Environmental Toxicology and Pharmacology 94 (2022) 103925

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Multi-biomarker approach and IBR index to evaluate the effects of bisphenol A on embryonic stages of zebrafish (*Danio rerio*)

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ARTICLE INFO

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Keywords:
Bisphenol-A
Embryotoxicity
Oxidative stress, teratogenicity, gene expression, IBR

ABSTRACT

This study assessed the effects of Bisphenol A in embryonic stages of zebrafish, applying an IBR multi-biomarker approach that included alterations in growth and oxidative status and relates it with the expression of *Nrf1, Nrf2, Wnt3a, Wnt8a, COX-2, Qdpra*, and *DKK1* genes. For this purpose, we exposed zebrafish embryos to eight environmentally relevant concentrations of BPA (220, 380, 540, 700, 860, 1180, 1340, and 1500 ng L^{-1}) until 96 h post-fertilization. Our results show that BPA induces several malformations in embryos (developmental delay, hypopigmentation, tail malformations, and on), leading to their death. The LC_{50}, EC_{50} of malformations, and teratogenic index (TI) were 1234.60 ng L^{-1}, 987.77 ng L^{-1}, and 1.25, respectively; thus, this emerging contaminant is teratogenic. Regarding oxidative stress and gene expression, we demonstrated BPA altered oxidative status and the gene expression in embryos of *Danio rerio*.

1. Introduction

Plastics are currently present in all areas of human life, widely used as packaging, plastic artifacts, and medical devices. However, their increasing production generates an environmental problem due to their abundance and persistence in the environment (Maldonado, 2012; US EPA, 2017). Every year about 400 million tons of plastic are produced worldwide, and only 9% is recycled (PlasticsEurope, 2020).

Plastic debris includes microplastics, a small group of plastic in the range of millimeters, which have gained scientific concern due to their ease distribution in the air, water, sediments, and biota (Elizalde-Velázquez and Gómez-Oliván, 2021; Sarria and Gallo, 2016). Microplastics can harbor toxic substances that remain adsorbed on their surface for years, such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and pesticides such as DDT (Hirai et al., 2011; Sarria-Villa and Gallo-Corredor, 2016). Additionally, some toxic chemicals, such as Bisphenol A (BPA), are used in the manufacture of polycarbonate plastics, epoxy resin, and other polymer materials.

Due to its continuous production and extensive applications, several authors have reported the presence of BPA in aquatic environments. Bossingham et al. (2005) and Yamazaki et al. (2015), for instance, reported the occurrence of BPA in several rivers of Japan (12 ng L^{-1}), China (23 ng L^{-1}), and India (380 ng L^{-1}). Similarly, Hu et al. (2019) found concentrations of 23 and 222 ng L^{-1} of BPA in sediments of the US. In addition, Chávez-Mejía et al. (2008) and Madera-Parra et al. (2018) reported that in wastewater from Colombia and Mexico, BPA reached concentrations of up to 319 and 1586 ng L^{-1}, respectively. As can be seen, the concentrations found in the environment are in the ng L-1 range; however, few references use this range to assess the toxic effects of BPA in aquatic environments. Thus, in this study, environmentally relevant concentrations were used to observe the toxic effect induced by BPA.

Upon BPA release into the environment, it can enter the body of aquatic organisms through different pathways, leading to different toxic

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https://doi.org/10.1016/j.etap.2022.103925
Received 19 January 2022; Received in revised form 23 June 2022; Accepted 29 June 2022
Available online 12 July 2022

responses. Qiu et al. (2020), for instance, showed that BPA, at concentrations of 100 μg L^{-1}, induced a decrease in gonadotropin levels of *Danio rerio* after 120 h of exposure. Similarly, Laing et al. (2016) demonstrated that BPA (0.01, 0.1, and 1 mg L^{-1} concentrations exposed for 15 days) decreased oviposition and reduced the number of oocytes fertilized in *Danio rerio*. In addition, Wang et al. (2013) showed that 3.42 mg L^{-1} of BPA at 72 hpf generated reactive oxygen species (ROS) and DNA damage, while at concentrations of 0.228–3.42 mg L^{-1} altered the locomotion of *Danio rerio* embryos. The authors pointed out that these effects could be related to the skeletal muscle structure damage by BPA; however, they did not observe teratogenic and embryotoxic effects on the organisms. Ultimately, Chen et al. (2022) demonstrated that BPA is able to affect signaling pathways involved in the defense against ROS, such as the Nrf2 gene in *Xenopus tropicalis* embryos exposed to concentrations of 5.72, 11.41, and 22.28 mg L-1. Previous studies have shown that Nrf2, under oxidative conditions, translocates to the nucleus where it dimerizes with small *Maf* proteins and then binds to the promoters of genes containing the sequence of antioxidant response elements (Johnsen et al., 1998). Therefore, this gene is an excellent indicator of oxidative damage. Other genes that allow us to clarify the presence of oxidative damage are *Wnt*, *Qdpra*, and *COX-2* which are related to the processes of organogenesis and pigmentation in the early stages of development (Fig. 1) (Steinhart and Angers, 2018).

Our research aimed to ascertain the effects of BPA in the early life stages of zebrafish by assessing the alterations in growth, oxidative status, and expression of genes involved in those responses through a multi-biomarker approach. Due to the above, the objective of this study was to relate gene expression to malformations induced by exposure to environmental concentrations of BPA in the embryonic stages of zebrafish.

2. Materials and methods

2.1. Test substance

Bisphenol-A (BPA, 2,2-bis (4-hydroxyphenyl) propane), was purchased from Merck (Darmstadt, Germany). The standard degree of purity was ≥ 99%, $C_{15}H_{16}O_2$, with a molecular weight of 228.29 (CAS Number 80–05–7).

A stock solution was prepared at a concentration of 1 mg L^{-1} by dissolving in ultrapure water. Dilutions were performed to reach the desired concentration (220, 380, 540, 700, 860, 1180, 1340, and 1500 ng L^{-1}).

2.2. Procurement and maintenance of test organism

Danio rerio adults, wild type (AB strain), were obtained commercially from Vega S.A. aquarium, Mexico. Before experiments, we allocated all fish in 100 L tanks at a rate of 1 fish per liter. All aquaria were provided with dechlorinated and UV-sterilized tap water (27 ± 1°C 14 h light: 10 h dark). The composition of tap water was determined to verify that it was suitable for the maintenance of *Danio rerio*, measuring dissolved oxygen, nitrates, nitrites, $CaCO_3$, Ca, Mg and pH (Table 1SM, Supplemental material). The acclimatization process lasted approximately two months. Fish were fed three times a day with spirulina flakes (Ocean Nutrition) and supplemented with crustaceans in brine (*Artemia sp. Napili*) to induce spawning.

2.3. Danio rerio embryo collection

The night before spawning, we placed 12 adult zebrafish at a ratio of 2 males:1 female in individual reproduction chambers. Spawning and fertilization took place in the morning by natural light stimulation. Following established protocols, embryos were collected after one hour of fecundation (hpf) and washed with water and saline solution (Westerfield, 2007). Next, we classified the embryos under a stereoscopic microscope as described by Kimmel et al. (1995), and we only selected those organisms at the blastula stage (2.5 hpf). Finally, blastula stage embryos were placed in plates with ultrapure water and incubated at a temperature of 28 ± 1°C until they reached the sphere stage (4 hpf).

