HEMATOLOGICAL AND PHYSIOLOGICAL CHANGES INDUCED BY SHORT-TERM EXPOSURE TO COPPER IN THE FRESHWATER FISH, *Prochilodus scrofa*

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ABSTRACT

Hematological and physiological changes in the blood of juveniles of the freshwater fish, *Prochilodus scrofa* were determined after acute exposure to 20, 25, and 29 μgCu L^{-1} in water (pH 7.5; hardness 24.5 mg L^{-1} as $CaCO_3$) for 96 h. Copper exposure to 25 and 29 μgCu L^{-1} caused significant increase in the hematocrit and red blood cell values. The increase in red blood cells was associated with increase in whole blood hemoglobin only in fish exposed to 29 μgCu L^{-1}. Leukocytes increased following copper exposure and were significantly higher in fish exposed to 29 μgCu L^{-1}. Differential leukocyte percentage displayed significant reduction in lymphocytes and an increase in neutrophils in fish exposed to 25 and 29 μgCu L^{-1}. The percentage of monocytes remained unchanged after copper exposure. The thrombocytes did not change. There was a significant decrease in plasma [Na^+] and [Cl^-] and a significant drop in blood pH in fish exposed to 25 and 29 μgCu L^{-1} while [K^+] showed significant increase in fish exposed to 29 μgCu L^{-1}. Copper exposure led to ionoregulatory impairment, although chloride cell hypertrophy was induced. The changes in red blood cells suggest a compensatory response to respiratory surface reduction of gills (tissue damage and cell proliferation) in order to maintain oxygen transference from water to the tissues, allowing the fish to survive during the so-called shock phase of LC_{50} exposure, at least while at rest.

Key words: copper, hematological parameters, plasma ions, gill histopathology, *Prochilodus scrofa.*

RESUMO

Alterações hematológicas e fisiológicas em *Prochilodus scrofa* induzidas durante exposição aguda ao cobre

As alterações hematológicas e fisiológicas em *Prochilodus scrofa* juvenis foram determinadas após exposição aguda a 20, 25 e 29 μgCu L^{-1} no meio aquático (pH 7,5; dureza 24,5 mg L^{-1} como $CaCO_3$) durante 96 h. A exposição a 25 e 29 μgCu L^{-1} causou aumento significativo nos valores de hematócrito e número de eritrócitos. O aumento no número de eritrócitos foi associado a um aumento na porcentagem de hemoglobina somente nos peixes expostos a 29 μgCu L^{-1}. O aumento nos leucócitos após exposição ao cobre foi significativamente maior nos peixes expostos a 29 μgCu L^{-1}. A porcentagem diferencial de leucócitos apresentou redução significativa nos linfócitos e aumento nos neutrófilos nos peixes expostos a 25 e 29 μgCu L^{-1}, entretanto nenhuma modificação ocorreu na porcentagem de monócitos e trombócitos após a exposição ao cobre. Houve decréscimo significativo na [Na^+] e [Cl^-] plasmática e redução significativa no pH sangüíneo em peixes expostos a 25 e 29 μgCu L^{-1}, enquanto a [K^+] mostrou aumento significativo em peixes expostos a 29 μgCu L^{-1}. A exposição ao cobre provocou distúrbios na regulação iônica, embora a hipertrofia das células-cloreto

tenha sido induzida, e as mudanças nos parâmetros hematológicos sugerem resposta compensatória à redução da superfície respiratória das brânquias (lesões no tecido branquial e proliferação celular) de forma a manter a transferência do oxigênio da água para o sangue, permitindo a sobrevivência dos peixes durante a fase de choque da exposição a CL_{50}, pelo menos, sob condições de repouso.

Palavras-chave: cobre, parâmetros hematológicos, íons plasmáticos, histopatologia branquial, *Prochilodus scrofa*.

INTRODUCTION

Water pollution has become a global problem. Some essential metal trace elements for animal life, such as copper, are continuously increasing in water which may result in toxic effects on aquatic organisms, including fish (Heath, 1995). In Brazil, as a result of increases in industrial development the Southeast Brazilian rivers have experienced increasing copper concentrations, a situation aggravated by the ocurrence of episodic ecological accidents. Previously, the copper concentration in these environments was usually lower than 5 $\mu g\ L^{-1}$ but is has increased during the last decade reaching occasionally, 50 $\mu g\ L^{-1}$ (CETESB, 1992-2000) although the Brazilian Environmental Bureau has adopted the copper limits recommended by the U.S. EPA (US EPA, 1984) for the protection of aquatic life (20 $\mu gCu.L^{-1}$). However, no toxicological studies have been done on the effects of copper on native fish of these environments.

