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Investigating the role of prostaglandin receptor isoform EP4b in zebrafish ovulation

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ABSTRACT

Prostaglandins (PGs) are a class of fatty acid-derived hormones that play an essential role in the regulation of ovulation of teleosts. This study investigated the various isoforms of ovarian PG receptors in the zebrafish ovary and their role in ovulation. Using real time qPCR, six PG receptor isoforms (*ptger1a, ptger1b, ptger2a, ptger4a, ptger4b,* and *ptgfr*) were shown to be expressed in the ovary. Only the PG receptor isoform *ptger4b* was upregulated at the time of ovulation *in vivo*, or following treatment *in vivo* with Ovaprim, which contains a gonadotropin releasing hormone analogue and a dopamine receptor antagonist and stimulates ovulation. Treatment of full-grown follicles with the maturation-inducing hormone 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) *in vitro* also induced expression of EP4b mRNA. Females ovulate *in vivo* after injection with Ovaprim, or injection with Ovaprim and inhibitors of EP1 (ONO-8130) or EP2 (TG4-155) function; they do not ovulate when injected with Ovaprim and an EP4 inhibitor (GW237368x). These findings suggest that the EP4 receptor, in particular the EP4b isoform, is essential for ovulation.

1. Introduction

Ovulation is the process by which mature oocytes exit their ovarian follicles in preparation for fertilization. In fishes, the process of ovulation is under the control of the ovulatory surge of gonadotropins. Numerous studies, including hormone replacement, gene knockout, and inhibition of downstream signalling have established that ovulation is mediated by the essential actions of luteinizing hormone (LH) (Crespo et al., 2013; Ogiwara et al., 2013; Tang et al., 2016; Zhang et al., 2015). Knockout of both zebrafish gonadotropin releasing hormone isoforms does not inhibit ovulation; however knockout of nuclear progesterone receptor, a mediator of luteinizing hormone activity, leads to anovulation (Marvel et al., 2018; Tang et al., 2016). Additionally, spawning is inhibited in LH knockout zebrafish; however females with knockout of the follicle stimulating hormone (FSH) gene are still fertile and able to reproduce (Zhang et al., 2015).

LH recruits a number of downstream hormones, receptors, and enzymes that mediate the ovulatory process (Goetz and Garczynski, 1997; Patiño et al., 2003; Takahashi et al., 2013; Zhang et al., 2015). The molecular species involved in ovulation in fishes include progestins such as 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) (Knight and Van Der Kraak, 2015; Tokumoto et al., 2011) progestin receptors (Hanna and Zhu, 2011; Tang et al., 2016; Zhu et al., 2015), immune regulators (Berishvili et al., 2006; Crespo et al., 2010), matrix metalloproteases and their inhibitors (Ogiwara et al., 2005; Russell et al., 2015; Shozu et al., 2005), and prostaglandins (Kim et al., 2014; Lister and Van Der Kraak, 2008).

Prostaglandins (PGs) are a class of fatty acid derivatives whose proper function is essential for ovulation (Goetz et al., 1991). PGs are also involved in a variety of additional physiological processes in vertebrates, including inflammation, immune system regulation, and smooth muscle contraction (Simmons et al., 2004). The role of PGs, particularly PGE_2, in ovulation have been well characterized (Goetz and Garczynski, 1997; Murakami and Kudo, 2004; Takahashi et al., 2013). Studies in mice have shown that knockout or inhibition of cyclooxygenase (COX) enzymes, which are essential in the production of PGs, leads to infertility in females by blocking ovulation (Lim et al., 1997). Additionally, inhibition of COX enzyme function leads to inhibition of ovulation in zebrafish, an effect which is ameliorated with subsequent addition of PGE_2 (Fujimori et al., 2011; Lister and Van Der Kraak, 2008). LH signaling has also been shown to upregulate expression of *ptgs2a* mRNA, one of the COX isoforms responsible for PG synthesis (Tang et al., 2017). PGs have also been shown to be involved in the ovulation of fishes, as seen in studies in the yellow perch (Goetz et al., 1989), brook trout (Goetz et al., 1982), medaka (Fujimori et al., 2012), and goldfish (Stacey and Pandey, 1975). While it is well established that PGs are essential for ovulation in vertebrates, the nature of the role played by PGs in this process is less well understood, such as which of

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the various isoforms of PG receptors play a role in mediating ovulation. Teleost fish have several isoforms of prostaglandin receptors – EP1a, EP1b, EP1c, EP2a, EP4a. EP4b and FP all of which are present in the ovary of zebrafish (Kwok et al., 2012; Villablanca et al., 2007). In mammals, there have been several studies investigating the roles of the various PG receptors with EP1, EP2, and EP4 all being implicated as being involved in regulating the process of ovulation (Harris et al., 2011; Kim et al., 2014; Peluffo et al., 2014; Sugimoto et al., 2015); however there has been comparatively less investigation into the roles of the PG receptor isoforms in fish. In medaka, expression of Ptger4 mRNA was upregulated by recombinant luteinizing hormone via a pathway involving the nuclear progesterone receptor (Hagiwara et al., 2014; Tang et al., 2016). Additionally, impairment of the EP4b receptor by a competitive antagonist *in vitro* led to anovulation in medaka follicles (Fujimori et al., 2012).

