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Acute exposure to 17-α-ethinylestradiol disrupt the embryonic development and oxidative status of *Danio rerio*

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ABSTRACT

17-Alpha-ethinylestradiol (EE2) is an estrogen derived from estradiol (E2). This compound and is one of the most widely used drugs both in humans and animals. Numerous studies have reported the ability of EE2 to alter sex determination and delay sexual maturity, but there are toxic effects that need to be explored. In this work, we analyzed the effect of EE2 on embryonic development and oxidative stress biomarkers in *Danio rerio*. For this effect, zebrafish embryos in the blastula period (2.5 h post fecundation) were exposed to different concentrations of EE2 (36–106 ng L^{-1}) until 96 hpf. Survival, alterations to embryonic development, and teratogenic effects were evaluated using a stereomicroscope. Furthermore, oxidative stress biomarkers: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) activities, lipid peroxidation (LPX), hydroperoxide content (HPX), and protein carbonyl content (POX) were evaluated at 72 and 96 hpf using spectrophotometric methods. LC_{50} and EC_{50} of malformations got values of 82 ng L^{-1} and 57.7 ng L^{-1}, respectively. The main teratogenic effects found were: chorda malformation, body malformation, and developmental delay. These alterations occurred at 86, 96, and 106 ng L^{-1}. Integrated biomarker index showed that the oxidative stress biomarkers that had the most influence on embryos were SOD, CAT, GPX, and LPX. Overall, our results allow us to conclude that low concentrations of EE2 may potentially alter the development and oxidative status in the early life stages of zebrafish. Therefore, this bio-active estrogen can be considered a hazardous substance for fish.

1. Introduction

According to the U.S. Environmental Protection Agency (2016), endocrine-disrupting chemicals (EDCs) are agents that disrupt the production, release, transport, metabolism, action, or elimination of the body's natural hormones (Archer et al., 2017). Depending on their origin, these can be classified into: natural (estrogens and androgens), semi-synthetic (contraceptives), or synthetic (xenoestrogens) (Houtman et al., 2011).

17-alpha-ethinylestradiol (EE2) is a synthetic hormone derived from the estradiol (E2) and is the most used estrogen in combine oral contraceptives (Adeel et al., 2017). Nonetheless, this hormone is also used for other indications such as hormone replacement therapies, treatment of female hypogonadism, palliative treatment for breast cancer and prostate cancer, and as a treatment for some women with acne and in Turner's Syndrome (Aris et al., 2014; Siegenthaler et al., 2017). Thus, the annual contribution of EE2 for the aquatic environment is high, with discharge values of this drug close to 700 kg $year^{-1}$ (Almeida et al., 2020).

This hormone is found in wastewater treatment plants (WWTPs) effluents, at concentrations of up to 100 ng L^{-1} (Desbrow et al., 1998), and in surface water at concentrations of up to 830 ng L^{-1} (Kolpin et al., 2002; Bögi et al., 2003; Vethaak et al., 2005; Li et al., 2007; Salehzadeh et al., 2009; Chang and Huang, 2010; Díaz-Torres et al., 2013). Even though the concentrations found for this hormone in the aquatic environment are not as high as those reported for other contaminants, the predicted no-effect concentration of EE2 is low, with values between 0.1 and 0.5 ng L^{-1} (Caldwell et al., 2012; Laurenson et al., 2014). Thus, the

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Table 1
Water quality parameters.

Parameter	Value ± SD
pH	7.6 ± 0.2
Hardness	113 ± 12 mg L^{-1} $CaCO_3$
Un-ionized ammonia	0.012 ± 0.007 mg L^{-1}
Alkalinity	107 ± 6.7 mg L^{-1} $CaCO_3$
Nitrate (NO^{3-})	3.1 ± 0.7 mg L^{-1}
Nitrite (NO^{2-})	0.035 ± 0.012 mg L^{-1}
Dissolved oxygen	13 ± 0.7 mg L^{-1}
Conductivity	3 84 ± 18 μS cm^{-1}
Salinity	0.9 ± 0.3 g L^{-1}

high chemical stability, the low predicted no-effect concentration, and its tendency to concentrate in biota make the presence of this hormone in the aquatic environment a great concern.

Among the toxic effects that EE2 may induce in aquatic organisms, it has been reported that this compound is responsible for generating endocrine disruption in fish. Jackson et al. (2019) for instance, demonstrated that the exposure of *Heterandria formosa* to 5 or 25 ng L^{-1} of EE2 resulted in severe intersex in fish, a slowdown of spermatogenesis in male fish, and a slowdown of oogenesis in the female fish. Thus, EE2 may lead to multiple impacts on fish reproduction and consequently affect fish populations. This hormone has been associated with severe alterations in the immune system, modification of the oxidative status, and liver damage in yellow catfish (Hussain et al., 2017). Moreover, Mo et al. (2019) showed that EE2 increased the levels of malondialdehyde and inhibited the levels of the antioxidant enzymes SOD and CAT in *Pelteobagrus fulvidraco*. Therefore, it is believed that EE2 exerts its toxic effects by inducing reactive oxygen species (ROS) generation, leading to LPX and immunosuppression.

Despite the ubiquitous presence of this hormone in aquatic environments, there is little or non-information about the regulatory framework of EE2 in water bodies. Gilbert (2012), for instance, reported that the European Community set an annual average threshold value of EE2 of 0.035 ng L^{-1}. However, this author also mentions that the pharmaceutical companies opposed such regulation, as they claim there is little evidence of damage to the fish population.

In light of these considerations, this study aimed to investigate whether EE2 is able to induce developmental toxicity in the early life stage of *D. rerio*, through the determination of teratogenic effects and oxidative status.

2. Method

2.1. Chemical reagents

17-Alpha-ethinylestradiol (17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol, CAS number: 57-63-6, analytical purity of ≥98%) was purchased from Sigma-Aldrich (Mexico). All other compounds used were also purchased from Sigma-Aldrich (Mexico).

2.2. Animal husbandry

Wild-type zebrafish (*D. rerio*) used for the experiments were free of macroscopic symptoms of infection and disease and were not subjected to any drug treatment (acute or prophylactic) two months before spawning. The brood fish were kept in tanks with a recommended loading capacity of 1 L of water per fish and a 12:16 h light-dark photoperiod. *D. rerio* were kept in tanks of 100 L of capacity (Elizalde-Velázquez et al., 2021a,b). All tanks were provided with dechlorinated, aerated, UV-sterilized, and charcoal-filtered tap water. Furthermore, water quality parameters were monitored and controlled along with the experiment (Table 1). Fish were fed with *Spirulina* flakes (Ocean Nutrition, US) and brine shrimp *Artemia salina* larvae to promote oviposition (*Test No. 236: Fish Embryo Acute Toxicity [FE0054] Test*, OECD, 2013).

