An agonist for membrane progestin receptor (mPR) induces oocyte maturation and ovulation in zebrafish in vivo

Thesis

An agonist for membrane progestin receptor (mPR) induces oocyte maturation and ovulation in zebrafish *in vivo*

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CONTENTS

LIST OF ABBREVIATION

LH	: Luteinizing hormone
MIH	: Maturation-inducing hormone
MIS	: Maturation-inducing steroid
EDC	: Endocrine-disrupting chemical
EEDC	: Environmental endocrine-disrupting chemical
DHP	: 17α, 20β-dihydroxy-4-pregnen-3-one
DES	: Diethylstilbestrol
mPR	: Membrane progestin receptor
nPR	: Nuclear progestin receptor
Org OD 02	: Org OD 02-0
GVs	: Germinal vesicles
GVBD	: Germinal vesicle breakdown
BSA	: Bovine serum albumin
DAPI	: 4,6-diamidino-2-phenylindole
ER	: Estrogen receptor
MPF	: Maturation promoting factor
GnRH	: Gonadotropin-releasing hormone
Gi	: Inhibitory G-protein
cAMP	: Cyclic AMP
Tes	: Testosterone

Abstract

Considering the near proximity of oocyte maturation and ovulation in fish, it's possible that these processes are intertwined and controlled by the same hormones. After being ovulated or released from the follicular layer, mature oocytes are being able to be spawned and fertilized. Luteinizing hormone (LH), its product maturation-inducing hormone (MIH), and several locally derived peptide growth factors play significant role in oocyte maturation and ovulation.

The effects of externally applied endocrine-disrupting chemicals (EDCs) or steroid hormones in zebrafish were investigated by simply adding the agents into water to resolve the effects of EDCs *in vivo* in zebrafish. As a result, a method for inducing fish ovulation *in vivo* was developed, which involved simply adding the maturation-inducing steroid 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) to the water. In zebrafish, adding steroids to the ambient water causes oocyte maturation within a few hours. *In vivo* assays, oocyte maturation was induced in a matter of hours (2 hours by DHP and 3 hours by Diethylstilbestrol (DES). Unlike *in vitro* studies, steroids penetrate the fish body quickly and induce nongenomic action in the ovary. As a result, we concluded that the effects of EDC hormones on nongenomic activities via the membrane progestin receptor (mPR) could be assessed using oocyte maturation in an *in vivo* zebrafish assay, as well as genomic actions via the nuclear progesterone receptor (nPR) via ovulation scoring.

In the present study, as a selective agonist for mPR, the effect of Org OD 02-0 (Org OD 02) on fish oocyte maturation and ovulation was evaluated through both *in vitro* and *in vivo* assays. At first, the female wild type *roy* zebrafish was collected by paring the male and female zebrafish as the only female fish was required for this experiment. Female fish with full-grown immature oocytes were gathered from a mixed group of 10-50 males and females. Later, females were placed in a glass tank with 100 ml of water per fish and added 10µl of 1 mM DHP into each of the 100 ml water and incubated at 28.5°C for 4 hours. Through this process all full-grown immature oocytes were ovulated those were observed by their posterior portion of the ovary by naked eye. These types of females were finally selected for proceeding the further experiment. All the confirmed females kept into same tank for 5-10 days prior to using for the experiment. After 5-10 days later, *in vitro* oocyte maturation assay was conducted. Gravid females were selected those are not contain ovulated eggs. Ovaries were segregated and washed in fresh zebrafish ringer solution from sacrificed female fish. Then ovaries were manually dissected into ovarian fragments. Fragments were treated with 4 ml of zebrafish ringer solution containing experimental agents in *in vitro* condition and incubated with a light agitation. Afterward the oocytes were observed under binocular microscope as transparent due to the germinal vesicle breakdown (GVBD) occurrence. Conversely, *in vivo* oocyte maturation and ovulation assay were also performed to evaluate the maturation and ovulation rate. In this

portion, gravid female zebrafish was selected with full-grown immature oocytes those were not contain ovulated eggs from the group of confirm females. Selected females were placed in a glass tank with 100 ml water per fish and added 10µl of 1 mM agent into each of the 100 ml water and incubated at 28.5°C for 4 hours. Then, by following the same procedure as described for *in vitro* methods, the oocyte maturation of the zebrafish was also observed. The oocytes that were transparent was determine GVBD and the presence of a transparent fertilization membrane was determine ovulation. To observed more briefly, we conducted *in vitro* ovulation assay also. Females were chosen by same way as describe previously. Oocytes were put in fresh 90 percent L-15 medium pH 9.0 supplemented with 0.5 percent bovine serum albumin (BSA) before being washed with the same solution. Fully grown immature oocytes were exposed *in vitro*. 4,6-diamidino-2-phenylindole (DAPI) was applied to the medium to determine ovulation processes, and blue-fluorescent follicle cell nuclei were studied under a fluorescent binocular microscope. Ovulation was evaluated when the follicle cell layers were absent, and GVBD also when translucent.

These results indicated that Org OD 02 triggered physiological ovulation in live zebrafish. In summary, we have demonstrated the effect of Org OD 02 on fish oocyte maturation and ovulation *in vitro* and *in vivo*. The results suggested that Org OD 02 acted as an agonist not only of mPR but also of nPR.

General Introduction

Endocrine Disrupting Chemical (EDC)

An endocrine-upsetting compound was characterized by the U.S. Ecological Insurance Office (EPA) as "an exogenous specialist that meddles with union, discharge, transport, digestion, restricting activity, or end of regular blood-borne chemicals that are available in the body and are liable for homeostasis, proliferation, and formative interaction. Endocrine-disturbing synthetic compounds (EDCs) were initially thought to apply activities basically through atomic chemical receptors, including estrogen receptors (trama centers), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors, among others. An endocrine-disturbing substance is a compound, either normal or manufactured, which, through ecological or improper formative openings, changes the hormonal and homeostatic frameworks that empower the creature to speak with and react to its current circumstance.

EDCs are exogenous specialists that meddle with hormonal guideline that is answerable for controlling numerous formative cycles just as the upkeep of homeostasis. EDCs can copy or impede the action of endogenous chemicals and in this manner can essentially affect regenerative turn of events and practicality if organic entities are uncovered during basic phases of advancement (Scholz and Mayer, 2008). EDCs can go about as agonists or opponents of oocyte development in fish (Tokumoto et al. 2005). Regardless of the aftereffects of studies exhibiting that bisphenol A (BPA), DES, and prochloraz are equipped for prompting oocyte development, numerous EDCs hinder development and impede regenerative turn of events (Baek et al. 2007; Ogawa et al. 2011; Rime et al. 2010).

Oocyte maturation

Oocyte maturation in fish completes the oogenesis process, resulting in a fertile female gamete or egg (Goetz, 1983, Guraya, 1986). Oocyte maturation is a remarkable metamorphosis from the prophase I oocyte, which is specialized for transcription, material uptake, is non-excitable, and unable to osmoregulate in fresh water, to the metaphase II egg, which is almost the polar opposite of the oocyte in many ways. Immature oocytes began meiosis and ended at prophase I of their meiotic cell cycle, resulting in immature oocytes. Hormones induce oocytes to resume meiosis until they reach metaphase II, which is referred to as a mature oocyte. Oocyte maturation is the process of transforming an immature oocyte into a mature oocyte. Many morphological changes occur during maturation, including chromosomal condensation,

germinal vesicle breakdown (GVBD), and initial polar ejection. The oocyte nucleus, or germinal vesicle (GV), is ordinarily found in the center of the oocyte; however, in response to hormonal stimulation, the GV migrates from the center to the animal pole. Following that, GVBD takes place, and the oocytes become transparent rather than opaque. After telophase I, the egg generates the first polar body in the meiosis division. Oocytes eject the first polar body to the outside and continue to divide in meiosis until metaphase II-arrest, when they are referred to as mature oocytes **(Figure 1).**