This study sought to determine whether any of the EP and FP series receptors were dynamically regulated in zebrafish and changed in expression during the ovulatory period. Additionally, this study examined if any of the EP series receptors are under the hormonal control of gonadotropins. Finally, we investigated whether inhibition of any of the EP1, EP2, or EP4 series receptors would block ovulation *in vivo*.

2. Materials and methods

2.1. Animals

Sexually mature zebrafish were purchased from AQUAlity Tropical Fish Wholesale (Mississauga, ON) and held in an environmentally controlled chamber with a photoperiod of 12 h light:12 h dark in the Hagen Aqualab (University of Guelph, Guelph, ON), with lights turned on at 7:00 am and turned off at 7:00 pm daily. Fish were held in aquatic habitat units (Aquatic Ecosystems, Apopka, FL) with recirculating Guelph well water held at 28 °C. Fish were fed either brine shrimp (AQUAlity) or salmon fry pellets (Martin Mills, Elmira, ON) twice daily. All experiments were performed using protocols approved by the University of Guelph Animal Care Committee.

2.2. Tissue extraction and in vitro assays

Gravid female zebrafish were sacrificed for tissue collection by anaesthetization with an overdose of MS-222 (tricaine methanosulfonate; 1 g/L buffered by sodium bicarbonate) followed by transcervical severance. Immediately after sacrificing the fish, whole ovaries were removed and placed into a petri dish containing 60% L-15 media (Invitrogen, Carlsbad, CA) containing 100 IU/ml penicillin G and 100 μg/ml streptomycin sulphate (Invitrogen). Using fine forceps and the aid of a light dissecting microscope, intact full-grown follicles (those which had fully undergone vitellogenesis but had not yet undergone germinal vesicle breakdown) were separated from the whole ovary and placed into 24-well plates before treatment with the appropriate test solution. Plates were then wrapped in foil and placed in an incubator at 28 °C for 1–4 h, at which time the follicles were collected and stored at −80 °C until analysis.

2.3. RNA extraction and quantitative polymerase chain reaction

RNA was extracted from either the whole ovary or isolated follicles and then treated with DNase as described in Knight and Van Der Kraak (2015). RNA was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and diluted to 300 ng/μl using DNase/RNase free water (Invitrogen, Carlsbad, CA). Reverse transcription and qPCR was then performed as described previously (Lister and Van Der Kraak, 2008). Primer sequences are shown in Table 1. A six-point standard curve for each gene was run with a pooled sample containing all treatment groups. Gene expression values were normalized to expression levels of the reference genes β-actin and ef1α using the reference residual normalization method developed by Edmunds et al. (2014). Expression of the reference genes did not change significantly across treatments. Coefficients of variation of the normalized relative quantities of all reference genes were below 25% for all experiments, indicating that expression of these genes was stable and thus the genes were appropriate for use as reference genes (Hellemans et al., 2007).

2.4. Temporal regulation of EP and FP receptors

Fish were held in mixed sex tanks containing 6 female and 3 male fish for two days prior to sampling. Fish were then sacrificed as described above, with groups sampled at 10:00 pm, 1:00 am, 4:00 am, 7:00 am, and 10:00 am. Whole ovary samples were collected at the time of sacrifice and mRNA expression was analyzed (N = 12).

2.5. Hormonal regulation of EP receptors

Zebrafish were held in beakers containing 2 females and 1 male for 48 h prior days prior to experiments and males were removed 24 h before experiments. Water was changed daily. Female fish were injected with either 20% DMSO in water or a 20% Ovaprim solution (sGnRH analogue and dopamine antagonist; Syndel, Nanaimo, BC). Female fish that had ovulated on the final morning were removed from the experiment and not injected. Female fish were injected with 10 μl of the appropriate test solution using a syringe with a 30-gauge Ultra-Fine needle (Becton Dickinson, Franklin Lakes, NJ) before being returned to their beakers. Whole ovary tissues were taken 1 or 3 h post injection and were either analyzed as whole ovaries or had full-grown follicles isolated for analysis. Gene expression was quantified from these tissues as described above.

