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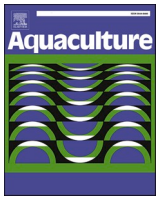

Overcoming dysfunctional gametogenesis in hatchery-produced greater amberjack, *Seriola dumerili* using recombinant gonadotropins, and spawning induction using gonadotropin releasing hormone agonist-loaded implants

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ABSTRACT

Female greater amberjack, *Seriola dumerili* reared in tanks have been inconsistent in entering or completing vitellogenesis. As a result, established spawning induction protocols employing gonadotropin-releasing hormone agonist (GnRHa) fail to induce oocyte maturation, ovulation and spawning. Males also exhibit reproductive dysfunctions resulting in low sperm production of relatively low quality. To ameliorate these problems, homologous *Seriola dumerili* single-chain recombinant Follicle stimulating hormone (sdrFsh) and Luteinizing hormone (sdrLh) were produced and administered to adult 4.8-year-old hatchery-produced (F1 generation) greater amberjack held in tanks, in order to enhance gametogenesis and increase reproductive performance (fecundity and fertilization success) upon spawning induction with GnRHa. Beginning at the onset of gametogenesis (27 April 2022), injections of sdrFsh and sdrLh were given for 6 consecutive weeks (a total of 7 treatments) in different combinations in males and females, and increasing doses. All fish treated with sdrFsh/Lh underwent complete gametogenesis, with females having oocytes in post-vitellogenesis and males producing releasable sperm at the time planned for the GnRHa spawning induction (6 June 2022). Furthermore, five of the six females initiated oocyte maturation prior to the GnRHa treatment, and at least one female also spawned spontaneously earlier (week 4). All but one untreated Control females did not complete vitellogenesis and exhibited follicular apoptosis/atresia, making them ineligible for GnRHa-induced spawning. Untreated Control males completed spermatogenesis and produced releasable sperm, but of somewhat lower spermatozoa density and significantly lower motility percentage and velocity compared to sdrFsh/Lh-treated males. GnRHa-induced spawning of the sdrFsh/Lh-treated fish enhanced mean daily relative fecundity and fertilization success, with embryo and larval survival >50%. These results are encouraging, and we expect that further optimization of dosage and administration timing can provide a reliable protocol for enhancing gametogenesis and promoting maturation in dysfunctional greater amberjack broodstocks maintained in aquaculture indoor facilities.

1. Introduction

In fish, as in other vertebrates, reproduction is under the control of the brain-pituitary-gonad (BPG) axis (Zohar et al., 2010). Follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), the two gonadotropins (Gth) produced in the pituitary, play a pivotal role in regulating the endocrine and developmental processes taking place in the gonads during gametogenesis (Levavi-Sivan et al., 2010; Rosenfeld et al., 2007). While Fsh promotes spermatogenesis and vitellogenesis (the synthesis by the liver and uptake by the secondary oocytes of vitellogenin, the precursor of the yolk phosphor-lipo-proteins) (Lubzens et al., 2010; Schulz et al., 2010), Lh stimulates oocyte maturation, ovulation, and spermiation (Nagahama and Yamashita, 2008; Rosenfeld et al., 2007).

Advancing the aquaculture of greater amberjack, *Seriola dumerili* to a

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commercial scale in the Mediterranean region has proven to be challenging, due to difficulties encountered in establishing a steady supply of fertilized eggs, since breeders are often found to be dysfunctional (Micale et al., 1999; Mylonas et al., 2004; Zupa et al., 2017a; Zupa et al., 2017b). Up to date, spontaneous spawning in tanks is very rare and has been reported only for one or two individuals (Jerez et al., 2006; Sarih et al., 2018). In many species under captive conditions, the cascade of endocrine events regulated by the BPG axis might be disrupted due to improper husbandry practices or because of poor acclimatization of the fish in captivity; fish may undergo gametogenesis, but do not enter oocyte maturation, with a consequent lack of spawning (Mylonas et al., 2010). Improper practices may result from our lack of knowledge of the full environmental requirements for proper reproductive function of some species, or because it is not easy to simulate the required environmental conditions (*e.g.* river substrate for nest building in salmonids, macrophyte structures for adhering the eggs in cyprinids or lower salinity in euryhaline species than spawn in estuaries and river mouths). Therefore, existing protocols for inducing spawning and the production of fertilized eggs in many fishes are based on the administration of pituitary extracts containing Lh or gonadotropin-releasing hormone agonists (GnRHa) -which act *via* the release of endogenous Lh stores from the pituitary- at the completion of vitellogenesis, resulting in the induction of oocyte maturation, ovulation and spawning in females and the enhancement of sperm production and spawning in males (Mylonas et al., 2010; Mylonas and Zohar, 2001; Zohar and Mylonas, 2001).

In greater amberjack, GnRHa treatment induces oocyte maturation, ovulation and spawning in females with post-vitellogenic oocytes with a diameter of at least 600 μm (Fakriadis et al., 2020a; Fernández-Palacios et al., 2015; Mylonas et al., 2004; Nyuji et al., 2019). This treatment has been successful with wild-caught, captive-reared females maintained in sea cages during the year. However, when administered to wild-caught breeders reared in tanks supplied with borehole seawater (which is used for biosecurity reasons) or when attempting to induce hatchery-produced breeders, GnRHa treatment resulted in unreliable and poor spawning success (*i.e.* low fecundity and fertilization success) (Corriero et al., 2021a; Fakriadis et al., 2020b). This was due to failure to enter or complete vitellogenesis spontaneously or due to the onset of extensive follicular apoptosis (Corriero et al., 2021b) with the approaching spawning season. Furthermore, when some hatchery-produced breeders completed vitellogenesis, treatment with GnRHa failed to induce spawning (Lancerotto et al., 2024), presumably because of dysfunctional vitellogenesis that prevented oocytes from developing competence for maturation. In males, spermatogenesis has been significantly inhibited in captivity regardless of the origin of the fish (*i.e.* wild or hatchery-produced), rearing conditions, or seawater supply, resulting in poor sperm production and spermatozoa motility (Fakriadis et al., 2019; Fakriadis et al., 2020a; Fakriadis and Mylonas, 2021; Fakriadis et al., 2020b; Zupa et al., 2017a).

