EFFECTS OF 17α - ETHINYLESTRADIOL ON *DANIO RERIO* HEPATOSOMATIC INDEX AND LIVER COMPOSITION

A Report of a Senior Study

by

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Spring, 2018

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ABSTRACT

The synthetic estrogen 17α-ethinylestradiol (EE2) is an endocrine disrupting contaminant that is commonly used in the medical and pharmaceutical fields. Exposure to EE2 is known to cause problems in developing animals. This study examines the effects of EE2 exposure on the hepatosomatic index and liver composition of adult *Danio rerio*. Hepatic tissue of 6 controls and 5 EE2 individuals was analyzed using histology to record hepatocyte counts, hepatosomatic index and structural differences. A t-test indicated that there was no significant evidence of a difference for cell counts using imagej ($p=0.14753$), while there was significant difference for hepatocyte counts analyzed through manual counts ($p=0.02195$). Gross structural differences were seen in exposed fish, as they had less distinct hepatic cell membranes and nuclei. Behavioral differences were noted in the treated fish as they consistently resided in the top of the water column. In conclusion, there appears to be some effects of EE2 to *Danio rerio*, but further studies on hepatic effects are warranted.

ACKNOWLEDGEMENTS

I would like to thank Dr. Andrew Crain and Dr. Jerilyn Swann for their guidance and help with this project. I would like to think Maryville College for providing the resources to perform this experiment.

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CHAPTER I

INTRODUCTION

ENDOCRINE DISRUPTION

Endocrine disruption, a branch of toxicology, is caused by exposure to endocrine-disrupting contaminants (EDCs) that alter the function of the endocrine system (Bateman et al. 2017). An EDC can be from numerous types of anthropogenic or natural compounds that have been introduced or concentrated in the environment including pesticides, plasticizers, pharmaceuticals etc. (Uzumcu et al. 2012). It was not until the mid-1990s that EDCs became intensely studied due to the hormone mimicking actions of the xenobiotic chemicals (Guillette and Crain 2000). The presence of EDCs in our environment has been on the rise, causing a multitude of questions being asked, mainly relating to how this is going to alter animals, including ourselves. Much of the modern research can no longer rely on having a set control or reference because it is difficult to detect everything that is contaminating the control area. The exposure of EDCs has been shown to cause modifications in both humans and other organisms (Guillette and Crain 2000; Toparri and Skakkebaek et al. 2000).

Exposure to EDCs can cause organisms and offspring to experience adverse health effects either in reproductive, metabolic, or neural control. These health effects arise due to the various mechanisms used by EDCs to alter normal physiology (Frye et al. 2012). It is important to understand the type of mechanism a specific EDC uses when conducting research to better understand the predicted outcomes. Some EDCs use epigenetic mechanisms – DNA methylation or post-translational modifications of histones and non-coding RNA – where there is no change to the DNA sequence, but the gene expression changes (Uzumcu et al. 2012). These disruptions can alter gene expression, gene activation or silencing, or inactivate particular functions. The main function of EDCs is to mimic a hormone and successfully interfere by altering synthesis, metabolism, binding, and cellular action within an organism (Sharma et al. 2017).

The resulting interference with hormonal actions can lead to organizational defects during development or even cause effects when exposed during adulthood (Frye et al. 2012). Due to the ubiquitous presence of EDCs in the environment, most animals are being exposed to EDCs during embryonic development. Male reproductive disorders have been linked to EDC exposure during critical life times, including embryonic development, infancy and puberty (Toppari and Skakkebaek et al. 2000). This exposure can cause a variety of issues including, but not limited to, cryptorchidism, hypospadias, and even some cases of testicular cancer. This failure of reproductive abilities is rarely discovered before 30 or 40 years of age. Research of EDC exposure could lead to a better understanding of how these developmental issues are formed and better ways to combat the present issue. Exposure to

EDCs during adulthood can initiate mechanisms that alter components important for converting cholesterol to steroid hormones. Lacking steroid hormones can also induce changes to the reproductive organs and the developmental process that begin in adulthood rather than during developmental stages.

The increase of EDCs in the environment has been suggested to be partially responsible for some changes in adult metabolic health as well. The presence of EDCs in the environment has been suggested to lead to changes in mesenchymal cells (MSC), which contribute to the control of metabolism (Bateman et al. 2017). The role of MSCs in the body is to regulate regeneration and make repairs to damaged tissues that help maintain homeostasis of the human body. MSCs have become more important recently due to possible immunotherapy treatments for autoimmune disorders such as acute graft-versus-host disease and autoimmune encephalomyelitis. Because of this potential, it is important for the effects of EDCs on MSCs to better understand how the efficacy of the treatments would be effected. MSC exposure to EDCs *in vitro* results in increases in adipogenic differentiation, decreases in osteogenic differentiation, epigenetic changes, increase in oxidative stress and activation of pro-inflammatory responses. Adipogenic differentiation is particularly important because the development of preadipocytes into adipocytes is important for the human body metabolism. To have proper adipogenesis, the environment must fit certain criteria. Exposure to EDCs *in utero* and during development after birth have been connected to increased adipogenesis, leading to the obesity epidemic. These alterations also can result in fat deposition, decrease in bone stability, increase in inflammation among other issues. The consequences from MSC exposure to EDCs can lead to less effective immunotherapy treatments and the MSCs ability to regulate regeneration (Bateman et al. 2017). These types of alterations are testaments for the many reasons that the effects of EDCs should be evaluated more thoroughly because of the high content of EDCs currently in the environment.

