Gene Expression to Genetical Genomics

SHORT REPORT

Effects of Copper and Cadmium Exposure on mRNA Expression of Catalase, Glutamine Synthetase, Cytochrome P450 and Heat Shock Protein 70 in Tambaqui Fish (*Colossoma Macropomum*)

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Abstract: In this work the authors evaluated the expression of *cat*, *glu*, *cypP450, hsp70* and *18S* RNA in *Colossoma macropomum* following exposure to copper and cadmium by semi-quantitative RT-PCR and quantitative qRT-PCR. In RT-PCR and qRT-PCR *hsp70* expression was highest after exposure to copper for 3 hours (h). *glu* expression increased after exposure to cadmium, regardless of time. *cat* expression increased after the first hour of exposure to both metals, and decreased after 3 hs. *cyp450* expression was higher when compared to controls in exposed fish regardless of metal and exposure time. *cyp450* expression after exposure to copper for 3 h was twice that recorded after 1 h. In the presence of cadmium, *cyp450* expression was higher in the animals exposed for 1 h and lower at 3 h. Therefore, toxicology tests and biomonitoring based on molecular markers should focus on a set of molecular and/or environmental parameters, rather than isolated markers.

Keywords: copper and cadmium exposure, Amazon, *Colossoma macropomum*, antioxidants, *hsp70*

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Introduction

The Amazon basin has an average discharge of 134,119 m^3/second (s), corresponding to 73% of the discharge volume for the entire Brazilian territory.[1] It also has the largest diversity of fish in the world, with around 5,000 species.[2] The tambaqui (*Colossoma macropomum)*, a ubiquitous Amazonian fish, is often used as a model for the study of physiological processes in response to environmental changes in the Amazon.[3] Besides being present throughout the Amazon basin, the tambaqui is also an important species for use in biomonitoring environments affected by various xenobiotics.

The presence of xenobiotics in the environment probably causes alterations and a decline in certain species of organisms by altering the physicochemical characteristics of water, a parameter that is especially important in the complex Amazon biome. In the Amazon basin, aquatic environments are highly susceptible to metal contamination resulting from intense industrial and mining activities and deforestation, among other factors.

Metals such as copper (Cu) and cadmium (Cd) can have a severe impact on physiological processes in fish, because of the large gill area exposed to the environment.[4] For example, they can inhibit ion transport proteins such as Na^+K^+ATPase (Cu) and Ca^{2+}ATPase (Cd).[5,6] Although Cu, a naturally occurring trace element in many organisms, is essential for a number of biochemical processes,[7] significant amounts of waterborne Cu are toxic. Cu is found in the water mostly as a result of seepage or natural soil erosion, or discharge of from urban or industrial effluents.[8]

The exposure of organisms to sublethal Cu concentrations may trigger a series of cellular and physiological changes and compromise the survival of this species. In aquatic environments, Cu acts in vertebrates as an endocrine modulator; in fish, it induces an increase in the rate of oxygen consumption, reduces swimming speed, causes upregulation of ions, decreases lymphocyte levels, increases the number of neutrophils, alters enzymatic activity, and induces the proliferation of epithelial cells in the gills or intestine.[9]

Unlike Cu, Cd is considered a non-essential trace metal. The presence of Cd in the environment is the result of the use of fossil fuels, dumping of solid waste, manufacturing of iron, steel or non-ferrous metals, and mining. Like other metals, cadmium is stored in the liver, kidney and gills of fish.[10–12] Cd is potentially more toxic than Cu, and even low doses Cd can disrupt the behavior of fish.[13]

Technological advances and the frequent replacement of electronic equipment that uses nickel and cadmium batteries have contributed greatly to the contamination of aquatic environments. In Brazil, no measures have been taken to ensure the appropriate disposal of this highly toxic material. Manaus, capital of the state of Amazonas, has around 1.7 million inhabitants and a daily production of up to two thousand metric tons of waste. Waste is dumped in sanitary landfills without any previous treatment or separation. The region has one of the largest industrial parks in Brazil, with a wide range of economic activities (steelmaking, chemicals, electronics, food and drinks, and pharmaceutical industries). Oil extraction, refining and transport are also carried out in Manaus. The region has seen an increase in the amount of waterborne metals, which have directly and indirectly affected organisms. Although pollution biomarkers have been used to study contamination in other species,[14,15] their use in neotropical fish is still a challenge.