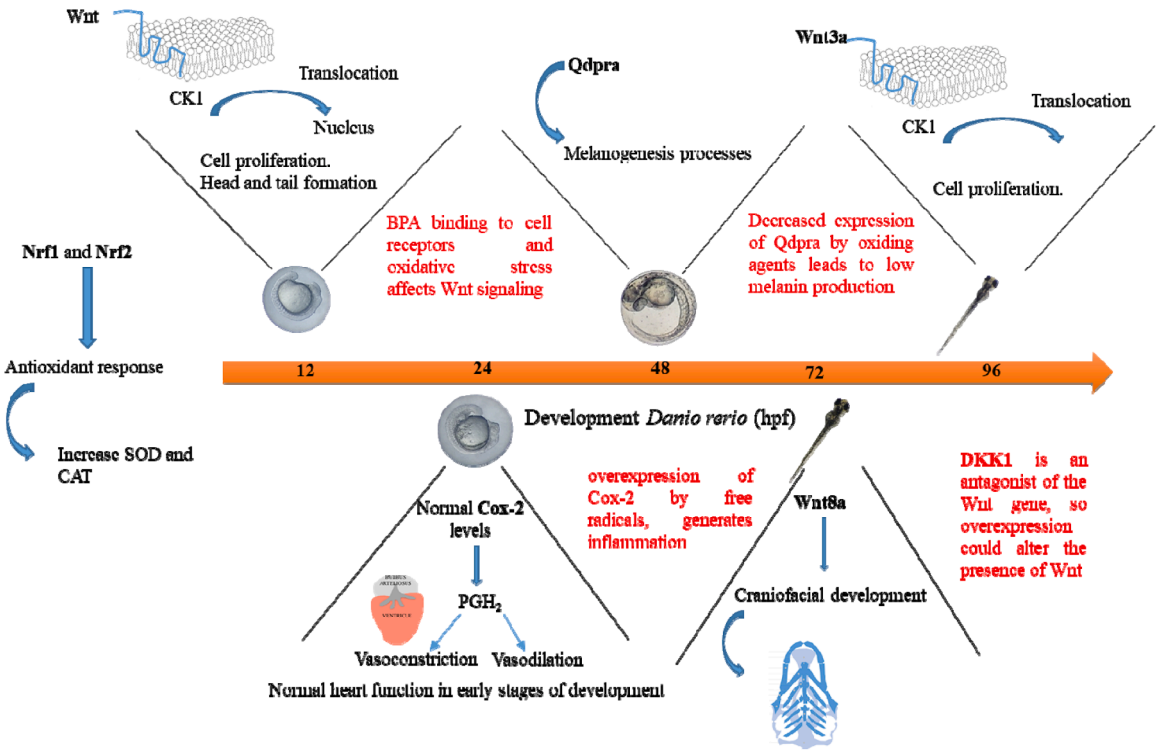

Fig. 1. Relationship between target genes and embryonic development of *Danio rerio*.

Table 1
Genes evaluated in *Danio rerio* embryos exposed to BPA.

Gen	Access number	Forward primer	Reverse primer	Reference
Nrf1a	NP_998020	TTTGGTTCCCGATGAAGACG	TGATTAGCGTGAGACTGAGC	Sant et al. (2017)
Nrf2a	NP_878309	ACCCAATAGATCTACAGAGC	GGTGTTTGGACATCATCTCG	Sant et al. (2017)
Wnt3a	AY613787.1	GGTACGCAGCCCATAATGTG	GGCCAGCTTGTCGTTGATAG	Renda et al. (2020)
Wnt8a	NM_130946.3	TGTAGACGCGCTGGAAAATG	ACTTCCGTGCTTGATCATGC	Renda et al. (2020)
COX-2	NM 001025504	TTTGGCCTGGGTTCACGT	TCGTCCTGCCCGTTTAGTT	Zheng et al. (2016)
Qdpra	NM_001110469.1	GTCATTCACCGATCAAGCCG	CTGCGATGTACTCCAGTGGT	Breuer et al. (2019)
Dkk1b	NM_131003.1	AGGATCACCACAAAACCAGA	GGAATATCTCCAAGCCATGA	Untergasser et al. (2011)
β-actin	NM_131031	GTTTAGGTTGGTCGTTCGTTTGA	AAGTGCGACGTGGACA	Gonzalez et al. (2005)

2.4. Toxicity test in Danio rerio embryos

Following the OECD (2013) guidelines [No. 236: Fish Embryo Acute Toxicity (FET) Test], we placed 72 embryos at the sphere stage (4 hpf) into 24-well plates (one embryo per well). For each experiment, we used three 24-well plates for each concentration of BPA. The environmentally relevant BPA concentrations used were 220, 380, 540, 700, 860, 1180, 1340, and 1500 ng L^{-1}. All plates were kept in incubation at 28ºC ± 1ºC with natural light/dark periods of 12 h. Toxicological endpoints, including embryonic mortality, hatching rate, heart rate, and malformations, were evaluated at different times along the exposure to BPA (12, 24, 48, 72, and 96 hpf). To evaluate the above endpoints, we took as reference the established guidelines of Kimmel et al. (1995). Malformations were expressed as the percentage of embryos with at least one malformation compared to the control group and assigned scores according to the scale (Hermsen et al., 2011). Graphs showing the major malformations induced by BPA exposure were constructed using IBM SPSS Statistics 22 software. At 96 hpf, live, dead and malformed organisms were counted to then carry out a maximum likelihood linear regression analysis and calculate the lethal concentration 50 (LC50) and the effective concentration of malformations (EC50) whit their 95% confidence intervals ($p < 0.05$).

2.5. Evaluation of oxidative stress by exposure to BPA on Danio rerio embryos

Oxidative stress systems were designed taking into account the established criteria of Elizalde-Velázquez et al. (2021). Nine systems were prepared separately in 5 L tanks. From each one, 1 g of oocytes were obtained and weighed on an electronic balance (Ohaus Explorer®). Eight of these systems were exposed to environmentally relevant concentrations of BPA: 220, 380, 540, 700, 860, 1180, 1340, and 1500 ng L^{-1}, while the last one was the control group. We maintained all systems at a constant temperature of 28 ± 1ºC until 96 hpf. At each endpoint (72 and 96 hpf), surviving organisms were selected and homogenized in phosphate buffer (PBS pH 7.4). The study was performed in triplicate.

To perform the oxidative stress tests, it was necessary to divide the homogenates into two Eppendorf tubes. Tube 1 contained 300 μL of homogenate and 300 μL of trichloroacetic acid (20%) solution and was used to evaluate biomarkers of cellular oxidation such as the levels of protein carbonylation (PCC), lipoperoxidation (LPX), and the hydroperoxide content (HPC). Tube 2 contained 700 μL of homogenate and was used to assess the antioxidant activity of the enzymes: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX). The level of each biochemical parameter was normalized to its protein content.

2.5.1. Evaluation of cellular oxidation biomarkers

The level of lipid peroxidation was assessed following the procedures reported by Buege and Aust (1978). 50 μL of supernatant from tube 1, 450 μL of Tris-HCl solution (150 mM), and 1 mL of trichloroacetic acid-thiobarbituric acid (TCA-TCB) solution were mixed in glass tubes. Heat shock was produced by immersing the tubes in boiling water. The tubes were incubated for 30 min at 37 °C and then centrifuged at 3500 g for 10 min. The absorbance was measured at 535 nm.

