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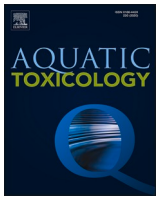

Multigenerational impacts of EE2 on reproductive fitness and immune competence of marine medaka

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ABSTRACT

Estrogenic endocrine disrupting chemicals (EEDC) have been suspected to impact offspring in a transgenerational manner via modifications of the germline epigenome in the directly exposed generations. A holistic assessment of the concentration/ exposure duration-response, threshold level, and critical exposure windows (parental gametogenesis and embryogenesis) for the transgenerational evaluation of reproduction and immune compromise concomitantly will inform the overall EEDC exposure risk. We conducted a multigenerational study using the environmental estrogen, 17α-ethinylestradiol (EE2), and the marine laboratory model fish Oryzias melastigma (adult, F0) and their offspring (F1-F4) to identify transgenerationally altered offspring generations and phenotype persistence. Three exposure scenarios were used: short parental exposure, long parental exposure, and a combined parental and embryonic exposure using two concentrations of EE2 (33ng/L, 113ng/L). The reproductive fitness of fish was evaluated by assessing fecundity, fertilization rate, hatching success, and sex ratio. Immune competence was assessed in adults via a host-resistance assay. EE2 exposure during both parental gametogenesis and embryogenesis was found to induce concentration/ exposure duration-dependent transgenerational reproductive effects in the unexposed F4 offspring. Furthermore, embryonic exposure to 113 ng/L EE2 induced feminization of the directly exposed F1 generation, followed by subsequent masculinization of the F2 and F3 generations. A sex difference was found in the transgenerationally impaired reproductive output with F4 females being sensitive to the lowest concentration of EE2 (33 ng/L) upon long-term ancestral parent exposure (21 days). Conversely, F4 males were affected by ancestral embryonic EE2 exposure. No definitive transgenerational impacts on immune competence were identified in male or female offspring. In combination, these results indicate that EEDCs can be transgenerational toxicants that may negatively impact the reproductive success and population sustainability of fish populations.

1. Introduction

Endocrine-disrupting chemicals (EDCs) have the potential to alter the normal endocrine function of wildlife and humans (Jürgens et al., 2002; Zhou et al., 2007; Lei et al., 2009; Aris et al., 2014). Estrogenic EDCs (EEDCs) affect estrogen hormone biosynthesis and pathways through mimicking natural estrogen, and/or interfering with estrogen receptors (ERs) (Guillette and Gunderson, 2001; Waring and Harris, 2005; Watson et al., 2011; Rosenfeld & Cooke, 2019) subsequently altering reproduction, growth, metabolism, and immune function (Johnson et al., 2013; Adeel et al., 2017). Synthetic estrogens, such as 17α-ethinylestradiol (EE2), are of serious environmental concern due to their similarity to natural estradiol, higher binding affinity for ERs, and relative stability in the marine environment (Tyler et al., 1998; Ying et al., 2003; Nagpal and Meays, 2009; Milla et al., 2011). The primary EE2 sources for the marine environment are waste-water treatment

Capsule: Xenoestrogens can transgenerationally impair reproductive competence up to the F4 generation with negative implications on fish populations in the absence of continued exposure.

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effluents (Reviewed by Lee et al., 2007; Liu et al., 2009; Nagarnaik et al., 2010; Aris et al., 2014). Environmental EE2 levels are estimated between 0.4 ng/L and 14 ng/L in U.S. waterways (Kostich et al., 2013) and can range from undetectable (< 0.1 ng/L) to 43 ng/L in sea water (Valdés et al., 2015).

EEDC exposed fish showed altered gonad and intersex development, impaired fertilization success, fecundity and hatching success at concentrations as low as 1 ng/L (Matthiessen, 2003; Robinson et al., 2003; Mills and Chichester, 2005; Burkhardt-Holm et al., 2008; Tyler et al., 2008; Leet et al., 2011; Fuzzen et al., 2015; Armstrong et al., 2016; Parrott and Blunt, 2005). Exposure duration, EE2 concentration and developmental stage at the time of exposure determine the extent of EE2 induced reproductive impacts (Scholz and Gutziet, 2000). Phenotypic sex change in males in addition to the development of a mixed testis-ova occurred in more than 50% of male Oryzias latipes after exposure to 29.3 ng/L EE2 for 21 days (Kang et al., 2002). A 7-year whole lake field study of chronic 5 – 6 ng/L EE2 exposure in wild fathead minnow (Pimephales promelas) showed intersex development in males and altered oogenesis in females (Kidd et al., 2007), eventually resulting in near depletion of the population in the lake (Blanchfield et al., 2015).

The immunomodulation of EE2 in fish is not as well understood as reproductive effects and appears to be species-, sex-, and exposure concentration-dependent. Estrogen may inhibit cell-mediated immunity but enhance humoral immunity (Iwanowicz et al.; 2008). Low levels of EE2 may be stimulatory, while higher levels show an inhibitory immune effect (Nalbandian and Kovats, 2005). Exposure of adult marine medaka (Oryzias melastigma) to 33 ng/L EE2 induced hormesis (immune enhancement) in the female immune response to bacterial challenge, while 113 ng/L EE2 impaired immune function in females and males (Ye et al., 2018). Estrogens can impact both the innate and adaptive immune systems through direct interaction with lymphocytes and macrophages, modulation of cytokine and chemokine production, and gene expression (Straub, 2007; Jin et al., 2010; Sun et al., 2011; Cabas et al., 2012; Shelley et al., 2012; Seemann et al., 2013, 2016).

A growing body of evidence has identified EDCs as potential modulators of the germ cell epigenome (Desaulniers et al., 2009; Casati et al., 2012; Manikkam et al., 2012; Bhandari et al. 2015; McBirney et al., 2017; reviewed in Robaire et al., 2022). EEDCs may impact reproduction in a transgenerational manner (Turusov et al., 1992; Anway et al., 2006; Meyer et al., 2018; Rattan & Flaws, 2019; Major et al., 2020). Transgenerational effects may occur through direct exposure induced alteration of the germ cells (sperm or oocyte) or maternal transfer of EEDCs into the developing F1 embryo. Stressor-induced alterations of the F1 germ-line epigenome could be transferred to the subsequent F2 generation and beyond. Any impacts present in the F3 generation or beyond without direct exposure would qualify as unexposed transgenerational events (Anway and Skinner, 2006; Skinner 2008; Ho and Burggren, 2010).

Hormones can induce DNA methylation in somatic tissues, histone modifications, and change micro-RNA expression (Contractor et al., 2004; Nugent et al., 2011; McCarthy and Nugent, 2013). Unlike mammals, there is unequivocal evidence that the paternal (sperm) methylome does not undergo reprogramming during embryogenesis in the teleosts O. latipes and D. rerio; instead, the paternal pattern is stably inherited, and the female methylation undergoes de novo demethylation and remethylation to match the paternal pattern (Walter et al., 2002; Jiang et al., 2013; Potok et al., 2013). This lack of remethylation suggests a higher likelihood of paternally inherited changes in methylation, and thus, a higher susceptibility to transgenerational impacts as a result of paternal EEDC exposure, which has been recently shown for atrazine, bisphenol A, and EE2 (Cleary et al., 2019; Bhandari et al., 2015, 2020). The higher potential for transgenerational impacts in fish makes it critical to evaluate the immune and reproductive competence in multiple generations to improve assessment of the risk of EEDC exposures on fish populations.

Evidence of unexposed transgenerational impacts of exogenous estrogens in fish remains sparse but suggests that both parental exposure and direct exposure during embryogenesis may impact the subsequent unexposed generations in a transgenerational manner. The direct exposure transgenerational impacts of EEDCs identified previously have been mostly limited to the F1 generation and include decreased hatching success, decreased fertilization success and decreased survival (Zillioux et al., 2001; Hill and Janz, 2003; Brown et al. 2008; Segner, 2009, Wei et al., 2018). Paternal exposure of zebrafish to 2.5 ng/L and 5 ng/L EE2 for 14 days led to increased esr1 and esr2b expression in the F0 testes and a significant increase in malformations in the F1 offspring (Valcarce et al., 2017). Embryonic and early life exposure (1 – 80 dpf) to 1.2 ng/L of EE2 resulted in reduced fertilization success in the F0 generation even after 82 days of exposure cessation and led to significantly increased anxiety and shoaling intensity in the F1 offspring (Volkova et al., 2015).

To date, limited studies have assessed EEDC induced reproductive impacts in fish in the F2 generation and beyond. Waterborne exposure during parental gametogenesis to 50 ng/L endosulfan for four hours caused decreased hatching success in the F2 offspring in Melanotaenia fluviatilis (Holdway et al., 2008). Additionally, lifetime exposure to 5.4 ng/L EE2 reduced survival in the F2 offspring of ancestrally exposed Pimephales promelas (Schwindt et al., 2014). It has also been observed in zebrafish that 14-day paternal exposure to 2000 ng/L BPA led to increased heart failures in the F1 and F2 generation, possibly linked to changes in early development mRNA levels in the sperm (Lombó et al., 2015). Ancestral exposure of Japanese medaka (O. latipes) for 7 or 12 days during embryogenesis revealed that 50 ng/L EE2 could decrease fertilization success in the F2 generation, as well as reduce hatching success in the F3 and F4 (Bhandari et al., 2015; Cleary et al., 2019; Bhandari et al., 2020).

