DEVELOPMENTAL AND REPRODUCTIVE EFFECTS OF 2-(2H-BENZOTRIAZOL-2-YL)-4-METHYLPHENOL (UV-P) ON THE MODEL FISH SPECIES, ZEBRAFISH (*DANIO RERIO*), FOLLOWING EMBRYONIC EXPOSURE

YAMIN RAZA
Bachelor of Science, University of Lethbridge, 2022

A thesis/project submitted
in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGICAL SCIENCES

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

DEVELOPMENTAL AND REPRODUCTIVE EFFECTS OF 2-(2H-BENZOTRIAZOL-2-YL)-4-METHYLPHENOL (UV-P) ON THE MODEL FISH SPECIES, ZEBRAFISH (*DANIO RERIO*), FOLLOWING EMBRYONIC EXPOSURE

YAMIN RAZA

Date of Defence: December 1, 2023

Dr. S. Wiseman Thesis Supervisor	Associate Professor	Ph.D.
Dr. N. Thakor Thesis Examination Committee Member	Professor	Ph.D.
Dr. M. Bogard Thesis Examination Committee Member	Assistant Professor	Ph.D.
Dr. D. Yevtushenko Chair, Thesis Examination Committee Member	Associate Professor	Ph.D.

DEDICATION

I would like to dedicate this thesis to my parents, Naila and Ahmad, who both have supported me in all my endeavours and taught me importance of hard work.

ABSTRACT

Benzotriazole ultraviolet stabilizers (BUVSs) are emerging contaminants of concern. They are added to a variety of products including building materials, personal care products, and paints to prevent degradation caused by UV light. Despite widespread occurrence in aquatic environments, little is known regarding effects of BUVSs on aquatic organisms. The aim of this study was to characterize effects of exposure to 2-(2H-Benzotriazol-2-yl)-4-methylphenol (UV-P) on reproductive success of adult zebrafish (*Danio rerio*) following embryonic exposure. Zebrafish embryos were exposed, by use of microinjection, to UV-P at a control, low, and high dose, then reared until sexual maturity, where reproductive performance was assessed, following which molecular and biochemical endpoints were analyzed. Exposure to UV-P did not have a significant effect on fecundity. However, there was a significant effect on fertilization success. Using UV-P exposed males and females, fertility was decreased in the low and high treatments, relative to control. Additionally, in a reproduction assay with UV-P exposed males and control females, fertility was decreased in the high treatment, relative to the control. Embryonic exposure to UV-P might have perturbed male sex steroid synthesis as indicated by small changes in blood plasma concentrations of E2 and 11-KT, and small decreases in mRNA abundances of *cyp19a1a*, *cyp11c1*, and *hsd17b3.* Additionally, decreased transcript abundances of genes involved in spermatogenesis was observed. Decreases in later stages of sperm development were observed, suggesting that embryonic exposure to UV-P impaired spematogenesis, resulting in decreased sperm quantity. This study is the first to demonstrate latent effects of BUVSs, specifically on fish reproduction.

CONTRIBUTION OF AUTHORS

The candidate is the main author of chapters 1-3. The candidate primarily designed/conducted experiments and analyzed data stated in chapter 2. Dr. Steve Wiseman and Dr. Jon Doering provided guidance and scientific input for chapters 1-3. Dr. Zhe Lu developed and conducted the method of chemical analysis in chapter 2. Emily Mertens and Lauren Zink both contributed to data collection and analysis in chapter 2.

Chapter 2 has been accepted in Environmental Toxicology and Chemistry as Raza, Y., Mertens, E., Zink, L., Lu, Z., Doering, J. A., & Wiseman, S. (2023). "Embryonic exposure to the benzotriazole UV stabilizer, UV-P, decreases fertility of adult zebrafish (*Danio rerio*)."

ACKNOWLEDGEMENTS

I would firstly like to express my gratitude to my supervisor Dr. Steve Wiseman for providing me with endless opportunities, unwavering support, and guidance as an undergraduate and graduate student. Your constant mentorship has not only propelled me to become a better researcher, but also contributed tremendously to my academic and personal development. Since starting in your lab four years ago, I have grown from being a quiet undergraduate student to someone who is able to confidently make decisions and conduct meaningful research – which is largely due to the time you have invested in me, for which I am truly appreciative. I would like to acknowledge my thesis committee members Dr. Nehal Thakor and Dr. Matt Bogard for all their feedback and encouragement. I also want to thank all the individuals involved in the Research Internship Concentration, specifically Dr. Elizabeth Schultz, for providing me with an opportunity to get involved with research, as well as a safe space to learn and grow.

I would like to acknowledge the past and current members of the Wiseman lab, for their continuous support, expertise, and assistance over the years. Although there are many highs and lows associated with research, we all tackled the challenges as a team and shared lots of laughs. You all have made my research experience truly enjoyable and your willingness to always lend a helping hand was very much appreciated. I would like to extend a big thank you to the staff in the Aquatic Research Facility for their efforts, kindness, and help with experimental setup and design. Also, I want to thank the wonderful friends I have made throughout my time at the UofL. Chloe, Emily, Kirsty, and Jaxon, thank you for all the support, guidance, and fun times throughout the years. I will always look back fondly on the experiences we had together.

Finally, I would like to thank my family for their unconditional love and support. To my siblings, Hassan and Sarah, I am extremely grateful to have the two of you as such wonderful role models. To my parents, you are the sole reason that I am where I am today. To my dad, Ahmad, you have taught me the meaning of hard work and have encouraged me to pursue my dreams. To my mom, Naila, you have been my biggest fan and supporter throughout the years. You both have provided me with guidance, celebrated my accomplishments, and consoled me during difficult times, all for which I cannot thank you enough.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

11-KT	11-ketotestosterone
A_{diff}	Differentiated type A spermatogonia
AhR	Aryl hydrocarbon receptor
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
AO	Adverse outcome
AON	Adverse outcome network
AOP	Adverse outcome pathway
AR	Androgen receptor
A_{und}	Undifferentiated type A spermatogonia
BP3	Benzophenone-3
BPA	Bisphenol A
bpm	Beats per minute
BUVS	Benzotriazole ultraviolet stabilizer
CYP11a	Cytochrome P450 cholesterol side-chain cleavage enzyme
CYP11C1	11b-hydroxylase
CYP17a	17a-hydroxylase
CYP19a	Aromatase
CYP1A	Cytochrome P450 1A
DEHP	Bis-(2-ethylhexyl) phthalate
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
DOHaD	Developmental origins of health and disease hypothesis
dpf	Days post-fertilization
E1	Estrone
E2	17b-estradiol

EDC	Endocrine-disrupting chemical
ELISA	Enzyme-linked immunosorbent assay
ELS	Early life-stage
EPA	United States Environmental Protection Agency
ER	Estrogen receptor
F-53B	6:2 chlorinated polyfluorinated ether sulfonate
F1	First filial generation
F2	Second filial generation
FLU	Flutamide
FSH	Follicle-stimulating hormone
FSHR	Follicle stimulating hormone receptor
GC-MS	Gas chromatography-mass spectrometry
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptors
GPCR	G-protein coupled receptor
GSI	Gonadosomatic index
HAT	Histone acetyltransferase
Hg	Mercury
hpf	Hours post-fertilization
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
HSD11b2	11b-hydroxysteroid dehydrogenase 2
HSD17b3	17b-hydroxysteroid dehydrogenase 3
HSD3b1	3b-hydroxysteroid dehydrogenase
HSI	Hepatosomatic index
IGF3	Insulin-like growth factor-3
INSL3	Insulin-like peptide 3
K	Fulton’s condition factor
KE	Key event
KISS	Kisspeptin

K_{OW}	Octanol-water partition coefficient
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MBD	Methylated DNA-binding domain proteins
MIE	Molecular initiating event
MIH	17a,20b-dihydroxy-4-pregnen-3-one
NAM	New approach method
OECD	Organization for economic co-operation and development
POP	Persistent organic pollutant
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin
SSC	Spermatogonial stem cells
StAR	Steroidogenic acute regulatory protein
T	Testosterone
TBCO	1,2,5,6-Tetrabromocyclooctane
UV	Ultraviolet
UV-090	2-[2-Hydroxy-5-[2-(methacryloyloxy)ethyl]phenyl]-2*H*-benzotriazole
UV-234	2-[3,5-Bis(1-methyl-1-phenylethyl)-2-hydroxyphenyl]-2*H*-benzotriazole
UV-320	2-(2'-hydroxy-3',5'-di-t-butyl-phenyl)benzotriazole
UV-326	2-(2-Hydroxy-3-*tert*-butyl-5-methylphenyl)-5-chlorobenzotriazole
UV-327	2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)-5-chlorobenzotriazole
UV-328	2-(2*H*-benzotriazol-2-yl)-4,6-di-*tert*-pentylphenol
UV-329	2-(2-Hydroxy-5-*tert*-octylphenyl)-benzotriazole
UV-350	2-(3-*s*-Butyl-5-*tert*-butyl-2-hydroxyphenyl)benzotriazole
UV-P	2-(2H-Benzotriazol-2-yl)-4-methylphenol
UV-PS	2-(5-*tert*-Butyl-2-hydroxyphenyl)benzotriazole
YAS	Yeast androgen screen
YES	Yeast estrogen screen

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Regulation of Reproduction in Male Teleosts

In male fish, reproduction is under control of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis regulates essential processes, including sex steroid synthesis, gametogenesis, and reproduction. A schematic illustration of the HPG axis is shown in **Figure 1.1**. Under normal regulation, the neuropeptide, kisspeptin (KISS) activates a G-protein coupled receptor (GPCR) which stimulates secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Tsukamura, 2022). GnRH reaches the anterior pituitary, where it activates gonadotropin-releasing hormone receptors (GnRHRs) thereby signaling the release of two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Yaron & Levavi-Sivan, 2011). Both LH and FSH bind to sex hormone binding globulin (SHBG) and travel via blood circulation to the gonads, where they bind to luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR), respectively (Miguel-Queralt et al., 2004; Yaron & Levavi-Sivan, 2011). In fish, these gonadotropin receptors have been reported to be less selective than in mammals, as FSHR can be activated by both LH and FSH (Zhang et al., 2015). In the gonads, LH and FSH stimulate the synthesis of sex steroid hormones involved in reproduction (Yaron & Levavi-Sivan, 2011). Additionally, LH and FSH play specific roles in gamete synthesis and production in male fish.

The gonads of male fish consist of several cell types. The testes are comprised of Leydig and Sertoli cells, which have LHR and FSHR, respectively, and are the sites of sex steroid synthesis (Fujisawa, 2006; Schulz et al., 2010). Synthesis of sex steroids, also known as steroidogenesis, involves conversion of cholesterol to progestins, androgens, and

estrogens – all of which are necessary for successful reproduction (Rajakumar & Senthilkumaran, 2020). An overview of the steroidogenesis pathway is shown in **Figure 1.2**. Initially, the transport of cholesterol from the outer mitochondrial membrane into the inner mitochondrial membrane of Leydig cells is mediated by the steroidogenic acute regulatory protein (StAR; Tengu et al., 2021). Once in the inner mitochondrial membrane, cholesterol is converted to pregnenolone by cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11a), marking this to be the first and rate-limiting step in steroidogenesis (Rajakumar & Senthilkumaran, 2020). Following this, pregnenolone is either converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3β1) or undergoes 17α-hydroxylase (CYP17a)-mediated conversion to form 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA; Tokarz et al., 2015). CYP17a is then responsible for the formation of 17α-hydroxyprogesterone from progesterone, and androstenedione (an androgen) from DHEA (Tokarz et al., 2015). 17α-hydroxyprogesterone is needed for the formation of maturation-inducing hormone (MIH; 17α,20β-dihydroxy-4-pregnen-3-one), which is critical for stimulating early stages for spermatogenesis and the maturation of sperm (Chen et al., 2013; Tokarz et al., 2015). Additionally, androstenedione is converted to testosterone (T) and the weak estrogen, estrone (E1), via 17β-hydroxysteroid dehydrogenase 3 (HSD17β3) and aromatase (CYP19a), respectively (Tokarz et al., 2015). Furthermore, T is converted to 11β-hydroxytestosterone following hydroxylation by 11β-hydroxylase (CYP11C1), which is then oxidized by 11β-hydroxysteroid dehydrogenase 2 (HSD11β2) to form 11-ketotestosterone (11-KT; Tokarz et al., 2015). 11-ketotestosterone can also be synthesized from androstenedione following various multi-step reactions using enzymes CYP11C1,

HSD11β2, and HSD17β3, to form the intermediate products 11β-hydroxyandrostenedione, 11β-hydroxytestosterone, and 11-KT (Rajakumar & Senthilkumaran, 2020; Tokarz et al., 2015). Testosterone is the active androgen in mammals and exerts action by binding to the androgen receptor (AR), whereas 11-KT, which is synthesised in response to FSH, is the ligand for AR in teleost fishes and plays a role in inducing spermatogenesis (Borg, 1994; de Waal et al., 2008; Kime, 1993; Olsson et al., 2005; Schulz et al., 2010). In addition to progestins and androgens, 17β-estradiol (E2) has been shown to be essential for reproduction in male fish (Amer et al., 2001; Miura et al., 1999; Song & Gutzeit, 2003). Furthermore, T can be converted to E2 by CYP19a (Tokarz et al., 2015).

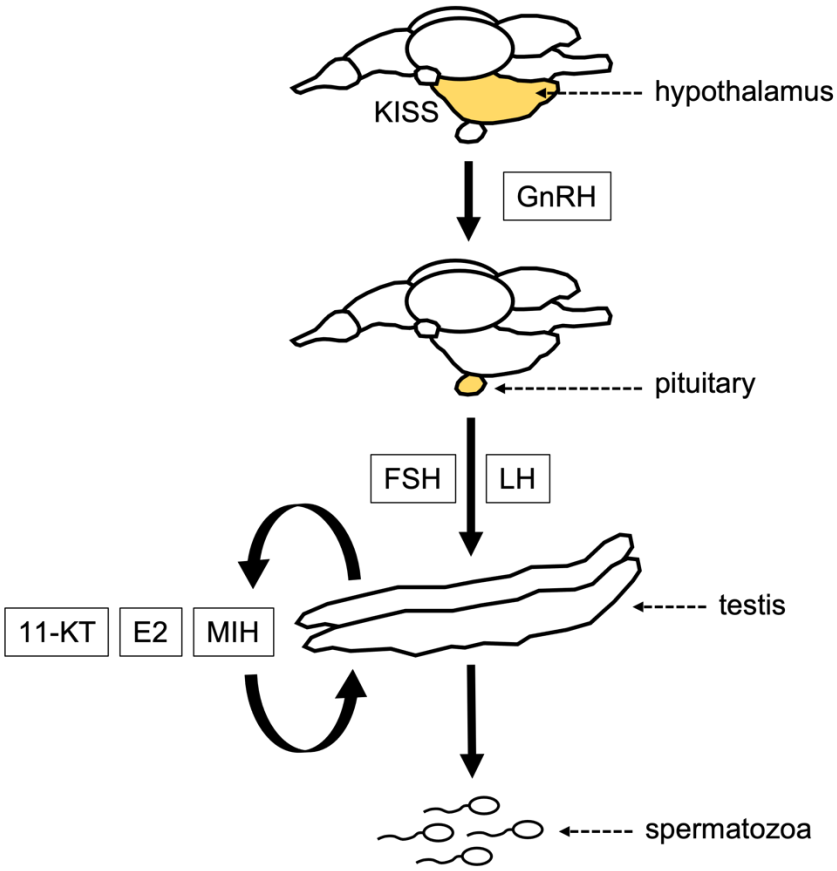

Figure 1.1 Simplified schematic of the hypothalamus-pituitary-gonadal axis in male zebrafish (*Danio rerio*). Figure not drawn to scale. The neuropeptide, kisspeptin (KISS) signals the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH binds to gonadotropin-releasing hormone receptors (GnRHRs) in the anterior pituitary, which then signals the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both FSH and LH travel to the testis via circulation and bind to their respective receptors, FSHR and LHR. Following activation of their receptors, LH and FSH regulate the formation of various essential sex steroids, such as 11-ketotestosterone (11-KT), 17β-estradiol (E2), and maturation-inducing hormone (MIH; 17a,20b-dihydroxy-4-pregnen-3-one). These steroids are critical for the process of spermatogenesis, during which production of mature spermatozoa occurs.

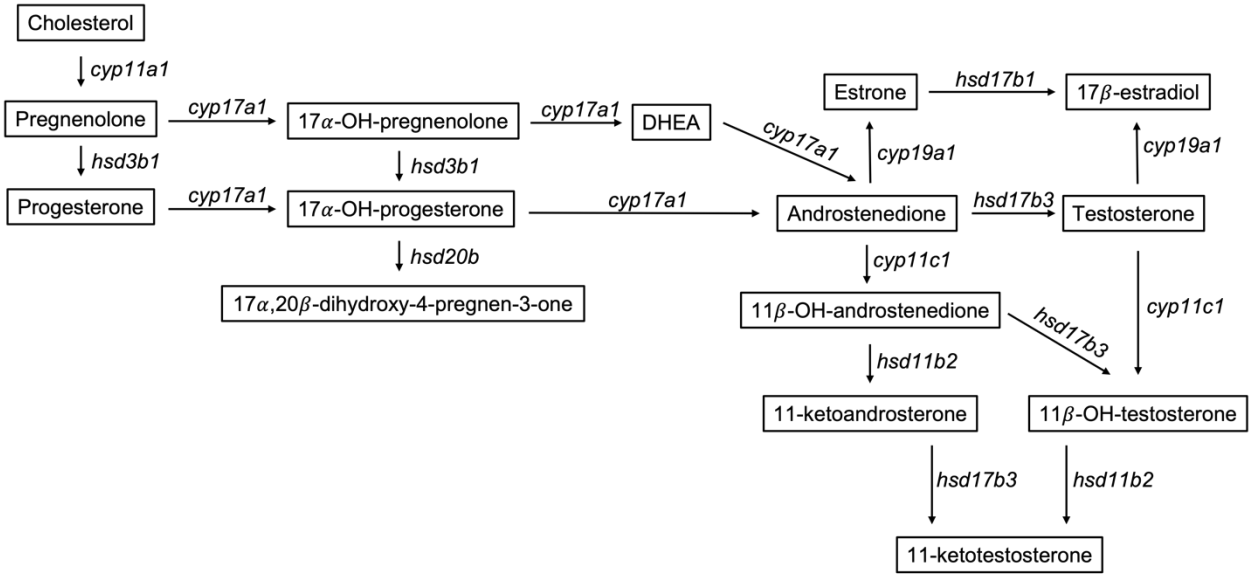

Figure 1.2 Schematic illustrating testicular steroidogenesis of sex hormones, to form 17α,20β-dihydroxy-4-pregnen-3-one (MIH), 17β-estradiol, and 11-ketotestosterone. Synthesized hormones are outlined in boxes, while italicized text represents enzymes responsible for catalyzing specific reactions. *Cyp11a1*: cholesterol side chain cleavage enzyme, *cyp17a1*: 17a-hydroxylase*, cyp11c1:* 11b-hydroxylase, *cyp19a1*: aromatase, *hsd3b1*: 3β-hydroxysteroid dehydrogenase, *hsd20b*: 20β-hydroxysteroid dehydrogenase, *hsd17b3*: 17β-hydroxysteroid dehydrogenase 3, *hsd11b2*: 11β-hydroxysteroid dehydrogenase 2*,* and *hsd17b1*: 17β-hydroxysteroid dehydrogenase 1.

1.2 Spermatogenesis

Spermatogenesis is the developmental process in which mature male gametes, also known as sperm, are produced. Normal functioning of spermatogenesis is often studied by investigating the quantity of sperm produced, however, the quality of sperm can also be assessed. In terms of assessing sperm quantity, there is often a focus on the different stages of sperm development (Schulz et al., 2010). In teleost fish, the various stages of sperm development are classified as: undifferentiated type A spermatogonia (A_{und}), differentiated type A spermatogonia (A_{diff}), type B spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa (Leal et al., 2009; Schulz et al., 2010). Progression through these stages of development is regulated by sex steroids – such as 11-KT, E2, and MIH – and spermatogenesis-specific hormones (Schulz et al., 2010). Spermatogenesis consists of three major stages: mitosis, meiosis, and spermiogenesis (Schulz et al., 2010). An overview of spermatogenesis is shown in **Figure 1.3**.