2.3. Egg production

From fish previously acclimated for 30 days, 24 males and 12 females (mean size 4–5 cm) were selected and placed in 3 individual breeding traps (4 × 4 mm mesh) at a ratio of 8 males to 4 females. The tanks contained 12 L of water that was aerated, dechlorinated, and reconstituted with 9 mg L^{-1} of commercial Instant Ocean® salts and 50 ppb of methylene blue as a fungicide (Elizalde-Velázquez et al., 2021a,b). Early next day, after the onset of light, the oocytes were collected and washed with saline solution according to the established protocols (Westerfield, 2007; Varga, 2011). Thereafter, using a stereomicroscope, blastula period oocytes (2.5 hpf) were selected and used for embryo-lethality and teratogenesis tests (Beekhuijzen et al., 2015).

2.4. Experimental set-up

Blastula period oocytes were randomly assigned and placed in 24-well plates (1 embryo per well). Each 24-well plate contained either a test solution of EE2 or a control solution. The concentrations of EE2 (36, 46, 56, 66, 76, 86, 96, and 106 ng L^{-1}) used in this study were environmentally relevant, as these concentrations have been previously reported in WWTPs effluents and surface waters (Kolpin et al., 2002; López de Alda et al., 2002; Vethaak et al., 2005; Salehzadeh et al., 2009; Chang and Huang, 2010; Díaz-Torres et al., 2013; Kanso et al., 2013; Nieto, 2014) and in some toxicity studies mentioned in the Introduction section. For each concentration, 72 embryos were used, as experiments were performed in triplicate to guarantee the reproducibility of results. 24-well plates were kept at 27 ± 1 °C and under same light/dark periods (14:10 h). At 96 hpf, the number of live, dead and malformed embryos was recorded. A maximum-likelihood multiple regression analysis using the Spearman-Karber method trimmed was performed to calculate the lethal concentration 50 (LC50) and effective malformation concentration (EC50m), 95% confidence intervals were also determined ($p < 0.05$, US-EPA software ver 1.5). The teratogenic index (TI) was calculated using the LC_{50}/CE_{50m} ratio. If this was greater than 1, EE2 was considered teratogenic, and if it was less than 1 embryo lethal (Tenorio-Chávez et al., 2020).

The evaluation of the morphological alterations of the embryos was performed using the methodology established by Kimmel et al. (1995) and Hermsen et al. (2011). The evaluation times were 12, 24, 48, 72, and 96 hpf. At these times, it was determined whether EE2 exposure caused alterations in the formation of the tail, somites, head, fins, and hatching, among other variables. The different cut-off times were scored according to the morphological alterations due to exposure to EE2 and contrasted with the control group ($p < 0.05$). With these results, a graph was made using IBM SPSS Statistics 22.

2.5. Evaluation of oxidative stress in zebrafish embryos

Cellular oxidation and antioxidant enzymes biomarkers were determined in zebrafish embryos at 72 and 96 hpf. These exposure times were chosen because embryos had already hatched, and their enzymatic system was already functioning. For oxidative stress determination, 500 mg of middle blastula stage embryos (average 750 ± 150) were exposed to 36, 46, 56, 66, 76, 86, 96, and 106 ng L^{-1} of EE2 in 6 L tanks. After 72 and 96 h of exposure, the obtained embryos were homogenized with phosphate buffer at pH 7.4, and the biomarkers of cellular oxidation and antioxidant defense were determined by different spectrophotometric methods. Oxidative stress biomarkers, for instance, were determined by the following methods: LPX by the method of Buege and Aust (1978), HPX by the method of Jiang et al. (1992) method, and POX by the method of Levine et al. (1994). Meanwhile, antioxidant enzymes were quantified by the following methods: SOD by the method of Misra and Fridovich (1972), CAT by the method of Misra and Fridovich (1972), Radi et al. (1991) and GPX by the method of Gunzler and Flohe-Clairborne (1985). Experiments were replicated three times in three

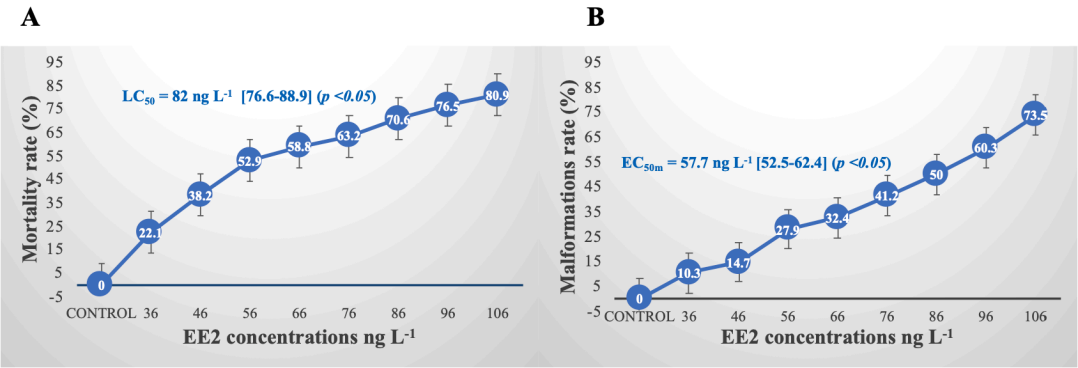


Fig. 1. Mortality and malformations rate in *D. rerio* embryos exposed to environmentally relevant concentrations of EE2 (ng L^{-1}). A represents the average of the mortality percentages obtained for each concentration of EE2 tested; B represents the average of the percentages of malformations obtained for each concentration of EE2 tested. Values are the mean of three replicates ± SE.

independent experiments.

2.6. Integrated biomarker response index (IBR)

Oxidative stress results were applied to the "Integrated Biomarker Response (IBR) Index" described by Sanchez et al. (2013). To calculate the IBR values, all biomarkers from each concentration of EE2 (Xi) were compared to biomarkers of the control group (Xo). The quotient between *Xi* and *Xo* was log-transformed (*Yi*), and then *Yi* values were standardized applying the following formula $Zi = (Yi - \mu) / s$. Finally, the biomarker deviation index (A) was calculated through the difference between Zi and Z0, and A values were pictured in a star plot that represents the integrated biomarker responses (Elizalde-Velázquez et al., 2021a,b).

