Dose Duration Effects of 17-α Ethynylestradiol in Zebrafish Toxicology

Decatur Foster and Kim Hanford Brown

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Abstract

Exposure of zebrafish to the synthetic estrogen 17-α ethynylestradiol (EE2) has been shown to cause a number of detrimental effects, including but not limited to feminization of male fish, reduced reproductive capabilities, and impaired embryonic development. This paper systematically reviews the effects of five environmentally relevant concentrations of EE2 on 12 measurements that are commonly selected when studying the effects of EE2 on zebrafish. Concentrations of 0.1 ng EE2/L, 1 ng EE2/L, 3 ng EE2/L, 10 ng EE2/L, 25 ng EE2/L, and 100 ng EE2/L were reviewed for their effects on sex ratio, vitellogenin induction, gonad morphology, spawning success, survival, bodily malformation, length/weight, swim-up success, fecundity, fertilization success, hatching success, and the reversibility of aforementioned effects. A greater occurrence of effects was observed as the dose of EE2 was increased, starting at exposure levels of 1 ng EE2/L. For exposures of 3 and 10 ng EE2/L, negative effects on sex ratio, morphology, and reproductive capabilities were reversible after zebrafish were able to recover in clean water for a period of time. Data for zebrafish exposed to 100 ng EE2/L was limited, as this concentration severely decreased survival.

Keywords: Danio rerio, zebrafish, 17-α ethynylestradiol, estrogen, endocrine disrupting chemical (EDC), toxicology

1. Introduction

Endocrine disrupting compounds (EDCs) are a class of chemical that have the ability to interfere with normal functions of the endocrine systems of living organisms. EDCs can affect organismal systems by mimicking, counteracting, or disrupting the synthesis and metabolism of endogenous hormones, as well as disturbing the synthesis of specific hormone receptors [1]. Among EDCs, estrogenic chemicals (ECs) are among the most extensively studied, primarily due to high
levels of environmental contamination and a wide range of effects on aquatic ecosystems that have come to light over the past several decades. ECs can be found in many common household items, but their primary modes of entry into the environment are through wastewater effluent from municipal treatment plants, hospital effluent, and livestock activities [2].

Among ECs, 17 α-ethynylestradiol (EE2) is of particular concern, as it has been shown to be 10–50 times more potent in fish than naturally produced estrogen, due to its longer half-life and tendency to bio-concentrate in tissues [3]. EE2 is a derivative of the natural hormone estradiol (E2) and is commonly used as the bioactive estrogen for human oral contraceptive pills. In terms of frequency of use, oral contraceptives containing EE2 rank among the top 15 U.S. active pharmaceutical ingredients [4]. In addition to being utilized in human birth control, EE2 is also widely used in livestock to prevent pregnancy. Beyond contraceptive use, EE2 is utilized as a medicine for alleviating menopausal and postmenopausal syndrome symptoms, physiological replacement therapy for estrogen deficient states, treatment of prostatic cancer and breast cancer, and osteoporosis [1].

Human urine is considered the main source of EE2 contamination in the environment, as excess EE2 in the body is excreted in urine and enters aquatic systems through wastewater effluent release. Prior to EE2’s excretion in urine, it is metabolized to become a biologically inactive, water-soluble sulfate or glucuronide conjugate [5]. Following excretion and subsequent transfer to wastewater treatment plants, EE2 may be activated into its free form due to bacterial modification. The activated EE2 remains relatively stable during the activated sludge process in sewage treatment plants, thus avoiding breakdown and elimination [6]. Because of EE2’s highly stable molecular structure (Figure 1), it has become a widespread problem in the environment. Given its high resistance to degradation, and its tendency to be absorbed by organic matter, accumulate in sediment, and concentrate in biota, EE2 can cause significant issues for aquatic organisms and populations once present in the environment [7].

