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Embryological Development and Mortality of *Danio rerio* in Long Term Exposure to 17α-Ethynylestradiol

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Abstract

Synthetic estrogens as well as other chemicals have become an increasing issue in the impact they have on marine species particularly those sensitive to the environment. A broadly used synthetic estrogen, 17α-ethynylestradiol, has become more widely known to become a growing problem to the ecology of marine wildlife due to increased usage and lack of removal in municipal sewage treatment facilities. This study observes the impact of low level EE2 in embryos following generational exposure. Zebrafish embryos, following three generations of exposure, were observed in rate of mortality, lethality, and hatch success. Concentrations as low as 1ng/L of EE2 has shown to have significant impact in the survival of zebrafish embryos. Removal of EE2 has shown ability of some recovery in zebrafish pointing to the possibility of recovery in areas impacted by EE2 pollution.

Dedication

I dedicate this to my mom and to my best friends, Jasmine Torres and Aye Myat Htoo Mon.

Acknowledgements

I would like to acknowledge my advisor Kim Brown, and the team, Decatur Foster and Emily Morse for their support and giving me the opportunity in being part of the team. I would also like to acknowledge my mother and my greatest friends, Jasmine Torres and Aye Myat Too Mon, for pushing me to always try my best and get out of my comfort zone. I appreciate all that they have done for me in becoming a better researcher and person.

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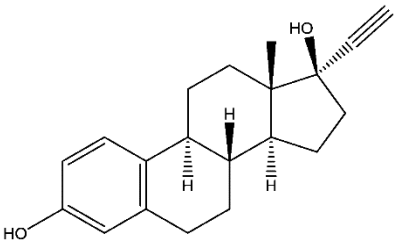
Introduction

Since the beginning of the Anthropocene humans have caused more environmental damage than any other species in history. These changes encompass a broad range of activities including the burning of fossil fuels, deforestation, and direct environmental contamination to both land and water. In aquatic ecosystems the introduction of various chemicals, directly or indirectly, is a major contributor to declines. Endocrine disrupting chemicals are one such class which included synthetic and natural estrogens. Estrogen is an important chemical in development and is crucial for proper bone and sexual development [1]. The importance of estrogen is so profound in animals that estrogen receptors have been identified even in invertebrate species, indicating a long-standing function for this hormone [17]. While all vertebrates are known to produce their own biological estrogen, synthetically produced estrogens, including 17α-ethynylestradiol (EE2) which is used for birth control and other medical treatments, are taken up when in the environment and can negatively affect organisms [1].

Synthetic representatives of estrogen are categorized under endocrine disrupting compound (EDCs). EDC are type of chemicals that are able to alter or disrupt the endocrine system in normally functioning organisms [1]. These chemicals interrupt the normal processes by inhibiting or modifying the production of endogenous hormones and their receptors [1]. Synthetic estrogen is classified as a type of estrogenic chemical (EC) which have been greatly studied due to its impacts in aquatic ecosystems [1,2,3,4]. Different types of ECs can be found in large concentrations with sewage effluents being the greatest source since the Anthropocene [3,5].

Figure 1. Molecular structure of 17 α-ethynylestradiol (EE2)



Of these ECs, 17 α-ethynylestradiol (EE2) has high potency, higher affinity, longer half-life, and ability to concentrate in tissues [1]. It is commonly found in birth control pills, menopausal medications and in livestock [6]. Given the importance and increased use of these synthetic estrogens for human contraception, their concentrations in aquatic environments have correspondingly increased due to our inability to properly filter these chemicals in sewage treatment facilities [1,2,5,6,7,10]. Copious amounts of deactivated EE2 is flushed into the environment largely through excretion from humans and livestock [5]. Due to bacteria, formerly inactivated EE2 can be reactivated in water treatment plants [7]. During this process, the compound can be trapped in sewage sludge preventing the further breaking down of this chemical [8]. Compared to its sister compounds, it is both more stable and long living [8] making it more difficult to degrade and deposits into sediments allowing large concentrations in areas where sewage meets the outside environment [9].

Approximately 700kg per year of synthetic estrogen is dumped into the environment from contraceptives [5] and the actual amount in the water can greatly vary from 0.05-830ng/L in surface waters [11]. Studies have reported as high as 830ng/L in the rivers of US [8]. There have been concerns raised over the concentration as previous studies report that concentrations as low as 1ng/L can affect the survival rate of male fish and offspring [9].

In fish, there is a two-fold higher binding affinity for EE2 compared to biologically produced estrogen which also causes a greater response than natural estrogen [1]. This higher binding affinity results in a potency that is about 10-50 times greater than natural estrogen [10] which can have major impacts to aquatic ecosystem. Known effects include shifts in sex ratio, decreased reproduction and clutch size, changes in reproductive behaviors, and reductions in male gonadal development [2,4,6,7,8,9,10,12,13,14].

