Neotropical Ichthyology, 15(4): e170054, 2017
DOI: 10.1590/1982-0224-20170054

Journal homepage: www.scielo.br/ni
Published online: 18 December 2017 (ISSN 1982-0224)
Printed: 18 December 2017 (ISSN 1679-6225)

Effects of manganese on fat snook *Centropomus parallelus* (Carangaria: Centropomidae) exposed to different temperatures

Bárbara Louise L. Tuzuki, Frederico Augusto C. Delunardo, Luciana N. Ribeiro, Caroliny P. de Melo, Levy Carvalho Gomes and Adriana Regina Chippari-Gomes

This study evaluates the effects of exposure to manganese (Mn^{2+}) for 96 hours at two different temperatures (24 and 27°C) on juveniles of *Centropomus parallelus* through the activities of glutathione S-transferase (GST) and catalase (CAT), micronuclei test (MN) and comet assay. The GST activity did not show any significant difference between the groups exposed to Mn^{2+} and the respective control groups; in contrast, a major increase in the CAT activity was observed at 27°C in the group exposed to Mn^{2+} compared to the control group. The genotoxic analyses showed that in all animals exposed to Mn^{2+}, the number of red cells with micronuclei increased significantly compared to the respective control groups. There was also a significant increase in the incidence of DNA damage in the groups exposed to Mn^{2+}. At a temperature of 24ºC, animals exposed to Mn^{2+} had more DNA damage than those at 27°C. It is likely that the increase in temperature can also induce oxidative stress. Thus, we conclude that manganese is toxic to the fat snook juveniles, causing genotoxic damage, and when associated with an increase in temperature, manganese can also provoke an increase in oxidative stress.

Keywords: Biomarkers, DNA damage, Enzymes, Genotoxicity, Metal, Micronuclei.

Este estudo avaliou os efeitos da exposição ao manganês (Mn^{2+}), após 96 horas, a duas temperaturas (24 e 27°C) em juvenis de *Centropomus parallelus* por meio de análises bioquímicas (atividade das enzimas glutationa S-transferase (GST) e catalase (CAT)) e genotóxicas (teste do micronúcleo e ensaio cometa). A atividade da GST não mostrou diferença significativa entre os grupos expostos ao Mn^{2+} e os seus respectivos grupos controle, enquanto que um aumento significativo na atividade da CAT foi observado a 27°C no grupo exposto ao Mn^{2+}, quando comparado ao grupo controle. As análises genotóxicas mostraram que os animais expostos ao Mn^{2+} tiveram aumento significativo na quantidade de células com micronúcleo em relação aos seus grupos de controles. Houve também aumento significativo na incidência de danos ao DNA nos grupos expostos a esse contaminante. Na temperatura de 24°C, os animais expostos ao Mn^{2+} tiveram maior quantidade de danos no DNA em relação a 27°C. É provável que o aumento da temperatura também possa induzir o estresse oxidativo. Assim, concluímos que o manganês é tóxico para os juvenis de robalo, causando dano genotóxico, e quando associado a um aumento da temperatura, também pode provocar um aumento no estresse oxidativo.

Palavras-chave: Biomarcadores, Danos no DNA, Enzimas, Genotoxicidade, Metal, Micronúcleo.

Introduction

Global climate change is one of the critical challenges that can affect ecosystem health and chemical safety. The current increases in sea surface temperature are considered as one of the new and significant threats to aquatic ecosystems (Daufresne *et al.*, 2009; IPCC, 2013). Water temperature by itself may act as a stressor since marine organisms inhabiting these environments are mostly ectotherms; as a result, the temperature affects the metabolic rate, thereby affecting energy metabolism and inducing various physiological changes (Eales, Brown, 1993; Caissie, 2006; Vergauwen *et al.*, 2013). Furthermore, many aquatic environments are affected by the release of human contaminants, resulting in increased metal concentrations (Gomiero, Viarengo, 2014). These facts can lead to severe impairments to aquatic organisms because the rise in water temperature may modify the chemistry of many pollutants (Schiedek *et al.*, 2007). These facts can also lead to increases in the bioavailability of pollutants in the environment and, consequently, the metal uptake rates and their toxicity (Bervoets *et al.*, 1996; Vergauwen *et al.*, 2013; Lee *et al.*, 2014).