Therefore, the objective of the present study was to evaluate the expression of catalase (*cat*), heat shock protein (*hsp70*), glutamine synthetase (*glu*) and cytochrome P450 (*cyp450*) in the liver of neotropical tambaqui fish following exposure to Cu and Cd.

Methods

Juvenile Tambaqui (*Colossoma macropomum*; mean mass: 12.0 ± 3.0 g; length: 6.5 ± 0.5 cm) obtained from a fish farm (Litiara farm, Manaus, AM, Brazil) were placed in 1000 L filter tanks in a laboratory, supplied with constant aeration, and fed ad libitum. During the experimental period, the water temperature was maintained at 29 °C ± 0.5 °C, under a photoperiod of 12 hours (h). Rates of dissolved oxygen were maintained close to 6.61 mg/L.

After acclimatization for one week, 15 fish were distributed in five 4.5 L plastic recipients (3 fish per recipient) with constant aeration for 24 h and without food. The fish were exposed to four treatments corresponding to four experimental groups, which were compared with the non-exposed group. The treatment groups were as follows: two groups exposed to water

containing $CuSO_4 \cdot 5H_2O$ [20 µg/mL] for 1 and 3 h, and two exposed to water containing $Cd(NO_3) \cdot 4H_2O$ [10 µg/mL] for 1 and 3 h. No mortality was observed in either the treatment or the control groups during the experimental period. The livers were removed from fifteen randomly selected tambaqui after 1 and 3 h of treatment. These were sampled, and then stored at −80 °C until the messenger RNA extraction occurred. The initial and final metal concentrations in the water samples were determined using flame atomic absorption spectrophotometry (AAnalyst 800, Perkin-Elmer). The Cd measurements (mean Cd µg/L ± standard error of the mean [S.E.M.]), followed by treatment type in parenthesis, were: 0.08 ± 0.03 (control), 5.62 ± 0.07 (initial), and 5.04 ± 0.32 (final). The Cu measurements (mean Cu µg/L ± S.E.M), followed by treatment in parenthesis, were: 2.51 ± 0.12 (control), 8.49 ± 0.17 (initial), and 6.87 ± 0.97 (final).

Total RNA was extracted from the livers of tambaqui not exposed and exposed to copper and cadmium for 1 and 3 h, using a TRIZOL Isolation kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Two micrograms of total RNA extracted from the livers of control, cadmium and copper-treated tambaqui were reverse-transcripted with M-MLV reverse transcriptase (USB Corporation, Cleveland, Ohio, USA) using oligo-$d(T)_{15}$ as primer (Promega, Madison, Wisconsin, USA).

The messenger ribonucleic acid (mRNA) sequences for *cat*, *hsp*70, *glu* and *cypP*450 were not available in the NCBI GeneBank at the time of the study. Reverse Transcriptase-polymerase chain reaction (RT-PCR) was conducted to determine the relative expression of *cat*, *hsp*70, *glu* and *cyp*450 and 18*S* ribosomal RNA in the tambaqui livers. PCR amplification was performed using Paq5000 deoxyribonucleic acid (DNA) polymerase (5 U/µL) (Stratagene, USA). PCR was achieved after 30 s at 94 °C, followed by 30 cycles of 20 s at 94 °C, 45 s at Δ* (Table 1) and 1 min at 72 °C, with a final extension at 72 °C for 5 minutes (m). 18S was amplified in each PCR reaction as a loading control. The PCR products from the amplification cycles were visualized with a UV transilluminator T26M (BioAgency) after 1% agarose gel electrophoresis with ethidium bromide (0.5 µg/µL). The signal intensity was quantified using the Sequentix software (www.sequentix.de/software.php). The PCR product was purified using a Concert Gel Extraction Systems Kit (Gibco/BRL, Grand Island, NY, USA), ligated into the pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA), and followed by heat-shock transformation into *E. coli* (MosBlue) competent cells. Samples were plated onto Lauria-Bertani medium agar plates with tetracycline (15 mg/mL) and carbenicillin (50 mg/mL). Colonies were transformed and plasmid DNA was extracted as described by Sambrook et al.[16] Clones were analyzed using an ABI 3130 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Sequences obtained from ABI 3130 were processed for quality attributes (Phred),[17] assembly (Phrap) and cluster generation (CAP3).[18] Clusters were locally analyzed with BLAST2GO[19] against the *Danio rerio* and *Ictalurus punctatus* expressed sequence tags (EST) database to find homology and similarity using BLAST, as described by Altschul et al,[20] with a cut-off of *e*-value 10^{-6}.

