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### Effects of endocrine-disrupting chemical exposure on zebrafish ovarian follicles

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## Effects of endocrine-disrupting chemical exposure on zebrafish ovarian follicles

### Abstract

The process of oocyte maturation involves both nuclear and cytoplasmic events that are essential for the production of viable eggs and subsequent fertilization in sexually reproducing animals. In zebrafish, oocyte nuclear maturation (meiotic resumption) is marked by a transition from opaque to translucent ovarian follicles. This transition is called clearing and results from germinal vesicle breakdown (GVBD) and the transformation of vitellogenin into yolk proteins. Oocyte maturation can be perturbed by endocrine-disrupting chemicals (EDCs). These EDCs have been broadly shown to interfere with reproductive development. Many herbicides and pesticides used agriculturally act as EDCs. These chemicals can enter aquatic ecosystems via runoff and erosion. To evaluate the effects of EDCs on zebrafish oocyte maturation, zebrafish ovarian follicles were cultured and exposed to various concentrations of either atrazine, 2,4-D, or diazinon. Follicles were evaluated at 2, 3, and 4 hours for size and viability, but clearing was assessed only at 4 hours post-exposure. Atrazine and diazinon significantly reduced follicle clearing at the tested concentrations. No effect was observed with 2,4-D exposure. At low enough concentrations, none of the chemicals produced endocrine-disrupting effects. The results suggest that at or above environmentally relevant concentrations, some EDCs impair oocyte maturation in exposed zebrafish follicles. The results support the need to monitor EDC exposure to prevent harmful effects not only on aquatic organisms, but also on humans and wildlife that are also exposed to these contaminants.

### Disciplines

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### Comments

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## **Research Article: Effects of endocrine-disrupting chemical exposure on zebrafish ovarian follicles**

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# Effects of endocrine-disrupting chemical exposure on zebrafish ovarian follicles

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*Abstract.* The process of oocyte maturation involves both nuclear and cytoplasmic events that are essential for the production of viable eggs and subsequent fertilization in sexually reproducing animals. In zebrafish, oocyte nuclear maturation (meiotic resumption) is marked by a transition from opaque to translucent ovarian follicles. This transition is called clearing and results from germinal vesicle breakdown (GVBD) and the transformation of vitellogenin into yolk proteins. Oocyte maturation can be perturbed by endocrine-disrupting chemicals (EDCs). These EDCs have been broadly shown to interfere with reproductive development. Many herbicides and pesticides used agriculturally act as EDCs. These chemicals can enter aquatic ecosystems via runoff and erosion. To evaluate the effects of EDCs on zebrafish oocyte maturation, zebrafish ovarian follicles were cultured and exposed to various concentrations of either atrazine, 2,4-D, or diazinon. Follicles were evaluated at 2, 3, and 4 hours for size and viability, but clearing was assessed only at 4 hours post-exposure. Atrazine and diazinon significantly reduced follicle clearing at the tested concentrations. No effect was observed with 2,4-D exposure. At low enough concentrations, none of the chemicals produced endocrine-disrupting effects. The results suggest that at or above environmentally relevant concentrations, some EDCs impair oocyte maturation in exposed zebrafish follicles. The results support the need to monitor EDC exposure to prevent harmful effects not only on aquatic organisms, but also on humans and wildlife that are also exposed to these contaminants.

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## Introduction

The process of oocyte maturation is essential for the production of viable eggs and subsequent fertilization in sexually reproducing animals. Oocyte maturation is initiated when the brain produces gonadotropin-releasing hormone (GnRH), which causes the pituitary to secrete luteinizing hormone (LH) and primes follicles to synthesize and respond to maturation-inducing hormone

(MIH) (Clelland and Peng, 2009; Rime et al., 2010). MIH production by ovarian follicles requires an interaction between the thecal and granulosa cell layers, the epithelial cell types of the vertebrate ovary (Nagahama and Yamashita, 2008). A natural MIH in many fish species is  $17\alpha$ -hydroxy,  $20\beta$ -dihydroprogesterone ( $17,20\beta$ -DHP; Tokumoto et al., 2005). MIH binds to receptors on the oocyte membrane and triggers a protein cascade that activates maturation-promoting factor (MPF) in the oocyte cytoplasm (Tokumoto et al., 2005; Tokumoto et al., 2008b). MIH binding induces MPF formation by initiating cyclin B production, a regulatory protein required for MPF activity (Nagahama

