Peroxisome proliferator-activated receptors (PPARs), phthalates and reproduction in the female zebrafish (Danio rerio)

by
Abiran Sritharan

A Thesis
Presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Integrative Biology (Reproductive Physiology)

Guelph, Ontario, Canada
© Abiran Sritharan, September 2017
ABSTRACT

Peroxisome proliferator-activated receptors (PPARs), phthalates and reproduction in the female zebrafish (*Danio rerio*)

Abiran Sritharan  Advisor: Dr. Glen Van Der Kraak
University of Guelph, 2017

Peroxisome proliferator-activated receptors are nuclear transcription factors that mediate a wide array of physiological processes in mammals including reproduction. The objective of this thesis was to characterize the presence, regulation and actions of members of the PPAR family in the ovary of the zebrafish (*Danio rerio*). Gene expression of all five PPARs, *ppara*, *pparb*, *pparbeta*, *pparbeta* and *ppary*, was detected in ovarian follicles at different stages of development. The expression of *ppara*, *pparbeta* and *ppary* declined as the follicles developed. Human chorionic-gonadotropin, a luteinizing hormone analog, was shown to decrease gene expression of *ppara*, while activators of the Protein Kinase C pathway downregulated expression of *ppara*, *ppara* and *pparbeta* in full grown ovarian follicles. Intraperitoneal injections of the known PPAR ligands, diethylhexyl phthalate (DEHP) and mono-(2-ethylhexyl) phthalate (MEHP) reduced the numbers of eggs spawned by adult female zebrafish and this was attributed to a disruption in ovulation. Fish injected with either DEHP or MEHP had reduced expression of the nuclear progesterone receptor (*npr*) gene, and fish injected with MEHP had reduced expression of a distintegrin and metalloproteinase with thrombospondin motifs 1 (*adamts1*) gene expression, two key players in the ovulation process. This study provides insight into the physiological pathways that are regulated by the PPAR family of transcription factors in teleost ovaries.
ACKNOWLEDGEMENTS

To my advisor, Glen thank you for giving me opportunity to study and undertake this project under your supervision. Your invaluable knowledge, advise and guidance throughout this project made it a great learning experience and have learnt much from you that will be immensely valuable in any future path that I will take. The occasional (and often sarcastic) pep talks gave me some much needed confidence at the low points of the project and is deeply appreciated. I would like to thank Joanna and Rob for sitting on my committee, and for the useful and constructive feedback on the project during our meetings. This was extremely beneficial in terms of focusing and fine-tuning the project as I progressed.

A huge thank you to the fearless second-in-command of the Van Der Kraak crew, Jacquie Matsumoto. From teaching me the different lab techniques that I used in this experiment, to providing me with valuable input on almost every aspect of my project, your constant support and advise was and still is greatly appreciated. I’d like to thank my present office mates Liz and Hailey for joining me in lamenting and laughing about the sorrows and joys of scientific research. I want to thank Cory, for the advice and companionship and Andy for the stress-relieving weekly squash sessions. I also want to thank the fine gentleman at the Hagen Aqualab, Matt Cornish and Mike Davies for not only assistance in the Aqualab but also for worldly advise and banter that was more than enjoyable and something I looked forward to at the end of the day. I wouldn’t have been able to go through graduate school without the help of my good friends, Mark, Vaibhav, Brian, Kyle, Imran and Aankit for providing me with constant support and laughter and keeping me grounded throughout this degree. Finally, I would like to thank my family: Appa, Amma, Nadha and Thanchi for being there for me and providing constant support and encouragement through the course of my Masters.
# TABLE OF CONTENTS

Acknowledgements...........................................................................................................iii
Tables and Contents...........................................................................................................iv
List of Tables and Figures.................................................................................................v
List of Abbreviations.........................................................................................................vii

## Introduction

- Discovery.........................................................................................................................1
- Evolution of PPARs in mammals and teleosts.................................................................3
- Receptor structure and mechanism of action...............................................................4
- PPARs in mammals: Expression, function, and ligands.................................................6
- PPARs in teleosts: Expression, function, and ligands...................................................8
- PPARs and mammalian reproduction...........................................................................10
- PPARs and teleost reproduction..................................................................................13

## Thesis: Goals and Objective

- .................................................................................................................................14

## Methods

- Experimental Animals.................................................................................................17
- Experimental Design....................................................................................................17
- Analytical Techniques.................................................................................................22

## Results

- .................................................................................................................................29

## Discussion

- Spatiotemporal expression of PPARs in the zebrafish ovary........................................47
- Regulation of PPARs in the zebrafish ovary...............................................................49
- Phthalates, PPARs and reproduction.........................................................................52
- PPARs: Negative regulators of ovulation in zebrafish.................................................57

## Future directions

- .................................................................................................................................59

## Conclusions

- .................................................................................................................................61

## References

- .................................................................................................................................62
LIST OF TABLES

Table 1. Summary of natural and synthetic ligands of PPARs in mammals .................. 5
Table 2. List of primers and their sequences used in the study ................................. 27

LIST OF FIGURES AND CAPTIONS

Figure 1. Depiction of the general mechanism of action of the PPAR: RXR complex during ligand-dependent activation .................................................................................. 5

Figure 2. Expression of PPARs across multiple stages of ovarian follicle development .......................................................................................................................... 33

Figure 3. Relative expression of PPARs within each stage of ovarian follicle development .......................................................................................................................... 35

Figure 4. Expression of PPARs in full grown follicles that were treated in vitro with hCG (20 IU/ml) or 17α-20βP (2ng/µl) for a period of six hours ........................................................................ 37

Figure 5. Expression of PPARs in full grown follicles that were treated in vitro with hCG (20 IU/ml), PMA (400 nm)/A23187 (10 µM) or hCG in combination with PMA/A23187 .................................................................................................................................. 38

Figure 6. Average % germinal vesicle breakdown in zebrafish full grown follicles that were treated in vitro with 17α-20βP (2 ng/ul), DEHP (1 µM) in combination with 17α-20βP (2 ng/µl) and DEHP (10 µM) plus 17α-20βP (2 ng/µl) ........................................................................................................ 39

Figure 7. Testosterone production in zebrafish full grown follicles that were treated in vitro with hCG (20 IU/ml), MEHP (50 µM) and hCG (20 IU/ml) in combination with MEHP (50 µM) ................................................................................................................................. 40
Figure 8. Comparison of the average number of eggs produced per female per day in zebrafish during the pre-exposure where fish were not injected with any chemicals and the seven-day exposure period during fish were injected with 0, 50 or 500 mg/kg of DEHP every other day.

Figure 9. Ovarian expression of npr in zebrafish after a seven-day exposure period during which they were injected with 0, 50 or 500 mg/kg of DEHP every other day.

Figure 10. Average ovarian T and E$_2$ content in zebrafish after a seven-day exposure period during which they were injected with 0, 50 or 500 mg/kg of DEHP every other day.

Figure 11. Comparison of the average number of eggs produced per female zebrafish per day during the pre-exposure where fish were not injected with any chemicals and the exposure period where fish were injected with 0, 35.6 or 356 mg/kg of MEHP.

Figure 12. Ovarian gene expression of PPAR isoforms in zebrafish after a seven-day exposure period during which were injected with 0, 35.6 or 356 mg/kg of MEHP every other day.

Figure 13. Ovarian gene expression of npr, adams1 and star in zebrafish after a seven-day exposure period during which were injected with 0, 35.6 or 356 mg/kg of MEHP every other day.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Proper name</th>
</tr>
</thead>
<tbody>
<tr>
<td>8(S)-HETE</td>
<td>8-Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>15 (S) – HETE</td>
<td>15-Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>17α-20βP</td>
<td>17α,20 βp dihydroxy-4-pregnen-3-one</td>
</tr>
<tr>
<td>17-βHSD</td>
<td>17-β-Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>A disintegrin and metalloproteinase with thrombospondin motifs 1</td>
</tr>
<tr>
<td>A-HAB</td>
<td>Aquatic Habitat</td>
</tr>
<tr>
<td>Acyl-CoA</td>
<td>Acetyl coenzyme A</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DEHP</td>
<td>Di-2-ethylhexyl phthalate</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DiDP</td>
<td>Diisodecyl phthalate</td>
</tr>
<tr>
<td>E₂</td>
<td>17 β-estradiol.</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine disrupting chemicals</td>
</tr>
<tr>
<td>EF1α</td>
<td>Elongation factor 1 α</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>EIOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>FSHr</td>
<td>Follicle stimulating hormone receptor</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LHr</td>
<td>Luteinizing hormone receptor</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>MEHP</td>
<td>Mono-(2-ethylhexyl)-phthalate</td>
</tr>
<tr>
<td>MIS</td>
<td>Maturation Inducing Steroid</td>
</tr>
</tbody>
</table>
mPRβ  Membrane progestin/progesterone receptor β
mRNA  Messenger ribonucleic acid
NTC  No template control
PBS  Phosphate Buffered Saline
PKC  Protein Kinase C
PMA  Phorbol 12-myristate 12-acetate
POC  Polycistic ovary syndrome
PPAR  Peroxisome proliferator-activated receptor
PPRE  Peroxisome proliferator-activated response element
15-PDGJ₂  15-deoxy 12,14- prostaglandin J₂
PG J₂  Prostoglanding J₂
qPCR  Real time quantitative polymerized chain reaction
RAR  Retinoic acid receptor
RXR  Retinoid– x-receptor
T  Testosterone
TR  Thyroid receptor
TZD  Thiazolidinedione
STAR  Steroidogenic acute regulatory protein
VLDL  Very low density lipoproteins
Introduction

The nuclear receptor superfamily consists of 48 distinct ligand-activated transcription factors and forms the largest family of transcription factors found in metazoans (Chawla et al, 2001; Bookout et al, 2006). This superfamily is made up structurally related proteins that provide a crucial link between transcriptional regulation and physiology. Several members of this family interact with endogenous steroid hormones or anthropogenic chemicals to influence expression of target genes that are involved in a diverse range of processes such as metabolism, development, immune response, and reproduction (Chawla et al, 2001). Members of this family such as the thyroid hormone receptors (TR), estrogen receptors (ER) and retinoic acid receptors (RAR) have been well studied and documented in most vertebrates. However, much less information exists on the peroxisome proliferator activated receptors (PPARs). PPARs have been most extensively studied in mammals, where they have been implicated in regulating reproductive processes such as ovarian follicle development, ovulation, and gonadal steroidogenesis (Komar et al, 2001; Komar et al, 2005). Furthermore, PPARs in the mammalian ovarian tissue have been shown to respond to gonadotropins such as luteinizing hormone (LH) which is an endogenous modulator of the reproductive cycle in vertebrates (Kim et al, 2008)

Relatively little is known about the presence of PPARs in teleosts and it was not until recently that existence of five different isoforms of PPARs were confirmed in the zebrafish genome (Den Broeder et al, 2015). Any insight into PPAR function in the ovarian tissue of teleost has come from experiments studying the effects of endocrine disrupting chemicals (EDC) on fish health (Carnavalli et al, 2010). EDCs like phthalates are thought to interact with PPARs in teleosts and have been shown to inhibit egg production and alter steroidogenesis through transcriptional regulation of genes involved in reproduction (Uren-Webster et al., 2008; Carnavali et al, 2010). However, research in this area is lacking and it remains unclear how specific pathways and target genes are connected and regulated through phthalate mediated PPAR activation. The initial objective of this thesis was to characterize PPARs in the zebrafish ovary in relation to follicle development and ovulation. A second objective was to determine if
PPARs were under the influence of classical regulators of reproduction in zebrafish such as LH, sex steroids and activators of the protein kinase C pathway. Finally, I set out to determine the effects of phthalates, known PPAR activators, on reproductive success, selected gene expression and steroidogenesis in the adult female zebrafish.

**Discovery**

The discovery of PPARs arose from the pharmacological research that was being carried out in the 1960s to the mid-1980s which was aimed at understanding the phenomenon of peroxisome proliferation. Peroxisomes are single-membrane organelles found in most animal and plant cells and they contain numerous enzymes involved in breakdown of various compounds such as uric acid, hydrogen peroxide and fatty acids (Reddy et al, 1987). Peroxisome proliferation was first observed in hepatocytes of rats that were fed a diet containing clofibrate, a lipid lowering agent (Hess et al, 1965). Since then, a wide variety of compounds that induce peroxisome proliferation have been identified including herbicides, industrial plasticizers, and several pharmaceuticals (Lalloyer and Staels, 2010). Apart from peroxisome proliferation, these chemicals can elicit a wide variety of responses that included upregulating transcription of genes involved in β-oxidation of fats and increasing the activity of several hepatic enzymes (Lalwani et al, 1983; Osumi et al, 1984; Reddy et al, 1986). It was hypothesized that the pleiotropic response brought about by peroxisome proliferators were mediated through a ligand-receptor mechanism (Reddy et al, 1986; Reddy et al, 1987).

