Histological alterations in gills of *Astyanax aff. bimaculatus* caused by acute exposition to zinc

Daiane Cristina Marques dos Santos\textsuperscript{a}, Sérgio Luis Pinto da Matta\textsuperscript{b,}\textsuperscript{a}, Juraci Alves de Oliveira\textsuperscript{b}, Jorge Abdala Dergam dos Santos\textsuperscript{a}

\textsuperscript{a} Federal University of Viçosa, Animal Biology Department. Av. Peter Henry Rolfs s/n, Campus Universitário, 36570–000 Viçosa, MG, Brazil

\textsuperscript{b} Federal University of Viçosa, General Biology Department. Av. Peter Henry Rolfs, s/n, Campus Universitário, 36570–000 Viçosa, MG, Brazil

\textbf{A R T I C L E  I N F O}

\textbf{Article history:}
Received 5 November 2010
Accepted 10 March 2011

\textbf{Keywords:}
Heavy metal
Aquatic contamination
Yellow tail lambari
Gill epithelium
Histopathology

\textbf{A B S T R A C T}

Increasing contamination of aquatic ecosystems by metals has caused various morphological, physiological and biochemical changes in aquatic organisms, and the gills of fish are recognized as indicators of environmental quality. In this context, the present work proposed to study the effects of different concentrations of zinc (Zn) in the histology of gills of yellow tail lambari (*Astyanax aff. bimaculatus*) after acute exposure. Seventy-two adult males of *A. aff. bimaculatus* were used, the treatments were six concentrations of Zn: 0; 3; 5; 10; 15; and 20 mg/L of water, by 96 h, and gills, muscle and bone fragments were removed. Fragments of gills were fixed and included, sectioned in a rotary microtome and stained with toluoidin blue. Fragments of bone, muscle and gills were dehydrated and digested to quantify the absorption of Zn. The median lethal concentration (LC\textsubscript{50}) 96 h after Zn acute exposure was 10 mg/L of water. Noteworthy, Zn was highly toxic in acute exposure trials starting at the concentration 5 mg/L. The exposure of fish to the metal caused branchial histopathological changes correlated with increasing concentration, caused the death of fish at concentrations of 10, 15 and 20 mg/L. The histological alterations observed in the gills were hyperplasia, lamellar fusion, aneurysm, destruction of the lamellar epithelium, rupture of membrane, deletion of secondary lamellar high, which presented more severity in treatments exposed to the highest concentrations. In conclusion, gills of *A. aff. bimaculatus* presented profound histological alterations as a result of Zn exposure, and hence, proved to be excellent indicators of environmental contamination.

© 2011 Elsevier GmbH. All rights reserved.

1. Introduction

The contamination of aquatic environment by Zn is usually caused by residues of agricultural, industrial and urban activities (Siraj Basha and Usha Rani, 2003). Increasing contamination of aquatic ecosystems by metals has caused various morphological, physiological and biochemical changes in aquatic organisms (Bordajandi et al., 2003; Siraj Basha and Usha Rani, 2003; Dautremepuis et al., 2004). Thus, damage to the structures of cells and tissues and histopathology of target organs constitute an important parameter to be considered in assessing the toxic potential of contaminants on living organisms (Fent, 1996), expressing the environmental conditions, and represent the time exposure to which organisms are subjected (Schmalz et al., 2002).

Zinc is classified as a micronutrient, is required to perform a process of osmoregulation (Verbost et al., 1994), acid-base balance (Hughes, 1982), they are also involved in the feeding process of some species by filtration. The gills of most fish and other aquatic organisms (Romani et al., 2003; Celik and Oehlenschlager, 2004). This happens because organisms can bioaccumulate heavy metals by incorporating them into the food chain and reaching a great part of the different strata that constitute the aquatic ecosystems (Viarengo, 1989).

The morphological changes may reveal the most affected target organs and detect the sensitivity of the organism to the levels of contaminants to which they were exposed (Wester and Canton, 1991). It may also, with the histopathological assessment, differentiate lesions promoted by internal failures of some physiological processes induced by other environmental factors, such as exposure to pollutants (Schwaiger et al., 1997).