The hydroperoxides content was evaluated with the technique of Jiang et al. (1992). 100 μL of the supernatant from tube 1 and 900 μL of the reaction mixture (10 mL of solution A ($FeSO_4 + H_2SO_4$)) and 20 mL of solution B (dehydroxytoluene butylate + xylenol orange) were mixed in a tube, left to stand for 60 min at room temperature and protected from light. The absorbance was measured at 560 nm.

The level of protein carbonyl was quantified using the method of Levine et al. (1994) modified by Burcham (2007); Parvez and Raisuddin (2005). Briefly, precipitate from tube 1, and 150 mL of dinitrophenylhydrazine (DNPH)/HCl 10 mM were mixed and left to stand for one hour in the dark at room temperature. After that time 500 mL of ATC (20%) was added, the tubes were incubated at 4 °C for 15 min before centrifugation at 11,000 g for 5 min. The supernatant was discarded, and the button was washed with a 1:1 ethanol: ethyl acetate solution to obtain a white button dissolved in 1 mL of 6 M guanidine and incubated for 30 min at 37 ºC. The absorbance was measured at 366 nm. A PBS blank with the same treatment as the samples were used in all assays.

2.5.2. Antioxidant activity assays

The activity of SOD in embryos was assessed following the protocols of Misra and Fridovich (1972). For the assay, 40 μL of the supernatant from tube two, 260 μL of carbonate buffer, and 200 μL of adrenaline (30 nM) were added directly into the cell, and absorbance was measured at 480 nm (30 s and 5 min).

CAT activity was measured with the method of Radi et al. (1991). 30 μL of supernatant from tube two, 420 μL of isolation buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES, and 5 mM KH_2PO_4), and 300 μL of hydrogen peroxide (20 mM) were added directly into the cell, and absorbance was measured at 240 nm (0 and 60 s).

The activity of GPx in whole embryos was ascertained using the technique of Flohé and Günzler (1984). 100 μL of the supernatant from tube two was placed directly into the cell, 290 μL of reaction buffer, 100 μL of hydrogen peroxide (20 mM), and 12 μL of glutathione reductase were added, absorbance was measured at 340 nm (0 and 60 s). A PBS blank with the same treatment as the samples were used in all assays.

2.5.3. Determination of total protein content

Total protein content was determined spectrophotometrically by the method described by Bradford (1976). 13 μL of supernatant were taken from tube two and mixed with 75 μL of distilled water and 1.25 mL of Bradford's reagent. This mixture was homogenized in a Vortex for one minute, protected from light and incubated at room temperature for 5 min. The absorbance was measured at 595 nm, using a PBS blank with the same treatment as the samples. The absorbances obtained were extrapolated to a standard curve (bovine serum albumin) to determine the total protein concentration.

2.6. qRT-PCR

Total RNA isolation was carried out from 40 zebrafish larvae at 96

hpf using the RNeasy® kit (QIAGEN, Hilden, Germany) as the manufacturer's indications. cDNA synthesis was performed using 1 μg total RNA and QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany, REF 205313). Real-time RT-PCR was carried out using SYBR Green Kit (QIAGEN, Hilden, Germany) on a Rotor-Gene Q instrument (Qiagen). For, RT-PCR, we use the following protocol: an initial activation step 15 min at 95 °C, and 35 amplification cycles,15 s at 94 °C, followed by 30 s at 60 °C and 30 s at 72 °C. The primers used in this study are shown in Table 1.

The target genes were normalized against the β-actin gene. The experiment was repeated three times, and the relative quantification of gene expression among the treatment groups was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Table 1 shows the characteristics of the evaluated genes (*Nrf1, Nrf2, Wnt3a, Wnt8a, COX-2, Qdpra,* and *Dkk1b*), while Fig. 1 shows the relationship of these genes in the development of *Danio rerio*.

2.7. Integrated biomarker response (IBR)

The oxidative stress and gene expression results were applied to an IBR analysis, as Elizalde-Velázquez et al., (2021b) previously described. Briefly, we split the biomarkers of each treatment group (Xi) against the biomarkers of the control group (Xo). To decrease the variance of the obtained result, the ratio (Xi/Xo) was log-transformed (Yi). Subsequently, was standardized Yi values by employing the following formula $Zi = (Yi - \mu)/s$. μ and s are the mean and the standard deviation of Yi, respectively. Next, it was calculated the biomarker deviation index (A) by performing a difference of Zi and Zo. Then, each absolute value of A was summed to obtain the IBR values. The A data was represented by a star chart where the integrated responses of each biomarker can be observed.

2.8. Statistical analysis

For the evaluation of embryonic development (Hermsen score-scale), a two-way ANOVA followed by a Student-Newman-Keuls post hoc test was performed. Oxidative stress and gene expression biomarkers data were examined using a two-way analysis of variance (ANOVA), considering time as factor A and concentration as factor B. Variations between the means were examined with the Student-Newman-Keuls method, using Sigma Plot 12.3 software. All oxidative stress biomarkers passed the normality test.

3. Results

3.1. Embryotoxicity and teratogenicity of Danio rerio embryos at environmentally relevant concentrations of BPA

The percentage of the dead and malformed embryos after BPA exposure is shown in Fig. 2. By using these data, we estimated the lethal concentration 50 (LC50) of 1234.60 ng L^{-1} with 95% confidence intervals of 1084.56–1462.32 and the effective concentration 50 of malformations (EC50) of 987.77 ng L^{-1} with 95% confidence intervals of 903.76–1090.37. Subsequently, we calculated the teratogenic index (TI) with the quotient of LC_{50} and EC_{50}, which was 1.25. According to Weigt et al. (2011), if the TI is greater than 1, the substance is considered teratogenic and if the TI is below 1, it produces mainly embryolethal effects. So, BPA must be categorized as teratogenic for *Danio rerio* (Weigt et al., 2011).

As can be seen in Fig. 2, the number of normal embryos decreases as the concentration increases. Consequently, we observed the highest number (94.44%) of normal embryos at the concentration of 220 ng L^{-1} and the lowest number of these (1.38%) at the concentration of 1500 ng L^{-1}. As the mortality rate, the rate of malformed embryos by BPA increased in a concentration-dependent manner, reaching a minimum value of 2.77% and a maximum value of 51.38%.

3.2. Main malformations observed in Danio rerio embryos exposed to environmentally relevant concentrations of BPA

From Fig. 3, we can observe the different malformations identified during the exposure of *Danio rerio* embryos to BPA, showing an increase in function of the concentrations, and observing significant differences with respect to the control group ($p < 0.05$). The major malformations we identified in the embryos exposed to BPA were developmental delay, hypopigmentation, pericardial edema, yolk sac deformation, tail deformation, scoliosis, and craniofacial malformation. It is noteworthy to mention we observed a tendency in malformations, which was that as concentration increased, the severity and number of malformations per embryo also augmented. Concerning the general embryo morphology score established by (Hermsen et al., 2011), we also observed a concentration- and time-dependent effect. So, as concentration increased and time lapsed, the morphology score of embryos exposed to BPA decreased (Fig. 4). The concentrations with the lowest scores are those where the exposed organisms did not meet all the developmental characteristics.