Along with fat deposition and various reproductive failures, EDCs can also cause a large amount of neurodevelopmental concerns (Frye et al. 2012). One mechanism that can be altered is the morphology of the sexually-dimorphic cerebral circuits. Alterations within these cause problems with sexual differentiation. Exposure for this particular modification is most concerning during development due to the organism being more sensitive to hormone changes. Estrogenic endocrine disruptors (EEDs) may contribute to the development of complex chronic human brain health disorders (Preciados et al. 2016). This is due to the brains reliance on estrogen levels in relation to the constant cellular generation, neurogenesis, the changes in EDCs and EEDs can modify how and when the brain is regenerating. Due to this variation in an otherwise very complex mechanism, neurological deficits can be formed which can change behavior, cause-learning difficulties or memory issues along with many more (Preciados et al. 2016). It is important for studies to begin evaluating how the EDCs are being introduced into the environment and ways that can resist having any physical changes done to the body from changes in EED levels.

Many EDCs are introduced to the environment by leaching from consumer products and industrial discharge (Sheikh et al. 2016 A; Archer et al. 2017). These EDCs can then enter the body through various pathways, including ingestion, inhalation, and direct contact

(Preciados et al. 2016). Many of the EEDs presence in the environment are derived from oral contraceptives, hormone replacement therapy, and other prescribed estrogens. Estradiol, a natural EED that is used in multiple oral contraceptives, is present in many aquatic environments due leaching of the consumer product (Archer et al. 2017).

ESTROGENS

Estrogens are steroid hormones, such as estradiol, that are secreted by female and male reproductive systems which promote the development of male and female secondary sex characteristics and the growth and maintenance of the female reproductive system (Guyton and Hall 2016). The effects of estrogens on other systems including adipose, nervous, cardiovascular, muscle and bone tissues are being studied as well (Monteiro et al. 2014). These effects on various systems are due to the signaling role in metabolic inflammation that estrogen has.

Exposure to various types of estradiol during development can have positive and negative effects. The presence of under-nutrition during development is reversed when estradiol was administered postnatal to rats (Carrillo et al. 2016). However, a positive correlation has been found between the use of hormone replacement therapy and an increase in various diseases, including breast cancer, stroke, etc. (Mauvaus-Jarvis et al. 2013). For instance, the presence of virtually all estrogens is associated with a higher risk of developing breast cancer (Fuhrman et al. 2012). Also, having low levels of estrogen during menopause has also been linked to enhancement of metabolic dysfunctions including but not limited to type 2 diabetes, predisposing women to obesity.

ETHINYLESTRADIOL

The common EDC, 17α-ethinylestradiol (EE2), is used in the medical and pharmaceutical fields (Budin and JieYnn 2015). EE2 is a synthetic compound that has a water solubility of 4.8 mg/L as well as a longer half-life than natural estrogens. This solubility causes concern due to the common use of EE2 in many therapeutic medical purposes, including the formulation of combined oral contraceptive pills and hormone replacement therapy. The presence of EE2 in drinking water can have negative impacts in humans, animals, and fish (Snyder et al. 2003; Archer et al. 2017). Concentrations as little as 2 ng/L can greatly affect fish reproduction, and this concentration are common after waste water treatment (Snyder et al. 2003). The residual EE2 that exits the body after treatment are not completely removed at wastewater treatment facilities and regularly feed into sources for drinking water treatment.

EE2 is classified as a contaminate candidate, but the United States Environmental Protection Agency (EPA. 2007) still does not require EE2 removal for drinking water treatment facilities. The concentration of EE2 in surface waters has recently been added to the European Union watch list (Laurenson et al. 2014), indicating that future elimination of EE2 from surface waters and drinking water. The removal of EE2 from water supplies can easily be completed by advanced treatment technologies, such as activated carbon and reverse osmosis (Snyder et al. 2003).

Table 1 indicates EE2 concentrations retrieved from surface waters after wastewater treatment facilities had completed treatment. The predicted no observable effect level

(NOEL) of EE2 for aquatic chronic toxicity of 0.1 ng/L (Laurenson et al. 2014). Within the sampling sites analyzed, Colorado is the only site that is below the NOEL. Surprisingly, samples taken from developed countries as well as less developed countries had an EE2 concentration above the NOEL. The EE2 concentration is higher in countries that are less developed than the US, which is attributed to higher quality wastewater treatment facilities.

Table 1: The 17α-ethinylestradiol concentrations found in surface waters in close proximity to waste water treatment facilities that received treatment prior to collected located in Malaysia, Poland, Europe, United States, and The Netherlands.

Location	EE2 concentration	Comments	Source
Sabah, Malaysia	12.28 ng/L	Average concentration from 3 sampling sites	Budin and JieYinn 2015
Kielce, Poland	3.2 ng/L to 6.28 ng/L	Average concentration range from eight sampling sites	Czerwonka and Kaca 2012
European waste water	1.4 ng/L to 8.9 ng/L	Mean EE2 concentration found in European waste waters.	Kuch and Ballschmiter 2001
Participating European Union Member States	0.53 ng/L to 17.9 ng/L	EE2 concentration range from 27 sampling sites throughout the EU member states and US.	Jarosova et al. 2014
European surface water	0.8 ng/L to 5.1 ng/L	Mean EE2 concentration found in European surface waters.	Kuch and Ballschmiter 2001
The Netherlands	0.3 ng/L to 4.3 ng/L	EE2 average concentration from 3 out of 11 surface water sampling sites.	Belfroid et al. 1999
US and Europe	0.2 ng/L to 0.3 ng/L	90^{th} percentile	Hannah et al. 2009
Colorado	0.067 ng/L	EE2 concentration found in one of three sampling sites.	Huang and Sedlak 2001

ZEBRAFISH

The tropical teleost zebrafish (*Danio rerio)* is used as a model organism to examine mammalian events occurring during embryonic development, organ formation, and adult physiology (Tiso et al. 2014). Zebrafish are used frequently in various studies within developmental biology and molecular genetics (Dubinska-Magiera et al. 2016). Through the use of zebrafish, the steps for formation of many endocrine glands have been interpreted and explained thoroughly (Tiso et al. 2014). Zebrafish are commonly used in developmental biology due to the rapid development after fertilization and short juvenile stage. Only recently have zebrafish been considered for *in vivo* experimentation to observe molecular cross-talks and effects on adult physiology. The small size of the zebrafish also reduces the cost of toxicants and reagents rather than if a larger organism was being tested.

