INHIBITION OF OOCYTE MATURATION BY ORGANOPHOSPHATE INSECTICIDES IN ZEBRAFISH (*DANIO RERIO*) AFTER *IN VITRO* AND *IN VIVO* EXPOSURE.

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INHIBITION OF OOCYTE MATURATION BY ORGANOPHOSPHATE INSECTICIDES IN ZEBRAFISH (DANIO RERIO) AFTER IN VITRO AND IN VIVO EXPOSURE

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DEDICATION

I dedicate this thesis to my parents Peter and Bev, and my siblings Jamie and Kelly. I would like to give a special dedication to my late grandfather Lloyd Surkan, who instilled my passion for nature and fish during my childhood.

ABSTRACT

Oogenesis is the process by which a primary oocyte develops into a fertilizable oocyte. During the final step in zebrafish (*Danio rerio*) oogenesis, oocyte maturation, the maturation inducing hormone 17α,20β-dihydroxy-4-pregnen-3-one (MIH) activates the membrane progesterone receptor (mPR), inducing germinal vesicle breakdown. Anthropogenic stressors can dysregulate oocyte maturation. However, it is unknown waterborne exposure to chemicals that perturb maturation after *in vitro* exposure also disrupt MIH induced maturation *in vitro*, or if the *in vitro* assay is predictive of reproductive performance. Here, we demonstrate that malathion and dimethoate inhibit oocyte maturation when oocytes are exposed *in vitro* or *in vivo*, suggesting that the *in vitro* zebrafish oocyte maturation assay might be predictive of alterations to reproductive performance. This research introduces the oocyte maturation assay as being predictive of the success of *in vitro* oocyte maturation after *in vivo* exposure, but it remains unclear whether this assay is predictive of reproductive impairment.

PREFACE

The candidate is the main author of chapters 1-3. The candidate primarily conceived, executed, and evaluated the experiments completed in chapter 2. Dr. Steve Wiseman provided funding for this research. Both Dr. Steve Wiseman and Dr. Jon Doering contributed to the scientific input and guidance of chapter 1-3. Dr. Jon Doering, Justin Dubiel, Kaden Fujita and Yamin Raza contributed to the experimental take downs involved in chapter 2. Dr. Markus Brinkmann developed the protocol and analysis pipeline for analytical chemistry evaluation in chapter 2.

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
AO	Adverse outcome
AOP	Adverse outcome pathway
AR	Androgen receptor
Bb	Balbiani body
CAK	Cyclin activating kinase
cAMP	Cyclic adenosine monophosphate
CDC2	Cyclin-dependent kinase 1
cGMP	Cyclic guanosine monophosphate
CYP	Cytochrome P450 enzyme
DEHP	Bis(2-ethylhexyl) phthalate
DES	Diethylstilbestrol
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
ER	Estrogen receptor
ERE	Estrogen responsive element
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G_i	G-inhibitory protein
GnRH	Gonadotropin releasing hormone
GNRHR	Gonadotropin releasing hormone receptor
GPCR	G-protein coupled receptor
GPER	G-protein coupled estrogen receptor
G_s	G-stimulatory protein
GSC	Germline stem cell
GSI	Gonadosomatic index
GV	Germinal vesicle

GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotropin
HPGL	Hypothalamus-pituitary-gonad-liver
HSI	Hepatosomatic index
K	Fulton's condition factor
KE	Key event
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LV	Lipovitellin
MAPK	Mitogen-associated protein kinase
MIE	Molecular initiating event
MIH	17α,20β-dihydroxy-4-pregnen-3-one
MPF	Maturation promoting factor
mPR	Membrane progesterone receptor
nPR	Nuclear progesterone receptor
OECD	Organization for economic co-operation and development
PAQR	Progesterone and adipoQ receptor
PDE	Phosphodiesterase
PVDF	Polyvinylidene difluoride
PGC	Primordial germ cell
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol-3,4,5-triphophate
PKA	Protein kinase A
PV	Phosvitin
SHBG	Sex hormone binding globulin
TGF-β	Transforming growth factor beta
VTG	Vitellogenin
VTGR	Vitellogenin receptor

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

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 The endocrine system

Reproduction in fish is a complex process that is under neuroendocrine control of the hypothalamus-pituitary-gonad-liver (HPGL) axis. The neuroendocrine system secretes hormones that have effects on peripheral glands or target tissues. The HPGL system is dynamic and regulated by use of positive and negative feedback loops to mediate the secretion of hormones. An overview of HPGL regulation of reproduction is provided below.

1.1.1 Initiation of GnRH signalling

Release of gonadotropin releasing hormone (GnRH) from the hypothalamus is a regulated and coordinated process beginning with kisspeptin signalling. Kisspeptin is a neuropeptide that has been highly studied in mammals and fishes since its significance in reproduction and the HPGL axis was elucidated, but less research has been conducted in the zebrafish (Roa et al., 2008). Kisspeptin activates a G-protein coupled receptor (GPCR) to trigger the release of GnRH from GnRH neurons. In zebrafish, there are two kisspeptins, KISS1 and KISS2, that interact with their cognate GPCRs, KISSR1a (GPR54-1) and KISS1Rb (GPR54-2; Biran et al., 2008; Lee et al., 2009; Gopurappilly et al., 2013). In contrast to mammals, knockout of both zebrafish kisspeptins does not affect reproductive capabilities, despite undoubted support of the role kisspeptins play in GnRH secretion (Skorupskaite et al., 2014; Tang et al., 2015).

There are upwards of 23 variants of GnRH that comprise three families of GnRH (GnRH 1-3). Most vertebrates express GnRH1, and expression of GnRH2 (also known as chicken GnRH) is conserved across all vertebrates (Fernald and White, 1999; Millar et al., 2004; Löhr and Hammerschmidt, 2011; Perrett and McArdle, 2013). Zebrafish have GnRH2 and GnRH3

(salmon GnRH), a GnRH that only teleosts are known to have (Miyamoto et al., 1984; Millar et al., 2004; Löhr and Hammerschmidt, 2011). Surprisingly, double knockout of either or both GnRHs does not impact reproduction in zebrafish as they have a redundant reproductive strategy involving differential expression of genes outside the GnRH cell system (Spicer et al., 2016; Marvel et al., 2018).

1.1.2 Gonadotropin signalling

Stimulation of the adenohypophysis by GnRH has long been known to control the release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), functions of which have been intensively studied in fishes (Schally et al., 1971). Gonadotropins are heterodimeric proteins that share a GTHα domain and have hormone specific LHβ or FSHβ domains (So et al., 2005). Two isoforms of GTHα have been identified in goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), and species of salmon, but zebrafish are only known to express a single GTHα isoform (So et al., 2005). Expression of *gthα*, *fshβ*, and *lhβ* has been demonstrated in the pituitary of zebrafish (So et al., 2005). In zebrafish, the promotor region of *LHβ* contains many binding sequences for regulatory elements including C/EBPb, which can be stimulated by cyclic adenosine monophosphate (cAMP; Park et al., 1990; Chen and Chiou, 2010). Teleost FSH is less well known in terms of regulatory element binding and initiation of transcription.

Upon release, FSH and LH are secreted into the bloodstream where they are bound to sex hormone binding globulin (SHBG) to travel to the gonads where they can bind to their respective receptors, FSHR and LHR (Miguel-Queralt et al., 2004). Gene knockout studies have revealed the promiscuous nature of LH and FSH binding to LH receptor (LHR) and FSH receptor (FSHR), respectively (Kawauchi et al., 1989). When zebrafish FSHR is expressed in Chinese

hamster ovary cells, FSH activates FSHR. However, LH can activate both the FSHR and LHR in the same cellular system, but a greater concentration of LH is required to activate FSHR in comparison to LHR (So et al., 2005; Zhang et al., 2015). Interestingly, follicle development, onset of puberty in females, and maintenance of female status are impaired in zebrafish deficient in *FSHR* (Zhang et al., 2015). Knockout of *LHR* in female zebrafish resulted in infertility, as indicated by the failure to spawn (Zhang et al., 2015). Expression of FSHR and LHR in oocytes is dynamic during stages of oogenesis. For example, *fshr* increases in stages I and II and peaks at stage III, whereas expression of the *lhr* is low during stages I-III and peaks just prior to maturation in stage IV (Kwok et al., 2005). Within the ovary of zebrafish, FSH stimulates folliculogenesis through synthesis of 17β-estradiol (E2), while LH stimulates oocyte maturation via synthesis of 17α,20β-dihydroxy-4-pregnen-3-one (MIH, maturation inducing hormone; Nagahama and Yamashita, 2008). Both FSH and LH play a role in regulation of steroidogenesis and thus are crucial in modulating oogenesis (Figure 1.1).

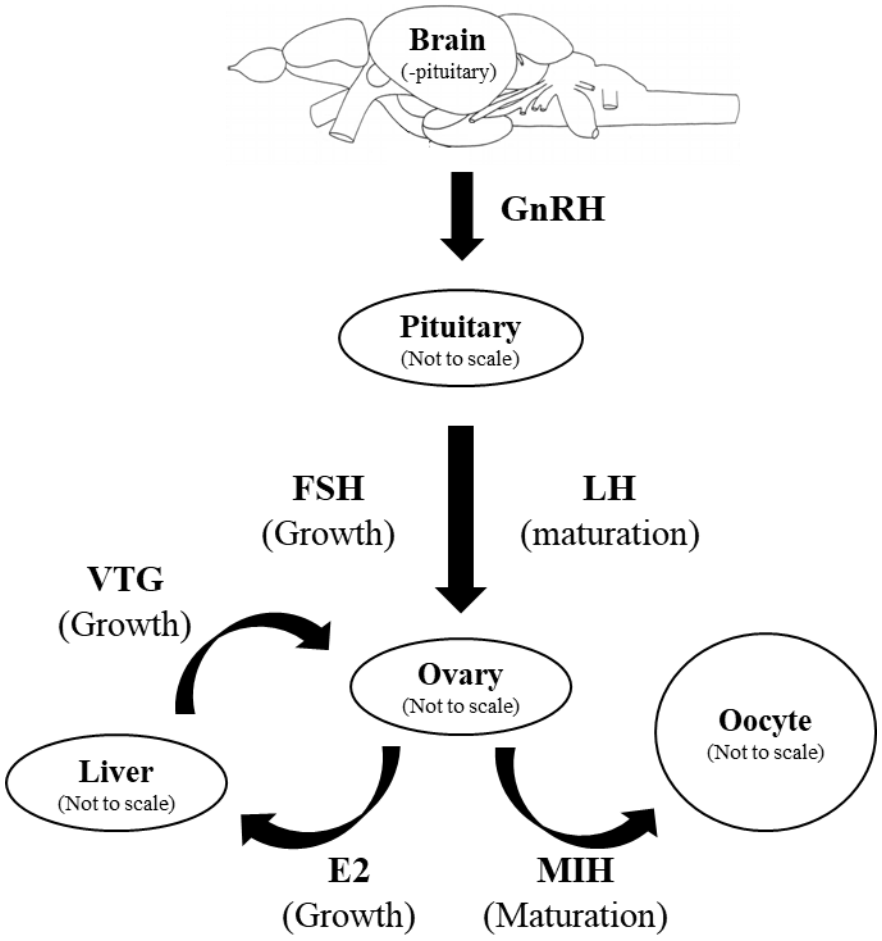

Figure 1.1: Schematic illustration of endocrine signalling in the hypothalamus-pituitary-gonad-liver (HPGL) axis in female zebrafish. The neuropeptide kisspeptin activates GnRH neurons in the hypothalamus (not pictured) to release GnRH to the pituitary. The gonadotropins follicle stimulating hormone (FSH) or luteinizing hormone (LH) are secreted from the pituitary to the bloodstream to regulate growth (FSH) or maturation of oocytes (LH) in the ovary. Once bound to its cognate receptor, FSH coordinates a steroidogenic pathway leading to the transport of 17β-estradiol (E2) to the liver to initiate synthesis of vitellogenin (VTG) for the growth oocytes. When the LH receptor is activated, it causes synthesis of 17α,20β-dihydroxy-4-pregnen-3-one (MIH) to induce maturation of immature oocytes.

1.2 Oogenesis in zebrafish

In zebrafish, oogenesis is a sequential and asynchronous process that is divided into major occurrences that result in the ovulation of an egg that was generated from primordial germ cells (PGC; Patiño and Sullivan, 2002). Seminal work completed in 1993 by Selman established five stages of development of the oocyte and initiated a stream of research that elucidated in fine detail the major steps of oogenesis in zebrafish (Selman et al., 1993). Each of these stages are marked by biochemical and morphological differences within the oocyte. Typically, the process of oogenesis can be parsed into three phases: oocyte growth (stages I-III), oocyte maturation (stage IV), and a mature oocyte (stage V). Following the differentiation of a PGC into a germ cell fate rather than a somatic one, oogonium progenitor cells set the stage for the development of the oocyte (Yoshizaki et al., 2002; Draper, 2012). Developing oocytes are locked in prophase I until maturation, allowing the oocyte to proceed through metaphase II (Nagahama and Yamashita, 2008). The intricacies of these phases are explained below.

1.2.1 Phase 1: Growth

1.2.1.1 Germline stem cells

Germline stem cells (GSC) are ~7-10 μm in size, immortal, and undifferentiated cells that are responsible for sustained egg production in fishes (Yoshizaki et al., 2002; Draper, 2012). During embryogenesis, germline arises from PGCs, which contain a germ plasm comprised of maternally deposited RNA and protein (Yoshizaki et al., 2002; Clelland and Peng, 2009). Zebrafish PGCs form before gastrulation and migrate from germinal ridges to the gonadal anlagen (presumptive gonad) to associate with somatic gonad cells (Clelland and Peng, 2009). This migration is highly passive but is regulated by the chemokine somatic cell derived-factor 4 (SDF1a) that attract PGCs with the SDF1a receptor, CXCR4b (Knaut et al., 2003). Complete

migration patterns of PGCs have been extensively studied and reviewed (Yoshizaki et al., 2002; Knaut et al., 2003). All zebrafish GSCs initially develop an ovary-like gonad that either gives rise to a mature ovary or develops into testis during sexual differentiation (Clelland and Peng, 2009). Like the mammalian GSCs, zebrafish GSCs display the molecular marker *vasa*, are unipotent (will differentiate into either spermatogonia or oogonia), and in females, reside within a stem cell niche to produce oocytes (Yoon et al., 1997; Beer and Draper, 2013). Evidence of GSC unipotency and sexual plasticity in zebrafish came in 2011 when pre-meiotic germ cells of females were transplanted into sterile male fish, resulting in sperm production (Wong et al., 2011).

1.2.1.2 Stage I

Oocytes in stage I can be divided into two substages, stages Ia and Ib, which are defined by the formation of a follicular cell layer. Stage I oocytes are pre-vitellogenic, entering the diplotene stage prophase I, and are 7-140 μm in size (Wallace and Selman, 1990; Selman et al., 1993; Patiño and Sullivan, 2002). The pre-follicular phase of primary growth of the oocyte is stage Ia. In this stage, oocytes reside within blastocysts of the ovary and are 7-20 μm in size. Germline stem cells undergo type II divisions producing a cystoblast, which is a cyst surrounded by a follicle layer allowing for the production of stage Ia oocytes within, eventually yielding multiple stage Ib oocytes enclosed in their own follicle layer (Selman et al., 1993; Saito et al., 2007). Stage Ia has been referred to as the chromatin-nucleolus phase and constitutes the first stage of development of the oocytes arisen from GSCs. The term ‘chromatin nucleolus phase’ was coined by Yamamoto in 1956 due to the appearance of chromosomes within the nuclei of the diplotene stage. Chromosome visibility due to condensation during later stages of development in Ia oocytes mark the zygotene and pachytene stages of prophase I. Stage Ia

oocytes do not enter pachytene until leaving the blastocyst nest. As they develop, oocytes gain a pre-follicular cell layer, which marks stage Ib.