The gills of fish have vital roles, because besides being the main site of gas exchange (Hughes, 1982), they are also involved in the process of osmoregulation (Verbost et al., 1994), acid-base balance (Goss et al., 1992), excretion of nitrogen compounds (Sayer and Oehlenschlager, 1987) and taste (Hughes, 1982), with participation in the feeding process of some species by filtration. The gills of most teleosts are typically composed of four pairs of gill arches, which are supported by a bone skeleton. Filaments come from the gill arches, supported by cartilage (primary lamellae), from which the...
secondary lamellae exit. The secondary lamellae are constituted by a simple epithelium, where gas exchanges occur (Rossi, 2008). The gill epithelium is found lining the arch, trails, primary lamellae and interlamellar regions. This epithelium is stratified and composed of various cell types, including squamous cells, mucus-secreting cells and cells rich in mitochondria (formerly described as chloride cells) (Evans et al., 2005), and gustatory buds and pillar cells. The second epithelium type is the respiratory epithelium, which covers the respiratory lamellae. It is usually composed of a single layer of squamous cells, through which gas exchange occurs between blood and the environment (Evans et al., 1982).

The mucus-secreting cells are usually found in the filaments, but the mucus can be found on the respiratory epithelium in fish exposed to stress conditions, suggesting that the mucus layer protects the lamellar surfaces against infectious agents, toxic and particulate matter (Powell et al., 1992). The gills are presented in the smooth lamella and microsclerites in the primary lamella region. Primary lamellae are supported by a cartilaginous tissue and have many blood vessels. The secondary lamellae, in turn, consist of three layers of cells: two of squamous cells (external) and an intermediate layer composed of pillar cells (Silva, 2004).

The multifunctionality of gills, the large surface area it occupies and its location, directly related to the external environment, make the gills a key organ for understanding the relation of fish with the environment and the action of pollutants in the aquatic environment. Thus, the histological changes of the gills are recognized as a valid and fast method to determine the damage caused in fish by the exposure to different pollutants (Arenellano et al., 1999). The effects of pollutants on gill structure have been widely studied in fish collected from polluted environments or exposed to laboratory tests, and the histological changes found have been used as a sensitive indicator of water pollution (Pacheco and Santos, 2002; Mazon et al., 2002; Fanta et al., 2003).

In the presence of pollutants, the gills may show changes that are considered a defense response, which decrease the surface area in contact with the pollutant. However, the difficulty for pollutant diffusion to the internal environment, at the same time, causes a reduction in the diffusion of respiratory gases, and may cause hypoxia in fish (Fernandes and Mazon, 2003). Heavy metals, such as Zn, stimulate the proliferation of cells rich in mitochondria, accelerate ion loss and affect the absorption by the epithelium in freshwater fish (Laurent and Perry, 1991).

In fish collected in the Piracicaba River, in the so-called Steel Valley region – MG, Brazil, high concentrations of metals were detected, especially Zn, due to the large amount of effluent released by mining activity. This situation, along with the scarcity of literature on toxicology of metals in tropical species and histopathological data of Zn toxicity in fish, encouraged us to carry out this work. In this context, the present work proposed to study the effects of different concentrations of Zn in the histology of gills of adult yellow tail lambari (A. aff. bimaculatus), after acute exposure, and to assess the species as a bioindicator.

2. Material and methods

The research was approved by the Ethics Committee of the Department of Veterinary Medicine of the Federal University of Viçosa, protocol 36/2008. Seventy-two males of A. aff. bimaculatus were used, randomly collected at 70 days after spawning, at Prata’s Fish Farm, Eugênópolis – MG, Brazil, in the basin of the Paraíba do Sul river. The study was conducted at the Laboratory of Fish Morphophysiology of the Department of Veterinary, in Laboratories of Structural Biology, and Application of Radioisotopes of the Department of General Biology, Laboratory of Plant Anatomy of the Department of Plant Biology, and in the Laboratory of Atomic Absorption Spectrometry of the Department of Chemistry, at the Federal University of Viçosa, MG, Brazil.

