REPRODUCTIVE EFFECTS FROM EARLY EXPOSURE OF ZEBRAFISH (*DANIO RERIO*) TO 17β-ESTRADIOL

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Abstract

The impact of a class of chemicals termed endocrine- disrupting chemicals (EDCs) has become a growing concern in light of recent studies elucidating their effects. They disrupt vertebrate hormone signaling and cause damage at very low concentrations. The zebrafish (*Danio rerio*) has been studied as a model organism. Thus far little data exists on effects of endocrine disrupting chemicals (EDCs) on zebrafish during the pre-hatching time period. It is known that EDCs affect neuronal and enzymatic function and disrupt endogenous hormone balance, but it is not known how early exposure with affect later reproductive fitness. I exposed zebrafish to environmentally relevant doses (1 and 10 ng/L) of 17β- estradiol in the post-fertilization, pre-hatching time period. Subsequently the exposed zebrafish were transferred to clean water and raised to sexual maturity. They were then bred with unexposed zebrafish and data on their fertility was collected. Exposure to steroidal estrogen during the pre-hatching period of time at 1 ng/L and 10 ng/L concentrations did not significantly affect zebrafish length, hatching, number of eggs produced, or egg fertilization.

Introduction

A species ability to live, grow and reproduce effectively relies on a fine-tuned, complex system of signaling and metabolic pathways. An interruption in this delicate metabolic web has negative consequences on the organism's ability to survive and reproduce in its environment. A group of chemicals termed endocrine-disrupting chemicals (EDCs) disrupts this delicate system and has become a concern within the last 40 years (Sonnenschein & Soto, 1998). The Endocrine Society defines EDCs as "a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment" (Diamanti-Kandarakis et al., 2009).

The present study sought to understand the effect of EDCs on vertebrate health by using zebrafish (*Danio rerio*) as a model organism. What effects will a pulsatile low dose exposure of 17β-estradiol (an EDC) during the pre-hatching time period have on zebrafish fertility once sexual maturity is reached? I expect there to be a reduction in reproductive ability in zebrafish subsequent to their early exposure to an EDC.

Background

EDCs affect many systems in the body including the thyroid, adrenals, and, in particular, the reproductive system (Diamanti-Kandarakis et al., 2009). Some are even considered to be obesogens—molecules that interfere with normal fat metabolism and storage in a way that promotes obesity (Grün & Blumberg, 2006).

Endocrine disruptors are a highly diverse and varied group. They include bisphenol A (BPA) in plastics, phthalates in plasticizers, pesticides like

dichlorodiphenyltrichloroethane (DDT), fungicides, pharmaceuticals like diethylstilbestrol, and industrial solvents and lubricants such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and dioxins (Diamanti-Kandarakis et al., 2009). EDCs can disrupt endogenous (naturally occurring) hormones through a variety of different mechanisms. Some act as hormone mimickers or agonists, which bind to and activate a receptor (Sonnenschein & Soto, 1998). Others act as antagonists (Sonnenschein & Soto, 1998) by binding to the receptor and not activating it, which prevents binding of the natural hormone. They also act as disruptors of the natural synthesis and metabolism of hormones and hormone receptors (Sonnenschein & Soto, 1998). These chemicals are affecting the ability of animals to thrive and reproduce on all levels of life (Pelch, Beeman, Niebruegge, Winkeler & Nagel, 2010; Vajda & Norris, 2011).

EDCs have been shown to induce breast cancer (Jenkins et al., 2009; Murray, Maffini, Ucci, Sonnenschein & Soto, 2007; Lee, Hwang, Park & Choi, 2012). They have also been linked to premature puberty (Rasier, Parent, Gerard, Lebrethon & Bourguignon, 2007), disturbed lactation (McLachlan, Simpson & Martin, 2006), increased prostate size (Gupta, 2000), and prostate cancer (Ho, Tang, Belmonte de Frausto & Prins, 2006). EDCs may also be underlying testicular dysgenesis syndrome (TDS), consisting of reduced sperm quality, hypospadias, cryptorchidism, and testicular cancer (Bay, Asklund, Skakkebaek & Andersson, 2006; Sharpe, 2006). The extent to which exposure will have an effect depends on numerous factors, including the timing of exposure, amount and length of exposure, as well as mixture of EDCs present (Diamanti-Kandarakis et al., 2009).

