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Copper induced alterations of biochemical parameters in the gill and plasma of *Oreochromis niloticus*

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Abstract

The main objective of this study was to determine the effects of copper exposure on copper accumulated in branchial tissue, gill Na^+/K^+-ATPase activity and plasma Na^+, Cl^-, osmolality, protein, glucose and cortisol, in *Oreochromis niloticus*. Fish were experimentally exposed to 40 and 400 μg L^{-1} of waterborne copper and sacrified after 0, 3, 7, 14 and 21 days. Copper accumulation and Na^+/K^+-ATPase activity were determined in branchial tissue, whereas osmolality, Na^+, Cl^-, protein, glucose and cortisol concentrations were measured in plasma samples. Gill copper accumulation increased linearly with exposure time and concentration, whereas gill Na^+/K^+-ATPase activity was maximally inhibited after 3 days of exposure and showed a significant negative correlation with copper tissue levels. Plasma Cl^- values decreased with time of exposure but only at 400 μg L^{-1} of copper. Plasma Na^+, protein and osmolality decreased with exposure time at the highest copper concentration tested, whereas at 40 μg L^{-1} of copper this effect was only observed after 21 days of exposure. Plasma glucose and cortisol levels increased in a dose and time dependent manner, while showing complex fluctuations during the intermediate exposure times. In conclusion, copper induces an early maximum inhibition of gill Na^+/K^+-ATPase activity in *O. niloticus*. The subsequent slow decrease in ion plasma levels was related to compensatory mechanisms involving a non-specific stress response that appeared overcome at long-term exposures.

Keywords: Copper toxicology; Fish; Gills; Na^+/K^+-ATPase; *Oreochromis niloticus*; Osmoregulation; Stress response; Teleostei

1. Introduction

Copper is one of the most abundant transition metals in nature and an essential constituent of all living tissues. However, when present at high concentrations, it becomes toxic to living organisms, including fish (Pelgrom et al., 1995b). Toxic concentrations of copper occur in nature mainly through mining and smelting, leaching from bedrock due to acid mine drainage and precipitation, and from industrial and agricultural activities (IPCS, 1998). Although the aquatic environment is frequently monitored, sublethal heavy metal concentrations may nevertheless be toxic to aquatic organisms. Subsequently, it has been suggested that the ideal aquatic pollution bio indicator relies in the study of heavy metal accumulation levels in fish organs (Handy, 1992).

Gills represent a thin and extensive surface (up to 90% of the total body surface) in intimate contact with water. They carry out three main functions, gas exchange, ion regulation and excretion of metabolic waste products. Due to the constant contact with the external environment, gills are the first target of waterborne pollutants (Perry and Laurent, 1993) and the main place for waterborne copper uptake (Campbell et al., 1999). Associated with copper accumulation, several histopathological changes occur in the branchial epithelium (Sola et al., 1995; Arellano et al., 2000; De Boeck et al., 2001; Mazon et al., 2002) followed by changes in systemic physiological parameters. The osmor-

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egulatory disturbances induced by copper were associated with an increased epithelial permeability and inhibition of active ion uptake, subsequently to the reduction of Na^+/K^+-ATPase activity and a decrease in the number of active chloride cells (Laurén and McDonald, 1985, 1987a,b; De Boeck et al., 2001).

Oreochromis niloticus is a widely used species in aquaculture for food supply and has been suggested as a bio indicator of water contamination (Almeida et al., 2002). The main purpose of this study is to determine the effects of copper exposure on copper accumulation, Na^+/K^+-ATPase activity in gill tissue, plasma ion levels, osmolality and protein and in stress response indicators such as plasma, glucose concentration and cortisol concentration, in *O. niloticus*.

