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RESEARCH ARTICLE

Embryonic developmental toxicity in marine medaka (*Oryzias melastigma*) caused by combined 17α-ethinylestradiol and hypoxic exposure

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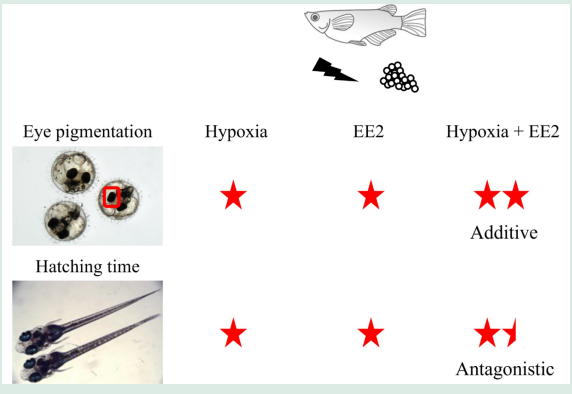
HIGHLIGHTS

- Hypoxic or EE2 exposure delay eye pigmentation stage of embryogenesis.
- Hypoxic exposure caused the delay of heart development.
- EE2 exposure affected embryo hatching.
- Combined hypoxia and EE2 exposure synergistically altered embryo development.
- Combined hypoxia and EE2 exposure adversely affected larval locomotion.



ABSTRACT: Hypoxia is one of the most pressing global challenges affecting aquatic ecosystems and is primarily driven by global warming and eutrophication. 17α-ethinylestradiol (EE2), a representative endocrine-disrupting chemical, is widely used in hormone therapy and contraceptives. Both hypoxia and EE2 affect embryonic development by disrupting endocrine signaling and their interactions may induce effects significantly different from their individual impacts. However, the combined exposure of aquatic animals to EE2 under hypoxic conditions remains poorly understood. In this study, marine medaka (*Oryzias melastigma*) were exposed to combined stressors of EE2 and hypoxia to investigate their interactive effects on embryonic development compared to individual exposures. The key parameters assessed were heart rate, hatching time, hatching rate, and larval locomotion. Our findings indicate that combined exposure to EE2 and hypoxia resulted in an additive effect eye pigmentation development and

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an antagonistic effect on hatching time. These results highlight the diverse trends in the effects induced by the interaction of multiple stressors, suggesting that in-depth omics-based analyses are required to explore the underlying molecular mechanisms.

KEYWORDS: 17α-ethinylestradiol, Hypoxia, Embryonic development, Locomotion, Endocrine disrupting chemicals

1 Introduction

Marine pollution has become a public health concern owing to its potential impacts on marine life and humans. Many studies have focused on the toxicological effects of various chemical and physical stressors, such as pH, temperature, heavy metals, and endocrine-disrupting chemicals (Donelson et al., 2010; Dos Santos et al., 2020; Delbès et al., 2022; Taslima et al., 2022). However, the natural marine environment is polluted not only by wastewater treatment plant effluents (Schwarzenbach et al., 2006; Loos et al., 2009) but also by agricultural chemicals (Moschet et al., 2014). Aquatic organisms are often exposed to complex mixtures of chemicals simultaneously (Thrupp et al., 2018). Consequently, an increasing number of studies started focusing on the effects and mechanisms of these chemical mixtures.

According to a review published in 2001, seasonal hypoxic conditions have occurred along the coastal seabed during the past 15–20 years (Diaz, 2001). Hypoxia is defined as a dissolved oxygen (DO) concentration of less than 2–3 mg O_2/L in marine environments (Farrell and Richards, 2009). A review of the threshold levels of hypoxia for marine organisms, such as fish, crustaceans, grastropods, bivalves, polychaetes, cnidarians, and ctenophores, concluded that the mean LC_{50} under hypoxia conditions for these species was 2.05 ± 0.09 mg O_2/L (Vaquer-Sunyer and Duarte, 2008). Moreover, studies have suggested that hypoxia can impair multiple physiologic systems. Hypoxia exerts effects similar to those of endocrine disruptors (Wu et al., 2003; Lai et al., 2021; Saha et al., 2022). It may cause embryonic developmental impairments in fish, such as increased embryonic mortality, altered hormone levels, and lowered hatching rates (Wu et al., 2003; Pollock et al., 2007; Wu, 2009). Hypoxia has also been reported to alter fish swimming activities, including swimming speed (Domenici et al., 2013).

Endocrine-disrupting chemicals (EDCs) are a diverse group of synthetic and natural compounds that are widespread in aquatic environments. 17α-ethinylestradiol (EE2), an EDCs, has been reported to impact embryonic development in fish, reducing embryonic survival rates, increasing teratogenesis rates, and delaying hatching (Brown et al., 2007; Hu et al., 2017; Qin et al., 2023). Furthermore, a study showed that EE2 uptake in killifish (*Fundulus heteroclitus*) is exacerbated under hypoxic conditions (Blewett et al., 2013), suggesting that the interactive effects of EE2 and hypoxia on organisms may be more severe than those of a single factor. However, the interaction effects of EE2 and hypoxia on embryonic development are poorly understood. Based on the aforementioned findings: (1) hypoxia may exert endocrine disrupting effects in organisms, and (2) the accumulation of EE2 increases under hypoxic condition. Therefore, it is hypothesized that combined exposure to the endocrine disruptor EE2 in hypoxic condition may cause interactive effects (including antagonistic, additive, or synergistic) that differ significantly from the sum of their individual effects on fish embryonic development. These effects may influence various aspects of embryonic development, including developmental patterns, heart rate, hatching, and larval locomotion.

In this study, two concentrations of EE2, 71 ± 5 and 668 ± 35 ng/L, were applied for exposure. Numerous studies have reported an unbalanced global distribution of EE2 concentrations. In freshwater samples from rivers, EE2 concentrations ranged from below detection limits to 33.5 ng/L in Austria, China, Hungary, Slovenia, and the USA, whereas Argentina, Brazil and Span reported much higher concentrations of more than 100 ng/L (Brossa et al., 2005; Klaic and Jirsa, 2022). For water samples near the estuary, EE2 concentrations ranged from below detection limits to 97.7 ng/L in the Douro River Estuary, Portugal (Ribeiro et al., 2009). In marine environments, EE2 concentrations have been reported from below the detection limit to less than 50 ng/L, with higher values of 75 ng/L reported in Italy (Pojana et al., 2004; Almeida et al., 2020). Therefore, concentrations below 100 ng/L are considered environmentally realistic for toxicological experiments (Lee et al., 2014). By contrast, the threshold of hypoxic conditions ranges from 2 to 5 mg/L depending on the organisms (Vaquer-Sunyer and Duarte, 2008; Gobler and Baumann, 2016). For marine fish, specifically, DO levels from 1.6 to 2.5 mg/L at pH 7.5 were considered hypoxic to assess early-life sensitivities (DePasquale et

al., 2015). Therefore, 71 ng/L EE2 was used as an environmentally realistic concentration, 668 ng/L EE2 served as a positive control, and a DO level of 1.8 mg/L was used as the hypoxia condition.

In the present study, we used marine medaka (*Oryzias melastigma*) as an animal model to address the embryonic developmental toxicity induced by the interaction between EE2 and hypoxic condition. Marine medaka is a fish species native to Asia. The transparency of its embryos facilitates the observation of morphological changes during embryogenesis, making it an ideal model for studying the biological impacts of various pollutants (Kim et al., 2016; Horie and Takahashi, 2021). Detailed differences in embryogenesis were observed and recorded, as the duration of marine medaka embryogenesis is approximately 9–13 d (Iwamatsu, 2004; Wang et al., 2020), compared to the shorter duration of approximately 3 d in zebrafish embryogenesis, another common fish model (Kimmel et al., 1995). This longer embryogenesis duration allows researchers to more easily record and compare morphological changes in marine medaka. Several endpoints were used to evaluate potential impairments during embryogenesis. First, developmental pattern was assessed. Second, heart rate was measured to determine developmental delays. Third, the hatching process including hatching time and hatching rate was evaluated to assess developmental delays and teratogenicity induced by combined exposure of EE2 and hypoxia. Finally, larval locomotion was measured to identify the lasting harmful effects on embryonic development. Our study indicated that combined exposure to EE2 under hypoxic conditions induced various effects compared to EE2 or hypoxia alone. Notably, an antagonistic effect was observed for hatching time under combined exposure to low EE2 concentrations in hypoxia, whereas an additive effect was observed for eye pigmentation development under combined exposure to high EE2 concentrations in hypoxic conditions.