This notion that PPARs act via a ligand-receptor mechanism was confirmed by Issemann and Green (1990) who identified and cloned a ligand-activated transcription factor in rats that interacted with peroxisome proliferators such as clofibrate and was highly localized to tissues that were especially sensitive to the effects of peroxisome proliferators such as the heart, liver, and kidney (Issemann and Green, 1990; Lalloyer and Staels, 2010). They aptly named this transcription factor a “peroxisome proliferator activated receptor” or “PPAR” and placed it into the nuclear hormone receptor superfamily (Issemann and Green, 1990). In the following year, Osumi et al, (1991) showed that the promoter region of the acyl coenzyme A oxidase gene, which is involved in fatty acid breakdown, contained a peroxisome-proliferator response element.
(or) PPRE. Activation of these PPARs was shown to upregulate promoter activity of the Acyl-CoA gene providing evidence for the functional transcription regulation of genes by PPARs (Dreyer et al, 1992). Future studies would later go on to show that PPARs can regulate the transcription of a wide variety of genes involved in diverse physiological processes and multiple biological pathways (Lalloyer and Staels, 2010).

**Evolution of PPARs in mammals and fish**

PPARs belong in the steroid nuclear receptor superfamily, a superfamily that also contains thyroid hormone (TR), retinoic acid (RAR), and vitamin D receptors (Laudet et al, 1992; Laudet, 1997). Phylogenetic analysis of nuclear receptor structures showed that the ancestral nuclear hormone receptor appeared early in eukaryote evolution around 500 million years ago and underwent two duplication events: one duplication event was before the arthropod/vertebrate split and another more recent duplication that was restricted to vertebrates (Escriva et al, 2000; Zhou et al., 2015). Along with other receptors such as TR and RAR, it is likely that the ancestral PPAR arose during the first duplication event which occurred before the vertebrate lineage diverged from arthropods (Escriva et al, 2000; Michalik et al, 2002). The second duplication event, which was vertebrate specific, occurred right before bony fish separated from mammals and birds and this likely resulted in the three types of PPARs shared among all vertebrates (Laudet et al, 1992; Bertrand et al, 2007). Following the second duplication event, rapid mutation, speciation events and evolution of independent ligand binding capacities resulted in a much more complex and diverse array of PPAR isoforms in teleosts than in mammals (Escriva et al, 2000; Zhou et al, 2015).

Presently, three major isoforms of PPARs are found in mammals: PPARα, PPARβ and PPARγ. Due to differential promoter usage and alternate splicing of the same gene, three isoforms of PPARγ (γ1, γ2 and γ3) are found in rodents and humans (Zhu et al, 1995; Fajas et al, 1999). Compared to mammals, the number, and types of PPARs varies considerably between different fish species. For example, sea bass, rabbit fish and brown trout possess only one isoform of PPARα, whereas the turbot, sea plaice and pufferfish have two different PPARα isoforms (Boukavala et al, 2004; Batist-Pinto et al, 2005; Cunha et al, 2013). The Atlantic salmon has four distinct isoforms of PPAR-β
(β1A, β1B, β2A, β2B) (Leaver et al., 2007). Currently only one PPARγ gene and isoform has been identified in teleosts but the number of species investigated is limited and a more comprehensive analysis of PPAR isoforms in fish species is required. Previously, Ibabe et al, (2005) identified three PPAR isoforms in zebrafish but more recent studies have shown the presence of five different genes for PPAR isoforms (αA, αB, βA, βB and γ) in the zebrafish genome (Den Broeder et al, 2015).

**Receptor structure and mechanism of action**

PPARs in both mammals and teleosts share a common molecular structure made up of multiple functional domains however certain domains are not well conserved between the two groups. The DNA binding domain (DBD) which binds specifically to PPREs in the promoter region of target genes is highly conserved among mammals and teleost (Robinson-Rechavi et al, 2003). The ligand binding (E/F) domain in PPARs is a characteristic feature of the subfamily due to it being 3-4 times larger than those of other nuclear hormone receptors (Grygiel-Gorniak et al, 2014). This ligand binding region is poorly conserved between mammals and teleosts, with fish like the zebrafish possessing a significantly larger and more hydrophilic domain compared to mammals (Boukalava et al, 2004; Leaver et al, 2005; Den Broder et al, 2015). The (A/B) domain mediates ligand independent activation of PPARs and is also not well conserved and is also found to be much larger in fish than in mammals (Werman et al, 1997; Leaver et al., 2005). Finally, the D-domain consists of a flexible hinge region and is responsible for receptor dimerization with the retinoid-x-receptor (RXR) (Werman et al, 1997). The D-domain of PPARs has two fewer amino acids than that of other nuclear receptors and serves as a distinguishing feature of PPARs in both mammals and fish.
Figure 1. Activation of PPAR by ligands at the ligand-dependent region (E/F) causes the flexible hinge region (D) to interact with the RXR and with the help of the DNA-binding region (C), this PPAR:RXR complex binds to PPREs of target genes. Along with the assistance of co-repressor or activator proteins PPARs can influence transcription of genes involved in different physiological processes. Figure is adapted from Goygiel-Gorniak, 2014.

PPARs upon activation combines with the RXR and the resultant PPAR:RXR complex then binds to PPREs in the promoter region of PPAR responsive genes (summarized in Figure 1; Laudet et al, 1992). Studies show that the ligands of RXR such as 9-cis retinoic acid can activate the PPAR:RXR complex and act in synergy with the PPAR specific ligands to influence gene transcription (Nagase et al, 1999). PPARs can also recruit either coactivator or corepressor proteins that bind to the PPAR:RXR complex to initiate or suppress transcription of target genes (Berger and Moller, 2002). The nature and type of cofactors that interact with PPARs is dependent on the ligands interacting with the PPARs (Oberfield et al, 1999; Berger an Moller, 2002). Different ligands can affect the affinity to which PPARs interact with other local factors and this
has implications on the downstream physiological process that are mediated by PPARs (Oberfield et al, 1999; Berger and Moller, 2002).

**PPARs in mammals: Expression, Function, and Ligands**

While PPARα and PPARγ have been well characterized in mammals in terms of tissue distribution, function and the ligands they interact with, much less work has been done on PPARβ (Schmidet et al, 1992, Amri et al, 1995, Aperlo et al, 1995). PPARα is found in metabolically active tissues such as heart, liver, intestine and skeletal muscle (Auboeuf et al, 1997; Mukherjee et al, 1997). Accordingly, PPARα influences transcription of genes involved in metabolic processes such as fatty acid uptake, triglyceride breakdown and glucose metabolism (Berger and Moller, 2002). PPARγ is also expressed in areas of high mitochondrial activity but is predominantly found in the adipose tissue, large intestine and spleen (Braissant et al, 1996; Lemberger et al, 1996). Studies show that PPARγ plays a critical role in adipogenesis, energy balance and insulin sensitization (Spiegelman et al, 1998; Medina-Gomez et al, 2007). Compared to the other isoforms, PPARβ expression is found to be much more ubiquitous and widespread in mammalian tissues, and is the predominant isoform found in the adult nervous system (Xing et al, 1995). Studies have implicated PPARβ in regulating genes involved in fatty acid oxidation, glucose metabolism and myelin sheath formation (Kersten et al, 2000; Woods et al, 2003; Barish et al, 2006). Apart from their metabolic role, PPARs have also been implicated in regulating non-metabolic physiological processes such as inflammation, blood vessel formation, apoptosis, and reproduction (Berger ad Moller, 2002).

As stated earlier, PPARs possess the largest ligand binding domain in the nuclear receptor family which allows them to interact with a wide range of endogenous and exogenous ligands (Grygiel-Gorniak, 2014). Endogenous ligands shared by all PPARs are primarily made up of fatty acids by-products released during lipid metabolism and this includes a range of unsaturated and saturated fatty acids (Grygiel-Gorniak, 2014). Both PPARγ and PPARα are also targeted by lipid metabolites produced by the breakdown of the fatty acid: arachidonic acid such as 15-Hydroxyeicosatetraenoic acid (15 (S) – HETE), Leukotriene-B4 and prostaglandins.
(Devchand et al, 1996; Campbell et al, 2009). Prostaglandins such as 15-deoxy 12,14-prostaglandin J₂ (15-PDГJ₂) and prostaglandin J₂ (PGJ₂) (Ray et al., 2006) are agonists that bind specifically with PPARγ, while omega-3 fatty acids such as eicosapentaenoic acid (EPA) have been shown to activate PPARα in a dose-dependent manner (Ray et al., 2006; Grygiel-Gorniak, 2014). Common modulators of PPAR-β/δ include very low-density lipoproteins (VLDL) and the prostaglandin I₂ (PGI₂) (Amri et al, 1995; Forman et al, 1997). A list of endogenous PPAR ligands can be found in Table 1.

Fibrates such as clofibrate, fenofibrate and bezafibrate, are activators of PPARα in mammals (Schoonjans et al, 1996). Thiazolidinediones (TZDs) are antidiabetic drugs that activate only PPARγ and are insulin-sensitizing agents which are used in the treatment of Type II diabetes and polycystic ovarian syndrome (POCs) (Saltiel and Olefsky, 1996; Olefsky and Saltiel, 2000). Growing research utilizing TZDs such as rosiglitazone and troglitazone have provided some insight into the reproductive role of PPARγ in mammals (Komar et al, 2001; Froment et al, 2006). Apart from pharmaceuticals, compounds used in PVC manufacturing such as phthalates and bisphenols have been shown to interact with both PPARα and PPARγ (Bility et al, 2004; Desvergne et al, 2009). Synthetic agonists for PPARβ are not as common and novel drugs such as L-165041 and GW501516 have been specifically developed in the lab for potential therapeutic use in metabolic syndrome (Berfer et al., 1999; Grygiel-Gorniak, 2014). A summary of exogenous PPAR agonists can be found in Table 1 below.
Table 1. Summary of both natural and synthetic ligands of PPARs. Information present in the table is summarized from Bensinger and Tontonoz., 2008 and Grygiel-Gorniak., 2012.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Natural Ligands</th>
<th>Synthetic Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>Saturated fatty acids&lt;br&gt;Unsaturated fatty acids&lt;br&gt;Omega – 3 fatty acids: DHA, EPA&lt;br&gt;Eicosanoids: Leukotriene B4, 15 (S)-HETE, 8(S)-HETE</td>
<td>Clofibrate, Fenofibrate, Gemfibrozil&lt;br&gt;Phthalates: DEHP, DiDP, MEHP, DIBP</td>
</tr>
<tr>
<td>PPARβ</td>
<td>Saturated fatty acids&lt;br&gt;Unsaturated fatty acids&lt;br&gt;Very low-density lipoproteins (VLDL)&lt;br&gt;PGI₂</td>
<td>L-165041&lt;br&gt;GW501516</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Unsaturated fatty acids&lt;br&gt;Eicosanoids: 15-HETE, COX 1/2-derived metabolites: 9-HODE, 13-HODE&lt;br&gt;Prostaglandins: 15d-PGJ₂, PGJ₂</td>
<td>TZDS: Rosiglitazone, Ciglitazone, Troglitazone, Pioglitazone&lt;br&gt;Phthalates: DEHP, MEHP</td>
</tr>
</tbody>
</table>

**PPARs in teleosts: Expression, Function, and Ligands**

Research on fish PPARs is far less extensive compared to mammals when it comes to characterizing the tissue expression, biological function, and ligand specificity of these nuclear receptors. The tissue distribution of PPARs in teleost tends to follow the same pattern seen in rodents and humans (Liu et al, 2005). In the brown trout, PPARα and β mRNA are highly expressed in heart, liver, white muscle, and testis where as PPAR-γ was only detected in the kidney (Batista – Pinto et al, 2005; Liu et al, 2005). In zebrafish, western blot analysis shows that PPARα and PPAR γ are
concentrated in tissues where there is high fat metabolism such as adipose tissue, liver, kidney, and pancreas while PPARβ expression is more widespread (Ibabe et al, 2002). Collectively the expression profiles seen in teleosts suggest that PPARs may perform similar regulatory roles in lipid metabolism like their mammalian counterparts. Analysis of the expression pattern of fatty acid genes during embryonic development in the marine fish, turbot reveals a strong correlation between transcription of these genes with PPAR (α1, α2, β and γ) mRNA levels (Cunha et al, 2013). In sea bass, both PPARα (α1 and α2) isoforms upregulate promoter activity and transcription of the fatty acid desaturase 2 (FADS2) gene which is involved in long chain fatty acid breakdown (Dong et al, 2017). The fatty acid binding proteins (FABP) are involved in adipocyte differentiation and studies show that their genes possess PPREs for all three isoforms in their promoter region in zebrafish (Venkatchelam et al, 2013).

Information on the endogenous activators of PPARs in fish is scarce but limited studies have suggested fatty acids and prostaglandins as potential ligands of PPARs (Boukalava et al, 2004; Ganga et al, 2006; Cunha et al, 2013). The fatty acid, linoleic acid was shown to significantly increase the transcription activity of both PPARα and PPARβ in sea bass while it had no effect on PPARγ (Boukalava et al, 2004). In zebrafish, metabolites of the arachidonic acid pathway are suggested to interact with PPARs to regulate cortisol production in the kidney (Ganga et al, 2006). In torafugu pufferfish, the omega-3 fatty acids, DHA and EPA were shown to bring about a 4-5 fold increase in PPARα1 and PPARα2 transcription activity whereas no change in PPAR-β and PPARγ was observed (Kondo et al, 2007). Synthetic activators of PPARs in fish have also been identified including pharmaceuticals, synthetic estrogens, herbicides, and chemicals used in PVC manufacturing such as phthalates (Desvergne et al, 2009; Huang and Chen, 2017).