Primary growth stage begins with oocytes becoming enclosed in a follicular layer and accordingly this stage is termed the follicular phase of primary growth. These stage Ib oocytes are 20-140 μm in size and upon follicle formation and chromosome decondensation are locked in the diplotene stage of prophase I where they will remain until oocyte maturation (Lyman-Gingerich and Pelegri, 2007). During this stage, organelles begin to proliferate, the balbiani body (Bb) forms, oocytes become enclosed in a follicular layer, and the germinal vesicle (GV; nucleus) becomes apparent (Selman et al., 1993; Lyman-Gingerich and Pelegri, 2007). Many nucleoli can be visualized within the GV at this stage but with growth of the oocyte comes nuclei movement to the periphery of the GV, which also becomes enlarged. These nuclei are proposed to produce ribosomal mRNA (Wallace and Selman, 1990). Squamous granulosa cells enclose the oocyte and represent the granulosa cell layer of the follicle, which lies upon a basement membrane of the oocyte. Microvilli extend from both the oocyte and granulosa layer to connections containing gap junctions to allow small molecules to pass (Kessel et al., 1985). The granulosa layer is then encased within the vascularized theca cell layer, completing the formation of the follicle (Figure 1.2A).

Many subcellular structures form in Stage I. These include organelles important to the production of maternal factors, such as mitochondria, golgi, and endoplasmic reticulum, and the Bb (Lyman-Gingerich and Pelegri, 2007). The Bb is a transient structure that contains organelles like mitochondria, golgi bodies, and endoplasmic reticulum, as well as maternal transcripts and proteins (Kloc et al., 2004; Langdon and Mullins, 2011). The Bb establishes the vegetal pole and marks the first step in establishing polarity of the animal and vegetal axes (Kloc et al., 2004;

Langdon and Mullins, 2011). During stage Ia of oogenesis the Bb forms adjacent to the nucleus, translocates to the future vegetal pole via action of BUCKY BALL, and establishes asymmetry of the oocyte through the release of its contents in stage Ib (Marlow and Mullins, 2008; Bontems et al., 2009; Langdon and Mullins, 2011).

1.2.1.3 Stage II

Stage II of oogenesis marks the completion of primary growth and entry into the cortical alveolus stage, or primary vitellogenesis (Selman et al., 1993; Lubzens et al., 2010). At this point in development, oocytes are 140-340 μm in size and are phenotypically characterized by developing cortical alveoli (yolk vesicles), that lack a membrane and increase in size due to the accumulation of glycoproteins and in number during this stage (Selman et al., 1993). Other morphological changes include the growth of the GV and increase in the number of nucleoli within, elongation of mitochondria within the ooplasm, and the formation of a tripartite vitelline envelope (zona radiata) between the oocyte and granulosa cell layer formed in stage I (Selman et al., 1993). During this stage, microvilli on the exterior of the oocyte protrude through the granulosa cell layer via pore canals (Lyman-Gingerich and Pelegri, 2007). Although vitelline envelope formation at stage II was reported in 1993, more recent studies contest that this morphological change occurs at stage III (Selman et al., 1993; Kaviani et al., 2013). Few genes important to vitelline envelope formation in zebrafish have been identified. Zebrafish vitelline envelope protein was identified to be vitelline envelope specific, the mRNA of which is found in stage I ooplasm and is translated to protein and translocated out of the oocyte via exocytosis (Xu et al., 2009).

1.2.1.4 Stage III

Stage III is the final growth stage in oogenesis and is termed vitellogenesis, or 'yolk deposition'. Vitellogenesis is the major growth stage in oocytes of zebrafish, resulting in variable sized oocytes ranging from 340-690 μm. Other morphological changes that occur within the oocyte during this stage include the centrally located GV of the oocyte beginning to migrate away from the opaque ooplasm. As yolk bodies from stage II accumulate, cortical alveoli distribute to the periphery from their association with the golgi, where they release their contents into the zona radiata during egg activation and hydration (Selman et al., 1993; Patiño et al., 2001; Lubzens et al., 2010). As oocytes proceed through early oogenesis (stage IIIa), they accumulate molecular factors that are critical for maturation of the oocyte. By mid- to late-vitellogenesis (stage IIIb), these oocytes have accumulated these factors and have gained the ability to maturate when induced by hormones (Zayed et al., 2020).

Induction of vitellogenesis in most fishes requires E2 synthesis in follicular cell layers, regulated by neuroendocrine control, specifically an increase in synthesis and release of FSH. Binding of FSH to FSHR activates the Δ4 and Δ5 steroidogenic pathways within theca and granulosa cell layers, both of which are essential to the production of E2 (Figure 1.2B). Induction of E2 synthesis begins in the thecal cell layer with increased transport of cholesterol to the mitochondria by steroidogenic acute regulatory protein (Stocco, 2000). Within the mitochondria, side chain cleavage of cholesterol by cytochrome P450 (CYP) side chain cleavage (P450scc) generates a common steroid precursor, pregnenolone, which can then react with 3β-hydroxysteroid dehydrogenase (3β-HSD) to generate progesterone, or be hydroxylated by CYP17-I (17α-hydroxylase) to 17α-hydroxypregnenolone. Both progesterone and 17α-hydroxypregnenolone can be converted to 17α-hydroxyprogesterone by 17α-hydroxylase activity

of CYP17-I or 3β-HSD, respectfully. Cytochrome P450C17 (CYP17) has dual functions within the oocyte, acting as a 17α-hydroxylase or 17,20-lyase (Nagahama and Yamashita, 2008). In the genomes of tilapia (*Nicotilus oreochromis*), medaka (*Oryzias latipes*), and zebrafish, there are two isoforms of CYP17, CYP17-I and CYP17-II, to delineate hydroxylase and lyase functions, respectively (Zhou et al., 2007a; Zhou et al., 2007b; Nelson and Van Der Kraak, 2010b). Dehydroepiandrosterone and 17α-hydroxyprogesterone can undergo enzymatic action by CYP17 to generate androstenedione, which is converted to testosterone (T) by 17β-hydroxysteroid dehydrogenase (17β-HSD) in the thecal cell layer and E2 is generated from T by aromatase (CYP19a) in the granulosa cell layer (Figure 1.2B; Clelland and Peng, 2009). Thus, aromatase conversion of T to E2 is an essential mediator of maintaining meiotic arrest. An alternative pathway to generate E2 involves 17α-hydroxypregnenolone being converted to dehydroepiandrosterone by CYP17-II, which is acted upon by 3β-HSD to generate androstenedione, from which E2 is generated by aromatase. Based on *in vitro* incubations of separated follicle layers by Nagahama and Yamashita (2008), it was determined that the thecal cell layer is responsible for T synthesis, while the granulosa layer converts T to E2 via aromatase (Figure 1.2B).

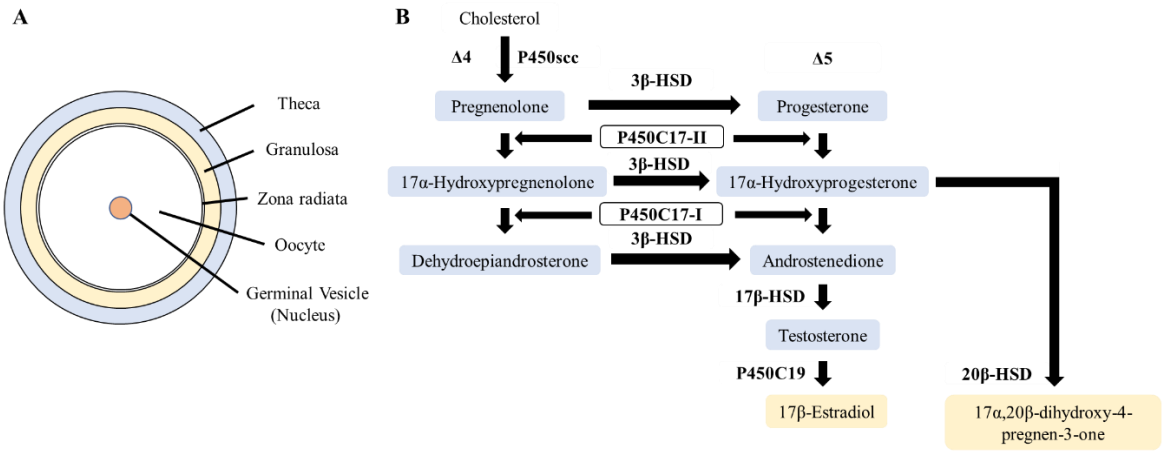

Figure 1.2: A simplistic diagram of an oocyte with outer theca and inner granulosa follicle layers (A) and the Δ4 and Δ5 steroidogenic pathways within the theca (blue) or granulosa (yellow) cell layers (B). The theca and granulosa cell layers are separated by a basement membrane (not pictured) and from the oocyte by the zona radiata (A). The Δ4 and Δ5 steroidogenic pathways generate 17β-estradiol for oocyte growth and 17α,20β-dihydroxy-4-pregnen-3-one (MIH) for oocyte maturation. Increased activity of cytochrome P450C19 (aromatase) is required to convert testosterone to E2, while increased activity of 20β-HSD is required for MIH synthesis. Panel B is adapted from Nagahama and Yamashita (2008).

Estrogen receptor (ER) signalling stimulates vitellogenin (VTG) synthesis. Once E2 is generated in the granulosa layer, it is bound by SHBG in blood plasma and diffuses into hepatocytes to induce VTG synthesis by activating a cytosolic ER (Miguel-Queralt et al., 2004). There are two ERs in zebrafish, ERα and ERβ, which form homo- or heterodimers to regulate gene transcription (in this case, *vtg* transcription; Sullivan and Yilmaz, 2018). Once ER is bound by E2, it undergoes a conformational change allowing it to recruit co-factors for transcription. This complex is translocated to the nucleus where it dimerizes, allowing it to bind an estrogen response element (ERE) in the promotor region of *VTG* (Clelland and Peng, 2009; Sullivan and Yilmaz, 2018). Next, VTG is synthesized in ribosomes of the rough endoplasmic reticulum from *vtg*, loaded into secretory vesicles and folded at the golgi, and secreted into the bloodstream for transport to the ovary (Sullivan and Yilmaz, 2018).

There are key molecular players involved during early vitellogenesis. Vitellogenin is a large (300-600 kDa) glycophospholipoprotein which is synthesized in the liver and considered the 'yolk-precursor protein'. Vitellogenin consists of an N-terminus, lipovitellin (LV) heavy chain, phosvitin (PV), a LV light chain, a β-component, and lastly a C-terminus domain (Sun and Zhang, 2015). In zebrafish, there are eight isoforms of VTG that constitute three major types of VTG (VTG-I, VTG-II, and VTG-III) based on subdomain structure (Yilmaz et al., 2018). Hepatic VTG is transported by blood and sequestered by oocytes to allow VTG to pass through the basement membrane of the follicle layer and through pore canals of granulosa cells to reach the oocyte surface and forms a complex with the VTG receptor (VTGR; Patiño and Sullivan, 2002). This complex is then internalized within clatherin-coated pits on the surface of the oocyte (Wallace and Selman, 1985; Sullivan and Yilmaz, 2018). Once internalized, the yolk protein domains PV and both LV chains are cleaved by cathepsin D to derive yolk proteins PV and LV

within the oocyte (Sullivan and Yilmaz, 2018). Both chains of LV dimerize to form the major VTG-derived yolk protein of the oocyte that carries structural phospholipids (Yilmaz et al., 2018). PV is the smaller VTG-derived yolk protein that stabilizes LV structure, enhance lipid loading and VTG solubility, inhibits radical-mediated oxidation of biomolecules, and both PV and LV serve nutritional and immune functions (Sun and Zhang, 2015; Yilmaz et al., 2018).

In addition to being essential for the synthesis of VTG, E2 released from the follicular layer acts on a G-protein coupled estrogen receptor (GPER) to maintain meiotic arrest through elevated levels of cAMP. The GPER is a 7-transmembrane domain receptor that is part of the G-protein coupled receptor (GPCR) superfamily (Thomas et al., 2010). A signalling cascade is initiated upon activation of the GPER in which the α domain of a stimulatory G protein (G_s) stimulates adenylyl cyclase activity, maintaining concentrations of cAMP in the oocyte (Figure 1.3A; Pang and Thomas, 2010). The activity of the βγ domain of this G_s is also involved in maintaining arrest via transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinases 1/2 (Pang and Thomas, 2010). This transactivation is proposed to be via a mitogen-activated protein kinase (MAPK) signalling pathway, which is activated by intracellular protein kinase (src) and matrix metalloproteinase, thereby stimulating phosphorylation of MAPK3/1 via activation of MAPK kinase (Peyton and Thomas, 2011). One effect of these signalling cascades is the downregulation of membrane progesterone receptor (mPR) α expression when GPER is activated. Interestingly, when the mPR is activated by MIH, *GPER* and GPER is downregulated, illustrating dual control of meiotic resumption by GPER and mPRα (Pang and Thomas, 2010).

