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Physiological and Biochemical Effects of Lithium in Rainbow Trout

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Abstract. The physiological responses of juvenile rainbow trout (*Oncorhynchus mykiss)* to lithium (as LiCl) in moderately hard freshwater ($CaCO_3$ = 120–140 ppm, Na^+ = ~0.6 mM) were studied. The study employed a 15-day step-up exposure regime; 66 μg/L Li for the first 9 days and 528 μg/L for the next 6 days. The concentrations of plasma ions, apolipoprotein AI, total cholesterol, and fatty acids, as well as metabolic enzyme citrate synthase (CS) and Na^+,K^+-ATPase activities in the gill were measured. Li affected fish by exacerbated diffusive Na^+ losses at the gills in the beginning of exposure and a decrease of branchial CS activity. Detrimental effects were shown in fish exposed to 528 μg Li/L. These included a reduction of gill Na^+,K^+-ATPase activity, possibly related to observed lower concentrations of free fatty acids and cholesterol in gill tissue.

Key words: Lithium—Fish gill—Plasma ions—Apolipoprotein AI—Lipids—Enzyme activity

Lithium (Li) is a light and highly reactive metal, discovered in Sweden by Gustav Arfvedson in 1817 (Andraos 2004) and is found in stable minerals such as pegmatite and many forms of brine (Kszos and Stewart 2003; Ober 2003). Industrial uses of Li include the coolant in nuclear reactors, components of batteries and accumulators, the building and pharmaceutical industries, and metallurgy (Ober 2003). In addition, since the 1950s, Li has been used for humans as an effective antimanic drug and it is therefore well known for its effects in neuropsychiatric disorders and neuronal communication. However, excessive weight gain is a recognized metabolic side effect (Corbella and Vieta 2003).

The global consumption of Li minerals was estimated to be around 195,000 tons in 2002, which amounts to ~5000 tons of pure Li. A review of Li resources, distribution, and toxicity in the aquatic environment has been provided by Kszos and Stewart (2003). For example; in a 5-year period (1994–1999), 0.62–1.41 tons/year of Li flowed into the Kenti river system (northwest Russia) as a result of water runoff from mining activities (Lozovik *et al.* 2001). The excessive concentration (Li up to 20 μg/L) has therefore recently aroused interest in the effects of Li on fish and the whole ecosystem structure and function (*e.g.*, Holopainen *et al.* 2003; Tkatcheva *et al.* 2004).

In spite of the many anthropogenic sources of Li to groundwater and surface water, such as nuclear-related waste-disposal areas, chemical manufacturing, and spills from manufacturing or recycling facilities, there is relatively little information on the toxicological effects on aquatic biota. However, it is known that Li has teratogenic effects in amphibians (Boğa *et al.* 2000) and could affect embryonic development in fish (Stachel *et al.* 1993) and the plasma membrane protein pattern in amphibian embryos (Lazou and Beis 1993). Li also exerts effects on prokaryotic organisms, such as causing the marine diatom *Pseudo-Nitzshia multiseries* to produce a higher level of the neurotoxin domoic acid (Subba Rao *et al.* 1998).

In fish, Li uptake is most likely via a putative Na channel in the gill (Grosell and Wood 2002; Bury *et al.* 2003). In the fish gill, the cell type primarily responsible for osmoregulation and ion regulation are mitochondria-rich cells (MRCs, also known as chloride cells). These are partially covered by pavement cells (PVCs, also known as respiratory cells). However, the apical surface areas of the MRCs are exposed to the ambient water, and the extent of this exposure can be adjusted by dynamic changes in the PVC coverage in response to acid–base and ionic disturbances (Goss *et al.* 1995; Laurent and Perry 1995; Perry 1997; Wood 2001). Increases of both the apical surface area of the chloride cells and the number and size of gill cell mitochondria were found by Tkatcheva et al. (2004) in perch from alkaline lake water. It was suggested that these effects were caused by high concentrations of K^+ and Li^+. In the same study, the gill lipid composition (phospholipids, free fatty acids, and cholesterol content) was also altered (Tkatcheva *et al.* 2004). Potassium is an essential mineral for animals due to its role in

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electrolyte and acid–base balance, which helps to maintain plasma viscosity and osmotic pressure (Emsley 2003). Li's physiological role is not very clear (Kszos and Stewart 2003).

