



# Investigating the roles behind four main corticosteroids in the reproductive cycle of female Eurasian perch *Perca fluviatilis* L. reared in RAS

Leila El Mohajer

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Par Leila EL MOHAJER

Sous la direction de Dr. Sylvain MILLA

**Investigating the roles behind four main corticosteroids in the reproductive cycle of female Eurasian perch *Perca fluviatilis* L. reared in RAS**

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***"I would like to dedicate this thesis to my mom, letting her know that every achievement in my life is hers"***



# ABSTRACT

## **Investigating the roles behind four main corticosteroids in the reproductive cycle of female Eurasian perch *Perca fluviatilis* L. reared in RAS**

Eurasian perch, *Perca fluviatilis* L. is a promising candidate for the freshwater aquaculture and is mainly cultivated in intensive monoculture in recirculating aquaculture systems (RAS). Such systems depend on the artificial control of both photoperiod and temperature to induce synchronous spawning. However, the production of eggs with optimal quality under such artificial conditions proved to be challenging. In several studies, the latter challenges in obtaining optimal reproductive performances in RAS-reared fish was accredited to the stress imposed by external stressors such as photoperiod and temperature manipulations. Corticosteroids are known to signal the internal physiological reflection of external stressors *via* the HPI system in vertebrates. Research defines four major corticosteroids (11-deoxycorticosterone, 11-deoxycortisol, corticosterone, and cortisol) that can interfere with the Hypothalamic-Pituitary-Gonadal (HPG) axis and could thereby lead to the observed reproductive drawbacks. Up to date, neither the basic roles nor the kinetics of these four corticosteroids during the whole reproductive cycle of female perch has been well defined. In this context, the present work aimed to first characterize the functions of the defined corticosteroids in female perch reproduction as a primary attempt to better combat the challenges faced in obtaining optimal reproductive outcomes in RAS.

The first part of this study allowed to first *in vivo* detect and monitor the plasma kinetics of the four corticosteroids along the reproductive cycle of domesticated female perch while monitoring the expression of the different receptors and enzymes mediating their production and possible functions. In this part, some of the corticosteroids revealed elevations in their plasma levels at the beginning and end of the reproductive cycle with slight elevations during

egg-yolk formation. In the later parts of this study, corticosteroids were thereby further investigated using *in vitro* cell culture to detect any direct role for corticosteroids at the time of their plasma rise. The latter *in vitro* investigations supported the existence of a minor role for some corticosteroids in the final stages of egg maturation before spawning while suggesting a possible role in egg yolk formation. Eventually, this study supported the involvement of corticosteroids in critical stages of Eurasian perch reproduction which could later explain the reproductive drawbacks observed in RAS systems.

**Keywords:** Corticosteroids, reproduction, plasma kinetics, stress, RAS, Eurasian perch.

# RESUME

## **Étude des rôles de quatre corticostéroïdes principaux dans le cycle de reproduction de la perche femelle *Perca fluviatilis* L. élevée dans RAS**

La perche eurasienne, *Perca fluviatilis* L. est un candidat prometteur pour le développement de l'aquaculture en eau douce et est principalement élevée en monoculture intensive dans des systèmes d'aquaculture avec recirculation de l'eau (RAS). De tels systèmes nécessitent le contrôle artificiel de la photopériode et de la température pour induire des pontes synchrones. Cependant, la production d'œufs de qualité optimale dans de telles conditions artificielles s'avère difficile. La littérature montre que l'obstacle à l'obtention de performances de reproduction optimales en RAS est attribué au stress induit par des facteurs externes tels que les manipulations de la photopériode et de la température. Chez les vertébrés, les corticostéroïdes sont connus pour refléter la perturbation physiologique induite par des facteurs de stress. La recherche définit quatre corticostéroïdes majeurs (11-déoxycorticostérone, 11-déoxycortisol, corticostérone, et cortisol) qui peuvent interférer avec l'axe hypothalamo-hypophyse-gonade (HPG) et qui pourraient être liés aux défauts de reproduction. A ce jour, ni les rôles fondamentaux, ni la cinétique de ces quatre corticostéroïdes au cours du cycle de reproduction de la perche femelle n'ont été bien définis. Dans ce contexte, ce travail vise d'abord à caractériser les fonctions de ces corticostéroïdes dans la reproduction de la perche femelle afin d'améliorer les performances de reproduction obtenues en RAS.

La première partie de cette étude a permis de détecter et de suivre *in vivo* la cinétique plasmatique des quatre corticostéroïdes tout au long du cycle de reproduction de la perche femelle domestiquée et d'analyser l'expression des gènes des différents récepteurs aux corticostéroïdes et ceux des enzymes impliqués dans leur métabolisme. Dans cette partie,

certaines des corticostéroïdes ont montré une hausse de leurs taux plasmatiques au début et à la fin du cycle de reproduction avec de légères élévations au cours de la vitellogénèse. Dans les dernières parties de cette étude, le rôle physiologique de ces corticostéroïdes a été approfondi en utilisant une culture folliculaire *in vitro*, ceci afin de détecter tout rôle direct des corticostéroïdes au moment de leur augmentation plasmatique. Ces études *in vitro* montrent un rôle mineur pour certains de ces corticostéroïdes dans les derniers stades de la maturation méiotique ovocytaire, avant la ponte. Elles suggèrent aussi un rôle possible dans la formation du vitellus. Par conséquent, ces données pourraient être en faveur de l'implication des corticostéroïdes dans les étapes critiques de la reproduction de la perche eurasiennne, ce qui pourrait expliquer les troubles de la reproduction des femelles dans les systèmes RAS.

**Mots clés :** Corticostéroïdes, reproduction, cinétique plasmatique, stress, RAS, perche Eurasiennne.

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# LIST OF ABBREVIATIONS

**11-D or 11-DC:** 11-Deoxycortisol

**11 $\beta$ -HSD2:** 11 $\beta$ -Hydroxysteroid dehydrogenase-2

**17OH:** 17 $\alpha$ -hydroxyprogesterone or 17 $\alpha$ -OH-progesterone

**17 $\alpha$ -OH-pregnenolone:** 17 $\alpha$ -hydroxy pregnenolone

**17 $\beta$ -HSD:** 17 $\beta$ -Hydroxysteroid dehydrogenase

**20 $\beta$ -HSD:** 20 $\beta$ -Hydroxysteroid dehydrogenase

**2-OHE<sub>2</sub>:** Catecholestrogen or 2-hydroxyestradiol-17 $\beta$

**ACTH:** Adrenocorticotrophic hormone

**ARA:** Arachidonic acid

**BSA:** Bovine serum albumin

**CA:** Cortical alveoli

**cAMP:** Cyclic adenosine monophosphate

**Co:** Cortisol

**CoS:** Corticosterone

**CR:** Corticosteroid receptor

**CR20 $\beta$ -HSD:** Enzyme carbonyl reductase-like 20 $\beta$ -HSD

**CRH:** Corticotropin-releasing hormone

**CV:** Coefficient of variation

**D1:** Dopamine receptor 1

**D2:** Dopamine receptor 2

**DA:** Dopamine

**DBD:** DNA binding domain

**DHP or 17,20 $\beta$ -P:** 17 $\alpha$ ,20  $\beta$  Dihydroxy-4-pregnen-3-one

**DOC:** 11-Deoxycorticosterone

**DRSP:** Drospirenone

**E2:** 17 $\beta$ -Estradiol

**ELISA:** Enzyme-linked immunosorbent assay

**EP2b:** PGE2 receptor

**ER:** Estrogen receptor

**FAA:** Free amino acid

**FOM:** Final oocyte maturation/Follicle ovarian maturation

**FSH:** Follicle stimulating hormone

**FSHR:** Follicle stimulating hormone receptor

**GH:** Growth hormone

**GnIH:** Gonadotropin-inhibitory hormone

**GnRH:** Gonadotropin-releasing hormone

**GnRHa:** Gonadotropin-releasing hormone agonist

**GPOR:** G protein-coupled estrogen receptor



**GR1:** Glucocorticoids receptor type 1

**GR1a:** Glucocorticoids receptor type 1 splice variant (a)

**GR1b:** Glucocorticoids receptor type 1 splice variant (b)

**GR2:** Glucocorticoids receptor type 2

**GSI:** Gonado-somatic index

**GTH:** Gonadotropin

**GTH-I:** Gonadotropin-I

**GTH-II:** Gonadotropin-II

**GTH-R1:** Gonadotropin-I receptor

**GTH-R2:** Gonadotropin-II receptor

**GV:** Germinal vesicle

**GVBD:** Germinal vesicle break down

**GVM:** Germinal vesicle migration

**hCG:** Human chorionic gonadotropin

**HD:** Hinge D region

**HPG:** Hypothalamic-pituitary-gonadal axis

**HPI:** Hypothalamic-pituitary-internal axis

**HSI:** Hepato-somatic index

**hsd17 $\beta$ 12:** 17  $\beta$ -Hydroxysteroid dehydrogenase type 12

**IGF:** Insulin-like growth factor

**LBD:** Ligand-binding domain

**LDH:** Lactate dehydrogenase

**LH:** Luteinizing hormone

**LHR:** LH follicular receptor

**LNG:** Human medication Levonorgestrel

**Lv:** Lipovitellin

**MAPK:** Mitogen-activated protein kinase

**MEK:** Mitogen-activated protein kinase kinase

**MEKK:** Mitogen-activated protein kinase kinase kinase/MEK kinase

**Mel:** Melatonin

**MIH:** Maturation inducing hormone

**MPF:** Maturation promoting factor

**mPR $\alpha$ :** Membrane bound progestogen receptor type  $\alpha$

**MR:** Mineralocorticoid receptor

**MTR:** Melatonin receptor

**OD:** Oocyte Diameter

**OV:** Ovulation

**P:** Progesterone

**P450arom:** Enzyme aromatase

**PG:** Prostaglandins

**PGC:** Primordial germ cells

**PGE1:** Prostaglandin E1

**PGE2:** Prostaglandin E2

**PGF2 $\alpha$ :** Prostaglandin F2 $\alpha$

**PKA:** Serine-threonine kinase

**PR:** Progestogens receptor

**Pv:** Phosvitin

**RAS:** Recirculating aquaculture system

**Sero:** Serotonin

**SHBG:** Steroid hormone-binding globulin

**SP:** Spawning

**Star:** Steroidogenic acute regulatory protein

**T:** Testosterone

**THP or 20 $\beta$ -S:** 17 $\alpha$ ,20  $\beta$  trihydroxy-4-pregnen-3-one

**Vtg:** Vitellogenin

**YG:** Yolk granules or globules

**YGD:** Yolk globules diameter

**YP:** Yolk protein

**$\beta'$ -c:**  $\beta'$ -Component

## LIST OF SPECIES MENTIONED

<b>Family</b>	<b>Species</b>	<b><i>Lattin name</i></b>
<b><i>Acanthuridae</i></b>	Blue tang fish	<i>Paracanthurus hepatus</i>
<b><i>Acipenseridae</i></b>	Stellate sturgeon	<i>Acipenser stellatus</i>
	White sturgeon	<i>Acipenser transmontanus</i>
	Starry surgeon	<i>Acipenser stellatus</i> Pallas
	Persian sturgeon	<i>Acipenser persicus</i>
	Sterlet sturgeon	<i>Acipenser ruthenus</i>
	Siberian sturgeon	<i>Acipenser baeri</i>
<b><i>Adrianichthyidae</i></b>	Medaka	<i>Oryzias latipes</i>
<b><i>Anabantidae</i></b>	Climbing perch	<i>Anabas testudineus</i>
<b><i>Anguillidae</i></b>	European eel	<i>Anguilla anguilla</i>
	Japanese eel	<i>Anguilla japonica</i>
<b><i>Callorhynchidae</i></b>	Elephant shark	<i>Callorhynchus milii</i>
<b><i>Centrarchidae</i></b>	Rock bass	<i>Ambloplites rupestris</i>
<b><i>Clupeidae</i></b>	Indian shad	<i>Tenulosa ilisha</i>
<b><i>Cyprinodontidae</i></b>	Salt-marsh killifish	<i>Fundulus heteroclitus</i> L.
<b><i>Cyprinidae</i></b>	Zebrafish	<i>Danio rerio</i>
	Chalcalburnus tarichi	<i>Chalcalburnus tarichi</i>
	Caspian kutum	<i>Rutilus frisii kutum</i>
	Goldfish	<i>Carassius auratus</i>
	Fathead minnow	<i>Pimephales promelas</i>
	Pearl mullet	<i>Alburnus tarichi</i>
	Common carp	<i>Cyprinus carpio</i>
<b><i>Cichlidae</i></b>	Blue tilapia	<i>Tilapia aurea</i>
	Nile tilapia	<i>Oreochromis niloticus</i>
	Mozambique tilapia	<i>Oreochromis mossambicus</i>
<b><i>Fundulidae</i></b>	Killfish	<i>Fundulus heteroclitus</i>
<b><i>Gobiidae</i></b>	Longchin goby	<i>Chasmichthys dolichognathus</i>
<b><i>Heteropneustidae</i></b>	Asian stinging catfish	<i>Heteropneustes fossilis</i>
	African sharptooth catfish	<i>Clarias gariepinus</i>
<b><i>Ictaluridae</i></b>	Channel catfish	<i>Ictalurus punctatus</i>
<b><i>Labridae</i></b>	Kyusen wrasse	<i>Halichoeres poecilopterus</i>
<b><i>Moronidae</i></b>	European seabass	<i>Dicentrarchus labrax</i>
	Striped bass	<i>Morone saxatilis</i>
	White perch	<i>Morone americana</i>
	White bass	<i>Morone chrysops</i>
<b><i>Notopteridae</i></b>	Bronze featherback	<i>Notopterus notopterus</i>
<b><i>Percidae</i></b>	Yellow perch	<i>Perca flavescens</i>
	<b>Eurasian perch</b>	<b><i>Perca fluviatilis</i> L.</b>
	Eurasian ruffe	<i>Gymnocephalus cernuus</i>
	Pike perch	<i>Sander lucioperca</i>
<b><i>Plecoglossidae</i></b>	Ayu	<i>Plecoglossus altivelis</i>
<b><i>Pleuronectidae</i></b>	Starry flounder	<i>Platichthys stellatus</i>
	European plaice	<i>Pleuronectes platessa</i>
	winter flounder	<i>Pseudopleuronectes americanus</i> Walbaum

<i>Salmonidae</i>	Amago salmon	<i>Oncorhynchus rhodurus</i>
	Rainbow trout	<i>Oncorhynchus mykiss</i>
	Brook trout	<i>Salvelinus fontinalis</i>
	Japanese huchen	<i>Parahucho perryi</i>
	Arctic char	<i>Salvelinus alpinus</i>
	Brown trout	<i>Salmo trutta</i>
	Coho salmon	<i>Oncorhynchus kisutch</i>
	Masu salmon	<i>Oncorhynchus masou</i>
	Pink salmon	<i>Oncorhynchus gorbuscha</i>
	Red salmon	<i>Oncorhynchus nerka</i>
	Kokanee salmon	<i>Oncorhynchus nerka kennerlyi</i>
<i>Sebastidae</i>	Rockfish	<i>Sebastes taczanowskii</i>
<i>Sciaenidae</i>	Atlantic croaker	<i>Micropogonias undulatus</i>
	Spotted seatrout	<i>Cynoscion nebulosus</i>
<i>Sparidae</i>	Yellow porgy	<i>Acanthopagrus latus</i>
	Red seabream	<i>Pagrus major</i>
<i>Stichaeidae</i>	Ribbed gunnel	<i>Dictyosoma burgeri</i>
<i>Syngnathidae</i>	Pipefish	<i>Syngnathus fuscus</i>
<i>Tetraodontidae</i>	Tiger puffer	<i>Takifugu rubripes</i>
<i>Triglidae</i>	Red gurnard	<i>Chelidonichthys cuculus</i>
<i>Mugilidae</i>	Grey mullet	<i>Mugil cephalus</i> L.

# GENERAL INTRODUCTION

The global fish consumption is on an ongoing increase along with the population growth. In fact, food fish consumption grew from 9.0 kg in 1961 to 20.2 kg in 2015, at an average rate of about 1.5 percent per year where fish accounted for about 17 percent of animal protein consumed by the global population during that same year (FAO 2018). Unfortunately, development of the aquaculture industry has been exclusively based on the farming of just a few major species (mostly cyprinids, tilapias, catfishes and salmonids) that were often introduced into new geographic areas. As a result, 85% of the world's production of finfish has come from only 15 species, which are farmed in numerous countries (Teletchea and Fontaine, 2014). The anticipation for increased durability in farms productivity encouraged the continued development of new farming strategies. One of the best means to maintain or increase the contribution of aquatic products to such global consumption is thereafter to farm new species of marine or freshwater origin in which their production is continuously enhanced. However, the farming of new animal species is faced with deleterious impacts on the reproductive performance of the newly farmed animals. These deleterious impacts are partly mediated by induced stressors due to certain farming practices. For instance, the two basic farming practices of the capture and handling of fish induce enough stress to have a profound effect on circulating levels of gonadal steroids, in particular androgens and estrogens in a variety of teleosts. Taking live examples from several fish species as red gurnard *Chelidonichthys cuculus*, spotted sea trout *Cynoscion nebulosus*, rainbow trout *Oncorhynchus mykiss*, stressors as capture, handling, and confinement have been seen to result in a rapid decrease in both 17 $\beta$ -estradiol (E2) and testosterone (T) levels in these fish (Pankhurst and Van Der Kraak, 2000). Still, such stressors do not necessarily lead to a decrease in all hormones where the plasma concentrations of the corticosteroid cortisol have been noted to increase as a result of such stressors (Clearwater and Pankhurst, 1997). This increase could resemble the naturally occurring physiological increase

in cortisol levels in wild fish as a response to the naturally occurring stressors encountered in the wild during the mating season (intensive mating behaviour, fights between dominant males, or spawning migration activities) (Schreck, 2010). In all cases, the plasma sex-steroidal levels have been always observed to fluctuate with the changes in fish surrounding environment. The strong urge of global fish farms to have more control over the latter environment as an attempt to better control, schedule, and ameliorate the different stages in fish reproduction constituted the need for controlled indoor aquaculture systems as the commonly known recirculating aquaculture system (RAS) adapted in most intensive fish farms today. The latter system may arrive with other stressors in addition to the ones previously defined such as the need to control temperature and light period to have more control over the reproductive cycle (Policar et al., 2015; Pyanov et al., 2014). It has been indeed documented that seasonally-changing pattern of daylength and temperature are responsible for the cueing and timing of reproduction in the majority of fish (Bromage et al., 2001). Being of economic importance to fish farms as it saves time and labor, attaining out-of-season and synchronous spawning through controlled reproduction has been of paramount importance to fish industry. It is believed that stressors resulting from such control of the environment can be badly reflected on the success of attaining optimal egg quality and synchronous spawning in some farmed fish today (Hamlin et al., 2008; Rocha de Almeida et al., 2019). Stress response is known to provoke alterations of the physiological levels of the stress-sensitive corticosteroids which might in turn be attributed to the control of certain aspects of fish reproduction. Indeed, the reported effects of corticosteroids on GnRH (Gonadotropin Releasing Hormone) and gonadotropins production have led to recent interest in their possible roles in fish reproduction (Kiilerich et al., 2018; Milla et al., 2009). However, absence of reports on the exact plasma variations of the main corticosteroids during oogenesis in most teleost limits the ability to define their possible reproductive roles.

Eurasian perch *Perca fluviatilis* L, is a teleost highly perceived as a valuable freshwater species that is being cultivated in RAS today with current challenges related to optimal egg quality and synchronisation of ovulations and spawning (Fontaine and Teletchea, 2019; Migaud et al., 2002; Rocha de Almeida et al., 2019). Being defined as the ability of eggs to be fertilized and subsequently develop into a normal embryo, egg quality is of high importance for fish farms, where the inability of produced eggs to be fertilized or develop into fully grown embryo would render the egg as being of bad quality (Bobe, 2015). As in most teleosts, the roles of the previously mentioned stress-sensitive corticosteroids are still undefined in female perch either. Studies highlight the possible involvement of four main corticosteroids (11-deoxycorticosterone or DOC, 11-deoxycortisol or 11-D, corticosterone or CoS, and cortisol or Co) in critical stages of perch reproduction (Khendek et al., 2017; Noaksson et al., 2005, 2004). During this thesis, we will therefore focus on defining the exact roles of these four main corticosteroids in female Eurasian perch reproduction in the context of partly understanding the reproductive drawbacks observed in female perch farmed using RAS.



# **STATE OF ART**

# STATE OF ART

## 1. Eurasian Perch

### 1.1. Ecology

As recently defined by Hirsch and Eckmann (2015), Eurasian perch *Perca fluviatilis* Linnaeus, commonly known as the common perch, is a popular model species in fresh water ecology which is extensively utilized in field studies, cultivated in controlled aquariums, and incorporated in mesocosm experiments (Hirsch and Eckmann, 2015). Eurasian perch is a teleost belonging to the percid genus *Perca*, of the Actinopterygii class, order Perciforms, and family *Percidae*.

Eurasian perch is native to Eurasia at an altitude from sea level to just over 1000m (Thorpe, 1977) and has a wide distribution over Eurasian rivers and lakes and some brackish waters (Pimakhin et al., 2015; Stepien et al., 2015). The optimal ecological conditions to allow moderate perch growth and high yield include large, not too shallow, weed-free mesotrophic waters with no high stream velocities that are rich in forage fish such as roach or smelt (Thorpe, 1977). The flexibility of Eurasian perch to adapt to new environments allowed a broad distribution of Eurasian perch throughout whole Europe.

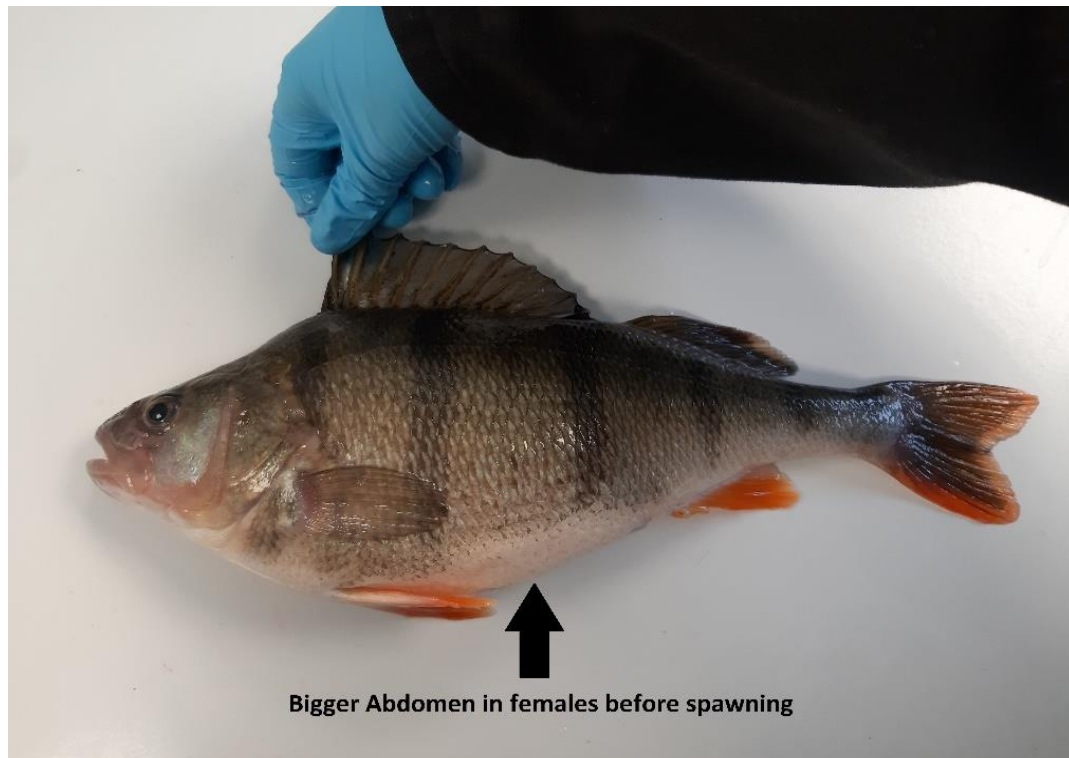
Eurasian perch is considered among fish species that can tolerate certain salinities and which migrate from bay into lower saline deltas and streams over the winter (Christensen et al., 2021). In autumn, wild perch are found to move to deeper waters for overwintering where they live at temperatures below 6°C for about 6 months at depths contours ranging between 42 and 69 m. At that time, perch is found to perform vertical migrations controlled by light where they would migrate between the bottom during the night to 25m off bottom during the day. As spawning

season approaches in the spring, they gradually vertically migrate to shallow warmer waters (0.25m/day) where adults are said to spawn by late May (Eckmann and Imbrock, 1996).

Perch is found to possess the ability to tolerate a maximum of 1% salinity during 3 weeks and a level of dissolved oxygen at 1.1-1.3 ppm at 16°C (Thorpe, 1977). In fact, perch has the ability to highly tolerate variations in both salinity and temperature that support its popular incorporation in European recreational and commercial fisheries (Stepien et al., 2015).

## **1.2. General morphology**

Eurasian perch are greenish with pelvis and caudal fins and are identified by their conspicuous pattern consisting of vertical darker pigmented stripes on their body flanks from just behind the operculum to the caudal peduncle (Hirsch and Eckmann, 2015). Their maximum recorded length and weight can reach 60cm and 607g respectively (Kestemont et al., 2015), although some studies recorded a higher weight of 1137g (Pirhonen et al., 2019). Eurasian perch displays sexual growth dimorphism in which females grow at a faster rate than males resulting in the bigger adult female body (Figure 1). Despite the numerous attempts for developing new mechanisms, it is still difficult to determine the sex of fish in perch (Rougeot et al., 2002; Ulićević et al., 2018).



**Figure 1: General morphology of female Eurasian perch.**

### **1.3. Life cycle and general reproductive cycle**

Eurasian perch start their life cycle as 4.8-6.0 mm larvae that survive without any parental care (Teletchea and Fontaine, 2010). Later during their life, males sexually mature in the wild at 2 years of age while females at 3-4 years of age (Thorpe, 1977). Perch is considered as a group-synchronous spawner. Once sexually mature, female perch ovaries start to form their pool of developing oocytes following a series of developmental stages. Briefly, these developmental stages involve, first, the entrance of oocytes into their primary growth stages after which they continue on growing as they accumulate egg-yolk in a process known as vitellogenesis until reaching an approximate size 0.99-1.08 mm. At the latter size, oocytes enter their final maturational stages known as “Final Oocyte Maturation (FOM)” in preparation for their later-on release from the ovary in a process commonly known as “ovulation” (Lubzens et al., 2010; Źarski et al., 2011a). Following ovulation, Eurasian perch oocytes, now known as eggs, are released into shallow waters during the night in a phenomenon known as “spawning”. In wild

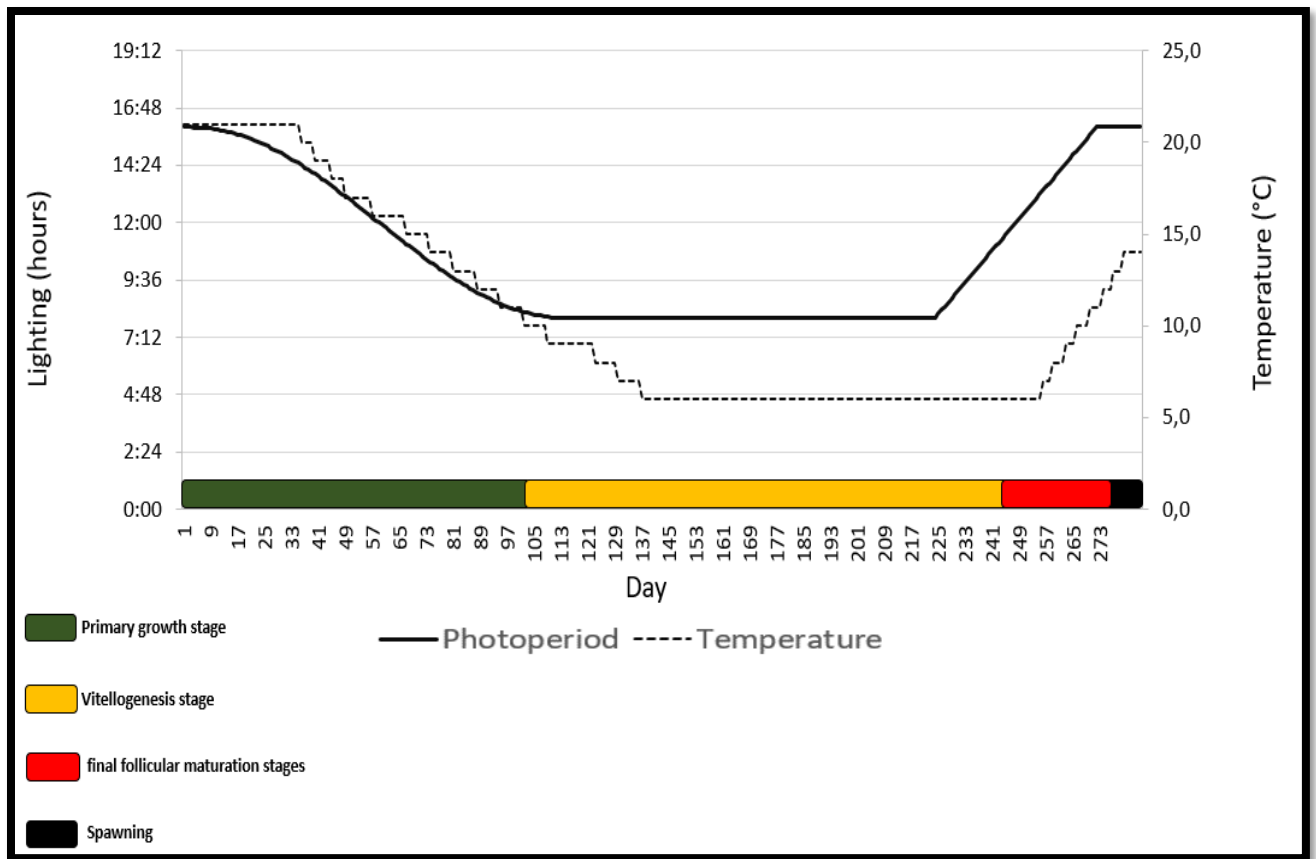
perch, the spawning season takes place annually during the spring (April and May) when water temperatures rises above 10°C where eggs are released within large cylindrical gelatinous ribbon-like strands (Probst et al., 2009; Thorpe, 1977; Żarski et al., 2012a). The latter strands get attached to submerged plants or fallen trees (Thorpe, 1977). As such, the female seasonal migratory behaviour from deep water in the winter to shallow waters during the spawning season in the spring is well justified.

#### **1.4. Common intensive production systems**

Although the market for perch is small by comparison to other species such as salmon, it is highly consumed in the alpine regions of Switzerland, Italy, Germany and France. Among the latter countries, Switzerland is the most important European market for perch where its production reached an average between 200 and 300 tons per year over the 2000–2010 period (Toner, 2015). Today, there is updates on a market increase in percid production to almost 1500 tons in RAS (Pascal Fontaine, communications). At the national level, the Lorraine territory scale in France and the flexibility of Eurasian perch to adapt to new environments, drove the interest of several stakeholders to this species. The human consumption of Eurasian perch in the Lorraine territory is estimated around 100 tons originating from three farms (Fontaine and Teletchea, 2019). Such increase in the aquaculture production of European perch is predominantly due to the utilization of Recirculating Aquaculture System(s) (RAS) (Toner, 2015). Cultivation of Eurasian perch using the latter system is possible thanks to the strong effect of water temperature and day/night duration on its reproductive developmental stages briefly mentioned earlier (Sulistyo et al., 1998). As such, a decrease in both day/light duration (photoperiod) and temperature can initiate the onset of their oocyte's developmental stages with a continued decrease later allowing the occurrence of the later-on vitellogenesis. However, a re-increase in both environmental cues is needed to allow oocytes to enter their final maturation stages (FOM) in preparation for their ovulation and later-on spawning (Abdulfatah et al., 2011;

Wang et al., 2006) (Figure 2). Such ability to environmentally control perch reproduction encouraged European perch production to shift from pond-based systems to the restricted RAS. This shift was in favour of the ability of RAS to implement the water temperature and photoperiod manipulation to obtain out-of-season spawning in seasonal fish as perch (Abdulfatah et al., 2013; Pimakhin et al., 2015). In other words, RAS serves as a method for the complete control of the whole perch reproductive cycle targeting their out-of-season spawning to eliminate the need to rely on the single annual reproduction that naturally occurs once in the spring (Fontaine and Teletchea, 2019; Sulistyo et al., 1998). In addition to water temperature and photoperiod, RAS also attempts to optimise other parameters as water salinity, pH, and dissolved oxygen with the monitoring of other related parameters as ionized and unionized (free) ammonia and nitrites to obtain optimal fish growth (Polcar et al., 2015).

Therefore, RAS always anticipates optimization of all the previously mentioned parameters to reach its goals. In order to best benefit from advantage of controlling photoperiod in RAS, the selection of appropriate light intensity could affect the sensitivity of fish to water tank colour (Polcar et al., 2015). It has been found that high light intensities (eg. 1100 lx) can eliminate the adverse effects of water tank colour on perch feed intake and growth as they are more able to detect feed in dark tanks when a higher light intensity is utilized. Otherwise, attention should be provided to the water tank colour when using low light intensities (Strand et al., 2007) which offer lower perceived stressors by cultured perch (Staffan, 2004). Nevertheless, a good RAS implements good water quality through appropriate oxygen supplementation which should be maintained above 5-6 mg/l for optimal feed intake and utilization by fish (Polcar et al., 2015). In the case of even short-term exposure to low oxygen, hypoxia could result leading to deleterious effects on fish physiology (Milla et al., 2015).



**Figure 2: Photothermal program adapted in RAS.**

## 2. The reproductive cycle in teleosts

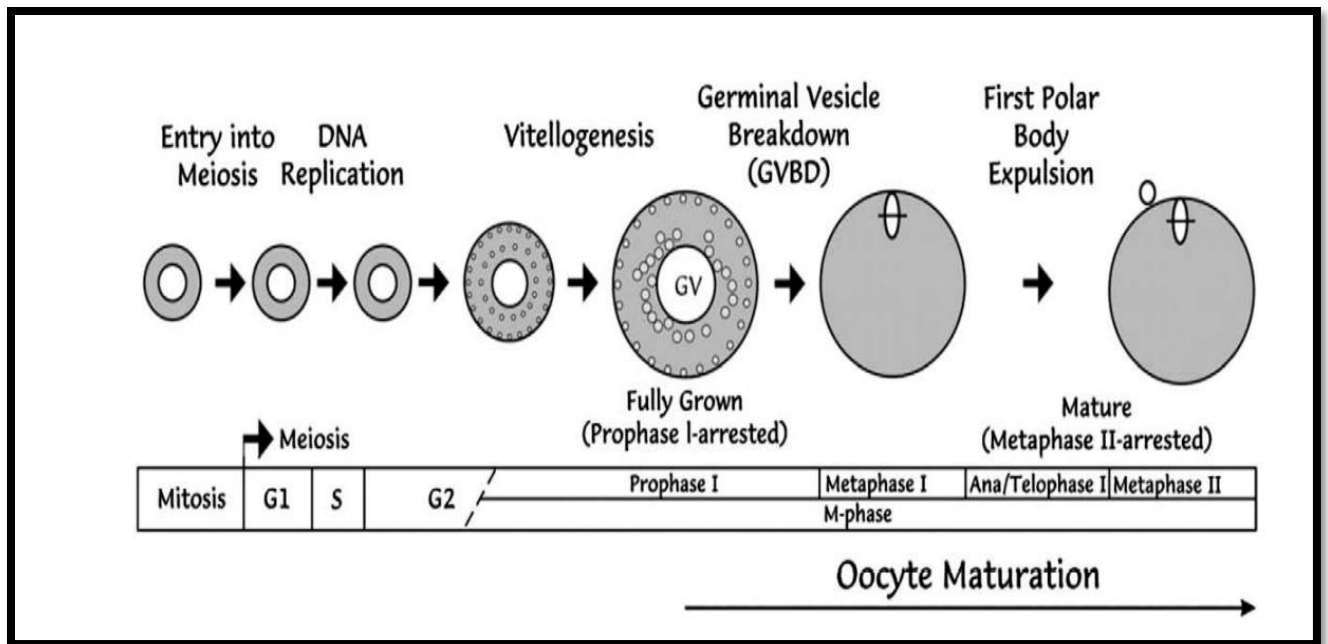
First of all, it should be well noted that not all teleosts share the same pattern of ovarian development. In fact, teleost have three different kinds of documented ovarian developmental patterns; namely Synchronous, Group-synchronous, and Asynchronous. In the synchronous type, the oocytes all undergo both their development and ovulation together where all eggs are shed all together in a single episode. This means that a single population of oocytes can be recognized in the ovary throughout the whole reproductive season. In the Group-synchronous, at least two populations can be recognized in the ovary throughout the reproductive season, which means that there are two cohorts of developing oocytes in the ovary at any time (a group of primary oocytes and a group of synchronously developing larger oocytes). In the Asynchronous type, eggs are recruited into maturation and ovulation from a heterogeneous

population of developing oocytes. Eggs are thus ovulated in several batches during each spawning season (Lubzens et al., 2010; Tyler and Sumpter, 1996). Eurasian perch falls into the category of group-synchronous spawners (Lubzens et al., 2010; Żarski et al., 2012a).

## **2.1. The different stages of the reproductive cycle in teleosts**

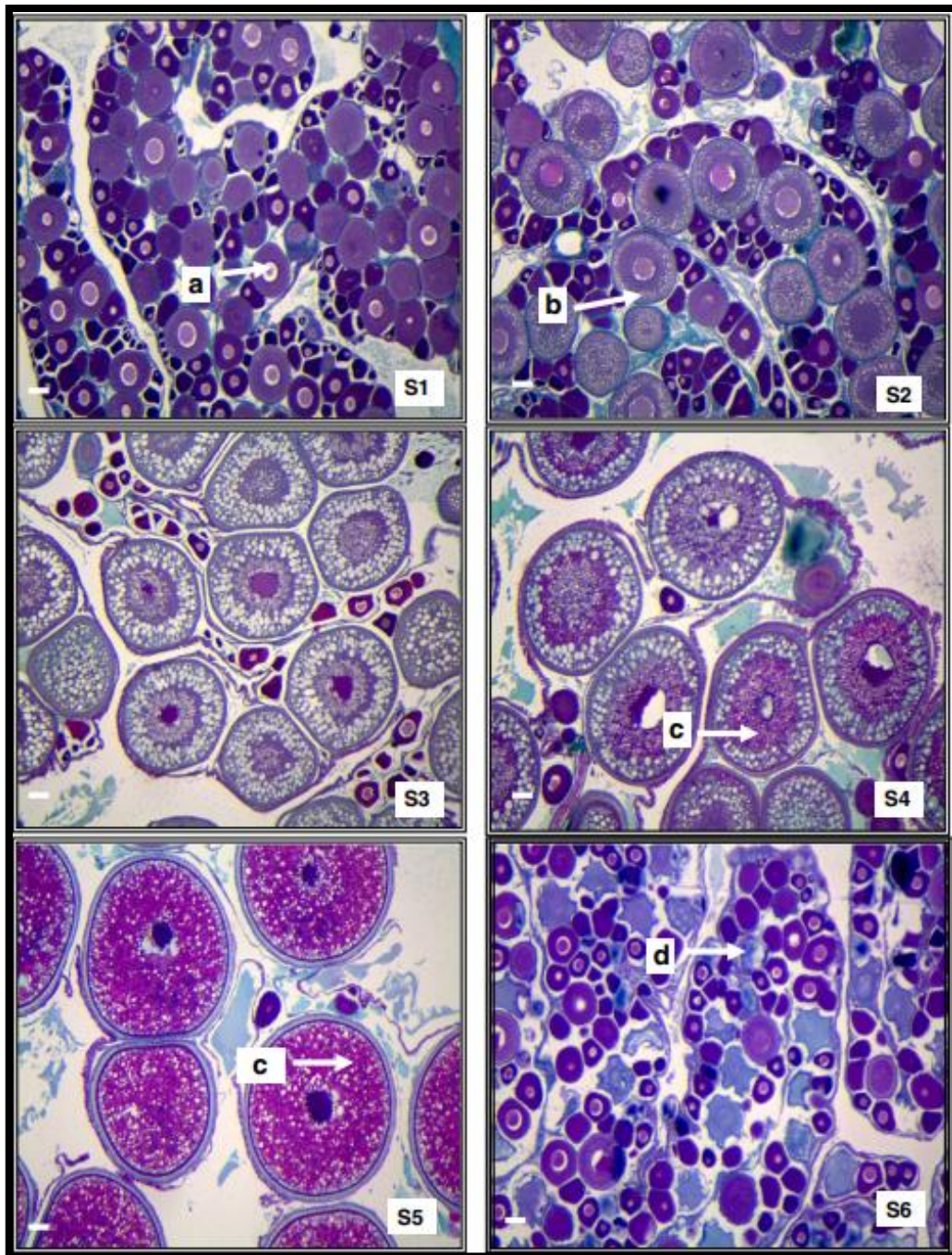
The reproductive cycle in teleosts, at the cellular level, begins by the transformation of the primordial germ cells into oogonia. These stocks of oogonia keep on proliferating in adult females thereby renewing the stocks of young oocytes and follicles through life. Oogonia later transform into primary oocytes, which then enter their first meiotic division at sexual maturity. This division is arrested twice; first at Diplotene stage of Prophase I of the first meiotic division, and second at Metaphase II of the second meiotic division (Figure 3). During the first arrest, the primary oocyte takes time to accumulate nutritional reserves needed by the later-on developing embryo thereby undergoing massive growth in a process known as Vitellogenesis. Additionally, the oocyte during this period accumulates maternal RNA and completes the differentiation of its cellular and non-cellular envelopes. Once reaching the appropriate size, the oocyte is said to resume its first meiotic division by releasing its first polar body during the final maturational stages (FOM) in preparation for ovulation (Jalabert, 2005; Lubzens et al., 2010).





**Figure 3: Different stages of oogenesis in teleosts (Lubzens et al., 2010).**

Each of the above illustrated developmental stages starting from the initial formation of oogonia and primary oocyte, followed by their growth, and ending by their final maturational stages just prior to their ovulation is shown in figure 4 for Eurasian perch and will be thoroughly explained in the following sections.



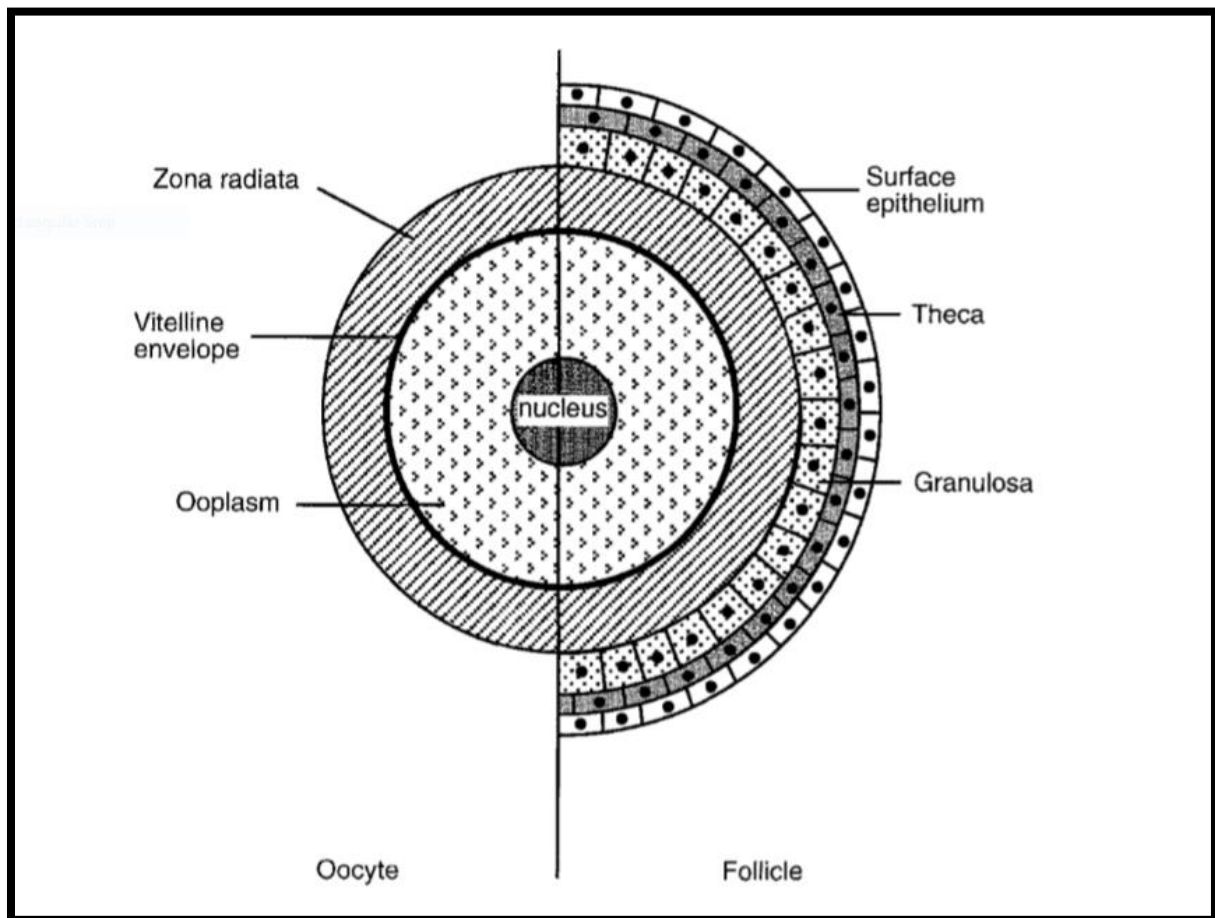
**Figure 4: The oocyte developmental stages developed by Abdulfatah et al. (2011) according to the description of Wallace and Selman (1981). S1 ( $\times 87$ ): primary growth phase, S2 ( $\times 87$ ): initiation of yolk vesicle (cortical alveoli), S3: late yolk vesicles stage, S4 ( $\times 87$ ): early vitellogenesis, S5 ( $\times 87$ ): maturation and S6 ( $\times 87$ ) atresia. Scale: 100  $\mu\text{m}$ . a: basophilic nucleolus, b: yolk vesicle, c: yolk protein, d: atretic oocyte.**

### **2.1.1. From primordial germ cell (PGC) to the formation of oogonia and primary oocyte**

Starting from the early stages of embryogenesis, all oogonia are derived from non-dividing totipotent primordial germ cell or PGCs inducing structural changes within the PGCs themselves. Proliferation of the latter cells gives rise to the stem cell populations of oogonia that reach a well-defined species-specific number in the embryonic ovaries. These oogonia multiply each by mitotic divisions forming oogonial nests in association with pre-granulosa cells (Lubzens et al., 2010; Tyler and Sumpter, 1996). The formed oogonia later enter into their first meiotic division which timing is species dependent. In most species for instance, entrance of oogonia into prophase one is at very early stages of embryogenesis as in the common carp *Cyprinus carpio* in which oogonia enter their first meiotic division 16 weeks post-fertilization (Miura et al., 2007).

Before leaving the oogonial nest, the transition from oogonium to primary oocyte takes place marking the start of the first meiotic division where cells are arrested at diplotene stage of Prophase I of the first meiotic division. As the primary oocytes are arrested at the first meiotic division, their growth starts, rendering the transition to the primary oocyte growth phase. This transition involves the envelopment of each oogonium by a monolayer of somatic granulosa cells which secretes a basement lamina separating the oogonium from the ovarian stroma cells. Somatic cells forming a monolayer outside the basement lamina, constitute the theca that becomes associated with blood vessels. At this stage, the primary oocyte is surrounded by granulosa cells, basement lamina (basal lamina) and theca somatic layer closely surrounding the plasma membrane or oolemma. At a later stage of oocyte growth, the extra-oocyte matrix between the latter oolemma and granulosa fills with fibrillar structures giving rise to the zona radiata as exhibited in figure 5. Within this matrix, oocyte microvilli protrude from the oocyte surface, that later becomes covered by the vitelline envelope, towards granulosa cells. It is

through these microvilli that the compounds (ex: steroids) move in and out of the blood to the oocyte (Lubzens et al., 2010; Tyler and Sumpter, 1996).



**Figure 5: Fully-grown primary oocyte within its follicle (Tyler and Sumpter, 1996).**

### **2.1.2. Growth stages of primary oocyte**

The early growth stage of a primary oocyte is marked by a period of intense RNA synthesis (meiotic chromatin nucleus stage) (Lubzens et al., 2010; Tyler and Sumpter, 1996). After which, a secondary growth follows marked by the appearance of cortical alveoli. This is the stage where the numbers of the cortical alveoli (CA) increase steadily until occupying almost the entire ooplasm. Cortical alveoli have been characterized in pipefish *Syngnathus fuscus* to contain each one or two eccentrically situated filamentous bodies suspended in a rather wispy component all residing within the thin membrane of a single cortical alveolus (Anderson, 1968).



The role for these alveoli is still unclear but they might later take part in the so-called cortical reaction at fertilization, where their contents are released to the egg surface. The start of the third growth stage is marked by the start of vitellogenesis in which primary oocytes continue on growing due to the deposition or incorporation of yolk granules (YG) into their ooplasm (Bazzoli and Godinho, 1994).

In wild Eurasian perch, the speed of GSI (Gonadosomatic Index) and OD (Oocyte Diameter) growth allowed the distribution of the above described growth stages over two major time periods; the first period characterized by the rapid growth of both GSI and OD and takes place between July and December. A second period during which growth slows down lasting from December until spawning in April (Sulistyo et al., 1998).

### Vitellogenesis

In oviparous female teleosts as Eurasian perch, vitellogenesis is the potent stage of the production and selective incorporation or packaging of extraovarian vitellogenin (Vtg) into the growing oocytes to formulate the egg-yolk which constitutes the nutritional source for the future developing embryo (Jalabert, 2005). In some teleosts, the egg-yolk comprises both neutral lipids referred to as oil/lipid droplets and the mentioned Vtg-associated polar lipids (Hiramatsu et al., 2015). Vtg can be thereby detected in the blood of females during their vitellogenesis through which Vtg migrates from the site of its production in the liver until reaching the oocytes (Lubzens et al., 2010). There are two kinds of Vtg in teleosts, namely Vtg1 and Vtg2, which are incorporated into the vitellogenic oocytes by receptor-mediated endocytosis after which they are further processed into the egg-yolk proteins (YP); lipovitellin (Lv), phosvitins, and  $\beta$ -component. The latter processing of Vtg into the YP is referred to as “first proteolysis” and is an indispensable step for egg-yolk formation and storage inside the developing oocyte (Fabra et al., 2006; Hara et al., 2016).

