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Physiological effects of waterborne lead exposure in spiny dogfish (*Squalus acanthias*)

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ABSTRACT

To broaden our knowledge about the toxicity of metals in marine elasmobranchs, cannulated spiny dogfish (*Squalus acanthias*) were exposed to 20 μM and 100 μM lead (Pb). Since we wanted to focus on sub lethal ion-osmoregulatory and respiratory disturbances, arterial blood samples were analysed for pH_a, PaO_2, haematocrit and total CO_2 values at several time points. Plasma was used to determine urea, TMAO, lactate and ion concentrations. After 96 h, Pb concentrations were determined in a number of tissues, such as gill, rectal gland, skin and liver. To further investigate ion and osmoregulation, Na^+/K^+-ATPase activities in gill and rectal gland were analysed as well as rates of ammonia and urea excretion. Additionally, we studied the energy reserves in muscle and liver. Pb strongly accumulated in gills and especially in skin. Lower accumulation rates occurred in gut, kidney and rectal gland. A clear disturbance in acid–base status was observed after one day of exposure indicating a transient period of hyperventilation. The increase in pH_a was temporary at 20 μM, but persisted at 100 μM. After 2 days, plasma Na and Cl concentrations were reduced compared to controls at 100 μM Pb and urea excretion rates were elevated. Pb caused impaired Na^+/K^+-ATPase activity in gills, but not in rectal gland. We conclude that spiny dogfish experienced relatively low ion-osmoregulatory and respiratory distress when exposed to lead, particularly when compared to effects of other metals such as silver. These elasmobranchs appear to be able to minimize the disturbance and maintain physiological homeostasis during an acute Pb exposure.

1. Introduction

As far back as 1919, reports of streams poisoned with lead (Pb) (mainly caused by mining activity and deposits of airborne lead from lead-based fuel) drew attention to this metal and its toxicity in aqueous ecosystems (Järup, 2003; Tao et al., 1999). Since then, researchers have studied adverse effects of Pb predominantly in freshwater fish, such as fathead minnows, stickleback, brown trout, rainbow trout and common carp (Alados and Weber, 1999; Bervoets et al., 2001; Birceanu et al., 2008). Neurological disorders, Pb induced muscle spasms, haematological effects, growth inhibition, reproductive problems, paralysis and mortality are some general effects of Pb on exposed freshwater fish (Grosell et al., 2006; Martinez et al., 2004). However, there is still limited information available about the effect of Pb on marine elasmobranchs. The few earlier toxicological studies illustrated that elasmobranchs could tolerate relatively high levels of copper (Cu), cadmium (Cd) and zinc (Zn) (mg/l range), but were remarkably sensitive to silver (Ag) which was accumulated quickly and extensively (De Boeck et al., 2001, 2007; Grosell et al., 2003; Taguchi et al., 1979). Our attention was drawn to the possible toxic effects of Pb, since an earlier study with spotted dogfish *Scyliorhinus canicula* indicated that Pb accumulated in high amounts especially in skin, but also in gill and rectal gland (De Boeck et al., 2010).

One of the adverse effects during Ag exposure appeared to be a failure in urea retention and consequently the occurrence of dehydration (De Boeck et al., 2001). Since elasmobranchs are known to be slightly hyperosmotic to their environment, urea, which circulates in the blood in high concentrations and permeates all cells, becomes vital in their strategy to retain water (De Boeck et al., 2010; Epstein, 1979; Hazon et al., 2003). Urea's tendency to destabilize proteins is counteracted by trimethylamine oxide (TMAO) which serves as a balancing osmolyte (Kajimura et al., 2008; Yancey, 2001). Despite tight and impermeable gill membranes (Pärt et al., 1998; Wood et al., 1995), with high cholesterol epithelia and basolateral Na^+-coupled urea back transporters (Fines et al., 2001), a continuous loss of urea exists due to the a high urea gradient between the internal and external environment (reviewed by

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Walsh and Smith, 2001). Approximately 90% of nitrogen excretion in *Squalus acanthias* consists of urea, although there has been speculation that TMAO attributes to the total nitrogen excretion as well (e.g. Kajimura et al., 2008). The kidney reabsorbs more than 90% of both urea and TMAO (Treberg and Driedzic, 2006; Wood et al., 1995, 2005). In contrast to urea, which is produced in both liver and muscle (Kajimura et al., 2006; Morgan et al., 2003), elasmobranchs are not able to produce TMAO. They are therefore obliged to eat every 5–6 days to maintain their nitrogenous balance (Kajimura et al., 2006, 2008). Besides urea and TMAO, inorganic ions such as Na^+ and Cl^- account for most of the other osmolytes in plasma (Kajimura et al., 2006, 2008).

Gills of elasmobranchs are the most important sites of passive NaCl uptake. Na^+ and Cl^- gained by diffusion are not primarily excreted at the gills but rather by the rectal gland. This little finger-shaped organ secretes an almost pure NaCl solution (Shuttleworth et al., 2006; Wood et al., 2007) with miniscule amounts of urea present (Epstein, 1979). Gills and rectal gland are involved in ionoregulation and are both equipped with Na^+/K^+-ATPases in their basolateral membranes (Epstein, 1979; Payan and Maetz, 1973), making these organs important targets for metal toxicity. The gill is also the main site of ammonia and H^+-ion excretion as well (Grosell et al., 2003; Payan and Maetz, 1973).

In this study, we assessed the physiology of Pb toxicity in *S. acanthias* to broaden our knowledge concerning toxicology of metals in these marine elasmobranchs. Spiny dogfish are widespread, relatively abundant, and of appropriate size, and their physiology has been extensively studied. To limit ecological impact, we avoided working with females which are much larger and often pregnant. We used cannulated spiny dogfish to investigate osmoregulatory and respiratory disturbances over time (effects on blood gasses, pH, plasma ions, lactate, urea and TMAO concentrations, Na^+/K^+-ATPase activity in gill and rectal gland) and assessed Pb accumulation as well as energy reserves in liver and muscle tissues, which might be indicative for changes in energy metabolism.