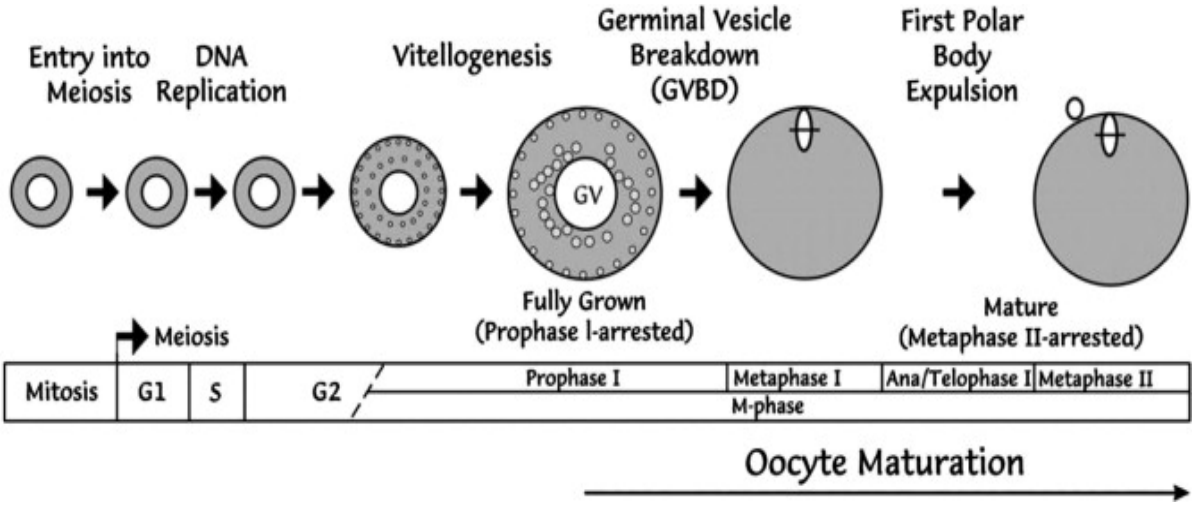


Figure 1. Lubzens et al, 2009. Oocyte maturation process

Ovulation

The creation of MIH by teleost ovarian follicles is additionally important to initiate the last advance of oogenesis, ovulation. However, in contrast to meiotic resumption, ovulation requires MIH-subordinate transcriptional initiation (Theofan and Goetz 1981; Pinter and Thomas 1999; Patino et al. 2003b) that is controlled by an atomic MIH receptor (nMIHR) (Pinter and Thomas 1999). This situation in fishes resembles that in rodents, where ovulation is by all accounts instigated by the limiting of LH-incited follicular progesterone to the traditional progesterone receptor (PR) (Pall et al. 2000). The area of nMIHR in teleost follicles has not been inspected, however the PR in rodent follicles is found in granulosa and maybe thecal cells (Pall et al. 2000). Albeit meiotic resumption can be incited by an assortment of boosts following up on various transduction pathways the acceptance of ovulation is more explicit and by and large restricted to improvements that expansion protein kinase C (PKC) action and amino corrosive (AA) digestion. Numerous examinations in the overall writing have analyzed the job in ovulation played by cyclooxygenase and lipoxygenase pathways of AA digestion. In spite of the fact that there is no agreement concerning the general commitment of these two pathways, late information acquired for Atlantic croaker shows that the two pathways are similarly significant for MIH-subordinate ovulation (Patino et al. 2003b). Ovulation includes measures identified with follicular debasement and crack and the ejection of the develop ovum (Goetz et al. 1991), however the components connecting ovulation-actuating elements to these cycles are yet indistinct for teleosts. In certain species, ovulation is profoundly synchronized with spermiation in guys through an arrangement of pheromonally controlled practices (Kobayashi et al. 2002). Ovulated ova in many teleosts are totally stripped of substantial cells and their vitelline envelope (VE) is presented to the outer climate. Sperm enter through the VE by means of at least one pores called micropyles. Contact with the egg surface (preparation) incites a cortical response, during which the substance of the cortical alveoli is delivered into the perivitelline space. Osmotically dynamic A lot of glycoproteins (polysialoglycoproteins) cause hydration, and metalloproteases hydrolyze VE proteins into more modest subunits. These subunits are then polymerized into insoluble high sub-atomic weight VE proteins (Iwamatsu et al. 1995; Ha and Luchi 1996; Shibata et al. 2000). The solidified VE is perceived as a treatment envelope. Treatment likewise instigates the culmination of the second meiotic division and removal of the second polar body. In some viviparous species, treatment is intrafollicular and 'ovulation' of the undeveloped organism is deferred until parturiti on (Venkatesh et al. 1992)

Many biological activities, such as Proteolysis, inflammation, coagulation, vasodilatation, and angiogenesis were suggested to be involve in ovulation. It means, the molecular pathway to induce ovulation are vary and complex **(Figure 2).**

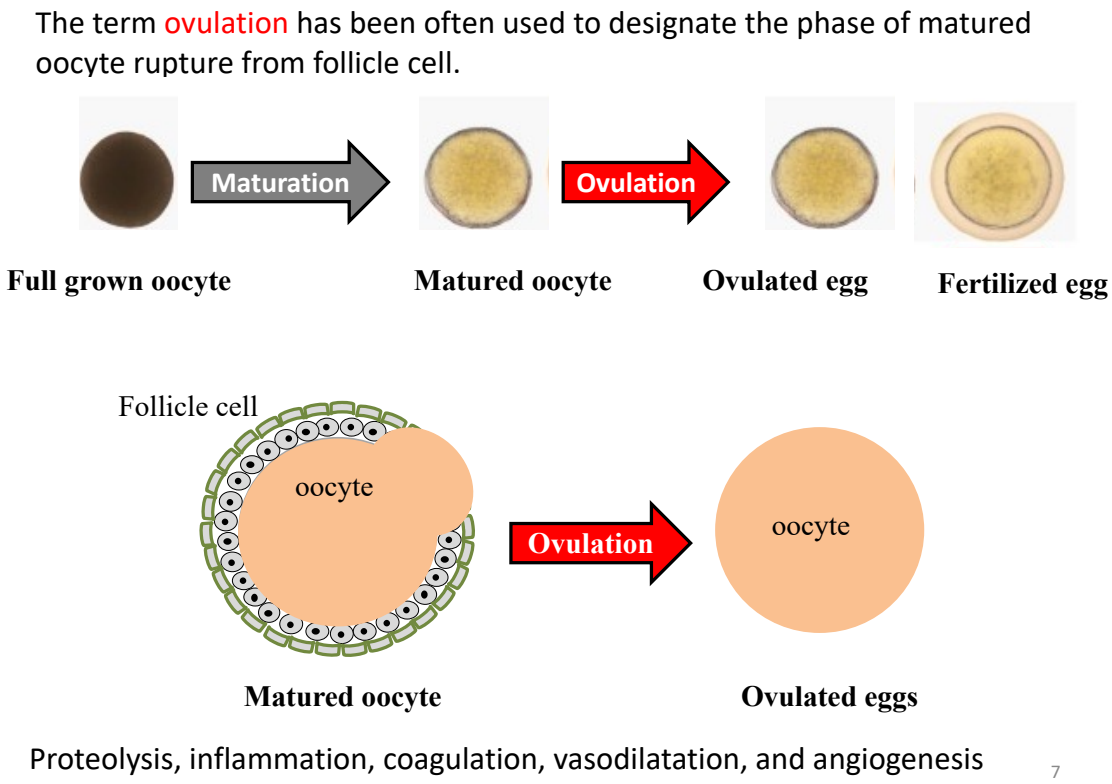


Figure 2. Ovulation process. Org OD induce maturation, then follicle cell degrades and matured oocyte rupture from follicle layer. This is ovulation. If egg cannot undergo ovulation, they also cannot fertilization.

Introduction

Hormonal signal is a natural phenomenon that controls the physiological characteristics of all living organisms. Several EDCs have been identified as environmental contaminants that interfere with exposed organism's endogenous hormone systems by disrupting hormone signaling or altering endogenous hormone synthesis, transport, and catabolism. EDCs that function as estrogen mimics, that is, they activate estrogen receptors (ERs) as ligands and modulate ER-regulated gene expression, have received the most attention. Many of the studies on endocrine disruption, especially in aquatic wildlife, have been published previously (Tyler et al., 1998; Vos et al., 2000; Sumpter, 2005).

Oocyte maturation and ovulation occur after the completion of female gametogenesis in the ovary and subsequent fertilization and embryogenesis, though it is a crucial process (Lane et al., 2014). In animal oocytes, meiosis is halted during the first division's prophase (ProI). In most vertebrates and invertebrates, hormonal activation causes meiosis to resume, and oocytes undergo nuclear maturation when nuclear disassembly occur (i.e., GVBD).

