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Recombinant gonadotropins in meagre (*Argyrosomus regius*): *In-vivo* effect on sexually undifferentiated fish.

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Contents

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Abstract

Meagre (*Argyrosomus regius*) is a teleost fish that has experienced an increase in Mediterranean aquaculture production in recent years. To improve productivity, it is necessary to establish a genetic selection program, but the generational interval in meagre is very high (2 and 3 years in males and females respectively). The objective of this survey is to evaluate the effect of specific *A. regius* single-chain recombinant gonadotropins (rGths) in prepubertal and sexually undifferentiated meagre. For this, 8 treatment groups were established, in which doses of 6, 12 or 18 μg / kg of rFSH or rLH were injected weekly for 3 weeks. Results did not show significant differences in GSI, but they did in plasma E2. Furthermore, early gonadal development was induced with the appearance of oocytes and spermatids in localized regions, while 100% of the fish in the saline group remained sexually undifferentiated. Our results on sexual differentiation show that with rGths therapy 1) males sexually differentiate earlier than females; 2) there is a higher proportion of males; 3) there are intersex fish. This is the first report of the presence of intersex fish and a male skewed ratio in this species. In conclusion, rGths therapy represent a potential solution to reduce the generation interval in prepubertal meagre, but more studies are needed to know how its administration affects during the period of sexual differentiation.

Keywords: *Argyrosomus regius, meagre, prepubertal, recombinant gonadotropins, intersex, sexual differentiation.*

Resumen

La corvina (*Argyrosomus regius*) es un pez teleósteo que ha experimentado un incremento en la producción acuícola mediterránea en los últimos años. Para mejorar la productividad es necesario establecer un programa de selección genética, pero el intervalo generacional en la corvina es muy alto (2 y 3 años en machos y hembras respectivamente). El objetivo de este estudio es evaluar el efecto de las gonadotropinas recombinantes (rGths) de cadena simple específicas de *A. regius* en corvina juvenil y sexualmente indiferenciada. Para ello se establecieron 8 grupos de tratamiento, en los que se inyectaron semanalmente durante 3 semanas dosis de 6, 12 o 18 µg / kg de rFSH o rLH. Los resultados no mostraron diferencias significativas en IGS, pero sí en E2 en plasma. Además, se indujo un desarrollo gonadal temprano con la aparición de ovocitos y espermátidas en regiones localizadas, mientras que el 100% de los peces del grupo salino permanecieron sexualmente indiferenciados. Nuestros resultados sobre diferenciación sexual demuestran que con un tratamiento de rGths, 1) los machos diferencian sexualmente antes que las hembras; 2) hay una mayor proporción de machos; 3) hay peces intersexuales. Este es la primera vez que se publica la presencia de peces intersexuales y una mayor proporción poblacional de machos en esta especie. En conclusión, un tratamiento con rGths representa una posible solución para reducir el intervalo generacional en la corvina juvenil, pero se necesitan más estudios para conocer cómo afecta su administración durante el período de diferenciación sexual.

Palabras clave: *Argyrosomus regius, corvina, juvenil, gonadotropinas recombinantes, intersexual, diferenciación sexual.*

1. Introduction

1.1. Meagre (*A. regius*) biology and perspectives in aquaculture

Meagre (*Argyrosomus regius*) (Asso, 1801) is a marine and migratory teleost fish species which belongs to *Sciaenidae* family that can be found in the Mediterranean and Black Sea, and along the Eastern coast of the Atlantic Ocean (Kružić *et al*., 2016; Ramos-Júdez *et al*., 2019). This fish species is morphologically characterized by having a large head with small eyes, as well as large ctenoid scales with a grey colour. It has a terminal mouth with a yellow internal colour and a multitude of small sharp teeth. The lateral line is easily identifiable by the black spots it presents (Fig.1) (García, 2012). In addition, it has a large swim bladder with which it produces drumming sounds like other fish of the *Sciaenidae* family, which is why they are commonly referred to as "croakers" or "drums" (Duncan *et al*., 2013).

Fig.1: Anesthetized meagre (*Argyrosomus regius*). (Photo by Álvaro González).

This is a gonochoristic species that reaches sexual differentiation between 10 and 12 months of age, although it begins to differentiate at 5 months (Schiavone *et al*., 2012). Females are asynchronous (Gil *et al*., 2013) or group-synchronous spawners (Duncan *et al*., 2013) and in most cases fail to undergo maturation, ovulation and spawning in captivity (Duncan *et al*., 2018). Males have an unrestricted, cystic and lobular type testis (Gil *et al*., 2013) and undergo spermatogenesis and spermiogenesis, but often produce low volume with reduced quality of sperm (Duncan *et al*., 2012; Ramos-Júdez *et al*., 2019). For this reason, the use of synthetic agonist of gonadotropin-releasing hormone (GnRHa) is necessary to induce final oocyte maturation (FOM), ovulation and spawning in females, and to ensure adequate milt production in males (Fakriadis *et al*., 2020). Vitellogenesis begins in March, and oocytes reach the fully vitellogenic stage between April and June; the spermiating period, when fluid sperm can easily be extracted is between March and June (Mylonas *et al*., 2013) The sex-ratio population is 1:1 (Gil *et al*., 2013).

The meagre has had an important increase in Mediterranean aquaculture in recent years for its good feed conversion ratio (FCR) (0,9-1,2) (Monfort, 2010) (1,8) (Duncan *et al*., 2013), fast growth rates reaching 1 kg per year, large size (above 2 m and 50 kg), capability to withstand diverse environmental conditions (Monfort, 2010; Duncan *et al*., 2013; Kružić *et* al., 2016), high resilience to stress factors (Monfort, 2010), low fat content (1,06 %) and of high nutritional value (Grigorakis *et al*., 2011) and high fecundity with GnRHa treatments, achieving up to 1.415.000 eggs per kg (Duncan *et al*., 2012; Mylonas *et al*., 2016).

For these reasons, *A. regius* Mediterranean production increased 6.6% in 2018 (APROMAR, 2019) and 10,5% in 2019 (Fig.2), with the main producing countries being Egypt (32.000 t), Spain (3.650 t), Turkey (2.600 t) and Greece (1.800 t) (APROMAR, 2020); while the global fisheries production ranges from 5.000 t to 10.000 t per year (Monfort, 2010). In Spain there was an increase of 44,9% in 2019 compared to the previous year (APROMAR, 2020).

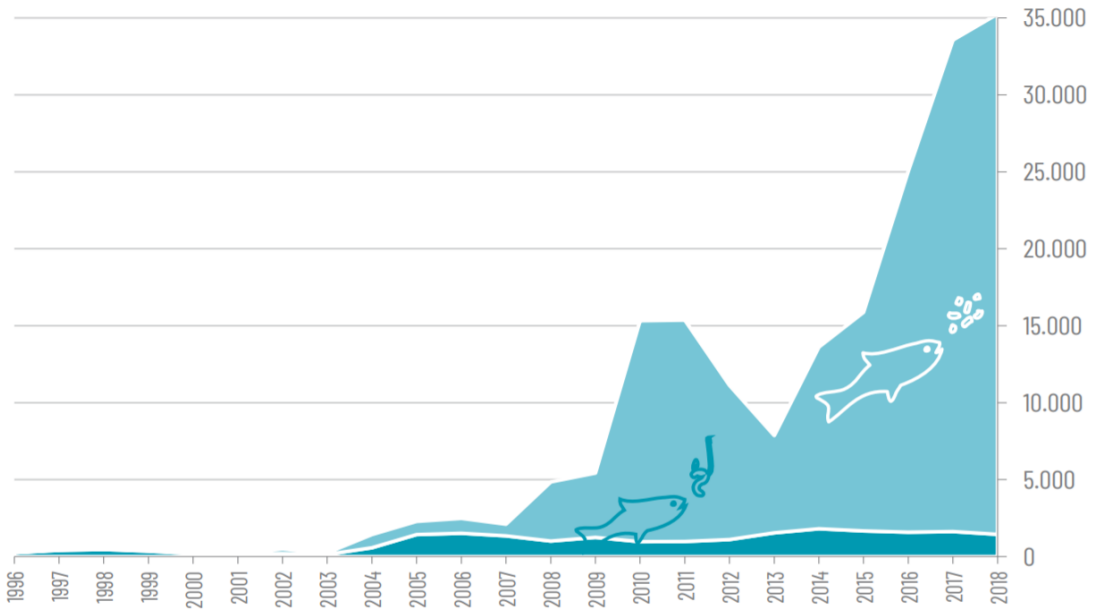

Fig.2: Evolution of the Mediterranean production of meagre (*A. regius*) in tons, through aquaculture (light blue) and fishing (dark blue), in the period 1996 - 2018 (APROMAR, 2020).

Selective breeding programmes enable an increase in production due to a higher growth rate, a reduction of production costs as a result of an improved FCR, a reduction in stress and mortality with better domestication and adaptivity to captivity, a raise in disease resistance and a better flesh quality (Gjedrem, 2000; Houston *et al*., 2020). A key factor of this process is the length of the generation interval (Houston *et al*., 2020); in *A. regius* sexual maturity is reached at two years old at 0.92 ± 0.08 kg in males, and three years old at 1.61 ± 0.09 kg in females (Schiavone

et al., 2012). One method that is being researched to reduce this generation interval is the isolation of the germ cells of the objective species at an early life stage and transplantation of these cells in a surrogate species with a shorter generation time (surrogate broodstock) (Houston *et al*., 2020). Nevertheless, some species possess low concentrations of germline stem cells in their genital glands and the efficiency of transplantation of germs cells can vary with the season (Yoshizaki and Yazawa, 2019; Goto and Saito, 2019).

Another innovative alternative to reduce the generation interval could be the use of recombinant gonadotropins (rGths) to stimulate spermatogenesis and oogenesis in immature prepubertal fish. The role of Gths in reproduction has been examined as a hormone therapy to treat infertility and other reproduction-related problems (Molés *et al*., 2020).

1.2. Fish gonadotropins and their receptors

Reproduction in vertebrates is controlled by the brain-pituitary-gonad (BPG) axis (Burow *et al*., 2019), which in turn is controlled by environmental factors (Fig.3) (Migaud *et al*., 2010). Within this system, the pituitary gonadotropins (Gths) and their receptors are the key to conveying the hormonal signals released by the BPG axis. Gths are two heterodimeric glycoproteins composed of a common alpha subunit and a specific beta subunit linked non-covalently (Schulz *et al*., 2001; Levavi-Sivan *et al*., 2010). They are synthesized and regulated by the pituitary gland through the main neuropeptide gonadotropin-releasing hormone (GnRH), in addition to others such as kisspeptines, dopamine (DA), gonadotropin-inhibitory hormone (GnIH), neuropeptide Y (NPY), serotonine, leptin, glutamate, ϒ-aminobutyric acid (GABA), norepinephrine (NA), secretoneurin, ghrelin and pituitary adenilate cyclase-activating peptide (PACAP) (Fig.3). These hypothalamic neurohormones act on the cells of the anterior lobe of the adenohypophysis (Levavi-Sivan *et al*., 2010; Carrillo *et al*., 2012; Paullada-Salmerón *et al*., 2016). Gths can stimulate sexual steroids secretion (Fig.3), which in turn positively or negatively regulate gonadotropin secretion depending on the stage of gonadal development. Estrogens and aromatizable androgens stimulate β-LH synthesis in juveniles of various teleost species, while an increase in 17β-estradiol (E2) or testosterone (T) inhibits β-FSH synthesis in rainbow trout (*Oncorhynchus mykiss*) (Yaron and Levavi -Sivan, 2011), coho salmon (*Oncorhynchus kisutch*) (Dickey and Swanson, 1998) and Atlantic salmon (*Salmo salar*) (Borg *et al*., 1998). Otherwise, progestins stimulate β-FSH synthesis in zebrafish (*Danio rerio*) (Wang *et al*., 2016). In addition, it has been observed that in in-vitro studies, the gonadal peptides activin A and B stimulate β-FSH synthesis and reduce β-LH

synthesis, although in turn, they are regulated by follistatin and activin-binding protein (Levavi-Sivan *et al*., 2010; Yaron and Levavi-Sivan, 2011).

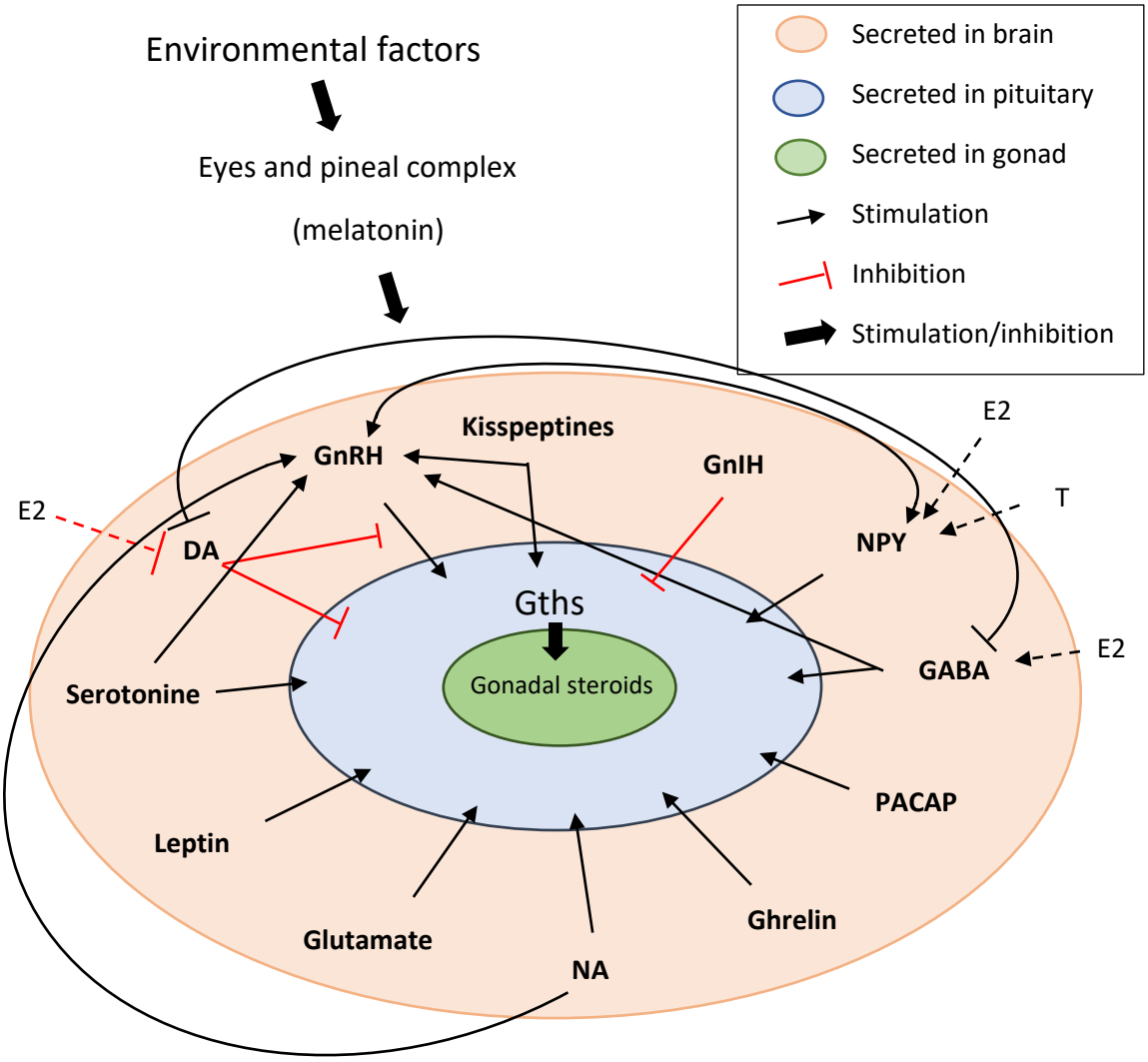

Fig.3: Representative diagram of the brain pituitary-gonad (BPG) axis that controls the reproductive process in fish. GnRH, gonadotropin-releasing hormone; GnIH, gonadotropin-inhibitory hormone; NPY, neuropeptide Y; GABA, Y-aminobutyric acid; PACAP, pituitary adenilate cyclase-activating peptide; NA, norepinephrine/noradrenaline; DA, dopamine; E2, 17β-estradiol; T, testosterone. By Álvaro González.