Laboratório de Ictiologia Aplicada (LAB PEIXE), Universidade Vila Velha - UVV, Rua Comissário José Dantas de Melo, 21, Boa Vista, 29102-770 Vila Velha, ES, Brazil. (BLLT) barbara.tuzuki@hotmail.com, (FACD) frederico.delunardo@gmail.com, (LNR) luciana.bio@live.com, (CPM) carol.patriciomelo@gmail.com, (LCG) levy.gomes@uvv.br, (ARCG) adriana.chippari@uvv.br (corresponding author)

Manganese (Mn^{2+}) is a constitutive element of a series of essential enzymes and cofactors that are fundamental to brain function, such as glutamine synthetase, superoxide dismutase and others (Yokel, 2009), but it can be very toxic at concentrations above the optimal threshold level (Vieira *et al.*, 2012). In natural waters, dissolved manganese from anthropogenic sources/influences associated with metal mining and other industrial activities may reach very high concentrations (McNeely *et al.*, 1979; Morillo, Usero, 2008). Recently, the Doce River basin (Minas Gerais, Brazil) suffered a severe environmental impact after the rupture of two dams controlled by a mining company (Escobar, 2015), and a significant amount of metal was released into the water body; notably, Mn^{2+} was one of the metals that presented higher concentrations.

The deleterious effects of either chronic or acute exposure to Mn^{2+} depend on the species and within a species, depend on the tissue and ambient water chemistry (Fish, 2009; Arndt *et al.*, 2012). Numerous studies have shown that the effects of manganese on fish include impaired functions of the gill epithelium, such as hydromineral imbalance (Gonzalez *et al.*, 1990) and histopathology of the gills (Dalzell, Macfarlane, 1999; Hedayati *et al.*, 2015; Dolci *et al.*, 2017). Impacts on hematology (Agrawal, Srivastava, 1980; Wepener *et al.*, 1992), immunomodulation (Cossarini-Dunier *et al.*, 1988; Hernroth *et al.*, 2004), and hormonal interference (Hoseini *et al.*, 2004) can also occur. Other damages on the metabolic system were also observed, such as impacts on carbohydrate metabolism (Nath, Kumar, 1987; Barnhoorn *et al.*, 1999; Partridge, Lymbery, 2009) and alterations to the antioxidant system (Falfushynska *et al.*, 2011; Vieira *et al.*, 2012; Dolci *et al.*, 2013; Gabriel *et al.*, 2013). However, the use of biochemical and genotoxic endpoints to understand the interaction between water temperature and manganese exposure in fish has not yet been investigated.

The most commonly used biochemical tests in fish studies are assays of liver enzymes that are involved in the detoxification of xenobiotics and their respective metabolites, such as the glutathione S-transferase enzymes (GST) and catalase (CAT) (Teles *et al.*, 2005; Halliwell, Gutteridge, 2006). The GST are phase II enzymes that play a major role in conjugation reactions and detoxification mechanisms by reducing organic hydroperoxides (ROOH). GST hydrolyzed lipophilic compounds are subsequently excreted as a water-soluble non-reactive conjugate (Simonato *et al.*, 2011; Azevedo *et al.*, 2013). Catalase is an essential enzyme that catalyzes the conversion of hydrogen peroxide (H_2O_2) into water and oxygen, thereby preventing its conversion to hydroxyl radicals and reducing oxidative stress levels (Gonçalves-Soares *et al.*, 2012). Catalase is usually regulated by complex and interconnected systems that are sensitive to the concentration of its substrates (Lushchak, 2011). Thus, these enzymes often show increased activities when the production of superoxide and peroxide anions increase up to a certain level (McCord, Fridovich, 1969; Beutler, 1975; Lushchak, 2011).