While pharmaceuticals such as fibrates have proven to be effective activators of PPARα in both teleosts and mammals, this is not necessarily the case for all synthetic ligands (Riu et al, 2011). In trout, the surfactant perfluorooctanoic acid (PFOA) was shown to bind with higher affinity to PPARγ than potent ligands such as rosiglitazone and GW1929 (Leaver et al., 2005). In transient transfection experiments, rosiglitazone
showed very weak binding affinity for the zebrafish PPARγ and was only able to activate the ligand binding domain (LBD) at extremely high doses which is contrast to what is seen in rodents and humans (Riu et al., 2011). In the same experiment, flame retardants such as tetrabromobisphenol A (TBBPA) and the phthalate, mono-(2-ethylhexyl) phthalate (MEHP), activated the LBD much more easily over a wide range of doses (Riu et al, 2011). Aside from MEHP, phthalates like disodecyl phthalate (DiDP) binds with high efficiency to all three PPARs in sea bream (Riu et al, 2011; Cocci et al, 2015). Though not necessarily always the case, ligands that are specific to certain PPARs in mammals can also interact with different isoforms in teleost. For example, the herbicide, clofibric acid which exclusively activates PPARα in mammals, was shown to increase PPARγ transcripts and acyl-CoA oxidase activity in salmon hepatocytes (Ruyter et al, 1997). These differences in ligand binding specificity between mammals and fish may have implications on the physiological process that are affected by PPAR activation in teleosts.

PPARs and mammalian reproduction

Understanding of the role that PPARs play in vertebrate reproduction has primarily come from research conducted in mammals. Immunohistochemistry and western analysis have shown that PPARs are expressed in the ovarian tissue of mice, cow, rats, pigs, mice, and humans (Komar, 2005). Among the three isoforms, PPARγ is the most extensively studied and has received considerable attention for its possible role as a critical regulator of various ovarian processes such as ovarian follicle development, ovulation, and gonadal steroidogenesis (Komar et al, 2001).

In vertebrates, ovarian follicle development is characterized by two phases: one which entails follicular growth where an immature oocyte initially grows in size, developing layers of follicular cells followed by a maturation stage where the immature oocyte transitions into a mature egg ready for ovulation (Selman et al, 1993). In the rat ovary, while PPARα and PPARβ gene expression remain stable throughout ovarian follicle development, PPARγ gene expression drastically drops in follicles that are undergoing oocyte maturation (Komar et al, 2001). The stable expression of PPARα and PPARβ during the entire process suggests that these transcriptions factors may be
involved in regulating both the follicular growth and maturation phases, while the suppression of PPARγ during maturation indicates that while this isoform might be involved in early follicular growth, it might have an inhibitory role in maturation and/or ovulation (Komar et al., 2001; Komar et al., 2005).

During ovulation, the follicular layer ruptures and mature oocyte or egg is released from the ovarian follicle to be fertilized (Takahashi and Ohnishi, 1995). The primary upstream inducer of ovulation is the luteinizing hormone (LH) and studies in certain mammals show that while PPARα and PPARβ do not appear to be affected by LH, PPARγ is regulated by LH although this occurs in a species-specific manner (Komar et al., 2001; Kim et al., 2008). In rats, there is a decrease in ovarian PPARγ gene expression in response to LH (Komar et al., 2001; Komar et al., 2005). In vitro studies utilizing the human chorionic gonadotropin (hCG), an LH analog, show that PPARγ gene expression is downregulated four hours after hCG treatment in rat and macaque granulosa cells (Komar et al., 2005). This downregulation suggests that PPARγ may play a negative regulatory role in ovulation in these species and its suppression by LH is required for the normal ovulatory cascade to take place (Komar et al., 2005; Funuhashi et al., 2017). However, mice, PPARγ gene expression in the ovary increases five hours following hCG treatment suggesting that this transcription factor may be necessary for ovulation in certain mammals (Kim et al., 2008).

Cui et al., (2002) showed that elimination of the PPARγ gene from mice ovary resulted in infertile mice which took longer periods to conceive and smaller litter size, however, the physiological mechanism underlying these phenomena remained unclear. Later, Kim et al., (2008) also created knock-out mice (PPARγ-null) which had the PPARγ gene deleted from the ovary and demonstrated a significant impairment to ovulation. PPARγ-null mice showed a reduction in the different factors involved in ovulation such as endothelin-2 (ET-2) and interleukin-6 (IL-6) (Kim et al., 2008). Unlike its effects in the wild type mice, hCG was incapable of inducing gene expression of these factors required for ovulation in PPARγ null mice (Kim et al., 2008). Cyclooxygenases (COX) such as COX-2 catalyze different aspects of the arachidonic acid pathway and COX-derived metabolites such as prostaglandins and fatty acids are endogenous activators
of PPARγ (Yang and Frucht, 2001). Treatment with a general COX inhibitor, indomethacin and the COX2, NS-398 specific inhibitor significantly reduced gene expression of ET-2 and IL-6 in the ovaries (Kim et al., 2008). This suppression of gene expression in wild type mice was rescued when the ovaries were treated with the PPARγ agonist, rosiglitazone, (Kim et al, 2008). Collectively, these results show that PPARγ is a critical regulator of ovulation in mice and this is primarily through its regulation of various genes that are active during ovulation (Kim et al, 2008).

Studies also show that PPARγ and PPARα activation affect the ovarian steroidogenic pathway in mammals (Komar et al, 2001). Most of the studies that have focused on PPARγ have utilized TZDs as the ligands and present conflicting results. For example, TZDs stimulate PPARγ to increase production of the female sex steroid, 17β-estradiol, in rat and sheep granulosa cells (Komar et al, 2001; Froment et al, 2003), whereas they inhibit 17β-estradiol secretion in porcine and human granulosa cells (Gasic et al, 1998; Mu et al, 2000). The steroidogenic acute regulatory protein (StAR) is a transport protein that is involved in transferring cholesterol across the mitochondrial membrane and is the rate limiting factor in the steroidogenic pathway (Stocco et al, 2001). In humans and mice, TZDs stimulate StAR promoter activity, gene and protein expression suggesting that in these organisms, PPARγ activation induces testosterone production and this might be due to its regulation of StAR (Seto-Young et al, 2007; Kowalewski et al, 2009).

Though there is less work on PPARα involvement in steroidogenesis, few studies show that activation of this isoform has effects on the production of the female sex steroid, 17β -estradiol, and this is primarily through its regulation of the P450 aromatase enzyme. Aromatase mediates the last step of estrogen synthesis and catalyzes the conversion of androstenedione to estrone and testosterone to estradiol (Simpson et al, 1994). Activation of PPARα by fibrates inhibits gene expression of aromatase and suppressed overall estrogen synthesis in the mouse ovary (Toda et al, 2003). Phthalates like MEHP can activate PPARα to inhibit aromatase gene expression and 17β-estradiol production in rat granulosa cells (Lovekamp-Swan et al, 2003). Collectively, these results show that the PPAR isoforms, especially PPARα and PPARγ,
can influence sex steroid production by regulating transcription of genes involved in the steroidogenic pathway (Stocco et al, 2001; Toda et al, 2003; Lovekamp-Swan et al, 2003).

**PPARs and teleost reproduction**

There is very little information on the expression and biological functions of PPARs in the reproductive tissues of teleost. Previously, Ibabe et al (2002) characterized the presence of three PPARs (α, β and γ) in the zebrafish ovary and showed that their protein expression decreases with follicular development. More recent studies have revealed the existence of five different PPAR isoforms, it is presently unclear if all five of these PPARs are found in the zebrafish ovarian cells and what function they may serve (Den Broeder et al, 2015).

Several studies have shown that phthalates affect the reproductive process in teleost and it is generally thought that the negative effects of phthalates on reproduction are primarily mediated through PPARs (Hurst and Waxman, 2003; Huang and Chen, 2017). A previous study on zebrafish showed that male fish which were injected with extremely high doses (5000 mg/kg) of diethylhexyl phthalate (DEHP) experienced an inhibition in sperm development, reduced fecundity and an increase in the transcripts of PPAR responsive genes such as acyl-coenzyme A oxidase 1 (Uren-Webster et al, 2008). Adult marine medaka that were exposed to both DEHP (0.1 and 0.5 mg/L) and MEHP (0.1 and 0.5 mg/L) experienced diminished fertilization, overall reduction in egg production and sex-specific changes in steroid production at all doses tested (Ye et al, 2014). In female fish, 0.5 mg/L of DEHP was shown to increase whole body levels of 17β-estradiol and testosterone, while 0.1 mg/L was shown to increase just testosterone levels while having no effect on 17β-estradiol (Ye et al, 2014). In male fish, both DEHP and MEHP increased 17β-estradiol at all doses measured, while no change in testosterone production was seen (Ye et al, 2014). An increase in gene expression of steroidogenic enzymes such as StAR, aromatase and 17β-HSD was observed in response to DEHP and low dose MEHP treatment groups in males but not in females (Ye et al, 2013).
A separate study in female zebrafish that were exposed to 2, 20 and 40 µg/L of DEHP experienced a significant reduction in ovulation and embryo production (Carnavalli et al., 2010). This was associated with a downregulation in ovarian cox2 and membrane progestin receptor β (mprβ) gene expression and increase in bone morphogenic protein 15 (BMP15) (Carnavalli et al., 2010). While cox2 and mprβ are facilitators of ovulation and oocyte maturation, respectively, studies show that BMP15 is an inhibitor of premature oocyte maturation in zebrafish follicles (Clelland et al, 2007; Clelland and Ping, 2009). In zebrafish and most teleosts 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) is the maturation inducing steroid (MIS) and interacts with mprβ to induce oocyte maturation in ovarian follicles (Patino and Sullivan, 2002). An in vitro maturation assay showed that 10 and 100 nM of DEHP negatively affect oocyte maturation by blocking 17α,20β-P induced maturation in zebrafish follicles (Carnavalli et al, 2010). Overall, while studies focusing on the ligand activation of PPARs by phthalates in fish are limited, they have shown that PPARs are involved in teleost reproduction and have also shed some light into potential reproductive genes that are regulated by these transcription factors.

**Thesis: Objectives**

The goal of this study was to identify the members of the PPAR family in the zebrafish ovary, describe their expression pattern across follicular development and up to and during the ovulation period. This project attempted to determine whether the activation of these PPARs by phthalates influenced different reproductive parameters of the female zebrafish. I hypothesized that PPARs were expressed in the zebrafish ovary and played a fundamental role in the control of ovarian follicle development, steroidogenesis, and ovulation. First, I predicted that expression pattern of PPARs would change as the ovarian follicle undergoes development and that this pattern would be dynamic with relation to ovulation. I also predicted that mRNA expression of PPARs are modulated by natural regulators of reproduction in zebrafish such as gonadotropins and sex steroids. Finally, I predicted that activation of PPARs by phthalates would affect reproduction in female zebrafish, specifically by inhibiting oocyte maturation, modifying gonadal steroidogenesis, and reducing egg production.
Zebrafish are asynchronous spawners which means that follicles at different stages of development can be found simultaneously in the ovary. Therefore, to test my first prediction, I used qPCR to measure the gene expression of each PPAR isoform in follicles collected at different developmental stages. In addition, I measured gene expression of PPARs in whole ovarian tissue collected at three-hour intervals during a 12-hour period leading to ovulation to determine if PPAR expression was dynamic with relation to ovulation. For my second prediction, I treated full grown follicles with the LH analog, hCG, and the MIS, 17α,20β-P, in vitro for three and six-hour periods after which gene expression of PPARs was measured. In a separate in vitro assay, I treated full grown follicles with activators of the protein kinase C (PKC) pathway: PMA and A23187 for a period of six hours to determine if PPARs are regulated by the PKC pathway. Previous studies in teleosts show that the PKC pathway is involved in different aspects of reproduction such as gonadal steroidogenesis, oocyte maturation and ovulation (Srivastava and Van Der Kraak, 1994; Mishra and Joy, 2006).

To test my third prediction, I started out with two in vitro studies using DEHP and MEHP to determine the downstream effects of PPAR activation on oocyte maturation and steroid production. In the first set of in vitro experiments, I incubated immature full-grown follicles with DEHP for a period of four hours after which 17α,20β-P was added and then incubated for a further six hours. At the end of the incubation, germinal vesicle breakdown (GVBD) was assessed to see if DEHP disrupted 17α,20β-P induced oocyte maturation. GVBD signals the resumption of meiosis during oocyte maturation and is used as a marker for successful maturation in teleosts (Clelland and Ping, 2009). In my second set of in vitro experiments, I treated follicles with MEHP alone and in combination with hCG for a period of six hours to determine whether MEHP had any influence on testosterone production in the zebrafish ovary. Following incubation, media was separated from the follicles and enzyme immune assays (EIAs) was used to measure testosterone content.