As these yolk globules are deposited, they start to replace the previously mentioned cortical alveoli in the center of the ooplasm which in turn tend to migrate towards the peripheral ooplasm (Bazzoli and Godinho, 1994; Miura et al., 2007; Tyler and Sumpter, 1996). It is at this exact stage during which the acellular vitelline membrane referred to as chorion vitelline envelope is deposited around the oocyte (Figure 5) and continues to differentiate and increase in complexity throughout the oocyte growth until forming the chorion (Tyler and Sumpter, 1996).

In Eurasian perch, the described early accumulation of yolk vesicles and the beginning of vitellogenesis, occur between August and September (Sulistyo et al., 1998). This whole process occurs in response to a hormonal stimulation controlled by an endocrine system which will be thoroughly discussed later.

### **Folliculogenesis**

During the primary growth stage, the follicular layers are still undifferentiated. However, the follicular layers begin to differentiate to granulosa and theca sublayers at the secondary growth stage. Thus, both granulosa and theca cell layers are known to constitute the follicle cell layers. As the oocyte grows, the acellular *zona radiata* thickens and is surrounded by a surface epithelium and connective tissue. The oocyte is then said to be surrounded by layers of steroid-secreting granulosa and thecal cells which both play an integral role later during vitellogenesis (Tyler and Sumpter, 1996). These follicles play an even more critical role in the steroidogenic control of the later up-coming FOM (Planas et al., 2000).

### **2.1.3. Final Oocyte Maturation (FOM) and Ovulation**

Following the above developmental stages in which the oocyte grows in diameter, FOM is initiated by the resumption of the first meiotic division followed by the release of the first polar body and ends by the arrest at metaphase II. During this arrest, the primary oocyte undergoes

complex cellular processes involving its Germinal Vesicle (GV) or nucleus starting by its migration (GVM) until its breakdown in a phenomenon commonly known as the Germinal Vesicle Break down (GVBD) (Jalabert, 2005; Nagahama and Yamashita, 2008).

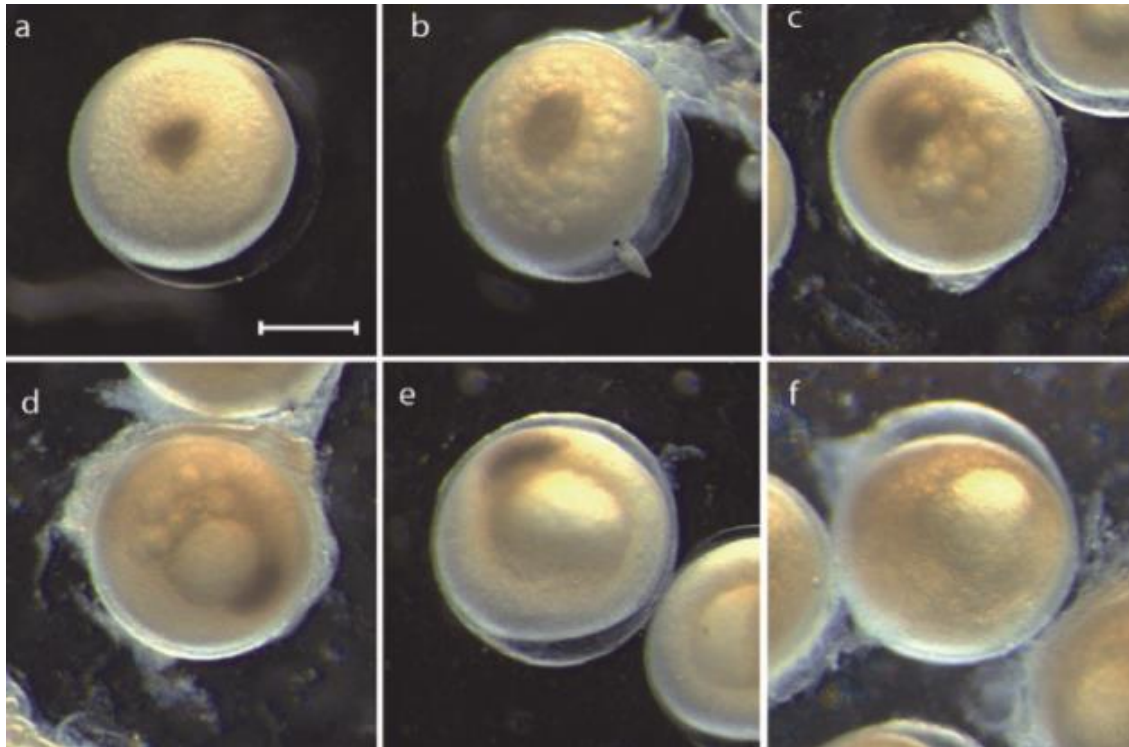
In pelagophil fish, oocytes gain 67–75% of their final volume during oocyte maturation due to oocyte hydration (Lubzens et al., 2010). This increase in volume is important in the production of pelagic (buoyant) eggs in such marine teleosts (Tyler and Sumpter, 1996). During such hydration, a second proteolysis of the previously mentioned YPs that form the prominent yolk globules during vitellogenesis further occurs during FOM. In fact, this second proteolysis occurs concomitant with the fusion of the yolk globules which is also observed during FOM in pelagophil fish (Fabra et al., 2006; Hara et al., 2016). In fresh water fish such as salmonids, this second proteolysis is not usually observed (Hiramatsu et al., 2002). However, significant hydration was observed using both *in vivo* and *in vitro* assays in freshwater fish as rainbow trout *Oncorhynchus mykiss* (Milla et al., 2006). This observation revived the possibility for hydration existence in fish that lay their eggs in fresh water such as salmonids after having been eliminated in these fish (Hiramatsu et al., 2002). Still, such hydration would contribute in a significant increase in oocyte diameter during FOM as has been seen in pelagophil fish (Lubzens et al., 2010). In Eurasian perch, developing oocytes maintain a quite constant diameter during the whole FOM which puts into question the occurrence of hydration in this teleost. In fact, Zarski et al. (2011) measured the diameter of the maturing Eurasian perch oocytes and found it to remain within the range of 0.99–1.08 mm during whole FOM (Żarski et al., 2011a). Following such findings, it would be first thought that Eurasian perch oocytes do not undergo oocyte hydration. However, these authors histologically exhibited in the following year that hydration did occur in perch but was estimated to be very minor (4%) as opposed to the much higher percentage obtained in pelagophil fish. In pelagophil fish, the higher hydration is logically linked to the need for buoyancy in the latter eggs that, unlike perch, are not laid in

ribbons that fall into the submerged substrate (Żarski et al., 2012b). As such, the absence of major increase in oocyte diameter during FOM can be justified.

FOM has been estimated in wild perch to occur during March (Sulistyo et al., 1998). Additionally, FOM has been further investigated in Eurasian perch resulting in the highly adapted classification depicted below. The latter classification includes a series of 6 maturational stages (Figure 6) (Żarski et al., 2011a):

- Stage I: the GV is situated in the oocyte centre with oil droplets being poorly visible (Figure 6a).
- Stage II: Beginning of migration of the GV (GV is less than half of the oocyte diameter) and coagulation of the oil droplets (Figure 6b).
- Stage III: GV migration extends to above half of the oocyte diameter with clearly visible oil droplets (Figure 6c).
- Stage IV: the GV reaches the oocyte periphery, and a large forming oil droplet becomes visible where its diameter is greater than that of the GV (reaching about 1/3 the oocyte diameter). Smaller droplets are also visible during this stage (Figure 6d)
- Stage V: the GV is situated at the oocyte edge and a clearly visible one large oil droplet is the size of about half the oocyte diameter (Figure 6e)
- Stage VI: includes the Germinal vesicle breakdown (GVBD) where after its migration to the edge of the oocyte, GV breaks down marking meiosis resumption from prophase I to metaphase I.





**Figure 6: Final oocyte maturation stages as developed by Zarski et al. (2011a).**

As shown in figure 6, this classification is based on monitoring the observation of two major cellular organelles; oil droplets and GV whose respective coalescence and migration occur over the three maturational stages (III, IV, and V) estimated in a period of 3-4 weeks if no hormonal stimulation is implemented (Żarski et al., 2011a). Indeed, the phenomenon of oil droplets formation and coalescence into one large droplet and the accompanied phenomenon of GV migration are used in the proper detection of the exact FOM stage. The latter classification can thus come in handy for studies monitoring the advancement of the primary oocytes through their FOM.

After completing its first meiotic division, the primary oocyte is now referred to as secondary oocyte and is arrested at metaphase II of the second meiotic division. At this point, the expulsion of the mature secondary oocyte from the ovarian follicles takes place marked by the collapse of the follicular layers signifying the occurrence of ovulation (Lubzens et al., 2010). Few days

later, the release of eggs from female body commonly known as spawning occurs. In the wild Eurasian perch of Eastern France, spawning was observed to occur around April coinciding with the rise in water temperature (Sulistyo et al., 1998).

## **2.2. Hormones and neurotransmitters involved in the control of the reproductive cycle in teleosts**

Reproductive hormones and neurotransmitters orchestrate the process of reproduction initiated at the level of the brain and translated to the anterior pituitary which in turn induces the release of hormones that possess direct effects on the gonads. Indeed, the hypothalamus takes the very first step in monitoring, initiating, and thus causing the release of the specific hormones and neurotransmitters in order to initiate and maintain teleosts reproductive cycle. This control proceeds for the sake of maturity and continuation of the reproductive performance throughout the animal's life.

In Eurasian perch, studies by Migaud illustrate how manipulations of both water temperature and photoperiod can control the onset of gametogenesis implying a strong effect for light/dark durations on the timing of reproduction in perch (Migaud et al., 2006). The pineal gland in fish is known to exhibit photoreceptive characteristics and is the major production site for the neurotransmitter melatonin which is the key hormone acting in the circadian system of vertebrates (Sánchez-Vázquez et al., 2019). With the demonstrated existence of specific melatonin receptor proteins on the hypothalamus–pituitary–gonadal (HPG) axis of fish, the pattern of how the provided information on day length and seasons by melatonin affects reproduction is more evident (Maitra and Hasan, 2016).

### **2.2.1. GnRH and gonadotropins (LH, FSH)**

Fish brain, mainly the hypothalamus, controls fish reproduction *via* the release of gonadotropin-releasing hormones (GnRHs). Following their production by the hypothalamus, these

decapeptides reach out to the anterior pituitary *via* hypothalamic neuronal processes allowing the direct neuronal control of the adenyhypophyseal function. The latter results in the discharge of two major gonadotropins; follicle stimulating hormone GTH-I or FSH and luteinizing hormone GTH-II or LH, as identified in teleosts (Yaron and Levavi-Sivan, 2011).

Monoamines such serotonin and melatonin have been known to highly interfere with the above mentioned gonadotropin axis (Prasad et al., 2015). Existence of day/night variations and melatonin inhibitory effects on the expression of GnRH and its respective receptors in the brain of European sea bass provided evidence on how melatonin can control the seasonality of reproduction via the HPG axis (Maitra and Hasan, 2016). In fact, the previously illustrated impact of environmental cues on the progress of the different reproductive stages of Eurasian perch is possible thanks to the existence of the neurotransmitters through which such environmental cues are reflected on the physiology of the reproductive hormones.

The biochemistry of fish FSH and LH defines them as two heterodimeric glycoproteins each consisting of an  $\alpha$  and a  $\beta$  subunit. The  $\alpha$  subunit is the same for both but the  $\beta$  subunit is specific to each hormone, both subunits being non-covalently linked in the two hormones (Swanson et al., 2003). To initiate their function, FSH and LH bind to their receptors in the gonads (FSHR and LHR respectively) resulting in the secretion of sex steroids critical for the initiation and progress of gametogenesis (Yaron and Levavi-Sivan, 2011). In the ovary of coho salmon, *Oncorhynchus kisutch*, GTH-RI (FSHR) has been localized on both the theca and granulosa cells and was found to have affinity to both FSH and LH. On the other hand, GTH-RII (LH-R) was found to specifically bind to LH and to be exclusively located on the granulosa cells (Kwok et al., 2005). The hormonal balance between the two gonadotropins seems to be the main mediator of oogenesis where during secondary oocyte growth, plasma levels of FSH are elevated by contrast to the low circulating levels of LH. At that time, the type I GTH receptor has been shown to be present in both theca and granulosa cells but perhaps the higher

concentration of FSH in blood during secondary oocyte growth would allow a higher binding affinity of FSH to GTH-RI (FSHR) by comparison to LH. As oocytes approach their FOM, the balance shifts to a plasma elevation in LH levels in parallel to a decline in FSH levels with the appearance of the type II GTH receptor in granulosa cells (Planas et al., 2000). Human chorionic gonadotropin (hCG) is an analogue of the gonadotropin LH and has been widely used for hormonal induction of spawning (Křišťan et al., 2013). Following the binding to their receptors on both theca and granulosa cells, these gonadotropins stimulate the production and release of gonadal sex steroids which are the direct mediators of the different reproductive stages in teleosts and will thus be thoroughly described in the following sections.

### 2.2.2. Sex steroids

Four major classes of sex steroids namely; progestogens, estrogens, androgens and corticosteroids are majorly involved in the orchestration of the different reproductive stages. As previously mentioned, these sex steroids are the end-products resulting from the GnRH stimulated adenohypophyseal secretion (Tenugu et al., 2020).

Estrogens as estradiol-17 $\beta$  (E2) have been implemented in the progression of germ cells through early oogenesis acting directly on oogonial proliferation and later vitellogenesis (Yaron and Levavi-Sivan, 2011). In fact, E2 originates from the androgen Testosterone (T) which has been also found to be involved in the regulation of meiosis (Goetz and Bergman, 1978; Miura et al., 2007; Nagahama and Yamashita, 2008).

In teleost, the two progestogens 17 $\alpha$ -hydroxyprogesterone (17OH), 17 $\alpha$ ,20  $\beta$  dihydroxy-4-pregnen-3-one (17,20 $\beta$ -P or DHP) and 17 $\alpha$ ,20  $\beta$  trihydroxy-4-pregnen-3-one (20 $\beta$ -S or THP) have been majorly prominent during FOM. Studies on Japanese huchen *Parahucho perryi* and common carp *Cyprinus carpio* suggest that DHP can be involved in the reproductive cycle even as early as during the development of the first stages of primary growth of oocytes (Lubzens et

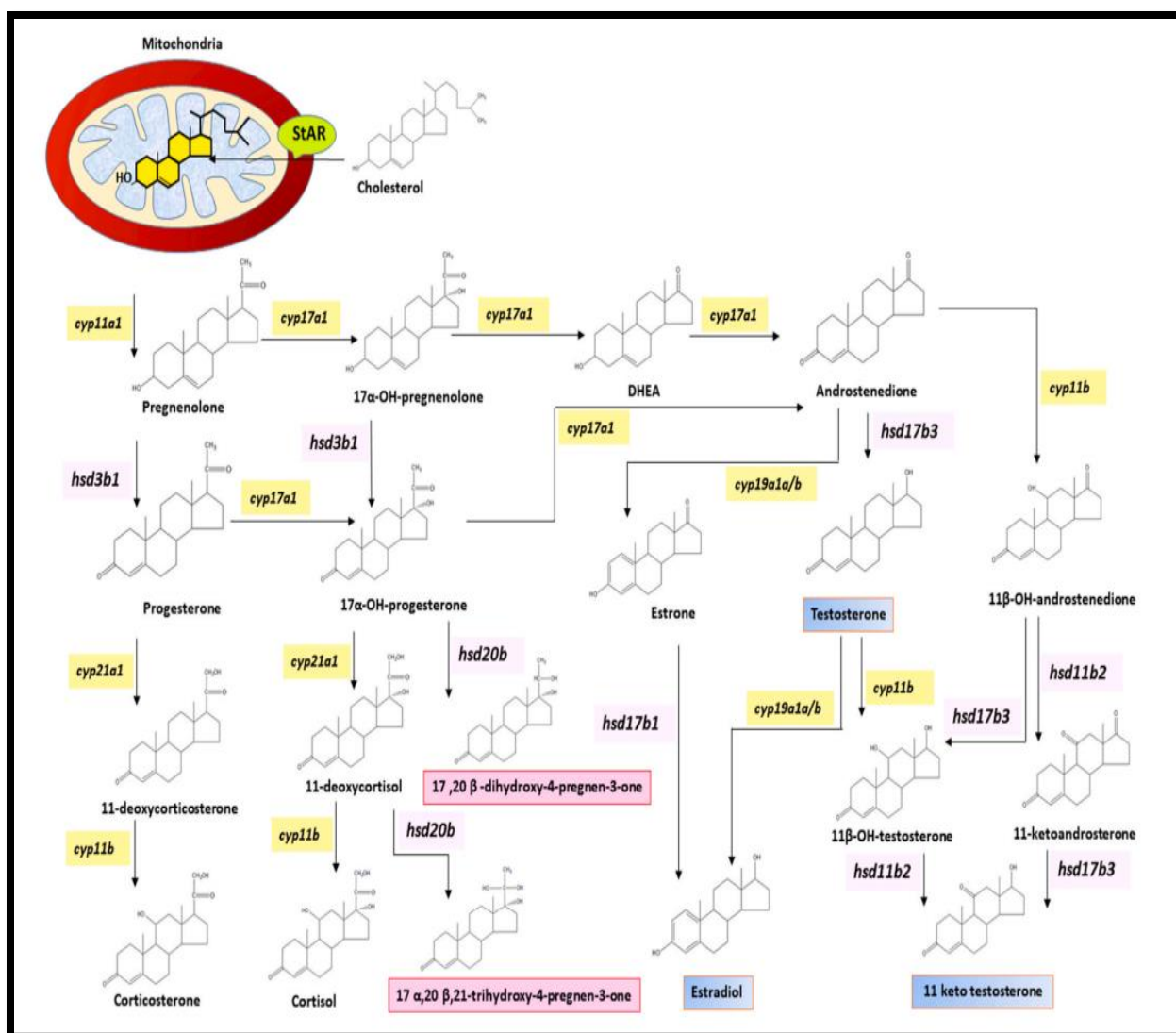
al., 2010). However, there are more studies suggesting DHP to be rather implemented in the last stages of development (FOM) as the maturation inducing hormone (MIH) which is the hormone that principally mediates FOM in teleosts (Berndtson et al., 1989; Migaud et al., 2003; Pinter and Thomas, 1999).

Finally, corticosteroids have been suggested by several studies to be incorporated in the mediation of certain stages in female teleost reproductive cycle. Literature defines four major corticosteroids (11-deoxycorticosterone or DOC, 11-deoxycortisol or 11-D, corticosterone or CoS, and cortisol or Co) that have been accredited in certain teleosts to the endocrinological regulation of stages as early as the initiation of oogenesis and regulation of puberty (Huang et al., 1999), all the way to vitellogenesis (Lethimonier et al., 2002), until FOM induction and spawning (Milla et al., 2009).

### **2.2.3. Steroidogenesis occurring during the reproductive cycle in teleosts**

Similar to the regulation of the initiation and progress of the reproductive cycle, the regulation of steroidogenesis is under the control of the hypothalamic-pituitary-gonadal (HPG) axis, which is in turn under the control of monoamines as melatonin and its precursor serotonin. Following its release from the hypothalamus, GnRH binds and activates its cognate receptor (GnRH receptor) on the pituitary gonadotropin cells and, in turn, stimulates the synthesis and secretion of gonadotropins (GtHs) (Maitra and Hasan, 2016) which trigger the consequent release of previously defined sex steroids. The latter biologically active sex steroids are primarily derived from cholesterol where they become abundantly produced in the gonads following GtHs stimulations. In fish however, steroids are actively produced by interrenal tissue and central nervous system (brain) in addition to the gonads with the major sites of steroidogenesis being limited to the gonads and interrenals (Tenugu et al., 2020).

At the intracellular level, studies in some teleosts as zebrafish, *Danio rerio*, and African sharptooth catfish, *Clarias gariepinus*, revealed an hCG-dependent increase in the expression of the steroidogenic acute regulatory protein (star) during FOM and spawning (Ings and Van Der Kraak, 2006; Sreenivasulu and Senthilkumaran, 2009). In vertebrates, star usually regulates cholesterol mobilization between inner and outer mitochondrial membranes which is a critical step during steroidogenesis. The latter finding implies the existence of a similar phenomenon taking place during fish steroidogenesis which highlights the indispensable role for mitochondria in the production of the different steroids from the main precursor cholesterol (Tenugu et al., 2020). Cholesterol gives rise to pregnenolone which is the precursor of the two principle steroids progesterone and 17 $\alpha$ -hydroxy pregnenolone (17 $\alpha$ -OH-pregnenolone) following a fundamental step catalysed by the enzymes 3 $\beta$  dehydrogenase and 17 $\alpha$  hydroxylase respectively. The latter enzymes also catalyse the transformation of these two steroids into 17 $\alpha$ -hydroxyprogesterone (17 $\alpha$ -OH-progesterone) which traverses the basal lamina of the LH stimulated follicular thecal cell layers. Within follicular granulosa, 17 $\alpha$ -OH-progesterone can be then converted into the two progestogens DHP and THP. In parallel, the other sex steroids (androgens and estrogens) can be derived from 17 $\alpha$ -OH-pregnenolone following its transformation into Androstenedione where E2 can be also produced from T in the granulosa of FSH stimulated vitellogenic oocytes (Figure 7) (Tenugu et al., 2020).



**Figure 7: Schematic representation of general steroidogenesis in teleosts (Tenugu et al., 2020).**

#### **2.2.4. Control of primordial germ cell (PGC) proliferation and the formation of primary oocytes**

The control of early oogenesis, from the proliferation of oogonia to the initiation of meiosis, involves a series of hormonal stimulations. As mentioned earlier, and as a rule for teleost species, the gonadal function is regulated by the two distinct gonadotropins GTH I (FSH) and GTH II (LH) (Yaron and Levavi-Sivan, 2011). FSH, in specific, mediates the regulation of oogonia proliferation *via* stimulating the release of E2. The produced subpopulation of oogonia

reaches a species-specific number at which meiosis commences and the oogonia become known as primary oocytes. In some teleosts, DHP was found to act on the induction of the latter meiotic division without any apparent effect on oogonial proliferation whatsoever (Yaron and Levavi-Sivan, 2011). Taking the example in the Japanese huchen, *Parahucho perryi*, histological observations of the early stages of oogenesis suggest that DHP is involved in the initiation of the first meiotic division in this species (Miura et al., 2007).

### **2.2.5. Control of primary oocyte growth and vitellogenesis**

As the primary oocyte enters meiosis during fish puberty, it is arrested at the prophase of the first meiotic division during which its growth phases are initiated. Several hormones and growth factors are integrated in the control of such growth phases. These hormones include gonadotropin (GTH), thyroxine, triiodothyronine, growth hormone, insulin and insulin-like growth factors (IGFs) (Tyler and Sumpter, 1996). As previously mentioned, DHP initiates the first meiotic division leading to the development of the first stage in the primary growth, namely the chromatin nucleolar oocytes in some teleosts (Lubzens et al., 2010).

During the transition from primary to secondary growth, plasma and pituitary levels of FSH are observed to increase to elevated levels. This increase, accompanied by plasma E2 increase, coincided with the cortical alveoli synthesis which usually takes place during the secondary growth of the primary oocyte. Elevated FSH hence stimulates these cells to release E2, which in turn triggers the formation of cortical alveoli during secondary growth of the primary oocyte. Such high FSH levels are maintained as follicles are recruited into vitellogenesis which in turn marks the third growth phase of the primary oocyte growth (Lubzens et al., 2010).

As such, early vitellogenesis would record a further increase in plasma FSH and E2 in addition to an increased expression of FSH ovarian receptor. FSH secreted into the bloodstream acts on the follicles to further induce synthesis of E2 which binds to sex steroid hormone-binding



globulin (SHBG) (Hara et al., 2016). According to an *in vitro* experiment performed on rainbow trout, *Oncorhynchus mykiss*, not only does FSH trigger E2 release to promote hepatic Vtg synthesis, but it also increases Vtg uptake by ovarian follicles (Lubzens et al., 2010). Vtg uptake by ovarian follicles has been shown to be stimulated by growth factors as insulin in rainbow trout suggesting the existence of a possible integrative interaction between E2 and the insulin growth factor in the mediation of such phenomenon (Tyler and Sumpter, 1996). Following Vtg uptake by the developing oocytes, the endocrinological control of the further processing of Vtg into the three egg-yolk proteins (YPs) or first proteolysis has not yet been investigated in teleosts. As soon as FOM arrives after vitellogenesis, the shift in the whole gonadotropic balance from being FSH dominated to being LH dominated takes place (Zohar et al., 2010).

#### **2.2.6. Control of final oocyte maturation (FOM) and ovulation**

The termination of vitellogenesis and progression towards meiosis resumption hosts major endocrine events starting by the increased expression of the LH receptor accompanied by an LH-derived switch in the ovarian follicle steroidogenic pathway, where follicular cells shift from the predominant production of E2 during vitellogenesis to the MIH production (Planas et al., 2000). As previously explained, the MIH is the maturation inducing hormone principally mediating the progress of the maturing oocytes throughout their FOM. Being the most critical hormone at this stage, MIH is subjected to several integrated hypophyseal, paracrine, and even autocrine stimulations that control its release from the maturing follicles (Maitra et al., 2013; Planas et al., 2000). Neurotransmitters as melatonin and dopamine conduct a time or season-dependent stimulatory effect on gonadotropins as LH (Dufour et al., 2010; Hong et al., 2014). As such, the latter neurotransmitters are said to be indirectly involved in the control of MIH production and release *via* LH stimulation.

In order to initiate its effect, an MIH should first bind to its receptor which has been classically referred to as the “MIH receptor” following the typical mode of action of a steroid on the target

cell (Patiño and Thomas, 1990). As the identity of this MIH is still undefined in all teleosts and can vary among the different teleosts in which it has been defined, the identity and molecular structure of such MIH receptor could also vary among different teleosts. In teleosts as salmonids, cyprinids, and fresh water percids including our E. perch, progestogens as DHP and THP have been suggested as the true MIH (Berndtson et al., 1989; Patiño and Thomas, 1990). As a consequence, membrane bound progestogens receptors (mPR $\alpha$ ) have been incorporated in some studies that aim at characterizing the mode of action of a typical MIH (Rezanujjaman et al., 2020). The maturing oocyte must nevertheless first express the MIH receptors on its cell membrane in a process termed maturational competence, which is highly regulated by LH (Lubzens et al., 2010). Once in the ooplasm, the MIH activates a series of signal transduction cascades leading to the activation of the maturation promoting factor (MPF) which triggers the dissolution of the germinal vesicle and reinitiates the second meiotic division (Li et al., 2015).

MIH is said to also control ovulation taking place just after FOM where *in vitro* studies on yellow perch have reported progestogens as DHP to induce not only oocyte maturation, but also ovulation. Additionally, the inhibition of ovulation as a result of prostaglandin synthase inhibitor in Longchin Goby, *Chasmichthys dolichognathus*, can somehow highlight the involvement of prostaglandins (PG) in the regulation of ovulation in this teleost (Baek and Lee, 2019). This actually comes in agreement with the past documentations of the involvement of prostaglandins (PG) in the ovulation of several other fish (Lubzens et al., 2010). Hence, both DHP and PG might somehow function together in stimulating the occurrence of ovulation. In E. perch, Henrotte et al. (2011) were able to explain this relation where they showed that arachidonic acid (ARA), *via* its conversion into PG (specifically prostaglandin E2 or PGE2), could indirectly enhance both the follicle final maturation and ovulation by stimulating the synthesis of DHP.

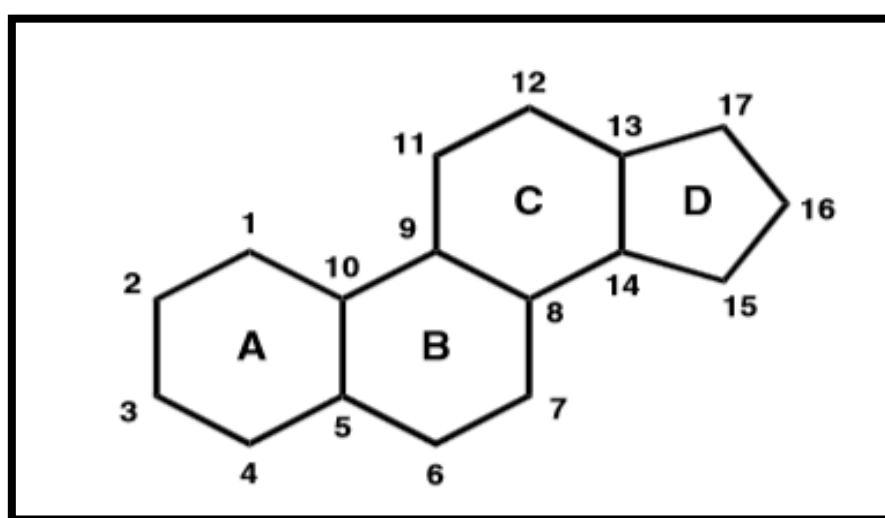
### 3. Corticosteroids

Corticosteroids are steroids that follow the same steroidal metabolic pathway originating from cholesterol and include the previously mentioned four main hormones; cortisol (Co), 11-deoxycortisol (11-D), corticosterone (CoS), and 11-deoxycorticosterone (DOC) in teleosts. Corticosteroids can be sub-grouped into two groups; glucocorticoids and the mineralocorticoids based on their receptor binding affinity (Milla et al., 2009). Similarly to other vertebrates, the four mentioned corticosteroids have been isolated from fish blood where cortisol can be detected at substantially measurable levels (Milla et al., 2015).

Corticosteroids are known to trigger diverse biological processes starting with carbohydrate, lipid and protein metabolism, and immune responses regulation, to the regulation of cardiovascular systems and hydromineral balance. In reproduction, corticosteroids have been documented to play a role in the regulation of follicular development in some species. In some mammals for instance, ovulation is directly followed by the preovulatory increase of cortisol follicular content which occurs during the LH surge. This was proven to be exactly true for humans, but completely irrelevant in the case of some other mammals as mice and pigs in which cortisol was proven ineffective or of inhibitory effect (Milla et al., 2009). In teleosts, corticosteroids functions in reproduction have been less focused on. However, the fact that these steroids are highly affected by fish farming induced stress which has been reflected on their reproductive outcomes, raises several hypotheses regarding the existence of an indispensable role for these hormones in perch reproduction. Hence, in this chapter, we will present some information on corticosteroids production, structure, and plasma levels linked to their possible functions in teleosts, with emphasis on Eurasian perch.

### 3.1. General structure

Corticosteroids share the basic steroid backbone of four fused rings; consisting of three six-membered rings (Figure 8; A, B, C) and one five-membered ring (Figure 8; D). The three six-membered rings together are referred to as phenanthrene, while the five-membered ring is referred to as cyclopentane, and together they give rise to cyclopentanoperhydrophenanthrene, which is the name for the basic steroid structure (Brueggemeier and Li, 2010).



**Figure 8: Basic steroid chemical structure (Brueggemeier and Li, 2010).**

Notably, corticosteroids exhibit common chemical groups attached to the basic steroidal backbone. These include: an oxygen at position C3 resulting from the oxidation of this carbon, a double carbon bond formed between C4 and C5 carbons, a free methyl (CH<sub>3</sub>) end at both positions C10 and C13, and a physiologically important ketol (COCH<sub>2</sub>OH) side chain at the C17 position. At the latter position, corticosteroids can differ where 11-deoxycortisol exhibit a common hydroxyl group (17-OH) at this position as opposed to corticosterone which exhibits it at position C11. Cortisol happens to exhibit the OH group at both C17 and C11 positions where DOC has no OH group at either positions (Brueggemeier and Li, 2010) (Figure 9).

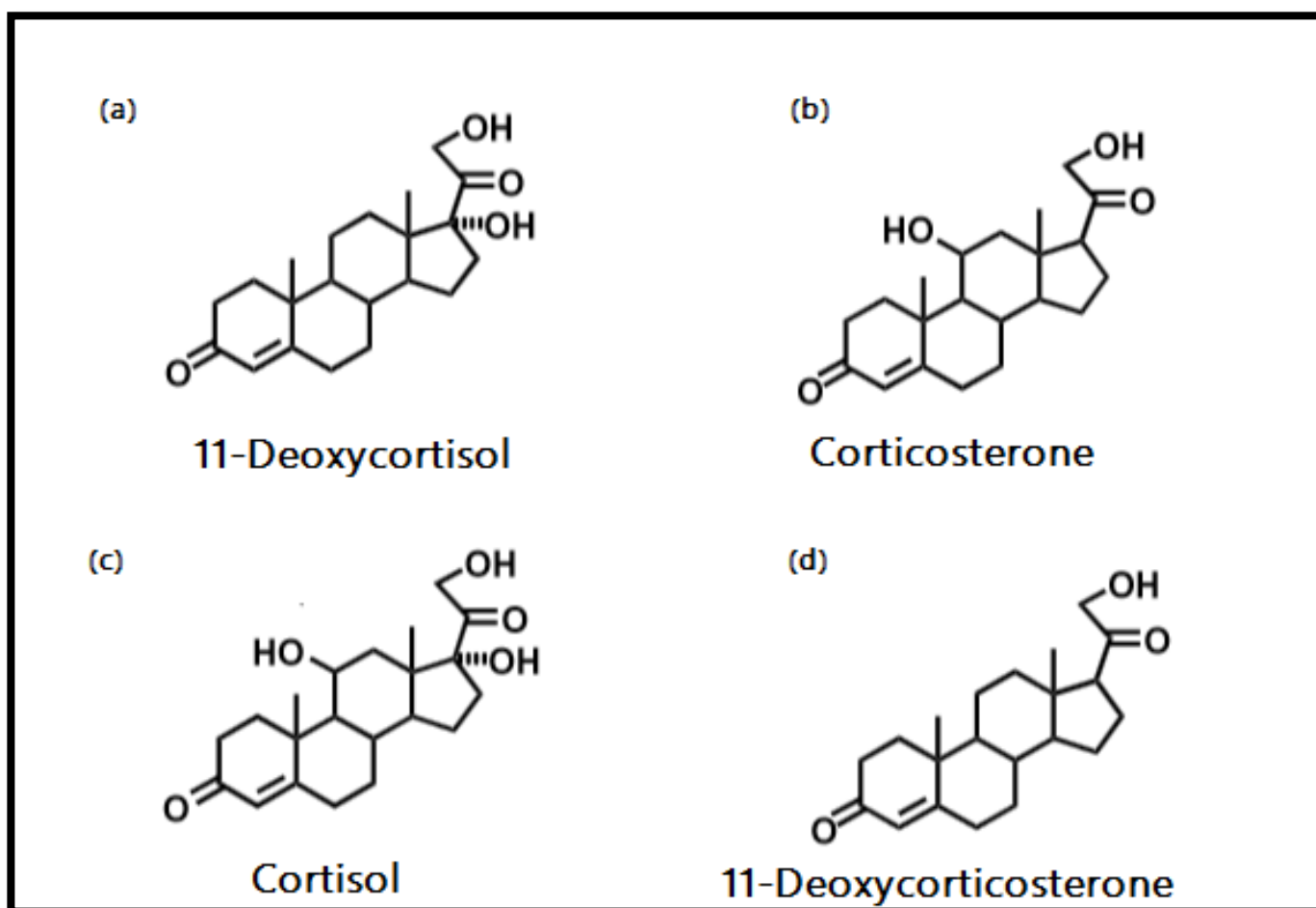
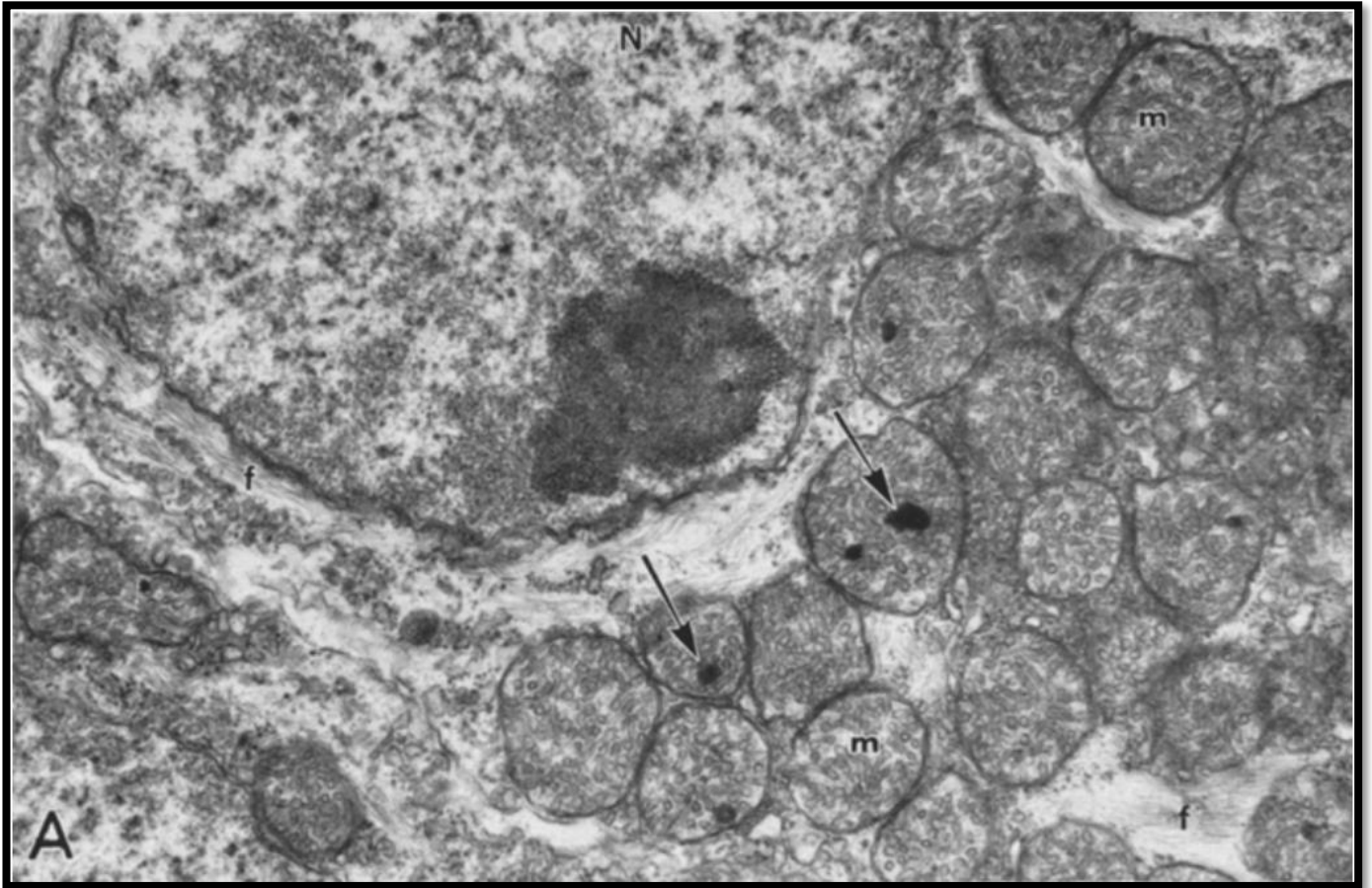


Figure 9: Structure of the four main corticosteroids (Brueggemeier and Li, 2010).

### 3.2. Production and metabolism

In teleost fish, the interrenal gland is the functional equivalent to mammalian adrenal cortex in being at the origin of adrenocortical steroids production. The anatomy of the interrenal gland has been thoroughly illustrated in adult goldfish *Carassius auratus* and found to be located in the head kidney consisting of groups of cells associated with post-cardinal veins or their associated smaller branches (Ogawa, 1967). These interrenal cells are known as the chromaffin cells (Madaro et al., 2015). The fine structure of such interrenal cells was found to be similar to that of adrenal cortical cells of higher animals exhibiting a central nucleus and loose outer membrane. Low electron opacity of these cells revealed a few granules with the visible

cytosolic mitochondria, endoplasmic reticulum, ribosomes, a prominent Golgi apparatus and cytoplasmic filaments (Figure 10) (Ogawa, 1967).

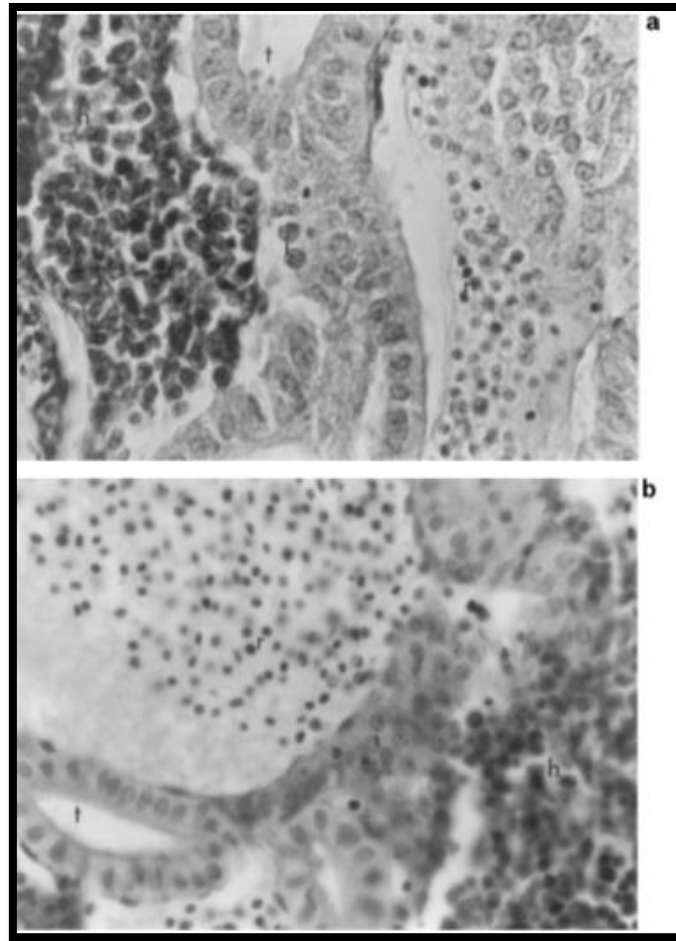


**Figure 10: Microscopic observation of a histological cut of the interrenal cells of goldfish (x16200) showing characteristic mitochondria (m), smooth surfaced endoplasmic reticulum, and cytoplasmic filaments (f). Some intramitochondrial deposits are indicated by arrows, as exhibited by Ogawa in 1967.**

The cytoplasmic filaments exhibited in figure 10 are projected from the mitochondria into the myofilament zone in the smooth muscle cells of the post-cardinal veins in interrenals located in the wall of these veins. Such tubular inner structure of mitochondria usually characterizes steroid secreting cells which are not only found in head kidney but in corpus luteum of the gonads as well. As such, interrenal glands can be also located in the head of the kidney and gonads of fish. In fact, it was shown that the interrenal tissue of gold fish is comparable in structure to the zona intermedia of the rat adrenal cortex especially with regard to the such

mitochondrial structure (Ogawa, 1967). In Eurasian perch, gonads were found to possess all the enzymes responsible for the four corticosteroids synthesis, adding to the fact that the two deoxycorticosteroids (DOC and 11-deoxycortisol) and cortisol were actually shown to be vital products of ovarian steroidogenesis in these species (Milla et al., 2009).

Fish ability to undergo such biosynthesis is however inactive until later stages in life where the high levels of cortisol detected in newly fertilized eggs of yellow perch *Perca flavescens* were found to be of maternal origin. It was later revealed that these fish acquire the ability to synthesize their own cortisol between day 5 and 18 post fertilization (day 8 post-hatch). Still, no interrenal cells were detected in newly hatched yellow perch until the first week after hatching (Figure 11) which raised doubts concerning the interrenal origin of the such early *de novo* production (Jentoft et al., 2002).



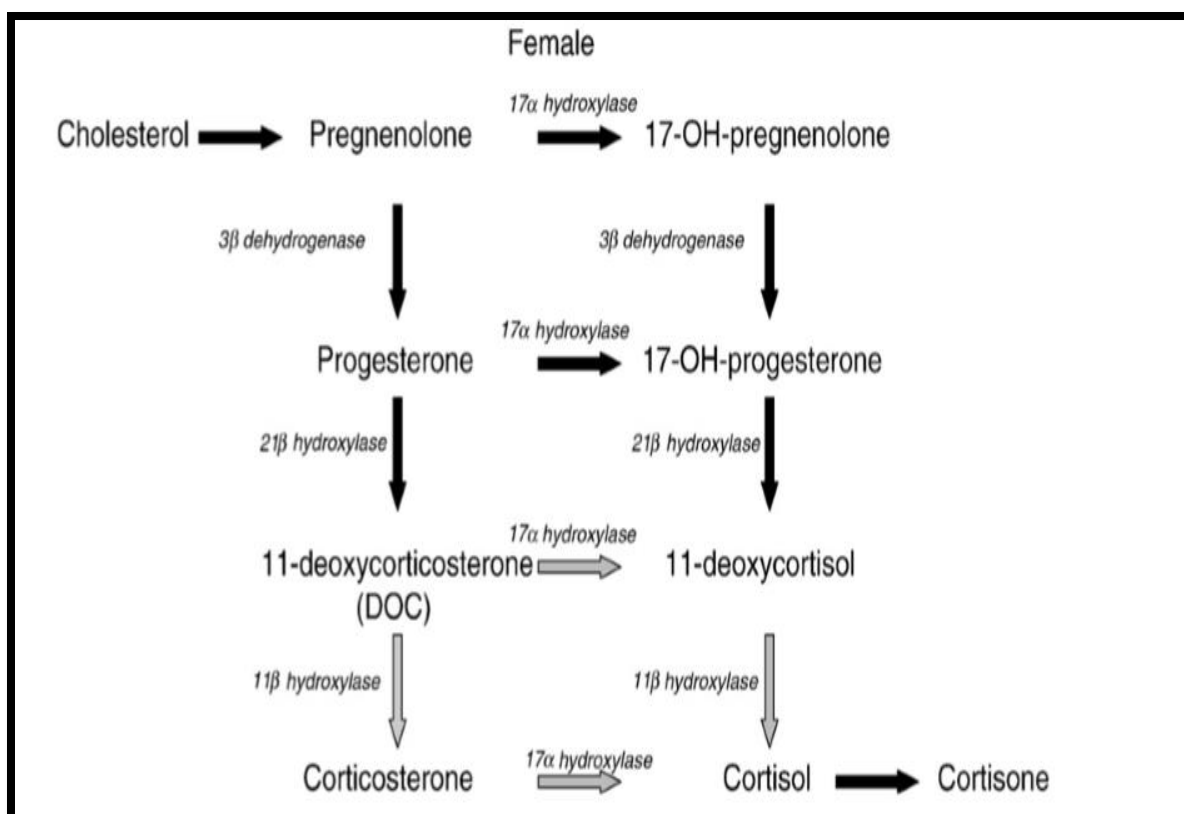
**Figure 11: Microscopic observation of a histological cut of the head kidney of post-hatch yellow perch (x50) exhibiting the hormonal biosynthesis by interrenal cells of one-week old (a) and three-week old (b) fish (Jentoft et al., 2002).**

In teleosts, the interrenal glands are under hypothalamic-pituitary-interrenal (HPI) axis regulation. From the hypothalamus, corticotropin-releasing hormone (CRH) stimulates the pituitary to release the adrenocorticotrophic hormone (ACTH) which reaches, through the blood stream, the interrenal gland located at the fish head kidney. After reaching the head kidney, ACTH activates star responsible for the transport of cholesterol into the mitochondrial membrane (Madaró et al., 2015).

As had been demonstrated in figure 7, corticosteroids are also descendants of cholesterol following the same metabolic pathway described for the other steroids leading to the two principle steroids progesterone and  $17\alpha$ -OH-pregnenolone. Once in the smooth endoplasmic



reticulum, DOC can then be directly produced from progesterone whereas 11-D can be produced from progesterone only following its transformation into 17 $\alpha$ -OH-progesterone. Only then, another hydroxylation occurs by 21 $\beta$  hydroxylase to produce 11-deoxycortisol containing the corticosteroids physiologically important ketol (COCH<sub>2</sub>OH) side chain at the 17 $\beta$ -position (Brueggemeier and Li, 2010; Tenugu et al., 2020). In parallel, the same enzyme catalysis the production of DOC from progesterone (Figure 12) (Milla et al., 2009; Tenugu et al., 2020). 11 $\beta$ -hydroxylase, a mitochondrial cytochrome enzyme complex, and 17 $\alpha$ -hydroxylase are two key enzymes that are thought to mediate the metabolic cycle connecting the four main corticosteroids after having been detected in the gonads of teleosts (Milla et al., 2009). As shown in Figure 11, corticosterone and cortisol could be hydroxylated from the two deoxycorticosteroids (DOC and 11-D respectively) at the C-11 position by the enzyme 11 $\beta$ -hydroxylase. On the other hand, studies imply that 11-D and cortisol could be also hydroxylated from DOC and corticosterone respectively at C-17 position by the enzyme 17 $\alpha$ -hydroxylase. Cortisol can be further transformed into the inactive metabolite cortisone by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase-2 (11 $\beta$ -HSD2) (Jiang et al., 2003). Hence, corticosteroids in teleosts appear to be metabolically linked by the two main enzymes 17 $\alpha$ -hydroxylase and 11 $\beta$ -hydroxylase which have been indeed detected in E. perch head kidney (Milla et al., 2015) with further studies required to confirm their metabolic activities (Milla et al., 2009).