While there is evidence of EEDCs as transgenerational reproductive toxicants, transgenerational immunotoxicity has not been investigated. There is a lack of knowledge as to whether traditional risk assessment approaches used for the determination of toxic effects from direct exposure, such as dose-response, threshold level, time for induction of effect, adaptation and persistence of changes are applicable for assessing transgenerational toxicity (Brandner et al., 2022).

This study has been designed to test the hypothesis that parental gametogenesis exposure to EE2 can cause transgenerational impairments of reproduction, immune function, and survival in fish. Using two parental exposure scenarios, a short-term parental exposure (SPE), a long-term parental exposure (LPE) and a combined parental and embryonic exposure (PEE)at a low environmentally relevant and a high EE2 concentration, this study aims

(i) to determine if EE2 elicits transgenerational impairments of fish reproduction and immune function (F1-F4 generations),
(ii) to investigate if EE2 induced transgenerational effects are influenced by exposure concentration (low vs high) and exposure duration (short-term vs long-term),
(iii) to decipher the potential critical windows for EE2 induced transgenerational reproductive and immune impairment;
(iv) assess whether embryonic exposure to EE2 can modulate the likelihood, nature, and severity of transgenerational reproductive impacts;
(v) to identify the induction (wash-in) and reversibility (wash-out) patterns of these impacts over the F1-F4 generations and differences in these dynamics between LPE and PEE.

Furthermore, it is hypothesized that EE2 can induce transgenerational epigenetic impacts through both alterations of the parental sperm/oocyte epigenome during gametogenesis and alterations in the epigenome during embryogenesis (embryonic development).

2. Methods

2.1. Marine medaka culture

The marine medaka Oryzias melastigma were obtained from the State Key Laboratory in Marine Pollution, City University of Hong Kong (Hong Kong, SAR). Six-months-old fish were randomly assigned as 15 pairs (15 males /15 females) into 40L × 24W × 28H cm tanks (3 replicates per treatment) filled with artificial sea water (ASW) (SeaTreasure, Japan) under the following husbandry conditions: pH 7.4, 25‰ salinity, 26 ± 1°C, 7.2 ± 0.2 ppm O_2 on a 12:12 hour light:dark cycle. The fish were acclimated for two weeks prior to exposure. Fish were fed twice daily in the morning and afternoon with hormone-free dry flake food (Ecosystems, USA), and once in the evening with newly hatched Artemia nauplii (Lucky Brand, O.S.I. USA)

2.2. Parental EE2 exposure and multigenerational experiments

Two sublethal EE2 concentrations at nominal 50 ng/L (33 ng/L measured; low concentration), which is at the high end of concentrations measured in the environment, and at nominal: 250 ng/L (measured: 113 ng/L measured; high concentration), which has been shown to induce immune compromise and reproductive impairment in female medaka were used (Ye et al., 2018) (Fig. 1). Male and female adult fish (F0) were subjected to two EE2 exposure durations: short-term exposure (SPE) for 7 days and long-term exposure (LPE) for 21 days (Ye et al., 2018). The SPE and LPE experiments (2 concentrations x 2 exposure durations) enabled the determination which of the EE2 treatment regimes (concentration and exposure duration) may trigger transgenerational inheritance of reproductive impairments in the F3 generation and beyond upon exposure during the reproductively active stage. The combined parental and embryonic exposure (PEE) experiment, using the same EE2 concentrations and offspring (F1) as during the 21-day parental exposure, was designed to identify potential critical windows of exposure during reproduction and embryonic development. The F1 eggs were reared in the same concentrations of EE2 until hatching (Fig. 1). The LPE and PEE experiments allow comparison of the phenotypes and dynamics of transgenerational inheritance of reproductive impairments in the F1-F4 offspring. All exposure and control tanks were run in triplicates.

The waterborne EE2 exposure was initially administered by adding 2 mL of an EE2 stock solution (0.5 or 2.5 mg/L EE2 dissolved in methanol, MeOH, A.C.S. grade) into the designated triplicate 50 ng/L and 250 ng/L tanks containing 20 L of artificial seawater (ASW). Every second day half of the seawater in each tank was removed and replaced with clean ASW. The tanks were then spiked with 1 mL of the stock EE2 solution to maintain the desired waterborne EE2 concentration. Control tanks were spiked with 2 mL methanol (MeOH, A.C.S. grade) and renewed every second day at the same time as the treatment groups. For the embryonic exposure, 0.5 mL of the same EE2 stock solutions (low and high concentration) was diluted into 5 L of ASW and 50 mL were added to 11 mm glass Petri dishes containing 50 eggs in a treatment dependent manner. The water was removed and replaced respectively with 50 mL of freshly prepared EE2 ASW every second day. The medaka eggs were exposed less than 30 seconds to the air. Water samples were randomly collected six times throughout the exposure experiment for analysis of the actual EE2 concentrations.

2.3. Multigenerational study: Rearing of F1-F4 generation

Upon completion of the EE2 exposure, the F1 generation was grown in ASW under optimal rearing conditions (Fig. 1). Embryos were reared at a density of 100 embryos per 11mm Petri dish following the standard rearing protocol for marine medaka (Peterson et al., 2019) at 26°C, 14:10 light:dark cycle. F1 Larvae were transferred into 2 L tanks (dimensions 15L × 15W × 15H cm) at a density of 100 larvae per tank. After one month, they were moved into 40L × 24W × 28H cm tanks with 20 L of ASW. At 6 months of age, the fish were removed from their tank and sexed, then 25 males and 25 females (25 mating pairs) were returned to their original tank and further reared until 8 months of age. At 8 months old, the eggs from the F2 generation were collected for 14 consecutive days and reared under the same conditions as the F1. To maintain similar breeding conditions as the F1 generation, every second day, half of the ASW was removed and replaced with new ASW. The subsequent F1-F4 generations were collected and reared under the same conditions as the F1.

2.4. 17α-ethinylestradiol exposure concentration analysis

Details on water EE2 chemical analysis follow the procedures described in Ye et al. (2018). Briefly, water was sampled from the EE2 and control water tanks every two days 1 hour before and 1 hour after spiking with EE2 or methanol for an estimate of the average EE2 concentration. The water samples for the EE2 and control groups were spiked with isotope-labelled EE2-d4 as an internal surrogate standard before extraction using Oasis HLB (Waters, USA) cartridges, following the manufacturer's protocol. Nitrogen gas was then used to evaporate the liquid from the extract. The dried extract was reconstituted with 50 μL pyridine and 50 μL bistrifluoroacetamide (+ 1% trimethylchlorosilane, Supelco, USA). The sample was heated for derivation for 1 hour at 70 °C. Gas chromatography–mass spectrometry (Agilent 7890/5975C) with an HP-5MS fused silica capillary column (Agilent Technologies, USA) following Zhang et al. (2011; Staring temperature 100°C for 1 min; ramp: 10°C/min to 200; ramp: 15°C/min to 260°C; ramp: 3°C/min to 300°C; hold: 2 min; carrier: helium; flow rate: 1 mL/min) was used for quantification of EE2 based on the retention time relative to that of the internal standard. The electron impact energy was 70 eV and temperatures for injector (280°C) and mass spectrometer (250°C) were constant. The spitless mode was set for the 1μL sample injection.

2.5. Reproductive fitness assessment

Three reproductive endpoints: fecundity, fertilization success and hatching success were measured to assess the reproductive performance of the F0 fish. Fecundity was determined by the number of eggs produced per female. F1 eggs were collected in all treatments during EE2 exposure on days 6-8 (SPE) and 19-21 (LPE and PEE). Unfertilized and unviable eggs were quantified and discarded. Fertilization success was calculated as the number of viable fertilized eggs over dead or unfertilized eggs. Throughout the embryonic development period, dead eggs were recorded and discarded. Hatching time and hatching rate of the collected eggs were monitored and recorded daily. Hatching success on each post-fertilization day was calculated as the number of hatched larvae divided by the number of fertilized eggs. For the subsequent F2-F4 generations, eggs were collected from eight-month-old adults for two weeks to assess fecundity, fertilization success and hatching success. Fertilized eggs were monitored for hatching time and rate similar to the F1 generation eggs. Egg collection was conducted one hour after the beginning of the daily light cycle. All the reproduction parameters are presented as the mean of the three replicate tanks for each treatment ± standard error of the mean (S.E.M). Raw data are available in supplementary data 1.