2. Materials and methods

In the summer of 2009, Pacific spiny dogfish (*S. acanthias*) were caught in the proximity of Bamfield, BC, Canada. These dogfish were housed in a large concrete indoor tank (~150,000 l) at Bamfield Marine Science Centre served with running aerated seawater (14 °C, 30‰ salinity) and kept there at least 1 week before experiments began. Fish were fed twice a week with a mixture of marine teleosts, but starved for 1 week prior to experiments.

2.1. Lead exposed cannulated fish

This experiment required repetitive blood sampling and in order to do so, dogfish were fitted with caudal artery catheters. Twenty four dogfish (1.94 ± 0.11 kg, 74.07 ± 1.34 cm (mean ± SEM)) were caught from the indoor tank and anaesthetized in a 100 mg/l MS222 (neutralized) seawater solution for surgery. Dogfish were placed on a V-shaped operating table, their gills were constantly irrigated with anaesthetic throughout surgery. A small incision was made, approximately 5 cm anterior to the caudal fin, to the vertebrae, exposing the cartilaginous haemal canal. This canal was punctured with a #22 needle creating a small hole for a PE50 polyethylene cannula, filled with heparinised 50 i.u./ml dogfish saline (6 mM $NaHCO_3$, 257 mM NaCl, 7 mM $NaSO_4$, 0.1 mM NaH_2PO_4, 4.1 mM KCl, 3 mM $MgSO_4$, 5 mM glucose, 2 mM $CaCl_2$, 350 mM urea, 15 mM TMAO). The cannula was held in place by a sleeve of PE160 secured with 2 sutures to the skin. Dogfish were revived by artificial ventilation with anaesthetic-free seawater and were left to recover overnight in covered wooden fish boxes. These boxes (length 105 cm, width 16.5 cm and height 25 cm) contained 32 l of Bamfield Marine Station seawater with a flow-through of 1 l/min and were fitted with perimeter aeration over the complete length of the box.

Three exposure series, for a period of 96 h each, were performed on 8 dogfish (8 for each exposure) using nominal total Pb concentrations of 0, 20 μM and 100 μM. Exposure started by adding Pb to each fish box as $Pb(C_2H_3O_2)_2{\cdot}3H_2O$ (from a stock solution). Since Pb precipitates very quickly, a lower dose was spiked at 6-h intervals so as to maintain the desired concentrations. Once the exposure started, the flow-through was inactivated (however, the aeration was maintained) in order to measure the urea and ammonia efflux to the water. Every 12 h, fish boxes were flushed 3 times with 67% renewal, allowing the dogfish to remain submerged. After renewal, fish boxes were spiked again with the appropriate amount of Pb. Water samples for determination of Pb concentration and urea and ammonia excretion were taken at the beginning and end of each 12 h exposure period.

During each exposure, blood samples (1 ml) were taken before the start of the exposure and after 12 h, 24 h, 48 h, 72 h and 96 h of exposure with a gas-tight Hamilton syringe. When blood was sampled, 1 ml of non-heparinised dogfish saline was injected to replace the volume of the blood. At the end of each exposure, dogfish were quickly killed by an overdose of neutralized MS222. Gill, muscle, skin, liver, rectal gland, gut and kidney were dissected and flash frozen in liquid nitrogen and subsequently stored at −80 °C for determination of Pb concentration. Gill and rectal gland samples for Na^+/K^+-ATPase were stored separately, also at −80 °C.

2.2. Analytical procedures

Pb concentrations were measured using inductive coupled plasma mass spectrometry. The decline in lead concentration between the start and end of the 12-h periods did not exceed 3.5% in the highest Pb concentrations, and was less than 7% in the lower exposure concentrations.

Immediately after blood sampling, arterial pH, PaO_2, and $PaCO_2$ were determined using a micro-capillary pH electrode (Radiometer G279/G2 plus E5021) coupled to a PHM71 meter for pH measurements, a micro-oxygen electrode (Radiometer E5046) for PaO_2 measurements, a Cameron chamber (Cameron, 1971) with a CO_2 electrode (Radiometer E5046) coupled to a PHM71 meter for total $[CO_2]$ (CCO_2) measurements. The Cameron chamber was kept at 37 °C. All other electrodes were kept at 14 °C by a water jacket perfused with ambient seawater. $PaCO_2$ was calculated using the solubility of carbon dioxide (αCO_2) and the apparent pK (pKapp) for dogfish plasma according to Boutilier et al. (1984):

$$PaCO_2 = CCO_2/(\alpha CO_2(10\mathrm{pH} - \mathrm{p}K\mathrm{app} + 1))$$

with CCO_2 being total plasma CO_2. Plasma HCO_3^- content was calculated as the difference between total plasma CO_2 and $\alpha CO_2{\cdot}PaCO_2$. Haematocrit was determined by centrifuging blood-filled capillary tubes (heparinized) at 2000 × g for 2 min and measuring the percentage of red blood cells present.

The remainder of the blood sample was centrifuged (5 min, 10,000 × g) and plasma samples were frozen at −80 °C for determination of $[Na^+]$, $[K^+]$, $[Cl^-]$, $[Ca^{2+}]$, [TMAO], [urea], [lactate] and [Pb].

Plasma ions were analysed using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium). Plasma urea and water urea were analysed with the diacetyl monoxime method (Price and Harrison, 1987). Plasma lactate was determined using an enzymatic kit (Cat. No. 11 112 821 035, R Biopharm, Boehringer Mannheim, Darmstadt, Germany). 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).

Frozen gill and rectal gland samples were thawed on ice and homogenized for determination of Na^+/K^+-ATPase activity using the method of McCormick (1993). The associated protein content was assayed using the technique of Bradford (1976).

Tissue samples for metal analysis were weighed, dried in a 60 °C drying oven for at least 1 week, weighed again, dissolved with 69% HNO_3 and 30% H_2O_2 (Merck, Darmstadt, Germany) and digested in a microwave oven. After digestion, samples were diluted to obtain a final acid concentration of 1%. Plasma samples were diluted to 1% HNO_3 with Milli-Q grade water (Millipore, Bedford, MA, USA). Standard curves were made by standard addition. Metal concentrations of tissue and water samples were analysed using Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Varian Ultra Mass 700, Victoria, Australia). Analytical accuracy was achieved by the use of blanks containing Milli-Q water and solutions used for digestion. Generally, the concentrations of the blanks were below detection limits and recovery rates of the standards were 97.3 ± 4.8%.

