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A stereological study of copper toxicity in gills of *Oreochromis niloticus* ☆, ☆☆

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Abstract

Stereological methods were used to estimate the volumetric density (V_V) of the filamentar epithelium (FE, 39%), lamellae (L, 28%), central venous sinus (CVS, 14%), central axis (16%), mucous cells (MC, 2%) and chloride cells (CC, 1%) in the gill filament of control Nile Tilapia. The relative volumes of FE and L, and the relative volumes of CVS and central axis, varied inversely under exposure to copper, with high copper toxic levels declanching a chronic defence mechanism that was, nevertheless, overcome, and low copper toxic levels causing adaptation within a moderate acute phase type of response. Copper also induced a decrease of the V_V (MC, gill filament) due to reduction of surface MC, despite the marked increase of stem MC at chronic exposure to high copper toxic levels. Diminution of the numerical density of filamentar CC was responsible for the decreased V_V (CC, gill filament), although lamellar CC significantly increased at chronic exposure to low copper toxic levels. The present results demonstrate that cell relative volumes, mean volumes and numerical densities are dependent on the variations of the FE and L, which without a quantitative approach may be misinterpreted, thus stressing the importance of using stereological tools for analyzing histopathological patterns.

Keywords: Copper toxicity; Gills; Histopathology; Stereology; Teleostei; Tilapia

1. Introduction

Gills are a critical organ to fish as they represent the primary site for gas exchange, ion regulation, and excretion of metabolic waste products. With a wide surface area open to the external milieu, gills are also the first target to waterborne pollutants (Mallatt, 1985; Perry and Laurent, 1993). When present at high concentrations, copper causes histopathological changes in gills of Teleost fish, such as epithelial necrosis, vasodilatation, epithelial lifting, hypertrophy of the respiratory epithelium and hyperplasia of the filamentar and lamellar epithelium (Bury et al., 1998; Olsson et al., 1998; Arellano et al., 1999; Cerqueira and Fernandes, 2002).

To quantitatively characterize the histopathological changes caused by waterborne toxicants, several studies have used morphometric methods to analyze the thickness of the filamentar epithelium (FE) (Cerqueira and Fernandes, 2002) as well as the size and number of chloride and mucous cells (MC) (Lock and Overbeeke, 1981; Wendelaar bonga et al.,1990; Pelgrom et al., 1995; Cerqueira and Fernandes, 2002; Lease et al., 2003; Alvarado et al., 2006), whereas others have used stereological methods to evaluate

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the thickness of the blood-oxygen diffusion barrier (Hughes and Perry, 1976; Pinkney et al., 1989; Pane et al., 2004). Stereology is a precise tool for acquiring quantitative three-dimensional information based on two-dimensional data obtained from microscopic structures in tissue sections (Gundersen et al., 1988a, b). Thus, knowing the relative volumes of the various gill filament constituents, the nature of the changes following an experimental exposure can be more precisely described.

The Nile tilapia *Oreochromis niloticus* is a teleost fish with wide distribution around the world, and economic importance for fisheries and aquaculture. Due to its easy handling, culture and maintenance in the laboratory, and because it promptly responds to environmental alterations, this species is also a well-established model for toxicological research (Almeida et al., 2002; Figueiredo-Fernandes et al., 2006). In the present study, we applied current stereological methods to the analysis of the histopathological changes caused by different concentrations of waterborne copper in gills of *O. niloticus*. The relative volumes of the various gill filament constituents, their variation under acute and chronic copper exposure, and the usefulness of the stereological parameter volume-weighted mean volume for detecting size-related changes in chloride cells are presented.

2. Materials and methods

2.1. Fish

Nile tilapia, *O. niloticus* (Linnaeus, 1758) were raised in the Aquaculture Station of the University of Trás-os-Montes and Alto Douro (UTAD, Vila Real, Portugal), kept in 100 L recirculating tanks (5 L/min) filled with dechlorinated tap water whose quality parameters (84/449/EEC Directives, Annex 5, method c1) were maintained by mechanical and biological filtration: pH 6.5–7.5, 60 mg HCO_3^-/L (alkalinity), 63 μS/cm (conductivity), 14 mg Na^+/L, 2.3 mg K^+/L, 4.1 mg Ca^{2+}/L, 6.5 mg Mg^{2+}/L, 19.5 mg Cl^-/L, 27 mg NO_3^-/L (nitrate), 0.5 mg NO_2^-/L (nitrite), 74.5 mg $CaCO_3$/L (hardness), 6.2 mg dissolved O_2/L, 21 mg CO_2/L, 0.1 mg H_2S/L (hydrogen sulfide), 0.7 mg NH_4^+/L (ammonia) and 12 mg suspended solids/L. Supplemental aeration was provided to maintain the dissolved oxygen near saturation, the temperature was kept at 25 ± 1 °C and the photoperiod was controlled (12D:12L). Fish were fed daily once to visual satiation, with commercial fish food dry pellets (Aquasoja-Sorgal, Ovar, Portugal): 1.9% fiber, 4.3% lipids, 37.2% crude proteins, 2.2% Ca^{2+}, 1.4% P and vitamins A, C, D_3 and E.

2.2. Experimental design

Experiments complied with European (86/609/EU) Guidelines for the correct use of laboratorial animals. Sixty sexually mature male and female fish of 36.3 ± 7.7 g mean body weight were randomly distributed among 12 tanks (5 fish/100 L). Four tanks served as controls and had water without added copper (3.9 μg Cu/L tap water). Copper (copper sulfate, $CuSO_4$, Merck, Darmstadt, Germany) was added to nominal concentrations of 40 μg Cu/L (mean 39.8, range 36–45) in 4 tanks and 400 μg Cu/L (mean 395.8, range 375–420) in 4 other tanks after preliminary results shown to be sublethal during a 21-day period of exposure. Experiments were carried out in semi-static systems, with a rate of 1/3 water renovation every 2 days. Water samples were taken at collecting days and actual copper concentrations were measured by graphite-furnance atomic absorption spectrophotometry (Unicam 939 AA Spectometer, equipped with a Unicam GF 90 furnance and FS 90 furnance autosamples, UK). Water quality parameters were also reassessed at collecting days during the experimental period, with no significant changes being observed. No mortality was observed during experiments. Fish were fasted for 24 h before collection at days 3, 7, 14 and 21. Five fish per treatment were anesthetized with 1 mL of 2-phenoxiethanol/L water (Sigma, Barcelone, Spain), euthanized by decapitation and the second gill arch of the right side of each fish collected (Bury et al., 1998; Handy et al., 2002; Pane et al., 2004; van Heerden et al., 2004).