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and Yamashita, 2008; Tokumoto et al., 2012). Once active, MPF initiates the resumption of the meiotic cycle by releasing immature oocytes from meiotic arrest that occurs during prophase I. Morphological hallmarks observed during oocyte maturation in the fish include germinal vesicle breakdown (GVBD) at the prophase/metaphase transition and also the transition from opaque ovarian follicles to transparent follicles (Tokumoto et al., 2004; 2008b; 2011). Follicle clearing occurs when vitellogenin is transformed into yolk proteins and this translucent appearance is indicative of maturation in follicle clearing assays (Clelland and Peng, 2009).

Zebrafish are an excellent model organism to study oocyte maturation because they undergo processes that are highly conserved among other vertebrate species and the zebrafish genome has been fully sequenced (Weber et al., 2013). By performing toxicological studies in zebrafish, the impact on human and environmental populations can be accurately assessed. Unlike mammalian models, zebrafish are oviparous making early exposure possible without affecting the mother and visualization of maturation and embryonic events is relatively easy (Yen et al., 2011). Zebrafish are considered sexually mature three months post-hatching, which allows for rapid experimentation (Clelland and Peng, 2009). In comparison to other small fish models, the number of eggs spawned by zebrafish is greater than that of all other models and oocytes can be collected during all seasons (Tokumoto et al., 2004). Spawning occurs under favorable environmental conditions every 4-7 days (Clelland and Peng, 2009). Zebrafish are appropriate for research involving EDC exposure studies because many of the factors involved in physiological processes during embryonic development and oocyte maturation have been identified (Osterauer and Köhler, 2008). Thus, the goal of this study was to assess the stimulatory, inhibitory, or neutral effects of atrazine, 2,4-D, and diazinon exposure on oocyte maturation in zebrafish.

The pathway involved in oocyte maturation can be perturbed by endocrine-disrupting chemicals (EDCs) causing them to interfere with

reproductive development. For this reason, identifying MIH receptors would allow for a better understanding of how EDCs elicit their effects in nontarget tissues and organisms. Membrane progesterin receptors (mPRs) have characteristics of G-protein-coupled receptors and are currently being investigated as potential MIH receptors because they are located on follicle-enclosed oocytes and have been shown to have high affinity for MIH (Nagahama and Yamashita, 2008; Tokumoto et al., 2011; 2012). In zebrafish, mPR $\alpha$  and mPR $\beta$  have been identified as potential candidates for this binding activity (Rime et al., 2010; Tokumoto et al., 2005). Studies have shown that blocking the mPR $\alpha$  with antisense oligonucleotides inhibits oocyte maturation in goldfish and zebrafish, supporting the idea that mPRs might be mediators for MIH and EDCs that block development in exposed aquatic species (Tokumoto et al., 2007; 2012). Despite EDC-specific differences in their mechanisms of action, disrupting cyclin B synthesis via the oocyte maturation pathway is one potential mechanism of action (Kortner and Arukwe, 2007). EDCs are prevalent in the environment and available literature proposes that reproductive development is susceptible to chemical pollutants, especially in aquatic organisms (Kortner and Arukwe, 2007). Understanding that mPRs provide a common link between the effects of some EDCs and MIH, oocyte maturation in fish species can be used as a model for understanding the effects of exposure to chemicals in the environment (Ogawa et al., 2011; Tokumoto et al., 2008b).

