INVESTIGATING IMPACTS OF ACUTE AND CHRONIC EXPOSURE TO ENDOCRINE DISRUPTING COMPOUNDS ON THE REPRODUCTIVE AND STRESS-RESPONSIVE AXES OF ZEBRAFISH (*DANIO RERIO*)

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INVESTIGATING IMPACTS OF ACUTE AND CHRONIC EXPOSURE TO ENDOCRINE DISRUPTING COMPOUNDS ON THE REPRODUCTIVE AND STRESS-RESPONSIVE AXES OF ZEBRAFISH (*DANIO RERIO*)

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ABSTRACT

Regulatory agencies aim to keep us safe from harmful chemical exposures by placing limits on levels of toxic compounds in our environment, food, consumer products, and more. However, these limits are set based on exposure levels where broad adverse effects are no longer observed. Additionally, these limits are usually set at much higher concentrations than those at which hormones work to control many critical biological functions. Endocrine disrupting compounds (EDCs), which can mimic or interfere with these functions, can act below the suggested safe levels and lead to adverse health effects. Therefore, exposure studies are needed to better understand the potential harmful effects of EDCs. The overall purpose of this dissertation research was to understand EDC exposure effects on the reproductive hypothalamic-pituitary-gonadal (HPG) and the stress responsive hypothalamic-pituitary-adrenal (HPA) hormonal axes. I studied the exposure effects of bisphenols and cadmium, common environmental and food contaminants, on zebrafish development, gene expression, hormone production, and behavior. To model environmental exposures, these investigations included environmentally relevant concentrations and mixtures. An acute bisphenol study was used to determine if exposure to a mixture of bisphenols augments effects of individual bisphenol exposures. The chronic bisphenol mixture exposure study investigated exposure effects on both reproductive and stress-related endpoints across multiple generations. An acute

cadmium exposure was also conducted to understand the effects of early developmental exposure on the stress response. Acute exposure to individual bisphenols led to changes in reproductive gene expression at environmentally relevant concentrations. Additive or synergistic effects of the mixture exposure were only seen in the highest concentration. In the acute cadmium exposure, however, environmentally relevant concentrations, did not lead to any changes in stress response. The environmentally relevant concentration in chronic bisphenol exposure impacted stress-related behavior in the F0 generation, and the highest concentration led to fertility deficits and stress axis dysregulation. Changes in both behavior and gene expression were seen in subsequent generations of offspring of fish exposed to environmentally relevant concentrations. Taken together, environmentally relevant exposures are still a concern, although the effects may not always be seen in the directly exposed group but may be seen in offspring.

BIOGRAPHICAL SKETCH

Cedric was born in Kansas City, KS into a family that prioritized education above many things. He understood very early on the importance of applying himself to his studies. He moved schools 8 different times between elementary school to graduation from high school, and each move he looked for ways to stay on track with his studies. In his sophomore year of high school, he was accepted into the North Carolina School of Science and Mathematics (NCSSM) boarding school. Here, he took an AP environmental science course that fostered his desire to be a better steward of the environment.

After graduating high school, he returned home to attend the University of Kansas as he continued to consider pursuing environmental research. He was accepted into the KU Initiative for Maximizing Student Development (IMSD) program that supported many students through research training. This program was instrumental in teaching him about the graduate school process as he applied himself in various labs, trying to find the best fit for his interests. It was not until he began looking at graduate school research that he stumbled upon the field of toxicology, and this field fit his interests very well.

By joining the Mukai Lab, he had the flexibility to explore his interests in the non-lethal toxicological effects of environmental contaminants. He gained an understanding of the role of food in exposures to toxic compounds. By going to conferences, he understood the breadth of research that connected to his initial goals of being a better steward of the environment. Whether or not he continues his career in research or elsewhere, he is confident that it will involve toxicology.

To my God for his unchanging grace. My family for instilling in me great values. My parents for their unending support. My (now) wife for her continued patience.

ACKNOWLEDGEMENTS

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I would also like to thank the many people in the food science department that helped to make the workplace a home. This includes administrators who have always been willing to point me in the correct direction when I need it. The Weidmann and Gibney Lab for giving me access to instruments critical for the success of my research. To my lab members who encouraged me along on this journey, mentored me, and assisted me on data collection. Lastly, to my friends who made it a joy to come to work, and who helped me stay sane.

I am grateful for the funding offered by Cornell University, including the SUNY diversity fellowship and the Provost Diversity Fellowship, and all the people who helped make my extended stay at Cornell possible. I am also thankful for the Cornell Statistical Consulting Unit (CSCU) for their workshops that helped me better understand how to use the JMP statistical software, and for the CSCU members for always being willing and able to break down various statistical concepts I was unsure about.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACC	American Chemistry Council
ACTH	Adrenocorticotropic hormone
BPA	Bisphenol A
BPAF	Bisphenol AF
BPAP	Bisphenol AP
BPB	Bisphenol B
BPF	Bisphenol F
BPS	Bisphenol S
BPZ	Bisphenol Z
$CdCl_2$	Cadmium chloride
CGA	Glycoprotein hormone a
CHO-K1	Chinese hamster ovary K1
CRH	Corticotropin releasing hormone
CYP19B	Aromatase b
DES	Diethylstilbestrol
DMSO	Dimethyl sulfoxide
DPF	Days post fertilization
dT	Oligo
E3	Embryonic culture media
EDC	Endocrine disrupting chemicals
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FSH	Follicle stimulating hormone
FSIS	Food and Safety Inspection Service

GnIH	Gonadotropin inhibitory hormone
GnRH	Gonadotropin releasing hormone
gnrh3	Gonadotropin releasing hormone 3
GR	Glucocorticoid receptor
GSI	Gonadosomatic index
H295R	Human adrenocortical carcinoma
HPA	Hypothalamic pituitary adrenal
HPF	Hours post fertilization
HPG	Hypothalamic pituitary gonadal
HPI	Hypothalamic pituitary interrenal
HPP	Hypothalamic pituitary prolactin
HPS	Hypothalamic pituitary somatic
HPT	Hypothalamic pituitary thyroidal
HSI	Hepatosomatic index
LC	Lethal concentration
LH	Luteinizing hormone
LOAEL	Lowest observed adverse effect level
MEPS	Mass Embryo Production System
MPF	Month post fertilization
MR	Mineralocorticoid receptor
NOAEL	No observed adverse effect level
PCOS	Polycystic ovary syndrome
Pomca	Proopiomelanocortin
TRAP	Toxicity Relationship Analysis Program
TSCA	Toxic Substances Control Act
USDA	US Department of Agriculture

USEPA	U.S. Environmental Protection Agency
VTG1	vitellogenin

CHAPTER ONE: LITERATURE REVIEW - ENDOCRINE DISPRUTION BY ENVIRONMENTAL AND FOOD CONTAMINANTS

1.1. Introduction

Each day we are exposed to a wide range of chemicals in our environment that can potentially be harmful, and regulatory agencies are responsible for determining which compounds are safe enough for our use. There are currently over 80,000 chemicals that have commercial use in the US, according to the Toxic Substances Control Act (TSCA) inventory (EPA 2022a), with around 500 new submissions each year (EPA 2023). This act requires the Environmental Protection Agency (EPA) to review the possible risks for each chemical that is manufactured in, processed in, or imported to the US, and determine any necessary restrictions. The EPA is also responsible for pesticide regulation under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) which is in place to ensure the safety of pesticide use. Pesticide residue, or other chemicals in food, drugs, and certain consumer goods, however, are controlled by the Food and Drug Administration (FDA). Pesticides and chemical contamination in meat, poultry, and egg products are overseen by the US Department of Agriculture (USDA) with their Food and Safety Inspection Service (FSIS). The goals of these programs is to protect human and environmental health, however, the EPA has a substantial backlog of chemicals already on the market that must be assessed, in addition to new chemicals, according to TSCA (EPA 2022b). Under FIFRA, multiple pesticides that are banned, or are being banned, in the EU and China that are still being produced in upwards of millions pounds each year in the US (Donley 2019). This means there is a high likelihood of environmental contamination of potentially dangerous compounds, and this is without considering the contamination from consumer products and medication. Not all toxic exposures are immediately lethal to humans and animals, however sometimes harm can come over a longer period. When we are exposed to low levels of endocrine disrupting compounds, for example, effects are not always apparent.

1.2. Endocrine disrupting chemicals

The World Health Organization broadly defines an endocrine disrupting chemical (EDCs) as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". Although EDCs have only been defined within the last 30 years, the deleterious impacts of EDCs have been observed for multiple decades [reviewed in (Marty et al. 2011)], and effects of xenoestrogens on animals and humans played a large role in their characterization. For instance, disruption of reproductive capacity has been documented in healthy sheep that consumed phytoestrogens found in wild clover (Bennetts et al. 1946), and in female pigs consuming mycoestrogens found in moldy feed (Pullar and Lerew 1937). In time, both synthetic and natural estrogens were used as contraceptives in humans (Christin-Maitre 2013). On the other hand, one use for a particularly potent synthetic estrogen, diethylstilbestrol (DES), was to aid pregnancy (Watkins 1948). However the carcinogenic effects of DES were not discovered until many years later when daughters of women who used DES had relatively high chances of having vaginal cancers (Herbst et al. 1971) and abnormal gonadal morphology (Kaufman and Adam 2002). However, it wasn't until years after these events that endocrine disrupting compounds began to be characterized.

EDCs can alter the function of the endocrine system as antagonists (Kelce et al. 1995) and agonists (Kuiper et al. 1998) to various hormone receptors. For example, genistein, an isoflavone found in soybeans, is an estrogen receptor agonist (Li et al. 2013). Metabolites of vinclozolin fungicide, however, are anti-androgenic compounds (Kelce et al. 1994), and can inhibit critical receptor activities. Various EDCs, like methoxychlor can also disrupt hormone synthesis by directly inhibiting enzymes responsible for steroid synthesis (Craig et al. 2010). These compounds can also affect enzymatic activity responsible for metabolizing hormones along the pathways for hormone production (Usmani et al. 2003). Examples of chemicals commonly associated with endocrine disruption include phthalates, parabens, and dioxins [reviewed in (Gore et al. 2015)], and understanding of their adverse effects in animal models have helped gauge safety of human exposure.

Animal studies have been used during these EDC investigations to elucidate the complexity of hormonal disruption. For instance, a study demonstrated that rats developed differently dependent on whether their mothers were exposed to DES *in utero* or exposed post-weaning (Odum et al. 2002), showing the time sensitivity of exposure studies. EDC exposures affecting progeny is common, and animal models allow researchers to observe impacts in both parents and offspring in rats (Crews et al. 2007), zebrafish [reviewed in (Horzmann and Freeman 2018)], or other models. In multigenerational exposures, for instance, generations of animals are raised, and each generation is exposed to the compound of concern. In transgenerational studies, however, only the first generation is exposed. In this case, research focuses on understanding effects observed in subsequent offspring, especially the effects seen in offspring that were not present as germ cells during the exposure. This would be the F3 generation in mammals subject to *in utero* exposures and the F2 generation in F0 exposed fish. Zebrafish, mice, or other animals models also allow researchers to also utilize genetic tools available to create transgenic organisms that can aid in visualizing locations of protein expression (Abdelmoneim et al. 2020), create models for knockouts or knockdowns of key endocrine receptors (Eddy et al. 1996). Utilizing these advantages with EDC studies has improved our understanding of many of these compounds, their mechanisms, and their toxicological outcomes.

Exposure concentrations play an important role in discovering what impacts an EDC may make on an organism. Initially, risk associated with a compound was determined by exposing organisms, often rats or mice, to high concentrations compounds in the ppm range until no observed adverse effect level (NOAEL) or the lowest observed adverse effect level (LOAEL) was reached (EPA 1988). Common endpoints include lethality, adverse effects on reproductive organs, or changes in bodyweight. Results from animal studies have been used to create legislation by setting the limits of exposure many folds lower than the NOAEL/LOAEL seen in animal studies (EPA 1988). With the discoveries of endocrine disrupting effects, researchers found that some exposures led to non-monotonic response and elicit adverse effects even in the µg/L and ng/L range. This led to experimental designs investigating low-dose exposures, or exposures below regulatory limits at ranges humans are expected to be exposed to. This

effect of low dose exposures leading to adverse outcomes had been attributed to the fact that endogenous hormones act at very low concentrations, and exposures to EDCs are shown to be able to do the same, affecting critical functions.

1.3. Endocrine system and hormones

EDCs can target endocrine processes including, but not limited to, development, reproduction, metabolism, and behavior (Hiller-Sturmhöfel and Bartke 1998). There are hormonal pathways in humans that have specific roles, but this current work focuses on pathways initiated by the hypothalamus and pituitary. Five axes are directly linked to the hypothalamus and the pituitary which control a subset of these responses: hypothalamic-pituitary-gonadal (HPG), hypothalamic-pituitary-thyroidal (HPT), hypothalamic-pituitary-adrenal (HPA; the adrenal gland is analogous to zebrafish interrenal gland), hypothalamic-pituitary-somatic (HPS), and the hypothalamic-pituitary-prolactin (HPP) axis (Padilla and Carmichael 2016). Of these axes, the HPG axis, primarily regulating reproduction and sex specific traits, is one of the most studied, and the system that has received much of the attention very early with the focus on synthetic estrogens.

Research surrounding the reproductive axis is important for humans to understand health problems such as increases in infertility [reviewed in (Mikhael et al. 2019)], farmers considering deficits in livestock reproductive productivity (Pullar and Lerew 1937), and understanding growth and decline of wildlife populations (Bryan et al. 1986). Gonadotropin releasing hormone (GnRH) neurons are key initiators of HPG axis activity where hypothalamic GnRH neuron pulses release GnRH which binds to pituitary receptors, leading to the production and release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Shupnik and Fallest 1994). These gonadotropins are released into the bloodstream and bind to receptors in the gonads to produce sex hormones estrogen, progesterone, or testosterone at concentrations dependent on the sex of the individual and the relative concentrations of LH and FSH (Chian et al. 1999; Lee et al. 1974). As sex hormones are produced, they travel through blood to bind to receptors throughout the body to elicit tissue specific effects. Production

of the sex hormones is regulated by negative feedback of estrogen and testosterone binding to receptors in the hypothalamus, inhibiting further GnRH production (Shupnik 1996a; 1996b), or to receptors in the pituitary, inhibiting LH and FSH production (Shaw et al. 2010). Relative expression and availability of various receptors and unbound hormones are important in the ultimate outcome of hormone responses, which is why the toxicity of EDCs must be investigated even at a range of biologically relevant concentrations.

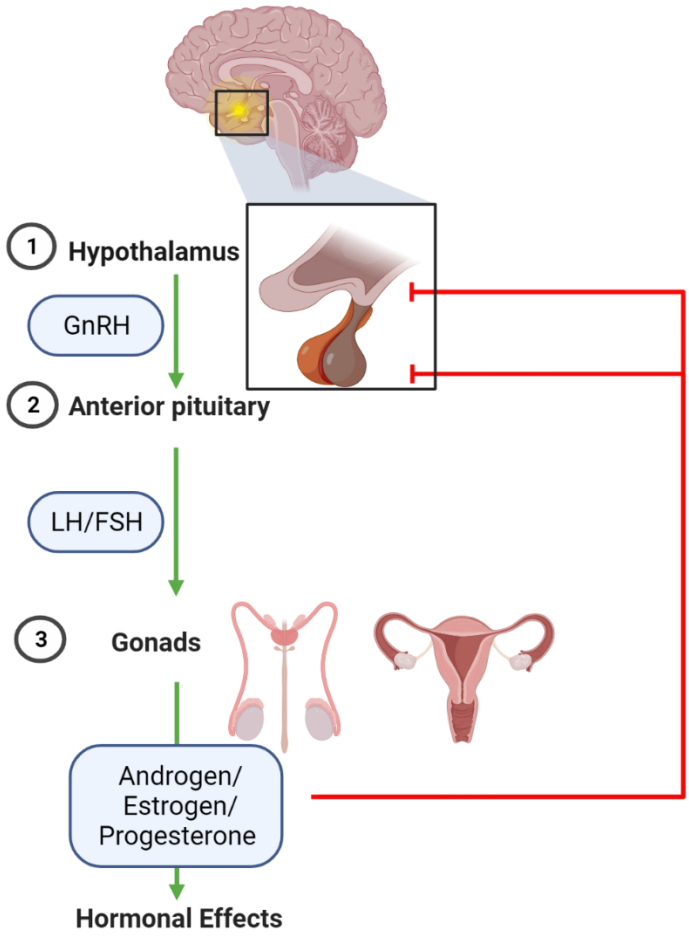

Fig. 1.1 – Hypothalamic-pituitary-gonadal axis (HPG axis)

The HPG axis is one of the most studied hormonal pathways, and there is a wide breadth of toxicological data on chemicals perturbing this reproductive axis. For males, articles have focused exposure effects on reproductive endpoints including semen quality (Meeker et al. 2004), anogenital

distance (Bornehag et al. 2015), steroidogenesis (Vitku et al. 2016), and behavior (Gore, 2019). Articles discussing female exposure to EDCs investigate polycystic ovary syndrome (Palioura and Diamanti-Kandarakis 2015), endometriosis (Cummings et al. 1996), steroidogenesis (Zhou et al. 2008), vaginal and uterine structure (Su et al. 2012), behavior (Gore, 2019), among other endpoints. Experimental studies have been conducted in a variety of model species including mice, rats, zebrafish, medaka, as well as various cell lines [reviewed in (Diamanti-Kandarakis et al. 2009)]. Some study designs investigate specific critical periods of HPG axis development, both acute (Jarema et al. 2015) and chronic (Wang et al. 2015) exposures, and transgenerational effects (Zhou et al. 2017). These studies support and expand our understanding of epidemiological evidence of reproductive disruption in humans, but less studied axes also subject to disruption deserve attention as well.

Only recently have studies increased on the effects of EDCs on the glucocorticoid stress response regulated by the HPA axis. Like the reproductive system, stress response is also primarily regulated by the hypothalamus and the pituitary, but it is the adrenal glands (interrenal gland in fish) that produce glucocorticoids "stress hormones", cortisol in humans and fish, but corticosterone in murine species (Poimenova et al. 2010). In a stressful situation, whether there is an actual stressor or a perceived stressor, cortisol is produced, increasing the heart rate, breaking down sugar stores, and distributing minerals throughout the body so it can respond to the stressor, known as 'fight-or-flight' response. High levels of cortisol are not intended to be continually maintained and should decrease after the stressor is removed. In some circumstances stressors are not removed for long periods, and the stress response remains active, which can lead to exhaustion, inflammatory and immune health issues, and HPA axis dysregulation. Despite there being less data on EDCs and the stress response, there is literature suggesting chemical exposures can affect the stress axis.

There are multiple points along the HPA axis that can affect cortisol production and our response to stress. Corticotropin releasing hormone (CRH) production in the hypothalamus (Spiess et al. 1981), is the first step in the glucocorticoid mediated stress response. This hormone travels through the blood of the

pituitary portal system to bind to CRH receptors in the pituitary (Aguilera et al. 1987). Adrenocorticotropic hormone (ACTH) is produced in the pituitary in response (Suda et al. 1992), which binds to a receptor in the adrenal glands, stimulating cortisol production (Keller-Wood et al. 1983). After cortisol is sent throughout the body, it binds primarily to the glucocorticoid receptor (GR) to activate the stress response (Griffiths et al. 2012), with different cell types having different responses. Similarly to the sex hormones, increased glucocorticoids in the blood stream decrease their own production by binding to GRs in the hypothalamus and pituitary to begin the negative feedback of this axis (Spencer et al. 1998). Cortisol levels can also be controlled by enzymes that convert active cortisol into the inactive cortisone (Chapman et al. 2013), or by being bound to proteins like corticosteroid binding protein or albumin (Bright 1995). There is a growing body of evidence of the different enzymes and receptors mentioned above as being targets for EDCs to disrupt cortisol production and stress response.

