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Recovery of fat snook, *Centropomus parallelus* (Teleostei: Perciformes) after subchronic exposure to copper

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ABSTRACT

We studied the recovery of juvenile fat snook (*Centropomus parallelus*) after subchronic exposure to different concentrations of copper. Healthy juveniles (1.98 g) were exposed to 25 or 50 μg Cu/L for 30 days (12 replicates with 5 fish in each one), and recovery was observed at 0, 4, 10, and 30 days after exposure (3 replicates with 5 fish in each one). Copper genotoxicity in exposed individuals was observed using a micronucleus assay, and recovery was not observed even 30 days post-exposure. Copper accumulation was observed in fish exposed to 25 or 50 μg/L of copper in the gills (14.4 and 34.4 μg/g, respectively) and muscle (5.7 and 5.5 μg/g, respectively), and a return to normal copper levels (6.0 μg/g for gills and 2.5 μg/g for muscle) was observed 4 and 30 days post-exposure in the gills and muscle tissues, respectively. Glutathione S-transferase (GST) was 80% inhibited in individuals exposed to copper and returned to normal levels for fish exposed to basal concentrations within 10 days. Although copper accumulation in tissues dispersed 30 days post-exposure, no recovery from genotoxicity was observed during this time. Thirty days was not enough to recover juvenile fat snook following subchronic exposure to copper.

1. Introduction

Pollution from metals such as copper is a significant issue in wildlife conservation, mainly due to the accumulation and persistence of the metals in the environment. Copper has several functions in the body including a role in metabolism, in which it is required in small concentrations (Oliveira et al., 2008; Yadav and Trivedi, 2009). However, copper is toxic at high concentrations and causes severe damage to many organisms, including fish (Liu et al., 2010). Among other deleterious effects, copper induces oxidative stress, promotes the formation of species highly reactive to oxygen, and affects lipid membranes and DNA integrity (Carvalho and Fernandes, 2008).

Many efforts have been made to restore bodies of water polluted by copper to their original states (Buck et al., 2007) with the aim of improving conditions for biota in the area. Recovery following copper toxicity begins with increased biosynthesis (mitosis and increased protein synthesis) that helps to repair biological damage and physiological disorders (McGeer et al., 2000). The metals are mobilized by proteins such as metallothionein and other processes that neutralize or compete with the deleterious effects of metal, for example, those related to ion regulation (McGeer et al., 2000) are up-regulated. Subsequently, the internal physiology of the exposed organisms may either return to pre-exposure conditions or establish new equilibria due to increased tolerance (McGeer et al., 2000).

The fat snook, *Centropomus parallelus*, is a valuable and commonly consumed (Tsuzuki et al., 2007) fish species native to the south-central Atlantic Ocean (Borges et al., 2010). It is an estuarine species that is found in both marine and freshwater environments at different stages of its life cycle (Tsuzuki et al., 2007). This species occurs in highly urbanized and industrialized areas, such as in the estuarine cities of northeast and southeast Brazil, and has been subjected to copper accidents, without information concerning their recovery ability after copper exposure. Therefore, the aim of this study was to investigate the recovery of juvenile fat snook (*Centropomus parallelus*) after subchronic exposure to different copper concentrations in freshwater.

2. Materials and methods

C. parallelus juveniles (1.98 ± 0.58 g in mass and 5.96 ± 0.64 cm in total length) were acquired from a private hatchery. In the hatchery, in brackish water (salinity — 20–30‰), fish larvae were reared up to approximately 2 g (starting juvenile phase). At this point, the water was manipulated to decrease the salinity down to 0‰ after four days. Fish were kept in this condition for 10 days prior to

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transportation to the laboratory. In the laboratory fish were acclimated for 15 days in 500-L indoor tanks with freshwater and continuous aeration at a density of 0.7 g/L. They were fed with commercial ration (INVE NRD®, 1.2 mm, 59% crude protein) twice daily.