EDCs are exogenous agents that interfere with hormonal regulation that is responsible for regulating many developmental processes as well as the maintenance of homeostasis. EDCs can mimic or block the activity of endogenous hormones and thus can have significant impacts on reproductive development and viability if organisms are exposed during critical stages of development (Scholz and Mayer, 2008). For the purpose of this paper, it is important to emphasize that maturation of oocytes occurs in adults, whereas migration of primordial germ cells occurs in embryos. This allows for the

same cells to be studied at different points in time to provide data about how EDCs affect reproduction in its entirety. EDCs can act as agonists or antagonists of oocyte maturation in fish (Tokumoto et al., 2005). Despite the results of studies demonstrating that bisphenol A (BPA), diethylstilbestrol (DES), and prochloraz are capable of inducing oocyte maturation, many EDCs inhibit maturation and impair reproductive development (Baek et al., 2007; Ogawa et al., 2011; Rime et al., 2010). EDCs shown to antagonize oocyte maturation include *o,p*-DDD (lysodren), methoxychlor, and ethynyl estradiol (Tokumoto et al., 2008a). The inability for fish to detoxify their environment and the lack of biodegradation of EDCs may contribute to the harmful effects EDCs have on fish development (Wiegand et al., 2001). The structural resemblance of some EDCs to endogenous hormones increases the likelihood that they employ mPRs to produce their stimulatory or inhibitory effects (Tokumoto et al., 2004). One study proposed that the mechanism of DES involves activation of mPR $\alpha$  to cause stimulatory effects on oocyte maturation (Tokumoto et al., 2007). The prevalence of EDCs worldwide makes them an important topic for research because EDCs invade aquatic habitats via many routes of entry and disrupt reproductive development in nontarget organisms (Wiegand et al., 2001). EDCs can have deleterious and even permanent effects on tissue formation and fecundity, and promote an increased likelihood of cancer development. Studies have identified notable alterations in the development of neuroendocrine and reproductive systems as a result of chemical exposure (Weber et al., 2013). It is by way of interfering with receptors or enzymes involved in steroid synthesis and metabolism that EDCs may impair various processes involved in reproduction (Ma et al., 2012). Aquatic organisms are not the only nontarget organisms affected. For example, there is concern that EDCs have the potential to harm human development if exposure occurs during critical developmental stages (Wiegand et al., 2001). Studies have associated the development of testicular dysgenesis syndrome in humans to EDC exposure

(Santos et al., 2007). For this reason, research that employs a vertebrate model, like zebrafish, is capable of demonstrating how severely development is affected, what aspects of development are affected, and how these effects might translate to human health. Using data collected from toxicological studies may initiate the development of systems that reduce the impact EDCs have on nontarget organisms. One current system being analyzed is sonophotocatalysis. This system combines the activities of sonolysis and photolysis, which have a synergistic interaction enabling the breakdown of organic molecules like some EDCs. By using this method, activities of EDCs can be reduced or completely lost, as was observed with DES (Tokumoto et al., 2008a). This study focuses on three of the most commonly used EDCs in agriculture, including atrazine (2-chloro-4-ethylamino-6-isopropylamine-s-triazine), 2,4-D (2,4-Dichlorophenoxyacetic acid), and diazinon (O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl]).

Atrazine is the most common herbicide pollutant of freshwater ecosystems. As a selective herbicide, it is used to increase yields of crops such as sugarcane and soy, but also to control weeds and grasses by reducing carbon fixation (Jin et al., 2009). Atrazine enters aquatic ecosystems by way of runoff and erosion, contaminating the habitats of many nontarget aquatic species. Increased exposure to atrazine creates a concern about bioaccumulation in nontarget organisms because it is very persistent and has biological effective levels that last several weeks. The approximate environmental concentration of atrazine is 5  $\mu\text{g/L}$ , which has been found to alter zebrafish behavior (Wiegand et al., 2001). In smaller aquatic environments, atrazine concentrations can reach as high as 1 mg/L, thus increasing the likelihood that nontarget organisms will experience deleterious effects following chemical exposure. These detrimental effects include degradation of gill epithelium and physiological consequences like increased respiration, reduced reflexes, and increased renal excretion of proteins in fish (Wiegand et al., 2001). Poor development of male gonads has also been observed in fish

exposed to atrazine (Ghodageri and Katti, 2013). Atrazine can also yield toxic effects in fish by increasing oxidative stress (Jin et al., 2009). Low doses of atrazine have been shown to demasculinize fishes and amphibians by interrupting reproductive development and function in such a way that viability is harmed (Hayes et al., 2002). Literature concerning atrazine exposure reveals some inconsistencies about whether it impacts reproductive development, the severity of its impact, and the mechanisms it uses to produce effects in aquatic organisms (Solomon et al., 2008). These inconsistencies have thus driven this study to help elucidate atrazine's potential to impair oocyte maturation in zebrafish.