The gill is the primary target organ for the toxic action of copper. Impairment of the respiratory and the ionoregulatory functions may occur due to the structural changes and an increased the ion permeability of the gill epithelia (Laurén & McDonald, 1985; Wilson & Taylor, 1993), and inhibition of the Na^+/K^+-ATPase activity (Li *et al*., 1998). Such toxic effects may result in biochemical and physiological changes in fish blood (Nussey *et al*., 1995a, b, c). These changes can be an indicator of the physiological state of fish, as it is well known that the blood's function is to maintain tissue stability by keeping the internal environment of the body constant (Banerjee & Homechaudhuri, 1990; Heath, 1995).

Prochilodus scrofa is an active species living in Southeastern Brazilian rivers. Juvenile specimens show high sensitivity to copper and can be a potential vertebrate bio-indicator organism for environmental monitoring in this region of Brazil (Mazon & Fernandes, 1999). Gill and kidneys accumulate high amounts of copper during acute exposure, and preliminary morphological examination of these organs detected pathological changes, even at low concentration of copper in water (Mazon, 1997; Mazon *et al*., 2002), suggesting possible respiratory and ion-osmoregulatory impairment. Thus, the purpose of this study was to determine the hematological and physiological changes of the blood of *P. scrofa*, as well as to examine gill tissue after exposure to different copper concentrations in water in order to evaluate the homeostatic status of the fish and possible adaptive responses to environmental copper exposure.

MATERIAL AND METHODS

Animals

Juvenile *Prochilodus scrofa,* Steindachner 1881, weighing 15-25 g were obtained from the Hydrobiology and Aquaculture Station of Furnas Hydroelectric Power Plant, Furnas, MG, Brazil. Following their transfer to the Zoophysiology and Comparative Biochemistry Laboratory, Federal University of São Carlos, São Carlos, SP, the fish were maintained at 25 ± 1°C in tanks (1,000 L) with continuously aerated and flowing dechlorinated tap water (pH 7.0 ± 0.22, hardness 24.5 ± 0.3 mg L^{-1} as $CaCO_3$; alkalinity 23.7 ± 1.9 mg L^{-1} as $CaCO_3$) at least one month prior to the experiments. Fish were fed *ad libitum* with balanced fish food for this species provided by the Aquaculture Research and Training Center CEPTA/IBAMA. Feeding was suspended 24 h before experiments. The laboratory photoperiod was 12D:12L.

Experiment protocol

Groups of 10 fish were exposed (96 h) to 20 $\mu gCu\ L^{-1}$ (copper limit for the protection of aquatic life), 29 $\mu gCu\ L^{-1}$, LC_{50} of copper calculated for this species (Mazon & Fernandes, 1999) and an

intermediate copper concentration (25 μgCu L^{-1}) in a 200 L glass aquarium, not exceeding 1 g fish.L^{-1} (with replicate), using a static test system. Each aquarium was continuously aerated (water PO_2 > 130 mmHg) and the same physical and chemical characteristics of the water as those in laboratory acclimation were maintained. The copper agent was $CuSO_4.5H_2O$ and its concentration in the water was measured using an atomic absorption spectrophotometer. Control fish were maintained under the same conditions in water devoid of copper detectable. Dead fish were removed from the aquarium. After 96 h, 10 control fish and 10 fish from each copper concentration exposure were randomly sampled, anaesthetized with 0.01% benzocaine (ethyl *p*-aminobenzoate), and in less than 1 minute their blood was withdrawn, from the caudal vein into heparinized plastic tubes. Sub-samples were used for hematological and ion analyses. The gills of each fish were rapidly excised and fixed for histological processing.

Blood analysis

Analyses of blood pH, hematocrit (Hct), red blood cell count (RBC), and hemoglobin concentration [Hb] were conducted immediately. The pH was measured using a Micronal B375 pHmeter (São Paulo, Brazil) and the electrode was adjusted with high precision buffer. Hct was determined by spinning the blood sample contained in heparinized capillary tubes in a microhematocrit centrifuge. The RBC count was carried out in a modified Neubauer chamber after saline (0.9% NaCl solution) dilution of the blood and the [Hb] was determined by the cyanomethaemoglobin method. The blood indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were then calculated using the blood measurements above. Blood smears were fixed with methanol and stained with Leishman solution for immature red blood cell counts, and thrombocytes and leukocytes by 5,000 cell count according to the method described by McKnight (1966). To prevent errors arising from uneven distribution of cells, the slides were divided into four segments and cells were counted in fields in a parallel row commencing from the outside edge of the slide to the inside. Differential leukocyte counts were made by identifying 200 leukocytes in each slide (Dick & Dixon, 1985). The leukocytes were classified according to their general form and affinity to the dye (Takashima & Hibiya, 1995).