Quantitative RT-PCR was conducted to determine the relative mRNA expression of all the genes described using total RNA. cDNA was synthesized from 2.0 µg of total RNA from each sample using M-MLV reverse transcriptase (Promega). First strand cDNA synthesis was conducted using an oligo-d(T) (1 µg) primer (Promega). mRNA quantification was normalized using *18S* ribosomal RNA (rRNA) gene expression.

Table 1. PCR primers utilized for RT-PCR and qRT-PCR assays.

Primer	Forward 5′–3′	Reverse 5′–3′	Δ*
cat	AGAGCGGATACCAGAGAG	GTGGATGAAAGACGGAAAC	48 °C
cyp450	AAGGGATGGCAGCTGACC	GTGATGAAGTCCTCCTCCAAGTT	60 °C
glu	ACTGTGGTGTGGGAGCGGAC	CATTGTCCAGCCCTCCTTT	50 °C
hsp70	CAGGTGGCCATGAATCCCC	CGTCTTCAATGGTCAGGATGG	54 °C
18S	AAGCATTTGCCAAGAATGTTTT	TTAAGTTTCAGCTTTGCAACCA	42 °C

Note: Δ*: Annealing temperature.
Abbreviations: *cat*, catalase; *cyp450*, cytochrome p450; *glu*, glutamine synthase; *hsp70*, heat shock protein 70; *18S*, RNA ribossomal 18S.

Real-time PCR was performed on an ABI 7300 (Applied Biosystems, Foster City, CA, USA), while data acquisition and analysis were carried out using the Real-Time Detection System Software (Applied Biosystems). The quantitative polymerase chain reaction (qPCR) was targeted at the gene and a double-stranded DNA-specific dye SYBR Green I was used. The 50 µL reaction was composed as follows: 25 µL of SYBR® Green (Applied Biosystems), 50 nM of each primer (Table 1), and, as previously described, 1 µL of extracted cDNA (200 ng).

The reaction was cycled with preliminary denaturation for 5 minutes at 95 °C, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing as indicated in Table 1, for 30 s, with primer extension at 72 °C for 30 s. This was followed by melting point analysis of the double-stranded amplicons consisting of 40 cycles of 1 °C decrements (45 s each) beginning at 95 °C. Relative expression control consisted of *18S* rDNA gene extracted from white muscle of *C. macropomum* as the constitutive gene. A significant change in fluorescence accompanies the melting curves of the double-stranded PCR products and the rate of change (d) of fluorescence (F) in the reaction with time (T) is given by the first derivative dF/dT, a plot of -dF/dT against temperature, with these changes being displayed as distinct peaks. RNA transcription levels were determined by the method of direct comparison of CT values, and relative quantities were calculated by the $\Delta\Delta C_T$ equation or transformed into linear form by $2^{-\Delta Ct}$.

Statistical analysis was performed using the software Statistic 6.0. The non-parametric Student's *t*-test was used to compare groups, and an alpha level of 0.05 was chosen.