Among the available genotoxicity tests, the alkaline comet assay and the micronuclei test (MN) are the most commonly used and recognized due to their robustness, sensitivity and statistical power in the evaluation of DNA injuries. Thus, these analyses can be considered complementary biomarkers of DNA damage based on two different endpoints (Tice *et al.*, 2000; Udroiu, 2006; Heuser *et al.*, 2008). The MN test is a fast method of detecting structural and numerical chromosomal alterations that are induced by clastogenic and aneugenic agents (Heddle *et al.*, 1991; Jha, 2008). By contrast, the comet assay measures strand breaks before the DNA repair systems intervene; these can include DNA single-and double-strand breaks, alkali-labile sites and excision-repair events caused by simple and bulky DNA adducts (Singh *et al.*, 1988; Tice *et al.*, 2000).

The fat snook (*Centropomus parallelus* Poey, 1860) can inhabit coastal waters, estuaries and freshwater environments (Cerqueira, Tsuzuki, 2009) that are commonly characterized by high anthropogenic pressure, including the occurrence of metal contamination. Moreover, because the fat snook is an ectothermic species, its susceptibility to metal toxicity can be modified by higher water temperatures through changes in its rates of biochemical and physiological processes and the stability of its biomolecules (Heugens *et al.*, 2001; Lannig *et al.*, 2006).

Thus, we conducted short-term thermal bioassays (96 h) to investigate the effects of different temperatures (24 and 27°C) on manganese toxicity in *C. parallelus*. To do this, we analyzed responses to the phase II biotransformation enzyme glutathione S-transferase (GST), the antioxidant enzyme catalase (CAT) and genotoxic analyses (*i.e.*, alkaline comet assay and the micronuclei test).

Material and Methods

Animals and acclimation. The fat snook, *C. parallelus* juveniles (Voucher # MBML 12877) were obtained from a hatchery and transferred to the laboratory. For temperature acclimatization, 72 fish (2.46 ± 0.54 g and 7.12 ± 0.52 cm) were equally divided into two clean tanks (300 L; fish density = 0.3 g L^{-1}) with water temperatures of either 24°C (similar conditions of seawater) or 27°C (approximately a 2.5°C increase in water temperature as devised by the moderate Intergovernmental Panel on Climate Change (IPCC) emission scenario). The water temperature was gradually increased by 1.0 ± 0.1°C/day using a heater coupled to a thermostat (Full Gauge, TIC-17RGT). The tanks were filled with aerated seawater that was biologically filtered and experienced a 12:12 h (light:dark) photoperiod. During the acclimatization period (three weeks), the fish were fed daily to satiation with commercial fish food that was composed of 1.2 mm pellets with 60% protein (NRD INVE, Belgium), and the seawater was replaced twice each week with seawater of the same temperature. Feeding

was suspended 24 h before the animals were transferred into the test aquaria. The physicochemical parameters were monitored daily using a multiparameter YSI (model 85, Yellow Springs Inc. Ohio, United States). The water quality parameters for tanks with water at 24ºC and 27ºC were as follows: temperature (24.2 ± 0.3 and 27.1 ± 0.2°C, respectively), dissolved oxygen (5.8 ± 0.5 and 6.0 ± 0.4 mg L^{-1}, respectively), salinity (25.4 ± 0.1 and 25.3 ± 0.1 ppt, respectively) and conductivity (25.4 ± 0.1 and 25.3 ± 0.1 µS cm^{-1}, respectively). The pH (8.1 ± 0.1 and 8.1 ± 0.1, respectively) was monitored with a pH meter YSI 100 (Yellow Springs Inc., OH, USA). The total ammonia (0.63 ± 0.3 and 0.63 ± 0.4, respectively) and total nitrites (0.3 ± 0.4 and 0.3 ± 0.3, respectively) were monitored according to APHA guidelines (2005).

