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Investigating Dose-Dependent, Multi-Generational, and Strain-Specific Effects of 17α-ethynylestradiol Exposure in Zebrafish *(Danio rerio)*

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by

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A dissertation submitted in partial fulfillment of the
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Abstract

Endocrine disrupting chemicals (EDCs) pose a threat to biodiversity at the individual, population, and ecosystem level, as they can interfere with processes that are responsible for regulating metabolism, development, behavior, and reproduction in living organisms. 17α-ethynylestradiol (EE2) is a synthetic estrogen and EDC utilized in the pharmaceutical and livestock industries; it has been found to contaminate waterways worldwide. This research explores the effects of dose-dependent and multi-generational exposure of EE2 in three strains of zebrafish. High dose (10-25 ng/L EE2) exposure led to complete reproductive failure, as well as significantly decreased survival and growth. A period in clean water (depuration) after exposure allowed for some recovery of growth, but zebrafish never regained reproductive abilities. Low dose (1 ng/L EE2) exposure over the course of three generations led to an increase in the number of eggs produced (clutch size) by Generations 1 and 2, but a reduction in embryo hatch success in all generations, and therefore an overall reduction in reproductive capability. Depuration allowed for a return to normal clutch size, but hatch success remained low. When these results were separated by strain of zebrafish (AB, TU, and WIK), the WIK strain experienced the greatest variance in response after exposure and depuration, suggesting greater sensitivity to EE2. The findings from this study show that in a laboratory setting, one generation of exposure to EE2 concentrations above 10 ng/L causes irreversible damage to zebrafish, while multi-generational exposure to low concentrations of EE2 may slowly diminish reproductive capability, most likely caused by alterations to sperm, impact to the quality of the egg, and genetic and/or epigenetic effects that interrupt embryo development.

Dedication

I dedicate this work to the students and scientists who don't see themselves represented in academia, who take non-traditional paths to pursue what they are passionate about, and who decide to pave their own way to change science for the better.

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I would like to thank my advisor and committee for their guidance, and Dr. Thomas Hancock for graciously loaning us the Blazka swim tunnel utilized to assess zebrafish swim performance for this research. Figures 3.1, 3.2, 3.3, 3.4, and 8.1 were created using BioRender (www.biorender.com).

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List of Abbreviations

ANOVA: analysis of variance

BL/s: body lengths per second

BPA: bisphenol A

DDT: dichloro-diphenyl-trichloroethane

dpf: days post fertilization

E1: estrone

E2: 17ß-estradiol

EDC: endocrine disrupting chemical

EE2: 17α-ethynylestradiol

GSI: gonadosomatic index

H&E: Hematoxylin and Eosin

HPG: hypothalamic-pituitary-gonad

hpf: hours post fertilization

IACUC: Institutional Animal Care and Use Committee

LOEC: lowest observed effect concentration

MS-222: tricaine mesylate

NP: nonphenol

OHSU: Oregon Health and Science University

PCB: polychlorobiphenyl

PNEC: predicted no-effect concentration

PSU: Portland State University

SE: standard error

SEM: standard error of the mean

Ucrit: critical speed

VTG: vitellogenin

ZIRC: Zebrafish International Resource Center

Chapter 1 : An Introduction

Endocrine disrupting chemicals (EDCs) are a well-studied class of substances that pose a threat to aquatic biodiversity, as they can activate, block, or alter hormone synthesis and degradation in living organisms (Aris et al., 2014). EDCs impact the endocrine system, and have been shown to cause both lethal and sublethal effects in aquatic species by interfering with processes that are responsible for regulating metabolism, growth, development, behavior, and reproduction.

The EDCs of greatest concern for aquatic wildlife are those that eventually enter surface waters, most often the result of treated and untreated discharge from municipal treatment plants, livestock activities, and industrial wastewaters (Ying et al., 2002). Chemicals found in municipal effluent pre- and post-treatment include industrial chemicals used in the creation of pesticides (e.g., p-DDT), plastic precursors (such as bisphenol A and phthalates), paints, detergents, polychlorobiphenyls (PCBs), and substances formed from their breakdown (like nonylphenol and octylphenyl), all of which are suspected to disrupt the endocrine system of animals (Combalbert and Hernandez-Raquet, 2010). The most potent EDCs contained in these effluents are natural and synthetic steroid estrogens, such as estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) (Xu et al., 2014). These estrogenic chemicals are among the most extensively studied EDCs, primarily due to high levels of environmental contamination and a wide range of observed exposure effects on aquatic ecosystems.

The synthetic estrogen EE2 (Figure 1.1), is of particular concern, given its higher binding affinity to estrogen receptors than endogenous E2 (Blair et al., 2000). This results in low concentrations of EE2 having higher estrogenic activity in organisms than naturally occurring estrogens. EE2 is commonly used as the bioactive estrogen for human oral contraceptive pills, and as a medicine for alleviating menopausal and postmenopausal syndrome symptoms, physiological replacement therapy for estrogen deficient states, and as a treatment for prostate cancer, breast cancer, and osteoporosis (Aris et al., 2014). EE2 is also widely utilized in livestock to regulate pregnancy and treat disease (Ying et al., 2002).

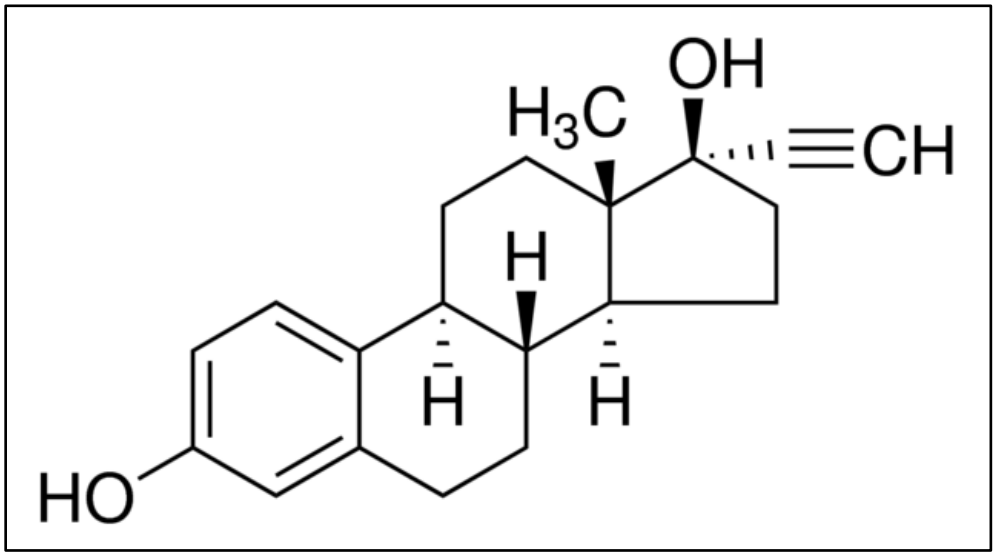

Figure 1.1 Chemical structure of 17α-ethynylestradiol (EE2).

Sources of EE2 contamination

Human urine is considered a major source of EE2 contamination in the environment, as excess EE2 in the body is excreted and enters aquatic systems through wastewater effluent release. Prior to excretion in urine, EE2 is metabolized to become a

biologically inactive, water-soluble sulfate or glucuronide conjugate (Desbrow et al., 1998). Following excretion and subsequent transfer to wastewater treatment facilities, EE2 may be activated into its free (unconjugated) form via bacterial modification. The free form of EE2 remains relatively stable during the activated sludge process that is meant to degrade biological content from human waste; thus, in some cases the concentration of free EE2 is increased during sewage treatment (Forrez et al., 2009). As EE2 takes longer to degrade than natural estrogens and tends to bio-concentrate in tissues, it has become a widespread problem in the environment, particularly in aquatic ecosystems (Ying et al., 2003; Larsson et al., 1999). EE2 pollution in many waterways is chronic, meaning aquatic species may live in this environment for multiple generations, which in turn can impact both local populations as well as higher trophic level organisms.

With a global human population of over seven billion, it is estimated that approximately 700 kg/year of synthetic estrogens are released into the environment from contraceptive usage alone (Combalbert and Hernandez-Raquet, 2010). This number does not take into account estrogen release from livestock, which has been shown to be at a rate of more than twice that of human discharge (Adeel et al., 2017). 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 (Kolpin et al., 2002). 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 of 4 ng/L (King County, 2007). Concentrations of 42 ng/L EE2 have been found in Canadian sewage treatment effluent (Ternes et al., 1999), while studies in

Europe have found concentrations generally below 5 ng/L (Figure 1.2) (Desbrow et al., 1998).

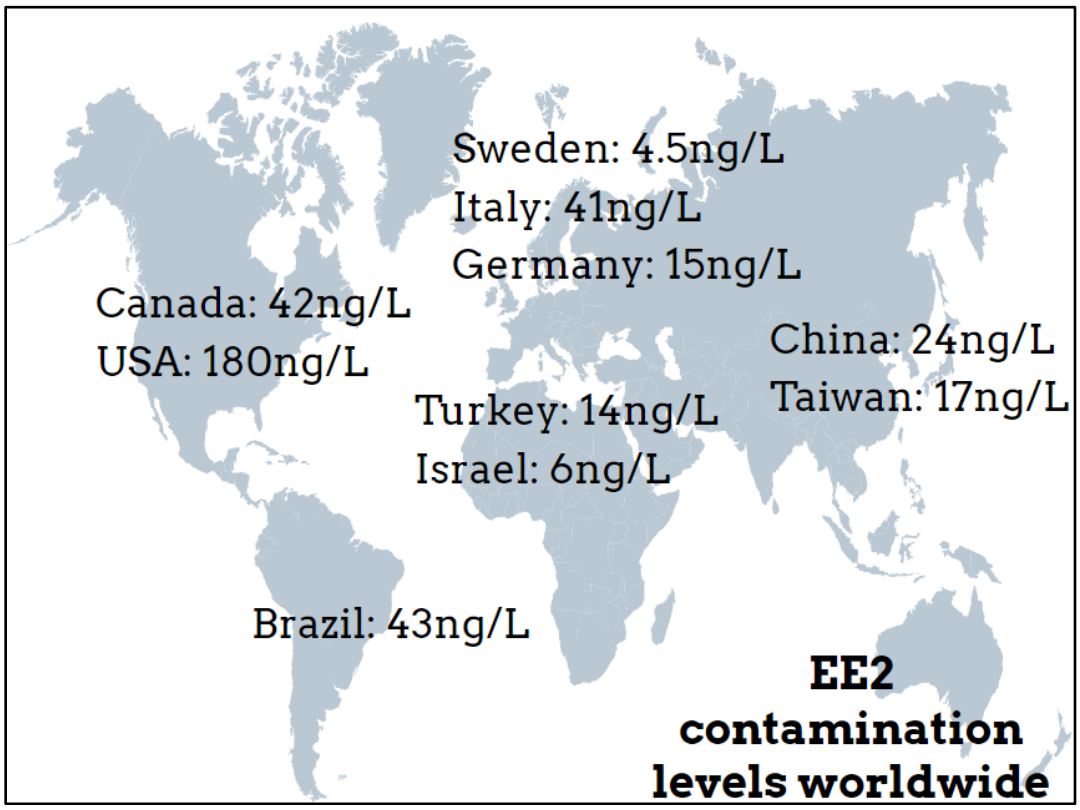

Figure 1.2. Published concentrations of EE2 found in surface water and wastewater effluent.

As the aquatic environment is a major repository for EDCs, increased attention has been given to toxicological research utilizing fish as a model organism. An inherent assumption of toxicology studies is that the biological effects of a chemical in a laboratory model organism are predictive of similar effects in humans. Thus, it is important to understand both the highly conserved and species-specific differences of endocrine system responses to toxicants like EE2.

Effects of EE2 in fish species

EE2 has been shown to concentrate in the body of fish species at a 332-fold higher rate than in the environment (Lai et al., 2002). In some fish species, the binding affinity of EE2 to estrogen receptors has been shown to be up to five times higher than E2 (Thorpe et al., 2003). This higher receptor affinity indicates that EE2 can be a more potent estrogenic compound in terms of eliciting an estrogenic response, as compared to naturally produced E2 (Aris et al., 2014). Under environmental and laboratory conditions, concentrations of EE2 at measurements as low as 5-50 ng/L (parts per trillion) have been reported to cause a wide variety of effects in multiple species of fish, including decreased fertility and fecundity, bias in the sex ratio toward female, vitellogenin (a female egg yolk precursor protein) induction in males, reduction of gonadal development, and impairment of reproductive behaviors (Brown et al., 2007; De Wit et al., 2010; Woodling et al., 2006). These effects were also found in a whole-lake experiment where a population of fathead minnow (*Pimephales promelas*) exposed to 5 ng/L EE2 over the course of seven years collapsed after the second season of EE2 exposure, likely due to severe reproductive impairment (Kidd et al., 2007).

Zebrafish as a model system to study the effects of EE2

Zebrafish (*Danio rerio*) are commonly used in laboratory settings to observe the effects of EE2 in aquatic species. They are small (3-4 cm) freshwater fish that can be easily kept in the laboratory and effectively exposed to toxicants via tank water. Given their rapid development from fertilization to reproductive maturity in only three to four

months, both short-term early life stage and chronic full life-cycle tests can be conducted in a relatively short amount of time. 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; the entire zebrafish genome has been published, and matches approximately 70% to human orthologs, allowing for in-depth genetic comparison and analysis (Ortiz-Zarragoitia and Cajaraville, 2005).

Current areas in need of study

Although the effects of EE2 exposure on aquatic species is a well-researched topic, further study is needed into the long-term, multi-generational effects of EE2 exposure. The majority of laboratory experiments utilize acute exposure periods in order to understand life-stage specific effects, or to investigate how EE2 affects gene regulation. To date, few studies have looked at full-life cycle, multi-generational exposure, which more closely resembles the exposure experienced by wild fish populations. Furthermore, while some studies have looked at full-life cycle exposure, there remain questions about whether the effects seen after chronic exposure can be alleviated by time in clean water (i.e. depuration).

Research into response differences between the multiple strains of zebrafish utilized in toxicant studies is also needed. Zebrafish researchers typically report that "wild type" zebrafish are utilized, but fail to specify which "wild type" they are utilizing.

Common laboratory “wild type” strains include AB, Tubingen (TU), Wild India Kolkata (WIK), and Tupfel long fin (TL), among others. The published zebrafish genome was generated from sequencing a single double-haploid TU strain fish (Ruzicka et al., 2019). Given the extensive genetic diversity between laboratory strains, several studies have described both physiological and behavioral differences between them. It has been reported that the AB strain displays significantly lower levels of anxiety-related behavior than two wild-derived lines (Wong et al., 2012). Furthermore, after characterizing AB, TU, WIK, and two fish farm strain (EKW and PKR) responses to PCB126 exposure, researchers found that of the five strains, the TU strain was the most sensitive and the PKR strain was the most tolerant (Waits and Nebert, 2011). AB, TU, and WIK also have different baseline mRNA expression, illustrating the fact that their molecular “normal” is slightly different (Holden and Brown, 2018). Therefore, it is likely that strain type has an impact on control and exposure outcomes in toxicant studies, as each differs in their initial method of establishment, course of selective breeding, and genetic background.

Aims of this study and rationale for chapters

The aims of this research are to (1) investigate the effects of multi-generational exposure to EE2 in zebrafish, (2) observe their capacity to recover from exposure effects when given access to clean water, and (3) identify dose-dependent and (4) strain-specific responses to EE2 exposure.

The current understanding of EE2 effects in zebrafish is summarized in Chapter 2. We review the effects of three environmentally relevant concentrations of EE2 on 12

measurements that are commonly selected when carrying out toxicology studies on estrogenic chemicals. Effects of full life-cycle exposure to concentrations of 0.1 ng/L, 3 ng/L, and 100 ng/L EE2 are reviewed for their effects on sex ratio, vitellogenin induction, gonad morphology, spawning success, survival, bodily malformation, length and weight, swim-up success, fertility, and fecundity. Furthermore, we review which of these exposure effects could be mitigated or recovered after a period in clean water.

In Chapter 3, we explain our experimental design for evaluating dose-dependent, multi-generational, and strain-specific effects of EE2 exposure in zebrafish, as well as data points that were collected. Furthermore, the Brown Aquatic Lab zebrafish husbandry protocols are explained, including system maintenance and depuration procedures.

In chapters four through seven, we elucidate the results of our research into the effects of full life-cycle exposure to EE2 concentrations of 1 ng/L, 10 ng/L, and 25 ng/L. Full life-cycle refers to exposure up until the organism is capable of reproduction, and thus creation of a new generation. These concentrations were chosen after a review of literature showed that exposure to 0.1ng/L EE2 appears to have no observable effect on the 12 common markers of endocrine disruption mentioned above, while exposure to 100 ng/L EE2 leads to severe mortality rates within 14 days (Örn et al., 2006). Furthermore, these are concentrations that are found in aquatic ecosystems worldwide (Figure 1.2) and are therefore environmentally relevant.

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Chapter 2 : Review – Dose-Dependent Effects of 17α-ethynylestradiol Exposure

Abstract

Exposure of the synthetic estrogen 17α-ethynylestradiol (EE2) to zebrafish has been shown to cause several effects, including but not limited to reduced reproductive capabilities, impaired embryonic development, and feminization of male fish. In this chapter, we review the effects of exposure to three environmentally relevant concentrations of EE2 (0.1 ng/L, 3 ng/L, and 100 ng/L) on 12 measurements commonly selected when studying the effects of EE2 on zebrafish: sex ratio, vitellogenin induction, gonad morphology, spawning success, survival, bodily malformation, length and weight, swim-up success, fecundity, viable eggs, hatching success, and the mitigation of aforementioned effects after access to clean water. Exposure to 0.1 ng/L had no impact on these measurements, while exposure to 100n ng/L severely impacted the survival, growth, and reproduction of zebrafish. Exposure to 3 ng/L EE2 affected the sex ratio, morphology, and reproductive capabilities of zebrafish, but after a period in clean water these measurements returned to normal levels.

Introduction

In this chapter, we focus on reviewing the impact that 17α-ethynylestradiol (EE2) exposure has on 12 measurements of fitness that are commonly selected when studying the effects of EE2 on zebrafish. They include: (1) sex ratio of exposure offspring (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 gonads) (4) spawning success (onset of spawning and number of successful spawns) (5) survival (6) bodily malformation (7) length and weight (8) swim-up success (successful inflation of the swim bladder by day seven post fertilization) (9) fecundity (number of eggs per spawn) (10) viable eggs (successful fertilization) (11) number of hatched eggs per spawn, and (12) mitigation of exposure effects after depuration (the ability of the previous 11 measurements to return to control levels after a period of time in clean water). This chapter summarizes two decades of inquiry into the above effects of EE2 exposure in zebrafish.

We focused on three concentrations of EE2 commonly used by researchers, all of which are readily found in the environment: 0.1 ng/L, 3 ng/L, and 100 ng/L. Exposure periods ranged from 120 hours to 180 days, followed by depuration periods of 25 to 80 days (Figure 2.1). Studies that did not begin exposure at day 1 (i.e. partial life-cycle exposures) were excluded from consideration. Furthermore, effects on second-generation exposure fish are not reported in this review. When findings in this review are reported as statistically significant, they were deemed so by the original authors, as compared to control, unless otherwise noted.

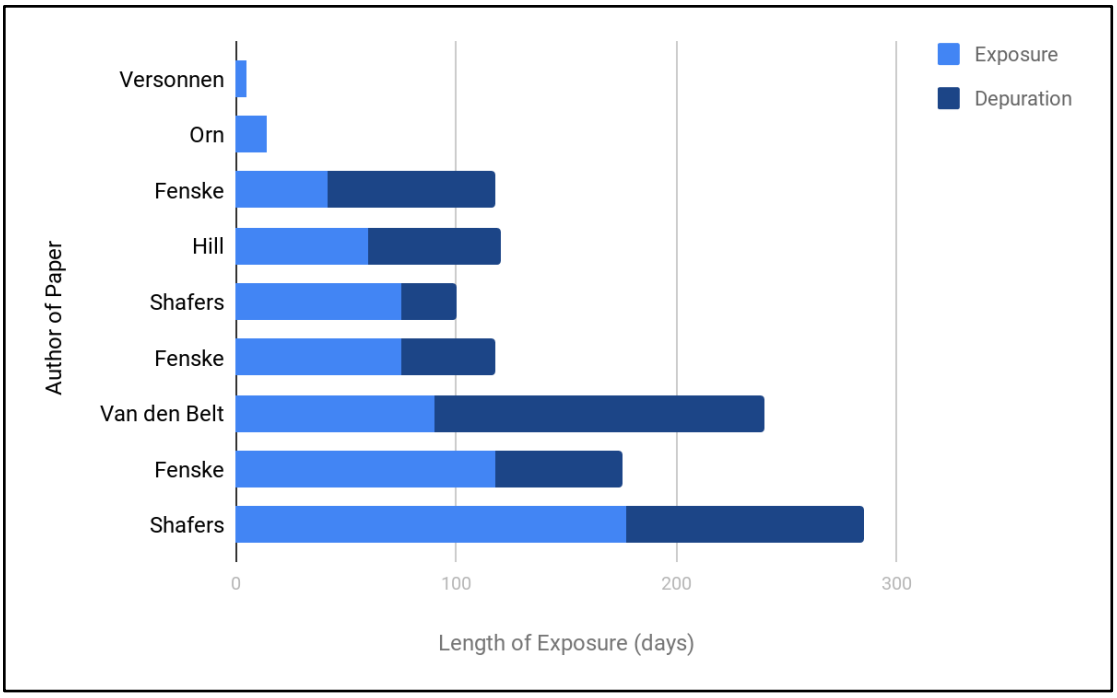

Figure 2.1. Length of EE2 exposure and depuration periods observed in each zebrafish study evaluated in this chapter.

Exposure Effects

A summary of published exposure effects can be seen in Table 2.1. Overall, exposure to 0.1 ng/L EE2 did not affect the 11 measurements of fitness in zebrafish. Exposure to 3 ng/L EE2 significantly increased VTG levels, caused abnormal gonad morphology, and decreased spawning success. Exposure to 100 ng/L EE2 led to reduced hatch success, swim up success, and eventually a 90-100% mortality rate.

Table 2.1. Observed effects of acute exposure to EE2 in zebrafish that were deemed statistically significant at varying concentrations. '-' indicates that the factor was not tested at the concentration. '↓' indicates that there was a significant decrease, while a '↑' indicates there was a significant increase in the measurement.

	0.1 ng/L EE2	**3 ng/L EE2**	**100 ng/L EE2**
Sex Ratio (% female)	no difference	no difference	-
VTG Levels in Males	no difference	↑	↑
Abnormal Gonad Morphology	-	observed	-
Spawning Success	-	↓	-
Survival	-	no difference	↓
Bodily Malformation	no difference	-	-
Length and Weight	no difference	no difference	-
Swim-up Success	-	-	↓
Hatching Success	-	no difference	↓
Fecundity	no difference	no difference	-
Viable Eggs	no difference	no difference	-

Exposure to 0.1 ng/L EE2

Overall, exposure to 0.1 ng EE2/L appears to have little or no observable effect on zebrafish. Two studies (Van den Belt et al., 2003; Shäfers et al., 2007) evaluated concentrations of EE2 at this level with no detrimental effects observed.

Van den Belt study

After 90 days of exposure, 40% of zebrafish were female, while 40% had undeveloped gonads; this did not significantly differ from control ratios. Furthermore, VTG was not detected in male fish, and no bodily malformation was observed in zebrafish. The total body length and weight of exposed zebrafish was not significantly different than the control group.

Shäfers study

After 177 days of exposure, 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). Furthermore, there was no difference in the number of successfully fertilized eggs between exposure and control zebrafish (exposure fish had a fertilization success rate of 91.6%).

Exposure to 3 ng/L EE2

Exposure to 3 ng/L EE2 significantly increased VTG levels and decreased spawning success in zebrafish. One paper (Fenske et al., 2005) was reviewed at this concentration of EE2, observing effects for exposure periods of 42 days, 75 days, and 118 days. While short-term exposure (42 days) had no effect on zebrafish, exposure for 75 days affected VTG levels, while exposure for 118 days impacted gonad morphology and inhibited spawning.

Fenske study

In the group exposed to EE2 for 42 days, the sex ratio of exposure fish was unaffected. The histological appearance of the ovaries in exposed fish was not different from the control fish, however, testes were less developed than in control fish; seven out of nine male fish had immature testes. Body homogenate VTG concentrations in the exposure group did not differ from control. The first spawning event in this group occurred at 83 days post fertilization (dpf), while control fish started spawning between

80-82 dpf. There was no statistically significant difference in number of eggs produced (fecundity), viable eggs (85.3% fertilization success in exposure fish compared to 90.1% in the control), or hatch success between the exposure and control group.

In the group exposed to EE2 for 75 days, all 20 individuals possessed ovaries; ovarian histology did not differ from the control group. Mean plasma VTG concentration in exposed fish was significantly elevated as compared to control values.

After 118 days of exposure, all 27 individuals examined possessed ovaries; 13 zebrafish had developed ovaries, while 14 fish had immature ovaries. In the 13 fish with mature ovaries, oocyte maturation was less progressed than in mature ovaries of the control group. Male zebrafish had significantly increased levels of VTG. Zebrafish in this exposure group did not spawn during the exposure period.

Exposure to 100 ng/L EE2

Exposure to 100 ng/L EE2 significantly decreased survival, swim-up success, and hatching success of zebrafish. Three papers were reviewed at this concentration of EE2, observing effects for a period of 120 hours (Versonnen and Janssen, 2004), 14 days (Örn et al., 2006), and 60 days (Hill and Janz, 2003).

Versonnen study

Mortality of zebrafish embryos exposed to EE2 for 120 hours did not differ from control (6.7% mortality in exposure, 1.7% in control). However, hatch rates were significantly lower (67% success in exposure, 95% in control). Hatching was also

delayed compared to control (50% at 72 hours post fertilization (hpf) in exposure as compared to 100% in control). Furthermore, swim-up success was significantly reduced (60% in exposure as compared to 91% in control).

Örn study

After 14 days of exposure, there was 0% survival of exposed zebrafish.

Hill study

After 60 days of exposure, less than 10% of exposed zebrafish survived. Among these fish, VTG induction in males was observed.

Mitigation of Exposure Effects via Depuration

In this review of depuration effects, only outcomes that the authors deemed statistically significant during the EE2 exposure period are considered below. For measuring the effects of depuration, successful mitigation of exposure effects was defined by two steps: (1) there was a statistically significant effect found during the EE2 exposure period and (2) that effect returned to control levels, or 'normal' levels, following a depuration period. With these parameters in mind, mitigation and recovery of effects was evaluated for zebrafish exposed to 3 ng/L EE2 in one paper (Fenske et al., 2005). Results of this section are summarized in Table 2.2.

Table 2.2. Mitigation and recovery of effects that were observed after acute exposure to 3 ng/L EE2 in zebrafish, followed by a period of depuration. 'N/A' means that the factor was not significantly affected by exposure, thus could not be measured for recovery.

Sex Ratio (% female)	N/A
VTG Levels in Males	Recovered
Abnormal Gonad Morphology	Recovered
Spawning Success	Recovered
Fecundity	N/A
Viable Eggs	Not recovered

VTG Levels

After 42 days of exposure and 76 days in clean water, there was no significant difference in VTG levels of males between groups, which indicates a mitigation of effects (during the exposure period, VTG levels of exposed fish were increased as compared to control). 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 the measurements taken immediately after the exposure period.

Gonad Morphology

After 42 days of exposure and a 76-day depuration period, 17 out of 30 zebrafish possessed ovaries and 13 possessed testes. This indicates that depuration allowed for a mitigation of exposure effects, as gonad morphology was underdeveloped and/or exclusively ovarian during the exposure period. The histological appearance of the ovaries varied: in 11 phenotypic females, mature ovaries were observed, whereas in six

of the 17 ovary-containing individuals, immature ovaries were found. Zebrafish with mature testes contained numerous spermatozoa, while one male had ovo-testis.

After 42 days of exposure and 134 days in clean water, 13 out of 29 fish of this treatment possessed mature testes, with all spermatogenesis stages being present. The remaining 16 fish examined showed gonads with ovarian morphology; eight had mature ovaries and the other eight had immature ovaries.

After 118 days of exposure and 58 days in clean water, six out of 27 fish mature testes, and one male displayed ovo-testis. The other 20 fish possessed ovaries, of which 19 were developed ovaries and one ovary was immature.

Spawning Success

After 118 days of exposure, reproduction was inhibited; 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 (days 0-42). However, the absence of spawning activity during the exposure period was successfully recovered.

Viable Eggs

After 118 days of exposure and a 58-day depuration period, fertilization success was significantly reduced in the exposure group at 21.7%, as compared to 91% in the control group. This indicates that EE2 exposure had a delayed negative impact on egg

viability, as there was no difference in viable eggs between the control and exposure group during the exposure period.

Conclusion

This literature review elucidated both lethal and sublethal effects of EE2 exposure over the course of one generation, which appear to be dependent on both concentration and length of exposure. Exposure to 0.1 ng/L EE2 appears to have no observable effect on zebrafish. This is consistent with the published predicted no-effect concentration (PNEC) for chronic exposures of EE2 on aquatic life (Caldwell et al., 2012). Furthermore, the findings of this review are consistent with a lowest observed effect concentration (LOEC) value of 1ng/L EE2 for Japanese medaka, fathead minnows, and zebrafish (Metcalfe et al., 2001; Pawlowski et al., 2004, Shäfers et al., 2007). Zebrafish exposed to 3 ng/L EE2 were able to recover the ability to spawn after a depuration period, yet experienced reduced fertilization success, even after depuration. Data for 100 ng/L EE2 exposure is limited, as zebrafish do not often survive to sexual maturity after exposure to this concentration.

This leads us to ask several questions: (1) What are the effects of EE2 exposure at environmentally relevant concentrations, when exposure occurs over the course of multiple generations? (2) Can these exposure effects be mitigated after access to clean water? And finally, in many of these studies, the strain of zebrafish utilized in the experiment was not specified, despite genetic variation in strains that might contribute to disease susceptibility or chemical exposure response differences (Balik-Meisner et al.,

2018). Deviations observed between results of these studies could be due to the presence of strain specific effects, as they differ in origin and genetic background. Thus leading us to ask (3) are there any differences in EE2 exposure effects between different zebrafish strains? Future studies that answer these questions 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.

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Chapter 3 : Experimental Protocol and Zebrafish Husbandry

Experimental Protocol

Figure 3.1 shows the experimental design of this research. Zebrafish were exposed to 17α-ethynylestradiol (EE2) for five months, during which the following data points were collected: survival from day 0-21, survival from day 21 through five months, length and weight at 21 days as well as five months of age, reproduction (clutch size and hatch success) at 3.5-4.5 months of age, swimming performance at 4-4.5 months of age, and a sperm cell count at five months of age. During a six-month depuration period, data points collected were survival, reproduction at 8.5-9.5 months, as well as length, weight, and a sperm cell count at 11 months of age.

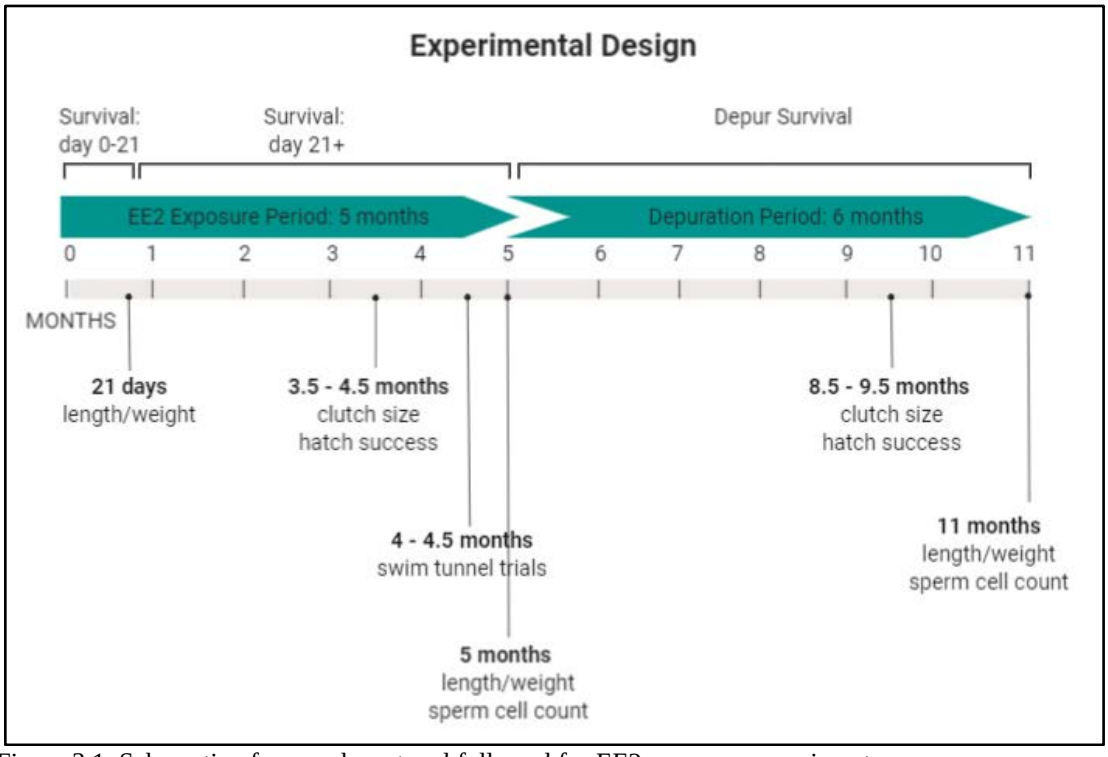


Figure 3.1. Schematic of research protocol followed for EE2 exposure experiments.

