

**EMBRYONIC EXPOSURE TO THE FLAME RETARDANT, 1,2,5,6-TETRABROMOCYCLOOCTANE (TBCO) INHIBITS MIH-INDUCED OOCYTE MATURATION AND ALTERS GENE EXPRESSION AND DNA METHYLATION IN ADULT JAPANESE MEDAKA (*ORYZIAS LATIPES*)**

**SODIQ AREMU OLAWOORE**  
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## **DEDICATION**

I dedicate this thesis to the Japanese medaka, whose lives were sacrificed for this study.

## ABSTRACT

Oocyte maturation, the final stage of oogenesis, occurs when maturation-inducing hormone (MIH) binds to its membrane progesterin receptor (mPR). A previous study demonstrated that reduced fecundity of female Japanese medaka (*Oryzias latipes*) exposed as embryos to maternally transferred 1,2,5,6-Tetrabromocyclooctane (TBCO), an endocrine-disrupting chemical, was caused by impaired MIH-induced oocyte maturation. However, the molecular mechanism(s) of this effect are unknown. The objective of the present study was to investigate the molecular basis of decreased oocyte maturation leading to decreased fecundity in Japanese medaka exposed as embryos to maternally deposited TBCO. Sexually mature F0 females were fed 100 or 1000  $\mu\text{g}$  TBCO/g diet for 21 days, and F1 embryos were reared to adulthood in clean water. F1 fecundity was decreased by 11.3% and 31.4% relative to the control. Using an *ex vivo* assay, MIH-stimulated maturation of fully grown stage IX oocytes to stage X matured oocytes was decreased by 16.1% and 22.3% relative to the control. To investigate the mechanistic underpinnings of this decreased oocyte maturation, mRNA sequencing and enzymatic methyl sequencing (EM-Seq) were used to compare the transcriptome and methylome of stage IX oocytes that matured to stage IX oocytes that failed to mature, from control and TBCO-exposed females. Pathway analysis revealed enrichment of the MAPK, insulin, GnRH, and oocyte-meiosis signalling pathways that are known to regulate oocyte maturation. Several genes that encode proteins in the AC/cAMP/PKA and PI3K/AKT signaling pathways had either gene body hypomethylation or altered transcript abundance. These changes likely explain the impaired MIH-induced oocyte maturation, leading to decreased fecundity, in medaka exposed to maternally transferred TBCO. This study provides novel insight into how early life exposure to endocrine-disrupting chemicals can lead to altered later life reproductive performance of fish.

## **ETHICS**

Japanese medaka were maintained following University of Lethbridge Animal Welfare Protocol #2023.

## **USE OF GENERATIVE AI**

ChatGPT and Google Gemini AI was used to search for academic literature used in chapters 1-3 of this thesis.

## PREFACE

The candidate is the main author of chapters 1-3. The Candidate primarily designed and conducted experiments and analyzed data for chapters 2. Dr. Steve Wiseman provided scientific input and guidance of chapters 1-3.

Chapter 2 will be submitted to the journal, Environmental Epigenetics, as Olawoore, S & Wiseman, S. (2026). “Embryonic exposure to the flame retardant, 1,2,5,6-tetrabromocyclooctane (TBCO) inhibited MIH-induced oocyte maturation and altered gene expression and DNA methylation in adult Japanese medaka (*Oryzias latipes*)”.

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## LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AMP	Adenosine monophosphate
AHR	Aryl hydrocarbon receptor
AR	Androgen receptor
BPA	Bisphenol A
cAMP	Cyclic adenosine monophosphate
CDC2	Maturation promoting factor catalytic unit
CDK1	Cyclin-dependent kinase 1
CYP11a	Cytochrome P450 cholesterol side-chain cleavage
CYP19a	Aromatase
E2	17 $\beta$ -estradiol- estrogen
EGFR	Epidermal growth factor receptor
Erk1/2	Extracellular-related kinase 1 and 2
F0	Parental generation
F1	First filial generation
FSH	Follicle-stimulating hormone
GPGR	G protein-coupled estrogen receptor
GnRH	Gonadotropin-releasing hormone
GS $\alpha$	G stimulatory protein $\alpha$
GS $\beta\gamma$	G stimulatory protein $\beta\gamma$ subunit
Gi $\alpha$	G inhibitory protein $\alpha$
Gi $\beta\gamma$	G inhibitory protein $\beta\gamma$ subunit
GVBD	Germinal vesicle breakdown
hCG	Human chronic gonadotropin
HPGL	Hypothalamus pituitary gonadal liver
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
LH	Luteinizing hormone
MIH	17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$ -DP)
MPF	Maturation promoting factor
MPR	Membrane progestin factor
PGRMC1	Progesterone receptor membrane component 1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5- bisphosphate
PIP3	Phosphatidylinositol 3,4,5- trisphosphate
PKA	Protein kinase A
PKAC	Protein kinase A catalytic unit
PKAR	Protein kinase A regulatory unit
VLDL	Very Low-Density Lipoprotein
VTG	Vitellogenin
wm	Wet mass

Throughout this thesis, gene names are given in uppercase italics, mRNA is reported as lower-case italics, and protein are upper case non-italicized.

## CHAPTER 1: LITERATURE REVIEW

### 1.1 Oogenesis

Successful reproduction by female fish is dependent on the coordinated actions of a plethora of molecular and biochemical pathways. Several of these pathways are required for oogenesis, the multistep process that results in development of a fertilizable egg. Oogenesis can be divided into two general phases, oocyte growth and oocyte maturation (Tyler & Sumpter, 1996). Oogenesis involves hormone synthesis and signalling via the hypothalamus-pituitary gonadal (HPG) axis, that is conserved across vertebrates (Lubzens et al., 2010). The neuropeptide, kisspeptin, stimulates hypothalamic secretion of gonadotropin-releasing hormone to the anterior pituitary while mediating feedback by sex steroids (Somoza et al., 2020; Yun et al., 2014). Activation of gonadotropin-releasing hormone receptors stimulates the anterior pituitary to produce and release the major gonadotropin hormones, luteinizing hormone and follicle-stimulating hormone (Lubzens et al., 2010; Yaron & Sivan, 2011). Luteinizing hormone and follicle-stimulating hormone are released into the bloodstream and bind to their respective receptors located in the gonads, where they stimulate gonadal steroidogenesis and gametogenesis (Miguel-Queralt et al., 2004). The C18 steroid,  $17\beta$ -estradiol (E2), and the C21 steroid,  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17,20\beta$ -DP), also termed maturing inducing hormone (MIH), are two key sex steroids that regulate oocyte growth and maturation, respectively, in female fishes (Nagahama & Yamashita, 2008). Two types of MIH have been identified in fish, the dominant  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17,20\beta$ -DP) that is synthesised in most species, including Japanese medaka (*Oryzias latipes*) (Nagahama & Yamashita, 2008), and  $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one ( $17,20\beta,21$ -P) documented in Atlantic croaker (*Micropogonias undulatus*) (Trant et al., 1986). Both types have a similar role in oocyte maturation.

### **1.1.1 Oocyte growth**

The primary oocyte is an immature egg cell, optimized for transcription and maternal uptake of vitellogenin (Lubzens et al., 2010). During oocyte growth, the oocyte undergoes massive growth through vitellogenesis that is stimulated by E2, which is synthesised from cholesterol in granulosa cells of the ovaries and transported to target tissues by sex hormone binding globulin (Miguel-Queralt et al., 2004). In the liver, E2 binds to estrogen receptors, which are ligand-activated transcription factors, triggering dimerization and binding to estrogen response elements in the promoter region of vitellogenin genes (Sullivan & Yilmaz, 2018). Following translation, vitellogenin is secreted into the blood and delivered to the ovaries where it enters the granulosa layer via theca capillaries and is deposited onto the oocyte surface and sequestered through receptor-mediated endocytosis (Lubzens et al., 2010; Nagahama & Yamashita, 2008). Throughout vitellogenesis, the oocyte maintains a state of meiotic arrest at prophase 1 (Thomas, 2017).

### **1.1.2 cAMP regulation of oocyte meiotic arrest**

Meiotic arrest of the primary oocyte at prophase 1 is regulated by elevated concentration of cyclic adenosine monophosphate (cAMP) within the oocyte. Concentration of cAMP increase when E2 binds to a G protein-coupled estrogen receptor (GPER), formerly known as GPR30, which signals via a G stimulatory protein consisting of  $GS\alpha$  subunit and a  $\beta\gamma$  heterodimer (Thomas, 2017). Activation of  $GS\alpha$  stimulates adenylyl cyclase activity that converts ATP into cAMP, which subsequently activates protein kinase A (PKA) (Takahashi & Ogiwara, 2023). The holoenzyme, PKA, is a serine/threonine kinase that consists of two regulatory subunits (PKARs), bound to two catalytic subunits (PKACs) (Das et al., 2017). Binding of a cAMP molecule to each PKAR induces a conformational change that leads to release of PKACs (Das et al., 2017).

Active PKA regulates multiple substrates to keep maturation promoting factor (MPF) in an inactive form, which prevents precocious oocyte maturation (Khan & Maitra, 2013).

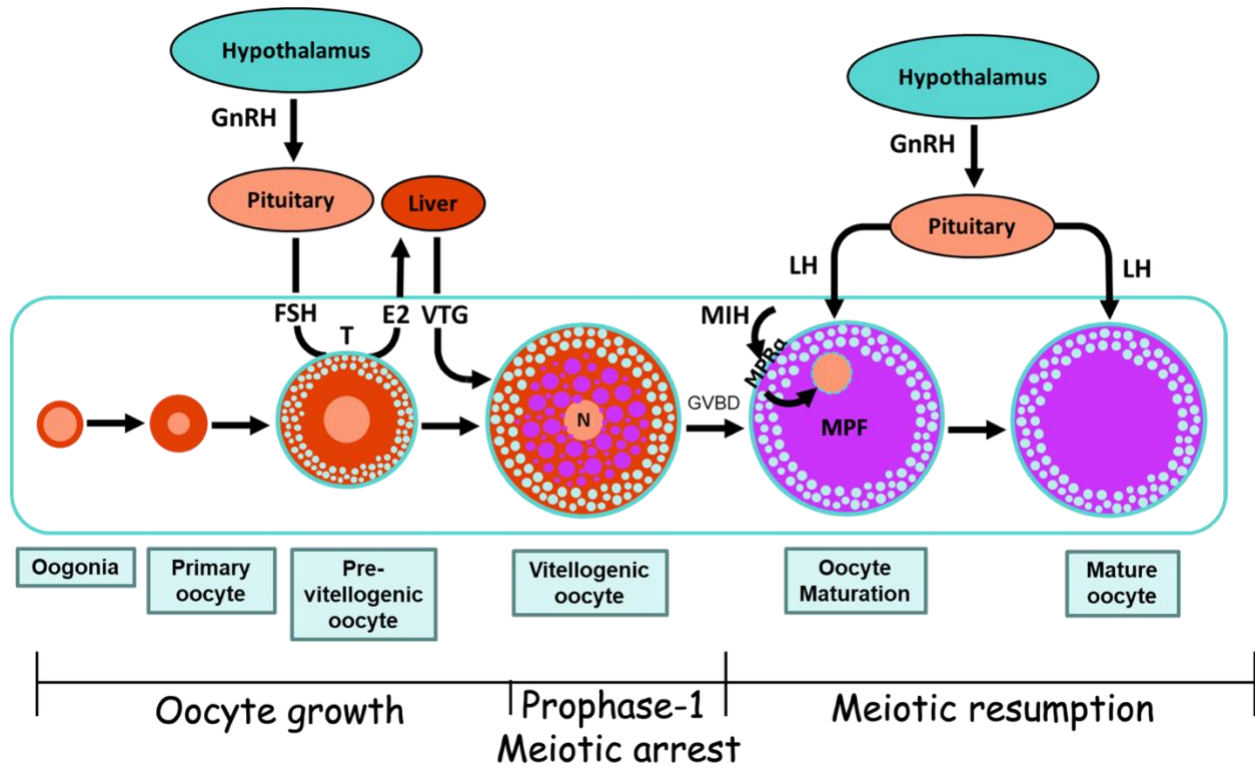
GPER acts as an intermediary to mediate E2 inhibition of oocyte maturation. In fish, GPER is mostly expressed on the oocyte surface and not in the follicle (Pang & Thomas, 2010). MIH-induced maturation was significantly decreased in zebrafish oocytes incubated with E2 or GPER agonist G-1 (Pang & Thomas, 2009). However, treatment with G-15, a GPER antagonist, led to spontaneous oocyte maturation in common carp (*Cyprinus carpio*) (Majumder et al., 2015). GPER antisense nucleotides injected into zebrafish oocytes attenuated the inhibitory effect of E2 on oocyte maturation via E2/GPER/cAMP signalling pathway (Pang et al., 2008). Overall, binding of E2 to GPER increases cAMP production, which leads to, and maintain oocyte arrest at prophase I during oocyte growth.

### **1.1.3 Oocyte maturation and cAMP regulation of meiotic resumption**

Oocyte maturation is characterized by the termination of vitellogenesis, meiotic resumption from prophase I to metaphase II, germinal vesicle breakdown, meiotic spindle formation and chromosome condensation, and first polar body formation (Nagahama & Yamashita, 2008). An overview of oogenesis, from oocyte growth to final maturation, is illustrated in Figure 1.1. This process requires a shift in the steroidogenic pathway, from the production of E2 to the production of the progestin, MIH, regulated by a surge in synthesis and release of luteinizing hormone from the anterior pituitary (Nagahama & Yamashita, 2008).

Oocyte maturation is divided into two stages: oocyte maturation competence and meiotic resumption (Picha et al., 2012). The potential of post-vitellogenin follicles to respond to MIH via upregulation of mPR $\alpha$  is characterized as oocyte maturation competence (Picha et al., 2012). Binding of MIH to mPR $\alpha$  on the oocyte surface leads to a decrease in cAMP that triggers

formation of MPF, a protein complex that contains a regulatory cyclin B unit and a catalytic cyclin-dependent kinase 1 (CDK1), also termed p34 CDC2 (Kotani & Yamashita, 2002). The decrease in intra-oocyte cAMP involves multiple intracellular, endocrine, and paracrine processes mediated by non-genomic actions stimulated by MIH. Binding of MIH to mPR $\alpha$  activates a pertussis toxin-sensitive inhibitory G protein (Gi), causing the G $\alpha$ i subunit to dissociate from the G- $\beta\gamma$ i dimer (Takahashi & Ogiwara, 2023). The G $\alpha$ i subunit inhibits adenyl cyclase activity, blocking cAMP production and thereby inhibiting PKA activity (Khan & Maitra, 2013). Inactivation of PKA lifts the inhibitory regulation of MPF, allowing resumption of meiosis. Several studies have demonstrated this mechanism. For example, PKA inhibitors led to MPF activation in the climbing perch (*Anabas testudineus*) and walking catfish (*Clarias batrachus*) (Haider & Baqri, 2002; Khan & Maitra, 2013). Catecholestrogen (2-hydroxyestradiol 17 $\beta$ , 2-OHE2), a GPER antagonist, induced oocyte maturation in zebrafish and Asian stinging catfish (*Heteropneustes fossilis*) by blocking E2-induced cAMP production (Chourasia et al., 2015; Mishra & Joy, 2006). Taken together, a decrease in intra-oocyte cAMP lifts the inhibitory actions of E2 on resumption of meiosis 1.