Plasma samples were obtained by blood centrifugation and cooled at –20ºC until ion analyses were done. Plasma sodium [Na^+] and potassium [K^+] concentrations were determined using a ZEISS M4Q2 flamephotometer and the plasma chloride concentration [Cl^-] was determined by the thiocyanate method using a commercial kit (SIGMA 461).

Gill morphology analysis

To assess the effects of copper on gill morphology, 20 random samples contained 5-7 filament pairs from the gill arches of the right side of each fish were fixed in 1% glutaraldehyde and 4% paraformaldehyde buffered to pH 7.3 with 0.1 M phosphate buffer and processed for light microscopy. Gill samples were dehydrated in graded ethanol solutions and embedded in historesin (LEICA). Sagittal sections were stained with toluidine blue which permits colored localization of mucous (pink), chloride cells (light blue), pavement cells, and nucleus (dark blue). Gill tissue and cell morphology were analyzed under an Olympus-Micronal CBA-K photomicroscope. In brief, every 10 sections were used and, at least 5 fields from each section were selected at random for analysis of histopathological changes.

Statistical analysis

The analysis of variance (ANOVA) was used to determine the significance of the data and the Tukey test with 95% confidence limit was applied to compare the means whenever the data were significant. All the tests were done using the software program InStat for Windows (GraphPads Software, San Diego, CA).

RESULTS

Hematological parameters

The *P. scrofa* exposed to 29 μgCu L^{-1} (LC_{50}) over 96 h showed a significant increase in Hct (26%) and RBC (50%), associated with a 23% increase in whole blood [Hb] (Fig. 1). Exposure to 25 μgCu L^{-1} also resulted in a significant

increase in Hct and RBC ($p < 0.05$) but no change was found in [Hb]. Lower copper concentration (20 µgCu L^{-1}) showed only a slight change of blood parameters that were maintained within the control range (Fig. 1). With increasing copper concentration in water, MCH tended to decrease but the change was not significant (Fig. 1). Circulating immature as opposed to mature red blood cells were very low (0.47 ± 0.02%) in control *P. scrofa* and did not increase in fish exposed to copper.

Total leukocyte number tended to increase in fish exposed to copper and was significantly higher following exposure to 29 µgCu L^{-1} (24.65 ± 0.23 10^3 mm^3 for controls and 28.93 ± 0.10, 31.63 ± 0.11, and 53.99 ± 0.16 10^3 mm^3 for fish exposed to 20, 25, and 29 µgCu L^{-1}, respectively). Differential leukocyte counts (Fig. 2) showed that lymphocytes were the most frequent white blood cells in control *P. scrofa* (75 ± 5%), and the proportion of these cells was reduced to 62% and 66% respectively in fish exposed to 25 and 29 µgCu L^{-1}. The percentage of neutrophils was low compared to that of monocytes. After copper exposure, the monocyte percentage showed a slight increase but resulted in a nonsignificant change, while neutrophils increased significantly in fish exposed to 25 and 29 µgCu L^{-1}. Basophils were not found in the prepared smears and eosinophils were very rare (less than 0.33%).

Thrombocytes were easily identified and did not show significant change following copper exposure. The mean thrombocytes number was 45.94 ± 2.18 10^3 mm^3.

