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# In vitro follicle culture shows that progestagens are the maturation-inducing hormones (MIH) and possible regulators of the ovulation-mediating hormone PGE2 in female Eurasian perch *Perca fluviatilis*

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## Keywords

Eurasian perch, corticosteroids, in vitro, DHP, FOM, MIH

## Abstract

In European aquaculture, Eurasian perch, *Perca fluviatilis* L., is perceived as one of the most highly valuable freshwater fish species and a strong candidate for the development of freshwater aquaculture. In the pursuit of improving the quality of reproduction in this domesticated species, investigating the hormones mediating the final oocyte maturation (FOM) is therefore indispensable. But, the exact nature of the maturation-inducing hormone (MIH) in Eurasian perch is unknown. To further validate the existence of a maturation-inducing activity behind potential hormonal candidates in this species, we in vitro tested a group of nine hormones: cortisol (Co), 11-deoxycortisol (11-D), corticosterone (coS), 11-deoxycorticosterone (DOC), 17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregn-3-one (DHP) and 17 $\alpha$ ,20 $\beta$ ,21 trihydroxy-4-pregn-3-one (THP), prostaglandin E2 (PGE2), estradiol-17 $\beta$  (E2) and testosterone (T), in their ability to trigger FOM advancement and the production of sex steroids potentially involved in FOM. Using mature female perch, two in vitro experiments were conducted with oocytes at the start of the FOM. The follicles were incubated for 62 h in Cortland media with and without human chorionic gonadotropin (hCG). By the end of the incubation, only DHP and THP triggered the full advancement in FOM even at low doses with the effect of DHP being in vivo validated. However, the de novo productions of E2 and DHP were not shown to be regulated by either of the MIH candidates. Progestagens are hence more credible candidates as MIH than corticosteroids in Eurasian perch. Our in vitro study also revealed that both PGE2 and DHP are strongly associated with ovulation and that PGE2 might have slightly contributed to such DHP activity.

## 1. Introduction

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Eurasian perch, *Perca fluviatilis*, is a newly introduced and highly valuable fish species that can serve in the diversification of European freshwater aquaculture (Fontaine and Teletchea 2019). Great efforts have been therefore made to control its reproductive cycle in order to both extend and synchronize its spawning (Żarski et al. 2011a; Abdulfatah et al. 2013). Such attempts involved the conduction of studies on the environmental control of the reproductive cycle of this percid via the manipulation of both thermal and photoperiodic conditions. Unfortunately, controlling reproduction exclusively through environmental control results in some unpredictable and unwanted asynchronous ovulations that are reflected in variabilities in egg quality and larval sizes (Żarski et al. 2011a; Rocha de Almeida et al. 2019). Hence, it might be relevant to couple hormonal manipulations with the environmental ones to anticipate a better control of reproduction.

Attempts for synchronization of reproduction by direct hormonal stimulations are strongly dependent on the detection of the natural maturation-inducing hormones (MIHs) that are known to control the stages of final oocyte maturation (FOM). However, detailed information concerning the endocrine control of the reproductive cycle in Eurasian perch, especially during the final maturation stages, is still limited (Migaud et al. 2003). So far, multiple experimental approaches have been conducted to investigate the hormonal regulation of oocyte maturation in teleosts, among which in vitro oocyte germinal vesicle breakdown (GVBD) bioassays have proven to be of most use (Nagahama and Yamashita 2008). In the percid highly related to Eurasian perch, the yellow perch (*Perca flavescens*), there were evidences of the role of progestagens such as 17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregn-3-one (17,20 $\beta$ -P or DHP) in this process (Goetz et al., 1989). In other teleosts as salmonids, the

highest plasma concentrations of DHP were recorded before and/or around ovulation (Fostier et al. 1981). In Eurasian perch, in vivo measurements of this progestagen revealed a slight peak (3.4–3.6 ng/ml) in its plasma concentrations during the pre-ovulatory period (Migaud et al. 2003). In other perciform species such as the spotted sea trout (*Cynoscion nebulosus*) and the Atlantic croaker (*Micropogonias undulatus*), another progestagen, namely 17 $\alpha$ ,20 $\beta$ ,21 trihydroxy-4-pregnen-3-one (20 $\beta$ S or THP), was proven to be an effective MIH (Thomas and Trant 1989; Patiño and Thomas 1990a; Pinter and Thomas 1999). The rise in the in vivo levels of progestagens is also accompanied by a rise in prostaglandin levels in some teleosts around ovulation (Juntti et al. 2016). In fact, inhibition of ovulation in vitro was reported to result from the exposure to a prostaglandin synthase inhibitor observed in yellow perch (Berndtson et al. 1989), which came in accordance with the previous reports about the involvement of prostaglandins (PGs) in the ovulation of fish (Lubzens et al. 2010; Baek and Lee 2019). It may be hence postulated that PGs somehow function in coherence in stimulating the occurrence of ovulation at least in percid fishes. There are two major prostaglandins in fish, namely PGE2 and PGF2a, that are known to induce both FOM and ovulation (Kestemont and Henrotte 2015). However, with more evidence on the presence of PGE2 receptor (EP2) in female Eurasian perch in relation with FOM regulation, PGE2 was favoured for being tested compared to PGF2a (Henrotte et al. 2011).

Corticosteroids are another group of steroids that might possess final maturation-inducing activities (Thomas and Trant 1989). Among them, 11-deoxycorticosterone (DOC) and 11-deoxycortisol (11D) were shown to be evident maturation inducers using in vitro studies performed by Goswami and Sundararaj (1974) on catfish (*Heteropneustes fossilis* Bloch). In that study, 11-D was proven to be even more effective than DOC. Also in vitro, 11-D induced almost 100% of GVBD at 0.5  $\mu$ g/ml and ovulation at higher concentrations (1  $\mu$ g/ml) in yellow perch in which DOC also proved to provoke GVBD at 0.1  $\mu$ g/ml and some ovulations at 1  $\mu$ g/ml. A third corticosteroid, corticosterone (coS), was able to induce a large percentage of ovulation in yellow perch but at high concentrations (10  $\mu$ g/ml) (Goetz and Bergman 1978). Cortisol (Co), the main corticosteroid in fish, had been recorded by Eurasian perch, *Perca fluviatilis*, is a newly introduced and highly valuable fish species that can serve in the diversification of European freshwater aquaculture (Fontaine and Teletchea 2019). Great efforts have been therefore made to control its reproductive cycle in order to both extend and synchronize its spawning (Żarski et al. 2011a; Abdulfatah et al. 2013). Such attempts involved the conduction of studies on the environmental control of the reproductive cycle of this percid via the manipulation of both thermal and photoperiodic conditions. Unfortunately, controlling reproduction exclusively through environmental control results in some unpredictable and unwanted asynchronous ovulations that are reflected in variabilities in egg quality and larval sizes (Żarski et al. 2011a; Rocha de Almeida et al. 2019). Hence, it might be relevant to couple hormonal manipulations with the environmental ones to anticipate a better control of reproduction.

