CHARACTERIZATION OF THE EFFECTS OF 2-(2H-BENZOTRIAZOL-2-YL)-4-METHYLPHENOL (UV-P) TO THE MODEL FISH SPECIES, JAPANESE MEDAKA (*ORYZIAS LATIPES*)

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DEDICATION

I would like to dedicate this thesis to all my friends and family who have supported me throughout my academic journey.

ABSTRACT

Environmental pollution is a major contributor to declines in populations of fish. Recently, benzotriazole ultraviolet stabilizers (BZT-UVs) have emerged as pollutants of concern. Although these compounds are detected globally in aquatic ecosystems, few studies have investigated the potential toxic effects of these chemicals to fish. The objectives of this study were to assess the potential adverse effects that a BZT-UV, specifically 2-(2H-Benzotriazol-2-yl)-4-methylphenol (UV-P), has to fish. To test this, effects of UV-P on reproduction were assessed. Effects of UV-P on the liver metabolome of Japanese medaka were assessed to investigate potential mechanisms of toxicity. Exposure to UV-P did not impair reproduction in Japanese medaka, however, qPCR suggested there was altered expression of genes related to the steroidogenesis pathway. Additionally, metabolomics analysis indicated potential perturbation of various biological pathways. Overall, this study fills a knowledge gap regarding the potential effects of BZT-UVs on fish populations.

PREFACE

Kaden Fujita is the primary author of chapters 1-3. Dr. Zhe Lu wrote section 2.8. Dr. Steve Wiseman, Dr. Jon Doering, and Mr. Tony Montina aided in experimental design and data interpretation of chapter 2 and edited chapters 1-3.

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throughout my academic journey. Lastly, I would like to like to thank my lovely girlfriend Katrina for all the love and support she has provided.

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LIST OF ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BFR	Brominated flame retardant
BZT-UV	Benzotriazole UV stabilizer
cDNA	Complimentary deoxyribonucleic acid
EC50	50% effect concentration
EDC	Endocrine disrupting compound
ELISA	Enzyme linked immunosorbent assay
ELS	Early life stages
ER	Estrogen receptor
E2	17β-Estradiol
FSH	Follicle stimulating hormone
GC-MS	Gas chromatography – mass spectrometry
GnRH	Gonadotropin releasing hormone
GSI	Gonadosomatic index
HPGL	Hypothalamic-pituitary-gonadal-liver
HSI	Hepatosomatic index
IC20	20% inhibition concentration
K	Condition factor
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
NBFR	Novel brominated flame retardants
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PXR	Pregnane X receptor
RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
T	Testosterone
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
VIAVC	Variable importance analysis based on random variable combination
VTG	Vitellogenin
XRE	Xenobiotic response element
YAS	Yeast androgen screen
YES	Yeast estrogen screen
11-KT	11-ketotestosterone

Note: Throughout this thesis, transcripts are written in lowercase italics, proteins are written in non-italicized uppercase, and genes are written in uppercase italics.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Environmental pollution and plastics

Threats to aquatic ecosystems around the world occur through a plethora of mechanisms including habitat disruption, overfishing/hunting, and environmental pollution. Environmental pollution is regarded as contamination that has adverse effects on the environment and can come from a variety of anthropogenic sources including, industry, mining, agriculture, and plastic waste.[3] Effects of plastic pollution on both marine and freshwater ecosystems, as a result of macro- and microplastics (<5mm) have garnered concern from the scientific community.[4, 5] Microplastics can either be categorized as primary microplastics, meaning they were intentionally created to be that size, or secondary microplastics, which are formed as a consequence of the degradation of larger plastics.[4] Effects of macro-plastics to large aquatic organisms such as fish include injury via ingestion or entanglement.[4] More recently, concerns regarding microplastics have increased, in large part due to the potential for plastics to act as a vector for exposure of wildlife to toxic compounds.[4]

Plastic additives such as brominated flame retardants (BFRs), plasticizers, and UV-stabilizers have been reported to have adverse effects on aquatic organisms.[6-8] Brominated flame retardants are commonly used in plastic products including electronics, car components, and building materials to improve fire resistance.[9] Brominated flame retardants can be divided into two groups, the first of which are known as legacy flame retardants such as hexabromocyclododecane (HBCD), polybrominated biphenyls (PBBs), and polybrominated diphenyl ethers (PBDEs).[6] These flame retardants have largely been phased out as a result of their persistence in the environment and toxic effects.[6] As a result, novel brominated flame retardants (NBFRs) have been used as replacements to legacy flame retardants, however, many

of these NBFRs are ubiquitous in the environment and can lead to a variety of adverse effects, including oxidative stress, endocrine disruption, and developmental toxicities.[6] Plasticizers are chemicals that are added to plastics to increase plasticity of the material.[10] The most widely used plasticizers are phthalates, which are ubiquitous in the environment and of which, the lower molecular weight phthalates have been reported to cause acute and chronic toxicities in various fish species.[7] A group of plastic additives of emerging concern and the focus of the research described in this thesis are benzotriazole UV stabilizers (BZT-UVs) which are added to plastics to prevent damage caused by UV-radiation.[11]

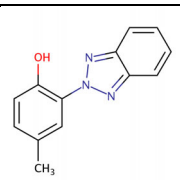
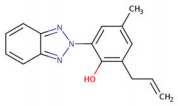
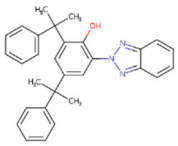
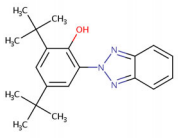
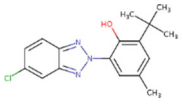
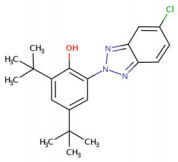
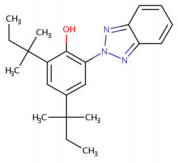
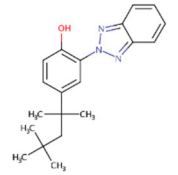
1.2 Properties of benzotriazole UV stabilizers

UV-light stabilizers are added to a variety of products to prevent damage from UV-radiation.[12] The most commonly used UV stabilizers are benzophenones and benzotriazoles, which are groups of chemicals containing either a benzophenone or benzotriazole moiety, respectively.[12, 13] More specifically, BZT-UVs consist of a benzotriazole moiety, and a 2-hydroxyphenol attachment, with various alkyl additions, and their structures allow for broad range UV protection (280-400 nm).[12, 13] BZT-UVs are found in a wide range of products including building materials, paint, wax, shoes, and other plastics. There are many different BZT-UVs which vary in their overall structure and properties such as molecular weight, functional groups, and octanol-water partition coefficient (logKow: 3.86-7.71).[14] BZT-UVs have been reported in the literature as Tinuvins® but are also commonly reported as UV-X, where X identifies the specific BZT-UV. Some BZT-UVs reported in literature include UV-P, UV-090, UV-9, UV-234, UV-320, UV-326, UV-327, UV-328, and UV-329 **(Table 1-1)**.[12, 15-17]

1.3 Benzotriazole UV stabilizers in the environment

Due to the widespread use of BZT-UVs in products and multiple routes of contamination, including wastewater effluent, sewage, and leaching from plastics, BZT-UVs are often detected in aquatic ecosystems.[11] BZT-UVs have been detected globally, including in Canada, USA, Japan, Germany, Spain, India, and the Philippines **(Table 1-1)** and are found in a variety of environmental matrices such as surface water, sediment, sewage, water treatment plant effluent, fish, human breast milk, and house dust.[11, 12, 16, 18-23] Additionally, BZT-UVs have been suggested to bioaccumulate, likely in part due to their hydrophobicity, as indicated by their log Kow that ranges from 3.86-7.71.[12, 14, 19] Because BZT-UVs are added to many high-usage products, it can be hypothesized that concentrations of these compounds in the environment are likely to increase. Therefore, it is critical that we identify any adverse effects that these chemicals might have in the environment, including effects on biota.

Table 1-1. Locations and matrices where BZT-UVs have been detected.

Name	Structure	Sediment	Surface water	Wastewater & Sewage	Biota
UV-P		USA[21], India[19], Japan[24], China[25], Canada[11], Germany[18]	India[19], Japan[24], China[26], Spain[20]	Portugal[27], Japan[24], China[26], Spain[20, 27]	India[19], China[28], Philippines[16]
UV-9					Philippines[16]
UV-234		Australia[29], Japan[24], China[25], Canada[11], Germany[18]	Australia[29]	Canada[30]	Philippines[16], China[28]
UV-320		USA[21], India[19], Japan[12]	India[19],	Portugal[27]	India[19], Japan[12], Philippines[16]
UV-326		USA[21, 31], India[19], Japan[24, 12], China[25, 31], Canada[11], Germany[18],	India[19], Australia[29], Japan[24]	Japan[24,17], Portugal[27], Canada[30], China[31], Spain[20, 27]	India[19], Japan[12], China[28], Philippines[16]
UV-327		USA[21, 31], India[19], Australia[29], Japan[12, 24], China[25, 31], Canada[11], Germany[18]	India[19], Australia[29], Japan[24]	Portugal[27], Japan[17, 24], Canada[30], China[31], Spain[20, 27]	India[19], Japan[12], China[28], Philippines[16]
UV-328		USA[21, 31], India[19], Australia[29], Japan[12, 24], China[25, 31], Canada[11], Germany[18]	India[19], Australia[29], Japan[24]	Portugal[27], Japan[17, 24], Canada[30], China[31],Spain[20]	India[19], Japan[12], China[28], Philippines[16]
UV-329		Japan[24], China[25], Canada[11], Germany[18]	Australia[29]	Canada[30], Spain[20, 27]	China[28], Philippines[16]

1.4 Adverse effects of Benzotriazole UV stabilizers to biota

Despite their high usage and ubiquitous distribution in the environment, little is known about adverse effects of BZT-UVs to biota, including fishes. Limited research to date suggests that BZT-UVs might cause adverse effects through endocrine disruption by alteration of the thyroid hormone pathway or through anti-androgenic activity, activation of the aryl hydrocarbon receptor (AHR), and immunotoxicity.[8, 32, 33]

1.4.1 Endocrine disruption

Reproductive development and function in fish is largely under control of the hypothalamic-pituitary-gonadal-liver (HPGL) axis (**Figure 1-1A**).[34] In brief, gonadotropin releasing hormones (GnRH) are released from the hypothalamus and act via GnRH receptors (GnRHr) located on the anterior pituitary.[34] Activation of GnRHrs leads to the release of two gonadotropins from the pituitary gland, follicle stimulating hormone (FSH) and luteinizing hormone (LH) which play key roles in the development of the reproductive system and reproductive function.[34, 35] Both FSH and LH a known act through binding to their respective receptors (FSH receptor and LH choriogonadotropin receptor), however, in studies using fish, it has been indicated that gonadotropin receptors are not as selective as in mammals.[36] FSH is reported to be involved in early gonadal development and gonadal steroidogenesis.[37] Alternatively, LH is reported to be responsible for regulation of gametogenesis including oocyte maturation and ovulation.[37] Steroidogenesis is a process through which key sex steroids such as E2, testosterone (T), and 11-ketotestosterone (11-KT) are produced from a cholesterol precursor through a series of enzyme catalyzed reactions beginning with the conversion of cholesterol to pregnenolone by CYP11A (cholesterol side chain cleavage enzyme) (**Figure 1-1B)**.[38] Overall, processes occurring along the HPGL axis are vital for both the development of the reproductive

system and reproductive performance, and disruption of these processes by exogenous compounds can be detrimental to fish development and health.[39]