To carry out a research project that more closely resembles EE2 exposure in nature, we exposed zebrafish to EE2 for multiple generations (Figure 3.2), as pollution due to endocrine disrupting chemicals (EDCs) is a long-term, chronic issue. After full life-cycle exposure (five months), zebrafish were placed in clean water for a depuration period of six months. This period was meant to mimic the experience of fish that are able to gain access to clean water after exposure to EDCs, either through migration to a new area or through human-led cleanup efforts.

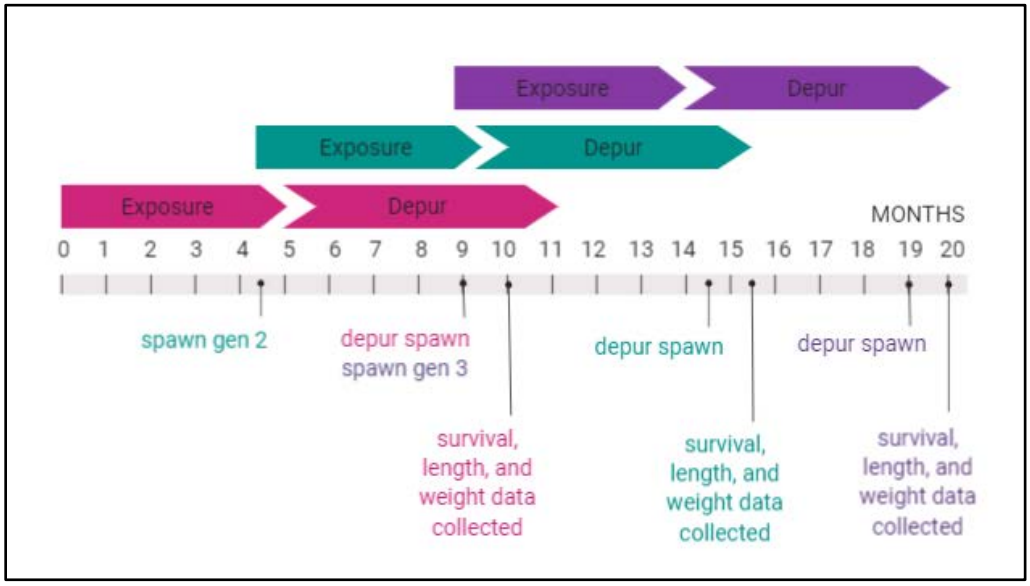


Figure 3.2. Protocol schematic of a five-month EE2 exposure, followed by a six-month depuration period, for three generations. Full exposure experiment took place over the course of 20 months, with staggered generations.

Zebrafish Husbandry

Three common laboratory strains of zebrafish (AB, TU, and WIK) were originally obtained from the Zebrafish International Resource Center (ZIRC) in Eugene, OR, and bred in the Brown Zebrafish Aquatics Facility at Portland State University (PSU) for

three years before research began. All fish were maintained in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC) of PSU.

Adult fish were maintained on a modular Aquaneering system with 4-stage central filtration providing continuous flow-through of recirculating water with a twice daily automated 10% water change. This system monitored and maintained a pH of approximately 7.4 and conductivity of 1100 µS. Water temperature was maintained at 27.5°C and fish were kept on a 16-hour light, 8-hour dark photoperiod. Zebrafish were housed in 2.8-liter baffled flow-through tanks at a ratio of 1 male:1 female with a stocking density of 10 or less fish per tank. Fish were fed commercial flake food twice daily ad libitum and supplemented with live brine shrimp (*Artemia salina*) and rotifers (*Brachionus plicatilis*).

Five pairs of sexually mature zebrafish (per strain) approximately one year of age were bred to obtain eggs for EE2 exposure experiments. Embryos were transferred to sterile petri dishes and incubated at 28.5°C until hatched. At five days post fertilization (dpf), 10 randomly chosen hatched larvae per breeding pair were transferred to 1000mL glass beakers containing 250 mL embryo media and started on concentrated rotifers twice daily, ad libitum. At nine dpf larvae were transitioned to live brine shrimp in addition to rotifers. Larval powder was introduced at 14 dpf. Fish were raised under these conditions, with manual embryo media changes every other day, until six weeks of age. At six weeks, 20 zebrafish were randomly chosen from the population and transferred to one-gallon fish bowls on the exposure system to create the experimental population (Figure

3.3). This process was carried out for each treatment (control, 1 ng/L, 10 ng/L, and 25 ng/L EE2) (Figure 3.4).

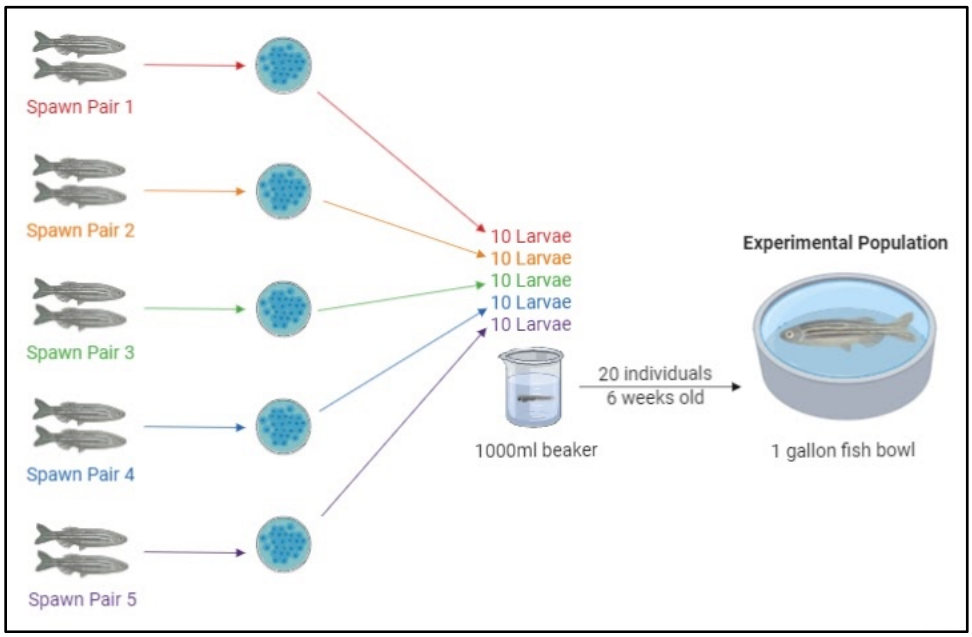


Figure 3.3. Process of spawning five pairs of fish in order to create a pool of 50 fry, from which 20 individuals are randomly chosen at 6 weeks of age to become the experimental population. This process was carried out for each strain (AB, TU, WIK), at each exposure concentration (control, 1 ng/L, 10 ng/L, and 25 ng/L EE2).

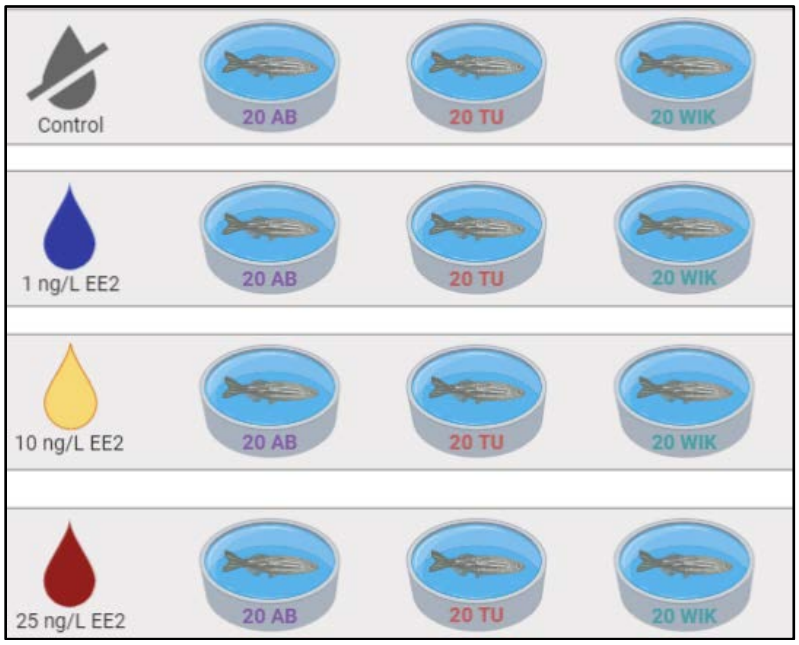


Figure 3.4. Number of fish per strain (AB, TU, WIK), per exposure concentration (control, 1 ng/L, 10 ng/L, and 25 ng/L EE2) for an entire population of one generation. One generation consisted of 240 total fish.

Fish were housed in an exposure system designed and built in-house until five months of age (Figure 3.5). PVC piping and a centrifugal pump allowed water to be routed into one-gallon fish bowls. Each tank had an isolated outflow directed into a common waste basin which was pumped through a triple filtration system before emptying into the common building drainage and city wastewater system. Manual 30-second flushes refreshed water every other day. Waste debris was removed via turkey baster.

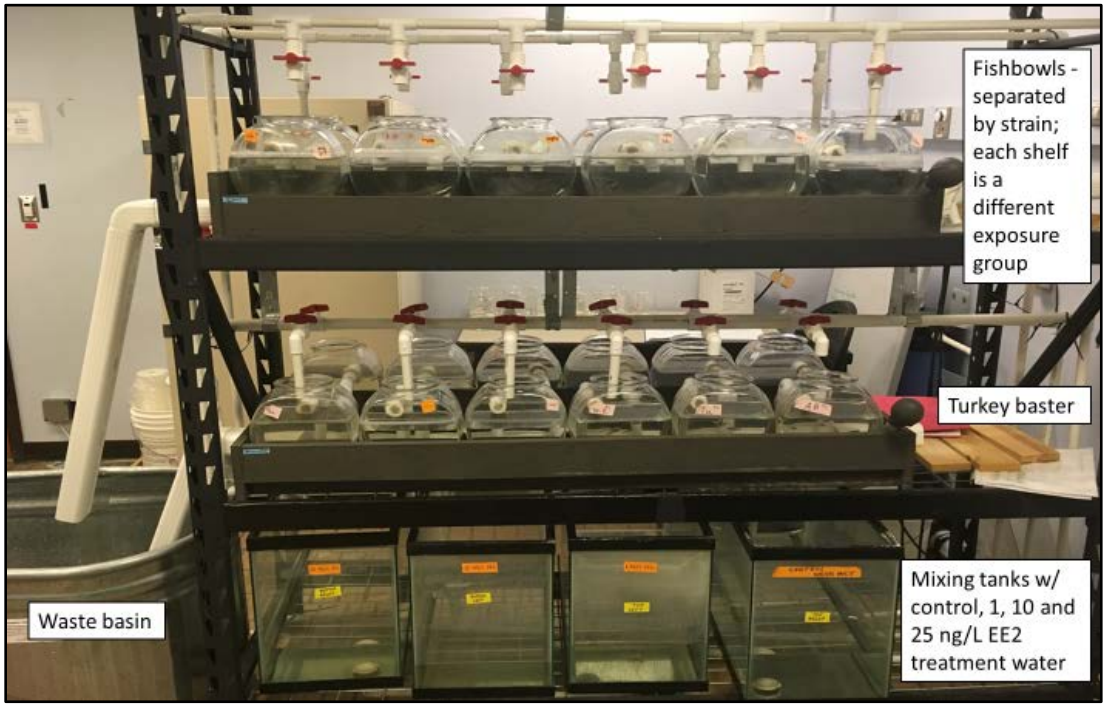


Figure 3.5. EE2 Exposure system.

Exposure to EE2

The test compound 17α-ethynylestradiol was obtained from Sigma-Aldrich (≥ 98% grade). EE2 was solubilized in 100% methanol at a ratio of 1mg/1mL. A final working concentration of 1 ng/µL EE2 in 10% methanol was prepared as the exposure

working stock for spiking treatment water. Control fish were exposed to methanol at the highest EE2 treatment concentration, 5µL 100% methanol/liter tank water, or ≤0.0005%. One-liter amber glass bottles were filled with embryo media and spiked with EE2 working stock at respective treatment concentrations 24-hours prior to treatment start.

Upon collection, zebrafish embryos were immediately submersed in pre-mixed embryo media from one of four treatment groups: control, 1 ng/L, 10 ng/L, or 25 ng/L EE2. After six weeks of exposure via embryo media, zebrafish were moved to the exposure system, where common mixing tanks were spiked with EE2 or 0.0005% methanol (for control) and allowed to equilibrate for 24 hours prior to transfer of juvenile fish.

Depuration

After five months of EE2 exposure, zebrafish were transferred to a clean water system. Laboratory water passed through a 4-stage central filtration process on an Aquaneering Modular System, which provided continuous flow-through of recirculating water and a twice daily automated 10% water change. Fish were kept on this system for six months. Following reproductive trials, the fish were euthanized utilizing an overdose of MS-222 (Sigma-Aldrich) at a concentration of 300 mg/L in water at pH 8 for a minimum of 15 minutes.

Chapter 4 : 17α-ethynylestradiol Effects on Survival, Growth, and Development

Abstract

The impact of 17α-ethynylestradiol (EE2) full life-cycle exposure on survival, growth, and development in zebrafish was evaluated utilizing three environmentally relevant concentrations: 1 ng/L, 10 ng/L, and 25 ng/L. Exposure effects to these concentrations were observed for one generation, as well as effects of 1 ng/L EE2 exposure over the course of three generations. Furthermore, three strains of zebrafish (AB, TU, and WIK) were utilized in this study to assess strain-specific EE2 exposure effects. Length and weight were recorded at 21 days and five months of age, and both short- and long-term mortality rates were calculated. Reduced survival rates during the first 21 days of development were observed in all three exposure concentration groups, but only the 25 ng/L EE2 exposure group exhibited a significant reduction in long term survival. Reduced growth at both 21 days and five months of age was observed in the 10 ng/L and 25 ng/L EE2 exposure groups, while many in the 25 ng/L EE2 group exhibited pericardial edema. Multi-generational exposure to 1 ng/L EE2 resulted in reduced survival rates during the first 21 days of age. Additionally, the TU stain of zebrafish appears to exhibit greater sensitivity to EE2 exposure at higher concentrations when considering survival and growth as endpoints, while the WIK strain exhibits a more varied morphological response to EE2 exposure. These findings suggest that full life-cycle exposure to 25 ng/L EE2 severely impacts the survival, growth, and development of zebrafish, while chronic, low dose exposure (1 ng/L EE2) may have a wide range of sublethal effects.

Introduction

Environmental toxicants like endocrine disrupting chemicals (EDCs) can be persistent or transient risks to aquatic wildlife and are found in many locations around the world. These xenobiotics have been shown to alter survival and growth in fish species, affecting the health and survival of organisms and the ecosystems they live in. Studies utilizing zebrafish have shown that exposure to certain toxicants results in reduced length and weight, including cadmium (Bresch, 1982), herbicides and insecticides like 3,4-dichloroaniline and lindane (Ensenbach and Nagel, 1997) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Elonen et al., 1998). Of particular concern are synthetic and endogenous estrogens, which have been shown to modulate development in various vertebrates. However, exposure to the synthetic estrogen 17α-ethynylestradiol (EE2) has not been shown to impair the growth of fish species that are commonly used in toxicology research, such as medaka (*Oryzias latipes*) or sheepshead minnows (*Cyprinidon variegates*) (Scholz and Gutzeit, 2000; Zillioux et al., 2001).

This chapter investigates both short- and long-term survival, growth, and development effects of EE2 exposure in zebrafish. In this study, zebrafish were exposed to three environmentally relevant concentrations of EE2 (1 ng/L, 10 ng/L, and 25 ng/L), and observed for significant differences in mortality, length, weight, condition factor, and abnormal morphology. Our study design also included three generations of full life-cycle exposure to 1 ng/L EE2, to detect any indications of compounding lethal or sublethal effects on development. Three strains of zebrafish (AB, TU, and WIK) were utilized in this study, to identify strain-specific effects of exposure to EE2.

Materials and Methods

Survival Parameters

Survival curves were calculated for two periods: days 0-21 and day 21 to five months. For the day 0-21 time period, a survival curve was calculated based on the population of 50 hatched zebrafish per strain, per exposure group. At day 21, 20 zebrafish per strain, per exposure group were chosen at random to continue as the experimental population, and the survival curve was reset in order to be calculated for day 21 to five months.

Growth and Development Parameters

Zebrafish length and weight was recorded at a juvenile stage (21 days) as well as an adult stage (five months). At 21 days of age, 10-20 zebrafish per strain, per exposure group were euthanized, and length and weight were recorded. Between 4-5 months of age, length and weight of 10-20 fish per exposure group was recorded while fish were anesthetized using MS-222. Condition factor (K) was calculated to measure the relationship between the weight of the fish and its length. K is a value used by scientists to describe the "condition" of fish, and often utilized as a general indicator of health. The formula is:

$$K = 10^N * W/L^3$$

where W = the weight of the fish in grams, and L is the length of the fish in mm. A species-specific scaling factor (N) is applied to bring the factor close to 1: for zebrafish N=5.

Statistical Analysis

Statistical analysis was performed with JMP Pro 14 software. To analyze survival data, we used a Kaplan-Meyer survival curve, with the log-rank test for differences between exposure groups and the control. For length, weight, and condition factor, all data were examined for homogeneity and normality using Levene's and Kolmogorov-Smirnov tests. If these assumptions were met, one-way analysis of variance (ANOVA) followed by a Tukey's test was utilized to identify differences between each exposure group and the control. If the homogeneity and normality assumptions were not met, the nonparametric Kruskal-Wallis test followed by multiple comparison was performed. For multi-generational data, t-tests were performed to identify differences between exposure groups and control. All values presented are mean ± SEM. The significance level for all the statistical analyses was set at $p<0.05$.

Results

Exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2

During the first 21 days of development, survival significantly decreased in all three exposure groups as compared to the control group. The 25 ng/L EE2 exposure group had the lowest survival rate (73%) as compared to survival in control (87%), while

the 1 ng/L EE2 exposure group had a survival rate of 77%, and the 10 ng/L EE2 exposure group had a survival rate of 78% (Figure 4.1).

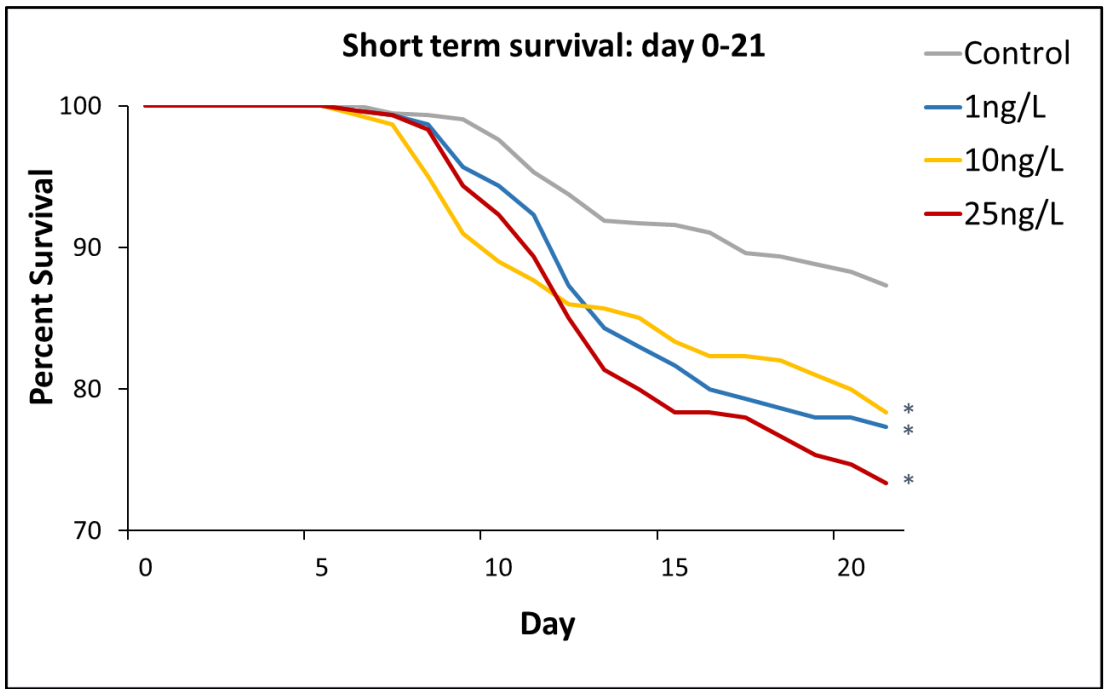


Figure 4.1. Survival curve of zebrafish, day 0-21, for control group and exposure groups to concentrations of 1 ng/L, 10 ng/L and 25 ng/L EE2. n=750 for control group, n=300 for exposure groups. * indicates significant difference from control, $p<0.01$.

Over the course of five months, the 25 ng/L EE2 exposure group experienced a significant decrease in survival as compared to the control group (Figure 4.2). After five months of exposure to EE2, the control group had a survival rate of 81%, the 1 ng/L EE2 and 10 ng/L EE2 exposure groups had a survival rate of 72%, and the 25 ng/L EE2 exposure group had a survival rate of 45%. Survival of the 1 ng/L and 10 ng/L EE2 exposure groups was not significantly different from the control.

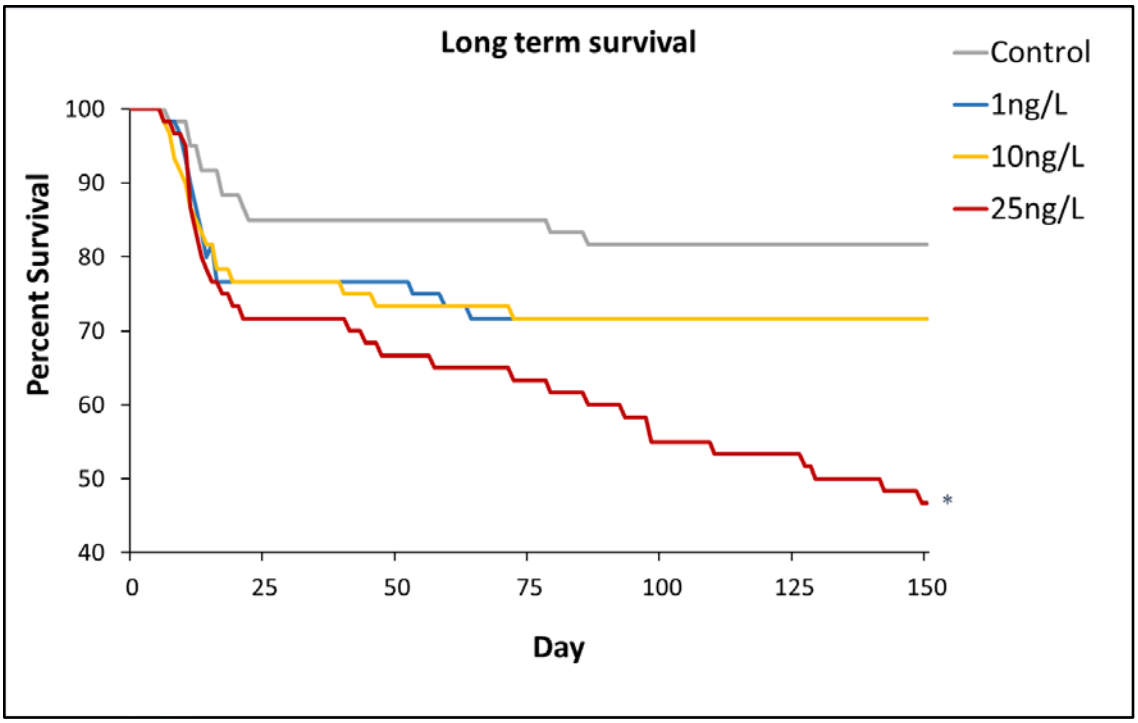
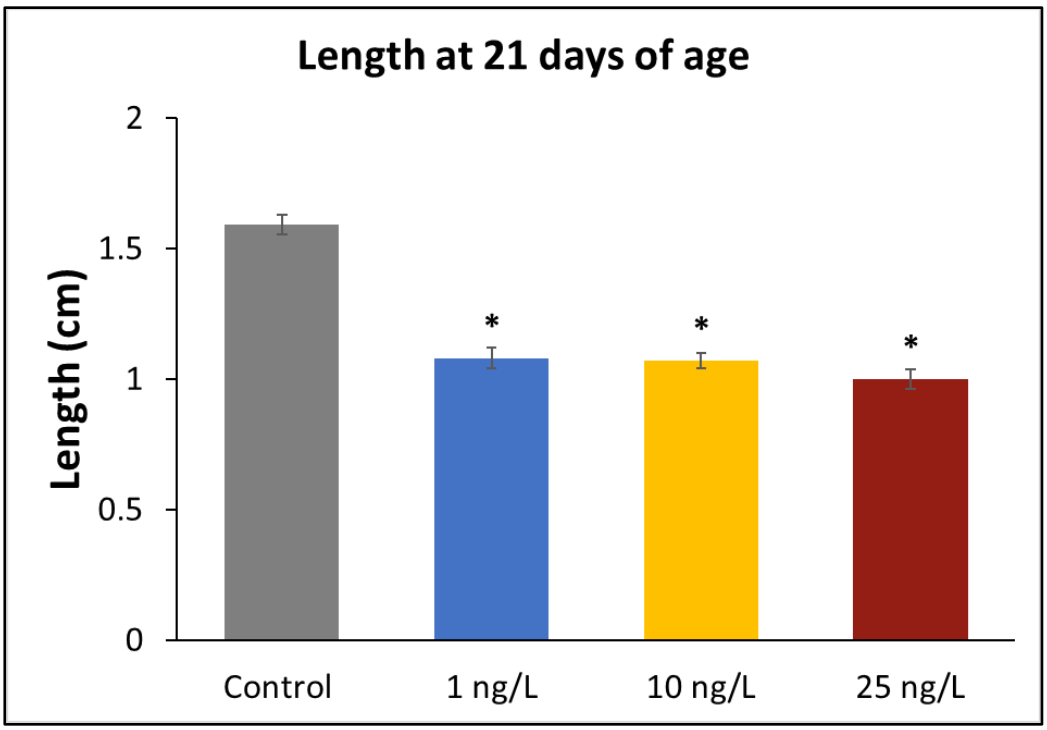


Figure 4.2. Survival curve of zebrafish exposed to EE2 concentrations of 1 ng/L, 10 ng/L and 25 ng/L for five months. n=60 per group. * indicates significant difference from control, $p<0.0001$.

Length and weight of all three exposure groups at 21 days of age was significantly reduced (Figure 4.3). Length of the control group was 1.59 cm, while the 1 ng/L group was 1.08 cm, 10 ng/L group was 1.07 cm, and 25 ng/L group was 1.00 cm. Weight of the control group was 0.060 g, while the 1 ng/L group was 0.014 g, 10 ng/L group was 0.013 g, and 25 ng/L group was 0.012 g. By five months, only the length and weight of the 25 ng/L EE2 exposure group was significantly reduced, as compared to control (Figure 4.4). Length of the control group was 3.08 cm, while the 1 ng/L group was 3.00 cm, 10 ng/L group was 2.97 cm, and 25 ng/L group was 2.42 cm. Weight of the control group was 0.30 g, while the 1 ng/L group was 0.28 g, 10 ng/L group was 0.24 g, and 25 ng/L group was 0.21 g.

A



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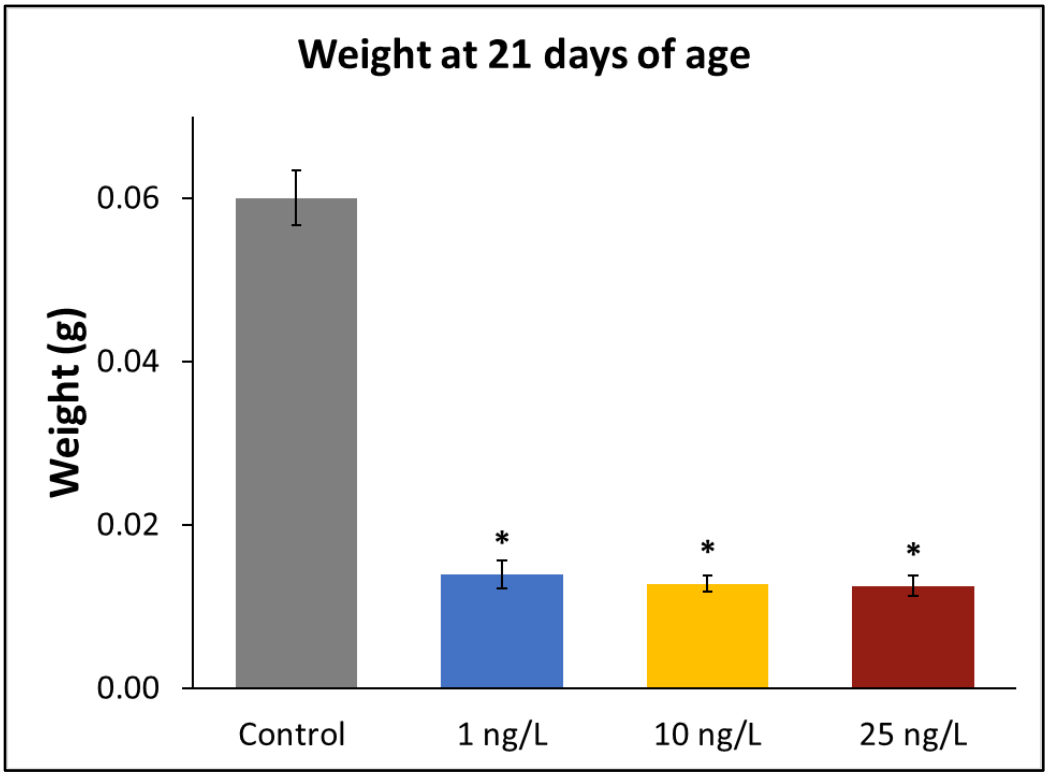
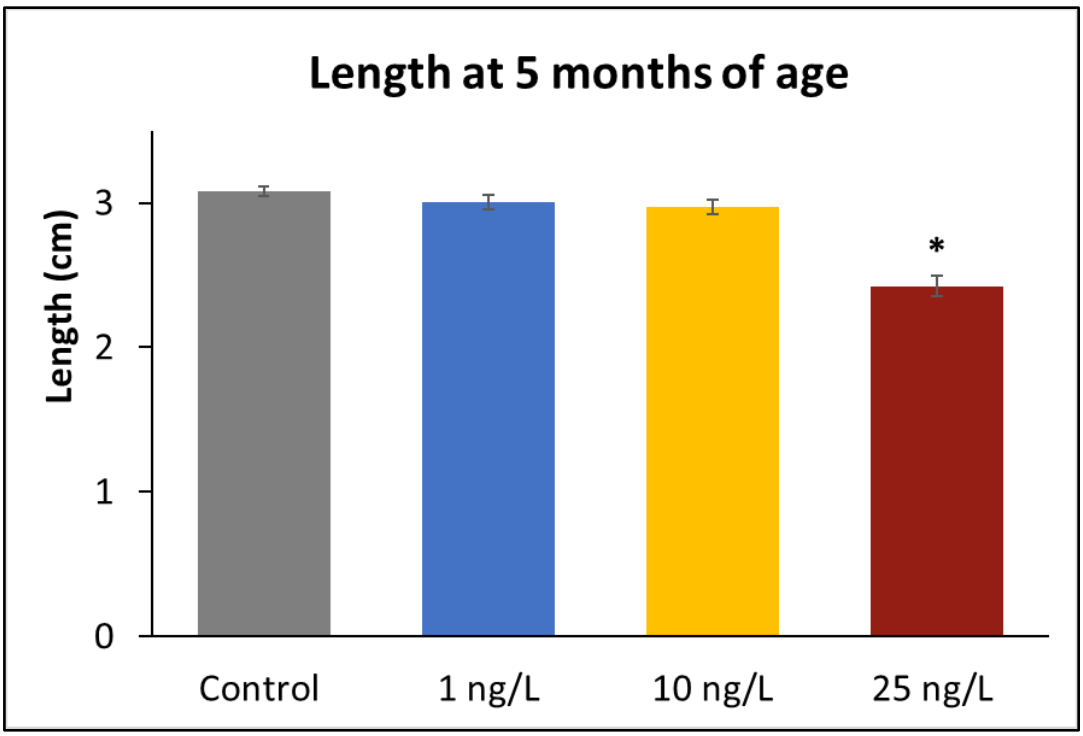


Figure 4.3. Growth of zebrafish at 21 days of age exposed to EE2 concentrations of 1 ng/L, 10 ng/L and 25 ng/L EE2. n=60 per group. * indicates significant difference from control, $p<0.05$ A) Mean length ± SEM B) Mean weight ± SEM.