Follicle stimulating hormone (FSH) stimulates the early stages of gametogenesis, including spermatogenesis in males and oogenesis in females through the synthesis of 17β-estradiol (E2) and 11-ketotestosterone (11-KT) (Melamed *et al*., 1998) via the membrane-bound receptors Fshra, which belongs to the family of G-protein-coupled receptors (GPCRs). In males, Fshra can be expressed in Sertoli and Leydig somatic cells; and in females in the follicular somatic cells of

granulosa and theca as well as in connective tissue (Yaron and Levavi-Sivan, 2011; Chauvigné *et al*., 2012; Ozaki *et al*., 2019).

Luteinizing hormone (LH) is related to the final oocyte maturation (FOM) and ovulation in females (Kwok *et al*., 2005), and the later stages of germ cell development and the differentiation of spermatids to spermatozoa in males (Chauvigné *et al*., 2012) by the production of maturation induction steroid (MIS) (Nagahama, 2008) through Lhcgrba membrane-bound receptors (GPCRs) activation. In males it is expressed in spermatids and in Leydig cells (Yaron and Levavi-Sivan, 2011; Chauvigné *et al*., 2012). García-Lópes *et al*., (2010) described the presence of Lhcgrba in Sertoli cells in zebrafish (*D. rerio*), being the first report of the presence of these receptors in Sertoli cells in vertebrates. In females, Lhcgrba are found in granulosa cells (Yaron and Levavi-Sivan, 2011). It has been observed that in some teleosts Fshra also respond to LH (Kobayashi *et al*., 2008; García-Lópes *et al*., 2010; Burow *et al*., 2019; Rajakumar and Senthilkumaran, 2020), but when there are peaks of this hormone as in the spawning season (Schulz *et al*., 2010). In rainbow trout (*O. mykiss*), Lhcgrba also responded to supraphysiological purified FSH, although at concentrations five times higher than LH (Sambroni *et al*., 2007). Both receptors can be expressed in non-gonadal tissues such as brain, gills, eyes, intestine, kidney or liver (Kwok *et al*., 2005; Kobayashi *et al*., 2008; Burow *et al*., 2019).

In females, Fshra mRNA expression (*fshra*) is detectable at low levels in immature ovaries. Its expression increases during vitellogenesis reaching its maximum expression in the midvitellogenic stage and decreasing in the postovulatory follicle state. The expression of *lhcgrba* is detectable in the previtellogenesis stage, although there is an increase in transcription with the onset of vitellogenesis, reaching the peak of expression at the full-grown stage (Kwok *et al*., 2005; Kobayashi *et al*., 2008; Carrillo *et al*., 2012) or maturation / ovulation (Sambroni *et al*., 2007; Rocha *et al*., 2009). In males, the expression of *fshra* and *lhcgrba* was found in all testis stage, from immature to fully mature, although their expression increased during the transition to fully mature stage (spermiation) (Kusakabe, 2006; Sambroni *et al*., 2007; Maugars and Schmitz, 2007; Rocha *et al*., 2009; García-Lópes *et al*., 2009; Burow *et al*., 2019). In contrast, in Shortfinned eel (*Anguilla australis*) with hCG-induced spermatogenesis, *fshra* expression increased in early stages, while *lhcgrba* increased since spermatids were observed (Ozaki *et al*., 2019). In Nile tilapia (*O. niloticus*) there is *fshra* and *lhcgrba* during sexual differentiation period (Yan *et al*., 2012).

1.3. Steroidogenesis in teleosts

Sex steroids stimulate gametogenesis and are related to sex differentiation in different vertebrate taxa (Nakamura, 2010; Morohashi *et al*., 2013). Sexual steroidogenesis is regulated by Gths, and begins with the transport of cholesterol to the mitochondria by the steroidogenic acute regulatory protein (StAR), where it is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc / Cyp11a1). Pregnenolone is the precursor of various steroids, including E2, 11-KT, T, progestogens, and corticosteroids (Rajakumar and Senthilkumaran, 2020).

In females, gonadal steroidogenesis is explained according to the two-cell model described by Young *et al*. (1986), in which FSH-stimulated theca cells synthesize precursors for their subsequent transformation into estrogens and progestogens in granulosa cells when stimulated with LH (Fig.4). Nevertheless, LH and FSH have similar potencies in stimulating E2 production during early phases of oogenesis; yet, LH is more potent during later stages of the process (Cerdá *et al*., 2007). First, pregnenolone is transformed into progesterone in theca cells by the enzyme 3β-hydroxy-steroid dehydrogenase (3β-HSD). Progesterone is converted back in theca cells to 17α-hydroxy-progesterone by the action of the enzyme 17-hydroxylase (P450c17 / Cyp17). The synthesis of 3β-HSD is stimulated by FSH and LH (Nakamura *et al*., 2003).

For E2 synthesis, 17α-hydroxy-progesterone is transformed into androstenedione by the enzyme P450c17 / Cyp17, which in turn is converted into T by the activity of the enzyme 17β-hydroxy-steroid dehydrogenase (17β-HSD). Finally, T can be catalyzed to E2 by the enzyme cytochrome P450 aromatase (P450arom / Cyp19a) in granulosa cells (Nagahama *et al*., 1994; Young *et al*., 2005; Nakamura *et al*., 2005; Nagahama *et al*., 2008; Carrillo *et al*., 2012). The synthesis of P450arom / Cyp19a is stimulated by FSH (Rocha *et al*., 2009), LH and insulin growth factor I (IGF-I) (Kagawa *et al*., 2003; Nakamura *et al*., 2003; Montserrat *et al*., 2004). Its expression in the ovary increases during vitellogenesis (Chang *et al*., 2005).

For progestogens synthesis, 17α-hydroxy-progesterone crosses the basement membrane and is converted in granulosa cells to MIS by the enzyme 20β-hydroxy-steroid dehydrogenase (20β-HSD). This enzyme is synthesized in granulosa cells in response to LH. Two progestins have been identified that act as MIS in teleosts, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP; 20βP) and 17, 20β, 21-trihydroxy-4-pregnen- 3-one (20βS) (Young *et al*., 1986; Nagahama *et al*., 1994; Thomas *et al*., 2001; Tanaka *et al*., 2002; Carrillo *et al*., 2012); although any steroid capable of breaking down the germ vesicle (GBVD) such as 11-deoxycorticosterone (DOC) or T are also considered as MIS (Nagahama *et al*., 2008). In *Sciaenidae* family, 20βS was shown to act as MIS (Peter and Yu, 1997; Senthilkumaran *et al*., 2002; Fakriadis *et al*., 2020).

Therefore, E2 is produced during oocyte growth, and the progestins (DHP or 20βS) during maturation. The change in the steroidogenic pathway occurs in fish ovarian follicles immediately prior to oocyte maturation (Nagahama and Yamashita, 2008). This change could occur due to the cellular levels of the enzymes P450c17 and 3β-HSD, so that when P450c17 is dominant, the synthesis of E2 is favoured and vice versa (Sakai *et al*., 1994; Nakamura *et al*., 2005).

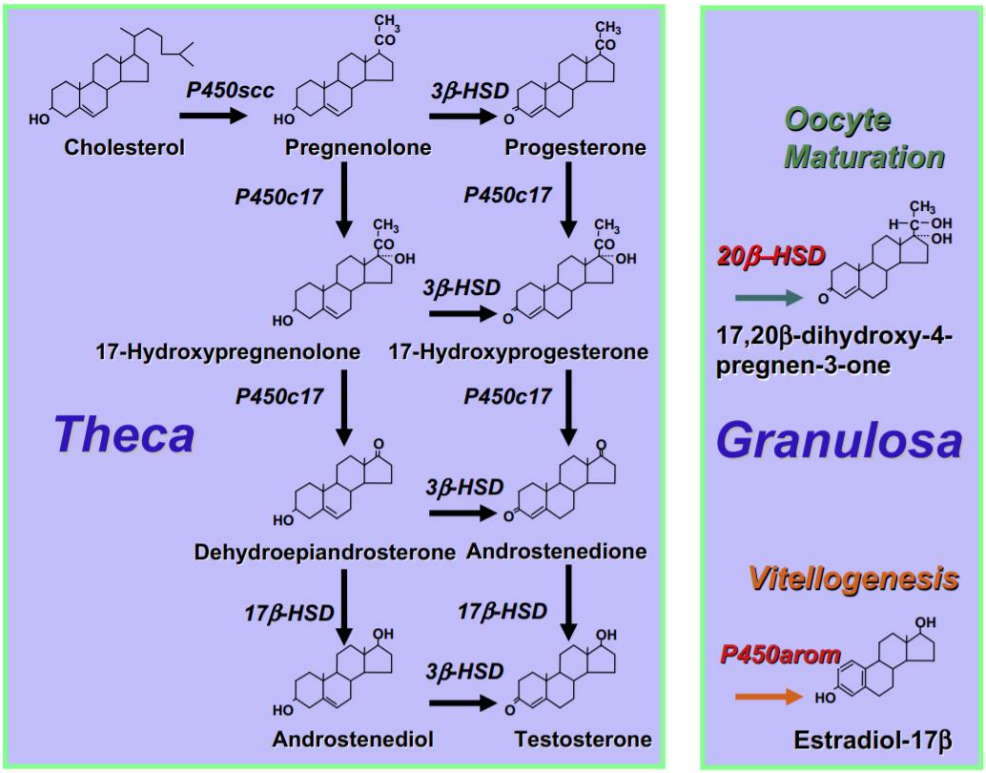

Fig.4: Representative diagram of the two-cell model described by Young *et al*., (1986) for female gonadal steroidogenesis. P450scc, P450 side-chain cleavage; P450c17, 17-hydroxylase/C17-C20-lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 20β-HSD, 20β-hydroxysteroid dehydrogenase; P450arom, P450 aromatase (Luzbens *et al*., 2010).

In males, gonadal steroidogenesis is not as well described as in females. The steroids required for spermatogenesis are E2, 11-KT, and DHP / 20βS. The P450arom / Cyp19a enzyme is expressed in the testis (Blázquez and Piferrer, 2004; Chang *et al*., 2005), allowing the synthesis of E2 from T.

For androgens synthesis (11-KT and T), FSH and LH are equipotent in stimulating its production by upregulating expression of steroidogenic enzyme genes (Suzuki *et al*., 2019). However, LH is more potent in stimulating DHP / 20βS (Planas and Swanson, 1995; Kazeto *et al*., 2008). For the synthesis of 11-KT, androstenedione is converted into 11β-hydroxyandrostenedione (11β-OHA) by the enzyme 11β-hydroxylase (P45011β / Cyp11b) (Rajakumar and Senthilkumaran, 2015). Then, 17β-HSD converts 11β-OHA on 11β-hydroxytestosterone (11β-OHT). Furthermore, T can be converted to 11β-OHT by P45011β / Cyp11b (Kusakabe *et al*., 2003). Finally, the enzyme 11-

beta-hydroxysteroid-dehydrogenase (11β-HSD) converts 11β-OHT into 11-KT (Fig.5) (Kusakabe *et al*., 2003; Ozaki *et al*., 2006; Suzuki *et al*., 2020). The steroid 11-KT stimulates its own production based on positive feedback, it enhanced *cyp11b* expression in testis (Ozaki *et al*., 2019). Furthermore, an increase in 11-KT following hCG injection elevated testicular *fshra* mRNA levels augmenting FSH sensitivity in the testis (Ozaki *et al*., 2016). Progestin production is catalyzed by 20β-HSD from 17α-hydroxy-progesterone in Leydig cells, spermatogonia and spermatocytes (Sreenivasulu *et al*., 2012). The DHP / 20βS stimulates the synthesis of 11-KT by increasing the expression of 11β-HSD (Ozaki *et al*., 2006).

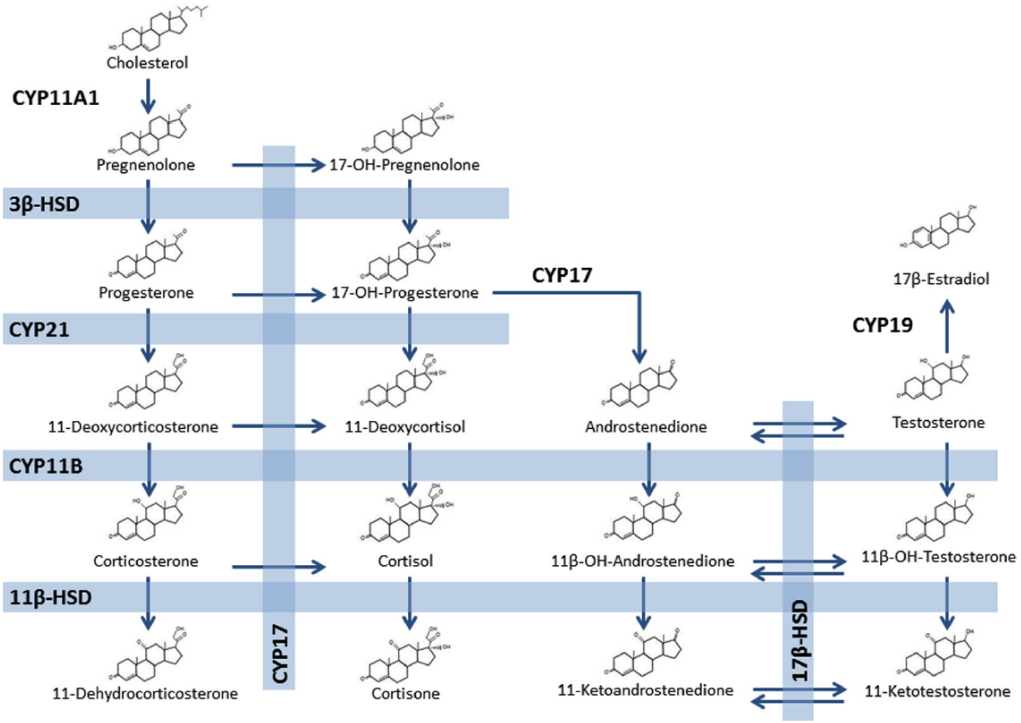

Fig.5: Simplified pathways of biosynthesis of sex steroids and glucocorticoids in fish. CYP11A1, cholesterol side-chain cleavage enzyme (P450scc); 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, P450 aromatase (P450arom); CYP17, 17-hydroxylase/C17-C20-lyase (P450c17); 11β-HSD, 11-beta-hydroxysteroid-dehydrogenase, CYP11B, 11β-hydroxylase (P45011β) (Fernandino *et al*., 2013).