Finally, I carried out two separate in vivo experiments utilizing, DEHP and MEHP to determine the downstream effects of PPAR activation on overall zebrafish fertility. Fish were injected with these chemicals over the course of 8 days during which egg
production was assessed. After each experiment, I collected the ovary and a portion of this ovary was taken to measure gene expression of PPARs and various local factors that play crucial roles in different aspects of zebrafish reproduction. These include but were not limited to genes that mediate oocyte maturation and ovulation such as mprβ, cox2, mmp9 and those that are involved in steroidogenic pathways such as star and 17βhsd (Clelland and Ping, 2009). Finally, EIAs were carried out to measure ovarian testosterone (T) and 17β-estradiol (E2) content.
Methods

Experimental Animals

Zebrafish were purchased from AQUAlity Tropical Fish Wholesalers (Mississauga, ON) and housed in an environmental chamber at the Hagen Aqualab (University of Guelph, Guelph, ON). Fish were held in A-HAB containment units (Aquatic Habitats, Apopka, FL) that were approximately 4L and were supplied with recirculating water maintained at 28°C. Photoperiod in the chamber was set to 12 hours light: 12 hours dark. Fish were fed twice daily to satiation with frozen brine shrimp (AQUAlity) in the morning and fish pellets (North Fin, Mississauga, ON) in the afternoon. Zebrafish used in experiments were anaesthetized by overdosing in a buffered tricaine methanesulfonate solution (MS-222; 300 mg/L) (Syndel Laboratories Inc, Vancouver, ON) and then euthanized by severing the spinal cord. In the case of female zebrafish, whole body weight and ovary weight were recorded. The experiments were conducted using protocols approved by the University of Guelph Animal Care Committee on behalf of the Canadian Council on Animal Welfare.

Experimental design

*Experiment 1: Expression of PPARs across follicular development*

This experiment was carried out to quantify the expression of PPARs in ovarian follicles at different stages of development. Ovaries were collected from ten to twelve female zebrafish (n = 10-12) at 10:00 AM and immediately placed in 500 mL of 60% Leibovitz L-15 culture medium (Invitrogen, Carlsbad, CA). This medium contained 10 ml of penicillin (10,000 units/mL)/streptomycin (10,000 µg/mL) (Invitrogen, Carlsbad, CA) per 500 ml of medium to prevent bacterial growth. Follicles at different stages of development (as described by Selman et al, 1993) were separated using fine forceps under a light microscope and distinguished by various morphological features such as size and opacity from yolk accumulation. The follicles were sorted into the following stages: (I) primary growth (II) early vitellogenic (III-A) mid-vitellogenic stage (III) late vitellogenic and (IV) full grown. Follicles from each stage were stored at -80°C for subsequent measurement of gene expression by quantitative real-time PCR (qPCR).
Experiment set up, follicle collection and RNA extraction in this experiment was previously carried out by previous lab members to determine expression of other genes active during ovarian follicle development. I used cDNA obtained from this experiment to determine the expression of PPARs across follicular development as well as the relative expression of each isoform within each follicular stage.

Experiment 2: Expression of PPARs with respect to ovulation.

This experiment was carried out to determine any change in the temporal expression patterns of PPARs in the zebrafish ovary with respect to the ovulatory process. Six female fish and three males were housed in a 3.8 L tanks. There were a total of ten tanks and they were assigned to five different time periods: 10 PM, 1 AM, 4 AM (preovulatory period), 7 AM (ovulation) and at 10AM (post ovulation) such that there were two tanks per period. Only female fish were sampled (n= 10 – 12). Whole ovaries were extracted and were snap frozen at -80C. The whole ovarian tissue was used for subsequent quantification of PPAR expression by qPCR. This experiment was previously carried out by previous lab members to determine expression of other genes active during different time frames leading up to and after ovulation. I used cDNA obtained from this experiment to determine the expression of PPARs with respect to ovulation.

Experiment 3: Regulation of PPARs: hCG and 17α-20βP

This experiment tested the in vitro effects of hCG and 17α-20βP on the mRNA expression of PPARs. The methods used in this experiment followed established protocols previously carried out by Nelson and Van Der Kraak, (2010) and Irwin and Van Der Kraak, (2012). Five to six adult female fish were sampled at 9AM and ovaries were removed for determination of GSI. Full grown follicles were separated from the rest of the ovary using fine forceps and pooled. From the pool of full grown follicles, 20 follicles were placed in a well containing 500 µL of 60% L-15 culture medium in a 24-well polystyrene plate (Corning Life Sciences, Corning, NY). Follicles were treated with control (Ethanol + water), hCG (20 IU/ml) or 17α-20βP (10 ng/ml). There were 4 replicates per treatment and the experiment was repeated 6 times. The control was treated with a mix of water and ethanol to account for the carriers for hCG (water) and
17α-20βP (ethanol), respectively. The final volume of ethanol added to each well was 2.5 µl such that the concentration of the ethanol was 0.5 % of the total volume. Following addition of the chemicals, plates were covered with aluminum foil and incubated in darkness with shaking on an Innova 4000 incubator shaker (LabX, Midland, ON) set to 40 rpm and at a temperature of 28°C. Follicles were incubated for a period of either three or six hours after which follicles were removed from the wells and frozen for subsequent quantification of PPAR expression by qPCR.

Experiment 4: Regulation of PPARs: PMA/A2378

This experiment was carried out to determine if PPARs are regulated by activators of the PKC pathway: PMA and A2387. The methods used in this experiment followed established protocols previously carried out by Wade and Van Der Kraak, (1991) and Irwin and Van Der Kraak, (2012). Similar to the previous in vitro experiment, full grown follicles from five to six adult zebrafish were pooled and 20 follicles were placed in a well containing 500 µL of culture medium and treatment. The treatments were Control, PMA (400 nM)/A23187 (10µM), hCG (20 IU/ml) and hCG plus PMA/A23187. There were four replicate wells per treatment and the experiment was repeated three times. The control was treated with a mix of water and ethanol to account for the carriers for hCG (water) and PMA/A23187 (ethanol), respectively. After incubation for 6h using the conditions described above, follicles were removed from the wells and frozen at - 80°C for RNA extraction and quantification of PPAR expression by qPCR.

Experiment 5: In vitro effects of DEHP on oocyte maturation

This experiment was carried out to determine if activation of PPAR by DEHP disrupted 17α-20βP induced oocyte maturation in the immature full-grown follicles of zebrafish. The methods used in this experiment followed established protocols previously carried out by Carnevali et al, (2010). Following the same protocol as the previous experiment, full grown follicles from five to six adult zebrafish were pooled and 20 follicles were placed in a well containing 500 µL of culture medium and treatment. The incubation period was 10 hrs including a pre-treatment with control or DEHP for 4 hrs followed by incubation with control or 17α-20βP (MIS), Follicles were preincubated
with 1 and 10 µM DEHP for 4 hrs. The medium was replaced with control (EtOH), 17α-20βP (10 ng/ml), DEHP (1 µM) + 17α-20βP (10 ng/ml) and DEHP (10 µM) + 17α-20βP (2 ng/µl). There were four replicates per treatment and the experiment was repeated only once (n=1). At the end of the six-hour incubation period, the proportion of follicles that completed germinal vesicle breakdown (GVBD) was determined. %GVBD is an indicator of successful maturation in follicles.

Experiment 6: In Vitro effects of MEHP on testosterone production

This experiment was carried out to determine the effects of the PPAR activator MEHP on testosterone production in zebrafish full grown follicles. Full grown follicles from five to six adult zebrafish were pooled and 20 follicles were placed in a well containing 500 µL of culture medium and treatment. The treatments were Control (EtOH), MEHP (50 µM), hCG (20 IU/ml) and MEHP (50 µM) + hCG (20 IU/ml). There were four replicates per treatment and the experiment was repeated four times (n=4). After incubation for six hours, media was removed from the wells and frozen at -80°C for subsequent determination of the amount of testosterone produced by the follicles.

Experiment 7: In Vivo effects of DEHP on zebrafish reproduction

This study determined the in vivo effects of the known PPAR ligand DEHP on egg production and the expression of selected genes involved in reproduction and the measurement of ovarian sex steroid levels in female zebrafish. Sexually mature fish were selected and placed into groups of five females and three males in 3.8 L tanks. Each tank was fitted with a breeding apparatus consisting of an artificial plant and marbles that were placed on top of a filter mesh. Spawned eggs from breeding would pass through the filter mesh and settle at the bottom of the tank for collection. Each aquarium was supplied with an air stone and water changed every morning. Fish were acclimated to the tank for five days during which egg production was monitored daily. At the end of the acclimation period, tanks were distributed to the different treatments such that the average number of eggs produced per groups of three tanks was similar. The fish then underwent a seven-day pre-exposure period during which they were not exposed to any chemicals. This was done to establish a baseline for egg production that
allowed comparison with egg production during the exposure period. There were three tanks of fish assigned to each treatment (n=3).

On day seven of the pre-exposure period, fish received intraperitoneal injections of either 0, 50 or 500 mg/kg of DEHP (Accustandard, Connecticut, USA) at 5:00 PM. Due to the extremely low solubility and very rapid breakdown of phthalates in water, exposure via injection is considered the optimal method to investigate phthalate exposure in fish (Oehlmann et al, 2009; Uren-Webster et al, 2010). DEHP that was used in the experiment was stored at 4°C and dissolved in 95% ethanol at a concentration of 100 µg/µl. The low dose of 50 mg/kg was chosen based on previous study on male zebrafish carried out by Uren-Webster et al, (2010). The high dose of 500 mg/kg is much higher than concentrations found in the environment but was chosen to evaluate the mechanisms through which DEHP disrupts reproduction in zebrafish. In order to reduce the amount of ethanol being injected into the fish, the DEHP: ethanol solution was diluted in Ultrapure DNase/RNase free distilled water (Invitrogen, Carlsbad, CA). Fish were injected with 20 µL of carrier containing DEHP or control with 2.8 µl of ethanol being injected into each fish through the abdomen. Injections were done with ½ cc syringe, with a 30G ultrafine II needle (Becton Dickinson, Mississauga, ON). The control (0 mg/kg) received a mixture of ethanol and water. The amount of DEHP injected into each fish for each treatment was calculated based on estimated average body mass of 0.8 g.

Including the injection on the last day of the pre-exposure, fish were injected once every two days (day 2, 4, and 6) such that they were injected a total of four times during the exposure period. Fish were injected in the afternoon every other day so as to allow the zebrafish to rest and recover from the stress induced by injection. Eggs were collected every morning and egg production per female per tank was calculated in order to account for any misidentification of sex or female mortalities that may have occurred during the course of the experiment. On day 8 fish were euthanized at 9:00 AM, ovaries were removed from female zebrafish, weighed and immediately frozen on dry ice in microfuge tubes and stored at -80°C. A section of these ovaries were used for the
analysis of gene expression. A section of the ovaries was used to measure T and E₂ levels using Enzyme Immunoassays (EIAs)

Experiment 8: In Vivo effects of MEHP on zebrafish reproduction

This experiment determined the effects of another PPAR activator, MEHP, on female reproduction. Similar to the DEHP experiment, changes in reproductive endpoints including egg production, gene expression and steroidogenesis were measured. Five females and three males were housed as described above for Experiment 7. Acclimation and exposure conditions followed the same protocol as the previous in vivo using DEHP. There were three tanks of fish assigned to each treatment (n=3).

On day seven of the pre-exposure period, fish were injected intraperitoneally 20 µl of 0, 35.6 or 356 mg/kg of MEHP (Accustandard, Connecticut, USA) at 5:00 PM. MEHP was stored at 4°C and was dissolved in olive oil at a concentration of 100 µg/µl. Since MEHP is a metabolite of DEHP, dosage of MEHP was tested at an equimolar concentration of that used in the previous experiment. Eggs per female per tank were measured daily. On day 8, fish were euthanized at 9:00 AM and the samples were collected and processed as described above.

Analytical techniques.

RNA extraction and revere transcription

Total RNA was extracted from the ovarian follicles using TRIxol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Approximately 20 mg of ovarian tissue was homogenized in 800 µL TRIxol reagent using a 3mL syringe with 21G needle (Becton Dickinson, Mississauga, ON). After a five minute incubation period, 160 µL of chloroform was added and mixed by rigorous inversion for 15 seconds. Followed by a three minute incubation period, samples were centrifuged at 12,000 xg for 15 minutes at 4°C. The aqueous phase was transferred to a new tube to which 400 µL of isopropyl alcohol / isopropanol was added. This new mix was pulse vortexed and allowed to incubate at room temperature before centrifugation at 12,000 xg for 10 minutes at 4C. A RNA pellet was produced and the isopropanol was discarded.
Ethanol (75%) was added to the pellet and centrifuged at 7500g for 5 minutes. The ethanol was discarded and the pellet was allowed to dissolve in 5 – 15 µL of Ultrapure water at 60°C for 10 minutes.