2.6. Immune competence assessment

Host resistance assay (HRA) against Edwardsiella tarda challenge was performed for each generation (F1–F4) following the standard operating procedures described in Peterson et al. (2019). The E. tarda strain PE210 was kindly provided by Dr. Shin-Ichi Kitamura (Song et al., 2012). Specifically, 1 mL of E. tarda from a -80°C stock was cultured with 5 mL brain heart infusion culture medium (BD, Hong Kong, 3%

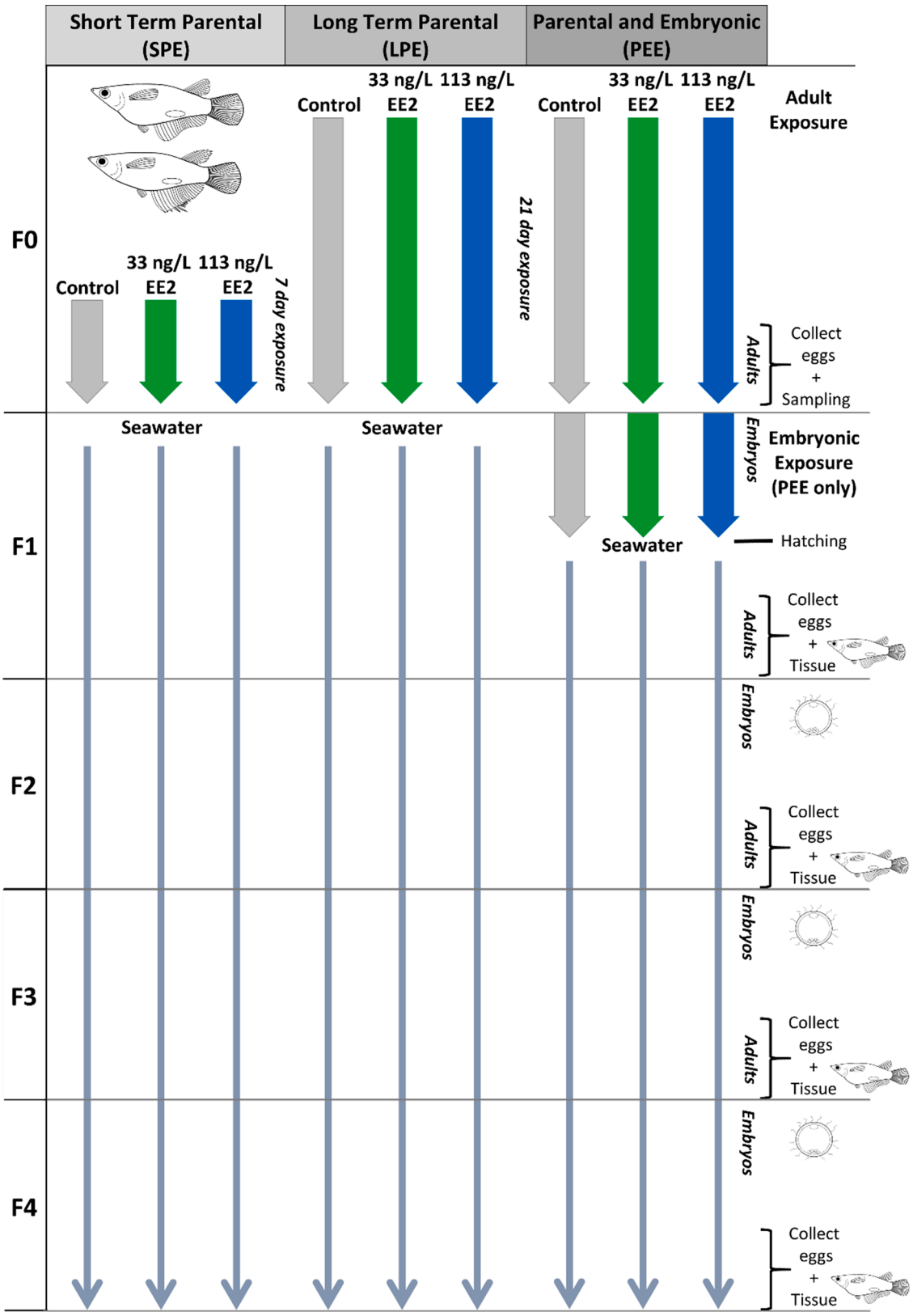

Fig. 1. An overview of EE2 exposure experimental design, rearing and sampling to the F4 generation. Six-month-old adult marine medaka were subjected to 33 ng/L (green arrow) and 113 ng/L (blue arrow) waterborne EE2 exposure for 7-day short term exposure (SPE) or 21-day long term exposure (LPE). Eggs were collected and reared in artificial sea water for the F1-F4 generations (Ye et al., 2018). For PEE, 6-month-old marine medaka were subjected to 33 ng/L (green arrow) and 113 ng/L (blue arrow) waterborne EE2 exposure for 21 days. Eggs were collected and reared in the same parental concentration (33 ng/L or 113 ng/L) of EE2 spiked artificial seawater until hatching. After hatching, F1 larvae were reared in clean artificial sea water until adulthood.

NaCl) at room temperature for 12 hours (overnight) in a 50 mL centrifuge tube (Biofil). 40 mL of brain heart infusion culture medium was added until the optical density (at 600nm) reached 0.5. The bacterial culture was centrifuged at 1317 * g for 10 minutes, and the supernatant was discarded and then re-suspended with 10 mL of sterile PBS. The bacterial suspension was centrifuged again at 1317 * g for 10 minutes, the supernatant was discarded, and the bacterial pellet was re-suspended with 5 mL of sterile PBS. The approximate concentration of the bacterial suspension was 1×10^9 CFU/mL. This bacterial suspension was serially diluted with PBS to 5×10^5 CFU/mL for injection. Microinjection of the bacterial suspension into the intraperitoneal cavity of the fish was used as the route of exposure (Kinkel et al., 2010). A micro-injection machine (PV820 Pneumatic PicoPump) was used with custom-pulled needles from 5 μl glass micropipettes (Drummond Scientific Company, USA, PC-10, NARISHIGE, USA). Prior to injection, fish were briefly anesthetized in 0.2% MS-222 in artificial seawater. One μL of the 5×10^5 CFU/mL E. tarda bacterial solution was carefully injected into the intraperitoneal cavity of 10–15 male and 10–15 female fish per replicate ($10 - 15 \times 3 = 30 - 45$ males and 30 – 45 females per treatment). Post-infection fish were returned to their respective tanks and the normal water change and feeding regime were continued. Post-infection mortality was monitored for up to 30 days, or until there were three subsequent days without mortality in any treatments. Fish were checked for mortality three times daily, and moribund/dead fish were removed immediately. The phenotypic and gonadal sex was identified and recorded. The raw data are available in supplementary data 2.

2.7. Reproductive competence index

The reproductive competence index (RCI) was proposed by Ye et al. (2018) to quantify the reproductive performance of EE2-exposed fish relative to the solvent control fish. This index uses the key reproduction endpoints that could potentially affect the number of viable offspring. For males, the RCI was calculated based on fertilization success (FS) and hatching success (H), and for females, the calculation was based on fecundity (FC) and hatching success (H) using the following formulas:

$$\text{Male } RCI = \frac{FS_i \times H_i}{FS_{ct} \times H_{ct}} \quad Female\ RCI = \frac{FC_i \times H_i}{FC_{ct} \times H_{ct}}$$

To account for hatching delay, hatching success was determined for the first day when the control group reached ≥ 90% of their total hatching success value. In the formulae, "i" is the EE2 treatment group, and "ct" is the control group. RCI values were calculated for each replicate and presented as the mean of the three replicate tanks (± S.E.M). An RCI > 1 indicates that fish in the EE2 group showed reproductive competence superior to that of the control. When the RCI < 1, the fish in the EE2 group were impaired in their reproductive competence relative to the control group. An RCI =1 indicates that fish in the EE2 group had a similar reproductive competence as the control. For ease of presentation, the RCI data were normalized to the control (control set to 0) as Δ RCI.

2.8. Immune competence index

The immune competence index (ICI) was calculated based on the survival curve from the HRA through determination of the hazard ratio (HR) using a Cox proportional hazard model (Kumar and Klefsjö, 1994). The EE2 group was set as the covariate for the different sex and experimental regimes. As proposed by Ye et al. (2018) the ICI was calculated as the reciprocal value of the HR relative to the male control group:

$$ICI_{EE2,sex} = 1/HR_{EE2,sex}$$

"EE2" is the specific EE2 exposure treatment (33 or 113 ng/L) compared to the control, and "sex" is either male or female. ICI values were calculated for each replicate and data presented as the mean of the three replicated tanks (± SEM). An ICI > 1 indicates that fish in the EE2 group have higher immune competence compared to the control; when ICI < 1, it means that fish in the EE2 group had lower immune competence compared to the control group. The ICI data were normalized to the control (control set to 0), and data presented as Δ ICI to simplify data presentation and highlight deviations from the control.

The RCI and ICI data were presented in two parts: (i) to determine the concentration/ exposure duration effect and threshold level of parental EE2 exposure to elicit transgenerational reproductive and/or immune impairment (using the SPE and LPE data for each generation) (Fig. 5) and (ii) to identify and decipher the parental and embryonic sensitive windows for inducing transgenerational reproductive and/or immune impacts (LPE vs PEE) (Fig. 6). Both, the concentration /exposure duration-response and the critical window in the F0-F4, were assessed for male and female fish separately.

2.9. Parallel reproductive and immune competence assessments

To assess the potential differences by sex and/or age, RCI and ICI of adult fish were categorized by sex (male: Fig. 6A, female: Fig. 6B) and presented as ΔRCI or ΔICI (using the control as the baseline). The significant alterations and trends of change in RCI and ICI, as compared to the control, were compiled, and sorted based on sex, exposure treatment, SPE, LPE, PEE, and exposure concentration (low and high EE2). RCI and ICI values that statistically significantly increased or decreased relative to the control ($p > 0.05$) are represented by an "*" (Fig. 6a–c). The methodology for assessing statistically significant differences between the treatment groups and the control is described in section 1.2.4. To identify increasing or decreasing trends of RCI and ICI, which may be indicative of phenotypic variability in the treatment population, the threshold for a trend was set as a ΔRCI or ΔICI ± 0.30 from the respective control and is representative of a ≥30% change from the mean control phenotype value, as a 1/3rd reduction or increase of individuals fitness is likely to affect the population level, notably if occurring in a transgenerational manner.

