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Oxidative stress and DNA damage responses to phenanthrene exposure in the estuarine guppy *Poecilia vivipara*

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ABSTRACT

Despite ubiquitous phenanthrene contamination in aquatic coastal areas, little is known regarding its potential effects on estuarine fishes. The present work evaluated the response of a large suite of oxidative stress- and DNA damage-related biomarkers to phenanthrene exposure (10, 20 and 200 μg L^{-1}, 96 h) using DMSO as the solvent in estuarine guppy *Poecilia vivipara* (salinity 24 psu). Phenanthrene affected oxidative stress-related parameters, and decreased antioxidant defenses and reactive oxygen species in the gills and muscle overall. Lipid peroxidation occurred in muscle at 200 μg L^{-1} phenanthrene. Genotoxicity was increased at 20 μg L^{-1}, while 200 μg L^{-1} caused a relative decrease in erythrocyte release into the bloodstream. These findings indicated that phenanthrene is genotoxic and can induce oxidative stress, depending on tissue and phenanthrene concentration analyzed. Thus, some of the biomarkers analyzed in the present study are sufficiently sensitive to monitor the exposure of the guppy *P. vivipara* to phenanthrene in salt water. However, further studies are required for a better interpretation of the dose–response patterns observed.

1. Introduction

Estuaries, fresh water and marine coastal environments have been used as natural deposits of chemical contaminants (Kennish, 1991), and many of these environments now exhibit deteriorated ecosystems. Thus, monitoring the deleterious effects of aquatic contaminants is crucial for the proper preservation of natural resources. The use of biomarkers in fish is a cost-effective strategy for the early assessment of the quality of the aquatic environment (Pathiratne and Hemachandra, 2010). However, limited knowledge on the responses of fish to chemical pollutants hampers the identification of suitable biomarkers for use in biomonitoring programs. Thus, it is important to better understand the response and function of biomarkers after exposure to common aquatic contaminants.

Polycyclic aromatic hydrocarbons (PAHs) are among the most problematic contaminants. They are organic pollutants that are widespread in aquatic ecosystems (Lazartigues et al., 2010), and their natural environmental concentrations are increased by the anthropogenic combustion of fossil fuel, oil spilling and burning of organic matter (Kennish, 1991; Kreitsberg et al., 2010). Once in the environment, these contaminants can be trapped in sediments, which form long-term contaminant reservoirs, thereby increasing the risks of contamination and toxicity to biota (Jee et al., 2004). Although it is generally well accepted that PAHs do not markedly biomagnify with the food web, their cellular effects can result in

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changes in organisms and their ecosystems (Kreitsberg et al., 2010). Phenanthrene is one typical example of these challenging compounds, which has been reported as the most abundant and ubiquitous PAHs in fresh, salt or brackish water, as well as in seafood and aquatic organisms (Jee et al., 2004; Lazartigues et al., 2010; Oliveira et al., 2008; Yin et al., 2007; Wenju et al., 2009).

In fish, phenanthrene has been shown to cause neurotoxicity (Barron et al., 2004), endocrine and reproductive disruptions (Han et al., 2010), cytotoxicity (Schirmer et al., 1998), genotoxicity (Oliveira et al., 2007), oxidative damage (Yin et al., 2007), impairment of growth (Jee et al., 2004), and carcinogenic and mutagenic effects (Pathiratne et al., 2010; Wenju et al., 2009). Its potential toxicity places phenanthrene among the most aggressive contaminants, in which it is considered as a priority pollutant in several countries. Unfortunately, there is still little information on its effects on tropical aquatic organisms (Sun et al., 2006; Pathiratne and Hemachandra, 2010). In addition, data on phenanthrene effects in estuarine tropical fishes are limited, and few studies have been performed focusing on the analysis of biomarkers of exposure and effects in these organisms (Oliveira et al., 2007). This type of information is rare, and Brazilian environmental regulators demonstrate no convincing scientific data on safe levels to native species on which to establish adequate water quality criteria for phenanthrene in national surface waters or sediments. Indeed, Brazilian water criteria do not limit phenanthrene concentration in any Brazilian environment (CONAMA, 2005).

The present work analyzed oxidative stress (reactive oxygen species, enzymatic and non-enzymatic antioxidants and lipid peroxidation in the liver, gills and muscle) and DNA damage (DNA strand breakage and clastogenicity in peripheral erythrocytes) in guppy *Poecilia vivipara*. This fish is one of the few test species present along the entire Brazilian coast and is found in fresh, brackish and salt waters (Gomes and Monteiro, 2008). Thus, this coastal fish species has been strongly recommended as one of most promising fish models for water quality monitoring in Brazilian coastal environments (INCT-TA, 2013). In addition, it has been previously used in ecotoxicological studies (Araújo et al., 2001), and recent studies have shown that *P. vivipara* is tolerant to chemical pollutants due to the activation of several sensitive biomarkers at environmentally relevant concentrations of contaminants (Ferreira et al., 2012; Harayashiki et al., 2013; Machado et al., 2013). Thus, the present study was designed to screen for the first time the effects of phenanthrene on a large suite of biochemical and genetic parameters in tropical estuarine guppy *P. vivipara* in salt water (salinity 24 psu).

2. Material and methods

2.1. Fish collection and exposure

Male *P. vivipara* were collected using sieve nets and minnow traps in the 'Arroio do Gelo' stream (Cassino Beach, Southern Brazil; 32°10′50.01″ S, 52°08′54.14″ O) between November 2010 and January 2011. After collection, the fish were in conditioned in buckets with air bubbling and taken to the laboratory of animal toxicology of Universidade Federal do Rio Grande (Rio Grande, RS, Brazil). Females were avoided because in this viviparous species, females are usually gravid throughout the year, indicating that they present a distinct response to pollutants than males (Harayashiki et al., 2013), and their physiology is largely influenced by gravidity status. For practical purposes, two experiments of phenanthrene exposures were performed to obtain sufficient biological material for the analyses of all biomarkers selected. Fish obtained after the first exposure (29.48 ± 5.27 mm total length; 0.649 ± 0.348 g wet body mass) were used for the analysis of antioxidant enzyme activities and nuclear abnormalities. Fish obtained after the second exposure (38.19 ± 6.85 mm total length; 0.818 ± 0.433 g wet body mass) were used for the comet assay, reactive oxygen species (ROS) and total antioxidant capacity against peroxyl radicals (ACAP). The difference in length of fish from both exposures reflected differences in the size of the animals caught from the field in November (spring time) and January (summer time). Nevertheless, all of the tested fish were at the same adult stage (sexually mature) and are considered of comparable physiological status. All fish used in this study were maintained at a fixed photoperiod (12 L: 12 D), temperature (20 °C), salinity (24 psu), and fed *ad libitum* with commercial fish food (Alcon Basic, Camboriú, SC, Brazil) for at least 10 days prior to the experiments.