Using wild-caught individuals to develop broodstocks for aquaculture should be avoided, as it does not allow the development of improved strains of fish with faster growth, greater disease resistance and better flesh characteristics through selective breeding (Janssen et al., 2016; Vandeputte et al., 2020). Therefore, the development of hatchery-produced breeders is imperative for a successful and sustainable aquaculture. Also, holding valuable broodstock in sea cages -which constitutes the most natural rearing environment- to enhance reproductive function, poses a significant biosecurity risk for a commercial hatchery. On the other hand, maintaining fish in land-based tanks has additional advantages in terms of allowing off-season spawning by exposure to shifted photothermal regimes, in order to expand the annual spawning season (Bromage et al., 2001). Therefore, it is imperative to develop methods to (a) control reproduction of hatchery-produced greater amberjack breeders and (b) achieve full control of gametogenesis and spawning of breeders maintained in indoor tanks.

Recently, the biotechnological synthesis of species-specific recombinant Fsh (rFsh) and Lh (rLh) in a steadily increasing number of fish species opened up new possibilities for better control of reproductive function of aquaculture fishes (Moles et al., 2020). Used at first as a tool to develop analytical assays for the measurement of endogenous hormones in plasma and pituitaries (Aizen et al., 2007; Chauvigne et al., 2015; Nyuji et al., 2016), the potential of recombinant gonadotropins (rGths) for practical applications in aquaculture is closer to being materialized and approachable economically, as it has been done for terrestrial livestock animals (Cabeza et al., 2024). For instance, spermatogenesis and spermiation were attained in immature Japanese eel, *Anguilla japonica* in which weekly administration of Japanese eel rLh allowed a drastic increase in the total amount of collectible sperm, sperm motility and velocity (Ohta et al., 2017). Also, in hatchery-produced Senegalese sole, *Solea senegalensis* the administration of specific rFsh/Lh induced spermatogenesis and spermiation (Chauvigné et al., 2022). The use of sdrFsh was also shown recently to be able to induce full spermatogenesis and spermiation in pre-pubertal greater amberjack (Lancerotto et al., 2022, September 27–30) and meagre, *Argyrosomus regius* (Zupa et al., 2023). Relevant results have been obtained not only in males but also in sexually immature females of different species. Weekly injections to Japanese eel with long-lasting rFsh/Lh with extra O-glycosylation sites were able to drive primary oocytes to the nuclear migratory stage (Kazeto et al., 2023). Moreover, the administration of rFsh/Lh was used to induce gametogenesis, maturation and spawning in flathead grey mullet, *Mugil cephalus* with the successful production of fertilized eggs and viable larvae (Ramos-Júdez et al., 2021; Ramos-Judez et al., 2022).

The objectives of the present study were (a) to use recently synthesized single-chain recombinant *Seriola dumerili* Fsh (sdrFsh) and Lh (sdrLh) to enhance gametogenesis in adult hatchery-produced greater amberjack maintained in tanks, and then (b) induce them to spawn using established protocols using GnRHa implants.

2. Materials and methods

2.1. *Seriola dumerili* gonadotropin production, detection and in vitro bioassay

Single-chain sdrFsh and sdrLh were produced by Rara Avis Biotec S. L. (Valencia, Spain) using in-house technology, as described previously (Chauvigné et al., 2017; Jéhannet et al., 2023; Penaranda et al., 2018; Ramos-Júdez et al., 2021; Zupa et al., 2023). The synthesized (a) cDNAs encoding either the sdFshβ subunit (GenBank accession no. XP_022606074.1) or Lhβ subunit (GenBank accession no. XP_022603873.1), (b) the 28 amino acid carboxyl-terminal sequence (SSSSKAPPPSLPSPSRLPGPSDTPILPQ) of the human chorionic gonadotropin β subunit (CTP) to be used as a linker and (c) the *Seriola dumerili* mature α glycoprotein hormone subunit (GenBank accession no. XP_022606074.1) (Fig. 1) were sub cloned into mammalian expression vectors. Chinese hamster ovary (CHO-S) cells were cultured in suspension (1 L volume, 37 °C, 5% CO_2, 95% HR) and transiently transfected with the expression constructs. After 120 h from transfection, the cell cultures were centrifugated and the supernatants were stored for 24 h at 4 °C. The secreted recombinant gonadotropins were purified from the supernatants by ion exchange chromatography, concentrated to 12 μg mL^{-1} (Bradford assay), and stored at −80 °C in 1 mL aliquots. A total of 10 L (7 and 3 L of sdrFsh and sdrLh, respectively) of cell cultures were transfected and processed in 1-L batch production.