The effects of Li on the physiology of juvenile rainbow trout (*Oncorhynchus mykiss)* were studied. Na^+, K^+, and Cl^- ions, osmolality, and apolipoprotein AI (apoAI) were measured in the blood plasma, and metabolic enzyme activities, cholesterol, and free fatty acid concentrations were measured in the gills (*i.e.*, the organ continuously exposed to ambient water) in order to reveal potential effects on ion transfer or acid–base regulation. Na^+,K^+-ATPase was chosen as a key enzyme in ion transport in the gills. Also studied was the activity of the mitochondrial enzyme citrate synthase (CS, a citric acid cycle enzyme) as an index of mitochondrial density in tissue and as an indicator of tissue aerobic capacity and chloride cells number (Le François and Blier 2003) because Na^+,K^+-ATPase activity is the most important ATP sink in the gill chloride cells (Wood 2001). Lipids and apoAI are important in cell membrane protection (Hochachka and Somero 2002; Smith *et al*. 2005). Specifically, based on the above-summarized information, it was hypothesized that Li exposure might inhibit active ion uptake or exacerbate diffusive ion losses at the gills, effects that would be reflected in plasma ion levels. It was also hypothesized that Li might reduce gill Na^+,K^+-ATPase activity, either by direct inhibition, reduced expression, or alteration of the lipid microenvironment provided by the gill cell membranes; therefore, activities were measured on gills either chronically exposed to Li in vivo or acutely in vitro.

Materials and Methods

Fish and Husbandry

Juvenile rainbow trout (mean weight = 13 ± 2 g, fork length = 10.5 ± 0.5 cm) were obtained from Humber Springs Trout Farm (Ontario, Canada). Prior to experimentation, these fish were acclimated for a minimum of 4 weeks to the laboratory and water conditions at McMaster University. This facility is supplied with dechlorinated Hamilton (Ontario, Canada) city tap water originating from Lake Ontario. This water contains ~0.6 mM Na^+, 0.7 mM Cl^-, 1.0 mM Ca^{2+}, 0.2 mM Mg^{2+}, and 0.05 mM K^+, with a hardness ≈ 120–140 ppm as $CaCO_3$, DOC ≈ 3 mg/L temperature = 11–12°C, and pH ≈ 8.0. Throughout this period, fish were fed at 1% daily ration (dry feed/wet body weight) of commercial trout chow (Silver Cup Fish Feed; South Murray, UT, USA) containing 48% (minimum) crude protein, 14% (minimum) crude fat, 3% (maximum) crude fiber, 24 mg/g Na^+, 22 mg/g Cl^-, 5.3 mg/g K^+, 1.3 mg/g Mg^{2+}, and vitamins (10,000 IU/kg vitamin A, 500 IU/kg vitamin D, and 380 IU/kg vitamin E). One week before the experiments, fish were transferred from the 11–12°C holding tank to four acclimation tanks where the experimental temperature of 14–15°C was gradually attained.

Lithium Exposure

Lithium exposure was carried out in 200-L plastic tanks. Seven days prior to the start of Li exposure, 27 fish were introduced into each tank. Food was withheld at this time, to minimize the effects of feeding on metabolic processes (Brett and Zala 1975), until the end of the experiment, (a total of 15 days).

The exposure was conducted in two steps: 9 days and 6 days. The Li dosing system consisted of one Marriotte bottle containing the stock solution of Li (LiCl; Fisher Scientific, Toronto, ON, Canada) at a concentration of 635 mg/L LiCl in the first 9 days of the experiment and 5077 mg/L in the last 6 days. A mixing tank received the stock solution of LiCl at 0.046 ± 0.001 L/h; which was continuously mixed by aeration with tap water flowing at a rate of 47.8 ± 0.3 L/h before directing it into the experimental tanks. The control tanks received an equal flow of dechlorinated tap water only. Water in the experimental tanks was sampled daily and preserved by acidification to 1% with concentrated nitric acid. Samples for measurement of water Li concentration was kept at +4°C in sealed polypropylene vials and analyzed ~1 month later by atomic absorption spectrometry (Perkin Elmer AAS) at the water laboratory of the University of Joensuu (Finland). The actual Li concentration was 7 ± 1 μg/L (mean ± SD, n = 11) in the control tanks and 66 ± 27 μg/L (n = 20) during the 9 days of lower concentration exposure, and 528 ± 165 μg/L (n = 12) during the 6 days of higher concentration. The concentration of chloride ions (Cl^-) was below 1 mM Cl^-, which is not toxic for fish (Nagpal *et al.* 2003). This system was repeated so that there were two replicates of the Li-exposure tanks and two replicates of the control tanks.