The realization that EDCs were affecting human health began with dichlorodiphenyltrichloroethane (DDT), the first commercially produced insecticide known to be estrogenic (Fry, 1995). However those affected by DDT were generally exposed to massive quantities. The story today with estrogenic EDCs is different. Most people realize that pesticides are generally toxic chemicals that should not be indiscriminately used. The issue today is what David Norris aptly calls "stealth pollution" (personal communication). EDCs can produce effects at extremely low concentrations (ng/ L) (Vandenberg et al., 2012). Some are even more potent in small quantities than they are in a large dose, which is known as a non-monotonic dose response (Vandenberg et al., 2012). Many of these EDCs cannot be detected by sight, taste, or smell, and are very difficult to measure accurately in the environment at low (ng/L) concentrations.

The brain is also affected by EDCs. The neuroendocrine system serves as the interface that coordinates between the brain and endocrine system (Zohar, Muñoz-Cueto, Elizur & Kah, 2010). While the neuroendocrine system is very intricate and multifaceted, containing multiple axes, the focus here will be exclusively on the hypothalamus-pituitary-gonadal (HPG) axis (see Walker & Gore, 2007) Activation of steroidogenesis (synthesis of steroid hormones) and gametogenesis (formation of sex cells or "gametes") in the gonads begins with neurons in the hypothalamus (Diamanti-Kandarakis et al., 2009). These neurons are responsible for control of the entire reproductive neuroendocrine system. They release gonadotropin-releasing hormone (GnRH), a decapeptide that orchestrates reproduction throughout a vertebrate's lifecycle (Gore, 2002). GnRH also serves as the primary stimulus to other parts of the reproductive axis, i.e., the pituitary and gonads (Diamanti-Kandarakis et al., 2009). When GnRH is released

from neurons in the basal hypothalamus, the pituitary gland is stimulated to release gonadotropins, activating gametogenesis and steroidogenesis in ovaries and testes (Diamanti-Kandarakis et al., 2009). Steroids produced by ovaries and testes then act on target tissues with steroidal hormone receptors (Diamanti-Kandarakis et al., 2009). Cytochromes are proteins involved in a wide variety of metabolic processes, with cytochrome P450 aromatase being the key enzyme responsible for regulating estrogen levels. The latter enzyme is the rate-limiting step in estrogen synthesis and is responsible for aromatizing androgens to yield estrogen (Diotel et al., 2010). Cytochrome P450 aromatase is a prime target for disruption by EDCs (Cheshenko, Pakdel, Segner, Kah & Eggen, 2008).

The focus of the present study was estrogenic EDCs that affect the body in a unique way because of their receptor. The intracellular estrogen receptor (ER) is the key receptor affected by estrogenic EDCs, although estrogen membrane receptors may be involved to some extent (see Pelch et al., 2010). Hormones and receptors generally are structurally and electronically complimentary, making their binding very specific (Pratt & Cornely, 2011). For example, even the difference of one hydrogen, hydroxyl or double bond may affect the binding ability of the hormone (Norris, 2007). In contrast, the ER binds various chemicals that only loosely resemble estrogen (Wiseman, 2005). The majority of EDCs that affect the ER are mimickers. The ER has been termed "promiscuous" because it is so non-specific to natural estrogens (Vajda & Norris, 2011). There is no complete explanation as to why this is the case for the ER. However, the receptor's promiscuity clearly makes it prone to disruption.