With a global human population of over 7 billion, it is estimated that approximately 700 kg/year of synthetic estrogens are released into the environment from contraceptive usage alone [8]. Environmental EE2 concentrations in water are highly variable, ranging from non-detectable levels to a maximum reported concentration of 830 ng/L in U.S. rivers [9]. As an example, a study in Washington State analyzed 266 surface water samples from lakes and streams in the Seattle area and detected EE2 in 66 samples, with a maximum concentration measuring 4 ng/L [10]. Other studies have observed concentrations of 42 ng/L in Canadian

![Figure 1. Chemical structure of 17-α ethynylestradiol (EE2).](image-url)
sewage treatment effluent [11], while studies in Europe have found concentrations generally below 5 ng/L [12]. This has raised concern, as concentrations as low as 1 ng/L have been observed to affect offspring survival of adult male fish exposed to EE2 [13].

In some fish species, the binding affinity of EE2 to the estrogen receptor has been shown to be up to five times higher than E2 [3]. This higher receptor affinity indicates that EE2 can be a more potent estrogenic compound in terms of eliciting an estrogenic response, compared to naturally produced E2 [1]. Under environmental and laboratory conditions, EE2 has been reported to cause a wide variety of negative effects in multiple species of fish, including bias in the sex ratio toward females, decreased fertility and fecundity, vitellogenin induction in males, reduction of gonadal development, intersex, and impairment of reproductive behaviors [1, 7, 12–27].

Zebrafish (Danio rerio) are commonly used in laboratories to observe the effects of EE2 in aquatic models, as they exhibit most of the measurements that have been detected in a variety of fish species and have high gene ontology with humans [28]. Given their rapid development from fertilization to reproductive maturity in only three to four months, both short-term early life stage tests and chronic life-cycle tests can be conducted in a relatively short amount of time [19]. The short life cycle is also beneficial when studying developmental and reproductive effects of endocrine disrupting compounds [20]. Their ability to breed year round makes zebrafish ideal for studies observing fecundity and fertility. Furthermore, zebrafish produce a large number of transparent eggs per spawn, which is preferable when collecting both quantitative and morphological data. Finally, zebrafish are well studied; embryogenesis in this species has been researched in detail, and the entire zebrafish genome has been published, allowing for in-depth genetic comparison and analysis [23].

In this paper, we focus on reviewing the impact that EE2 has on 12 measurements of fitness that are commonly selected when studying the effects of EE2 on zebrafish. They include: (1) skewed sex ratios from male to female; (2) the induction of vitellogenin (VTG) in male fish (an egg yolk precursor protein normally expressed only in females); (3) gonad morphology (undeveloped gonads, mature ova/testes or intersex - see Figure 2); (4) spawning success (onset of spawning and number of successful spawns); (5) survival; (6) bodily malformation; (7) length/weight; (8) swim-up success (successful inflation of the swim bladder by day 7 post fertilization) (9) fecundity (number of eggs per spawn); (10) number of viable eggs per spawn (fertilization success); (11) number of hatched eggs per spawn; and (12) reversibility of effects (the ability of these 11 measurements to return to control levels after a period of depuration). This review will help summarize the vast amount of zebrafish research that has been published over the past two decades pertaining to EE2 exposure.

We chose five concentrations of EE2 that were most commonly used by researchers, all of which are environmentally relevant: 0.1 ng/L, 1 ng/L, 3 ng/L, 10 ng/L, 25 ng/L, and 100 ng/L. These studies observed exposure periods of 5–180 days, followed by a depuration period of 25–150 days in order to test for reversibility of effects (Figure 3). Studies that did not begin exposure at day 1 (i.e. partial lifecycle exposures) were excluded from this review. Furthermore, effects on second-generation exposure fish are not reported in this review. All findings reported in this review were deemed statistically significant by the original authors, as compared to control tests, unless otherwise noted.
Figure 2. Histology image of intersex tissue from 8-week-old zebrafish larva (red circle indicating an oocyte and black arrows indicating testicular tissue) [17].

Figure 3. Length of EE2 exposure and depuration observed in each zebrafish study evaluated.
2. Control experiment parameters

It is important to consider that a number of factors outside of exposure to EE2 can affect the 12 measurements reviewed in this paper. The age and size of fish, interval between successful spawns, light cycle, and diet all have an effect on quality and quantity of egg production. There is often little continuity in these environmental factors between aquatic laboratories, which can lead to differences in control outcomes. One additional complication with cross evaluation of studies is the variety of solvents used between different laboratories to dissolve EE2 for exposure trials. Acetone, methanol, and ethanol are most commonly used in the reviewed papers we evaluated, but other solvents have been reported, which may have differing effects on organismal physiology.