Prior to exposure to phenanthrene, all glassware was washed several times with nitric acid (3%), ethanol (10%) and thoroughly rinsed with distilled water. The exposure medium was prepared with seawater diluted to salinity 24 ± 1 psu with overnight aerated tap water. This salinity was chosen because it is the reproductive optimum of this species in our laboratory (unpublished data), suggesting the salinity of physiological comfort. The seawater tap water mixture was filtered (0.45-μm mesh filter) and contaminated with phenanthrene immediately prior to the fish's introduction in the test chamber. Phenanthrene (purity >98%; Sigma–Aldrich, USA) was diluted in dimethylsulfoxide (DMSO) to obtain a stock solution of 2 mg phenanthrene.mL^{-1}, which was added into saltwater prepared as described above to achieve the desired exposure concentrations (nominal concentrations: 10, 20 and 200 μg phenanthrene L^{-1}). Two controls were also run in parallel: (1) fish exposed to saltwater without the addition of either DMSO or phenanthrene (referred hereafter as the 'control' group), and (2) fish exposed to DMSO (100 μL DMSO L^{-1}). This DMSO group was exposed to the same solvent concentration of the highest concentration of phenanthrene tested (200 μg L^{-1}) (referred hereafter as the 'DMSO' group). Phenanthrene concentrations in the exposure medium were measured (see results at Section 3.1) and were very similar to nominal values; consequently, the treatments described in the present work will be considered to involve exposure to the nominal concentration of this hydrocarbon. In addition, tested concentrations were selected taking into account three different scenarios of contamination with environmentally relevant concentrations of the hydrocarbon. Moreover, environmental concentrations of phenanthrene in estuarine and coastal contaminated areas are accepted within the range from 14 to more than 1000 μg L^{-1} (Oliveira et al., 2007; Wenju et al., 2009).

Adult male *P. vivipara* were acutely exposed to phenanthrene (96 h) in 30-L glass aquariums in duplicate under the acclimation conditions described above. The fish density at the exposure corresponded to 1 g fish L^{-1}. The exposure media received continuous gentle air bubbling to maintain the dissolved oxygen condition close to the saturation level. In addition, the exposure media were 100% renewed every 24 h to maintain a constant water pH (~7.8) and low levels of nitrogenous compounds. The number of fish tested for each biomarker measurement was determined on the basis of the fish availability in the laboratory, as well as our previous knowledge on the typical deviation shown by each specific biomarker analyzed (Machado et al., 2013).

After exposure, the fish were anesthetized with benzocaine (0.1 g L^{-1}), weighed (wet body mass), and blood was collected by puncture of the caudal vein and immediately used in comet or nuclear abnormalities assays. The liver, gills and muscle were then dissected and frozen (−80 °C) for further analyses. These procedures comply with the rules of the university ethical committee. The collection and maintenance of fish were registered at Brazilian

Environmental Ministry under SISBIO license numbers 31783, 15349-1 and 37129-1.

2.2. Phenanthrene concentration measurements

Water samples were collected immediately prior to the fish's introduction in the test chamber and after 24 h of exposure. They were pre-concentrated in a C18 (IST Isolute) activated solid-phase system according to Lanças (2004). Briefly, the method consists of filtering the water in a system that is able to bind to the PAHs, enabling sample storage for posterior elution and analysis. To achieve this, all material used, such as glass amber containers, silica gel (Merck, 60–200 mesh ASTM), neutral alumina (Al_2O_3) (Merck, 70–230 mesh ASTM) and granular sodium sulfate anhydrous were previously calcined at 450 °C for 6 h. Absorbent compounds were activated at 160 °C for 4 h, further deactivated with 5% ultrapure water (MilliQ®) and extracted with *n*-hexane. The extraction process occurred when, after C18 conditioning in methanol, water samples were slowly aspirated through the column, where the analyte remained trapped. The filtering cartridge was dried and the PAHs were eluted with ethyl acetate and *n*-hexane and were further concentrated by gentle N_2 bubbling and maintained at 10 °C until the fractionation step. This step was performed using adsorption liquid chromatography in an open column consisting of alumina and silica. Phenanthrene identification was based on the retention time and mass spectra. Analyte quantification was performed comparing the sample results with standard curves built using deuteron phenanthrene D10 (Sigma Aldrich, Sao Paulo, SP, Brazil). Both processes were performed at the Laboratory of Organic Contaminants and Ecotoxicology of the Universidade Federal do Rio Grande (Rio Grande, RS, Brazil) using gas chromatography (Perkin Elmer® Clarus 500) equipped with a mass spectrophotometry detector (CG-EM). All chemicals used were HPLC grade and the quantification limit was 0.125 ng L^{-1}.

2.3. Biomarker measurements

The number of fish tissues used for each analysis was dependent on the variability of the parameter to be analyzed, as previously determined (Harayashiki et al., 2013; Machado et al., 2013), and fish availability in the laboratory at time of the experiment. Due to the size of *P. vivipara*, some biomarker analyses required the entire liver or gills of one organism, which resulted in a total of 103 fish used in the experiments reported in this study.

2.3.1. Oxidative stress-related parameters

Oxidative stress-related parameters were assessed in the gills, liver, and muscle as examples of the uptake, detoxification and non-target organs, respectively. For most of the biochemical analyses, tissue was mechanically homogenized in phosphate 4 °C cooled buffer with pH varying from 7 to 7.5 (depending on the biomarker method). The homogenization buffer for lipid peroxidation was 1.15% KCl with 35 μM butylated hydroxytoluene (Sigma Aldrich, São Paulo, Brazil). For the antioxidant capacity, reactive oxygen species tissues were homogenized in 4 °C cooled buffer with Tris–HCl 100 mM (Sigma Aldrich, São Paulo, Brazil), ethylenediaminetetraacetic acid (EDTA) 2 mM (Sigma Aldrich, São Paulo, Brazil), $MgCl_2 \cdot 6 H_2O$ 5 mM, pH 7.75. For all methods, 1: 10 (tissue: buffer) were centrifuged at 300 rpm for 5 min (debris deposition) and the supernatant was diluted with homogenization buffer until the appropriate protein concentrations ranging from 0.7 to 3 mg protein.mL^{-1} were achieved. All protein concentrations of the tissue homogenates were determined using a commercial reagent kit (Microprote, Doles, Goiânia, GO, Brazil). Biomarkers were evaluated in total debris-free homogenate.

Reactive oxygen species (ROS) production and antioxidant capacity against peroxyl radicals (ACAP) assays were performed according to Amado et al. (2009) using tissue samples obtained from 4 to 6 fish for each treatment. In addition, because the experiments reported in the present work were performed concomitantly with other closely related studies, and control data for ROS and ACAP were previously presented in Machado et al. (2013). Metallothionein-like protein concentrations were measured using the DTNB reaction with sulfohydryl groups (Viarengo et al., 1997) using tissue samples from 4 fish. Lipid peroxidation (LPO) was estimated according to the procedures described by Oakes and Van Der Kraak (2003) using tissue samples from 9 to 15 fish for each treatment. Catalase (CAT) activity was evaluated according to the method previously described by Beutler (1975) using tissue samples from 5 to 11 fish for each treatment. Superoxide dismutase (SOD) activity was evaluated following procedures described by McCords and Fridovich (1969) using tissue samples from 5 to 9 fish for each treatment. Glutathione reductase (GR) activity was determined according to Carlberg and Mannervik (1975) using tissue samples from 4 fish. Glutathione S-transferase (GST) activity was measured according to the procedures described by Keen et al. (1976) ($N = 4$ for each tissue and treatment).