2.10. Sex ratio

To determine the sex ratio of a generation, approximately 200 6-months old fish were randomly selected per treatment. Based on phenotypic secondary sex characteristics (SSC), an enlarged urogenital papilla in females, and the longer anal and dorsal fins in males, the number of males and females was determined (Peterson et al., 2019).

2.11. Statistical analysis

All data were tested for normality and homogeneity of variances using a Kolmogorov–Smirnoff test and Bartlett's test. Data were normalized by log10 transformation prior to ANOVA to achieve equality of variance if necessary. Data on fecundity, fertilization success, hatching success and GSI were tested with a one-way analysis of variance (ANOVA) for the null hypothesis that the two concentrations of EE2 in each exposure scenario do not cause significant changes in each endpoint. If a significant difference ($p \leq 0.05$) was identified, pairwise comparisons among different groups were carried out using the Tukey HSD post-hoc test (Zar, 1999).

To determine any significant differences in the survival of the adult HRA, the mortality data from the three replicate tanks were pooled into single groups for the control, 33, and 113 ng/L treatments as each individually bacteria-injected fish is considered a biological replicate (Gehan, 1965). A log-rank test was performed to test the null hypothesis that there was no difference in survival between the control and the EE2 treatment groups, and a Pearson chi-squared test was used to determine significant differences ($p \leq 0.05$) between the control and EE2 treatment at specific time points.

For the critical window RCI data, a Student's t-test revealed no

significant difference between the control fish from the LPE and SPE treatments, therefore the ICI data from the control groups were pooled into a single control group. One-way ANOVA was used to test the null hypotheses that there were no changes in RCI between fish in the concentration/ exposure duration groups/ critical window groups and the control. If a significant difference ($p \leq 0.05$) was identified, pair-wise comparisons were made among different groups using the post-hoc Tukey's test. Data were normalized by log10 transformation prior to ANOVA to achieve equality of variance if necessary. Sex ratio statistical analysis was assessed via a Fisher's exact test to test the null hypothesis that there is no change in sex ratio relative to the control (Zar, 1984).

To assess the concentration/ exposure duration/ critical window ICI data, the SPE and LPE control ICI data were pooled into a single control group, as a Student's t-test found no significant difference between the control fish from the two treatments. One-way ANOVA was used to test the null hypothesis that there were no changes in ICI between fish in the EE2 concentration/ critical window groups and the control. If a significant difference ($p \leq 0.05$) was identified, pair-wise comparisons were made among different groups using the post-hoc Tukey's test. Statistical analyses were performed on the ICI data prior to normalization to the control.

3. Results

3.1. Measured EE2 concentrations and overall fish mortality

The measured low (50 ng/L) and high concentrations (250 ng/L) were 33.1 ± 12.1 ng/L EE2 and 112.8 ± 60.1 ng/L EE2 with a maximum of 90.4% and 69.2% one hour after exposure and a minimum of 42% and 21.1% before the water change respective to the nominal concentration, respectively. The EE2 concentration in the control water samples was below the detection limit. No mortality was observed in the directly exposed F0 generation. No abnormal mortality was found in the subsequent F2-F4 generations. Only the F1 SPE treatment suffered an unexpected mass mortality event prior to maturation to adulthood, due to a defective air supply. Therefore, replicate tanks were not available for the three F1 SPE groups. Changes in the gonadosomatic index (F3 low conc. PEE females ↓), hepatosomatic index (F2/F4 low/high conc. SPE/LPE females ↓; F1 low/high conc. PEE females ↑) and condition factor (F2 SPE low/high conc. males ↓; F3 low conc. SPE females ↓; F1 low conc.

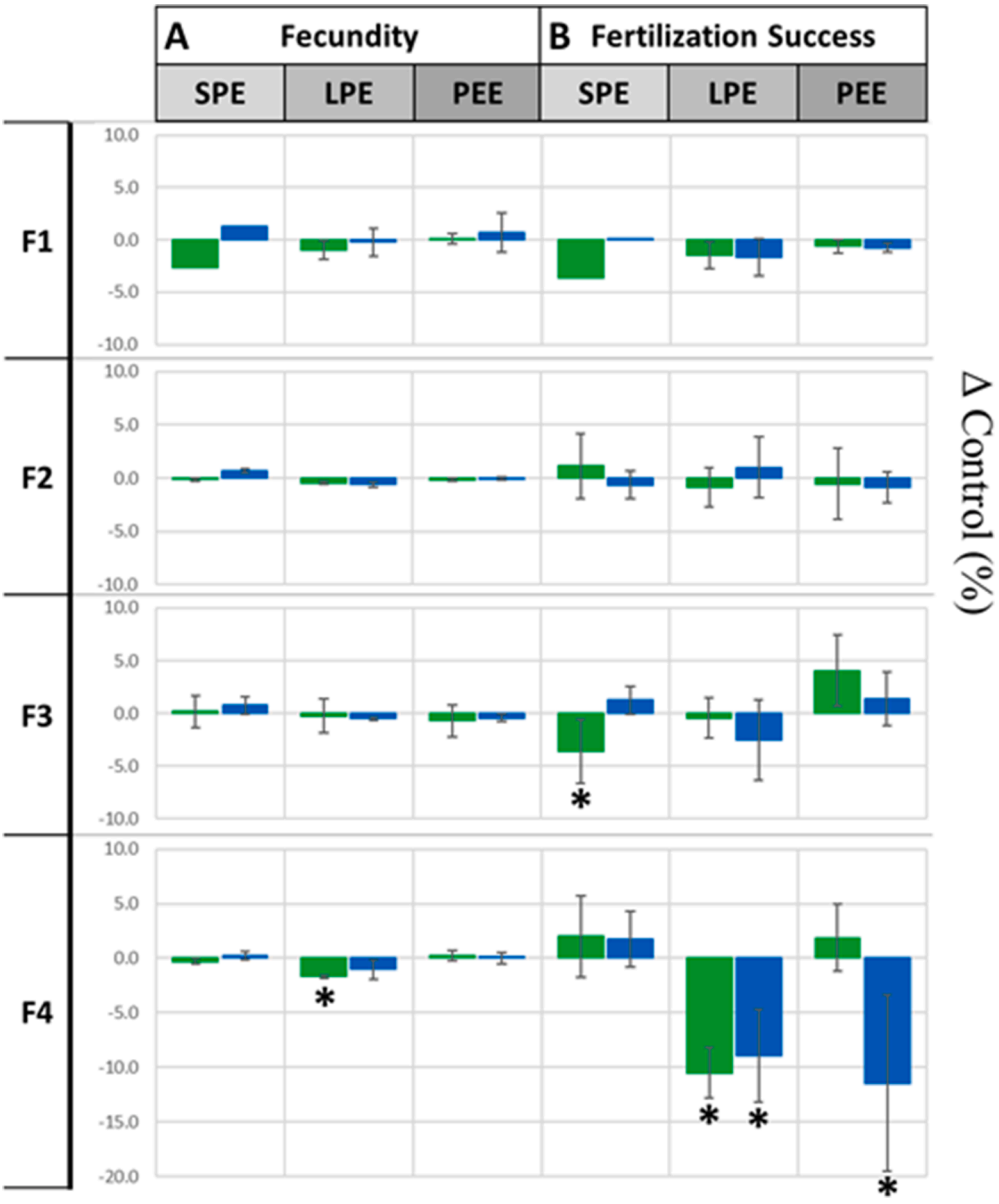

Fig. 2. Mean fecundity (A) and fertilization success (B) in the F1-F4 generations for the short-term parental exposure (SPE), long-term parental exposure (LPE), and parental and embryonic exposure (PEE) conditions. The 33 ng/L concentration (green) and 113 ng/L (blue) bars are normalized as delta % of the control value (n = 3 replicate tanks, except SPE F1, in which there were n = 1 tank (9 fish per treatment). (Left) Fecundity: Fecundity for F0 generation was measured over 3 days in 6-month-old fish. The F1-F4 fecundity was measured in 8-month-old fish for 14 days. (Right) Fertilization success for the F0 generation was measured over 3 days in 6 months-old fish. The F1-F4 fertilization success was measured in 8-month-old fish for 14 days. (n = 3 replicate tanks (25m/25f fish per tank), in triplicate; SPE F1 n = 1 (9 fish per treatment) Error bars are presented as ± SEM. One-way ANOVA was used to test the null hypothesis that there were no significant changes in each parameter between the EE2 groups and the control for each treatment. When significant differences were identified ($p < 0.05$), pairwise comparisons were made among different groups using Tukey's post-hoc test. The "*" indicates a significant difference from the control ($p < 0.05$)

LPE females ↑; F3 high conc. LPE males ↓; F1 high conc. PEE females ↑) are reported and discussed in S1.

3.2. Fecundity

No significant changes were found for either EE2 concentration in the fecundity of the F1 to F4 generations in the SPE experiment relative to that of the control (Fig. 2). A significant fecundity decrease was observed in the F4 generation in the low concentration LPE treatment group (Fig. 2) but not in the preceding F0-F3 generations. The PEE treatment revealed no significant differences in fecundity in the F1-F4 generations (Fig. 2).

3.3. Fertilization success

The fertilization success was affected by the SPE treatment in the F3 generation for the low exposure concentration as compared to the control (Fig. 2). Interestingly, both the low and high concentration LPE F4 fish showed a significant decrease in fertilization success as compared to that of the control (Fig. 2). A significant reduction in fertilization success was found in the F4 PEE high concentration relative to that of the control and low concentration groups (Fig. 2).