Tissue Sample Collection

At 0, 2, 4, 7, 13, and 15 days from the start of the experiment, 10 fish were sacrificed each time (except 7 fish on day 13) from the experimental and control treatments (5 per tank, n = 2). Fish were caught individually with a dip-net and immediately euthanized by an overdose of neutralized MS-222 (0.3 g/L). Blood (~0.5 mL from each fish), the whole liver and ~200 mg of gill tissue were immediately taken and frozen in liquid nitrogen. Blood samples were collected in 1-mL heparinized syringes from the caudal vein punctured with a 21-gauge needle. Whole livers were removed and weighed for the Hepato-Somatic Index (HSI): HSI = 100 × liver wet weight/total wet weight. Gill samples were collected separately for Na^+,K^+-ATPase, CS, and lipid analysis. The tissues were investigated singly for each fish. Tissues were frozen in liquid nitrogen and stored at −80°C before analysis.

Plasma Ion Concentrations

Blood was centrifuged and plasma samples were used for immediate measurements of osmolality, total Na^+,K^+ concentrations, and Cl^- concentrations. The plasma Cl^- concentration was measured by coulometric titration (Radiometer CMT10 chloridometer). Concentrations of K^+ and Na^+ in water and plasma were determined by atomic absorption spectrometry (Varian 1275 AA). Plasma osmolality (mosmol/kg) was measured by vapor pressure osmometry (Wescor 5100C). Plasma samples were kept on ice and then frozen at −80°C for lipid analysis.

Na^+,K^+-ATPase Activity

The activities of Na^+,K^+-ATPase in both the control and Li-exposed gills were measured using a kinetic microassay run in 96-well microplates at 25°C, as outlined by McCormick (1993). Approximately 10 mg of gill filament was homogenized in 500 μL of SEID (150 mM sucrose, 10 mM EDTA, and 50 mM imidazole, pH 7.3 with 0.1% deoxycholic acid) for 30 s in a Teflon-in-glass homogenizer. ADP, which was released from ATP by Na^+,

K^+-ATPase, was measured in two media. The first medium contained the optimal concentration of all ions. The second medium contained the first solution's composition but with 0.5 mM ouabain added. In addition, in order to test whether Li^+ had a direct effect on gill Na^+,K^+-ATPase activity in vitro, LiCl was added directly to both media at various concentrations ranging from 0.1 pM to 1 mM.

Lipid Analysis

For lipid analysis, the frozen gill tissue was powdered in a liquid-nitrogen-cooled mortar and transferred into preweighed glass tubes. The samples (about 100 mg) were homogenized in a chloroform–methanol (2:1, v/v) solution (Bligh and Dyer 1959). Lipids were extracted at room temperature over a 40-min time period in the dark. The bottom layer was collected with a Pasteur pipette and placed into a glass vial, and the chloroform was evaporated under nitrogen. Dry samples were concentrated 2:1 when they were redissolved in a hexane–isopropanol mixture (3:4, v/v). Extracts were stored in glass vials at −80°C before analyses. Lipid compositions (the quantity of total free fatty acids and total cholesterol) in the gill tissue extract and in serum were measured spectrophotometrically with enzymatic colorimetric methods using commercially available diagnostic kits (NFFA C and ChoE; Wako Chemicals, USA). The manufacturer's recommendations were used to perform the assays in 96-well microplates.

Citrate Synthase

For the CS assay, about 50 mg of the frozen gill powder (prepared as for the lipids analysis) was transferred into 1 mL of homogenization buffer (20 mM HEPES, 1 mM EDTA, 0.1% Triton) and then homogenized with a glass-on-glass homogenizer. CS was determined as described previously (McClelland *et al.* 2005). Protein concentrations were determined by the Bradford method (Bio-Rad) using bovine serum albumin (BSA) as a standard at 595 nm.