Zebra fish (*Danio rerio*) is a model organism extensively used for observing embryological development given their transparent eggs, ease of spawning, large clutch size, short life span, ability to breed continuously, and rapid development time, approximately 72 hours from fertilization to hatching [,12]. Given their rapid development they are commonly used to analyze the effect of various chemical compounds during early development [12]. Such developmental studies are aided by zebrafish's extensively detailed embryological developmental stage maps. Identification of zebrafish stages in eggs are possible due to the transparent membranes surrounding the embryo which allow for close microscopic observation and determination of cell abnormality based on morphology and cell apoptosis [12].

This study evaluated the embryological development and mortality among multiple zebrafish strains following three generations of lifetime exposure to EE2. The goal of the study was to observe and identify the extent of the embryological developmental changes resulting from long-term 17α-ethynylestradiol exposure and characterize strain variation and differential mortality. Previous studies have shown that the effects of estrogen on development and mortality typically occurs between 8 and 24 hours post fertilization (hpf) in single generation exposures [4]. The concentration used in this experiment fall within the range (0.05-831 ng/L) found in U.S. surface waters, thus mimicking potential environmental effects [4]. With the growing concerns about

environmental pollution, this study aims to establish the effects of EE2 on embryonic survival and success following multi-generational exposure.

Methods and Materials

Three strains of zebrafish were used in this experiment, AB, WIK and Tübingen (TU). Embryos were collected from adult zebrafish exposed to either methanol (MeOH; control fish) or 1ng/L EE2 over three (3) complete generations from a parallel experiment [18] In addition to embryos from exposed parents, embryos from 5 adult unexposed AB pairs (5 males and 5 females) and 200 embryos of each WIK and TU obtained from Zebrafish International Resource Center. All zebrafish husbandry and experiments were maintained following the guidelines by Holden and Brown (2018) and in accordance to the Institutional Animal Care and Use Committee (IACUC) of Portland State University [15].

Adult fish from each group were paired and placed in breeding containers with removable barriers to separate the male and female until just prior to spawning. The breeding containers have false bottoms with slits to allow fertilized eggs to pass through.

Mature Zebrafish were placed in breeding containers overnight separated by a divider with a removable mesh. Containers were filled to one inch from the bottom using water from the zebrafish housing system. One female and male were placed in each side of the container and covered with a lid. Containers were labeled and dated before being left overnight. At 7:00 AM the dividers were removed, and fish were allowed to court and breed for one (1) hour. After one (1) hour containers were checked and in containers where breeding had not occurred pairs were given additional increments of 15 minutes for successful breeding for a max of two (2) total hours. Once bred, the fish are carefully taken out of the container using a fish net and are put

back in their respective tanks with the spawning time recorded. The eggs were collected using a strainer for respective type (i.e., TU, WIK, AB) and the containers were flushed with embryo media to remove all eggs. The eggs were strained and washed a second time with embryo media[18] or 1ng/L EE2 solution. Harvested eggs were placed in petri dishes filled with embryo media or 1ng/L EE2 solution. Each egg was then placed in a single well of a labeled (date, strain, time of fertilization) 96-well plate filled with embryo media and placed in an incubator at 28.5°C. Every hour for the first 8 hours, embryos are observed under a stereo microscope (Zeiss Stemi 2000-C) to assess for dead eggs and the presence abnormalities. Wells containing dead or abnormal embryos were marked and the hour the condition was initially observed was noted. Mortality rates were recorded each hour. Critical timepoints in development include first cleavage (1hpf-2hpf), blastula formation (2hpf-5hpf), gastrulation (5hpf-10hpf), segmentation (10hpf-24hpf), pharyngula (24hpf-48hpf), and hatching (48-72hpf) [16]. Abnormalities observed during each developmental stage were recorded for each trial. After the first 8 hourly observations embryos were evaluated at 24hpf, 48hpf, and 72hpf. At the completion of 72 hpf the number of successfully and unsuccessfully hatched fish were recorded.

Results and Conclusion

Current water pollution in US rivers far exceed the 1ng/L EE2 in addition to other EDCs. From the exposure of long term EE2, results show higher rates of morphological abnormality, lower rate of hatch success as well as a higher rate of death in the 1ng/L 3rd Gen EE2 group compared to the naïve control.