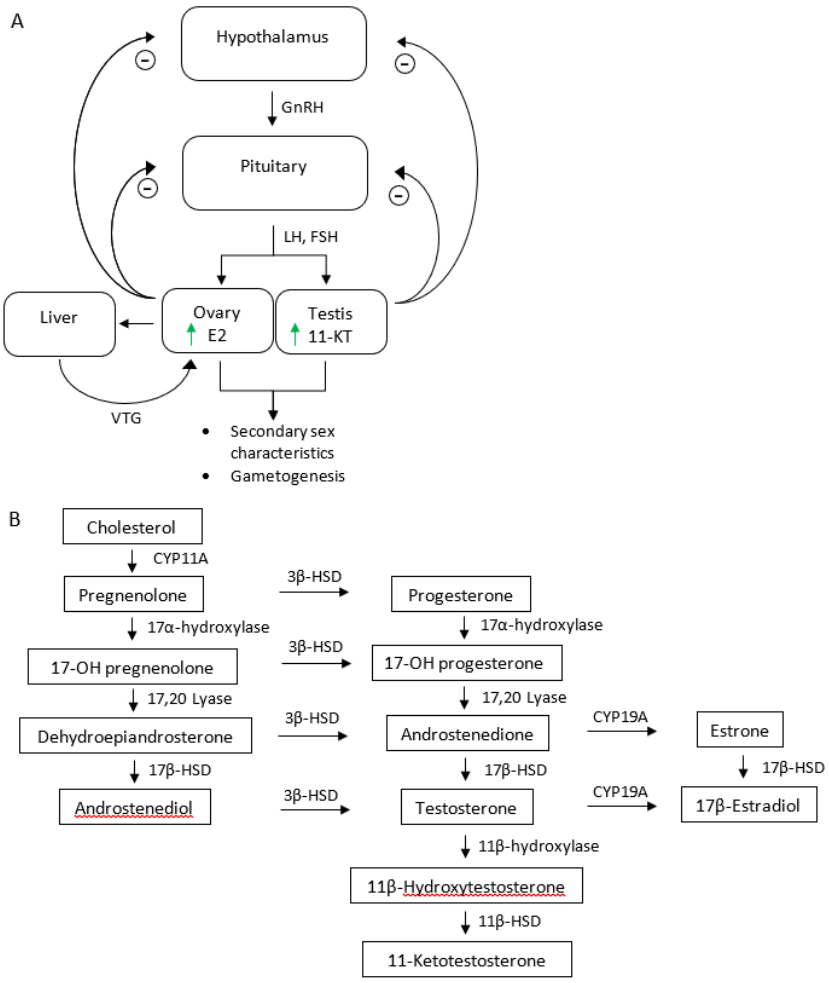


Figure 1-1: (A) Simplified schematic of the hypothalamic-pituitary-gonadal-liver (HPGL) axis. "-" indicate negative feedback loops. GnRH: gonadotropin releasing hormone, LH: luteinizing hormone, FSH: follicle stimulating hormone, E2: 17β-estradiol, 11-KT: 11-ketotestosterone, VTG: Vitellogenin. (B) Schematic illustrating sex hormone steroidogenesis, to produce 17β-estradiol and 11-ketotestosterone. Boxed names indicate small molecules, while names above and beside arrows represent enzymes responsible for catalyzing specific reactions. CYP11A: cholesterol side chain cleavage enzyme, CYP19A: aromatase, 3β-HSD: 3β-hydroxysteroid dehydrogenase, 17β-HSD**:** 17β-hydroxysteroid dehydrogenase, and 11β-HSD: 11β-hydroxysteroid dehydrogenase**.**

Endocrine disrupting compounds (EDCs) are compounds which can disrupt the normal functioning of the endocrine system and can result in various adverse outcomes.[40] La Merrill et al[40] developed a list of 10 key characteristics which are indicative of an EDC, including actions as a hormone receptor agonist or antagonist, as well as perturbation of hormone synthesis, transport, or breakdown, among other actions. As many physiological functions are under endocrine control, studies of endocrine disruption are quite diverse. However, within the field of aquatic ecotoxicology, the vast majority of studies are concerned with effects of anthropogenic chemical stressors on reproductive performance.[41] Mechanisms of reproductive impairment by EDCs include ER and androgen receptor (AR) activation and antagonism, and disruption of steroidogenesis.[41] The toxicity resulting from inhibition of aromatase (CYP19A), which is responsible for the conversion of T to E2, has been well characterized in fish.[42] Inhibition of aromatase leads to decreased concentrations of E2 in blood plasma, resulting in decreased synthesis of VTG, and a subsequent reduction in fecundity via disruption of oogenesis.[42] This mechanism is generally well understood and has been shown to be conserved across different fish species.[42] EDCs can have significant effects on the reproductive success of fish populations and therefore when assessing the effects of toxic compounds, it is important investigate potential endocrine disrupting effects.

Studies suggest that BZT-UVs are EDCs that might impact reproductive performance via an anti-androgenic mode of action, through antagonism of the AR.[8, 43] Specifically, Fent et al[8] assessed the estrogenic, androgenic, and anti-androgenic activity of UV-P, UV-320, UV-326, UV-327, and UV-328 in the yeast estrogen screening (YES) and yeast androgen screening (YAS) assay. In these assays, yeast strains have the DNA sequence of either the human ER (hER) or AR (hAR) integrated in the genome, as well as expression plasmids containing the *lac*-

Z reporter gene under control of hER or hAR response elements.[44, 45] Upon ligand activation of hER or hAR, β-galactosidase (encoded by *lac-Z*) is synthesised, which converts a yellow chromogenic substrate from to a red product that is measured at 540 nm.[44, 45] Although no estrogenic or androgenic activity was reported, UV-P exhibited anti-androgenic activity.[8] Another study which measured the anti-androgenic activity of BZT-UVs indicated that UV-P showed potent anti-androgenic activity (IC_{20} = 0.365 μM), while UV-234, UV-326, UV-328, UV-329, and UV-350 showed weak anti-androgenic activity at higher concentrations (50μM).[43] Additionally, this study demonstrated the potential for metabolic activation of UV-328 and metabolic deactivation of UV-P as an AR antagonist.[43]

1.4.2 Aryl hydrocarbon receptor pathway dysregulation

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is localized in the cytoplasm as a complex bound to co-chaperones including two HSP90 proteins, hepatitis B virus X-associated protein (XAP-2), and p23 (**Figure 1-2**).[1] In the canonical AHR pathway, after activation by a ligand, the AHR complex undergoes translocation to the nucleus, where the complex dissociates and the AHR heterodimerizes with ARNT (AHR nuclear translocator).[1] After dimerization, the heterodimer binds to xenobiotic response elements (XRE), and activates transcription of target genes, including the cytochrome p450 enzymes, CYP1A and CYP1B that metabolize xenobiotics as a part of phase I metabolism.[1, 46] Increased expression of *CYP1A* measured as an increase in *cyp1a* transcript or CYP1A protein is a widely used biomarker for AHR activation, and is therefore useful to identify AHR agonists.[47]

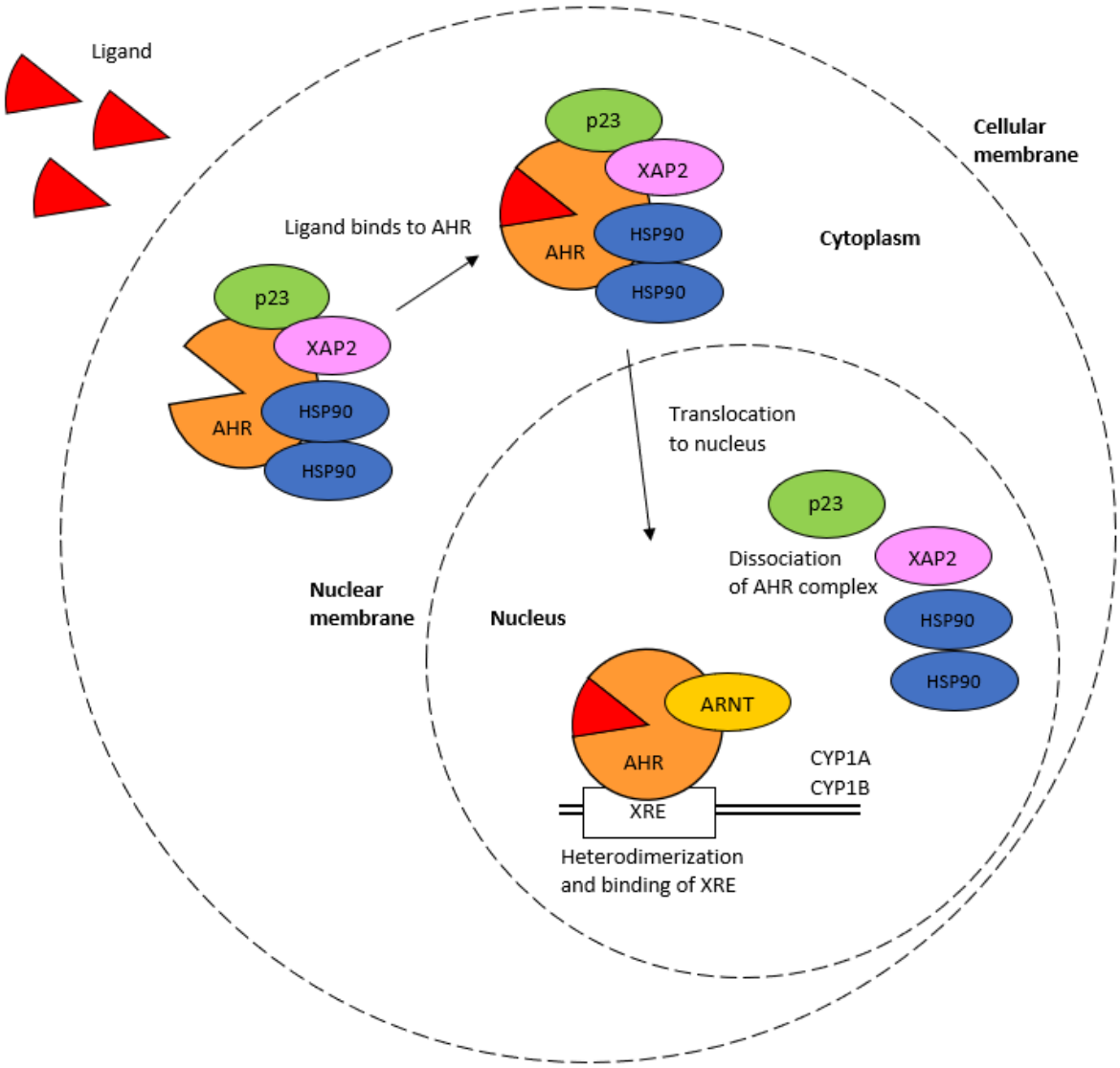


Figure 1-2: Simplified schematic of the AHR pathway modified from Larigot et al[1] showing the process through which an AHR ligand activates the AHR. First, the ligand enters the cell and binds to the AHR which exists in the cytoplasm as a complex with p23, XAP2, and an HSP90 dimer. The complex then moves into the nucleus where it dissociates. The AHR then heterodimerizes with ARNT (AHR Nuclear Translocator), and binds to the xenobiotic response element (XRE) resulting in transcription of target genes, including CYP1A and CYP1B.[1]

The endogenous role of the AHR has been studied in fish and has been suggested to be important for various aspects of development as well as reproductive health; however, the AHR pathway has been most well understood for its role in mediating toxicity of a variety of chemicals including dioxins, polychlorinated biphenyls (PCBs), and polycyclic aromatic

hydrocarbons (PAHs).[48, 49] Early life stages of fish are particularly susceptible to AHR mediated toxicity as dysregulation of this pathway is linked to increased mortality and developmental malformations (spinal curvature, pericardial edema, yolk sac edema, craniofacial malformations).[49] Additionally, dysregulation of the AHR pathway by compounds such as the 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), which is the prototypical AHR ligand, has been reported to result in decreased reproductive success.[40, 49]

There is evidence that some BZT-UVs might act as ligands of the AHR. Multiple studies have investigated activation of the AHR pathway by BZT-UVs in ELS of zebrafish (*Danio rerio*).[8, 33] Li et al[33] reported the abundance of *cyp1a* transcripts was increased after exposure to UV-P and UV-329, while Fent et al[8] reported greater abundance of *cyp1a* transcripts after ELS exposure to UV-P and UV-326. *In vitro* studies have also investigated the potency of BZT-UVs as ligands of human AHR and found that UV-P, UV-9, UV-090, and UV-326, all activated the AHR.[15] However, the BZT-UVs are much less potent than TCDD as the 50% effect concentration (EC_{50}) of UV-P, UV-9, and UV-090 was 130, 460, and 5.1 x 10^3 nM respectively while the EC_{50} of TCDD was 10 nM.[15]