To investigate the effects of subchronic exposure, fish (end of larvae phase and start of juvenile phase), were exposed to copper for 30 days. Fish ($n = 5$ for each replicate) were transferred to 36 tanks with 25-L capacity in fresh water with constant aeration (fish density = 0.4 g/L). During exposure, fish were fed twice daily (INVE NRD®, 1.2 mm, 59% crude protein). The photoperiod was controlled (14 h light and 10 h dark), and 80% of the water was renewed every 2 days, adding drinking water with adjusted concentration of copper for each respective treatment. To reach the desired concentrations copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, Sigma®), was diluted in the water. Fat snook were exposed to 0 (control group, without copper addition), 25 or 50 μg Cu/L. A total of 12 replications (tanks) per treatment were used.

Fish mortality was observed, and dead fish were removed every 24 h. After 30 days of exposure, fish from three tanks of each group were euthanized with 1% benzocaine, and their blood was taken from caudal veins with heparinized syringes. Next, samples were collected from muscle (~200 mg), gills, and liver.

The remaining nine tanks in each group were divided into nine recovery groups, resulting in three replications (tanks) of each treatment (0, 25 or 50 μg Cu/L) followed by recovery periods of 4, 10, and 30 days. The recovery groups were placed in tanks and maintained under conditions similar to those during the exposure period but without the addition of copper.

After their respective recovery periods, the fish were euthanized, and their blood and tissues removed, as described above. Observation of a recovery period was not possible for the group exposed to 50 μg Cu/L, given its near-total mortality rate.

The collected blood was used to quantify micronuclei levels. The gill and muscle samples were used to verify metal accumulation and quantify liver glutathione S-transferase (GST; EC 2.5.1.18) levels.

Micronuclei were counted using a smear of blood on a microscope slide, were fixed with methanol for 30 min, and were then stained with 5% Giemsa for 40 min (Grisolia et al., 2005). The slides (2 per fish) were then examined under an optical microscope, 1000 blood cells (erythrocytes) were counted from each fish to quantify the micronuclei present.

To measure copper accumulation in gill and white muscle, tissues (~100 mg) were washed with deionized water, weighed, and digested with 10% nitric acid in an oven at 80 °C for 24 h. The total copper in the samples was measured using atomic absorption spectrophotometry and a graphite furnace (Perkin Elmer AAnalyst 800). The results are expressed as μg/g wet mass. The amounts of copper in the exposure water was determined weekly from each replicate with water filtered with a 0.45-μm Millipore Millex filter. The copper from the water of each replicate of recovery group was measured in the start and in the end of the recovery period.

Glutathione S-transferase activity levels in liver tissue were measured using the technique described by Habig et al. (1974) and Habig and Jakoby (1981), which employs a 50 mM phosphate buffer containing 1 mM GSH (reduced glutathione) at pH 7.2 and 25 °C. The reactions were initiated by the addition of 1 mM CDNB (1-chloro-2,4-dinitrobenzene). The absorbance was measured at 340 nm. Absolute activity was estimated using the CDNB extinction coefficient.

During the exposure, water quality parameters were measured every 7 days, and during the recovery period the water from each replicate was measured in the start (after fish addition in the tank) and in the end of the trial. Dissolved oxygen, temperature, and conductivity were measured using the YSI 85 Multiparameter meter, and pH was measured using a digital pH meter (Quimis Q400). The hardness and total ammonia were measured according to APHA (1998).

The significance of the mean differences was determined using analysis of variance (ANOVA) and Tukey's test ($p<0.05$), comparing both the different concentrations of copper and the various recovery times. The SYSTAT 12 software package was used for the statistical analyses.

3. Results

The water used in the trials was similar among treatments during exposure and recovery. Water quality parameters were: pH 7.2 ± 0.1, temperature 22.7 ± 0.2 °C, conductivity 69.2 ± 6.7 μs/cm, and oxygen concentration 7.9 ± 0.1 mg/L. The measured hardness was 24.4 ± 0.6 $mgCaCO_3$/L, and the total ammonia concentration was 0.3 ± 0.2 mg/L. The baseline concentration of copper in the water was 12.7 ± 2.7 μg/L. For the 25 μg Cu/L group, the final copper concentration in the water was 37.2 ± 1.7 μg/L. For 50 μg Cu/L group, the final copper concentration was 63.2 ± 3.8 μg/L. The water used for recovery contained 13.3 ± 3.6 μg/L of copper. These data indicate that copper was already present in the water used in the experiments, which came from the water company of Vitoria—ES, prior to the addition made in the experimental tanks.

