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Abstract

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Using zebrafish (*Danio rerio*) to study the behavioral impacts of early bisphenol F exposure reveals decreased swim speed, increased distance between fish, and increased freezing behaviors

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Abstract. The widespread use of endocrine disrupting chemicals (EDCs) has been a source of concern because of their various effects on the endocrine system. These effects include metabolic disorders, complications in reproductive health, hormone-related cancers, and neurodevelopmental disorders. Of particular concern is bisphenol A (BPA), a synthetic compound commonly found in consumer products such as water bottles, thermal receipt paper, and epoxy resins used in processed food packaging. Previous studies have shown that BPA can mimic estrogen through a variety of mechanisms and thus elicit an endocrine response. Some manufacturers have responded by removing BPA from their products; however, studies using a replacement compound bisphenol S have reported it to be just as, if not more, dangerous. The use of zebrafish (*Danio rerio*) larvae as a model organism allows for the effects of bisphenol exposure to be rapidly quantified through a simple behavioral assay. In studies involving bisphenol exposure, the use of zebrafish has demonstrated reproductive, developmental, endocrine, and behavioral effects. The study of bisphenol F, yet another endocrine disruptor that has become a replacement for BPA in consumer products, is highly important to public safety.

Introduction

Endocrine disrupting chemicals (EDCs) are substances that have widespread effects on the endocrine systems of humans and other animals at very low doses. EDCs elicit their effect through a variety of different mechanisms, including the disruption of hormone synthesis, binding, or elimination. These disruptions can lead to alterations in development and behavior. Many EDCs are found in household and industrial products that

humans come into contact with every day, making them particularly relevant to study. Due to the numerous different systems of the body that are influenced by hormones, EDCs can lead to metabolic disorders, reproductive health issues, hormone-related cancers, and neurodevelopmental disorders (Erler and Novak, 2010; Giulivo et al., 2016). When cells are growing and differentiating during the critical period of development, specific doses of hormones are imperative for proper maturation of the developing fetus. The introduction of an EDC that either blocks or increases the dosage of a certain hormone can have enormous consequences on animal growth and development. One of the

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Figure 1. Structure of bisphenol A (BPA) and its common analogues, bisphenol S (BPS) and bisphenol F (BPF). Each structure shares the common feature of two phenol rings, leading to concerns over the way these replacement compounds (BPS and BPF) may act as endocrine disruptors.

most prolific EDCs in recent years is bisphenol A (BPA).

BPA was first developed in the 1890s and was recognized as a synthetic estrogen in the 1930s because it was shown to have similar effects to estrone in female rats (Dodds, 1936). Since the 1950s, BPA has been used in the manufacture of polycarbonate plastics, including water bottles, food containers, the linings of metal food cans, medical equipment, children's toys, and thermal receipt paper (Vandenberg et al., 2007). It has been shown to mimic endogenous estrogen and bind the estrogen receptor to elicit responses, and can also act as an anti-estrogen by blocking the estrogenic response through competition with endogenous estradiol (Bonefeld-Jorgensen et al., 2007). Studies have also shown that BPA can bind directly to androgen receptors to activate or block endogenous androgen action (Kinch et al., 2015; Wetherill et al., 2007). In addition, BPA can bind thyroid receptors and has been shown to have both agonistic and antagonistic effects on thyroid function (Moriyama et al., 2002). Likely due to the numerous ways in which BPA can interact with hormone receptors in the body, BPA exposure has been linked to a number of disorders, including cancer, reproductive problems, early puberty, obesity, neurodevelopmental disorders, and heart disease (Vogel, 2009). These widespread effects on the human body make bisphenol exposure particularly relevant to study.

Early BPA exposure has been implicated in numerous neurodevelopmental disorders, including attention-deficit/hyperactivity disorder (ADHD; Li et al., 2018), impaired social function (Miodovnik et al., 2011), autism (Stein

et al., 2015), and behavioral problems (Roen et al., 2015). Further exploration of the link between early bisphenol exposure and neurodevelopmental disorders is necessary to understand the specific mechanism of bisphenols on the nervous system. Bisphenols, like other EDCs, are known to act through a nonmonotonic dose curve, meaning that there is a nonlinear relationship between the dose and the effect (Vandenberg et al., 2012). EDCs can elicit an effect at very low doses and very high doses, and yet may show no effect at a mid-range level. This makes them particularly concerning given how ubiquitous they are in the environment.