2.3.2. Erythrocyte DNA damage

Two types of DNA damage were evaluated in the present study. Reversible damage (single or double DNA strand breaks) was scored by the comet assay (Tice et al., 2000) using erythrocytes from 4 to 6 fish. This procedure was adapted for red gel use and to calculate the comet score as described by Machado et al. (2013). In addition, control data for the comet assay were previously reported in Machado et al. (2013). This assay provided a comparative analysis, in which increasing comet classes and scores were correlated to a higher level of DNA single or double strand breaks.

Non-reversible DNA damages (clastogenotoxicity) were accessed via nuclear abnormalities analysis (frequencies of micronucleated cells (MN), nuclear buds, bi-nuclear cells, apoptotic fragments and bilobed cells) according to the procedure described by Barsiene et al. (2006) and adapted by Machado et al. (2013). Analysis was performed using erythrocytes obtained from 9 to 15 fish.

2.4. Statistical analyses

A Bayesian approach was used for data analysis (Gelman et al., 2004). Conjugated families of probability were used to calculate the posteriori averages and standard deviation for each biomarker according to the analytic solutions presented by Kinas and Andrade (2010). The priori values and conjugate family of probability for each biomarker analyzed and all procedures adopted in the present work are described in details in Machado et al. (2013). These Bayesian methods were used because the present study was introduced in a research network devoted to aquatic toxicology (INCT-TA, 2013), which aims to compare and provide models for the effect of different chemicals on biomarkers for environmental monitoring. Thus, the Bayesian approach is a powerful statistical tool used to extract data information, which can be stored in models of posterior distribution of probability, for use in future environmental studies. Thus, posterior distributions may be analyzed by comparing several other parameters in addition to the average, which enables a deeper and fine-tuned probabilistic understanding on how each of the chemicals affects each biomarker.

Significant differences were accessed by hypothesis testing with Bayes' decision, which is based on the posteriori odds ratio and Bayes Factor (Jeffreys, 1961). The structure of the test has also been described in detail in Machado et al. (2013). Bayes Factor (BF) is

shown in the results section for every time significant difference detected. Interestingly, BF expresses how many times H_1 is more likely than H_0, and BF higher than 3.14 is considered substantial evidence against H_0 (Jeffreys, 1961). In this context, the present work assumed a conservative posture, rejecting H_0 only when BF was greater than 5. The BF also includes the effect of experimental N, enabling the power of analysis. Data were expressed as the average ± standard deviation for all biomarkers.

The potential effect of the solvent (DMSO) used to dissolve phenanthrene in saltwater was confirmed by comparing the responses of fish from the 'DMSO' group with those from the 'control' group. Because some of the parameters analyzed were affected by a single exposure to DMSO, the potential effects of phenanthrene were subsequently identified by comparing the responses of fish exposed to any concentration of phenanthrene to fish obtained from the 'DMSO' group.

3. Results

3.1. Phenanthrene concentrations and toxicity

Measured concentrations of phenanthrene in the exposure media and respective 24-h degradation rates for the different experimental treatments are shown in Table 1. Measured concentrations of phenanthrene varied from 97.3 to 104.5% of the desired nominal concentrations. In turn, the phenanthrene degradation rate ranged from 1.15 to 4.51% over the 24-h period depending on the exposure concentration (Table 1). Higher degradation rates were observed at higher phenanthrene concentrations. No fish mortality was observed over the 96-h experimental period in any phenanthrene concentration tested.

3.2. Oxidative stress biomarkers

First, considering the effect of the solvent, fish singly exposed to DMSO showed no significant change in ROS production in the liver, while increased mean values were found in the gills (BF > 597) and muscle (BF > 5) (Fig. 1A). ACAP was also increased after DMSO exposure in the gills (BF > 38) (Fig. 1B). The MT concentration decreased in the liver (BF > 14) and increased in the gills (BF > 5) and muscle (BF > 10) of fish exposed to DMSO for 96 h (Fig. 1C). Regarding antioxidant enzymes activities, GST activity in the liver, gills and muscle was not affected by a single exposure to DMSO (Fig. 2A), while SOD activity was reduced in the muscle (BF > 7) (Fig. 2B), and GR activity was decreased in the liver (BF > 31) and gills (BF > 5) (Fig. 2C). Moreover, CAT was not disturbed by DMSO exposure (Fig. 2D). Exposure to DMSO induced a slightly reduced LPO in the gills (BF > 13) (Fig. 3) and increased DNA damage [comet score (BF > 13) (Fig. 4) and frequency of micronucleated cells (BF > 5) (Fig. 5B)] in erythrocytes.

In phenanthrene-exposed fish, ROS generation (Fig. 1) and ACAP (Fig. 2) were not affected in the liver by any concentration of

Table 1
Nominal and measured concentrations (mean ± standard deviation) and degradation rate of phenanthrene during exposure of *Poecilia vivipara* in saltwater (salinity 24 psu). Density fish constant at 1 g fish L^{-1}. Phenanthrene concentrations in control treatments were below detection limits (BDL).

Phenanthrene concentration (μg L^{-1})			
Nominal	Measured		Degradation (%)
	Before exposure	After 24 h of exposure	
Control	BDL	BDL	NA
DMSO	BDL	BDL	NA
10	9.85 ± 0.85	9.73 ± 0.83	1.15 ± 0.21
20	20.90 ± 1.44	20.26 ± 1.46	3.09 ± 0.39
200	206.65 ± 6.71	197.35 ± 7.20	4.51 ± 0.58

BDL: Below detection limits.
NA: Non applicable.