**Figure 1.1.** Schematic of the general process of oogenesis in teleost fish. Gonadotropin-releasing hormone (GnRH) from the brain binds to GnRH receptors in the anterior pituitary, which leads to release of follicle stimulating hormone (FSH) to the bloodstream promoting oocyte growth. Testosterone (T) is converted to  $17\beta$ -estradiol (E2), which is transported to the liver via the bloodstream, where it binds to E2 receptors initiating the expression of vitellogenin (VTG), that is released into the bloodstream and incorporated into the growing oocyte. The oocyte is arrested at meiotic prophase-1 while growing. Following oocyte growth, GnRH signals the release of luteinizing hormone (LH) from the anterior pituitary, which promotes maturation of oocytes. Binding of LH to the LH receptor stimulates synthesis of maturation-inducing hormone ( $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; MIH) which binds to the membrane progesterin receptor ( $mPR\alpha$ ) on the oocyte surface to generate maturation-promoting factor (MPF). Finally, MPF signals resumption of meiosis and causes germinal vesicle breakdown (GVBD) resulting in a mature, fertilizable oocyte. This figure is taken from (Devoy, 2023) with author permission.

## 1.2 Membrane progesterin receptors

The mPRs mediate progesterin activation of non-genomic signalling, that can lead to a genomic response required for oocyte maturation. These receptors attain their final 3-dimensional conformation in the rough endoplasmic reticulum and undergo post translational chemical modification before being localized to the plasma membrane (Thomas et al., 2023).

mPRs belong to the progestin adipoQ receptor (PAQR) family, which include five mPR subtypes in vertebrates: mPR $\alpha$  (PAQR7), mPR $\beta$  (PAQR8), mPR $\gamma$  (PAQR5), mPR $\delta$  (PAQR6), and mPR $\epsilon$  (PAQR9) (Thomas, 2022). Co-immunoprecipitation studies and G protein inhibition studies showed that mPR $\alpha$ , mPR $\beta$ , and mPR $\gamma$  are coupled to inhibitory G (Gi) proteins, while mPR $\delta$  and mPR $\epsilon$  are coupled to the stimulatory G (Gs) proteins (Kelder et al., 2022). Among these isoforms, mPR $\alpha$  and mPR $\beta$  regulate oocyte maturation in vertebrates, by binding MIH (Josefsberg Ben-Yehoshua et al., 2007; Thomas et al., 2023). Studies by Zhu et al. (2003) and Tokumoto et al. (2006) showed that mPR $\alpha$  antisense oligonucleotide microinjected into zebrafish and goldfish (*Carassius auratus*) inhibited MIH-induced oocyte maturation. Impairment of oocyte maturation also occurred from the injection of mPR $\beta$  antisense oligonucleotides in zebrafish (Thomas et al., 2004). mPR $\beta$  appears to be more the dominant MIH receptor in amphibians (*Xenopus laevis*), while mPR $\alpha$  has been characterized as the dominant mPR subtype in reproductive tissue of many fish, including Japanese medaka (Josefsberg Ben-Yehoshua et al., 2007; Nagahama & Yamashita, 2008). The progesterone receptor membrane component (PGRMC1), a single transmembrane heme binding protein containing a cytochrome b5 motif, acts as a chaperone and adapter protein that forms a membrane receptor complex with mPR $\alpha$  aiding proper localization to the oocyte surface (Aizen et al., 2018; Thomas, 2022). In addition to the non-genomic interaction between MIH and mPR $\alpha$  that initiate oocyte maturation, other factors have also been documented to aid the regulation of this process.

### **1.3 Autocrine and paracrine factors regulating oocyte maturation**

In addition to endocrine regulation by E2 and MIH, oocyte maturation is regulated by autocrine or paracrine factors. Growth factors such as the insulin-like growth factors (IGFs), which belong to the insulin peptide family, act as autocrine or paracrine factors in the gonad to

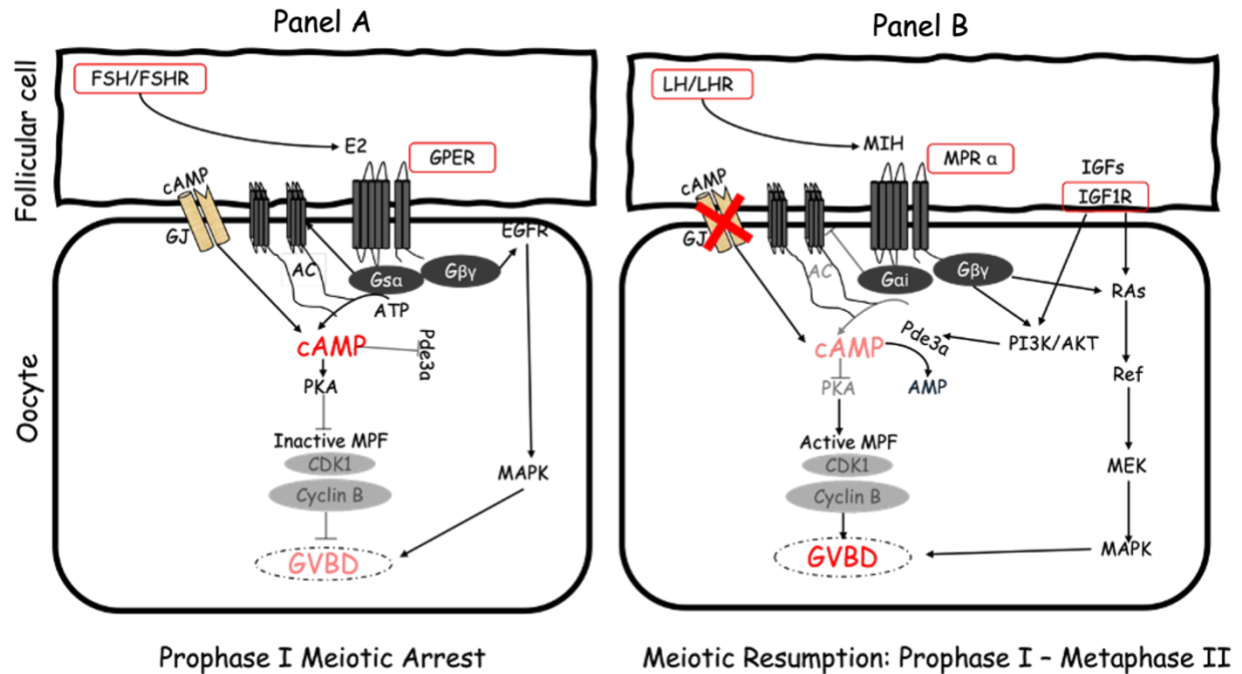
promote oocyte maturation in a manner that can act synergistic or parallel to MIH (Das et al., 2016a). IGFs are documented in most fish species, and their actions are mediated by IGF1 receptors (IGF1R) (Irwin & Van Der Kraak, 2012). In zebrafish, induction of oocyte maturation by insulin is partially mediated by IGF1R through a pathway that is parallel to MIH signaling, likely by upregulating expression of mPRs and 20 $\beta$ -hydroxy-steroid dehydrogenase proteins (Das et al., 2013; Das et al., 2016b). Ovarian IGFs can activate two downstream signalling cascades via IGF1R, the phosphatidylinositol-3-kinase PI3K/AKT signaling pathway and the extracellular-signal regulated kinase RAS/ERK signaling pathway (Das et al., 2016a).

Oocyte maturation can be stimulated by activation of the P13K/AKT pathway. Activation of PI3K occurs by converting phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>), that activates the serine/threonine kinase AKT to phosphorylate oocyte specific phosphodiesterase, PDE3a, that mediates insulin/IGF1 induced oocyte maturation by hydrolyzing cAMP to AMP (Das & Arur, 2017; Das et al., 2013). Human insulin-like growth factor-I (IGF-I) induced GVBD in Common Carp (*Cyprinus carpio*) oocytes independent of MIH action (Paul et al., 2009). Interestingly, inhibition of PI3K does not block MIH-induced oocyte maturation and can only delay it by few hours in zebrafish (Das et al., 2013). Although MIH stimulates the PI3K/AKT/PDE3a cascade, stimulation by IGF triggers a rapid activation (Das et al., 2016a). Oocyte maturation can also be stimulated by activation of Ras–Raf–MEK–ERK1/2 (MAPK3/1) pathway. The MAPK extracellular signal regulated kinases ERK1/2 are rapidly activated in response to various growth factors and steroid hormones (Das et al., 2018). Haccard and Jessup (2006) showed that, in xenopus, activation of the CDC2 catalytic unit of MPF requires either the MOS /ERK pathway or cyclin B synthesis to induce oocyte maturation. However, in fish, the MOS/ERK pathway is not required for progesterone induced

oocyte maturation, as shown in goldfish and Atlantic croaker (Kajiura-Kobayashi et al., 2000; Pace & Thomas, 2005). Insulin induced-oocyte maturation via ERK 1/2 signalling in zebrafish and common carp is activated within 30-90 min of insulin/IGF1R stimulation (Das & Arur, 2017; Paul et al., 2009). The MEK/MAPK pathway precedes MPF activation and GVBD (Maitra et al., 2014). MEK inhibition delays insulin stimulated GVBD in zebrafish (Maitra et al., 2014), however, it completely blocks insulin/IGF1-induced oocyte maturation in carp (Paul et al., 2009).

The PI3K/AKT and RAS/ERK signaling pathways are contributory but not obligatory for MIH-induced oocyte maturation (Das et al., 2013; Khan & Maitra, 2013). MAPK is not necessary for activation of CDC2 induction of GVBD in IGF-I or insulin-stimulated oocyte maturation in carp (Paul et al., 2009). MIH-induced PI3k/AKT and MAPK/ERK activation precedes the onset of GVBD in cAMP sensitive manner and plays a role in downregulating PKA, possibly by stimulating PDE3a in zebrafish (Das et al., 2018). Collectively, activation of IGFR can activate the Ras/Raf/MEK /ERK pathway in parallel to PI3K/Akt pathway in fish, to resume meiosis.

Other growth factors such as member of the transforming growth factor Beta (TGF $\beta$ ) family also regulate oocyte maturation. Inhibin A and activin A in zebrafish oocytes increased GVBD in a concentration-dependent manner, however, the activin-binding protein, follistatin, blocked human chorionic gonadotropin induced oocyte maturation (Li & Ge, 2013; Wu et al., 2000). Tan et al. (2009) showed that TGF- $\beta$  and bone morphogenetic protein (BMP-15) represses activin-induced oocyte maturation in zebrafish follicles, likely by regulating expression of mPR $\alpha$ . Taken together, growth factors may function either as auxiliary pathways or exert an additive effect, rather than being strictly required for the optimal induction of GVBD.

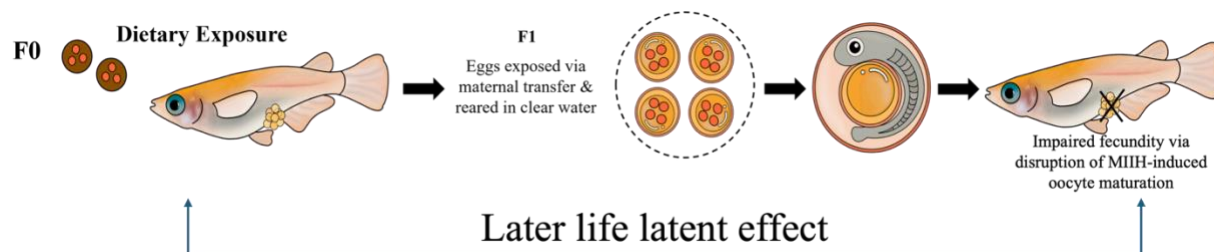


**Figure 1.2.** Schematic of the general process of oocyte meiotic arrest and resumption. Panel A:  $17\beta$ -estradiol (E2) synthesized under the regulation of follicle stimulating hormone (FSH) binds to G protein-coupled estrogen receptor (GPER) to mediate prophase-I meiotic arrest. Binding of E2 to GPER activates the G stimulatory protein  $\alpha$  ( $G_{s\alpha}$ ) subunit, which stimulates adenylyl cyclase (AC) to convert ATP into cAMP. Additional cAMP produced in the follicle is transported into the oocyte via gap junctions (GJ). Elevated levels of cAMP inhibit phosphodiesterase 3A (PDE3A) and activates protein kinase A (PKA) which leads to the inhibitory effect of maturation promoting factor (MPF) on oocyte maturation. Transactivation of the epidermal growth receptor (EGFR) via G protein  $\beta\gamma$  subunit also aids oocyte meiotic arrest via downstream MAPK signalling. Panel B: Maturation inducing hormone (MIH), under luteinizing hormone (LH) regulation, binds to  $mPR\alpha$  to activate the pertussis toxin-sensitive inhibitory G protein ( $G_i$ ). The  $G_{\alpha i}$  subunit dissociates from the G- $\beta\gamma$  dimer and inhibits (AC), blocking cAMP production. Closing of the gap junction blocks diffusion of additional cAMP into the oocyte. This decrease in cAMP leads to activation of PDE3a and inhibition of PKA activity. Inactive PKA lifts the inhibitory regulation of MPF, and meiosis resumes. Parallel activation of insulin-like growth factor (IGF) signalling via insulin like growth factor receptor (IGFR) aids oocyte maturation via downstream MAPK/ERK and PI3K/AKT signalling.

#### 1.4 Impairment of oocyte maturation by endocrine disrupting chemicals

Many natural and synthetic chemicals can impair endocrine processes in fish. These chemicals, termed endocrine-disrupting chemicals (EDC), disrupt the synthesis, transport, actions, and elimination of hormones (Carnevali et al., 2018). A major concern is the ability of

EDCs to impair the reproductive performance of fish. For female fish, this presents as a decrease in the number of eggs laid, which is termed fecundity. Most studies on reproductive impairment in fishes focus on disruption of vitellogenesis leading to reduced fecundity, which has mechanistically been linked to inhibition of E2 synthesis and ER signalling (Chen et al., 2016; Doering et al., 2019). However, there has been little focus on effects of EDCs on oocyte maturation. Exposure of zebrafish oocytes *in vitro* to pentachlorophenol (PCP), an organochlorine pesticide and disinfectant, inhibited MIH-induced oocyte maturation (Tokumoto et al., 2005). Additionally, exposure of zebrafish oocytes *in vitro* to various pesticides led to a decrease in human chorionic gonadotropin induced oocyte maturation (Maskey et al., 2019). Furthermore, *in vitro* exposure of zebrafish oocytes to the insecticides, malathion and dimethoate, inhibited MIH-induced oocyte maturation (Miller et al., 2022). There is some evidence that inhibition of oocyte maturation is a mechanism of decreased fecundity. Van Essen et al. (2021b) showed decreased maturation of oocytes from zebrafish exposed to the brominated flame retardant, 1,2,6,8-tetrabromocyclooctane (TBCO). Based on this, it was suggested that impaired oocyte maturation might be a mechanism of decreased fecundity observed previously in Japanese medaka exposed to TBCO (Saunders et al., 2015). In another study, TBCO decreased fecundity in Japanese medaka by a magnitude that was similar to decreased *in vitro* oocyte maturation (Raza et al., 2023).