Teleosts have a tri-lobed (2 lateral and 1 ventral) liver that functions very similarly to that of mammals, which have four-lobed livers (Bastiaan et al. 2014, White et al. 2016). There are, however, differences in the structural organization of hepatosomatic tissue between mammalian liver and teleost (see Figure 1). Whereas large bile ducts, portal veins, and hepatic arteries are organized into portal tracts in the mammalian liver, in the teleost liver these are randomly located throughout the liver parenchyma. Hepatocytes are location in tubules in teleost liver rather than plates as in the mammalian liver. The cellular hepatocyte plates in mammalian livers are two cells thick (Guyton and Hall 2016). Between each of the hepatocyte plate cell layers are small bile canaliculi. This differs from the location of bile canaliculi in teleost liver, which radiate centripetally from the hepatocytes

(Bastiaan et al. 2016). In both the mammalian liver and the teleost liver the bile canaliculi lead to bile ducts, which connect to the gallbladder (Bastiaan et al. 2016; Guyton and Hall 2016).

Table 2: Comparison of hepatic structures located in the teleost livers and mammalian livers

	teleost liver	mammalian liver
large bile ducts	present; randomly throughout liver parenchyma (Bastiaan et al. 2016)	present; organized in portal tracts (Guyton and Hall 2016)
central vein	present; scattered throughout parenchyma (Akiyoshi and Inoue 2004)	present; liver lobule formed around (Guyton and Hall 2016)
portal veins	present; randomly throughout liver parenchyma (Bastiaan et al. 2016)	present; organized in portal tracts (Guyton and Hall 2016)
hepatic arteries	present; randomly throughout liver parenchyma (Bastiaan et al. 2016)	present; organized in portal tracts (Guyton and Hall 2016)
hepatocytes	present; located in tubules (Bastiaan et al. 2016)	present; located in plates (Guyton and Hall 2016)
bile cacaliculi	present; radiate centripetally from the hepatocytes (Bastiaan et al. 2016)	present; located between hepatic plate cell layers (Guyton and Hall 2016)
endothelial cells	present (Ferri and Sesso 1981)	present (Guyton and Hall 2016)
Kupffer cells	present (Akiyoshi and Inoue 2004)	present; resident macrophages lining the sinusoids (Guyton and Hall 2016)
hepatic sinusoids	present; located within hepatic tubules (Akiyoshi and Inoue 2004)	present; lie between hepatic plates and into central vein (Guyton and Hall 2016)

