MULTIGENERATIONAL EFFECTS OF THE NOVEL BROMINATED FLAME RETARDANT 1,2,5,6- TETRABROMOCYCLOOCTANE ON REPRODUCTIVE PERFORMANCE IN JAPANESE MEDAKA (ORYZIAS LATIPES)

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DEDICATION

I dedicate this thesis to my parents Trenna and Daniel, who support me in all that I do.

ABSTRACT

The brominated flame retardant, 1,2,5,6-tetrabromocyclooctane (TBCO), is an endocrine disrupting chemical (EDC) that's usage is predicted to increase. It is not known if early-life stage exposure of fish to TBCO causes reproductive impairments, as is the case when sexually mature fish are exposed to this chemical. This thesis demonstrates that TBCO is maternally transferred in Japanese medaka (*Oryzias latipes*), causing multigenerational reproductive impairment. Following parental (F0) exposure, F1 fecundity was decreased by 26.0-56.8%. Plasma concentrations of 17β-estradiol were not significantly decreased. A small decrease in transcript abundance of vitellogenin was observed, but the magnitude was unlikely to have decreased fecundity. However, *ex vivo* maturation of oocytes from the F1 generation was significantly decreased by 20.0-23.4%, and transcript abundances of genes that regulate oocyte maturation were consistently decreased. This is the first study to demonstrate that early-life stage exposure to an EDC caused decreased fecundity via inhibition of oocyte maturation.

PREFACE

The candidate is the main author of chapters 1-4. The candidate primarily designed and conducted experiments and analyzed data for chapters 2-3. Dr. Steve Wiseman and Dr. Jon Doering contributed to the scientific input and guidance of chapter 1-4. Dr. Paul Jones completed chemical analysis for chapters 2-3.

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LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AON	Adverse outcome networks
AOP	Adverse outcome pathway
AR	Androgen receptor
BDE-209	Decabromodiphenyl
BFR	Brominated flame retardant
BPA	Bisphenol A
bpm	Beats per minute
CH_3Hg^+	Methylmercury
CYP11a	Cytochrome P450 cholesterol side-chain cleavage
CYP19a	Aromatase
DCM	Dichloromethane
DHEA	Dehydroepiandrosterone
DOHaD	Developmental origins of health and disease
dpf	Days post-fertilization
E1	Estrone
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
ELISA	Enzyme-Linked Immunosorbent Assay
ELS	Early-life stage
EPA	Environmental protection agency
ER	Estrogen receptor
ERE	Estrogen responsive element
F0	Parental generation
F1	First filial generation
F2	Second filial generation
F3	Third filial generation

FR	Flame retardant
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GC/MS	Chromatography mass spectrometry
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GSI	Gonadosomatic index
GVBD	Germinal vesicle breakdown
HAT	Histone acetyltransferase
HBCD	Hexabromocyclododecane
HFFR	Halogen-free flame retardant
Hg^{2+}	Mercury
hpf	Hours post-fertilization
HPG	Hypothalamic-pituitary-gonadal
HPGL	Hypothalamus-pituitary-gonad-liver
HSI	Hepatosomatic index
IGF	Insulin-like growth factor
K	Fulton's condition factor
KISS	Kisspeptin
Kow	Octanol-water partition coefficient
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MIH	17α,20β-dihydroxy-4-pregnen-3-one
MPF	Maturation promoting factor
mPR	Membrane progestin receptor
MTBE	Methyl tertiary-butyl ether
OECD	Organization for economic co-operation and development
PBDE	Polybrominated diphenyl ether
PFBS	Perfluorobutanesulfonate
PGRMC	Progesterone receptor membrane component

PTV	Programmed temperature vaporization
qAOP	Quantitative adverse outcome pathway
SHBG	Sex hormone binding globulin
StAR	Steroidogenic acute regulatory protein
T	11-ketotestosterone
TBBPA	Tetrabromobisphenol A
TBCO	1,2,5,6-tetrabromocyclooctane
TBDD	2,3,7,8-tetrabromodibenzo-p-dioxin
VTG	Vitellogenin
wm	Wet mass

Gene names are given in uppercase italics, mRNA is reported as lower-case italics, and protein are upper case non-italicized

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Flame retardants

Flame retardants (FRs) are synthetic chemicals that are added to a wide range of products such as clothing, furniture, electronics, and vehicles to prevent the start and/or spread of fire (Aschberger et al., 2017). Stricter legislation and increased fire regulations have driven the development and production of FRs (UNEP, 2008). Since the introduction of regulations in the 1970s requiring the presence of FRs in nearly all industrial products, fire incidence has dropped significantly (Brown & Cordner, 2011; Birnbaum & Staskal, 2004). Flame retardants reduce fire damage, injuries, and deaths by increasing the ignition resistance of a material (Purser, 2014). If ignition resistance is overcome, they can decrease the size and rate of propagation of the fire, thereby increasing the time available for escape and survival rate of occupants (Purser, 2014). Mechanistically, the majority of FRs work by capturing the free radicals that are produced during the combustion process which destroy organic material by means of oxidation (Alaee et al., 2003).

Halogenated flame retardants are widely used by manufactures due to their high efficiency at capturing free radicals and their low impact on other material properties however, not all halogens are suitable for use as flame retardants (Alaee et al., 2003; Aschberger et al., 2017). To be an effective FR, the chemical must deliver its halogens during decomposition (Alaee et al., 2003). Iodinated compounds are unstable because they decompose at temperatures lower than most organic matter burns and fluorinated compounds decompose at temperatures higher than most organic matter burns. As such, only organochlorine and organobromine compounds are effective halogenated FRs due to their stability and decomposition temperatures close to that at which most organic matter burns. However, brominated flame retardants (BFRs) are most abundantly used due

to their lower cost and higher efficiency (Alaee et al., 2003; Horrock, 2020). The higher atomic weight of bromine ensures that BFRs are comprised mainly of bromine which in turn reduces the total amount of flame retardant that is needed (Horrock, 2020).

1.1.1 Brominated flame retardants

Brominated flame retardants can be classified as monomer, reactive, or additive depending on their mode of incorporation into polymeric products (Alaee et al., 2003; Stubbings & Harrad, 2014). Monomeric BFRs are mixed directly with products prior to polymerization whereas reactive BFRs form covalent bonds with materials (Andersson et al., 2006; Stubbings & Harrad, 2014). Nevertheless, both methods of incorporation ensure little potential for chemical leaching. Conversely, additive BFRs that are used in greater volumes and in a greater diversity of products than monomer or reactive BFRs (likely due to their lower cost), do not chemically bind to materials and are thus more likely to separate from the surface of their product and ultimately enter the environment (Alaee et al., 2003; Birnbaum & Staskal, 2004). Other sources of BFR emission include effluent and flue gases generated by BFR factories and other BFR processing facilities (Watanabe & Sakai, 2003). Due to their hydrophobicity, BFRs can accumulate in soils and sediments serving as sources and sinks of contamination in the aquatic environment (Yu et al., 2016). Additionally, BFRs are highly resistant to degradation via chemical, biological, or photolytic processes and are therefore highly likely to accumulate in the environment increasing their potential for long-range transport (Stubbings & Harrad, 2014). As such, despite their obvious value in protecting against fire, there is concern about the effects of additive BFRs that enter the environment.

Since their implementation, many BFRs have been banned due to environmental and public health concerns. However, as flammability standards remain a concern for most commercial products and building materials, the banning of one BFR often leads to the introduction of a replacement BFR (Melymuk et al., 2015). The implementation of novel BFRs has proven to be problematic, as chemicals introduced into commerce are often as toxic as those they replace. Polybrominated diphenyl ethers (PBDEs), which are the earliest class of BFRs, were first produced in the 1960s but were gradually phased out due to concerns about their persistence, bioaccumulation, and toxicity (US EPA, 2017). Because of these concerns, PBDEs were the first brominated chemicals added to the Stockholm Convention on Persistent Organic Pollutants, a convention designed to identify and mitigate the use of various organic chemicals of concern (Siddiqi et al., 2003; Sindiku et al., 2015). Following the banning of PBDEs, novel BFRs that were considered less environmentally persistent and less toxic were introduced. Among them, hexabromocyclododecane (HBCD) became the most widely used additive BFR in Europe and North America, with production rates as great as 31,000 tons/year (De Wit 2002; Law et al., 2014, Wang et al., 2018). As a result of widespread use and lipophilicity, HBCD is ubiquitous in the environment, and has been shown to bioaccumulate in wildlife and humans (Covaci et al., 2006; Sellström et al., 1998). Currently, usage of HBCD is being phased out and will likely be replaced by the novel additive BFR, 1,2,5,6 tetrabromocyclooctane (TBCO) (Saunders et al., 2015, Sun et al., 2016).

1.1.2 Ecotoxicology of TBCO

Little is known about the environmental occurrence and toxicity of TBCO. Based on chemical properties, TBCO has been characterized as persistent and bioaccumulative, and has been characterized as a potential aquatic hazard based on screening-level assessments using EU criteria

(Fisk et al., 2008; Zhou et al., 2011). Consequently, environmental concentrations of TBCO are likely to increase. Concentrations as great as 1.2 ng/g dry weight have been reported in the German Bight (Sühring et al., 2016). Additionally, β-TBCO was detected in more than 75% of atmospheric samples from the West Antarctic at concentrations as great as 0.21 pg/m^3 (Zhao et al., 2020). Studies have demonstrated that TBCO causes toxicity to early-life stage (ELS) fishes. Japanese medaka (*Oryzias latipes*) embryos exposed to TBCO were delayed in their time to hatch and suffered impaired vision and reduced heart rate (Sun et al., 2016). Exposure of zebrafish (*Danio rerio*) embryos to TBCO resulted in a concentration dependent increase in spinal curvature and swim bladder malformation as well as a concentration dependent decrease in heart rate (Van Essen et al., 2021a). In embryos of both Japanese medaka and zebrafish, exposure to TBCO resulted in a concentration dependant increase in mortality (Sun et al., 2016; Van Essen et al., 2021a).

While little is known about the toxicity of TBCO, there is evidence that TBCO is an endocrine disrupting chemical (EDC). An EDC is any natural or synthetic xenobiotic agent that affects synthesis, secretion, transport, metabolism, binding action, or elimination of natural hormones that are present in the body and are responsible for homeostasis, reproduction, and other developmental process (Crisp et al., 1988). In a series of *in vitro* bioassays, TBCO disrupted synthesis of sex steroids and elicited a dose-dependent antiandrogenic response (Saunders et al., 2013; Mankidy et al., 2014). Fecundity (number of eggs produced) of female Japanese medaka exposed via their diet to TBCO was significantly reduced compared to that of control fish (Saunders et al., 2015). Effects on reproduction were attributed to organ-specific and dose-dependent alterations in the expression of genes involved in sex hormone steroidogenesis, metabolism of cholesterol, and estrogen signaling (Saunders et al., 2015). Inhibition of reproduction by TBCO might also be due to impaired oogenesis (Van Essen et al., 2021b). Dietary

exposure of sexually mature female zebrafish to TBCO impaired oocyte maturation, which is the final step in oogenesis, as demonstrated by an *ex vivo* oocyte maturation assay, decreased mRNA abundances of genes known to regulate oocyte maturation, and altered abundances of microRNAs that regulate expression of genes involved in oocyte maturation (Van Essen et al., 2021b).

1.2 Potential for TBCO to be maternally transferred

While TBCO can cause toxicity to fishes, less is known about routes of exposure. In fishes, maternal transfer is a common mechanism by which embryos are exposed to toxicants (Khadra et al., 2019). Hydrophobic compounds are more efficiently transferred from parent to offspring than less hydrophobic compounds (Nyholm et al., 2008; Zhou et al., 2011). The octanol-water partition coefficient (Kow) is a chemical measurement used to estimate the tendency of a substance to favour a non-aqueous or oily environment as opposed to water thus indicating its hydrophilic/lipophilic balance (Sangster, 1997). Chemicals with high logKow values are lipophilic and are often of greater concern due to their potential to bioaccumulate in organisms. Guidelines suggest that chemicals with a logKow > 5 are likely to bioaccumulate (Gustavsson et al., 2016; UNEP, 2001). 1,2,5,6-tetrabromocyclooctane has a logKow of 5.24. A structurally similar BFR, HBCD with a similar logKow of 5.60 has been shown to be maternally transferred in fish (Nyholm et al., 2008; Reindl & Falkowska, 2015).

Lipophilic contaminants can be transported from maternal tissue to developing oocytes, via circulating lipoproteins, thereby bioaccumulating readily in lipid-rich fish eggs (Ungerer & Thomas, 1996; Petersen & Kristensen, 1998; Nyholm et al., 2008). Through maternal transfer, embryos can be exposed to the same effective internal concentration as the maternal organisms from which the eggs originated (Russell et al., 1999). A study by Nyholm et al. (2008) measured the extent of maternal transfer of BFRs in zebrafish and reported that eight out of eleven BFRs

delivered via spiked feed were detectable in all egg samples. Eggs from fish exposed to high-dose feed (100 nmol/g) of each compound contained roughly an order of magnitude higher concentrations than eggs from fish exposed to low dose feed (10 nmol/g) suggesting dose-related transfer (Nyholm et al., 2008). While many BFRs have been demonstrated to bioaccumulate in eggs, studies to date have yet to examine whether TBCO is maternally deposited in fish.

1.3 Control of reproduction in teleost fish

Reproduction in fish is regulated by a complex signaling pathway along the hypothalamus-pituitary-gonadal-liver (HPGL) axis (Hachfi et al., 2015). Under normal circumstances, the neuropeptide, kisspeptin (KISS), signals hypothalamic secretion of gonadotropin-releasing hormone (GnRH) to the anterior pituitary of the brain (Hachfi et al., 2012). Activation of gonadotropin-releasing hormone receptors (GnRHR) in the pituitary of females causes the adenohypophysis to produce and release two gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH; Yaron, 2011). These gonadotropins are secreted into the bloodstream where they attach to sex hormone binding globulin (SHBG) and are transported to the gonads where they bind to their respective receptors, luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR; Miguel-Queralt et al., 2004). Gonadotropins stimulate gonadal development as well as synthesis of sex hormones. Steroid hormones released from gonads regulate the production and secretion of LH and FSH via positive and negative feedback mechanisms that vary depending on the gonadal phase of reproductive development (Yaron, 2011). Together, theca cells and granulosa cells form the connective tissue known as the stroma of the female gonad (Hannon & Flaws, 2015). In the theca cells of the ovary, LH binds to LHR, inducing the synthesis of androgens and ovulation (Hachfi et al., 2012). In the granulosa cells of the ovary, FSH binds to the FSHR stimulating 17β-estradiol (E2) synthesis by a series of

systematic enzymatic conversions including the aromatization of androgens to estrogens (Nagahama and Yamashita, 2008; Hannon & Flaws, 2015; **Figure 1.1**)

In the ovaries, cholesterol, the precursor of most sex hormones, undergoes enzyme mediated reactions to produce estrogens, including E2 (Nakamoto et al., 2010). The rate-limiting step of steroidogenesis is the movement of cholesterol across the inner mitochondrial membrane of theca cells which is mediated by the steroidogenic acute regulatory (StAR) protein. (Hannon & Flaws, 2015). Cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11a) catalyzes the first step of steroidogenesis by converting cholesterol to pregnenolone (Lian et al., 2018). Next, pregnenolone diffuses out of the mitochondria and is transported to the smooth endoplasmic reticulum where it is converted to progesterone or dehydroepiandrosterone (DHEA; Hannon & Flaws, 2015). Progesterone and DHEA are then converted to the androgen, androstenedione (Hannon & Flaws, 2015). Androstenedione can be converted to the weak estrogen, estrone (E1) in the ovary's granulosa cells by aromatase (CYP19a) or it can remain in the theca cells where it is converted to testosterone (Hannon & Flaws, 2015). Testosterone and estrone are then converted to the most potent estrogen, E2, in the granulosa cells of the ovary (**Figure 1.2;** Hannon & Flaws, 2015). A key function of E2 is regulation of the synthesis of vitellogenin (VTG) that is required for oocyte growth (Hannon & Flaws, 2015).

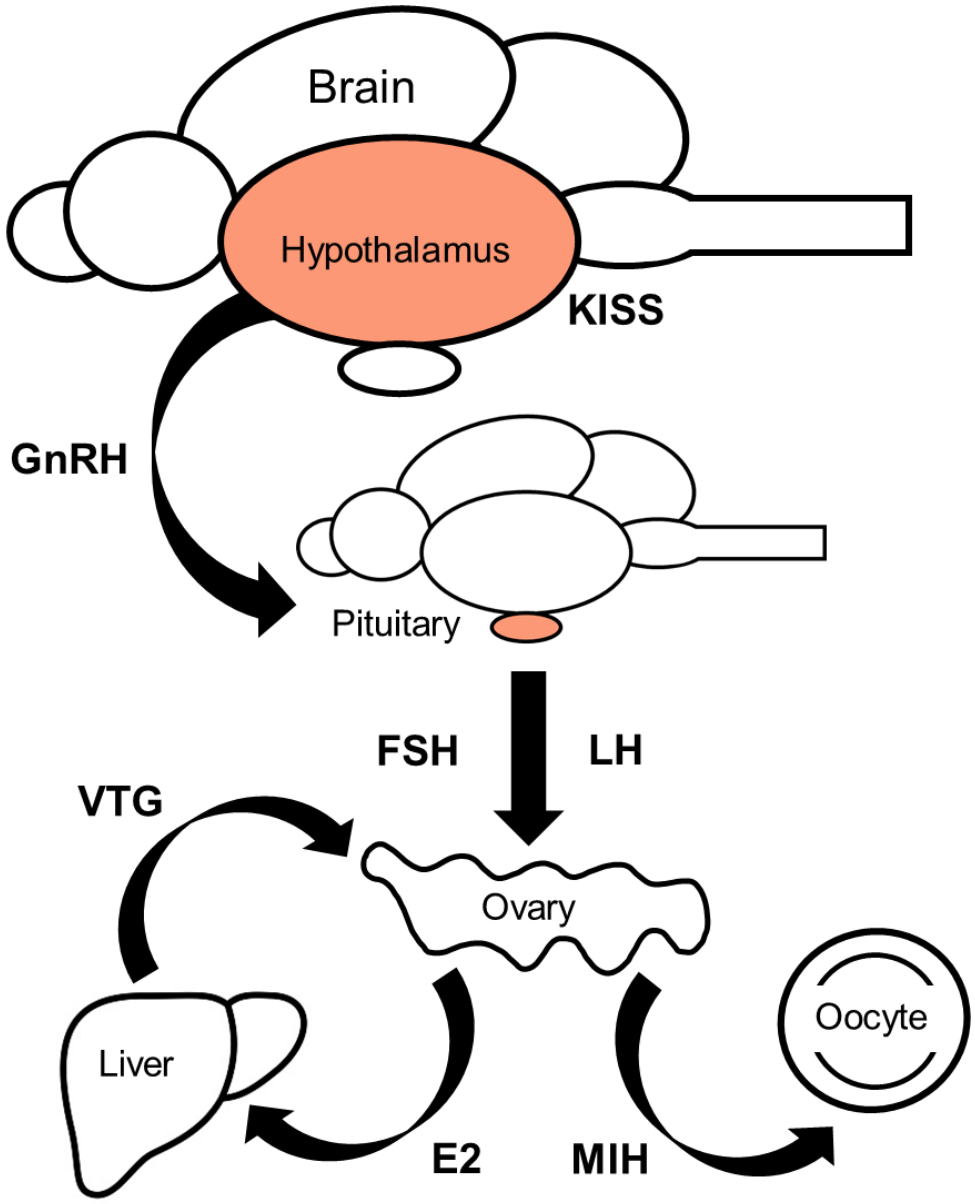

Figure 1.1 Simplified schematic of hypothalamus-pituitary-gonad-liver (HPGL) axis signalling in female Japanese Medaka (not drawn to scale). The neuropeptide, kisspeptin, signals the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which binds to GnRH receptors (GnRHR) located in the anterior pituitary. Activation of GnRHR signals the release of follicle stimulating hormone (FSH) and/or luteinizing hormone (LH) into to the bloodstream which promote growth (FSH) and maturation of oocytes (LH) in the ovary. When the FSH receptor is activated, it signals the synthesis and secretion of 17β-estradiol (E2) which is transported to the liver where it binds to E2 receptors initiating the synthesis of vitellogenin that is released into blood and incorporated into the growing oocyte. When the LH receptor is activated, 17α,20β-dihydroxy-4-pregnen-3-one (MIH) is produced, inducing oocyte maturation.

