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Expression of genes related to metal metabolism in the freshwater fish *Hyphessobrycon luetkenii* living in a historically contaminated area associated with copper mining

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ABSTRACT

Copper (Cu) mining in Minas do Camaquã-Brazil, released significant amounts of metals into the João Dias creek, where *Hyphessobrycon luetkenii* inhabit. Because the involvement of Cu in biological processes its concentration and availability is regulated by molecules as the metal regulatory transcription factor (MTF-1), metallothionein (MT) and transporters (ATP7A and CTR1). These genes were whole sequenced and their expression (GE) evaluated in gills, liver and intestine. Were collected fish in non-contaminated and contaminated (Cu 3.4-fold higher) sites of the creek (CC and PP) and respectively translocated (CP and PC) for 96 h.

The GE of the non-translocated groups evidenced that MT, MTF-1 and CTR1 have organ specific differences between both communities. Additionally the translocation allowed to identify organ specific changes associated with the activation/inactivation of protective mechanisms. These findings indicate that MTF-1, MT and CTR-1 GE play an important role in the tolerance of *H. luetkenii* to Cu contamination.

1. Introduction

Metals such as Cu, Fe and Zn are all essential micronutrients. They are involved in electron transference in biological processes like respiration and photosynthesis. Also, they can be part of the structure of several proteins. However, they can be toxic to organisms if present at excessive concentrations in the environment (Zhao et al., 2014). Indeed, anthropogenic activities can release great amounts and complex mixtures of metals into the environment. Therefore, organisms must regulate the internal level of these metals to avoid the potential injuries they can induce to cells and tissues.

The metal transcription factor (MTF) is a metalloregulatory protein (Andrews, 2001). MTF is a structurally highly conserved molecule from fish to mammals, especially in the zinc finger DNA binding domains, which ensures a conserved functionality to this molecule (Maur et al., 2005). MTF-1 is considered as the most important transcription factor implicated in the metallothionein (MT) gene regulation in response to metal (Zn, Cd, Cu, Ni and Pb) exposure (Heuchel et al., 1994). Actually, it is accepted that MTF-1 is essential for both basal MT expression and metal-induced MT expression (Otsuka, 2001).

The role of MTF-1 in metal homeostasis is well established. It can up regulate the expression of exporters and scavengers and repress the expression of importers in response to metal exposure (Günther et al., 2012). MTF-1 activates transcription in response to metal load by binding to short DNA sequence motifs, the metal responsive elements (MREs) found in metallothionein gene promoters (Maur et al., 2005). However, the effects of MTF-1 are not limited to gene induction. In fact, it has been demonstrated that several genes can be down-regulated by this transcription factor in the freshwater fish *Danio rerio* (Hogstrand et al., 2008). Also, the relationship between metal homeostasis and the regulatory mechanism of response to oxidative stress can alter the expression of genes associated with this process (Giedroc et al., 2001). Additionally, MTF-1 has been suggested as a Zn sensor that coordinates the expression of genes involved in Zn homeostasis, as well as the protection against metal toxicity and oxidative stress (Heuchel et al., 1994).

Despite the important role of MTF-1 in Zn homeostasis, it has been demonstrated that this transcription factor is also implicated in Cu balance, handling both intake and excretion of Cu. In flies, high Cu levels activate MT and ATP7 genes (Günther et al., 2012), while MTF-1 activates the gene encoding for the Cu importer Ctr1B at low levels of Cu (Selvaraj et al., 2005; Balamurugan and Schaffner, 2006). ATP7A

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and CTR1 are integral membrane proteins. CTR proteins are exclusive of eukaryotes. They do not use ATP as energy source, but the intracellular concentration gradient to carry preferentially Cu(I). ATPases are ubiquitous, being present in all organisms. They are multi-domain proteins with at least two Cu binding sites (Rubino and Franz, 2012). These proteins mediate the translocation of Cu across cellular membranes, having a key function in managing the excretion of Cu excess from the cell (La Fontaine and Mercer et al., 2007).

Fish are interesting and important organisms for monitoring the aquatic environmental health, including the impact of metals (Santos et al., 2010). In fact, they share similar metabolic pathways with other vertebrates. General effects of metal contamination in fish include changes in body composition (Craig et al., 2007), as well as in the levels of expression, quantity and activity of MT (Atli and Canli, 2008), metal-transporting proteins (da Silva et al., 2014; Minghetti et al., 2010) and proteins associated with oxidative stress (Atli and Canli, 2008; Machado et al., 2013). Also, it is important to note that the genomic variation among locally adapted populations can account for the phenotypic plasticity regarding gene expression (Whitehead et al., 2011). Indeed, this can be taken as an evolutionary alternative for populations inhabiting dynamic and/or contaminated environments.

