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Effects of atrazine on the gill cells and ionic balance in a neotropical fish, *Prochilodus lineatus*

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ABSTRACT

The effects of the herbicide atrazine on the gill of the freshwater fish *Prochilodus lineatus* were evaluated after exposure of fish to 2, 10 and 25 μg L^{-1} atrazine during 48 h (acute exposure) and 14 d (subchronic exposure). Ions and osmolality were measured in plasma and gill samples were taken to determine the Na^+/K^+-ATPase (NKA) and carbonic anhydrase (CA) activities and for morphological analysis. Plasma osmolality and Na^+ and Cl^- ions changed depending on atrazine concentration, but atrazine exposure had no effect on the Na^+/Cl^- ratio. NKA activity did not change after atrazine exposure, but CA activity decreased in fish exposed to 25 μg L^{-1} for 14 d. Gill MRC density decreased after acute exposure but did not change in fish exposed to the subchronic treatment. The MRC density at the epithelial surface increased in fish exposed to 25 μg L^{-1}, and the MRC fractional area (MRCFA) increased in fish exposed to 10 μg L^{-1}. The changes in MRCs provide evidence of morphological adjustments to maintain ionic homeostasis in spite of the inhibition of CA activity at the highest atrazine concentration.

1. Introduction

The intense use of pesticides and herbicides on agricultural fields to control diseases and increase food production has contributed to increase environmental pollution in terrestrial and aquatic ecosystems. Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine), one of the most widely used herbicides in the world, acts by inhibiting photosynthesis (Solomon et al., 1996, 2008). This pesticide is considered moderately toxic but, due to its relative persistence in soil (41–230 d for degradation) and solubility in water (33 mg L^{-1} at 22 °C), environmental atrazine contamination has increased in recent years, and the herbicide has become a common contaminant of superficial and underground water (Brodeur et al., 2009; Dong et al., 2009).

Although atrazine is a selective herbicide, studies have demonstrated its toxic effects in animals, including amphibians and fish (Solomon et al., 2008; Rohr and McCoy, 2010). Most of the fish killed in North American atrazine incidents were in spring when atrazine is applied (USEPA, 2006). In fish, the 50% lethal concentration varies from 3 to 45 mg L^{-1}, depending on exposure conditions and species sensitivity (Neskovic et al., 1993). Numerous sublethal effects have been reported after long exposure such as locomotor activity increased, olfactory and immune response decreased (Rymuszka et al., 2007; Tierney et al., 2007), oxidative stress on blood cells (Fatima et al., 2007) and mutagenic and genotoxic effects (Moron et al., 2006; Ventura et al., 2008). Concentrations as low as 3 μg L^{-1} of atrazine also affect the fish behavior and health (Steinberg and Speiser, 1995; Dong et al., 2009).

Earlier studies described atrazine effects on osmoregulation and ion balance in animals. In mammals, atrazine promoted ion and protein loss via urine (Santa Maria et al., 1986), and, in fish, ion imbalance was reported in *Oreochromis niloticus* (Prasad and Reddy, 1994; Hussein et al., 1996), *Chrysichthyes auratus* (Hussein et al., 1996), and *Salmo salar* (Waring and Moore, 2004). Recently, Matsumoto et al. (2010) showed that atrazine did not cause ionic disturbance in pre-smolt salmon exposed to atrazine in freshwater before transfer to sea water free of atrazine.

Ionic and osmotic regulation is essential for life. In freshwater fish, the gills are the principal organs for ion uptake and the kidney plays a role in ion reabsorption. The gills are also the main organ for respiration and play a role in acid–base equilibrium and nitrogen excretion. They are the first organs to contact and respond to xenobiotics. Changes in the physical and chemical characteristics of water result in changes to the cells of the gill epithelium and may result in disruption of gill functions. The mitochondria-rich cells (MRCs) in the gill epithelium are directly involved in Na^+, Cl^- and Ca^{2+} uptake in freshwater fish to maintain ionic homeostasis (Perry et al., 1992; Mazon et al., 2002b). Metals and pesticides affect MRC density and function (Mazon et al., 2002a; Camargo et al., 2009; Ceyhun et al., 2010; Ekinci and Beydemir, 2010). MRCs contain large amounts of Na^+/K^+–ATPase (NKA) and carbonic

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anhydrase (CA) enzymes, which activities are measured as indicators of iono-regulatory performance. The NKA is localized in the tubular membrane system of MRCs, and its activity creates an electrochemical force for Na^+ and Cl^- uptake at the cell apical surface, whereas the cytosolic CA provides the counterions H^+ and HCO_3^- for the incorporation of Na^+ and Cl^- (Hwang and Lee, 2007).

In this context, the main goal of this study was to evaluate the effect of sublethal concentrations of atrazine on osmo- and ionic regulation concomitantly with analyses of MRC morphology and NKA and CA activity in the gill epithelium of *Prochilodus lineatus*. In Brazil, atrazine has been used all over the country to control broadleaf weeds in fields of sugar cane, corn and soy; aquatic environments near crop fields areas already have atrazine levels higher (Armas et al., 2007) than those permitted in water by the Brazilian Environment National Council resolution (CONAMA 354/2005) for preservation of biota. *P. lineatus* is a native species widely distributed throughout the Southeast and South regions of Brazil, where most of sugar cane and corn crops are grown. This species has been considered as a model for environmental toxicological studies due to its moderate-to-high sensitivity to changes in water quality and its detritivorous feeding habits, which put the animal in contact with xenobiotics from water and sediment (Mazon et al., 2002a,b; Almeida et al., 2005; Camargo et al., 2009).