The most representative malformations induced by each

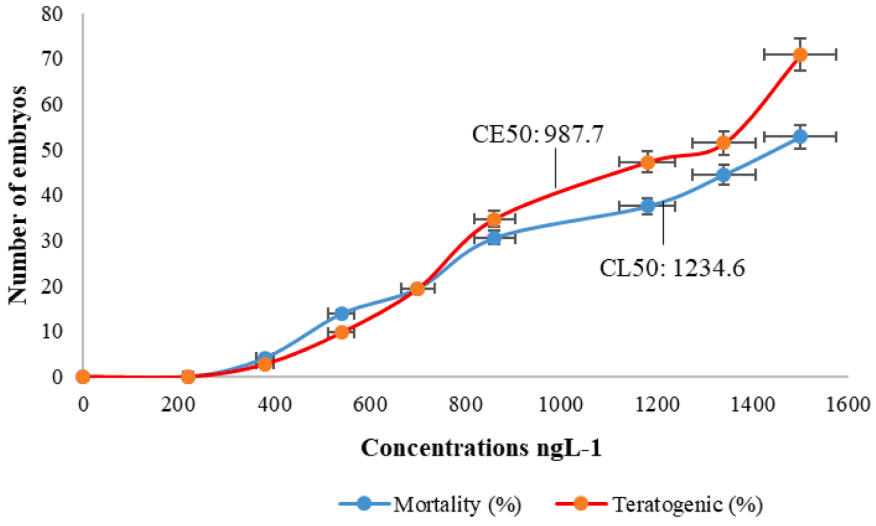

Fig. 2. Percentage of normal, dead, and teratogenic *Danio rerio* embryos exposed to BPA at environmentally relevant concentrations.

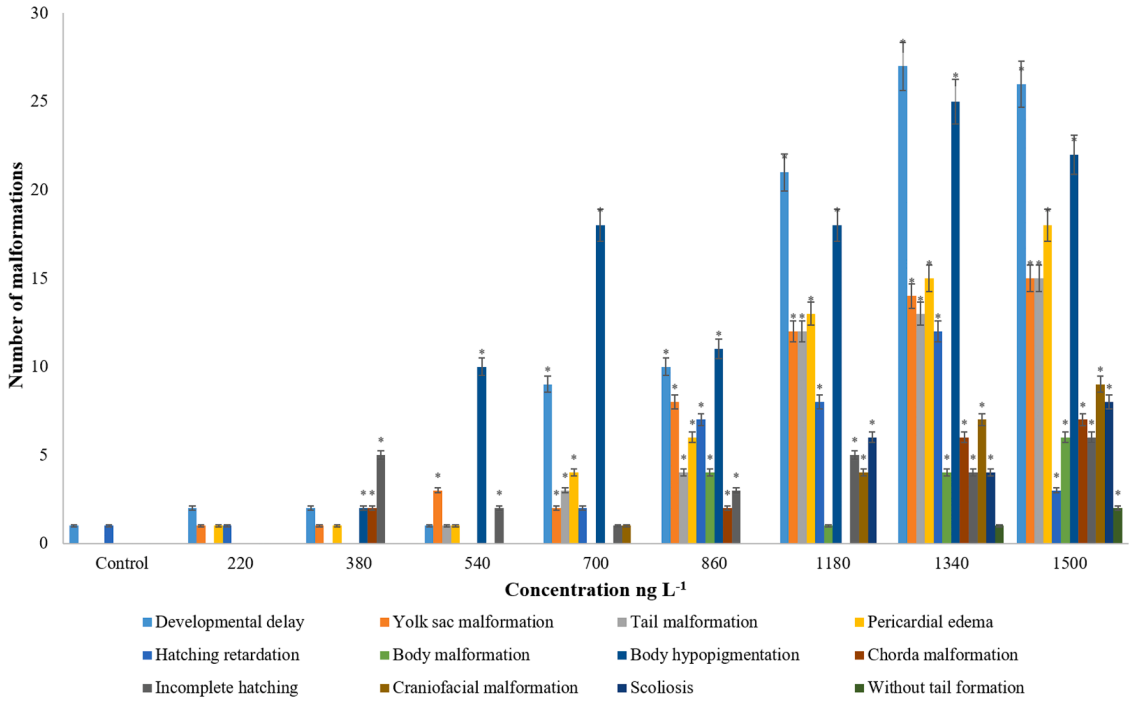

Fig. 3. Number of malformations observed in *Danio rerio* embryos exposed to BPA at environmentally relevant concentrations with significant differences concerning the control group ($p < 0.05$).

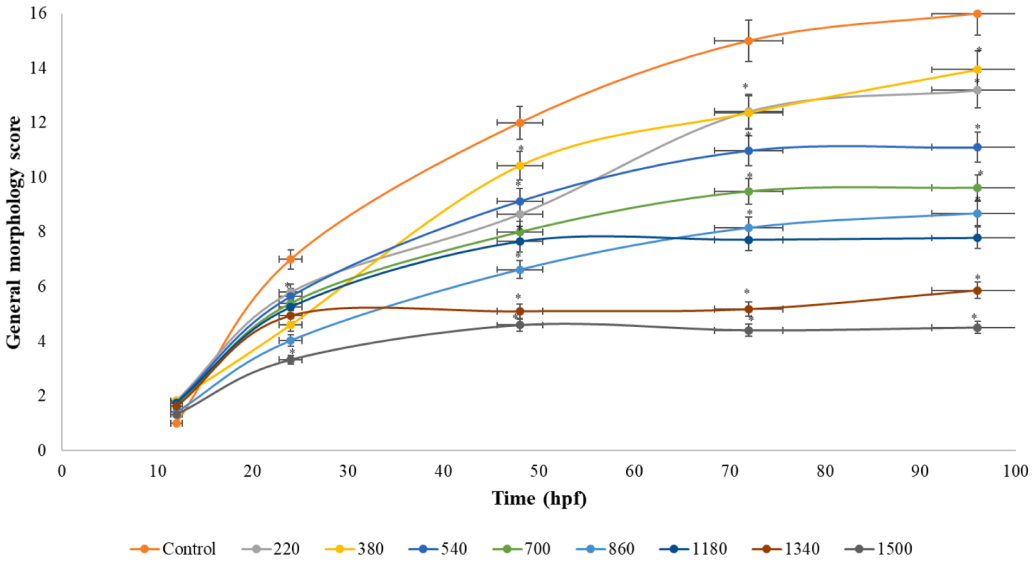

Fig. 4. General morphology scores of *Danio rerio* embryos exposed to BPA at environmentally relevant concentrations. (*) Significant differences concerning the control group ($p < 0.05$), value ± SD n = 72 embryos for concentration.

concentration of BPA at different exposure times are shown in Fig. 4. We observe a normal development of the control group at all exposure times ($p < 0.05$). At 12 hpf, we observed that some embryos suffered a delay in their development as they did not develop eye and tail. At 24 h, embryos presented pigmentation, heartbeat, and movement. At 48 h, the major malformations were hypopigmentation and pericardial edema. At 72 h, most of the embryos had already hatched, and some of them presented structural malformations, such as lack of pectoral fin formation, yolk sac deformation, and scoliosis. Finally, at 96 h, we verified that the embryos did not present any alteration in the hatching process.