1.4. Oogenesis and spermatogenesis in teleosts

Spermatogenesis is the process during which immature diploid spermatogonia develop into mature, fertile haploid spermatozoa (Ozaki *et al*., 2019). In spermatogenesis there is a self-renewal of spermatogonia stimulated by E2 released by Leydig cells. The steroid E2 interacts with its receptors on Sertoli cells (Miura *et al*., 1999), which synthesize spermatogonial stem-cell renewal factor (Gonadal soma-derived growth factor; GSDF) (Fig.6) (Satawari *et al*., 2007; Yaron and Levavi -Sivan, 2011). Undifferentiated A spermatogonia (StgA_{und}) have the capacity

for self-renewal, however, it is not clear whether differentiated A spermatogonia (StgA_{diff}) in teleosts have the capacity to return to a more undifferentiated state of development (StgA_{und}) (Lacerda *et al*., 2014) ; although Huckins (1971) showed that in mammals they have this capacity. The secretion of 11-KT in Leydig cells stimulates in Sertoli cells the synthesis of mediators that regulate the proliferation of more differentiated spermatogonia cells (spermatogonia B; Stg*B*) for their subsequent entry into meiosis (Schulz *et al*., 2010; Lacerda *et al*., 2014). Activin B and insulin growth factor I (IGF-I) are the mediators that stimulate differentiation to Stg*B*, while anti-Müllerian hormone (AMH) and eSRS21 inhibit it (Halm *et al*., 2007; Carrillo *et al*., 2012). Induction of meiosis (formation of spermatocytes to develop into spermatids) is stimulated by progestins (Miura *et al*., 2006; Yaron and Levavi-Sivan, 2011). Sperm maturation (capability of motility and fertilization) is also produced by DHP, which increases the pH of seminal plasma by increasing the cAMP content in sperm after activation of carbonic anhydrase (CA / eSR22) (Fig.6) (Miura and Miura, 2003). Spermiation (sperm are released into the lumen of the seminiferous tubule) is induced by DHP and 11-KT (Miura *et al*., 2006; Schulz *et al*., 2010).

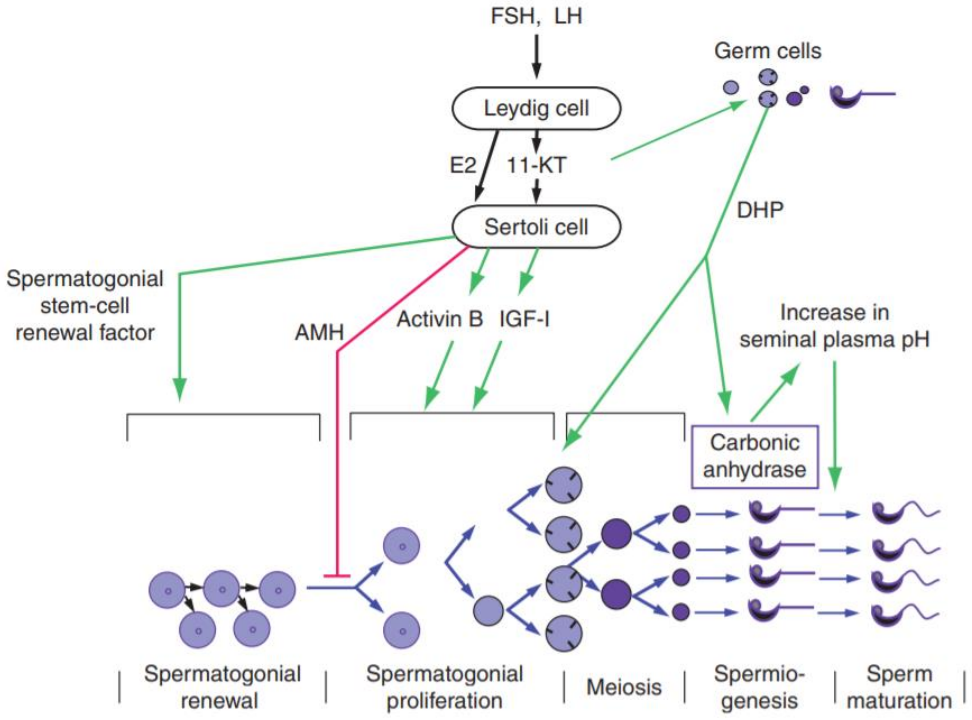

Fig.6: Endocrine mechanisms regulating spermatogenesis in the Japanese eel (*Anguilla japonica*). 11-KT, 11-ketotestosterone; AMH, a peptide homologous to anti-Müllerian hormone; DHP, 17α,20β-dihydroxy-4-pregnen-3-one; IGF-I, insulin-like growth factor I. Cells at the upper right corner depict the germ cells as the source of DHP acting upon themselves in a paracrine or autocrine manner. Modified from Yaron and Levavi-Sivan (2011).

Oogenesis is the formation, development and maturation of the female gamete and ovum (Arukwe and Goksøyr, 2003). In oogenesis there is mitotic renewal of oogonia stimulated by E2, while the induction of meiosis is stimulated by progestogens (Fig.7) (Miura *et al*., 2007; Luzbens *et al*., 2010). The expression of IGF-I has also been observed in oocytes and somatic cells at the onset of meiosis in Nile tilapia (*Oreochromis niloticus*) (Berishivili *et al*., 2006). The role of FSH in regulating the accumulation of cortical alveoli is currently unknown, however, the synthesis of cortical alveoli is related to an increase in plasma and pituitary FSH, Fshra, plasma E2, *cyp19a* mRNA, AMH, *StAR* mRNA and GSDF (Kwok *et al*., 2005; Rodríguez-Marí *et al*., 2005; Campbell *et al*., 2006). In the oocyte lipidation there is an accumulation of lipids from the precursor triacyl glyceride (TAG)-rich serum lipoprotein such as very-low-density lipoprotein (VLDL) (Hiramatsu *et al*., 2015). In rainbow trout (*O. mykiss*) VLDL is sufficient to induce lipidation, while in Shortfinned eel (*A. australis*) and Japanese eel (*A. japonica*) VLDL and 11-KT are required (Lokman *et al*., 2007; Endo *et al*., 2011).

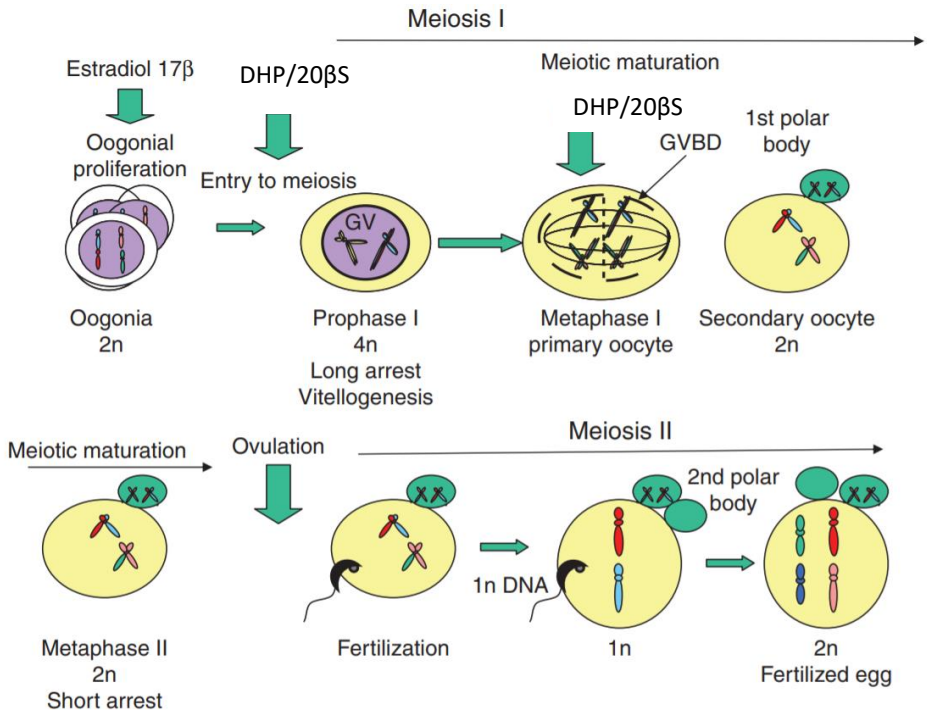

Fig.7: Stages of fish oogenesis and their endocrine regulation. DHP, 17α,20β-dihydroxy-4-pregnen-3-one; 20βS, 17,20β, 21-trihydroxy-4-pregnen- 3-one; GVBD, germinal vesicle breakdown. Modified from Yaron and Levavi-Sivan (2011).

Vitellogenesis begins with the synthesis of vitellogenin, a phosphoglycoprotein synthesized in the liver and regulated mainly by E2 (Fig.8) (Miura *et al*., 2007). After their release and transport into blood plasma, vitalogenins (Vtg) are incorporated into the oocyte through the Vtg receptor (Vtg-R) in the ovary, resulting in the formation of yolky eggs from oocytes (Cohen and Smith

2014). Yolk is a source of nutrients for embryogenesis and facilitates hydration in buoyant eggs (Kwon *et al*., 2001).

The final maturation of the oocyte (FOM) is related to an increase in the synthesis of LH, Lhcgrba and MIS. MIS activates its receptors on the oocyte surface (GPCRs) reducing the levels of cAMP and protein kinase A (PKA), which in turn activates the maturation promoting factor (MPF) (Fig.8) (Nagahama *et al*., 1994; Nagahama *et al*., 2008; Luzbens *et al*., 2010). The MPF is activated by phosphorylation of the Cdc2 subunit on threonine 161 (T161) by cdk-activating kinase (CAK). Phosphorylation occurs when Cdc2 is bound to cyclin B, and induces GVBD and the continuation of meiosis (until now the oocyte was arrested in metaphase II by the activity of the Mos complex) (Nagahama *et al*., 2008; Carrillo *et al*., 2012). During ovulation, the oocyte is released from its follicle into the ovarian cavity or into the abdominal cavity (Luzbens *et al*., 2010). It is induced by MIS (Nagahama and Yamahita, 2008) and prostaglandins (PGs) (Fig.8) (Peter and Yu, 1997; Takahashi *et al*., 2013). In the hydration of the oocyte in pelagophil teleosts, water enters through the aquaporins due to the increase in osmotic pressure. This osmotic pressure is generated by free ions (K^+, Cl^-, NH_4^+…) and free amino acids (FAAs) pool, product of the hydrolysis of vitalogenins (Finn *et al*., 2002; Cerdá *et al*., 2007).

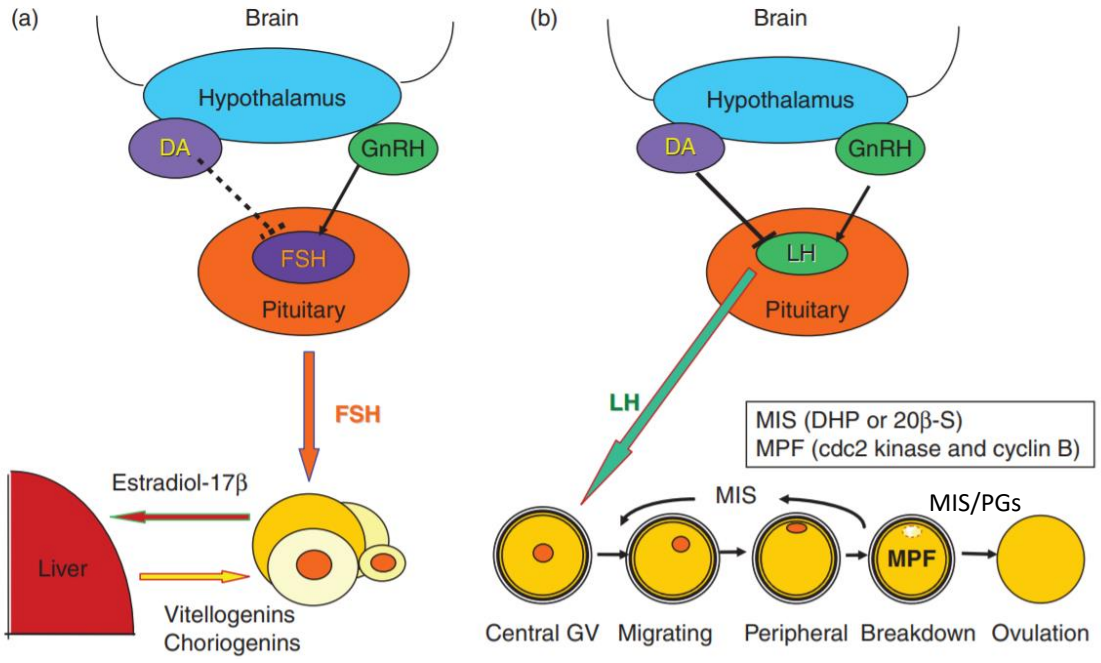

Fig.8: (a) an overview of the endocrine chain, brain–pituitary–gonadal axis (BPG axis) in model female fish during the vitellogenic phase. (b) An overview of the BPG axis during final oocyte maturation and ovulation. DA, dopamine; GnRH, gonadotropin-releasing hormone; MIS, maturation induction steroid; DHP, 17α,20β-dihydroxy-4-pregnen-3-one; 20βS, 17,20β, 21-trihydroxy-4-pregnen- 3-one; MPF, maturation promoting factor. Modified from Yaron and Levavi-Sivan (2011).

1.5. Fish recombinant gonadotropins

Recombinant hormones (rGths) have the advantage that they guarantee no cross-contamination with other related glycoproteins and can be produced continuously without depending on the purification of the native hormones from fish pituitary glands, which is costly and time-consuming process (Levavi-Sivan *et al*., 2010). For the production of rGTHs, the isolated cDNA is cloned in expression vectors (plasmids or viruses) that are subcloned in heterologous prokaryotic or eukaryotic systems (Molés *et al*., 2020). There are several expression systems such as *Escherichia coli* (Hew *et al*., 1989; Cao *et al*., 2009), the yeast *Pichia pastoris* (Kamei *et al*., 2003; Kasuto and Levavi-Sivan, 2005; Sanchís-Benlloch *et al*., 2017; Nocillado *et al*., 2019), *Drosophila* S2 cells (Zmora *et al*., 2007; Kazeto *et al*., 2008), Chinese hamster ovary (CHO) cells (Chauvigné *et al*., 2012; Chauvigné *et al*., 2017; Ramos-Júdez *et al*., 2019), silkworm larvae (Hayakawa *et al*., 2009; Kobayashi *et al*., 2010), the soil amoeba *Dictyostelium discoideum* (Vischer, 2003), Sf9 insect cells (Cui *et al*., 2007; Molés *et al*., 2011; Morita *et al*., 2004) and FreeStyle 293-F cells (Kazeto *et al*., 2019; Suzuki *et al*., 2019). The choice of the expression system depends on cost, hormone secretion, correctly folding and glycosylation, which is essential for the biological activity of the rGths (Levavi-Sivan *et al*., 2010).