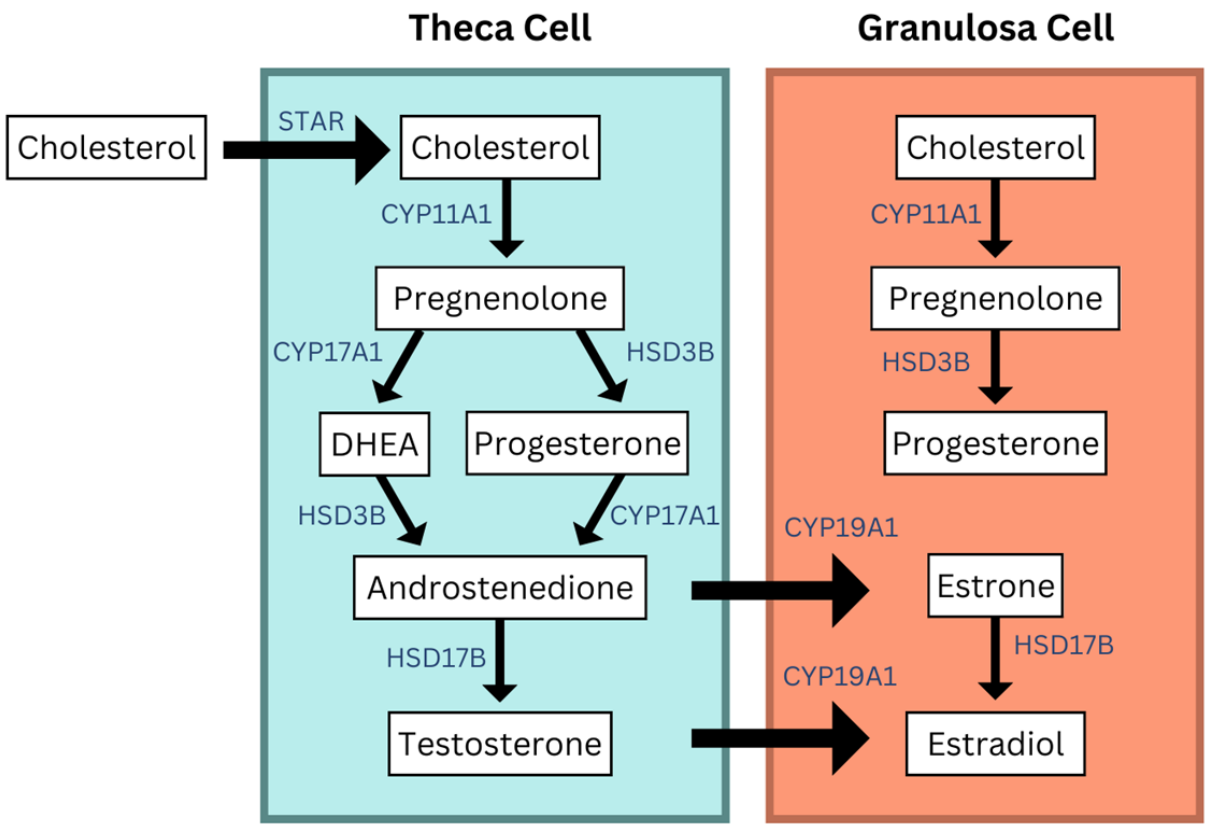

Figure 1.2 Schematic of ovarian steroidogenesis showing the enzymatic conversion of cholesterol to 17β-estradiol (E2) and other sex steroid hormones in the theca cells and granulosa cells of the gonad. Hormones synthesised in the ovaries are given in text boxes while enzymes responsible for hormonal conversions are given in between text boxes in smaller blue font.

1.3.1 Oocyte development and maturation

Oogenesis, the process by which germ cells develop into fertilizable oocytes, is a multi-stage process under control of the HPGL axis. Oogenesis commences when oogonia first undergo meiosis to become oocytes (Uribe & Grier, 2011). Oocytes then enter arrest at the first meiotic prophase prior to entering their growth phase (Sen & Caiazza, 2013). Production of E2 promotes oocyte growth (Lubzens et al., 2010). 17β-estradiol is produced via aromatization of 11-ketotestosterone (T) and is secreted from the ovaries into blood and binds to estrogen receptors (ERs), which are ligand-activated transcription factors located in the liver. In Japanese Medaka there are two estrogen receptor types, ERα and ERβ (Sullivan and Yilmaz, 2018). Once E2 binds to the receptors, they form homo- or heterodimers that are translocated to the nucleus where they bind to estrogen response elements (EREs) in the promoter region of the VTG genes. (Sullivan and Yilmaz, 2018). Estrogen receptor signalling stimulates the synthesis of VTG which is secreted into blood and is taken up by developing oocytes (Lubzens et al., 2010; Nagahama and Yamashita, 2008). Japanese medaka have two isoforms of VTG, VTGI and VTGII, both of which are essential for oocyte development.

Oocyte growth is followed by oocyte maturation. Following the oocyte growth phase, a surge of the ovulatory hormone, LH, from the anterior pituitary, promotes the resumption of meiosis (Sen & Caiazza, 2013). Additionally, LH signals the release of MIH from follicular cells, which act upon membrane progestin receptors (mPRs) located within the oocyte, signalling the synthesis of maturation-promoting factor (MPF; Nagahama and Yamashita, 2008). Maturation-promoting factor causes germinal vesicle breakdown (GVBD) representing the final step of oocyte maturation (Nagahama and Yamashita, 2008). Oocytes then remain arrested during the second meiotic cycle at metaphase II until fertilization (Sen & Caiazza, 2013**; Figure 1.3**).

Several isoforms of mPRs are expressed in oocytes, but the precise role of each in oocyte maturation is not known. Membrane progestin receptor α (mPRα) is thought to mediate nongenomic progestin signaling as shown by knockdown studies (Wu et al., 2020). Little is known on the role of membrane progestin receptor β (mPRβ), but impaired oocyte maturation has also been observed in fish lacking mPRβ (Wu et al., 2020). Expression of mPRs is believed to be regulated by progesterone receptor membrane components (PGRMC) (Wu et al., 2018). It has been suggested that PGRMC acts as an adapter and chaperone protein that forms a receptor complex with mPRα to mediate progesterone signaling in different vertebrate cells (Thomas, 2022). Impaired oocyte maturation has been observed in pgrmc1−/− female zebrafish (Wu et al., 2018). Additionally, insulin-like growth factors (IGF) are thought to play a regulatory role in teleost oocyte maturation (Picha et al., 2012). Ovarian mPRα expression has been shown to be upregulated by IGF (Picha et al., 2012). Additionally, mRNA abundance of insulin-like growth factor 3 (IGF3) has been shown to be decreased in zebrafish that experienced *ex vivo* impaired oocyte maturation following dietary exposure to TBCO (Van Essen et al., 2021).

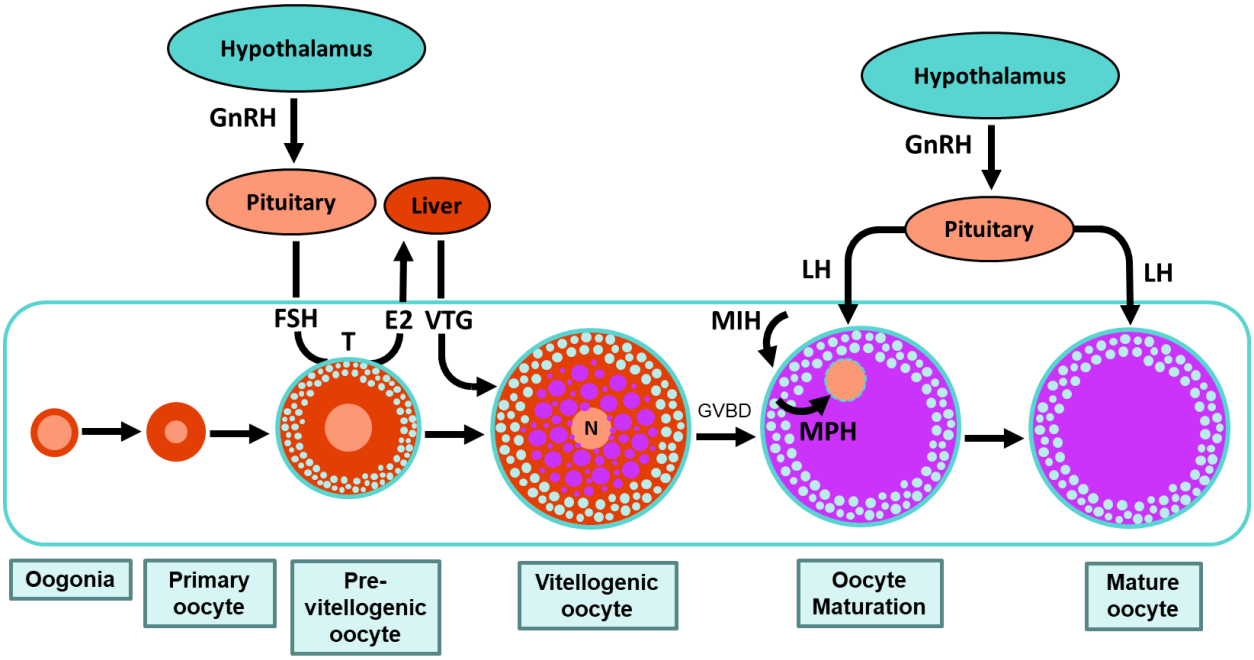

Figure 1.3 Schematic of the general process of oogenesis in teleost fish adapted from Alix et al., (2020). Kisspeptin (KISS) first initiates the secretion of gonadotropin-releasing hormone (GnRH) from the brain which binds to GnRH receptors in the pituitary which in return release follicle stimulating hormone (FSH) to the bloodstream promoting oocyte growth. 11-ketotestosterone (T) is converted to 17β-estradiol (E2) which is transported via the bloodstream to the liver where it binds to E2 receptors initiating the synthesis of vitellogenin (VTG) that is released into the bloodstream and incorporated into the growing oocyte. Following oocyte growth, GnRH signals the release of luteinizing hormone (LH) which promotes maturation of oocytes. Binding of LH to the LH receptor stimulates synthesis of maturation-inducing hormone (17α,20β-dihydroxy-4-pregnen-3-one; MIH) which interacts with the membrane progestin receptor (mPR) on the oocyte surface to generate maturation-promoting factor (MPF). Finally, MPF causes germinal vesicle breakdown (GVBD) resulting in a mature, fertilizable oocyte.

1.4 Multigenerational and transgenerational toxicity

Traditionally, the field of ecotoxicology has focused primarily on identification and characterization of apical effects such as developmental malformations or reproductive impairment that manifests during acute and chronic exposure to chemical stressors. For example, the gold standard assay to determine whether chemicals impact reproductive performance of fishes is the Organisation for Economic Co-operation and Development (OECD) Test No. 229, in which fecundity of female fish and fertilization of eggs by male fish is assessed in a 21-day reproduction assay during which fish are exposed to the chemical of interest (OECD, 2012). Other assays such as OECD Test No. 210 are used to determine ELS toxicity by exposing fertilized eggs to a range of concentrations of a chemical dissolved in water for up to 30 days post-hatch (OECD, 2013). However, a rapidly expanding body of literature is demonstrating that exposures can have long-lasting and/or latent effects on phenotype that are not evident during the period of exposure. The developmental origins of health and disease (DOHaD) hypothesis, which has its origins in the field of epidemiology, suggests that exposure to stressors, including chemical stressors, at sensitive periods of development can cause developmental reprogramming, causing long-term changes in phenotype, including development and reproduction (Hanson, 2015). Developmental effects are often caused by conditions that organisms experience during embryogenesis with earlier disturbance being linked to more pronounced long-term effects (Jonsson & Jonsson, 2014).

As studies on the direct and immediate effects of exposure to chemical stressors are the primary focus in risk assessment, little is known about how ELS exposure can lead to effects later in life, and even less is known about the potential for chemical exposure in parental generations to affect subsequent generations (Decourten et al., 2020; Bhandari, 2016). However, chemical stressors can induce latent effects that do not manifest until specific stages of development or when

specific physiological processes such as reproduction are ‘activated’, long after an exposure has ceased. Moreover, chemical stressors can have effects that manifest in subsequent generations of progeny, including generations with no life-history of exposure. As such, there is increasing recognition that assessments across multiple generations are crucial to determine the risk of contaminant exposure during ELS (Decourten et al., 2020). When a phenotype arises due to direct exposure of multiple generations to an environmental stressor inducing toxicity only in those generations directly exposed, then a multigenerational effect has occurred (Skinner & Guerrero-Bosagna, 2009). In oviparous vertebrates such as fish, multigenerational effects can result from direct exposure of the parental generation (F0) and germ line/first filial generation (F1) to a toxicant, or when a chemical is maternally transferred from the mother (F0) to the offspring (F1), resulting in the second filial generation (F2) being exposed as germ cells (**Figure 1.4**). The ability of an environmental factor to induce toxicity in subsequent unexposed generations, is known as transgenerational inheritance (Skinner & Guerrero-Bosagna, 2009; Head et al., 2012). As parental traits are passed to offspring via gametes, environmental stressors must affect germ cells for a transgenerational phenotype to occur (Bhandari, 2016). In fish, transgenerational effects manifest in the F2 generation when only the parental generation (F0), and its germ cells (F1) have been directly exposed to test chemicals (Bhandari, 2016). When maternal transfer has occurred, a transgenerational phenotype can first be observed in the third filial generation (F3). Current risk assessment protocols have yet to include assessment of multigenerational and/or transgenerational effects of EDCs on human and wildlife health (Bhandari, 2016).

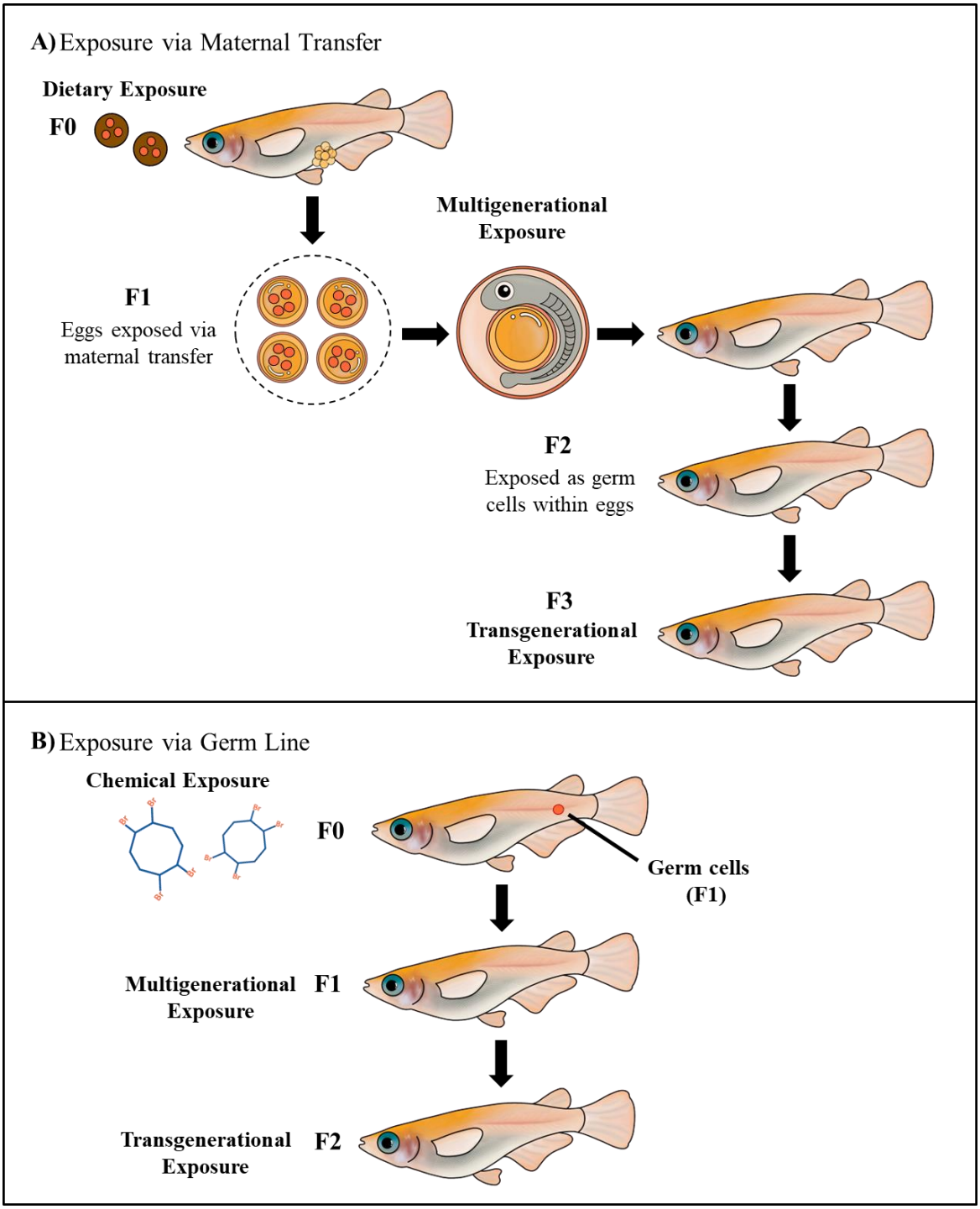

Figure 1.4 Exposure to a chemical stressor in teleost fish by A) maternal transfer or B) germ line exposure can induce multigenerational and transgenerational effect. Effects are considered multigenerational when the organism was directly exposed to the stressor A) via maternal transfer (F1 and F2) or B) via germ cells (F1). Effects are considered transgenerational when they are inherited in an organism that was never directly exposed to the stressor via A) maternal transfer (F3) or B) via germ cells (F2).

1.4.1 Multigenerational and transgenerational effects of endocrine disrupting chemicals

While the multigenerational and transgenerational effects of exposure of fishes to anthropogenic chemicals are largely uninvestigated, their consequences have the potential for broad ecological impacts (Bhandari et al., 2015). Fish are particularly sensitive to EDCs during ELS as exposure can disrupt the HPGL axis which can result in genital defects, abnormal gamete development, infertility, or complete phenotypic sex reversal (Liao et al., 2014). Fish fecundity is thought to be most affected by exposures to chemicals during the earliest stages of gametogenesis (Sumpter, 1996). Exposure of ELS of zebrafish to inorganic mercury (Hg^{2+}) impaired reproduction of females and their progeny by disrupting the balance of sex hormones and gametogenesis because of changes in abundances of mRNA of genes that regulate the hypothalamic-pituitary-gonadal (HPG) axis (Xie et al., 2020). In a study examining the effects of exposure to dietary methylmercury (CH_3Hg^+) on killifish *(Fundulus heteroclitus),* it was demonstrated that male survival was decreased, and offspring experienced reduced fecundity, fertility, and altered sex ratios (Matta et al, 2001). Additionally, exposure of zebrafish to the BFR, decabromodiphenyl ether (BDE-209), via maternal transfer induced developmental toxicity and dysregulated expression of several genes involved in the hypothalamic–pituitary–thyroid axis (Han et al., 2017). It was also demonstrated that while exposure of ELS Japanese medaka to 100 mg/l bisphenol A (BPA) or 0.05 mg/l ethinylestradiol (EE2) resulted in no phenotypic abnormalities in the parental generation (F0) or offspring (F1), transgenerational adverse effects were observed as F2 and F3 adults showed a significant reduction in fertilization capacity and resultant F3 and F4 embryos showed a reduced rate of survival (Bhandari et al., 2015). Transgenerational effects resulting from chemical exposure are often the result of inheritance of chemical induced alterations to the epigenome (Uller, 2019).

1.4.2 Epigenetics as a mechanism of multigenerational toxicity

Epigenetics is the study of heritable changes in gene expression, unrelated to mutation, that are propagated without continued exposure to the original stimulus (LeBaron et al., 2010). These changes in gene expression can be mitotically and meiotically heritable and allow for an additional level of biological regulation beyond the strict base-coding contained within DNA (LeBaron et al., 2010). There are three main mechanisms by which gene expression can be heritably altered without any change in DNA sequence; DNA methylation, histone modifications, and microRNA gene silencing or activation (Best et al., 2018). DNA methylation and histone modifications regulate gene expression at the levels of chromatin structure and DNA, while microRNAs function post-transcriptionally by regulating mRNA abundance and translation (Best et al., 2018).

DNA methylation is the most studied epigenetic mechanism in the field of ecotoxicology as the methylome is responsive to many environmental contaminants (Head, 2014). Parental exposure to toxicants can result in changes in DNA methylation that can be passed down to other exposed generations (multigenerational effects) or unexposed generations (transgenerational effects; Brander et al., 2022). While epigenetic changes such as DNA methylation can contribute to the adaptation of a population by promoting advantageous changes in gene expression, it can also have detrimental effects on an organism by promoting the expression of deleterious genes or silencing expression of genes essential for survival (Šrut, 2021). As such, there is considerable interest in the field of ecotoxicology in developing changes in DNA methylation to develop biomarkers of adverse effects (Šrut, 2021). Methylation of DNA is catalyzed by DNA methyltransferase (DNMT) enzymes and occurs via the addition of a methyl group to the carbon 5 position of the cytosine ring (Singal & Ginder, 1999). In vertebrates, DNA methylation targets the CG dinucleotide sequence, which is the only dinucleotide sequence to contain a palindromic

cytosine that can be transferred by DNMTs from a parental strand onto a daughter strand (Razin & Riggs, 1980; Singal & Ginder, 1999). The enzyme responsible for maintenance of the existing pattern of methylation during DNA replication is DNMT1 whereas DNMT3 catalyses *de novo* methylation of DNA (Mortusewicz et al., 2005).

During embryonic development, the genome undergoes extensive changes in DNA methylation. In fish, this has been best characterized in Japanese medaka and zebrafish. Japanese medaka have a DNA methylation reprogramming process comparable to that of humans (Wang & Bhandari, 2020). The medaka oocyte genome is hypomethylated whereas the sperm genome is hypermethylated (Wang & Bhandari, 2020). However, within the first cell cycle the paternal methylome is erased (Wang & Bhandari, 2020). Embryos then maintain hypomethylation until the 16-cell stage where methylation levels increase until the blastula stage which occurs from approximately 6-8 hpf (Wang & Bhandari, 2020; Iwatmasu, 2004). Following this, methylome reprogramming occurs in two stages. From the blastula stage to ~8 days post-fertilization (dpf), a first round of global demethylation occurs in both males and females (Wang & Bhandari, 2020). Next, from 10-dpf to 12-dpf a second demethylation phase takes place (Wang & Bhandari, 2020). At ~25-dpf, *de novo* DNA methylation takes place in male primordial germ cells (PGC) but not in females (Wang & Bhandari, 2020). For both humans and Japanese medaka, PGC development is one of the largest windows of susceptibility to chemical contaminants due to drastic changes in DNA methylation (Wang & Bhandari, 2020). In contrast, zebrafish display relatively stable DNA methylation patterns that undergo minimal change during embryogenesis (Wang & Bhandari, 2020). Changes in DNA methylation due to chemical exposure during methylome reprogramming can have effects that last long after exposure has ended.