In a separate experiment, full-grown follicles were collected from five female fish and pooled before being treated *in vitro* with 5 nM 17,20β-P in 60% L-15 (Wisent, St-Bruno, QC) media for 1 h in darkness at 28 °C. Follicles were treated in a 24 well flat bottom tissue culture plate (Becton Dickinson) with 20 follicles per well. Each plate contained 4 wells each of 17,20β-P treatment or control solution, and the incubations were repeated 3 times using follicles pooled from new females each time. Gene expression was then quantified by the procedure described above.

2.6. In vivo inhibition of EP receptors

Two adult female zebrafish were put into tanks containing a single male fish for 24 h. The male fish was then removed, and females remained in the tanks for an additional 24 h before the experiment. Female fish were injected with 10 μl of a sham injection (20% DMSO in water), 20% Ovaprim in water, or an EP4 receptor inhibitor (GW627368x; Cedarlane, Burlington, ON) at concentrations of 5 μM, 10 μM, or 20 μM (N = 16 per treatment). Injections were performed as above. After injection, females were returned to their tanks for 8 h before ovulation was assessed by applying light pressure to the abdomen of the fish and visually assessing the presence or absence of eggs, with the presence of eggs indicating that the fish had ovulated. When female fish are held in absence of male fish, they will ovulate but not spawn. This procedure was repeated, injecting female fish with 10 μl of sham injection, 20% Ovaprim in water, an EP1 or EP2 receptor inhibitor alone at a concentration of 20 μM (ONO-8130 for EP1, TG4-155 for EP2, or GW627368x for EP4; Cedarlane, Burlington, ON) or a combination of 20% Ovaprim and 20 μM inhibitor (N = 16 per treatment).

2.7. Statistical analyses

Gene expression data were analyzed by one-way ANOVA. When necessary, data were log or square root transformed in order to satisfy

Table 1
Primer sequences used for qPCR.

Primer Name	Gene	Sequence	Annealing Temperature (°C)	Accession No./Reference
Ef1α	ef1a	GAT CAC TGG TAC TTC TCA GGC TG GGT GAA AGC CAG GAG GGC	60	NM_131263.1 (Nelson et al., 2010)
B-actin	actb	ACA GGG AAA AGA TGA CAC AGA TCA CAG CCT GGA TGG CAA CGT A	59	NM_181601.4 (Lister et al., 2009)
EP1a	ptger1a	AAA TGT CAC CTC GAG CAG AC ACA GGA GAA AGG CCT TGG AT	63.2	NM_001166330.1
EP1b	Ptger1b	AGC ACG GTT TTA GCT GAG GA CAG CGC TAT GAT GTT GGA GA	63.2	NM_001166333.1
EP1c	Ptger1c	GAC ACG GAT CTT TCT CGT C CCA GCT TGC CAA ACG CAG	63.2	NM_001166291.1
EP2a	Ptger2	TAA CGG GAA CCT GTC TGA CC GTG ATA CGG ATA CCC GAT GG	63.2	NM_200635.1
EP4a	Ptger4a	TGC TCA ATC CCG CTT GTT GTC C CGA AGC GGA TGG CCA GAA GAT	64.4	NM_001039629.1
EP4b	*Ptger4b*	ATC GTT CTC ATA GCC ACG TCC ACT CCG GGT TTG GTC TTG CTG ATG AAT	64.4	NM_001128367.1
FP	ptgfr	CTG TCA GCT TTT GGC AAT CA AAC AGC CTT GCG TAG AAG GA	61	NM_001185071.1

the requirements of Levine's homogeneity of variance test. Tukey's Honestly Significant Difference test was used to assess significant differences between groups. Ovulation rate data were analyzed by Fisher's Exact Test. All statistical analyses were performed using SPSS software or Rstudio (Boston, MA) and $p < 0.05$ was considered to be significant.