In zebrafish, the MIH DHP can cause GVBD (Tokumoto *et al.* 2004). As the brain secretes gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to secrete LH and primes follicles to synthesize and respond to MIH, oocyte maturation or GVBD occurs (Clelland and Peng, 2009; Rime *et al.* 2010). The interaction between the theca and granulosa cell layers, the epithelial cell types of the vertebrate ovary, is needed for maturation inducing hormone development by ovarian follicles (Nagahama and Yamashita, 2008). DHP is a naturally occurring MIH found in a variety of fish species (Tokumoto *et al.* 2005). MIH binds to the oocyte membrane's mPR and activates the maturation promoting factor (MPF) (Tokumoto *et al.* 2008). Oocytes undergo dramatic morphological changes associated with progression of the meiotic cell cycle during maturation, one of which is the breakdown of the oocyte nuclear envelope GVBD, which occurs at the prophase/metaphase transition and is generally regarded as a hallmark of oocyte maturation development. By binding with MIH, inhibitory G-protein (Gi) is activated, resulting in a decrease in intracellular cyclic AMP (cAMP) and a reduction in protein kinase A activity (PKA). The MPF is required for GVBD and oocyte maturation to occur.

MIH binding causes MPF to form by triggering the development of cyclin B, a regulatory protein necessary for MPF action (Nagahama and Yamashita, 2008; Tokumoto *et al.* 2012). Once activated, MPF triggers the restart of the meiotic cycle by releasing immature oocytes from prophase I meiotic arrest. GVBD at the prophase/metaphase transition, as well as the transition from opaque to translucent ovarian follicles, are all part of oocyte maturation in fish (Tokumoto *et al.* 2004; 2008a; 2011). When vitellogenin is converted into yolk proteins, follicle clearing occurs, and this translucent appearance indicates maturation in follicle clearing assays (Clelland and Peng, 2009).

In most fishes, the DHP, a steroid hormone, is reported as the MIH (Nagahama and Yamashita, 2008). The DHP can induce oocyte maturation in zebrafish *in vitro* (Tokumoto *et al.* 2004). DES, a non-steroidal estrogen, is an EDC that induces oocyte maturation in zebrafish. *In vitro* treatment of oocytes with DES resulted in germinal GVBD. *In vitro* analysis of oocytes treated with DES revealed the presence of cyclin B, an essential factor in the development of MPF (Tokumoto *et al.* 2004).

Hydration is caused during oocyte maturation in fish, and the oocyte becomes translucent. After oocyte maturation, ovulation (or extrusion from surrounding follicle cells) is triggered. Before spawning, ovulated eggs remain in the female body. When spawned fish eggs are released into the water, they produce a fertilization membrane almost immediately, regardless of whether fertilization has occurred. In freshwater fish, the same MIS (DHP) causes both oocyte maturation and ovulation. In fish, the mPR is thought to mediate the MIS signal in the oocyte to induce oocyte maturation (Zhu *et al.* 2003; Tokumoto *et al.* 2006). The

cytoplasmic nPR has been shown to be a receptor for ovulation induction (Zhu *et al.* 2003; Tang *et al.* 2016). Thus, it is thought that the same hormone induces both oocyte maturation and ovulation, but that they are activated by two separate pathways, mPR and nPR, respectively.

The *in vivo* assay is a novel method for inducing oocyte maturation without ovulation using DES or testosterone (Tes), as well as a method for inducing ovulation using DHP in the same way (Tokumoto *et al.* 2011). This is the first time that comparing the expression levels of genes between DHP treated samples and DES or Tes treated samples has enabled researchers to compile a list of genes that are explicitly up or downregulated in order to induce ovulation. As a result, *in vivo* assays would be extremely useful in identifying main regulators of ovulation, and this approach can also be used to assess the impact of EEDCs on progestin genomic actions by ovulation assessment (Tokumoto *et al.* 2011).

Hormones and other chemical compounds, including endocrine disruptors, have been studied *in vitro* using fish oocytes dissected from ovaries (Das *et al.* 1999; Tokumoto *et al.* 2005). We previously demonstrated that the natural MIS (DHP) binds to mPR in a highly specific manner. Furthermore, there was a lot of competition for mPR binding between DES and 17,20b- DHP. The agonistic effect of DES on mPRα is associated with the oocyte maturation-inducing activity of DES on goldfish and zebrafish oocytes *in vitro* (Tokumoto *et al.* 2004). *In vitro* assays using oocytes can thus be used to assess the agonistic and antagonistic effect of substances on mPRα.

The improvement of medium conditions led to the creation of an *in vitro* ovulation-inducing assay (Seki *et al.* 2008). It was possible to assess the direct effects of chemicals on oocyte maturation and ovulation in zebrafish oocytes using this technique.

Zebrafish are an ideal model organism for studying oocyte maturation because they go through processes that are highly conserved across vertebrate organisms, and their genome has been fully sequenced (Weber *et al.* 2013). It's a fantastic model for learning about the molecular processes of reproduction and growth at the genetic stage. Zebrafish have many advantages over other models, including rapid development (only two days are needed for hatching), oocyte maturation (takes only 2-3 hours), and visible morphological changes of oocytes during oocyte maturation (from opaque to transparent). Three months after hatching, zebrafish are considered sexually mature, allowing for fast experimentation (Clelland and Peng, 2009). The number of eggs spawned by zebrafish is greater than those of all other small fish models, and oocytes can be obtained at any time during the year (Tokumoto *et al.* 2004). Every 4-7 days, spawning occurs in favorable environmental conditions (Clelland and Peng, 2009). Since many of the factors involved in physiological processes during embryonic development and oocyte

maturation have been established, zebrafish are an excellent choice for EDC exposure studies (Osterauer and Kohler, 2008).

Moreover, two steroid signaling pathways to induce maturation and ovulation have been described in zebrafish. It named genomic and non-genomic steroid signaling. It has been revealed that DHP bind to nuclear progestin receptor (nPR) and mediated ovulation through the genomic steroid signaling pathway. Homozygous nPR knock out females cannot undergo ovulation and oocyte development stops at maturation. It means that even nPR was knock out but, maturation is still occurred. It leads scientist to search other receptor to mediate maturation.

Later, researcher found that 17, 20β-DHP also binds to a membrane progestin receptor (mPR) and acts via the non-genomic steroid signaling pathway to induce oocyte maturation. Oocyte maturation and ovulation are coordinated process induced by the genomic and non-genomic pathways which activated by 17, 20β-DHP.

Not only DHP but DES and Org OD also bind to mPR and induce maturation. But DES cannot induce ovulation whereas Org OD and can also induce the ovulation **(Figure 3A and 3B)**.