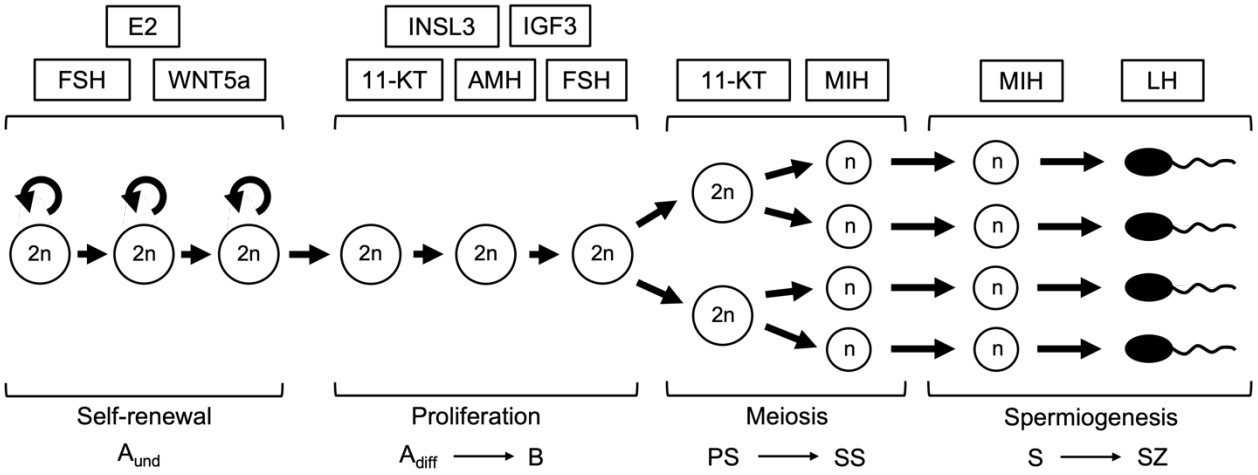

Figure 1.3 Simplified schematic of spermatogenesis in male teleost fish. Major phases of self-renewal, proliferation, meiosis, and spermiogenesis are illustrated with respective developmental stages listed below: A_{und} – type A undifferentiated spermatogonia; A_{diff} – type A differentiated spermatogonia; B – type B spermatogonia; PS – primary spermatocytes; SS – secondary spermatocytes; S – spermatids; SZ – spermatozoa. Germ cells are labelled as being diploid (2n) or haploid (n). Hormones involved in regulating each phase of spermatogenesis are outlined in boxes.

1.1.1.1 Self-Renewal

In the testis, primordial germ cells, also known as spermatogonial stem cells (SSCs), have the potential to become sperm (Schulz et al., 2010; Safian et al., 2019). Activity of SSCs is regulated by various endocrine factors and cell-to-cell communication (Xie et al., 2020b). The SSCs, also known as A_{und}, can either self-renew to produce more SSCs or produce differentiating daughter cells which are committed to develop into spermatozoa via meiosis (de Rooij, 2017; Safian et al., 2019). It is critical to maintain the balance between SSCs that are undergoing self-renewal and SSCs that are differentiating towards meiosis, as this balance works to maintain the stem cell pool for the male's lifespan, which is essential for long-term fertility (Safian et al., 2019). The process of self-renewal is regulated by various spermatogonial renewal factors, such as low doses of E2 and the protein-coding gene, WNT5a (Amer et al., 2001; Miura et al., 1999; Safian et al., 2018b; Song & Gutzeit, 2003).

1.1.1.2 Mitosis & Meiosis

Spermatogenesis is initiated once there is a switch from the SSC self-renewal phase to the irreversible commitment to mitosis (Schulz et al., 2010). This is marked by the progression of Type A_{und} spermatogonia into Type A_{diff} spermatogonia and Type B spermatogonia (Leal et al., 2009; Safian et al., 2019). During cell development, cytoplasmic bridges connect germ cells to allow for synchronized development of cells arising from the same SSC (Safian et al., 2019). The mitotic divisions during this phase are controlled by several molecular regulators, where the overarching regulator is FSH (Schulz & Miura, 2002; Schulz et al., 2010). Another major regulator is 11-KT, which not only activates ARs, but also stimulates the differentiation of Type A_{und} spermatogonia and the expression of

genes involved in spermatogenesis (Cavaco et al., 1998; Golshan & Alavi, 2019; Safian et al., 2019; Schulz et al., 2010). Additionally, proteins that promote differentiation include insulin-like peptide 3 (INSL3) and insulin-like growth factor-3 (IGF3), however, anti-Müllerian hormone (AMH) works to inhibit differentiation and maintain the balance between self-renewal and mitosis (Assis et al., 2015; Morais et al., 2017; Safian et al., 2018a). Following mitotic proliferation, MIH induces type B spermatogonia to enter meiotic prophase and become primary spermatocytes (Chen et al., 2013; Schulz et al., 2010). However, the mechanism through which MIH initiates meiosis is unknown. Completion of meiosis I gives rise to secondary spermatocytes, while completion of meiosis II subsequently gives rise to immature spermatids (Schulz et al., 2010).

1.1.1.3 Spermiogenesis

The final step in spermatogenesis is spermiogenesis, where haploid spermatids undergo events that transform them into spermatozoa capable of fertilizing eggs (O'Donnell, 2014). During this phase, a surge of LH from the pituitary gland signals the production of MIH and 11-KT (Chen et al. 2013). The release of MIH is indispensable for maturation of spermatids into mature spermatozoa (Chen et al., 2013; Schulz et al., 2010). Maturation of sperm is accompanied by the development of a flagellum, nuclear condensation, and rearrangement and elimination of cellular organelles (Schulz et al., 2010). Although the mechanism(s) are unclear, it has been suggested that 11-deoxycorticosterone, LH, and MIH all potentially play a role in sperm migration to the sperm duct and milt hydration, which are critical for producing sperm capable of motility and fertilization (Milla et al., 2008; Schulz et al., 2010).

1.2 Chemically Induced Disruption of Reproduction in Male Fish

Disruption of reproduction in male fish can result in impaired sperm quantity and quality via altered spermatogenesis, which can ultimately impact fertility. Anthropogenic chemicals that are classified as endocrine-disrupting chemicals (EDCs) – chemicals that interfere with the normal functioning of the endocrine system – have been shown to impair spermatogenesis. Following EDC exposure, teleost fish have been shown to display reduced testes growth and delays in maturation, ova-testis development, changes in abundance of male sex cell types, production of spermatozoa, and alterations in levels of estrogens and androgens (Carnevali et al., 2018; Kime, 1999). For instance, chronic exposure of zebrafish to environmentally relevant concentrations of bis-(2-ethylhexyl) phthalate (DEHP) has been shown to reduce embryo production by inducing mitotic arrest during spermatogenesis, which was associated with an increase in spermatozoa DNA fragmentation (Corradetti et al., 2013). Exposure of male zebrafish to bisphenol A (BPA) caused alterations in spermatogenesis, via impairment of meiotic progress and decreased abundance of spermatocytes (González-Rojo et al., 2019). Additionally, BPA exposure dysregulated expression of genes involved in testicular cell development, as well as DNA hypermethylation and histone hyperacetylation in the testis (González-Rojo et al., 2019). In a study where male adult zebrafish were exposed to diethylstilbestrol (DES) and flutamide (FLU) individually and in combination, it was found that both DES and FLU impaired spermatogenesis in a similar manner and displayed an additive effect when exposed simultaneously (Yin et al., 2017). Additionally, genes involved in the HPG axis were supressed, alongside decreased 11-KT plasma concentrations, decreased expression of genes involved in meiotic regulation, and enhanced apoptosis in the testis (Yin et al., 2017). It is evident that male reproduction can be targeted by EDCs, however, majority of

studies focus on exposure and direct effects on adult stages. There is potential for the effects of EDCs to persist across multiple life stages and generations.

1.3 Effects of Early Life-Stage Exposure

Traditionally, studies in ecotoxicology have focused on the direct effects of exposure to contaminants on different life stages, with early life-stages (ELS) and adult life-stages being the most common. However, there is growing interest in the long-term/latent effects of contaminants following exposure during ELS. Exposure of organisms to chemical stressors during critical stages of embryo development has been demonstrated to have greater adverse effects on biological processes relative to effects on adult life stages (Russell et al., 1999). As suggested by the developmental origins of health and disease (DOHaD) hypothesis, exposure to stressors at sensitive developmental periods might result in developmental reprogramming, which can cause alterations in phenotype including development and reproduction (Heindel et al., 2015). Exposure of fish to anthropogenic chemicals during ELS has been shown to disrupt various physiological processes, resulting in abnormal gamete development, reduced fecundity, infertility, or sex reversal. For instance, exposure of ELS Japanese medaka to letrozole – a triazole-containing drug that acts as an aromatase inhibitor – caused impairments at adult life stages, including decreased reproductive capacity, decreased maturation of gonads, altered transcription of genes that regulate reproduction, and altered sex ratio (Liao et al., 2014). Exposure of zebrafish embryos to benzophenone-3 (BP3), a UV filter, impaired development of ovaries, egg production, and expression of reproduction-related genes in mature female adults (Tao et al., 2023).

Compared to female fish, there are fewer studies of effects of ELS exposure to toxicants on reproductive performance of male fish, however, studies have demonstrated impaired reproduction of adult male fish following embryonic exposure. Exposure of embryonic zebrafish to mercury (Hg) for five days disturbed the balance of E2 and 11-KT in plasma of male fish, altered the expression of genes involved in the HPG axis, delayed gonadal development, and decreased gonadosomatic index (Xie et al., 2020a). However, this effect caused by embryonic exposure was recovered in subsequent (F1, F2) generations (Xie et al., 2020a). Another study investigated the effects of 6:2 chlorinated polyfluorinated ether sulfonate (F-53B) on zebrafish eggs that were exposed via maternal transfer following parental exposure. It was found that male F1 adult fish displayed decreased body weight and condition factor (K), decreased serum T concentrations, disruption of HPG-axis-related gene expression, and disruption of spermatogenesis as indicated by transcriptome analysis (Shi et al., 2018). Finally, exposure of zebrafish embryos to benzo(a)pyrene for 96h, has been demonstrated to supress spermatogenesis in adult fish, as indicated by decreased percentage of later stages of sperm development, impaired fertility, and decreased expression of genes involved in spermatogenesis and the HPG axis (Xu et al., 2023).

The mechanism(s) by which latent effects persist are not fully known, but evidence suggests a role for the epigenome, including changes in DNA methylation, histone modifications, and noncoding RNA activity (Groh et al., 2015). The development of embryos tends to be tightly controlled by epigenetic regulation, therefore, disruption of epigenetic remodelling induced by environmental stressors has the potential to persist into adulthood (Bernal & Jirtle, 2010; Groh et al., 2015; Szyf, 2009). Exposure of embryonic zebrafish to the aryl hydrocarbon receptor (AhR) agonist, benzo(a)pyrene, has been

suggested to impair reproductive performance later in life via methylation of gonadotropin-releasing hormone receptors (GnRHRs), following increased expression of DNA methyltransferases (DNMTs) mediated by the AhR (Aluru et al., 2015; Gao et al., 2018). Methylation of GnRHR promoter regions can down-regulate GnRH expression, thus decreasing synthesis of downstream sex steroids, such as E2, which is essential for the development and production of eggs (Gao et al., 2018). Another study investigated exposure of ELS inland silverside fish *(Menidia beryllina*) to various EDCs – including bifenthrin, levonorgestrel, ethinylestradiol, and trenbolone – and assessed DNA methylation patterns in F0, F1, and F2 adults (Major et al., 2020). Differential methylation of promoters/gene bodies was observed for all EDC exposures, therefore, causing phenotypic alterations and changes in gene expression across all generation (Major et al., 2020). Several genes in the endocrine system (involved in processes such as steroidogenesis, prostaglandin synthesis, sexual development, and cell signalling) were found to be differentially methylated across all treatments and generations – indicating the role of epigenetics in long-term effects that span life stages and generations (Major et al., 2020). In zebrafish exposed as embryos to benzo(a)pyrene, hypermethylation of the promoter methylation of various genes related to testicular development was linked to suppression of spermatogenesis in adult fish (Xu et al., 2023). Understanding the effects of ELS exposure on reproductive performance of fishes, and the molecular and biochemical mechanisms underpinning these effects, is needed to improve ecological risk assessments.

1.4 Benzotriazole Ultraviolet Stabilizers

The presence of anthropogenic chemicals in the environment is a major concern due to their potential adverse effects on biota. These pollutants can enter aquatic environments

from a variety of sources, including mining, agriculture, stormwater, industrial and municipal wastewater effluent, and improper disposal of waste (Wu et al., 2020). One class of chemical pollutants of emerging concern are benzotriazole ultraviolet stabilizers (BUVSs; Nakata et al., 2009; Parajulee et al., 2018). They consist of a benzotriazole moiety, 2-hydroxyphenol attachments, as well as various alkyl functional groups that form unique BUVSs (Cantwell et al., 2015b). Structures of several BUVSs are shown in **Figure 1.4**. Due to their photochemically stable structure, BUVSs are capable of absorbing and providing protection from UV-A and UV-B light, specifically at 280-400nm (Cantwell et al., 2015a; Parajulee et al., 2018; Peng et al., 2019). These anthropogenic compounds are added to a variety of products including building materials, waxes, personal care products, and paints (Zhang et al., 2011). Ultraviolet-stabilizers prevent UV-induced cracking, yellowing, and degradation of products and materials following sun exposure (Zhang et al., 2011). Mechanisms of UV-stabilization include preventing UV radiation from reaching the polymer matrix by absorbing/blocking light and deactivating excited species and/or free radicals generated by UV radiation by converting them into stable forms (Chin, 2007). BUVSs undergo a reversible proton transfer reaction, specifically by absorbing UV light energy, and converting this to thermal vibrational energy, thus preventing various photochemically initiated reactions (Cantwell et al., 2015a; Kramer, 2003). During the production process, they are commonly incorporated into various synthetic polymers, such as polyvinyl chloride, polyesters, acrylics, and polycarbonates (Cantwell et al., 2015a). The amount of BUVS incorporated into products largely depends on the product being manufactured and the BUVS being used (Cantwell et al., 2015a).

BUVSs are produced at high volumes globally, thus increasing their potential to contaminate the environment. In 2012, the United States Environmental Protection Agency (EPA) reported that BUVSs were imported or produced in the USA in quantities greater than 10,000 lbs (5 tonnes) annually (Cantwell et al., 2015a). More recently it was reported that BUVSs production volumes in the US reached up to 9,072 tonnes/year (Castilloux et al., 2022). Similarly, European production volumes reached 10,000 tonnes/year (Castilloux et al., 2022). Due to the high and increasing production volumes of BUVSs, contamination of aquatic environments can occur through wastewater effluent or leaching from plastic waste (Parajulee et al., 2018). BUVSs have been detected in rivers, lakes, sediment, sewage sludge, wastewater effluent, marine ecosystems, and aquatic organisms across several countries, including the USA, Canada, China, and Japan (Cantwell et al., 2015b; Lu et al., 2016, 2017, 2019; Nakata et al., 2009; Parajulee et al., 2018; Peng et al., 2019; Zhang et al., 2011). Their environmental concentrations range from 0.19 to 3422 ng/g dry weight in sediment, and 0.7 to 701 ng/L in surface waters (Castilloux et al., 2022; Kameda et al., 2011; Lu et al., 2018; Nakata et al., 2009; Parajulee et al., 2018; Peng et al., 2017; Vimalkumar et al., 2018). Additionally, they have been found in various aquatic biota, including northern pike (*Esox Lucius*) and lake sturgeon (*Acipenser fulvescens*) at concentrations ranging from 0.23 ng/g to 259 ng/g wet weight (Castilloux et al., 2022; Lu et al., 2018; Peng et al., 2017). BUVSs have octanol-water partition coefficients (log K_{OW}) ranging from 3.00-7.67, and therefore have greater solubility in lipid than water, indicating potential for bioaccumulation, biomagnification, and maternal transfer (Castilloux et al., 2022; Provencher et al., 2022). Due to the various properties of BUVSs, many have been classified as persistent organic pollutants (POPs). Therefore, some countries have limited

their usage, as well as placed certain BUVSs under regulation (e.g., added UV-328 on the Stockholm Convention of POPs; Nakata et al., 2009; Sheriff et al., 2022).

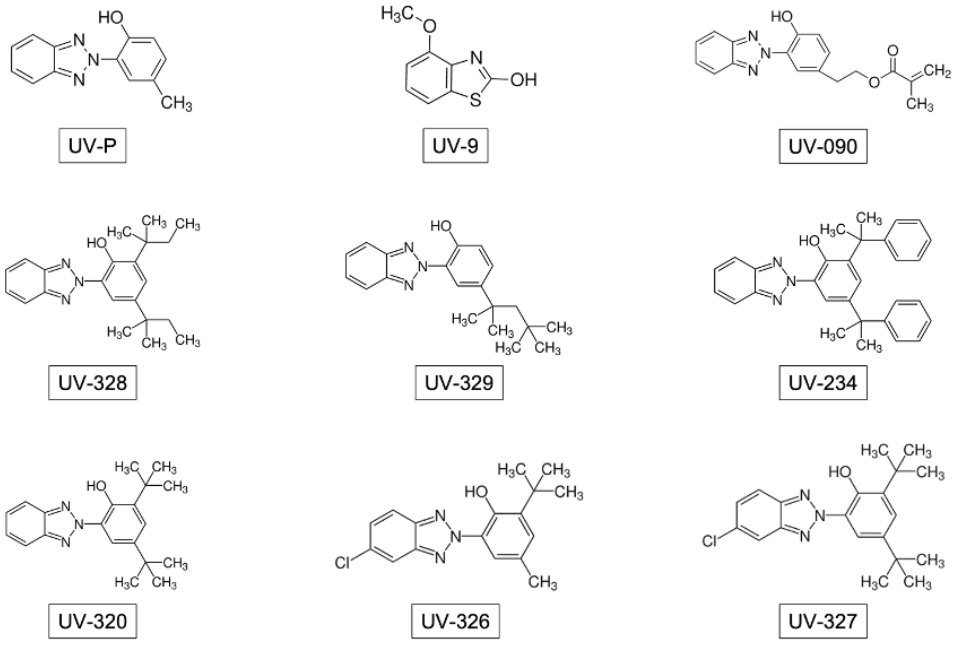

Figure 1.4 Structures of unique benzotriazole ultraviolet stabilizers.

1.5 Adverse Effects of BUVSs

Although BUVSs are implemented in commonly used products and are ubiquitous in the environment, little is known regarding their effects on aquatic organisms. In fish, studies have reported that BUVSs can interfere with various biological processes/pathways, thus, causing adverse effects. For instance, BUVSs have been shown to activate the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor which is essential in regulating expression of genes that are important for numerous physiological processes, including detoxification of xenobiotics and immunomodulation (Dubiel et al., 2022; Larigot et al., 2021). It has been shown that UV-329 activates the AHR in early life-stages of zebrafish, and that UV-P, UV-9, UV-090 and UV-326 activate the human AHR, *in vitro* (Li et al., 2020; Nagayoshi et al., 2014). Similarly, UV-326 and UV-P significantly activated the AHR pathway as indicated by the induction of a commonly used biomarker for AHR activation, cytochrome P450 1A transcript (*cyp1a*; Fent et al., 2014). Benzotriazole ultraviolet stabilizers also have the potential to disrupt the thyroid system. In zebrafish embryos, it was found that UV-234, UV-326, UV-329, and UV-P altered the expression of genes involved in the hypothalamic-pituitary-thyroid (HPT) axis, thus suggesting thyroid disrupting mechanisms of BUVSs (Liang et al., 2017). Additionally, BUVS have been shown to impair processes related to immune function and inflammation. In juvenile zebrafish exposed to various BUVSs, hepatic damage was displayed following exposure to UV-234 and UV-329, which was attributed to activation of inflammation pathways (as indicated by transcriptomic and gene expression analyses), specifically associated with immunotoxicity via the AHR-IL17/IL22 pathway (Li et al., 2020). A study that performed global transcriptome sequencing in the brain of juvenile zebrafish following exposure to UV-234, UV-236, UV-329, and UV-P, demonstrated that pathways related to

inflammation and immune function were commonly expressed across all BUVSs (Li et al., 2018). Finally, embryonic exposure of zebrafish to UV-328 has been shown to promote G_1 phase cell cycle arrest and inhibit genes related to oxidative stress and apoptosis, ultimately accelerating embryonic/larval development to abnormal levels (Zhang et al., 2023).

Several lines of evidence from *in vitro* and *in vivo* studies suggest that BUVSs are EDCs that might impair reproduction in fishes. When the endocrine disrupting potential of various BUVSs was assessed *in vitro* by utilizing yeast estrogen and androgen screening (YES and YAS; screens used to detect estrogenic and androgenic activities, respectively, of chemicals/samples), as well as investigating responses of zebrafish embryos, it was found that UV-P displayed significant antiandrogenic activity (Fent et al., 2014). This was consistent with findings in Japanese medaka (Fujita et al., 2022). Additional studies have shown that UV-P, UV-9, UV-234, UV-326, UV-328, UV-329, and UV-350 acted as antagonists of ARs (Sakuragi et al., 2021; Zhaung et al., 2017). When assessing the human ER, UV-P, UV-PS, UV-090, and UV-329 displayed agonistic activity, while UV-320, UV-350, UV-329, and UV-328 displayed antagonistic activity (Sakuragi et al., 2021). Similarly, in a study using an *in vitro* estrogen-inducible MVLN luciferase assay – an assay using MCF-7 breast cancer cell lines expressing estrogen receptors (ERs) and transfected with luciferase reporter genes to detect the estrogenic/antiestrogenic activity of molecules – UV-P, UV-PS, and UV-9 displayed partial estrogenic activity (Feng et al., 2020). Biotransformation can influence the endocrine disrupting potential of BUVSs. For instance, metabolized UV-328 displayed increased antiandrogenic activity, while metabolites of UV-P had decreased antiandrogenic activity (Zhaung et al., 2017). Despite the abundance of

evidence that BUVSs can act as EDCs, almost nothing is known about effects on reproductive performance of fish exposed to these chemicals.

