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**TESI DI LAUREA**

# Induction of gametogenesis of pre-pubertal greater amberjack (*Seriola dumerili,* Risso 1810) using recombinant gonadotropins (GtHs)

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**Abstract**

The greater amberjack (*Seriola dumerili*) is a candidate species for enhancement and diversification of Mediterranean aquaculture production due to its rapid growth, until ten times higher than European seabass *Dicentrarchus labrax*, reaching 800g in their first year. The leading producer is Japan, reaching ~40.000 tons in 2013, relying mainly on wild rearing or imported juveniles, because of the insufficient number of hatcheries. This strategy is justified by the difficulty of wild greater amberjack to adapt to captive condition, resulting in limited reproductive capacity. Greater amberjack reproductive dysfunctions are characterized by females failing to achieve ovarian maturation. Indeed, they show a lower plasma steroid profile if compared to wild fish, which is a consequence of their altered ability to release luteinizing hormone (LH). Males' reproductive dysfunctions are instead correlated to a reduced ability to enter meiosis and undergo spermatogenesis; moreover, the eventual milt quality is reported low, probably due to a lack in sperm hydration. To overcome these challenges a protocol exists, based on a treatment with a gonadotropin release hormone agonist (GnRHa). The fish are reared in sea cages and transferred inshore for the treatment. Mediterranean greater amberjack does not mature consistently in tanks and must be held in cages before the transfer to tanks for spawning induction. The aim of the present study was to use *Seriola dumerili* recombinant follicle stimulating hormone (FSH) and LH (SdrFSH and SdrLH), to induce maturation in 2-year-old greater amberjack kept in tanks. Fish were divided into control group (n=10) and treated group (n=15) and recombinant gonadotropins were administrated by weekly injection to induce puberty onset, vitellogenesis, spermatogenesis and improve sperm quality. At the end of the treatment, after histological analysis, the adjusted oocyte diameter was evaluated in both groups, showing no statistically significant differences between control group, and treated group ($P \leq 0.05$), and remaining completely immature. Further steroid profile analysis will help to clarify if and where the hormonal mechanism failed. On the other hand, males' results were encouraging, showing spermatogenesis in 100% of treated males (n=8) and sperm quality, assessed by the computer- assisted

sperm analysis (CASA), comparable to the one of adult individuals. As for females, also for males further steroid profile analysis will help to clarify the relationship between sex steroid level and sperm production and quality. This study could lay the foundation for future understanding of the mechanism responsible for greater amberjack puberty onset and completion, and the relationship between valuable milt production and steroid hormones, thus enhancing the knowledge on puberty hormonal mechanisms and the *Seriola dumerili* production.

# 1 Introduction

## 1.1 Greater amberjack

The greater amberjack *Seriola dumerili* (Risso, 1810) is a teleost fish belonging to the family Carangidae, genus *Seriola*. This genus includes 12 species distributed in tropical and temperate waters (Corriero, 2021). Adults present an elongated body laterally compressed with blue-olive colour and the typical amber band, silver colour ventrally and a black lateral band from the eye to the base of the dorsal fin (Fig.1).

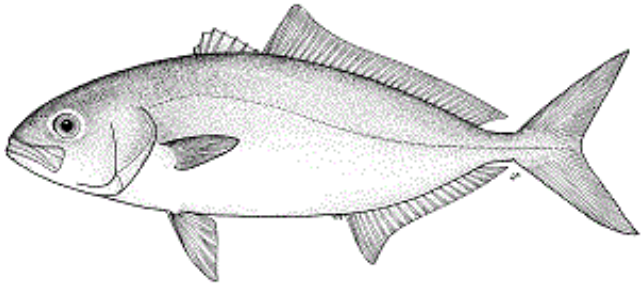

**Fig. 1 Greater amberjack, *Seriola dumerili* (FAO, 2022)**

The greater amberjack is distributed in tropical and subtropical areas in both the Atlantic and the Indo-Pacific Ocean, the Mediterranean Sea, Senegal and the United Kingdom. It can also be found in South Africa, Australia and Hawaii (Fig. 2). Greater amberjack is a pelagic species, living both inshore and offshore, usually found between 18 and 360 m depth.

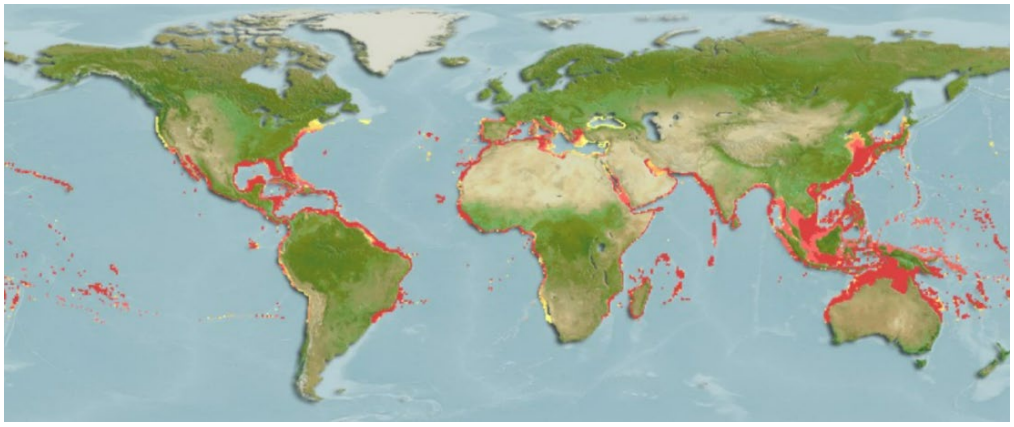

**Fig. 2. Worldwide distribution of the greater amberjack, *Seriola dumerili*, (AquaMaps, 2018)**

The greater amberjack is a gonochoristic species without sexual dimorphism (Zupa, 2017). It is reported that gonads start to differentiate at about three months of age or when the standard length (SL) reaches from 23 to 26 cm (Corriero et al., 2021). From the fifth month (28-32 cm SL) it is possible to observe ovigerous folds containing oogonia and few chromatin-nucleus stage oocytes in ovaries, whereas spermatogonial cysts and seminiferous tubules appear in testes (Corriero, 2021). Complete ovarian differentiation in hatchery-reared fish is completed at 408 days post hatching (dph), at a mean total length of 41.22 ± 3.83 cm (Papadaki et al., 2021). Maturity is attained by 50% of the males at 109 cm SL, 50% of females at 113 cm SL, and 100% of the fish over 128 cm, or after the fifth year of age (Kožul et al., 2001; Murie and Parkyn, 2008). Greater amberjack is an asynchronous spawner with indeterminate fecundity (Harris et al., 2007).