Laboratory zebrafish typically attain sexual maturity in the 3rd month of their development, but initial spawns can be observed in fish at ages as young as 2.5 months. Once sexual maturity is reached, prime reproductive performance is maintained for several months, but decreases with advancing age. Optimal zebrafish reproduction through natural mating occurs when the fish are aged 6 months to 1 year [24].

Figure 4. Survival from fertilization to 6 weeks of three different strains of zebrafish (AB, TU, WIK) under control conditions (no EE2 present). Survival of two cohorts per strain (CH1 and CH2) is shown, exhibiting substantial intra- and inter-strain variation [17].
Finally, and perhaps most importantly, few papers specify which strain of zebrafish are used. Zebrafish researchers typically report that “wild type” zebrafish are used, but fail to specify which “wild type” they are referring to. Many different laboratory “wild type” strains are available, including AB, Tuebingen (TU), WIK, and Tupfel long fin (TL), among others. The specific “wild type” strain used could have an impact on control and exposure outcome, given the extensive genetic diversity between laboratory strains [25]. Among the array of laboratory strains available, the three most common laboratory strains include AB, TU, and WIK [17]. Each of these strains differs in their initial method of establishment, historic degree of selective breeding, and genetic bottlenecks that likely affect physiological performance under exposure. Figure 4 represents such strain variability in survival of zebrafish, based solely on vehicle control conditions.

3. Exposure to 0.1 ng EE2/L

Overall, exposure to 0.1 ng EE2/L appears to have little or no effect on zebrafish. Two studies evaluated concentrations of EE2 at this level with no detrimental effects observed. Zebrafish in these studies were evaluated for a period of 90 days [18] and a period of 177 days [19].

3.1. Sex ratio

After 90 days of exposure, 40% of zebrafish were female, while 40% had undeveloped gonads [18]. This did not significantly differ from control ratios.

3.2. VTG levels

After 90 days of exposure, VTG was not detected in male fish [18].

3.3. Bodily malformation

After 90 days of exposure, no bodily malformation was observed in zebrafish [18].

3.4. Length/weight

After 90 days of exposure, the total body length and weight of zebrafish was not significantly different than that of the control fish [18].

3.5. Fecundity

After 177 days of exposure (at 0.05 ng/L), there was no statistically significant difference in number of eggs produced per day between exposure and control zebrafish. Exposure fish produced 32.6 eggs per day [19].

3.6. Viable eggs

After 177 days of exposure (at 0.05 ng/L), there was no statistically significant difference in the number of successfully fertilized eggs between exposure and control zebrafish. Exposure fish had a fertilization success rate of 91.6% [19].
4. Exposure to 1 ng EE2/L

Exposure to 1 ng EE2/L impacted onset of spawning, fecundity, as well as the number of viable eggs per spawn. Spawning did not occur during the exposure period for fish that were exposed for 177 days. The percentage of eggs laid per day by exposed fish was reduced by 24%, while the percentage of viable eggs reduced from approximately 95% in control fish to 41–51% in exposure fish. Three papers were reviewed at this concentration of EE2, observing effects for a period of 60 days [20], 90 [18], and 177 days [19].

4.1. Sex ratio

After 60 days of exposure, 77% of zebrafish were female, while 5% had undeveloped gonads [20]. After 90 days of exposure, 50% of zebrafish were female, while 40% had undeveloped gonads [18]. None of these data points were deemed statistically significant.

4.2. VTG induction

After 90 days of exposure, VTG in males was measured at approximately 150 ng/mg, which was not deemed statistically significant compared to the control [18].

4.3. Spawning success

After 75 and 177 days of exposure, there was a delay in onset of spawning [19]. For the group that was exposed for 177 days, spawning occurred at day 120, while control fish spawned at day 112 [19]. These data points were not deemed statistically significant.

4.4. Survival

After 6 weeks of exposure, there was no significant difference in survival between exposure fish and control fish. The greatest loss occurred within the first 10 days post fertilization, which was attributed to normal larval mortality. Figure 5 shows this survival curve, with other major losses shown at the end of the 6 weeks attributed to miscounts and/or cannibalism [17]. There was no statistically significant difference in survival between exposure fish and control fish at day 42 [19], day 60 (survival rate of 60% was reported) [20], or day 75 of exposure [19].