3. Results

3.1. Regulation of EP and FP receptors

3.1.1. Temporal

Initial studies examined if prostaglandin receptors were dynamically expressed across the ovulatory period. Whole ovaries of females were isolated at 10:00 pm, 1:00 am, 4:00 am, 7:00 am, and 10:00 am. The gonadosomatic index of the females used in this experiment was 7.28 ± 0.29 (N = 55). No females had ovulated at 1:00 am, 4:00 am, or 10:00 am. All females (N = 11) had ovulated at 7:00 am, while 1 out of 11 females had ovulated at 10:00 pm. Using qPCR, relative expression levels of *ptger1a, ptger1b, ptger2a, ptger4a, ptger4b,* and *ptgfr* were determined. The mRNA transcripts of all six receptor subtypes were detected in ovarian tissue; *ptger1c* was not detected. *ptger1a* had the highest relative expression of the EP receptor genes at 10:00 pm, 1:00 am, and 4:00 am, with *ptger4b* showing the lowest expression at these time points as well. The other EP genes had similar relative expression levels to each other at these time points. Relative expressions of the EP receptor genes are shown in Fig. 1. At 7:00 am, *ptger2a* had the highest relative expression of the EP genes and *ptgfr* had the lowest relative expression, with other genes showing similar relative expressions of moderate levels. At 10:00 am, *ptgfr* had the highest relative expression and *ptger2a* had the lowest relative expression. Of the EP receptor genes, only *ptger4b* expression showed significant changes over the ovulatory period ($p = 0.003$), with expression elevated at 7:00 am and 10:00 am compared to 10:00 pm, 1:00 am, and 4:00 am. The level of expression at 7:00 am was approximately 4 times higher than at 10:00 pm, 1:00 am, and 4:00 am, and 5 times higher at 10:00 am (Fig. 2). There were no significant differences in levels of ovarian mRNA expression for of *ptger1a, ptger1b, ptger2a, ptger4a*, or *ptgfr* over the ovulatory period, though *ptger2a* showed a general increase to 7:00 am, while *ptgfr* showed a general increase to 10:00 am (data not shown).

3.1.2. Ovaprim treatment

To investigate if hormonal regulation leads to dynamic expression of prostaglandin receptors, adult female fish were injected with either Ovaprim or water and relative mRNA expression was quantified using qPCR in both whole ovary samples and in isolated full-grown follicles. No females were observed to have spawned on the day of injections, and any females which had ovulated were removed from the experiment. There were no significant differences in mRNA expression for *ptger1a, ptger1b, ptger1c, ptger2a, ptger4a*, or *ptgfr* either 1 or 3 h post injection in full-grown follicles or in whole ovary tissues (Data not shown). In full-grown follicles, *ptger4b* expression was significantly increased after both 1 and 3 h (approximately 3.2-fold and 3.8-fold, respectively; $p = 0.033$ and $p = 0.014$). Whole ovary *ptger4b* mRNA expression was also increased after 1 (3.7-fold; $p = 0.066$) and 3 h (2.8-fold; $p = 0.005$), respectively (Fig. 3).

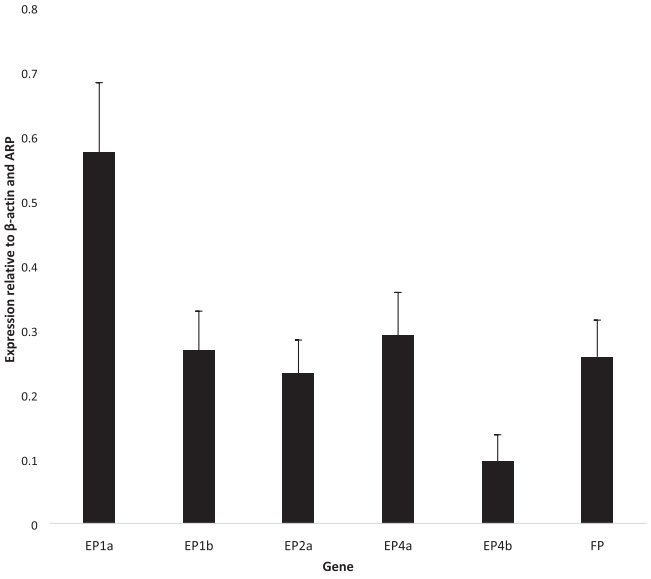

Fig. 1. mRNA expression levels of seven prostaglandin receptor genes in zebrafish ovaries isolated at 10:00 pm. Expression levels are normalized to β-actin and ARP. Data represent means ± SEM. No significant differences were detected.

3.1.3. 17,20β-P treatment

In another experiment examining the hormonal control of the EP4b receptor, full-grown follicles were isolated from adult female fish and treated with 17,20β-P. Treatment with 17,20β-P led to significant up-regulation of *ptger4b* mRNA expression compared to controls (approximately 2.5-fold; $p = 0.017$; Fig. 4).