Table 1.1 Time of Onset Abnormality of 1 ng/L 3rd Gen EE2 v. Naïve

			Times of Onset Morphologic Abnormality (frequency)											
Exposure	Strain Type	Total # of Individuals	1 (hpf)	2 (hpf)	3 (hpf)	4 (hpf)	5 (hpf)	6 (hpf)	7 (hpf)	8 (hpf)	24 (hpf)	48 (hpf)	72 (hpf)	Total Abnormality
1ng/L 3rd Gen EE2	TU	96	0.417	0.469	0.469	0.469	0.479	0.479	0.489	0.489	0.499	0.509	0.542	0.542
	AB	168	0.625	0.750	0.792	0.810	0.810	0.810	0.810	0.810	0.810	0.810	0.821	0.821
	WIK	96	0.510	0.552	0.552	0.552	0.552	0.552	0.552	0.552	0.562	0.562	0.573	0.573
	Total	360	0.539	0.622	0.641	0.649	0.652	0.652	0.655	0.655	0.661	0.664	0.681	0.681
Naïve	TU	168	0.101	0.119	0.137	0.137	0.137	0.137	0.137	0.137	0.137	0.137	0.137	0.137
	AB	96	0.063	0.167	0.209	0.209	0.209	0.209	0.209	0.209	0.251	0.303	0.344	0.344
	WIK	96	0.052	0.146	0.188	0.188	0.188	0.188	0.188	0.188	0.198	0.198	0.198	0.198
	Total	360	0.078	0.139	0.170	0.170	0.170	0.170	0.170	0.170	0.184	0.198	0.208	0.208

Table 1.1 shows the accumulative frequency of abnormal morphology per strain type per hour for 1ng/L 3rd Gen EE2 versus Naïve. The first two hours post fertilization show the highest increase in frequency for onset abnormality in both groups. The total frequency for abnormality in 1ng/L 3rd Gen EE2 is 0.681 and the naïve control has a total frequency at 0.208.

Figure 2. Time of Onset Morphological Abnormality for 1ng/L 3rd Gen v. Naïve (Frequency)

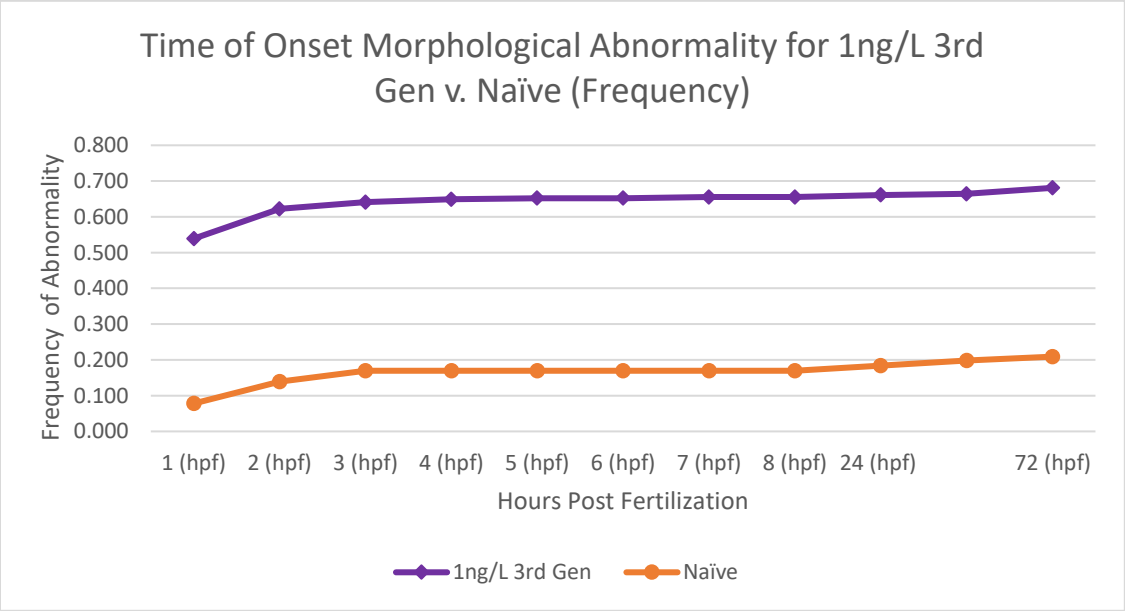


Figure 2 plots the accumulative frequency of abnormal morphology in 1ng/L 3rd EE2 v. Naïve per hour. The frequency of the rate of abnormality at each hour for 1ng/L 3rd Gen v. Naïve shows a significance in the rate of mutation from the generational exposure to 1ng/L EE2.