Zebrafish, *Danio rerio*, were used as the model of study for several reasons. The hormonal, cellular and genetic mechanisms through which reproduction occurs is highly conserved in vertebrates (Vajda & Norris, 2011; Hill, Teraoka, Heideman & Peterson, 2005; Kah et al. 2007), and extrapolations can be made about other vertebrates by studying these fish (Ankley et al., 2009). Zebrafish thus make an excellent model for the present study in particular because genetic research has shown that the human and zebrafish estrogen receptor is exceptionally highly conserved (Lam et al., 2011). Why zebrafish as opposed to any other fish? Thanks to the vast amount of research that has been done using zebrafish as models, a lot is known about what constitutes normal in zebrafish (Hill et al., 2005). Understanding normal functioning allows researchers to distinguish when things are not normal due to manipulated variables. It is beneficial to the scientific community to do more research on zebrafish to understand every aspect of its development, such as critical periods in development of certain systems. There is a mass of information on effects of estrogens on zebrafish and reproduction, especially in regard to effects of estrogens (Hill & Janz, 2003; Xu et al., 2008) However, most of this literature examines chronic effects of exposure of older animals, and developmental sensitivity of zebrafish to estrogenic EDCs is less well understood. The present study sought to add to the understanding of estrogens on early developmental periods in zebrafish and possible appearance of effects later in life.

This area of study is important not only in the interest of the health of human populations, but also in preserving fish populations. EDCs commonly end up in aquatic systems via wastewater runoff (Sumpter, 1998). In many densely populated areas, wastewater effluent contributes half of the flow of some rivers (Sumpter, 1998). During

periods of little rainfall, wastewater can contribute 90% or more of the river flow (Sumpter, 1998). Aquatic environments are thus "the ultimate sink" for EDCs (Sumpter, 1998). Fish are important for biodiversity and their role within ecosystems is central. They are also extremely valuable from an economic point of view because they comprise a large commercial and recreational industry (Mills & Chichester, 2005).

The present study focused on the subgroup of EDCs that are steroidal estrogens (see Leet, Gall & Sepúlveda, 2011). Fish in natural environments exposed to EDCs are typically exposed to a number of different estrogenic compounds including 17β-estradiol (E_2), estrone, estriol and 17α-ethynylestradiol (EE_2) (Desbrow, Routledge, Brighty, Sumpter & Waldock, 1998; Snyder et al., 1999; Baronti et al., 2000). However, because virtually all estrogenic EDCs mimic E_2 and activate the ER, their effects are additive. The concentrations at which steroidal estrogenic EDCs produce an effect are not all the same: some are more potent than others. The ability of a mixture of estrogenic EDCs to produce an effect can be calculated using their equivalency to E_2, called estrogen equivalency (EEq) (see Vajda et al., 2008)

It is well established that chronic exposure of developing fish to estrogenic EDCs can lead to effects like intersex and sex reversal (Vajda & Norris, 2011). But only a few studies so far have assessed effects of exposure between fertilization and hatching (e.g., Vosages et al., 2010, Lam et al., 2011). From those studies, it is known that early exposure to EDCs affects the GnRH axis (Vosages et al., 2010) as well as the cytochrome enzyme aromatase in the brain (Fenske & Segner, 2004) during early exposure. But does early exposure have effects on reproductive success later? Research focusing on reproductive effects has in the past focused on spermatogenesis (the generation of sperm)

and vitellogenesis in males (the production of vitellogenin—a protein precursor to egg yolk proteins, that is not naturally produced in males), but not actual reproductive function (Page, Vosages, Servili, Brion & Kah, 2011; Sumpter & Jobling, 1995). It is also known that perinatal exposure to BPA in rodents affects their fertility later in life (Salian, Doshi & Vanage, 2009). However, using rodents as model organisms for developmental time period research is hindered by their inaccessibility during early development (Lam et al., 2011).

The original rationale behind the present experiment was to do a multigenerational study. Such studies have been done in mice, showing that EDCs induce epigenetic effects (Bernal & Jirtle, 2010). Epigenetic (literally meaning "above genetics") are alterations not of the genome itself, but of the way it is expressed. Studies in mice have shown that the first generation exposed *in utero* passes on these changes for multiple future generations, not themselves exposed (Salian et al., 2009).

The choice of which steroidal estrogen EDC to expose the zebrafish to in the present study was arbitrary since all EDCs affect ER in the same way. I chose to use E_2 itself to eliminate the need for EEq calculation, which made the experiment more simple.