1.6 Routes of Exposure to BUVSs in Aquatic Environments

Aquatic organisms can be exposed to contaminants via a variety of mechanisms. In adult fish, common routes of exposure include uptake through the gills and diet, as well as absorption through the skin (Pickford et al., 2003). The physiochemical properties of the contaminant and the developmental stage of the organism influence the route of exposure. The $\log K_{OW}$ differs among various BUVSs, making certain BUVSs more hydrophilic or hydrophobic/lipophilic than others (Castilloux et al., 2022; Provencher et al., 2022). Organisms tend to be exposed to hydrophilic chemicals via the water column, whereas hydrophobic/lipophilic chemicals can be taken up from sediment or pore water, and can be maternally transferred to eggs, both which provide potential for embryonic stages to be exposed (Russell et al., 1999; Streit, 1998). The process of maternal transfer occurs via circulating lipoproteins that transport contaminants from maternal tissue to developing oocytes during gonad development (Ungerer & Thomas, 1996). Because of maternal transfer, concentrations of bioaccumulated contaminants in embryonic stages have been demonstrated to be equivalent to concentrations to which the maternal organism was exposed (Russell et al., 1999). The maternal transfer of xenobiotics has been shown to alter the development of ELS and has been proposed to be a mechanism of impaired reproductive success in offspring (Khadra et al., 2019; Ostrach et al., 2008). Furthermore, due to the hydrophilicity/hydrophobicity of certain BUVSs, they hold the capacity to expose fish at adult stages as well as embryonic stages.

1.7 Zebrafish as a Model Species

Various species of fish are utilized in the field of ecotoxicology to study endocrine disruption and reproductive toxicity. The most common are zebrafish, Japanese medaka, and fathead minnow (*Pimephales promelas*). Zebrafish are the most used model species of fish due to their structural simplicity, ability to be manipulated, relatively small and fully annotated genome, external fertilization, and ability to produce a large quantity of transparent embryos (Ribas & Piferrer, 2013). Additionally, zebrafish have a relatively short generation time, with the developmental time from embryo to sexually mature adult being 3 to 6 months, depending on water temperature and feeding (Ribas & Piferrer, 2013; Singleman & Holtzman, 2014). Moreover, zebrafish are sensitivity to many chemicals and are, therefore, a useful model for toxicity studies (Ribas & Piferrer, 2013). Additionally, knowledge surrounding male reproduction and spermatogenesis is well described and understood in zebrafish.

1.8 Objectives and Hypothesis

Due to the increasing production and usage of various everyday products and materials, there is concern surrounding the potential for anthropogenic contaminants, many of which are EDCs, to leach from these sources and enter aquatic environments. These chemicals can cause a great number of adverse health effectt on aquatic organisms, including fish. In the field of ecotoxicology there often is a focus on the direct effects of acute exposure on a single life stage, or chronic exposure across multiple life stages. However, there is potential for adverse effects to span multiple life stages and generations, following transient exposure to a contaminant during critical stages of early development.

Furthermore, there is growing realization that latent effects need to be studied to better understand the impacts of these contaminants.

The presence of BUVSs in aquatic ecosystems is predicted to increase due to increasing plastic production and usage. The BUVS, UV-P, has been previously shown to disrupt the endocrine system through anti-androgenic and estrogenic modes of action, however, the majority of these findings were from *in vitro* studies with mammalian receptors (Feng et al., 2020; Sakuragi et al., 2021; Zhuang et al., 2017). Furthermore, there is a limited knowledge about the effects of UV-P on fish, specifically, latent effects following exposure during ELS. Therefore, the overall goal of this thesis was to determine whether embryonic exposure of the model teleost, zebrafish, to UV-P can induce adverse effects in adult life stages. It was hypothesized that since UV-P has been shown to display endocrine disrupting properties and that exposure to EDCs in early life have been linked to adverse effects later in life, embryonic exposure of zebrafish to UV-P will cause reproductive impairments at adult stages. Specific objectives of this research were to:

1. determine the effects of UV-P exposure on the reproductive success of adult zebrafish following embryonic exposure.
2. determine molecular mechanism(s) of effect(s) of UV-P on reproductive performance in zebrafish.

CHAPTER 2: EMBRYONIC EXPOSURE TO THE BENZOTRIAZOLE UV STABILIZER, UV-P, DECREASES FERTILITY OF ADULT ZEBRAFISH (*DANIO RERIO*)

Yamin Raza[1], Emily Mertens[1], Lauren Zink[1], Zhe Lu[2], Jon A. Doering[3], & Steve Wiseman[1]

[1] Department of Biological Sciences, University of Lethbridge, Lethbridge, AB, T1K 3M4, Canada

[2] Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, Rimouski, Québec G5L 3A1, Canada

[3] Department of Environmental Sciences, Louisiana State University, Baton Rouge, Louisiana, 70803, United States

*This manuscript was accepted for publication in November, 2023

*Y. Raza was the main author, primarily designed/conducted experiments, and analyzed data stated in this manuscript.

Citation: Raza, Y., Mertens, E., Zink, L., Lu, Z., Doering, J. A., & Wiseman, S. (2023). Embryonic exposure to the benzotriazole UV stabilizer, UV-P, decreases fertility of adult zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry*. doi: 10.1002/etc.5790

2.1 Introduction

Benzotriazole ultraviolet stabilizers (BUVSs) are a class of chemicals used in personal care products, paints, textiles, and plastics (Nakata et al., 2009; Parajulee et al., 2018; Zhang et al., 2011). By absorbing light at 280-400nm, these chemicals prevent degradation, cracking, and yellowing induced by UV light (Parajulee et al., 2018; Peng et al., 2019; Zhang et al., 2011). They consist of a benzotriazole moiety, a phenol group, and chemical specific alkyl functional groups (Cantwell et al., 2015). The octanol-water partition coefficients (log K_{OW}) of BUVS ranges from 3.00-7.67 (Castilloux et al., 2022; Provencher et al., 2022). Because of this property, some BUVSs have been classified as potential persistent organic pollutants (POPs). For instance, the Environmental Ministry of Japan classified BUVSs as potential POPs and either banned or limited the usage of certain BUVSs (e.g., 2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)-5-chlorobenzotriazole (UV-327), 2-(2'-hydroxy-3',5'-di-t-butyl-phenyl)benzotriazole (UV-320); Nakata et al., 2009). Additionally, 2-(2*H*-benzotriazol-2-yl)-4,6-di-*tert*-pentylphenol (UV-328) was added to Annex A of the Stockholm Convention on Persistent Organic Pollutants (Sheriff et al., 2022).

Benzotriazole ultraviolet stabilizers have been detected in aquatic environments. They have been detected in lakes, rivers, streams, sediment, sewage sludge, wastewater effluent, and aquatic organisms, globally (Lu et al., 2016, 2018; Montesdeoca-Esponda et al., 2019; Nakata et al., 2009, 2012; Parajulee et al., 2018; Peng et al., 2017, 2019; Zhang et al., 2011). Although concentrations of BUVSs in the environment are frequently below detection limits, when detected, concentrations of certain BUVSs can be as great as 720 ng/g (dry wt.) in sediment, 4780 ng/L in heavily polluted surface waters, and 255 ng/g (lipid

wt.) in muscle tissue of fish, globally (Castilloux et al., 2022; Kameda et al., 2011; Kim et al., 2011; Nakata et al., 2009). One BUVS that has been detected in aquatic environments is 2-(2H-benzotriazol-2yl)-4-methylphenol (UV-P). Annual production of UV-P has ranged from 227 to 454 tonnes in the United States, and 1,000 to 10,000 tonnes in Europe (Castilloux et al., 2022). Concentrations of UV-P up to 23 ng/L have been detected in surface waters, and up to 15 ng/g (dry wt.) in sediment from various locations globally (Kameda et al., 2011; Vimalkumar et al., 2018). Additionally, UV-P has been detected in muscle tissues from 26 species of freshwater and marine fish, with maximum detected concentrations of 222 ng/g (lipid wt; Kim et al., 2011; Vimalkumar et al., 2018). Although UV-P does accumulate in lipid, it can be biotransformed in fish (Zhang et al., 2021). Since BUVSs are additives in plastic, it is plausible that increases in both plastic use and waste could increase concentrations of BUVSs in the environment, including in biota (Borrelle et al., 2020; MacLeod et al., 2021). Therefore, it is critical to understand any adverse effects these chemicals might have on aquatic environments.

Little is known about adverse effects of BUVS, but there is evidence that some BUVS, including UV-P, can act as endocrine disrupting chemicals (EDCs). Using reporter gene assays, UV-P was classified as an antagonist of the of human androgen receptor (AR; Sakuragi et al., 2021). In another study of transactivation of the human AR, UV-P was determined to be a potent antiandrogen, and metabolites from CYP3A4-mediated biotransformation of UV-P also acted as antiandrogens (Zhuang et al., 2017). Significant antiandrogenic activity of UV-P was also observed in the yeast androgen screening assay (YAS; Fent et al., 2014). These findings are consistent with observations in UV-P-exposed Japanese medaka (*Oryzias latipes*), where an antiandrogenic mode of action was proposed

based on changes in expression of steroidogenic genes and plasma concentrations of 17β-estradiol (E2) and testosterone (T; Fujita et al., 2022). Since most research about the endocrine disrupting effects of BUVSs, including UV-P, has been conducted using mammalian models, further research is needed to understand the endocrine disrupting potential of BUVSs on aquatic biota, including fishes.

Exposure to chemical stressors during early life-stages (ELS) can have greater adverse effects on biological processes relative to impacts of exposures during adult life stages, specifically due to heightened sensitivity to contaminants during critical developmental periods (Russell et al., 1999). Embryonic exposure of fish to EDCs can result in disruption of physiological processes later in life, including abnormal gamete development, reduced fecundity, and infertility. For instance, embryonic exposure of Japanese medaka to the EDC, letrozole, impaired reproductive capacity, gonad maturation, and expression of reproductive genes at sexual maturity (Liao et al., 2014). Embryonic exposure of zebrafish (*Danio reri*o) to benzophenone-3 (BP3) impaired the development and maturation of ovaries and reduced egg production, and decreased chasing behaviour (Tao et al., 2023). Exposure of Japanese medaka embryos to the brominated flame retardant 1,2,5,6-Tetrabromocyclooctane (TBCO), via maternal transfer, resulted in decreased fecundity and impairment of oocyte maturation (Devoy et al., 2023a, 2023b). To date, no study has investigated whether embryonic exposure to BUVSs impacts physiological performance of fishes at sexual maturity, including effects on reproduction.

Studies of effects of ELS exposure to anthropogenic contaminants on later-life physiological performance are needed to understand the risk that contaminants pose to organisms. Therefore, the present study aimed to characterize the effects of embryonic

exposure to UV-P, a BUVS with anti-androgen activity, on the reproductive performance of zebrafish. Zebrafish embryos were microinjected with UV-P, reared to sexual maturity in clean water, and effects on reproductive performance were assessed, including molecular mechanism(s) of effect. Results provide novel insight into the long-term effects of ELS exposure to UV-P on fish reproduction which fills critical knowledge gaps about the potential risks of this class of chemicals to the aquatic environment.

2.2 Material and Methods

2.2.1 Chemicals

2-(2H-benzotriazol-2yl)-4-methylphenol (UV-P; CAS#: 2440-22-4; purity > 97%), was from MilliporeSigma (Oakville, ON, Canada). This chemical has a molecular weight of 225.3 $g.mol^{-1}$, water solubility of 25.6 $mg.L^{-1}$, and estimated log K_{ow} of 3.0 (US EPA, 2009). Deuterated UV-328 (UV328-d_4) was obtained from ASCA GmbH (Berlin, Germany). HPLC grade n-hexane and DMSO were purchased from MilliporeSigma.

2.2.2 Animals

Use of animals was approved by the University of Lethbridge Animal Care Committee (Protocol #2105). Adult zebrafish were held in an active breeding group, in a 1:1 ratio of male to female fish, maintained in a ZebTEC Active Blue System (Tecniplast, Toronto, ON, Canada) at the Aquatic Research Facility within the Alberta Water and Environmental Science Building at the University of Lethbridge (Lethbridge, AB, Canada). Fish were under a 14:10-h light:dark photoperiod and were supplied with processed City of Lethbridge municipal tap water at 28°C. Diets consisted of adult zebrafish feed (Ziegler Bros., Gardners, Pennsylvania, USA), brine shrimp (*Artemia salina,* Brine Shrimp Direct, Ogden, Utah, USA), and Gemma Micro 300 feed (Skretting, Fontaine-lès-Vervins, France),

at a feeding rate of approximately 2% bodyweight per day. Water quality parameters, including dissolved O_2, pH, ionized and unionized ammonia, NO_3^-, and NO_2^-, were measured daily.

2.2.3 Microinjections

Zebrafish embryos were microinjected with UV-P based on a previously described protocol (Lane et al., 2019) with minor modification (Dubiel et al., 2022). This method of exposure allows for precise doses of UV-P to be delivered into each embryo, therefore reducing any differences in exposure dosage between embryos, and allowing for full absorption of the chemical by the fish (Lane et al., 2019). To obtain embryos, two male and two female zebrafish were placed in 1.7 L sloped breeding tanks (Tecniplast) the evening prior to microinjections. Embryos were collected 1 h following a breeding event, and dead and unfertilized embryos were discarded. A batch of embryos was used only if greater than 80% of eggs were fertilized. Using an IM-400 Electric Microinjector (Narishige Group, Tokyo, Japan), zebrafish embryos were injected with 1.5 nL of either a solvent control (100% DMSO), or UV-P at nominal doses of 100 and 1000 ng/g–egg. All microinjections were completed prior to gastrulation, at approximately 6 h post-fertilization (hpf). The highest nominal exposure dose was approximately the dose causing 20% lethality (LD_{20}) based on a previously determined dose-response to zebrafish embryos (Johnson et al., unpublished data). Exposures were replicated three times, each with eggs from a discrete breeding event. Approximately 200 embryos per replicate were injected per dose. After injections, embryos were placed in a glass petri dish containing dechlorinated water, for 24 h, after which any dead embryos were discarded. Mortalities were assumed to be caused by the microinjection procedure and therefore were not included in the final mortality

determination. Remaining embryos were used for the embryotoxicity assay and reproduction assays, as described in the next sections. For chemical quantification of dose, 1 g of embryos were injected per treatment, and immediately frozen at -80°C (Section 2.2.9).

2.2.4 Embryotoxicity Assays

Twenty-four of the surviving embryos were placed into 24-well plates (one embryo per well) with 2mL of dechlorinated municipal tap water in each well. These embryos were reared until 15 days post-fertilization (dpf) to ensure complete yolk sac absorption and exposure to the entire dose of UV-P. A 50% water renewal was performed daily. To assess embryos, a dissecting microscope was used. Heart rate as the number of beats per minute (bpm) was assessed at 48 hpf, where the number of beats in 30 sec was recorded, then doubled, for eight randomly selected embryos. Mortality (based on lack of heartbeat) was scored daily, and malformations – including spinal curvature, yolk sac edema, and pericardial edema – was recorded as cumulative totals at 15 dpf.

2.2.5 Reproduction Assays

Approximately 150 embryos per replicate were reared at 28°C until sexual maturity (approx. 6 months; Singleman & Holtzman, 2014) without further exposure to UV-P. During rearing, fish were initially fed a diet consisting of brine shrimp and Gemma Micro 300 feed beginning at 7 dpf. Once fish were of sufficient size (approx. 1 month) their diet was supplemented with adult zebrafish feed (Ziegler Bros.). Delaying initiation of feeding until 8 dpf does not impact growth or survival of zebrafish (Hernandez et al., 2018). Two assays were performed to assess the effects of embryonic exposure to UV-P on reproductive performance of adults. The assays were based on OECD Test number 229 (Fish Short Term

Reproduction Assay; OECD, 2012) except one male and one female were used per tank so that fecundity and fertilization success of individual fish could be determined. Due to aggression between male and female fish across all treatments, including the controls, assays were terminated at 14-days or 12-days, rather than 21-days. The first reproduction assay (terminated at 14-days) was conducted using UV-P exposed male and female fish. The second reproduction assay (terminated at 12-days) was conducted using UV-P exposed male fish and DMSO exposed (control) female fish to eliminate the possibility that the decrease in fertilization success was due to decreased egg quality from female fish that had been exposed to UV-P, as embryos. To perform the reproduction assays, sexually mature adult zebrafish were placed into 3.5L tanks in groups of two (one male and one female) in a ZebTEC Active Blue System (Tecniplast), with ten or eight replicate tanks per treatment for the first and second assay, respectively. Fish were fed adult zebrafish feed at approximately 2% bodyweight daily, as described in Section 2.2.2. Following a one-week acclimation period, fecundity was assessed daily as the number of eggs per tank and fertilization success was assessed daily using a light microscope. Eggs were collected and assessed for successful fertilization within 5 h of being laid. Upon termination of the reproduction assays, fish were euthanized using MS-222 (250 mg/L, buffered with sodium bicarbonate), and gonads and blood were collected for analysis of molecular and biochemical endpoints. Fulton's condition factor (K), gonadosomatic index (GSI), and hepatosomatic index (HSI) were determined from the mass (g) and length (mm) of the fish, gonad mass (g), and liver mass (g), respectively. Due to the lack of effect on fecundity of females, gene expression, steroid hormone concentrations, and histological analyses were determined only with male fish (section 2.2.6 - 2.2.8). During the rearing period and reproduction assays, average daily water temp was 28.0 ^{o}C (min = 27.9, ^{o}C max = 28 ^{o}C)

and average daily DO was 6.3mg/L (90% saturation). Total Ammonia, nitrite, and nitrates were recorded weekly and average values were 0.01mg/L, 0.007mg/L and 3.0 mg/L, respectively.

2.2.6 mRNA Abundances of Genes Involved in Reproduction

Abundances of transcripts of genes involved in steroidogenesis and spermatogenesis were quantified in testes from six to eight male fish per treatment, from the first reproduction assay. Total RNA was isolated using TRIzolä reagent (ThermoFisher Scientific, Ottawa, ON, Canada) according to the manufacturer's protocol. RNA concentrations were quantified by use of a Nanodrop™ One© spectrophotometer (ThermoFisher Scientific), following which complementary DNA (cDNA) was synthesized from 2.5 mg of RNA by use of Superscriptä IV First-Strand Synthesis System (Thermofisher Scientific). The cDNA synthesis protocol included a DNase step. The protocol for qPCR was identical to what was described previously (Fujita et al., 2022). No-template controls were run for each primer set to ensure there was no contamination in primers. Additionally, melt curves were generated to ensure a single product was amplified. Transcript abundance of each target gene was normalized to *18S rRNA* and using the efficiency corrected method, changes in mRNA abundance was calculated relative to the control (Pfaffl, 2001). Efficiencies of qPCR reactions using different primer sets were calculated using 5-fold serial dilutions of cDNA templates (**Table 2.1**).

Table 2.1 Gene function, efficiencies, sequences of oligonucleotide primers used in real-time PCR (qPCR).

Target mRNA	Function	Primer Sequence (5' – 3')	Efficiency (%)	Reference
18S rRNA	Housekeeping	F: CCACTCCCGAGATCCAACTA R: CAAATTACCCATTCCCGACA	95	Meng et al. (2018)
ar	Sex steroid synthesis	F: CACTACGGAGCCCTCACTTGCGGA R: GCCCTGAACTGCTCCGACCTC	86	Hossain et al. (2008)
cyp11a1	Sex steroid synthesis	F: TGCTGTGGACGCTTTATGA R: CAATACGAGCGGCTGAGAT	109	Hoffman et al. (2006)
cyp19a1a	Sex steroid synthesis	F: CTGAAAGGGCTCAGGACAA R: TGGTCGATGGTGTCTGATG	114	Hoffman et al. (2006)
cyp11c1	Sex steroid synthesis	F: TGTGCTGAAGGTGATTCTCG R: GCTCATGCACATTCTGAGGA	101	Nesan & Vijayan (2016)
hsd17b3	Sex steroid synthesis	F: ACATTCACGGCTGAGGAGTTT R: ATGCTGCCATACGTTTGGTC	109	Liang et al. (2015)
hsd11b2	Sex steroid synthesis	F: TGCTGCTGGCTGTACTTCAC R: TGCATCCAACTTCTTTGCTG	82	Alsop & Vijayan (2008)
nanos2	Spermatogenesis	F: AAACGGAGAGACTGCGCAGAT R: CGTCCGTCCCTTGCCTTT	81	Assis et al. (2016)
piwil1	Spermatogenesis	F: ATACCGCTGCTGGAAAAAGG R: GCAAGACACACTTGGAGAACC	105	Safian et al. (2016)
dazl	Spermatogenesis	F: ACTGGGACCTGCAATCATGA R: AATACAGGTGATGGTGGGGC	105	Oakes et al. (2019)
insl3	Spermatogenesis	F: TCGCATCGTGTGGGAGTTT R: TGCACAACGAGGTCTCTATCCA	103	Assis et al. (2016)
igf3	Spermatogenesis	F: TGTGCGGAGACAGAGGCTTT R: CGCCGCACTTTCTTGGATT	98	Assis et al. (2016)
amh	Spermatogenesis	F: CTCTGACCTTGATGAGCCTCATTT R: GGATGTCCCTTAAGAACTTTTGCA	98	Assis et al. (2016)
wnt5a	Spermatogenesis	F: TGGAGATCGTGGACGCAAA R: CACTTCAGGAATCAGCAGAGGATT	82	Oakes et al. (2020)

2.2.7 Quantification of Steroid Hormone Concentrations

Enzyme-linked immunosorbent assays (ELISAs) were used to quantify concentrations of 17β-estradiol (E2) (Item No: 501890) and 11-ketotestosterone (11-KT) (Item No: 582751) in blood plasma from five to eight male zebrafish per treatment from the first reproduction assay, following the manufacturers protocols (Cayman Chemical, MI, USA).