As above, in Fig. 1SM, Supplement material, we can observe that as BPA concentration increased, malformations were more severe for the organisms. Thus, at the lower concentrations of BPA, for instance, we observed the main malformations in the embryos were: developmental delay and malformations of the yolk sac and body; while, at the higher concentrations of this compound, embryos showed a higher incidence of malformations of the tail, skull, and pericardial edema.

3.3. Evaluation of oxidative stress in Danio rerio embryos at environmentally relevant concentrations of BPA at 72 and 96 h

Concerning enzymatic activity of SOD, we observed a significant difference at all concentrations in comparison with the control group ($p < 0.05$). The highest enzymatic activity of SOD was observed at the concentration of 860 ng L^{-1}. However, after this concentration, the

activity of SOD significantly decreased in a concentration-dependent manner (Fig. 5). When comparing the two times of exposure at each of the concentrations, we observed that SOD activity decreases at 96 hpf compared to 72 hpf. In the case of enzymatic activity of CAT, we observed that the activity increased as a function of the concentrations showing significant differences compared to the control ($p < 0.05$) and reaching the highest activity of this enzyme at the concentration of 1500 ngL-1. In addition, we observed that CAT activity increases at 96 h at all concentrations. GPX activity increased as concentrations increased, with a significant difference concerning the control group ($p < 0.05$). Between concentrations, the highest activity is observed at 1340 ng L^{-1} and subsequently a decrease. Furthermore, we observed a significant increase in LPX, HPC, and PCC remaining enzymes at 96 hpf compared to 72 hpf.

3.4. Evaluation of gene expression of Nrf1, Nrf2, Wnt3a, Wnt8a, COX-2, Qdpra and DKK1b genes

The relative expression of all genes evaluated in this study showed a significant increase compared to the control group (Fig. 6). Nonetheless, each of the genes showed a different tendency. For example, for *Nrf1* and *Nrf2*, we saw that the highest fold was at the concentration of 860 ng L^{-1}, but after this concentration, the relative gene expression decreased in a concentration-dependent manner. Unlike antioxidant activity-related genes, the gene expression, *Wnt3a* and *Wnt8a* reached their highest fold at the lowest concentration of BPA. Moreover, we observed as BPA concentration increased, the expression of these two genes remained almost constant. We saw the highest fold of the gene expression of *DKK1b* at the highest concentration of BPA, which may explain the low fold change of *Wnt3a* and *Wnt8a* at the higher concentrations of this pollutant. For *COX-2*, we saw gene expression increased in a concentration-dependent manner, and in the case of *Qdpra*, we observed a significant decrease in a concentration-dependent manner. Significant differences between 72 hpf and 96 hpf were found for all genes but *Wnt3a* and *COX-2*.

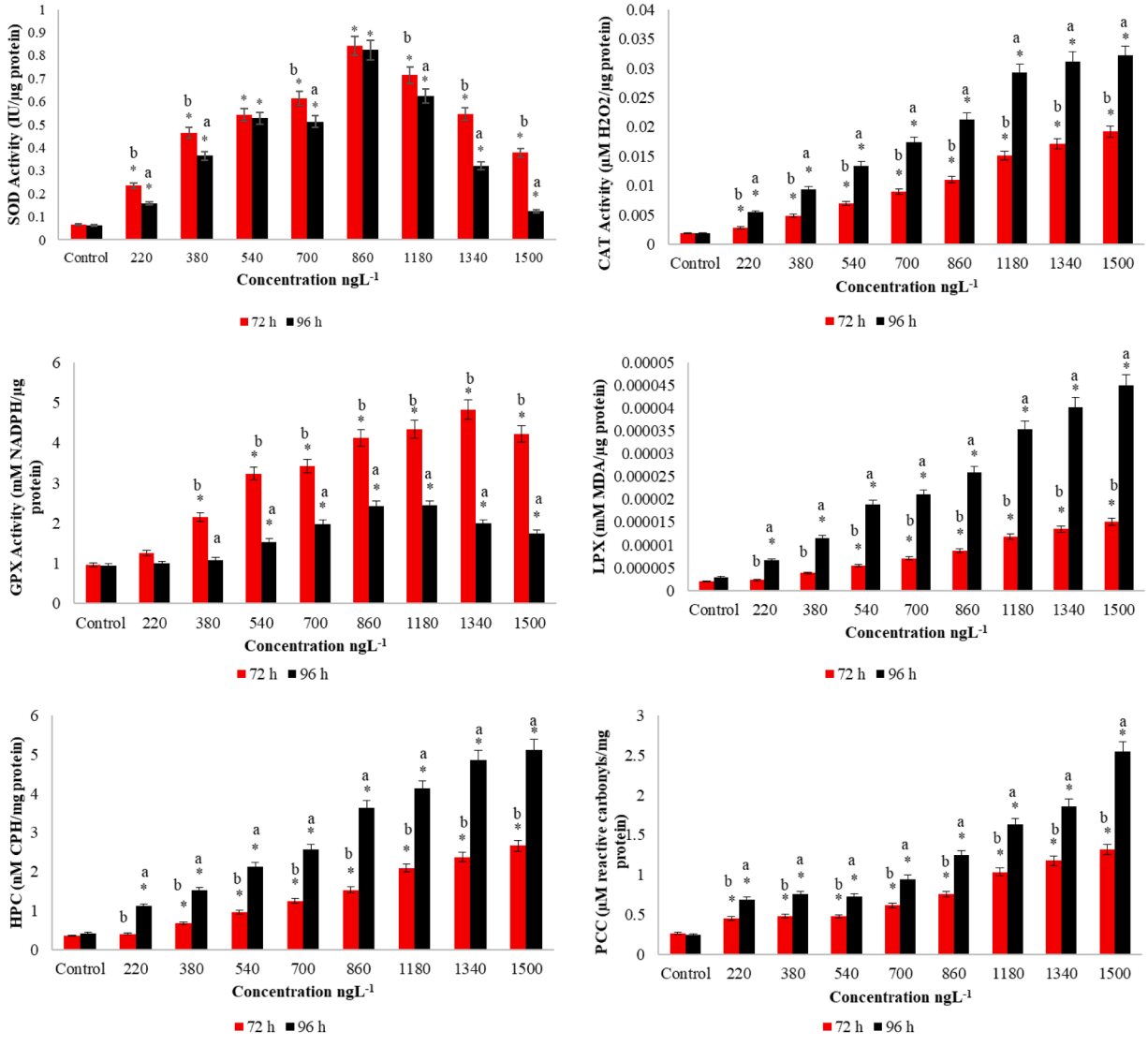

Fig. 5. Biomarkers of oxidative stress in *Danio rerio* embryos exposed to BPA at environmentally relevant concentrations at 72 hpf and 96 hpf. Significantly different of: *control group; [a]72 h; [b]96 h ($p < 0.05$). The statical values: SOD (F (8) = 144.7; n = 9; $p < 0.001$), CAT (F (8) = 131.924; n = 9; $p < 0.001$) and GPX (F (8) = 106.336; n = 9; $p < 0.001$). For all oxidative damage biomarkers LPX (F (8) = 108.663; n = 9; $p < 0.001$), HPC (F(8) = 124.281; n = 9; $p < 0.001$) and PCC (F (8) = 139.234; n = 9; $p < 0.001$).