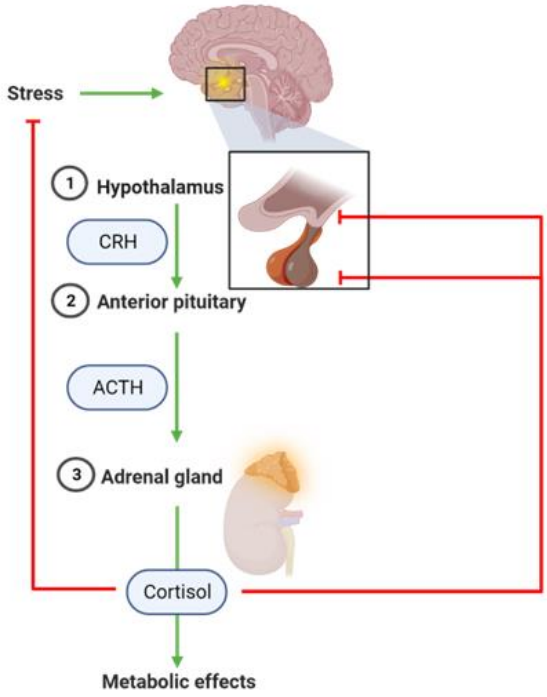

Fig. 1.2 – Hypothalamic-pituitary-adrenal axis (HPA axis)

Despite data being available on the function of the stress axis, there is still a need for more research in how chemical exposure may affect stress axis function. Arsenite and cadmium are examples of compounds that can prevent glucocorticoids from binding to the GR (Simons et al. 1990), and bacterial aflatoxin exposure has been shown to inhibit the activated GR's ability to bind to DNA in rat nuclei *in vitro* and *in vivo* (Kensler et al. 1976). Data from Chinese hamster ovary K1 (CHO-K1) and human adrenocortical carcinoma (H295R) cells suggest that multiple herbicides and pesticides disrupt glucocorticoid production (Shen et al. 2020), although mechanisms of action were unclear. Other groups have shown glucocorticoid concentrations can also be affected by bisphenols in rats (Panagiotidou et al. 2014) and zebrafish (Wei et al. 2020). Consequences of these perturbations can lead to prolonged elevation of GC levels which can lead to anxiety issues (Korte 2001; Nicolaides et al. 2014), and blunted GC stress responses which are associated with the development of anxiety and depressive disorders (Fiksdal et al. 2019; Petrowski et al. 2021). Although the impact on the HPA axis alone underscores the importance of EDC research in this area, the potential for HPA dysregulation to affect other axes, like the reproductive axis, increases the importance of the research.

There is a clear link between the stress and reproductive axes in both animal studies and human studies. Embryos from mice forced into restraints for multiple days had significantly reduced survival and liter sizes compared to embryos of unstressed mice (Gao et al. 2016). Another investigation found that mice subjected to auditory stress had increased fetal resorption and smaller liters than non-stressed control mice (Jafari et al. 2017). Follicular fluid from patients undergoing *in vitro* fertilization showed significant increases in unfertilized eggs corresponding to higher levels of cortisol (Jimena et al., 1992). Males who had higher anxiety or depression scores also had reductions in serum testosterone and reduced sperm quality (Bhongade et al. 2015). These effects on fertility can be traced back to hormone secretion.

At the hormonal level, components of the HPA axis have been shown to have inhibitory effects on the HPG axis. Data supports that CRH and cortisol can inhibit GnRH neurons and stimulate gonadotropin inhibitory hormone (GnIH) neurons that inhibit gonadotropin release (Tsutsui et al. 2012). Inhibition of gonadotropin releasing hormone results in decreased levels of gonadotropins, particularly

decreases in LH levels. Other research shows the disrupted productivity of the HPG axis ultimately affecting both estrogenic activity (Joseph and Whirledge 2017) and testosterone production (Gao et al. 1996) necessary for key reproductive processes. With the above information, the activation of the HPA axis has a clear effect on the HPG axis in certain contexts. This link has led to the question of whether endocrine exposures insulting the HPA axis require more attention when their effects could lead to dysregulation of the HPG axis as well.

1.4. Current gaps in EDC research

As the field of toxicology continues to explore the effects of endocrine disrupting compounds on the various axes, there are areas that require more attention. As mentioned previously, classic toxicological studies were evaluated at concentrations many folds higher than low environmental exposures. Any exposures "lower" than these traditional exposure concentrations are typically referred to as 'low-dose exposures' or 'environmentally relevant exposures' (Vandenberg et al. 2012). Further understanding is still needed on effects of environmentally relevant exposures, particularly EDC investigations to help determine which compounds continue to have adverse effects at environmentally relevant concentrations.

There is a need to understand the toxicity of a given compound individually, but because naturally occurring exposures are to multiple compounds at a time, there is also a need to understand the toxicity of mixtures. Chemicals modeling more realistic mixture exposures can potentially lead to effects not seen in singular exposures. Effects of a mixture can be additive (Scholz et al. 2006), where the toxicity of the mixture is the sum of the effects of the exposure to the individual compounds. Effects of a mixture of compounds can be synergistic (Arnold et al. 1996), where the toxicity of the mixture is more than the additive effects of exposure to the individual compounds. A mixture can have potentiating effects (Stepic et al. 2013), where a compound that does not lead to adverse effects in a singular exposure does so when combined with other chemicals. Lastly, compounds in a mixture can be antagonistic, where toxic effects of a compound at an endpoint can be countered with the addition of another compound (Ekins et

al. 1985). Results from mixture exposures may better inform regulators of the potential complex nature of toxicity.

Although there are studies investigating effects of stress on reproductive endpoints, there are comparatively fewer investigations of EDC exposure effects on HPA axis. There are even fewer exposure studies that investigate exposure effects on both the HPA and HPG axis simultaneously (Chen et al. 2021; Liu et al. 2011). BPA, for example, has been shown to affect the reproduction (Gámez et al. 2015; Wisniewski et al. 2015) in one study, and the stress response (Panagiotidou et al. 2014) in a separate study. BPA has not, however, been investigated in a cohesive study apart from one study that investigates the HPG axis as well and the HPA-associated *pomc* gene (Eckstrum et al. 2018). Conducting a study with endpoints in both stress and reproduction will help identify whether exposure effects on the stress axis can lead to impacts on the reproductive axis, which can determine the necessity for increased research of the glucocorticoid axis.

1.5. Filling the research gap

Part of my dissertation research seeks to fill these gaps by investigating exposure effects of multiple bisphenols, a common environmental and food contaminant, using zebrafish. This study investigates the effect of acute exposure to a mixture of seven bisphenols at environmentally relevant concentrations so the observed effects can model everyday exposures and add to literature needed in this area. Additionally, zebrafish will be exposed across early development, peri-pubertal stage, into early adulthood to cover multiple critical periods of development, and to resemble chronic exposures commonly found in nature. Offspring from this exposed generation will be raised up for two additional generations to study transgenerational effects. The study is separated into two chapters, one consisting of the assessment on reproductive effects (Chapter 2) and the other on stress response effects (Chapter 3). The two assessments include both behavioral and gene expression endpoints.

The third study (Chapter 4) seeks to fill the mentioned gaps by adding to literature investigating exposure effects of EDCs on the stress response. Cadmium is the EDC of focus in this chapter, which will

be exposed to zebrafish larvae at environmentally relevant concentrations during early development only, as important hormonal axes are developing. Endpoints of this acute exposure will focus on gene expression along the HPA axis, hormonal responses to stress, and behavior. The hypothalamic-pituitary-interrenal gland is the analogous structure in zebrafish. Data from each of these exposures will add to a growing body of literature focused on understanding the effects of EDCs we are exposed to each day through the environment and in our food.

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CHAPTER TWO: ACUTE, CHRONIC, AND TRANSGENERATIONAL EFFECTS OF BISPHENOL ANALOGUE MIXTURE EXPOSURES ON ZEBRAFISH REPRODUCTIVE AXIS

2.1. Abstract

In the past decade, concerns of Bisphenol A (BPA) effects on reproductive health have resulted in the introduction of several BPA-substitutes for food packaging. These products marketed as 'BPA-free', still contain a variety of bisphenol analogues that remain to be fully tested for their endocrine-disrupting activity. Adverse health effects of any of these compounds, either individually or in mixtures, need careful assessment as a public health priority. In this study, we evaluated acute, chronic, and transgenerational effects of exposures to 7 bisphenol mixtures (BPA, BPB, BPF, BPS, BPZ, BPAF, and BPAP) using zebrafish (Danio rerio). LC10 of each bisphenol were divided by 10, 100, and 1000 to achieve a range of mixture concentrations for exposure, including low environmentally relevant concentrations. F0 embryos were exposed to mixtures until 120 days-post-fertilization (dpf). Expression of hypothalamic-pituitary-gonadal (HPG) axis-related mRNA, fertility, and reproductive behaviors were evaluated at early developmental, peri-pubertal, and adult stages, in F0, F1, and F2. The exposure to LC10/10 resulted in severe sex skewing towards males, and F0 females were unable to breed, precluding LC10/10 progeny in F1 or F2. At 8 dpf, multiple HPG genes were significantly increased in the mixture group compared to controls, significance did not show in brain/pituitary of older fish, although there were visible trends towards expression increase in mixture exposed vs. control with F0 and F2. Notably, both *gnrh3* and follicle-stimulating hormone (*fshb*) expression in brain/pituitary at 85 dpf was decreased with treatment but this effect was only observed in the F2 generation and not in F0 or F1. Liver vitellogenin 1 (*vtg1*) expression was also increased with treatment in F2 only. Our results demonstrate that the effect of bisphenol mixtures is more complex than a simple additive effect. LC10/10 concentration caused severe reproductive deficits in F0 females. Transgenerational effects on mRNA expression were present with LC10/100 although it did not cause overt reproductive problems in the F1 and F2 generation at environmentally relevant concentrations.

2.2. Introduction

Bisphenol A (BPA) is known for its uses in a range of products including polycarbonate plastics, epoxy resins, and food containers (Hoekstra and Simoneau 2013; Munguia-Lopez et al. 2005). Much of human exposure comes from ingestion, due to BPA's use in food contact materials and its capability to leach out into food (Geens et al. 2012). In response to a food additive petition submitted by the American Chemistry Council (ACC), in July 2012 the Food and Drug Administration (FDA) amended its regulations and removed BPA from approved use in baby bottles and sippy cups as well as in coatings in packaging intended for infant formula due to manufacturers abandonment of its use. Still, high production of BPA and widespread use in other products has led to its detection in a variety of environmental samples such as surface water, wastewater, and even indoor dust samples [reviewed by (Chen et al. 2016)]. The prevalence of this compound is concerning considering it has been well-established as an endocrine disrupting compound (EDC).

Data demonstrates that the thyroidal (Moriyama et al. 2002), adrenal (Poimenova et al. 2010), and gonadal steroid pathways (Qiu et al. 2016), can be affected by BPA exposure. Effects exhibited on these endocrine pathways have been observed even at low environmentally relevant doses in fish (Mandich et al., 2007; Zhang et al., 2014) and rodents (Doshi et al. 2011; Newbold et al. 2009). Epidemiological studies suggest a positive association with BPA concentrations and follicle stimulating hormone (FSH) concentrations and an inverse association with the ratio of estradiol:testosterone (Meeker et al. 2010) in blood samples. Exposures to BPA demonstrate changes in regulation of key hormones involved in the hypothalamic-pituitary-gonadal (HPG) axis including gonadotropin-releasing hormone 3 (GnRH3), follicle stimulating hormone (FSH), luteinizing hormone (LH), and estrogens and androgens in fish (Qin et al. 2013; Qiu et al. 2016; Wang et al. 2019) and rats (Gámez et al. 2015; Wisniewski et al. 2015). With BPA demonstrating endocrine disruptive effects, there is also concern for bisphenols that may be used to replace it.

Products that do not contain BPA are considered ‘BPA-free’, ‘non-toxic’ or even ‘safe’ despite containing alternative bisphenol compounds that have not been evaluated for their safety in terms of endocrine disrupting activity. These alternatives have been found in food (Gonzalez et al. 2020; Morgan and Clifton 2021; Russo et al. 2019) and are prevalent in the environment (Chen et al. 2016; Wang et al. 2020). Multiple studies have reported variable toxicity of the alternatives using various models, including zebrafish (Moreman et al. 2017), *C. elegans*, and mammalian fibroblast cells (Hyun et al. 2021) and reproductive effects in zebrafish and mice (Qiu et al. 2019; Shi et al. 2015; Shi et al. 2019a). However, information on the combined effects of multiple bisphenols in a single exposure, to closer resemble real-life exposures, remains limited. There are limited reports of how chronic low-dose exposures to individual BPA alternatives affect gene expression, behavior, and other endpoints (Salahinejad et al. 2020; Ullah et al. 2018); moreover, studies on transgenerational effects of chronic exposures to their mixtures are lacking. Transgenerational effects on gene expression, behavior, and other endpoints have been observed in EDC exposures of rats to vinclozolin (Anway et al. 2005), medaka (*Orysias latipes*) to BPA (Bhandari et al. 2015), and mice to phthalates and bisphenols (Hatcher et al. 2019; Shi et al. 2019b). It is likely that compounds similar to BPA exposed as a mixture will also affect multiple endpoints after chronic exposure.

The purpose of this study is to understand the toxicological effects of exposures to environmentally relevant bisphenols in complex mixtures. General developmental toxicity and effects on HPG gene expression were evaluated with exposure to individual bisphenol compounds as well as to complex mixtures of seven bisphenol analogues. Additionally, long term effects on reproductive axis and behavior are evaluated in the F0 generation exposed to the mixture to the F2 generation to focus on the transgenerational effects. Among the many analogues synthesized, this investigation will focus on seven bisphenols which have been detected in the environment (Cesen et al. 2018; Wang et al. 2020), food (Liao and Kannan 2013; Morgan and Clifton 2021), and in humans (Chen et al. 2016; Zhang et al. 2020). We hypothesize that exposures to bisphenol mixture would pose reproductive effects even at environmentally

relevant concentrations, and that some of these effects may persist in the unexposed F2 generation. Here we report, the F0 generation chronically exposed to LC10/10 dose mixture demonstrated delays in gonadal development, a skew toward male populations, and reproductive delays in females. Transgenerational effects in gene expression along the HPG axis were evident in F2 fish compared to their control counterparts. Our study shows that some cases of larval exposures to bisphenol mixtures can result in effects greater than simple additive effect. In addition, these effects can have a lasting effect on future generations at higher concentrations.

2.3. Materials and Methods

2.3.1. Animals:

Sexually mature wild-type zebrafish raised in the Cornell University (Ithaca, NY, USA) facility were bred using Pentair mini-Mass Embryo Production System (MEPS) to obtain embryos for the study. Larvae were fed 3 times/day with Larval AP100 (Zeigler, Gardners, PA) and Golden Pearl powder (Brine Shrimp Direct, Ogden, UT) starting 4 dpf, and *Artemia nauplii* and commercial fish flakes (Tetra, Blacksburg, VA) were also added twice/day from 10 dpf. From 46 dpf onward, fish were only fed with *Artemia nauplii* and fish flakes. Fish were kept under a 14h light:10h dark photoperiod, water temperature was maintained at 28 ± 1 °C, conductivity at 550 ± 150 µS, and at a pH of 7.4 ± 0.2. Water quality measurements were taken weekly to ensure ammonia, nitrate, and nitrite were at safe levels.

2.3.2. Chemicals:

All bisphenols selected for this study were purchased from Sigma-Aldrich (St. Louis, MO): bisphenol A (BPA ≥ 99.0 %), bisphenol AF (BPAF ≥ 99.0 %), bisphenol AP (BPAP ≥ 99.0 %), bisphenol B (BPB ≥ 98.0 %), bisphenol F (BPF ≥ 98.0 %), bisphenol S (BPS ≥ 98.0 %), and bisphenol Z (BPZ ≥ 98.0 %). All bisphenols were dissolved in 100% dimethyl sulfoxide (DMSO) to create stock solutions. The final treatment solutions (containing 0.002-0.1% v/v DMSO) were made fresh each day by diluting

the stock solution with embryonic culture media (E3; 5 mM NaCl, 0.18 mM KCl, 0.33 mM $CaCl_2 \cdot 2H_2O$, 0.40 mM $MgCl_2 \cdot 6H_2O$, pH 7.2).

2.3.3. Exposure Studies:

Embryos were collected and dechorionated using Pronase® (Sigma, St. Louis, MO; 0.1 mg/mL for 15 min) at 3 hours post fertilization (hpf), rinsed repeatedly, and 25-30 healthy embryos were randomly distributed into (100 x 15mm) glass petri-dishes containing 30 mL treatment solutions at 4 hpf. An acute study to generate complete lethality dose-response curves of the individual seven bisphenols was done using the following dose ranges: BPA, 0.2-22 mg/L; BPAF, 0.05-7 mg/L; BPAP, 0.15-7 mg/L; BPB, 0.099-20 mg/L; BPF, 0.098-60 mg/L; BPS, 4.95-500 mg/L; and BPZ, 0.1-5 mg/L (n=6 replicate dishes/treatment). Treatment solutions were changed daily, and dead embryos were removed. At 120 hpf (5 dpf), zebrafish survival was recorded per plate to construct a dose-response curve where LC50 and LC10 were estimated using Toxicity Relationship Analysis Program (TRAP, USEPA).

To determine the sublethal toxicity after exposure to individual and mixtures of bisphenol, both acute and long-term studies were conducted, with exposures starting at 4 hpf. For the acute study, exposure media was refreshed daily, and larvae were euthanized and sampled in pools of 10 for gene expression at 8 dpf (n=6). The long-term study, from 4 hpf to 120 dpf, was focused on effects of complex mixture exposure. Exposure media was exchanged daily from 0 – 45 dpf. Subsequently, fish were moved to 20-L glass aquarium tanks and 50% of tank water was exchanged twice a week, until the termination of the exposure at 120 dpf, after which 50% of the tank water was exchanged once a week. This exposed group of zebrafish was considered the F0 generation, and F0 females were bred against unexposed WT males to produce the F1 generation 10 weeks after the cessation of the exposure (around 6 mpf). The F2 generation was produced by inbreeding the F1 generation. The LC10 of each bisphenol was divided by a factor of 1000 (LC10/1000) and 10 (LC10/10), to derive concentrations for single or complex mixture exposures for the sublethal acute study and LC10/100 dosage were also added for the chronic study.

2.3.4. Egg Production and Fertilization rate:

In order to assess reproductive capacity of F0, F1, and F2, adult fish between 5 and 7 months post-fertilization (mpf) each generation were placed in 2-L breeding tanks (Aquatic Habitats) the afternoon prior to egg collection and were allowed to breed in the morning. For F_0 generation breeding, each experimental fish was bred against a WT fish of the opposite sex (n=6-13). For F_1 and F_2 generation breeding, 2 male and 2 female fish of the same experimental group were bred (n=10-13). Number of eggs laid were counted at 2-hrs after light onset and fertilization rate was determined by (total live embryos at 24-hpf)/(total eggs laid).

2.3.5. Tissue Collection:

For each generation, prepubertal female fish were euthanized with MS-222 and dissected at 85 dpf. Body weight and body length were measured. Ovary was inspected under dissection microscope to determine the maturity of ovarian follicles (data not shown), ovary, and liver weight. Brain/pituitary, liver, and ovaries were collected, and flash frozen in liquid nitrogen. Frozen tissues were stored at -80°C until samples were processed for gene expression.

2.3.6. Reproductive Behavior:

Reproductive behavior was recorded in adult F0 fish. All behavior was recorded for an hour using a camera (DBPower EX5000) situated above the tanks. The fish were recorded in a completely enclosed white box illuminated by an LED light screen (1100-1300 lux) from the side. Each tank (30 cm x 18 cm) was divided into thirds where one of the thirds was used as a breeding region designated with an artificial plant and a 15.5 x 6.25 cm egg insert (Aquatic Habitats), shown in Fig. 2.1. Reproductive behavior was of a male or female experimental fish was recorded with a WT fish of the opposite sex in the F0 generation. Each fish was added to the tank in the late afternoon of the day before behavior analysis. Behavioral recording began the following day within five minutes of the light onset.

All behavioral recordings were analyzed using Behavior Cloud (Columbus, OH). Behaviors such as spawning quivers (rapid tail movement along female body) and chases (males following females) were

manually tagged and counted using the software. The duration of time fish spent in the breeding zone was also measured.