2. Materials and methods

2.1. Animals

Nile *O. niloticus* (Bouaké strain) were originally obtained from the Institute Nationale de Recherche Agronomique (Rennes, France) and raised in the Aquaculture Station of the University of Trás-os-Montes and Alto Douro (UTAD, Vila Real, Portugal) for three generations. Fish were kept in 100 L recirculating tanks (water flow rate of 5 L min^{-1}) filled with dechlorinated tap water. Water composition was in agreement with European Community instructions (84/449/EEC Directives, Annex 5, method c1) and quality parameters maintained by mechanical and biological filtration: pH 6.5–7.5; alkalinity 60 mg HCO^{3-} L^{-1}; conductivity 63 μS cm^{-1}; 14 mg Na^+ L^{-1}; 2.3 mg K^+ L^{-1}; 4.1 mg Ca^{2+} L^{-1}; 6.5 mg Mg^{2+} L^{-1}; 19.5 mg Cl^- L^{-1}; 27 mg NO_3^- L^{-1} (nitrate); 0.5 mg NO_2^- L^{-1} (nitrite); hardness 74.5 mg $CaCO_3$ L^{-1}; 6.2 mg dissolved O_2 L^{-1}; 21 mg CO_2 L^{-1}; 0.1 mg H_2S L^{-1} (hydrogen sulfide); 0.7 mg NH_4^+ L^{-1} (ammonia); and 12 mg suspended solids L^{-1}. Supplemental aeration was provided to maintain dissolved oxygen near saturation, the temperature was kept at 25 ± 1 °C and the photoperiod controlled (12 D : 12 L). Fish were fed once or more times daily to visual satiation with commercial fish food dry pellets (Aquasoja-Sorgal, Ovar, Portugal). The composition of the commercial pellets was: fiber, 1.9%; lipid, 4.3%, crude protein, 37.2%, Ca^{2+}, 2.2%, P, 1.4% and vitamins A, C, D_3 and E.

2.2. Experimental design

The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the UTAD for the use of laboratory animals. Both control and experimental tanks were submitted to a rate of water renovation of 1/3 every 2 days. The water quality parameters mentioned above were assessed at collection days during the experimental period, with no significant changes being observed.

Sexually mature male and female *O. niloticus* ($n = 120$, 36.3 ± 7.7 g of mean body weight) were randomly distributed through 12 tanks of 100 L. There were 10 fish per tank and 4 tanks for each treatment. Fish from 4 tanks containing water without additional copper (3.9 μg Cu L^{-1} copper in tap water) served as the control group. Fish from the remaining tanks were exposed to water copper concentrations of 40 μg Cu L^{-1} (mean: 39.8; range: 36–45) and 400 μg Cu L^{-1} (mean: 395.8; range: 375–420) supplied as copper sulphate ($CuSO_4$; Merck, Darmstadt, Germany). Water copper concentrations were selected based on preliminary results shown to be sublethal after a 21 day period of exposure. No fish mortality was observed during the experiments.

Fish were fasted for 24 h before collection at days 0 (before copper exposure), 3, 7, 14 and 21 (2 fish per tank; 8 fish per treatment: control, 40 μg Cu L^{-1} and 400 μg Cu L^{-1} tanks). They were anaesthetized with 2-phenoxiethanol (Sigma, Barcelona, Spain) (1 mL L^{-1} water), weighed and sampled. Blood (1.5–2 mL) was extracted from the caudal peduncle artery using 1 mL (10,000 U) ammonia-heparinized syringes (ammonium salt heparin, Sigma; sodium salt heparin interferes with ATPase activity determinations). Plasma was separated from cells by centrifugation (Sigma ultracentrifuge 3K30) of whole blood (5 min, 10,000 ×*g*, 4 °C), immediately frozen on liquid nitrogen (100 μl aliquots) and stored at −80 °C until use. According to Siegler et al. (1996), there are differences between arch and size in respect to Na^+/K^+-ATPase activity. For this reason, and trying to evaluate the copper effect on Na^+/K^+-ATPase activity of the complete gill and not only one specific gill arch, a random sampling was performed. Biopsies of the gill tissue were placed in 100 μl ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3; Sigma) and then stored at −80 °C. The remaining gill tissue was frozen at −80 °C.

2.3. Analytical techniques

To determine the copper concentration in gill tissue, 2 gill arches were excised and thoroughly rinsed with distilled water to remove the adhering blood mucus and water. The soft tissue was scraped off with glass slides and its wet weight determined. It was then dried at 60 °C during 24 h until a constant weight was reached, and digested with 500 μl of H_2SO_4 (65%; Merck). After complete tissue digestion (visual inspection), 50 μl samples were diluted with distilled water to 2 mL and stored at 4 °C until use. All samples were completely clear and not filtered. Copper concentrations, expressed as μg Cu g^{-1} of dry weight, were determined by atomic absorption spectrophotometry (Unicam 939, Kassel, Germany).