1.5 Metabolism of BZT-UVs

Metabolism of xenobiotic compounds is vital for detoxification and clearance to lessen the potential for adverse effects. This process occurs through phase I, II, and III detoxification and removal of xenobiotic compounds.[50] A major mechanism of phase I detoxification is the enzymatic conversion of substrates by cytochrome p450 enzymes (CYP450) such as CYP1A, CYP1B, and CYP3A4, while phase II detoxification occurs through a variety of enzyme mediated conjugation reactions, such as conjugation of glutathione, sulfate, glycine, or

glucuronic acid to increase solubility of products of phase I reactions.[50,51] Phase III primarily entails the removal of xenobiotics from the organism via efflux proteins.[51] Various BZT-UVs have been studied with respect to metabolism and biotransformation, and it has been reported that some BZT-UVs are bio-transformed by CYP3A4, and can become more (i.e. bioactivation) or less active antagonists of the AR.[43] Additionally, it has been reported that UV-328 is metabolized when incubated with human liver microsomes, which contain cyp450 enzymes, through oxidative phase I metabolism, resulting in the production of multiple metabolites.[52] These preliminary results indicate metabolism of BZT-UVs, resulting in activation or deactivation, and the production of multiple metabolites, suggesting that metabolism of these compounds might play a role in mediating toxicity.[43, 52]

1.6 Omics in ecotoxicology

Recently there has been an increase in the use of high-throughput omics techniques in the field of ecotoxicology. The use of omics technologies can provide significant information regarding the effects of chemicals on the transcriptome, proteome, metabolome, and epigenome of fish species which can provide further insight into potential mechanisms of toxicity at their respective biological levels.[53] At the transcriptomic level, it can be difficult to determine whether changes to transcript abundance result in functional effects, as altered transcript expression may not translate to changes in protein amount.[54] Alternatively, proteomics allows for direct measurement of proteins.[54] Metabolomics assesses changes in the concentrations of small biologically relevant molecules such as amino acids.[53] In comparison to transcriptomics and proteomics, metabolomics has the advantage of being further downstream, and therefore changes to the metabolome can be considered more closely linked to changes in the phenotype of the organism.[55]

1.7 Japanese medaka as a model organism

Three species of small-bodied fish, the fathead minnow (*Pimephales promelas*), zebrafish, and Japanese medaka, have been used extensively in the field of aquatic ecotoxicology to understand potential adverse effects, and associated mechanisms, of chemical pollutants.[54] These species have been instrumental in advancing understanding of effects of EDCs on reproductive performance of fishes. These fish are useful as model species due to there short generation time, easy handling, hardiness, as well as their well understood physiology and development, and sequenced genome.[56] Japanese medaka are particularly useful for studying reproductive toxicities because they spawn consistent numbers daily and do not cannibalize eggs, allowing for accurate measurements of changes in fecundity.[56] They are morphologically sexually dimorphic, allowing for easy identification of male and female fish to ensure even spawning ratios.[56] Therefore, Japanese medaka have been commonly used to test the endocrine disrupting potential of various chemicals in the Organization for Economic Co-operation and Development (OECD) 229 Protocol, Fish Short-Term Reproduction Assay and US EPA endocrine disruptor screening program.[56, 57]

1.8 Research rationale, objectives, and hypothesis

Among the BZT-UVs currently in production that have been studied for their adverse effects, 2-(2H-Benzotriazol-2-yl)-4-methylphenol (UV-P) is suggested to have anti-androgenic activity and to be the most potent activator of the AHR.[8, 15] Additionally, UV-P has been detected in sediment, waterbodies, and fish.[12, 16, 19] For these reasons, UV-P is a leading candidate for studies investigating the effects of BZT-UVs.

Objectives

The overarching objective of this research is to characterize effects of UV-P, as a representative BZT-UVs, to the laboratory fish model species, Japanese medaka. Specific objectives of this research were to:

1. Assess the effects of foodborne exposure to UV-P on reproductive success of adult Japanese medaka.

2. Assess the effects of UV-P exposure on the metabolome of female Japanese medaka livers to better understand potential mechanisms of toxicity.

Hypothesis

Due to UV-P's proposed action as an AHR agonist and an AR antagonist, it is hypothesized that the reproductive success of Japanese medaka will be negatively impacted, and that changes will be observed in the metabolome of Japanese medaka livers.

CHAPTER 2: EFFECTS OF 2-(2H-BENZOTRIAZOL-2-YL)-4-METHYLPHENOL (UV-P) ON THE REPRODUCTIVE SUCCESS OF THE MODEL FISH SPECIES, JAPANESE MEDAKA (*ORYZIAS LATIPES*).

Abstract

Benzotriazole ultraviolet stabilizers (BZT-UVs) are a class of chemicals that are added to various products to prevent damage caused by UV light, and have emerged as pollutants of concern. Although BZT-UVs are detected in aquatic biota globally, very few studies have assessed the potential toxic effects of these chemicals. The objective of this study was to assess effects of 2-(2H-Benzotriazol-2-yl)-4-methylphenol (UV-P) on the reproductive success of Japanese medaka (*Oryzias latipes*) by use of a 21-day reproduction assay. Japanese medaka were exposed to UV-P through a foodborne exposure to nominal concentrations of 0, 25, 125, and 625 ng UV-P/g food, for a total of 28 days which included 7 days of exposure prior to the start of the 21-day reproduction assay. No significant effect on egg production or fertilization success was observed. Abundances of transcripts of *erα*, *vtgI, cyp1a*, or *cyp3a4* were not significantly different in livers of male or female fish exposed to UV-P. However, abundances of transcripts of *cyp19a* and *cyp11a* were significantly lower in the gonads from female fish. There was a trend of increasing concentrations of E2 and a non-significant increase of T in the 625 ng/g treatment in blood plasma from female fish exposed to UV-P but concentrations of 11-KT were unchanged in blood plasma from males. The responses suggest weak perturbation of the steroidogenesis pathway, consistent with an antiandrogenic mode of action, but that is insufficient to impair reproductive performance. Metabolomics analysis suggested altered concentrations of various metabolites and biological pathways, including glutathione metabolism, suggesting that UV-P might be causing responses related to oxidative stress or phase II metabolism; however, there was no obvious mechanism of toxicity. Overall, results of this study indicate that UV-P does not

significantly impact reproductive success of Japanese medaka; however, impacts on the steroidogenesis pathway could indicate a potential mechanism of toxicity which might lead to reproductive impairment in more sensitive species.

2.1. Introduction

Benzotriazole ultraviolet stabilizers (BZT-UVs) are a class of pollutants that have garnered increased attention and public concern.[12, 58] BZT-UVs contain a benzotriazole moiety with 2-hydroxyphenol attachments, and various alkyl additions, that allows them to absorb both ultraviolet-A and -B (UV-A and UV-B) light (280-400nm).[11] Therefore, these compounds are added to a variety of products, including building materials, waxes, paints, shoes, and glasses to prevent UV damage and yellowing because of sun exposure.[31] BZT-UVs enter the environment through wastewater effluent, sewage and leaching from plastic waste.[31] As a result, BZT-UVs are ubiquitous in the environment and have been detected in the USA, Canada, China, Japan, India, and the Philippines.[12, 16, 19, 31, 59] BZT-UVs are frequently detected in rivers, lakes, sediment cores, wastewater, sewage sludge, and marine ecosystems as well as biota including fish, dolphins, turtles, and birds.[11, 12, 19, 30, 31,59, 60]

Despite their frequent detection in aquatic ecosystems and biota, few studies have investigated the toxicity of BZT-UVs to aquatic organisms, including fish.[19] Initial studies of the toxicity of BZT-UVs to fish indicated that BZT-UVs can activate the aryl hydrocarbon receptor (AHR) pathway in early life stages (ELS) of zebrafish and in *in vitro* bioassays.[8] Results from *in vitro* assays also suggest that some BZT-UVs are androgen receptor (AR) antagonists.[8, 43] It has also been suggested that BZT-UVs could cause toxicity through dysregulation of the thyroid hormone pathway.[32] In fish, dysregulation of the AHR and thyroid hormone pathway have been linked to adverse effects on development of early life stages, and impaired reproductive success

in sexually mature fish, while anti-androgenic compounds have been shown to impair reproduction.[49, 61-63]

Studies to date provide important information regarding potential adverse effects of BZT-UVs to aquatic organisms; however, there are still significant knowledge gaps with respect to adverse effects on fishes, including effects on reproductive performance. One BZT-UV in particular, UV-P, has been reported to be a potent AHR agonist as well as an antiandrogenic compound.[8, 15] To this end, the objectives of this study were to perform a standardized 21-day reproduction assay to determine the effects of foodborne exposure to UV-P on the reproductive success of the model laboratory species, Japanese medaka (*Oryzias latipes*), and to determine effects of any maternally deposited UV-P on development of ELS medaka. Fecundity, fertilization success, concentrations of plasma sex steroids, abundances of transcripts of genes that regulate reproduction, and profiles of metabolites in livers were investigated in adults while survival to hatch was measured in ELS medaka.

2.2. Methods

2.2.1. Japanese Medaka

Adult Japanese medaka (6-7 months old, approximately 0.3 - 0.5 g) were acquired from a culture maintained at 25°C at the Aquatic Research Facility at the University of Lethbridge (Lethbridge, AB, Canada). All procedures were approved by the University of Lethbridge Animal Welfare Committee (protocol #2014).

2.2.2. Food preparation

Food was prepared using methods adapted from Saunders et al.[64] TetraMin® tropical flakes were ground to fine granules using a Betty Crocker 4 Cup Glass Bowl Food Chopper and

60 g were allocated into four separate Pyrex dishes. Food was spiked with a 150 ml solution of 0, 0.01, 0.05, or 0.25 µg UV-P/mL in acetone. Food was mixed periodically and left to dry in the dark for 28 h to produce food containing nominal concentrations of 0, 25, 125, and 625 ng UV-P/g food.

2.2.3. Reproduction Assay

Experimental procedures were adapted from the Organization for Economic Co-operation and Development (OECD) 229 Protocol, Fish Short-Term Reproduction Assay.[57] Ten fish, five males and five females were allocated to 9 L tanks in a recirculating system with four replicate tanks across four different treatments. The fish were given a 21-day acclimation period prior to initiation of the assay. During the acclimation period, oxygen concentrations and water temperature were measured daily through a built-in monitoring system, and background fecundity data was collected to ensure that reproduction was consistent among all tanks prior to initiation of the experiment. Fish were fed 100 mg of UV-P spiked food twice daily, accounting for roughly 5-6% of their body mass throughout the course of the exposure. Exposure to the 0, 25, 125, and 625 ng UV-P/g food began 7 days prior to the start of the 21-day reproduction assay to allow for any bioaccumulation to occur. Throughout the 21-day assay, fecundity was assessed daily. Fertilization success was quantified on day 1, 5, 9, 13, and 17 of the reproduction assay, by collecting eggs from each replicate tank and assessing presence of a visible cell mass 24 h after collection, by use of a SteREO Discovery V12 microscope (Zeiss, North York, ON, Canada). To assess maternal transfer (section 2.2.8.3), eggs were collected daily throughout the 21-day reproduction assay, and 4 days' worth of eggs were pooled across all tanks from their respective treatments (day 1-4, 5-8, 9-12, 13-16, 17-20). Oxygen concentrations and water temperature were measured daily while manual measurements of pH, ammonia, nitrate, and nitrites were

performed weekly. On the final day of the assay, fish were euthanized using MS-222 and liver and gonads were dissected, weighed, and flash frozen in liquid N_2 to be analysed using quantitative qPCR (section 2.2.5). Gonadosomatic index (GSI), hepatosomatic index (HIS), and condition factor (K) were calculated using measurements taken on the final day of the assay. Blood was collected and centrifuged at 13,000 x g for 10 mins at 10 °C and plasma was removed and stored at -80 °C until it was used for quantification of steroid hormone concentrations (section 2.2.6).