Short-term toxicity test. After acclimatization, groups of six fish were transferred to 12 polyethylene aquaria containing 20 L of seawater (fish density = 0.75 g L^{-1}; salinity = 32) under constant aeration, and temperature was controlled by a heater coupled to a digital thermostat (0.1°C precision). Fish were kept under the respective temperatures they were acclimated to (*i.e*., 24.2 ± 0.3°C and 27.1 ± 0.2°C). For each temperature, the fish groups were divided into two treatments: i) control (no contaminant addition) or ii) exposed to a nominal manganese concentration (3.18 mg L^{-1} or 26.426 nM Mn^{2+} as $MnCl_2 \cdot 4H_2O$), and the experiment lasted for 96 h. A total of four treatments were conducted (*i.e*., the two temperatures versus the two Mn^{2+} concentrations) (three replicates for each treatment; 18 fish per treatment). After contamination, the concentration of dissolved Mn^{2+} was measured in the water of all aquaria at the start of the experiment, and the average values were 2.269 mg L^{-1} (treatment group) and 0.00 mg L^{-1} (control group). Values of 0.00 were considered as the control group since the Mn^{2+} measurements in this group were below of the limit of quantification of AAS. The manganese treatments were chosen according to previous studies conducted with *Lithobates catesbeianus* (Veronez *et al*., 2016). During the experiment, one-third of the water (of the appropriate temperature and contaminant level) was renewed after 48 h. No mortality was observed during the experiment. All procedures performed in the present study were approved by the Ethics Committee for Animal Use of the Universidade Vila Velha (CEUA-UVV), number 198-2011.

Biological material sampling. Immediately after removing the fish from the aquaria, they were anesthetized with benzocaine (0.1 g L^{-1}), and blood samples were taken from the caudal vein using heparinized syringes; blood samples were used in the alkaline comet assay and the micronuclei test (MN). Then, animals were weighed, measured and euthanized by cervical sectioning. A fragment of the liver from each animal was removed and stored at -80°C until enzymatic analyses.

Enzyme assays. Assays of the activity of the biotransformation enzyme, glutathione S-transferase (GST - EC 2.5.1.18), and the antioxidant enzyme, catalase (CAT - EC 1.11.1.6), were carried out according to well-established protocols. Initially, the frozen liver samples were weighed and homogenized (1:4 - w/v) in 20 mM Tris buffer (pH 7.4) with 0.5 mM sucrose, 0.15 mM KCl and 1 mM protease inhibitor (PMSF). The homogenates were then centrifuged at 10,000 g for 20 min at 4°C, and the enzyme assays were run on a SpectraMax Plus 384 spectrophotometer (Molecular Devices). The GST activity was determined using a phosphate buffer solution (pH 7.0) containing 1-chloro-2,4-dinitrobenzene (CDNB; 1 mM) and glutathione (GSH; 1 mM) as substrate. Enzyme activity was determined based on the extinction coefficient of CDNB (Habig *et al.*, 1974; Habig, Jakoby, 1981). Catalase activity was determined by the continuous evaluation of the decrease in the concentration of hydrogen peroxide (H_2O_2) (Aebi, 1984). The reaction medium was prepared with a buffer solution (1 M Tris HCl and 5 mM EDTA) containing hydrogen peroxide (10 mM). The results are reported as µmol min^{-1} mg $protein^{-1}$. The protein quantification of all samples was determined at a controlled room temperature (25°C), according to Lowry *et al.* (1951).