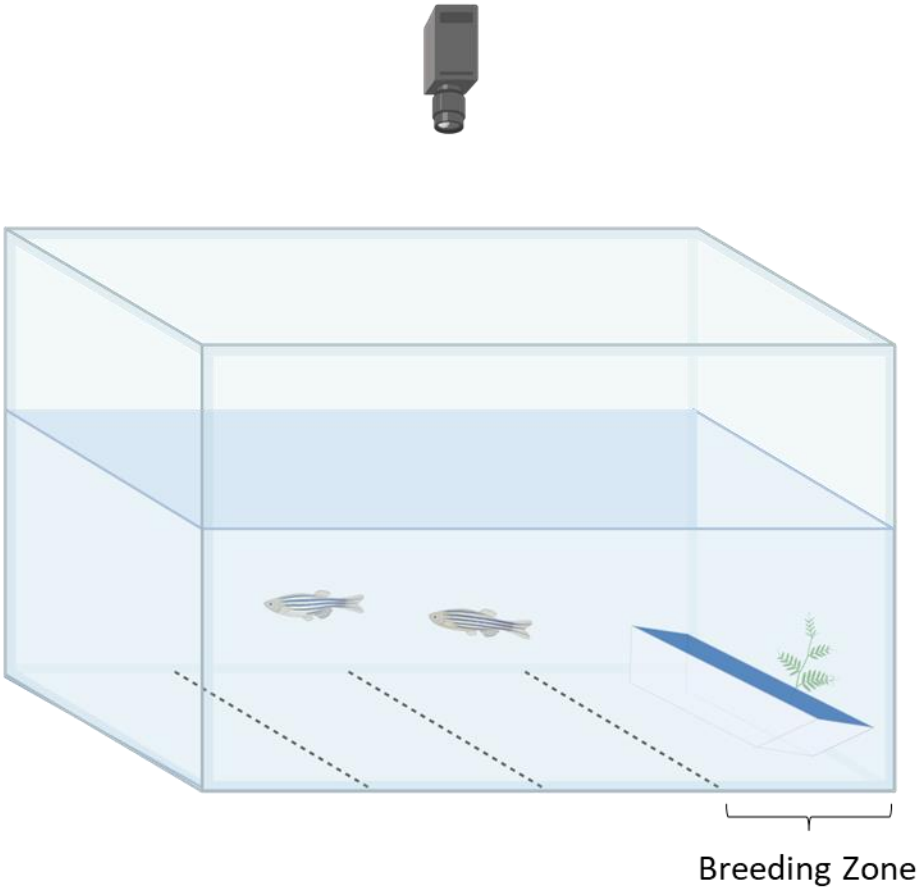


Fig. 2.1 – Reproductive behavior, experimental setup

2.3.7. Gene Expression:

Total RNA was extracted from frozen tissues using TRIzol reagent (Invitrogen) according to manufacturer's instructions. All samples were reversed transcribed using a high-capacity reverse transcription kit (Applied Biosystems) and Oligo$(dT)_{18}$ primers (Thermo Fisher Scientific). Each qPCR reaction well contained 10 µL and included: 2 ng total RNA equivalent of cDNA template (10 ng for low expressing genes), containing 0.3 µM forward and reverse primers, SYBR Select Master Mix (Applied

Biosystems), and 0.2 µm filtered MilliQ water. Quantstudio 6 Flex Real-Time PCR System (Applied Biosystems) was used to determine C_t (threshold cycle) value. The amplicon amount was calculated using the standard curve method.

Target genes (Table 2.1) *gnrh3, fshb, lhb, cga, cyp19b*, and *vtg1* in larvae at 8 dpf were normalized to *rpl13a*. Target genes for *gnrh3, fshb, lhb, cga, cyp19b* in fish at 85 dpf and 9 (mpf) fish brain/pituitary samples were normalized against *b-actin*, and *vtg1* in liver sample against *rpl13a*.

Table 2.1

Gene Name (Abbreviation)	Primer Sequence
Gonadotropin releasing hormone (*gnrh3*)	F: 5'-GGTCATACGGTTGGCTTCCC-3' R: 5'-CCCCGTCTGTCTGGAAATCTT-3'
Follicle stimulating hormone (*fshb*)	F: 5'-AGAGCGAAGAATGTGGGAGC-3' R: 5'-AATCAACCCCTGCAGGACAG-3'
Luteinizing hormone (*lhb*)	F: 5'-GCTGTCCAAAATGCCTGGTG-3' R: 5'-CAGTCGGGCAGGTTAATGGT-3'
Glycoprotein hormone a (*cga*)	F: 5'-CGAACGTTTCTCCAAACCCG-3' R: 5'-GGGATATTCGTGGCAACCATTT-3'
Aromatase (*cyp19b*)	F: 5'-GTGTGTGCTGGAGATGGTGA-3' R: 5'-AGTCAGCCGACTCTACGTCT-3'

2.3.8. Statistics:

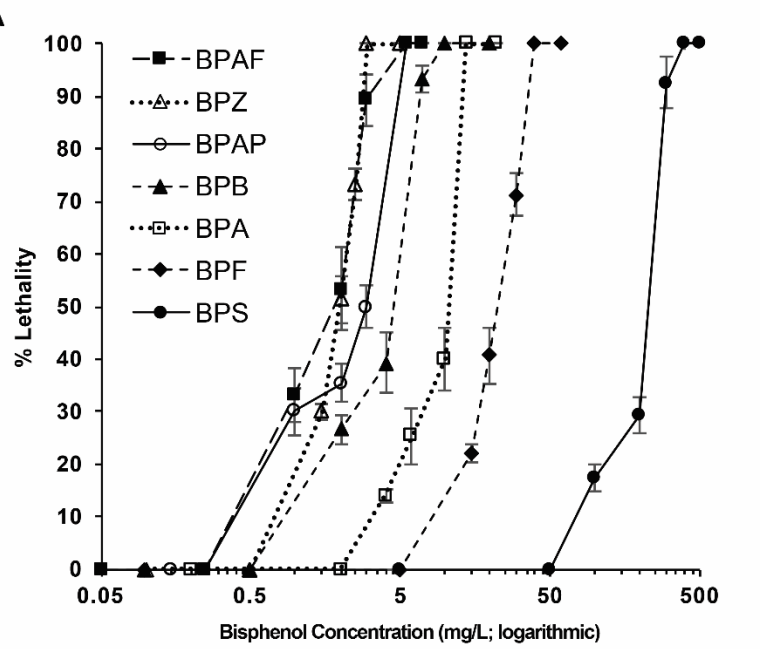
Statistical analysis was conducted using JMP Pro 16 software. Normality and homogeneity of variance were evaluated, some data were transformed as needed, followed by either a one-way or two-way ANOVA. A Tukey HSD post-hoc test was performed when ANOVA showed significance. In some instances, custom comparisons were made between means and adjusted using Bonferroni correction. Differences were considered statistically significant at $p \leq 0.05$. Gene expression 8 dpf: Reproductive capacity and reproductive behavior, One-Way ANOVA + Tukey HSD.

2.4. Results

2.4.1. Effects of bisphenol exposure on larval lethality

Dose-response lethality curves were generated at 5 days post-fertilization (dpf) using zebrafish larvae (Fig. 2.2A), and the LC10 and LC50 were estimated using EPA's TRAP (Fig. 2.2B). No-observed adverse effect level (NOAEL) was assigned as the highest experimental dosage that was not statistically significant from the vehicle group. A wide range in toxicity was observed with the seven bisphenols tested, from most toxic BPAF to least toxic BPS, with an LC50 of 1.83 ± 0.07 to 209.70 ± 5.13 mg/L, an LC10 of 0.69 ± 0.15mg/L to 112.62 ± 6.96 mg/L, and NOAEL of 0.25 mg/L to 50.00 mg/L, respectively. The toxicity gap was the widest between the least toxic BPS and the second least toxic BPF, with an 8.9-fold difference in LC50, 209.70 ± 5.13 mg/L vs. 23.59 ± 0.46 mg/L. The subsequent experiments were done using dosages at fractions of the each LC10 values – an environmentally-relevant low-dose range of LC10/1000 (0.69-112.62 µg/L), mid-dose range LC10/100 (6.89-1,126.20 µg/L) and high-dose range for LC10/10 (68.87-11,262.00 µg/L). All exposure dosages (including LC10/10) were well-below the NOAEL of each bisphenol (Fig. 2.2B; note – NOAELs are shown in mg/L vs. experimental exposure dosages are shown in µg/L).

A



B

Bisphenol	LC50	LC10	NOAEL	LC10/1000-LC10/10
	mg/L			µg/L
BPAF	1.83	0.69	0.25	0.69-68.87
BPZ	1.93	1.13	0.50	1.13-112.94
BPAP	2.59	0.81	0.25	0.81-81.40
BPB	4.18	1.67	0.50	1.67-166.73
BPA	8.97	4.44	2.00	4.44-444.41
BPF	23.59	12.85	5.00	12.85-1,285.00
BPS	209.70	112.62	50.00	112.62-11,262.00

Fig. 2.2: Lethal effects of individual bisphenol exposure on zebrafish larvae from 4 hours post-fertilization to 124 hours post-fertilization. Concentrations ranged from 0.05 to 500 mg/L, and were dependent on the bisphenol. A) Complete lethality curve B) LC50 and LC10 determined by Toxicity Relationship Analysis Program (TRAP; US EPA) for seven different bisphenols, listed in the order of toxicity (most to least). Values derived from LC10 were used for subsequent exposures (range are listed from LC10/1000 to LC10/10), which are lower than no-observed adverse effect levels (NOAEL). Data points are shown as mean ± SEM (n=6/treatment, 25 larvae per replicate).

2.4.2. Effects of bisphenol exposure on HPG genes in early developmental stage

Both individual bisphenols and their mixtures led to changes in key genes along the HPG axis at LC10/1000 and LC10/10 groups after the sublethal exposure. Individual exposures did not lead to significant changes in *gnrh3* expression overall (Fig. 2.3A), although there was a trend toward a decrease (p-value = 0.0969) in response to LC10/1000 BPA exposure compared to the vehicle control. *Fshb* expression increased in response to BPAP exposure at both dose groups compared to vehicle (Fig. 2.3B) but was not affected with other bisphenols or mixture. BPF exposure led to a trend toward an increase of *lhb* expression (p-value = 0.0817; Fig. 2C) compared to the vehicle at LC10/10, and BPA and BPF each

led to significant dose-dependent increases. Significant increases in *cga* expression (Fig 2.3D) were observed in response to positive control (E2) and LC10/10 exposure of BPAP, BPB, BPF, BPS, BPZ, and the mixture. With the lower dose of LC10/1000, only BPF, BPZ, and the mixture significantly affected *cga* expression similarly, although trends toward an increase were observed with BPAP and BPS (p-value = 0.0574 and 0.0926, respectively). A significant dose-dependent increase was also seen in response to BPF exposure. *Cyp19b* expression (Fig. 2.3 E) significantly increased in larvae exposed to BPA, BPAP, and BPZ at both LC10/1000 and LC10/10, the mixture at LC10/10 and with E2 (272.8 ± 32.4.7-fold) compared to vehicle control, with a trend toward increase with BPF (p-value = 0.0719 and 0.0556, for LC10/1000 and LC10/10, respectively). Mixture exposure at LC10/10 increased *cyp19b* expression most prominently with a 109.0 ± 13.9-fold expression compared to the vehicle control although expression was not affected at lower mixture dose of LC10/1000. *Vtg1* expression (Fig. 2.3F) was significantly increased with BPF and the mixture at LC10/10, and with E2 (4,351.4 ± 685.5, 562.0 ± 81.9, and 11,169.0 ± 1860.0-fold, respectively), and showed a trend toward increase with BPA exposure at LC10/10 (p-value=0.0811) compared to the vehicle. On the other hand, significant decreases of *vtg1* expression were observed with BPAP and BPF exposure at the lower LC10/1000 dose. Dose-dependent increases in *vtg1* were observed with BPA, BPAP, BPF and the mixture at LC10/10 (Fig. 2.3F). BPAF exposure did not result in significant changes compared to the vehicle in any of the studied genes despite being the most toxic.

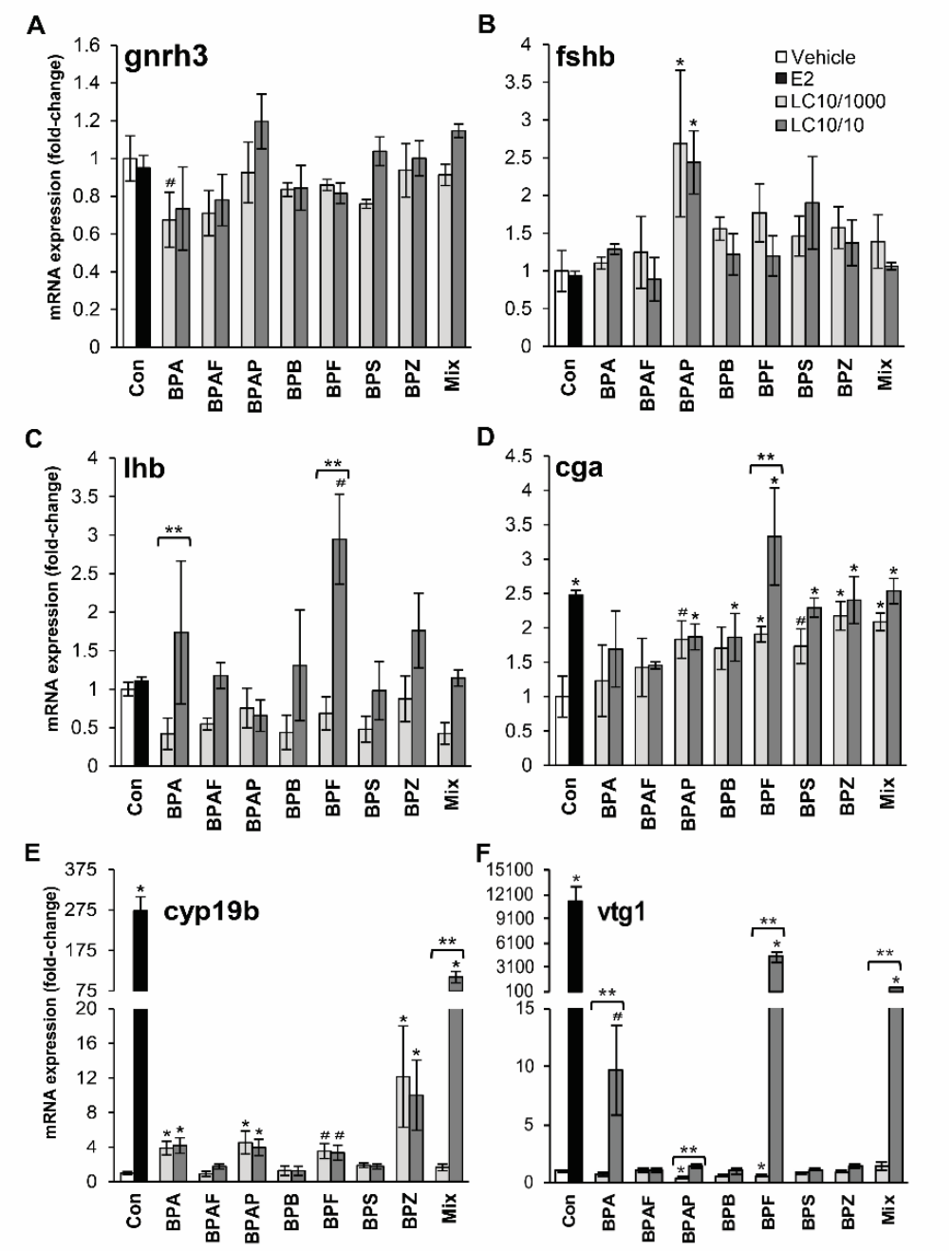


Fig. 2.3: Effects of early developmental exposures to seven individual bisphenols and their mixture, on gene expression related to hypothalamic-pituitary-gonadal axis at 8 days post-fertilization (dpf). Zebrafish larvae were exposed from 4 hours post-fertilization (hpf) to 146 hpf (total 144 hours) at fractions of the individual LC10 (LC10/10 and LC10/1000) for each bisphenol, and estradiol (E2, 0.5 µg/L) as positive control. Expression of A) *gnrh3* (gonadotropin-releasing hormone 3), B) *fshb* (follicle stimulating hormone β), C) *lhb* (luteinizing hormone β), D) *cga* (α-glycoprotein), E) *cyp19b* (aromatase β), and F) *vtg1* (vitellogenin 1) were normalized to *rpl13a* (ribosomal protein l13a) and shown as fold-changes compared to the vehicle control. All data are shown as mean ± SEM (n=6/group, 10 larvae per replicate). *p<0.05 compared to vehicle (ANOVA, pairwise Dunnett's post-hoc test), **p< 0.05 differences between the two concentrations groups (ANOVA, Dunnett's post-hoc test).

2.4.3. Effects of bisphenol exposure on development

The developmental effects of chronic bisphenol mixture exposure were investigated in females in each generation using body weight, body length, gonad, and liver weight with calculated gonadosomatic index (GSI), and hepatosomatic index (HSI; Table 1). Male and female fish were placed into separate tanks (sexed-out) based on external secondary sex phenotypes prior to tissue collection at 85 dpf and periodically during the rest of the experiment until 9 mpf. Upon the tissue collection of F0 at 85 dpf, however, only four of seven dissected fish exposed to the LC10/10 dosage with external-phenotypic female characteristics had identifiable gonads under the dissection microscope. LC10/10 exposure in F0 also led to significantly reduced body weight compared to LC10/100 exposed fish but was not significantly different from the control. Gonad weight in the LC10/10 group and GSI were both significantly decreased compared to control as well as lower dose groups. HSI was significantly increased compared to the control group, although there was no significant difference in liver size among treatment groups. At 9 mpf in the F0 generation, only five of the 14 externally phenotypic females of LC10/10 treated fish actually had ovaries (9 had testis). Only externally phenotypic females with ovaries were included in the data reported. LC10/100 exposed fish had significantly higher body weights compared to LC10/1000 treated fish and significantly longer body lengths compared to control and LC10/1000 groups. The F2 generation LC10/1000 group GSI was significantly higher than fish in the control group, and the HSI of the LC10/100 group was significantly higher than fish from the control group.

Table 2.2: Gonadosomatic Index (GSI) and hepatosomatic Index (HSI) of F0, F1, and F2 generation female zebrafish at 85 dpf and (F0) 9 mpf

	Control	LC10/1000	LC10/100	LC10/10
F0 - 85 dpf	n = 6	n = 6	n = 6	n = 7
total fish	29	30	28	31
body weight (mg)	211.43 ± 15.48ab	223.57 ± 15.59ab	243.62 ± 21.79a	164.87 ± 13.16b
body length (mm)	26.85 ± 0.25	27.14 ± 0.38	27.42 ± 0.63	25.94 ± 1.12
gonad weight (mg)	19.47±2.62a	22.97±2.03a	23.35±4.71a	5.33±0.32b*
liver weight (mg)	8.88 ± 0.66	10.42 ± 0.99	11.85 ± 1.04	9.33 ± 1.39
GSI	9.06 ± 0.73a	10.34 ± 0.81a	9.22 ± 1.21a	3.39 ± 0.46b*
HSI	4.26 ± 0.3a	4.63 ± 0.15ab	4.9 ± 0.22ab	5.48 ± 0.5b
F0 - 9 mpf	n = 6	n = 6	n = 6	n = 5
male fish	9	10	13	13
female fish	13	13	7	5
body weight (mg)	394.12 ± 35.41ab	365.22 ± 21.90a	501.22 ± 39.77b	415.42 ± 37.50ab
body length (mm)	32.10 ± 0.52a	31.93 ± 0.58a	34.99 ± 0.73b	33.79 ± 1.05ab
gonad weight (mg)	60.05 ± 5.57a	62.38 ± 5.83a	103.42 ± 9.47b	65.75 ± 14.29a**
liver weight (mg)	15.85 ± 1.29	16.17 ± 1.05	20.22 ± 1.76	19.14 ± 1.82
GSI	15.57 ± 1.58	17.26 ± 1.59	20.63 ± 1.01	15.92 ± 2.15**
HSI	4.10 ± 0.36	4.52 ± 0.41	4.07 ± 0.29	4.77 ± 0.57
F1 - inbred	n = 6	n = 8	n = 6	
male total fish	20	22	24	
female total fish	24	22	24	
body weight (mg)	170.48 ± 15.78	131.49 ± 14.99	145.70 ± 13.79	NA
body length (mm)	26.18 ± 0.62	24.47 ± 0.83	25.37 ± 0.82	NA
gonad weight (mg)	12.45±2.91	9.25±1.79	9.98±1.72	NA
liver weight (mg)	4.38±0.63	2.98±0.38	3.18±0.5	NA
GSI	6.96 ± 1.13	6.70 ± 0.68	6.83 ± 0.86	NA
HSI	2.57 ± 0.28	2.32 ± 0.22	2.26 ± 0.41	NA
F2 - inbred	n = 7	n = 7	n = 7	
male total fish	18	20	19	
female total fish	21	19	19	
body weight	202.64 ± 15.39	195.39 ± 10.45	168.74 ± 15.6	NA
body length	27.25 ± 0.50	26.56 ± 0.39	25.96 ± 0.71	NA
gonad weight	12.50 ± 1.99	19.76 ± 3.33	16.00 ± 3.16	NA
liver weight	5.90 ± 0.38	5.67 ± 0.58	6.30 ± 0.52	NA
GSI	6.00 ± 0.71a	9.81 ± 1.21b	8.98 ± 1.07ab	NA
HSI	2.96 ± 0.18a	2.94 ± 0.32ab	3.79 ± 0.19b	NA

Each exposed fish's length was measured, and body was weighed before tissue collection. Gonads and liver were weighed before being flash frozen in liquid nitrogen. None of the F0 exposed females from LC10/10 group laid eggs thus LC10/10 concentration were eliminated from F1 and F2 study. Measurement counts (n) are displayed in parenthesis when measurements taken deviate from total measurements; a, b - different alphabet denotes significant difference ($p<0.05$); dpf (days post-fertilization), mpf (months post-fertilization) *-ovaries identified in only four of seven dissected fish, **-only the right ovary found in fish n = 4

2.4.4. Effects of bisphenol exposure on reproductive capacity and behavior

Reproductive capacity was assessed in each generation (F0, F1, and F2) at 6 mpf to determine if the chronic bisphenol exposure causes adverse effects on reproduction in the exposed F0 generation and offspring. The exposure effects were most pronounced in the LC10/10 group where females (n=5) were unable to lay eggs when bred with an unexposed male, which prevented further generations of LC10/10 fish. In LC10/10 exposed males, two of the four parings of a male bred with unexposed female, yielded eggs. Overall, all bisphenol treatment groups had lower breeding success in the F0 generation (ranging from 0-79%) compared to the control (85% success with exposed females, and 100% success exposed male; Table 2). Among fish that had bred successfully, the number of eggs laid per female, fertilization rate, and embryo survival at 4 dpf, showed no significant difference between bisphenol treated fish and fish from the respective control groups in all generations. However, in the F1 generation, the rate which the eggs were fertilized of LC10/1000 females had decreased compared to the LC10/100 group, but not when compared to controls (Table 2). Due to either reduced or complete lack of breeding success of the exposed groups, reproductive behavior was as an additional reproductive assessment. However, chronic bisphenol exposure did not have a significant effect in the behavioral endpoints analyzed, including quivers (Fig. 2.4A), chases (Fig. 2.4B), entries into the breeding zone (Fig. 2.4C), and time spent in the breeding zone (Fig. 2.4D).