For the detection of sdrFsh and sdrLh, Western blot analysis was

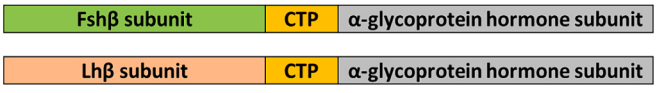

Fig. 1. Schematic representation of the single-chain recombinant greater amberjack, *Seriola dumerili* Follicle stimulating hormone (sdrFsh) and Luteinizing hormone (sdrLh) cDNA.

carried out using as primary antibodies, serum (1:500) from mice immunized *Argyrosomus regius* rFshβ subunit (arrFshβ) (Zupa et al., 2023) (Fig. 2A), and rLhβ subunit (arrLhβ) (Fig. 2B), respectively, expressed in *E.coli*. Shortly, protein samples were mixed with an equal volume of 2× Laemmli sample buffer and boiled for 5 min at 95 °C. Samples and a prestained protein ladder (Precision Plus Protein Standards, Bio-Rad) were loaded onto a 10% SDS-PAGE gel. Electrophoresis was performed and proteins were transferred to a PVDF membrane (Millipore) using a wet transfer system (Bio-Rad). Membranes were blocked with 5% BSA in PBS-T (Phosphate-buffered saline plus 0,1% Tween-20) for 2 h at room temperature, washed three times for 5 min each with PBS-T and incubated with primary antibody (serum from mice immunized against arrFsh and arrLh diluted 1:500 in PBS-T with 5% BSA) (Zupa et al., 2023) overnight at 4 °C with gentle shaking. Membranes were washed three times for 5 min each with PBS-T and then incubated with Anti-Mouse IgG-horseradish peroxidase-conjugated (diluted 1:1000 in PBS-T with 5% BSA) as secondary antibody for 1 h at room temperature. Membranes were washed again three times for 5 min each with PBS-T and detection was done with chemiluminescence (ECL) substrate (Thermo Fisher Scientific) and imaged with a Rx films (Agfa). Band intensities were quantified using Image J software.

The biological activities of sdrFsh and sdrLh were determined by an *in vitro* bioassay based on the intracellular increase of 3′,5′ cyclic adenosine monophosphate (cAMP) after the activation of gonadotropin receptors transiently expressed in mammalian cells as described earlier (Chauvigné et al., 2012). Briefly, the sdFsh receptor (sdFshR) and sdLh receptor (sdLhR) cDNAs (GenBank accession no. BAR43498.1 and BAR43499.1, respectively) were synthesized and sub cloned into mammalian expression vectors (pcDNA3). Human embryonic kidney (HEK293T) cells were transfected with three expression vectors: one vector containing the sdFshR or sdLhR cDNAs, a second vector containing the firefly luciferase gene under the control of cAMP response element (CRE-Luc) and a third vector containing the β-galactosidase cDNA for luminometry data normalization. Sixteen hours after transfection, the cells were seeded in 48 well plates. Forty-eight hours later, new culture medium with different concentrations ranging from 0.0005 to 1.2 μg mL^{-1} and 0.018 to1.2 of the purified sdrFsh or sdrLh, respectively, were added to the wells. After six hours, the cells were washed in PBS and lysed. Cell lysates were frozen (−80 °C) for sixteen hours, thawed and luciferase and β-Galactosidase activities were measured by luminescence or colorimetric detection using Luciferase Assay Kit (BioTherma, Sweden) or *o*-nitrophenyl βD-galactopyranoside, respectively. Luciferase activity data were normalized to those of β-Galactosidase.

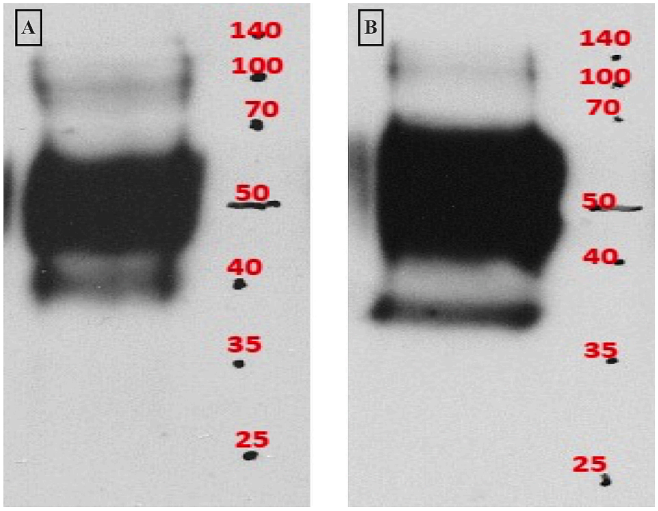

Fig. 2. Western blot of the purified sdrFsh/Lh (100 ng/lane). A) sdrFsh, primary antibody: mice serum Anti-meagre recombinant Fsh (1:500), *sec*ondary antibody: Anti-Mouse IgG horseradish peroxidase (HRPO) conjugate (1:1000). B) sdrLh, primary antibody: mice serum Anti-meagre recombinant Lh (1:500), secondary antibody: Anti-Mouse IgG horseradish peroxidase (HRPO) conjugate (1:1000).

2.2. Fish and experimental design

Ethical approval for the experimental protocol was provided by the National Veterinary Services, Region of Crete under Protocol No 31326, 07/02/2022. All procedures involving animals were conducted in accordance with the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 2020), the Ethical justification for the use and treatment of fishes in research: an update (Metcalfe and Craig, 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

Hatchery-produced greater amberjack juveniles grown from eggs (June 2017) at the hatchery facilities of the Hellenic Centre for Marine Research (HCMR) were reared in sea cages at the Argosaronikos Fishfarms S.A. (Greece) until February 2022, under typical commercial rearing conditions. They were fed with extruded feed from IRIDA S.A. (Greece) for the first two years and then with Vitalis Repro broodstock diet (Skretting, Spain). In February 2022, sexed and PIT-tagged greater amberjack ($n = 24$) were taken from the sea cage to form two experimental groups. Each group contained six females and six males, maintained in a separate indoor 23-m^3 tank until July 2022 when the breeders were returned to the sea cage at the end of the study. A mixture of borehole and filtered surface seawater was used to supply the tanks, and fish were exposed to simulated/natural photoperiod with open windows and additional Aquaray Ocean Blue LED lights (TMC, UK) (Fig. 3). Fish were fed at 1% body weight 3 times a week (Monday, Wednesday and Friday) with a commercial broodstock diet (Vitalis Cal, Skretting, Spain).