## 1.2 Production and market landscape

Greater amberjack is a candidate species for the enhancement and diversification of Mediterranean aquaculture production, showing growth rates until ten times higher than the European seabass, *Dicentrarchus labrax* (Muraccioli et al., 2000). It can reach 180-190 cm length and 80 kg of weight, reaching in its first year 40 cm of length and 800 g of weight (FAO, 2020). In recent years, interest for greater amberjack in the aquaculture industry is expanding, due to its high demand and market price (Sicuro & Luzzana, 2016; Nijssen et al, 2019), rapid growth, excellent fillet quality, and its capacity to accept inert food (FAO dataset, FishstatJ, 2020; Freitas J et al., 2019). European countries have tried since 1980 to rear this species through the fattening of juveniles. With its exponential growth, the aquaculture industry soon recognized the need to close the biological cycles of farmed species to improve their productivity (FAO, 2020).

Today, at worldwide level, the leading producer is Japan, reaching 38,770 tons in 2013 (Rigos, 2020), followed by Saudi Arabia, the United States and Mexico. Around the Mediterranean Sea, some countries, such as Spain, Italy, Malta, Croatia and Greece (Sicuro & Luzzana, 2016), have begun to produce the greater amberjack. There is a limited but gradually growing commercial activity with hatchery-produced individuals, and the first market-sized fish reached the European

market in 2018. Malta presents a small hatchery production estimated at 500 tons (Corriero et al., 2021; Fakriadis et al., 2020b; Pérez et al., 2020).

A representative market price can vary between 14€/kg and 20€/kg in Europe, whereas in Japan the price could reach 30$/kg (about 30€/kg) (DIVERSIFY, European Union's 7th framework programme). For comparison, gilthead sea bream (*Sparus aurata*) and European sea bass average price nowadays is 6€/kg (Bjørndal et al., 2019).

### 1.3 Larval rearing

As mentioned earlier, in Japan, the main producing country for greater amberjack, wild or imported juveniles are reared, due to the scarce number of hatcheries currently producing juveniles and the lack of a rearing protocol (FAO, 2022).

Eggs are pelagic with a diameter of about 1 mm, with a single oil droplet of about 0.25 mm (Mylonas et al., 2004, Papandroulakis et al., 2005). Embryos appear about 10 h post fertilization and hatching occur after 30-34 h at 23.5°C. Yolk absorption is completed at about 72 h post hatching, followed, at 120 h post hatching, by the swim bladder inflation (Papandroulakis et al., 2005). Water temperature about 23°C, flow rate 12 L $min^{-1}$ and aeration 1.5 L $min^{-1}$ are suitable for a proper hatching rate (≥60%) (Jerez et al, 2005).

Larval rearing in large tanks and low stocking density of eggs-larvae can improve growth and survival rate, whereas photoperiod has an essential role, starting from a photo phase of 24L:00D and then switching to 18L:06D after 21 dph. Light intensity and water flow must be carefully adjusted according to the fish's stage of development. Dissolved oxygen should be maintained >6.0 mg $L^{-1}$, salinity between 35 and 40 psu, pH between 7.8 and 8.5, and temperature between 23.5 and 25.0ºC.

Larvae are generally fed first with microalgae (150-300 x $10^3$ cell $ml^{-1}$), then they are switched to enriched rotifers (Arachidonic acid: ARA; 20:4n-6), artemia at 12 dph, enriched artemia at 18 dph and finally to commercial diets (FAO, 2022). Larval performance could be optimized supplementing taurine, histidine and protein (0.3 to 1.1%) to commercial diets: this practice, when combined with semi-intensive (instead of intensive) systems, may also improve survival rates. (Djiellata, 2021).

For grow out phase, where fish are raised and maintained until they reach the desired size, sea cages are used. The greater amberjack can accept inert commercial feed, as long as it contains the right ratio of nutrients, high- value proteins included. Stocking density should be maintained at 5kg $m^{-3}$. The environmental conditions have been shown to play an important role in growth performance; for example, juveniles have been shown to achieve a better growth at 21°C compared to 26 and 16°C, whereas when the body weight increases (≥500 g) temperature becomes less relevant, with fish showing no differences in growth performance between 20 and 23°C (Fernandez-Montero et al., 2017; FAO, 2022).

## 1.4 Fish reproduction

In fish, reproduction is controlled by the brain-pituitary-gonad axis. Gonadotropin releasing hormone (GnRH), a brain synthetized neuropeptide triggered by external environmental factors, such as photoperiod, temperature etc, to directly stimulate pituitary gland gonadotropins (GtHs) secretion. GtH stimulation (mostly FSH) causes the secretion of androgens such as T and 11-KT in males and $E_2$ in females, which operate concurrently with FSH in the control of gametogenesis (Moles et al., 2020). In females, $E_2$ stimulates vitellogenin synthesis from the liver, essential for female gametogenesis and oocyte maturation. At the end of this phase, under the action of LH, the secretion of the maturation-inducing steroid (MIS) from follicle cells is induced. At the same time, FSH and steroid levels in blood decrease. Once gonadal maturation is completed, a peak of LH induces final oocyte maturation (FOM) and ovulation in females, whereas in males a lower peak induces spermiation (Mañanós et al., 2009).

Gonadotropins are fundamental hormones in vertebrate reproduction. They are produced by the anterior pituitary gland and secreted in the bloodstream after the action of GnRH (Levavi-Sivan et al., 2010). Teleost fish species present two different GtHs, comparable to mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Kawauchi et al., 1989).

GtHs are glycoproteins composed by a non-specific alpha subunit and a specific beta subunit, encoded by different genes (Moles et al., 2020). Their complex structure synthesis starts from a cysteines series primary structure, during

biosynthesis passages it goes through the rough endoplasmic reticulum to the Golgi apparatus, where specific disulphide bonds modify the overall cysteines structure in a core with a central cysteines knot with peripheral loops (Cahoreau et al., 2015). The carbohydrate structure attached to the protein core can be very variable, and the terminal residues affect the protein folding, secretion, half-life in circulation and receptor-binding (Cahoreau et al., 2015; Moles et al., 2020).