Alkaline comet assay. Blood samples were diluted 1:120 (v/v) in RPMI 1640 medium (RPMI-Roswell Park Memorial Institute) and used immediately. The alkaline comet assay was performed as described by Tice *et al.* (2000) and Andrade *et al.* (2004), with some modifications. Briefly, 5 µl of each diluted blood sample was added to 95 µl of 0.75% (w/v) molten low-melting-point agarose, and an aliquot of the mixture was spread on a microscope slide that was pre-coated with 1.5% (w/v) normal melting point agarose and topped with a coverslip. After agarose solidification, coverslips were removed, and the slides were immersed in a lysis solution (2.5 M NaCl,100 mM EDTA and 10 mM Tris, pH 10.0-10.5) containing 1% Triton X-100 and 20% DMSO. Slides were maintained in this lysis solution (4°C) and kept in the dark for 2-3 h. Slides were then incubated in a freshly prepared alkaline buffer solution (300 mM NaOH and 1 mM EDTA, pH ≥13) for 20 min for DNA unwinding. Electrophoresis (15 min at 300 mA and 25 V) was performed using the same buffer solution. Each step was performed under indirect yellow light. After electrophoresis, slides were neutralized in a Tris solution (400 mM; pH 7.5), rinsed three times with distilled water, and dried overnight at room temperature. Slides were fixed for 10 min in trichloroacetic acid (15% w/v), zinc sulfate (5% w/v), and glycerol (5% v/v), and then rinsed three times with distilled water and dried at 37°C for 2 h. Dry slides were rehydrated for 5 min in distilled water and stained under constant shaking for 35 min using a solution containing sodium carbonate (5% w/v), ammonium nitrate (0.1% w/v), silver nitrate (0.1% w/v), tungstosilicic acid (0.25%), and formaldehyde (0.15% w/v), which was freshly prepared in the dark. Stained slides were rinsed twice

with distilled water, submerged in the stop solution (acetic acid 1%), rinsed again with distilled water, and immediately coded for analysis. A total of 100 cells from each replicate (*i.e.*, 50 from each duplicate slide) were randomly analyzed under an optical microscope (100× magnification) to measure the length of the comet's tail. The analysis of the slides involved 100 cells/fish using a visual classification based on the degree of DNA fragment migration from the nucleus. Cells were classified into class 0 (no damage), class 1 (little damage - when the tail length was smaller than the nucleus), class 2 (medium damage - when the tail length was between 1 and 2 times the nucleus diameter), class 3 (extensive damage - when the tail length was over 2 times the nucleus diameter), and class 4 (presence of apoptosis) (Kobayashi *et al.*, 1995; Speit, Hartmann, 1999). The DNA damage index (DI) was calculated for each fish as the sum of the number of nucleoids that were observed for each damage class multiplied by the value of its respective damage class (0, 1, 2, 3 or 4). The results were expressed as the mean DNA damage index for each experimental group, where 0 represented the absence of damage and 400 indicated the highest damage score.

Micronuclei (MN) frequency. Peripheral blood samples were obtained from the caudal vein and smeared onto clean slides. The slides were air-dried after fixation in pure ethanol for 20 min, and the smears were stained with a 10% Giemsa solution for 25 min. Each MN was identified according to the following criteria: spherical cytoplasmic inclusions with a sharp contour, a diameter smaller than one-third the diameter of the nucleus, a color and texture resembling the nucleus, and no contact with the nucleus (Al-Sabti, Metcalfe, 1995; Kirsch-Volders *et al.*, 2003). A total of 2000 erythrocytes per fish were examined under an Olympus optical microscope (1000× magnification), and the mean frequencies of the MN found in each experimental group were calculated and expressed per 1000 cells (‰). Only intact cells with distinct nuclear and cellular membranes were scored.

Statistical analyses. The acute toxicity test data were analyzed for normality and homoscedasticity through Shapiro-Wilk and Levene's test, respectively. Data were analyzed by two-way ANOVA to determine significant differences in Mn^{2+} exposure, temperatures and the interaction between these two factors, followed by the Bonferroni test for post hoc comparisons. All data are expressed as the mean ± standard error of the mean (SEM), and the differences were considered significant at $p \geq 0.05$. GraphPad Prisma 5.0 software was used for statistical analyses.