3.4. Hatching success and hatching time

The SPE F1 embryos showed a significantly decreased total hatching success for both the high and low concentration groups. A significant delay in hatching from 11 to 16 dpf as compared to the control occurred in both low and high EE2 SPE groups, and the high concentration was significantly delayed compared to the low concentration group from 11 to 13 dpf (Fig. 3). The SPE F2 embryos showed a trend of decreased hatching success and delayed hatching, which was not statistically significant (Fig. 3).

The LPE high concentration F1 embryos showed a significantly decreased hatching success and delay in hatching from 9 dpf onward as compared to the control, and the low concentration (Fig. 3). There were no significant changes in F2-F5 embryo hatching success or hatching time (Fig. 3).

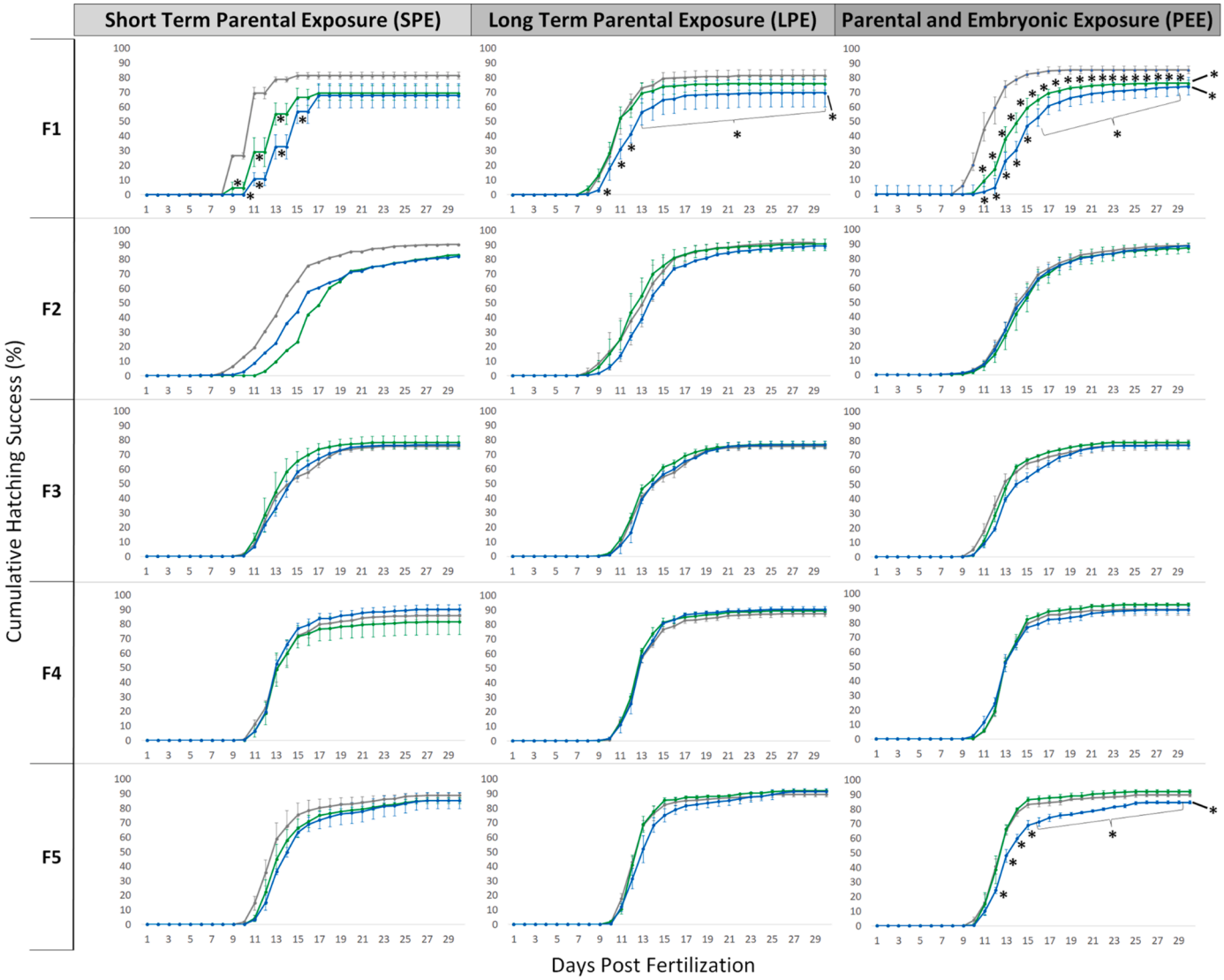

Fig. 3. Mean cumulative hatching success of the F1-F5 generations for the control (gray) 33 ng/L (green) and 113 ng/L (blue) short-term parental exposure (SPE), long-term parental exposure (LPE) and parental and embryonic exposure (PEE) treatment groups (n = 3 replicate tanks). One-way ANOVA was used to test the null hypotheses that there were no significant changes in hatching success between fish in the EE2 groups and the control for each treatment. When significant differences were identified ($p < 0.05$), pairwise comparisons were made among different groups using Tukey's post-hoc test. The "*" indicates a significant difference from the control ($p < 0.05$).

The PEE F1 embryos from the high and low concentration groups exhibited significantly decreased hatching success and delay in hatching time from 11 dpf onward as compared to that of the control (Fig. 3). The high concentration hatching time was also significantly delayed as compared to the low concentration from 12 to 15 days post-hatching (dph) (Fig. 3). In the F5 embryos, a significant decrease in hatching success and delay in hatching time in the high concentration group compared to that of the control and low concentration was observed (Fig. 3).

3.5. Adult host resistance assay survival analysis

Ancestral SPE resulted in a significant survival reduction in the F2 low EE2 males as compared to the control males (Fig. 4). No changes were observed in the SPE F3 males, but interestingly, in the F4 generation, the males from the low EE2 concentration group showed a significantly increased survival as compared to the control and high concentration groups. A similar biphasic pattern was observed in the females, the survival of both the F2 low and high concentration females was significantly reduced as compared to the control. This impact was no longer observed in the F3 generation (Fig. 4), and a significantly increased survival was measured in the subsequent F4 generation low concentration as compared to the control and high EE2 groups (Fig. 4).

In the LPE treatment, the low EE2 females showed a significant transgenerational increase in survival in the F4 generation as compared to the control and high EE2 (Fig. 4). No significant differences in survival were present in any of the other generations for males or females.

As for the PEE treatment, the F1 low EE2 females experienced a significantly higher survival than the high EE2, and a trend of increasing survival as compared to that of the control (Fig. 4). While this disappeared in the F2 generation, the low EE2 the F3 generation again showed a significant increase in survival compared to both the control and high EE2 groups.

3.6. Reproductive competence index (RCI) and immune competence index (ICI)

3.6.1. Threshold and concentration/ exposure duration effect of parental exposure of EE2 on RCI and ICI in F0-F4 males and females

Four cumulative EE2 doses (concentration x exposure duration) were calculated from the SPE and LPE treatments as follows: 231 ng/L (7 d x 33 ng/L), 693 ng/L (21 d x 33 ng/L), 791 ng/L (7 d x 113 ng/L) and 2373 ng/L (21 d x 113 ng/L). In the F4 generation, there was a wash-in of significant reduction in the 2373 ng/L dose RCI as compared to the control (Fig. 5A). The F4 females showed a significant reduction in RCI in the 693 ng/L group as compared to the control (Fig. 5A). Additionally, the RCI of F4 2373 ng/L females was significantly reduced compared to that of the control (Fig. 5A). Overall, prolonged ancestral exposure to EE2 for 21 days induced significant transgenerational impairment in both male and female RCI in the F4 generation. The threshold dose for EE2-induced RCI impairment in the F4 generation was 2373 ng/L for males, and 693 ng/L for the females.

In the adults, ancestral EE2 exposure did not induce significant impairment or enhancement in immune competence for males or females in the transgenerational F1 – F3 generations (Fig. 5B). In the F4 generation, the 231 ng/L males demonstrated a non-significant trend of enhancement in ICI (Fig. 5B). The F4 231 ng/L females showed a non-significant trend of increased survival relative to the control (Fig. 5B). No transgenerational positive dose-response of ICI impairment was measured; however, the F4 generation males and females did show a