Baculovirus-infected insect cells produce higher amounts of rGths (Cui *et al*., 2007). However, post-transcriptional modification, as in *P. pastoris*, does not allow the production of complex products with terminal sialic acids, generating a reduction in the half-life of the protein (Levavi-Sivan *et al*., 2008). CHO cells are able to produce glycosylated proteins with terminal sialic acids, which makes them excellent for in vivo administration (Molés *et al*., 2020), however CHO cells are expensive expression systems too (Cui *et al*., 2007). Silkworm larvae, soil amoeba, fish embryos or *P. pastoris* are less expensive (Morita *et al*., 2004; Levavi-Sivan *et al*., 2010). Prokaryotic expression systems (*E. coli*) are also less expensive, but they are not capable of performing N-glycosylations (Molés *et al*., 2020).

For the expression of the dimer there are two possibilities: a) transfecting a single plasmid with a cDNA encoding both subunits joined by a linker (single-chain) (Cui *et al*., 2007), which is usually composed of histidine amino acids (Aizen *et al*., 2007a), the carboxy-terminal peptide (CTP) from the human (hCG) or equine chorionic gonadotropin (eCG) (Morita *et al*., 2004; Chauvigné *et al*., 2017), three Gly-Ser pairs (or other variations of Gly- Ser) (Palma *et al*., 2019; Kazeto *et al*., 2019) or a synthetic N-linked glycosylation sequence (NCS) (Kim *et al*., 2012); b) co-transfecting two expression plasmids, one with the alpha subunit and the other with the beta subunit (Kobayashi *et al*., 2006). However, the fusion of the two monomers without a linker or the expression of the

two monomers separately usually generates misfolding, low yield in protein production or impaired bioactivity (Molés *et al*., 2020).

The rGths have been successfully used in various fish species. Until now, at the steroidogenic level it has been tested in 18 species (Table 1), while at the level of gonadal development it has been tested in 16 species (Table 2).

Table 1: Review of publications where rGths have been used to stimulate steroid secretion. All significant data are compared to control groups unless otherwise indicated. The best results obtained from all the treatments used in each survey are shown. Recombinant hormones are species specific unless otherwise indicated. Data is displayed in chronological order of publication. E2, 17β-estradiol; T, testosterone; 11-KT, 11-ketotestosterone; OHA, 11β-hydroxyandrostenedione; SGv, vitellogenic stage of secondary growth; OM, maturation stage.

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Steroidogenesis**	**Reference**
Japanese eel (*Anguilla japonica*)	Male	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Stimulation of E2 and 11-KT secretion while control did not stimulate steroid production (in-vitro)	Kamei *et al*., 2003
African catfish (*Clarias gariepinus*)	Male	rGths	Alone	Amoeba (*Dictyostelium discoideum*)	MB12n	Stimulation of OHA (in-vitro)	Vischer, 2003
Nile tilapia (*Oreochromis niloticus*)	Male	rLH	Single-chain/alone (just βLH)	Yeast (*Pichia pastoris*)	pGEM-T Easy Vector and pPIC9K	Single-chain gonadotropin showed a stimulation of 11-KT secretion (in-vitro) βLH did not show any stimulation of 11-KT secretion (in-vitro)	Kasuto and Levavi-Sivan, 2005
Japanese eel (*Anguilla japonica*)	Female	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Significant stimulation of E2 and T secretion (in-vitro)	Kamei *et al*., 2006
Goldfish (*Carrassius auratus*)	Both	rGths	Single-chain	silkworm (*Bombyx mori*) larvae	Pyng and baculovirus	Significant increase in plasma concentration of E2 and T in females and males respectively	Kobayashi *et al*., 2006
Orange-spotted grouper (*Epinephelus coioides*)	Female protogynous	rLH	Alone	Sf9 insect cells	pFastBacDual bacmid transfer vector and baculovirus	Significant increase in E2 and T secretion compared with hCG treatment group (in vitro)	Cui *et al*., 2007
Rainbow trout (*Oncorhynchus mykiss*)	Female	rGths (*Brachymystax lenok*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Stimulation of E2 and T secretion (in-vitro) No significant differences in E2 and T stimulation (in-vivo)	Ko *et al*., 2007

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Steroidogenesis**	**Reference**
Nile tilapia (*Oreochromis niloticus*)	Both	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Stimulation of E2 and 11-KT secretion in ovarian and testes respectively (in-vitro)	Aizen *et al*., 2007b
Channel catfish (*Ictalurus punctatus*)	Both	rGTHs	Single-chain	Drosophila S2 cell line	pMT	Stimulation of E2 and OHA secretion in ovary and testes respectively (in-vitro)	Zmora *et al*., 2007
Japanese eel (*Anguilla japonica*)	Male	rGths (*Carrassius auratus)*	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Significant increase in plasma 11-KT concentration	Hayakawa *et al*., 2008a
Japanese eel (*Anguilla japonica*)	Female	rGths (*Brachymysta x lenok*)	-	-	-	Significant increase in plasma E2 and T concentration	Kim *et al*., 2008
Japanese eel (*Anguilla japonica*)	Male	rGths (*Carrassius auratus*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	No significant differences in plasma 11-KT concentration	Hayakawa *et al*., 2008b
Japanese eel (*Anguilla japonica*)	Male	rGths	Alone	Drosophila S2 cell line	pMT/V5-His	Significant increase in 11-KT secretion (in vitro)	Kazeto *et al*., 2008
Zebrafish (*Danio rerio*)	Male	rGths	-	-	-	Stimulation of 11-KT and OHA secretion (in-vitro) Significant increase in plasma concentration of 11-KT	García-Lópes *et al*., 2010
Flatfish Senegalese sole (*Solea senegalensis*)	Male	rGths	Single-chain	CHO cells	pcDNA3	Stimulation of T and 11-KT secretion (in-vitro) Significant increase in plasma T and 11-KT concentration	Chauvigné *et al*., 2012
Cinnamon clownfish (*Amphiprion melanopus*)	Both	rGths	Single-chain	*Escherichia coli*	pYNG	Significant increase concentration of E2 (in vitro)	Kim *et al*., 2012
Orange-spotted grouper (*Epinephelus coioides*)	Female protogynous	rFSH	Single-chain/alone	Yeast (*Pichia pastoris*)	pPICZαA	Single-chain and alone rFSH showed stimulation of E2 and T (in-vitro) Single-chain and alone rFSH showed a significant increase in plasma E2 and T concentration	Chen *et al*., 2012a
Carp (*Cyprinus carpio*)	Female	rGths (*Oreochromis niloticus*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Very low stimulation of E2 in early SGv stage oocytes (in-vitro) No significant stimulation of E2 in mid SGv stage oocytes Significant increase of E2 in post SGv stage oocytes	Aizen *et al*., 2012

Species	Sex	rGth	Subunit construct	Expression system	Expression vector	Steroidogenesis	Reference
						Significant increase of E2 in OM stage oocytes No significant stimulation of DHP in early and mid SGv stage oocytes Significant increase of DHP in post SGv stage and OM stage oocytes	
Carp (*Cyprinus carpio*)	Female	rGths (*Brachymistax lenok*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Very low stimulation of E2 in early SGv oocytes (in-vitro) No significant stimulation of E2 in mid SGv stage oocytes Significant increase of E2 in post SGv stage oocytes No significant stimulation of E2 in OM stage oocytes No significant stimulation of DHP in early, mid and post SGv stage, and OM stage oocytes	Aizen *et al*., 2012
Carp (*Cyprinus carpio*)	Female	rGths (*Anguilla japonica*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Very low stimulation of E2 in early SGv stage oocytes (in-vitro) Stimulation of E2 in mid SGv stage oocytes Significant increase of E2 in post SGv stage oocytes No significant stimulation of E2 in OM stage oocytes No significant stimulation of DHP in early and mid SGv stage oocytes Significant increase of DHP in post SGv stage and OM stage oocytes	Aizen *et al*., 2012
European Sea Bass (*Dicentrarchus labrax*)	Male	rFSG	Single-chain	CHO cells	pGEM-T Easy Vector	Significant increase in plasma 11-KT concentration	Mazón *et al*., 2013
Shortfinned eel (*Anguilla australis*)	Both	rFSH	Alone	Drosophila S2 cell line	pMT/V5-His	No significant differences in stimulation of E2, 11-KT, OHA, T and DHP (in-vitro)	Reid *et al*., 2013
Russian sturgeon (*Acipenser gueldenstaedtii*)	Both	rGths	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Stimulation of E2 and 11-kt secretion in ovarian and testes respectively (in-vitro)	Yom-Din *et al*., 2016
Carp (*Cyprinus carpio*)	Female	rLH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Significant increase in plasma concentration of E2 and DHP	Aizen *et al*., 2016
European eel (*Anguilla anguilla*)	Male	rGths	Single-chain	CHO cells	-	Significant increase in plasma 11-KT and T concentration compared to the initial levels	Peñaranda *et al*., 2017
Yellowtail kingfish (*Seriola lalandi*)	Both	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Significant increase of E2 and 11-KT concentration compared to the control group in ovarian and testes respectively (in-vitro)	Sanchís-Benlloch *et al*., 2017

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Steroidogenesis**	**Reference**
						Significant increase in plasma E2 concentration in females and significant decrease in plasma concentration of 11-KT in males	
Flatfish Senegalese sole (*Solea senegalensis*)	Male	rGths	Single-chain	CHO cells	pcDNA3	Significant increase in plasma 11-KT concentration	Chauvigné *et al*., 2017
Spotted scat (*Scatophagus argus*)	Both	rGths	Single-chain	*Escherichia coli*	pMD18-T	Significant increase in plasma concentration of E2 and 11-KT in females and males respectively	Zhang *et al*., 2018
Japanese eel (*Anguilla japonica*)	Male	rGths	Single-chain	FreeStyle 293-F cells	pCAGGS	Significant increase in 11-KT secretion (in vitro)	Suzuki *et al*., 2019
Brown-marbled grouper (*Epinephelus fuscoguttatus*)	Female protogynous	rFSH (*Epinephelus lanceolatus*)	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Significant increase in plasma concentration of E2 and T	Palma *et al*., 2019
Flathead grey mullet (*Mugil cephalus*)	Both	rGths	Single-chain	CHO cells	pGEM-T Easy vector	Significant increase in plasma concentration of E2 and 11-KT in females and males respectively	Ramos-Júdez *et al*., 2020

Table 2: Review of publications in which rGths are used to stimulate gonadal development. All significant data are compared to control groups unless otherwise indicated. The best results obtained from all the treatments used in each survey are shown. Recombinant hormones are species specific unless otherwise indicated. Data is displayed in chronological order of publication. The terminology used for the germ cells maturation stages has been homogenized according to the terminology used in this survey. Stg*A*, type A; Stg*B*, type B spermatogonia; Spc, spermatocytes; Spd, spermatids; Spz, spermatozoa; PGcn, oocyte at primary growth of chromatin nucleolar stage; PGps, oocyte at perinucleolar stage of primary growth; SGca, cortical alveoli stage of secondary growth; SGv, vitellogenic stage of secondary growth; GVBD, germinal vesicle breakdown. Alone refers to the fact that the two subunits are not expressed together by a linker (single-chain).

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Gonadal development stimulation**	**Reference**
Japanese eel (*Anguilla japonica*)	Male	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Increased in germ cell proliferation with the appearance of Stg*B*, spc and spd; while control group just had Stg*A* (in-vitro)	Kamei *et al*., 2003
Goldfish (*Carrassius auratus*)	Both	rGths	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Females were in vitellogenesis, and ovulation did not occur Induction of milt production while control group did not	Kobayashi *et al*., 2006
Bitterling (*Rhodeus ocellatus ocellatus*)	Female	rGths (*Carrassius auratus)*	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Induced ovulation	Kobayashi *et al*., 2006
Rainbow trout (*Oncorhynchus mykiss*)	Female	rGths (*Brachymysta x lenok*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Significant increase in GSI and follicular diameters Oocytes at early SGca stage were presented in treated females while control females presented oocytes at PGcn and PGps stages	Ko *et al*., 2007
Rainbow trout (*Oncorhynchus mykiss*)	Female	rGths (*Brachymysta x lenok*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	No significant differences in GSI Significant increase in follicular diameters There was no induction of ovulation	Park *et al*., 2007
Goldfish (*Carrassius auratus*)	Male	rGths (*Brachymysta x lenok*)	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Induced milt production	Ko *et al*., 2007
Japanese eel (*Anguilla japonica*)	Male	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	More percentage of cysts of late Stg*B* than control group (in-vitro)	Ohta *et al*., 2007
Japanese eel (*Anguilla japonica*)	Male	rGths (*Carrassius auratus)*	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Significant increase in GSI Induced spermatogenesis, with the appearance cysts containing spz, while control group had Stg*B*	Hayakawa *et al*., 2008a

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Gonadal development stimulation**	**Reference**
						Induced milt production while control group did not	
Japanese eel (*Anguilla japonica*)	Male	rGths (*Carrassius auratus)*	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	No significant differences in GSI Induced spermatogenesis, with the appearance cysts containing spz, while control group had Stg*A* There was no induction of milt production	Hayakawa *et al*., 2008b
Japanese eel (*Anguilla japonica*)	Female	rGths (*Brachymysta x lenok*)	-	-	-	Significant increase in GSI Significant increase in follicular diameters	Kim *et al*., 2008
Japanese eel (*Anguilla japonica*)	Both	rGths	Alone	Drosophila S2 cell line	pMT/V5-His	No significant differences in GSI Oocytes at SGv stage were presented in treated females while control females presented oocytes at SGca stage Entire process of spermatogenesis was presented in treated group while control group presented Stg*A* or Stg*B* (in-vitro) Late Stg*B* were presented in treated males while control males presented germ cells before proliferation	Kazeto *et al*., 2008
Goldfish (*Carrassius auratus*)	Male	rGths	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Induced milt production	Hayakawa *et al*., 2008b
Bitterling (*Rhodeus ocellatus ocellatus*)	Female	rGths (*Carrassius auratus)*	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Induced ovulation	Hayakawa *et al*., 2008b
Japanese eel (*Anguilla japonica*)	Both	rGths	Single-chain	silkworm (*Bombyx mori*) larvae	pYNG and baculovirus	Significant increase in GSI Induction of GVBD of oocytes (in-vitro) Induced spermatogenesis with the appearance cysts containing spz, while control group had Stg*A*	Kobayashi *et al*., 2010
Orange-spotted grouper (*Epinephelus coioides*)	Female protogynous	rFSH	Single-chain/alone	Yeast (*Pichia pastoris*)	pPICZαA	Single-chain and alone rFSH stimulated early ovarian development with the appearance of oocytes at PGcn and PGps, while control group had no oocytes	Chen *et al*., 2012a
European Sea Bass (*Dicentrarchus labrax*)	Male	rFSH	Single-chain	CHO cells	pGEM-T Easy Vector	Increased in germ cell proliferation and cysts of spc and spd were observed, while control group presented just Stg*A*. Furthermore, the treated groups had a Sertoli cell proliferation.	Mazón *et al*., 2013
Medaka (*Oryzias latipesr*)	Female	rLH	Single-chain	CHO cells	pEB Multi-Neo vector	Induced GVBD of oocytes (in-vitro)	Ogiwara *et al*., 2013