## 1.5 Reproduction, reproductive dysfunctions in captivity and spawning induction in greater amberjack

In nature, greater amberjack spawns in spring or summer, depending on the geographical latitude. Spawning has been reported to occur in the United States eastern coast tropical waters from April to June (Murie & Parkyn, 2008), in the Gulf of Mexico and the north-west Atlantic from April to May (Murie & Parkyn, 2008; Harris et al., 2007) and in the Pacific Ocean (Hawaii) from March to April (Kikawwa & Everson, 1984). In the Mediterranean Sea reproductive recrudescence starts in early May and by the end of May, when the water surface reaches 19-20°C, spawning events may occur (1.3-4.2 million eggs) (Kikawwa & Everson 1984, Harris et al. 2007). The reproductive peak is recorded when the water surface temperature reaches 23-24°C, in June-July (Mandich et al., 2004; Zupa et al., 2017b; Pousis et al., 2018, 2019). However, greater amberjack spawning events in the wild have never been recorded, information on preferential depth comes from fisheries-based observations (Corriero et al., 2021), for example, between 25 and 35 meters of depth have been reported in the Pelagie Islands and Tunisia (Lazzari & Barbera, 1989), and between 20 and 100 meters of depth have been reported in the north-western Atlantic Ocean (Harris et al., 2007). Information on preferred spawning time is derived from the same kind of observations; greater amberjack collected in the morning in the north-western Atlantic Ocean were expected to spawn several hours later, based on observations of the oocyte maturation stage (Harris et al., 2007). A reproductive behaviour event, recognized as sign of imminent spawning, was recorded at the sunset in a Caribbean coral reef (Heyman & Kjerfve, 2008). In captivity, wild-caught greater amberjack shows difficulties to adapt to captive conditions, with limited capacity to reproduce spontaneously

(Corriero et al., 2021). From the first studies on greater amberjack reproduction, it was clear that captive individuals had difficulties to spawn (Kawabe et al., 1996, Micale et al., 1999), exhibiting different types of reproductive dysfunctions (Mylonas et al., 2004).

Captive-reared females gonadosomatic index (GSI) during spawning phase is lower than in wild females, with the ovaries exhibiting more than 50% of atretic vitellogenic follicles and, during the reproductive season peak, females fail to undergo complete oocyte maturation (OM), exhibiting 100% of atretic oocytes (Zupa et al., 2017b). In the same females, an altered plasma steroid profile was observed, with testosterone (T), 17β-estradiol (E2) and 17α, 20β-dihydroxy-4-pregnen-one (17,20β-P) concentration being lower than in wild fish (Zupa et al., 2017b; Pousis et al., 2018). Additionally, there was no difference between wild and captive-reared females in terms of the liver expression of vitellogenins (*vtga, vtgb, and vtgc*), but there was a decrease in the gene expression of vitellogenin receptors (*vtgr, lrp13*) in captive-reared females, which resulted in a decrease in the number of vitellogenic oocytes during the vitellogenesis phase. This suggests that the early oogenesis phase is affected under captivity, thus reducing the reproductive potential of the fish (Pousis et al., 2019; Fakriadis et al., 2020b, Corriero et al., 2021).

Nyuji et al. (2016) conducted research on follicle stimulating hormone (FSH) and luteinizing hormone (LH) subunit β (FSHβ and LHβ), and their ovarian receptors (*FSHr, LHr*), reporting a peak in FSHβ and FSHr expression, followed by a FSH plasma level peak at the end of the reproductive season, possibly related to the role of FSH in preparing the gonad for the next reproductive cycle. Pituitary gonadotropin hormones (Gths) can be produced by captive-reared females, but their altered ability to release LH prevents oocytes from entering the oocyte final maturation (OM), leading to atresia and preventing spawning (Corriero et al., 2021). This is supported by several trials, where it has been demonstrated that external stimulation of LH release can allow greater amberjack to overcome this dysfunction, undergoing OM and completing its reproductive cycle in captivity, i.e. with gonadotropin release hormones agonist (GnRHa) administration, increasing the LH plasma concentration 24 hours after the treatment and inducing spawning (Nyuji et al., 2019; Mylonas et al., 2010; Zupa et al., 2017; Mylonas et al., 2004, Fakriadis et al., 2019; Corriero et al., 2021).

Captive-reared males present low GSI, reduced seminiferous lobule diameters, early interrupted spermatogenic activity and gonads in regressing phase during the spawning season, compared to wild individuals (Zupa et al., 2017b). Spermatogonia of captive-reared fish present reduced ability to enter meiosis and undergo spermatogenesis, resulting in an early end of reproductive activity (Zupa et al., 2017a). These fish present altered steroid plasma concentrations, specifically of 11-KT, T and 17,20β-P, and very high concentrations of E2. This hormonal profile is hypothesized to be the cause of meiosis limitations. However, sperm samples can be obtained, but with low quality, most likely because of a lack in sperm hydration (Zupa et al., 2017a, b). These aspects of male reproductive dysfunctions can partly explain the relative lower fertilization observed in captive-reared fish, compared to wild individuals (Fakriadis et al., 2019, 2020a; Jerez et al., 2018).

Greater amberjack sperm parameters are reported to be highly variable; sperm motility ranges of 0.5 to 8 min (Zupa et al., 2017a; Fakriadis et al., 2020b, 2021); spermatozoa density between 18 to 70 X $10^9$ spermatozoa $mL^{-1}$ (Fakriadis et al., 2020b; Zupa et al., 2017a; Jerez et al., 2018), and survival under cold storage (4°C) from 5 to 14 days (Fakriadis et al., 2020b, 2021; Jerez et al., 2018).

Considering computer-assisted sperm analysis (CASA), a semi-automatic system to study sperm quality, that evaluate the sperm head speed related to the considered path, the different parameters measured involve: curvilinear velocity (VCL), the average speed of a sperm head along its actual two-dimensional path, straight-line velocity (VSL), the average speed along the straight line between first and last detected head position, average path velocity (VAP), the average speed along the average path, all computed by an algorithm (Lu et al., 2013). In a recent study, it has been shown that greater amberjack presents lower VCL than other marine fish, such as European seabass and gilthead seabream (Fakriadis et al., 2021).