2.7. Quantification of EE2 in zebrafish embryos

To determine EE2 concentrations in zebrafish embryos, systems for each exposure time were placed with the EE2 concentrations used in the toxicity studies (36–106 ng L^{-1}) and a control system free of EE2. Systems were supplemented with 1 g of zebrafish embryos. Before the quantification, extraction was performed. Briefly, 1 g of tissue was digested using 0.4 mg of the protease subtilisin for 1 h. Subsequently, 5 mL of methanol was added. The mixture was shaken for 1 min and centrifuged at 2800 ×*g* for 10 min. To the supernatant 2 mL of hexane were added, it was mixed for 1 min and the mixture was centrifuged again at the same speed and the same time to eliminate the hexane. 5 mL of deionized water was added and the mixture was preconcentrated on a Supel™-Select SPE HLB (6 mL, 200 mg) column. The cartridge was washed using an acetone/water mixture (20:80, v/v) and hexane. EE2 was eluted with methanol. The dry residue was dissolved in a methanol/water mixture (65:35, v/v). 5 μL of this solution were injected into the HPLC–MS system. For EE2 quantification, an Agilent Infinity 1260 chromatograph with a binary pump, degasser, autosampler, and UV detector connected to LC-MS was used. A Poroshell 120C18, 100 × 3 mm, 2.7 μm particle size column was used. The elution mixture was 0.1% formic acid in Millipore grade water (A) and 0.1% formic acid in LC-MS grade acetonitrile (B). The ratio of eluent B was increased to 100% for 3.5 min and maintained at this level for the same time. The column equation was performed for 12 min. The analysis time was 36 min. The flow rate was set at 0.5 mL min^{-1}, the column oven was maintained at 25 °C, and the sample storage box was at 4 °C. The conditions for the EE2 analysis were as follows: $m/z = 297$, fragment (V) = 100, LOQ (ng g^{-1}) = 0.09, LOD (ng g^{-1}) = 0.03, Rt (min) = 15.03. All measurements were taken in triplicate, and the results were presented as mean values ± S.E.M of ten subsamples ($n = 10$).

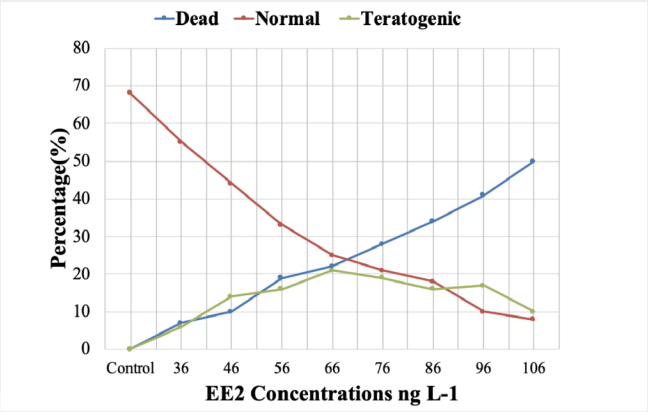


Fig. 2. Percentage of normal, dead and teratogenic embryos of *D. rerio* exposed to EE2.

2.8. Ethics approval

The protocols for this experiment were previously submitted to the Ethics and Research Committee of the Universidad Autónoma del Estado de México, Toluca, Mexico (Approval ID: CEI.UAEMCQ.REC.125.2019).

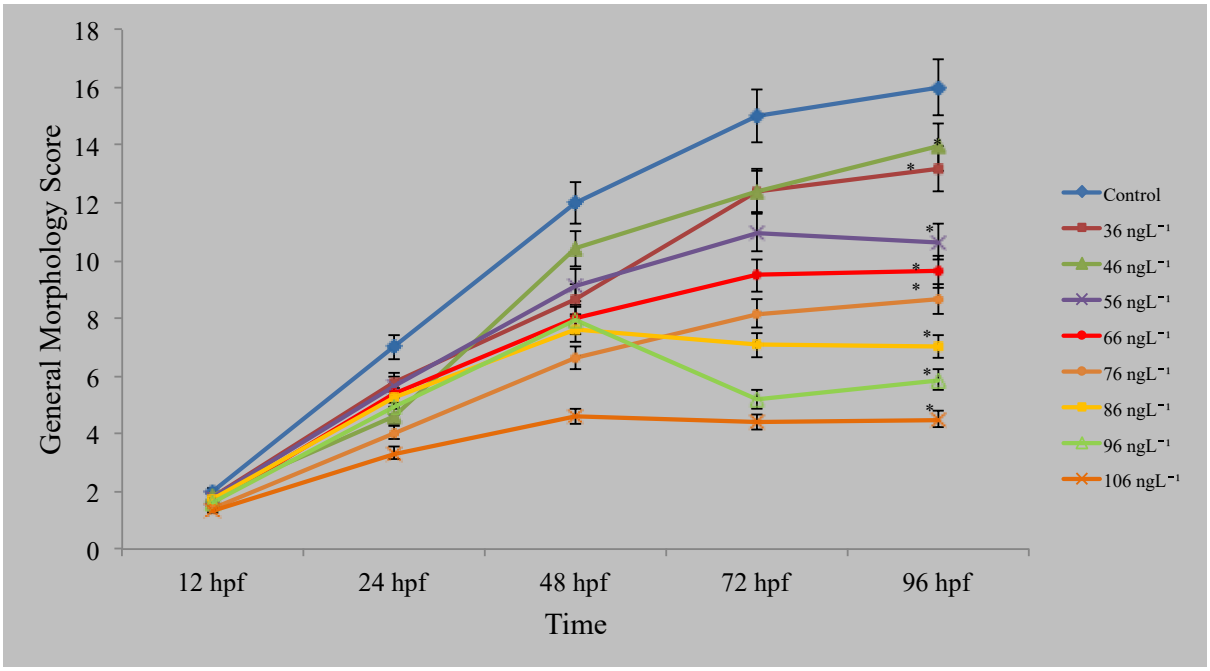
2.9. Statistical analysis

LC_{50} and EC_{50m} values were calculated using US-EPA software ver. 1.5 based on regression analysis. Morphological and teratogenic alterations identified by EE2 exposure were contrasted with controls by Fisher's test (IBM SPSS Statistics 22 software). Biomarkers of oxidative stress were analyzed by a two-way analysis of variance (ANOVA), followed by a Tukey-Kramer multiple comparisons test with a 95% confidence limit of 95%. To guarantee the traceability of results, in the embryotoxicity tests, the degree of fertilization was ≥90%, and mortality in the control groups was not greater than 10%.

3. Results

3.1. Survival test

Fig. 1 shows the data on mortality and malformations presented in *D. rerio* embryos exposed to environmentally relevant concentrations of EE2. Both mortality and malformations increase in a concentration-dependent manner. The teratogenic index of EE2 in *D. rerio* was 1.42. According to this result and the criteria of Weigt et al. (2011), EE2 should be classified as teratogenic. Several of the morphological



All EE2 concentrations were significantly different from the control group ($p<0.05$). The differences are not indicated so that the

Fig. 3. Concentration-response curves of EE2 in *D. rerio* embryos. Values are the mean of three replicates ± SE.
All EE2 concentrations were significantly different from the control group ($p < 0.05$). The differences are not indicated so that the figure can be easily appreciated (Student's *t*-test).

alterations presented in *D. rerio* affected the integrity of the fish and led to their death.