2.3. Immunohistochemistry

Tissues were fixed for 24 h in Bouin fluid (Panreack, Barcelone, Spain), dehydrated and embedded in paraffin wax. Five paraffin blocks per treatment were serially sectioned (5 μm thick) parallel to the long axis of the gill filament. Two consecutive sections were chosen and mounted on poly-L-lysine-coated glass slides (Sigma). The first section was used for MC localization using the periodic acid-Schiff (PAS) reaction (Merck) and the second section was used for chloride cell (CC) identification using a mouse monoclonal antibody raised against a chicken synthetic peptide corresponding to part of the highly conserved region of the Na^+/K^+-ATPase α-subunit (Ura et al., 1996). After deparaffinization and rehydration, slides were rinsed in tap water and dipped for 15 min in 0.3% H_2O_2 for endogenous peroxidase blocking (Merck). After rinsing 5 min each in tap water, distilled water and Tris buffer (Merck), sections were incubated for 18 h at 22 °C with 1:500 primary antibody (Department of Biological Sciences, University of Iowa, USA). After 3×5 min washes in Tris buffer, sections were incubated for 1 h with 1:40 goat-anti-mouse IgG1 secondary antibody and then for 45 min in 1:100 PAP complex (Sigma). After rinsing in Tris buffer, sections were immersed for 10 min in 0.05% of 3,3′-diaminobenzidine in Tris buffer containing 0.03% H_2O_2 (Sigma). Subsequently, sections were rinsed for 10 min in tap water, dehydrated, cleared and mounted. Antisera and PAP complex were diluted in Tris buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin, lambda carrageenen, 0.5% Triton X-100 and 0.02% sodium azide (Sigma). For incubations, Coplin jars were used for antisera, whereas, PAP was carried out in a closed dark moist chamber. For negative controls, the primary antibody was omitted.

2.4. Stereological analysis

Volume density (V_V) is defined as the percentage of the total volume of a well-defined reference space occupied by any given component within it. Accordingly, a stereological approach was designed to estimate the V_V of the different structural elements within the gill filament (the reference space): central axis (cartilaginous and subepithelial connective tissue), central venous sinus (CVS), lamellae (L) and FE and, within the latter, CC and superficial and deep MC. The V_V were estimated by point counting (Freere and Weibel, 1967) using the formula

$$V_v(\text{structure}, \text{reference}) = [P(s) \times 100]/P(r)$$

where $P(s)$ is the number of test points within each structural component and $P(r)$ is the total number of test points lying over the reference space. Counting was done in a microscope (Olympus, BX-50) equipped with a motorized stage (Prior) for stepwise displacements in x–y directions (1 μm accuracy) and a CCD camera (Sony) connected to a 17″ PC monitor (Sony). The whole system was controlled by CAST-Grid software version 1.5 (Olympus Denmark A/S, Albertslund, Denmark). The first field of vision was randomly selected. Following fields were systematically sampled by stepwise movements of the stage in the x- and y-directions ($\text{step}_{x,y} = 165$ μm). A software-generated counting frame, with two sets of points (ratio 1:12), was superimposed on the monitor live image (Fig. 1). To estimate the V_V of central axis, CVS, FE, and L, the 16-encircled points of the lattice were used, whereas, for the V_V of CC and MC, the 192 points of the lattice were used. Counting was made at a final magnification of ×400, with analysis of an average of 230 fields per fish.

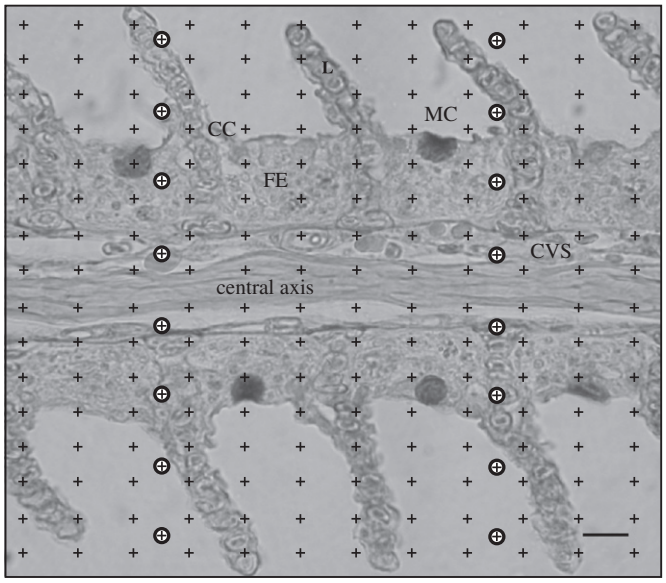

Fig. 1. Software-generated counting frame, with two sets of points (ratio 1:12), superimposed on the monitor live image. To estimate the V_V of the central axis, central venous sinus (CVS), filamentar epithelium (FE) and lamellae (L), the 16 encircled points of the lattice were used, whereas for mucous cells (MC) and chloride cells (CC) the 192 points of the lattice were used. Bar = 10 μm.

When the V_V values are referred to the same reference space, they might be mathematically combined, allowing the derivation of new stereological parameters (Howard and Reed, 1998). Accordingly, the values of V_V (CC_{FE}, FE), V_V (CC_L, L) and V_V (MC, FE) were also determined in agreement with the example formula:

V_V (CC, FE) = CC volume/FE volume = (CC volume/filament volume)/(FE volume/filament volume) = V_V (CC, filament)/V_V (FE, filament).