Na^+/K^+-ATPase activity in gill tissue was determined using a microassay method (McCormick, 1993). Briefly, a small scrap of gill tissue was homogenized (Kontes pellet pestle motor 0.5 mL; Fisher Scientific, USA) in 125 μl of SEID buffer (SEI buffer with 0.1% deoxicholic acid; Sigma) and centrifuged at 4 °C, 3000 ×*g* for 30 s. Duplicate 10 μl

supernatant samples were added to 200 μl of the assay mixture with or without 0.5 mM ouabain (Sigma) in 96-well microplates (Costar, Corning, NY, USA) and read at 25 °C, 340 nm for 10 min, with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as μmol ADP/mg protein/h. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Smith et al., 1985) (Pierce, Rockford, IL, USA), with bovine serum albumin (Sigma) as standard. Both assays were run on a microplate reader (EL3401, Bio-Tek Instruments, Winooski, VT, USA) using the DeltaSoft3 software for Macintosh (BioMetallis Inc., NJ, USA).

Plasma osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, VT, USA) and expressed as mOsm kg^{-1}. Plasma Na^+ was measured on 5 μl samples by atomic absorption spectrophotometry (Unicam) after 1:400 dilution with deionized water. Plasma Cl^- was measured by the thiocyanate method using the Chloride Sigma kit (no 461). Plasma protein concentration was determined using the BCA protein assay kit for microplates after diluting the plasma 1:40. Plasma glucose was measured by the Sigma enzymatic coupling kit with hexokinase and glucose-6-phosphate dehydrogenase (Glucose HK, 16-20UV) adapted to microplates (Stein, 1963), and expressed as mmol L^{-1}. Assays in microplates were run on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments) using the DeltaSoft3 software for Macintosh (BioMetallics). Plasma cortisol levels were determined by competitive immunoassay with chemiluminescent substrate (Immulite; Diagnostic Products Corporation, Los Angeles, CA, USA).

2.4. Statistics

Data are expressed as means ± SE. Differences among groups were tested by one-way or two-way ANOVA. The Tukey test, with 95% confidence limits, was applied to compare the means whenever there was a significant difference (SigmaStat for Windows, version 2.03, Statistical software, SPSS Inc., San Rafael, CA, USA). If conditions for ANOVA were not fulfilled, the Kruskal–Walis and Dunn's tests were used. Correlations were calculated using Spearman's correlation analysis. The level of significance was set at $P<0.05$.

3. Results

3.1. Gill dry weights

Gill dry weights were kept constant between groups at each time of exposure, except at day 21 where a significant decrease to controls was observed in the 40 μg Cu L^{-1} group ($P=0.003$). Gill dry weights were also kept constant within each group along the time of exposure, with the exception of day 21 for the 40 μg Cu L^{-1} group, where significant decreases were observed from day 3 to day 14 ($P=0.043$) and day 21 ($P<0.001$) and between day 7 and day 21 ($P=0.003$).

3.2. Gill copper accumulation

Copper accumulation in gill tissue significantly increased in a time ($P=0.031$) and concentration dependent manner during the exposure period. Exposure to 40 μg Cu L^{-1} caused a significant rise in gill copper levels of about 325% at day 3 and 501% at day 7, stabilized during day 14 and finally reached a maximum increase of about 771% at day 21. On the contrary, exposure to 400 μg Cu L^{-1} caused a significant progressive rise in gill copper levels from about 392% at day 3 to 1263% at day 21. Differences in gill copper accumulation between fish exposed to 40 and 400 μg Cu L^{-1} became significant from day 14 until the end of the exposure (Table 1).

3.3. Gill Na^+/K^+-ATPase activity

Gill Na^+–K^+-ATPase activity significantly decreased during the exposure period, but this inhibition was time independent ($P=0.078$). Exposure to 40 μg Cu L^{-1} caused a significant decrease of about 59% in Na^+–K^+-ATPase activity at day 3, which then became stabilized except for a small but significant increase at day 7. On the contrary, exposure to 400 μg Cu L^{-1} caused a significant decrease in Na^+–K^+-ATPase activity of about 70% at day 3, with no further significant decreases being observed during the remaining exposure period. Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant at all times except at day 21 of exposure (Table 1).

3.4. Plasma Na^+ concentration

There was a significant dose and time ($P=0.0004$) dependent decrease in plasma Na^+ concentrations over the entire period of copper exposure. At 40 μg L^{-1} of copper, plasma Na^+ levels were kept within the range of control values, decreasing significantly by about 14% only at day 21 of exposure. On the contrary, at 400 μg Cu L^{-1}, the reduction in plasma Na^+ levels was significant and progressive, about 9% at day 3 and 17% at day 7, then became stabilized at day 14 and finally reached a maximum decrease of about 29% by day 21 of exposure. Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant from day 7 of exposure (Table 1).