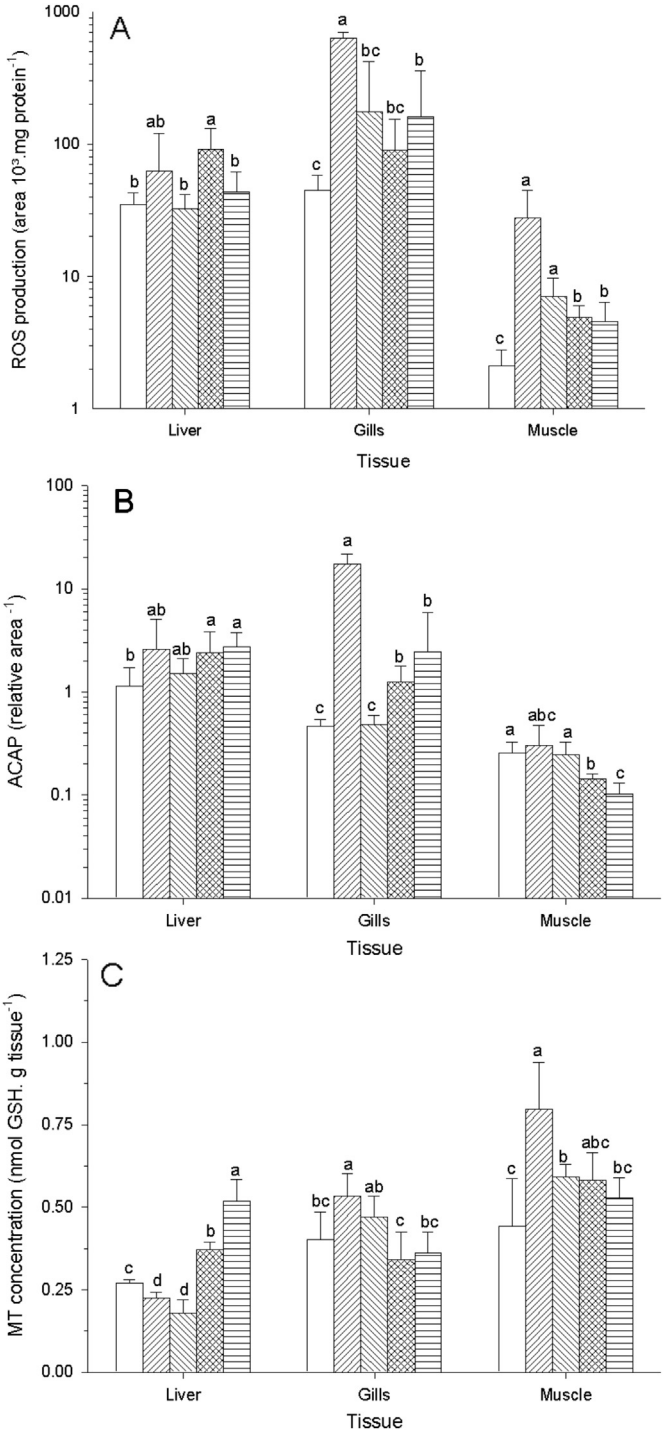

Fig. 1. (A) Reactive oxygen species (ROS) production, (B) total antioxidant capacity against peroxyl radicals (ACAP), and (C) metallothionein-like proteins (MT) concentration in the liver, gills and muscle of *Poecilia* maintained for 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 μg L^{-1} (horizontally hatched bars) of waterborne phenanthrene in saltwater (salinity 24 psu). Data were expressed as the mean ± standard deviation. Different letters indicate significant difference among treatments for each tissue within the same parameter, and means sharing the same letter are not significantly different.

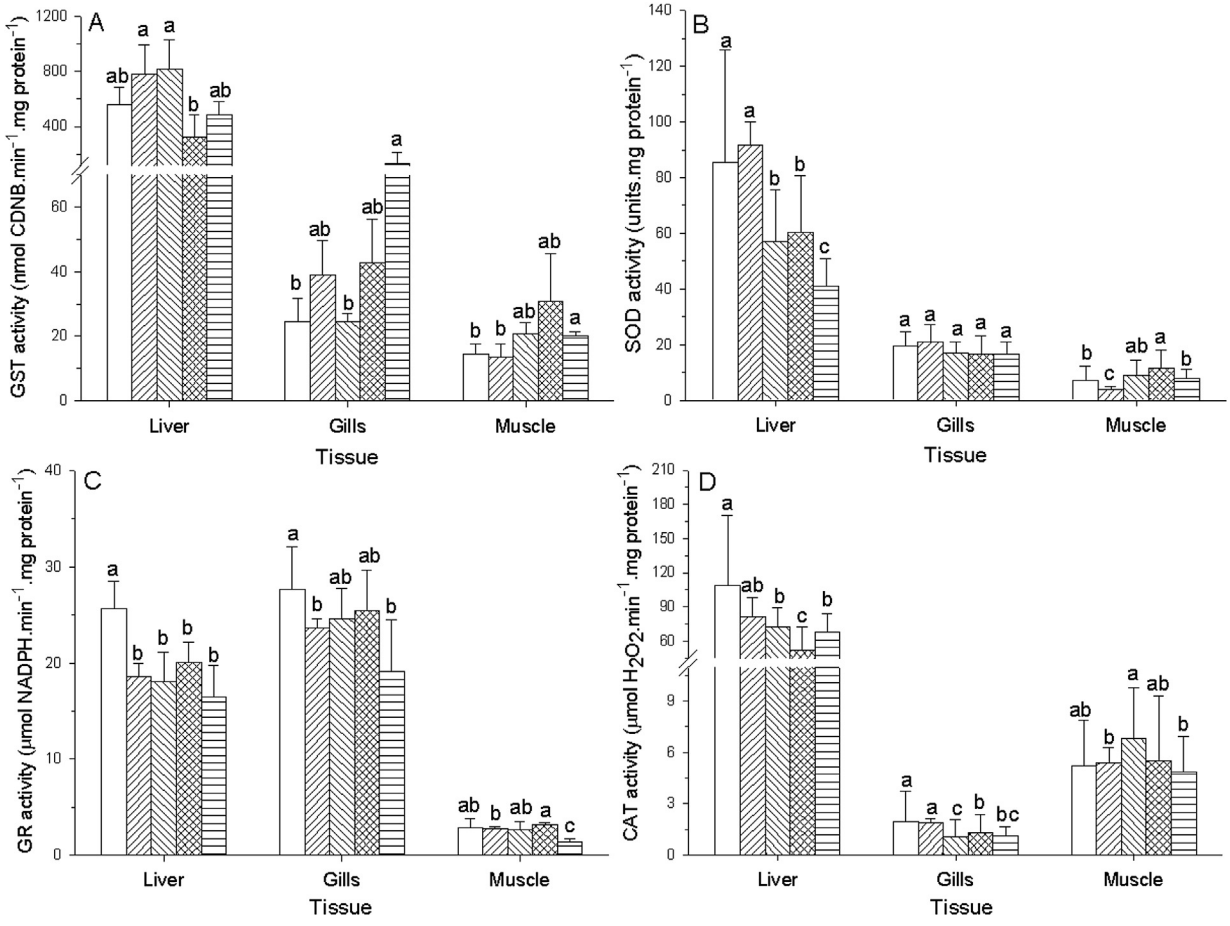

Fig. 2. (A) Glutathione S-transferase (GST), (B) superoxide dismutase (SOD), (C) glutathione reductase (GR) and (D) catalase (CAT) activity in the liver, gills and muscle of *Poecilia vivipara* maintained for 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 µg L^{-1} (horizontally hatched bars) of waterborne phenanthrene in saltwater (salinity 24 psu). Data were expressed as the mean ± standard deviation. Different letters indicate significant difference among treatments for each tissue within the same parameter, and means sharing the same letter are not significantly different.