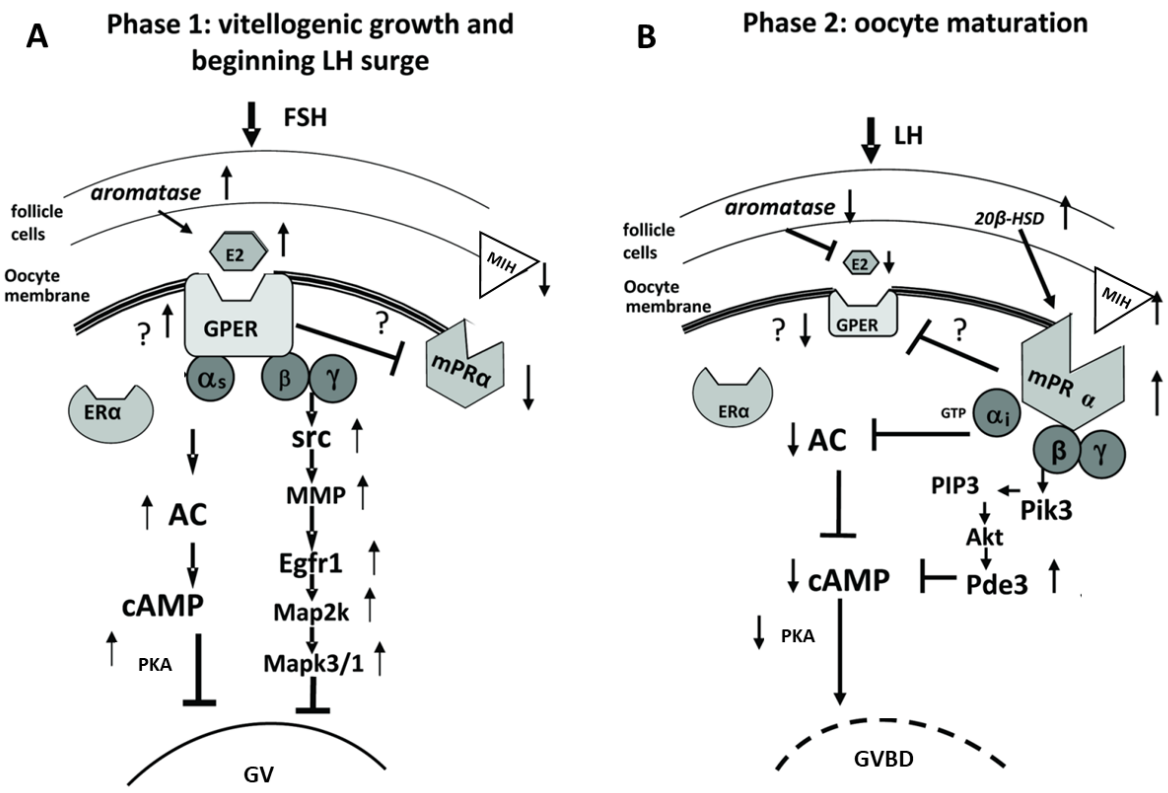

Figure 1.3: Regulation of meiotic arrest during phase 1 (A) and induction of maturation during phase 2 (B). During phase 1 (A), FSH from the pituitary acts on the theca cell layer (not pictured) to cause the synthesis of 17β-estradiol (E2) in the granulosa layer (not pictured). Activation of a stimulatory G-protein coupled estrogen receptor (GPER) causes the G-protein's α subunit to stimulate adenylate cyclase (AC) to maintain high concentrations of inter oocyte cyclic adenosine monophosphate (cAMP). The G protein β-subunit initiates an intracellular protein kinase (src) and matrix metalloproteinase (MMP), thereby stimulating phosphorylation of mitogen activated protein kinase (MAPK) 3/1 via activation of MAPK kinase (MAP2k). Upon LH stimulation of theca cells in phase 2 (B) there is an increase in 20β-hydroxysteroid dehydrogenase and decrease in aromatase, causing synthesis of 17α,20β-dihydroxy-4-pregnen-3-one (MIH) that activated the membrane progesterone receptor (mPR). Activation of mPR induces the α-subunit of an inhibitory G-protein to reduce AC activity, thereby reducing cAMP and PKA. The β-subunit recruits a phosphatidylinositol 3-kinase (PiK3) to the plasma membrane, initiating the formation of phosphatidylinositol-3,4,5-triphophate (PIP3) that binds protein kinase B (AKT) to reduce cAMP concentrations via cAMP-specific phosphodiesterase 3 (PDE3). Figure adapted from Thomas (2012).

Members of the transforming growth factor (TGF-β) superfamily, specifically bone morphogenic protein 15 (BMP15) and TGF-β, are involved in maintaining oocyte meiotic arrest (Kohli et al., 2003; Clelland et al., 2006; Zayed et al., 2020). Treatment of zebrafish oocytes with BMP15 caused a decrease in the number of oocytes that could undergo oocyte maturation *in vitro*. Further, overexpression of *BMP15* inhibited human chorionic gonadotropin (hCG)-induced oocyte maturation (Clelland et al., 2006). *In vitro* exposure of zebrafish oocytes to TGF-β completely inhibits hCG-induced maturation, and partially inhibits MIH-induced oocyte maturation (Kohli et al., 2003).

By mid- to late vitellogenesis, the developing oocyte has accumulated molecular factors needed for maturation, and can maturate when induced by hormones (Zayed et al., 2020). One family that is able to induce maturation in mid-vitellogenic oocytes are insulin-like growth factors (IGF; Nelson and Van Der Kraak, 2010a). There are three isoforms of teleost IGFs that are spatially and temporally expressed within the oocyte. Both *igf-II's* are expressed in the follicle layers and oocyte, but *igf-IIa* is more highly expressed in the oocyte but not the follicle layer, and the expression pattern of *igf-IIb* is the exact opposite (Nelson and Van Der Kraak, 2010a) However, *igf-III* expression is confined to the follicle layer (Nelson and Van Der Kraak, 2010a). In mid-vitellogenic oocytes there is an increase in *igf3*, suggesting it might play a role in gaining maturational competence. In another study, *in vitro* exposures of mid-vitellogenic oocytes to 100 nM IGF-I or IGF-II induced maturation whereas early-vitellogenic oocytes did not undergo MIH-induced maturation (Nelson and Van Der Kraak, 2010b). In that study it was found that pre-exposure of mid-vitellogenic oocytes to IGF-I, but not IGF-II, increased the oocytes maturational competency in responding to MIH (Nelson and Van Der Kraak, 2010b).

This suggests that IGF-I signalling is important to generating maturationally-competent oocytes (Nelson and Van Der Kraak, 2010b).

1.2.2 Phase 2: Oocyte maturation

1.2.2.3 Stage IV

Oocyte maturation generates fertilizable oocytes. Major changes during oocyte maturation include GV breakdown (GVBD), formation of the first polar body, and chromosome condensation (Nagahama and Yamashita, 2008). The major molecular events responsible for the shift from oocyte growth to oocyte maturation include the steroidogenic shift from FSH to LH, an enzymatic shift from aromatase to 20β-hydroxysteroid dehydrogenase (20β-HSD), synthesis of MIH, and the subsequent activation of mPR that activates maturation promoting factor (MPF; more commonly known as M-phase promoting factor), causing GVBD.

Generation of MIH occurs in the follicle layers of the oocyte. During the shift from oocyte growth to oocyte maturation, there is increased synthesis of 17α-hydroxyprogesterone from progesterone or 17α-hydroxypregnenolone in the thecal layer, which is converted to MIH by 20β-HSD in the granulosa layer (Figure 1.2B; Clelland and Peng, 2009). 20β-hydroxysteroid dehydrogenase functions to reduce the 20-carbonyl group to 20β-hydroxylated groups, and is *de novo* upregulated in follicle layers just prior to maturation (Nagahama and Yamashita, 2008). Interestingly, Young et al. (1986) found that MIH was produced in co-cultures of thecal and granulosa cell layers but was not generated in monocultures of either follicle layer. Based on this finding, Nagahama and Yamashita (2008) proposed the two-cell type model in which 17α-hydroxyprogesterone is generated in the outer thecal cell layer, transverses the basement membrane separating the follicle layers, and increased activity of 20β-HSD causes the synthesis

of MIH in the granulosa cell layer (Figure 1.4). MIH interacts with the mPR on the oocyte surface to initiate a signalling cascade to generate MPF, and induce GVBD (Hanna and Zhu, 2009).

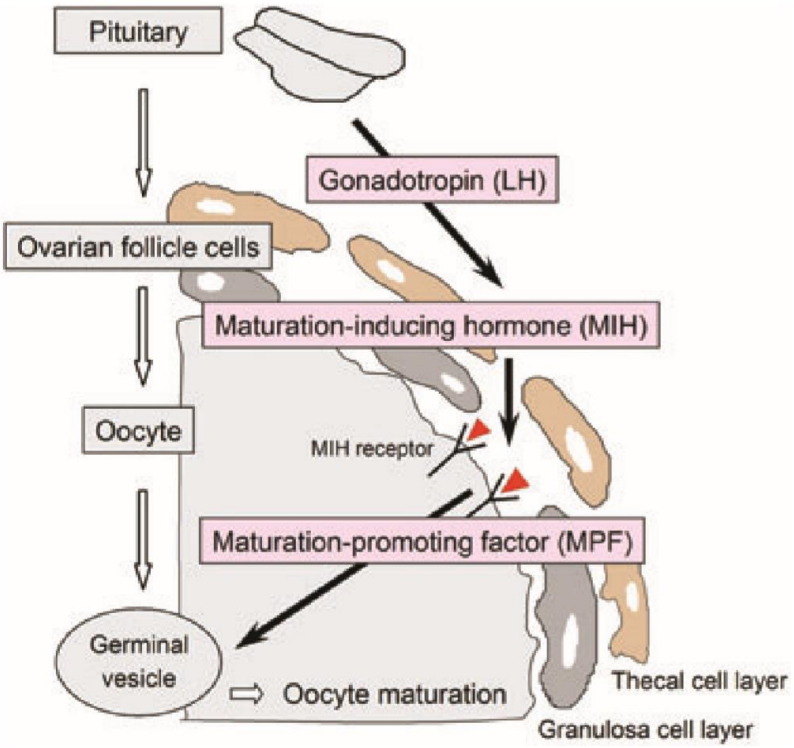

Figure 1.4: A simplistic diagram illustrating the overarching steps in oocyte maturation. Luteinizing hormone (LH) stimulates the synthesis of maturation-inducing hormone in the granulosa cell layer, which transverses a basement membrane and binds to the membrane progestin receptor (mPR). Activation of the mPR induces the synthesis of maturation promoting factor (MPF), which causes germinal vesicle breakdown (GVBD). Figure adapted from Nagahama and Yamashita, 2008.

The mPR is an essential mediator between follicle cell layers and the oocyte during maturation. The family of mPRs include mPRα, mPRβ, and mPRγ, which are members of the progesterone and adipoQ receptor (PAQR) family, of which zebrafish have mPRα (PAQR7) and mPRβ (PAQR8; Zhu et al., 2003; Hanna et al., 2006; Hanna and Zhu, 2009). During the steroidogenic shift to LH, *mprα* and *mprβ* are upregulated and localized to the oocyte membrane to mediate the actions of MIH (Hanna and Zhu, 2009). Interestingly, only *mprβ* is located on the oocyte surface until activation of mPRβ by MIH, at which time mPRα is recruited from cortical granules just below the membrane by progesterone receptor membrane component 1 (PGRMC1; Hanna and Zhu, 2009; Aizen et al., 2018). Zebrafish possess a nuclear progesterone receptor (nPR) that can be activated by MIH in dual luciferase reporter assays, however the localization of nPR mRNA and protein to the follicle cell layers of maturationally competent oocytes implicates that the nPR is not involved in maturation (Hanna et al., 2006; Hanna et al., 2010).

When mPRα is activated by MIH, it induces an inhibitory G protein (G_i) signaling cascade that ultimately causes reduction of concentrations of cyclic guanosine monophosphate (cGMP) in follicular layers, and cAMP within the oocyte, releasing the oocyte from prophase I arrest (Figure 1.3B; Nagahama and Yamashita, 2008; Maitra et al., 2014; Aizen et al., 2018; Li et al., 2018, 2020). Specifically, the α-subunit of the G_i functions to downregulate adenylyl cyclase, in turn lowering concentrations of cAMP and protein kinase A (PKA; Thomas, 2012). When the mPR is activated by MIH, the β-subunit recruits a phosphatidylinositol 3-kinase (PiK3) to the plasma membrane, initiating the formation of phosphatidylinositol-3,4,5-triphophate (PIP3) that binds protein kinase B (AKT) to reduce cAMP concentrations via cAMP- or cGMP-specific phosphodiesterases (PDE) (Figure 1.3B; Peyton and Thomas, 2011).

There are cGMP and cAMP specific PDEs in zebrafish oocytes. Three PDEs, PDE5, PDE6, and PDE9 are cGMP-specific, and PDE3 is cAMP specific (Das et al., 2013; Li et al., 2018, 2020; Li and Bai, 2020). During the steroidogenic shift to LH, cGMP is rapidly degraded by PDE3 in the follicular layer, causing cGMP to diffuse out of the oocyte via gap junctions (Li et al., 2018; Li and Bai, 2020). Because PDE action in the oocyte is repressed due to high levels of cGMP, this rapid diffusion of cGMP out of the oocyte relieves its repression of PDE5, PDE6, and PDE9 to breakdown inter-oocyte cAMP (Das et al., 2013; Li et al., 2018, 2020; Li and Bai, 2020). The reduction of cyclic nucleotides results in a concomitant decrease in PKA via the cAMP/PKA pathway, a crucial step in the initiation of MPF synthesis (Nagahama and Yamashita, 2008).

Although not revealed in zebrafish, activation of MPF in the oocyte is the prevailing mechanism that induces GVBD (Figure 1.5; Nagahama and Yamashita, 2008; Clelland and Peng, 2009). Maturation promoting factor is an M-phase promoting factor and is crucial to meiotic and mitotic progression, therefore it is found in all eukaryotic cells (Nagahama and Yamashita, 2008). This reduction of PKA allows for the *de novo* translation of masked cyclin B mRNA. It has been suggested that a microfilament complex is responsible for masking cyclin B mRNA, as treatment with a microfilament disruptor cytochalasin B causes GVBD through liberation of cyclin B mRNA (Nagahama and Yamashita, 2008). Further, injection of cyclin B into oocytes can induce GVBD without activation of mPR signalling cascades (Kondo et al., 1997). The translated cyclin B then forms a duplex with cyclin-dependent kinase 1 (CDC2). The active form of MPF is generated when this duplex is phosphorylated by cyclin activating kinase (CAK) on threonine 161 (Figure 1.5; Nagahama and Yamashita, 2008; Clelland and Peng, 2009). Activation of MPF is typically mediated by MIH activation of the mPR, however a cAMP-

independent pathway of MPF activation has been proposed. When maturationally competent oocytes are exposed to recombinant human insulin, a phosphoinositide 3-kinase (PI3K)/AKT pathway is initiated to activate CDC2 (Das et al., 2013). In that study, inhibitors of PI3K were unable to inhibit MIH-induced maturation, suggesting that insulin induced maturation is independent of cAMP and functions through PI3K/AKT activation of CDC2 (Das et al., 2013).

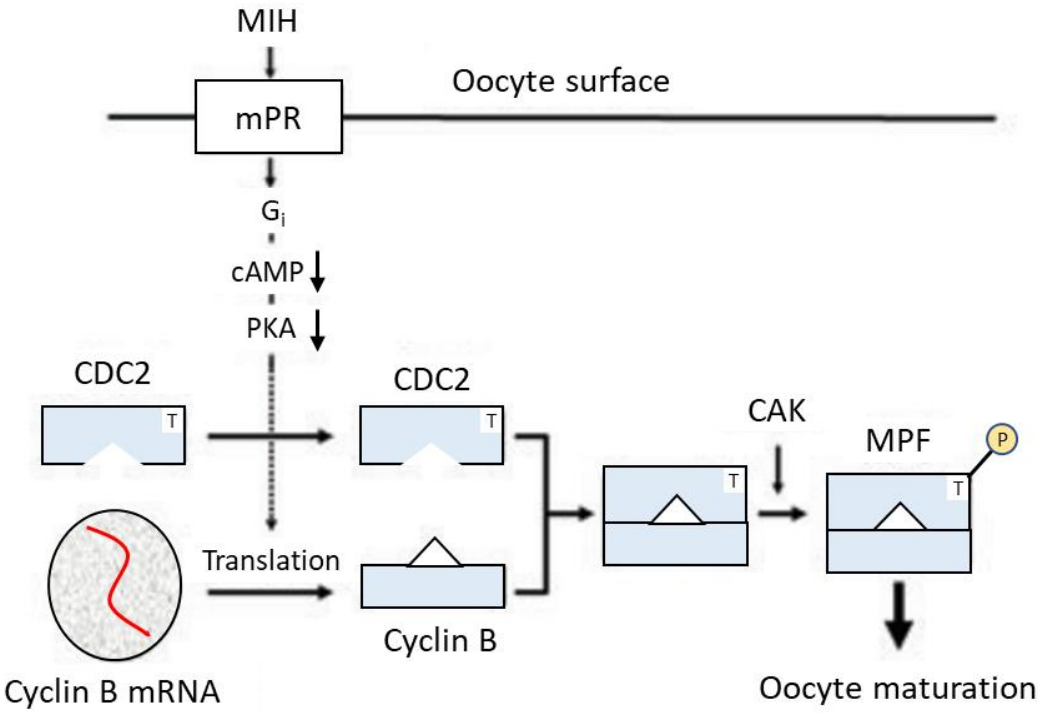

Figure 1.5: Schematic of the activation of maturation promoting factor (MPF). The membrane progesterone receptor (mPR) is activated by 17α,20β-dihydroxy-4-pregnen-3-one (MIH) to induce a G_i signalling cascade that reduces inter-oocyte concentrations of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). Reduction in PKA activity alleviates translational repression of masked cyclin B mRNA, allowing for dimerization with cyclin-dependent kinase 1 (CDC2). When the cyclin B-CDC2 heterodimer is phosphorylated on threonine 161 by cyclin activating kinase (CAK), MPF can induce maturation by germinal vesicle breakdown. Adapted from Nagahama and Yamashita (2008).