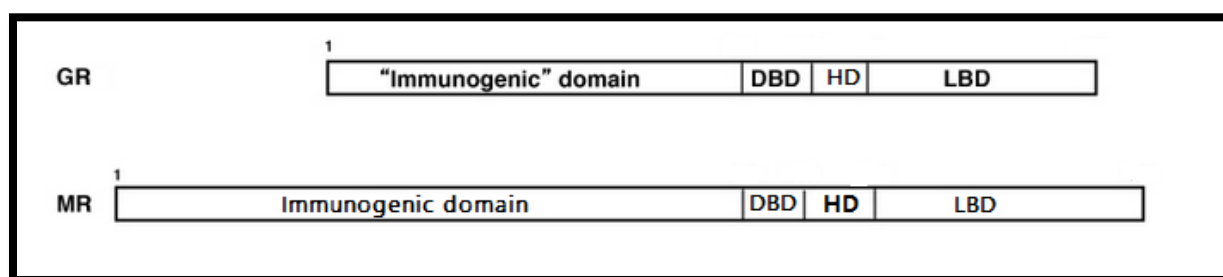


**Figure 12: Schematic representation of the assumed pathways of corticosteroid biosynthesis in female teleost. Solid black arrows show that the enzyme catalyzing the steroid metabolism is active in the gonad; the grey arrows indicate that the enzyme required for that step is expressed in the gonad but its activity needs to be confirmed (Milla et al., 2009).**

### 3.3. Corticosteroid receptors (CRs)

Following their production, corticosteroids are released into the blood circulation where they can be easily detected in blood circulation following their release. At the cellular level, the uptake of the lipophilic corticosteroids can occur by passive diffusion through the cell membrane allowing corticosteroids to follow the genomic pathway. Following this pathway, corticosteroids enter the cell and bind to their specific corticosteroid receptors (CR) that act as ligand-dependant transcriptional factors present in the cytoplasm of the different target cells (Das et al., 2018; Sadoul and Geffroy, 2019). There are two identified types of CR in teleosts; glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). In Eurasian perch and

most teleosts, studies identify two genes each coding for the two distinct GRs (GR1 & GR2) while one gene exists for the single identified MR. Both receptors have the common general morphology of all nuclear and steroid receptors with four distinct regions referred to as domains: AB domain (Immunogenic domain) responsible for transcription activity modulation and is highly variable among species, the DNA binding domain (DBD or C domain) which is highly conserved among species, the hinge D region (HD) involved in conformational changes, and the ligand-binding domain (LBD, E domain) at the carboxyl terminal which binds to the hormone (Prunet et al., 2006) (Figure 13).



**Figure 13: General schematic representation of the four basic domains in both corticosteroid receptors (GR and MR).**

Cortisol can bind to both GRs and MRs while DOC possess the highest affinity for MR which justifies the classification of DOC as a potential mineralocorticoid while cortisol can be classified as both gluco- and mineralocorticoid in teleosts (Mathieu et al., 2014, 2013). 11-deoxycortisol and corticosterone are able to transactivate GRs but at a lower extent compared to cortisol (Bury et al., 2003). In addition to CRs, *in vitro* studies on spotted seatrout revealed that DOC and 11-D can additionally bind and even outcompete with DHP to MIH receptor described earlier (Patiño and Thomas, 1990).

### **3.4. Corticosteroids and reproduction reviewed and linked to stress in captivity**

Up to date, there is still scarce information on the roles of corticosteroids in reproduction. Evidence collected so far on their probable roles are still faced by the fact that it is still unclear whether they inhibit or stimulate reproductive functions. The latter hence would limit the ability to anticipate the effect of corticosteroids-induced stress on reproduction (Milla et al., 2009). In this chapter, we therefore illustrate some of the information available linking corticosteroids to reproduction. This part will help to formulate several hypotheses based on the roles of corticosteroids in female Eurasian perch reproduction.

#### **3.4.1. Roles in early growth stages of primary oocytes**

In European eel *Anguilla anguilla* and rainbow trout, a positive role for cortisol had been suggested in first sexual maturation following the observed increase in pituitary LH resulting from cortisol treatment (Crim et al., 1981; Dufour et al., 1983; Huang et al., 1999). The latter suggestion can be further supported in rainbow trout by the observed rise in cortisol plasma levels between immature and mature trout (Koldkjær et al., 2004). Another corticosteroid (DOC) also highly increased from immaturity to maturity in blue tilapia *Tilapia aurea* (Katz and Eckstein, 1974). Such rise could be linked to the possible effects for corticosteroids on the onset of the very first oogenesis occurring at sexual maturity. The latter could be linked to the possible documented effect of cortisol on gonadotropins secretion necessary for the initiation of oogenesis or in early steroidogenesis (Faught and Vijayan, 2018).

We have previously defined the early growth stages to be marked by intense RNA synthesis and increased appearance of cortical alveoli. In the female freshwater bronze featherback *Notopterus notopterus*, cortisol injection lead to the increase in nucleoli formation apparently reflecting an amplification of genes (Shankar and Kulkarni, 2006). The latter gene amplification

could resemble the intense RNA synthesis occurring during the early growth stages of oocytes. This study also revealed large number of cortical alveoli following cortisol treatment where fish were found to be activated for vitellogenesis based on the appearance of vacuoles and the increased thickness of the follicular layer above the vitelline membrane (Shankar and Kulkarni, 2006). Cortisol also increased the expression of ACTH in zebrafish in which ACTH induced strong vacuolization. The same study also reported cortisol to affect nucleic acid stability in follicles undergoing their cortical alveolar stage (Sousa et al., 2015).

All the latter information demonstrating the effect of cortisol treatment in such freshwater fish could perhaps explain the elevations of some corticosteroids during the onset of oogenesis. Indeed, similar to what had been shown in the bronze featherback, it could be hypothesized that corticosteroids might possess a potent role in early growth stages of oocytes including RNA amplification, cortical alveolar stage, and the initiation of vitellogenesis.

### **3.4.2. Roles in vitellogenesis**

We have previously explained the endocrinological control of vitellogenin production by hepatocytes which is principally mediated by E2. Studies on rainbow trout indicate an interference for cortisol with the binding of E2 to its estrogen receptor (ER) thereby blocking the hepatic Vtg production during vitellogenesis (Lethimonier et al., 2002). To further investigate the exact status of such effect, Berg et al. (2004) performed an *in vivo* study on Arctic char *Salvelinus alpinus* where they compared the single introduction of cortisol to its introduction in combination with E2. Their results showed that high concentrations of cortisol intensified Vtg production rather than blocking it as shown in rainbow trout. However, such effect was observed only when cortisol was introduced alone. When cortisol was introduced with E2, it provided the same results observed in rainbow trout in which it similarly reduced the amount of circulating plasma Vtg (Berg et al., 2004; Lethimonier et al., 2002). The latter results propose two perspectives; first, the possible role of cortisol on teleost vitellogenesis

could be highly species dependent, second, cortisol effect is highly modulated by whether it is introduced alone or in combination with other hormones.

In Eurasian perch, *in vitro* studies which rather focused on late vitellogenesis revealed the two corticosteroids cortisol and DOC to highly depress such *de novo* E2 production (Mandiki et al., 2017). The latter finding can further suggest that the previously exhibited effects for cortisol on vitellogenin production could be resulting from its effects on E2 production by follicles as shown in Eurasian perch. The latter could be true, except that, in the same study on Arctic char, western blot of plasma vitellogenin additionally provided some evidence that cortisol might be acting on the conformation of plasma-bound Vtg (Berg et al., 2004). It has been previously demonstrated how Vtg is usually transported in its intact form comprising the three egg-yolk proteins (Lv, phosvitins, and  $\beta$ -component) after which it undergoes “first proteolysis” in order to be stored inside the developing oocyte (Fabra et al., 2006; Hara et al., 2016). The results revealed by the western blot of Berg and his colleagues (2004) revealed a disturbance in plasma-bound Vtg when fish were treated with cortisol in combination with E2. The latter could perhaps imply the possibility for an effect of cortisol on the first proteolysis. The latter hypothesis is however not enough supported and would require further investigations but can as well propose a role for cortisol on plasma-bound Vtg structure.

### **3.4.3. Roles in FOM and ovulation**

By comparison to progestogens, corticosteroids are less focused on in literature when it comes to the control of FOM and ovulation. Despite that, among all the mentioned stages of oogenesis, FOM remains the most highlighted stage during which corticosteroids effects have been tested. In Asian stinging catfish, *Heteropneustes fossilis* Bloch, *in vitro* assays investigating the effects of corticosteroids on FOM revealed the two corticosteroids DOC and 11-D to be evident maturation inducers with 11-D being even more effective than DOC (Goswami and Sundararaj, 1974). Another *in vitro* study in yellow perch revealed 100% GVBD by 11-D at 0.5  $\mu\text{g/ml}$  with

ovulation at higher concentrations (1 µg/ml). This same study revealed the occurrence of GVBD and some ovulations by DOC at 0.1 µg/ml and 1 µg/ml respectively, while corticosterone required much higher concentrations (10 µg/ml) to achieve such ovulations (Goetz and Bergman, 1978). Cortisol has been also found to be highly effective in inducing the *in vitro* FOM in yellow perch and several other teleosts (Theofan and Goetz, 1983). In fact, a peak in plasma concentration of 11-D and DOC which usually synchronises with plasma MIH levels in several teleosts, and the surge in cortisol levels recorded around ovulation in several teleosts as the rainbow trout, brown trout *Salmo trutta*, goldfish, and common carp, also implies the existence of a crucial role for these corticosteroids in the final stages of reproduction (Mandiki et al., 2017; Milla et al., 2009). It should be nevertheless noted that in some studies, it is suggested that 11-D and DOC may displace DHP bound to plasma and cortisol may lower oocyte sensitivity to DHP (Shankar and Kulkarni, 2006).

In wild Eurasian perch, a peak in 11-D was indeed recorded during FOM and ovulation which also coincided with DHP (the proposed MIH in several teleosts) peak (Noaksson et al., 2004). However, the effects recorded in studies about the displacement of DHP by these corticosteroids can equivocally speculate whether the rise in corticosteroids levels coinciding with DHP peak in perch would be to induce or inhibit FOM. The latter is still faced by the other mentioned *in vitro* studies demonstrating a stimulatory effect for corticosteroids on the FOM which could still propose their positive role in inducing FOM advancement in perch. Up to date, the study performed by Mandiki and colleagues (2017) gave some clues about the possible nature of corticosteroids effect during early FOM where both cortisol and DOC were found to deeply depress the *in vitro* steroidogenic ability of maturing oocytes to produce DHP (Mandiki et al., 2017). Therefore, it appears that the effect of corticosteroids in female Eurasian perch should be further investigated during FOM to further characterise their involvement in this stage.

#### **3.4.4. Roles in spawning and mating behaviour**

In species which undergo migration during spawning, some corticosteroids were found to increase during fish migration (Carruth et al., 2000; Truscott et al., 1986). Some studies link the rise in corticosteroids levels during migration, to the behavior of fish during that period (olfaction, maternal aggressiveness, metabolic needs to reach the spawning grounds...) (Carruth et al., 2002; Faught and Vijayan, 2018; McConnachie et al., 2012). However, the recorded rises in some corticosteroids around spawning could also imply other reproductive roles for these hormones during spawning. In semelparous pacific salmon exposed to the same challenges encountered during its migration induced an increase in plasma cortisol (Hinch et al., 2006). It was interpreted that such cortisol rise could have affected the levels of androgens and estrogens in that species in which a decline was observed in the latter hormones along the increase in cortisol (Hinch et al., 2006; Young et al., 2006). In female pacific salmon, cortisol plasma increases following induced stress in spawning fish did not alter reproductive hormone titers. Instead, it was observed that cortisol treatment resulted in high egg retention during the migration for spawning (McConnachie et al., 2012). All these finding imply that cortisol increase at spawning, which could be naturally induced, could be linked to a role for cortisol either in reproductive hormone oscillations or in other processes of spawning as egg release. In all cases, it can be evident that chronic cortisol elevations on spawning grounds can highly influence reproductive functions and success.

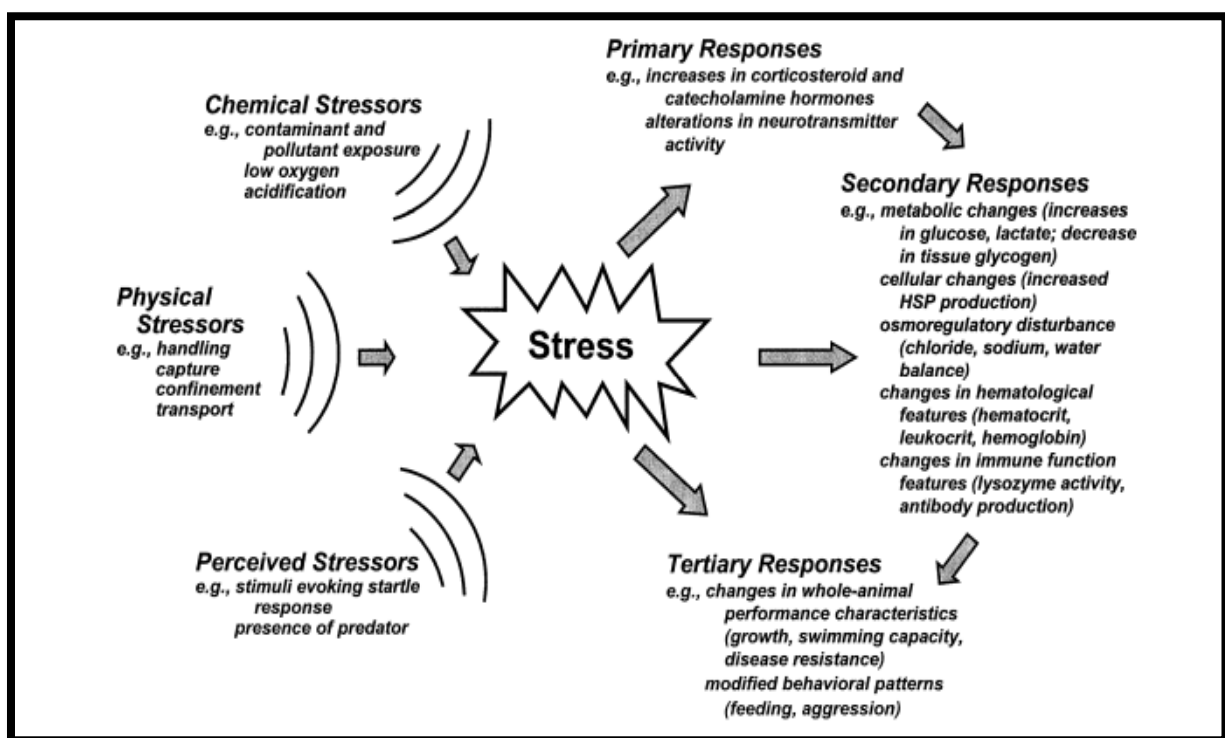
#### **3.4.5. Corticosteroids and the stress response linked to their roles in reproduction**

Being under control of the HPI axis, corticosteroids are known to play an essential role in regulation of the stress response in all vertebrates including teleosts (Kiilerich et al., 2018). Consequently, corticosteroids plasma levels would be highly dependent on the stress perception by the fish (Milla et al., 2015, 2009). When considering the above hypothesized roles for



corticosteroids on reproduction, it makes good sense to draw a link between stress and reproduction. We therefore find it equivocally vital to discuss the possible effects of stress on reproduction due to the effects of stress on corticosteroids.

In the review by Barton (2002), stressors are defined as being either chemical (e.g. contaminants), physical (e.g. handling, confinement, capture...), or other kind of stimuli that provokes stress. The resulting stress responses to such stressors can be; primary (exhibited in the form of disturbed catecholamines and corticosteroids levels), resulting in secondary (exhibited in the form of cellular or metabolic changes), eventually leading to a tertiary responses that can lead to changes in the whole animal performance (Figure 14) (Barton, 2002).



**Figure 14: Physical, chemical and other perceived stressors act on fish to evoke physiological and related effects, which are grouped as primary, secondary, and tertiary (Barton, 2002).**

In the review by Milla et al. (2015), the secondary response was later justified as the animal's mechanism to resist changes in the external environment in order to withstand stress. This secondary stress is reflected in the anatomical, histological, and physiological changes as an attempt for the animals to survive the stressful situation. In the latter review, authors insist on the fact that such attempt for animals to survive the stressful situation is highly dependent on the magnitude and duration of the stressor (Milla et al., 2015). This brings us to the concept of chronic (continuous) and acute (short-term) stress where studies investigating the effects of induced stress on corticosteroid-related functions highly differentiate between the two kinds of stress. One of these studies is a very old study in brown trout showing that the effect of chronic stress can highly differ from that of acute stress (Pickering et al., 1987). Today, studies insist on indicating the nature of the induced-stress as either chronic or acute when documenting the effects of stress on reproduction.

Among the four corticosteroids, cortisol is known to be the most stress-sensitive (Kiilerich et al., 2018) where its plasma level is estimated in the range of 1-200 ng/mL during stressful conditions as opposed to a much lower basal level in the range of 2-40 ng/mL during low or mild stressful situations in percids. Eurasian perch females exposed to leachate (considered as a kind of environmental stressor) have revealed levels of cortisol ranging between 200-600 ng/g plasma along the whole reproductive season (Noaksson et al., 2004). We still insist on the fact that the levels of corticosteroids and their response to a stressor is highly dependent on the studied percid (Milla et al., 2015). In yellow perch, cortisol/stress relation starts to become apparent as early as one-week post-hatch (Jentoft et al., 2002). Concerning the other corticosteroids (DOC, 11-D, and corticosterone), there is much less information on the effect of stress on their plasma levels in teleosts. In a study by Kiilerich et al. (2018), the effects of applying confinement stressors were assessed on the levels of DOC along the levels of cortisol in rainbow trout. It was revealed that such stressors induced the upregulation of DOC plasma

levels along an upregulation in pituitary MR. As such, a physiological role for DOC in fish stress endocrinology was suggested (Kiilerich et al., 2018).

In mature female fish, stress effect on reproduction starts to become more prominent due to the corticosteroid/stress relation. In the example on female Atlantic croaker *Micropogonias undulatus* subjected to sublethal concentration of chemical pollutants as to mimic the impact of an acute environmental stressor on fish reproduction during the period of their ovarian recrudescence, fish exhibited highly elevated cortisol levels (Thomas, 1989). The impact of such elevations were later further assessed in another study and were observed to be reflected on cumulative vitellogenin production (Murphy et al., 2005). As explained earlier, the primary stress response exhibited in the form of increased corticosteroid levels (increase cortisol levels), and the resulting secondary response exhibited in the form of cellular changes (cumulative vitellogenin production), would have eventually led to the tertiary response observed on whole animal performance (changes in produced egg-yolk). Although the kind of stressors experimented in the latter study falls into the category of acute chemical stressors (acute exposure to a pollutant or contaminant), it can provoke the same primary and secondary stress responses resulting from the acute physical stressors frequently encountered in fish reared in RAS (handling, tagging,...) (Barton, 2002). This is due to the fact, as stated by Dourson et al. (2011); stress is one of the unavoidable main problems in modern breeding practices of captive fish.

# **OBJECTIVES AND HYPOTHESES**

## OBJECTIVES AND HYPOTHESES

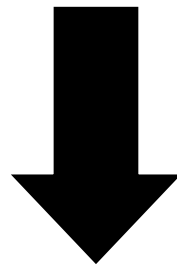
The economics of fish culture rely on the maximization of fish production year-round. The year-round production of European perch is increasing thanks to the development of intense indoor systems as RAS (Toner, 2015). An indispensable element of such intensive aquaculture systems is the human control over the entire reproductive cycle of fish (Żarski et al., 2019). For Eurasian perch, manipulation of both photoperiod and temperature in RAS is highly used to obtain out-of-season spawns (Abdulfatah et al., 2011; Wang et al., 2006). One main challenge that is still faced in controlled aquaculture production of this percid is the synchronization of its spawning (Żarski et al., 2012b). In several studies performed in percids, photoperiod and temperature manipulations can alter the timing of gonadal development in fish (Migaud et al., 2004). In captivity, fish are exposed to multiple simultaneous stressors including repeated handling, transport and crowding especially during spawning season to assess readiness of fish to spawn (Madaro et al., 2015; Żarski et al., 2021). Alterations in reproductive outcome of several fish due to such captivity-induced stressors is also observed (Milla et al., 2015; Schreck, 2010). The changes in fish gonadal development and synchronization has been hypothesized to occur over the course of domestication of Eurasian perch in RAS (Khendek et al., 2017; Rocha de Almeida et al., 2019).

There have been several studies that recorded the effect of captivity-induced stress on fish corticosteroids levels from one side (Jentoft et al., 2005; Kiilerich et al., 2018; Schreck, 2010), and another study that have recorded the effect of Eurasian perch domestication on cortisol levels from another side (Khendek et al., 2017). We recall that plasma levels of cortisol and other corticosteroids are under the control of the HPI axis (Milla et al., 2015). With both stress and domestication revealing alterations in reproductive performances, it appears as if the bidirectional interactions between the hypothalamus-gonadal-interrenal axis (HPI) and the

hypothalamus-pituitary-gonadal axis (HPG) could explain the observed alterations in reproductive performance of domesticated Eurasian perch.

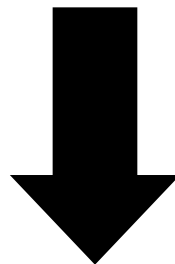
## **Thesis Hypothesis**

**Corticosteroids possess a highly potent role in reproduction that could explain the reproductive drawbacks in domesticated female Eurasian perch**



## **Major Bottleneck**

**To discuss the above hypothesis, the limitation related to the scarce available data on corticosteroids role in reproduction constitutes one major bottleneck**



## **Thesis objective**

**The major aim of this thesis is thus to detect the putative roles for the four most studied corticosteroids (DOC, 11-D, corticosterone, and cortisol) in the reproductive cycle of domesticated female Eurasian perch *Perca fluviatilis* L.**

**To reach the above objective, we test the following working hypotheses:**

**H1:** Corticosteroids basal plasma levels would fluctuate along the whole reproductive cycle where an increase from the basal levels at a certain point of the female reproductive cycle could imply a putative function at this point.

**H2:** Corticosteroids role might be linked to vitellogenesis which is a very critical stage in the formation of egg-yolk. The latter being a crucial quality indicator of the produced egg and larva.

**H3:** Corticosteroids role might be linked to FOM. As such, a disturbance in their function during FOM would explain the observed uneven ovulations and resulting asynchrony in spawning of captive Eurasian perch.

1. To test the first hypothesis (**H1**), we conducted a descriptive approach in **chapter 1** where we measured the plasma levels of the four studied corticosteroids along the whole female reproductive cycle. The measurements were based on frequent samplings at precise points of the reproductive cycle (onset of oogenesis, early and late cortical alveolar stage, early and late vitellogenesis, FOM, and spawning). To better link the plasma kinetics of corticosteroids to their assumed roles in the different stages of the reproductive cycle, we measured in parallel the relative gene expression of the gonadal corticosteroids receptors to describe their kinetics as well. To monitor the metabolic pathways linking the corticosteroids together along the reproductive cycle, we additionally measured the relative gene expression of some of the metabolic enzymes involved in corticosteroids metabolism.
2. To test the second hypothesis (**H2**), we added a more direct approach in **chapter 1** during which we *in vitro* test the direct effects of corticosteroids on late vitellogenic oocytes. During this approach, we focused on hypothesizing a direct role for corticosteroids on the

formed yolk globules which would be important quality indicators in the quality of the formed egg-yolk.

3. To test the third hypothesis (**H3**), we first conducted a systematic review during which we collected more information from previous publications to discuss the possibility of nominating corticosteroids, rather than progestogens, as MIH (**chapter 2**). We later *in vitro* tested the direct effect of corticosteroids as possible MIH (**chapter 3**) and as hormones affecting the activity of the MIH (chapter 4) during FOM.

Testing the first hypothesis (**H1**) would allow to observe the kinetics of corticosteroids along the reproductive cycle to better spot the periods of their potential physiological activity. Testing the second hypothesis (**H2**) would help in later discussing whether corticosteroids activity during late vitellogenesis could perhaps explain the alterations in egg quality in captive fish. testing the third hypothesis (**H3**) would allow to discuss whether the corticosteroids role in FOM could be behind reproductive defects during the spawning period.

By the end, the three working hypotheses would serve in reaching the thesis objective concerning the realization of the potent roles for the four corticosteroids in female Eurasian perch reproduction. Once reaching the latter objective, it would become easier to discuss the stated thesis hypothesis assuming that the alterations in fish corticosteroids due to domestication or captivity-induced stressors could be behind the alterations in reproductive performance in captive fish.



# CHAPTER 1

# CHAPTER 1

**Chapter 1: What would Corticosteroids plasma kinetics and gonadal receptor expression tell about their possible roles in the reproductive cycle of domesticated female Eurasian Perch *Perca fluviatilis*?**

**(Submitted to Aquaculture Journals on January, 21, 2022)**

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## **Abstract**

To improve the quality of reproduction in Eurasian perch, *Perca fluviatilis* L., a promising candidate for the Eurasian freshwater aquaculture that is currently cultivated in RAS, investigating the hormones mediating and affecting its reproduction is indispensable. Literature defines a group of four major corticosteroids (11-deoxycorticosterone, 11- deoxycortisol, corticosterone and cortisol) that might be mediating critical stages in female perch reproduction. Unfortunately, neither the basic roles nor the kinetics of these four corticosteroids during the whole reproductive cycle of female perch has been well defined up to date. In this study, we therefore exhibit the plasma kinetics of the four corticosteroids along the reproductive cycle of domesticated female perch while monitoring the expression of the different receptors and enzymes mediating their production and possible functions. Additionally, we perform an *in vitro* experiment during late vitellogenesis to investigate any direct role for these steroids during that stage. Our results revealed that the four corticosteroids are in detectable levels along the whole reproductive cycle along which most of them fluctuated significantly depending on the stage of reproduction. Interestingly, our results opened the possibility for the existence of rather side metabolic pathways for corticosteroids production other than the defined principle ones. Effectively, our *in vitro* analysis revealed some of these corticosteroids to have possible physiological effects during late vitellogenesis.

**Keywords:** Corticosteroids, Eurasian perch, plasma levels, late vitellogenesis, receptors, reproductive cycle

## 1. Introduction

Eurasian perch, *Perca fluviatilis*, is one of the carnivorous fishes that has been appreciated by anglers and consumers particularly in western Europe and is mainly cultivated in intensive recirculating aquaculture systems (RAS) (Fontaine and Teletchea., 2019). The latter system would allow for rapid fish growth rate within shorter periods of production year round under optimal human-controlled conditions notably by controlling both photoperiod and temperature (Polcar et al., 2015). However, attaining such productivity with optimal egg quality remains challenging (Rocha de Almeida et al., 2019). To better understand the deterioration in reproductive performance of perch in RAS, further information is required regarding the whole physiological regulation of their reproduction.

One of the known sources for reproductive failure in fish is the physiological internal response to the perception of stressors which is hormonally controlled by corticosteroid hormones. The reported effects of corticosteroids on GnRH and gonadotropins production have led to recent interest in their possible roles in fish reproduction (Kiilerich et al., 2018; Milla et al., 2009). But up to date, scattered information is available on the exact roles for all these steroids in female fish reproduction. To illustrate this, no study has so far reported the plasma variations of the main corticosteroids during oogenesis in any teleost. Eurasian perch is a group-synchronous spawner. Its reproductive cycle is initiated with the primary oocyte growth, followed by vitellogenesis, and ends with final oocyte maturation (FOM) in preparation for ovulation (Lubzens et al., 2010; Źarski et al., 2012). In yellow perch, *Perca flavescens*, a percid sharing strong phylogeny with Eurasian perch, the involvement of four main corticosteroids (11-deoxycorticosterone or DOC, 11-deoxycortisol or 11-D, cortisol or Co, and corticosterone or CoS) in FOM induction as maturation inducing hormones (MIH) had been proposed following some *in vitro* assays (Goetz and Bergman, 1978; Theofan and Goetz, 1983). Receptor-binding affinity assays have indeed highlighted the link between corticosteroids

receptors (CRs) and FOM (Liu et al., 2018). It was revealed that 11-deoxycortisol, corticosterone and, to a higher extent, cortisol are able to transactivate two of the identified CRs namely glucocorticoid receptors (GR1) and (GR2) (Bury et al., 2003) whereas only DOC and, to a lower extent, cortisol can activate the other identified CR namely mineralocorticoid receptor (MR) (Mathieu et al., 2014; Sturm et al., 2005). Interestingly, *in vitro* studies also showed that DOC and 11-D can additionally bind to MIH receptors on maturing oocytes from spotted seatrout (Patiño and Thomas, 1990). The latter information and the previous findings in yellow perch were both sufficient to suggest corticosteroids as MIH. However, our recent study using domesticated female Eurasian perch revealed that none of the suspected four corticosteroids is the actual MIH (El Mohajer et al., 2021). When reviewing available data on the plasma levels of cortisol, the most abundant circulating corticosteroid in teleosts (Sturm et al., 2005), and of 11-D in wild female perch, their highest levels is measured around FOM and spawning (Noaksson et al., 2005). This limits us from completely eliminating other possible roles for these corticosteroids during the peri-ovulatory period. Besides these final stages, studies suggest certain roles for cortisol during vitellogenesis where it was found to inhibit E2-mediated vitellogenesis in the liver of rainbow trout *Oncorhynchus mykiss*, by suppressing the expression of hepatic estrogen receptors (ER) mediated *via* its hepatic GR (Lethimonier et al., 2002). By contrast, cortisol was found to upregulate hepatic vitellogenin production in Arctic char *Salvelinus alpinus*. The physiological effect would remain to be species-dependent as cortisol mediates the variable stress-coping mechanisms in fish (Berg et al., 2004). In domesticated perch, cortisol levels were found to be higher than its basal levels (>10 ng/ml) around vitellogenesis (Khendek et al., 2017). Hence, it would make sense to suggest a possible involvement for cortisol at this stage. Finally, at the onset of oogenesis, cortisol has been shown to participate to the endocrine regulation of puberty in eels (Huang et al., 1999). Altogether, the

current literature suggests that corticosteroids, notably cortisol, can exert some roles during critical stages of female perch reproductive cycle.

Besides cortisol, three other corticosteroids (11-D, DOC and CoS) are detected in the blood circulation of teleost but their levels or even presence highly depend on the fish species and on the reproductive stage (Milla et al. 2009). In studies performed on wild female perch, 11-deoxycortisol is usually assessed along cortisol and is found in detectable levels along the whole reproductive cycle (Noaksson et al., 2004). 11-deoxycortisol has been previously detected in several other teleosts as salmonids (Milla et al., 2009) such as rainbow trout (Campbell et al., 1980). In fact, 11-deoxycortisol is produced from DOC where both are highly upregulated during reproduction (Katz and Eckstein, 1974; Milla et al., 2009). Finally, corticosterone is detected at high levels that fluctuate during the reproductive cycle in elasmobranchs (Manire et al., 2007), but its variations along the reproductive cycle in teleost are unknown. All the previous information proposes a possible physiological function for these corticosteroids which might come in handy in resolving the reproductive drawbacks of teleosts in RAS later on. The objective of the present study is thus to investigate the involvement of the four main corticosteroids (Co, 11-D, DOC, CoS) adjacently to other classical sex hormones of more known function; 17-beta Estradiol (E2), testosterone (T), prostaglandin E2 (PGE2) and 17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one (DHP), during the course of oogenesis in Eurasian perch. To address this question, i) we measured the plasma levels of these corticosteroids along the whole reproductive cycle, ii) we measured the relative gonadal expression of the so far identified CRs, and iii) we tested the *in vitro* effects of these corticosteroids on the vitellogenesis given the sparse information of a possible role for these steroids at this step of the reproductive cycle.

## **2. Materials and Methods**

### **2.1. Ethics**

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: 23871- 2020013017452359) by the local ethics committee in France (name: CELMEA).

### **2.2. Experimental animals and rearing conditions**

One population of 2-year-old domesticated female Eurasian perch with an average weight of  $417.5 \pm 42.1$ g, standard length of  $28.6 \pm 1.3$ cm was used in this experiment. The population was provided by Lucas Perches company (Lorraine, France) and transferred to the Aquaculture Experimental Platform (AEP, registration number for animal experimentation C54-547-18) belonging to the URAFPA lab of the University of Lorraine (Nancy, France). In this platform, fish were maintained in 2m<sup>3</sup> tanks under recirculating water systems (RAS) (renewal rate of 150% per hour) supplied originally with the city water. All fish tanks were equally exposed to a single photothermal program which aims at initiating the reproductive cycle in female perch from sexual rest through active oocyte growth and vitellogenesis until final oocyte maturation and spawning (Fontaine et al., 2015). The photothermal program was initiated at T0 (photoperiod: 16h light (L): 8h dark (D); temperature 22°C) with fish being at sexual rest period. Later, both temperature and light duration were gradually decreased (T1-T2) to allow fish to enter into the phase of active oocyte growth and vitellogenesis. Following such decrease, both environmental conditions were held steady (photoperiod: L:D 8:16; temperature 6 °C) for a period of 4.5 months (T3 until T5) to allow the progress through vitellogenesis. Both temperature and light duration were increased again around T6 to allow progress through final oocyte maturation (T7) and the consequent spawning (T8) to take place later on (Figure 1). The

temperature, photoperiod and light intensity (10 lx at the water surface) were controlled automatically by a centralized technical management.

During this photothermal program, frequent measurements of pH and oxygen in water were performed three times a week; pH had been maintained between 7 and 7.5 (using NaCO<sub>3</sub> additions) and dissolved oxygen above 6 mg/L. Total ammonia and nitrite nitrogen had been also frequently measured on weekly basis and assessed below 1 mg/L using indophenol blue colorimetric methods and CARY 1 spectrophotometer. Fish feed was purchased from Le Gouessant (B-repro 32 Astrax) and was used to feed fish until satiation using automated feeders throughout the whole experiment.

### **2.3. *In vivo* blood and organ sampling**

12 different fish were sampled for blood and gonads every 6 weeks starting from T0 until T6. One week later (T7), fish were checked for their FOM advancement where only the 12 fish which were advanced through FOM (only stages 3-4, see Zarski et al. 2011) were sampled for blood. During the spawning period, blood and eggs were sampled from 12 of the spawned females 48 h after the spawning (T8).

#### **2.3.1. Blood plasma sampling**

At each sampling time, the 12 sampled fish were anesthetized by immersion into Ethyl 3-aminobenzoate methanesulfonate solution for 2-3 min (150 mg/L, Sigma). Blood was then removed from the caudal vein using 2ml heparinized syringe within 5 min (Milla et al., 2010) at least for 6 of the 12 sampled fish. Collected blood was then centrifuged at 3000rpm for 10min (Centrifuge Jouan C-412). Plasma aliquots were then directly stored at -80°C pending ELISA hormonal analysis.



### 2.3.2. Gonadal histology and estimation of FOM advancement throughout oogenesis

After blood sampling, fish were euthanized by overdose of Ethyl 3-aminobenzoate methane-sulfonate solution (300 mg/L, Sigma). Fish were then measured (cm), weighed (g), and dissected for gonad and liver weights (g) to calculate the gonado-somatic ( $GSI = \frac{100 \times \text{gonad weight}}{\text{body weight}}, \%$ ) and hepato-somatic ( $HSI = \frac{100 \times \text{liver weight}}{\text{body weight}}, \%$ ) indices respectively.

Ovarian fragments of only 9 of the 12 sampled fish from T0 until T5 were stored in Bouin solution for one week before they were washed once with water and twice with 70% ethanol. Follicles were further dehydrated (95 and 100% ethanol) after which they were cut and immersed in toluene then embedded in paraffin blocks. Sections of 7  $\mu\text{m}$  were cut from the paraffin blocks using Leica microtome (RM2125 RTS) and were then stained with Masson's trichrome-hematoxylin Gill (Merck, Darmstadt, Germany), 0.5% phloxine B (VWR, California, USA), and 0.5% light green (Sigma, Saint-Quentin-Fallavier, France) according to Rinhard and Kestemont (1996). The stages of oocytes development were visualized using an upright light optical microscope (Nikon Eclipse Amsterdam, Netherlands), Nikon BR software (Nikon France, Champigny sur Marne, France) and were assessed following the classification system developed by Abdulfatah et al. (2013). At times T3, T4, and T5, Yolk Globules diameter (YGD,  $\mu\text{m}$ ) of the vitellogenic oocytes was also measured for 5 of the biggest yolk globules per 10 oocytes randomly selected per fish using the same light upright optical microscope.

During T6 and T7, the entrance and advancement for fish through FOM was checked using Serra's solution (ethanol:formaldehyde:acetic acid in the proportion 6:3:1, respectively) following the classification developed by Źarski et al. (2011).

### 2.4. *In vivo* Hormone assays

All hormonal assays were carried out on plasma aliquots after validation of the signal specificity by making plasma hormone doping consisting in addition of known concentration of the

hormone and then calculation of the recovery rate and/or by checking the proportionality between the dilution factors of serially diluted plasma aliquots and the calculated hormonal levels after the ELISA. Dilution was performed for some samples when necessary to obtain an absorbance signal included within the standard curve. Detailed information regarding each ELISA is shown in table 1. Hormonal levels of the four corticosteroids (DOC, 11-D, CortS, and Cort) were assayed from the plasma aliquots of the six fish sampled within less than 5min whereas the hormonal levels of the other hormones (E2, T, DHP and PGE2) were assayed from the plasma aliquots of other six fish not necessarily sampled within less than 5min.

## **2.5. *In vivo* Total RNA extractions and cDNA synthesis**

Pieces of intact ovarian follicles from the sampled gonads of each of the 12 fish sampled at all times (except at T7) and the ova collected from the 12 spawned fish at the spawning time were preserved at -80°C until the day of RNA extraction. On the day of RNA extraction, total RNA was extracted from the preserved pieces of intact ovarian follicles and ova using TRIzol reagent (15596026, Invitrogen) according to the manufacturer's protocol. The integrity of the extracted RNA was assessed using a Nanodrop ND-1000 Spectrophotometer (DS-11, DeNovix). Our extracted RNA samples exhibited a 260:230 nm and 260:280 nm ratios averages of 1.8 and 2.1 respectively. Reverse transcription was performed using Reverse Transcriptase kit (RT-RTCK-05, Eurogentec) following DNase treatment (DNase I, Thermo fisher Scientific, EN0521) according to the manufacturer's recommendations. For each sample, 200ng of total RNA were used and the reaction mixtures were prepared according to the manufacturer's recommendations. Controls without RNA (replaced by a volume of ultrapure water), without the reverse transcriptase enzyme, and without DNase treatment were each included in the analysis. Each reverse transcription mix was preserved at -20°C until Gene expression analysis.

## 2.6. Gene expression analysis

The relative gene expression for the different corticosteroids receptors (GR1, GR2 and MR), progesterone receptor (PR), and metabolic enzymes (17 $\alpha$ hydroxylase and 11 $\beta$ -hydroxysteroid dehydrogenase or 11 $\beta$ HSD2) involved in corticosteroids metabolism was assayed using Real-time PCR (qPCR, quantitative Polymerase Chain Reaction). The primers for the two GR1 splice variants (a and b) identified in perch (Mathieu et al., 2013) and those of the other assayed genes are presented in table 2. Primers for housekeeping genes, GR (GR1a, GR1b, GR2), MR, and 11 $\beta$ HSD2 were selected from previous studies (Mathieu et al., 2014, 2013; Milla et al., 2010; Rocha de Almeida et al., 2019). Primers for 17 $\alpha$ hydroxylase and PR were designed based on multiple alignment of sequences available in the NCBI database which targets the conserved regions of intended mRNA. When possible, primers at inter-exon junction were selected to avoid potential signal contamination by genomic DNA. Efficiency for each of the used primers was validated prior to analysis and is also presented in table 2. The RT-qPCR was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using SsoAdvanced<sup>TM</sup> Universal SYBR<sup>®</sup> Green Supermix (Bio-Rad) reagent according to manufacturer's instructions in a final reaction volume of 20  $\mu$ l (5  $\mu$ l of 40-fold diluted cDNA, 625 nmol/L for the reverse and forward primers of GR1a, GR1b, GR2 and MR, and 125nmol/L for those of 11 $\beta$ HSD2 - 11 $\beta$ -hydroxysteroid dehydrogenase 2). The reaction carried was as follows; 95°C for 10min, 40 cycles; 95°C for 15sec + T<sub>m</sub> of each gene for 1min, followed by an increase between 65°C and 95°C (+0.5°C/5 sec) to attain the fusion curve. A melting curve was added to each well to verify that a single DNA fragment was amplified. In addition to the three controls mentioned above, a control without cDNA was included (replaced by a volume of ultrapure water). The expression of the mRNA was determined by the comparative CT method considering a standard curve of samples diluted in cascade (mix of cDNA from several samples). After failing to attain no significant variation for the selected housekeeping genes (18S RNA,  $\beta$ -actin, EF-1 $\alpha$ ,

Cathepsin-D) between the different sampling times, samples were expressed relatively to the amount of reverse-transcribed template RNA for each sample that went into the real-time PCR as validated in Campbell et al. (2006) and Breton et al. (2012) in a study about the seasonal changes of transcript levels during fish reproductive cycle.

## **2.7. *In vitro* follicle culture, histology, and estimation of hormonal effects during late vitellogenesis**

At late vitellogenesis (T5), additional ovarian fragments from 6 of the sampled fish at that time were dissected in Cortland medium at 6°C to reach an average weight of  $1081.5 \pm 106.3$  mg. Each of these ovarian fragments was incubated into each well of the sterile 12-well plastic plates (STARLAB, Sarstedt, Germany) containing 2 ml of Cortland medium (prepared from the components in 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) per well. To reach their desired final concentration (ng/ml); E2 (10, 100), DOC (10, 100), 11-D (10, 100), Co (100, 1000), CoS (10, 100), 10 µl of each of the five hormones initially dissolved in ethanol were added into its assigned well containing the culture medium just before adding the dissected ovarian fragment. In addition to the four corticosteroids, E2 was also selected based on its major known role in teleost vitellogenesis process. For each female, two sets of controls were used: CTet; Cortland medium plus 10 µL standard ethanol, and CT; Cortland medium alone. All chemicals were purchased from Sigma-Aldrich (France) and the utilized hormonal doses were selected to reach physiological and supra-physiological levels according to available information on their recorded circulating plasma concentrations during late vitellogenesis (Berg et al., 2004; Hara et al., 2016; Khendek et al., 2017; Noaksson et al., 2005; Pankhurst, 1998; Pankhurst and Van Der Kraak, 2000; Sulistyo et al, 1998). All plates were then incubated at 6°C for 24h under agitation in the dark. Following the 24h incubation period, each of the incubated ovarian

fragments was gently transferred to Bouin solution for 6 days before they were subjected to the same series of inclusions described in part 3.2. The effect of the hormonal treatment on some of the histomorphological features of late vitellogenic oocytes was evaluated on 10 randomly selected follicles using three parameters:

1. Red region ratio represented by the length of the red region (RR) occupied by yolk globules (YG) over half the diameter of the whole oocyte (OD/2)
2. Density of YG represented by the number of YG per fixed unit area of  $137517,49 \pm 417,47 \mu\text{m}^2$  (b)
3. Diameter ( $\mu\text{m}$ ) of the largest five yolk globules per follicle within the fixed unit area indicated above.

The latter parameters were visualized also using the same light upright optical microscope used in part 3.2.

## **2.8. Statistical analysis**

For all the variables of the *in vivo* assay (independent sample comparison), after checking the normality of the data and the homogeneity of variances using Shapiro-Wilk and Levenes test respectively, when the assumptions of the ANOVA test were not validated, a Kruskal-Wallis test was carried out followed, when significant, by a Conover test. For all the variables of the *in vitro* assay (pairwise sample comparison), after checking the normality of the data and the homogeneity of variances using Shapiro-Wilk and Levenes test respectively, when the assumptions of the ANOVA test were validated, a one-way ANOVA was performed followed, when significant, by the Fisher's LSD test. Differences between modalities were considered statistically significant if  $p \leq 0.05$ . If necessary, the data were transformed to log or square root, to reach the assumptions of ANOVA and they were also corrected using Bonferroni. Using Pearson's correlation, correlations between changes in plasma levels of different hormones and

the relative expressions of the measured receptors and metabolic enzymes were all tested together at all times. Correlation was also verified between the *in vitro* hormonal effects on yolk globule diameter and their densities. Free R software version 3.3.1 was used. All results are represented as mean  $\pm$  standard deviation.

### **3. Results**

#### **3.1. *In vivo* gonadal developmental stages and FOM advancement**

During the first two months of the experiment (T0 and T1), the GSI was low (below 2%). From T1, the GSI started to rise significantly ( $p < 0.05$ ) until reaching its maximum of 16.1% at T6 (Table 3). Meanwhile, the HSI significantly ( $p < 0.05$ ) increased from T0 to reach its maximum at T2-T3 after which it started to drop significantly ( $p < 0.05$ ) (Table 3). Histological analysis of the ovaries revealed that the gonads were still at early oogenesis stages (primary oocyte, early and late cortical alveoli stages) between sampling times T0 and T1 where they entered into vitellogenesis at T2 (Figure 2). Between sampling times T4 and T5, the percentage of oocytes at the late vitellogenic stage recorded its highest values. At T6, fish were shown to be at stage 1 of FOM. One week later (T7), fish were shown to advance in their FOM until stages 3-4 and asynchronous spawning was recorded for a group of other fish at T8 (Figure 2).

During the vitellogenesis period, the YGD ( $\mu\text{m}$ ) measured at T3, T4, and T5 was shown to significantly ( $p < 0.005$ ) increase between T4 and T5 but was almost stable between T3 and T4 (Table 3).

#### **3.2. *In vivo* sex hormonal levels**

As exhibited in Figure 3, plasma E2 levels increased significantly to 3 ng/ml at T2 ( $p < 10^{-5}$ ) and then dropped significantly between T3 and T5 ( $p < 0.05$ ) where it continued to drop significantly until reaching a significant minimal level (0.05 ng/ml) during the spawning period (T8). Plasma

testosterone (T) levels increased gradually until reaching its highest significant levels ( $p<0.05$ , 40 ng/ml) as follicles entered into the FOM (T6) after which it dropped sharply to a significantly ( $p<0.05$ ) low level 48hrs after spawning (T8). Similarly, DHP recorded its highest, yet insignificant, levels (0.07 ng/ml) at the start of FOM (T6) after which it remained detectable (0.024 ng/ml) until spawning at T8. PGE2 levels increased significantly between T5 and the start of FOM ( $p<0.05$ ) where it remained almost stable (0.6-0.8 ng/ml) until spawning at T8.

Both DOC and 11-D recorded their highest levels, 1.8 ng/ml and 58 ng/ml respectively, at the beginning of the reproductive cycle (T1). Following this period, 11-D gradually decreased until reaching their lowest significant levels ( $p<10^{-2}$ ) at T4, while DOC strongly decreased until reaching its lowest significant levels at T2 and T4. Following T4, 11-D started to gradually increase returning to its highest initial concentration (around 58 ng/ml) (Figure 4). Corticosterone plasma kinetics did not reveal any significant change ( $p>0.05$ ) during the whole reproductive cycle where its low plasma levels remained in the detectable range of 0.04-0.01 ng/ml (Figure 4). Cortisol started to increase significantly ( $p<0.05$ ) from T6 to reach its highest significant levels at T6, T7, and T8 (Figure 4). When testing the correlations between all corticosteroids, the only significant correlation positively linked 11-D and DOC ( $p<0.05$ ,  $r=0.31$ ).

### **3.3. Gene expression analysis of the CR and PR receptors and corticosteroidogenic enzymes**

The relative gene expression for each of the identified corticosteroid receptors (GR1a and b, GR2, and MR) was found to be at its highest at the beginning of the photothermal program, after which it started to decrease until the maintenance at the lowest levels of expression (Figure 5). None of the corticosteroid receptors revealed significant correlation with any of their ligand corticosteroids but revealed significant correlations with each other (MR and GR1a:  $p<10^{-3}$ ,  $r=0.28$ ; MR and GR2:  $p<10^{-3}$ ,  $r=0.29$ ; GR1a and GR1b:  $p<10^{-14}$ ,  $r=0.7$ ; GR2 and GR1a:  $p<10^{-$

<sup>31</sup>,  $r=0.9$ ; GR2 and GR1b:  $p<10^{-22}$ ,  $r=0.84$ ). The relative gene expression of progesterone receptor (PR) dropped significantly ( $p<0.05$ ) between T5 and T8 at which it recorded its lowest relative expression (Figure 6). The relative gene expression of the enzyme 17 $\alpha$ hydroxylase reached its highest levels at T2 and T6, significantly higher ( $p<10^{-3}$ ) than its lowest level at T8 (Figure 6). Finally, the gene expression of 11 $\beta$ HSD2 was the highest at T0-T1 and then dropped to a constant low level from T3 to T8.

The correlation between the relative gene expression of PR and 17 $\alpha$ hydroxylase with each of the four plasma corticosteroids showed that it was only significant between PR and DOC ( $p<0.05$ ,  $r=-0.33$ ). However, the correlation between the relative gene expression for both 17 $\alpha$ hydroxylase and PR was shown to be highly significantly positive ( $p<10^{-11}$ ,  $r=0.4$ ) where neither was correlated to any of the CRs. 11 $\beta$ HSD2 was the only enzyme to be significantly correlated but to only two of the CRs namely the MR ( $p<10^{-4}$ ,  $r=0.36$ ) and GR1a ( $p<0.05$ ,  $r=0.15$ ) with which it showed similar kinetics (Figure 7).

### **3.4. The *in vitro* effects of the four corticosteroids and E2 on late vitellogenic oocytes**

The effect of subjecting late vitellogenic oocytes to each of the four corticosteroids and E2 treatment revealed that only DOC and 11-D (at both tested doses of 10 and 100 ng/ml) induced a significant 2-fold increase in average yolk globules diameter ( $p<10^{-2}$  and  $p<10^{-3}$  respectively) compared to the negative control CTet (Figures 8 and 9). Both corticosteroids at their two tested doses also induced a significant decrease ( $p<10^{-6}$ ) in the density of formed yolk globules. However, no effect was realized for any of the tested hormones on the ratio of red region (Figures 8 and 9). After realizing the significant effects of DOC and 11-D, the average yolk globules diameters and densities correlation was tested and was found to be significantly negative ( $p<10^{-4}$ ,  $r=-0.49$ ).



#### 4. Discussion

This *in vivo* study describing the plasma levels of the four corticosteroids during the whole reproductive cycle of female Eurasian perch revealed all four corticosteroids to be within detectable levels. Previous studies on female perch have already exhibited high levels of cortisol in the range of 80-550 ng/ml which fluctuated according to the stage of reproduction and experimental conditions (Khendek et al., 2017; Noaksson et al., 2005). Consistently, our study also revealed the highest cortisol levels in the range of 100-125 ng/ml. 11-Deoxycortisol (11-D) levels have also been recorded in the range of 1-10 ng/ml in wild female perch (Noaksson et al., 2005), but our study has shown that it can reach 6 times higher levels. This difference between the absolute levels recorded in wild perch and different domesticated perch populations can be attributed to the differences in the experimental conditions and fish populations. In all cases, both studies do support the existence of 11-D in huge levels in female perch. Studies on mature female rainbow trout have already exhibited 11-D to be measured at high levels (18ng/ml) (Campbell et al., 1980). With such consequent levels, we thus support that 11-D is a major corticosteroid in teleosts and may deserve as much attention as cortisol in the physiological studies. In our study, DOC was also found to be within a range of 0.5-1.8 ng/ml. This mild level has ever been detected in other mature females in teleosts (Milla et al., 2009) which implies that DOC is not a minor corticosteroid during the reproductive cycle. However, we were able to detect corticosterone only at very low levels (0.03-0.08 ng/ml) which were found to be a bit close to the low levels detected in female winter flounder *Pseudopleuronectes americanus* Walbaum (0.3 ng/ml) (Campbell et al., 1976). To our knowledge, this study is the first to report the absolute levels of these four corticosteroids in any maturing female teleost. Indeed, this study allows to nominate both cortisol and 11-D as two major corticosteroids while the barely detectable levels of corticosterone do not favor its strong implication in perch

oogenesis. In addition, the significant fluctuations of 3 corticosteroids (DOC, 11-D, and cortisol) during the reproductive cycle suggest their involvement in perch oogenesis.