A subsample of liver and muscle tissue was analysed for its lipid, glycogen and protein concentration using slightly modified protocols of Bligh and Dyer (1959), Roe and Dailey (1966) and the Bradford method (Bradford, 1976) respectively.

2.3. Statistics

Using Statistica, One way analysis of variances (ANOVA), followed by a Tukey HSD post hoc test, was used to determine differences between control and exposed values for all measured parameters (statistically different results were indicated with "*"). Furthermore, repeated measures ANOVA was used for all blood, plasma and excretion parameters determined (statistically different results were indicated with "°"). Experimental values of each group were compared to its own pre-exposure control value within the repeated measures statistics. All results are given as mean values (±SEM). The significance level was 5%.

3. Results

No mortality occurred during the 96 h experiment. Measured metal concentrations were 24.51 ± 4.29 μM for the 20 μM Pb exposure and 107.34 ± 2.58 μM for the 100 μM Pb exposure. Control values (0 μM Pb) were 0.21 ± 0.10 μM Pb. Pre-exposure blood and plasma values and excretion rates at 0 h from all cannulated dogfish are reported in Table 1.

Due to confinement and repeated sampling, some of these parameters changed over time in control dogfish. Repeated sampling reduced the Hct levels over time from approximately 20% to 11–12%. This decrease in Hct was similar in all exposure groups. Other changes in control dogfish included modest changes in plasma ions and bicarbonate.

3.1. Pb accumulation

In all tissues studied (Table 2), including control tissues, Pb accumulation was found. It appeared that, even without an experimental Pb exposure, control dogfish had traceable amounts of Pb present, especially in skin and gills. At the end of the 96-h exposure period, dogfish exposed to 20 μM Pb accumulated Pb mostly in skin and gill. Gut, kidney and rectal gland showed moderate increases while Pb concentrations in liver and muscle did not increase significantly. However, when dogfish were exposed to 100 μM Pb, all organs (including liver and muscle) displayed higher Pb concentrations compared to control tissues. Nonetheless, the same trend was found: skin and gills were the tissues with the highest Pb accumulation and liver and muscle showed the lowest Pb accumulation during this acute exposure period.

Table 1
Pre-exposure values at 0 h from control and exposed dogfish ($N = 24$) for parameters with repeated measurements.

	Mean value	S.E.M.	Unit
pH	7.88	0.01	
$PaCO_2$	1.30	0.05	Torr
PaO_2	111.87	3.02	Torr
Total [CO_2]	4.55	0.13	mM
[HCO_3^-]	4.44	0.12	mM
Hct	19.64	0.56	%
[Pb] Plasma	0.20	0.03	μg/ml
[Na^+]	235.07	1.42	mM
[Cl^-]	228.76	1.80	mM
[Ca^{2+}]	2.47	0.01	mM
[K^+]	3.17	0.06	mM
[Urea] plasma	432.03	7.13	mM
[TMAO] plasma	76.05	3.1	mM
[Lactate] plasma	1.84	0.21	mM
Urea excretion	204.14	20.96	μM/kg/h
Ammonia excretion	278.20	38.31	μM/kg/h

Table 2
Pb accumulation in tissues of dogfish exposed to 0, 20 and 100 μM Pb (96 h). ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.).

	0 μM Pb	20 μM Pb	100 μM Pb
Gill	2.70 ± 0.46	7.36 ± 0.74*	21.39 ± 4.24***
Liver	0.09 ± 0.01	0.13 ± 0.03	0.15 ± 0.03*
Muscle	0.11 ± 0.01	0.76 ± 0.18	1.13 ± 0.34**
Gut	0.11 ± 0.01	0.65 ± 0.17**	1.30 ± 0.29***
Kidney	0.94 ± 0.13	2.39 ± 0.59*	5.74 ± 0.58***
Rectal gland	0.63 ± 0.15	1.23 ± 0.13**	1.78 ± 0.33**
Skin	4.45 ± 0.42	232.69 ± 33.15**	513.83 ± 86.88***

Control plasma Pb concentrations were very low during the entire exposure period. Exposure to 20 μM Pb resulted in a peak of Pb concentration after 24 h of exposure, shortly followed by some recovery but yet still significantly higher concentrations compared to control dogfish (Fig. 1). Dogfish exposed to 100 μM Pb were not able to clear their plasma as quickly and showed no signs of recovery within the first days. Only at the end of the exposure, plasma Pb levels dropped to levels comparable to the 20 μM exposure group, bur remained significantly elevated compared to the control values at the same time points.

3.2. Plasma ion concentration

In both the 20 and 100 μM Pb exposures, a drop of [K^+] occurred at 96 h (Fig. 2) to a level which was significantly different to the own

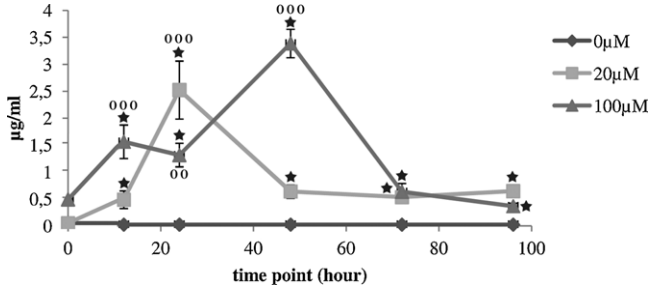

Fig. 1. Plasma [Pb].
[Pb] in plasma of dogfish exposed to 0, 20 and 100 μM Pb over time (12–96 h). ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$/'°' indicates a significant difference between exposed ($N = 8$) and their own pre-exposure value at time point 0 h:°°: $p < 0.01$;°°°: $p < 0.001$.)