Results

Enzyme activities. The biotransformation enzyme GST activity did not show any significant changes as a result of the heat stress treatments (P=0.905; F=0.01) and Mn^{2+} exposure (Fig. 1a; P=0.178; F=1.85). In addition, there was no significant interaction between the two factors (temperature and Mn^{2+}) (Fig. 1a; P=0.7611; F=0.09).

For antioxidant enzyme CAT activity, temperature did not affect the activity of this enzyme (P=0.1154; F=2.55), and CAT activity was not affected by exposure to Mn at 24°C (Fig. 1b; P=0.1246; F=2.42). However, at 27°C, the enzymatic activity of CAT was significantly higher in fat snook exposed to Mn^{2+} compared to the respective control (Fig. 1b; P=0.0034; F=4.67).

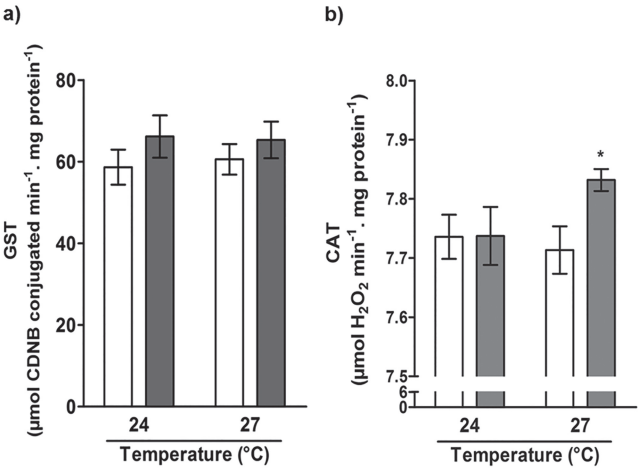

Fig. 1. Activities of glutathione S-transferase (GST) (**a**) and catalase (CAT) (**b**) in liver of *Centropomus parallelus*, after 96 h exposure to manganese (Mn^{2+}) (grey bars) at two different temperatures, and their respective control groups (white bars). Values represent the means ± standard error. Each treatment was performed in triplicate (n = 6 per aquarium).

Comet assay. The result of the DNA damage or fragmentation, measured as the damage index (DI), demonstrated that Mn^{2+} provoked damage on DNA in both temperatures compared with the respective control group (Fig. 2a; P<0.0001; F=56.06). The temperature increase from 24 to 27°C, by itself, did not interfere with the results since the control groups were similar. However, there was a significant interaction between the two factors, Mn^{2+} and temperature (Fig. 2a; P=0.0435; F=4.24). At 27ºC, it was observed that there was a decrease in the amount of DNA damage caused by exposure to Mn^{2+} compared to the group acclimated to 24°C.

Micronuclei (MN) test. The fat snook exposed to Mn^{2+} showed a significant increase in the frequency of micronuclei in the erythrocytes of the fat snook acclimatized in two temperatures (Fig. 2b; P<0.0001; F=48.61) when compared to their respective control groups. In addition, it was observed that there was a significant interaction between the two factors, Mn^{2+} and temperature (Fig. 2b; P=0.0073; F=7.64), since the fish acclimated to 27°C and exposed to Mn^{2+} showed more micronuclei in relation to fish acclimated to 24°C and exposed to Mn^{2+}.