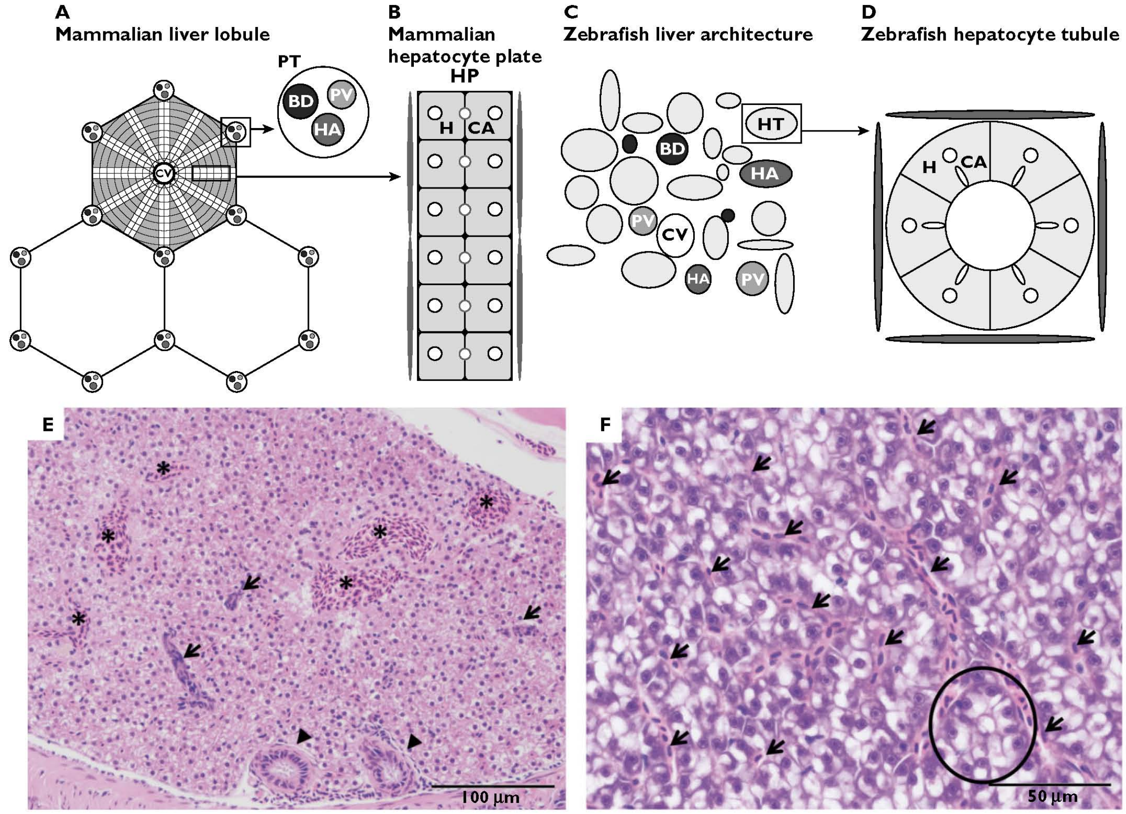

Figure 1: Schematic transverse representation of mammalian and zebrafish liver architecture. (A) The mammalian liver lobule, arranged with plates of hepatocytes radiating outward from a central vein (CV). At the corners of each lobule are portal tracts (PT), containing a portal vein (PV), a hepatic artery (HA) and a bile duct (BD). (B) Mamalian bilayered hepatocyte plate. Bicellular canaliculi (CA) are located adjacent to the hepatocytes (H) in the hepatocyte plate (HP). A basal hepatocyte membrane allows transport of oxygen, proteins, and different macromolecules to the hepatocytes. Blood enters the liver through the portal vein and hepatic artery, after which it enters the central vein through sinusoid vessels, located between the plates. (C) The zebrafish liver architecture. The portal vein (PV) hepatic artery (HA), bile duct (BD) hepatocyte tubule (HT) and the central vein (CV) are scattered throughout the parenchyma. (D) Zebrafish hepatocytes (H) are arranged in tubules around small bile duct, which receive bile from the hepatocyte canaliculi (CA). Sinusoids are located at the periphery of these tubules. (E) Histological image of male zebrafish liver (haemotoxylin and eosin staining at X200 magnification). Note the presence of several biliary ducts (arrows), bile ducts (arrowheads) and blood vessels (*), with lack of lobular arrangement. (F) Histological image of female zebrafish liver (haemotoxylin and eosin staining at X400 magnification). This high-power image displays sinusoidal spaces between hepatocytes (arrows) and an instance of the tubular arrangement of hepatocytes (encircled), which is frequently not visible histologically. Note the difference in staining of male and female zebrafish liver. Taken from Vligenthart et al. 2014.

Even with the structural distinctions between teleost and mammalian liver anatomy, the function of hepatic tissues is very similar in both organisms (Bastiaan et al 2016; Goessling and Sadler 2015). In both mammalian and teleost livers, the hepatocytes are the functional center of the liver (Akiyoshi and Inoue 2004). The cellular function, composition and cellular processes that mediate liver diseases are similar between teleost and human livers (Goessling and Sadler 2015). This is partially due to the fact that genes are highly conserved between zebrafish and humans. Also, the enzymes used for metabolizing a range of endogenous and exogenous compounds are comparable between enzymes in teleost livers and human livers (Huang and Sedlak 2001). Mammalian livers use multiple growth hormones which regulate growth of body tissues (Guyton and Hall 2016). Zebrafish growth hormones, specifically insulin-like growth factor (IGFs) have been used to evaluate function of the liver (Chou et al. 2008). Similarities between other teleost and mammalian growth hormones have been indicated (Dai et al. 2015). Zebrafish are considered a model organism because of these similarities.

The noted similarities between both mammalian livers and zebrafish livers support the use of zebrafish livers in experimenting for changes caused by exposure to various hormones, both natural and synthetic. Exposure to natural estrogens can have positive effects throughout the body. When adult individuals are exposed to natural estrogens, the hormone has an activational effect, which is an effect that is transient and occurs throughout life, of positively altering insulin sensitivity as well as preventing inflammation (Arnold and Breedlove 1985; Mauvais-Jarvis et al. 2013). Due to the activational effect of natural estrogen, a natural estrogen deficiency can also lead to predisposition to obesity and type 2 diabetes. However, exposure of adult individuals to synthetic estrogen is not believed to have the same activational effect (Metzler et al. 1990). Research has shown that nonmammalian species, like humans, are susceptible to liver disease caused by environmental and dietary insults, among other factors (Goessling and Sadler 2015). Exposure to synthetic estrogens, such as EE2, can lead to altered levels of sex steroid hormones and impairment of gonad development, among other adverse health effects (Teng et al. 2013). EE2 is also considered a known strong tumor promoter, specifically in hepatic cells (Wan and O'Brien 2013).

Increase in hepatic growth is more prominent during the initial time of activational exposure to EE2, compared to subsequent periods of exposure (Yager et al. 1994). The increase in hepatic growth, which can lead to a malignant tumor, can be caused by adult exposure to EE2 (Miceli et al. 2011). When adult individuals are exposed to EE2, changes to estrogen signaling can occur due to variation in DNA methylation. Along with variation in DNA methylation, exposure to EE2 is also believed to cause an increase in epidermal growth factor receptor (Vickers and Lucier 1991). The epidermal growth factor receptor is associated with the actions of promoters, which can lead to promotion of hepatic tumors by estrogens. Chronic exposure to EE2, largely through prolonged use of oral contraceptive steroids, has been indicated to induce emergence of new hepatocyte populations. Although an oral contraceptive has a much higher amount of EE2, surface water that contains leached EE2 is used for drinking water around the world, even in the US. The amount of EE2 exposure that is needed to produce adverse side effects is still being researched.

QUESTION

The majority of research on the effects of EE2 on zebrafish has been conducted during embryonic, organizational development but very few studies have addressed adult activational exposure. The evaluation of 17α-ethinylestradiol exposure on adult zebrafish will provide insight on the effects of EDC exposure when the organism is fully grown rather than developing. Based on the predicted characteristics of EE2 in relation to tumors and growth factors found in livers that were exposed, exposure to an organism as an adult might have changes in the relative size, while the developmental structure of the liver may go unharmed.

This study examines the effects of EE2 exposure on the hepatosomatic index and liver composition of adult *Danio rerio*. After controlled exposure to 17α-ethinylestradiol, it is hypothesized that the liver composition and the hepatosomatic index will increase with exposure to a higher concentration of 17α-ethinylestradiol. The liver is hypothesized to increase in size based on the aforementioned effects of exposure to EE2 and due to the tumor promoting nature of EE2 (Wan and O'Brien 2013).

CHAPTER II

METHODS AND MATERIALS

DANIO RERIO HUSBANDRY

Two ten gallon aquatic tanks were used to house 6 adult *Danio rerio* each. Each tank was filled with approximately eight gallons of dechlorinated water maintained at a temperature of 26˚C - 27.5˚C with two heaters in each tank and a pH between 6.8-7.5 (Westerfield 2000). Temperature and pH were checked daily. Water was continually aerated and filtered with a carbon filter. A quarter water change was performed every three days immediately before treatment. *Danio rerio* were kept on a twelve-hour light cycle followed by a twelve-hour dark cycle (12:12) and fed tropical fish flakes once a day. All experimental animal protocols were approved by the Maryville College IACUC (see Appendix).

