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Metal accumulation and expression of genes encoding for metallothionein and copper transporters in a chronically exposed wild population of the fish *Hyphessobrycon luetkenii*

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ABSTRACT

In the present study, metal (As, Cd, Cu, Fe, Mn, Pb and Zn) accumulation and expression of genes involved in metal metabolism (metallothioneins, ATP7A and CTR1) were evaluated in gills and muscle of the fish *Hyphessobrycon luetkenii* living in the João Dias creek, a site historically (~1870–1996) contaminated with a metal mixture associated with copper mining (Minas do Camaquã, southern Brazil). Fish were collected in a metal impacted site of the João Dias creek and kept in a cage at this site (PP fish) or translocated to a non-metal impacted reach of this creek (PC fish). Gill metal concentrations and metallothionein gene expression were lower in PC fish than in PP fish at any experimental time (24, 48 and 72 h). In muscle, no significant changes were observed. These findings indicate that metal accumulated in gills of wild fish chronically exposed to the metal mixture are more easily excreted than those accumulated in the muscle. In this case, expression of gene encoding for metallothionein is shown to play a key role in the regulation of metal accumulation in gills of *H. luetkenii* living in an area historically contaminated with a metal mixture associated with copper mining.

1. Introduction

Mechanisms involved in the regulation of cellular levels of metals include several pathways for metal uptake, distribution, utilization, storage, detoxification, and excretion (La Fontaine and Mercer, 2007). Due to the key biological role played by essential metals such as Cu, Fe and Zn, specific transporting proteins are implicated in the regulation of cellular metal uptake and internalization. In this context, the high-affinity copper transporter of the plasma membrane (CTR1) is the main transporting protein responsible for Cu uptake (Zhao et al., 2014). Considering the oxidative damage associated with the redox potential of some transition metals, such as Cu and Fe, protective mechanisms have emerged in order to scavenge and/or inactivate these metals. In this context, metallothioneins (MT) are the most representative example. These proteins have low molecular weight and are rich in cysteine, thus showing ability to bind metals (Wood et al., 2011). Indeed, their abundance is directly regulated by the external availability of metals (Nunes et al., 2014). The expression of the gene encoding for MT is regulated by the metal regulatory transcription factor 1 (MTF-1). This gene is responsive to metals, which bind at the promoter region and stimulate MT expression (Balamurugan and Schaffner, 2006). Although MT can bind the excessive amount of metals and store them, they are not able to excrete it from the cell. In turn, ATP7-type transporters are mainly involved in the intracellular distribution of Cu (Balamurugan and Schaffner, 2006) and its subsequent excretion (Zhao et al., 2014). This transporter has the ability of translocating Cu across cellular membranes (La Fontaine and Mercer, 2007). Like CTR1 and MT, ATP7 levels can be also modulated by Cu exposure (Zhao et al., 2014).

The analysis of metal accumulation and gene expression after exposure of fish to a single metal is already widely explored (Farrell et al., 2011; Wood et al., 2011). However, studies on the accumulation and effects of metal mixtures in fish are seldom highlighted. In fact, the evaluation of the possible interactions among metals is of extreme importance for a better understanding of the effects of dissolved metals in wild fish (Craig et al., 2009). Classical research on the effects of metal contamination is focused on laboratory studies with fish acclimated to controlled conditions (Farrell et al., 2011; Wood et al., 2011). However, studies performed with fish inhabiting areas chronically contaminated with metal mixtures in the field, such as those associated with mining and smelting, are more realistic than those performed under controlled laboratory conditions (Hamilton et al., 2016). The data generated from field studies are of great ecological relevance.

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Studies on freshwater fish inhabiting environments historically contaminated with metals can provide key evidences on how phenotypic plasticity can help these animals handle these stressing environments in order to achieve an ecological success (Whitehead et al., 2011; Uren Webster et al., 2013; Hamilton et al., 2016). In fact, divergences among protein sequences and genetic expression patterns are essential mechanisms for survival (Romero et al., 2012) and diversity (Warnefors and Kaessmann, 2013). According to Hamilton et al. (2016), wild fish show phenotypic plasticity and local adaptations can occur that adds geographic and temporal variance on responses. In this context, some studies have evaluated metal adapted fish in different field conditions, including those performed in lakes around the mining town of Sudbury in Canada, as well as in rivers in the South-West of England and North of Australia (Pyle et al., 2005; Couture and Pyle, 2008; Durrant et al., 2011; Uren Webster et al., 2013; Jeffree et al., 2014).

According to Hamilton et al. (2016), increasing abundance or reproductive success of resident fish genetically tolerant to contaminated environments compared with immigrant fish is considered as a local adaptation. In this context, Pyle et al. (2005) showed that diversity of wild fish populations from 12 lakes near Sudbury (Ontario, Canada) was associated with the level of sediment contamination with industrial (mining and smelting) metals. Additionally, Durrant et al. (2011) reported local adaptation to metals in the brown trout *Salmo trutta* from the River Hayle (Cornwall, southwestern England), an environment heavily contaminated with a waterborne mixture of metals associated with the historical mining activity in the surrounding area. Although metals were not a barrier to gene flow within rivers, these authors identified genetic differences between populations separated by river distances of little more than 1 km.