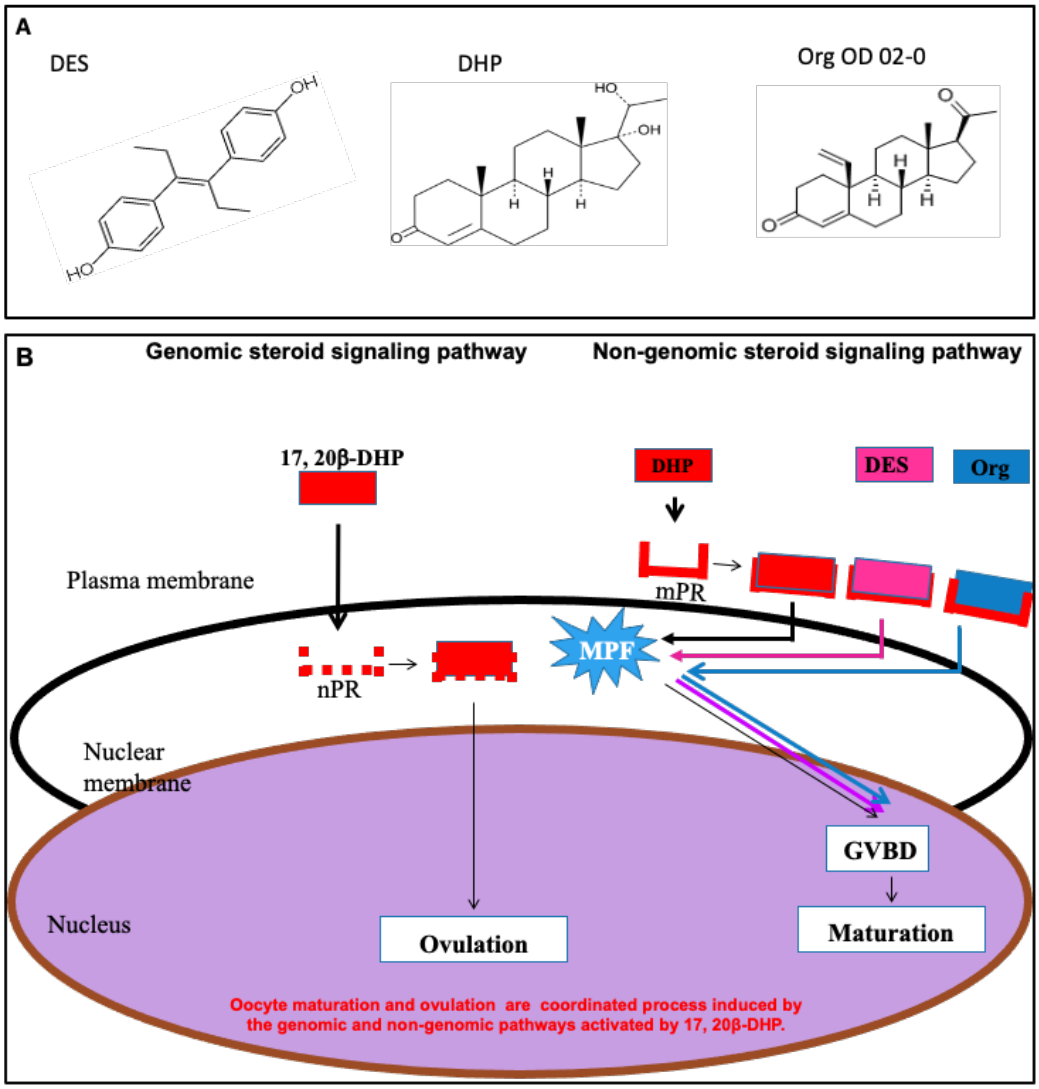


Figure 3. Oocyte maturation and ovulation signaling pathway. (A) The structure of different types of EDCs those are used in this experiment (except DES). (B) Genomic and non-genomic signaling pathway in zebrafish. This panel shows the maturation and ovulation process of oocytes in zebrafish.

The chemical Org OD 02, which induces oocyte maturation *in vitro*, was recently released (Aizen *et al.* 2018). In this research, I tried to show how Org OD 02 exposure stimulates oocyte maturation and ovulation in zebrafish and acts as an agonist for both nPR and mPR.

Materials and Methods

Matetials

Zebrafish **(Figure 4)** were raised and cared for in a laboratory environment. The fish used in the experiments were held in a flow-through culture system at 28.5 degrees Celsius with a 14-hour light/10-hour dark period (Westerfield *et al.* 1995). Org OD 02 was obtained from AXON Medchem BV (Groningen, Netherlands). 17,20β-DHP was purchased from Sigma Chemical Co. (St. Louis, MO). This study uses of zebrafish, and the experimental protocol for the use of animal was approved (approval no. 2019F-5) by the Institutional Ethics Committee of Shizuoka University, Japan.

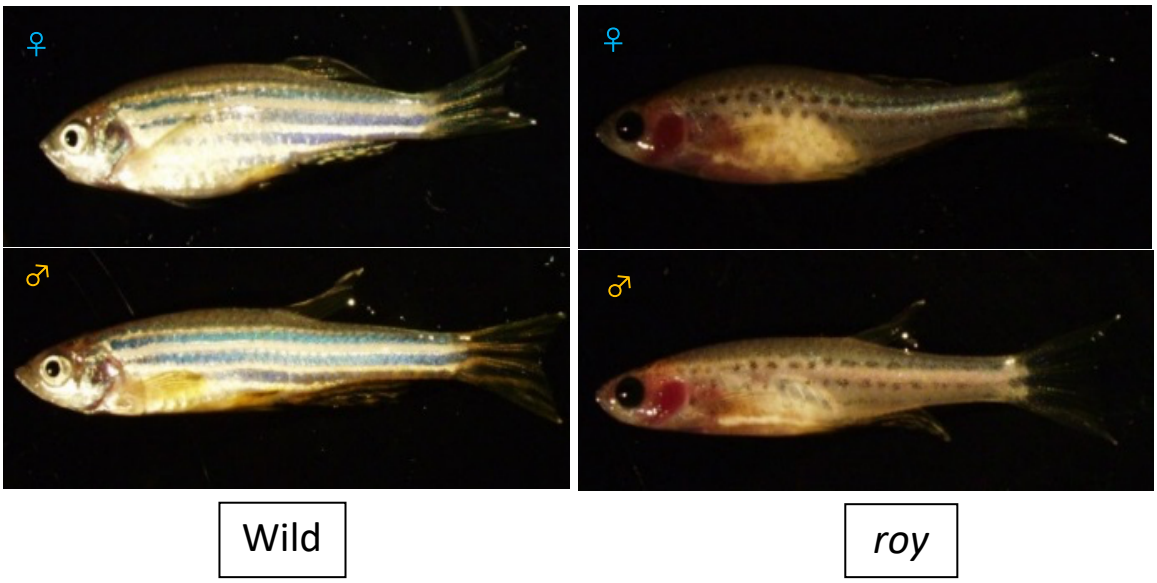


Figure 4. Experimental species (*roy* zebrafish). Comparison between normal and *roy* zebrafish strain. Normal zebrafish is opaque with white and black stripes liked zebra, while *roy* strain was established by removing the iridophores that are silver color. It affected that *roy* strain has transparent body and easily observe inside their body.

Confirmed female preparation

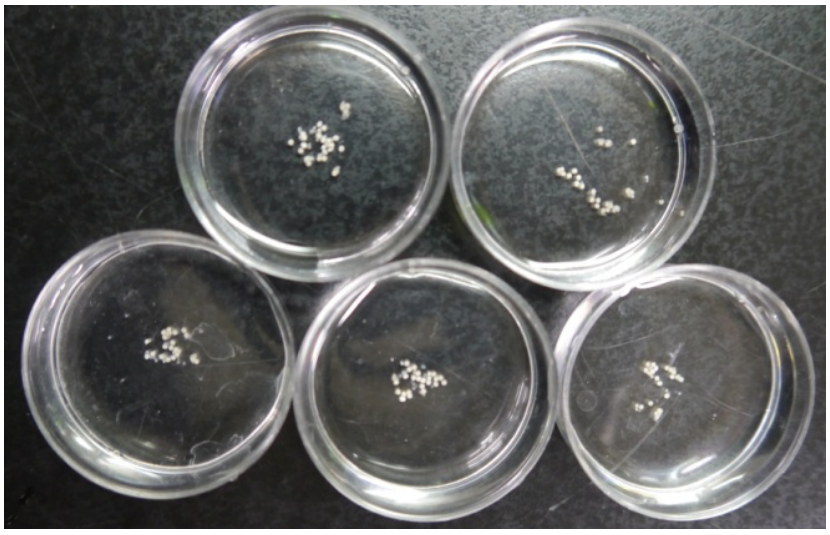
Ovulation assessment has been conducted on a group of females those are able to ovulate. Individuals who were unable to ovulate were excluded from the analysis. Female fish with full-grown immature oocytes were chosen from a mixed group of 10-50 males and females held in a 20cm x 25cm x 25cm square acryl case in our lab using the standard water method. Females were placed in a glass case with 100 milliliters of water per fish. *In vivo* exposure to agents was achieved by putting 10µl of 1 mM DHP into each fish's water. For 4 hours, the fish were incubated at 28.5°C. Confirmed females have translucent oocytes that can be seen with the naked eye in the posterior portion of the ovary **(Figure 5)**. Oocytes that have been ovulated may be extracted from the ovaries. Before being used in tests, all confirmed females were placed in the same tank for 5 days under normal water conditions. Each bio-replicate was obtained from the same batch of confirmed females for the transcriptome analyses.

Figure 5. Confirmed female preparation. Agents add into the water and incubate. After 4 hours later oocyte is ovulated. Red circle indicates ovulated eggs.