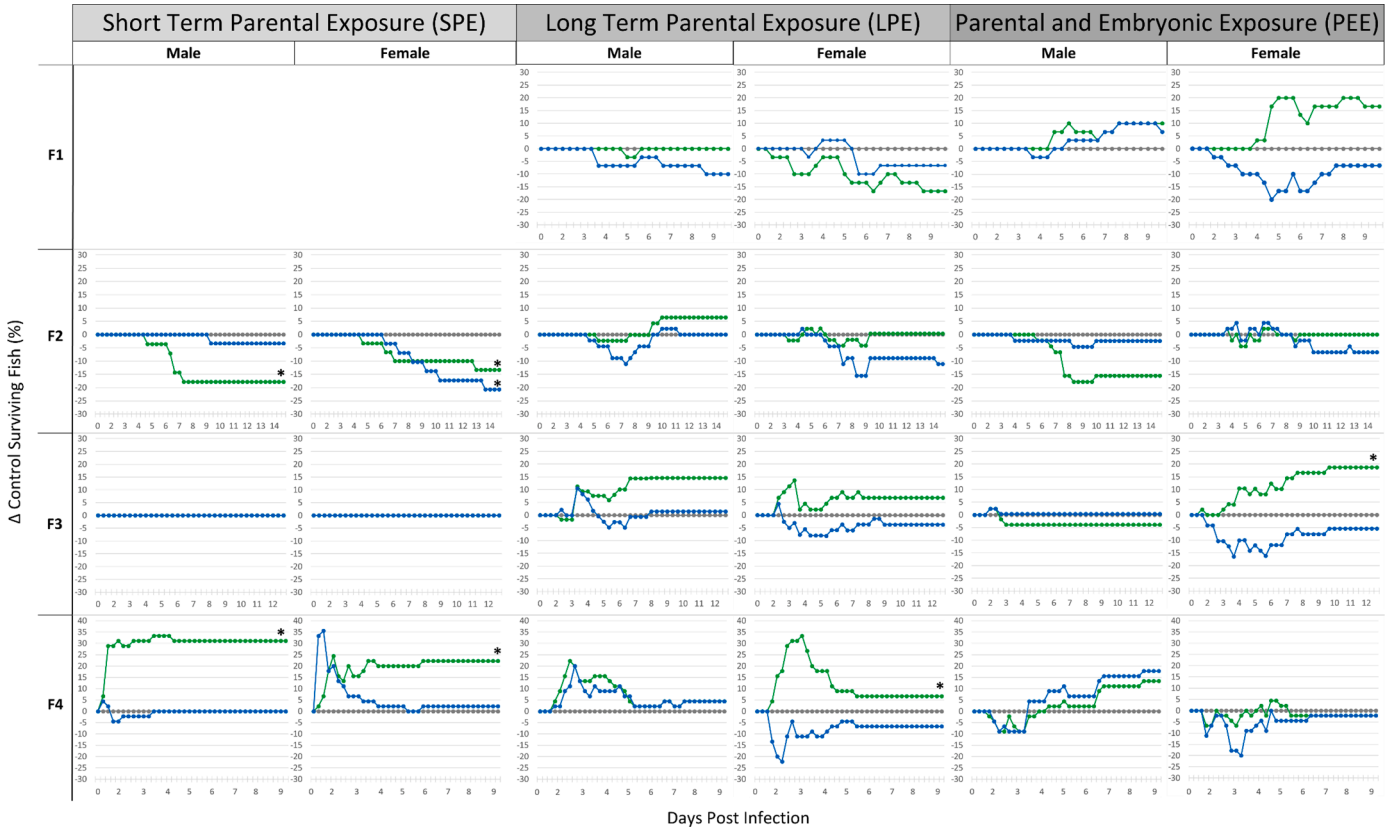

Fig. 4. Adult host-resistance to pathogenic bacteria of the F1 to F4 for the control (grey line) 33 ng/L (green) and 113 ng/L (blue) groups from the SPE, LPE, and PEE conditions. Data presented as mean post infection survival ± SEM (n = 3 replicate tanks (15 M/15 F per tank)). A log rank followed by a chi-squared test was used to test the null hypothesis that there was no change in survival between the treatment group and its respective control. Statistically significant differences between the treatment and control are presented with an "*" ($p < 0.05$).

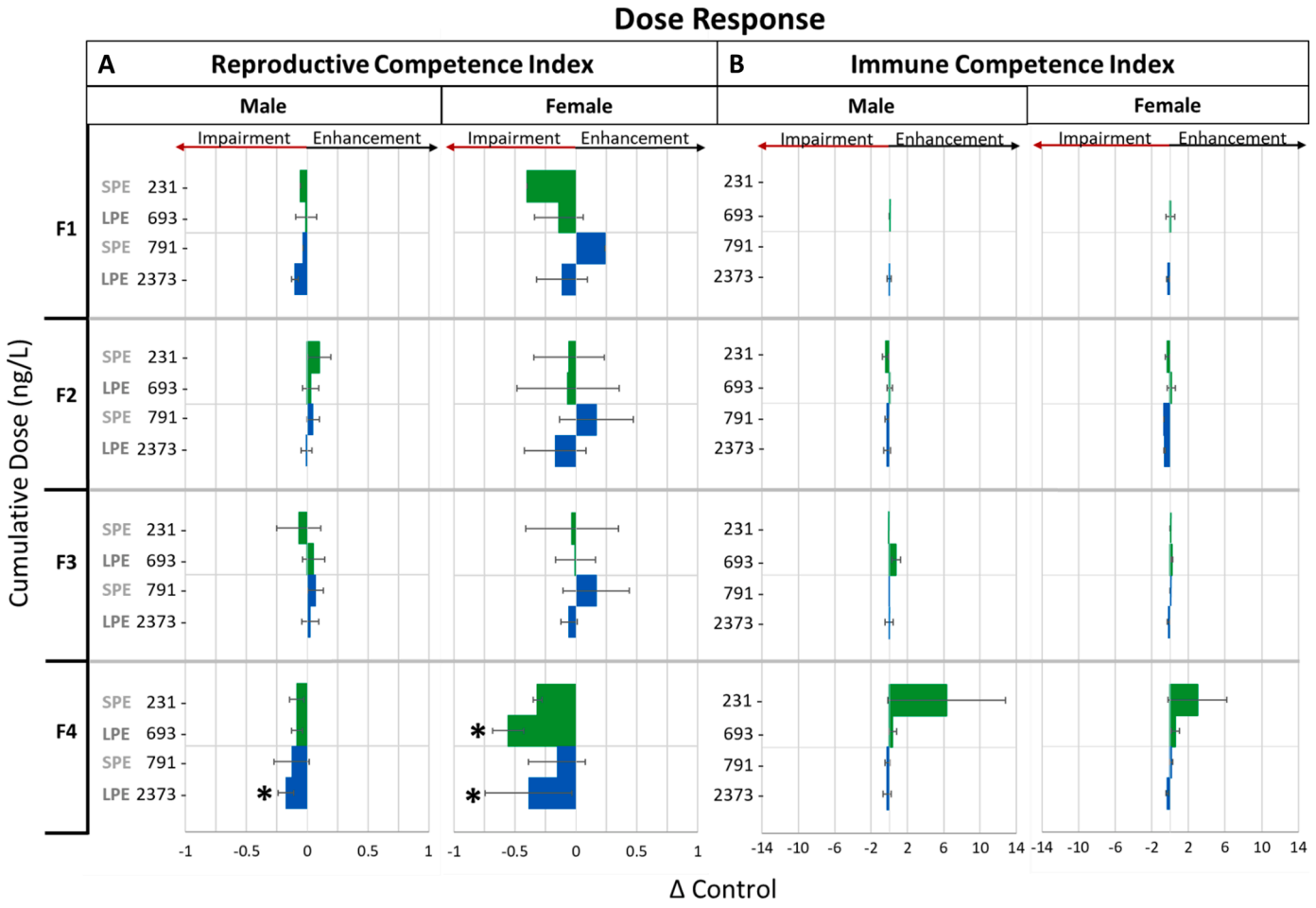

Fig. 5. Dose dependent change of (A) reproductive competence index (RCI) in the SPE and LPE treatments and (B) adult immune competence index (ICI) in the SPE and LPE treatments, in the F1–F4 generations of marine medaka ancestrally exposed to EE2 as compared to the control. Green bars represent the low concentration and blue bars represent the high concentration group. Impairment is identified as RCI or ICI < 1, and enhancement as RCI or ICI < 1. Both RCI and ICI values were normalized to the control, and the control was set at 0 and values presented as Δ control ($n = 3$ replicate tanks). Error bars represent ± S.E.M. normalized to the control. One-way ANOVA was used to test the null hypotheses that there were no changes in between fish in the four EE2 dose groups and the control. When significant differences were identified ($p < 0.05$), pairwise comparisons were made among different groups using Tukey's post-hoc test. The "*" indicates a significant difference from the control ($p < 0.05$). Four cumulative EE2 doses were calculated from the SPE and LPE treatments as follows: 231 ng/L (7 d x 33 ng/L), 693 ng/L (21 d x 33 ng/L), 791 ng/L (7 d x 113 ng/L) and 2373 ng/L (21 d x 113 ng/L).

trend of ICI enhancement associated with decreasing ancestral EE2 dose (Fig. 5B).

3.6.2. *Critical windows of EE2 exposure induced transgenerational reproductive competence and immune competence impacts*

The male and female RCI for both the PEE and LPE treatments were not significantly impacted until the F4 generation (Fig. 6A). For F4 male fish, exposure during the critical parental gametogenesis and embryogenesis windows were able to trigger a significant transgenerational impairment of RCI (Fig. 6A). The extent of RCI reduction due to exposure during the embryogenesis window (PEE) appeared to be more severe than the parental gametogenesis (LPE) only (Fig. 6A). For F4 female fish, the exposure during parental gametogenesis alone could induce a transgenerational reduction of the RCI in the low concentration group. Conversely, a subsequent exposure during embryogenesis alleviated the transgenerational RCI impact (Fig. 6A). In summary, EE2 exposure during parental gametogenesis and embryogenesis affect the reproductive competence of the F4 generation with embryonic exposure being the critical window for male reproduction, and exposure during gametogenesis being the critical window for female reproduction.