Along the oogenesis stages, the significantly positive correlation between DOC and 11-D is consistent with the fact that 11-D is produced from DOC. However, the relative gene expression of 17 $\alpha$ hydroxylase, the enzyme catalyzing this conversion in teleosts (Milla et al., 2009), did not show to be correlated to the plasma kinetics of either hormones (DOC or 11-D) which cannot support this metabolic pathway. We assume that the production of DOC and 11-D during perch reproductive cycle may stem-out from other hormones like progesterone and 17-OH-progesterone respectively. In this way, the synchronously correlated levels of DOC and 11-D attained could be due to the synchronous activation of the enzyme 21 $\beta$ hydroxylase synchronously catalyzing both reactions (Milla et al., 2009).

The high plasma levels of DOC and 11-D at the start of the reproductive cycle (T1) relative to the other stages of this cycle strongly highlight a potent role for these corticosteroids perhaps in the primary growth stages or the onset of oogenesis. The absence of high significant levels for the other two corticosteroids (corticosterone and cortisol) eliminates a similar role for these steroids in the primary oocyte growth stages. The overall plasma levels of cortisol, as for 11-D and DOC, however did fluctuate during the upcoming stages of the reproductive cycle implying possible roles for these steroids in the other perch reproductive stages as well.

Within the period extending between T4 and T5, DOC and 11-D levels started to increase again, this period coinciding with late vitellogenesis according to both histological and E2 plasma analysis. As such, we hypothesize that the marked elevation of yolk globules diameter between T4 and T5 is mediated by these two corticosteroids. Our *in vitro* results attained during late vitellogenesis (T5), supported this hypothesis where both corticosteroids (DOC and 11-D) stimulated the significant increase in yolk globules diameters and the consequent decrease in their densities. As this elevation of yolk globules diameters is negatively correlated with their

intra-cytoplasmic density, we assume that these corticosteroids triggered the fusion of existing yolk globules. These yolk globules are usually formed of the protein Vitellogenin (Vtg) which is produced in the liver and secreted into blood circulation in its intact form before it is incorporated into oocytes. Once in the oocytes, Vtg must be cleaved into the multiple egg yolk proteins namely lipovitellin (Lv), phosvitin (Pv), and  $\beta'$ -component ( $\beta'$ -c) in order to form the yolk globules which are stored in the cell. The three egg yolk proteins have been previously isolated and purified in rainbow trout (Hara et al., 2016). In Arctic char *Salvelinus alpinus*, there has been evidence that cortisol affects the intact form of Vtg that migrates in the blood where it induced the cleavage of Vtg from its intact form (Berg et al., 2004). However, our realized steroidal effect was rather on the coalescence of these yolk globules and was only realized for DOC and 11-D. Perhaps, such mechanism of yolk globules fusion resembles that of lipid droplets coalescence which occurs during the final oocyte maturation. In fact, it has been documented that in pelagophil teleosts the yolk globules fuse into a single mass of fluid yolk during FOM resulting in increased concentration of free amino acids (FAA) and small peptides in the oocyte ending-up with the osmotic mechanism for water influx into oocyte (Fabra et al., 2006). Presumably, such coalescence of yolk globules is initiated in the rapid transition between late vitellogenesis and postvitellogenesis in perch where the *in vitro* stimulation of late vitellogenic oocytes with DOC and 11-D might have induced this mechanism. Meanwhile, both the low relative expression for all CRs (all GRs and MR) and the absence of any significant correlation between these receptors and the plasma kinetics of either DOC or 11-D do not argue the possibility for the involvement of the corticosteroid receptors in such possible physiological effect. Instead, the PR relative expression was shown to be elevated at T5. Also, the relative mRNA abundance for PR was found to be maximal during late vitellogenesis in rainbow trout (Mourot et al., 2006). The fact that PR shares similar structural domains with CRs (both GR and MR) in some vertebrates (Leo et al., 2004) and that PR can be activated by DOC and 11-D

in elephant shark *Callorhynchus milii* in which progesterone was in turn able to activate the MR as well (Katsu et al., 2019; Lin et al., 2021), strengthens the structural-ligand similarity between the corticosteroids and progestogen ligands. This encourages us to hypothesize the possibility of PR mediation of such yolk globules fusion which is probably induced by the two corticosteroids during late vitellogenesis in perch. The latter information is further supported by the realized significant correlation between PR and DOC yet limited by the absence of significant correlation between 11-D and PR. To demonstrate the involvement of these two corticosteroids through PR, further *in vitro* biological tests would be needed by adding PR antagonists to DOC and 11-D and by observing the consequences on the yolk globules.

Corticosterone levels did not change significantly during the whole reproductive cycle with its slight rise starting around late vitellogenesis without reaching any significant levels later on. The absence of any significant correlation between cortisol and corticosterone or between both hormones and the enzyme 17 $\alpha$ hydroxylase (transforming corticosterone into cortisol) implies that the metabolic pathway involving the production of cortisol from corticosterone using this enzyme (Milla et al., 2009) is not very efficient at this stage. Despite that, cortisol levels do reach their highest significant levels at FOM and spawning compared to the start of the reproductive period which implies the possible existence of an alternative metabolic pathway for its production. The absence of correlation between cortisol and 11 $\beta$ HSD2 (transforming cortisol into cortisone) on the other hand signifies that the produced cortisol is not being transformed into its inactive metabolite cortisone during FOM and spawning. The latter information can further support the indispensable necessity for maintaining cortisol in its active form perhaps to mediate a putative role during FOM and spawning. During FOM and spawning, 11-D also returns to its highest significant plasma levels. Both corticosteroids (11-D and cortisol) thus display elevation at the final stages of the reproductive cycle which could have nominated the latter two corticosteroids as possible active MIH. In fact, the latter possibility

had been eliminated by our last *in vitro* study in which DHP, and none of the corticosteroids, was confirmed as the potent MIH in female perch (El Mohajer et al., 2021). In Tiger puffer *Takifugu rubripes*, 11-D contributes, along with DHP, for the production of THP (the other identified MIH by El Mohajer et al., 2021) through the enzymes 20 $\beta$ hydroxylase and 21-hydroxylase respectively (Matsuyama et al., 2001). Using the latter information, we can somehow link our attained high levels of 11-D to the possible crucial production of THP between FOM and spawning. Also, DHP levels are shown to reach their highest at the start of FOM after which their levels slightly drop perhaps to contribute with 11-D in the production of THP until spawning. Besides the latter possible role, the peaks of cortisol and 11-D may be attributed to other physiological roles during perch mating. In other teleost species, cortisol has been also shown to increase during these final stages and to be implicated in oocyte hydration (Milla et al., 2006), reproductive behaviour (Bry, 1985; Shankar and Kulkarni, 2006) and broodstock immune defense (O'connor et al. 2009). The exact role of cortisol and 11-D would thereafter deserve further investigations for example by examining the effects of hormone injections on the above physiological processes.

In conclusion, our study clearly shows that the four corticosteroids can be measured at variable levels in female maturing perch. For the first time in any teleost fish species, this study demonstrates that DOC, cortisol and 11-D exhibit significant variation along the reproductive cycle, which support their involvement in oogenesis. The detected possible functions for DOC and 11-D during late vitellogenesis were evident but would still require further analysis to understand the exact molecular aspect of such role in the fusion of yolk globules possibly by measuring FAA and small peptides in the oocyte which result from the fusion of yolk globules as explained earlier. Given the high levels for some corticosteroids at FOM, we therefore insist on a possible role for these hormones during FOM and spawning even if we have previously excluded their roles as MIH.

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## 6. Tables and Figures

**Table 1 Information on the different ELISA kits utilized in blood plasma analysis.**

Hormone	Company	ELISA kit	CV%		Assayed Volume μl	Samples Dilution (times)	Sensitivity	Recovery Rate %
			Intra	Inter				
<b>DHP</b>	MyBioSource (San Diego, USA)	MBS2602842	<5	<10	100	none	0.06 ng/ml	81%-99%
<b>E2</b>	Diasource (Louvain-La-Neuve, Belgium)	KAP0621	<4	<5	50	1:10 T1 to T5	5 ± 2 pg/ml	91%-104%
<b>T</b>	Diasource (Louvain-La-Neuve, Belgium)	KAPD1559	<4	<9	25	1:5 T4  1:10 T5 to T7	0.083 ng/ml	88-96%
<b>PGE2</b>	Thermo Fisher Scientific (Illkirch-Graffenstaden, France)	KHL 1701	<5	<6	100	1:5 All Samples	31.3 pg/ml	---
<b>Cort</b>	Diasource (Louvain-La-Neuve, Belgium)	KAPDB270	<6	<9	20	1:2 All Samples	0,4 μg/dl	95% to 102%
<b>DOC</b>	MyBioSource (San Diego, USA)	MBS749262	<8	<3	100	none	1 pg/ml	81.09
<b>11-D</b>	BlueGene (Pudong New District, Shanghai)	E12D0070	<7	<12	100	1:4 All Samples	0,1 ng/ml	75.37
<b>CortS</b>	R&Dsystems (Rennes, France)	KGE009	<4	<9	50	none	0,047 ng/ml	75.48

**Table 2 Primer sequences used for real-time PCR analysis.**

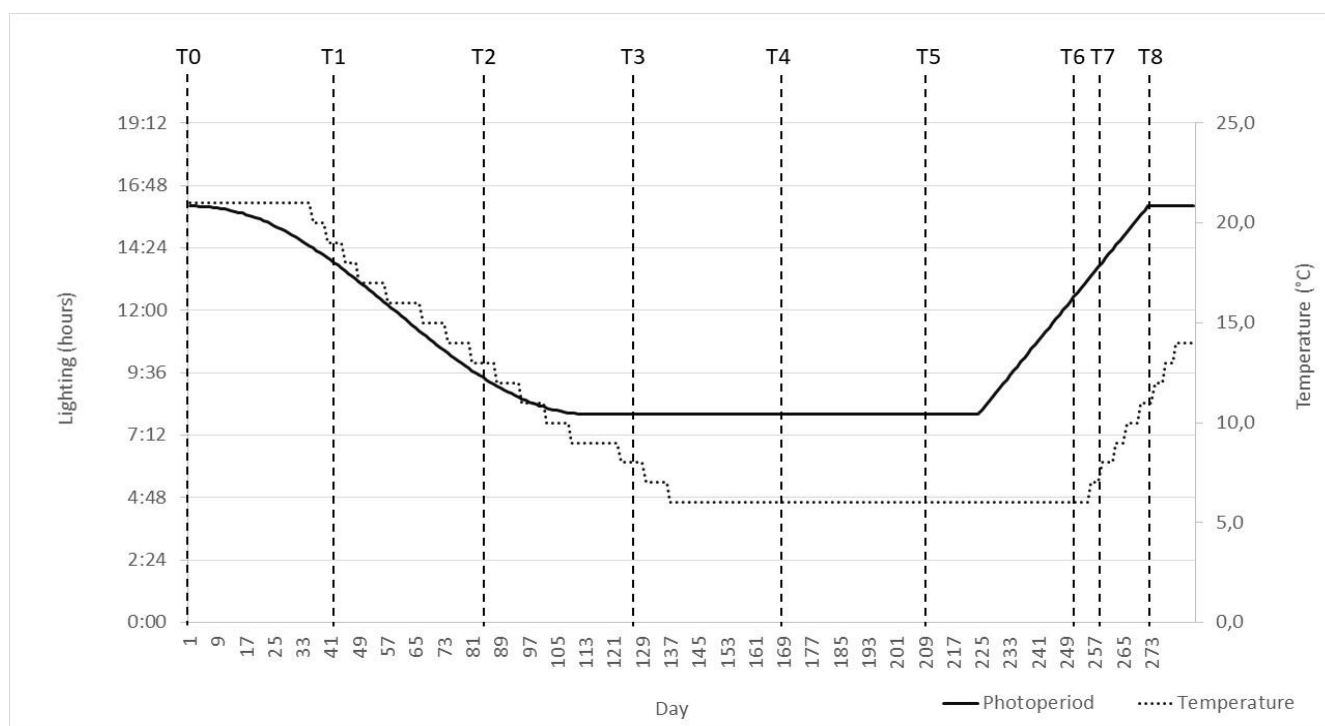
Target gene	NCBI/ GenebankGene ID	Sequence	Annealing temperature T <sub>m</sub> (°C)	PCR efficiency (average)
<b>GR1a</b>	EU861040	<b>Forward</b> CTGTTTGGTGTGTTTCAGACGAG	60.8	101.5%
		<b>Reverser</b> ATCTGTATTTTGTCTGCTCTCCATC	63.8	
<b>GR1b</b>	EU861040	<b>Forward</b> CTGTTTGGTGTGTTTCAGACGAG	60.8	94.5%
		<b>Reverser</b> AGGTAGTTGTGCTGTCCTTCGA	61.2	
<b>GR2</b>	FJ946991	<b>Forward</b> GCAACGGCAACATGCTCTGCTTCGCA	65.9	101.5%
		<b>Reverser</b> TCGTCGTTGGAAACCTGCAGCCGGA	65.8	
<b>MR</b>	FJ946992	<b>Forward</b> AGCGCTGCCAATGCCCTGGT	64.5	90.6%
		<b>Reverser</b> TGCTTTCCGGCCAGGCGGTTGA	65.5	
<b>PR</b>	XM_039782254.1	<b>Forward</b> GGGACTCAAAAGTCAGGCCG	60.96	79.5%
		<b>Reverser</b> ATGCTGCTGTACTCAGATCCAC	60.16	
<b>17<math>\alpha</math>hydroxylase</b>	XM_039783344.1	<b>Forward</b> CCAGAGTTTGAGGCCATGCT	60.32	99.7%
		<b>Reverser</b> GTCAGTGTAGTCTGCCTTGTGT	60.22	
<b>11<math>\beta</math>HSD2</b>	FJ946994	<b>Forward</b> GTACGGAGCCTCCAAGGCTGCTCTG	64.9	57%
		<b>Reverser</b> TCGGCATGCTGCGATTCCCA	61.5	

**Table 3 Gonadosomatic (GSI) and Hepatosomatic (HSI) indices measured for each time (n=9) and the Diameter of Yolk Globules (YGD) measured only at T3, T4 and T5.**

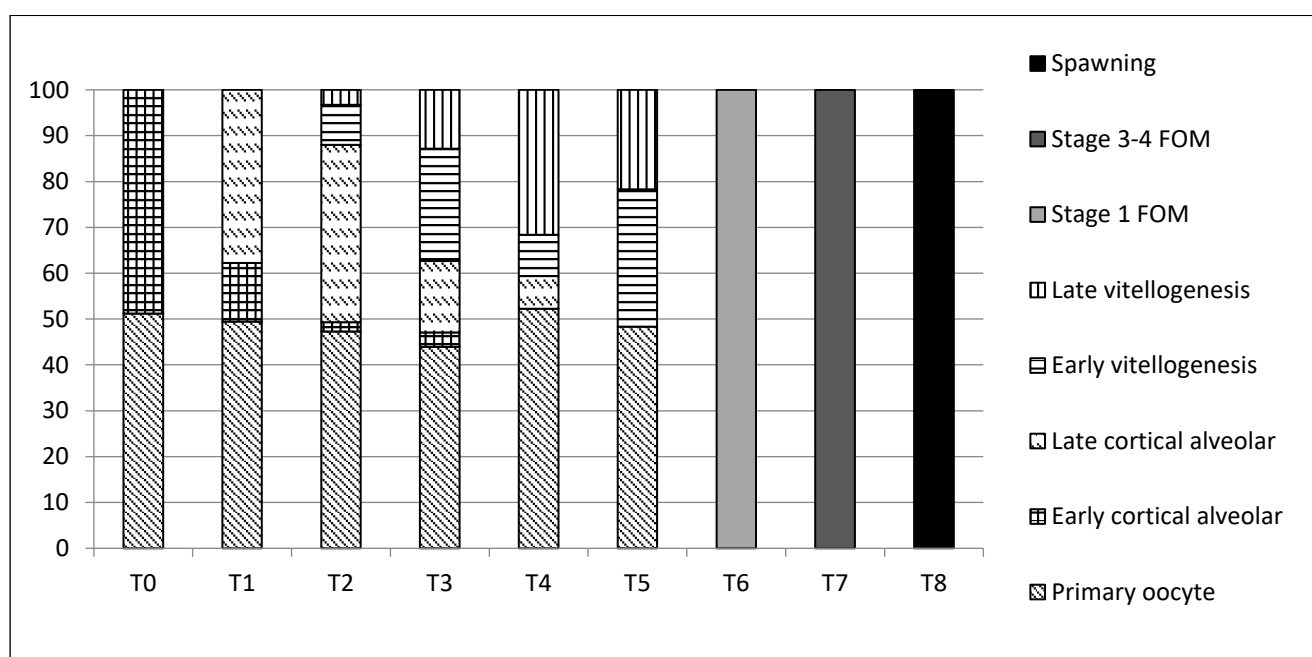
The values recorded in this table represent the mean $\pm$ SD for each of the measured parameters alone where values for each parameter were compared to each other and statistically different values ( $p < 0.05$ ) are marked by different letter.

	T0	T1	T2	T3	T4	T5	T6
GSI%	1,19 $\pm$ 0,33 <sup>a</sup>	1,48 $\pm$ 0,88 <sup>a</sup>	5,05 $\pm$ 1,84 <sup>b</sup>	7,30 $\pm$ 1,43 <sup>b</sup>	11,54 $\pm$ 4,15 <sup>c</sup>	13,47 $\pm$ 3,08 <sup>cd</sup>	16,14 $\pm$ 5,1 <sup>d</sup>
HSI%	1,62 $\pm$ 0,24 <sup>a</sup>	2,32 $\pm$ 0,35 <sup>b</sup>	4,17 $\pm$ 1,00 <sup>e</sup>	3,91 $\pm$ 0,48 <sup>de</sup>	3,20 $\pm$ 1,00 <sup>cd</sup>	2,81 $\pm$ 0,83 <sup>bc</sup>	2,35 $\pm$ 0,32 <sup>b</sup>
Diameter of Yolk Globules (YGD) in $\mu$ m				7,54 $\pm$ 1,52 <sup>a</sup>	6,60 $\pm$ 3,75 <sup>a</sup>	15,60 $\pm$ 5,82 <sup>b</sup>	

**Figure 1 Photothermal program and sampling times followed during the experiment.**

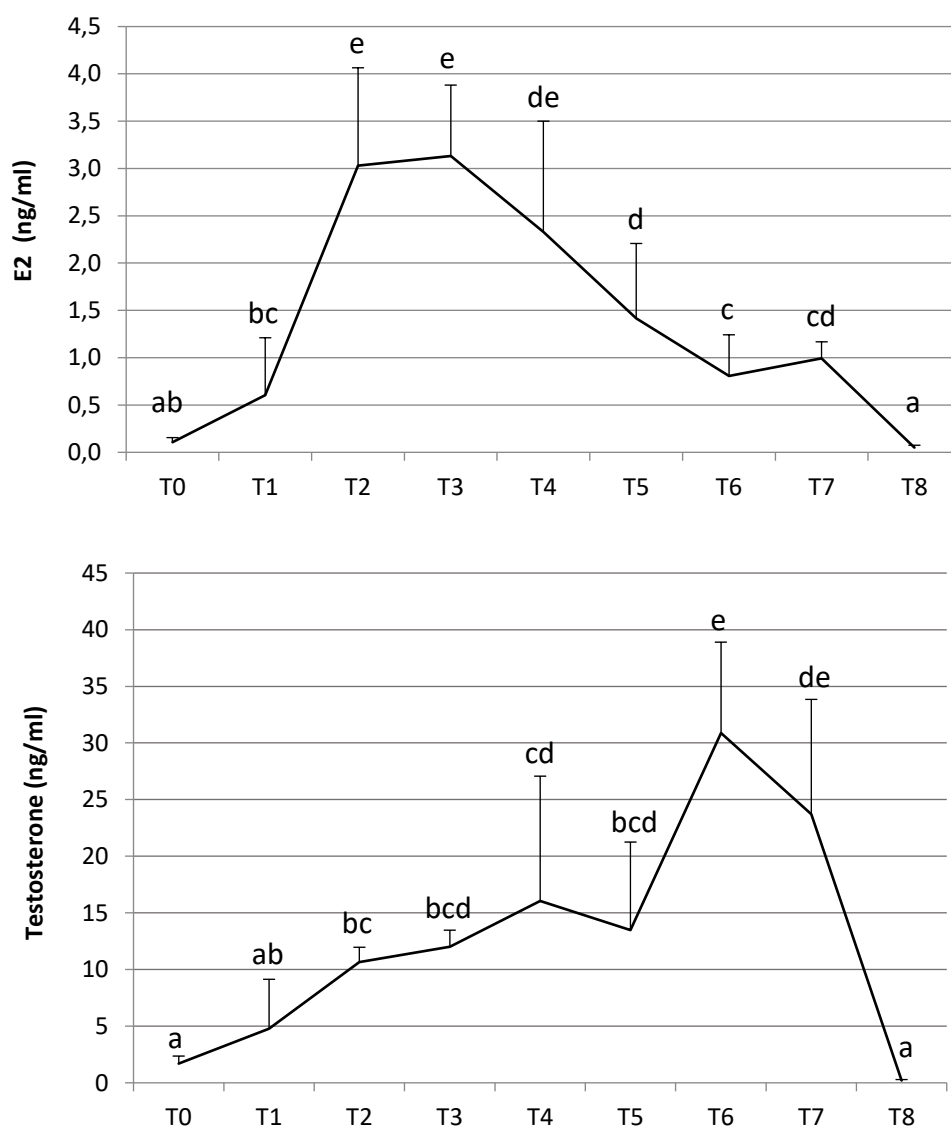


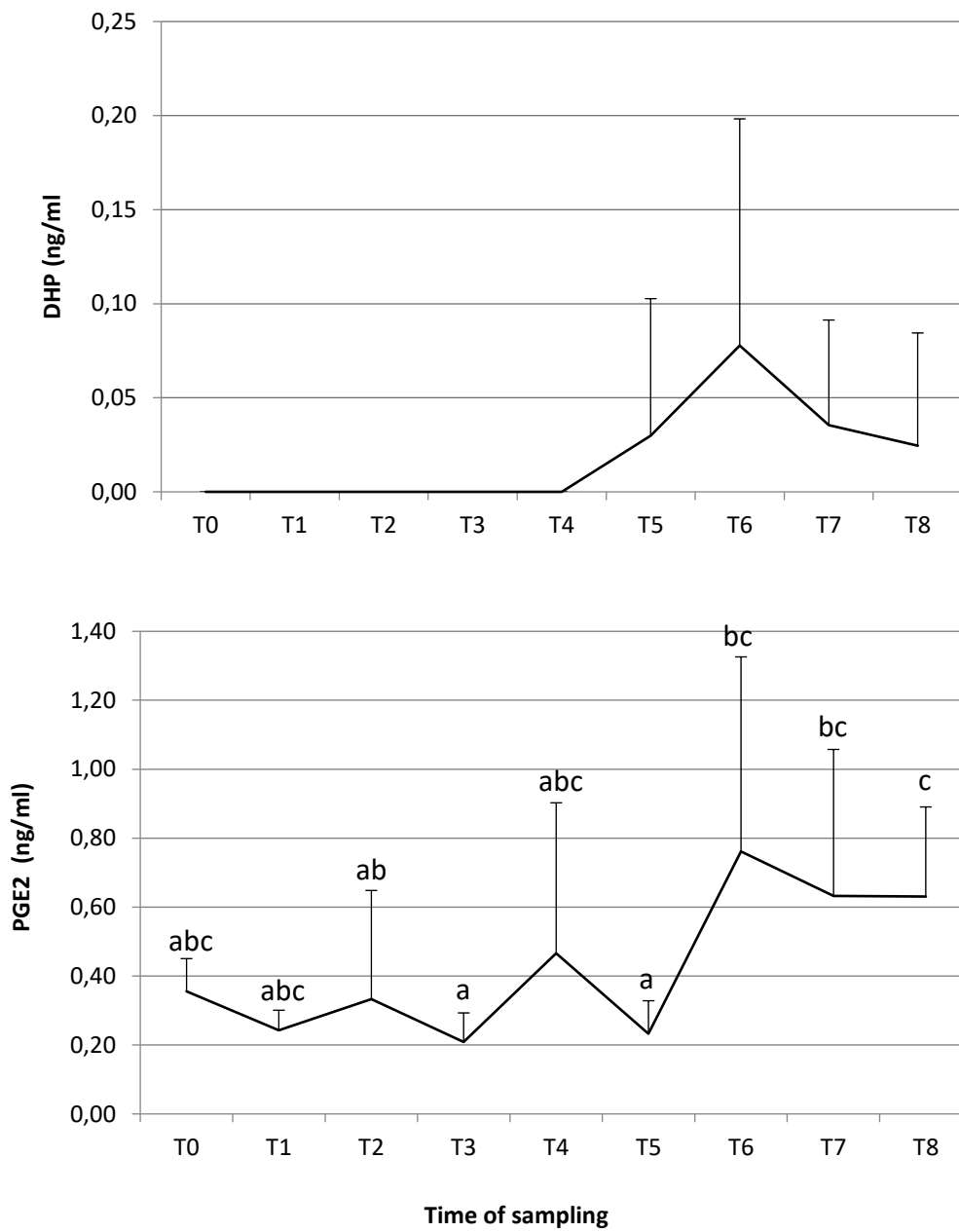
**Figure 2 Developmental stages of oogenesis in fish sampled at each time (n=9).**



**Figure 3 Variability in the plasma sexual hormones levels variation (mean+SD) in fish (n=6) measured for each hormone (E2; 17-beta Estradiol, T; Testosterone, DHP; 17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one, PGE2; Prostaglandin E2) at each sampling time.**

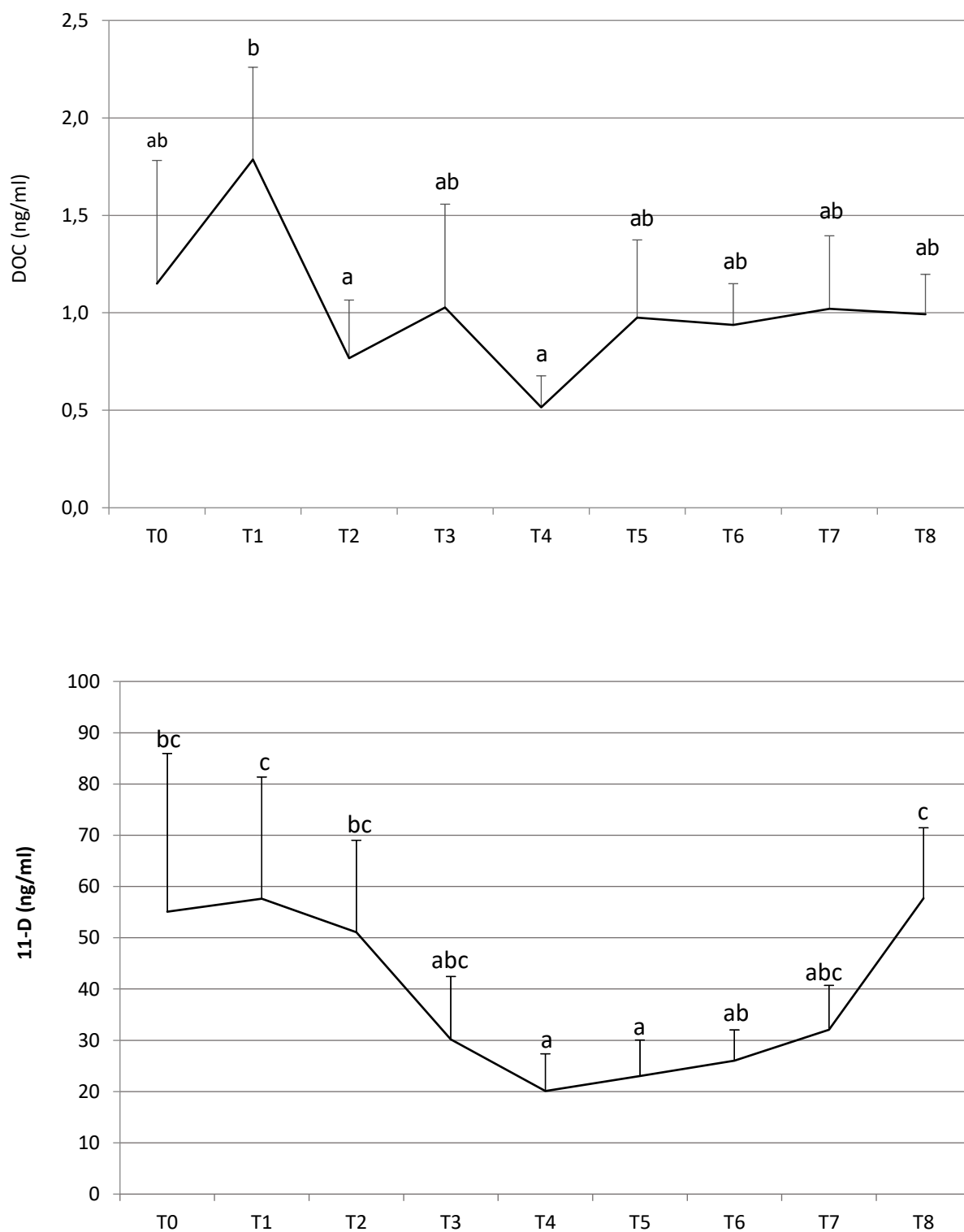
For each hormone, value at the different sampling times were compared to each other and statistically different values ( $p < 0.05$ ) are marked by different letters.



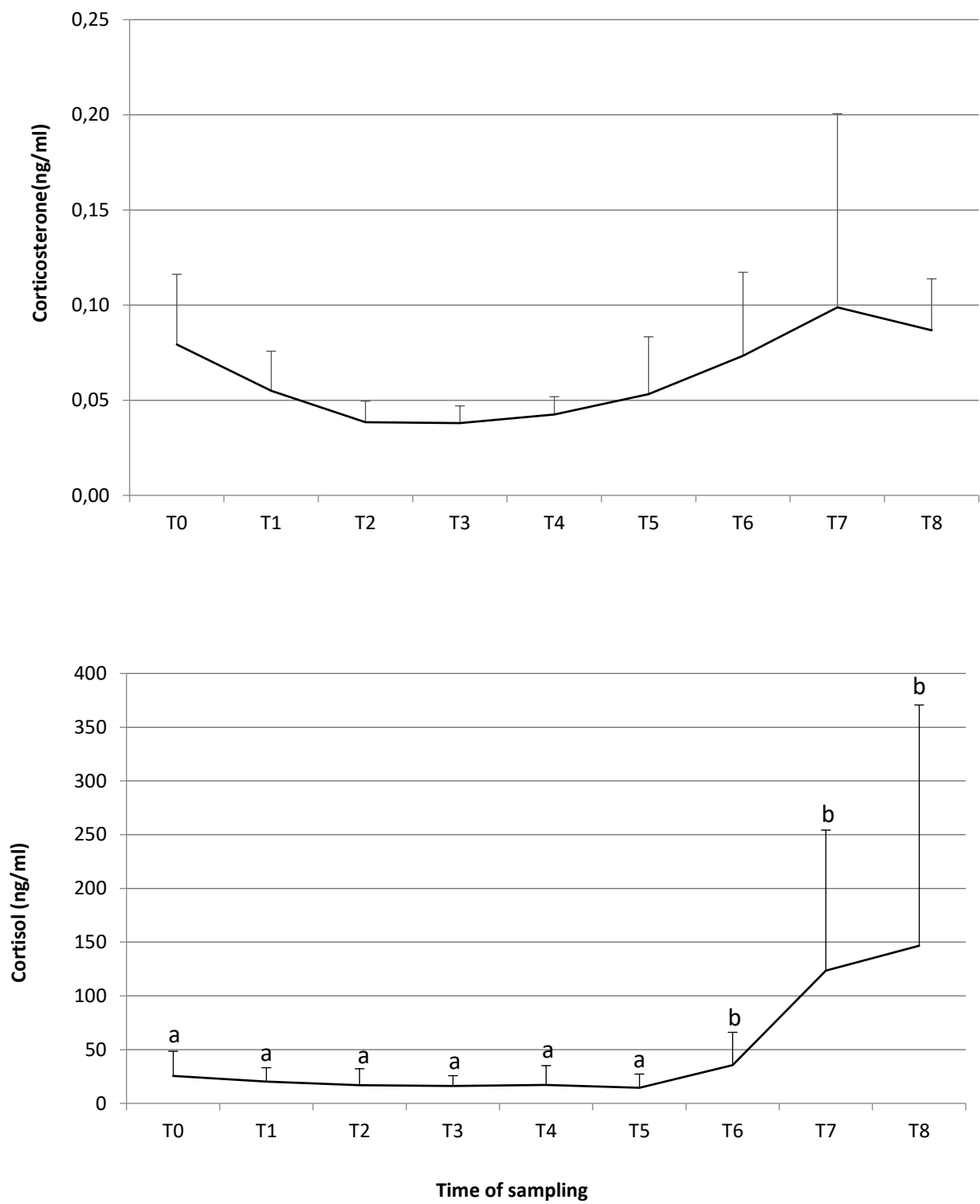


**Figure 4 Variability in the plasma sexual corticosteroids levels variation (mean+SD) in fish (n=6-12) measured for each corticosteroid (11-Deoxycorticosterone; DOC, 11-Deoxycortisol; 11-D, Corticosterone, and Cortisol) at each sampling time.**

For each hormone, value at the different sampling times were compared to each other and statistically different values ( $p<0.05$ ) are marked by different letters.

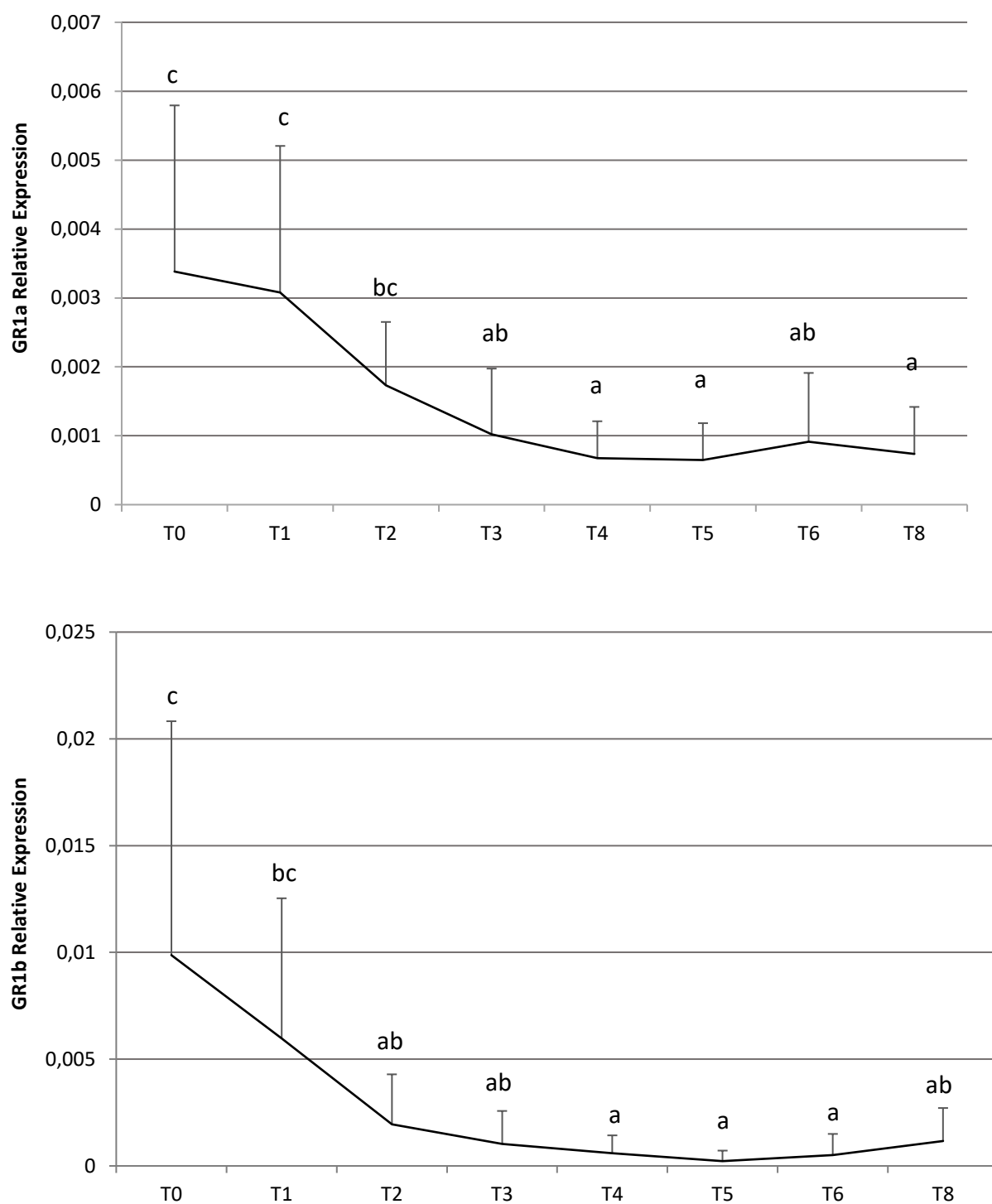


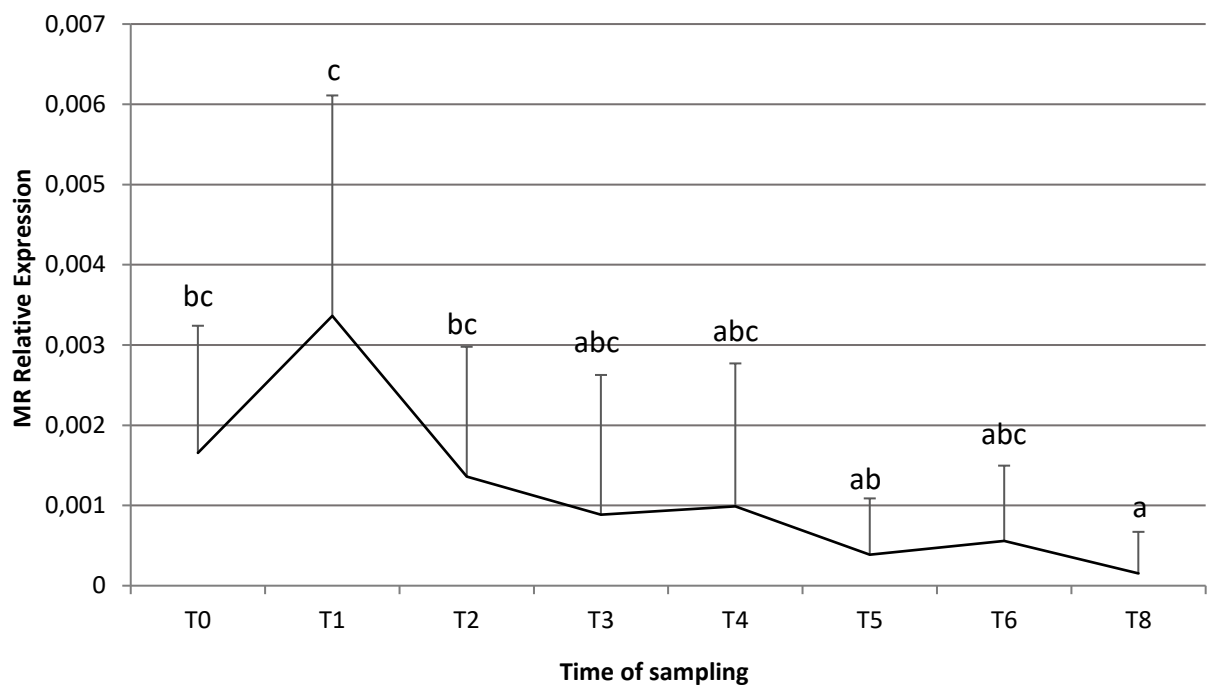
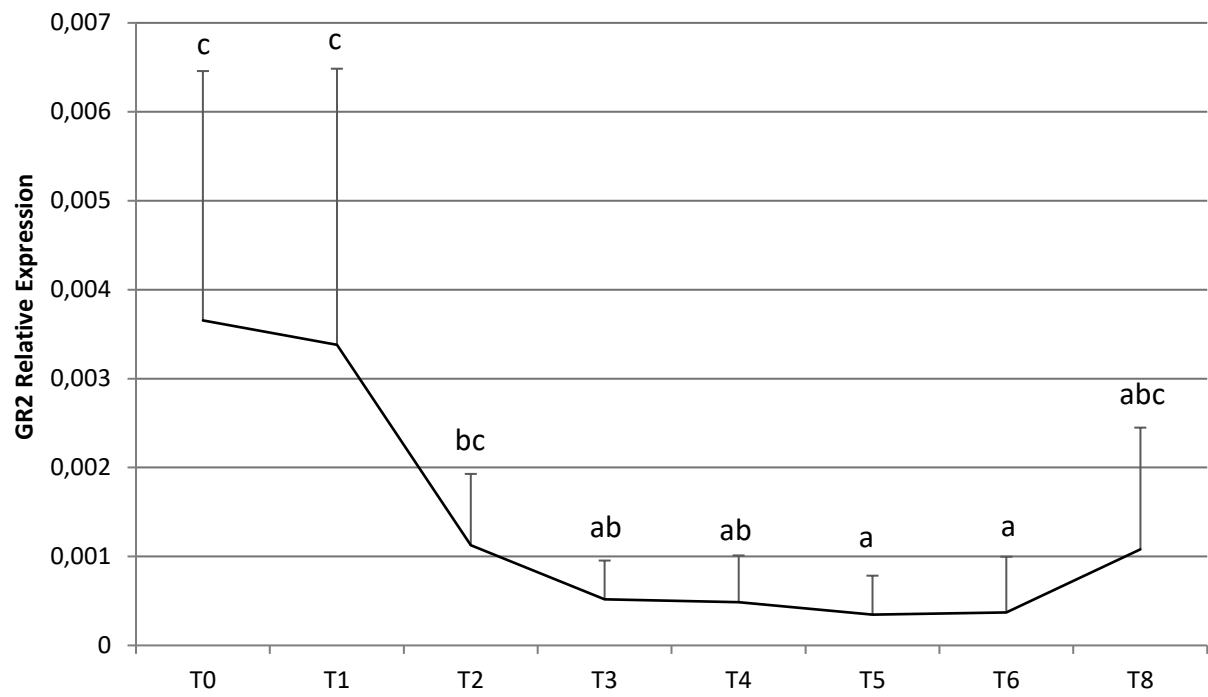




**Figure 5** Variability in the relative expression (mean+SD) in fish (n=12) measured for each of the four corticosteroid receptors (Glucocorticosteroid receptors; GR1a, GR1b and GR2, and mineralocorticosteroid receptor; MR) at each sampling time (except at T7).

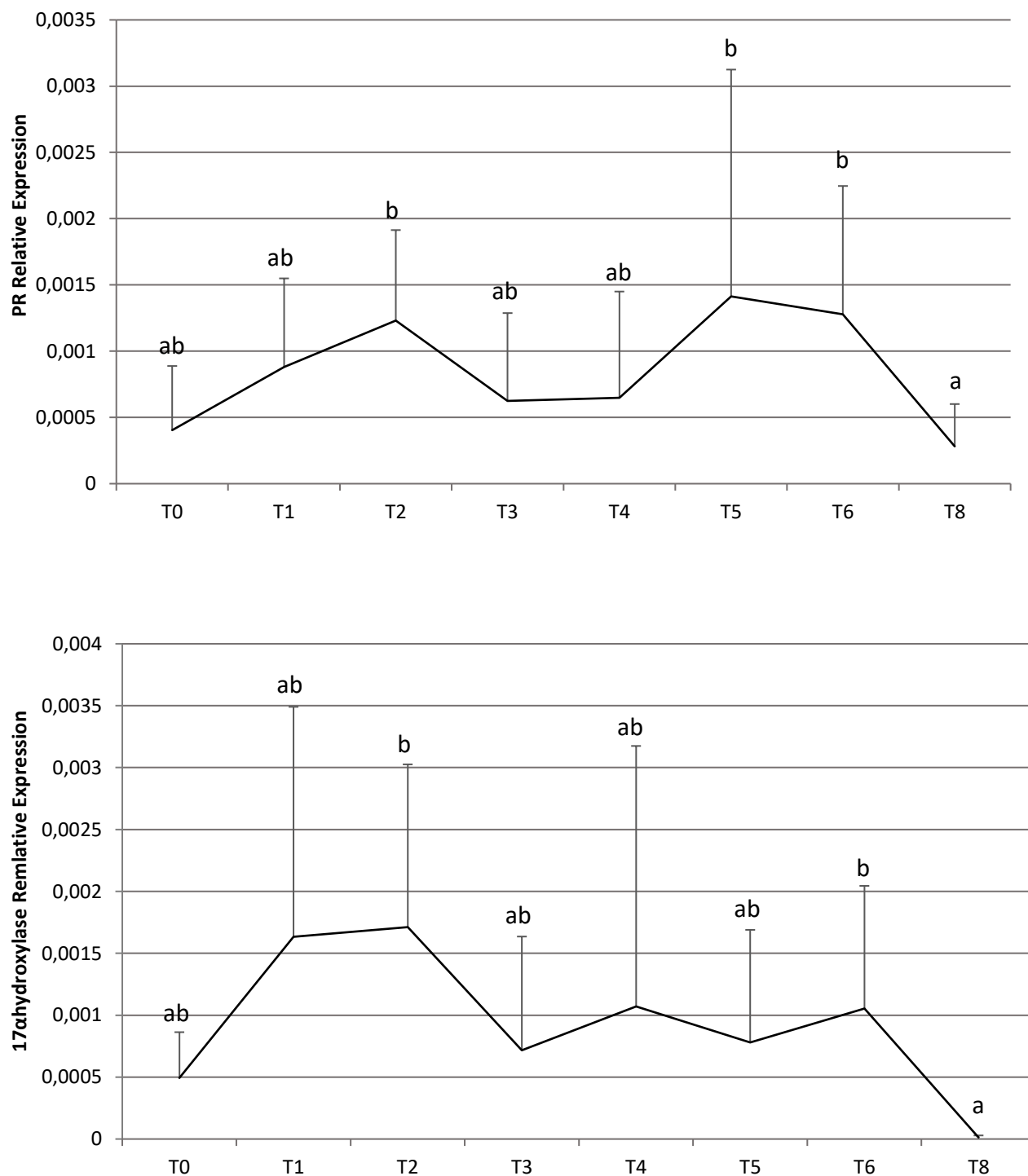
For each receptor, value at the different sampling times were compared to each other and statistically different values ( $p < 0.05$ ) are marked by different letters.

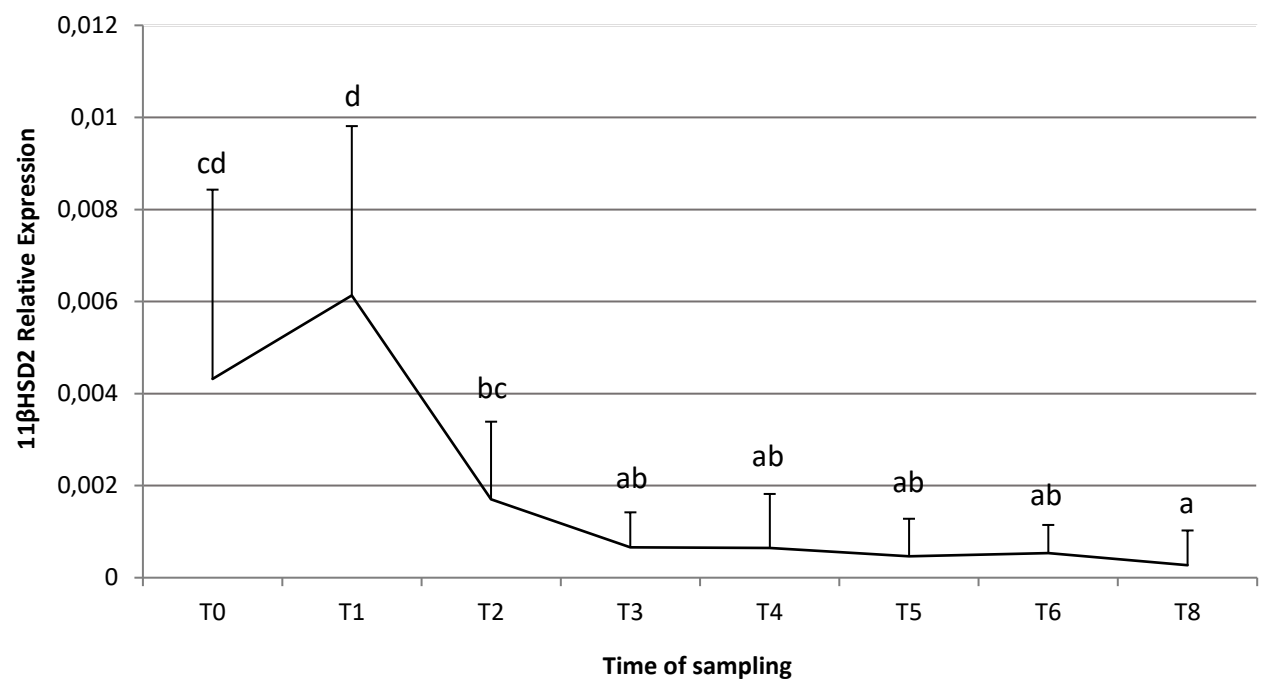




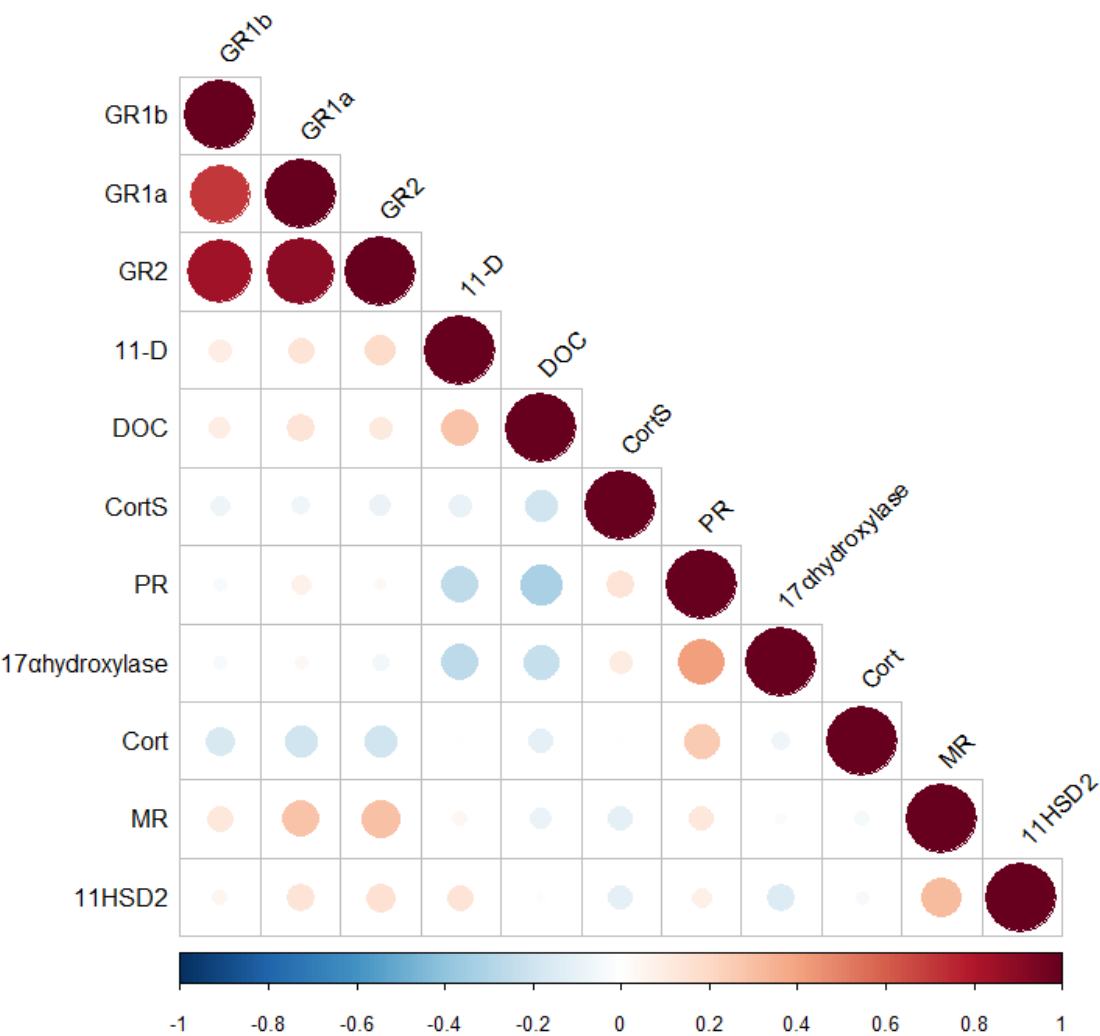
**Figure 6 Variability in the relative expression (mean+SD) in fish (n=12) measured for each of Progesterone receptor (PR) and the enzymes 17 $\alpha$ hydroxylase and 11 $\beta$ HSD2 at each sampling time (except at T7).**

For each of receptor and enzyme, value at the different sampling times were compared to each other and statistically different values ( $p<0.05$ ) are marked by different letters.