T able 1.2 Rate of Mortality by Hour in Frequency of 1ng/L 3rd Gen v. Naïve

Rate of Mortality by Hour (Frequency)													
Exposure	Strain Type	Total # of Individuals	1 (hpf)	2 (hpf)	3 (hpf)	4 (hpf)	5 (hpf)	6 (hpf)	7 (hpf)	8 (hpf)	24 (hpf)	48 (hpf)	72 (hpf)	Total Death
1ng/L 3rd Gen EE2	TU	96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.500	0.500	0.500	0.500
	AB	168	0.000	0.000	0.000	0.006	0.006	0.006	0.006	0.006	0.786	0.786	0.786	0.786
	WIK	96	0.000	0.000	0.000	0.000	0.021	0.063	0.094	0.198	0.563	0.563	0.563	0.563
	Total	360	0.000	0.000	0.000	0.003	0.009	0.020	0.028	0.061	0.650	0.650	0.650	0.650
Naïve	TU	168	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.137	0.137	0.137	0.137
	AB	96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.240	0.240	0.250	0.250
	WIK	96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.188	0.188	0.188	0.188
	Total	360	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.178	0.178	0.181	0.181

Table 1.2 shows the rate of mortality by hour from the exposure to EE2. The rate of mortality is highest between 8 hpf-24 hpf. The overall total death in 1ng/L 3rd Gen EE2 is significantly higher than the control. The earliest time of death for 1ng/L 3rd Gen is at 4 hpf, and for naïve, it is between 8 hpf-24 hpf.

The exposure of EE2 has shown earlier times of cell aptosis. Sample embryos show deaths starting as early as four hours post fertilization (Table 1.2). The naïve groups have earliest deaths starting at 7 hours post fertilization (Table 1.2). While hour of highest death cannot be determined, it is approximated within the 8-24 hours post fertilization stage when cell speciation occur (Table 1.2, 2.2).

Figure 3. Rate of Mortality per Strain for 1ng/L 3rd Gen v. Naïve

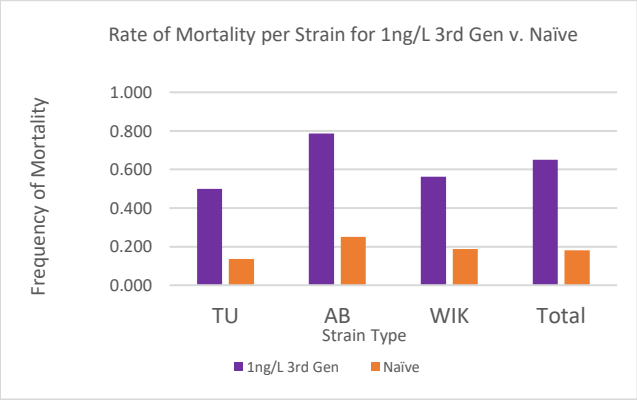


Figure 3 shows overall mortality per strain for 1ng/L 3rd EE2 and naïve group. All strains exposed with 1ng/L EE2 depicts a higher rate of mortality compared to the naïve group. The 1ng/L 3rd Gen EE2 does show an earlier time of death and an overall higher rate of death with AB strain resulting in the highest rate of death.

Table 1.3 Ratio of Survival in Frequency of 1ng/L 3rd Gen v. Naïve

Ratio of Survivial (frequency)				
Exposure	Strain Type	Normal	Mutative	Dead
1ng/L 3rd Gen	TU	0.458	0.042	0.500
	AB	0.179	0.036	0.786
	WIK	0.427	0.010	0.563
	Total	0.319	0.031	0.650
Naïve	TU	0.696	0.000	0.137
	AB	0.656	0.094	0.250
	WIK	0.979	0.010	0.188
	Total	0.761	0.028	0.181

Table 1.3 show the overall survival of both exposure groups. Generational exposure of 1ng/L EE2 is shown with higher rates of death. The impact of EE2 has a higher rate of mortality than resulting of abnormal morphology in hatchlings perhaps indicating EE2 effect in earlier stages of development leading to mortality.