A Nanodrop 8000 (Thermo Scientific, Waltham, MA) was used to quantify RNA present in each sample. All final RNA samples were diluted to 500 ng/µL. Following quantification, samples underwent DNAase treatment and reverse transcription as described by Nelson and Van Der Kraak (2010). DNAase treatment was done using a DNase treatment kit (Sigma, St. Louis, MO) in order to remove any trace amounts of DNA remaining from previous steps. A cocktail consisting of DNAse enzyme, buffer and water is added to each sample. To ensure no external DNA contamination from reagents used during the entire process, a “No template / water control” (NTC) was run alongside the samples. Following DNase treatment, samples were subject to reverse transcription to generate a first strand cDNA. In addition, “no-RT” controls were also run to confirm absence of genomic DNA contamination. A no-RT control contained all the reagents used in reverse transcription except for the reverse transcriptase. In each experiment, approximately 10% of the samples were randomly chosen for no-RT controls. Final samples were incubated in Thermocycler C100 Touch (Bio-Rad, Mississauga, ON) at 37°C for 1 hour followed by 5 minutes at 95°C. At the end of the incubation, undiluted cDNA was obtained and these samples were then stored at -20°C for subsequent dilution and preparation for RT-qPCR.

Quantitative real-time PCR

Relative levels of gene expression were measured using RT-qPCR. Primer sequences used for qPCR are shown in Table 1 and were purchased from Sigma Aldrich (St.Louis, MO). In order to confirm the specificity of the PPAR primers, final PCR product was purified and primers were sequenced at the Advanced Genomics Facility, Guelph, ON and confirmed using BLAST (NCBI). A standard curve to determine efficiency of primers and relative abundance of cDNA was made by serially diluting pooled cDNA from all samples. Each reaction compromised of 3.75 µl of 10x diluted cDNA, 1.875 µl of forward and reverse primer and 7.5 µl of Perfecta SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD). Then 15 µl of this mix was added to a
well in a 96 Fast PCR plate (Starstedt. Numbrecht, Germany) and each plate was run in the C1000 Touch Thermocycler (Bio-Rad, Mississauga, ON). An interassay control containing pooled cDNA was run in the case of genes covering multiple plates in order to adjust for any differences between plates. The cycling protocol for each gene was 2 min at 50°C, 5 min at 95°C, followed by 40 cycles of 1 sec at 95°C and 30 seconds at the specific annealing temperature of each primer pair. A temperature gradient test was carried out to determine the optimal annealing temperature for each primer.

The housekeeping/reference genes used in majority of the experiments were β-actin (β-actin) and elongation factor 1 alpha (ef1α). β-actin and ef1α were chosen because the expression pattern of these two genes were identical and did not significantly change across treatment groups. Results from the qPCR were normalized to both β-actin and ef1α based on the reference residual normalization method described by Edmunds et al, (2014). In the case of experiment 1, the expression value of both β-actin and ef1α was not consistent among follicular stages and thus were not appropriate for the Edmunds et al, (2014) normalization method. Among all treatments, both ef1α and β-actin appeared to decrease with development. Therefore, expression of PPARs across follicle development and within each stage of development was determined by using raw arbitrary input (non-normalized) (Ings and Van Der Kraak, 2006)

Steroid extraction, purification and enzyme immunoassays (EIAs)

For the in vitro assay in experiment 6, duplicates of 50 µL of the medium was used to measured T production using EIA kits (Cayman Chemical, MI, USA) as per the manufacturer’s instructions. For steroid measurement in the in vivo experiments, ovarian tissue that was collected underwent steroid extraction and purification before they were subjected to EIA. Approximately 20 mg of ovarian tissue collected from fish used in experiments 7 and 8 were sonicated in 1.5 ml microfuge tubes with a solution made up of 100 µl of PBS (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl; pH 7.4), 1 mM of EDTA and 10µM of indomethacin. After sonication, 400 µL of methanol was added and samples were incubated at 4°C for 1 hour. Samples were pulse
vortexed every 20 minutes. Following incubation, samples were centrifuged at 3000 g for 5 minutes at 4°C. This produced pellets which were snap frozen on dry ice and the methanol supernatant formed during the centrifugation was decanted into 7 ml glass scintillation vials. The pellets are then allowed to thaw and re-suspended in 400 µl of methanol, incubated at 4°C for 30 minutes with intermittent vortexing and centrifuged at 3000 g for 5 minutes. Pellets are once again snap frozen and the methanol layer collected. This process is repeated three times and total methanol collected in combined into a single vial and then dried under a stream of N₂.

The dried samples were stored in 300 µL of 50 mM acetate buffer (pH = 4.0) prior to column purification (Lister and Van Der Kraak, 2009). Samples were carefully passed through C-18 SPE columns (VWR International, Missassauga, ON) according to the manufacturer’s instructions and collected in glass test tubes. The final eluate was transferred into scintillation vials where they were eluted with 2 ml of ethyl acetate + 1% methanol. These samples are then dried again with N₂ for 4 – 6 hours after which they are reconstituted in EIA buffer (Cayman Chemicals, Ann Arbour, MI) and stored at 20°C. T and E₂ concentrations are analyzed by EIA (Cayman Chemical) as per manufacturer’s instructions. The analytes were tested for parallelism to determine if the samples contained any compounds that interfere with the assays.

**Statistical Analysis.**

All statistical analysis was done using SPSS V24 (IBM, San Jose, CA) where significance was set to p = 0.05. Differences in eggs produced per female per tank between pre-exposure and exposure period were compared using a t-test. Gene expression, egg production and steroid levels were tested for homogeneity of variance with Levene’s test and Kolmogorov-Smirnov test for normality. If both conditions were met a one-way analysis of variance (ANOVA) was carried out to determine if there was an overall difference among treatment groups, time points and follicular stages. A multiple comparison post-hoc Tukey’s test was carried out to confirm significant differences between treatment groups, time points and follicular stages. In cases where data failed the Levene’s test and normality was not achieved, data was log transformed.
for further parametric testing. If normality was still not achieved after transformation, an independent sample Kruskal-Wallis non-parametric analysis followed by a post hoc multiple comparison Mann-Whitney’s test was carried out.
Table 2. List of nucleotide sequences and associated accession numbers from GenBank.

<table>
<thead>
<tr>
<th>Gene (Annealing T°C)</th>
<th>Sequence (5’ – 3’)</th>
<th>Database ID</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>βactin (59°C)</td>
<td>F: ACAGGGAAAAGATGACACAGATCA R: CAGCCTGGATGGCAACGTA</td>
<td>NM_181601.4</td>
<td>~101%</td>
</tr>
<tr>
<td>ef1α (60C)</td>
<td>F: GATCACTGGTACTTTCAGGCTG R: GGTGAAAGCCAGGAGGC</td>
<td>NM_131263.1</td>
<td>~102%</td>
</tr>
<tr>
<td>lhr (55.7C)</td>
<td>F: Aacctgacctccatctttctccc R: TGGATGGTACTGAGCCGAGA</td>
<td>NM_205622.2</td>
<td>~104%</td>
</tr>
<tr>
<td>fshr (61.4C)</td>
<td>F: GCCCCGTCGTCTGATAT R: CAGAGAGTAGACTGAAACGC</td>
<td>NM_001001812.1</td>
<td>~108%</td>
</tr>
<tr>
<td>mprβ (61.9C)</td>
<td>F: GCCAGTTGCTGCTACTCCAAM R: TGGCAGATCTTCCCGTGAA</td>
<td>NM_183344.1</td>
<td>~110%</td>
</tr>
<tr>
<td>npr (61.9C)</td>
<td>F: GATACACACCCGATTGCCTTATC R: CCGGAGATGCGGTGTATG</td>
<td>NM_001166335.1</td>
<td>~95%</td>
</tr>
<tr>
<td>cox2 (60C)</td>
<td>F: GTTTAAAGATGGAAAGCTTTAA R: GGGTACACCTCACCACACCACA</td>
<td>NM_153657.1</td>
<td>~92%</td>
</tr>
<tr>
<td>cpla2 (61.9C)</td>
<td>F: TGCTCTTGGAAGTTTGCG R: TCTGCGTGTCTGCATGAGA</td>
<td>NM_131295.2</td>
<td>~99%</td>
</tr>
<tr>
<td>adams1 (60C)</td>
<td>F: GTTTGGCACAATGACACTC R: CAGCCTTCACACACTGTCTC</td>
<td>XM_688443.8</td>
<td>~109%</td>
</tr>
<tr>
<td>mmp9 (61.4C)</td>
<td>F: TGGGCACCTGCTCGTGGA R: TTGGAGATGACCGCCCTGC</td>
<td>NM_213123.1</td>
<td>~98%</td>
</tr>
<tr>
<td>cyp19a (62C)</td>
<td>F: CGTTACTGCTG TGACGGG R: CCAACCGGG ACCTGGAG</td>
<td>NM_131642.1</td>
<td>~97%</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Accession</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Star (59C)</td>
<td>F: ACCTTTTCTGGCTGGGATG</td>
<td>R: GGTCATTTCTCAGCCCTTAC</td>
<td>NM_131663.1</td>
</tr>
<tr>
<td>17βhsd (58C)</td>
<td>F: GGCACCATTCCGACCA</td>
<td>R: CTCGGGGAATGGCAACCT</td>
<td>NM_205584.1</td>
</tr>
<tr>
<td>pparaA (55.7C)</td>
<td>F: CTGGGAGACCCGATTA</td>
<td>R: TGCTGGCTGAGAACAC</td>
<td>NM_001161333.1</td>
</tr>
<tr>
<td>pparaB (57C)</td>
<td>F: TCGGACCATTCCGCTCA</td>
<td>R: CCACGGACAGGCACTTCT</td>
<td>NM_001102567.1</td>
</tr>
<tr>
<td>pparβA (61.4C)</td>
<td>F: GCCAAACCCTTCAGCGAGAT</td>
<td>R: TGCGGCGACGAACAGAG</td>
<td>XM_694808.4</td>
</tr>
<tr>
<td>pparβB (57C)</td>
<td>F: GCTGGTGCTGGACTA</td>
<td>R: CACTGGCGACGGTAGA</td>
<td>NM_131468.2</td>
</tr>
<tr>
<td>pparγ (59C)</td>
<td>F: GCCATCAGCGAAGTAC</td>
<td>R: TCAGGGTCCCGTCTTAT</td>
<td>NM_131467.1</td>
</tr>
</tbody>
</table>
Results

Experiment 1: Expression of PPARs across follicular development

Experiment 1 was carried out to determine the expression pattern of PPARs across ovarian follicle development. The expression of *ppary*, *pparaA* and *pparβB* was significantly higher in earlier follicular stages compared to late stage follicles. Overall, *ppary* expression was significantly higher in primary stage and early vitellogenic (p<0.01) than in full grown follicles (Figure 2E). Whereas *pparaA* expression was significantly higher (p<0.05) in early vitellogenic follicles compared to full grown follicles (Figure 2A). Similar to *ppary*, *pparβB* expression was also significantly higher in primary and early vitellogenic stages (p<0.05) compared to full grown follicles (Figure 2D). The relative expression of each PPAR isoform within each stage of follicle development is shown in Figure 3. While *pparβB* expression was generally much higher than other isoforms in all stages of development, a significant difference was seen only in the primary stage where *pparβB* was significantly higher (p<0.05) than *pparaB* (Figure 3A).

Experiment 2: Expression of PPARs up to and after ovulation

This experiment was carried out to determine the mRNA expression pattern of PPARs during the time period leading up to and after ovulation. No significant changes were seen in any of the PPARs across all five time periods (10 PM, 1 AM, 4 AM, 7 AM and 10 AM) (Figure not shown).

Experiment 3: Regulation of PPARs- hCG and 17α-20βP

Full grown follicles were treated with key regulators of the zebrafish reproduction, hCG and 17α-20βP, for either 3 or 6 hours in order to determine if they had any effect on the mRNA expression of PPARs. There was no change in expression of PPARs after 3 hours of treatment with either hCG or 17α-20βP (data not shown). Similarly, there was no change in expression after 6 hours of incubation with 17α-20βP (Figure 4). However, *pparaA* expression was significantly downregulated (p=0.008) after 6 hours of hCG exposure (Figure 4)
Experiment 4: In vitro regulation of PPARs - PMA/A23187

The effects of protein kinase C activation and hCG on PPAR expression in full grown follicles are shown in Figure 5. There was a significant downregulation in pparaA, and pparaB expression after 6 hours of treatment in both PMA/A23187 and PMA/A23187 + hCG groups compared to the follicles that were treated with either control or hCG while pparβA was significantly lower compared to the hCG group alone (p<0.005) (Figure 5).

Experiment 5: In Vitro effects of DEHP on oocyte maturation

This experiment was carried out to determine if PPAR activation disrupted the progression of oocyte maturation induced by the MIS, 17α-20βP in zebrafish. There was a significant increase in GVBD% in full grown follicles that were treated with 17α-20βP, DEHP (1 µM) plus 17α-20βP and DEHP (10 µM) plus 17α-20βP alone compared to the control (p<0.05). There was no significant difference in GVBD% between the 17α-20βP group and follicles that were preincubated with DEHP (Figure 6).