Chlorophenols are utilized as biocides, wood treatments, and as methods for bleaching paper mills. 2,4-D is one of the most common chlorophenols found in the environment. It is a synthetic auxin used in agriculture to regulate plant growth, such as to pasture land, corn, sugarcane and other crops (Koç and Akbulut, 2012). 2,4-D is an herbicide used worldwide, and similar to atrazine, is relatively persistent with a half-life of 20-200 days (Bukowska, 2006). The by-product of 2,4-D is 2,4-dichlorophenol (2,4-DCP), both of which have been observed to act as potential threats to humans and wildlife. The mechanism by which 2,4-D disrupts reproduction in nontarget organisms involves the hypothalamic-pituitary-gonadal axis (HPG) and steroidogenesis, both of which play major roles in reproductive development. A study of zebrafish exposed to 2,4-DCP observed a decrease in the number of eggs spawned and also a decrease in the number of eggs that hatched successfully (Ma et al., 2012). Another study observed slower rates of oogenesis and an increase in the number of nonviable oocytes. The delay in oogenesis corresponded to growth inhibition and slowed development of oocytes (Koç and Akbulut, 2012). Similar to atrazine, 2,4-D is associated with increased production of free radicals that can cause oxidative stress, cell damage, and tissue transformation in nontarget organisms (Bukowska, 2006).

Diazinon is an organophosphorus pesticide that was originally used in agriculture and in household environments for controlling insects. It has been restricted from household use due to its potential threat to human health, but it is still widely used agriculturally and industrially (Yen et al., 2011). Diazinon is an appealing choice for a pesticide because it is degraded quickly, can be applied in low volumes, and has variable chemical structures compared to other pesticides (Osterauer and Köhler, 2008). Despite rapid degradation, the acute toxicity attributed to organophosphates makes it an environmental concern (Ghodageri and Katti, 2013). In fish, glutathione-S-transferases, monooxygenases, and oxonases are involved in diazinon metabolism (Keizer et al., 1991). The metabolized form of diazinon serves as an acetylcholinesterase (AChE) inhibitor, which allows for the build-up of acetylcholine (ACh). Excess ACh in neuronal synapses results in excessive firing of cholinergic receptors that eventually paralyzes target organs. Following application of diazinon in the environment, the chemical is easily washed into surface and groundwater, thus threatening nontarget organisms in these aquatic environments (Yen et al., 2011). Diazinon can enter the body via skin contact, feeding, and inhalation. The effects of exposure to toxic chemicals can also be worsened by physical stressors, including increased temperature. One study in zebrafish demonstrated that elevated temperatures increased fish metabolism and solubility of diazinon, acting synergistically to cause higher rates of early hatching and mortality (Osterauer and Köhler, 2008). The purpose of diazinon in this study is to further assess how exposure affects oocyte maturation and reproductive development since it is currently used in agriculture, but is known to have harmful effects.

## Materials and Methods

Zebrafish were purchased from a local pet store (Rochester, NY) and were acclimated to the housing system for at least two weeks prior to experimentation. All experimentation was

reviewed and approved by the Institutional Animal Care and Use Committee at St. John Fisher College. Mixtures of male and female zebrafish were maintained in tanks containing 4-8 fish (1-2 males for every 3-4 females) with a constant outflow of water. Zebrafish used in experiments were between 6-18 months old. They were maintained at 27-29°C on a 14 h light/10 h dark cycle. The salinity of the system was monitored every other day and was kept between 1400-1600 microsiemens. The pH was measured twice per week and was maintained between 6.9-7.4. Fish were fed twice daily; once with live brine shrimp (Utah Red Shrimp from Artemac L.L.C.) and once with either freeze-dried bloodworms (San Francisco Bay Brand) or flake food (Ocean Star International freshwater aquarium flake food).

The maturation inducing steroid, 17, 20 $\beta$ -DHP was purchased from Steraloids, Inc. (Q1850-000). Atrazine (#49085), 2,4-D (#49083), and diazinon (#45428) were purchased from Supelco/Sigma. Leibovitz (L-15) medium without L-Glutamine was purchased from Sigma-Aldrich Co. (SLBG8614) and used at 90%, pH 9 (Seki et al., 2008).

