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Biomarkers of waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to salt water

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ABSTRACT

The responses of a large suite of biochemical and genetic parameters were evaluated in tissues (liver, gills, muscle and erythrocytes) of the estuarine guppy *Poecilia vivipara* exposed to waterborne copper in salt water (salinity 24 ppt). Activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, and glutathione S-transferase), metallothionein-like protein concentration, reactive oxygen species (ROS) content, antioxidant capacity against peroxyl radicals (ACAP), and lipid peroxidation (LPO) were evaluated in liver, gills, and muscle. Comet assay score and nuclear abnormalities and micronucleated cell frequency were analyzed in peripheral erythrocytes. The responses of these parameters were evaluated in fish exposed (96 h) to environmentally relevant copper concentrations (5, 9 and 20 $\mu g\,L^{-1}$). In control and copper-exposed fish, no mortality was observed over the experimental period. Almost all biochemical and genetic parameters proved to be affected by waterborne copper exposure. However, the response of catalase activity in liver, ROS, ACAP and LPO in muscle, gills and liver, and DNA damages in erythrocytes clearly showed to be dependent on copper concentration in salt water. Therefore, the use of these parameters could be of relevance in the scope of biomonitoring programs in salt water environments contaminated with copper.

1. Introduction

Metals are environmental contaminants of great concern (Phillips, 1980; Nemerow, 1991; Heath, 1995; Langston and Bebianno, 1998). They are naturally ubiquitous in aquatic environments, but their concentrations can increase as a result of the growing discharges into the environment from domestic and industrial effluents, as well as fossil fuel burning (Neto et al., 2008; Paytan et al., 2009). Mining, groundwater use, and soil waterproofing and erosion are also considered as secondary sources of metal input into aquatic environments (Heath, 1995). Nevertheless, recent work suggests that metal concentrations in the aquatic environment may actually be decreasing (Mahler et al., 2006).

Metals can be considered as harmful contaminants to aquatic animals, especially if one considers their environmental persistency, bioavailability in water, potential for bioaccumulation, and toxicity (Kennish et al., 1991). Thus, water contamination with these chemicals may result in both sublethal and lethal effects (Santos et al., 2000; Pinho et al., 2007), modifying populations and the structure of the impacted ecosystems (Langston and Bebianno, 1998; Paytan et al., 2009).

Copper is a transition metal essential to metabolism (Heath, 1995), becoming toxic to estuarine and marine invertebrate and fish when at elevated concentrations (Santos et al., 2000; Pinho et al., 2007; Martins and Bianchini, 2008; Lopes et al., 2011a). It is well accepted that the key mechanism of acute copper toxicity in fish is associated with ionic and osmotic disturbances due to impairments especially on Na^+ and Cl^- regulation linked to the inhibition of the gill Na^+-K^+-ATPase activity. Furthermore, it is reported

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that these disturbances can lead to death in both freshwater and seawater fish (Grosell et al., 2002, 2007; Alsop and Wood, 2011). However, it is not understood if these disorders are resulting from a direct inhibitory effect on the enzyme activity or are related to damages associated with a copper-induced oxidative stress. Therefore, the evaluation of the response of oxidative stress parameters (biomarkers) would help to better understand the biochemical basis of these disturbances in fish. In addition, it would also help to explain some detrimental biological effects observed after fish exposure to waterborne copper. For example, it has been reported that copper can induce endocrine disruption and changes in metabolic rates (Handy, 2003), behavior (Phillips, 1980), immunological function, swimming performance (Heath, 1995), enzyme activities, and liver histology (Langston and Bebianno, 1998).

As far as we know, studies evaluating concomitantly the responses of a large suite of biochemical and genetic biomarkers to characterize the sublethal effects of copper other than those associated with the ionic and osmotic regulation in a single marine fish species are lacking in the literature. Biomarkers are considered useful alternative tools in biomonitoring programs and have usually been employed in environmental health assessment studies (Rose et al., 2006). They are being pointed out as potential tools for an early detection of environmental pollution, as well as for preventing the biological effects associated and providing information for management. Biomarkers can be considered as measurable responses on body fluid, cells, tissues or organism indicating the presence of contaminants (for review: Monserrat et al., 2007). In this context, biomarkers of exposure can be measured to evaluate the presence of contaminants. They include chemicals, or chemical metabolites, that can be measured in the body or after excretion from the body. In turn, biomarkers of effect can be used to evaluate the quantifiable changes that an individual endures after exposure to a compound. These biomarkers may thus indicate a resulting health effect.

Despite their recognized importance in biomonitoring programs, biomarkers are still not being used to derive water quality criteria. Also, there is not a general agreement on the level of the biomarker response characterizing an environment as chemically polluted or biologically impacted. This likely arises from the fact that there is controversial information on how the biomarker response is specific to the contaminant exposure. Therefore, studies involving a large suite of biomarkers at different levels of the biological organization are necessary to identify which biomarkers are good for biomonitoring purposes, as well as to establish the relationship between the degree of the biomarker response and the environmental health condition.

With this background in mind, the present study was performed to describe, for the first time, the comparative response of a large set of biochemical and genetic biomarkers in the guppy *P. vivipara* exposed to sublethal and environmentally relevant concentrations of waterborne copper (Chester, 2003; Nayar et al., 2003; Azetsu-Scott et al., 2007; D'Adamo et al., 2008). This Brazilian euryhaline teleost fish is commonly found in both fresh and coastal water bodies along the South Atlantic Ocean (Gomes and Monteiro, 2008). It has been pointed as a promising fish species to monitor the health condition of tropical and sub-temperate coastal waters (INCT-TA, 2012). Endpoints evaluated in the present study included oxidative status parameters (reactive oxygen species, antioxidant capacity against peroxyl radicals, activities of antioxidant enzymes and concentration of non-enzymatic antioxidants), as well as the oxidative damage to biomolecules (lipid peroxidation and oxidative damage to DNA) in several fish tissues (liver, gills, and muscle or peripheral erythrocytes). The responses of these parameters were evaluated considering their suitability to detect biological effects associated with exposure to low and high environmental concentrations of copper.

Table 1
Nominal and measured (total and dissolved) copper concentrations employed in experiments with the guppy *P. vivipara* in salt water (24 ppt). Relevance according to the Brazilian environmental regulation is also presented. WQC: water quality criterion. BDL: below detection limit (1 μg Cu L^{-1}). Data are mean ± standard deviation (n = 8).

