GENE EXPRESSION EFFECT OF EARLY DEVELOPMENTAL EXPOSURES TO BISPHENOL A AND ITS ALTERNATIVES ON THE REPRODUCTIVE REGULATORY AXIS

A Thesis

Presented to the Faculty of the Graduate School of Cornell University

In Fulfillment of the Requirements for the Degree of Master of Food Sciences

By

Jennifer Kusumah

2021

ABSTRACT

Bisphenol A (BPA) is a known endocrine-disrupting chemical (EDC) and also a food safety concern due to leaching of BPA from food and beverage containers into the foodstuff. Substitutes such as BPB, BPAF, BPAP, BPS and BPZ were developed but there is still concern over their toxicity due to their structural similarity to BPA. The objective of this study is to perform direct comparison of six bisphenol analogues to BPA and control on their effects on the gene expression of the hypothalamic-pituitary-gonadal (HPG) axis in the early developmental phase. Acute toxicity and gene expression studies were performed using early developmental-stage zebrafish larvae. Additionally, literature review was performed to further understand the result of our toxicity exposure study. Our study found BPAF to be the most and BPF and BPS as the least toxic in terms of lethality. However, only one gene (*lhb)* was significantly induced by BPAF at below sublethal doses, while three genes (*vtg1, lhb and gnrh3*) were induced by both BPF and BPS. The extent of induction of *arom-b* and *vtg1* in the complex mixture group cannot simply be explained by an additive effect of 7 bisphenols and may indicate a significant synergistic effect occur with combined exposures. The result for BPS and BPF was consistent with data from literature, although there were some inconsistencies with BPAF effects. In conclusion, our study indicated that while BPS were found to be less toxic compared to BPA in terms of lethality, they may still trigger endocrine-disrupting activity, especially when it exists in complex mixtures, which is demonstrated by their effect on the gene expression of reproductively relevant genes on the HPG axis.

BIOGRAPHICAL SKETCH

Jennifer Kusumah was born and raised in Jakarta, Indonesia. She came to the United States in 2016 to attend the University of Illinois at Urbana-Champaign (UIUC) where she double-majored in Food Science and Linguistics. In UIUC, she had the opportunity to work on an independent research project on mung bean protein, which was then presented virtually at both the Undergraduate Research Symposium held in UIUC and the American Chemical Society national conference. She graduated in 2020 and continued her education at Cornell University where she joined Dr. Motoko Mukai's lab as a Master's student.

To my parents and my sister, for all your support and unwavering belief in me

ACKNOWLEDGEMENTS

I would like to extend many thanks to Cedric Clark who has taken the time to guide me around the lab. Thank you for showing me the ropes and helping me with technical support during the experiment part. Although I did not end up going forward with the benchtop experiment as previously planned, but your guidance helped me in learning new lab skills that will surely be valuable in the future.

The bisphenol exposure experiment and gene expression study reported in this thesis was performed and figures 4 and 5 as well as table 1 were generated by a former PhD student in the Mukai Lab, Austin Martini, as part of his thesis project. However, I wrote all parts of this thesis (including materials/methods, results and discussion section summarizing data generated by a former student) with the help of Dr. Mukai.

Lastly, I would like to extend my thanks to Dr. Motoko Mukai for her guidance and constant support during the writing of this thesis. Without your insight and mentoring, this thesis would not be possible. Thank you for giving me the opportunity to join your lab and learn under your tutelage. Thank you for your constant support, encouragement and understanding throughout this challenging academic year.

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LIST OF ABREVIATIONS

EDC	Endocrine-disrupting compounds
BPA	Bisphenol A
PCB	Polychlorinated biphenyl
DDT	Di-chloro di-phenyl tri-chloroethane
DES	Diethylstilbestrol
FDA	Food and Drug Administration
BPAF	Bisphenol AF
BPAP	Bisphenol AP
BPB	Bisphenol B
BPS	Bisphenol S
BPZ	Bisphenol Z
BPF	Bisphenol F
HPG	Hypothalamic-pituitary-gonadal
GnRH	Gonadotropin releasing hormone
FSH	Follicle stimulating hormone
LH	Luteinizing hormone
ER	Endocrine receptor
AR	Androgen receptor
hpf	hour post fertilization
dpf	day post fertilization
PCR	Polymerase chain reaction
NOAEL	No observed adverse effect level

INTRODUCTION

1.1. Endocrine Disrupting Compounds (EDCs)

Endocrine-disrupting chemicals (EDCs) are a collection of exogenous substances that disrupt the normal function of the endocrine system. Some of these substances occur naturally, such as phytoestrogens, mycoestrogens and heavy metals, but most are man-made, created and used during the time when there was little knowledge of their harmful effects (Darbre et al. 2019). Many of the well-known EDCs are ubiquitous in our environment and have been found in various consumer goods. Polychlorinated biphenyls (PCBs), Bisphenol A (BPA), phthalates, dioxins (TCDD) and dichloro-diphenyl-trichloroethane (DDT) are some of more well-known examples.

The concern over the negative effects of these compounds started in the 1990's, although the negative effect of EDCs have been documented long before that. The earlier signs of detrimental effects of EDCs were observed in wildlife, before it was documented in human beings. Ecologists first noticed that in the Great Lakes, domesticated minks stopped producing pups, while in Lake Apopka in Florida, many male alligators were observed with physically disabled genitalia, and in England, fish with severe reproductive abnormalities were found. All of these point to a larger pattern of chemicals that specifically disrupt the reproductive functions and raises a question of whether similar effects would be observed in humans (Reviewd by (Schug et al. 2016). Diethylstilbestrol (DES)—molecular structure illustrated in **Fig 1**—is a synthetic estrogen that was prescribed as a drug to prevent miscarriages and premature births before it was found out to increase the risk of clear-cell carcinoma in daughters of the pregnant mothers who were prescribed DES, and also increased the risk of breast

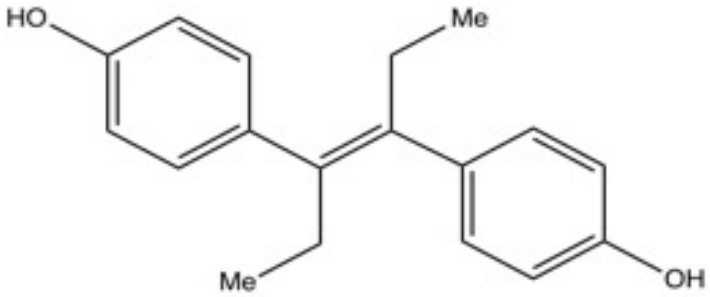

Fig 1. Molecular structure of diethylstilbestrol (DES)

cancer in those pregnant mothers. This prompted the Food and Drug Administration (FDA) to advise physicians to stop the prescription of DES (Marty et al. 2011). Rachel Carson's *Silent Spring,* published in 1962, was a major landmark that brought the harmful effects of the chemical dichlorophenyltrichloroethane (DDT), a widely used pesticide, toward the environment, wildlife and humans to the mainstream public attention (NDRC. 2018). DDT was used by the allied forces during the World War II as insecticides to combat malaria, and later, it was made available for civilian commercial use. The effectiveness of DDT was mostly due to its high chemical stability that allows it to persist in the environment, long after its initial application, so that frequent use of it was not needed. However, this led to DDT being accumulated in the food chain and eventually ending up exposed to human through its residues in the food chain. Animal studies in the mid-1950s demonstrated its adverse effects, particularly for the reproductive functions, and these results led to the increasingly restricted use, and later banning, of DDT in the 1970s (Beard. 2006).