Apolipoprotein AI

Purified trout apoAI is not commercially available for a fully quantitative assay. Therefore, blood plasma apoAI concentrations were assessed by a semiquantitative enzyme-linked immunosorbent assay (ELISA). Twenty microliters of rainbow trout blood plasma were mixed with 100 μL ELISA sensitizing buffer (18 mM Na_2CO_3 + 32 mM $NaHCO_3$) and added to the first well of a row on a 96-well Reacti-bind™ ELISA plate (Pierce Scientific). The remaining wells in the row were filled with 100 μL sensitizing buffer only. The initial plasma/buffer mixture was then serially diluted 1:5 along the row of wells by removing 20 μL of the plasma/buffer mixture and adding it to the next well in the row until well 11. Well 12 contained sensitizing buffer only. An anti-rainbow trout apoAI antibody is not commercially available. Therefore, a delipidated and defibrinated anti-human apoAI polyclonal antibody (Capricorn Products Inc., USA), raised in goats, was used. Thus, each plate also contained a human blood plasma positive control dilution curve (1:10 serial dilutions) and two specific negative control dilution curves to accommodate the consideration of nonspecific binding of the human antibody against a piscine antigen (*i.e.*, 1:1 serial dilutions of fetal bovine serum [FBS] (known to contain bovine apoAI; Smith *et al.* 2005) and 1.0 mg/mL BSA, prepared in the same manner as described in the above process). These plates were then incubated overnight at 4°C. The wells were then thoroughly washed with phosphate-buffered saline containing 2.5% Tween-20 (PBST) (pH 7.5) and allowed to air-dry. A fixed amount of the primary anti-human apoAI antibody (100 μL of a 1:500 dilution in PBST) was added to each well and the plate incubated at 20°C for 2 h. The plates were washed with PBST, dried, and treated with a goat ExtrAvidin® peroxidase staining kit (Sigma), containing an anti-goat IgG biotin conjugate and peroxidase conjugate, according to the manufacturer's instructions. After washing and drying (as previously described), a substrate containing 4 mg/mL of *O*-phenylenediamine and 2 μL/mL of 30% H_2O_2, in a phosphate/citrate buffer (97 mM citric acid + 205 mM PO_4, as $NaHPO_4$), was added. Color development was stopped using 2 M H_2SO_4 and the absorbance read at 405 nm. All assays were run on a SPECTRAmax microplate reader using SOFTmax software (Molecular Devices, USA).

Statistics

The effect of Li on fish morphometric parameters, concentrations of plasma Na^+, K^+, and Cl^- ions, osmolality, free fatty acids, cholesterol, and CS values in the Li-exposed fish were compared (each group versus values before exposure as well as each group against others) by one-way analysis of variance (ANOVA) followed by the least significant difference test (LSD). Na^+,K^+-ATPase were compared by the Kolmogorov–Smirnov test, and the repeated measures (RM ANOVA) test was used in each Li-exposed and control group for determining statistical significance against time to lock in changes caused by starvation. The Student's *t*-test was used for the 405-nm absorbances at each dilution in the ELISA assay to compare the mean area and describe the effect of Li along the plasma dilution curve. In all statistical analyses, $p < 0.05$ was considered to be significant.

Results

Mortalities and Growth

In total, four fish died during this investigation: one in the control group and three in the experimental group, two of which died on the last day of the Li exposure.

The fork length did not change over the exposure period in either the control or experimental treatments. The body mass of Li-exposed fish (which began the experiment at a similar size to nonexposed fish) remained unaltered, whereas the control body mass tended to fall. It was therefore higher in the Li-exposed fish than the controls by the end of the exposure period (Table 1).

Osmolality and Ion Concentrations

Exposure to Li was found to cause very few effects on plasma osmolality or ion concentration. After day 2, the plasma osmolality of nonexposed fish had declined, whereas that of Li-exposed fish remained at preexposure levels (Table 2). By day 4, the osmolality of Li-exposed fish had also declined and was similar to nonexposed fish (Table 2). Thereafter, there were no further effects on either the nonexposed or Li-exposed fish (Table 2). In Li-exposed fish, only plasma Na concentrations declined on day 2 into the exposure regime but then returned to preexposure values after day 4 (Table 2). From day

Table 1. Morphological parameters (mean ± SE) of rainbow trout during 9 days of exposure at 66 μg Li/L and 6 days at 528 μg Li/L