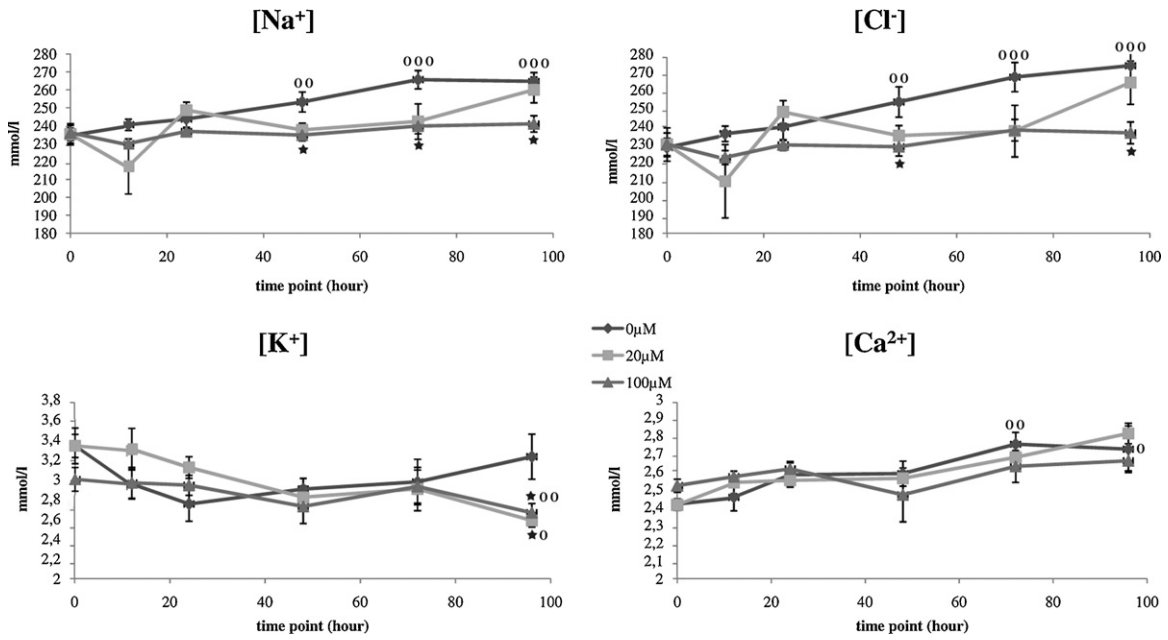

Fig. 2. Plasma [Ion].
Ion concentrations in plasma of dogfish exposed to 0, 20 and 100 μM Pb over time (12–96 h) ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$): *: $p < 0.05$/'°' indicates a significant difference between exposed ($N = 8$) and their own pre-exposure value at time point 0 h:°: $p < 0.05$;°°: $p < 0.01$;°°°: $p < 0.001$). Dark grey ● represents 0 μM Pb; light grey ■ represents 20 μM Pb; grey ▲ represents 100 μM Pb.

pre-exposure values (Table 1) and to the control fish at 96 h. The exposure to 100 μM Pb resulted in plasma [Na^+] and [Cl^-] levels which were statistically below the values of control fish at the same time points (Fig. 2), mainly because levels of these ions increased in control dogfish during the 96-h experiment. Throughout the experiment, no differences between control and exposed dogfish were noticed in [Ca^{2+}] concentrations, however the level of Ca^{2+} was increased at 72 h and 96 h (Fig. 2) compared to the pre-exposure values at 0 h in control dogfish (Table 1.).

3.3. Na^+/K^+-ATPase activity in gill and rectal gland

At the end of the exposure the Na^+/K^+-ATPase activity in gill tissue of dogfish exposed to 100 μM Pb (Fig. 3A) was reduced by half compared to its activity in control dogfish. In contrast, we found no differences between the groups in Na^+/K^+-ATPase activity in the rectal gland (Fig. 3B).

3.4. Plasma urea and TMAO/ammonia and urea excretion

In plasma of the Pb exposed dogfish (Fig. 4A) [TMAO] showed increased levels after 12 h, 48 h and 96 h of exposure compared to values of control dogfish at the same time points, however, these increased values were not statistically different from their own pre-exposure control values.

The [urea] in plasma as well as the urea and ammonia excretion rates were not influenced by the control conditions or by exposure to 20 μM Pb (Fig. 4B (urea plasma), Fig. 5A (urea excretion), and Fig. 5B (ammonia excretion)). Only when the dogfish were exposed to 100 μM Pb, plasma [urea] increased briefly at 72 h of exposure (Fig. 4B), accompanied by an increased urea excretion in the second half of the exposure (Fig. 5A). Furthermore, ammonia excretion in dogfish exposed to 100 μM Pb increased at the end of the exposure period at the highest exposure level (Fig. 5B).

3.5. Blood acid–base status

Although there appeared to be a drop in the PaO_2 after 12 h of exposure, no significant differences could be found, indicating that oxygen transport was not compromised during Pb exposure (Fig. 6).

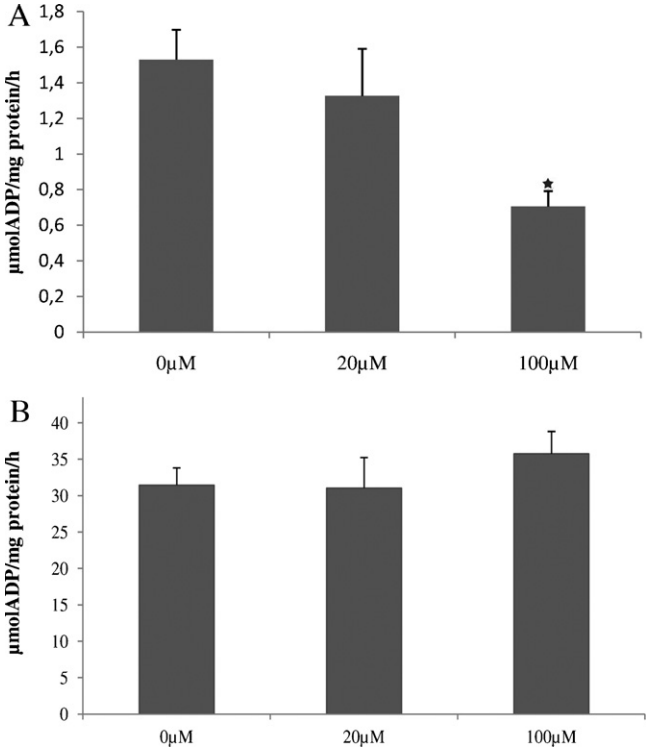

Fig. 3. Na^+/K^+-ATPase activity in gill and rectal gland.
Na^+/K^+-ATPase activity in gill (A) and rectal gland (B) of dogfish exposed to 0, 20 and 100 μM Pb. ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$.)