The effects and mechanism of EDCs continue being studied today due to these compounds, not limited only to DES and DDT, still persisting in the environment and affecting both wildlife and human beings. Human exposure to these compounds occur mostly through ingestion, inhalation and dermal contact including through food and beverages, cosmetics use, pesticides, and everyday items such as toys, detergents,

plastic containers and bottles (NIEHS. 2021). Heavy metals, such as lead, also act as an EDC and its mode of exposure to human beings are mainly through lead contamination in drinking water and lead content in paint commonly used in houses built before 1980 (Brown & Margolis. 2012). The possible exposure of human to EDCs through ingestion make EDCs a food safety concern, particularly due to some EDCs being used as components to make food contact materials. The main concern is due to the possible migration of these EDCs from food containers and packaging into the food itself that are consumed by humans and also through bioaccumulation of the compounds in the food chain.

Phthalates, polychlorinated biphenyls (PCBs) and Bisphenol A (BPA) are just few examples of the well-known EDCs that are found in foodstuff. Phthalates are esters of phthalic acid, and due to its low melting point and relatively high boiling point are used widely as plasticizers and thus see diverse applications in plastic-based customer goods. The major source of exposure of phthalates to human is food, and this is due to the migration of the compounds from the packaging into the food over time due to temperature or mechanical stress (Fasano et al. 2012). A study had shown that usage of plastic food containers contributed to phthalate exposure as evidenced by the urinary phthalate level (Dong et al. 2017). Exposure to phthalates have been linked to endocrine and reproductive dysregulation such as early puberty, endometriosis, infertility and altered fetal development. It has also been linked to testicular dysgenesis syndrome in males due to its ability to interact with the hypothalamic-pituitary-gonadal (HPG) axis (Giulani et al. 2020). Like phthalates, food is also a major source for human exposure to PCBs, but unlike phthalates, PCB contamination in food are largely due to the bioaccumulation of PCB in the food chain. Fish in particular contributes to exposure of PCBs to human through food (Fromberg et al. 2011). Food from farm animals such as

meat, poultry, eggs, and dairy products are also found to be contributors of PCB exposure to human due to the PCB contamination in soil (Weber et al. 2018). Exposure of PCB to human has been linked to reproductive dysfunctions such as decreased sperm motility, decreased fecundity, earlier menarche and altered gonadal hormones in newborns (Ross. 2014).

Finally, BPA is another EDC that is also a major food safety concern due to ingestion and contamination in food being some of the major sources of human exposure (Kang et al. 2006). Growing concern over the toxicity of BPA and its detrimental health effects in human exposed to it through food caused many food packaging manufacturers to create products that are claimed to be "BPA-free". However, the label "BPA-free" does not immediately guarantee that the product does not release with their own estrogenic activity (Bittner et al. 2014). Thus, there is a growing research interest in examining alternatives used for BPA substitute for their potential toxicity, especially compared to BPA, to determine if they are safer alternatives. Therefore, this group of compounds were selected for this review and previous data obtained in the Mukai Lab (unpublished) will also be summarized.

1.2. Bisphenol A

Bisphenol A (BPA) is a synthetic xenoestrogen compound that contains two aromatic benzene rings, two hydroxyl groups and two methyl side-chains. The molecular structure of BPA is illustrated in **Fig 2** along with several of its analogues. BPA has some structural similarities with DES, another synthetic xenoestrogen that was prescribed to pregnant women before it was banned after its endocrine-disrupting effects was found out (Vogel. 2009), as elaborated in the previous section above. DES

was found to have stronger estrogenic activity, hence it was favored over BPA as drugs with estrogenic properties (Dodds et al. 1936), but BPA was discovered by the plastic industry beginning in the 1940's where it is used in the manufacture of many consumer goods such as food and beverage containers, coating of DVDs, CDs and electronic and electrical equipments, and also as components in dental sealants (Welshons et al. 2006). It has the advantages of being lightweight, easy to mold, colourable and resistant to heat and light, all of which are desirable quality in the making of many commercial goods, hence its widespread use (Dodds et al. 1936).

BPA has been found to be able to leach from food and beverage containers into the food itself, which makes it a food safety concern (Vandenberg et al. 2007). The leaching of BPA from baby bottles in particular had gained much attention. A study found that used baby bottles underwent higher BPA leaching compared to new baby bottles and that higher temperature caused more BPA leaching (Tan & Mustafa. 2003). There is also evidence that repeated use of baby bottles increase BPA leaching (Nam et al. 2010). In another study, BPA was found to leach from the linings of food can, especially upon heating (Takao et al. 2002). A population study conducted among college students also found that the amount of BPA leaching from polycarbonate drinking bottles is enough to significantly raise BPA level in urine (Carwile et al. 2009). BPA has also been shown to be released from reusable aluminum drinking bottles lined with epoxy resins, especially when heated to high temperature (Cooper et al. 2011).

As reviewed by Murata and Kang (2018), BPA has been linked to numerous health defects including carcinogenesis, reproductive toxicity, abnormal inflammatory or immune response, and developmental disorders of brain and nervous system. Reproductive function is a central theme within the field of endocrinology and even more so when it comes to the study of EDC, as the earliest and most commonly

observed effects of EDC manifest as reproductive function disorder. Thus, it is not surprising that the reproductive toxicity effects of BPA have gained much interest.

In rats, exposure to BPA has been shown to impair male fertility for multiple generations (Salian et al. 2009). It has also been shown to reduce sperm count and quality (Dobrynzska and Radzikowska. 2012). Male zebrafish exposed to BPA also showed reduction in sperm density and quality, and the effect carried over to the offsprings, resulting in delayed hatching, increase in malformation and mortality (Chen et al. 2015). In female zebrafish exposed to BPA, reduction of expression of *cyp19b* as well as hypertrophy and hyperplasia of gonadotroph cells are observed (Molina et al. 2018). In medaka, BPA exposure is associated with decreased ratio of estrogen to testosterone, slower ovary development and decreased egg production (Huang et al. 2018).

A study conducted in China showed that in utero parental exposure of BPA resulted in shortened anogenital distance in male offsprings (Miao et al. 2011). Another study conducted in Korea showed that higher plasma BPA level has significant association with occurrence of hypospasdias in newborn males (Choi et al. 2012). Higher BPA level was also observed in women with polycystic ovary-like syndrome (PCOS) and is associated with higher level of testosterone and androstenesdione, which pointed to the possibility of the role of BPA in PCOS pathophysiology (Kandaraki et al. 2011). Higher BPA level in human blood serum has also been suggested to potentially play a role in the development of endometriosis (Cobellis et al. 2009).