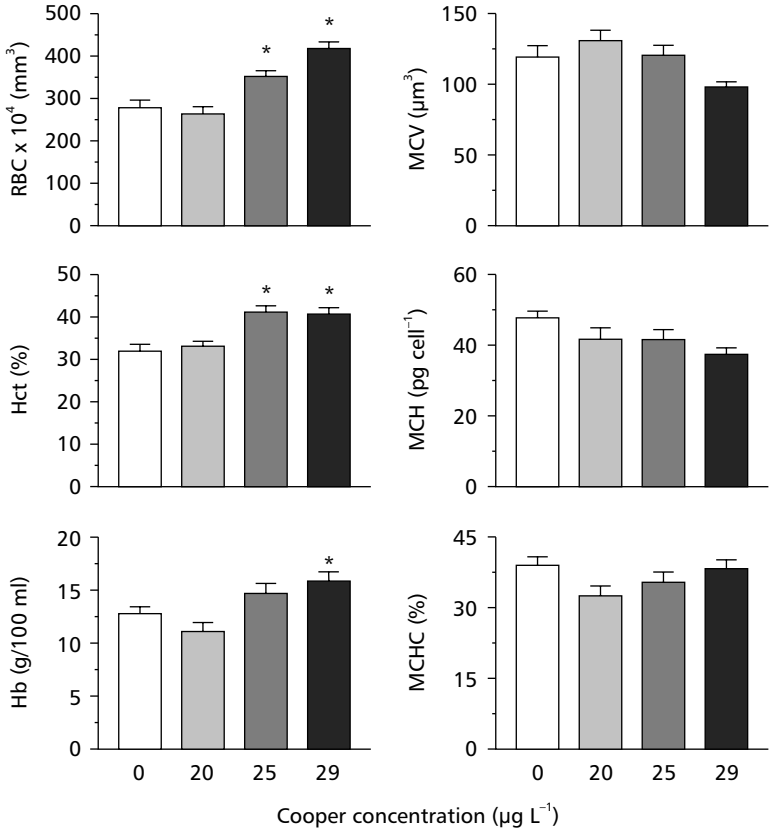

Fig. 1 — Changes in hematocrit (Hct), red blood cells (RBC), whole blood hemoglobin concentration [Hb], mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin content (MCHC) of *P. scrofa* (n = 10) after acute exposure to different copper concentrations. Points are means ± SEM. * Indicates significant difference ($p < 0.05$) from controls.

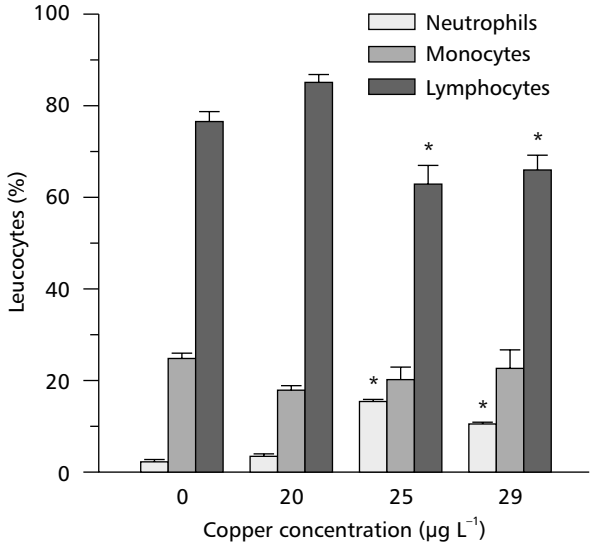

Fig. 2 — Changes in the percentage of differential leukocyte counts of *P. scrofa* blood after exposure to different copper concentrations. Points are means ± SEM. * Indicates significant difference ($p < 0.05$) from controls.

Physiological parameters

Copper exposure induced ionoregulatory disturbances in *P. scrofa*. Plasma [Na^+] and [Cl^-] decreased significantly ($p < 0.05$) in fish exposed to lethal and sublethal copper concentration (Fig. 3). The reduction in plasma [Cl^-] was 44% higher than the corresponding fall in plasma [Na^+] and the Na/Cl ratio consequently increased significantly in fish exposed to 25 and 29 µgCu L^{-1} ($p < 0.05$). Plasma [K^+] increased with increasing copper in the water, reaching significant values at 29 µgCu L^{-1} exposure ($p < 0.05$) (Fig. 3). The percentage of increased plasma [K^+] was similar to the percentage of plasma [Na^+] lost (approximately 13%). Blood pH decreased significantly ($p < 0.05$) in fish exposed to 25 and 29 µgCu L^{-1}.

Gill histophology

When compared with gills of control *P. scrofa* (Fig. 4A), several distinct histopathologies were observed in fish exposed to copper on both the epithelia and blood vessels, even at the low copper concentrations in water recommended by the US EPA (1984). The exposure to copper induced intense proliferation of pavement cells and hypertrophy of both pavement and chloride cells (Fig. 4A-E). Filament epithelium height of fish exposed to copper was higher than that of control fish (3-5 cell layers, 0.0134 to 0.0280 mm in height), usually consisting of 5-15 hypertrophied cells layer (0.0284 to 0.0408 mm in height) and evidenced a dose-dependent response increasing with copper concentration in the water (Fig. 4B-D). Cell proliferation resulted in incomplete fusion of several lamellae in 28%, 46%, and 58% of examined samples of fish exposed to 20, 25, and 29 µgCu L^{-1} (LC_{50}) respectively, and in complete lamellar fusion (Fig. 4E) in 1%-4% of samples of fish exposed to 20 and 25 µgCu L^{-1}, reaching 27% in fish exposed to 29 µgCu L^{-1}. Detachment of lamellar epithelium and necrosis were common and increased with increasing copper in the water.