Despite the numerous scientific studies showing otherwise, the FDA maintains that BPA is safe to consumers at the current exposure level (Center for Food Safety and Applied Nutrition, 2018). However, in response to the numerous studies implicating BPA in developmental problems, many manufacturers have removed BPA from their products in response to consumer concerns. This has only lead to the replacement of one harmful chemical with another. Specifically, several bisphenol analogs exist, including bisphenol F and bisphenol S (Fig. 1). These chemicals all share the common feature of two phenol rings, and it is their similar structures that raise concerns that bisphenol analogs may act on the body in much the same way that BPA does. BPF is a BPA analog that is structurally identical except for the absence of the two methyl groups, as highlighted in Figure 1. BPF has comparable estrogen and anti-androgen activity to that of BPA (Fic et al., 2014). BPS is commonly used in the production of polycarbonate plastic and

especially in the production of thermal paper (Liao et al., 2012). Studies have found that BPS was not detected in human urine samples collected in earlier years, but more recent samples (between 2009 and 2014) contained significant amounts of BPS (Ye et al., 2015). This indicates that exposure to BPS is on the rise in more recent years, as it is added to products from which BPA has been removed. It is clear that if manufacturers will continue to use alternative bisphenols in “BPA-free” products, further research must be done to evaluate the safety of these structurally-similar compounds.

The use of zebrafish (*Danio rerio*) as a vertebrate organism for developmental processes has become an emerging model system for human disease in the past two decades (Zon, 1999). There is evidence of synteny, or conservation of blocks of genes between chromosomes in different species, between the human genome and the zebrafish genome, which allows for their use in numerous studies as a model organism for human disorders (Barbazuk et al., 2000). The use of zebrafish is advantageous due to their small size, their short life span, the ease of rearing, and their well-defined embryonic development, as described in Kimmel et al. (1995). Zebrafish are an ideal model organism for research into how specific chemicals may affect vertebrate development.

The effects of EDCs on zebrafish development have been explored by several research groups in recent years. In particular, the effect of BPA exposure on brain development at low doses has been an emerging topic of interest. Developmental BPA exposure has implications for adverse behavior and learning deficits, including neurological disorders such as anxiety and hyperactivity, but the mode of action that underlies these effects is unclear. A study performed using larval zebrafish found that neurodevelopmental BPA exposure (0.01, 0.1, and 1 μM) led to larval hyperactivity and learning deficits in adult zebrafish when exposed from 8 hours post fertilization to 5 days post fertilization (Saili et al., 2012). This study also found that exposure to 0.1 μM 17 β -

estradiol led to larval hyperactivity, implicating increased estrogen levels in adverse zebrafish developmental effects. A more definitive study by Kinch et al. expanded on this work by exposing larvae to very low doses (0.0068 μM) of BPA and BPS. The results of this study showed a 180% (BPA-exposed) and 240% (BPS-exposed) increase in neurogenesis in the hypothalamus, a region of the brain involved in hyperactivity (Kinch et al., 2015). BPA and BPS exposure caused the birth of neurons earlier in development, providing for the first time a possible mechanism for bisphenol effects on neurodevelopmental disorders. Exposure occurred during the specific window of 24-36 hours post fertilization, when peak neurogenesis is occurring. The Kinch group hypothesized that the mechanism of precocious neurogenesis was BPA acting through estrogen receptors; however, the results of their study revealed that bisphenols did not act in this manner, but rather through androgen receptors (Kinch et al., 2015). The significance of the Kinch study was to provide support that the neurogenic period is a vulnerable window in development and to suggest a mechanism for how BPA and BPS affect early brain development.