3.5. Plasma Cl^- concentration

Cl^- concentrations also decreased over the entire period of copper exposure in a time ($P=0.0016$) and concentration dependent manner. However, at 40 μg Cu L^{-1}, plasma Cl^- levels were kept constant and within the range of control

Table 1
Biochemical parameters measured in gills and plasma of *O. niloticus* before and after copper exposure

Exposure days	Waterborne Cu concentration (μg L^{-1})		
	Control [0]	[40]	[400]
Gill copper (μg Cu/g dry wt)*			
0	1.95±0.26		
3	2.32±0.26^{a}	9.85±1.21$^{b(A)}$	11.41±0.81$^{b(A)}$
7	2.69±0.27^{a}	16.18±1.01$^{b(B)}$	18.04±1.13$^{b(B)}$
14	2.59±0.34^{a}	15.49±1.14$^{b(B)}$	28.04±1.54$^{c(C)}$
21	2.42±0.03^{a}	21.08±2.29$^{b(C)}$	32.99±0.91$^{c(D)}$
Gill Na^{+}/K^{+}-ATPase activity (μmol/mg protein/h)			
0	1.92±0.10		
3	1.95±0.05^{a}	0.80±0.06$^{b(A)}$	0.59±0.04^{c}
7	1.82±0.05^{a}	1.14±0.13$^{b(B)}$	0.62±0.09^{c}
14	1.88±0.09^{a}	0.99±0.09$^{b(AB)}$	0.57±0.07^{c}
21	1.88±0.07^{a}	0.81±0.08$^{b(AB)}$	0.56±0.10^{b}
Plasma Na^{+} (mmol/l)*			
0	200.00±4.74		
3	203.17±3.39^{a}	195.25±3.92$^{ab(A)}$	185.63±3.84$^{b(A)}$
7	201.50±4.69^{a}	198.00±3.18$^{a(A)}$	167.38±2.84$^{b(B)}$
14	202.33±6.01^{a}	201.88±2.06$^{a(A)}$	160.25±4.68$^{b(B)}$
21	197.83±3.88^{a}	170.00±5.14$^{b(B)}$	140.71±8.42$^{c(C)}$
Plasma Cl^{-} (mmol/l)*			
0	149.07±2.43		
3	152.23±6.12^{a}	149.55±4.77^{ab}	133.61±4.34$^{b(A)}$
7	148.04±4.15^{a}	141.59±5.04^{a}	120.68±4.32$^{b(A)}$
14	145.38±5.75^{a}	143.99±6.14^{a}	103.78±2.82$^{b(B)}$
21	144.11±4.93^{a}	143.63±4.06^{a}	73.23±7.91$^{b(C)}$
Plasma osmolality (mOsm/kg)*			
0	297.86±3.65		
3	301.60±2.97^{ab}	307.14±3.91$^{a(A)}$	286.00±5.22$^{b(A)}$
7	288.40±8.72^{ab}	294.71±5.96$^{a(AB)}$	271.86±7.41$^{b(AB)}$
14	284.40±2.49^{a}	279.63±7.98$^{a(B)}$	253.29±6.94$^{b(B)}$
21	289.60±2.97^{a}	256.86±10.48$^{b(C)}$	164.43±7.75$^{c(C)}$
Plasma protein (mg/mL)*			
0	34.76±0.84		
3	34.46±0.85^{a}	35.78±1.07$^{ab(A)}$	37.61±0.93$^{b(A)}$
7	36.30±0.74^{a}	37.51±0.75$^{a(A)}$	34.58±0.74$^{b(B)}$
14	36.69±0.89^{a}	36.32±0.20$^{a(A)}$	35.92±1.08$^{a(AB)}$
21	36.08±1.01^{a}	25.13±0.64$^{b(B)}$	26.81±0.71$^{b(C)}$
Plasma glucose (mg/dl)*			
0	2.38±0.19		
3	2.67±0.12^{a}	5.45±0.48^{b}	5.38±0.52$^{b(A)}$
7	2.86±0.29^{a}	4.81±7.52^{b}	5.98±0.51$^{b(B)}$
14	2.74±0.38^{a}	4.29±0.49^{a}	9.29±0.56$^{b(B)}$
21	2.90±0.21^{a}	5.46±0.50^{b}	6.28±0.58$^{b(A)}$
Plasma cortisol (ng/mL)*			
0	33.25±5.57		
3	28.55±4.88^{a}	198.83±17.26$^{b(AC)}$	257.55±18.38$^{c(AC)}$
7	31.96±3.19^{a}	278.81±15.09$^{b(B)}$	72.02±4.75$^{a(B)}$
14	31.93±5.10^{a}	163.62±2.85$^{b(C)}$	301.40±12.69$^{c(CD)}$
21	34.00±8.77^{a}	239.39±22.74$^{b(AB)}$	320.21±24.92$^{c(D)}$