Experiment 6: In vitro effects of MEHP on testosterone production

This experiment was carried out to determine if PPAR activation had any influence on testosterone production in zebrafish ovarian follicles. There was no significant difference in T production between the control group and follicles treated with MEHP. Testosterone production between follicles that were exposed to hCG alone and a combination of hCG plus MEHP was also not significantly different. However, the testosterone produced by both groups was significantly higher (p<0.05) than follicles that were exposed to the control or MEHP alone (Figure 7).

Experiment 7: In vivo effects of DEHP on zebrafish reproduction

Experiment 7 was designed to determine whether DEHP, through its interaction with PPAR, had any effect zebrafish reproduction. There was no significant change in average egg production per female per day between the three groups during the pre-exposure period (Figure 7). After the exposure period, the control fish did not
experience any significant change in egg production compared to the pre-exposure period. Fish injected with either 50 or 500 mg/kg of DEHP had significantly reduced egg production (p<0.05) compared to their pre-exposure production (Figure 8). At the end of the exposure period, fish injected with either 50 or 500 mg/kg of DEHP had significantly lower egg production (p=0.008) per female per day compared to the control group (Figure 8). Among the genes measured, npr was significantly downregulated in 500 mg/kg compared to the control group (Figure 9). Gene expression of other local factors involved in reproduction such as lhr, fshr, mprβ3, cox2, cpla2, ADAMTS1, and mmp9 did not change across treatment. There was no significant change in gene expression in any of the PPAR isoforms in response to any of the doses. Furthermore, no significant changes in expression of the steroidogenic genes, star, cyp19a and 17βhsd were seen with treatment. Finally, there was no significant change in the T or E₂ steroid levels across treatments (Figure 10).

Experiment 8: Effects of MEHP on zebrafish reproduction

Experiment 8 was conducted to determine the effects of the DEHP metabolite, MEHP, on zebrafish reproduction. Eggs produced per female per day were not significantly different between treatment groups during the pre-exposure period (Figure 11). After the exposure period, the control fish did not experience any significant change in their egg production compared to the pre-exposure period. After exposure, egg production was significantly reduced (p<0.05) in fish injected with either 35.6 or 356 mg/kg of MEHP compared to the pre-exposure production. During the exposure period, eggs produced per female per tank were significantly reduced in fish injected with 35.6 and 356 mg/kg of MEHP (p=0.003) compared to the control group (Figure 11). There was a significant upregulation (p<0.05) in two PPAR isoforms, pparγ and pparaA, in fish injected with 356 mg/kg of MEHP compared to the control group (Figure 12). There was a significant reduction in npr expression (p=0.045) in the 35.6 mg/kg group compared to the control dose (Figure 13). In addition, there was a significant downregulation of ADAMTS1 (p=0.04) in fish injected with 356 mg/kg compared to the control group (Figure 13). Gene expression of lhr, fshr, mprβ3, cox2, cpla2, mmp9 and cyp19a, 17βhsd did not change across treatment. A significant decrease in star expression
(p=0.042) was seen in fish injected with 35.6 mg/kg of MEHP compared to both the control group (Figure 13).
Figure 2. Expression of PPAR family members across multiple stages of ovarian follicle development in zebrafish. Follicles were separated into five stages: primary (P), early vitellogenic (EV), mid-vitellogenic stage (MV), late vitellogenic stage (LV), and full grown (FG) follicles. Gene expression is non-normalized and data represents the mean ± S.E.M. Data was generated from multiple follicles belonging to each developmental stage collected from different fish (n=10-12). Differences in the mRNA expression of PPARs with respect to follicular stage were determined by a one-way ANOVA and post-hoc Tukey’s test with significant differences indicated by different letters.
Figure 3. Relative expression of PPAR family members within each stage of ovarian follicle development in zebrafish. Follicles were separated into five stages: primary (P), early vitellogenic (EV), mid-vitellogenic stage (MV), late vitellogenic stage (LV), and full grown (FG) follicles. Gene expression is non-normalized, and data represents the mean ± S.E.M. Data was generated from multiple follicles belonging to each developmental stage collected from different fish (n=10-12). Differences in the mRNA expression of PPARs within follicular stage was determined by a one-way ANOVA and post-hoc Tukey’s test with significant differences indicated by different letters.
Figure 4. Expression of PPARs in full grown ovarian follicles that were treated in vitro with control (EtOH), hCG (20 IU/ml) or MIS (17α-20βP) (10 ng/ml) for a period of six hours. Gene expression was normalized to βactin and ef1α and expressed as a fold change relative to the expression of the control group. Data represents the mean ± S.E.M of expression values generated from multiple replicates per treatment repeated six different times (n=6). Differences in the mRNA expression of PPARs after the six-hour treatment period were determined by a Kruskal Wallis Test and post-hoc Mann-Whitney’s test. Different letters indicate significant differences.
Figure 5. Expression of PPARs in full grown ovarian follicles that were treated *in vitro* with Control (water + EtOH), hCG (20 IU/ml), PMA (400 nm)/A23187 (10 µM) and hCG (20 IU/ml) plus PMA (400 nm)/A23187 (10 µM) for six hours. Gene expression was normalized to βactin and ef1α and expressed as a fold change relative to the expression of the control group. Data represents the mean ± S.E.M of expression values generated from multiple replicates per treatment repeated three different times (n=3). Differences in the mRNA expression of PPARs after the six-hour treatment period were determined by a one-way ANOVA Test and post-hoc Tukey’s test. Different letters indicate significant differences.
Figure 6. Average % germinal vesicle breakdown in zebrafish full grown follicles that were treated with control (EtoH), control plus 17α-20βP (10 ng/ml), DEHP (1 µM) + 17α-20βP (10 ng/ml) and DEHP (10 µM) + 17α-20βP (10 ng/ml). Data represents the mean ± S.E.M of expression values generated from multiple replicates per treatment repeated once (n=1). Differences in % GVBD among treatments were determined by a one-way ANOVA Test and post-hoc Tukey’s test. Different letters indicate significant differences.
Figure 7. Average testosterone production in zebrafish full grown follicles that were treated in vitro with control (water plus EtoH), hCG (20 IU/ml), MEHP (50 µM) and hCG (20 IU/ml) plus MEHP (50 µM). Data represents the mean ± S.E.M of expression values generated from multiple replicates per treatment repeated four times (n=4). Differences in testosterone production among treatments were determined by a one-way ANOVA Test and post-hoc Tukey’s test. Different letters indicate significant differences.
Figure 8. Average number of eggs produced per female zebrafish per day during the pre-exposure where fish were not injected with any chemicals and the exposure period where fish were injected with 0, 50 or 500 mg/kg of DEHP. Data represents the mean ± S.E.M of three tanks per treatment (n=3). Differences in egg production among treatment groups during the exposure period were determined by a one-way ANOVA and Tukey’s test. Significant differences in egg production are indicated by different letters. Different letters indicate significant differences.
Figure 9. Ovarian expression of npr from ovarian follicles extracted from female zebrafish after a seven-day exposure period during which they were injected with 0, 50 or 500 mg/kg of DEHP every other day. Gene expression was normalized to βactin and ef1α and expressed as a fold change relative to the expression of the control group. Data represents mean ± S.E.M of three tanks per treatment (n = 3). Significant differences in gene expression among treatments were detected by a one-way ANOVA and Tukey’s test. Different letters indicate significant differences.
Figure 10. Average ovarian T and E$_2$ content per mg of ovarian tissue extracted from zebrafish after a seven-day exposure period during which they were injected with 0, 50 or 500 mg/kg of DEHP every other day. Data represents mean ± S.E.M of three tanks per treatment (n = 3). No significant differences in steroid production among treatments were seen detected among treatments by a one-way ANOVA.
Figure 11. Average number of eggs produced per female zebrafish per day during the pre-exposure where fish were not injected with any chemicals and the exposure period where fish were injected with 0, 35.6 or 356 mg/kg of DEHP. Data represents the mean ± S.E.M of three tanks per treatment (n=3). Differences in egg production among treatment groups during the exposure period were determined by a one-way ANOVA and Tukey’s test. Significant differences in egg production are indicated by different letters.
Figure 12. Ovarian gene expression of PPAR isoforms in zebrafish after a seven-day exposure period during which they were injected with 0, 35.6 or 356 mg/kg of MEHP every other day. Data represents mean ± S.E.M of three tanks per treatment (n = 3). Gene expression was normalized to βactin and ef1α and expressed as a fold change relative to the expression of the control group. Significant differences in gene expression were detected by a one-way ANOVA and confirmed with Tukey’s test. Significant differences indicated by different letters.
Figure 13. Ovarian gene expression of \textit{npr}, \textit{adams1} and \textit{star} in zebrafish after a seven-day exposure period during which were injected with 0, 35.6 or 356 mg/kg of MEHP every other day. Data represents mean ± S.E.M of three tanks per treatment (n = 3). Gene expression was normalized to \textit{βactin} and \textit{ef1α} and expressed as a fold change relative to the expression of the control group. Significant differences in gene expression among treatments were detected by a one-way ANOVA and Tukey’s test. Different letters indicate significant differences.
General Discussion

My thesis sought to determine the presence, regulation, and function of the PPAR subfamily of nuclear transcription factors in the ovary of the female zebrafish. I found that all five PPAR isoforms are present in the zebrafish ovary, their gene expression is negatively regulated by signalling molecules that induce ovulation (see Fig 4) and that in vivo activation of PPARs by synthetic ligands disrupts ovulation and inhibits spawning success (See Fig 7 & 10). Collectively, these results suggest that PPARs may be negative regulators of ovulation in the zebrafish. Gene expression of select PPAR isoforms (ppary, pparaA and pparβB) tends to decrease with follicular development suggesting a potential involvement of PPARs in early follicular development and a more inhibitory role towards the final stages of development (see Fig 2). LH-analog, hCG, was shown to suppress pparaA expression which suggests that this isoform is negatively regulated by LH during the zebrafish reproductive cycle (See Fig 3). Moreover, activators of the PKC pathway, PMA and A23187 also downregulated pparaA, pparaB and pparβB expression indicating that these isoforms are also under regulation by the PKC pathway in zebrafish ovaries (See Fig 4). In vivo activation of PPARs by DEHP and MEHP resulted in impairment to reproduction and this can be attributed to disruption in the ovulation process (See Fig 7 & 10). Both phthalates caused a suppression of the nuclear progestin receptor (nPR) gene which is a critical regulator of ovulation in zebrafish (See Fig 8 & 11). In addition, MEHP was shown to downregulate gene expression of a protease, ADAMTS1 (see Fig 11), which is involved in follicular rupture during ovulation and is also regulated by nPR further suggesting activation of PPARs by phthalates impairs reproduction in zebrafish through disruption of ovulation.

Spatiotemporal expression of PPARs in the zebrafish ovary

The initial portion of this thesis confirmed the presence of the PPAR isoforms in zebrafish ovary, specifically their mRNA expression in each stage of ovarian follicle development and also how their gene expression changes in whole ovarian tissue in time points leading up to, during and after ovulation. As transcription factors, the primary
function of PPARs would be as regulators of different genes that are involved in processes that take place during different periods of follicle development. An analysis of PPAR expression pattern as the ovarian follicle progresses through development and with respect to ovulation provides insight into what processes that PPARs might regulate.

Spatial expression pattern of PPARs:

This study demonstrates the presence and relative gene expression levels of all five PPARs in the zebrafish ovary. Specifically, mRNA of each isoform was found in each stage of ovarian follicle development and the expression pattern of certain PPARs tends to decrease with development. Expression of *ppary*, *pparaA* and *pparβB* were significantly higher in early stages of follicular development such as the primary growth and early vitellogenic stages compared to the full-grown follicles which are approaching maturation and ovulation. While the *pparaB* and *pparβB* appeared to follow a similar pattern of decreasing expression with successive follicular stage, the differences in gene expression between stages was not statistically significant. Expression of *pparaB* was significantly lower than *pparβA* and *pparβA* in primary oocytes suggesting that this isoform might not be involved in the initial follicular development when it begins in primary follicle. The results of this study are consistent with previous studies in mammals and zebrafish which show similar trends in PPAR gene expression with respect to follicle development (Ibabe et al, 2005). In zebrafish, western blot analysis has shown that protein levels of PPAR-α, PPARβ and PPARγ were moderately to strongly expressed in early stage oocytes while the expression decreased in late stage follicles (Ibabe et al, 2005), similar to what was seen in the present study. Studies on sheep ovarian cells, show that PPARγ mRNA levels were much higher in early (antral) follicles than in pre-ovulatory follicles (Froment et al, 2003), consistent with what has been observed in zebrafish. Collectively these studies suggest that PPARs may be involved in regulating processes that are involved in early follicular development, such as follicular growth and formation of the follicular layers.
Temporal expression pattern of PPARs:

There was no change in the whole-ovary expression of PPARs at any of the time points leading up to and after ovulation. Temporal characterization of ovarian PPARs during the reproductive cycle has not yet been documented in either teleosts or mammals. The lack of downregulation at the time of ovulation and the consistent expression of PPARs across all time points measured suggests that PPARs may be involved in pathways that are taking place in the zebrafish ovary separate from regular ovarian pathways. This may include processes such as inflammation and blood vessel formation which both take place during the reproductive cycle in vertebrates. Studies in mammals have shown that PPARs such as PPARα and PPARγ regulate transcription of inflammatory proteins such as interleukins and cytokines (Clark, 2002; Lee et al, 2011). In mammals, it is suggested that PPARγ is involved in blood vessel formation in the ovary through its regulation of angiogenic factors such as vascular endothelial growth factor (VEGF), and nitric oxide synthase (NOS) (Bishop-Bailey, 2000; Milvae, 2000). While the findings of this study do not shed light into the involvement of PPARs during ovulation, they do suggest that PPARs may be involved in separate biological processes that occur during the same timeframe.