To further disclose the differences observed in the V_V (CC, filament) of fish exposed to copper, CC number and size were also analyzed. The mean number-weighted cellular volume ($\bar{v}_N$) is a stereological parameter that provides an unbiased estimate of the mean volume of a given population of particles in the number-weighted distribution, without any assumptions about their shape and with each unit being proportionally sampled to their volume (Howard and Reed, 1998). The procedure requires an arbitrary-fixed point within a unique defined subspace of the particle. For practical purposes, this was chosen as the center of the nucleolus. According to the dissector principle, for an isolated cell with a single nucleolus, the selection of the unique point does not need to be made by uniform random probability (Howard and Reed, 1998). In this case, the estimate of the cell volume might be performed with a very small bias by sampling all cells in one independent section. Therefore, in each section of every fish, all cells with an evident nucleolus were sampled and their $\bar{v}_N$ estimated using the nucleator method (Gundersen et al., 1988b). Briefly, two isotropic orthogonally oriented lines were generated from a random point within the nucleolus (for practical purposes the nucleolus centre), and the distances from the nucleolus centre to the cell limit were recorded along the isotropic lines. From a series of these measurements, the mean cell volume (μm^3) in the so-called number-weighted distribution was estimated from

$$\bar{v}_N = \frac{4\pi}{3}\bar{l}_n^3 = \frac{4\pi}{3n}\sum_{i=1}^{n} l_{n,i}^3$$

where l_n refers to the distances from the sampling point to the edge of the cell. All estimating procedures were carried out under oil immersion (× 100 objective lens) using the CAST-Grid software. At the PC monitor, a final magnification of × 4024 allowed an accurate recognition of CC and their nucleoli.

The numerical density of CC per boundary length of the gill filament (N_{BL}) was estimated using the formula

$$N_L(\text{CC, filament}) = \Sigma Q^- / \text{BL}$$

where $\sum Q^-$ is the sum of the number of CC with an evident nucleolus counted in each filament of a section, and BL is the estimated total boundary length of all gill filaments present in the section. Although this procedure is not strictly unbiased, it was, here, adopted as a practical approach for comparative purposes among groups. The BL was estimated using the CAST-Grid software, by evaluating the filament length at every fifth-sampled filament, counted from a random start. The sum of the values obtained by this procedure was multiplied by five to make the estimate of the filaments BL in a section.

2.5. Statistical analysis

Statistical analysis was performed with the software SigmaStat (Statistical software, SPSS Inc.). The values of V_V, $\bar{v}_N$ and N_L determined for each animal were used to calculate the mean and standard deviation (SD) at each concentration and time group. Once normality and variance homogeneity were confirmed, these parameters were analyzed with two-way ANOVA to test the isolated and interactive effects of copper concentration and exposure time. After a significant ANOVA, the Tukey post-hoc test was used to study differences among groups. The level of significance (α) was set at 0.05.

3. Results

The stereological analysis of control fish showed that of the structural components of the gill filament, the FE (39%) and L (28%) had the major relative volumes, whereas the central axis (16%) and the CVS (14%) occupied an intermediate relative volume. In the gill filament, the relative volumes were 2% for MC and 1% for CC, whereas in the FE they were 4.6% for MC and 2.8% for CC. MC were only observed in the FE, with the superficial population contributing to 99.6% of the total MC relative volume, either referred to the gill filament or the FE. Contrary to fish exposed to copper, only one control animal had CC in L.

The relative volume of the FE was decreased in relation to controls during the whole period of exposure to 40 μg Cu/L, although without reaching statistical significance. On the contrary, exposure of fish to 400 μg Cu/L caused a slight decrease of the relative volume at 3 days of exposure, followed by a progressive increase that became significant to 40 μg Cu/L by day 14 and to both controls and 40 μg Cu/L by day 21 of exposure (Fig. 2). The relative volume of the L was increased by exposure to 40 μg Cu/L during the whole period of exposure, reaching significant values at 14 days of exposure to copper. On the contrary, exposure of fish to 400 μg Cu/L caused a slight increase of the relative volume at day 3, followed by a significant increase at day 7 of exposure in relation to controls. After that, it progressively decreased and became significant at day 14 in relation to 40 μg Cu/L and to both controls and 40 μg Cu/L at day 21 of exposure (Fig. 2).

The relative volume of the CVS remained slightly decreased in relation to controls during the first 2 weeks of exposure to 40 μg Cu/L and then increased, although