Means in the same line with different small caps are significantly different.
Different caps lock in the same column indicates significant differences between exposure times (ANOVA, $P<0.05$).
* Interaction between waterborne Cu concentration and exposure time is statistically significant.

values during all the 21 days of exposure. On the contrary, at 400 μg L^{-1} of copper, plasma Cl^{-} levels were significantly reduced by about 12% at day 3, then remained constant at day 7 and finally progressively decreased up to about 49% at day 21 of exposure. Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant from day 7 of exposure (Table 1).

3.6. Plasma Na^{+}/Cl^{-} ratios

Constant Na^{+}/Cl^{-} ratios were kept between groups at each time of exposure, except at day 21 where significant differences existed between controls and experimental groups. Na^{+}/Cl^{-} ratios were also kept constant within each group along the time of exposure, with the exception of day

21 where at 40 μg Cu L^{-1} there were significant differences to day 14 ($P=0.013$) and day 7 ($P=0.014$) and at 400 μg Cu L^{-1} to all other days ($P<0.001$).

3.7. Plasma osmolality

During waterborne copper exposure period, there was a significant time ($P=0.0024$) and dose dependent decrease in plasma osmolality. Although fish submitted to copper evidenced a progressive decrease in plasma osmolality, especially from day 14 of exposure, this became significant in relation to controls only at day 21 (about 11% of reduction) at 40 μg L^{-1} of copper and at day 14 of exposure at 400 μg L^{-1} of copper (reaching about 43% of reduction at day 21). Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant at all times of exposure (Table 1).

3.8. Plasma protein concentration

There was a significant time ($P=0.018$) and concentration dependent decrease of plasma protein concentrations along the exposure period to copper, while exhibiting a complex and delayed pattern of decrease. At 40 μg L^{-1} of copper, plasma protein levels were kept constant and within the range of control values up to 14 days of exposure, decreasing significantly by about 30% only at day 21. On the contrary, at 400 μg Cu L^{-1}, there was a significant increase of the plasma protein concentration at day 3, then a significant decrease at day 7 followed by a stabilization at day 14, and finally a significant decrease of about 26% at day 21 of exposure. Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant only at day 7 of exposure (Table 1).

3.9. Plasma glucose concentration

Plasma glucose concentrations significantly increased with water copper exposure. Although plasma glucose levels did not rise linearly, the interaction between time and concentration were statistically significant ($P=0.0004$). At 40 μg Cu L^{-1}, plasma glucose levels significantly increased by about 104% at day 3 and then remained stabilized up to 21 days of exposure. Although in relation to controls differences were kept significant except at day 14 of exposure, a relative decrease in plasma glucose concentrations was observed at days 7 and 14 of exposure. On the contrary, at 400 μg L^{-1} of copper, there was a significant and progressive increase in plasma glucose levels in relation to controls. This rise was continuous (101% at day 3 and 109% at day 7), stabilized at day 14 at its maximum value (about 239%) and then decreased to 117% at day 21 of exposure. Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant only at day 14 of exposure (Table 1).

3.10. Plasma cortisol concentration

Plasma cortisol concentrations significantly increased during the water copper exposure period. Although plasma cortisol levels did not rise linearly, the interaction between

Table 2
Correlation between biochemical parameters in gills and plasma of *O. niloticus* before and after copper exposure