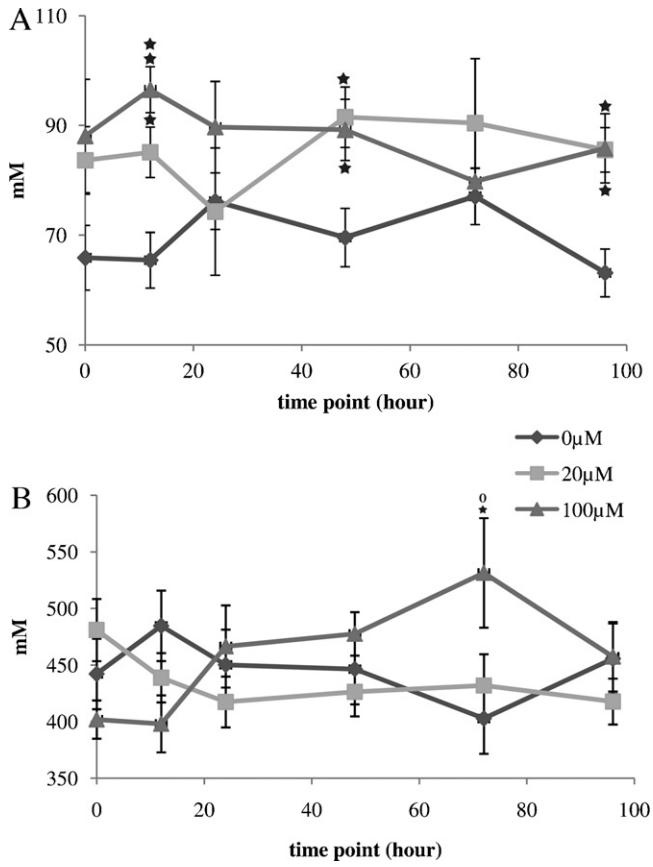

Fig. 4. Plasma [TMAO] and [Urea].
[TMAO] (A) and [Urea](B) in plasma of dogfish exposed to 0, 20 and 100 µM Pb over time (12–96 h). ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$, **: $p < 0.01$.) '°' indicates a significant difference between exposed ($N = 8$) and their own pre-exposure value at time point 0 h:°: $p < 0.05$). Dark grey ● represents 0 µM Pb; light grey ■ represents 20 µM Pb; grey ▲ represents 100 µM Pb.

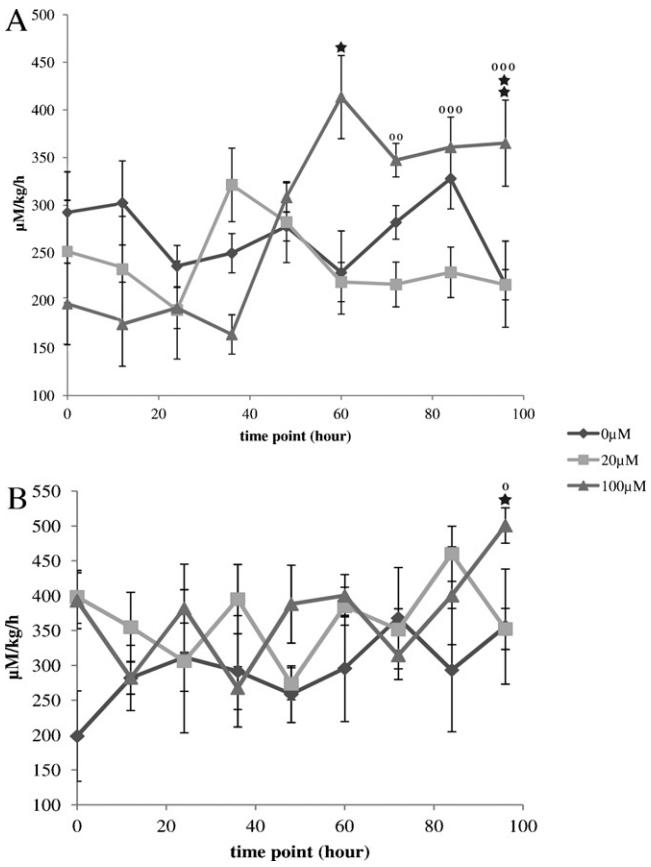

Fig. 5. Urea and Ammonia excretion.
Urea excretion rate (A) and ammonia excretion rate (B) of dogfish exposed to 0, 20 and 100 µM Pb over time (12–96 h). ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$, **: $p < 0.01$/'°' indicates a significant difference between exposed ($N = 8$) and their own pre-exposure value at time point 0 h:°: $p < 0.05$;°°: $p < 0.01$;°°°: $p < 0.001$.) Dark grey ● represents 0 µM Pb; light grey ■ represents 20 µM Pb; grey ▲ represents 100 µM Pb.

The arterial pH_a in both Pb-exposed groups exhibited an increase (Fig. 7). When dogfish were exposed to 20 µM Pb, this transient but steep increase appeared after 24 h and was followed by recovery thereafter. A different scenario was seen when dogfish were exposed to 100 µM Pb; here pH_a was significantly different from control values and/or their own pre-exposure values during the entire exposure period.

A clear decrease of $PaCO_2$ was seen after 24 h and to a lesser extent after 96 h of Pb exposure. No significant changes were found between total [CO_2] and [HCO_3^-] of control and exposed dogfish (Fig. 7). When compared to their own pre-exposure values, control dogfish and dogfish exposed to 100 µM Pb showed increased concentrations of total [CO_2] and [HCO_3^-] near the end of the exposure period.

In dogfish exposed to 100 µM Pb, lactate concentrations in plasma were lower compared to concentrations in control dogfish over the entire exposure period. However this drop was not significant when compared to their own pre-exposure value (Fig. 8).

