Characterizing the Role of Eicosanoids in Maturation-Inducing Steroid-Mediated Ovulation and Spawning in the Zebrafish (*Danio rerio*)

By

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ABSTRACT

This study employed a hormone bioassay to characterize the eicosanoids involved in zebrafish ovulation and spawning, in particular the prostaglandin (PG) products of cyclooxygenase (COX) metabolism and the leukotriene (LT) products of lipoxygenase (LOX) metabolism. Exposure to the teleost progestogen 17α, 20β-dihydroxy-4-pregnen-3-one (17,20βP) induced ovulation, but not spawning, in solitary females and both ovulation and spawning in male-female pairs. Transcription of the eicosanoid-synthesizing enzymes cytosolic phospholipase A2 (cPLA2) and COX-2 increased and LTC4 synthase decreased in peri-ovulatory ovaries of 17,20βP-exposed fish. Ovarian PGF2α levels increased post-spawning in 17,20βP-exposed fish, but there was no difference in LTB4 or LTC4. Pre-exposure to cPLA2 or LOX inhibitors reduced 17,20βP-induced ovulation rates, while a COX inhibitor had no effect on ovulation or spawning. Collectively, these findings suggest that eicosanoids, in particular LOX metabolites, mediate 17,20βP-induced ovulation in zebrafish. COX metabolites also appear to be involved in ovulation and spawning but their role remains undefined.
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# TABLE OF CONTENTS

Abstract.........................................................................................................................ii

Acknowledgements......................................................................................................iii

Table of Contents.........................................................................................................iv

List of Tables and Figures.............................................................................................v

List of Abbreviations.......................................................................................................vi

CHAPTER 1 – General Introduction..............................................................................1

  Oocyte maturation, ovulation and spawning in female teleosts..............................1
  Ovulation as an inflammatory response.................................................................7
  Eicosanoids and the variability among teleosts.........................................................9
  The zebrafish model..................................................................................................14
  Research applications..............................................................................................15
  Thesis objectives, hypotheses and predictions........................................................16

CHAPTER 2 – The role of eicosanoids in 17α, 20β-dihydroxy-4-pregnen-3-one-induced ovulation and spawning in *Danio rerio*.........................................................19

  Introduction...............................................................................................................19
  Materials and Methods............................................................................................23
  Results......................................................................................................................29
  Discussion...............................................................................................................44

CHAPTER 3 – General Discussion.................................................................................53

  Future directions.....................................................................................................58
  Conclusions.............................................................................................................60

References...................................................................................................................61

APPENDIX – Mechanisms of ovulation.....................................................................69
LIST OF TABLES AND FIGURES

CHAPTER 1

Figure 1. Generalized schematic of oocyte maturation, ovulation and spawning...........4

Figure 2. Generalized schematic of the eicosanoid biosynthesis pathway including diagrams of chemical structures.................................................................10

CHAPTER 2

Table 1. Forward and reverse qPCR primer pair sequences for eicosanoid-synthesizing enzymes and the reference genes in the zebrafish (Danio rerio), and associated accession numbers from GenBank.................................................................32

Figure 1. Mean proportion of females that had undergone ovulation and spawning in response to either 17,20βP (10 nM) or a solvent control under solitary or paired conditions after 4 hours.................................................................33

Figure 2. Time course of spawning during exposure to 17,20βP (10 nM), showing the cumulative proportion of fish that had spawned at a given time relative to the total proportion of fish that had spawned after 6 hours.................................................................35

Figure 3. Temporal expression of cpla2, ptgs2, alox5, alox5ap, lta4h, ltc4s and cysltr1 in ovarian tissue from zebrafish sampled at 1, 2 and 4.5 hours of exposure to 17,20βP (10 nM)..............................................................................................37

Figure 4. Mean ovary concentrations of PGF2α, LTB4, and LTC4 immediately post-spawning in female zebrafish housed with a male exposed to 17,20βP (10 nM) or a time-matched solvent control.................................................................40

Figure 5. Mean ovulation rates among solitary female zebrafish after a 2 hour pre-exposure to either a solvent (<0.001% EtOH) or one of the eicosanoid synthesis inhibitors QUIN, INDO or NDGA, and mean spawning rates among female zebrafish from mixed-sex pairs after 2 hour pre-exposure to a solvent (<0.001% EtOH) or INDO, followed by a 4 hour exposure to either a solvent or 17,20βP (10 nM).................................................................42

APPENDIX

Table 1. Summary of changes in mRNA expression for 8 genes with putative ovulatory functions in zebrafish ovarian tissue after 2 hours exposure to 17,20βP (10 nM) in vivo relative to control fish exposed to 0.0001% EtOH, n=10 fish per treatment..............74

Table 2. Forward and reverse qPCR primer pair sequences for putative ovulatory genes and the reference genes in the zebrafish (Danio rerio), and associated accession numbers from GenBank...............................................................................75
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Proper name</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
<td>Smooth muscle marker</td>
</tr>
<tr>
<td>15-keto-PGF$_{2\alpha}$</td>
<td>15-keto-prostaglandin F$_{2\alpha}$</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>17αHP</td>
<td>17α-hydroxy-progesterone</td>
<td>Steroid intermediate</td>
</tr>
<tr>
<td>17,20βP</td>
<td>17α, 20β-dihydroxy-4-pregnen-3-one</td>
<td>Steroid hormone</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
<td>Enzyme</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
<td>Cyclooxygenase isoform</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
<td>Signalling molecule</td>
</tr>
<tr>
<td>cPLA$_2$</td>
<td>Cytosolic phospholipase A$_2$</td>
<td>Enzyme</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
<td>Alcohol</td>
</tr>
<tr>
<td>FLAP</td>
<td>Five-lipoxygenase activating protein</td>
<td>Enzyme co-activator</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
<td>Hormone</td>
</tr>
<tr>
<td>GVBD</td>
<td>Germinal vesicle break down</td>
<td>Meiotic event</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
<td>Lipid signalling molecule</td>
</tr>
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<td>HPETE</td>
<td>Hydroperoxyeicosatetraenoic acid</td>
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</tr>
<tr>
<td>INDO</td>
<td>Indomethacin</td>
<td>Cyclooxygenase inhibitor</td>
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<td>LOX</td>
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<td>Enzyme</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
<td>Hormone</td>
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<td>LT</td>
<td>Leukotriene</td>
<td>Lipid signalling molecule</td>
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<td>LTB$_4$</td>
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<td>LX</td>
<td>Lipoxin</td>
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<tr>
<td>MIS</td>
<td>Maturation-inducing steroid</td>
<td>Steroid hormone</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix-metalloprotease</td>
<td>Protease</td>
</tr>
<tr>
<td>MPF</td>
<td>Maturation promoting factor</td>
<td>Cell cycle initiator</td>
</tr>
<tr>
<td>mPR</td>
<td>Membrane progesterone receptor</td>
<td>Receptor</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nordihydroguaiaretic acid</td>
<td>Lipoxygenase inhibitor</td>
</tr>
<tr>
<td>nPR</td>
<td>Nuclear progesterone receptor</td>
<td>Receptor</td>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
<td>Inflammatory mediator</td>
</tr>
<tr>
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<td>Prostaglandin</td>
<td>Lipid signalling molecule</td>
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<td>Prostaglandin</td>
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<td>Prostaglandin E$_2$</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>Prostaglandin F$_{2\alpha}$</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
<td>Enzyme</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
<td>Enzyme</td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>Phospholipase A$_2$</td>
<td>Enzyme</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
<td>Protein kinase C activator</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
<td>Gene expression assay</td>
</tr>
<tr>
<td>QUIN</td>
<td>Quinacrine</td>
<td>Phospholipase A$_2$ inhibitor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloprotease</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
<td>Lipid signalling molecule</td>
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CHAPTER 1

General Introduction

Oocyte maturation, ovulation and spawning in female teleosts

The female reproductive cycle in teleosts is regulated by the interactions of a variety of hormones and local factors. Gonadotropin-releasing hormone from the hypothalamus stimulates the production of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary (Clelland and Peng, 2009). FSH regulates the growth phase of oogenesis: the progression from pre-vitelligenic to full grown ovarian follicle. A subsequent surge in LH causes the granulosa cells of the ovary to produce the maturation-inducing steroid (MIS) 17α, 20β-dihydroxy-4-pregnen-3-one (17,20βP) and the oocyte to become responsive to 17,20βP, or maturationally competent (Figure 1; Fabra et al., 2006; Clelland and Peng, 2009). This activates the maturation phase of follicular development. 17,20βP binds to membrane-associated progesterone receptors (mPRs) on the oocyte and initiates a downstream cascade involving a decrease in adenylyl cyclase and protein kinase A activity, leading to the production of maturation promoting factor (MPF) (Nagahama and Yamashita, 2008; Clelland and Peng, 2009). MPF instigates germinal vesicle breakdown and reinitiates meiosis (Clelland and Peng, 2009). Mature ovarian follicles then undergo ovulation, where the oocyte exits the ovary through a localized digestion of the follicle layer and descends into the reproductive tract (Clelland and Peng, 2009). Following ovulation, the eggs are spawned, releasing them from the body into the external environment to be fertilized by the male. It has been postulated that 17,20βP and its downstream products
also regulate ovulation and spawning, through a distinct pathway and receptor type than oocyte maturation (Patino and Sullivan, 2002; Nagahama and Yamashita, 2008).

Previous studies have suggested that 17,20βP-induced ovulation is mediated through binding of nuclear progesterone receptors (nPRs) and requires de novo transcription, as well as an increase in protein kinase C (PKC) activity (Theofan and Goetz, 1981; Patino and Sullivan, 2002; Hanna and Zhu, 2011). Unlike the well-characterized consequences of mPR activation that lead to oocyte maturation, research on the downstream effects of nPR activation is still in its infancy (Nagahama and Yamashita, 2008; Hanna et al., 2010). Research by Hanna et al. (2010) in zebrafish (Danio rerio) profiled the expression of the nPR across tissue types and found it was highly expressed in stage IV (mature) follicle cells. They also identified 17,20βP as the most potent ligand of the nPR among the steroids tested, which included various estrogens, androgens and progestogens. This was the first research to characterize the nPR in a teleost. Another study by Hanna and Zhu (2011) showed that the nPR in zebrafish acts through a genomic pathway, whereas the mPR which acts through a non-genomic pathway. This lends support to the theory that the nPR is the progesterone receptor isoform that mediates ovulation, as ovulation can be inhibited in vitro in many teleosts through the use of Actinomycin D, a transcriptional inhibitor (Theofan and Goetz, 1981; Pinter and Thomas, 1999; Patino et al., 2003). The evidence for a role by PKC in facilitating ovulation is derived primarily from studies that showed a PKC activator, phorbol myristate acetate (PMA), is capable of eliciting ovulation of isolated follicles in vitro in the yellow perch (Perca flavescens) and the Atlantic croaker (Micropogonias undulatus) though its mechanism of action is not known (Berndtson et al., 1989; Patino et al., 2003). Beyond activation of a progesterone
receptor and an increase in PKC activity, there are still major gaps in our understanding of the physiological cascade that leads to ovulation and spawning. However, there is mounting evidence that ovulation in teleosts, as in mammals, resembles an inflammatory response.
Maturation pathway

Oocyte surface

17,20βP

mPR

cAMP/PKA

GVBD
Resume meiosis

Mature oocyte

Ovulation and spawning pathway

Follicle cell surface

17,20βP

nPR?