Because of this reprogramming of the methylome, early embryogenesis is a sensitive window of exposure of fishes to environmental contaminants (Wang and Bhandari, 2019). Altered patterns of DNA methylation caused by disruption of reprogramming can lead to altered methylation that can be inherited in somatic and germ cells. As such, changes in DNA methylation pattern caused by exposure to an environmental agent can be maintained through DNA replication cycles resulting in a long-lasting altered phenotype (Szyf, 2011). In a study using inland silverside (*Menidia beryllina*), ancestral exposure to environmentally relevant levels of EDCs (bifenthrin, levonorgestrel, EE2, and trenbolone) caused impaired reproduction and transgenerational inheritance of altered methylation of genes involved in sex steroid signalling (androgen receptor (AR) and ER) and in sex hormone synthesis (17ß-hsd, 3ß-hsd; Major et al., 2020). One-time exposures to bifenthrin, levonorgestrel, EE2, and trenbolone were predicted to result in population declines and lagged recovery over many years due to lagged epigenetic effects (Brander et al., 2022). In another study, environmentally relevant concentrations of the aquatic pollutant, perfluorobutanesulfonate (PFBS), were shown to skew the sex ratio of marine medaka toward male dominance and greatly impair female reproduction as characterized by shrunken ovaries, impaired oocyte development, and decreased fecundity (Chen et al., 2019). Female reproduction was impaired via antiestrogenic activity leading to disruption of critical hormones along the HPGL axis as E2 levels were decreased in a concentration-dependent manner (Chen et al., 2019). Additionally, plasma FSH levels were significantly higher and 11-ketotestosterone (11-KT) was significantly lower in both sexes exposed to PFBS (Chen et al., 2019). Perfluorobutanesulfonate was found to be maternally transferred to F1 offspring eggs and impaired reproduction persisted in F1 and F2 offspring (Chen et al., 2019). Global DNA methylation analysis revealed hypermethylation of the

ovarian genome in exposed F0 female fish and abnormal hypomethylation of the F1 embryonic genome (Chen et al., 2019).

1.5 Japanese medaka as a model species

Several model species of fishes are used in ecotoxicology to study endocrine disruption. These include the Japanese medaka, zebrafish, and fathead minnow (*Pimephales promelas*), each of which are reliable indicators of endocrine disruption in aquatic environments (Celino-Brady et al., 2021). Japanese Medaka are a small (3-4cm) freshwater fish whose eggs are fertilized and develop externally (Wittbrodt et al., 2002). Their reproductive physiology has been extensively characterized, making them an excellent species for studies of endocrine disruption (Ankley & Johnson, 2004). Their high reproductive capacity allows for short term reproduction assays to be conducted where sexually mature male and spawning female fish are exposed to a chemical for 21 days and their reproductive performance is assessed in terms of fecundity and fertility (OECD, 2012).

Medaka have relatively short life cycles, reaching sexual maturity in as little as two months under ideal conditions (Shima & Mitani, 2004). Because of this, they are an ideal species for studies of multigenerational and transgenerational effects of chemical stressors (Bhandhari, 2016). Reprogramming of the DNA methylome during early development has been characterized, facilitating studies on the role of the methylome in multigenerational and transgenerational effects of EDCs (Wang & Bhandari, 2020). From an epigenetic perspective, Japanese medaka have DNA methylation and demethylation related genes that are near identical to humans (Wang & Bhandari, 2020).

Japanese Medaka are distinct in terms of their large number of oocyte developmental stages. For example, in zebrafish, there are five stages of oocyte development (Selman et al., 1993). In contrast, Japanese medaka have ten stages of oocyte development that can be distinguished by size and visual parameters, such as a semitransparent surface and the presence of oil droplets (**Figure 1.5**). The ten stages of oocyte development were first described by Iwamatsu et al. (1988). Little research has been conducted on oocytes of this species, however, it is known that oocyte development in Japanese Medaka is under a natural circadian rhythm, where endogenous MIH is uniquely released within 2 hrs of light onset (Iwamatsu, 1978). While an *in vitro* assay to assess MIH-stimulated oocyte maturation has been developed for zebrafish, such an assay does not exist for Japanese medaka.

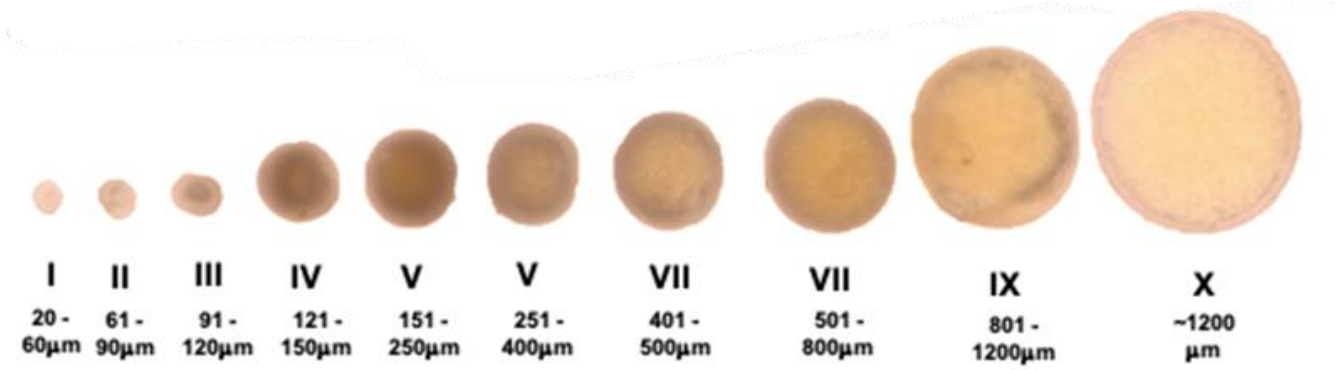

Figure 1.5 Stages of Japanese medaka oocyte development. Stages I-IX depict immature oocytes while stage X depicts a fully mature egg prior to fertilization. Representative images are shown. Size (in μm) of oocyte is noted below each stage. Image from Raza et al., (unpublished).

1.6 Research goals and objectives

There is great concern surrounding the effects of chemicals that enter aquatic environments on the health of aquatic organisms, including fish. There is an immense body of literature describing effects of contaminants on fishes, including molecular and biochemical mechanisms of these effects. These studies have focused almost exclusively on effects that manifest during acute exposures at discrete life-stages or during or chronic exposures that might span multiple life-stages. Recently, with the realization that exposure to toxicants can cause multigenerational and transgenerational effects, there is a growing realization that these effects need to be characterized, and that risk assessment frameworks need to consider how to incorporate such effects. This is particularly true of EDCs that can impact the reproductive capacity of fishes.

Usage of the novel BFR, TBCO, is predicted to increase, which will likely result in increased concentrations in aquatic environments. Previous research has shown that TBCO is an EDC, and that it impairs reproduction in Japanese medaka (Saunders et al., 2015; Sun et al., 2016; Mankidy et al., 2014; Van Essen et al 2021b). As a persistent lipophilic compound, TBCO is likely to bioaccumulate in lipid-rich tissues, such as fish eggs, because of maternal transfer (Gustavsson et al., 2016; Nyholm et al., 2008). However, it is not known whether exposure to TBCO during critical stages of early development can impact reproductive success within or across generations. Therefore, this program of study aimed to determine whether TBCO can disrupt reproduction and induce adverse effects that are multigenerational and/or transgenerational in the model teleost, Japanese medaka. Specific objectives of the proposed research are to:

1. Investigate the effects of maternally deposited TBCO on measures of reproductive success in three generations of fish (F1, F2, F3).

2. Determine molecular and biochemical mechanism(s) of effect(s) of TBCO on reproductive performance in three generations of fish (F1, F2, F3).

Several studies have reported that exposure to EDCs during critical early stages of development can cause multigenerational or transgenerational effects on reproductive performance. Therefore, it is hypothesised that exposure of Japanese medaka to TBCO via maternal transfer will result in multigenerational and transgenerational reproductive impairment caused by dysregulation of the expression of genes that regulate processes critical for successful reproduction.

CHAPTER 2: THE BROMINATED FLAME RETARDANT, 1,2,5,6-TETRABROMOCYCLOOCTANE (TBCO), CAUSES MULTIGENERATIONAL EFFECTS ON REPRODUCTIVE CAPACITY OF JAPANESE MEDAKA (*ORYZIAS LATIPES*).

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2.1 Introduction

1,2,5,6-tetrabromocyclooctane (TBCO) is a brominated flame retardant (BFR) that is a potential replacement for the widely used BFR, hexabromocyclododecane (HBCD). TBCO has been characterized as a potential aquatic hazard based on screening-level assessments using European Union (EU) criteria and has been measured in environmental matrices at concentrations as great as 1.2 ng/g (Fisk et al., 2008; Suhring et al., 2016). It has been demonstrated that TBCO is an endocrine disrupting chemical (EDC) (Saunders et al., 2013, 2015; Mankidy et al., 2014). In a series of *in vitro* bioassays, TBCO disrupted synthesis of sex steroids and elicited a dose-dependent antiandrogenic response (Saunders et al., 2013; Mankidy et al., 2014). Fecundity of female Japanese medaka (*Oryzias latipes*) exposed via their diet to TBCO was significantly reduced compared to that of control fish (Saunders et al., 2015). Effects on reproduction were attributed to organ-specific and dose-dependent alterations in the expression of genes involved in sex hormone steroidogenesis, metabolism of cholesterol, and estrogen signaling (Saunders et al., 2015). In embryos of Japanese medaka exposed to TBCO, abundances of proteins that regulate oocyte meiosis were significantly decreased, but effects on reproduction at maturity were not assessed (Sun et al., 2016). Dietary exposure of sexually mature zebrafish (*Danio rerio*) to TBCO impaired oocyte maturation and altered abundances of mRNAs and microRNAs with roles in the regulation of oogenesis (Van Essen et al., 2021b).

Chemical stressors can have latent effects that manifest later in life or in subsequent generations of progeny, including generations with no life-history of exposure (Kimberly & Salice, 2014). When a phenotype arises due to coincident direct exposure of multiple generations to a chemical stressor inducing toxicity only in those generations directly exposed, then a multigenerational effect has occurred (Skinner & Guerrero-Bosagna, 2009). In oviparous

vertebrates such as fish, multigenerational effects can result from direct exposure of the parent (F0) and germ line (F1) to a toxicant, or when a chemical is maternally transferred from the mother (F0) to the offspring (F1), resulting in the F2 generation being exposed as germ cells. The ability of an environmental factor to induce toxicity not only in the exposed generation, but in subsequent generations with no life-history of exposure, is known as transgenerational inheritance (Skinner & Guerrero-Bosagna, 2009; Head et al., 2012). In fish, transgenerational effects manifest in the F2 generation when only the parental generation (F0), and its germ cells (F1) have been directly exposed to a chemical stressor (Bhandari, 2016). When maternal transfer has occurred, a transgenerational phenotype can first be observed in the F3 generation. Multigenerational and transgenerational effects resulting from chemical exposure are often the result of inheritance of chemical induced alterations to the epigenome, including DNA methylation, histone modifications, and noncoding RNAs (Best et al., 2018; Uller, 2019).

Studies of exposure to EDCs have focused primarily on direct and immediate effects on reproduction. However, there is a growing body of literature showing that exposure of parental generations to EDCs can induce multigenerational and transgenerational effects on the reproductive capacity of fishes (Decourten et al., 2020; Bhandari, 2016). Exposures of early-life stage (ELS) inland silversides (*Menidia beryllina*) to environmentally relevant concentrations of ethinylestradiol (EE2), bifenthrin, trenbolone, and levonorgestrel, were shown to negatively impact subsequent generations (Decourten et al., 2020). Specifically, exposure of the F0 generation to EE2 led to a female-biased sex ratio and reduced egg production in the F1 generation while exposure to bifenthrin resulted in decreased egg production in both the F0 and F1 generations (Decourten et al., 2020). Exposure of F0 embryos to trenbolone or levonorgestrel had no effect on reproductive performance but decreased egg production in the F1 generation (Decourten et al.,

2020). In a study where marine medaka (*Oryzias melastigma*) were exposed as eggs (F0) to environmentally relevant concentrations of perfluorobutanesulfonate (PFBS) continuously until they reached sexual maturity, PFBS was found to be maternally transferred to the F1 generation and female reproduction was impaired as characterized by shrunken ovaries, impaired oocyte development, and decreased fecundity in the F0, F1, and F2 offspring (Chen et al., 2019). Short-term exposure of sexually mature F0 zebrafish (*Danio rerio*) to phytosterols resulted in masculinization of the F1 generation and increased concentrations of vitellogenin in blood plasma of males and females (Nakari & Erkomaa, 2003). In contrast, in the F2 generation, the sex ratio was biased towards females and plasma vitellogenin levels were elevated in males (Nakari & Erkomaa, 2003). Exposure of Japanese medaka to 100 mg/L bisphenol A (BPA) or 0.05 mg/L EE2 did not cause reproductive effects in the parental (F0) or F1 generations, but fertilization capacity of F2 and F3 adults was decreased (Bhandari et al., 2015).

Maternal transfer is an important mechanism by which ELS fish can be exposed to lipophilic chemicals, such as BFRs (Haldén, 2010). Studies to date have not examined whether TBCO is maternally deposited in eggs but based on its log octanol–water partition coefficient (Kow) value of 5.24 (Gustavsson et al., 2016), it likely is maternally deposited. Studies have shown that dietary exposure of sexually mature Japanese medaka to TBCO can decrease fecundity, but it is unknown whether exposure to TBCO during ELS development can induce multigenerational or transgenerational toxicities. Thus, this study investigated the multigenerational and transgenerational effects on reproduction of exposure to TBCO. Sexually mature F0 Japanese medaka were exposed to TBCO via their diet, maternal transfer to F1 embryos was assessed, and effects on reproductive performance of F1, F2, and F3 generations were investigated, including mechanisms that regulate steroidogenesis and vitellogenesis. Results of the present study expand

our understanding of the potential effects of TBCO on reproductive performance of fishes across multiple generations which is crucial when assessing the long-term risks associated with exposure to chemical stressors.

2.2. Methods

2.2.1 Preparation of TBCO contaminated food

TetraMinR tropical flakes fish food (Tetra, Brampton, ON, Canada) was spiked with TBCO (SynQuest Laboratories, Inc. Alachua FL, purity = 98%) according to methods described previously (Saunders et al., 2015; Van Essen et al., 2021a). Briefly, flakes were ground using a food processor and mixed with TBCO dissolved in 150 mL of acetone to make nominal concentrations of 100 μg TBCO/g, wm (wet mass) food (low dose), and 1000 μg TBCO/g, wm food (high dose). These nominal concentrations are equivalent to concentrations used in a previous study that reported effects of dietary TBCO on reproduction of Japanese medaka (Saunders et al., 2015). Spiked food was stirred every 20 min for 4 h to ensure thorough mixing of food and chemical and was subsequently air dried for 12 h in a dark fume hood. The same protocol, excluding the addition of TBCO, was used to prepare control food.

2.2.2. Animal care

Adult Japanese medaka (F0) were from a culture maintained in the Aquatic Research Facility in the Alberta Water and Environmental Science Building at the University of Lethbridge (Lethbridge, AB, Canada). Japanese medaka were maintained in flow-through racks (Tecniplast, Toronto, ON, Canada) supplied with dechlorinated city of Lethbridge water (average water quality: conductivity 378 μS/cm, alkalinity 128 mg as CaCO3/L, hardness 165 mg as CaCO3/L, > 90% oxygen saturation, pH 8.01) at 25°C and kept on a 16:8 light:dark photoperiod. Adults were

fed to satiety with TetraMinR tropical flake fish food and live Artemia (Brine Shrimp Direct, Ogden, TU, USA) three times daily. Medaka were maintained in accordance with University of Lethbridge Animal Welfare Protocol #2014.

2.2.3 Exposure of F0 generation to TBCO

Five sexually mature male and female medaka were randomly assigned to 10 L tanks under flow-through conditions and were allowed to acclimate for seven days prior to the initiation of exposure. Four replicate tanks were used per treatment. Fish were fed approximately 5% of their body mass each day, with half of the food provided in the morning and the other half in the afternoon. Water temperatures were maintained at 25°C during the experiment. On days 14, 16, and 18, 40 eggs per treatment tank were collected, rinsed in dechlorinated water, and stored at -80 °C for quantification of maternal transfer of TBCO. Fertilized eggs from days 14-20 of the exposure were collected and reared as described in section 2.2.4.

2.2.4 Rearing of the F1, F2, and F3 generations

Fertilized eggs from the F0, F1, and F2 generation were grown in freshwater (dechlorinated city of Lethbridge water) for approximately four months until they reached sexual maturity. Eggs were separated by treatment and grown in 3.5 L tanks in a standalone rack (Tecniplast) under flow-through conditions, at a water temperature of 25°C. Artemia (Brine Shrimp Direct) was introduced immediately post-hatch and fish were fed three times daily until sexual maturity. Fish were grown at a density of 20 fish per tank at a daily water renewal rate of 15%.

2.2.5. Reproduction assays

Once the F1, F2, and F3 generations reached sexual maturity, reproductive performance was assessed by use of a 21-day short term reproduction assay according to OECD Test Guideline

229, and using the same conditions described in section 2.3 (OECD, 2012). None of the F1, F2, or F3 generations were re-exposed to TBCO, although the F1 generation were exposed to maternally deposited TBCO (section 3.1). TetraMinR tropical flake fish food was gradually introduced to fish and artemia was phased out during a two-week acclimation period prior to initiation of reproduction assays. During reproduction assays, fish were fed approximately 5% of their body mass daily in TetraMinR tropical flakes fish food, with half of the food provided in the morning and the other half in the afternoon. Fecundity was assessed daily and was calculated as the cumulative number of eggs per female per tank. Eggs from days 14-21 were collected and reared as described in section 2.2.4. Upon termination of each reproduction assay, mass and length of each fish was measured to determine Fulton's condition factor. Liver and gonad tissue from each female was collected, weighed to determine hepatosomatic index (HSI) and gonadosomatic index (GSI), and frozen in liquid nitrogen. Tissues were stored at -80 °C until used for molecular analysis. Blood from each female fish per replicate tank was collected in a heparinized microcapillary tube from the caudal vein. Immediately following collection, blood samples were centrifuged at 8000×g for 10 min at 4 °C and plasma was removed and stored at −80 °C until analysis.

2.2.6 Assessment of embryotoxicity

On days 15 and 20 of the F0, F1, and F2 reproduction assays, 24 eggs per replicate tank from each treatment were selected at random for assessment of embryotoxicity. Heart rate recorded in beats per minute (bpm) was assessed at 48 hours post-fertilization (hpf). Malformations including spinal curvature, yolk sac edema, pericardial edema, and uninflated swim bladder were assessed at 384 hpf. Mortality was assessed daily until 24 hours post-hatch. Assays were performed in 24 well plates with one egg per well with four replicated plates per treatment. A 50% water

change was conducted every third day. Plates were kept in an incubator at 25°C until termination of the assay at 384 hpf.

2.2.7 Real-time PCR

Abundances of mRNA of genes that regulate reproductive performance were quantified in sexually mature female medaka from the F1, F2, and F3 generations. Total RNA was isolated from gonad and liver by use of TRIzol™ reagent following the manufacturers protocol (Life Technologies, Burlington, ON, Canada). Concentration of RNA was determined using a Nanodrop™ One© spectrophotometer (Thermo Scientific, Ottawa, ON, Canada). Complementary DNA (cDNA) was synthesized from 2 µg of RNA by use of superscript IV VILO Master Mix with ezDNAse, following the protocol provided by the manufacturer (Life Technologies). For each cDNA sample and primer combination, a 25 µL reaction mixture containing SensiFAST™ SYBR© No-ROX Mix (Bioline, FroggaBio Inc., Toronto, ON, Canada), 1.25 µL of cDNA, 1.25 µL of primers (final concentration of 10 pM), and nuclease-free water was prepared. qPCR reactions were duplicated with 10 µL volumes in 96 well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Prior to the first cycle, the reaction mixture was heated at 95 °C for 2 min. Thermal cycle conditions were denaturation for 5 s at 95 °C and extension for 10 s at 60 °C for a total of 40 cycles. Melt curves were generated to confirm that a single product was amplified for all samples by increasing 0.5 °C every 5 s from 65 °C to 95 °C. mRNA abundance of target genes that regulate reproduction was normalized to abundances of mRNA of the housekeeping genes RPL7 and 18S rRNA. Changes in abundances of mRNA were calculated relative to the control using the efficiency corrected method (Pfaffl, 2001). Before calculating mRNA abundance, efficiencies of PCR reactions with were established using 5-fold serial dilutions of cDNA template. Details of target mRNAs and primers are provided below (**Table 2.1**).

Table 2.1 Details of oligonucleotide primers used in semi-quantitative real-time PCR (qPCR).