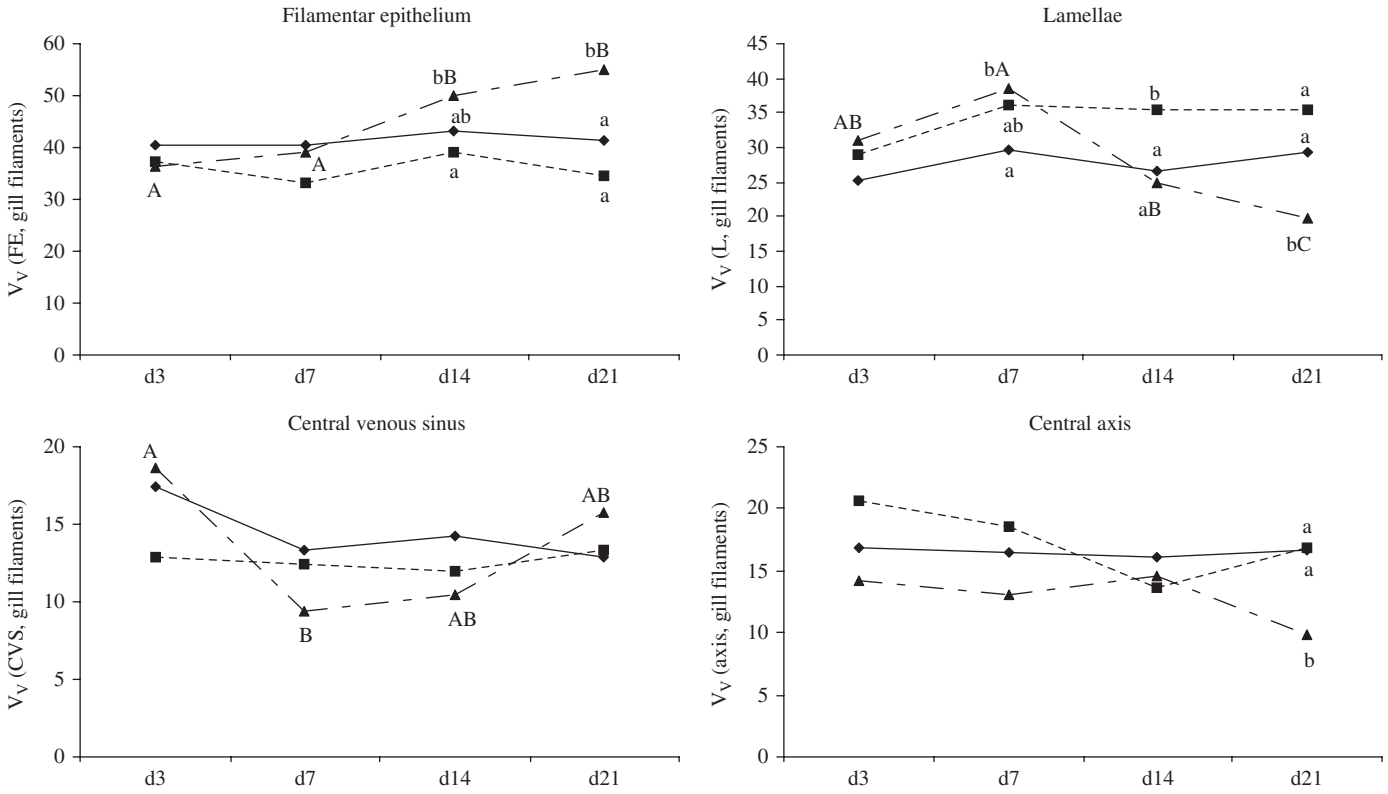

Fig. 2. Relative volumes (V_V) of the gill main structural elements in controls (continuous line) and in fish exposed to 40 μg Cu/L (dotted line) and 400 μg Cu/L (hatched line). At the same exposure time, different low case letters represent significant (ANOVA, $P<0.05$) differences between concentrations. Within the same copper concentration, different upper case letters represent significant differences between exposure times.

without reaching statistical significance. On the contrary, intra-group analysis of fish submitted to 400 μg Cu/L showed an increase of the relative volume at day 3, followed by a significant decrease at day 7, and then by a progressive rise until day 21 of exposure, although without reaching statistical significance to controls and 40 μg Cu/L (Fig. 2). The relative volume of the central axis was slightly increased in relation to controls during the first week and then decreased during the second and third week of exposure to 40 μg Cu/L, although without reaching statistical significance. On the contrary, in fish submitted to 400 μg Cu/L, the relative volume remained slightly decreased during the first 2 weeks in relation to controls and 40 μg Cu/L, and then significantly decreased in relation to both controls and 40 μg Cu/L at 21 days of exposure (Fig. 2).

The relative volume of MC was studied in relation to the gill filament and the FE, once the latter showed changes during exposure to copper. We also analyzed two different populations of MC, the superficial pool (mature MC) and the deep pool (MC precursors). In fish exposed to 40 μg Cu/L, intra-group analysis revealed that in relation to controls the relative volume of total MC in the gill filament was slightly increased at day 3 and then significantly decreased, although since day 7 of exposure it showed a progressive recover. Similar changes were observed for the relative volume of MC in the FE. On the contrary, exposure of fish to 400 μg Cu/L caused a constant decrease in the relative volume of MC. At day 3 of exposure, this decrease was slight in relation to controls but significant to 40 μg Cu/L, and then became significant only to controls during the second and third week of exposure. Similar changes were observed for the relative volume of MC in the FE, although the significant decrease observed during the second and third week of exposure to copper were relative both to controls and 40 μg Cu/L, probably in relation with the increase of the relative volume of the FE (Fig. 3).

Changes in the relative volume of MC were due to divergent variations in the superficial and deep populations. In fish exposed to 40 μg Cu/L, intra-group analysis revealed that the superficial pool-relative volume in the gill filament followed a similar pattern of variation as described above for the total MC relative volume. A similar trend was observed in the FE, although the increase observed at day 3 of exposure was significantly higher. On the contrary, although the precursor pool-relative volumes in the gill filament and FE showed a similar decrease after day 3 of exposure, the relative volumes were, thereby, held slightly higher than controls. In animals exposed to 400 μg Cu/L, the superficial pool-relative volumes in the gill filament and FE followed a similar pattern of variation as described above for the total MC relative volumes. On the contrary, the precursor pool-relative volumes in the gill filament and FE also slightly decreased during the first