Do novo transcription

PKC

Proteases, Inflammatory factors

Eicosanoids (PGs)

Ovulation, Spawning
Fig. 1. Generalized schematic diagram of oocyte maturation, ovulation and spawning in teleosts. The box at the top left shows a simplified ovarian follicle, consisting of an oocyte and the surrounding follicle layers made up of outer theca cells and inner granulosa cells. The centre of the diagram depicts a magnified view of theca and granulosa cells and the production of the maturation inducing-steroid 17α, 20β-dihydroxy-4-pregnene-3-one (17,20βP). The lower left side shows a magnified view of the oocyte and the pathway leading to oocyte maturation; the lower right side shows a magnified view of a follicle cell and the pathway leading to ovulation and spawning.

Luteinizing hormone (LH) plays a dual role, causing cholesterol to be converted into 17α-hydroxy-progesterone (17αHP) within the theca cells, as well as upregulating the enzyme that converts 17αHP into 17,20βP within the granulosa cells. In oocyte maturation, 17,20βP binds to the membrane type progesterone receptor (mPR) located on the oocyte plasma membrane, inhibiting cyclic AMP (cAMP) and protein kinase A (PKA) and enabling the formation of a complex called the maturation promoting factor (MPF), which then acts on the oocyte to facilitate germinal vesicle breakdown (GVBD) and the resumption of meiosis, ultimately generating a mature oocyte. In ovulation and spawning, 17,20βP is thought to bind to a nuclear progesterone receptor (nPR) located within the follicle layer, though it has not been localized to a specific cell type (i.e., theca vs. granulosa). There is de novo transcription of genes including cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2), and subsequently the production of eicosanoids (specifically prostaglandins (PGs)). There is also evidence of a role for protein kinase C (PKC), as well as proteases and other inflammatory factors, but whether
these mediators are regulated directly by the nPR, through an alternative receptor, or through the actions of eicosanoids has yet to be determined.
Ovulation as an inflammatory response

In mammals, the peri-ovulatory surge in LH leads to changes in ovarian vasculature that facilitate an influx of inflammatory mediators and an increase in the intra-follicular pressure (Espey, 1980; Brannstrom and Enskog, 2002; Richards et al., 2002). The inflammatory mediators, including chemokines, cytokines, eicosanoids, and many others, are involved in the production and regulation of proteases that degrade the apex of the follicle layer, the contraction of smooth muscle tissue within the ovary, and ultimately the successful rupture of the follicular tissue and release of the mature egg into the oviduct. Research has suggested these inflammatory mediators may also contribute to post-ovulatory luteal formation and regression (e.g., Arosh et al., 2004; Ricciotti and FitzGerald, 2011). Our understanding of the system in teleosts is less complete. While the literature on teleostean ovulation spans many different species and employs many different experimental designs, the majority of studies present evidence that ovulation in teleosts may be achieved through similar mechanisms (Goetz and Garczynski, 1997; Lubzens et al., 2010). As in mammals, ovulation in teleosts involves changes in the vasculature of the ovary, the production of inflammatory mediators, increases in intra-oocyte pressure, and targeted degradation at the apex of the follicular layer by specific proteases (Lubzens et al., 2010). Recent large scale, transcriptomic studies in rainbow trout and fathead minnow have illustrated peri-ovulatory increases in mRNA expression of genes involved in angiogenesis, tissue remodelling, oocyte hydration, and proteolysis (Bobe et al., 2006; Villeneuve et al., 2010). Studies on brook trout, yellow perch, and medaka have all provided evidence for a role of proteolytic enzymes, specifically from the metalloprotease family, in facilitating ovulation (Berndtson et al., 1989; Berndtson
A role for metalloproteases in ovulation has also been established in rodents, ungulates, monkeys, and humans (Curry and Osteen, 2003; Peluffo et al., 2011). An increase in intra-oocyte pressure during the peri-ovulatory period via hydration of the oocyte that may contribute to follicular rupture has also been observed in many teleost species (Cerda 2009; Lubzens et al., 2010). A considerable portion of the literature on teleost oocyte maturation and ovulation focuses on a particular subset of inflammatory mediators – the eicosanoids, specifically the prostaglandins (e.g. Stacey and Peter, 1979; Stacey and Goetz, 1982; Sorensen et al., 1988; Berndtson et al., 1989; Sorbera et al., 2001; Patino et al., 2003; Lister and Van Der Kraak, 2008). Studies on other inflammatory mediators are limited to a few studies on the effects of tumor necrosis factor alpha on ovulation in brown trout (Salmo trutta) (e.g. Crespo et al., 2010) and a broad scale microarray study in rainbow trout (Oncorhynchus mykiss) indicating upregulation of chemokine mRNA during ovulation (Bobe et al., 2006). While most teleosts do not form structures homologous to the corpora lutea found in mammals, it is conceivable that inflammatory mediators play a role in regulating tissue remodelling in the post-ovulatory ovary, though this is entirely speculative given the limited research on this topic (Drummond et al., 2000; McMillan, 2007). Of course, mammals do not spawn their eggs to be fertilized externally as teleosts do. However there is evidence that the same inflammatory mediators that function internally to regulate ovulation also function externally as pheromones to coordinate spawning related sexual behaviours and ultimately, spawning (Stacey, 1976; Sorensen et al., 1988; Stacey and Kobayashi, 1993). Such action has been thoroughly demonstrated in the goldfish (Carassius auratus), and is discussed below.
Eicosanoids and the variability among teleosts

Among the inflammatory mediators, the eicosanoids, and in particular the prostaglandins (PGs), have received the most attention in the teleost literature. The eicosanoid family is a large one – it consists of PGs, prostacyclins, thromboxanes, leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), lipoxins, and other derivatives of the common precursor arachidonic acid (AA) (McCracken, 2005). In the classical eicosanoid pathway model, AA is cleaved from membrane phospholipids by the enzyme phospholipase A2, specifically the cytosolic isoform (cPLA$_2$) (Figure 2; Leslie, 2004; Burke and Dennis, 2009). Cyclooxygenase (COX) enzymes then synthesize PGH$_2$ from AA, which is metabolized into PGs, thromboxanes or prostacyclins by specific synthases or non-enzymatic reactions. Similarly, lipoxygenase (LOX) enzymes synthesize hydroperoxyeicosatetraenoic acids, which are metabolized into LTs, HETEs, or lipoxins. While their specific functions differ, all play a role in inflammation.
Figure 2. Generalized schematic diagram of the eicosanoid biosynthesis pathway including diagrams of chemical structures. The major metabolites of the cyclooxygenase (COX) and lipoxygenase (LOX) pathways are shown. Abbreviations: PG, prostaglandin; TX, thromboxane; HPETE, hydroperoxyeicosatetraenoic acid; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; LX, lipoxin. Figure adapted from Heckmann et al., 2008.
The role and regulation of eicosanoids in teleost oocyte maturation, ovulation and spawning is somewhat contested. The specific eicosanoids involved, mechanism of action and position in the physiological cascade appears to vary between species. An in vitro study on European sea bass (*Dicentrarchus labrax*) showed that cultured oocytes could be induced to undergo maturation by exposure to gonadotropin and AA, and that gonadotropin-induced maturation could be inhibited by the addition of the COX inhibitor indomethacin (INDO) (Sorbera et al., 2001). Further, this inhibition could be reversed by addition of AA or PGs. Both PGE$_2$ and PGF$_{2a}$ were also capable of inducing oocyte maturation. Conversely, a similar study in Atlantic croaker indicated AA metabolites exerted their effects downstream of maturation (Patino et al., 2003). In this study, neither AA nor PGF$_{2a}$ induced maturation of oocytes *in vitro*. However, both AA and PGF$_{2a}$ induced ovulation in MIS-matured ovarian follicles. The same study also showed that treatment of matured oocytes with INDO or the LOX inhibitor nordihydroguaiaretic acid (NDGA) inhibited MIS-dependent ovulation. However, only INDO inhibited ovulation induced using the PKC activator PMA. These findings support a role for both LOX and COX products in ovulation, upstream and downstream of PKC activation respectively. In contrast, *in vitro* studies on yellow perch ovarian follicles suggested the opposite, where both NDGA and INDO inhibited 17,20βP-induced ovulation but only NDGA inhibited ovulation induced using PMA (Berndtson et al., 1989). Studies in goldfish strongly support a role for PGs, in particular the isoform PGF$_{2a}$ and its metabolite, 15-keto-PGF$_{2a}$, in ovulation and as pheromones that regulate spawning behaviour (Stacey 1976; Stacey and Peter, 1979; Sorensen et al., 1988; Kobayashi and Stacey, 1993). PGF$_{2a}$ has also
been seen to induce ovulation in vitro in rainbow trout, brook trout (Salvelinus fontinalis), and yellow perch, however the PGE\textsubscript{2} isoform was the more potent inducer of ovulation in the latter. Both PGF\textsubscript{2\alpha} and PGE\textsubscript{2} were capable of inducing ovulation in vitro in Atlantic croaker (Patino et al., 2003). Research on the other eicosanoids in a reproductive context is lacking in the teleostean literature, but there is evidence to suggest a role for leukotrienes in mammals, in particular rodents and ungulates (Priddy and Killick, 1993). Studies on the rat, the rabbit, and the pig demonstrated that ovulation was inhibited by injection with a LOX inhibitor (Yoshimura et al., 1991; Downey et al., 1998; Mikuni et al., 1998). Additionally, studies on the rat and the rabbit demonstrated that LT levels increased during the peri-ovulatory period, and that inhibition of ovulation by a LOX inhibitor could be reversed by injection with LTB\textsubscript{4} (Yoshimura et al., 1991; Higuchi et al., 1995; Mikuni et al., 1998). The putative function of LTs in mammalian ovulation is to regulate the infiltration and distribution of leukocytes in the ovary, which secrete proteases and vasoactive substances critical to the ovulatory process (Brannstrom and Enskog, 2002; Oakley et al., 2011). The mammalian literature points to a complex, interconnected relationship between the COX and LOX pathways. For example, in one study, PGE\textsubscript{1} and PGE\textsubscript{2} both inhibited LTB\textsubscript{4} production in rat neutrophils (Ham et al., 1983). However, Lotzer et al. (2007) demonstrated that LTD\textsubscript{4} induced COX-2 transcription in human endothelial cells. As well, PGs and LTs may exert some actions through a common receptor type, the peroxisome proliferator-activated receptors (McCracken, 2005). Cross-talk between the LOX and COX pathways could also be present in teleosts and might explain some of the apparent conflict between different studies.
The zebrafish model

The zebrafish is recognized as a powerful model for studying a wide range of subjects, including developmental biology, reproductive physiology, and toxicology (Briggs, 2002; Ge, 2005). Zebrafish are easily maintained due to their small size and minimal habitat requirements. The zebrafish genome has been sequenced and they are amenable to an array of genetic and physiological manipulation techniques (Briggs, 2002). They are well-suited to research on reproductive physiology, gametogenesis in particular, due to their short spawning cycle, asynchronous ovarian development, and abundant egg production. However, until recently, studies on zebrafish ovulation and spawning were hindered by two factors: 1) zebrafish do not ovulate in vitro and 2) there is sufficient variability in the frequency of ovulation and spawning among females under laboratory conditions in vivo to introduce confounding error into physiological studies (Eaton and Farley, 1974; Selman et al., 1994; Gerlach, 2006). A solution to both of these challenges was provided by Tokumoto et al. (2011), where they demonstrated that a high percentage (80-100%) of female zebrafish exposed to waterborne 17,20βP ovulated independent of time of day or stage of light cycle. Thus, Tokumoto’s method enables maximization of the number of females undergoing ovulation at a given time and thus collection of samples with reduced confounding variability. Interestingly, under Tokumoto’s conditions, female fish did not spawn but retained their eggs within the body cavity. This is likely due to the absence of male fish, as previous research has indicated
female zebrafish are unable to proceed from ovulation to spawning in the absence of a cue from male fish (Spence et al., 2008).