Nominal	Total	Dissolved	Environmental relevance
Copper concentration (μg Cu L^{-1})			
0	BDL	BDL	Control
5	4.25 ± 1.25	3.45 ± 0.75	WQC – salt water
9	10.25 ± 1.75	6.70 ± 1.00	WQC – fresh water
20	21.50 ± 0.05	20.75 ± 0.25	Non-conforming

2. Materials and methods

2.1. Fish collection and copper exposure

Male fish (*P. vivipara*) were collected from May 2010 to December 2011 at the 'Arroio do Gelo', a small creek running into the Cassino Beach (Rio Grande, RS, Southern Brazil). Fish were transferred to the laboratory and kept under controlled conditions (photoperiod: 12 L:12 D; temperature: 20 °C; and salinity: 24 ppt) for two weeks. Fish were daily fed until satiation with commercial food for omnivorous fish. This food is a complex mixture containing minerals, vitamins, animal and vegetal protein, algae, lipids and several probiotics (Alcon Basic, Camboriú, SC, Brazil).

Full natural sea water was diluted with dechlorinated tap water to prepare the salt water to be employed in the experiments (salinity: 24 ± 1 ppt). Salt water was filtered (0.45-μm mesh filter) and copper ($CuCl_2$; Vetec Química Fina, São Paulo, Brazil) was added from a stock solution (1 mg Cu L^{-1}). All glassware and exposure chambers were previously acid washed and thoroughly rinsed with distilled water. Contamination of the exposure media was performed 24 h before fish introduction into the test chambers to allow copper equilibration with salt water. Three copper concentrations (nominal values: 5, 9 and 20 μg L^{-1}) were tested along with a control (no copper addition into the experimental medium). The concentrations of 5 and 9 μg L^{-1} actually represent the Brazilian water quality criteria for sea water and fresh water, respectively. In turn, 20 μg L^{-1} was selected as a non-conforming copper concentration according to the Brazilian regulation (CONAMA, 2005). It is important to note that these concentrations can also be considered as representative of non-polluted, moderately contaminated and highly contaminated environment, respectively (Chester, 2003; Nayar et al., 2003; Azetsu-Scott et al., 2007; D'Adamo et al., 2008).

Total and dissolved copper concentrations were measured immediately before fish introduction into the exposure medium and 24 h after exposure. Copper concentrations were measured in non-filtered (total copper) and filtered (0.45-μm mesh filter; dissolved copper) water samples by atomic absorption spectrophotometry (AAS 932 Plus, GBC, IL, USA) following procedures previously described (Pinho et al., 2007; Martins and Bianchini, 2008; Lopes et al., 2011b). Results obtained are shown in Table 1. For practical reasons, copper concentrations will be referred hereafter considering the nominal values.

Dissolved oxygen was kept close to the saturation level by continuously and gently bubbling air in the experimental medium, which was totally renewed every 24 h to keep the water pH (~7.8) and other water physicochemical parameters constant. Adult males of *P. vivipara* were kept under control condition or exposed to copper in 30-L glass aquaria for 96 h. Fish stocking density was 1 g fish L^{-1}.

After copper exposure, fish were anesthetized with benzocaine (0.1 g L^{-1}), weighed (wet body weight), and measured (total body length). Blood was collected by puncture of the caudal vein and immediately used for comet and nuclear abnormalities assays.

Liver, muscle and gill samples were then dissected and immediately used for ROS and ACAP measurements or frozen (−80 °C) for further measurements. For practical reasons, the exposure procedure was repeated two times. Tissue samples of fish from the first exposure (wet body weight: 1.07 ± 0.31 g; total body length: 41.6 ± 4.4 mm) were used for enzyme activity measurements, lipid peroxidation (LPO) and nuclear abnormalities analyses. Tissue samples of fish from the second exposure (wet body weight: 1.01 ± 0.31 g; total body length: 43.1 ± 4.3 mm) were used for Comet assay, ROS, antioxidant capacity against peroxyl radicals (ACAP) and metallothionein-like protein (MT) measurements. The number of fish exposed in each experiment was dependent on the variability of the parameter to be analyzed, as previously determined, and fish availability in the laboratory, as described below.

2.2. Biochemical and genetic analyses

2.2.1. Oxidative stress parameters

Biomarkers of oxidative status were measured in gills (target organ), liver (detoxification organ) and muscle (non-target organ) samples. ROS and ACAP were determined (n = 4–6 fish for each experimental group) following the analytical procedures described in Amado et al. (2009). MT concentration was measured (n = 7–9 fish for each experimental group) based on the DTNB reaction with sulfhydryl groups according to the method described by Viarengo et al. (1997) and following the analytical procedures previously described (Amado et al., 2006; Martins and Bianchini, 2009).

Protein concentration in the tissue homogenate was determined using a commercial reagent kit based on the Bradford reagent method (Sigma, São Paulo, SP, Brazil). Catalase (CAT) activity was evaluated (n = 7–9 fish for each experimental group) based on the H_2O_2 degradation as described by Beutler (1975) and following the analytical procedures previously described (Geracitano et al., 2002; Amado et al., 2006). Superoxide dismutase (SOD) activity was evaluated (n = 4–6 fish for each experimental group) measuring the cytochrome C reduction (McCord and Fridovich, 1969), following the analytical procedures previously described (Geracitano et al., 2002; Amado et al., 2006). Glutathione reductase (GR) activity was measured (n = 5 fish for each experimental group) following the NADPH consumption in the presence of oxidized glutathione (GSSH), following the analytical procedures described by Carlberg and Mannervik (1975). Glutathione S-transferase (GST) activity was assessed (n = 5–7 fish for each experimental group) measuring the conjugation of the reduced glutathione (GSH) with CDNB (Keen et al., 1976), following the analytical procedures previously described (Geracitano et al., 2002; Amado et al., 2006). It is important to note that the term "activity" employed here for a given enzyme in fact corresponds to its concentration and not its in vivo activity, i.e., the conditions of the enzymatic assays employed were chosen so as to measure the V_{max} value for the enzyme, and not its true activity in the original tissue.

2.2.2. Oxidative damage parameters

LPO was measured (n = 6–10 fish for each experimental group) using the thiobarbituric acid reactive substances method (TBARS), following the analytical procedures described by Oakes and Van Der Kraak (2003).

Fresh erythrocytes were used to analyze the damages (reversible and non-reversible) to DNA. Single or double reversible DNA strand breaks were scored (n = 4–6 fish per experimental group) using the Comet assay and following the procedures described by Tice et al. (2000), with some modifications. In this case, analysis was performed using red gel as fluorescent marker. The tail of each comet was classified into 4 types of damage, with indexes varying from 0 to 3 (class 0, class 1, class 2 and class 3). Increasing class index corresponded to augmented DNA damage. Approximately 100 nucleoids were observed per fish. Measurements were performed in duplicate. Comet score was calculated multiplying the number of nucleoids belonging to each class by the respective class index. Therefore, higher comet scores corresponded to higher DNA strand breaks.