Fig. 2 shows that the health status of the embryos that survived EE2 exposure. As can be seen from this figure, the health of fish decreased as the drug concentration increased. Furthermore, the number of dead embryos increased in a concentration-dependent manner ($p < 0.05$). Regarding the organisms that presented teratogenic alterations, it was observed that the maximum peak was reached at the concentration of 46 ng L^{-1}. After this concentration, no very drastic variations were observed in the percentage of organisms with teratogenic effects. Even, at the highest concentration of EE2 tested in this study, a decrease was observed.

3.2. Evaluation of embryonic development due to exposure to EE2

The concentration-response curves for each EE2 concentration tested are shown in Fig. 3. From the Kimmel et al. (1995) and Hermsen et al. (2011) evaluation (applied to the *D. rerio* embryos exposed to different concentrations of EE2), we observed that the most severe alterations in embryonic development occurred at 72 and 96 hpf and in a concentration-dependent manner. Regarding the general morphology score, this was significantly reduced with regard the control group. Moreover, this decrease was in a ratio from 17.3% to 71.9% ($p < 0.05$).

3.3. Teratogenic effects induced by EE2

The most severe EE2-induced alterations to embryonic development were identified at concentrations of 86, 96, 106 ng L^{-1}. These were: body malformation, lack of tail formation, developmental delay, chorda malformation, yolk sac malformation, and scoliosis. Most of these alterations occurred after 48 hpf, leading to the death of the embryos at 96 hpf. The less frequent malformations were: lordosis, kyphosis, craniofacial deformities, reduces heartbeat, delayed hatching at 36, 46, 56, 66 ng L^{-1}, as can be seen in Fig. 4.

Table 2 shows the differences in the embryonic development between the control group and the embryos exposed to different concentrations of EE2. Exposure to low concentrations (36, 46, 56 ng L^{-1}) of these hormones induced alterations such as yolk-sac malformation, craniofacial malformation, tail malformation, lordosis, pericardial edema, kyphosis, scoliosis, lack of fins formation, and body hypopigmentation. For the concentrations of 66, 76, 86 ng L^{-1}, the same malformations were observed, in addition to the delay of hatching, body malformation, and tail malformation. Finally, for 96 and 106 ng L^{-1}, late hatching was additionally observed. Most of these malformations were visible in the embryos from 24 hpf.

3.4. Oxidative status of D. rerio embryos exposed to EE2

The results of oxidative stress show the values of the different oxidative stress biomarkers measured in the control group and the embryos exposed to the EE2. The oxidative stress biomarkers presented statistically significant differences concerning the control group. These differences were in a concentration- and time-depended manner.

3.4.1. Antioxidant activity induced by EE2

Fig. 5 shows the activity of the antioxidant enzymes SOD, CAT, and GPX of *D. rerio* exposed to EE2. All enzymes showed a significant increase concerning the control ($p < 0.05$). The behavior of these biomarkers was concentration and time-dependent with respect to the control group ($p < 0.05$).

3.4.2. Oxidative damage induced by EE2

Fig. 6 shows the cellular oxidation biomarkers LPX, HPX, and POX in *D. rerio* embryos exposed to EE2. All cellular oxidation biomarkers showed significant increases concerning the control ($p < 0.05$). The behavior of these biomarkers was concentration and time-dependent with respect to the control group ($p < 0.05$).

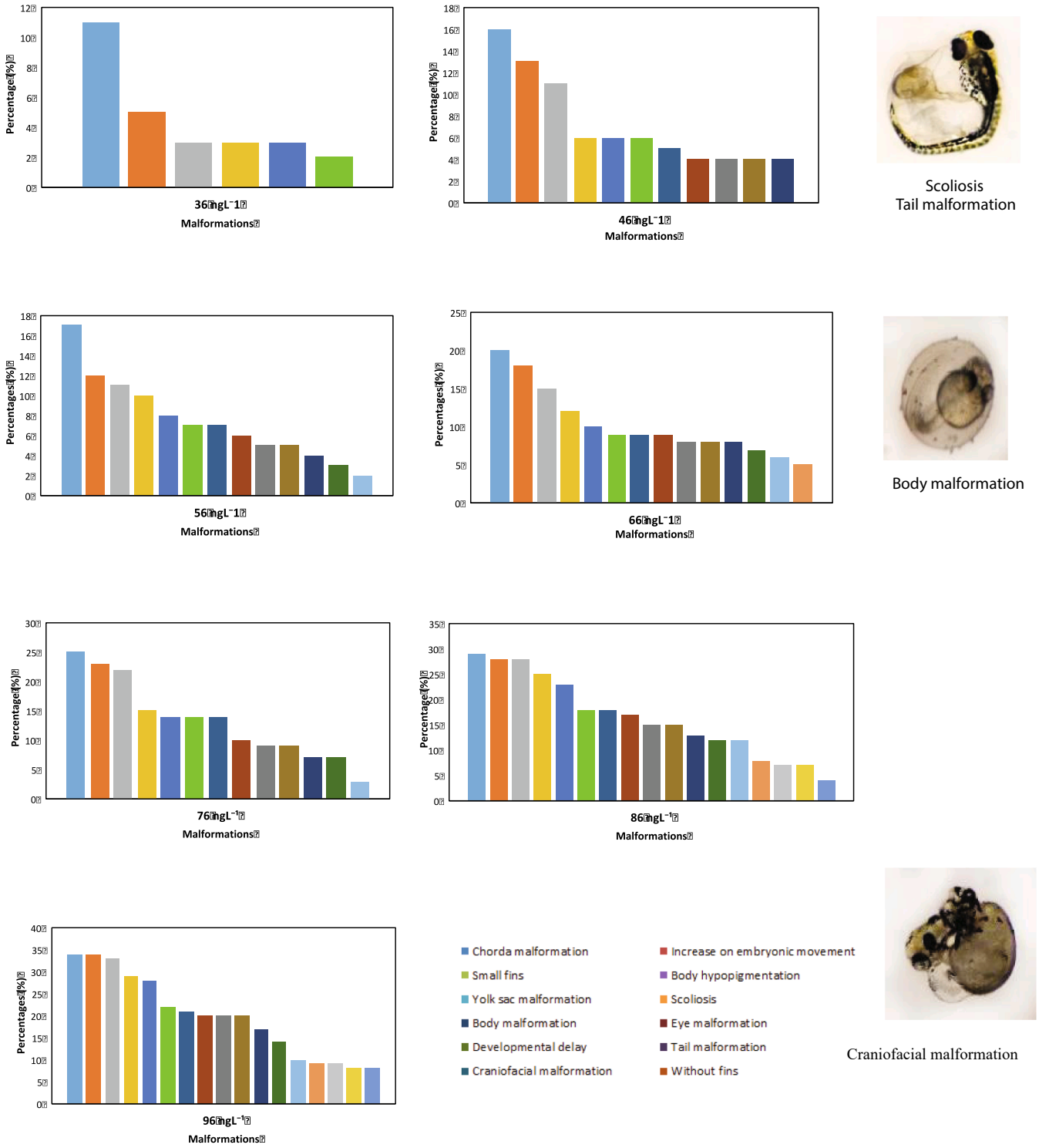
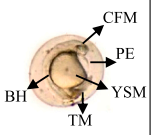
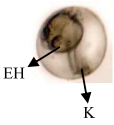
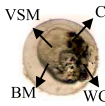
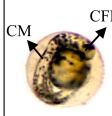
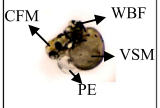
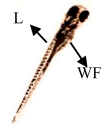
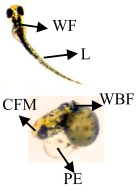
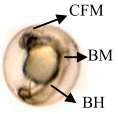
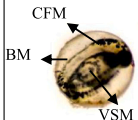
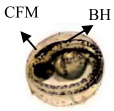
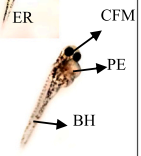
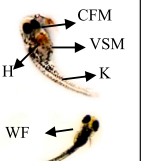
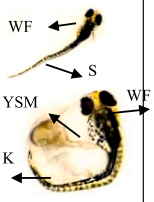
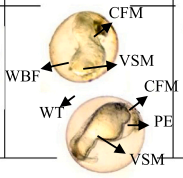
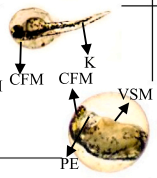
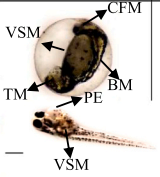
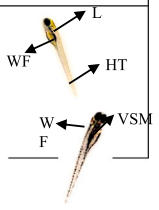


