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Relation between metallothionein, RNA/DNA and heavy metals in juveniles of *colossoma macropomun* (Cuvier, 1818) in natural conditions

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Abstract. Metal content, Metallotionein and physiological condition (RNA/DNA ratio) were analyzed in Cachama, *Colossoma macropomum* from the Orinoco River. Juvenile samples were dissected and one gram of white muscle gills, kidney and liver was taken. Cu, Cd, Pb, Ni and Fe were analyzed by atomic absorption spectrophotometer using TORT and LUTSI as reference material. Metallothioneins were determined with Ellman´s reagent and RNA/DNA ratio was analyzed by fluorometric method. Results showed that Cd, Cu, Ni, Fe, and Pb levels were significantly higher in liver than muscle. There was no difference statistically between Mts concentrations in liver, kidney and gills; muscle showed the lowest Mts concentrations. Multivariate analysis ACP determined relationship between Mts-L and Cu and Mts-G and Cd. The Mts in muscle was related with Pb and Cd. There was no association between physiological condition and metals concentrations, however, the RNA/ DNA showed relationship with Mts-M and Mts-L

Key Words*:* Metallotionein, RNA/DNA ratio, heavy metals.

Introduction

Metals induced in organisms, including fishes, synthesis of low molecular weight proteins named metalothineins (Mts) which are molecules rich in cystein groups. These proteins have physiological functions such as detoxification and regulation of intracellular concentrations of heavy metals. Mts rise with increasing inside the cells of metal concentrations principally essential metals like zinc, copper and nonessential metals like Cd and Hg. (Bebianno and Langston, 1991; Domouhtsidou et al., 2004).

Many authors have proposed the use of MT like bioindicators of heavy metal exposition due the initial effect of heavy metal pollution maybe evident only at cellular or tissue levels before significant changes can be identified in organisms (Leung and Furness, 1999; Oliveira et al, 2010). On the other hand, Mts are easy to determinate in tissues.

Mts determinations in experimental aquatic organisms exposed to heavy metal and organisms in natural condition have demonstrated its expression, which supports its use as a biomarker, however in natural environments is not always possible to establish associations between this protein and levels of metals in tissues (Mieiro et al., 2011). This is associated with multiple factors involved in physiological and biochemical responses of organisms.

On the other hand, high concentration of metals in aquatic environment can compromise long-term ecological stability of populations because they affect the reproduction and growth rate. There are no studies that indicate the relationship between levels of metals and Mts in tissue with RNA/DNA ratio in natural environments, some work done in laboratory conditions have been controversial in this regard (Anton et al., 2009).

In Venezuela have been reported moderate heavy metal concentrations in rivers and basins (Gamboa y Bonilla 1983;Martinez, 2002), and moderate concentration of toxic metals such as cadmium, lead and Hg in fish and other aquatic species (Rojas et al., 2009; Salazar-Lugo, 2009; Lemus et al., 2010). Bauxite and gold exploitation near of the Orinoco River, the principal basin of Venezuela, are important source of heavy metal discharges in the ecosystem. It has been reported moderate metals concentrations in Orinoco River and in some fish species. The freshwater fish *C. macropomum* is abundant in the Orinoco River and it is very important for the local riverside economy. In this study we analyzed metal concentrations in different organs of *Colossoma macropomum* of lakes of low Orinoco River and their association with metalothionins and RNA/DNA condition.

Materials and Methods

Biological samples the *C. macropomus* juveniles were captured in the Caigual lagoon, Tucupita, Delta Amacuro (09° 03' 33" latitude N. y 62° 04' 05" longitude O), the fishes were transferred to laboratory where the morphometric parameters were measured and they were dissected to take up the tissues (liver, gills, kidney and muscle).

Determination of heavy metal in muscle and liver: the metal concentration (Cu, Cd, Ni, Pb and Fe) in muscle and liver were determinate using Flame Atomic Absorption Spectrophotometriy (Perkin Elmer 3110). The quality of the metal measurements was assured by the use of DORM-2 dogfish muscle, Certified Reference Material for Trace Metals (National Research Council of Canada). The average values of metals obtained by analytical process agreed with the certified values 90-97%.