Target mRNA	Function	Primer Sequence (5′–3′)	Efficiency (%)
rpl7	Housekeeping	**F**- GTCGCCTCCCTCCACAAAG **R**- AACTTCAAGCCTGCCAACAAC	88.0
18s *rRNA*	Housekeeping	**F**- GACAAATCGCTCCACCAACT **R**- CCTGCGGCTTAATTTGACCC	111.0
lhr	sex steroid synthesis	**F**- GTCCTGGTCATCCTGCTCGTT **R**-AACCGGGAGATGGTCAGTTTGT	100.0
fshr	sex steroid synthesis	**F**-TTCAGGCCACTGATGATGTTAT **R**-CCTTCGTGGGTTCCAGTGAGT	100.0
cyp11a	sex steroid synthesis	**F**-GCTGCATCCAGAACATCTATCG **R**-GACAGCTTGTCCAACATCAGGA	110.0
cyp19a	sex steroid synthesis	**F**-CTCTTCCTGGGTGTTCCTGTTG **R**-GCTGCTGTCTTGTGCCTCTG	112.0
vtgI	Egg yolk precursor protein	**F**-ACTCTGCTGCTGTGGCTGTAG **R**-AAGGCGTGGGAGAGGAAAGTC	109.0
vtgII	Egg yolk precursor protein	**F**-TCGCCGCAAGAGCAACAC **R**-CTGGAGGACCTGGAAGAACTG	100.0

2.2.8 Quantification of E2

Concentrations of E2 were quantified in blood plasma from female F1, F2, and F3 fish using the Enzyme-Linked Immunosorbent Assay (ELISA) following the manufacturers protocol (Item #: 501890, Cayman Chemical, MI, USA). Due to small plasma volumes in adult Japanese medaka, plasma from all five females in each replicate tank were pooled (n=4).

2.2.9 Analysis of TBCO

Concentrations of TBCO in food and eggs were determined at the University of Saskatchewan (Saskatoon, SK, Canada), using gas chromatography mass spectrometry (GC/MS). To assess concentrations of TBCO in food, approximately 0.4 g of each sample was combined with 1.0 g of anhydrous Na_2SO_4 and incubated at room temperature for 1 h. Samples were then mixed thoroughly with 40 ml of a 1:1 mixture of hexane: methyl tertiary-butyl ether (MTBE) and incubated at room temperature overnight. The following day, 2 ml of each sample were placed into a disposable tube and blown dry under a stream of nitrogen. The resulting residue was dissolved in 4 ml of hexane, mixed with 1 g of neutral alumina (6% deactivated) and left to sit at room temperature for 1 h. Next, samples were mixed with 1 ml of dichloromethane (DCM) and left to sit at room temperature for 1 h. A 1 ml aliquot of the supernatant was transferred to a GC vial for analysis. A spiked sample was created by adding 5 µl of a 50 µg/ml bTBCO standard (Wellington Laboratories, Guelph, ON, Canada) to 0.4 g of control food and extracted using the process described above. A Q-Exactive GC OrbiTrap (Thermo Scientific) interfaced to a Trace 1310 Gas Chromatograph (Thermo Scientific) was used to perform the analysis. A Programmed Temperature Vaporization (PTV) injector was fitted to the GC and analysis was performed on a 60m 13 DB-5ms column 0.1 mm ID with a film thickness of 0.1 micron. The mass spectrometer

was operated at 60,000 nominal resolution at 200 m/z in full scan mode (150-300 m/z). A standard curve using the TBCO standard (Wellington Laboratories, Guelph, ON, Canada) with concentrations of 0, 5, 50, 500, and 5000 ng/ml was used. The method detection limit was 0.0005 ug/g.

To assess maternal transfer of TBCO, each sample of eggs was combined with 1.0 g of anhydrous Na_2SO_4 along with 10 ml of 1:1 Hexane:MTBE. Samples were then sonicated for 30 min and left in the dark overnight. The following day, samples were mixed and sonicated again for another 30 min. Samples were then allowed to settle following which 10 ml of the supernatant was placed into a disposable tube and blown dry under a stream of nitrogen. Once dry, 1 ml of 20% DCM in hexane and 0.1g of neutral alumina (6% deactivated) were added to the residue and samples were sonicated for 30 min. One ml of the resulting supernatant was transferred to GC vials for analysis. Analysis was conducted as described for the food samples with the exception that a 0.5 ng/ml standard was used in place of a 500 ng/ml standard. The method detection limit ranged from 8 to 29 ng/g ww depending on the amount of tissue available for analysis.

2.2.10 Statistical analysis

Data were tested for normality and equality of variance using a Shapiro Wilk test and Bartlett test, respectively. Data which did not conform to parametric assumptions was log10 transformed. Data was analyzed using a t-test or one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's test. A value of $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA).

2.3 Results

2.3.1 Concentrations of TBCO in food and eggs

At nominal concentrations of 100 and 1000 μg TBCO/g food, measured concentrations of TBCO were 40.6 and 1034.4 μg TBCO/g food, respectively. Concentrations in the acetone control were less than the limit of detection. Concentrations of TBCO were 711.3 ± 234.0 and 2535.5 ± 423.1 ng/g ww in eggs from females given the low and high concentration diet, respectively. Concentrations of TBCO in eggs from control fish were less than the limit of detection.

2.3.2 Embryotoxicity

No effects on mortality or incidences of pericardial or yolk sac edema were observed in the F1, F2, or F3 generation (data not shown). However, heart rate was decreased by 13.8% and 16.9 % in F1 embryos from F0 adults given the low and high TBCO diet, respectively (**Table 2.2**). Incidences of uninflated swim bladder and spinal curvature in F1 embryos from adults given the high TBCO diet were significantly increased to 13.10% and 26.92%, respectively (**Table 2.2**). No effects on heart rate, swim bladder inflation, or spinal curvature were observed in the F2 or F3 generations (**Table 2.2**).

Table 2.2. Average heart rate (bpm) and incidences of pericardial edema, yolk sac edema, swim bladder malformation, and spinal curvature in three generations (F1-F3) of Japanese medaka (*Oryzias latipes*) embryos following exposure of the F0 generation to < 0.0005 μg/g, wm food (control), 40.6 μg/g, wm food (low) and 1034.4 μg/g, wm food (high). Data are expressed as the mean ± standard error. Data was analyzed by use of a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test. An asterisk (*****) indicates a significant difference from the control ($p \leq 0.05$).

Generation	Treatment	Heart Rate (bpm)	Pericardial Edema (%)	Yolk Sac Edema (%)	Swim Bladder Malformation (%)	Spinal Curvature (%)
	Control	132.50 ± 1.04	4.17 ± 4.17	1.17 ± 0.34	3.63 ± 3.63	1.23 ± 1.23
F1	Low	114.29 ± 3.21*	2.12 ± 2.12	5.52 ± 0.98	1.28 ± 1.28	4.55 ± 4.55
	High	110.33 ± 3.58*	6.63 ± 0.68	4.15 ± 1.77	13.1 ± 2.45*	26.9 ± 6.92*
	Control	136.30 ± 1.00	5.01 ± 4.01	0.00 ± 0.00	0.95 ± 0.48	6.00 ± 4.71
F2	Low	134.10 ± 0.80	0.00 ± 0.00	0.00 ± 0.00	2.08 ± 1.04	5.73 ± 4.45
	High	132.64 ± 2.88	2.42 ± 1.42	2.42 ± 1.42	6.25 ± 3.12	5.20 ± 3.12
	Control	132.73 ± 2.31	4.25 ± 1.56	2.95 ± 0.17	1.39 ± 1.39	4.25 ± 1.42
F3	Low	139.10 ± 1.48	5.45 ± 1.39	1.67 ± 1.67	1.25 ± 1.25	5.45 ± 2.06
	High	130.92 ± 1.88	7.29 ± 1.60	0.00 ± 0.00	2.64 ± 1.52	7.29 ± 1.28

2.3.3 Multigenerational effects on fecundity

Parental exposure to TBCO caused a significant decrease in the cumulative fecundity of the F1 generation (**Figure 2.1**). F1 progeny from adults given the low and high treatment experienced a 33.9% and 33.3% decrease in fecundity compared to the control (**Figure 2.1**). In the F2 generation, cumulative fecundity of the high treatment fish was significantly decreased by 29.8% compared to the control however no significant difference was observed in the low treatment (**Figure 2.1**). In the F3 generation, there were no significant differences in fecundity (**Figure 2.1**). The gonadosomatic index (GSI) of F3 female fish in the high treatment was significantly lower than in the control, whereas the hepatosomatic index (HSI) was greater in the high treatment (**Table 2.2**). There were no effects of TBCO on fertilization of eggs in the F1 generation, therefore this endpoint was not assessed in the F2 of F3 generations (data not shown).

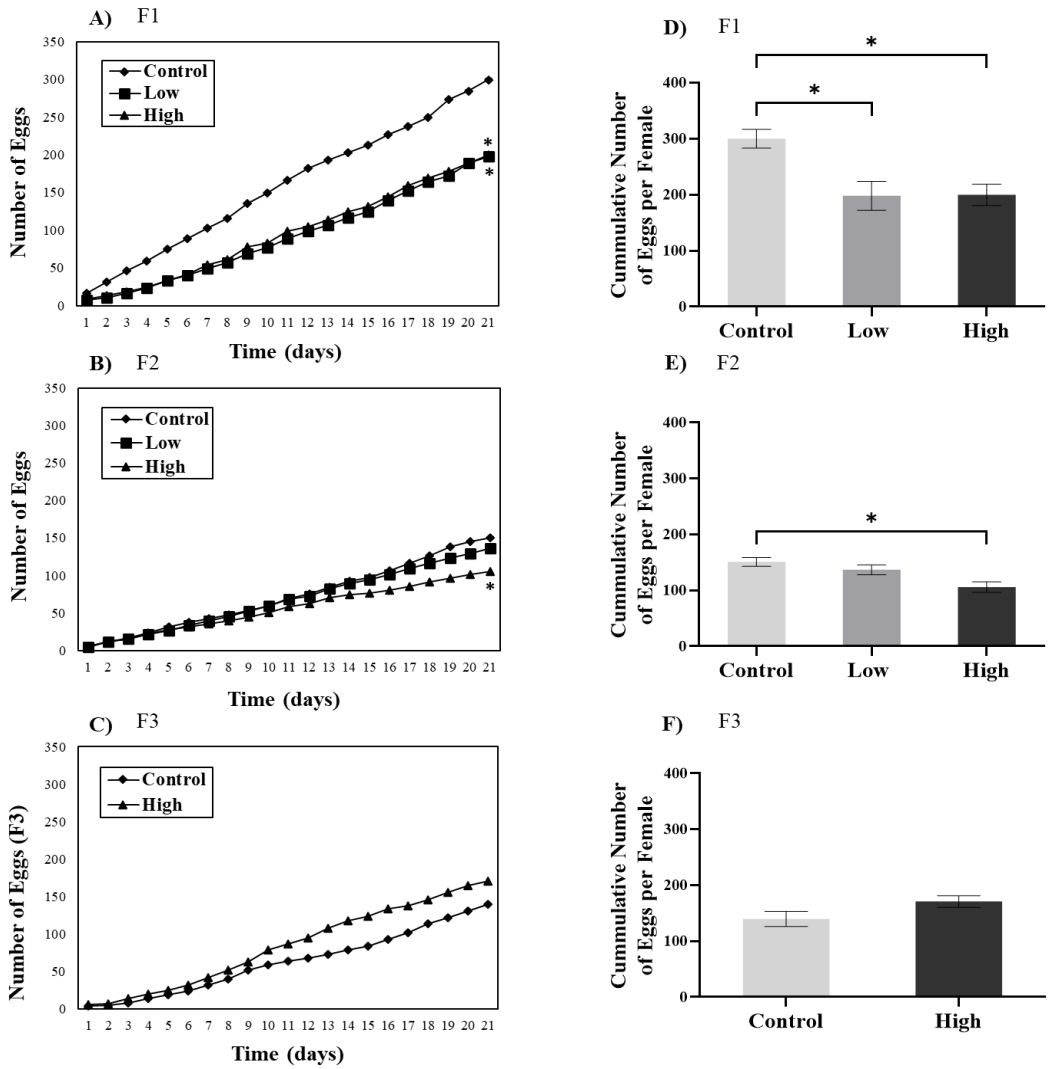

Figure 2.1 Effect of ancestral (F0) exposure to TBCO on cumulative fecundity of female Japanese medaka (*Oryzias latipes*) in the **A)** F1 generation **B)** F2 generation and **C)** F3 generation. The F0 generation was exposed to either a control diet (< 0.0005 µg/g, wm food) or TBCO at 40.6 µg/g, wm food (low) or 1034.4 µg/g, wm food (high) for 21 days during reproduction. Fertilized eggs were collected on days 14-20 of reproduction, reared to sexual maturity without additional exposure to TBCO, and fecundity of the F1 generation (**A)** was assessed. The same protocol was used for the F2 and F3 generations. The F3 low treatment was not reared to maturity as no reproductive effects were observed for the F2 low treatment. Values represent mean cumulative number of eggs per female over a 21-day period. All exposures were conducted in quadruplicate (n = 4). There were five female and five male medaka per tank for the F1 & F2 reproduction assays and four female and four male medaka per tank for the F3 reproduction assay. Differences among treatments in the F1 and F2 generation were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. An unpaired t-test was used to assess differences between treatments in the F3 generation. (*) indicates significant differences from the control determined by values of $p \leq 0.05$. The y-axis scale was kept consistent across fecundity plots to facilitate comparisons across generations of fish.

Table 2.3 Mean body weight, length, Fulton's condition factor (K), gonadosomatic index (GSI), and hepatosomatic index (HSI) of four generations (F0-F3) of female Japanese medaka (*Oryzias latipes*) following exposure of the F0 generation to < 0.0005 μg/g, wm food (control), 40.6 μg/g, wm food (low) and 1034.4 μg/g, wm food (high) for 21 days. Data are expressed as the mean ± standard error and are relative to the control treatment. Data was analyzed by use of a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test (F0 – F2 generation) or an unpaired t-test (F3 generation). An asterisk (*****) indicates a significant difference from the control ($p \leq 0.05$).

Generation	Treatment	Mass (g)	Length (mm)	Condition factor (K)	GSI	HSI
F0	Control	0.39 ± 0.06	32.6 ± 0.48	1.14 ± 0.03	10.60 ± 0.56	4.70 ± 0.42
	Low	0.44 ± 0.02	33.7 ± 0.20	1.15 ± 0.03	8.31 ± 2.24	4.38 ± 0.33
	High	0.43 ± 0.27	33.3 ± 0.55	1.16 ± 0.03	8.92 ± 1.24	5.41 ±0.28
F1	Control	0.30 ± 0.01	30.3 ± 0.59	1.07 ± 0.04	9.95 ± 0.54	3.17 ± 0.10
	Low	0.33 ± 0.01	31.4 ± 0.28	1.08 ± 0.02	9.14 ± 0.89	2.87 ± 0.15
	High	0.31 ± 0.02	30.4 ± 0.87	1.11 ± 0.06	8.04 ± 0.38	4.31 ± 0.85
F2	Control	0.30 ± 0.01	29.9 ± 0.50	1.10 ± 0.01	9.95 ± 0.71	3.45 ± 0.38
	Low	0.30 ± 0.01	30.6 ± 0.24	1.04 ± 0.03	9.02 ± 1.02	3.95 ± 0.40
	High	0.29 ± 0.01	29.2 ± 0.16	1.15 ± 0.03	8.43 ± 0.77	3.10 ± 0.32
F3	Control	0.34 ± 0.03	32.60 ± 1.25	1.01 ± 0.13	10.80 ± 0.73	2.65 ± 0.16
	High	0.31 ± 0.01	31.10 ± 0.46	1.02 ± 0.04	8.20 ±0.45*	3.03 ±0.59*

2.3.4 Multigenerational effects on mRNA abundance of steroidogenesis genes

Fecundity in the F1 generation was decreased by a similar magnitude in both the low and high treatments, but abundances of transcripts involved in steroidogenesis did not show the same trend. In the high treatment, mRNA abundance of *lhr* was significantly increased by approximately 8-fold, and mRNA abundance of *fshr* was decreased by approximately 10-fold, but this change was not significant (p=0.07) (**Figure 2.2**). In the high treatment, *cyp11a* mRNA abundance was significantly increased by approximately 10-fold, and in the low treatment, *cyp19a* mRNA abundance was significantly increased by approximately 6-fold. (**Figure 2.3**). There were no significant changes in mRNA abundances in the F2 or F3 generations.

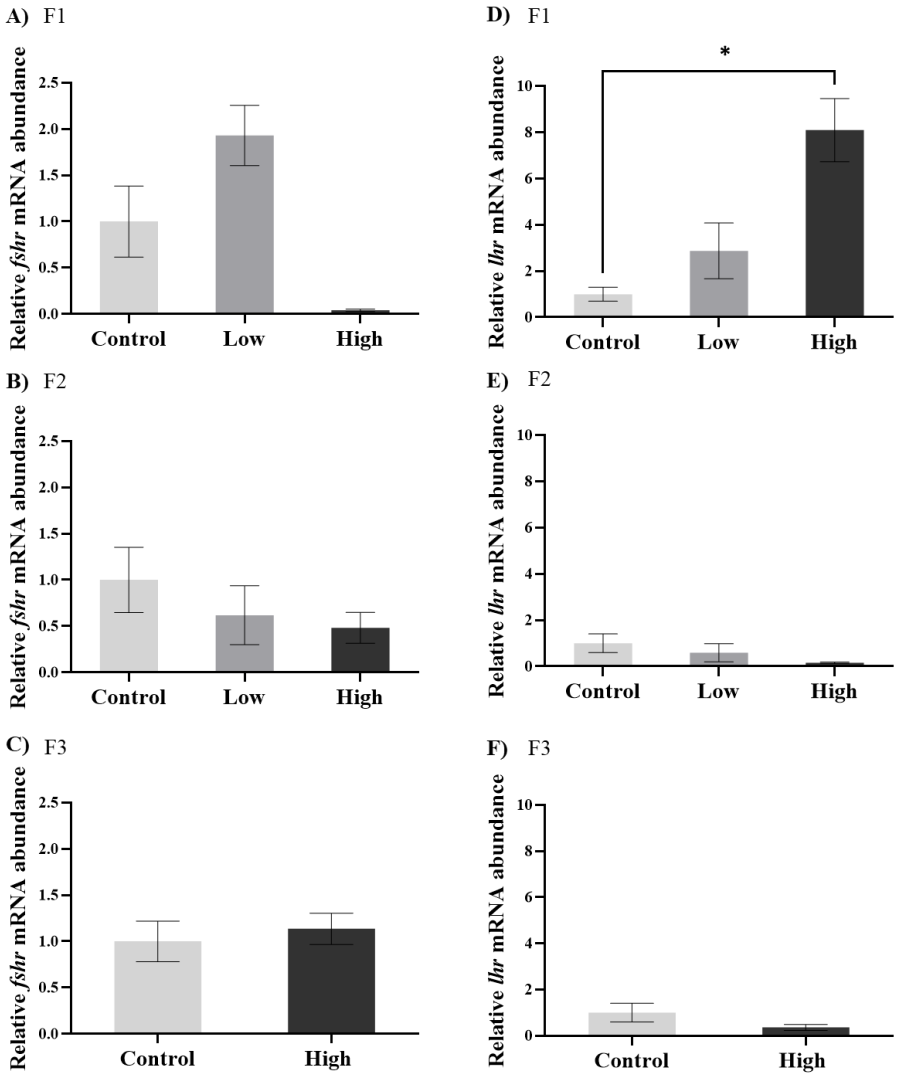

Figure 2.2 Effect of ancestral (F0) exposure to TBCO on mRNA abundance of follicle-stimulating hormone receptor *(fshr)* **(A, B, C)** and luteinizing hormone receptor *(lhr)* **(D, E, F)** in female Japanese medaka (*Oryzias latipes*) in the F1 generation, F2 generation, and F3 generation. The F0 generation was exposed to either a control diet (< 0.0005 μg/g, wm food) or TBCO at 40.6 μg/g, wm food (low) or 1034.4 μg/g, wm food (high) for 21 days. Fertilized eggs were collected on days 14-20 of reproduction, reared to sexual maturity without additional exposure to TBCO. The same protocol was used for the F2 and F3 generations. The F3 low treatment was not reared to maturity as no reproductive effects were observed for the F2 low treatment. mRNA was quantified from liver tissue from two fish per each of the four replicate tanks (n=8). Data are expressed as the mean ± standard error and are relative to the acetone control. Differences among treatments in the F1 and F2 generation were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. An unpaired t-test was used to assess differences between treatments in the F3 generation. (*) indicates significant differences from the control determined by values of $p \leq 0.05$. The y-axis scale was kept consistent across the plots for each gene to facilitate comparisons across the generations of fish.