Given the similarities in structure between BPA and BPF, and knowing that BPS was shown to work through a similar mechanism, one might logically assume that BPF will act through a similar mechanism. However, studies into the harmful effects of BPF are only beginning to emerge in recent years. A study found that BPA may disrupt neural differentiations in human neural progenitor cells, leading to abnormal development of the brain after gestation; the same study found no changes in neural development with BPF treatment, suggesting a different mechanism or less adverse effects (Fujiwara et al., 2018). Novel evidence in mice suggests that BPF induces anxiety and depression behaviors that are more significant than those induced by BPA exposure in offspring when the mother is treated orally (Ohtani et al., 2017). BPF has been shown to significantly alter 56 different genes involved in the serotonin-dopamine pathway in the prefrontal cortex of female rats, changing the transcrip-

Table 1. Common behaviors that can be measured using a behavioral assay.

Common behavior	How measured in a behavioral assay
Avoidance	Time spent in the upper or lower half of the well in response to a stimulus (ex: moving red bar).
Thigmotaxis	Edge preference; amount of time spent on the edge of the well. Can be a measure of anxiety in larval zebrafish.
Phototaxis	Orientation towards or away from a light stimulus.
Freezing	Time spent not moving.

tion of these genes (Castro et al., 2015). Studies with zebrafish embryos have revealed decreased heart rates, hatching inhibition, and disrupted development when treated with BPF (Mu et al., 2018). Similar to the mechanism proposed by the Kinch lab, BPF was shown to upregulate aromatase in the brain of embryonic zebrafish through estrogen receptors by acting as a full agonist *in vivo* (Le Fol et al., 2017).

Given the proposed mechanism of bisphenol A exposure on larval zebrafish, coupled with the structural similarities of bisphenol F and previous studies suggesting similar adverse effects, it is hypothesized that larval zebrafish exposure to BPF at a window of 24-48 hours post fertilization will result in behavioral changes like increased freezing behaviors and thigmotaxis (Table 1). These are established markers of anxiety-like behaviors in zebrafish (Colwill and Creton, 2011a; Colwill and Creton, 2011b; Gerlai et al., 2000; Maximino et al., 2010; Richendrfer et al., 2012).

Materials and Methods

Adaption of the imaging system

The Kinch lab uses a commercially available platform, Zebrafish (ViewPoint Life Sciences), to observe larval zebrafish swimming behaviors. Use of this system was not feasible at our institution, so the literature was consulted for a more practical solution. Richendrfer and Creton provide the basis for an imaging assay involving a cabinet, a camera, and a laptop screen to provide a stimulus for zebrafish larvae swim-

ing in agarose lanes (2013). Upon request, Dr. Creton (Brown University) generously provided the necessary macro and Excel file to simulate the imaging system developed in his laboratory. We were able to build a system modeled after Dr. Creton's system for less than \$1,000, making it feasible for research at the undergraduate level. The imaging system can be used to determine behavioral differences, such as increased anxiety-like behaviors, between chemically-treated and untreated larval zebrafish.

Collection of zebrafish embryos, treatment of embryos, and growth of larvae

Adult wild-type zebrafish were obtained from a local pet store and housed at St. John Fisher College in female/male mixed tanks with a 14:10 hour light:dark cycle. They were fed flake food at least twice per day. IACUC Animal Care and Use Guidelines were followed for embryos. Egg collection procedures follow those described in Richendrfer and Creton's paper (2013). Egg-collection containers consisting of a glass Pyrex dish and cover threaded with fake "grass," made of yarn, were placed into the zebrafish tanks after the final feeding of the day. The lights turning on at 8AM the next day signaled the fish to lay their eggs, and the collection containers were removed between 9:30-10:30AM. To check for embryos, the Pyrex dishes were poured over a handheld strainer and rinsed with system water (salinity = 1400-1600 microsiemens; pH = 6.9-7.4; temperature = 27-29°C). Embryos were grown in a 24-well plate (Corning Incorporated Costar #3527; polystyrene, nonpyrogenic) in an incubator with system water at 28°C on a 14:10 light:dark cycle. At 24 hours post fertilization, embryos were treated with either 0.0068 μ M bisphenol F (Sigma-Aldrich Co.) dissolved in ethanol (treatment group) or only ethanol (control group). At 48 hours post fertilization, embryos were removed from treatment and transferred to a 6-well plate for further development in the absence of BPF. Embryos were then grown until 7 days post

fertilization under the temperature and light conditions already described.