Non-reversible DNA damages were accessed in 10^3 cells per fish (n = 16) by counting the frequency of micronucleated cells, nuclear buds, binucleated cells and cells with nucleus presenting apoptotic fragments. These parameters were evaluated following the procedures described by Barsiene et al. (2006). The frequency of cells with bilobed nucleus was also considered. It is known that hematopoietic organs can release immature cells into the fish bloodstream (Heath, 1995). These immature erythrocytes may show a nucleus still in division, which has the appearance of a cell with bilobed nuclei. Therefore, the frequency of erythrocytes with bilobed nucleus was considered as an index of immature cells release into the blood stream.

Table 2
Conjugated families of 'probability distribution' and 'a priori parameters' used to obtain the 'posteriori distributions' for each biomarker analyzed in the guppy *P. vivipara* acclimated to salt water (salinity 24 ppt) exposed (96 h) to copper.

Parameter	Conjugated family	*A priori* parameter
Oxidative status	Normal-gamma & normal	Jeffreys's non informative[a]
Score comet	Normal-gamma & normal	Jeffreys's non informative[a]
Comet class 0	Gama & Poisson	α = 13.0, β = 0.18
Comet class 1	Gama & Poisson	α = 0.28, β = 0.03
Comet class 2	Gama & Poisson	α = 3.57, β = 0.59
Comet class 3	Gama & Poisson	α = 2.17, β = 2.17
Micronucleus	Gama & Poisson	α = 1.79, β = 5.97
Nuclear buds	Gama & Poisson	α = 0.41, β = 2.72
Apoptotic fragments	Gama & Poisson	α = 2.45, β = 24.49
Binuclear cells	Gama & Poisson	α = 3.61, β = 6.23
Bilobed nucleus	Gama & Poisson	α = 1.00, β = 0.01

[a] According to mathematical solution presented by Kinas and Andrade (2010).

2.3. Statistical analyses

Statistical analyses were performed using Bayesian methods as described by Gelman et al. (2004). All mathematical basis for statistical procedures used in the present study is explained in details by Kinas and Andrade (2010). Briefly, the model of probability (normal or abnormal distributions) most appropriate for average and standard deviation calculation was generated for each parameter, allowing a deeper and more adjusted inference. It is worth to note that the Bayesian approach came up as the most adequate method to analyze the probability of alternative hypothesis, given the ecotoxicological context in which the present study was performed, as well as the data effectively generated. Therefore, data and their distribution models reported allow the incorporation of new data from future laboratory and even environmental studies, constituting a tentative tool to be used for environmental management.

Conjugated families of probability were used to calculate a posteriori distributions for averages and standard deviations. In terms of variables, parameters analyzed are expressed as concentration, content, rate, or counting. This type of data tend to follow a normal distribution, while counting data are likely described by Poisson distribution (Gelman et al., 2004). Therefore, the conjugated family of normal-gamma a posteriori distributions with non-informative priori was used to estimate averages and standard deviations for most of the parameters analyzed, which were assumed to follow a normal distribution (Table 2). For nuclear abnormalities and comet assay data, the conjugated family of gamma distribution was used, since they were obtained by counting and were assumed to follow a Poisson distribution. These gamma distributions have two prior hyper-parameters, which are α and β. These parameters can

be derived from prior averages and standard deviation since there is a strict relationship between them (Gelman et al., 2004). In this case, α is the square of average divided by the square of standard deviation, while β is described as being the average divided by the square of standard deviation. In the present study, prior average and deviation values used for DNA damage parameters were obtained from Barsiene et al. (2006), Amado et al. (2006), and Negreiros et al. (2011). Values reported in these studies for control animals were compiled and used to adjust the a priori parameters for a gamma probability distribution for each DNA damage parameter, where the a priori expectancies were the mean values found in those studies, while a priori standard deviations were the values reported in these studies multiplied by 3 in order to increase the prior open-mindedness. The respective hyper-parameters (α and β) for each DNA damage parameter are shown in Table 2.

To access the strength of evidence for differences among treatments, frequentist hypothesis testing was replaced with Bayes's decision, based on the a posteriori odds ratio and Bayes factor (Jeffreys, 1961). The posterior distributions of differences (i.e. average group 1 and average group 2) for the most likely averages for each treatment and parameter were obtained from simulations. Then, distributions of differences were evaluated for the position around zero. Posteriori differences concentrated around zero favor hypothesis zero (H_0), negating the difference between averages. In turn, a posteriori distributions of differences away from zero favor hypothesis 1 (H_1), accepting the difference between averages. For these tests, we used a priori odds ratio = 1 (i.e. giving the same a priori probability to both H_0 and H_1) and a loss $w_0 = 5$ and $w_1 = 1$ for erroneously rejecting H_0 and H_1, respectively. Bayes factor (BF) is only reported when significant differences are pointed out in Section 3. It may be worth to mention that BF express how many times H_1 is more likely to occur than H_0, and a BF value higher than 3.14 is considered as a substantial evidence against H_0 (Jeffreys, 1961). According to the methodology adopted, H_0 was rejected only when BF was higher than 5. Relationship between parameters analyzed was evaluated using the Pearson linear correlation coefficient.

3. Results

No mortality was observed in control and copper-exposed fish, but almost all parameters evaluated were significantly affected by waterborne copper exposure. However, not all of them showed a clear concentration-dependent response in the range of copper concentrations tested.

Reactive oxygen species content was increased (1.3-fold) in liver of fish exposed to 20 μg Cu L^{-1} (BF > 22). Also, gills of fish exposed to 9 μg Cu L^{-1} (BF > 8) and 20 μg Cu L^{-1} (BF > 7) showed higher ROS content (1.6-fold) when compared to the ROS content observed in gills of fish exposed to 5 μg Cu L^{-1}. In the other hand, ROS content was slightly reduced (0.8-fold) in muscle of fish exposed to 5 μg Cu L^{-1} (BF > 5) (Fig. 1A).

ACAP was significantly increased (2.6-fold) in liver of fish exposed to 20 μg Cu L^{-1} (BF > 54) and reduced (0.5-fold) in muscle of those exposed to 5 μg Cu L^{-1} (BF > 77) (Fig. 1B).

MT concentration was reduced in liver of fish exposed to 5 μg Cu L^{-1} (0.6-fold; BF > 5) and 20 μg Cu L^{-1} (0.7-fold; BF > 7). Similar reduction (0.6-fold) was observed in gills of those exposed to 5 μg Cu L^{-1} (BF > 7). However, significant increase in MT concentration was observed in gills of fish exposed to 9 μg Cu L^{-1} (1.4-fold; BF > 8) and 20 μg Cu L^{-1} (1.5-fold; BF > 6) (Fig. 1C).