PREPARATION OF TREATMENT

To prepare the experimental treatment solution, 0.01 mg of 17α-ethinylestradiol (CAS 57-63-6, Sigma E4876) was dissolved into 1ml of 100% ethanol. This solution was vortexed to dissolve EE2 completely. This 1ml of EE2 and 100% ethanol solution was then added to 1 L of deionized, double distilled (ddH_2O) water and placed on a stir plate with a large magnetic stir bar. Once the ethanol and EE2 mixture had dissolved into the water, twenty replicates of 1 mL were aliquoted into a cryovial. In case of future experimental error, 20 of these were prepared. To prepare the control treatment solution, 1mL of 100% ethanol was added to 1 L of deionized, double distilled water. After mixing with a stir rod, 1 mL of the solution was transferred into a cryovial. As with the previous concentration, 20 1 mL aliquots were made. Cryovials were placed into the -80°C freezer for storage between treatment.

One ten-gallon tank, the control, received 302 µL of a 1mg/L ethanol and water solution. The second ten-gallon tank, the experimental, received 302 µL of a 1mg/L ethanol, EE2, and water solution to yield a 0.1 µg/L in 8 gallons, or 30.28 L, of water (Notch et al. 2007). These treatments were added every 3 days for the course of 20 days.

ENDPOINTS MEASURED

Throughout the treatment, videos were taken of the water column activity and reactivity of the fish to sudden movements were recorded for comparison. After the duration of the 20-day treatment, each fish was euthanized with 400 mg/L of Tricaine Mesylate, or MS-222. After euthanasia, each fish was weighed to obtain an overall body weight. The liver was then removed from the body cavity through dissection (Gupta and Mullins 2010). The liver was then weighed and the weight recorded in correspondence with the body weight. This produced the hepatosomatic index, comparison of liver weight and body weight. This process was repeated for all zebrafish.

After obtaining all mass data, each liver was placed into Bouin's fixative as instructed in the histology manual (Presnell and Schreibman 1997). The livers remained in Bouin's fixative for 1 week. The livers were then cleared of fixative and embedded into wax to prepare for histology of the organ. The organs were then sectioned, fixed to microscope slides and stained for analysis. Both the process of embedding and sectioning were performed following the histology manual protocol (Presnell and Schreibman 1997).

For each specimen, 3 images were taken and used in analysis. The tissue filled the image completely and folds, organ structures, and holes were avoided to maintain consistency. Each image was then analyzed using imagej software. Once the image was imported, the image type was changed from RGB to 16-bit, a black and white image. The threshold was adjusted to highlight the structures that should be counted. After trial and error, the most accurate count obtained was through measuring the nucleus. Once the threshold was applied, outliers of 16 pixels or less were removed. The holes in the image were then filled and the image went through the watershed process, which separates cells that were touching. The image was then analyzed and the data as well as final image were exported.

The data collected were analyzed by comparison of the control and experimental groups to evaluate for hepatic structure shape or morphology using a T-Test in Microsoft Excel. Hepatic structures, such as the hepatocytes and epithelial cells, were measured for cell count within a given area and irregularity between the experimental and the control group.

CHAPTER III

RESULTS

Out of the 12 *Danio rerio* that were a part of the study, only 11 of the fish samples contained the liver. The sample that did not contain liver tissue was from the experimental group. To help balance this flaw, the data set of one of the control groups was omitted through random selection.

The hepatosomatic index for both the fish exposed to 17α-Ethinyl estradiol (experimental) as well as the fish not exposed to the EDC (control) can be found in Figure 2. The average imagej cell counts taken for both the experimental and control specimens were 536.40 and 595.67, respectively (Figure 3; $P=0.14753$, $n=5$ for each group). The average manual cell counts taken for both the experimental and control specimens were 536.40 and 595.67, respectively (Figure 3; $P=0.02195$, $n=5$ for each group). Upon analysis, the liver cells observed for the experimental and control specimens displayed structural differences (Figure 5). The overall appearance of the tissue collected from the control group had very distinct plasma membranes and nuclei in comparison to the liver samples collected for the experimental group, which appear to have less distinct plasma membranes and nuclei. Throughout the experiment, behavioral differences were observed as well. The experimental specimen consistently resided in the top section of the water column while the control specimen swam throughout the water column (Figure 6). When the reactivity of the fish was recorded with sudden movements, the experimental fish did not react while the control fish reacted with frantic swimming and movements.

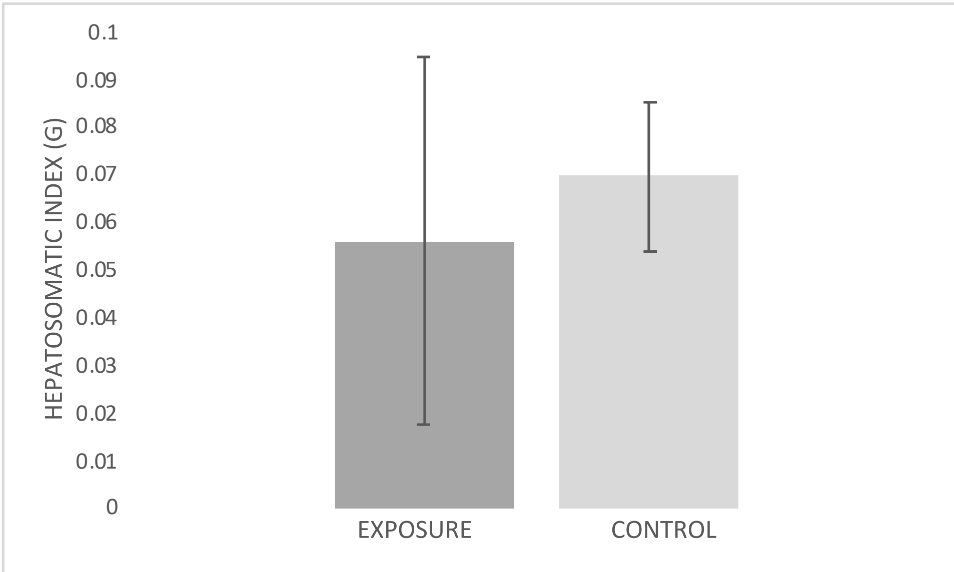

Figure 2: A graph depicting the hepatosomatic index collected for fish exposed to 17α-Ethinylestradiol (exposure; +/- 0.0386 SD) and for fish not exposed to 17α-Ethinylestradiol (non-exposure; +/- 0.0157 SD). The average hepatosomatic index for the exposure group was 0.05595 and the average hepatosomatic index for the control group was 0.06963.