During the adaptation period, glass tanks were used, with a capacity of 1501 of chlorine-free water (by evaporation), with 12 fish per aquarium, which were maintained for 40 days. All the aquarium had a filtration system using gravel of different sizes and an acrylic mesh, with a filtration system with pumps and continuous aeration of the water. Every day, about 20% of the volume of the water in the tanks was siphoned for the removal of the remains of organic matter, and immediately replaced, maintaining the same initial characteristics.

Water quality was monitored every 24 h for measuring the dissolved oxygen, toxic ammonia, total hardness and nitrite by colorimetric kits, pH by portable digital pH meter and electrical conductivity by portable digital conductivimeter. The pH and total hardness were stable, according to the database published by FishBase for the species (Froese, 2009). The dissolved oxygen and electrical conductivity were measured as described by (Oyakawa et al., 2006) and the toxic ammonia was determined according to (Kubitz, 1999). The temperature was controlled (26 ± 1 °C) (Froese, 2009) through heaters and thermostats. The photoperiod was established at 12-h-dark/12-h-light controlled by timer. The fish were daily fed “ad libitum” or until apparent satiation with commercial feed (45% crude protein), offered three times a day: at 8 a.m., 1 p.m. and 5 p.m. This ration consisted of 4 mm beads, and due to incompatibility with size of the oral cavity of the fish, it was crushed and sieved using a sieve with 1 mm mesh.

The animals were exposed to concentrations of 3, 5, 10, 15, and 20 mg/L of Zn in the water, provided as ZnSO4·7H2O and one control group, during 96 h, in which each fish was an experimental unit, with 12 repetitions. After using the treatment, observations were made every hour and the animals which died in this interval, were removed from the aquarium, and submitted to the same procedures of those who survived, until the end of the exposure time, except for euthanasia. At the end of the exposure, the animals were previously anesthetized with benzocaine solution of 1:10,000, which was followed by the deepening of the anesthesia application, until they were euthanized. Following, weighing with digital analytical balance with accuracy 0.0001 g, measurements with a digital caliper with accuracy 0.02 mm, and the removal of tissues (scissors and surgical scalpel) were carried out for the histological analyses and Zn concentration. The medial region of the second branchial arches were used for the histological analyses.

Samples of bone, gills and muscle were incubated at 70 °C, after weighing in digital analytical balance with accuracy 0.0001 g, until they reached dry constant weight. The pre-dried samples were placed in Erlenmeyer flasks (25 mL), and received the addition of 1.5 mL of concentrated HNO3, 0.5 mL of HClO4 (70%), 2 drops of hydrogen peroxide 30% and 1 drop of kerosene to reduce foam formation. The temperature of the digester block was gradually increased, starting at 70 °C until 90 °C, so that complete digestion lasted about 30 minutes. After the dilution of the digested material, the concentrations of mineral elements were determined by an atomic absorption spectrophotometer. After the incision in the opercular region with surgical scissors, the gills were removed, and fixed in Karnovsky solution for 24 h and then transferred to 70% alcohol. The fragments of gills were then placed in increasing ethanol series (70%, 80%, 90%, 95% and 100%), with exchanges every 30 min, and embedding in methacrylate (Historesin®, Leica). After inclusion, semi-serial sections (3 μm thick) were made with a rotary microtome, using a glass knife. The preparations were stained with toluidine blue and sodium borate 1%, mounted with Entellan® (Merck), and analyzed under light microscopy. The digital images were captured by Olympus-AX70) photomicroscope, in the Laboratory of Plant Anatomy of the Department of Plant Biology (UFV), and processed using the Adobe Photoshop software system.
For histological analyses, the following pathologies were qualified and quantified using ten fields per animal: hyperplasia, lamellar fusion, aneurism, destruction of the lamellar epithelium, rupture of membrane, detachment of lamellar epithelium, deletion of secondary lamellar epithelium and excessive mucus production. There was not a scoring system for lesions, if the pathology was present in at least one of the fields displayed, it would be considered present in the animal. In the end, a link was made between the animals with the pathology and the total treatment (n = 12), expressing the result as a percentage. The results of the Zn accumulation in the gills were subjected to the analysis of variance and the means were compared by the Tukey test at 5% probability and expressed in mean ± standard deviation. The other results were analyzed descriptively.