2 Materials and methods

2.1 Chemicals and equipment used

HPLC grade dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and stored at 4 °C. EE2 (purity ⩾ 98%; CAS No. 57-63-6) was purchased from Sigma Chemical Company (St. Louis, MO, USA). The EE2 stock solution was dissolved in DMSO, and the working EE2 solutions were prepared in 1 L glass bottles with nominal concentrations of 125 and 1000 ng/L by diluting the stock solution with Milli-Q water. The DMSO control solution was prepared using the same procedure but without the addition of EE2. A Leica S6 E Stereo Zoom Microscope and Nikon SMZ1270 were used, along with the imaging software NIS-Elements. A Heal Force Tri-Gas Incubator (HF100) was purchased from Heal Force International Trading Co., Ltd. (Shanghai, China) to maintain hypoxic conditions. An RNA extraction kit (TransZol Up Plus RNA Kit, CAS No. ER501) was purchased from TransGen Biotech (Beijing, China). PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (CAS No. RR047A) and TB Green® Premix Ex Taq^{TM} (Tli RNaseH Plus) (CAS No. RR420A) were purchased from Takara Bio Inc. (Kyoto, Japan) for cDNA synthesis and PCR amplification.

2.2 Fish embryo maintenance and experimental setup

Marine medaka embryos were obtained from the City University of Hong Kong. Embryos were collected from the bottom of the tank within 1 h of morning light activation using a fine mesh net. They were then washed under running tap water by gentle kneading along the outer surface of the net before being transferred to Petri dishes. Embryos were cleaned under a microscope. Unfertilized and unviable embryos were discarded, and only fertilized, healthy embryos no older than 8 h post-fertilization (hpf) were used for exposure. In this study, an environmentally relevant concentration (71 ng/L) and a high EE2 concentration (668 ng/L) were used for exposure. A Heal Force Tri-Gas Incubator (HF100) was set to 95% N_2 and 5% CO_2 in a hypoxic environment. After exposure, the DO levels of the exposure solutions were detected by a DO meter, and were determined to be 1.8 mg/L. The embryos were exposed to six conditions until hatching: (1) control (0.004% of DMSO), (2) low concentration of EE2 (71 ng/L in 0.004% of DMSO, EE2_L), (3) high concentration of EE2 (668 ng/L in 0.004% of DMSO, EE2_H), (4) hypoxia group (1.8 mg/L DOfor the first 2 d of the experiment), (5) low concentration EE2 + hypoxia (EE2_L + hypoxia), and (6) high concentration EE2 + hypoxia (EE2_H + hypoxia). Each experiment included four replicates with 100 embryos per replicate. After 48 h of hypoxia exposure, all hypoxic groups were removed from the chamber. The embryos were reared under the following husbandry conditions: pH 7.4, 26 ± 1 °C, 7.2 ± 0.2 mg/L O_2, 25‰ salinity, and a 14-h light/10-h dark cycle. The exposure solution for each group was renewed daily. All treatments were maintained until hatching. Larvae were reared in cubic

tanks (n = 4, dimensions: 15 cm × 15 cm × 15 cm) for locomotion measurements at 3 d post-hatching (dph).

2.3 Measurement of EE2 exposure concentration

Water samples were collected from Petri dishes at 1, 3, 5, and 7 d of exposure and analyzed by liquid chromatography-tandem mass spectrometry. Water samples were subjected to solid-phase extraction using Oasis® WAX cartridges (500 mg, 6 mL). Methanol (5 mL × 2) and Milli-Q water (5 mL × 2) were sequentially added to precondition the cartridge. After loading the water samples into each cartridge (flow rate: one drop per second), the cartridge was washed with 10 mL of Milli-Q water, dried under vacuum for 30 min, and eluted with 10 mL of methanol. A gentle stream of high-purity nitrogen was used to concentrate the eluate to 20 µL at 40 °C. Replicate procedural blanks (Milli-Q water) and procedural recoveries (Milli-Q water spiked with 50 ng of EE2) were included in each batch of sample analyses. The average procedural recovery of EE2 was 76% ± 0.07%.

An Agilent 1290 Infinity ultra-performance liquid chromatograph (Agilent, Palo Alto, CA, USA) interfaced with a 5500 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) using multiple reaction monitoring in negative ion electrospray mode was used to determine EE2 concentrations. The Agilent Eclipse Plus C18 guard column (2.1 mm i.d. × 5 mm L., 1.8 µm) and the Agilent Eclipse Plus C18 analytical column (2.1 mm i.d. × 50 mm L., 1.8 µm) were employed. Briefly, a 10 µL aliquot of the extract was injected into the analytical column. The mobile phase consisted of 0.1% NH_4OH in Milli-Q water (solvent A) and acetonitrile (solvent B), with a flow rate of 0.3 mL/min. The gradient program was as follows: 50% solvent B stable for 1 min, increased to 90% over 3 min, maintainedfor 4 min, and returned to 50% within 0.1 min. During instrumental analysis, a quality control sample (10 ng/mL EE2 standard solution) was injected every 12 samples to monitor instrument stability. The standard deviation of the quality control was 0.55 ng/mL, indicating good instrument stability.

2.4 Determination of embryonic development

The observation period spanned from 0 d post-fertilization (dpf) to 7 dpf, with data collection during the entire embryogenesis period, defined as the time from fertilization to hatching (Iwamatsu, 2004; Kinoshita et al., 2009). This period was divided into three stages for comparing embryonic development: (1) pre-eye pigmentation period (0 dpf to the day when less than half of the embryos displayed eye pigmentation), (2) eye pigmentation period (from the day more than 50% of embryos displayed eye pigmentation to the day no embryos hatched), and (3) hatching period (from the day embryos began hatching to the day hatching ceased). The embryos were observed daily in the morning using a Leica S6 E Stereo Zoom Microscope. The developmental periods of the control were used as standards for comparison.

2.5 Measurement of heart rate, hatching delay, and hatching rate

The heart rate was measured at 3 dpf and on the first day of hatching. For each treatment replicate, a 40-s video was recorded using a Nikon SMZ1270 with the imaging software NIS-Elements. Ten embryos were randomly selected from each treatment replicate, and their heart rates were recorded for 30 s. The recorded data were multiplied by two to express the results as beats per minute.The hatching delay and hatching rate of the embryos were recorded from 0 to 20 dpf. Hatching delay was recorded, and the percentage of hatched embryos relative to the total number of embryos was calculated as the final hatching success.

2.6 Larval locomotion assessment

Twenty-four 3-d-old larvae were randomly selected from each treatment group for larval locomotion assessment. Larval locomotion data were captured using the Noldus behavior recording system equipped with Ethovision software version 3.1 (Noldus Information Technology, Leesburg, VA, USA) and analyzed using R software (version 4.2). The active area of the larvae was set as the area of the bottom of the wells in 24-well plates, and the coordinates of origin point 0 were adjusted to the center of the well. Onelarva and 1 mL of clean artificial seawater were placed in each well. The program for larval locomotion was set as follows: (1) acclimation: 10 min, 100% intensity level of light, no data collection, (2) lasting stimulation: 15 min, 100% intensity level of light, with data collection, (3) transitional stimulation, 10 min, 5 min in dark switching to 5 min of 100% intensity level of light, with data collection. These three periods of behavioral data were analyzed and presented as the means of speed.

2.7 Total RNA extraction, cDNA cloning and RT-qPCR

Thirty 5-d-old embryos from each replicate were randomly collected and pooled in RNAlater™

Stabilization Solution (Thermo Fisher Scientific) for storage at −80 °C until total RNA extraction (n = 4, total 4 × 6 × 30 = 720 embryos). Total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech), following the manufacturer's instructions. Briefly, frozen samples were thoroughly ground into a powder under in liquid nitrogen. Subsequently, 1 mL of TransZol Up and 0.2 mL RNA Extraction Agent were added for homogenization. After a 5-min incubation at room temperature and a 15-min centrifugation at 10000 × g, the upper transparent aqueous phase was collected. This phase was mixed with an equal volume of absolute ethanol, washed with the kit washing solution, and dissolved in RNase-free water for subsequent analysis. The total RNA concentration was quantified using a spectrophotometer (Thermo Scientific NanoDrop™ 2000 Spectrophotometers). RNA with an A260/A280 ratio of no less than 1.8 was used for cDNA synthesis. Subsequently, 1 μg of RNA was used for cDNA synthesis using PrimeScript™ RT Reagent Kit (Takara Bio Inc) and gDNA Eraser (Perfect Real Time), following the product manual. Quantitative polymerase chain reaction (qPCR) was performed with TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio Inc) using a C1000 Touch Thermal Cycler under CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Germany). The cycling conditions were as follows: (1) denaturation: 95 °C for 30 s, (2) annealing and extension: 95 °C for 5 s and 60 °C for 30 s, for a total of 40 cycles. *O. melastigma* 18S rRNA served as the reference gene for normalization (Kim et al., 2013). The threshold cycle (C_t) value was determined using CFX96™ Real-Time System CFX Maestro™ software version 2.3 (Bio-Rad), and ΔC_t values were calculated as $\Delta C_t = C_t$ (reference gene) – C_t (target gene). The fold change in relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2021).