2.2.4. Embryotoxicity

Embryotoxicity was assessed for embryos collected on day 7, 14, and 21 of the 21-day reproduction assay. Embryos were transferred to 24 well plates (1 egg per well) containing dechlorinated city of Lethbridge water at 25 °C and reared until 18 days post fertilization. Fifty percent water renewals were performed every 3-4 days. At 6 days post collection, the heart rate of 6 randomly chosen embryos per replicate was assessed using a SteREO Discovery V12 microscope (Zeiss, North York, ON, Canada) by counting beats for 30 seconds and multiplying by two. Survival to hatch was assessed at 18-days post fertilization (dpf).

2.2.5. Quantitative PCR

Total RNA was isolated from livers and gonads of eight males and females per treatment (two males and two females per replicate tank) using TRIzol™ reagent (Thermofisher Scientific Ottawa, ON, Canada), and RNA concentrations were measured using a NanoDrop One spectrophotometer (ThermoFisher Scientific). Synthesis of cDNA was performed using 2 µg of RNA using a QuantiNova™ Reverse Transcription Kit (Qiagen, Inc. Mississauga, ON, Canada). A 25 µL reaction mixture was created with 1.25 µL cDNA, 1.25 µL primers (10 pM), and

RNAse free water, and SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience, Cincinnati, OH, United States). Reactions were performed in duplicate (10 µL) in 96 well plates using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada). An initial denaturation step was performed at 95 °C for 2 min followed by 40 cycles under the following conditions: denaturation 95 °C for 5 s, annealing and elongation at 60 °C for 10 s. Following completion, a melt curve was performed to ensure only a single product was amplified. Transcript abundances were normalized to *rpl7* and *18S rRNA* and changes in abundance were calculated relative to the control using the Pfaffl method.[65] A no template control was used to ensure there was no contamination of the samples. Efficiencies of primer sets were generated by performing reactions using serial dilutions of cDNA **(Table 2-1)**.

Table 2-1: Forward and reverse primer sequences and efficiency of primer sets used for quantitative real-time PCR (RT-qPCR). Annealing temperature of all primers was 60 °C.

Gene	Sequence	Efficiency	Reference
18s rRNA	F - GACAAATCGCTCCACCAACT R - CCTGCGGCTTAATTTGACCC	111.1%	66
rpl7	F - GTCGCCTCCCTCCACAAAG R - AACTTCAAGCCTGCCAACAAC	88.7%	64
cyp1a	F - GAGCACCTGGTCAAAGAGATAG R - AGCACATGCCACAGATAACA	103.4%	67
cyp3a4	F - GAGATAGACGCCACCTTCC R - ACCTCCACAGTTGCCTTG	117.4%	64
era	F - CGGACCAGCACTCAGATCCA R - CAGGGGAGCAGAGTAGTAGC	92.2%	64
vtgI	F - ACTCTGCTGCTGTGGCTGTAG R - AAGGCGTGGGAGAGGAAAGTC	108.8%	64
cyp11a	F - GCTGCATCCAGAACATCTATCG R - GACAGCTTGTCCAACATCAGGA	110.1%	64
cyp19a	F - CTCTTCCTGGGTGTTCCTGTTG R - GCTGCTGTCTTGTGCCTCTG	112.7%	64

2.2.6. Plasma steroid hormones

Blood from male and female fish was collected in blood collection capillary tubes by making a small incision on the tail of the fish. Whole plasma for use in ELISA assays was isolated from blood by centrifuging at 13000 x g for 10 mins at 10 °C and removing the top fraction. Estradiol (E2) (Item No. 501890), and testosterone (T) (Item No. 582701) ELISA kits (Cayman Chemicals; Ann Arbor, MI, United States) were used according to manufacturer's instructions to assess concentrations of E2 and T in plasma from females. An 11-ketotestosterone (11-KT) (Item No. 582751) ELISA kit was used according to manufacturer's instructions to assess concentrations of 11-KT in plasma from males.

2.2.7. Untargeted metabolomics

Metabolites were extracted from livers of female fish based on the protocol outlined by Fujita et al.[68] Briefly, samples were thawed and homogenized in methanol (8µL/mg of tissue) using a handheld homogenizer. Next, Milli-Q H_2O (7.2 µL/mg of tissue) and chloroform (8 µL/mg of tissue) were added to the homogenized livers, vortexed, and the samples were left for 10 mins at -80 °C. Samples were centrifuged at 6000 x g and 4 °C for 15 minutes, the top layer was removed and transferred to a clean 1.5 mL microfuge tube, and then samples were placed into an N_2 gas drying box until solvents had evaporated. After drying, water-soluble metabolites were resolubilized in 120 µL of D_2O with 0.02709% (v/v) trimethylsilyl propanoic acid (TMSP) and 480 µL metabolomics buffer (0.125 M KH2PO4, 0.5 M K2HPO4, 0.00375 M NaN3, and 0.375 M KF: pH ~7.4). 550 µL of resolubilized sample was aliquoted into a 7mm NMR tube for spectral acquisition.

Data acquisition and processing was identical to that of Fujita et al.[68] In brief, Spectra were acquired on a Bruker 700 MHz Avance Spectrometer (Bruker, Milton, ON, Canada) and phase corrected in topspin and binned in MATLAB.[69] Data was normalized to the whole metabolome, pareto scaled, and both univariate and multivariate statistics were performed. The appropriate univariate test was applied based on data normality according to the decision tree algorithm outlined by Goodpaster et al.[2] In the case of normal and non-normal data a Welch's t-test or Mann-Whitney U test was applied, respectively. For multivariate testing, variable importance analysis based on random variable combination (VIAVC) was performed to assess variable significance. VIAVC takes subsets of variables in the data and creates random combinations to determine if certain variables have greater influence on classification when considered in the context of other variables as a result of synergistic effects.[70] By doing so, VIAVC can identify variables that may be seemingly unimportant on their own but in the context of other variables have significant effects on group classification.[70] MetaboloanalystR was utilized to create hierarchal clustering heatmaps, PCA plots and, OPLS-DA plots.[71] Metabolites were identified using Chenomx NMR suite and pathway topology analysis was done against the zebrafish KEGG database.[72, 65] Pathway topology analysis was performed using an open access Metaboanalyst online software, with hypergeometric test selected for metabolic set analysis and betweenness centrality test selected for topology analysis.[72-74]

2.2.8 UV-P quantification in food and eggs

2.2.8.1. Chemicals

2-(2-Hydroxy-5-methylphenyl) benzotriazole (UV-P) (purity: 97%), anhydrous sodium sulfate, Supelclean ENVI-Florisil glass solid-phase extraction (SPE) tubes, HPLC grade acetonitrile, water, hexane, acetone, and dichloromethane were purchased from Sigma-Aldrich

(Oakville, ON, Canada). The surrogate standard 2-(2H-benzotriazol-2-yl)-4,6- di-tert-pentylphenol-d4 (UV328-d_4) (purity: 99.8%) was purchased from ASCA GmbH (Berlin, Germany). The dispersive SPE kits "QuEChERS Enhanced Matrix Removal – Lipid" (EMR) were purchased from Agilent (Mississauga, ON, Canada).

2.2.8.2. Concentrations in food

Fish food (0.1-0.5 g) was transferred to a glass test tube and surrogate standard UV328-d_4 was spiked into the sample. The fish food sample was vortexed with 5 mL of hexane/dichloromethane (1/1) for 1 min, followed by 10 min of sonication and 5 min of centrifugation at 1167 x g. The extraction was repeated 3 times, and the extracts were combined. The solvent extract was concentrated to 1 mL under gentle N_2. The 1 mL fish food extract was cleaned using Florisil SPE and EMR. The Florisil SPE was conditioned using 12 mL of hexane/dichloromethane (1/1) before use. After loading the 1 mL extract on the SPE, 10mL of dichloromethane/acetone (1/1) was used to elute the target compounds. Elutes were concentrated to dryness using a gentle stream of N_2 and then reconstituted in 5mL acetonitrile. Sorbents and salts in the EMR kits were transferred to glass tubes before use. Then, 4 mL of water was added to the EMR dispersive SPE tube and vortexed for 30s to activate the sorbents. The 5 mL acetonitrile sample was added to the EMR dispersive SPE tube, followed by 1 min of vortexing, 1 min up-down shaking, and 5 min centrifugation at 1167 x g. The top supernatant was transferred to the EMR polish tube to remove water by 30s of vortexing, 1 min up-down shaking, and 5 min centrifugation at 1167 x g. The top acetonitrile was transferred to a glass centrifuge tube and concentrated to dryness using a gentle stream of N_2. The sample was reconstituted in 0.5 mL of hexane for instrument analysis.

2.2.8.3 Maternal transfer

To assess maternal transfer of UV-P, fish egg sample (0.5-1 g) was homogenized with 2 g of Na_2SO_4 in a mortar. The sample was transferred to a glass test tube and surrogate standard UV328-d_4 was spiked in the sample. Next, the fish egg sample was vortexed with 5 mL of acetonitrile for 1 min, followed by 10 min of sonication and 5 min of centrifugation at 1167 x g. The extraction was repeated 3 times, and the extracts were combined and concentrated to 5 mL under a gentle stream of N_2. The sample was then cleaned using the EMR method, described above.

A gas chromatography-mass spectrometry (GC-MS) was used for sample analysis. The GC-MS consisted of a Thermo Trace GC and an Ultra-PolarisQ MS. The GC column was a 30m RXI-5MS column (Chromatographic Specialties, Brockville, Canada) (0.25mm in diameter) with 0.25µm diameter for the film thickness. Three µL of sample was injected using a Programmable Temperature Vaporization (PTV) injector. Initial injector temperature was set at 50°C, held for 0.1 min and ramped to 120 °C at a rate of 14 °C /min, then the temperature was increased to 300 °C at a rate of 2.5 °C /min (held for 10 min), followed by a cleaning process by increasing temperature to 330 °C and held for 5 min. The oven temperature program started at 80 °C and was maintained for 2 min. The temperature then raised to 160 °C with a rate of 10 °C / min, then to 230 °C with a rate of 5 °C / min and finally to a temperature of 300 °C with a rate of 10 °C / min, followed by an isothermal hold of 15 min. Helium gas (1 mL/min) was used as the carrier gas. The GC-MS transfer line temperature was set to 300 °C. The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and the ion source temperature was 200 °C. The quantification ions was *m/z* 225 for UV-P. The qualification ions was *m/z* 168 for UV-P. The quantification ion for the surrogate standard UV-328-d_4 was *m/z* 326.

Quantitation was done by relative response to the surrogate standard using a solvent standard curve. Procedural blanks were included in each batch of the experiment, but none of these target compounds was detected. The recovery of surrogate standard was 83 ± 31% in fish egg samples and 62 ± 22% in fish food samples.

2.2.9. Statistical analysis

Statistical analysis was performed using R studio version 1.2.5042 with R-4.0.0.[75] All data was tested for normality using the Shapiro Wilk test and for equal variance using the Bartlett test. Parametric data were assessed by a one-way ANOVA followed by a post-hoc Tukey test. Non-parametric data was log transformed and if still non-parametric were assessed by a Kruskal Wallis test followed by a post-hoc Dunn test. Differences with a $P \leq 0.05$ were considered statistically significant.

2.3. Results

2.3.1. UV-P concentration in spiked food

Concentrations of UV-P in fish food were determined to be 0 ± 0 ng/g, 36 ± 9.5 ng/g, 158 ± 16.8 ng/g, and 634 ± 119.3 ng/g in the 0, 25, 125, and 625 ng/g treatment, respectively.