To overcome these challenges, a GnRHa-based treatment protocol has been developed: breeders are reared in sea cages during the year to be then transferred in tanks and receive a spawning induction treatment with GnRHa slow- release implants (ideal: 50 μg $kg^{-1}$ BW, seawater temperature 19-24°C) (Fakriadis et al., 2020b), or directly reared in outdoor large tanks, and treated with GnRHa implants for spawning (Jerez et al., 2018).

## 1.6 Recombinant GtHs (rGtHs)

The ability to isolate the cDNAs coding for GtHs and the composition of GtHs from two subunits coded by different genes, allowed the production of single subunit (monomeric) GtHs or dimers, comprising of two subunits (Moles et al., 2020). To produce rGtHs subunits, the isolated cDNAs are cloned in a chosen vector and expressed in a heterologous system. Vectors are molecules used to carry DNA segments into host cells, helping also in the replication or in the expression processes (NIH, glossary of genetic term). Common vectors include plasmids and viruses, and vector selection is particularly important for the success of the expression (Moles et al., 2020). The chosen host can vary, i.e., yeast (*Pichia pastoris*), *E. coli*, the fruit fly (*Drosophila melanogaster*) and mammalian cells (i.e. CHO-Chinese hamster ovary) affecting the protein production and quality (Moles et al., 2020).

rGtHs applications in aquaculture are several; *in vitro* i.e. in gonad tissues to evaluate the steroidogenic activity and gene expression (Aizen et al., 2017), and *in vivo*, mainly by injection, to evaluate sex steroid profiles, GSI, spawning induction, milt production and sperm quality, after both short-term and long-term treatment (Aizen et al., 2017; Palma et al., 2019; Penaranda et al., 2018, Moles et al., 2020).

## 1.7 Aim of the study

As reported earlier, Mediterranean greater amberjack does not mature consistently in tanks and must be held in cages before transfer to tanks for spawning induction. The aim of the present study was to use recombinant FSH and LH (SdrFSH and SdrLH), produced by a private company as single chain gonadotropins in heterologous expression system to induce puberty in 2-year-old greater amberjack kept in tanks. With this aim, two greater amberjack populations have been treated with a combination of homologous SdrFSH and SdrLH to induce vitellogenesis and spermatogenesis.

## 2 Materials and methods

### 2.1 Fish maintenance

The experiment was conducted in the Aqualabs of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre of Marine Research (HCMR) in Heraklion (Crete, Greece).

**Fig. 3. Aqualabs IMBBC with the used 2-m$^3$ cylindrical tanks**

Four stocks of greater amberjack were formed with 2-year-old fish born and reared at the facilities of Argosaronikos Fish Farm S.A. (Salamina Island, Greece) and transferred to HCMR. A total of 25 fish were used (mean wet weight± SD, 1016.4 g ± 197.13 g), divided randomly into four 2-m$^3$ cylindrical tanks (Fig.3), assuming a sex ratio of 1:1. The control group consisted of 10 fish held in two tanks, control group 1 (CG1) (n=5; mean wet weight± SD: 998.4 g ± 300 g) and control group 2 (CG2) (n=5; 1006.2 g ± 270.2 g), whereas the treated group consisted of 15 fish held in other two tanks, fshlh group 1 (FSHLH1) (n=8; 995.5 g ± 107.84g) and fshlh group 2 (FSHLH2) (n=7; 1060.4g ± 172.9g). Fish were held under natural seasonal photoperiod and temperature (18-20°C) between March and June 2022. They were fed with industrial feed (Zoonomi, 6 mm) daily. Dissolved oxygen (85-101%), temperature, pH (7.2-7.7) and salinity (32-35‰) were measured daily and flow rate (1200%) was measured once a week.

### 2.2 Samplings

Weekly samplings were conducted from March until June 2022, for a total of 84 days. Fish were starved one day before the sampling. The water volume in the tank was reduced to 0.85 $m^3$, closing the waterflow and turning on the oxygenation system, in order to proceed with a clove oil- anaesthetic treatment (0.01 mL $L^{-1}$) to tranquilize the fish. Then a solution with a higher concentration (0.03 mL $L^{-1}$) was used in an anaesthetic bath for complete fish sedation.

Samplings of weight and blood were conducted from day 0 (22$^{nd}$ of March) every 21 days (D0, D21, D42, D63, D84). Over the samplings, fish were injected using hypodermic needles (21Gx1½″) along the dorsal muscle close to the vertebral column (Fig.4), with the determined dose of rGtHs (treated group) or saline solution (control group). Individuals belonging to the control group were administered with 1 mL $Kg^{-1}$BW of isotonic physiological solution (NaCl 0.9%), whereas the treated groups were administered in Week 0 with rFSH, at a dose of 8 µg $Kg^{-1}$, and then from Week 1 until the end of the experiment with both FSH and LH at a dose of 12 µg $Kg^{-1}$ rFSH and 10µg $Kg^{-1}$ rLH. Doses were decided following a previous study conducted at HCMR (Mylonas, personal communication).

**Fig. 4: rGtHs administration**

Fish were weighed and blood was collected from their caudal vein using heparinized syringes. The blood was then poured into heparinized tubes, centrifuged at 4°C, 4500 rpm for 15 min (Centrifuge5804R, Eppendorf) (Fig.5) and the obtained plasma was stored, in two aliquots, at −80°C (SANYO, Japan) until solid phase extraction.