**Figure 1.3.** Schematic of later life effects of embryonic exposure to TBCO via maternal transfer. Sexually mature Japanese medaka were fed a diet of TBCO. Maternal transfer of TBCO to eggs resulted in exposure of the F1 generation. Fecundity and MIH-stimulated oocyte maturation were decreased in sexually mature F1 females, despite being reared to sexual maturity in clean water, without re-exposure to TBCO. This figure is adapted from (Devoy, 2023) with author permission

### 1.5 Later life latent effects of EDCs

The Developmental Origins of Health and Disease (DOAHD) phenomenon suggests that early life experience can exert health influence that shows up in later in life. This scenario, where embryonic exposure to a stressor leads to a phenotypic change later in life without presence of the initial stressor can be referred to as intragenerational latent effect. It is becoming apparent that embryonic exposure to EDCs can lead to intragenerational effects on reproduction. Short term embryonic exposure to benzo(a)pyrene decreased fecundity of adult female zebrafish (Gao et al., 2018). Exposure of zebrafish embryos to benzophenone-3 inhibited development of ovaries and reduced egg production in later life (Tao et al., 2023). Embryonic exposure of Japanese medaka to TBCO via maternal transfer resulted in decreased fecundity of female fish at reproductive maturity (Devoy et al., 2023a). This effect was likely caused by decreased MIH-induced oocyte maturation (Devoy et al., 2023b). However, the molecular mechanism(s) of decreased oocyte maturation were not identified. Indeed, understanding the mechanistic basis for later life effects following embryonic exposure to EDCs have presented challenges for the field of ecotoxicology over recent decades (Seemann et al., 2015; Tang & Ho, 2007).

## 1.6 Epigenetic regulation as a mediator of embryonic exposure

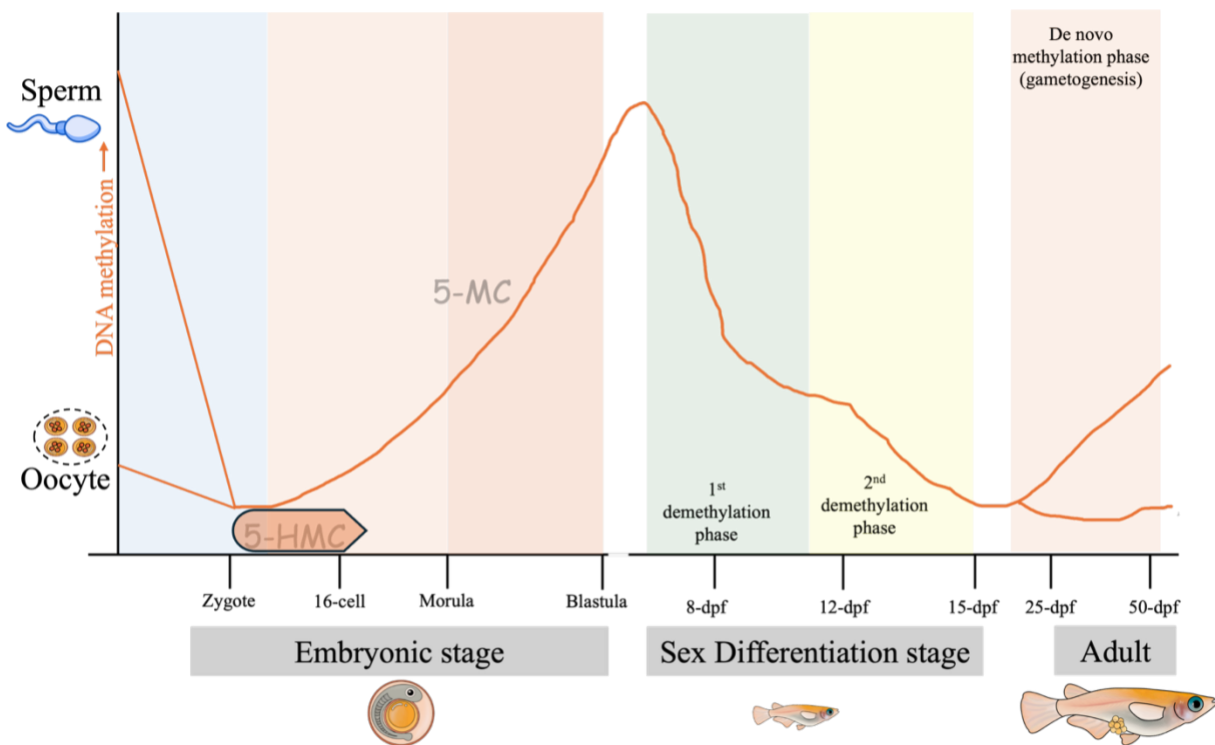
Later life effects of EDCs via embryonic exposure can be caused by disruption of the epigenome. Epigenetics refers to mitotically and meiotically heritable changes in gene expression that are not due to changes in the primary sequence of DNA (Tang & Ho, 2007). The major mechanisms of epigenetic modification are DNA methylation, histone modifications, and non-coding RNAs (Head et al., 2012; Miller & Grant, 2013). Among these, DNA methylation is the most researched epigenetic mechanism in the field of ecotoxicology.

DNA methylation regulates gene expression and therefore is critical for proper development and function of an organism (Schübeler, 2015). In vertebrates, DNA methylation mostly occurs at cytosine residues that are followed by a guanine residue, characterized as CpG methylation (Head, 2014). Clusters of unmethylated CpGs are termed a CpG island, which are mainly found in a gene's promoter region, promoting transcription. However, an unmethylated promoter region does not guarantee activation of gene expression, as surrounding regulatory networks also regulate gene transcription (Labbé et al., 2017). DNA methylation can also occur within gene-bodies, comprising both exons and introns. DNA methylation in the gene body is often inversely correlated with promoter methylation, whereas loss of DNA methylation in the gene body is associated with gene silencing (Jones, 2012; Lou et al., 2014). DNA Methylation can regulate gene expression by regulating binding of transcription factors to target sequences, altering DNA shape, recruiting methyl CpG binding region proteins to hinder binding of RNA polymerase to the template strand, and changing the structure of chromatin (Zhang et al., 2023). Additionally, changes in DNA methylation can be characterized as hypermethylation (increased DNA methylation) or hypomethylation (reduced methylation) (Zhang et al., 2018).

Regulation of DNA methylation is critical for the development of an organism. Members of the DNA methyltransferase (DNMT) family, which includes DNMT1, DNMT2, DNMT3a and DNMT3b in fish, work in coordination to regulate DNA methylation (Head, 2014; Montjean et al., 2022). De novo methylation is catalysed by DNMT3a and DNMT3b by adding a methyl group from the universal methyl donor S-adenosylmethionine (SAM) to DNA and is maintained by DNMT1 (Head, 2014; Miller & Grant, 2013). DNA also undergoes demethylation via removal of the methyl group from 5-methylcytosine, which can occur actively or passively (Schübeler, 2015; Tahiliani et al., 2009). Passive demethylation occurs through the absence of DNA methylation maintenance (Best et al., 2018). Conversely, there are two pathways for active demethylation of DNA, in fish (Best et al., 2018). First, members of the ten-eleven translocation (TET) methyl-cytosine dioxygenase family oxidize 5-methylcytosine (5mc), yielding 5-hydroxymethylcytosine (5hmC) and subsequently break it down to 5-formyl-cytosine and 5-carboxyl-cytosine (López-Moyado & Rao, 2022; Zhang & Wu, 2010). Alternatively, the AICDA/APOBEC pathway that was first discovered in mammals (Morgan et al., 2004), converts 5mc to thymine, resulting in unmethylated cytosine via deamination (Nabel et al., 2012).

Reprogramming of DNA methylation occurs after fertilization and is critical for proper development. Variation exists in DNA methylation reprogramming pattern among eukaryotes, including different species of fish. Reprogramming of DNA methylation in Japanese medaka occurs during early embryonic stages (from zygote to blastula) and during primordial germ cell (PGC) specification, similar to that in humans and mice (Wang & Bhandari, 2020a). Following fertilization, the Japanese medaka oocyte genome is hypomethylated, while the sperm genome is hypermethylated (Wang & Bhandari, 2019). The paternal genome undergoes active DNA demethylation within the first cell cycle stage, while the maternal genome remains

hypomethylated. As a result, both paternal and maternal genome converge to a similar hypomethylated state after several rounds of early cell divisions, before undergoing global de novo DNA methylation from the 16 cell stage to the blastula stage (Wang & Bhandari, 2019). Afterwards, PGCs undergo the first wave of global demethylation which is completed before eight days post fertilization (dpf) followed by second demethylation phase from 10 dpf -12 dpf. Male PGCs undergo de novo methylation at 25 dpf while female PGCs remain hypomethylated at a ground state level similar to 15-dpf (Wang & Bhandari, 2020b).



**Figure 1.4.** Schematic of DNA methylation reprogramming during early developmental stage in Japanese medaka. The sperm genome is hypermethylated, while the oocyte genome is hypomethylated. Following fertilization, paternal DNA methylation is actively erased in the zygote. A global hypomethylated state is maintained across several cleavage events during early embryonic development. Global DNA methylation levels progressively rise from the 16-cell stage to the blastula stage. During sexual differentiation, primordial germ cells (PGC) complete the first demethylation phase before eight days post-fertilization (dpf), followed by a second demethylation phase between 10 and 12 dpf. After that, global methylation increases from 15 to 25 dpf in male PGCs, while female PGCs retain a hypomethylated profile. This figure was adapted from (Wang & Bhandari, 2020a).

Disruption of DNA methylation is the most researched epigenetic mechanism of toxicity in the field of ecotoxicology. During embryonic development, environmental stressors, including toxicants, can cause aberrant DNA methylation marks that escape reprogramming events (Wang & Bhandari, 2020b). Once formed, altered DNA methylation marks can be mitotically and meiotically inherited, even in the absence of the contaminant (Labbé et al., 2017). Exposure of fish to environmental contaminants during embryogenesis can alter DNA methylation, affecting gene expression and resulting in phenotypic changes in adulthood and future generations (Labbé et al., 2017). In another study, embryonic exposure of inland silversides (*Menidia beryllina*) to multiple EDCs, including ethinylestradiol, bifenthrin, trenbolone, and levonorgestrelm from eight hours post fertilization hpf to 21 days post hatch (dph) resulted in differentially methylated regions (DMRs) in the genome of all generations (F0, F1, F2), relative to the control treatment (Major et al., 2020). Lastly, early life exposure of zebrafish embryos to the organophosphorus flame-retardant tris (1,3-dichloro-2-propyl) led to later life delayed neurotoxicity associated with DNA methylation in adulthood (Li et al., 2020). Overall, exposure to EDCs during critical stages of epigenome reprogramming are detrimental to fish health.

### **1.7 Japanese medaka as a model species**

Japanese medaka is a small (2-4cm) egg-laying freshwater fish often found in shallow water, and are native to Japan, Korea, Taiwan and China (Hilgers & Schwarzer, 2019). Medaka is an ideal vertebrate for studies of latent intragenerational effects as they reach sexual maturity in 2-3 months, have well-characterized embryo development, and are economical and easy to maintain (Padilla et al., 2009). In addition, Japanese medaka has a fully sequenced and annotated genome, and the pattern of methylome reprogramming following fertilization has been characterized (Wang & Bhandari, 2019). The reproductive physiology of Japanese medaka is

well characterized, making them an ideal animal for studying endocrine disruption and reproductive toxicology (Ankley & Johnson, 2004). A short-term reproduction assay that assesses fecundity and fertility for 21 days during exposure to a chemical has been established and is widely used in regulatory toxicology (OECD, 2012). Furthermore, an *in vitro* assay to assess MIH-stimulated oocyte maturation has been developed for this species (Raza et al., 2023). There are 10 stages of oocyte development in Japanese medaka, and each can be identified by size and visual characteristics such as a semitransparent surface and oil droplets (Iwamatsu, 1978; Raza et al., 2023).

### **1.8 Objectives and Hypothesis**

Embryonic exposure to maternally deposited TBCO impairs the reproductive capacity of Japanese medaka via a decrease in MIH-stimulated oocyte maturation, but the molecular mechanism is not known (Devoy et al., 2023b). In this study, Japanese medaka embryos were exposed to maternally transferred TBCO and reared in clear water until they reached sexual maturity. A 21-day short-term reproduction assay that assesses fecundity was performed, after which an *ex vivo* assay was performed to assess MIH-stimulated oocyte maturation. High-throughput mRNA sequencing (mRNA-seq) was used to compare the transcriptome of the stage IX oocytes that fail to mature and the stage IX oocytes that mature to stage X from control and TBCO-exposed female fish. Further, enzymatic methyl sequencing (EM-seq) was used to determine if changes in DNA methylation might be altering the expression of genes that regulate oocyte maturation. We hypothesize that decreased oocyte maturation in sexually mature female medaka exposed as embryos to TBCO is caused by gene expression reprogramming due to alterations of DNA methylation during the early developmental stages. The overall objective of this research is to elucidate the mechanism by which exposure to maternally transferred TBCO

causes an intragenerational decrease in fecundity of female Japanese medaka by impairment of MIH-stimulated oocyte maturation:

The specific objectives of this research are to:

1. Use of RNA-seq to identify genes whose expression is altered by embryonic exposure to TBCO that might be related to decreased oocyte maturation.
2. Use of EM-seq to investigate the role of DNA methylation in decreased oocyte maturation of fish exposed as embryos to TBCO.

## **1.9 Significance**

Inputs of chemicals into water bodies pose a continuous challenge for aquatic organisms. Some of these chemicals are EDCs that decrease the reproductive performance of fish. Traditionally, most studies on the effects of EDCs on reproductive performance have focused on exposure to sexually mature fish and disruption of the growth/vitellogenesis stage of oogenesis as a mechanism of reproductive impairment. However, there is a growing appreciation that exposure during critical stages of embryonic development can have effects that result in decreased reproductive performance in adulthood. Furthermore, research has shown that embryonic exposure of fish to EDCs can lead to decreased fecundity via disruption of oocyte maturation. Successful completion of this research endeavor will provide novel knowledge of the threat that EDCs pose to fishes. More specifically, it that will improve our understanding of how embryonic exposure of EDCs during ELS of fish leads to later-life effects. In addition, it will give a greater understanding of oocyte maturation as a target of EDCs. Epigenetics plays a significant role in intragenerational-induced disease due to the alteration of gene expression regulation. This study will generate new information about the role of DNA methylome in effects of EDCs on oocyte maturation and reproductive performance. If a complete analysis of

this study suggests that epigenetics plays a role in the intragenerational disruption of MIH-induced oocyte maturation, it will provide evidence that EDCs may be of bigger concern than anticipated due to their widespread occurrences in aquatic ecosystems. Overall, this research offers novel and valuable insights into the mechanisms of EDC toxicity in the aquatic ecosystem.