A



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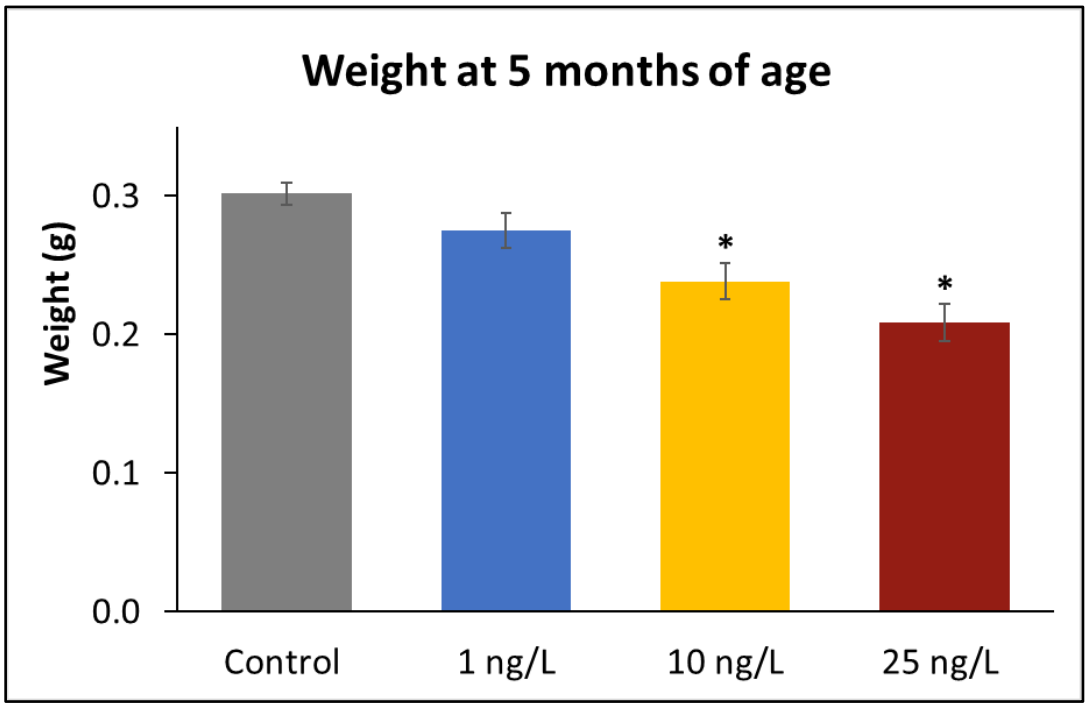

Figure 4.4. Growth of zebrafish at five months of age, exposed to EE2 concentrations of 1 ng/L, 10 ng/L, and 25 ng/L EE2. n=60 per group. * indicates significant difference from control, $p<0.05$ A) Mean length ± SEM B) Mean weight ± SEM.

At five months, the 10 ng/L EE2 group exhibited a significantly lower condition factor than the control group (0.90 g/cm^3 compared to 1.04 g/cm^3, respectively), while the 25 ng/L EE2 group had a significantly higher condition factor (1.49 g/cm^3) than the control group (Figure 4.5). Exposure to 1 ng/L EE2 had no significant effect on the condition factor of zebrafish (0.98 g/cm^3).

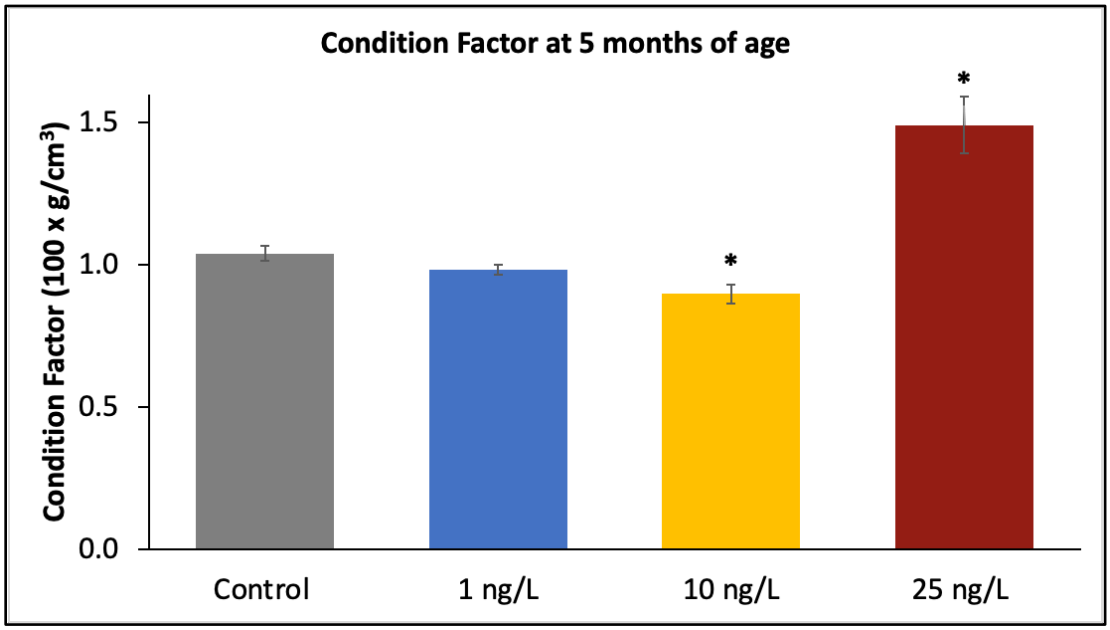

Figure 4.5. Condition factor of zebrafish exposed to EE2 concentrations of 1 ng/L, 10 ng/L and 25 ng/L EE2 for five months. n=60 per exposure group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

Multi-Generational Exposure to 1 ng/L EE2

Exposure to EE2 significantly decreased survival during the first 21 days for all three generations (Figure 4.6). Survival rate for the generation 1 exposure group was 77.33% (as compared to 87.33% in control), for generation 2 was 77.42% (as compared to 92.09% in control), and for generation 3 was 76% (as compared to 81.11% in control). There was no significant difference between long-term survival of control and 1 ng/L EE2 exposure groups in any generation (data not shown).

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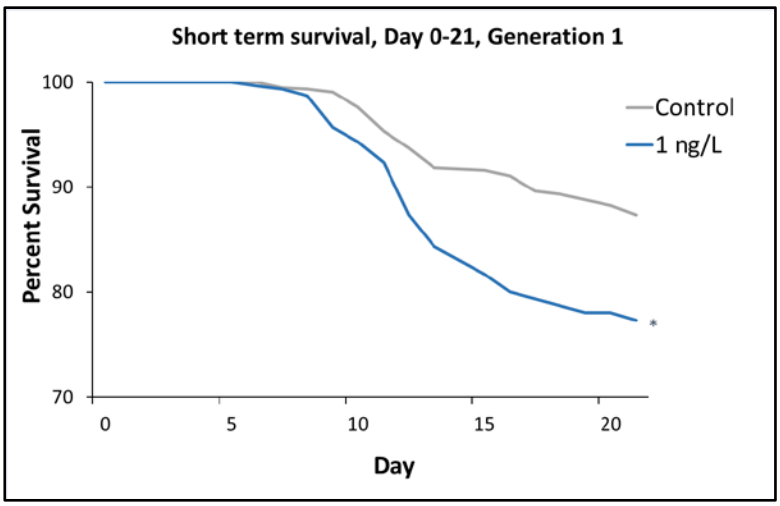

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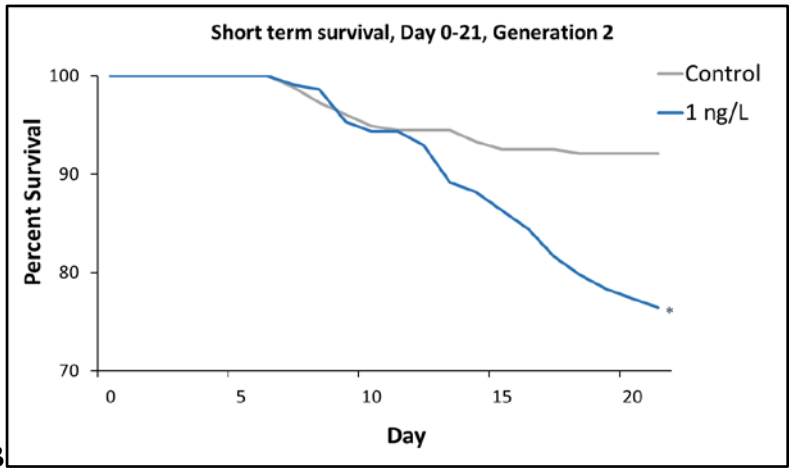

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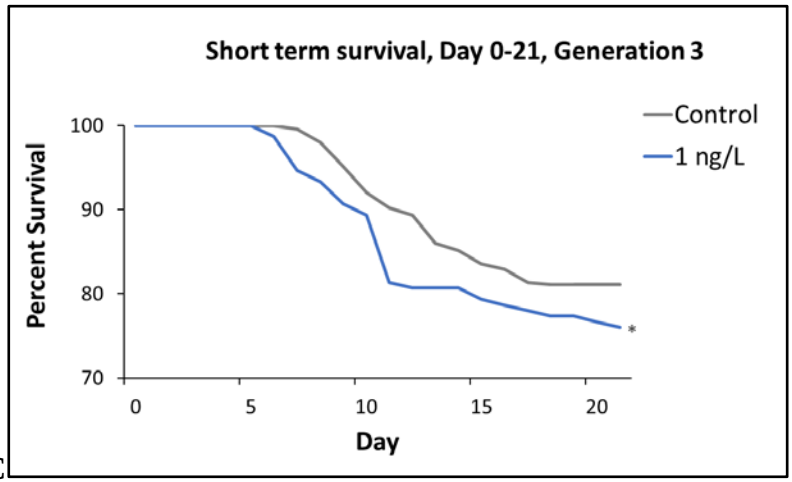


Figure 4.6. Survival curve of zebrafish exposed to EE2 concentration of 1 ng/L for the first 21 days of development, for three generations. * indicates significant difference from control, $p<0.05$. A) Generation 1 (n=750 for control group, n=300 for exposure group). B) Generation 2 (n=300 per group) C) Generation 3 (n=450 for control group, n=150 for exposure group).

Exposure to 1 ng/L EE2 for five months resulted in a significantly increased length and weight of the second generation of zebrafish (Table 4.1). By the third generation of exposure, there was no difference between the exposure group and the control group. Exposure to 1 ng/L EE2 had no significant effect on the condition factor of zebrafish for three generations, as compared to the control.

Table 4.1. Weight, length, and condition factor of zebrafish exposed to EE2 concentrations of 1 ng/L for three generations. n=60 per exposure group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

	Weight (g)		**Length (cm)**		**Condition factor (100 x g/cm^3)**	
Exposure	Control	1ng/L EE2	Control	1ng/L EE2	Control	1ng/L EE2
Generation 1	0.30 ± 0.01	0.27 ± 0.01	3.08 ± 0.04	3.00 ± 0.05	1.04 ± 0.03	0.98 ± 0.02
Generation 2	0.24 ± 0.01	0.28 ± 0.01*****	2.99 ± 0.03	3.16 ± 0.03*****	0.88 ± 0.01	0.91 ± 0.02
Generation 3	0.19 ± 0.01	0.19 ± 0.01	2.81 ± 0.03	2.79 ± 0.03	0.84 ± 0.01	0.88 ± 0.01

AB, TU, and WIK Strain-Specific Effects of EE2 Exposure

When comparing strain differences between exposure groups there was no difference in the day 0-21 survival between the AB and WIK strains in any of the groups. However, the TU strain experienced the lowest survival rate in all groups, including control (Figure 4.7). Furthermore, in the 25 ng/L exposure group, the TU strain experienced the greatest decrease in survival as compared to control (a 24% decrease), while the AB and WIK strains both experienced an 8% decrease as compared to control.

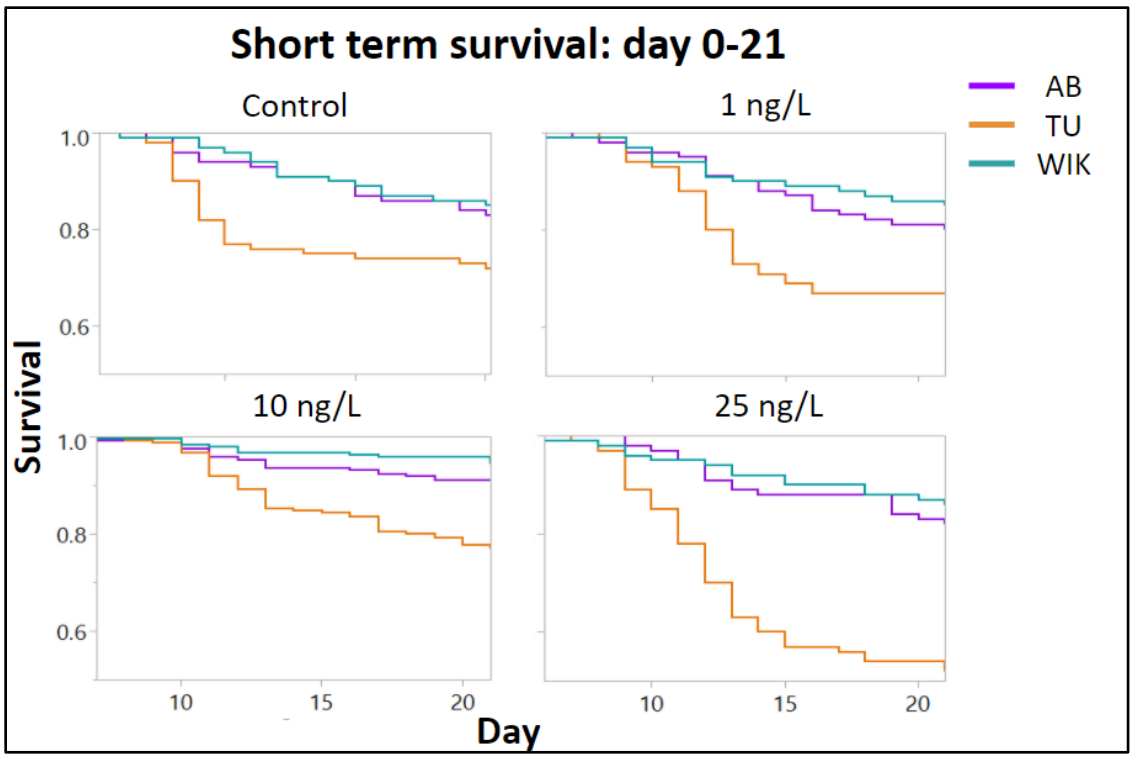


Figure 4.7. Survival curves of zebrafish, day 0-21, for control group and groups exposed to EE2 concentrations of 1 ng/L, 10 ng/L, and 25 ng/L EE2, separated by strain. n=250 per strain for control group, n=100 per strain per exposure group. No significant difference from control.

There was no difference in long-term survival between the three strains in the control, 1 ng/L, and 10 ng/L EE2 exposure groups (data not shown). In the 25ng/L EE2 exposure group, the TU strain experienced the lowest survival rate (33%), followed by AB (42%), and WIK (64%) (Figure 4.8).

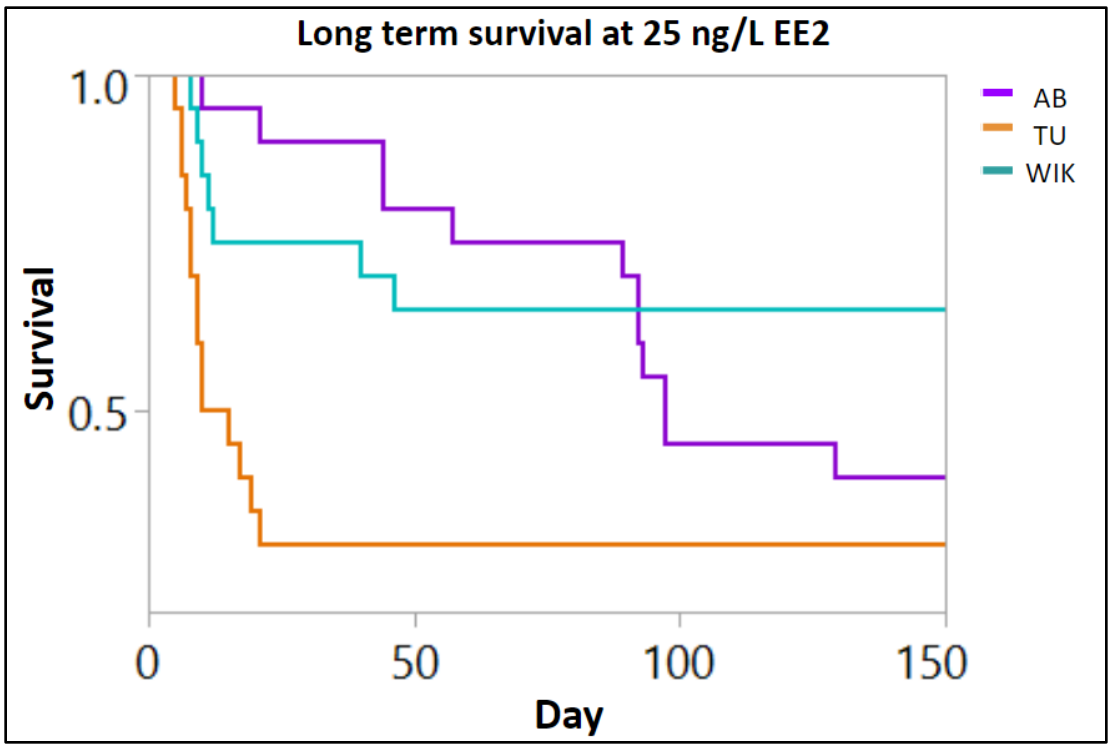


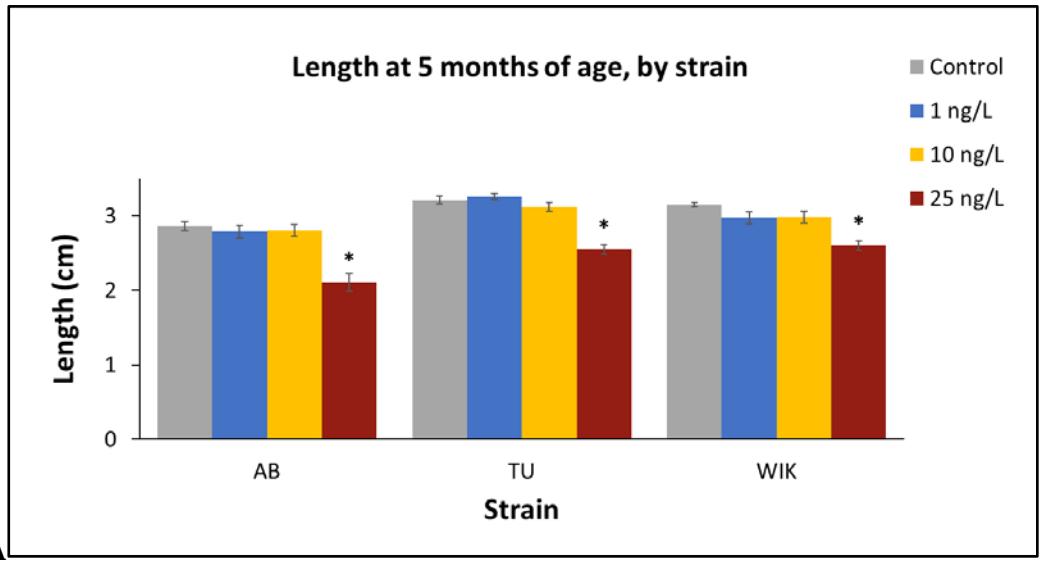
Figure 4.8. Survival curve of zebrafish exposed to 25 ng/L EE2 for five months, separated by strain. n=20 per strain.

When exposed to 1 ng/L EE2, all three strains experienced a significant decrease in survival during Generation 1. During Generation 2, only the TU and WIK strains experienced a significant decrease in survival. During Generation 3, the TU control group experienced a significant decrease in survival (Table 4.2).

Table 4.1. Survival rates for the first 21 days of development of zebrafish exposed to 1 ng/l EE2 for three generations, separated by strain. Generation 1 – control, n=250 per strain; 1 ng/L, n=100 per strain. Generation 2 - n=100 per strain, per group. Generation 3 – control, n=150 per strain; 1 ng/L, n=50 per strain. * indicates significant difference from control, $p<0.05$.

	Generation 1		**Generation 2**		**Generation 3**	
Strain	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*
AB	90.8%	80.2%*	90.4%	82.9%	84.0%	81.0%
TU	76.9%	67.0%*	91.0%	80.6%*	47.0%	94.0%*
WIK	94.4%	85.0%*	95.7%	64.5%*	84.0%	89.0%

At five months, length and weight of all three strains was significantly decreased in the 25 ng/L EE2 exposure group, as compared to control. Furthermore, the TU and WIK strains experienced a significant decrease in weight in the 10 ng/L EE2 exposure group (Figure 4.9).



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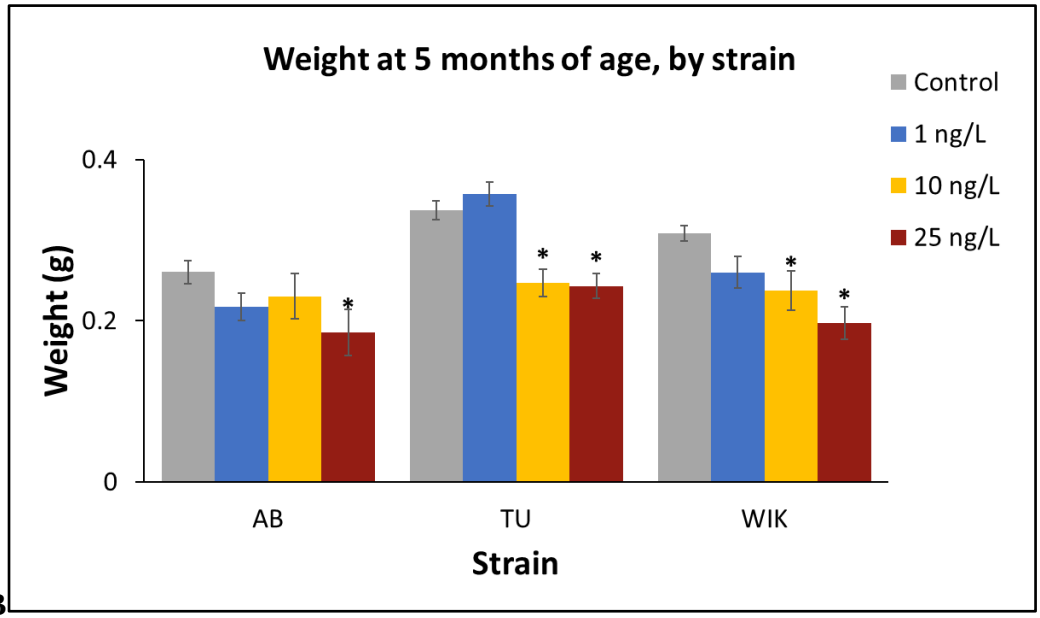

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Figure 4.9. Growth at five months of zebrafish exposed to EE2 concentrations of 1 ng/L, 10 ng/L, and 25 ng/L EE2, separated by strain. n=20 per strain, per group for control and 1 ng/L EE2 groups; n=10 per strain, per group for 10 ng/L and 25 ng/L EE2 groups. *indicates significant difference from control, $p<0.05$ A) Mean length ± SEM B) Mean weight ± SEM.

Mean body length did not differ between control and exposure groups for any generation in the AB and TU strains. The WIK strain experienced a significant increase in length during Generation 2 (Table 4.3).

Table 4.2. Body length (cm) of zebrafish exposed to 1 ng/L EE2 for three life cycles, separated by strain. n=20 per strain, per exposure group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

	Generation 1		**Generation 2**		**Generation 3**	
Strain	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*
AB	2.86 ± 0.06	2.79 ± 0.08	2.91 ± 0.06	3.07 ± 0.06	2.64 ± 0.04	2.68 ± 0.07
TU	3.21 ± 0.05	3.26 ± 0.04	3.11 ± 0.04	3.16 ± 0.03	2.96 ± 0.03	2.91 ± 0.03
WIK	3.15 ± 0.03	2.97 ± 0.08	2.97 ± 0.06	3.24 ± 0.04*	2.86 ± 0.05	2.78 ± 0.04

Mean body weight did not differ between control and exposure groups for any generation in the AB and TU strains. The WIK strain experienced a significant increase in weight during Generation 2 (Table 4.4).

Table 4.3. Body weight (g) of zebrafish exposed to 1 ng/L EE2 for three life cycles, separated by strain. n=20 per strain, per exposure group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

	Generation 1		**Generation 2**		**Generation 3**	
Strain	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*
AB	0.26 + 0.01	0.22 ± 0.02	0.24 ± 0.02	0.29 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
TU	0.34 ± 0.01	0.36 ± 0.02	0.25 ± 0.01	0.26 ± 0.01	0.22 ± 0.01	0.21 ± 0.01
WIK	0.31 ± 0.01	0.26 ± 0.02	0.24 ± 0.02	0.31 ± 0.01*	0.19 ± 0.01	0.19 ± 0.01

Discussion

In this study, we conducted full life-cycle exposures of three environmentally relevant concentrations of EE2: 1 ng/L, 10 ng/L, and 25 ng/L. Furthermore, we looked at the effects of three generations of 1 ng/L EE2 full life-cycle exposure, as well as strain-specific effects of these exposures. Our findings show that exposure to 25 ng/L EE2 adversely affected the short and long-term survival, growth, and development of

zebrafish, while multi-generational low dose exposure (1 ng/L EE2) had no significant long-term effects on these parameters. Strain-specific effects were observed with the TU and WIK strains; both strains experienced a decrease in weight after five months of exposure to 10 ng/L EE2, while the AB strain was unaffected.

Survival

We observed a significant decrease in long-term survival in zebrafish exposed to 25 ng/L EE2. Mortality rates in the 1 ng/L and 10 ng/L EE2 exposure groups did not differ from the control group. Previous studies support these results. After 60 days (Hill and Janz, 2002), 90 days (Van den Belt et al., 2003), and 177 days (Shäfers et al., 2007) of exposure to 1 ng/L EE2 and 10ng/L EE2, there was no significant difference in survival of exposure groups, as compared to control.

While multi-generational low dose exposure (1 ng/L EE2) had no significant long-term effect on mortality, it did reduce the survival of exposed zebrafish during the first 21 days of development in all three generations. An increase in mortality between 8-15 dpf is often observed in zebrafish, due to starvation after the yolk has been completely absorbed (Strähle et al., 2012). Larvae that are unable to switch to exogenous feeding do not survive. While we observed this trend in our results, there was an increased rate of mortality as compared to the control for all three generations exposed to EE2. This suggests that EE2 exposure as low as 1 ng/L significantly impacts the survival of zebrafish during an already vulnerable time-period.

While we did not observe strain-specific survival responses during the first 21 days of development, the TU strain consistently had the lowest short-term survival rate for all groups, including control. The TU strain also had the highest rate of long-term mortality after exposure to 25 ng/L EE2, as compared to the AB and WIK strains. This suggests that the TU strain may generally have lower survival rates than the AB and WIK strains, independent of EE2 exposure.

Growth and Development

In our study, the weight and length of zebrafish exposed to 10 ng/L and 25 ng/L EE2 for five months were significantly lower than those of the control. These findings are supported by previous studies, where zebrafish exposed to 10 ng/L EE2 for 60 (Hill and Janz, 2003) and 75 days (Shäfers et al., 2007), as well and 25 ng/L EE2 for 90 days (Van den Belt et al., 2003) experienced a reduction in body length. The 25 ng/L EE2 exposure group had a significantly higher condition factor (K) as compared to the control group, which can be explained by interpreting condition factor: a larger K value translates to a thicker body. While K is normally used as an indication of health (a thicker body translates to a healthier, more robust fish), in this case it was an indication of pericardial edema. Pericardial edema was observed primarily in the AB and TU strains of zebrafish, and began developing within two months (Figure 4.10). Pericardial edema has been observed in other EE2 exposure studies: after 90 days of exposure to 25 ng/L EE2, 17% of zebrafish exhibited pericardial edema, and 51% exhibited lordosis and/or scoliosis (Van den Belt et al., 2003). After 180 days of exposure, edema in the body

cavity and bulging eye were observed. The development of pericardial edema in response to xenobiotic exposure or environmental stress is a known response, and has been seen in carp (*Cyprinus carpio*) exposed to the endocrine disrupting PCB 126 (Stouthart et al., 1998), as well as European minnows (*Phoxinus phoxinus*) exposed to fungicide triphenyltin chloride (Fent and Meier, 1994). Therefore, we consider these observations to be a general EDC toxicity response, not specifically related to the estrogenic action of EE2.

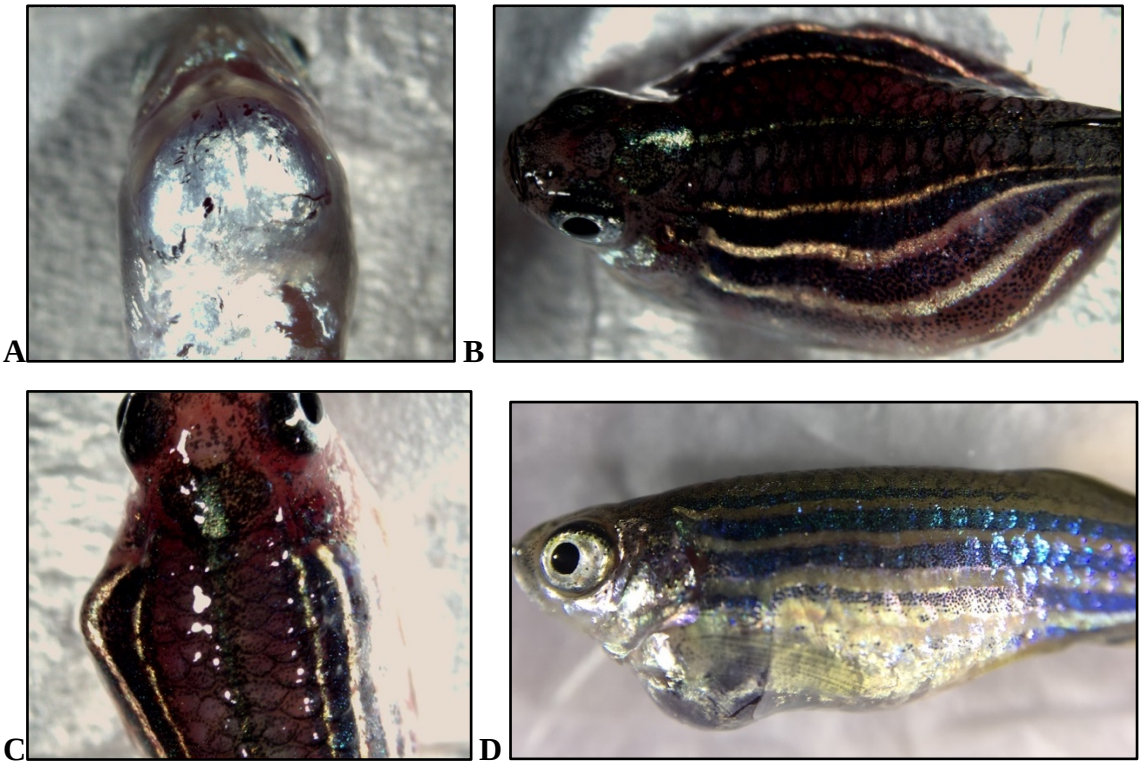

Figure 4.10. Abnormal physiology observed in fish exposed to EE2 for five months A) 25 ng/L EE2 exposure, AB strain: pericardial edema B) 25 ng/L EE2 exposure, AB strain: curved spine, water retention, and pericardial edema C) 25 ng/L EE2 exposure, AB strain: tumor D) 10 ng/L EE2 exposure, TU strain: pericardial edema.