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main mentioned corticosteroids (11-deoxycorticosterone, 11-deoxycortisol, corticosterone and cortisol) in the regulation of FOM in Eurasian perch.

In addition to the mentioned progestagens and corticosteroids, Fontaine et al. (2003) found the two sex steroids (17 $\beta$ -estradiol (E2) and testosterone (T)) to be possibly involved in FOM in Eurasian perch. The latter possibility was based on the high plasma concentrations of E2 obtained during the stages of vitellogenesis and the migration of the germinal vesicle, and plasma testosterone was quite high before spawning (Sulistyo et al. 2000; Fontaine et al. 2003). This finding was in agreement with the findings observed in the spotted sea trout, where both E2 and T were the major steroidal products of ovarian incubations prior to FOM (Thomas and Trant 1989). Generally, testosterone was able to induce GVBD in vitro in fish only at high concentrations (Nagahama and Yamashita 2008). In the case of yellow perch, testosterone was effective in the induction of high percentage of GVBD (88% GVBD) at high dose (10  $\mu$ g/ml) but only very minimal ovulation was

monitored (9% ovulation) at such dose (Goetz and Bergman 1978). Before the MIH can commence with its known activity, FOM is said to be initiated by a rise in plasma gonadotropin concentrations, which is, in turn, thought to induce the synthesis of the MIH by the ovarian follicles (Trant and Thomas 1989). Previous reports mentioned hCG, an LH analogue, to have a boosting activity of the MIH production (Patiño and Thomas 1990b; Nagahama and Yamashita 2008; Křišť'an et al. 2013; Das et al. 2014; Mandiki et al. 2017). Ovulation has also been successfully reached via the injection of mammalian gonadotropins in a wide range of species (Lim 2016). It is thereafter fair to note that hCG might be a strong inducer of the typical MIH activity.

All the mentioned reports suggest a wide range of possible MIH or MIH inducers in Eurasian perch. However, the exact nature of this MIH is still unknown in the latter teleost. Hence, in this study, we aim at detecting the putative MIH among a wide range of possible hormones in Eurasian perch as a future attempt to synchronize spawning in this species.

## 2. Material and Methods

All experimental manipulations were carried out in agreement with the European and French national legislations on animal welfare after evaluation and approval of the experimental project (protocol number: APAFIS1 530 7-2 018 053 110 404 570 and 238712020013017452359) by the local ethics committee in France (name: CELMEA). Two independent in vitro experiments and one in vivo experiment were carried out; in the first in vitro experiment, the most putative MIH among 8 hormones was tested on intact follicles from 6 mature Eurasian perch females at the start of maturation. In the second in vitro one, the two MIHs that were suspected to be the most promising were tested over a wider range of doses. In the in vivo experiment, the MIH which proved to be the most potent in the first two in vitro manipulations was injected in live fish at two different doses to confirm its MIH activity.

### 2.1. Experimental animals and rearing condition

A group of 2-year-old **female** Eurasian perch was used with an average weight of 518.4 g, standard length of 27.8 cm and GSI of 21.5%. Fish were obtained from Lucas Perches company (Lorraine, France) and transferred to the Aquaculture Experimental Platform (AEP, registration number for animal experimentation C54547-18) belonging to the URAFPA lab of the University of Lorraine (Nancy, France). In this facility, fish were kept in tanks (2 m<sup>3</sup>) with recirculating water system (RAS) exposed to one photothermal program able to induce and drive gonadogenesis and to reach the final stages of reproduction (Fontaine et al. 2015) The photothermal program was initiated at day 1 (photoperiod: 16 h light (L):8 h dark (D); temperature 22 °C) where fish were at the sexual rest period after which both temperature and exposure to light duration were gradually decreased to allow fish to enter into the phase of active oocyte growth and vitellogenesis. Then, the temperature and light conditions were maintained steady (photoperiod: 8 h L:16 h D; temperature 6 °C) during 4.5 months to allow the progress of vitellogenesis. Later, both temperature and light duration were increased to allow fish to enter into FOM and later

spawn. During this photothermal program, water was measured three times a week. pH had been maintained between 7 and 7.5 by NaCO<sub>3</sub> additions, and dissolved oxygen had also been maintained above 6 mg/L. The concentrations of total ammonia and nitrite nitrogen were measured using indophenol blue colorimetric methods in which nitroprusside was used as the catalyst and the water sample containing the ammonium ions from each tank was treated with a solution of chlorine and phenol to give indophenol blue which can be detected by the spectrophotometer (CARY I). The concentration assessed in each tank remained always below 1 mg/L. Fish had been fed to satiety with artificial feed supplied from Le Gouessant (B-repro 32 Astax) from the initiation of the program until spawning.

### 2.2. Chemical

All chemicals and fatty acids were purchased from Sigma-Aldrich (France) and Cayman Chemical (Ann Arbor, MI, USA). Cortland medium used for follicular incubations was prepared under sterile conditions and contained the following components (g/L): 7.25 NaCl, 0.38 KCl, 0.23 CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.00 NaHCO<sub>3</sub>, 0.41 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.23 MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.23 MgSO<sub>4</sub>·7 H<sub>2</sub>O, 5.20 HEPES, 1.00 BSA, 0.03 penicillin and 0.05 streptomycin at pH 7.3–7.4. All hormones used in the in vitro incubations were initially dissolved in ethanol. Aliquots of these concentrated ethanolic solutions were then dissolved directly in Cortland medium to reach the desired concentration in the medium. The volume of ethanol was 0.5% of the final incubation volume.

### 2.3. In vitro fish follicle preparation

Each female was anaesthetized (MS-222; Sigma-Aldrich, Lyon, France), catheterized, using a catheter (DAHLHUSEN; REF 07.020.06.600; 1.2 mm internal diameter and 2 mm external diameter), and checked to be at stage 1 (out of the 6 stages in Eurasian perch) according to the classification of Źarski et al. (2011a). The average

diameter of oocytes at stage 1 was measured to be at  $1.01 \pm 0.22$  mm. Sampled oocytes were then placed in Serra's solution (ethanol/formalin/glacial acetic acid, 6:3:1; v/v/v) in which they were slowly mixed and left for 5 min until their cytoplasm became clarified (Żarski et al. 2011b). The oocyte maturation stage was then evaluated under a binocular microscope, magnification  $\times 4$  (Olympus, SZX7). Fish proven to be at stage 1 or 3 were immersed in a bath containing 300 mg/L of the anaesthetic MS-222 to provoke the fish euthanasia. Then, fish were decapitated and the whole ovary was removed, weighed and dissected, in Cortland medium at 13 °C, into small pieces containing 10–15 prematurational follicles attached to the surrounding extrafollicular tissue (Henrotte et al. 2011).