1.2.3 Phase 3: The mature oocyte

Stage IV maturation culminates in a mature and fertilizable stage V oocyte. Stage V oocytes are 730-750 μm in size and must become hydrated to be ovulated prior to fertilization. Hydration of zebrafish oocytes is critical to their survival in the hypotonic freshwater environment. Although the underlying mechanism of oocyte hydration in zebrafish is not known, teleostean hydration is thought to be stimulated by MIH, and involve inorganic ions like K^+ and Na^+ that attract water through aquaporins (Skoblina, 2010). Ovulation is governed by genomic mechanisms, but it is proposed that MIH might be involved through the activation of the nuclear progesterone receptors (Nagahama and Yamashita, 2008). Oocytes must rupture the follicular layers to be ovulated, which is achieved through the dissolution of the extracellular matrix of the follicular layer (Nagahama and Yamashita, 2008). Once ovulated, the newformed egg (ovulated oocyte) is able to be fertilized via the micropyle, a pore-like structure within the vitelline envelope that allows the penetration of spermatozoa (Selman et al., 1993; Nagahama and Yamashita, 2008).

1.3 Endocrine disrupting chemicals

Endocrine disrupting chemicals (EDC) are chemicals found in the environment that perturb homeostasis or reproduction by disrupting tissues, glands, or hormones involved in the endocrine system. These exogenous chemicals can be of anthropogenic or natural sources and disrupt male and female reproduction through alterations in estrogenic pathways, membrane and nuclear receptors, steroid action and biosynthesis, activity of steroidogenic enzymes, and disruption of the neuroendocrine system, but effects of EDC are not limited to these pathways or targets (Diamanti-Kandarakis et al., 2009). Recently, a consensus on the 10 key characteristics of EDCs has been established (La Merrill et al., 2020). These key characteristic include but are not

limited to interaction with, activation or antagonization of, and alteration in expression of hormone receptors, and perturbation in synthesis, transport, distribution, and metabolism of hormones (La Merrill et al., 2020).

There are many factors that determine the extent of the adverse effect(s) that EDCs might have on organisms. One important factor is the timing of life stage at which an organism is exposed to an EDC. The timing of an organisms susceptibility to be negatively impacted by chemical exposure is known as a 'critical window' (Mueller, 2018). One EDC that received great attention for its feminization effects on male fathead minnows (*Pimephales promelas*) is the potent synthetic estrogen used in birth control, 17α-ethinylestradiol (EE2). Wastewater treatment plants do not remove EE2 and remains in the environment due to consistent input, therefore concern grew over the potential impact this potent estrogenic compound might have (Kidd et al., 2007). To investigate these effects, fathead minnows were exposed for 7 years in individual lakes of the experimental lakes area in Northwest Ontario and were subsampled in spring, summer, and fall each year to analyze abundances of *vtg* and VTG. Male fish have incredibly low expression of VTG, whereas VTG is highly expressed in females. After exposure, male fish expressed VTG an order of magnitude higher than females and concentrations of VTG were three orders of magnitude greater than reference fish, resulting in intersex gonads. Females also expressed higher *vtg* leading to delayed ovarian development. These effects on males and females lead to a significant population collapse due to failure to reproduce (Kidd et al., 2007). This is only one mechanism of many EDCs that might cause population level effects due to chronic exposure.

1.4 Disruption of oocyte maturation

Major processes that regulate reproduction are targeted by EDCs via interaction with specific protein targets. Targets of EDCs that result in impairment of oogenesis occur through impacts on vitellogenesis, which include antagonism (block interaction of natural estrogens) of the ER, agonism (bind receptor to mimic natural compound) of the androgen receptor (AR), and inhibition of aromatase activity (Ankley et al., 2002; Luccio-Camelo and Prins, 2011; Shanle and Xu, 2011). Through these interactions there is a decrease in plasma E2 and VTG, contributing to decreased oocyte growth (stages 1-3), resulting in less oocytes that can mature. These are well understood mechanisms by which EDCs impact oogenesis, but there are other targets of EDCs such as the mPR that might be an underlying and understudied mechanism of inhibition of oocyte maturation.

Oocyte maturation in fishes can be altered by anthropogenic stressors, including EDCs. Maturation of oocytes from many species of fishes, including goldfish, carp, striped dwarf catfish (*Mystus vittatus*), rainbow trout (*Oncorhynchus mykiss*) and spotted sea trout (*Cynoscion nebulosus*) have been reported, but alteration of oocyte maturation has been studied in greatest detail in zebrafish (Haider and Upadhyaya, 1986; Haider and Moses Inbaraj, 1988; Tokumoto et al., 2004; Rime et al., 2010). It is proposed that chemicals interact with the mPR to alter the number of oocytes able to mature after *in vitro* exposure interact with the mPR (Das and Thomas, 1999; Tokumoto et al., 2004, 2007; Carnevali et al., 2011). This phenomenon has been demonstrated in spotted sea trout, where the organochlorines kepone and O,P'-DDT competitively inhibited binding of the maturation-inducing steroid of spotted seat trout, 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) to its membrane receptor (Das and Thomas, 1999). Alternatively, non-competitive binding of the goldfish mPR by diethylstilbestrol (DES)

has been observed in transfected human MDA-MB-231 cells (Tokumoto et al., 2007). In zebrafish, *in vitro* exposure of stage IV oocytes to malathion, Bis(2-ethylhexyl) phthalate (DEHP), tamoxifen, 4-hydroxytamoxifen, pentachlorophenol, methoxychlor, and genistein decreases the magnitude of success of oocyte maturation, whereas *in vitro* exposure to DES, melatonin, and iprodione induce oocyte maturation (Tokumoto et al., 2004, 2005, 2007; Carnevali et al., 2010, 2011; Maskey et al., 2019). Inhibition of oocyte maturation by malathion has also been demonstrated in other fish species including carp and catfish (Haider and Upadhyaya, 1986; Haider and Moses Inbaraj, 1988; Maskey et al., 2019).

Most knowledge regarding inhibition of oocyte maturation has been investigated *in vitro*, yet very few studies have examined alterations to oocyte maturation after *in vivo* exposure to anthropogenic chemical stressors. However, Carnevali et al (2010) observed that exposure of zebrafish to 2 μg/L DEHP for 21 days decreased expression of *bmp15*, a mediator in prevention of maturation (Carnevali et al., 2010). This group also showed that exposure of zebrafish to 1μM of melatonin increased abundance of *kiss1*, *kiss2*, and *gnrh3* in the brain, and decreased *Tgf-β* and *bmp15*, which was accompanied by increased expression of *mPRα* and *mPRβ* (Carnevali et al., 2011). This suggests that *in vivo* or *in vitro* exposure of maturationally competent oocytes could cause failure of oocyte maturation. However, no study to date has examined the ability of oocytes to mature *in vitro* after *in vivo* exposure.

1.5 Animal research and toxicology

Toxicological testing has experienced a shift toward the use of predictive models for many reasons, including ethics over the use of animals in research as well as the financial and time commitments needed to generate data on a plethora of chemicals that must be evaluated for their potential to cause adverse effects on non-target receptor organisms. Alternative testing

methods and development has been extensively focussed on development of *in vitro* models, assays using established cell lines, and lastly *in silico* models that circumvent laboratory assays (Zhang et al., 2014).

A product of predictive toxicology is the increased importance placed upon the development of adverse outcome pathways (AOPs). These AOPs provide dose-response and time-course information to foster regulatory decision making through the organization of new and existing data into a pathway detailing the events that lead to an adverse outcome (Villeneuve et al., 2014; Conolly et al., 2017). There are three overarching steps involved in an AOP, beginning with a chemical binding or interacting with a biological target, termed a molecular initiating event (MIE). After the MIE has occurred, a series of key events (KE) are induced beginning at the macromolecular level, for example protein oxidation, that causes directly related KE's at the cell and organ level. The KEs are sequential to result in adverse outcome (AO), which can have a population-level effect, such as lethality or impaired reproduction (Figure 1.5; Ankley et al., 2010; Villeneuve et al., 2014).

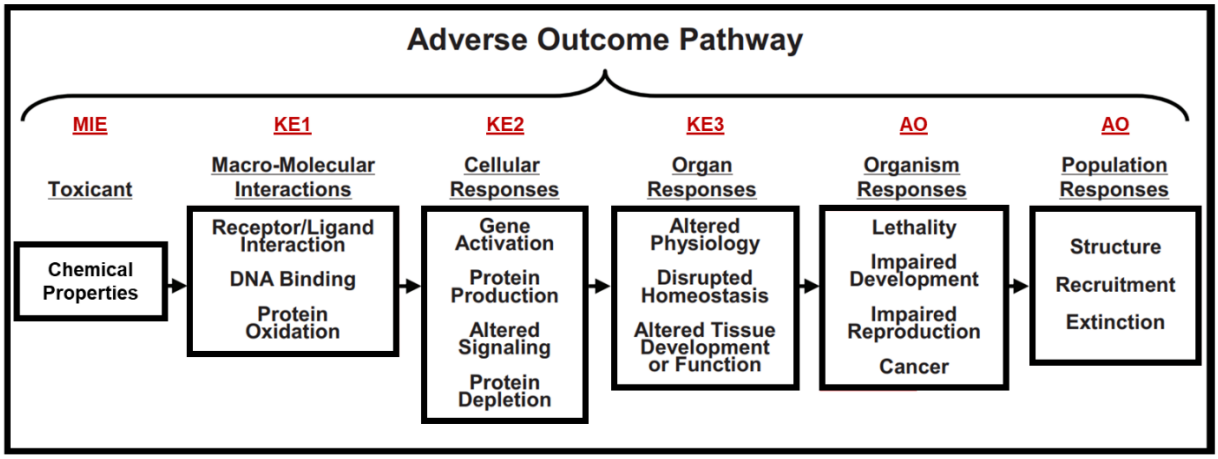

Figure 1.6: A diagram of an example of an adverse outcome pathway (AOP). A molecular initiating event (MIE) from chemical exposure initiates a sequential series of key events (KE) that alter macromolecular interactions, and in turn, processes at the cellular, organ, and organismal responses, leading to an adverse outcome (AO) at the population level. This diagram is adapted from Ankley et al. (2010).

One of the first established AOPs links aromatase inhibition with population level declines in fathead minnow (*Pimephale promelas*; Conolly et al., 2017). In that AOP, aromatase is inhibited (MIE), causing reduced synthesis (KE1) and circulation (KE2) of E2, in turn causing reduced synthesis (KE3) and circulation (KE4) of hepatic VTG. Reduced VTG concentrations lead to impaired oogenesis (KE5) and ovulation (KE6), ultimately manifesting in the AO of a declining population (Conolly et al., 2017). There is a spectrum of completeness that AOPs exist as, beginning with a putative AOPs that do not necessarily identify all overarching steps of an AOP, moving to a true AOP that identifies the link between MIE-KE's-AO, which culminates in a quantitative AOP that contains information about degrees of change needed in a biological parameter to initiate the following KE.

Assays that use animal models to identify chemicals that cause a reproductive impairment are currently in use. These assays are validated and quantify the structure-activity relationship of a variety of chemicals and their biological activity in signal transduction or metabolic pathways (Ellison et al., 2016). Several *in vitro* assays have been developed to model embryotoxicity, teratogenicity, or genotoxicity, but fewer methods have been validated to predict reproductive toxicity, especially in aquatic vertebrates. In fish, Organization for Economic Co-operation and Development (OECD) assay #229: fish short term reproductive assay (OECD, 2012) is the gold-standard *in vivo* assay used to assess reproductive toxicity. Assay #229 is not a predictive assay. This assay involves a 21-day exposure of at least 120 fish to 4 separate treatments (1 control and 3 serial concentrations of the chemical of interest) to evaluate the reproductive parameters fecundity (how many eggs do females deposit per day) and fertilization success (how many eggs deposited are fertilized), and the concentrations of circulating steroids vitellogenin VTG and E2

(OECD, 2012). Ideally, an *in vitro* predictive assay could be used to generate information regarding potential reproductive impairment due to chemical exposure that uses far fewer fish.

Ethical concerns surround animal use in research. A push to better establish principles in ethics of animal research began in 1959 when Russel and Burch established a set of guidelines to improve the life quality of laboratory animals. These guidelines were defined as the three R's: reduction, refinement, and replacement (Russel and Burch, 1959). In modern animal research these are the guiding principles and, in Canada, are regulated by the Canadian Council on Animal Care (CCAC). Reduction in animal use is centered on attaining high-quality results using fewer animal or maximizing that amount of data one experimental animal can provide. Refinement is based upon the quality of life of the experimental animal and the researcher's ethical responsibility to refine procedures to enhance the animals' life. The final of the three R's is the replacement of higher animal models for animals that have a lower potential for pain perception, which is exemplified by the increased use of *in vitro* and *in silico* modelling. The increased use and development of predictive toxicological screening tools strongly align with the ethical principles developed by Russel and Burch (1959) and the evolution of these principles in animal research science.

There is a need to develop alternative screening methods to identify EDCs that use fewer animals. There are established *in vitro* alternative screening assays for a number of EDC endpoints, such as androgen or ER transactivation, and steroidogenesis alteration (Cosnefroy et al., 2009; Higley et al., 2010). As the establishment of these EDC-identifying screening assays continues, non-typical targets of EDCs need to be identified and developed in non-animal models. One non-typical target of EDCs is the mPR. Examinations of oocyte maturation inhibition via mPR interaction occur *in vitro.* A knowledge gap exists regarding if these

chemicals also cause maturation failure after *in vivo* exposure, or if oocyte maturation inhibition is occurring *in vivo*. The failure of oocyte maturation could lead to a decrease in reproductive performance and needs to be further investigated.

1.6 Research goals and objectives

The effects of EDCs are well established, but effects of EDCs on non-typical targets needs to be characterized. Assays of oocyte maturation are used to establish whether a chemical can inhibit maturation, but a structure-activity relationship between chemicals and the mPR has not been established. Importantly, it is unknown if chemicals that inhibit oocyte maturation after *in vitro* exposure will inhibit *in vitro* maturation of oocytes after fish are exposed *in vivo*. Lastly, it remains to be determined if *in vitro* assays of oocyte maturation can be used to predict reproductive successes (fecundity, fertilization) of fish exposed to chemicals *in vivo*.