3. Results and discussion

The Zn in the aquaria was daily monitored, and it was observed that concentrations had a slight decrease with the development of the experiment, which was approximately stable until the end, or until the animals reached 100% mortality (Table 1).

In control treatments, and where Zn concentrations were 3 and 5 mg/L, no mortality of fish was observed until the end of the experiment. With 24 h of exposure, the treatment that received 20 mg/L showed 100% mortality. In the treatment that received 15 mg/L, after 24 h of application, 67% of fish died, and after 72 h of exposure, 75%, indicating the high level of toxicity of Zn for the species under study. In the treatment where concentration was 10 mg/L, the fish mortality reached 50% after 24 h of exposure, without any more death to the end of 96 h (Table 2). Thus, it is possible to estimate the Lethal Concentration of 50% of the organisms (LC50–96 h) on acute exposure for Astyanax aff. bimaculatus as 10 mg/L of Zn, in water for animal breeding, which is twice the value allowed by the Brazilian legislation (5 mg/L) according to CONAMA–Brazilian National Council of the Environment (Conama, 2005).

In treatments with 10, 15 and 20 mg/L, both the fish that died and those who remained alive at the end of 96 h of exposure showed abnormal behavior. They remained near the surface of the aquaria, with intense opercular movement, uncoordinated swimming and lethargy, while the animals that did not receive Zn or those receiving the metal at concentrations of 3 or 5 mg/L, presented the characteristic behavior of the species, with active movements in pods, and fed normally. In treatments where the concentration in the water was higher than 10 mg/L, the accumulation of this metal was higher in gills, followed by bones.

The muscles did not present differences in concentration between treatments, perhaps because there had been sufficient time to cause such accumulation (Fig. 1). In gills, the treatments whose dosage was 5 mg/L or less did not differ significantly from each other in concentration. In subsequent treatments, with the increase in the concentration in water, the accumulation in the gills also increased, characterizing dose dependence. Mcgeer et al. (2000) verified the accumulation of cadmium, copper and zinc in different tissues of rainbow trout (Oncorhyncus mykiss), in an experiment of chronic exposure, and observed significant accumulation of metals in gills, liver and kidney of the exposed fish compared to the controls.

In bones, the treatments which were equal to or less than 5 mg/L were not statistically different from each other with the accumulation of this metal, probably because of the essential trace element to animal nutrition. Thus, in lowest concentrations, it was metabolized or excreted, not characterizing toxicity to fish. However, as the concentration of Zn in the water of the aquaria was increased, there was greater accumulation in bone. Hogstrand and Wood (1995) showed that one of the most important sublethal effects of Zn2+ in fish is the inhibition of calcium absorption (Ca2+), since Zn competes for the same absorption sites in gills. Consequently, zinc excess can lead to hypocalcaemia. The reduction in the concentration of Ca2+ would be harmful to the organism, since this element is essential for the integrity of the cell membrane and stabilization of branchial permeability (De La Torre et al., 2000).

The gills of the control group presented normal distribution of cellular constituents and organization pattern of lamella.
primary, secondary and blood vessels (Fig. 2A). The histopathological changes found were hyperplasia, lamellar fusion, aneurysm, destruction of the lamellar epithelium, cellular rupture, and secondary lamellar detachment followed by the deletion of the secondary lamellar epithelium (Table 3 and Figs. 2B–D and 3A–D).

It was verified the emergence of edema in the lamellar and filament epithelia in treatments with concentrations above 3 mg/L, with higher incidence on the surface of secondary lamellae, leading to intense epithelial detachment, destruction and deletion of secondary lamellae. These structural changes are considered the main causes of exposure to heavy metals (Mallatt, 1985; Ostrenski et al., 2001; Oliveira Ribeiro et al., 2005; Nero et al., 2006).