We took several measurements to quantify how fertility was affected by early exposure to E_2. We assessed total number of eggs produced, percentage of eggs fertilized, percentage that hatched, and length of the mature fish at time of spawning. The length measurement was for the purpose of estimating growth rate. We used length as opposed to weight because weight is notoriously variable for small fish and difficult to accurately determine (Norris D., personal communication). Furthermore, some studies have shown

that growth in length is stimulated by a large dose of E_2 (van der Ven, van den Brandhof, Vos & Wester, 2007).

Materials and Methods

Source of Embryos

Embryos were collected from spawning zebrafish from the Tuebingen line, an established inbred line of wild type zebrafish. This allowed for genetic homogeny. In preparation of the spawn, the fish were put on a regular light schedule with 14 hours of light and 10 hours of dark. The fish were kept at 28.5 °C. Special spawning tanks with a mesh bottom were used that allowed eggs to drop into the bottom part of the tank. This protected the eggs from being eaten by the parents. Six pairs of fish were spawned. Each tank held one male and one female, which were divided by a removable barrier. The fish were held overnight in the spawning tanks to allow them to acclimate. In the morning, barriers were removed, allowing the fish to interact and spawn. After four to five hours, eggs were collected in petri dishes.

Treatment

Sixty fertilized eggs were selected for two treatment groups and one control group (180 eggs total). Fertilization was determined by examination under a dissecting microscope. Two groups of sixty eggs each were exposed to a concentration of either 1 or 10 ng/L of 17β-estradiol, respectively, dissolved in 30% Danieau saline solution. 17β-estradiol was obtained from Sigma Chemical and serially diluted. A third group of 60 eggs was not exposed (control group). The concentrations of E_2 were maintained by changing the solutions daily. The embryos were kept in petri dishes in an incubator at 28.5°C with a 14L: 10D photoperiod. After 5 days, the hatchlings from each group were

separated into two groups, and each subgroup was transferred to continuous flow-through aquaria that employed water purified through reverse osmosis and a pad filter, an FSI particle filter, a fluidized bed biofilter and a UV unit. The aquaria were kept at a pH between 7.0 and 7.45, with conductivity between 300-1500μS. When fish were transferred to the flow-through aquaria, fish were fed commercial food (First Bites fry food by Kyorin). After 10 days fish were fed brine shrimp in addition to First Bites.

Spawning of Treated Fish

When the fish were between 108 and 129 days old, they were bred with wild type zebrafish from the Tubigenin line. The exposed fish were spawned with wild type fish instead of other exposed fish in order to assess whether estrogen affected males and females differently. The gonadal tissue was harvested for histological examination. Gonads were embedded in paraffin (Paraplast®) and sectioned with a microtome at 10 μm thickness. Sections were mounted on glass microscope slides and stained with hematoxylin and eosin. If a fish did not spawn during their first opportunity, it was given another opportunity a week later. If after the second attempt the fish failed to spawn, the fish was fixed in Bouin's fixative. The spawning took place over a four-week period. Fertilization was determined by examination under a dissecting microscope. Adult fish were terminated by immersion in MS-222 (200-300 mg/L) and fixed in Bouin's fixative 24 hr post-spawning. Fertilized eggs were kept for a week to determine hatching numbers, and then terminally anesthetized.

The animal use protocol was approved by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

Statistical Analysis

Separate two-way ANOVAs were performed on the data collected for length, percent hatching, percent fertilized eggs, and total number of eggs produced. Gender and exposure were used as the independent variables.

Results

For each of the dependent variables measured, the null hypothesis was supported. An assumption of a two-way ANOVA is that all treatment groups are a uniform sample size. This was not the case in our study. The statistical program used, SPSS, automatically adjusts a two-way ANOVA when unequal N's across groups are used, which reduces statistical power. When executing four separate ANOVAs the probability of falsely obtaining a significant result when no significance actually exists is increased. To mitigate this problem a Bonferroni adjustment was made on the alpha level (P= 0.16) required for significance. Even when the male fish was the one exposed and the female was unexposed, this number still gives evidence as to the male's effectiveness in stimulating the female to produce eggs. There were no significant differences in any of the parameters measured.