### Test chemicals and concentration

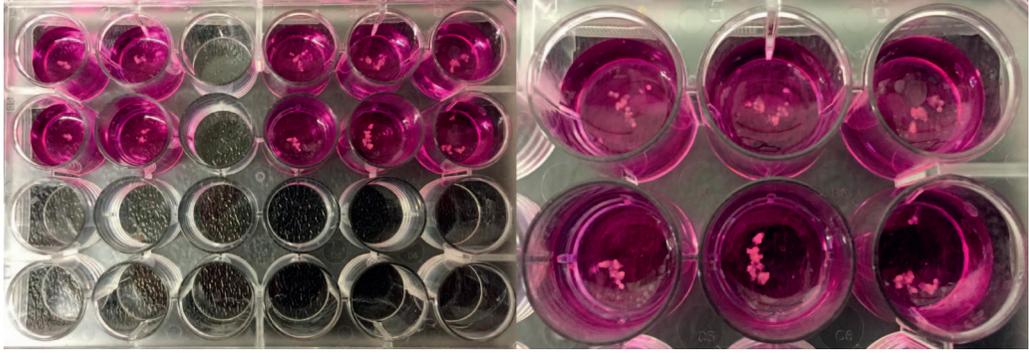
Atrazine was prepared as a stock solution with a concentration of 15 mM. The final concentrations of atrazine in the experiments were 500 nM, 1  $\mu$ M, and 10  $\mu$ M. A stock solution of 0.1 mM was made for 2,4-D and from this stock, 0.42 nM, 20 nM, and 1 nM working solutions were prepared. Diazinon was provided as a neat solution with a concentration of 3.67 mM. The final working concentrations for diazinon in this experiment were 2.44  $\mu$ M and 24.45  $\mu$ M.

### Oocyte preparation and *in vitro* culture

From the mixture of zebrafish in each tank, female zebrafish were selected and placed in an ice water bath to be anesthetized. Zebrafish stopped swimming after approximately 10-20 sec and were removed from the ice water bath after approximately 45 sec. Upon removal

from the ice water bath anesthetized zebrafish were placed into a petri dish. The zebrafish were then decapitated with a razor blade and microdissection tools were used to perform the remainder of the dissection. Fine forceps and scissors were used to make parallel incisions on each side of the ventral body wall to the paired pelvic fins. The ventral body wall covering the zebrafish ovaries was removed to expose the ovaries and forceps were used to carefully collect and transfer ovarian tissue to a petri dish that contained fresh zebrafish Ringer's solution (115.98 mM NaCl, 2.89 mM KCl, 1.79 mM CaCl, 5.00 mM HEPES, pH 7.2). The ovarian tissue was gently agitated with forceps to remove loosely bound fatty globules and blood vessels. The tissue was then washed as it was transferred to another petri dish containing fresh Ringer's solution. The agitation and transfer was repeated once more. A Leica S8AP0 dissecting scope was then used to dissect the ovaries after the third wash in Ringer's solution.

Under 1.25X magnification, forceps were used to dissect the follicles into fragments containing 2-10 oocytes each (Tokumoto et al., 2005). The remaining blood vessels and fatty globules were removed from the zebrafish follicles to ensure they would not interfere with oocyte maturation. Oocyte fragments were placed in a petri dish containing fresh Ringer's solution as they were dissected. Once all fragments of immature oocytes were placed into the petri dish of Ringer's solution, they were dispersed randomly in a 24-well plate. The number of follicles per well was carefully estimated to achieve relatively similar quantities in the control and experimental groups. For each female fish sacrificed, experimental and control wells (two types: clearing and carrier controls) were generated from the follicles. The first control (clearing) had 17, 20 $\beta$ -DHP in L-15/Ringer's solution to ensure that follicle clearing occurred normally. The second control (carrier) had 1 or 10  $\mu$ L ethanol in L-15/Ringer's to confirm that the experimental results were due to the specific chemical being tested and not the carrier (ethanol). For atrazine, the control wells contained 1.5 mL