4.5. Bodily malformation

After 90 days of exposure [18] and 180 days of exposure [17], there was no bodily malformation.

4.6. Length/weight

After 60 days of exposure [20], and 90 days of exposure [18], the total body length and weight of exposure fish was not significantly different than that of the control fish.

4.7. Swim up success

There was no statistically significant difference in swim up success between exposure and control fish [26].
4.8. Hatching success

There was no statistically significant difference in hatching success between exposure and control fish [26].

4.9. Fecundity

When fish had been exposed for 75 days, fecundity was not affected [19]. After 177 days of exposure, the number of eggs laid per day by exposure fish was reduced to 23.3 eggs per day, as compared to 30.4 eggs per day in control fish [19]. This was deemed a statistically significant difference.

4.10. Viable eggs

When fish had been exposed for 75 days, the percentage of viable eggs was reduced from approximately 95% in control fish to 41% in exposed fish [19]. After 177 days of exposure, the percentage of viable eggs was reduced from 95% in control fish to 51.8% in exposed fish [19]. These data points were deemed statistically significant.
5. Exposure to 3 ng EE2/L

Exposure to 3 ng EE2/L significantly impacted VTG levels and spawning success in zebrafish. VTG levels were greater in exposed fish than in control fish after 75 days of exposure. Spawning did not occur during the exposure period for fish that were exposed for 188 days. One paper was reviewed at this concentration of EE2, observing effects for a period of 42 days, 75 days, and 118 days [21].

5.1. Sex ratio

After 42 days of exposure, the sex ratio of exposed fish was unaffected [21].

5.2. VTG levels

After 42 days of exposure, no difference was observed in the body homogenate VTG concentrations between the EE2 exposed and control fish. A range of 0.05 to 7.75 μg/ml was detected [21]. After 75 days of exposure, mean plasma VTG concentration in exposed fish was elevated over control values to a level that was deemed statistically significant. Inter-individual variation was high, with VTG concentration in exposed fish ranging between 14.76 and 1356.21 μg/ml. This variation could have been caused by the selection sample, which was of unknown sex [21], as females are known to show less increase in VTG compared to males. After 118 days of exposure, male fish had significantly increased levels of VTG [21].

5.3. Gonad morphology

After 42 days of exposure, the histological appearance of the ovaries was not different from the control fish. However, testes were less developed than in control fish; only two out of nine male fish possessed mature testes, while the other seven fish had immature testes [21]. After 75 days of exposure the ovarian histology did not differ from the control group [21]. After 118 days of exposure, all 27 individuals examined possessed ovaries, and none of the fish had gonads of testicular morphology. Both mature and immature ovaries were present: thirteen individuals had developed ovaries, with all oocyte stages and post-ovulatory follicles, while 14 fish had immature ovaries with exclusively oogonia and primary growth stage oocytes. In the 13 fish with mature ovaries, oocyte maturation was less progressed than in mature ovaries of control fish [21].

5.4. Spawning success

When fish were exposed for 42 days, the initiation of spawning was not altered. The first spawning event occurred at 83 days post fertilization (DPF), while control fish started spawning between 80 and 82 DPF [21]. However, when fish were exposed for 118 days, they did not spawn during the exposure period [21].
5.5. Survival

After 28 days of exposure, survival of exposed fish was 56–84%, which was not statistically different than control fish [21].

5.6. Length/weight

After 28 days of exposure, exposed fish were longer in length compared to control fish [21]. However, after 45 days of exposure and 75 days of exposure, there was no statistically significant difference [21].

5.7. Hatching success

The number of successfully hatched fish per spawn was not affected [21]

5.8. Fecundity

When fish were exposed for 42 days, fecundity was not statistically different compared to control fish [21].

5.9. Viable eggs

When fish were exposed for 42 days, there was no statistically significant difference in number of viable eggs between exposed and control fish (85.3% fertilization compared to 90.1% in the control) [21].