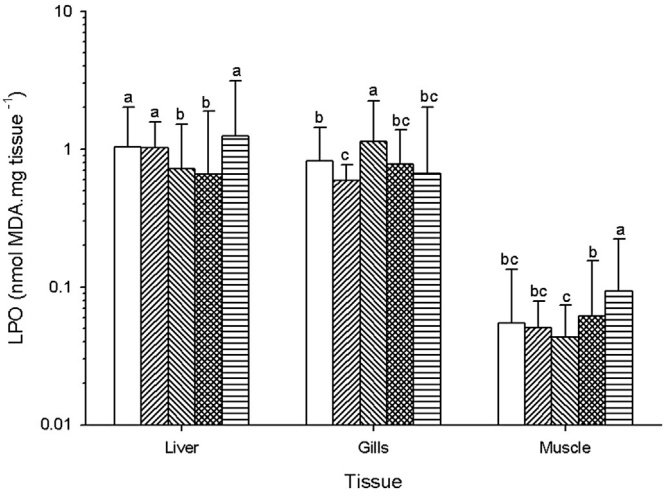

Fig. 3. Lipid peroxidation (LPO) on the liver, gills and muscle of guppy *Poecilia vivipara* after 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 µg L^{-1} (horizontally hatched bars) of waterborne phenanthrene in saltwater (salinity 24 psu). Data were expressed as the mean ± standard deviation. Different letters indicate significant differences among atrazine concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

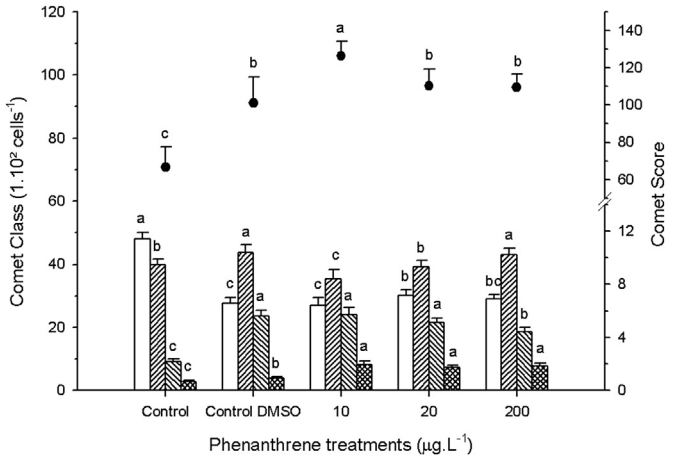

Fig. 4. DNA damage measured using the comet assay in erythrocytes of *Poecilia vivipara* exposed to phenanthrene for 96 h in saltwater (salinity 24 psu). For the comet class (left *y*-axis) (class 0: open bars; class 1: diagonally hatched bars; class 2: diagonally inverted hatched bars; class 3: double hatched bars), the bars represent the mean and the lines indicate the standard deviation. For the comet score (right *y*-axis), data were expressed as the mean (closed circles) ± standard deviation (vertical lines). Different letters indicate significant difference among treatments for each parameter, and means sharing the same letter are not significantly different.

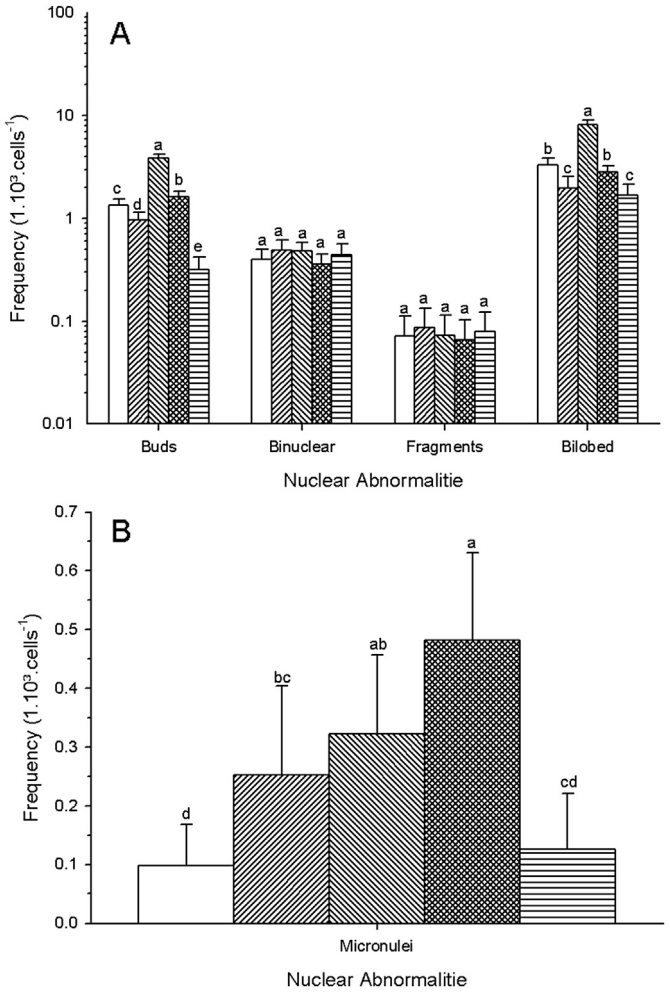

Fig. 5. DNA damage measured via the frequency of nuclear abnormalities (A), and micronucleated cells (B) in the erythrocytes of *Poecilia vivipara*. The organisms were maintained for 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 μg L^{-1} (horizontally hatched bars) of waterborne phenanthrene in saltwater (salinity 24 psu). The bars indicate the mean and the vertical lines represent the standard deviation. Different letters indicate significant difference among treatments for each parameter, and means sharing the same letter are not significantly different.

phenanthrene. However, an apparent concentration-dependent reduction in both ROS generation (BF > 383) (Fig. 1A) and ACAP (BF > 38) (Fig. 1B) was observed in the gills of fish exposed to this hydrocarbon. In muscle, reduced ROS generation was observed in fish exposed to 200 μg phenanthrene L^{-1} (BF > 5) (Fig. 1A), while no significant change was observed in ACAP (Fig. 1B). Furthermore, MT concentration was increased in the liver of fish exposed to 20 (BF > 79) and 200 μg phenanthrene L^{-1} (BF > 63), while decreased mean values were observed in the gills of these fish (BF > 14). In muscle, a significant reduction in MT concentration was observed in fish exposed to 10 (BF > 5) and 200 μg phenanthrene L^{-1} (BF > 7) (Fig. 1C).