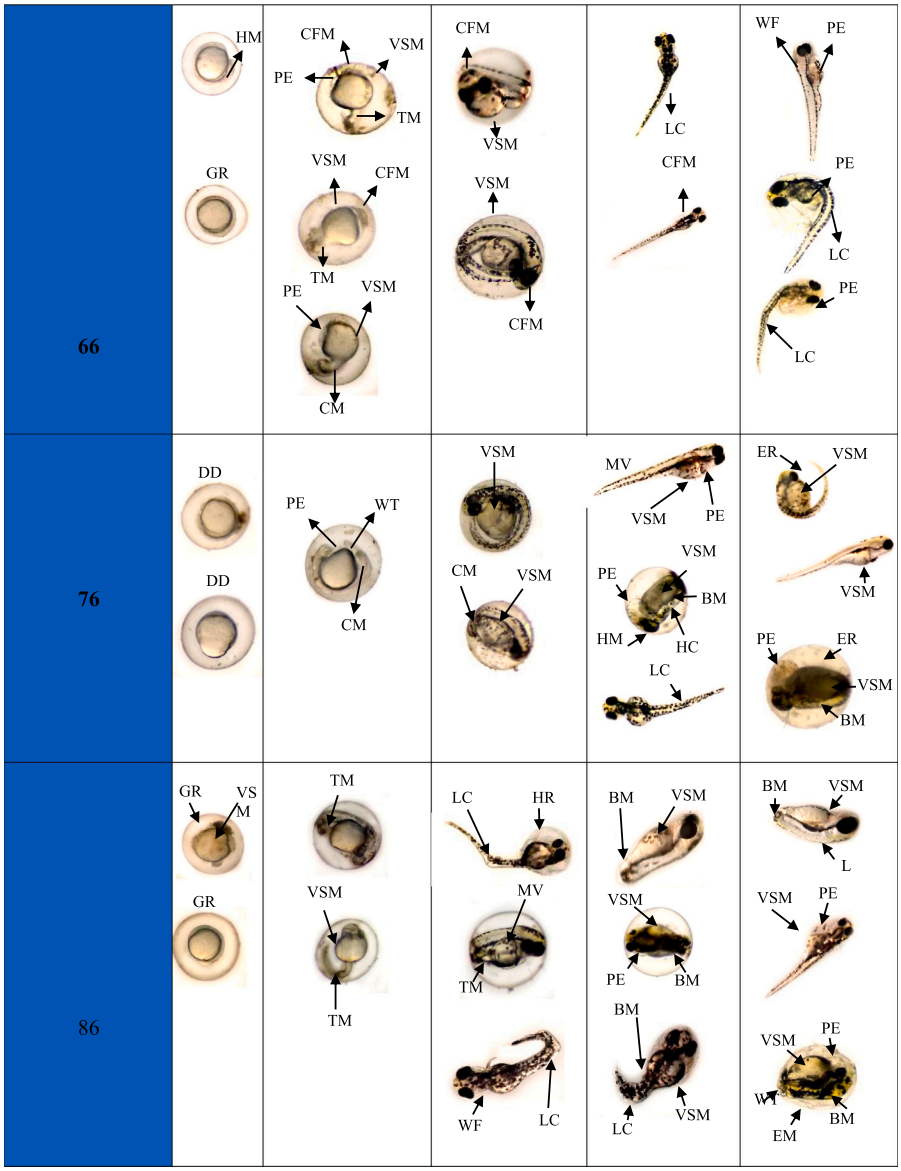
Fig. 4. Percentages of teratogenic effects induced by exposure of *D. rerio* embryos to relevant environmental concentrations of EE2.

3.5. IBR

As can be seen in Fig. 7, oxidative stress biomarkers that got more influence over the embryos were SOD, CAT, GPX, and LPX, at both 72 hpf and 96 hpf. For instance, at concentrations of 36, 46, 56, and 66 ng L^{-1} of EE2, star plots showed a deviation both towards cellular oxidation biomarkers (LPX and HPX) and antioxidant defense biomarkers (SOD, CAT, and GPX) in embryos. Moreover, at concentrations of 76, 86, 96, and 106 ng L^{-1} of EE2, star plots showed the same deviation towards antioxidant defense biomarkers as those presented at lower concentrations. Nonetheless, at these concentrations, star plots did not show a deviation towards HPX, but only towards LPX. Regarding IBR values, at lower concentrations (36, 46, 56, and 66 ng L^{-1}) of EE2, these got a value of 7.07, while at higher concentrations (76, 86, 96, and 106 ng L^{-1}) of EE2, these reached a value of 5.65. Thus, EE2 could experience a biphasic dose-response.

Table 2
Teratogenic effects induced by EE2 in *D. rerio* embryos.