The assessment of *in situ* effects of metals is very important to a better understand on how these chemical elements can alter the metabolism of aquatic organisms in a real world, once the influence of potential changes in water chemistry are also taken into account over the experimental period (Walker et al., 2008). Classically, laboratory mechanistic and toxicity tests allow us to access a limited number of parameters. However, the interactive effect of complex metal mixtures may alter the biological effects of these chemical elements in the field. In this context, the Minas do Camaquã district (Caçapava do Sul city, Rio Grande do Sul state, southern Brazil) is a natural scenario that bring us the opportunity to study how long-term metal mining can affect aquatic populations. The mining activity in this region began in ~1870 and ended in 1996 (Ronchi and Lobato, 2000). Mining operations released great amounts of Cu, Fe, Mn, and other metals in the João Dias creek for more than a century (Bidone et al., 2001). In fact, despite the mining activities has stopped for approximately two decades, metal contamination can still be detected in the old mining area. Studies developed over the last 20 years have reported variable levels of Cu in waters of the João Dias creek. Concentrations are dependent on the distance from the mining area, and can vary from 9 to 179 µg/L (Bidone et al., 2001; Aldrovandi, 2012). Our study takes as experimental scenery two areas of this region, with differences in the Cu dissolved levels. Therefore the area with significantly higher values of Cu is named Polluted (P) area and the other one named control (C) area. *Hyphessobrycon luetkenii* is a small freshwater fish species (maximum body length = 7 cm) inhabitant subtropical rivers (http://www.fishbase.us/summary/Hyphessobrycon-luetkenii.htmlitation), including the João Dias creek (Konrad and Paloski, 2000). Considering its small size and likely high metabolism, *H. luetkenii* can be a suitable model organism to understand the biochemical/physiological/genetic adjustments in fish exposed to historically contaminated sites. Indeed, alterations in the expression of the afore named genes in the intake (gills, intestine) and metabolism (liver) organs, can give the account of the genic mechanism used by these fish to face environmental changes in the metal levels, which one will be addressed in this study as field translocations.

2. Material and methods

2.1. Environmental parameters

Water physicochemical parameters (temperature, pH and dissolved O_2) were monitored once a day during fish collection and the translocation experiments. Once a day, non-filtered and filtered (0.45-µm mesh filter) water samples were collected, acidified (final concentration: 1%) with 65% HNO_3 (Suprapur, Merck, Darmstadt, Germany), and stored at 4 °C until analysis. Total (non-filtered water samples) and dissolved (filtered water samples) concentrations of carbon, major cations (Na, K and Ca) and metals (Cd, Cu, Fe, Mn, Pb and Zn) were analyzed. Carbon concentration was determined using a Total Organic Carbon (TOC) analyzer (5050 A, Shimadzu, Japan) while cations and metals were analyzed using a High-Resolution Continuum Source Graphite Furnace Atomic Absorption Spectrometer (HR-CS GF AAS, model Control-A 700 Analytik Jena, Germany), equipped with AGS-GF and a transversely heated graphite tube atomizer. Pyrolytic graphite tubes with platforms were used. A Xe short-arc lamp (GLE, Berlin, Germany) in "hot-spot" mode was used as radiation source. Standard curves were built with standard solutions prepared by serial dilution of 1000 mg/L stock solutions (Multi-Element Standards Certipur®, Merck, Darmstadt, Germany). All reagents used were of high-purity grade. Water used for preparing all reagents and reference solutions was deionized and further purified using a Milli-Q system (Millipore Corp., Bedford, USA).

2.2. Fish collection and translocation

Male and female *H. luetkenii* were caught in a metal non-contaminated (n = 100; mean body length: 4.1 cm)) and in a metal contaminated (n = 90; mean body length: 4.3 cm) sites of the João Dias creek using a fish trap. The two sites are approximately 7 km apart and separated by a man-made dam. Fish were then split into four experimental groups for the translocation experiment. Fish collected at the metal non-contaminated site were caged and kept at the site of collection (CC) or translocated, caged and kept at the metal contaminated site (CP) for 96 h. In turn, fish collected at the metal contaminated site were caged and kept at the site of collection (PP) or translocated, caged and kept at the metal non-contaminated site (PC) for 96 h. Fish cages (volume: 5000 cm^3) were built with PVC pipes and nylon nets. Fish were stocked at a density of < 0.1 g fish/L. After the 96-h exposure time, fish were anesthetized with benzocaine, decapitated and had their tissues (gills, liver and intestine) dissected and immediately stored in RNA-later (Ambion, USA) following the manufacturer instructions.

2.3. Identification of gene partial sequences and relative gene expression

Based on the conserved regions of the EF1α, MTF-1, MT, ATP7A and CTR1 gene sequences reported for the freshwater fish *Astyanax mexicanus* and other characiformes, primers were designed for quantitative polymerase chain reaction (qPCR). Designed primers were used to determine the partial sequence, as well as the relative expression of target genes in gill, liver and intestine of fish kept at their respective collection site or translocated between the two fish collection sites (metal non-contaminated and contaminated sites). Total mRNA was extracted with Trizol (Ambion, USA), reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) and RNAse inhibitor (Applied Biosystems, North America). All analyses were performed using the same amount of mRNA (1 µg/µL). The relative gene expression was quantified using real-time PCR using the GoTaq Kit (Promega, USA) and an ABI PRISM® 7300 machine (Applied Biosystems, USA). Results were normalized based on the EF1α gene expression and analysed using the 2^{-DCT} method (Schmittgen and Livak, 2008).

2.4. Data presentation and statistical analysis

Data on physicochemical parameters in the two fish collection sites at the João Dias creek were compared using the Student *t* test. Data on total and dissolved carbon, major cation and metal concentrations were compared using One-Way Analysis of Variance (ANOVA) followed by Tukey test. Data on relative gene expression for each tissue were analyzed using the non parametric Kruskall Wallis test followed by the