**Figure 1.** Representative images of typical experimental setup. Immature follicles are cultured in a 24-well plate. The leftmost image displays control (left) and experimental (right) wells separated by a single row. The rightmost image shows only the six experimental wells; the third well in the top row, and all wells in the bottom row demonstrate follicles being exposed to DHP and 1 or 10  $\mu\text{L}$  of EDC.

of Ringer's solution with 1  $\mu\text{L}$  of ethanol. Carrier controls were not performed for diazinon or 2,4-D. Specifically, diazinon was provided as a neat solution and methanol controls for 2,4-D were unnecessary since no effect on follicle clearing was noted following 2,4-D exposure.

In a 24-well plate, control and experimental wells were populated with follicles. Follicles exposed to atrazine and 2,4-D were cultured in zebrafish Ringer's solution (Tokumoto et al., 2005). Follicles exposed to diazinon were cultured in either Ringer's solution or L-15 media (Selman et al., 1994). Both culture media have been shown to be effective for follicle clearing studies (Selman et al., 1994; Tokumoto et al., 2005). The number of wells used for oocyte distribution was based on the amount of immature oocytes collected from each female. Females carrying more follicles required more wells to allow for ample space to score each fragment since overcrowded follicles could potentially contribute to scoring errors. The follicles in the control group and in the experimental groups were separated by a single well in the 24-well plate to avoid confusion. A standard plate used in experimentation can be found in Figure 1.

The 24-well plate was maintained in a water bath at 26°C for a 4-hour incubation period. The follicles were evaluated at 2, 3, and 4 hours using the Leica S8AP0 dissecting scope at

1.25X magnification. The follicles were carefully evaluated to ensure that they were of size ( $\geq 0.65$  mm); those that met the size requirement were scored for transparency, which is indicative of oocyte maturation. A count of the opaque, or immature follicles, was also noted at each time point. The data collected were charted and used to calculate clearance rates at 4 hours in the absence and presence of either atrazine, diazinon, or 2,4-D.

### Statistical analysis

Statistical analyses were performed to assess the significance of the test results. Specifically, One-way ANOVA was performed using SPSS software. Means, standard deviations (SD), and significance levels were determined and are reported in Table 1.

## Results and Discussion

The impact of EDC exposure on oocyte maturation was investigated with follicle clearing assays performed using zebrafish oocytes. Follicle clearing has been associated with the resumption of meiosis in the zebrafish and is therefore used as an assay of oocyte maturation (Selman et al., 1994). Figure 2 shows the physical appearance of both cleared (meiotic resumption) and uncleared (no meiotic resumption) follicles.

**Table 1.** Experimental results for clearance rates of follicles treated with an EDC or 17, 20 $\beta$ -DHP (control) only.

Treatment <sup>1</sup>	Dose	Replicates	Mean $\pm$ SD of clearance rate in EDC treated follicles	Mean $\pm$ SD of clearance rate in 17, 20 $\beta$ -DHP control follicles	Significance	Conclusion regarding oocyte maturation <sup>2</sup>
Atrazine	500 nM	3	50.7 $\pm$ 13.7	69.7 $\pm$ 6.6	0.101	No effect
Atrazine	1 $\mu$ M	3	39.7 $\pm$ 8.7	73.3 $\pm$ 4.7	0.008*	Reduced
Atrazine	10 $\mu$ M	5	39.2 $\pm$ 14.8	74.8 $\pm$ 5.6	0.011*	Reduced
2,4-D	0.42 nM	4	72.6 $\pm$ 6.2	74.3 $\pm$ 2.3	0.643	No effect
2,4-D	1 nM	2	70.5 $\pm$ 19.1	69.6 $\pm$ 9.1	0.920	No effect
2,4-D	20 nM	4	71.1 $\pm$ 7.9	70.1 $\pm$ 5.3	0.816	No effect
Diazinon	2.44 $\mu$ M	7	47.8 $\pm$ 12.2	77.0 $\pm$ 5.3	0.000*	Reduced
Diazinon	24.45 $\mu$ M	7	47.3 $\pm$ 11.7	77.6 $\pm$ 5.4	0.002*	Reduced

<sup>1</sup>Chemical treatment and dose that follicles were exposed to are listed next to their corresponding P values.