Although BPA is valuable in the plastic industy, its adverse health effects cause growing concern among consumers, prompting for the search for compounds that could potential act as BPA subtitute. Bisphenol analogues are such compounds, and some of

them, such as BPF and BPS, are seeing increasing usage within the industry as BPA replacement.

1.3. Bisphenol Analogues

Bisphenol analogues are a group of compounds which have structural similarity to BPA. These compounds were synthesized in attempt to create substitutes for BPA that are less toxic but with all the advantages. The analogues that we will be focusing in this research are BPAF, BPAP, BPB, BPF, BPS, and BPZ. These compounds were chosen due to their presence in human urine samples and have been studied before by a previous member of our lab (Chen et al. 2016).

BPS and BPAF are two substitutes that are seeing increasing use—BPS in the production of thermal paper, epoxy glue, and plastic goods, and BPAF in the production of synthetic rubbers for uses such as O-rings, seals and gaskets in processing plants, as well as food contact polymers and electronic materials (NIEHS. 2008; Liao et al. 2012; OEHHA. 2012; Li et al. 2020a). BPS residues have been found in surface water, indoor household dust, sewage sludge and marine sediments, which suggest its ubiquitous presence in the environment. It has also been found in food products, especially in canned goods, and neurobehavioural study on the effect of exposure of BPS in the early life stages of zebrafish suggested that it could decrease locomotor behaviour, increase oxidative stress, promote apoptosis and altered retinal structure, as well as downregulating the expression of neurodevelopment genes (Gu et al. 2019). Similarly, BPAF residues have also been found in rivers, sediments, soil, indoor household dust and groundwater (Song et al. 2012). BPAF exposure to zebrafish has also been found to

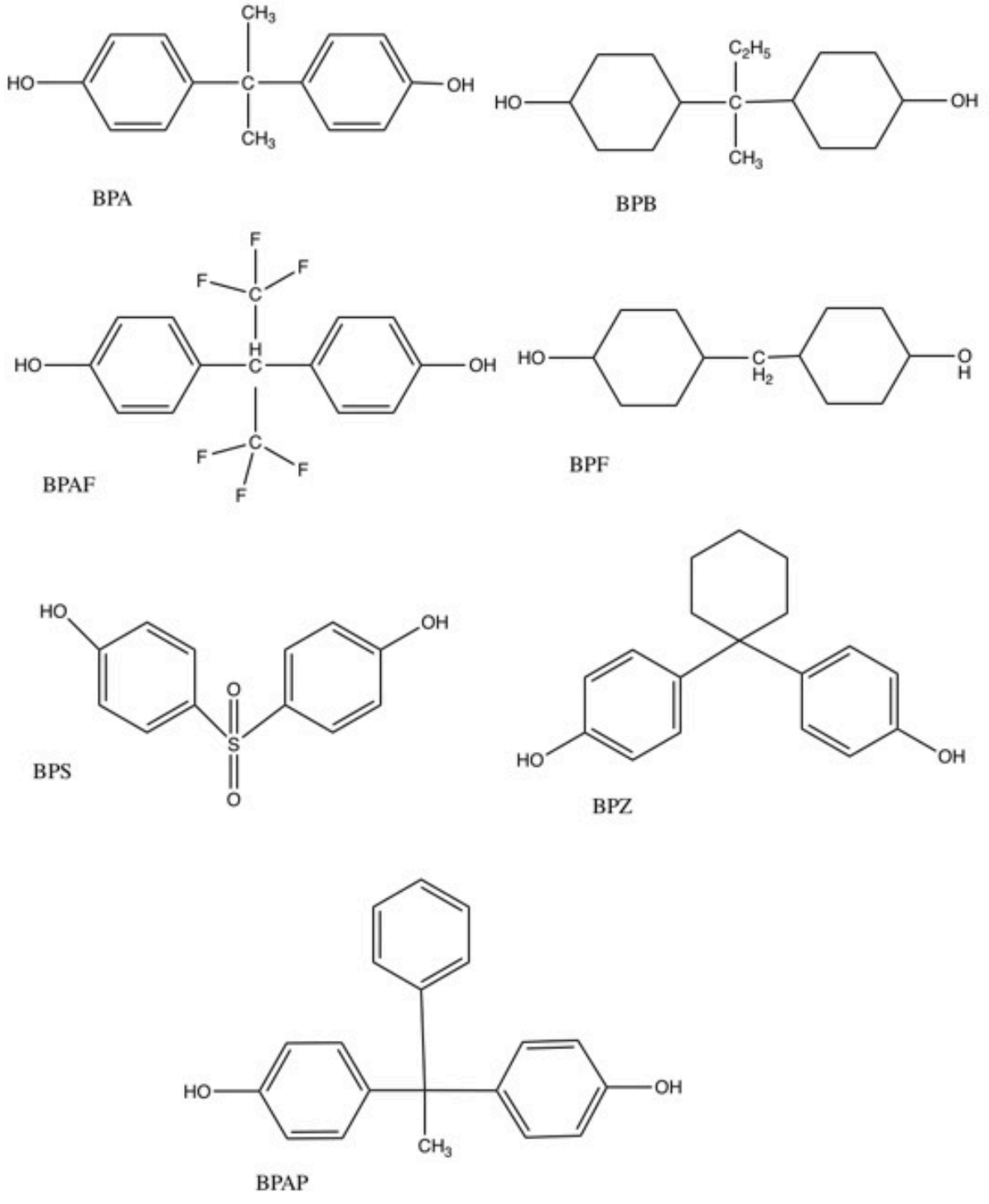

Fig 2. Molecular structures of bisphenol A and bisphenol analogues BPAP, BPAF, BPB, BPZ, BPS and BPF.

induce cardiotoxicity, increase oxidative stress, as well as downregulating expression of genes important for cardiac development (Gu et al. 2020).

BPB and BPF are another analogs commonly used as BPA substitutes. BPB is used in the production of phenolic resins, while BPF are used in the manufacture of lining and flooring materials, as well as pharmaceuticals (Cunha and Fernandes, 2011; Qiu et al, 2018). Residues of BPB has also been found as contaminant in canned foods such as canned tomatoes, which make it a food safety concern (Grumetto et al. 2008). Similarly, BPF residues has also been found in food items such as canned foods, candies, fruits, vegetables, meat and beverages (Gallart-Ayala et al. 2011). Study has found that exposure of BPB and BPF caused alteration in reproductive functions, impaired follicles, imbalance in steroid hormones and increasing oxidative stress (Ijaz et al. 2020).