Regarding the activity of antioxidant enzymes, phenanthrene exposure slightly reduced the GST activity in the liver of fish exposed to 20 μg phenanthrene L^{-1} (BF > 6), while no significant change was observed in the gills. In muscle, increased GST activity was observed in fish exposed to 20 μg phenanthrene L^{-1} (BF > 5) (Fig. 2A). In addition, SOD activity was reduced in the liver of fish exposed to any concentration of phenanthrene (BF > 143), while no significant change was observed in the gills. However, exposure significantly increased the SOD activity in muscle (BF > 34) (Fig. 2B). Furthermore, GR activity in both the liver and gills was not altered by phenanthrene exposure, while enzyme activity was enhanced in the muscle of fish exposed to 20 (BF > 6) and decreased in fish exposed to 200 μg phenanthrene L^{-1} (BF > 26) (Fig. 2C). CAT activity was reduced in the liver of fish exposed to 20 μg phenanthrene L^{-1} (BF > 7) and in the gills of fish exposed to any concentration of phenanthrene tested (BF > 33). Moreover, no significant change in CAT activity was observed in the muscle of fish exposed to any concentration of phenanthrene (Fig. 2D).

LPO values were decreased in the liver of fish exposed to 10 (BF > 6) and 20 μg phenanthrene L^{-1} (BF > 7), while increased values were observed in the gills of fish exposed to 10 μg phenanthrene L^{-1} (BF > 341) and in the muscle of fish exposed to 200 μg phenanthrene L^{-1} (BF > 24) (Fig. 3).

A higher comet score was observed in the erythrocytes of fish exposed to 10 μg phenanthrene L^{-1} (BF > 5) due to higher frequencies of cells demonstrating comets in class 3 after fish exposure to the hydrocarbon (Fig. 4). Nuclear buds and bilobed cells followed a similar pattern, in which higher frequencies of these nuclear abnormalities were observed in fish exposed to 10 and 20 μg phenanthrene L^{-1} (BF ~ ∞) (Fig. 5A). In addition, a higher frequency of micronucleated cells was observed in the erythrocytes of fish exposed to 20 μg phenanthrene L^{-1} (BF > 6) (Fig. 5B).

4. Discussion

The present study evaluated several biochemical and DNA damage responses after phenanthrene exposure in guppy *P. vivipara*. The degradation rates of phenanthrene in saltwater (salinity 24 psu) over a 24-h period were low (<4.51%), and measured concentrations in the exposure media were maximally 2.7% lower and 4.5% higher than nominal concentrations. These phenanthrene exposures were acutely sublethal to the guppy *P. vivipara* in saltwater (salinity 24 psu), and no mortality was observed during a 96 h period. However, several biochemical and genetic parameters analyzed in the present study were responsive to phenanthrene exposure. The response of these parameters is discussed in terms of the physiological mechanisms and consistency for potential biomarkers.

First, the present work was planned according to the guidelines for aquatic toxicity testing of substances with difficult solubility (Organization for Economic Cooperation and Development; Hutchinson et al., 2006). The maximum solvent (DMSO) concentration in the experimental media was 0.01% (100 μL L^{-1}), which also comply with the experimental intern protocol of the INCT-TA project (INCT-TA, 2013). However, the safety limits for solvent exposure in these guidelines were estimated on the basis of endpoints such as mortality and growth, which are less sensitive compared to the biochemical biomarkers used in the present study. In fact, DMSO alone was able to affect some biomarkers under the experimental conditions tested. This indicated that the responses discussed below might be caused by phenanthrene after DMSO dissolution. Although, it does not simplify the isolation of single phenanthrene effects, it constitutes important information because it also clarifies the potential effects of the DMSO vehicle. Several studies have reported only a positive solvent control and have used an even higher DMSO concentration than that tested in the present study (Hutchinson et al., 2006). Only recent studies have addressed the effect of DMSO on recent sensitive biochemical biomarkers (Turner et al., 2012). From a physiological perspective, performing only a positive control mandatorily indicates that the solvent does not interfere in the responses or there is a single additive effect of the compounds. However, DMSO is also a lipophilic xenobiotic similar to phenanthrene (Turner et al., 2012), and the data

presented here suggest that some effects of the DMSO and phenanthrene mixture may not be additive.

4.1. Physiological mechanisms

The lipophilic nature of phenanthrene enables it to be easily taken up from water and metabolized in fish (Cheikyula et al., 2008), thereby eliciting its cytotoxicity via multiple modes of action (Schirmer et al., 1998; Sun et al., 2006). The diversity in the modes of action might reflect a variety of impairments in general health. Moreover, Brinkmanna et al. (2013) recently proposed that a mixture of PAHs, including phenanthrene remobilized from sediments, promoted activation and deactivation in a time-dependent cascade of biomarker responses. Indeed, the data presented here support that phenanthrene exposure provokes several biochemical responses, which can vary greatly according to the concentration and tissue analyzed. In this sense, the different tissues studied in the present work are expected to take up phenanthrene at distinct times and are subject to the delay on the cascade of multiple modes of action, and thus, each tissue should be separately considered.

4.1.1. Phenanthrene effects on the liver

In the liver, no significant LPO was observed in *P. vivipara* exposed to a higher concentration of phenanthrene tested (200 μg L^{-1}), and even reduced LPO values were observed in fish exposed to 10 and 20 μg phenanthrene L^{-1}. These findings were consistent with the general lack of changes in ROS generation, ACAP and CAT activity, and the clear reduction in SOD activity observed in the liver of *P. vivipara* exposed to phenanthrene. Ferreira et al. (2012) also reported an absence of oxidative damages when *P. vivipara* was exposed to other organic compounds. Moreover, Brinkmanna et al. (2013) found evidence of lipid peroxidation only when hydrocarbon exposure was associated with a nearly lethal high temperature. Notwithstanding the physiological ability of PAH bioaccumulation in causing oxidative damage, their ability to cause lipid peroxidation at environmental levels is often found to be less conclusive than body accumulation (Hudjetz et al., 2013). Thus, we hypothesized that at environmentally tested concentrations, phenanthrene was not able to disrupt homeostasis in *P. vivipara* in terms of oxidative stress parameters. However, more importantly, there was a dynamic physiological rearrangement rather than an absence of a response. Indeed, the present results suggested a downregulation of most oxidative stress parameters with increasing phenanthrene concentrations. This was consistent with a control in the metabolic rates and pathways with increasing PAH. Moreover, the GST activity in the liver of *P. vivipara* should be sufficient to overcome phenanthrene accumulation and metabolism, which has been suggested by the lack of increase in the liver of fish exposed to 10 and 200 μg phenanthrene L^{-1}, and a reduction in enzyme activity was found in fish exposed to 20 μg phenanthrene L^{-1}. Similarly, it was also supported by a lack of change in the GR activity in fish exposed to any concentration of phenanthrene.