Against this backdrop, the overall goal of this thesis is to explore if assays of oocyte maturation are predictive of reproductive impairments. To this end, this research will focus on two overarching objectives:

1. Determine whether *in vitro* assays of oocyte maturation are predictive of reproductive performance.
2. Investigate the structure-activity relationship of chemical induced inhibition of oocyte maturation

CHAPTER 2: INHIBITION OF OOCYTE MATURAITON BY ORGANOPHOSPHATE INSECTICIDES IN ZEBRAFISH (*DANIO RERIO*) AFTER *IN VITRO* AND *IN VIVO* EXPOSURE

2.1 Introduction

Reproduction in fishes is a highly complex and tightly regulated process. Oogenesis is crucial to successful reproduction in female fishes and is regulated by the hypothalamic-pituitary-gonadal-liver (HPGL) axis. In zebrafish (*Danio rerio*), there are five stages of oogenesis constituting three major events: oocyte growth (stages I-III), oocyte maturation (stage IV), and a mature, fertilizable oocyte (Stage V; Selman et al., 1993, Patiño and Sullivan, 2002). Growth stages of oogenesis are dependent on follicle stimulating hormone (FSH) secreted from the pituitary, cytochrome P450 (CYP) mediated synthesis of 17β-estradiol (E2) in follicular cells, and synthesis of vitellogenin in the liver and its subsequent transport to developing oocytes (Selman et al., 1993; Lubzens et al., 2010). Regarding oocyte maturation, immature oocytes are arrested at prophase I of meiosis by elevated levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), along with a multitude of other molecular factors (Maitra et al., 2014; Das et al., 2017; Li et al., 2018). In zebrafish, maturation occurs approximately 2 h prior to the onset of light and requires a steroidogenic shift from FSH to luteinizing hormone (LH). The surge of LH induces synthesis of maturation inducing hormone (MIH; 17α,20β-dihydroxy-4-pregnen-3-one) in follicular cells which then binds to and activates an inhibitory G-protein coupled receptor, the membrane progesterone receptor (mPR; Zhu et al., 2003; Hanna and Zhu, 2009; Tokumoto et al., 2011). Activation of the mPR initiates signalling cascades, including the phosphorylation of phosphodiesterase (PDE) enzymes that specifically degrade cAMP or cGMP (Das et al., 2017; Li et al., 2018, 2020; Li and Bai, 2020). This decrease

in cyclic nucleotides indirectly inactivates protein kinase A, allowing the formation of maturation promoting factor (MPF, a duplex of CDC2 and *de novo* translated cyclin B mRNA), which ultimately induces germinal vesicle breakdown (GVBD) resulting in a fertilizable egg (Nagahama and Yamashita, 2008; Das et al., 2018).

Use of *in vitro* oocyte maturation assays has demonstrated that some anthropogenic chemicals have the potential to alter oocyte maturation. In zebrafish, *in vitro* exposures of stage IV oocytes to malathion, Bis(2-ethylhexyl) phthalate (DEHP), and genistein decreases oocyte maturation success, whereas *in vitro* exposures to diethylstilbestrol, melatonin, and iprodione can induce oocyte maturation (Tokumoto et al., 2005, 2007; Carnevali et al., 2010, 2011; Maskey et al., 2019). Exposures of maturationally-competent oocytes *in vitro* to malathion, an organophosphate insecticide, causes a dose-dependent decrease in success of oocyte maturation in common carp (*Cyprinus carpio*) and zebrafish (Haider and Moses Inbaraj, 1988; Maskey et al., 2019). Alteration to success of oocyte maturation by anthropogenic chemicals is proposed to be mediated by dysregulation of or interaction with the mPR (Das and Thomas, 1999; Tokumoto et al., 2007, 2011; Nagahama and Yamashita, 2008; Kjeldsen et al., 2013; Maskey et al., 2019). Dietary exposure of zebrafish to 1,2,5,6-tetrabromocyclooctane for 14 days caused a significant reduction in the number of oocytes able to mature *in vitro*, but *in vitro* exposures were not performed in that study (Van Essen et al., 2021). It is currently unknown whether chemical inhibition of MIH-stimulated oocyte maturation quantified using *in vitro* assays is indicative of the success of oocyte maturation in fish exposed *in vivo*, or if inhibition of oocyte maturation decreases reproductive performance. Further, the structure-activity relationship of the mPR to interact with chemical stressors has not been determined.

Against this backdrop, the primary goal of this study was to determine whether *in vitro* assays of oocyte maturation are predictive of reproductive performance. The secondary goal was to determine the structure-activity relationship of chemical induced inhibition of oocyte maturation. To this end, high quality stage IV oocytes excised from sexually mature zebrafish were exposed *in vitro* to malathion and dimethoate, as well as the oxidative desulphurized degradation products of malathion and dimethoate, malaoxon and omethoate, respectively. Due to their structural similarity, the degradation products and parent compounds serve as excellent candidates to help evaluate the structure-activity relationship of chemical interaction with mPR leading to altered oocyte maturation in the assay (Figure 2.1). Additionally, MIH-stimulated maturation of oocytes was quantified *in vitro* following waterborne exposure of zebrafish to each chemical and fecundity was assessed after a single breeding event after 10-day exposure. To examine reproductive performance, a 21-day reproduction assay was performed using malathion. Results of this study increase understanding of chemical inhibition of oocyte maturation as a possible mechanism of reproductive impairment in fishes and the ability to predict reproductive performance from *in vitro* assays of oocyte maturation.

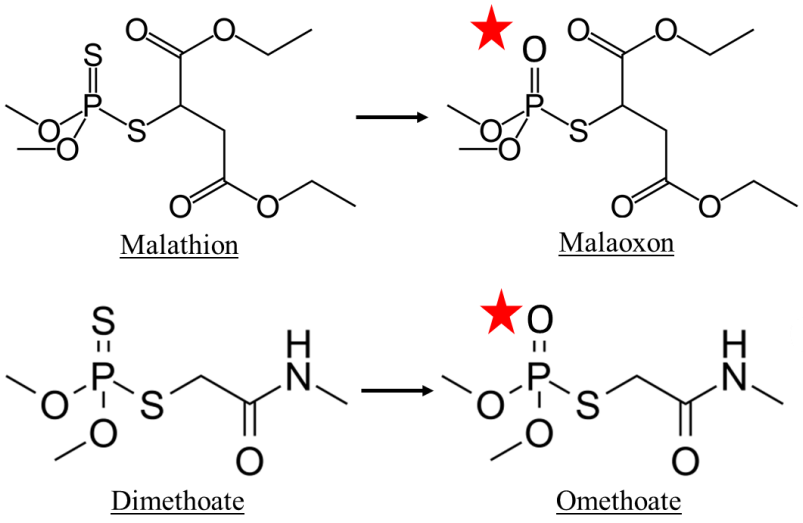

Figure 2.1: An illustration of the cytochrome P450-dependent oxidative desulphuration of malathion and dimethoate to the active oxon metabolites, malaxon and omethoate, which are anticholinergic agents in insects. The red star identifies the structural change.

2.2 Methodology

2.2.1 Chemicals

Maturation inducing hormone (batch # 0591978-1) was purchased from Cayman Chemical (Ann Arbour, MI, USA). The Pestanal™ analytical standards of malathion (diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate, purity: 99.2%), dimethoate (*O,O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] dithiophosphate, purity: 99.8%, malaoxon (diethyl 2-(dimethoxyphosphoylsulfanyl)butanedioate, purity: 99.9%), and omethoate (2-[(Dimethoxyphosphoryl)sulfanyl]-*N*-methyl-acetamide, purity: 98.4%) were from Millipore-Sigma (Mississauga, ON, Canada). Working solutions of each exposure chemical were prepared in anhydrous ethanol (Commercial Alcohols, Brampton, ON, Canada). Gibco™ Leibovitz's L15 media and Gibco™ penicillin-streptomycin was purchased from Fisher Scientific (Ottawa, ON, Canada).

2.2.2 Fish husbandry

Adult zebrafish (*TL* strain) were maintained in a ZebTEC Active blue system (Techniplast, Toronto, ON, Canada) in the Aquatic Research Facility at the Alberta Water and Environmental Science Building at the University of Lethbridge (Lethbridge, AB, Canada). Female zebrafish were part of an active breeding group and were housed with male fish to maintain breeding condition. Water temperature was 28 °C and a 14:10 h light:dark photoperiod was used. Fish were fed a diet of adult zebrafish feed (Ziegler Bros., Inc., Gardners, PA, USA), Gemma 300 micro feed (Skretting France, Le Pont de Pierre, France), and newly hatched brine shrimp (*Artemia salina*, Brine Shrimp Direct, Ogden, Utah, USA) equalling a feeding rate of approximately 2% bodyweight daily. Water quality parameters (pH, dissolved O_2, ionized and unionized ammonia, NO_3 and NO_2^-) were tested daily to abide by Canadian Council on Animal

Care guidelines. Studies were approved by the University of Lethbridge Animal Welfare Committee (protocol #1909).

2.2.3 *In vitro* exposure and oocyte maturation

Female zebrafish were collected from the breeding colony 2-4 h after the onset of light and euthanized by an overdose of buffered tricaine methanesulfonate (MS-222, Millipore-Sigma). Both lobes of the ovary were gently excised and placed in 90% Leibovitz's L15 media containing 1% penicillin-streptomycin (hereby referred to as L15) and adjusted to pH = 9.0 with 1N NaOH.

High-quality stage IV oocytes were dissociated from the ovary mass using micro tweezers to carefully manipulate singular stage IV oocytes from connective tissues and other early-stage oocytes, with the aid of a dissecting microscope (Motic, Richmond, BC, Canada). No effort was made to defolliculate oocytes. After isolation, dissociated stage IV oocytes were maintained in a covered Pyrex Petri dish containing L15 at 28 °C in the absence of light until they were needed for the maturation assay. Stage IV oocytes were pooled from 2-5 fish to ensure an adequate number of high-quality oocytes and were used in the assay only if the GV was intact.

To assess maturation, 20 pooled stage IV oocytes were placed in 1 mL of L15 in individual wells of a 24-well plate (Corning Inc, Corning, NY, USA). Oocytes were exposed in triplicate for 4 h to malathion, dimethoate, malaoxon, or omethoate at nominal concentrations of 0, 0.5, 5, or 50 μg/L. All wells contained 0.01% ethanol and each plate included a negative vehicle control (-MIH), and a positive control (50 mg/L malathion) adapted from Maskey et al., (2019) which demonstrated 19.8 mg/L inhibits maturation. Oocytes successfully completing

GVBD in each well was scored visually by use of a SteREO Discovery V12 Stereo microscope (Zeiss, North York, ON) 4 h after addition of 0.1 mg/mL MIH according to Selman (1993) and the average success of maturation of three replicate wells was used for statistical analysis. At least 70% maturation of control oocytes per replicate well was needed for the replicate to be included for analysis. *In vitro* exposures were repeated three times for each chemical. At the end of the exposure all mature and immature oocytes from each well were collected into 1.5 mL microfuge tubes and stored at -80 °C for later analysis.

At the end of the 10-day (section 2.2.4.1) and 21-day exposure (section 2.2.4.2), stage IV oocytes were isolated from exposed females, as described in section 2.2.3, but there was no pooling of oocytes from different fish nor was there any secondary chemical exposure. From each female fish per exposure, two wells of a 24 well plate received 5-20 stage IV oocytes and maturation was stimulated by addition of 0.1 mg/mL MIH. Five oocytes from each fish served as a negative control (-MIH). Maturation was assessed as the presence or absence of GVBD 4 h after addition of MIH by use of a SteREO Discovery V12 Stereo microscope (Zeiss, North York, ON).

2.2.4 Zebrafish exposures

2.2.4.1 10-day chemical exposure

Adult zebrafish were transferred from the breeding colony (section 2.2.2) to 9 L tanks (Aquabiotech) containing 5 L of dechlorinated City of Lethbridge water and acclimated for 3 days. Each tank contained six females and two males. Tanks were placed in water baths at 28 ±1.0 °C with a 14:10 h light:dark photoperiod. A 50% water renewal was conducted on each tank every 12 h. Fish were fed an identical diet as the breeding stock (section 2.2.2), excluding newly

hatched brine shrimp. Water quality parameters (pH, dissolved O_2, ionized and unionized ammonia, NO_3 and NO_2^-) were tested daily.

Following the 3-day acclimation, zebrafish were exposed for 10 days to malathion, dimethoate, malaoxon, or omethoate at nominal concentrations of 0, 5, or 50 μg/L. There were two replicate tanks for each concentration of chemical and 50% of the solution was renewed every 12 h. At the end of the 10-day exposure period, every male fish and three of the female fish from each replicate tank (n=6) were euthanized by an overdose of buffered MS-222 and the length (mm) and mass (g) of each female was recorded to determine Fulton's condition factor (K). The ovary and liver were excised and weighed to determine gonadosomatic index (GSI) and hepatosomatic index (HSI). Stage IV oocytes from excised ovaries were used immediately in an *in vitro* oocyte maturation assay (section 2.2.3). After completion of the *in vitro* maturation assay, mature and immature oocytes were pooled from each fish and used for SDS-PAGE and Western blotting analysis (section 2.2.5).

At the end of 10-day exposure, the remaining three exposed female fish from each replicate tank (n = 6) were used in a single breeding event to evaluate reproductive success measured as fecundity and percentage of eggs fertilized. Females from each exposure concentration were removed from exposure tanks, rinsed with dechlorinated City of Lethbridge water to wash away any chemical, then transferred to breeding tanks containing a perforated insert within the ZebTec Active blue zebrafish unit under identical parameters outlined in section 2.2.2. Each tank received two sexually mature male zebrafish from the breeding colony so that any effects on fertilization were not due to effects of the chemicals of interest on males. The following morning, number of eggs deposited and fertilization success, measured as the percentage of eggs fertilized, were scored visually 2 h after the onset of light. Females were then

sacrificed by an overdose of neutralized MS-222. The average number of eggs deposited and fertilized for all six fish per treatment were used for statistical analysis.

2.2.4.2 21-day malathion exposure

A 21-day reproduction assay was performed based on standardized test guidelines described under the Organisation for Economic Cooperation and Development (OECD) Test No. 229: Fish Short Term Reproduction Assay (OECD, 2012), with minor modifications. Specifically, four female and four male zebrafish were transferred from the breeding colony (section 2.2.2) to 9 L tanks (Aquabiotech) fitted with a plug to contain 9 L of dechlorinated City of Lethbridge water. Each tank was fitted with a perforated plastic cage to prevent cannibalism of the eggs. Tanks were placed in water baths at 28 ± 1.5 °C with a 14:10 h light:dark photoperiod. Fish were fed an identical diet as the breeding stock (section 2.2.2). Water quality parameters (pH, dissolved O_2, ionized and unionized ammonia, NO_3 and NO_2^-) were tested daily. A 66% water renewal was conducted on each tank daily. Fecundity and fertilization success were evaluated in each tank daily.

Prior to initiation of the exposure, the zebrafish were acclimated to the exposure tanks for 21 days. Zebrafish were then exposed for 21 days to malathion at nominal concentrations of 0, 0.5, 5, or 50 μg/L. Three replicate tanks were used for each concentration tested. At the end of 21-day exposure, the mass (g) and length (mm) of each female fish were recorded to evaluate K, HSI, and GSI. Stage IV oocytes were isolated from both lobes of the ovary for use in an MIH-stimulated *in vitro* maturation assay (section 2.2.3). After completion of the *in vitro* maturation assay, mature and immature oocytes were pooled from each fish and used for SDS-PAGE and Western blotting analysis (section 2.2.5).