2.2.8 Histological Analysis

Following the second reproduction assay, testes from six males per treatment were extracted and fixed in 10% formalin for 24 h for histological analysis. Following fixation, tissues were dehydrated in a series of ethanol dilutions, cleared in xylene, and embedded in paraffin blocks. Six sections per sample were sectioned at 5 mm and stained with hematoxylin and eosin at Prairie Diagnostic Services (University of Saskatchewan, Saskatoon, SK, Canada). Histological changes were observed using a brightfield Olympus CX43 microscope (Olympus Corporation, Shinjuku City, Tokyo, Japan). High-resolution images were analyzed using ImageJ Version 1.53 (National Institutes of Health). Proportions of area of the following stages of sperm development were determined - Type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (B), primary spermatocytes (PS), secondary spermatocytes (SS), and spermatids (S). Five to six sections per sample were analyzed.

2.2.9 Analysis of UV-P in Eggs

Fish egg samples (1 g wet mass per dose) were homogenized and transferred to a glass test tube and spiked with 20 ng of UV328-d_4. A commercially available isotope labeled UV-P standard was not available at the time of the present study. A previous study

showed similar recovery of UV-P and UV328-d4 from fish tissues (Castilloux et al., 2022). The sample was extracted using 5mL of *n*-hexane in an ultrasonic water bath for 10 min, followed by 1 min of vortexing and 5 min of centrifugation at 1167 ×g. The top *n*-hexane extract was transferred to a new glass tube. The extraction was repeated 3 times, and the solvent extracts were combined. The extract was concentrated to dryness using N_2. The sample was reconstituted in 1 mL of *n*-hexane for gas chromatography-mass spectrometry (GC-MS) analysis. Details of GC-MS analysis parameters are previously published (Fujita et al., 2022). UV-P was quantified using *m*/*z* 225. The *m*/*z* 168 was used as qualification ions. The recovery of UV328-d_4 was 113±6% (mean ± standard error; five samples). UV-P was not detected in procedure blanks (*n*=3). The limit of detection (LOD), which was estimated using 3 times of signal to noise ratio in the control fish egg extract, was 1.5 ng/g (wet mass).

2.2.10 Statistical Analysis

Initial statistical analyses were performed in R, version 4.2.1 (R Core Team, 2022). Multivariate Analysis of Variance (MANOVA) were completed for all endpoints utilizing the same individuals; for example, all transcript abundance data were analyzed together, and all histopathological endpoints compared to each other. The MANOVA was constructed to eliminate experimental design aspects such as different microplates or different fish housing units as being a source of differentiation in the data. Following the MANOVA analysis, data were transferred to GraphPad Prism 9.3.1 for Mac for subsequent analysis. Data were analyzed for normality and homogeneity of variance using the Shapiro-Wilk and Bartlett's test, respectively. Data that conformed to parametric assumptions was further tested by using a single-factor analysis of variance (ANOVA), followed by a

Dunnett's post-hoc test to determine significance between the treatments and control. Data that did not conform to parametric assumptions was $\log_{10}$ transformed. Data that failed to meet the parametric assumption following $\log_{10}$transformation was analyzed using Kruskal-Wallis test and post-hoc Dunn's test. All data were represented as mean ± standard error of the mean (SEM). Differences were considered significant when $p \leq 0.05$.

2.3 Results

2.3.1 Concentration of UV-P in Eggs

Measured doses of UV-P in embryos were < 1.5 ng/g-egg, 2.77 ng/g-egg, and 24.3 ng/g-egg in the 0, 100, and 1000 ng/g-egg treatment, respectively. Each treatment is hereafter referred to as control, low, and high. Measured doses of UV-P were approximately 40-fold less than the intended nominal dose.

2.3.2 Effect of UV-P on Embryotoxicity of Zebrafish

Embryonic exposure of zebrafish to UV-P did not cause any significant changes in cumulative survival, which was greater than 80% across all treatments (**Figure 2.1**), or heart rate (data not shown). Mean prevalence of both spinal curvature and uninflated swim bladder was significantly increased from 1.7% in controls to 12.1% in the low treatment (**Figure 2.1**). No significant difference in the prevalence of pericardial or yolk sac edema was observed among treatments (data not shown).

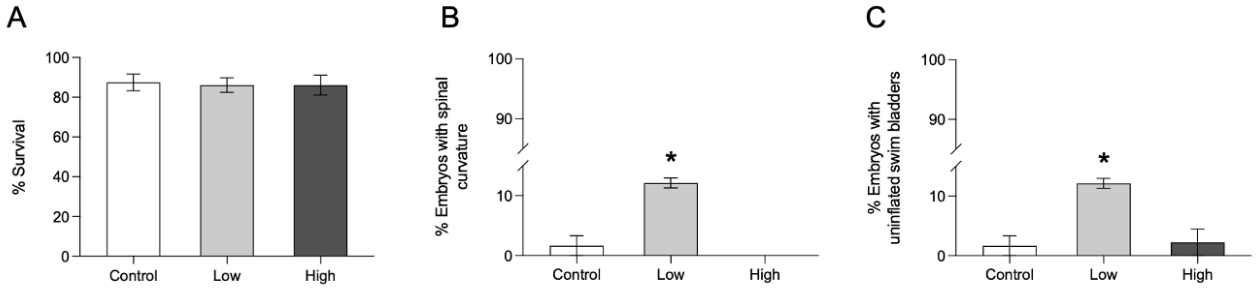

Figure 2.1 Effect of UV-P on ELS zebrafish, measured between 24 hpf to 360 hpf. Zebrafish embryos were exposed via microinjection at doses of < 1.5 ng/g-egg (control), 2.77 ng/g-egg, and 24.3 ng/g egg. (A) Cumulative survival at 360 hpf, (B) spinal curvature, and (C) uninflated swim bladder. Data are represented as mean (± SEM) of three independent replicates. Differences from the control were measured using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. (*) indicates significant differences ($p \leq 0.05$).

2.3.3 Effect of Embryonic Exposure to UV-P on Reproductive Performance of Adult Zebrafish

Reproductive capacity of zebrafish was altered following embryonic exposure to UV-P. In the first reproduction assay with UV-P-exposed male and female fish, fecundity in the controls (35.7 ± 7.4) was not statistically different than the low (38.4 ± 3.8) and high (38.2 ± 5.1) treatments. However, fertilization success was significantly decreased by 8.8% and 15.0% relative to controls in the low and high treatments, respectively (**Figure 2.2**). In the second reproduction assay with UV-P-exposed males and control females, fecundity in the controls (55.1 ± 9.7) was not statistically different than the low (55.7 ± 9.7) and high (34.5 ± 8.8) treatments, however, fertilization success was significantly decreased by 11.5% in the high treatment relative to the control (**Figure 2.2**). There were no significant differences in GSI, HSI, or K of male fish among treatments for either reproduction assay (**Table 2.2**).

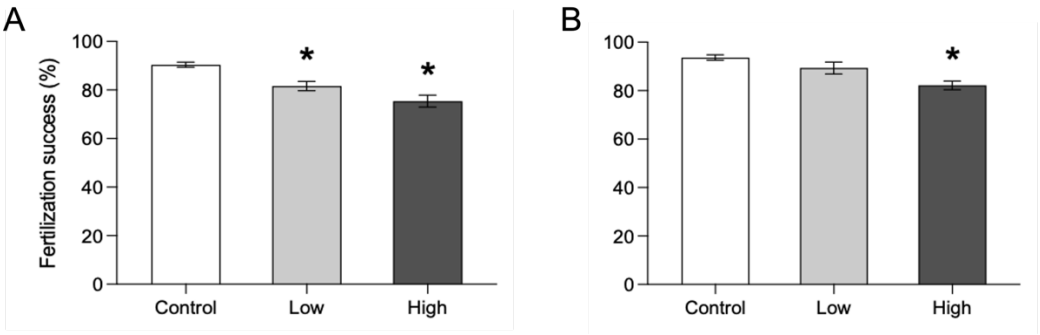

Figure 2.2 Effect of embryonic exposure to UV-P on the fertilization success of adult male zebrafish. Embryos were exposed via microinjection to doses of < 1.5 ng/g-egg (control), 2.77 ng/g-egg, and 24.3 ng/g egg and reared to sexual maturity in clean water. Once at sexual maturity, one male and one female zebrafish were assigned to eight to ten replicate tanks per treatment. Fertilization success of (A) UV-P-exposed male and female zebrafish, following 14-day reproduction assay, and (B) UV-P-exposed male and unexposed female zebrafish, following 12-day reproduction assay, was measured. Data are represented as mean (± SEM) of six to nine replicate tanks. Differences from the control were measured using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. (*) indicates significant differences ($p \leq 0.05$).

Table 2.2 Mean Fulton's condition factor (K), gonadosomatic index (GSI), and hepatosomatic index (HSI) of male adult zebrafish, following an embryonic exposure to UV-P at doses of 0, 100, or 1000 ng UV-P/g-egg. Data are represented as mean (±SEM) of seven to ten biological replicates. Differences among treatments were analyzed using a one-way analysis of variance (ANOVA). No significant differences were observed.

Treatment	Condition Factor (K)	Gonadosomatic Index (GSI)	Hepatosomatic Index (HSI)
1st Reproduction Assay			
Control	0.83 ± 0.04	1.09 ± 0.16	0.64 ± 0.09
Low	0.78 ± 0.03	1.78 ± 0.40	0.92 ± 0.24
High	0.85 ± 0.03	1.57 ± 0.17	0.98 ± 0.21
2nd Reproduction Assay			
Control	0.96 ± 0.05	1.53 ± 0.25	1.01 ± 0.21
Low	0.96 ± 0.09	1.44 ± 0.14	1.36 ± 0.14
High	0.97 ± 0.10	1.34 ± 0.15	0.93 ± 0.05

2.3.4 Effect of UV-P on Blood Plasma Steroid Hormone Concentrations of Male Zebrafish

Concentrations of E2 and 11-KT in blood plasma of sexually mature male fish exposed as embryos to UV-P were not significantly different from control male fish (**Figure 2.3**).

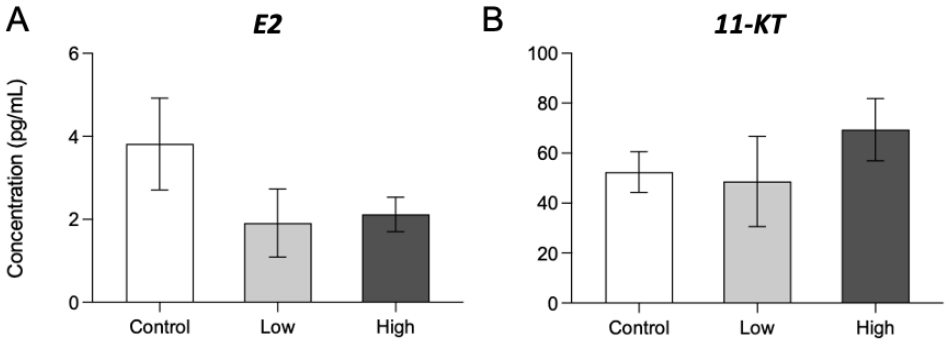

Figure 2.3 Effect of embryonic exposure to UV-P on concentrations (pg/mL) of (A) E2 and (B) 11-KT, in blood plasma from adult male zebrafish. Embryos were exposed via microinjection to doses of < 1.5 ng/g-egg (control), 2.77 ng/g-egg, and 24.3 ng/g egg UV-P/g-egg and reared to sexual maturity in clean water. Blood was collected and analyzed from five to eight adult male fish per treatment. Data are represented as mean (± SEM). Differences from the control were measured using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test.

2.3.5 Effect of UV-P on Transcript Abundance of Steroidogenic Genes in Male Zebrafish

Abundances of transcripts of *ar, cyp11a1, cyp19a1a, cyp11c1,* and *hsd11b2* were not significantly different in testes of male fish in either treatment, relative to the control (**Figure 2.4**). Transcript abundance of *hsd17b3* was significantly decreased by 2.5-fold in testes of male fish exposed to the high treatment, relative to the control (**Figure 2.4**). Although not statistically significant, transcript abundances of *cyp11a1, cyp19a1a,* and *cyp11c1* in testes from male fish were decreased by 1.4- ($p = 0.60$), 1.8- ($p = 0.18$), and 1.5-fold ($p = 0.60$), respectively, in the high treatment relative to the control.

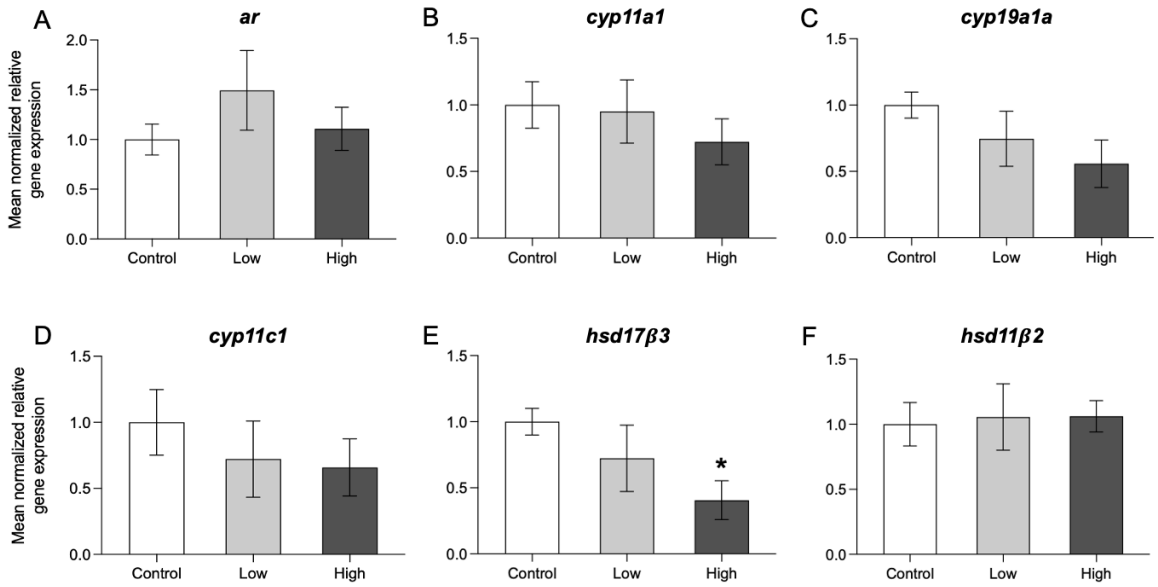

Figure 2.4 Effects of embryonic exposure to UV-P on abundances of transcripts of steroidogenic genes in testes from male adult zebrafish. Embryos were exposed via microinjection to doses of < 1.5 ng/g-egg (control), 2.77 ng/g-egg, and 24.3 ng/g egg and reared to maturity in freshwater. (A-F) Abundances of *ar, cyp11a1, cyp19a1a, cyp11c1, hsd17b3,* and *hsd11b2* transcripts in testes of male zebrafish. Parametric data was analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. Non-parametric data was $\log_{10}$ transformed, and if data remained non-parametric, was analyzed using a Kruskal-Wallis test, followed by a Dunn's post hoc test. Data are represented as mean (± SEM) of six to eight replicates. (*) indicates significant differences from control ($p \leq 0.05$).

2.3.6 Effect of UV-P on Morphometry of Zebrafish Testicular Cells

In the testes of male zebrafish exposed as embryos to either dose of UV-P, there was no change in proportion of area of type A_{und} spermatogonia, type A_{diff} spermatogonia, type B spermatogonia, or primary spermatocytes relative to the control (**Figure 2.5**). The proportion of area of secondary spermatocytes was non-significantly increased by 1.3-fold in the low treatment, and significantly increased by 1.6-fold in the high treatment, relative to the control (**Figure 2.5**). The proportion of area of spermatids was decreased by 1.5-fold ($p = 0.07$) in the high treatment, relative to the control, but was not statistically significant (**Figure 2.5**). There were no incidences of ova-testis in males from either treatment group, which is consistent with gross-observations of the testis during dissections.

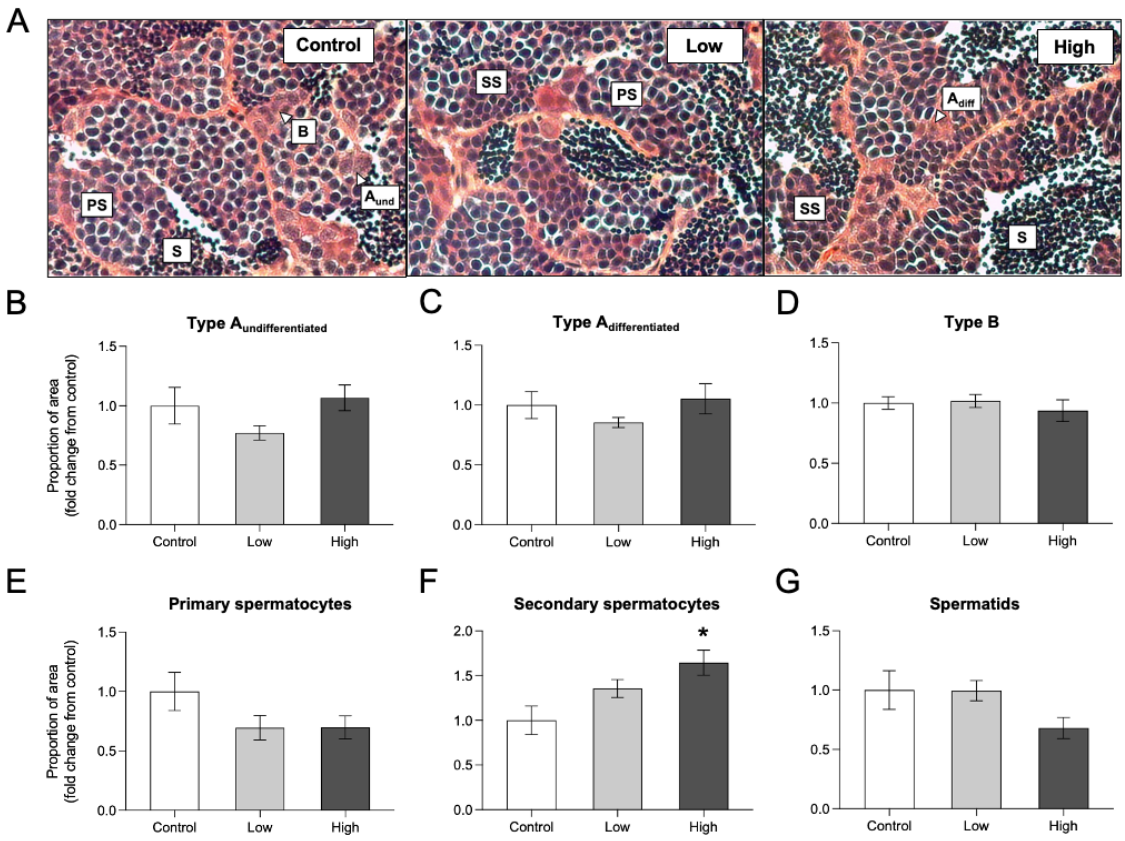

Figure 2.5 Effects of embryonic exposure to UV-P on spermatogenesis of adult male zebrafish. Embryos were exposed via microinjection to doses of < 1.5 ng/g-egg (control), 2.77 ng/g-egg, and 24.3 ng/g egg and reared to maturity in freshwater. (A) Representative histological sections of male zebrafish testes, and (B) proportion of testicular components. Type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (B), primary spermatocytes (PS), secondary spermatocytes (SS), and spermatids (S) are indicated. Parametric data was analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. Non-parametric data was $\log_{10}$ transformed, and if data remained non-parametric, it was analyzed using a Kruskal-Wallis test, followed by a Dunn's post hoc test. Data are represented as mean (± SEM) of six replicates. (*) indicates significant differences from control ($p \leq 0.05$).

2.3.7 Effect of UV-P on Transcript Abundance of Spermatogenic Genes

Transcript abundance of *nanos2* was significantly decreased by 2.0-fold in the low treatment, relative to the control, whereas the 1.88-fold decrease in the high treatment was near statistical significance ($p = 0.06$; **Figure 2.6**). Statistically significant 3.0- and 3.1-fold decreases in transcript abundance of *dazl* was observed in the low and high treatments, respectively, relative to the control (**Figure 2.6**). Abundances of transcripts of *piwil1, insl3, igf3, amh,* and *wnt5a* were not statistically different in UV-P exposed male fish, from the controls.