Results and Discussion

Exposure of tambaqui fish to low concentrations of Cd and Cu increased the expression of all biomarkers in liver fragments proportionally to exposure time (Figs. 1 and 2). *hsp70* expression was highest after exposure to Cu for 3 h. *glu* expression was highest after exposure to Cd, regardless of time. Catalase mRNA expression increased after the first h of exposure to both metals, when compared with the controls, and decreased after 3 h. *cyp450* expression was also higher compared with the controls in fish exposed to both metals, regardless of exposure time. *cyp450* expression after exposure to Cu for 3 h was twice that recorded after 1 h. Conversely, in the presence of Cd, *cyp450* expression was higher after 1 h and lower after 3 h.

The exposure of invertebrates and fish to Cd and Cu has been shown to induce *hsp* expression.[21,22] These proteins are responsible for maintaining the integrity of tertiary and quaternary protein structures, acting as chaperones.[23] Increased expression of *hsp* in the cell may be the result of physical or chemical stress.[24] This gene can also be expressed in a tissue-specific manner.[25] This study found similar conclusions to those of De Boeck et al,[26] who reported that exposure

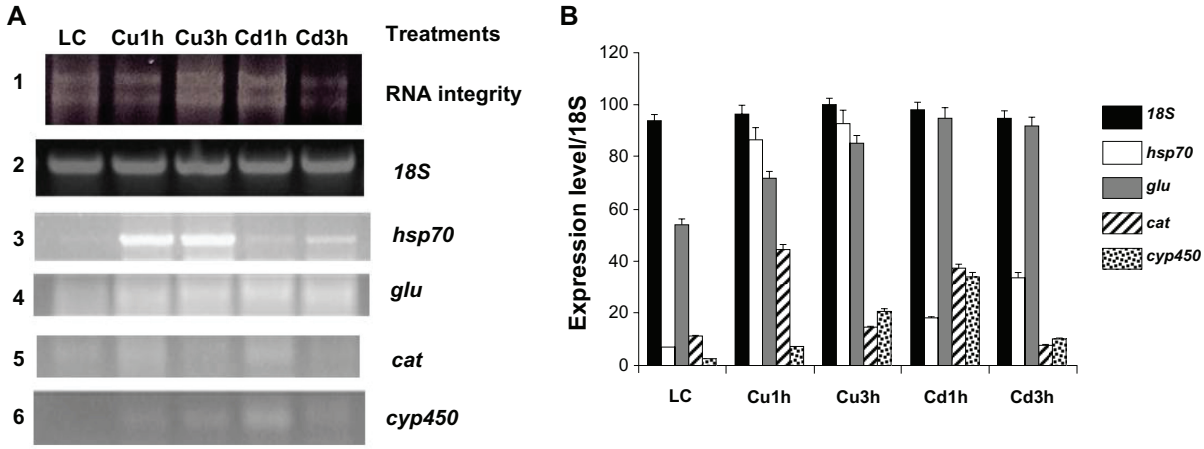

Figure 1. RT-PCR in *C. macroporum* exposed to Cu and Cd for determining expression of heat shock protein (*hsp70*), glutamate synthetase (*glu*), catalase (*cat*) and cytochrome P450 (*cyp450*). (**A**) 1, Integrity of experimental RNA; 2, Amplification of *18S* ribosomal RNA for control; 3, Tissue (liver)-specific expression of *hsp70* mRNA; 4, Tissue (liver)-specific expression of *glu* mRNA; 5, Tissue (liver)-specific expression of *cat* mRNA; 6, Tissue (liver)-specific expression of *cyp450* mRNA; (**B**) Comparison of expression levels between *hsp70*, *glu*, *cat*, *cyp450* in fish exposed to different treatments.
Abbreviations: LC, liver control; Cu1h, Exposure to Cu for 1 h; Cu3h, Exposure to Cu for 3 h; Cd1h, Exposure to Cd for 1 h; Cd3h, Exposure to Cd for 3 h.