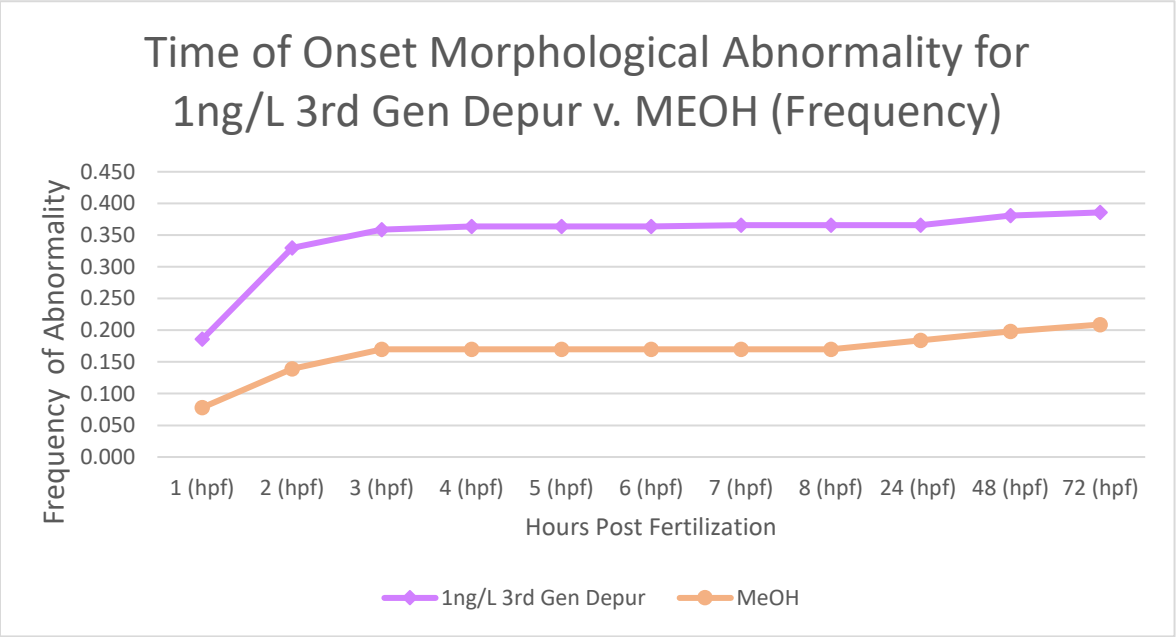
Table 2.1 Time of Onset Abnormality of 1ng/L 3rd Gen Depur v. MeOH

Time of Onset Morphological Abnormality (Frequency)														
Exposure	Strain Type	Total # of Individuals	1 (hpf)	2 (hpf)	3 (hpf)	4 (hpf)	5 (hpf)	6 (hpf)	7 (hpf)	8 (hpf)	24 (hpf)	48 (hpf)	72 (hpf)	Total Abnormality
1ng/L 3rd Gen Depur	TU	127	0.189	0.220	0.236	0.236	0.236	0.236	0.236	0.236	0.236	0.244	0.252	0.252
	AB	121	0.231	0.619	0.669	0.686	0.686	0.686	0.694	0.694	0.694	0.727	0.727	0.727
	WIK	161	0.149	0.199	0.224	0.224	0.224	0.224	0.224	0.224	0.224	0.230	0.236	0.236
	Total	409	0.186	0.330	0.359	0.364	0.364	0.364	0.366	0.366	0.366	0.381	0.386	0.386
MeOH	TU	140	0.579	0.636	0.643	0.643	0.643	0.643	0.643	0.643	0.664	0.693	0.700	0.700
	AB	96	0.354	0.406	0.406	0.406	0.406	0.406	0.406	0.406	0.416	0.426	0.447	0.448
	WIK	113	0.124	0.133	0.133	0.133	0.133	0.133	0.133	0.133	0.133	0.151	0.151	0.150
	Total	349	0.370	0.370	0.370	0.370	0.370	0.370	0.370	0.370	0.370	0.370	0.370	0.453

In Table 2.1, the total abnormality of 1ng/L 3rd gen depur is not significant to the control.

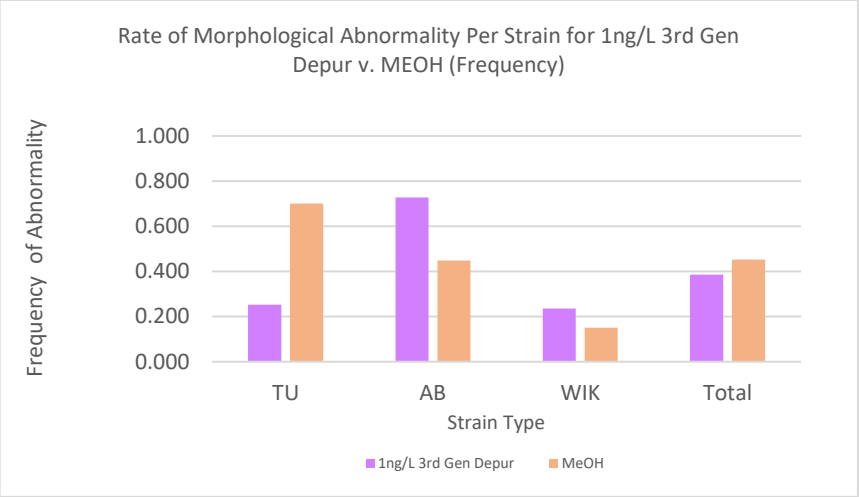
However, the AB strain in both the 1ng/L 3rd Gen and 1 ng/L 3rd Gen depur have a higher rate of mutation compared to the WIK and TU strains. In the MeOH control group, TU shows the highest rate of abnormality at 0.700.