In vitro Oocyte Maturation Assay

As previously reported, an *in vitro* zebrafish oocyte maturation assay was performed (Tokumoto *et al.* 2005). Gravid females who did not have ovulated eggs were chosen by pressing their abdomens after the onset of light to squeeze the eggs (9 a.m. in our laboratory). Ovaries of sacrificed female zebrafish were separated and washed in fresh zebrafish ringer solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.2). Using fine forceps, the ovaries were manually dissected into ovarian fragments (each containing 2-10 oocytes). The ovarian fragments were exposed *in vitro* by incubating them at 25°C or room temperature with gentle agitation in 4 ml of zebrafish Ringer's solution containing each experimental agent (diluted from a 1000-fold stock in ethanol) (40 rpm). Germinal vesicles (GVs) were studied under a binocular microscope to determine maturation processes (SMZ645, Nikon, Tokyo, Japan). The oocytes that were translucent were scored to determine GVBD **(Figure 6).** In each experimental condition, the percent GVBD was calculated in more than twenty oocytes of stage 3 in each fish.

A



B

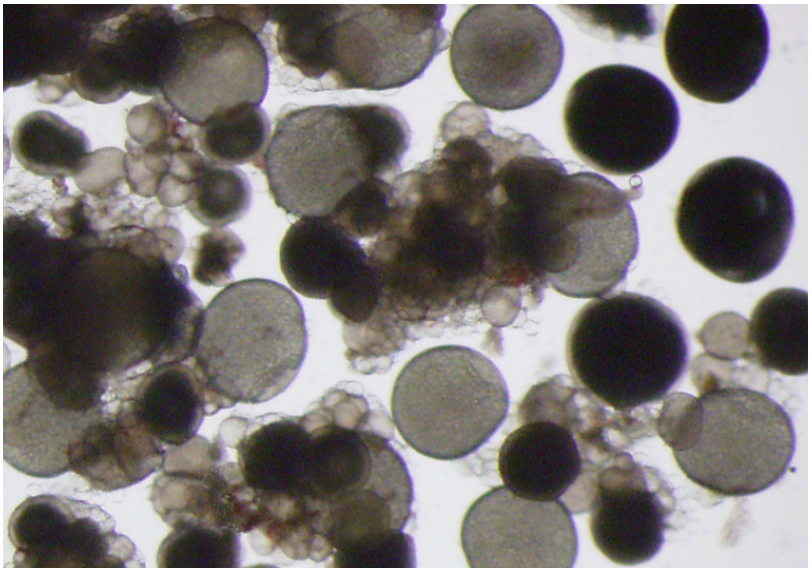


Figure 6. *In vitro* oocyte maturation assay. Oocytes are separated into different petri dish with zebrafish ringer solution and add experimental agent (panel A). Then incubate 4 hours and observed under microscope (panel B).

In vivo Oocyte Maturation and Ovulation Assay

In zebrafish, an *in vivo* oocyte maturation and ovulation assay was performed as previously mentioned (Tokumoto *et al.* 2011). From a mixed group of males and females held in a 20 cm 25 cm square acryl case that was 25 cm high and allowed continuous flow-through, gravid female zebrafish with full-grown immature oocytes were chosen. Females who were determined to be fertile were chosen by pre-spawning with DHP (Tokumoto, 2014). Females that had been pre-spawned were held for 7-10 days after spawning before they formed full-grown immature oocytes. On the day of the experiment, females who had not ovulated eggs were chosen by attempting to squeeze the eggs by moving the abdomen of the fish after the onset of light (9 am in our laboratory). Selected females were placed in a glass case with 100 milliliters of water per fish. Agents were administered to the fish *in vivo* by dissolving each agent in water at 28.5°C (from a 10,000-fold stock in ethanol) **(Figure 7).** The treatment of the fish began 1-2 hours after the onset of light (10-11 am in our laboratory). Zebrafish ovaries were separated from sacrificed females and deposited in fresh zebrafish Ringer's solution after 4 hours of incubation (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.2). Using fine forceps, the ovaries were manually dissected into ovarian fragments (each containing 1-10 oocytes). A binocular microscope was used to photograph the oocyte morphology (SZX12, Olympus, Japan). The oocytes that were translucent were scored to determine GVBD. The presence of a transparent fertilization membrane on the oocytes was used to determine ovulation. In each experimental condition, the rates of GVBD and ovulation were calculated in at least twenty oocytes.

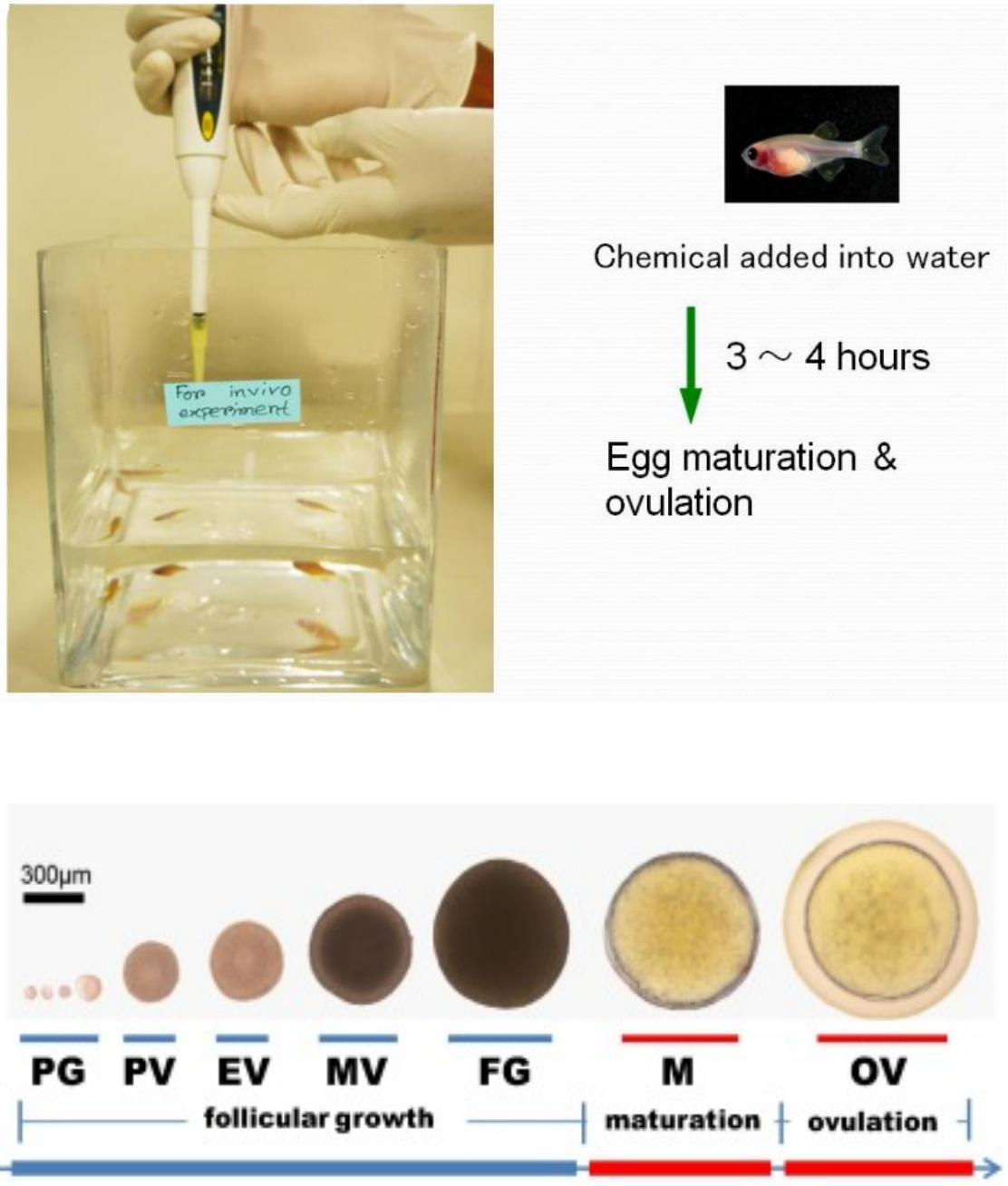


Figure 7. *In vivo* maturation and ovulation assay. (A)The assay is very simple. Just add the solution into the water and incubate for several hours. For assay spawning push the abdomen of tested fish. For assay maturation and ovulation, sacrifice the fish and dissect the ovary and observe under microscope. (B) Stage of morphological change in oocyte during follicular growth, oocyte maturation to ovulation. There are indicated in many stages, including primary growth stage (PG), previtellogenic stage (PV), early vitellogenic stage (EV), Mid vitellogenic stage (MV), full-grown stage (FG), maturation (M) and ovulation (OV).