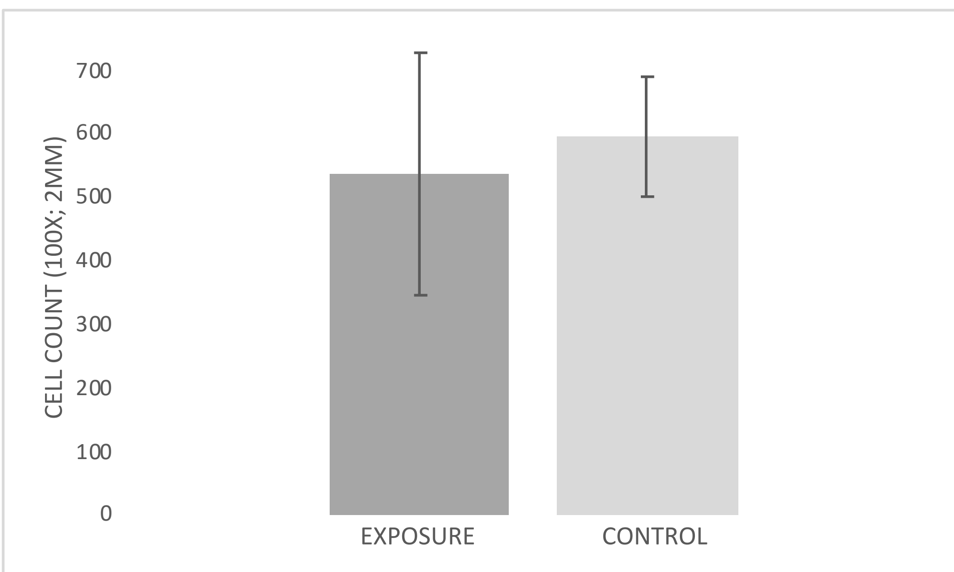

Figure 3: A graph depicting average imagej cell counts collected for fish exposed to 17α-Ethinylestradiol (exposure; +/- 192.1170 SD) and for fish not exposed to 17α-Ethinylestradiol (non-exposure; +/- 95.1502 SD). The average cell count for the exposure group was 544.95 and the average cell count for the control group was 613.2.

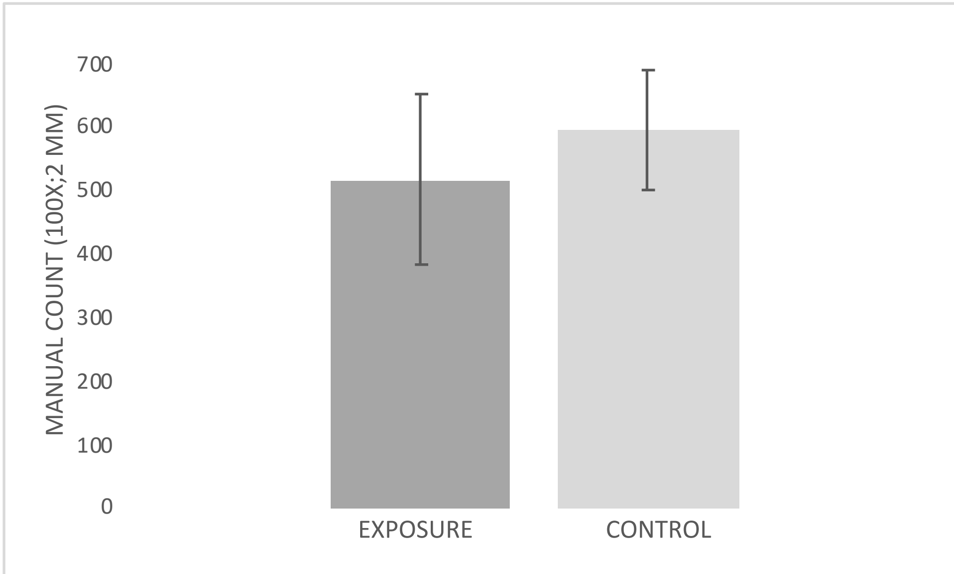

Figure 4: A graph depicting the manual cell counts collected for fish exposed to17α-Ethinyl estradiol (exposure; +/- 133.9887 SD) and for fish not exposed to 17α-Ethinylestradiol (non-exposure; +/- 95.1502 SD). The average cell count for the exposure group was 515.53 and the average cell count for the control group was 595.47.

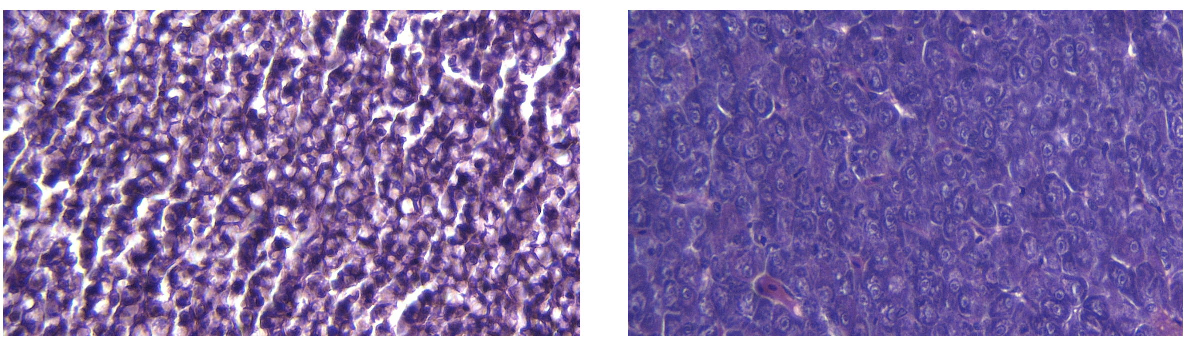

Figure 5: Histology slide of liver cells observed in *Danio rerio* not exposed to 17α-Ethinyl estradiol (left). Histology slide of liver cells observed in *Danio rerio* exposed to 17α-Ethinyl estradiol(right).