While the numbers give us some information, they do not elucidate the whole picture. For example, there was one control male bred against wild type female that produced eggs but no fertilized eggs. This could mean that the male was not able to produce sperm, or did produce sperm but the sperm was not viable, the male was not ready to produce sperm yet, or the male was physiologically fit but was unable to elicit viable behavior to induce the female to lay eggs. There was another male in the group exposed to 1 ng/L of E_2 that produced 164 fertilized eggs, none of which hatched. Several

treated females produced eggs, but none were fertilized. This could be due to a physiological issue or could be due to behavior.

Length

Exposure	Gender	Mean	Std. Deviation	N
Control	M	26.8889	3.00809	9
	F	28.9167	2.03511	6
	Total	27.7000	2.77617	15
1x	M	29.5833	3.05641	6
	F	32.1667	2.75379	3
	Total	30.4444	3.06639	9
10x	M	28.0833	1.71513	6
	F	27.7500	1.06066	2
	Total	28.0000	1.51186	8
Total	M	28.0000	2.82843	21
	F	29.5909	2.57700	11
	Total	28.5469	2.80943	32

Table 1 Length of Treated and Untreated Fish ± Standard Deviation. The group treated with 1 ng/L of 17β-estradiol is designated 1x, the group treated with 10 ng/ L is designated 10x. The control group was untreated. Also shown is the combined total of the treated groups plus the control group. Exposure: $F = 3.853$, $p = 0.034$; Gender: $F = 1.928$, $p = 0.177$.

Total # Eggs

Exposure	Gender	Mean	Std. Deviation	N
Control	M	38.5556	50.56706	9
	F	59.8333	49.80529	6
	Total	47.0667	49.63361	15
X1	M	46.1667	67.46382	6
	F	24.3333	21.07922	3
	Total	38.8889	55.45143	9
X10	M	57.6667	50.60303	6
	F	55.5	51.6188	2
	Total	57.125	47.01804	8
Total	M	46.1905	53.54028	21
	F	49.3636	43.09124	11
	Total	47.2813	49.5048	32

Table 2 Total # Eggs Produced by Treated and Untreated Fish ± Standard Deviation The group treated with 1 ng/L of 17β-estradiol is designated 1x, the group treated with 10 ng/ L is designated 10x. The control group was untreated. Also shown is the combined total of the treated groups plus the control group Exposure: F = 0.311, p = 0.735; Gender: F =0.002, p = 0.966.

%Fertile Eggs

Exposure	Gender	Mean	Std. Deviation	N
Control	M	43.0444	47.39272	9
	F	35.6833	50.11788	6
	Total	40.1000	46.84522	15
X1	M	49.8990	54.66205	6
	F	33.3333	57.73503	3
	Total	44.3771	52.62510	9
X10	M	60.6500	35.34401	6
	F	34.0500	33.30473	2
	Total	54.0000	34.67510	8
Total	M	50.0330	44.87218	21
	F	34.7455	45.10777	11
	Total	44.7779	44.83103	32

Table 3 Percentage of Fertile Eggs of Treated and Untreated Fish ± Standard Deviation. The group treated with 1 ng/L of 17β-estradiol is designated 1x, the group treated with 10 ng/ L is designated 10x. The control group was untreated. Also shown is the combined total of the treated groups plus the control group Exposure: F = 0.059, p = 0.943; Gender: F = 0.772, p = 0.388.

% Hatching

Exposure	Gender	Mean	Std. Deviation	N
Control	M	17.8000	30.28172	9
	F	14.2667	26.43018	6
	Total	16.3867	27.86905	15
X1	M	23.9167	40.05219	6
	F	.0000	.00000	3
	Total	15.9444	33.84690	9
X10	M	33.2500	25.20546	6
	F	21.5500	15.62706	2
	Total	30.3250	22.75997	8
Total	M	23.9619	31.13894	21
	F	11.7000	20.93136	11
	Total	19.7469	28.31804	32

Table 4 Percentage of Eggs That Hatched of Treated and Untreated Fish ± Standard Deviation. Percent hatching was found by diving the number of hatched eggs by the total number eggs produced, both fertilized and unfertilized. The group treated with 1 ng/L of 17β-estradiol is designated 1x, the group treated with 10 ng/ L is designated 10x. The control group was untreated. Also shown is the combined total of the treated groups plus the control group. Exposure: $F = 0.504$, $p = 0.610$; Gender: $F = 1.239$, $p = 0.734$.