	Fork length (cm)	Weight of fish (g)	Fulton condition coefficient	Liver index %
Before exp.	10.5 ± 0.2	13.0 ± 0.6 [a]	1.10 ± 0.02 [a]	1.8 ± 0.05 [a]
2 days cont.	10.5 ± 0.2	12.8 ± 0.4 [a]	1.12 ± 0.03 [aa]	1.8 ± 0.1 [a]
2 days exp.	10.8 ± 0.2	13.3 ± 0.6 [aa]	1.04 ± 0.03 [ab]	1.9 ± 0.1 [a]
4 days cont.	10.4 ± 0.1	12.4 ± 0.3 [a]	1.10 ± 0.01 [a]	1.8 ± 0.1 [a]
4 days exp.	10.7 ± 0.1	13.0 ± 0.5 [a]	1.05 ± 0.02 [ab]	1.7 ± 0.1 [a]
7 days cont.	10.4 ± 0.2	11.6 ± 0.6 [ab]	1.00 ± 0.03 [b]	1.8 ± 0.05 [a]
7 days exp.	10.7 ± 0.2	12.6 ± 0.7 [a]	1.03 ± 0.02 [b]	1.6 ± 0.1 [b]
13 days cont.	10.5 ± 0.2	11.3 ± 0.7 [a]	0.97 ± 0.02 [bb]	1.9 ± 0.1 [a]
13 days exp.	10.7 ± 0.1	12.0 ± 0.5 [a]	0.98 ± 0.03 [b]	1.9 ± 0.1 [a]
15 days cont.	10.3 ± 0.1	10.6 ± 0.5 [b]	0.99 ± 0.02 [b]	2.0 ± 0.1 [c]
15 days exp.	10.7 ± 0.1	12.2 ± 0.4 [a]	0.99 ± 0.02 [b]	1.8 ± 0.1 [a]

Note: Values with no common letter (superscript) differ at least at the $p < 0.05$ level, ANOVA followed by post hoc test (LSD); $df = 10$. exp. = exposure; cont. = control.

Table 2. The plasma osmolality and sodium, chloride, and potassium concentrations (mean ± SE) in rainbow trout during 9 days of exposure at 66 μg Li/L and 6 days at 528 μg Li/L

	Plasma osmolality (mosmol/kg)	Plasma sodium (mmol/L)	Plasma chloride (mmol/L)	Plasma potassium (μmol/L)
Before exp.	288 ± 1 [a]	143 ± 5 [a]	130 ± 2 [a]	2.9 ± 0.4
2 days cont.	279 ± 1 [b]	143 ± 3 [a]	122 ± 1 [b]	3.2 ± 0.4
2 days exp.	283 ± 1 [ac]	120 ± 4 [b]	123 ± 1 [b]	3.2 ± 0.5
4 days cont.	281 ± 2 [bc]	139 ± 2 [a]	127 ± 1 [aa]	2.7 ± 0.5
4 days exp.	278 ± 2 [b]	148 ± 3 [a]	130 ± 1 [a]	2.8 ± 0.4
7 days cont.	277 ± 1 [b]	143 ± 2 [a]	125 ± 1 [ba]	3.5 ± 0.8
7 days exp.	277 ± 1 [b]	139 ± 3 [a]	125 ± 1 [ba]	2.5 ± 0.3
13 days cont.	280 ± 1 [bc]	173 ± 4 [c]	120 ± 2 [bc]	3.7 ± 0.3
13 days exp.	278 ± 2 [b]	165 ± 6 [c]	123 ± 1 [ba]	3.6 ± 0.7
15 days cont.	281 ± 2 [bc]	169 ± 3 [c]	131 ± 1 [a]	2.7 ± 0.2
15 days exp.	280 ± 3 [bc]	168 ± 3 [c]	127 ± 2 [aa]	3.4 ± 0.3

Note: Values with no common letter (superscript) differ at least at the $p < 0.05$ level, ANOVA followed by post hoc test (LSD); $df = 10$. exp. = exposure; cont. = control.

4 to day 7, plasma Na concentrations of both nonexposed and Li-exposed fish were the same as preexposure (Table 2). However, from day 13 to day 15, plasma Na increased equally in both groups (Table 2). Plasma Cl concentrations in nonexposed and Li-exposed fish declined after day 2 but thereafter returned to preexposure values for the remainder of the exposure period (Table 2). Li exposure had no effect of plasma K concentrations (Table 2).

Na^+,K^+-ATPase

No effect of Li exposure was observed until day 15, when Na^+,K^+-ATPase in the gills was significantly reduced in the Li-exposed group compared to the control group: 0.27 ± 0.06 and 0.58 ± 0.1 mmol ADP/mg protein/h, respectively (Fig. 1).

However, when LiCl was added directly to the Na^+,K^+-ATPase assay media in concentrations of Li from 0.1 pM to 1 mM, there was no significant inhibitory effect for gills from fish that had never been exposed to Li (data not shown). This indicated that the impact of Li exposure in vivo in depressing Na^+,K^+-ATPase activity was likely an indirect effect rather than a direct blockade of the enzyme.