Preparing agarose molds for the behavioral assay

A plastic mold was 3D printed based on specifications provided in Richendrfer and Creton's paper (2013). The mold was used to create the agarose lanes used in the behavioral assay. Agarose (1 g/100 mL system water) was melted in a microwave, allowed to cool for 2-3 min, and then poured into a glass Pyrex dish (17.8 cm x 12.7 cm x 3.8 cm). The mold was inserted slowly on top of the melted agarose before it had polymerized. A single lane of the gel measured 12.4 cm x 8.1 cm x 1.2 cm. The agarose molds were made before each assay was performed and used only once.

Image capture

Twenty-five larvae (five control (ethanol-treated) in lane 1; twenty BPF-treated, five in each of the next four lanes) were placed into the agarose lanes in the gel with enough system water for them to move around freely. A transfer pipette with the tip cut off was used to move the larvae from the 6-well plate to the agarose lanes. The Pyrex dish was placed on top of a tablet screen (SONY Xperia Z) at the bottom of the imaging cabinet. The cabinet measures 206 cm high, 87.6 cm wide, and 31.8 cm deep. The camera was positioned 138.4 cm away from the tablet screen. A plastic diffuser sheet purchased from Staples was placed between the tablet screen and the Pyrex dish to prevent moiré patterns.

A Canon EOS Rebel T5i camera was placed on top of the shelf of the imaging cabinet so that it was facing the gel lanes. The focus was adjusted using the Live Shoot Mode on the Image Capture software which was previously downloaded onto the laptop connected to the camera for image storage. All images were sent directly to the laptop.

A Microsoft PowerPoint presentation was used to display a blank white background on the tablet screen for 15 min. During this time, the

camera took an image every 6 sec. The PowerPoint then displayed a moving red (RGB value = 255, 0, 0) bar stimulus that slowly rose at a rate of 2 cm per sec from the bottom of the lanes to the halfway point of all five lanes simultaneously, before lowering. The purpose of the red bar was to act as a stimulus to test for avoidance, freezing, and thigmotaxis behaviors in the larvae in response to a moving object that loomed overhead, simulating a predator. This red bar stimulus was displayed for another 15 min, during which time an image was taken every 6 sec. A total of 300 images were taken for each experiment.

Image analysis

The images were analyzed through ImageJ (National Institute of Health, 2017) using a macro designed and provided by the Creton lab (Zebrafish_macro25k). The prompts in the macro can be used to set the number of images and subtract the background by setting a threshold. After the images were run through the ImageJ macro, the results file was displayed as an Excel file and contained x,y coordinates of the individual larvae for each image along with the image number and lane number. The results file was sorted based on blank background vs. red bar background and then lane number. A second Excel file, designed by the Creton lab, determined placement of larvae in the wells, distance between larvae, speed of movement, and number of fish at rest. Values from the results file were sorted into the second Excel template based on treatment group and whether or not the red bar stimulus was present. Graphs showing each variable, such as swim speed or percent together, with unpaired t-tests for comparison were built into the Excel sheet.

Results

In the analysis of the data collected from five replicates of the experiment, including a total of 24 control (ethanol-treated) fish and 89 BPF-treated fish, the macro revealed three variables of significance in the comparison of behaviors between the two groups. In the comparison of

Figure 2. Experimental results for the percentage of time the larvae spent at rest. There is a significant increase ($p = 0.002$) in the amount of time spent resting/not moving between the control fish (Control, Red Bar) and the BPF-treated fish with the red bar stimulus applied (BPF, Red Bar), and also between the control larvae (Control, No Stimulus) and the BPF larvae (BPF, No Stimulus) without the stimulus ($p = 0.04$). This analysis was performed on five replicates (24 control fish; 89 experimental fish). Error bars show SEM.

the percentage of time the larvae spent at rest (i.e. not moving), significance was found when comparing the control and BPF groups without the stimulus, and again when comparing the two groups with the red bar stimulus (Fig. 2). Within each group (control vs. BPF), the larvae spent less time at rest when the stimulus was added; however, the BPF-treatment group had very similar values both with and without the stimulus (59% and 62%, respectively), whereas the control fish showed more movement with the red bar (35% rest with red bar, 45% rest without). The overall trend of the data is that the BPF-treated group spends more time at rest, regardless of whether the stimulus is present or not. However, the control fish spend a lower percentage of time at rest when the stimulus is present, while the BPF fish show a similar percentage even when the stimulus is present.