Table 2.3: Reproductive capacity of F0, F1, and F2 generation zebrafish at 6-month post-fertilization (6-mpf) after chronic exposures to bisphenol mixtures in F0.

	Control	LC10/1000	LC10/100	LC10/10
F0 - outcrossed to unexposed fish				
breeding success (%) with F:M=1:1 pairing/tank				
Exposed Female x Unexposed Male	85 (11/13)*	33 (4/12)	67 (4/6)	0 (0/5)
Exposed Male x Unexposed Female	100 (9/9)	64 (7/11)	79 (11/14)	50 (2/4)
number of eggs laid/female				
Exposed Female x Unexposed Male	177 ± 16	171.3 ± 22	243.8 ± 35	N/A
Exposed Male x Unexposed Female	150.3 ± 16	163.1 ± 29	151.5 ± 20	172 ± 12
fertilization rate (%)				
Exposed Female x Unexposed Male	84.9 ± 3	82.5 ± 6	86.3 ± 10	N/A
Exposed Male x Unexposed Female	82.2 ± 5	81.8 ± 5	69.1 ± 8	81.6 ± 16
survival at 4-dpf				
Exposed Female x Unexposed Male	84.2 ± 7	97.4 ± 1	95.6 ± 1.5	N/A
Exposed Male x Unexposed Female	99.1 ± 0.4	98.7 ± 0.6	98.0 ± 0.9	98.9 ± 0.2
F1 - inbred				
breeding success (%) with F:M=2:2 pairing/tank	91 (11/12)	90 (9/10)	72 (8/11)	N/A
number of eggs laid/female	42.7 ± 7.4	68 ± 7.3	50.75 ± 12	N/A
fertilization rate (%)	83.0 ± 3^{ab}	78.2 ± 5^{b}	92.6 ± 2^{a}	N/A
survival at 4-dpf	97.8 ± 0.7	96.4 ± 1	98.9 ± 0.6	N/A
F2 - inbred				
breeding success (%) with F:M=2:2 pairing/tank	75 (9/12)	100 (10/10)	72 (8/11)	N/A
number of eggs laid/female	79.7 ± 9.2	73.6 ± 10.7	81.5 ± 9.6	N/A
fertilization rate (%)	68.8 ± 12	83.7 ± 7	62.7 ± 13	N/A
survival at 4-dpf	78.1 ± 7	68.5 ± 13	79.1 ± 4	N/A

Each exposed F0 fish was outcrossed with an unexposed fish of the opposite sex. None of the F0 exposed females from LC10/10 group laid eggs thus LC10/10 concentration were eliminated from F1 and F2 study. Following F1 and F2 generations were inbred within the treatment groups. * - (number of tanks with eggs/number of tanks setup); a, b - different alphabet denotes significant difference ($p<0.05$); dpf (days-post-fertilization)

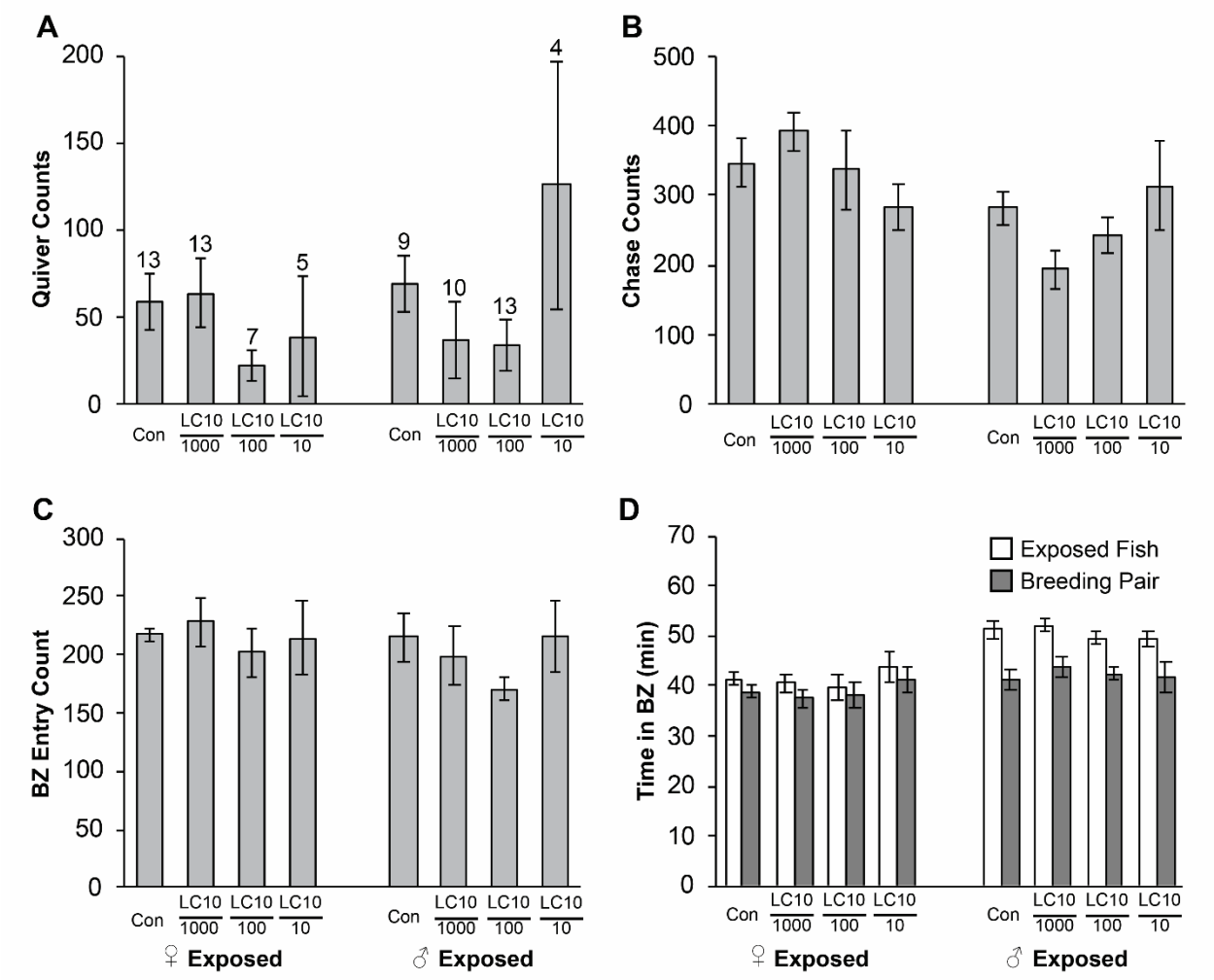


Fig. 2.4: Effects of chronic exposures to bisphenol mixtures of seven bisphenols on reproductive behaviors of 7-month-old F0 zebrafish. Zebrafish larvae were exposed from 4 hours post-fertilization (hpf) to 120 dpf to mixtures of bisphenols, at fractions of the individual LC10 (LC10/10, LC10/100, and LC10/1000). Bisphenol exposed fish were outcrossed to unexposed zebrafish of the opposite sex and behaviors were recorded for one hour within five minutes of lights turning on. Behaviors include A) quiver, B) chase count, C) number of breeding zone (BZ) entries by the exposed fish, D) time exposed fish or breeding pair spent in BZ. All data are shown as mean ± SEM (n=4-13 recordings/treatment). Comparisons made using One-way ANOVA.

2.4.5. Effects of bisphenol exposure on HPG genes expression in peri-pubertal and adult female

Key genes along the HPG axis were quantified to assess the impact of chronic bisphenol mixture exposure in directly exposed fish and in their indirectly exposed (F1) and unexposed (F2) offspring. Although there were no changes in the expression of gonadotropin releasing hormone (*gnrh3*) at 85 dpf in F0 or F1 compared to respective control groups, there was a significant decrease in expression in the F2 LC10/100 group at 0.58 ± 0.08-fold that of the control (Fig. 2.5A). F0 aromatase (*cyp19b*) expression was

significantly higher in the LC10/100 group, but only statistically significant compared to the LC10/10 group and not with controls. There was a trend toward an increase in *vtg1* expression (p-value = 0.072) in F2 liver samples of the LC10/100 treatment group which was 1.66 ± 0.12-fold higher than respective control, despite there being no significant changes in *vtg1* in either F0 or F1 generation females at 85 dpf (or 9 mpf for F0) (Fig. 2.5F).

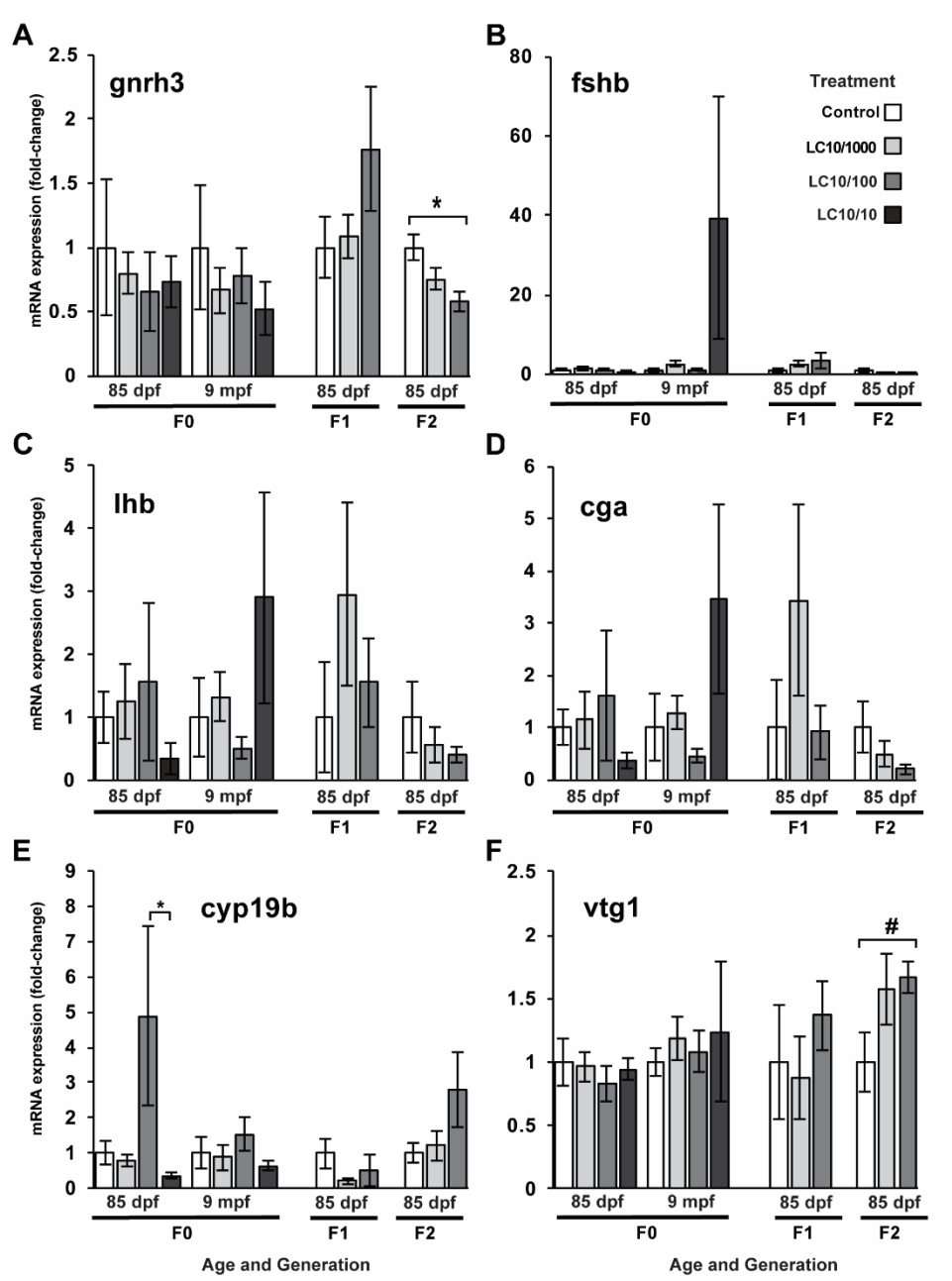


Fig. 2.5: Effects of chronic exposures to bisphenol mixtures of seven bisphenols on gene expression related to hypothalamic-pituitary-gonadal axis in brain/pituitary and liver at 85 days post-fertilization (dpf) and 9 months post-fertilization. Zebrafish larvae were exposed from 4 hours post-fertilization (hpf) to 120 dpf to mixtures of bisphenols, at fractions of the individual LC10 (LC10/10, LC10/100, and LC10/1000). Expression of A) *gnrh3* (gonadotropin-releasing hormone 3), B) *fshb* (follicle stimulating hormone β), C) *lhb* (luteinizing hormone β), D) *cga* (α-glycoprotein), and E) cyp19b (aromatase β) in brain/pituitary were normalized to b-actin. Expression of F) *vtg1* (vitellogenin 1) in the liver was normalized to rpl13a (ribosomal protein l13a). All data shown as fold-changes of the control of respective generation/age group as mean ± SEM (n=5-7/sample). *p<0.05 (One-way ANOVA by respective generation/age group, Tukey HSD post-hoc test).

2.5. Discussion

Bisphenols can be found in the environment and in biological samples, usually as a mixture of multiple analogues (Chen et al. 2016; Liao and Kannan 2013; Wang et al. 2022). Prevalent bisphenols found in these samples were explored here, where the effects of individual and complex bisphenol mixtures on multiple endpoints were investigated. A lethality curve was generated to assess toxicity at lethal concentrations, which determined subsequent exposure concentrations. At sublethal concentrations, body and tissue weights were taken to assess development, and HPG axis gene expression, reproductive capacity, and reproductive behavior were used to assess reproduction. Bisphenol exposure affected multiple genes related to reproduction, affected fish development in multiple generations, and led to poor breeding outcomes.

Toxicity of the seven bisphenols investigated in this study were ranked by LC50 from most toxic to least toxic with results consistent with previous zebrafish studies. Two groups using zebrafish found similar toxicity rankings as our study, with BPAF being the most toxic (Gao et al. 2022; Moreman et al. 2017). One group reports BPF to be the least toxic among BPAF, BPAP, BPC, BPB, and BPA at 4 dpf (Gao et al. 2022), while another similarly found BPS to be the least toxic at a magnitude higher than BPAF, BPA, and BPF at 4 dpf (Moreman et al. 2017). Similarities between these studies and our study were found regardless of dechorionation status or the day of sample collection. Human and rat stem cells exposed to BPAF, BPA, and BPS also lead to the same toxicity ranking found in vivo (Harnett et al. 2021). However, when using H295R cells, BPS exposure was determined to be more toxic than BPF (Feng et al. 2016). Discrepancies may be a result of the model used, but BPAF consistently leads to the highest toxicity, and BPF and BPS are consistently among the least toxic across models. Environmental concentrations vary considerably as some polluted surface water BPA concentrations reach 14.8 µg/L (Lalwani et al. 2020), dormitory dust BPA concentrations can be 32.5 ug/g (Zhang et al. 2020), thermal paper can reach 16,300 ug/g BPA and 7,800 ug/g BPS (Pivnenko et al. 2015), and BPA concentrations in some food items can reach 48.0 ng/g. For this reason, the LC10/1000 bisphenol concentrations at parts per billion (µg/L) levels are considered environmentally relevant.

To investigate effects on reproductive function, important genes related to gonadal health were assessed in zebrafish during early development. Expression of hypothalamic *gnrh* is necessary to stimulate production of gonadotropin subunits *fshb*, *lhb*, and *cga* in the pituitary (Haisenleder et al. 1991), which are important for gonadal health of males and females. Although *gnrh3* expression was only decreased in response to BPA exposure, the different gonadotropin subunits were not affected by the exposures in the same way. The differences are likely due to changes in pulsatility of GnRH neurons rather than expression, where longer or shorter pulses lead to differential expression of *fshb*, *lhb*, and *cga* (Ferris and Shupnik 2006). CGA, the alpha subunit required by *fshb*, *lhb*, and other hormones to exert their biological effects (Pierce and Parsons 1981), was the most impacted by the various bisphenol exposures. *Cga* is also associated with *tsh* activity so upregulation may be tied to its expression as well (Bargi-Souza et al. 2015). Although *lh* expression was not significantly affected in most cases, the dose-dependent effects seen with BPA and BPF, are less pronounced with other bisphenols, suggesting non-monotonic dose responses common to EDC exposures.

Previous reports indicate increases in *gnrh3*, *fshb*, and *lhb* expression in response to BPA and BPS exposure (1, 10, 100 and 1000 µg/L) at 25 hpf and 120 hpf (Qiu et al. 2016), and to BPA, BPAF, BPB, BPF, and BPS (1 µg/L and 100 µg/L) at 120 hpf (Qiu et al. 2021). Whereas the only exposure to lead to increased expression in our study among these three genes was *lhb*. Differences in exposure concentrations and age can be a factor in the differences between studies. Considering age, a 0.25 µM BPF zebrafish exposure at 2 dpf led to significantly lower *gnrh3* neurons in the terminal nerve and preoptic area compared to the control, but there were no differences at 3 dpf (Weiler and Ramakrishnan 2019). Additionally, medaka expressing TN-GnRH3-GFP that were exposed to 200 µg/L BPA had significantly more fluorescence at 3 dpf but significantly less at 5 dpf, compared to respective controls (Inagaki et al. 2016).

Cyp19b (Fenske and Segner 2004) and *vtg1* (Muncke and Eggen 2006) were additional genes investigated that are used as indicators of estrogenic activity. CYP19B is the predominate aromatase that converts androgens to estrogens in the brain (Menuet et al. 2005), and *vtg1* codes for an egg yolk liver

protein produced in response to estrogen (Wahli 1988). *Cyp19b* expression increased with multiple individual bisphenol exposures similar to other studies (Cano-Nicolau et al. 2016; Qiu et al. 2021), but our study is the first to document a mixture exposure leading to synergistic increases. This synergistic effect was only seen at the LC10/10 concentration despite both LC10/1000 and LC10/10 concentrations of the individual bisphenol exposures leading to comparable *cyp19b* expression. This may be a result of the complex involvement of hormonal feedback loops. Dose-dependent increases of *vtg1* may come from non-monotonic responses common to EDC exposures. In the case of BPAP and BPF LC10/1000 exposures, reductions in *vtg1* expression were significant. Seeing reductions at environmentally relevant concentrations suggests estrogenic compounds can also act as anti-estrogens at low concentrations, which one group has seen with bisphenols outside our study (Cao et al. 2022).

The weights of the body, gonad, liver, and the body length of female fish were measured throughout the chronic bisphenol exposure to assess developmental outcomes of the exposures. Decreases of the gonad weight and GSI endpoints suggest reproductive and developmental delays in the LC10/10 at 85 dpf. Delays at this stage contributed to the inability to locate gonads in multiple LC10/10 treated fish during tissue collection. Only upon gonad tissue collection at 9 mpf was it determined that 9 of 14 fish placed in the LC10/10 female tank were actually males, as testes were found instead of ovaries. However, these adult males had large deposits of adipose tissue in their abdomen compared to control fish (data not shown), which likely contributed to the incorrect sexing. The larger size of LC10/100 females at 9 mpf, is likely due to a low density of fish (n=7) in the tank, when other female tanks had 12-14 fish. The combination of lower GSI and higher HSI in LC10/10 at 85 dpf was also observed in zebrafish exposed to 100 µg/L BPS (Naderi et al. 2014) and rainbow trout exposed to 85.6 µg/L nonylphenol (Harris et al. 2001). Harris et al. suggests increases in *vtg1* to be the main cause of increased HSI, we see something similar with HSI and *vtg1* increases in the F2 generation.