## 2.4. Part 3

In both experiments, three of the ovarian pieces (each containing 10–15 follicles) per female were placed into each well of the sterile 12-well plastic plates (STARLAB, Sarstedt, Germany) containing 2 ml of culture medium per well. A volume of 10  $\mu$ l of dissolved hormone in ethanol was added in the culture medium just before adding the follicles.

### 2.4.1. Experiment 1

Two different sets of Cortland media were prepared under sterile conditions to be used as culture media, either containing hCG (25 IU/ml) or not. Eight hormones were tested: the four corticosteroids (11-deoxycortisol, cortisol, corticosterone and 11-deoxycorticosterone), the progestagen DHP, the two sex steroids (17 $\beta$ -estradiol and testosterone) and prostaglandin E2, each at three different doses selected according to the circulating plasma concentrations during the FOM in Eurasian perch and/or previously reported in vitro activities in close fish (Goetz and Bergman 1978; Pinter and Thomas 1999; Planas et al. 2000; Migaud et al. 2003; Miura et al. 2007; Milla et al. 2009; Henrotte et al. 2011; Mandiki et al. 2017). The desired doses of each hormone (in ng/ml) are presented in Table 1.

### 2.4.2. Experiment 2

Cortland without hCG was used in all culture wells, and the two progestagens (DHP and THP) were only tested but at 5 different doses including lower doses than the ones tested in the first experiment. The desired doses of each hormone (in ng/ml) are presented in Table 2.

For each female, follicles were placed in three sets of control wells:

1. CTet: Cortland medium plus 10  $\mu$ l standard ethanol
2. hCG: hCG in Cortland medium plus 10  $\mu$ l standard ethanol (used in experiment 1)
3. CT: Cortland medium alone

After the addition of the follicles to all wells containing the culture media and the appropriate dose of each hormone, all plates were incubated at 13 °C for 62 h under agitation (Berndtson et al. 1989; Goetz 1997; Henrotte et al. 2011) in the dark until possible advancement of the oocyte maturation

takes place (Berndtson et al. 1989; Żarski et al. 2011b). The media were replaced once after 18 h with new experimental media, and the treatments were renewed as well. Media after 62 h (end of incubation) were placed in Eppendorf tubes to be stored at  $-20$  °C for later steroidal enzyme-linked immunosorbent assay (ELISA).

## 2.5. Estimation of the FOM advancement following the in vitro hormonal treatments

Estimation of the FOM advancement following the in vitro hormonal treatments

After 62 h of incubation, agitation was stopped. The wells were emptied, and Serra's solution (ethanol: formaldehyde: acetic acid in the proportion 6:3:1, respectively) was added to each well to clarify the oocytes. After 5 min, the oocytes were observed under the binocular to monitor for each well: (1) the most represented (MR) stage of FOM (represented by the biggest number of follicles among selected populations), (2) the highest reached (HR) stage of FOM (represented by the most advanced follicles among the same selected populations) following the classification of Żarski et al. (2011b) and (3) ovulation (represented by counting the number of ovulated follicles) with expulsion of the oocytes from the follicular layers.

## 2.6. Cytotoxic assay following the in vitro incubations

The Cytotoxicity LDH Assay Kit-WS T (BCCC2868) from Sigma (Darmstadt, Germany) was used to evaluate the cell viability of the follicles after the end of the follicle incubation with or without hormonal treatments (6 fish per treatment). This test is based on the determination of lactate dehydrogenase (LDH) activity released. The manufacturer's recommendations were followed using lysed follicles. The percentage of cytotoxicity estimated at 4% was considered as low.

## 2.7. Hormone assays following the in vitro incubations

All hormone measurements were carried out in the culture medium after validation of the signal specificity by testing the absence of bias caused by fresh culture medium and by testing the hormonal recovery after supplementations of hormones in the culture media and/or by checking the proportionality between the dilution factors of the culture medium and the calculated hormonal level.

Levels of DHP were assayed on 100  $\mu$ l of the incubation culture media of all treatments in experiment 1 by ELISA using fish 17 $\alpha$ , 20 $\beta$  dihydroxyprogesterone (17 $\alpha$ , 20 $\beta$ OH-PROG) ELISA Kit (MBS 2602842) obtained from MyBioSource (San Diego, USA). Sensitivity was 0.06 ng/ml. Intra-assay coefficient of variation (CV) was less than 5%, and inter-assay coefficient was less than 10%.

Levels of E2 were assayed on 50  $\mu$ l of the incubation culture media of all treatments in experiment 1 by ELISA using E2-EASIA Kit (KAP0621) obtained from Diasource (Louvain-La-Neuve, Belgium); 1:50 dilutions of the samples were made. Sensitivity was  $5 \pm 2$  pg/ml. Intra-assay CV was less than 4%, and inter-assay coefficient was less than 5%.

Both DHP and E2 were measured in the culture media after their respective additions to estimate the recovery rate of these hormones at the end of the incubation period (62 h).

Levels of PGE2 were assayed on 100 µl of the incubation culture media obtained in experiment 1 from both controls and DHP treatments, with or without hCG, using PGE2 ELISA Kit (KHL 1701) obtained from Thermo Fisher Scientific (Illkirch-Graffenstaden, France). Sensitivity was 31.3 pg/ml. Intra-assay CV was less than 10%, and inter-assay coefficient was less than 5 %.

## 2.8. In vivo DHP injection

Another group of 2-year-old female Eurasian perch was used with an average weight of 650.5 g and standard length of 32.17 cm. The latter fish were similarly obtained from Lucas Perches company (Lorraine, France) and transferred to the same Aquaculture Experimental Platform exposed to the same photothermal program utilised for fish belonging to the first two in vitro manipulations. Each fish was anaesthetized and catheterized, and its FOM stage was determined using the same principles as in section "In vitro fish follicle preparation". Fish proven to be at stage 1 or 2 were injected intraperitoneally with one of the following treatments: (1) negative control with saline solution, the vehicle of all molecules (NaCl 0.9%, n = 12); (2) low DHP dose (0.2 mg/kg BW, n = 7); (3) and high DHP dose (2 mg/kg BW, n = 7). Fish were then placed back into their tank, and FOM advancement was recorded for each fish 2 days (day 2) and

4 days (day 4) following the injections (day 0). Spawning was checked daily until 14 days post injection, and every 2 days, all fish were stripped manually by gently massaging the lower abdomen. The success of spawning was evaluated based on (1) the spawning rate (100 × number of spawned fish/total number of fishes for each group of control and treated fish) and (2) the average latency time of spawned fish (duration in hours between injection and spawning) for each group of control and treated fish.