## **CHAPTER 2: EMBRYONIC EXPOSURE TO THE FLAME RETARDANT, 1,2,5,6-TETRABROMOCYCLOOCTANE (TBCO) INHIBITS MIH-INDUCED OOCYTE MATURATION AND ALTERS GENE EXPRESSION AND DNA METHYLATION IN ADULT JAPANESE MEDAKA (*ORYZIAS LATIPES*)**

### **2.1 Introduction**

Oogenesis is the process that produces a fertilizable egg. Oocyte maturation, the terminal step of oogenesis, is mediated by non-genomic action of the progestin, maturation inducing hormone (MIH) (Nagahama & Yamashita, 2008). During vitellogenesis, immature oocytes are arrested in meiotic prophase 1 by an elevated intracellular concentration of cyclic adenosine monophosphate (cAMP), due to non-genomic actions of  $17\beta$ -estradiol (E2) binding to a G protein-coupled estrogen receptor (GPER) on the oocyte surface (Pang & Thomas, 2010; Thomas, 2017). Meiosis resumes as a response to a surge in luteinizing hormone (LH) that shifts steroidogenesis from synthesis of E2 to MIH (Nagahama & Yamashita, 2008).

While it is well established that exposure of sexually mature fish to endocrine disrupting chemicals (EDCs) can be detrimental to their reproductive performance, exposure of early life stage (ELS) fish to EDCs can also be detrimental to their reproductive fitness. ELS exposure of zebrafish to benzophenone-3 inhibited development of ovaries and reduced egg production in later life (Tao et al., 2023). Additionally, ELS exposure of fathead minnows (*Pimephales promelas*) to the flame retardant 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) reduced fecundity at sexual maturity in the absence of the initial stressor (Thornton et al., 2016). Furthermore, early life stage exposure via maternal transfer of TBCO to Japanese medaka led to a reduction in fecundity and MIH-induced oocyte maturation (Devoy et al., 2023b). This scenario whereby fish

are exposed only during ELS but experience a phenotypic effect in adulthood, is termed an intragenerational effect (Al-Griw et al., 2017).

Mechanistically, intragenerational effects can be a result of contaminant induced epigenetic alterations during susceptible developmental windows, including reprogramming of the methylome (Aluru et al., 2015; Wang & Bhandari, 2020a). Embryonic exposure of inland silversides (*Menidia beryllina*) to trenbolone from eight hours post fertilization (hpf) to 21 days post hatch (dph) altered DNA methylation pattern of *TGFB* and *GPR30* in 21dph larvae, each of which in regulate oocyte maturation (DeCourten et al., 2020). In another study, inland silversides exposed to EDCs from fertilization to 21dph also experienced altered methylation of genes in the KEGG oocyte meiosis pathway (Major et al., 2020). Because DNA methylation is mitotically stable, changes in DNA methylation can persist into adulthood, altering gene expression and altering physiological performance, including reproduction (Labbé et al., 2017; Major et al., 2020). Embryonic exposure to phenanthrene led to hypermethylation of gonadotropin releasing hormone receptor 3 (*GNRH3*) and fewer mature (stage V) oocytes in adult zebrafish (Chen et al., 2021). Similarly, 96 hours exposure of zebrafish embryos to benzo(a)pyrene led to a hypermethylation of GNRH and GNRHR3, decreased abundances of *gnrh3* and *gnrhr3* mRNA, and a significant decrease in plasma concentrations of luteinizing hormone (LH $\beta$ ). These changes were associated with decreased fecundity and reduced matured oocyte based on histological examination of sexually mature adults (Gao et al., 2018). Taken collectively, it is becoming increasingly evident that ELS exposure of fish to EDCs can alter the epigenome, including DNA methylation, resulting in altered later life phenotypes due to altered gene expression.

The brominated flame retardant, 1,2,5,6-tetrabromocyclooctane (TBCO), is an EDC (Saunders et al., 2015; Sun et al., 2016). Studies have demonstrated that exposure of sexually mature fish to TBCO impairs MIH-stimulated oocyte maturation and decreases fecundity (Raza et al., 2023; Van Essen et al., 2021b). Further, embryonic exposure of Japanese medaka to TBCO via maternal transfer decreases fecundity via impairment of MIH-induced oocyte maturation (Devoy et al., 2023b). However, the molecular mechanism(s) of impaired oocyte maturation in embryonically exposed Japanese medaka were not clear (Devoy et al., 2023b). In the present study, we hypothesised that embryonic exposure to maternally deposited TBCO causes changes to the DNA methylome that escape DNA methylation reprogramming during embryogenesis leading to changes in expression of genes essential for oocyte maturation regulation. To test this hypothesis, Japanese medaka were exposed as embryos to maternally deposited TBCO and reared to sexual maturity in clean water. Fecundity and oocyte maturation were assessed, and mRNAseq and EMseq were used to characterize the transcriptome and methylome of fully grown oocytes from control and TBCO exposed females.

## **2.1 METHODOLOGY**

### **2.2.1 Preparation of TBCO-contaminated food**

TetraMin<sup>®</sup> tropical flakes fish food (Tetra, Brampton, ON, Canada) was spiked with TBCO (SynQuest Laboratories, Inc. Alachua FL, purity = 98%) according to the protocol previously described (Saunders et al., 2015). Flakes were ground using a food processor and mixed with TBCO dissolved in 150 mL of acetone to make low (100 µg TBCO/g, wet mass (WM)) and high (1000 µg TBCO/g WM) concentration food. These nominal concentrations are equivalent to concentrations used in previous studies that reported effects of embryonic exposure

to TBCO on reproduction and oocyte maturation of Japanese medaka (Devoy et al., 2023b). The same protocol, excluding the addition of TBCO, was used to prepare control food.

### **2.2.2 Animal care**

Parental generation adult Japanese medaka 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) in accordance with OECD guidance for medaka testing (OECD Test No. 240). Average water quality was conductivity 882  $\mu\text{s}/\text{cm}$ , alkalinity pH 8.53 at 26°C, ammonia at 0.01mg/L, nitrite at 0.011, and nitrate at 2.2. A 16:8 light:dark photoperiod was used. Adults were fed to satiety with TetraMin<sup>®</sup> tropical flake fish food and live Artemia (Brine Shrimp Direct, Ogden, TU, USA) three times daily. Japanese medaka were maintained following University of Lethbridge Animal Welfare Protocol #2023.

### **2.23 Exposure of Parental generation to TBCO**

Five sexually mature male and female medaka were randomly assigned to 10L tanks under flow-through conditions and allowed to acclimate for 14 days before the initiation of exposure. During the acclimation period, medaka were transitioned from Artemia to TetraMin<sup>®</sup> tropical flake fish food. Three replicate tanks were used per treatment. During the experimental phase, fish were fed approximately 5% of their body mass daily with control or TBCO spiked flaked food, with half of the food provided in the morning and the other half in the afternoon. Water temperature was maintained at 25°C during the experiment. On days 14, 16, and 18, 50 eggs per treatment tank were collected, rinsed in dechlorinated water, and stored at -80°C to quantify maternal transfer of TBCO. Fertilized eggs collected on days 14-20 of the exposure were reared to sexual maturity as described in the next section.

## 2.24 Reproduction F1 Japanese medaka

Fertilized eggs from the parental generation exposures were grown in freshwater (dechlorinated city of Lethbridge water), without additional exposure to TBCO, for approximately four months until they reached sexual maturity. Artemia was introduced immediately post-hatch. Rearing conditions for these F1 generation medaka were identical to conditions described in the animal care section. Once the maternally exposed F1 medaka reached sexual maturity, reproductive performance was assessed using a 21-day reproduction assay conducted according to OECD Test Guideline 229 (OECD, 2012). Three sexually mature male and female fish were randomly assigned to 3.5L tanks under flow-through conditions with four replicate tanks per treatment. Fish were acclimated for 14 days prior to the start of the assay. Throughout the reproduction assay, fish were fed approximately 5% of their body mass daily with TetraMin<sup>®</sup> tropical flake fish food, divided equally between morning and afternoon feedings. The F1 generation was not re-exposed to TBCO during the reproduction assay. Fecundity was assessed daily by collecting and counting eggs from each tank and was expressed as the number of eggs per female per tank per day. At the conclusion of the assay, all fish were euthanized in buffered MS-222 and measured for total length and body mass to calculate Fulton's condition factor ( $K = 100 \times [\text{mass (g)}] / [\text{length (cm)}]^3$ ) (Table 2.1). Ovaries were collected to assess MIH-stimulated oocyte maturation and for molecular analysis.

Statistical analyses were performed using R version 4.5.1. Homogeneity of variance was assessed using a Levene's test and normality of residual was assessed using a Shapiro-wilk test. A non-parametric Kruskal-Wallis with Dunn's post-hoc test was performed to assess differences in fecundity among treatment groups. Differences in body mass, length, and K among treatment groups were analyzed using a Welch's ANOVA.

## 2.25 Oocyte maturation

After the 21-day F1 reproduction assay, ovaries from three female fish were used in an ex-vivo oocyte maturation assay where the response of stage IX immature oocytes to MIH was assessed. The protocol was performed according to previously described methods (Devoy et al., 2023b; Raza et al., 2023). Briefly, ovaries were excised at the onset of light and placed in 90% Gibco Leibovitz L-15 medium (L-15: containing pH 7.0; Fisher Scientific, Waltham, MA, USA) as endogenous MIH is released within 2 hrs of light onset in Japanese medaka (Iwamatsu, 1978). Stage IX immature oocytes were distinguished using a Zeiss discovery V12 stereo microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) based on size, oocyte surface opacity, and the presence of postovulatory follicles and oil droplets (Iwamatsu, 1978; Raza et al., 2023). Stage IX immature oocytes from each of the three female medaka per tank were pooled (n =4 pools of oocytes per treatment). After pooling, 12 randomly selected stage IX immature oocytes per treatment were placed in individual wells of two six well plates containing 1 ml of L-15 medium containing 1000 mg/L MIH. Plates were incubated for 24 hours at room temperature, after which oocyte maturation was scored based on the presence or absence of GVBD.

Statistical analyses were performed using R version 4.5. Homogeneity of variance was assessed using a Levene's test and normality of residual was assessed using a Shapiro-wilk test. A one-way ANOVA with Tukey's-HSD post-hoc test was used to determine differences in oocyte maturation between treatment groups

## 2.27 Transcriptome and Methylome Analysis

### 2.27.1 RNA and DNA isolation

At the end of the ex-vivo oocyte maturation assay, control stage IX oocytes that matured and stage IX oocytes that failed to mature because of embryonic exposure to TBCO were pooled separately. Genomic DNA and total RNA were extracted from all four replicates per treatment using a Norgen DNA/RNA/Protein purification kit according to manufacturer's protocol ([Norgen Biotech, Thorold, ON Canada](#)). Genomic DNA concentration was determined using a Qubit 4 Fluorometer (Thermo Fisher Scientific), and genomic DNA samples meeting the requirement of quantity > 50ng, volume  $\geq 10\mu\text{L}$ , Concentration  $\geq 5 \text{ ng}/\mu\text{L}$ , and  $\text{OD}_{260/280} \geq 2.0$  and  $\text{OD}_{260/230} \geq 2.0$ , were selected for enzymatic methyl-sequencing (EM-seq). Total RNA concentration was determined using a Qubit 4 fluorometer and samples meeting the requirement of quantity > 100ng, volume  $\geq 10\mu\text{L}$ , concentration  $\geq 10 \text{ ng}/\mu\text{L}$ ,  $\text{OD}_{260/280} \geq 1.8$  and  $\text{OD}_{260/230} \geq 2.0$  were selected for RNA sequencing. Sequencing of DNA and RNA was performed by [Novogene](#) (Beijing, China). DNA and RNA were sequenced on an Illumina platform, using 150bp paired end reads. All RNA samples had a RIN  $\geq 4.0$ , as determined by Novogene.

### 2.27.2 Transcriptome responses analysis

Raw sequencing data were analyzed on the Galaxy public server (<https://usegalaxy.org/>). The raw read quality was assessed using FastQC, and low-quality bases (Phred score < 30) and adapter sequence were trimmed using Fastp. Two of mRNA samples from TBCO exposed fish did not pass quality control (Phred score < 30) and were excluded from further analysis. Cleaned RNA-seq reads were mapped to the *O. latipes* reference genome (HdrR strain, assembly

ASM223467v1) using RNA-STAR, with default parameters, and feature quantification was performed with feature Counts to generate a raw gene count matrix.

For statistical analysis, differential gene expression analysis was conducted in R (v4.5.1) using the DESeq2 package. Genes with zero counts across all samples were removed prior to differential expression analysis. Gene counts were normalized using DESeq2 size-factor normalization and modeled using a negative binomial generalized linear model with treatment condition as the design factor. Differentially expressed genes (DEGs) were identified based on an absolute log<sub>2</sub> fold change ( $|\log_2FC|$ ) > 1.2 and adjusted p-value (padj) < 0.05. For data visualization, volcano plots were generated with the ggplot2 package in Rstudio. Statistically significant differentially expressed genes (DEGs) were annotated by mapping Ensembl IDs to gene symbols and Entrez IDs using biomaRt package. KEGG Pathway enrichment analyses were performed with gProfiler2 using mapped Entrez IDs. Enrichment results were visualized as gene-pathway networks (cnetplots), showing altered biological processes.

### **2.27.3 Methylome response analysis**

Raw sequencing data were analyzed on the Galaxy public server (<https://usegalaxy.org/>). The raw read quality was assessed using FastQC, and low-quality bases (Phred score < 30) and adapter sequence were trimmed using Fastp. One of genomic DNA samples from the control, and two of the TBCO treated fish did not pass quality control and were excluded from further analysis. For each replicate, trimmed reads were aligned independently to the *O. latipes* reference genome (Hdr strain, Ensembl ASM223467v1) using Bwameth. Per-base methylation metrics in CpG context were extracted using MethylDackel in a MethylKit format.

For statistical analysis, coverage files were analyzed in R (v 4.51) using the Methylkit package (v1.28). CpG sites with < 10× coverage or above 99.9th percentile were removed.

Distribution of global CpG methylation between samples was analyzed using a Wilcoxon test. Differential methylation analysis was performed at both the single-CpG and tiled-region levels (1 kb windows) to identify differential methylated regions (DMRs). A Benjamini–Hochberg adjusted p-value (q-value) of  $< 0.01$  and methylation difference of  $\geq 25\%$  was used to control false discovery due to small sample size. DMRs were annotated using genomation package (v1.36.0) on gene features derived from the *O. latipes* reference genome (Hdr strain, Ensembl ASM223467v1). For data visualization, volcano plots were generated showing DMRs with the ggplot2 package in Rstudio. Genes overlapping with significant DMRs were used for KEGG pathway enrichment using gProfiler. Enrichment results were visualized as gene-pathway networks (cnetplots), showing altered biological processes.

## 2.3 RESULTS

### 2.3.1 Concentration of TBCO in food and eggs

Concentrations of TBCO in food and eggs will be analyzed by use of ultra-high resolution gas chromatography mass spectrometry, as outlined in (Van Essen et al., 2021).