2.2.5 SDS-PAGE and Western blotting

Abundances of phosphorylated PDE5a, a PDE important for oocyte maturation, was quantified according to Li and Bai (2020) with minor modifications. In brief, pooled mature and immature oocytes induced to mature after 10-day and 21-day exposure were removed from -80 °C and slow-thawed on ice. Thawed samples were lysed with 125 µL RIPA lysis buffer (Millipore) including SIGMA*FAST*™ protease inhibitor (Millipore-Sigma) and phosSTOP™ phosphatase inhibitor (Millipore-Sigma). Concentration of proteins in lysates was determined by use of a bicinchoninic acid kit (BCA, Millipore-Sigma) with bovine serum albumin as the standard. For each sample, 40 µg of protein was mixed with 4X Laemelli's buffer, supplemented with 10% β-mercaptoethanol, and incubated at 95 °C for 5 min. After denaturation, 20 µL of sample was added to one well of a 10% mini-PROTEAN TGX stain free gel, and proteins were separated for 1.5 h at 100 V in a Mini-PROTEAN® Tetra System (Bio-Rad) powered by a Power-Pac™ Basic system (Bio-Rad). Protein was transferred onto 0.2 µm Mini polyvinylidene fluoride (PVDF) membranes by use of a Trans-Blot® Turbo™ Transfer System (Bio-Rad). The membrane was blocked in EveryBlot blocking buffer (Bio-Rad) at room temperature with gentle agitation for 5 min, washed twice (5 min each) in tris-buffered saline with 0.05% Tween-20 (TTBS), and incubated with the PDE5a (phospho Ser92) 1° antibody (GeneTex, Irvine, California, USA) diluted 1:1,000 in blocking buffer, for 1 h at room temperature with gentle agitation. Next, the membrane was washed twice (5 min each) in TTBS and incubated with the 2° antibody (Goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate, Bio-Rad, 1:3,000 dilution) and Precision Protein™ StrepTactin HRP conjugate (Bio-Rad, 1:10,000 dilution) in blocking buffer for 1 h. The membrane was then washed twice in TTBS (5 min each), once for 10 min in tris buffered saline (TBS), and exposed to Clarity ECL substrate (Bio-Rad) for 5

minutes prior to final development. Stain-free images of the membrane were taken using a 45 sec. auto exposure after protein transfer to determine the total protein in each lane and chemiluminescent images of detected phosphorylated PDE5a were taken using a 30 sec. signal accumulation by use of a ChemiDoc™ imager (Bio-Rad). Densitometric analysis of the abundances of phosphorylated PDE5a were normalized to the total protein in each lane using Image Lab (Bio-Rad, version 6.1.0, build 7) according to Taylor et al. (2013). Densitometric peaks were verified using the band analysis tool function and a rolling disk size between 5-6.2 was sufficient to eliminate background before normalization. Data was collected from n=3-4 samples per treatment and are represented as fold-change from the control.

2.2.6 Statistical analysis

Statistical analysis was conducted using IBM® SPSS® (version 20, release 20.0.0). Normality and homogeneity of variance were assessed by use of a Shapiro-Wilk test and Levene's test, respectively. Data that did not meet parametric assumptions were $\log_{10}$ transformed. Comparisons were evaluated using one-way ANOVA followed by a Tukey's post-hoc test. Data that did not have homogeneity in variance were evaluated using a Welch's test followed by a Tukey's post-hoc. All data are represented as mean ± standard error of the mean (SEM). Differences from the control were considered significant at $p \leq 0.05$.

2.3.0 Results

2.3.1 Oocyte Maturation

In vitro exposure to malathion or dimethoate at 5 and 50 µg/L significantly inhibited oocyte maturation in comparison to control oocytes ($p < 0.05$; Figure 2.2A, 2.2C; white bars). Neither malaoxon nor omethoate affected oocyte maturation after *in vitro* exposure ($p > 0.05$;

Figure 2.2B, 2.2D; white bars). *In vivo* exposure to malathion or dimethoate at 5 or 50 μg/L for 10 days caused a significant decrease in MIH-stimulated *in vitro* oocyte maturation in comparison to oocytes from control zebrafish ($p < 0.05$; Figure 2.2A, 2.2C; grey bars). There was no difference in the success of MIH-stimulated maturation of oocytes from zebrafish exposed to either concentration of malaoxon or omethoate in comparison to controls ($p > 0.05$; Figure 2.2B, 2.2D; grey bars). *In vitro* MIH-stimulated maturation of oocytes from zebrafish exposed to 0.5 or 50 μg/L of malathion for 21-days was significantly decreased compared to maturation of oocytes from control fish ($p < 0.05$; Figure 2.3;).

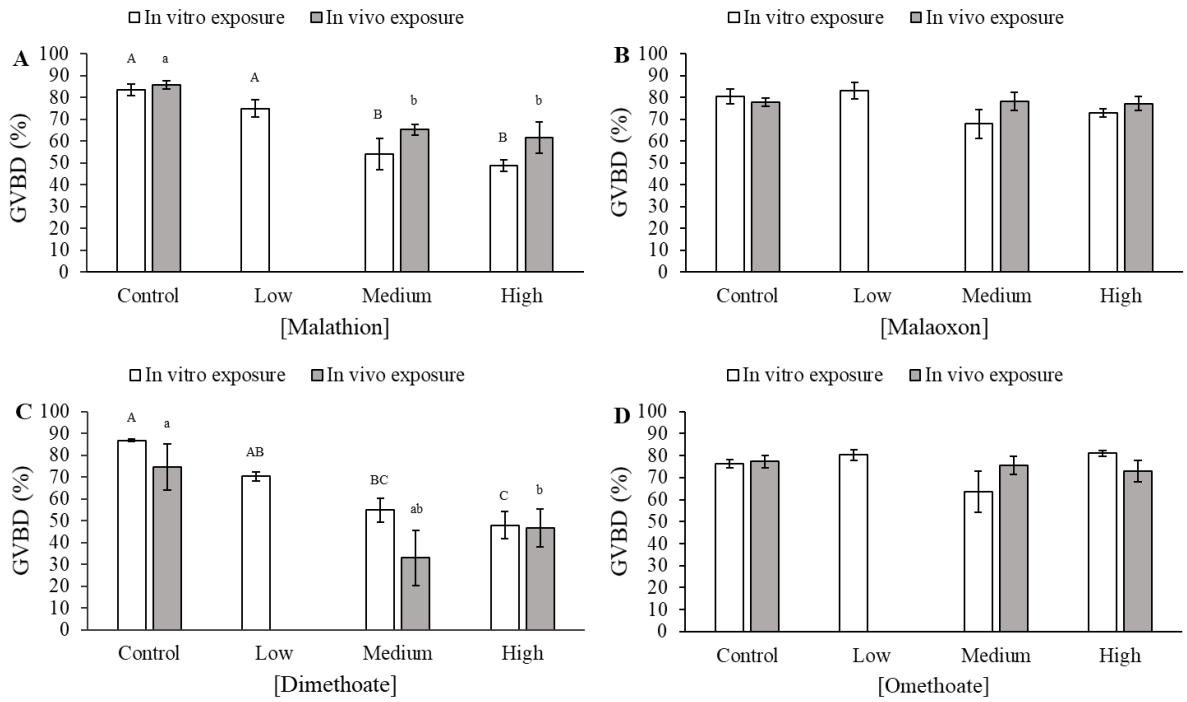

Figure 2.2: Effect of exposure to (A) malathion, (B) dimethoate, (C) malaoxon, and (D) omethoate on maturation (germinal vesicle breakdown, GVBD) of stage IV zebrafish oocytes. For *in vitro* exposures (white bars), oocytes were either exposed to malathion, dimethoate, malaoxon, or omethoate at 0, 0.5, 5, or 50 µg/L for 4 h. For *in vivo* exposures (grey bars), female fish were exposed for 10 days to malathion, dimethoate, malaoxon, or omethoate at 0.0, 5.0, or 50 µg/L. Maturation of oocytes was induced by addition of MIH at a final concentration of 0.1 mg/mL. Data are represented as the mean (±SEM) of 3 biological replicates using oocytes from 2-5 pooled zebrafish (*in vitro* exposure) or from 4-6 biological replicates (*in vivo* exposure). No comparisons were made between maturation data from *in vitro* and *in vivo* exposures. Differences among treatment groups were analyzed by use of a one-way ANOVA followed by a Tukey's post-hoc test. Differences were considered significant at $p \leq 0.05$. Different uppercase letters indicate significant differences among treatments in the *in vitro* exposure, and different lowercase letters indicate significant differences among treatments in the *in vivo* exposure.

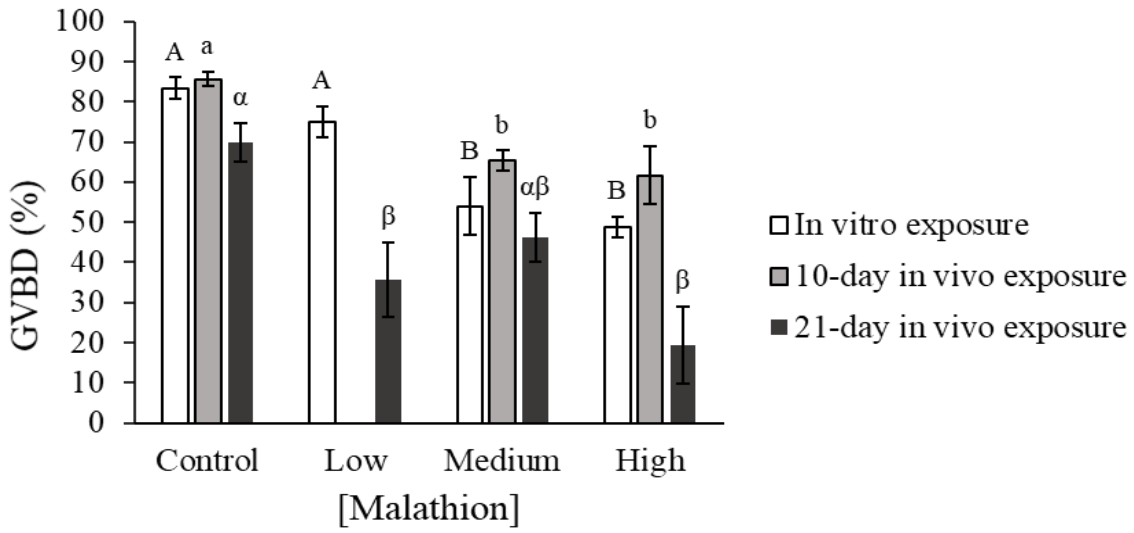

Figure 2.3: Effect of 21-day exposure to malathion on maturation (germinal vesicle breakdown, GVBD) of stage IV zebrafish oocytes. Data from *in vitro* and 10-day *in vivo* exposures are replicated from figure 2.2A for comparison. Female fish were exposed for 21 days to 0, 0.5, 5.0, or 50 μg/L malathion. Immediately following exposures, maturation of oocytes was induced by 0.1 mg/mL exogenous MIH. Data are represented as the mean (±SEM) of 4-6 biological replicates. Differences among treatment groups were analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. Differences were considered significant at $p \leq 0.05$. Different uppercase letters indicate significant differences among treatments in the *in vitro* exposure, different lowercase letters indicate significant differences among treatments in the *in vivo* exposure, and different Greek letters indicate significant differences among treatments in the 21-day *in vivo* malathion exposure.

2.3.1.1 Abundances of phosphorylated PDE5a

Ten-day exposure to malathion, dimethoate, malaoxon, or omethoate at concentrations as great as 50 µg/L did not significantly alter the abundance of phosphorylated PDE5a after oocytes were induced to mature with MIH *in vitro* (Figure 2.4). Similarly, exposure to malathion at concentrations as great as 50 µg/L malathion for 21 days had no affect on the abundance of phosphorylated PDE5a after *in vitro* MIH-stimulated maturation of oocytes (Figure 2.4).

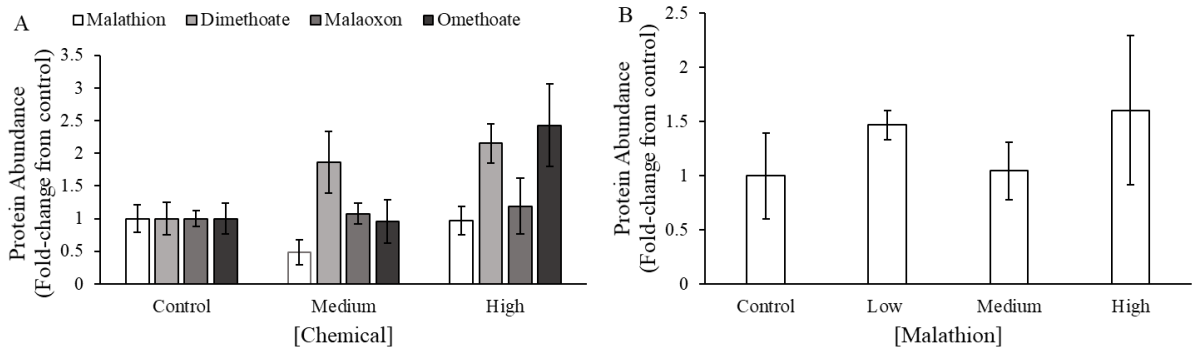

Figure 2.4: Effect of 10-day exposure to malathion, dimethoate, malaoxon, or omethoate (A) and 21-day exposure to malathion (B) on the abundance of phosphorylated PDE5a. Data was generated from *in vivo* exposed oocytes after MIH-induced *in vitro* maturation. Data is presented as fold-change from the control (±SEM) of n=3-4 biological replicates. Differences were analyzed by use of one-way ANOVA with a Tukey's post hoc test and were considered significant at $p \leq 0.05$.

2.3.2 Reproductive effects

2.3.2.1 10-day exposure

There were no differences in K across chemicals and treatments after 10-days of exposure to any chemical examined. However, compared to controls, zebrafish exposed for 10 days to malaoxon at 50 μg/L had significantly decreased HSI, and fish exposed to 5 μg/L dimethoate had significantly decreased GSI and HSI ($p < 0.05$; Table 2.1). Fecundity from a single breeding event of female zebrafish exposed to dimethoate at 50 μg/L for 10 days was significantly reduced compared to control fish ($p < 0.05$; Figure 2.5C). Exposure to malathion, malaoxon, or omethoate for 10 days did not significantly alter fecundity from a single breeding event ($p > 0.05$; Figure 2.5A, 2.5B, 2.5D). Fertilization success in zebrafish exposed to 50 μg/L dimethoate for 10 days was significantly reduced in comparison to control fish ($p < 0.05$; Figure 2.6C). However, fertilization success in fish exposed malathion, malaoxon, or omethoate for 10 days was not significantly different from the control ($p > 0.05$; Figure 2.6A, 2.6B, 2.6D).

Table 2.1: Effects of 10-day exposure to malathion, dimethoate, malaoxon, or omethoate on condition factor (K), gonadosomatic index (GSI) and hepatosomatic index (HSI) in female zebrafish after waterborne exposure. Data are presented as the mean (±SEM) of 4-6 fish. Differences among treatment groups were made by use of a one-way ANOVA with a Tukey's post-hoc test. Differences were considered significant at $p \leq 0.05$, and are indicated by different letters.