Zebrafish exposed to low dose EE2 (1 ng/L) for multiple generations experienced an increase in length and weight during the Generation 2. A previous study showed that

the second generation of males exposed to 1 ng/L EE2 experienced a significant increase in body length, but no other parameters were affected (Soares et al., 2009).

Strain-specific growth effects occurred, both dose-dependent and multi-generational. The TU and WIK strains experienced a decrease in weight after exposure to 10 ng/L EE2, while the AB strain was unaffected. Furthermore, the WIK strain of Generation 2 exposure to 1 ng/L EE2 experienced an increase in length and weight, while the other strains were unaffected. This suggests that the TU and WIK strains are more susceptible to morphological responses to high concentrations of EE2 exposure, while the WIK strain specifically displays a more varied morphological response.

Conclusion

These findings together suggest that full life-cycle exposure to 25 ng/L EE2 has a severe detrimental effect on the survival, growth, and development of zebrafish. Chronic, low dose exposure (1 ng/L EE2) also appears to have a wide range of sublethal effects, in particular a negative impact on the survival of zebrafish during the vulnerable early life stage period. Additionally, the TU stain of zebrafish appears to exhibit greater sensitivity to EE2 exposure at higher concentrations when considering survival and growth as endpoints, while the WIK strain exhibits a more varied morphological response to EE2 exposure. Further research is needed into the underlying mechanisms of sublethal effects caused by chronic, low dose exposure to EE2, as this most accurately represents the experience of fish species in the wild.

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Chapter 5 : 17α-ethynylestradiol Exposure Effects on Reproduction

Abstract

In this chapter, the impact of 17α-ethynylestradiol (EE2) on reproduction in zebrafish was evaluated using female egg production (clutch size), embryo hatch success, and sperm cell production as endpoints. Three strains of zebrafish (AB, TU, and WIK) were exposed to solvent control or EE2 at 1ng/L, 10ng/L, and 25 ng/L for five months, as well as 1 ng/L EE2 over the course of three generations. Zebrafish exposed to 10 ng/L and 25 ng/L EE2 experienced total reproductive failure. Zebrafish exposed to 1 ng/L EE2 experienced a statistically significant increase in clutch size during the first and second generation, but a significant decrease in hatch success in all three generations, resulting in an overall reduction in reproductive success. Furthermore, the TU strain of zebrafish experienced the greatest variability in response to low dose EE2 exposure; the second generation experienced the greatest increase in clutch size, and the second and third generation experienced the lowest hatch success. These findings together suggest that chronic, low dose (1 ng/L) exposure to EE2 stimulates parental fecundity, but decreases both short-term and generational fertility. Overall, multi-generational exposure to low dose EE2 reduces reproductive success in zebrafish.

Introduction

Endocrine disrupting compounds (EDCs) are known to interfere with the sexual differentiation, development, and reproduction of vertebrates. Previous studies have found evidence that EDCs alter sexual differentiation and negatively impact reproductive

capabilities of a wide array of species. Dogwhelk sea snails (*Nucella lapillus*) exposed to tributyltin, a compound found in anti-fouling paint applied to the hulls of boats and ships, experienced a reduction in reproductive success due to masculinization of females (Gibbs et al., 1991). Studies on reproductive impairment caused by EDCs are often conducted on fish species, as they can experience full life-cycle exposure in polluted aquatic environments. Wild roach (*Rutilus rutilus*) exposed to sewage treatment work effluents containing a complex mixture of EDCs experienced disruption in gonadal development and altered gamete production (Jobling et al., 2002); white perch (*Morone americana*) collected from lakes containing effluent runoff from treated domestic sewage containing EDCs experienced a high prevalence of gonadal intersex individuals (Kavanagh et al., 2004); eelpout (*Zoarces viviparus*) collected along a coast containing pulp mill effluent experienced male-biased sex ratios (Larsson et al., 2000). Lab based research has shown that guppies (*Poecilia reticulata*) exposed to low levels of tributyltin and bisphenol A (BPA) experienced reduced sperm counts (Haubruge et al., 2000). Although examples of detrimental effects in fish species dominate the literature, effects in terrestrial vertebrates like the Italian wall lizard (*Podarcis sicula*) (Verderame and Limatola, 2015) and Long-Evans rats (Akingbemi et al., 2004) are similar to the EDC exposure effects seen in aquatic vertebrates and invertebrates.

Zebrafish are a model organism often chosen for toxicology studies on reproductive outcomes, as their process of sex determination is affected by genetic factors and can be secondarily influenced by environmental factors like xenoestrogens (Santos et al., 2017). During gonad differentiation, zebrafish initially develop immature

ovarian tissue regardless of genetic sex (i.e. juvenile hermaphroditism), prior to differentiation into mature ovaries or testes (Takahashi, 1977). This unique method of sexual development makes zebrafish a well-suited model for evaluating the effects of EDCs like environmental estrogens during the critical transition from immature ovarian tissue to ovary, or degeneration and development of testes. While the mechanisms controlling zebrafish gonad differentiation and sex determination remain complex and not fully understood (Liew and Orban, 2013), the hypothesis has been made that synthetic estrogens like EE2 can disrupt reproductive and developmental functions by mimicking the effects of endogenous estrogen. Past studies have shown that short-term EE2 exposure in zebrafish can induce both short- and long-term impacts on zebrafish development and reproductive function (Fenske et al., 2005).

One major gap in our interpretation of environmental toxicant exposure effects is full life-time exposures starting at fertilization and continuing through sexual maturity, as well as the effects of multi-generational exposure. The present study goes beyond our current understanding of short-term EE2 exposure responses in zebrafish. To assess these effects in our long-term, multi-generational exposure trials we evaluated female egg production (clutch size), embryo hatch success, and sperm cell production. Additionally, this study addresses strain-specific responses to EE2 exposure, utilizing AB, TU, and WIK strains of zebrafish, which differ in their initial method of establishment, course of selective breeding, and genetic background. Although laboratories utilize different strains of zebrafish in their studies, strain-specific responses are an area of research that has

received little attention, but could provide useful information on the genetic variability of exposure response.

Materials and Methods

Spawning and Embryo Collection

Ten spawning pairs per strain were set up in the evening in Aquaneering Crossing Tanks with dividers separating each individual of the spawning pair. The following morning, dividers were removed within one hour of the beginning of the daily light cycle (as zebrafish are dawn spawners) and zebrafish were allowed up to five hours to complete spawning. Embryos were collected and rinsed twice in embryo media using a metal sieve, and then transferred to sterile petri dishes at a density of less than 50 eggs per dish. Non-viable eggs were removed at 24 and 48 hours, and embryo media was changed at 48 hours. Hatching occurred between 3-5 days post fertilization (dpf). At five dpf, zebrafish fry were transferred to 1000ml beakers containing 250ml embryo media, and the number of successfully hatched embryos was recorded. Effect endpoints included onset of spawning, number of eggs spawned per female (clutch size), viability of clutch (successful fertilization), and embryo hatch success.

Reproductive Parameters

At 3.5 months of age, randomly chosen spawning pairs were set up on a weekly basis until successful spawning occurred. All reproduction parameters are presented as the mean of multiple trials for each treatment and endpoint. For a trial to be counted, the

spawning pair must have produced at least one viable embryo (i.e. the egg was successfully fertilized, able to develop, and hatched). The following equations were used to calculate reproduction parameters per exposure group:

$$\text{Clutch size} = \frac{\text{total number of collected eggs per spawning pair}}{\text{number of spawning pairs}}$$

$$\text{Embryo hatch success} = \frac{\text{cumulative hatched larvae on each post fertilization day}}{\text{total number of eggs}}$$

Quantification of Spermatogenesis

Zebrafish were euthanized at five months of age, and testes were removed using dissecting needles under a stereoscopic microscope (Leica Microsystems). Testes were placed in 10X volume of 10% neutral buffered formalin for 48 hours, then rinsed with phosphate buffered saline. Individual testes were placed in a histology cassette and preserved in 70% ethyl alcohol at 35°C. Tissue preparation was carried out by the OHSU Histopathology Shared Resource in Portland, Oregon. Testes were embedded in paraffin wax, sliced, and stained with Hematoxylin and Eosin (H&E). Each finished slide contained three slices of testes from one zebrafish.

Using a Leica DM IRB Inverted Microscope at a magnification of 1000X, one digital image was taken from each of the three tissue slices per slide. The following criteria were used when selecting which part of tissue sample to capture: (1) each image should be taken from a different part of the three different tissue sections to avoid overlap (2) each image should contain three full cysts of cells (3) at least one of each cell type

that is being counted should be present in the image (4) damaged/torn tissue should be avoided as much as possible (5) image should be completely filled with tissue.

To quantify spermatogenesis, the number of spermatogonia, spermatocytes, spermatids, and spermatozoa were counted per image. This was achieved using ImageJ: each cell was marked with a dot, the color of the dot designating what phase of spermatogenesis the cell was in. After the full image had been marked, the image would be adjusted with a color threshold. This would make a specific color turn black while making the rest of the image become white. A particle counter was then used to count the dots on the black and white image, which would provide the cell count for that specific sperm cell type. This process was then repeated for each color used to mark a sperm cell type on the original image (Figure 5.1). Excel was used to record cell counts.

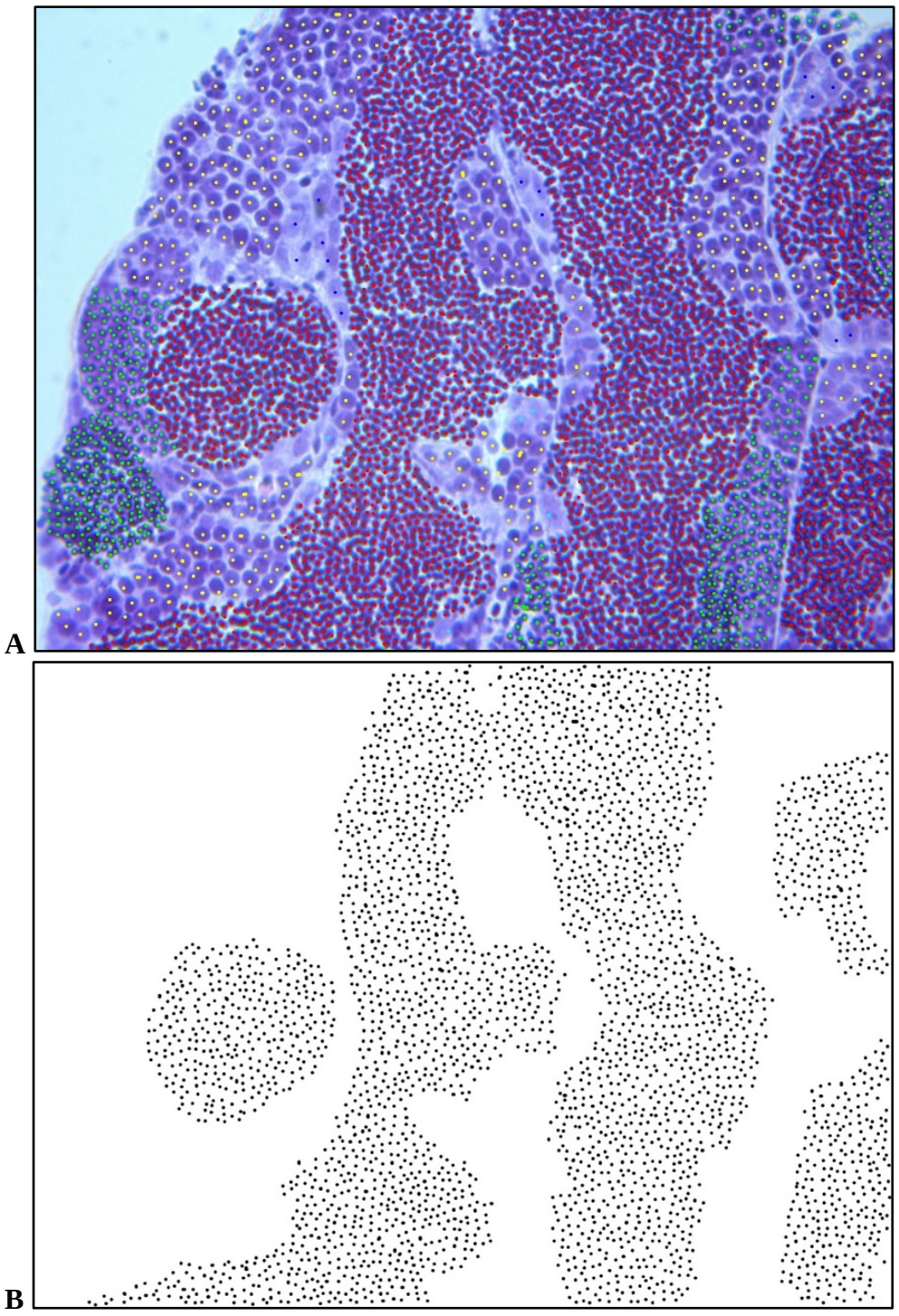

Figure 5.1. Example images of zebrafish testes used to quantify spermatogenesis A) H&E stained testes identifying spermatogonia (blue), spermatocytes (yellow), spermatids (green), and spermatozoa (red) B) Color threshold adjusted image used by particle counter, indicating spermatozoa.

Statistical Analysis

Statistical analysis was performed with JMP Pro 14 software. All data were examined for homogeneity and normality using Levene's and Kolmogorov-Smirnov tests. If these assumptions were met, one-way analysis of variance (ANOVA) followed by a Tukey's test was utilized to identify differences between each exposure group and the control. If the homogeneity and normality assumptions were not met, the nonparametric Kruskal-Wallis test followed by multiple comparison was performed. For multi-generational data, t-tests were performed to identify differences between exposure groups and control. All values presented are mean ± SEM. The significance level for all the statistical analyses was set at $p<0.05$.

Results

Exposure to 10 ng/L and 25 ng/L EE2

Zebrafish exposed to 10 ng/L and 25 ng/L EE2 exhibited complete reproductive failure and were unable to spawn. This eliminated multi-generational analysis at these exposure concentrations. Spawning trials began at 3.5 months, and continued until eight months of exposure, with no viable eggs produced.

Multi-Generational Exposure to 1 ng/L EE2

The clutch size of the first generation of zebrafish exposed to 1 ng/L EE2 doubled in size as compared to control (64.21 and 31.47 eggs, respectively), while the clutch size of the second generation was nearly five times that of the control (143.8 and 29.8 eggs,

respectively). Clutch size of the third generation of exposed fish did not significantly differ from the control group (42.8 and 39.8 eggs, respectively) (Figure 5.2).

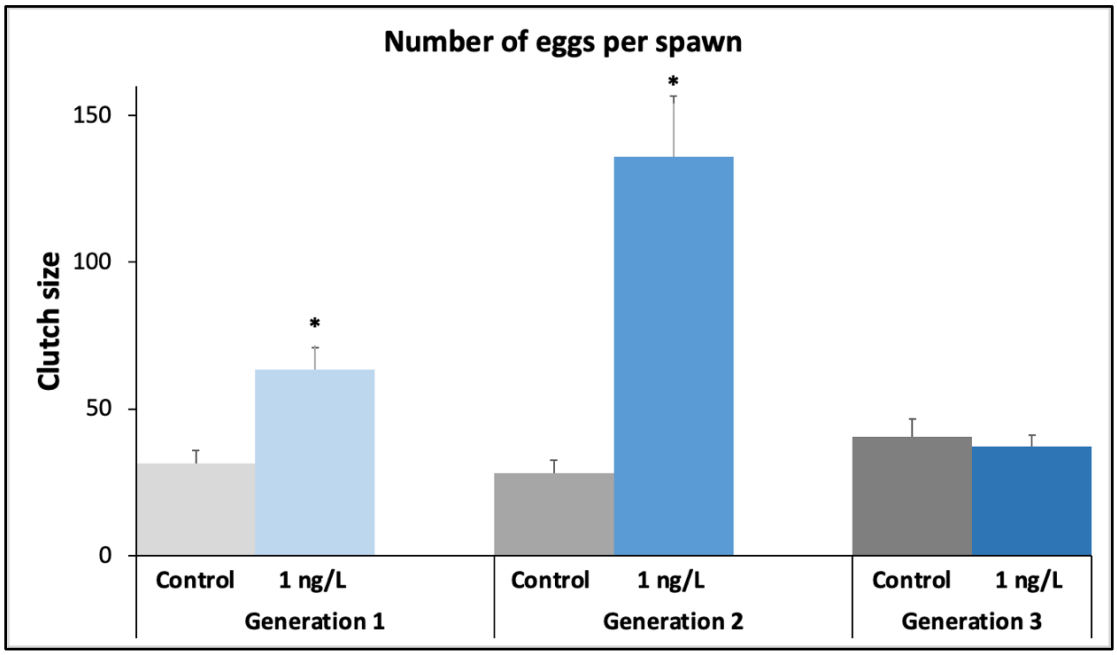

Figure 5.2. Number of eggs per clutch spawned by zebrafish after five months exposure to 1 ng/L EE2, for three generations. n=15 spawning pairs per group, one clutch per spawning pair. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

While hatch success of the control group was between 72-82% over three generations, exposure to 1 ng/L EE2 significantly reduced hatch success for all three generations to rates between 19-27% (Figure 5.3).

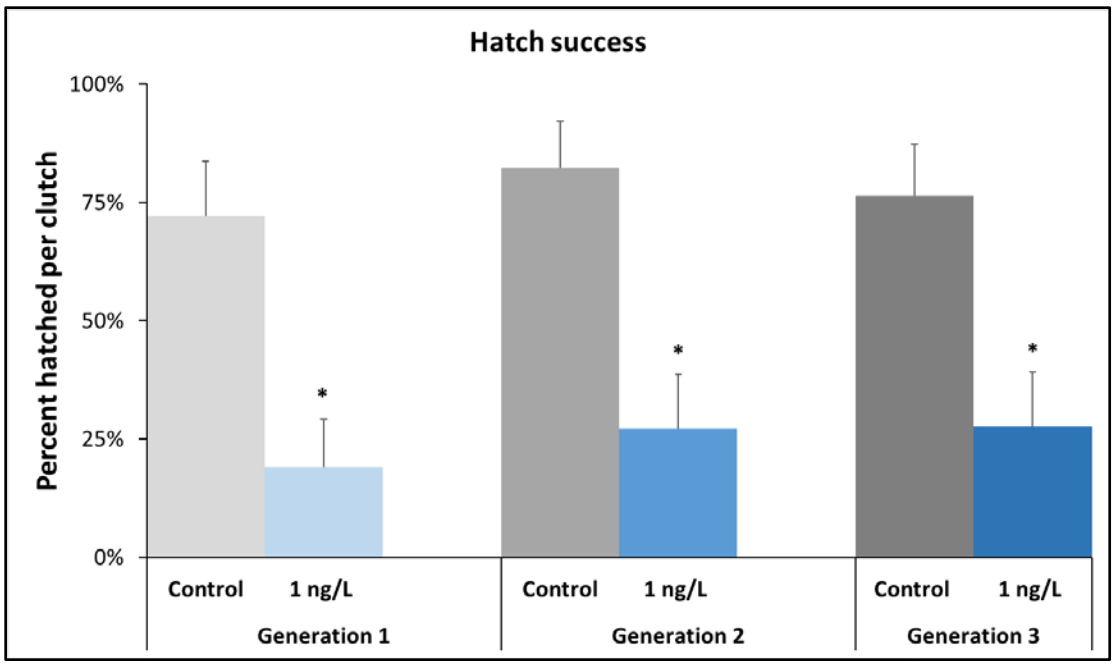

Figure 5.3. Percent of zebrafish embryos that hatched after five months of parental exposure to 1 ng/L EE2, for three generations. n=15 spawning pairs per exposure group, one clutch per spawning pair). Bars indicate SE. * indicates significant difference from control, $p<0.01$.

When clutch size and hatch success data are combined, the number of successfully hatched eggs per clutch can be calculated as a measure of reproductive success (Figure 5.4). Generation 1 and 3 exposure groups had significantly low reproductive success (12.21 and 10.86 eggs, respectively) as compared to control (22.73 and 32.66 eggs, respectively). The Generation 2 exposure group, despite the boost to clutch size, experienced similar reproductive success as control (39.12 eggs for exposure group, 24.53 eggs for control group).

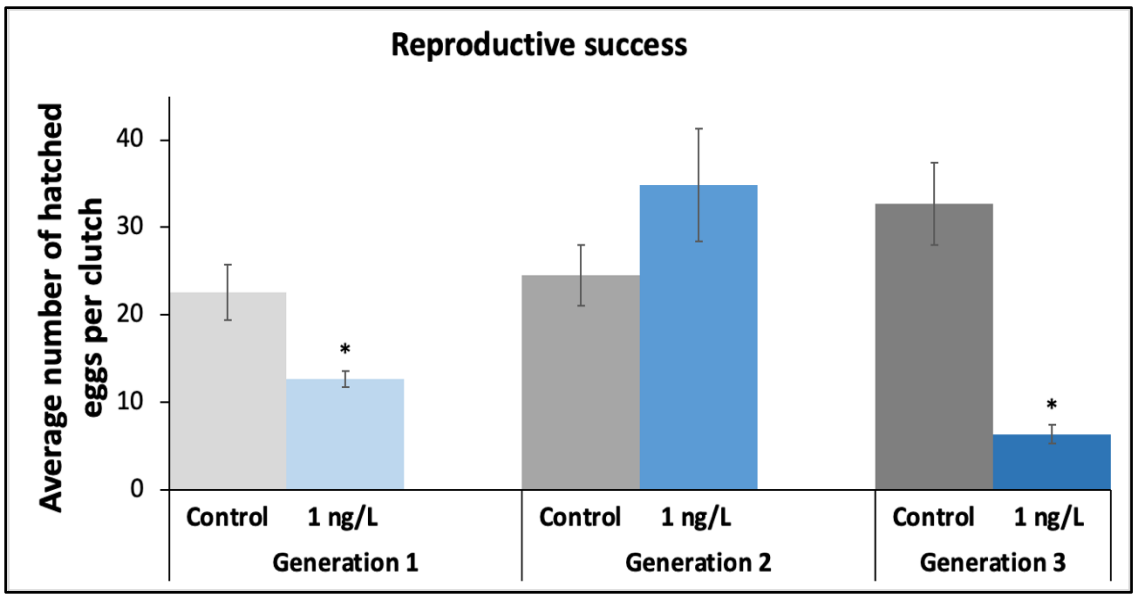

Figure 5.4. Number of successfully hatched eggs per clutch after parental exposure to 1 ng/L EE2, for three generations, n=15 spawning pairs per exposure group, one clutch per spawning pair. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

The exposure groups also experienced a delay in spawning in all three generations when compared to control, as well as a greater increase in non-viable clutches (clutches with 0% hatch success) (Table 5.1). Non-viable clutches were counted until the spawning pairs were able to produce five clutches that had at least one egg successfully hatch.

Table 5.1. Onset of spawning (first instance of successfully fertilized eggs, leading to a hatched embryo) and number of non-viable clutches (counted until five viable clutches occurred) for zebrafish exposed to 1 ng/L EE2 for five months, for three generations.

	Generation 1		**Generation 2**		**Generation 3**	
	Control	**1 ng/L EE2**	**Control**	**1 ng/L EE2**	**Control**	**1 ng/L EE2**
Onset of spawning	110 days	117 days	102 days	134 days	105 days	108 days
Non-viable clutches	3	22	0	4	0	13

The number of spermatogonia, spermatocytes, spermatids, and spermatozoa did not differ significantly between the exposure and control groups (Figure 5.5).

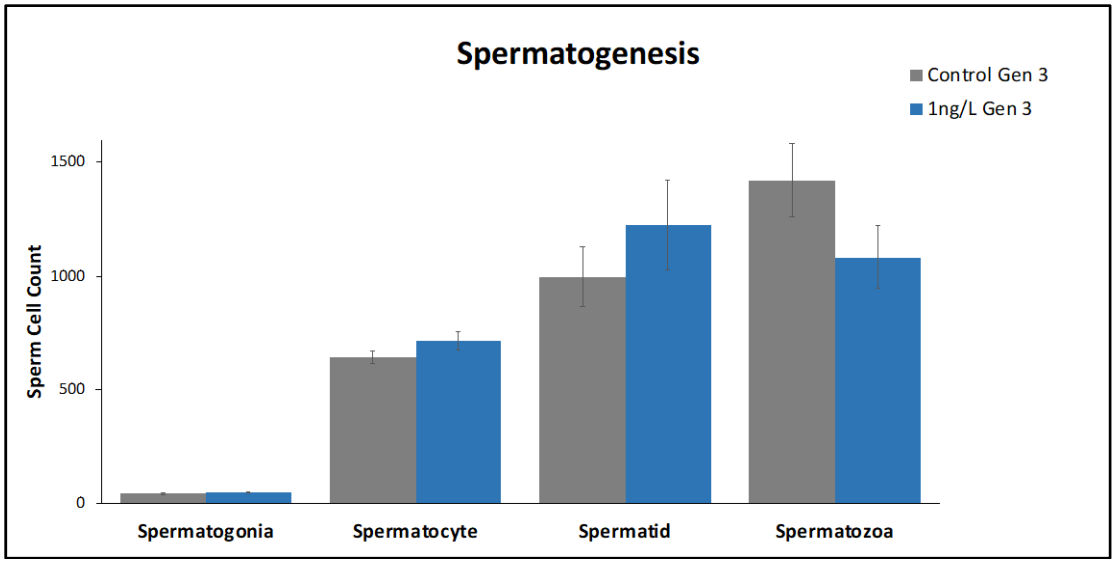

Figure 5.5. Number of zebrafish spermatogonia, spermatocytes, spermatids, and spermatozoa per observed slide, in the third generation of control group and groups exposed to 1 ng/L EE2 for five months. n=38 for control group, n=24 for exposure group. Values are mean ± SEM. There was no significant difference between control and exposure groups.

Strain-Specific Effects of EE2 Exposure

While the number of eggs per spawn did not significantly differ between the strains in each exposure group, all three strains experienced an increased clutch size in the second generation 1 ng/L EE2 exposure group, as compared to the control (Figure 5.6). The TU strain experienced the greatest increase in clutch size (198.6 eggs), as compared to AB (105.6 eggs) and WIK (127.8 eggs) strains.

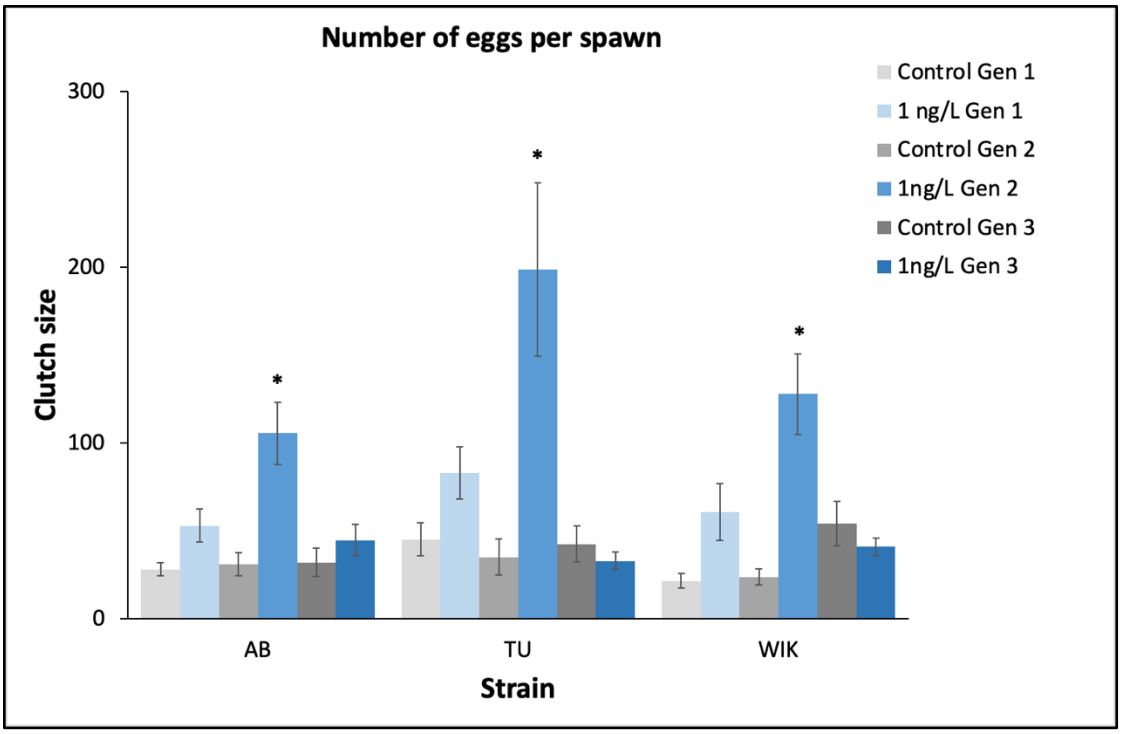

Figure 5.6. Number of eggs spawned by zebrafish exposed to 1 ng/L EE2 for three generations, separated by strain. n=5 spawning pairs per strain, per exposure group; one clutch per spawning pair. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

All three strains exposed to 1 ng/L EE2 experienced a statistically significant decrease in hatch success, in all three generations, as compared to control. In the first generation, the TU strain experienced the highest hatch success of the three strains. In the second and third generation, the TU strain experienced a lower hatch success than the AB and WIK strains (Figure 5.7).

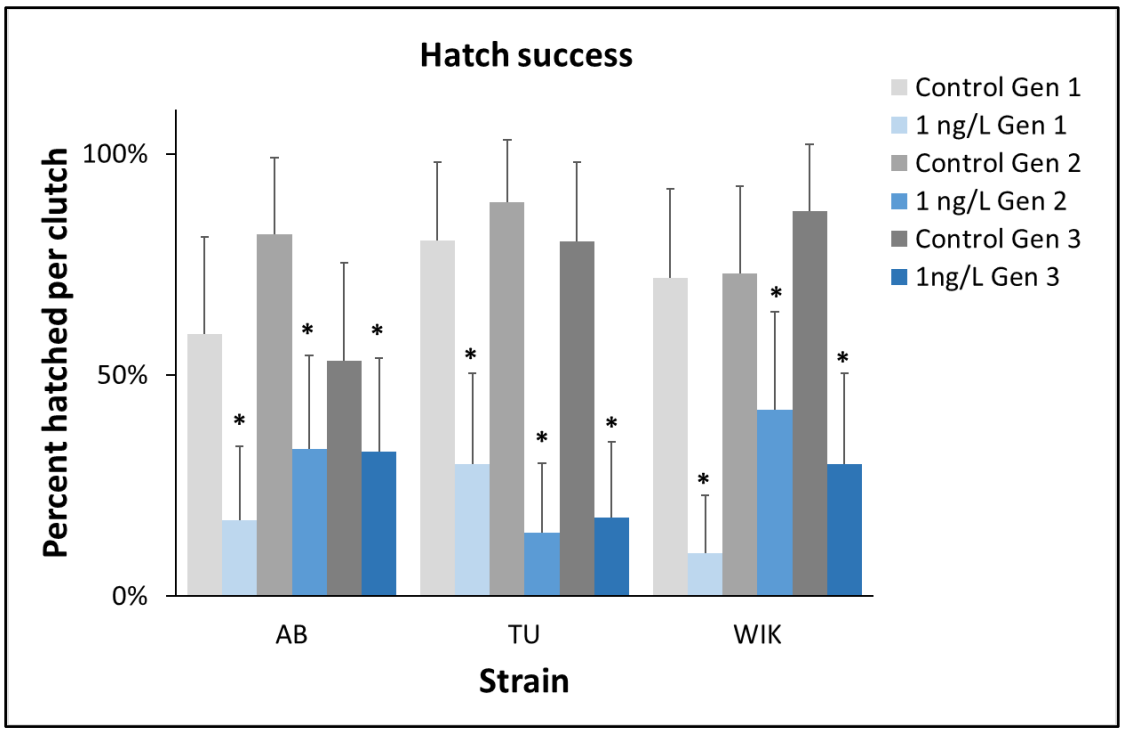

Figure 5.7. Percent of zebrafish embryos that successfully hatched after five months of parental exposure to 1 ng/L EE2, for three generations, separated by strain. n=5 spawning pairs per exposure group, per strain; one clutch per spawning pair. Bars indicate SE. * indicates significant difference from control, $p<0.05$.