Regulation of PPARs in the Zebrafish ovary.

I used an in vitro approach to determine whether ovarian PPARs were regulated by the LH (hCG), sex steroids and activators of the PKC pathway. In vitro experiments are commonly used to investigate the regulation of reproductive processes in the ovary. Previous in vitro studies have used hCG to shed some light into the regulation of processes such as oocyte maturation, ovulation and steroidogenesis by LH in ovarian follicles (Pang et al, 2002; Skoblina, 2009; Li et al, 2015). In vitro incubations have helped establish the oocyte maturation inducing effect of 17α-20βP in immature follicles and have also shown that 17α-20βP regulates the expression of select genes involved in prostaglandin production, oocyte maturation and ovulation (e.g. Irwin and Van Der Kraak, 2012). Data from in vitro assays also show that PKC activators such as PMA and A23187 are involved in the regulation of the prostaglandin pathway and steroid
production in teleost ovaries (Wade and Van Der Kraak, 1991; Goetz et al, 1991; Srivastava and Van Der Kraak, 1994).

Regulation of PPARs BY hCG and 17α-20βP:

As mentioned earlier, there are numerous studies that have used hCG to determine the role of LH as a regulator of reproduction in teleosts. In studies both in vitro and vivo hCG induces progesterone, T, and E₂ production, regulates different aspects of prostaglandin production in teleosts and induces oocyte maturation and ovulation in teleosts (Sorbera et al, 1990; Zuberi et al, 2011). In the present study, after six hours of incubation with hCG, there was a significant downregulation pparaA mRNA in full grown follicles. This is similar to previous studies in mammals that show that while PPARα and PPARβ expression aren’t affected by hCG stimulation, significant changes in PPARγ gene expression are observed (Komar et al., 2005; Kim et al., 2008; Funuhashi et al, 2017). However, the mammalian data available on hCG regulation on PPARγ is conflicted and varies with model organism studied. In rats and macaque granulosa cells, PPAR-y gene expression decreases after five hours following the LH surge or exposure to exogenous hCG treatment, while in mice hCG was shown to stimulate gene expression after five hours (Komar et al, 2005; Kim et al, 2008). Given the suppression of its gene expression following hCG treatment in the present experiment, it is possible that pparaA in zebrafish is regulated by hCG in a similar manner to that of the macaque or rat PPARγ. The suppressive regulation of pparaA expression by hCG is potentially necessary for the normal reproductive cascade in zebrafish to continue.

Results from the present study shows that 17α-20βP failed to affect gene expression of any of the PPAR after either a three or six hour incubation period. 17α-20βP regulates oocyte maturation which is the final step of follicular development and the production of 17α-20βP is in turn induced by LH (Clelland and Ping, 2009). It is possible that the zebrafish ovarian PPARs might be either functioning upstream of the MIS and are not under regulation by17α-20βP but by LH.
Regulation of PPARs by the PKC pathway:

The PKC pathway has been shown to inhibit processes such as oocyte maturation (Cerda et al., 1993), stimulate prostaglandin synthesis (Kellner and Van Der Kraak, 1993; Berndtson et al., 1989), induce ovulation (Berndstat et al., 1989) and inhibit steroidogenesis (Srivastava and Van der Kraak, 1993) in teleost ovarian follicles. In the present study, there was a significant downregulation in pparaαa, pparaβa and pparβa after a six hour incubation with PMA and A23187 which suggests that the ovarian PPARs in zebrafish are regulated by the PKC pathway. There is little to no information on PPAR gene expression in response to the PKC pathway in vertebrates, however a few studies show that PKC regulates PPARα through post-translational modifications (Blanquart et al., 2004; Gray et al., 2005). The PKC pathway was shown to phosphorylate PPARα at specific amino acid residues which causes the transcription factor to switch between transactivation and transrepression activity (Blanquart et al, 2004). The present study shows that PKC pathway also regulates transcription of PPARs in zebrafish and the negative regulation of PPAR gene expression might be required for processes that are regulated by PKC signalling pathway in the zebrafish ovaries to take place.

Past studies show that PMA and A23187 can inhibit hCG induced T and E₂ production, and diminish hCG mediated oocyte maturation competence in teleost ovarian follicles (Srivastava and Van Der Kraak, 1993; Yoshizaki et al, 2001). Studies show that PKC activators inhibit cyclic AMP formation that is induced by hCG, however the molecular pathways and underlying mechanisms of this phenomenon are not well understood (Srivastava and Van Der Kraak, 1993). In the current in vitro study, the PKC activators did not appear to modify any hCG effect on gene expression of PPARs since there was no significant difference in PPAR gene expression between PMA/A23178 and PMA/A23178 plus hCG groups. This suggests that any inhibition or interference of the PKC activators on end points that are induced by hCG are not mediated by and/or do not involve PPARs. It must be noted that in the current experiment while there appeared to be a decrease in pparaα expression with hCG exposure, it was not significant. This might be due to the smaller sample size (n=3) that was employed in this experiment.
compared to the previous *in vitro* experiment. Overall, the downregulation of *pparaA*, *pparaB* and *pparβA* by PMA/A23178 suggests that the suppression in PPAR expression might be required for processes that are regulated by PKC such as ovulation and/or steroidogenesis.

**Phthalates, PPARs and Reproduction**

I utilized both *in vitro* and *in vivo* methods to investigate the effects of phthalate induced PPAR activation on overall spawning success, oocyte maturation, ovulation and ovarian steroidogenesis. Studies investigating the effects of environmental contaminants on fish health have utilized phthalates to determine the downstream effects of PPAR activation on different aspects of teleost reproduction (Carnavalli et al, 2010; Ye et al, 2014). Results from the present study show that fish injected with both DEHP and MEHP experienced a significant reduction in egg production indicating that PPAR activation is detrimental for reproduction in zebrafish. Analysis of gene expression of different factors indicate that this dysfunction in reproduction caused by PPAR activation might not be due to disruption in processes such as ovarian follicle development or oocyte maturation but can be attributed instead to changes to ovulation.

**Phthalates and PPARs**

I utilized the plasticizing agent, DEHP and its metabolite, MEHP as PPAR ligands to determine the effects of PPAR activation on zebrafish reproduction. Both phthalates induce a wide variety of hormonal and biochemical modifications in teleost production and it is generally thought that this is mediated by PPARs (Huang and Chen, 2017). DEHP is considered to be a potent ligand of all three PPAR isoforms in fish, while transactivation studies have shown that MEHP interacts with both PPARα and PPARγ in teleosts (Riu et al, 2011; Huang and Chen, 2017). The activation of PPARs in the second *in vivo* experiment is supported by upregulation of expression of both *pparaA* and *ppary* observed in response to MEHP. Though not necessarily conclusive, the gene expression of PPARs is a good indicator of their activation due to the ability of nuclear receptors to autoregulate. Autoregulation is the transcriptional regulation of its own gene by the protein upon ligand activation and is a common feature shared by nuclear
receptors (Chawla et al, 2001; Cocci et al, 2015). In both mammals and fish, PPARs such as PPARα possess their own PPRE and are capable of modulating transcription of their own genes (Torra et al, 2002; Handschin et al, 2003; Cocci et al, 2015).

There was no change in gene expression of any PPARs to either dose of DEHP making it unclear which PPAR was targeted by DEHP in these experiments. A past study showed in vitro DEHP upregulated gene expression of PPARα, PPARβ and PPARγ in zebrafish hepatocytes (Maradonna et al, 2013). Male zebrafish that were injected with DEHP experienced an increase in peroxisomal enzymes such as acyl-CoA which are transcriptionally regulated by both PPARα and PPARγ (Uren-Webster et al, 2008). Taken together, results from the past studies and gene expression data from the present study show that phthalates like DEHP and MEHP are effective ligands to determine the function of PPARs in teleost ovary.

While PPARs are considered the primary nuclear receptors that interact with and mediate the downstream effects of phthalates, studies show that in certain cases phthalates can also activate estrogen receptors (ER) to elicit an estrogenic response (Moore et al, 2000). However, these studies are not conclusive and have demonstrated that the ability of diester phthalates like DEHP or DiDP to induce oestrogenic endpoints appears only at concentrations that past their solubility in water which ranges anywhere from 270 to 280 µg/L (Moore et al, 2000). Receptor binding assays have shown that the smaller monoester phthalates like MEHP are incapable of activating the estrogen receptors at concentrations as high as 1 mM (Moore et al., 2000; Corton and Lapinskas, 2005). A characteristic estrogen-responsive endpoint that is observed in teleosts is the downregulation of the LHR gene (Cosme et al, 2015), which was not observed in response to both phthalates in either in vivo experiment. This suggests that in the present in vivo experiments, DEHP and MEHP are most likely targeting and interacting with PPARs to influence reproduction in zebrafish.

**PPAR activation and ovarian follicle development**

Studies show that the negative effects of phthalates on reproduction can start even in the early follicular developmental stages like folliculogenesis (follicle growth) (Kim et al, 2002; Bonilla et al, 2010). In Japanese medaka, exposure to 10 and 50 µg/l
of DEHP for 3 months was shown to stunt early oocyte development by decreasing the amount of early to mid-vitellogenic follicles that were formed (Kim et al., 2002). In mouse and rats, in vitro assays show that MEHP treatment for 24 -48h results in reduced viability of follicles and increase apoptosis of the follicular layer cells (Bonilla et al., 2010; Inada et al., 2012). In the present experiment, I measured gene expression of follicle stimulating hormone receptor (fshr) which interacts with follicle stimulating hormone to regulate folliculogenesis (Clelland and Ping, 2009). While studies in teleost are lacking, DEHP and MEHP have shown to downregulate fshr expression in mammalian ovarian cells (Ernst et al., 2014; Hannon et al., 2015). In the present experiments, no change in fshr expression was seen in response to DEHP and MEHP, suggesting that the detrimental effects of both phthalates may not be through negative modulation of folliculogenesis.

The final phase of follicular development is oocyte maturation which takes place in the immature full-grown follicles prior to the ovulatory process (Patino and Sullivan, 2002). Initially I tested the in vitro effects of DEHP on 17α-20βP induced oocyte maturation in full grown follicles and found that there DEHP failed to disrupt the maturation induction by 17α-20βP. This seemed to suggest that PPAR activation during the oocyte maturation stage might not be detrimental to maturation success. The results from the in vitro assay are consistent with the gene expression pattern from the in vivo experiments in which key factors that regulate the maturation process were measured. LH binds to its respective receptor, lhr, to induce production of 17α-20βP by the follicular layers which in turns interacts with mprβ to initiate oocyte maturation (Patino and Sullivan, 2002; Clelland and Ping, 2009). In both in vivo experiments, expression of lhr, and mprβ were unaffected by phthalate exposure indicating that phthalate disruption of reproduction might not be through interference with the oocyte maturation process. It appears that phthalate-induced activation impairs reproduction through modification of processes that are downstream of oocyte maturation such as ovulation.

**PPAR activation and ovulation**

Gene expression of several factors that are involved in ovulation in zebrafish such as prostaglandin enzymes, nuclear progesterone receptor and metalloproteinases was
measured in response to DEHP and MEHP. Previous studies show that exposures to inhibitors of Cox2 and Cpla2, two enzymes involved in prostaglandin synthesis, diminished spawning success in zebrafish highlighting the critical nature of these enzymes for ovulation (Lister and Van der Kraak, 2009; Knight and Van Der Kraak., 2015). In zebrafish, DEHP exposure diminishes Cox2 gene (Carnavalli et al., 2010) expression while studies in mammals, MEHP was shown to do the opposite (Tetz et al., 2015). However, in the current experiments no change in gene expression of these enzymes were seen in response to both phthalates suggesting that any disruption in ovulation caused by PPAR activation might not be through changes in the prostaglandin pathway.