Chemical	[Chemical]	K	GSI	HSI
Malathion	Control	3.7 ± 0.08	8.2 ± 0.91	3.4 ± 0.20
	Medium	3.9 ± 0.14	6.6 ± 0.64	2.8 ± 0.38
	High	3.9 ± 0.14	7.2 ± 0.91	3.3 ± 0.23
Dimethoate	Control	4.1 ± 0.14	8.2 ± 0.71 **a**	2.0 ± 0.21 **a**
	Medium	3.6 ± 0.19	6.7 ± 0.43 **b**	1.3 ± 0.11 **b**
	High	4.2 ± 0.33	7.5 ± 0.69 **ab**	2.0 ± 0.21 **a**
Malaoxon	Control	3.9 ± 0.17	7.5 ± 1.43	0.8 ± 0.15 **a**
	Medium	3.8 ± 0.12	8.9 ± 0.72	1.5 ± 0.14 **ab**
	High	3.8 ± 0.12	5.9 ± 0.66	1.8 ± 0.32 **b**
Omethoate	Control	4.2 ± 0.13	6.3 ± 1.13	2.4 ± 0.27
	Medium	4.1 ± 0.21	6.6 ± 1.3	1.3 ± 0.33
	High	4.0 ± 0.09	5.8 ± 0.37	2.6 ± 0.40

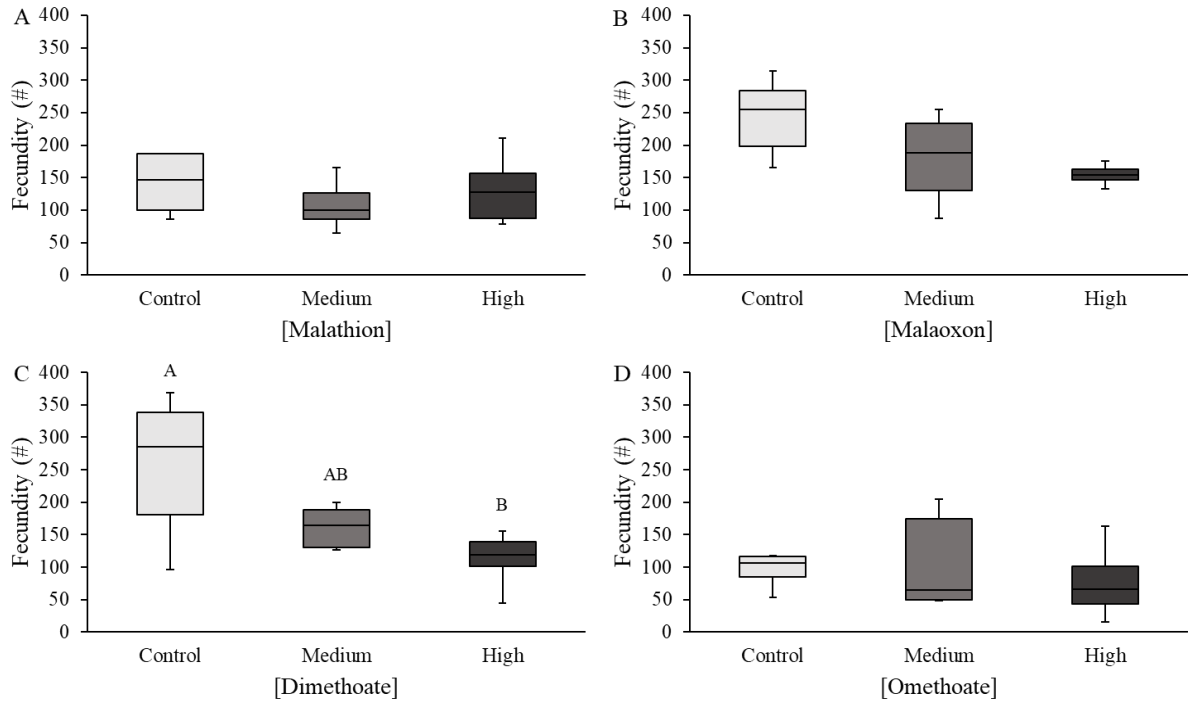

Figure 2.5: Effect of 10-day exposure to (A) malathion, (B) dimethoate, (C) malaoxon and (D) omethoate on fecundity in zebrafish. Sexually mature female zebrafish were exposed *in vivo* to malathion, malaoxon, dimethoate, or omethoate at 0, 5, or 50 µg/L for 10 days, after which fecundity was assessed following a single breeding event that used unexposed males. Data are represented as the mean (± SEM) of number of eggs deposited per female. Differences among treatment groups were analyzed by use of a one-way ANOVA followed by a Tukey's post-hoc test. Data that did not meet homogeneity of variance requirements were evaluated by using a Welch's test with a Tukey's post-hoc. Differences were considered significant at $p \leq 0.05$. Different letters indicate significant differences among treatments.

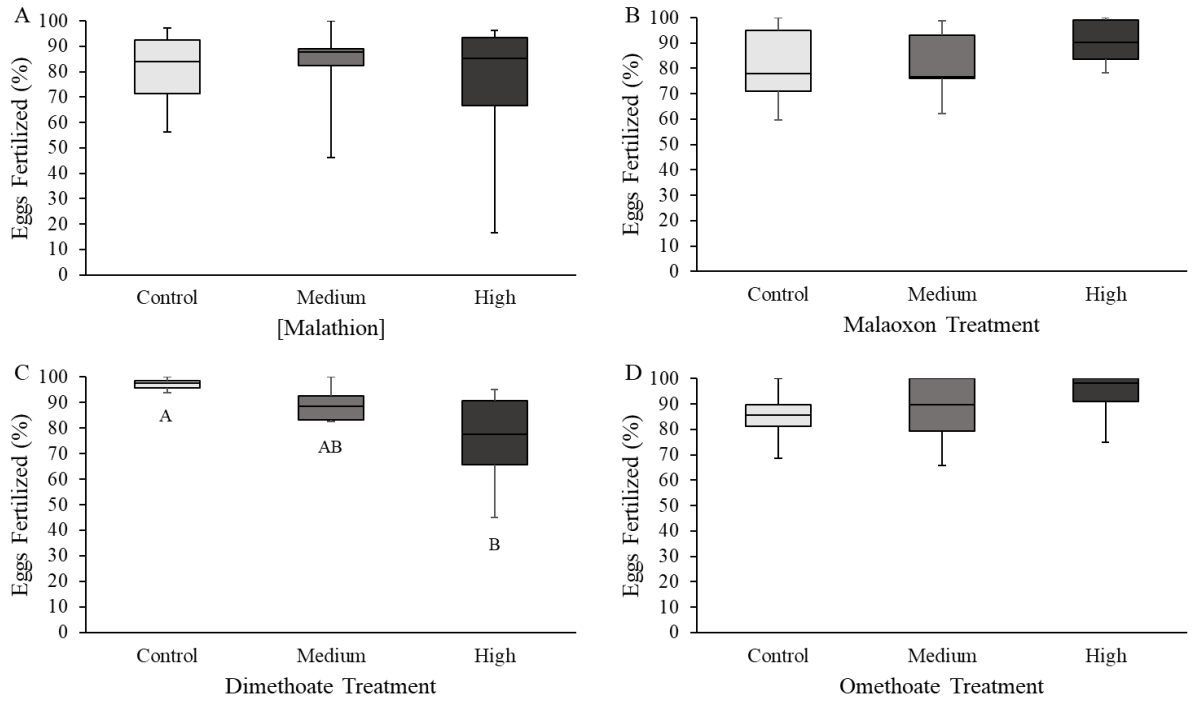

Figure 2.6: Effect of 10-day exposure to (A) malathion, (B) dimethoate, (C) malaoxon and (D) omethoate on success of fertilization of eggs. Sexually mature female zebrafish were exposed *in vivo* to malathion, malaoxon, dimethoate, or omethoate at 0, 5, or 50 µg/L for 10 days, after which fertilization success was assessed following a single breeding event. Data are represented as the mean (± SEM) of eggs deposited. Differences among treatment groups were analyzed use of a one-way ANOVA followed by a Tukey's post-hoc test. Differences were considered significant at $p \leq 0.05$. Different letters indicate significant differences among treatments.

2.3.2.2 21-day malathion exposure

Condition factor and GSI of zebrafish exposed to 50 μg/L malathion for 21 days were significantly decreased in comparison to controls ($p < 0.05$; Table 2.2). However, there was no change in HSI after 21-day exposure to either concentration of malathion ($p > 0.05$; Table 2.2). There was no significant effect on the average fecundity per female or the fertilization success per tank at either concentration of malathion ($p > 0.05$; Figure 2.7A, 2.7C). Finally, trends of cumulative egg production per tank over 21 days did not differ among treatment groups (Figure 2.7B).

Table 2.2: Effects of 21-day exposure to malathion on condition factor (K), gonadosomatic index (GSI) and hepatosomatic index (HSI) in female zebrafish after waterborne exposure. Data are presented as the mean (±SEM) of 10-11 fish. Differences among treatment groups were made to the control by use of an ANOVA with a post-hoc Tukey's test. Differences were considered significant at $p \leq 0.05$, and are indicated by different letters.

Chemical	[Chemical]	K	GSI	HSI
Malathion	Control	4.0 ± 0.20 **a**	8.8 ± 1.25 **a**	1.6 ± 0.40
	Low	4.0 ± 0.14 **ab**	7.8 ± 1.10 **ab**	2.3 ± 0.57
	Medium	3.8 ± 0.14 **abc**	7.6 ± 1.17 **ab**	2.6 ± 1.12
	High	3.2 ± 0.14 **c**	4.0 ± 0.69 **b**	1.0 ± 0.13

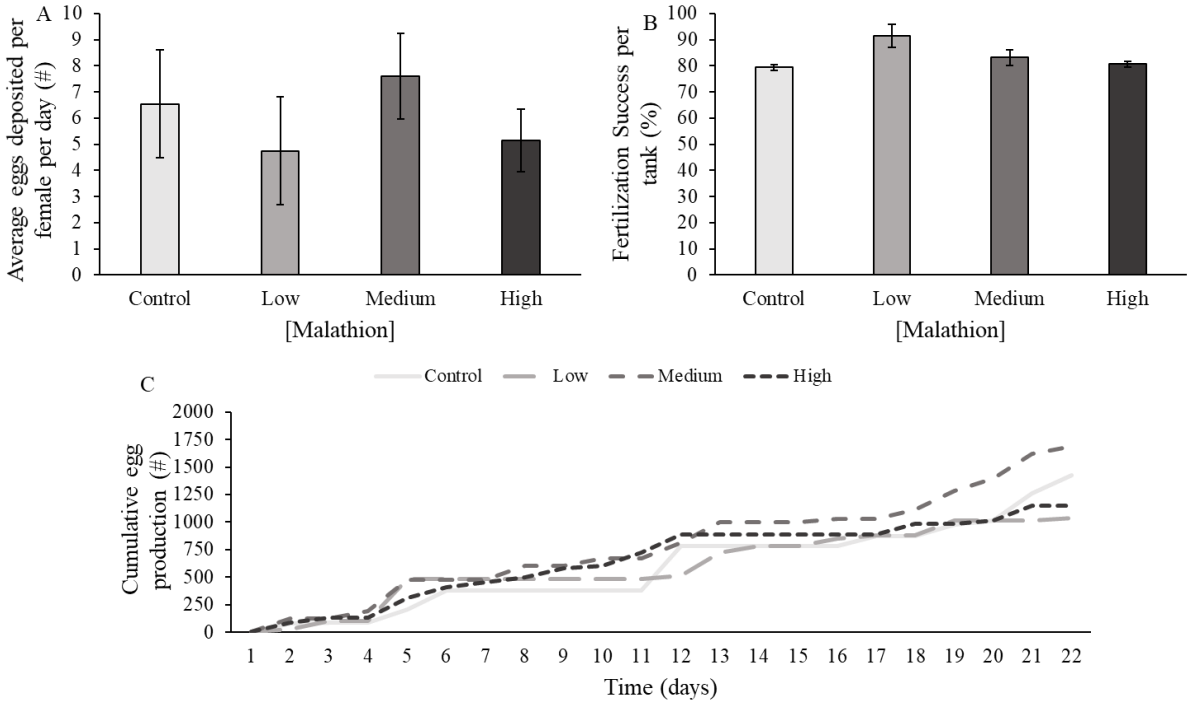

Figure 2.7: Effect of 21-day exposure to 0, 0.5, 5, or 50 µg/L malathion on reproduction in zebrafish. Egg production data was attained from four mature male and female zebrafish per tank per concentration of malathion, in triplicate. Data are represented as the mean (± SEM) of eggs deposited (A) or successfully fertilized (B). Differences in the average amount of eggs produced per female per day or successfully fertilized eggs in comparison to the control were analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. No statistical tests were performed on cumulative egg production (C). Differences were considered significant at $p \leq 0.05$.

2.4.0 Discussion

Environmental chemicals can disrupt oocyte maturation after *in vitro* exposure (Tokumoto et al., 2005, 2007; Carnevali et al., 2010; Maskey et al., 2019). Objectives of this study were to investigate the structure-activity relationship between oocyte maturation and chemical stressors, and to evaluate whether in vitro assays of oocyte maturation are predictive of reproductive performance. Ten-day exposures were completed to determine whether *in vitro* assays of oocyte maturation are predictive of the ability of oocytes from *in vivo* exposed fish to mature *in vitro*. To evaluate the use of assays of oocyte maturation to predict impairments in reproductive parameters like fecundity and fertility, a 21-day exposure to malathion was completed.

2.4.1 Structure-activity relationship between oocyte maturation and chemical stressors

Dimethoate has a high degree of structural similarity to malathion, and thus was predicted to disrupt oocyte maturation *in vitro*, as demonstrated previously for malathion (Maskey et al., 2019). Malathion and dimethoate contain identical phosphorothioate groups that undergo CYP-mediated oxidative desulphuration to the primary metabolites malaoxon and omethoate (Błasiak et al., 1999). These oxidation reactions generate malaoxon or omethoate by substitution of a sulphur for an oxygen atom on the phosphorothiol group (Figure 2.1; Tony et al., 1995; Lartiges and Garrigues, 1998; Hernandez et al., 2017). Due to their structural similarity, malaoxon and omethoate were also used in the present study to examine the structure-activity relationship of chemicals that might inhibit MIH induced oocyte maturation. However, neither malaoxon nor omethoate altered MIH-stimulated oocyte maturation after *in vitro* exposure (Figure 2.2B, 2.2D). Based on the *in vitro* results, the sulphur atom of the parent phosphorothiol moiety is integral to

the ability of malathion and dimethoate to inhibit MIH induced oocyte maturation, and oxidation of this phosphorothiol moiety substituting sulphur for an oxygen atom leads to a loss of effect.

The phosphorylation of PDE5a activates its specific activity in decreasing concentrations of cGMP in the oocyte during maturation, alleviating prophase I arrest (Li and Bai, 2020). However, neither 10-day exposure to malathion, malaoxon, dimethoate or omethoate, or 21-day exposure to malathion altered the abundance of phosphorylated PDE5a after MIH-induced maturation (Figure 2.4). Previous studies have demonstrated that inhibition of PDE5a in zebrafish oocytes has a stimulatory role in inducing oocyte maturation due to follicular levels of cGMP, but inhibition of the protein kinase that phosphorylates PDE5a inhibits maturation (Li and Bai, 2020). Based on these results, it appears that *in vivo* exposure to these treatments do not target the cGMP/PKG pathway to alter oocyte maturation.