Concentration ng L^{-1}	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control					
36	 	 	 	 	 
46	 	 	 	 	 
56					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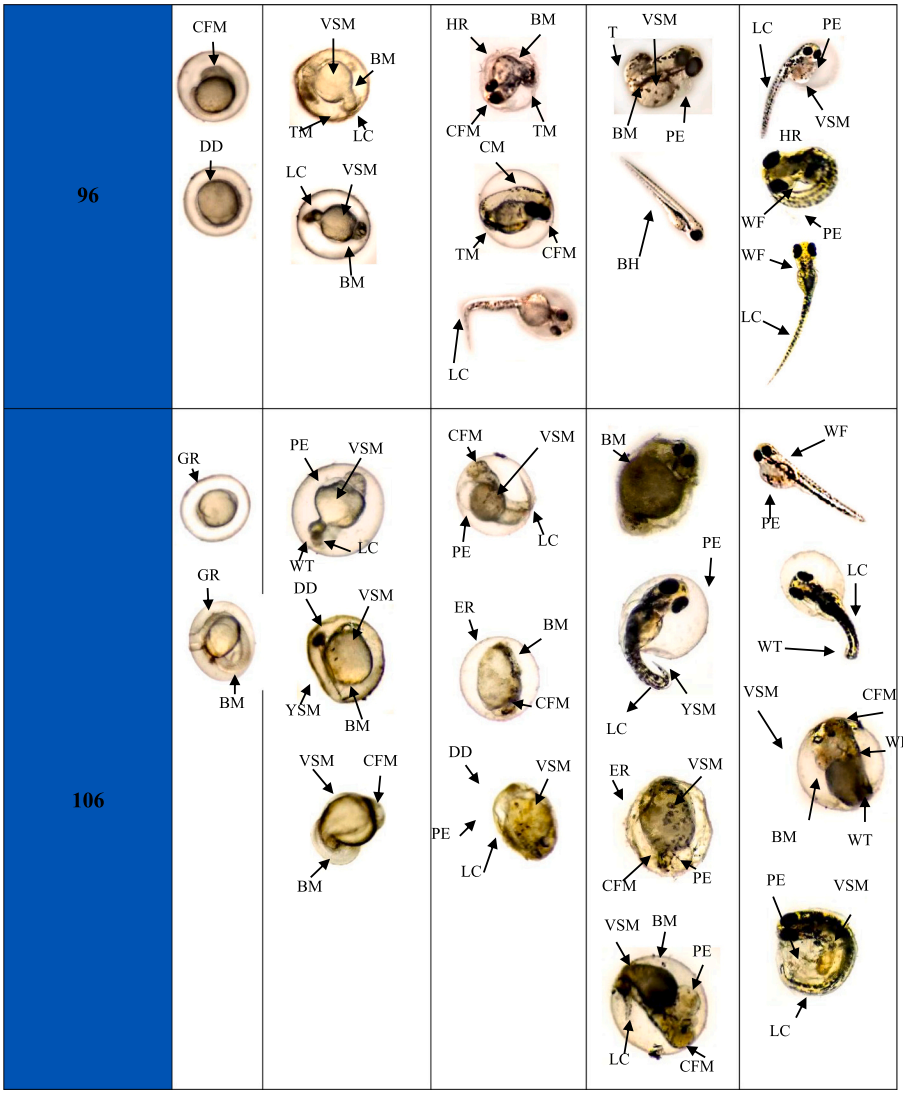

PE = pericardial edema, RHL = reduce heart late, CFM = craniofacial malformation, GR = growth retardation, K = kyphosis, L = lordosis, BH = Body hypopigmentation, YSM = Yolk sac malformation, TM = tail malformation, EM = eye malformation, BM = body malformation, WT = without tail, HR = hatching retardation, WF = without fin, CH = chorda hemorrhage, SM = small fins, DD = developmental delay, WCF without chorda formation, WBF without body formation, ER eclosion retardation, VSM vitelline sac malformation, S scoliosis, H hemorrhage, WS without somites, HT hypopigmentation tail, LC lordosis column, HM head malformation, CM column malformation, HC hypopigmentation of column.

3.6. EE2 concentration in zebrafish embryos

Table 3 shows the EE2 concentrations measured in zebrafish embryos at the different concentrations tested in the study and at different exposure times. The concentration of EE2 increases at each concentration according to the exposure time. This shows that EE2 is uptake by the embryo and this is related to the toxic responses found in the study.

4. Discussion

In this study, we demonstrated that environmentally relevant concentrations of EE2 may alter the oxidative status and affect the embryonic development of *D. rerio* by generating teratogenic effects that put their life at risk. Integrated biomarker index showed that the oxidative stress biomarkers that had the most influence on embryos were SOD, CAT, GPX, and LPX. No significant difference between 72 and 96 hpf in IBR biomarkers. Currently, there is none or little information about the regulation of EE2, due to the lack of information about the risks generated by this substance for being present in the environment. Therefore, the findings of this study may contribute to complement the information on the toxicity of EE2 in aquatic environments.

The EE2 toxicity is intimately linked to its ability to be resistant to degradation processes, as well as to the fact that this hormone is a substance with a high degree of lipophilicity (Almeida et al., 2020). Moreover, the toxicity of this hormone has been linked to its great absorption capacity in water body sediments and biota (Aris et al., 2014). For example, several studies have reported that EE2 has a long half-life (t1/2 = 92 days) and a high tendency to bioconcentrate in aquatic organisms such as fish (Al-Ansari et al., 2013; Huang et al., 2015; Ricciardi et al., 2016). In addition, studies conducted by Schultz et al. (2003), mention that EE2 is subjected to enterohepatic circulation in fish, amplifying its toxic effect due to continuous re-dosification.

The high lipophilicity of EE2 (log Kow 3.67–4.2), allows this compound to bind to the vitellogenin, a serum phospholipoglycoprotein

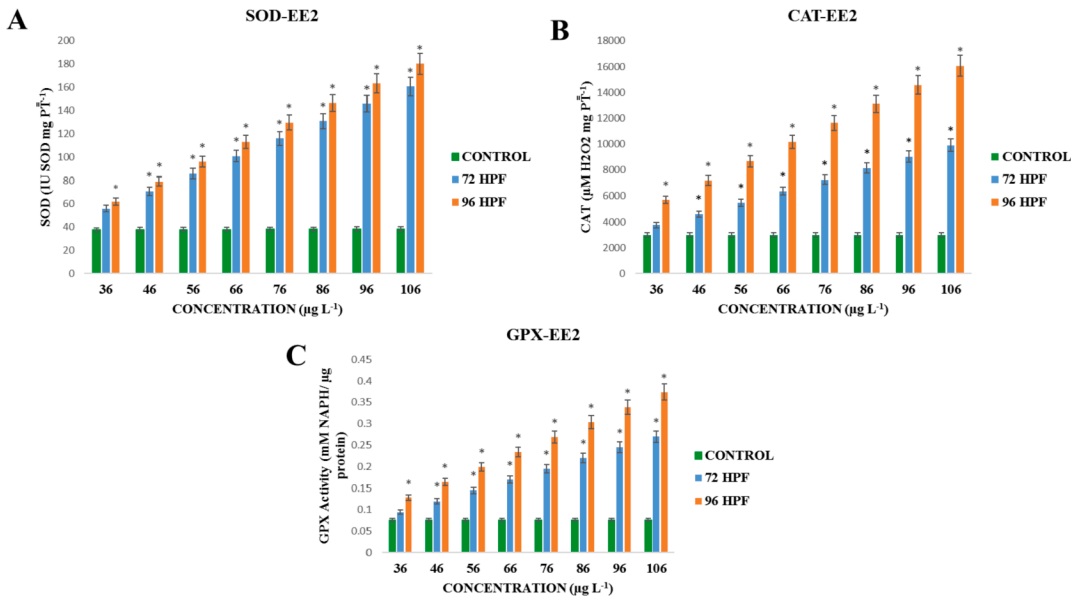

Fig. 5. Enzymatic activity of SOD (A), CAT (B), and GPx (C) in *D. rerio* embryos exposed to EE2. Values are the mean of three replicates ± SE. The control group did not show any significant differences between 72 and 96 hpf. The control group bar represents the results obtained at 96 hpf.