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Gonadal development stimulation**	**Reference**
Japanese eel (*Anguilla japonica*)		rGths	Single chain	CHO cells	pcDNA3.1	Induction of GVBD of oocytes (in-vitro)	Kim *et al*., 2016
Medaka (*Oryzias latipesr*)	Female	rLH	-	-	-	Induced ovulation (in-vitro)	Takahashi *et al*., 2013
Zebrafish (*Danio rerio*)	Male	rGths	Single-chain	Drosophila S2 cell line	pMT	2-fold increase of the mitotic index of Stg*A*	Nóbrega *et al*., 2015
Carp (*Cyprinus carpio*)	Female	rLH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	Induced spawning while control group did not	Aizen *et al*., 2016
European eel (*Anguilla anguilla*)	Male	rGths	Single-chain	CHO cells	-	Significant increase in GSI Induced spermiation in 80% of eels	Peñaranda *et al*., 2017
Yellowtail kingfish (*Seriola lalandi*)	Female	rFSH	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	No significant differences in GSI Oocytes at early SGca were presented in treated females while control females presented oocytes at PGcn and PGps Spz in lumen of the lobules were presented just in treated males	Sanchís-Benlloch *et al*., 2017
Flatfish Senegalese sole (*Solea senegalensis*)	Male	rGths	Single-chain	CHO cells	pcDNA3	Significant increase in GSI Enhanced spermatogenesis, reducing the number of Stg and progressively increasing the number of spc, spd and spz compared to the control group. Furthermore, the treated groups had a Leydig cell proliferation. Increased sperm production up to 7 times	Chauvigné *et al*., 2017
Spotted scat (*Scatophagus argus*)	Both	rGths	Single-chain	*Escherichia coli*	pMD18-T	Late SGv oocytes were presented in treated females while control fish presented early SGv oocytes. Male fish presented more mature spd than control group.	Zhang *et al*., 2018
Brown-marbled grouper (*Epinephelus fuscoguttatus*)	Female protogynous	rFSH (*Epinephelus lanceolatus*)	Single-chain	Yeast (*Pichia pastoris*)	pPIC9K	No significant changes in GSI Oocytes at early SGca stage were presented in treated fish while control fish presented oocytes at PGcn and PGps (8 weeks of treatment) Stg were presented in treated fish while control fish presented oocytes at SGca stage (38 weeks of treatment)	Palma *et al*., 2019
Shortfinned-eel (*Anguilla australis*)	Female	rFSH (*Anguilla japonica*)	Single-chain	FreeStyle 293-F cells	pCAGGS	Significant increase in GSI Significant increase in follicular diameters Oocytes at SGca stage were presented in treated fish while just a few of oocyte at SGca stage were presented, mostly PGcn and PGps stage	Tuan Nguyen *et al*., 2020

Species	**Sex**	**rGth**	**Subunit construct**	**Expression system**	**Expression vector**	**Gonadal development stimulation**	**Reference**
Flathead grey mullet (*Mugil cephalus*)	Both	rGths	Single-chain	CHO cells	pGEM-T Easy vector	Induced the entire process of gametogenesis in sexually immature male and females producing viable larvae. While control groups remained arrested as immature fish	Ramos-Júdez *et al*., 2020

2. Objectives

2.1. General objective

Study the effect of different single-chain recombinant gonadotropin-based hormones therapies (rFSH or rLH) produced in CHO cells heterologous system in prepubertal and sexually undifferentiated meagre (*A. regius*).

2.2. Specific objectives

- Evaluate the effect of rGths therapy on the gonadosomatic index (GSI).
- Evaluate the effect of rGths therapy on the synthesis of estradiol (E2).
- Evaluate the effect of rGths therapy on gametogenesis at histological level.
- Evaluate the effect of rGths therapy on sexual differentiation.

3. Material and methods

The present experimental study has been approved by IRTA's Ethics Committee for Animal Experimentation and the Animal Experimentation Commission from the Local Government (*Dpt. de Territori i Sostenibilitat from the Generalitat de Catalunya*). The study was conducted in accordance with the European Union, Spanish and Catalan legislation for experimental animal protection (European Directive 2010/63/EU of 22 September on the protection of animals used for scientific purposes; Spanish Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado (BOE), 2013; Catalan Law 5/1995 of June 21th, for protection of animals used for experimentation or other scientific purposes and Catalan Decree 214/1997 of July 30th for the regulation of the use of animals for the experimentation or other scientific purposes).

3.1. Study animals and maintenance

A total of 89 juvenile meagre fish (captivity reared fish) with approximately 10-months old of 222 ± 36 g (mean ± SD) were obtained from the research centre Estação Piloto de Piscicultura de Olhão (EPPO) / Aquaculture Research Station (Olhão, Portugal), which is part of the Instituto Portugués do Mar e da Atmosfera / Portuguese institute for the Ocean and Atmosphere (IPMA) (Lisboa, Portugal). The meagre arrived in IRTA research facilities at Sant Carles de la Rápita (Tarragona, Spain) on the 26th November 2020. Each fish was implanted with a PIT tag (Trovan, Spain) for identification. Fish were fed to satiety five days a week with commercial feed (BroodFeed, Sparos, Portugal) and were kept under natural photoperiod. The mean temperature during the experiment was 16,1 ± 0,4°C based on previous experiments with meagre (Duncan *et al*., 2013).

Juvenile fish were held in rectangular 10 m^3 fiber glass tank (3 m × 3 m × 1.5 m depth) with a biomass of 15,1 kg connected to a recirculation system (IRTAmar®).

Tanks were covered with a strong top net (multi-monofilament knotless nylon netting with 30 mm mesh suitable for seine fishing, Badinotti, Milan, Italy) as descrived by Duncan *et al*. (2013) to avoid any impact damage as a result of meagre's jump.

3.2. Recombinant Gths production of *A. regius*

Argyrosomus regius single-chain recombinant gonadotropins were made by Rara Avis Biotec S.L. (Valencia, Spain). The pituitary gland was removed from a sacrificed fish and RNA was purified; subsequently, the alpha subunit of FSH and beta subunits of FSH and LH were sequenced, synthesizing the cDNA as a single-chain with the entire coding sequence of meagre βFSH or βLH followed by six His residues, the carboxyl-terminal 28 amino acid peptide hCGβ as a linker and the α subunit previously sequenced; rFSH` and rLH` had the same structure except the linker, which has a sequence that is protected intellectual property (Rara Avis Biotec S.L., Valencia, Spain) (Fig.9). The sequences were subcloned into the expression vector pcDNA3.1, which was transfected into CHO cells. For recombinant gonadotropins production, clones were cultured for 8 days at 37°C in 225 cm^3 flasks with 5% CO_2 and Dulbecco modified Eagle medium (DMEM). After that, the medium was centrifuged at 15.000 rpm for 15 min and the supernatant (containing the recombinant gonadotropins) was purified by a chromatography nickel column for their affinity for histidine tags. Purified recombinant gonadotropins were released from the column with imidazole (the final concentration of imidazole was reduced with successive washes with phosphate-buffered saline). During this process rGTHs were concentrated to 12 µg/mL and stored in 1 mL vials at -80°C.

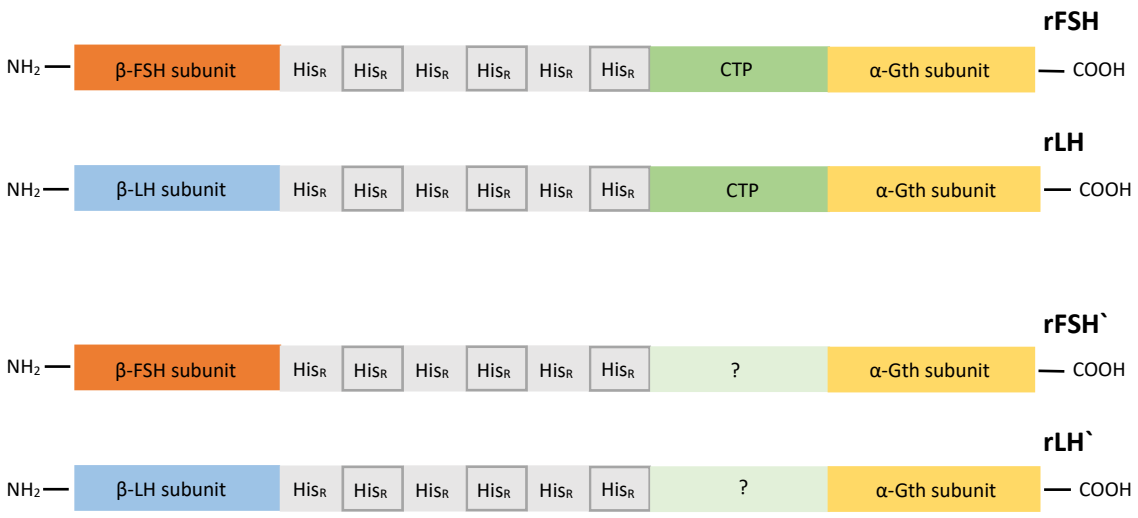

Fig.9: Schematic representation of the structure of the single chain recombinant gonadotropins used in this study. His_R, histidine residues; CTP, carboxyl-terminal 28 amino acid peptide hCGβ (linker), and ?, protected linker. By Álvaro González.

3.3. Experimental setup, fish manipulation and samplings

The experiment to investigate the effect of rFSH and rLH on immature fish was performed over 3 weeks, from 15th of February to 8th of March. Ten groups of fish were stablished. All groups were made up of 8 fish, except for group 1 (21 fish) and groups 9 and 10, wich were made up of 6 fish. All groups were injected intramusculary (constant concentration throughout the experiment). Group 1 (control) was sacrificed on the first day to know the initial gonadal development. Group 2 (saline) was injected with saline serum (ERN S.A.). Groups 3, 4 and 5 received rFSH doses of 6, 12 and 18 µg kg^{-1} respectively. Groups 6, 7 and 8 received rLH doses of 6, 12 and 18 µg kg^{-1} respectively. Group 9 (rFSH`) and group 10 (rLH`) included a new protected linker that differentiated these rGths from the rGths used in groups 3 – 8, which used a previously proven linker. Throughout the experiment, 8 blood samples were drawn and 3 doses of hormone/ saline serum were injected in all groups (Fig.10).

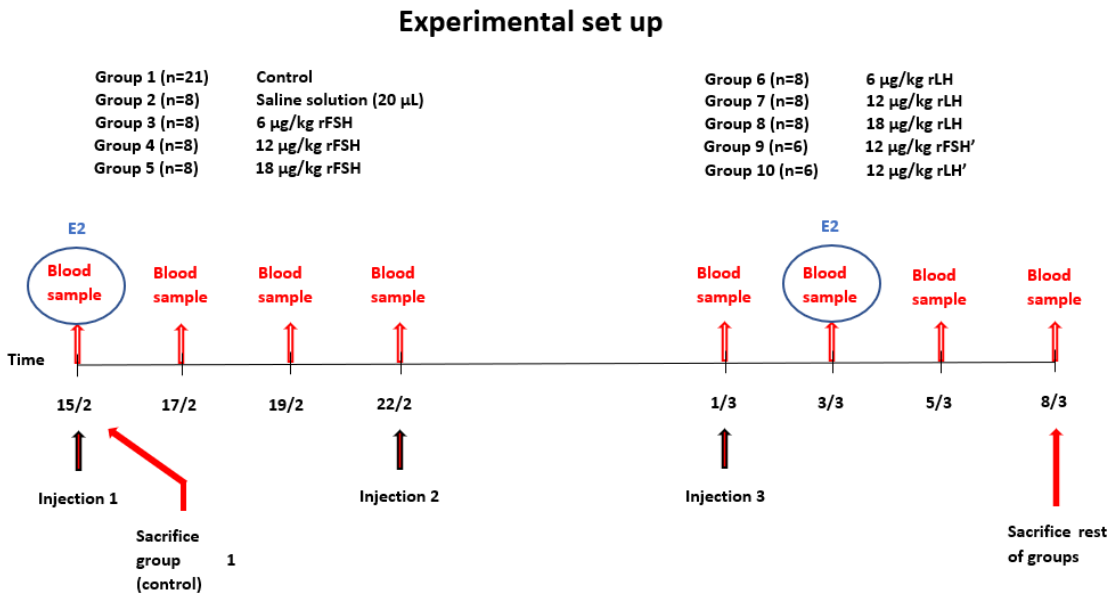

Fig.10: Shematic representation of the experimental set up. The effect of different doses of rGths (rFSH or rLH) on steroid production and gonadal development was tested over 3 weeks. The rGths treatments were administered once a week. The effect on the level of 17β-estradiol was measured on the first day of the experiment (control) and three days after the third treatment The effect on gonadal development (oogenesis and spermatogenesis) was tested at the end of the third week. By Álvaro González.

Before any manipulation for blood extraction or hormone administration fish were anaesthetised with 90 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma Aldrich) as in previous studies (Duncan *et al*., 2013; Chauvigné *et al*., 2017; Ramos-Júdez *et al*., 2020). Blood samples (400 µL) were collected from caudal vein with a syringe previously coated with lithium heparin (Deltalab S.L., Tarragona) to prevent coagulation during blood extraction. On days when blood

samples were taken and rGths administered the blood samples were taken immediately before hormone administration. The blood was placed into a 1,5 mL eppendorf containing 5 µL lithium heparin, centrifuged at 3000 rpm for 5,5 min at 4˚C; and plasma was aliquoted 3 times (replicas for sex steroid hormones analysis) and stored at -80˚C.

Fish were sacrificed by anaesthetic overdose (180 mg L^{-1} MS-222; Sigma Aldrich) followed by bled out and pithing to destroy the brain according to Directive 2010/63/EU guidelines. Gonads and liver were weighed to calculate for gonadosomatic index (GSI) and hepatosomatic index (HSI) (gonad and liver weight respectively divided by total body weight, expressed in porcentage).