### 2.3.2 Condition factor

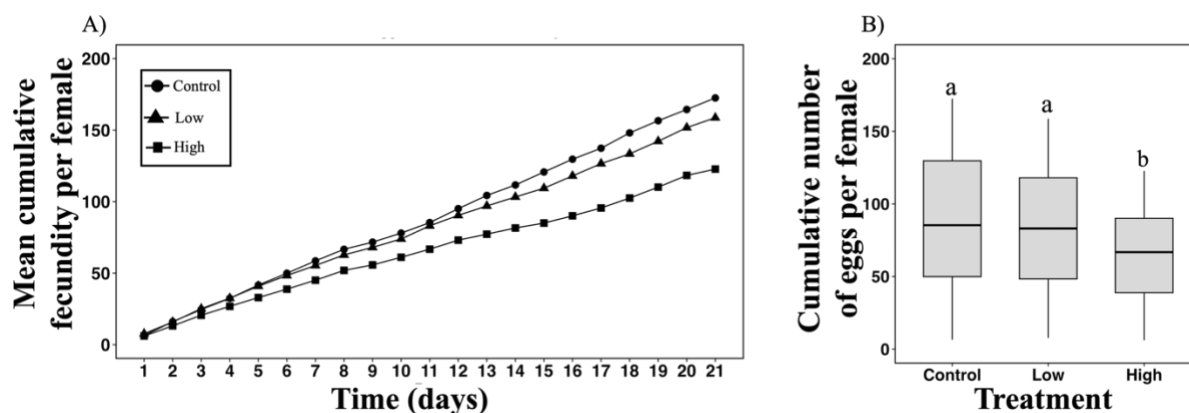
Neither length, mass, nor K of adult Japanese medaka exposed embryonically to either the low or high TBCO diet was significantly different from that of control fish (Table 2.1).

**Table 2.1:** Mean body length, mass and Fulton condition factor (K) of adult Japanese medaka exposed embryonically to TBCO via maternal transfer. Data are expressed as the mean  $\pm$  standard error and are relative to the control treatment. The parental generation was exposed to either a control diet (no TBCO) or a diet containing TBCO at 100  $\mu\text{g/g}$ , wm food (low) or 1000  $\mu\text{g/g}$ , 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. Fulton’s condition factor (K) was calculated as  $(K = 100 \times [\text{mass (g)}] / [\text{length (cm)}]^3)$ . Data was analyzed using a Welch ANOVA. There was no significant difference seen in length, mass, and K of control and TBCO-exposed fish ( $p > 0.05$ ).

Treatment	Length (mm)	Mass (g)	Condition factor (K)
Control	29.8 ± 1.95	0.19 ± 0.01	0.92 ± 0.15
Low	26.3 ± 0.97	0.21 ± 0.01	1.26 ± 0.16
High	24.7 ± 1.95	0.20 ± 0.01	1.81 ± 0.34

### 2.3.3 Fecundity of F1 Japanese medaka

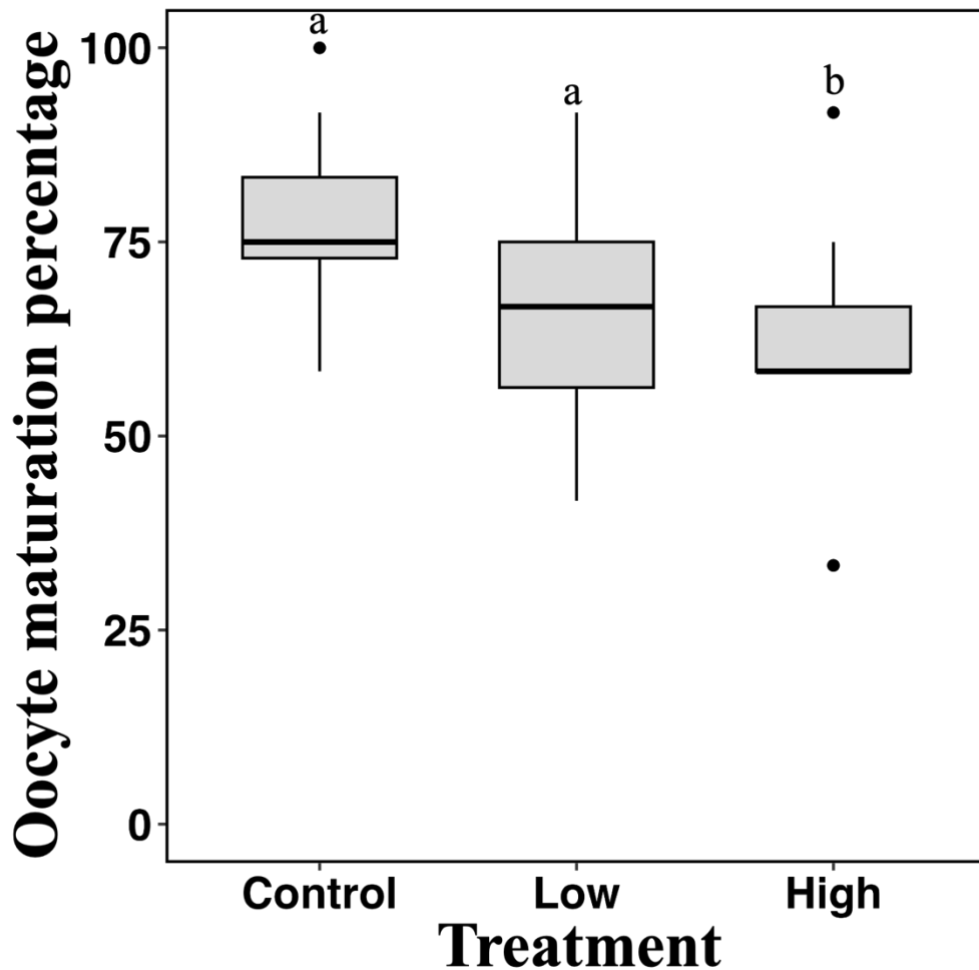
The F1 generation Japanese medaka exposed as embryos to low and high TBCO experienced a 11.3% and 31.4% decrease in fecundity, compared to the controls (Figure 3.1). High TBCO exposed females had significant lower fecundity than control ( $p < 0.05$ ). There were no significant differences between fecundity of low TBCO exposed fish and controls, or between high and low TBCO exposure groups ( $p \geq 0.05$ ).



**Figure 2.1.** Effect of embryonic exposure to TBCO via maternal transfer on cumulative fecundity of F1 adult female Japanese medaka. Panel A shows mean cumulative fecundity per female during the 21-day assay. Panel B shows cumulative number of eggs per female in each treatment. The parental generation was exposed to either a control diet (no TBCO) or a diet containing TBCO at 100  $\mu\text{g/g}$ , wm food (low) or 1000  $\mu\text{g/g}$ , 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 was assessed. Exposures were conducted with 4 replicate tanks per treatment, with 3 male and 3 female fish per tank. The boxplot shows the median (horizontal line in each box), interquartile range (the box) and non-outlier values (whiskers). Differences among treatments were determined using a Kruskal-Wallis test, followed by a Post hoc Dunn test with Bonferroni adjustment. Significant differences between treatments are indicated by different letters ( $p < 0.05$ ).

### 2.2.4 Ex vivo oocyte maturation

Stage IX oocytes from F1 medaka exposed as embryos to low and high TBCO via maternal transfer experienced a 16.1% and 22.3% decrease in *ex vivo* MIH-stimulated maturation, compared to the control (Figure 2.2). However, only maturation of oocytes from females of the high TBCO treatment was significantly decreased compared to the control ( $p < 0.05$ ).

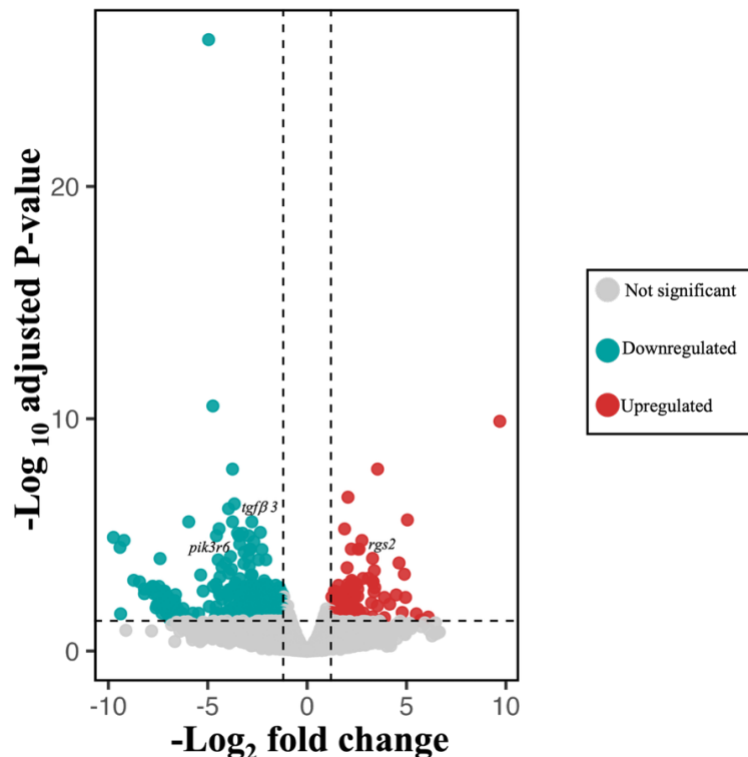


**Figure 2.2.** Effect of embryonic exposure to TBCO via maternal transfer on *ex vivo* maturation of stage IX oocytes from adult female Japanese medaka. The parental generation was exposed to either a control diet (no TBCO) or TBCO at 100  $\mu\text{g/g}$ , wm food (low) or 1000  $\mu\text{g/g}$ , wm food (high) for 21 days during reproduction. Fertilized eggs were collected on days 14-20 of reproduction and reared to sexual maturity without additional exposure to TBCO. Following the reproduction assay, percentage of stage IX oocytes undergoing GVBD after 24-hour incubation with MIH was measured. The boxplot shows the median (horizontal line in each box),

interquartile range (the box), outlier (solid dot) and non-outlier values (whiskers). Differences among treatments were determined by use of a one-way ANOVA and Tukey HSD post hoc analysis. Significant differences between treatments are indicated by different letters ( $p < 0.05$ ).

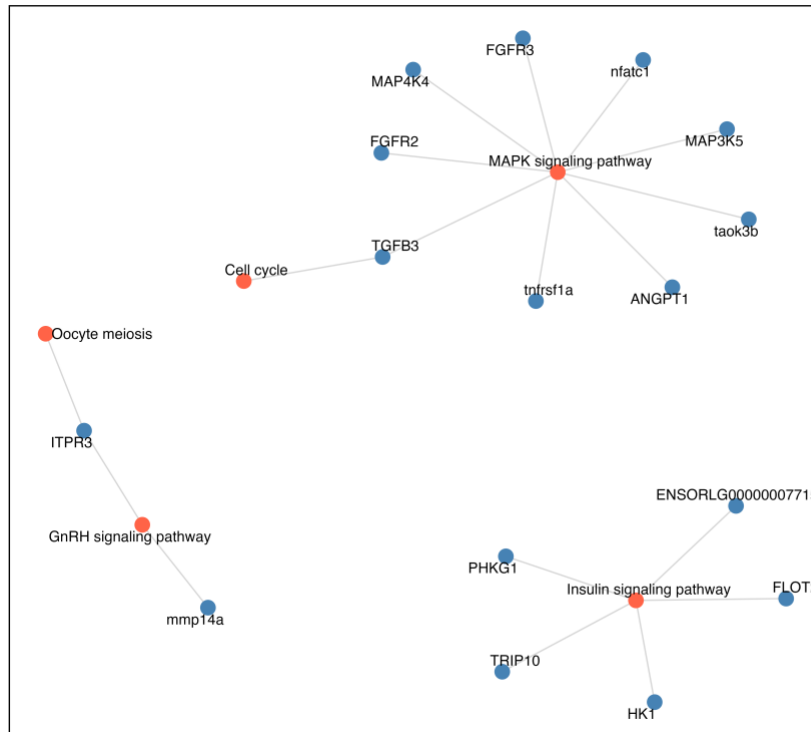
### 2.2.5 Effect of embryonic TBCO exposure on adult Japanese medaka oocyte transcriptome

There were 664 significant differentially expressed genes (DEGs) between control stage IX oocytes that matured and stage IX oocytes that failed to mature because of embryonic exposure to TBCO ( $p \leq 0.05$ ,  $\log_2FC \geq 1.2$ ) (Fig 2.3). Out of 664 DEGs, 357 were annotated, including 290 downregulated and 67 upregulated genes. Among those, *tgfb3*, *pik3r6*, and *rgs2* are suggested to play a role in oocyte maturation. Gene-KEGG pathway network analysis using annotated DEGs were not significantly enriched after multiple testing corrections ( $p < 0.01$ ). However, several genes mapped to pathways involved in oocyte maturation, including oocyte meiosis, cell cycle, insulin signaling, MAPK, and GnRH signaling.



**Figure 2.3.** Volcano plot showing annotated differentially expressed genes between control stage IX oocytes that matured and stage IX oocytes that fail to mature because of embryonic exposure to TBCO, in F1 adult Japanese medaka. Differentially expressed genes had a  $\log_2$  fold change  $\geq$

1.2 and adjusted p value  $\leq 0.05$ . Downregulated genes are in green, upregulated genes are in red, and non-significant genes are in grey. *tgfb3* and *pik3r6* were downregulated while *rgs2* was upregulated.

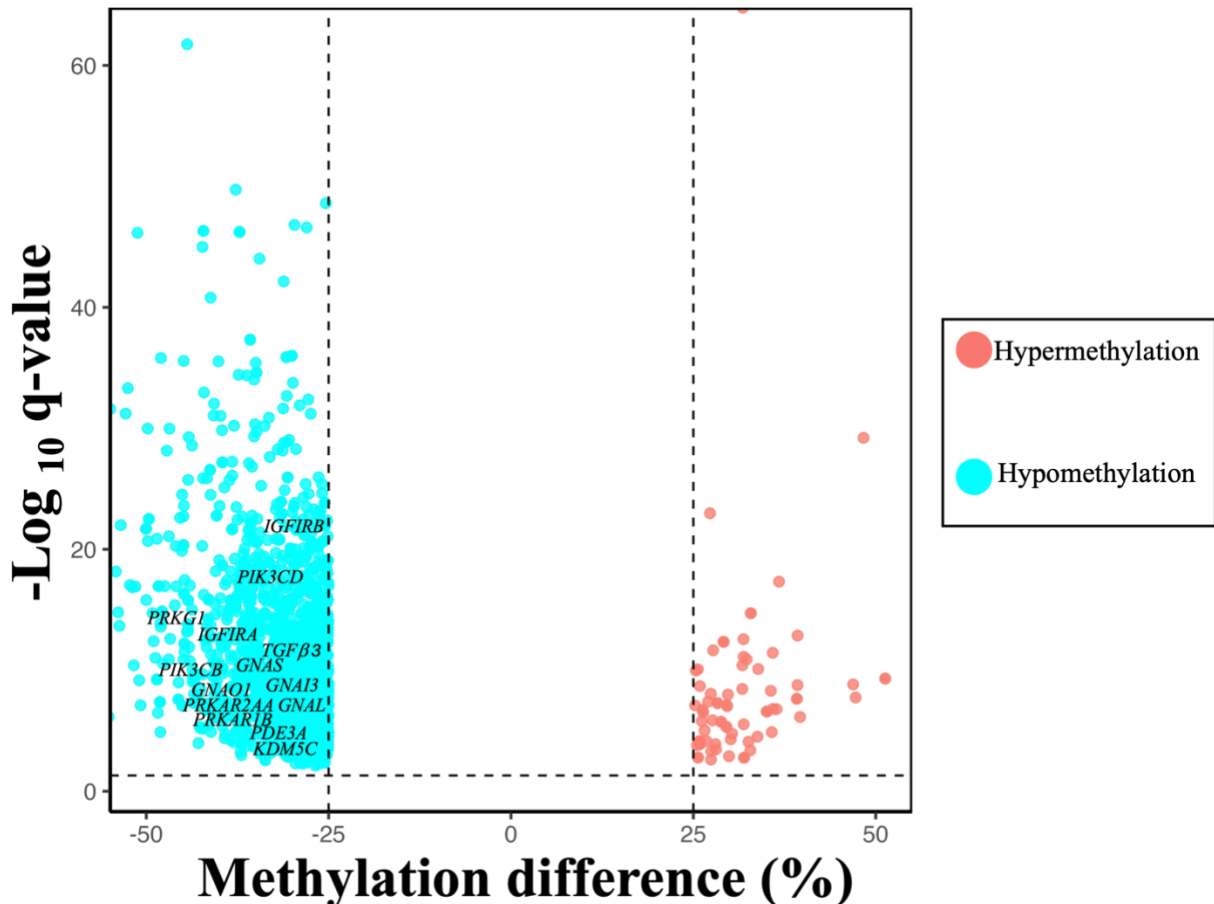


**Figure 2.4** Gene-KEGG pathway network showing KEGG pathways associated with the differentially expressed genes (DEGs) between control stage IX oocytes that matured and stage IX oocytes that fail to mature because of embryonic exposure to TBCO, in F1 adult Japanese medaka. Blue nodes represent DEGs mapped to KEGG pathways, and red nodes represent KEGG pathway terms. Pathways were obtained from g:Profiler (KEGG database). Differentially expressed gene associated with oocyte meiosis, insulin, MAPK, and GnRH signalling pathways were represented. Although, these pathways were not significantly enriched.