In addition to cell changes in the filament and lamellar epithelium, several histopathologies in the vascular system were identified in the gills (Fig. 5). Erythrocyte congestion was common in the marginal channel (telangiectasis) (Fig. 5A) in fish exposed to 20, 25, and 29 µgCu L^{-1}. Erythrocyte congestion throughout the entire lamella (aneurysm) (Fig. 5B) was usually observed in fish exposed to 25 and 29 µgCu L^{-1}, and rupture of the lamellar epithelium and the pillar cell system indicating hemorrhage foci was mainly observed in fish exposed to 29 µgCu L^{-1} (Fig. 5C).

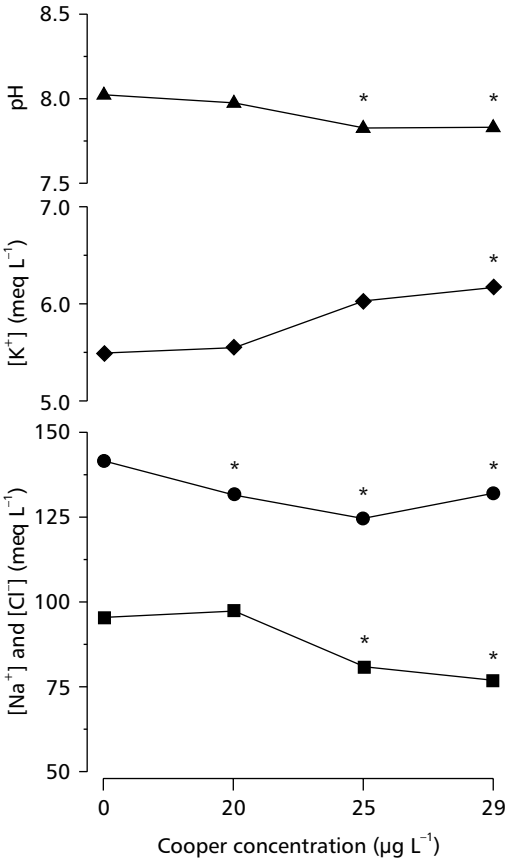

Fig. 3 — Blood pH (▲) and plasma ions Na^+ (●), K^+ (◆), Cl^- (■) of *P. scrofa* after exposure to different copper concentrations. Points are means ± SEM. * Indicates significant difference ($p < 0.05$) from controls.

DISCUSSION

Heavy metal exposure is known to induce changes in blood parameters in fish (see review Heath, 1995). The direct effects of copper on blood parameters are usually associated with increased erythrocytes disintegration or, in the case of more sensitive species, damage of the hemopoietic system (Svobodová *et al*., 1994). In *P. scrofa* the increase of Hct, RBC, and [Hb] may indicate a compensatory response of this species to increase the blood's O_2 carrying capacity. The changes in gill epithelia of *P. scrofa* caused by copper, such as cell hypertrophy, cell proliferation, and epithelial lifting may represent a defense response, as pointed out by Mallatt (1985), because these changes increase the distance across which copper must diffuse to reach the bloodstream. However, they also increase the water-blood distance for O_2 diffusion whereas lamellar fusion reduces the respiratory area. Although *P. scrofa* has a large respiratory surface (Mazon *et al.*, 1998, 2002) the changes observed in its gill tissue probably impair branchial gas transfer, generating an internal hypoxia which may stimulate erythrocyte release stored in organs into the blood circulation by adrenergic stimulation (Pilgaard *et al*., 1994). Furthermore, since copper is required for hemoglobin synthesis, a mild excess may stimulate erythrocyte formation or release from hemopoietic tissue (Heath, 1995). The increase in Hct alone is usually related to a stress response causing red blood cell swelling (Soivio & Nikinmaa, 1981) or hemoconcentration due to plasmatic volume reduction (Wilson & Taylor, 1993).