4.1.2. Phenanthrene effects on gills

Gills of the guppy *P. vivipara* showed reduced ROS production after exposure to any of the tested concentrations of phenanthrene. The authors hypothesized (1) a lack of increasing phenanthrene metabolism in gills within tested concentrations, and (2) potential phenanthrene-induced dysfunctions in mitochondrial respiratory function in gill cells. The lack of increased phenanthrene metabolism in the gill is based on the absence of a significant effect of phenanthrene on GST activity. GST plays a major role in phase II of PAH detoxification (Pathiratne and Hemachandra, 2010) and its non-responsive activity may indicate a lack of an increased PAH metabolism in gills. Unfortunately, phase I detoxification enzymes were not evaluated in the present study. Nevertheless, oxydegradation of PAHs per se would generate an excess of ROS production, which is not consistent with the reduction of ROS observed in this study. However, lower mitochondrial respiratory rates result in consequently reduced rates of ROS production. Thus, the combination of a lack of increased phenanthrene metabolism at higher concentrations and mitochondrial dysfunction may explain the reduced ROS in phenanthrene-exposed fish. The reduced ACAP and the absence of major changes in LPO observed in gills of fish exposed to phenanthrene were consistent with the lowered ROS production previously discussed. On the basis of the results reported in the present study, reduced ACAP resulted in part from the reduced contributions of both non-enzymatic (MT) and enzymatic (CAT) antioxidants.

4.1.3. Phenanthrene effects on muscle

In muscle, as observed in gills, a lower ROS production was also found after *P. vivipara* exposure to phenanthrene, particularly at the highest concentration tested. However, in contrast to gills, no significant change in ACAP was observed. Because a lower level of non-enzymatic antioxidants, such as MT, was observed in association with lower GR activity, the lack of change in ACAP was compensated by the contribution of enzymatic antioxidants, specifically by a consistent increase in SOD activity. The sum of these physiological changes was a slight increase in muscle LPO, which was observed in fish exposed to the highest concentration of phenanthrene tested (200 μg L^{-1}). To the best of our knowledge, there have been no other studies reporting the effects of phenanthrene on the oxidative responses in fish muscle because most of the studies examining this tissue have focused on measurements of tissue PAH concentrations. However, the biochemical alterations reported in the present study have also suggested that muscle may be a target organ of phenanthrene toxicity. Not only was oxidative status modified by this chemical, but phenanthrene might also have been metabolized in this tissue. This finding was based on the increased GST activity observed in fish exposed to 10 and 200 μg phenanthrene L^{-1}. Indeed, some authors have suggested that phenanthrene is one of the most bioaccumulated-bioconcentrated PAH in muscle, where PAH tissue levels are comparable to the liver (Cheikyula et al., 2008).

4.1.4. Phenanthrene effects on erythrocytes

Despite the apparent minor direct or ROS-mediated effects of phenanthrene on membrane lipids in terms of LPO, the present data support the hazards of phenanthrene and/or its metabolites to DNA integrity. In this context, several indirect effects of phenanthrene exposure have been reported in salmon, such as enzymatic and proteomic dysfunctions in antioxidant scavenging capacity, energy metabolism, cell communication, cell reproduction, homeostasis maintenance, among other factors (Sanchez et al., 2009). Such a large spectrum of effects may indicate a more fundamental and general cause, such as DNA damage. In fact, a strong reduction in the activity of nuclear RNA polymerases I and II has been described in the mussel digestive gland after phenanthrene exposure (Viarengo and Moore, 1982). Moreover, Woo et al. (2006) highlighted that bioaccumulation of PAHs in intracellular medium causes modifications in DNA, inducing incomplete transcription of DNA to mRNA, which culminate in metabolic dysfunctions such as an impairment of enzymatic reactions.

In the present study, potential DNA damage induced by phenanthrene has been measured using the comet assay and the frequency of genotoxic indices, such as micronucleated cells. Increases in the frequency of micronucleated cells have been associated with higher mutation rates and clastogenicity (Mitchelmore and

Chipman, 1998; Barsiene et al., 2006). Moreover, DNA strand breaks measured via the comet score, as well as the frequency of nuclear abnormalities are highly correlated to xenobiotic-initiated carcinogenesis and mutagenesis rate (Mitchelmore and Chipman, 1998; Barsiene et al., 2006). While the comet assay offers information at the molecular level, micronucleus and nuclear abnormalities provide relevant integrative information on the dynamic interaction of chromosome breakage (observed in comet assay) and chromosome loss. In addition, DNA strand breakage, such as nuclear abnormalities has been reported as an important effect of PAHs in both laboratory and field studies (Santos et al., 2010; Brinkmanna et al., 2013). Thus, an integrative analysis of the data generated using the comet assay and nuclear abnormalities evaluation appear to be the best strategy to interpret DNA damage caused by xenobiotics and to predict the effects on populations or communities in the marine environment (Woo et al., 2006).

DNA damage may result in disturbances in the proper transcription of DNA into proteins. Thus, other physiological effects caused by phenanthrene may be attributed to direct effects of phenanthrene and its metabolites on the activity of enzymes, and may also be related to indirect effects on DNA transcription into their respective proteins. However, DNA expression was not evaluated in the present study, and a consistent and significant increase in the frequencies of erythrocytes in class 3 (higher damage) of the comet assay was observed in *P. vivipara* exposed to any concentration of phenanthrene. Interestingly, this increase was paralleled by a decrease in the frequencies of erythrocytes in class 1 in fish exposed to 10 and 20 μg phenanthrene L^{-1} and in erythrocytes in class 2 in fish exposed to 200 μg phenanthrene L^{-1}, resulting in an increased comet score only in *P. vivipara* exposed to 10 μg phenanthrene L^{-1}. These findings also suggest that the exposure concentration affects the release of young cells as discussed below. However, the absence of a clear dose response in the comet score and phenanthrene exposure is consistent with the fact that PAH metabolites may interact with DNA and other biomolecules forming DNA adducts, which is a kind of damage that cannot be interpreted using the comet assay (Mitchelmore and Chipman, 1998). In fact, DNA adducts have been reported to be associated with phenanthrene and other PAH exposure (Willett et al., 1997).

A consistent effect of phenanthrene exposure was observed in the frequency of nuclear buds. An increased frequency of nuclear buds has been reported in the golden gray mullet *Liza aurata* following short-term exposure to phenanthrene (Oliveira et al., 2007). These DNA damage indicators may also be related to genotoxicity (Çavas and Ergene-Gozukara, 2005). Moreover, the trend in the decrease in the frequency of cells with a bilobed nucleus suggests a reduced release of young erythrocytes into peripheral circulation in *P. vivipara* exposed to higher concentrations of phenanthrene. Because most erythrocyte division occurs in hematopoietic organs, where clastogenicity processes occur, it is likely that phenanthrene-induced inhibition of erythrocyte release into the blood stream may reflect changes in the frequency of micronucleated cells and other nuclear abnormality indices. Interestingly, during the microscopic evaluation and quantification of nuclear abnormalities, erythrocytes from *P. vivipara* exposed to higher phenanthrene concentrations showed several membrane lesions and nuclear deformities that were not integrated in the indices employed to measure DNA damage in the present work. The amount of cells on the slides was decreased (data not shown), suggesting lower densities of peripheral cells. Similar findings were reported in erythrocytes of flounder *Paralichthys olivaceus* exposed to phenanthrene (Jee et al., 2004). These studies also found several other hematological responses in flounders exposed to phenanthrene, including a significant decrease in the number of erythrocytes at high concentrations of phenanthrene. Moreover, these findings may suggest disruptive activity of PAH on the erythropoietic tissue, resulting in lower values of cell viability, which is consistent with the previously reported data in the present study.