### 2.2.6 Effect of embryonic TBCO exposure on adult Japanese medaka oocyte methylome

There was no statistically significant difference in global CpG methylation between control stage IX oocytes that matured (43.34%) and stage IX oocytes that failed to mature because of embryonic exposure to TBCO (34.93%) (Wilcoxon rank-sum test,  $p = 0.20$ ; Figure S2.1). A comparison of the control and embryonically TBCO exposed groups revealed 5053 DMRs, of which 4867 were hypomethylated and 186 were hypermethylated. Annotation of these regions showed distribution across genomic features with 15% of DMRs in introns, 12% in

exons, 7% in promoter regions, and 66% in the intergenic region. Several DMRs were linked to same gene, while some were not linked to any. DMRs were uniquely mapped to 1075 genes, several of which are suggested to play a role in oocyte maturation. These genes are *PDE3A*, *IGF1RA*, *IGF1RB*, *PIK3CB*, *PIK3CD*, *GNAI3*, *GNAS*, *PRKAR1B*, *PRKAR2AA*, *KDM5C*, and *PRKG1* (Figure 2.5). KEGG pathway network analysis performed using annotated DEGs were not significantly enriched after multiple testing corrections ( $p < 0.01$ ). However, several genes overlap with pathways involved in oocyte maturation including oocyte meiosis, insulin signaling, MAPK, GnRH signaling, cell cycle, and progesterone mediated oocyte maturation signalling.



**Figure 2.5.** Volcano plot showing distribution of significantly differentially methylated regions (DMRs) between control stage IX oocytes that matured and stage IX oocytes that fail to mature because of embryonic exposure to TBCO, in F1 adult Japanese medaka. Only those regions with

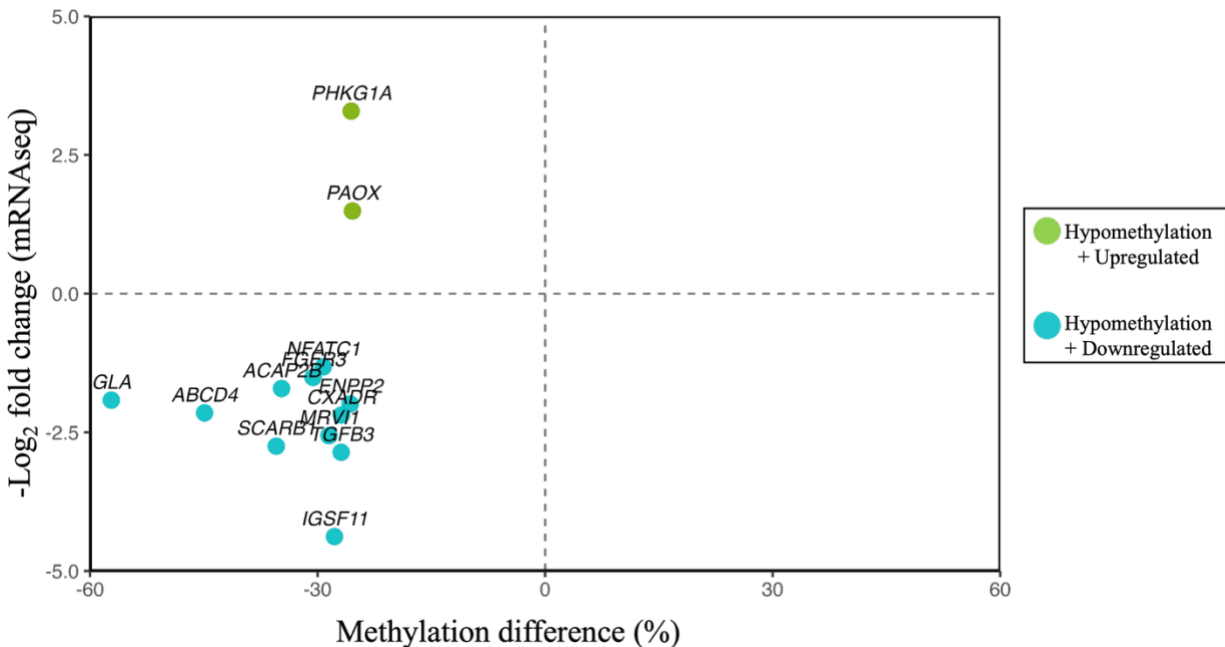


**Table 2.2.** Genes with significant changes in DNA methylation (q-value < 0.05, methylation change > ± 25% and gene expression (mRNA) (p-value < 0.05, Log2fold > 1.2) involved in oocyte maturation

Gene symbol	Full gene name	Role of protein product	Methylation and expression context
<b>DNA METHYLATION</b>			
<i>PDE3A</i>	Phosphodiesterase 3a	Regulate intracellular level of cAMP	Gene body hypomethylated
<i>PRKG1</i>	Protein kinase G type 1	Mediates effect of cGMP	Gene body hypomethylated
<i>IGF1RA</i>	Insulin-like growth factor 1 receptor a	Mediates signalling of the PI3K/AKT pathway	Gene body hypomethylated
<i>IGF1RB</i>	Insulin-like growth factor 1 receptor b	Mediates signalling of the PI3K/AKT pathway	Gene body hypomethylated
<i>PIK3CB</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta	Encodes the p110 $\beta$ catalytic subunit of PI3K	Gene body hypomethylated
<i>PIK3CD</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	Encodes the p110 $\delta$ catalytic subunit of PI3K	Gene body hypomethylated
<i>GNAS</i>	Guanine nucleotide binding protein (G protein) alpha stimulating activity polypeptide	Encodes subunit of stimulatory G-protein G $\alpha$ protein, which stimulate adenylyl cyclase-cAMP activity	Gene body hypomethylated
<i>GNAI3</i>	G protein subunit alpha i3	Gnai3 encodes the inhibitory G-protein G $\alpha$ protein, which suppress adenylyl cyclase-cAMP activity	Gene body hypomethylated
<i>PRKAR2AA</i>	Protein kinase cAMP-dependent type II regulatory subunit alpha	Encodes protein kinase A regulatory subunit II regulated by cAMP, involved in controlling PKA activity	Gene body hypomethylated
<i>PRKAR1B</i>	Protein kinase cAMP-dependent type I regulatory subunit beta	Encode protein kinase A regulatory subunit I regulated by cAMP, involved in controlling PKA activity	Gene body hypomethylated
<b>GENE EXPRESSION (mRNA)</b>			
<i>pik3r6</i>	PI3K $\gamma$ regulatory subunit)	Isoform of Pi3k and contribute to pi3k activation	Downregulated
<i>rgs2</i>	Regulator of G protein signalling 2	Functions as a negative regulator of G-protein signalling and reduce activity of various isoform of adenylyl cyclase	Upregulated
<b>OVERLAPPING DIFFERENTIALLY EXPRESSED AND METHYLATED GENE</b>			
<i>TGFB3</i>	Transforming growth factor $\beta$ 3	Promotes embryonic development	Hypomethylated and downregulated

### 2.2.7 Shared differentially expressed and methylated genes

Fourteen genes were both differentially expressed and differentially methylated between control stage IX oocytes that matured and stage IX oocytes that failed to mature because of embryonic exposure to TBCO (Figure 2.7). Among these, *TGFB3*, which has been shown to play a role in oocyte maturation, was hypomethylated in the gene body by -26.70% and was downregulated by -2.8-fold.



**Figure 2.7.** Scatter plot showing genes that were significantly differentially expressed and differentially methylated between control stage IX oocytes that matured and stage IX oocytes that fail to mature because of embryonic exposure to TBCO, in F1 adult Japanese medaka. Hypomethylated and upregulated genes are shown in green. Hypomethylated and downregulated genes are shown in cyan. *ENPP3*, *GLA*, *SCARB1*, *MRV11*, *ACAP2B*, *CXADR*, *IGSF11*, *NFATC1*, *TGFB3*, *ABCD4*, *FGFR3*, *PCYOXI* were hypomethylated in the gene body and downregulated. *PHKG1A* and *PAOX* were hypomethylated in the gene body and upregulated. Genes were differentially expressed if log<sub>2</sub> fold change was  $\geq 1.2$  and were differentially methylated if percentage methylation difference was greater than  $|25\%|$ .

## 2.4 DISCUSSION

F1 progeny of Japanese medaka exposed to dietary TBCO experienced significantly decreased fecundity. This is consistent with previous studies using the same route of exposure

and nominal concentrations of TBCO (Devoy et al., 2023b; Devoy et al., 2023a). In this study, the concentration of TBCO in F1 embryos was not quantified. However, in separate studies using the same dietary concentrations of TBCO, Devoy et al. (2023a) reported that the concentrations of TBCO in eggs were  $711.3 \pm 234.0$  and  $2535.5 \pm 423.1$  ng/g ww and  $46.1 \pm 25$  and  $422.6 \pm 240.6$  ng TBCO/g ww. While there was variation between these two studies, both observed maternal transfer of TBCO to F1 eggs.

Decreases in fecundity of fish exposed as embryos to EDCs can be caused by reduced levels of E2 and VTG. Microinjection of zebrafish embryos with serial doses of benzo[a]pyrene and benzo[k]fluoranthene caused a dose dependent decrease in plasma E2, plasma VTG, and fecundity at sexual maturity (Doering et al., 2024). In another study, embryonic exposure of zebrafish to benzophenone-3 decreased plasma concentrations of E2 and decreased fecundity at adulthood (Tao et al., 2023). However, Devoy et al. (2023b) demonstrated that the decreased fecundity of Japanese medaka exposed to maternally deposited TBCO led to impairment of MIH-stimulated oocyte maturation, arguing that decreased plasma concentrations of E2 and VTG were not sufficient to have caused the observed decreases in fecundity. In the present study, MIH-stimulated maturation of fully grown stage IX oocytes from F1 adult Japanese medaka that were the progeny of a F0 generation exposed to the same nominal concentrations used by Devoy et al. (2023b) was decreased by 16.1% and 22.3% respectively, which is similar to the magnitude of reduction in that prior study.

In the present study, we hypothesized that decreased MIH-stimulated oocyte maturation in female medaka exposed as embryos to TBCO might be due to changes in expression of genes that regulates oocyte maturation resulting from altered DNA methylation. Japanese medaka undergo two DNA methylation reprogramming events, one in early embryogenesis and the other

during primordial germ cell specification (Wang & Bhandari, 2020a). Contaminant exposure during embryonic development can alter patterns of DNA methylation and these alterations can escape reprogramming and persist to later life-stages, altering phenotypic outcomes (Bhandari et al., 2015; Manikkam et al., 2013). Results of the present study supports this hypothesis as more than 5000 DMRs were found in the genome of oocytes that failed to mature in response to MIH. Of those DMRs, 96.3% were hypomethylated, primarily in the gene body. Similar to this, 71% of differentially methylated genes in testis of zebrafish exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) during gonad development were hypomethylated in adulthood, with many of these changes occurring within 100kb of linked genes (Akemann et al., 2019). Furthermore, embryonic exposure of zebrafish to bisphenol A caused extensive changes to DNA methylation, with 13,379 of the 20,474 differential methylated sites localized in the gene body (Penglase et al., 2019). Additionally, other studies that investigated effects of ELS exposure of fish to EDC have also shown greater changes to DNA methylation in gene bodies than in promoter regions (DeCourten et al., 2020; Major et al., 2020). The interplay between DNA methylation and gene expression is complex. Gene body methylation is often inversely correlated with promoter methylation and has been shown to be a better indicator of gene expression, as intragenic methylation is frequently associated with transcriptional activation whereas gene body hypomethylation is associated with gene silencing (Jones, 2012; Lou et al., 2014). In the present study, 14 genes were both differentially expressed and methylated. Of these, 12 genes were hypomethylated in the gene body with methylation difference between (-25 to -50), and decreased transcript abundance within the range of (-1.3 to -4.4). This further supports the suggestion that loss of methylation in the gene body might be positively correlated with gene silencing (Yang et al., 2014). The remaining two genes, *PKHGIA* and *PAOX*, were

hypomethylated in their gene body, but expression was upregulated. PHKG1A had a methylation difference of -25.6 and transcript abundance of 3.3 fold, whereas, PAOX had a methylation difference of -25.1 and transcript abundance of 1.5 fold. Jjingo et al. (2012) reported that gene body DNA methylation in the human genome exhibits a non-linear relationship with gene expression. They showed that moderately expressed genes display the highest levels of gene body methylation, whereas both lowly and highly expressed genes exhibit lower levels of methylation. Among the shared genes, TGF $\beta$ 3 belongs to the TGF $\beta$  superfamily and encodes the transforming growth factor  $\beta$ 3 protein. TGF $\beta$ 3 had decreased transcript abundance and was hypomethylated in the gene body. TGF $\beta$ 3 is highly conserved in bony fish (Laing et al., 1999), however, little is known about its role in oocyte maturation in fish. In humans, TGF $\beta$ 3 can influence oocyte maturation by regulating follicular thromboxane production (Hsuan Lai et al., 2024). Other members of the TGF $\beta$ 3 gene family have been shown to regulate oocyte maturation in fish. Recombinant TGF $\beta$ 1 protein added to culture medium inhibited MIH-induced oocyte maturation in zebrafish follicle (Kohli et al., 2003). Future studies should investigate the role of TGF $\beta$ 3 in fish oocyte maturation.