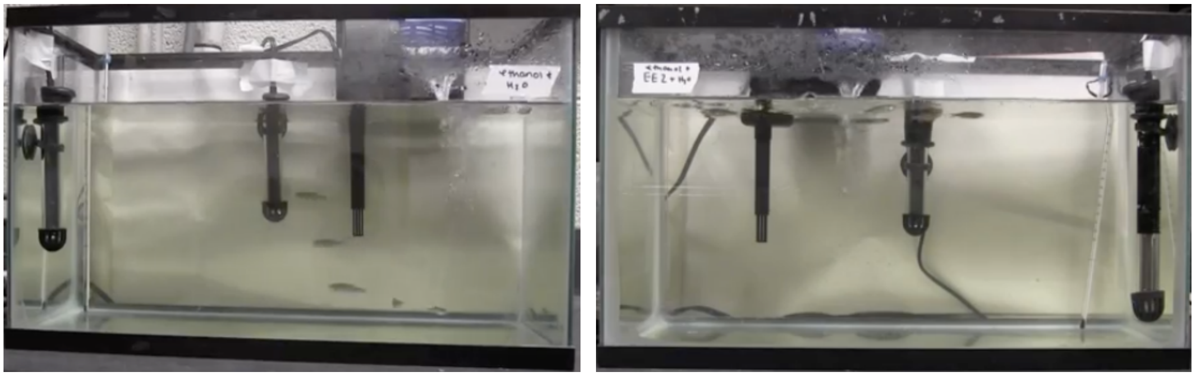

Figure 6: Image captured from behavior analysis footage of *Danio rerio* not exposed to 17α-Ethinyl estradiol (left). Image from behavior analysis footage of *Danio rerio* exposed to 17α-Ethinyl estradiol (right).

CHAPTER IV

DISCUSSION

Endocrine-disrupting contaminants (EDCs) can be found in a multitude of medications and chemicals that are produced. Until recently, as late as the 1990s, EDCs were not studied extensively. Estrogens, which are used in a lot of hormonal medications such as combined oral contraceptives, can cause extensive problems for human health due to its presence in drinking water (Archer et al. 2017). It is crucial to understand more about this chemical's characteristics and pathway to better approach removing EDCs, such as this one, from the environment. In previous studies of EE2 and the effects of *Danio rerio*, juvenile fish were used. These studies revealed changes in sexual physiology as well as organ developmental differences (Lange et al. 2009; Chen et al. 2015). Previous studies have found that exposure to EE2 causes the hepatic estrogen receptor-α mRNA was significantly increased when exposed to EE2 (Caspillo et al. 2014).

Out of the 12 *Danio rerio* livers collected, only 11 of the samples were analyzed due to collection error. To balance the use of only 5 experimental samples, one control sample data set was randomly omitted. After analysis, the cell counts recorded indicated no significant difference ($p=0.148$) between the *Danio rerio* exposed to EE2 and *Danio rerio* not exposed to EE2. The experimental group appears to have a less defined cell shape and seems to have different cytoplasmic density or makeup. The method of analysis, the imagej software, relies heavily on contrast for the process of counting cells. Due to this error in methodology, the data collected for the experimental group is inconclusive. However, the data collected for the control group was relatively reliable after multiple manual count checks. This is important to note for future studies that might want to explore a similar type of study. TEM might provide a more informative picture of what is or is not happening in these hepatocytes with and without EE2 exposure. It is also important to note the small sample pool of the study. Due to time and space constraints, the number of specimens that could be used in the experiment was limited. The data were also impacted by the reduction to 10 samples used for analysis rather than 12.

Along with having a small sample size, 2 of the experimental fish died before the experiment was over. These specimens were dissected and the livers were collected at the time of death. One of the histology slides produced from the deceased samples showed less damage in the hepatic tissue, while the other sample did not contain the liver. The use of these data in the set might have impacted the results of analysis.

After the livers underwent the embedding and sectioning, it was evident that during dissection and collection, a different organ composition was collected for some of the specimens. Although the weights for the specimens were taken, they could not be used in this analysis. For further studies, it would be beneficial to attempt to measure the weights of only the liver to better understand if there is a link between adult exposure to EE2 and tumor development in the liver.

Throughout the study, the behavior of the *Danio rerio* was observed periodically. From the middle to the end of the study, the specimens in the experimental group exhibited a strange behavior of remaining in the top of the water column for the majority of the time, while the control group traveled throughout the water column in the tank without any tendency to remain in a particular area of the water column extensively. The set-up of both tanks had the same type of water filtration and circulation set-up. Along with the odd behavior regarding the water column, the fish in the experimental group also did not respond to a rapid movement of hands in front of the tank while the control group responded consistently. Similarly, a study on locomotor behavior of zebra fish as a biological warning system for toxicants in the water found that there were changes in how the fish responded to rapid hand movements and the different directions and speed of swimming (Fernandes et al. 2016). Another study determining the affects of EE2 on anxiety and shoaling behavior in adult male zebra fish, found that as the EE2 exposure increased in duration and percentage, the fish were less likely to leave the shoal, which is the shallow part of the water. Although the ten-gallon water tank used for this experiment was not similar to the depth of a wild habitat for zebra fish, the fish in the experimental group displayed a behavior of staying at the top of the tank while the control group swam throughout the water (Reyhanian et al. 2011).