Studies investigating the eicosanoid pathway in zebrafish ovulation and spawning have previously demonstrated an increase in transcription of the enzymes cpla2 and ptgs2, which code for the enzymes cPLA2 and COX-2 (the inducible isoform of the COX enzyme in teleosts), at approximately the time of ovulation (Lister and Van Der Kraak, 2009). As well, an increase in the ovarian concentration of PGF2α was detected during the spawning period, and exposure to a COX inhibitor, INDO, was observed to inhibit egg production (Lister and Van Der Kraak, 2008; Lister and Van Der Kraak, 2009).

Research applications

The main goal of this research was to provide novel insight into the regulation of ovulation and spawning in zebrafish, with the intention of contributing to a comprehensive model of teleost reproduction. Such a model would enable comparisons across fishes, as well as with higher vertebrates, to enhance our understanding of reproduction at both a proximate and evolutionary scale. But beyond furthering our knowledge of the physiology of reproduction, there are also practical applications of this research. Zebrafish are widely used in developmental studies as a result of their external fertilization, embryonic transparency, and other beneficial features (Briggs, 2002). An optimized version of Tokumoto’s 17,20βP bioassay could provide a method to easily obtain embryos on demand for such studies. This research could also have implications for aquaculture practices. The capacity to induce spawning could improve captive breeding programs in species of economic or conservation interest, as many fishes
experience reproductive dysfunction in captivity (Reid and Hall, 2003). It could also overcome the research limitations imposed by reproductive seasonality in some species, such as the goldfish and other economically important cyprinid fishes. Additionally, the bioassay might be used in toxicology studies, to assess the effects of environmental contaminants on reproductive success and ultimately fitness, which could be of considerable use for informing water quality regulations and maintaining aquatic ecosystem health. Overall, this research has the potential to contribute to both basic and applied reproductive science.

**Thesis objectives, hypotheses and predictions**

The majority of studies that investigate the physiological changes underlying maturation and ovulation in teleosts have been conducted *in vitro*. There are several drawbacks to studies of this nature, namely that isolating the ovary does not allow an understanding of the physiological communication between different organs, in particular the complex relationship between the gonad and the brain, where reproductive physiology and sexual behaviour are integrated. In order to link ovulation with spawning, an event that involves a suite of behaviours and likely incorporates a pheromonal aspect as well, it is important to study the whole organism under *in vivo* conditions.

The objectives of this thesis were to provide a simple, effective *in vivo* framework within which to conduct studies on zebrafish ovulation and spawning and to use that framework to begin to characterize the role and regulation of the eicosanoid family in these events. The second chapter of this thesis involves the modification of Tokumoto’s
bioassay to create this framework, in order to facilitate comparisons between the ovulatory physiology of solitary females and the spawning physiology of females in the presence of males. The modified bioassay was then used to investigate the regulation of the eicosanoid pathway by 17,20\(\beta\)P and to begin to characterize the role of this system in ovulation and spawning. It was hypothesized that 17,20\(\beta\)P initiates the reproductive cascade that leads to ovulation but that 17,20\(\beta\)P is insufficient to elicit spawning in solitary females; spawning requires 17,20\(\beta\)P as well as a male cue. In accordance with this hypothesis, exposure to 17,20\(\beta\)P should consistently induce ovulation but not spawning in isolated females, and induce both ovulation and spawning in mixed-sex pairs. It was also hypothesized that eicosanoids mediate 17,20\(\beta\)P-induced ovulation and spawning. To address this hypothesis, the transcription profile of various members of the eicosanoid biosynthesis pathway were investigated, a technique of particular relevance to the study of ovulatory physiology. Given that de novo transcription is obligatory for ovulation to occur, the critical mediators of this event should be reflected by changes in the transcriptome. Thus it was predicted that 17,20\(\beta\)P-exposed fish would exhibit an increase in transcription of members of the eicosanoid biosynthesis pathway. Ovarian levels of eicosanoids were measured post-spawning and eicosanoid biosynthesis inhibitor exposures were conducted to further clarify the involvement of this pathway. It was predicted that 17,20\(\beta\)P-exposed fish would exhibit an increase in the ovarian concentration of eicosanoids during the peri-spawning period and that pre-exposure to inhibitors of eicosanoid biosynthesis would block 17,20\(\beta\)P-induced ovulation and spawning.
The third chapter reviews the findings of this study and examines them within the broader context of the reproductive model in teleosts and in mammals. The best avenues of research to build on the findings of this study are also discussed in order to clarify the function of eicosanoids in teleost reproduction.
CHAPTER 2

The role of eicosanoids in 17α, 20β-dihydroxy-4-pregnen-3-one-induced ovulation and spawning in Danio rerio

Introduction

Reproduction in female teleost fishes is regulated by combination of hormones and local factors (Clelland and Peng, 2009). Once the ovarian follicle is fully grown, a surge in luteinizing hormone from the pituitary stimulates the production of a teleost-specific progestogen, 17α, 20β-dihydroxy-4-pregnen-3-one (17,20βP), triggering a cascade that leads to the maturation of the egg, ovulation, and spawning (Nagahama and Yamashita, 2008). However, the physiological changes downstream of oocyte maturation that underlie the rupture of the oocyte from the ovary and its subsequent release from the body cavity into the external environment are poorly understood. A few studies have provided evidence that proteolytic enzymes may be involved in facilitating follicular rupture in brook trout, yellow perch, and fathead minnow (Berndtson and Goetz, 1990; Ogiwara et al., 2005). Some preliminary work has been conducted to characterize the zebrafish nuclear progesterone receptor (nPR), which is regarded as the receptor that mediates the effects of 17,20βP on ovulation (Hanna et al., 2010); and there have been a couple of transcriptomic studies in select species (e.g., Bobe et al., 2006; Villeneuve et al., 2010). These broad-scale approaches have suggested that ovulation is an incredibly dynamic process that involves transcriptional changes of hundreds, if not thousands, of genes. The most robust body of research on ovulation implicates a group of inflammatory mediators called prostaglandins (PGs) (e.g. Stacey and Peter, 1979; Stacey and Goetz, 1982; Sorensen et al., 1988; Berndtson et al., 1989; Sorbera et al., 2001;
PGs belong to a larger family of fatty acid products called eicosanoids, which also includes leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), thromboxanes, and prostacyclins, among others. These compounds are derived from the precursor arachidonic acid (AA), which is liberated from membrane phospholipids by the enzyme cytosolic phospholipase A2 (cPLA2). PGs are cleaved from AA by cyclooxygenase (COX) enzymes and have a diversity of known functions in higher vertebrates including roles in muscle contraction, inflammation, and cell signalling (McCracken, 2005). PGs have been shown to play a role in female teleost reproduction in a number of species, including goldfish, zebrafish, Atlantic croaker, yellow perch, and others (Stacey, 1976; Berndtson et al., 1989; Patino et al., 2003; Lister and Van Der Kraak, 2008). However, the nature of their role is somewhat contested, with the apparent position of PGs within the physiological cascade (upstream or downstream of ovulation) and the relevant isoform(s) varying from species to species (Stacey and Kobayashi, 1976; Berndtson et al., 1989; Sorbera et al., 2001; Patino et al., 2002). Further, the mechanism of PG action remains poorly characterized in most species, though studies demonstrated that in goldfish, PGs function as pheromones, coordinating spawning by regulating spawning behaviour (Kobayashi and Stacey, 1993).

Although not previously investigated in teleosts in a reproductive context, another family of eicosanoids has been demonstrated to play a role in mammalian ovulation: the leukotrienes (LTs) (Priddy and Killick, 1993). LTs are synthesized from AA by the enzyme 5-lipoxygenase (5-LOX) and it’s co-activator, 5-lipoxygenase activating protein (FLAP) (McCracken, 2005). LTA₄ is the precursor of LT biosynthesis; it is highly unstable and is readily metabolized into LTB₄ by the enzyme LTA₄ hydrolase or to LTC₄.
by the enzyme LTC₄ synthase. LTC₄ can be metabolized further to generate other LT isoforms. LTs possess inflammatory and immune functions, with roles in regulating vascular permeability and chemotaxis of leukocytes (Samuelsson, 1987). Leukocytes have been localized to the theca layer in mammals, and putative functions include production of proteolytic enzymes, extracellular matrix degradation, and roles in follicle maturation, ovulation and luteal formation (Oakley et al., 2011). LTB₄ and LTC₄ have been observed to increase during the ovulatory period of the rat, and specific inhibitors for LTB₄ receptor inhibit ovulation in the rat (Higuchi et al., 1995; Matousek et al., 2001). Studies in yellow perch and Atlantic croaker demonstrated that ovulation could be inhibited by treatment with nordihydroguaiaretic acid (NDGA), a LOX inhibitor, in vitro, suggesting that LOX products may play a role in teleost ovulation (Berndtson et al., 1989; Patino et al., 2003). Additionally, inhibition of the LOX pathway by NDGA has been shown to inhibit ovulation in rats and pigs (Downey et al., 1998; Kurusu et al., 2009).

To study teleost reproduction, the zebrafish is often employed as a model. Zebrafish are well-suited for reproductive studies as they possess asynchronous ovaries with ovarian follicles at all stages simultaneously and are reproductively active year-round rather than seasonally. Gravid females kept in mixed-sex tanks exhibit a peak in endogenous 17,20βP around 3:30 AM, and typically spawn in conjunction with the onset of light in the morning, on a 2-5 day cycle (Gerlach, 2006; Blanco-Vives and Sanchez-Vazquez, 2009; Lister and Van Der Kraak, 2009). Research has shown that zebrafish exhibit an increase in ovarian transcription of the eicosanoid-synthesizing enzymes cpla2 (cPLA₂) and ptgs2 (COX-2) during the ovulatory period and an increase in the ovarian
concentration of PGF$_{2\alpha}$ at the time of spawning (Lister and Van Der Kraak, 2008; Lister and Van Der Kraak, 2009). In addition, exposure to the COX inhibitor indomethacin (INDO) inhibited egg production in zebrafish (Lister and Van Der Kraak, 2008).