Variables		Gill Na^+–K^+-ATPase activity	Plasma [Na^+]	Plasma [Cl^-]	Plasma osmolality	Plasma [protein]	Plasma [glucose]	Plasma [cortisol]
Gill [Cu]	*R*	−0.717	−0.687	−0.581	−0.580	−0.420	0.696	0.729
	P value	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	n	76	83	76	76	83	76	56
Gill Na^+–K^+- ATPase activity	*R*		0.616	0.514	0.420	0.212	−0.743	−0.749
	P value		0.000	0.000	0.000	0.064	0.000	0.000
	n		77	73	72	77	72	54
Plasma [Na^+]	*R*			0.71	0.539	0.466	−0.669	−0.507
	P value			0.000	0.000	0.000	0.000	0.000
	n			77	77	86	76	56
Plasma [Cl^-]	*R*				0.505	0.283	−0.503	−0.473
	P value				0.000	0.013	0.000	0.000
	n				73	77	73	54
Plasma osmolality	*R*					0.265	−0.383	−0.507
	P value					0.020	0.000	0.000
	n					77	74	55
Plasma [protein]	*R*						−0.203	−0.281
	P value						0.078	0.036
	n						76	56
Plasma [glucose]	*R*							0.637
	P value							0.000
	n							55

R, correlation coefficient.
Pairs of variables with positive *R* and $P<0.05$ tend to increase together.
For pairs of variables with negative *R* and $P<0.05$, one tends to decrease while the other increases.
For pairs of variables with $P>0.05$, there is no significant relationship between the two variables.

time and concentration were statistically significant ($P=0.0002$). At 40 μg Cu L^{-1}, plasma cortisol levels significantly increased 596% at day 3 to its maximum at day 7 (about 772%), then decreased at day 14 (412%) and finally increased again at day 21 of exposure (604%). On the contrary, at 400 μg Cu L^{-1}, although there was a significant increase in plasma cortisol concentrations (802% at day 3 to 842–844% at days 14 and 21), there was a significant decrease at day 7 of exposure. Differences between fish exposed to 40 and 400 μg Cu L^{-1} were significant at all times of exposure (Table 1).

3.11. Correlations between the different biochemical parameters

Gill copper and plasma Na^+ concentrations showed significant correlations above 25% (R^2) with all other parameters. No significant correlations above 25% were found between gill Na^+–K^+-ATPase activity and plasma osmolality, plasma Cl^- and cortisol, plasma glucose and osmolality, and plasma protein levels with any other biochemical parameter. No significant correlations were found between plasma protein levels and gill Na^+–K^+-ATPase activity, and between plasma protein and glucose levels (Table 2).

4. Discussion

4.1. Gill copper accumulation

The basal copper concentrations found in *O. niloticus* gills were similar to those reported in *Oreochromis mossambicus* (Pelgrom et al., 1995a,b) and lower than those found in *Tilapia zillii* (Ay et al., 1999). Under exposure to sublethal copper concentrations, metal deposition levels were similar to those reported in *Anguilla anguilla* (Grosell et al., 1998b) and higher than those observed in *O. mossambicus* (Pelgrom et al., 1995a,b). A steady state in copper accumulation was observed after 2–3 days in *Oncorhyncus kisutch* (Buckley et al., 1982) and *Solea senegalensis* (Arellano et al., 2000), after 6 days in *O. mossambicus* (Pelgrom et al., 1995a) and within 14–21 days in the juvenile trout (Dixon and Sprague, 1981). The copper steady state has been suggested to occur when the net copper uptake at the apical membrane is equalised by transport across the basolateral membrane into the blood (Grosell et al., 1996). On the contrary, copper accumulation was here found to increase with time in *O. niloticus*, similarly as previously described for *A. anguilla* (Grosell et al., 1998b).

The apical membrane of the gill epithelium has two types of copper binding sites, saturable high affinity-low capacity binding sites, found at low metal concentrations, and lower affinity-higher capacity binding sites, which bind copper in a linear fashion at higher exposure levels (MacRae et al., 1999; Taylor et al., 2002). However, when saturation of gill surface binding sites occurs, the metal is allowed to pass into the intercellular compartment, favouring its accumulation (Wepener et al., 2001). The intracellular transport of bound copper is carried out by P-type ATPases (Campbell et al., 1999) through Na^+-sensitive and Na^+-insensitive components, both exhibiting saturation kinetics at low copper concentrations, but also a linear component above 5.6 μg Cu L^{-1} (Grosell and Wood, 2002). Inside the cell there is a great variety of binding sites (Wepener et al., 2001), but ultimately copper is transported into the blood through Cu-ATPases at the basolateral membrane (Campbell et al., 1999).