The overall swim speed, in mm/min, of the

Figure 3. Experimental results for swim speed (mm/min). There was a significant decrease ($p = 0.018$) in swim speed between the control group (Control, Red Bar) and the BPF-treated group (BPF, Red Bar) when the aversion stimulus was applied. There was also a decrease ($p = 0.006$) between the control (Control, No Stimulus) and BPF-treated (BPF, No Stimulus) groups when no stimulus was applied. This analysis was performed on five replicates containing a total of 24 control fish and 89 experimental fish. Error bars show SEM.

zebrafish was found to be significant between the control fish without a stimulus and the BPF-treated fish without a stimulus, and again between the control stimulus group and the BPF stimulus group (Fig. 3). In the presence of no stimulus, the control fish swim at an average speed of 87 mm/min, while the BPF-treated fish swim at an average 40 mm/min. When the red bar stimulus is added, the average swim speed increases to 102 mm/min for the control fish and 55 mm/min for the BPF-treated fish. The trend of the data shows two things: first, the addition of the red bar causes both groups to move at a faster speed, and second, the BPF-treated fish move at an overall slower speed compared to the control group whether the stimulus is applied or not. In contrast with the data in Figure 2, these data seem to indicate that the treated fish move faster in response to the

Figure 4. Experimental results for the distance between two fish in a single lane, or fish-fish distance (in mm). There was a significant increase ($p = 0.016$) in the distance between the BPF-treated fish (BPF, Red Bar) when compared to the control fish (Control, Red Bar) in a single lane when the red bar stimulus is applied. The Control Red Bar group, Control No Stimulus group, and BPF No Stimulus group showed no significant differences. This analysis was performed on five replicates (24 control fish; 89 experimental fish). Error bars show SEM.

stimulus, whereas their greater time spent at rest indicates they do not react to the stimulus. In both cases (Control No Stimulus compared with BPF No Stimulus and Control Red Bar compared with BPF Red Bar), the BPF larvae move about half as fast as their untreated cohort. Not only do these fish exhibit a larger percentage of time spent at rest, but when they move, it is at a much slower speed.

When observing the differences in the distance between fish in each lane, also called fish-fish distance, there is no significant difference between the Control No Stimulus group (34 mm), the Control Red Bar group (32 mm), and the BPF No Stimulus group (36 mm; Fig. 4). However, there is a difference when the BPF-treated group was exposed to the red bar stimulus. These fish had a significantly larger distance between one another within each lane, an average of 42 mm, indicating that the fish

may be actively moving away from one another when the stimulus is applied. This behavior was only revealed in the presence of the red bar stimulus.

Discussion

The findings of this study indicate that embryonic zebrafish treatment, with the EDC bisphenol F, leads to significantly more time spent at rest, increased distance between fish in a single well in response to a stimulus, and overall decreased swim speed. Measures of anxiety in zebrafish have been well characterized, including freezing, thigmotaxis, and phototaxis (Maximino et al., 2010). It was hypothesized that the BPF-treated zebrafish would exhibit increased thigmotaxis and freezing behaviors in response to a moving stimulus. However, thigmotaxis was not a significant variable in this experiment. Freezing behavior increased significantly in both the presence and absence of the stimulus, as well as distance between fish when the red bar stimulus was applied.