Zebrafish Spawning and Survival Data

Exposure	Gender	% Survival to Spawning	% That Spawned	% That Spawned the First Time
Control	M	-	66.7	55.6
	F	-	83.3	50
	Total	25	73.3	53.3
X1	M	-	50	33.3
	F	-	66.7	66.7
	Total	15	55.6	44.4
X10	M	-	83.3	50
	F	-	100	50
	Total	13.3	87.5	50

Table 5 Percentage of zebrafish exposed to E_2 that survived to sexual maturity, the percentage of treated fish that spawned with wildtype fish as well as the percentage of treated fish that spawned on their first try. Each treatment group shows the data for each individual sex as well as the total. Data for percent survival to spawning was unavailable for each individual sex because sex could not be determined until sexual maturity was reached, and large majority of die off happened early on, much before sexual maturity was reached.

Discussion

Since treatment of the zebrafish at 1ng/L and 10ng/L of E_2 during the post-fertilization, pre-hatching period of time supported the null hypothesis in every parameter measured, I conclude that there is no effect in reproductive fitness.

Vosages et al. (2010) demonstrated significant effects of a similar dose of the pharmaceutical estrogen, ethinylestradiol (EE_2), on GnRH (see also Page et al., 2011) activity in brains following exposure of fertilized eggs to hatching. However, studies in fishes have shown EE_2 to be 5 to 50 times more potent than the natural estrogen E_2

(Thorpe et al., 2003; Nash et al., 2004; Tilton, Foran & Benson, 2005). Their results indicate a developmental sensitivity to estrogens is present in the brain at this early stage of development. However, this was not reflected in the spawning features measured in the present study.

No effect of E_2 on zebrafish body length was evident in the present study. Another study done exposing zebrafish from fertilization to the time for completion of gonad differentiation (day 42) with approximately 300 ng/L E_2 (which is thirty times as much E_2 as the highest dose in my study) did produce an increase in body length. However, lower doses were ineffective (van der Ven et al., 2007). No effect on length from exposure to E_2 in the present study is not surprising.

There is a tremendous amount of variability in the data. For example, in the data for percent fertile eggs and percent hatching (see tables 3 and 4), the majority of standard deviations are larger than the mean. This could mean that the sample sizes simply were not large enough to reveal anything statistically significant. However, the sample sizes were large enough to say something about length. Having a larger N value probably would not have decreased the variability of the data. In endocrinological studies, a standard sample size is usually only between six and ten individuals, and is typically a large enough treatment group to get significance in hormone studies.

Future studies should be done to better understand the developmental sensitive periods of zebrafish. The more we understand about zebrafish, the better we can understand effects of EDCs during development, which is especially useful and applicable to the understanding of EDCs in regards to the prenatal period of human development. This study could be redone with a higher N in order to completely rule out

the possibility of no significant differences between the means because of the low N and uneven numbers in the treatment groups. Additionally this experiment could be repeated while examining fish mating behavior and gonad tissue analysis to understand the sources of variability in the data. This same study could be done with a higher concentration of E_2 to find out what exposure dose would produce a response. A multigenerational study could then be conducted to see if reproductive effects from exposure to E_2 would be passed down through generations.

Limitations of the Present Study

The present study encountered a few difficulties. Due to scheduling issues, the embryos were not checked before they died. The offspring of one male in the 10x treatment group was difficult to obtain hatching data for because there was a large number of hatched embryos that had died and decayed somewhat by the time the petri dish containing the embryos was checked.

To distinguish which zebrafish produced which offspring, so we had to set up the breeding tanks with one male and one female. An ideal breeding situation for zebrafish would be to have more than one mating pair in a single tank (Linbo, 2009). We might have obtained a higher spawning percentage had multiple mating pairs been set up per tank. However, we would have sacrificed the ability to match the histology of certain fish with the parameters we measured.

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