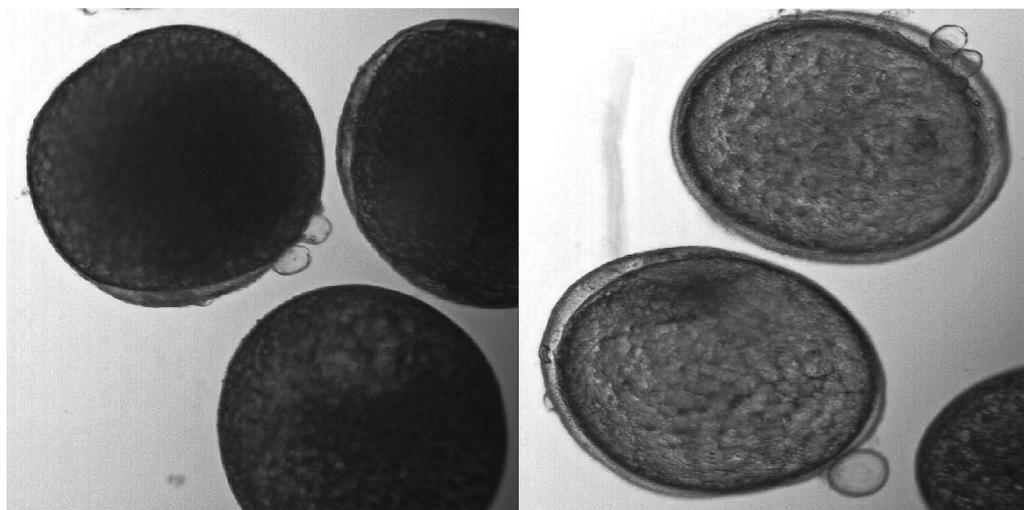
<sup>2</sup>The tests revealed a significant increase in follicle clearing when compared to ethanol (vehicle) controls and a significant reduction in clearing with EDC exposure where indicated.

\* indicates significant differences at  $p < 0.05$ .

Atrazine and diazinon significantly reduced follicle clearing, while no effect was observed for 2,4-D exposure (Table 1). Atrazine decreased clearing rates at exposures of 10  $\mu$ M and 1  $\mu$ M, but no reduction in clearing rates was observed at 500 nM atrazine. Diazinon impaired clearing at both 2.44  $\mu$ M and 24.45  $\mu$ M. 2,4-D, the other herbicide used in our studies, did not significantly alter follicle clearing rates following low dose exposure (0.42 nM, 1 nM, 20 nM) compared to clearing rates for unexposed control follicles.

Results obtained for atrazine and diazinon exposure align with the literature indicating that deleterious developmental effects are induced by chemical exposure (Osterauer and Köhler, 2008; Wiegand et al., 2001). Surprisingly however, 2,4-D did not affect follicle clearing rates in this system contrary to what has been reported in the literature (Koç and Akbulut, 2012; Ma et al., 2012).

Developmental alterations in nontarget organisms exposed to atrazine include teratogenic



**Figure 2.** Follicle clearing is marked by the transition from uncleared or opaque (left) to cleared or translucent (right) ovarian follicles. This transition occurs as a result of GVBD and transformation of vitellogenin into yolk proteins.

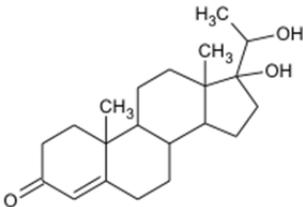
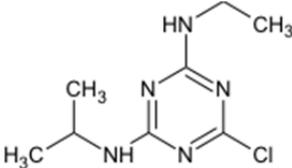
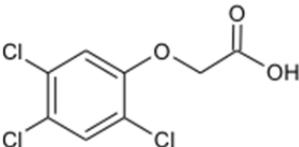
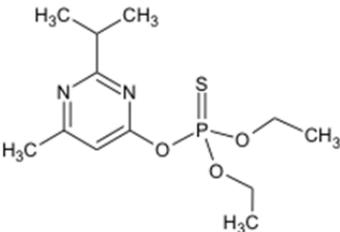
effects on developing zebrafish embryos, specifically during organogenesis, which can impact the survival and reproduction of future generations (Wiegand et al., 2001). Despite the dose-dependent increase in detoxification metabolism that occurs post-atrazine exposure, proteins involved in detoxification can denature at high atrazine concentrations, yielding acute developmental defects (Wiegand et al., 2001). The results of this study, combined with what is already known about atrazine's effects on organogenesis, supports atrazine's ability to disrupt organismal development and reproduction. The inability of atrazine at 500 nM to produce a significant change in zebrafish follicle clearing may be attributable to research suggesting that the effects of atrazine exposure are reversible once exposure is terminated (Solomon et al., 2008). Specifically, Solomon et al. demonstrated that higher concentrations of atrazine are required to produce long-lasting effects, but that low doses of atrazine do not pose a significant threat to exposed nontarget organisms.