Regarding key enzymes involved in the antioxidant defense system (Fig. 2), SOD activity was reduced in liver of fish exposed to 20 μg Cu L^{-1} (0.6-fold; BF > 6) and in gills of those exposed to 5 μg Cu L^{-1} (0.7-fold; BF > 6) or 9 μg Cu L^{-1} (0.6-fold; BF > 14). In muscle, no significant change was observed (Fig. 2A). A linear increase

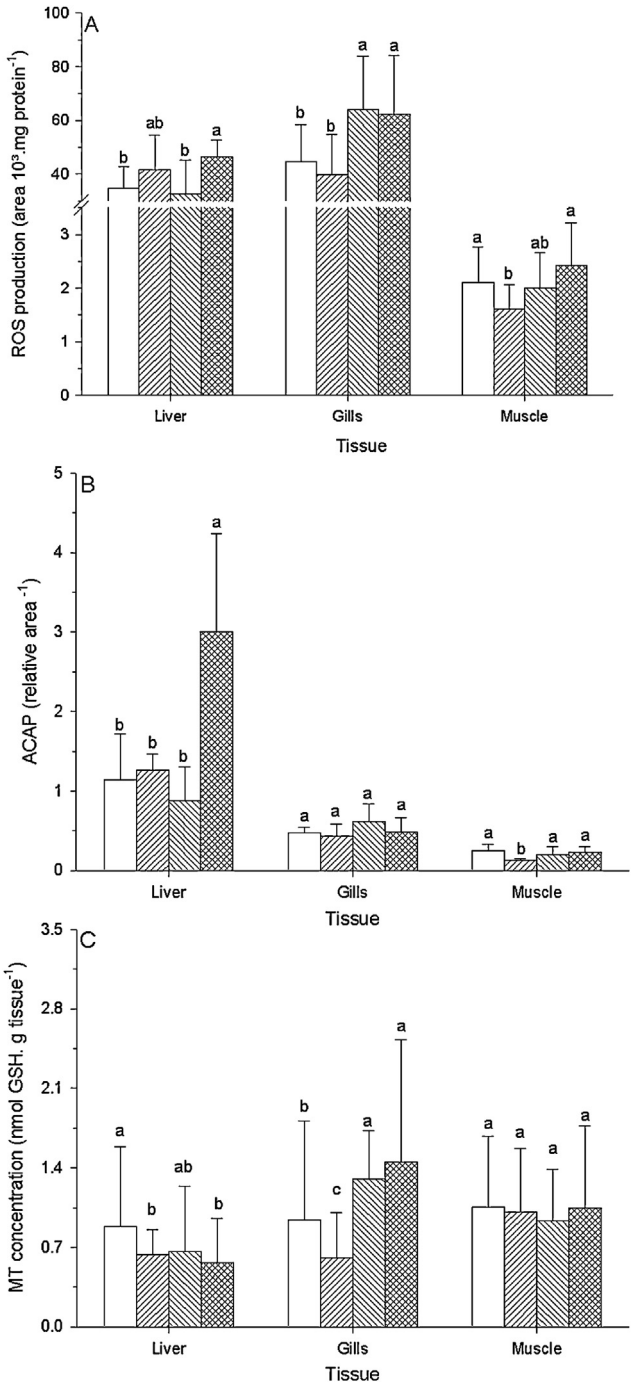

Fig. 1. (A) Reactive oxygen species (ROS) production, (B) total antioxidant capacity against peroxyl radicals (ACAP), and (C) metallothionein-like proteins (MT) concentration in liver, gills, and muscle of the guppy *P. vivipara* kept under control conditions (open bars) or exposed (96 h) to 5 μg Cu L^{-1} (diagonally hatched bars), 9 μg Cu L^{-1} (diagonally inverted hatched bars) and 20 μg Cu L^{-1} (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation of posterior distribution (ROS: $n = 4$–6; ACAP: $n = 4$–6; MT: $n = 7$–9). Different letters indicate significantly different mean values among treatments for each tissue.

in CAT activity was observed in liver of fish exposed to copper ($r^2 = 0.89$), with enzyme activity being significantly different from control values in those exposed to 5 μg Cu L^{-1} (1.2-fold; BF > 14) and 20 μg Cu L^{-1} (4.4-fold; BF > 833). No significant change was observed in muscle CAT activity (Fig. 2B). GR activity was increased in liver of fish exposed to 5 (2.6-fold; BF > 13) and 9 μg Cu L^{-1} (2.4-fold; BF > 10) (Fig. 2C). A linear increase in GST activity was observed