3.4. Plasma steroid analysis

Levels of E2 were determined by commercial enzyme inmunosorbent assay (EIA, Cayman chemical company, USA) in all prepubertal fish. Before this analysis, free steroids were extracted from plasma with methanol. Firstly, the plasma samples were thawed at room temperature. Then, 100 µL of plasma was extracted and mixed with 500 µL of methanol (previously cooled to 4˚C), subsequently centrifuged at 6000 rpm and 4˚C for 10 minutes. The supernatant was pipetted and the process was repeated with 250 µL of methanol. The resulting supernatant (about 750 µL) was allowed to incubate in the oven overnight at 37˚C. The pellet resulting from evaporation (steroids) was resuspended in 0,5 mL of Elisa buffer (1:5 dilution).

3.5. Histological preparation

Ovarian, testis and undifferentiated gonads biopsy samples were fixed in bouin`s fluid for 24 hours at room temperature and mantained in ethanol 70% until being dehydrated through ethanol series. Dehydrated samples were oriented in the molds and embedded in paraffin. Immature fish samples were oriented to obtain a longitudinal section. Histological sections (3 µm) (RM 2155, Leica) were stained with hematoxylin and eosin (Casa Álvarez, Spain). Gonadal tissue structure was observed under a light microscope (Leica DMLB, Houston, USA).

Germ cells developmental stage was classified according to the relative size, appearance of structures and morphological changes. The gonadal development of prepubertal meagres was established by evaluating the presence of germ cells in six random optical areas at 20x magnification, although a maximum gonadal development was also established by evaluating the entire gonad due the low presence of sex specific germ cell stages of development.

Female germs cells were classified as: *chromatin nucleolar stage of primary growth (PGcn)* characterized by small oocytes with a large single nucleolus surrounded by a thin layer of citoplasm*; perinuclear stage of primary growth (PGps)* with a bigger oocyte due to the enlargement of the nucleus and the appearance of multiple nucleoli; *cortical alveoli stage of secondary growth (SGca)* characterized by the presence of small lipid droplets and cortical alveoli at the periphery of the cytoplasm*; vitellogenic stage of secondary growth (SGv)* with the initiation of the vitellogenesis and the presence of yolk globules*; mature stage (OM)* characterized by the coalescence of the lipid droplets and the yolk globules, the peripheral migration of the germinal vesicle (nucleus) and the dissolution of its membrane, and the hydration of the oocyte; *ovulation stage (OV)* when there is a unique large yolk globule and the size is maximum (West, 1990; Ramos-Júdez *et al*., 2020).

Male germs cells were classified as: *type A undifferentiated spermatogonia* (StgA_{und}) characterized by being the largest male germ cells and having a large nucleus in adittion to one or two nucleoli; *type A differentiated spermatogonia* (StgA_{diff}), smaller than *StgA*$_{und}$ and present in cysts in groups of 2 to 8 germs cells linked by cytoplasmic bridges; *type B spermatogonia* (Stg*B*) with a smaller size than the previous cells and present in cysts in groups of 16 or more germs cells; *spermatocyte* (*spc*) characterized by an increase in cell and nucleus size with respect to *StgB* due to entry into meiosis (condensing chromosomes), moreover an increase in germs cells per cyst; *spermatid* (*spd*) with a significant reduction in cell size and increase of germs cells per cyst; and *spermatozoa* (*spz*) with the presence of a flagellum and reduction in cell size (Leal *et al*., 2009; Schulz *et al*., 2010; Lacerda *et al*., 2014).

Gonad development was determined according to the criteria established by Gil *et al*., (2013) for wild specimens, adapted to the germ cell classification used in this study. Seven stages of development were described based on histological examination: a) Stage I (*incompletely differentiated*) when female fish present oogonia, PGcn and PGps. Males lack a well-defined tubular system with numerous espermatogonia b) Stage II (*differentiated immature*) in which female fish present oogonia, PGcn and PGps too, while male fish has numerous tubules filled with spermatogonia and some spermatogenic cysts in all developmental stages; c) Stage III (*developing*) with very few oogonia but abundant PGcn and PGps; SGca can be observe too. Spermatogenesis activity is generalised with numerous Stg*A* and Stg*B*, and spz present in some tubules; d) Stage IV (*ripening*) when there are oocytes at all stages of development, but postovulatory follicules are not seen; vitellogenic oocytes remain low (<30%). Males present cysts at all stages of development with spz in the majority of tubules, but not in all; e) Stage V (*running*) represent females with oocytes at all stages of development and postovulatory

follicules. Males present enlarged tubules and the sperm duct (vas deferens) full of spz; f) Stage VI (*spent*) with SGv, atretic and postovulatory follicules. Males have tubules and sperm duct full of spz; f) Stage VII (recovering) present oogonia, PGcn, PGps, corpus albicans (from atretic oocytes and postovulatory follicules), no SGv are present. Tubules of testes are full of Stg*A* and Stg*B* with residual spz at the lumen of tubules and sperm duct; f) Stage VIII (*resting*) represent females with oogonia, PGcn and PGps, and males with tubules full of Stg*A* and Stg*B* indicating the beginning of the resting period.

3.6. Statistical analysis

Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. A one-way repeated-measures analysis of variance (ANOVA) followed by the Holm-Sidak test for pairwise comparisons was used to compare the GSI and HSI between the immature meagre treatment groups. Differences in E2 secretion between the beginning and the end of the experiment and between treatments, a Two Way repeated measures ANOVA followed by the Holm-Sidak test were performed to compare plasma E2 concentration amongst treatments at the beginning and end of the experiment. The Chi-square test was used to compare sex-ratio of immature meagres amongst the treatment groups. Analyses were performed using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA).

4. Results

4.1. Gonadosomatic and hepatosomatic index

After three weeks of treatment with weekly intramuscular injections, no significant differences ($P < 0.05$) were found in GSI between group 2 (Saline) and the rest of the treatment groups. Slight differences, although significant ($P < 0.05$), were found between group 5 (FSH 18) and group 10 (LH` 12) (Fig.11). At HSI level, no significant differences were found between treatment groups (Fig.12).

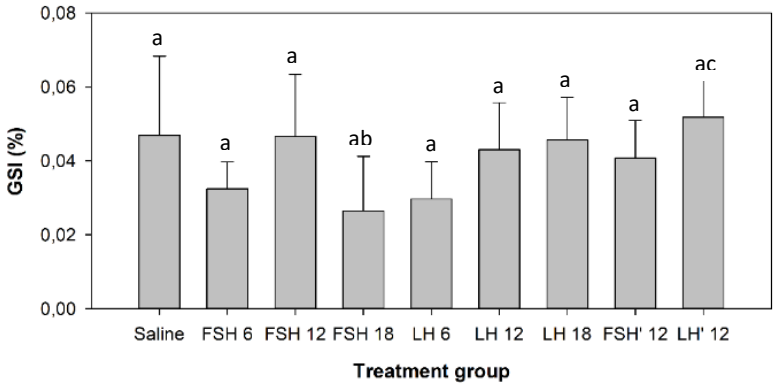

Fig.11: Effect of rGths treatments on GSI of immature meagre (*A. regius*) of 220 grams. Data are the mean ± SD. "a" denote significant difference between treatment groups and saline group. "b" and "c" denote significant differences among treatment groups. $P < 0.05$.

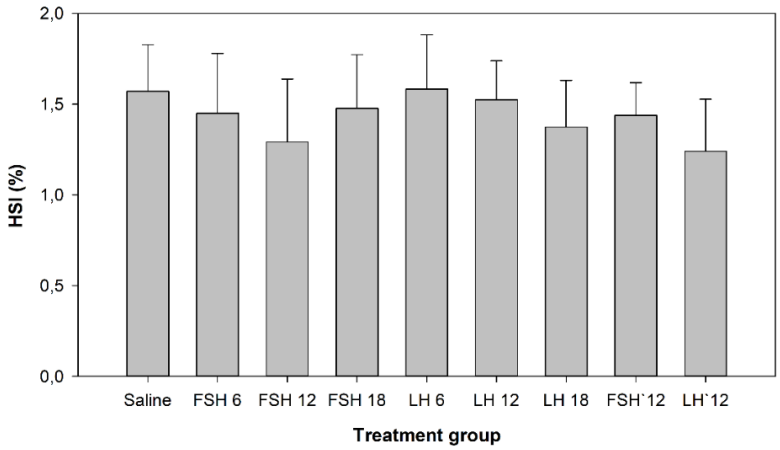

Fig.12: Effect of rGths treatments on HSI of immature meagre (*A. regius*) of 222 ± 36 grams. Data are the mean ± SD.

4.2. Estradiol

Weekly injections of rGths during three weeks to prepubertal and sexually undifferentiated fish generated a significant increase in plasma E2 in all treatment groups considered ($P < 0.05$), while saline showed a slight decrease, although not significant between week 0 and week 3. However, there was no significant differences between treatment groups at the third week, including saline, maybe due to the small sample size considered. There were no significant differences among treatments at week 0 (Fig.13).

Mean E2 levels in the treatment groups at the third week ranged between 2400 and 3600 pg / mL while in the saline group it was about 60 pg / mL. There was a direct relationship between the concentration of the rGths treatments and the plasma E2 level, although the differences were not significant. FSH` 12 was the most stimulated treatment group for the synthesis of E2 from the groups considered. FSH 18 treatment group was more stimulating than FSH 12 and FSH 6.

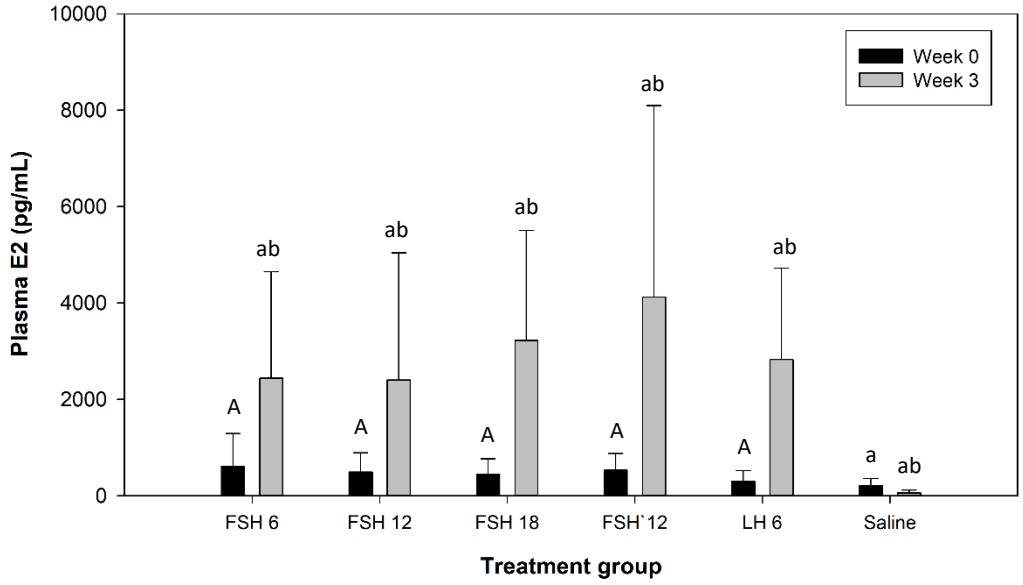

Fig.13: Comparison plasma E2 levels of rGths treatment groups and saline prepubertal meagre (*A. regius*). Data was normalized. Data are the mean ± SD. "a" denotes significant differences between week 0 and week 3. "b" denotes significant differences among groups at week 3. Treatment groups 7 (LH 12), 8 (LH 18) and 10 (LH`12) were not considered for statistically study due to lack of data or small sample size. $P < 0.05$. FSH 6 (n=8); FSH 12 (n=8); FSH 18 (n=8); FSH` 12 (n=6); LH 6 (n=4); Saline (n=2).

4.3. Histological observations

In general, the gonad had somatic tissue on the outside and germ cells in the middle, with a space for the duct in the area of germ cells. The duct indicated sexual differentiation had initiated, but actually gonads had not completed differentiation. Moreover, most cells were completely undifferentiated either somatic cells or germ cells that could not be identified as oogonia or spermatogonia (Fig. 15A and B). Amongst the germ cell were isolated cells that had differentiated into either oocytes or spermatogonia B. These few cell we searched for to define male or female, however, differentiated had just initiated and was far from complete.

Recombinant gonadotropins short-term therapy induced early gonad development with the appearance of oocytes at PGcn and PGps stage (Fig.15B and E), and the increase in the percentage of fish that presented spc and Stg*B* compared to the control and saline groups. Group 1 (control) and group 2 (saline) did not show any oocyte development, while treatments with rFSH, rFSH` and rLH showed oocytes; specifically group 3 (FSH 6; 12.5%; n = 8), group 4 (FSH 12; 12.5%; n = 8), group 7 (LH 12, 37.5%; n = 8) , group 8 (LH 18; 12.5%; n = 8) and group 9 (LH` 12; 16.7%; n = 6) (Fig.14E). At level of male germ cells development, all treatment groups showed Stg*B* (Fig.14B) (Fig.15E), and spc (Fig.14C) (Fig.15D) while saline did not. Treatment groups 3 (FSH 6), 4 (FSH 12), 6 (LH 6) and 10 (LH`12) showed one fish with spd each, however, control group showed two fish with spd (n = 17) (Fig.14D) (Fig.15C) (4 fish from the control group were discarded due to the small amount of gonadal tissue). Fish with all completely undifferentiated gonadal tissue were found in group 1 (control; 88%), group 2 (saline; 100%), group 4 (FSH 12; 62,5%) and group 8 (LH 18; 62,5%) (Fig.14A).

The most satisfactory treatment to induce gonadal development was FSH 6 followed by FSH` 12 and LH 12. FSH 6 treatment group showed 75% of the fish (n = 8) with some germ cells in the stages of spc, spd or oocytes. FSH 12, FSH 18 and LH 18 treatments showed less ovarian and testis development, each only had 37.5% of fish (n = 8) in these stages of germinal development. FSH` 12 treatment group (n = 6) presented 66.7% of fish with a development of spc, spd or oocyte; while the LH 12 treatment group (n = 8) accounted for 50% of the fish. Treatments with LH 6 (n = 8) and LH` 12 (n = 6) only showed one fish with some of the commented gonadal states.

Germ cell development was highly localized, most of the gonadal tissue did not have any development and all fish were classified as Stage I (incompletely differentiated). In fact, all of them showed a large amount of Embryonic germ stem cells (EGSC) and connective tissue (CT) (Fig.15A,B and E). Actually, in groups 1 (control), 2 (saline), 6 (LH 6) and 10 (LH` 12) only EGCS,

oogonia or spermatogonia were found in the first six fields observed. The rest of the group showed more advanced stages of development.