## 2.9. Statistical analysis

For all dependent variables, after checking the data normality and the homogeneity of variances, if the assumptions of the ANOVA test were not validated, the effects of the different hormonal treatments were determined using Friedman test followed, when significant, by Conover test. If the assumptions of the ANOVA test were validated, the effects of the different hormonal treatments were determined using one-way ANOVA or two-way ANOVA for paired samples, followed, when significant, by LSD tests. The comparison of in vitro ovulation rates was performed using chi-square test. The differences between the treatments were considered to be statistically significant if  $P \leq 0.05$ . Data were log or square root transformed when necessary and were also corrected using the Bonferroni test or the Benjamini and Hochberg method. Statistica software (StatSoft, Tulsa, OK, USA) and the free software R version 3.3.1 were used. In case of parametric tests, the data showed the mean ± SD while the data showed median ± quartiles for non-parametric tests.

## 3. Results

### 3.1. In vitro hCG effect on FOM advancement and ovulation

The effect of the incorporation of hCG with the hormonal treatments was evaluated on the progress of FOM. The results revealed no significant effect ( $P > 0.05$ ) for inserting hCG in the culture media when considering all hormonal treatments confounded ( $P > 0.05$ ). Such non-significant effect was attained when considering both the most represented stage (MR) and the highest reached stage (HR) by follicles.

### 3.2. In vitro hormonal treatment effects on FOM advancement and ovulation

#### 3.2.1. Experiment 1

By comparing the MR and HR stages between negative control (CTet) and each treatment (Fig. 1a and b), it was revealed that  $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one (DHP) at all doses (10 ng/ml, 100 ng/ml and 1000 ng/ml) gave a significant effect ( $P < 10^{-4}$  for both MR and HR) where it induced all follicles to advance through all stages of the FOM until ovulation.

Prostaglandin E2 (PG) induced slight progress in FOM (stage 3 for MR and stages 3–4 for HR) which was significant ( $P < 0.05$ ) compared to the negative control (except for the dose 1000 ng/ml) (Fig. 1a and b).

Concerning corticosteroids, Co managed to significantly ( $P < 0.01$ ) induce a rather slight advancement in FOM at all tested doses (10 ng/ml, 100 ng/ml and 1000 ng/ml) in most of the follicles (MR) and at only two doses (10 ng/ml, 100 ng/ml) in most advanced follicles (HR), with  $P < 0.05$  (Fig. 1a and b). Similarly, corticosterone (coS) also revealed the same effect ( $P \leq 0.01$ ) in MR and HR stages but at all its tested doses (0.1 ng/ml, 1 ng/ml, 10 ng/ml). Finally, the other corticosteroids (11-D and DOC) and both sex steroids (E2 and T) failed to provoke the advancement of FOM (Fig. 1a and b), except 11-D at the highest dose (1000 ng/ml) for the HR stage.

Among all tested hormones, only PGE2 and DHP triggered some ovulations (Fig. 2,  $P < 0.05$ ). Regarding PGE2, 60–100% and 40–50% ovulations occurred at such slight advancements in the FOM (stages 3–4 by most follicles) with and without hCG, respectively (Fig. 2). DHP, on the other hand, induced consequent ovulation (65–100%) following a normal advancement of FOM, whereas PGE2 induced ovulation without any marked advancement of FOM.

#### 3.2.2. Experiment

The results revealed that when subjecting follicles to even a lower dose of both DHP and THP at 1 ng/ml, follicles were still able to significantly ( $P \leq 0.01$ ) advance from stage 2 (stage reached by negative controls) to stage 3 regarding both MR and HR stages of the follicles. Below this dose (0.1 ng/ml), neither progestagens could significantly ( $P > 0.05$ )

induce FOM progress regarding both MR and HR stages (Fig. 3a and b). Concerning the other doses (10 ng/ml, 100 ng/ml, 1000 ng/ml), FOM was also significantly induced ( $P < 10^{-4}$ ) considering both MR and HR stages, reaching the stage 7 (ovulation) in some cases, and DHP being much more effective than THP (Fig. 3a and b).

### 3.3. In vitro hormonal treatment effects on DHP, E2 and PGE2 production

None of the treatments significantly induced DHP or E2 production compared to the negative control CTet ( $P > 0.05$ ). Thus, none of the hormonal treatments could affect either DHP or E2 production by the follicles within the 62 h of their incubation. At the end of the incubation, the recovered DHP or E2 concentration, in DHP-treated or E2-treated wells, respectively, was very low, probably due to steroid binding to the plastic of the culture plate along the time course of follicle incubation.

Our results showed that there was no significant overall effect for applying hCG treatment on PGE2 production. However, the two-way ANOVA revealed the existence of a significant interaction ( $P < 0.01$ ) between the two factors: "hCG treatment" and "DHP dose". Indeed, in the presence of hCG, the effect of DHP on PGE2 production ( $P < 10^{-4}$ ) by comparison to the negative control (CT) was significant from the lowest dose (10 ng/ml) while this DHP effect was only observed at the highest dose (1000 ng/ml) without hCG ( $P < 10^{-5}$ ). In fact, at the lowest dose of DHP (10 ng/ml), hCG-treated follicles were significantly induced to produce PGE2 at a level similar to non-hCG-treated follicles at the highest dose of DHP (1000 ng/ml) (Fig. 4).