2.8 Statistical analysis

All data were presented as the mean ± SEM. Data were shown as bar plots generated using GraphPad Prism 8 software (GraphPad, USA). Different letters indicated a significant differences among multiple comparisons performed using a one-way ANOVA followed by Duncan's test ($p < 0.05$).

3 Results

3.1 EE2 and hypoxia exposure altered embryonic development

The nominal low- and high-dose EE2 concentrations of 125 and 1000 ng/L, the actual measured concentrations were 71 ± 5 and 668 ± 35 ng/L, respectively. The DO in the exposure solution was 1.8 mg/L. Eye pigmentation started at 2 dpf in the control group (Fig. 1A), whereas both EE2_L and EE2_H delayed this onset by 1 d (starting at 3 dpf). Moreover, exposure to hypoxia and EE2_L + hypoxia resulted in a 2-d (starting at 4 dpf), whereas EE2_H + hypoxia caused a significant 3-d delay in eye pigmentation (starting at 5 dpf).

To further determine the alterations in embryogenesis caused by EE2 and hypoxia exposure, embryonic

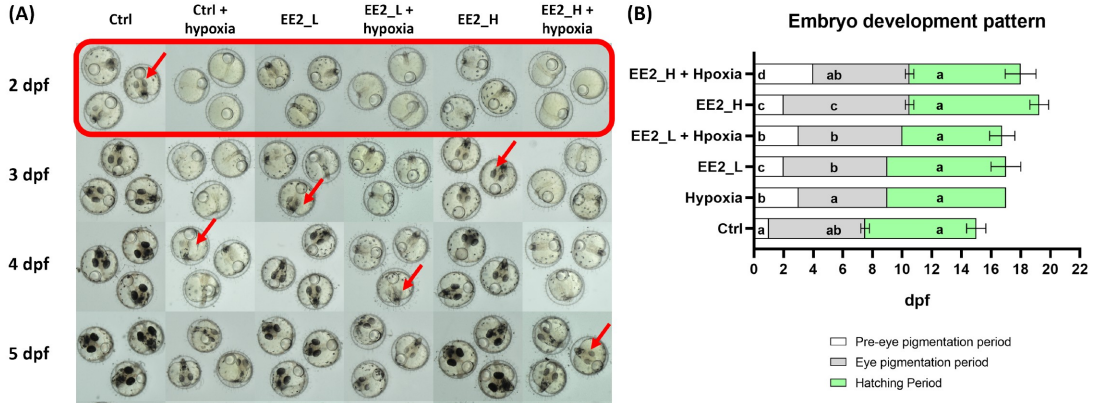

Fig. 1 EE2 and hypoxia exposure altered embryonic development. (A) Hypoxic and EE2 exposures delayed the onset of eye pigmentation, The red arrow indicates the apparent difference in the onsetof eye pigmentation, (B) hypoxic and EE2 exposures altered the embryonic development pattern, including the pre-eye pigmentation period, eye pigmentation period, and hatching period. Different letters indicate significant differences among multiple comparisons by a one-way ANOVA followed by Duncan's test ($p < 0.05$). EE2, 17α-ethinylestradiol; dpf, days post-fertilization.

development was assessed at three stages. Our results showed that exposure to either hypoxia or EE2 prolonged the pre-eye pigmentation period compared with that in the control group (Fig. 1B). This aligns with the observed delay in onset of eye pigmentation (Fig. 1A). During the eye pigmentation period, only the high dose of EE2 caused a significant extension compared to the controls. There were no significant changes observed during the hatching period. Collectively, these results suggest that EE2 and hypoxia mainly affect the pre-eye pigmentation period of embryogenesis.

3.2 EE2 and hypoxia exposure led to a reduction in embryonic heart rate

Our results showed that at 3 dpf, EE2, hypoxia, and their combination significantly decreased the heart rate of embryos (to an unobservable heartbeat) compared with that in the control groups (Fig. 2A). However, on the day of the first fish hatching, the EE2_L and EE2_L + hypoxia groups exhibited a significant reduction in the heart rate (8.71% and 11.87% with $p = 0.0170$ and $p = 0.0026$, respectively), whereas the hypoxia groups showed no significant change ($p = 0.1614$) compared to the control group (Fig. 2B). Furthermore, comparing the high EE2 concentration with the control group, EE2_H group showed no significant difference ($p = 0.0823$), but the EE2_H + hypoxia group showed a significant 11.35% reduction ($p = 0.0033$).

3.3 The hypoxic and EE2 exposures reduced the hatching rate of embryos

Our results showed that hypoxic exposure significantly delayed the initiation of hatching by 2 d (Fig. 3A). EE2 exposure induced a dose-dependent hatching delay, and combined EE2 and hypoxia exposure induced a 1 d delay compared to EE2 exposure alone (Fig. 3A). Moreover, our results showed the EE2 exposure caused a reduction in the hatching rate at high concentrations. The combined exposure of a high concentration of EE2 and hypoxia significantly increased the hatching rate (83.26% ± 5.72%) compared with that of the EE2_H group (60.10% ± 5.55%), but no significant changes were observed compared to the control group (Fig. 3B).

3.4 Effects of EE2 and hypoxia exposure on larval locomotion

Three larval speed periods were recorded for assessment. In the first 15 min of the light stable period, only the high-dose EE2 group showed a significant 33.85% decrease in larval speed compared with that in the control groups (Fig. 4A). The hypoxic and EE2-H + hypoxia groups showed a decreasing trend but this was not statistically significant. No significant changes were observed during the second and third periods (Figs. 4B

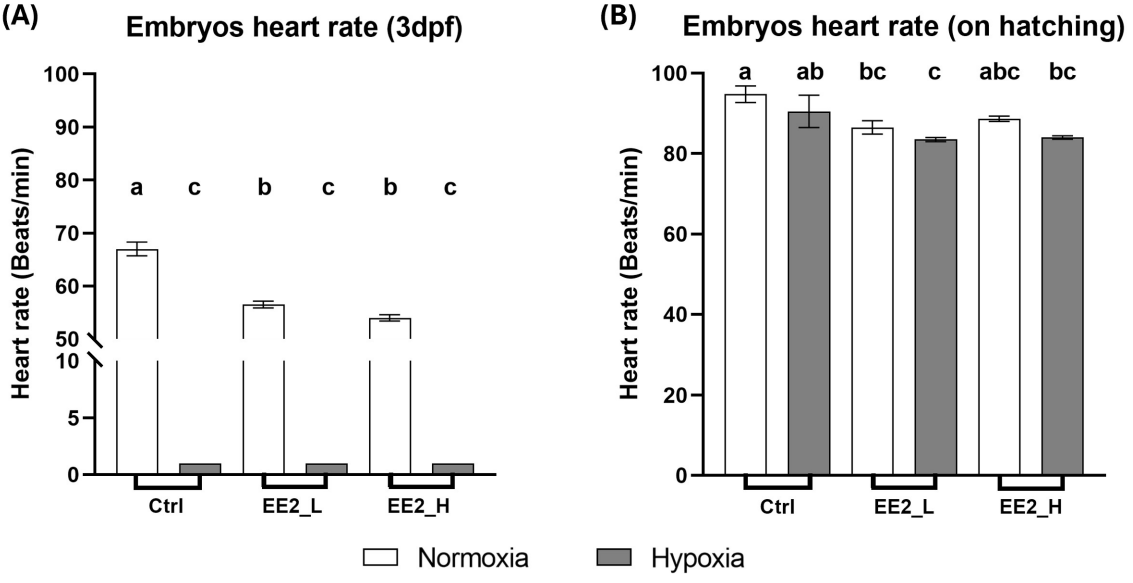

Fig. 2 EE2 and hypoxia exposure reduced the heart rate of embryos at different developmental stages. The exposure of EE2 and hypoxia reduced the heart rate (A) at 3 dpf and (B) on the day of the first fish hatching. Different letters indicate a significant differences among multiple comparisons determined by one-way ANOVA followed by Duncan's test ($p < 0.05$). EE2, 17α-ethinylestradiol; dpf, days post-fertilization.