Figure 4. Time of Onset Morphological Abnormality for 1ng/L 3rd Gen Depur v. MeOH (Frequency)



The highest rate of abnormality was in the first two hours post fertilization for all four groups. While the rate of abnormality is higher in the 1ng/L 3rd Gen EE2 Depur, the rate has decreased following the removal of EE2.

Figure 5. Rate of Morphological Abnormality per Strain for 1 ng/L 3rd Gen Depur v. MeOH



The rate of mutation was overall higher in the 1ng/L 3rd Gen Depur than the control. After depurification, the 1ng/L 3rd Gen. Depur is not statistically significant from the control. TU shows a higher morphological abnormality in the MeOH that the 1ng/L 3rd Gen EE2 depur. The AB and WIK strains in the 1ng/L 3rd Gen EE2 Depur has a slightly higher rate of mutation.

Table 2.2 Rate of Mortality by Hour in Frequency of 1ng/L 3rd Gen v. MeOH

Rate of Mortality by Hour (Frequency)													
Exposure	Strain Type	1 (hpf)	2 (hpf)	3 (hpf)	4 (hpf)	5 (hpf)	6 (hpf)	7 (hpf)	8 (hpf)	24 (hpf)	48 (hpf)	72 (hpf)	Total Death
1ng/L 3rd Gen Depur	TU	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.236	0.236	0.236	0.236
	AB	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.008	0.669	0.669	0.669	0.669
	WIK	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.012	0.224	0.224	0.224	0.224
	Total	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.007	0.359	0.359	0.359	0.359
MeOH	TU	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.650	0.657	0.657	0.657
	AB	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.062	0.417	0.417	0.417	0.417
	WIK	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.133	0.133	0.133	0.133
	Total	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.018	0.419	0.421	0.421	0.421

The rate of mortality following depurification lowered. The earliest time of death for the 1ng/L 3rd Gen Depur and MeOH is at 7 hpf. The rate of mortality is highest between the 8hfp-24hpf for both 1ng/L 3rd Gen EE2 and MeOH.

Figure 6. Rate of Mortality per Strain for 1ng/L 3rd Gen Depur v. MeOH

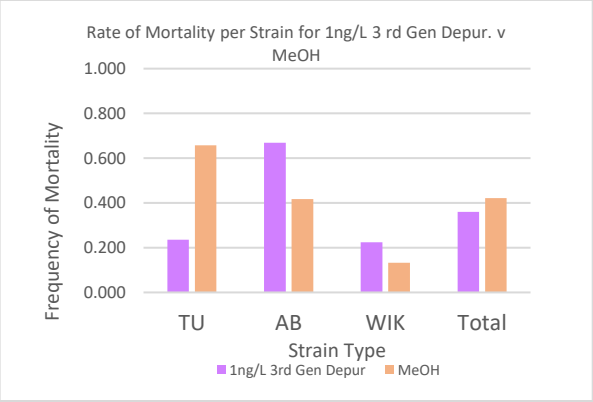


Figure 6 depicts the rate of mortality per strain for 1ng/L 3rd Gen EE2 Depur and MeOH. The overall death was higher in the MeOH compare with the 1ng/L EE2 Depur. Both the AB and WIK strain exposed with MeOH has a lower rate of mortality where as the TU strain has a higher rate of mortality.

Table 2.3 Ratio of Survival in Frequency of 1ng/L 3rd Gen Depur v. MeOH

Ratio of Survivial (Frequency)				
Exposure	Strain Type	Normal	Mutative	Dead
1ng/L 3rd Gen Depur	TU	0.748	0.016	0.236
	AB	0.273	0.058	0.669
	WIK	0.764	0.012	0.224
	Total	0.614	0.027	0.359
MeOH	TU	0.500	0.043	0.657
	AB	0.552	0.031	0.417
	WIK	0.699	0.018	0.133
	Total	0.579	0.032	0.421

Table 2.3 depicts the ratio of survival in frequency for the three strains following depurification and compared to the MeOH exposure. Overall results in the two exposure groups result in similar ratios of survival.