KEGG enrichment analysis identified the MAPK, Insulin, GnRH, oocyte meiosis, and progesterone-mediated oocyte maturation signalling pathways. Several of the hypomethylated genes within these pathways encode proteins that regulate oocyte maturation. Resumption of meiosis from arrest at prophase I marks the start of oocyte maturation (Nagahama & Yamashita, 2008). This resumption involves interplay among multiple signalling pathways that decrease intraoocyte concentrations of cAMP (Takahashi & Ogiwara, 2023). In most fish species, including Japanese medaka, oocyte maturation occurs via the AC/cAMP/PKA signalling pathway. Binding of MIH to the  $\alpha$  isoform of the membrane progesterin receptor (mPR $\alpha$ ) activates

a pertussis toxin-sensitive inhibitory G protein (Gi), from which the G $\alpha$ i subunit dissociates from the G- $\beta\gamma$  dimer (Takahashi & Ogiwara, 2023). The G $\alpha$ i subunit inhibits adenylyl cyclase thereby suppressing cAMP production, resulting in inactivation of protein kinase A (PKA), thereby lifting the inhibitory regulation of maturation promotion factor (MPF), a protein complex that initiates oocyte maturation by inducing germinal vesicle breakdown (GVBD) and first polar body formation (Khan & Maitra, 2013; Nagahama & Yamashita, 2008).

Results of the present study suggest that cAMP synthesis might have been dysregulated in oocytes that failed to mature because of embryonic exposure to TBCO. Two genes, *GNAI3* and *GNAS*, were hypomethylated in the gene body. *GNAI3* encodes the G $\alpha$ i protein, which inhibits adenylyl cyclase thereby reducing production of cAMP, allowing for meiotic resumption (Das et al., 2016a). The imprinted gene, *GNAS*, codes for several proteins, including the G $\alpha$ s isoform XL $\alpha$ s (Weinstein et al., 2007). Activation of the G $\alpha$ s coupled to GPER stimulates adenylyl cyclase activity to increase cAMP production, leading to meiotic arrest (Das et al., 2016a). Because protein products of *GNAI3* and *GNAS* have opposing effects on adenylyl cyclase activity, it is not clear what the effect on oocyte maturation would be. Additional evidence of potential adenylyl cyclase mediated dysregulation of intracellular cAMP is the increased abundance of *rgs2*. RGS2 negatively regulates G-protein signalling and reduces activity of various isoforms of adenylyl cyclase (Kehrl & Sinnarajah, 2002; Salim et al., 2003). If RGS2 protein levels were increased in oocytes that failed to mature, that would inhibit G $\alpha$ s activity thereby decreasing synthesis of cAMP, which in turn allows for oocyte meiotic resumption. Studies on RGS2 in teleost are limited, however, in mice, loss of RGS2 does not impair meiotic resumption, as measured by GVBD (Jiang et al., 2016). Our analysis also identified hypomethylation in the gene bodies of *PRKAR1B* and *PRKAR2AA*, two genes that

encode regulatory subunits of cAMP-dependent protein kinase A (PKA). These subunits, also termed R1 and R2, are bound to two inactive catalytic subunits (Duncan et al., 2006). Binding of cAMP to the R1 and R2 activates the catalytic subunits via phosphorylation (Khan & Maitra, 2013). The specific role of these regulatory subunits in fish has not been determined, but PKA is an important regulator of oocyte maturation. PKA inhibitors induced resumption of meiosis in climbing perch (*Anabas testudineus*) (Khan & Maitra, 2013), zebrafish (Maitra et al., 2014), and Ayu (*Plecoglossus altivelis*) (Yamamoto et al., 2008). Hypomethylation of the genes encoding PKA regulatory subunits in the current study might have affected expression of PKA. Taken together, hypomethylation of *GNAS*, *PRKAR1B*, and *PRKAR2AA*, as well as increased *rgs2* transcript abundance, suggest that dysregulation of the AC/cAMP/PKA signalling pathway might have led to the inhibition of oocyte maturation in stage IX oocytes that failed to mature in response to MIH.

In addition to the AC/cAMP/PKA signalling pathway, MIH can also stimulate oocyte maturation via the IGFR/PI3K/AKT/PDE3A signalling cascade (Das et al., 2018; Das et al., 2016a). Ovarian insulin-like growth factors (IGFs) regulate the phosphatidylinositol-3-kinase (PI3K)/AKT pathway (Das & Arur, 2017). Activation of the G $\beta$  $\gamma$  subunit coupled to mPR $\alpha$  after binding by MIH can transactivate the IGF1 receptor to stimulate PI3K/AKT signalling (Baloucouné et al., 2012; Schafer & Blaxall, 2017; Tan & Thomas, 2014). MIH-induced PI3k/AKT signaling precedes the onset of GVBD in a cAMP sensitive manner and plays a role in downregulating PKA, possibly by stimulating phosphodiesterase (PDE3a) to lower intra-oocyte concentrations of cAMP in zebrafish (Das et al., 2013; Das et al., 2018). Nelson and Van Der Kraak (2010) reported that recombinant human IGF-i and IGF-ii stimulated GVBD in fully grown follicles of zebrafish. Studies with zebrafish and walking catfish (*Clarias batrachus*) have

also shown that stimulation of oocyte maturation by IGF-1 can occur independent of MIH (Hajra et al., 2016; Nelson & Van Der Kraak, 2010). However, in white bass (*Morone chrysops*) and white perch (*Morone americana*), MIH is needed to stimulate IGF-1 induced oocyte maturation (Weber et al., 2007; Weber & Sullivan, 2005).

In the present study, methylation and transcript abundance of several genes along the IGFR/PI3K/AKT/PDE3A signalling cascade were altered in stage IX oocytes that failed to mature in response to MIH. Two genes, *IGFR1A* and *IGFR1B* were hypomethylated in the gene bodies. These genes encode the IGF receptors that regulate IGF signaling in zebrafish follicular cells and oocytes (Li et al., 2015). In addition, *PIK3CB* and *PIK3CD*, both of which encode components of the PI3K/AKT signaling pathway, were hypomethylated in their gene bodies. These genes encode the class 1A PI3K catalytic subunits p110 $\beta$  and p110 $\delta$ , respectively (Vanhaesebroeck et al., 2010). Activation of receptor tyrosine kinase, such as IGF1R, activates class 1 PI3K to generate PIP3, thereby activating AKT (Vanhaesebroeck et al., 2010). Additionally, transcript abundance of *pik3r6* was decreased by 3.6-fold in stage IX oocytes that failed to mature in response to MIH. *PIK3R6*, also known as p87, encodes the regulatory subunit of class 1B PI3K (PI3K $\gamma$ ) (Vanhaesebroeck et al., 2010). It has been proposed that MIH can stimulate AKT phosphorylation in zebrafish by rapid activation of PI3K $\gamma$  via the  $\beta\gamma$  subunit of a heterotrimeric G-protein coupled to mPR $\alpha$  (Das et al., 2018). Wortmannin, a PI3K inhibitor, reversed insulin/IGF1R induced oocyte maturation in the walking catfish follicles, demonstrating PI3K/AKT signaling as a key downstream effector of Igf1r activation (Hajra et al., 2016). PI3K/AKT signaling is required for IGF1/IGF1R induced oocyte maturation, however, PI3K inhibition can only delay MIH-induced oocyte maturation in denuded zebrafish oocytes, which suggests that MIH induced oocyte maturation is not dependent on the PI3K/AKT pathway (Das

et al., 2016a). Nonetheless alteration of PI3K in this study might have alter the synergism between PI3K/AKT and MIH induced oocyte maturation in regulation of cAMP. Gene body hypomethylation also occurred in *PDE3a*. In zebrafish, AKT phosphorylation stimulates PDE3A enzymatic activity to break down intraoocyte cAMP, which allows for meiotic resumption (Das et al., 2018). Overall changes in DNA methylation of the genes encoding IGF1 receptors might have altered PI3K/AKT signalling, disrupting oocyte maturation.

In addition to cAMP, cyclic guanosine 5'-monophosphate (cGMP) also regulates oocyte maturation in fish (Li et al., 2018). In zebrafish, cGMP regulated oocyte maturation is mediated by a cGMP-dependent protein kinase (PKG), which is a serine/threonine specific protein kinase (Li & Bai, 2020). The cGMP analog, 8-Br-cGMP, stimulates oocyte maturation via PKG in zebrafish preovulatory follicles in a dose dependant manner, and PKG inhibitors block cGMP's stimulatory effect on oocyte maturation (Li et al., 2018). PKG is encoded by multiple genes, including *PRKG1A* that is expressed in the ovaries of most teleost (Li et al., 2018). In our study, *PRKG1A* was hypomethylated in the gene body. Dysregulation of cGMP signalling may contribute to the impaired maturation of stage IX oocytes following embryonic EDC exposure.

Although hypomethylation in the gene body suggests gene silencing, DNA methylation does not always correlate to gene expression (Mattei et al., 2022). However, these changes shown in our study suggest that maternal deposition of TBCO during these sensitive periods of embryogenesis and sex PCGs specification might have altered DNA methylation patten of these genes that escaped the reprogramming phases, thereby causing latent effect leading to adulthood phenotypic outcome.

## **Conclusion**

We have previously demonstrated decreased fecundity of sexually mature female medaka that were exposed as embryos to maternally deposited TBCO (Devoy et al., 2023b; Devoy et al., 2023a). Mechanistically, this was demonstrated to be caused by impairment of MIH-stimulated oocyte maturation. The current study confirms those previous findings and expands our mechanistic understanding of this effect. Specifically, we show extensive changes to DNA methylation and gene expression (changes in transcript amounts) in stage IX oocytes that failed to mature because of embryonic exposure to TBCO. Embryonic TBCO exposure caused gene body hypomethylation and changes in transcript abundance of key genes along the AC/cAMP/PKA and IGFR/PI3K/AKT/PDE3A signalling cascades that regulate MIH-stimulated oocyte maturation. These studies provide additional evidence that early life stage exposure of fish to chemical stressors can affect the DNA methylation leading to somatic epigenetic transmission that affects phenotypic outcome in later life.

## CHAPTER 3: DISCUSSION

### 3.1 INTRODUCTION

There is growing awareness that exposure of fish to endocrine disrupting chemicals (EDCs) during early developmental windows can impair physiological performance of later life stages. This later life phenotypic effect in the absence of the initial stressor is termed an intergenerational effect (Al-Griw et al., 2017). Multiple studies have shown that short term exposure to EDCs can have long lasting effects on organisms (Heard & Martienssen, 2014; Tao et al., 2023). DNA methylation is an important regulator of gene expression (Lou et al., 2014; Yang et al., 2014). Because patterns of DNA methylation are mitotically stable, changes to DNA methylation caused by early life-stage exposure to EDCs have been proposed as the cause of intergenerational effects of EDCs (Gao et al., 2018; Labbé et al., 2017; Schübeler, 2015).

Previous research from our lab demonstrated that embryonic exposure of Japanese medaka (*Oryzias latipes*) to the brominated flame retardant, TBCO, decreased fecundity of sexually mature female fish (Devoy et al., 2023a). Mechanistically, reduced fecundity is well-known to be caused by reduced level of E2 and VTG synthesis (Doering et al., 2024; Doering et al., 2019). However, Devoy et al (2023b) demonstrated that impairment of oocyte maturation, the final stage of oogenesis that gives rise to a fertilizable oocyte, was the mechanism by which embryonic exposure to TBCO impaired the reproductive performance of adult female medaka. The objective of the research presented in this thesis is to identify the molecular mechanism(s) of this impairment of oocyte maturation. Because DNA methylation is an important determinant of gene expression and is mitotically stable, we hypothesized that embryonic exposure to TBCO alters DNA methylation patterns, resulting in altered gene expression, and impairment of oocyte maturation. To test this hypothesis, 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. F1 eggs were collected during the final week of exposure and reared to sexual maturity and fecundity was assessed using a 21-day reproduction assay. At the end of the assay, an *ex vivo* oocyte maturation was used to determine whether maturation inducing hormone (MIH)-stimulated oocyte maturation was impaired. Additionally, RNA-seq and EM-seq was used to investigate t DNA methylation and gene expression in stage IX oocyte that fail to mature because of embryonic exposure to TBCO.

### **3.2 PHENOTYPIC EFFECT OF EMBRYONIC EXPOSURE TO TBCO IN ADULT JAPANESE MEDAKA**

The present study shows that embryonic exposure of Japanese medaka to TBCO inhibited fecundity. Specifically, fecundity of Japanese medaka at sexual maturity following embryonic exposure to TBCO was reduced by 11.3% and 31.4% relative to the control group as shown in (Figure 2.1). Our lab has previously shown the same effect with similar magnitude in Japanese medaka exposed embryonically to TBCO via maternal transfer (Devoy et al., 2023a). Other studies have shown similar effects of embryonic exposure to EDCs. Embryonic exposure to benzophenone-3 led to inhibition of fecundity in adult zebrafish (*Danio rerio*) (Tao et al., 2023). Additionally, early life stage (ELS) exposure of fathead minnows (*Pimephales promelas*) to 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) caused reproductive impairment leading to reduced fecundity (Thornton et al., 2016).

Egg production in fish requires successful completion of oogenesis. A decrease in fecundity is usually associated with a reduction in E2 and VTG that are critical for the growth phase of oogenesis (Doering et al., 2024; Doering et al., 2019). Chen et al. (2021) suggested that reduced transcription of VTG and reduced concentrations of E2 in blood plasma level might have led to a decrease in fecundity and matured oocytes in adult zebrafish exposed during ELS to phenanthrene. However, Devoy et al. (2023b), demonstrated that decreased MIH-stimulated

oocyte maturation, but not decreased plasma concentrations of E2 or decreased expression of vitellogenin, likely caused the decreased fecundity of sexually mature female Japanese medaka exposed as embryos to TBCO. Consistent with this mechanism, results of the current study demonstrate that MIH-stimulated oocyte maturation was decreased by 16.1% and 22.3% in Japanese medaka exposed as embryos to low and high doses of TBCO.

### **3.3 MOLECULAR EFFECT OF EMBRYONIC EXPOSURE TO TBCO IN ADULT JAPANESE MEDEKA**

Oocyte meiotic arrest and resumption are regulated by intra-oocyte cAMP levels (Craig et al., 2011). For example, in the Asian stinging catfish (*Heteropneustes fossilis*), 2-hydroxyestradiol-17 decreased the total cAMP level and induced germinal vesicle breakdown (GVBD), the oocyte maturation indicator, and IBMX, a cAMP elevating drug inhibited 2-OHE2-induced oocyte maturation (Mishra & Joy, 2006). In MIH-induced oocyte maturation, adenylate cyclase (AC) activity is decreased resulting in a reduction of cAMP levels via the AC/cAMP signalling pathway (Takahashi & Ogiwara, 2023). Additionally, studies have shown that MIH-induced oocyte maturation can be regulated via PI3K/AKT signalling pathway by acting independent or synergistically with AC/cAMP signalling pathway to induce GVBD (Das et al., 2018; Pace & Thomas, 2005). Given their essential role in regulation of oocyte maturation, changes in expression of genes along these pathways would likely lead to disruption of oocyte maturation. TBCO induced disruption of DNA methylation could lead to changes in expression of these genes.