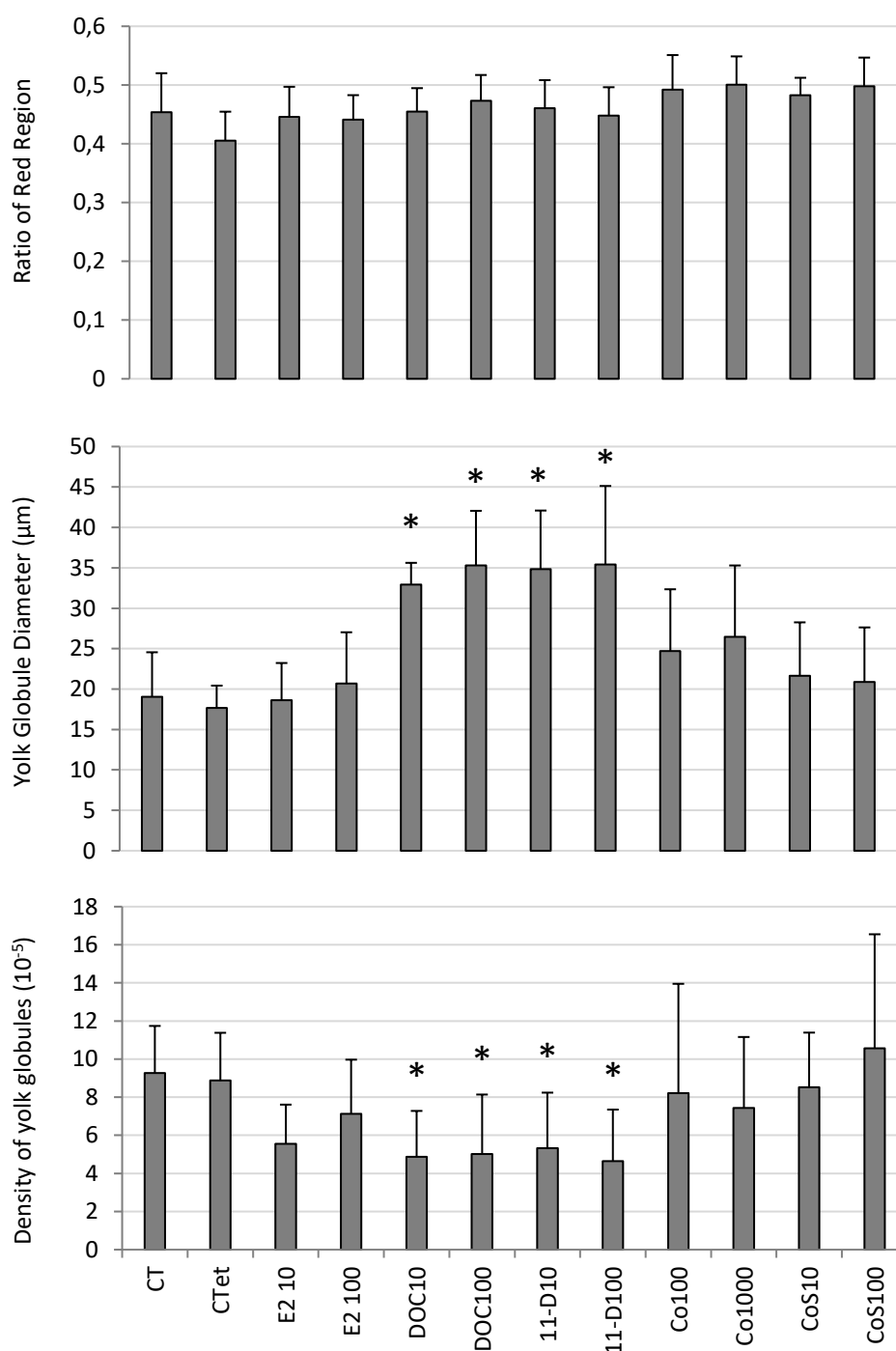


**Figure 7 Pearson’s Correlation between the plasma kinetics of the four corticosteroids (11-Deoxycorticosterone; DOC, 11-Deoxycortisol; 11-D, Corticosterone, and Cortisol), the relative expression of different receptors (GR1a and b, GR2, MR, PR), and the relative expression of the enzymes (11βHSD2 and 17αhydroxylase).**



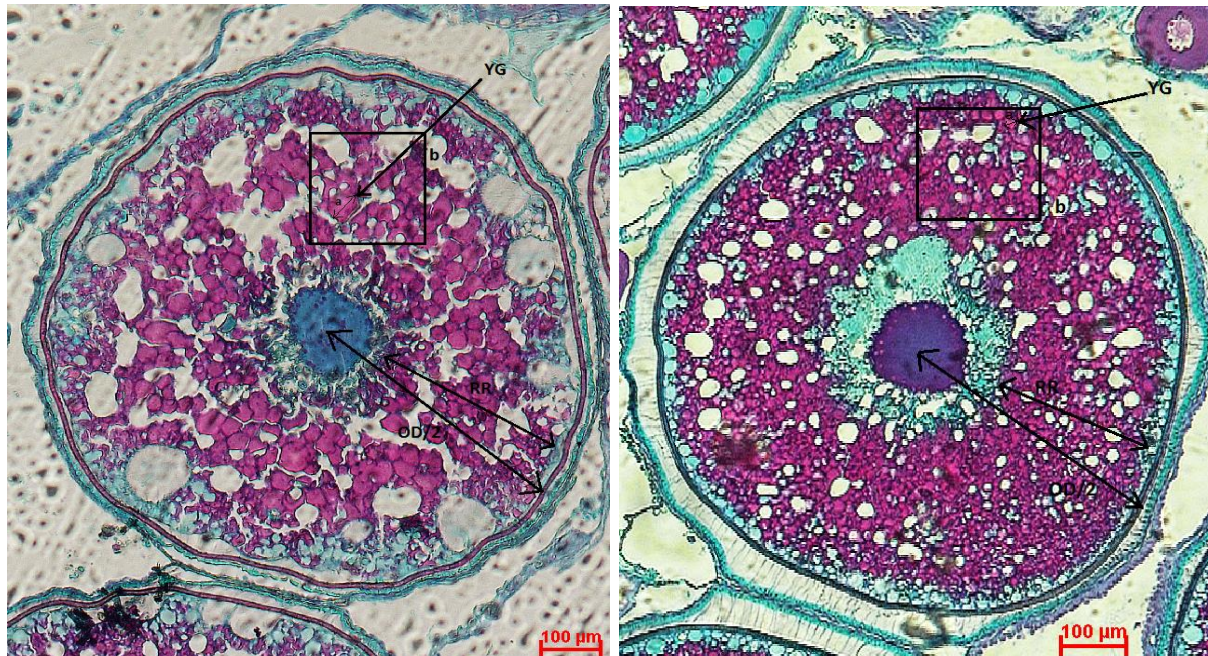
**Figure 8** The *in vitro* effect of E2 and of the corticosteroids (DOC, 11-D, Co, and CoS) hormonal treatments on the three parameters; (1) Ratio of red region (length of red region divided by half the oocyte diameter) and (2) Yolk globule diameter and (3) Density of yolk globules (number of yolk globules within a fixed unit area) (n=6 fish, 10 oocytes per fish).

The treatments are the following: CT (control), CTet (ethanol vehicle only), E2 (17-beta Estradiol; 10ng/ml, 100ng/ml), DOC (11-deoxycorticosterone; 10ng/ml, 100ng/ml), 11-D (11-deoxycortisol; 10 ng/ml, 100 ng/ml), (cortisol; 100ng/ml, 1000ng/ml), CoS (corticosterone; 10ng/ml, 100ng/ml). For each of the three measured parameters, the values recorded represent mean+SD which were compared with the negative control CTet, and statistically different values ( $p<0.05$ ) are marked by stars.



**Figure 9 Histological cross-sections of oocytes showing the effect of DOC and 11-D treatments on yolk globules diameter and density (left image) by comparison to the negative control CTet (right image).**

Magnification x4 and scale 100 $\mu$ m was used to attain both images. (YG: yolk globules, a: diameter of YG, b: fixed unit area, RR: length of red, OD/2: half oocyte diameter).





## **7. Conflict of interest**

The authors declare that they have no conflict of interest

## **8. Ethics approval**

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: 23871- 2020013017452359) by the local ethics committee in France (name: CELMEA).

## **9. Consent to participate**

The authors declare that they agree of participating in this study with no conflict of interest

## **10. Consent for publication**

The authors declare that they agree of publishing in this study with no conflict of interest

## **11. Availability of data and material**

Non-applicable.

## **12. Code availability**

The only codes used in this study were used to perform the statistics using the free R software version 3.3.1.

## **13. Authors' contributions**

**Leila El Mohajer:** *In vivo* experiment - conceptualization, experimental design, formal analysis, software, data curation. *In vitro* experiment - conceptualization, experimental design, formal analysis. Writing - original draft.

**Céline Chevalier:** *In vivo* experiment - formal analysis, software, data curation.

**Dominique Chardard:** *In vivo* experiment - formal analysis.

**Bérénice Schaerlinger:** *In vivo* experiment - Resources.

**Sylvain Milla:** *In vivo* experiment - conceptualization, experimental design, formal analysis. *In vitro* experiment - conceptualization, experimental design. Writing - review & editing.

**Pascal Fontaine:** *In vivo* experiment - conceptualization, experimental design. *In vitro* experiment – conceptualization. Writing - review & editing.

### ***“Major conclusions and up-coming approaches”***

The results of chapter 1 exhibited, for the first time, the absolute plasma levels of the four stress-sensitive corticosteroids (DOC, 11-D, corticosterone, and cortisol) along the whole reproductive cycle of female Eurasian perch reared under a controlled environment. The significant kinetics of DOC, 11-D, and cortisol suggest their implication in perch oogenesis while this was not the case for corticosterone which exhibited resting kinetics along whole oogenesis.

The highest plasma levels for DOC and 11-D were recorded at the start of the reproductive cycle, FOM, and spawning, while those of cortisol were observed at FOM and spawning. The results in chapter 1 could thus support the first working hypothesis (**H1**).

However, the results in chapter 1 could not validate the second working hypothesis (**H2**) where the *in vitro* direct effect of some corticosteroids (DOC and 11-D) could only suggest the existence of a role for corticosteroids in late vitellogenesis but this was highly limited by the insignificant rises for these corticosteroids during that period.

Interestingly, the results of chapter 1 allowed to further insist on a potential role for corticosteroids in FOM and ovulation. Further, the available data in chapter 1 suggest that cortisol is not deactivated by being transformed into cortisone even when reaching high levels during FOM and spawning. The latter conversion has been believed to be as an attempt to reduce the effects of high cortisol levels following a stressful situation (Pottinger and Moran, 1993). The results of chapter 1 thus allow us to hypothesize that cortisol may possess an indispensable role during FOM and spawning which favored its preservation in its active form even after reaching relatively high levels.

We may thus suggest that the above interpretations of the data provided by chapter 1 still let open a potential role for corticosteroids during FOM. So far, our interpretations agree with the previous information illustrated in the bibliography on the probable involvement of corticosteroids in FOM as has been recorded in other close teleosts (e.g. yellow perch). In the above state of art, it was suggested that corticosteroids can be potent maturation inducing hormones (MIH). We recall that an MIH usually mediates the final maturation stages that take place during final oocyte maturation (FOM). As such its identity has been a key element for the control of FOM and synchronization of spawning (Migaud et al., 2003). However, we acknowledge that corticosteroids are less focused on in literature when it comes to the control of FOM and ovulation by comparison to progestogens as DHP and THP. In the following chapter 2, we propose to collect more information on previous reports that suggest corticosteroids as potential MIH or as possible mediators of MIH action. Generally, we believe that the following chapter could serve for validating the third working hypothesis (**H3**) which suggested that corticosteroids are involved in FOM.

# CHAPTER 2

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Review

## Maturation Inducing Hormones in teleosts: Are progestogens always the first to be nominated?



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### **Chapter 2: Maturation Inducing Hormones in teleosts: are progestogens always the first to be nominated?**

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**Running title:** MIH in teleost fish

## **Abstract**

Attempts to increase fish production and restocking capacities have made synchronous out-of-season spawning necessary. Achieving such spawning is, however, highly dependent on mastering the fundamental control of reproduction during critical reproductive periods in a variety of teleosts. In all teleosts, maturation-inducing hormones (MIHs) are the hormones that mediate the critical stages of final oocyte maturation (FOM) in preparation for ovulation and spawning. Importantly, the identities of MIHs are still unknown or unconfirmed in a number of teleosts, especially those newly introduced into the fish market. Therefore, we here attempt to provide an overview of the most likely MIHs in a wide range of teleosts to aid in confirmation of their identities in already studied species and to suggest their identities in still-unstudied species. In addition to progestogens, various *in vitro* and/or *in vivo* studies on a variety of teleosts have yielded other MIHs, such as corticosteroids, prostaglandins, and even melatonin, that are not commonly suspected to be MIHs. Such nonprogestogens can at least interfere with MIH production or function. In all cases, however, both *in vitro* and *in vivo* assays should agree on the nomination of a certain MIH before its identity can be confirmed. Altogether, the existing studies strongly suggest that the identity of an MIH can vary among different species, even within the same family of teleosts. Finally, we list the main methods for MIH detection while focusing on *in vitro* MIH ones considering the paramount importance of such a pursuit in achieving future hormonal treatments for production synchronization in additional teleost species.

**Keywords:** MIH, FOM, progestogens, corticosteroids, teleosts

## **1. Introduction**

Fish and fish products are some of the most traded food items in the world today. In 2016, 88% of the total fish production was utilized for direct human consumption, and the percentage has increased significantly in recent decades (FAO, 2016). Such growth in market demand is always accompanied by the need for expansion of production (FAO, 2014). Aquaculture can offer systems that aim to expand such capacity of fish production with more limited use of land and water, allowing better control of the reproductive cycle by fish farmers. Additionally, aquaculture provides elements for restocking which adds to the diversification and fish abundance within a particular water zone (Aprahamian et al., 2003). Nevertheless, 85% of the world's production of finfish has come from farming of only 15 species in numerous countries (Teletchea and Fontaine, 2014). Hence, a major way to maintain or increase the contribution of aquaculture systems to global consumption is to farm or domesticate new species of marine or freshwater fishes. Control of the reproductive cycle and achievement of out-of-season spawning for farmed species are major issues in fish farming that must be addressed in the continuous attempts to increase production capacity. Such control can be achieved through the different applications of artificial reproduction which include the monitoring of the environmental parameters or the exogenous introduction of sexual hormones to induce or hinder fish maturation and to attain more synchronized spawning. Fortunately, specific studies have so far demonstrated that residues of such hormonal treatments do not accumulate or at least disappear within few days due to their metabolism by fish tissues (Hoga et al., 2018). For such out-of-season spawning, complete control of reproduction in captivity is a prerequisite (Ben Ammar et al., 2015). However, the diversity in cultured fish species is accompanied by extreme diversity in modes of reproduction that involves numerous adaptations and reproductive strategies (Jalabert, 2005). Diverse as they might be, these strategies must be compatible with



aquaculture procedures, which requires understanding of the fundamental control of reproduction in a wide range of teleosts.

In all teleosts, the primary oocytes are first arrested at the diplotene stage of prophase I, during which the oocytes enter growth stages marked by the appearance of cortical alveoli and vitellogenic granules, which leads to enormous increases in oocyte size (Tyler and Sumpter, 1996; Miura et al., 2007; Lubzens et al., 2010). The most critical stage just prior to ovulation follows the growth stages and is known as Final Oocyte Maturation (FOM), which is characterized primarily by the breakdown of the oocyte nuclear envelope in a phenomenon known as Germinal Vesicle Breakdown (GVBD) (Migaud et al., 2003; Tokumoto et al., 2005). FOM is under endocrinological control *via* the hypothalamic-pituitary-gonadal (HPG) axis (S (Yaron and Levavi-Sivan, 2011; Planas et al., 2000) and is marked by the predominant production of Maturation-Inducing Hormones (MIHs). MIHs mediate the final maturational stages during FOM. Hence, investigation into the natures of these mediators in several species of teleosts has been a key element for anticipation of ovulation in diverse species in captivity. For instance, synchronization of reproduction in some captive fish, such as percids, is an industrial target to reach higher productivity in a limited amount of time (Migaud et al., 2003). Synchronization of production can be pursued through direct hormonal stimulation, which is in turn strongly dependent on the detection of MIHs. Attaining more synchronous production is not the only purpose behind the detection of MIHs; mitigation of reproductive dysfunction in some groups of cultured teleosts, such as cyprinids, is also a goal (Podhorec and Kouril, 2009). However, while 17,20  $\beta$ -dihydroxy-4-pregnen-3-one (DHP) has traditionally been considered to be the MIH in teleost fish, the updated literature highlights a more complex scheme.

In this review, we explain the endocrinological routes leading to MIH release and the modes of action of a typical MIH while utilizing the information available on the most often characterized MIH (DHP). We also list available information on previously detected classical and

nonclassical MIHs in studied teleost species, including those relevant for further aquaculture production. We eventually discuss some of the common assays for MIH detection while focusing on the *in vitro* ones, with the aim of enhancing the latter methods and thus improving synchronization of egg production in additional teleost species.

## **2. Endocrinological route of MIH release and the environmental and paracrine factors controlling this release**

In essence, FOM takes over following vitellogenesis which is accompanied by a surge in circulating LH released from the anterior pituitary; all of these events are under the control of gonadotropin-releasing hormone (GnRH) released from the hypothalamus in the brain (Plant, 2015; Zohar et al., 2010). Following LH release, LH binds to its follicular receptor (LHR) located on both the granulosa cells and theca cells of the ovaries (Fig. 1). Several paracrine factors acting on at least one of the hormonal elements of the HPG axis have been found to primarily affect FOM.

For instance, there exists an important paracrine effect for the time-keeping hormone melatonin on the release of MIH given that the addition of melatonin to an *in vivo* treatment commercially used for artificial FOM induction has proven to be very efficient in enhancing the treatment. Such indirect mechanism by which this monoamine acts is believed to involve time-dependent effects on either GnRH or gonadotropin-inhibitory hormone (GnIH) levels that affect LH according to the season (Fig. 1) (Maitra et al., 2013; Moniruzzaman et al., 2016). Some studies also recorded Melatonin to inhibit 17 $\beta$ -estradiol (E2) production in fish ovaries and to directly induce the *in vitro* production of significant levels of progestogen in ovarian fragments. The latter effect was eventually also accredited to the probable effect of melatonin on LH-induced progestogen synthesis (Hong et al., 2014; Takahashi et al., 2018). As described in a review by Maitra et al. (2013), melatonin affects reproduction by acting on several components of the HPG axis in fish, and, importantly, these effects are photoperiod-dependent (Yumnamcha et

al., 2017). After all, melatonin mediates the internal physiological reflection of the day-night patterns taking place in the outside environment, which thereby confines its effect, ranging between induction and inhibition of FOM, to the day length in seasonal breeders (Carnevali et al., 2011).

Dopamine (DA) is a neurotransmitter that indirectly contributes to MIH production in teleosts *via* the neurohormonal signaling pathway (Dufour et al., 2010). DA can exert an inhibitory effect on LH release from the pituitary, particularly through DA receptors from families D1 and D2 (Fig. 1) (Dufour et al., 2010; Popescu et al., 2010; Roche et al., 2018). Hence, the combination of D2-family DA receptor antagonist with GnRH agonist (GnRHa) has often been used to enhance the reproductive effectiveness of GnRHa therapy for FOM stimulation (Fontaine et al., 2015; Zadmajid and Butts, 2018). This effect of DA on GnRH was enough to encourage the use of Ovaprim®, a product commonly used in fish farming that is a combination of both a DA receptor antagonist and GnRH. Interestingly, when the previously mentioned melatonin treatment is combined with Ovaprim® in carp, the effects of Ovaprim® on the cascade leading to FOM are augmented (Moniruzzaman et al., 2016). These data shed light on the crucial interaction of these season-dependent factors in FOM control.

Following its release, LH stimulates the release of MIH by granulosa cells, resulting in the replacement of FSH-stimulated E2 secretion by MIH secretion (Jalabert, 2005; Nagahama and Yamashita, 2008; Yaron and Levavi-Sivan, 2011). In addition to vitellogenin production, FSH-stimulated E2 secretion is known to be crucial for maintaining the meiotic arrest required during vitellogenesis (Thomas, 2018). Meiotic arrest at prophase of meiosis I is made possible primarily by the ability of growing oocytes to maintain high intraoocyte levels of cAMP *via* membrane-bound adenylyl cyclases (Das et al., 2017). In the croaker (*Micropogonias undulatus*) and zebrafish (*Danio rerio*), E2 has been revealed to be possibly responsible for this high intraoocyte cAMP level, as E2 treatment leads to increases in both cAMP production and

GPER expression (Thomas, 2018). This phenomenon explains the indispensable shift from the predominant production of E2 from T catalyzed by the enzyme aromatase (P450arom) to the predominant production of MIH (Fig. 1) (Zhou et al., 2007).

In most teleosts, DHP has been often nominated as the MIH, the production of this MIH is catalyzed by the enzymes belonging to the 20 $\beta$ -hydroxysteroid dehydrogenase (20 $\beta$ -HSDs) family, which usually catalyze the production of DHP from its precursor 17OHP. *In vitro* studies on zebrafish and masu salmon (*Oncorhynchus masou*) have isolated the enzyme carbonyl reductase-like 20 $\beta$ -HSD (CR20 $\beta$ -HSD) which can be structurally attributed to the 20 $\beta$ -HSDs family (Aranyakanont et al., 2020). However, another finding in masu salmon raised doubt as to whether this isolated enzyme is the enzyme actually possessing the 20 $\beta$ -HSDs activity responsible for DHP production from 17OHP. In this finding, it was suspected that another enzyme which belongs to the 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) family namely 17  $\beta$ -hydroxysteroid dehydrogenase type 12 (hsd17 $\beta$ 12) is the enzyme that actually catalyzes DHP production and hence would possess the real 20 $\beta$ -HSD activity (Ijiri et al., 2017).

### **3. Mode of action of an MIH**

After being produced, an MIH binds to its membrane progesterin receptor (mPR $\alpha$ ) at the oocyte surface (Fig. 1), leading to the potential activation of various signal transduction cascades that ultimately end with the activation of the MPF (Maturation promoting factor) (Li et al., 2015). After its activation, the MPF, a heterodimer of the regulatory component cyclin B and the catalytic component cdc2, promotes a series of intraoocyte mechanisms characterizing FOM, such as chromosome condensation, spindle formation, GVBD, release of the first polar body, and completion of meiosis I (Das et al., 2017). However, the success of the binding of the MIH to its receptor is highly dependent on the stage of oocyte maturation. As previously mentioned, the E2/GPER system (G protein-coupled estrogen receptor) is known to mediate an intraoocyte

signaling pathway before FOM that serves to block the synthesis of the latter MIH receptor by increasing the level of cAMP (Thomas, 2018). In catfish, the agent catecholesterogen (2-hydroxyestradiol-17 $\beta$ , 2-OHE<sub>2</sub>) has been reported to act as an antagonist of GPER. By antagonizing GPER, catecholesterogen might relieve the E2-mediated inhibition of mPR $\alpha$  synthesis by blocking the production of E2-induced cAMP, thereby allowing the MIH to bind to its receptor (Das et al., 2017). In addition to the rise in cAMP, however, previous evidence has established that the MPF is also affected by another factor, the active serine-threonine kinase PKA, in the oocytes of most species (Das et al., 2017). Existing evidence on the link between the rapid decrease in intraoocyte cAMP levels and the inhibition of PKA activity suggests that cAMP and PKA cooperate to diminish intraoocyte MPF action. Although high-cAMP/PKA-mediated inhibition of FOM is almost universal, the intracellular targets at which PKA imposes its action in fish oocytes are not clearly understood (Das et al., 2017). In a study performed on the climbing perch, *Anabas testudineus*, by Khan and Maitra (2013), mitogen-activated protein kinases (MAPKs) were found to play a very large role in the process of PKA-mediated inhibition of MPF. That study revealed that endogenous PKA inhibits MPF production *via* inhibition of MEK kinase (MEKK), which is critical in the activation of MAPK through MEK. This finding leads to the conclusion that, in climbing perch, inhibition of PKA may enable activation of the MEKK/MEK/MAPK cascade to ultimately activate MPF (Khan and Maitra 2013). Therefore, it is reasonable to hypothesize that MIH, after binding to its receptor (mPR $\alpha$ ), is able to lower the level of intraoocyte cAMP, thereby lowering PKA levels, which activates the MEKK/MEK/MAPK cascade to ultimately activate MPF.

#### **4. Progestogens as MIHs**

The first evidence that progestogens are major MIHs in fish is the peak in their circulating plasma levels during the periovulatory period. Several studies have recorded peaks in the plasma levels of progestogens such as progesterone (P), DHP, and 17,20 $\beta$ ,21-trihydroxy-4-

pregnen-3-one (THP) in numerous families of teleosts, such as Acanthuridae, Acipenseridae, Percidae, and Cichlidae. In Table 1, we list examples of several teleost species for which progestogens have been measured *in vivo* in blood plasma and/or progestogen activity has been investigated during FOM or spawning. In some studies, revealing peaks in progestogens during certain periods of FOM or spawning, *in vitro* biological assays of follicle culture have been conducted in an attempt to confirm the MIH activity. These *in vitro* assays have been intended to validate that FOM is provoked *in vitro* at a certain physiological dose at which a certain progestogen peak during FOM. However, several *in vitro* assays suggesting that certain progestogens are MIHs have not been backed up with corresponding *in vivo* records to validate their effective doses. For example, in the striped bass, *Morone saxatilis*, plasma DHP levels during FOM have been found to be almost 10-fold lower than the recorded *in vitro* dose that effectively induces GVBD (Table 1). Thus, it seems rather unreasonable to consider DHP as a potent MIH in this species. Similar conclusions have been made for DHP in Japanese eel, *Anguilla japonica*; European seabass, *Dicentrarchus labrax*; white sturgeon, *Acipenser transmontanus*; yellow perch, *Perca flavescens*; goldfish, *Carassius auratus*; and white perch, *Morone americana*. In these species, plasma measurements of DHP have not validated DHP as a potent MIH, even in the two latter species, in which fish have been injected with hCG (Table 1). The *in vitro* assays have not been validated by the corresponding circulating levels of other progestogens that have been suspected to be MIHs, such as P, THP, and 17 $\alpha$ -hydroxyprogesterone (17OHP), as has been the case for *Alburnis tarichi* and Eurasian ruffe, *Gymnocephalus cernuus* (Table 1). Nevertheless, progestogens have been shown to be MIHs in some other teleosts in which an *in vitro* effective progestogen dose is close to the recorded plasma levels during FOM, as in red seabream, *Pagrus major*; kyusen wrasse, *Halichoeres poecilopterus* and Eurasian perch, *Perca fluviatilis* (Table 1). Finally, in rainbow trout, *Oncorhynchus mykiss*, the DHP concentration that induces GVBD was recorded to be 10 ng/ml

in one study (Nagahama et al., 1983) and 40 ng/ml in a more recent study (Milla et al., 2006). In both studies, the *in vitro* active dose was much lower than the DHP plasma level measured during the reproductive period, which was in the range of 70-380 ng/ml; these studies were the first to validate DHP as an MIH (Table 1). Notably, the very high DHP plasma levels measured in rainbow trout and Amago salmon, *Oncorhynchus rhodurus*, compared to the active doses are excellent examples of plasma levels that are high enough to eliminate all doubt about whether a certain progestogen is an MIH (Table 1).

Another interesting conclusion based on the data in Table 1 regards the extent of interspecies differences that are encountered when nominating the most potent MIH. As discussed earlier, DHP and THP can be considered MIHs in a range of species, such as rainbow trout, red seabream and kyusen wrasse. On the other hand, in sterlet sturgeon, *Acipenser ruthenus*, circulating levels of P are fully compatible with the *in vitro* effective doses that induce GVBD (Table 1). In Caspian kutum, *Rutilus frisii kutum*, 17OHP is usually considered to affect FOM indirectly *via* its transformation into DHP, which is another way to interpret the peak recorded in Table 1 during FOM. In fact, studies have mentioned that the 17OHP plasma peak is concurrent with the DHP plasma increase at FOM and ovulation in several other cyprinid teleosts (Heidari et al., 2010). Consequently, studies may still tend to nominate either DHP or THP as the most potent MIH rather than the precursor progestogen P or 17OHP. A comparative analysis of the levels of all circulating progestogens and their respective potencies in triggering GVBD would enable clarification of the most prominent progestogens that serve as MIHs in each species.

Additional information stemming from Table 1 is a comparison between DHP and THP in some species (red seabream and kyusen wrasse) for which the *in vitro* effective doses for both compounds were exactly the same and were similar to their measured plasma levels, which were slightly higher for THP than for DHP. These data not only enable conclusions to be drawn

regarding the potency of both progestogens as MIHs but can also be used to show that THP is more potent than DHP in some species. The reason underlying the uncertainty regarding whether THP or DHP is the more potent MIH is also related to the abilities of these molecules to actively bind to MIH receptors on the oocyte membrane. A slightly stronger activity of a progestogen at a lower dose or similar dose to another progestogen (as has been exhibited for THP and DHP) might reflect a stronger ability to bind to the typical MIH membrane receptor (Thomas, 2012). Returning to the example of striped bass, in which both THP and DHP have been shown to be active inducers of GVBD, THP was revealed to be a more potent MIH than DHP through examination of the binding affinities of the two produced progestogens to the MIH receptor. In this example, the binding affinities of the two progestogens was measured using THP and DHP as ligands in combination with aliquots of ovarian membrane suspension which would normally contain the MIH receptors (King et al., 1997). It was thus concluded that the pool of DHP produced was destined to be utilized as a substrate for THP production (King et al., 1997; Ohta et al., 2002). This finding supports the hypothesis that both progestogens are MIHs but that, in some species, DHP is transformed into THP, a more potent MIH that binds more strongly to the MIH receptor. This idea might explain the low plasma DHP levels recorded in Table 1 for the striped seabass. After all, both progestogens, DHP and THP, are 20 $\beta$ -hydroxylated progestins; their only difference resides in the hydroxyl group at the 21 position (Matsuyama et al., 2002). Given that these progestogens are structurally similar and are produced from the same precursor, 17OHP (Brueggemeier and Li, 2010), it is reasonable to expect that the affinities of these progestogens for their receptor might result in strong competition. Due to their low or mild affinities to this MIH receptor, other nonprogestogen MIHs, such as corticosteroids, or nontypical MIHs, such as androgens and estrogen, have been hypothesized to be MIHs less frequently than progestogens (Thomas et al., 2002). However, these hormones have been studied and sometimes found to actively induce FOM.



## 5. Corticosteroids as MIHs

Despite all the information listed above, there exists some fish species with minimal or no increases in the plasma concentrations of either or both of the so-called progestogens during FOM (Alvarado et al., 2015; Roche et al., 2018; Ünal et al., 2008). Furthermore, *in vivo* or *in vitro* assays might sometimes contradict with regards to the nomination of progestogens as MIHs. In the latter case, the findings may suggest another group of steroids, namely, corticosteroids, which do exhibit some MIH properties. Indeed, studies have identified three corticosteroids (11-deoxycortisol, 11-DC; 11-deoxycorticosterone, DOC; and cortisol) that appear to be credible candidate MIHs instead of or in addition to progestogens in some teleosts. Receptor-binding affinity assays, *in vivo* plasma monitoring of corticosteroids levels, *in vitro* testing of MIH activities on maturing ovarian follicles, and *in vitro* analysis of ovarian steroidogenesis products support that corticosteroids might be MIHs (Baker and Vynne, 2014; Milla et al., 2009).

Starting with the receptor-binding affinity assays, an old study using the ovaries of spotted sea trout, *Cynoscion nebulosus*, revealed that corticosteroids such as the mineralocorticoid hormone DOC and, to a much lower extent, 11-DC, can bind to MIH receptors *in vitro*. Interestingly, DOC was able to compete with THP and even outcompete DHP for the MIH receptor (Patiño and Thomas, 1990a). Additionally, RU486, a molecule recognized for its antagonistic activity towards both progestogen and glucocorticoid receptors, has recently been shown to trigger GVBD without counteracting the effect of DHP (Liu et al., 2018). This finding strengthens the link between corticosteroid receptors (CRs; mineralocorticoid and/or glucocorticoid receptors) and FOM, but further studies are required to determine whether the maturation-inducing activity of corticosteroids is mediated by either CRs or progestogen receptors. In any case, these findings strengthen the hypothesis that corticosteroids can act as MIHs instead of or in addition to the mentioned progestogens. Moving on to the *in vivo* and *in*

*vitro* assays that were used to investigate the MIH activity of corticosteroids, these provide several evidences on the MIH activity of corticosteroids.

### **5.1. 11-deoxycortisol (11-DC)**

In the cyprinid *Alburnis tarichi* caught in lakes, the plasma levels of 11-DC ( $\approx 3.8$  ng/ml) were found to significantly surpass the levels of both progestogens (DHP and THP) during FOM before the migration of germinal vesicles. When this corticosteroid was tested alongside the two progestogens DHP and THP *in vitro*, it induced GVBD at a much lower dose than the progestogens (Ünal et al., 2008). Notably, *in vitro* activity of 11-DC on GVBD has been previously exhibited in other species, such as the catfish *Heteropneustes fossilis* Bloch and yellow perch, at high doses of 500 ng/ml and 5000 ng/ml (Goswami and Sundararaj, 1974; Goetz and Bergman, 1978), respectively. However, 11-DC is effective at a much lower dose (20 ng/ml) in yellowfin porgy (Jeng et al., 2012).

### **5.2. 11-deoxycorticosterone (DOC)**

Another corticosteroid, DOC, also induces oocytes to mature at 5000 ng/ml in catfish (Goswami and Sundararaj 1974) and provokes GVBD at 100 ng/ml in yellow perch (Goetz and Bergman, 1978). However, recent *in vitro* assays conducted at the beginning of FOM in wild or captive Eurasian perch have revealed that the DHP productions are decreased by DOC and that DOC is not capable to induce GVBD at physiological doses (Mandiki et al., 2017; El Mohajer et al., 2021), which seems to be quite contradictory to the maturation-inducing activity of DOC recorded for yellow perch, a species phylogenetically close to Eurasian perch. This contradiction raises the question of whether variations exist among close species in the identities of corticosteroids such as DOC as either putative MIHs or inhibitors of FOM. The contradiction might be linked to the dose-dependent action of a steroid, as has been depicted in *Alburnis tarichi*, in which a fivefold elevation in the dose of 11-DC (1000 ng/ml) reverses the stimulatory effect of 11-DC on GVBD (Ünal et al., 2008). We may thus hypothesize that the

inhibitory or null effect of DOC is likely reversed to a rather stimulatory effect at higher doses. Indeed, the active dose of a corticosteroid must be maintained at a proper level for the corticosteroid to play its proper physiological role (Zhang et al., 2020). But in the case of DOC, the *in vitro* effective doses are higher than physiological levels (Milla et al., 2009). Although it is unreasonable to definitively eliminate DOC as an MIH in some teleosts, stimulatory effects on FOM or DHP production are thus still expected at low doses.

### **5.3. Cortisol**

Among corticosteroids, cortisol is the most strongly suspected to be involved in FOM, as it has been the subject of greater focus than other corticosteroids in *in vitro* assays conducted on several teleosts. Perhaps the first evidence to support the involvement of corticosteroids such as cortisol in reproduction, and specifically in FOM, was evidence related to the effects of stress on reproduction and FOM. The corticosteroid most strongly linked to stress and linked to reproduction by the greatest number of published studies is cortisol (Gennotte et al., 2012; Small, 2004). Table 2 shows documented *in vivo* plasma cortisol levels coinciding with the final stages of the reproductive cycle in some teleosts. According to this table, studies on coho salmon, *Oncorhynchus kisutch*, and goldfish provide the best examples of *in vivo* cortisol levels that are higher than or in the range of *in vitro* active doses. Nevertheless, similar to what has been deduced for progestogens, the two types of assays (*in vitro* and *in vivo*) might contradict in that the measured *in vivo* cortisol levels might be significantly lower than the *in vitro* active dose of the hormone. In white sturgeon, cortisol has been revealed to be able to induce GVBD *in vitro* starting from a dose of only 480 ng/ml within a minimum of 14 hr of incubation (Webb et al., 2000). In another study, when this species was treated with carp pituitary homogenate to provoke GVBD, the *in vivo* plasma levels of all corticosteroids combined increased to a maximum of 150 ng/ml in fish that successfully reached the GVBD stage (Lutes et al., 1987). The *in vitro* findings showing cortisol as an MIH in sturgeon would thus be put into question

following the above *in vivo* findings. Perhaps the best examples of the extent to which *in vivo* studies fail to support *in vitro* results suggesting corticosteroid as an MIH have been obtained in catfish. In this species, the plasma cortisol level measured around spawning is 100 ng/ml, which is tenfold less than the effective dose needed to induce FOM *in vitro* (Table 2). Such evidence should be enough to indicate that cortisol is not an MIH in this fish. Nevertheless, the circulating levels of cortisol in teleosts, often in the range of tens or even hundreds of ng/ml, are comparable to doses often found to induce GVBD in some species (Table 2).

Metabolic evidence may also support the identity of cortisol as an MIH. For instance, in zebrafish, in which plasma cortisol levels are usually high, with a minimum level of 1250 ng/ml at FOM (Shang et al., 2019), mutation of the gene 11 $\beta$ hydroxylase, a key enzyme in cortisol production, fails to promote GVBD (Zhang et al., 2020). Together, the findings of these two recent studies provide strong evidence of the involvement of cortisol in FOM occurrence in this species. Additionally, comparisons of *in vivo* plasma cortisol levels between males and females during critical reproductive periods may support the assumption that cortisol is an MIH. Such evidence has been obtained in several studied species of Pacific salmon (*Oncorhynchus spp.*), in which the plasma cortisol levels in females have been found to be much higher than those in males, especially at spawning (Baker et al., 2013). These findings reflect possible roles for cortisol in female FOM and spawning, as has been reported in much earlier studies (Jalabert, 1976; Bry, 1985; Baker and Vynne, 2014).

All the mentioned information regarding the high plasma levels or *in vitro* FOM-inducing activity of cortisol around FOM and ovulation allow us to consider accepting cortisol as potential MIHs in some species. However, two observations lead us to criticize its physiological mechanism of action as classical MIH. On the one hand, cortisol has been shown to be one of the most vulnerable corticosteroids to mechanical or environmental stresses similar to those encountered in fish farms (Faltermann et al., 2020; Milla et al., 2015). On the other hand,

reproductive dysfunctions often recorded in fish raised in captivity are more severe in female broodstocks than in male broodstocks and are associated with FOM, ovulation and spawning (Alvarado et al., 2015). Taking both observations into account, it is reasonable to relate the resulting asynchronous ovulation and other reproductive failures to the disturbed cortisol levels. Hence, the hypothesis that cortisol is an MIH is limited by the possibility that abnormal stress-induced cortisol release might affect FOM or ovulation indirectly by affecting the signal transduction pathway leading to the release of the MIH rather than by directly mediating its effect as an MIH (Baker and Vynne, 2014; Mandiki et al., 2017). Cortisol thus mediates the stress-coping mechanisms in these species while probably causing some cases of reproductive failure.

## **6. Nonclassical MIHs**

In addition to classical MIHs (mainly DHP and THP) and other strongly suspected steroids, such as corticosteroids, some other hormones have been shown to exert stimulatory or inhibitory effects on the induction of FOM. Other sex steroids are regulators of FOM even if their potency is lower than that of progestogens. Estrogens have long been suspected to be ineffective in the regulation of FOM (Nagahama and Yamashita, 2008; Skoblina, 2009). However, more recently, they have been shown to inhibit the entry of oocytes into the FOM stage *in vitro* in a range of fish species (Pang and Thomas, 2009, 2010; Majumder et al., 2015; Das et al., 2016). This inhibitory role is consistent with the low circulating levels of E2 at the time of FOM, as the levels of this hormone drop at the end of the vitellogenesis stage. This drop corresponds to a shift towards testosterone (T) production, which is known to be involved in the final stages (Nagahama and Yamashita, 2008). It has been suggested that androgens may play roles during FOM in fish. Circulating T levels vary markedly during the final maturation stage in female teleosts (Mylonas et al., 1997). In addition, in contrast to E2, T is an inducer of FOM, although pharmacological doses are often necessary to observe *in vitro* triggering of

GVBD (Goetz and Cetta, 1985; Nagahama and Yamashita, 2008; Das et al., 2014). The anti-androgen CPA dramatically inhibits the *in vitro* LH-induced oocyte maturation and MIH synthesis in trout (Rime et al., 2015), which strengthens the link between the androgenic pathway and FOM mechanisms.

Prostaglandins (PGs) also display some MIH activities, which explains why their precursor arachidonic acid (ARA) is of primary importance in the quality of fish reproduction. Through *in vitro* tests, PGF2 alpha and PGE2 have recently been shown to induce FOM in a wide range of teleost fish species (Skoblina and Minin, 2016; Kim et al., 2017; Takahashi et al., 2018). However, similar to the case for T, doses in the range of  $\mu\text{g/ml}$  are sometimes necessary to provoke FOM *in vitro* (Skoblina and Minin, 2016), which raises the question of whether the effect on FOM is direct or indirect. Indeed, the finding that a prostaglandin can induce DHP production in longchin Goby *Chasmichthys dolichognathus* (e.g. Baek and Lee, 2019) raised the possibility that PGs effects on FOM induction are mediated through the production of the progestogens DHP and THP. Conversely, in Eurasian perch, DHP induced the *in vitro* production of PGE2 in maturing follicles which highlight the strong bilateral link between DHP and prostaglandins (El Mohajer et al., 2021). Otherwise, given the capacity of PGs to be effective at low doses (from 1 to 10 ng/ml) *in vitro* in other studies (Sorbera et al., 2001; Joy and Singh, 2013), we cannot rule out any direct effect of PGs on FOM induction, primarily because the circulating levels of PGs in the plasma of fish in the peri-ovulation stage are within the ranges of the mentioned effective doses, at least in goldfish and brook trout (Cetta and Goetz, 1982; Sorensen et al., 2018). This direct effect may be mediated through gonadotropin action as the effect of PGE2 on the FOM induction is hCG-dependent (Sorbera et al., 2001). Altogether, the evidence indicates that while the roles of PGs have long been attributed to ovulation, the existence of a direct effect on GVBD induction cannot be excluded during this

time period. Many more interspecies studies are needed to draw conclusions about the ability of PGs to induce oocyte maturation in teleosts.

The third category of hormones that are likely to directly regulate FOM includes the monoamines. The roles of monoamines in teleost fish reproduction have been thoroughly reviewed and are related to many stages of the reproductive cycle, with strong interference with the gonadotropin axis (Prasad et al., 2015). Among the monoamines, serotonin and melatonin exhibit local actions targeting the ovaries; notably, they regulate FOM. However, unlike that of the hormones in the abovementioned categories, the involvement of these hormones appears to differ depending on the fish species. Serotonin has been shown to be a stimulator of FOM *in vitro* in the medaka (*Oryzias latipes*) in the range of 10-100 ng/ml, and the stimulation is accompanied by hypersecretion of DHP (Iwamatsu et al., 1993). In contrast, serotonin has been shown to be an inhibitor of induced FOM *in vitro* in salt marsh killifish (Cerdà et al., 1998). This discrepancy could be due to the level of DHP released in the culture medium, as serotonin at 100 pg/ml stimulates FOM, while it inhibits DHP action (Chattoraj et al., 2008). Similar to that of serotonin, the status of melatonin as an MIH remains unclear. While melatonin may also be an effective MIH in carp (*Catla catla*) and zebrafish at 100 pg/ml *in vitro* (Carnevali et al., 2011; Chattoraj et al., 2008), it seems to be ineffective in other fish species (e.g. Chattoraj et al., 2005). In addition to direct effects, indirect effects cannot be totally excluded. In carp, for instance, it has been revealed that *in vitro* incubation of oocytes with melatonin for four hours prior to the addition of the MIH to the medium accelerates the action of the MIH by accelerating the formation of maturation-promoting factor (MPF) to induce GVBD (Maitra et al., 2013).

Finally, the somatotrophic hormones insulin-like growth factor (IGF) and growth hormone (GH) have been demonstrated to efficiently regulate FOM in some teleosts. In some species, GH, IGF-I or IGF-II triggers GVBD *in vitro* (Sarang and Lal, 2005; Das et al., 2014; Li et al., 2015). While IGF-1 mostly optimizes maturational competence, i.e., the ability to respond to an MIH,

in white perch (Weber et al., 2007), IGF-3 may also mediate LH action on FOM or act synergistically with DHP to overcome the E2 inhibition of FOM, as shown *in vitro* in zebrafish oocytes (Das et al., 2016; Li et al., 2015). Contrary to those of IGF, the mechanisms of action of GH on FOM induction remain unexplained. In both cases, the doses used in the biological tests performed to investigate some mechanisms have been in the range of 25 nM-2  $\mu$ M (Das et al., 2014; Li et al., 2015; Weber et al., 2007). The use of such high doses compared with the physiological concentrations in the plasma or ovarian fluid prevents us from making firm conclusions about the direct physiological actions on FOM events.

## **7. Overview of MIH detection methods with major emphasis on the most common *in vitro* assay and its optimization**

### **7.1. *In vivo* monitoring of MIH fluctuations**

As depicted in the previous sections, *in vivo* plasma measurements of physiological concentrations of sex steroids have been ultimately utilized to investigate the possible roles for these steroids in fish reproduction and particularly to detect MIHs during FOM. However, they display two major limitations. The first is that changes in circulating hormone levels during FOM do not formally demonstrate that a hormone is an MIH. Second, as blood sampling itself can be a source of stress, the plasma levels of some stress-sensitive hormones as cortisol in fish may not reflect the actual plasma hormonal kinetics (Bertotto et al., 2010). Therefore, alternative cortisol measurement assays are being developed to allow more accurate assessment of natural fish cortisol secretion (Sadoul and Geffroy, 2019). All attempts nevertheless remain to allow caution when interpreting *in vivo* plasma level fluctuations as evidence for a potent endocrine role in reproduction. In all cases, this descriptive approach may complement an experimental approach that involves administration of the hormone followed by evaluation of its effects on FOM induction. The different methods of MIH administration may range between the injection of the MIH into the muscles or peritoneal cavity of the fish and the direct



administration of the hormone into the water containing the fish (El Mohajer et al., 2021; Tokumoto et al., 2011). However, direct hormonal injection imposes risks of unresponsiveness to treatment or injuries to the fish which can affect the success of confirming the MIH identity. Concerns also rise with regards to the effects of hormonal administration into water despite the advantage of its rapid effect (Adeel et al., 2017; Tokumoto et al., 2011). Therefore, alternative methods as Ovarian lavage is otherwise proposed in some fish species (Müller et al., 2018). In pikeperch, ovarian lavage with carp pituitary extract proved to be 10% more successful at inducing ovulation than the commonly used intramuscular injection (Németh et al., 2012). Indeed, the methodology of ovarian lavage only requires the insertion of a catheter which induces much less discomfort to the animal by comparison to the commonly used intramuscular or peritoneal injections whilst resulting in higher outcome success of the same hormonal treatment. Above all, the considerable advantage of these *in vivo* explorations is that the integrity of the fish is conserved at the organ, tissue, cellular and molecular levels. The latter advantage still cannot compensate for the limitations concerning the accuracy of frequent *in vivo* assays due the stress factors mentioned above and considerable time investments, labor, cost, difficulty, and ethical considerations associated with animal maintenance in the laboratory throughout the *in vivo* experimental period (Beitel et al., 2014). Hence, direct *in vitro* application of hormonal agents that are suspected to play roles in FOM has been widely used for the last 40 years as an attempt to validate the roles of these agents as MIHs. In this section, we will list some of the commonly used *in vitro* bioassays for MIH detection while focusing on the most commonly used one and the practices that can optimize it.