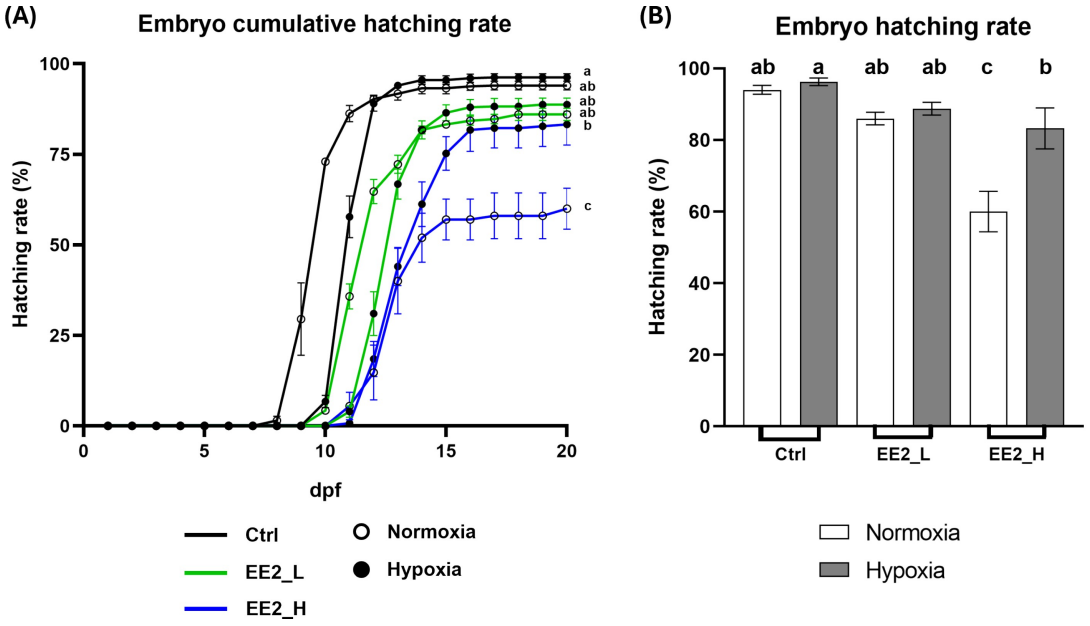

Fig. 3 EE2 and hypoxia exposures reduced the hatching rate of embryos. (A) EE2 and hypoxia exposure induced a hatching delay and (B) reduced the embryonic hatching rate. Different letters indicate significant differences among multiple comparisons determined by one-way ANOVA followed by Duncan's test ($p < 0.05$). EE2, 17α-ethinylestradiol.

and 4C). These results suggest that the reduction in larval locomotion primarily result from EE2 exposure.

3.5 EE2 or hypoxia exposure altered the gene expression related to embryonic development, heart development, nervous function and behavior

Based on the observed phenotypic changes, we summarized the alterations in embryonic development, heart rate, and larval locomotion caused by EE2 and hypoxic exposure. qPCR analysis was performed to understand the molecular changes underlying these adverse effects. Genes related to heart development (Ephrin B2 [*EFNB2*]), synaptic function and behavior (leucine-rich repeat transmembrane neuronal 1 [*LRRTM1*] and Syntaxin binding protein 1 [*STXBP1B*]), and eye development and function (Nuclear receptor subfamily 2 group E member 3 [*NR2E3*], Syntaxin 3 [*STX3*], and Thioredoxin-related transmembrane protein 3 [*TMX3*]) were also included. Our results showed that EE2 exposure had no effect on the gene expression of *EFNB2*, but hypoxia exposure

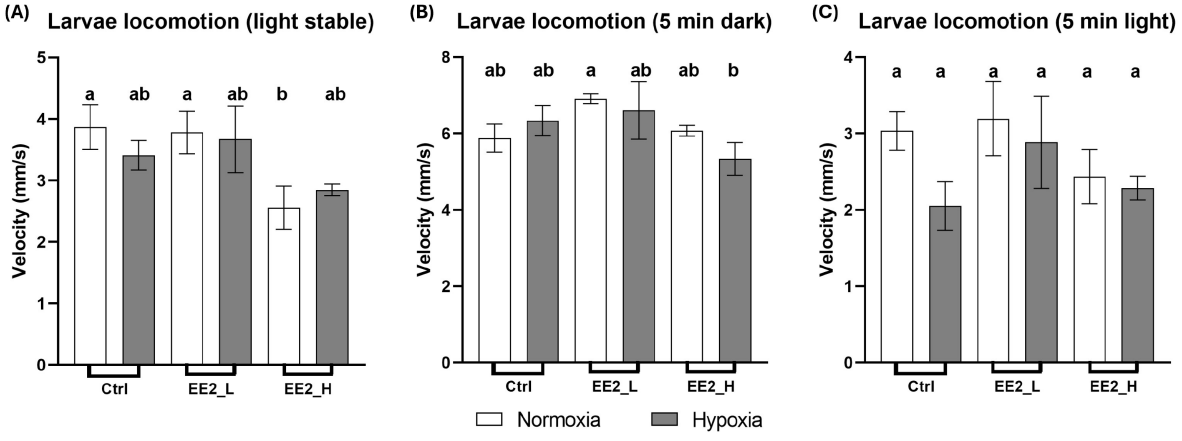

Fig. 4 Effects of EE2 and hypoxia on larval locomotion. The effects of larval locomotion under hypoxia, EE2, and combined exposure to EE2 and hypoxia during different periods: (A)–(C). Different letters indicate a significant differences among multiple comparisons determined by one-way ANOVA followed by Duncan's test ($p < 0.05$). EE2, 17α-ethinylestradiol.

significantly inhibited its expression, with fold changes of 0.295 ± 0.211, 0.285 ± 0.271, and 0.196 ± 0.088 in the hypoxia, EE2_L + hypoxia, and EE2_H + hypoxia groups, repectively (Fig. 5A). For synaptic function and behavior, both EE2 and hypoxia exposure suppressed the expression of *LRRTM1* and *STXBP1B* with fold changes below 0.5 (Fig. 5B). A similar result was observed in eye development and function-related genes; EE2 and hypoxia exposure reduced the expression of *NR2E3*, *STX3*, and *TMX3*, with fold changes below 0.6 (Fig. 5C).

3.6 Effects induced by the combined exposure of EE2 and hypoxia

The effects of the combined exposure of EE2 and hypoxia were further compared to the control, hypoxia, and EE2 exposure groups. a: indicates a significant difference between exposure groups (hypoxia, EE2_L, EE2_L + hypoxia, EE2_H, and EE2_H + hypoxia) and control groups; b: indicates a significant difference between exposure groups (EE2_L, EE2_L + hypoxia, EE2_H, and EE2_H + hypoxia) and hypoxia groups; c: indicates a significant difference between the combined exposure and EE2 exposure (EE2_L vs. EE2_L + hypoxia, EE2_H vs. EE2_H + hypoxia) (Tables 1 and 2). Our results showed that the EE2_H + hypoxia group presented a significant delay in the appearance of eye pigmentation compared with the other three groups (control, hypoxia, and EE2_H). Additionally, the EE2_L + hypoxia group showed a significant delay in hatching time compared with the other three groups (control, hypoxia, and EE2_L) (Table 1). The additive, synergistic, and antagonistic effects are defined as follows, additive: the combined effect is equal to the sum of the individual effects of each pollutant; synergistic: the combined effect is greater than the sum of the individual effects of each pollutant, and