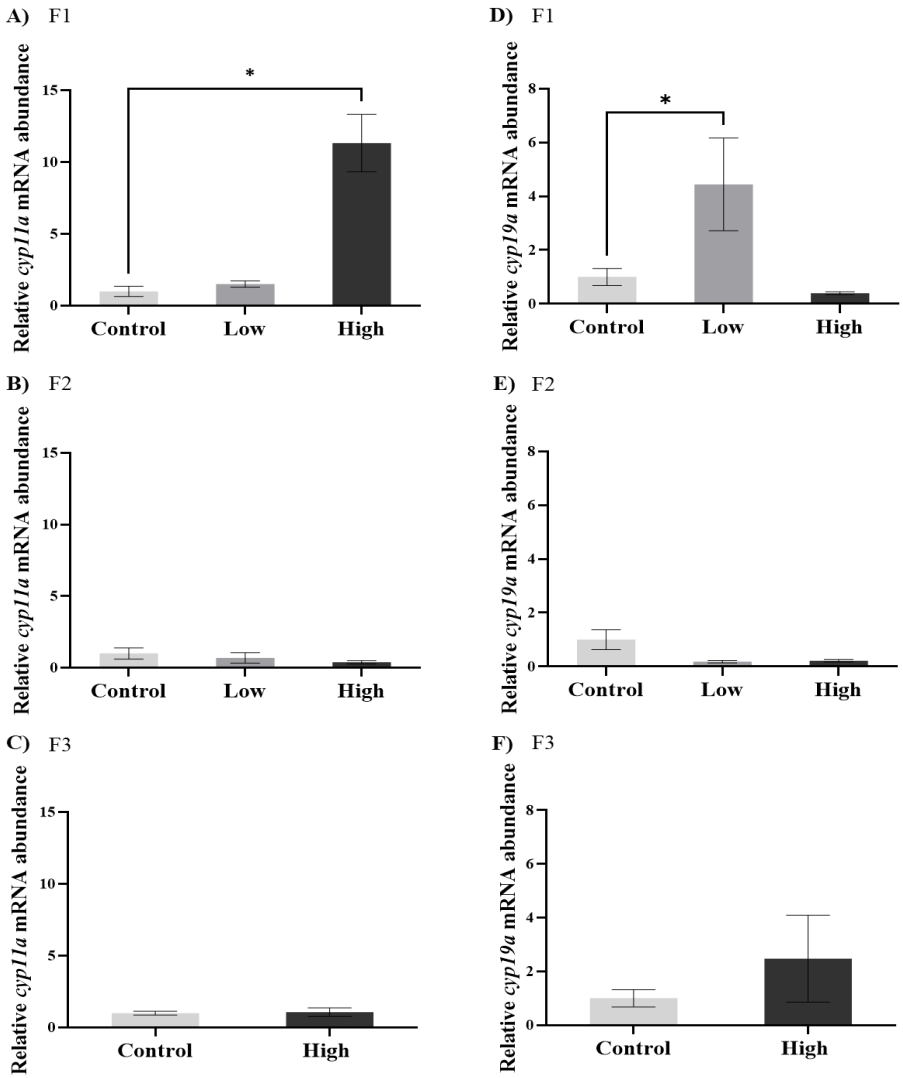

Figure 2.3 Effect of ancestral (F0) exposure to TBCO on the mRNA abundance of *cyp11a* **(A, B, C)** and *cyp19a* **(D, E, F)** in female Japanese medaka (*Oryzias latipes*) in the F1 generation, F2 generation, and F3 generation. The F0 generation was exposed to either a control diet (< 0.0005 μg/g, wm food) or TBCO at 40.6 μg/g, wm food (low) or 1034.4 μg/g, wm food (high) for 21 days. Fertilized eggs were collected on days 14-20 of reproduction, reared to sexual maturity without additional exposure to TBCO. The same protocol was used for the F2 and F3 generations. The F3 low treatment was not reared to maturity as no reproductive effects were observed for the F2 low treatment. mRNA was quantified from liver tissue from two fish per each of the four replicate tanks (n=8). Data are expressed as the mean ± standard error and are relative to the control. Differences among treatments in the F1 and F2 generation were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. An unpaired t-test was used to assess differences between treatments in the F3 generation. (*) indicates significant differences from the control determined by values of $p \leq 0.05$. The y-axis scale was kept consistent across the three plots for each gene to facilitate comparisons across the generations of fish.

2.3.5 Multigenerational effects on concentrations of E2 and mRNA abundance of vitellogenin

There were no significant differences in mRNA abundances of either isoform of vitellogenin (*vtgI* and *vtgII*; **Figure 2.4**) or concentrations of E2 in female medaka from the F1, F2 or F3 generations (**Figure 2.5**).

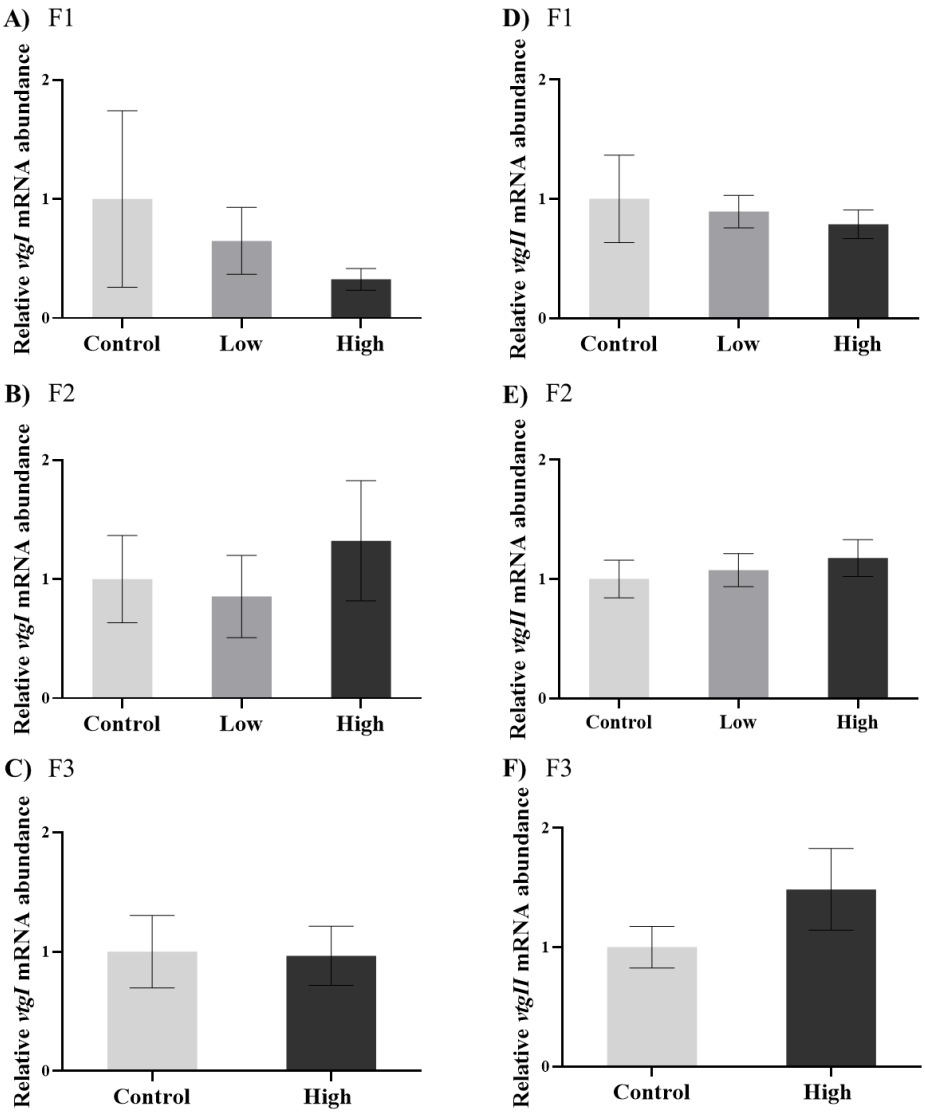

Figure 2.4 Effect of ancestral (F0) exposure to TBCO on the mean normalized mRNA abundance of *vtgI* **(A, B, C)** and *vtgII* **(D, E, F)** in female Japanese medaka (*Oryzias latipes*) in the F1 generation, F2 generation, and F3 generation. The F0 generation was exposed to either a control diet (< 0.0005 µg/g, wm food) or TBCO at 40.6 µg/g, wm food (low) or 1034.4 µg/g, wm food (high) for 21 days. Fertilized eggs were collected on days 14-20 of reproduction, reared to sexual maturity without additional exposure to TBCO. The same protocol was used for the F2 and F3 generations. The F3 low treatment was not reared to maturity as no reproductive effects were observed for the F2 low treatment. mRNA was quantified from fish liver tissue in two fish per tank for four replicate tanks (n=8). Data are expressed as the mean ± standard error and are relative to the control. Differences among treatments in the F1 and F2 generation were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. An unpaired t-test was used to assess differences between treatments in the F3 generation. The y-axis scale was kept consistent across the three plots for each gene to facilitate comparisons across the generations of fish.

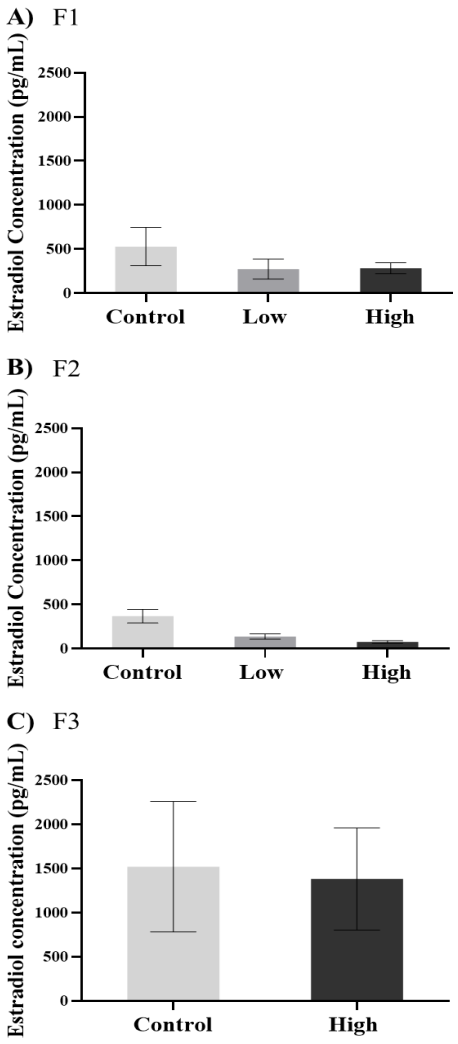

Figure 2.5 Effect of ancestral (F0) exposure to TBCO on concentrations of estradiol (E2) in blood plasma (pg/mL) of female Japanese medaka (*Oryzias latipes*). The F0 generation was exposed to either a control diet (< 0.0005 µg/g, wm food) or TBCO at 40.6 µg/g, wm food (low) or 1034.4 µg/g, wm food (high) for 21 days. Fertilized eggs were collected on days 14-20 of reproduction, reared to sexual maturity without additional exposure to TBCO. The same protocol was used for the F2 and F3 generations. The F3 low treatment was not reared to maturity as no reproductive effects were observed for the F2 low treatment. **A)** E2 plasma concentration of the F1 generation. **B)** E2 plasma concentration of the F2 generation. **C)** E2 plasma concentration of the F3 generation. For the F1 and F2, plasma was pooled from five female fish per tank. For the F3 generation, plasma was pooled from four female fish per tank. There were four tanks per treatment (n=4) for each generation. Data are expressed as the mean ± standard error and are relative to the control. Differences among treatments in the F1 and F2 generation were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. An unpaired t-test was used to assess differences between treatments in the F3 generation. The y-axis scale was kept consistent across the three plots to facilitate comparisons across the generations of fish.

2.4 Discussion

2.4.1. Maternal transfer and embryotoxicity

This is the first study to show maternal transfer of TBCO. Hydrophobic chemicals, including many BFRs, can be transported from maternal tissue to developing oocytes via circulating lipoproteins (Ungerer & Thomas, 1996). Nyholm et al. (2008) reported that 8 out of 11 BFRs delivered to zebrafish via spiked feed were maternally deposited into eggs. While TBCO (logKow = 5.24) was not assessed in that study, HBCD (logKow = 5.6), which is structurally similar to TBCO, was maternally transferred (Nyholm et al., 2008; Reindl & Falkowska, 2015). Maternal transfer of TBCO is consistent with guidelines suggesting that chemicals with a log Kow > 5 are likely to bioaccumulate (Gustavsson et al., 2016; UNEP, 2001). Eggs exposed via maternal transfer can have concentrations that exceed those of the adult fish (Foekema et al., 2012). Early-life stages of many species exhibit a greater toxicological sensitivity to chemicals than adult life stages (Russell et al., 1999). As such, maternal transfer is a particularly relevant route by which ELS fishes are exposed to lipophilic persistent organic pollutants.

Embryotoxicity in the F1 generation (**Table 2.1**) was likely caused by exposure to maternally deposited TBCO. A similar magnitude of reduction in heart rate was observed in Japanese medaka embryos exposed to waterborne TBCO (Sun et al., 2016). Interestingly, Sun et al (2016) did not observe other developmental malformations whereas spinal curvature and uninflated swim bladders were observed in the present study. These effects are likely concentration dependent. In the study by Sun et al (2016) concentrations in embryos exposed to the least concentration increased from 3.2 ± 1.5 μg/g wm on day 1 of exposure to 37.4 ± 4.4 μg/g wm on day 9, and from 394.7 ± 212.2 μg/g wm on day 1 of exposure to 647.7 ± 24.1 μg/g wm on day 9. In the present study, concentrations in F1 embryos collected on the day of deposition were 711.3

± 234.0 and 2535.5 ± 423.1 ng/g ww. In another study, exposure of zebrafish embryos to waterborne TBCO resulted in a concentration dependent increase in spinal curvature and swim bladder malformation as well as a concentration dependent decrease in heart rate (Van Essen et al., 2021a). However, concentrations of TBCO in embryos were not quantified in the study by Van Essen et al (2021a). Impaired cardiac function has been postulated to cause impaired swim bladder development as a secondary effect suggesting that the two developmental effects observed in the F1 generation could be linked (Yue et al., 2015).

Ancestral chemical exposure can induce multigenerational embryotoxicity. Exposure of adult F0 Gulf killifish (*Fundulus grandis*) to crude oil has similarly been shown to induce spinal curvature in the F1 generation, although maternal transfer of chemicals was not reported (Hess, 2022). In a multigenerational study where adult zebrafish (F0) were exposed to benzo[a]pyrene (BaP) via diet, the F1 generation experienced a significant increase in the incidence of swim bladder malformation and spinal curvature (Corrales et al., 2014). Morphological deformities of the body, tail, and pectoral fins were most severe in the F1 but still present in the F2 but not the F3 generation. Additionally, a significant increase in craniofacial deformities were observed in the F2 generation despite not being present in the F1 generation (Corrales et al., 2014). In these studies, the effects were proposed to be due to activation of the aryl hydrocarbon receptor (AhR). Studies have shown that TBCO does not activate the AhR (Saunders et al., 2013; Van Essen et al., 2021a).

2.4.2. Reproductive performance of the F1 generation

This study shows that dietary exposure of F0 adults to TBCO during reproduction decreased fecundity in two subsequent generations of progeny. (**Figure 2.1**). Although the amount of TBCO in the diets differed by 25.5-fold, and the amount maternally deposited into eggs was different by 3.6-fold between the low and high treatments, the magnitude of decrease in fecundity

in the F1 generation was equal. This suggests that both dietary concentrations of TBCO, and the resulting concentrations in eggs, exceeded the threshold for causing the maximal effect on fecundity. Although the trend of decreased E2 matched the trend of decreased fecundity in the F1 generation, this decrease was not sufficient to cause a decrease in fecundity based on a quantitative adverse outcome pathway (qAOP) that can predict decreased fecundity as a function of decreased E2 in plasma of Japanese medaka (Conolly et al., 2017; Doering et al., 2019). In the present study, E2 and VTG do not correlate with fecundity, and therefore it is highly unlikely that disrupted steroidogenesis is the mechanism responsible for the decrease. Numerous studies have shown that decreased fecundity is intricately linked to decreased E2 and VTG when steroidogenesis is impacted (Ankley et al., 2010). Alterations in gene expression in the F1 generation were observed for LHr (**Figure 2.2**), which induces ovulation (**Figure 2.2**), CYP11a (**Figure 2.3)**, which catalyzes the first step of steroidogenesis by converting cholesterol to pregnenolone, and CYP19a which catalyzes the conversion of androgen to estrogen (**Figure 2.3**) (Piferrer & Blázquez, 2005; Lian et al., 2018). However, these changes in gene expression are not consistent with any known steroidogenic mechanism resulting in decreased fecundity (Ankley et al., 2011; Ankley et al., 2020; Skolness et al., 2011; Villeneuve et al., 2007). It has been demonstrated that parental exposure of rare minnow (*Gobiocypris rarus*) to BPA inhibits reproduction of the F1 generation by reducing the number of mature oocytes (Zhu et al., 2021). Exposure to EDCs during ovarian development can delay meiotic progression and primordial follicle assembly affecting the quality of the oocytes that are produced (Delbes et al., 2022). Further, dietary exposure of sexually mature zebrafish to TBCO has been shown to impair oocyte maturation, as well as decreased mRNA abundances of genes known to regulate oocyte maturation and altered abundances of microRNAs that regulate expression of genes involved in oocyte maturation (Van Essen et al., 2021b).

Abundances of proteins that regulate oocyte meiosis were also significantly decreased in ELS Japanese medaka exposed to waterborne TBCO (Sun et al., 2016). During maternal transfer, hydrophobic chemicals can be carried via circulating lipoproteins that are transported from maternal tissue to the oocytes during oocyte maturation (Ungerer & Thomas, 1996; Nyholm et al., 2008). Therefore, impaired oocyte maturation might be the mechanism by which fecundity is decreased in the F1 generation of the present study. However, no histological analysis of the gonads or assays of oocyte maturation were conducted to support this hypothesis.

2.4.3. Reproductive performance of the F2 generation

Fecundity of the F2 low treatment group completely recovered from the decrease observed in the F1 generation (**Figure 2.1)**. However, fecundity of the F2 high treatment remained significantly decreased compared to the control, but the magnitude of effect was comparable to the F1 (**Figure 2.1**). Although the effects of exposure in the F1 generation to the low treatment are comparable to the high treatment, parental exposure to the higher dose might result in multigenerational effects that are longer lasting. In the F1 generation, both treatments had the same magnitude of effect on fecundity. However, in the F2 generation, the low treatment was able to recover while the high treatment did not, suggesting a dose dependent effect. As no changes were observed in either treatment at the molecular level, it is difficult to speculate mechanistically what might be responsible for the observed decrease in fecundity in the F2 high treatment. The lack of change in expression of genes involved in steroidogenesis, expression of vitellogenin, or plasma E2 in the F2 generation (**Figure 2.2-2.5**) provides further support for another mechanism – likely the same mechanism responsible for the decrease in fecundity in the F1 generation.

2.4.4. Reproductive performance of the F3 generation

In the F3 generation, fecundity of the high treatment was shown to completely recover from the decrease observed in both the F1 and F2 generation (**Figure 2.1**). Only the reproductive capacity of the high treatment group was examined in comparison to the control in this generation as fecundity of the low treatment was recovered in the F2 generation. No alterations in expression of genes that regulate steroidogenesis, expression of vitellogenin, or plasma E2 were observed which is consistent with observations in the F1 and F2 generations (**Figures 2.2-2.5**). Full recovery of fecundity by the F3 generation suggests that the reproductive effects caused by TBCO, and the mechanism that drives decreased fecundity, might not be transgenerational. However, reproductive effects might persist past the F2 at greater exposure doses. Regardless, the present study demonstrates that TBCO can induce multigenerational effects in fishes that might have lasting impacts on populations.

2.5. Perspectives and future directions

Ancestral exposure to dietary TBCO resulted in multigenerational reproductive impairment in a model fish species, the Japanese medaka. Exposure of the F0 generation to a diet containing 40.6 μg TBCO/g food resulted in a reduction in F1 cumulative fecundity by 33.9%. However, reproduction was shown to recover by the F2 generation. F0 exposure to a diet containing 1034.4 μg TBCO/g food resulted in decreased fecundity in the F1 and F2 generation by 33.3% and 29.8% respectively. However, reproduction was shown to recover by the F3 generation. Fecundity was decreased by approximately 30% in the F1 low treatment, F1 high treatment, and F2 high treatment suggesting this could potentially be the maximal magnitude of response to TBCO using this exposure regime. The present study suggests that the dose of an

exposure might influence the number of subsequent generations that experience impairment. In a study where sexually mature Japanese medaka were fed a diet containing either 58 or 607 μg TBCO/g, wm food, cumulative fecundity was decreased by 18% in the low treatment (Saunders et al., 2015). However, fecundity of Japanese medaka exposed to the higher concentration of TBCO food was not significantly different from that of controls (Saunders et al., 2015). Results from Saunders et al. (2015) are counterintuitive and suggest that interpreting responses to chemical exposures can be complex. Likewise, results from the present multigenerational study are not clear-cut. As such, a variety of mechanisms might be responsible for the observed multigenerational differences in fecundity, and they might be differentially dose dependent.

Ancestral EDC exposure can cause epigenetic changes in DNA methylation, histone modifications, and noncoding RNAs which can persist in future generations of fish (Best et al., 2018; Major et al., 2020). DNA methylation, which regulates DNA expression, is the most studied epigenetic mechanism in the field of ecotoxicology (Head, 2014). In a study where inland silverside were exposed to bifenthrin, levonorgestrel, ethinylestradiol, or trenbolone, inheritance of altered methylation of genes relevant to impaired phenotypes was observed across multiple generations (Major et al., 2020). In a study where marine medaka were exposed to PFBS and multigenerational reproductive impairment was observed, global DNA methylation analysis revealed hypermethylation of the ovarian genome in F0 female fish and abnormal hypomethylation of the F1 embryonic genome (Chen et al., 2019). Exposure of rare minnow to BPA resulted in suppressed methylation of the ovarian cyp19a1a gene where there was a significant negative correlation between *cyp19a1a* mRNA expression and methylation level (Liu et al., 2014). However, changes in DNA methylation of genes involved in steroidogenesis and vitellogenesis are unlikely to be the cause of multigenerational reproductive impairment in the present study.

While the decrease in fecundity was the same for both the low and high treatment in the F1 generations, changes in gene expression were inconsistent between the two treatments. Such changes could be compensatory responses as a result of rapid and possibly transient fluctuations in gene expression to restore impaired reproduction. Whatever the basis of the changes in gene expression is, altered expression of genes that regulate steroidogenesis is unlikely to be responsible for the observed F1 reproductive impairment and therefore another mechanism must be responsible. Impaired oocyte maturation might be a potential mechanism by which reproduction is impaired in the F1 generation of this study. As such, future studies should investigate the role of oocyte maturation in multigenerational effects of TBCO on fecundity of Japanese medaka, including expression of genes known to regulate oocyte maturation.