## **7.2. Characterization of the ligand-receptors interactions**

Similar to *in vivo* assays, *in vitro* molecular assays can serve for the detection of MIH and/or MIH receptors. Starting with the less frequently used, cytoplasm microinjection involves the direct injection of intact oocytes with MIH (Torres et al., 2016). However, due to the fact that

an MIH usually follows a membrane-receptor mediated intracellular action, this *in vitro* assay was proven to be a failure. In fact, this assay helped further confirm that an MIH action is usually mediated through cell surface receptors (Thomas, 2012). As such, isolation of membrane MIH receptors have been developed for identifying the MIH through the molecular characterization of these receptors (Zhu et al., 2003). Notably, ovarian membrane suspensions incubated with the putative MIH were used to compare the binding affinities of the potential ligands to MIH membrane receptors (e.g. King et al., 1997). Conversely, photoaffinity labeling combined with immunochemical detection, using oocyte membrane fractions incubated with radioactive MIH analogues, are proposed in starfish to isolate the membrane receptors (Toraya et al., 2017) and it may be possibly utilized in teleosts. With such membrane isolation, electrophysiological studies have been also proven to be effective in revealing the MIH effects (Marshall et al., 2011). However, it might be unfair to definitely exclude the nuclear progesterone receptors from the FOM mechanisms. For instance, their gene expression increases during FOM in zebrafish and their gene knockout suggest their implication in the final stages, especially in the ovulatory event (Tang et al., 2016). If the involvement of nuclear progesterone receptors in the FOM remains to be demonstrated in fish (Hanna et al., 2009; Tang et al., 2016), appealing to transactivation studies of these receptors after appropriate transfection in cell lines may be a future guide to discover the most potent endogenous agonists to activate these receptors (Hanna et al., 2009). Once the profile of progesterone receptors involved in FOM is validated, pharmacological approaches using chemicals as agonists or antagonists of these receptors can also be useful to define the chemical structure of the active ligands and thus to propose potential MIH (Rezanujjaman et al., 2020; Tang et al., 2016).

### **7.3. *In vitro* biological test of follicle incubation to discover the MIH**

One of the most rapid and less complex *in vitro* MIH detection assay remains to be the direct administration of the hormones suspected to be the MIHs into culture media containing intact

maturing follicles. The activity of this MIH would then be assessed according to its impact on the advancement of oocytes through the FOM leading to their ovulation. Most notable advantage of such *in vitro* assay includes the possibility of testing quickly and efficiently a wide range of hormonal treatments. Still, there are different ways to further optimize this type of *in vitro* assays. First, the sensitivity of oocytes to *in vitro* treatments varies according to the stage of morphological maturation of the oocytes, such as the migration status of the nucleus, under treatment (Baek and Lee, 2019). Hence, detection of an MIH necessitates that oocytes are at a stage early enough to give an unbiased and fair reflection of the putative effect of the *in vitro*-applied MIH but late enough to be sensitive to this applied MIH. In many studies, great care has been taken to classify the different stages of FOM in different teleosts. These classifications aim to enable detection of the initial stage at which the MIH can be adequately tested. In zebrafish, as in most teleosts, adoption of a translucent appearance by follicles is live evidence of the transition to FOM (Sintaha, 2013). In sturgeon, the classification of FOM is based upon the distances between the nucleus and the animal/vegetal poles (Chapman and Eenennaam, 2007). In Eurasian perch, a more advanced and specific classification system has been developed to detect oocytes during each of the maturation stages taking place during FOM. This classification system is based on the diameter, migration, positioning of Germinal vesicle (GV) and on observation of oil droplets (Żarski et al., 2011). Such FOM monitoring involving the surveillance of GVs and oil droplets is an improved strategy to accurately detect the different stages of FOM in various fish species.

In addition to the choice of the most reliable starting point for assessment of the putative activity of an MIH *in vitro*, the success of *in vitro* assays is reliant on optimization of the choice of culture medium, pH, osmolality, hormone dose adapted to the cellular content in the medium (e.g., density and sizes of follicles, denuded or intact follicles), and ideal duration of incubation to achieve useful results while avoiding a high risk of cytotoxicity. First, the medium in which

oocytes are incubated with an MIH plays an important role in the success of MIH action. The most suitable culture medium might vary among species. For instance, for zebrafish, the most common culture medium is Leibovitz L-15 medium (Sintaha, 2013). For Eurasian perch, Cortland medium has been adapted as a reliable medium for assessment of MIH activity (Henrotte et al., 2011; Mandiki et al., 2017). The choice of the most reliable culture medium has been proven to be highly dependent on the osmolality of the medium (Seki et al., 2008). Indeed, the medium should be isosmotic (Silva et al., 2018). In a recent study performed on sturgeon, it was revealed that the percentage of oocytes that were successfully ovulated *in vitro* increased after the concentration of sodium bicarbonate in the culture medium was decreased. This study successfully highlighted the importance of optimizing the osmolality of the culture medium in order to optimize *in vitro* assays on maturation and ovulation (Skoblina and Goncharov, 2012).

Aside from determination of the ideal composition of the culture medium, the use of various additives in this medium has been suggested to promote the activity of an MIH. Many previous reports have mentioned that hCG, an LH analog, increases MIH production (Das et al., 2014). In addition, other additives, such as transforming growth factor- $\beta$ , transforming growth factor- $\alpha$ , epidermal growth factors, activin, and cyclin B, have also been suggested to optimize the performance of maturing follicles during *in vitro* assays. However, the efficiency of the additives, the medium pH and the incubation period are closely related, and it is believed that the relationship affects the optimization of the assays (Sintaha, 2013). Considerable attention should be given to optimization of the medium pH. It has been revealed in Atlantic croaker (*Micropogonias undulatus*) that alkaline conditions, similar to those of natural ovarian fluid, facilitate the *in vitro* maturation of oocytes (Seki et al., 2008). One major element that should be adjusted with caution to facilitate the search for MIHs *in vitro* is the duration of incubation; this factor should be adjusted to accurately and optimally exhibit the efficiency of an MIH. In

Persian sturgeon, the *in vitro* success of oocyte maturation was measured using the fertilization rate, which was revealed to decrease with increasing incubation duration (Azarin et al., 2016). On the other hand, the risk of cytotoxicity associated with increased durations of *in vitro* incubation in culture medium should be carefully considered. The term “cytotoxicity” refers to cell death resulting from toxicant-induced disruptions of cell membrane integrity and mitochondrial energy production, which is believed to result from prolonged periods of incubation (Rehberger et al., 2018). Excessive cytotoxicity in culture medium may call into question the follicular receptivity to hormones and in turn the discovery of potential MIHs.

Several *in vitro* assay protocols for MIH detection propose defolliculation of oocytes in an attempt to reflect the solitary action of an MIH. As the two ovarian follicle layers in teleosts are involved in the production of MIHs such as DHP, some researchers prefer to separate the follicles to enable more credible assessment of the effects of MIH treatment. This is possible because, in several fish species, the oocytes host receptors for MIHs (Nagahama and Yamashita, 2008). However, not separating follicles from oocytes provides the advantage of maintaining the integrity of whole follicles, thus enabling assessment of the follicle-oocyte interaction during FOM; it also enables the potential discovery of MIHs other than progestogens that need the presence of the follicular layers to act.

Finally, the hormone doses applied during *in vitro* incubation are of primary importance. As can be concluded from both Tables 1 and 2, pharmacological doses of putative MIHs are sometimes used. The fact that these concentrations are completely different from the levels measured in the blood or in the ovarian fluid may call into question their involvement as MIHs. In contrast, a number of studies have shown effects at doses that are sufficiently low to be physiologically realistic for triggering of FOM *in vivo*. Comparisons of the *in vitro* effective doses and the circulating levels *in vivo* should thus be taken into consideration when a given hormone is considered as an MIH. The differences residing between the recorded hormonal

levels in plasma and ovarian fluid should also be considered taking the example on the blue tang fish (*Paracanthurus hepatus*) in which the recorded plasma levels of DHP shown in Table 1 were found to be five-fold lower than its levels in ovarian fluid (Sang et al., 2019). In this case, this latter should be taken into consideration when assessing the true MIH identity as the maturing oocytes are in more direct contact with the ovarian fluid than with the blood.

With all the conditions of the *in vitro* assays being optimized, the assays can enable hormonal tests that are difficult to perform *in vivo*. With the absence of animal ethics considerations as well, *in vitro* assays would be even more preferred over *in vivo* assays (Rehberger et al., 2018). After all, the whole purpose from pointing out the most potent MIH is the attempt to optimize the direct hormone-induced spawning techniques using either corticosteroids, melatonin, PGs, or progestogens. In fish farms however, medication containing synthetic GnRH analogues associated or not with antagonists of dopamine receptors (e.g Ovaprim® or Ovopel®) or containing hCG, which follow the indirect upstream-FOM induction pathway, are the commonly used hormonal therapies for maturation and spawning induction (Hoga et al., 2018). The reason for the unencouraged use of commercial progestogens as direct inducers of maturation and spawning is perhaps the documented unexpected deleterious effects of the human medication Levonorgestrel (LNG) or Drospirenone (DRSP) in fathead minnow (*Pimephales promelas*) (Zeilinger et al., 2009, Runnalls et al., 2015) and zebrafish (Hua et al., 2015). The latter findings can possibly justify why fish farmers would rather use the commonly used indirect upstream-acting hormonal treatments over the proposed direct progestogen hormonal treatments.

In conclusion, both *in vitro* and *in vivo* assays will be crucial in further studies to confirm whether a certain hormone can be validated as true MIHs. Additionally, deeper investigation on the application of readily available synthetic true MIHs which possess direct positive effects on fish FOM are further required.

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## 9. Tables and figures

**Table 1** *In vivo* progestogens concentrations in unstressed fish during the peri-ovulatory period and the minimal efficient progestogen doses to *in vitro* induce the final oocyte maturation (FOM), ovulation (OV), germinal vesicle breakdown (GVBD) and spawning (SP) for different families of teleost.

Family	Specie	Latin name	Progestogens	<i>In vitro</i> Concentration (ng/ml)	<i>In vivo</i> Concentration (ng/ml)	Maturation stage Reached	Reference
<i>Acanthuridae</i>	Blue Tang Fish	<i>Paracanthurus hepatus</i>	DHP	–	0.2-0.24	SP	Sang et al. (2019)
<i>Acipenseridae</i>	White sturgeon	<i>Acipenser transmontanus</i>	P DHP	62 31	<sup>a</sup> 0.35	FOM GVBD SP	Lutes (1985); Webb et al. (2002)
	Persian sturgeon	<i>Acipenser persicus</i>	DHP P	1000 –	– 0.03	FOM	Azarin et al. (2016); Khara et al. (2014)
	Starry surgeon	<i>Acipenser stellatus</i> Pallas	P	–	1.2	OV	Barannikova et al. (2002)
	Sterlet sturgeon	<i>Acipenser ruthenus</i>	P DHP	30 1000	40 –	GVBD OV	Azarin et al. (2020); Mohammadzadeh et al. (2020); Skobolina and Goncharov (2012)
	Siberian sturgeon	<i>Acipenser baeri</i>	DHP	1000	–	GVBD OV	Azarin et al. (2020)
<i>Anguillidae</i>	Japanese eel	<i>Anguilla japonica</i>	DHP	100	<0.05	FOM OV	Abe et al. (2010); Kim et al. (2007)
<i>Centrarchidae</i>	Rock bass	<i>Ambloplites rupestris</i>	DHP	0.5-2.0	–	GVBD	Goetz and Cetta (1985)
<i>Clupeidae</i>	Indian shad	<i>Tenulosa ilisha</i>	DHP	–	3.76 4.6 <sup>b</sup>	FOM	Pramanick et al. (2013)
<i>Cyprinidae</i>	Zebrafish	<i>Danio rerio</i>	DHP	3.15	–	GVBD OV	Tokumoto et al. (2011)
	Chalcalburnus tarichi	<i>Chalcalburnus tarichi</i>	17OHP THP	1000 1000	0.19 0.26	GVBD SP	Ünal et al. (2008)
	Caspian Kutum	<i>Rutilus frisii kutum</i>	17OHP P	– –	4 2.6	FOM	Heidari et al. (2010)
	Goldfish	<i>Carassius auratus</i>	DHP	100	1-10 <sup>c</sup>	GVBD FOM	Nagahama et al. (1983); Wu et al. (2020)
<i>Labridae</i>	Kyusen Wrasse	<i>Halichoeres poecilopterus</i>	DHP THP	1 1	0.5 0.7	GVBD OV	Matsuyama et al. (2002)
<i>Moronidae</i>	European seabass	<i>Dicentrarchus labrax</i>	THP DHP	10 10	1.4 40 (feral fish)	FOM FOM SP	Asturiano et al. (2000); Sorbera et al. (1999) Rocha and Reis-Henriques (1999); Sorbera et al. (1999)
	Striped bass	<i>Morone saxatilis</i>	DHP THP	10 10	1.87 –	GVBD FOM	Berlinsky and Specker (1991); King et al. (1994)
	White perch	<i>Morone americana</i>	DHP	1000	1 <sup>c</sup>	GVBD	King et al. (1995)
	White bass	<i>Morone chrysops</i>	DHP	–	3.5 <sup>c</sup>	GVBD	King et al. (1995)

<b>Percidae</b>	Yellow perch	<i>Perca flavescens</i>	DHP	0.24 0.48	– –	GVBD GVBD OV	Goetz and Theofan (1979); Rinchard et al. (2002); Skobolina (2009)
			P	– 100 1000	0.027~ –	SP GVBD OV	Goetz and Bergman (1978)
	Eurasian perch	<i>Perca fluviatilis</i>	DHP	1	1.4-1.5 0.2-0.6	FOM GVBD OV	Fontaine et al. (2003); Noaksson et al. (2004); Sulisty (1998)
	Eurasian ruffe	<i>Gymnocephalus cernuus</i>	THP DHP	1 10	– –	FOM GVBD	EL Mohajer et al. (2021) Sorensen et al. (2004)
<b>Plecoglossidae</b>	Ayu	<i>Plecoglossus altivelis</i>	THP DHP	10 10	3 <sup>c</sup>		
<b>Pleuronectidae</b>	Starry flounder	<i>Platichthys stellatus</i>	DHP	10	–	GVBD	Nagahama et al. (1983)
<b>Salmonidae</b>	Amago salmon	<i>Oncorhynchus rhodurus</i>	DHP	–	2.5	SP	Lim (2016)
	Rainbow trout	<i>Oncorhynchus mykiss</i>	DHP	10	50	GVBD	Nagahama et al. (1983); Young et al. (1983)
	Brook trout	<i>Salvelinus fontinalis</i>	DHP	10-40	70-380	GVBD OV	Espinosa et al. (2013); Milla et al. (2006); Nagahama et al. (1983); Scott and Baynes (1982)
<b>Sciaenidae</b>	Atlantic croaker	<i>Micropogonias undulatus</i>	P	100	–	GVBD	Goetz and Bergman (1978)
	Spotted seatrout	<i>Cynoscion nebulosus</i>	DHP	10-15	–	GVBD	Mathews et al. (2002); Thomas and Das (1997); Trant and Thomas (1988)
	Yellow porgy	<i>Acanthopagrus latus</i>	THP	–	1	FOM	Thomas (1988)
<b>Sparidae</b>	Red seabream	<i>Pagrus major</i>	THP	1.7	–	GVBD	Thomas and Das (1997)
			DHP	2	–	GVBD	Jeng et al. (2012)
			THP	2	–	GVBD	
<b>Stichaeidae</b>	Ribbed Gunnel	<i>Dictyosoma burgeri</i>	DHP	3	1.4	GVBD FOM	Ohta et al. (2002)
<b>Tetraodontidae</b>	Tiger Puffer	<i>Takifugu rubripes</i>	THP	3	2.4		
<b>Mugilidae</b>	Grey mullet	<i>Mugil cephalus</i> L.	DHP	–	5	FOM	Hwang et al. (2012)
			THP	10	–	GVBD	Matsuyama et al. (2001)
			DHP	200	–	GVBD	Das et al. (2014)

DHP: 17, 20  $\beta$ -dihydroxy-4-pregnen-3-one, THP: 17,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one, P: Progesterone, 17OHP: 17 $\alpha$ -hydroxyprogesterone.

– Absence of records.

~ Low recorded plasma values with no peak at all.

<sup>a</sup> Effect was studied but levels were not shown.

<sup>b</sup> Values recorded when plasma level Peaked twice.

<sup>c</sup> Levels after injection of fish or priming of cells with hCG.

**Table 2** *In vivo* cortisol concentrations in unstressed fish during the peri-ovulatory period and the minimal efficient cortisol doses to *in vitro* induce the final oocyte maturation (FOM), ovulation (OV), germinal vesicle breakdown (GVBD) and spawning (SP) for different families of teleost.

Family	Specie	Latin name	<i>In vitro</i> Concentration (ng/ml)	<i>In vivo</i> Concentration (ng/ml)	Maturation stage Reached	Reference
<i>Acipenseridae</i>	Stellate sturgeon	<i>Acipenser stellatus</i>	–	85 <sup>a</sup>	FOM	Roosta et al. (2019)
	White sturgeon	<i>Acipenser transmontanus</i>	480	151 <sup>a</sup>	GVBD	Lutes et al. (1987); Webb et al. (2000)
	Starry surgeon	<i>Acipenser stellatus</i> Pallas	–	57.3	OV	Barannikova et al. (2002)
	Persian sturgeon	<i>Acipenser persicus</i>	–	70	OV	Khara et al. (2014)
<i>Adrianichthyidae</i>	Medaka	<i>Oryzias latipes</i>	100	–	OV	Hirose (1976)
<i>Cichlidae</i>	Nile tilapia	<i>Oreochromis niloticus</i>	–	2.3	FOM	Gennotte et al. (2012)
<i>Cyprinidae</i>	Goldfish	<i>Carassius auratus</i>	500	300	FOM   OV	Cook and Peter (1980); Jalabert (1976)
	Zebrafish	<i>Danio rerio</i>	–	1250	FOM	Shang et al. (2019)
<i>Fundulidae</i>	Killfish	<i>Fundulus heteroclitus</i>	–	70	OV	Bradford and Taylor (1987)
<i>Heteropneustidae</i>	Catfish	<i>Heteropneustes fossilis</i>	1000-5000	100	FOM   SP	Lamba et al. (1983); Sundararaj et al. (1979)
<i>Ictaluridae</i>	Channel catfish	<i>Ictalurus punctatus</i>	–	6.6	SP	Small (2004)
<i>Moronidae</i>	European bass	<i>Dicentrarchus labrax</i>	–	60 (ferral)  90 (captive)	SP	Rocha and Reis-Henriques (1999)
<i>Percidae</i>	Eurasian perch	<i>Perca fluviatilis</i>	–	550	SP	Noaksson et al. (2005)
	Eurasian ruffe	<i>Gymnocephalus cernuus</i>	1000~	–	GVBD	Sorensen et al. (2004)
	Yellow perch	<i>Perca flavescens</i>	10 000	–	GVBD	Goetz and Bergman (1978)
<i>Pleuronectidae</i>	European plaice	<i>Pleuronectes platessa</i>	–	147	SP	Wingfield' and Grimm (1977)

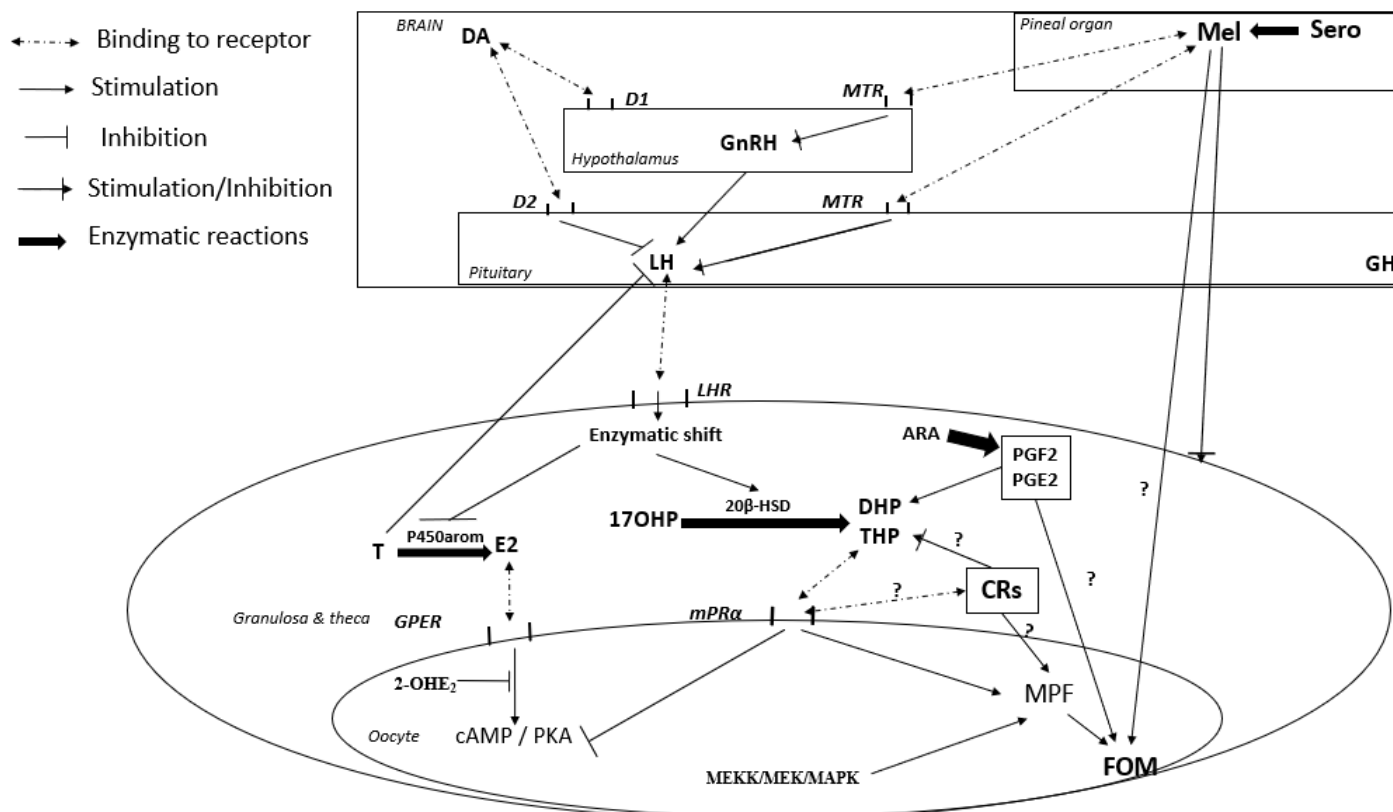
<i>Salmonidae</i>	Brook trout	<i>Salvelinus fontinalis</i>	1000 –	– 10-44	GVBD FOM OV	Goetz and Bergman (1978); Pickering and Christie (1981)
	Coho salmon	<i>Oncorhynchus kisutch</i>	50	100	GVBD	Sower and Schreck (1982a, 1982b)
	Masu salmon	<i>Oncorhynchus masou</i>	–	325	SP	Westring et al. (2008)
	Pink salmon	<i>Oncorhynchus gorbuscha</i>	–	297	OV	McConnachie et al. (2012)
	Rainbow trout	<i>Oncorhynchus mykiss</i>	–	22~ 7.7-10.5~	OV FOM	Bry (1985); Pankhurst and Van Der Kraak (2000)
	Red salmon	<i>Oncorhynchus nerka</i>	–	10-53 50-200	FOM SP	Baker and Vynne (2014)
<i>Sciaenidae</i>	Atlantic croaker	<i>Micropogonias undulatus</i>	105	–	GVBD	Patiño and Thomas (1990b)
<i>Stichaeidae</i>	Ribbed gunnel	<i>Dictyosoma burgeri</i>	–	2081	SP	Hwang et al. (2012)
<i>Sebastidae</i>	Rockfish	<i>Sebastes taczanowskii</i>	1000	–	GVBD	Takemura et al. (1989)

~ Low recorded in vitro activity or plasma levels.

– Absence of records.

<sup>a</sup> Levels after injection of fish or priming of cells with hCG or other LH analogues.

**Figure 1 Endocrinological route of MIH release and the environmental and paracrine factors controlling this release with brief representation of the mode of action of an MIH.**



Dopamine (DA) / Dopamine Receptor (D1,D2) – Melatonin (Mel) / Melatonin Receptors (MTR) – Serotonin (Sero) – Gonadotropin Releasing Hormone (GnRH) – Luteinizing Hormone (LH)/ Luteinizing Hormone Receptor (LHR)- Growth Hormone (GH)– Testosterone (T) – 17β-estradiol (E2) / G-coupled estrogen membrane receptor (GPER) – Aromatase (P450arom) 17α – Hydroxyprogesterone (17OHP) – 20β-hydroxysteroid dehydrogenase (20β-HSD) – 17, 20 β-dihydroxy-4-pregnen-3-one (DHP), 17,20β,21-trihydroxy-4-pregnen-3-one (THP) / membrane progestin receptor (mPRα) – Corticosteroids (CRs) – Arachidonic Acid (ARA)/ Prostaglandins (PGF2, PGE2) – Catecholestrogen (2-OHE<sub>2</sub>) – Cyclic adenosine monophosphate (cAMP) – Serine threonine kinase (PKA) – MEK kinase cascade (MEKK/MEK/MAPK) – Maturation promoting factor (MPF) – Follicle Ovarian maturation (FOM)

## **10. Conflict of interest**

The authors declare that they have no conflict of interest

## **11. Ethics approval**

No ethical approval is necessary as it is a review article

## **12. Consent to participate**

The authors declare that they agree of participating in this review article with no conflict of interest

## **13. Consent for publication**

The authors declare that they agree of publishing in this review article with no conflict of interest

## **14. Availability of data and material**

**Non-applicable**

## **15. Code availability**

We haven't used any software for statistics as we haven't done statistics

## **16. Authors' contributions**

Leila El Mohajer: Conceptualization, data curation, writing

Rose Bulteau: data curation

Sylvain Milla: Conceptualization, writing

Pascal Fontaine: writing



### ***“Major conclusions and up-coming approaches”***

The collected data in chapter 2 were able to strongly nominate other hormones, besides the commonly nominated progestogens, as MIH in several teleosts. More importantly, the collected data in chapter 2 highlighted a major condition in considering a hormone as an MIH. The latter condition was that both *in vitro* active doses and *in vivo* physiological levels of the suspected MIH must be consistent in nominating a certain hormone as true MIH.

In this respect, the collected data in chapter 2 revealed that *in vivo* or *in vitro* assays might contradict with regards to the nomination of progestogens as MIHs in several teleosts. The latter would thereby speculate the common assumption of progestogens being the MIH in most teleosts. On the other hand, the collected data in chapter 2 potentially nominated corticosteroids as potential MIH. Indeed, receptor-binding affinity assays, *in vivo* plasma monitoring of corticosteroids levels, *in vitro* testing of MIH activities on maturing ovarian follicles, and *in vitro* analysis of ovarian steroidogenesis products support that corticosteroids might be MIHs (Baker and Vynne, 2014; Milla et al., 2009). So far, we may postulate that the results of chapter 2 agree with the assumptions of chapter 1 in supporting the third working hypothesis (H3) which assumed a potent role for corticosteroids in FOM.

Starting from the latter assumption, we hence suggested to go deeper into investigating the maturation inducing activity of corticosteroids. Indeed, the latter step will serve more in addressing our third working hypothesis (**H3**).

In the following chapter (**chapter 3**), we conducted an *in vitro* experiment during FOM to test the direct activity of corticosteroids as potential MIH in Eurasian perch. To respect the condition set in chapter 2 about the consistency between both applied *in vitro* doses and *in vivo* plasma levels, we made sure to select the applied *in vitro* doses within the range of the physiological plasma levels as revealed in chapter 1.

# CHAPTER 3

## CHAPTER 3

Fish Physiol Biochem  
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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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#### **Chapter 3: *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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## 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-pregnen-3-one (DHP) and 17 $\alpha$ ,20 $\beta$ ,21 trihydroxy-4-pregnen-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.

**Keywords:** Eurasian perch, corticosteroids, *in vitro*, DHP, FOM, MIH.

## 1. Introduction

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 (MIH) that are known to control the final stages of 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-pregnen-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 are 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 functions together in stimulating the occurrence of ovulation at least in percid fishes. There are two major prostaglandins in fish namely PGE<sub>2</sub> and PGF<sub>2</sub> $\alpha$  that are known to induce both FOM and ovulation (Kestemont and Henrotte 2015). However, with more evidence on the presence of PGE<sub>2</sub> receptor (EP<sub>2</sub>) in female Eurasian perch in relation with FOM regulation, PGE<sub>2</sub> was favoured for being tested compared to PGF<sub>2</sub> $\alpha$  (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 (11-D) 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 previous reports to show a

surge in its plasma concentration around ovulation in fish species such as the rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*) (Mandiki et al. 2017), and even Eurasian perch (Noaksson et al. 2005). In yellow perch, both cortisol and DOC were effective in inducing the *in vitro* oocyte maturation (Theofan and Goetz 1983; Milla et al. 2009). Therefore, we may postulate a high possibility of the involvement of the four 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. Materials 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: APAFIS15307-2018053110404570 and 23871-2020013017452359) by the local ethic 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 were tested on intact follicles from 6 mature Eurasian perch females at the start of maturation. In the second *in vitro* one, the two MIH 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 conditions**

A group of 2 years-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), were transferred to the Aquaculture Experimental Platform (AEP, registration number for animal experimentation C54-547-18) belonging to the URAFFPA 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: 16L:8D; 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: 8L:16D; 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. Chemicals

All chemicals and fatty acids were purchased from SigmaAldrich (France) and Cayman Chemical (Ann Arbor, Michigan, United States). 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> 6 H<sub>2</sub>O, 0.23 MgSO<sub>4</sub> 7H<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 follicles preparation**

Each female was anesthetized (MS-222; Sigma-Aldrich, Lyon, France), catheterized, using a catheter (DAHLHUUSEN; 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 Zarski 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 binocular microscope, magnification 4× (OLYMPUS, SZX7). Fish proven to be at stage 1 or 3 were immersed in a bath containing 300 mg/L of the anesthetic 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. *In vitro* ovarian Follicles incubations**

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 µl of dissolved hormone in ethanol was added in the culture medium just before adding the follicles.

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.

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 62h 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; Źarski 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**

After 62 h 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 Zarski et al. (2001a), (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-WST (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 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 hormones 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 µl of the incubation culture media of all treatments in Experiment 1 by ELISA using Fish 17alpha,20beta dihydroxyprogesterone (17alpha, 20betaOH-PROG) ELISA Kit (MBS2602842) 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 µl 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 ThermoFisher 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 years-old female Eurasian perch was used with an average weight of 650.5 g, 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 utilized for fish belonging to the first two *in vitro* manipulations. Each fish was anesthetized, catheterized, and its FOM stage determined using the same principles as in part 2.3. 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 two (Day 2) and four days (Day 4) following the injections (Day 0). Spawning was checked daily until 14 days post injection and every two days all fish were stripped manually by gently massaging the lower abdomen. The success of spawning was evaluated based on: (1) the spawning rate ( $100 \times \text{number of spawned fish} / \text{total}$

number of fish 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 khi2 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 Bonferroni or Benjamini & Hochberg. 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 $\pm$ SD while the data showed Median $\pm$ 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

*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, 100, 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 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, cortisol (Co) managed to significantly ( $P < 0.01$ ) induce a rather slight advancement in FOM at all tested doses (10, 100, and 1000 ng/mL) in most of the follicles (MR) and at only two doses (10, 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, 1, 10 ng/mL). Finally, the other corticosteroids 11-deoxycortisol (11-D) and 11-deoxycorticosterone (DOC), and both sex steroids  $17\beta$ -estradiol (E2) and testosterone (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.

*Experiment 2.* The results revealed that when subjecting follicles to even a lower dose of both DHP and  $17\alpha,20\beta,21$  trihydroxy-4-pregnen-3-one (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, 100, 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 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 (10ng/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 (10ng/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).

### **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 post-injection 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 were 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  $154.8\pm54.7$  h,  $148.5\pm18.7$  h and  $207.5\pm54.7$  h for the high dose, low dose and control spawned fish, respectively.

#### 4. Discussion

In the current study, the *in vitro* effect of exposing postvitellogenic 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 postvitellogenic 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), and 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 Seat trout (Thomas and Trant 1989). In our study as well, both DHP and THP induced FOM in a dose 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 DHP production by maturing follicles after the whole incubation period (62 h). They also did not exert any significant effect on 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 down-regulation of E2 and DHP production in perch post-vitellogenic oocytes (Mandiki et al. 2017). Altogether, it is thought that 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 for 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 for 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 the 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). 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 PGE<sub>2</sub>. 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*), oocytes response to the MIH in inducing FOM is highly dependent on the pretreatment with gonadotropin (Patiño and Thomas 1990b; Pang and Ge 2002). The elevated efficiency of DHP treated follicles to induce PGE<sub>2</sub> production in 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 PGE<sub>2</sub> where DHP was found to be produced, though unsignificantly, by PGE<sub>2</sub>. DHP up-regulation by another prostaglandin (namely PGE<sub>1</sub>) and by arachidonic acid (the PGE<sub>2</sub> precursor) were 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 either direct effect on ovulation but also slight indirect effect on ovulation through the *de novo* production of PGE<sub>2</sub>. At the follicular level, PGE<sub>2</sub> 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 PGE<sub>2</sub> receptors (EP4b) increases dramatically as the follicles approach their destined ovulation. The latter finding thereafter signifies the putative role of PGE<sub>2</sub> 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 PGE<sub>2</sub> are inducers of ovulation and our data support that PGE<sub>2</sub> 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.

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perch, *Perca fluviatilis* L. Reprod Biol 11:194-209. [https://doi.org/10.1016/S1642-431X\(12\)60066-7](https://doi.org/10.1016/S1642-431X(12)60066-7).

## 6. Tables and Figures

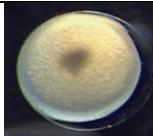
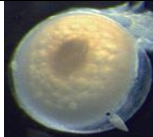

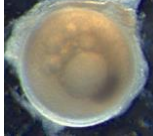

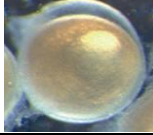

**Table 1 Hormonal doses (ng/mL) tested in experiment 1.**

Doses ng/mL	D1	D2	D3	References
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**

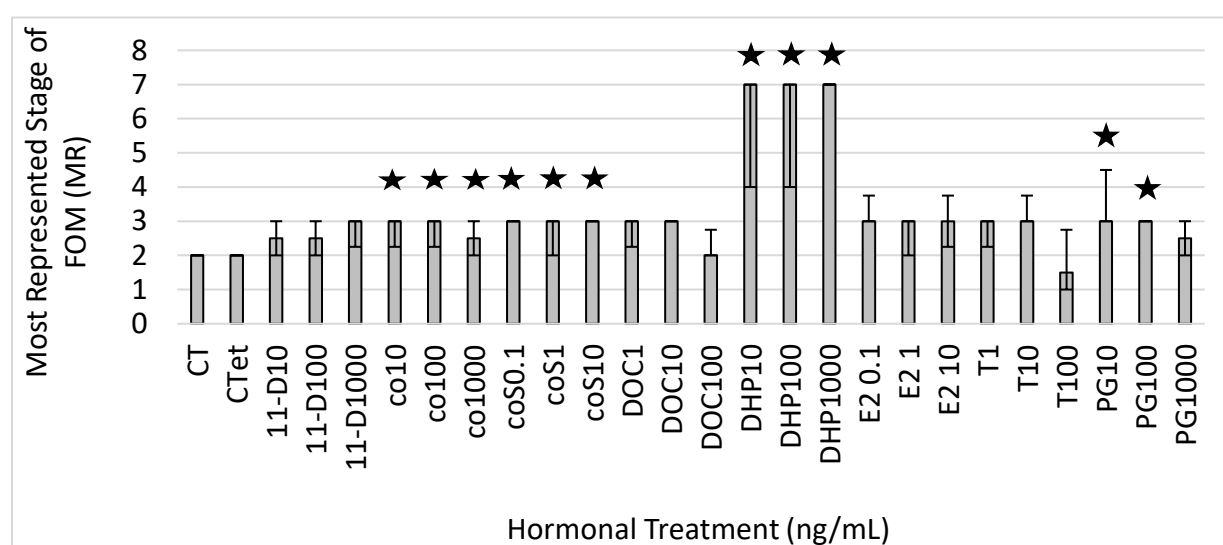
Doses ng/mL	D 1	D2	D 3	D 4	D5	References
17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one (DHP)	0.1	1	10	100	1000	Miura et al. (2007); Pinter and Thomas (1999)
17 $\alpha$ ,20 $\beta$ ,21 trihydroxy-4-pregnen-3-one (THP)	0.1	1	10	100	1000	Pinter and Thomas (1999)

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

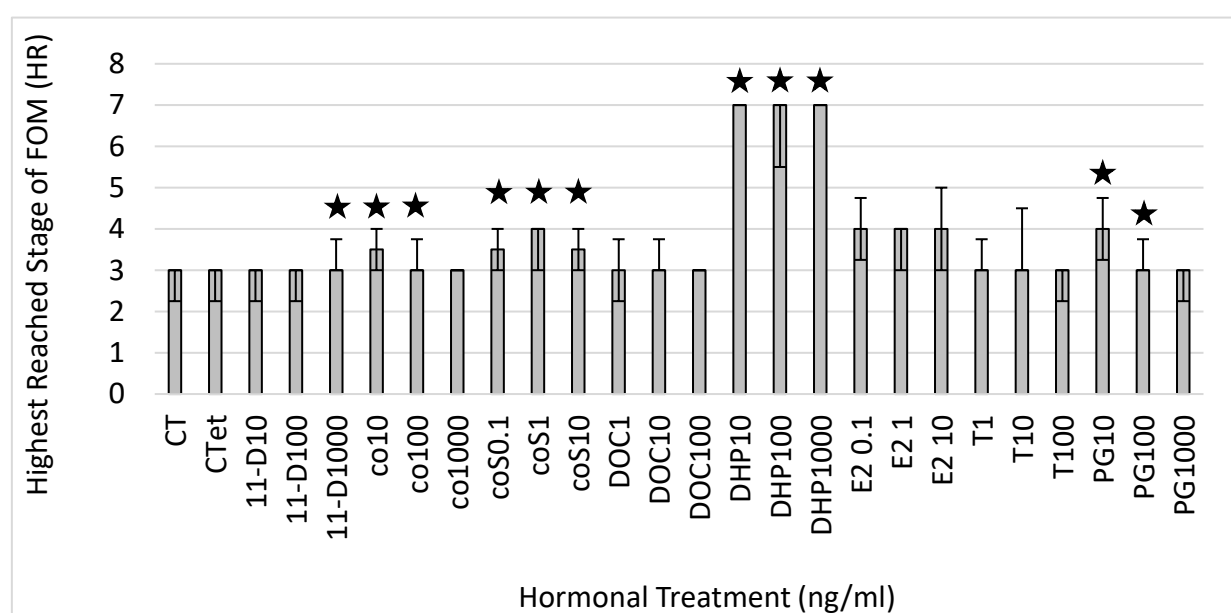
Stages		
1	The Germinal Vesicle (GV) is situated in the oocyte centre, oil droplets are poorly visible	
2	Beginning of GV migration, beginning of coagulation of clearly visible oil droplets	
3	Migration of GV (above half of the oocyte diameter), oil droplets are clearly visible	
4	The GV is at the oocyte 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, 100 and 1000 ng/mL), Co (cortisol; 10, 100 and 1000 ng/mL), coS (corticosterone; 0.1, 1 and 10 ng/mL); DOC (11-deoxycorticosterone; 1, 10 and 100 ng/mL), DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one; 10, 100 and 1000 ng/mL); E2 (17 $\beta$ -estradiol; 0.1, 1 and 10 ng/mL); T (testosterone; 1, 10 and 100 ng/mL); PG (prostaglandin E2; 10, 100 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 errors bar 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.



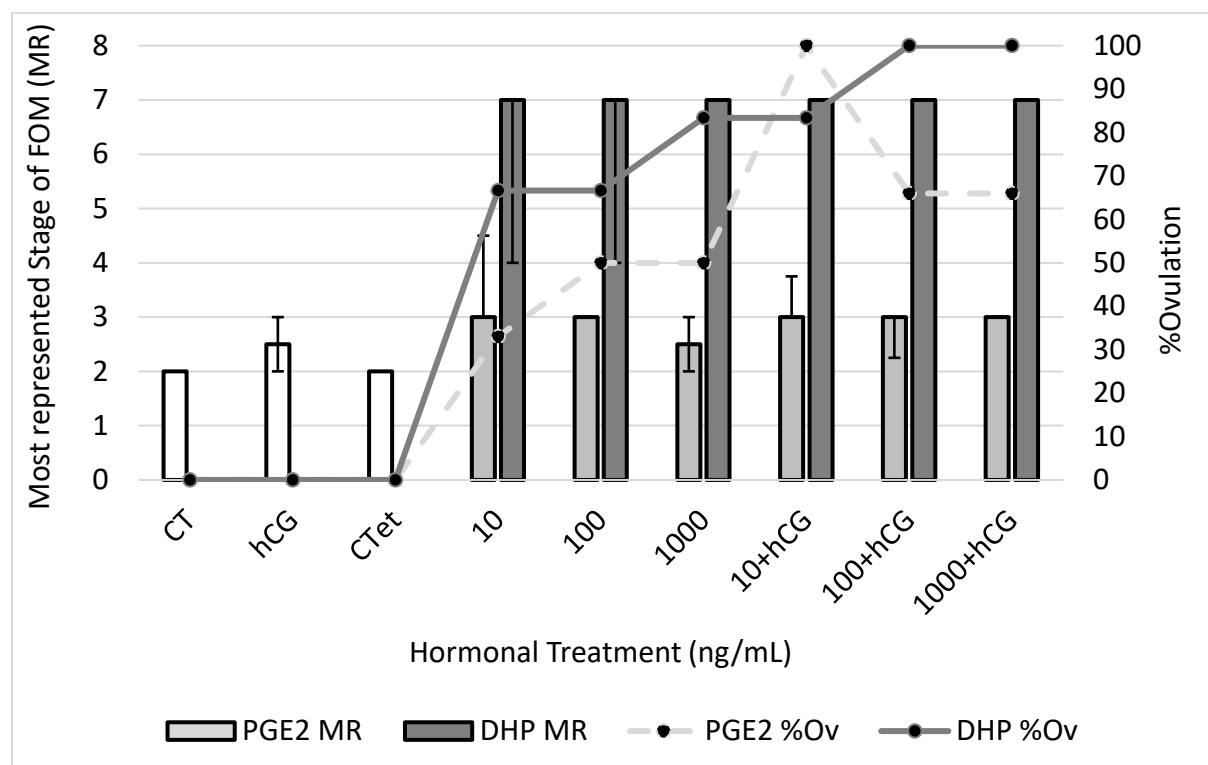
**a**



**b**

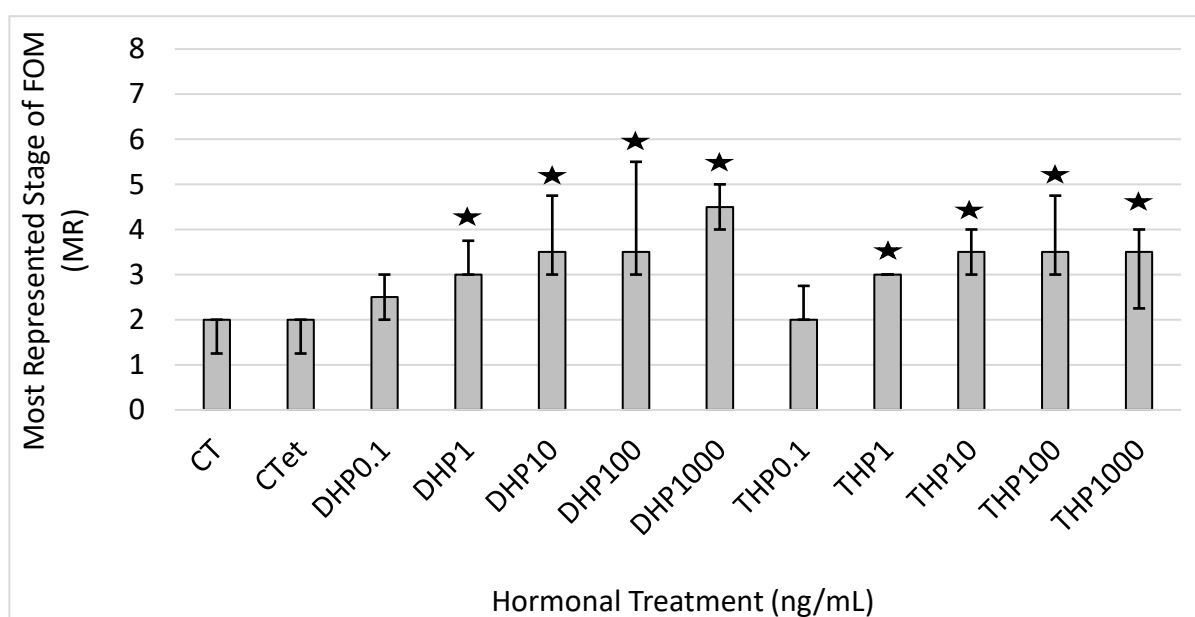
**Figure 2 The *in vitro* effect of DHP and PGE2 hormonal treatments on FOM advancement and ovulation with and without hCG.**

The treatments are the following: CT (control), hCG (hCG plus ethanol vehicle), CTet (ethanol vehicle only), PGE2 (prostaglandin E2; 10, 100 and 1000 ng/mL) and DHP ( $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one; 10, 100 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 (MR). The upper tip of the errors bar 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 were compared with the three negative controls: CT, hCG and CTet.

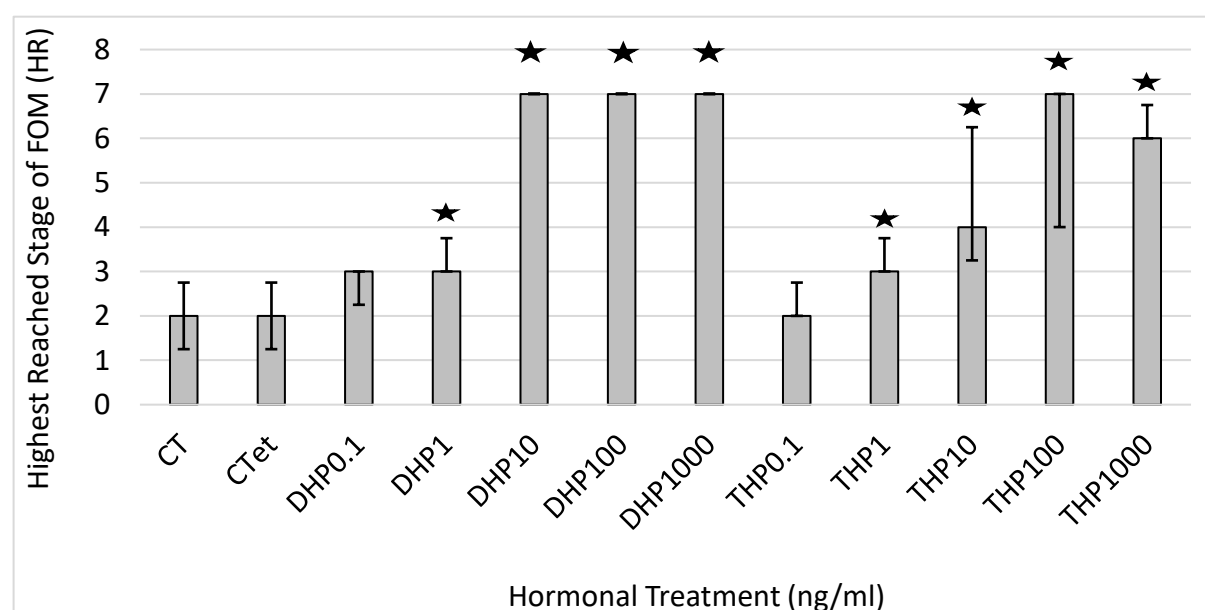


**Figure 3 The *in vitro* effect of the two progestagens (DHP and THP) hormonal treatments on FOM advancement.**

The treatments are the following: CT (control), CTet (ethanol vehicle only), DHP ( $17\alpha,20\beta$  dihydroxy-4-pregnen-3-one; 0.1, 1, 10, 100 and 1000 ng/mL), THP ( $17\alpha,20\beta,21$  trihydroxy-4-pregnen-3-one, 0.1, 1, 10, 100 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 errors bar 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.