The first group (Control, $n = 12$, sex ratio 1:1, mean ± SD body weight of 9.9 ± 0.4 kg) was held undisturbed in the tank, from February until 6 June 2022. Fish from the Control group were sampled only during the last sampling in order to assess their reproductive stage, at the time when greater amberjack are expected to mature and enter the spawning season in the Mediterranean Sea, which is early June (Fakriadis et al., 2020b; Zupa et al., 2017b). At this time -decided *a priori* according to the experimental design- fish were evaluated for eligibility for spawning induction with GnRHa (week 6, Table 1); samples of blood, sperm and ovarian biopsies (see below) were collected. The second group (sdrFsh/Lh, n = 12, sex ratio 1:1, 10 ± 0.2 kg) was treated with sdrFsh and sdrLH injections over a period of 6 weeks beginning on 27 April 2022, at the expected time of the onset of gametogenesis. This was done because previous work suggested that females were not responsive if the treatment started before they entered "secondary growth" (unpublished data), which includes the stages of lipid vesicle, cortical alveoli and early vitellogenin sequestration by the developing follicle (Selman et al., 1986; Wallace and Selman, 1981). Duplicate tanks for each treatment were not considered necessary since replication in this experiment was achieved by each male and female participating in the study ($n = 6$ per sex and treatment). Each fish in the sdrFsh/Lh study was given the treatment individually (injection on a body weight basis), and ovarian biopsies, sperm samples, hormone treatments and blood hormone data were obtained from each individual (tagged with a PIT tag) and obtained at multiple times when necessary (Table 1).

The range of sdrFsh/Lh doses used (6–12 μg kg^{-1}) was chosen based on to their effectiveness in inducing the reproductive function in other teleost species, such as European eel, *Anguilla anguilla* (Penaranda et al., 2018), Senegalese sole (Chauvigne et al., 2018; Chauvigné et al., 2022; Chauvigné et al., 2017), flathead mullet (Ramos-Júdez et al., 2021; Ramos-Judez et al., 2022), pre-pubertal greater amberjack (Lancerotto et al., 2022) and meagre (Zupa et al., 2023). The treatments were given at weekly intervals with increasing doses and differential timing of

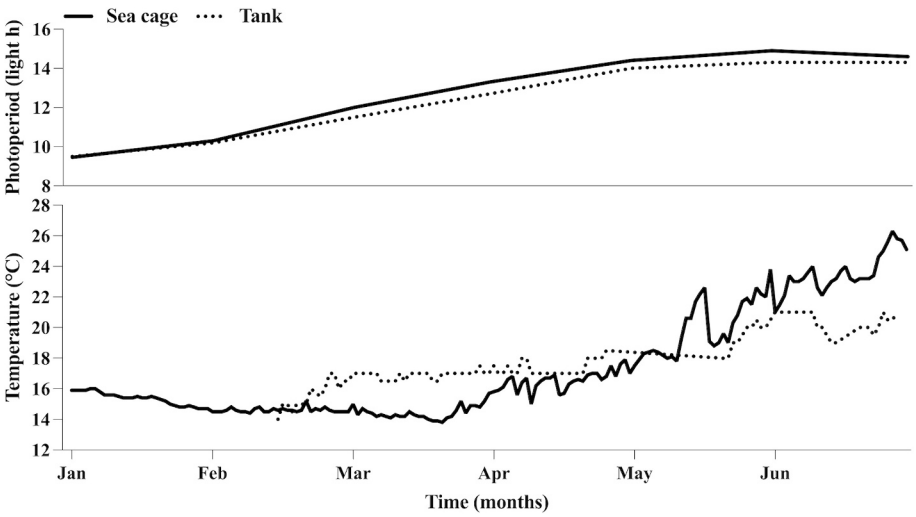

Fig. 3. Photoperiod and thermal profiles at sea cage (plain lines), and provided to greater amberjack (*Seriola dumerili*) of both Control and sdrFsh/Lh in tanks (dotted lines) from February to July 2022.

Table 1
Experimental protocol for the enhancement of gametogenesis in greater amberjack, *Seriola dumerili* using combinations of sdrFsh and sdrLh, administered in weekly injections. Ovarian biopsies and blood samples were taken at the start of the experiment (27 April 2022) and in week 3 from females in the sdrFsh/Lh group only, while in males only blood was collected at these times (green check symbol). At week 6 of the experiment (6 June 2022) ovarian biopsies, sperm and blood samples were taken from all fish in both the sdrFsh/Lh and Control groups (black check symbol).

Week	0	1	2	3	4	5	6
Females							
sdrFsh (μg kg^{-1})	6	9	12	12	12	12	–
sdrLh (μg kg^{-1})	–	–	–	–	12	12	6
GnRHa (μg kg^{-1})	–	–	–	–	–	–	50
Ovarian biopsy	✓	–	–	✓	–	–	✓
Bleeding	✓	–	–	✓	–	–	✓
Males							
sdrFsh (μg kg^{-1})	–	–	6	12	12	12	12
sdrLh (μg kg^{-1})	–	–	–	–	–	12	12
GnRHa (μg kg^{-1})	–	–	–	–	–	–	50
Sperm collection	–	–	–	–	–	–	✓
Bleeding	✓	–	–	✓	–	–	✓

administration for females (sdrFsh: 6, 9, 12 μg kg^{-1} on week 0, 1, 2–6, respectively. sdrLh: 12 μg kg^{-1} on weeks 4–6) and males (sdrFsh: 6, 12 μg kg^{-1} on weeks 2, 3–6, respectively. sdrLh: 12 μg kg^{-1} on weeks 5–6) (Table 1). Ovarian biopsies and blood were collected from the females every 3 weeks (weeks 0, 3, and 6). Sperm samples were collected only at week 6, while blood was obtained at weeks 0, 3 and 6, as for the females in the same group. At week 6 (6 June 2022), all females from both groups were examined for eligibility for hormonal spawning induction using GnRHa implants (Table 1).