3.6. Energy status of muscle and liver: [glycogen], [lipid], [protein]

Analysis revealed decreases in liver protein and muscle glycogen in dogfish exposed to 100 µM Pb (Fig. 9A liver and Fig. 9B muscle).

4. Discussion

In seawater, Pb speciation is in function of the chloride concentration; $PbCl_3^-$, $PbCO_3$, $PbCl_2$, $PbCl^+$, $Pb(OH)^+$ and even uncomplexed Pb^{2+} can be present (Fernando, 1995). In normal circumstances, seawater contains only trace amounts of Pb (0.03–0.05 µg/l), however, there have been reports of Pb concentrations from 10 up to around 25 mg/l Pb (Fatoki and Mathabatha, 2001; Yilmaz and Sadikoglu, 2011). The concentrations used in this manuscript are therefore environmentally relevant (20 µM:

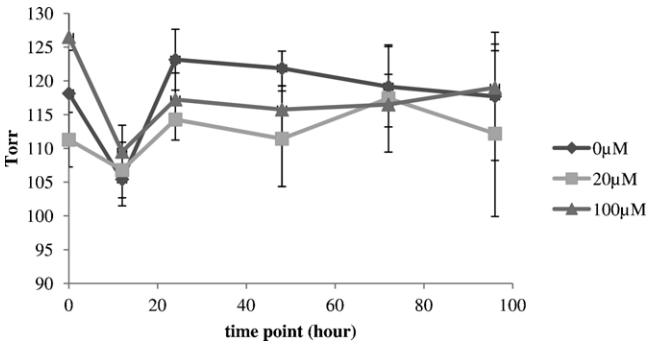

Fig. 6. Blood [PaO_2].
PaO_2 in blood of dogfish exposed to 0, 20 and 100 µM Pb over time (12–96 h). No significant differences were observed.

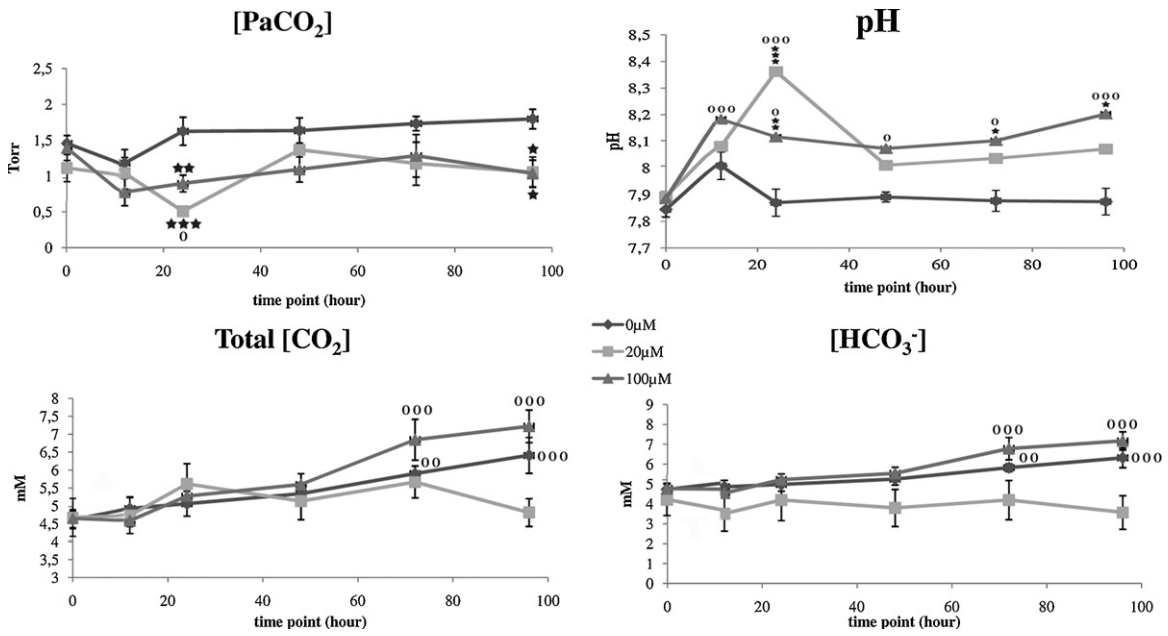

Fig. 7. Blood pH, [$PaCO_2$], Total [CO_2] and [HCO_3^-].
pH, [$PaCO_2$], Total [CO_2] and [HCO_3^-] in blood of dogfish exposed to 0, 20 and 100 µM Pb over time (12–96 h). ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$/'°' indicates a significant difference between exposed ($N = 8$) and their own pre-exposure value at time point 0 h: °: $p < 0.05$; °°: $p < 0.01$; °°°: $p < 0.001$.) Dark grey ● represents 0 µM Pb; light grey ■ represents 20 µM Pb; grey ▲ represents 100 µM Pb.

4.144 mg/l and 100 µM: 20.72 mg/l). For freshwater fish, Pb^{2+} is more available in acidic and ion-poor waters than in more neutral waters (Birceanu et al., 2008) and Pb^{2+} toxicity is attributed to its similar hydrated radius as Ca^{2+}, so that it likely competes with Ca^{2+} for binding sites on the gill which eventually can lead to hypocalcaemia and death of the fish (Birceanu et al., 2008; MacDonald et al., 2002; Rogers et al., 2003; Rogers and Wood, 2004; Stouthart et al., 1994). In our research, there was no sign of disturbance in plasma Ca^{2+} levels, and overall few effects were observed on plasma ions. Only a clear decrease of [K^+] was seen in both Pb-exposed groups. In humans, a decreased plasma [K^+] can cause muscle spasms and we did indeed notice more post mortem muscle spasms during dissection in comparison with control dogfish. Furthermore, gill Na^+/K^+ ATPase activity was inhibited by 50% at the highest Pb concentration, but since rectal gland Na^+/K^+ ATPase activity was not affected, the effect of the decrease was limited. Possibly, a long-term exposure to high concentrations of Pb could lead to a high Pb accumulation in the rectal gland as well and negatively influence the ion homeostasis. In De Boeck et al. (2010), exposure of European dogfish to 10 µM Pb did not cause any osmoregulatory disturbance either, although Pb accumulated in all studied organs. During our study in the spiny dogfish, the high accumulation of Pb in dogfish skin and gills was prominent. Since the animals were exposed only to waterborne Pb, those organs naturally made up the first contact area with this toxic metal. Pb accumulation in liver