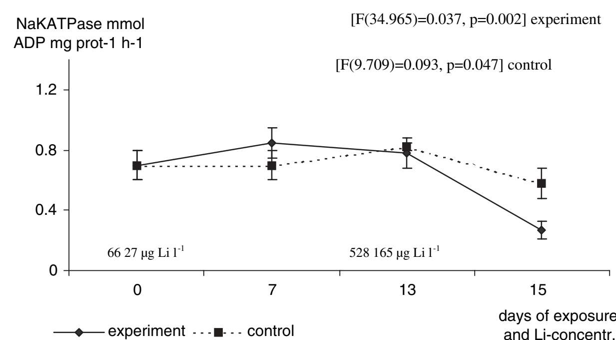

Fig. 1. Na^+,K^+-ATPase activities (mean ± SE) in rainbow trout gills during Li exposure. Temporal differences were observed in the experimental group of fish by the RM ANOVA test

Citrate Synthase Activity

In nonexposed fish, gill CS activity increased during the experiment (Table 3). Although there was a decline in Li-exposed fish on day 7 (0.74 ± 0.06 vs 1.18 ± 0.19 μmol/mg protein in the control), on day 13 the Li exposure caused an additional increase in CS activity. At day 15, CS activity in

Table 3. Concentrations of CS (mean ± SE) in rainbow trout gill tissue after 9 days of exposure at 66 μg Li/L and 6 days at 528 μg Li/L

	Gills CS (μmol/mg protein)	Gills protein (μg/mL)
7 days control	1.18 ± 0.19 [a]	3.50 ± 0.16 [a]
7 days exp.	0.74 ± 0.06 [a]	3.97 ± 0.24 [aa]
13 days control	2.47 ± 0.19 [b]	3.04 ± 0.24 [ab]
13 days exp.	3.29 ± 0.29 [c]	2.93 ± 0.20 [ab]
15 days control	2.09 ± 0.23 [b]	3.01 ± 0.18 [ab]
15 days exp.	2.38 ± 0.26 [b]	2.92 ± 0.23 [ab]

Note: Values with no common letter (superscript) differ at least at the $p < 0.05$ level, ANOVA followed by post hoc test (LSD); $df = 5$. exp. = exposure.

Li-exposed fish was similar to nonexposed fish (Table 3). At day 13, the higher CS activity might have been due (in part at least) to a decline in total protein (Table 3). Similarly, it is noteworthy that at day 15, CS activity in Li-exposed fish was similar to nonexposed fish despite a decline in total gill protein (Table 3).

Lipid Analysis

Lithium had no effect on blood plasma free fatty acids (FFAs) or cholesterol (Cho) and very little effect on FFAs and cholesterol in the gill tissue (Table 4). The apparent decrease in gill FFAs on day 15 in Li-exposed fish was in fact due to higher FFAs in the nonexposed gills of fish sampled at the same time (Table 4).

Free fatty acids in the gills of in Li-exposed fish were lower than in corresponding control fish; this was because of an increase in FFAs in nonexposed gills tissue. Day 15 exposure to Li resulted in a decline in total FFAs, and day 13 exposure resulted in an increase in cholesterol (Table 4) when compared with nonexposed fish at the same time period. Total FFAs and cholesterol decreased on day 15 in the gills of Li-exposed fish (FFAs = 0.233 ± 0.133 mmol/L compared to 0.455 ± 0.303 mmol/L in the control and Cho = 53.8 ± 19.1 mmol/L compared to 79.3 ± 39.1 mmol/L in the control; Table 4). Overall, there was a significant correlation between the CS activity and FFAs in fish gills (Pearson's correlation coefficient [PC] = 0.52, $p < 0.01$). Plasma lipids did not show any significant change during the experiment. Gill lipids correlated significantly with the plasma FFAs, and FFAs and Cho were interdependent on in gills (PC for gills Cho and FFAs was 0.45, $p < 0.01$; PC for plasma FFAs and gills FFAs was 0.59, $p < 0.01$).