Discussion

In this study, we exposed zebrafish to a low dose of EE2 (1 ng/L), over the course of three generations. Our findings suggest that even at this minimal concentration, the reproductive capabilities of zebrafish were significantly impacted. While the first two generations exposed to EE2 experienced a slight increase in clutch size, hatch success was greatly reduced in all three generations, therefore the overall reproductive success was reduced for Generation 1 and 3. The distribution of spermatogonia, spermatocytes, spermatids, and spermatozoa in testes of male zebrafish exposed to EE2 did not differ from control, suggesting that cell proliferation in the seminiferous tubules was not significantly affected by low dose exposure to 1 ng/L EE2. Furthermore, the TU strain of

zebrafish experienced the greatest variability in exposure response, suggesting more sensitivity to EE2 with regards to reproductive endpoints than the AB and WIK strains.

Female Egg Production

Laboratory zebrafish typically attain sexual maturity in the third month of 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 six months to one year of age (Nasiadka and Clark, 2012).

In this study, zebrafish exposed to 10 ng/L and 25 ng/L EE2 exhibited complete reproductive failure and were unable to spawn a second generation. This could be due to the impact EE2 has on developing gonads in zebrafish, as previous studies have shown that after 60 days of exposure to 10 ng/L EE2, 16 out of 20 zebrafish possessed undeveloped gonads, as compared to only one fish in the control group with undeveloped gonads (Hill and Janz, 2003). In a different study, after 177 days of exposure to 10 ng/L EE2, all individuals displayed gonads with ovarian morphology, but no mature ovaries - fish with testes were not found among all 27 individuals (Shäfers et al., 2007). When exposed to 25 ng/L EE2 for 90 days, 100% of zebrafish had undeveloped gonads, and there was a complete absence of spawning activity (Van den Belt et al., 2003).

Zebrafish exposed to 1 ng/L EE2 experienced a significant increase in mean number of eggs per clutch in the first two generations: a two-fold increase after one

generation, and a five-fold increase in the second generation. By the third generation, there was no significant difference between the control and exposure group. This suggests that low dose exposure (1 ng/L EE2) provides a slight, short-term fecundity boost to the parental generation, specifically with regards to clutch size, which disappears by the third generation of exposure. A similar response was observed in marine medaka (*Oryzias melastigma*), where long-term, low dose exposure to EE2 resulted in increased fecundity (egg production per female per day) (Ye et al., 2018). Furthermore, fathead minnows (*Pimephales promelas*) exposed to 0.32 and 0.96 ng/L EE2 produced more eggs in total than control fish (Parrott and Blunt, 2005). This initial boost to clutch size may be due to a compensatory mechanism in response to estrogenic stimulation, or an example of hormesis, where low dose exposure can provide beneficial effects, while higher doses lead to impairment.

The TU strain experienced the greatest increase in clutch size during Generations 1 and 2, but by the third generation the TU strain experienced the lowest clutch size. This suggests that the TU strain may have greater sensitivity in reproductive response to low dose EE2 exposure, as compared to the AB and WIK strains.

Embryo Hatch Success

While zebrafish exposed to 1 ng/L EE2 over the course of three generations may have experienced a slight increase in clutch size, the hatch success of those clutches stayed significantly low in each generation, as compared to control. This, coupled with the observation that zebrafish exposed to 1 ng/L EE2 experienced an increase in non-

viable clutches (zero eggs successfully hatched), suggests that low dose exposure to EE2 significantly impedes the ability of an embryo to develop in a way that leads to successful hatching. Similar results have been found in studies utilizing medaka (Ye et al., 2018) and fathead minnows (Parrott and Blunt, 2005). Furthermore, it appears that zebrafish able to successfully hatch in and survive sublethal EE2 exposure levels are unable to pass on adaptive characteristics to their offspring, as the chronically low hatch success rates continued for all three generations.

The TU strain of zebrafish experienced the greatest variability in hatch success after exposure to 1 ng/L EE2 for three generations. While the first TU exposure generation experienced a greater hatch success than AB and WIK strains, the TU strain had the lowest hatch success in exposure generations 2 and 3.

Spermatogenesis

Fish spermatogenesis is largely regulated by androgens (such as testosterone), but estrogens and estrogen receptors are known to play a role as well (Betka and Callard, 1998). Previous studies have reported that increased xenoestrogen levels can disrupt spermatogenesis in multiple aquatic species. Male Nile tilapia (*Oreochromis niloticu*s) exposed to the herbicide tebuthiuron experienced a decreased diameter of the seminiferous tubules and lumen, as well as impaired release of sperm into the lumen (de Almeida et al., 2018).

In this study, exposure to 1 ng/L EE2 appeared to have no effect on the distribution of spermatogonia, spermatocytes, spermatids, and spermatozoa in male

zebrafish testes. These findings are similar to that observed in rainbow trout (*Oncorhynchus mykiss*) (Brown et al., 2008). Previous research showed that adult male zebrafish exposed to 5.62 ng/L EE2 for 60 days experienced stimulated germ cell proliferation and meiosis in the testes, but no effect on the gonadosomatic index (GSI) or sperm count (Wang et al., 2019). Zebrafish exposed to 10 ng/L EE2 for 15 days experienced a disruption to spermatogenic cell proliferation, but no gross alterations in gonad histology (Ortiz-Zarragoitia and Cajaraville, 2005). This suggests that chronic, multi-generational, low dose exposure to EE2 may not have an impact on zebrafish sperm cell production, but could have the ability to disrupt underlying mechanisms like mitosis and meiosis, or alter the genetic content of sperm.

Conclusion

These findings together suggest that chronic, low dose exposure to EE2 appears to stimulate zebrafish fecundity (i.e. increased clutch size), but that there is no boost to progeny, therefore an overall reduction in reproductive success. Parental exposure to EE2 may decrease egg quality through epigenetic effects, a mechanism that is seen in mammals (Anway et al., 2005). Our laboratory findings in zebrafish mirror the results of a field experiment utilizing fathead minnow: in this whole-lake study, the population collapsed after the second season of exposure to 5 ng/L EE2, likely due to severe reproductive impairment (Kidd, 2007). Therefore, in wild populations, it is becoming increasingly evident that low dose EE2 exposure has the potential to greatly reduce

population size in fish species, as each generation exposed to EE2 may see a compounded decline in embryos that successfully hatch.

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Chapter 6 : 17α-ethynylestradiol Exposure Effects on Swim Performance

Abstract

While exposure of 17α-ethynylestradiol (EE2) to zebrafish in a laboratory setting has been shown to result in a variety of sublethal effects (abnormal sexual development and differentiation, decreased fertility and fecundity, vitellogenin induction in males, and impairment of reproductive behaviors), no investigation has been conducted into the effects of EE2 on zebrafish swim performance. Three strains of zebrafish (AB, TU, and WIK) were exposed to 1 ng/L, 10 ng/L, and 25 ng/L EE2 for five months. Additionally, individuals from each strain were also exposed to 1 ng/L EE2 for three generations. Following exposure, all individuals were assessed in a swim tunnel to determine exposure effects on critical swim speed (Ucrit). No significant effects were observed in these trials.

Introduction

Scientists have long utilized locomotor performance as an indicator of fitness in both aquatic and terrestrial species. When quantifying locomotor performance, scientists often use the categories of burst, prolonged, and sustained locomotion. Burst locomotion is characterized as a fast-start sprint that lasts for less than 15 seconds in duration, is typically performed anaerobically, and ends due to exhaustion of intracellular energy supplies (Beamish, 1978). Prolonged locomotion is often maintained between 20 and 200 minutes, is aerobic, and characterized by periods of cruising and occasional bursts, ending in fatigue (Webb, 1975). Sustained locomotion is defined as a speed that is

maintained for long periods of time (greater than 200 minutes) without fatigue, is aerobic, with metabolic demand matching supply and a balance between waste production and disposal (Jones and Randall, 1982).

Understanding how a species performs in burst, sustained, and prolonged locomotion can give us insight into their abilities to evade predators, mate, feed, and migrate. Previous studies have shown that Caribbean *Anolis* lizards sprint very close to their maximum tested speed during predator escape (Irschick and Losos, 1998), and garter snakes (*Thamnophis sirtalis fitchi*) with a greater laboratory tested burst speed are more likely to survive in the wild (Jayne and Bennett, 1990). Fish species like gudgion (*Gobio gobio*) have been shown to possess both a high maximum and prolonged speed that allows them to pass physical barriers and overcome fast flowing water while migrating (Tudorache et al., 2007).

In aquatic species, swim performance is a widely studied, minimally invasive endpoint used to investigate the effects of sublethal exposure to contaminants (Cheng and Farrell, 2007; Beecham et al., 2014). Altered swim performance can impact foraging behavior, reproduction, and predator avoidance, and can be a measure that links individual exposure effects to ecosystem level consequences (Weis et al., 2000). In fish species, a special category of sustained speed called ‘critical speed’ (Ucrit) is often utilized to measure swim performance, and is characterized as the maximum velocity a fish can maintain until fatigue (Brett, 1964). Ucrit has become an important endpoint in assessing sublethal effects of toxicant exposure on fish species, and is accepted as an ecologically relevant measure of locomotor performance (Plaut, 2001). Past studies have

shown that exposure to a variety of toxicants can reduce Ucrit in fish species. Examples include Mahi-Mahi (*Coryphaena hippurus*) and puffer fish (*Takifugu rubripes*) exposed to crude oil (Mager et al., 2014; Yu et al., 2015).

Ucrit can be expressed absolutely or relatively. Absolute Ucrit measures the velocity achieved by a fish, often expressed in centimeters per second (cm/s). As larger fish generally swim faster than smaller fish and therefore have a higher absolute Ucrit, relative Ucrit is a way to compare speeds attained by differently sized species, or between differently sized individuals within the same species. Relative Ucrit takes into consideration the body length of a fish and, utilizing a conversion equation, is expressed as body lengths per second (BL/s). Generally, smaller fish exhibit a faster relative Ucrit, as they are able to travel the distance of their smaller body length more quickly than a larger fish travels the distance of their larger body.

Zebrafish are a species that can be used to investigate the effects of toxicant exposure on Ucrit, as they are a multi-disciplinary vertebrate model and are easy to breed and maintain in a laboratory setting (Palstra et al., 2010). Zebrafish embryos exposed to hydraulic fracturing wastewater for 24-48 hours experienced a significant reduction in aerobic capacity and Ucrit (Folkerts et al., 2017), while adult zebrafish exposed to selenite for 14 days also experienced a significant decrease in Ucrit (Masse et al., 2013). Furthermore, zebrafish embryos exposed to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and then raised in clean water also experienced a decrease in Ucrit (Marit and Weber, 2012).

17α-ethynylestradiol (EE2) is a man-made estrogenic chemical found in aquatic environments worldwide and is known to have lethal and sublethal effects on fish species. These include disruption of normal sexual development and differentiation, decreased fertility and fecundity, and impairment of reproductive behaviors (Nash et al., 2004). To date, no studies have been conducted on the effects of EE2 exposure on Ucrit in zebrafish. In this chapter, we investigate the effects of 1 ng/L, 10 ng/L, and 25 ng/L EE2 full life-cycle exposure on zebrafish Ucrit, as well as the effects of multiple generations of low dose (1 ng/L EE2) exposure. Furthermore, we utilize three common laboratory strains of zebrafish (AB, TU, and WIK) to elucidate if there are differences in exposure responses between strains.

Materials and Methods

Swim Performance

Swimming performance was assessed by measuring the maximum aerobic speed, or critical speed (Ucrit) of zebrafish. Swim trials were carried out on individual zebrafish using a Blazka type swim tunnel in the Brown Aquatic Laboratory (Figure 6.1). Water in the tunnel was supplied from the calibrated Aquaneering system, and the tunnel was set up as a closed loop with continuous aerated flow of water. Zebrafish were anesthetized utilizing MS-222, weighed, measured, and fasted overnight. The following day, zebrafish acclimated to the swim tunnel (Figure 6.2) for one hour at 2 BL/s before the trial. Upon acclimation, zebrafish were subjected to step-wise increments in swimming velocity (0.5 BL/s every five minutes) until fish experienced complete fatigue. Fatigue was defined as

resting against the back mesh of the swim tunnel for five seconds or more. Visual observation ensured that no erratic swimming behavior or premature fatigue occurred during the swimming trial.

The swim tunnel was operated utilizing a frequency controller connected to a variable speed direct current (DC) motor; when the user adjusted the hertz (Hz) of the frequency controller, the motor would adjust the speed of the propellor, which in turn adjusted the water velocity. The equation used for this calibration was:

$$Y = 0.6667X - 1.667$$

where Y is Hz (cycles/second), and X is velocity (cm/sec) of the water flow. Utilizing this equation, we were able to apply velocity increments that were corrected to the size of the fish (i.e. step-wise increments occurred via relative speed instead of absolute speed).

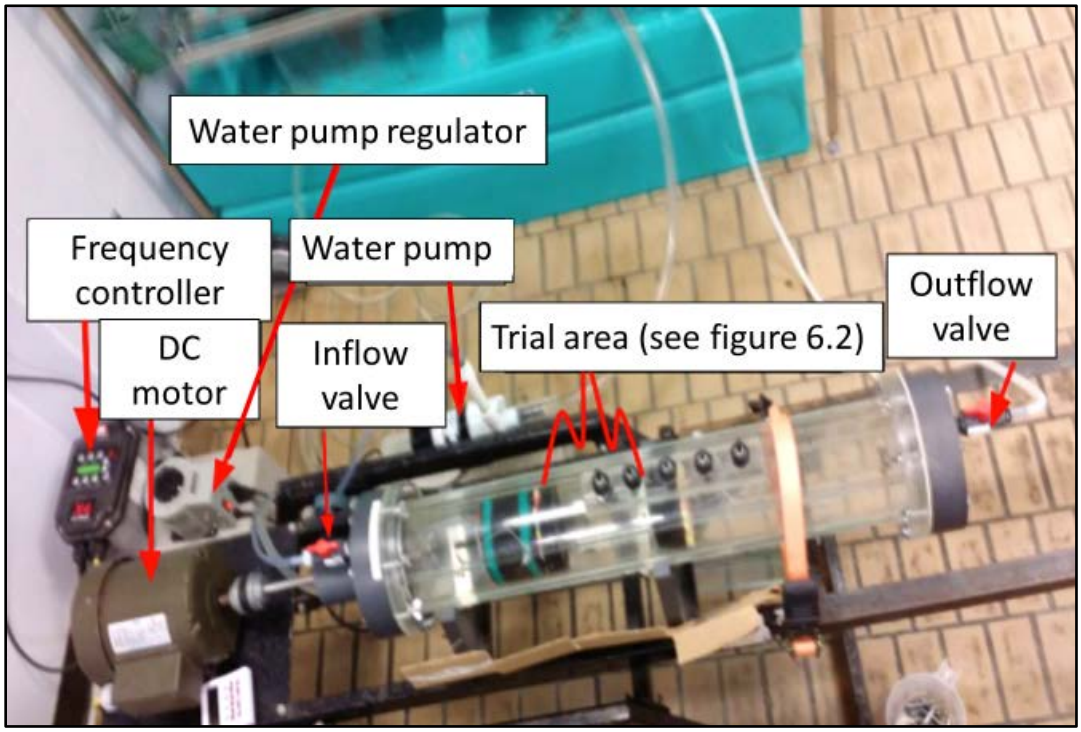

Figure 6.1. Blazka-type swimming tunnel.

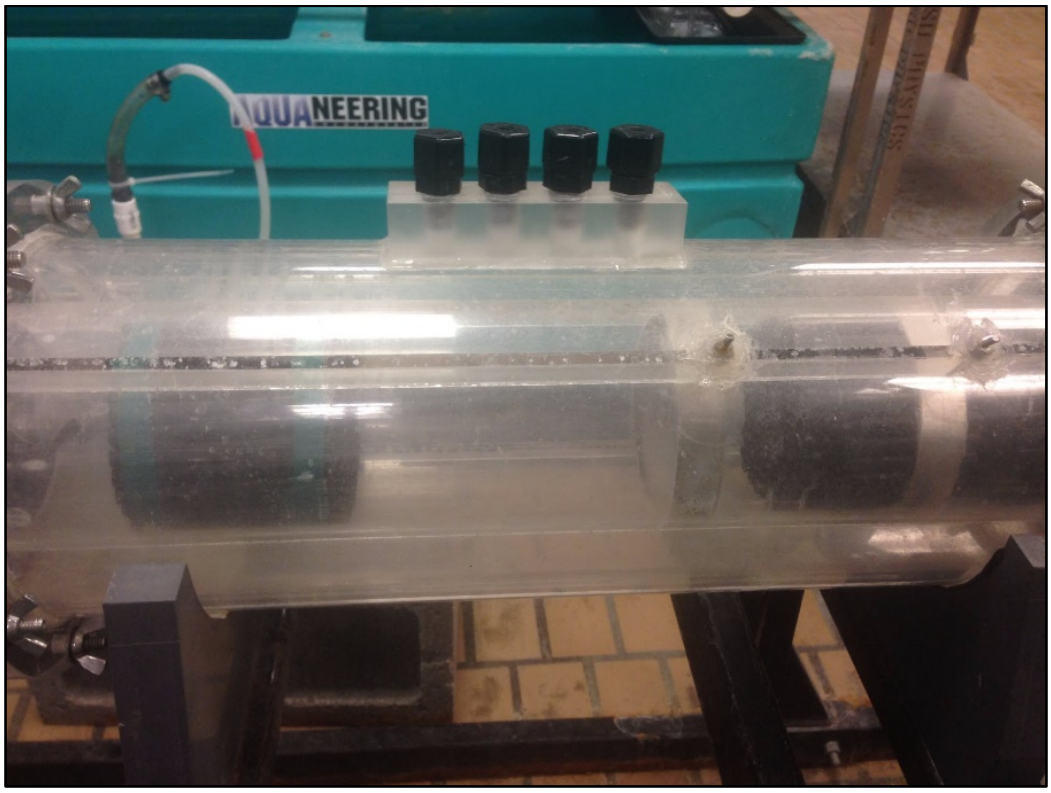

Figure 6.2. Trial area of swimming tunnel.

Critical Swim Speed (Ucrit)

Critical swimming speed was calculated using the following equation (Brett, 1964):

$$Ucrit = Ui + (Uii\ [Ti/Tii])$$

where Ui is the highest velocity maintained for the entire interval (cm/s), Uii is the velocity increment (cm/s), Ti is the time elapsed at fatigue velocity (s), and Tii is the prescribed interval time (s). The critical swim speed values (Ucrit) for each fish were calculated to present Ucrit as a velocity (absolute Ucrit, in cm/s), as well as adjusted for individual fish length to present Ucrit relative to body length (relative Ucrit, in BL/s).

These values were not adjusted for the solid blocking effect since all fish had a cross sectional area less than 5% of the swim tunnel diameter.

Additional Data Points

Additional endpoints included trial duration, length, weight, and condition factor. Trial duration was measured in minutes and began as soon as the first step-wise velocity increment occurred, after the acclimation period. Trial duration ended when the fish experienced complete fatigue. Generally, a higher absolute or relative Ucrit correlates to a longer trial duration.

Condition factor (K) was calculated utilizing the length and weight of each zebrafish. K is a value used by scientists to describe the "condition" of fish, and often utilized as an indicator of health; generally, a higher K value translates to a thicker, more robust fish. The formula is:

$$K = 10^N * W/L^3$$

where W = the weight of the fish in grams, and L is the length of the fish in mm. A species-specific scaling factor (N) is applied to bring the factor close to 1: for zebrafish N=5.

Statistical Analysis

Statistical analysis was performed with JMP Pro 14 software. All data were examined for homogeneity and normality using Levene's and Kolmogorov-Smirnov tests. If these assumptions were met, one-way analysis of variance (ANOVA) followed by a Tukey's test was utilized to identify differences between each exposure group and

the control. If the homogeneity and normality assumptions were not met, the nonparametric Kruskal-Wallis test followed by multiple comparison was performed. For multi-generational data, t-tests were performed to identify differences between exposure groups and control. All values presented are mean ± SEM. The significance level for all the statistical analyses was set at $p<0.05$.

Results

Exposure to 1ng/L, 10ng/L, and 25ng/L EE2

After five months of exposure to varying concentrations of EE2, there was no significant difference in relative Ucrit (BL/s) between the exposure groups and control (Figure 6.3).

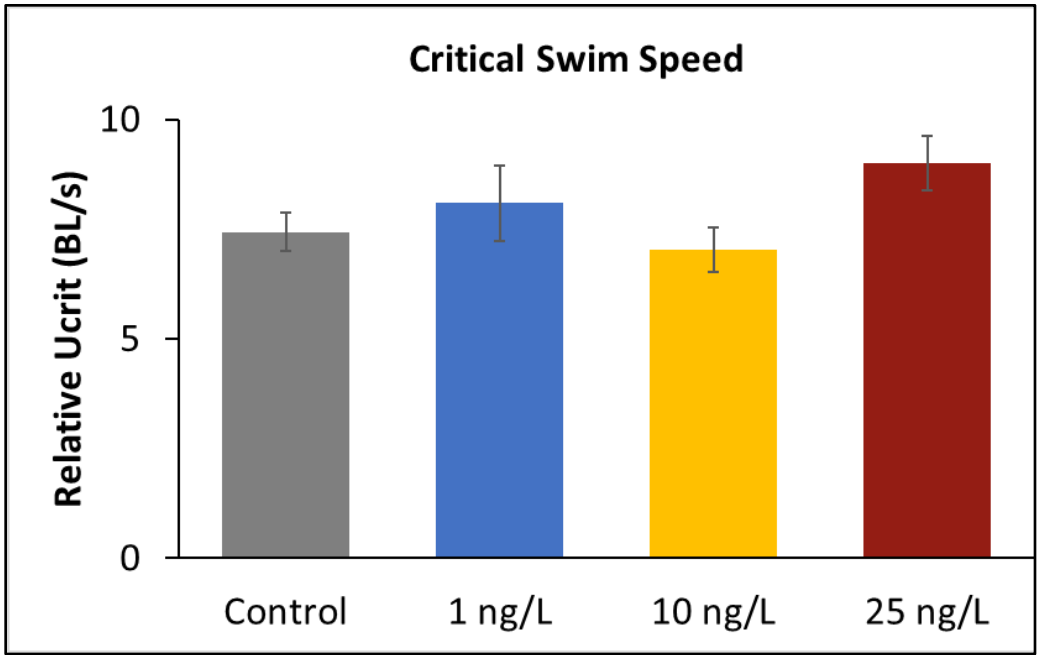

Figure 6.3. Relative Ucrit (BL/s) of zebrafish exposed to 1 ng/L, 10 ng/L, and 25 ng/L EE2 for five months. n=21 per group. Values are mean ± SEM. There was no significant difference between exposure groups and control, $p<0.05$.

There was no significant difference in absolute Ucrit or trial duration for any exposure group, as compared to control (Table 6.1). Zebrafish exposed to 25 ng/L EE2 had a significantly smaller length and weight as compared to the control group, as well as a higher condition factor. The 10 ng/L EE2 exposure group experienced a significantly smaller weight and condition factor than control.

Table 6.1. Relative Ucrit, absolute Ucrit, trial duration, length, weight, and condition factor of zebrafish exposed to 1 ng/L, 10 ng/L, and 25 ng/L EE2 for five months. n=21 per group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

	Relative Ucrit (BL/s)	**Absolute Ucrit (cm/s)**	**Trial Duration (min)**	**Length (cm)**	**Weight (g)**	**Condition factor (K)**
Control	7.43 ± 0.44	22.20 ± 1.37	51.04 ± 4.06	2.99 ± 0.04	0.30 ± 0.01	1.12 ± 0.04
1 ng/L	8.09 ± 0.87	24.66 ± 2.62	58.62 ± 8.62	3.07 ± 0.05	0.30 ± 0.02	1.02 ± 0.02
10 ng/L	7.02 ± 0.51	20.79 ± 1.51	48.44 ± 5.01	2.97 ± 0.05	0.24 ± 0.01*	0.90 ± 0.03*
25 ng/L	9.00 ± 0.62	21.40 ± 1.28	68.23 ± 6.26	2.42 ± 0.07*	0.21 ± 0.01*	1.49 ± 0.10*

A multivariate analysis of the entire experimental population (see supplemental data - Figure S.1) showed that the length of the fish had a significant negative correlation with relative Ucrit, as well as a positive correlation with absolute Ucrit (not statistically significant) (Figure 6.4). However, when a multivariate analysis was performed on individual exposure groups and the control group, these correlations were only significant in the 25 ng/L EE2 group (see supplementary data – Figure S.2, Figure S.3, Figure S.4, and Figure S.5).

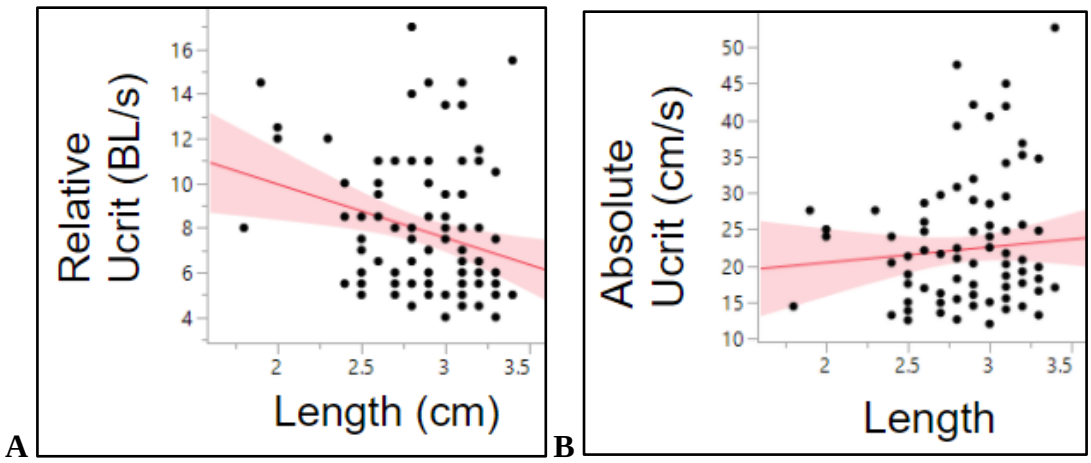

Figure 6.4. Scatterplot matrix of relative and absolute Ucrit by length. n=84 per plot. A) Negative correlation for relative Ucrit by length, statistically significant, $p<0.01$ B) Positive correlation for absolute Ucrit by length, not statistically significant.

Multi-Generational Exposure to 1 ng/L EE2

Exposure of zebrafish to 1 ng/L EE2 for three generations had no significant effect on relative Ucrit, as compared to the control, in any generation (Figure 6.5). Furthermore, there was no significant difference in absolute Ucrit or trial duration between exposure groups and control (data not shown).

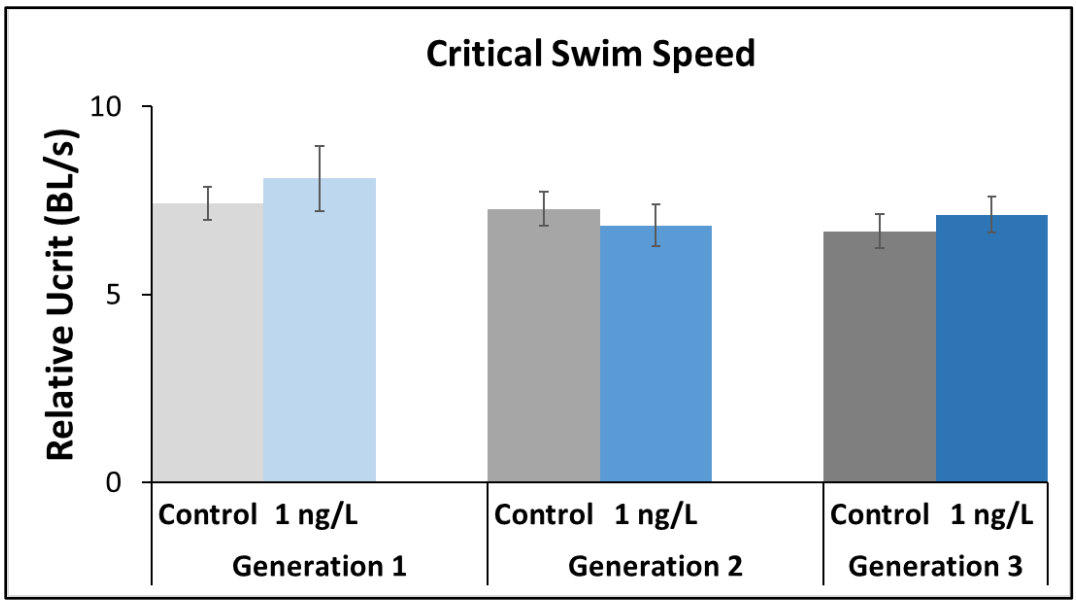

Figure 6.5. Relative Ucrit (BL/s) of zebrafish exposed to 1 ng/L for five months, for three generations. n=30 per group. Values are mean ± SEM. No significant difference between control and exposure groups, $p<0.05$.

AB, TU, and WIK Strain-Specific Effects of EE2 Exposure

When the first generation of exposure groups were analyzed by strain, the AB strain experienced a significant increase in relative Ucrit after exposure to 1 ng/L EE2 and 25 ng/L EE2, while the WIK strain experienced a decrease in relative Ucrit in these two exposure groups (Figure 6.6). The TU strain only experienced an increase in relative Ucrit after exposure to 25 ng/L EE2.

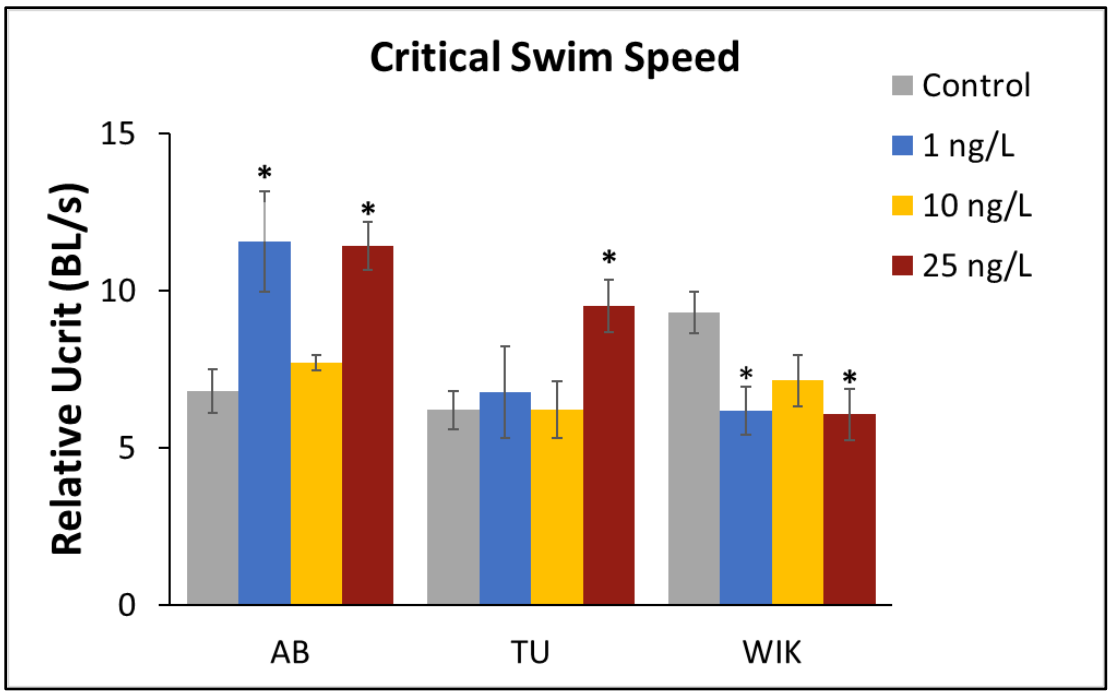

Figure 6.6. Relative Ucrit (BL/s) of zebrafish exposed to 1 ng/L, 10 ng/L, and 25 ng/L EE2 for five months, separated by strain. n=7 per group, per strain. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

With regards to absolute Ucrit, the AB strain experienced a significant increase after exposure to 1 ng/L EE2, while the WIK strain experienced a significant increase after 1 ng/L and 25 ng/L EE2 exposure, as compared to control (Table 6.2). The TU and WIK strains had a significantly lower weight in the 10 ng/L and 25 ng/L EE2 exposure groups, as compared to control. AB and TU were the only strains to experience pericardial edema in the 25 ng/L EE2 exposure group, which is reflected in their significantly increased condition factor, as compared to control.