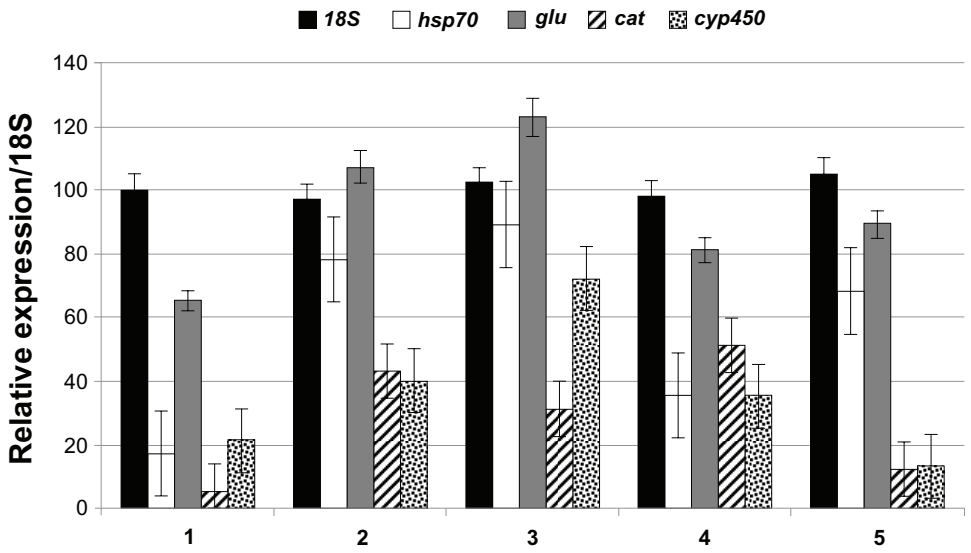

Figure 2. qRT-PCR in *C. macroporum* exposed to Cu and Cd for determining expression of heat shock protein (*hsp70*), glutamate synthetase (*glu*), catalase (*cat*) and cytochrome P450 (*cyp450*).
Notes: Comparison of expression levels between *hsp70*, *glu*, *cat*, *cyp450* in fish exposed to different treatments and control (LC).
Abbreviations: (1) LC, liver control; (2) Cu1h, Exposure to Cu for 1 h; (3) Cu3h, Exposure to Cu for 3 h; (4) Cd1h, Exposure to Cd for 1 h; (5) Cd3h, Exposure to Cd for 3 h.

of *Cyprinus carpio* to a Cu concentration of 1.9 mM in water induced *hsp70* expression in the gills, erythrocytes and liver. Using the mRNA differential display technique, Carginale et al[27] also described changes in *hsp70* expression in the liver of *Chionodraco hamatus* submitted to sublethal doses of Cd. Analyses using RT-PCR in samples extracted from the liver of *Danio rerio* exposed to Cu (10–30 µM) and Cd (10–100 µM) for 4 h revealed that although Cd is known to induce *hsp*, the gene expression was not significantly affected in this tissue. On the other hand, others have found copper to induce gene expression[28] in the same way as described by us for tambaqui. Still, despite the differences in experimental conditions, these findings underscore the importance of *hsp* expression as a useful parameter for toxicity tests involving Cu and Cd, due to the high sensitivity of this marker, even with low metal concentrations.

Glutamine synthase catalyzes the synthesis of glutamine from ammonia and glutamate. Due to its important role in the metabolism of nitrogen, including the biosynthesis of nucleotides, amino acids and urea, this enzyme has a long evolutionary history.[29] According to Iwata et al,[30] *glu* is commonly found in the liver, which plays an important role in the process of ammonia detoxification in fish. In general, all physiological stress-response processes generate ammonia, which is partially responsible for the activation of *glu*.

In the present work, *glu* was expressed in all treatments, that is, there was an increase in the expression of mRNA transcribed from this enzyme in the first and third h, in response to both Cu and Cd exposure, suggesting ammonia formation in all treatments. Tanguy et al[31] reported different *glu* expression patterns in Pacific oysters (*Crassotrea gigas*) following exposure to bicarbonates, hypoxia and pesticides. Those authors suggest that this difference in expression may be explained, at least in part, by the different mechanisms of detoxification that are triggered in response to different stressing agents. Boutet et al[32] have also described the observation that the presence of bicarbonates in *C. macropomum* causes a change in energy metabolism and regulation of *glu* activity. Many xenobiotic agents can also affect carbohydrate metabolism, causing an increase in energy consumption, which may lead to an increase in the ability of enzymes to metabolize proteins involved in catabolism for glucose production. The *glu* pathway is also an alternative for the synthesis of small amounts of cellular glucose. In organisms under stress, there is a tendency to activate this pathway, which shares with the Krebs cycle a potential to increase cellular oxidation.