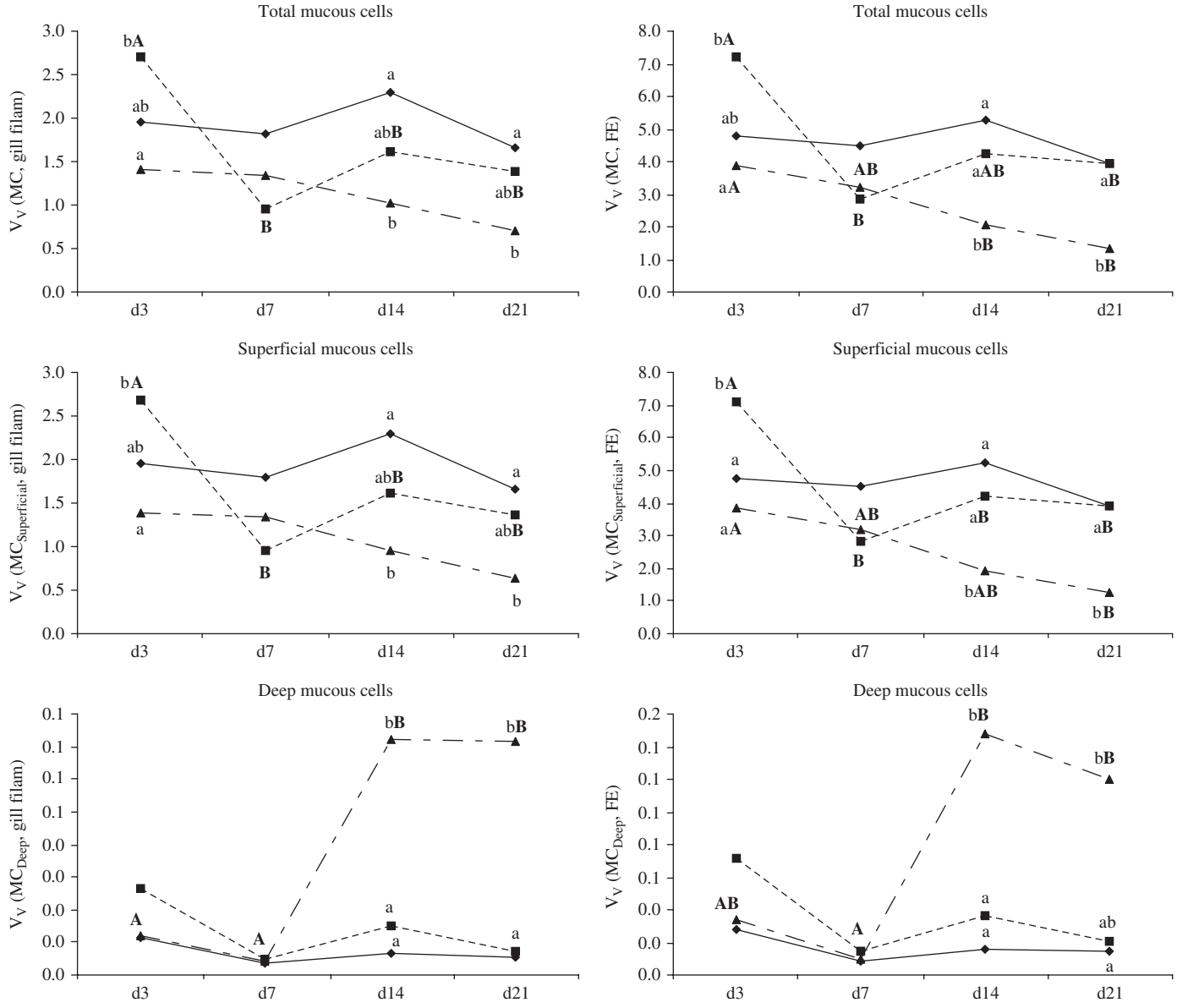

Fig. 3. Relative volumes of total, superficial and deep mucous cells per gill filaments and per filamentar epithelium in controls (continuous line) and in fish exposed to 40 μg Cu/L (dotted line) and 400 μg Cu/L (hatched line). At the same exposure time, different low case letters represent significant (ANOVA, $P<0.05$) differences between concentrations. Within the same copper concentration, different upper case letters represent significant differences between exposure times.

week but thereafter significantly increased in relation to controls and 40 μg Cu/L (Fig. 3).

Per gill filament, the relative volume of total CC was significantly decreased by exposure to 40 μg Cu/L in relation to controls, with the minimum reached at day 7. Thereafter, there was a slight relative increase up to 21 days of exposure. This was due to a similar decrease of the relative volume of CC located in the FE, as the relative volume of CC located in L was slightly increased during the first week, and significantly increased in the second and third week of exposure in relation to controls (Fig. 4). At 400 μg Cu/L, the relative volume of total CC was significantly decreased to a minimum at day 3 in relation to controls and 40 μg Cu/L. Thereafter, it increased until day 14, reaching values similar to controls and significantly higher than at 40 μg Cu/L, and then again significantly decreased in relation to controls and 40 μg Cu/L. This was due to a similar decrease of the relative volume of CC located in the FE, as the relative volume of CC located in L was significantly increased in relation to controls and 40 μg Cu/L at day 7 and to controls at day 14, after which it significantly decreased to control values (Fig. 4). Per FE, the relative volumes of CC located in the FE exhibited the same patterns of decrease, although without significant differences between both copper concentrations. Similar patterns of increase were also observed for the relative volumes of CC located in L. However, in fish exposed to 40 μg Cu/L the significant rise in relation to controls was