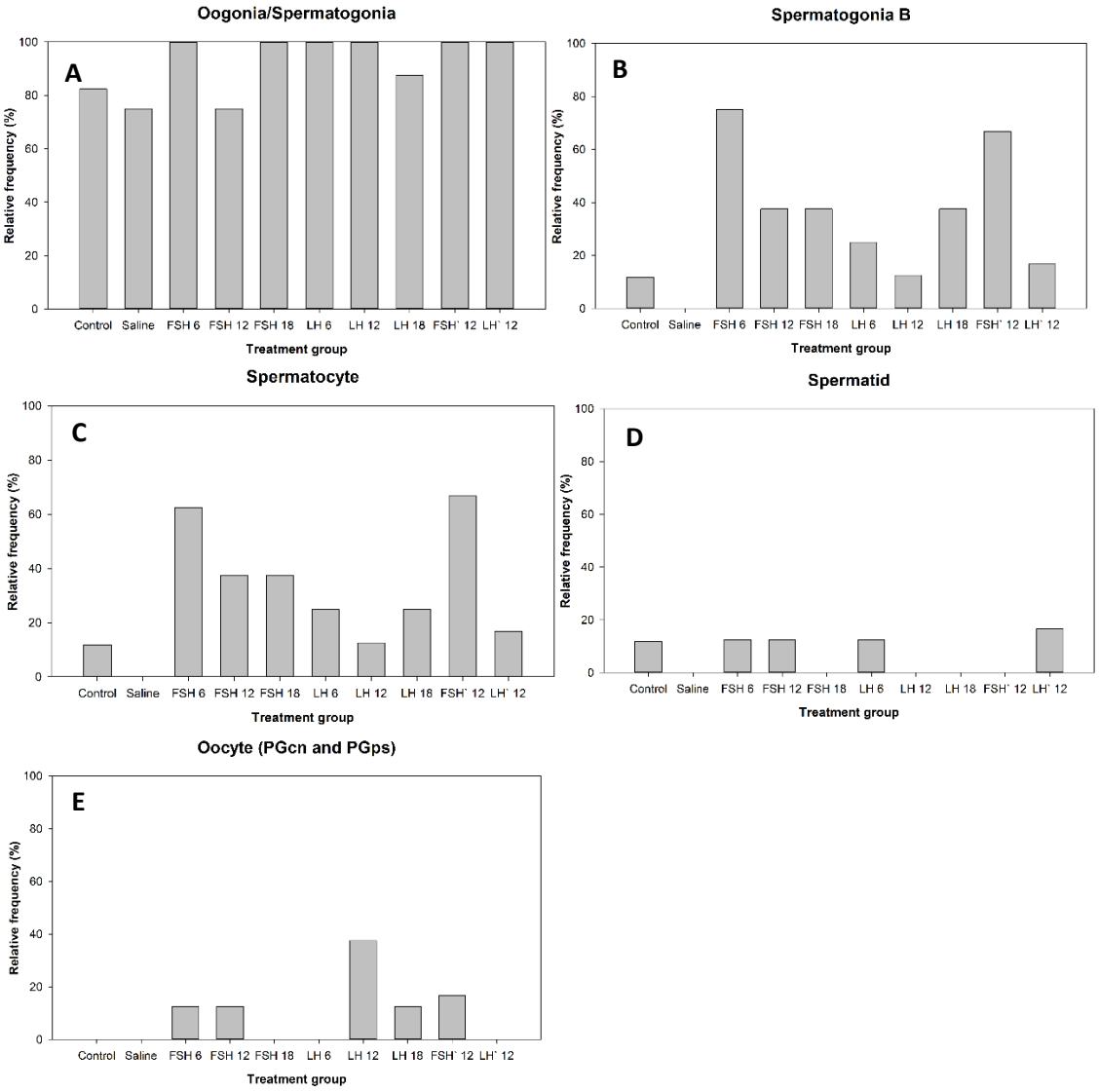

Fig.14: Comparison of the relative frequency of fish per treatment group that presented the developmental stage of A) Oogonia or spermatogonia; B) Spermatogonia B; C) Spermatocyte; D) Spermatid and E) Oocyte at chromatin nucleolar stage (*PGcn)* and perinucleolar stage (*PGps*). Control (n=17); Saline (n=8); FSH 6 (n=8); FSH 12 (n=8); FSH 18 (n=8); LH 6 (n=8); LH 12 (n=8); LH 18 (n=8); FSH` 12 (n=6); LH` 12 (n=6).

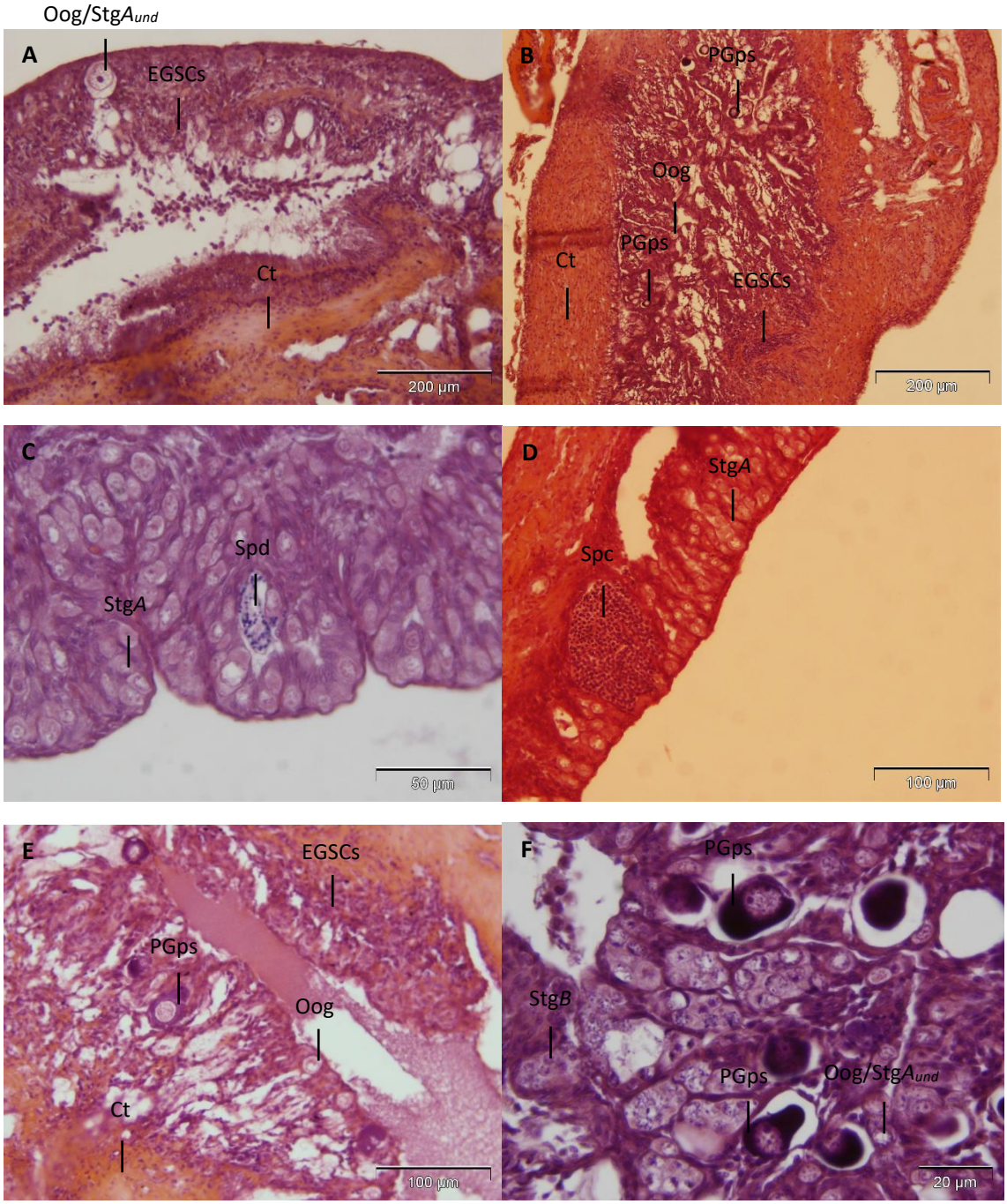

Fig.15: Representative photomicrographs of histological sections of immature meagre stained with hematoxylin and eosin after three weeks of treatment. A) sexually undifferentiated fish; B) female fish; C) male fish; D) male fish; E) female fish; F) intersex fish. EGCS, Embryonic Germ Stem Cells; Oog/Stg, Oogonia or spermatogonia; Oog, oogonia; StgA_{und}, type A undifferentiated spermatogonia; Stg*B*, type B spermatogonia; Spc, spermatocytes; Spd, spermatids; PGps, oocyte at perinucleolar stage of primary growth; Ct, connective tissue.

4.4. Sexual differentiation

Fish were sexed at cellular level because no difference was found in the morphology of the ovaries and testes in this stage of sexual differentiation, so fish that presented oocytes were classified as female, those that presented Stg*B* or higher development stages were classified as males, those with both developed stages were classified as intersex, and those with all germ cells in a developmental stage lower than Stg*B* or oocyte were classified as sexually undifferentiated.

Gonadotropin treatment significantly altered the sex ratio, increasing the proportion of males and developing intersex fish ($P < 0.05$). The only treatment group that presented females was LH 12. All fish in saline treatment group and 88% of fish in control group (n = 17) were sexually undifferentiated due to low gonadal development. However, in all treatment groups, except LH 12, no females were found, although oocytes were found (Fig.16). Fish that presented oocytes from the treatment groups FSH 6, FSH 12, FSH` 12 and LH 18 also presented male germ cells (Stg*B*, spc or spd) (Fig.15F). The recombinant hormone that showed the most intersex fish was FSH (n = 30), with 10% fish; specifically, one fish from each treatment of FSH 6, FSH 12 and FSH` 12 (Fig. 16).

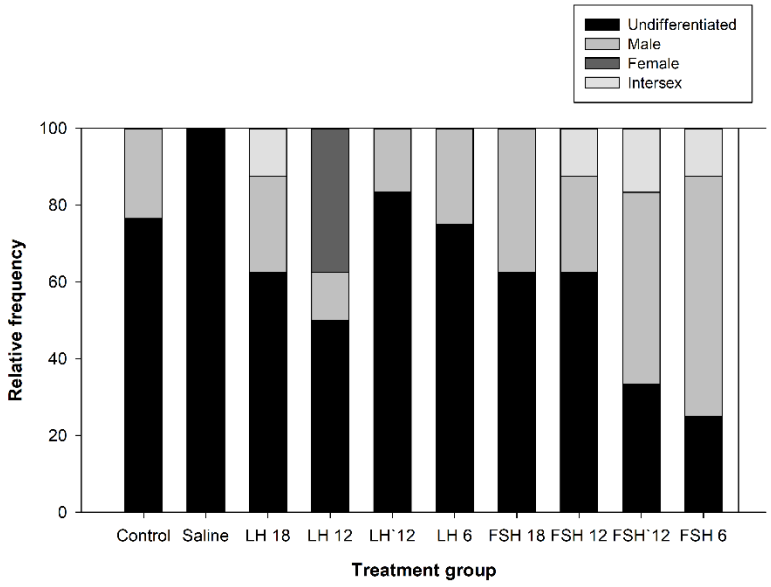

Fig.16: Comparison of the relative frequency of fish per treatment group that were classified as sexually undifferentiated, male, female or intersex based on the development of germ cells in the histological sections.

5. Discussion

The present survey shows that the rGths produced by a CHO system are biologically active and their half-life is long enough to induce *in vivo* effects in prepubertal and sexually undifferentiated meagre (*A. regius*). Both gonadotropins, rFSH and rLH, were able to stimulate E2 secretion, the onset of meiosis in female germ cells, and the onset and resumption of meiosis in male germ cells. These findings demonstrate that there is *fshra* and *lhcgrba* expression in gonadal tissue at this stage of development, and single-chain rGths therapy could be a useful tool to induce gametogenesis in prepubertal fish.

5.1. Gonadosomatic index

In our study, no treatment group showed a significant increase in GSI. In contrast, recombinant gonadotropins therapy has increased it in various fish species, such as Japanese eel (*A. japonica*) (Kobayashi *et al*., 2010), European eel (*A. anguilla*) (Peñaranda *et al*., 2017) or Shortfinned eel (*A. australis*) (Tuan-Nguyen *et al*., 2020). However, the effectiveness of treatments on the gonad growth depends on the concentration of recombinant hormone, the initial stage of development or the duration of the experiment. In the Tuan-Nguyen *et al*. (2020) survey, only the group treated with the dose with the highest concentration of rFSH (500 µg / kg) for 3 weeks showed significant differences; while the doses of 20 µg / kg and 100 µg / kg did not show significant differences. Moreover, in contrast with our survey, all of them were sexually differentiated. Nevertheless, in our study, the treatment with the best histological result was the one with the lowest concentration of rFSH (6 µg / kg). This data may indicate that the doses used were correct, but fish were too young to response at GSI level.

5.2. Estradiol

Both FSH and LH were able to stimulate E2 secretion. All of our treatment groups showed significant differences in plasma E2 secretion between week 0 and week 3, while saline did not. The initial stage of development of fish was not enough to stimulate an increase in gonadal size, but actually it was enough to stimulate the synthesis of plasma E2; doses with less concentration are enough to stimulate its secretion. Similar results were obtained in Shortfinned eel (*A. australis*), in which 100 µg / kg rFSH treatment for 3 weeks did not show an increase in GSI, but showed an increase in E2 (Tuan-Nguyen *et al*., 2020).

5.3. Histological observations

Data demonstrate that rGths treatments were effective in inducing early gonad development, although not enough to differentiate all germ cells. In females, gonad development was similar to that obtained with rFSH in Orange-spotted grouper (*E. coioides*), where control group resulted in an undifferentiated ovary, while treatment group in an ovary with oocytes in PGcn and PGps (Chen *et al*., 2012a). Both surveys studied the effects of rGths treatment on fish that had not reached sexual maturity (prepubertal or juvenile fish). However, the Orange-spotted grouper (*E. coioides*) is a protogyny hermaphroditic fish (sexually differentiated) (Liu and Mitcheson., 2009) (Chen *et al*., 2012a), while meagre (*A. regius*) is a gonochoristic fish that was at sexual differentiation period. Otherwise, in our experiment, males had a greater development of germ cells than females, with the resumption of meiosis and development of spermatids in some cases, while females initiated meiosis but did not reach SGca stage. In flathead grey mullet (*M. cephalus*) rFSH and rLH therapy induced the entire process of gametogenesis, however, 11 and 5 weeks of treatment for females and males respectively and the combination of both gonadotropins were necessary. Furthermore, the initial stage of development in female germ cells were PGps or SGca (Ramos-Júdez *et al*., 2020). In giant grouper (*E. fuscoguttatus*) after 8 weeks of treatment with a weekly injection of rFSH at 100 µg / kg, the oocyte in PGps stage developed to SGca (Palma *et al*., 2019). In Japanese eel (*A. japonica*) 5 weeks of treatment with a weekly injection of rFSH or rLH were able to develop spermatogonia into spermatids and spermatozoa respectively (Hayakawa *et al*., 2008). In Shortfinned eel (*A. australis*) the duration of the experiment was 3 weeks like in our survey; and weekly injections of rFSH developed oocytes to SGca from PGps, although there were some oocytes at SGca stage in initial control group (Tuan-Nguyen *et al*., 2020). This data demonstrates that at histological level there are differences depending on the initial stage of development and the duration of the treatment.