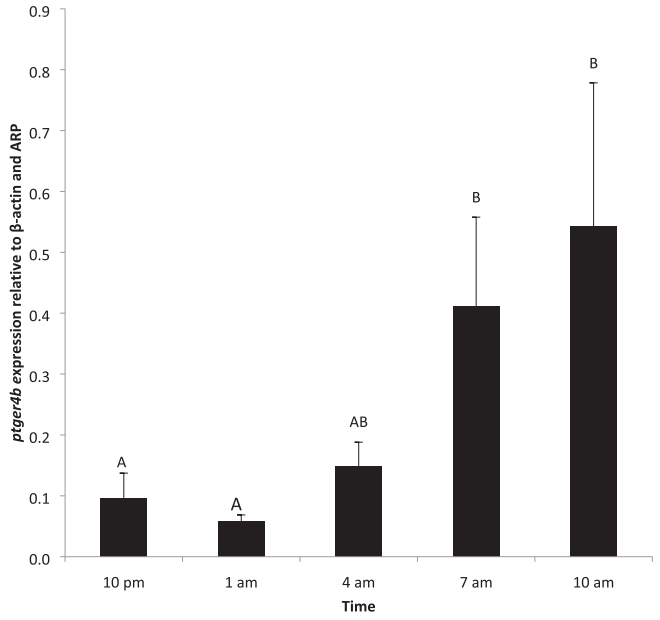

Fig. 2. *ptger4b* mRNA expression at various timepoints over the ovulatory period in zebrafish ovaries. Expression levels are normalized to β-actin and ARP. Data represent means ± SEM. Groups which do not share a letter are significantly different ($p < 0.05$; ANOVA, Tukey's Test).

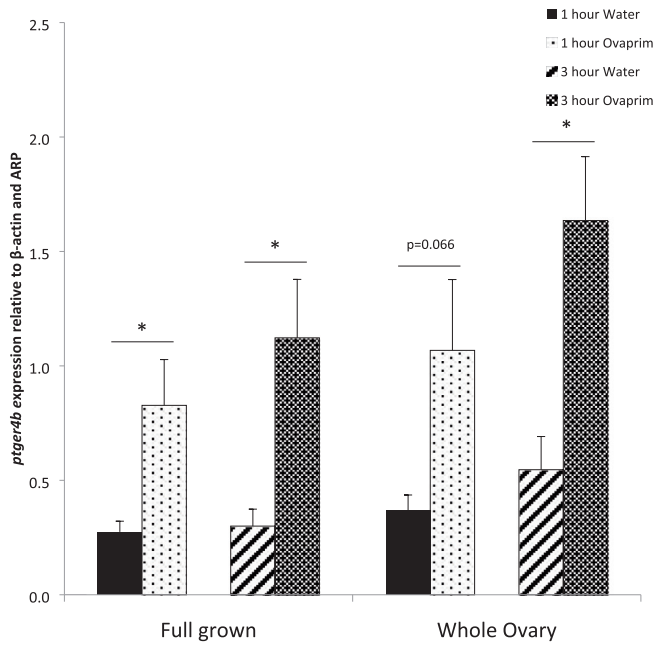

Fig. 3. *ptger4b* mRNA expression in zebrafish full-grown follicles or whole ovary after injection of either water or a solution of 20% Ovaprim in water. Samples were either taken 1 or 3 h post injection. Expression levels are normalized to β-actin and ARP. Data represent means ± SEM. Asterisks indicate a significant difference between Ovaprim and water injections in the same tissue type at the same time point ($p < 0.05$; ANOVA).

3.2. Function of EP receptors

3.2.1. EP1, EP2, EP4 inhibitors

To investigate how function of EP receptors can impact ovulation, adult female fish were injected with either Ovaprim, Ovaprim and an EP1 inhibitor, Ovaprim and an EP2 inhibitor, Ovaprim and an EP4 inhibitor, or water. Ovulation was significantly induced in females injected with a solution of 20% Ovaprim (87% ovulation compared with

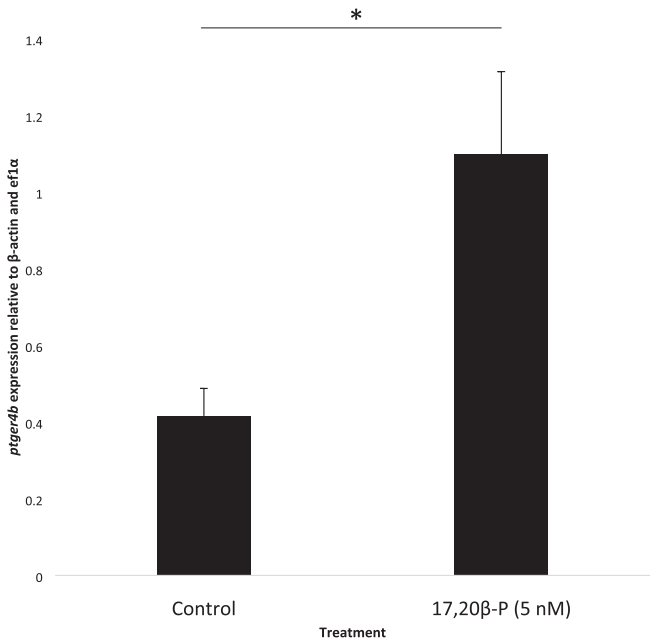

Fig. 4. *ptger4b* mRNA expression in zebrafish full-grown follicles treated with 5 nM 17,20β-P for 1 h in darkness at 28 °C. Expression levels are normalized to β-actin and ef1α. Data represent means ± SEM. An asterisk indicates that groups are significantly different ($p < 0.05$; ANOVA).