The present results suggest that a steady state in gill copper accumulation levels was not achieved and that ionoregulatory disturbances were sustained. Accordingly to the damage–repair hypothesis (McDonald and Wood, 1993), structural damage to gills results in biochemical/physiological disturbances early in the exposure followed by recovery. On the contrary, the present results indicate that *O. niloticus* is tolerant to copper and that the structural damage, if any, that occurred, was inadequate to induce the repair phase and recovery. This lack of agreement thus suggests that other factors are likely in play to keep animals alive during the whole period of toxicant exposure. It is also possible that the exposure period was not long enough for recovery to occur in this species. Longer times to reach a steady state were also previously demonstrated for the rainbow trout (Dixon and Sprague, 1981; Marr et al., 1996).

4.2. Na^+/K^+-ATPase activity

In *O. niloticus*, copper accumulation significantly inhibited gill Na^+/K^+-ATPase activity. This inhibition was an early event (within 3 days) and steadily sustained, thus confirming the absence of compensatory processes going on at the gill. Inhibition of gill Na^+/K^+-ATPase activity has also been described in *O. mossambicus* (Pelgrom et al., 1995b; Li et al., 1998) and *T. zillii* (Ay et al., 1999), and shown to occur by covalent binding of copper to SH-groups and interaction with Mg^{2+} binding sites, although there was an increase in the number of Na^+/K^+-ATPase-rich chloride cells in *O. mossambicus* after 5–6 days of exposure to copper (Pelgrom et al., 1995b; Dang et al., 1999). On the contrary, recovery of gill Na^+/K^+-ATPase activity (>2 times control values) with continued exposure to copper has been shown in trouts (McGeer et al., 2000).

4.3. Plasma Na^+ and Cl^- concentrations

In fish, copper has been shown to cause a decrease in plasma Na^+ and Cl^- concentrations due to loss of gill ion regulatory functions (Nussey et al., 1995; Pelgrom et al., 1995b). At low copper concentrations, the active uptake of ions appears suppressed by inhibition of Na^+/K^+-ATPase activity (Li et al., 1998), although other studies could not confirm such a linear relationship (Laurén and McDonald, 1987a; Sola et al., 1995). At higher copper concentrations, there is also a marked ion efflux due to weakening of tight junctions brought on by displacement of membrane bound

calcium (Laurén and McDonald, 1985). The decrease in plasma Na^+ concentrations has also been attributed to loss of chloride cells (De Boeck et al., 2001), to direct inhibition of the apical Na^+-channel or to inhibition of carbonic anhydrase (Grosell and Wood, 2002).

The present results show that at 40 μg Cu L^{-1} the decrease of plasma Na^+ concentrations only occurred after 21 days of exposure to copper, while Cl^- concentrations were kept constant and at control levels during the whole exposure period. On the contrary, at higher copper concentrations, there was a significant and progressive decrease in both plasma ion concentrations. According with the significant correlations observed between copper accumulation levels, Na^+/K^+-ATPase activity and plasma ion concentrations, the decrease in plasma Na^+ and Cl^- concentrations may be attributed in part to copper inhibition of gill Na^+/K^+-ATPase activity.

4.4. Plasma osmolality and protein levels

In tilapia, decreased plasma ion concentrations induce vascular water efflux and, subsequently, hemoconcentration and a decline in plasma volume (Nussey et al., 1995). This compensatory loss of water from the vascular bed may be exacerbated by decreased water influx due to reduction of the gill exchange surface caused by lamellar fusion, a common injury observed after heavy metal exposure (Mallatt, 1985; De Boeck et al., 2001). Since freshwater fish are hyperosmotic to the surrounding water, disruption of the gill epithelium that occurs after long-term copper exposures will increase epithelium permeability, water influx and salt efflux (Laurén and McDonald, 1985; Richards and Playle, 1999), and thus lead to a final significant decrease of plasma osmolality, protein and ion concentrations.

In *O. niloticus*, plasma osmolality was significantly reduced after 3 (40 μg Cu L^{-1}) or 2 (400 μg Cu L^{-1}) weeks of exposure to waterborne copper. Similar results were obtained regarding plasma protein concentrations, with significant decreases being observed only after 3 weeks of exposure to both copper concentrations. At 400 μg Cu L^{-1}, hyponatremia was probably compensated by increased renal Na^+ absorption (Grosell et al., 1998a) and vascular water efflux, which may thus explain the observed delayed decrease in plasma osmolality and the higher and then normalized plasma protein levels. However, at 21 days of exposure to both waterborne copper levels, a highly significant simultaneous decrease occurred in plasma osmolality, ion and protein concentrations, which suggests a severe disruption of the gill epithelium or, alternatively, as fish remained alive, an increase in vascular permeability (Nussey et al., 1995).