In the group exposed to 50 μg Cu/L, mortality was observed after 9 days of exposure, and 91.6% of the fish were dead after 30 days (Fig. 1). In the 25 μg Cu/L group, mortality was observed after 28 or 29 days of exposure, and 3.3% of fish died after 30 days (Fig. 1). No mortality was observed in the control group.

Significant differences were observed in the micronucleus counts of the various treatments (Fig. 2). Immediately after 30 days of exposure (i.e., 0 days of recovery), the micronucleus frequency was 1.09 ± 0.83 in the fish from the control group, while in the fish from the 25 and 50 μg Cu/L groups, the observed micronucleus frequencies were 3.8 ± 2.1 and 10.0 ± 5.6, respectively. The micronucleus frequencies did not show significant variation within the treatment groups at different recovery times and remained significantly higher in fish from the 25 μg Cu/L group.

Immediately after 30 days of exposure, the copper concentrations in the gills of the fish from the 50 μg Cu/L group were significantly higher than those in the other two groups (Fig. 3A). After 30 days of recovery, there was a significant decrease in the copper concentrations in the gills of the fish from the 25 μg Cu/L group (Fig. 3A). The copper concentrations in the gills of the control group did not vary significantly over time.

At day 0 of recovery, the muscle tissues from fish exposed to 25 or 50 μg Cu/L had significantly higher amounts of copper than the control group. However, after four days recovering, no significant difference remained between the concentrations observed in fish from the 25 μg Cu/L group and the control group (Fig. 3B). The copper concentrations in the muscle tissues of the control group did not vary significantly over time.

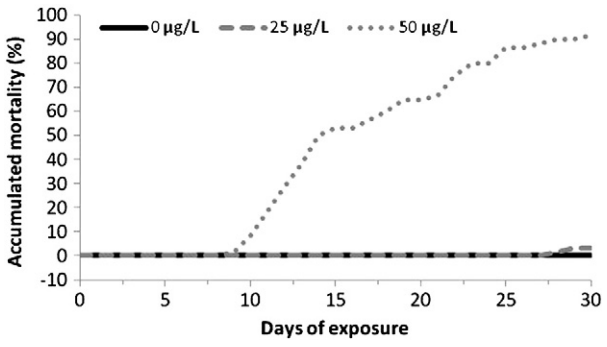

Fig. 1. Accumulated mortality of fish exposed to various concentrations of copper for 30 days.

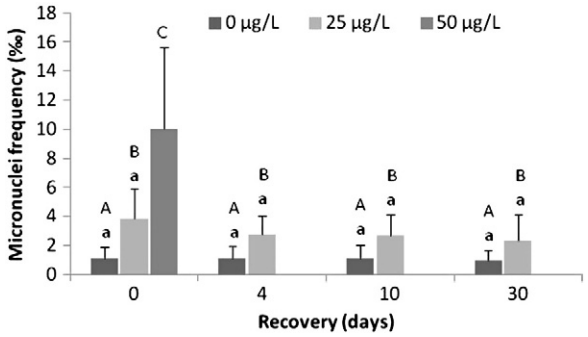

Fig. 2. Micronuclei frequency in fish exposed to copper for 30 days at various recovery intervals (0, 4, 10, and 30 days). Capital letters indicate significant differences among or between groups at a given recovery time ($p<0.05$). Lowercase letters indicate significant differences in a given group at various recovery times ($p\leq0.05$).

There was significantly greater GST activity in fish from control group than in fish from the 25 and 50 µg Cu/L groups (Fig. 4) at day 0 of recovery. After 10 and 30 days of recovery, the GST levels in fish from the 25 µg Cu/L group did not differ significantly from those of the control group (Fig. 4).