0% in sham injected females and females injected with EP4 antagonist alone; $p < 0.001$). The proportion of females that ovulated following treatment with Ovaprim and the EP4 antagonist was reduced as the concentration of EP4 inhibitor was increased, with 60% of females injected with 20% Ovaprim and 5 μM EP4 antagonist ovulating, 50% of females injected with 20% Ovaprim and 10 μM EP4 antagonist ovulating, and 20% of females injected with 20% Ovaprim and 20 μM EP4 antagonist ovulating. The highest concentration of EP4 antagonist used (20 μM) led to a significant reduction in ovulation compared to Ovaprim alone ($p < 0.001$) (Fig. 5). Females injected with either EP1 antagonist or EP2 antagonist alone did not ovulate. In this experiment, 75% of females injected with 20% Ovaprim ovulated. Females treated

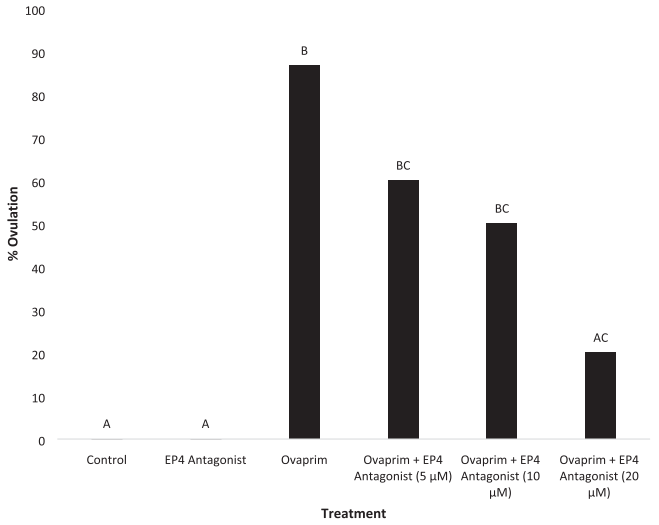

Fig. 5. *In vivo* ovulation rates of female zebrafish injected with EP4 antagonist (5 μM), 20% Ovaprim solution in water, or a combination of Ovaprim and EP4 antagonist (GW627368x) at various doses. Data represent percentage of females that ovulated 8 h post injection. Groups which do not share a letter are significantly different ($p < 0.05$, Fisher's Exact Test).

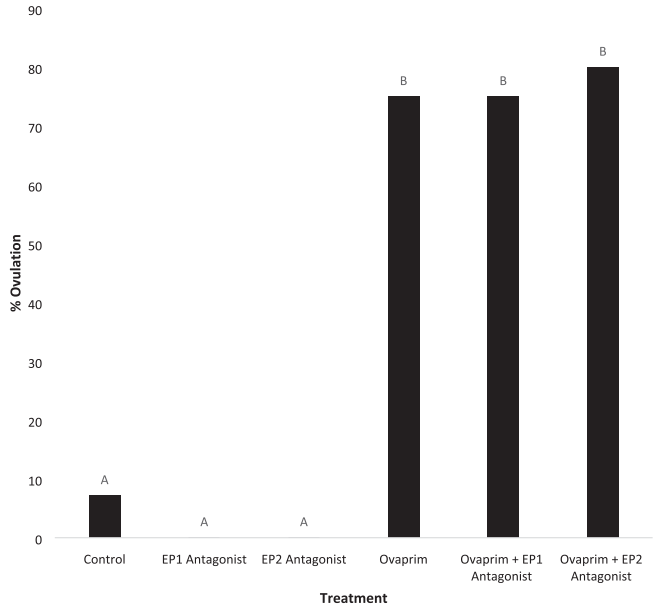

Fig. 6. *In vivo* ovulation rates of female zebrafish injected with 20 μM EP1 antagonist (ONO-8130), 20 μM EP2 antagonist (TG4-155), 20% Ovaprim solution in water, both Ovaprim and EP1 antagonist, or both Ovaprim and EP2 antagonist. Data represent percentage of females that ovulated 8 h post injection. Groups which do not share a letter are significantly different ($p < 0.05$, Fisher's Exact Test).

with either 20% Ovaprim and 20 μM EP1 antagonist or 20% Ovaprim and 20 μM EP2 antagonist had ovulation rates of 75% and 80% respectively, which was not significantly different from the ovulation rates of females injected with Ovaprim alone (Fig. 6).