A recent study by Tokumoto et al. (2011) showed that ovulation could be induced in groups of female zebrafish independent of time of day or stage of light cycle, by \textit{in vivo} exposure to 17,20\textbeta P. This bioassay has the potential to enhance reproductive studies in zebrafish as it enables maximization of the number of females undergoing ovulation at a given time and thus collection of samples with reduced physiological variability. Notably, females under this experimental design did not proceed to spawn but retained the eggs within the body cavity. Thus, this bioassay also provides a unique opportunity to investigate the physiology of ovulation in isolation from spawning.

Previous research has indicated that female zebrafish require a male cue to proceed from ovulation to spawning, which may explain the absence of spawning in this design (Spence et al., 2008).

The purposes of this study were to validate and modify Tokumoto’s 17,20\textbeta P bioassay for studying both ovulation and spawning in zebrafish, and to manipulate the bioassay to clarify the role and regulation of eicosanoids in those reproductive events. To address the first objective, female zebrafish were exposed to 17,20\textbeta P and housed either in isolation or with a male, and ovulation and spawning rates were recorded. The time course of spawning under bioassay conditions was also determined. To address the second objective a variety of approaches and techniques were employed, including transcription profiling by qPCR, measurement of ovarian eicosanoid concentrations by enzyme immunoassay, and \textit{in vivo} exposures to eicosanoid biosynthesis inhibitors.
mRNA expression of enzymes in the eicosanoid pathway, specifically cpla2, ptgs2, alox5 (5-LOX), alox5ap (FLAP), lta4h (LTA4 hydrolase) and ltc4s (LTC4 synthase), as well as a receptor for cysteinyI leukotrienes, cysltr1, was quantified at 3 time points during the bioassay, to represent pre-ovulatory, peri-ovulatory and peri-spawning stages. The ovarian concentrations of PGF2α, LTB4 and LTC4 were compared between 17,20βP-exposed spawning fish and non-spawning controls. The effect of eicosanoid biosynthesis inhibitors on 17,20βP-induced ovulation and spawning rates was investigated by pre-exposure to quinacrine (QUIN), a cPLA2 inhibitor; INDO, a COX inhibitor; or NDGA, a LOX inhibitor.

Materials and Methods

Experimental Animals

Zebrafish were obtained from AQUAlity tropical fish wholesaler (Mississauga, ON) and housed at the Hagen Aqualab (University of Guelph, ON). The zebrafish were kept in aquatic habitat units (A-HAB; Aquatic Eco-Systems, Apopka, FL) supplied with re-circulated well water at 27°C. The fish were maintained under a 12 hour light: 12 hour dark cycle (from 08:00 to 20:00) and fed a diet of salmon fry pellets (Martin Mills, Elmira, ON) or brine shrimp (AQUAlity) to satiation 2-3 times daily. Fish were raised to sexual maturity before use in experiments. Individuals were sacrificed as required by anesthetization overdose in buffered tricaine methanesulfonate (MS-222; 1 g/l) (Syndel Laboratories Inc., Vancouver, BC) followed by spinal severance. All procedures were
carried out in accordance with the University of Guelph Animal Care Committee guidelines.

**Experimental Protocol**

17,20βP bioassay for solitary females

Gravid female zebrafish were individually assessed for the presence of ovulated eggs in the body cavity by gently squeezing the abdominal region. In the absence of ovulated eggs, a female was randomly allocated to a 4L glass beaker containing 3.5L of well water at 27°C, an airstone, a round plastic container with a mesh bottom (hereafter a “breeder”) and an artificial substrate (a plastic plant and 10-15 glass marbles). The breeder functioned to create a barrier between the fish and the bottom of the beaker, enabling any spawned eggs to fall through the mesh to the bottom of the beaker and avoid consumption or disruption by the fish. At 12:00, each of the beakers containing a single female zebrafish (n=16-20) received either 17,20βP (Sigma Aldrich, Oakville, ON) to a final concentration of 10 nM or the equivalent volume of solvent control, ethyl alcohol (EtOH, final concentration <0.001%; Commercial Alcohols, Brampton, ON). At 16:00, females were removed from the beakers and re-assessed for the presence of ovulated eggs. Beakers were also checked for the presence of spawned eggs.

17,20βP bioassay for mixed-sex pairs

This experiment was set up as described above for the 17,20βP bioassay for solitary females, but with each beaker containing a mixed-sex pair instead of a solitary
female. In this experiment, rather than a final assessment for ovulated eggs, beakers were checked for the presence of spawned eggs at 10-minute intervals from 14:00 to 18:00.

**Temporal expression of the eicosanoid pathway**

To investigate the ovulatory transcription profile, the 17,20βP bioassay for solitary females was conducted as described above. Fish were sampled at 1 hour (13:00) and 2 hours (14:00) of exposure in two respective experiments, to obtain ovary samples from females in pre-ovulatory and peri-ovulatory states. To investigate the spawning transcription profile, the 17,20βP bioassay for mixed-sex pairs was conducted as described above. Fish were sampled at 4.5 hours (16:30) of exposure to obtain ovary samples from females in a peri-spawning state. Ovaries were collected and frozen on dry ice. In those individuals where ovulated eggs were present in the body cavity, they were included with the ovary sample and measured in conjunction with the ovary as a single sample. Samples were stored at -80°C until the time of RNA extraction.

**Tissue concentrations of eicosanoids in the zebrafish ovary post-spawning**

The 17,20βP bioassay for mixed-sex pairs was conducted as described above. All beakers were surveyed for the presence of spawned eggs at 10-minute intervals from 14:00 to 18:00 and treated fish were sampled immediately post-spawning. A random control beaker was sampled concurrently with each treated beaker to obtain time-matched control and treatment samples. Ovaries and ovulated eggs (if applicable) were dissected out and frozen on dry ice. Samples were stored at -80°C until the time of steroid extraction.
Eicosanoid-synthesizing enzyme inhibitor exposures

To determine the effects of inhibiting eicosanoid production on ovulation, gravid female zebrafish assessed and confirmed to lack retained ovulated eggs were allocated in groups of n=8 fish to 20L aquaria containing 15-20 marbles and 5 plastic plants as substrate. During the pre-exposure period from 10:00 to 12:00, females were exposed to one of: a high concentration of QUIN (200 nM; Cayman Chemical, Ann Arbor, MI), a low concentration of QUIN (2 nM), a high concentration of INDO (300 nM; Cayman Chemical), a low concentration of INDO (3 nM), a high concentration of NDGA (5 µM; Cayman Chemical), a low concentration of NDGA (0.5 µM), or a solvent control of equivalent volume (EtOH <0.001%), n=1 aquarium per condition, with each condition replicated three times. At 12:00, 17,20βP (10 nM) was added to all aquaria. At 16:00, fish were re-assessed for ovulation.

To determine effects on spawning, gravid female zebrafish assessed and confirmed to lack retained ovulated eggs were paired with male zebrafish and allocated to beakers as described for the 17,20βP bioassay for mixed-sex pairs. The use of individual mixed-sex pairs instead of mixed-sex groups served to eliminate the confounding issue of how to identify the specific spawner(s) within a large group, as well as to reduce the influence of a hierarchical social structure on determining which females obtained the opportunity to interact with the male and occupy/defend a position on the substrate for spawning to take place. From 10:00 to 12:00, the pairs were exposed to one of either a high concentration of INDO (300 nM) or a solvent control of equivalent volume (EtOH...
<0.001%), n=8 pairs per condition. At 12:00, 17,20βP (10 nM) was added to all beakers. At 18:00, beakers were re-assessed for the presence of spawned eggs.

**Sample Analyses**

*Real-time primer design*

Primers not previously tested in zebrafish were designed using Primer Express v.3.0 (Applied Biosystems, Forster, CA) to cross exon-exon boundaries from nucleotide sequences available in GenBank. Primer sets were tested on standard curves made from whole ovary tissue. Specificity of the primer was verified by the presence of a single peak in the resulting dissociation curve and primer efficiency was evaluated using the equation $\%E = (10^{(-1/slope)} - 1) \times 100$. Only trials where primer efficiency was 75-110% were included in data analysis. All primer sequences and accession numbers are listed in Table 1.

*Quantification of gene expression*

Total RNA was extracted from whole ovary tissue using TRIzol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Samples were quantified using the Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA) and diluted to a final concentration of 2 µg/µl. Samples were treated for possible DNA contamination using a DNase treatment kit (Sigma Chem. Corp, St. Louis, MO) according to manufacturer’s instructions. Reverse transcription of samples was conducted according to methods employed by Lister (2008). A standard curve for each gene was generated from pooled cDNA and used to determine the appropriate dilution of
cDNA samples that would enable extrapolation of relative amounts of template from cycle threshold values. Diluted samples were measured using semi-quantitative real-time PCR (qPCR). Each reaction contained 3.75 µl of sample, 7.5 µl PerfeCTa SYBR green fast mix (Quanta Biosciences; Gaithersburg, MD), 1.875 µl forward primer (Sigma), and 1.875 µl reverse primer (Sigma). qPCR was conducted using an ABI Prism 7000 sequence detector system (Applied Biosystems) under the following cycle conditions: 50°C for 2 minutes, 95°C for 5 minutes, and then 40 cycles at 95°C for 1 second and 60°C for 30 seconds. All results were normalized to the expression of the reference gene, β-actin (bactin2), which did not change significantly across treatments. Results were also normalized to a second reference gene, elongation factor 1 α (ef1α), which showed the same trends as seen when normalized to bactin2 (data not shown).

Quantification of ovarian eicosanoid concentration

Ovarian PGF$_{2α}$, LTB$_4$ and LTC$_4$ were extracted and purified as described by Lister and Van Der Kraak (2008) and measured using an enzyme immunoassay kit according to manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI). Diluted samples were run in duplicate, and absorbance of samples was read using a SpectraMaxPlus microplate reader (Molecular Devices, Sunnyvale, CA) at 420 nm. Serial dilutions of pooled ovary samples and the assay standards exhibited parallelism indicating no interfering contaminants were present in the samples.

Statistical Analyses
Each experiment was conducted in triplicate, and the values reported in the results are means of the three replicate experiments. Ovulation and spawning rates were reported as the mean percentage of fish that underwent each event after 4 hours of exposure to 17,20βP, although spawning rates continued to be recorded until 6 hours of exposure. Cumulative spawning rate was calculated as the cumulative percentage of fish that had undergone spawning at a given time relative to the total percentage of fish that had spawned by 6 hours of exposure to 17,20βP. Normalized average gene expression data was presented in the results as fold change relative to the control. A Levene’s test for homogeneity of variances was employed to evaluate the distribution of data. For normally distributed data, a Student’s t-test was conducted to test for a significant effect of treatment. For non-normally distributed data, a Mann-Whitney U test was conducted. All tests were conducted using SPSS statistical software (v.20.0.0), accepting significance at \( p < 0.05 \).

**Results**

Exposing solitary female zebrafish to 17,20βP (10 nM) for 4 hours led to a significant induction of ovulation, with a mean ovulation rate of 77%, whereas none of the control females ovulated (Fig. 1). Spawning did not occur among solitary females under either treatment or control conditions. By comparison, 86% of females in mixed-sex pairs ovulated and 73% spawned after 4 hours of exposure to 17,20βP, whereas only 18% of control females ovulated and spawned. The earliest appearance of spawned eggs was after 130 minutes of exposure (Fig. 2). The average time to spawning was 192 minutes and maximum spawning rate was reached at 310 minutes of exposure. Of the
fish that spawned, 68% spawned between 130 and 190 minutes of exposure, the remaining 32% spawned between 190 and 310 minutes of exposure.