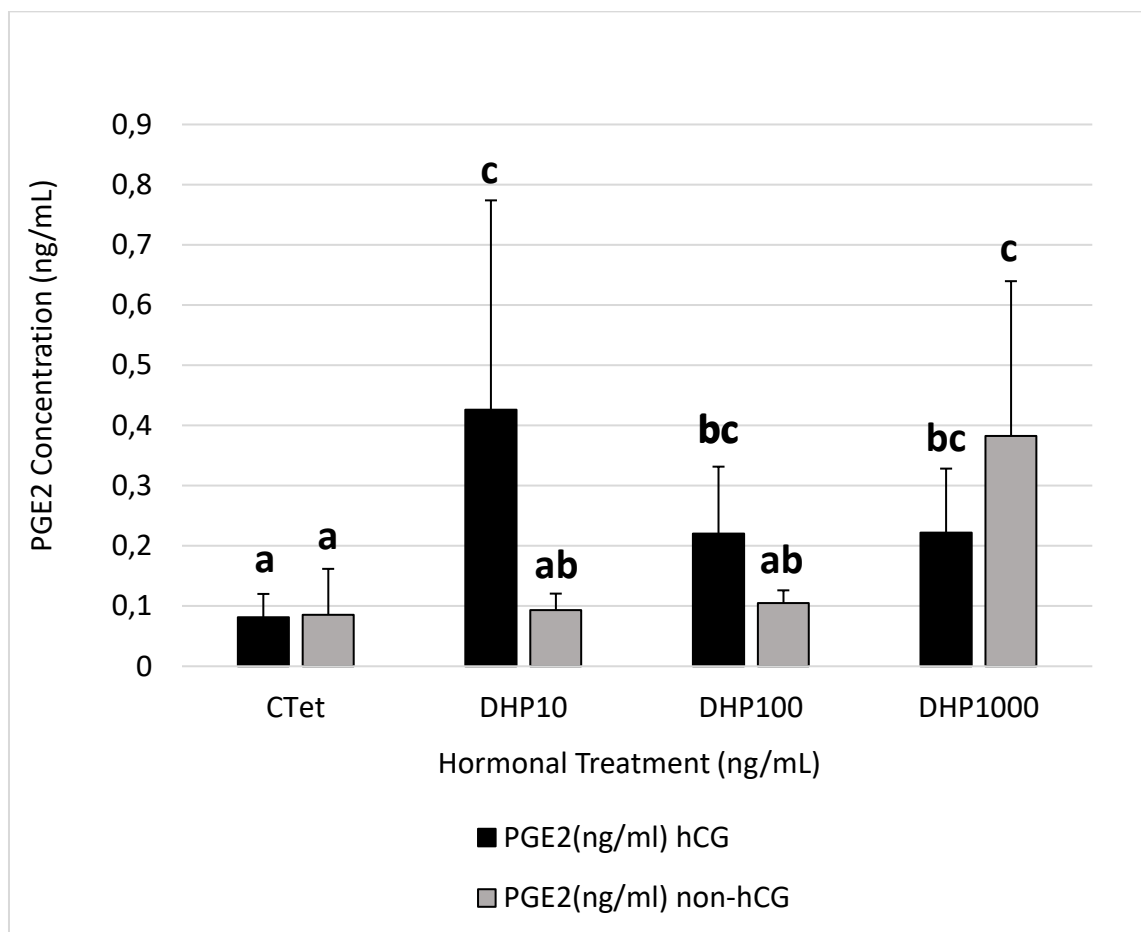
**a**



**b**

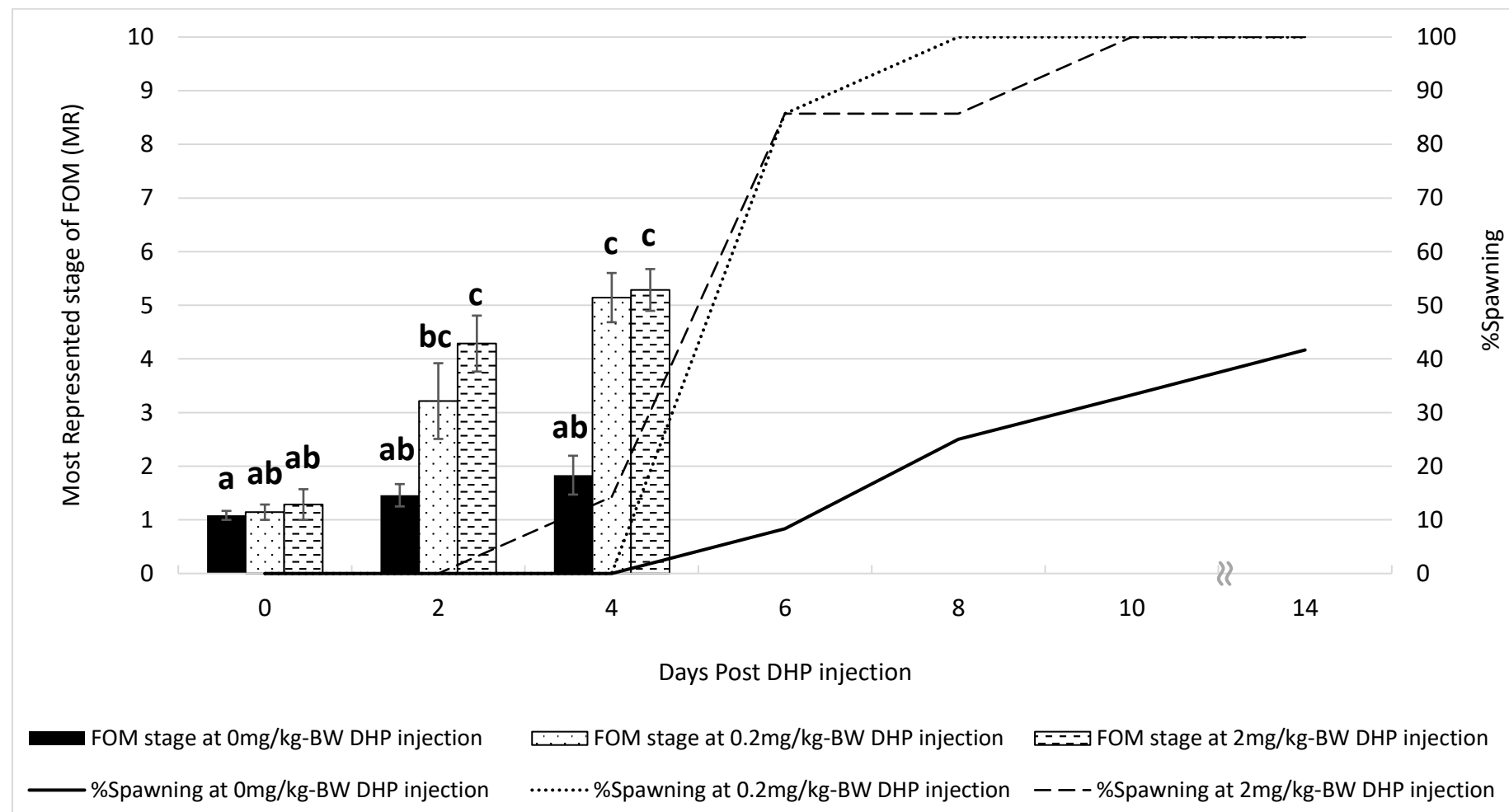
**Figure 4 The levels of PGE2 produced by follicles under the different DHP treatments with and without hCG.**

The treatments are the following: CTet (ethanol vehicle with or without hCG), DHP ( $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one; 10, 100 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 ( $P<0.05$ ) were 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 ( $17\alpha,20\beta$ dihydroxy-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 (MR) and (2) the spawning rate of fish under each treatment.





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## **8. Conflict of interest**

The authors declare that they have no conflict of interest.

## **9. Availability of data and material**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## **10. Code availability**

Not applicable.

## **11. Authors's contributions**

**Leila El Mohajer:** conceptualization, culture experiments, *in vivo* experiment, analysis, writing

**Rose Bulteau:** analysis

**Céline Chevalier:** *in vivo* experiment

**Sirine Selmi:** culture experiments

**Pascal Fontaine:** conceptualization

**Sylvain Milla:** conceptualization, culture experiments, *in vivo* experiment, writing

## **12. Ethics approval**

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: APAFIS15307-2018053110404570 and 23871-2020013017452359) by the local ethic committee in France (Name: CELMEA).

### ***“Major conclusions and up-coming approaches”***

The results in chapter 3 confirmed DHP as a true MIH in female Eurasian perch. Corticosteroids failed to induce, *in vitro*, a strong advancement in FOM by comparison to the advancement obtained with DHP treatments. The results in chapter 3 did not either reveal any effect for corticosteroids on the realized MIH (DHP) production by the maturing follicles (data were removed by referees of the FPB journal). The latter came in contrast to what has been published in the study of Mandiki and colleagues (2017) on the inhibiting effects of corticosteroids on steroidogenic activity of follicles at the start of their FOM (Mandiki et al., 2017). Despite that, we still recall the results in chapter 1 which exhibited elevations in plasma levels of some corticosteroids (11-D and cortisol) at FOM together with the information in chapter 2 assuming that corticosteroids can indirectly affect FOM. Recalling the latter information, a certain role for corticosteroids in FOM is not to be completely eliminated. **As such, the third raised working hypothesis (H3) assuming a role for corticosteroids in FOM is still not completely addressed.** Therefore, to further address the third working hypothesis (**H3**), the new question was postulated:

**“Do corticosteroids possess an effect on the activity of the realized MIH during FOM?”**

Starting from the above question, the same three different doses of the four corticosteroids (DOC, Cortisol, Corticosterone, 11-deoxycortisol) tested in chapter 3 were also tested in a second *in vitro* experiment in the following chapter (chapter 4). Instead that, this time the corticosteroids were tested in combination with each of the two revealed MIH (DHP and THP) at their two minimal effective doses (1 and 10 ng/ml). Following the latter treatments, corticosteroids effects on FOM, ovulation, and even PGE2 production by the MIH (DHP) were also tested in the following chapter.

# CHAPTER 4

## CHAPTER 4

**Chapter 4: Deeper *in vitro* investigation reveals a possible depressing activity for corticosteroids on the maturation inducing activity of DHP in female Eurasian perch**

***Perca fluviatilis* L.**

**(In preparation)**

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## Abstract

To improve the quality of reproduction in Eurasian perch, *Perca fluviatilis* L., a promising candidate for the freshwater aquaculture, investigating the hormones mediating and affecting the final oocyte maturation (FOM) is indispensable. Up to date, the progestagens 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (DHP) and THP or 20 $\beta$ -S (17,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one) were postulated to be a possible MIH in some perciforms. However, some corticosteroids have shown peaks in their concentrations which synchronized with the peri-ovulatory period and might signify the existence of a stimulatory or inhibitory role for these steroids in the FOM. As such, corticosteroids were tested in combination with the two MIH (DHP and THP) realized in our last *in vitro* investigation for the potent MIH in Eurasian perch. The results showed that most of the tested corticosteroids possessed a slight inhibitory effect on DHP effect as an MIH suggesting a negative effect for these steroids on FOM advancement. Therefore, the results of this study propose that the role of corticosteroids might be rather inhibitory of FOM advancement.

**Keywords:** Eurasian perch, *in vitro*, corticosteroids, Final oocyte Maturation, MIH, DHP.

## 1. Introduction

The maintenance of a healthy broodstock population is mandatory for attaining ideal fish productivity including egg production and spawning performances. Today some broodstock holding facilities implement protocols that manipulate both temperature and light in recirculating aquaculture systems (RAS) in order to control the reproductive cycle in seasonal fish (Fontaine et al., 2015). Such system also involves confining fish to a more restricted water body during which they experience frequent nettings and samplings as part of their maintenance routine. Such systems and practices thus lead to disturbances in fish reproductive performances by stimulating fish response to both acute and chronic stressors (Hamlin et al., 2008). This may be the case in percid fishes whose egg quality is highly variable among females (Żarski et al., 2012). Stress is commonly perceived by the hypothalamus and translated as corticotrophin-releasing hormone (CRH) into the pituitary. The pituitary in turn releases adrenocorticotrophic hormone (ACTH) into the blood stream which triggers the synthesis of corticosteroid hormones (Kiilerich et al., 2018). Environmental stress could thereby be reflected in the form of alterations of the physiological levels of these corticosteroids. Literature defines four major corticosteroids (cortisol, 11-deoxycortisol, corticosterone, and deoxycorticosterone) which are products of female fish gonadal steroidogenesis possessing gonadal receptors (Milla et al., 2009) thereby linking them to their possible reproductive roles. We therefore first emphasize on investigating such potential mechanisms whose disturbance could be at the origin of the reproductive drawbacks in most farmed fish today.

Eurasian perch, *Perca fluviatilis*, is one of the fish cultivated using intensive RAS with existing challenges related to attaining optimal egg quality and synchronisation of spawning (Fontaine and Teletchea, 2019; Rocha de Almeida et al., 2019). However, literature is still vague with regards to the exact roles for the four previously mentioned corticosteroids in female perch reproduction. Most records published today on other percids or teleosts emphasize on a possible

role for corticosteroids in the most critical stage for attaining eggs and spawning namely the Final Oocyte Maturation (FOM). In all teleosts, the release of mature oocytes from ovary, commonly known as ovulation, is preceded by FOM which had been previously characterized in female perch by Żarski et al. (2011) and involves a set of six maturational stages. During these stages the germinal vesicle (encompassing maternal genetic material) migrates gradually until reaching the edge of the oocyte where it is breakdown in a phenomenon known as Germinal Vesicle Breakdown (GVBD) (Żarski et al., 2011). The whole FOM is known to be under specialized control by a hormone defined as the Maturation Inducing Hormone (MIH) (Migaud et al., 2003). Several *in vitro* receptor-binding affinity and direct MIH activity investigation assays together with *in vivo* plasma assessment of corticosteroids levels strongly supported the involvement of certain corticosteroids in FOM possibly as MIH (El Mohajer et al., 2021a). However, our *in vitro* study conducted on mature female perch confirmed that the progestogens DHP and THP are more likely to be the potent MIH in perch (El Mohajer et al., 2021b). However, when referring to the significant elevations in the plasma levels of most corticosteroids in mature female perch around FOM and spawning (chapter 1 unpublished data), inquiries about the possible roles behind such plasma elevations around FOM is raised again. Also, there had been one study in perch where corticosteroids as cortisol and DOC were found to drastically *in vitro* depress the steroidogenic capacities of late vitellogenic oocytes, notably DHP production (Mandiki et al., 2017). According to the existing literature, it is thus unclear whether corticosteroids display positive or negative effects on the physiological processes linked to FOM. To address this question we thereafter investigate, using the same *in vitro* approach, the effect of these corticosteroids on the maturation inducing activity of the two MIHs (DHP and THP) previously realized by our study in order to find out whether corticosteroids would affect the activity of the MIH in inducing a full advancement in FOM until ovulation (El Mohajer et al., 2021b).

## **2. Materials and Methods**

### **2.1. Experimental animals and rearing conditions**

A group of 2 years-old female Eurasian perch was used with an average weight of 560.81 g, standard length of 28.9 cm and GSI of 23.2%. Fish were obtained from Asialor company (Dieuze, France), were transferred to the Aquaculture Experimental Platform (AEP, registration number for animal experimentation C54-547-18) belonging to the URAFPA lab of the University of Lorraine (Nancy, France). In these facilities, 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 (Abdulfatah et al., 2013) during which 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 a spectrophotometer and remained always below 0.8 mg/L. Fish had been fed to satiety with artificial feed supplied from Le Gouessant from the initiation of the program until the FOM start.

### **2.2. Chemicals**

All steroids were purchased from SigmaAldrich (France). Cortland medium was used as the medium 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> 6 H<sub>2</sub>O, 0.23 MgSO<sub>4</sub> 7H<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 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. Fish follicles preparation**

Each female was anesthetized (MS-222; Sigma-Aldrich, Lyon, France), catheterized, using a catheter (DAHLHUUSEN; 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 Zarski et al. (2011). 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., 2011). The oocyte maturation stage was then evaluated under binocular microscope, magnification 4× (OLYMPUS, SZX7). Fish proven to be at stage 1 or 2 were immersed in a bath containing 300 mg/L of the aesthetic 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 pre-maturational follicles attached to the surrounding extrafollicular tissue (Henrotte et al., 2011).

### **2.4. *In vitro* ovarian Follicles Incubations**

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 µl of dissolved hormonal treatment in ethanol was added in the culture medium just before adding the follicles.

Three doses of each of the four corticosteroids (11-deoxycortisol or 11-D, cortisol or Co, corticosterone or CoS and 11-deoxycorticosterone or DOC) were tested in combination with each of the two progestogens DHP and THP at each of their two minimal active maturation inducing doses recently revealed by our study. We used the same latter study to choose our three desired doses of each corticosteroid (El Mohajer et al., 2021b). The desired doses used to prepare each hormonal treatment (in ng/mL) are presented in Table 1.

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

2. CTet: Cortland medium plus 10  $\mu$ L standard Ethanol
3. CT: Cortland medium alone

After the addition of the follicles to all wells containing the culture media and the appropriate dose of each hormonal treatment, all plates were incubated at 13°C for 62h 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., 2011). The media were replaced once after 18h with new experimental media and the treatments were renewed as well. Media after 62h (end of incubation) were placed in Eppendorf tubes to be stored at -20°C for later steroidal ELISA assays.

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

After 62h 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 minutes, the oocytes were observed under the binocular to monitor for each well, following the same principle as in our previous study (El Mohajer et al., 2021b) and the classification of Zarski et al. (2011):

- 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).
- 3-Ovulation (represented by counting the number of ovulated follicles) with expulsion of the oocytes from the follicular layers.

## **2.6. Hormone assays following the *in vitro* incubations**

Levels of PGE2 were assayed on 100 µl of the incubation culture of only the treatments which showed significant effect on ovulation. The assay was performed using PGE2 ELISA Kit (KHL 1701) obtained from ThermoFisher scientific (Illkirch-Graffenstaden, France). Sensitivity was 31.3 pg/mL. Intra-assay coefficient of variation (CV) was less than 10% and inter-assay coefficient was less than 5%. The ELISA was 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.

## **2.7. Statistical analysis**

After checking the data normality and the homogeneity of variances, when 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. The comparison of *in vitro* ovulation rates was performed using khi2 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 Bonferroni or Benjamini & Hochberg. Free software R version 3.3.1 was used. In case of parametric tests, the data showed the Mean $\pm$ SD while the data showed Median $\pm$ quartiles for non-parametric tests.

## **3. Results**

### **3.1. Hormonal treatment effects on FOM advancement and ovulation**

When considering the most represented stages of FOM, only the combination of DHP at its minimal MIH active dose of 1 ng/ml with corticosterone at each of its implemented doses of

0.1, 1, and 10ng/ml slightly yet significantly ( $p<0.05$ ) depressed FOM advancement by comparison to DHP effect alone (1ng/ml) (Figure 1). However, the latter finding was not realized when considering the highest reached stages of FOM where no significant effect was realized for combining any of the corticosteroids with DHP at 1 ng/ml (results not shown). All additions of corticosteroids (except for 11-D at its lowest tested dose of 10ng/ml) to DHP (1 ng/ml) managed to strongly depress ovulation by comparison to DHP (1 ng/ml) alone which induced 100% ovulation (Figure 1).

When combining DHP at a higher dose of 10ng/ml with each of the corticosteroids, all corticosteroids (except for corticosterone), at all their three doses, revealed a significant ( $p<0.05$ ) depressing effect on FOM advancement by comparison to DHP (10 ng/ml) effect alone when only considering the most represented stages of FOM (Figure 2). Such significant effect was not at all realized for any of the combined treatments when considering the highest reached stages of FOM where they induced similar FOM advancement to DHP (10 ng/ml) alone (results not shown). Concerning ovulation, none of the corticosteroids were able to prominently affect ovulation by comparison to DHP (10 ng/ml) individual effect in inducing ovulation (Figure 2).

No significant effect on FOM advancement was revealed when combining the corticosteroids with THP at either of its implemented active MIH doses of 1 or 10 ng/ml when considering either most represented or highest reached stages by follicles. The corticosteroids combined with THP (at both doses) were able to induce FOM advancement and ovulation to a similar extent as THP alone.

### **3.2. Hormonal treatment effects on PGE2 production**

PGE2 levels were measured in the medium culture after the incubation and showed extreme inter-individual variability for some treatments. Despite that, PGE2 levels were found to be significantly ( $p<0.001$ ) depressed in culture media subjected to the combination of 11-

Deoxycortisol (at each of the three implemented doses of 10, 100, and 1000 ng/ml) with DHP (1 ng/ml). When combining this dose of DHP with cortisol at its two doses of 10 and 100 ng/ml, the same depressing effect was significantly realized ( $p < 0.01$ ). Such effect was also observed for the other two corticosteroids corticosterone and DOC but only at their lowest implemented dose of 0.1 ng/ml ( $p < 10^{-4}$ ) and 1 ng/ml ( $p < 0.01$ ) respectively (Figure 3).

As we did not have an important effect for the other treatments on ovulation, PGE2 was not measured in the other culture media.

#### **4. Discussion**

We have previously confirmed using both *in vitro* and *in vivo* assays, that DHP is the true MIH in female Eurasian perch with THP activity being tested and realized only *in vitro* (El Mohajer et al., 2021b). Using the same *in vitro* approach, we tested the effect of combining each corticosteroid with each of the two realized MIHs (DHP and THP). At its minimal active dose (1 ng/ml) for FOM induction (El Mohajer et al., 2021b), DHP maturation inducing activity in most follicles was significantly but slightly depressed only when treated with a combination of corticosterone and DHP. Conversely, when increasing the dose of DHP to 10 ng/ml, all corticosteroids (at all their implemented doses), except corticosterone, were able to significantly but mildly depress DHP activity in inducing a full FOM advancement. A MIH usually binds to the cellular MIH receptors expressed on oocyte plasma membranes (Lubzens et al., 2010). The first time different steroidal potencies to MIH membrane receptors had ever been investigated was in 1990 by Patiño and Thomas who realized that corticosteroids as cortisol, 11-D, and DOC do possess the ability to bind to membrane MIH receptor and sometimes even compete with the progestogens over such receptor (Patiño and Thomas, 1990). However, many elements of the mechanisms involved remains to be understood. First, these corticosteroids are supposed to be agonists of the MIH receptors (Patiño and Thomas, 1990; Sugimoto et al., 2016) while their depressing effects on the DHP activity revealed by our study rather support antagonistic

activities. Second, this depressing effect contradicts with their slight but significant stimulatory effect on the FOM advancements when administered alone (El Mohajer et al., 2021b). Third, the ability of corticosteroids to disrupt DHP action was apparently dependent on the dose of DHP implemented with a strikingly stronger effect to depress DHP with a higher dose of the latter progestogen.

Usually, corticosteroids bind to nuclear receptors after traversing the cellular membrane to execute their functions (Faught and Vijayan, 2016; Prunet et al., 2006). Still, the increase in hepatocytes plasma membrane rigidity is detected following the *in vitro* treatment of hepatocytes by cortisol in rainbow trout (Dindia et al., 2012). The latter finding implies that *in vitro* treatments with corticosteroids could induce rigidity of plasma membranes. With an increase in the rigidity of the plasma membrane, we assume that corticosteroids diffusion through plasma membrane would be more limited. The latter would perhaps result in the preferred binding of corticosteroids to membrane MIH receptors. Perhaps in our previous study, the *in vitro* treatment with the corticosteroids alone induced the same *in vitro* rigidity in the plasma membrane as observed in hepatocytes of rainbow trout making it easier for the corticosteroids to bind to membrane MIH receptors by default. Activation of the MIH receptor rather than the corticosteroid nuclear receptor, with the addition of corticosteroids alone, would thus induce the slight advancement of FOM. After all, activation of MIH receptor provokes the ultimate mediation of intracellular mechanism leading to FOM initiation and induction (El Mohajer et al., 2021a). In our current study, the combined treatment of corticosteroids with DHP could have as well induced rigidity of plasma membrane allowing corticosteroids to rather bind to the membrane MIH receptors. Using low DHP levels (1 ng/ml), it was perhaps still possible for corticosteroids (except corticosterone) to slightly outcompete DHP to such receptors and hence follow the same stimulatory effect *via* MIH receptors after the failure of DHP to saturate the MIH receptors at such low levels (Figure 4.a). On the other hand, when

using a higher dose of DHP (10 ng/ml), DHP would have been more able to saturate the MIH membrane receptors. Consequently, with such hypothesis, corticosteroids (except corticosterone) would be left to traverse the rigid plasma membrane and classically bind to their nuclear receptors possibly stimulating a rather depressive effect on FOM (Figure 4.b). Perhaps the binding to such nuclear receptors signals the phosphorylation of Serine threonine kinase (PKA) in hepatocytes (Faught and Vijayan, 2016) possibly triggering the PKA-mediated inhibition of MPF that eventually leads to slight inhibition of FOM (El Mohajer et al., 2021a). These corticosteroids were nevertheless unable to affect DHP-induced ovulation at a DHP dose of 10 ng/ml but were able to strongly depress DHP-induced ovulation at the lower dose of DHP (1 ng/ml) at which they did not inhibit FOM. We have previously shown that FOM advancement and ovulation could act as two quite independent phenomena where follicles at unadvanced stages of FOM can be unreluctantly ovulated (El Mohajer et al., 2021b). As such, the corticosteroid effects on DHP-induced FOM which seems to contradict with their effects on ovulation can be somehow explained as two independent phenomena in which the corticosteroids effect on FOM advancement doesn't necessarily have to be linked to ovulation success. When interpreting the effects of corticosteroids on DHP-induced PGE2 production, we realize a significant depressing effect for corticosteroids on PGE2 production by DHP at 1 ng/ml. Additionally, PGE2 can simply bind to its receptor previously detected in perch (Henrotte et al., 2011) following its production. This could imply that corticosteroids depress DHP-induced ovulation *via* affecting the production of PGE2 by DHP treated follicles rather than affecting PGE2 action itself. However, we have previously suggested that *de novo* PGE2 production by oocytes following DHP treatment can only slightly contribute to ovulation success (El Mohajer et al., 2021b). This could only mean that corticosteroids, by affecting PGE2 production, probably mask such slight contribution of PGE2 to ovulation. The latter thus leads

to the observed depression in ovulation success when DHP treated follicles are co-treated with corticosteroids.

In conclusion, our results propose a potential effect for most corticosteroids on the maturation inducing activity of DHP. We also assume that the latter effect could be dependent on both type of the receptor (MIH receptor or CR) and DHP dose implemented. Our results revealing possible effects for such stress-sensitive steroids on the two most critical mechanisms affecting the success and quality of spawning in fish would somehow be able to explain why stressed female broodstock display lower reproductive performances. Nevertheless, due to the high biological variations in our fish, especially with regards to the PGE2 levels, we highly recommend that this experiment is repeated to reflect more accurate results. We additionally propose the use of CR antagonists to confirm the involvement of CR in the above proposed effects of corticosteroids. Once the effect of corticosteroids is confirmed *in vitro*, it would be crucial to test the activity of corticosteroids *in vivo* while monitoring the advancement of FOM in live fish and the quality of produced eggs following their spawning.



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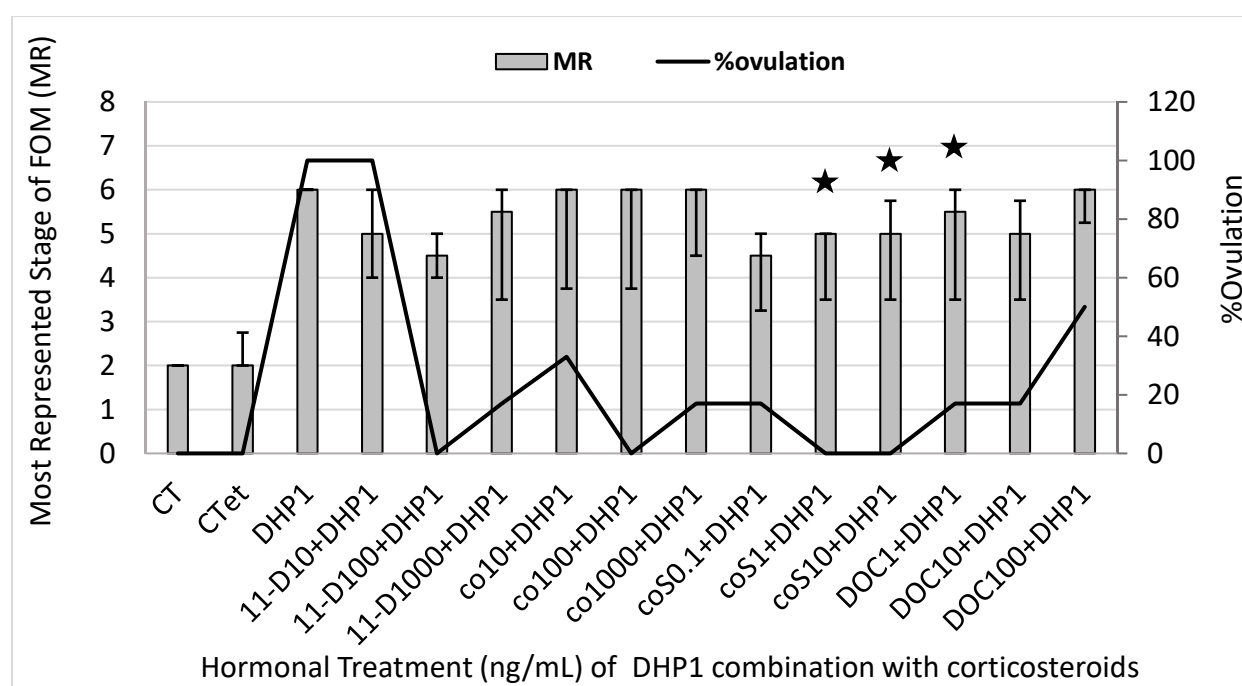
## 6. Tables and Figures

**Table 1 Hormonal doses (ng/mL) tested.**

MIH	DHP 1 ng/ml			DHP 10 ng/ml			THP 1 ng/ml			THP 10 ng/ml		
Doses ng/mL	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3
<b>11-deoxycortisol (11-D)</b>	10	100	1000	10	100	1000	10	100	1000	10	100	1000
<b>Cortisol (Co)</b>	10	100	1000	10	100	1000	10	100	1000	10	100	1000
<b>Corticosterone (coS)</b>	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10
<b>11-deoxycorticosterone (DOC)</b>	1	10	100	1	10	100	1	10	100	1	10	100

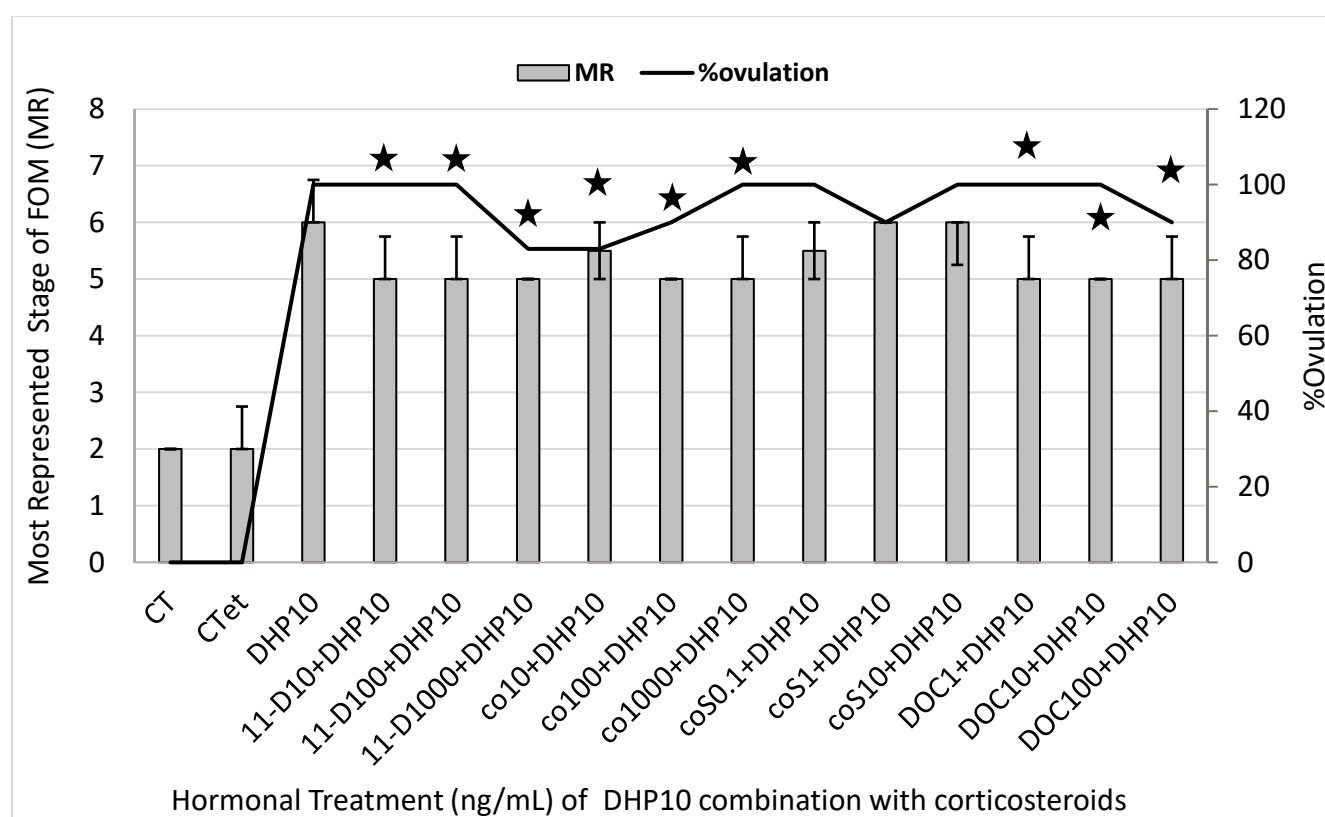
**Figure 1** The *in vitro* effect the different corticosteroids treatments with DHP (1 ng/ml) on ovulation and FOM advancement by most represented follicles (30-45 follicles/fish, 6 fish).

The treatments are the following: CT (control), CTet (ethanol vehicle only), DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one) at 1ng/ml alone and in combination with each of the following corticosteroids: 11-D (11-deoxycortisol at doses; 10 ng/ml, 100 ng/ml and 1000 ng/ml), Co (cortisol at doses; 10 ng/ml, 100 ng/ml and 1000 ng/ml), coS (corticosterone at doses; 0.1 ng/ml, 1 ng/ml and 10 ng/ml), DOC (11-deoxycorticosterone at doses; 1 ng/ml, 10 ng/ml and 100 ng/ml). The values recorded represent the median of the stages reached by the biggest population of follicles thereby representing the most represented stage (MR). 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 positive control DHP1, and statistically different values ( $P < 0.05$ ) are marked by stars. The ovulation rate obtained under the different hormonal treatments was also compared with the positive control DHP1 ( $P < 0.05$ ).



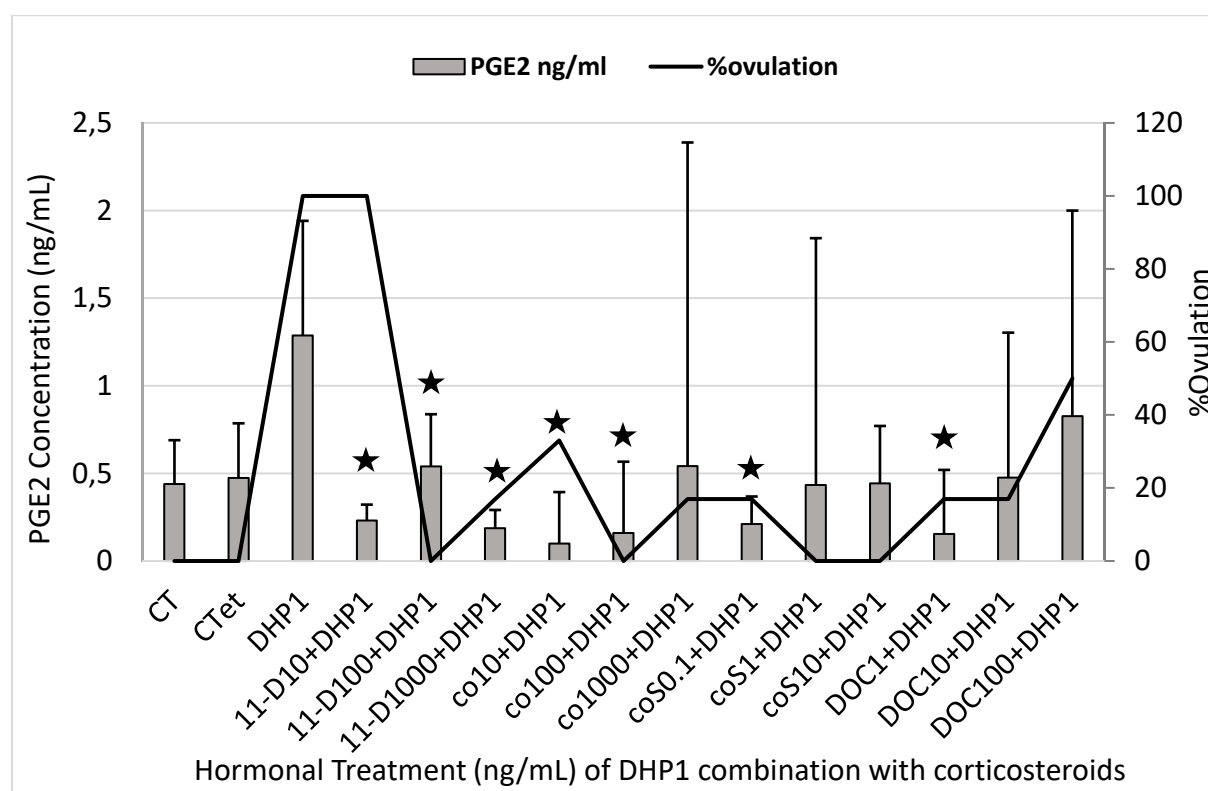
**Figure 2** The *in vitro* effect the different corticosteroids treatments with DHP (10 ng/ml) on ovulation and FOM advancement by most represented follicles (30-45 follicles/fish, 6 fish).

The treatments are the following: CT (control), CTet (ethanol vehicle only), DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one) at 10ng/ml alone and in combination with each of 11-D (11-deoxycortisol at doses; 10 ng/ml, 100 ng/ml and 1000 ng/ml), Co (cortisol at doses; 10 ng/ml, 100 ng/ml and 1000 ng/ml), coS (corticosterone at doses; 0.1 ng/ml, 1 ng/ml and 10 ng/ml), DOC (11-deoxycorticosterone at doses; 1 ng/ml, 10 ng/ml and 100 ng/ml). The values recorded represent the median of the stages reached by the biggest population of follicles thereby representing the most represented stage (MR). 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 positive control DHP10, and statistically different values ( $P < 0.05$ ) are marked by stars. The ovulation rate obtained under the different hormonal treatments was also compared with the positive control DHP10.



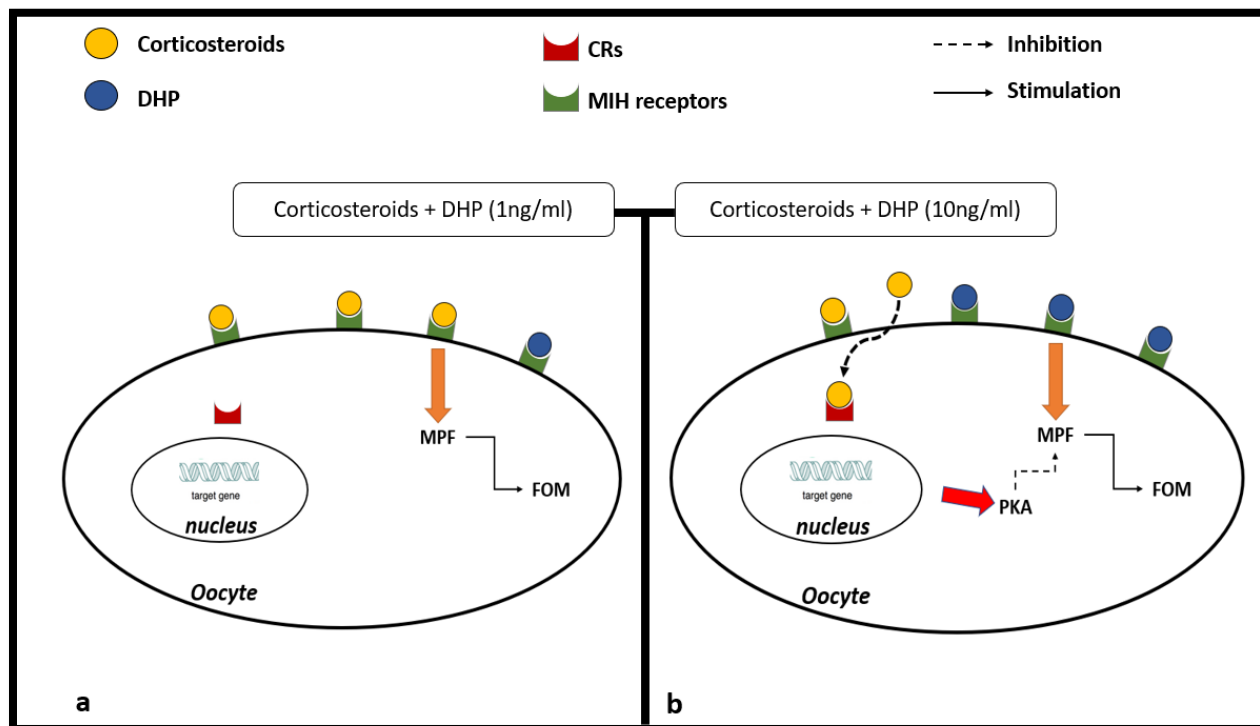
**Figure 3** The *in vitro* effect the different corticosteroids treatments with DHP (1 ng/ml) on ovulation and PGE2 production (ng/ml) by follicles (30-45 follicles/fish, 6 fish).

The treatments are the following: CT (control), CTet (ethanol vehicle only), DHP (17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one) at 1ng/ml alone and in combination with each of the corticosteroids: 11-D (11-deoxycortisol at doses; 10 ng/ml, 100 ng/ml and 1000 ng/ml), Co (cortisol at doses; 10 ng/ml, 100 ng/ml and 1000 ng/ml), coS (corticosterone at doses; 0.1 ng/ml, 1 ng/ml and 10 ng/ml), DOC (11-deoxycorticosterone at doses; 1 ng/ml, 10 ng/ml and 100 ng/ml). The values recorded represent the median of PGE2 levels produced under different hormonal treatments. The upper tip of the error bars represents the third quartile (Q3), and the lower tip represents the first quartile (Q1). PGE2 levels produced under corticosteroids hormonal treatments were compared with the positive control DHP1, and statistically different values ( $P < 0.05$ ) are marked by stars. The ovulation rate obtained under the different hormonal treatments was also compared with the positive control DHP1.



**Figure 4** The schematic representation explaining the possible receptor mediated effect of corticosteroids on DHP based on the implemented dose of DHP (1 or 10 ng/ml).

17 $\alpha$ ,20 $\beta$ dihydroxy-4-pregnen-3-one (DHP)/ Maturation inducing hormone receptors (MIH receptors) – Corticosteroids receptors (CRs)– Maturation Promoting Factor (MPF) – Final Oocyte Maturation (FOM) – Serine threonine kinase (PKA).



# **GENERAL DISCUSSION**



## GENERAL DISCUSSION

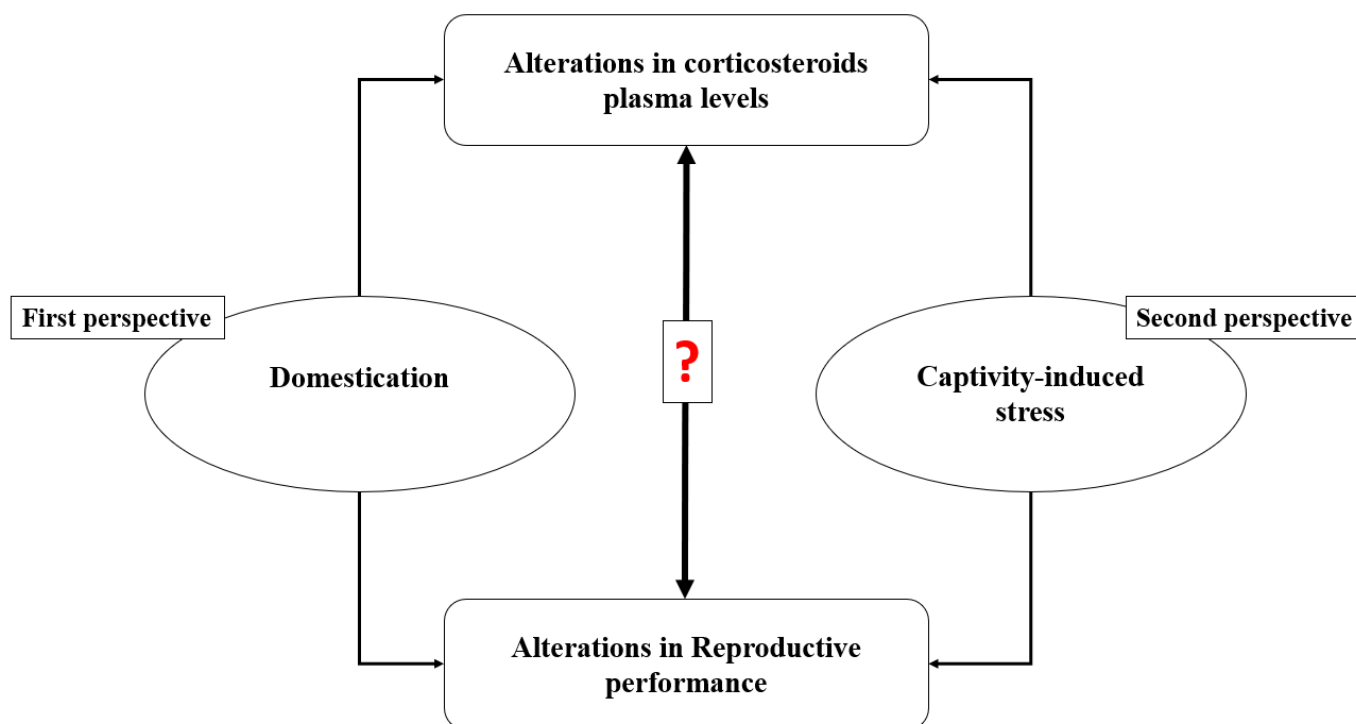
Aquaculture is one of the most growing food industries in the world today where it has been the driving force behind the growth in sea-food industry (Toner, 2015). However, 85% of the world's production of finfish has come from farming of only 15 species in numerous countries (Teletchea and Fontaine, 2014). To increase the contribution of aquaculture to the world's fish production, farming of new fish species appears to be crucial. Eurasian perch, *Perca fluviatilis*, is one of the newly introduced and highly valuable fish species that can serve in the diversification of European freshwater aquaculture (Fontaine and Teletchea, 2019). The growing interest by anglers and consumers at the European scale to increase its production year-round encouraged its out-of-season spawning (Szczerbowski et al., 2009). To accomplish out-of-season spawning in Eurasian perch, its intensive cultivation using controlled environments in RAS has been suggested (Fontaine and Teletchea, 2019; Pimakhin et al., 2015). Indeed, RAS can allow the control of environmental factors (e.g. water temperature, photoperiod, water quality) to obtain out-of-season spawning in Eurasian perch (Abdulfatah et al., 2013; Pimakhin et al., 2015). Along the domestication of perch using such environmentally controlled systems, asynchrony in spawning among different fish is observed. In addition, farming of Eurasian perch using environmentally controlled systems fastens their gonadal development with deteriorations in fecundity, spawning quality, egg hatching rate, and gamete quality (Khendek et al., 2017; Křišť'an et al., 2012; Migaud et al., 2004; Rocha de Almeida et al., 2019). Wang et al. (2003) linked such changes in fish reproductive performance to the high levels of cortisol observed following the fish adaptation to environmentally controlled conditions in RAS. Corticosteroids, mainly cortisol, are known to be the main mediators of a stress response in fish where their release results from stimulation of HPI (hypothalamic-pituitary-interrenal) axis following a stressful situation (Bruijn and Romero, 2018; Kiilerich et al., 2018). When newly introducing fish to a human controlled environment (RAS), stressing of these fish would result

from failure to mimic the natural environmental cues in artificially controlled systems (Milla et al., 2020). However, fish possess the ability to eventually adapt to a stressful human controlled environment during the process of domestication. This process is nevertheless accompanied by changes in animal morphology, physiology and behavior with gradual transformation process over many generations (Douxflis et al., 2011). As such, studies assessing the effect of human controlled environments as RAS on the reproductive performance of Eurasian perch usually analyze two populations differentiated by their number of generations spent in captivity; (1) previously domesticated fish population that has been domesticated over several generations (e.g. F7+), and (2) another population that has been much more recently introduced to RAS (Khendek et al., 2017; Křišť'an et al., 2012; Migaud et al., 2004; Rocha de Almeida et al., 2019). When the reproductive performance was monitored along cortisol plasma variations in some of the latter studies, cortisol plasma levels were also observed to be highly altered in domesticated fish (Khendek et al., 2017). This came in agreement with an older study on juvenile perch which revealed that cortisol rise was significantly higher in more recently domesticated fish by comparison to fish domesticated for longer generations (F4) (Douxflis et al., 2011). The latter information would thereafter suggest that captive perch would exhibit much less deleterious rises in cortisol levels resulting from culture stressors along their domestication process. However, perch that have been domesticated for several generations (e.g. F7+) still exhibit lower reproductive performances by comparison to more recently domesticated populations (Khendek et al., 2017; Křišť'an et al., 2012; Rocha de Almeida et al., 2019). The drop of reproductive capacity along the domestication process puts into question the adaptative success of perch in captivity. In all cases, the domestication process appears to be accompanied by both alteration of reproductive capacity and drop of corticosteroids levels.

Considering the well-known effects of corticosteroids on reproductive success in some other teleosts (Milla et al., 2009), we may hypothesize, from one perspective, that both phenomenon

are linked to explain the deteriorations in reproductive performance in domesticated female Eurasian perch. To address this question, it is crucial to first of all define the roles of corticosteroids in the reproductive processes of captive Eurasian perch.

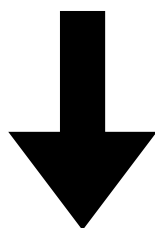
In captivity, fish are subjected to a range of stressors linked to husbandry practices and the artificial control of environmental conditions. The poor reproductive performances observed in domesticated fish imply that increased levels of domestication would not absolutely reduce the sensitivity of domesticated fish to culture practices. After all, stressors resulting from the indispensable frequent handling of fish in captivity would strengthen the disruption of reproductive success even if domestication is running (Milla et al., 2020). Thus, from an additional perspective, we should also understand the exact role of stress-induced corticosteroids in reproduction to understand the persisting effect of such stressors on reproductive performance of Eurasian perch in culture systems as RAS (Figure 15).



**Figure 15: Illustration of the two perspectives proposing a link between corticosteroids and reproduction.**

Starting from the above two perspectives, we hypothesize the existence of a potent role for four major corticosteroids (11-deoxycorticosterone, 11- deoxycortisol, corticosterone and cortisol) in the reproductive cycle of female Eurasian perch. The latter could explain the observed reproductive drawbacks in captivity. The main bottleneck is that no study has ever investigated in parallel the physiological roles of the above four corticosteroids in the oogenesis of Eurasian perch. In fact, no study has ever exhibited the basal plasma levels of these corticosteroids along the whole reproductive cycle of female Eurasian perch.