Overall, findings from this study suggest TBCO can cause multigenerational effects on reproductive performance in Japanese medaka. However subsequent generations can recover from these effects. Japanese medaka have short generation times, but for numerous native species, multigenerational effects might not become evident for years. While maternal transfer is an environmentally relevant mechanism by which exposures can occur, environmental exposures are more likely to be long-lasting where multiple generations are continuously exposed. Re-exposure of progeny might extend the recovery time beyond the F2 generation. Assessment across multiple generations is crucial when determining the long-term risks associated with exposure to chemicals and further investigation of this subject should be conducted.

CHAPTER 3: 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

3.1 Introduction

Early-life stage (ELS) exposure of fishes to endocrine disrupting chemicals (EDCs) has been linked to later-life effects on physiological performance, including reproductive impairment (Voisin et al., 2019). Mechanisms of ELS susceptibility to environmental stressors leading to latent adverse effects are still poorly understood (Voisin et al., 2019). In a study where marine medaka (*Oryzias melastigma*) were exposed as eggs (F0) to environmentally relevant concentrations of perfluorobutanesulfonate (PFBS) via maternal transfer, shrunken ovaries, impaired oocyte development, and decreased fecundity of the F1 generation was observed (Chen et al., 2019). One-time exposure of inland silverside (*Menidia beryllina)* embryos to environmentally relevant concentrations of bifenthrin has been shown to induce reproductive impairment later in life (MeCourten et al., 2020).

1,2,5,6-tetrabromocyclooctane (TBCO) is a novel brominated flame retardant (BFR) and a lipophilic EDC (Saunders et al., 2013, 2015; Mankidy et al., 2014). Dietary exposure to TBCO has been shown to decrease the fecundity of adult Japanese medaka (Saunders et al., 2015; Raza et al., unpublished). It has also been shown that TBCO is maternally transferred by Japanese medaka (*Oryzias latipe*s) exposed via their diet (Devoy et al., 2022). When sexually mature Japanese medaka (F0) were given a diet of 40.6 or 1034.4 μg TBCO/g for 21 days, average concentrations of TBCO in eggs (F1 generation) collected during the final week of exposure were 711.3 and 2535.5 ng/g wet weight, respectively (Devoy et al., 2022). Despite being reared to adulthood in clean water, the F1 generation from each exposure experienced a 33% decrease in cumulative fecundity, compared to the control (Devoy et al., 2022). Mechanistically, the decrease

in fecundity was not caused by impairment of steroidogenesis or vitellogenesis as changes in concentrations of 17β-ethinylestradiol (E2) in blood plasma or mRNA abundances of isoforms of vitellogenin (*vtgI, vtgII*) in the liver was not significantly different compared to controls, thus suggesting another mechanism is responsible (Devoy et al., 2022). Studies suggest that impairment of reproduction in adult fish exposed to TBCO via their diet is driven by impairment of oocyte maturation (Raza et al., unpublished; Van Essen et al., 2021). In zebrafish (*Danio rerio*), there are five stages of oocyte development (Selman et al., 1993) whereas in Japanese medaka there are ten stages of oocyte development (Iwamatsu et al.,1988). The final stage of oogenesis is the maturation of the immature stage IV (zebrafish) or stage IV (Japanese medaka) oocyte to a mature oocyte that can be fertilized. When sexually mature female zebrafish were exposed to dietary TBCO for 14 days, a smaller percentage of stage IV oocytes matured into stage V oocytes (Van Essen et al., 2021). Additionally, when sexually mature female Japanese medaka were exposed to dietary TBCO for 21 days, a smaller percentage of stage IX oocytes matured into stage X oocytes (Raza et al., unpublished). Further, the abundance of proteins that regulate oocyte meiosis was decreased in Japanese medaka exposed to waterborne TBCO as embryos (Sun et al., 2016).

Fish reproduction is dependent on the successful completion of oogenesis. During the growth stages of oogenesis, follicle stimulating hormone (FSH) is released from the pituitary signalling the production of E2 that binds to estrogen receptors (ERs), ERα and ERβ, in the liver, stimulating the synthesis of the egg yolk precursor protein, vitellogenin, that is incorporated into the growing oocyte (Lubzens et al., 2010; Nagahama and Yamashita, 2008; Sullivan and Yilmaz, 2018). Vitellogenin is responsible for the growth of oocytes and promotes the development of primary (previtellogenic) oocytes into secondary (vitellogenic) oocytes (Lubzens et al., 2010). Oocyte growth is followed by oocyte maturation (Nagahama and Yamashita, 2008; Lubzens et al.,

2010). Luteinizing hormone (LH) is released from the pituitary and binds to the luteinizing hormone receptor (LHR), stimulating the release of maturation-inducing hormone (MIH; 17α,20β-Dihydroxy-4-pregnen-3-one) from follicular cells (Sen & Caiazza, 2013). Binding of MIH to membrane progestin receptors (mPRs) signals the formation of maturation-promoting factor (MPF) that induces the last step of oocyte maturation, breakdown of the oocyte germinal vesicle, resulting in a fertilizable oocyte (Nagahama and Yamashita, 2008).

Several proteins play critical roles in MIH-stimulated maturation of oocytes. Maturation-inducing hormone binds to mPRs located on the oocyte surface (Nagahama & Yamashita, 2008). Membrane progestin receptor α (mPRα) mediates nongenomic progestin signaling that induces oocyte maturation (Wu et al., 2020). Little is known about the role of membrane progestin receptor β (mPRβ), however, impaired oocyte maturation has been observed in mPRβ mutants (Wu et al., 2020). Progestin receptor membrane components (PGRMC) have two paralogs: PGRMC1 and PGRMC2 that regulate oogenesis and ovulation (Wu & Zhu, 2020). Knockout of PGRMC1 or PGRMC2 suggests that both regulate mPR (Wu et al., 2018). There is evidence that pgrmc1 regulates mPRα expression and localization to the oocyte cell surface, and impaired oocyte maturation has been observed in *pgrmc1*$^{-/-}$ female zebrafish (Wu et al., 2018). Lastly, the insulin-like growth factor (IGF) system is thought to play a regulatory role in teleost oocyte maturation by up-regulating expression of ovarian mPRα (Picha et al., 2012). Stage IV oocytes from zebrafish exposed to TBCO via their diet for 10 days had significantly decreased mRNA abundance of IGF3 and impaired MIH-stimulated oocyte maturation (Van Essen et al., 2021).

The objective of the present study was to determine whether decreased fecundity in Japanese medaka reared from embryos exposed to maternally deposited TBCO is caused by inhibition of oocyte maturation and to investigate potential mechanisms of this effect. Sexually

mature F0 fish were exposed to dietary TBCO as described by Devoy et al. (2022) as a means of exposing the F1 generation via maternal transfer. Embryos were collected and reared in clean water and upon reaching sexual maturity, fecundity was assessed using a standard 21-day reproduction assay. Upon termination of the assay, gonad tissue was excised from female fish and an *ex vivo* oocyte maturation assay was performed to determine whether exposure to TBCO via maternal transfer impairs MIH-stimulated oocyte maturation at sexual maturity. Additionally, mRNA abundances of genes involved in MIH-stimulated oocyte maturation (*mPRα*, *mPRβ*, *pgrmc1*, *pgrmc2*, and *igf3*) was quantified.

3.2. Methods

3.2.1. Preparation of TBCO contaminated fish food

Preparation and analysis of food spiked with TBCO has been described previously (Devoy et al., 2023). Nominal concentrations of TBCO were 100 and 1000 ug/g food. Samples are currently being analysed at the University of Saskatchewan to determine actual concentrations.

3.2.2. Animal care

Sexually mature F0 generation Japanese medaka were from a culture maintained at the University of Lethbridge Aquatic Research Facility (Lethbridge, AB, Canada), following Animal Welfare Protocol #2014. Animals were maintained in flow-through tanks (Tecniplast, Toronto, ON, Canada) supplied with dechlorinated municipal tap water (average water quality: conductivity 378 μS/cm, alkalinity 128 mg as CaCO3/L, hardness 165 mg as CaCO3/L, > 90% oxygen saturation, pH 8.01). Fish were kept on a 16:8 light: dark photoperiod at 25°C and fed three times daily using a combination of live Artemia (Brine Shrimp Direct, Ogden, TU, USA) and TetraMinR tropical flakes (Tetra, Brampton, ON, Canada).

3.2.3. Exposure of F0 generation to TBCO

Five sexually mature Japanese medaka of each sex were randomly assigned to each of four 10 L replicate tanks per treatment and housed under flow-through conditions. Fish were allowed to acclimate for seven days before exposure during which they were fed approximately 2.5% of their body mass twice daily with TetraMin R tropical flakes. Water temperature was maintained at 25 ± 1 °C throughout the experiment. During the exposure, fish were fed TBCO spiked or control TetraMinR tropical flakes (2.5% of their body mass) twice daily. Fertilized eggs collected during the last week of the exposure were reared as described in section 3.2.2.

3.2.4. Rearing and reproductive performance of the F1 generation

Fertilized eggs (F1) were separated by treatment in 3.5 L tanks in a Tecniplast rack and grown for approximately four months at 25°C in dechlorinated city of Lethbridge water until sexual maturity was reached. Fish were grown at a density of 20 fish per tank under flow-through conditions where 15% of the water was renewed daily. Fish were fed live Artemia (Brine Shrimp Direct) twice daily, once in the morning and once in the afternoon, and received an additional feeding of TetraMinR tropical flakes at mid-day following one-month post-hatch. Reproductive performance was assessed by use of a standardized 21-day reproduction assay, according to OECD Test Guideline 229 (OECD, 2012). Conditions were the same as described in section 3.2.3 apart from there being three tanks per treatment, with four males and four females per tank, and the diet did not contain TBCO. Each day, all eggs were collected from each tank and fecundity was determined as the mean number of eggs per female per tank. Upon termination of the reproduction assay, the mass and length of each female fish was recorded to calculate Fulton's condition factor (k). Liver and gonad tissue was collected from each female and weighed to determine

hepatosomatic index (HSI) and gonadosomatic index (GSI). Ovaries from three female fish per tank were used for assessment of oocyte maturation (section 2.5). Remaining tissues were snap frozen in liquid nitrogen and stored at -80 °C until needed. Blood from each female fish was collected from the caudal vein by use of a heparinized microcapillary tube, pooled for each replicate tank, and immediately centrifuged at 8000×g for 10 min at 4 °C, to collect plasma for quantification of concentrations of E2.

3.2.5. F1 Oocyte maturation assay

Upon termination of the 21-day F1 reproduction assay, gonads from 3 female fish per replicate tank were excised and dissociated. An oocyte maturation assay where the response of stage IX immature oocytes to maturation inducing hormone (MIH) was performed according to methods previously described (Raza et al., unpublished). Ovaries were excised immediately at the onset of light and placed in 90% Gibco Leibovitz L-15 medium (L-15: containing 1X penicillin-streptomycin; pH 7.0; Fisher Scientific, Walthan, MA, USA) as endogenous MIH is released within 2 hrs of light onset in Japanese medaka (Iwamatsu, 1978). Stage IX oocytes were distinguished using a dissecting microscope (Motic Instruments, Richmond, BC, Canada) based on size, oocyte surface opacity, and the presence of postovulatory follicles and oil droplets (Iwamatsu et al., 1988; Raza et al., unpublished). Stage IX oocytes collected from females from the same tank were pooled and 25 were each placed into three replicate wells of a 24-well plate containing 1 ml of L-15 and 1000 mg/L MIH, resulting in three biological replicates per treatment. Plates were incubated for 24 hrs following which oocyte maturation was scored based on breakdown of the germinal vesicle (the final stage of oocyte maturation).

3.2.6. Real-time PCR

Changes in relative abundances of mRNAs of *vtgI* and *vtgII* in livers, and *mPRα*, *mPRβ*, *pgrmc1*, *pgrmc2*, and *igf3* in ovaries of sexually mature F1 female medaka were determined according to methods previously described (Devoy et al., 2022). Abundances of these mRNAs were determined relative to abundances of housekeeping genes 18s ribosomal RNA (*18s rRNA*) and ribosomal protein L7 (*rpl7*), and correcting for reaction efficiencies (Pfaffl, 2001). Target mRNAs and primer details are provided below (**Table 3.1**). Initial attempts at quantification of mRNA abundances of *mPRα*, *mPRβ*, *pgrmc1*, *pgrmc2*, and *igf3* were performed using oocytes collected at the end of the oocyte maturation assay. However, qPCR was not successful because of poor RNA quality. Therefore, intact ovaries from one female fish per tank from each replicate were used for qPCR analysis, which resulted in a sample size of three per treatment.

Table 3.1 Details of oligonucleotide primers used in semi-quantitative real-time PCR (qPCR).

Target mRNA	Function	Primer Sequence (5′–3′)	Efficiency (%)
rpl7	Housekeeping	**F**- GTCGCCTCCCTCCACAAAG **R**- AACTTCAAGCCTGCCAACAAC	88.0
18s *rRNA*	Housekeeping	**F**- GACAAATCGCTCCACCAACT **R**- CCTGCGGCTTAATTTGACCC	111.0
vtgI	Egg yolk precursor protein / oocyte growth	**F**-ACTCTGCTGCTGTGGCTGTAG **R**-AAGGCGTGGGAGAGGAAAGTC	109.0
vtgII	Egg yolk precursor protein / oocyte growth	**F**-TCGCCGCAAGAGCAACAC **R**-CTGGAGGACCTGGAAGAACTG	100.0
mPRa	Oocyte maturation	**F**-CCACGTTCTCTACGGCTACA **R**-CCAATCTGCTCCATCACAAC	108.8
mPRb	Oocyte maturation	**F**-GGAAGAGAGTCGGTCTGAGGTT **R**-CAAGGACGAGGTCTGAGATGTG	81.1
pgrmc1	Oocyte maturation	**F**-CTCTCCGCTAAACCTCACGC **R**-TCAGTTTAGGTAGCGGCGTT	91.3
pgrmc2	Oocyte maturation	**F**-AGGCTTCCTGAGAGACGAGT **R**-AACCTCCCCACGTAGTCGTA	95.4
igf3	Oocyte maturation	**F**-GCACGACCACCTCTCACAT **R**-CTGAACAGAAAGCGGCACTT	104.0

3.2.7. Quantification of E2

Blood plasma E2 concentrations were quantified in F1 female fish using an Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturers protocol (Item #: 501890, Cayman Chemical, MI, USA). Plasma from all four females in each replicate tank were pooled (n=3) due to small plasma volumes in adult Japanese medaka.

3.2.8 Statistical analysis

All data was analyzed using a one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's test. Data normality and variance was assessed using a Shapiro Wilk test and a Bartlett test, respectively. A log10 transformation was conducted when data was non-parametric. Differences were considered statistically significant at $p \leq 0.05$. Statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA).

3.3 Results

3.3.1. Effect on F1 fecundity

Offspring of adult Japanese medaka (F1) fed the low and high TBCO spiked food experienced a 26.0% and 56.8% decrease in cumulative fecundity compared to the control (**Figure 3.1**). However, effects were not statistically significant ($p = 0.059$ low, $p = 0.058$ high). No effects of TBCO on fertilization success were observed (data not shown). Additionally, no effects on mean body weight, length, K, GSI, or HSI were observed in F1 female Japanese medaka (**Table 3.1**).

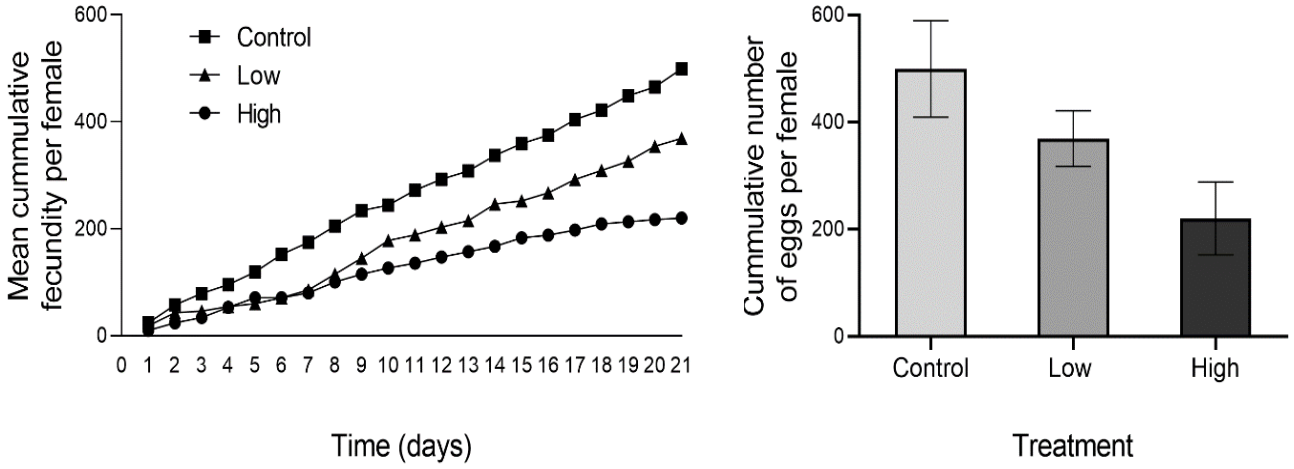

Figure 3.1 Effect of maternal (F0) exposure to TBCO on cumulative fecundity of F1 female Japanese medaka (*Oryzias latipes*). The F0 generation was exposed to a control diet (< 0.0005 µg/g, wm food) or TBCO at 100 µg/g, wm food (low) or 1000 µg/g, wm food (high) for 21 days during reproduction. Fertilized eggs were collected on days 14-21 of reproduction, reared to sexual maturity without additional exposure to TBCO, and fecundity of the F1 generation was assessed. Values represent mean cumulative number of eggs per female over a 21-day period. Exposures were conducted in triplicate (n = 3). There were four female and four male fish per tank. Differences among treatments were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. No significant differences were observed.

Table 3.2 Mean body weight, length, Fulton's condition factor (K), gonadosomatic index (GSI), and hepatosomatic index (HSI) of F1 female Japanese medaka (*Oryzias latipes*) following exposure of the F0 generation to < 0.0005 μg/g, wm food (control), 100 μg/g, wm food (low) and 1000 μg/g, wm food (high) for 21 days. Data are expressed as the mean ± standard error and are relative to the control treatment. Data was analyzed by use of a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test. No significant differences were observed.

Treatment	Mass (g)	Length (mm)	Condition factor (K)	HSI	GSI
Control	0.36 ± 0.08	30.8 ± 3.08	1.31 ± 0.57	4.00 ± 0.74	8.92 ± 1.34
Low	0.35 ± 0.08	31.2 ± 1.50	1.16 ± 0.25	3.75 ± 0.89	9.51 ± 1.15
High	0.34 ± 0.09	31.4 ± 2.60	1.10 ± 0.26	4.11 ± 0.88	10.5 ± 3.18

3..3.2 Oocyte maturation

Ex vivo MIH-stimulated maturation of stage IV oocytes from female F1 Japanese medaka from the low and high TBCO treatments were decreased by 23.4% and 20.0% respectively, compared to the control (p = 0.011 low, p = 0.023 high; **Figure 3.2**).

3.3.3 Effects on F1 plasma E2

Concentrations of E2 in blood plasma from female F1 Japanese medaka exposed to maternally deposited TBCO were not significantly different from concentrations in controls (p=0.964 low, p=0.905 high; **Figure 3.3**).

3.3.4 mRNA abundance of VTG, mPR, IGF, and PGRMC

Abundance of *vtgI* and *vtgII* mRNAs were decreased in a concentration dependent manner. Specifically, mRNA abundance of *vtgI* was significantly decreased by 4.5-fold in the low treatment and by 8.7-fold in the high treatment (p = 0.029 low, p = 0.036 high; **Figure 3.3**). No significant differences were observed for *vtgII* mRNA abundance in the low or high treatment compared to the control (p = 0.535 low, p = 0.208 high; **Figure 3.3**). Abundances of mRNAs of *mPRα* (p = 0.320 low, p = 0.298 high), *pgrmc1* (p = 0.403 low, p = 0.288 high), *pgrmc2* (p = 0.410 low, p = 0.348 high), and *igf3* (p = 0.378 low, p = 0.265 high) were all decreased in a concentration dependent manner, but effects were not statistically significant (**Figure 3.4**). No concentration dependent trends or significant changes in transcript abundance were observed for mPRβ (p = 0.752 low, p = 0.836 high; **Figure 3.4**).

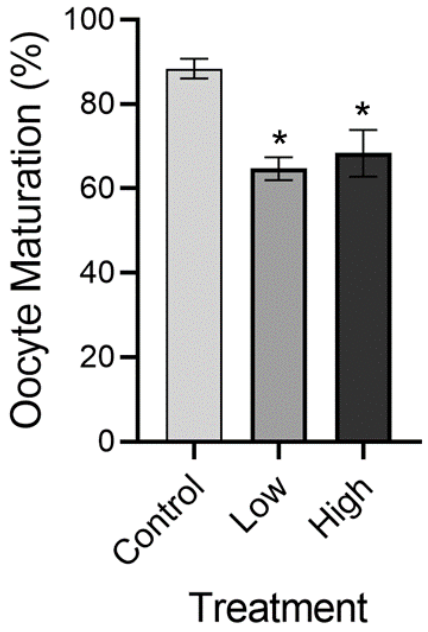

Figure 3.2 Effect of 21-day exposure to dietary TBCO on *ex vivo* maturation (germinal vesicle breakdown [GVBD]) of stage IX oocytes. The F0 generation was exposed to a control diet (< 0.0005 µg/g, wm food) or TBCO at 100 µg/g, wm food (low) or 1000 µg/g, wm food (high) for 21 days during reproduction. Fertilized eggs were collected on days 14-21 of reproduction, reared to sexual maturity without additional exposure to TBCO, and fecundity of the F1 generation was assessed. Values represent mean cumulative number of eggs per female over a 21-day period. Exposures were conducted in triplicate (n = 3). There were four female and four male fish per each of the three replicate tanks. Following exposure, stage IX oocytes were isolated and pooled from each female per replicate tank. Oocytes were divided into three wells of a 24 well plate and exposed to 1000mg/L MIH. The average number of mature oocytes (GVBD) per replicate was determined across the three wells. Data are expressed as the mean ± standard error and are relative to the control. Differences among treatments were analyzed by using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. (*) indicates significant differences from the control ($p \leq 0.05$).