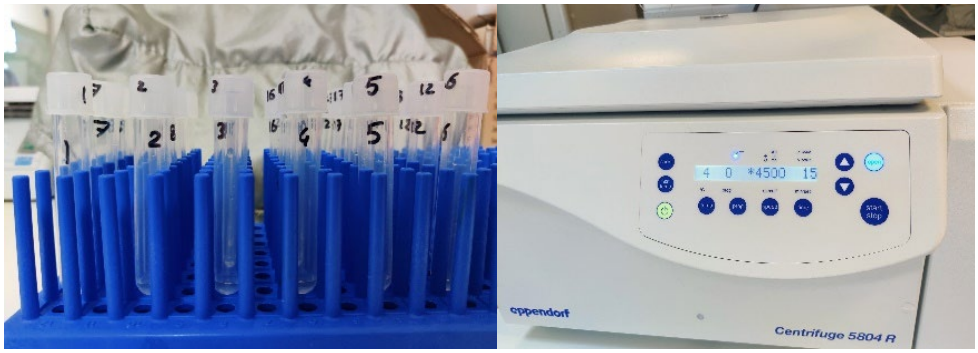


**Fig.5: left) heparinized tubes for bleeding; right) centrifuge**

At the last day of the experiment (D84), fish were checked for sex and maturation stage. First, gentle abdominal pressure was applied to check for the presence of sperm, after cleaning and drying of the genital pore to avoid potential contaminations (Mylonas et al., 2013). If fish were releasing sperm, the spermiation index was evaluated using a subjective scale from 0 to 3, S0 = no milt release, S1= drops of milt after multiple attempts, S2 = milt release easily after first attempt, S3 = copious amount of milt with little pressure (Mylonas et al., 2016), If no milt was obtained with stripping, a biopsy was collected using a plastic catheter inserted into the genital pore and applying gentle aspiration, followed by observation of the sample in a compound microscope (40 and 100 x). The maturation stage of the ovarian biopsies was assessed by observing the wet mount (Brown-Peterson, 2011, Mylonas et al., 2013). After sexing and maturity stage evaluation, 10 fish were killed, 8 from the control group and 2 from the treated group and gonads were excised, weighted, and kept in a solution of 4F:1G (formaldehyde: glutaraldehyde) for histological processing.

2.3 Plasma hormones solid-phase extraction

Custom-made Solid Phase Extraction (SPE) cartridges were prepared by dry-packing 10 mg of polymer-based C18 sorbent (Strata-X 33µm polymeric reversed phase, Phenomenex) into 1 mL polypropylene pipette tips, the lower end of which were stoppered with a small piece of wool. Packed cartridges were mounted on a vacuum manifold (VM12 12-port vacuum SPE manifold, Phenomenex) and

conditioned with 500 µL of methanol and 500 µL of water. Subsequently, a 200 µL aliquot of each plasma sample was diluted 1:1 with water and loaded onto a SPE cartridge. After a two-step washing procedure with 500 µl of water and 350 µL of methanol 40% v/v, the hormones were selectively eluted using 450 µL of pure methanol and collected in amber glass vials. The flow rate during SPE procedure was adjusted to 0.5 drop $sec^{-1}$.

*2.4 Histological analysis*

The excised gonads were dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany) (Fig.6).

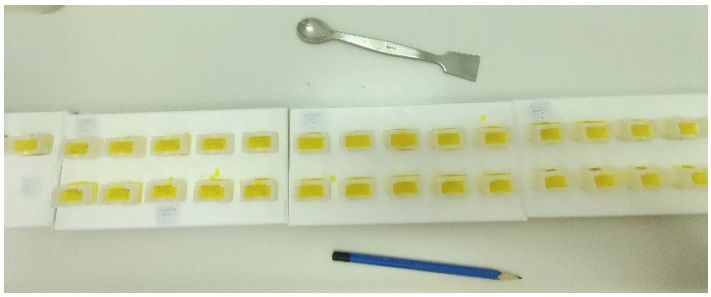

**Fig. 6: glycol methacrylate resin embedding**

A semi-automatic microtome (Leica RM2245, Germany) was used to obtain serial sections of 3–5 µm using disposable blades. Slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976), they were examined under a light microscope (50i Eclipse, Nikon, Japan) and photographed using a digital camera (Progres, Jenoptik AG, Germany).

**Fig. 7: semi-automatic microtome (Leica RM2245, Germany), light microscope (50i Eclipse, Nikon, Japan) with digital camera (Progres, Jenoptik AG, Germany)**

The adjusted oocyte diameters were recorded using the scientific image analysis program ImageJ (Schneider, 2012), by evaluating and computing the mean of the longitudinal and the transversal length for each sample under analysis (Mylonas et al., 2013) (n=8 oocytes per sample). A total of 13 samples were used, 6 from the control groups and 7 from the treated groups.

2.5 Sperm quality evaluation

Sampled sperm was stored in 1.5 ml micro-centrifuge tubes, which were then placed on ice and transferred to a 4°C refrigerator until evaluation. Sperm quality parameters that were evaluated included sperm motility (seconds), initial percentage of spermatozoa showing forward motility immediately after activation (spermatozoa motility, %), and survival of spermatozoa under cold storage at 4°C (spermatozoa survival, days) according to published and validated methods (Fauvel et al., 1999; Mylonas et al., 2016; Papadaki et al., 2008). Spermatozoa motility was evaluated on a microscope slide (400× magnification) after mixing 1 μl of sperm with a drop of about 50 μl of saltwater (in duplicate). Activated sperm samples were observed under the compound light microscope for the first time 10 sec after activation. Spermatozoa motility (%) was determined subjectively using increments of 10%. Sperm was stored at 4°C for the days after collection, and was examined

every two days for spermatozoa motility, until no forward motility was observed, in order to estimate spermatozoa survival time (days). A total of 11 samples were analysed, 3 from the control group and 8 from the treated group, evaluating the presence/absence of milt, computing the absolute and relative frequencies of the two variables, and the expected frequencies.

Milt quality was also assessed using CASA (ISAS, Spain). Immediately after milt collection, samples were activated in seawater containing 2% bovine serum albumin to obtain 200-300 cells in the field. Spermatozoa movement was recorded on a disposable counting chamber with a fixed depth (Leja) 15 sec after activation using a digital camera (The Imaging Source DMK 22BUC03) with a resolution of 744 × 480 pixels at 30 frames per second (fps) attached to a light microscope (Zeiss Primo Star) under 200× magnification, using dark field microscopy. The analysed parameters were curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) (μm $sec^{-1}$), motile cells, progressive cells (> 80% straightness (STR)), rapid cells, and straightness (STR (%)).

### 2.6 Statistical analysis

The statistical analysis was carried out using an additional Microsoft Excel component, XLSTAT software and the statistics graphic interface R Commander (Fox J, 2017).

Oocyte adjusted diameter independence of the two groups was tested with a z-test after normalizing the data, a level of $P \leq 0.05$ was set as statistically significant.

A Fisher exact test was computed to evaluate how strong the relationship between the treatment and the presence of milt was. A level of $P \leq 0.05$ was set as statistically significant.

## 3 Results

### 3.1 Females

Ovarian biopsies and gonad sections from females (n=13) studied with histological examination exhibited only oogonia and primary oocytes (Fig. 8). Control group females (n=7) presented an average adjusted oocyte diameter of 55.9±6.19 µm, with the maximum recorded diameter being 62.8 µm and the minimum 50.8 µm. Treated females (n=6) presented an average adjusted oocyte diameter of 53.9±8.29 µm, with the maximum recorded diameter 61.9 µm and the minimum 43.01 µm (Fig.8, b). No difference was found between the two groups in oocyte diameters (P>0.05).