4. Discussion

Durrieu et al. (2005) observed that fish species with long residence periods in the Gironde estuary (France) were more contaminated with copper than fish with short residence time. The main reason for this result is the high concentration of this metal in the estuary. Copper was carried to the estuary from both marine and freshwater environments. The mortality of long time residence estuarine juvenile fat snook exposed to 50 µg Cu/L indicates the vulnerability of this species to this metal. Mortality began 9 days after exposure,

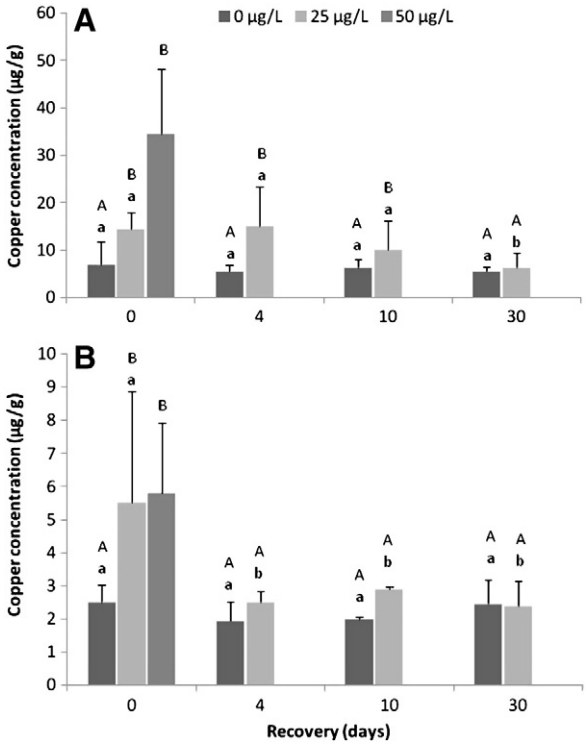

Fig. 3. Copper concentrations in the gills (A) and muscle (B) of fish exposed to copper for 30 days at various recovery intervals (0, 4, 10, and 30 days). Capital letters indicate significant differences among or between groups at a given recovery time ($p<0.05$). Lowercase letters indicate significant differences in a given group at various recovery times ($p\leq0.05$).

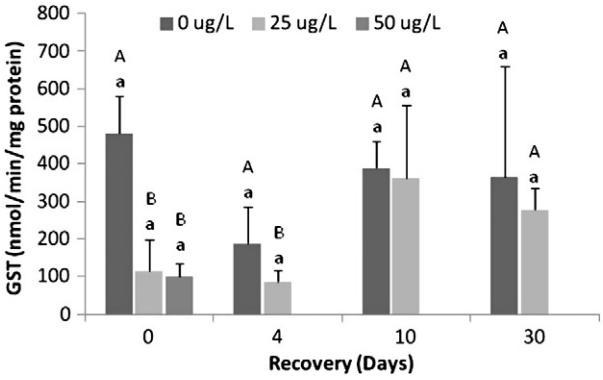

Fig. 4. Glutathione S-transferase activity in fish exposed to copper for 30 days at various recovery intervals (0, 4, 10, and 30 days). Capital letters indicate significant differences among or between groups at a given recovery time ($p<0.05$). Lowercase letters indicate significant differences in a given group at various recovery times ($p\leq0.05$).

indicating the inability of the organism to tolerate the exposure concentrations of metal. Importantly, the mortality rate did not stabilize or fall, suggesting that this fish at the starting juvenile phase was not able to adapt to these concentrations. In the present work, this lack of adaptation led to total lethality after a longer exposure period. As suggested by Handy (2003) this mortality may not be a simple matter of copper toxicity to a specific organ, it may rather involve a series of physiological effects that impair long-term survival due to some endocrine disruption. Therefore, subchronic exposure to 50 µg Cu/L may have severe consequences for fat snook populations.

Studies have shown that the fish micronucleus test is a valid technique for monitoring genotoxic and mutagenic effects induced by heavy metals, including copper (Bombail et al., 2001; Çavaş and Konen, 2008). Genotoxic effects were observed for the tested copper concentrations, as the groups exposed to copper had higher micronuclei levels than the control group. It was observed that the addition of 50 µg Cu/L produced a higher rate of erythrocytic nuclear abnormalities in fish. The presence of cells with this abnormality is a reflection of structural failures and/or numerical chromosomal aberrations that arise during mitosis (Çavas et al., 2005). Oliveira et al. (2008) found similar results in their study with *Anguilla anguilla* L. exposed to 27 µg Cu/L for 7 days, as a significant increase in erythrocytic nuclear abnormalities was observed. The same was observed in studies of *Cyprinus carpio*, *Carassius gibelio*, and *Corydoras paleatus* exposed to copper (10–250 µg Cu/L) for 21 days, in which individuals exposed to the highest concentrations of copper showed significant increases in micronucleus frequencies, furthermore, copper genotoxicity was observed in these species. *Channa punctata*, exposed to 100 µg Cu/L for 7 days, showed increased micronucleus frequencies before the end of the treatment (after 1, 2, 3, and 4 days), with the number of micronuclei rising in proportion to the exposure time (Yadav and Trivedi, 2009).