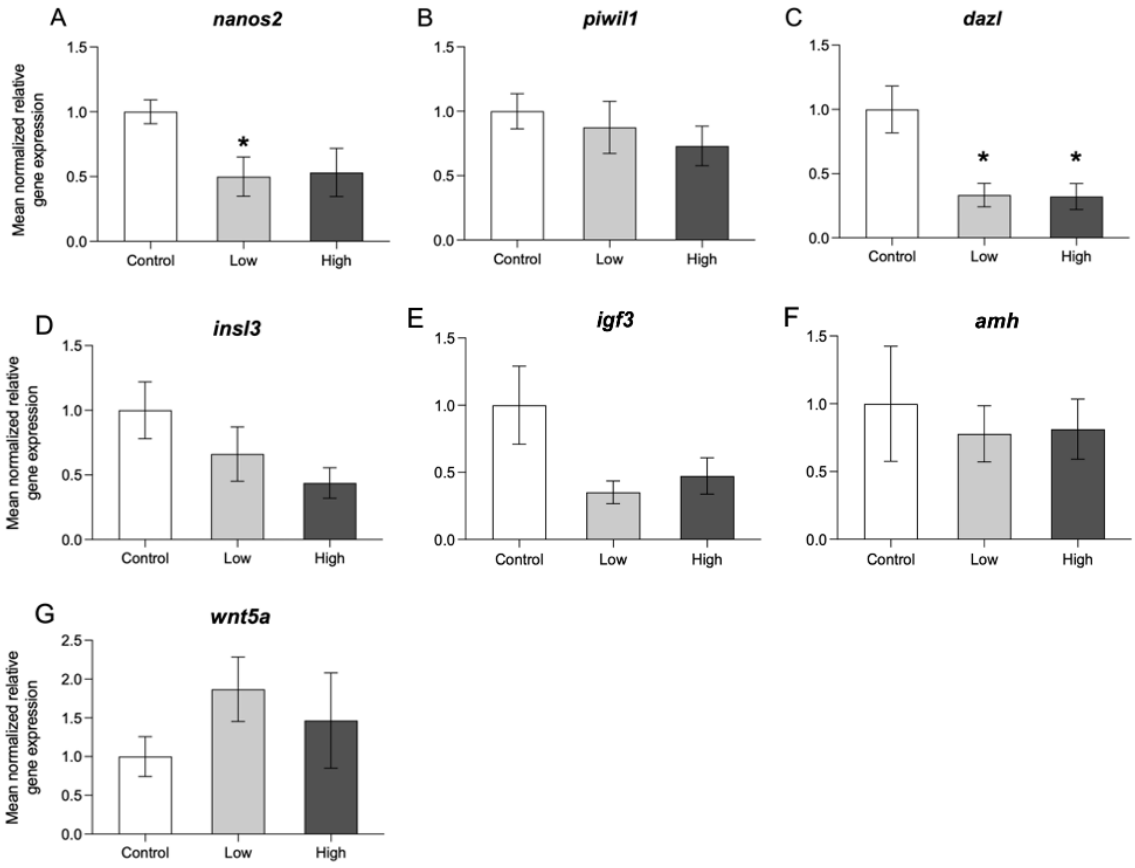

Figure 2.6 Effect of embryonic exposure to UV-P on transcript abundances of genes involved in spermatogenesis in adult male zebrafish. Embryos were exposed via microinjection at doses of < 1.5 ng/g-egg (control), 2.77 ng/g-egg, and 24.3 ng/g egg and reared to sexual maturity in freshwater. (A-G) Abundances of *nanos2, piwil1, dazl, insl3, igf3, amh,* and *wnt5a* transcripts in testes of male zebrafish. Parametric data was analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. Non-parametric data was $\log_{10}$ transformed, and if data remained non-parametric, it was analyzed using a Kruskal-Wallis test, followed by a Dunn's post hoc test. Data are represented as mean (± SEM) of six to eight replicates. (*) indicates significant differences from control ($p \leq 0.05$).

2.4 Discussion

Benzotriazole ultraviolet stabilizers are environmental contaminants that have been detected in a variety of environmental matrices, including aquatic biota. Some BUVSs have endocrine-disrupting potential, however, there is a lack of knowledge regarding the effects of BUVSs on reproduction. The present study investigated the effects of embryonic exposure to environmentally relevant concentrations of UV-P on the reproductive performance of adult zebrafish. In the environment, UV-P has been found at concentrations as great as 81.5 ng/g lipid weight in muscle tissues of fish, however, concentrations of UV-P in fish eggs have not been reported (Kim et al., 2011; Vimalkumar et al., 2018). Thus, doses measured in embryos in the present study – 2.77 ng/g-egg and 24.3 ng/g-egg – fall into the range of reported environmental concentrations of UV-P in biota (Kim et al., 2011; Vimalkumar et al., 2018). It is not clear why measured doses are approximately 40-fold less than nominal. Eggs were frozen at -80°C immediately after injections, meaning biotransformation of UV-P would not have occurred. In addition, there was no evidence that UV-P had precipitated out of solution. Exposure of zebrafish embryos to UV-P impaired fertilization success of adult male zebrafish, however, fecundity of female zebrafish was unaffected. Although a clear mechanism of this effect was not identified, evidence suggests that altered expression of genes involved in sex steroid synthesis and spermatogenesis, as well as decreased abundance of later stages of sperm development, might be playing a role.

Microinjection of embryos with UV-P had negligible effect on embryos. There were no significant effects on cumulative survival or heart rate. There was significant, albeit small (6.7%) increase in prevalence of spinal curvature and uninflated swim bladder in UV-

P-exposed embryos, in comparison to the controls. However, these increases occurred only in embryos exposed to the low treatment of UV-P. In previous studies, UV-P has been reported to be an agonist of the aryl hydrocarbon receptor (AhR) in zebrafish embryos based on greater transcript abundance of cytochrome P450 1A (*cyp1a),* a commonly used biomarker for AhR activation, as well as activation of the zebrafish AhR in a luciferase reporter gene assay (Fent et al., 2014; Johnson et al., unpublished). Activation of the AhR by some xenobiotics can cause developmental malformations, such as spinal curvature, yolk sac edema, and pericardial edema (Dubiel et al., 2022; Elonen et al., 2009; Larigot et al., 2018). In a previous study where zebrafish embryos were injected with UV-P at nominal doses of 4167, 12500, and 37500 ng/g-egg, there was a 13%, 14% and 18% incidences of malformed larvae, respectively (Johnson et al., unpublished). The lower incidences of malformations in the present study are likely due to lower doses injected into the embryos.

Following embryonic exposure of zebrafish to UV-P, fish were reared to sexual maturity in clean water, and reproductive performance was assessed. First, reproductive performance was assessed using male and female fish that had been exposed to UV-P. There were no impacts on fecundity, but fertilization success was significantly decreased by both doses of UV-P. Since fertility is usually associated with males, fertilization success was re-assessed in a second reproduction assay that paired UV-P-exposed males with unexposed females to determine whether the males were responsible for this decline in fertility. Similar to the first reproduction assay, results of this second reproduction assay showed a dose-dependent decrease in fertilization success, though the decrease was statistically significant only in the high treatment. This is the first study that demonstrates impaired reproduction of fish exposed to a BUVS. In a previous study where sexually

mature Japanese medaka were exposed to UV-P via their diet, there was no impacts on fecundity or fertilization success, despite evidence of an antiandrogenic effect based on changes in gene expression and plasma concentrations of E2 and T (Fujita et al., 2022). Reasons for the differences in effects between these two studies are unknown, but it has been demonstrated that critical stages of embryo development are more sensitive to contaminants relative to adult life stages, thus these differences are likely the result of differences in the exposed life stage (Russell et al., 1999).

Previous studies have linked impaired fertilization success in zebrafish to disruption of steroidogenesis resulting in decreased E2 or T, following contaminant exposure (Meng et al., 2023; Qian et al., 2020). Results of the current study suggest that decreased fertility of male zebrafish exposed as embryos to UV-P might be a result of disruption of steroidogenesis. Sex steroids are synthesised from cholesterol, through a series of enzyme mediated reactions. First, cholesterol side-chain cleavage enzyme (CYP11A1) converts cholesterol to pregnenolone, which is a precursor for the synthesis of sex steroids (Bacila et al., 2021; Young et al., 2005). Testosterone can be converted to E2 by aromatase (CYP19A), or it can be converted to 11-KT in a multi-step pathway involving CYP11C1 and HSD11β2 (Bacila et al., 2021; Young et al., 2005). However, the dominant pathway for synthesis of 11-KT in zebrafish involves HSD17β3 (de Waal et al., 2008; Oakes et al., 2020; Tokarz et al., 2015). Binding of 11-KT to ARs initiates spermiation, allowing immature spermatids to differentiate and become mature, flagellated spermatozoa (Golshan & Alavi, 2019; Schultz et al., 2010; Schulz & Miura, 2002). Transcript abundances of *cyp11a1, cyp19a1a,* and *cyp11c1* were decreased by 1.4- to 1.8-fold in testes from males exposed to UV-P, relative to the control, although these changes were not statistically

significant. Transcript abundance of *hsd17β3* was decreased by 1.48- and 2.5-fold in testes of male fish exposed as embryos to the low and high dose of UV-P, respectively, with the decrease being statistically significant in the high treatment. There were no changes in abundances of transcripts of *hsd11β2* or *ar* in testes of fish exposed to UV-P. Decreases in transcript abundances were small (< 3-fold) and generally not statistically significant, but there was a maximal 50.0% decrease in E2. However, there was a maximal 32.4% increase in 11-KT, which is opposite of what would be expected if the decreased transcript abundances of *cyp11c1* and *hsd17β3* resulted in decreased protein abundances. The absence of any changes in abundances of transcripts of *hsd11β2* and *ar* suggests that embryonic exposure to UV-P likely did not inhibit synthesis of 11-KT or decrease AR signaling (Tokarz et al., 2015). In sexually mature male three-spot wrasse (*Halichoeres trimaculatus*) exposed to exemestane, an aromatase inhibitor, concentrations of E2 were decreased, concentrations of 11-KT were increased, and although there was an impairment in spermatogonial proliferation of the testis which was able to be rescued by exogenous E2, fertility was not determined (Kobayashi et al., 2010). These patterns in hormone concentrations are like what were observed in the present study and could result from the decreased transcript abundance of *cyp19a1a*. However, a study with aromatase-deficient zebrafish found no significant changes in fertilization success (Tang et al., 2017). Other studies involving chemical inhibition of aromatase also found no impairment of fertility, although impairments in hatching rate of embryos fertilized from exposed males, irreversible damage to sperm, and sperm motility, thus impacting sperm quality were observed (McAllister & Kime, 2003; Thresher et al., 2011).

Another mechanism by which embryonic exposure to UV-P might have impaired fertilization success is by interfering with the expression of genes regulating sperm development and quantity. Expression of several genes, such as PIWIL1, WNT5A, and NANOS2, is essential for early spermatogenesis. PIWIL1 is predominantly expressed in Type A_{und} and type A_{diff} spermatogonia and is a marker for early stages of spermatogenesis because it has major functions in germ cell maintenance (Almeida et al., 2022; Houwing et al., 2007; Ye et al., 2023). Abundance of transcripts of *piwil1* was decreased by 1.4-fold in the high treatment, but the change was not statistically significant. Additionally, expression of *wnt5a*, which is involved in spermatogonial self-renewal, was not different in UV-P exposed males (Safian et al., 2018b). The RNA-binding protein, NANOS2, is required for the maintenance and renewal of germline stem cells and is highly expressed in type A_{und} spermatogonia (Sada et al., 2009; Suzuki & Saga, 2008). Embryonic exposure to UV-P decreased transcript abundance of *nanos2* in the low and high treatments by 2.0-fold and 1.9-fold, respectively, where the change in the low treatment was statistically significant. However, the abundance of neither type A_{und} nor type A_{diff} spermatogonia was altered in testes of UV-P-exposed males. The quantity of type A_{und} and A_{diff} spermatogonia is controlled by balancing increased self-renewal of type A_{und} spermatogonia, and spermatogonia proliferation forming type A_{diff} and type B spermatogonia (Schulz et al., 2010). Given the lack of any change in abundance of type A spermatogonia, either there was no corresponding change in protein levels or larger decreases in transcript abundance are needed to drive decreases in type A_{und} spermatogonia. Overall, these results suggest that decreased fertility of male zebrafish exposed as embryos to UV-P are not due to decreases in spermatogonial self-renewal.

There is evidence that embryonic exposure to UV-P might have impacted the mitotic phase of spermatogenesis. Insulin-like peptide 3 (INSL3) and insulin-like growth factor-3 (IGF3) are both involved in recruiting and signalling spermatogonia into differentiation (Assis et al., 2015; Morais et al., 2017; Safian et al., 2018a). Although not statistically significant, transcript abundance of *insl3* was decreased by 1.5-fold and 2.3-fold in the low and high treatment groups, respectively. Additionally, transcript abundance of *igf3* was decreased by 2.8-fold and 2.1-fold in testes of males from the low and high treatments, respectively, however these were not statistically significant. Based on these findings, a decrease in the number of type A_{und} spermatogonia differentiating into type A_{diff} spermatogonia, thus decreasing the number of later developmental stages, would be expected, but was not observed. The function of AMH in the testis is to inhibit the action of INSL3 and 11-KT in promoting differentiation/proliferation of spermatogonia (Safian et al., 2019; Skaar et al., 2011). Transcript abundance of *amh* was not different in zebrafish exposed to UV-P. Previous studies have shown that knockdown of expression of the IGF3 gene in Common Carp (*Cyprinus carpio*) increased concentrations of 11-KT in blood plasma, increased in the proportion of earlier stages of sperm development present in testis and led to a lesser number of spermatids (Song et al., 2021). This is consistent with the decreased abundance of spermatids and increased concentrations of 11-KT in UV-P-exposed fish, relative to the control, observed in the present study. Type B spermatogonia represent the most proliferated stage prior to meiosis (Schulz et al., 2010). Abundance of the RNA-binding protein, DAZL, is increased in type B spermatogonia relative to abundance in early stages of primary spermatocytes (Chen et al., 2013; Oakes et al., 2019; Saunders et al., 2003). Previous research has determined that expression of DAZL is critical for entry of germ cells into meiosis during germ cell development (Bertho et al., 2021;

Chen et al., 2013; Li et al., 2016; Saunders et al., 2003). In mice, knockout of *dazl* inhibited progression of germ cell development following meiosis (Saunders et al., 2003). In the present study, transcript abundance of *dazl* was significantly decreased by 3.0- and 3.1-fold in testes of males from the low and high treatments, respectively. However, there was no change in abundance of type B spermatogonia in testes of males exposed to UV-P, relative to the control. This suggests that embryonic exposure to UV-P could be impairing spermatogenesis by preventing entry into meiosis, which could be reflected by decreased abundance of later developmental stages, including primary and secondary spermatocytes, as well as spermatids. In the present study, abundance of primary spermatocytes was non-significantly decreased by 1.4-fold in both treatment groups, abundance of secondary spermatocytes was significantly increased by 1.6-fold, and abundance of spermatids was non-significantly decreased by 1.5-fold, in fish exposed as embryos to the high dose of UV-P. The decrease in primary spermatocytes could potentially be due to decreases in *dazl* expression, thus, preventing type B spermatogonia from entering meiosis and forming primary spermatocytes 11-ketotestosterone is critical for the maturation of gametes, as it ultimately signals the formation of spermatids (Schulz et al., 2010). The present study observed a small increase in blood plasma concentrations of 11-KT in fish exposed to UV-P, however, this change was not consistent with the observed decrease in the abundance of spermatids. Therefore, results suggest this impairment is likely not due to changes in 11-KT synthesis. Consequently, the production of secondary spermatocytes might be increased to compensate for the decrease in primary spermatocytes and spermatids.

The present study determined that embryonic exposure to UV-P decreased reproductive performance of male zebrafish, and therefore attempted to identify the

potential mechanism(s) of this effect by investigating disruption of spermatogenesis via changes in steroidogenesis and expression of genes related to sperm development and quantity, but the precise mechanism remains unclear. The observed impairment in reproduction aligns with the small and often not statistically significant changes in gene expression and plasma hormone concentrations, thus making it difficult to elucidate a specific mechanism of effect. However, these small changes in gene expression and hormone concentrations are likely due to the use of doses that have been measured in biota in some locations, as well as the relatively small change in fertilization success observed. Thus, future studies are needed to pinpoint the mechanism(s) by which embryonic exposure to UV-P impairs zebrafish reproduction. As indicated by the present study, there is potential for UV-P to act on multiple pathways, all of which might contribute to impaired fertility. To elucidate the mechanism(s) by which UV-P acts, future research should explore the molecular regulation of post-mitotic stages of spermatogenesis, such as quantifying LH and MIH. Also, although it was observed that sperm quantity is likely being decreased (as indicated by decreased abundance of primary spermatocytes and spermatids), decreased sperm quality can also play a role in decreased fertility. Effects of embryonic exposure to UV-P on sperm quality parameters, such as sperm motility, sperm volume and concentration, seminal plasma pH, osmolality, membrane stability, and enzymatic activity should be measured in future studies (Kowalski & Cejko, 2019). Regardless of the precise mechanism(s), this study demonstrates the potential for BUVSs to impair reproductive success in fishes via decreased fertility of male fish. However, the 9-15% decrease in fertilization success observed in the present study would be unlikely to cause declines in populations, as 20-30% decreases in number of offspring have been proposed as being required for population level effects (Conolly et al., 2017). However, if concentrations of

UV-P in aquatic systems continue to increase, there could be potential for population level effects via decreased fertility of males. Further, other BUVSs, such as 2-(5-*tert*-Butyl-2-hydroxyphenyl)benzotriazole (UV-PS), 2-[2-Hydroxy-5-[2-(methacryloyloxy)ethyl]phenyl]-2*H*-benzotriazole (UV-090), 2-[3,5-Bis(1-methyl-1-phenylethyl)-2-hydroxyphenyl]-2*H*-benzotriazole (UV-234), 2-(2-Hydroxy-3-*tert*-butyl-5-methylphenyl)-5-chlorobenzotriazole (UV 326), 2-(2-Hydroxy-5-*tert*-octylphenyl)-benzotriazole (UV-329), 2-(3-*s*-Butyl-5-*tert*-butyl-2-hydroxyphenyl)benzotriazole (UV-350), and UV-328 can act as anti-androgens (Sakuragi et al., 2021; Zhuang et al., 2017). Thus, compared to single compounds, exposure to multiple BUVSs might have greater impacts on fertility of male fish. Additionally, differences in sensitivity to endocrine disruption are known to exist among species of fishes, so species that are more sensitive than zebrafish to anti-androgenicity could be more greatly impacted by doses used in the present study (Ankley & Johnson, 2004). Thus, assessments of the effects of UV-P on reproductive performance of adult male fish following embryonic exposure should be extended to other fish species. Finally, the role of the epigenome in the observed decreases in fertilization success should be investigated. Specifically, the DNA methylome of zebrafish undergoes a period of remodelling following fertilization, where the maternal methylation pattern is erased and the paternal pattern is inherited (Wang et al., 2019; Potok et al., 2013). This reprogramming is sensitive to disruption by contaminants, and because changes in DNA methylation are mitotically stable, alterations to the methylome can have effects that extend across life-stages and generations (Chen et al., 2019; Major et al., 2020; Xu et al., 2023). Future studies should also assess whether embryonic exposure to UV-P causes multigenerational or transgenerational effects via alterations to the DNA methylome.

2.5 Conclusion

Overall, embryonic exposure of zebrafish to UV-P impaired the reproductive performance of male zebrafish by decreasing fertilization success. Small but consistent decreases in abundances of transcripts that regulate sex steroid synthesis indicate disruption of the steroidogenic pathway. Additionally, there was evidence that ELS exposure to UV-P disrupted spermatogenesis, potentially reducing the quantity of sperm produced. However, further research is needed to determine the mechanism by which embryonic exposure to UV-P impairs fertility, specifically, the molecular regulation of post-mitotic stages of spermatogenesis, as well as the investigation of sperm quality. This study is the first to demonstrate that an embryonic exposure to an environmentally realistic dose of a BUVS impaired reproductive performance of a fish species, the zebrafish. Because of the ubiquitous presence of BUVS in freshwater ecosystems, and the strong likelihood that concentrations of these chemicals will continue to increase, future research is needed to fully understand the risk BUVSs pose to fishes and other aquatic biota.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSION

3.1 Introduction

The leaching of anthropogenic chemicals into aquatic ecosystems poses a threat to various aquatic biota, such as fish. These contaminants can adversely affect various life stages and latent effects can occur following a single exposure. However, the majority of studies in aquatic ecotoxicology focus on the direct effects of exposure on either early life or adult stages. It has been proposed that early life stages, specifically critical stages of embryo development, are more sensitive to toxicants than adult stages (Russell et al., 1999). Additionally, there is growing recognition that exposure to environmental stressors during early life can have long-term effects that persist until or manifest later in life, such as impairments in the development and reproductive performance of adults (Heindel et al., 2015; Jonsson & Jonsson, 2014). Embryonic exposure to endocrine disrupting chemicals (EDCs) – compounds that interfere with the normal functioning of the endocrine system – have been shown to impair essential biological processes later in life (Liao et al., 2014; Tao et al., 2023; Xu et al., 2023). Benzotriazole ultraviolet stabilizers (BUVSs) are a class of anthropogenic chemicals incorporated into personal care products, plastics, and textiles, and works to prevent degradation induced by UV light (Zhang et al., 2011). The BUVS, 2-(2H-benzotriazol-2yl)-4-methylphenol (UV-P), has been shown to act as an EDC by displaying estrogenic and antiandrogenic activity in *in vitro* studies using mammalian receptors (Feng et al., 2020; Fent et al., 2014; Sakuragi et al., 2021; Zhaung et al., 2017). The objectives of this research were to investigate the effects of UV-P on the reproductive success of adult zebrafish following embryonic exposure, as well as to determine the molecular mechanism(s) of effect(s). Since UV-P has been shown to display endocrine disrupting properties and early life exposure of EDCs have been linked to adverse effects

later in life, it was hypothesized that embryonic exposure to UV-P will cause reproductive impairments at adult stages.