There were no significant differences in ovarian mRNA levels between females exposed to 17,20βP and control females for any of the genes measured after 1 hour of exposure (Fig. 3A). After 2 hours cpla2 and ptgs2 levels were significantly higher in 17,20βP-exposed fish than controls, on average 1.9-fold and 5.0-fold higher respectively (Fig. 3B). The ltc4s levels were significantly lower in 17,20βP-exposed fish than controls, and on average decreased by 3.9-fold.

Ovary tissue from females exposed to 17,20βP and sampled immediately post-spawning had a significantly higher mean concentration of PGF₂α, with a mean of 4.21 pg/mg tissue compared to 2.72 pg/mg tissue in paired control fish (Fig. 4). There was no difference observed in ovarian LTB₄ in 17,20βP-exposed fish immediately post-spawning compared to control fish, with mean concentrations of 4.51 pg/mg tissue and 4.05 pg/mg tissue, respectively (Fig. 4). Similarly, there was no difference in ovarian LTC₄ in 17,20βP-exposed fish and control fish, with mean concentrations of 1.19 pg/mg tissue and 1.26 pg/mg tissue, respectively (Fig. 4).

Mean ovulation rate among solitary females after exposure to 17,20βP was reduced significantly by pre-exposure to quinacrine (200 nM), from a mean of 79% to 21% (Fig. 5A). A lower concentration of quinacrine (2 nM) had no significant effect on ovulation rate. Mean ovulation rate was not affected by pre-exposure to indomethacin (3 nM or 300 nM). Pre-exposure to the higher of the two tested concentrations of NDGA (5 µM) significantly reduced ovulation rate from an average of 79% to 42%, but ovulation was not affected by the lower concentration (0.5 µM). Mean spawning rate among
mixed-sex pairs after exposure to 17,20βP was not inhibited by pre-exposure to indomethacin (Fig. 5B).
Table 1. Forward and reverse qPCR primer pair sequences for eicosanoid-synthesizing enzymes and the reference genes in the zebrafish (Danio rerio), and associated accession numbers from GenBank

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Accession #</th>
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</thead>
<tbody>
<tr>
<td>bactin</td>
<td>F-ACAGGGAAAAAGATGACACAGATCA R-CAGCCTGGATGGCAACGTGA</td>
<td>AF025305</td>
</tr>
<tr>
<td>eflα</td>
<td>F-GATCACTGGTACTTCTCAGGCTGA R-GGTGAAAGCCAGGAGGGC</td>
<td>NM_131263</td>
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<td>cpla2</td>
<td>F-TGCTCTTGGAAAGTTTGCACG R-TCTGTGGTCTGAGCATGAACAG</td>
<td>NM_131295</td>
</tr>
<tr>
<td>ptgs2</td>
<td>F-GTTTAAAGATGGAAAGCTTAATACCAGG R-GGGTACACCTCACCATCCACA</td>
<td>NM_153657</td>
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<tr>
<td>alox5</td>
<td>F-AGAGATGCGAGGCTCAGAT R-TTGCCGCTGTGCTTTTC</td>
<td>XM_001923650</td>
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<tr>
<td>alox5ap</td>
<td>F-CGTTGTTTCAGAATGTGTGTTTTTGC R-CTCGAAAGCTGCAAGACGTTT</td>
<td>NM_200061</td>
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<tr>
<td>lta4h</td>
<td>F-CCTCATTACCAGTCTGACTTTGA R-TCCAGGTCAGAAGAAGAATTTATC</td>
<td>NM_213286</td>
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<tr>
<td>ltc4s</td>
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<tr>
<td>cysltr1</td>
<td>F-TGCAACCTGTGAGGAGTCAAT R-GCCAAGCAAAGCGTGATGA</td>
<td>NM_001020648</td>
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Mean proportion of females undergoing event (%)

- **Solvent control (0.001% EtOH)**
- **10 nM 17,20βP**

<table>
<thead>
<tr>
<th></th>
<th>Ovulation</th>
<th>Spawning</th>
<th>Ovulation</th>
<th>Spawning</th>
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<tr>
<td><strong>Mixed-sex pairs</strong></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
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<tr>
<td><strong>Solitary females</strong></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
<td><img src="chart-data" alt="Bar chart data" /></td>
</tr>
</tbody>
</table>
Figure 1. Mean proportion of females that had undergone ovulation and spawning in response to either 17,20βP (10 nM) or a solvent control under solitary or paired conditions after 4 hours. Standard error bars represent s.e.m; n=3 experiments with 8-10 fish per treatment per experiment. Asterisks denote a significant difference from the solvent control (p<0.05; Student’s t-test).
**Figure 2.** Time course of spawning during exposure to 17,20βP (10 nM), showing the cumulative proportion of fish that had spawned at a given time relative to the total proportion of fish that had spawned after 6 hours. Cumulative spawning rate was calculated from the pooled data of 3 experiments, representing n=22 fish. Cumulative spawning rate increases rapidly from 120 minutes to 190 minutes of exposure, and then increases at a reduced rate from 200 to 310 minutes, when it reaches the maximum.
Fold change in expression normalized to β-actin (arbitrary units)

Gene

cpla2  ptgs2  alox5  alox5ap  lta4h  ltc4s  cysltr1

1 hr

- Control
- 10 nM 17,20βP

Gene

cpla2  ptgs2  alox5  alox5ap  lta4h  ltc4s  cysltr1

2 hr

- Control
- 10 nM 17,20βP
Fold change in expression normalized to β-actin (arbitrary units)

Gene

cpla2  ptgs2  alox5  alox5ap  lta4h  ltc4s  cysltr1

C

4.5 hr

Control

10 nM 17,20βP
**Figure 3.** Temporal expression of *cpla2*, *ptgs2*, *alox5*, *alox5ap*, *lta4h*, *ltc4s* and *cysltr1* in ovarian tissue from zebrafish sampled at 1 (A), 2 (B) and 4.5 (C) hours of exposure to 17,20βP (10 nM). Data are normalized to *bactin* expression and expressed as fold change relative to the control condition. Error bars represent s.e.m; values are the mean of n=3 experiments with 8-10 fish per treatment per experiment. Asterisks indicate significant differences from the control (p<0.05; Student’s t-test, Mann-Whitney U test).
Ovarian concentration (pg/mg tissue)

- **PGF2α**
  - Control
  - 10 nM 17,20βP

- **LTB4**
  - Control
  - 10 nM 17,20βP

- **LTC4**
  - Control
  - 10 nM 17,20βP
Figure 4. Mean ovary concentrations of PGF$_{2\alpha}$, LTB$_4$, and LTC$_4$ immediately post-spawning from female zebrafish, housed with a male, exposed to 17,20βP (10 nM) or a time-matched solvent control. Error bars represent s.e.m; values are the mean of n=3 experiments with 6-8 fish per treatment per experiment. Asterisks indicate a significant difference from the control (p<0.05, Student’s t-test).
A

**Mean ovulation rate (%)**

<table>
<thead>
<tr>
<th>Pre-exposure condition</th>
<th>Solvent</th>
<th>2 nM QUIN</th>
<th>200 nM QUIN</th>
<th>3 nM INDO</th>
<th>300 nM INDO</th>
<th>0.5 µM NDGA</th>
<th>5 µM NDGA</th>
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</thead>
<tbody>
<tr>
<td>Exposure condition (+/- 17,20βP)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

B

**Mean spawning rate (%)**

<table>
<thead>
<tr>
<th>Pre-exposure condition</th>
<th>Solvent</th>
<th>Solvent</th>
<th>300 nM INDO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure condition (+/- 17,20βP)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5. Mean ovulation rates among solitary female zebrafish after a 2 hour pre-exposure to either a solvent (<0.001% EtOH) or one of the eicosanoid synthesis inhibitors QUIN, INDO or NDGA (A), and mean spawning rates among female zebrafish from mixed-sex pairs after 2 hour pre-exposure to a solvent (<0.001% EtOH) or INDO (B), followed by a 4 hour exposure to either a solvent or 17,20βP (10 nM). Error bars represent s.e.m; values are means of n=3 experiments, with 8 fish per treatment per experiment. Asterisks indicate a significant difference from the group that received solvent for pre-exposure and 17,20βP for the exposure (p<0.05; Student’s t-test).
Discussion

Exposure to waterborne 17,20βP induces ovulation in female zebrafish, and in mixed-sex pairs, induces spawning as well. These findings suggest that ovulation is likely regulated primarily by internal physiological changes downstream of 17,20βP, whereas spawning requires a combination of physiological and behavioural cues. There are foreseeable benefits for a female to retain ovulated eggs until stimulated by a male cue – this would enable the synchronization of spawning and increased fertilization success. It has been proposed that the cue that functions to synchronize spawning is the release of pheromones (Kobayashi et al., 2002). These pheromones induce the fish to perform a suite of sexual behaviours that lead to the coordinated release of gametes by both sexes. Although the mechanism of 17,20βP action was not investigated in this study, it has been proposed that in addition to autocrine functions on ovarian physiology, it may also function as a pheromone to coordinate gamete production, maturation and release by the opposite sex in goldfish and zebrafish (Dulka et al., 1987, van den Hurk et al., 1987). Future studies could use the 17,20βP bioassay with anosmic fish to investigate the relative role of the olfactory detection of 17,20βP and its downstream products in mediating its effects on ovulation and spawning. The progression to spawning in the bioassay occurred on a similar time scale to that observed under natural spawning conditions, with an approximate time of 3.5 hours between the endogenous peak in 17,20βP and spawning (Lister and Van Der Kraak, 2008) compared to an average of 3.2 hours between the addition of 17,20βP to the water and the occurrence of spawning in the bioassay. This suggests that 17,20βP is likely acting through the same pathways under bioassay conditions as under natural spawning conditions.
Some of the genes investigated showed transcriptional changes that support a role for eicosanoids in ovulation and spawning. Significant elevations in ovarian cpla2 and ptgs2 mRNA coincide with the approximate time of ovulation. This finding is in agreement with previous studies in zebrafish that demonstrated elevated ovarian cpla2 and ptgs2 mRNA at 06:00, one hour prior to the onset of the lights and the anticipated spawning time (Lister and Van Der Kraak, 2009). By 4.5 hours of exposure, during the peri-spawning to post-spawning period, cpla2 and ptgs2 levels had returned to control levels. This extremely transient change in mRNA expression suggests a tightly regulated system, which is reasonable given the damage that sustained inflammation can cause. Interestingly, in vitro studies on zebrafish showed that incubation of full grown ovarian follicles with 17,20βP for 6 hours led to elevated levels of cpla2 (Melnyk, 2011), which considered in concert with the findings from this study has a number of potential implications. It is likely that ovarian follicles in vitro are not subject to the same regulatory/feedback systems as those in the intact organism, in this case potentially enabling cpla2 transcription to continue for an extended period or causing a shift in the timing of transcription relative to what is observed in vivo. Alternatively, it is possible that there is a second surge in cpla2 transcription during the course of reproduction. This might occur if elevated transcription of cpla2 is required upstream of ovulation, corresponding to the synthesis of cPLA2 and subsequently eicosanoids involved in pro-inflammatory functions, and then is required again downstream of ovulation and spawning, corresponding to the production of anti-inflammatory eicosanoids involved in the resolution of the inflammatory reaction. There is some support for this model: Gilroy et al. (2004) determined that inflammation in Wistar rat pleural exudate was regulated by
biphasic production of arachidonic acid and its subsequent differential metabolism into either pro- or anti-inflammatory eicosanoids. However, no evidence of a second peak in cpla2 transcription was observed in naturally spawning zebrafish; rather, cpla2 expression appeared to peak during the ovulatory period and gradually decrease to a minimum by 00:30 (Lister and Van Der Kraak, 2009).