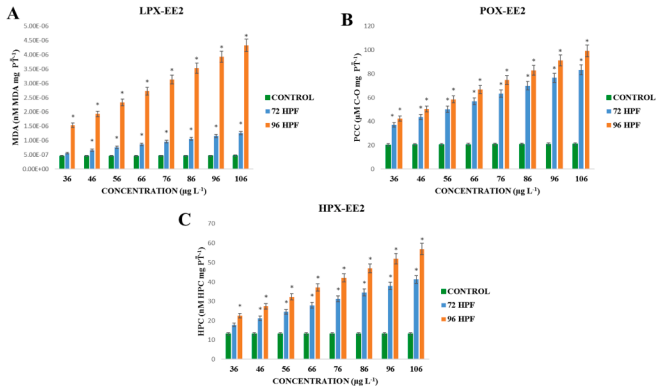

Fig. 6. Levels of oxidative damage biomarkers: LPX (A), POX (B), HPX (C) in *D. rerio* exposed to EE2. Values are the mean of three replicates ± SE. The control group did not show any significant differences between 72 and 96 hpf. The control group bar represents the results obtained at 96 hpf.

precursor of oocyte yolk formation (Soares et al., 2009; Zimmermann et al., 2019). Souder and Gorelick, 2017, pointed out that EE2 diffuses through cell membranes of zebrafish through passive diffusion with a concentration gradient, which favors its absorption by lipophilic tissues such as embryonic yolk. Furthermore, they established that from the 24 to 72 hpf, this compound is absorbed by embryos in a 40 to 60%, to be further accumulated in the lipid-rich areas of the embryo, and consequently induces alterations in their development.

Several studies have shown that EE2 may induce diverse toxic effects on different aquatic species (*Gasterosteus aculeatus*, *Radix balthica*, *Bithynia tentaculate*, *D. rerio*, *Sparus aurata*) at concentrations that range from 5 to 100 ng L^{-1}. Among the main effects found until now included increased levels of plasma vitellogenin, increased intersexuality in fish, decreased germ cell production and quality (egg cells and sperm), reduced fertility and fecundity, and also behavioral alterations (Andersson et al., 2007; Reyhanian et al., 2011; Cabas et al., 2012; Hallgren et al., 2012; Larcher et al., 2012; Lara-Severino et al., 2017). This is noteworthy as these alterations may lead to reduced survival rates, fertility, and hatching success.

EE2 has also been related to inducing oxidative stress in fish such as *Pelteobagrus fulvidraco*, *Carassius auratus*, *Aphanius fasciatus*, and *D. rerio* (Kessabi et al., 2010; Q. Chen et al., 2017; X. Zhou et al., 2019). Mo et al., 2019 for instance, showed that 1 µg L^{-1} of EE2 induced an increase in levels of malondialdehyde (MDA) in yellow catfish (*P. fulvidraco*), leading to oxidative damage. Furthermore, Y. Chen et al. (2017) identified that yellow catfish exposed to 0.1 and 1 ng L^{-1} of EE2 for 56 days significantly modified the immune response and altered the antioxidant capacity of this fish. These results obtained are consistent with the findings obtained in our study, in which we observed that the concentrations of MDA and cumene hydroperoxides (CHP) increased significantly at 72 and 96 hpf in zebrafish embryos and a concentration-dependent manner. Likewise, in this study, we observed that the antioxidant enzymes SOD and CAT increased in a concentration-dependent manner with respect to the control ($p < 0.05$). Oxidation-reduction (REDOX) processes are important for embryogenesis. For example, during embryonic development, there are changes in redox potential that determine the fate of cells towards proliferation, differentiation, apoptosis, or necrosis (Jensen et al., 2001). Therefore oxidative damage as observed in this study can alter the processes of proliferation, differentiation, or apoptotic activity.

During the growth of the embryo, there is a great demand for energy due to the overload of the aerobic metabolism, which leads to an imbalance of the oxidative state. In consequence, the early life stages of embryonic development are highly susceptible to ROS damage (Parlak, 2018). The increase in ROS in fish embryos has been linked to a reduction in fertilization, a decrease in the quality of the embryo, and an increase in the abnormalities that can be observed in the later life of the offspring (Lord and John Aitken, 2013). Some studies have demonstrated the toxic effects induced by EE2 in embryonic and juvenile stages of aquatic organisms. For example, Bhandari et al. (2015) investigated the transgenerational consequences that 50 ng L^{-1} of EE2 induces in *Oryzias latipes*. According to their results, EE2 induced a significant reduction in the fertilization rate in offspring two generations later (F2) as well as a reduction of embryo survival in offspring three generations later (F3). Thus, EE2 exposure during embryonic development induces transgenerational phenotypes of reproductive impairment and compromised embryonic survival in fish of subsequent generations.

The embryotoxic and teratogenic effects observed in this work may be due to the capacity of EE2 to bind the GPR30 transmembrane protein of the chorion, activating phospholipase C, and consequently,