Ovarian biopsies were obtained by inserting an endometrial catheter (Pipelle de Cornier, Laboratoire CCD, France) and applying gentle aspiration. A fraction of the biopsy was examined immediately for reproductive stage, as a wet mount under a compound light microscope (x40 magnification), and the mean diameter of the most advanced vitellogenic oocytes was measured (n = 10, at x100 magnification) (Micale et al., 1999), and microphotographs were taken. The remaining part of the biopsies were preserved in a 4% formaldehyde: 1% glutaraldehyde solution and stored for further histological processing (see below). On week 6, the eligibility of females for spawning induction with GnRHa was determined based on the presence in the ovarian biopsies of vitellogenic oocytes with a diameter > 600 μm. Blood was collected using heparinized 2-mL syringes with a 21G1.5-in. needle inserted in the caudal vasculature, and was stored on ice until separation of the plasma after centrifugation at 4500 rpm for 15 min at 4 °C. The obtained plasma was aliquoted to microfuge vials and was maintained at −80 °C until hormonal analysis.

Stripping of sperm using abdominal pressure cannot be usually implemented in greater amberjack in captivity, due to the limited amount of sperm produced and the fish's thick abdominal musculature (Fakriadis et al., 2019; Fakriadis and Mylonas, 2021). Therefore, sperm for quality evaluation was collected using a biopsy catheter (Pipelle de Cornier, Laboratorie CCD, France) inserted into the genital pore and then applying gentle suction. Sperm samples (50–100 μL) were stored at 4 °C until evaluated for sperm and spermatozoa quality, as soon as the sampling was completed (see below).

For spawning induction, a slow-release solid Ethylene-Vinyl Acetate implant loaded with GnRHa (Des-Gly^{10}, DAla^{6}-Pro-NEth^{9}-mGnRHa, H-4070, Bachem, Switzerland) at an intended dose of 50 μg kg^{-1} body weight was given to each eligible female selected from the Control (n = 1 of 6) and sdrFsh/Lh groups (n = 6 of 6), and to spermiating males from the Control (n = 2 of 6) and sdrFsh/Lh groups (n = 6 of 6) to enhance sperm production. Not all males from the Control group were used for the spawning induction experiment, since only a single female was eligible and induced to spawn. Therefore, we were concerned that too much male-to-male aggressiveness could adversely affect the fertilization of any eggs, if the female succeeded in maturing, ovulating and spawning. The GnRHa-induced fish from the Control and sdrFsh/Lh groups were placed again in separate 23-m^3 tanks for spawning and continued to be monitored for the following 14 days (see later).

2.3. Histological analysis

Ovarian biopsies were dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraus Kulzer, Germany). Serial sections of 4 μm were obtained using a semi-automatic microtome (Leica RM2245, Germany), and histology slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976) and examined under a light microscope (50i Eclipse, Nikon, Japan). Eventually, microphotographs of the stained content of the ovary were taken

using a digital camera (Progres, Jenoptik AG, Germany).

2.4. Measurement of plasma sex steroids

The extraction and analysis of steroid hormones was performed according to (Papadaki et al., 2021) with a few modifications. The sex steroids that were included in the panel of analytes were testosterone (T), 17β-estradiol (E2), 11-ketotestosterone (11-KT) and 17α,20β-dixydroxy-4-pregnen-3-one (17,20β-P). Furthermore, instead of *N*,*N*-dimethyl-*L*-phenylalanine, 13C-labeled E2, T, and 17,20β-P (>98% purity) purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA), were used as internal standards for better quality control and more accurate quantification of the hormones. A mixture of those four compounds in varying concentrations (10 to 85 pg μL^{-1}) was prepared in methanol:water 1:1 and 10 μL of this solution were added to the serum samples prior to solid phase extraction. Subsequently, the preparation of samples and the analysis of hormones by LC-MS/MS was implemented following the same procedures as those described in our previous study.

2.5. Sperm quality analysis

For Computer Assisted Sperm Analysis (CASA, ISAS, Spain), sperm was activated in seawater containing 2% bovine serum albumin (1:201 or 1:334) to obtain 200–300 cells in the field. Fifteen sec after activation, a reusable counting chamber with a fixed depth (SpermTrack, ISAS) was used to record spermatozoa movement using a digital camera at 100 frames per second (fps) attached to a light microscope (Primo Star, Zeiss, Germany) under 100× magnification. Spermatozoa movement recording was stopped when <5% of the spermatozoa in the field of view were showing forward motility. The CASA included the following parameters: sperm density (number of spermatozoa ml^{-1} of sperm), duration of forward spermatozoa motility of ≥5% of the spermatozoa in the field of view (motility duration, min), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) (μm sec^{-1}), motile cells, progressive cells (> 80% straightness (STR)), rapid cells, and STR (%). The software settings were adjusted to 1 to 90 μm for the head area; VCL < 10 μm sec^{-1} to classify a spermatozoon as immotile; and spermatozoa were considered rapid when VCL was higher than 100 μm sec^{-1}.