4. Discussion

The purpose of this study was to investigate the role of prostaglandin receptors in the process of ovulation in zebrafish using both *in vivo* and *in vitro* methods. Prostaglandins and their receptors have been demonstrated to be essential to properly functioning ovulation in teleosts (Goetz et al., 1989; Goetz and Garczynski, 1997; Lister and Van Der Kraak, 2008; Patiño et al., 2003). In particular, prostaglandin E2 and its EP4b receptor have been highly implicated as playing a crucial role in the regulation of ovulation in medaka (Fujimori et al., 2012, 2011).

After sampling ovarian tissue at a variety of time points from female zebrafish over the ovulatory period, mRNA for the six receptor subtypes of interest (EP1a, EP1b, EP2a, EP4a, EP4b, and FP) were identified. All receptor subtypes were expressed at relatively similar levels, ranging from about 0.1–0.6x the expression levels of the reference genes β-actin and ARP. No comparisons were made between the expression levels of the EP receptor genes as absolute expression levels were not measured; expression was quantified relative to reference genes expressed at stable levels between treatments. It was found that EP4b mRNA expression increases over the time period leading up to ovulation; none of the other prostaglandin receptors examined (EP1a, EP1b, EP2a, EP4a, FP) significantly changed in expression over the ovulatory period. This pattern was mirrored *in vivo* when female zebrafish were injected with Ovaprim, a potent stimulator of ovulation comprised of a GnRH analogue and dopamine antagonist that functions by promoting the release of luteinizing hormone from the pituitary. The increase in *ptger4b* transcripts at the time of ovulation suggests the *ptger4b* gene is upregulated at time of ovulation. The changes in *ptger4b* mRNA expression in the zebrafish parallel the work by Fujimori et al., (2012), who reported that *ptger4b* mRNA was significantly upregulated in the time leading up to ovulation in the medaka, both *in vivo,* and *in vitro* in response to pregnant mare serum gonadotropin. It is likely that the changes in *ptger4b* mRNA expression correspond with an increase in EP4b receptor function and the fact that expression peaks at the time of ovulation indicates that this receptor likely plays an important role in the process of ovulation. Ovulation is a tightly controlled process and proper timing of the events leading up to ovulation is essential to maintain proper reproductive viability. Dynamic regulation of receptors via hormonal signalling is one method by which this can be achieved.

The production of 17,20β-P, the maturation inducing steroid in zebrafish, is stimulated by release of luteinizing hormone from the pituitary (Clelland and Peng, 2009; Nagahama and Yamashita, 2008). To more specifically investigate which hormones regulate expression of prostaglandin receptors, full-grown follicles were treated *in vitro* with 17,20β-P, which was found to increase EP4b mRNA expression *in vitro* in full-grown follicles in a manner similar to what was seen with Ovaprim, though the fold-increase in gene expression was slightly smaller (about 3.5-fold for *in vivo* Ovaprim injections vs 2.5-fold for full-grown follicles treated *in vitro* with 17,20β-P). In the zebrafish, there are two follicular receptors for 17,20β-P: a membrane bound receptor (mpr) and a nuclear receptor (npr) (Hagiwara et al., 2014; Hanna and Zhu, 2011; Tang et al., 2016; Tokumoto et al., 2011). Expression of npr increases over the follicular growth period, peaking just prior to ovulation (Hanna and Zhu, 2011; Tang et al., 2016). 17,20β-P induces maturation via the membrane progestin receptor (Tokumoto et al., 2011), but can also induce ovulation in solitary females (Knight and Van Der Kraak, 2015), likely via the actions of the nuclear receptor, which is directly associated with the promoter region of the *ptger4b* gene (Hagiwara et al., 2014). The timing of the events leading to ovulation are under hormonal control, as the LH surge leads to an increase in both 17,20β-P and npr expression, the latter of which primes the follicles to be responsive to 17,20β-P in zebrafish (Hagiwara et al., 2014; Pang and Ge, 2002). In the *in vitro* treatment of full-grown follicles with 17,20β-P, there was no LH surge to cause upregulation of the nuclear 17,20β-P receptor. The lack of upregulation of the nuclear receptor in the *in vitro* experiment is likely why the fold-increase in *ptger4b* transcripts is not as large as the *in vivo* Ovaprim injections. The two actions of 17,20β-P (inducing both maturation and ovulation) are likely controlled by the membrane-bound and nuclear receptors respectively. Maturation occurs rapidly in response to 17,20β-P, likely via a non-genomic mechanism, while the ovulatory effects caused by 17,20β-P occur after a time delay, likely due to the need to synthesize new protein (Zhu et al., 2015). Hagiwara et al. (2014) found that recombinant LH increased *ptger4b* mRNA expression, likely through a pathway involving 17,20β-P, as RU486, an antagonist of npr, suppressed the increase in *ptger4b* previously seen when follicles were treated with 17,20β-P. Additionally, knockout of nuclear progestin receptor leads to follicles that mature but do not ovulate in response to 17,20β-P, indicating that a genomic response to 17,20β-P is necessary for follicles to properly ovulate after maturation (Tang et al., 2016; Zhu et al., 2015). The increase in *ptger4b* expression seen in the present study is likely one of these essential genomic responses required for ovulation in zebrafish.