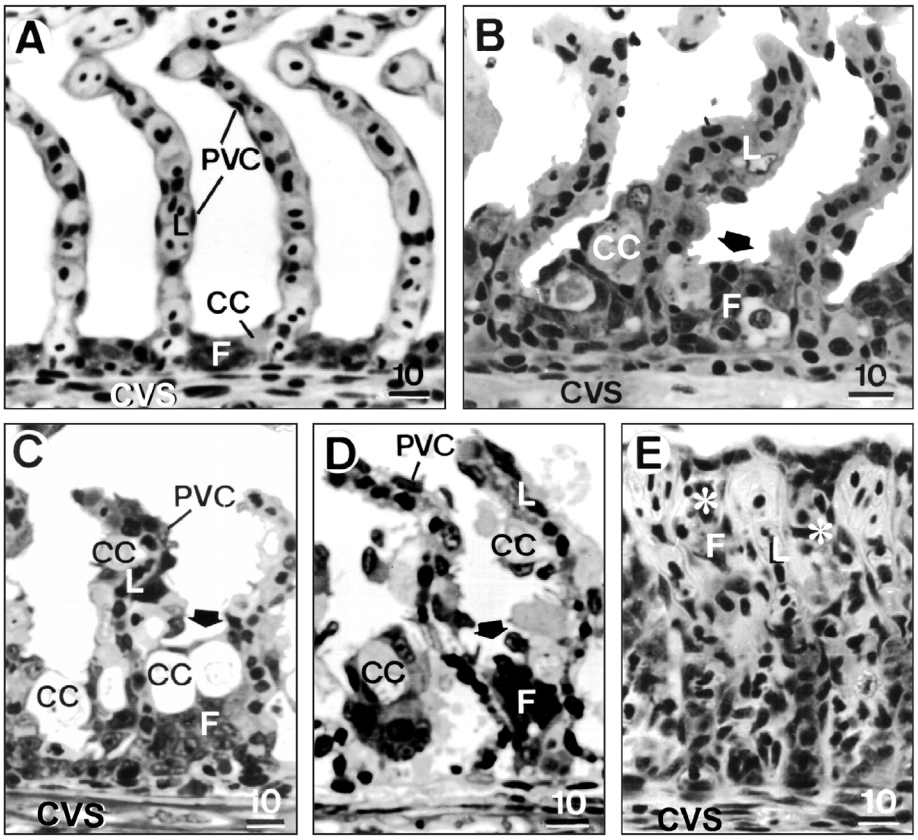

Fig. 4 — Representative sections of gill filament of *P. scrofa.* **A** – Control fish. **B** – Fish exposed to 20 µgCu L^{-1}. Note filament epithelium (F) hypertrophy (arrow). **C** – Fish exposed to 25 µgCu L^{-1}. Note lamellar epithelium and pavement (PVC) and chloride (CC) cell hypertrophy. **D** and **E** – Fish exposed to 29 µgCu L^{-1}. Note CC hypertrophy (arrows) in D and total fusion of several lamellae (*) due to intense cell proliferation. CC chloride cell, CVS central venous sinus, L lamella, PVC pavement cell. Scale bar in µm.

Nevertheless, the increase in Hct coupled with the increase in the RBC and [Hb], with no significant changes in the MCH and MCHC blood indices, cell size, and circulating immature red blood cells suggest the body's attempts to absorb more oxygen from the external environment so as to supply the oxygen requirement of tissue.

Leukocytopenia, an overall reduction in leukocytes, has been demonstrated in teleosts exposed to copper (Mishra & Srivastava, 1980; Dick & Dixon, 1985; Svobodová *et al*., 1994) and other heavy metals (Srivastava & Agrawal, 1979; Mishra & Srivastava, 1980; Gill & Pant, 1987). Leukocytopenia is a nonspecific response to a variety of stressors mediated by corticosteroid hormones (Ellis, 1981) and cannot be considered a specific cytotoxic action of copper (Dick & Dixon, 1985). However, the leukocytosis reported in *O. mossambicus* after acute exposure to copper (Nussey *et al*., 1995a, b) and found in *P.* scrofa in the present study may be attributed to increased leukocyte mobilization to protect the body against infections in copper-damaged tissue.

Among the leukocytes, the lymphocytes were the most frequent cells in *P. scrofa* and other teleosts (Takashima & Hibiya, 1995). However, exposure to copper caused a reduction in lymphocyte percentage such as that found in some other species (Mishra & Srivastava, 1980; Dick & Dixon, 1985; Svobodová *et al*., 1994).