2.6. Reproductive performance evaluation

Spawning and egg production were monitored for daily relative fecundity (eggs kg^{-1} female body weight) and the eggs were evaluated for fertilization success (%). Briefly, a cylindro-conical passive egg collector fitted with a 300-μm mesh was connected to the water overflow of each tank and was checked twice a day (8.00 a.m. and 8.00 p.m.). Harvested eggs were removed from the collector and placed into a 10-L bucket, from which a sub-sample of 10 mL was taken with a volumetric pipette. All sampled eggs were counted and checked for fertilization in a round wheel-counter using a stereoscope (Stemi 305, Zeiss, Germany).

Egg quality was evaluated using individual egg incubation in 48-well microtiter plates (48-mct), by monitoring 24-h embryo development, hatching, and larval survival to yolk absorption (day 5 post-spawning) from the spawns with a fertilization success ≥25% (n = 5). Spawns with lower fertilization success were not used, as we usually have very poor survival results. The 48-mct method of egg and larval survival was a modification of an earlier method with 96-well mct (Panini et al., 2001), which uses a larger water volume per egg (700 μL per well) to prevent water quality deterioration due to the high (relatively) incubation temperature of greater amberjack eggs/larvae (21–24°C).

2.7. Statistical analysis

Mean oocyte diameter and plasma sex steroid level data from the sdrFsh/Lh group among the three samplings of the experiment were compared using a one-way ANOVA followed by Tukey's test. Mean oocyte diameter and plasma sex steroid level data between the sdrFsh/Lh and Control group were compared using a *t*-Test, while for the sperm quality analysis a Welch's-t-Test was used. Results are presented as mean values ± standard error of the mean (SEM) unless mentioned otherwise. In all the statistical tests performed, *p*-values below 0.05 were considered statistically significant. Statistical analyses were run using GraphPad Prism 9.4.1 for Mac OS, GraphPad Software, San Diego, California USA, www.graphpad.com.

3. Results

3.1. Recombinant gonadotropin validation in vitro assay

Western blot analysis of the purified product showed reactive bands of 45–65 kDa for sdrFsh and 40–70 kDa for sdrLh. Purified sdrFsh/LH reacted against mice serum immunized with the specific arrFsh or arrLh (Zupa et al., 2023). No cross-reactivity between sdrFsh or sdrLh, and normal mice serum was observed. Therefore, the bands observed were considered to be sdrFsh and sdrLh. In the *in vitro* bioassay, both purified sdrFsh and sdrLh increased the luminescence signal in a concentration-dependent manner (Fig. 4). Moreover, both sdrFsh and sdrLh showed a high specificity in the activation of their cognate receptor and no cross-activation (promiscuity) was observed at any hormone concentration tested. These results indicate that both sdrFsh and sdrLh are expressed

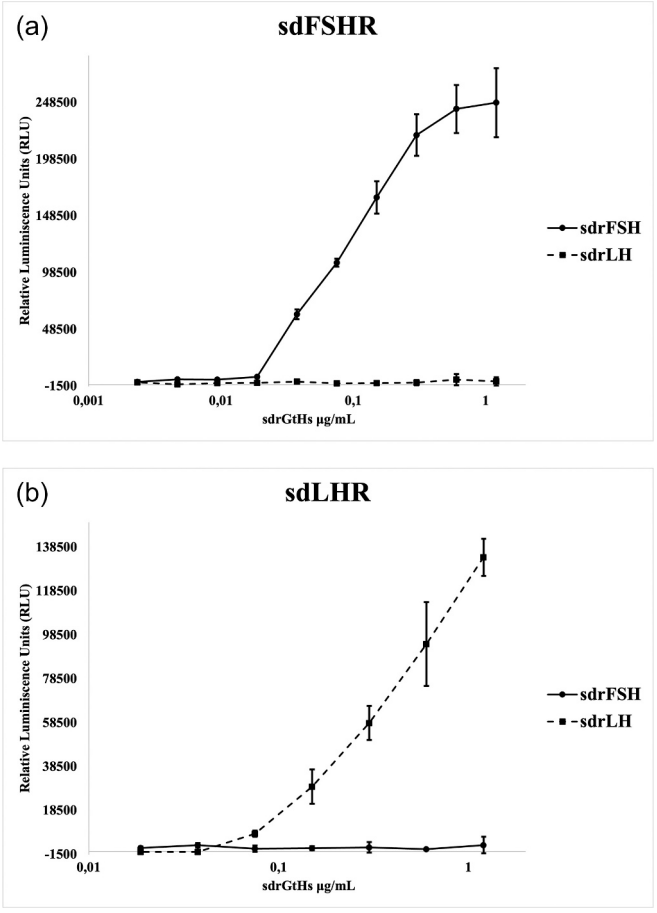

Fig. 4. Relative luminescence units (RLUs) normalized to β-galactosidase activity in an *in vitro* receptor (R) bioassay evaluating the efficacy of the synthesized sdrFsh (solid lines) and sdrLh (broken lines) at different concentrations (μg mL^{-1}). The HEK293T cells were transiently transfected with sdFshR (left graph) or sdLhR (right graph) and CRE-Luc.

and secreted efficiently as biologically active gonadotropins by CHO-S cells. In total 11,760 and 5040 μg of sdrFsh and sdrLh, respectively, were produced. The purity of the sdrFsh/Lh produced was assessed by SDS-PAGE and Coomassie blue staining (>90%) (Fig. 5).