Micronucleus frequencies did not decrease after 30 days of recovery, suggesting that fat snook, even in copper-free water, do not recover after 30 days. This prospect of long-term damage makes the protection of aquatic environments even more crucial. According to Campana et al. (1999), variations in micronucleus frequency are related to the kinetics of blood cell replacement. Therefore, a recovery period may not be sufficient for blood cell replacement, making it impossible to eliminate cells with abnormalities.

The accumulation of copper in the gills of fish exposed to the higher concentrations of copper is similar to the results reported by Grosell et al. (2004), who found a linear and continuous increase of copper in the gill tissue of *Opsanus beta* exposed to copper for 30 days. After 4 and 10 days of recovery, the levels of copper accumulation in the gills were the same as those observed immediately after exposure to 25 µg Cu/L, indicating that these tissues do not undergo

recovery during this period. Recovery was, however, observed 30 days after the exposition had ended.

Muscle tissue recovery differed from that of the gills. Individuals exposed to copper media showed increased metal concentrations in muscle tissue. However, the muscle-tissue copper levels decreased compared to those found in the control group just 4 days after exposure had ended.

Gill tissue is of greater importance for metal uptake (Gale et al., 2003). The gills tend to accumulate higher copper concentrations than muscle tissue due to their greater permeability (Grosell et al., 2004). The lower levels of metal bioaccumulation in muscle tissue may be related to the induction of metal-binding proteins such as metallothioneins (Uysal et al., 2008). While exposed to copper, however, the muscle tissues showed high levels of copper accumulation and presented a risk of biomagnification, which could increase copper levels in consumers. This possibility points to a potential risk to human health and represents another argument for increased efforts to reduce and control the release of copper in water bodies.

Even at low concentrations, it is possible that copper can be accumulated by fish and may generate medium- and long-term deleterious effects, not only in the directly exposed organisms but also throughout the entire food web. Papagiannis et al. (2004) found concentrations of 0.12 μg Cu/L at Pamvots Lake in Greece that resulted in copper bioaccumulation in *C. carpio*. Joyeux et al. (2004) observed the accumulation of several metals, including copper, in *Centropomus* sp. and *Mugil* sp. in the Vitoria Bay, Brazil.

Glutathione S-transferase plays an important role in both detoxification and the prevention of lipid peroxidation. Several types of action mechanisms and contradictory effects have been reported in the interactions of metals with this enzyme. Vieira et al. (2009) found an increase in the gill GST activity of another species of estuarine fish *Pomatoschistus microps* exposed to similar copper concentrations and suggested that this result is due to the fact that the gill is the first barrier against the entrance of toxicants in the fish body. Cunha et al. (2007) found significant reductions in GST activity in the gastropod *Nucella lapillus* exposed to copper. Similarly, Dautrememepuits et al. (2002) reported that GST was inhibited in *C. carpio* during acute exposure to copper concentrations of 100 and 250 μg Cu/L for 96 h. Similar inhibition of GST activity was observed in the present work. The accumulation of metal in cells can result in decreased levels of GSH, the substrate of GST, due to both binding and oxidation (Cunha et al., 2007). At 10 and 30 days post-exposure, recovery of the GST activity levels was observed, after this period, the GST activities of the exposed fish were similar to those of the control group.

In conclusion subchronic exposure to 50 μg/L of Cu was lethal to the species. The exposure to 25 μg/L of Cu can cause mortality, bioaccumulation of the metal, oxidative stress and was genotoxic to *C. parallelus*. Elevated numbers of micronuclei were maintained even 30 days post-exposure to 25 μg/L, demonstrating that the fish did not fully recover.

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