2.3.2. Reproductive success

Mean GSI, HSI, and K of female and male fish were not significantly different among any treatment groups (**Table 2-2**). Mean cumulative fecundity was 350 ± 25, 392 ± 54, 355 ± 40, and 353 ± 29 eggs/female in the 0, 25, 125, and 625 ng/g treatments, respectively (**Figure 2-1A**). The mean daily fecundity per female was 17.0 ± 1.6, 18.7 ± 2.6, 16.9 ± 1.9, and 17 ± 1.3 eggs/female/day in the 0, 25, 125, and 625 ng/g treatments respectively (**Figure 2-1B**). Neither the cumulative fecundity nor mean daily fecundity were significantly different among any of the

treatment groups. Mean fertilization success was not significantly altered in any treatment group and was 88.7 ± 4.1%, 84.8 ± 5.5%, 80.5 ± 9.9%, and 84.9 ± 4.7% in the 0, 25, 125, and 625 ng/g treatments, respectively (**Figure 2-1C**).

Table 2-2: Body parameters of male and female Japanese medaka after 28-day of exposure to food spiked with 0, 25, 125, and 625 ng UV-P/g food. Parametric data were assessed by a one-way ANOVA followed by a post-hoc Tukey test. Non-parametric data was log transformed and if non-parametric were assessed by a Kruskal Wallis test followed by a post-hoc Dunn test. No significant differences were found among any treatment for any of the measured endpoints ($P > 0.05$).

Treatment	Sex	K	GSI	HSI
0 ng/g	F	1.11 ± 0.06	10.73 ± 1.52	4.69 ± 1.06
25 ng/g	F	1.17 ± 0.10	9.79 ± 1.38	4.11 ± 1.11
125 ng/g	F	1.08 ± 0.04	9.26 ± 1.67	4.46 ± 0.49
625 ng/g	F	1.14 ± 0.07	9.40 ±0.82	3.84 ± 0.75
0 ng/g	M	0.97 ± 0.05	1.00 ± 0.28	1.73 ± 0.13
25 ng/g	M	1.02 ± 0.06	1.23 ± 0.11	2.25 ± 0.42
125 ng/g	M	1.04 ± 0.03	1.33 ± 0.51	2.24 ± 0.34
625 ng/g	M	0.99 ± 0.05	0.85 ± 0.59	2.54 ± 1.14

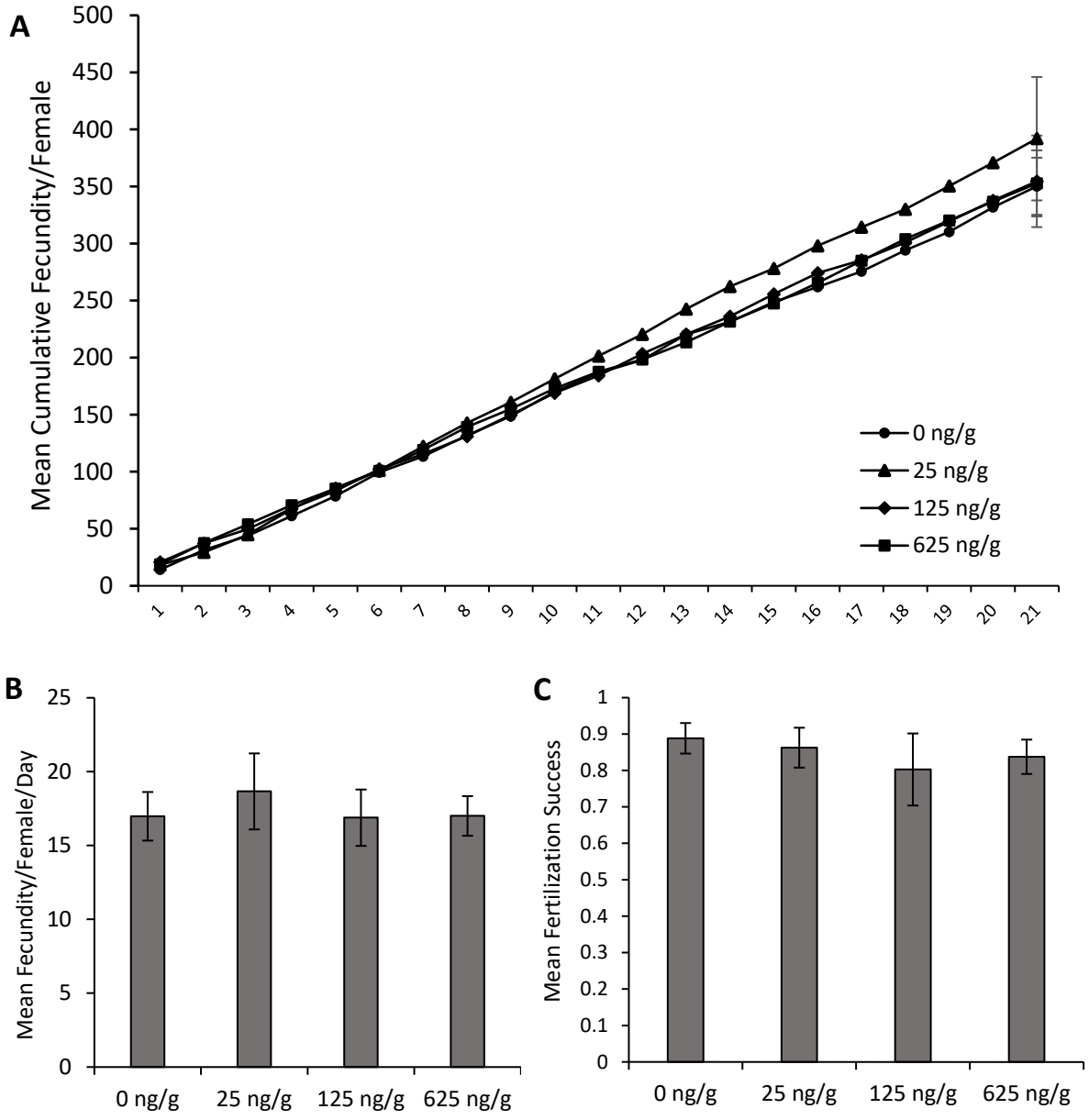


Figure 2-1: Reproductive success of Japanese medaka (*Oryzias latipes*) exposed for 28 days to food spiked with 0, 25, 125, and 625 ng UV-P/g food. **(A)** Mean cumulative fecundity of Japanese medaka females throughout the 21-day reproduction assay. **(B)** Mean fecundity per day of Japanese medaka during the 21-day reproduction assay. **(C)** Mean fertilization success measured at 5 intervals during the 21-day reproduction assay. Parametric data were assessed by a one-way ANOVA followed by a post-hoc Tukey test. Non-parametric data was log transformed and if non-parametric were assessed by a Kruskal Wallis test followed by a post-hoc Dunn test. No significant differences were found among any treatment for any of the measured endpoints ($P > 0.05$). Data represent the mean across 4-6 replicates, and error bars represent standard deviation.

2.3.3 Gene expression

Abundances of transcripts of *cyp3a4, cyp1a,* and *erα* were not different in liver of female or male fish among any treatment (*erα* in males below detection) (**Figure 2-2**). Abundances of transcripts of *vtgI* in liver of female fish were lower in the 125 ng/g treatment compared to the 25 or 625 ng/g treatment; however, these abundances were not significantly different from the 0 ng/g control (*vtgI* in males below detection) **(Figure 2-2)**. Abundances of transcripts of *cyp19a* and *cyp11a* were significantly lower in gonads of female fish exposed to any UV-P treatment compared to the 0 ng/g control but were not significantly different from any other UV-P treatment. Abundances of these transcripts were not different in gonads of male fish exposed to UV-P across all treatments (**Figure 2-2**).

2.3.4 Plasma hormones

There was a trend of increasing concentration of E2 in blood plasma of female fish exposed to UV-P. Concentrations of E2 were 178.6 ± 135.6, 298.6 ± 316.3, 307.8 ± 116.4, and 758 ± 302.8 ng/mL in the 0 ng/g, 25 ng/g, 125 ng/g, and 625 ng/g treatments respectively (**Figure 2-3A**). Concentrations of T were 80.0 ± 28.0, 74 ± 35.1, 76.2 ± 41.03, and 141.0 ± 86.9 ng/mL in the 0 ng/g, 25 ng/g, 125 ng/g, and 625 ng/g treatments respectively (**Figure 2-3B**). None of the changes to concentrations of E2 or T were significant among any treatment group. Concentrations of 11-KT were not significantly different in blood plasma of male fish exposed to UV-P, nor was there a trend of increasing or decreasing concentrations. Concentrations of 11-KT in male plasma were 15.5 ± 5.1, 18.2 ± 2.1, 16.3 ± 1.4, 20.6 ± 2.2 ng/mL in the 0 ng/g, 25 ng/g, 125 ng/g, and 625 ng/g treatment respectively (**Figure 2-3C**).

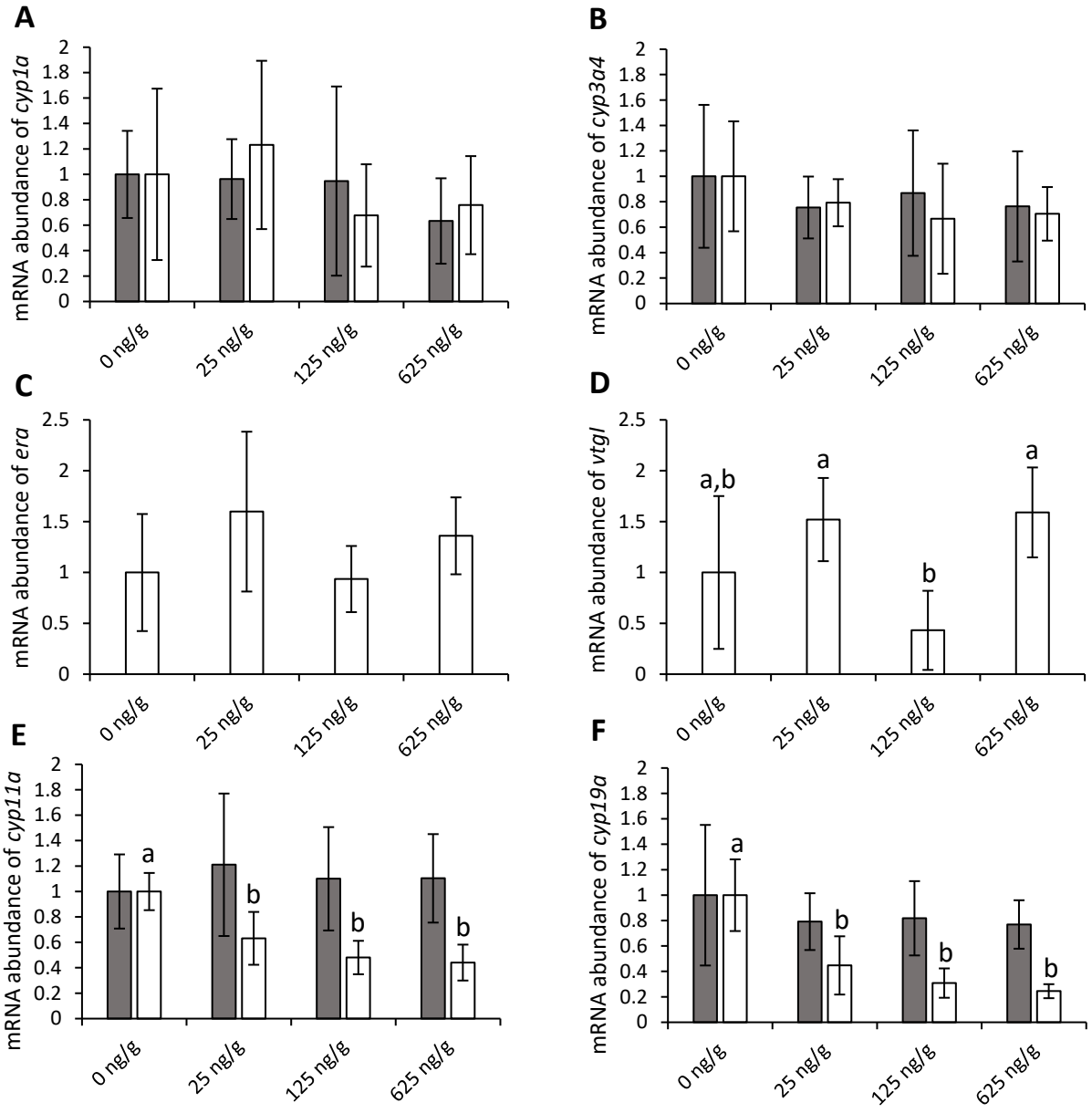


Figure 2-2: Abundances of transcripts in male (grey bar) and female (open bar) Japanese medaka as a fold change from the control (0 ng/g)after a 28-day exposure to food spiked with 0, 25, 125, and 625 ng UV-P/g food. **(A-D)** Abundances of *cyp1a*, *cyp3a4*, *erα*, and *vtgI* transcripts in livers of female and male Japanese medaka. **(E-F)** Abundances of *cyp19a* and *cyp11a* transcripts in gonads of female and male Japanese medaka. Parametric data were assessed by a one-way ANOVA followed by a post-hoc Tukey test. Non-parametric data was log transformed and if non-parametric were assessed by a Kruskal Wallis test followed by a post-hoc Dunn test. No significant differences were observed in male fish comparisons. Letters are used to indicate significance in the female treatments by data having different letters (ie. a vs b) ($P < 0.05$). Data represent the mean across 8 replicates, and error bars represent standard deviation.