3.2. Enhancement of vitellogenesis

All sdrFsh/Lh-treated females ($n = 6$) were considered eligible for GnRHa spawning induction at week 6. Four out of six females were found in a late development stage as indicated by the diameter of the largest oocyte batch (>700 μm) on week 6 (Figs. 6A and 7), and by the histological appearance of the ovarian biopsies (Fig. 8); these females also possessed oocytes undergoing maturation. Moreover, the remaining two females had already ovulated and spawned, and only eggs were obtained in the ovarian biopsies (Fig. 7). For this reason, vitellogenic oocytes could not be collected through biopsies from these females and therefore oocyte diameter data were obtained only from four females on week 6.

Conversely, on week 6 only one female from the Control group was found eligible for GnRHa spawning induction with oocytes of 650 μm (Fig. 7). The other five females possessed mainly primary oocytes, and few oocytes in early vitellogenesis having a significantly lower mean diameter of the largest oocyte batch than in sdrFsh/Lh-treated females (Fig. 6B). Moreover, two females out of these five had an ovary containing only primary oocytes, thus oocyte diameter data were obtained only from four females (Figs. 7 and 8).

The increase in the diameter of the oocytes found in all females from the sdrFsh/Lh group was accompanied by a significant increase in the mean plasma levels of E2 that doubled by the end of the sdrFsh/Lh treatment and was significantly higher than the mean levels of Control females (Fig. 9). The other measured sex steroid hormones did not show any significant change over the course of the study, or between the sdrFsh/Lh and Control females.

3.3. Enhancement of spermatogenesis

Sperm was successfully collected using a catheter from all males from both sdrFsh/Lh-treated and Control groups (Fig. 10). Surprisingly, one of the six sdrFsh/Lh-treated males could be even stripped by applying abdominal pressure, which is rare in greater amberjack males in culture. No significant differences in terms of spermatozoa density were found between the two groups, though a tendency for higher values was observed in sdrFsh/Lh-treated males (Fig. 10A). On the other hand, sdrFsh/Lh induced highly significant increases in the number of motile spermatozoa, progressive spermatozoa and rapid spermatozoa with the

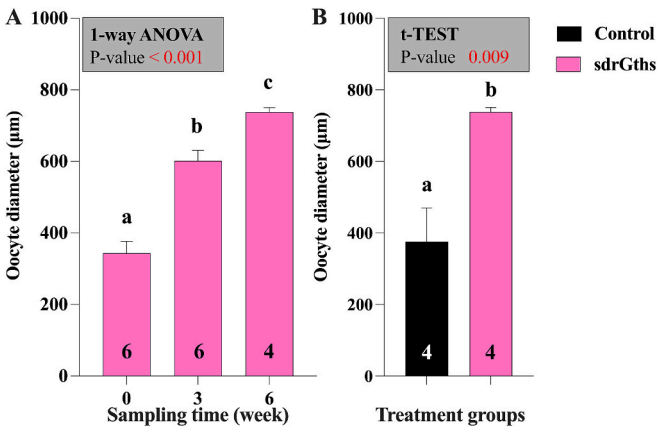

Fig. 6. Mean (± SEM) oocyte diameter of the most advanced vitellogenic oocytes in hatchery-produced greater amberjack in response to six weekly treatments with sdrFsh/Lh for the enhancement of gametogenesis. **A.** Mean oocyte diameter at 0, 3 and 6 weeks after weekly sdrFsh/Lh treatment (see Table 1). **B.** Mean oocyte diameter of Controls and sdrFsh/Lh-treated females at week 6 (6 June 2022), the time of evaluation for GnRHa administration to induce spawning. Letter superscripts indicate significant differences (**A**, one-way ANOVA, Tukey HSD, $P < 0.05$ and **B**, *t*-Test, $P < 0.05$). Numbers inside the columns indicate the number of females from which ovarian biopsies were obtained at each sampling.

mean values obtained, being more than three times higher than those of the Controls (Fig. 10B). Moreover, the administration of sdrFsh/Lh increased the mean velocity parameters of spermatozoa from the treated males, and VCL, VSL and VAP were significantly higher than in Controls (Fig. 10C). Concomitantly with increased spermatozoa quality parameters in sdrFsh/Lh-treated males, gradually increased levels of both androgens were observed over the course of the study (Fig. 9). On week 6, mean plasma T and 11-KT increased significantly compared to the levels measured on weeks 0 and 3, and were >6-fold higher from the Control group (Fig. 9). The other measured sex steroid hormones did not show any significant change over the course of the study, or between the sdrFsh/Lh and Control males.

3.4. Spawning evaluation

In the Control group, although the one and only of the six females found to be eligible for induction was treated with GnRHa, no spawning was observed. On the contrary, spawning in the sdrFsh/Lh group began

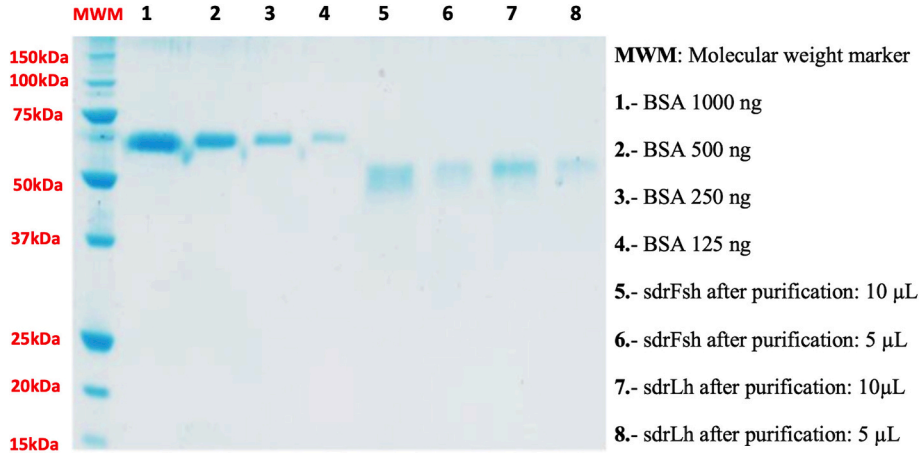

Fig. 5. SDS-PAGE (15%) stained with Coomassie Blue of the purified sdrFsh/Lh 2× concentrated. MWM: Precision Plus Protein™ Unstained Protein Standards (Bio-Rad). Lanes 1–4: BSA standards 1000, 500, 250 and 125 ng (Bio-Rad). Lanes 5–6: 230 and 115 ng of purified sdrFsh, respectively. Lanes 7–8: 220 and 110 ng of purified sdrLh, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