3.2 Summary of Study

In this study (Chapter 2), zebrafish embryos were injected with either a control (DMSO), a low (100 ng/g-egg), or high (1000 ng/g-egg) dose of UV-P. Following injections, a subset of embryos were reserved for embryotoxicity assessments, including measurements of survival, heart rate, and developmental malformations. The remaining embryos were reared in clean water until sexual maturity, where reproductive performance was assessed using short-term reproduction assays where fecundity and fertility were measured daily. Upon termination of reproduction assays, fish were euthanized, and tissues and blood plasma were stored for analysis of molecular and biochemical endpoints. Enzyme-linked immunosorbent assays (ELISAs) were used to measure concentrations of 17β-estradiol (E2) and 11-ketotestosterone (11-KT) in blood plasma, and quantitative real-time PCR was performed to determine the expression of genes responsible for the regulation of reproduction, specifically, spermatogenesis-related genes and genes involved in the hypothalamic-pituitary-gonadal (HPG) axis. Gonads were used for histological analysis. Doses of injected embryos were determined using gas chromatography-mass spectrometry (GC-MS).

Measured doses of UV-P in embryos were 2.77 ng/g-egg and 24.25 ng/g-egg for the low and high treatments, respectively. In terms of embryotoxicity, there was no change in survival and heart rate across all treatments of embryos exposed to UV-P. However, both incidences of swim bladder inflation and spinal curvature were significantly increased in the low treatment embryos, although the observed magnitude of change was small (less

than 15%) Embryonic exposure to UV-P did not affect fecundity of female fish. However, when exposed male and female fish were used in the reproduction assay, fertilization success was significantly decreased by 8.75% and 15.02% in the low and high treatments, respectively, relative to controls. Due to this decrease in fertilization success, a second reproduction assay was performed with males exposed to UV-P and unexposed females, to determine whether the males were responsible for the decrease in fertility. There was no change in fecundity, however, there was an 11.47% decrease in fertility in the high treatment, relative to the control.

Mechanistically, transcript abundances of genes involved in the HPG axis indicated slight disruption of steroidogenesis. Specifically, gene expression of 17b-hydroxysteroid dehydrogenase 3 (*hsd17b3*) was significantly decreased in the high treatment, whereas expression of cholesterol side-chain cleavage enzyme (*cyp11a1*), aromatase (*cyp19a1a*), and 11b-hydroxylase (*cyp11c1*) were also decreased in the testes from male fish in the high treatment, although not statistically significant. Additionally, plasma concentrations of E2 were maximally decreased by 50%, although the changes were not statistically significant. Overall, consistent decreases, albeit small, in abundances of transcripts that regulate sex hormone synthesis were observed – pointing to alterations in steroidogenesis in male fish exposed to UV-P, as embryos.

Further analyses indicated that UV-P might have impaired spermatogenesis by interfering with the expression of genes involved in sperm development and quantity. Transcript abundance of *nanos2*, a gene required for the maintenance of germline stem cells, was decreased in the low and high treatments by 2.00-fold and 1.88-fold, respectively, following UV-P exposure. Additionally, transcript abundances of *piwil1*, a gene also

critical for germ cell maintenance, was shown to decrease following exposure to UV-P, however, was not statistically significant. These changes in gene expression were not reflective of the quantity of early stages of sperm development since abundances of Type A undifferentiated and Type A differentiated spermatogonia were not changed among treatments. Transcript abundances of genes involved in the mitotic phase of spermatogenesis – including *igf3* and *insl3* – were decreased, albeit not significantly, following UV-P exposure. However, the relative expression of *dazl*, which is predominantly expressed in Type B spermatogonia and is critical for entry of germ cells into meiosis, was significantly decreased by 3.00- and 3.11- fold in males from the low and high UV-P treatments, respectively. Although there were no changes in the abundances of Type B spermatogonia in fish exposed to UV-P, there were non-significant decreases of later stages of development, including primary spermatocytes and spermatids. This suggests that decreases in primary spermatocytes could be due to decreases in *dazl* expression, preventing type B spermatogonia from entering meiosis and forming primary spermatocytes. A significant increase in secondary spermatocytes was observed, which could be a compensatory response to the decreased abundance of primary spermatocytes and spermatids.

Taken together, this study demonstrated that exposure of early life stage zebrafish to UV-P impairs fertility of adult male fish. While the precise mechanism of action is not known, this study suggested that UV-P caused this effect by disrupting spermatogenesis via alterations in steroidogenesis and the expression of genes related to sperm development and quantity.

3.3 Exposure to Antiandrogens in Early Life

Antiandrogens exert their action by antagonizing androgen receptors (ARs), or inhibiting the synthesis/signalling of androgens, thus, decreasing or preventing their biological effects (Barakat & Ko, 2018). The BUVS, UV-P, has been reported to display antagonistic activity toward the human AR (Fent et al., 2014; Sakuragi et al., 2021; Zhaung et al., 2017). Additionally, adult Japanese medaka exposed to UV-P exhibited patterns of gene expression and plasma hormone concentrations consistent with an antiandrogenic mode of action (Fujita et al., 2022). This thesis did not investigate the binding affinity of UV-P to zebrafish ARs, however, expression of *ar* remained unchanged in male testes across treatments, following UV-P exposure. A small number of studies have investigated antiandrogen exposure and the manifestation of latent effects at later life stages in fish. Exposure of juvenile zebrafish to the antiandrogenic compound, vinclozolin, resulted in sex ratios skewed towards females, and impaired gonad development and fertility later in life (Lor et al., 2015). Additionally, exposure of juvenile guppies (*Poecilia reticulata*) to the antiandrogens, vinclozolin, *p,p'*-DDE (a persistent DDT metabolite), and flutamide, was associated with adverse effects at sexual maturity, such as skewed sex ratio, reduced sexual courtship behaviour, changes in sexual display coloration, and decreased sperm count – however, mechanism(s) of action were not investigated (Bayley et al., 2002). It has been proposed that underlying mechanisms of antiandrogen toxicity following juvenile exposure can not only be related to antagonism of the AR, but also imbalances of critical hormones, such as estrogens and androgens (Lor et al., 2015). There is a lack of studies surrounding the effects of antiandrogenic chemicals on embryonic stages of fish, and the implications of early life exposure, later in life. Consequently, mechanism(s) by which latent effects of antiandrogenic contaminants occur following exposure in early life, are not

known. Although the mechanism of action of UV-P is unclear in the present study, the decrease in fertility and impairment of spermatogenesis observed are common adverse outcomes of antiandrogen exposure in adult fish. Future studies should not only assess the antiandrogenic activity of UV-P in zebrafish via assessment of binding affinity to the AR, but also further investigate the mechanism through which UV-P exerts latent effects in fish. Additionally, embryonic exposure studies using model antiandrogens (e.g., flutamide, vinclozolin) should be conducted to not only understand effects later in life, but also the mechanism(s) of action of antiandrogens in causing latent effects. Comparisons can be drawn between model antiandrogens and chemicals of interest, such as UV-P, to understand common mechanisms of antiandrogenic activity.

3.4 Mixture Effects on Reproductive Performance

Aquatic biota are rarely exposed to a single chemical. Rather, due to the leaching of anthropogenic chemicals from various sources into aquatic ecosystems, biota, such as fish, are usually exposed to complex mixtures of contaminants. Therefore, studies on the effects of complex mixtures are important. Depending on the mechanism(s) of action of individual contaminants, mixtures can result in synergistic or additive effects. Additive effects refer to the effect(s) of a mixture equalling the action of each individual compound, whereas synergistic effects refer to the combined effect(s) being greater than each compound acting individually (Olszowy-Tomczyk, 2020). Studies have reported the effects of various contaminant mixtures on the endocrine system, specifically, the capability of mixtures to impair reproductive performance of fish. For instance, waterborne exposure of fathead minnow (*Pimephales promelas*) to a mixture of copper and nickel resulted in additive effects on cumulative egg production, decreased vitellogenin expression, and

alterations in ovarian development (Driessnack et al., 2017). Similarly, exposure of brown trout (*Salmo trutta fario*) to an environmentally relevant mixture of estrogens (estrone, 17β-estradiol, 4-nonylphenol) resulted in an increase in male vitellogenin production and significantly impaired fertilization success (Schubert et al., 2008). In aquatic ecosystems, the BUVS, UV-P, often co-exists with other BUVSs, several of which have antiandrogenic activity (Sakuragi et al., 2021; Zhaung et al., 2017). Therefore, future studies should investigate whether exposure of embryos to mixtures of antiandrogenic BUVSs cause synergistic or additive effects in fish, resulting in greater effects on fertilization success than observed in the present study.

3.5 The Epigenome as a Target of UV-P

Further investigation is needed to pinpoint the mechanism(s) through which embryonic exposure to UV-P caused decreased fertility of male zebrafish. One hypothesis is that this effect is caused by UV-P-induced changes in the epigenome, which can lead to decreases in expression of regulators critical in spermatogenesis, such as *igf3, nanos2,* and *dazl*.

The term "epigenome" refers to the record of chemical modifications that occur "above" the genome, therefore, not altering the DNA sequence itself, but instead altering how the sequence is read (Head et al., 2012). The environment an organism resides in and the experiences it has are largely influential over the presence of epigenetic marks. In the field of ecotoxicology, there is growing recognition that the epigenome can be altered by anthropogenic contaminants, and these modifications or epigenetic marks have the potential to persist long-term (Head et al., 2012; Lempradl, 2020). Exposure to chemical stressors during critical periods of development have been shown to have long-lasting

effects that span multiple life stages, and potentially even generations (Kundakovic & Champagne, 2015). Among the epigenetic pathways that can be altered following chemical exposure, histone modifications and DNA methylation are important for spermatogenesis (Ge et al., 2017; Günes & Kulaç, 2013).

DNA-histone complexes, also known as chromatin, are important regulators of gene expression. Chromatin exists as either heterochromatin that is a condensed and "closed" structure that represses transcription, or euchromatin that is a more "open" structure that is highly transcriptionally active (Morrison & Thakur, 2021). Modifications to histones can alter their physical interactions with DNA, thus promoting or repressing transcription (Lempradl, 2020). These modifications consist of the addition or removal of chemical groups on histone amino-acid tails, which include acetyl, phosphate, and methyl groups (Bannister & Kouzarides, 2011; Kundakovic & Champagne, 2015). Addition of acetyl groups by histone acetyltransferases (HATs) to the positively charged lysine group of histone tails, masks the positive charge of histones thus reducing the attraction to the negatively charged DNA backbone (Bannister & Kouzarides 2011; Imhof, 2006). Reduced attraction between histones and DNA will allow chromatin to adapt a more open confirmation, promoting transcription (Imhof, 2006). Similarly, histone phosphorylation adds a negatively charged phosphate group to a hydroxyl group of an amino-acid in the histone tail, which promotes electrostatic repulsion between DNA and the histone (Bannister & Kouzarides 2011). Finally, histone methylation often results in decreased gene expression, which is due to the addition of methyl groups onto histones, preventing transcriptional machinery from accessing DNA (Bannister & Kouzarides 2011). Decreases

in gene expression following toxicant exposure is more likely due to histone methylation, as opposed to histone acetylation and phosphorylation.

In the field of ecotoxicology, out of the epigenetic mechanisms involved in gene expression regulation, DNA methylation is the most studied (Head, 2014). It involves the transfer of a methyl group, by DNA methyltransferases (DNMTs), to the C5 position of cytosine which exists in a cytosine-guanine dinucleotide (CpG; Jin et al., 2011). Methylation of promoters can silence gene expression by blocking the binding of transcription factors, or by recruiting methylated DNA-binding domain proteins (MBD) which can further recruit histone-modifying complexes resulting in inactive chromatin configurations (Szyf, 2011). Alternatively, the removal of normal methylation patterns, also referred to as DNA hypomethylation, has been associated with genomic instability, chromosomal rearrangements, and increased expression of unfavorable genes (Baccarelli & Bollati, 2011). Exposure of organisms to contaminants can alter DNA methylation patterns, in turn, causing genome instability or altering gene expression patterns.

Epigenetic modifications, including DNA methylation and histone modifications, are essential regulators of spermatogenesis and are necessary for differentiation to mature spermatozoa (Ge et al., 2017; Günes & Kulaç, 2013). For instance, hypomethylation of promoters of genes involved in stages of sperm development allows for expression during critical periods (Ge et al., 2017; Günes & Kulaç, 2013; Rajender et al., 2011). Modifications that alter the DNA-binding capacity of histones are also essential for regulating gene expression, such as methylating histone tails (Ge et al., 2017; Günes & Kulaç, 2013). Additionally, testis-specific histone variants are expressed during certain phases of spermatogenesis, such as histone H2B expressed in mature sperm, suggesting important

roles during developmental changes (Ge et al., 2017; Günes & Kulaç, 2013; Rajender et al., 2011). Since the process of spermatogenesis is heavily regulated by epigenetic mechanisms, it is particularly susceptible to disruption by environmental factors (e.g., toxicant exposure). Although limited, studies have linked chemically induced impairment of spermatogenesis and fertility to alterations in the epigenome. For instance, exposure of male zebrafish to bisphenol A significantly impaired fertilization capacity, decreased the number of spermatocytes, and dysregulated genes involved in sperm development – all of which contributed to disruption of spermatogenesis (Gonźalez-Rojo et al., 2019). This alteration in spermatogenesis was suggested to be linked to changes in the epigenome, such as, dysregulation of epigenetic remodelling enzyme transcripts, promotion of DNA methylation and demethylation, hyperacetylation of histones (H3K9ac, H3K14ac, H4K12ac), and chromatin fragmentation (Gonźalez-Rojo et al., 2019). Further investigation is needed on the role of the epigenome when investigating disruptions of spermatogenesis in fish caused by chemical exposures.

In early life stage organisms, extensive changes in the epigenome occur. This is due to the transfer of epigenetic marks from parents to offspring, where epigenetic modifications from parental gametes are usually cleared and re-established following fertilization (Jiang et al., 2013). However, the process of epigenetic reprogramming differs depending on the species. Out of all the epigenetic mechanisms, changes to patterns of DNA methylation are most extensively studied. Zebrafish embryos undergo a unique pattern of DNA methylation reprogramming, where both maternal and paternal DNA with oocyte and sperm methylomes, respectively, are stably inherited until the 16-stage cell (Jiang et al., 2013). Following this, the oocyte methylome is erased via passive DNA

demethylation, and then reprogrammed to patterns similar to the sperm methylome via *de novo* methylation (Jiang et al., 2013; Potok et al., 2013). By the mid-blastula stage, the embryo has inherited the sperm methylome (Jiang et al., 2013; Potok et al., 2013). Since the blastula period of zebrafish embryo development lasts until 5.25 hours post-fertilization (hpf), mitotically and meiotically stable alterations in the methylome (induced by anthropogenic chemicals) during this period of reprogramming can result in long-lasting and heritable effects (Kimmel et al., 1995). The present study exposed zebrafish embryos to UV-P and observed potential disruption of spermatogenesis, leading to impaired fertility. Embryos were exposed between 1-4 hpf, therefore, UV-P could have impacted the process of methylation reprogramming. Additionally, decreased expression of spermatogenesis-related genes, including *igf3, nanos2,* and *dazl,* was observed. Hypermethylation of promoter regions of these genes might have been induced by UV-P, thus, decreasing their expression and disrupting spermatogenesis. Therefore, future studies should investigate DNA methylation patterns of genes involved in spermatogenesis in zebrafish exposed as embryos to UV-P or mixtures of antiandrogenic BUVSs. Due to the inheritance of sperm methylomes in zebrafish embryos, potential hypermethylation of the germline following UV-P exposure might be transferred to offspring (Stewart et al., 2016). Due to this, UV-P has the potential to cause multigenerational and transgenerational effects via the passing of epigenetic marks through the sperm methylome. Additional research should be conducted to investigate the potential of UV-P to cause multigenerational and transgenerational effects following embryonic exposure, and the associated changes in the epigenome.

3.6 An Adverse Outcome Pathway for Inhibition of Expression of Spermatogenesis-Related Genes, Resulting in Decreased Fertility

In the field of toxicology, there has been a shift from animal testing to the use of new approach methods (NAMs) to replace, reduce, and refine toxicity testing using animals and meet demands of assessing chemicals quickly and effectively (Lambert, 2022). Adverse outcome pathways (AOPs) are mechanistic tools designed to link the interaction of a chemical with a molecular target, to toxicological effects across different biological organization levels, and ultimately adverse outcome(s) at an individual or population level (Vinken et al., 2017). Specifically, AOPs consist of a molecular initiating event (MIE; interaction between chemical and biomolecule), a series of key events (KEs) at various levels of biological organization, and one or more adverse outcomes (AOs) at individual and population levels (Ankley et al., 2010; Villeneuve et al., 2014; **Figure 3.1**). These independent events are connected to one another by key event relationships. An AOP is not chemical specific and can describe the effects of many chemicals – as long as the same MIE is activated, it is assumed that the same KEs and AOs can occur (Villeneuve et al., 2014). Multiple AOPs with the same MIE, KEs, or AOs can be aggregated to form adverse outcome networks (AONs; Villeneuve et al., 2014; **Figure 3.1**). Adverse outcome networks allow for prediction of real-world situations since mixtures of chemicals are found in the environment (thus, expanding across multiple AOPs), and if only a single chemical was found, it is unlikely that only a single pathway would be impacted (Ankley et al., 2010; Villeneuve et al., 2014). Furthermore, AOPs and AONs can be utilized to predict the adverse effects of chemicals relevant to risk assessment and regulatory policies (Villeneuve et al., 2014).

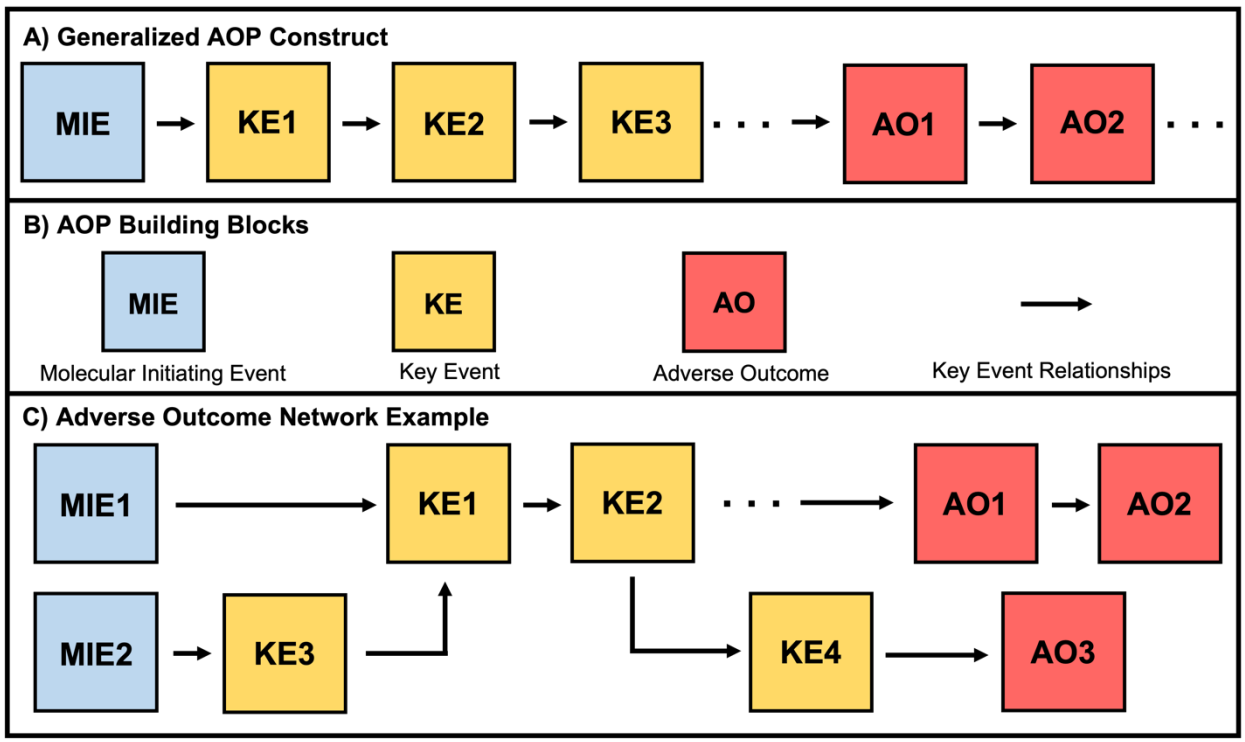

Figure 3.1 Depiction of a generalized adverse outcome pathway (**A**). The essential components of AOP are molecular initiating events (MIEs), key events (KEs), and adverse outcomes (AOs; **B**). Separate AOPs can be combined at points where MIEs, KEs, and AOs are shared, forming adverse outcome networks (AONs; **C**). Figure adapted from Villeneuve et al. (2014).

Adverse outcome pathways that link 11β-hydroxysteroid dehydrogenase (11β-HSD) inhibition (AOP #348, AOP-Wiki), peroxisome proliferator-activated receptor alpha (PPARα) activation (AOP #323, AOP-Wiki), or mammalian AR antagonism (AOP #19, AOP-Wiki) to impaired fertility of males have been proposed. However, there are no proposed AOPs that link epigenetic alterations to disruption in spermatogenesis and impaired fertility. Therefore, a putative AON, comprising three AOPs, is proposed for decreased fertility of male zebrafish following embryonic exposure to UV-P (**Figure 3.2**). In this AON, exposure of embryos to UV-P led to alterations in DNA methyltransferase activity causing hypermethylation of spermatogenesis-specific genes (*igf3, nanos2, dazl*), thus decreasing their expression, disrupting spermatogenesis, and ultimately leading to impaired fertility. However, further research should be conducted to determine whether decreases in gene expression of spermatogenesis-related genes is due to hypermethylation. Ultimately, hypermethylation of genes can result in decreased gene transcription leading to decreased protein abundance, resulting in insufficient development and quantity of sperm. This AON, following further investigation, has potential to be applied to different chemicals and species.