Determinaton of metallothionein the Mts were quantified according to the methodology of Viarengo (1997). After homogenizing (1g/3ml) the tissue, muscle, liver, gills and kidney with 20 mmol/l of Tris-HCl buffer containing 0.5 mol/l saccharose, 0.05 mmol/l PMSF, 6 mmol/l leupeptina and 0.05 mmol/l DTT, pH 8, the homogenate was centrifuged at 30.000 g for 20 min at 4°C and the supernatant was mixed with 1 mg RNA, 40µl HCl (37%) and three volumes of ethanol (87%). The samples were centrifuged at 6.000g for 10 min. The supernatant was mixed with 3 ml of cold ethanol and kept for 1 hour at 20°C and was centrifuged at 6000 g for 10 min. The pellet was washed with a mixture of ethanol, chloroform and 20 mmol/l of Tris-HCl buffer (87:1:12), centrifuged at 6000 g for 10 min and drying under a nitrogen atmosphere. The pellet was resuspended in 300 µl of 2mmol/l HCL and 1 mmol/l EDTA mixture. Each sample was added 1 ml of 0.43 mmol/l DTNB prepared with 0.2 mol/l PBS pH 8. Sulfhydryl groups were measured for the content of sulphyhydryl residues according to the method of Ellman (1958). Therefore, the amount of Mts was defined assuming a cysteine content in mussel Mts of 20%.

Determination of RNA/DNA ratio Twenty five mg of muscle dorsal put into a vial containing 0.4 m1 of Tris-NaC1 buffer: 0.05 mmol Tris, 0.1 mmol NaCl, 0.01 mmol EDTA and 2% SDS adjusted to pH 8.0 with HCl and was homogenized with a glass homogenizer and a motor-driven Teflon pestle in ice-cold. Homogenates were centrifuged at 15.000 g for 30 min at 4°C in to obtain crude supernatants. Aliquots of supernatants were used for ARN and DNA analyses following the methodology of Canino and Calderone (1995).

Statistical analyses Statistical analysis was performed with one-way analysis of variance (ANOVA) in Mts and heavy metal in tissue. The results are presented using box and whisker plot. Multivariate analysis (CPA) was performed between Mts in tissue and RNA/DNA ratio with Cu, Cd, Fe, Ni and Pb in muscle and liver. Analyses were performed using the statistical program Statgraphic plus 5.0

Results and Discussion

The content of Cu, Cd, and Fe analyzed in muscle and liver of juvenile cachama showed significant differences, the highest concentrations are shown in the liver. The Pb and Ni showed no significant differences between tissues (Fig 2).

The results obtained in this investigation show that the Cu levels for muscle and liver were lower than permissible limits, while the Pb and Cd exceed it. If we would consider that the tissue analyzed in this work presented 70% of water, the heavy metal values could compared with permissible limit in wet weight. The muscle showed 1.08± 1.01 µg Cu/g d w concentration and liver 9.83± 5.68 d w. These values were lower than permissible legal limit for fish products (10; ≈30 µg Cu/g d w). in Venezuela there is no legal regulation to Fe and Ni. These metals may vary depending of species, growth stage and reproductive condition.

Cadmium concentrations was higher in liver (10.46 ± 5.76 µg/g d w) than muscle (7.75 ± 3.81 µg/g d w) exceeding the Venezuela the legal limit (0.01 µg/g d w; ≈ 0.023 µg/g w w), however, other countries have limits between 0.1 and 5.5 µg/g w w . Similar results were found to Pb; the muscle presented 7.19 ± 3.15 µg/g d w and liver 12.24 ± 11.77 (Venezuela legal limit is 2.00 µg/g w w; ≈ 4.6 µg/g d w). The origin of these metals probably is related to metallurgy and Bauxite activities located upstream of the discharge of the Orinoco Delta.