Patterns of DNA methylation regulate gene expression, and therefore it is critical for proper development and physiological performance. Following fertilization, Japanese medaka embryos undergo two waves of demethylation (embryogenesis and primordial germ cell

specification) to erase inherited methylation marks and establish de novo methylation patterns crucial for proper development (Wang & Bhandari, 2020a). Therefore, because patterns of DNA methylation patterns are mitotically stable, gene expression in later life is influenced by patterns of gene expression established during early development (Labbé et al., 2017). Several studies have shown that chemical exposure during methylome reprogramming lead to phenotypic alteration in later life, even in the absence of the initial stressor. For example, embryonic exposure of zebrafish to phenanthrene led to reduced mature oocyte and fecundity, altered transcript abundance of several reproductive genes, and hypermethylation of gonadotropin-releasing hormone 3 (*gnrh3*), essential for successful reproduction (Chen et al., 2021). In addition, embryonic exposure of zebrafish to benzo(a)pyrene without further re-exposure resulted in hypermethylation of gonadotropin-releasing hormone 3 and its receptor, with a decrease in mature oocyte and fecundity at adulthood (Gao et al., 2018).

In the present study, methylation and expression (measured as transcript abundance) of multiple genes along pathways that regulate oocyte maturation were altered IN fully grown stage IX oocytes that failed to mature in response to MIH. Most differentially methylated genes were hypomethylated in the gene body. DNA methylation is usually categorized as hypomethylation indicating methylation loss and hypermethylation indicating addition of methylation. Methylation in the promoter region of a gene often correlates to gene silencing (Jones, 2012). Conversely, methylation in the gene body has been suggested to be positively correlated with gene expression (Jjingo et al., 2012; Yang et al., 2014).

The current study shows hypomethylation in the gene body of genes that code for proteins in the AC/cAMP/PKA signalling cascade that regulates oocyte maturation. *GNAS* and *GNAI3*, which encode the stimulatory and inhibitory G-protein  $\alpha$  subunits that regulate adenylyl

cyclase activity on cAMP production, respectively, were hypomethylated; GNAS promotes cAMP synthesis, whereas GNAI3 suppresses it. Inhibition of adenylyl cyclase activity is sufficient to promote oocyte meiosis in zebrafish (Das et al., 2016a). Since it has been suggested that gene body hypomethylation correlates positively with gene silencing, the results suggest that both the stimulatory and inhibitory action of adenylate cyclase could have both been downregulated in oocytes from female fish exposed as embryos to TBCO. Although these effects would appear contradictory, they suggest that changes in methylation of genes encoding adenylyl cyclase activity might have caused dysregulation of intra-oocyte cAMP levels. Additionally, increased transcript abundance of the *rgs2* further suggest that the effects on oocyte maturation might have been caused by altered cAMP levels. Roy et al. (2006) suggested that *rgs2* acts as a negative regulator of adenylyl cyclase activity by inactivating G-protein  $\alpha$  subunits. This further shows that changes in adenylyl cyclase activity might have led to inhibition of MIH-induced oocyte maturation.

Results of this study also suggest that impaired oocyte maturation might have been caused by hypomethylation in the gene body of genes that encode proteins of the PI3K/AKT pathway. Das et al. (2016a) suggested that the PI3K/AKT can act as an auxiliary pathway independent of MIH or synergistically with MIH signaling via upstream activation of IGF receptor to induce oocyte maturation in fish. In addition, several studies have reported that activation of the PI3K/AKT pathway can induce oocyte maturation in fish (Das et al., 2013; Pace & Thomas, 2005; Tan & Thomas, 2014). PI3K/AKT signaling can be activated by the receptor tyrosine kinase, IGF1R (Vanhaesebroeck et al., 2010). IGF1R is encoded by two paralogous genes, *IGF1Ra* and *IGF1Rb*, both of which exhibited gene body hypomethylation in the present study. The G-protein  $\beta\gamma$  heterodimer coupled to mPR $\alpha$  can transactivate IGF1R to activate the

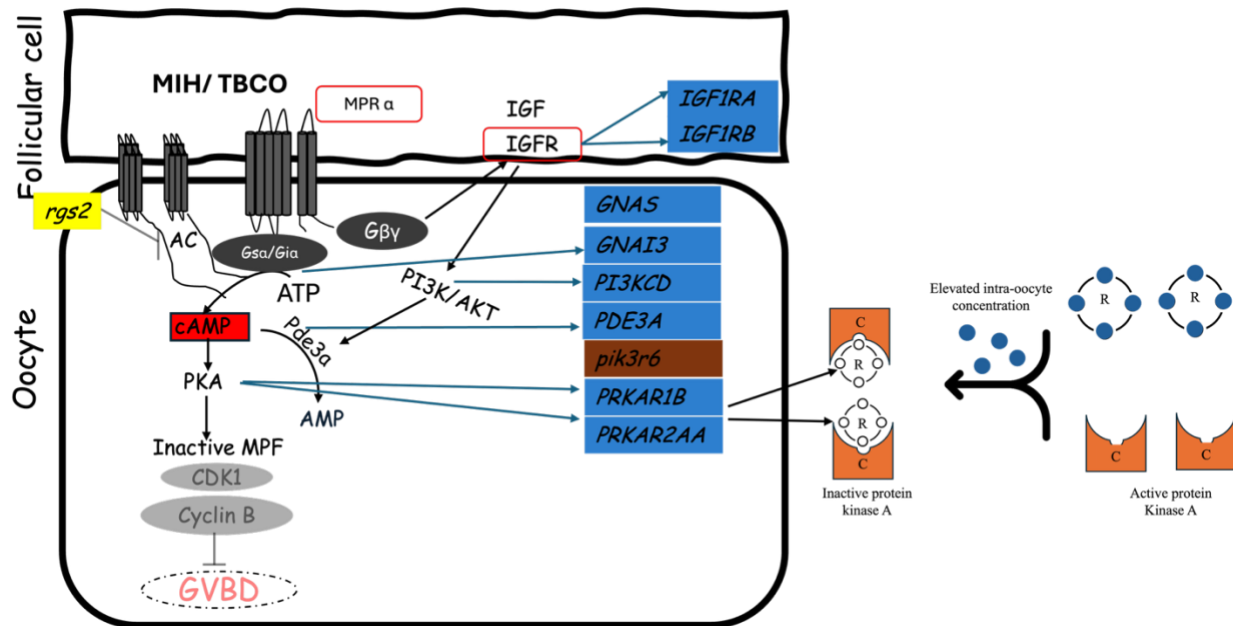
PI3K/AKT pathway. Furthermore, two class 1 PI3K genes, *PIK3CB* and *PIK3CD* coding for their catalytic subunit p110 $\beta$  and p110 $\delta$  protein respectively, showed gene body hypomethylation in the present study. In addition, *Pik3r6* transcript abundance was decreased in oocytes that failed to mature in response to MIH. *Pik3r6*, also known as p87, encodes the class 1B PI3K (PI3K $\gamma$ ) regulatory subunit (Vanhaesebroeck et al., 2010). Treatment of oocytes with a PI3K inhibitor has been shown to impede oocyte maturation (Das et al., 2013; Tan & Thomas, 2014). Furthermore, PI3K/AKT stimulation of oocyte maturation have been suggested to induce oocyte maturation via activation of PDE3A, which breaks down cAMP allowing for meiotic resumption (Das et al., 2013; Das et al., 2018). Remarkably, *PDE3A* also exhibited gene body hypomethylation in our study. Changes along the PI3K/AKT pathway might yet present another mechanism that explains why oocyte maturation was decreased in response to embryonic exposure to EDC.

Results of this study also suggest that protein kinase A (PKA) signalling was disrupted by embryonic exposure to TBCO. The AC/cAMP/PKA and PI3K/AKT pathways regulate intra-oocyte concentrations of cAMP. When intracellular cAMP levels are high, cAMP binds to the regulatory subunits of protein kinase A (PKA), releasing active catalytic subunits. Activated PKA maintains maturation-promoting factor (MPF) in an inactive state through inhibitory phosphorylation of its catalytic CDK1 subunit, thereby preventing germinal vesicle breakdown and enforcing oocyte meiotic arrest (Das et al., 2017). Conversely, a reduction in cAMP signaling leads to MPF activation and resumption of oocyte maturation (Yamamoto et al., 2008). In the current study, PKA regulatory genes (*PRKAR1B*, *PRKAR2AA*) showed hypomethylation in the gene body. These gene code for the regulatory subunit, R1 and R2 of PKA (Duncan et al., 2006). It has been demonstrated in mammals that knockdown of the regulatory subunit of PKA

led to loss of PKA activity (Duncan et al., 2006; Kovo et al., 2006). PKA serves as the final effector of cAMP -mediated regulation of MIH-induced oocyte maturation via MPF, suggesting that disruption of PKA signalling may arise from perturbations in AC/cAMP and IGFR/PI3K/AKT pathways.

### **3.4 PROPOSED MODEL OF OOCYTE MATURATION INHIBITION**

Based on the results of the current study, we propose a model in which embryonic exposure to TBCO impairs oocyte maturation at sexual maturity. We suggest that maternally deposited TBCO alters DNA methylation patterns of several regulatory genes during early developmental stages, and a subset of these epigenetic changes escape DNA methylation reprogramming during embryogenesis and primordial germ cell specification. These mitotically stable alterations persist into later life stages, leading to changes in transcript abundance of genes, thereby reducing mature oocyte following MIH induction at adulthood, ultimately resulting in decreased fecundity. Specifically, *GNAS*, *GNAI3*, *IGF1RA*, *IGF1RB*, *PI3KCD*, *PIK3CB*, *PDE3A*, *PRKAR1B* and *PRKAR2AA* exhibited gene body hypomethylation, while *rgs2* and *pik3r6* showed altered transcript abundance within the AC/cAMP/PKA and IGFR/PI3K/AKT/PDE3A/PKA signalling pathway that are essential for oocyte maturation.



**Figure 3.1** Proposed model of impaired MIH-stimulated oocyte maturation in sexually mature Japanese medaka exposed as embryos to TBCO. The model proposes that inhibition of oocyte maturation results from disruption of the AC/cAMP and IGF1R/PI3K/AKT/PDE3A signalling pathways due to altered expression of genes encoding proteins in these pathways. Genes that are hypomethylated in the gene body are in blue boxes, downregulated genes are in brown boxes, and upregulated genes are in yellow box. *GNAS* and *GNAI3*, whose protein products regulate adenylyl cyclase (AC) were hypomethylated in the gene body, while *rgs2*, a negative regulator of  $G\alpha$ -dependent adenylyl cyclase signalling was upregulated. Altered expression of these genes is predicted to disrupt the AC/cAMP signalling cascade. Additionally, *IGF1RA*, *IGF1RB*, *PI3KCB*, *PI3KCD*, and *PDE3A* were hypomethylated in the gene body and *pik3r6* transcript was downregulated, suggesting perturbation of IGF1R/PI3K/AKT signalling. Alteration of both pathways is hypothesised to alter intra-oocyte cAMP concentrations. cAMP regulates oocyte maturation through its downstream effector, protein kinase A (PKA). *PRKAR1B* and *PRKAR2AA*, encoding PKA regulatory subunits, were hypomethylated in the gene body. High cAMP levels promote PKA activity by releasing the holoenzyme catalytic subunit, thereby blocking GVBD formation leading to meiotic arrest and inhibiting oocyte maturation. Taken together, these molecular changes may contribute to reduced oocyte maturation success following MIH stimulation.

### 3.5 ROLE OF OTHER EPIGENETIC FACTORS IN REGULATING GENE EXPRESSION

DNA methylation is the most studied mechanisms of gene expression regulation. Because patterns of DNA methylation are mitotically stable, a mechanism for how chemically induced

changes to DNA methylation patterns in early life-stages of fish can lead to intragenerational effects was investigated. However, other epigenetic mechanisms regulate gene expression, including histone modifications and non-coding RNAs (Cavalieri & Kathrein, 2022; LeBaron et al., 2010). Our DNA methylation study revealed hypomethylation of KDM5C, a histone lysine specific demethylase 5C in the promoter region, suggesting that expression of histone modifiers might be altered following embryonic exposure of fish to EDC. Environmental chemicals can alter histone modifications, which can have long lasting effects on gene expression (Aluru, 2017). Interestingly, our transcriptomic data revealed decreased *hdac7b* transcript abundance, the histone deacetylase 7b. The most common histone modifiers are acetylation and deacetylation. Acetylation favours transcriptional activation by relaxing DNA coiling from histone cores, allowing transcription factors to engage with regulatory region (He et al., 2014). Histone deacetylase is positively correlated with transcriptional repression which occurs by tightening DNA-histone complex (Wang et al., 2023). These modification involves the addition of their functional group to a lysine on a specific histone tail, which can alter the chromatin structure to favor gene expression or silencing (Aluru, 2017). In recent years, non-coding RNA (ncRNAs) have been shown to regulate gene expression regulation. ncRNA includes microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-associated RNAs (piRNAs) and long coding RNAs (Brander et al., 2017). These ncRNAs can act by interfering with transcribed mRNA by binding to it and by remodeling chromatin structure to silence genes (Brander et al., 2017). piRNA aid in silencing retrotransposons in germline and act as a key component in spermatogenesis (Chuma & Nakano, 2013). Furthermore, ethanol treatment of two cell stage zebrafish embryos disrupts the miRNA, miR-9 role in gene expression (Pappalardo-Carter et al., 2013).

Multiple studies suggest that DNA methylation, histone modification and ncRNA work together to regulate gene expression by influencing chromatin structure (Brander et al., 2017). Gene activity is primarily modulated through modification of chromatin structure. Chromatins exist in two states, heterochromatin, where DNA is wrapped tightly by proteins and euchromatin where DNA is loosely wound and support gene expression. ELS exposure of TCDD to zebrafish, without further re-exposure led to reduced fecundity and fertility at adulthood (Baker et al., 2013). This effects was attributed to differential methylation of subset of DNMTs, histone methylases and demethylases, and miRNA suggesting roles of other epigenetic factor in later-life effects of ELS exposure to EDCs (Akemann et al., 2019). In the present study, we quantified DNA methylation and mRNA to understand the mechanistic underpinning behind intragenerational latent effect due to embryonic exposure to EDC. Future studies should quantify RNAseq to determine changes in expression of ncRNAs and investigate other epigenetic factor alongside DNA methylation to further understand what role they might play in later life phenotypic outcome in fish due to early life stage exposure to EDC.

### **3.6 CONCLUSION**

In this study, we show that embryonic exposure to the EDC, TBCO via maternal transfer led to reduced fecundity via MIH-induced oocyte maturation in adult Japanese medaka. This phenotypic outcome was likely caused by differential methylation and transcript abundance of genes mechanistically relevant to the AC/cAMP and IGFR/PI3K/AKT signaling pathways regulating oocyte maturation in fish. This study provides further evidence that early life exposure of fish to environmental chemical can cause disrupt the epigenome altering gene expression leading to phenotypic outcome in adulthood. Future studies should investigate the role of other epigenetic factors following early life stage exposure to fish to better understand the correlation

between epigenome alteration and gene expression along with how they lead to phenotypic outcome.

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