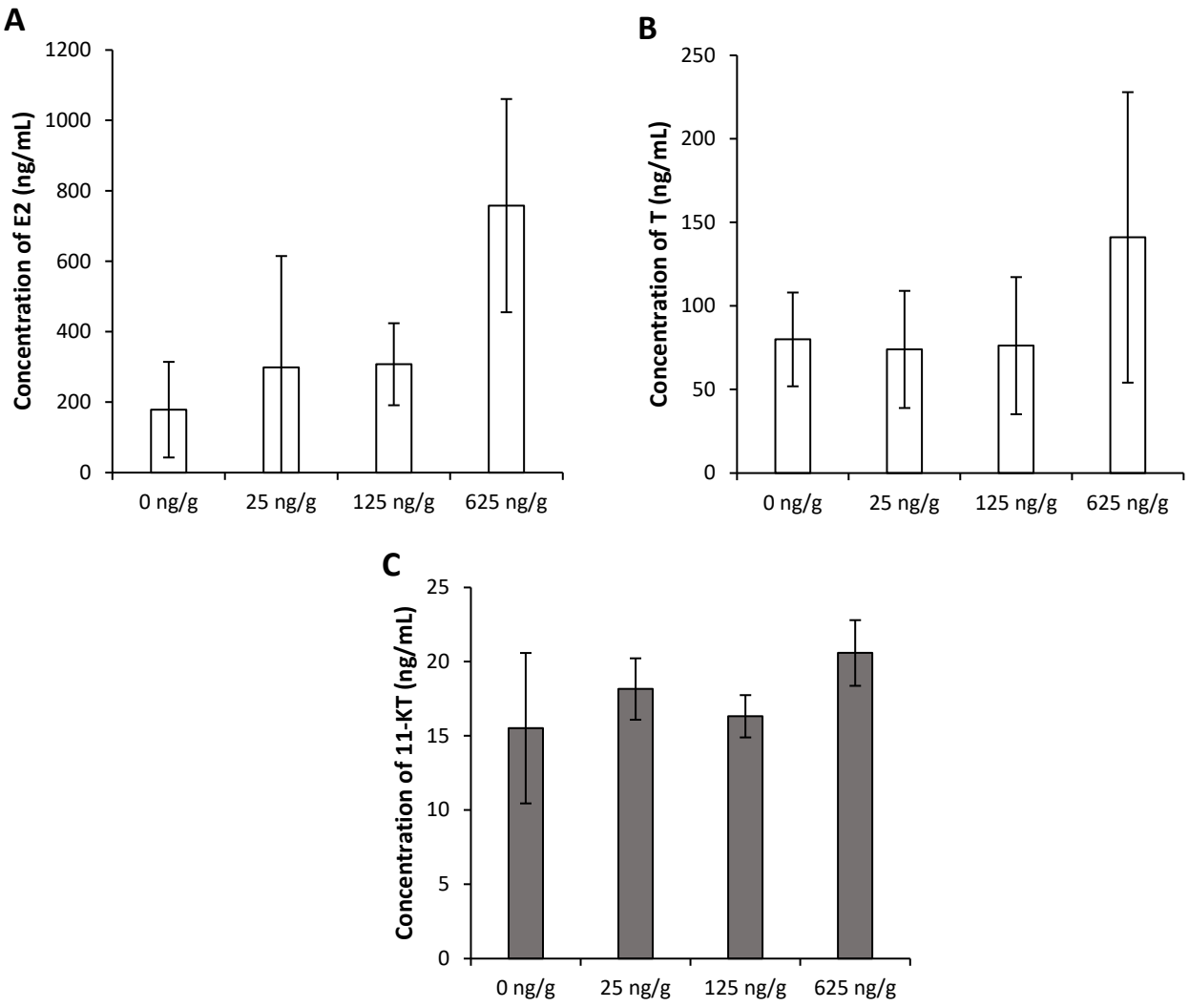


Figure 2-3: Concentrations of steroid hormones in plasma of female (open bars) and male (grey bars) adult Japanese medaka after a 28-day exposure to food spiked with 0, 25, 125, and 625 ng UV-P/g food. **(A)** concentration of estradiol (E2) in plasma of Japanese medaka females. **(B)** concentration of testosterone (T) in plasma of Japanese medaka females. **(C)** concentration of 11-ketotestosterone in Japanese medaka males. Parametric data were assessed by a one-way ANOVA followed by a post-hoc Tukey test. Non-parametric data was log transformed and if non-parametric were assessed by a Kruskal Wallis test followed by a post-hoc Dunn test. No significant differences were found among any treatment for any of the measured endpoints ($P > 0.05$). Data represent the mean across 4 replicates, and error bars represent standard deviation.

2.3.5 Maternal transfer and embryotoxicity

Mean chemical burden of UV-P in embryos of Japanese medaka collected throughout the 21-day reproduction assay were 0.41 ± 0.72, 0.65 ± 0.55, 0.20 ± 0.31, and 0.60 ± 0.15 ng/g in the 0, 25, 125, and 625 ng/g treatment, respectively. Mean survival to hatch of ELS Japanese medaka collected from the 25 ng/g exposure, but not the 125 or 625 ng/g exposures, was significantly less compared to the 0 ng/g control (**Figure 2-4)**.

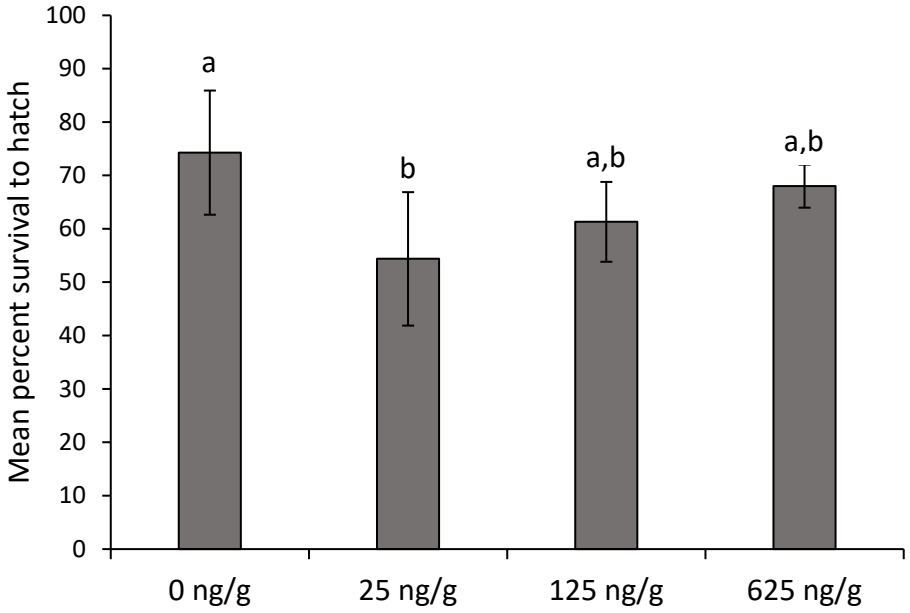


Figure 2-4: Mean survival to hatch of ELS Japanese medaka collected from adult Japanese medaka exposed to food spiked with 0, 25, 125, and 625 ng UV-P/g food. Eggs were collected on days 13, 20, and 27 of the 28-day exposure. Parametric data were assessed by a one-way ANOVA followed by a post-hoc Tukey test. Non-parametric data was log transformed and if non-parametric were assessed by a Kruskal Wallis test followed by a post-hoc Dunn test. Significant differences are indicated by data having different letters (ie. a vs b) ($P < 0.05$) Data represent the mean across eggs taken from 4-6 replicate tanks on 3 different sampling days, and error bars represent standard deviation.

2.3.6 Metabolomics

There were 319 bins identified through NMR analysis. Of these, 71, 31, and 36 bins were identified as significantly altered in the 0 ng/g vs 25 ng/g, 0 ng/g vs 125 ng/g, and 0 ng/g vs 625 ng/g comparisons, respectively. In the 0 ng/g vs 25 ng/g comparison, 66 bins were identified by univariate analysis and 10 bins were identified by multivariate analysis, with five bins in common. In the 0 ng/g vs 125 ng/g comparison, 26 bins were identified by univariate analysis and 14 bins were identified by multivariate analysis, with nine bins in common. In the 0 ng/g vs 625 ng/g comparison, 36 bins were identified by univariate analysis and one bin was identified by multivariate analysis, with one bin in common. Hierarchical clustering heatmaps generated using bins identified as significantly altered suggest improved class separation between 0 ng/g control and treatment groups with increased concentration of UV-P (**Figure 2-5)**. Principal component analysis (PCA) plots suggested partial group separation in the 0 ng/g vs 25 ng/g, 0 ng/g vs 125 ng/g, and 0 ng/g vs 625 ng/g comparisons while OPLS-DA indicated partial group separation in 0 ng/g vs 25 ng/g and complete group separation in the 0 ng/g vs 125 ng/g and 0 ng/g vs 625 ng/g comparisons (**Figure 2-6 A-C**).

In total, across all treatments, 13 metabolites were identified as significantly altered from the 0 ng/g control (**Figure 2-7**). Of the 13 metabolites, seven were the amino acids aspartate, glutamate, glycine, isoleucine, proline, tyrosine, and valine. Other metabolites identified as significantly altered were choline, glucose, lactate, phosphorylcholine, s-adenosylhomocysteine, and taurine. Pathway topology analysis of the identified metabolites suggested that 14 pathways were significantly altered based upon $P < 0.05$ (**Figure 2-7**). Of these 14 pathways, eight had an impact factor greater than 0. These pathways were: arginine biosynthesis; alanine, aspartate and glutamate metabolism; glutathione metabolism; glyoxylate and dicarboxylate metabolism;

glycine, serine and threonine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; glycerophospholipid metabolism; arginine and proline metabolism.