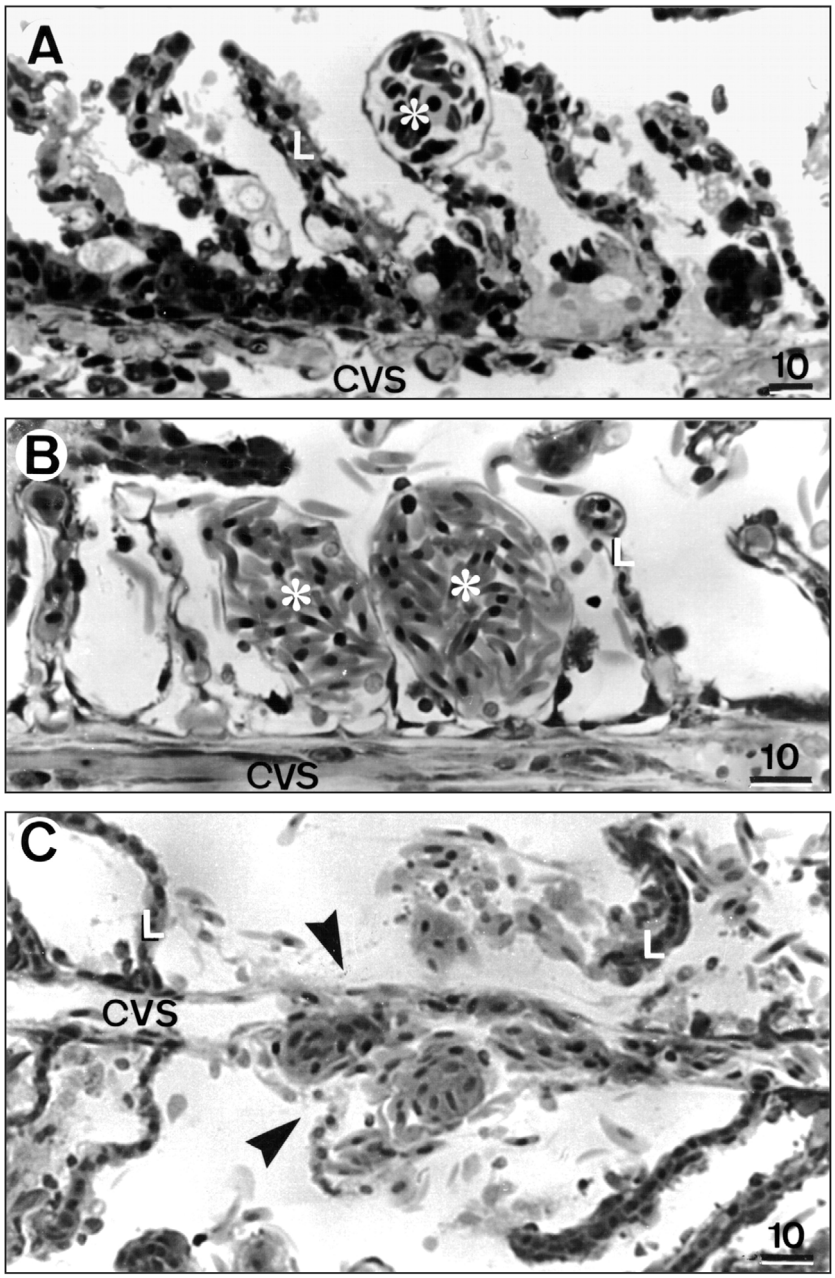

Fig. 5 — Representative blood vessel anomalies of the gill of *P. scrofa* exposed to copper (25 and 29 µgCu L^{-1}). **A** – Telangiectasis (*). **B** – Stasis throughout the entire lamella (*) **C** – Hemorrhage foci (arrowhead). Note the rupture of lamellar epithelium and pillar cell system. CVS central venous sinus, L lamella. Scale bar in µm.

Neutrophils and monocytes are important white blood cells to protect the body, through their elevated phagocytic activity, against bacterial infection in damaged tissue. The percentage of these cell types generally decreases during acute exposure to copper (Nussey *et al*., 1995b; Svobodová *et al*., 1994), and in situations of chronic copper exposure, the neutrophil percentage has been reported to increase (Dick & Dixon, 1985). In *P. scrofa*, the monocyte percentage did not change following acute copper exposure but neutrophil percentage increased significantly which may also be related to gill tissue damage. This would reflect such direct deleterious effects of copper on gill epithelia as cell degeneration and the intense rupture and peeling of lamellar epithelial after 96 h exposure to 25 and 29 μgCu L^{-1}. Basophils and eosinophils were not found in *P. scrofa* although they have been identified in some fish species (Takashima & Hibiya, 1995). In *O. mossambicus* increased counts of eosinophils were found during copper exposure (Nussey *et al*., 1995b).