Apolipoprotein AI

The results of the apoAI ELISA assay are indicated in Figure 2. After 13 days of Li exposure, there was an increased antigenic response in the blood plasma of Li-exposed fish when compared to nonexposed fish after the same time period; whereas after 15 days of Li exposure, the antigenic response was similar to comparable nonexposed fish. However, the ELISA assay also suggested an increase in plasma apoAI between 13 and 15 days from the onset of the experiment (*i.e.*, after the withdrawal of food). This daily variation in apoAI could mask the effect of Li exposure and illustrates the value of this study in conducting assays at more than one time point after Li exposure commenced. In all cases, human blood gave a positive reaction with the anti-human apoAI antibody, whereas there was no antigenic reaction with FBS and BSA or, in the case of omitting the primary antibody, treatment with human plasma (data not shown).

Discussion

The concentrations of 0.066 and 0.528 mg Li/L used in the present study include the concentration range reported in field situations: 0.15–0.5 mg Li/L (Kszos *et al.* 2003). Given that 96-h lethality tests yielded fish LC_{50} values from 13 to >100 mg Li/L (Colorado squawfish [*Ptychocheilus lucius*], bonytail [*Gila elegans*], and razorback sucker [*Xyrauchen texanus*] [Hamilton 1995]; fathead minnow [*Pimephales promelas*] [Long *et al.* 1998]), whereas a 26-day exposure gave LC_{50} values for fathead minnow larvae of 1.2 mg/L and the unobserved effect concentration of 0.20 mg Li/L, the concentrations used in the present study are clearly sublethal.

Kszos *et al.* (2003) reported that Li toxicity might be reduced by high levels of Na^+ because at a concentration of 10 mg/L Na^+, there was no effect on minnow survival at 1 mg Li/L, whereas at 2.5 mg Na/L, the survival rate was reduced to 80% at 1.0 mg Li/L and was only 2.5% at 2 mg Li/L. In the present study, the Na^+ concentration was ~14 mg/L (0.6 mM). The Na/Li log ratio (ion concentration in mmol/L) was used to prove the similarity of water conditions in the field and lab studies. It was 0.1 at the lower concentration of 0.066 mg Li/L (0.0086 mM) and 0.2 at 0.528 mg Li/L (0.076 mM). Similarly, the latter concentration of Li duplicated the ambient composition in Poppalijärvi Lake (Na/Li log = 0.2) from the Kenti River system (Tkatcheva *et al.* 2004). Our results show that Li is potentially detrimental to the juvenile rainbow trout at concentrations of 0.528 mg Li/L, with sublethal effects on the physiology because gill Na pump activities of gills were dramatically decreased, even when mortalities were low (12%).

Based on observed increases of the mitochondria-rich cells' sizes and their apical surface areas, as well as increases in mitochondria numbers and size found by Tkatcheva *et al.* (2004) in perch, we originally hypothesized that Li exposure might inhibit ion uptake or increase ion losses at the gills, with the observed structural changes representing compensatory responses. However, in the present study on rainbow trout, only slight differences were found in blood plasma osmolality and Cl^- and Na^+ ion concentrations during exposure to the lower of the Li concentrations. Apparently, no other studies have measured plasma ions after Li exposure in fish, but after the increase in Li concentration to 528 μg Li/L, our data are in agreement with the results of Abdel-Zaher and Abdel-Rahman (1999) and of Abdel-Zaher (2000) in mammals. They also reported that the plasma sodium (and calcium) levels were not changed in experiments with rats and rabbits as a result of intravenous administration of LiCl.

Our second hypothesis was that Li affected Na^+,K^+-ATPase by altering the gill cell membrane lipid dynamics. At

Table 4. Concentrations of total FFAs and Cho in rainbow trout gill tissue (weight of the tissue 0.100 ± 0.02 g) and blood plasma after 9 days of exposure at 66 μg/L and 6 days at 528 μg/L of Li (mean ± SE)

	Plasma		Gills tissue	
	FFA (mmol/L)	Cho (mmol/L)	FFA (mmol/L)	Cho (mmol/L)
7 days cont.	0.573 ± 0.033	138.3 ± 9.6 [a]	0.154 ± 0.035 [aa]	75.1 ± 7.8 [aa]
7 days exp.	0.540 ± 0.039	137.5 ± 8.8 [a]	0.271 ± 0.071 [a]	69.2 ± 6.5 [aa]
13 days cont.	0.552 ± 0.041[a]	103.3 ± 6.0 [b]	0.249 ± 0.039 [a]	67.5 ± 2.5 [ac]
13 days exp.	0.495 ± 0.031	91.2 ± 6.4 [b]	0.362 ± 0.057 [ab]	94.1 ± 6.5 [bb]
15 days cont.	0.522 ± 0.033	120.4 ± 11.0 [ac]	0.455 ± 0.086 [bb]	79.3 ± 12.4 [ab]
15 days exp.	0.557 ± .036	112.0 ± 6.8 [bc]	0.233 ± 0.037 [a]	53.8 ± 6.0 [ac]

Note: Values with no common letter (superscript) differ at least at the $p < 0.05$ level, ANOVA followed by post hoc test (LSD); $df = 5$.
exp. = exposure; cont. = control.