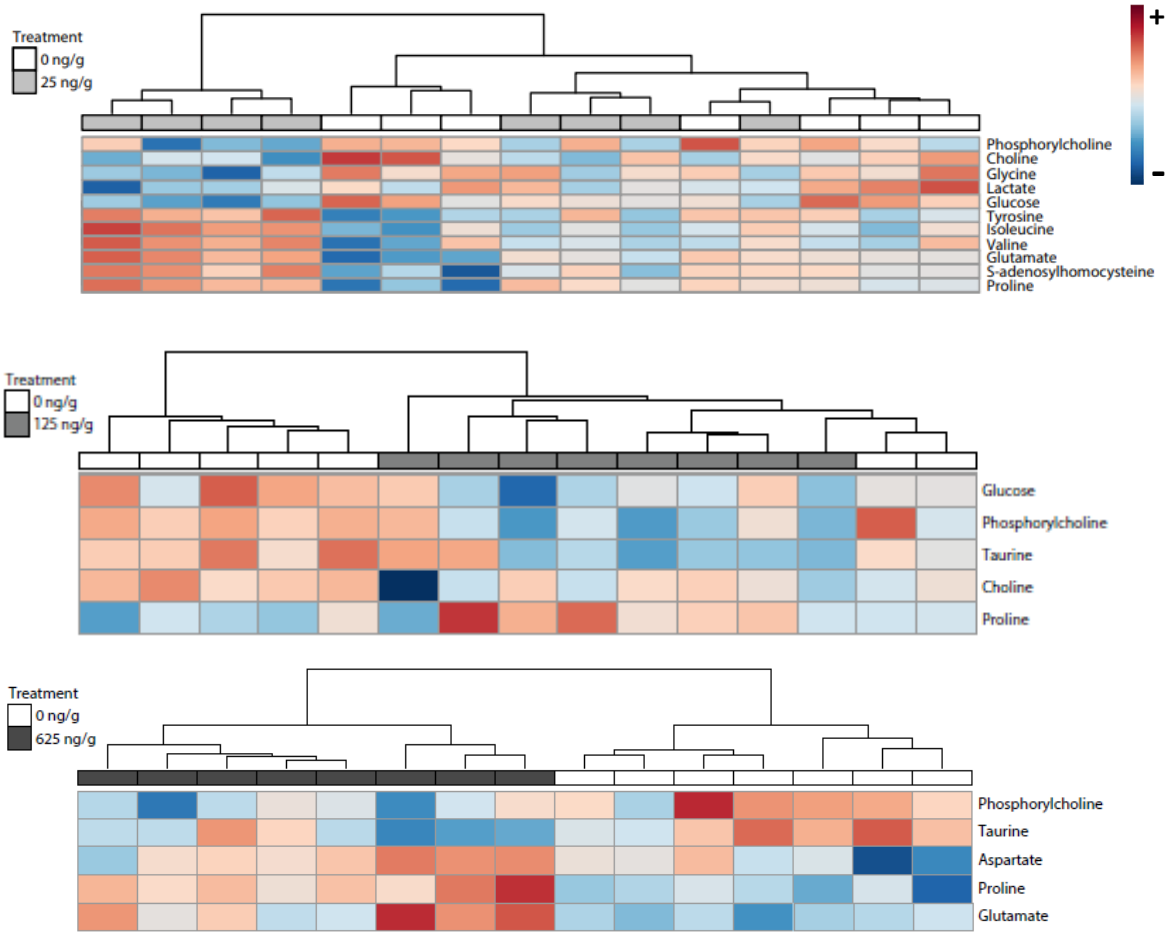


Figure 2-5: Hierarchical clustering heatmaps comparing the metabolome of livers from female Japanese medaka after a 28-day exposure to 25, 125, and 625 ng UV-P/g food to the metabolome of fish exposed to a control (0 ng/g). Comparisons made were performed using bins which were determined to be significant ($P < 0.05$) using Mann Whitney U or Welch's T test following the decision tree algorithm outlined by Goodpaster et al[2] and variable importance analysis based on random variable combination (VIAVC). Data were generated from 7-8 replicates per treatment.

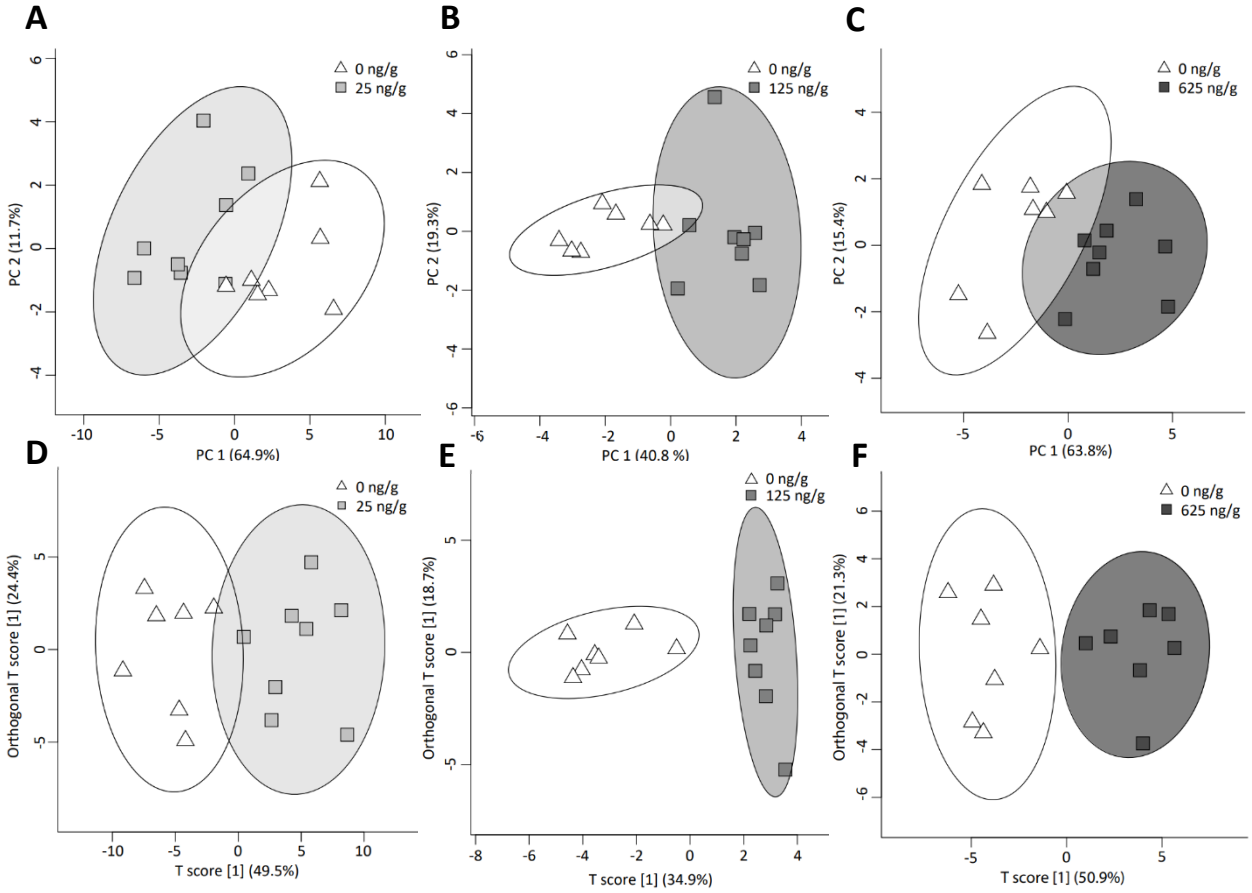


Figure 2-6: (A-C) Principal component analysis (PCA) plots and **(D-F)** orthogonal partial least squares discriminant analysis (OPLS-DA) plots comparing the metabolome of livers from female Japanese medaka after a 28-day exposure to 25, 125, and 625 ng UV-P/g food to the metabolome of fish exposed to a control (0 ng/g). Comparisons made were performed using bins which were determined to be significant ($P < 0.05$) using Mann Whitney U or Welch's T test following the decision tree algorithm outlined by Goodpaster et al[2] and variable importance analysis based on random variable combination (VIAVC). OPLS-DA was validated using double 10-fold cross validation and permutation testing (2000 permutations). 0 ng/g vs 25 ng/g Q^2: 0.719 R^2: 0.814, 0 ng/g vs 125 ng/g Q^2: 0.724 R^2: 0.905, and 0 ng/g vs 625 ng/g Q^2: 0.715, R^2: 0.881. Data were generated from 7-8 replicates per treatment.

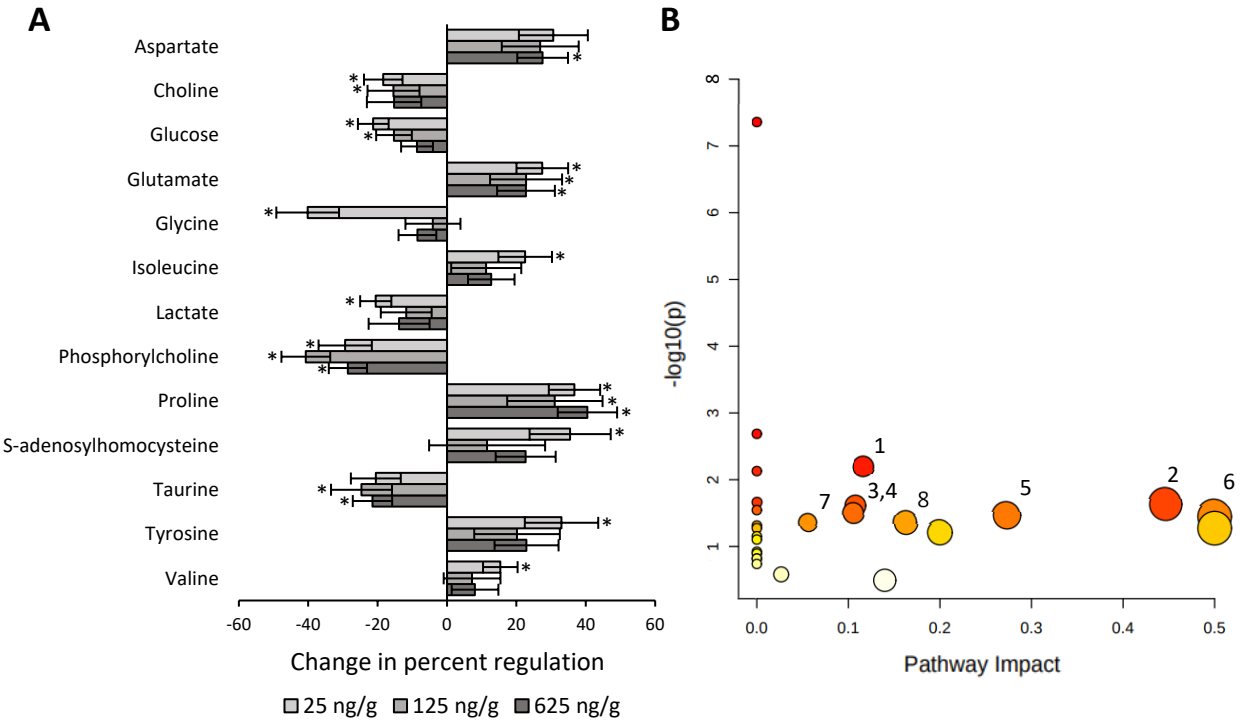


Figure 2-7: (A) Change in percent regulation of 13 metabolites in livers from female Japanese medaka after a 28-day exposure to 25, 125, and 625 ng UV-P/g food. Differences from the 0 ng/g control were determined to be significant if $P < 0.05$ (indicated by "*") using Mann Whitney U or Welch's T test following the decision tree algorithm outlined by Goodpaster et al[2] and variable importance analysis based on random variable combination (VIAVC). Data are the mean change in percent regulation from control and error bars represent standard error. **(B)** Pathway analysis overview indicating potentially perturbed pathways in female Japanese medaka livers after 28-day foodborne exposure to UV-P. Identified pathways were: 1) arginine biosynthesis, 2) alanine, aspartate, and glutamate metabolism, 3) glutathione metabolism, 4) glyoxylate and dicarboxylate metabolism, 5) glycine, serine, and threonine metabolism, 6) phenylalanine, tyrosine, and tryptophan biosynthesis, 7) glycerophospholipid metabolism, 8) arginine and proline metabolism. Data were generated from 7-8 replicates per treatment.