6. Exposure to 10 ng EE2/L

Exposure to 10 ng EE2/L significantly impacted sex ratio (up to 100% female), VTG levels (increased), gonad morphology (no mature ovaries), spawning success (delay in onset of spawning), length/weight of zebrafish (reduced), fecundity (reduced) and number of viable eggs (reduced). Six papers were reviewed at this concentration of EE2, observing effects for a period of 7 and 14 days [23], 60 days [19, 21], 90 days [18], 177 days [19], and 180 days [17].

6.1. Sex ratio

After 60 days of exposure, 18% of zebrafish were female, while 82% had undeveloped gonads, which was found to be statistically significant as compared to control fish [20]. A different study reported that 100% of zebrafish were female with well-defined ovaries after 60 days of exposure (compared to the mean percentages of male and female zebrafish of 33% and 67% in control) [22]. After 90 days of exposure, a sex ratio of 30% female and 70% undeveloped gonads was reported [18]. After 180 days exposure, 80% of zebrafish were female, which was statistically higher than the control ratio [17].
6.2. VTG levels

After 7 days of exposure, plasma VTG levels were measured at 760 ug/mg protein, while at day 14, levels were measured at 1272 ug/mg protein [23]. Two other papers reported that VTG induction was observed after 60 days of exposure [20], with levels being higher in exposure fish (4900 μg VTG/g) compared to control (0.5 μg/g) [22]. After 90 days of exposure, one study found VTG levels of approximately 575 μg/g [18]. All of these data points were deemed statistically significant.

6.3. Gonad morphology

After 60 days of exposure, 16 out of 20 zebrafish possessed undeveloped gonads, as compared to only one fish in the control group with undeveloped gonads. In these fish, only a small mass of primordial gonadal cells were located at the genital ridge lining the edges of the liver and swim bladder. Thus, although gonadal tissue was present, gonads were classified as undeveloped when no discernable cells characteristic of either sex were observed [20]. After 177 days of exposure, all individuals displayed gonads with ovarian morphology, but no mature ovaries. Three fish possessed ovaries containing vitellogenic and mature oocytes, while the ovaries of the remaining 24 fish contained immature pre-vitellogenic oocytes only, mostly at the perinucleolar stage and in a few cases at the cortical alveolar stage. Fish with testes were not found among all 27 individuals [19].

6.4. Spawning success

When fish were exposed for 75 days, spawning was delayed [19]. When fish were exposed for 90 days, there was a reduction in the number of spawning females within 3 separate spawning periods that were observed [18]. No mating behavior or spawning occurred during a 177 day exposure [19].

6.5. Survival

After 6 weeks of exposure, there was no significant difference in survival between exposure fish and control fish. The greatest loss occurred within the first 10 days post fertilization, which was attributed to normal larval mortality. Figure 6 shows this survival curve, with other major losses shown at the end of the 6 weeks attributed to miscounts and/or cannibalism [17]. After 60 days of exposure, a 42% survival rate was observed, which was not statistically different than control fish [20]. After 75 days of exposure, survival of exposure fish was slightly lower than in control fish, but not statistically significant [19].

6.6. Bodily malformation

After 90 days [18] and 180 days [17] of exposure, no bodily malformation was observed.

6.7. Length/weight

After 42 days of exposure, no difference in length/weight was observed [19]. After 60 days of exposure, body length of females had decreased compared to control [20]. Separate papers
observing 75 and 90 day exposure periods both found that body length of exposed fish was reduced compared to control fish [17–18]. These data points were deemed statistically significant.

6.8. Swim-up success

No significant difference was observed [26].

6.9. Hatching success

No significant difference was observed [26].

6.10. Fecundity

After 75 days of exposure, fecundity was not affected [19]. However, after 90 days of exposure, total egg production was significantly reduced, down from approximately 70 eggs per female in the control group to approximately 45 eggs per female in the exposed group [18].

6.11. Viable eggs

After 75 days of exposure, the number of viable eggs was reduced from approximately 95% in the control group to 41% in the exposed group [19]. This was deemed statistically significant.
7. **Exposure to 25 ng EE2/L**

Exposure to 25 ng EE2/L impacted sex ratio (increase in % females) VTG levels (increased), gonad morphology (no developed gonads), spawning success (absence of activity), and length/weight of zebrafish (reduced). Two papers were reviewed at this concentration of EE2, observing effects for a period of 90 days [18] and 180 days [17].