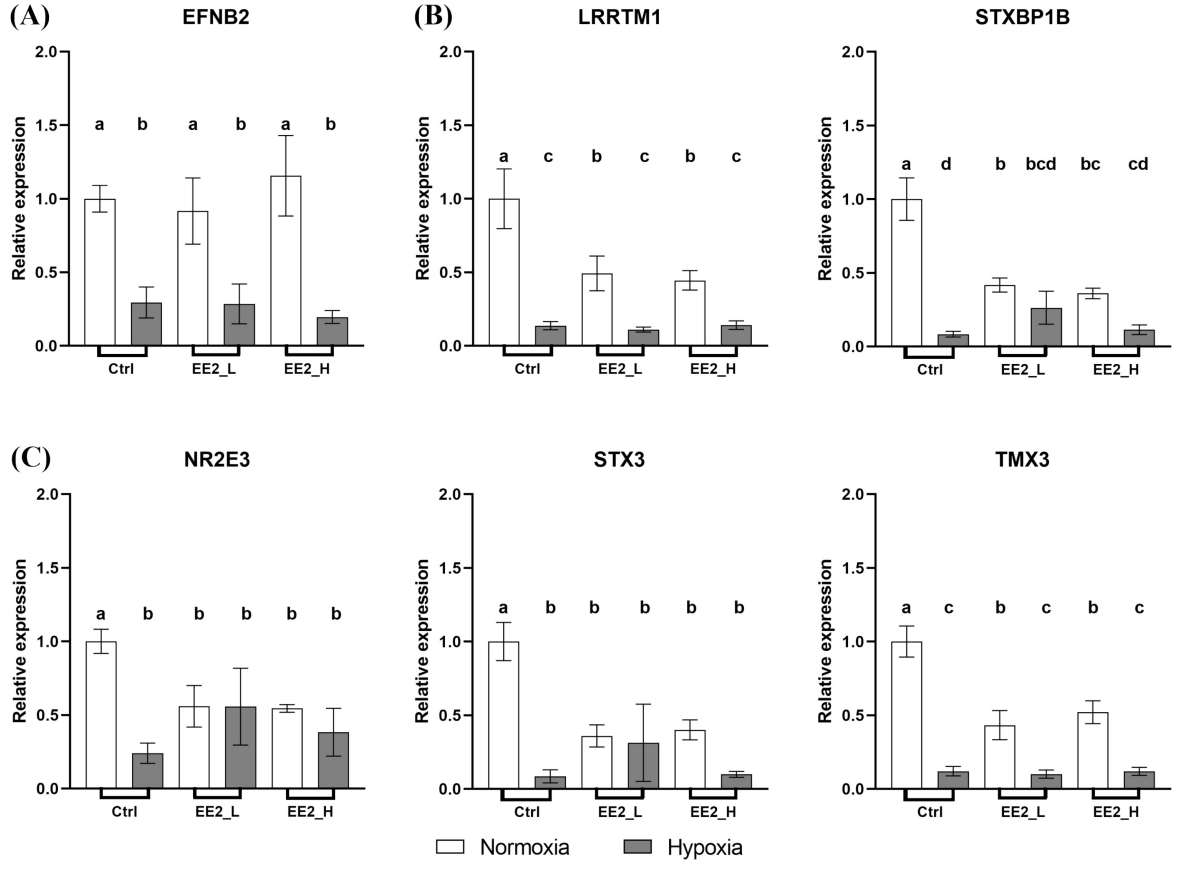

Fig. 5 Effects of EE2 and hypoxia on development-related gene expression. The changes in gene expressions related to (A) heart development, (B) synaptic function and behavior, and (C) eye development and function caused by the combined exposure of EE2 and hypoxia. Different letters indicate significant differences among multiple comparisons determined by one-way ANOVA followed by Duncan's test ($p < 0.05$). EE2, 17α-ethinylestradiol; *STX3*, Syntaxin 3; *NR2E3*, Nuclear receptor subfamily 2 group E member 3.

Table 1 Values for development and hatching in fish. Data are presented as mean ± SEM

Endpoints		Ctrl	Hypoxia	EE2_L	EE2_L + hypoxia	EE2_H	EE2_H + hypoxia
Development (dpf)	Eye pigmentation	2.0±0.0	4.0±0.0^{a}	3.0±0.0^{ab}	4.0±0.0^{ac}	3.0±0.0^{ab}	5.0±0.0^{abc}
Hatching (dpf, %)	Hatching time	7.5±0.3	9.0±0.0^{a}	9.0±0.0^{a}	10.0±0.0^{abc}	10.5±0.3^{ab}	10.5±0.3^{ab}
	Hatching rate	93.9±1.3	96.2±1.0	85.8±1.7	88.7±1.7	60.1±5.5^{ab}	83.3±5.7^{bc}

Note: a: $p < 0.05$ versus the control; b: $p < 0.05$ versus the hypoxia groups; c: $p < 0.05$ versus the EE2 groups (EE2_L + hypoxia VS EE2_L, EE2_H + hypoxia VS EE2_H); EE2, 17α-ethinylestradiol; dpf, days post-fertilization.

Table 2 Value for gene expression in fish. Data are presented as mean ± SEM

Endpoints		Ctrl	Hypoxia	EE2_L	EE2_L + hypoxia	EE2_H	EE2_H + hypoxia
Gene relative expression	*EFNB2*	1.000±0.18	0.295±0.21^{a}	0.916±0.45^{b}	0.285±0.27^{ac}	1.156±0.55^{b}	0.196±0.088^{ac}
	LRRTM1	1.000±0.41	0.138±0.056^{a}	0.492±0.24^{ab}	0.110±0.034^{ac}	0.445±0.13^{ab}	0.141±0.059^{ac}
	STXBP1B	1.000±0.29	0.084±0.039^{a}	0.417±0.096^{ab}	0.263±0.22^{a}	0.361±0.072^{ab}	0.114±0.065^{a}
	NR2E3	1.000±0.16	0.240±0.14^{a}	0.559±0.28^{a}	0.557±0.52^{a}	0.545±0.051^{a}	0.383±0.33^{a}
	STX3	1.000±0.26	0.086±0.088^{a}	0.360±0.15^{a}	0.314±0.52^{a}	0.401±0.14^{a}	0.099±0.04^{a}
	TMX3	1.000±0.21	0.121±0.065^{a}	0.433±0.2^{ab}	0.101±0.056^{ac}	0.521±0.16^{ab}	0.120±0.055^{ac}

a: $p < 0.05$ versus the control; b: $p < 0.05$ versus the hypoxia groups; c: $p < 0.05$ versus the EE2 groups (EE2_L + hypoxia VS EE2_L, EE2_H + hypoxia VS EE2_H); EE2, 17α-ethinylestradiol; *EFNB2*, Ephrin B2; *NR2E3*, Nuclear receptor subfamily 2 group E member 3; *STX3*, Syntaxin 3; *LRRTM1*, leucine-rich repeat transmembrane neuronal 1; *STXBP1B*, Syntaxin binding protein 1; *TMX3*, Thioredoxin-related transmembrane protein 3.

antagonistic: the combined effect is less than the sum of the individual effects of each pollutant (Jin et al., 2019; Mainka and Magdalena, 2022; Sures and Nachev, 2022). The delayed times of hypoxia, EE2_H, and EE2_H + hypoxia groups in the appearance of eye pigmentation were 2, 1, and 3 d (= 1 + 2), respectively. The delayed times in hatching were 1.5, 1.5, and 2.5 d (< 1.5 + 1.5). These results indicate an additive effect induced by the combined exposure of EE2 and hypoxia at high concentrations in eye pigmentation development and an antagonistic effect induced by the combined exposure of EE2 and hypoxia at low concentrations during hatching.

4 Discussion

4.1 Combined exposure of EE2 and hypoxia induced an antagonistic effect on the hatching time and affected the hatching process

Although increasing attention is being paid to the combined harmful effects of pollutants, few studies have focused on the combination of EE2 and hypoxia. A study using male Fathead minnows (*Pimephales promelas*), exposed to a mixture of EE2, 17β-estradiol (E2), 4-tert-nonylphenol, 4-tert-octylphenol, and bisphenol A (BPA) under hypoxia (DO level: 1.44 to 1.75 mg/L) for 14 d, showed no significant change in the level of vitellogenin (Brian et al., 2009). However, our results showed that newly fertilized embryos exposed to EE2, hypoxia, and combination of EE2 and hypoxia exhibited a significant delay in embryonic development, especially in the appearance of eye pigmentation. The difference in results may be because of (a) different fish species (Li et al., 2015; Spurgeon et al., 2020), fathead minnows are freshwater species, whereas marine medaka are seawater species used in our study; (b) different exposure scenarios (Dourson et al., 2004; Wang et al., 2021), Brian's study used sexually mature fish, but newly fertilized embryos were used in our study, which are more sensitive during exposures; (c) different concentrations used (Dornberger et al., 2016; Connell et al., 2024), highest concentration of EE2 used in Brain's study was 1.68 ng/L, which is lower than the concentrations used in our study (71 ± 5 and 668 ± 35 ng/L of EE2).