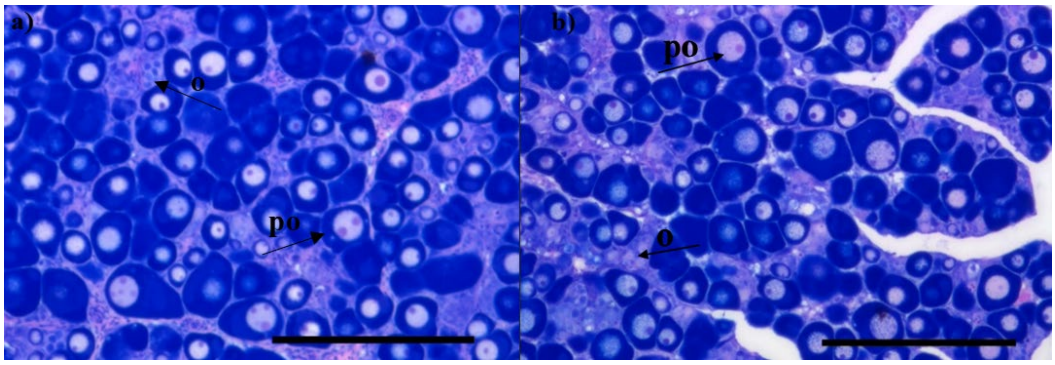


**Fig. 8: Histological sections of female greater amberjack ovaries: a) control group, b) treated group, obtained at the end of the trial, after treatment with rGths (rFSH: 12 µg $Kg^{-1}$ and rLH: 10µg $Kg^{-1}$) for 12 weeks. The ovary contains oogonia (o) and primary growth oocytes (po), with primary growth oocyte adjusted diameters ranging from 43.01µm to 61.9 µm. Bar = 200 µm.**

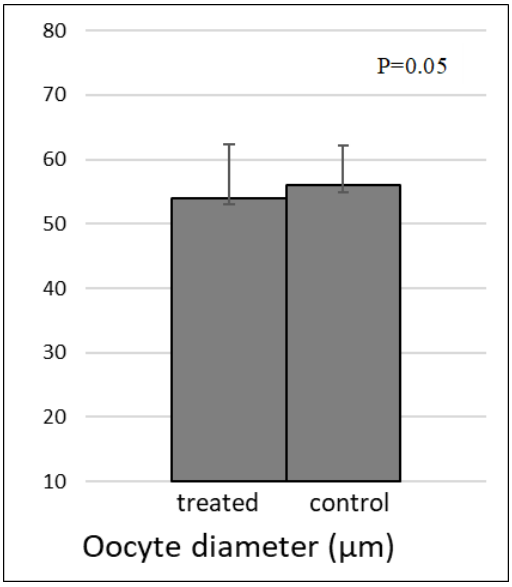


**Fig. 9: Comparison between adjusted oocyte diameter means (± SEM) after rGths treatment of the treated (53.9±8.29 µm) and the control group (55.9±6.19 µm), not showing statistically significant difference (z- test, P ≤ 0.05).**

3.2 Males

Considering males (n=11), 9 males were able to release sperm with a spermiation index of S2, from which 8 belonged to the treated group and 1 to the control group. Of the three control fish tested, only 1 was found to be spermiating, with the remaining 2 males exhibiting a spermiation index of S0. The treated group presented 8 spermiating males from a total of 8 fish. The independence between the treatment and the presence of milt has been rejected (P ≤ 0.05), showing the effect of rGtHs treatment on spermiation induction.

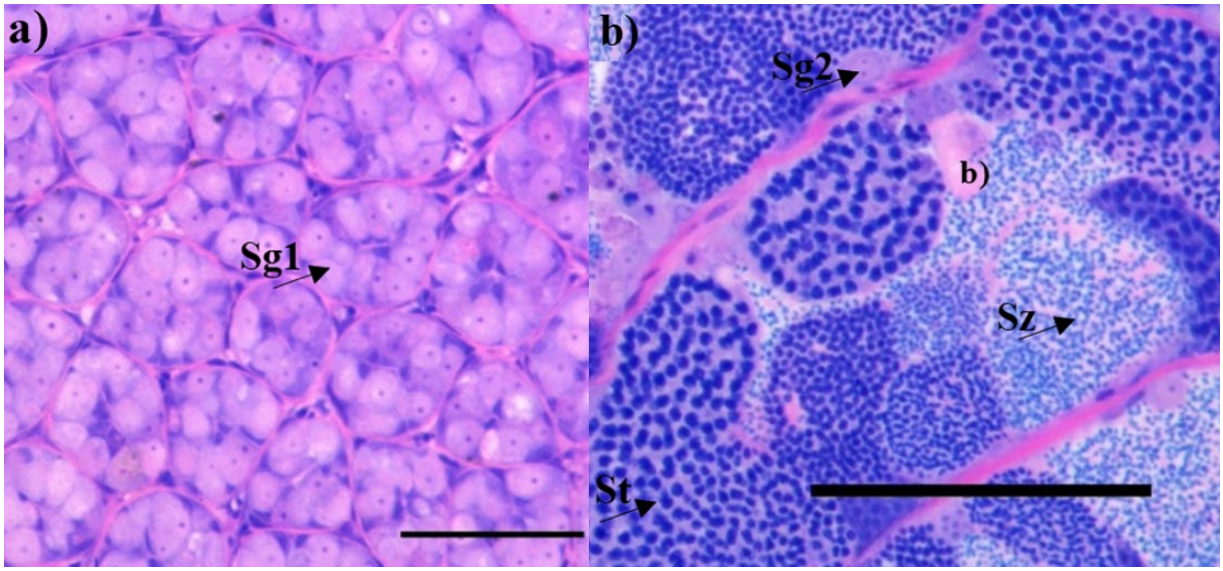


**Fig. 10: Histological sections of male greater amberjack testes collected after the end of the trial. a) Section from a male (control group) treated with 1 mL $Kg^{-1}$ BW of NaCl 0.9%, containing primary spermatogonia (Sg1). b) Section from a male (treated group) treated with rGths (rFSH: 12 µg $Kg^{-1}$ and rLH: 10µg $Kg^{-1}$) for 12 weeks, containing secondary spermatogonia (Sg2), spermatids (St) and spermatozoa in the lumen (Sz). Bars: a) 50 µm; b) 200 µm.**