Studies show that 17α,20β-P can interact with the nPR to induce ovulation in zebrafish and it is thought that nPR acts primarily through a genomic pathway to induce ovulation (Hanna and Zhu, 2011). In addition, expression of nPR is found to be highest in full grown follicles ready for ovulation and this expression is further regulated both in vivo and in vitro by LH signalling (Tang et al, 2016). In the present study, both DEHP (500 mg/kg) and MEHP (35.6 mg/kg) were shown to downregulate nPR gene (npr) expression in female zebrafish suggesting that the disruption of reproduction by PPAR activation is potentially through negative modulation of nPR-mediated ovulation in fish. While both the medium dose of DEHP (50 mg/kg) and high dose of MEHP (356 mg/kg) appeared to suppress nPR gene expression as well, they were not statistically significant and it may instructive to repeat the experiment with a larger sample size. These results suggest that the nPR gene is targeted for transcriptional regulation by PPARs and that activation of PPARs by phthalates results in downregulation of npr resulting in an impairment in ovulation.

A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS1) belongs to the metalloprotease family and is one of the several proteases that is involved in follicular wall degradation during ovulation and in zebrafish is shown to be under regulation by nPR (Zhang et al, 2016). The suppression of its gene expression at 356 mg/kg of MEHP suggests that npr downregulation by PPARs can negatively affect the follicular rupture process diminishing ovulation success. This therefore could prevent release of the mature egg from the follicular layers resulting in diminished ovulation.
Unlike MEHP, both doses of DEHP failed to have any effect on ADAMTS1 gene expression. This could possibly be due to the fact that PPAR activation by MEHP and DEHP might affect different end points due to different ligand specificities. MEHP activation of PPARs might directly affect transcription of ADAMTS1 independent of nPR and this in addition to the suppression of nPR negatively affects ovulation. Studies in mammals show that PPARγ agonists (TZDs) suppress transcription of different members of the ADAMTS family such ADAMTS4, ADAMTS5 (Worley et al, 2003; Boileau et al, 2007) and a similar phenomenon could be occurring in zebrafish with MEHP and/or DEHP. Collectively, the results from the in vivo experiments suggest that PPAR activation by phthalates impairs reproduction and this is due to disruption in the ovulatory process in zebrafish.

PPAR activation and ovarian steroidogenesis.

No changes in T or E<sub>2</sub> was observed in fish that were exposed to DEHP. Due to lack of samples, the in vivo effects of MEHP on ovarian steroid production could not be explored. The in vitro assay showed that MEHP failed to have any effect on T production after six hours of exposure alone. This conflicts with previous studies in teleosts. In female marine medaka, 0.1 mg/L of DEHP exposure was shown to increase whole body estradiol levels while MEHP had no effect (Ye et al, 2014). Similarly, both 0.5 mg/L of DEHP and 0.1 mg/L of MEHP were shown to increase whole body testosterone levels (Ye et al, 2014). Exposure to 4.0 and 37.5 ug/L of MEHP has been shown to increase plasma T and E<sub>2</sub> levels in female zebrafish (Zhu et al, 2016). While there was also no change in steroidogenic gene expression in zebrafish that were injected with DEHP, MEHP was shown to downregulate StAR gene expression in the 35.6 mg/kg. MEHP has been shown to have mixed effects on the StAR gene in mammals. In rats studies show that MEHP stimulates gene expression of StAR but has the opposite effect in mouse ovaries (Ye et al, 2014; Komar et al, 2005).

The lack of changes in the steroidogenic pathway in response to both phthalates could be due to the shorter exposure period in both the in vivo and in vitro experiments carried out in present study. The exposure period in previous in vivo teleost studies that have utilized phthalates have ranged from 60– 80 days and such a chronic exposure
might be required to see changes in steroidogenesis (Ye et al, 2014; Zhu et al, 2016). Similarly, treatment duration with MEHP in *in vitro* studies in rodents and fish ranges from 24 – 48 h before changes in steroid production and gene expression are observed (Svechnikova et al, 2011; Savchuk et al, 2015)

**PPARs: Negative regulators of ovulation in Zebrafish**

The results from the *in vitro* assays and *in vivo* exposure experiments carried out in the present study suggest that PPARs act as negative regulators of the ovulatory process in zebrafish. Firstly, the low levels of PPAR gene expression observed in pre-ovulatory full-grown follicles that are at the end of development and approaching ovulation, indicate that the upregulation and/or activation of PPARs in these follicles might have negative implications for ovulation. The gonadotropin, LH and PKC signalling pathway can induce ovulation in teleosts through regulation of local factors involved in steroid production and prostaglandin synthesis (Bernsdadt et al, 1989; Lister and Van Der Kraak., 2009). In the *in vitro* assays, both hCG and PMA plus A23178 were shown to suppress gene expression of PPARs in full grown follicles suggesting that this suppression of PPARs is potentially necessary for ovulation. Finally, *in vivo* activation of PPARs by phthalates resulted in an impairment to reproduction and this is primarily attributed to a decrease in expression of two critical factors involved in ovulation in zebrafish: nPR and ADAMTS1. This notion of negative regulation of ovulation by PPARs is supported by studies in the mammalian literature (Komar, 2005; Long et al, 2009).

In mammals, the corpus luteum is an endocrine gland that is formed after the oocyte ruptures from the follicular walls and is an indicator of successful ovulation in mammals (Komar et al, 2001). In rats, PPARγ gene expression was shown to drastically drop during corpus luteum formation suggesting that either the upregulation or activation of PPARγ might be detrimental to the ovulation process (Komar et al, 2001; Long et al, 2009). A much more recent study supported this hypothesis by showing that treatment with PPARγ specific ligand, rosiglitazone reduced the size of corpus luteum and also diminished fertility in female rats (Funahashi et al, 2017). In addition, rosiglitazone was also shown to block the induction of *cox2* and *star* expression by hCG
in full grown follicles further highlighting the negative relationship between PPARγ and ovulation.

In mammals, PMA can induce gene expression of metalloproteinases such as MMP-9 and ADAMTS4 in human monocyte-like cells and this induction is inhibited by synthetic and endogenous PPARγ agonists like rosiglitazone and 15d-PGJ2 respectively (Worley et al, 2003). As stated earlier, in both mammals and fish, proteinases modulate ovulation by facilitating the degradation of the follicular layer which allows successful release of the egg from the follicle thereby completing ovulation (McIntush et al, 1999; Ogiwara et al, 2005). It is possible that the zebrafish activation of PPARs decreases transcription of genes that code for proteases during ovulation thereby having a negative effect on the process. This hypothesis was supported by the downregulation of ADAMTS1 in fish that were injected with 356 mg/kg of MEHP indicating that metalloproteinases are targeted for negative transcriptional regulation by PPARs. In addition, previous studies in teleosts show that nPR mediated ovulation is associated with an increase in PKC activity (Patino and Sullivan, 2002; Hanna and Zhu, 2011). The downregulation of PPARs by activators of the PKC pathway in the *in vitro* assay and the decrease in nPR expression by PPARs in the *in vivo* experiments establishes a negative relationship between PPARs and nPR in zebrafish ovaries and indicates that suppression of PPARs by the PKC signalling pathway is required for ovulation.

A novel finding of the present study was the suppression of nPR by fish exposed to both MEHP and DEHP. Through its interaction with 17α,20βP, nPR induces ovulation in zebrafish (Patino and Sullivan, 2001) and it appears that this pathway is disrupted by suppressive transcription of the nPR gene by PPARs. There is little to no information on the relationship between progesterone receptors and PPARs in teleosts. In mice, knock-out of the ovarian progesterone receptor was shown to diminish PPARγ mRNA levels and suppress ovulation and overall fertility in female mice (Kim et al, 2008). However, this is conflicting with what is seen in the present study which show that PPAR activation by phthalates is detrimental for reproduction and this is through its negative regulation of nPR. It appears, that zebrafish PPARs behave more like their counterparts in the rat ovaries and are negative regulators of ovulation.
Future Directions

The present study sheds some light into the role of PPARs in reproductive process in zebrafish, however there remain several unexplored questions regarding their expression, regulation, and function. While this study confirms the presence of the five members of PPAR sub-family in the zebrafish ovary, it would be beneficial to determine the localization of the isoforms within the ovarian follicle i.e. follicular layers vs oocyte. Characterization of the PPAR family within different follicular layers either by immunohistochemistry or qPCR would provide more insight into the processes that PPARs are involved inside ovarian follicle.

Gene expression data shows that PPARs are highly expressed in earlier stage follicles like the primary and early vitellogenic oocytes. Inducing PPAR activation in vitro in these early stage follicles and examining changes in select gene expression, steroid production and follicular growth would be useful in understanding how PPARs are involved in folliculogenesis. Though there was no change in expression of PPARs in the time-points measured up to and after ovulation, insertion of additional time points and narrowing the time interval between measurements might provide a more accurate understanding of the temporal expression pattern of PPARs with respect to ovulation. Another consideration when analyzing the temporal expression data from the present study is that the total RNA was extracted from whole ovaries which is made up of follicles from all stages and this may mislead PPAR expression with respect to ovulation. Thus, it would be suitable to isolate follicles of a particular stage at each time point instead of whole ovaries in order to fully determine how PPAR gene expression changes in the time periods leading up to and after ovulation. Ideally, full grown follicles that are immature and approaching maturation or mature follicles that are ready for ovulation would be suitable for such experiments. Furthermore, instead of qPCR, quantification of protein levels in the ovaries via immunohistochemistry or western-blot would provide a more accurate understanding of PPAR involvement in ovulation.

The present study used the phthalates EDCs, DEHP and its metabolite, MEHP to determine the downstream effects of PPAR activation on reproduction. The ligand specificity of PPARs is the most diverse among the nuclear receptor family and studies
show that different synthetic and endogenous ligands activate PPARs to influence transcription of different set of target genes and subsequently have varied effects on physiological processes (Huang and Chen, 2017). Fibrates such as gemfibrozil and clofibrate are potent ligands of PPARα in both mammals and fish and are ideal candidates to determine the effects of PPARα activation in teleosts (Prindiville et al, 2001; Den Broeder et al, 2015). Halogenated analogs of the plasticizer, bisphenol A, such as tetrabromobisphenol A have been shown to efficiently bind to and activate the ligand binding region of PPARγ in zebrafish cells (Riu et al, 2011). Tributyltin (TBT) and Perfluorononanoic acid, (PFOA) are industrial chemicals that can have been shown to activate all three PPAR isoforms in zebrafish and juvenile salmon cell cultures (le Maire et al, 2009; Yang et al, 2014). Future studies would benefit from utilizing different ligands to determine the different molecular and physiological processes PPARs are involved in teleost reproduction.

Reproduction in all vertebrates is primarily controlled by the hypothalamic-pituitary gonadal (HPG) axis. Neuroendocrine stimulus from the hypothalamus results in gonadotropins release from the pituitary gland and these in turn stimulates gonadal development (Thackray et al, 2010). The present study focused on PPARs in the ovary which is at the downstream end of the HPG axis and thus it would be useful to look at PPAR involvement further up the HPG axis i.e. within the hypothalamus and pituitary. PPAR have been identified in human, rodent and bird hypothalamus and pituitary glands but an understanding of their function in the brain is limited (Froment et al, 2003; Bogazzi et al, 2005). Studies in mammals show that knock out of the pituitary PPARγ in rats increases the production of LH, decrease FSH levels in the blood and diminishes fertility indicating regulation of gonadotropins by the pituitary PPARs (Heaney et al, 2003; Sharma et al, 2011). LHβ is a polypeptide that combines with the α-subunit to form LH and its production has been shown to be inhibited by PPARα, PPARβ and PPARγ agonists further implicating PPARs in the regulation of gonadotropin synthesis in vertebrates (Takeda et al, 2007; Shiu et al, 2013; Vitti et al, 2016). Currently, little to no information exits on the presence of PPARs in teleost hypothalamic and/or pituitary
tissue. The HPG axis is well conserved among vertebrates and therefore it would be beneficial to see if PPARs play similar roles in teleosts like the zebrafish.

**Conclusion**

This study aimed to characterize all five members of the PPAR subfamily in terms of gene expression, regulation and function in teleost ovaries. The expression of *ppary*, *pparaA* and *pparβB* is much higher in primary and early vitellogenic oocytes suggesting an involvement in regulating early follicular development. The LH analog, hCG was shown to downregulate *pparaA* expression while activators of the PKC pathway, PMA and A23187 in combination were shown to suppress *pparaA*, *pparaB* and *pparβB*. This inhibition of gene expression by natural regulators of reproduction in teleosts might be required for processes such oocyte maturation and ovulation to naturally take place. *In vivo* activation of PPARs by the phthalates, DEHP and MEHP supports this notion and shows that fish exposed to either phthalates experience diminished fertility caused by a disruption in ovulation. Both phthalates were shown to downregulate expression of the nPR gene which induces ovulation in zebrafish and MEHP alone was shown to downregulate gene expression of the protease, ADAMTS1 which facilitates follicular degradation during ovulation. While there are still several missing pieces in the PPAR signalling pathway in teleost ovaries, this study has shed some light into characterizing the PPAR family with respect to their expression, regulation and function in a teleost ovary.
References


63


Sabatini, M., Bardiot, A., Lesur, C., Moulharat, N., Thomas, M., Richard, I., & Fradin, A. (2002). Effects of agonists of peroxisome proliferator-activated receptor γ on proteoglycan degradation and matrix metalloproteinase production in rat cartilage in