In vitro Ovulation Assay

As previously described, an *in vitro* zebrafish oocyte maturation and ovulation assay was performed (Klangnurak *et al.* 2018). Females who had not ovulated eggs were chosen by pressing the abdomen of the fish after the advent of light to pinch the eggs (9 am in our laboratory). Zebrafish ovaries were extracted from sacrificed females and put in fresh 90 percent L-15 medium pH 9.0 supplemented with 0.5 percent BSA before being washed with the same solution. Fine forceps were used to manually separate intact follicles from ovarian parts. Fully grown immature oocytes were exposed *in vitro* by incubating at room temperature with gentle agitation in 4 ml of medium containing each experimental agent (diluted from a 1000-fold stock in ethanol) (40 rpm). DAPI was applied to the medium to determine ovulation processes, and blue-fluorescent follicle cell nuclei were studied under a fluorescent binocular microscope (SZX12, Olympus, Japan). Ovulation was determined by grading the oocytes after the follicle cell layers were separated. The oocytes that were translucent were also scored to determine GVBD. a percentage in each fish's experimental state, ovulation was calculated in more than twenty oocytes **(Figure 8).**

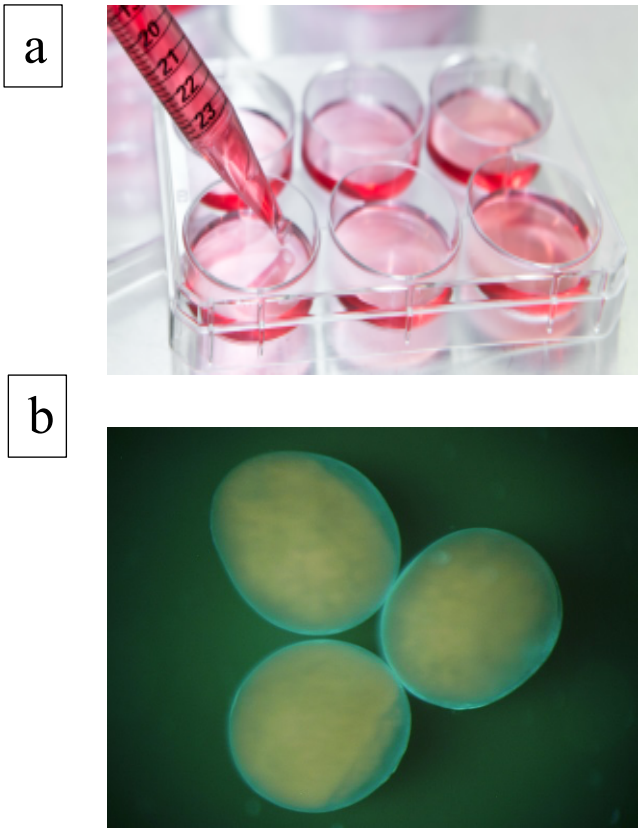


Figure 8. *In vitro* ovulation assay. (a) L-15 medium where the whole experiment was executed, every petri dish contains 4 ml L-15 medium with experimental agents, and (b) here the follicle cell layer of the oocyte was disrupted, and the ovulation was occurred.

Fertilization

In vitro insemination was used to evaluate the ability of the ovulated eggs to fertilize using standard methods (Seki *et al.* 2008). The fertilization rate (%) was determined by counting the number of embryos that matured to the 4-cell stage or later. At least twenty embryos were used to determine the rate of fertilization.

Statistical Analysis

All the experiments were carried out three times. GraphPad Prism was used to do a one-way study of variance (ANOVA) (San Diego, CA). Statistical significance was described as a P value of less than 0.05.

Result

Externally applied org OD 02 induces oocyte maturation and ovulation *in vivo*

In the first step, we used an *in vitro* assay to confirm Org OD 02's oocyte maturation-inducing activity on zebrafish oocytes. Org OD 02 mediated zebrafish oocyte maturation **(Figure 9A)**, as previously stated (Aizan *et al.* 2018). After treatment with Org OD 02 or DHP, oocytes became translucent, but after treatment with ethanol, they remained opaque. We tested the effect of Org OD 02 on fish oocyte maturation and ovulation by putting it directly into the water where live zebrafish were held. Externally applied Org OD 02 induced oocyte maturation *in vivo*, as predicted. When DHP was added to the water, oocyte maturation was induced *in vivo* in a similar way to when the hormone was given directly to oocytes *in vitro*. The morphology of the oocytes after 4 hours of treatment with ethanol, DHP, or Org OD 02 is shown in **(Figure 9B)**.

After receiving Org OD 02 or DHP, the oocytes became translucent, but after receiving ethanol, they remained opaque (Fig. 7B). Org OD 02, to our surprise, caused both oocyte maturation and ovulation. Immediately after transfer into medium, eggs from fish treated with Org OD 02 or DHP shaped fertilization membranes (**Figure 9B**).

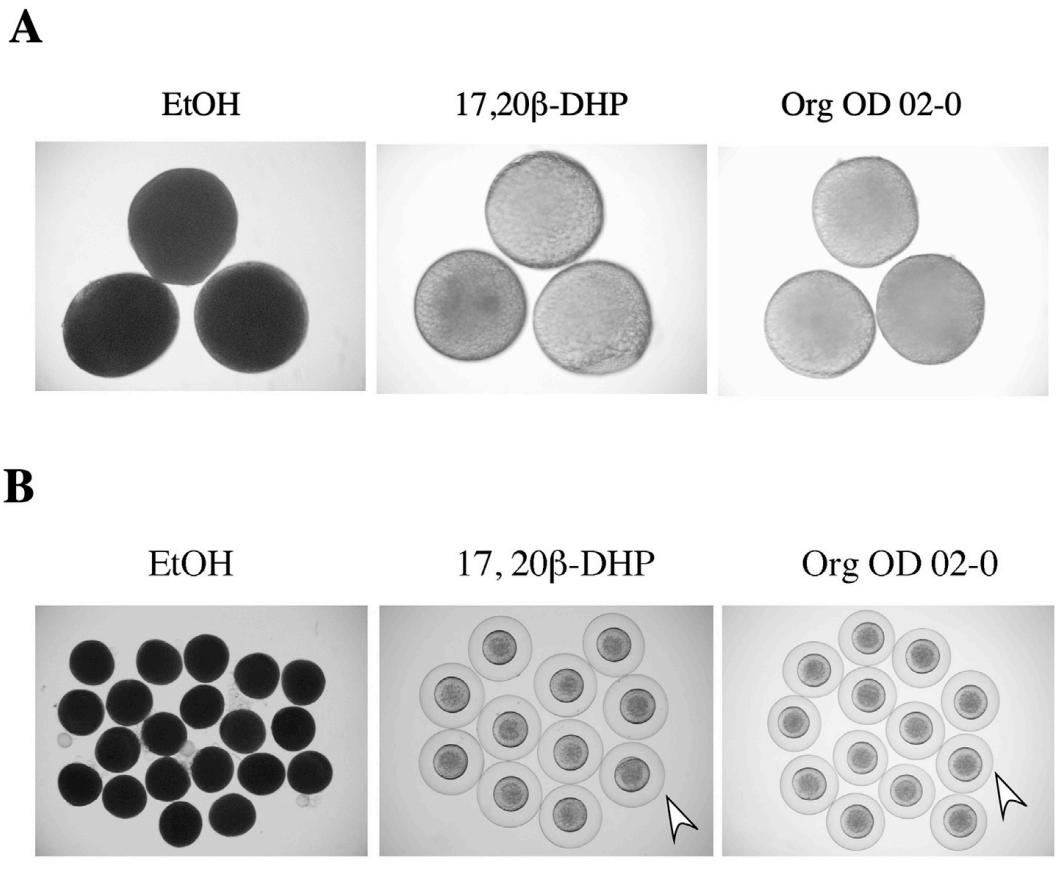


Figure 9. Org OD 02 induced oocyte maturation and ovulation in zebrafish. (A) In vitro assay: three oocytes that showed typical morphological changes were selected and photographed after the oocytes were incubated with each compound (0.1% ethanol, 0.1 mM 17,20β-DHP or 0.1 mM Org OD 02) for 2 h. The oocytes became transparent after treatment with 17,20β-DHP or Org OD 02 but remained opaque following treatment with ethanol. (B) In vivo assay: the morphology of oocytes and eggs from fish treated with 0.01% ethanol, 0.1 mM 17,20β-DHP or 0.1 mM Org OD 02 was photographed. After 4 h of incubation, ovarian samples containing oocytes and eggs were excised from the females by dissection. The oocytes remained opaque following treatment with ethanol. A fertilization membrane developed around eggs whose ovulation was induced by 17,20β-DHP or Org OD 02 treatment, as indicated by the arrow.