4.5. Plasma glucose and cortisol concentrations

Fish exposed to toxicants activate several compensatory mechanisms, of which some are mediated by a non-specific stress response (Wendelaar Bonga, 1997). Cortisol protects the gill epithelium to ion losses by inducing chloride cell proliferation (Flik and Perry, 1989; Goss et al., 1992) and decreasing epithelial permeability (Kelly and Wood, 2002). By binding to its receptor, cortisol inhibits cell necrosis (Bury et al., 1998), stimulates Na^+–K^+-ATPase activity, the levels of ion transport enzymes (Flik and Perry, 1989; Shrimpton and McCormick, 1999) and the gill ion transporting capacity (Goss et al., 1992). On the contrary, copper downregulates the glucocorticoid receptor, causing opposite effects (Dang et al., 2000). However, sustained levels of stress are also capable of inducing tissue injury (Mallatt, 1985), disruption of ion regulatory mechanisms and osmotic imbalance (Wendelaar Bonga, 1997). This has been tested by using administration of cortisol (De Boeck et al., 2001) and adrenaline (McDonald and Milligan, 1997), air exposure and long-term confinement (Arends et al., 1999), acclimation to different temperatures or sudden temperature changes, use of anesthetics and handling (Eddy, 1981).

Cortisol affects carbohydrate metabolism and a rise in cortisol levels is frequently followed by hyperglycemia in fish (Wendelaar Bonga, 1997). Although the mechanisms involved remain unclear, the rapid rise in plasma glucose concentration following an acute stressor has been associated with the activation of the hypothalamus–sympathetic–chromaffin cell (HSC) axes (McDonald and Milligan, 1997), rather than with the cortisol rise mediated by the hypothalamus–pituitary–interrenal (HPI) axes (Arends et al., 1999). Plasma glucose levels have been shown to correlate with gill copper concentrations, even after long-term exposures, and thus have been suggested as a better indicator of stress (Laurén and McDonald, 1985) than plasma cortisol levels (De Boeck et al., 2001). Accordingly, the high plasma levels of cortisol and glucose observed in the present study may be indicative of the simultaneous activation of the HPI and HSC axes by copper and induction of different compensatory responses.

In *O. niloticus*, plasma cortisol and glucose levels significantly increased during the water copper exposure period. Although plasma levels did not rise linearly, the interaction between time and concentration were statistically significant. Both concentrations also correlated with each other and with the other biochemical parameters. Increases in plasma glucose concentrations were previously described in *Ictalurus nebulosus* (Christensen et al., 1972), *Salmo gairdneri* (Laurén and McDonald, 1985), *Oncorhynchus mykiss* (Richards and Playle, 1999) and *O. mossambicus* (Pelgrom et al., 1995b). However, and contrary to *O. mykiss* (Dethloff et al., 1999) and *Cyprinus carpio* (De Boeck et al., 2001), in *O. niloticus* plasma cortisol concentrations remained high during all the exposure period. The present results also revealed a significant drop in plasma cortisol concentrations at 14 (40 μg Cu L^{-1}) and 7 (400 μg Cu L^{-1}) days of exposure, after which cortisol levels raised again. Although unexpected, holding and exposure conditions had not changed and all animals showed concordant results, thus suggesting that somehow an early adaptation to stress of this species occurred, followed by a sustained decompensation

in long-term exposures such as occurred with all previous analyzed parameters.

4.6. Conclusions

O. niloticus is tolerant to copper. Waterborne copper exposure induced an early maximum inhibition of gill Na^+/K^+-ATPase activity although, a continuous increase in gill copper accumulation and sustained osmoregulatory disturbances, proved by the decreasing levels of plasma Na^+, Cl^- and osmolality, were observed. As the correlations observed between gill copper accumulation levels, Na^+/K^+-ATPase activity and plasma ion concentrations were statistically significant, the decline in plasma Na^+ and Cl^- may be, in part, attributed to the direct effect of copper in osmoregulation and Na^+/K^+-ATPase activity inhibition. Plasma glucose and cortisol levels, used as stress indicators, increased during the waterborne copper exposure period, indicating the simultaneous activation of the HPI and HSC axes, inducing different compensatory responses. Plasma glucose and cortisol concentrations were significantly correlated with each other and with osmoregulatory parameters suggesting that the slow decrease in ion plasma levels was related to compensatory mechanisms involving a non-specific stress response that appeared overcome at long-term exposures.

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