Table 6.2. Relative Ucrit, absolute Ucrit, trial duration, length, weight, and condition factor of each exposure group, separated by strain. n=7 per strain, per exposure group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

	Relative Ucrit (BL/s)	**Absolute Ucrit (cm/s)**	**Trial duration (minutes)**	**Length (cm)**	**Weight (g)**	**Condition factor (K)**
AB						
Control	6.80±0.69	18.72±1.86	45.26±6.56	2.77±0.07	0.26±0.02	1.22±0.09
1 ng/L	11.57±1.59*	33.60±4.91*	93.01±16.07*	2.87±0.10	0.25±0.02	1.03±0.04
10 ng/L	7.71±0.24	21.58±0.81	55.71±2.39	2.80±0.08	0.23±0.03	1.02±0.07
25 ng/L	11.42±0.77*	24.10±1.82	93.56±7.78*	2.11±0.12*	0.19±0.03	1.92±0.19*
TU						
Control	6.20±0.62	19.18±1.93	39.88±6.25	3.10±0.06	0.31±0.01	1.05±0.04
1 ng/L	6.78±1.46	22.38±5.08	45.34±14.34	3.27±0.05	0.36±0.02	1.02±0.02
10 ng/L	6.21±0.91	19.57±3.02	40.93±8.84	3.12±0.06	0.25±0.02*	0.80±0.03*
25 ng/L	9.50±0.83*	24.22±2.11	72.91±8.09*	2.55±0.06*	0.24±0.02*	1.44±0.04*
WIK						
Control	9.30±0.65	28.71±1.94	67.97±5.25	3.09±0.04	0.32±0.02	1.09±0.04
1 ng/L	6.19±0.69*	18.83±1.97*	40.13±6.83*	3.06±0.06	0.29±0.02	1.01±0.06
10 ng/L	7.14±1.24	21.21±3.53	48.65±12.22	2.99±0.08	0.24±0.02*	0.87±0.03*
25 ng/L	6.07±0.25*	15.85±0.99*	38.22±2.38*	2.60±0.06*	0.20±0.02*	1.09±0.06

When analyzing relative Ucrit by strain for the multi-generational exposure to 1 ng/L EE2 groups, there was no difference between exposure groups and control, in all three generations (data not shown).

Discussion

A positive correlation between length and absolute Ucrit (cm/s) was observed when conducting a multivariate analysis on the experimental population as a whole. There is an established correlation between length and absolute Ucrit within the field of fish physiology (Hammer, 1995), as larger fish generally swim faster than smaller fish, and this was found to be true within our dataset. Furthermore, a significant negative correlation between length and relative Ucrit (BL/s) was observed. This is to be expected, as small fish swim faster than larger fish relative to body length. These population level observations give us confidence in the statistical significance of our data set as whole; however, when multivariate analyses were conducted on individual exposure groups and the control group, only the 25 ng/L EE2 exposure group (which also had the widest range in size) exhibited these significant correlations. This could mean that the individual data sets may not have a large enough sample size to draw significant conclusions from.

Of note, the 25 ng/L EE2 exposure group had the highest relative Ucrit of the three exposure groups, as well as the highest condition factor. The high condition factor of the 25 ng/L EE2 exposure group can be explained by the pericardial edema experienced by many in that exposure group, a buildup of excess fluid in the sac-like structure around the heart (called the pericardium) (Figure 6.7). K is traditionally utilized to measure body thickness as it relates to health, though in this experiment it showed that the 25 ng/L EE2 exposure fish had a thicker body than other groups due to pericardial edema (Froese, 2006).

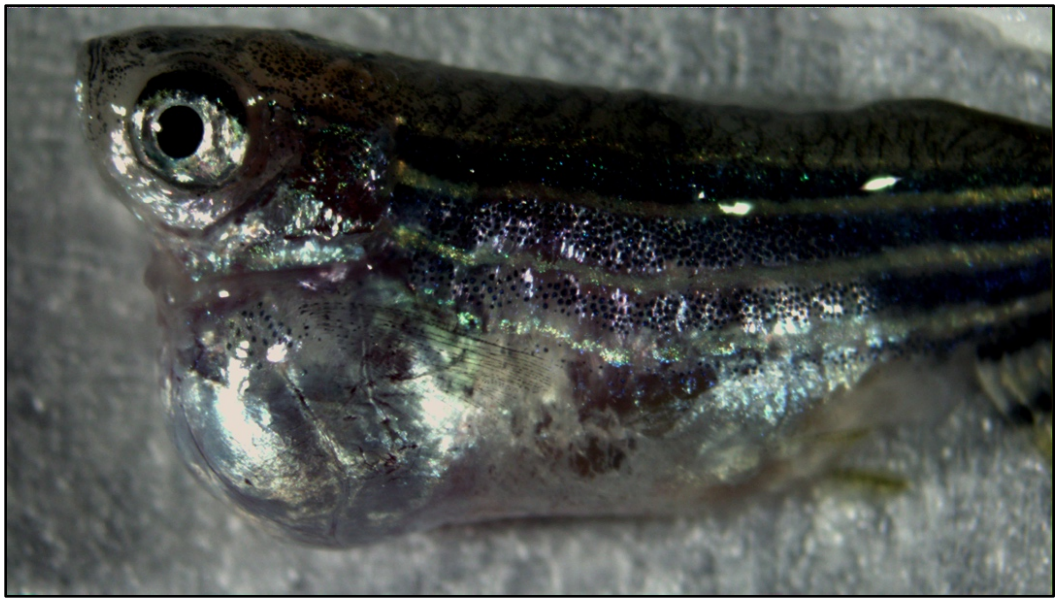

Figure 6.7. Zebrafish exposed to 25 ng/L EE2 for five months, exhibiting pericardial edema

When body length is corrected for and made relative (utilizing BL/s), there was no significant difference in relative Ucrit between exposure groups (1 ng/L, 10 ng/L, and 25 ng/L EE2) and control. Furthermore, multi-generational low dose exposure to 1 ng/L EE2 also appears to have no significant effect on relative Ucrit, as compared to control. Our findings suggest that EE2 exposure does not have a significant effect on relative or absolute Ucrit. As EE2 is known to primarily effect measures like reproduction and behavior, zebrafish may be able to overcome morphological effects induced by exposure (like pericardial edema) in order to maintain high maximum sustained swim speeds. This is consistent with the concept of Ucrit being a measure of aerobic capacity and oxidative stress, rather than a measure of general estrogenic effects (Thomas and Janz, 2011).

Further studies could be conducted into the effects of EE2 exposure on different types of swimming performance. As zebrafish are a species that commonly exhibit schooling behavior (Miller and Gerlai, 2012), testing Ucrit for a group of fish instead of

an individual may provide insight into how exposure to endocrine disrupting compounds affects group swimming behavior. Observing aerobic metabolism during swim performance could also give insight into the effects of EE2 exposure on zebrafish. When coupled with Ucrit values, utilizing a respirometer within the swim tunnel can elucidate muscle performance as it relates to oxygen consumption and fatigue.

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Chapter 7 : Effects of Depuration on 17α-ethynylestradiol Exposure

Abstract

Xenoestrogens, including 17α-ethynylestradiol (EE2), are known to have significant morphological and reproductive effects on aquatic species during direct exposure, yet limited information is available on the permanency of these effects. In this study, we investigated the ability of zebrafish to recover from the effects of prolonged EE2 exposure after transfer to clean water (i.e. depuration). After five months of exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2, followed by a six-month depuration period, endpoints of survival, growth, sperm cell production, and female reproductive success were assessed. Furthermore, we evaluated the ability of zebrafish to recover from exposure to low dose EE2 (1 ng/L) over the course of three generations. Results indicate that depuration following exposure to concentrations of EE2 above 10 ng/L allowed for a return to normal length and weight, but female reproductive success was permanently inhibited. Zebrafish exposure to 25 ng/L EE2 led to a decline in survival that was not mitigated by depuration. Multi-generational 1 ng/L EE2 exposure resulted in an irreversible decline in female reproductive success, despite a stabilization of fecundity following depuration.

Introduction

The vertebrate endocrine system regulates the processes of reproduction, metabolism, and growth in tissues throughout the body by utilizing hormones and their receptors (Norris and Carr, 2013). A primary part of the endocrine system is the hypothalamic-pituitary-gonad (HPG) axis, which can be altered by chemicals that mimic

or block the activity of endogenous (natural) hormones. These endocrine disrupting chemicals (EDCs) are known to cause abnormalities in the reproductive behavior, sexual differentiation, fertility, and fecundity of vertebrates (Guillette and Gunderson, 2001). For affected organisms, disruptions can be temporary and reversible, or permanent and irreversible in nature, depending on the concentration and time point in development of exposure (Bigsby et al., 1999).

As wildlife exposure to EDCs is often intermittent, additional research investigating whether species can recover from toxicological effects induced by a period of exposure is needed. Laboratory experiments using fish models have shown an ability to recover from exposure to certain EDCs after a period in clean water (i.e. depuration). Female eelpout (*Zoarces viviparus*) exposed to 4-tert-octylphenol (a chemical utilized in manufacturing detergents and fungicide) for 14 days experienced an increase in hepatosomatic index (liver size), as well as vitellogenin (VTG) and calcium levels; after depuration, the liver recovered to normal size, while VTG and calcium levels decreased towards control values (Jespersen et al., 2010). Male marine medaka (*Oryzias melastigma*) exposed to 17α-ethynylestradiol (EE2) for 21 days were able to recover from immune and reproductive impairment after seven days in clean water (Ye et al., 2018). While these studies indicate the potential ability of organisms to recover from short-term exposure effects after depuration, long-term exposure may induce additional, irreversible damage.

EE2 is a well-researched and ubiquitous environmental pollutant, however few studies have focused on the effects of depuration after exposure. Zebrafish were chosen

as the model in this study, given their rapid development from fertilization to sexual maturity within three months - short generation time makes zebrafish ideal for full life-cycle and multi-generational exposure experiments. Furthermore, multiple developmental and reproductive endpoints can be monitored in a lab setting. Using this model, we investigated morphological and reproductive outcomes from EE2 exposure in zebrafish, identifying temporary (reversible) or permanent (irreversible) effects.

Materials and Methods

Exposure Parameter: Survival

For each exposure group (control, 1 ng/L, 10 ng/L, and 25 ng/L) fifteen male (five of each strain) and fifteen female (five of each strain) zebrafish were transferred to a clean water system after five months of exposure to EE2 (with the exception of the 25 ng/L group; due to low survival during the exposure period, sixteen total zebrafish were transferred to the clean water system). Date of death was recorded for individual zebrafish, and survival curves were created utilizing this data over the six-month depuration period.

Exposure Parameter: Growth

At eleven months of age (five months of exposure to EE2 and a six-month depuration period), length and weight of each surviving zebrafish was recorded. Condition factor (K) was calculated utilizing the relationship between the weight of a fish

and its length, with the intention of describing the "condition" of each individual. The formula is:

$$K = 10^N * W/L^3$$

where W = the weight of the fish in grams, and L is the length of the fish in millimeters. A species-specific scaling factor (N) is applied to bring the factor close to 1: for zebrafish N=5.

Exposure Parameter: Reproduction

After five months of exposure to EE2 and five months of depuration, zebrafish from the control group and each exposure group were spawned. For each breeding trial five male/female pairs per strain were randomly selected and placed into Aquaneering Crossing Tanks with dividers separating each individual of the spawning pair overnight. The following morning, dividers were removed, and zebrafish were allowed up to five hours to complete spawning. Embryos were collected and counted to quantify egg production. At five days post fertilization (dpf), zebrafish fry were counted to quantify hatch success. After each trial, fish were returned to their original tanks and rested for seven days before the next trial. The breeding trials lasted until five spawning pairs per strain produced viable eggs (i.e. at least one egg was successfully fertilized, developed, and hatched). The following equations were used to calculate reproduction parameters:

$$\text{Clutch size} = \frac{\text{total number of collected eggs per spawning pair}}{\text{number of spawning pairs}}$$

$$\text{Embryo hatch success} = \frac{\text{cumulative hatched larvae on each post fertilization day}}{\text{total number of eggs}}$$

To quantify spermatogenesis, zebrafish were euthanized utilizing MS-222 after the six-month depuration process, and testes were removed using dissecting needles under a stereoscopic microscope (Leica Microsystems). Tissue preparation was carried out by the OHSU Histopathology Shared Resource in Portland, Oregon. Testes were embedded in paraffin wax, sliced, and stained with Hematoxylin and Eosin (H&E). Using a Leica DMIRB Inverted Microscope at a magnification of 1000X, the number of spermatogonia, spermatocytes, spermatids, and spermatozoa were counted. This was achieved by creating a digital image of each slide, using ImageJ to mark each cell with a dot, and using a particle counter to count the dots on the image. Excel was used to keep track of sperm cell counts.

Statistical Analysis

Statistical analysis was performed with JMP Pro 14 software. To analyze survival data, we used a Kaplan-Meyer survival curve, with the log-rank test for differences between exposure groups and the control. All data were examined for homogeneity and normality using Levene's and Kolmogorov-Smirnov tests. If these assumptions were met, one-way analysis of variance (ANOVA) followed by a Tukey's test was utilized to identify differences between each exposure group and the control. If the homogeneity and normality assumptions were not met, the nonparametric Kruskal-Wallis test followed by multiple comparison was performed. For multi-generational data, t-tests were performed to identify differences between exposure groups and control. All values presented are mean ± SEM. The significance level for all the statistical analyses was set at $p<0.05$.

For measuring the effects of depuration, 'reversibility of exposure effects' was defined by two steps: (1) there was a statistically significant effect found during or after the exposure period (see supplementary data – Table S.1 and Table S.2) and (2) that effect returned to non-significant differences between control and exposure groups following depuration.

Results

Exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2

Single generation exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2 only affected survival at the highest concentration level, causing a significant decrease over the course of five months. This pattern continued during the depuration period, as 57% of the 25 ng/L EE2 exposure group survived, compared to 100% survival in control (Figure 7.1). Furthermore, zebrafish in the 10 ng/L and 25 ng/L EE2 exposure groups experienced total reproductive failure during the exposure period and were unable to spawn. This inability to produce viable eggs continued throughout the depuration period and eliminated multi-generational analysis at these exposure concentrations.

No strain-specific effects on survival were observed during the depuration period, as 100% of the 1 ng/L EE2 exposure group survived, and only one zebrafish (TU strain) in the 10 ng/L EE2 group did not survive (data not shown). Strain-specific survival could not be calculated for the 25 ng/L EE2 exposure group, as the experimental population was too small due to low survival rates during the five-month EE2 exposure period.

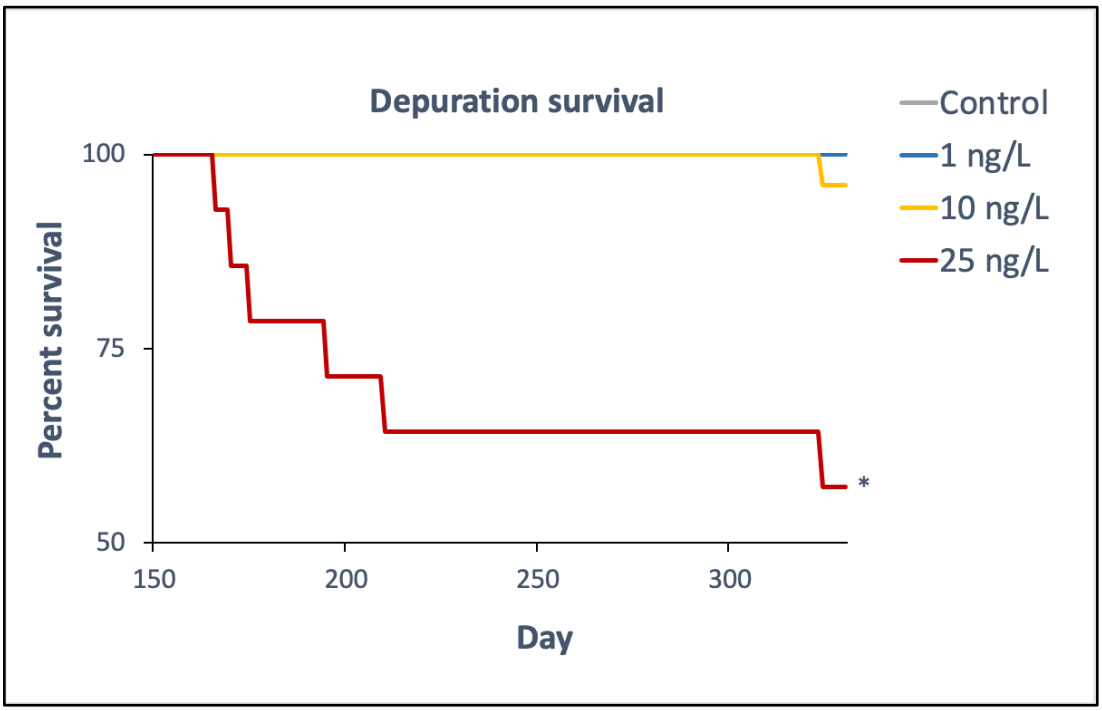

Figure 7.1. Survival curve of zebrafish during a six-month depuration period, after five months of exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2. n=30 for control and 1ng/L EE2 groups; n=25 for 10ng/L EE2 group; n=16 for 25ng/L EE2 group. * indicates significant difference from control, $p<0.01$.

Exposure to 25 ng/L EE2 followed by a depuration period resulted in a significantly higher weight than control (Table 7.1), as well as a higher condition factor (1.2 g/cm^3 and 0.86 g/cm3, respectively) (Figure 7.2). There was no difference in growth between the 1 ng/L and 10 ng/L EE2 as compared to control, with the exception of the 1ng/L EE2 exposure group which had a slightly higher condition factor than control.

Table 7.1. Weight and length of zebrafish exposed to EE2 concentrations of 1 ng/L, 10 ng/L, and 25 ng/L EE2 for five months, followed by a six-month depuration period. n=30 for control and 1 ng/L EE2 groups; n=25 for 10 ng/L EE2 group; n=10 for 25 ng/L EE2 group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

Exposure Group	**Weight (g)**	**Length (cm)**
Control	0.33 ± 0.01	3.39 ± 0.04
1 ng/L EE2	0.37 ± 0.01	3.37 ± 0.04
10 ng/L EE2	0.36 ± 0.02	3.37 ± 0.04
25 ng/L EE2	0.46 ± 0.05*	3.37 ± 0.07

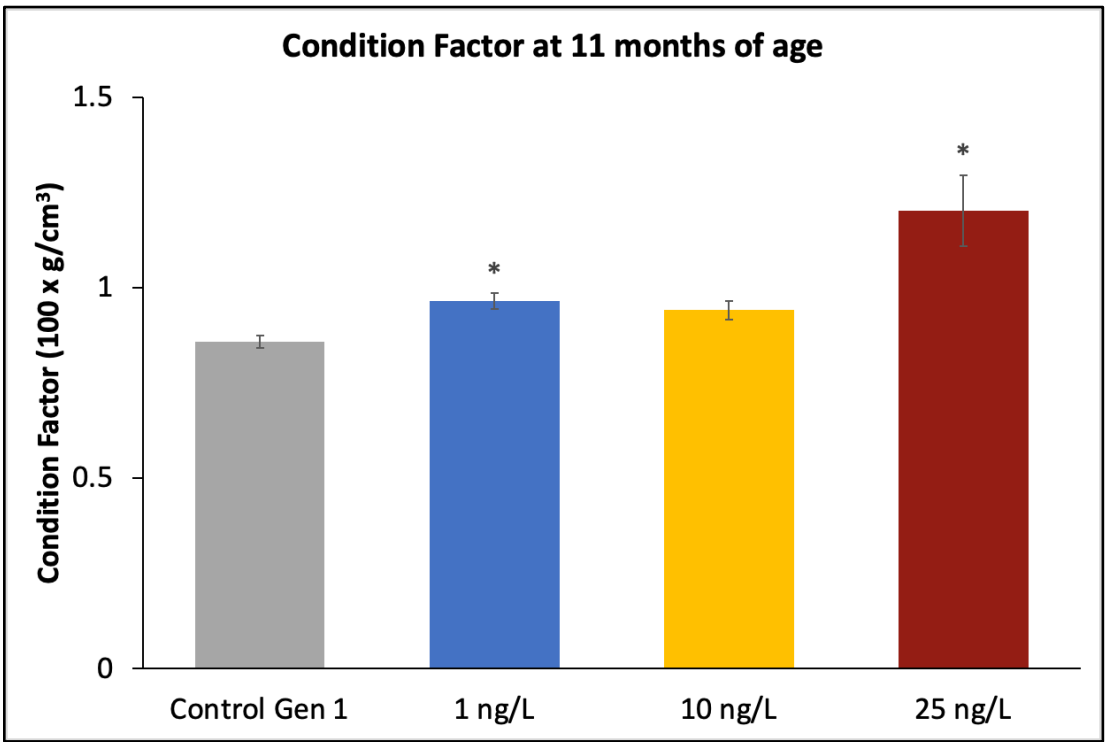

Figure 7.2. Condition factor of zebrafish exposed to EE2 concentrations of 1 ng/L, 10 ng/L, and 25 ng/L EE2 for five months, followed by a six-month depuration period. n=30 for control and 1 ng/L EE2 groups; n=25 for 10 ng/L EE2 group; n=10 for 25 ng/L EE2 group. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

Multi-Generational Exposure to 1 ng/L EE2

Depuration after multi-generational exposure to 1 ng/L EE2 had no significant effect on survival (data not shown). Both control and exposure fish had a 100% survival rate during the exposure and depuration periods, for all three generations.

After one generation of exposure to 1 ng/L EE2, followed by a depuration period, the exposure group experienced a slight increase in condition factor (0.96 g/cm^3, compared to 0.86 g/cm^3 for control) (Figure 7.3). There was no significant difference in growth of the Generation 1 and 2 exposure groups, compared to their respective control groups (Table 7.2). However, after depuration, Generation 3 had a significantly larger length and weight than control.

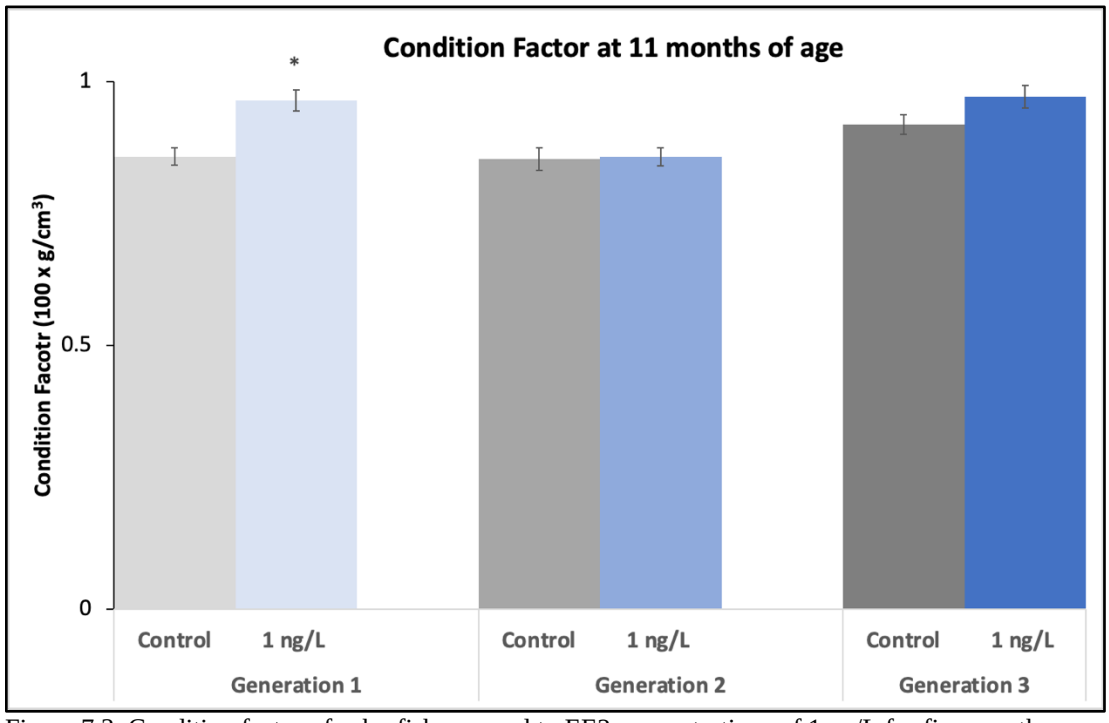

Figure 7.3. Condition factor of zebrafish exposed to EE2 concentrations of 1 ng/L for five months, followed by a six-month depuration period, for three generations. n=30 per exposure group. Values are mean ± SEM. * indicates significant difference from control, $p<0.01$.

Table 7.2. Weight and length of zebrafish exposed to EE2 concentrations of 1 ng/L for five months, followed by a six-month depuration period, for three generations. n=30 per exposure group. Values are mean ± SEM. * indicates significant difference from control, p<0.05.

	Weight (g)		**Length (cm)**	
Exposure	*Control*	*1 ng/L EE2*	*Control*	*1 ng/L EE2*
Generation 1	0.34 ± 0.01	0.37 ± 0.01	3.40 ± 0.04	3.37 ± 0.04
Generation 2	0.29 ± 0.01	0.30 ± 0.01	3.24 ± 0.04	3.26 ± 0.04
Generation 3	0.27 ± 0.01	0.34 ± 0.01*	3.09 ± 0.04	3.26 ± 0.03*

There was no difference in length between strains after the six-month depuration period (Table 7.3), although weight was significantly increased in the 1 ng/L EE2 Generation 1 and Generation 3 TU strain (Table 7.4).

Table 7.3. Body length (cm) of zebrafish exposed to 1 ng/L EE2 for five months, followed by a six-month depuration period, for three life cycles, separated by strain. n=10 per strain, per exposure group. Values are mean ± SEM. No difference between exposure and control groups, p<0.05.

	Generation 1		**Generation 2**		**Generation 3**	
Strain	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*
AB	3.16 ± 0.08	3.13 ± 0.04	3.15 ± 0.09	3.09 ± 0.07	2.92 ± 0.05	3.14 ± 0.04
TU	3.48 ± 0.04	3.51 ± 0.08	3.31 ± 0.05	3.32 ± 0.03	3.17 ± 0.03	3.33 ± 0.03
WIK	3.55 ± 0.05	3.51 ± 0.02	3.28 ± 0.04	3.37 ± 0.03	3.20 ± 0.06	3.29 ± 0.05

Table 7.4. Body weight (g) of zebrafish exposed to 1 ng/L EE2 for five months, followed by a six-month depuration period, for three life cycles, separated by strain. n=10 per strain, per exposure group. Values are mean ± SEM. * indicates significant difference from control, p<0.05.

	Generation 1		**Generation 2**		**Generation 3**	
Strain	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*	*Control*	*1ng/L EE2*
AB	0.30 ± 0.03	0.31 ± 0.01	0.29 ± 0.03	0.28 ± 0.01	0.24 ± 0.02	0.32 ± 0.02
TU	0.33 ± 0.01	0.41 ± 0.03*	0.29 ± 0.01	0.30 ± 0.01	0.28 ± 0.01	0.35 ± 0.01*
WIK	0.38 ± 0.02	0.40 ± 0.01	0.29 ± 0.01	0.32 ± 0.01	0.30 ± 0.02	0.34 ± 0.02

Clutch size of the first and second generation of zebrafish exposed to 1 ng/L EE2 was significantly higher than control; after a depuration period, there was no significant

difference in clutch size between control and exposure groups for all three generations (Figure 7.4).

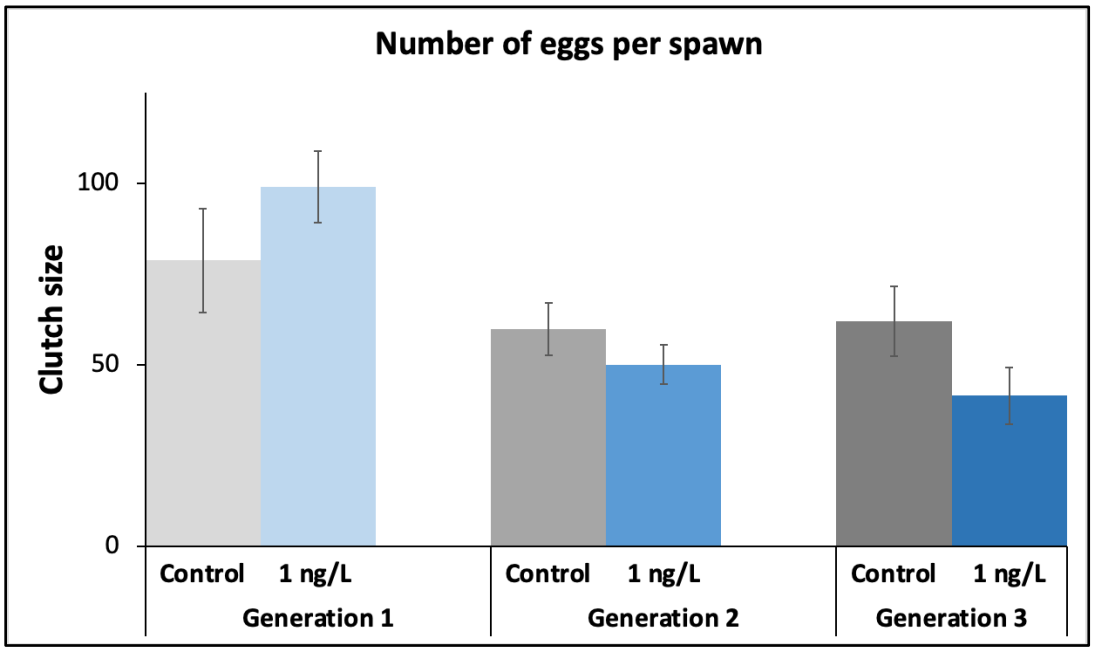

Figure 7.4. Number of eggs per clutch spawned by zebrafish after five months exposure to 1 ng/L EE2 and a five-month depuration period, for three generations. n=15 spawning pairs per exposure group, one clutch per spawning pair. Values are mean ± SEM. No difference between exposure and control, $p<0.05$.

Embryo hatch success was low for all three generations during the exposure period to 1 ng/L EE2; after depuration, all three generations of exposure groups had a significantly lower hatch success than control (Generation 1: 75% in control, 29% in exposure; Generation 2: 63% in control, 26% in exposure; Generation 3: 55% in control, 42% in exposure) (Figure 7.5).

When clutch size and hatch success data are combined, overall reproductive success can be assessed as the number of successfully hatched eggs per clutch (Figure 7.6). All three generations of exposure groups experienced a significantly low number of successfully hatched eggs per clutch, compared to control.

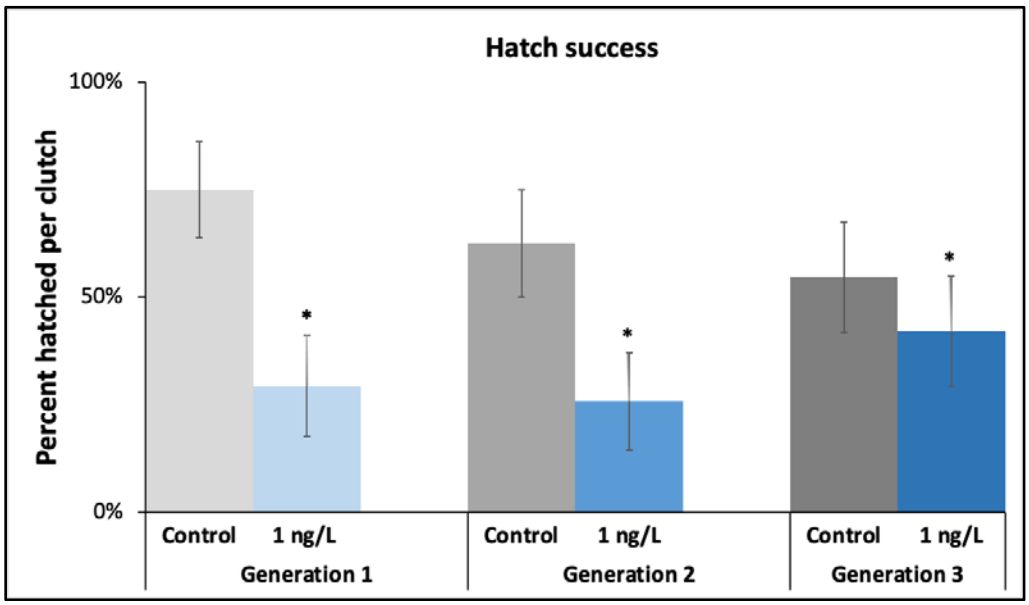

Figure 7.5. Percent of zebrafish embryos that hatched after five months of parental exposure to 1 ng/L EE2 followed by a five-month depuration period, for three generations. n=15 spawning pairs per exposure group, one clutch per spawning pair. Bars indicate SE, * indicates significant difference from control, p<.001.