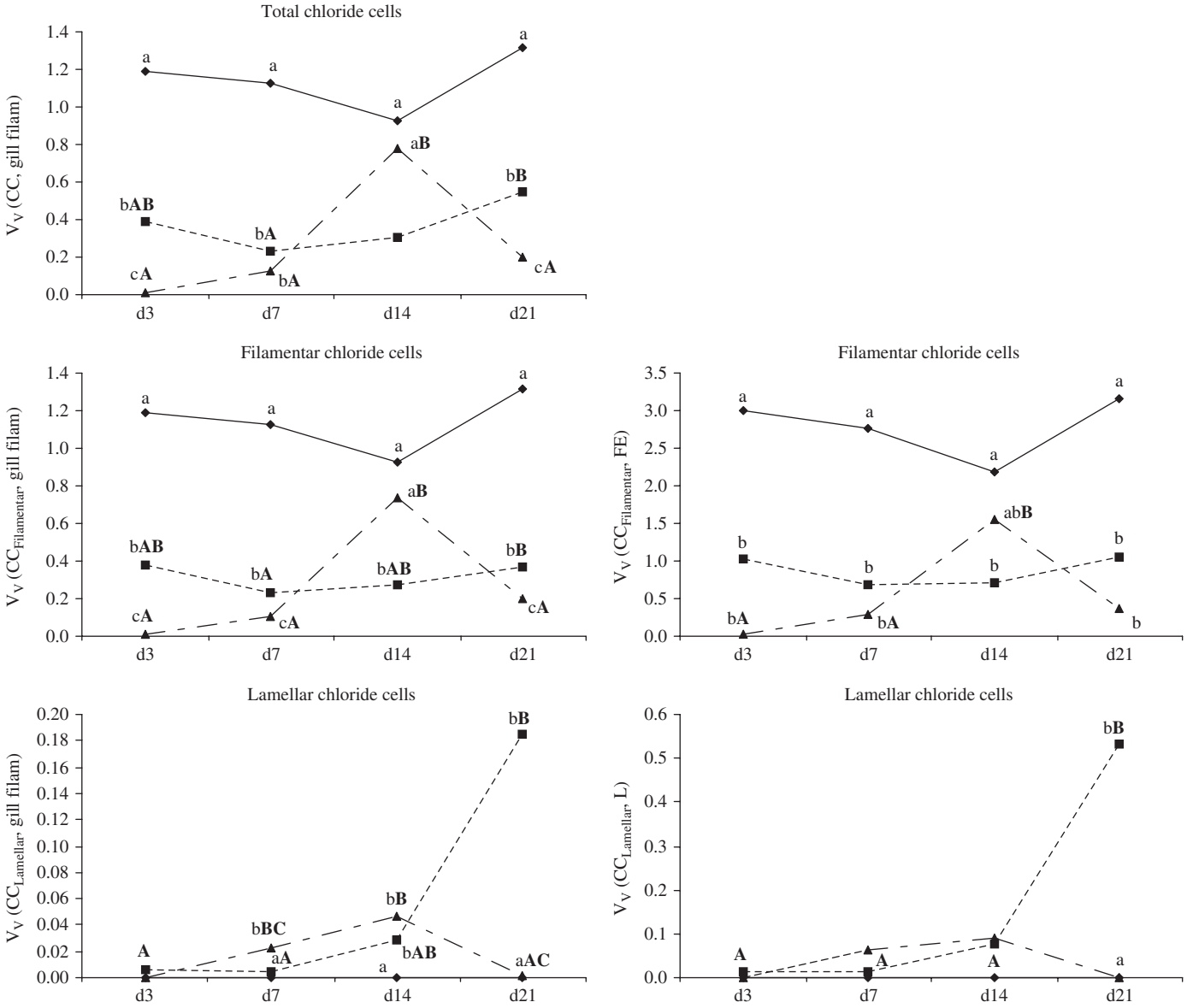

Fig. 4. Relative volumes of total, filamentar and lamellar chloride cells per gill filaments, and of filamentar and lamellar chloride cells per filamentar epithelium and lamellae, in controls (continuous line) and in fish exposed to 40 μg Cu/L (dotted line) and 400 μg Cu/L (hatched line). At the same exposure time, different low case letters represent significant (ANOVA, $P<0.05$) differences between concentrations. Within the same copper concentration, different upper case letters represent significant differences between exposure times.

only observed at 21 days, whereas, at 400 μg Cu/L values never reached significance in relation to controls (Fig. 4).

In controls, the mean volume ($\overline{v}_N$) of CC varied between 331 and 391 μm^3 and the numerical density of CC per boundary length of the gill filament (N_{BL}) varied between 0.175 and 0.223/μm. Copper caused a sustained slight increase of the mean volume of CC, although only fish exposed to 400 μg Cu/L showed a significant increase in relation to controls at day 7 (Fig. 5). On the contrary, at 40 μg Cu/L, the numerical density of CC was slightly higher than controls at day 3, then significantly decreased at day 7, and finally progressively increased reaching again slightly higher numbers than controls at day 21 of exposure. At 400 μg Cu/L, the numerical density of CC was significantly decreased at day 3 in relation to controls and 40 μg Cu/L, and then progressively increased to control values at day 14 and to significant higher values than controls and 40 μg Cu/L at 21 days of exposure (Fig. 5).

4. Discussion

To our knowledge, this is the first stereological study that determines the volumetric density of all structural components of the gill filament with evaluation of waterborne copper toxic effects. In *O. niloticus*, near 70% of the relative volume of the gill filament was occupied by the FE and L, including mucous and CC, which were also the major components affected by copper. The present results

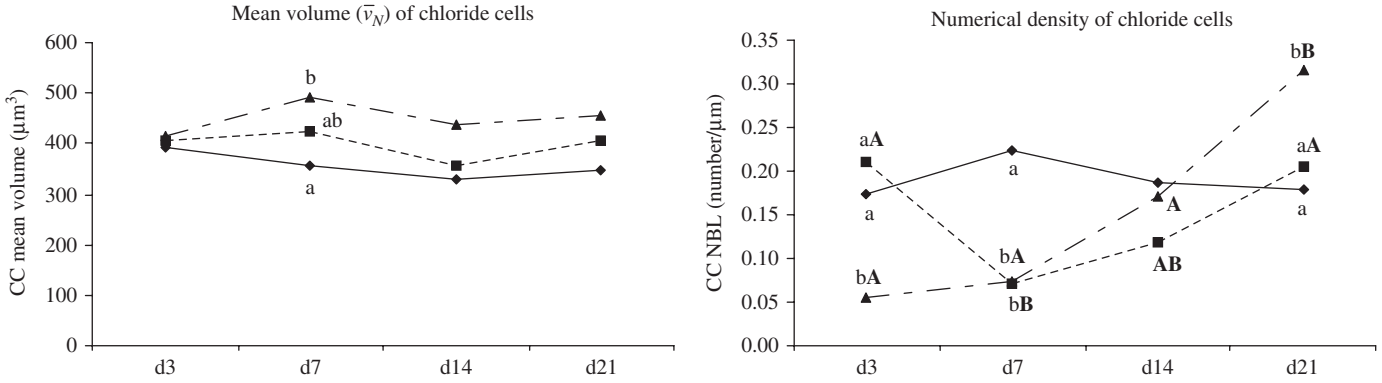

Fig. 5. Mean volume ($\bar{v}_N$) and numerical density per boundary length of the gill filament (N_{BL}) of chloride cells in controls (continuous line) and in fish exposed to 40 µg Cu/L (dotted line) and 400 µg Cu/L (hatched line). At the same exposure time, different low case letters represent significant (ANOVA, $P<0.05$) differences between concentrations. Within the same copper concentration, different upper case letters represent significant differences between exposure times.

also show that the relative volumes of these two components varied inversely under exposure to copper. These changes are in accordance with the previously described acute and chronic histopathological defence responses to pollutants (Arellano et al., 1999; Fanta et al., 2003; Pane et al., 2004). Thus, for fish exposed to high copper toxic levels, the slight decrease of the relative volume of the FE might have been caused by desquamation and necrosis (Fig. 6), whereas, the significant increase in the relative volume of L was probably related to edema, as well as to lamellar epithelial lifting and hypertrophy (Fig. 6), all known to occur during the acute defence phase. Similarly, changes occurring during the chronic defence phase, such as proliferation of the FE and fusion of L (Fig. 6), could have caused the significant increase of the relative volume of the FE and decrease of the volumetric density of L. On the contrary, fish exposed to low copper toxic levels did not reveal this dual pattern of defence mechanisms, as the relative volume of the FE remained slightly decreased and the relative volume of L slightly increased during the whole period of exposure, thus, suggesting adaptation within a moderate acute phase-type of response. Additionally, the variations observed in the relative volumes of the main gill filament constituents, after copper exposure, emphasize the different kinds of responses induced, in fish, by different pollutant concentrations. De Boeck et al. (2007) testing two copper concentrations, intermediate to the ones used here, also observed different responses that inclusively varied with the fish species and their sensitivity to copper, thus, stressing the importance of realistic unbiased quantitative methods that allow easy and direct comparisons among the results.