Both BPAP and BPZ have been detected in the urine samples of Chinese university students as well as detected in the indoor household dust from the same environment along with other bisphenol analogues (Zhang et al. 2020). BPZ in particular was also commonly detected in wastewater collected from wastewater treatment plans (Cesen et al. 2018). A study have shown that estrogenic effect of BPAP could delay uterine development in female mice at very low doses and at doses relevant to human exposure, could potentially disrupt blood glucose homeostasis (Xiao et al. 2018). In contrast to other bisphenol analogues that have been much more studied, there is a knowledge gap for the toxicity of BPZ, but at least one study have shown that like other bisphenol analogues investigated, BPZ showed agonistic and antagonistic activity against estrogen, androgen and glucorticoid receptors. The study also showed that BPZ was an especially potent pregnane X receptor agonist (Kojima et al. 2019).

Looking at all of these data, we could see that these substitutes are not necessarily less toxic compared to BPA as some of these research have suggested that some

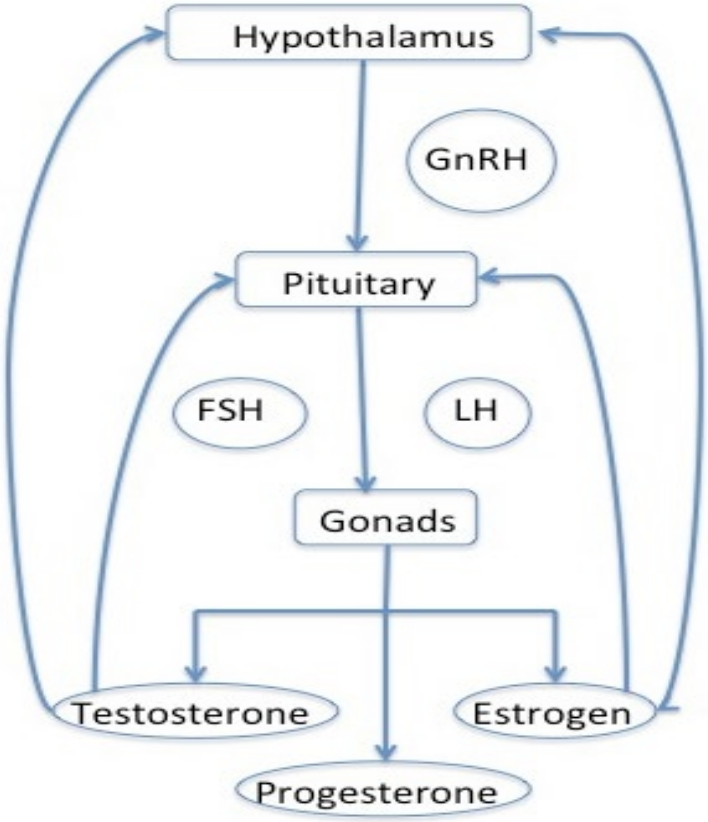

Fig 3. Diagram of the human hypothalamic-pituitary-gonadal (HPG) axis. It consists of the organs hypothalamus, pituitary and the gonads (testis in males, ovary in females). Hypothalamus releases the gonadotropin hormone (GnRH), which stimulates the pituitary to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn stimulate the gonads to secrete testosterone, estrogen and progesterone. The HPG-axis is regulated by negative feedback mechanism, where secretion of testosterone, progesterone and estrogen regulates the hypothamalus from further release of GnRH.

substitutes are just as toxic or more toxic when compared to BPA. However, direct comparisons of all of these substitutes to each other and BPA, particularly in terms of their effects on reproductive gene expressions, have yet to be done.

1.4. Effects of EDCs on the Hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis describes the interaction between the hypothalamus, the pituitary and the gonads as well as the hormones produced by

these organs in their function to regulate the reproductive system. The hypothalamus produces the gonadotropin releasing hormone (GnRH) which stimulates the pituitary gland to release the follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Plant. 2015). LH acts on ovaries in females to stimulate ovulation and development of corpus luteum, and stimulates testosterone synthesis and secretion in males. FSH promotes follicular maturation and estrogen synthesis in females, and promote spermatogenesis in males (Wang et al. 2015; Jin et al. 2014). The gonads—testes in males, and ovaries in females—produces estradiol, progesterone and testosterone that regulates the feedback loop that enables the HPG axis to control reproductive functions (Meethal and Atwood. 2005).

As reviewed by Wirbisky and Freeman (2015), research in the mechanism of the action of EDCs on the reproductive system has focused on how EDCs interact with the hormone receptors of the nuclear receptor family such as estrogen receptors (ER) and androgen receptors (AR). Xenoestrogens such as BPA and DDT are able to bind to the hormone-binding sites of ER and their interactions are depending on their size and chemical structure with smaller size compounds having lower binding affinity and larger sized EDCs having affinity on par with the natural endogenous estrogens (Delfosse et al. 2014). The action of EDCs on AR has also been studied due to the importance of AR in development and differential of male features, and studies, as reviewed by Toporova and Balaguer (2020), have identified anti-androgenic activity of EDCs such as BPA, BPC and α-zearalanol.

Many EDCs have been shown to display estrogenic activity and interfere with normal estrogen signaling that are mediated by the ERα and ERβ (Shanle and Xu. 2011). They were thus able to act as agonist or antagonist for steroid hormone receptors. As reviewed by Lee et al. (2013), there are several ways that EDCs can exert their

estrogenic actions through ER-mediated signaling including disrupting ERα-mediated transcriptional activity and stimulation of ERα-dependent kinase pathways through membrane Erα or G protein coupled receptor. The ability of EDCs to bind to ER is crucial in its ability to affect cells in dose-dependent manner.

1.5. Zebrafish (*Danio rerio*) as model organism

In this project, zebrafish (*Danio rerio*) was selected as model organism to study the effect of bisphenols on gene expression on the HPG axis. Zebrafish is a freshwater tropical fish that is native to the northern Indian subcontinent, originating in the Ganges region in the eastern India (Tavares & Santos Lopes. 2013). It has been used as an animal model in biomedical research such as genetic studies, vertebrate embryonic development studies and adult stem cell as well as regenerative studies due to its high fecundity, rapid embryonic development and transparency (Major & Poss. 2007). Another advantage that made zebrafish a valuable model in research is its ability to allow for excellent *in vivo* and *ex vivo* imaging (Jaffe et al. 2010) and its fully sequenced genome, well-characterized behavior and availability of several mutants and transgenic strains such as the *casper* mutant (White et al. 2008). In addition, the shorter breeding time and its relatively small size makes the studies more cost- and time-effective, and zebrafish also has most of the endocrine organs found in humans, including the HPG-axis (Löhr and Hammerschmidt. 2011), which is the focus of this research.

1.6. Objective

The objective of this study is to perform a systemic literature review in order

examine and compare the effects of six different bisphenol analogues to BPA on the gene expression of the HPG axis during developmental period using zebrafish as the model organism.Previous research on the effect of BPA and bisphenol analogues on the HPG-axis have mostly focused on adult fish (>45-dpf), but it is also critical to evaluate the effect during developmental phase in order to fill in the knowledge gap. Therefore, in addition, the yet unpublished results from a exposure study and gene analysis study done in our lab by a former student will be summarized as well to supplement the review.