The antioxidant defense system is a complex mechanism involving many enzymes, including *cat*, which plays an important role in the maintenance of cellular homeostasis and in antioxidant defense, by removing reactive oxygen species (ROS).[33] Catalase is an oxidoreductase that transforms two hydrogen peroxide molecules into two water and oxygen molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$), reducing the toxicity of hydrogen peroxide.[34] The oxidative stress caused by ROS leads to lipid peroxidation, protein denaturation and DNA damage. This, in turn, leads to change, inhibition and a variety of enzymatic activations, causing cellular damage and metabolic imbalance, resulting, for example, in apoptosis.[35] The antioxidant defense system can be either activated or inhibited in the presence of chemical stress;[36] activation or deactivation depends on the intensity and duration of the stress applied to the organism, as well as on the susceptibility of the specific organism to the stressing agent.

RT-PCR results for *cat* in tambaqui fish exposed to Cu and Cd for 1 h suggest that *cat* expression may be an immediate response mechanism to environmental stress. After 3 h, a decrease in transcriptional levels was noted, which may have resulted from the activation of additional processes used to overcome environmental stress, or else it may suggest greater susceptibility to chemical contamination, in which case adverse effects should be expected. Another explanation for the increase in catalase mRNA expression in tambaqui fish in the first h after exposure, followed by a decline, has been proposed by Jo et al.[37] The authors describe an increase in H_2O_2 concentration after longer exposure time to the toxic agent; this leads to the hypothesis that extreme oxidative stress is induced by the generation of ROS and that the decrease in mRNA levels after a specific interval may have been caused by a decrease in the organism's metabolic capacity to react against the toxic agent. Zhang et al[38] have stated that although defense mechanisms may be activated by weak stress, strong stress leads to a loss of metabolic functions, resulting in decreased mRNA expression. Despite the many works indicating that increased *cat* activity is a preventative mechanism against the action of ROS, many investigators have also shown inhibition of *cat* activity as a result of exposure to specific types of environmental pollutants.

Cytochrome P450 is not constitutively expressed in many tissues of mammals and fish.[38] Nevertheless, as shown in this study with tambaqui fish, exposure to Cd for 1 h has resulted in an increase in transcribed levels followed by a decrease after 3 h. Similarly, qPCR analyses in *Platichthys flesus* exposed to Cd revealed decrease in *cyp450* expression.[39] A gradual increase in *cyp450* levels in the liver has been described in *Anguilla anguilla* exposed to Cu at a concentration of 2.5 µM,[40] a response that is also similar to what the authors observed. This suggests that different metals may induce expression of specific biomarkers in different ways and intensities. In conclusion, the expression of all the biomarkers analyzed—*hsp70*, *glu*, *cat*, *cyp450*—in tambaqui fish exposed to Cu and Cd, was increased in comparison to non-exposed fish after both 1 and 3 h. These results suggest that different metals may induce different patterns and intensities of gene expression. Therefore, toxicology tests and biomonitoring based on molecular biomarkers should consider a set of, rather than isolated, molecular and environmental parameters.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Author Contributions

Conceived and designes the experiments: FMC, RTH, PHRA, SRN. Analysed the data: FMC, RTH, SRN. Wrote the first draft of the manuscript: SRN, FMC. Contributed to the writing the manuscript: FMC, MSFN, SRN. Agree with manuscript results and conclusions: FMC, RTH, MSFN, SRN. Jointly developed the structure and arguments for the paper: SRN. Made critical revision and approved final versions: SRN.

Disclosures and Ethics

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