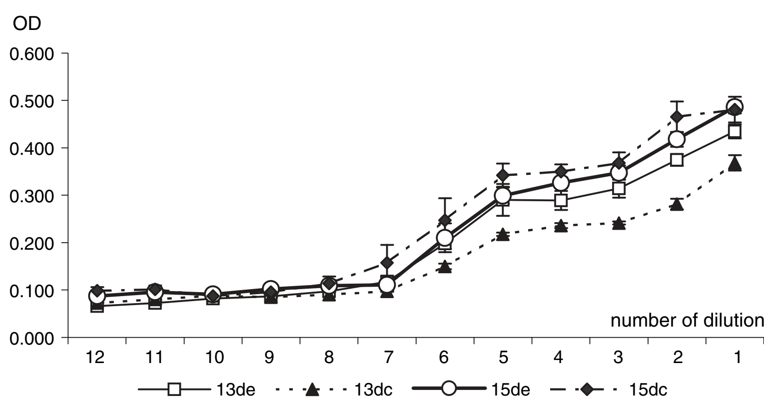

Fig. 2. Apolipoprotein optical densities (ODs) of rainbow trout blood plasma after 3 and 5 days of exposure in 528 ± 165 μg Li/L. Plasma dilution as $5n$ dilution (5 = the dilution factor and n = the number of dilutions; listed on the x axis). Blank was well #12. The t-test is significant with $p < 0.05$ in the range from 2 to 5 on day 13

the end of the exposure period, the increase in anti-apoAI antibody binding suggests the fish responded with increases of plasma apoAI concentration (Fig. 2), FFA and Cho concentrations (Table 3), and CS activity (Table 4) in the gill tissue. Overall, these data suggest an inverse relationship between blood plasma apoAI and gill Cho. It is possible that these changes were directed at gill apical membrane protection. The importance of apoAI as a determinant of the barrier properties of the freshwater fish gill has been recently demonstrated by Smith *et al.* (2005), where the synthesis of apoAI increased in response to the osmotic challenge (*i.e.*, imposing asymmetrical conditions on gill cells, cultured on membrane supports, as intact epithelia; refer to Smith *et al*, 2005). This increase in apoAI synthesis increased overall transepithelial resistance (*i.e.*, a measurement of epithelial integrity) by specifically acting on the transcellular properties of the cell membrane (Smith *et al.* 2005) and is associated with a previous finding of increased gill cell lipid metabolism (Hansen *et al.* 2002). Accordingly, the higher apoAI concentration in blood plasma might have been directed at the introduction of lipids into the membranes to enhance barrier properties of the gill epithelium after exposure to 0.528 μg Li/L. Therefore, it was hypothesized that the increase in plasma apoAI and gill Cho concentration on day 13 could indicate that rainbow trout react protectively to low Li concentrations by reducing membrane fluidity, therefore potentially also reducing permeability. Exposure to the higher Li concentration might have caused this protective reaction to diminish, with gill cell membrane lipids concentration declining, as has been reported in fish gills from L. Poppalijarvi (Tkatcheva *et al.* 2004).

The Na^+,K^+-ATPase activity in the gills declined significantly (Fig. 1) in the actual in vivo exposure to waterborne Li on day 15 (after 6 days of exposure at 0.528 mg Li/L; Fig. 1). Because we found that Li does not affect gills' Na^+,K^+-ATPase activity directly (*i.e.*, after adding Li to gill cell homogenates in vitro), this decrease might be a secondary effect related to the lower lipids concentrations on day 15 compared to day 13 (Table 4). Total FFA and Cho do exert an effect on ion regulation; for example, Else *et al.* (2003) found that decreases in lipids and phospholipid molecules are linked to a reduced sodium pump activity. It is therefore possible that the action of this enzyme is dependent on membrane fluidity.

Taking the above-mentioned data into consideration, it was proposed that Li affected fish by exacerbated diffusive Na^+ losses at the gills in the beginning of the Li-exposure regime and by reduced gill enzymatic activity due to reductions to the lipid microenvironment in gill cell membranes toward the end of exposure.

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