MATERIALS AND METHODS

2.1. Materials

Chemicals

Bisphenol A (BPA)(Sigma-Aldrich, Saint Louis, MO, 99% purity) Bisphenol F (BPF), Bisphenol S (BPS), Bisphenol Z (BPZ) (Sigma-Aldrich, Saint Louis, MO, 98%), Bisphenol AF (BPAF), Bisphenol AP (BPAP) and BPB (Sigma-Aldrich, Saint Louis, MO, analytical standard) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Saint Louis, MO). Exposure solutions were prepared by serially diluting stock concentrations into buffered 1x E3 embryo medium (pH 7.2) at a final vehicle concentration of 0.1% DMSO.

Fish

All wild-type zebrafish used in this study were bred and raised in the fish facility at Cornell University under a 14h:10h light:dark photoperiod, with pH maintained in the range of 7.2 - 7.4, temperature in the range of 26 – 30 C, and water conductivity in the range of 400 – 700. Broodstock of about 250 fish were maintained in a mini mass embryo production system (mini MEPS, Aquatic Habitats, Apopka, FL) with at a 1:2 male:female ratio and fed twice daily with a combination of hatched *Artemia nauplii* and ground fish flakes (TetraMin, Tetra USA, Blacksburg, VA). In the evening before the embryo collection, a spawning insert was placed allowing the fish to breed the following morning soon after the light onset. During the 6-dpf lethality study, larvae were not

fed. For the gene expression study, the larvae were fed with AP100 (Zeigler Gardens, PA) and Golden Pearl Active Spheres (Brine Shrimp Direct, Ogden, UT) three times a day and with *Artemia nauplii* starting at 6-dpf. All maintenance and experimental protocols used have received approval from the Institutional Animal Care and Use Committee at Cornell University.

2.2. Methods

Literature Review

Bibliographical search of available literature was conducted from January 2021 to July 2021 using Web of Sciences, Scopus, and Google Scholar. Keywords used are "bisphenol analogues", "BPA", "BPZ", "BPF", "BPS", "BPAF", "BPAP", "BPB", "gene expression effect", "HPG axis", "endocrine disrupting", "early developmental', "zebrafish", "reproductive toxicity" and combinations of all of those. No limit was placed on the time range for the publications, but most recent studies published after 2010 were prioritized. Articles found using those keywords were first screened based on their abstract for their relevance to the topic.

Exposure

Adult zebrafish were allowed to spawn in the MEP for 30 min after the light onset and ~10,000 embryos were collected. At 3-hpf, embryos were chemically dechorionated by exposing the embryos to 0.1 mg/mL pronase for 10 min under gentle agitation. Viable healthy embryos were then selected under a dissection scope (Zeiss,

Carl Zeiss Microimaging, Thornwood, NY). Subsequently, at 4-hpf, 25 healthy dechorionated zebrafish embryos were randomly assigned, 5 at a time, into 60-mL glass petri dishes containing 30mL of treatment media (n=6 replicate dishes/treatment, 7 concentrations per bisphenol). The 7 concentrations used for each bisphenol were selected based on the pilot study and to capture the complete lethality curve. Final concentration ranges used for the acute lethality study were 0.02-22 mg/L for BPA, 0.005-7 mg/L for BPAF, 0.015-7 mg/L for BPAP, 0.099-20 mg/L for BPB, 0.098-60 mg/L for BPF, 4.95 -500 mg/L for BPS, and 0.1 – 5 mg/L for BPZ.

The number of surviving larvae for each treatment was counted each day up to 5-dpf to generate lethality curve. Mortality was confirmed by a lack of visible heartbeat under a dissecting scope (Zeiss, Carl Zeiss Microimaging, Thornwood, NY). Malformations (tail curve, pericardial edema, yolk sac edema, brain bleed, pericardial bleed) were also recorded.

LC10 was calculated from each lethality curve and was used for further chronic exposure experiment to assess the effect of sublethal exposure to bisphenols. The same exposure procedure was followed with a few modifications. Larvae were transferred from petri-dish to 1-L glass beakers at 4-dpf (n=25/beaker) and feeding was initiated (see previous section for the feeding protocol) to allow survival beyond 6-dpf. At 8-dpf, subset of larvae were collected for gene expression analysis to assess the effects of BPA and bisphenol analogues on gene expression during early-developmental phase. Exposure continued until 120-pdf to assess effects of a chronic exposure in the FO generation as well as in the subsequent generations (not included in this thesis).

Gene Expression Analysis

A pooled larvae samples (10 larvae/replicate, n=6/group) were collected in 2-mL RNase-free microtubes (Ambion, Carlsbad, CA) containing chilled 500 μL Trizol (Qiagen, Hilden, Germany) and zirconia beads (0.7 mm diameter, Biospec Products, Bartlesville, OK) They were then immediately homogenized using a TissueLyser II (Qiagen, Hilden, Germany) and stored at –80°C. Total RNA was then extracted using Trizol then the concentrations were quantified using Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). The 260/280 and 260/230 ratios of the extracted RNA samples were >1.8, indicating purity of the RNA. The total RNA was then reverse transcribed using high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) and oligo(dt) primers (Thermo Scientific), according to the manufacturer's protocol. Real-time polymerase chain reaction (PCR) was performed using a 96-well plates with 20 µL total reaction volume per well and QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The 20 µL reaction mixture was comprised of 5.4 µl of Millipore water, 0.6 µl of primer mix, 10 µl of SYBR Green Supermix (Biorad), and 4 µl of the cDNA sample. All primers used (GnRH3, GnRH2, lhb, fshb, VTG1, and AROM-b) as well as housekeeping gene (rpl13a) were intron spanning - and the housekeeping gene were confirmed to be not significantly different between the treatment groups.

Statistical Analysis

One-way ANOVA test was run for the results to determine their statistically significant differences. P value <0.05 was considered statistically significant with Dunnet's post-hoc test.

With the result generated previously in our laboratory, it was found that BPAF, BPZ, BPAP and BPB were more toxic in terms of lethality compared to BPA, with BPAF being the most toxic. BPF and BPS were found to be less toxic compared to BPA, and out of all the seven bisphenol analogues tested, BPS was found to be the least toxic in terms of lethality.