## 4. Discussion

In the current study, the in vitro effect of exposing post-vitellogenic intact follicles to a set of nine hormones on the progress of FOM was evaluated in sexually mature female Eurasian perch. Both DHP (with and without hCG) and THP were able to induce final oocyte maturation by induction of GVBD (Table 3). Among all the sex hormones tested, DHP and THP were the only inducers of GVBD (stage 6) and ovulation (stage 7) in Eurasian perch. Both DHP and THP have been reported to be involved in the in vitro induction of oocyte maturation of post-vitellogenic follicles in various teleost species (Migaud et al. 2003). Also, DHP appears to be the MIH of salmonids and cyprinids and other fish families (reviewed by Scott and Canario 1987). For example, DHP was revealed as the most potent MIH in the grey mullet (*Mugil cephalus* L.) (Das et al. 2014), striped sea bass (*Morone saxatilis*) (Weber and Sullivan 2001), amago salmon (*Oncorhynchus rhodurus*) (Patiño and Thomas 1990a), rainbow trout (Fostier et al. 1973) and goldfish (Jalabert et al. 1977). THP was also reported to be the MIH in both perciforms, the Atlantic croaker (Patiño and Thomas 1990a) and spotted seatrout (Thomas and Trant 1989). In our study as well, both DHP and THP induced FOM in adose-dependent manner. Our in vitro data thus support that both progestagens are MIH in Eurasian perch. In addition, the effectiveness of in vivo DHP injection to promote clear advancement of FOM and the triggering of rapid and quite synchronous spawning is a further argument to nominate DHP as an MIH in Eurasian perch.

On the other hand, corticosteroids and the two sex steroids (E2 and T) induced none or very slight FOM advancement and hence cannot be considered as putative MIH in Eurasian perch. In addition, they did not show any regulatory activity of

### 3.4. In vivo DHP injection effect on FOM advancement and spawning

Our results of injecting DHP into maturing female perch revealed that there was a significant overall effect of DHP injection on FOM advancement ( $P < 10^{-6}$ ) by comparison to the negative control (fish injected with saline solution). Interestingly, the two-way ANOVA revealed a significant interaction ( $P < 0.01$ ) between the two factors: dose of injected DHP and postinjection time. Indeed, the advancement of FOM was more progressive for fish injected with the low dose of DHP (0.2 mg/kg BW) than for fish injected with the high dose (2 mg/kg BW) (Fig. 5).

After DHP injection, whatever the dose, the vast majority of fish had spawned at day 6 post injection and complete spawning was attained at day 10 (Fig. 5). In both cases, however, we noticed the absence of egg ribbons and follicles for some females was still at stages 5–6. Only 33.3% of the control fish spawned at day 10 post injection where their spawning rate had been increasing at a much slower rate compared to the DHP-treated fish. The spawning rate in those control fish managed to reach only 42% at day 14 post injection. Such delay between DHP-treated fish and control fish was fairly demonstrated by a significant difference in the latency time ( $P < 0.01$ ) estimated at  $154.8 \pm 54.7$  h,  $148.5 \pm 18.7$  h and  $207.5 \pm 54.7$  h for the high dose, low dose and control spawned fish, respectively.

DHP production by maturing follicles after the whole incubation period (62 h). They also did not exert any significant effect on the regulation of E2 production despite the latter being considered as a FOM blocker in a range of fish species (Majumder et al. 2015; Das et al. 2017). Still, at a very short term (6 h) in vitro, the corticosteroids DOC and cortisol elicited downregulation of E2 and DHP production in perch post-vitellogenic oocytes (Mandiki et al. 2017). Altogether, it is thought that the corticosteroids E2 and T are not involved markedly in the final achievement of FOM in Eurasian perch even if early transitory effects on DHP attenuation remain possible. However, corticosteroids were shown to induce FOM in the close species yellow perch (Goetz and Bergman 1978). Therefore, even within the percid family, we cannot rule out any inter-species specificity concerning the nature of the MIH.

Our study also revealed the inexistence of an effect of combining hCG with the MIH (DHP) on FOM progress. This came in agreement with what had been shown by a recent study on the induction of oocyte maturation and steroidogenesis by gonadotropins in grey mullet (Das et al. 2014). Hence, it is possible that in our study, hCG did not have an effect on FOM progress in media which were subjected to DHP treatments due to the fact that DHP had already solely mediated the follicular progress

through FOM and GVBD until ovulation. Indeed, as realised by Das et al. (2014), hCG with DHP rather significantly stimulates steroidogenesis. It is thereafter possible that the function of hCG is to solely stimulate the synthesis of an MIH as proposed by Thomas et al. in 2002 and also recently shown in Eurasian perch (Mandiki et al. 2017). In addition,



the induction of FOM by gonadotropin could be mimicked by MIH alone, supporting even more the putative role of gonadotropin in solely stimulating the synthesis of the MIH (Thomas et al. 2002). As hCG acts on the enzymatic production of DHP rather than amplifying the MIH activity, perhaps in our experiment, hCG was almost useless when considering its effect on MIH activity.

Besides detecting the nature of the MIH in Eurasian perch, another finding of this study was that PGE2 was the only hormone besides DHP and THP to unleash a rather astonishing impact on incubated follicles where it induced ovulation in follicles still at slightly advanced stages of maturation. This is not so surprising since the involvement of prostaglandins, notably PGE2, in ovulation has been previously documented in several fish species including the yellow perch (Goetz et al. 1989; Lubzens et al. 2010; Fujimori et al. 2011). Generally, prostaglandins like PGE2 can stimulate the ovulation of follicles that had been in vitro stimulated to mature by gonadotropins or MIH (Baek and Lee 2019). In our study, however, the individual treatment of PGE2 was enough to stimulate ovulation regardless of FOM completion. Additionally, it was concluded in yellow perch that once oocytes are induced to be ovulated, their advancement through final maturation would be disrupted, resulting in oocytes being ovulated before completion of the FOM (Goetz and Bergman 1978). Considering the latter conclusion in yellow perch and the previously mentioned potent effect of PGE2 on ovulation, it might be possible to explain why we obtained ovulations at stages 3 and 4 of FOM under exclusive treatments with PGE2. Such findings may offer two conclusions: first, PGE2 cannot be considered as an MIH but rather an ovulation inducer as has been recorded in many other teleosts (Baek and Lee 2019), and second, ovulation and FOM may be considered as two independent phenomena in terms of endocrine regulation where the progress of follicles through the FOM completion might not be a crucial pre-requisite for ovulation to occur.