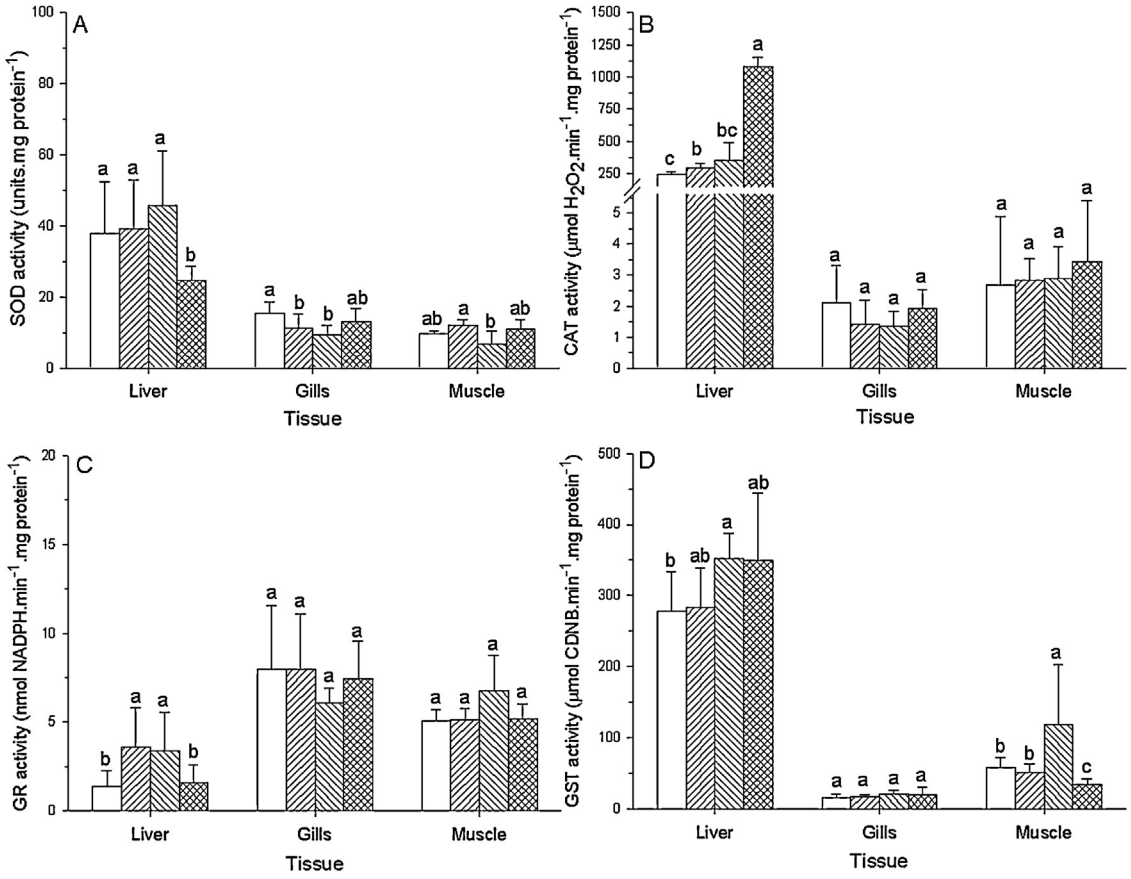

Fig. 2. (A) Superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione reductase (GR) and (D) glutathione S-transferase (GST) activity in liver, gills, and muscle of the guppy *P. vivipara* kept under control (open bars) or exposed (96 h) to 5 μg Cu L^{-1} (diagonally hatched bars), 9 μg Cu L^{-1} (diagonally inverted hatched bars) and 20 μg Cu L^{-1} (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation of posterior distribution (SOD: n = 4–6; CAT: n = 7–9; GR: n = 5; GST: n = 5–7). Different letters indicate significantly different mean values among treatments for each tissue.

as a function of copper concentration in liver (r^2 = 0.72). However, a significant increased enzyme activity was only observed in liver of fish exposed to 9 μg Cu L^{-1} (1.3-fold; BF >5). An increased (2.1-fold) GST activity was observed in muscle of fish exposed to 9 μg Cu L^{-1} (BF > 21), while a decreased (0.6-fold) enzyme activity was observed in muscle of those exposed to 20 μg Cu L^{-1} (BF > 9) (Fig. 2D).

In liver, exposure to all copper concentrations induced increases in TBARS value as high as 3.1-fold (BF > 1387), suggesting significant increases in LPO. In gills, significant increases in TBARS values were observed in fish exposed to 9 μg Cu L^{-1} (2.1-fold; BF > 123) and 20 μg Cu L^{-1} (2.2-fold; BF > 138) (Fig. 3). In fact, a linear increase as a function of copper concentration in the water was observed in TBARS values in liver (r^2 = 0.84) and gills (r^2 = 0.76). A very strong positive correlation was observed between ROS content and TBARS levels in gills (r^2 = 0.97) and between TBARS levels in gills and liver (r^2 = 0.99). In muscle, only fish exposed to 9 μg Cu L^{-1} showed significantly increased TBARS values (1.2-fold; BF > 21) (Fig. 3).

Regarding recoverable DNA damage (strand breakage), class distribution and score values from the comet assay indicated a significant increase in DNA single and double breaks in peripheral erythrocytes of fish exposed to any copper concentration tested (BF > 919) (Fig. 4). It is worth to note that fish exposed to 20 μg Cu L^{-1} showed a significant reduction (0.8-fold) compared to those exposed to 9 μg Cu L^{-1} (BF > 10). They showed less nucleoids in classes 2 and 3, and a consequently lower Comet score. This pattern is similar to that observed for nuclear buds and apoptotic fragment frequencies (Fig. 5A), which were significantly higher (5.5-fold) in fish exposed to 9 μg Cu L^{-1} (BF ∼ ∞). However, micronucleated cells frequency was increased in fish exposed to 9 μg Cu L^{-1} (2.5-fold; BF > 45) and 20 μg Cu L^{-1} (3.0-fold; BF > 171) (Fig. 5B). In fact, a highly significant and positive linear correlation was observed between micronucleated cell frequency and copper concentration

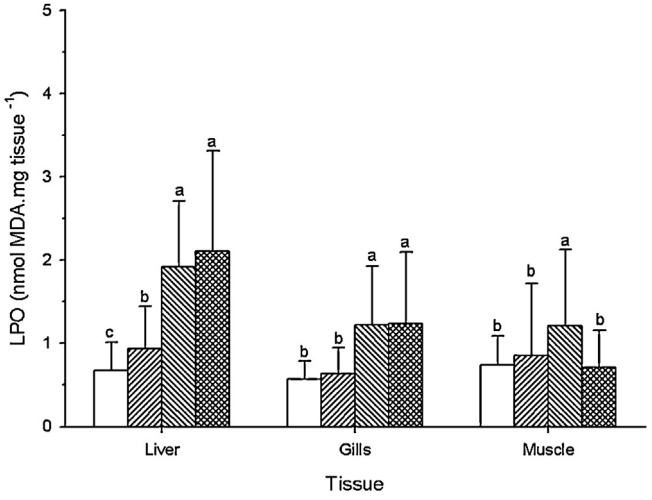

Fig. 3. Lipid peroxidation (LPO) in liver, gills, and muscle of the guppy *P. vivipara* kept under control conditions (open bars) or exposed (96 h) to 5 μg Cu L^{-1} (diagonally hatched bars), 9 μg Cu L^{-1} (diagonally inverted hatched bars) and 20 μg Cu L^{-1} (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation of posterior distribution (n = 6–10). Different letters indicate significantly different mean values among treatments for each tissue.

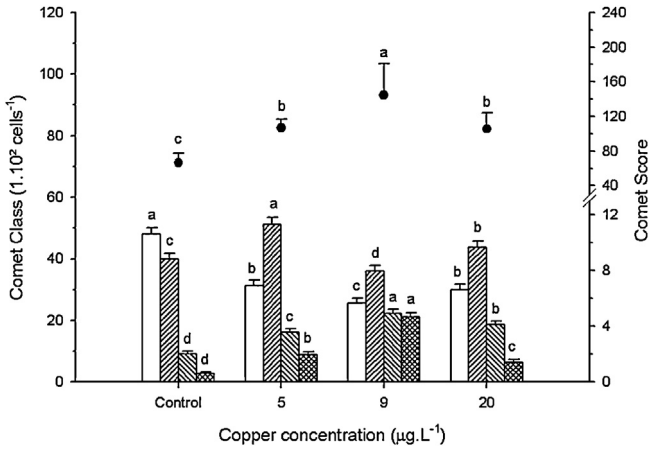

Fig. 4. DNA damage measured through Comet assay in erythrocytes of the guppy *P. vivipara* kept under control conditions (control) or exposed (96 h) to different concentrations of waterborne copper in salt water (salinity 24 ppt). For Comet class (left *y*-axis) (class 0: open bars; class 1: diagonally hatched bars; class 2: diagonally inverted hatched bars; class 3: double hatched bars), bars represent means and vertical lines correspond to standard deviation of posterior distribution. For comet score (right *y*-axis), data are expressed as mean (closed circles) ± standard deviation (vertical lines) of posterior distribution ($n = 4$–6). Different letters indicate significantly different mean values among treatments for each parameter analyzed.

in the water ($r^2 = 0.90$). The frequency of erythrocytes with bilobed nuclei decreased (0.8-fold) in fish exposed to 5 μg Cu L^{-1} (BF > 3400), while it increased (1.1-fold) in those exposed to 20 μg Cu L^{-1} (BF > 28). The frequency of binucleated cells did not change after exposure to the copper concentrations tested (Fig. 5A).