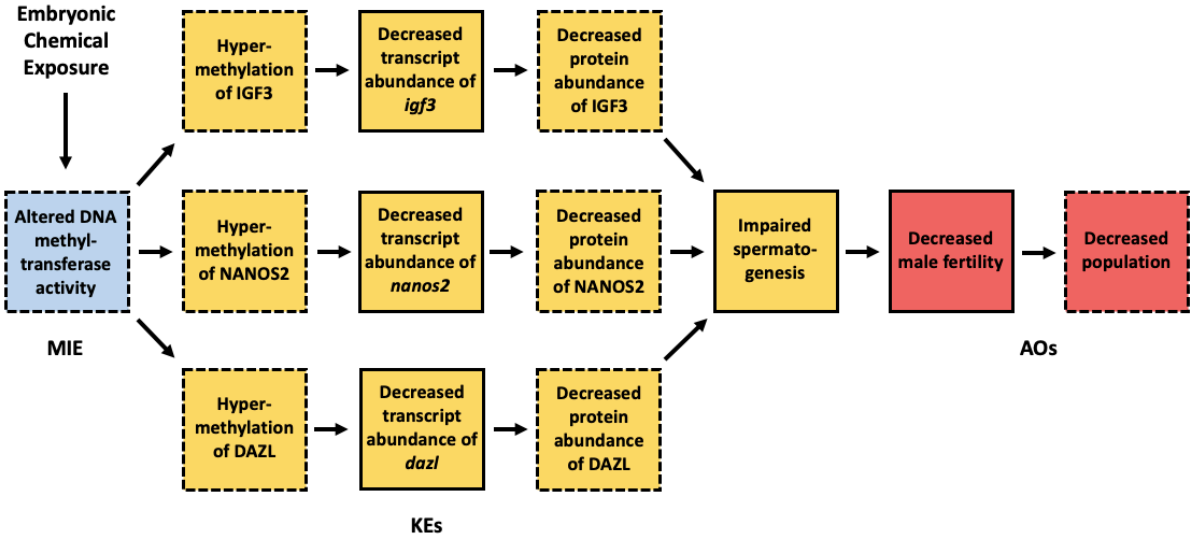

Figure 3.2 A putative adverse outcome network (AON) for decreased fertility resulting from impairment of spermatogenesis. The MIE is predicted to be altered DNA methyltransferase activity, ultimately resulting in hypermethylation of genes involved in spermatogenesis (IGF3, NANOS2, and DAZL). Hypermethylation can then lead to decreased transcript and protein abundances, which can lead to disruptions in spermatogenesis via irregular sperm development and production. Inadequate quantity and development of sperm would result in decreased fertilization capacity of male fish. Boxes outlined with solid lines represent events confirmed by the present study, whereas boxes outlined with dashed lines represent unknown/hypothesized events.

3.7 Conclusion

This study expands our understanding on the potential adverse effects of UV-P on aquatic biota. Although environmental concentrations of UV-P have been reported to be low – up to 31 ng/L in surface waters and 81.5 ng/g lipid weight in aquatic biota – there is potential for them to rise alongside increased usage and production (Kim et al., 2011; Kameda et al., 2011; Vimalkumar et al., 2018). Therefore, it is critical to understand the potential threats of UV-P, and other BUVSs, on the health of fishes. This research provides insight into the adverse effects UV-P can induce in fish, specifically via disruption of the endocrine system. Studies have suggested that UV-P can act as an EDC by exhibiting estrogenic and antiandrogenic activity, however, this was largely determined by *in vitro* studies using mammalian receptors (Feng et al., 2020; Fent et al., 2014; Sakuragi et al., 2021; Zhaung et al., 2017). The present study found that although UV-P caused very little embryotoxicity, a more substantial effect – impaired fertility of sexually mature male fish – was observed. This reproductive impairment was linked to small and consistent changes in the expression of genes involved in steroidogenesis and spermatogenesis. This change in gene expression was reflective of observed decreases in abundances of post-meiotic stages of sperm development. Overall, it was demonstrated that changes in spermatogenesis are likely responsible for impairments in fertilization success following UV-P exposure. Future studies should pinpoint the mechanism by which UV-P acts and investigate the long-term effects of UV-P by conducting multigenerational and transgenerational studies. When existing in the environment, EDCs have the potential to cause large ecosystem impacts, such as decreased population size (Brander et al., 2016). If early life exposure to UV-P can decrease reproductive performance of male fish, there is potential for these effects to persist for generations, ultimately, impacting the health of the ecosystem and leading to population

collapse. Aquatic organisms, such as fish, have important cultural and financial value for many individuals globally. Therefore, toxicity studies using model organisms, such as zebrafish, can give insight into how contaminants might affect other fish species, providing opportunities for cross-species comparison studies. Additionally, by understanding the threat chemical stressors have on model organisms, important information can be gained that is relevant to protecting aquatic wildlife globally.

LITERATURE CITED

Almeida, F. L., Skaftnesmo, K. O., Andersson, E., Kleppe, L., Edvardsen, R. B., Norberg, B., Fjelldal, P. G., Hansen, T. J., Schulz, R. W., & Wargelius, A. (2022). The Piwil1 N domain is required for germ cell survival in Atlantic salmon. *Frontiers in Cell and Developmental Biology, 10*, 977779.

Aluru, N., Kuo, E., Helfrich, L. W., Karchner, S. I., Linney, E. A., Pais, J. E., & Franks, D. G. (2015). Developmental exposure to 2,3,7,8-tetrachlorobenzo-*p*-dioxin alters DNA methyltransferase (*dnmt*) expression in zebrafish (*Danio rerio*). *Toxicology and Applied Pharmacology, 248*(2), 142-151.

Amer, M. A., Miura, T., Miura, C., & Yamauchi, K. (2001). Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (Hucho perryi). *Biology of Reproduction, 65*(4), 1057-1066.

Ankley, G. T., & Johnson, R. D. (2004). Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *Institute for Laboratory Animal Research, 45*(4), 469-483.

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrano, J. A., & Villeneuve, D. L. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry*, 29(3), 730-741.

Assis, L. H. C., Crespo, D., Morais, R. D. V. S., França, L. R., Bogard, J., & Schulz, R. W. (2015). INSL3 stimulates spermatogonial differentiation in testis of adult zebrafish (*Danio rerio*). *Cell Tissue Res, 363,* 579-588.

Baccarelli, A., & Bollati, V. (2011). Epigenetics and environmental chemicals. *Current Opinion in Pediatrics, 21*(3), 243-251.

Bacila, I., Cunliffe, V. T., & Krone, N. P. (2021). Interrenal development and function in zebrafish. *Molecular and Cellular Endocrinology, 535*, 111372.

Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Research, 21,* 381-395.

Barakat, R., & Ko, C. J. (2018). Female Antiandrogens. In M. K. Skinner (Ed.), *Encyclopedia of Reproduction - Second Edition* (pp. 748-751). Academic Press.

Bayley, M., Junge, M., & Baatrup, E. (2002). Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. *Aquatic Toxicology, 56,* 227-239.

Bernal, A. J., & Jirtle, R. L. (2010). Epigenomic disruption: the effects of early developmental exposures. *Birth Defects Research Part A: Clinical and Molecular Teratology, 88*(10), 938-944.

Bertho, S., Clapp, M., Banisch, T. U., Bandemer, J., Raz, R., & Marlow, F. L. (2021). Zebrafish *dazl* regulates cystogenesis and germline and stem cell specification

during the primordial germline stem cell specification during the primordial germ cell to germline stem cell transition. *Development, 148*(7), dev187773.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry & Physiology, 109*(3), 219-245.

Borrelle, S. B., Ringma, J., Law, K. L., Monnahan, C. C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G. H., Hilleary, M. A., Eriksen, M., Possingham, H. P., De Frond, H., Gerber, L. R., Polidoro, B., Tahir, A., Bernard, M., Mallos, N., Barnes, M., & Rochman, C. M. (2020). Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science (American Association for the Advancement of Science), 369*(6510), 1515-1518.

Brander, S. M., Gabler, M. K., Fowler, N. L., Connon, R. E., & Schlenk, D. (2016). Pyrethroid pesticides as endocrine disruptors: molecular mechanisms in vertebrates with a focus on fishes. *Environmental Science & Technology*, 50(17), 8977-8992.

Cantwell, M. G., Sullivan, J. C., & Burgess, R. M. (2015a). Chapter 16 – Benzotriazoles: History, Environmental Distribution, and Potential Ecological Effects. *Comprehensive Analytical Chemistry, 67,* 513-545.

Cantwell, M. G., Sullivan, J. C., Katz, D. R., Burgess, R. M., Hubeny, J. B., King, J. (2015b). Source determination of benzotriazoles in sediment cores from two urban estuaries on the Atlantic Coast of the United States. *Marine Pollution Bulletin, 101*(1), 208-218.

Carnevali, O., Santageli, S., Forner-Piquer, I., Basili, D., & Maradonna, F. (2018). Endocrine-disrupting chemicals in aquatic environment: what are the risks for fish gametes? *Fish physiology and biochemistry, 44*(6), 1561-1576.

Castilloux, A. D., Houde, M., Gendron, A., De Silva, A., Soubaneh, D., & Lu, Z. (2022). Distribution and fate of ultraviolet absorbents and industrial antioxidants in the St. Lawrence River, Quebec, Canada. *Environmental Science & Technology, 56*(8), 5009-5019.

Cavaco, J. E. B., Vilrokx, C., Trudeau, V. L., Schulz, R. W., & Goos, H. J. T. (1998). Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology, 275*(6), R1793-R1802.

Chen, L., Lam, J. C., Hu, C., Tsui, M. M., Lam, P. K., & Zhou, B. (2019). Perfluorobutanesulfonate exposure skews sex ratio in fish and transgenerationally impairs reproduction. *Environmental Science & Technology,* 53(14), 8389-8397.

Chen, S. X., Bogerd, J., Schoonen, N. E., Martijn, J., de Waal, P. P., & Schulz, R. W. (2013). A progestin (17a,20b-dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. *General and Comparative Endocrinology, 85,* 1-9.

Chin, J. W. (2007). 5 – Durability of composites exposed to ultraviolet radiation. *Durability of Composites for Civil Structural Applications,* 80-97.

Conolly, R. B., Ankley, G. T., Cheng, W., Mayo, M. L., Miller, D. H., Perkins, E. J., Villeneuve, D. L., & Watanabe, K. H. (2017). Quantitative adverse outcome pathways and their application to predictive toxicology. *Environmental Science & Technology, 51*(8), 4661-4672.

Corradetti, B., Stronati, A., Tosti, L., Manicardi, G., Carnevali, O., & Bizzaro, D. (2013). Bis-(2-ethylexhyl) phthalate impairs spermatogenesis in zebrafish (*Danio rerio*). *Reproductive Biology, 13*(3), 195-202.

de Rooij, D. G. (2017). The nature and dynamics of spermatogonial stem cells. *Development, 144*(17), 3022-3030.

de Waal., P. P., Wang., D. S., Nijenhuis, W. A., Schulz, R. W., & Bogerd, J. (2008). Functional characterization and expression analysis of the androgen receptor in zebrafish (Danio rerio) testis. *Reproduction, 136*(2), 225-34.

Devoy, C., Raza, Y., Kleiner, M., Jones, P. D., Doering, J. A., & Wiseman, S. (2023a). The brominated flame retardant, 1,2,5,6-tetrabromocyclooctane (TBCO), causes multigenerational effects on reproductive capacity of Japanese medaka (*Oryzias latipes*). *Chemosphere, 313,* 137561.

Devoy, C., Raza, Y., Jones, P. D., Doering, J. A., & Wiseman, S. (2023b). Japanese medaka (*Oryzias latipes*) exposed via maternal transfer to the brominated flame retardant, 1,2,5,6-tetrabromocyclooctane (TBCO), experience decreased fecundity and impaired oocyte maturation. *Aquatic Toxicology*, 106761, https://doi.org/10.1016/j.aquatox.2023.106761.

Driessnack, M. K., Jamwal, A., & Niyogi, S. (2017). Effects of chronic exposure to waterborne copper and nickel in binary mixture on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). *Chemosphere, 185,* 964-974.

Dubiel, J., Green, D., Raza, Y., Johnson, H. M., Xia, Z., Tomy, G. T., Hontela, A., Doering, J. A., & Wiseman, S. (2022). Alkylation of benz[a]anthracene affects toxicity to early-life stage zebrafish and in vitro aryl hydrocarbon receptor 2 transactivation in a position-dependent manner. *Environmental Toxicology and Chemistry, 41*(8), 1993-2002.

Elonen, G. E., Spehar, R. L., Holocombe, G. W., Johnson, R. D., Fernandez, J. D., Erickson, R. J., Tietge, J. E., & Cook, P. M. (2009). Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin to seven freshwater fish species during early life-stage development. *Environmental Toxicology and Chemistry,* 17(3), 472-483.

Feng, H., Cao, H., Li, J., Zhang, H., Xue, Q., Liu, X., Zhang, A., & Fu, J. (2020). Estrogenic activity of benzotriazole UV stabilizers evaluated through in vitro assays and computational studies. *Science of the Total Environment, 727,* 128549.

Fent, K., Chew, G., Li, J., & Gomez, E. (2014). Benzotriazole UV-stabilizers and benzotriazole: Antiandrogenic activity in vitro and activation of aryl hydrocarbon receptor pathway in zebrafish eleuthero-embryos. *Science of the Total Environment, 482-483,* 125-136.

Fujisawa, M. (2006). Regulation of testicular function by cell-to-cell interaction. *Reproductive Medicine and Biology, 5*(1), 9-17.

Fujita, K. K., Doering, J. A., Stock, E., Lu, Z., Montina, T., & Wiseman, S. (2022). Effects of dietary 2-(2H-benzotriazol-2-yl)-4-methylphenol (UV-P) exposure on Japanese medaka (Oryzias latipes) in a short-term reproduction assay. *Aquatic Toxicology, 248,* 106206.

Gao, D., Lin, J., Ou, K., Chen, Y., Li, H., Dai, Q., Yu, Z., Zuo, & Wang, C. (2018). Embryonic exposure to benzo(a)pyrene inhibits reproductive capability in adult female zebrafish and correlation with DNA methylation. *Environmental Pollution, 240*, 403-411.

Ge, S. Q., Lin, S. L., Zhao, Z. H., & Sun, Q. Y. (2017). Epigenetic dynamics and interplay during spermatogenesis and embryogenesis: implications for male fertility and offspring health. *Oncotarget, 8*(32), 53804-53818.

Golshan, M., & Alavi, S. M. H. (2019). Androgen signaling in male fishes: example of anti-androgenic chemicals that cause reproductive disorders. *Theriogenology, 139*, 58-71.

González-Rojo, S., Lombó, M., Fernández-Díez, C., & Herráez, M. P. (2019). Male exposure to bisphenol a impairs spermatogenesis and triggers histone hyperacetylation in zebrafish testes. *Environmental Pollution, 248,* 368-379.

Groh, K. J., Carvalho, R. N., Chipman, J. K., Denslow, N. D., Halder, M., Murphy, C. A., Roelofs, D., Rolaki, A., Schirmer, K., & Watanabe, K. H. (2015). Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: I. Challenges and research needs in ecotoxicology. *Chemosphere, 120,* 764-777.

Günes, S., & Kulaç, T. (2013). The role of epigenetics in spermatogenesis. *Turkish Journal of Urology, 39*(3), 181-187.

Head, J. A., Dolinoy, D. C., Basu, N. (2012). Epigenetics for ecotoxicologists. *Environmental Toxicology and Chemistry, 31*(2), 221-227.

Heindel, J. J., Balbus, J., Birnbaum, L., Brune-Drisse, M. N., Grandjean, P., Gray, K., Landrigan, P. J., Sly, P. D., Suk, W., Slechta, D. C., Thompson, C., & Hanson, M. (2015). Developmental origins of health and disease: integrating environmental influences. *Endocrinology*, *156*(10), 3416-3421.

Hernandez, R. E., Galitan, L., Cameron, J., Goodwin, N., & Ramakrishnan, L. (2018). Delay of initial feeding of zebrafish larvae until 8 days postfertilization has no impact on survival or growth through the juvenile stage. *Zebrafish, 15*(5), 515-518.

Houwing, S., Kamminga, L. M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., Filippov, D. V., Blaser, H., Raz, E., Moens, C. B., Plasterk, R. H. A., Hannon, G. J., Draper, B. W., & Ketting, R. F. (2007). A role of Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell, 129*(1), 69-82.

Imhof, A. (2006). Epigenetic regulators and histone modification. *Briefings in Functional Genomics and* Proteomics*, 5*(3), 222-227.

Jiang, L., Zhang, J., Wang, J. J., Wang, L., Zhang, L., Li, G., Yang, X., Ma, X., Sun, X., Cai, J., Zhang, J., Huang, X., Yu, M., Wang, X., Liu, F., Wu, C. I., He, C., Zhang, B., Ci, W., & Liu, J. (2013). Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. *Cell, 153*, 773-784.

Jin, B., Li, Y., & Robertson, K. D. (2011). DNA methylation: superior or subordinate in the epigenetic hierarchy? *Genes & Cancer, 2*(6), 607-617.

Jonsson, B., & Jonsson, N. (2014). Early environment influences later performance in fishes. *Journal of Fish Biology*, *85*(2), 151-188.

Kameda, Y., Kimura, K., & Miyazaki, M. (2011). Occurrence and profiles of organic sun-blocking agents in surface waters and sediments in Japanese rivers and lakes. *Environmental Pollution, 159*(6), 1570-1576.

Khadra, M., Caron, A., Planas, D., Ponton, D. E., Rosabal, M., & Amyot, M. (2019). The fish or the egg: Maternal transfer and subcellular partitioning of mercury and selenium in Yellow Perch (*Perca flavescens*). *Science of The Total Environment*, 675, 604-614.

Kim, J. W., Isobe, T., Ramaswamy, B. R., Chang, K. H., Amano, A., Miller, T. M., Siringan, F.P., & Tanabe, S. (2011). Contamination and bioaccumulation of benzotriazole ultraviolet stabilizers in fish from Manila Bay, the Philippines using an ultra-fast liquid chromatography–tandem mass spectrometry. *Chemosphere*, *85*(5), 751-758.

Kime, D. E. (1993). 'Classical' and 'non-classical' reproductive steroids in fish. *Reviews in Fish Biology and Fisheries, 3*, 160-180.

Kime, D. E. (1999). A strategy for assessing the effects of xenobiotics on fish reproduction. *Science of The Total Environment, 225*, 3-11.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics, 203,* 253-310.

Kobayashi, Y., Nozu, R., & Nakamaura, M. (2010). Role of estrogen in spermatogenesis in initial phase males of the three-spot wrasse (*Halichoeres trimaculatus*): Effect of aromatase inhibitor on the testis. *Developmental Dynamics, 240*(1), 116-121.

Kowalski, R. K., & Cejko, B. I. (2019). Sperm quality in fish: Determinants and affecting factors. *Theriogenology, 134,* 9-108.

Kramer, H. E. A. (2003). Chapter 16 – Tautomerism by Hydrogen Transfer in Salicylates, Triazoles, and Oxazoles.

Kundakovic, M., & Champagne, F. A. (2015). Early-life experience, epigenetics, and the developing brain. *Neuropsychopharmacology, 40,* 141-153.

Lambert, J. C. (2022). Adverse outcome pathway 'footprinting': a novel approach to the integration of 21st century toxicology information into chemical mixtures risk assessment. *Toxics, 11*(1), 37.

Lane, T., Green, D., Bluhm, K., Raes, K., Janz, D.M., Liber, K., & Hecker, M. (2019). In ovo exposure of fathead minnow (*Pimephales promelas*) to selenomethionine via maternal transfer and embryo microinjection: A comparative study. *Aquatic Toxicology, 216*, 105299.

Larigot, L., Juricek, L., Dairou, J., & Coumoul, X. (2018). AhR signaling pathways and regulatory functions. *Biochimie Open, 7*, 1-9.

Leal, M. C., Cardoso, E. R., Nóbrega, R. H., Batlouni, S. R., Bogerd, J., França, L. R., & Schulz, R. W. (2009). Histological and stereological evaluation of zebrafish (Danio rerio) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction, 81*(1), 177-187.

Lempradl, A. (2020). Germ cell-mediated mechanisms of epigenetic inheritance. *Seminars in Cell & Developmental Biology, 97,* 116-122.

Li, M., Zhu, F., Li, Z. Hong, N., & Hong, Y. (2016). Dazl is a critical player for primordial germ cell formation in medaka. *Scientific Reports, 6,* 28317.

Li, Z., Li, W., Zha, J., Chen, H., Martyniuk, C. J., & Liang, X. (2018). Transcriptome analysis reveals benzotriazole ultraviolet stabilizers regulate networks related to inflammation in juvenile zebrafish (*Danio rerio*) brain. *Environmental Toxicology, 34*(2), 112-122.

Li, Z., Liang, X., Liu, W., Zhao, Y., Yang, H., Li, W., Adamovsky, O., & Martyniuk, C. J. (2020). Eludicating mechanisms of immunotoxicity by benzotriazole ultraviolet stabilizers in zebrafish (*Danio rerio*): Implication of the AHR-IL17/IL22 immune pathway. *Environmental Pollution, 262,* 114291.

Liang, X., Li, J., Martyniuk, C. J., Wang, J., Mao, Y., Lu, H., & Zha, L. (2017). Benzotriazole ultraviolet stabilizers alter the expression of the thyroid hormone pathway zebrafish (*Danio rerio*) embryos. *Chemosphere, 182*, 22-30.