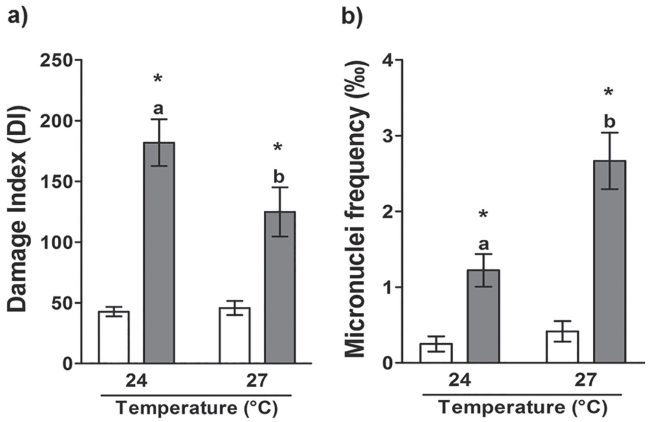

Fig. 2. Damage index (DI) (**a**) and Micronuclei frequency (**b**) in erythrocytes of *Centropomus parallelus*, after a 96 h exposure to manganese (Mn^{2+}) (grey bars) at two different temperatures, and their respective control groups (white bars). Values represent the means ± standard error. Different lowercase letters indicate significant differences ($p \geq 0.05$) between the temperature treatments, asterisks mark (*) indicate significant differences ($p \geq 0.05$) in relation to controls. Each treatment was performed in triplicate (n = 6 per aquarium).

Discussion

In the present study, the interaction between short-term exposure to a sublethal concentration of Mn^{2+} and an increase in water temperature did not affect the GST activity. Some GST changes were expected in fish exposed to Mn^{2+} since GST plays a significant role in metabolism, acting on the detoxification of some electrophilic compounds, and changes in the activities of this enzyme directly reflect metabolic disturbances and cell damage in specific organs of fish (Carvalho-Neta, Abreu-Silva, 2013); however, this response was not verified in this study. The present results might indicate that the metabolism of Mn^{2+} occurs by another biotransformation pathway. Instead, the biochemical responses of biotransformation found in this study are in accordance with the previous investigations of thermal stress by other authors. These studies reported that the levels of GSH-dependent antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST), in the different tissues of *Carassius auratus* (at 3 and 23°C) (Bagnyukova *et al.*, 2007), *Morone saxatilis* (at 7 and 25°C) *(*Grim *et al.*, 2013) and *Notothenia coriiceps* (at 0 and 8°C) (Machado *et al.*, 2014) were slightly affected by the increase in temperature.

On the other hand, the 27°C treatment resulted in a stimulation of the CAT activity, which was not observed at 24°C; it is possible that the combination of both increased temperature and Mn^{2+} exposure could be related to changes in the oxidative stress levels. According to Daoud *et al.* (2007), increased CAT activity is usually associated with rising temperatures, which, in turn, further accelerate the metabolic rate of the organisms. In the present study, this was not observed, since the CAT activity of fat snook acclimated to 27°C did not differ from those acclimated to 24°C (control groups); however, when Mn^{2+} was added to the water at 27°C, CAT activity increased significantly in the group exposed to Mn^{2+}. These results suggest that the interaction between elevated temperature and Mn^{2+} influences CAT activity. Nevertheless, there are several reports concerning patterns of CAT expression under oxidative-stress-inducing conditions, such as chemical toxicity and thermal stress. These antioxidant defenses may be increased (Khessiba *et al.*, 2005; Vinagre *et al.*, 2012, 2014), inhibited (Kaur *et al.*, 2011; Sabatini *et al.*, 2011) or unaffected (Mueller *et al.*, 2012; Gabriel *et al.*, 2013; Machado *et al.*, 2014) by agent stressors. The occurrence of one type of response or another depends on the intensity and duration of the stress applied, the susceptibility of the species that are exposed and/or the route of exposure (Bebianno *et al.*, 2005; Sanchez *et al.*, 2005). Furthermore, CAT activity may be species-specific (Fonseca *et al.*, 2011; Madeira *et al.*, 2013, 2014) and tissue-specific (Viera *et al.*, 2012; Vinagre *et al.*, 2014). In the present study, it can be observed that the association between the two conditions (temperature and Mn^{2+}) to which the fat snook specimens were exposed induced the activation of the catalase enzyme, and therefore, induced the activation of the oxidation system.