None of the exposure scenarios, low/high EE2 exposure concentration and LPE/PEE, affected the immune competence of the male fish. The F1 female fish from the PEE scenario experienced a trend of enhanced ICI in response to the low EE2 exposure concentration as compared to the high EE2 concentration and both concentrations of the LPE treatment. This trend was no longer observable in the F2 generation (Fig. 6B). However, this trend of enhanced immune competence was observed again in the F3 generation with the same exposure history (PEE, low EE2 concentration), which was significantly different from that of the female fish in the high EE2 concentration PEE group. This trend was no longer present in the female fish of the F4 generation (Fig. 6B).

3.7. *Sex ratio*

The SPE and LPE treatments, with direct F0 adult exposure to EE2, did not experience any significant changes in sex ratio in the F1-F4 generations (Fig. 7A, B). As a result of the direct F1 embryonic exposure in the PEE treatment, the F1 PEE adults from both exposure concentrations were significantly skewed towards females compared to the control (Fig. 7C) in a concentration/ exposure duration-dependent manner with almost 20% more females in the high concentration group relative to the low concentration group. Notably, in the F2 generation, both the PEE high concentration and low concentration groups

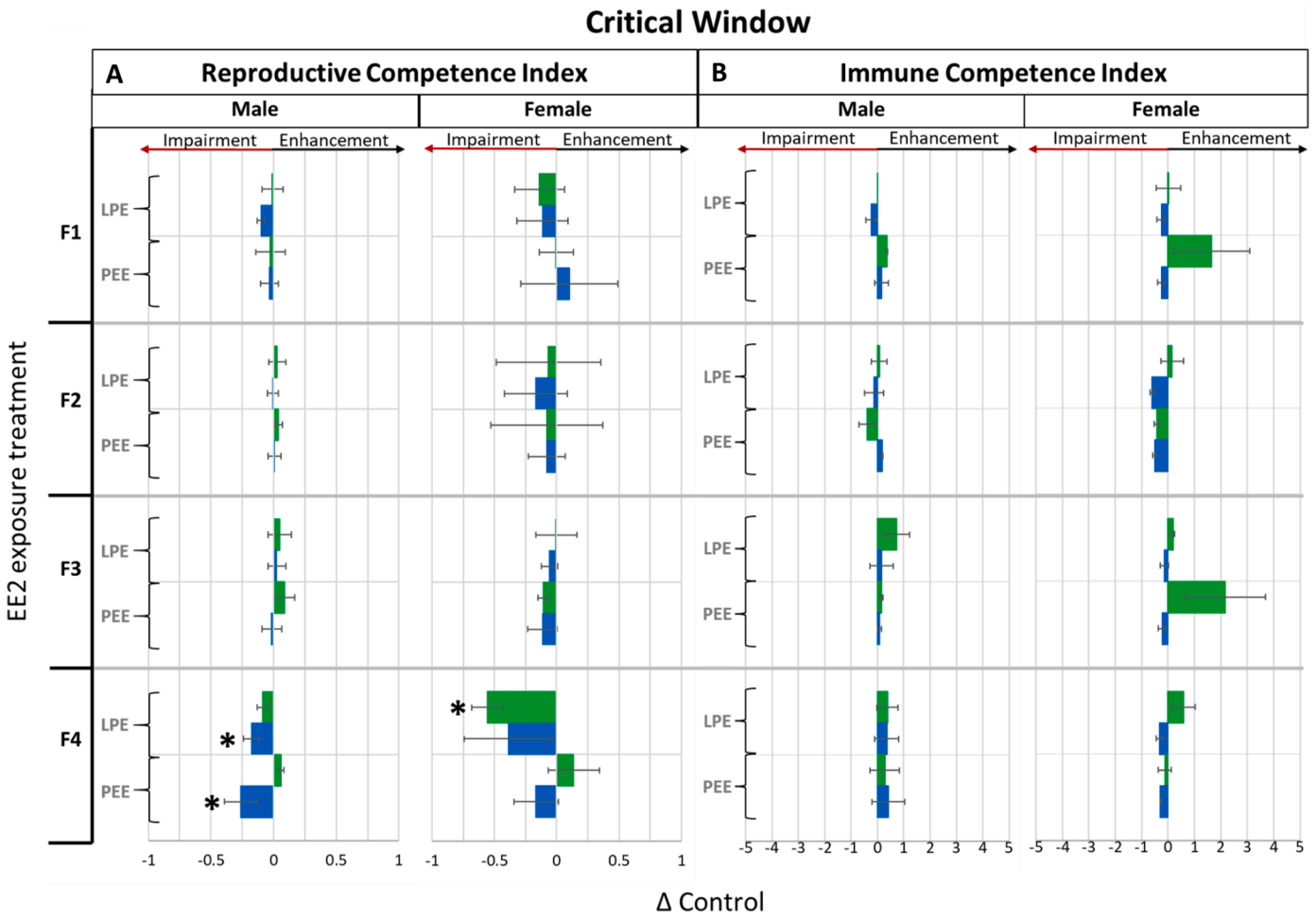

Fig. 6. Comparison of LPE and PEE treatment to identify the critical windows for altered (A) reproductive competence (RCI) and (B) immune competence index (ICI) in marine medaka ancestrally exposed to EE2 (F1-F4 generations). Green bars represent the low concentration group and blue bars represent the high concentration group. Impairment is identified as RCI or ICI < 1, and enhancement as RCI or ICI < 1. Both RCI and ICI values were normalized to the control, the control was set as 0 and values presented as Δ control (n =3 replicate tanks). Error bars represent ± S.E.M. normalized to the control. One-way ANOVA was used to test the null hypotheses that there were no changes in RCI or ICI between fish in the EE2 critical window groups and the control. When significant differences were identified ($p < 0.05$), pairwise comparisons were made among different groups using Tukey's post-hoc test. The "*" indicates a significant difference from the control ($p < 0.05$).

showed a significant bias in sex ratio towards males in the treatment population relative to the control (Fig. 7C). This significant bias towards male sex continued into the PEE F3 generation for the high concentration group, but not the low concentration group (Fig. 7C). By the F4 generation, the sex ratio in the PEE offspring was fully recovered to a normal sex ratio (≈1:1) for both the high and low concentration treatments (Fig. 7C). Of the 9 males and 9 females sampled for GSI measurements from each treatment in each generation, one "female" fish (1/9) from the F1 PEE high concentration treatment exhibited the female SSCs of shortened dorsal and anal fins, but also a testis sized gonad (data not shown). The phenotypic SSCs and gonadal sex were consistent for all F2-F4 fish of the PEE and the F1-F4 fish of the SPE and LPE treatments.

4. Discussion

This study is the first to assess the multigenerational impacts of the xenoestrogen EE2 on immune competence and reproductive success of fish (F1-F4) under multiple ancestral (F0) exposure regimes. Prolonged parental (21 days) and combined parental (21 days) and embryonic exposure to environmentally relevant levels of EE2 could induce transgenerational (F4) impairment in male and female reproductive competence. For parentally exposed fish, 33 ng/L EE2 triggered transgenerational reproductive impairment in the F4 generation upon parental exposure for 21 days. Furthermore, both (i) parental gametogenesis and (ii) embryogenesis are critical windows for EE2 -induction of transgenerational (F4) reproductive impacts. However, the nature of EE2-induced transgenerational effects differed based on the critical window of exposure:

(i) parental gametogenesis exposure induced impairment of fecundity and fertilization success,
(ii) Exposure during embryogenesis adversely affected hatching success and hatching time.

In addition to transgenerational RCI impairment in the F4, embryonic exposure to EE2 altered sex ratios in the F1, F2 and, at the high concentration (113 ng/L), in the F3 generation, suggesting further detrimental impacts on population reproductive output and sustainability. These results further confirm EE2 as a transgenerational reproductive toxicant in fish and highlight the necessity of monitoring multiple reproductive endpoints beyond the F1 and F2 generations to better understand the risks of environmental estrogens on fish reproduction and population sustainability.

The holistic reproductive, immune, and growth assessment compiled in this study indicates that long duration (21 days) parental exposure to EE2 at high concentrations (113 ng/L) can cause significant transgenerational impacts on the reproductive success in the F4 male and female medaka, as shown in the LPE and PEE treatments, which is in line