Another finding in this study was that follicles treated with a pharmacological dose of DHP, in the absence of hCG, exhibited a significant rise in the production of PGE2. This significant rise was interestingly similar to the significant rise obtained with 100 times lower DHP dose but which had been

coupled to hCG treatment. In some species, as in the Atlantic croaker and red seabream (*Pagrus major*), oocyte response to the MIH in inducing FOM is highly dependent on the pre-treatment with gonadotropin (Patiño and Thomas 1990b; Pang and Ge 2002). The elevated efficiency of DHP-treated follicles to induce PGE2 production in the presence of hCG may be similarly due to the fact that this oocyte response to the MIH is more sensitive after gonadotropin treatment. In longchin goby (*Chasmichthys dolichognathus*) and Eurasian perch, there had been findings about the reciprocal of such relation between DHP and PGE2 where DHP was found to be produced, though insignificantly, by PGE2. DHP upregulation by another prostaglandin (namely PGE1) and by arachidonic acid (the PGE2 precursor) was nevertheless shown to be highly significant in longchin goby and Eurasian perch, respectively (Henrotte et al. 2011; Baek and Lee 2019). Up to date, few information is available on the progestagen-prostaglandin interaction during FOM (Baek and Lee 2019). It is now possible to believe that DHP exerts not only direct effect on ovulation but also slight indirect effect on ovulation through the de novo production of PGE2. At the follicular level, PGE2 exerts their effects through membrane receptors that have

been previously identified on the follicular membranes of medaka fish (*Oryzias latipes*) and Eurasian perch as well (Fujimori et al. 2011; Henrotte et al. 2011). In medaka, it was shown that the expression of the identified PGE2 receptors (EP4b) increases dramatically as the follicles approach their destined ovulation. The latter finding thereafter signifies the putative role of PGE2 and its receptors in ovulation (Fujimori et al. 2011). Further investigations are thus needed to understand the role of prostaglandin receptors in the perch ovulatory process.

In conclusion, our study strongly nominates the two progestagens (DHP and THP) rather than the four tested corticosteroids as the most potent MIH in Eurasian perch. Also, our study revealed that both DHP and PGE2 are inducers of ovulation and our data support that PGE2 might have contributed a little to such DHP activity. Nevertheless, such relation should be further investigated in an attempt to understand the endocrinological relation linking these two hormones especially at ovulation.




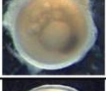
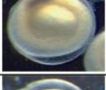


**Table 1. Hormonal doses (ng/ml) tested in experiment 1**

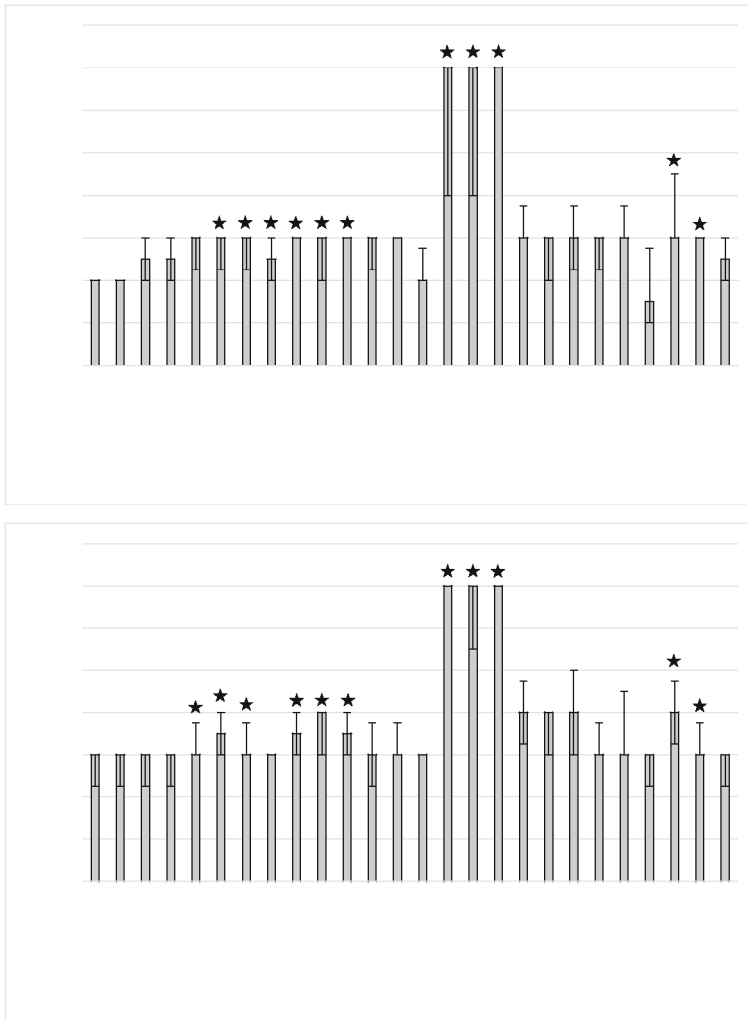
	Doses (ng/ml)			References
	D1	D2	D3	
11-Deoxycortisol (11-D)	10	100	1000	Goetz and Bergman (1978)
Cortisol (Co)	10	100	1000	Mandiki et al. (2017); Goetz and Bergman (1978)
Corticosterone (coS)	0.1	1	10	Milla et al. (2009); Goetz and Bergman (1978)
11-Deoxycorticosterone (DOC)	1	10	100	Mandiki et al. (2017); Pinter and Thomas (1999)
17 $\alpha$ ,20 $\beta$ Dihydroxy-4-pregnen-3-one (DHP)	10	100	1000	Planas et al. (2000); Miura et al. (2007); Pinter and Thomas (1999)
17 $\beta$ -Estradiol (E2)	0.1	1	10	Migaud et al. (2003); Planas et al. (2000); Miura et al. (2007); Goetz and Bergman (1978)
Testosterone (T)	1	10	100	Migaud et al. (2003); Planas et al. (2000); Goetz and Bergman (1978)
Prostaglandin E2 (PG)	10	100	1000	Henrotte et al. (2011)

**Table 2. Hormonal doses (ng/ml) tested in experiment 2**

	Doses (ng/ml)					References
	D1	D2	D3	D4	D5	
17 $\alpha$ ,20 $\beta$ Dihydroxy-4-pregnen-3-one (DHP)	0.1	1	10	100	1000	Miura et al. (2007); Pinter and Thomas (1999)
17 $\alpha$ ,20 $\beta$ ,21 Trihydroxy-4-pregnen-3-one (THP)	0.1	1	10	100	1000	Pinter and Thomas (1999)

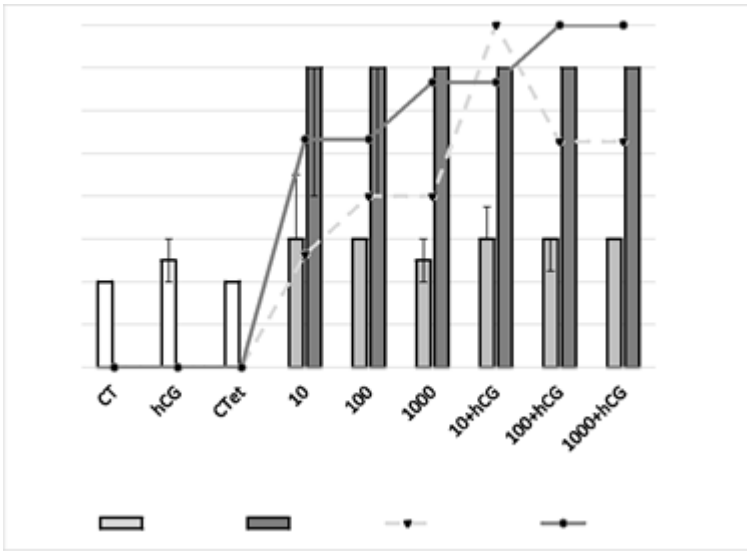
**Table 3. Classification of the final oocyte maturation (6 stages) in Eurasian perch according to Źarski et al. (2011b)**