Reproductive capacity is a common endpoint that is used to determine if exposures have any apparent effects on reproduction. At the time of this assay, it was unknown that 9 out of 14 fish sexed as females were males, as discussed earlier. These incorrectly sexed fish did not have the opportunity to

breed with females which led to a low sample size in reproductive capacity data for LC10/10 males. Despite the differences in breeding success, there were no differences in the other reproductive capacity endpoints investigated compared to control. Similar to our study, another group found no changes in fecundity while investigating female rats exposed *in utero*, offspring of 20 or 200 µg BPA/kg body weight (bw) per day exposed pregnant rats bred with unexposed male rats (Ryan et al. 2010). Another group also observed lower mating success, similar to the present study, when female mice exposed to 0.5 and 50 ug/kg bw per day BPA, 50 ug/kg bw per day BPE, or 0.5 ug/kg bw per day BPS *in utero* were paired with unexposed male mice (Shi et al. 2019a). Most of the pairings with lower mating success also had smaller liters (Shi et al. 2019a), similar to smaller egg clutches observed in 10 and 100 µg/L BPS exposed zebrafish (Naderi et al. 2014), although this was not the case in our study. Despite most reproductive endpoints in this current study being similar across treatments, the stark drop in breeding success in the LC10/10 is notable, even with a smaller sample size. The range of effects across species in multiple studies does suggest bisphenols have an effect on reproduction, however the complex mixtures may ameliorate some but not all of the effects seen elsewhere.

To determine if the differences in breeding success in the exposed F0 generation was related to reproductive behavior, fish behavior was recorded and compared. Of the endpoints studied, there were no significant difference between the treatment groups and the control. Although three of the four LC10/10 breeding pairs between an exposed female and an unexposed male had a relatively low quiver counts compared to the control mean, the low sample size makes it difficult to draw conclusions. The delayed development of LC10/10 fish at 85 dpf, the ambiguous phenotypic sex as late as 9 mpf, and lowest breeding success during the fertility assay, indicate the chronic LC10/10 exposure has detrimental effects on important components of development that can tie into reproduction, but not necessarily reproductive behavior.

Gene expression was also quantified after the chronic exposures and repeated for multiple generations. The affected genes in F0 larvae were largely diminished after the chronic exposure, however, the lack of significance was in part due to high variability in samples. Both *cyp19b* (Cano-Nicolau et al.

2016; Qiu et al. 2021) and *vtg1* (Gao et al. 2022; Notch and Mayer 2013) are reported to increase with estrogen and bisphenol exposure, but were largely unobserved in F0 fish at 85 dpf and 9 mpf. However, at 9 mpf, there was simultaneous elevation of *fshb*, *lhb*, and *cga* expression levels in response to the LC10/10 mixture exposure, but with high variability. Although it's not significant it may be a display of some HPG axis disruption in the LC10/10 females at the molecular level in the pituitary.

The effects of chemical exposures can have further consequences in offspring, which means it is important to consider subsequent generations in exposure studies. Despite some of the changes in gene expression that occurred in F0 larvae, there were no changes in expression in the genes tested in F1 compared to the control. However, in the F2 generation *gnrh3* expression decreased in the LC10/100 group. Although *fshb*, *lhb*, and *cga*, had a similar tendency to decrease with increased concentration, they were not considered trends. *Vtg1* expression, however, increased in the F2 LC10/100 group compared to the control. Knowing these two genes showed changes in expression in this generation, additional genes and hormone receptors should be tested to continue exploring potential transgenerational effects of bisphenol exposure. In terms of development, there were no differences observed in F1 85 dpf fish among the endpoints investigated. In the F2 85 dpf fish, the LC10/1000 fish had a higher GSI, and the LC10/100 fish had a higher HSI compared to control. These differences are unique to the F2 generation, but contrast the significant decrease in GSI only in the F2 generation of fish exposed to 20 µg/L BPA in another study (Santangeli et al. 2019). In this study, F0 exposure, started in adulthood and lasted four weeks, unlike ours which was four months. Additionally, the increase in HSI may have contributed to the increase in *vtg1* expression in this LC10/100 F2 group, although *vtg1* and HSI appeared disconnected in the F0 generation. The LC10/100 group from the F1 generation had a significantly higher fertilization rate than LC10/1000, but the remaining endpoints in F1 and each measured in F2 showed minimal differences. Although transgenerational inheritance cannot be assumed from the data collected, the effects seen in the offspring of the exposed F0 generation suggest that the chronic bisphenol mixture is affecting both gene expression and development in completely unexposed F2 fish.

2.6. Conclusion

The purpose of this study was to determine how bisphenol exposures affect the reproductive access by looking at development, HPG gene expression, reproductive behavior, and fertility using both acute and chronic exposures, and to observe effects across generations. We also sought to determine if mixture exposures lead to any augmentation of individual bisphenol exposures. In some cases, there were synergistic effects of the bisphenol mixture at 8 dpf. The reproductive deficits were only experienced at the high dose, and they were severe in females, but the environmentally relevant dose did not appear to affect reproduction at the endpoints tested. This is the first study to investigate these endpoints in this multi-bisphenol mixture, so additional mixture exposures are necessary for a fuller understanding of the effects of environmentally relevant bisphenol mixtures.

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CHAPTER THREE: TRANSGENERATIONAL EFFECTS OF BISPHENOL ANALOGUES MIXTURES ON ZEBRAFISH STRESS AXIS

3.1. Abstract

Disruption of this stress axis is associated with multiple psychiatric disorders in humans including anxiety and depression. Chronic stress is commonly associated with dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, a regulatory endocrine mechanism of a stress response. Endocrine disrupting compounds (EDCs) may also disrupt HPA axis function. In this study, we evaluated chronic (4 hpf - 120 dpf) exposure effects of a 7-bisphenol mixture (BPA, BPB, BPF, BPS, BPZ, BPAF, and BPAP) on stress response in peripubertal and adult zebrafish across 3 generations (F0-F2). LC10 of each bisphenol was divided by 10, 100, and 1000 for exposure concentrations. Expression of HPA axis mRNA was evaluated in females at 85 dpf and >5 mpf. At four months, novel tank test and group preference test were conducted to evaluate anxiety and social behavior, respectively. In the directly-exposed generation (F0), mRNA expression of corticotropin releasing hormone (*crhb*), glucocorticoid receptors (*gr*), mineralocorticoid receptor (*mr*) were downregulated in only LC10/10 exposed group at 85 dpf, not the lower concentrations. In F1 however, *mr* expression was inhibited in both the lower LC10/1000 and the LC10/100 concentrations. In F2, a trend towards a decrease was seen in the LC10/100 group with proopiomelanocortin (*pomca*). Female anxiety increased and male social behaviors decreased at the LC10/1000 level, whereas anxiolytic effects were observed in both LC10/1000 and LC10/100 in F2 males. Our results demonstrate both the highest and lowest concentrations affected fish at endpoints tested, and even impacted the F2 generation.

3.2. Introduction

The HPA axis is a hormonal axis critical for responding to stress and adjusting to changing environments. A primary role of this axis is to quickly catabolize and mobilize glucose energy stores so an organism can respond appropriately to a stressor (Kuo et al. 2015). Cortisol, the primary glucocorticoid in humans produced as a result of the positive stimulation of the HPA axis, is the key to activating this

stress response. Increased levels of cortisol is also responsible for inhibiting its own production through negative feedback at the hypothalamic (Edwardson and Bennett 1974) and pituitary (Buckingham and Hodges 1974) level, maintaining homeostasis. The HPA axis can be dysregulated, however, which can lead to abnormal cortisol production. Cushing's syndrome is an example where the pituitary produces too much ACTH leading to hypercortisolism, typically as result of a benign pituitary tumor (Liddle 1977) or hyperplasia (Ferri et al. 2022). Addison's disease is an example of a hypocortisolic condition, where adrenal tissues are not able to produce sufficient cortisol (Nieman and Chanco Turner 2006). Another source of HPA dysfunction occurs when a chronic stressor forces this axis to remain highly active for long periods of time.

Psychological chronic stress can lead to changes in HPA axis function including increased CRH expression (Bartanusz et al. 1993), delayed negative feedback from reduced glucocorticoid receptors levels (Sapolsky et al. 1984), and enlarged adrenal gland (Burchfield et al. 1980). These changes are also associated with depression, where ineffective inhibition of the HPA axis (Carrol et al. 1981) and adrenal hypertrophy (Rubin et al. 1995) are common. Data measuring HPA endpoints and anxiety has been inconsistent in part because of the range of anxiety disorders. There are reports demonstrating children with multiple anxiety disorders have lower basal cortisol production (Dieleman et al. 2015), whereas patients with panic disorder had elevated basal cortisol levels (Wedekind et al. 2000). In addition to psychological stressors, the HPA axis can be affected by chemical exposure.

The body of literature supporting the HPA axis as a target for EDCs is growing. High usage of drugs containing fluoxetine, an active ingredient in anti-depressants, has become an environmental contaminant which has experimentally been shown to decrease basal levels of cortisol in zebrafish at environmentally relevant concentrations (Vera-Chang et al. 2019). Cadmium has been cited to affect melanocortin 2 receptor in the interrenal gland (analogous to adrenal gland in humans) of rainbow trout (Sandhu, 2019), and multiple pesticides screened in Chinese hamster ovary (CHO-K1) cells were determined to be GR antagonists (Zhang, 2016). Bisphenol S has been shown to impact gene expression of multiple genes along the HPA axis including, *crhb*, *gr*, *mr*, and *pomca* after exposure to 1, 10, and 100

µg/L in zebrafish (Wei et al. 2020). Impacts of EDCs on the stress axis are not limited to molecular techniques as behavioral endpoints can be sensitive to chemical exposures as well.

Zebrafish have become useful models to study behavior (Egan et al. 2009). For example, larval photomotor response test (Burgess and Granato 2007; Ortmann et al. 2022) has been implemented to test for neurotoxicity. Predator avoidance (Ahmed et al. 2011) and novel tank tests (Blaser and Rosemberg 2012) have been used to gauge fear and anxiety. Zebrafish have also been used to test sociability during the group preference (or social preference) test (Ogi et al. 2020). These tests have led to insights including how BPS exposure leads to decreased sociability in zebrafish (Salahinejad et al. 2020), and that BPF exposure increases anxiety-like behavior in zebrafish (Kim et al. 2022).

This is the first study to investigate exposure effects of a bisphenol mixture on HPA axis dysregulation, as most studies focus on individual compound exposure. Critical genes related to cortisol production and stress response along the HPA axis will be assessed in response to a mixture of seven bisphenols commonly found in the environment. Exposure effects on sociability and anxiety will be observed using group preference and novel tank tests, respectively. These endpoints will be evaluated across the three generations (F0~F2). It is hypothesized that the bisphenol mixture will increase anxiety related behavior which will be closely associated with changes in gene expression. This study demonstrated that bisphenol mixture exposures affect both behavioral and molecular endpoints associated with the stress axis.

3.3. Materials and Methods

3.3.1. Animals:

Sexually mature wild-type zebrafish raised in the Cornell University (Ithaca, NY, USA) facility were bred using mini-Mass Embryo Production System (MEPS; Pentair, Apopka, FL) to obtain embryos for the study. Larvae were fed 3 times/day with Larval AP100 (Zeigler, Gardners, PA) and Golden Pearl powder (Brine Shrimp Direct, Ogden, UT) starting 4 dpf, and *Artemia nauplii* and commercial fish flakes (Tetra, Blacksburg, VA) were also added twice/day from 10 dpf. From 46 dpf onward, fish were fed with

Artemia nauplii and fish flakes. Fish were kept under a 14h light: 10h dark photoperiod, water temperature was maintained at 28 ± 1 °C, conductivity at 550 ± 150 µS, and at a pH of 7.4 ± 0.2. Water quality measurements taken weekly to ensure ammonia, nitrate, and nitrite were at safe levels.

3.3.2. Chemicals:

All bisphenols selected for this study were purchased from Sigma-Aldrich (St. Louis, MO): bisphenol A (BPA purity of ≥ 99.0 %), bisphenol AF (BPAF ≥ 99.0 %), bisphenol AP (BPAP ≥ 99.0 %), bisphenol B (BPB ≥ 98.0 %), bisphenol F (BPF ≥ 98.0 %), bisphenol S (BPS ≥ 98.0 %), and bisphenol Z (BPZ ≥ 98.0 %). All bisphenols were dissolved in 100% dimethyl sulfoxide (DMSO) to create stock solutions. The final treatment solutions (containing 0.002-0.1% v/v DMSO) were made fresh each day by diluting the stock solution with embryonic culture media (E3; 5 mM NaCl, 0.18 mM KCl, 0.33 mM $CaCl_2 \cdot 2H_2O$, 0.40 mM $MgCl_2 \cdot 6H_2O$, pH 7.2).

3.3.3. Exposure Studies:

Embryos were collected and dechorionated using Pronase® (Sigma, St. Louis, MO; 0.1 mg/mL for 15 min) at 3 hours post fertilization (hpf), rinsed repeatedly, and 25-30 healthy embryos were randomly distributed into (100 x 15mm) glass petri-dishes containing 30 mL treatment solutions at 4 hpf. An acute study to generate complete lethality dose-response curves of individual 7 bisphenols was done using the following dose ranges: BPA, 0.2-22 mg/L; BPAF, 0.05-7 mg/L; BPAP, 0.15-7 mg/L; BPB, 0.099-20 mg/L; BPF, 0.098-60 mg/L; BPS, 4.95-500 mg/L; and BPZ, 0.1-5 mg/L (n=6 replicate dishes/treatment). Treatment solutions were changed daily, and dead embryos were removed. At 120 hpf (5 dpf), zebrafish survival was recorded per plate to construct a dose-response curve where LC50 and LC10 were estimated using Toxicity Relationship Analysis Program (TRAP, USEPA).

To determine the sublethal toxicity after exposure to individual and mixtures of bisphenol, both acute and long-term studies were conducted, with exposures starting at 4-hpf. Exposure media was refreshed daily, and larvae were euthanized and sampled in pools of 10 for gene expression at 8 dpf (n=6). The long-term study, from 4 hpf to 120 dpf, was focused on effects of complex mixture exposure.

Exposure media was exchanged daily during from 0 – 45 dpf. Subsequently, fish were move to 16-L glass aquarium tanks and tank water containing bisphenols or vehicle was exchanged at 50% twice a week, until the termination of the exposure at 120 dpf, after which 50% of the tank water was exchanged once a week. This exposed group of zebrafish was considered the F0 generation, and F0 females were bred against unexposed WT males to produce the F1 generation 10 weeks after the cessation of the exposure (around 6 mpf). The F2 generation was produced by inbreeding the F1 generation. The LC10 of each bisphenol was divided by a factor of 1000 (LC10/1000) and10 (LC10/10), to derive concentrations for single or complex mixture exposures for the sublethal acute study and LC10/100 dosage were also added for the chronic study.

3.3.4. Egg Production:

In order to assess reproductive capacity of F0, F1, and F2, adult fish between 5 and 7 mpf of each generation were placed in 2-L breeding tanks (Aquatic Habitats) the afternoon prior to egg collection and were allowed to breed in the morning. For F_0 generation breeding, each experimental fish was bred against a WT fish of the opposite sex (n=6-13). For F_1 and F_2 generation breeding, 2 male and 2 female fish of the same experimental group were bred (n=10-13). Number of eggs laid were counted at 2-hrs after light onset and fertilization rate was determined by (total live embryos at 24-hpf)/(total eggs laid).

3.3.5. Tissue Collection:

For each generation, prepubertal female fish were euthanized with MS-222 and dissected at 85 dpf. Brain/pituitary were collected, and flash frozen in liquid nitrogen. Frozen tissues were stored in -80°C until samples were processed for gene expression.

3.3.6. 4 mpf and 5.5 mpf, Behavior Assays:

Novel tank test was recorded at 4 mpf for F0 through F2, and F0 had an additional recording around 5.5 mpf, 45 days post exposure. Fish were placed in 1.5-liter tanks (7 cm x 22 cm base area, 7 cm x 26 cm water surface area, 11.5 cm water column height), and the tank height was divided into halves.

Tank position and fish velocity was recorded for around 21 minutes using a camera (DBPower EX5000) situated in front of the tanks and analyzed using EthoVision XT 8.5 fish tracking software. The first 10 minutes was used to record depth, and the latter 10 minutes was used to record swim velocity. Two separate tanks with one fish per tank was recorded at a time in a completely enclosed white foam board box (50.5x71x45cm) illuminated by an LED light screen (each box within 200 lux from one another). During the recording, the two tanks were separated by a white partition to ensure fish were unable to see one another (Fig. 3.1).

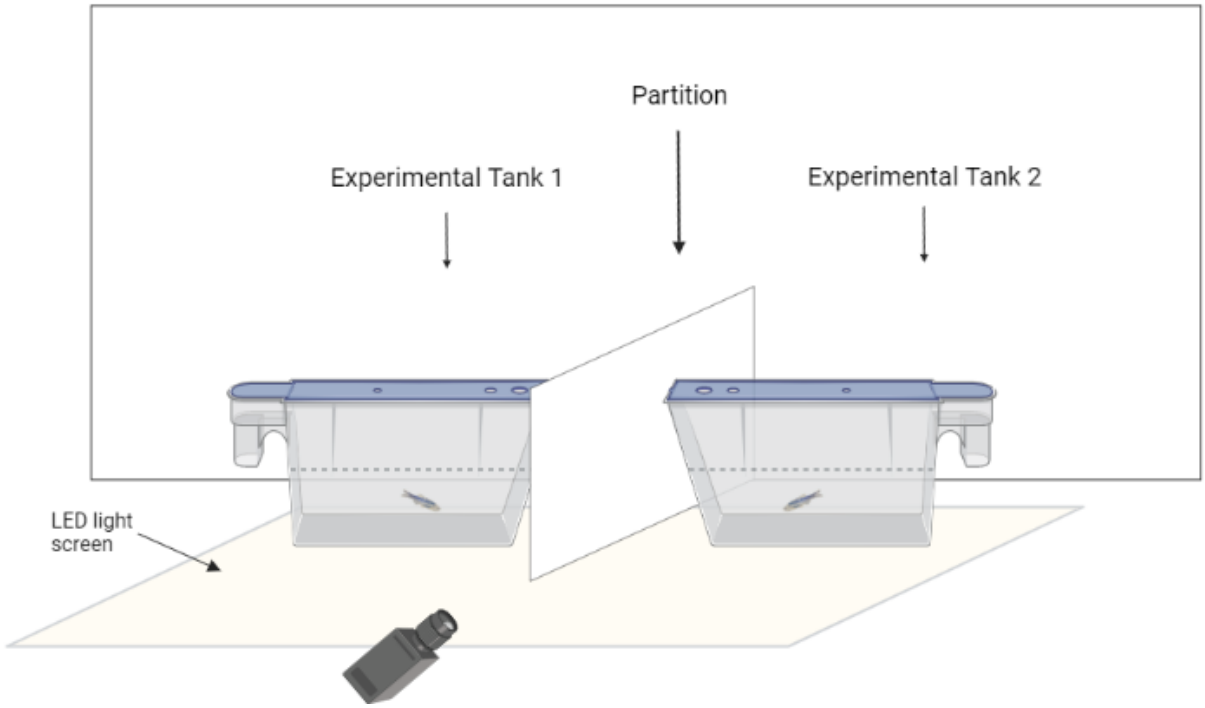

Fig. 3.1 – Novel Tank Test Setup

For group preference behavior, three tanks were placed in the fully enclosed white foam board box. The test fish was placed in an all-glass tank (30 cm x 18 cm base; 10 cm water column) divided into thirds across the length of the base, situated between two 3-liter tanks filled with water. One 3-L tank had 10 fish and the second had no fish. The DBPower EX5000 camera recording the fish was placed level in front of the fish tanks. The first 10 minutes the three tanks were separated by foam board partitions (Fig.

3.2A), and test fish were allowed to acclimate to the tank, and the partitions were removed at the ten-minute mark (Fig. 3.2B).