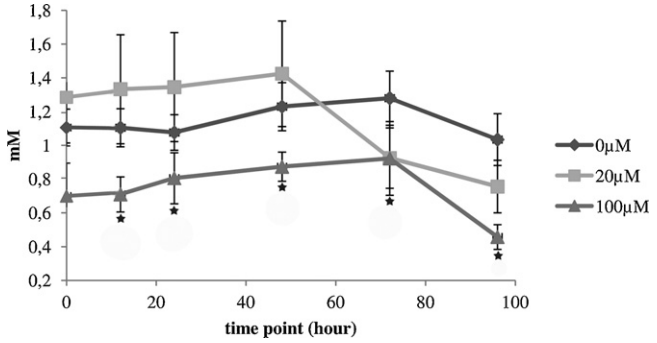

Fig. 8. Plasma [lactate].
[Lactate] in plasma of dogfish exposed to 0, 20 and 100 µM Pb over time (12–96 h). '*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); *: $p < 0.05$). (No differences were found using repeated measures ANOVA.)

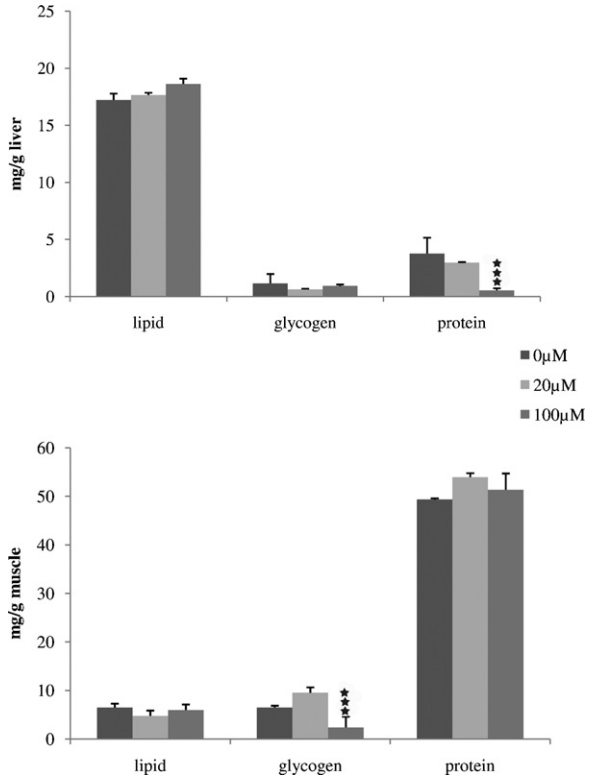

Fig. 9. Energy reserves in liver and muscle.
Energy reserves in liver (A) and muscle (B) tissue of dogfish exposed to 0, 20 and 100 µM Pb (96 h). ('*' indicates a significant difference between exposed ($N = 8$) and control dogfish ($N = 8$); ***: $p < 0.001$.)

during acute exposure conditions was relatively low, certainly when compared to accumulation levels of other non-essential metals (Ay et al., 1999). Heier et al. (2009) observed a similar pattern in brown trout, exposed to 15–20 μg/l in a natural stream, where Pb was first deposited in gill tissue and, over time, more in liver tissue. Besides the short acute exposure period in our experiment, the large size of the liver in spiny dogfish would also minimize the impact of a small hepatic influx of Pb.

Pb concentrations in skin and gills of control dogfish indicated previous encounters with Pb contamination in the wild. The placoïd scales and high collagen content of the skin seem to have an elevated affinity for Pb (De Boeck et al., 2010; Jeffree et al., 2006). Jeffree et al. (2006, 2008) studied Pb absorption on spotted dogfish eggs and its accumulation in embryos. Similar to the skin of dogfish, the egg case is made of collagen, which may be responsible for the high accumulation in dogfish skin and eggs (Jeffree et al., 2006). As such, the Pb burden in the organs of control dogfish suggested a rapid accumulation and slow excretion for Pb (De Boeck et al., 2010), especially since Pb levels in nature usually remain below the detection limit (Blanco et al., 2008). When looking at metal contamination in fish destined for food industry, skin should be considered a tissue for investigation.

As for the high accumulation rate in gills, Grosell et al. (2003), who investigated Cu exposure in clear nosed skate, suggested that this might be a common feature of marine elasmobranchs during waterborne metal exposure. In the present study, gill tissue accumulated 70 (exposed to 20 μM) to 200 (exposed to 100 μM) times more Pb than liver tissue. Fish gills can react with an excessive epidermal mucus secretion when confronted with metals or other particles (Carpenter 1927 in Coello and Khan, 1996). Components of glycoproteins in the mucus can bind Pb and thereby prevent, for a short period of time and until a certain concentration, further intrusion into the organism (Coello and Khan, 1996). However, since Pb was considerably elevated in plasma when dogfish were exposed to 100 μM Pb, it is obvious that Pb also crossed the gills and was able to significantly accumulate in other organs (Rogers et al., 2003). Exposure to 100 μM of Pb induced an accumulation of Pb in gut, rectal gland, skin, liver, muscle and kidney. With exposure to 20 μM Pb, accumulation occurred in the same organs, however not in liver and muscle. The slightly elevated Pb concentrations in gut tissue might be indicative of a stress-induced drinking response, which resulted in intestinal accumulation of Pb (Grosell et al., 2003). Although marine elasmobranchs are thought not to have a drinking response, Anderson et al. (2002) demonstrated that, under the appropriate environmental conditions, these fish species will drink as a part of their overall iso/hyperosmoregulatory strategy.