The very similar percentages of time spent at rest between the BPF, No Stimulus group, and the BPF, Red Bar group, could be indicative of freezing behaviors in the fish (Fig. 2). The control fish, overall, spent less time at rest, but when the stimulus was applied, they appeared to spend more time moving. On the other hand, the BPF fish spent almost the same amount of time not moving whether the stimulus was applied or not. This could be for a variety of reasons, including that the fish may not recognize the stimulus, or may not be able to move in response to it. This is contradictory to the findings summarized in Figure 3, in which the treated fish do move faster in response to the stimulus. A possible explanation for this is that the ability of the fish to move is altered, causing them to spend more time at rest, but when they are able to swim, they move faster in response to the stimulus. This indicates that the larvae are aware of the stimulus and may perceive it as a threat. The inclusion of the red bar in these experiments is necessary to observe if a predatory stimulus has the ability to return the

treated zebrafish back to a normal level of movement. In Figure 3, the zebrafish move faster in response to the stimulus, but they do not recover movement back to control levels. The same is true for Figure 2, in which even a predatory stimulus is not enough to restore the BPF-treated zebrafish to untreated levels of movement. Further investigation would be necessary to determine why these zebrafish are unable, or unwilling, to move as quickly as non-BPF-treated zebrafish.

In Figure 4, the similarity between the control fish with and without the stimulus indicates that there is no grouping behavior seen; the fish do not cluster closer together in response to a predatory stimulus. The BPF group, however, shows increased distance between the fish when the stimulus is applied. Not only are these fish not clustering together, they move away from other fish. This behavior is only seen when the stimulus is applied, highlighting the importance of using a predatory stimulus in these experiments. The addition of the stimulus can reveal behaviors that are not present, or are not as extreme, when the treated larvae are merely swimming around on the white background. The implications of this study are that BPF, at a very low dose, induces a behavioral change similar to what has been reported for other bisphenols, through an unidentified mechanism.

The mechanism proposed by the Kinch lab for bisphenol A involving precocious hypothalamic neurogenesis is a logical mechanism for bisphenol F's action as well (Kinch et al., 2015). The hypothalamus is a highly conserved region of the brain, and is involved in hyperactivity. A population of dopaminergic neurons in the zebrafish hypothalamus has been implicated in the regulation of swimming behaviors (McPherson et al., 2016). It is therefore possible that the disruption of these neurons during development, due to an EDC, could cause alterations in swimming behaviors, such as those observed in our experiment. The Kinch group found a dramatic increase in locomotor activity for both BPA and BPS (Kinch et al., 2015); however, we observed a decrease in activity. Further studies would need to be performed to determine the

exact mechanism of action for BPF, as compared to other bisphenols.

The flexibility of this imaging system allows for many different variables to be explored, including different compounds, doses, or exposure windows. At the undergraduate level, we were able to build the imaging cabinet and produce data similar to that shown in other published studies. The future directions of this system involve using different BPA analogues, such as bisphenol AF (BPAF), as well as other EDCs, to treat larval zebrafish. Due to the ubiquitous nature of both BPA, and more recently BPF, in nature, it would also be relevant to see the effects of both BPA and BPF in conjunction on larval zebrafish behavior. Because each assay takes a short time to set up and collect data, many different assays could be performed in a given time period, allowing for rapid collection of data. Additionally, different doses could be utilized. Bisphenols, like other EDCs, follow a nonmonotonic dose curve, making it difficult to predict the response at any given dose (Vandenberg et al., 2012). The dose selected in this experiment ($0.0068 \mu\text{M}$) was chosen because of an environmentally relevant dose based on the level of BPA found in a local water supply of another laboratory (Kinch et al., 2015). The exposure window of 24-48 hours was also selected because peak neurogenesis occurs between 24-36 hours, with possible neurogenesis occurring beyond that time point (Kinch et al., 2015). Future studies could examine if BPF's effect is time-specific in the same way as the effect of BPA.

One limitation of the study must be addressed, and that lies in the nature of the imaging assay: there is no way to account for the mechanism of action of BPF, only the behavioral impact. However, the results of the high-throughput imaging assay suggest a link between BPF and anxiety-like behaviors, and therefore merit further study into the effect of this bisphenol, as well as other BPA analogues.

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ImageJ macro and Excel file used to perform and analyze the imaging assay. The imaging system was initially set up and utilized by Amanda Wahl and Nathaniel Hinman, former undergraduate students in E.F.'s laboratory.

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