2. Materials and methods

2.1. Animals

Juvenile *P. lineatus* (Valenciennes, 1836) (body mass- = 29.3 ± 2.8 g and total length = 13.7 ± 0.6 cm) were provided by the Aquaculture Station of Furnas Hydroelectric Power Station, São José da Barra, MG, Brazil. Fish were acclimated for 30 d in a 1000-L aquarium with continuous aeration and dechlorinated water flow (dissolved oxygen = 7.0–7.5 mg L^{-1}, pH = 7.1–7.4, conductivity = 125–130 μS cm^{-1}, alkalinity = 35–43 mg L^{-1} as $CaCO_3$ and hardness = 39–50 mg L^{-1} as $CaCO_3$) at 24 ± 2 °C and natural photoperiod (12 h light:12 h dark). Fish were fed with commercial fish food.

2.2. Experimental Design

After the acclimation period, fish were randomly divided in eight groups. Three groups (n = 10 in each group) were exposed to atrazine for 48 h (acute exposure) and the other three groups were exposed for 14 d (subchronic exposure) in a 200 L aquarium with dechlorinated and continually aerated water. Two groups were used as controls and were kept in water free of atrazine for 48 h and 14 d. All experiments were done in duplicate using semi static system in which 80% of water was renewed every 24 h. Fish were exposed to atrazine at 2 μg L^{-1}, the maximum permitted in water by CONAMA resolution 357/2005 (CONAMA, 2005), 10 μg L^{-1}, an intermediate concentration similar to that applied to crop fields and 25 μg L^{-1}, the highest concentration indicated for use in agriculture (Ventura et al., 2008). Atrazine (CAS 1912-24-9, Sigma Aldrich, USA) concentrations were obtained from a stock solution prepared in methanol at 10 mg L^{-1}. Previous tests with methanol in the final test dilution did not show any detected effect in fish (data not shown). During experiments, the physical and chemical water characteristics were maintained similar of those of the acclimation period. The experimental procedures were approved by local animal and environmental ethics committees.

At the end of each experiment, a sample of blood was taken from each animal via the caudal vein to measure plasma ions and osmolality. The fish were then killed by medullar section, and the gills were removed. Gill samples were either fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 and Bouin fixative solution for morphological analyses or frozen at −80 °C for determination of CA and NKA activities. The latter samples were frozen in SEI buffer (300 mM sucrose, 0.1 mM Na_2-EDTA, 30 mM imidazolpH 7.4) + β-mercaptoethanol.

2.3. Determination of plasma ions and osmolality

Each blood sample was centrifuged at 4 °C to separate the plasma. Plasma Na^+ and K^+ concentrations were determined using a Flamer photometer (Digmed DM-61, Digimed, Brazil). Cl^- concentration was determined by the thiocyanate method with a commercial kit (Labtest, Brazil) and a microplate reader (MRX-HD, Dynex Technologies Inc., USA) at 490 nm. Osmolality was determined on the basis of freezing point in a μ-osmometer (μOSMETTE PRECISION SYSTEM, Precision System Inc., USA).

2.4. Determination of NKA and CA enzymes activity

Thawed gill filaments were homogenized in SEI buffer 0.1 M (pH 7.4) and centrifuged at 10000*g* at 4 °C. The supernatant was used to determine protein concentration and NKA activity. NKA activity (μmol Pi mg $protein^{-1}$ h^{-1}) was determined as described by Quabious et al. (1997), by measuring the phosphate released by samples incubated in buffer (100 mM NaCl, 8 mM $MgCl_2$, 30 mM imidazole, 0.1 mM EDTA, 3 mM ATP, pH 7.6) containing KCl (5 mM) or ouabain (2.5 mM). A solution of 0.65 mM phosphate (Sigma) was used as a standard, and the samples were analyzed in triplicate at 620 nm in a microplate reader. Protein concentration was determined using the Bradford method (Kruger, 1994) at 595 nm in a microplate reader.

To determine carbonic anhydrase activity, thawed gill filaments were homogenized (10% (W/V) in cold reaction medium (225 mM Mannitol; 75 mM Sucrose; 10 mM Tris–phosphate, pH 7.4) and centrifuged at 10000*g* at 4 °C. The supernatant was used to determine protein concentration and CA activity at 2.5 °C, as described by Vitale et al. (1999). Briefly, the CA activity was determined by adding 7.5 mL of reaction medium on 0.05 mL of tissue homogenate and 1 mL of CO_2-saturated distilled water at 2.5 °C. The change in pH was measured over 20 s using a pH meter (Jenway 3510, UK). CA activity was determined by the ratio between the slopes of linear regressions of catalyzed and un-catalyzed reaction and was expressed in mg protein.

2.5. Immunohistochemistry of NKA

Gill samples fixed in Bouin solution were serially dehydrated into absolute ethanol, rinsed in xylene and embedded in paraffin. Sections (8 μm in thickness) were deparaffinized, re-hydrated, washed in Tris-saline buffer + triton (TBS-T 0.5 mM, pH 7.4) diluted 1:10 and incubated in 20% normal goat serum (Gibco, Invitrogen) to block unspecific sites. Sections were incubated overnight in a humid chamber at 20 °C with a 1:300 dilution of the first antibody, α5 anti NKA (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA). The next day, the sections were incubated with the second antibody, peroxidase-conjugated goat anti-mouse (GAMPO, Chemicon International, USA) diluted 1:100. The antibody complex was visualized by staining the sections with DAB-Ni (3,3′-diaminobenzidina in ammonium nickel-(II) sulfate hexahydrate) in Tris-buffer with 0.0125% H_2O_2 added immediately before use. Negative controls were obtained by omission of either the first or the second antibody and were incubated and stained as described above. The slides were analyzed using an Olympus BX51 light microscope (Olympus, Denmark) with a digital video camera and the Motic Image Plus 2.0 software. The immune-positive NKA-rich MRC density were