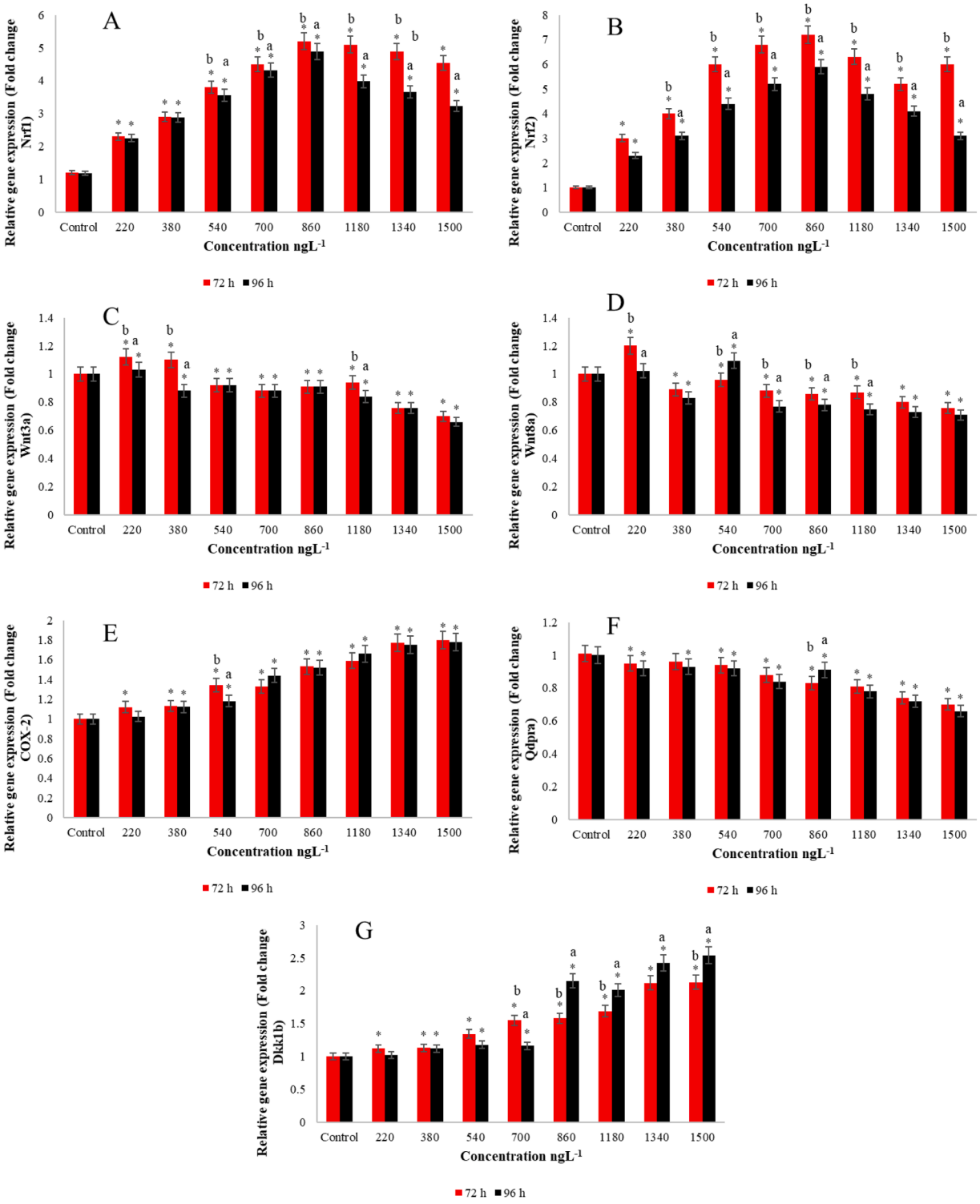

Fig. 6. Expression of *Nrf1, Nrf2, Wnt3a, Wnt8a, COX-2, Qdpra* and DDK1b (A, B, C, D, E, F, G) in the larvae of *Danio rerio* embryos exposed to BPA at environmentally relevant concentrations at 72 and 96 hpf. Values were normalized against β-actin (used as a house-keeping gene) and represent the mean mRNA expression value ± SD (n = 3) relative to those of the controls. Significantly different of: *control group; [a]72 h; [b]96 h ($p < 0.05$). The statical values: Nrf1(F (8) = 45.253; n = 3; $p < 0.001$), *Nrf2* (F (8) = 41.027; n = 3; p < 0.001), *Wnt3a* (F (8) = 43.680; n = 3; $p < 0.001$), *Wnt8a* (F (8) = 44.021; n = 3; $p < 0.001$), DKK1b (F (8) = 49.934; n = 3; $p < 0.001$), *COX-2* (F (8) = 55.253; n = 3; $p < 0.001$), *Qdpra* (F (8) = 42.124; n = 3; $p < 0.001$).

3.5. Integrated biomarker response index (IBR) of oxidative stress in Danio rerio larvae exposed to BPA at environmentally relevant concentrations

IBR values and star plots for each concentration of BPA are depicted in Fig. 7. As can be seen in this figure, star plots show a specific tendency towards each of the biomarkers. For example, at the concentration of 220 ng L^{-1}, we can see that the star plot shows a trend towards SOD, CAT, and GPX. Nonetheless, as the concentration of BPA increased, star plots inclined more towards oxidative damage biomarkers, showing that