Regarding physiological responses, Pyle et al. (2005) reported that body condition in yellow perch *Perca flavescens*, the only fish species common to 12 lakes contaminated with industrial metals near the mining town of Sudbury, was negatively affected by liver Cd accumulation. Additionally, they suggested that reduced reproductive condition of female yellow perch was related to an interaction between dietary Se and Cu uptake. In turn, Couture and Pyle (2008) reported that wild yellow perch from 10 lakes of the industrial regions of Sudbury (Ontario, Canada) grew faster, expressed higher aerobic capacities, and died younger than those from Rouyn-Noranda (Québec, Canada). Authors suggested that these differences were related to a better ability of fish from Sudbury to limit the accumulation of some metals. Jeffree et al. (2014) suggested that populations of bony bream *Nematalosa erebi* and black catfish *Neosilurus ater* living in the Finniss River (northern Australia) have modified kinetics within their metal bioaccumulation physiology, via adaptation or tolerance responses, to reduce their body burdens of metals. The Finniss River is a site historically polluted with metals associated with drainage of the Rum Jungle copper/uranium mine. These findings clearly indicate that adaptation in the mechanisms involved in metal uptake, storage and excretion are crucial for fish handle chronic exposure in environments heavily and historically contaminated with metals. In this context, Uren Webster et al. (2013) showed that brown trout *S. trutta* chronically exposed to the waterborne mixture of metals in the River Hayle can tolerate considerable metal accumulation, highlighting the importance of different tissues in metal uptake (gills), storage and detoxification (liver and kidney). Based on molecular studies, these authors pointed out metal- and ion-homeostasis pathways as being the most important mechanisms contributing to the metal tolerance exhibited by this fish population.

As described above, several studies have reported the responses of tolerant fish to environments historically contaminated with metals. However, studies evaluating how fish chronically exposed to metal mixtures in the wild would respond to reduced concentrations of waterborne metals are seldom highlighted. In the present study, we have evaluated the temporal dynamics of the expression of genes encoding for the main proteins involved in Cu uptake (CRT1), detoxification (MT) and distribution and/or excretion (ATP7A) through a 72-h field translocation experiment with a wild fish species inhabiting the João Dias creek (Minas do Camaquã, southern Brazil), a site historically (~1870–1996) contaminated with a waterborne mixture of metals associated with copper mining (Ronchi and Lobato, 2000). Gene expression analysis was paralleled by metal accumulation in fish gills and muscle. We hypothesized that fish collected in a metal impacted site in the João Dias creek and translocated to a non-metal impacted reach of the same creek will show adaptive changes in the expression of genes involved in metal metabolism, especially MT. Also, we expect that gene expression response will be reflected by a significant excretion of the excessive amount of metals accumulated in fish tissues, especially in the gills.

2. Materials and methods

2.1. Fish translocation experiment

The present experiment was performed in two sites of the João Dias creek (Minas do Camaquã, southern Brazil): (1) a historically metal impacted site within the old copper mining area (P site; 30°52′55″S - 53°27′11″W), and (2) a non-metal impacted site 7 km upstream from the old copper mining area (C site; 30°53′47″S - 53°25′28″W). Male and female fish (*Hyphessobrycon luetkenii*; mean total body length = 4.32 cm; $n = 90$) were caught using a fish trap in the P site and divided into two groups. One group ($n = 45$) was kept in one cage at the fish collection site (PP fish) for up to 72 h. The other group ($n = 45$) was translocated and kept in one cage at the C site (PC fish) for 72 h. Cages (volume = 5000 cm^3) were built with PVC pipe structure and mesh. Fish were stocked at a density < 0.1 g fish/L. After 24, 48 and 72 h of experiment, five fish of each group (PP and PC) were randomly collected, anesthetized with benzocaine, decapitated and had their gills and muscle dissected. The left pairs of gills and one muscle sample of each fish were immediately frozen in liquid nitrogen, transferred to the laboratory and kept in an ultrafreezer (−80 °C). These samples were employed for metal concentrations analysis. The right pairs of gills and another sample of muscle were immediately placed in RNA-later (Ambion), following the manufacturer instructions. These samples were employed for gene expression analysis. Remaining fish in each cage were employed in a conjoint study focused on the translocation effect on the whole body ionic regulation and oxidative stress in *H. luetkenii*.

2.2. Environmental parameters

Water physicochemical parameters (temperature, pH and dissolved O_2) were monitored once a day during fish collection and the translocation experiment. Also, filtered (0.45-μm mesh filter) and non-filtered water samples were collected, acidified (final concentration: 1%) with 65% HNO_3 (Suprapur, Merck, Darmstadt, Germany), and stored at 4 °C until analysis. Dissolved (filtered water samples) and total (non-filtered water samples) concentrations of carbon (Total Organic Carbon analyzer; 5050A, Shimadzu, Japan), Na, K, Ca (flame photometer, model B262, Micronal, São Paulo, Brazil), Mg, Cd, Cu, Fe, Mn, Pb and Zn (High-Resolution Continuum Source Graphite Furnace Atomic Absorption Spectrometer; HR-CS GF AAS; model Control-A 700; Analytik Jena, Germany) were determined. 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 in the present study 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).