4. Discussion

Copper concentrations tested in the present study (0–20 μg Cu L^{-1}) can be considered as being acutely sublethal to the guppy *P. vivipara* acclimated to salt water (salinity 24 ppt). In fact, no fish mortality was observed at any experimental condition tested. A lack of fish mortality was also previously observed in another experiment performed under the same experimental conditions (salinity, time of exposure, and copper concentrations) with *P. vivipara* in our laboratory. In this study, no significant copper accumulation was observed in whole-body (control fish = 1.45 ± 0.30 μg g^{-1} wet weight), gills (control fish = 16.42 ± 3.87 μg g^{-1} wet weight) and liver (control fish = 99.15 ± 22.02 μg g^{-1} wet weight) of saltwater guppies acutely (96 h) exposed to 5, 9 and 20 μg Cu L^{-1}. However, copper was significantly accumulated in the gut of these fish (control fish = 18.77 ± 3.82 μg g^{-1} wet weight) (Silva et al., 2013). Taken altogether, these findings indicate that the internal concentrations of copper are well under tight homeostatic control within the range of concentrations tested (0–20 μg Cu L^{-1}). Also, they point that the actual Brazilian environmental regulation (CONAMA, 2005) is effectively protecting the guppy *P. vivipara* in salt water if we consider only acute exposure (96 h) and lethal effects. However, as demonstrated in the present study and discussed below, acute exposure to copper concentrations as low as 5 μg Cu L^{-1} induced several relevant biochemical and genetic effects in *P. vivipara* acclimated to salt water.

All biomarkers analyzed in the present study are reported to respond to copper or other metals present in the aquatic environment (Heath, 1995; Handy, 2003; Amado et al., 2006; Monserrat et al., 2007; Nordlie, 2009; Alsop and Wood, 2011; Wang et al., 2011). It is accepted that they integrate several molecular and cellular processes, providing a general picture on the organism's health. On the other hand, it is recognized that they can be influenced by distinct physiological processes, which may mask their response to

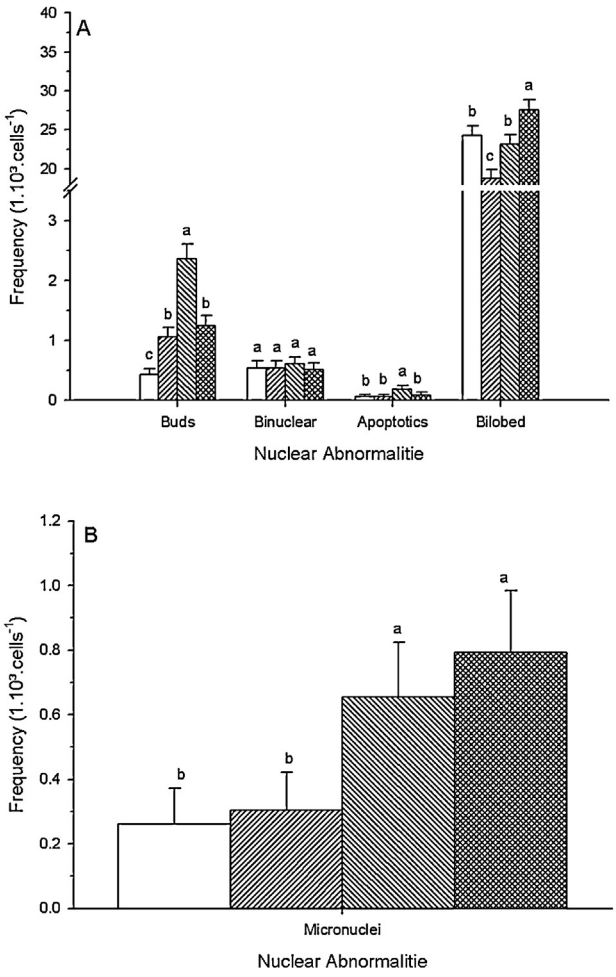

Fig. 5. DNA damage measured through nuclear abnormalities in erythrocytes of the guppy *P. vivipara* kept under control conditions (open bars) or exposed (96 h) to 5 μg Cu L^{-1} (diagonally hatched bars), 9 μg Cu L^{-1} (diagonally inverted hatched bars) and 20 μg Cu L^{-1} (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). (A) Frequency of nuclear abnormalities: nuclear buds, binucleated cells, cells with nucleus presenting apoptotic fragments, cells with bilobed nucleus. (B) Frequency of micronucleated cells. Bars indicate means and vertical lines represent standard deviation of posterior distribution ($n = 16$). Different letters indicate significantly different mean values among treatments for each parameter analyzed.

low concentrations of contaminants, thus hampering their use as suitable tools in biomonitoring programs (for review: Monserrat et al., 2007).

Findings from the present study show that not all of the analyzed biomarkers were able to respond to the environmentally relevant concentrations of copper tested (Chester, 2003; Nayar et al., 2003; Azetsu-Scott et al., 2007; D'Adamo et al., 2008). Furthermore, only few of them showed a clear relationship between the degree of response and the concentration of copper in water. After considering the consistency of the response of each parameter evaluated, as well as their physiological relevance, sensitivity to copper, and our previous knowledge on their response to high copper concentrations, it was possible to classify the potential of each biomarker response as a suitable biomarker to monitor the salt water contamination with low and high copper concentrations using the guppy *P. vivipara* (Table 3). Arguments supporting the selection of some biomarkers as potential tools for biomonitoring purposes and their physiological meaning are discussed below.

Let us first consider our findings on tissue MT concentration in the guppy *P. vivipara* exposed to waterborne copper in salt water, since this biomarker is generally used to identify exposure

Table 3
Responsive biomarkers to copper exposure in the guppy *P. vivipara* acclimated to salt water. Sensitivity level, physiological relevance and potential as a useful tool to monitor low and high levels of copper in aquatic environments are presented.