In the treatment with 3 mg/L, there was 83.3% of destruction of the lamellar epithelium, and in subsequent treatments, this destruction was 100%. The deletion of the lamellar epithelium, a result of the destruction of the secondary lamellae, increased gradually according to the concentrations of Zn in treatments. In the treatment with 5 mg/L, the deletion of secondary lamellae was 50%, indicating toxicity for the fish of the species under study. In treatments with 10 and 15 mg/L, the percentage of secondary lamellar deletion was 83.3, affecting the physiological functions of the gills, which explains the high mortality rate presented during the exposure to metal. The treatment of 20 mg/L caused secondary lamellar deletion of 91.7% of gills, leading to the mortality of all fish in treatment in a short period of exposure (24 h).

The proliferation of the lamellar epithelium led to a partial or complete fusion of secondary lamellae, in treatments with concentrations above 3 mg/L. In the control treatment, the percentage that appeared as lamellar fusion (25%) can be explained by the inclusion process, due to the high mobility of the secondary lamellae that easily overlaps. As the cellular components of the lamellae of animals from the control group were normal, it was discarded the possibility of considering few mergers as pathological changes. From the treatment using a concentration of 5 mg/L, the fusion of lamel-

Table 3
Pathological alteration percentage detected in the gills of male A. aff. bimaculatus submitted to different concentrations of zinc.

<table>
<thead>
<tr>
<th>Zn concentration (mg/L)</th>
<th>Gill histopathology (%)</th>
<th>HY</th>
<th>LF</th>
<th>A</th>
<th>LD</th>
<th>CR</th>
<th>SLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>25</td>
<td>16.6</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>83.3</td>
<td>91.6</td>
<td>75</td>
<td>83.3</td>
<td>83.3</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>91.6</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>91.6</td>
<td></td>
</tr>
</tbody>
</table>

HY, hyperplasia; LF, lamellar fusion; A, aneurysm; LD, lamellar deletion; CR, cellular rupture; SLD, secondary detach lamellar.
The presence of edema along with the detachment of the lamellar epithelium is the first sign of pathology in fish (Thophon et al., 2003). Edema of the filament and lamellar epithelium has also been described in fish exposed to different pollutants (Arellano et al., 2000). This thickening may be due to the proliferation of cells rich in mitochondria and stem cells (Dang et al., 1999), which causes partial or complete fusion of secondary lamellae. This effect can be a protection mechanism, because it decreases the exposure area of lamellae secondary to the toxic agent (Cengiz and Unlu, 2002; Cruz, 2005). Similar results were observed in tilapia-do-Nilo (Oreochromis niloticus) exposed to copper by Monteiro (2001) and Monteiro et al. (2005). The expansion of vascular axis of the lamellae was also observed, leading to the rupture of pillar cells, with loss of ability to support. This fact can be conducted to the emergence of lamellar aneurysms. Similar results were observed in Asian seabass (Lates calcarifer) submitted to exposure to cadmium (Thophon et al., 2003).

The CONAMA resolution number 357, dated 03/17/2005, sets the maximum value of 5 mg/L of Zn$^{2+}$ for water to be used for animal breeding (Conama, 2005). However, this concentration of Zn in the water clearly compromised the gill functions of the studied species, as demonstrated in this study, thus indicating the need for a review of the Brazilian legislation. The gills proved to be an excellent indicator of environmental contamination by Zn, because it is in direct contact with water and its histological composition, in addition to the large surface area and high permeability, related to gas exchanges.

4. Conclusion

Zinc was highly toxic for A. aff. bimaculatus in acute exposure, even in the concentration allowed by the Brazilian legislation. The exposure of A. aff. bimaculatus caused gill histopathological changes, and these pathologies were positively correlated with increasing concentration, thus compromising their respiratory function and causing the death of fish. There was higher Zn...
acumulation in gill tissues, followed by bones. Muscles presented no significant accumulation. It was possible to estimate the LC₅₀ 96h after acute exposure for A. aff. bimaculatus to be 10 mg/L of Zn in the water. The species A. aff. bimaculatus demonstrated sensitiveness when even submitted to low concentrations of Zn in the water. Therefore, it is an adequate biondicator.

Acknowledgements

This work was financed by FAPEMIG (Foundation of Support to the Research of Minas Gerais), as part of the project CBB-7704 – 03/12/2007.

References


Cézanne - Conselho Nacional do Meio Ambiente. Resolução CONAMA n°357 de 17/05/2005.