Duration of forward motility (min) exhibited a mean (± SEM) value of 5'23'' ± 0.26 min; initial percentage of spermatozoa showing forward motility immediately after activation (spermatozoa motility, %) ranged from 12.5% to 92.5%; spermatozoa survival under cold storage (4°C) ranged between 4 and 8 days (5.5 ± 1.25 days). Spermatozoa density ranged between 92.4 to 151.2 x $10^9$ szoa $ml^{-1}$, whereas results on the velocity parameters measured were: VCL of 138.4 ± 4.4 µm/s, VSL of 115.26 ± 5.3 µm $sec^{-1}$ and VAP of 119.2 ± 5.0 µm $sec^{-1}$. Progressive (%) spermatozoa mean was 30.59 ± 2.2%, rapid (%) spermatozoa mean was 26.25 ± 2.6% and STR (%) mean was 96.5 ± 0.73%.

## Discussion

The goal of the current study was to determine the gametogenic and steroidogenic activity in males and females greater amberjack by examining their gonad samples histology, using the CASA system to evaluate the milt quality, and monitoring their hormone plasma levels throughout the treatment period. Due to the fact that the hormonal analysis has not been completed yet at HCMR, the effect of GtHs administration on sex steroid production will not be discussed. Therefore, discussion will focus on the gametogenic and not on the steroidogenic properties of rGtHs.

The aim of the present study was to evaluate the possibility of inducing puberty and to analyse the effect of rGtHs administration on gametogenic and steroidogenic activities in pre-pubertal greater amberjack. The induction of puberty in females would result in an increase in oocyte diameter and an advancement of the maturation stage, whereas in males it would enhance spermatogenesis and spermiation and improve sperm quality.

The hormonal mechanism leading to puberty is a research topic not fully explored. In the past, ovarian primary growth phase appeared to be GtH-independant (Khoo et al., 1979; Billard, 1992); however, recent studies have shown how gonadotropins and their receptors, in particular FSH, could have a regulatory role in gonadal stimulation of steroidogenesis (11-KT and $E_2$) during primary growth (Nguyen et al., 2022, Luckenbach et al., 2011, 2013; Lokman et al., 2007; Campbell et al., 2006). In our trial, the effect of rFSH and rLH in inducing puberty was tested in 2-year-old pre-pubertal greater amberjack of an initial BW of 1016.4 ± 197.13 g (final BW of 1124.2 ± 59.5 g), while normally sex maturation in females is reached at 3-4 years and 7 kg of BW (Pousis et al., 2018; Zupa et al., 2017a; Zupa et al., 2017b). After observing the collected biopsy (wet mount) sample (Mylonas et al. 2013), no difference between the groups (control and treated) was found, whereas the diameter of the largest oocyte was less than 150 μm, associated with the early cortical alveoli stage or late perinucleolar stage (Marino et al., 1995b; Grau et al., 1996; Micale et al., 1999; Sley et al., 2014, Corriero et al., 2021). The possible

reasons for the lack of effect of GtHs in inducing puberty are multiple, such as a potential block in estrogen biosynthesis due to lack in sex steroid positive feedback, considering that estrogens of gonadal origin are likely not able to pass the blood-brain barrier. Moreover, it could be related to cytochrome P450 aromatase conversion of estrogens to androgens (Callard et al., 2001; Trudeau et al., 1993; Montero et al., 1995; Filby et al., 2008), or a failure in growth hormone and insulin like growth factor (Igf1) gonadal stimulation and expression, both supposed to have key roles in puberty onset due to a lack in hormonal feedback (Gray et al. 1990; Jalabert et al., 2000, Gioacchini et al., 2005, Kamangar et al., 2006, Taranger et al., 2010). After further histological analysis, adjusted oocyte diameters were in both groups less than 60 μm, a diameter associated with mid-perinucleolar stage. Relatively recent studies have shown how the deletion of the gene that encodes the FSH subunit did not abolish follicle growth but retarded it (Britt and Findlay, 2002; Barnett et al., 2006). On the other hand, the FSH-receptor (FSHr) deletion stops ovarian follicle development even at earlier stages (Zhang et al., 2015) showing small and atretic primary oocytes. Females of the present study were completely immature at the beginning of the experiment and remained completely immature after 12 weeks of rGtHs administration. This could also mean that the FSH-receptor was not functional so early in life. In a similar study using rFSH and rLH to induce maturation in flathead grey mullet (*Mugil cephalus*) treated females that had already entered pre-vitellogenesis and early vitellogenesis entered and complete vitellogenic growth, while control group did not show any further maturation. During completion of vitellogenesis, is recorded an increase in $E_2$ plasma levels (Ramos-Jùdez et al., 2022), showing the efficacy of rGths treatments and the correlation with steroids plasma levels, underlining the importance in understanding pubertal onset mechanisms and relationship with gonadotropins in *Seriola dumerili*. In accordance with our study, it has recently been shown that in FSHr-Knock-Out (KO) and LHr; FSHr double-KO medaka (*Oryzias latipes*) folliculogenesis was arrested at the pre-vitellogenic stage, with low level of $E_2$ (Kitano et al., 2022).

When hormonal analysis will be complete, results , taking into consideration plasma concentrations of 11-KT, $E_2$, MIS (17a, 20β-dihydroxy-4-pregnen-one), and their change in concentration during the trial, will clarify where and if the hormonal

feedback system failed in inducing maturation of the ovary. In cod (*Gadus morhua*), both *in vitro* and *in vivo*, androgen treatment promoted primary follicle development (Kortner et al., 2008, 2009), whereas in sea bass androgens may participate in primary growth regulation, increasing androgen receptor β (ARβ) transcript in early stages of development and then declining in early vitellogenic stage, supporting the importance of androgenic signalling during ovarian development (Garcìa-Lòpez et al., 2011).

As for females, also for males the mechanism related to the puberty onset is still poorly understood.