Thrombocytes are comparable to mammal blood platelets and play an important role in the blood clotting which prevents blood loss from hemorrhaging. A high number of thrombocytes reduces clotting time (Srivastava, 1969), by as much as 50% in cases where the number of circulating thrombocytes was found to be 1 to 2 times higher than normal (Cassilas & Smith, 1977). Some species exposed to copper displayed a high increase in the thrombocyte percentage (Mishra & Srivastava, 1980; Dick & Dixon, 1985) although, the thrombocytes increase did not entail clotting time reduction in fish exposed to copper due to thrombocyte malfunction (Nussey *et al*., 1995c). In *P. scrofa*, the thrombocytes did not change following copper exposure, although several hemorrhage foci identified as vessel ruptures in the lamellae were found in gills of fish exposed to 29 μgCu L^{-1}.

Copper causes serious ion imbalance in *P. scrofa*. Chloride cell hypertrophy on the gills of *P. scrofa* exposed to copper appear to result from a failure to compensate for ion losses. Copper concentrations as low as 20 μg L^{-1} decreased the concentration of sodium in the plasma and at a concentration of 29 μg L^{-1} (96 h LC_{50} (Mazon & Fernandes, 1999)), plasma sodium and chloride were significantly lower than the control, with a concomitant increase in plasmatic potassium concentration. Previous investigations have also found ion imbalance in *Salvelinius fontinalis* (McKim *et al*., 1970), *Ictalurus nebulosus* (Christensen *et al.*, 1972), *Lepomis macrochirus* (Heath, 1991), and *O. mossambicus* (Nussey *et al*., 1995a; Pelgrom *et al.*, 1995). Ionregulatory disruption induced by copper is related to inhibition of branchial Na^+-K^+-ATPase and ion uptake with concomitant stimulation of ion efflux in freshwater fish (Laurén & McDonald, 1985; Pelgrom *et al.*, 1995). The increased membrane permeability favoring ion efflux may be due to disruption of the membrane integrity of gill cells (Stagg & Shuttleworth, 1982; McDonald & Wood, 1993). In general, plasma ions increased in marine teleosts (Stagg & Shuttleworth, 1982) and decreased in freshwater fish (Christensen *et al.*, 1972; McKim *et al*., 1970). The imbalance of Na/Cl ratio may also reflect disturbed acid-base regulation (McDonald & Wood, 1993).

The reason for K^+ increases in plasma (K^+/Na^+ ratio from 0.03 in controls to 0.05 in fish exposed to 29 μg L^{-1}) may be due to increased cell membrane permeability, as pointed out by Laurén & McDonald (1985), allowing the intracellular K^+ to diffuse passively to extracellular fluid (Perry & Laurent, 1993). The significant blood acidosis found in *P. scrofa* has also been reported in *O. mykiss* (Wilson & Taylor, 1993) and *O. mossambicus* (Pelgrom *et al.*, 1995). It is generally related to increase in lactic acid or other acid production as a result of metabolism increase. In *P. scrofa* the acid-base imbalance may be related to decreased H^+ excretion due to gill cell damage or other acid production since no change in blood lactate was reported by Mazon *et al*. (2000) in this species exposed to 29 μgCu L^{-1} for 96 h in the same water conditions and temperature as those of the present study. Blood lactate increase was found only during the first 24 h- copper exposure and returned to control value in 48 hours.

In conclusion, the blood parameters of *P. scrofa* exposed to copper revealed ionoregulatory disturbances but also compensatory responses to allow fish to survive. Ionoregulatory disturbances may be related to the direct effect of copper on the gill tissue and probably on the permeability of cell membranes. The leukocyte increase implies a mobilization of cell defenses although the reduction of the lymphocyte percentage in the differential counts suggests a secondary effect of copper. On

the other hand, the changes in red blood cell parameters suggest a compensatory response to the disruption of structural integrity of gills with consequent reduction of respiratory surface, in order to increase O_2-carrying capacity and maintain the level of oxygen transference from water to tissues, allowing fish to survive during the nominated shock phase of LC_{50} exposure, at least under restful conditions. However, the blood changes found in the present study may be more drastic for mature *P. scrofa,* particularly during upstream migration for breeding which requires more energy expenditure.

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