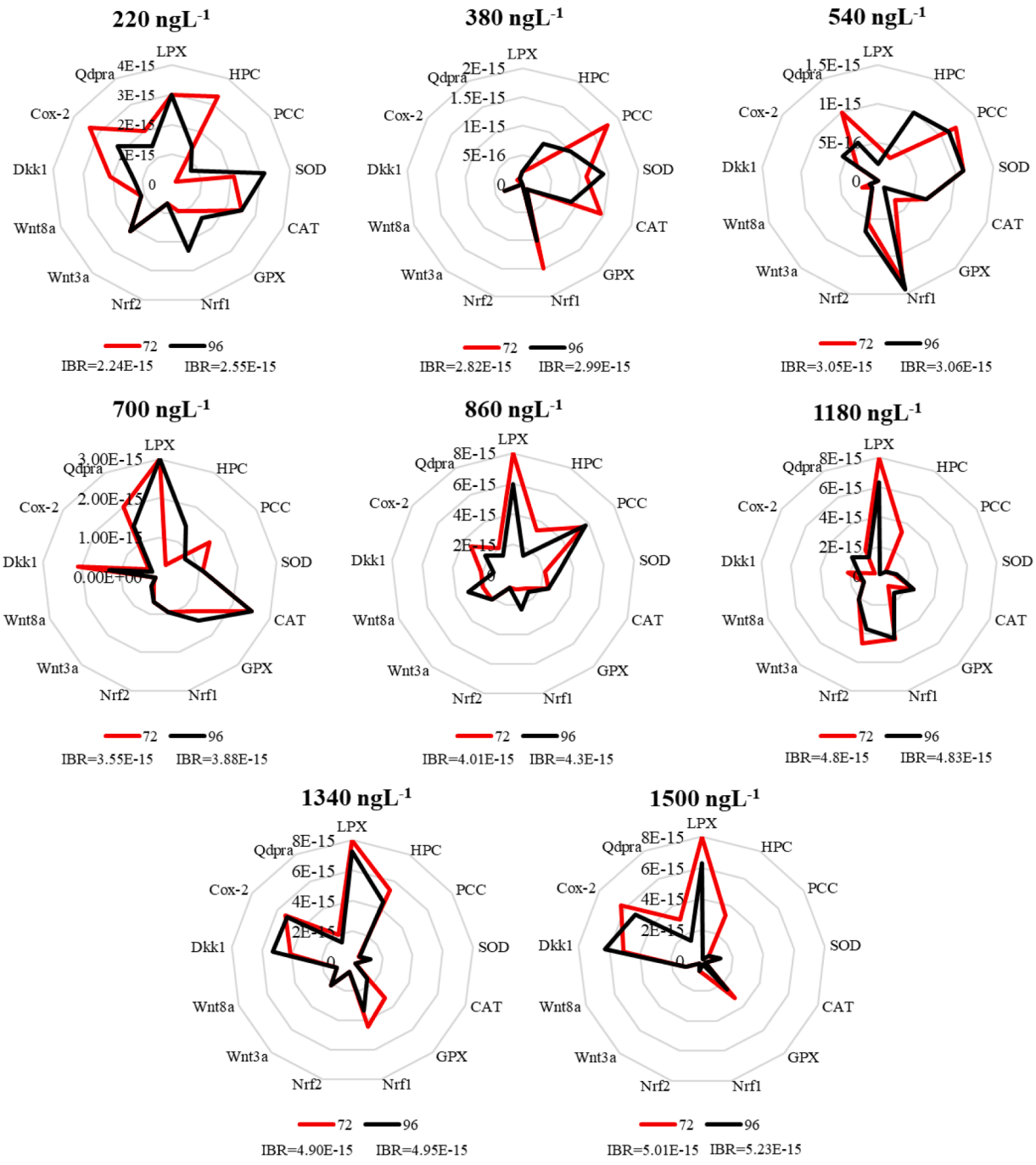

Fig. 7. Relationship between oxidative damage biomarkers and antioxidant enzymes in *Danio rerio* embryos exposed to environmentally relevant concentrations of BPA.

this pollutant can disrupt the redox status of *Danio rerio* embryos. In addition, to the above, we can observe that IBR values between 72 and 96 hpf also differ from one to another, with IBR values at 96 hpf higher than those at 72 hpf (Fig. 8).

4. Discussion

As observed above, BPA affected the embryonic development of *Danio rerio*, generating several malformations in embryos and altering their redox status and the gene expression of *Nrf1, Nrf2, Wnt3a, Wnt8a, COX-2, Qdpra*, and *DKK1b*.

In our study, BPA had an LC_{50} of 1234.60 ng L^{-1}, EC_{50} of 987.77 ng $L^{-1}$and TI of 1.25, which makes it a teratogenic compound (Weigt et al., 2011). Other studies have reported TI values greater than ours. For example, in the amphibian *Rhinella arenarum,* Wolkowicz et al. (2014) reported a TI of 4.7 (exposed to concentrations of 1.25 and 40 mg L^{-1} of BPA). On the other hand, Moreman et al. (2017) reported a LC_{50} of 12 mg L^{-1}, EC_{50} 5.7 mg L^{-1}, and therefore, a value of TI of 2.1 in *Danio rerio* exposed for 96 h to BPA (1–12.5 mg L^{-1}). It is important to note that although the authors conclude that the concentrations of bisphenol required to induce toxic and developmental effects were several orders of magnitude higher than concentrations commonly measured in the environment, in this study, we found that at environmentally relevant concentrations (in the ng L^{-1} range) similar acute toxic effects can also be seen in *Danio rerio.*

The incidence of malformations is in the function of concentration;

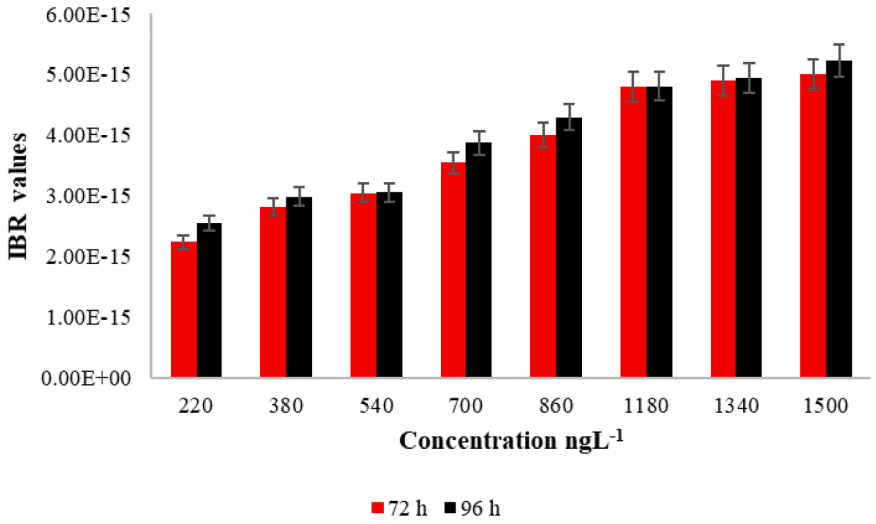

Fig. 8. IBR values.

consequently, as concentration increases, the severity of malformations also increases. Concordantly with our findings, Wang et al. (2013) observed a disruption in locomotion of *Danio rerio* after BPA exposure (0.228–3.42 mg L^{-1}) and concluded that alterations were due to damage to the skeletal structure. Also, in Fig. 2, we can observe that for all concentrations, the dominant malformations in the embryos were hypopigmentation and pericardial edema. Nonetheless, we also observed other severe malformations such as pericardial edema, yolk sac deformation, and scoliosis in the embryos exposed to BPA. Moreman et al. (2017) reported similar sublethal effects, including pericardial edema, craniofacial abnormality, pigment reduction, spinal malformation, and yolk sac deformity. These effects occurred for lower exposure concentrations, these results may suggest similar mechanisms of toxicity. Oxidative damage to cells is one of the mechanisms that can explain the alterations observed in the embryos. Even though oxidative stress is involved in protein synthesis and organogenesis (Zhang et al., 2020), ROS (superoxide anion O_2 * and hydroxyl radical *OH) have an affinity to molecular sites with high electron density and can abstract protons from defined molecular structures, triggering the propagation of secondary ROS (Valavanidis et al., 2006; Zaremba and Oliński, 2010). Sahoo et al. (2020) reported that BPA, at low concentrations (ng L^{-1}), can alter cellular responses in different tissues of organisms, and thus, generate reactive oxygen species (ROS). Furthermore (Ferguson et al., 2016; Kim and Hong, 2017; Wu et al., 2011) indicated that BPA caused damage to macromolecules, such as lipids and DNA.

Fig. 5 shows a significant increase concerning the control group ($p > 0.05$) in the content of hydroperoxides, lipid peroxidation, and content of carbonylated proteins at 72 and 96 hpf. This may be due to the fact that after BPA binds cell receptors, it may block different signaling lines that are crucial for cell proliferation during the early stages of embryonic development (Huang et al., 2018; Vauti et al., 2020; Wang et al., 2019). El-Demerdash et al. (2018), for instance, concluded BPA can generate O_2 * and OH* free radicals in response to an impairment of gene signaling pathways. Another process of ROS generation is through the scavenging processes of BPA, which is bio transformed into BPA-glucuronide by the action of cytochrome P450. (Geens et al., 2012; Matthews et al., 2001; Völkel et al., 2002).