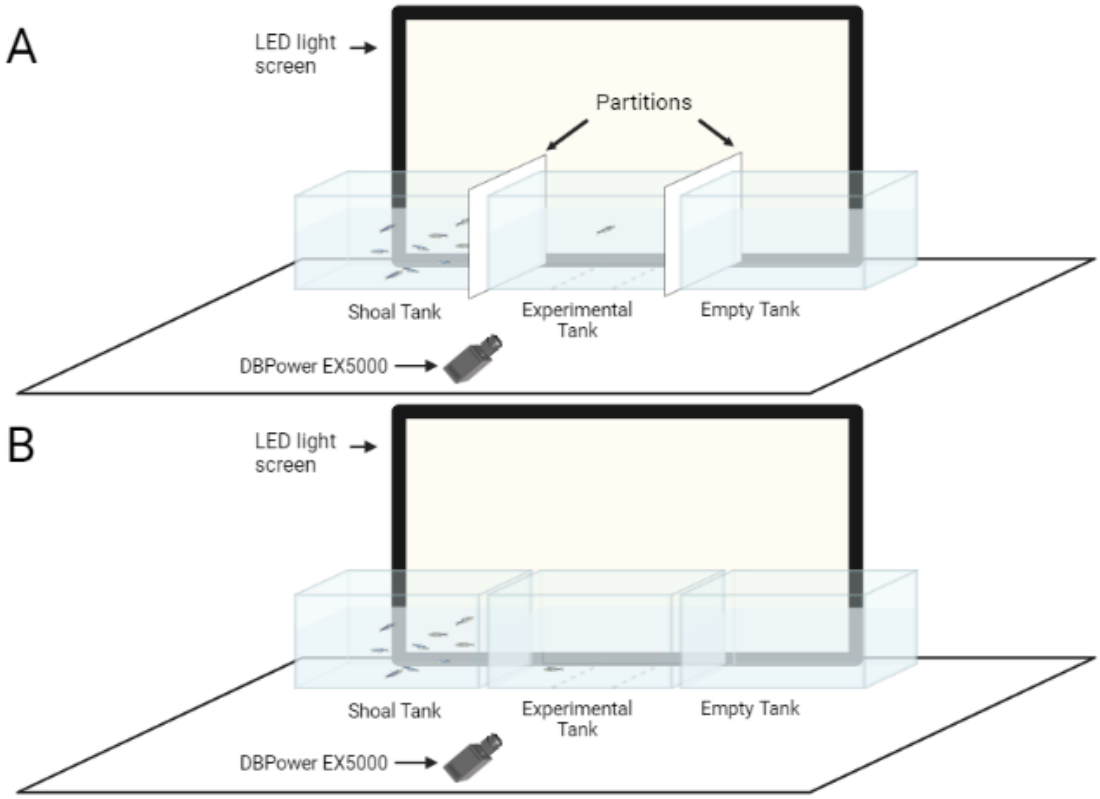

Fig. 3.2 – Group Preference Test Setup

Recordings for each test occurred between 2-5 pm window across multiple days until all fish were recorded. Fish-eye lenses of the DBPower EX5000 cameras were corrected using Adobe Premier Pro, and EthoVision XT 8.5 software used fish recordings to accurately determine fish depth and velocity.

3.3.7. Gene Expression:

Total RNA was extracted from frozen tissues using TRIzol reagent (Invitrogen) according to manufacturer's instructions. All samples were reversed transcribed using a high-capacity reverse transcription kit (Applied Biosystems) and Oligo$(dT)_{18}$ primers (Thermo Fisher Scientific). Each qPCR reaction well contained 10 µL and included: 2 ng total RNA equivalent of cDNA template (10 ng for low

expressing genes), containing 0.3 µM forward and reverse primers, SYBR Select Master Mix (Applied Biosystems), and 0.2 µm filtered MilliQ water. Quantstudio 6 Flex Real-Time PCR System (Applied Biosystems) was used to determine C_t (threshold cycle) value. Amplicon amount was calculated using the standard curve method. Target genes *crhb*, *pomca*, *cyp11c1*, *gr*, and *mr* in fish brain/pituitary samples were normalized against *b-actin*.

Table 3.1

Gene Name (Abbreviation)	Primer Sequence
Corticotropin releasing hormone (*crhb*)	F: 5'-AGCAACTAGAAGACAGACGC-3' R: 5'-CTACATTCATACGGCGGTGG-3'
Proopiomelanocortin (*pomca*)	F: 5'-TTGGCTCTGGCTGTTCTCTG-3' R: 5'-GATGGCTTTCTCCGGGGTAG-3'
Glucocorticoid receptor (*gr*)	F: 5'-TAAAAGGGCTGTTGAAGGGC-3' R: 5'-ATTACCTTTCCGGCTTGACG-3'
Mineralocorticoid receptor (*mr*)	F: 5'-TGCCACTACGGGGTTGTTAC-3' R: 5'-GTGCCCCAAGATTCATCCCA-3'

3.3.8. Statistics

To determine if there are differences between experimental groups for behavioral tests, one-way ANOVA was conducted separately among males and females. To determine if there are differences between experimental groups for gene expression, one-way ANOVA was conducted separately for each age/generation. Data not fitting a normal distribution was transformed until distributions were normal, where applicable. For each endpoint upon a significant, or very close to significant (within 0.002) ANOVA result, Tukey's HSD post-hoc test was then used to determine differences between the control group and the treatment groups. Trends were considered with $p \leq 0.08$ and significance at $p \leq 0.05$. Analyses were conducted using JMP Pro 16 software.

3.4. Results

3.4.1. The effect of chronic bisphenol exposure on anxiety-like behavior

The novel tank test is useful as a metric for anxiety because fish that are more anxious spend observably more time at the bottom of a new environment (Blaser and Rosemberg 2012). At 4 months post-fertilization (mpf), F0 female fish exposed to LC10/1000 bisphenol mixture spent more time in the lower portion of the tank than both control and LC10/100 females (Fig. 3.3 A). After ceasing the exposure and testing the same fish again 45 days later (at ~5.5 mpf), the LC10/1000 females still spent significantly more time at the bottom of the tank than the LC10/100 group, but not more than controls. However, the LC10/100 trended toward spending less time than the controls in the bottom half of the tank ($p = 0.0718$) (Fig. 3.3 A). These effects in females did not continue into the subsequent generations. At no point did the exposed F0 males show any changes in behavior (Fig. 3.3 B). However, in offspring of the exposed F0 female crossed with unexposed WT males, F2 males in the LC10/1000 and LC10/100 groups, did spend significantly less time in the lower tank compared to the control (Fig. 3.3 B).

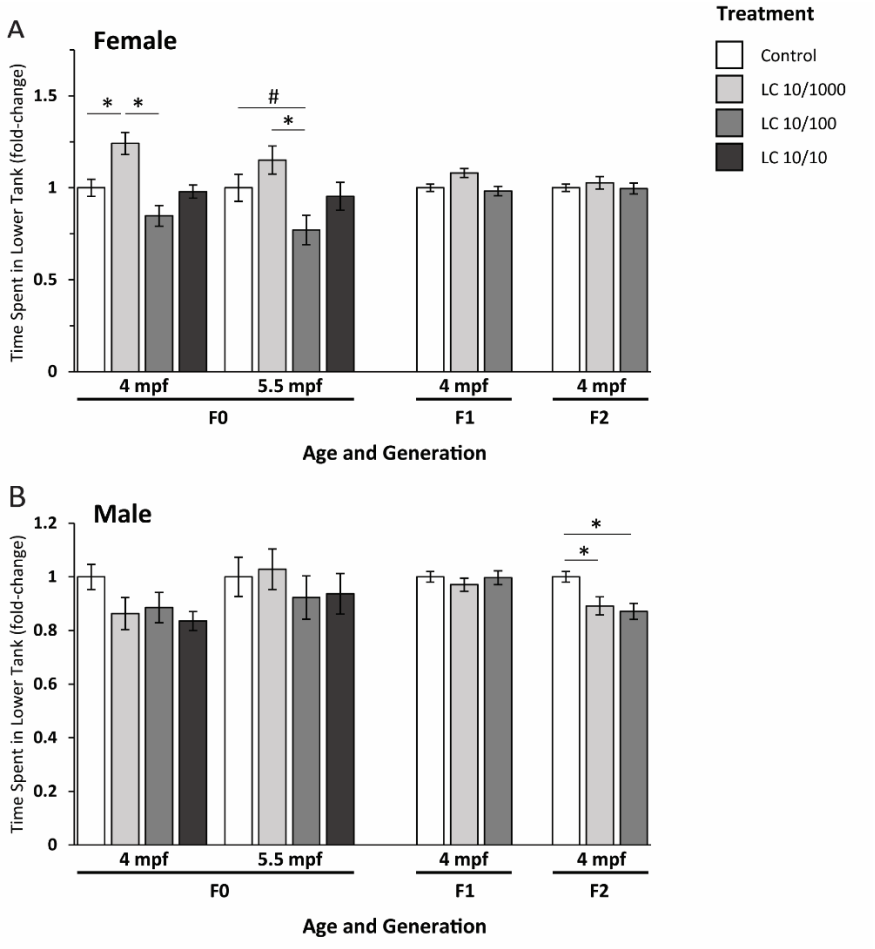

Fig. 3.3: Effects of chronic exposures to bisphenol mixtures of seven bisphenols on anxiety related behavior of zebrafish at 4 months post-fertilization (mpf); and 5.5 mpf in F0 only. Zebrafish larvae were exposed from 2 hours post-fertilization (hpf) to 120 dpf (4 mpf) to mixtures of bisphenols, at fractions of the individual LC10 (LC10/10, LC10/100, and LC10/1000). Experimental fish were individually placed in a novel environment in an enclosed box, exploration was tracked for 10 minutes, and the tendency to remain in the lower half of the tank was measured for each fish. Data is displayed as fold-change to control. All data are shown as mean ± SEM (n= 5-13 recordings/treatment each sex). #$p<0.08$, *$p<0.05$. Comparisons made using One-way ANOVA by respective generation/age group and sex, Tukey's HSD post-hoc test.

3.4.2. The effect of chronic bisphenol exposure on social behavior

The group preference test gave fish freedom to swim toward or away from social stimulus. Bisphenol treatment did not affect the time female fish spent near the shoal tank (Fig. 3.4 A). Males exposed to the LC10/1000 concentration, however, spent significantly less time near the shoaling tank than control fish (Fig. 3.4 B).

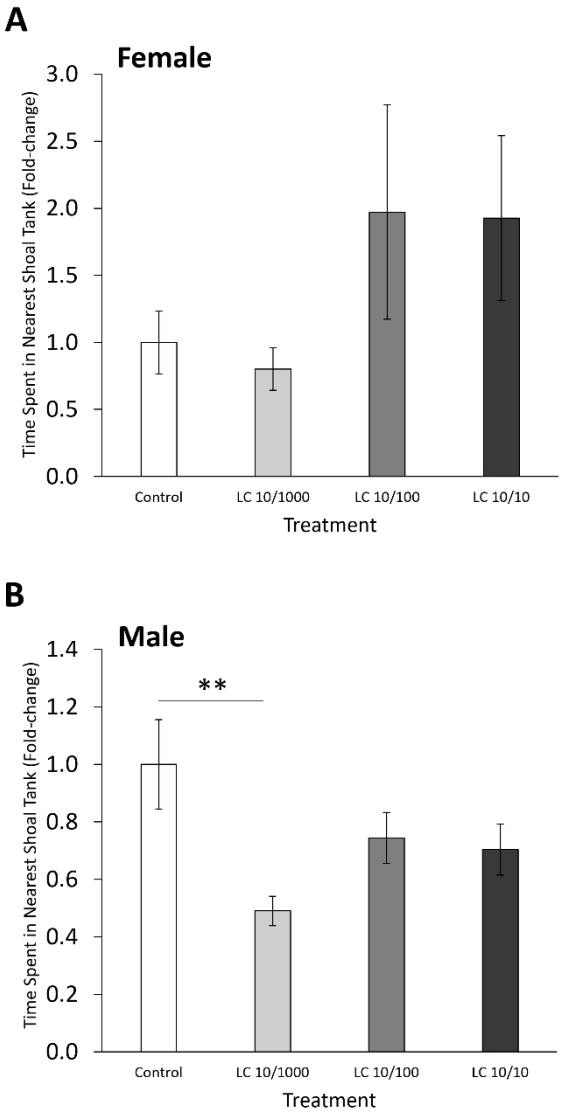

Fig. 3.4: Effects of chronic exposures to bisphenol mixtures of seven bisphenols on social behavior of zebrafish around 5.5 months post-fertilization (mpf) in F0 only. Zebrafish larvae were exposed from 2 hours post-fertilization (hpf) to 120 dpf (4 mpf) to mixtures of bisphenols, at fractions of the individual LC10 (LC10/10, LC10/100, and LC10/1000). Fish were placed in a tank neighboring both an empty tank and a tank with shoaling fish. Time **A)** female fish and **B)** male fish spent nearest the shoal tank was tracked for 10 minutes with and without a partition. Data was analyzed as a ratio of time spent near shoal tank "without partition/with the partition" and displayed as fold-change to control. All data are shown as mean ± SEM (n= 5-13 recordings/treatment each sex). **p<0. Comparisons made using One-way ANOVA by separated by sex, Tukey's HSD post-hoc test.

3.4.3. The effect of chronic bisphenol exposure on female stress axis gene expression

Gene expression studies checked the basal levels of genes associated with the stress response. In F0 females, *crhb* expression at 85 dpf was significantly lower in LC10/10 exposed groups compared to the control and trended toward decreased expression compared to LC10/1000 (p=0.073) (Fig. 3.5 A). However, this difference was not observed at 9 mpf, after the bisphenol exposure had ceased for 3 months, or in subsequent generations. *Pomca* expression was highly variable, and no differences in expression were observed until the F2 generation, where LC10/100 significantly decreased compared to the control (p=0.0445, One-Way ANOVA p-value = 0.0514) (Fig. 3.5 B). Similar to *crhb*, expression of *gr* showed a significant decrease in response to LC10/10 mixture exposure at 85 dpf in F0, but not at 9 mpf (Fig. 3.5 C). Expression of *mr* was significantly lower in LC10/10 fish compared to LC10/1000, and LC10/100 trended toward decreased expression compared to LC10/1000 (p= 0.0789) (Fig 3.4 D). This trend was not present at 9 mpf, however, in F1, fish in both the LC10/1000 and LC10/100 group expressed significantly less *mr* than controls. No changes were seen in *mr* the F2 generation (One-way ANOVA p = 0.089) F2 females (Fig 3.4 D).

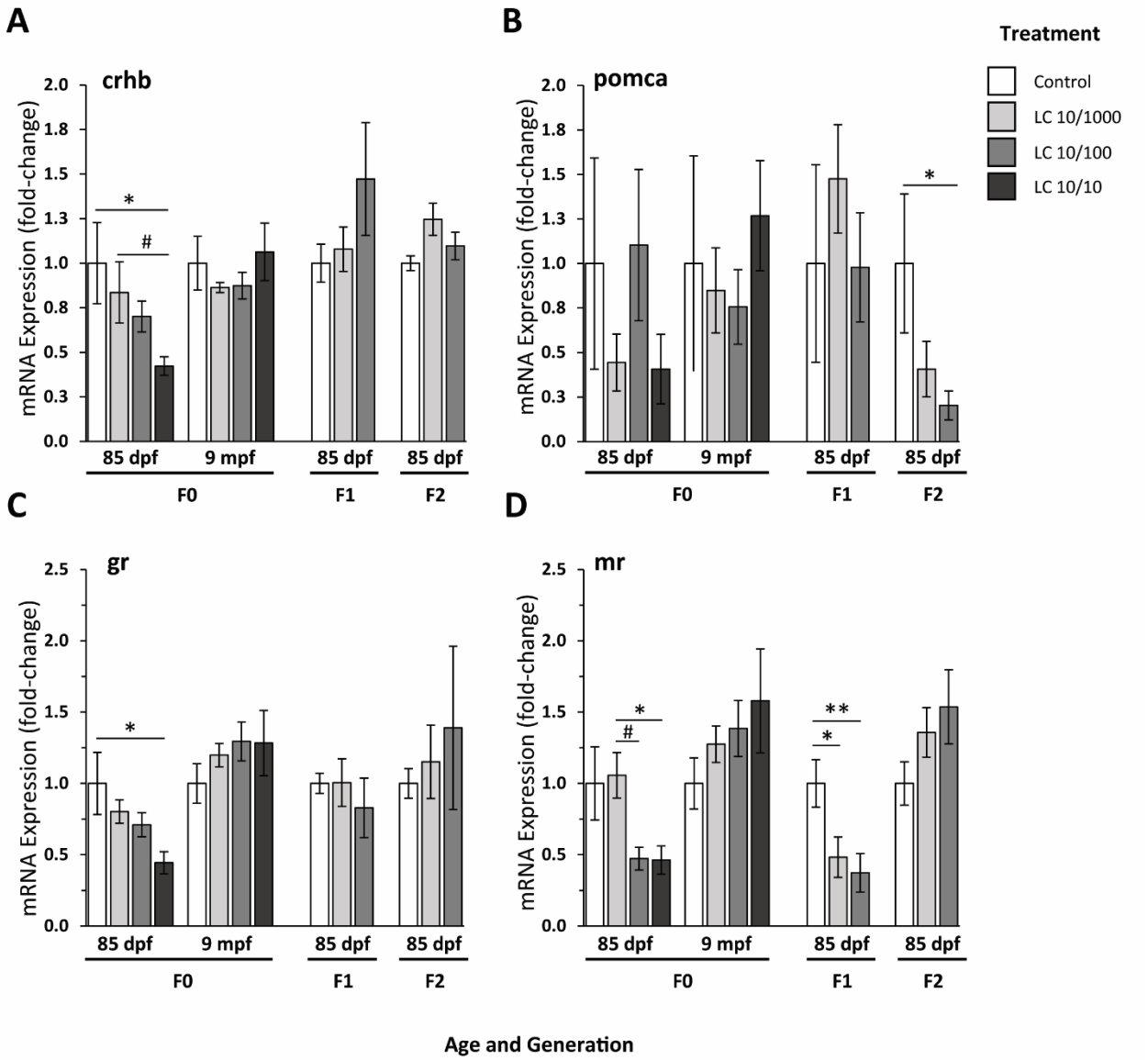

Fig. 3.5: Effects of chronic exposures to bisphenol mixtures of seven bisphenols on gene expression related to hypothalamic-pituitary-adrenal axis in brain/pituitary at 85 days post-fertilization (dpf) and 9 months post-fertilization in female zebrafish. Zebrafish larvae were exposed from 2 hours post-fertilization (hpf) to 120 dpf to mixtures of bisphenols, at fractions of the individual LC10 (LC10/10, LC10/100, and LC10/1000). Expression of **A)** *crhb* (corticotropin releasing hormone b), **B)** *pomca* (proopiomelanocortin a), **C)** *gr* (glucocorticoid receptor), **D)** *mr* (mineralocorticoid receptor) in brain/pituitary were normalized to *b-actin*. All data shown as fold-changes of the control of respective generation/age group as mean ± SEM (n=5-7/sample). #p<0.08, *p<0.05, **p<0.01 (One-way ANOVA by respective generation/age group, Tukey HSD post-hoc test).

3.5. Discussion

Detection of multiple bisphenols in environmental samples has created the need to investigate the effects of bisphenols as a mixture, however most studies investigate individual bisphenols. By incorporating multiple bisphenols in exposure media, this study seeks to create an exposure model that better resembles natural exposures (Chen et al. 2016). The study focuses on understanding the impacts of these endocrine disruptors on the HPA axis by observing fish behaviors and quantifying gene expression associated with HPA axis dysregulation.

Many behavioral endpoints in zebrafish are well established and are useful to determine deviations from a standard in response to chemical exposure. The novel tank test is one example, based on the concept that zebrafish entering an unfamiliar environment will immediately swim the floor of that environment (Collier et al. 2017). This test assessed anxiety-like behavior, as fish that remain in lower portion of the tank for longer correspond to increased anxiety. At 4 mpf, LC10/1000 showed more anxious tendencies than both the control and the LC10/100, showing an example of the non-monotonic nature of endocrine disrupting compound at concentrations so close to one another. In the second iteration of the test, the same fish at 5.5 mpf, both LC10/1000 and the LC10/100 group were less anxious, shown by their decreased time spent in the lower tank relative to controls. This suggests that treatment removal can lead to decreased anxiety. The only other group to exhibit exposure effects was the F2 male fish. These fish are offspring of the exposed F0 females crossed with unexposed WT males and subsequent inbreeding of F1 generation. Both the LC10/1000 and LC10/100 time spent in the lower tank suggested both groups were less anxious and more willing to explore, despite the differences in anxious behavior in their F0 parent. A few other groups observed a range of behaviors after exposure to bisphenols, and there was a mix of anxiogenic and anxiolytic effects.