In the previously mentioned study of De Boeck et al. (2010) similar results concerning Pb accumulation were found in Pb-exposed European dogfish. No physiological disturbances were seen in gill tissue of these sharks exposed to 10 μM Pb. Our exposure of spiny dogfish to 20 μM Pb gave the same results: no effects on Na^+/K^+-ATPase activity in gill tissue. However, when spiny dogfish were exposed to 100 μM Pb, this enzyme activity was decreased in gill tissue, despite the fact that there were no obvious Na^+ and Cl^- increases in plasma. We have also investigated the Na^+/K^+-ATPase activity in rectal gland, though the concentration of Pb accumulated in this organ did not cause any change in this enzyme's activity. This resembles earlier Cu exposures to spiny dogfish where the enzyme activity of Na^+/K^+-ATPase was not influenced either (De Boeck et al., 2007) although here, no significant accumulation was seen in the rectal gland. In contrast, exposure to Ag, did cause a steep increase of Ag concentration in the rectal gland and this rise was accompanied by a decreased activity of Na^+/K^+-ATPase (De Boeck et al., 2001).

Decreased plasma urea concentrations and elevated urea fluxes to the environment were likely indicative of changes in the diffusive permeability of gills. De Boeck et al. (2001) were able to link Ag exposure of dogfish to the aforementioned physiological effects. When dogfish were exposed to 100 μM Pb, plasma urea increased slightly after 72 h of exposure, only to return to normal 24 h later. The urea excretion was elevated around the same time point, but remained high until the end of the experiment. Since no decrease of urea concentration in plasma was seen and no loss of TMAO occurred, breaches in membrane permeability seemed unlikely. If anything, TMAO was slightly increased compared to control values at the same time points. Stress-induced breakdown of proteins can still be the reason of the small plasma urea elevation in sharks exposed to 100 μM Pb and the concurrent rapid excretion or loss of urea.

The results indicated no signs of respiratory stress, since no changes occurred in Pa_{O_2}. When compared to their own pre-exposure values, lactate remained unchanged, however, if we compared the control values at similar time points with values of dogfish exposed to 100 μM of Pb, lactate concentrations in the latter seemed slightly decreased indication lactate oxidation. Despite the stable Pa_{O_2}, the temporary drop in Pa_{CO_2} and the increased blood pH, both at 24 h after exposure to 20 μM Pb, could be indicative of a slight hyperventilation. A coughing reflex caused by an overproduction of mucus which can lead to asphyxiated gills, could also be a possible explanation (De Boeck et al., 2001). Concurrently at 24 h of exposure, plasma Pb concentration was elevated. This was also seen in dogfish exposed to 100 μM, where blood pH stayed high from 12 h after the start until the end of the exposure period. Plasma [Pb] also increased after 12 h of exposure, but returned to lower concentrations 36 h later. Interestingly, a high pH makes formation of lead precipitates easier, thereby decreasing Pb availability. Gill tissue is an important organ for acid excretion (Payan and Maetz, 1973) and since Pb deposited quickly in this organ, this function might be compromised, resulting in a higher internal pH. Rogers et al. (2003) observed similar stabilities in Pa_{O_2}, Pa_{CO_2}, pH_a, plasma lactate, and ventilation rates in Pb-exposed trout, indicating that the mechanism of acute toxicity for Pb in freshwater fish was not respiratory based either.

When confronted with stress situations, fish release stress hormones into the bloodstream, which can cause hyperglycemia, lactacidosis, depletion of glycogen, an increase of catabolism of the muscle proteins and inhibition of protein synthesis. Brain, heart, rectal gland and red muscle all use glucose as their main energy source (Walsh et al., 2006; Wood et al., 2010) and do need to maintain the right amount of glucose to function properly. Since elasmobranchs are not capable of oxidizing long chain fatty acids in extra hepatic tissues, fatty acids are transformed into ketone bodies in the liver. Those ketone bodies, such as β-hydroxybutyrate and acetoacetate, are then released into the blood stream to serve as important additional energy sources in other tissues (Richards et al., 2003; Speers-Roesch et al., 2006). As such, the liver seems to act as the principal site for lipid storage (Kajimura et al., 2008) although this high concentration of lipids in the liver is not entirely metabolically available since lipid stores are used for buoyancy as well (Rosell et al., 1989). The latter might explain why, together with the short exposure time, there was no change detectable in lipid concentrations in liver and muscle as a result of exposure to Pb. Liver protein and muscle glycogen however decreased in dogfish exposed to 100 μM Pb. Similar results were obtained in fresh water fish *Channa punctatus* exposed to Zn (Srivastava and Srivastava, 2008) and *Prochilodus lineatus* exposed to Pb showed a hyperglycaemia associated with a decrease in lipid and protein concentrations (Martinez et al., 2004). Since exhaustive exercise is partly fuelled by glycolysis (Richards et al., 2003), the decrease of glycogen in the muscle of spiny dogfish exposed to 100 μM of Pb, together with the decreased protein concentrations in these livers, can indicate a decreased fitness and potentially decreased

predatory success during long term exposure to high concentrations of Pb. If the decrease of Na^+/K^+-ATPase activity in gill tissue is considered together with the decrease in energy reserves, the exposure to 100 μM Pb might be harmful for overall survival of the spiny dogfish. However, the proteolytic effect of Pb is not exclusive as other metals and other stressors can likewise enhance this process (Srivastava and Srivastava, 2008). It seems that effects elicited by Pb were associated with a more general stress response resulting in muscle carbohydrate use and liver protein breakdown, as described previously in *P. lineatus* by Martinez et al. (2004), representing a classical general adaptation syndrome to stress during Pb exposure.

This study confirmed the high resistance to Pb exposure in the spiny dogfish, despite high Pb accumulation rates. Gill and especially skin seem to be able to form an effective first barrier for Pb, as Pb is found in those organs in control dogfish as well. When dogfish were exposed to 20 μM of Pb (already a relatively high concentration), almost no adverse effects occurred. Even the exposure to 100 μM of Pb was not able to thoroughly disturb ion-osmoregulation, despite a decreased Na^+/K^+-ATPase activity in the gills. Ca^{2+} homeostasis, which is connected to Pb toxicity in freshwater fish, was not affected since plasma [Ca] was stable and gill membrane permeability was not compromised. Pb exposure to environmentally relevant concentrations would therefore not likely pose an equally great threat to dogfish compared to Ag or Cu exposure. However, since elasmobranchs are confronted with diverse hazards, accumulated Pb can still be an important factor influencing survival capacities in elasmobranchs.

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