Considering our results at plasma E2 level and at histological level with the onset of meiosis in germ cells of males and females, we can suggest that i) there was expression of *fshra* and *lhcgrba* in gonads during the period of sexual differentiation, ii) rFSH and rLH, both, were able to stimulate the synthesis of 11-KT, iii) rFSH and rLH, both, were able to stimulate the synthesis of progestin 20βS.

i) There was *fshra* and *lhcgrba* expression in meagre (*A. regius*) tissue during the period of sexual differentiation, due to greater gonadal development and E2 secretion in the treatment groups than in the control and saline groups. In fact, in control and saline

groups the gonad in most cases remained undifferentiated and with a lower level of plasma E2, although not significant. This is in agreement with Baron *et al*. (2005), where there was expression of *lhcgrba* in female rainbow trout (*O. mykiss*) during the period of sexual differentiation; or as in the medaka (*O. latipes*), where *fshra* and *lhcgrba* expression was found in all testis stage (Burow *et al*., 2019). Moreover, in Nile tilapia (*O. niloticus*) there is *fshra* and *lhcgrba* in undifferentiated gonads during sexual differentiation period (Yan *et al*., 2012).

ii) Studies in Japanese huchen (*Hucho perryi*) (Amer *et al*., 2001), Japanese eel (*A. japonica*) (Miura *et al*., 2006), Atlantic salmon (*S. salar*) (Chen *et al*., 2012b) or Nile tilapia (*O. niloticus*) (Liu *et al*., 2014) suggest that MIS 20βP induces entry into meiosis of germ cells. In our results, females and males treated with rFSH and rLH initiated meiosis, suggesting that both rGths were able to activate Lhcgrba for the synthesis of MIS 20βS. This was in accordance with the results obtained by Burow *et al*. (2019), where both rGths activated the Fshra and Lhcgrba receptors in medaka (*O. latipes*).

iii) Otherwise, according to Miura and Miura (2003), 11-KT stimulates the differentiation of spermatogonia priors to initiation of meiosis. Our results suggest that both rFSH and rLH were able to stimulate the synthesis of 11-KT as it was reported before (Suzuki *et al*., 2019).

5.4. Sexual differentiation

About sexual differentiation, our histological observations demonstrate that with recombinant gonadotropin treatment: 1) the onset on meiosis in female germ cells occurs at the same time as de appearance of the ovarian cavity 2) males sexually differentiate earlier than females; 3) there is a higher proportion of males during the stage of sexual differentiation, 4) there are intersex fish during the stage of sexual differentiation. This is the first report of a male skewed ratio and the presence of intersex fish in meagre during the stage of sexual differentiation

In gonochoristic teleost fish such as meagre (*A. regius*), the first indicator of sexual identification is made according to the ovarian ontogeny. It depends on whether the ovarian lumen / ovarian cavity develops earlier or not than the entry into meiosis of the oogonia with the consequent development of the oocytes (Nakamura *et al*., 1998). In Olive flounder (*Paralichthys olivaceus*), Southern flounder (*P. lethostigma*), Nile tilapia (*Oreochromis niloticus*) or Blue tilapia (*O. aureus*), meiotic division occurs after the formation of the ovarian lumen, thus, it is used as the earliest recognizable sign of female differentiation (Liu and Mitcheson, 2009). In European eel

(*A. anguilla*), Silver-stripe round herring (*Spratelloides gracilis*) or *Cichlasoma dimerus*, meiotic division occurs before the formation of the ovarian lumen, so the appearance of oocytes is the earliest recognizable sign of female differentiation (Colombo and Grandi, 1995; Hatakeyama *et al*., 2005; Meijide *et al*., 2005). In Fathead minnow (*Pimephales promelas*), Viviparous eelput (*Zoarces viviparus*) or Roach (*Rutilus rutilus*), meiotic division of the oogonia and the formation of the ovarian lumen occurs at the same time (Van Aerle *et al*., 2004; Rasmussen *et al*., 2006; Paull *et al*., 2008). According to our results, in meagre (*A. regius*), meiosis of the oogonia occurs at the same time as the appearance of the ovarian lumen, as described by Schiavone *et al*. (2012). For this reason, oocyte development was used as a classification criterion for females.

Moreover, according to Schiavone *et al*. (2012), females sexually differentiate earlier than males, and according to Gil *et al*. (2013), wild meagre has a population sex ratio of 1: 1. Both ideas are in disagreement with our results, in wich male sexually differentiate earlier than females and there is a male skewed ratio. The control group presented 2 males and 15 undifferentiated fish, the LH` 12 treatment group showed 1 male and 6 undifferentiated fish, the LH 6 treatment group showed 2 males and 6 undifferentiated fish, and the FSH 18 treated group had 3 males and 5 undifferentiated fish. In addition, the rest of the treatments had males in a higher percentage than intersex fish. The only group that presented females was the one treated with LH 12, which showed 3 females, 1 male and 4 undifferentiated fish (Fig.16). We must consider that the population sample of Gil *et al*., (2013) was composed of completely sexually differentiated and wild individuals.

Sex determination is defined as the forces that determine whether a fish will become a male or a female, whereas sex differentiation refers to molecular and cellular processes that make a bipotential gonadal primordium develop into a testis or ovary after sex has been determined (Devlin and Nagahama, 2002). The sex determination mechanisms in vertebrates include Genotypic Sex Determination (GSD) and Environmental Sex determination (ESD), or a combination of both (Nakamura *et al*., 1998; Sandra and Norma, 2009; Navarro-Martín *et al*., 2011; Fernandino *et al*., 2013). Sex differentiation in egg-laying vertebrates is a hormone-dependent process that involves complex interactions among a large network of genes (Baron *et al*., 2005), such as *cyp19a* or *hsd11b*. Exposure to exogenous hormones and endocrine disruptors throughout sex differentiation can alter the expression of these genes, causing alterations in the sex differentiation process that ultimately leads to reproductive abnormalities, including intersex (Abdel-Moneim *et al*. 2015). There are two hypotheses about the influence of steroids on the gonad, the first one maintains that the ovaries develop due to the action of estrogens, while the testes develop due to the action of androgens (Nakamura, 2010). The other

hypothesis proposes that the testes develop by inaction of Cyp19a / P450arom (Guiguen *et al*., 2010). In any case, the balance between E2 and 11-KT have a key role on sexual differentiation processes (Sandra and Norma, 2009; Navarro-Martín *et al*., 2011; Bahamonde *et al*., 2013). Our results support the first hypothesis because treatment groups showed a male skewed ratio and an increase in E2 secretion, although not significant. Moreover, according to Kagawa *et al*. (2003), in Red seabream (*Pagrus major*), LH but not FSH, stimulated both aromatase activity and P450arom gene expression. This could explain why our treatment group 7 (LH 12) was the only one that presented females (37.5%; n = 8); however more data about E2 production in treatment groups would be necessary.

Intersex is defined as the presence of male and female gonadal tissue in a gonochoristic species (Bahamonde *et al*., 2013). The presence of intersex fish during the stage of sexual differentiation can have four main reasons. The first is that the development of juvenile intersex is a passive phenomenon and perhaps it is an advantage to achieve a reproductive partner in severe ecological conditions, since it temporarily maintains bipotentiality to develop in ovary or testis (Tricas and Hiramoto, 1989; Sandra and Norma, 2009). The second possible cause is that there is a "basal rate" of intersex in the meagre species although it is rarely (Grim *et al*., 2007; Abdel-Moneim *et al*., 2015). The third possible cause is that all the gonads develop initially as ovaries and that later half of them degenerate giving rise to testes (Juvenile hermaphroditism) (Deblin and Nagahama, 2002). The fourth possible cause is that some environmental factor has altered the normal development of sexual differentiation in this survey. Since the development of intersex fish during sexual differentiation and during adulthood has not been previously documented in meagre (*A. regius*) (Schiavone *et al*., 2012; Gil *et al*., 2013), and that the third possible cause is in disagreement with our results and those of previous studies, it is most likely that it is the fourth cause. Environmental factors include: a) pollution and exogenous steroids (endocrine-disrupting chemicals), b) temperature and other physical variables, and c) social interactions (Deblin and Nagahama, 2002). Although we should include d) recombinant gonadotropins treatment as a potential cause of intersex and male skewed sex ratio.

a. The development of intersex fish as a consequence of endocrine-disrupting chemicals (EDCs) has been widely studied in freshwater and marine fish (Kavanagh *et al*. 2004; Barucca *et al*., 2006; Blazer *et al*., 2007; Vajda *et al*. 2008; Diaz de Cerio *et al*., 2012; Rochman *et al*., 2014; Bahamonde *et al*., 2014). However, prepubertal fish in our study do not come from the marine environment, so they should not have been affected by EDCs. Furthermore, they have not received a diet with a rich source of phytoestrogens

or any compound with any estrogenic or androgenic activity that can alter the process of sexual differentiation (Grim *et al*., 2007; Rzepkowska *et al*., 2014).

b. Temperature is probably the most studied factor in ESD. It has been observed that in species with Temperature Sex Determination (TSD), high temperatures during the sexual differentiation process are associated with masculinization processes, intermediate ones with mixed-sex populations, and low temperatures with feminization processes (Strüssmann *et al*., 1997; Sandra and Norma, 2009). According to Fernandino *et al*. (2012), thermal stress of high temperatures during the period of sexual differentiation in TSD species generates an increase in cortisol levels, which in turn induces an increase in *hsd11b* levels (codes for the enzyme 11β-hsd), leading to a higher synthesis of 11-KT and a higher sex ratio of males. High temperatures also increase the methylation levels of the *cyp19a* promoter region in European seabass (*D. labrax*), which were found to be sexspecific and highly influenced by changes in temperature during early life stages, causing signs of masculinization as a result of the down-regulation of the expression of this gene (Navarro-Martín *et al*., 2011). In medaka (*O. latipes*), which is a GSD species with XX / XY sex determination, it has been observed that a high temperature treatment or a cortisol treatment at intermediate temperatures during the period of sexual differentiation also increases the sex ratio of males, suppresses the expression of *fshra* and female-type proliferation of germ cells (by upregulating of *amh*) (Selim *et al*., 2009; Hayashi *et al*., 2010). In Japanese flounder (*Paralichthys olivaceus*) (GSD with XX / XY sex determination) and Silverside (*Odontesthes bonariensis*) (TSD), a high temperature treatment during sex differentiation period stimulates the synthesis of cortisol, which inhibits the expression of *cyp19a*, increasing the sex ratio of males (Hattori *et al*., 2009; Yamaguchi *et al*., 2010; Guiguen *et al*., 2010; Yang *et al*., 2020). Therefore, an increase in cortisol levels is related to masculinization processes due to *cyp19a* downregulation, increased synthesis of 11-KT, reducing the number of PGC cells (Goikoetxea *et al*., 2017; Miller *et al*., 2018), or downregulation of *fshra*.

Further studies are necessary to elucidate the sex determination mechanism in meagre (*A. regius*). In any case, our fish were kept around 16°C, which should not be too high temperature to induce strong thermal stress, compared to the average water temperature between the months of February and May in the Western Mediterranean and the Eastern Atlantic (data not shown) (MITMA, 2021).

c. Population density is one of the social factors that affect sexual differentiation (Fernandino *et al*., 2013). In Japanese eel (*A. japonica*) or European seabass (*D. labrax*), high population densities in captivity were related to the male-biased sex ratio (Davey and Jellyman, 2005; Saillant *et al*., 2006), and an increase in cortisol levels (Chiba *et al*., 2002 cited in Fernandino *et al*., 2013). Prepubertal fish in our study were fished from the tank eight times in three weeks, and introduced into 300 dm^3 tanks with a population density of 50.3 kg m^{-3}. In addition, in each of these periods, blood extraction on hormone injection were performed. Is a possibility that intensive sampling during three weeks of the sexual differentiation period could increase cortisol levels and induce masculinization.

d. Never before rGths have been injected into sexually undifferentiated fish, so little is known about how they can affect sexual differentiation of gonochoristic fish. In rainbow trout (*O. mykiss*) and Nile tilapia (*O. niloticus*) during the stage of sexual differentiation there is expression of βFSH and βLH (Baron *et al*., 2005; Yan *et al*., 2012), however, it is possible that the increase in the level of gonadotropins by supraphysiological doses during this period could affect the expression of one or more genes related to sexual differentiation.

   Sox transcriptional factors are characterized as Sry-related high-mobility group (HMG) box proteins. *Sox* genes have been reported to be involved in sex determination and differentiation, formation of neuronal system, gonad, eye, pancreas, and cartilage (Wei *et al*., 2016). *Sox30* has been proven to be critical for mammalian and teleostean spermatogenesis. There is expression of *sox30* in testes and ovary of carp (*C. carpio*) (Arumugam and Balasubramanian, 2020) and Nile tilapia (*O. niloticus*) (Han *et al*., 2010; Wei *et al*., 2016). In fact, in carp (*C. carpio*), *sox30* expression is high during spermatogenesis and spawning / spermiation (Arumugam and Balasubramanian, 2020). In Nile tilapia (*O. niloticus*), *sox30* expression was reported during sexual determination and differentiation period in ovary and testes (Han *et al*., 2010). These data support that Sox30 plays a key role in gonadal differentiation and testes development (Han *et al*., 2010; Wei *et al*., 2016; Arumugam and Balasubramanian, 2020). Furthermore, *sox30* expression is influenced by gonadotropins. There is a substantial increase in *sox30* mRNA transcripts post-hCG induction, and hCG is proven to bind Lhcgrba (Arumugam and Balasubramanian, 2020).

   Taken together these results, we speculate that the increase in gonadotropin levels during the period of sexual differentiation with respect to basal secretion could increase

sox30 expression and alter sexual differentiation, however, there are no results that support this hypothesis. There are also other genes related to sexual differentiation such as *sox3* (Takehana *et al*., 2014).

Further studies are necessary to elucidate the possible alteration in gene expression by the administration of gonadotropins during the period of sexual differentiation.

6. Conclusion

1. Weekly intramuscular injections of specific *Argyrosomus regius* single-chain rGths for 3 weeks in prepubertal and sexually undifferentiated meagre (*A. regius*) are not able to stimulate a significant increase in GSI comparing with saline group.

2. Both rFSH and rLH are able to stimulate E2 secretion.

3. Both FSH and LH are able to stimulate early gonadal development with the onset of meiosis in female germ cells, and the onset and resumption of meiosis in male germ cells.

4. Our histological observations in sexual differentiation demonstrate that with recombinant gonadotropin treatment 1) the onset of meiosis in female germ cells occurs at the same time as de appearance of the ovarian cavity 2) males sexually differentiate earlier than females; 3) there is a higher proportion of males during the stage of sexual differentiation, 4) there are intersex fish during the stage of sexual differentiation.

Further studies are necessary to elucidate what kind of sex determination mechanisms has the meagre, how the administration of exogenous gonadotropins can influence sexual differentiation during this period and, to evaluate the effect of rGths treatment with a longer therapy and in older fish.

7. References

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