Liao, P. H., Chu, S. H., Tu, T. Y., Wang, X. H., Lin, A. Y. C., & Chen, P. J. (2014). Persistent endocrine disruption effects in medaka fish with early life-stage exposure to a triazole-containing aromatase inhibitor (letrozole). *Journal of Hazardous Materials*, *277*, 141-149.

Lor, Y., Revak, A., Weigand, J., Hicks, E., Howard, D. R., & King-Heiden, T. C. (2015). Juvenile exposure to vinclozolin shifts sex rations and impairs reproductive capacity of zebrafish. *Reproductive Toxicology, 58,* 111-118.

Lu, Z., De Silva, A. O., McGoldrick, D. J., Zhou, W., Peart, T. E., Cook, C., Tetreault, G. R., Martin, P. A., & De Solla, S. R. (2018). Substituted diphenylamine antioxidants and benzotriazole UV stabilizers in aquatic organisms in the Great Lakes of North America: Terrestrial exposure and biodilution. *Environmental Science & Technology, 52*, 1280-1289.

Lu, Z., De Silva, A. O., Peart, T. E., Cook, C. J., Tetreault, G. R., Servos, M. R., & Muir, D. C. G. (2016). Distribution, partitioning and bioaccumulation of substituted diphenylamine antioxidants and benzotriazole UV stabilizers in an urban creek in Canada. *Environmental Science & Technology, 50*, 9089−9097.

Lu, Z., De Silva, A. O., Provencher, J. F., Mallory, M. L., Kirk, J. L., Houde, M., Stewart, C., Braune, B. M., Avery-Gomm, S., & Muir, D. C. G. (2019). Occurrence of substituted diphenylamine antioxidants and benzotriazole UV stabilizers in Arctic seabirds and seals. *Science of the Total Environment, 663*, 960-957.

Lu, Z., Smyth, S. A., Peart, T. E., & De Silva, A. O. (2017). Occurrence and fate of substituted diphenylamine antioxidants and benzotriazole UV stabilizers in various Canadian wastewater treatment processes. *Water research*, *124*, 158-166.

MacLeod, M., Arp, H. P. H., Tekman, M. B., & Jahnke, A. (2021). The global threat from plastic pollution. *Science* (*American Association for the Advancement of Science*), *373*(6550), 61-65.

Major, K. M., DeCourten, B. M., Li, J., Britton, M., Settles, M. L., Mehinto, A. C., Connon, R. E., & Brander, S. M. (2020). Early life exposure to environmentally relevant levels of endocrine disruptors drive multigenerational and transgenerational epigenetic changes in a fish model. *Frontiers in Marine Science*, 7, 471.

McAllister, B. G., & Kime, D. E. (2003). Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinization and irreversible sperm damage in zebrafish (Danio rerio). *Aquatic Toxicology, 65*(3), 309-316.

Meng, X., Su, S., Wei, X., Wang, S., Guo, T., Li, J., Song, H., Wang, M., & Wang, Z. (2023). Exposure to bisphenol A alternatives bisphenol AF and fluorene-9-bisphenol induces gonadal injuries in male zebrafish. *Ecotoxicology and Environmental Safety, 253,* 114634.

Miguel-Queralt, S., Knowlton, M., Avvakumov, G. V., Al-Nouno, R., Kelly, G. M., & Hammond, G. L. (2004). Molecular and functional characterization of sex hormone binding globulin in zebrafish. *Endocrinology, 145*, 5221–5230.

Milla, S., Terrien, X., Sturm, A., Ibrahim, F., Giton, F., Fiet, J., Prunet, P., & LeGac, F. (2008). Plasma 11-deoxycorticosterone (DOC) and mineralocorticoid receptor testicular expression during rainbow trout *Oncorhynchus mykiss* spermiation: implication with 17a,20b-dihydroxyprogesterone on milt fluidity? *Reproductive Biology and Endocrinology, 6,* 19.

Miura, T., Miura, C., Ohta, T., Nader, M. R., Todo, T., & Yamauchi, K. (1999). Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. *Biochemical and Biophysical Research Communications, 264*(1), 230-234.

Montesdeoca-Esponda, S., Álvarez-Raya, C., Torres-Pardón, M. E., Sosa-Ferrera, Z., & Santana Rodríguez, J. J. (2019). Monitoring and environmental risk assessment of benzotriazole UV stabilizers in the sewage and coastal environment of Gran Canaria (Canary Islands, Spain). *Journal of Environmental Management, 233,* 567-575.

Morais, R. D. V. S., Crespo, D., Nóbrega, R. H., Lemos, M. S., Van de Kant, H. J. G., De França, L. R., Male, R., Bogerd, J., & Schulz, R. W. (2017). Antagonistic regulation of spermatogonial differentiation in zebrafish (Danio rerio) by Igf3 and Amh. *Molecular and Cellular Endocrinology, 454*, 112–124.

Morrison, O., & Thakur, J. (2021). Molecular complexes at euchromatin, heterochromatin and centromeric chromatin. *International Journal of Molecular Sciences, 22*(13), 6922.

Nagayoshi, H., Kakimoto, K., Takagi, S., Konishi, Y., Kajimura, K., & Matsuda, T. (2014). Benzotriazole ultraviolet stabilizers show potent activities as human aryl hydrocarbon receptor ligands. *Environmental Science & Technology, 49,* 578-587.

Nakata, H., Murata, S., & Filatreau, J. (2009). Occurrence and concentrations of benzotriazole UV stabilizers in marine organisms and sediments from the Ariake Sea, Japan. *Environmental Science & Technology, 43*(18), 6920-6926.

Nakata, H., Shinohara, R. I., Nakazawa, Y., Isobe, T., Sudaryanto, A., Subramanian, A., Tanabe, S., Zakaria, M. P., Zheng, G. J., Lam, P. K. S., Kim, E. Y., Min, B. Y., We, S. U., Viet, P. H., Tana, T. S., Prundente, M., Frank, D., Lauenstein, G., & Kannan, K. (2012). Asia–Pacific mussel watch for emerging pollutants: Distribution of synthetic musks and benzotriazole UV stabilizers in Asian and US coastal waters. *Marine Pollution Bulletin, 64*, 2211-2218.

O'Donnell, L. (2014). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis, 4*(2), e979623.

Oakes, J. A., Barnard, L., Storbeck, K. H., Cunliffe, V. T., & Krone, N. P. (2020). 11b-Hydroxylase loss disrupts steroidogenesis and reproductive function in zebrafish. *Journal of Endocrinology, 247*, 197-212.

Oakes, J. A., Li, N., Wistow, B. R. C., Griffin, A., Barnard, L., Storbeck, K. H., Cunliffe, V. T., & Krone, N. P. (2019). Ferredoxin 1b deficiency leads to testis disorganization, impaired spermatogenesis, and feminization in zebrafish. *Endocrinology, 160*(10), 2401-2416.

OECD. (2012). Test No. 229: Fish Short Term Reproduction Assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Olsson, P. E., Berg, A. H., von Hofsten, J., Grahn, B., Hellgvist, A., Larsson, A., Karlsson, J., Modig, C., Borg, B., & Thomas, P. (2005). Molecular cloning and

characterization of a nuclear androgen receptor activated by 11-ketotestosterone. *Reproductive Biology and Endocrinology, 3,* 37.

Olszowy-Tomczyk, M. (2020). Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. *Phytochemistry Reviews, 19,* 63-103.

Ostrach, D. J., Low-Marchelli, J. M., Eder, K. J., & Zinkl, J. G. (2008). Maternal transfer of xenobiotics and effects on larval striped bass in the San Francisco Estuary. *Proceedings of the National Academy of Sciences, 105*(49), 19354-19359.

Parajulee, A., Lei, Y. D., Kananathalingam, A., Mitchell, C. P., & Wania, F. (2018). Investigating the sources and transport of benzotriazole UV stabilizers during rainfall and snowmelt across an urbanization gradient. *Environmental Science & Technology*, *52*(5), 2595-2602.

Peng, X., Fan, Y., Jin, J., Xiong, S., Liu, J., & Tang, C. (2017). Bioaccumulation and biomagnification of ultraviolet absorbents in marine wildlife of the Pearl River Estuarine, South China Sea. *Environmental Pollution*, *225*, 55–65.

Peng, X., Zhu, Z., Xiong, S., Fan, Y., Chen, G., & Tang, C. (2019). Tissue distribution, growth dilution, and species-specific bioaccumulation of organic ultraviolet absorbents in wildlife freshwater fish in the Pearl River catchment, China. *Environmental Toxicology and Chemistry, 39*(2), 343-351.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research, 29*(9), e45-e45.

Pickford, K. A., Thomas-Jones, R. E., Wheals, B., Tyler, C. R., & Sumpter, J. P. (2003). Route of exposure affects the oestrogenic response of fish to 4-*tert*-nonylphenol. *Aquatic Toxicology, 65*(3), 267-279.

Potok, M. E., Nix, D. A., Parnell, T. J., & Cairns, B. R. (2013). Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. *Cell, 153*(4), 759-772.

Provencher, J. F., Malaisé, F., Mallory, M. L., Braune, B. M., Pirie-Dominix, L., & Lu, Z. (2022). 44-year retrospective analysis of ultraviolet absorbents and industrial antioxidants in seabird eggs from the Canadian Arctic (1975 to 2019). *Environmental Science & Technology, 56*(20), 14562-14573.

Qian, L., Qi, S., Zhang, J., Duan, M., Schlenk, D., Jiang, J., & Wang, C. (2020). Exposure to boscalid induced reproductive toxicity of zebrafish by gender-specific alterations in steroidogenesis. *Environmental Science and Technology, 54*(22), 14275-14287.

Rajakumar, A., & Senthilkumaran, B. (2020). Steroidogenesis and its regulation in teleost – a review. *Fish Physiology and Biochemistry, 46,* 803-818.

Rajender, S., Avery, K., & Agarwal, A. (2011). Epigenetics, spermatogenesis and male infertility. *Mutation* Research – *Reviews in Mutation Research, 727*(3), 62-71.

Ribas, L., & Piferrer, F. (2013). The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture, 6*(4), 209-240.

Russell, R. W., Gobas, F. A., & Haffner, G. D. (1999). Maternal transfer and in ovo exposure of organochlorines in oviparous organisms: a model and field verification. *Environmental Science & Technology*, *33*(3), 416-420.

Sada, A., Suzuki, A., Suzuki, H., & Saga, Y. (2009). The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science, 325*(5946), 1394-1398.

Safian, D., Bogerd, J., & Schulz, R. W. (2018a). Igf3 activates b-catenin signaling to stimulate spermatogonial differentiation in zebrafish. *Journal of Endocrinology*, *238*, 245-257.

Safian, D., Bogerd, J., & Schulz, R. W. (2019). Regulation of spermatogonial development by Fsh: The complementary roles of locally produced Igf and Wnt signaling molecules in adult zebrafish testis. *General and Comparative Endocrinology, 284,* 113244.

Safian, D., Ryane, N., Bogerd, J., & Schulz, R. W. (2018b). FSH stimulates Leydig cell Wnt5a production, enriching zebrafish type A spermatogonia. *Journal of Endocrinology, 239,* 351-363.

Sakuragi, Y., Takada, H., Sato, H., Kubota, A., Terasaki, M., Takeuchi, S., Ikeda-Araki, A., Watanabe, Y., Kitamura, S., & Kojima, H. (2021). An analytical survey of benzotriazole UV stabilizers in plastic products and their endocrine-disrupting potential via human estrogen and androgen receptors. *Science of the Total Environment, 800,* 149374.

Saunders, P. T. K., Turner, J. M. A., Ruggiu, M., Taggart, M., Burgoyne, P. S., Elliot, D., & Cooke, H. J. (2003). Absence of m*Dazl* produces a final block on germ development at meiosis. *Reproduction, 126,* 589-597.

Schubert, S., Peter, A., Burki, R., Schönenberger, R., Suter, M. J. F., Segner, H., & Burkhardt-Holm, P. (2008). Sensitivity of brown trout reproduction to long-term estrogenic exposure. *Aquatic Toxicology, 90*(1), 65-72.

Schulz, R. W., & Miura, T. (2002). Spermatogenesis and its endocrine regulation. *Fish physiology and Biochemistry, 26*, 43-56.

Schulz, R. W., de França, L. R., Lareyre, J. J., LeGac, F., Chiarini-Garcia, H., Nobrega, R. H., & Miura, T. (2010). Spermatogenesis in fish. *General and Comparative Endocrinology, 165,* 390-411.

Sheriff, I., Debela, S. A., & Mans-Davies, A. (2022). The listing of new persistent organic pollutants in the Stockholm convention: Its burden on developing countries. *Environmental Science & Policy, 130*, 9-15.

Shi, G., Guo, H., Sheng, N., Cui, Q., Pan, Y., Wang, J., Guo, Y., & Dai, J. (2018). Two-generational reproductive toxicity assessment of 6:2 chlorinated polyfluorinated

ether sulfonate (F-53B, a novel alternative to perfluorooctane sulfonate) in zebrafish. *Environmental Pollution, 243,* 1517-1527.

Singleman, C., & Holtzman, N. G. (2014). Growth and maturation in the zebrafish, *Danio rerio*: A staging tool for teaching and research. *Zebrafish*, *11*(4), 396-406.

Skaar, K. S., Nobrega, R. H., Magaraki, A., Olsen, L. C., Schulz, R. W., & Male, R. (2011). Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology, 152*(9), 3527–3540.

Society for Advancement of AOPs. (2023). AOP-Wiki. (October 22, 2023). Available from http://aopwiki.org

Song, F., Wang, L., Yu, Z., Wenbin, Z., Fu, J., & Dong, Z. (2021)., Knockdown of igf3 gene by RNA interference causes gonadal development inhibition of common carp (*Cyprinus carpio*). *Aquaculture Research, 53,* 3006-3014.

Song, M., & Gutzeit, H. O. (2003). Effect of 17α-ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (Oryzias latipes) testis. *Development, Growth & Differentiation, 45*, 327–337.

Stewart, K. R., Veselovska, L., & Kelsey, G. (2016). Establishment and functions of DNA methylation in the germline. *Epigenomics, 8*(10), 1399-1413.

Streit, B. (1998). Bioaccumulation of contaminants in fish. In: Braunbeck, T., Hinton, D.E., Streit, B. (eds) Fish Ecotoxicology. EXS, vol 86. Birkhäuser, Basel.

Suzuki, A. & Saga, Y. (2008). Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes & Development, 22*(4), 430-435.

Szyf, M. (2009). The early life environment and the epigenome. *Biochimica et Biophysica Acta (BBA) – General Subjects*, *1790*(9), 878-885.

Szyf, M. (2011). The implications of DNA methylation for toxicology: toward toxicomethylomics, the toxicology of DNA methylation. *Toxicological Sciences, 120*(2), 235-255.

Tang, H., Chen, Y., Liu, Y., Yin, Y., Li, G., Guo, Y., Liu, X., & Lin, H. (2017). New insights into the role of estrogens in male fertility based on findings in aromatase-deficient zebrafish. *Endocrinology, 158*(9), 3043-3054.

Tao, J., Yang, Q., Jing, M., Sun, X., Tian, L., Huang, X., Huang, X., Wan, W., Ye, H., Zhang, T., & Hong, F. (2023). Embryonic benzophenone-3 exposure inhibited fertility in late-life female zebrafish and altered developmental morphology in offspring embryos. *Environmental Science and Pollution Research, 30,* 49226-49236.

Tengu, S., Pranoty, A., Mamta, S. K., & Senthilkumaran, B. (2021). Development and organisation of gonadal steroidogenesis in bony fishes – A review. *Aquaculture and Fisheries, 6*(3), 223-246.

Thresher R, Gurney R, & Canning M. (2011). Effects of lifetime chemical inhibition of aromatase on the sexual differentiation, sperm characteristics and fertility of medaka (Oryzias latipes) and zebrafish (Danio rerio). *Aquatic Toxicology, 105*(3-4), 355-60.

Tokarz, J., Möller, G., de Angelis, M. H., & Adamski, J. (2015). Steroids in teleost fishes: A functional point of view. *Steroids, 103,* 123-144.

Tsukamura, H. (2022). Kobayashi Award 2019: The neuroendocrine regulation of the mammalian reproduction. *General and Comparative Endocrinology, 315*, 113755.

Ungerer, J. R., & Thomas, P. (1996). Role of very low-density lipoproteins in the accumulation of p′-DDT in fish ovaries during gonadal recrudescence. *Aquatic toxicology*, 35(3-4), 183-195.

US EPA, 2009. Estimation Programs Interface Suite! for Microsoft! Windows, v. 4.00. Environmental Protection Agency, Washington, DC, USA. http://www.epa.gov/oppt/exposure/pubs/episuite.htm.

Villeneuve, D. L., Crump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T. H., LaLone, C. A., Landesmann, B., Lettieri, T., Munn, S., Nepelska, M., Ottinger, M. A., Vergauwen, L., Whelan, M., (2014). Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicological Sciences*, 142, 312–320.

Vimalkumar, K., Arun, E., Krishna-Kumar, S., Poopal, R. K., Nikhil, N. P., Subramanian, A., & Babu-Rajendran, R. (2018). Occurrence of triclocarban and benzotriazole ultraviolet stabilizers in water, sediment, and fish from Indian rivers. *Science of the Total Environment*, *625*, 1351-1360.

Vinken, M., Knapen, D., Vergauwen, L., Hengstler, J. G., Angrish, M., & Whelan, M. (2017). Adverse outcome pathways: a concise introduction for toxicologists. *Archives of toxicology,* 91, 3697-3707.

Wang, X., & Bhandari, R. K. (2019). DNA methylation dynamics during epigenetic reprogramming of medaka embryo. *Epigenetics, 14*(6), 611-622.

Wu, Y., Rene, E. R., Zhou, M., & Tiwari, A. (2020). Non-point source pollution control and aquatic ecosystem protection – An introduction. *Bioresource Technology, 316,* 123956.

Xie, D., Chen, Q., Gong, S., An, J., Li, Y., Lian, X., Liu, Z., Shen, Y., & Giesy, J. P. (2020a). Exposure of zebrafish to environmentally relevant concentration of mercury during early life stages impairs subsequent reproduction in adults but can be recovered in offspring. *Aquatic Toxicology, 229,* 105655.

Xie, X., Nóbrega, R., & Pšenička, M. (2020b). Spermatogonial stem cells in fish: Characterization, isolation, enrichment, and recent advances of in vitro culture systems. *Biomolecules, 10*(4), 644.

Xu, K., Gao, D., Lin, J., Dai, Q., Zhou, Q., Chen, Y., & Wang, C. (2023). Benzo(a)pyrene exposure in early life suppresses spermatogenesis in adult male zebrafish and

association with methylation of germ cell-specific genes. *Aquatic Toxicology,* 258, 106504.

Yaron, Z., & Levavi-Sivan, B. (2011). Endocrine regulation of fish reproduction. *Encyclopedia of Fish Physiology: from Genome to Environment, 2*, 1500-1508.

Ye, D., Liu, T., Li, Y., Wang, Y., Hu, W., Zhu, Z., & Sun, Y. (2023). Identification of fish spermatogenic cells through high-throughput immunofluorescence against testis with an antibody set. *Frontiers in Endocrinology, 14*, 1044318.

Yin, P., Li, Y. W., Chen, Q. L., & Liu, Z. H. (2017). Diethylstilbestrol, flutamide and their combination impaired the spermatogenesis of male adult zebrafish through disrupting HPG axis, meiosis and apoptosis. *Aquatic Toxicology, 185,* 129-137.

Young, G., Kusakabe, M., Nakamura, I., Lokman, P. M., & Goetz, F. W. (2005). Gonadal steroidogenesis in teleost fish. *Hormones and their Receptors in Fish Reproduction,* 128-154.

Zhang, L., Shen, L., Huang, Y., Cui, S., Zhao, Q., Zhang, C., Zhuang, S., & Jiang, G. (2023). Embryonic exposure to UV-238 impairs the cell cycle in zebrafish (*Danio rerio*) by inhibiting the p38 MAPK/p53/Gadd45a Signaling Pathway. *Environmental Science & Technology, 57*(27), 9965-9974.

Zhang, S., Wang, Z., Chen, J., Xie, Q., Zhu, M., Han, W. (2021). Tissue-specific accumulation, biotransformation, and physiologically based toxicokinetic modeling of benzotriazole ultraviolet stabilizers in zebrafish (*Danio rerio*). *Environmental Science and Technology, 55(17)*, 11874-11884.

Zhang, Z., Lau, S. Q., Zhang, L., & Ge, W. (2015). Disruption of zebrafish follicle-stimulating hormone receptor (fshr) but not luteinizing hormone receptor (lhcgr) gene by TALEN leads to failed follicle activation in females followed by sexual reversal to males. *Endocrinology, 156*(10), 3747-3762.

Zhang, Z., Ren, N., Li, Y. F., Kunisue, T., Gao, D., & Kannan, K. (2011). Determination of benzotriazole and benzophenone UV filters in sediment and sewage sludge. *Environmental Science & Technology, 45*(9), 3909-3916.

Zhaung, S., Lv, X., Pan, L., Lu, L., Ge, Z., Wang, J., Wang, J., Liu, J., Liu, W., & Zhang, C. (2017). Benzotriazole UV-328 and UV-P showed distinct antiandrogenic activity upon human CYP3A4-mediated biotransformation. *Environmental Pollution, 220,* 616-624.