The classification of diazinon as an EDC was supported by our experimental results as diazinon reduced the frequency of oocyte maturation. Negative effects have also been observed in zebrafish exposed to diazinon wherein lower hatching rates and increased mortality were reported (Osterauer and Köhler, 2008). There is also data supporting reduced motility in zebrafish exposed to 10  $\mu$ M diazinon, but no significant increase in mortality was noted (Yen et al., 2011). This study demonstrated the potential for diazinon to impair reproduction, but research suggests that many developmental systems can be affected by EDC exposure. The ability for EDCs, specifically diazinon, to cause harm and affect an array of different functions implies that there may be common targets found, in the various systems, which help to explain the observed effects.

The use of 2,4-D in this experiment was supported by literature demonstrating detrimental effects of 2,4-D on reproduction in fish (Koç and Akbulut, 2012; Ma et al., 2012). 2,4-D has been shown to disrupt steroidogenesis

and reduce the number of eggs spawned and hatched by zebrafish (Ma et al., 2012). 2,4-D exposure has also been associated with delayed oogenesis, marked by fewer oocytes in the ovaries and an increase in deformed/atretic oocytes (Koç and Akbulut, 2012). Mature and viable oocytes are required for eventual fertilization and hatching, providing convincing evidence that changes in follicle clearing would be another likely target for the EDC effects of 2,4-D. Unexpectedly however, no negative effects were observed following 2,4-D exposure in our study suggesting the need for additional investigation with a wider range of chemical concentrations. Chemical structures for each molecule can be found in Figure 3.

The impairments to oocyte maturation when zebrafish were exposed to atrazine and diazinon in this study demonstrated the potential of these chemicals to act as EDCs. Taken together with the available literature, there are ample data to suggest that exposure to EDCs can have severe perturbations on the functioning, reproduction, and overall health of nontarget organisms. Discrepancies in the results regarding EDC exposure may be due to the wide variety of factors that influence oocyte maturation and also on the concentrations to which the organisms were exposed. Reproductive processes, including those involved in oocyte maturation, are complex and can be influenced by both external and internal factors. It is important to note that at very low concentrations the harmful effects of EDC exposure may be lost, even if effects are present at higher concentrations, which is demonstrated by atrazine in this study. These follicle clearing studies supplement the existing literature demonstrating negative effects following EDC exposure. Specifically, our work adds the follicle clearing assay as another tool to evaluate the EDC activity of atrazine and diazinon. Understanding the effects of EDCs is important since humans and wildlife are exposed to EDCs on a daily basis and both humans and wildlife have the potential to suffer severe implications that have not yet been identified for most chemi-

Treatment	Chemical Structure
17, 20 $\beta$ -DHP	
Atrazine	
2,4-D	
Diazinon	

**Figure 3.** Chemical structures for 17, 20 $\beta$ -DHP, atrazine, 2,4-D, and diazinon. 17, 20 $\beta$ -DHP is a Maturation Inducing Hormone (MIH) commonly used in zebrafish follicular maturation studies. Atrazine and 2,4-D are herbicides. Diazinon is a pesticide. Despite structural similarity, chemical effects on nontarget organisms are difficult to predict based on the varied mechanisms of EDC action.

icals. In addition to broadening the list of organisms exposed in EDC studies, future investigation should increase the range of concentrations tested to generate data for EDC effects at different doses.

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