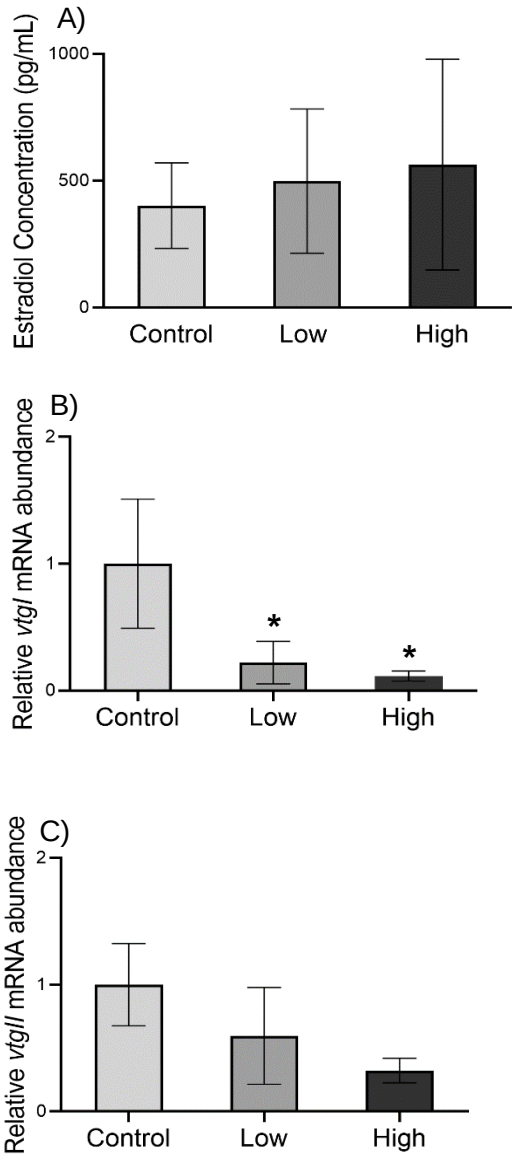

Figure 3.3 Effect of maternal (F0) exposure to TBCO on blood plasma concentration of estradiol (**A**) and mRNA abundance of vitellogenin isoform I *(vtgI)* **(B)** and vitellogenin isoform II *(vtgI)* **(C)** in female Japanese medaka (*Oryzias latipes*). The F0 generation was exposed to a control diet (< 0.0005 µg/g, wm food) or TBCO at 100 µg/g, wm food (low) or 1000 µg/g, wm food (high) for 21 days during reproduction. Fertilized eggs were collected on days 14-21 of reproduction, reared to sexual maturity without additional exposure to TBCO, and fecundity of the F1 generation was assessed. Values represent mean cumulative number of eggs per female over a 21-day period. mRNA was quantified from fish liver tissue in two fish per tank for three replicate tanks (n=6). Data are expressed as the mean ± standard error and are relative to the control. Differences among treatments were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. (*) indicates significant differences from the control determined by values of $p \leq 0.05$.

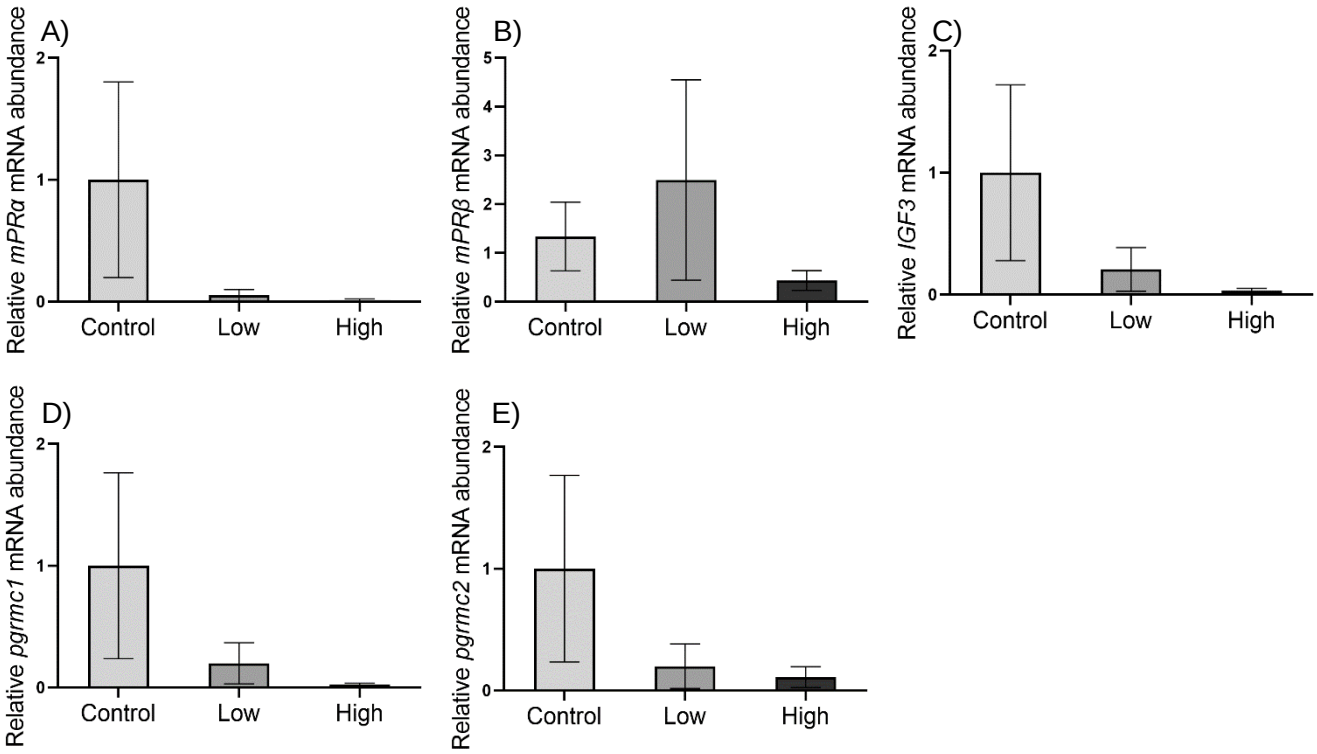

Figure 3.4 Effect of maternal (F0) exposure to TBCO on mRNA abundance of membrane progestin receptor α *(mPRα)* **(A)** progestin receptor β *(mPRβ)* **(B)** insulin growth factor 3*(igf3)* **(C)** progestin receptor membrane component I *(pgrmc1)* **(D)** progestin receptor membrane component II *(pgrmc2)* **(E)** in female Japanese medaka (*Oryzias latipes*). The F0 generation was exposed to a control diet (< 0.0005 µg/g, wm food) or TBCO at 100 µg/g, wm food (low) or 1000 µg/g, wm food (high) for 21 days during reproduction. Fertilized eggs were collected on days 14-21 of reproduction, reared to sexual maturity without additional exposure to TBCO, and fecundity of the F1 generation was assessed. Values represent mean cumulative number of eggs per female over a 21-day period. mRNA was quantified from fish liver tissue in two fish per tank for three replicate tanks (n=6). Data are expressed as the mean ± standard error and are relative to the control. Differences among treatments were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. No significant differences were observed.

3.4. Discussion

3.4.1. Impaired reproduction of the F1 generation

This is the second study to demonstrate reductions in fecundity of the F1 generation following F0 exposure to TBCO (Devoy et al., 2022). Maternal transfer is a mechanism by which ELS are exposed to lipophilic EDCs (Haldén, 2010; Khadra et al., 2019). Lipophilic compounds bioaccumulate quickly in the yolk of fish eggs (Petersen & Kristensen, 1998; Ungerer & Thomas, 1996; Nyholm et al., 2008). Japanese medaka exposed to nominal concentrations of 100 μg/g or 1000 μg/g of TBCO via maternal transfer experienced a 26.0% and 56.8% decrease in fecundity, respectively, reflecting a concentration-dependant effect. Although the decrease in cumulative fecundity was not statistically significant, this effect is consistent with what was observed previously where fish exposed to 40.6 μg/g or 1034.4 μg/g of TBCO via maternal transfer experienced a 33.9% and 33.3% decrease in fecundity, respectively (Devoy et al., 2022). Mechanistically, decreased fecundity is often linked to decreased synthesis of E2 and VTG that are required for oogenesis. Tributyltin, an organotin biocide, has been found to decrease fecundity of female zebrafish by disrupting oogenesis (Xiao et al., 2018). Exposure decreased expression of *vtgI* in the liver, increased expression of genes involved in oocyte meiosis, increased the number of early-stage oocytes, and decreased the number of late-stage oocytes (Xiao et al., 2018). In zebrafish exposed to 2,3,7,8-tetrabromodibenzo-p-dioxin (TBDD), decreased fecundity was observed along with reduced mRNA abundance of *vtgI* and decreased oocyte maturity (Haldén et al., 2011). When decreases in fecundity are the result of disrupted steroidogenesis, drastic decreases in plasma concentrations of E2 and VTG protein are typically observed (Doering et al., 2019). However, concentrations of E2 in plasma from F1 female Japanese medaka were not decreased, and therefore disruption of steroidogenesis is unlikely to be the mechanism of decreased

fecundity. Conversely, mRNA abundance of *vtgI* was significantly decreased in a concentration dependant manner, and a trend of concentration dependent decreases in *vtgII* was observed. While mRNA abundance of *vtgI* was significantly decreased by 4.5-fold in the low treatment and by 8.7-fold in the high treatment, these decreases are unlikely to be of biological significance as similar decreases in VTG protein have not resulted in decreased fecundity (Doering et al., 2019). Because of the small volume of blood collected from each female fish, it was not possible to measure concentrations of VTG after measuring E2. However, quantification of *vtg* by qPCR is often more sensitive than the use of ELISA to quantify VTG protein abundance, meaning that changes in VTG protein amount likely are less than mRNA abundance (Navas & Segner, 2006).

3.4.2. Impaired oocyte maturation

Fecundity of female fish is dependent on completion of oogenesis. The final step of oogenesis is oocyte maturation (Nagahama & Yamashita, 2008; Lubzens et al., 2010). Stimulation of oocyte maturation by MIH was impaired in F1 female Japanese medaka as indicated by a 23.4% and 20.0% reduction in GVBD observed in the low and high treatment respectively (**Figure 3.3**). Germinal vesicle breakdown is the final step of oocyte maturation which enables oocytes to become fertilizable eggs (Lubzens et al., 2010). Binding of MIH to mPRs located on the oocyte surface triggers a series of events culminating in germinal vesicle breakdown (Nagahama & Yamashita, 2008). Localization and expression of mPRs is dependent upon actions of PGRMCs (Wu et al., 2018). Insulin growth factor 3 is thought to play an important role in oocyte maturation by upregulating expression of mPRα (Picha et al., 2012). While not statistically significant, concentration dependant decreases in mRNA abundances of *mPRα*, *pgrmc1*, *pgrmc2*, and *igf3* (**Figure 3.5**) were observed. Lack of statistical significance is likely due to small sample size (n=3) as ovaries from three of four fish per tank were used for oocyte maturation assays. Additionally,

there could have been a dilution effect as entire ovaries containing a mix of oocytes at different stages of development were used for molecular analysis, and not just stage IX oocytes. Transcript abundance of *mPRα* was decreased by approximately 19-fold in the low treatment and 70-fold in the high treatment. Similarly, *pgrmc1* and *pgrmc2* were both decreased 5-fold in the low treatment and 41-fold and 9-fold in the high treatment, respectively. Lastly, *igf3* was decreased 5-fold in the low treatment and 30-fold in the high treatment. These decreases in expression of genes that are essential for MIH-stimulated oocyte maturation are consistent with a decrease in GVBD, and further support the conclusion that impairment of oocyte maturation contributes to decreased fecundity in F1 fish.

The mechanism of decreased fecundity in Japanese medaka exposed to maternally deposited TBCO appears to be decreased oocyte maturation, potentially caused by decreased expression of genes that regulate oocyte maturation. In the present study, all stage IX oocytes from control and TBCO exposed fish were of similar size. Oocytes only reach this stage of development if they have accumulated adequate VTG (Nagahama & Yamashita, 2008). Thus, differences in oocyte maturation of treated fish are unlikely to have been caused by decreased accumulation of VTG. Future studies could investigate in greater detail whether there are differences in VTG accumulation in oocytes from fish exposed to maternally deposited TBCO, and whether this impacts oocyte maturation in the *in vitro* assay.

3.4.3 Epigenetic mechanisms

Mechanistically, fish that are exposed to xenobiotics during ELS, can experience epigenetic changes such as DNA methylation, histone modification, and microRNA gene silencing or activation, that heritably alter gene expression without changing DNA sequence (Best et al.,

2018). During critical windows of development, the methylome undergoes extensive change where post-fertilization reprogramming erases previous epigenetic marks transferred through gametes and new marks become established (Wang & Bhandari, 2019). In Japanese medaka, a first round of global demethylation occurs from the blastula stage to ~8-days post-fertilization (dpf) followed by a second round from 10-12-dpf (Wang & Bhandari, 2020). As TBCO was maternally deposited, the chemical could have been present disrupting developmental reprogramming during this time.

Decreased mRNA abundances of genes involved in oocyte growth and maturation might be the result of changes in DNA methylation. Exposure to EDCs during embryo development disrupt the epigenome, including altered DNA methylation, leading to changes in gene expression (Head, 2014; 2019). Toxicant exposure is most likely to alter DNA methylation profiles during embryogenesis when DNA methylation patterns are being re-established (Falisse et al., 2018). As such, fish are incredibly sensitive to chemical exposure during this time. Molecular analysis of marine medaka exposed to PFBS via maternal transfer, that experienced reproductive impairment, revealed abnormal global DNA hypomethylation of the embryonic genome (Chen et al., 2019). Additionally, in a study where ELS zebrafish were exposed to the antibacterial agent triclosan during embryogenesis, differential DNA methylation was observed reflecting dose-dependent specific responses (Falisse et al., 2018). Lastly, reproductive effects in Japanese medaka exposed during ELS to BPA or EE2, were suspected to be due to disruption of the epigenome in germ cells as embryos were exposed 5-7 days post-fertilization, during the critical period for sex determination and germ cell development (Bhandari et al., 2015). During ELS exposure of fish to environmental stressors, the hypothalamus-pituitary-gonadal-liver (HPGL) axis can be disrupted resulting in a variety of reproductive effects (Liao et al., 2014). In a study where ELS inland silversides were exposed to waterborne bifenthrin,

expression of the genes *17β-hsd, ERa,* and *igf2* was decreased (Decourten et al., 2020). Exposure resulted in decreased fecundity at sexual maturity and was reflected in differences in DNA methylation in multiple genes involved in steroidogenesis (Decourten et al., 2020). In the present study, differences in expression of genes involved in oocyte growth and maturation observed in the F1 generation is unlikely to be due to antagonism of the receptor by TBCO as decreased expression was observed several months after exposure. As such changes in gene expression could be the result of altered DNA methylation that can cause effects long after chemical exposure has ceased.

3.5. Conclusions

Overall, findings from this study confirm that maternal exposure to TBCO results in decreased fecundity at sexual maturity. The decrease in fecundity of the F1 generation was shown to be concentration dependent as were decreases in oocyte maturation and gene expression suggesting that dose of TBCO could play an important role in causing reproductive impairment of Japanese medaka exposed to TBCO. While transcript abundance of *vtg* was decreased in female fish exposed to TBCO via maternal transfer, it is unlikely that this change is fully responsible for the observed decreases in fecundity as similar decreases in VTG have not been reflected at the level of fecundity and no corresponding decreases in E2 were observed (Doering et al., 2019). Impaired oocyte maturation of Japanese medaka exposed to TBCO via maternal transfer is likely the cause of decreases in fecundity as demonstrated by results of the ex *vivo* oocyte maturation assay which showed a significant decrease in the number of stage IX oocytes that matured into stage X oocytes (eggs). This mechanism is further supported by the observation that expression of genes involved in oocyte maturation was consistently downregulated. These changes in gene expression might be the result of epigenetic mechanisms such as altered DNA methylation. Future

studies should explore DNA methylation patterns of genes involved in oocyte growth and maturation in fish exposed to TBCO via maternal transfer.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1 Introduction

There is concern that anthropogenic chemicals in aquatic ecosystems can have adverse effects on resident biota, including fishes. Most studies in aquatic ecotoxicology are designed to assess effects that manifest during acute or chronic exposures of fishes to toxicants. However, there is growing recognition that exposure of fishes during critical stages of embryogenesis can have long-term effects on physiological performance, including multigenerational effects in generations with a life history of exposure (e.g., exposure via germ cells) and transgenerational effects in unexposed generations. However, much less is known about these effects compared to those that manifest during an exposure. Endocrine disrupting chemicals (EDCs) are natural or synthetic agents that can alter homeostasis, reproduction, and other developmental processes (Crisp et al., 1988). Exposure to EDCs during embryo development has been shown to cause multigenerational and/or transgenerational effects on reproductive performance (Bhandari et al., 2015). The novel brominated flame retardant (BFR), 1,2,5,6-tetrabromocyclooctane (TBCO), is an EDC that has been shown to impact hormone signaling, sex steroid synthesis, and reproductive performance of sexually mature Japanese medaka exposed to this chemical via their diet (Saunders et al., 2013, 2015; Mankidy et al., 2014). Objectives of this research were to investigate effects of maternally deposited TBCO on development and reproductive success in three generations of Japanese medaka (F1, F2, F3) and to determine molecular mechanism(s) of effect(s). Based on previous studies, it was hypothesized that TBCO would be maternally transferred to eggs and would induce multigenerational and transgenerational reproductive effects caused by dysregulation of expression of genes known to regulate reproduction. Two consecutive studies were conducted to test this hypothesis.

4.2 Summary of study #1

In the first study (Chapter 2), sexually mature fish (F0 generation) were fed either a control diet or a low (40.6 μg/g) or high (1034.4 μg/g) diet containing TBCO for 21 days and three generations of embryos were reared without additional exposure to determine effects of TBCO on reproductive performance. Once the F1, F2, and F3 generations reached sexual maturity, reproductive performance was assessed by use of a 21-day short-term reproduction assay where fecundity and fertility were measured daily. Additionally, embryotoxicity was assessed during the final week of each reproduction assay. Upon termination of each assay, molecular mechanism(s) of effect were investigated using an enzyme-linked immunoassay (ELISA) to measure concentrations of 17β-estradiol (E2) in blood plasma, and quantitative real-time PCR was performed to quantify expression of genes important in the hypothalamus-pituitary-gonadal-liver (HPGL) axis that regulate reproduction. Concentrations of TBCO in F1 generation eggs were determined by use of gas chromatography mass spectrometry (GC/MS) to assess maternal transfer.

Concentrations of TBCO in eggs (F1 generation) from F0 fish given the low and high diets were 711.3 and 2535.5 ng/g wet weight, respectively, confirming maternal transfer of TBCO. Embryotoxicity was evident in the F1 generation, but not the F2 or F3 generation. Heart rate of the F1 generation was decreased by 13.8% and 16.9 % in embryos from F0 adults given the low and high TBCO diet, respectively. Incidences of swim bladder malformation and spinal curvature in F1 embryos from adults given the low and high TBCO diet were significantly increased to 13.10% and 26.92%, respectively. Cumulative fecundity of the F1 generation in the low and high treatments was reduced by 33.9% and 33.3%, respectively, compared to the control. In the F2 generation, cumulative fecundity of the low treatment returned to the level of the controls, but

cumulative fecundity in the high treatment was decreased by 29.8%. Interestingly, recovery of fecundity was concentration dependent. There was no decrease in cumulative fecundity in the F3 generation compared to controls. Mechanistically, mRNA abundances of several genes that are regulators of sex hormone synthesis, specifically cholesterol side chain cleavage enzyme (*cyp11a*), aromatase (*cyp19a*), and luteinizing hormone receptor (*lhr*), were differentially expressed in gonads from F1 females. However, these changes were not consistent with observed decreases in fecundity. Additionally, concentrations of E2 in plasma and mRNA abundances of vitellogenin (*vtg*) in liver were decreased but the effects were small and not statistically significant compared to controls. No effects on mRNA abundances of target genes or concentrations of E2 were observed in the F2 or F3 generation. Overall, findings from this study suggest TBCO is maternally deposited into eggs, causes developmental toxicities, and multigenerational, but not transgenerational, decreases in fecundity of female Japanese medaka.