The central axis and the CVS comprised about 30% of the relative volume of the gill filament and showed less significant variations to copper exposure that were on the dependence of the changes observed in L. Although fish anaesthetics may induce changes in gill vasculature (Hill et al., 2002), as-control and exposed fish were submitted to the same sampling conditions, we assumed that the changes observed in the relative volume of CVS were due to the copper effect. Indeed, in fish exposed to high copper toxic levels, the volume density of CVS decreased from day 3 to 7 of exposure, increasing thereafter. This late increase might be related with redirection of blood flux from the arterial (lamellar) to venous system, since during this period the lamellar relative volume decreased. Although, there are no stereological studies to corroborate these assumptions, Campbell et al. (1999) also referred the redirection of the blood flux to the venous system, and Pinkney et al. (1989) and Pane et al. (2004) observed a significant contraction of the lamellar vasculature following tributyltin compounds and nickel exposure, respectively. Fish exposed to low copper toxic levels evidenced a continued slight decrease of the CVS relative volume, which might be interpreted as adaptation within a moderate acute phase type of response. Copper exposure did not induce significant changes in central axis relative volume, except the decrease observed after 21 days of exposure to 400 µg/L, which is probably related to the coincident chronic expansion of the CVS relative volume.

We, here, show that the relative volume of MC varied in a time and concentration-dependent manner under exposure to waterborne copper toxic levels. The slight increase of the relative volume of MC observed at day 3 of exposure to 40 µg Cu/L was especially due to a significant increase of the volumetric density of the superficial pool. This was followed by a significant reduction of the relative volumes at day 7 of exposure, probably caused by massive mucin release (Miller and Mackay, 1982; Bols et al., 2001). On the contrary, fish exposed to 400 µg Cu/L showed decreased MC relative volumes since 3 days of exposure, which suggests that an early mucus hypersecretion response is induced by high copper toxic levels. In fact, the mucus layer is known to efficiently uptake metal cations to impair epithelial penetration. Release of the attached metal ions is then promoted by mucous hypersecretion, which also serves as a stimulus to the turnover of the mucous layer (Varanasi and