One study testing a 75-day adult exposure of 1, 10, and 30 µg/L BPS on both male and female zebrafish, found the exposures to have anxiolytic effects when conducting the novel tank test (Salahinejad

et al. 2021). This group did not assess the stress axis, but they attributed the observed changes to oxidative stress and neural pathway disruption because of the antioxidative genes that were reduced upon BPS exposure. Another group saw the opposite effect after exposing zebrafish embryos to BPS for 120 days at 1, 10, and 100 µg/L, fish had increased anxiety in both males and females among each concentration (Wei et al. 2020). They determined that bisphenols affected HPA axis gene expression, with females having upregulated *crhb* and *gr*, but downregulated *mr* and *pomc* (Wei et al. 2020). The changes in gene expression led to increased basal cortisol levels which the group attributed to the behavioral changes observed. The results of the two groups highlighted the range at which bisphenols can affect behavior and gene expression, while demonstrating the importance of how the life stage of an exposure affects the outcome. The latter study also highlights the utility in collecting data on hormone production during investigations of the HPA axis for a more complete picture of endocrine changes (Wei et al. 2020).

Zebrafish are naturally social fish because of the value provided when looking for food and avoiding predators (Miller and Gerlai 2012). The group preference test, also known as the social preference test, is useful in determining if exposures changed the propensity of exposed fish to shoal with others. F0 males in the LC10/1000 group spent comparatively less time near shoaling fish, suggesting they were less social than control fish. This behavior shows the potential impact this mixture could have on fitness of fish who rely on groups for survival. Another investigation also observed increased distance between shoaling zebrafish exposed to 30 µg/L BPS, and consider hesitancy to shoal to be an anxiolytic outcome because of the willingness of treated fish to forgo the benefits of a shoal (Salahinejad et al. 2020). Another group exposing zebrafish to 500 µg/L BPA for 6-months had a similar result (Wang et al. 2015), showing the consistency of bisphenols ability to alter behavior during animal studies. Although males were impacted by this exposure in this present study, only exposed females were bred with unexposed males to produce offspring for subsequent generations. Therefore, without any effects observed in F0 females this assay was only conducted in the F0 generation.

A key endpoint in determining HPA axis regulation is the formation of hormone products. Although the stress response and glucocorticoid levels were not tested in the present chronic bisphenol study, basal levels of key genes involved in the glucocorticoid stress response were. The corticotropin releasing hormone (CRH), originating in the hypothalamus, and adrenocorticotropin releasing hormone (ACTH), released from the pituitary, are two initial hormones involved in the pathway to cortisol production along the HPA axis. The glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) are key receptors found throughout the body that assist in eliciting cortisol stress responses. The downregulation of *crhb*, *gr*, and *mr* after LC10/10 exposure clearly showed that high doses of bisphenols as a mixture are active in disrupting the HPA axis. However, 5 months after the bisphenol exposure ceased, the relative expression of each treatment was comparable to the control at 9 mpf. This demonstrated that the changes in expression of those genes initiated by the bisphenol exposure were not permanent. Despite no longer being significant at 9 mpf, bisphenol exposure still affected each generation.

Cortisol concentration was not measured in the fish of this study, therefore it is difficult to pinpoint specific consequences of these changes in gene expression in relation to systemic cortisol concentrations. However, hypercortisolism is a common condition accompanying decreased GR binding and functionality [reviewed in (Pariante and Miller 2001)] and MR receptor downregulation (Medina et al. 2013). These phenotypes are often experienced in patients with depression [reviewed in (Medina et al. 2013; Pariante and Miller 2001)]. Similarly to depressed patients, chronically increased levels of glucocorticoids have also led to decreased glucocorticoid receptor levels in both rat hippocampi (Tornello et al. 1982) and AtT-20/D-1 mouse pituitary tumor cells (Svec and Rudis 1981), two sites initiating negative feedback of glucocorticoid production. Although we did not measure protein levels, if GR and MR protein levels in our study would have resembled the basal expression levels we observed, it could suggest a similar depression-related condition. There are available examples of changes in protein levels, such as a study where mothers were exposed to 40 µg BPA/kg body weight, offspring exposed *in utero*

and through breast milk exhibited decreased GR protein levels (Panagiotidou et al. 2014). Although depression or anxiety were not measured, it is concerning that GR protein levels are impacted by bisphenol exposure.

The inhibition of *crhb* however, is not consistent with other reports of bisphenol exposure. For instance, another group reported increased *crhb* mRNA expression in zebrafish larvae exposed 10 µg/L and 30 ug/L BPS for 7 days immediately after fertilization (also 1 and 3 µg/L) (Zhang et al. 2017). In their case, they tested individual compounds with different timepoints than those tested in our study. When BPS was exposed to zebrafish from the embryonic stage to 120 dpf, females showed *crhb* increased at 10 µg/L, but not at 1 or 100 µg/L (Wei et al. 2020). Although, it could be possible that the age of the fish plays a role in this difference, even mice fed a high fat diet with or without 50 ug/kg BPA showed increased basal levels of *crhb* (Lama et al. 2023). The three available studies using three different bisphenols, two different model organisms, and exposed at various timepoints, led to increased *crhb* expression. This may mean that the bisphenol mixture may be the cause of *crhb* inhibiting. With both the receptors (*gr* and *mr*) and *crhb* expression low, this may be a sign that the HPA axis function is diminished at multiple levels by the bisphenol mixture.

3.6. Conclusion

The present study demonstrated for the first time that a bisphenol mixture affects the HPA axis at the gene expression level. This was accompanied by changes in behavior that have been closely tied to HPA function. Future studies exploring glucocorticoid hormone production and protein levels will be necessary to determine the specific level at which bisphenols impact the HPA axis. This study also indicates that bisphenol mixtures have potential to lead to changes in the HPA axis at the molecular level and subsequently lead to alteration in how they respond to novel environment and socially interact, and this effect could have long-term implications across generations. Therefore, daily bisphenol mixture exposures through food and the environment should be further investigated for potential impacts on future generations.

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CHAPTER FOUR: EARLY DEVELOPMENTAL EFFECTS OF ACUTE CADMIUM EXPOSURE ON ZEBRAFISH STRESS AXIS

4.1. Abstract

Cadmium is a toxic heavy metal that is naturally found in the environment, and is readily taken up by vegetables and grains, a primary food source for babies and infants. Early-life exposures could negatively impact developing hypothalamic-pituitary-adrenal (HPA) axis due to cadmium's neurotoxic effects and accumulation in the kidneys. To determine if HPA axis dysregulation occurs after acute and early developmental cadmium exposure, larvae were exposed to 0, 0.1, 1, 5, or 50 µg/L cadmium from 24 hours post-fertilization (hpf) to 6 days post-fertilization (dpf). Development, photomotor response, cortisol hormone production, and genes related to the stress axis were measured at 6 dpf. Larvae exposed to 50 µg/L were shorter in length than controls, but cadmium levels did not affect stress response, behaviorally or hormonally. However, cadmium exposure did increase *pomca* in both non-stressed and stressed fish than respective non-stressed and stressed controls. Overall, cadmium exposures minimally affected stress response at these concentrations, however, stunted growth this early in development may mean other components of the fish body may also be undeveloped or grow to be underdeveloped.

4.2. Introduction

Cadmium is one of many heavy metals commonly found in food and environment, and one of high for concern for adverse health effects with early developmental exposures. Primary exposures to cadmium, for non-smoking people removed from industrial areas, comes from food. Cadmium exposures from foods differ from region to region, but consistent sources of cadmium include grains, potatoes, leafy vegetables, and cereals that naturally take up cadmium found in soil (Satarug et al. 2017). This is of particular concern in babies and infants whose diet primarily consists of vegetables and grains. In fact, an investigation reported considerably high levels of heavy metals, including cadmium, in multiple baby food samples (Gardener et al. 2019). Exposure effects of these toxic metals during early development

require thorough investigation, especially considering effects on the brain and body and potential for metals.

Cadmium is a toxic heavy metal known for its widespread effects on multiple organ systems and its very long half-life. Kidneys are of interest because cadmium accumulates highest in this organ (Lech and Sadlik 2017), in both mice (Kollmer 1980) and fish (Woo et al. 1993). Renal tube dysfunction (Buchet et al. 1990) and a range of issues stemming from renal toxicity [reviewed in (Satarug 2018)] result from cadmium exposure. Although the HPG (Tilton et al. 2003) and HPT (Tian et al. 2020) axes are known to be impacted by cadmium exposure, the close proximity of the mammalian adrenal glands and fish interrenal glands to the kidneys makes cadmium accumulation a concern for the HPA axis stress response. Occupational exposures have linked cadmium with the slowing of visuomotor function and deficits in a battery of neurological tests in workers who refined cadmium (Vianene et al. 2000). This heavy metal is a neurotoxin that negatively affects critical aspects of neurogenesis in zebrafish (Chow and Cheng 2003; Chow et al. 2008) and cognition in rats (da Costa et al. 2017), among other issues. Cadmium exposure problematic for both the adrenal (interrenal gland in fish) and the nervous system, this underscores the need to investigate further impacts on the function of the stress axis.

Stimulation of the stress axis is critical for the body to produce cortisol to mount a response to the stressful stimulus. Cortisol is a member of the glucocorticoid hormone family that is responsible for releasing energy stores of glucose and initiating transport of other nutrients to muscles for “fight or flight” reaction. As discussed previously in Chapter 1 and Chapter 3, this glucocorticoid response requires corticotropin releasing hormone (CRH) (Spiess et al. 1981) and adrenocorticotropic hormone (ACTH) (Suda et al. 1992) for cortisol production and glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) for eliciting responses to cortisol, among other components. Examples of effects in adult rainbow trout include acute cadmium exposures (0.75 or 2 µg/L) leading to diminished cortisol stress response (Sandhu et al. 2019), and sub-chronic (Sandhu et al. 2014) exposures (0.75 or 2 µg/L) leading to decreases in GR and MR levels. The psychological changes, including increased aggression under stress

seen in cadmium exposed rats (Tercariol et al. 2011) or increase time responding to predatory threats in zebrafish (Kusch et al. 2008), show that cadmium can also lead to changes in behavior in animals.

The present study will investigate effects of cadmium exposures on the stress axis during early development using zebrafish larvae. Early development is a sensitive period for chemical exposures as the hypothalamic-pituitary-interrenal (HPI; analogous to human HPA) axis in zebrafish is forming from fertilization to 6 dpf (Alsop and Vijayan 2008). This investigation will determine whether acute exposures early in life impede zebrafish larvae's ability to respond to stressors. It was hypothesized that acute exposures would dysregulate the stress response in a way that prevented zebrafish from responding to stressors. Changes in gene expression of hormones along the stress axis: *crhb*, *pomca*, *cyp11c1*, *gr*, and *mr* were determined using real-time qPCR as changes to these genes are likely to affect cortisol production and/or stress response. Cortisol production was measured using an ELISA assay in both non-stressed and stressed larvae. The photomotor behavior responses were used to gauge startle responses in larvae by mean velocity during assay. The cadmium exposure did not significantly impact the stress response, and at 50 µg/L led to stunted growth and induction of basal *pomca* expression.

4.3. Materials and Methods

4.3.1. Animals:

Zebrafish embryos were obtained by either placing spawning inserts into a 10 L tank containing sexually mature wild-type breeders (in 2:1 female to male ratio) raised in the Cornell University (Ithaca, NY, USA) aquatic facility or by transferring breeders into a separate 2-L breeding tanks (2:1 female to male ratio) with artificial plants placed in them. These breeder fish were fed with *Artemia nauplii* and fish flakes (TetraMin, Tetra USA, Blacksburg, VA) twice a day, and kept under a 14h light: 10h dark photoperiod. Water temperature was maintained at 28 ± 1 °C, conductivity at 550 ± 150 µS, and at a pH of 7.4 ± 0.2. Water quality measurements taken weekly to ensure ammonia, nitrate, and nitrite were at safe levels. All embryos were kept separately in petri-dishes containing embryonic culture media (E3)

media following the egg collection in the same room as the breeders during the experiments. All fish maintenance and experimental protocols were approved by the Institutional Animal Care and Use Committee at Cornell University.

4.3.2. Chemicals:

Cadmium chloride ($CdCl_2$) selected for this study was purchased from Sigma-Aldrich (St. Louis, MO). $CdCl_2$ stock solutions were diluted to create the working solutions used for the treatments. These working solutions were made up of embryonic culture media (E3; 5 mM NaCl, 0.18 mM KCl, 0.33 mM $CaCl_2 \cdot 2H_2O$, 0.40 mM $MgCl_2 \cdot 6H_2O$, pH 7.2) and various concentrations of $CdCl_2$. Solutions were remade every 2 or 3 days and were protected from light until used to expose larvae.

4.3.3. Exposure Study:

After embryos were collected, they were dechorionated using Pronase® (Sigma, St. Louis, MO; 0.1 mg/mL for 18 min) at 4 hours post fertilization (hpf), rinsed repeatedly, and 100 healthy embryos were randomly distributed into (100 x 15mm) glass petri-dishes containing preheated E3 media. At 24 hpf, healthy larvae were transferred into petri-dishes with preheated solutions of E3 with no $CdCl_2$, 0.1 µg/L, 1 µg/L, 5 µg/L, or 50 µg/L $CdCl_2$ (n=6 replicate dishes/treatment; 60 larvae/dish). Treatment solutions were changed daily, and dead embryos were removed.

4.3.4. Tissue Sampling:

At 6 dpf between 12 pm and 4 pm, larvae from each treatment were collected for cortisol and mRNA extraction. Whole larvae were gently dispensed into a 1.7 mL microfuge tube with a transfer pipette. From each plate, two sets of 12 larvae (non-stressed and stressed) were sampled, one for mRNA extraction, one for cortisol extraction. Half of the larvae were moved from the petri-dish into a nursery tube within a 1000 mL beaker with ~100 mL of E3 media, then swirled for 1 minute on an orbital shaker at 150 rpm, and were collected into microfuge tubes 10 minutes after the stressor. In each case, all media was removed from the microfuge tubes containing 12 larvae, and the larvae were flash frozen in liquid

nitrogen. Sampling from collection to flash freezing larvae was complete within 2 minutes. Larvae were kept in -80 °C freezer until extraction.

4.3.5 Length Measurements:

At 6 dpf, 36 larvae were sampled from each treatment, selecting the same number of fish from each petri-dish, and anesthetized in MS-222. While anesthetized they were gently pulled into capillary tubes and the tubes were placed in a petri-dish and visualized under a Zeiss Stemi 305 dissection microscope. Photos were taken using Axiocam 105 color and the lengths of the larvae were measured using ZEN 2.3 lite (Carl Zeiss Microscopy, White Plains, NY).

4.3.6. Larval Behavior:

At 6 dpf between 12 pm and 4 pm, larvae movement was tracked using DanioVision (Noldus, Leesburg, VA). From each petri-dish, 3 larvae were placed into each 9x9 mm well (with 200 µL exposure media) of a 96-well plate and allowed to sit in the well for ~1 hour. All larvae were tracked under alternating light and dark cycles, starting with 10 minutes of dark acclimation followed by three sets of 10 minutes light and 10 minutes dark (70 minutes total). During the recording, the environment was kept at 28.5 ± 0.5 °C.

4.3.7. ELISA Assay:

Frozen larvae (containing 12 larvae) were transferred into a 700 µL tube (source) containing 250 µL chilled 1x PBS buffer with 0.7 mm zirconia beads, and immediately homogenized using a Tissue Lyser II (Qiagen, Valencia, CA) for 10 seconds at 30 beats/sec, allowed to rest on ice briefly, and homogenized a second time. The homogenates were pipetted into separate 1.7 mL microfuge tubes and tube/beads were then rinsed with additional 250 µl PBS, resulting in total 500 µl homogenate in 1x PBS. Subsequently, 800 µL of diethyl ether was added to each 500 µL homogenate, vortexed for 10 sec, and centrifuged at 2000 g for 5 minutes. The organic layer containing cortisol was then separated into another separate 1.7 mL tube. This extraction process was repeated 5 times per sample. Between each extraction,

the diethyl ether was allowed to continuously evaporate in a 45 °C heat block and upon the final extraction, samples were kept for an hour underheat block at 45 °C for complete evaporation.

Using 500 µL of ELISA buffer, made according to instructions from the Cayman Chemical: Cortisol ELISA Kit (Item No. 500360; Ann Arbor, MI USA), was added to each dry tube with extracted cortisol. The ELISA assay was performed according to the manufacturer's instructions. Each 96-well plate was read at 414 nm after about 1 hour and 25 minutes. Cortisol content in pg/mL was calculated based on the 414 nm reads using the Cayman Chemical Analysis tools (https://www.caymanchem.com/analysisTools/ELISA).

4.3.8. Gene Expression:

Frozen larvae homogenates were homogenized in 500 µL of TRIzol reagent (Invitrogen) with 0.7 mm zirconia beads in a 700 µL tube using a Tissue Lyser II (Qiagen, Valencia, CA) for 10 seconds at 30 beats/sec and allowed to rest on ice briefly before being homogenized a second time. Total RNA was extracted from frozen tissues according to TRIzol reagent manufacturer's instructions. All samples were reversed transcribed using a high-capacity reverse transcription kit (Applied Biosystems) and Oligo$(dT)_{18}$ primers (Thermo Fisher Scientific). Each qPCR reaction well contained 10 µL and included: 2 ng total RNA equivalent of cDNA template, containing 0.3 µM forward and reverse primers, SYBR Select Master Mix (Applied Biosystems), and 0.2 µm filtered MilliQ water. Quantstudio 6 Flex Real-Time PCR System (Applied Biosystems) was used to determine C_t (threshold cycle) value. Amplicon amount was calculated using the standard curve method. Target genes *crhb*, *pomca*, *cyp11c1*, *gr*, and *mr* 6-dpf larvae were normalized to *rpl13a*.

Table 4.1

Gene Name (Abbreviation)	Primer Sequence
Corticotropin releasing hormone (*crhb*)	F: 5'-AGCAACTAGAAGACAGACGC-3' R: 5'-CTACATTCATACGGCGGTGG-3'
Proopiomelanocortin (*pomca*)	F: 5'-TTGGCTCTGGCTGTTCTCTG-3' R: 5'-GATGGCTTTCTCCGGGGTAG-3'
11-beta-hydroxylase (*cyp11c1*)	F: 5'-CGCAGGATAGCAGAGAACG-3' R: 5'-CTGAAGGTGATTCTCGGTGG-3'
Glucocorticoid receptor (*gr*)	F: 5'-TAAAAGGGCTGTTGAAGGGC-3' R: 5'-ATTACCTTTCCGGCTTGACG-3'
Mineralocorticoid receptor (*mr*)	F: 5'-TGCCACTACGGGGTTGTTAC-3' R: 5'-GTGCCCCAAGATTCATCCCA-3'

4.3.9. Statistics:

To determine if there are differences between experimental groups for behavioral tests, one-way ANOVA was conducted separately among stressed and non-stressed larvae. To determine if there are differences between stressed and non-stressed groups for photomotor response, cortisol production, or stress axis, contrast mean comparisons were conducted with a Bonferroni correction. Data not fitting a normal distribution was log transformed. Upon a significant ANOVA result, Tukey HSD post-hoc test was then used to determine differences between the experimental groups. Data were considered significant at $p \leq 0.05$. Analyses were conducted using JMP Pro 16 software.

4.4. Results

4.4.1. Effects of acute cadmium exposure on larval length

Larval length measurements (n = 38 larvae/treatment) were taken at 6 dpf to determine if acute exposure to cadmium led to any deficits in early development. Mean body length ± SEM for the larvae were 3.94 cm ± 0.013 (control), 3.92 cm ± 0.018 (0.1 µg/L), 3.92 cm ± 0.018 (1 µg/L), and 3.89 cm ± 0.020 (5 µg/L), and 3.84 cm ± 0.027 (50 µg/L). Larvae exposed to 50 µg/L had significantly shorter body lengths than controls (p=0.0021), 0.1 µg/L (p=0.0313), and 1 µg/L (p=0.0243) according to the Tukey's HSD post-hoc test.

4.4.2. Effects of acute cadmium exposure on photomotor response

The photomotor response assay focused on larval swimming velocities during both the light and dark periods and was used to understand behavioral consequences of cadmium exposure on startle response. There were no changes in mean velocity during the light or dark periods when comparing the treatments (Fig. 4.1).