17β-estradiol regulates fish reproduction as it signals the synthesis of VTG which is required for oocyte growth (Hannon & Flaws, 2015). In this study, decreased fecundity is unlikely to have been caused by developmental reprogramming of steroidogenesis and/or impairment of vitellogenesis as larger decreases in E2 and expression of VTG than those that were observed are required to decrease fecundity (Ankley et al., 2010). While one of the most investigated mechanisms of female reproductive impairment is disruption of steroidogenesis and vitellogenesis, inhibition of oocyte maturation, the process by which an immature oocyte matures into a fertilizable egg, is an understudied mechanism by which anthropogenic chemicals can impair reproduction in fish. Anthropogenic chemicals have been demonstrated to disrupt oocyte maturation in several fish species (Carnevali et al., 2010; Haider et al., 1988; Maskey et al., 2019; Miller et al., 2022; Tokumoto et al., 2005, 2007). Notably, dietary exposure of zebrafish to TBCO

has been shown to impair oocyte maturation (Van Essen et al., 2021b). Additionally, in Japanese medaka embryos exposed to TBCO, abundances of proteins that regulate oocyte meiosis were significantly decreased (Sun et al., 2016).

4.3 Summary of study #2

Based on findings from the first study, the goal of the second study (Chapter 3) was to determine if impaired oocyte maturation is responsible for the observed decrease in fecundity following early-life stage (ELS) exposure to TBCO via maternal transfer. Sexually mature fish (F0) were fed either a control diet or a low (100 μg/g) or high (1000 μg/g) diet containing TBCO for 21 days. Eggs (F1) were collected during the final week of exposure and reared to sexual maturity at which point fecundity was assessed using a 21-day reproduction assay. Upon termination of the assay, an *ex vivo* oocyte maturation assay was used to determine whether maturation inducing hormone (MIH)-stimulated oocyte maturation was impaired. Additionally, expression of genes involved in oocyte maturation was quantified via qPCR.

The F1 generation experienced a 26.0% and 56.8% decrease in cumulative fecundity confirming effects observed in the first study (Chapter 2) that exposure to TBCO at ELS impairs reproduction. *Ex vivo* MIH-stimulated maturation of stage IX oocytes from females in the low and high TBCO treatments were decreased by 23.4% and 20.0% respectively. Transcript abundances of membrane progestin receptor α (*mPRα*), progesterone receptor membrane component 1 (*pgrmc1*), progesterone receptor membrane component 2 (*pgrmc2*), and insulin-like growth factor 3 (*igf3*) were decreased in a concentration dependent manner but effects were not statistically significant, potentially due to low sample size. Transcript abundances of *vtgI*, but not *vtgII*, was significantly decreased in a concentration dependent manner. However, it is unlikely that this change is responsible for the observed decrease in fecundity as decreases in *vtgI* expression of

similar magnitude have not caused impairments in fecundity in previous studies (Doering et al., 2019). Taken together, these results suggest that impaired oocyte maturation is likely the mechanism causing decreases in fecundity of adult Japanese medaka exposed as embryos to maternally deposited TBCO. The mechanism by which exposure to maternally deposited TBCO leads to impaired oocyte maturation is not clear. One hypothesis is that TBCO is antagonizing the mPR preventing MIH from binding and triggering oocyte maturation. However, this is unlikely given that reproduction assays were performed approximately 4 months following exposure of embryos to maternally deposited TBCO. Nevertheless, this possibility cannot be ruled out as chemical analysis of F1 adult fish was not performed. Another hypothesis is that exposure to maternally deposited TBCO caused alterations to the epigenome, leading to decreases in expression of *mPRα*, *pgrmc1*, *pgrmc2*, and *igf3* which are critical regulators of oocyte maturation, therefore lessening the response to MIH.

4.4 The epigenome as a target of TBCO

There is growing recognition within the field of ecotoxicology that the epigenome can be disrupted by anthropogenic chemicals, and that some alterations can be inherited in generations of progeny (Head at al., 2012). While most are familiar with the concept of passing down of one's genetic makeup, less are familiar with the idea that a parent's environment and experiences during early life can be inherited in the form of chemical marks on DNA – an idea which is becoming more prevalent (Head at al., 2012). Early-life stage exposure to contaminants can affect the health of future generations when epigenetic alterations such as DNA methylation occur in the germ line (Head, 2014). This is one possible explanation for the decrease in fecundity that was observed in F2 high treatment individuals in the first study. MicroRNA mediated gene silencing, histone modification, and alterations in DNA methylation are potential epigenetic mechanisms by which

maternally deposited TBCO caused decreased expression of genes critical for oocyte maturation in sexually mature females, resulting in impairment of oocyte maturation.

MicroRNAs (miRNAs) are small RNA molecules produced from endogenous precursor molecules that induce post-transcriptional gene silencing (Carthew & Sontheimer, 2009). For this to occur, part of the miRNA known as the "seed" base-pairs with the target mRNA allowing for mRNAs to be targeted for degradation (Chu & Rana, 2007). miRNA is incorporated into an RNA-induced silencing complex (RISC) which can either block the ribosome subunit from binding or facilitates degradation of the target mRNA, in either case translation is prevented thus impairing gene expression (Chu & Rana, 2007). Toxicant exposure can alter expression of miRNAs (Lema & Cunningham, 2010). For example, early-life stage red drum (*Sciaenops ocellatus*) exposed to crude oil experienced neuro-cardio toxicity because of abnormal expression of microRNAs (miR-18a, miR-27b, and miR-203a) and associated target genes (Xu et al., 2019). Additionally, in stage IV oocytes of zebrafish exposed to dietary TBCO, abundances of microRNAs that regulate expression of proteins involved in oocyte maturation were significantly different from control treatments (Van Essen et al., 2021b). The role of miRNAs in teleost oogenesis has not been extensively studied, but there is growing evidence that miRNAs regulate oocyte growth and maturation as they have been shown to be differentially expressed between oocyte stages (Bizuayehu & Babiak, 2014).

Association of chromosomal DNA with histones is another important regulator of gene expression (Annunziato, 2008). When the structure of this DNA-histone complex (chromatin) is closed, transcription is repressed whereas an open chromatin structure favours transcription (Annunziato, 2008). Post-translational modifications in chromatin structure such as histone acetylation, phosphorylation, and methylation are frequently associated with changes in gene

expression (Imhof, 2006; Bannister & Kouzarides, 2011). In the case of histone acetylation, histone acetyltransferases (HATs) catalyze the transfer of an acetyl group from the cofactor acetyl CoA to the amino group of a lysine side chain on the histone tail (Bannister & Kouzarides 2011; Imhof, 2006). When this occurs, the lysine's positive charge is masked, weakening the electrostatic attraction between the histone and the negatively charged DNA backbone causing the chromatin to adopt a more open conformation (Imhof, 2006). During histone phosphorylation, a phosphate group is transferred from ATP to the hydroxyl group of an amino-acid side chain (Bannister & Kouzarides, 2011). This similarly adds negative charge decreasing the electrostatic attraction between the histone and the DNA backbone (Bannister & Kouzarides, 2011). Histone modification can occur as a response to chemical exposure. For example, European sea bass (*Dicentrarchus labrax*) exposed to dietary butyrate experienced a two-fold increase in the acetylation level of histone H4 at lysine 8 as well as differential expression of genes involved in inflammatory response (Terrova et al., 2016). However, in the present study, mRNA abundances of mPR*α*, *pgrmc1*, *pgrmc2*, and *igf3* were decreased in ovaries of fish exposed to maternally deposited TBCO. This suggests that chromatin structure is unlikely to have adopted a more open conformation. As such histone methylation, where methyl groups are added to histones and prevent transcriptional machinery from accessing DNA, is a more likely mechanism for the observed decreases in gene expression (Bannister & Kouzarides, 2011).

Like histone methylation, DNA methylation can influence gene transcription. As previously mentioned, DNA methylation is the most studied epigenetic mechanism in the field of ecotoxicology (Head, 2014). Methylation occurs primarily on cytosine residues connected to a guanine (CpG) which decreases access of key proteins which initiate gene expression (Head, 2012). DNA methyltransferase (DNMT) enzymes add methyl groups to the carbon 5 position of

the cytosine ring (Singal & Ginder, 1999). Methylation is typically absent in CpG-rich regions (CpG islands) near the 5' end of the DNA strand where gene promoters are often located (Dahl & Guldberg, 2003). Transcriptional silencing is correlated with the methylation level within the CpG island of a gene's promoter (Dahl & Guldberg, 2003). While DNA methylation can either increase or decrease gene expression, expression is usually decreased in heavily methylated genes (Head, 2012). In Japanese medaka, the methylome undergoes extensive reprogramming following fertilization where previous epigenetic marks transferred through gametes are erased and new marks become established (Wang & Bhandari, 2019). Japanese medaka are particularly sensitive during this time as chemical exposure can interfere with methylome reprogramming. Decreased oocyte maturation in female medaka exposed to maternally deposited TBCO might have been caused by disruption of this reprogramming resulting in hypermethylation of genes involved in oocyte maturation therefore decreasing their expression.

Future studies should investigate DNA methylation patterns of genes involved in oocyte growth and maturation in fish exposed to TBCO via maternal transfer. To explore this, genome wide DNA methylation analysis or gene-specific DNA methylation analysis could be conducted. As the present study showed changes in expression of genes involved in oocyte maturation, methylation-specific PCR could be used to compare the methylation status of *mPRα*, *pgrmc1*, *pgrmc2*, and *igf3* within the gonads of control and exposed fish (Dahl & Guldberg, 2003). Methylation-specific PCR discriminates between methylated and unmethylated DNA. To do so, DNA must first undergo bisulphite-conversion whereby sodium bisulphite deaminates unmethylated cytosine residues to uracil while 5-methylcytosine residues remain unaffected (Dahl & Guldberg, 2003). Bisulphite-treated DNA can then be used directly as a template in a standard PCR (Dahl & Guldberg, 2003). While the genes examined in this study are likely candidates for

changes in DNA methylation, whole genome bisulphite sequencing of gonads of from control and exposed fish could be useful to identify other genes that might be differentially methylated, and that might contribute to decreased oocyte maturation. As this study shows that TBCO causes multigenerational but not transgenerational effects on the reproduction of Japanese medaka, it is unlikely that any epigenetic effects such as DNA methylation are meiotically inherited.

4.5 An adverse outcome pathway for inhibition of oocyte maturation leading to decreased fecundity

The field of ecotoxicology is moving towards the development of alternative testing and predictive tools as a means on increasing the efficiency of toxicity testing while decreasing the costs and use of animals in research (Zhang et al., 2014). One such tool, adverse outcome pathways (AOPs), have been developed to link molecular disruption of a biological system with adverse effects relevant to risk assessment (Villeneuve et al., 2014). In an AOP, adverse effects are predicted following chemical exposure beginning with an interaction between the chemical and a biomolecule, known as a molecular initiating event (MIE), followed by a series of key events (KEs) that occur at the cellular level to produce an adverse outcome (AO) that can have population level effects (Ankley et al., 2010; Villeneuve et al., 2014; **Figure 4.1**). Top-down AOP development where adverse phenotypic effects are observed and researchers work backwards to determine underlying mechanisms of effects is common (Villeneuve et al., 2014). Adverse outcome pathways are never chemical specific as the underlying assumption is that any stressor that triggers the MIE can elicit the chain of downstream effects (Villeneuve et al., 2014).

Networks of AOPs can be formed where MIEs, KEs, and AOs are shared. Expanded adverse outcome networks (AONs) are most likely to be representative of real-world scenarios as

organisms are often exposed to complex mixtures rather than individual chemicals (Villeneuve et al., 2014; **Figure 4.1**). As such, AONs are often extended to regulatory policies (Villeneuve et al., 2014). Both AOPs and AONs exist in three main stages: putative, formal, and quantitative (Vinken et al., 2017). While AOPs can advise chemical regulatory policies at any level of development, those most often applied to risk assessment are quantitative adverse outcome pathways (qAOPs; Vinken et al., 2017). In this case, the probability or severity of an AO can be predicted based on the magnitude of change in an upstream KE such as an MIE (Doering et al., 2019). As such, risk assessment using qAOPs can be conducted in such a way that dose-response and time-course predictions are made and policies are created based on these relationships (Conolly et al., 2017).

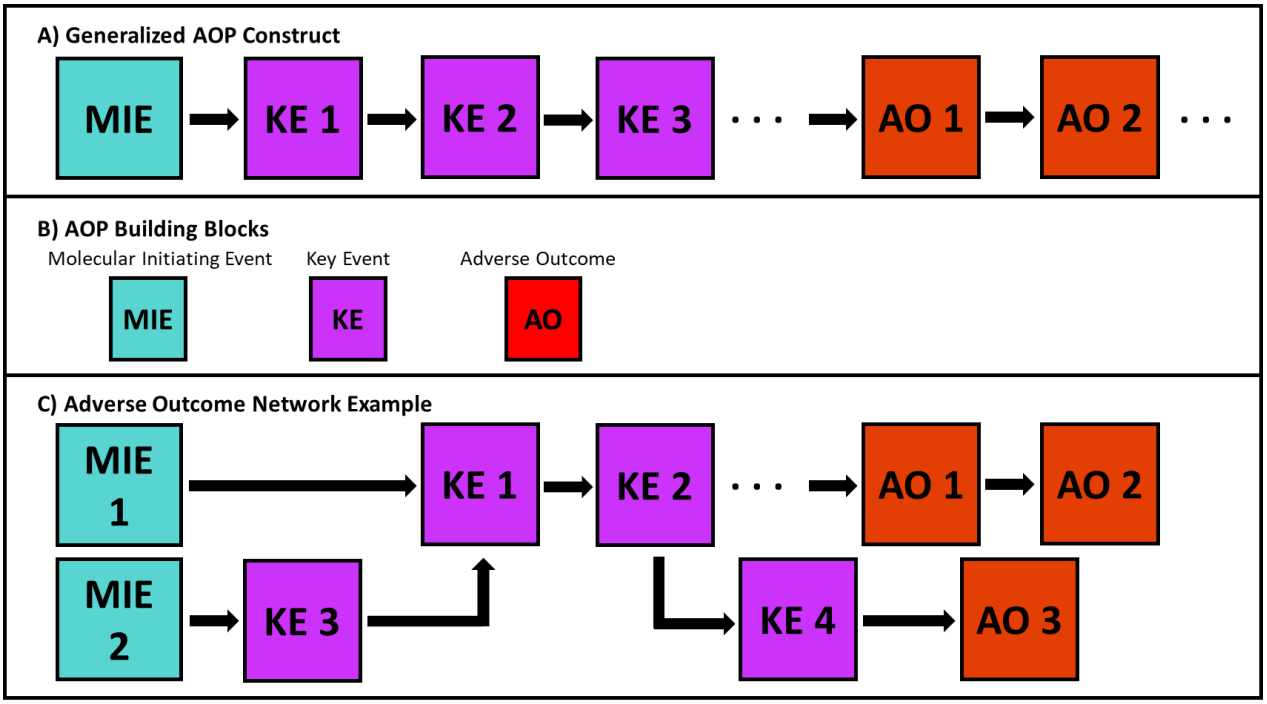

Figure 4.1 Illustration of a generalized adverse outcome pathway (AOP) adapted from Villeneuve et al. (2014; **A**). All AOPs are composed of three components: at least one molecular initiating event (MIE), at least one key event (KE), and at least one adverse outcome (AO; **B**). Individual AOPs can share MIEs, KEs, and AOs forming adverse outcome networks (AONs; **C**). The depicted AON is comprised of three individual AOPs.

While AOPs have been developed for disruption of steroidogenesis and vitellogenesis resulting in reduced fecundity, no AOP has been proposed for impaired oocyte maturation leading to reductions in fecundity (Ankley et al., 2010; Villeneuve et al., 2014). However, this study provides evidence that impaired oocyte maturation can result in adverse outcomes at the level of fecundity. In the following putative AON (**Figure 4.2**), it is hypothesized that TBCO exposure led to altered DNA methyltransferase activity causing hypermethylation of genes involved in oocyte maturation (*mPRα*, *pgrmc1*, *pgrmc2*, and *igf3*) and therefore decreased expression resulting in impaired oocyte maturation. However, the MIE leading to reproductive impairment as well as whether genes were hypermethylated should be investigated. As the *in vitro* oocyte maturation assay used for the second study (Chapter 3) was reflective of reductions in fecundity as well as concentration-dependent decreases in expression of genes involved in oocyte maturation, there is potential for this assay to be utilized as a predictive tool for the screening of endocrine disrupting chemicals that could cause decreases in fecundity due to impaired oocyte maturation in Japanese medaka.

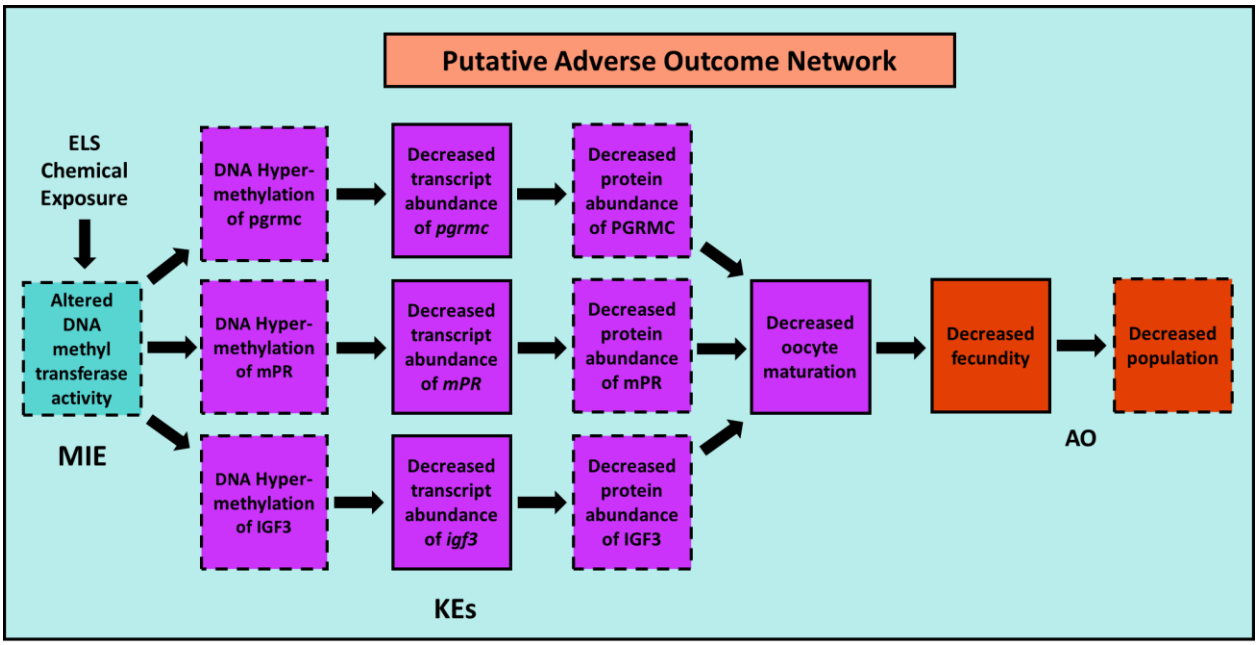

Figure 4.2 A putative adverse outcome network (AON) for decreased fecundity resulting from impairment of oocyte maturation. In this network, the molecular initiating event (MIE) is predicted to be altered DNA methyltransferase activity resulting in hypermethylation of genes involved in oocyte maturation (*mPRα*, *pgrmc1*, *pgrmc2*, and *igf3*) following ELS chemical exposure. Hypermethylation of the genes would then result in a series of key events including repressed gene transcription leading to decreased protein abundance resulting in impaired MIH stimulated oocyte maturation. As such fewer oocytes will mature to eggs thus causing a reduction in fecundity. Solid boxes represent aspects of the pathway confirmed by study results, while dashed boxes represent aspects of the pathway that remain to be investigated.

4.6 Conclusions

This study ultimately expands our understanding of the potential threats of TBCO usage and could be useful for future risk assessment. Concentrations of TBCO in the environment are low, with the highest environmental concentration reported being 1.2 ng/g dry weight in the German Bight (Sühring et al., 2016). However, environmental concentrations are likely to increase as usage increases. Identifying the potential adverse effects of TBCO is crucial to understanding whether the chemical might be a threat to fishes. This research provides additional evidence that TBCO could be a threat to the health of fish. Exposure to waterborne TBCO causes toxicity in ELS fishes as demonstrated by visual impairment, reduced heart rate, spinal curvature, swim bladder malformation, and increased mortality (Sun et al., 2016; Van Essen et al., 2021a). In the present study, exposure to TBCO via maternal transfer induced similar developmental effects such as reduced heart rate and increased incidences of spinal curvature. Several studies have demonstrated that TBCO is as an EDC that can disrupt synthesis of sex steroids and impair estrogen and androgen receptor signalling (Saunders et al., 2013; Saunders et al., 2015; Mankidy et al., 2014). Additionally, dietary exposure to TBCO has been demonstrated to reduce fecundity of adult Japanese medaka (Saunders et al., 2015). Results from the present study further demonstrate the potential for TBCO to impair reproductive performance of fishes. Maternally transferred TBCO caused multigenerational reductions in fecundity of Japanese medaka. In the F1 generation, decreased fecundity was caused by impaired oocyte maturation. Future studies should investigate potential effects on oocyte maturation in the F2 generation. Additionally, endocrine disrupting effects at the molecular and/or organismal level often result in reduced population size (Brander et al., 2016). Results suggest that if TBCO were to induce multigenerational effects in a fish species, it could lead to potential population collapse resulting in possible trophic cascades that could disturb an entire ecosystem.

This research also demonstrates that impaired oocyte maturation is a mechanism by which fecundity can be decreased in fish exposed to EDCs during critical stages of embryogenesis. Over 1,000 fish species are native to Canadian freshwater and marine systems, with countless Canadians making a living directly or indirectly from fishing and related activities. As a model fish species, Japanese medaka have the potential to signal effects that toxicants could induce in wild fish species. 1,2,5,6-tetrabromocyclooctane could be used as a model chemical to investigate oocyte maturation as a mechanism of impaired reproductive success in other species.

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