Parameter	Sensitivity[a] ($\mu g\ Cu\ L^{-1}$)	Physiological relevance	Concentration response	Potential as biomarker	
				Low levels	High levels
SOD	5	Exposure biomarker	No	Low	Medium
CAT	5	Exposure biomarker	Yes	High	High
GR	5	Exposure biomarker	No	Low	Low
GST	9	Exposure biomarker	No	Low	Medium
MT	5	Exposure biomarker	No	Low	High
ROS	9	Exposure biomarker	No	Medium	High
ACAP	5	Health condition	No	Medium	High
LPO	5	Effect biomarker	Yes	High	High
CA	5	Effect biomarker	Yes	High	High
NB	5	Effect biomarker	Yes	Medium	Medium
BN	5	Effect biomarker	No	Low	Low
AF	9	Effect biomarker	No	Low	Medium
MC	9	Effect biomarker	Yes	High	High

[a] The lower copper concentration inducing a significant biomarker response. SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; GST: glutathione S-transferase; MT: metallothionein-like proteins; ROS: reactive oxygen species; ACAP: antioxidant capacity against peroxyl radicals; LPO: lipid peroxidation; CA: comet assay; NB: nuclear buds frquency; BN: bilobed nucleus frequency; AF: apoptotic fragments frequency; and MCF: micronucleated cells frequency.

of aquatic animals to metals, including copper (Langston and Bebianno, 1998). MT is a family of cysteine-rich proteins, showing low molecular weight and high capacity for chelating metals, including copper (Valko et al., 2005). Also, their role as antioxidant agent is reported, since they are able to bind free radicals (for review: Monserrat et al., 2007). Therefore, these cytosolic proteins can contribute to decrease the deleterious effects of metals and ROS (Viarengo et al., 1997; Martins and Bianchini, 2009). In fact, an increased ROS content associated with an augmented MT level was observed in gills of fish exposed to 9 and 20 $\mu g\ Cu\ L^{-1}$. In turn, this increased level of MT would be related to higher levels of whole-body copper accumulation, since copper is shown to induce MT synthesis in aquatic animals (for review: Monserrat et al., 2007). As opposed to gills, liver showed an increased ROS content which was paralleled by a decreased MT content. Taken altogether, these findings suggest that MT would be 'mobilized' at some extent from the liver to support in some way the gills to keep increased MT levels in order to avoid or reduce the direct effect of waterborne copper on this multifunctional organ.

ROS are generated naturally as side products of certain metabolic pathways (Lushchak, 2011), especially during aerobic metabolism. Therefore, all aerobic animals show mechanisms to scavenge ROS (Geracitano et al., 2002; Valko et al., 2005). Such mechanisms are crucial in the whole-organism homeostasis, since ROS can react with biomolecules causing damages to DNA, proteins and lipids (Stohs and Bagchi, 1995). Genotoxicity and disturbances in membrane fluidity are some consequences from these injuries, respectively (Heath, 1995). However, several environmental and anthropogenic stressors, such as salinity changes and chemical pollution, can induce an excessive ROS production. In this case, toxicity would be directly associated with the metal effect on the balance between ROS production and antioxidant (enzymatic and non-enzymatic) defenses (for review: Monserrat et al., 2007). However, these stressors can also indirectly affect ROS production and toxicity by increasing metabolic rates (Martins and Bianchini, 2009). In this case, some organisms might show a depressed metabolism in certain tissues as a strategy to reduce ROS production and to better deal with the contaminant exposure. This could explain the lack of change in ROS content in muscle of *P. vivipara* exposed to 9 and 20 $\mu g\ Cu\ L^{-1}$. In turn, the reduced ROS content observed in muscle of guppies exposed to 5 $\mu g\ Cu\ L^{-1}$ is consistent with the concomitant reduced ACAP value found in this tissue under this experimental condition.

It is important to note that alterations in ROS content are associated with changes in the activity of several enzymes and concentration of antioxidant molecules to adequately scavenge the distinct types of ROS and avoid the subsequent deleterious oxidative damages in tissues (Lushchak, 2011). Some of these enzymes, such as SOD and CAT, are usually regulated by complex and interplaying systems sensitive to the concentration of their substrates (Lushchak, 2011). Thus, these enzymes usually show increased activity when production of superoxide and peroxide anions are increased up to some level, respectively (McCord and Fridovich, 1969; Beutler, 1975; Lushchak, 2011). The reactions catalyzed by these two enzymes are likely to occur one followed by the other. However, in the present study SOD activity was slightly reduced in liver of *P. vivipara* exposed to copper while CAT activity was strongly increased, suggesting the existence of uncoupled reactions for superoxide and peroxide radicals. Some authors suggest that contaminants may cause peroxisomal proliferation as a process related to an increased CAT activity after exposure to the contaminant (Valko et al., 2005; Amado et al., 2006). In fact, CAT activity has been reported to be more sensitive to other water contaminants (atrazine and phenanthrene) than SOD activity in the guppy *P. vivipara* acclimated to salt water (unpublished data). Therefore, CAT activity seems to be a more suitable tool as a biomarker in water quality monitoring programs than SOD activity, especially because it is cheaper and easier to measure.

GR and GST are also among the main enzymatic antioxidants (Roche and Bogé, 1993). While GR recycles the GSSH to GSH, GST conjugates the GSH with toxicants or ROS, allowing their metabolism by the P-450 complex (for review: Monserrat et al., 2007). In the present study, activities of these enzymes in gills and muscle of the guppy *P. vivipara* were not clearly responsive to copper exposure. However, GR activity was increased in liver of fish exposed to 5 and 9 $\mu g\ Cu\ L^{-1}$, while GST activity seems to respond only at concentrations higher than 9 $\mu g\ Cu\ L^{-1}$, when GR activity starts to decrease. These findings suggest that fish exposed to lower copper concentrations recycles GSSH, thus leading to a lower consumption of GSH. In turn, a larger consumption of this antioxidant agent would occur through the GST route when fish are exposed to higher copper concentrations. The increased GST activity observed in the liver as a function of copper concentration in the exposure medium can also be related to the higher levels of DNA breaks recorded in erythrocytes of fish exposed to copper. In fact, GST has been pointed out as being involved in the defense mechanism against the DNA peroxidative products (Amado et al., 2006).

Although the activities of the enzymes discussed above may provide important information on which mechanism of detoxification are turned on, they were not sensitive or did not show a clear copper concentration-dependent response, except in the case of

liver CAT activity. It may be possible that the activities of these enzymes are not monotonically responsive to low copper concentrations, mainly due to the integrated and complex way on how these enzymes are activated or deactivated within the whole chain of reactions and interplaying gene activator factors (Valko et al., 2005; Lushchak, 2011). Furthermore, their responses are not easily extrapolated at higher levels of biological organization, decreasing their applicability in biomonitoring programs. Therefore, we suggest that the measurement of a single exposure biomarker, such as liver catalase activity, may provide enough information on the exposure of the guppy *P. vivipara* to waterborne copper in salt water.