A study that examined zebrafish embryos exposed to a combination of EDCs and hypoxia revealed similar changes in embryonic development. In this study, zebrafish embryos were subjected to combined exposure to BPA and hypoxia (2.0 ± 0.5 mg/L O_2) from 1 hpf to the late hatching period. The results indicated significant decreases incardiac parameters, including heart rate and red blood cell velocity (Kubo et al., 2004; Cypher et al., 2018). Additionally, marine medaka embryos exposed to perfluorobutane sulfonate (PFBS) and hypoxia (DO level: 1.7 mg/L) from fertilization to 15 dpf exhibited significant reductions in survival, heart rate, and hatching rate (Tang et al., 2020). Although the EDCs studied differed from those in our study, both BPA and perfluorobutane sulfonate

have been reported to possess estrogenic properties in various organisms (Seachrist et al., 2016; Li et al., 2020), suggesting that combined exposure to estrogenic EDCs and hypoxia may elicit similar developmental effects.

4.2 Combined exposure of EE2 and hypoxia leads to an interactive effect in embryonic development compared with that of EE2 or hypoxia exposure alone

Considering the complexity of the natural environment, researchers have highlighted the necessity of chemical mixture exposure studies rather than focusing on the effects of single chemicals (Carlin et al., 2013). In particular, studies of chemical mixture exposure must address several important questions to understand their effects, including: (a) the impacts of individual chemicals, (b) interacting effects of chemical mixture, and (c) cumulative exposure effects of the chemical mixture (Braun et al., 2016). In this study, we evaluated the effects of individual factors and factor mixtures to understand their interaction.

In a typical chemical mixture exposure experiment, it is important to determine how the chemicals interact and whether their effects are additive, antagonistic, or synergistic (Lazarevic et al., 2019). An *in vivo* study on pejerrey fish (*Odontesthes bonariensis*) demonstrated that the larval survival rate showed a declining trend at 4 dph under single E2 or EE2 exposure, and combined exposure to E2 and EE2 showed a significant decline in larval survival rate, indivative of an additive effect (Gárriz et al., 2015). Our study showed that the combined exposure to EE2 and hypoxia induced additive effect on eye development (eye pigmentation) and antagonistic effects on hatching time. These results suggest that the effects induced by the combined exposure to EE2 and hypoxia cannot be simply defined as additive, antagonistic, or synergistic astheir impact varies in different biosystems. Previous studies have reported different EE2 accumulation patterns in fish organs. For example, EE2 accumulates in the gall bladder, followed by the carcass, gut, liver, gill, and spleen (Blewett et al., 2013). Another study showed that hypoxic conditions can significantly change the tissue-specific accumulation of EE2 in fish (Blewett et al., 2013). Moreover, a study showed that hypoxia inhibited the expression of the estrogen receptor (ER) in ER-positive cell lines of breast cancer (Wolff et al., 2017). Additionally, the 20-h estradiol and hypoxia exposure has been reported to synergistically degrade the expression of ERα in MCF-7 human breast cancer cells (Yi et al., 2009). Although these results were obtained from breast cancer cells, they suggest that hypoxia can interact with estrogen receptors and may further affect organisms. Hypoxic exposure may alter the accumulation and distribution patterns of EE2 in fish and could potentially affect the expression of estrogen receptors. This interaction may alter estrogenic activity of EE2 in specific organs, leading to differential gene expression and adverse effects in fish. However, the gene expression of ERα in marine medaka embryos may not be detected during early embryogenesis. One study detected its expression only as early as 6 dpf (Chakraborty et al., 2011). Therefore, the function of estrogen receptors in the early embryonic stage under hypoxic exposure remains poorly understood This hypothesis requires further evidence, particularly through embryonic exposure experiments, to enhance our understanding in future studies.

4.3 Exposure to EE2 and hypoxia altered the gene expression related to embryonic development and behavior

Based on the phenotypic results, we found that combined exposure to EE2 and hypoxia induced an additive effect on eye development during embryogenesis. Therefore, we hypothesized that this exposure might also alter gene expression related to embryonic development, eye development, and eye function. We conducted a qPCR analysis of genes associated with these observed phenotypes. *EFNB2* belongs to ephrin protein family and is highly affected by hypoxic conditions (Himanen et al., 2007). This proteins family is essential for neurous system function and angiogenesis (Brantley-Sieders and Chen, 2004; Butler and Tear, 2007; Reber et al., 2007). *EFNB2* is primarily expressed in arteries (Gerety et al., 1999), and *EFNB2* knockout mice have defects in arteries and veins at the capillary linkage between the head and yolk sac during angiogenesis (Wang et al., 1998). This finding supports our result that exposure to EE2 and hypoxia reduced heart rate during embryogenesis.

For genes related to eye function and behavior, *LRRTMA1* is highly expressed in the adult human brain and is involved in the stimulation of excitatory synaptogenesis (Linhoff et al., 2009; Winther and Walmod, 2014). More importantly, targeted deletion of *LRRTM1* results in a chronic reduction in excitatory synaptic function and may further affect cognitive, social, and sensorimotor gating deficits reminiscent of schizophrenia (Karimi et al., 2021). *STXBP1B* is a homolog of *STXBP1* in zebrafish (Suo et al., 2021). A study on *STXBP1* haploinsufficient mice showed a reduction in cortical inhibitory neurotransmission and

dysfunctional in cognitive, psychiatric, and motor functions (Chen et al., 2020). Furthermore, homozygous *STXBP1B* mutants in zebrafish caused neurodevelopmental abnormalities and decreased locomotor activity under a "dark-flash" visual stimulus system (Grone et al., 2016; Liu et al., 2021). These findings suggest that *LRRTM1* and *STXBP1B* may play a role in the reduced larval locomotion observed under EE2 or hypoxic exposure.

In addition, we investigated eye development and functional genes. *NR2E3* is a photoreceptor-specific orphan receptor involved in rod differentiation and is related to rod photoreceptor-specific genes (Cheng et al., 2004). An *NR2E3* knockout mouse study showed a defect in retinal morphology and abnormalities within the outer nuclear layer of the retina (Webber et al., 2008). *STX3* is also associated with eye functions, such as the fusion of vesicles in the photoreceptor plasma membrane, transportation of rhodopsin, and release of synaptic vesicles (Zulliger et al., 2015). The deletion of *STX3* in retinal cells results in a rapid degeneration and a mis-trafficking of photoreceptor outer segment proteins (Kakakhel et al., 2020). Another study using *STX3* knockout mice indicated that *STX3* is necessary for retinal photoreceptor cell survival (Janecke et al., 2021). *TMX3* was reported to play a role in delaying eye growth (Guerra and Molinari, 2020). This gene is expressed in the retinal neuroepithelium and lens epithelium, and TMX haploinsufficiency can affect eye morphology (Chao et al., 2010). The reduction in these genes aligns with our findings that exposure to EE2 and hypoxia led to a delay in eye pigmentation. Impairments in larval locomotion may also be attributed to dysfunction of the eye structure. However, it should be noted that there was no synergistic alteration in these genes under the combined exposure to EE2 and hypoxia, suggesting that more complex molecular mechanisms may be involved in the combined effects.

5 Conclusions

Exposure to EE2 or hypoxia impaires embryonic development, resulting in delayed the developmental processes, reduced heart rates, decreased hatching rates, and suppressed larval locomotion. Further gene expression analysis highlighted the involvement of genes such as *EFNB2*, *LRRTM1*, *STXBP1B*, *NR2E3*, *STX3*, and *TMX3* in the observed phenotypic changes. This gene cluster could be added to the gene list for evaluating the effects of EE2 or hypoxia.

Conflict of Interests 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.