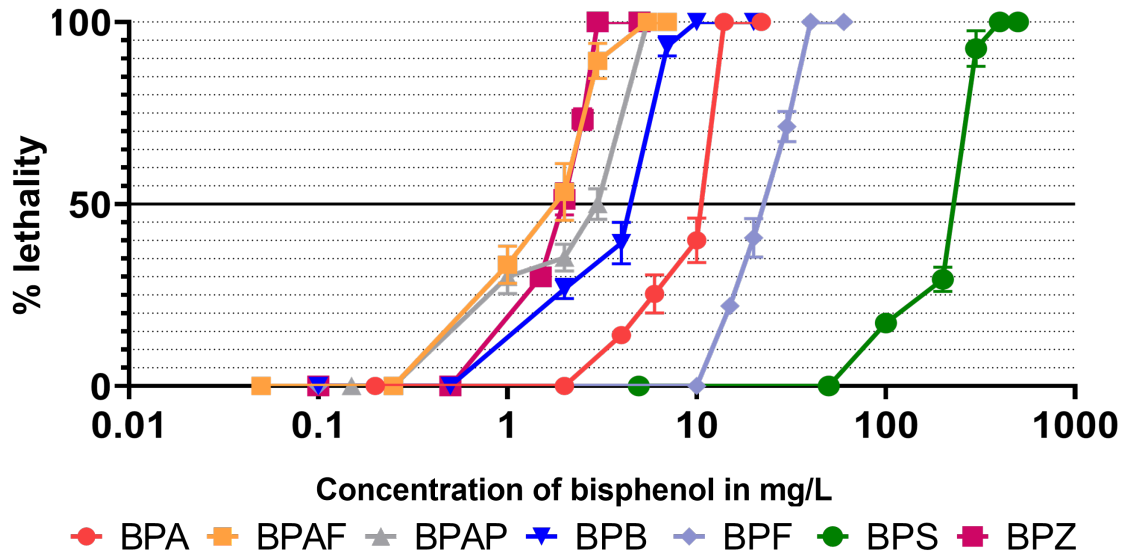

Fig 4. Lethality in percentage of zebrafish at 5-dpf for seven different bisphenol analogues at six different concentrations (Martini, et al., unpublished)

Most toxic

Least toxic

Bisphenol	LC_{50}	LC_{10}	NOAEL
BPAF	1.83 ± 0.07	0.69± 0.14	0.25
BPZ	1.93 ± 0.04	1.2± 0.10	0.5
BPAP	2.86± 0.12	0.81± 0.25	0.25
BPB	4.18± 0.14	1.67± 0.26	0.5
BPA	8.97 ± 0.20	4.44± 0.27	2
BPF	23.59± 0.46	12.85± 0.62	5
BPS	209.7± 5.13	112.62± 6.96	50

Table 1. LC_{50}, LC_{10} and NOAEL values for each of the bisphenol analogues to compare their toxicity (Martini, et al., unpublished)

Literature review was subsequently performed to gain insight on the toxic effects of each of the bisphenol analogues evaluated in this study. Research conducted on the toxic effects of the alternatives in comparison to BPA have shown that BPS is the least toxic alternative, which is in agreement with the data from our lab (Tisler et al. 2016; Li et al. 2015; Ren et al. 2017b). However, BPS was still found to have endocrine-disrupting effects such as upregulation of the gene CYP19a1, which encodes for aromatase that converts androgen to estrogen, and HSD7b1, which is regulates the level of estradiol in tissues, in zebrafish on par with BPA, BPAF and BPF (Mu et al. 2018; Kaewlert et al. 2018; Drzewiecka et al. 2014). Additionally, BPS, along with BPF, had also been found to increase the level of brain aromatase (Le Fol et al. 2017). Studies has also suggested that continuous exposure to BPS at higher than 0.5 ug/L could lead to decrease in egg production rate, hatching rate and sperm count, as well as increase in embryo malformation, hatching time and male-female ratio (Ji et al. 2013; Naderi et al. 2014; Rochester and Bolden. 2015). Other research had suggested that BPS exposure could also result in malformation in adult zebrafish, such as hunched back, pericardial edema and tail shortening when exposed to 0.5 – 50 ug/L of BPS for 21 days, and higher dose at 100 ug/L and longer exposure up to 75 days could result in decreased body weight and length in the males (Ji et al. 2013; Moreman et al. 2017; Ren et al. 2017a; Naderi etal. 2014). All of these suggest that while BPS might be the least toxic, and arguably the safest, alternative among the bisphenol analogues compared in this study, it still has endocrine-disrupting effects that one should be aware of when considering using it as BPA substitute.

BPF is another alternative that we found to be less toxic than BPA in terms of lethality, but it is not without its own endocrine-disrupting activity, some of which had

been mentioned above. Literature review done indicated that one research had found that exposure to 10 ug/L of BPF could lead to decrease in the level of testosterone and increase in the level of estradiol in adult male zebrafish (Yang et al. 2018). BPF had also been found to inhibit embryo hatching rate proportional to its dose exposure concentration and increase GnRH, LH and FSH hormone levels in the brain of zebrafish (Mu et al. 2018; Qiu et al. 2018). In addition to that, in a review by Liu et al (2021), it was found that BPF poses the second greatest ecological risk just behind BPA, perhaps due to it being one of the most commonly used BPA substitute, and the pollutant with highest risk in countries such as China, South Korea and Japan.

Our study found that BPAF to be the most toxic among the bisphenol analogues surveyed in terms of lethality and with higher toxicity compared to BPA (Fig 1 and Table 1), which is consistent with the results of other published studies (Qiu et al. 2021; Mu et al. 2018; Matsushima et al. 2010). Literature review performed indicated that BPAF had been found to decrease hatching rate of zebrafish, increase oxidative stress, as well as affecting the early development and immune system of the zebrafish larvae (Li et al. 2021). It had also been found to have an antagonistic estrogenic effect during the early development phase of zebrafish, stimulating the level of estradiol and vitellogenin, and upregulating the expression of ERα (Chen et al. 2018). One research also showed that BPAF exposure affect embryonic development of zebrafish, causing defects in the heart and swimming bladder, but zebrafish chorion was found to develop overtime as an effective barrier against BPAF (Yang et al. 2020). Study showed that human exposure to BPAF is lower compared to exposure to other bisphenol analogues, as seen through the urinary excretion data and wastewater analysis (Wang et al. 2020). This is could be due to BPAF not being as widely used as BPA substitute, perhaps owing to its higher toxicity and more potent estrogenic activity.

There has not been much data on the toxicity of BPZ and how it compares to BPA, according to the literature review performed, but a study done by Lin et al. (2020) suggested that it had the second highest ERα binding activity after BPAF and above BPA. BPZ had also been found to decrease testosterone hormone level while increasing estradiol hormone level in adult male zebrafish (Yang et al. 2018).

BPB had been found to impair reproductive function in dose-dependent manner, in which higher dose exposure to BPB significantly lowered hatching rate, number of eggs laid and decreased embryo survival (Yang et al. 2017). Compared to BPA, an *in vitro* study of the effect of BPB exposure on steroid hormone level found lowered level of testosterone, androstenedione, cortisol and estrone in cells exposed to BPB, similar to in cells exposed to BPA (Rosenmai et al. 2014). As reviewed by Braver-Sewradj et al. (2020), there has not been much data on the reproductive toxicity of both BPB and BPAP, however, a study had shown that BPAP exhibited neurodevelopmental toxicity similar to BPAF where exposure to both bisphenol analogues significantly decreased moving distance and speed of zebrafish larvae in addition to suppressing relevant neurodevelopment genes *mbp* and *syn2a* (Gu et al. 2019).

In addition to acute toxicity and exposure study, we also conducted systematic review of the effect of the bisphenol analogues on the gene expression of the HPG axis during zebrafish early developmental period, the results of which are summarized in **Table 2**.