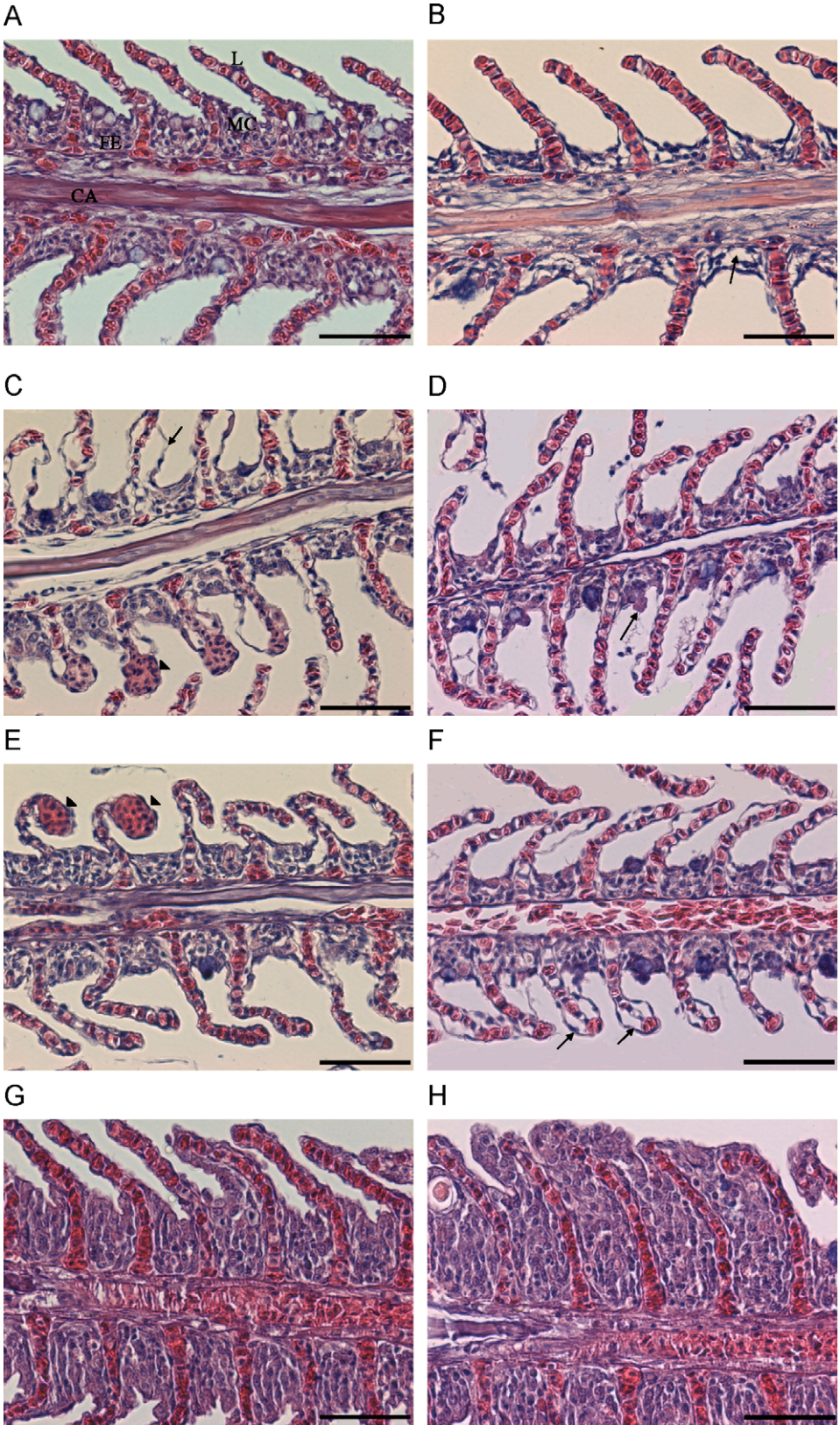

Fig. 6. Overview of histopathological changes in gills of *O. niloticus*. Images represent damage patterns rather than showing all changes present at each copper concentration or exposure time. (A) Control fish. Central axis (CA), filamentar epithelium (FE), mucous cell (MC), lamella (L). (B–D) Exposure to 40 μg Cu/L. (B) Day 3 or 7. Decreased FE thickness and FE edema (arrow). (C) Day 14. Lamellar lifting (arrow) and aneurisms (arrowhead). (D) Day 21. Epithelial desquamation (arrow). (E–H) Exposure to 400 μg Cu/L. (E) Day 3. Aneurisms (arrowhead). (F) Day 7. Lamellar lifting (arrow). (G) Day 14. Proliferation of the FE. (H) Day 21. Lamellar fusion. Scale bar corresponds to 50 μm.

Markey, 1978; Lock and Overbeeke, 1981; Reid and McDonald, 1991; Lichtenfels et al., 1996). After the acute defence phase, a compensatory chronic response was obvious for fish exposed to 400 μg Cu/L as evidenced by the significant increase of the precursor pool-relative volumes, although the superficial pool exhibited a further significant decrease. This suggests that at high copper toxic levels, compensation depends on cell differentiation from the adult stem cell pool, although complete renewal of surface MC was never achieved probably due to continuous cell sloughing and phagocytosis to avoid further accumulation of copper in the FE (Dang et al., 1999; Alvarado et al., 2006). On the contrary, fish exposed to 40 μg Cu/L had a more successful compensatory response, showing recovery of the superficial MC pool. Although this approach proved to be useful in distinguishing the diverse effects induced by different copper levels and exposure times, it does not allow to absolutely discern whether this is due to a reduced number or a reduced size of MC. Though this should be further studied in the future, in the present work this was not possible since the identification of an unique point, required for stereological analysis, was practically impossible to establish in PAS-stained cells.

Previous studies have shown that CC hypertrophy and hyperplasia help to maintain the osmoregulatory capacity of fish exposed to waterborne copper (McDonald and Wood, 1993; Pelgrom et al., 1995; Dang et al., 2000), although this capacity is frequently lost by the toxic action of pollutants on those cells (Wendelaar Bonga and Lock, 1992). The present study demonstrates that copper exposure caused a time and concentration-dependent decrease of the relative volume of CC, and that this was accompanied by similar decrease of the relative volume of CC located in the FE and increase of the relative volume of CC located in L. Copper accumulation in gills was shown to increase linearly with time, causing a sharp rise in plasma cortisol levels at 14 days of exposure to 400 μg Cu/L (Monteiro et al., 2005). As copper induces CC necrosis and apoptosis, and cortisol protects the gill epithelium to ion losses by inducing CC proliferation (Flik and Perry, 1989; Goss et al., 1992), the acute-defence phase decrease of the volumetric density of CC located in the FE could be caused by the direct toxic effects of copper, whereas, the recovery observed at 14 days of exposure to high copper toxic levels and the increase in the relative volume of CC located in L could be induced by the correspondent rise in plasma cortisol levels. Nevertheless, adaptation of fish submitted to high copper toxic levels was overcome at later chronic exposure times due to loss of both L and FE-CC, which is in agreement with the osmoregulatory changes observed in gills of *O. niloticus* under the same high copper toxic levels (Monteiro et al., 2005). On the contrary, adaptation of fish exposed to low copper toxic levels was more successful due to the significant increase of the relative volume of CC located in L during the chronic phase of exposure.

The present results also show that the decrease of the relative volume of CC was not related to a decrease of the mean volume ($\bar{v}_N$) of CC but to a decrease of the numerical density of CC per boundary length of the gill filament (N_{BL}), which is in accordance to previous results showing hypertrophy of CC in response to waterborne copper toxic levels (Pelgrom et al., 1995; Cerqueira and Fernandes, 2002). The apparent discrepancy observed between the present results and those showing an increase in CC density (McDonald and Wood, 1993; Pelgrom et al., 1995; Dang et al., 2000; Cerqueira and Fernandes, 2002) is probably related to the use of different methods in its determination. However, the method used here revealed to be consistent with unbiased stereological data, since CC numerical density followed the variation observed in the relative volume of CC, with exception of day 21 of exposure. This might be explained by the reduction of the superficial area, and thus, of the boundary length of the gill filament, due to the proliferation of the FE and fusion of L that occur at this stage of chronic exposure. Thus, the present results bring another possible explanation for the low levels of the gill Na^+/K^+-ATPase activity in view of the apparent proliferation of CC in fish exposed to heavy metals (Wendelaar Bonga and Lock, 1992; Pelgrom et al., 1995). Alternatively, proliferation of CC could participate in the increase of the relative volume of the FE at chronic exposure to 400 μg Cu/L without causing a correspondent increase in the relative volume of CC. In this case, impaired gene expression or protein function would explain the lower Na^+/K^+-ATPase activity.

5. Conclusion

The present results showed that the relative volumes of the two major structural components of the gill filament varied inversely under exposure to copper, with high copper toxic levels declanching a chronic defence mechanism that is, nevertheless, overcome, and low copper toxic levels causing adaptation within a moderate acute phase-type of response. Data also demonstrated that cell relative volumes, mean volumes and numerical densities are dependent on the variations of the FE and L, which without a quantitative approach may be misinterpreted, thus, stressing the importance of using stereological tools for analyzing histopathological patterns.

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Appendix A. Supplementary materials

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.ecoenv.2008.02.008.

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