The Mts showed significant differences between tissues, the muscle (Mts-M) had the lowest average, while Mts in liver (Mts-L), gills (Mts-G) and kidney (Mts-K) were statistically similar (Fig 3).

Many studies suggest that Mts are found greater concentration in parenchymal tissues because they have a very active mechanism for accumulation, metabolizing and depuration of metals (Viarengo et al., 1999; Domouhtsidou et al., 2004) and these tissues are mostly studied. The muscle heavy metal concentration was not more than 2% of values determined in liver, kidney and gills suggesting a low rate of synthesis of Mts.

The CPA analysis showed that the Mts liver was related with Cu-L, while Mts gills was related with Cd and Fe (Fig 3). Two Mts sulfhydryl groups bind Cu, we could find a physiological relationship between Cu and

Mts. Metallotionein maintains homeostasis of bioesenciales metals, particularly Cu and Zn. This protein

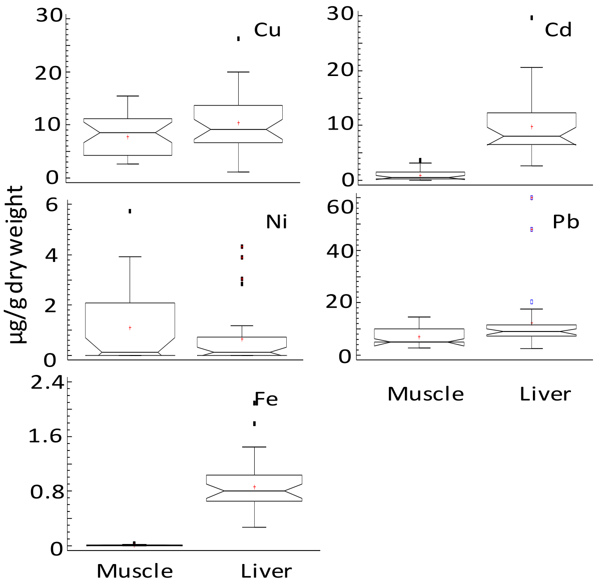

Fig. 1. Metal concentrations in muscle and liver of *C. macropomun* juvenile from Caigual lagoon, Tucupita, Delta Amacuro, Venezuela. Cu: Fs= 62.36, p<0.001; Cd: Fs= 5.65, p<0.02; Ni: Fs=3.84, p>0.05; Pb: Fs=0.04, p<0.084; Fe: Fs=115.19, p<0.001.

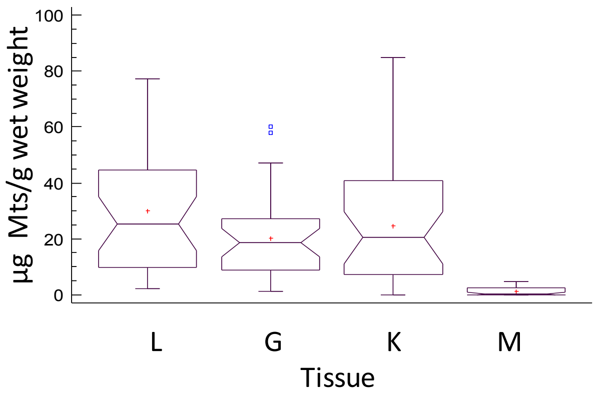

Fig. 2. Metallothionein concentration in tissues of *C. macropomun* juvenile from Caigual lagoon, Tucupita, Delta Amacuro, Venezuela. Fs= 29.8, p<0.01.

acts like donor of such metals to metalloenzymes. The metal-binding properties of Mts have been well investigated, these proteins are capable of binding 7 divalent (Zn^{2+}) and up to 12 monovalent (Cu^{+}) metal ions in vivo through two distinct metal-thiolate clusters, termed the α- and β- domains (Kaji and Kojima, 1983).

Multivariate analysis of CPA determined the relationship between Mts-M and Cd and Pb in this tissue (Fig 4).