Regarding the frequency of micronucleated cells, phenanthrene was able to induce clastogenic DNA damage of up to 20 μg L^{-1}. However, in the highest phenanthrene tested concentration, the micronucleus frequency decreased, which might be correlated to cell death or to the decrease (not significant) observed in the release of peripheral erythrocytes (bilobed cells index). A comparison of low and high temperature stress by Brinkmanna et al. (2013) revealed that the micronucleus occurrence was only affected by PAH exposure in the low temperature stress condition. In addition, Hudjetz et al. (2013) demonstrated that the micronuclei ability to represent different contamination levels was inconclusive. Taken together, both studies and the present results support the idea that phenanthrene induces micronuclei at low concentrations or stress conditions. At high PAH concentration, other factors, such as a decreased number of cells in the bloodstream, might interfere in the response of this biomarker.

Taken together, data from the comet classes and the frequency of some nuclear abnormalities, such as nuclear buds, indicate that phenanthrene exposure can induce DNA damage in erythrocytes of guppy *P. vivipara* in saltwater. However, the comet score may be less sensitive in detecting DNA damage induced by phenanthrene exposure compared to analysis of comet class distribution. Moreover, the clastogenicity is characterized by increasing nuclear abnormalities in low phenanthrene concentrations and increasing cell death and/or cell release into the bloodstream at high concentrations. Consequently, it has been suggested that a more deep and detailed analysis of data generated by the comet assay (e.g., frequency of cells in each comet class) combined with the evaluation of other genotoxic indices, such as nuclear abnormalities, should be considered in future studies on the genetic effects of phenanthrene in fish.

4.2. Response consistency of potential biomarkers

The most informative model on phenanthrene toxicity is provided by the Alkyl Phenanthrene Model (Barron et al., 2004). In this model, interactions of phenanthrene and its metabolites with biomolecules have been described to cause both sublethal and lethal toxicity. The underlying mechanism involving phenanthrene-induced physiological disturbances is still open in this model due to the complexity of the multiple modes of action previously described above.

In terms of the oxidative status, previous studies in other fish report a variety of responses of the antioxidant system to phenanthrene exposure. For example, increased ROS production (OH^-) was observed in freshwater goldfish *Carassius auratus*, which is associated to phenanthrene bioaccumulation in the liver (Sun et al., 2006; Yin et al., 2007). Decreases in CAT activity and enhanced LPO were observed in the golden gray mullet *L. aurata* (Oliveira et al., 2008) and *C. auratus* (Yin et al., 2007) exposed to high concentrations of phenanthrene (~1 mg L^{-1}). However, opposed results were found in tilapia (female *Oreochromis niloticus* and male *Oreochromis aureus*) and flounder (*P. olivaceus*), where there was an increase in CAT, SOD, GR and glutathione peroxidase activity after exposure to phenanthrene (Wenju et al., 2009; Jee and Kang, 2005). Importantly, variations in the response of oxidative stress-related enzymes were reported to occur as a function of the time and concentration of exposure in mullet *L. aurata* and goldfish *C. auratus* (Oliveira et al., 2008; Sun et al., 2006). Some studies have reported these responses as oscillating quantitatively and qualitatively with time (Brinkmanna et al., 2013). Moreover, according to the results presented here, the effects of phenanthrene are highly variable

depending on the tissue and concentration. For example, the SOD activity was consistently decreased in the liver; however, no alteration was observed in the gills, and increased activity was observed in the muscle in the present work.

The general consensus of all authors and present data is that even low phenanthrene concentrations can cause several physiological adaptations and changes. Thus, we hypothesized that tested concentrations in the present study are able to perturb without disrupting the homeostasis in terms of oxidative stress, indicating a dynamic physiological rearrangement instead of a pure absence of effects. Ferreira et al. (2012) compared biomarkers in the liver and gills of *P. vivipara* and another related species exposed to organic contaminants and found different gene inductions among organs and species. These studies attributed such divergences to potential differences in the detoxification/bioactivational processes and characteristic intervals of time in which each organ/species shows a "peak" of induction. More recently, Brinkmanna et al. (2013) proposed a concept involving biomarker cascade activation in rainbow trout exposed to PAH contaminated sediments. According to the cascade idea, there is a temporal dynamics of biomarker reactions (i.e., different induction intensity and different biomarker responses observed). Thus, because the different tissues studied here are exposed in a specific order to phenanthrene (i.e., gills in contact with waterborne are exposed earlier) and the fact that they present different metabolic rates, it was expected that they specifically react to phenanthrene exposure.

In addition, to compare the different effects of phenanthrene in oxidative stress biomarkers, it is important to consider the physiological aspects of fish. The physiology and behavior of species affect uptake and the effects in fish (Cheikyula et al., 2008; Hudjetz et al., 2013). *P. vivipara* was acutely (96 h) exposed to phenanthrene in a hyporegulating condition (salinity 24 psu). Freshwater fish are hyperegulating in freshwater and demand higher oxygen and energy consumption in conditions of low salinity. Thus, higher basal metabolic levels and ROS production are expected in freshwater compared to saltwater fishes. Consistent with these findings, the environmental levels of phenanthrene tested in the present work combined with the low metabolic demands of *P. vivipara* most likely result in lower phenanthrene accumulation in the liver, resulting in little or moderate phenanthrene metabolism. Thus, the physiological status of fish might also play an important role in the biomarker response.

Considering all of the responses of *P. vivipara* evaluated in this study, none of the biomarkers was specific to phenanthrene. However, the combination of (1) the decreased in ROS and ACAP and increased LPO in muscle, (2) increased MT and decreased SOD in the liver, and (3) DNA damage at low levels, which reduce the density of peripheral erythrocytes at high levels appear to be the best suite of biomarkers to represent phenanthrene contamination. Notwithstanding this complexity, biomarkers can still be meaningful in biomonitoring if careful interpretation is provided.

5. Conclusions

Biomarkers in guppy *P. vivipara* were affected by exposure to phenanthrene at environmentally relevant concentrations. The ability to overcome the changes of several biochemical parameters reinforces *P. vivipara* as a reliable, practical and biologically relevant biomonitor in the detection of phenanthrene exposure in saltwater. The findings reported in this study provided evidence that the effects on oxidative parameters were observed in all tissues studied (liver, gills and muscle). However, these responses varied in intensity and type according to the tissue analyzed and phenanthrene concentration tested. In addition, DNA damage was also observed in fish exposed to this hydrocarbon. DNA strand breaks, release of young cells into peripheral circulation and clastogenicity were increased after exposure to lower concentrations of phenanthrene. However, at higher phenanthrene levels, the release of young erythrocytes into the bloodstream and/or increased cell death masked the genotoxicity indices. Indeed, more studies on the consistency of the dose–response in terms of concentration, tissue and time of exposure are required to precisely employ these biomarkers in environmental monitoring programs with *P. vivipara.*

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