7.1. **Sex ratio**

After 180 days exposure, approximately 75% of zebrafish were female, which was statistically higher than the control ratio [17].

7.2. **VTG levels**

After 90 days of exposure, VTG levels in males were observed at approximately 1100 ng/mg, which was significantly higher than VTG levels in control males [18].

7.3. **Gonad morphology**

After 90 days of exposure, 100% of zebrafish had undeveloped gonads [18].

7.4. **Spawning success**

After 90 days of exposure, there was a complete absence of the spawning activity [18].

7.5. **Survival**

After 6 weeks of exposure, there was no significant difference in survival between exposure fish and control fish. The greatest loss occurred within the first 10 days post fertilization, which was attributed to normal larval mortality. **Figure 7** shows this survival curve, with other major losses shown at the end of the 6 weeks attributed to miscounts and/or cannibalism [17]. Between 6 weeks and 6 months of exposure, exposed fish exhibited survival rates of 70–90% (**Figure 8**), which was not statistically different from control values. Phenotypically, fish had a similar appearance to other treatment groups, despite survival patterns not demonstrating a similar pattern.

7.6. **Bodily malformation**

After 90 days of exposure, 17% of zebrafish suffered from pericardial edema, and 51% exhibited lordosis and/or scoliosis [18]. After 180 days of exposure, edema in body cavity (**Figure 9**) and bulging eye was observed [18].

7.7. **Length/weight**

After 90 days of exposure, the total body length and weight of exposed fish was significantly lower than that of the control fish [18].
Figure 7. Percent survival from 0 days post fertilization to 6 weeks for 25 ng EE2/L treatment groups of three zebrafish strains: AB (green), TU (blue), and WIK (orange). Two cohorts per strain (CH1 and CH2) are shown. Results were not statistically different from control cohorts [17].

Figure 8. Percent survival from 6 weeks to 6 months for 25 ng EE2/L treatment groups of three zebrafish strains: AB (green), TU (blue), and WIK (orange). Two cohorts per strain (CH1 and CH2) are shown. Results were not statistically different from control cohorts [17].
8. Exposure to 100 ng EE2/L

Exposure to 100 ng EE2/L impacted survival (decreased), swim-up success (decreased), and hatching success (delayed and decreased). Three papers were reviewed at this concentration of EE2, observing effects for a period of 120 hours [26], 14 days [22], and 60 days [20].

8.1. VTG levels

After 60 days of exposure, VTG induction in males was observed [20].

8.2. Survival

After 14 days of exposure, there was 0% survival [22]. Another paper observed that after 60 days of exposure, less than 10% of exposed fish survived [20].

8.3. Swim-up success

Swim up success was reduced to 60%, compared to 91% in control [26]. This was deemed statistically significant.

8.4. Hatching success

Hatching success was significantly reduced to 67%, compared to 95% in control. Hatching was also delayed compared to control (50% at 72 hours post fertilization (HPF) compared to 100% in control at 72HPF) [26].

9. Reversibility of Effects

When considering reversibility of effects, only measurements that authors had deemed significantly affected by exposure (see Table 1) are considered below. A statistically significant difference between exposed fish and control fish indicates that the aforementioned effect could not be reversed after a period of depuration. For reversibility to successfully occur, there must be no difference between control and exposure groups following depuration. This indicates that the measurement has reached control levels after a period of exposure followed by depuration. Results of this section are summarized in Table 2.
9.1. Sex ratio

9.1.1. 10 ng/L

After 60 days of exposure and 60 days in clean water, 25% of zebrafish were female and 75% male [20]. After 90 days of exposure and 150 days in clean water, 65% of zebrafish were female and 35% male [18]. This was not significantly different than control values and indicates a reversal of effects.