Expression of ltc4s was significantly lower in 17,20βP-exposed fish than controls at 2 hours of exposure. This may suggest a suppression of cysteinyl leukotriene production at this time, which could serve a protective function, reducing the damage incurred by leukocyte infiltration; alternatively it could serve to shuttle the common precursor LTA₄ towards LTB₄ production. Although an increase in LTB₄ among 17,20βP-exposed fish was not detected post-spawning, it is possible that LTB₄ is elevated upstream, during the peri-ovulatory period. Future studies could clarify this by quantifying ovarian LTB₄ at earlier times in the bioassay. Notably, alox5 expression was unaffected by 17,20βP exposure, possibly suggesting that the 5-LOX pathway is not involved in ovulation and spawning, but also possibly due to regulation at a post-transcriptional level. Given that NDGA exposure significantly reduced ovulation, the latter explanation seems more likely. Similarly, alox5ap, lta4h, and cysltr1 expression data did not reflect a transcriptionally regulated role in ovulation or spawning.

The concentration of ovarian PGF₂α was higher in 17,20βP-exposed fish immediately post-spawning than it was in controls. This is consistent with the literature on naturally spawning zebrafish, which showed an increase in ovarian PGF₂α 1 hour after the onset of spawning relative to other sampling times, whereas non-spawning fish showed no such increase (Lister and Van Der Kraak, 2008). Interestingly, in a separate
study, zebrafish sampled 1 hour prior to onset of spawning did not exhibit the same increase in ovarian PGF$_{2\alpha}$ relative to other sampling times, indicating that the increase in PGF$_{2\alpha}$ may be extremely transient (Lister and Van Der Kraak, 2009). That theory was supported by this study, as the difference in PGF$_{2\alpha}$ between 17,20βP-exposed fish and control fish is eliminated when sampling is conducted at a common time point, rather than tailored to individual spawning times (data not shown). It would appear that individual variation in the time of spawning leads to a loss of signal if sampling is delayed. This strongly suggests that PGF$_{2\alpha}$ levels increase rapidly at the time of spawning and then decline back to control levels within a period of 0.5-2 hours.

Research on PGF$_{2\alpha}$ production by 17,20βP-treated zebrafish ovarian follicles in vitro showed no difference in PGF$_{2\alpha}$ levels between treated and control follicles after 6 hours, but a significant increase after 18 hours of incubation (Melnyk, 2011). It is difficult to interpret these findings in relation to the findings of this study, as it is likely that both internal and external factors are involved in regulating 17,20βP-induced PG production in vivo that are absent under in vitro conditions. However, in a general sense, both studies demonstrated the capacity of 17,20βP to induce PGF$_{2\alpha}$ production in the ovary.

In contrast to the changes observed in PGF$_{2\alpha}$, the levels of LTB$_{4}$ and LTC$_{4}$ were not different between 17,20βP-exposed and control fish. This would suggest that the LTs are not involved in mediating spawning, although it does not necessarily negate a possible role in ovulation, especially if these eicosanoids are under similarly tight regulation as PGF$_{2\alpha}$. If the eicosanoids do exhibit distinct, non-overlapping roles in these events, it is plausible that they would each exhibit a unique temporal profile, similar to
what has been observed in the rat (Higuchi et al., 1995). It would be useful for future studies to measure ovarian LTs upstream of spawning to investigate this possibility.

The inhibitor exposures strongly suggest that some metabolite(s) of AA are critical to ovulation, as the PLA$_2$ inhibitor QUIN almost completely abolished the response to 17,20βP. However, determining which eicosanoids were the key mediators of this effect was more complicated. The exposure to INDO had no effect on ovulation or spawning rates, in contrast with what would be expected based on both the changes in transcription and PG concentration, as well as much of the literature (e.g., Stacey and Goetz, 1982; Lister and Van Der Kraak, 2008). This finding likely implies one of two things: that INDO does not inhibit PG production in vivo as it does in vitro (Lister and Van Der Kraak, 2008), or that PGs have a non-essential role in zebrafish ovulation and spawning. This study did not attempt to verify the inhibition of PG production by INDO exposure. INDO has been used to investigate the role of COX metabolites on reproduction in teleosts for many years, though studies with direct demonstrations of PG inhibition by INDO in vivo are rare (e.g., Stacey, 1976; Goetz and Theofan, 1979; Berndtson et al., 1987). This is likely due to the expense and challenges associated with conducting such studies. Given the transient nature of PGs in circulation, there is a risk of falsely confirming inhibition – that is, that due to experimental design or individual variability, INDO would appear to have suppressed PG production when, in reality, the change in PGs was simply not detected. Nevertheless, it might be worthwhile for future studies to attempt to quantify ovarian PGs in fish immediately post-spawning after exposure to INDO, to confirm that PGs are not elevated in spawning fish compared to controls.
It is conceivable that the role of PGs in reproduction is redundant, with other eicosanoid products exerting a similar function, such that inhibition of the COX pathway simply causes the common precursor to be diverted into the production of the other eicosanoids (McCracken, 2005) and therefore ovulation and spawning still occur at a similar rate. Alternatively, it is possible that the role of PGs is not obligatory in an acute sense, but becomes essential over time. This could be the case if PGs function as a sort of “clean-up crew” in the ovary, directing the digestion and reabsorption of damaged follicular tissue and regulating the resolution of the inflammatory reaction. PGF$_{2\alpha}$ is involved in the process of luteolysis in mammals, which could be considered analogous to post-ovulatory follicle atresia in teleosts (Ricciotti and FitzGerald, 2011). There is very limited research on this topic in teleosts, so this is purely speculative, but such a mechanism might also explain the discrepancy between the results of this study and one by Lister and Van Der Kraak (2008), which found chronic exposure to INDO led to a reduction in egg production. If PGs are responsible for post-ovulatory maintenance, then over time, inhibition of PGs would lead to an accumulation of damaged tissue in the ovary and possibly a reduction in the number of healthy, viable ovarian follicles capable of reaching oocyte maturation and undergoing ovulation.

NDGA inhibited ovulation, but not to the same magnitude as QUIN. This implies that LOX products are important to ovulation, but may work in concert with other AA metabolites as ovulation still occurred in some fish when the LOX pathway was blocked. The identity of these key LOX metabolites remains elusive. Given that LTB$_4$ and LTC$_4$ levels were not different between treated and control fish post-spawning, it is plausible that the other major LOX metabolite group, the HETEs, are playing a critical role. One
study in yellow perch found that exposure to 11-HETE was capable of restoring NDGA-
inhibited ovulation in vitro (Berndtson et al., 1989). In mammals, there is some evidence
for a role of HETEs in ovulation (Espey et al., 1991; Downey et al., 1998). Changes in
the levels of 5-, 12- and 15-HETE occurred during the peri-ovulatory period in the rat
(Espey et al., 1991), and 15-HETE was observed to increase prior to ovulation in the pig
(Downey et al., 1998). Thus, investigating the profile of ovarian HETEs during ovulation
and spawning might be a worthwhile avenue of future research. Outside of the COX and
LOX pathways, epoxides are compounds derived from cytochrome P450 epoxygenase
metabolism of AA, whose functions remains largely unknown (McCracken, 2005).
Another inflammatory mediator produced by cPLA₂ is platelet activating factor (PAF)
(McCracken 2005). There has not been any research on the role of this mediator in
teleost reproduction to date, but PAF has been implicated in mammalian ovulation, as it
is produced in ovarian leukocytes, and injection with a PAF-inhibitor has been shown to
decrease ovulation success in rats (Abisogun et al., 1989; Brannstrom and Enskog, 2002).

It is important to consider that some proportion of the impact of QUIN on
ovulation rate may have been due not to its effects on the eicosanoid pathway but rather
its effects on protease activity. A study on human leukocytes showed that QUIN
inhibited matrix-metalloprotease (MMP) production through a PLA₂-independent
mechanism (Stuhlmeier and Pollaschek, 2006). If zebrafish ovulation requires functional
MMPs as it does in some other teleosts (medaka) and mammals, and QUIN is exerting a
similar effect on MMP production here, that might account for a component of the
inhibitory effect of QUIN exposure (Curry and Osteen, 2003; Ogiwara et al., 2005).
There were a number of limitations to this study, in large part challenges inherent to the study of physiological systems. One of the major limitations to work investigating eicosanoids is their transiency – with such a short time in circulation, there is the risk of missing the changes in tissue levels. There is also the classical problem of interpreting the results from inhibition-style experiments. The eicosanoid-synthesizing enzyme inhibitors may cross-react with other enzymes, making it difficult to conclude which pathways are truly critical (Downey et al., 1998; Kurusu et al., 2009). There are also instances, as seen in the eicosanoid network, of different pathways with a common precursor exerting similar downstream effects, such that inhibition of an important pathway would not necessarily correspond to an inhibition of function (McCracken, 2005). In addition, there are the typical limitations of using transcriptomic analyses. Many of the members of the eicosanoid pathway have not yet been annotated in zebrafish, restricting the investigation at the mRNA level. Even for those sequences that are available, gene expression does not tell the whole story, as many molecules are regulated at a post-transcriptional level. To overcome the limitations of each type of analysis, this study combined these techniques to derive more meaningful, robust conclusions. By this method, this study was able to generate a useful foundation for future research into the role and regulation of eicosanoids. However, this study was still limited in the scope of analysis that was possible. Beyond using the mammalian literature to select candidate eicosanoids for investigation, identifying the specific eicosanoids involved from such a large family of compounds is a considerable challenge. Future studies may address this issue by employing broad-scale techniques such as transcriptomics and metabolomics to focus in on the more relevant eicosanoids. Once the
likely candidates have been identified, future work should be directed toward revealing the specific functions and modes of action of eicosanoids in ovulation and spawning. Direct exposures to eicosanoids or injections of eicosanoids would help to clarify their function in reproduction. Studies on sexual behaviours and the role of the olfactory system would be beneficial to explore the putative pheromonal mechanism of eicosanoids. Furthermore, based on substantial evidence from the mammalian literature that suggests eicosanoids regulate the proteases responsible for follicular rupture, it would be worthwhile to investigate a parallel mechanism in teleosts (Tsafiri, 1995).