Also, in Fig. 5, we can see the activity of SOD and GPX increased at 72 hpf, which may indicate an antioxidant response of the organism as the first line of defense against free radicals (Köktürk et al., 2020). SOD is responsible for removing O_2 * and the formation of H_2O_2 (Bourque et al., 2008). Nonetheless, as time elapses, the production of H_2O_2 increases, and excessive formation of this ROS may damage the histidine residue that is essential for the activation of SOD (Bray et al., 1974). Thus, H_2O_2 excessive production may explain the inhibition of SOD at 96 hpf. Unlike SOD, CAT and GPX activity increased at 96 and 72 hpf, respectively, which may be related to the excessive formation of H2O2, as CAT and GPX are the enzyme in charge of degrading this ROS.

The increased expression of *Nrf1* and *Nrf2* genes is observed in Fig. 6 (A, B), which suggests that *Danio rerio* had a response to the presence of ROS after BPA treatment. Both *Nrf1* and *Nrf2* are probably the most important pathways for cells to cope with ROS (Shi and Zhou, 2010). Subsequently, we observed a reduction in gene expression at the highest concentrations of BPA (1180, 1340, and 1500 ngL^{-1}) concerning the highest peak at the concentration of 860 ngL^{-1}. Both *Nrf1* and *Nrf2* are involved in the antioxidant response, which is why we observed an increase in the presence of SOD and CAT.

Fig. 4 shows how the scores assigned to *Danio rerio* decreased as the concentrations of BPA increased. For example, in the control group, fish achieved the highest score (16), while at the highest concentration of BPA (1500 ng L^{-1}), embryos barely achieved a score of 4.2. The above because the higher the concentration of BPA, the greater the severity of the malformations. The control group had normal development along all exposure time. Nonetheless, at the concentration of 220 ng L^{-1}, embryos showed an important incidence of tail deformation, incomplete hatching, and delayed development. Buckles et al., (2004) and Knippschild et al., (2005) pointed out that *Wnt3a* and casein kinase 1 and 2 proteins are involved in ocular development, processes that regulate the formation of the dorsal-central axis, and tail development of *Danio rerio*. Moreover, (Karimaian et al., 2017) mention that ROS are involved in *Wnt* signaling pathways affecting normal cell function. This affectation is generated from carbonyl protein interactions, which lead to a chain reaction in proteins. These studies could explain the incidence of developmental delay, tail deformations, and incomplete hatching in the embryos. The above agrees with our results, where we observe that the expression of the *Wnt3a* (Fig. 7 (C)) is affected at the highest exposure concentrations and at the highest exposure time of 96 h.

At the 380 ng L^{-1} and 540 ng L^{-1} concentrations, embryos showed a high incidence of pericardial edema, yolk sac deformation, body, and tail deformation. The presence of pericardial edema may be due to tissue super hydration attributed to osmotic regulation problems (Hill et al., 2004). Previous studies have indicated that this super hydration is given by an alteration of the permeability barrier of the embryo during the development of the gills and the digestive system (Kimmel et al., 1995). BPA could alter one of these barriers allowing the passage of water into the interior and induce super hydration. Fei et al., (2010), also reported that BPA induced damage to permeability in *Danio rerio* embryos, concluding that this effect is due to the fact that BPA is capable of

entering the cytoplasm and affecting protein synthesis.

On the other hand, Dong et al., (2010) relate the expression of cyclooxygenases (*COX-2*) to abnormal heart development. A higher expression of *COX-2* is due to the increased production of free radicals during oxidative stress (Valavanidis et al., 2006). The above is remarkable as the overexpression of *COX-2* by BPA exposure and other genes related to inflammation resulted in abnormalities in the heart ventricles of *Oryzias melastigma* in the embryonic stage (Huang et al., 2012). Also, in Fig. 6 (E), we can observe the overexpression of *COX-2*, which is also related to the development of the embryos.

In addition to the malformations mentioned above, at the concentrations of 700, 860, 1180, 1340, and 1500 ng L^{-1}, BPA induced hypopigmentation, no head and tail development, hemorrhages, and cranioencephalic deformation in the embryos. Melanin is biosynthesized through melanogenesis. Studies such as that of Mu et al., (2020 and 2018) suggest that BPA can alter melanin synthesis during *Danio rerio* development by reducing the expression of genes related to the production of this compound, as is the case of the *Qdpra* gene. In this study, we demonstrated the gene expression of *Qdpra* decreased in a concentration-dependent manner.

Craniofacial development in Danio rerio is regulated by several genes (*Wnt*, *Wnt8a*, and *sonic hedgehog* (Shh)), in addition to proteins such as SOX and Alx1 and deubiquitinases (B Lajis, 2018; LeCorgne et al., 2018; Mork and Crump, 2015). In Fig. 6 (D), we observe that there was an inhibition of the *Wnt8a* gene, which may explain the presence of skull malformations or even the non-formation of the head. As previously mentioned, ROS are capable of altering the signaling of genes such as *Wnt* as well as generating an overexpression of the *Dkk1* gene, which is a *Wnt* antagonist (Grotewold and Rüther, 2002; Shin et al., 2004). The study of Knobloch et al. (2007) reported that the *Dkk1* gene was overexpressed and generated a decrease in *Wnt* gene expression. The above agrees with our study, where we can observe a higher expression of the *Dkk1b* gene concerning the control group.

5. Conclusions

Environmentally relevant concentrations of BPA altered the redox status of the embryos, showing a significant increase in levels of LPX, HPC, PCC, and CAT. Oxidation of DNA and proteins in the early developmental stages of fish is potentially harmful to these organisms, leading to death or malformations. Exposure to BPA induced several malformations in fish, demonstrating that this compound at concentrations of ng L^{-1} can generate teratogenic effects on *Danio rerio* embryos. Developmental delay, hypopigmentation, and edema of the heart and yolk sac were the major malformations observed in the embryos exposed to this compound. Thus, BPA may negatively impact the embryonic development of *Danio rerio* through a mechanism in which oxidative stress is involved. Collectively, this research lets us warn about the harmful effects of BPA at concentrations found in the environment. Gene expression assessment allowed us to establish the relationship between the *Nrf1* and *Nrf2* genes and the defense mechanisms in the presence of reactive oxygen species, as well as to explain some of the malformations presented in *Danio rerio* through the expression of the *Wnt3a, Wnt8a, DKK1b, COX-2*, and *Qdpra* genes.

CRediT authorship contribution statement

Gerardo Heredia-García, Gustavo Axel Elizalde-Velázquez, Jesús Daniel Cardoso-Vera, José Manuel Orozco-Hernández performed all the exposure experiments; Leobardo Manuel Gómez Oliván and Gerardo Heredia-García were involved in the conception; Leobardo Manuel Gómez Oliván, Gerardo Heredia-García, Hariz Islas-Flores and Sandra García-Medina were involved in the design and interpretation of the data and the writing of the manuscript with input from Marcela Galar-Martínez and Octavio Dublán-García.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was made possible by financial support from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Project 300727).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2022.103925.

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