| 0.1 ng EE2/L | 1 ng EE2/L | 3 ng EE2/L | 10 ng EE2/L | 25 ng EE2/L | 100 ng EE2/L |
|--------------|------------|------------|-------------|-------------|-------------|
| Sex ratio (% female) | no difference | no difference | no difference | ↑ | ↑ |
| VTG levels in males | no difference | no difference | ↑ | ↑ | ↑ |
| Abnormal gonad morphology | X | X | X | |
| Spawning success | ↓ | ↓ | ↓ | ↓ |
| Survival | no difference | no difference | no difference | no difference | ↓ |
| Bodily malformation | no difference | no difference | no difference | X |
| Length/weight | no difference | no difference | no difference | ↓ | ↓ |
| Swim-up success | no difference | no difference | no difference | ↓ |
| Hatching success | no difference | no difference | no difference | ↓ |
| Fecundity | no difference | ↓ | no difference | ↓ |
| Viable eggs | no difference | ↓ | no difference | ↓ |

Blank spaces indicate that the measurement was not tested at a specific level. An ‘X’ indicates that the measurement was observed. A “↓” indicates that there was a significant decrease, while a “↑” indicates there was a significant increase in the measurement.

**Table 1.** Observed effects of acute exposure to EE2 in zebrafish that were deemed statistically significant at varying concentrations.

| 3 ng EE2/L | 10 ng EE2/L | 25 ng EE2/L |
|------------|-------------|-------------|
| Sex ratio (% female) | N/A | X | X |
| VTG levels in males | X | not reversible |
| Abnormal gonad morphology | X | X |
| Spawning success | X | X |
| Fecundity | N/A | X |
| Viable eggs | not reversible | not reversible |

Blank spaces indicate that the factor was not tested at a specific level. An ‘N/A’ means that the factor was not significantly affected by exposure, thus could not be measured for reversibility. An ‘X’ indicates that the measure was reversible.

**Table 2.** Reversibility of effects that were observed after acute exposure to EE2 in zebrafish, followed by a period of depuration.

9.1. Sex ratio

9.1.1. 10 ng/L

After 60 days of exposure and 60 days in clean water, 25% of zebrafish were female and 75% male [20]. After 90 days of exposure and 150 days in clean water, 65% of zebrafish were female and 35% male [18]. This was not significantly different than control values and indicates a reversal of effects.
9.1.2. 25 ng/L

After 90 days of exposure and 150 days in clean water, 35% of zebrafish were female and 65% male [18]. This was not significantly different than control values, and indicates a reversal of effects.

9.2. VTG levels

9.2.1. 3 ng/L

After 42 days of exposure and 76 days in clean water, there was no significant difference in VTG levels of males, which indicates a reversal of effects. After 118 days of exposure and 58 days in clean water, plasma VTG concentrations were approaching control levels in most exposed fish, and the agreement between the gonadal sex and the VTG level of individual fish was much higher than at 118 DPF [21].

9.2.2. 10 ng/L

After 177 days of exposure and 108 days in clean water, mean plasma vitellogenin levels were significantly higher at 6.7 ug VTG/ml plasma in male fish, compared with plasma VTG concentrations on average below detection limit in control males [19].

9.3. Gonad morphology.

9.3.1. 3 ng/L

After 42 days of exposure and 76 days in clean water, 17 out of 30 zebrafish possessed ovaries and 13 possessed testes. The histological appearance of the ovaries showed pronounced inter-individual variation: in 11 phenotypic females, mature ovaries were observed, whereas in six of the 17 ovary containing individuals, immature ovaries were found, containing oogonia and primary growth stage oocytes but no vitellogenic or mature oocyte. The occurrence of two types of ovaries was visible macroscopically during dissection of the fish: while in the case of mature ovaries numerous eggs were externally visible, immature ovaries appeared small, with no macroscopically recognizable substructure. The differentiated, mature testes contained numerous spermatozoa. One male had testis-ova, with a low number of primary growth stage oocytes dispersed in differentiated testicular tissue [21]. After 42 days of exposure and 134 days in clean water, 13 out of 29 fish of this treatment possessed normally differentiated testes, with all sperm stages being present. The amount of sperm cells among spermatocytes varied between individual males. The remaining 16 fish examined showed gonads with ovarian morphology, whereby eight individuals had mature and the other eight had immature ovaries [21]. After 118 days of exposure and 58 days in clean water, six out of 27 fish had fully differentiated testes, and one male displayed testis-ova. The other 20 fish possessed ovaries, of which 19 were developed as ovaries and one ovary was immature, containing oogonia and primary growth stage oocytes but with no further stages of oocyte maturation present [21].
9.3.2. 10 ng/L

After 60 days of exposure and 60 days in clean water, none of the exposed fish possessed undeveloped gonads or ovatestes/testis-ova, as was observed at 60 days post hatch (dph) [20]. After 177 days of exposure and 108 days in clean water, 5 out of 20 fish displayed gonads with the morphology of mature testes, and the remaining 15 fish had gonads with the morphology of mature ovaries [19].