The RNA/DNA ratio was not related with metals in tissues, but Mts-L and Mts-M levels presented relationship with RNA/DNA (Fig 5). Although metals concentration, particularly Cu and Cd were significantly higher in muscle and liver, physiological condition of juvenile cachama does not depend on heavy metals. Mts-

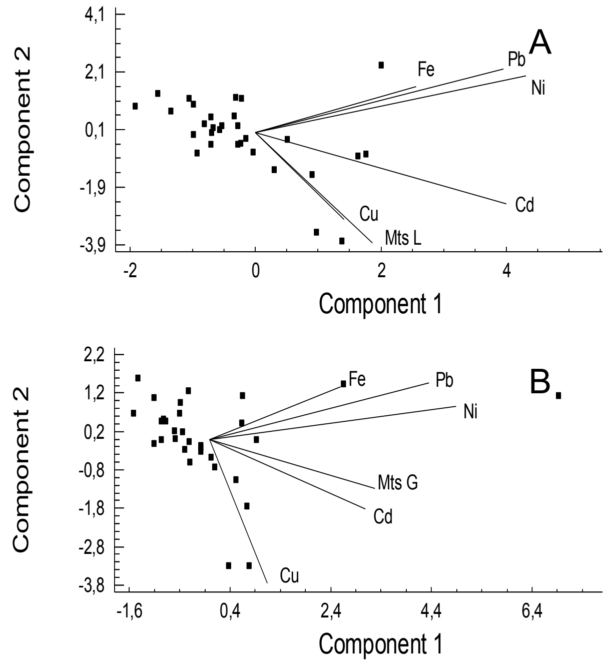

Fig. 3. Principal component analysis identifies: **A** Mts-L associated with Cu in liver; **B**: Mts-G associated with Cd in liver. Percentage of explained variance by two first axes: Mts-L 77.70%, Mts G 61.78%.

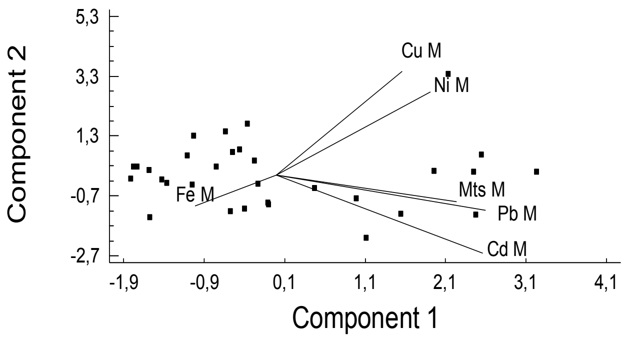

Fig. 4. Principal component analysis identifies the Mts-M associated with Cd and Pb in muscle. Percentage of explained variance by three first axes: 76.04%

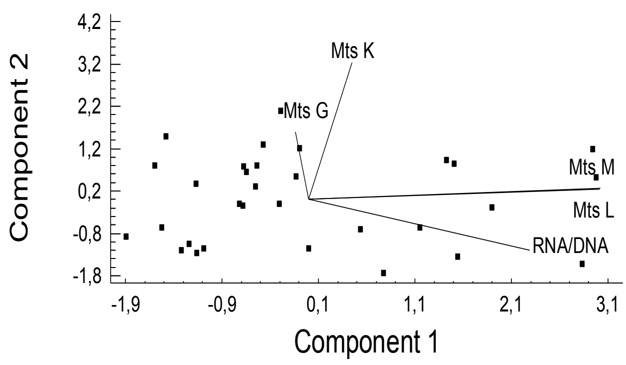

Fig. 5. Principal component analysis identifies the RNA/DNA ratio associated with Mts-L, and Mts-M Percentage of explained variance by three first axes: 81.19%

M and Mts-L seem to play a key role in the synthesis of specific new proteins to nucleic acid metabolism.

Conclusion

The study of relations between Mts with metals in various tissues of juvenile *C. macropomum* from a natural lagoon, using PCA analysis determined that these proteins are associated with physiological processes, given the particular relationship between Mts-L and Cu in liver. Additionally, Mts-L, Mts-M and RNA/DNA ratio are related in the same component suggesting its importance in bioessential metals regulating.

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