Stages		
1	The Germinal Vesicle (GV) is situated in the oocyte centre, oil droplets are poorly visible	
2	Beginning of GV migration, beginning of coagulation of clearly visible oil droplets	
3	Migration of GV (above half of the oocyte diameter), oil droplets are clearly visible	
4	The GV is at the oocyte periphery, a large forming oil droplet is clearly visible; the droplet diameter is greater than the GV diameter and it reaches the size of about 1/3 of the oocyte diameter; smaller droplets are also visible	
5	The GV is situated at the oocyte edge, one large (size of about half the oocyte diameter) oil droplet is clearly visible	
6	Oocyte samples taken for analysis are macroscopically transparent; no visible GV after they were placed in Serra's solution (following GVBD), oocytes at the pre-ovulation stage	
7	Ovulated eggs, FOM is completed, follicular layers released from the oocyte	



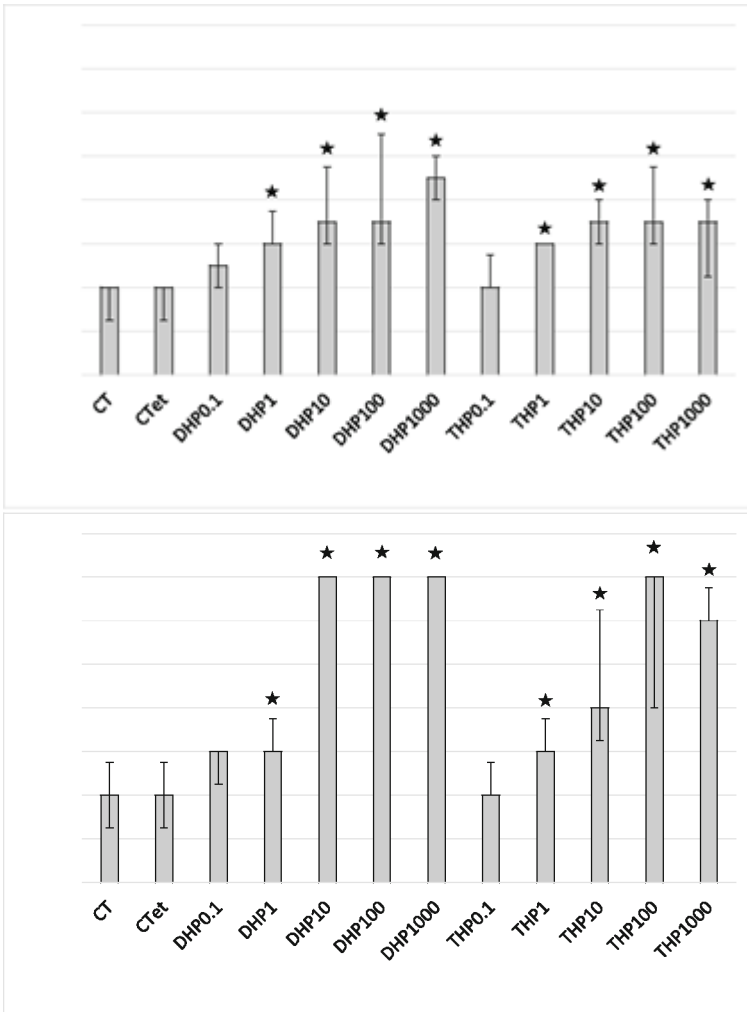
**Figure 1.**

The in vitro effect of the different hormonal treatments on FOM advancement in culture media into which hCG was not incorporated. The treatments are the following: CT (control), CTet (ethanol vehicle only), 11-D (11-deoxycortisol; 10 ng/ml, 100 ng/ml and 1000 ng/ml), Co (cortisol; 10 ng/ml, 100 ng/ml and 1000 ng/ml), coS (corticosterone; 0.1 ng/ml, 1 ng/ml and 10 ng/ml), DOC (11-deoxycorticosterone; 1 ng/ml, 10 ng/ml and 100 ng/ml), DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one; 10 ng/ml, 100 ng/ml and 1000 ng/ml), E2 (17 $\beta$ -estradiol; 0.1 ng/ml, 1 ng/ml and 10 ng/ml), T (testosterone; 1 ng/ml, 10 ng/ml and 100 ng/ml) and PG (prostaglandin E2; 10 ng/ml, 100 ng/ml and 1000 ng/ml). The values recorded in this figure represent a the median of the stages reached by the biggest population of follicles thereby representing the most represented stage  $\emptyset$  and b the median of stages reached by the most advanced follicles representing the highest reached stage (HR). The upper tip of the error bars represents the third quartile (Q3), and the lower tip represents the first quartile (Q1). The stages reached by follicles under hormonal treatment were compared with the negative control CTet, and statistically different values ( $P < 0.05$ ) are marked by stars