Most fish's ovulated oocytes (eggs) were programmed to form a fertilization membrane as soon as they had come into touch with water in some way. Org OD 02's ovulation-inducing activity was also demonstrated *in vitro*. By directly administering Org OD 02 to zebrafish oocytes *in vitro*, it was able to induce ovulation (**Figure 10**).

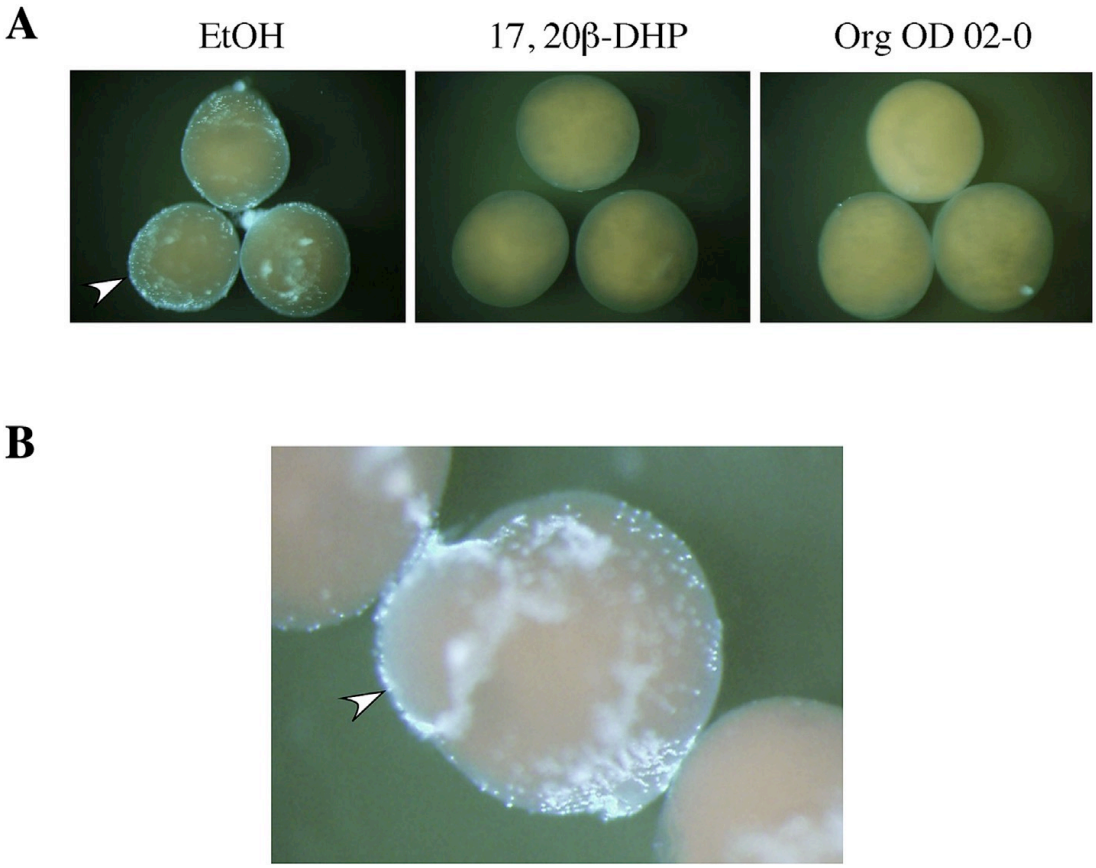


Figure 10. Org OD 02 induced ovulation of zebrafish oocytes in vitro. (A) In vitro assay of ovulation: three oocytes that showed typical morphological changes were selected and photographed after the oocytes were incubated with each compound (0.1% ethanol, 0.1 mM 17,20β-DHP or 0.1 mM Org OD 02) for 4 h. Follicle cell layers glowing by DAPI in blue were removed after treatment with 17,20β-DHP or Org OD 02 but remained following the treatment with ethanol. Follicle cell layer surrounding oocytes is indicated by the arrow. (B) The morphology of oocytes during ovulation was photographed after the induction of ovulation with Org OD 02. The edge of follicle cell layers during ovulation is indicated by the arrow.

In vitro, a dosage of 0.01 mM Org OD 02 was enough to induce oocyte maturation, and *in vivo*, the same concentration was enough to induce oocyte maturation and ovulation (**Figure 11**).

In addition, the concentration needed to induce ovulation *in vitro* was the same (**Figure 11C**). *In vitro* and *in vivo*, Org OD 02 had almost the same potency as DHP in inducing oocyte maturation and ovulation.

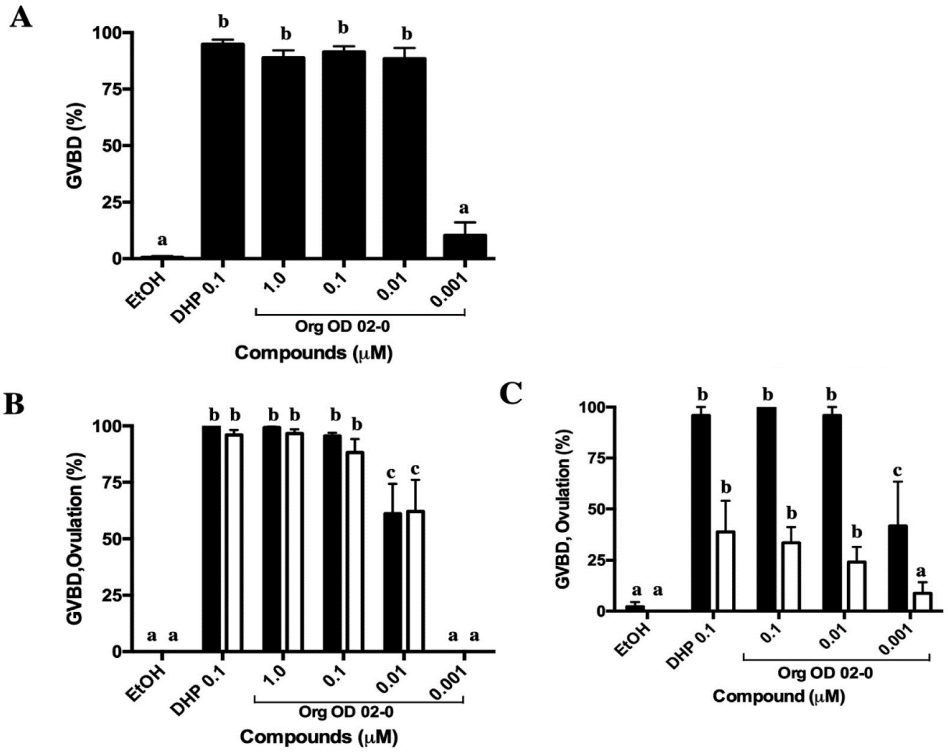


Figure 11. Dose dependency of Org OD 02 induced oocyte maturation and ovulation. (A) In vitro assay: ovarian fragments were dissected from sacrificed females and incubated with 0.1 mM 17,20β-DHP or the indicated concentrations of Org OD 02. Percentage of oocytes that induced germinal vesicle breakdown (%GVBD) was determined by scoring the oocytes that had become transparent. Each value represents the mean of data from nine different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % GVBD. The analysis was significant, $F(5,48)$. 151.9, $p < 0.0001$. (B) In vivo assay: for each concentration, ovarian fragments were dissected from sacrificed females, and % GVBD (closed column) and % ovulation (open column) were determined by scoring the oocytes or eggs that had become transparent or formed fertilization membranes. Each value represents the mean of data from six to ten different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % GVBD. The analysis was significant, $F(5,43)$. 60.74, $p < 0.0001$. A one-way ANOVA was calculated on % ovulation. The analysis was significant, $F(5,43)$. 41.38, $p < 0.0001$. (C) In vitro ovulation assay: oocytes were isolated from ovarian fragments and incubated with 0.1 mM 17,20β-DHP or the indicated concentrations of Org OD 02. % GVBD (closed column) and % ovulation (open column) was determined by scoring the

oocytes or eggs that had become transparent and removed with follicular cell layers. Each value represents the mean of data from three different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % GVBD. The analysis was significant, F (4,10). 18.58, p. 0.0001. A one-way ANOVA was calculated on % ovulation. The analysis was significant, F (4,10). 3.560, p. 0.047. Values with different subscripts within the same criterion were significantly different ($p < 0.05$).