Table 2. Review of different bisphenol analogues used as BPA substitutes and their gene expression effect on the HPG axis of early developmental phase zebrafish

Bisphenol analogue evaluated	Method	Result	Authors
BPF	Zebrafish embryo exposed to 0, 1, 10, 100, 1000 ug/L of BPF during 0 – 60 dpf; RNA extraction and real-time PCR to measure gene expression	10 and 1000 ug/L of BPF caused significant upregulation of *cyp19a1* expression; >10 ug/L BPF caused vtg expression	Yang et al. 2018
BPA, BPB, BPS, BPF, BPAF	Zebrafish embryo exposed to 1 or 100 ug/L of bisphenol analogues during 2 - 120 hpf (~5 dpf); RNA extraction and real-time PCR to measure gene expression	100 ug/L of BPA, BPB, BPS, and BPAF increases kiss1 expression; 1 ug/L BPF and 100 ug/L BPS increases kiss2 expression; 100ug/L BPA increases gnrh3; 100 ug/L BPAF increases fshβ expression; 1 ug/L BPS, BPF and 100 ug/L BPA, BPS, BPF and BPAF increases lhβ	Qiu et al. 2021
BPS, BPF, BPAF, BPAP	Zebrafish embryos were exposed to bisphenols analogues up to 7-dpf (wild-type) or 4-dpf (cyp19a1b-GFP transgenic line); RNA extraction then real-time PCR to measure gene expression; *in situ*	Significant induction of *cyp19a1b* by BPA, BPS, BPAF (36-, 410, 43-fold respectively compared to control), but no effect from BPAP; these results were confirmed by *in vivo* and *in situ* methods;	Cano-Nicolau et al. 2016

	hybridization; *in vivo* imaging	BPF found to induce *cyp19a1b* gene expression but not significant; BPAP has no estrogenic activity that affects brain aromatase	
BPAF, BPF, BPS, BPA	Zebrafish embryos were exposed up to 96 hpf; RNA extraction then real-time PCR for gene analysis	0.2, 1.0 and 10 mg/L of BPAF, BPA and BPF respectively significantly induce ER transcription; significant upregulation of *cyp19a1* by 0.1 mg/L of BPA and 10 mg/L of BPF; no significant gene expression effect observed from BPS exposure	Mu et al. 2018
BPS, BPA	Zebrafish embryos exposed to 100 ug/L of BPA and 100 ug/L of BPS up to 25 hpf	Environmentally relevant dose of BPA and BPS exposure caused increase in hypothalamic GnRH3 neurons and *kiss1, kiss2, gnrh3, erα, lhβ, fshβ* genes	Qiu et al. 2016

We compared the data on **Table 2** with the gene expression effect data from the study conducted in our lab. A strength of our gene expression study that made it unique compared to other studies of similar nature is that we used mixture of all the bisphenol analogues. The mixture of bisphenol analogues was found to have interesting results compared to the individual bisphenol analogue (**Fig 5**). In the result for the effect on the gene expression of arom-b which encodes for aromatase, the gene expression of arom-be affected by the mixture is much higher compared the individual analogues, more than double that of BPZ, which is the highest for individual bisphenol analogue. The effect of each of bisphenol analogue do not add up as high as the effect of the mixture, which is interesting. Another interesting result is the gene expression of vtg1, where the gene expression effect as caused by BPF far exceed the gene expression effect caused by the mixture. This is worth looking further into as BPF, according to our exposure study, was found to be the one of the least toxic analogues in terms of lethality. It is also worth looking more into as it suggests that there could be more thana simple additive effect for the result of the gene expression effect of the mixture.

To fill in the gap in knowledge, our study included BPB, BPZ and BPAP, which are three of the less studied bisphenol analogues, and our results indicate that BPB, BPAP and BPZ, along with BPF, BPS and the mixture, significantly upregulate the gene expression of cga, for both the medium and low dose. We also found BPAP to be the only one to significantly upregulate the gene expression of fshb compared to the control for both medium and low dose. BPAP and BPZ, along with the mixture and BPA, were also found to significantly upregulate the gene expression of arom-b in comparison to the control.