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References

Almeida Â, Silva M G, Soares A M, Freitas R (2020). Concentrations levels and effects of 17α-ethinylestradiol in freshwater and marine waters and bivalves: a review. Environmental Research, 185: 109316

Blewett T A, Robertson L M, MacLatchy D L, Wood C M (2013). Impact of environmental oxygen, exercise, salinity, and metabolic rate on the uptake and tissue-specific distribution of 17α-ethynylestradiol in the euryhaline teleost *Fundulus heteroclitus*. Aquatic Toxicology, 138: 43–51

Brantley-Sieders D M, Chen J (2004). Eph receptor tyrosine kinases in angiogenesis: from development to disease. Angiogenesis, 7(1): 17–28

Braun J M, Gennings C, Hauser R, Webster T F (2016). What can epidemiological studies tell us about the impact of chemical mixtures on human health? Environmental Health Perspectives, 124(1): A6–A9

Brian J V, Beresford N, Walker J, Pojana G, Fantinati A, Marcomini A, Sumpter J P (2009). Hypoxia does not influence the response of fish to a mixture of estrogenic chemicals. Environmental Science & Technology, 43(1): 214–218

Brossa L, Marcé R M, Borrull F, Pocurull E (2005). Occurrence of twenty-six endocrine-disrupting compounds in environmental water samples from Catalonia, Spain. Environmental Toxicology and Chemistry, 24(2): 261–267

Brown K H, Schultz I R, Nagler J J (2007). Reduced embryonic survival in rainbow trout resulting from paternal exposure to the environmental estrogen 17α-ethynylestradiol during late sexual maturation. Reproduction, 134(5): 659–666

Butler S J, Tear G. Getting axons onto the right path: the role of transcription factors in axon guidance (2007). *Development*, 134 (3): 439-448

Carlin D J, Rider C V, Woychik R, Birnbaum L S (2013). Unraveling the health effects of environmental mixtures: an NIEHS priority. National Institute of Environmental Health Sciences, 12(1): a6–a8

Chakraborty T, Shibata Y, Zhou L Y, Katsu Y, Iguchi T, Nagahama Y (2011). Differential expression of three estrogen receptor subtype mRNAs in gonads and liver from embryos to adults of the medaka, *Oryzias latipes*. Molecular and Cellular Endocrinology, 333(1): 47–54

Chao R, Nevin L, Agarwal P, Riemer J, Bai X, Delaney A, Akana M, JimenezLopez N, Bardakjian T, Schneider A, et al. (2010). A male with unilateral microphthalmia reveals a role for TMX3 in

eye development. PLoS One, 5(5): e10565

Chen W, Cai Z L, Chao E S, Chen H, Longley C M, Hao S, Chao H T, Kim J H, Messier J E, Zoghbi H Y, et al. (2020). Stxbp1/Munc18–1 haploinsufficiency impairs inhibition and mediates key neurological features of STXBP1 encephalopathy. eLife, 9: e48705

Cheng H, Khanna H, Oh E C, Hicks D, Mitton K P, Swaroop A (2004). Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Human Molecular Genetics, 13(15): 1563–1575

Connell W, Garcia K, Goodarzi H, Keiser M J (2024). Learning chemical sensitivity reveals mechanisms of cellular response. Communications Biology, 7(1): 1149

Cypher A D, Fetterman B, Bagatto B (2018). Vascular parameters continue to decrease post-exposure with simultaneous, but not individual exposure to BPA and hypoxia in zebrafish larvae. Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP, 206-207: 11-16

Delbès G, Blázquez M, Fernandino J I, Grigorova P, Hales B, Metcalfe C, Navarro-Martín L, Parent L, Robaire B, Rwigemera A, et al. (2022). Effects of endocrine disrupting chemicals on gonad development: mechanistic insights from fish and mammals. Environmental Research, 204: 112040

DePasquale E, Baumann H, Gobler C J (2015). Vulnerability of early life stage Northwest Atlantic forage fish to ocean acidification and low oxygen. Marine Ecology Progress Series, 523: 145–156

Diaz R J (2001). Overview of hypoxia around the world. Journal of Environmental Quality, 30(2): 275–281

Domenici P, Herbert N, Lefrançois C, Steffensen J, McKenzie D (2013). The Effect of Hypoxia on Fish Swimming Performance and Behaviour. In: Palstra A, Planas J, eds. Swimming Physiology of Fish. Berlin: Springer

Donelson J, Munday P, McCormick M, Pankhurst N, Pankhurst P (2010). Effects of elevated water temperature and food availability on the reproductive performance of a coral reef fish. Marine Ecology Progress Series, 401: 233–243

Dornberger L, Ainsworth C, Gosnell S, Coleman F (2016). Developing a polycyclic aromatic hydrocarbon exposure dose-response model for fish health and growth. Marine Pollution Bulletin, 109(1): 259–266

dos Santos J A, Soares C M, Bialetzki A (2020). Effects of pH on the incubation and early development of fish species with different reproductive strategies. Aquatic Toxicology, 219: 105382

Dourson M, Charnley G, Scheuplein R, Barkhurst M (2004). Differential sensitivity of children and adults to chemical toxicity. Human and Ecological Risk Assessment, 10(1): 21–27

Farrell A P, Richards J G (2009). Defining hypoxia: an integrative synthesis of the responses of fish to hypoxia. Fish Physiology, 27: 487–503

Gárriz Á, Menéndez-Helman R J and Miranda L A (2015). Effects of estradiol and ethinylestradiol on sperm quality, fertilization, and embryo–larval survival of pejerrey fish (*Odontesthes bonariensis*). Aquatic Toxicology, 167: 191–199

Gerety S S, Wang H U, Chen Z F, Anderson D J (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Molecular Cell, 4(3): 403–414

Gobler C J, Baumann H (2016). Hypoxia and acidification in ocean ecosystems: coupled dynamics and effects on marine life. Biology Letters, 12(5): 20150976

Grone B P, Marchese M, Hamling K R, Kumar M G, Krasniak C S, Sicca F, Santorelli F M, Patel M, Baraban S C (2016). Epilepsy, behavioral abnormalities, and physiological comorbidities in syntaxin-binding protein 1 (*STXBP1*) mutant zebrafish. PLoS One, 11(3): e0151148

Guerra C, Molinari M (2020). Thioredoxin-related transmembrane proteins: TMX1 and little brothers TMX2, TMX3, TMX4 and TMX5. Cells, 9(9): 2000

Himanen J P, Saha N, Nikolov D B (2007). Cell–cell signaling via Eph receptors and ephrins. Current Opinion in Cell Biology, 19(5): 534–542

Horie Y, Takahashi C (2021). Development of an *in vivo* acute bioassay using the marine medaka *Oryzias melastigma*. Environmental Monitoring and Assessment, 193(11): 725

Hu S, Zhang H, Shen G, Yuan Z, Xu T, Ji R (2017). Effects of 17β-estradiol and 17α-ethinylestradiol on the embryonic development of the clearhead icefish (*Protosalanx hyalocranius*). Chemosphere, 176: 18–24

Iwamatsu T (2004). Stages of normal development in the medaka *Oryzias latipes*. Mechanisms of Development, 121(7−8): 605–618

Janecke A R, Liu X, Adam R, Punuru S, Viestenz A, Strauß V, Laass M, Sanchez E, Adachi R, Schatz M P, et al. (2021). Pathogenic STX3 variants affecting the retinal and intestinal transcripts cause an early-onset severe retinal dystrophy in microvillus inclusion disease subjects. Human Genetics, 140(8): 1143–1156

Jin P, Overmans S, Duarte C M, Agusti S (2019). Increasing temperature within thermal limits compensates negative ultraviolet-B radiation effects in terrestrial and aquatic organisms. Global Ecology and Biogeography, 28(11): 1695–1711

Kakakhel M, Tebbe L, Makia M S, Conley S M, Sherry D M, Al-Ubaidi M R, Naash M I (2020). Syntaxin 3 is essential for photoreceptor outer segment protein trafficking and survival. Proceedings of the National Academy of Sciences of the United States of America, 117(34): 20615–20624

Karimi B, Silwal P, Booth S, Padmanabhan N, Dhume S H, Zhang D, Zahra N, Jackson M F, Kirouac G J, Ko J H, et al. (2021). Schizophrenia-associated LRRTM1 regulates cognitive behavior through controlling synaptic function in the mediodorsal thalamus. Molecular Psychiatry, 26(11): 6912–6925

Kim B M, Kim J, Choi I Y, Raisuddin S, Au D W, Leung K M, Wu R S S, Rhee J S, Lee J S (2016). Omics of the marine medaka (*Oryzias melastigma*) and its relevance to marine environmental research. Marine Environmental Research, 113: 141–152

Kim R O, Kim B M, Hwang D S, Au D W, Jung J H, Shim W J, Leung K M Y, Wu R S S, Rhee J S, Lee J S (2013). Evaluation of biomarker potential of cytochrome P450 1A (CYP1A) gene in the marine medaka, *Oryzias melastigma* exposed to water-accommodated fractions (WAFs) of Iranian crude oil. Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP, 157(2): 172–182

Kimmel C B, Ballard W W, Kimmel S R, Ullmann B, Schilling T F (1995). Stages of embryonic development of the zebrafish. Developmental Dynamics, 203(3): 253–310