Collectively, this study showed that 17,20βP regulates ovulation and spawning in female zebrafish through an eicosanoid-mediated pathway. It supported the notion that the nature of the role of the eicosanoid pathway in reproduction varies among teleosts. It provided evidence that COX products play a role in 17,20βP-induced ovulation and spawning, though the significance of this role was undetermined. It also indicated that one or more LOX products are critical to successful ovulation, however the identities of these critical LOX products remain unknown. Further, it suggested that other cPLA₂ metabolites, beyond those derived from the COX and LOX pathways, might be important to ovulation, and some possible candidates have been discussed. This study has provided a foundation upon which future research can build to further characterize the eicosanoid network, uncover the mechanism of action of eicosanoids and ultimately, improve our understanding of the physiology of ovulation and spawning in zebrafish and other teleosts.
CHAPTER 3

General Discussion

There are remarkable similarities between the physiological underpinnings of ovulation in mammals and teleosts. Both involve regulation by a complex system of hormones and local factors, including luteinizing hormone and progestogens (Robker et al., 2000; Richards et al., 2002; Clelland and Peng, 2009). Both require a localized digestion of the follicular layer by specialized proteases to allow the expulsion of the egg into the oviduct (Richards et al., 2002; Lubzens et al., 2010). Both appear to resemble an inflammatory reaction and involve a number of inflammatory products, including eicosanoids (Espey, 1980; Richards et al., 2002; Lubzens et al., 2010). Moreover, in both instances, the role of eicosanoids remains contested (Priddy and Killick, 1993; Lubzens et al., 2010). It seems clear that studies on mammalian reproduction have the potential to inform teleostean studies and vice versa. It is to the advantage of the teleost biologist that models like the zebrafish possess short generation times, are easy to maintain, and provide the opportunity for large scale studies that are considerably more difficult to undertake in mammals. Given that studies in both mammals and teleosts indicate that eicosanoids are critical to successful ovulation, and thus reproduction, investigations into their regulation and function are clearly warranted.

Studies on the role of eicosanoids in female reproduction have produced conflicting results, both in mammals and in teleosts. While the general consensus exists that an eicosanoid or a combination of eicosanoids play a critical role in this event, the nature of that role and the specific eicosanoids involved vary from study to study. Some studies indicate a COX product is at play, some a LOX product, some both (e.g.,
Berndtson et al., 1989; Patino et al., 2003; Downey et al., 1998; Mikuni et al., 1998). Studies vary in their specificity – sometimes the specific eicosanoid metabolites involved are identified; sometimes investigations are limited to the level of the major enzymatic pathway (i.e., COX vs. LOX pathways). In those studies that do make comparisons between different eicosanoid metabolites, even the relevant metabolite varies from species to species, i.e. PGE$_2$ in yellow perch vs. PGF$_{2\alpha}$ in goldfish (Stacey and Goetz, 1982). Some studies suggest the eicosanoid is exerting its role at the maturation stage, some at the ovulation stage, and others at the spawning stage (Kobayashi and Stacey, 1993; Sorbera et al., 2001; Patino et al., 2003). These discrepancies are further clouded by possible non-specific actions of INDO and NDGA (Downey et al., 1998; Kurusu et al., 2009). In addition, in teleosts, studies conducted in vitro make up the majority of the literature on the topic, which cannot adequately represent intact organisms with interacting organ systems. Given that the zebrafish is such a pivotal model organism in modern biology, it seems a prudent place to begin the process of clarifying the role and regulation of the eicosanoid pathway in ovulation and spawning. While the findings from this study may not be universally applicable, it is at least an important addition to a widely employed animal model, and a useful foundation from which to clarify the role of eicosanoids in reproduction and for comparisons with other teleost species and mammals.

This study used a 17,20βP bioassay to investigate the role of eicosanoids in zebrafish ovulation and spawning. Consistent with the original hypotheses, it provided in vivo evidence that 17,20βP regulates ovulation, and that an interaction between the downstream effects of 17,20βP and an unknown male cue regulate spawning. Also consistent with the hypotheses, it indicated that 17,20βP regulates eicosanoid production
in the female ovary. It provided insight into the relative importance of intact \( \text{PLA}_2 \), \( \text{COX} \) and \( \text{LOX} \) pathways to ovulation and spawning \textit{in vivo}. Overall, this study provided support for an indispensible role of eicosanoid(s) in ovulation (and therefore spawning), but also demonstrated that the role of eicosanoids in zebrafish is more complex than previously imagined, and that there are likely species-specific differences in the roles these compounds play and their place in the physiological cascade.

Many \textit{in vitro} studies have indicated that 17,20\( \beta \)P was the locally produced endocrine regulator of ovulation and spawning in teleosts, but few have demonstrated this in the intact organism. This study has not only demonstrated this, but also hinted that the mechanism of 17,20\( \beta \)P action might be more complex than in some other species. While many species are capable of undergoing ovulation \textit{in vitro} when treated with 17,20\( \beta \)P, zebrafish are not. Thus, the findings of this study may imply that the effects of 17,20\( \beta \)P on ovulation are not exclusively mediated at the ovary level, but involve interactions with other organ systems within the intact body. Further, this study provided strong evidence that an interactive effect of 17,20\( \beta \)P and male cue(s) lead to spawning. Given that female zebrafish are capable of retaining eggs in the ovary for periods of time, it seems likely that ovulation might be regulated entirely by internal physiological changes whereas in order to synchronize spawning and maximize fertilization success, release of the eggs is initiated by a male cue.

The regulation of the eicosanoid pathway by 17,20\( \beta \)P has been suggested by a number of \textit{in vitro} studies but it has rarely been explored \textit{in vivo}. This study provides strong evidence that eicosanoids mediate 17,20\( \beta \)P-induced ovulation and spawning in zebrafish. This study has thus revealed some details about the place of eicosanoids in the
physiological network that underlies ovulation and spawning – downstream of 17,20βP activation but upstream of ovulation. There may also be eicosanoids that function downstream of ovulation and upstream of spawning, but those were not revealed by this study. This is important information given that the position of eicosanoid function within the physiological cascade seems to differ from species to species.

This study provided in vivo evidence that an intact PLA₂ pathway is obligatory for 17,20βP-induced ovulation and spawning. This is a valuable addition to the body of evidence concerning this pathway, demonstrating that this pathway is similarly important to the real events as it is under cultured conditions. In contrast with expectations, the products of the PLA₂ pathway that are essential to facilitating ovulation and spawning do not appear to be COX metabolites. This does not negate a role for PGs in these events, but may imply that redundant pathways are in place such that inhibition of the COX pathway is insufficient to inhibit reproduction. Integrating these findings with previous research demonstrating that COX inhibition via INDO led to a decrease in egg production under a chronic exposure design (Lister and Van Der Kraak, 2008) may suggest that the essential actions of COX metabolites are not detectable over an acute period but rather manifest over the long term. Given that COX metabolites may have pro- or anti-inflammatory actions (Gilroy et al., 2004), we speculate that some COX products function to minimize damage to the ovary during ovulation by regulating the distribution of inflammatory factors and the resolution of the inflammatory response, and that without these metabolites the condition of the ovary deteriorates over time such that it is incapable of sustaining egg production. It is conceivable that PGs exert dual functions, with certain pro-inflammatory isoforms produced at the onset of ovulation acting in
concert with other eicosanoids to initiate follicular rupture, and anti-inflammatory isoforms produced at the conclusion of ovulation acting to resolve the inflammatory response. Further characterization of the profile of different PGs over the reproductive period would help to inform these speculations.

We obtained evidence from the eicosanoid biosynthesis inhibitor exposures that the role of the PLA$_2$ pathway in ovulation and spawning might be through the production of LOX products, though specifically which products remains elusive. Transcriptional data did not provide any insight into the identity of the LOX products that could be involved, and ovarian eicosanoid measurements suggested that LTB$_4$ and LTC$_4$ were not likely candidates. In rats, LTB$_4$, LTC$_4$ and PGF2$_\alpha$ have unique profiles (Higuchi et al., 1995), but given the highly condensed timing between maturation, ovulation, and spawning in fish, it seems less likely that distinct, non-overlapping profiles would occur. It is not appropriate to rule them out entirely; if the levels of LTs were very strictly regulated, measurements immediately post-spawning might have missed peaks at ovulation or before. More likely though, another LOX product, possibly from the HETE family, is playing an important function. An in vitro study on yellow perch ovarian follicles indicated that 11-HETE restored ovulation after inhibition with NDGA (Berndtson et al., 1989). Thus, the HETEs are a pertinent direction of exploration for future work.

Interestingly, the difference in magnitude of inhibition by NDGA and QUIN may suggest that an additional PLA$_2$ product, not synthesized through the COX or LOX pathways, is playing an important role in ovulation and spawning. Epoxides or platelet activating factor are proposed as worthwhile candidates.


*Future directions*

The current study expanded on what was previously known in the zebrafish concerning the role of eicosanoids in ovulation and spawning, forming hypotheses about critical members of the family based on the teleostean and mammalian literature. However, while the findings from this study were able to provide a foundation upon which to narrow down the likely candidates, it was not sufficient to identify them. Future research on this subject should endeavour to clarify which specific eicosanoids are playing the important roles. Given the size of the eicosanoid family, broad-scale techniques such as transcriptomics or metabolomics would be beneficial to highlight the likely candidates. Once identified, exposure or injection experiments could be undertaken to determine if these eicosanoids induced ovulation and/or spawning. In addition, co-exposure to candidate eicosanoids and QUIN or NDGA to assess if ovulation rates could be restored would be telling.

A question that this study did not attempt to address, but one that should be explored once the eicosanoid pathway has been adequately described, is that of mechanism. The function of eicosanoids has not been definitively established in teleosts or mammals. However, in mammals it has been proposed that eicosanoids might serve to regulate digestion of the follicular apex via follicular protease activity (Tsafriri, 1995). Recent mammalian models have proposed that leukocytes recruited to the ovary at the time of ovulation synthesize follicular proteases (Oakley et al., 2011). The regulation of follicular protease activity through leukocyte activity may be the function of eicosanoids – to regulate infiltration via regulation of the ovarian vasculature, to restrict proteolysis to the appropriate regions and protect the other portions of the ovary from degradation, and
to eventually repel leukocytes from the ovary and enable healing to commence. This model of ovulation would serve to integrate a large portion of the literature on teleost ovulation. It would accommodate the newer microarray studies that have highlighted cytokines, proteases, and angiogenic factors, along with the traditional literature focusing on the eicosanoids (Bobe et al., 2006; Villeneuve et al., 2010). In support of this theory, Goetz and Gareynszki (1997) detected an abundance of what appeared to be antileukoproteinases (enzymes that protect from nonspecific degradation by proteases from infiltrating leukocytes) during ovulation in brook trout. Recent studies in freshwater medaka have indicated that the follicular protease family matrix-metalloproteases play an essential role in ovulation (Ogiwara et al., 2005). A limitation of this model is that it only addresses the role of eicosanoids in ovulation – in teleosts, they may also play a role in spawning. Studies in goldfish suggested that eicosanoids with pheromonal actions are produced post-ovulation as a result of the distension of the body cavity (Stacey, 1976; Sorensen et al., 1988). It is conceivable that the different eicosanoids play distinct roles then, with specific upstream eicosanoids regulating ovulation, and eicosanoids produced downstream of ovulation functioning as a pheromonal cue to synchronize spawning. It seems plausible to imagine that leukotrienes, with leukocyte chemoattractive properties, would function as the former, and prostaglandins, previously shown to act as pheromones, function as the latter, but it would also be likely that these roles and the particular isoforms involved would vary from species to species. To investigate these speculations, future work should explore the putative link between eicosanoids and follicular proteases in teleosts.
Ultimately, the goal of this field of research is to obtain an integrated understanding of the physiological cascade and the mechanisms that underlie ovulation and spawning, to produce a comprehensive reproductive model. Such a model would be invaluable to making comparisons across teleost species and with other vertebrates, in particular mammals.