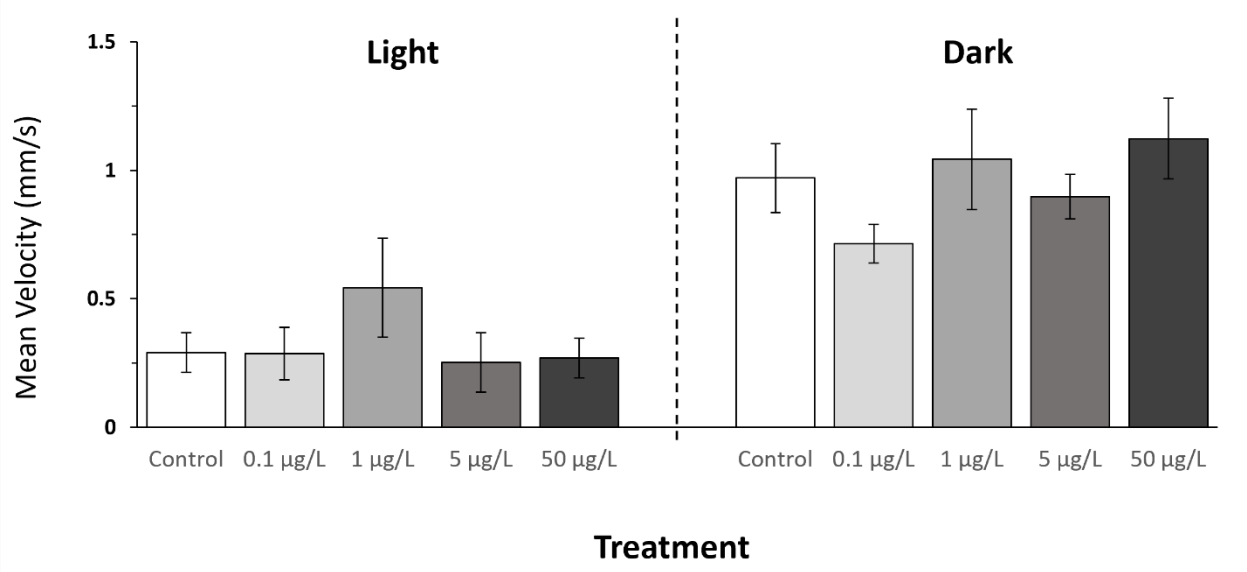

Fig. 4.1: At 6 days post-fertilization (dpf), effects of acute cadmium exposure on larval mean velocity (mm/s) was averaged across three transitions from light to dark. Each light/dark period was 10 minutes. Zebrafish larvae were exposed from 24 hours post-fertilization to 6 dpf to cadmium chloride. Data is displayed as. All data are shown as mean ± SEM (n = 18 larvae/treatment and stress group). Comparisons made using One-way ANOVA by respective light/dark period.

4.4.3. Effects of acute cadmium exposure on CORT response

Cortisol hormone was measured from treatment group to determine if cadmium diminishes an appropriate response to stress. Comparing non-stressed and stressed fish among the different exposures, each exposure's stressed larvae had significantly higher cortisol compared to the non-stressed group, apart from 5 µg/L exposed larvae which had a trend toward and increase (p-value 0.087). When comparing basal cortisol levels, there was no difference among the treatments. This was also the case comparing treatment cortisol levels after stress response.

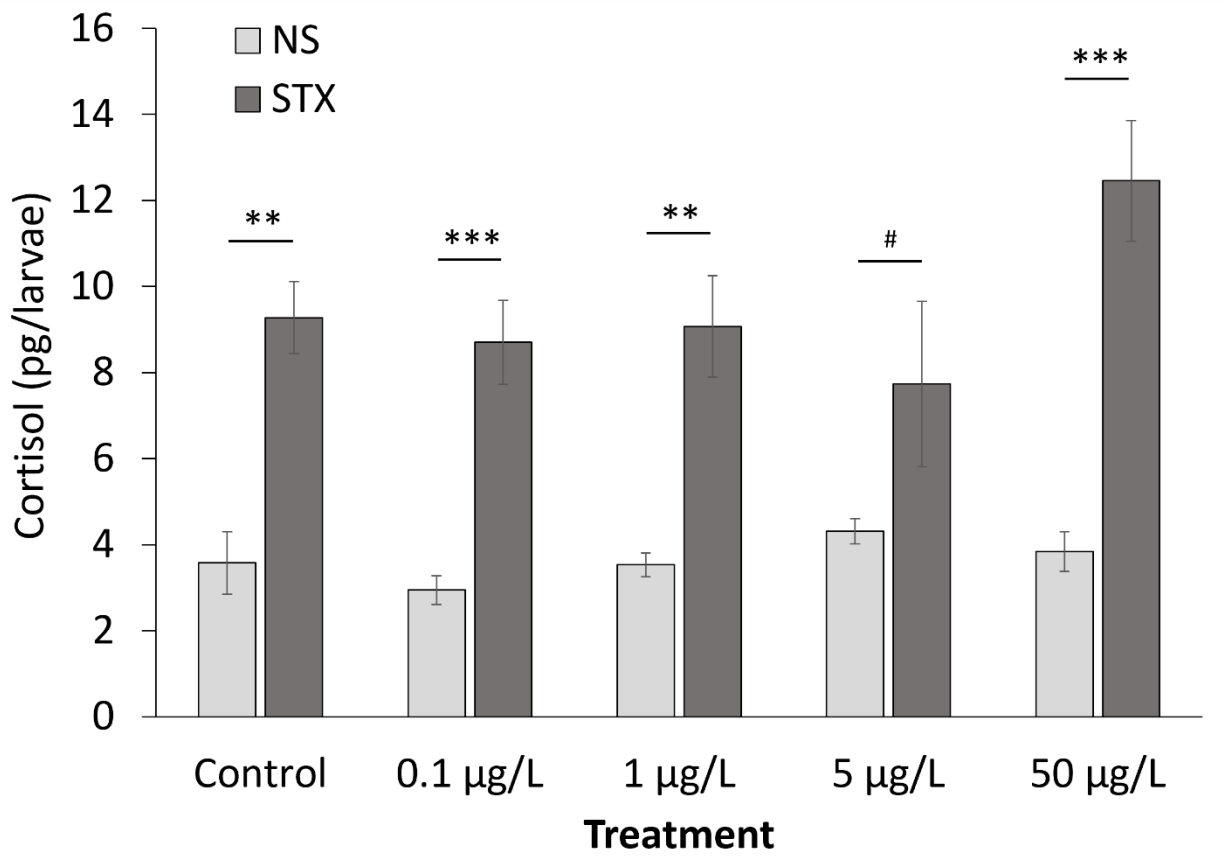

Fig. 4.2: At 6 days post-fertilization (dpf), non-stressed (NS) and stressed (STX) fish were flash frozen and their cortisol was extracted to test effects of acute cadmium exposure on cortisol production. Zebrafish larvae were exposed from 24 hours post-fertilization to 6 dpf to cadmium chloride at 0.1, 1, 5 and 50 µg/L. All data shown as mean pg cortisol per larvae ± SEM (n = 6 homogenates of 12 larvae/treatment and stress group). Comparisons made by One-way ANOVA by respective stress group; Contrast comparison between NS and STX each treatment with Bonferroni correction (#p<0.1, **p<0.001, ***p<0.0005).

4.4.4. Effects of acute cadmium exposure on HPI axis

The effect of cadmium exposure on key genes used in regulating stress response were measured in larvae homogenates. Among all the genes tested, proopiomelanocortin a (*pomca*), prohormone for adrenocorticotropic hormone (ACTH), was the only gene significantly affected by the cadmium exposure. Both stressed and non-stressed larvae exposed to 50 µg/L, had significantly higher *pomca* expression compared to the controls overall, although the stressed and non-stressed larvae were not significantly different from each other in this group. Apart from *pomca*, the non-stressed versus stressed treatment was not affected among the genes, nor did expression change from among the different exposures.

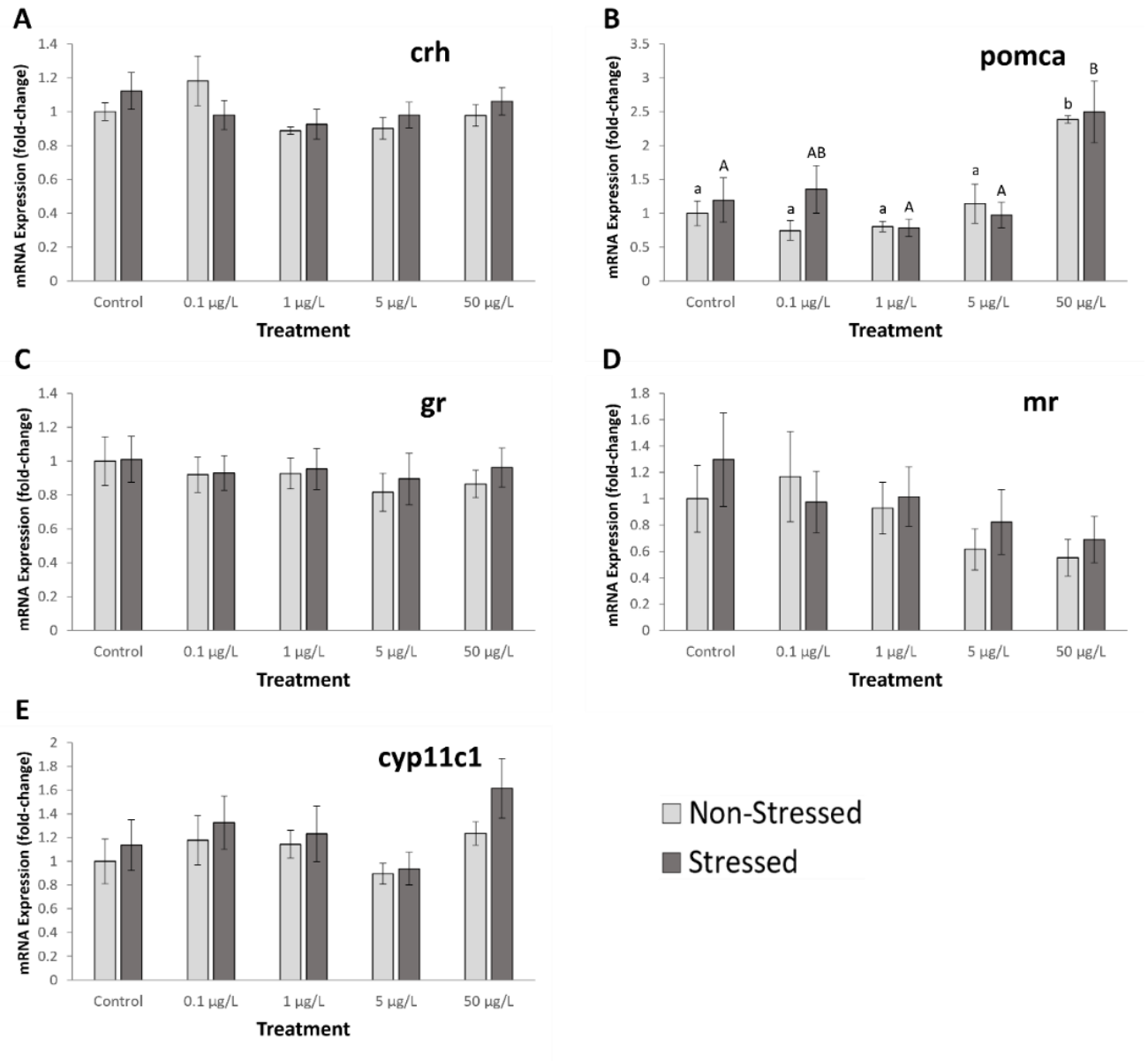

Fig. 4.4: At 6 days post-fertilization (dpf), effects of acute cadmium exposure on gene expression related to hypothalamic-pituitary-adrenal/interrenal axis in larval homogenates. Zebrafish larvae were exposed from 24 hours post-fertilization to 6 dpf to cadmium chloride at 0.1, 1, 5 and 50 µg/L. Sample fish were either Non-stressed (NS) or stressed (STX). Expression of **A)** *crhb* (corticotropin releasing hormone b), **B)** *pomca* (proopiomelanocortin a), **C)** *gr* (glucocorticoid receptor), **D)** *mr* (mineralocorticoid receptor), **E)** cyp11c1 (cytochrome P450 11c1) normalized to *rpl13a*. All data shown as fold-changes of the NS control group as mean ± SEM (n = 6 homogenates of 12 larvae/treatment and stress group). One-way ANOVA by respective stress group, followed by Tukey HSD post-hoc test. Treatments not connected by the same letter are significantly different, lowercase for NS, uppercase for STX ($p < 0.05$)

4.5. Discussion

Cadmium is an abundant environmental contaminant, and although industrial areas may have higher environmental cadmium levels, the general population is also exposed. Early-life exposure of babies and infants is a concern as their brains are in the early stages of development. The focus for this paper is whether short term cadmium exposures negatively impact somatic development and stress response during early stages of development.

Exposure to cadmium led to significant changes to larval length where fish exposed to 50 µg/L concentration were shorter in body length. Another zebrafish study exposed larvae to a slightly higher 56.2 µg/L cadmium exposure, and larvae also had shorter body lengths as early as 4 dpf (Di Paola et al. 2022). Zebrafish exposed to 38 µg/L, on the other hand, had no change in fish length at 10 dpf (Kim et al. 2011). Although on the surface level this suggests a lower concentration is safer, catfish exposed to 6 µg/L for 6 months, developed shorter body lengths from 3 months up to 5 months (Paul and Small 2021). This stunted development is not restricted to animal studies. Increased cadmium levels in mothers in North Carolina were associated with lower birth weights in humans (Johnston et al. 2014). Of three blood cadmium concentrations groups (low ≤0.28 mg/L, medium 0.29–0.49 mg/L, high 0.50 – 2.52 mg/L), the high group, ≥ 0.50 µg/L, had significantly more infants of lower birth weights according to gestational age. Cadmium's association with impaired development at 50 µg/L cadmium is not considered environmentally relevant, however the outcome was still relevant in human outcomes in terms of this endpoint.

The photomotor response assay is a test of the neuroactivity and startle response (Basnet et al. 2019). At 6 dpf, the distance zebrafish travel in a given interval increases when their environment goes from light to dark (shown in the results as "Dark" phase), and fish are less active when the light returns (MacPhail et al. 2009). It is postulated sudden darkness marks the presence of a predator, leading to a startle response, and increased activity. The differences are typically measured during the dark phase, when the startle response is initiated, but in the case of anxiolytic drug diazepam, this movement is not

initiated (Kokel et al. 2010). The assay in this study demonstrated that there were no differences in mean velocity in any larvae due to cadmium exposure during the light period or dark period. These results suggest the zebrafish behavioral stress response was maintained despite the cadmium exposures.

Zebrafish larvae in another investigation exposed to cadmium between 15.33 µg/L to 61.32 µg/L had decreased mean velocity compared to controls during the period of darkness (Han et al. 2019). This may have been because our light transitions were every ten minutes, whereas their transitions were every five minutes. Han et al. associated the decreases in mean velocity they saw with the underdeveloped otoliths they observed. The otolith structure aids in balance and movement (Riley and Moorman 2000), and the group suggested its underdevelopment affected the fish's ability to swim at the higher speeds.

Cadmium exposures conducted in zebrafish larvae have not studied cortisol production. In this study, an increase in cortisol was observed similarly in all experimental groups after being subject to swirling stress. This is another example of acute cadmium exposure not affecting the cortisol stress response. Chronic cadmium exposure affects the cortisol response, in some species. For example, a study exposing rainbow trout to 0.75 and 2.0 µg/L cadmium chloride for 28 days found that exposed fish had significantly lower cortisol levels before stressor and significantly lower cortisol levels after an hour after an acute stressor compared to the respective controls (Sandhu et al. 2014). In rare minnow, a five week exposure to 25 and 100 µg/L led to significant increases in basal cortisol levels compared to controls as well (Liu et al. 2017). The length of exposure may therefore be a factor, demonstrating that the acute exposure conducted during this study may not have been long enough to see such effect in zebrafish, or the larvae were not old enough to see the adverse effects of the acute 6-day exposure.

Risks toward cortisol production were largely unseen, however this may be a result of changing gene expression to support appropriate cortisol production. For this reason, genes critical to cortisol production were assessed. Hypothalamic CRH and pituitary ATCH (coded by *pomca*) are responsible for the hormonal initiation of cortisol production upstream of the adrenal glands. Cyp11c1 is the final enzyme in the pathway in the production of cortisol. Lastly, GR and MR are both receptors that elicit cell specific

responses as a result of cortisol expression. Stress did not affect any of the genes in question compared to non-stressed samples, however *pomca* levels were significantly higher after 50 µg/L exposure than respective stressed and non-stressed samples of other experimental groups. Despite *pomca* levels being the same for both stressed and non-stressed groups, the cortisol production in both of these groups was not affected, meaning cortisol production does not strictly correlate with *pomca* levels in this case. This was also seen in a relatively high 2 mg/L cadmium exposure to adult rare minnow fish, where *pomca* expression and ACTH serum concentrations were the same after an acute stressor, despite there being changes to serum cortisol compared to control fish (Liu et al. 2016). POMC is a prohormone that is cleaved into multiple hormones of various functions, not just ACTH peptide, but also opioid peptides, melanotropic peptides, among others (Hadley and Haskell-Luevano 1999). Therefore, although *pomca* expression is increased, ACTH production itself, still depends on how the prohormone is cleaved, perhaps leading to high expression of *pomca* but ACTH levels do not differ from controls.

4.6. Conclusion

The purpose of this study was to determine if acute cadmium exposure leads to impairment of cortisol production, abnormal behavior, or gene expression. This is the first study to investigate these endpoints in zebrafish conducting exposures starting near fertilization. Overall, there were no signs that this cadmium exposure significantly affects the endpoints tested in zebrafish. However, raising larvae to adulthood after early-life exposure may reveal additional consequences of this exposure that are not yet developed.

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CHAPTER FIVE: CONCLUDING REMARKS AND SUGGESTIONS FOR FUTURE RESEARCH

The overall purpose of this study was to understand exposure effects of endocrine disrupting compounds (EDCs) on the reproductive and stress endocrine axes. Chapter 2 does this by focusing on bisphenol exposure effects on reproduction related endpoints in both acute and chronic exposures. This chapter also discusses the transgenerational effects in offspring of chronically exposed fish. Chapter 3 also reports the effects of the chronic bisphenol mixture and transgenerational effects but with the focus on the hypothalamic-pituitary-interrenal (HPI) axis responsible for the hormonal stress response. Chapter 4 aimed to determine if acute cadmium exposure impacted the stress response in zebrafish larvae. In larvae, we found that acute exposure to non-lethal doses of bisphenol mixtures showed signs of having additive or synergistic effects in some genes. Chronic exposure in the F0 generation led to male skew and impaired reproductive capacity in the highest exposure concentration tested, but not in fish at the environmentally relevant concentration. HPI axis related genes were disrupted in F0 at the highest mixture concentration, and the subsequent generations showed effects in the lower exposure concentrations. Fish displayed anxiety-like behavior at the environmentally relevant concentration F0 adult female fish, reduction in anxiety-like behavior in F2 adult male offspring at environmentally relevant concentrations. Although the highest dose of the cadmium exposure showed developmental deficits, the stress responses, stress hormone production, and stress axis gene expression was largely unaffected among the treatments.

This study laid an important foundation for future studies investigating endocrine disruption endpoints using bisphenol mixtures and low levels of cadmium on the HPI axis in larvae. This was the first study to investigate this complex mixture of bisphenols in an animal model, or the effects of cadmium exposure on stress response in zebrafish larvae. The larval bisphenol mixture exposure was shown to augment effects of the individual bisphenols in some instances, although it was not consistently the case. Additionally, early-life exposures effects were not indicative of the effects seen in older fish, as seen when comparing the acute and chronic bisphenol exposures. In the chronically exposed fish from the bisphenol study, the environmentally relevant concentrations did not often affect the F0 generation but

did in the highest concentration in both reproductive and stress axis endpoints. However, effects were observed in the F2 generation in the lower concentrations with both axes. The acute cadmium exposure, however, did not lead to expected changes in the stress axis or stress response, despite other aspects of development being impacted.

Future investigations in larvae should pursue stress axis gene expression in both individual and mixture bisphenol exposures. This would determine if expression of genes associated with another hormonal axis, such as the HPA axis, shows signs of synergistic effects, and if acute exposure studies have a similar effect on the stress axis as seen in the chronic exposures. The infertility seen in the highest bisphenol dose should be explored further, perhaps starting with gauging the differences in gonadotropin releasing hormone (GnRH) neuron pulsatility compared to corticotropin releasing hormone (CRH) peptide production. Also, the dysregulation of the stress axis in response to the bisphenol exposure should be further investigated by quantifying cortisol during a stress responsive assay, to determine if the dysregulation is also reflected in the hormone response. Lastly, the larvae acutely exposed to cadmium should be raised into adulthood to determine if the absence of a response is dependent on life-stage of the fish, and if effects appear as fish age.

Zebrafish are a versatile animal model to investigate endocrine disruption. These fish produce the same hormones as humans do for reproduction and stress response. Behavior patterns have also been established that allow researchers to test for any changes that may result from endocrine disruption. The vast knowledge of the zebrafish genome also allows researchers to manipulate gene expression, which would be helpful to further elucidate any impacts that changes in the stress axis may have on the reproductive axis. Lastly, zebrafish development happens outside the mother, which allows manipulation of the embryonic environment so transgenerational effects can be determined in the F2 generation rather than waiting until the F3 generation as is required mammalian models.