9.4. Spawning success

9.4.1. 3 ng/L

After 118 days of exposure, spawning resumed after 22 days in clean water. This was a significant six week delay in the initiation of spawning compared to temporary, acute exposures performed only during the early life history stage [21]. However, the absence of spawning activity during exposure was successfully reversed.

9.4.2. 10 ng/L

After 177 days of exposure, spawning resumed after approximately 75 days in clean water [19]. The absence of spawning activity during exposure was successfully reversed.

9.5. Fecundity

9.5.1. 10 ng/L

After 75 days of exposure and 25 days in clean water, female fish laid 95 eggs per day [19]. After 177 days of exposure and 108 days in clean water, female fish laid 20.7 eggs per day, as compared to control fish that laid 30.4 eggs per day [19]. The authors did not indicate if there was a statistically significant difference between these two values.

9.6. Viable eggs

9.6.1. 3 ng/L

After 118 days of exposure and 58 days in clean water, fertilization success was significantly reduced from 91% in the control group to 21.7% in the exposed fish [21].

9.6.2. 10 ng/L

After 60 days of exposure and 60 days in clean water, 82% of eggs were viable, which was significantly reduced compared to 90% for control fish [20]. After 75 days of exposure and 25 days in clean water, the success rate was significantly reduced to 41% [19]. After 177 days of exposure and 252 days clean water, only 3% of eggs were successfully fertilized [19].
10. Conclusion

Table 1 summarizes the effects of the 6 concentrations of EE2 on each of the 12 measurements. As expected, we see a greater occurrence of effects as the dose of EE2 is increased, starting at exposure levels as low as 1 ng/L EE2. Data for 100 ng EE2/L exposure is limited, as zebrafish do not often survive at this concentration. It can be concluded that the impact of EE2 on zebrafish sexual development and reproductive functions, as well as the reversibility of effects, varies with exposure concentration, timing, and duration. In studies that had a short duration of EE2 exposure, negative effects persisted only if the exposure occurred during sexual differentiation and gametogenesis [20]. Male zebrafish pass through a stage of juvenile hermaphroditism, developing juvenile ovaries which are then transformed into testes between 20 and 60 dph [22]. This gonad transition stage is critical with respect to persisting effects of EE2 exposure on reproduction. Additionally, these experiments also illustrate the plasticity of gonadal differentiation and development in zebrafish, as a period of depuration was able to reverse many of the observed effects [20].

Though we can draw numerous conclusions from this data, a number of questions remain. First, what could be considered the toxic lethal dose (LD50) for zebrafish? It appears that the LD50 may be dependent on not only concentration, but length of exposure as well. Second, are there any differences in effects between strains? Deviations observed between evaluated studies could indicate the presence of strain specific effects. This is difficult, if not impossible, to determine since the preponderance of published papers do not identify which specific strain of zebrafish is being utilized. Without this information, reproducibility of the experiments is difficult as different strains may result in different outcomes. Additional factors, including zebrafish age and general husbandry techniques may differ between experiments and also compound the inability to replicate data.

Despite these assessments of life history exposure periods, there is a significant gap in our knowledge when it comes to chronic exposures longer than one life cycle. Studies on non-zebrafish species have shown that continuous exposure over extended generations can drive fish populations to near extinction [27]. While we have observed these long-term exposure effects, we still do not understand the mechanisms that underlie the dramatic population crashes. This knowledge gap could be filled using zebrafish, since they have a relatively short lifespan (less than three years) and generation time (~3 months). These characteristics make zebrafish an ideal model to test for the possibility of compounding effects over multiple generations. Such future studies will be particularly important for understanding long-term environmental impacts that result from continuous exposure of native populations and the mechanisms that cause such dramatic population declines.

Author details

Decatur Foster and Kim Hanford Brown*
*Address all correspondence to: kibr2@pdx.edu
Portland State University, Portland, OR, USA
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