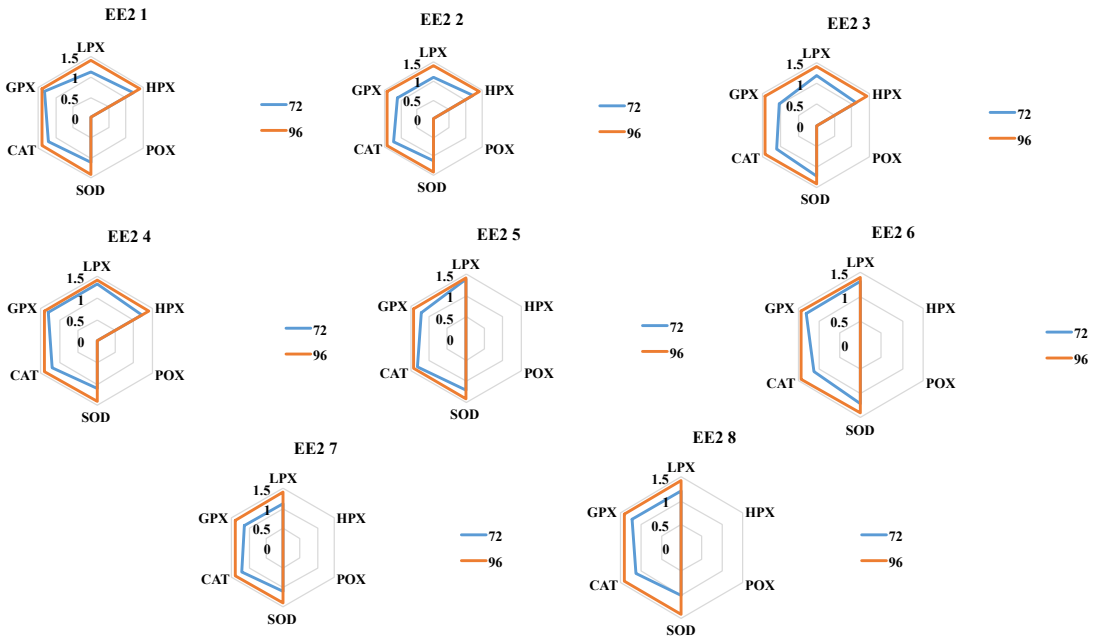

Fig. 7. Evaluation of the influence of the oxidative stress induced by EE2 on *D. rerio* embryos, using the IBRv2 method. The higher the IBR value, the greater the damage on the embryos.

Table 3
Measured concentrations of EE2 in the zebrafish embryos.

Nominal concentrations of MET	Measured MET concentrations at different exposure times (ng g^{-1})					
	0 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
36 ng L^{-1}	1.2 ± 0.4	2.1 ± 0.8	3.3 ± 0.6	4.1 ± 0.3	4.5 ± 0.6	5.1 ± 0.4
46 ng L^{-1}	1.5 ± 0.7	2.9 ± 0.7	3.9 ± 1.1	5.3 ± 0.9	6.1 ± 0.9	7.6 ± 1.3
56 ng L^{-1}	1.9 ± 0.5	3.3 ± 0.5	4.2 ± 1.0	5.9 ± 1.1	7.3 ± 1.3	8.4 ± 1.6
66 ng L^{-1}	2.2 ± 0.8	4.1 ± 0.3	5.1 ± 0.8	6.3 ± 1.3	8.6 ± 1.5	9.1 ± 1.5
76 ng L^{-1}	2.7 ± 0.3	4.7 ± 0.9	6.0 ± 1.3	7.5 ± 1.0	9.7 ± 1.2	10.6 ± 1.4
86 ng L^{-1}	3.1 ± 0.7	5.1 ± 1.0	6.8 ± 0.9	8.3 ± 0.9	10.5 ± 1.4	14.5 ± 1.7
96 ng L^{-1}	3.6 ± 0.5	5.9 ± 0.9	7.4 ± 0.7	9.1 ± 1.2	13.1 ± 1.6	16.7 ± 1.5
106 ng L^{-1}	4.1 ± 0.6	6.3 ± 1.2	8.1 ± 1.4	10.7 ± 1.4	15.4 ± 1.1	20.1 ± 1.1

Values represent mean ± S.E.M. of each concentration of ten subsamples (n = 10).

generating toxic effects in the embryos of zebrafish (Cohen et al., 2014). In a study conducted by Lin et al. (2018), it was shown that once the EE2 enters the cell, this is biotransformed by the CYP, leading to increased production of ROS. This increased production of ROS may lead to a disruption in the calcium homeostasis, DNA damage, and cell apoptosis, triggering different toxic effects in the embryos such as morphological alterations, oxidative stress, and dead (Larsson et al., 2016; Shen et al., 2019).

Our results of embryo lethality showed that the LC_{50} of EE2 in embryos was de 82 ng L^{-1}. Siegenthaler et al. (2017), showed that the LC_{50} of EE2 for adult zebrafish was 950 ng L^{-1}. Boudreau et al. (2004), found that LC_{50} was 1000 ng L^{-1} for *Fundulus heteroclitus*. This shows that embryos are life stages more sensitive to EE2.

In a study carried out by Boudreau et al. (2004), they showed that concentrations of EE2 between 1000 and 10,000 ng L^{-1} are capable of generating craniofacial alterations, scoliosis, lordosis, malformation of the chorda, malformations in the fins and eyes of *Fundulus heteroclitus*. In our study, we identified similar malformations in *D. rerio* but at lower concentrations (36–106 ng L^{-1}). These findings allow us to conclude that zebrafish are a more sensitive bioindicator of exposure to EE2.

The skeletal alterations observed in this study may be related to the presence of ROS that generates LPX, which leads to the inhibition of osteoblast differentiation and the promotion of osteoclast differentiation through the increased expression of interleukin-6 (Tseng et al., 2010), which contributes to bone resorption causing skeletal alterations (Boglione et al., 2013).

One of the malformations with the greatest occurrence in our study was craniofacial alteration (protruding mouth, eye development, otoliths malformation). These malformations can be explained because the EE2 is an agonist of one of the G proteins found in the embryo and is responsible for regulating the proliferation of chondrocytes, precursors of the skeletal system, causing craniofacial effects (Cohen et al., 2014). The same authors determined the up-regulation of the MMP3, a gene that codes for collagenase and directly causes the phenotypic defects in cartilage such as protruding mouth. Furthermore, the alterations presented in fins and chorda may be related to the alterations in the ossification process.

Another malformation identified in this study was pericardial edema, which is consistent with a study conducted by Sun et al. (2015), who reports that EE2 in zebrafish produces an up-regulation of CYP26A1 which is important for retinoic acid metabolism. Its deficiency is a causative factor of pericardial edema.

EE2 is a thyroid hormone agonist, which generates changes in the behavior of embryos by increasing their movement (Zimmermann et al., 2019). In our study, we also identified increasing in embryonic movement.

As explained previously, several studies have shown that the oxidative stress phenomenon is induced by EE2, which generates alterations in genetic expression, in transcription factors, and in the cell cycle (Hansen, 2006; Dennery, 2007). This would also explain the morphological modifications found in this study.

In summary, in this study, we find that EE2 generates alterations to embryonic development and teratogenic effects at low concentrations (36–106 ng L^{-1}) in zebrafish. This is very useful because we can use

these tests as good biomarkers of toxicity for estrogens. In addition, we proved that *D. rerio* is a good bioindicator for exposure to estrogenic compounds.

5. Conclusions

EE2 was shown to be embryotoxic and teratogenic, causing oxidative damage in zebrafish at environmentally relevant concentrations of 36–106 ng L^{-1}. The LC_{50} of EE2 for embryos was 82 ng L^{-1}, while the EC_{50} of malformations was 57.7 ng L^{-1}. The EE2 presented a teratogenic index of 1.42. Teratogenic alterations identified in this study were chorda malformation, small fin, and increasing in embryonic movement, body hypopigmentation, yolk sac malformations, tail malformations, and scoliosis. Survival and development alterations are good biomarkers and predictors of EE2 toxicity in *D. rerio* and possibly other fish species.

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.

Acknowledgements

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