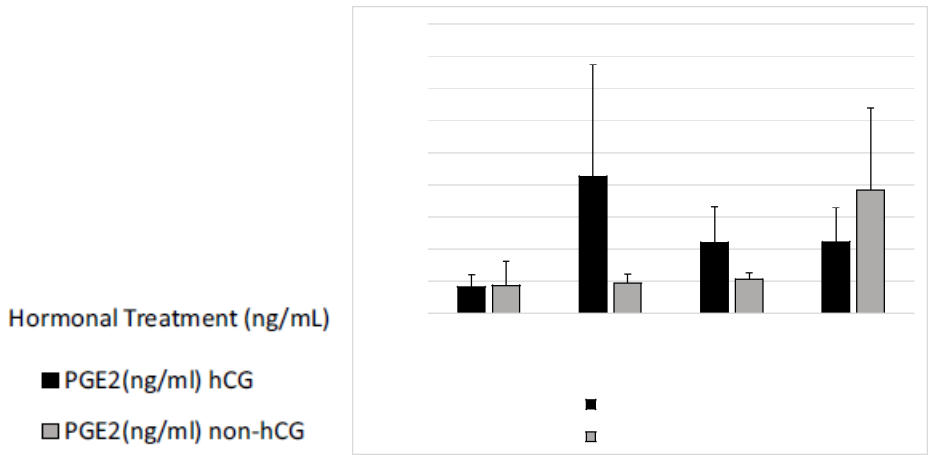
**Figure 2.**

The in vitro effect of DHP and PGE2 hormonal treatments on FOM advancement and ovulation with and without hCG. The treatments are the following: CT (control), hCG (hCG plus ethanol vehicle), CTet (ethanol vehicle only), PGE2 (prostaglandin E2; 10 ng/ml, 100 ng/ml and 1000 ng/ml) and DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one; 10 ng/ml, 100 ng/ml and 1000 ng/ml) with and without hCG. The values recorded represent the median of the stages reached by the biggest population of follicles thereby representing the most represented stage  $\emptyset$ . The upper tip of the error bars represents the third quartile (Q3), and the lower tip represents the first quartile (Q1). The ovulation rate obtained under the separate treatments of DHP and PGE2 was compared with the three negative controls: CT, hCG and CTet



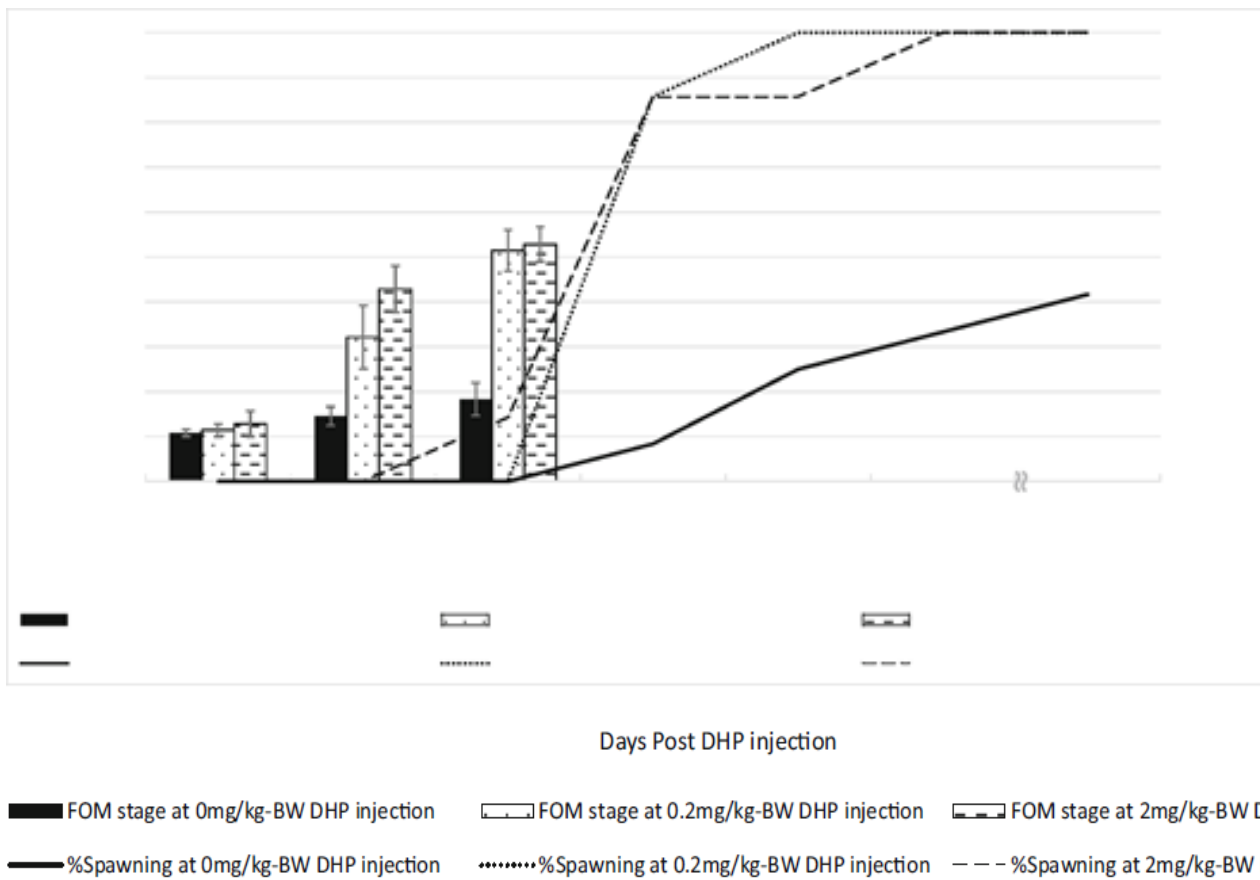
**Figure 3.**

The in vitro effect of the two progestagens (DHP and THP) hormonal treatments on FOM advancement. The treatments are the following: CT (control), CTet (ethanol vehicle only), DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one; 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml and 1000 ng/ml), THP (17 $\alpha$ ,20 $\beta$ ,21 trihydroxy-4-pregnen-3-one; 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml and 1000 ng/ml). The values recorded in this figure represent a the median of the stages reached by the biggest population of follicles thereby representing the most represented stage (MR) and b the median of stages reached by the most advanced follicles representing the highest reached stage (HR). The upper tip of the error bars represents the third quartile (Q3), and the lower tip represents the first quartile (Q1). The stages reached by follicles under hormonal treatment were compared with the negative control CTet, and statistically different values ( $P < 0.05$ ) are marked by stars



**Figure 4.**

The levels of PGE2 produced by follicles under the different DHP treatments with and without hCG. The treatments are the following: CTet (ethanol vehicle with or without hCG), DHP (17 $\alpha$ ,20(3dihydroxy-4-pregnen-3-one; 10 ng/ml, 100 ng/ml and 1000 ng/ml with or without hCG). The values recorded represent the mean + SD of concentration of PGE2 (ng/ml). The PGE2 levels produced under each hormonal treatment were compared to each other, and statistically different values (P < 0.05) are marked by different letters



**Figure 5.**

The in vivo effect of injecting DHP at 0.2 mg/kg BW or 2 mg/kg BW doses on FOM advancement and spawning. The treatments are the following: DHP (17  $\alpha$ ,20(3dihydroxy-4-pregnen-3-one) injection at 0 mg/kg BW (representing the negative control with saline solution), 0.2 mg/kg BW (representing the low dose of DHP) and 2 mg/kg BW (representing the high dose of DHP). The values recorded in this figure represent (1) the mean  $\pm$  SD of the stages reached by the biggest population of follicles thereby representing the most represented stage  $\emptyset$  and (2) the spawning rate offish under each treatment

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