Normally sexual maturity in greater amberjack males is reached at the 3rd years of age, with BW ranging from 7 to 10 kg (Pousis et al., 2018; Zupa et al., 2017a; Zupa et al., 2017b). In the present trial, 11 2-year-old males were used (initial BW 1016.4 g ± 197.13g; final BW 1124.2g ± 59.5g), 8 in the treated group and 3 in the control group. Nine of the males studied released milt with an evaluated spermiation index of S2, 8 (100%) belonging to the treated and 1 belonging to the control group, showing the efficacy of the treatment in inducing spermatogenesis. Use of recombinant gonadotropins has already been tested in different trials, in pre-pubertal fish, such as the Japanese eel, the European sea bass and the yellowtail kingfish (Sanchis-Benlloch et al., 2017) with similar results. However, the triggering role of FSH in spermatogenesis is not always considered essential (Britt and Findlay, 2002; Barnett et al., 2006). Its presence is associated with increases in spermatogonia and spermatocytes number (Mazon et al., 2014, Sanchis-Benlloch et al., 2017), and with structural, nutritional and regulatory support on germ cell development (Chauvigné et al., 2012). In the European sea bass, gonadotropins, and especially FSH, could act on Leydig cells, triggering androgen (11-KT) release (Mazòn et al., 2014). The same effect has been shown in other studies on Japanese eel, African catfish (*Clarias gariepinus*), zebrafish (*Danio rerio*), and Senegalese sole (*Solea senegalensis*) (Garcia-Lopez et al., 2008, 2010; Chauvigné et al., 2012; Ohta et al., 2007). In pre-pubertal yellowtail kingfish rFSH was administered in a long-time treatment (60 days) resulting in the production of spermatozoa in seminiferous tubules (Sanchis-Benlloch et al., 2017). LH role is commonly associated with the final stages of maturation, stimulating the synthesis and secretion of maturation inducing steroids (MIS) (Levavi-Sivan et al., 2010; Palma

et al., 2018), and triggering the differentiation of spermatids into spermatozoa (Chauvigné et al., 2012). It is considered strictly correlated with spermiogenesis, affecting milt volume and quality (Mazon et al., 2013; Ohta et al., 2017; Moles et al., 2020). In adult Senegalese sole, rFSH induced testes maturation improving the tubules formation, with a final optimal rLH injection promoting spermatogenesis, whereas a non-optimal tested rLH dose causes Leydig cell apoptosis and maturation failure (Chauvigné et al., 2017). This stresses out the importance of the correct dosage, underlining how low dosage could have any or slight efficacy on maturation, while high dose could be disrupting for steroidogenic activity or sperm quality (Chauvigné et al., 2017). In our experiment, we used in Week 0 only rFSH, at a dose of 8 µg $Kg^{-1}$, and then from Week 1 until the end of the experiment both rFSH and rLH at a dose of 12 µg $Kg^{-1}$ rFSH and 10µg $Kg^{-1}$ rLH, obtaining average sperm motility duration of 5'23'' ± 0.26 min, a value which seems to be in line with other studies, where average motility values ranged from 0.5 to 8 min (Zupa et al., 2017a; Jerez et al., 2018; Fakriadis et al., 2020b, 2021). Moreover, survival under cold storage ranged from 4 to 8 days (5.5 ± 1.25 days), similar to other studies, where average survival ranges from 5 to 14 days (Fakriadis et al., 2020b, 2021; Jerez et al., 2018). In another study on flathead grey mullet males received a rGths treatment with increasing doses; initially only of rFSH (6, 9, 12 µg $Kg^{-1}$), then both rFSH and rLH (12 µg $Kg^{-1}$) during maturation phase, to eventually induce the spawn with rLH (12 or 24 µg $Kg^{-1}$). Spermiation was obtained in all the treated group (n=9), with breed-eligible sperm quality, similar to mature adult wild *Mugil cephalus*; 11-KT levels in plasma increased gradually with a dose-response correlation, and high volume and density parameters (desirable) were attested, related to the rLH effect (Ramos-Jùdez et al., 2022). Those results underline the importance of administration pattern and the LH effect on quality. In the present study milt quality was also evaluated using CASA. Spermatozoa density (92.4 - 151.2 x $10^9$ szoa $mL^{-1}$) appeared to be higher than in other studies, in which it ranged between 18 and 70 x $10^9$ szoa $mL^{-1}$ (Fakriadis et al., 2020b). Velocity parameters evaluations exhibited values comparable to previous studies and other marine species. For example, it was similar to the sperm velocity of GnRHa-induced adult greater amberjack (VAP ~ 103 µm $s^{-1}$), European seabass (VCL ~ 140 µm $sec^{-1}$ and VSL ~ 100 µm $sec^{-1}$) and gilthead seabream (VCL ~ 160 µm $sec^{-}$

[1] and VSL ~ 110 μm $sec^{-1}$) (Fakriadis et al., 2020b). Therefore, treatment of male greater amberjack for 12 weeks with rGtHs induced spermiation in 100% of males with the exhibited sperm quality being comparable to the one of adult individuals. Steroids profile, and its modification during the trial, will clarify the effect of the treatment on sex steroid release and the relationship with sperm quality.

**Conclusion**

Greater amberjack is a promising species for aquaculture diversification. Despite the fact that some of its reproductive dysfunctions have been overcome, leading to the increase of hatchery facilities in Malta and Greece, reproduction in captivity is still a limiting factor, which necessitates reliance on wild caught specimens to fulfil the market demand. The aim of the present study was to increase current knowledge on fundamental aspects related to the endocrinological mechanism leading to puberty onset and completion, and to develop a method to induce and/or enhance puberty in 2-year-old fish. Treatment with rGtHs had no effect on females, as the examined ovaries exhibited oocytes in primary growth stage and oogonia, showing no difference in comparison with the control females. The completion of our analysis will clarify whether the recombinant gonadotropins treatment had any steroidogenic effect, enabling us to redesign the treatment protocol by evaluating possible solutions as doses modifications and administration period. On the other hand, males' results were encouraging, with all of treated males reaching spermiation and milt release after stripping, indicating that the treatment have an effect on gonadal maturation. Sperm quality seems to be similar to the quality in adult individuals. Eventually, our steroid analysis will elucidate the steroidogenic effects of the treatment.

In conclusion, the use of recombinant gonadotropins seems to have completed gametogenesis in males while having no apparent effects on females.

Further works should focus on the hormonal mechanism responsible for female greater amberjack puberty onset and completion, improving the treatment with recombinant gonadotropins, and evaluating its application for inducing puberty in females.

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