The role of the EP4 receptor in ovulation was further demonstrated *in vivo* when females were injected with Ovaprim in conjunction with inhibitors of EP1, EP2, or EP4 receptors. Females injected with either EP1 inhibitor (ONO-8130) or EP2 inhibitor (TG4-155) and Ovaprim were able to ovulate at a rate similar to that of females injected with Ovaprim alone. In the EP4 antagonist experiment, the ovulation rate of controls was 0% and the ovulation rate of females injected with Ovaprim was 86.7%. In the EP1/EP2 antagonist experiment, the ovulation rate of controls was 7% and the ovulation rate of Ovaprim-injected females was 75%. Conversely, females injected with Ovaprim and an antagonist of EP4 function showed diminishing levels of ovulation as the concentration of the antagonist was increased. While this does suggest that the EP4 receptor is essential to ovulation, it does not rule out the possibility that the other prostaglandin receptors are also involved as follicle viability after females were injected with EP1 or EP2

inhibitors in combination with Ovaprim was not assessed.

There is limited knowledge regarding how EP antagonists affect ovulation in zebrafish, especially *in vivo*. Previously, Fujimori et al. (2012) found that treatment of full-grown follicles *in vitro* with GW627368x one hour before the time of ovulation completely inhibited both ovulation and the increase in intracellular cAMP associated with stimulation of EP4 receptors by PGE_2 and Tang et al. (2016) found that blockage of *ptger4b* could block ovulation *in vitro;* however to our knowledge, no other researchers have examined the effects of inhibitors of other EP receptors, either *in vitro* or *in vivo*. Regardless, our results demonstrate that proper EP4 receptor function is essential to facilitate proper ovulation *in vivo*, however inhibition of EP1 or EP2 series receptors is not inhibitory towards ovulation. Only ovulation was measured during the injection experiment, so it is possible that while EP1 and EP2 function is not required for ovulation, these receptors may play a role in maturation of the oocyte or reproductive competency. Future work investigating the specific roles of each receptor subtype would provide valuable information towards understanding how ovulation is regulated in teleost fish.

While both PGE_2 and $PGF_{2\alpha}$ are thought to play a role in the reproduction of zebrafish, the present study focused mainly on the EP4b receptor, which is specific for PGE_2. We did not find any evidence that the FP receptor (specific for PGF2α) is dynamically regulated and as such much of our work focused on regulation of the EP series of prostaglandin receptors. While both prostaglandins likely play a role in teleost ovulation, their roles may differ. We have shown that the function of the EP4 receptor is essential to proper ovulation and as such, PGE_2 likely also plays a crucial role in the regulation of ovulation. Previous studies investigating $PGF_{2\alpha}$ have suggested that $PGF_{2\alpha}$ functions as a pheromone that drives courtship and spawning behaviour in fishes (Munakata and Kobayashi, 2010; Sorensen et al., 2018, 1988; Yabuki et al., 2016), though other studies have found that $PGF_{2\alpha}$ may also be involved in ovulation (Goetz et al., 1989; Jalabert and Szöllösi, 1975). The major prostaglandin responsible for ovulation appears to differ from species to species (Takahashi et al., 2013). The dual functions of $PGF_{2\alpha}$ are not resolved in the present study.

5. Conclusions

In the present study, we have showed that levels of the mRNA transcript for the prostaglandin receptor EP4b increase in the time period leading up to ovulation and spawning *in vivo*. This increase in *ptger4b* expression is also seen when female fish are injected with Ovaprim, a potent stimulant of ovulation. Consistent with this, we saw high rates of ovulation in fish injected with Ovaprim. The rate of ovulation was drastically reduced when fish were injected with both Ovaprim and GW627368x, a selective competitive inhibitor of the EP4 receptor. There was no reduction in ovulation rate when fish were injected with Ovaprim and an inhibitor of either EP1 or EP2 receptors. Additionally, incubation of follicles *in vitro* with 17,20β-P led to upregulation of *ptger4b*. Based on these findings, we conclude that the EP4b receptor is both dynamically regulated in order to properly time the events leading up to and including ovulation, and that proper function of the EP4b receptor is essential for ovulation.

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The authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113228.

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