Figure 7. Total Hatch Success

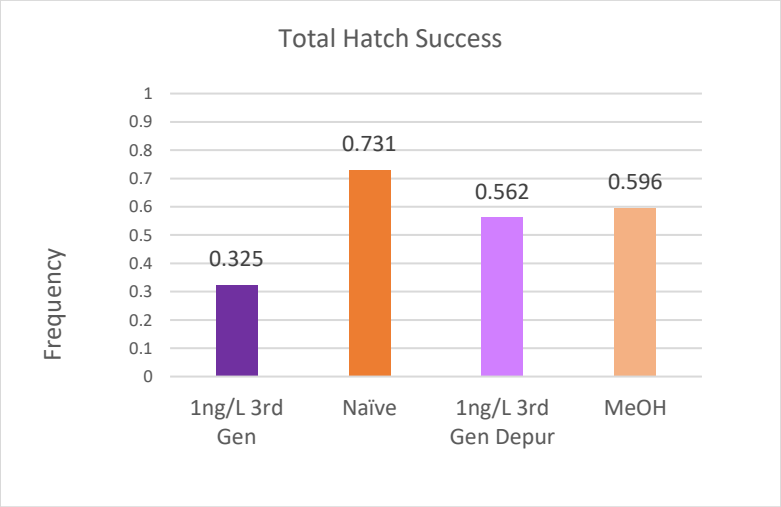
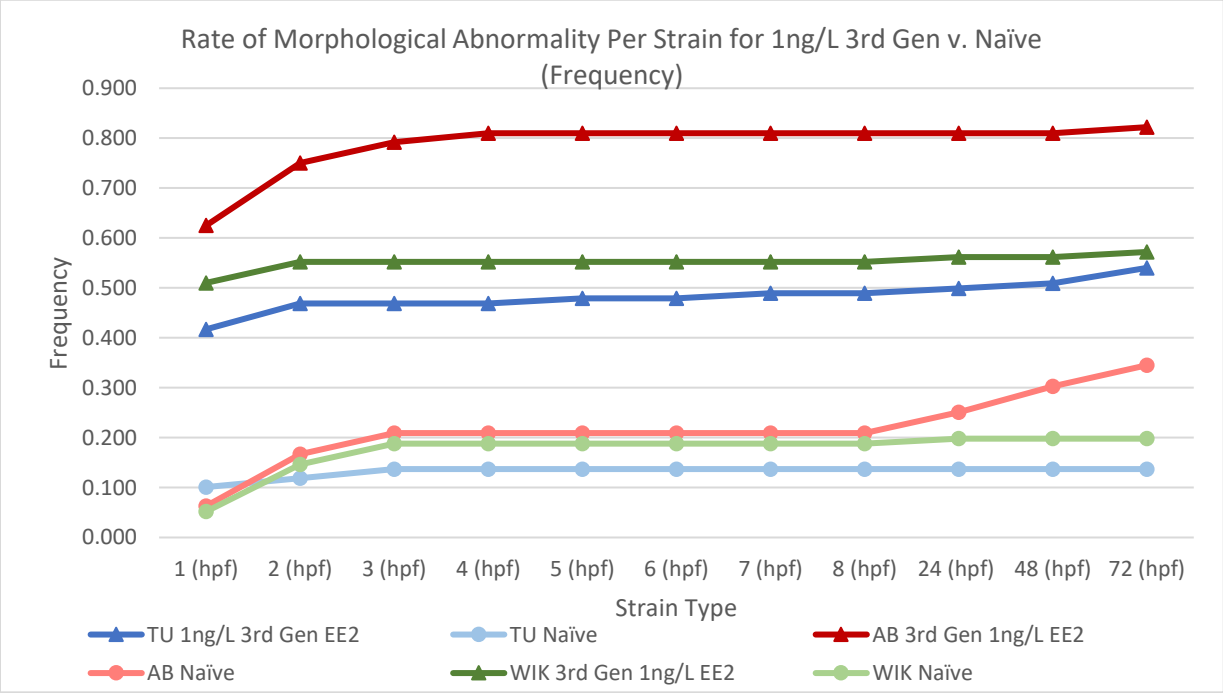


Figure 7 shows the overall hatch success in all four groups. The rate of hatching in 1ng/L 3rd Gen is statistically significantly less than the control. Following depurification, there is recovery in the hatch success for depur and is not statistically significant to the MeOH control.

Figure 8. Rate of Morphological Abnormality per Strain for 1 ng/L 3rd Gen v. Naïve per hour



The rate of morphological abnormality in 1ng/L is higher in all strain types and overall compared to the control. There is significance in the rate of mutation in the 1ng/L 3rd Gen compared to the control.

The time of onset abnormal development is marked highest within the first two hours following fertilization during rapid cell division (Fig 2). Both 1ng/L 3rd Gen EE2 and the naïve control show the highest rate of abnormal morphological development within the first two hours (Table 1.1, 2.1 & Fig. 2, 4) with the higher intensity in the 1ng/L 3rd Gen EE2 at two hours post-fertilization with a frequency of 0.083 (Table 1.1 & Fig. 2).