2.4 Discussion

BZT-UVs are a class of chemicals of increasing concern, that have been detected in various environmental matrices, including biota.[19, 60] However, little is known about the potential toxic effects these chemicals could have to organisms, including fish. UV-P, a representative BZT-UV, has been suggested to activate the AHR pathway, disrupt the thyroid hormone pathway, and be an anti-androgenic compound.[8, 15, 32, 43] This study investigated the effects of a dietary exposure to UV-P on the reproductive success of a model fish species, Japanese medaka. This study measured reproductive success (fecundity, fertilization success) and impacts on mechanisms that regulate reproduction, effects on the liver metabolome, and maternal transfer of UV-P to embryos. Results of this study indicate no clear apical effects of UV-P on reproductive performance and suggest there was not significantly greater maternal transfer of UV-P to eggs of fish in the UV-P treatments compared to the 0 ng/g control.

Dietary exposure to UV-P did not impact fecundity or fertility in Japanese medaka. Cumulative fecundity throughout the 21-day reproduction assay remained consistent in all treatment groups, and the fertilization success were similar across all treatments. However, changes to molecular endpoints were observed in female Japanese medaka exposed to UV-P. A decrease in the abundances of transcripts of *cyp11a* and *cyp19a* in female gonads, as well as a non-significant trend of increasing concentrations of E2 and non-significant increase of T in the 625 ng/g treatment in blood plasma of female fish indicate that UV-P might be impacting the steroidogenesis pathway. Abundances of transcripts of both *cyp11a* and *cyp19a* were significantly decreased in the ovaries of Japanese medaka exposed to any of the dietary concentrations of UV-P compared to those in the 0 ng/g control group. *Cyp11a* encodes the cholesterol side chain cleavage enzyme that catalyses the initial conversion of cholesterol to

pregnenolone, and *cyp19a* encodes aromatase, which converts T into E2.[38] *In vitro* studies have indicated that UV-P has anti-androgenic properties, which might explain the trends of changes in concentrations of E2, non-significant change in concentration of T in the 625 ng/g treatment, and changes in gene expression.[8, 43] In comparison, a previous study of the effects of flutamide, a known anti-androgen, demonstrated an increase in T, and trend of increasing concentration of E2 in blood plasma from female fathead minnows (*Pimephales promelas*) after a 21-day reproduction assay.[61] That study also reported a significant decrease in fecundity of fathead minnows throughout the 21-day assay.[61] In contrast, fecundity was not impacted in Japanese medaka exposed to similar concentrations of flutamide, which suggests that species differ in their sensitivity to anti-androgens.[76] Additionally, the *in vitro* studies which reported UV-P as an anti-androgen utilized human AR constructs, and therefore sensitivity could differ in Japanese medaka.[8] Although there were no significant changes to the reproductive success of Japanese medaka, it remains possible that different species of fish could be more sensitive to anti-androgenic effects of UV-P, leading to reproductive impairment.

Studies with ELS zebrafish and human AHR constructs have reported that UV-P activates the AHR signaling pathway, suggesting that this could be a mechanism of toxicity of UV-P.[8, 15] Activation of the AHR pathway leads to the production of various transcripts, including, cytochrome P450 1A (*cyp1a*), which is a biomarker for activation of the AHR in fish.[1, 47] In this study, *cyp1a* transcript abundance was not significantly greater in livers of male or female Japanese medaka, indicating that foodborne exposure to UV-P did not lead to activation of the AHR after 28 days. This could be a result of the transient nature of gene expression and perhaps levels of *cyp1a* had decreased to basal levels while abundance of protein was greater. Additionally, it is possible that UV-P is not a potent ligand of the Japanese medaka

AHR as there are known differences in species sensitivity to AHR ligands.[77] ELS fish tend to be more sensitive to AHR mediated toxicities; however, ELS survival to hatch was only significantly altered in the 25 ng/g treatment compared to the 0 ng/g treatment but not in the 125 ng/g or 625 ng/g treatment.

UV-P, along with many other BZT-UVs, is detected in aquatic biota and has been reported to have the potential to bioaccumulate, which is supported by UV-P's hydrophobic properties (LogKow: ~ 3.42-4.31) and predicted bioconcentration factor of 32.3.[14, 19, 78] Previous work has reported that chemicals which are lipophilic can bioaccumulate in fish and be maternally transferred into the eggs; however, the potential for maternal transfer of BZT-UVs has not been measured.[79] It was hypothesized that UV-P would be maternally transferred to the eggs; however, the present study indicates no significant maternal transfer of UV-P as concentrations of UV-P in eggs was not significantly different in any treatment. It is possible that UV-P was not accumulating in the fish and might have been metabolized before it could be maternally transferred to the eggs. UV-P was not measured in fish tissues in this study but previous work has indicated that juvenile rainbow trout (*Oncorhynchus mykiss*) can eliminate UV-234 and UV-328, which are more likely to bioaccumulate than UV-P.[80] Previous work has suggested that biotransformation of UV-P is mediated by CYP3A4, which is induced by activation of the PXR.[43] Results of this study indicate that the abundance of transcripts of *cyp3a4* in Japanese medaka livers was not altered after a 28-day foodborne exposure to UV-P; however, it remains possible that due to the transient nature of gene expression the levels of *cyp3a4* transcripts had decreased to basal levels while abundance of protein was greater.

Metabolomic analysis suggested changes to the liver metabolome of female Japanese medaka after a 28-day foodborne exposure to UV-P. The hierarchical clustering heatmap, PCA,

and OPLS-DA plots indicated differences in the metabolomic profile of livers of Japanese medaka that were exposed to UV-P compared to the 0 ng/g control. Of the pathways identified, glutathione metabolism is of particular interest as glutathione is involved in the oxidative stress response pathway and phase II metabolism of xenobiotics.[81] This pathway was potentially perturbed by alterations to the concentration of glutamate and glycine. Additionally, the glycine, serine, and threonine metabolism pathway was potentially perturbed by changes to choline and glycine, and this pathway has been reported to be linked with glutathione metabolism.[82] S-adenosylhomocysteine was increased in the 25 ng/g treatment providing another link to glutathione metabolism as this compound is needed for glutathione synthesis.[82] Previous studies have reported an increase in glutathione-S-transferase (*gstp1*) transcript abundance in zebrafish eleuthero-embryos after a waterborne exposure to UV-P.[8] These findings suggest that UV-P might be causing effects related to oxidative stress; however, any effects that UV-P might have did not result in effects on the reproductive success of adult Japanese medaka in this study.

This study indicates that a foodborne exposure to UV-P does not lead to reproductive impairment in adult Japanese medaka. Throughout the 21-day reproduction assay, fecundity and fertility remained consistent across all treatments. A significant decrease in transcript abundances of *cyp11a* and *cyp19a* which are related to steroidogenesis were observed, as well as a trend toward increasing E2 and a non-significant increase in concentration of T in the 625 ng/g treatment in the plasma of female Japanese medaka exposed to UV-P. Previous studies have indicated that UV-P might be an antagonist of the human AR, and this could be linked to the observed changes in the steroidogenesis pathway.[8] Previous studies have indicated that UV-P activates the AHR, however, this study did not indicate an increase in abundance of *cyp1a* transcripts, a common biomarker for AHR activation.[8, 15, 47] Future studies should investigate

activation of the AHR pathway in a variety of phylogenetically diverse species of fishes to determine if they differ in their sensitivity to UV-P, as has been shown for well-established ligands of the AHR.[77] Additionally, although it was expected that UV-P would be maternally transferred to eggs, this study found no significant evidence to support maternal transfer. Metabolomic analysis suggested changes in the concentration of several amino acids and other metabolites in the liver of female Japanese medaka exposed to UV-P. Pathway analysis identified glutathione metabolism as a potentially perturbed pathway, which could be linked to oxidative stress response as well as xenobiotic metabolism due to glutathione's role in those processes.[81] Overall, although no apical effects on reproductive success were observed, results of this study provide insight to potential mechanisms through which UV-P could impact reproductive performance of fishes. Future studies could be performed to assess the impact of UV-P on other species of fish which could be more sensitive to UV-P.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

Recently, BZT-UVs have emerged as contaminants of concern. BZT-UVs have been detected in the environment globally, and it can be expected that concentrations will increase because of their widespread use in many consumer products.[11, 12, 21] To date, there is limited information regarding potential effects of BZT-UVs to fish; however, it has been suggested that UV-P might activate the AHR and be an AR antagonist, suggesting that UV-P could have effects on reproduction in sexually mature fish.[8, 33, 43] Therefore, the objective of this study was to investigate the potential effects of UV-P on the reproductive success of Japanese medaka.

This study assessed the effects of a foodborne exposure to UV-P on Japanese medaka (*Oryzias latipes*) reproductive success throughout a standard 21-day reproduction assay.[57] This study measured fecundity throughout the assay and assessed fertilization success on a subset of days. Molecular endpoints related to reproductive performance were measured. Overall, this study indicates that UV-P does not impact the reproductive success of Japanese medaka at the concentrations tested. However, changes to abundances of *cyp11a* and *cyp19a* transcripts and a trend of increasing concentration of E2 and non-significant increase in concentration of T in the 625 ng/g treatment in female fish suggest that UV-P might be impacting the steroidogenesis pathway. This study also investigated effects on expression of enzymes that are important for biotransformation of xenobiotics. There were no changes in the abundances of either *cyp1a* indicating that the AHR pathway was not being activated by UV-P. Although previous studies have suggested that CYP3A4 might play a role in biotransformation of UV-P, levels of *cyp3a4* transcript were unaltered in the livers of medaka exposed to UV-P.[43] Metabolomics analysis of the livers indicate changes to pathways related to glutathione, which is known to be involved in phase II metabolism of xenobiotic compounds, and thus indicates that UV-P might be

metabolized by the liver. Although there were some changes to abundances of transcripts in the steroidogenesis, the lack of changes to either fecundity or fertilization throughout the study suggest that UV-P does not pose a significant risk to Japanese medaka at the concentrations tested.

Because of UV-P's proposed action as an AR antagonist, it was hypothesized that there could be reproductive impairment.[8] Although the current study indicates minimal reproductive toxicity of UV-P to Japanese medaka, other species of fish might be more sensitive to UV-P and therefore research should be performed to determine potential effects in phylogenetically diverse species such as the fathead minnow. Additionally, studies which proposed AR antagonism were performed *in vitro* using human AR, and therefore might not translate to effects in fish.[8] To address this, *in vitro* studies using AR constructs from phylogenetically diverse species should be performed to assess the antagonistic activity of UV-P against fish ARs.

Previous work has suggested that UV-P activates the AHR pathway in ELS zebrafish and has also been shown to activate the human AHR *in vitro.* Results of this study showed that exposure to UV-P did not result in an increase in *cyp1a* transcript abundance indicating that the AHR pathway was not activated. However, it is known that species vary in their sensitivity to AHR activating compounds.[77] For example, it has been shown that the sensitivity of species to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) can vary by 40-fold.[77] Thus, a comprehensive investigation of AHR activation as a mechanism of toxicity of UV-P would require studies with a diversity of fishes.

It was hypothesized that due to UV-P's hydrophobicity, it would bioaccumulate and be maternally deposited into the eggs throughout the 21-day reproduction assay. However, chemistry analysis showed that UV-P was not maternally deposited. Previous studies using liver

microsomes have reported that UV-328 is metabolized into multiple metabolic products.[43] Perhaps, rather than accumulate in the fish and subsequently be deposited, UV-P was metabolized in the liver of Japanese medaka and subsequently excreted. Future studies should investigate the metabolism of UV-P to better understand how UV-P is removed from the fish.

Using Japanese medaka as a model fish species, this study fills a gap in knowledge with respect to the potential risk that BZT-UVs might pose to fish in the environment. It has been previously understood that BZT-UVs are present in aquatic ecosystems and biota, however, there is still very little known about the potential effects that these chemicals could have on fish. This study provides important information regarding the potential toxicity of UV-P to Japanese medaka and suggests that UV-P has minimal toxicity to these fish. However, this study only assessed the effects of a single benzotriazole UV stabilizer, UV-P, and there are multiple other BZT-UVs which vary in their properties that should also be assessed to determine potential risks to aquatic ecosystems.

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