2.4.2 *In vitro* oocyte maturation predicts success of oocyte maturation after *in vivo* exposure

Maskey et al., (2019) determined that the organophosphate insecticide, malathion, inhibits MIH induced maturation of maturationally competent oocytes from zebrafish, *in vitro*, yet whether *in vivo* exposure to malathion causes inhibition of oocyte maturation was not determined. Malathion and dimethoate at 5 and 50 μg/L, but not 0.5 μg/L, significantly inhibited MIH-stimulated oocyte maturation after *in vitro* exposure (Figure 2.2A; 2.2C). This is the first study to report that dimethoate inhibits oocyte maturation, *in vitro*, but results from *in vitro* malathion exposures is consistent with other studies. In one study, MIH stimulated maturation of stage IV oocytes from zebrafish was inhibited by 50% compared to controls following *in vitro* exposure to malathion at 19.8 mg/L (Maskey et al., 2019). However, significant inhibition of oocyte maturation was not observed when oocytes were exposed to 1.98 mg/L malathion (Maskey et al., 2019), which is approximately 40-fold greater than concentrations that

significantly inhibited oocyte maturation in the current study (Figure 2.2A). Further, the concentration that inhibited oocyte maturation, 19.8 mg/L, is approximately 400-fold greater than the maximal concentration used here (Figure 2.2A). Differences in the concentration of malathion able to significantly inhibit oocyte maturation *in vitro* between the present study and that of Maskey et al. (2019) might be attributed to several differences between the two studies. First, Maskey et al. (2019) used 60% L-15 media supplemented with antibiotic-antimycotic which differs from our use of 90% L-15 supplemented with penicillin-streptomycin based upon established methods (Stewart, 2015). Secondly, our oocytes were induced to mature by addition of MIH in fresh L-15 after 4 h of exposure to the chemicals of interest, compared against methods used in Maskey et al. (2019) which utilized coincubation of malathion with human chorionic gonadotropin (hCG) over an 18 h period. In a study investigating maturation of oocytes from carp (*Cyprinus carpio L.*), 50 μg/L malathion significantly impaired LH-induced GVBD in maturationally competent oocytes when exposed for 36 h (Haider and Moses Inbaraj, 1988).

The magnitude of oocyte maturation inhibition from *in vitro* exposures aligns with the results of *in vitro* oocyte maturation after *in vivo* exposures. When female zebrafish were exposed *in vivo* to malathion or dimethoate at 5 and 50 μg/L for 10 days, success of MIH stimulated oocyte maturation was significantly less compared to control oocytes (Figure 2.2A; 2.2C). However, *in vivo* exposures to malaoxon and omethoate for 10 days at concentrations as great as 50 μg/L did not significantly alter oocyte maturation in comparison to respective controls (Figure 2.2B; 2.2D). Consistent with results of the 10-day exposure, success of MIH stimulated oocyte maturation was also significantly inhibited by malathion when exposed to 0.5 or 50 μg/L for 21 days (Figure 2.3). Surprisingly, 5 μg/L malathion did not significantly alter

oocyte maturation, but it should be noted that oocyte maturation was significantly inhibited by exposure to 0.5 μg/L malathion, a concentration an order of magnitude less than what caused the failure of MIH stimulated oocyte maturation after 10-day exposure. Dimethoate caused a more severe inhibition of maturation than did malathion, thus it appears dimethoate is a more potent inhibitor of oocyte maturation than malathion based on *in vitro* and *in vivo* exposure results. Regardless, a similar concentration-dependent decrease in oocyte maturation was observed in both *in vitro* and *in vivo* exposures to malathion and dimethoate, suggesting that the *in vitro* MIH-stimulated oocyte maturation assay is predictive of the success of MIH-stimulated oocyte maturation after *in vivo* exposure.

2.4.3 Assays of oocyte maturation as a tool to predict reproductive alterations in zebrafish

To determine if assays of oocyte maturation are indicative of reproductive performance, female zebrafish were evaluated for their ability to produce mature and fertilizable eggs after 10-days of exposure and during 21-days of exposure to malathion. Based on results of the MIH stimulated oocyte maturation assays after *in vitro* and 10-day *in vivo* exposure, it was hypothesized that malathion and dimethoate, but not malaoxon and omethoate, would cause reduction in single-event fecundity and fertility of female zebrafish. However, only dimethoate caused a significant decrease in fecundity and fertility of eggs after 10-day exposure (Figure 2.5C; 2.6C). Reproductive parameters including the average amount of daily eggs deposited per female per tank (Figure 2.7A), fertilization success per tank (Figure 2.7B), and trends of cumulative egg production (Figure 2.7C), were not altered in zebrafish exposed to 0, 0.5, 5, or 50 μg/L malathion for 21 days. Therefore, it appears that the assay of oocyte maturation after *in vitro* exposure cannot be used to predict reproductive alterations in zebrafish. Although results from the single event reproduction assays almost exactly align with the results from the MIH

stimulated maturation assays following *in vitro* and *in vivo* exposure, they should be interpreted with some caution. It is possible that the reproductive effects of sub-chronic exposure to malathion, malaoxon, and omethoate might take longer than 10 days to manifest. Although dimethoate was a more potent inhibitor of oocyte maturation and caused reduced single-event fecundity and fertility after 10-days of exposure in this study, the ability of dimethoate to reduce fecundity of zebrafish has been demonstrated after 96 h exposure to 24.68 μg/L dimethoate (Ansari and Ansari, 2011). However, an assessment of fecundity following exposure to malathion had not been made previously. To fully evaluate the reproductive effects of exposure to dimethoate, omethoate, and malaoxon, a complete assessment would be needed using standardized reproductive toxicity tests according to OECD protocols (OECD No. 229).

Reproductive alterations in zebrafish exposed to malathion and dimethoate have been identified previously, although the mechanisms underlying these changes are not fully elucidated. Zebrafish exposed for 4 months to malathion at 500 μg/L had reduced GSI, resorption of oocytes, and likely due to resorption, the fish failed to spawn (Ansari and Kumar, 1986). In common tank goby (*Glossogobius giuris*) exposed to malathion at 500 μg/L, secondary growth of primary oocytes was significantly repressed due to suppression of vitellogenesis (Narayanaswamy and Mohan, 2013). Exposure to dimethoate at 24.68 μg/L for 96 h reduced fecundity of adult zebrafish (Ansari and Ansari, 2011). In that study, no molecular, biochemical, or endocrine endpoints were investigated. In another study, long term (≥ 6 months) exposure of walking catfish (*Clairius batrachus*) and common carp (*Cyprinus carpio communis*) to formulations of dimethoate resulted in decreased GSI (Begum and Vijayaraghavan, 1995; Mir et al., 2011).

Prior investigations have demonstrated that environmental contaminants have the potential to inhibit MIH-stimulated oocyte maturation when exposed *in vitro* (Tokumoto et al., 2005, 2007; Carnevali et al., 2010, 2011; Maskey et al., 2019). Despite this, there was a complete lack of investigation regarding whether results from *in vitro* oocyte inhibition assays are predictive of impaired oocyte maturation *in vivo* and whether fecundity is decreased. This knowledge is important because decreased fecundity resulting from the failure of oocytes to mature due to exposure to anthropogenic stressors has the potential to cause population-level declines (Carnevali et al., 2010). Although in the present study there was no clear impact of inhibition of oocyte maturation on fecundity in zebrafish, zebrafish might not be the best model to evaluate impacts on reproduction. Zebrafish spawn asynchronously and will not produce eggs every day, which might explain the highly variable results from the single day reproduction assay. Also, due to the zebrafish's dynamic and asynchronous ovary during oogenesis, it is unlikely that all stage IV oocytes mature prior to a spawning event. Therefore, it is possible that the magnitude of chemical inhibition of oocyte maturation observed would not impact fecundity over 21 days, or more apically, be able to predict any substantial population level effect in this model (Tyler and Sumpter, 1996; Patiño and Sullivan, 2002; Lubzens et al., 2010). This is not the case for all fishes. For example, inhibition of maturation in oogenesis could potentially cause detrimental effects on fecundity in synchronous spawning fishes that spawn only once a year or once a lifetime. The differential sensitivity of oocyte maturation in species that spawn asynchronously provides an interesting avenue for further research, as the underlying mechanism might be relevant to the failure of oocyte maturation in all teleosts, regardless of their reproductive strategy. Efforts should be made to develop the oocyte maturation assay in other

species of fish, including species with synchronous oocyte maturation, to determine whether inhibition of oocyte maturation, *in vitro*, is predictive of impacts on reproductive performance.

2.5 Conclusion

The organophosphate insecticides malathion and dimethoate, but not their oxidized metabolites malaoxon or omethoate, inhibit maturation of oocytes that are exposed both *in vitro* and *in vivo*. Thus, the sulphur group on the phosphorothiol moiety of malathion and dimethoate is crucial to these chemicals ability to inhibit oocyte maturation. Assays of MIH-stimulated oocyte maturation after exposure *in vitro* predict the success of maturation of oocytes *in vitro* after *in vivo* exposure, but do not predict reproductive impairment after organophosphate insecticide exposure. Therefore, inhibition of oocyte maturation and how it could cause ecologically relevant effects on fishes needs to be further investigated, including in phylogenetically distinct species that might differ in their sensitivity to chemicals that inhibit maturation of oocytes as well as in species with synchronous spawning.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

It is well established that anthropogenic chemicals can negatively impact reproductive performance in fishes, and several mechanisms of impaired reproduction have been characterized. One mechanism that has not received great attention is the inhibition of oocyte maturation, the fourth step in oogenesis in the model teleost, the zebrafish (*Danio rerio*). Assays of oocyte maturation have been used to establish whether a chemical can inhibit maturation, but a structure-activity relationship between chemicals and interaction with or antagonism of the mPR has not been established. Further, it was unknown if some chemicals that inhibit oocyte maturation *in vitro* will inhibit *in vitro* maturation of oocytes after fish are exposed *in vivo*. Lastly, it had to be determined if *in vitro* assays of oocyte maturation can be used to predict reproductive successes of fish exposed to chemicals *in vivo*. To gain a better understanding of the utility of assays of oocyte maturation, the overall goals of this thesis were to explore if assays of oocyte maturation in sexually mature female zebrafish are predictive of reproductive impairments after *in vivo* exposure and to investigate the structure-activity relationship of chemical induced inhibition of oocyte maturation.

In the research described in this thesis, exposure of oocytes either *in vitro* or *in vivo* to the organophosphate insecticides malathion or dimethoate, but not their metabolites malaoxon or omethoate, inhibited MIH-stimulated oocyte maturation. These results demonstrate a structure-activity relationship between organophosphate insecticides and oocyte maturation, whereby substitution of a sulphur for oxygen results in loss of effect. The major finding of this research is that success of oocyte maturation *in vitro* after *in vivo* exposure can be predicted using assays of

in vitro oocyte maturation, but this cannot yet be extrapolated to predict reproductive success of fishes.

Although inhibition of oocyte maturation was not predictive of reproductive performance of zebrafish, it is possible that this assay might be predictive of reproductive performance in other species. One model fish species in toxicology that should be used to develop oocyte maturation assays is the fathead minnow due to its relevance in North American environments. However, future research should focus on developing assays of oocyte maturation in multiple species of determinate (fixed number of oocytes) synchronous spawning fishes. The negative effect that oocyte maturation inhibition might have on determinate synchronous spawning fish could manifest at the population level, because these fish have a fixed number of oocytes that develop over a longer period than in asynchronous species, such as zebrafish. By using multiple species of fish, data can be collected regarding interspecies variation in sensitivities to chemical-induced maturation failure.

Assays of oocyte maturation appear to demonstrate a novel mechanism of EDC disruption via antagonism of or interaction with the mPR (Das and Thomas, 1999). Based on results of this study, a putative AOP for reproductive failure resulting from antagonism of the mPR is proposed (Figure 3.1). While this AOP requires additional research, it is hypothesised that quantifying chemical interaction with or antagonism of the mPR could be used to predict inhibition of oocyte maturation and reproductive failure. If this AOP can be validated, an *in vitro* assay using non-animal models could be developed. Further validation and refinement of this relationship and its utility to predicting reproductive impairments could result in this assays' use in tier 1 testing in the endocrine disruptor screening program (EDSP) by the United States Environmental Protection Agency (EPA). Tier 1 screening in the EDSP identifies chemicals that

have potential to interact with the ER, AR, or thyroid hormone systems. With further validation of the mPR as a target like the ER or AR for EDCs, this assay might be refined and optimized to cell-based, high-throughput chemical screening systems that align with the goals and objectives of toxicology in the 21st century that aims to reduce the number of animals used in research by replacing them with predictive high-throughput *in vitro* bioassays.

There were shortcomings in this study. Reproduction data from the 21-day reproduction assay might not be representative of reproduction alterations, as every tank was disturbed each day to collect eggs for quantification of fecundity and fertility. Zebrafish can also deposit immature or undeveloped eggs (personal observation), which can skew fecundity and fertility data. Assays of oocyte maturation are established and validated in zebrafish, but this asynchronous model fish might not demonstrate the potential negative effects of oocyte maturation failure. For example, it is possible that zebrafish resorb oocytes that have or will fail to mature, which would skew the reproductive success data. Due to how frequently zebrafish spawn, it is unlikely that levels of oocyte maturation failure observed here culminate in population declines.

Although the oocyte maturation assay requires the use of animal lives, it significantly reduces the number of fish used, reduces cost and time investment needed to generate reliable data in predicting the success of oocyte maturation after exposure. The work presented here is a crucial step in the development of novel techniques used to predict reproductive impairments by anthropogenic chemical stressors.

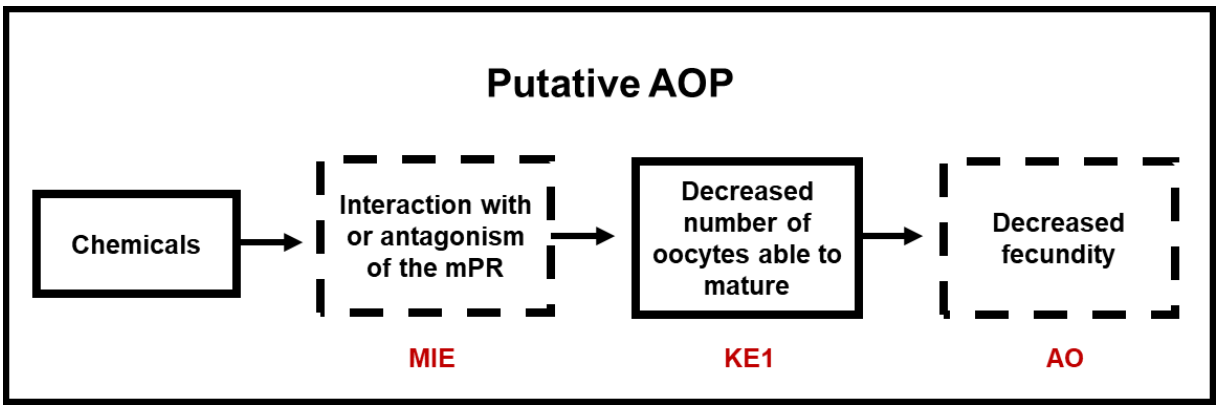

Figure 3.1: A proposed putative adverse outcome pathway (AOP) representing the failure of oocyte maturation as a mechanism of reduced fecundity. Dashed boxes represent not yet determined aspects of this pathway, while solid boxes are proposed mechanisms leading to an adverse outcome (AO). Anthropogenic chemicals are proposed to interact or antagonize the mPR as the molecular initiating event (MIE), which causes a decrease in the number of oocyte able to mature (KE1). Fewer oocytes able to mature ultimately might cause a decrease in fecundity (AO).

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