**Our objective behind this thesis is thus to investigate the role of corticosteroids using Eurasian perch reared under RAS conditions**



**In order to achieve our goal in getting closer to detecting the putative roles of corticosteroids in female Eurasian perch reproduction**

We first proposed to monitor the plasma kinetics of the four major corticosteroids while monitoring the relative gene expression of their different gonadal receptors and metabolic enzymes along the reproductive cycle

Second, we aimed to further investigate the direct role of corticosteroids at the time of their plasma rise using *in vitro* follicle culture assays

## **Plasma kinetics of corticosteroids and the relative gene expression of the different corticosteroids receptors (CRs)**

In chapter 1, cortisol was revealed to be the most elevated corticosteroids in female perch. The other corticosteroid 11-Deoxycortisol (11-D) also exhibited high levels as had been observed in other studies on female perch (Noaksson et al., 2005), with its precursor (DOC) detected only at mild levels. Such mild levels were nevertheless similar to the levels detected in other mature female teleosts (Milla et al., 2009) which implies that DOC would not a minor corticosteroid during the reproductive cycle of female perch. Corticosterone was considered to be the least detectable corticosteroid with very low levels (0.03-0.08 ng/ml). Corticosterone has ever been detected in low levels in female winter flounder (0.3 ng/ml) (Campbell et al., 1976) and is rather measured in elasmobranch fishes (sharks, skates and rays) (Manire et al., 2007). In all cases, we may say that our study originally shows the four corticosteroids within detectable levels in mature female Eurasian perch with only cortisol, 11-D and DOC reflecting significant kinetics along the reproductive cycle. Our study in chapter 1 was hence able to suggest the implication of cortisol, 11-D and DOC in female perch reproduction with no emphasis given to corticosterone due to its insignificant resting kinetics.

At the beginning of the reproductive cycle, DOC and 11-D exhibited high plasma levels as opposed to their significantly lower levels around early and mid-vitellogenesis. The latter finding suggests a role for the two hormones in early stages of oogenesis which is supported by the highest relative expression of both gluco- and mineralocorticoid receptors around the onset of the reproductive cycle (chapter 1). However, there is no study reporting rigorously higher level of 11-D or DOC at the onset of oogenesis. In immature perch, DOC plasma levels are around 1-3 ng/ml (Mathieu et al., 2013), a range which is similar to our recorded levels at the early oogenesis. Regarding 11-D, the plasma levels in wild Eurasian perch are recorded at basal levels during the onset of oogenesis (Noaksson et al., 2004). To our knowledge, we thus report,

for the first time, higher levels of DOC and 11-D plasma concentrations during the first step of fish oogenesis. Such gonadal development could refer to the proliferation of oogonia responsible for the renewal of the stocks of young oocytes and follicles which takes place for the first time at sexual maturity and later at the start of each oogenesis (Lubzens et al., 2010). Milla et al. (2009) reviewed that cortisol might be involved in the stimulation of fish puberty by enhancing pituitary gonadotropins at the onset of oogenesis in some species (Crim et al., 1981; Dufour et al., 1983; Huang et al., 1999; Milla et al., 2009). In captive Eurasian perch, DOC and 11-D elevation at the onset of oogenesis could be thus related to the previously reported effects of corticosteroids on pituitary gonadotropins (LH and FSH). As such, we preserve the possibility that such corticosteroids might be indirectly controlling some biological mechanisms linked to the onset of oogenesis (Brann and Mahesh, 1991). There have been reports on the effects of introducing cortisol implants on gonadal development and oocyte formation in several fish as rainbow trout and Mozambique tilapia *Oreochromis mossambicus* (Clearwater and Pankhurst, 1997). As far as it could be, the two deoxycorticosteroids in our study might induce the same effects. To investigate that question, we could introduce DOC and 11-D implants into Eurasian perch broodstock at their sexual rest. Such introduction would be just before the resumption of a new cycle and its impacts should be later assessed on oocyte formation and proliferation.

Early gonadal development stages could also include the early growth stages during which oocytes are in their cortical alveoli stage. As such, we had also hypothesized the existence of a role for both corticosteroids (DOC and 11-D) during cortical alveolar stage as has been suggested for cortisol in the female freshwater fish *Notopterus notopterus* (Shankar and Kulkarni, 2006). However, the plasma levels of corticosteroids in our captive Eurasian perch populations stabilize or even start to decline at the cortical alveolar stage, which do not support their strong involvements during that period. Cortisol reported effects on E2 could also help in

anticipating how corticosteroids might affect cortical alveolar stage. In fact, E2 production during follicular growth is not only linked to its role during vitellogenesis where it has been also linked to its role in the formation of cortical alveoli during cortical alveolar stage (Faught and Vijayan, 2018). In Eurasian perch, E2 appears to play the same role during cortical alveolar stage where we observe a strong increase in its plasma levels starting at the end of this stage. This increase was observed at the time where the levels of both deoxycorticosteroids start to drop. If comprehended this way, both deoxycorticosteroids might possibly downregulate the production of E2 as has been reported for cortisol on E2 production (Berg et al., 2004; Mandiki et al., 2017). Considering the assumed indispensable role for E2 in the formation of cortical alveoli, a physiological urge for the downregulation in both deoxycorticosteroids would be justified. The latter interpretation would more importantly suggest a role for both deoxycorticosteroids in the downregulation of E2 perhaps at periods of the reproductive cycle where E2 secretion is not desired. In this way, a disturbance in corticosteroids levels due to the persisting captivity-induced stressors might probably impact this oogenesis stage. To elucidate that, we propose to apply chronic stressors at this stage and to measure the consequences on the E2 and corticosteroid levels and on the progress from early cortical alveolar stage to the early vitellogenesis stage.

We have eliminated the possibility for a potent role for any of the corticosteroids around early and mid-vitellogenesis due to the significantly lower plasma levels of the two deoxycorticosteroids (DOC and 11-D) and the maintained relatively low levels of cortisol around these periods. However, the three corticosteroids initiated a rise in their plasma levels starting from late vitellogenesis with that of DOC and 11-D being a bit more prominent than that of cortisol. Such rise reached a constant level for DOC while it continued until its highest significance around FOM and spawning for 11-D and cortisol. Our results in chapter 1 thus allowed us to assume the possible initiation of a certain function for the three corticosteroids

(DOC, 11-D, and cortisol) during late vitellogenesis and an even more prominent function for 11-D and cortisol during FOM and spawning. Additionally, our result suggests the possibility of preserving cortisol in its active form where the relative gene expression of the enzyme 11 $\beta$ HSD2, transforming cortisol into its inactive form cortisone, was relatively low during FOM and spawning. The latter result further supports the existence of a role for cortisol in FOM and spawning. Consequently, we conducted a series of *in vitro* experiments during late vitellogenesis and FOM where the direct effects of the corticosteroids were further investigated.

### **Investigating the direct roles for corticosteroids during late vitellogenesis**

As in most oviparous species, vitellogenesis is the period during which egg-yolk, the nutritional reserve for the later-on growing embryo, is produced and continues to accumulate in the developing oocytes during late vitellogenesis (Jalabert, 2005). Our conducted *in vitro* assay testing the effects of the four corticosteroids during late vitellogenesis in chapter 1 allowed us to assume a role for DOC and 11-D on yolk-globules coalescence. We also proposed that the latter effect could be physiological after realizing that the significant increase in egg-yolk globules coalescence observed *in vivo* coincides with the slight start in the rise of the plasma levels of both deoxycorticosteroids from early to late vitellogenesis. A limitation to such hypothesis would be that the exhibited rises in plasma levels at late vitellogenesis were not significant enough to defend such possible potent role for the two corticosteroids at this stage of reproduction.

In the example on Arctic char, *in vivo* co-injection of juvenile fish with cortisol and E2 reduced Vtg production by comparison to E2 injection alone. Interestingly, this effect was later found to be rather on the molecular structure of circulating Vtg than on its production. As Vtg undergoes the first changes in its molecular structure during its first proteolysis, we assume that cortisol could possess the ability to affect first proteolysis occurring during vitellogenesis in rainbow trout (Berg et al., 2004). Similarly, the effect of DOC and 11-D on the yolk globule



coalescence in Eurasian perch of our study could highlight a role for these hormones on the possible further changes occurring on Vtg molecular structure at late vitellogenesis. The latter assumed role would perhaps help us anticipate the possible effect of a change in the levels of DOC and 11-D following a stressful situation. In RAS, stressors of handling, tagging, or feeding, can fall into the category of physical or mechanical stressors that are necessarily encountered during fish culture (Barton, 2002; Milla et al., 2020, 2015). However, there is very few information about the stimulation of DOC and 11-D release following the exposure to husbandry stressors. A recent study in rainbow trout revealed that confinement stress induced a rise in plasma DOC (Kiilerich et al., 2018). If the latter role of these corticosteroids is further validated, the primary stress response marked by disturbed corticosteroids levels would result in secondary stress response exhibited in the form of cellular changes (assumed increase in yolk-globules coalescence). The end result would be a tertiary response observed on whole animal performance (changes in produced egg-yolk) over the long term. In the example on Arctic char, authors anticipated that the effect for cortisol would be rather reflected on egg shell proteins of the future produced egg (Berg et al., 2004). In Eurasian perch, the introduction of DOC and 11-D implants into farmed vitellogenic females could perhaps confirm whether DOC and 11-D hypothesized role in egg-yolk formation is leading to the observed deterioration in egg quality.

Again, the above hypothesized physiological role for the deoxycorticosteroids on egg-yolk coalescence would still be limited by their insignificant rises at late vitellogenesis. By contrast, the observed high significant levels for 11-D and cortisol during FOM and spawning were much more in support of an existing role for both corticosteroids during both stages. We therefore insisted even more on the direct *in vitro* testing of the corticosteroids during FOM.

## **Reviewing and investigating the direct roles for corticosteroids during FOM**

Starting from the realized increase in plasma 11-D and cortisol levels during FOM in chapter 1, we attempted to formulate several hypotheses regarding their potent roles during FOM. But before that, we attempted to collect more information in chapter 2 on previous reports which had investigated the role of corticosteroids during FOM. Interestingly, the collected information in chapter 2 nominated 11-D and cortisol as potential MIH in several teleosts allowing us to assume the same role for these corticosteroids in Eurasian perch. For instance, both *in vitro* and *in vivo* studies in the cyprinid *Alburnis tarichi* suggested 11-D as a more potent MIH than progestogens themselves. In this cyprinid, plasma 11-D levels exceeded those of progestogens during FOM (Ünal et al., 2008) as has been realized for 11-D in our study (chapter 1; 11-D exceeded the plasma levels of DHP). Additionally, later *in vitro* assays also showed that 11-D managed to induce GVBD at lower doses than those required by the other progestogens further supporting 11-D as an MIH in that species (Ünal et al., 2008). Concerning cortisol, our collected data in chapter 2 also hypothesized this corticosteroid as MIH in several fish belonging to acipenserids, cyprinids, and salmonids.

Despite all the information illustrated in both chapters 1 and 2, our *in vitro* assay in chapter 3 testing the direct MIH activity of corticosteroids revealed very slight FOM advancement by cortisol and no effect for 11-D. In fact, cortisol induction of FOM was much lower than the advancement recorded when follicles were incubated with DHP, the hormone we later confirmed to be the true MIH following its injection in the live fish. After confirming DHP as the MIH in female E. perch (chapter 3), we hypothesized whether the high levels of 11-D and cortisol could be implying a role for the two corticosteroids rather on MIH (DHP) activity. In chapter 4, we therefore tested the direct *in vitro* effect of corticosteroids on our revealed MIH in perch (DHP). Our results later revealed the two corticosteroids (11-D and cortisol) to reflect a rather slight, yet significant, depressing effect on DHP. This information revived the possible

involvement of 11-D and cortisol in FOM. Hence, we still support an existence of a role for the two corticosteroids 11-D and cortisol in FOM even if this effect was later concluded to be highly dependent on the implemented dose of DHP and was hypothesized to be highly dependent on the type of receptor to which corticosteroids bind.

In Nile tilapia, the injection of supraphysiological doses of cortisol accelerated ovulation (Gennotte et al., 2012). However, it is still difficult to anticipate the same effect on ovulation as has been observed in tilapia due to our *in vitro* results where even the application of supraphysiological doses of corticosteroids failed to show a strong effect on FOM. On the contrary, *in vitro* co-exposure to such corticosteroids with DHP slightly slowed down the advancement of perch FOM (chapter 4) which would contradict with the acceleration of FOM observed in Nile tilapia. It should be also well noted that the reproductive strategy of tilapia, which is asynchronous, differs from than that of Eurasian perch with the latter being group synchronous. Thus, an extra limitation to anticipating the same results as in tilapia further exists.

The possible role for 11-D and cortisol could be also linked to the second proteolysis which occurs concomitantly with oocyte hydration that was proven to occur even in fresh water fish (Fabra et al., 2006; Hara et al., 2016; Milla et al., 2006). This hypothesis is strengthened by the *in vitro* effect of cortisol on trout oocyte hydration even if the proteolytic mechanisms were not obvious (Milla et al., 2006). We recall that a second proteolysis is the further degradation of the YPs that were first produced and incorporated into the developing oocyte during vitellogenesis. Such proteolysis necessarily occurs with oocyte hydration during which the oocyte absorbs water (Hara et al., 2016). Although such hydration is very minor (4%) in Eurasian perch (Żarski et al., 2012b), it does occur in the latter fish and would necessarily mean the existence of second proteolysis as well. The increased concentration of free amino acids (FAA) and small peptides in the oocyte leading to the osmotic mechanism for water influx into oocyte (Fabra et al., 2006) could be helpful as a parameter to investigate the secondary proteolysis in Eurasian perch. We

thereafter suggest assessing the amplitude of oocyte hydration and the quantities of produced FAA and small peptides in the oocyte following injections with 11-D and cortisol.

Another way to interpret our results is by recalling the indirect effects of corticosteroids, mainly glucocorticoids, on pituitary gonadotropins and other steroids (androgens and progestogens) that have been discussed in several reports (Milla et al., 2009). Reasonably, our *in vitro* assays would not be able to eliminate or confirm whether such high corticosteroids plasma levels imply a role for corticosteroids on the regulation of gonadotropins. To examine that, co-culture of pituitary and follicles would have been useful to investigate such indirect regulation. The only information that can be provided by our *in vitro* assays is whether there exists a direct effect on the follicles for the studied corticosteroids at the periods of their plasma elevations. In the latter respect, our *in vitro* assays failed to reveal a strong effect for the corticosteroids on inducing FOM either when introduced alone or co-introduced with DHP. **However, from our collected data in chapter 2, the high significant plasma levels at FOM, and even the slight *in vitro* effects on FOM advancement are still in support for a role for 11-D and cortisol on FOM that will need further investigation.**

The further investigation behind the roles for 11-D and cortisol during FOM is even more important for understanding the asynchrony in ovulation and the later-on spawning which is one of the main problems in controlled Eurasian perch reproduction. According to Zarski and colleagues (2012), the problem with asynchronous ovulation stems from the uneven final oocyte maturation in individual fish past after having completed their vitellogenesis (Żarski et al., 2021, 2012b). The elevations in 11-D and cortisol plasma levels at FOM thus deserve further investigation even if our *in vitro* results fail to provide a solid explanation for such high levels. Another way to understand our results is by hypothetically linking 11-D and cortisol to later functions occurring during spawning of Eurasian perch. In fact, the plasma 11-D and cortisol levels continued to increase past FOM after which they reached their highest significance at

spawning (chapter 1). The latter result provided by this thesis highlights the existence of a potent role for corticosteroids during spawning and even past FOM that deserves to be tested.

### **Interpreting the high plasma levels for some corticosteroids at spawning**

Following FOM, the continued significant rise in the levels of both corticosteroids 11-D and cortisol during spawning could be related to other functions that take place mainly around spawning. In upstream migratory sockeye or red salmon and Kokanee salmon *Oncorhynchus nerka kennerlyi*, 11-D and cortisol increased respectively over the course of migration until reaching their highest significant levels at the fish spawning locations (Carruth et al., 2000; Truscott et al., 1986). Wild Eurasian perch females are known to exhibit migratory behavior to shallow and warmer water around their spawning period during which both cortisol and 11-D have been observed to reveal their highest plasma levels (Ben Khadher et al., 2015; Eckmann and Imbrock, 1996; Noaksson et al., 2005). As such, an elevation in both 11-D and cortisol plasma levels in perch might be linked to the proposed site fidelity in wild Eurasian perch and their patterns of distribution during the spawning season (Ben Khadher et al., 2015), or simply, the migratory behavior as has been realized in both migratory salmons (Carruth et al., 2000; Truscott et al., 1986). In perch reared in captivity as our fish, the stimulation of a migratory behavior appears useless as temperature is controlled and is increased while fish “stay foot” in the same aquarium. In other non-migrating fish species or even under controlled environmental conditions, elevation of plasma cortisol is observed at the spawning period. In catfish, cortisol levels were significantly elevated at spawning even under controlled environmental conditions (Joy and Kumar, 2017). The latter example supports the assumption that the high levels of cortisol obtained in our study around spawning is not exclusively linked to fish migratory behavior. We therefore hypothesize that the high 11-D and cortisol levels might be as well linked to other reproductive and behavioral functions. We assume that such functions, such as

the release of eggs in the form of intact ribbons, release of pheromones, or the timing of spawning itself, would be spawning related-functions that are necessarily maintained even in cultured fish. In pink salmon, cortisol treatment effect on the spontaneous spawning of fish was assessed where stripping was not practiced. The results revealed that cortisol treatment induced higher egg-retention during the period of their spawning (McConnachie et al., 2012) indicating that cortisol can affect the release of eggs during spawning. In Eurasian perch, the elevated levels of cortisol might also imply a role for this corticosteroid on egg-retention during the spawning season. Whether the latter effect delays or induces spawning in perch is dependent on testing cortisol injections in live female perch. To test that, we propose injecting cortisol during FOM and then monitor the progress of FOM, the possibility to strip the ribbon, and the egg quality. Assessing the quality of the produced ribbons is highly recommended considering the observed fragmentation in produced egg strands obtained in Eurasian perch reared under controlled environments (Szczerbowski et al., 2009). Cortisol injection could perhaps explain the observed fragmentation in produced egg strands in the latter study which could be also linked to the previously proposed effect of cortisol on egg-retention. Besides corticosteroid injections, another way to assess the impact of elevated corticosteroids levels on spawning would be *via* the introduction of both capture and transport stressors as has been performed in adult lake sturgeon (Genz et al., 2014). In principle, the latter strategy proposes that capture and transport stressors, which are normally encountered during spawning seasons of farmed fish, would provoke a plasma rise in corticosteroids levels. Following such rise, the reproductive performance is monitored providing information on the potential role for the elevated corticosteroids levels.

### **A take home message**

The results provided by this thesis pointed out a potential role for corticosteroids at the onset of oogenesis and spawning and an existing role during late vitellogenesis and FOM that could be useful in:

1. Linking the alterations in the levels of corticosteroids in domesticated female Eurasian perch to the deteriorations in their reproductive performance in captivity.
2. Linking the stress-induced alterations in the levels of corticosteroids in female Eurasian perch to the alterations in their reproductive performance in captivity.

**The results of this thesis assume a link between the roles of corticosteroids and the reproductive alterations in Eurasian perch reared in captivity as illustrated in Figure 16**

We recall that the reproductive performance and the final reproductive outcomes of recently domesticated (F1) female Eurasian perch were found to be much better than those that had been domesticated for several generations. Indeed, fish domesticated for more generation produced a poor quality of eggs with a higher percentage of malformations in produced larvae. Such deteriorations in the quality of the ova and produced larvae were linked to the faster gametogenesis occurring in the more domesticated population. In the latter population, it was suggested that eggs were unable to accumulate enough nutrients for the proper embryonic development resulting in such malformations in the produced larvae (Khendek et al., 2017). In agreement with these findings, other studies that compared the performance between F1 and more domesticated fish populations (e.g. F7+) also revealed better overall egg quality for F1 fish (Křišť'an et al., 2012; Rocha de Almeida et al., 2019). In the study of Khendek et al. (2017), higher cortisol levels were observed in the F1 population than in the more domesticated one. The latter study was thereafter successful at suggesting a putative link between the observed

domestication-induced alterations in cortisol levels and the effects of domestication on reproductive performance. In this thesis, we were able through both *in vivo* and *in vitro* assays to further support the previously suggestion link. Indeed, this thesis highlighted the potential involvement of corticosteroids in critical stages of female Eurasian perch reproduction as onset of oogenesis, late vitellogenesis, FOM, and spawning. Adding to that, our results revealing significant variations in the relative expression of CRs with respect to the stage of the reproductive cycle insist even more on the involvement of these corticosteroids in critical stages of oogenesis. However, we always recall the fact that the first elevation in a hormonal level is not a proof but an indication of an endocrinological role. Integrating the latter with *in vitro* biological tests is neither enough as a confirmed proof on the endocrinological role of a hormone as these tests do not fully reflect the physiological reality.

In the study by Rocha de Almeida et al. (2019), the accumulation of maternal mRNA, which usually takes place during early growth stages of oogenesis (Lubzens et al., 2010), was found to be highly affected by the domestication process (Rocha de Almeida et al., 2019). The results of this thesis suggesting a possible role for DOC and 11-D at the early growth stages of oogenesis could perhaps explain the observed impacts of domestication on the accumulation of maternal mRNA. The effect for the two deoxycorticosteroids on mRNA could perhaps be by affecting the nucleic acid stability as has been realized for cortisol in zebrafish where cortisol had been found to affect nucleic acid stability in follicles (Sousa et al., 2015). Still, the latter interpretation is limited by the unavailability of studies showing the effect of domestication on either deoxycorticosteroids or showing their effect on mRNA accumulation in fish oocytes. Nevertheless, the suggested roles for both deoxycorticosteroids at early growth stages of oogenesis could perhaps partly explain the effects of domestication on the gonadal growth of female perch reported in several studies (Křišť'an et al., 2012; Rocha de Almeida et al., 2019). More specifically, we hypothesize that the effects of domestication could probably result from



changes in the levels of the two deoxycorticosteroids over the course of domestication as had been previously realized for cortisol (Khendek et al., 2017). Additionally, an effect for domestication on E2 levels was also reported in the same study where domestication was found to affect cortisol levels (Khendek et al., 2017). The effect of domestication on E2 levels could be thus also linked to our previously suggested role for the two deoxycorticosteroids on the production of E2. This hypothesis seems to be logical considering the previously reported effect for corticosteroids as cortisol on E2 levels in Arctic char (Berg et al., 2004). The results of this thesis concerning the effects of DOC and 11-D on the coalescence of egg-yolk globules also assumes that the two deoxycorticosteroids can affect the quality of the formed yolk inside the egg. In the review by Reading et al. (2018), poor egg quality is usually accredited to alterations in the cleavage of vitellogenin proteins which is necessary for the formation of yolk globules and their coalescence (Fabra et al., 2006; Reading et al., 2018). We can hence assume that our realized effect of the two deoxycorticosteroids on yolk-globules coalescence is perhaps linked to the observed poor egg quality in domesticated Eurasian perch. In Eurasian perch, the produced egg quality has been also correlated to the produced larval quality (Żarski et al., 2011b). Therefore, we also anticipate that the poor egg quality that could be linked to the role of the two deoxycorticosteroids in yolk-globules formation is at the origin of the bad larval quality in farmed Eurasian perch.

Above all, FOM remains to be the most critical stage of oogenesis that is most crucial for the preparation of the maturing oocyte for ovulation and spawning. With the observed high levels of 11-D and cortisol at FOM and the available *in vitro* assays conducted during the latter reproductive stage, the results of this thesis were mostly able to highlight a possible slight role for corticosteroids during FOM and even ovulation. The latter could be useful in explaining the persisting impact of domestication on offspring quality and synchronization of spawning. Even if minor or indirect, an existing role for the two corticosteroids 11-D and cortisol in FOM

advancement and ovulation is agreed on by both *in vivo* and *in vitro* results of this thesis. After all, asynchrony in ovulation and spawning has been frequently encountered in farmed Eurasian perch (Rocha de Almeida et al., 2019; Żarski et al., 2011b) in which disturbances in plasma cortisol levels have been observed (Khendek et al., 2017). To test that idea, it would be relevant to monitor the levels of cortisol and 11-D during the final stages of reproduction in both wild and domesticated populations.

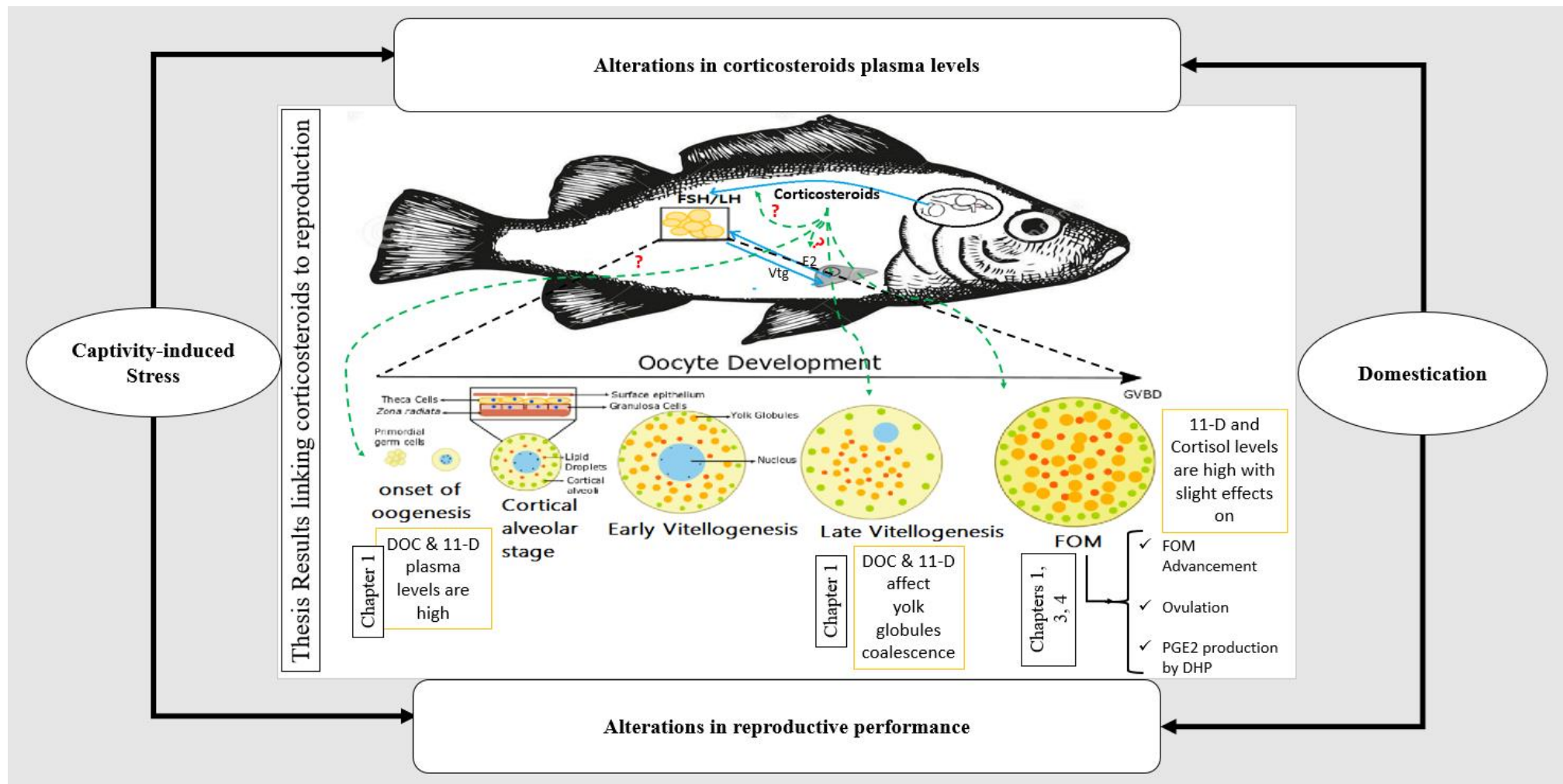
When reviewing all the above information, it appears that the deteriorations in the reproductive performance in farmed fish could be somehow explained by the suggested roles for corticosteroids in this thesis. We always insist on hypothesizing that an alteration in the plasma levels of corticosteroids over the course of domestication could be the reason behind the observed reproductive deteriorations with domesticated populations reared since a high number of generations in captivity. With such hypothesis, the persisting speculation remains on the exact cause for persisting alterations in the levels of corticosteroids over the course of domestication. What is agreed on in all studies is that corticosteroids levels are highly sensitive to stress which has always allowed their common use as indicators of the *primary response to stress* in fish (Barton, 2002). However, no direct stressors were introduced in either of the mentioned studies where they assessed the effects of domestication on Eurasian perch reproductivity. It would be biased and far out of the scope of this thesis to relate the altered reproductive performances in domesticated fish to the stress encountered in captivity especially considering the ability of fish to gradually adapt to farming conditions. So far, this thesis can only agree with our first perspective suggesting a link between alterations in corticosteroids levels and the observed alteration in fish reproductive performance over the course of domestication.

From our second perspective, we recall the strong endocrinological relation between stress and plasma corticosteroids elevation which could also explain the reproductive alterations in farmed

fish. With the results obtained in this thesis, it becomes easier to better explain the potential effects of stress on reproduction assuming that DOC and 11-D are also stress-induced corticosteroids in Eurasian perch. For maximizing production in captivity, fish would be subjected to many acute and chronic stressors such as crowding, handling, transport or even the sudden change in surrounding lightning and water conditions. As a result, interest in assessing the impact of stress on reproduction became intriguing in most aquaculture studies (Gennotte et al., 2012). In Eurasian perch, stress is nominated as one of the main causes for spawning problems (Castets et al., 2012). Generally in teleosts, gamete quality and quantity is known to be dependent on the trophic conditions and stress (Schreck, 2010). Controlled reproduction in fish necessarily involves stressful handlings of fish. A very recent study on the impact of the stress induced by alterations of light conditions in farmed female Eurasian perch revealed that such stressors can affect ovulation process (Żarski et al., 2021). It was revealed through both *in vivo* and *in vitro* assays in this thesis that 11-D and cortisol could be involved in ovulation (chapters 1, 3, and 4). With the information revealed in this thesis, we are hence more capable of explaining the bidirectional interactions between the hypothalamus-pituitary-interrenal axis and the hypothalamus-pituitary-gonadal axis. When it comes to understanding the effects of direct captivity-induced physical stressors on the reproductive performance of farmed Eurasian perch, no studies have been conducted up to date. On the other hand, several other studies exist on other teleosts where captivity-induced physical stressors have been tested and observed to highly affect fish reproductive performance. With the results attained in this thesis, we could attempt to explain such potential effects by captivity-induced physical stressors on fish reproductive performance that would also occur in captive perch.

The exposure of tilapia and rainbow trout to mild physical stressors as the ones encountered in captivity was observed to disturb the timing of ovulation in both fish. In tilapia, the stressors were applied during early ovarian development, while in trout, stressors were applied during

late vitellogenesis (Contreras-Sánchez et al., 1998; Schreck et al., 2001). The results in this thesis highlighting a potential role for DOC and 11-D during early ovarian growth stages and late vitellogenesis could perhaps explain the effects of such mild stressors on the timing of ovulation. Also in trout, more severe stressors applied prior to spawning resulted in small eggs and delayed ovulation (Campbell et al., 1992). It is possible that the latter stressors might have affected FOM during which the oocyte undergoes hydration and ovulation just prior to spawning. The results of this thesis implying a potential role for 11-D and cortisol in FOM and ovulation could thereby link both stress-sensitive corticosteroids to the observed alterations in egg-size and timing of ovulation in these species. In another study on tilapia, ovulation was more precocious in fish injected with cortisol in a dose within the estimated cortisol surge following an acute stress (Gennotte et al., 2012). Our realized results implying a role for cortisol in ovulation could perhaps explain the precocious ovulation obtained in the latter study. In addition, our revealed elevated plasma 11-D and cortisol levels around FOM and spawning, together with the observed higher egg retention in cortisol-treated pink salmon (McConnachie et al., 2012), could perhaps explain why fewer oocytes can be usually stripped from stressed fish (Schreck, 2010). Finally, along this manuscript, we have proposed a role for the two deoxycorticosteroids DOC and 11-D in the downregulation of E2 production which was *in vitro* illustrated for DOC and cortisol by Mandiki et al. (2017). The latter information could perhaps explain the effect of induced stress by capture and/or handling on the decrease in E2 levels in some teleosts (Clearwater and Pankhurst, 1997). In fact, the recorded effect of stress in some teleosts on the follicular-steroidogenic capacity during early stages of oogenesis might be explained by the results of this thesis which highlight a potent role for DOC and 11-D during early growth stages (Schreck, 2010).



**Figure 16: Schematic representation of how Corticosteroids suggested functions by this thesis could be linked to reproductive alterations in Eurasian perch reared in captivity.**

## **Limitations related to the performed work and more general future perspectives**

Our findings could represent a primary attempt to explain the observed reproductive drawback in farmed populations of Eurasian perch. However, to attain these findings, we used data from domesticated fish that have been subjected to regular RAS conditions without introducing any sudden additional stressor. Our findings thus are only capable of partly explaining the link between alterations in fish corticosteroids plasma levels and their reproductive performance over the course of domestication or as a result of stressful situation. Adding to that, our findings exhibiting, for the first time, the baseline plasma levels of the four stress-sensitive corticosteroids during more precised stages of farmed female Eurasian perch reproductive cycle. The latter finding could be highly appreciated in future studies on the effect of stress in this fish. Indeed, a common problem in the study of the effect of stress on fish is the lack of information on the baseline plasma levels in unstressed fish which is highly species dependent (Pankhurst and Sharples, 1992). The information on the different effect of stress on reproduction in different fishes clearly shows how stress response is highly dependent on the stage of oogenesis at which fish were stressed in addition to both duration and intensity of a stressor. Our results providing more clear information on the effects of corticosteroids on identified stages of oogenesis provide crucial information on what exact stages to pay attention to when fish are stressed. For instance, critical attention should be provided to stages where corticosteroid levels are high or when they display any activities in order to avoid an alteration in reproductive performance.

This thesis provided more information on the possible roles for corticosteroids in the reproductive cycle of female Eurasian perch. This can only help in formulating further hypothesis to explain the observed alterations in reproductive performances of domesticated or stressed Eurasian perch in captivity. To make this work more useful for aquaculture studies, we

wish to implement Eurasian perch populations that are subjected to acute physical stressors other than the ones routinely encountered in RAS. In other words, fish cultivated in controlled environments might be subjected to unexpected sampling or confinement periods when fish will be suddenly stressed. It would be hence crucial to assess the impact of such stressful situations on corticosteroids levels considering the information proposed by this thesis on their assumed critical roles in reproduction. To estimate the persisting effects of such stressors on reproductive performance of female Eurasian perch in culture systems as RAS, attention should be paid to periods of reproduction during which corticosteroids functions are highlighted. For example, it would have been better if we had tested the effect of netting, confinement, or crowding, as occurs during the spawning season, in one extra population of fish. The effect of such stressors would be later assessed on corticosteroids levels in the stressed population along the assessment of quality of spawning and produced eggs. We also wish to test the *in vitro* direct effects of DOC and 11-D on follicles during the onset of oogenesis at which they recorded their highest plasma levels with highest relative CRs expression. For selecting such population of fish, attention should be paid to their level of domestication where the history and the origin of the fish used in this thesis were not fully traceable.

Starting from the information provided in this thesis, the discussed role of some corticosteroids in critical stages of female Eurasian perch reproduction could recommend the use of corticosteroids treatments to enhance reproduction. For instance, treatment of fish with DOC and 11-D injections during late vitellogenesis could allow faster accumulation of yolk globules and a resulting more precocious ovulation. Our results assuming a role for 11-D and cortisol in spawning could suggest the use of the latter hormonal treatments in fish around spawning for better synchronization of production. Again, we insist on the fact that our results regarding the documented elevations in hormonal levels and/or the *in vitro* biological tests still do not constitute a sufficient proof but an indication of an endocrinological role. Indeed, further

attempts including the live injection of the hormone during the period of its assumed function could be more complementary in reflecting the physiological reality of a hormonal action.



# **GENERAL CONCLUSION**

## GENERAL CONCLUSION

The primary purpose behind this thesis was to show how stress sensitive corticosteroids can be implemented in fish reproduction in order to better understand the current reality of fish reproductive performances in captivity. This thesis did indeed exhibit how the plasma kinetics of most studies corticosteroids can vary according to the stage of oogenesis. This thesis equivocally showed how the relative expression of gonadal receptors, through which corticosteroids might impose their potential functions, can vary according to the stage of oogenesis. This thesis also showed that corticosteroids could be implemented in late vitellogenesis with further analysis required to understand the exact molecular aspect of such action. This thesis mainly focused on the potential role for corticosteroids during FOM where an activity could still be supported. Their activity does not necessarily have to be as true MIH but as potential hormones that could indirectly affect FOM. This thesis cannot anticipate whether corticosteroids possess a positive or negative effect on the reproductive stages to which they have been linked through this thesis. However, through providing some threads of evidence on the involvement of corticosteroids in reproduction, this thesis could be helpful for new studies that will deal with assessing the impacts of mechanical or environmental stressors similar to those encountered in fish farms. In fact, this thesis provided, for the first time, the unstressed basal levels of corticosteroids along highly precised stages of oogenesis in female Eurasian perch. The latter could constitute the basis for future studies which will attempt to assess the extent of plasma levels increases in the level of corticosteroids following the perception of a stressor at different stages of oogenesis. After all, corticosteroids are known to mediate the stress-coping mechanisms in fish whose impact on reproduction should be further investigated in Eurasian perch.

If the roles for corticosteroids are further validated using live injections in fish, this thesis could allow proposing corticosteroids as possible treatment to perhaps allow better synchronization

of spawning which would replace the regular treatment involving gonadotropins. Therefore, this thesis would perhaps contribute to the ultimate purpose of more optimized aquaculture production under controlled systems as RAS.

# LIST OF PUBLICATIONS

## Articles published or submitted

- Milla, S., Pasquet, A., **El Mohajer, L.**, Fontaine, P., 2020. How domestication alters fish phenotypes. *Rev. Aquacult.* 13, 388–405. <https://doi.org/10.1111/raq.12480>
- **El Mohajer, L.**, Bulteau, R., Fontaine, P., Milla, S., 2021. Maturation Inducing Hormones in teleosts: Are progestogens always the first to be nominated? *Aquaculture*. 546, 737315. <https://doi.org/10.1016/j.aquaculture.2021.737315>
- **El Mohajer, L.**, Bulteau, R., Chevalier, C., Selmi, S., Fontaine, P., Milla, S., 2021. *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*. *Fish Physiol Biochem.* <https://doi.org/10.1007/s10695-021-00946-5>
- **El Mohajer, L.**, Chevalier, C., Chardard, D., Schaerlinger, B., Fontaine, P., Milla, S. What would Corticosteroids plasma kinetics and gonadal receptor expression tell about their possible roles in the reproductive cycle of domesticated female Eurasian Perch *Perca fluviatilis*? Submitted to *Aquaculture* (Jan, 21, 2022).

## Oral communications

- The Search for The Maturation Inducing Steroid (MIS) In Eurasian Perch *Perca fluviatilis*. **EL Mohajer L, Selmi S, Fontaine P, Milla S.** European Aquaculture Society (EAS congress). **07-10 October 2019. Berlin-Germany.**
- Roles of corticosteroids in the progress of Final Oocyte Maturation in female Eurasian perch. **EL Mohajer L, Selmi S, Fontaine P, Milla S.** **Séminaire de l'école doctorale**

**SIReNa - Université de Lorraine - 13 February 2020. Vandoeuvre-Lès Nancy, France.**

- Deeper in vitro investigation into the possible roles for four major corticosteroids in FOM in relation to their plasma kinetics along the whole reproductive cycle of female Eurasian Perch *Perca fluviatilis* L. L. El Mohajer, C. Chevalier, P. Fontaine, S. Milla. European Aquaculture Society (EAS congress). **04-07 October 2021. Madeira-Portugal.**

# ACTIVITIES

- **Doctoral school courses.** Total participation: 344 hrs / 19 modules (main courses included trainings on Advanced Studio-R and LATEX softwares. French courses with Intermediate level achieved).
- **Training course in laboratory animal science for scientists responsible for the design or conduct of animal experiments.** UAB - Universitat Autònoma de Barcelona (Barcelona, Spain). Sep-Oct 2021.
- **Demi-ATER** - Université De Lorraine (Nancy, France). Sep 2020-Aug 2021.
- **Participation in the supervision of the Masters students.**
  - Master (M1); Serine Selmi. April-June 2019.
  - Master (M2); Rose Bulteau. March-July 2020.
  - Masters (M2); Céline Chevalier. Jan-June 2021.

# RÉSUMÉ SUBSTANTIEL EN FRANCAIS

Le développement de l'industrie aquacole a été exclusivement basé sur l'élevage de quelques espèces majeures (principalement des cyprinidés, des tilapias, des catfish et des salmonidés) qui ont souvent été introduites dans de nouvelles zones géographiques. En conséquence, 85% de la production mondiale de poissons à nageoires provient de seulement 15 espèces, qui sont élevées dans de nombreux pays. L'anticipation d'une durabilité accrue de la productivité des exploitations a encouragé le développement continu de nouvelles stratégies agricoles. L'un des meilleurs moyens de maintenir ou d'augmenter la contribution des produits aquatiques à cette consommation mondiale est par la suite d'élever de nouvelles espèces d'origine marine ou d'eau douce dans lesquelles leur production est continuellement améliorée. Cependant, l'élevage de nouvelles espèces animales est confronté à des impacts nuisibles sur les performances de reproduction des animaux nouvellement élevés. Ces impacts nuisibles sont en partie médiés par des facteurs de stress induits par certaines pratiques agricoles. Par exemple, les deux pratiques agricoles de base de capture et de manipulation des poissons induisent suffisamment de stress pour avoir un effet considérable sur les niveaux de stéroïdes gonadiques, en particulier les androgènes et les œstrogènes chez une variété de téléostéens. En prenant des exemples de plusieurs espèces de poissons comme le grondin rouge *Chelidonichthys cuculus*, la truite de mer tachetée *Cynoscion nebulosus* et la truite arc-en-ciel *Oncorhynchus mykiss*, les facteurs de stress tels que la capture, la manipulation et le confinement ont entraîné une diminution rapide à la fois du  $17\beta$ -estradiol (E2) et taux de testostérone (T) chez ces poissons. Pourtant, ces facteurs de stress ne conduisent pas nécessairement à une diminution de toutes les hormones où les concentrations plasmatiques du corticostéroïde cortisol ont tendance d'augmenter à la suite de tels facteurs de stress. Cette augmentation pourrait ressembler à l'augmentation physiologique naturelle des niveaux de cortisol chez les poissons sauvages en réponse aux facteurs de stress naturels rencontrés dans la nature pendant la saison du rut (comportement

d'accouplement intensif, combats entre mâles dominants ou activités de migration de frai). Dans tous les cas, on a toujours observé que les taux plasmatiques de stéroïdiens sexuels fluctuaient avec les changements dans l'environnement des poissons.

La forte demande des fermes piscicoles mondiales d'avoir plus de contrôle sur l'environnement d'élevage dans le but de mieux contrôler, programmer et améliorer les différentes étapes de la reproduction des poissons a constitué le besoin de systèmes d'aquaculture intérieures contrôlés comme le système d'aquaculture en recirculation (RAS) communément connu et adapté dans la plupart des fermes piscicoles intensives aujourd'hui. Ce système peut induire d'autres facteurs de stress en plus de ceux précédemment définis tels que la nécessité de contrôler la température et la période d'éclairage pour avoir plus de contrôle sur le cycle de reproduction. Il a en effet été documenté que les variations saisonnières de la durée du jour et de la température sont responsables des signaux et du moment de la reproduction chez la majorité des poissons. Étant d'une importance économique pour les piscicultures car cela permet d'économiser du temps et de la main-d'œuvre, la réalisation d'un frai hors saison et synchrone grâce à une reproduction contrôlée a été d'une importance primordiale pour l'industrie de poisson. On pense que les facteurs de stress résultant d'un tel contrôle de l'environnement peuvent avoir un impact négatif sur l'obtention d'une qualité optimale des œufs et d'une reproduction synchrone chez certains poissons d'élevage. La réponse au stress est connue pour provoquer des altérations des niveaux physiologiques des corticostéroïdes sensibles au stress, qui pourraient à leur tour être attribuées au contrôle de certains aspects de la reproduction des poissons. En effet, les effets rapportés des corticostéroïdes sur la production de GnRH (Gonadotropin Releasing Hormone) et de gonadotrophines ont reçu un intérêt récent pour leurs rôles possibles dans la reproduction des poissons. Cependant, l'absence de rapports sur les variations plasmatiques exactes des principaux corticostéroïdes au cours de l'ovogenèse chez la plupart des téléostéens limite la capacité de définir leurs éventuels rôles reproductifs.



La perche eurasienne *Perca fluviatilis* L est un téléostéen très perçu comme une espèce d'eau douce précieuse qui est aujourd'hui cultivée dans RAS avec les défis actuels liés à la qualité optimale des œufs et à la synchronisation des ovulations et du frai. Définie comme la capacité des œufs à être fécondés et à se développer ensuite en un embryon normal, la qualité des œufs est d'une grande importance pour les fermes piscicoles, où l'incapacité des œufs produits à être fécondés ou à se développer en un embryon pleinement développé rendrait l'œuf comme étant de mauvaise qualité. Comme chez la plupart des téléostéens, les rôles des corticostéroïdes sensibles au stress mentionnés précédemment ne sont pas non encore définis chez la perche femelle. Des études mettent en évidence l'implication possible de quatre principaux corticostéroïdes (11-désoxycorticostérone ou DOC, 11-désoxycortisol ou 11-D, corticostérone ou CoS, et cortisol ou Co) dans les étapes critiques de la reproduction de la perche. Au cours de cette thèse, nous nous attacherons donc à définir les rôles exacts de ces quatre principaux corticoïdes dans la reproduction des perches eurasiennes femelles dans le cadre de la compréhension en partie des troubles reproducteurs observés chez les perches femelles élevées en RAS.

Par conséquent, l'objectif principal de cette thèse était de montrer comment les corticostéroïdes sensibles au stress peuvent être mis en œuvre dans la reproduction des poissons afin de mieux comprendre la réalité actuelle des performances de reproduction des poissons en captivité. Cette thèse a montré comment la cinétique plasmatique de la plupart des corticoïdes étudiés peut varier selon le stade de l'ovogenèse. Cette thèse a montré de manière équivoque comment l'expression relative des récepteurs gonadiques, par lesquels les corticoïdes pourraient imposer leurs fonctions potentielles, peut varier selon le stade de l'ovogenèse. Cette thèse a également montré que les corticostéroïdes pouvaient être mis en œuvre à la fin de la vitellogenèse avec une analyse plus approfondie nécessaire pour comprendre l'aspect moléculaire exact d'une telle action. Cette thèse s'est principalement concentrée sur le rôle potentiel des corticoïdes au cours

de la FOM où une activité pouvait encore être soutenue. Leur activité ne doit pas nécessairement être en tant que véritable MIH mais en tant qu'hormones potentielles qui pourraient indirectement affecter la FOM. Cette thèse ne peut pas anticiper si les corticostéroïdes possèdent un effet positif ou négatif sur les stades de reproduction auxquels ils ont été liés à travers cette thèse. Cependant, en fournissant des éléments de preuve sur l'implication des corticoïdes dans la reproduction, cette thèse pourrait être utile pour de nouvelles études qui traiteront de l'évaluation des impacts de facteurs de stress mécaniques ou environnementaux similaires à ceux rencontrés dans les piscicultures. En fait, cette thèse a fourni, pour la première fois, les niveaux basaux non stressés de corticostéroïdes à des stades très précis de l'ovogenèse chez la perche eurasiennne femelle. Cette dernière pourrait constituer la base d'études futures qui tenteront d'évaluer l'ampleur des augmentations plasmatiques du taux de corticoïdes suite à la perception d'un facteur de stress à différents stades de l'ovogenèse. Après tout, les corticostéroïdes sont connus pour médier les mécanismes d'adaptation au stress chez les poissons dont l'impact sur la reproduction devrait être étudié plus en détail chez la perche eurasiennne. Cependant, pour rendre ce travail plus utile pour les études aquacoles, nous souhaitons mettre en œuvre des populations de perches eurasiennes qui sont soumises à des stress physiques aigus autres que ceux couramment rencontrés dans les RAS. En d'autres termes, les poissons élevés dans des environnements contrôlés pourraient être soumis à des périodes d'échantillonnage ou de confinement imprévues lorsque les poissons seront soudainement stressés. Il serait donc crucial d'évaluer l'impact de ces situations stressantes sur les niveaux de corticostéroïdes compte tenu des informations proposées par cette thèse sur leurs rôles critiques supposés dans la reproduction. Pour estimer les effets persistants de tels facteurs de stress sur les performances de reproduction de la perche eurasiennne femelle dans des systèmes d'élevage comme le RAS, il convient de prêter attention aux périodes de reproduction durant lesquelles les fonctions des corticostéroïdes sont mises en évidence. Par exemple, il

aurait été préférable de tester sur une population supplémentaire de poissons l'effet des filets, du confinement ou du surpeuplement, de la même manière que ceux-là se produisent pendant la saison de frai,. L'effet de ces facteurs de stress serait ensuite évalué sur les niveaux de corticostéroïdes dans la population stressée, parallèlement à l'évaluation de la qualité du frai et des œufs produits. Nous souhaitons également tester les effets directs *in vitro* du DOC et du 11-D sur les follicules au début de l'ovogenèse, au cours desquels ils ont enregistré leurs taux plasmatiques les plus élevés avec une expression relative des CR la plus élevée. Pour sélectionner une telle population de poissons, il convient de prendre en considération le niveau de domestication mais l'histoire et l'origine des poissons utilisés dans cette thèse n'étaient pas entièrement traçables.

À partir des informations fournies dans cette thèse, le rôle discuté de certains corticostéroïdes dans les étapes critiques de la reproduction de la perche eurasiennne femelle pourrait recommander l'utilisation de traitements aux corticostéroïdes pour améliorer la reproduction. Par exemple, le traitement des poissons avec du COD et des injections de 11-D à la fin de la vitellogenèse pourrait permettre une accumulation plus rapide de globules vitellins et une ovulation plus précoce. Nos résultats supposant un rôle du 11-D et du cortisol dans la ponte pourraient suggérer l'utilisation de ces derniers traitements hormonaux chez les poissons autour de la ponte pour une meilleure synchronisation de la production. Encore une fois, nous insistons sur le fait que nos résultats concernant les élévations documentées des niveaux hormonaux et/ou les tests biologiques *in vitro* ne constituent toujours pas une preuve suffisante mais une indication d'un rôle endocrinologique. En effet, d'autres tentatives incluant l'injection vivante de l'hormone pendant la période de sa fonction supposée pourraient être plus complémentaires en reflétant la réalité physiologique d'une action hormonale.

Si les rôles des corticoïdes sont davantage validés à l'aide des injections vivantes chez les poissons, cette thèse pourrait permettre de proposer des corticoïdes comme traitement possible

pour éventuellement permettre une meilleure synchronisation de la ponte qui remplacerait le traitement régulier impliquant des gonadotrophines. Par conséquent, cette thèse contribuerait peut-être à l'objectif ultime d'une production aquacole plus optimisée dans des systèmes contrôlés comme les RAS.

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