**Conclusions**

This study explored the regulation of zebrafish ovulation and spawning by $17,20\beta P$ and the role of eicosanoids in those processes. The findings from this study indicate that $17,20\beta P$ regulates ovulation through internal physiological changes, whereas spawning is controlled by an interaction between downstream effects of $17,20\beta P$ and a male cue. This study showed that $17,20\beta P$-induced ovulation and spawning is mediated by the eicosanoid pathway, in part through production of a LOX product. $17,20\beta P$ also induced the COX pathway, but the significance of this pathway to facilitating ovulation and spawning was not determined. This study has provided the foundation from which to understand the physiological network that underlies ovulation and spawning and the place of the eicosanoids within that network. Studies of this nature are key to providing the framework for future studies into mechanistic questions and ultimately, to providing a comprehensive reproductive model.
REFERENCES


APPENDIX

Mechanisms of ovulation

Ovulation appears to require a complex combination of structural and mechanical changes in the ovary. Rupture of the follicle layer surrounding the oocyte by follicular proteases has been indicated to play a critical role in facilitating ovulation in representatives from all vertebrate classes (Anderson and Yatvin, 1970; Yoshimura and Koga, 1982; Jones et al., 1988; Curry and Osteen, 2003; Lubzens et al., 2010). The family of proteases that has likely received the most attention in recent years are the MMPs. The subgroups within the MMPs have unique substrate specificities; for example, the gelatinases, MMP2 and MMP9, specifically degrade collagen type IV/V, while the collagenases, MMP1, MMP8, and MMP13, degrade type I collagen (Robker et al., 2000). Understanding these specificities provides insight into the role that different MMPs may play in follicular rupture, as distinct regions of the follicle layer are composed of different substrate types. Specifically, the granulosa cell basement membrane is primarily composed of collagen type IV, whereas the theca is primarily composed of collagen type I, implying that enzymes of each subgroup would be required to induce the necessary rupture (Ogiwara et al., 2005). The involvement of MMPs in ovulation is usually assessed through either enzyme activity assays or gene expression analyses. Both of these techniques have supported a role for MMPs in ovulation of rodents, ungulates, apes, and humans (Curry and Osteen, 2003; Peluffo et al., 2011). While most studies investigating the role of MMPs in ovulation have used mammalian models, Ogiwara et al. (2005) also demonstrated an indispensable role of MMPs in ovulation of the freshwater medaka, Oryzias latipes. They showed that incubating
medaka ovarian tissue with inhibitors of MMP function reduced the number of ovulated eggs by as much as 90%. The role of matrix-modifying enzymes in zebrafish ovulation has never been investigated previously, but 25 MMPs and 5 tissue inhibitors of MMPs (TIMPs) have been identified in this species (Wyatt et al., 2009). Of these, MMP2, MMP9, MMP13, MT1-MMP and TIMP2 are known to be expressed during zebrafish embryonic development (Zhang et al., 2003a; 2003b; 2003c; Hillegass et al., 2007; Hillegass et al., 2008). Although most studies in mammals have suggested functional redundancies exist among the MMPs and this may account for the absence of ovulation-related defects in MMP knockouts (Peluffo et al., 2011), zebrafish possess only one collagenase paralogue (MMP13) (Wyatt et al., 2009), which may make it a useful model to clearly elucidate the role of these enzymes.

In addition to the well-established dogma on localized follicular digestion by proteases, other putative mechanisms of ovulation have been proposed including an aquaporin-mediated increase in intra-oocytic pressure. During oocyte maturation in marine teleosts the hydrolysis of vitellogenin into yolk proteins and the accumulation of inorganic ions create an osmotic gradient that draws water into the oocyte (Clelland and Peng 2009). While originally this movement of water was believed to occur via passive diffusion, Fabra et al. (2005) showed that it was mediated by aquaporins. Although the purpose of oocyte hydration in marine teleosts has been suggested to be increased buoyancy and protection against the hyperosmotic ocean environment, some researchers have proposed that it may provide an intra-oocytic pressure that aids in ovulation (Lubzens et al., 2010; Skoblina, 2010). Microarray studies in rainbow trout (Bobe et al., 2006) and the fathead minnow (Villeneuve et al., 2010) showed an increase in aquaporin
4 mRNA during ovulation and aquaporin 8 mRNA during oocyte maturation, respectively. Research on the role of aquaporins in ovulation is not restricted to teleosts; studies have started to include mammalian species (e.g., Sun et al., 2009). Recently, Thoroddsen et al. (2011) demonstrated that aquaporin 2 and aquaporin 3 mRNA expression in the human ovary increase during the early ovulatory period, and may thus play a role in human ovulation. However, research into aquaporin-mediated oocyte hydration as a mechanism of ovulation is still very much in its infancy.

Another putative mechanism of ovulation that has been the subject of much controversy involves the contraction of smooth muscle-like cells located in the follicle layers (Choi et al., 2011). The presence and function of such cells has been speculated for decades, but research efforts have been largely limited to a select few mammalian species and, in the absence of a contractile trigger, both the existence and the relative importance of this mechanism are still debated (Schroeder and Talbot, 1985; Choi et al., 2011). Jalabert and Szollosi (1975) first cited the existence of smooth muscle-like cells in the ovary of a teleost species, the rainbow trout, from their own unpublished findings. They also implicated prostaglandins as the “trigger” for ovulatory smooth muscle contraction, but while research into the role of prostaglandins in ovulation has proliferated, this function remains to be confirmed. Van Nassauw et al. (1991) investigated the presence of smooth muscle cells in the ovary of three ectothermic vertebrates; the zebrafish, the axolotl and the red-eared turtle, using immunohistochemistry to test for reactivity with an antibody for the mammalian smooth muscle marker, α-smooth muscle actin (α-SMA). However, the merit of their results was called into question by Georgijevic et al. (2007) when the latter group determined that the
mammalian antibody for α-SMA uses an epitope not found in the zebrafish α-SMA sequence. Recently, Ren et al. (2009) showed that smooth muscle marker genes were upregulated in the pre-ovulatory mouse ovary, and Choi et al. (2011) demonstrated the presence of smooth muscle cells within the theca externa of human ovarian follicles. Ultimately, however, other than these and a few frequently cited studies in rodents (e.g., Martin and Talbot, 1981), the role of contractile smooth muscle cells within the follicle layer in facilitating ovulation remains to be firmly established.

This study endeavoured to collect some preliminary data on the transcription of candidate proteases, aquaporins, and smooth muscle cell markers during the peri-ovulatory period (Table 1). Specifically, qPCR was conducted for the matrix-metalloproteases 2, 9, and 13, for aquaporins 1b, 4 and 8aa, and for the smooth muscle cell markers α-SMA (acta2) and transgelin 1. Peri-ovulatory ovary samples were acquired from solitary females (n=10 fish) exposed to either 17,20βP (10 nM) or a solvent control (EtOH, <0.001%) for 2 hours. RNA extraction, quantification, reverse transcription and qPCR were conducted as described above in the Methods subsection: “Sample Analyses”. Primer sequences for these genes are described below (Table 2). Expression of all 8 genes was readily detectable in the ovary. There were no significant differences in expression between 17,20βP-exposed and control fish for any of the genes measured. These findings do not necessarily exclude the possibility of a reproduction-related function for the protein products of these genes, as their actions may be regulated post-transcriptionally, or alternatively they may be upregulated at a different time than that represented by these samples. Further, biological variability may have masked trends that would become apparent with a larger sample size or additional experimental
replication. Although these results are not conclusive, it is hoped that they may provide a starting point for future research investigating the mechanisms of 17,20βP-mediated ovulation.
Table 1. Summary of changes in mRNA expression for 8 genes with putative ovulatory functions in zebrafish ovarian tissue after 2 hours exposure to 17,20βP (10 nM) *in vivo* relative to control fish exposed to 0.0001% EtOH, n=10 fish per treatment.

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>mRNA expression relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmp2</td>
<td>no difference</td>
</tr>
<tr>
<td>mmp9</td>
<td>no difference</td>
</tr>
<tr>
<td>mmp13</td>
<td>no difference</td>
</tr>
<tr>
<td>aqp1b</td>
<td>no difference</td>
</tr>
<tr>
<td>aqp4</td>
<td>no difference</td>
</tr>
<tr>
<td>aqp8aa</td>
<td>no difference</td>
</tr>
<tr>
<td>acta2</td>
<td>no difference</td>
</tr>
<tr>
<td>tgl1n1</td>
<td>no difference</td>
</tr>
</tbody>
</table>
**Table 2.** Forward and reverse qPCR primer pair sequences for putative ovulatory genes and the reference genes in the zebrafish (*Danio rerio*), and associated accession numbers from GenBank

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>bactin</td>
<td>F-ACAGGGAAAAAGATGACACAGATCA&lt;br&gt;R-CAGCCTGGATGGCAACGTA</td>
<td>AF025305</td>
</tr>
<tr>
<td>mmp2</td>
<td>F-TTCTTGCTTCCCTGCAAACCT&lt;br&gt;R-TGCATTTTCTTTCAGCGTGTC</td>
<td>NM_198067</td>
</tr>
<tr>
<td>mmp9</td>
<td>F-GCTGTCTCATGAGTTTTGGAACA&lt;br&gt;R-AGGCCAGTCTTAGGTCAT</td>
<td>NM_213123</td>
</tr>
<tr>
<td>mmp13</td>
<td>F-ATGGTGCAAGGGCTATCCCCAAGAGT&lt;br&gt;R-GCCTGTTGGTTGAGCCAAAACATCAA</td>
<td>AF506756</td>
</tr>
<tr>
<td>aqp1b</td>
<td>F-CTGGTTCTCTGCGCTTTTGG&lt;br&gt;R-GGAGCCGGAAACATCAAGTTC</td>
<td>EU327345</td>
</tr>
<tr>
<td>aqp4</td>
<td>F-ACCTGTGACCCCCAAGCGTAA&lt;br&gt;R-CGATGCAACACAGACAGACAA</td>
<td>NM_001003749</td>
</tr>
<tr>
<td>aqp8aa</td>
<td>F-TGGAGGAAATGGAGGTATCCCT&lt;br&gt;R-GGCAGCAATCACTCCACCTAA</td>
<td>NM_001004661</td>
</tr>
<tr>
<td>acta2</td>
<td>F-ACAACATCTGCATGTCGGCTTTT&lt;br&gt;R-TGCTGAGCTTTATTCCCTATATGA</td>
<td>NM_212620</td>
</tr>
<tr>
<td>tgl1</td>
<td>F-GCATCGCACTCAACAAAGAG&lt;br&gt;R-GTCTCCTCTGCCTTTCTGAAG</td>
<td>NM_001045467</td>
</tr>
</tbody>
</table>