Exposure of EE2 is indicative to higher rate of mutation leading to death rather than resulting in abnormal phenotypic features. As for abnormal morphological development in the fish, there is no significance between the two groups (Table 1.3, 2.3). In both 1ng/L 3rd EE2 and naïve control group, samples with onset time of abnormality later in the hours post fertilization has shown to survive as the same rate (Table 1.1,2.1). The 1ng/L EE2 attribute to higher rates of embryonic death rather than morphologically abnormal hatchlings (Table 1.3). In all four exposure, the rate of abnormal hatchlings were consistent ranging between 0.027-0.032 in frequency (Table 3.1, 3.2). The lethality of 1ng/L 3^{rd} Gen is significant using a chi square test with a p value of $p<0.0001^*$ (Fig 3) compared to the control.

Following three generations of EE2 exposure, the 1ng/L 3^{rd} Gen group were placed in three months of clean water and compared to the MeOH control group. The observation done following depurification was to determine the ability of recovery from the effects of EE2 in embryos. There was no significance in the data between the 1 ng/L 3rd Gen Depur and the

control MeOH group in both lethality and hatch success using the chi square test with p values of $p>0.335$ and $p>0.401$ respectively (Table 2.1, 2.2, 2.3 & Fig. 5, 6, 7). Three months of depurification has shown recovery in rate of abnormal morphology (Fig. 5), mortality (Fig 6), and hatch success (Fig. 7).

Hatch success was also decreased in the 1 ng/L EE2 3rd Gen fish (Fig. 7). Hatch success was determined as the exit of the chorion. The frequency of hatch success is 0.324 for 1ng/L 3rd Gen EE2 which is statistically significant compared to the naïve group with a frequency of 0.731. The decreased rate of hatch success in 1ng/L 3rd Gen EE2 was significantly higher than the control using the chi square test with a p value of $p<0.0001$* (Table 1.3 & Fig. 7). The frequency of 1ng/L 3rd Gen EE2 Depur is 0.562 which is not statically significant from the frequency of the MeOH group.

Within strain types, AB was determined higher sensitivity to EE2 as rates of abnormal morphology increased in the exposure and after the depurification (Table 1.1, 2.1). The high rate of mutation also coincides with its high rate of death (Table 1.2, 2.2). Likewise, the AB strain had the lowest rate of hatch success (Table 1.3, 2.3). Exposure to 1ng/L EE2 has shown lasting effects in the AB strain as it still had a higher rate of abnormal morphology, death, and hatch success following depurification (Fig. 3, 5, 6, 8). An outlier of the results is the rate of abnormality and death rate in TU exposed to MeOH which is likely attributed to a possible error.

Discussion

In current studies of EE2 in marine species, there is a lack of broad-wide investigation of long-term effects of exposure in embryonic development. Within those studies, there has been a

significance in the effects of embryonic development and hatch success in a variety of marine species.

This study performed an investigation in the morphological developments of *Danio rerio* embryos exposed to three generations of EE2 using 1ng/L EE2 and allowed 3 months depurification.

Comparing the data between the 3rd Gen 1ng/L EE2 control group and naïve group, there is a significance in long term exposure in low concentrations. Exposure to 1ng/L EE2 increases embryological mortality and decreases hatch success of zebrafish embryos. Likewise, the comparison in the data between the 3rd gen 1ng/L Depur and MeOH control group show no significance using the chi square test in the rate of mortality and hatch success indicating that there is possibility of recovery after the removal of EE2 in the water. Depurification shows possible recovery from EE2 effects in embryos.

Of the three strains that were exposed to EE2, AB had the least success, resulting in both the highest mutation during development and in mortality rate. This is not reflected in the data when comparing the Depur and MeOH. Rather, results indicate that TU with lower success in the MeOH control group leading to a possibly in error.

Limitations in this experiment could result in possible influence on the data produced. Due to the age of the MEOH group (5 months) at the start of the study, the control group for the 1ng/L EE2 (3 months) were compared with a naïve group (3 months) which were sourced from a different gene pool. Likewise, unexpected results with TU in the comparison of 1ng/L 3rd Gen Depur and MeOH control group could have possibly stemmed from the limited size of pairs and test size.

Regarding the current EE2 concentration in the environment, it would be necessary to observe the effect of long-term exposure to EE2 in low quantities in other marine species particularly those that reside near or around sewage dumps. Further investigation observing rate of mortality between the 8-24 hours post fertilization is required to make final conclusion on what developmental stage is most impacted by EE2 in regard to cell aptosis and to observe possibility in EE2 resistance in long-term exposure.

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