^+ and Cl^- no increase in rectal gland Na^+/K^+-ATPase activity was observed, thus the rectal gland did not seem to compensate for the increase in plasma ions. However, an adequate response may have been inhibited by the systemic acidosis which can affect the ability of the gland to excrete NaCl (Wood et al., 2007).

Urea excretion increased significantly and almost doubled in the highest exposure group. As a consequence plasma urea levels dropped. This finding confirms that normal urea production only just parallels regular urea loss (Carrier and Evans, 1972; Wood et al., 1995), and extra urea loss is not immediately compensated by extra production. Also TMAO levels dropped proportionally to the urea loss. Since spiny dogfish depend on their food for the repletion of lost TMAO (Treberg and Driedzic, 2006), levels of this osmolyte were not restored either. Urea loss seemed to occur primarily from the plasma; plasma levels dropped about five times faster than could be predicted for whole body based on excretion rates. As mentioned above, changes in osmolarity due to plasma urea and TMAO loss (approximately 100 mM) were compensated by the Na^+ and Cl^- gain (about 50 mM each) so that total plasma osmolarity did not change.

Several mechanisms might play a role in the observed urea loss. Our original hypothesis was that this increase in urea excretion was caused by a decreased functioning of the urea back-transporter in the gill epithelia of the fish. This basolateral transporter is Na-coupled (Fines et al., 2001), and several factors could result in a lower efficiency of this transporter. These might include a reduction of the gill Na^+/K^+-ATPase activities, a general disturbance of cellular Na homeostasis leading to a reduced functioning of Na coupled transporters, and the capacity of Cu to compete with Na in transporters However, analysis of the plasma samples for TMAO proved instructive. Plasma TMAO loss was proportional to plama urea loss. This indicates that the whole gill permeability, which is extremely low under normal circumstances, was compromised. Morphological damage to the gill lamellae support the possibility that besides disturbances in the Na^+/K^+-ATPase activities,

leakage of osmolytes could play a role. In contrast with the study by Grosell et al. (2003), we did not observe an effect on ammonia metabolism. This difference is possibly time-related since differences in plasma ammonia in the clear-nosed skate were not yet present after 3 days of exposure but did occur after 8 days of exposure. Our exposures lasted for only 3–4 days.

Cu accumulation rates in tissues were low. At the gills, Cu levels increased with increasing exposure concentrations, and this rise was significant at the two highest Cu exposure levels. The increase in gill Cu levels was about 50-fold, comparable to the results observed for *R. erinacea* at much lower exposure levels (Grosell et al., 2003). Undoubtedly, given enough time, significant Cu accumulation would have occurred in the gills at the lower exposure concentration as well. The same tendency existed in the kidney, with the increase being significant only at the two highest Cu exposure levels. Although Cu was taken up into the plasma at the two highest exposure levels, there was no indication of Cu accumulation in any of the other tissues.

The difference in sensitivity of spiny dogfish to Cu or Ag, two metals with similar toxic actions in teleosts, is striking. The Cu ions that are considered to be bioavailable are Cu^{2+} and $CuOH^{+}$ (Paquin et al., 2002), and these are still abundantly present in full strength seawater (Grosell et al., 2007) making it unlikely that Cu speciation explains the lower sensitivity to this element. In contrast all of the silver is complexed as various forms of $AgCl_n^{1-n}$ at 30 ppt (Webb and Wood, 2000), However, Ag uptake in spiny dogfish was impressive; both in quantity and uptake rate (De Boeck et al., 2001). In contrast with the present study Ag accumulation occurred in all tissues except muscle at the highest exposure level. Even at the lowest exposure level of 30 $\mu g\,L^{-1}$ Ag, a significant accumulation was observed in both gill and liver over a 5-day exposure. The high bioavailability for Ag was also reflected in relatively high control levels. Despite the fact that Cu accumulation in elasmobranchs is higher than in teleosts (Grosell et al., 2003), it seems to be the exceptionally high bioavailability of Ag that explains the difference between the two elements (Webb and Wood, 2000; De Boeck et al., 2001).

We conclude that Cu causes both respiratory and ionoregulatory distress to spiny dogfish, and an estimated 96 h LC_{50} in the 800–1000 $\mu g\,L^{-1}$ range shows them to be just slightly more sensitive compared to the few marine fish for which data are available. Ionoregulatory perturbations only occurred at the two highest exposure levels (1000 and 1500 $\mu g\,L^{-1}$), and effects included a decrease in gill Na^{+}/K^{+}-ATPase followed by an increase in plasma Na^{+} and Cl^{-} as well as an increase in urea excretion (and probably also in TMAO excretion) followed by a decrease in plasma urea and TMAO levels. The rise in plasma Na and Cl counteracted the fall in the two organic osmolytes thereby resulting in no change in plasma osmolarity. Cu accumulation rates were moderate considering the high exposure concentrations, with an increased Cu burden in the plasma, gill and kidney at the two highest exposure levels only. In this elasmobranch, the toxic action of waterborne Cu exposure is similar to the effects seen in the marine teleosts as far as Na transport in the gills is concerned. An additional toxic effect is caused by the compromisation of urea and TMAO retention. Our short-term study did not provide an indication for disturbances in ammonia metabolism or excretion.

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