In this study, we also elucidated the modulatory effects on genotoxicity of an increase in temperature associated with Mn^{2+} exposure in *C. parallelus* through the alkaline comet assay and the micronuclei test. Both assays are useful biomarkers of environmental genotoxicity testing (Barsiene *et al.*, 2012; Dar *et al.*, 2015). Our results indicated that the exposure of *C. parallelus* to sublethal concentrations of Mn^{2+}, combined with the increase in water temperature, induced significantly higher DNA damage in the temperature treatments than in the control; thus, these results indicated the genotoxic potential of these experimental conditions. Earlier studies, which also used the comet assay method, have associated thermal stress with genotoxic effects in aquatic organisms, such as freshwater fish (*Carassius auratus*), crayfish *(Astacus leptodactylus*) and mussels (*Dreissena polymorpha*) (Anitha *et al.*, 2000; Buschini *et al.*, 2003; Malev *et al.*, 2010).

In the present study, although a significant increase in the DI was achieved in both temperature treatments compared to the control, we also observed a decrease in the DI values as the water temperature increased. Such depletion could be explained by the cytotoxic potential exerted by thermal stress, leading to changes in the blood cell kinetics and erythrocyte replacement (Çavas, Ergene-Gözükara, 2003). This observation finds support in the literature (Polard *et al.*, 2011; Vera-Candioti *et al.*, 2013); nevertheless, the decrease in DI values might be caused by other cytotoxic agents that also affected circulating blood cell populations.

The alkaline comet assay detects DNA damage, such as DNA single-strand breaks, DNA double-strand breaks

or DNA-DNA/DNA-protein cross-linking (Guilherme *et al.*, 2012), that could have originated from the interaction between the free radicals formed as a result of the oxidative stress and the DNA of the blood cells (Azqueta *et al.*, 2011; Nwani *et al.*, 2013). Moreover, unlike the micronuclei induction, the DNA damage detected by the alkaline comet assay is relatively minor and often transient (Dixon *et al.*, 2002). Furthermore, we also may suggest that the enhancement of CAT activity allows a significant decrease in DI values as the water temperature increases; repairs damaged macromolecules, such as DNA, and alleviates oxidative stress.

The micronuclei test is considered to be a sensitive and informative marker of cytogenetic damage caused by mutagenic compounds (Arslan *et al.*, 2015) and has been applied to identify the adverse potential of various genotoxic agents (Barsiene *et al.*, 2012), both in freshwater and marine sentinel species (Guidi *et al.*, 2010). The increase in MN frequency was observed in both treatment groups compared to the control. The increase in MN frequency is an indirect marker of numeric and structural chromosomal irregularities in the cells caused by many agents (Arslan *et al.*, 2015); thus, this indicates the clastogenic and/or aneugenic capacity of Mn^{2+} exposure combined with increased temperature. Furthermore, it is well documented that temperature modulates aquatic organisms' sensitivity to metals by affecting their physiological tolerance, energy demand, oxygen supply, and/or mitochondrial biogenesis (Sokolova, Lanning, 2008).

The results show that the combination of Mn^{2+} exposure and thermal stress caused significant DNA damage in *C. parallelus*, and the oxidative system can also be induced. Our findings indicate that the identified endpoints provide useful information for biomonitoring studies; additionally, they underline the need for comprehensive research on the possible influence of increased temperatures on the mechanism of Mn^{2+} and heavy metal toxicity in fish.

Acknowledgments

This work was supported by a research grant from FAPES (Proc. # 61902861). B.L.L Tuzuki was a master fellow from FAPES. F.A.C. Delunardo is a Ph.D. fellow from FAPES (Proc. # 61636509/2013). The authors thank the Instituto Nacional da Mata Atlântica (INMA) for accepting the deposit of voucher specimen in the Museu de Biologia Professor Mello Leitão.

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Submitted June 30, 2017
Accepted November 14, 2017 by Bernardo Baldisserotto