When determining the proper concentration to use for this experiment, a 0.1 μg/L. Due to time limitations, it was not possible to determine if the EE2 was completely decayed after 3 days or if there was residual chemical left in the water. Although a half water change was completed on the days of exposure to minimize the chance of overdose, it is still possible that the chemical was over concentrated and might have had more of an effect on the fish than would be in the wild.

In conclusion, the results of this study indicate that there is no significant effect on *Danio rerio* liver composition or hepatosomatic index, but methodology and experimental errors reduce our confidence in this conclusion. For future study opportunities, it would be important to have a more accurate dissection and collection protocol to ensure that only the liver is collected or at minimum a more consistent organ composition is collected for analysis. Also the method for analysis would need to be better understood and practiced for this particular type of cell counting.

APPENDIX

APPENDIX 1: IACUC APPROVAL OF STUDY

MARYVILLE COLLEGE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE (IACUC)
Application for Use of Vertebrate Animals in Faculty Research or Teaching

Faculty at Maryville College that use vertebrate animals in teaching or research are required to complete an IACUC proposal for each project.

Provide information after each bold item
Faculty Name: Drew Crain

Email Address: drew.crain@maryvillecollege.edu

Date: December 1, 2015

Species to be used: *Danio rerio*

Age of animals: zygote to adult

Number of animals in study: 500

Brief description of use (teaching or research): These zebrafish will be used to teach students the techniques involved in developmental biology research.

Duration of use: continuous

Location of animals (building and room) : Sutton 114

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Drew Crain	981-8238	292-8737	292-8737

What will happen to the animals at the end of the use? If euthanasia is required, state the methods.
Animals will be sedated with 400 mg/L MS222 (Tricane methanesulfonate) and frozen at the end of tissue collection.

(Do not write below line: For MC IACUC Use)

Maryville College IACUC Approval Number: 201506
Date Approved: Nov. 30, 2015
Signed: Irene Guerinot

Is it likely that pain/discomfort will be experienced by animals in this protocol?
YES____ NO_x__If "YES", describe:

Pain or Distress Category:B_(See listing of Pain or Distress Categories below)

For categories C,D, or E, USDA regulations require that the investigator consider alternative procedures. Please provide a narrative (for instance the end of Chapter 1) describing the methods and sources used to determine that alternatives are not available. If a computer assisted literature search was conducted, provide the names of the database(s) and date(s) of the search.

Pain or Distress Categories

A. ACUTE STUDIES
Studies performed under anesthesia from which the animals are not permitted to regain consciousness, or performed on excised animal tissues collected under anesthesia or following euthanasia.

B. PAIN OR DISTRESS - NONE OR MINOR
Chronic studies that DO NOT involve survival surgery, induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (so long as the tumors do not cause pain and the animals are terminated prior to becoming seriously ill), administration of mildly toxic substances or drugs that cause no significant disease or distress, and antibody production as long as significant disease does not result and antigen booster doses do not include Complete Freund's Adjuvant (CFA).

C. PAINFUL PROCEDURES WITH ANESTHESIA/ANALGESIA
a. Survival surgical procedures.
b. Painful or potentially painful non-surgical procedures; e.g. bone marrow taps, injections into particularly sensitive areas such as foot pads, cardiac punctures, or traumatic procedures such as burns (burns may be category D, depending on severity).

D. MODERATE DISTRESS OR PAIN GENERALLY WITHOUT ANESTHESIA/ ANALGESIA/ TRANQUILIZERS
Induction of moderately distressful or painful disease conditions (examples: arthritis, administration of toxic chemicals, infectious challenges, immunosuppression resulting in infectious disease, peritonitis, severe inflammation, especially of weight bearing surfaces or resulting in external sores), whole body irradiation, stress models, septic shock, hypotensive shock, moderate painful stimuli (examples: low level electrical shock or heat), survival surgical procedures that have the potential to result in long term distressful illness (organ transplants, for example), induction of cardiac ischemia, booster immunizations with CFA, tumor induction or animal cultures that cause significant distress or pain, sight deprivation, restraint for periods longer than 12 hours.

E. INTENSE SUSTAINED OR REPEATED PAIN WITHOUT ANESTHESIA/ANALGESIA
Direct stimulation of CNS pain tracts, nociceptor stimulation by physical or chemical means that causes severe pain (e.g., corneal abrasions), or any category C (see above) procedure if performed without chemical relief of pain.

Investigator Assurance

Check all boxes that apply.

X The information provided in this protocol form accurately reflects the intended use of animals for this research activity. Significant changes in procedures will not be undertaken without prior notification and approval of the Maryville College IACUC.

X All persons involved in the use of animals on this protocol have been informed of the experimental objectives and methods. Each has received training in the execution of animal-related procedures he/she will perform prior to participation in the protocol, and will participate in any educational or training programs deemed appropriate or necessary by the Maryville College IACUC.

X I agree to follow the provisions of the Animal Welfare Act and the guidelines of the National Institutes of Health on the care and use of laboratory animals.

X I agree to use anesthesia, analgesia and tranquilization to relieve pain or distress whenever use of these agents will not jeopardize the scientific validity of the data. I have specifically consulted with the Maryville College IACUC regarding any experiments that are classified in pain/distress categories C, D, or E.

X I will take appropriate steps to avoid exposure of persons working with these animals to any biohazard agents used in the study.

For any unchecked box above, explain the reason it does not apply.

Purpose for animal use: Briefly describe your proposed research project or teaching use of the animals. Be sure to include a justification for the species and number.

Danio rerio are the most utilized model in developmental biology research. Students taking Bio414 and Bio412, as well as some senior study students

Animal Husbandry: Briefly describe the basic animal husbandry requirements for the animals.

Animals will be maintained at a density no greater than 0.5 gal per fish and fed daily.

Potential Benefits: State the value of using the animals, with respect to human or animal health, advancement of knowledge, or good of society.

Danio rerio are required to teach students about animal models used in research.

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