Kinoshita M, Murata K, Naruse K, Tanaka M (2009). Medaka: biology, management, and experimental protocols: John Wiley & Sons

Klaic M, Jirsa F (2022). 17α-Ethinylestradiol (EE2): concentrations in the environment and methods for wastewater treatment–an update. RSC Advances, 12(20): 12794–12805

Kubo T, Maezawa N, Osada M, Katsumura S, Funae Y, Imaoka S (2004). Bisphenol A, an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1α (HIF-1α): structural requirement of bisphenol A for degradation of HIF-1α. Biochemical and Biophysical Research Communications, 318(4): 1006–1011

Lai K P, Tam N, Wang S Y, Tse W K F, Lin X, Chan T F, Tong Y, Zhang J, Au D W T, Wu R S S, et al. (2021). Proteomic response of the brain to hypoxic stress in marine medaka fish (*Oryzias melastigma*). Frontiers in Marine Science, 8: 618489

Lazarevic N, Barnett A G, Sly P D, Knibbs L D (2019). Statistical methodology in studies of prenatal exposure to mixtures of endocrine-disrupting chemicals: a review of existing approaches and new alternatives. Environmental Health Perspectives, 127(2): 026001

Lee P Y, Lin C Y, Chen T H (2014). Environmentally relevant exposure of 17α-ethinylestradiol impairs spawning and reproductive behavior in the brackish medaka *Oryzias melastigma*. Marine Pollution Bulletin, 85(2): 338–343

Li J, Cao H, Feng H, Xue Q, Zhang A, Fu J (2020). Evaluation of the estrogenic/antiestrogenic activities of perfluoroalkyl substances and their interactions with the human estrogen receptor by combining *in vitro* assays and *in silico* modeling. Environmental Science & Technology, 54(22): 14514–14524

Li J J, Wang X H, Wang Y, Wen Y, Qin W C, Su L M, Zhao Y H (2015). Discrimination of excess toxicity from narcotic effect: influence of species sensitivity and bioconcentration on the classification of modes of action. Chemosphere, 120: 660–673

Linhoff M W, Laurén J, Cassidy R M, Dobie F A, Takahashi H, Nygaard H B, Airaksinen M S, Strittmatter S M, Craig A M (2009). An unbiased expression screen for synaptogenic proteins identifies the LRRTM protein family as synaptic organizers. Neuron, 61(5): 734–749

Liu J, Salvati K A, Baraban S C (2021). *In vivo* calcium imaging reveals disordered interictal network dynamics in epileptic stxbp1b zebrafish. iScience, 24(6): 102558

Livak K J, Schmittgen T D (2021). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25: 402–408

Loos R, Gawlik B M, Locoro G, Rimaviciute E, Contini S, Bidoglio G (2009). EU-wide survey of polar organic persistent pollutants in European river waters. Environmental Pollution, 157(2): 561–568

Mainka A, Żak M (2022). Synergistic or antagonistic health effects of long-and short-term exposure to ambient NO_2 and $PM_{2.5}$: a review. International Journal of Environmental Research and Public Health, 19(21): 14079

Moschet C, Vermeirssen E L, Seiz R, Pfefferli H, Hollender J (2014). Picogram per liter detections of pyrethroids and organophosphates in surface waters using passive sampling. Water Research, 66: 411–422

Pojana G, Bonfà A, Busetti F, Collarin A, Marcomini A (2004). Estrogenic potential of the Venice, Italy, lagoon waters. Environmental Toxicology and Chemistry, 23(8): 1874–1880

Pollock M, Clarke L, Dubé M (2007). The effects of hypoxia on fishes: from ecological relevance to physiological effects. Environmental Reviews, 15(NA): 1–14

Qin X, Lin H, Cao Y, Wu R S S, Lai K P, Kong R Y C (2023). Embryo developmental toxicity in marine medaka (*Oryzias melastigma*) due to parental and embryonic 17α-ethinylestradiol exposure. Science of the Total Environment, 861: 160594

Reber M, Hindges R, Lemke G (2007). Eph receptors and ephrin ligands in axon guidance. Axon Growth and Guidance, 621: 32–49

Ribeiro C, Tiritan M E, Rocha E, Rocha M J (2009). Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River Estuary, Portugal. Archives of Environmental Contamination and Toxicology, 56(1): 1–11

Saha N, Koner D, Sharma R (2022). Environmental hypoxia: a threat to the gonadal development and reproduction in bony fishes. Aquaculture and Fisheries, 7(5): 572–582

Schwarzenbach R P, Escher B I, Fenner K, Hofstetter T B, Johnson C A, Von Gunten U, Wehrli B (2006). The challenge of micropollutants in aquatic systems. Science, 313(5790): 1072–1077

Seachrist D D, Bonk K W, Ho S M, Prins G S, Soto A M, Keri R A (2016). A review of the carcinogenic potential of bisphenol A. Reproductive Toxicology, 59: 167–182

Spurgeon D, Lahive E, Robinson A, Short S, Kille P (2020). Species sensitivity to toxic substances: evolution, ecology and applications. Frontiers in Environmental Science, 8: 588380

Suo G, Cao X, Zheng Y, Li H, Zhang Q, Tang J, Wu Y (2021). A *de novo* nonsense mutation of STXBP1 causes early-onset epileptic encephalopathy. Epilepsy & Behavior, 123: 108245

Sures B, Nachev M (2022). Effects of multiple stressors in fish: how parasites and contaminants interact. Parasitology, 149(14): 1822–1828

Tang L, Liu M, Song S, Hu C, Lam P K, Lam J C, Chen L (2020). Interaction between hypoxia and perfluorobutane sulfonate on developmental toxicity and endocrine disruption in marine

medaka embryos. Aquatic Toxicology, 222: 105466

Taslima K, Al-Emran M, Rahman M S, Hasan J, Ferdous Z, Rohani M F, Shahjahan M (2022). Impacts of heavy metals on early development, growth and reproduction of fish: a review. Toxicology Reports, 9: 858–868

Thrupp T J, Runnalls T J, Scholze M, Kugathas S, Kortenkamp A, Sumpter J P (2018). The consequences of exposure to mixtures of chemicals: something from 'nothing'and 'a lot from a little' when fish are exposed to steroid hormones. Science of the Total Environment, 619-620: 1482–1492

Vaquer-Sunyer R, Duarte C M (2008). Thresholds of hypoxia for marine biodiversity. Proceedings of the National Academy of Sciences of the United States of America, 105(40): 15452–15457

Wang H U, Chen Z F, Anderson D J (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell, 93(5): 741–753

Wang R F, Zhu L M, Zhang J, An X P, Yang Y P, Song M, Zhang L (2020). Developmental toxicity of copper in marine medaka (*Oryzias melastigma*) embryos and larvae. Chemosphere, 247: 125923

Wang Y Y L, Li P, Ohore O E, Wang Y, Zhang D, Bai Y, Su T, You J, Jin X, Liu W, et al. (2021). Life stage and endpoint sensitivity differences of fathead minnow (*Pimephales promelas*) to chemicals with various modes of action. Environmental Pollution, 290: 117995

Webber A L, Hodor P, Thut C J, Vogt T F, Zhang T, Holder D J, Petrukhin K (2008). Dual role of Nr2e3 in photoreceptor development and maintenance. Experimental Eye Research, 87(1): 35–48

Winther M, Walmod P S (2014). Neural cell adhesion molecules belonging to the family of leucine-rich repeat proteins. Cell Adhesion Molecules. Advances in Neurobiology. New York: Springer

Wolff M, Kosyna F K, Dunst J, Jelkmann W, Depping R (2017). Impact of hypoxia inducible factors on estrogen receptor expression in breast cancer cells. Archives of Biochemistry and Biophysics, 613: 23–30

Wu R S (2009). Effects of hypoxia on fish reproduction and development. Fish Physiology, 27: 79–141

Wu R S, Zhou B S, Randall D J, Woo N Y, Lam P K (2003). Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science & Technology, 37(6): 1137–1141

Yi J M, Kwon H Y, Cho J Y, Lee Y J (2009). Estrogen and hypoxia regulate estrogen receptor alpha in a synergistic manner. Biochemical and Biophysical Research Communications, 378(4): 842–846

Zulliger R, Conley S M, Mwoyosvi M L, Stuck M W, Azadi S, Naash M I (2015). SNAREs interact with retinal degeneration slow and rod outer segment membrane protein-1 during conventional and unconventional outer segment targeting. PLoS One, 10(9): e0138508