Regarding the most integrative parameters of the tissue oxidative status, it is important to note that ACAP integrates the response of most enzymatic and non-enzymatic antioxidants, while LPO is an indicative of the ROS/ACAP balance. Moreover, some contaminants such as copper may interact with biomolecules, thus causing direct oxidative damages to these molecules, which in turn are reflected in part by increased LPO values (Stohs and Bagchi, 1995). Therefore, LPO and ACAP responses have been pointed as useful biomarkers of effect and exposure, respectively (Rose et al., 2006). In fact, metal-induced oxidative stress is well reported in estuarine and marine vertebrates and invertebrates (Roche and Bogé, 1993; Heath, 1995; Geracitano et al., 2002; Ferreira-Cravo et al., 2009). In general, copper is considered as having a high oxidative potential (Stohs and Bagchi, 1995). Its effectiveness in inducing oxidative stress is in fact linked to its ability to participate in Fenton-like reactions and cycle between Cu(II) and Cu(I) oxidation states within the cell (Jomova et al., 2012). This characteristic could explain the toxic effects of this metal in estuarine animals (Ferreira-Cravo et al., 2009).

In the present study, ACAP was increased in liver of fish exposed to 20 μg Cu L^{-1}, while LPO showed a clear tendency of increase as copper concentration augmented in salt water. The observed increase in ACAP is likely a result from the response of the biochemical and physiological mechanisms in attempt to avoid or reduce the tissue damage induced by the increased ROS content. It can be explained, at least in part, considering the observed increase in liver CAT activity. However, it is important to note that the observed increase in ACAP was not enough to completely avoid the oxidative damage in the liver, since higher LPO levels were concomitantly observed. The clear copper concentration-dependent response showed by liver LPO and its integrative relationship with ACAP suggest the use of this biologically relevant biomarker of effect in the scope of biomonitoring programs in salt water environments contaminated with copper. However, its likely lack of specificity to copper must be taken into account.

Let us now consider the oxidative damage to DNA induced by copper in the guppy *P. vivipara*. Waterborne copper exposure significantly induced DNA strand breaks and nuclear abnormalities in fish erythrocytes. These oxidative damages are consistent with the increased ROS content and LPO values observed in gills and liver of copper-exposed fish, as discussed above. These findings have at least two major implications, one related to the physiology of the guppy *P. vivipara*, and another linked to the use of these parameters as biomarkers in biomonitoring programs.

At the physiological point of view, genetic damage observed indicate that exposure to low concentrations of waterborne copper can induce DNA damage in *P. vivipara*. This statement is based on the consistent response of the large suite of parameters analyzed, i.e., Comet score and frequency of nuclear buds, apoptotic fragments and micronucleated cells. Although Comet scores were similar in 5 and 20 μg Cu L^{-1}, erythrocytes from fish exposed to 20 μg Cu L^{-1} are likely to show significantly higher mutation levels, which are indicated by the increased frequency of micronucleated cells. The recovered condition observed in Comet score, as well as in the frequency of nuclear buds and apoptotic fragments in erythrocytes of fish exposed to 20 μg Cu L^{-1} could be explained considering an increased release of young cells into the bloodstream of fish. This statement is supported by the significant increase in bilobed cells observed in fish exposed to this copper concentration. Indeed, the release of young cell into the fish bloodstream appears to be reduced in fish exposed to 5 μg Cu L^{-1} and stimulated in those exposed to 9 μg Cu L^{-1}. An integrative analysis of the release rate of young cells and the efficiency of processes involved in DNA break recovery is reflected by the micronucleated cell frequency. Data reported in the present study suggest that even when more cells are provided into the bloodstream, fish erythrocytes cannot fully reverse the occurrence of single or double DNA breaks induced by copper exposure. This statement is supported by the significant increase in micronucleated cell frequency observed in erythrocytes of fish exposed to 9 and 20 μg Cu L^{-1}. The highly significant and positive linear correlation found between the micronucleated cell frequency and the copper concentration in water also supports this idea.

Considering the biomonitoring aspect, DNA breaks and clastogenicity parameters are considered as being closely related to genotoxicity and carcinogenesis (Mitchelmore and Chipman, 1998), putting them amongst the most relevant biological parameters. Therefore, findings reported and discussed above regarding these parameters would suggest their use as useful biomarkers in biomonitoring programs in saltwater environments contaminated with copper. The sensitivity of these parameters to waterborne copper exposure and the relationship observed between the degree of response and copper concentration in the water support this idea. However, further studies are necessary to verify the specificity of the response of these parameters only to waterborne copper.

In the sections above, we have discussed the responses of the various biomarkers in terms of sensitivity, consistency, dose–response and biological relevance. However, toxicant specificity is also an important factor to be considered regarding the use of biomarkers in environmental quality assessment. Although several of the biomarkers evaluated in the present study have been considered as being specific to certain classes of contaminants (Heath, 1995; Langston and Bebianno, 1998), the most accepted current idea is that none of them are really toxicant-specific. Taking results from the present study together with those from other similar studies performed with other contaminants, such as the herbicide atrazine and the polycyclic aromatic hydrocarbon phenanthrene (unpublished data), liver catalase activity, LPO and DNA damages appear to better characterize the fish exposure to copper under the experimental conditions employed, especially when these biomarkers are considered jointly. It might be possible to infer which is the major contaminant or environmental stressor using the biomarker approach, especially when appropriate statistical analyses are employed. In fact, adding more information in a posteriori distribution using the Bayesian approach would certainly increase the accuracy of the environmental assessment performed. Therefore, further studies using different copper concentrations in a variety of water chemistry conditions, as well as deeper modeling and exploratory approaches would certainly help to improve the selection of a biomarker or a combination of biomarkers that can better identify copper as the major stressor.

In summary, the responses of a large suite of biochemical and genetic biomarkers after acute exposure to environmentally relevant concentrations of copper were evaluated in the estuarine guppy *P. vivipara* acclimated to salt water (24 ppt). Findings reported in the present study clearly indicate that waterborne copper can significantly disturb biologically relevant parameters in *P. vivipara* exposed to copper concentrations as low as 5 μg L^{-1} in salt water. Considering the fact that *P. vivipara* shows a wide distribution, high capability to face a wide range of salinities (0–30 ppt),

high tolerance to inorganic and organic water contaminants, easy maintenance and cultivation in laboratory, small size, and response of several biomarkers to environmentally relevant concentrations of waterborne copper, we can suggest it as a potential biomonitor of copper contamination in salt water. It is important to note that some biomarkers seem not to respond monotonically to the copper concentrations tested. According to the characteristics considered in the present study (Table 3), catalase activity in liver, ROS, ACAP and lipid peroxidation in muscle, gills and liver, and DNA damages in erythrocytes could be considered as important tools for biomonitoring programs in salt water environments contaminated with copper. Despite the fact that these parameters could also be responsive to other aquatic contaminants, such as organic compounds or other metals than copper, it is worth to note that they showed a response clearly dependent on copper concentration in the water. Therefore, further studies would be important to analyze the response of these biomarkers to other contaminants and other environmental stressors for a better understanding of the relationship between each environment factor and the biomarker response.

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