Org OD 02 induces natural ovulation *in vivo*

In vitro fertilization was used to show that the ovulated oocytes generated by Org OD 02 treatment were normal. Hormonal stimulation for oocyte maturation and ovulation (with a burst of luteinizing hormone) starts the night before spawning, preparing the egg to be spawned after the onset of light (Hanna and Zhu, 2011). We chose gravid females who did not have ovulated eggs by pressing their abdomens after the advent of light to squeeze the eggs. As a result, the females in this study could have been outside of the normal spawning period, and the compounds applied to the water may have been used to artificially trigger oocyte maturation and ovulation. The eggs ovulated after treatment with Org OD 02 were fertile, much like the eggs ovulated after treatment with DHP (**Figure 12**). The fertilization rates of the ovulated eggs were not substantially different after treatment with Org OD 02 and DHP. *In vitro* fertilized eggs grew normally, and the fertileness of grown juveniles was confirmed. These findings demonstrated that ovulation caused by Org OD 02 was like physiological ovulation.

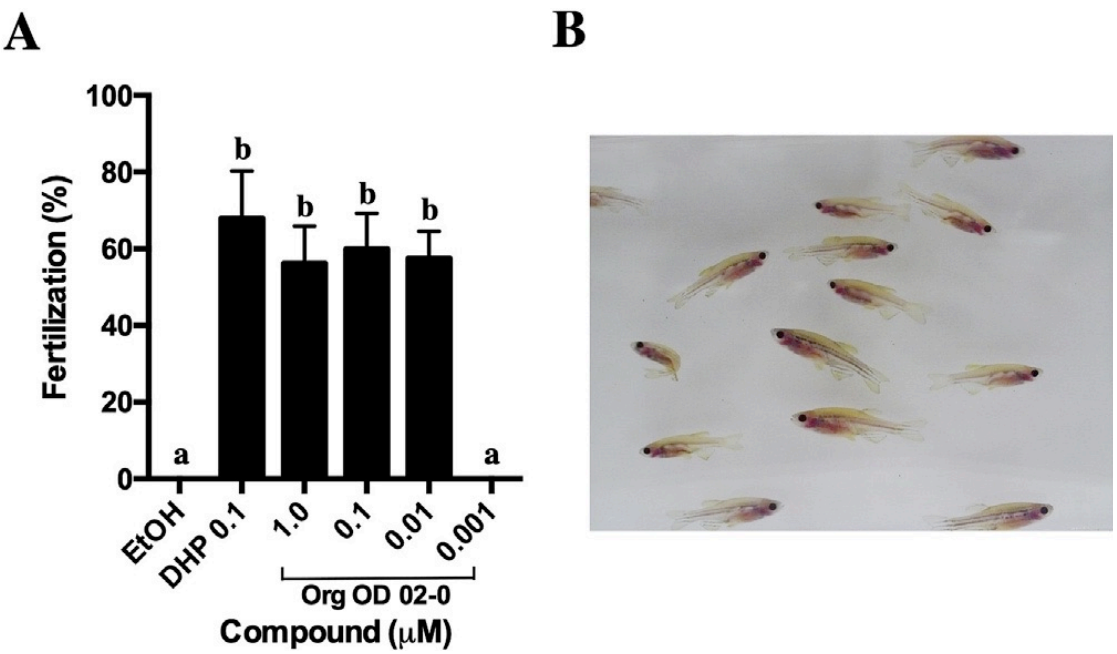


Figure 12. Externally applied Org OD 02 induced natural spawning. (A) Fertilization rates were compared between eggs extruded from females treated with 17,20β-DHP in vivo and those extruded from females treated with Org OD 02 *in vivo*. Fish were treated with the

indicated concentration of 17,20β-DHP or Org OD 02. Artificial fertilization of the extruded eggs was conducted using sperm obtained from the males. The fertilization rate was calculated by counting the number of fertilized eggs. Each value represents the mean of data from six to ten different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % Fertilization. The analysis was significant, $F(5,12)$. 46.53, $p < 0.0001$. Values with different subscripts within the same criterion were significantly different ($p < 0.05$). (B) Fishes developed normally from eggs ovulated by Org OD 02-treatment. A group of two-month-old juveniles that developed from the eggs whose ovulation was induced by Org OD 02 was photographed.

Discussion

The effect of Org OD 02, a selective mPR agonist, on oocyte maturation in zebrafish *in vitro* and *in vivo* was investigated. *In vitro* and *in vivo*, Org OD 02 induced oocyte maturation, as predicted. In addition, we discovered that incubation with Org OD 02 resulted in ovulation *in vivo*. Zebrafish that had been treated with Org OD 02 for more than 3 hours could be squeezed for eggs. After coming into contact with water, the squeezed eggs successfully formed a fertilization membrane. This is a normal feature of ovulated fish eggs, which are triggered by water contact and begin developing without the need for insemination. Org OD 02-induced ovulation resulted in fertilized eggs that formed normally and were fertile (data not shown). Org OD 02-induced ovulation was found to be similar to physiological ovulation. The findings of this study indicate that Org OD 02 may be used to induce fish spawning. Since injecting DHP is one of the most effective methods for inducing fish spawning, the method of inducing spawning by injecting Org OD 02 should be tried as well.

Org OD O2 is being studied to see whether it can act as a selective mPR agonist (Kelder *et al.* 2010). It's been used in studies that demonstrate mPR involvement in things like sperm motility (Tan *et al.* 2019), Ca^{2+} ion influx into T cells (Lin *et al.* 2016), and breast cancer cell apoptosis inhibition (Lin *et al.* 2016). (Dressing *et al.* 2012). Progesterone facilitates glioblastoma migration and proliferation, and its activity is mediated by mPR, according to this definition (Gonzalez-Orozco *et al.* 2018 and Castelnovo *et al.* 2020). Org OD O2 had a similar effect to progesterone, implying that this action was mediated by mPR rather than nPR.

In this study, we showed that Org OD 02 can induce oocyte maturation and ovulation *in vitro* and *in vivo*. DES has been shown to have oocyte maturation inducing activity in zebrafish. While DES causes oocyte maturation, it does not cause ovulation in humans (Tokumoto *et al.* 2011). The disparity in these findings is believed to be caused by a difference in nPR affinity. Org OD 02 has a high affinity for nPR (Ki= 33.9 nM), according to research (Kelder *et al.* 2010). DES, on the other hand, has a slightly lower sensitivity for nPR (Ki= 11 mM) (Laws *et al.* 2000). This knowledge could explain why Org OD 02 could cause ovulation *in vivo* but not DES.

Org OD 02 was found to be a selective mPR ligand that induced oocyte maturation both *in vitro* and *in vivo*, according to our findings. Org OD 02, on the other hand, is a nPR ligand that induces ovulation under physiological conditions. To discover a mPR-specific ligand, further research is needed (Lisanova *et al.* 2013 and Policarpova *et al.* 2017).

In conclusion, Org OD 02 has been shown to induce oocyte maturation and ovulation *in vitro* and *in vivo*. Org OD 02 is a mPR ligand that is selective. *In vitro* and *in vivo*, it is

predicted to cause only oocyte maturation. Our findings, on the other hand, showed that Org OD 02 has the ability to cause ovulation under physiological conditions. *In vivo*, these findings indicated that Org OD 02 may be a ligand for both mPR and nPR.

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