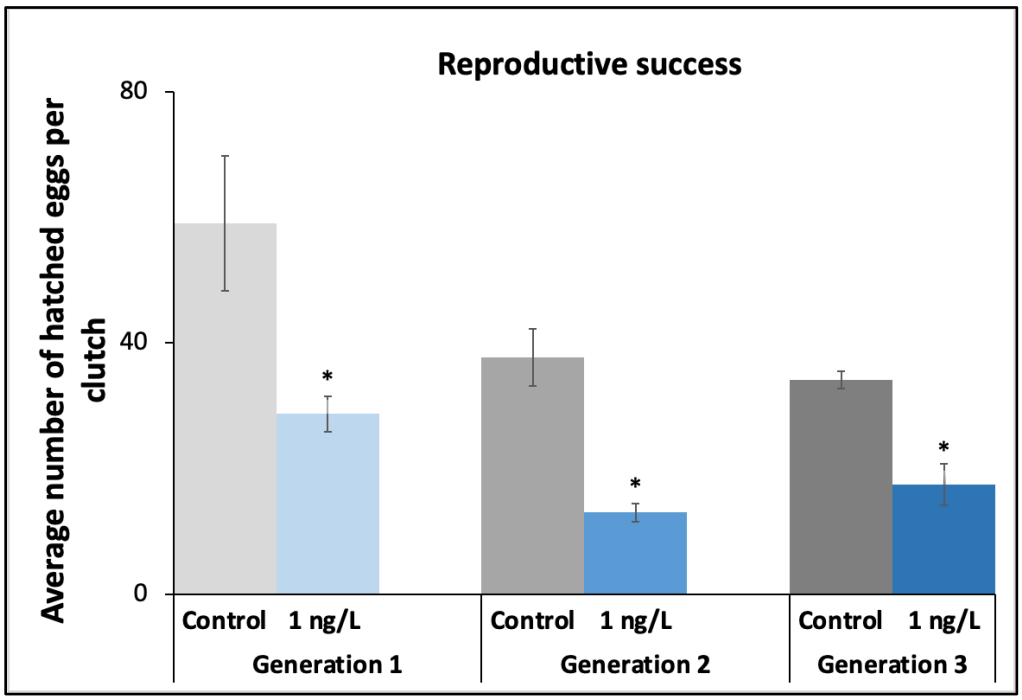

Figure 7.6. Number of successfully hatched eggs per clutch after parental exposure to 1 ng/L EE2 followed by a five-month depuration period, for three generations. n=15 spawning pairs per exposure group, one clutch per spawning pair. Values are mean ± SEM. * indicates significantly different from control, p<005.

After one generation of exposure to 1 ng/L EE2 and a five-month depuration period, the AB strain experienced a statistically significant decrease in clutch size (76 eggs for exposure, 115 eggs for control), while TU and WIK both experienced an increase in clutch size (120 eggs for exposure and 54 eggs in control for TU; 100 eggs for exposure and 66 eggs for control for WIK) (Figure 7.7). In Generation 2 and 3, there was no significant difference between the control and exposure group in any strain.

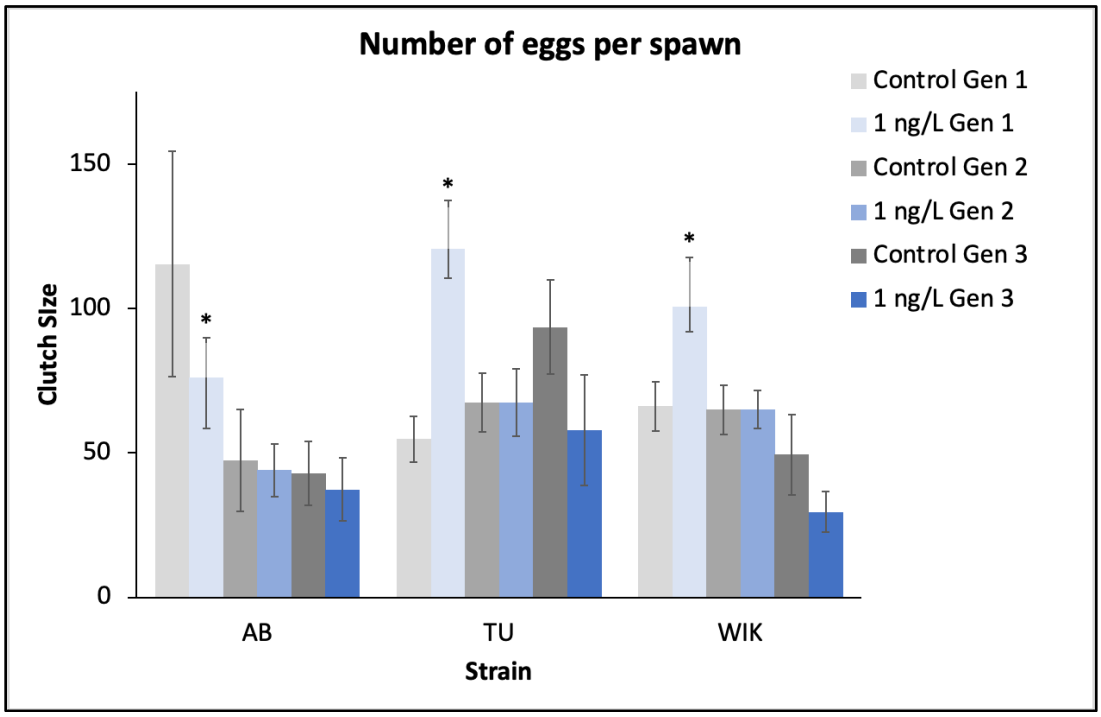

Figure 7.7. Number of eggs per clutch spawned by zebrafish after five months exposure to 1 ng/L EE2 and a five-month depuration period, for three generations, by strain. n=5 spawning pairs per exposure group, per strain; one clutch per spawning pair. Values are mean ± SEM. * indicates significant difference from control, $p<0.05$.

After one generation of exposure to 1ng/L EE2 and a five-month depuration period, the AB and WIK strains experienced a statistically significant decrease in hatch success, while the TU strain exposure group did not differ from control (Figure 7.8). In

Generation 2, all three strains experienced a significant decrease in hatch success, and by Generation 3 the only significant difference between control and exposure was a decrease in clutch size in the WIK strain.

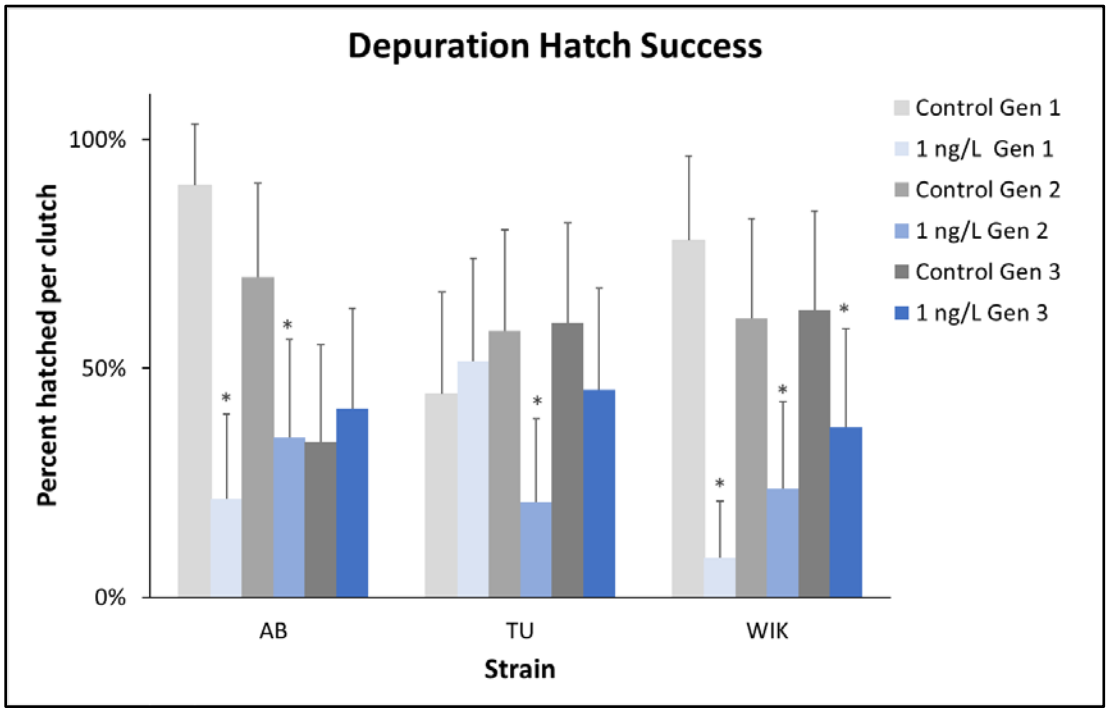

Figure 7.8. Percent of zebrafish embryos that hatched after five months of parental exposure to 1 ng/L EE2 and a five-month depuration period, for three generations, separated by strain. n=5 spawning pairs per exposure group, per strain; one clutch per spawning pair. Bars indicate SE. * indicates significant difference from control, $p<0.05$.

After five months of exposure to 1 ng/L EE2, the number of spermatogonia, spermatocytes, spermatids, and spermatozoa did not differ significantly between the third generation of exposure and control group. This pattern continued after six months in clean water (Figure 7.9).

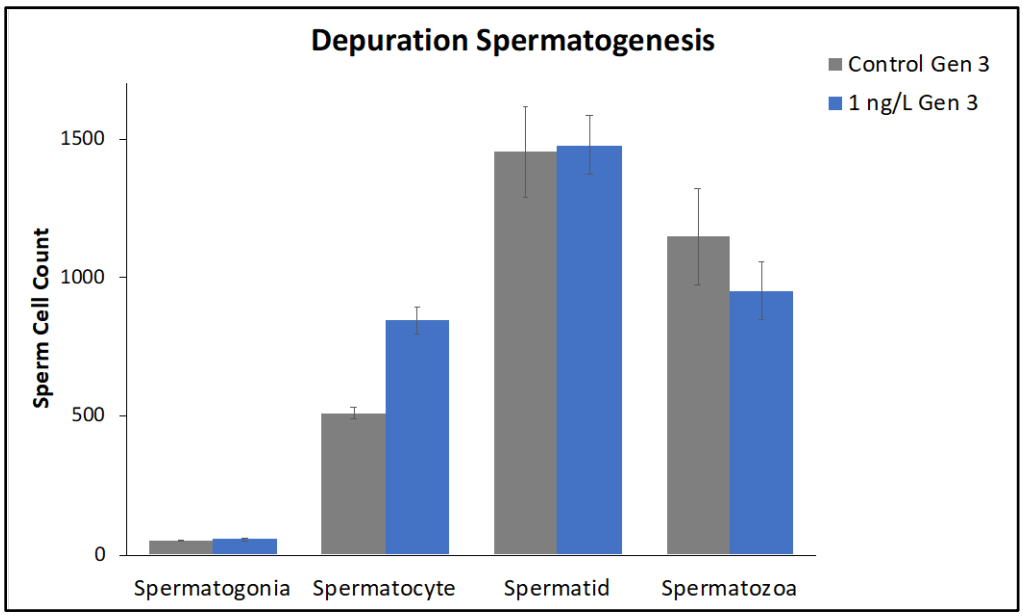

Figure 7.9. Number of spermatogonia, spermatocytes, spermatids, and spermatozoa per observed slide in the third generation of control group and zebrafish exposed to 1 ng/L EE2 for five months, followed by six months in clean water. n=28 for control group, n=29 for exposure group. Values are mean ± SEM. No significant difference from control, $p<0.05$.

A summary of depuration recovery can be seen in Tables 7.5 and 7.6.

Table 7.5. Summarized depuration findings after a five-month exposure period to EE2 and six-month period in clean water. Data is shown for one generation of exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2, as well as three generations of exposure to 1 ng/L EE2. Values given are as compared to statistically significant findings after exposure, where 'Recovered' means there is no difference between depuration value and exposure value (after a statistically significant finding during the exposure period), '↓' indicates a decrease in value, and '↑' indicates an increase in value; ''-' indicates that there was no significant difference between exposure and depuration, 'N/A' indicates that data could not be collected.

Exposure Group	**1 ng/L EE2 Gen 1**	**1 ng/L EE2 Gen 2**	**1 ng/L EE2 Gen 3**	**10 ng/L EE2 Gen 1**	**25 ng/L EE2 Gen 1**
Survival	-	-	-	-	Did not recover
Length	-	Recovered ↓	↑	-	Recovered ↑
Weight	-	Recovered ↓	↑	Recovered ↑	Did not recover
K	↑	-	-	Recovered ↑	Did not recover
Clutch Size	Recovered ↓	Recovered ↓	-	N/A	N/A
Hatch Success	Did not recover	Did not recover	Did not recover	N/A	N/A

Table 7.6. Summarized statistically significant findings for three strains of zebrafish (AB, TU, and WIK) from a five-month exposure period to EE2, followed by a six-month depuration period. Data is shown for one generation of exposure to 1 ng/L, 10 ng/L, and 25 ng/L EE2, as well as three generations of exposure to 1 ng/L EE2. Values given are as compared to statistically significant findings after exposure, where 'Recovered' means there is no difference between depuration value and exposure value (after a statistically significant finding during the exposure period), '↓' indicates a decrease in value, and '↑' indicates an increase in value; ''-' indicates that there was no significant difference between exposure and depuration, 'N/A' indicates that data could not be collected.

Parameter	Exposure Group	AB	TU	WIK
Survival	*1 ng/L EE2 Gen 1*	-	-	-
	1 ng/L EE2 Gen 2	-	-	-
	1 ng/L EE2 Gen 3	-	-	-
	10 ng/L EE2 Gen 1	-	-	-
	25 ng/L EE2 Gen 1	N/A	N/A	N/A
Length	*1 ng/L EE2 Gen 1*	-	-	-
	1 ng/L EE2 Gen 2	-	-	Recovered ↓
	1 ng/L EE2 Gen 3	-	-	-
	10 ng/L EE2 Gen 1	N/A	N/A	N/A
	25 ng/L EE2 Gen 1	N/A	N/A	N/A
Weight	*1 ng/L EE2 Gen 1*	-	↑	-
	1 ng/L EE2 Gen 2	-	-	Recovered ↓
	1 ng/L EE2 Gen 3	-	↑	-
	10 ng/L EE2 Gen 1	N/A	N/A	N/A
	25 ng/L EE2 Gen 1	N/A	N/A	N/A
Clutch Size	*1 ng/L EE2 Gen 1*	↓	↑	↑
	1 ng/L EE2 Gen 2	Recovered ↓	Recovered ↓	Recovered ↓
	1 ng/L EE2 Gen 3	-	-	-
Hatch Success	*1 ng/L EE2 Gen 1*	Did not recover	Recovered ↑	Did not recover
	1 ng/L EE2 Gen 2	Did not recover	Did not recover	Did not recover
	1 ng/L EE2 Gen 3	Recovered ↑	Recovered ↑	Did not recover

Discussion

In this study we assessed the ability of zebrafish to recover from exposure to 1ng/L, 10 ng/L, and 25 ng/L EE2, focusing on the endpoints of survival, growth, spermatogenesis, and female reproductive success. We utilized three common zebrafish strains (AB, TU, and WIK) for both single and multiple generations of exposure, followed by depuration. Our results show that zebrafish exposed to 25 ng/L EE2 for five months experienced a statistically significant decline in survival and alteration of growth; a six-month depuration period did not allow zebrafish to return to control values. A large number of zebrafish exposed to 25 ng/L EE2 began developing pericardial edema, or excess fluid between the heart and the sac that surrounds the heart (the pericardium). After two months of exposure, nearly two-thirds of the population had developed severe pericardial edema. This condition stayed severe throughout the six-month depuration period (Figure 7.10). Edema led to a significant increase in weight, evidenced by a significantly higher condition factor (g/cm^3) for the 25 ng/L EE2 exposure group. Fish with a high condition factor have thicker, less fusiform morphology, and are less hydrodynamic. This suggests that exposure to 25 ng/L EE2, despite a depuration period, has an irreversible impact on the survival and growth of zebrafish.

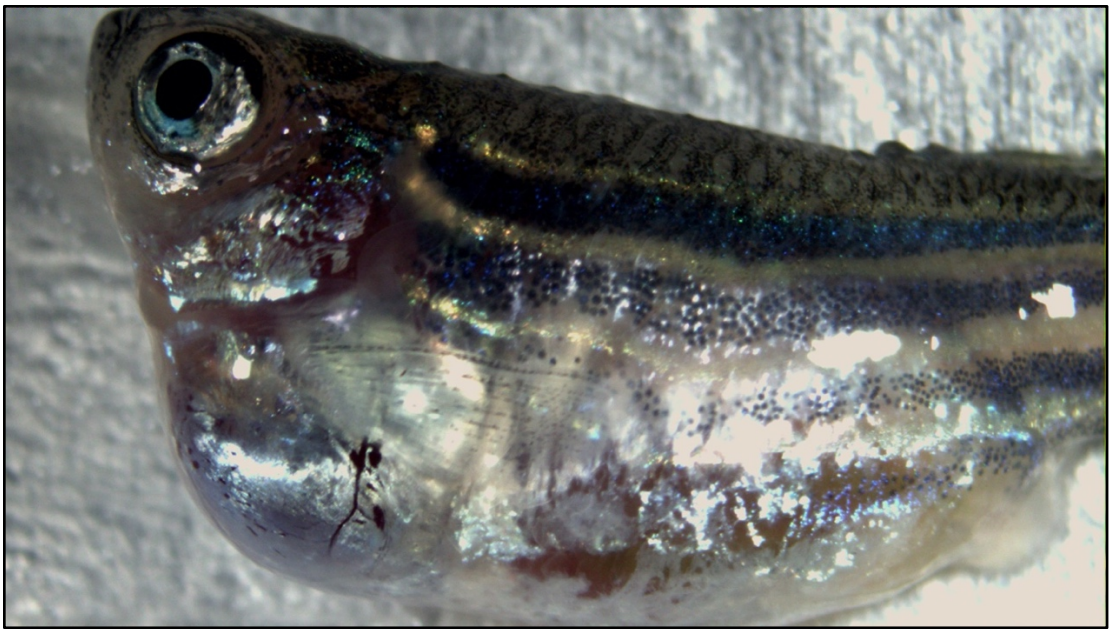

Figure 7.10. Zebrafish after five months of exposure to 25 ng/L EE2 and a six-month depuration period. Pericardial edema is evident as the enlarged sac around the heart. Also note the general fluid build-up in the body cavity of the fish.

Depuration allowed the weight of zebrafish exposed to 10 ng/L EE2 to return to control level. After five months of exposure to 10 ng/L EE2, zebrafish were lighter than control, but after a six-month depuration period there was no difference between the exposure group and control. These findings mirror previous research, where exposure to 10 ng/L EE2 for three months significantly reduced the length and weight of zebrafish, but after a three-month period in clean water there was no difference between control and exposure groups (Xu et al., 2008). This points to an overall trend that exposure above 10 ng/L EE2 negatively impacts the growth of zebrafish, but depuration may allow for some recovery.

In this study, exposure to EE2 at or above 10 ng/L resulted in total reproductive failure, even after a depuration period. Previous studies have shown that disruption and

recovery of reproductive function is possible at concentrations lower than 10 ng/L EE2. After 118 days of exposure to 3 ng/L EE2, zebrafish experienced reproductive failure, but recovered reproductive abilities after a depuration period. However, clutch size and fertilization rate were significantly reduced, compared to control (Fenske et al., 2005). In another study, zebrafish experienced reproductive failure after 177 days of exposure to 10 ng/L EE2, but spawning activity returned after 2.5 months in clean water. However, clutch size was small compared to control, and hatch success of the exposure group was less than 3% (Shäfers et al., 2007). Taken together, these results suggest that exposure to concentrations of 3 ng/L EE2 may temporarily alter endocrine homeostasis, while exposure to EE2 levels of 10 ng/L or higher may pathologically and permanently alter tissue structure and function.

Multi-generational low dose exposure to 1 ng/L EE2, followed by a depuration period, resulted in a significant post-exposure impact on growth in the first and third generation of zebrafish. While there was no difference in growth between control and exposure groups during the exposure period, after depuration zebrafish in the first generation had a higher condition factor than control and zebrafish in the third generation had a larger length and weight than control. Similarly, a study found that zebrafish exposed to 1 ng/L EE2 for 60 days followed by a depuration period of 40 days were heavier than control (Baumann et al., 2014). This suggests a possible compensatory mechanism for increased growth in zebrafish recovering after a period of exposure to low dose EE2.

Although zebrafish exposed to 1 ng/L EE2 for multiple generations experienced a return to normal clutch size after a depuration period, overall reproductive success declined in all three generations. This diminished reproductive success was driven primarily by the continued reduction in embryo hatch success, which persisted even after the depuration period. This is consistent with past research exposing male zebrafish to 5 ng/L EE2 for four months, followed by eight months in clean water. Fertilization rate in the exposed group was 23%, significantly lower than the 90% fertilization rate of the control group (Larsen et al., 2009). Comparing this result to our spermatogenesis data would indicate that the lower fertilization success rate was not due to a lack of sperm production, but more likely due to an alteration of sperm, leading to impaired embryo development.

While strain-specific differences among zebrafish strains have been identified for other estrogenic and environmental contaminants (e.g. PCBs) (Holden, 2018), we observed only subtle differences among the strains after a depuration period. Morphologically, the TU strain experienced the only increase in an endpoint after depuration - an increase in weight in both Generation 1 and Generation 3; the WIK strain experienced the only depuration recovery, a decrease in Generation 2 length and weight to return to control levels.

Reproductive endpoints after depuration varied between strains. While the TU and WIK strains experienced an increase in clutch size after one generation of exposure, the AB strain experienced a slight decrease. Furthermore, the WIK strain failed to recover successful embryo hatching in all three generations and was the only strain to fail

to recover in Generation 3. This suggests increased long-term sensitivity to low dose EE2 exposure in the WIK strain, compared to the AB and TU strains.

Conclusion

The results from this study suggest that the severity and permanence of EE2 exposure effects is dose-dependent, with a dose at or below 1 ng/L having significant permanent effects on reproductive success, regardless of depuration. While some effects were slightly alleviated by a depuration period, the induced effects appear to exhibit a strain specificity, with the WIK stain being the least capable of recovering reproductive capabilities, likely due to genetic differences between the strains. Although depuration after exposure to concentrations of EE2 above 10 ng/L allowed some morphological endpoints like length and weight to recover, inhibitory effects sustained from full life-cycle exposure resulted in complete, irreversible reproductive failure and an inability to produce additional generations. This is most likely due to disruption of sexual differentiation (e.g. changes to gonadal tissue structure and function) paired with genetic or epigenetic modifications to sperm or ova that reduce fertilization success and normal embryo development capabilities.

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Chapter 8 : Conclusions

Background

In the first chapter, we introduced the concept of endocrine disrupting chemicals (EDCs), which are natural or synthetically produced compounds that can mimic, block, or alter endogenous hormones. Natural EDCs like estrogens, androgens, phytoestrogens (derived from plants), as well as industrial chemicals like pharmaceuticals, polychloro-biphenyls (PCBs), nonyphenols (NPs), and pesticides are often released into the environment, and negatively impact organisms and ecosystems. Sources of these EDCs include industrial, hospital, and domestic waste filtered through municipal wastewater treatment plants, as well as livestock and agricultural runoff. EDCs in the aquatic environment are of particular concern, as there is intentional release of these chemicals into rivers, lakes, and oceans, as well as accidental release through spills and run-off. Research into EDC pollution initially began to focus on estrogenic chemicals contained in effluent in the 1990s, with 17α-ethynylestradiol (EE2) being identified as a major source in domestic effluent (Sumpter, 1995). As aquatic wildlife is disproportionately affected by EDC pollution, we utilized zebrafish, a long-established laboratory model in the field of toxicology, as our model organism to research the effects of EE2 exposure. Furthermore, we carried out this study utilizing three common laboratory strains of zebrafish (AB, TU, and WIK), as each strain differs in genetic background and course of selective breeding and therefore may respond differently to EE2 exposure.

Review of Findings

Dose-Dependent Effects of EE2 Exposure on Zebrafish

The results of this research support the hypothesis that 1 ng/L EE2 is the lowest observed effect concentration (LOEC) for EE2 exposure in zebrafish (Shäfers et al., 2007). When considering dose-dependent effects, the phenomenon of hormesis is helpful in explaining zebrafish reproductive responses to EE2. Hormesis is considered an adaptive response that is characterized by biphasic dose-responses to a toxicological stimulus (Calabrese and Baldwin, 2002). These responses can be either directly induced by the stimulus, or the result of compensatory biological processes that follow a disruption in homeostasis. Often, low dose exposure exhibits a beneficial response, while higher doses to the same stimulus become toxic (Figure 8.1). This is what is meant by 'biphasic'. We can see this occur with EE2 exposure in zebrafish, as exposure to 1 ng/L EE2 results in a reproductive boost for some characteristics (e.g. increase in fecundity), while exposure to 10 ng/L or higher results in complete reproductive failure (i.e. inability to spawn viable eggs).

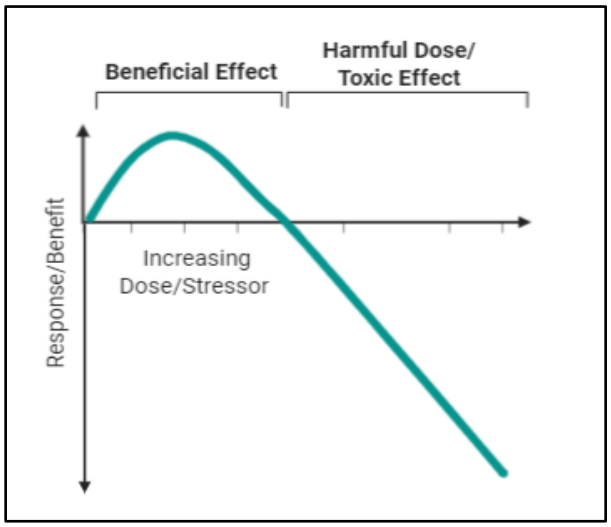

Figure 8.1. Biphasic dose-response of hormesis.

Zebrafish also experience a dose-dependent effect on growth and survival. Whereas exposure to 1 ng/L EE2 has no effect on length, weight, or survival of the zebrafish, exposure to 10 ng/L decreased zebrafish weight, while exposure to 25 ng/L EE2 induced pericardial edema, decreased length, and significantly reduced survival in zebrafish.

Multiple studies have found EE2 contamination of rivers, lakes, and streams worldwide at levels of 1 ng/L or higher (Tiedeken et al., 2017). As the published predicted no-effect concentration (PNEC) for chronic exposures of aquatic life to EE2 is estimated to be 0.1 ng/L, there is great cause for concern (Caldwell et al., 2012). LOEC values of 1 ng/L EE2 for Japanese medaka (Metcalfe et al., 2001) and fathead minnows (Pawlowski et al., 2004), as well as 0.1 ng/L for rainbow trout (Purdom et al., 1994) are well below the majority of predicted wastewater EE2 concentrations across the globe.

Low Dose Generational Effects of EE2 Exposure on Zebrafish

EDCs like EE2 can be considered a form of novel selective pressure, when exposure occurs over the course of multiple generations. As the resilience of natural populations depends on whether or not they can quickly adapt to such pressure, it is important to understand how an aquatic species like zebrafish responds to multi-generational, low level exposure to EE2. Our research shows that this type of sublethal exposure has both morphological and reproductive impacts on zebrafish.

Zebrafish exposed to 1 ng/L EE2 experienced an increase in growth in Generation 2, indicating the possibility of a compensatory response to toxicological stimulation by

EE2. By the third generation, however, there was no difference between the exposure group and control. This could potentially be due to selection for resistance genes in the population, similar to adaptation seen in the Atlantic killifish (*Fundulus heteroclitus*) after exposure to PCBs (Nacci et al., 1999). While adaptation to stressors can be seen as a biological advantage, it is important to also consider that survival of a species within a polluted environment can lead to an enhanced risk of bio-accumulation with each successive generation. When aquatic species are able to adapt and survive in polluted waterways, it can lead to an increase in EDCs within the food web, and a large burden in higher trophic-level organisms that normally would not be exposed to the EDCs found in aquatic ecosystems.

We also observed a compensatory response in the reproductive capabilities of zebrafish exposed to 1 ng/L EE2 for multiple generations. Both the first and second generation experienced an increase in clutch size, most likely due to an estrogen-induced boost to fecundity. However, the embryo hatch success remained low for all three generations, indicating that the compensatory response in fecundity may not be indicative of an ability to overcome the genotoxic effects of EE2 exposure. The overall result is a decrease in reproductive success. In natural populations, this could eventually lead to a total population collapse, as was seen in a seven-year, whole-lake experiment exposing fathead minnow to low levels of EE2 (Kidd et al., 2007).

When considering the causes of these observed exposure effects, differentiating between effect pathways is helpful. While environmental factors like EDCs do not normally modify DNA and the genome sequence directly, they can alter the epigenome,

and therefore modify genome activity (Head, 2014). If this epigenetic pathway leads to the modification of a somatic cell, disease may occur in the individual exposed, but will not be transmitted to offspring. If the modification occurs in a germ cell, then disease or susceptibility can be transmitted to the next generation. Consequently, these epigenomic changes can be the cause of population-level impacts within natural ecosystems, due to cumulative adverse effects after multiple generations (Bernal and Jirtle, 2010). The inability of zebrafish in our experiments to fully recover reproductive success following depuration supports the likelihood that genetic and/or epigenetic modifications are occurring within the zebrafish genome during exposure. Should similar effects occur in wild aquatic populations, population loss in exposed environments would likely ensue.

Strain-Specific Effects of EE2 Exposure in Zebrafish

Generally, all three strains of zebrafish (AB, TU, and WIK) responded similarly to both varying concentration and multi-generational exposure to EE2. Morphologically, the WIK strain experienced a greater increase in length and weight than the AB and TU strains during the second generation of 1 ng/L EE2 exposure. Reproductively, there was no difference between strains during the exposure period. After a period of depuration however, the WIK strain exhibited an increase in clutch size during Generation 1 but was unable to recover hatch success as consistently as the AB and TU strains. Therefore, the data may suggest that the WIK strain has a higher sensitivity to EE2 exposure, as well as a more varied capacity to compensate for exposure effects.

Recovery of Zebrafish after Exposure to EE2 followed by Depuration

Zebrafish experienced a wide variety of effects after exposure to EE2 for five months. We were curious to see if those effects could be mitigated or recovered if zebrafish were given access to clean water for a period of time. We tracked morphological and reproductive endpoints after a six-month depuration period that occurred after a five-month EE2 exposure period to 1 ng/L, 10 ng/L, and 25 ng/L, as well as depuration after three generations of exposure to 1 ng/L EE2.

Morphologically, zebrafish exposed to 10 ng/L EE2 or less were able to recover, and in some cases experienced an increase in growth, after a depuration period. Zebrafish exposed to 1 ng/L EE2 for multiple generations experienced a return to normal length and weight after the second generation of depuration, and an increase in length and weight after the third generation of depuration. This suggests the existence of a compensatory and/or adaptive mechanism in zebrafish following depuration after low dose EE2 exposure.

Reproductively, zebrafish clutch size stabilized after access to clean water, suggesting that the reproductive boost experienced after low dose exposure to EE2 was only temporary. This also suggests that fecundity is not permanently altered by low dose EE2 exposure. Clutch size data must be considered along with hatch success in order to give an overall picture of zebrafish reproductive success. As zebrafish hatch success was unable to recover after a depuration period, this suggests a permanent modification to

fertilization and embryo development pathways, and a permanent impairment of reproductive success. This could be due to alteration of sperm, impact to the quality of the egg, and/or epigenetic effects that interrupt embryo development.