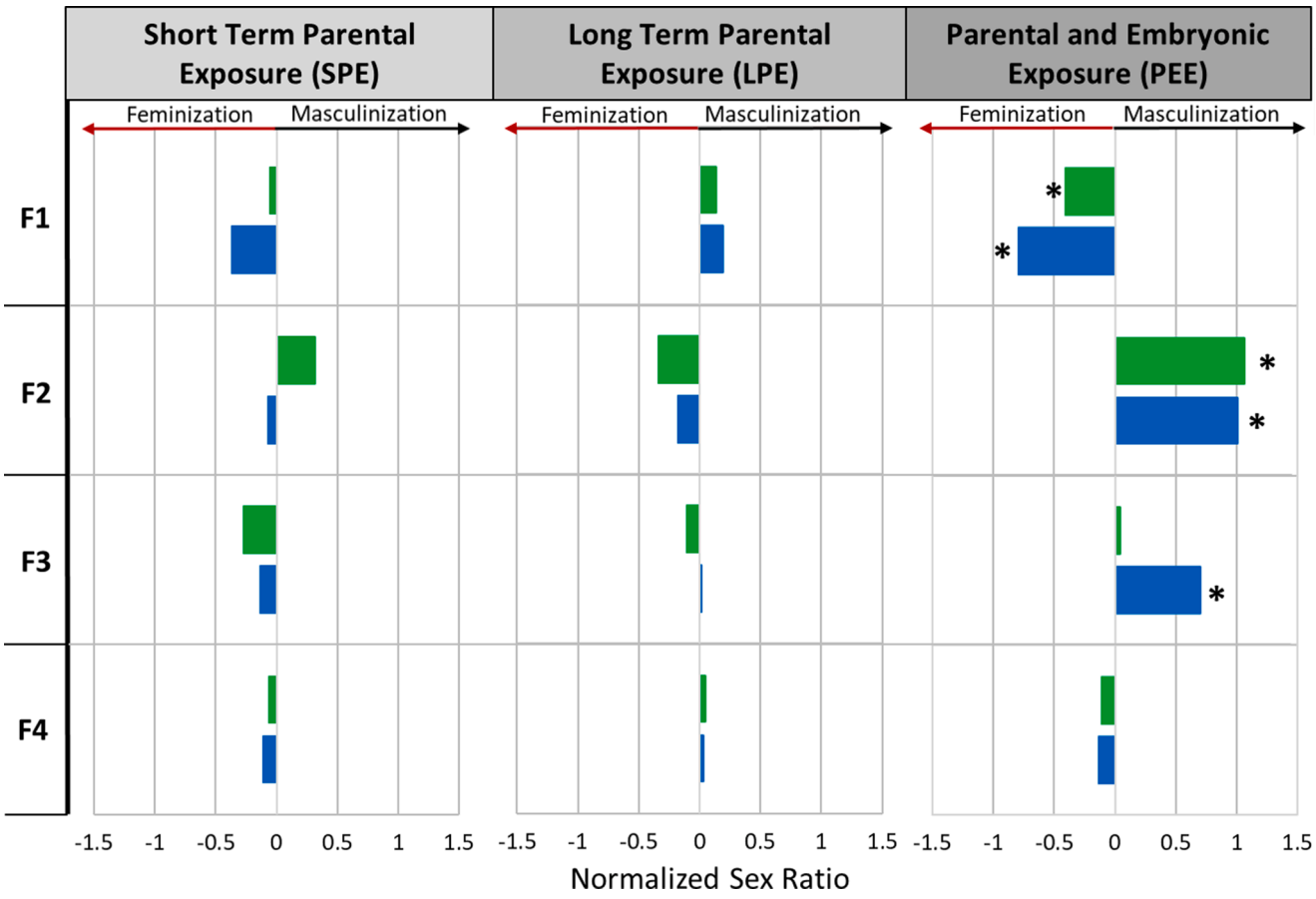

Fig. 7. Change of sex ratio (male: female) in marine medaka directly exposed to three different EE2 scenarios Short Term Parental, Long Term Parental and Parental and Embryonic in the F1 - F4 generations. The sex ratio was normalized to the control (n ≥ 200 fish). A sex ratio > 1 indicates the feminization of the population, and a sex ratio < 1 indicates the masculinization of the population. Statistical analysis was determined via a Fisher Exact Test with significance to the respective control presented as: * p ≤ 0.05.

with previous findings (Bhandari et al., 2015).

The medaka displayed a potential multigenerational sex difference in impaired reproductive competence in response to ancestral EE2 exposure dependent on the ancestral exposure treatment: PEE, M > F; and LPE, F > M. This result is consistent with previous studies in which the sex difference of impaired reproductive competence (M > F) occurred as a result of direct EE2 exposure (Ye et al., 2018; Bhandari et al., 2020; Bhandari et al., 2015). The reproductive competence in the parentally exposed F4 generation female medaka was more sensitive to the low EE2 treatment than the males after 21 days of parental exposure. This higher sensitivity led to significant reproductive impairment at both low and high EE2 concentrations, as compared to only the high EE2 concentration for the F4 males. However, the F4 male descendants were more sensitive to high ancestral EE2 embryonic exposure (PEE), showing a significant reduction in reproductive competence, which was not shown in the F4 females. The data on female reproduction impairment in response to ancestral EE2 exposure support previous findings of population level effects (Kidd et al., 2007; Schwindt et al., 2014; Jackson & Klerks, 2020) and are in line with current predictive models (Brander et al., 2022).

Embryonic exposure to EE2 altered sex ratios in the F1, F2 and, at the high concentration (113 ng/L), the F3 generation, suggesting further detrimental impacts on population reproductive output and sustainability. A skewed sex ratio due to embryonic estrogen exposure is not novel, as EE2 can initiate the transition from undifferentiated early gonad of genetic (XY) males into a functional mature ovary (Maack and Segner 2004). Few studies have investigated how a female skewed sex ratio of a single generation may impact the reproductive success of future generations, which is pertinent when assessing the population stability. White et al. (2017) highlights the importance of not only fertilization success, hatching success and fecundity on population sustainability, but also skewed sex ratios. White et al. (2017) uses a combination of predictive models and wild inland silverside (*Menidia beryllina*) populations to postulate that, for a non-monogamous pair mating species, such as marine medaka, feminization of a population (without reproductive impairment) to a certain level (>80%) can actually increase the population size of the subsequent generation. White et al. (2017) emphasizes that masculinization is more detrimental, maintaining the population replacement threshold due to loss of overall viable egg output. These results further confirm EE2 as a transgenerational reproductive toxicant in fish, and highlight the necessity of monitoring multiple reproductive endpoints beyond the F1 and F2 generation to better understand the risks of environmental estrogens on fish reproduction and population sustainability.

Overall, the significant transgenerational reproductive impairment displayed in the LPE and PEE high EE2 F4, as well as the LPE low and high EE2 F4 females, suggests that ancestral exposure conditions (LPE or PEE) can result in different, but always negative, impacts on male and female reproductive output and thus, population sustainability.

Exposure to EE2 during the critical parental gametogenesis and embryogenesis windows induced multiple patterns of altered phenotype inheritance in the F1-F4 generations. The presence and variability of the altered reproductive phenotype patterns appears to be dependent on exposure duration, concentration, and phenotypic endpoint assessed. This study highlights the importance of assessing multiple reproductive endpoints separately as they may show different temporal wash-in/

wash-out patterns and may be indicative of a combination of various multigenerational epigenetic and/or genetic mechanisms. Consistent with reviews by Burggren (2016) and O'dea et al. (2016), we were able to identify both wash-in and wash-out patterns of altered phenotypes over multiple generations. A wash-in of significantly reduced GSI was found in the PEE low concentration F3 females, which washed out in the F4 generation. The clearest wash-in pattern was the appearance of reduced fertilization success in the low and high LPE and high PEE concentration groups in the F4 generation. The explanation behind the lack of RCI impairment in the first transgenerational (F3) generation and subsequent delayed, altered phenotypes found in the F4 generation is unclear.

Despite the absence of significant changes of the ICI, improvement of pathogen survival has been evidenced in female offspring. It remains to be elucidated, if the enhanced immune competence in females upon low EE2 SPE, LPE and PEE exposure scenarios is associated with an improved pathogen recognition and complement cascade (Metcalf & Graham, 2018; Dong et al., 2017) or may be associated with an inheritance of an enhanced T-helper cell 2 phenotype (Lang, 2004) and, if and how epigenetic modifications of the X chromosome may play a role (Pinheiro et al., 2011).

5. Conclusion

The different wash-in and wash-out patterns of altered phenotypes found in this study may be indicative of the multiple genetic or epigenetic mechanisms altered by ancestral EE2 exposure that are endpoint specific, such as second-level epigenetic modifications and epialleles and requires further investigation (O'dea et al., 2016). EE2 exposure has been shown to change DNA methylation in the brains and livers of zebrafish, but whether these EE2 induced changes in methylation could be epigenetically inherited was not investigated (Strömqvist et al., 2010). Bhandari et al. (2020) demonstrated the absence of genomic changes in the male testes upon embryonic EE2 exposure, clearly supporting the epigenetic inheritance hypothesis. The non-monotonous dose-response pattern of EEDCs further complicates the nature of the impacts of direct exposure and unexposed transgenerational epigenetic inheritance and epigenetic reprogramming over generations. The mechanisms behind these varied inheritance patterns are still not well-understood. The varied temporal wash-in/wash-out patterns identified in this study necessitate further in-depth assessment, and genetic and epigenetic analysis is needed to elucidate the mechanisms through which the multigenerational altered phenotypes may occur.

These baseline data provide valuable insights into the potential multigenerational impact of EEDCs on the reproduction of wild fish populations. As the sensitivity to exogenous estrogens is species dependent (Lange et al., 2012), further confirmation of the EE2 induced reproductive impairment identified in this study using commercially important, and wild fish populations is necessary. Future toxicological and risk assessment studies of environmental toxicants/stresses should consider utilizing a multiple effects assessment at a multigenerational scale.

CRediT authorship contribution statement

Drew R. Peterson: Data curation, Investigation, Writing – original draft, Visualization. **Frauke Seemann:** Conceptualization, Project administration, Investigation, Supervision, Writing – review & editing, Methodology. **Miles T. Wan:** Investigation, Data curation. **Roy R. Ye:** Investigation, Data curation. **Lianguo Chen:** Investigation, Data curation, Methodology. **Keng P. Lai:** Visualization, Validation, Writing – review & editing. **Peter Yu:** Formal analysis, Writing – review & editing. **Richard Y.C. Kong:** Validation, Writing – review & editing, Supervision. **Doris W.T. Au:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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.

Data availability

Raw data are provided in the supplementary material.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106584.

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