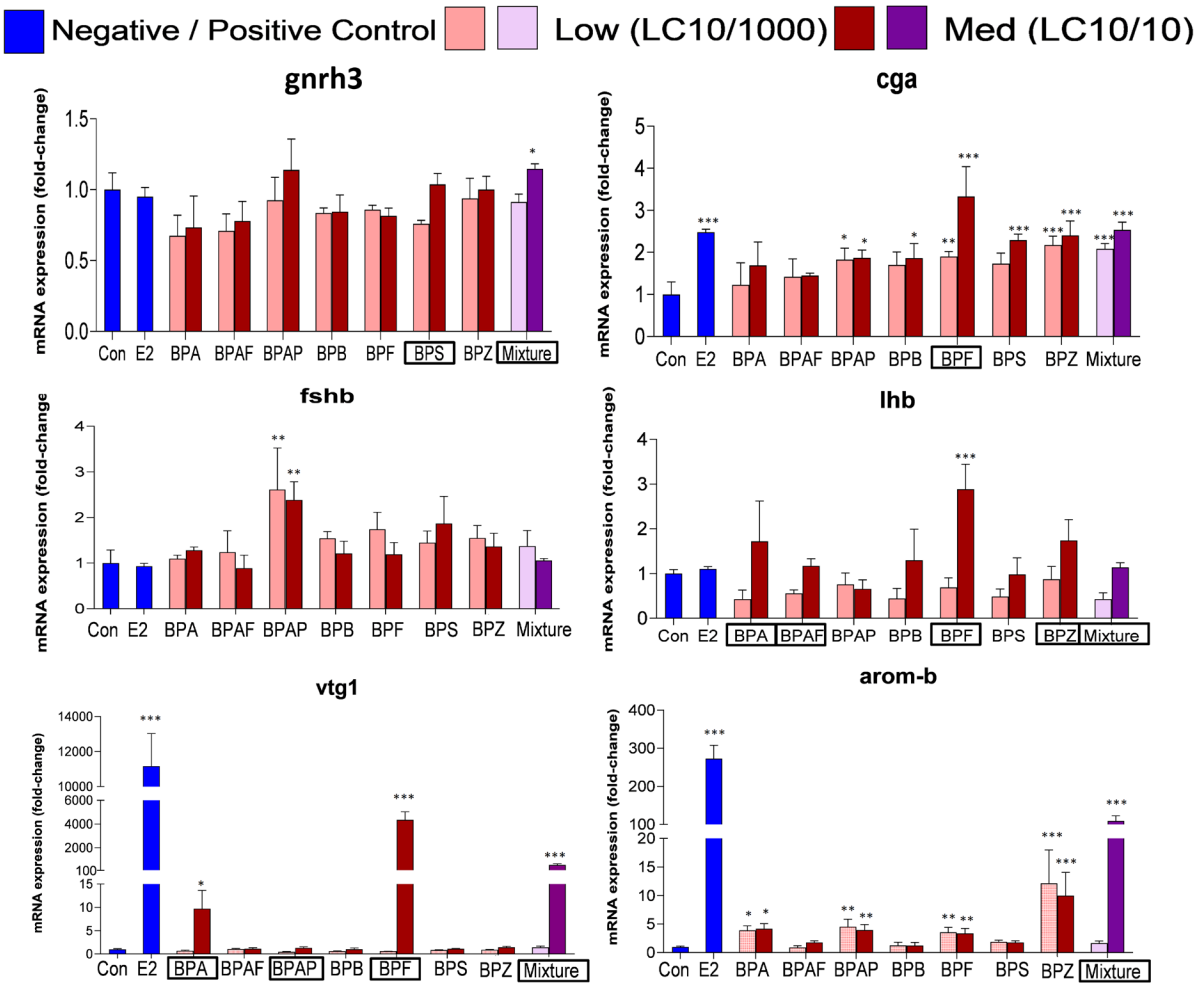

Fig 5. Results of gene analysis done on the gene expression effect of seven bisphenol analogues at 8-dpf (Martini et al., unpublished). Squares indicate significant difference between low and medium dose ($p<0.05$). Asterisks indicate significant difference between the analogue evaluated and the control (*$p<0.05$, **$p<0.01$, ***$p<0.001$ by ANOVA, Dunnet's as a posthoc).

Comparing the result of our study to the data obtained from literature review, we found that BPF and BPS consistently cause changes in gene expression of reproductive relevant neuroendocrine genes such as *lhb* and *gnrh3*, where our results indicate that there are significant differences between the doses, consistent with the results reported by published research. BPF in particular seems to have a significant gene expression effect toward reproductive relevant genes in the HPG axis, as both the literature review and our own gene analysis have shown, despite being less toxic than BPA in terms of

lethality. Also consistent with the results in published literatures, we found BPS to have the least gene expression effect as both of our gene analysis and data from published research showed that it took a much higher dose for BPS to have a significant effect. It is also worth noting that our gene analysis data does not show any significant change brought by BPAF exposure despite BPAF being consistently found to be the most toxic of bisphenol analogues including from our own exposure study in terms of lethality. Another unexpected result was the extent of induction of *vtg1* and *arom-b* with the complex mixture exposure. The effect on these genes appeared to be magnitudes of order higher than an additive effect of the 7 bisphenols. The complex mixtures were made based on the LC10 of each bisphenols. Due to high LC10 value of BPS (indicating lowest lethal toxicity), concentration of BPS was significantly much higher in the complex mixture compared to other bisphenols. Therefore, the contribution of BPS towards this induction in a complex mixture still needs to be evaluated, especially considering wide-use of BPS in recent years. We found no other studies reporting the effect of complex bisphenol mixtures,

Most literature data came from studies done on zebrafish larvae that are less than 5-dpf old, while the our lab used 8-dpf zebrafish larvae. Thus, the data generated by our lab could be used to fill in the knowledge gap of how bisphenol continue to affect early development of zebrafish as it transformed from embryo to larval stage. This difference in the age of the zebrafish larvae used could explain the difference between our gene analysis data and the data obtained from literature review.

We also looked at data from the results of studies using different animal models including human embryonic stem cells, marine mussels, Japanese medaka, goldfish, chicken embryo, rats and tadpoles to supplement our data in effort to study the toxic effect of bisphenol during the early development phase. BPAF, BPF, BPS, BPZ and BPB

exposure was found to disrupt embryonic development and lipid metabolism in human embryonic stem cells (Liang et al. 2021). BPAF had also been found to significantly upregulate the gene expressions of *cyp3a7* and *cpt1a* genes as well as decreasing embryonic viability and increasing gallbladder mass in chicken embryo (Sharin et al. 2021). In amphibians, BPAF exposure was found to induce disruption in testicular differentiation and development, resulting in morphological and histological abnormalities, as well as inhibiting the expression of sex-dimorphic genes *dmrt1, sox9* and *amh* in male African clawed frog tadpoles (Cai et al. 2020). BPA exposure in neonatal rats was also found to decrease *kiss1* gene expression especially in females, and significantly decrease ERβ expression in both males and females by postnatal day 10 (Cao et al. 2012).

There are three other aquatic animal models that we found interesting to look at to compare to zebrafish. Japanese medaka is an aquatic animal model that has been used t evaluate the effects of bisphenol exposures in aquatic environment and it was found that BPB and BPP exhibited high toxicity at early life stages of medaka and also inhibit egg hatching rate (Yokota et al. 2008). Marine mussel is another aquatic animal that is affected by bisphenol exposure and it was found that exposure of bisphenol analogues to it induces changes in gene expressions responsible for many of its key regulatory pathways and also associated with irregularities in shell formation (Balbi et al. 2016). In goldfish, it was found that the reproductive relevant genes of sgnrh, fshb, and lhb were decreased in females, and maturation of the testis and spermatogenesis were disrupted in males (Wang et al. 2019).

All in all, looking at the data from our own lab experiment as well data obtained from literature review, bisphenol analogues exert toxic effects during early developmental period for multiple animal models. We evaluated the toxicity of seven

different bisphenol analogues in terms of lethality and found that BPF and BPS, consistent with other research results, were the least toxic substitutes, but as gene analysis and exposure studies had found, they still exhibited negative impact during early developmental period which include morphological abnormalities, changes in reproductive relevant neuroendocrine genes and decreased hatching rate. BPAF was also found to be the most toxic in terms of lethality among the analogues evaluated, consistent with results of other published studies and environmentally relevant dose had been found to exert toxic effects which include morphological abnormalities and changes in gene expressions. There had not been much data published for BPB, BPZ and BPAP, therefore not much comparison could be made between the results of our study and those that had been published. Future research on bisphenol analogues could be done on these three to fill in the knowledge gap of their toxic effects. Future research on bisphenol analogues should also focus on mixtures of bisphenol analogues in order to evaluate their toxic effects as bisphenols are occurring more in environment as mixtures instead of individual analogues.

CONCLUSION AND FUTURE RESEARCH DIRECTION

With increasing concern over the toxic effects of BPA, bisphenol analogues such as BPAF, BPAP, BPZ, BPF, and BPB are synthesized in hope of finding a less toxic substitute with the same functionality. However, due to their structural similarities to BPA, there is still concern about their toxic effects. This study is conducted in order to fill in the knowledge gap in direct comparison of the effects of all the seven most ubiquitous bisphenol analogues on the gene expression of the HPG axis during the early developmental period using zebrafish. Our study indicated that BPAF is the most toxic among the analogues evaluated, with higher toxicity than BPA, and BPS is the least toxic in terms of lethality. However the overall gene expression effect after complex mixture expression suggest there may be more than a simple additive effect of toxicity at the molecular level and overall role of BPS (which was the highest concentration among all bisphenol used) to the toxicity of complex mixture remains unknown. Further studies are necessary as environmental exposures usually occur in complex mixture. The literature review performed suggest that bisphenol analogues exerted toxic effects during early developmental period on different animal including mortality, morphological abnormalities and disrupted reproductive functions. However, more data is needed to evaluate the toxic effects of BPB, BPZ and BPAP.

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