While physiological recovery from EE2 exposure effects is encouraging, reproductive capacity plays a larger role in the survival of a population. The findings from these studies show that in a laboratory setting, one generation of exposure to concentrations above 10 ng/L EE2 lead to total population collapse, while multi-generational exposure to concentrations as low as 1 ng/L EE2 slowly diminished the reproductive capability of a population to the point of being unable to spawn successive generations, and therefore eventual collapse.

Recommendations moving forward

Update wastewater treatment infrastructure

Municipal sewage treatment plants should be a focal point for efforts to reduce EDC contamination in waterways. Most treatment plants are currently not equipped to remove the majority of EDCs in the effluent (Larcher and Yargeau, 2013). This often leads to discharge of EDCs directly into the natural environment. Municipal landfills must also be considered, as they are a source of steroid hormone contaminants that can leach into groundwater (Li, 2014).

Research has been conducted into more efficient and effective methods of removing EE2 from wastewater. A variety of approaches have been established, ranging from physical techniques like activated carbon, reverse osmosis, and nanofiltration, to

chemical and biological techniques like ferric chloride coagulant and use of bacteria (Aris et al., 2014). Studies have shown that nearly 100% of EE2 and E2 can be removed by utilizing various mixtures of microbes (Yoshimoto et al., 2004). While each approach has its advantages and disadvantages, there is a clear need for the implementation of better treatment methods at wastewater facilities. In order to ensure the methods being used are appropriate for each location, decisions on how to treat effluent according to the mixture of EDCs should be informed by scientific studies.

More data on agricultural practices

EDC pollution originating with livestock and agriculture industries is of great concern. Steroid hormones are used to regulate growth and treat disease in cattle, which can then seep into surface and groundwater via urine excretion (Gadd et al., 2010). Furthermore, synthetic and natural estrogens leach into the ground from manure and sewage sludge utilized as fertilizer on agricultural land (Chen et al., 2010). It has been argued that the amount of estrogenic hormone excreted by livestock meets or exceeds the amount excreted by humans, making it the largest source of estrogenic hormones in the natural environment (Liu et al., 2012). Studies in the UK have shown that daily estrogen excretion from swine is more than twice that of humans, and if combined with sheep and poultry, generates almost four times more estrogen than the human population (Johnson et al., 2006). More must be done to consistently monitor and track EDCs that originate with agricultural industries, and to mitigate the movement of this pollution into waterways.

Change to policy, monitoring, and regulation

While updating wastewater treatment infrastructure and addressing livestock and agricultural sources seem like the most viable ways to reduce EE2 pollution in the environment, updating policy and regulation with regard to EE2 usage may prove to be useful as well. Limiting or banning the production of synthetic hormones is not possible, as they are a critical means to regulate the endocrine system (Combalbert and Hernandez-Raquet, 2010). However, putting systems in place to monitor EE2 runoff from agricultural sources and wastewater effluent would be a step in the right direction. One study showed that EE2 concentrations were reduced by half when sampled 25 km away from a sewage effluent source, but were still above PNEC values (Barel-Cohen et al., 2006). Measuring hormone levels in waterways is a rapid and inexpensive way to determine the source of pollution, evaluate the effectiveness of effluent management, and pinpoint areas in need of intervention.

Future Studies

Future studies are needed into the molecular and cellular mechanisms that are responsible for the effects we see in aquatic species exposed to EE2. A basic understanding into these mechanisms will strengthen risk assessment, as well as provide pathways for diagnosis and treatment of exposure effects. Understanding both the role genetics play in susceptibility, as well as the epigenetic component of response is critical in future research.

Studies into the potential toxic effect of EE2 on species at higher trophic levels is also crucial to understanding the ecosystem effects of EDC pollution. Terrestrial organisms that do not live in or around aquatic ecosystems can be exposed to EDCs via bio-accumulation through the trophic chain. Additionally, research into EE2 exposure effects on longer-lived species is needed. The average lifespan of zebrafish is 3.5 years, which allows for relatively quick generational turnover, and thus a greater chance for adaptation. Many aquatic species are longer-lived than zebrafish, and therefore may have a lower ability to adapt to toxicological impacts. Further research into a wider variety of taxa is needed, as the focus has been on aquatic species due to the direct and chronic exposure threat.

A note on birth control

The majority of published papers cite birth control as a main source of EE2 pollution in the environment, but it is critical to have a larger perspective on the issue. Birth control is undoubtedly one of the greatest medical inventions of the 21st century when it comes to bodily autonomy and family planning. When citing birth control as a major factor in EE2 pollution, one must also consider that it is a vital medication for a large proportion of the world's population, and has social and cultural implications beyond laboratory science. I believe it is critical to focus on the less regulated and under-studied sources of EE2 contamination, namely the agriculture and livestock industries. While we have infrastructure in place to alleviate the burden of EE2 pollution caused by human urine (i.e. wastewater treatment facilities), we currently have no way of mitigating

the entry of livestock urine into groundwater, or leaching of estrogen rich manure into farming fields. Regulating the use of hormones in livestock, as well as fertilizer usage and cleanup within agriculture, are critical goals to focus on.

The lack of a larger perspective on science and its connection to society is indicative of a greater issue in science, namely the narrow scope by which we collectively consider the implications of our research, and simultaneously overlook or ignore its social and cultural implications. My hope is that scientists currently coming of age will normalize taking the time to understand that every scientific research project has an impact on the lives of people and the environment. It is our responsibility as scientists to actively think about and take steps to identify and mitigate social harms that our research can cause. We need to do a better job at learning how to communicate our science at a level that is accessible and impactful. We have a social responsibility as scientists to consider how our research is interpreted by the general public, rather than thinking the science simply speaks for itself.

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Appendix: Supplementary Data

Chapter 6

Correlations

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	1.0000	0.9215	0.9953	-0.2829	-0.1340	0.3010
Absolute Ucrit (cm/s)	0.9215	1.0000	0.9098	0.0887	0.1406	0.0302
Trial Duration (min)	0.9953	0.9098	1.0000	-0.2952	-0.1475	0.3145
Length (cm)	-0.2829	0.0887	-0.2952	1.0000	0.7702	-0.6597
Weight (g)	-0.1340	0.1406	-0.1475	0.7702	1.0000	-0.0759
Condition Factor (K)	0.3010	0.0302	0.3145	-0.6597	-0.0759	1.0000

The correlations are estimated by Row-wise method.

Correlation Probability

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	<.0001	<.0001	<.0001	0.0057	0.1978	0.0032
Absolute Ucrit (cm/s)	<.0001	<.0001	<.0001	0.3952	0.1766	0.7725
Trial Duration (min)	<.0001	<.0001	<.0001	0.0039	0.1559	0.0020
Length (cm)	0.0057	0.3952	0.0039	<.0001	<.0001	<.0001
Weight (g)	0.1978	0.1766	0.1559	<.0001	<.0001	0.4673
Condition Factor (K)	0.0032	0.7725	0.0020	<.0001	0.4673	<.0001

Scatterplot Matrix

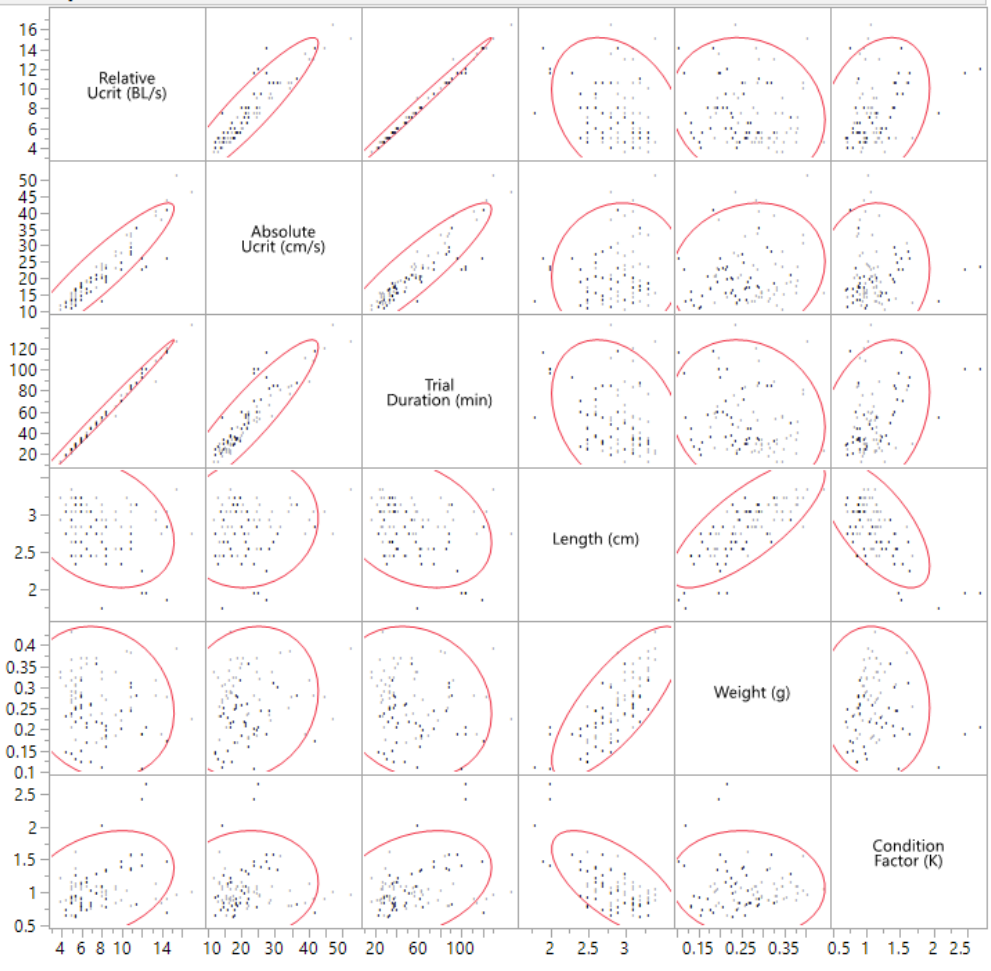

Figure S.1. Multivariate analysis of relative Ucrit (BL/s), absolute Ucrit (cm/s), trial duration (min), length (cm), weight (g), and condition factor (K), of entire experimental population after five months of exposure to EE2. n=30 for control group; n=21 for 1 ng/L, 10 ng/L and 25 ng/L exposure groups.

Correlations

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	1.0000	0.9727	0.9851	-0.0199	0.0441	0.0295
Absolute Ucrit (cm/s)	0.9727	1.0000	0.9612	0.2047	0.2113	-0.0714
Trial Duration (min)	0.9851	0.9612	1.0000	-0.0095	0.0230	-0.0065
Length (cm)	-0.0199	0.2047	-0.0095	1.0000	0.7054	-0.5195
Weight (g)	0.0441	0.2113	0.0230	0.7054	1.0000	0.2225
Condition Factor (K)	0.0295	-0.0714	-0.0065	-0.5195	0.2225	1.0000

The correlations are estimated by Row-wise method.

Correlation Probability

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	<.0001	<.0001	<.0001	0.9168	0.8171	0.8768
Absolute Ucrit (cm/s)	<.0001	<.0001	<.0001	0.2778	0.2623	0.7075
Trial Duration (min)	<.0001	<.0001	<.0001	0.9604	0.9039	0.9729
Length (cm)	0.9168	0.2778	0.9604	<.0001	<.0001	0.0033
Weight (g)	0.8171	0.2623	0.9039	<.0001	<.0001	0.2373
Condition Factor (K)	0.8768	0.7075	0.9729	0.0033	0.2373	<.0001

Scatterplot Matrix

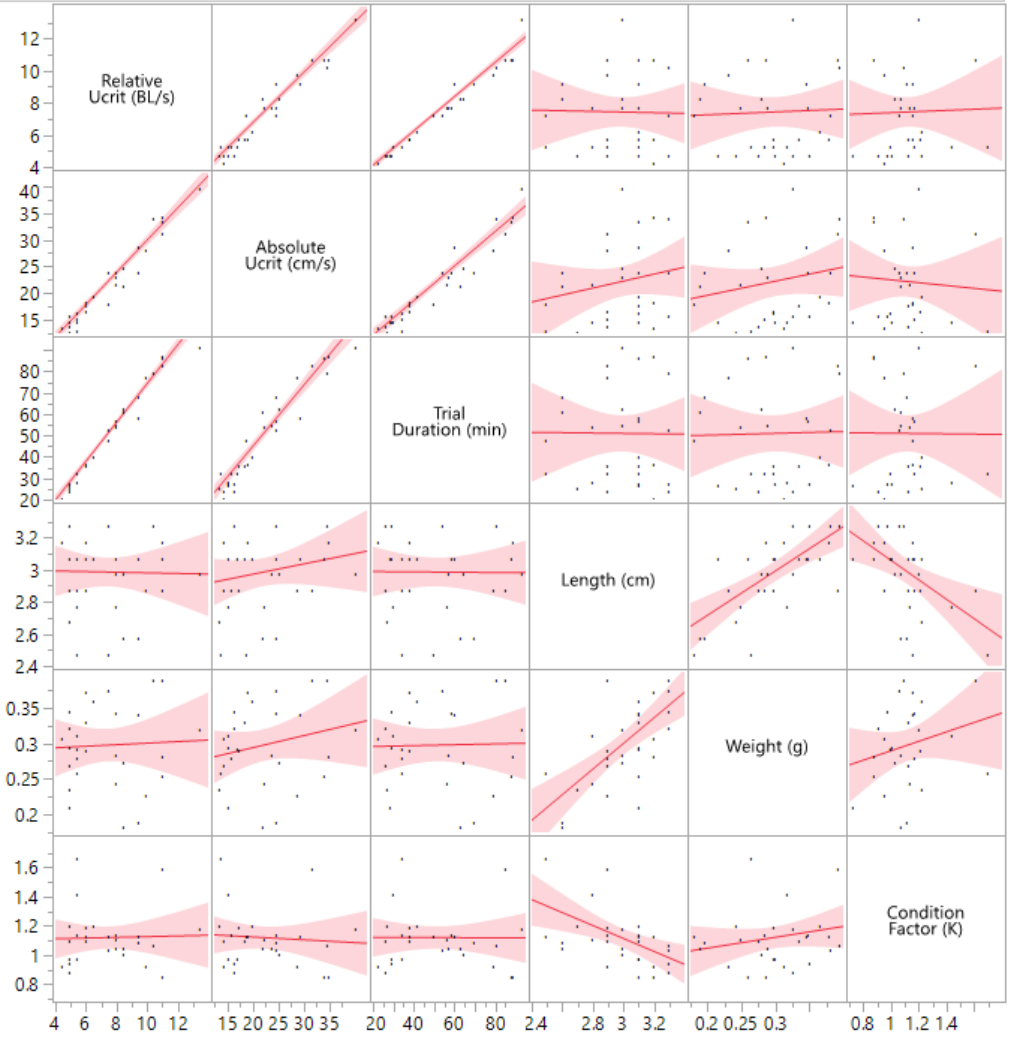

Figure S.2. Multivariate analysis of relative Ucrit (BL/s), absolute Ucrit (cm/s), trial duration (min), length (cm), weight (g), and condition factor (K) for the control group, Generation 1. n=30.

Correlations

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	1.0000	0.9841	0.9993	-0.1751	-0.2567	-0.2205
Absolute Ucrit (cm/s)	0.9841	1.0000	0.9833	-0.0156	-0.1091	-0.2434
Trial Duration (min)	0.9993	0.9833	1.0000	-0.1738	-0.2574	-0.2238
Length (cm)	-0.1751	-0.0156	-0.1738	1.0000	0.8926	-0.2210
Weight (g)	-0.2567	-0.1091	-0.2574	0.8926	1.0000	0.2325
Condition Factor (K)	-0.2205	-0.2434	-0.2238	-0.2210	0.2325	1.0000

The correlations are estimated by Row-wise method.

Correlation Probability

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	<.0001	<.0001	<.0001	0.4358	0.2489	0.3240
Absolute Ucrit (cm/s)	<.0001	<.0001	<.0001	0.9451	0.6288	0.2750
Trial Duration (min)	<.0001	<.0001	<.0001	0.4393	0.2476	0.3168
Length (cm)	0.4358	0.9451	0.4393	<.0001	<.0001	0.3230
Weight (g)	0.2489	0.6288	0.2476	<.0001	<.0001	0.2977
Condition Factor (K)	0.3240	0.2750	0.3168	0.3230	0.2977	<.0001

Scatterplot Matrix

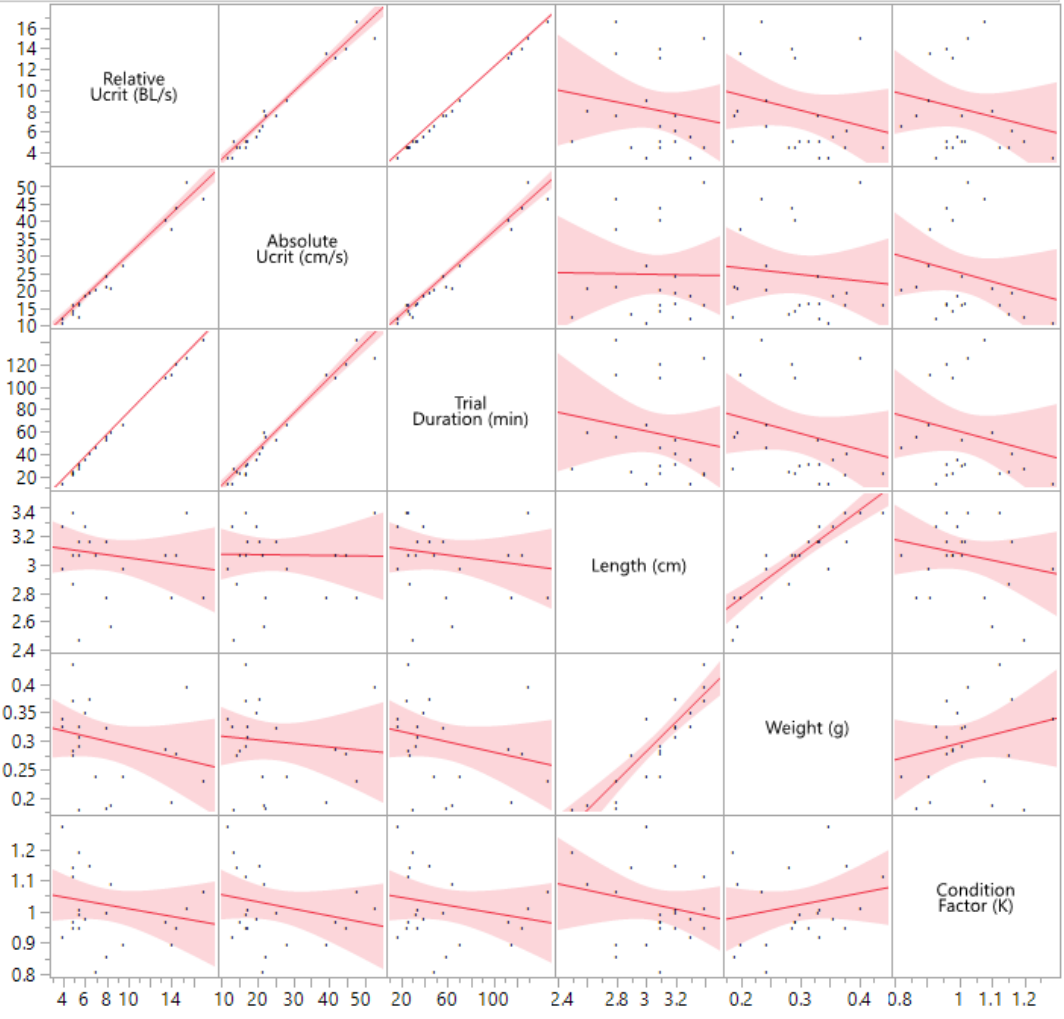

Figure S.3. Multivariate analysis of relative Ucrit (BL/s), absolute Ucrit (cm/s), trial duration (min), length (cm), weight (g), and condition factor (K) for the 1 ng/L EE2 exposure group, Generation 1. n=21.

Correlations

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	1.0000	0.9721	0.9985	-0.1746	-0.0842	0.1126
Absolute Ucrit (cm/s)	0.9721	1.0000	0.9730	0.0558	0.0936	0.0733
Trial Duration (min)	0.9985	0.9730	1.0000	-0.1633	-0.0683	0.1241
Length (cm)	-0.1746	0.0558	-0.1633	1.0000	0.7626	-0.1708
Weight (g)	-0.0842	0.0936	-0.0683	0.7626	1.0000	0.4980
Condition Factor (K)	0.1126	0.0733	0.1241	-0.1708	0.4980	1.0000

The correlations are estimated by Row-wise method.

Correlation Probability

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	<.0001	<.0001	<.0001	0.4491	0.7168	0.6269
Absolute Ucrit (cm/s)	<.0001	<.0001	<.0001	0.8102	0.6866	0.7521
Trial Duration (min)	<.0001	<.0001	<.0001	0.4793	0.7685	0.5919
Length (cm)	0.4491	0.8102	0.4793	<.0001	<.0001	0.4593
Weight (g)	0.7168	0.6866	0.7685	<.0001	<.0001	0.0216
Condition Factor (K)	0.6269	0.7521	0.5919	0.4593	0.0216	<.0001

Scatterplot Matrix

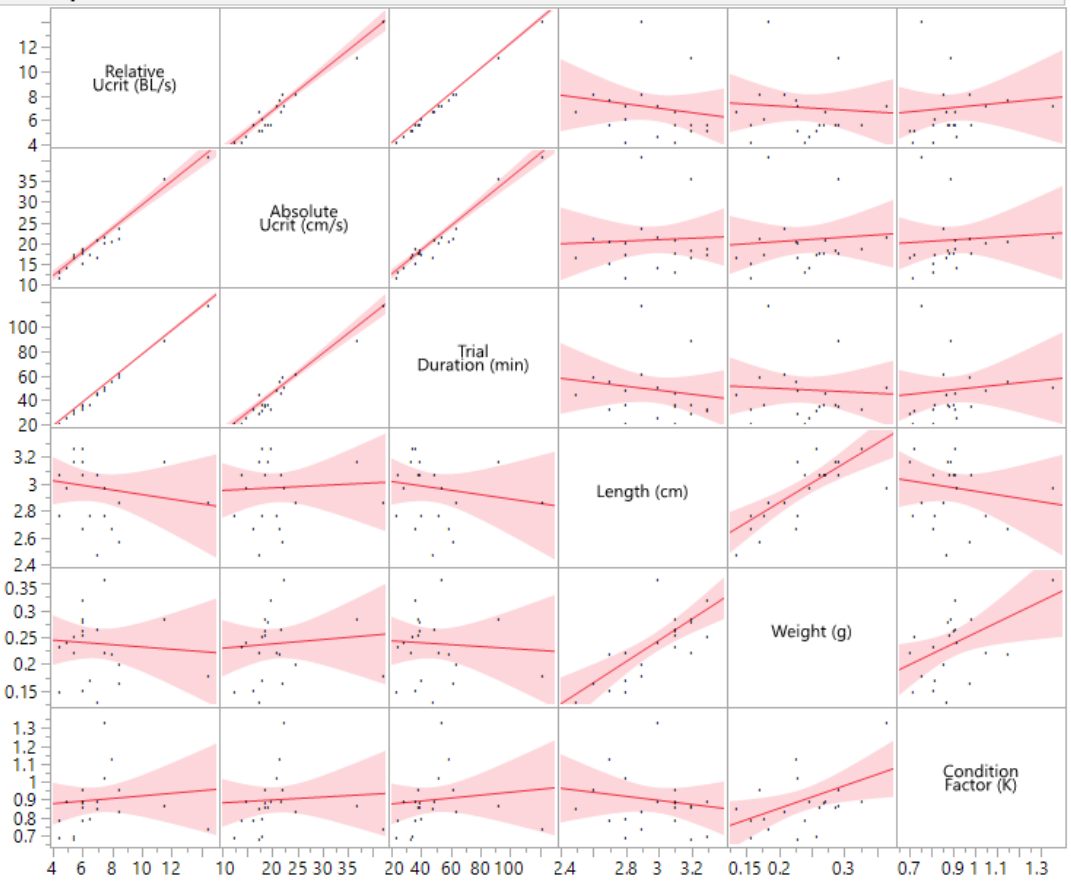

Figure S.4. Multivariate analysis of relative Ucrit (BL/s), absolute Ucrit (cm/s), trial duration (min), length (cm), weight (g), and condition factor (K) for the 10 ng/ L EE2 exposure group, Generation 1. n=21.

Multivariate

Correlations

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	1.0000	0.8796	0.9970	-0.5056	0.0070	0.6363
Absolute Ucrit (cm/s)	0.8796	1.0000	0.8685	-0.0498	0.4171	0.4061
Trial Duration (min)	0.9970	0.8685	1.0000	-0.5211	0.0072	0.6666
Length (cm)	-0.5056	-0.0498	-0.5211	1.0000	0.7352	-0.6385
Weight (g)	0.0070	0.4171	0.0072	0.7352	1.0000	0.0209
Condition Factor (K)	0.6363	0.4061	0.6666	-0.6385	0.0209	1.0000

The correlations are estimated by Row-wise method.

Correlation Probability

	Relative Ucrit (BL/s)	Absolute Ucrit (cm/s)	Trial Duration (min)	Length (cm)	Weight (g)	Condition Factor (K)
Relative Ucrit (BL/s)	<.0001	<.0001	<.0001	0.0194	0.9758	0.0019
Absolute Ucrit (cm/s)	<.0001	<.0001	<.0001	0.8304	0.0600	0.0678
Trial Duration (min)	<.0001	<.0001	<.0001	0.0154	0.9754	0.0010
Length (cm)	0.0194	0.8304	0.0154	<.0001	0.0001	0.0018
Weight (g)	0.9758	0.0600	0.9754	0.0001	<.0001	0.9283
Condition Factor (K)	0.0019	0.0678	0.0010	0.0018	0.9283	<.0001

Scatterplot Matrix

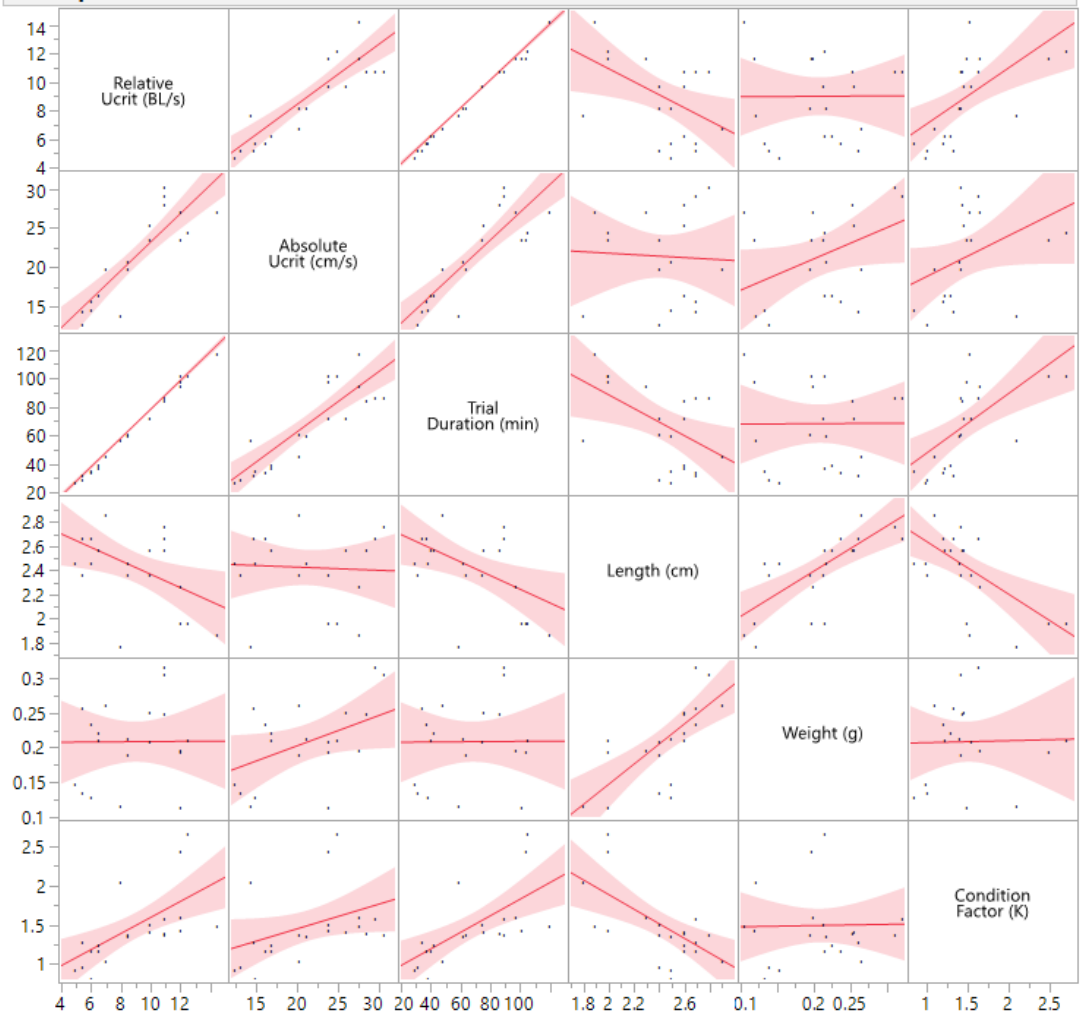

Figure S.5. Multivariate analysis of relative Ucrit (BL/s), absolute Ucrit (cm/s), trial duration (min), length (cm), weight (g), and condition factor (K) for the 25 ng/ L EE2 exposure group, Generation 1. n=21.

Chapter 7

Table S.1. Summarized statistically significant findings from a five-month exposure period to EE2. Survival, length, weight, condition factor (k), clutch size, and hatch success are shown for one generation of exposure to 10ng/L, and 25ng/L EE2, as well as three generations of exposure to 1ng/L EE2. Findings indicate a statistically significant difference from control, where '↓' indicates a decrease, and '↑' indicates an increase; ''-' indicates that there was no significant difference between exposure group and control, 'N/A' indicates that data could not be collected.

Exposure Group	**1ng/L EE2 Gen 1**	**1ng/L EE2 Gen 2**	**1ng/L EE2 Gen 3**	**10ng/L EE2 Gen 1**	**25ng/L EE2 Gen 1**
Survival	-	-	-	-	↓
Length	-	↑	-	-	↓
Weight	-	↑	-	↓	↓
K	-	-	-	↓	↑
Clutch Size	↑	↑	-	N/A	N/A
Hatch Success	↓	↓	↓	N/A	N/A

Table S.2. Summarized statistically significant findings for three strains of zebrafish (AB, TU, and WIK) from a five-month exposure period to EE2. Survival, length, weight, condition factor (k), clutch size, and hatch success are shown for one generation of exposure to 10ng/L, and 25ng/L EE2, as well as three generations of exposure to 1ng/L EE2. Values given are a statistically significant percentage increase or decrease in the parameter as compared to control, where '↓' indicates a decrease, and '↑' indicates an increase; '-' indicates that there was no significant difference between exposure group and control, and 'N/A' indicates that data could not be collected.

Parameter	**Exposure Group**	**AB**	**TU**	**WIK**
Survival	*1ng/L EE2 Gen 1*	-	-	-
	1ng/L EE2 Gen 2	-	-	-
	1ng/L EE2 Gen 3	-	-	-
	10ng/L EE2 Gen 1	-	-	-
	25ng/L EE2 Gen 1	N/A	N/A	N/A
Length	*1ng/L EE2 Gen 1*	-	-	-
	1ng/L EE2 Gen 2	-	-	9.09%↑
	1ng/L EE2 Gen 3	-	-	-
	10ng/L EE2 Gen 1	-	-	-
	25ng/L EE2 Gen 1	26.23% ↓	20.57% ↓	17.47% ↓
Weight	*1ng/L EE2 Gen 1*	-	-	-
	1ng/L EE2 Gen 2	-	-	29.41% ↑
	1ng/L EE2 Gen 3	-	-	-
	10ng/L EE2 Gen 1	-	26.48% ↓	22.58% ↓
	25ng/L EE2 Gen 1	26.93% ↓	29.42% ↓	35.49% ↓
Clutch Size	*1ng/L EE2 Gen 1*	-	-	-
	1ng/L EE2 Gen 2	242.54% ↑	467.43% ↑	441.52% ↑
	1ng/L EE2 Gen 3	-	-	-
Hatch Success	*1ng/L EE2 Gen 1*	71.25% ↓	62.93%↓	86.71% ↓
	1ng/L EE2 Gen 2	59.27% ↓	83.85% ↓	42.24% ↓
	1ng/L EE2 Gen 3	38.38% ↓	77.96% ↓	65.65% ↓