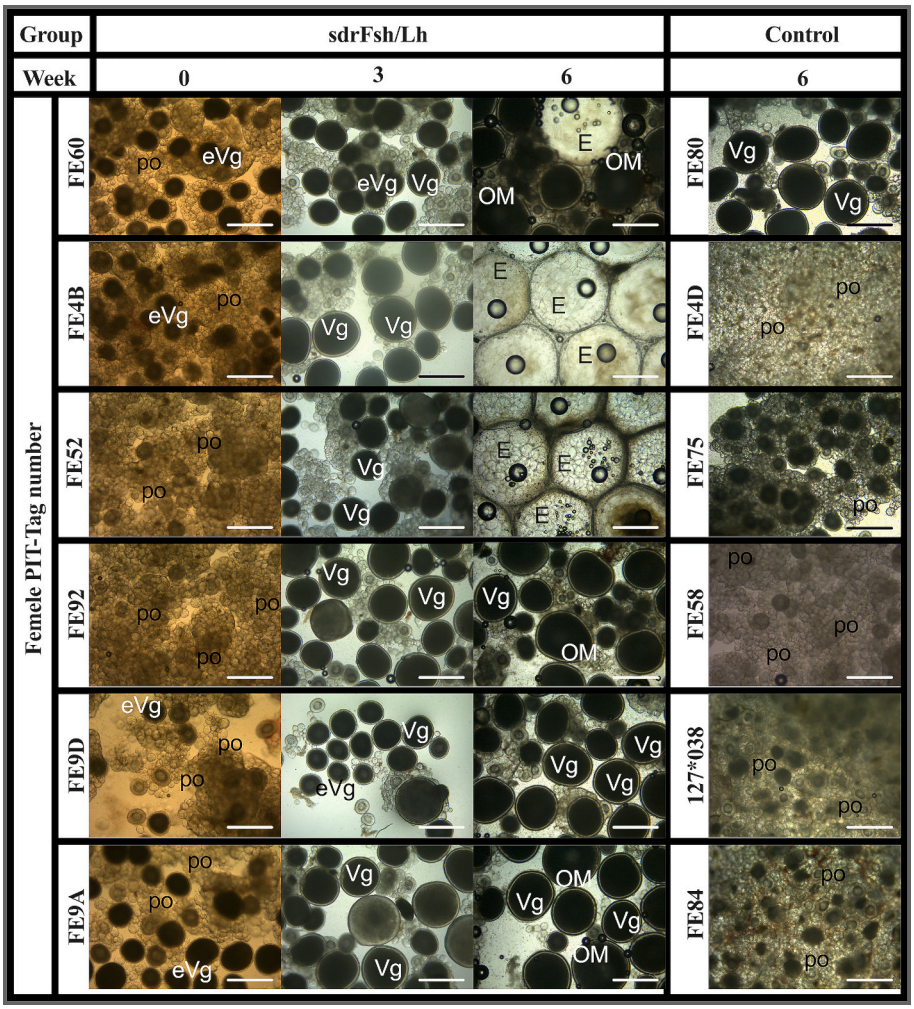

Fig. 7. Microphotographs of ovarian biopsies (wet mounts) from individual hatchery-produced female greater amberjack, *Seriola dumerili* in the Control group (sampled on week 6) and the group treated with sdrFsh/Lh (sampled on weeks 0, 3 and 6). Females from each group ($n = 6$) are indicated with their corresponding PIT-Tag and their oocytes and ova are labeled as primary oocytes (po), oocytes in early vitellogenesis (eVg), oocytes in advanced vitellogenesis (Vg), oocytes in maturation (OM), oocytes undergoing follicular atresia (AT) and ova/ eggs (E). Bars = 500 μm.

before the time designated for GnRHa induction (Fig. 11A), at week 4 of the treatment with sdrFsh and after the 1st injection of sdrLh, which was 9 days before the planned GnRHa administration. Nine additional spawns were obtained after GnRHa administration, which resulted both in higher mean (±SEM) relative fecundity and fertilization success from 2283 ± 508 to 5961 ± 1568 eggs kg^{-1} and from 9 ± 7 to 25 ± 9%, respectively. It is not possible to know how many females participated in these nine spawns, but based on their stage of ovarian development at the time of the GnRHa treatment, we expect that at least five of the six females spawned at least once. Egg quality was evaluated by monitoring survival to different embryo development and larval stages from the spawns with a fertilization success equal or above 25% ($n = 5$). In these spawns, embryo survival 24 h after spawning, hatching, and larval survival up to yolk reabsorption (5 days after spawning) were all above 50% (Fig. 11B).

4. Discussion

The results provided by the present study may improve greatly the control of reproductive function of greater amberjack under aquaculture conditions, where they have been shown to exhibit significant dysfunctions in gametogenesis when breeders are hatchery-produced, farmed individuals and/or maintained in tanks, especially when these are supplied with borehole seawater (Fakriadis et al., 2020b; Lancerotto et al., 2024). We demonstrated that administration of combinations of sdrFsh/Lh over 6 weeks can enhance both vitellogenesis and spermatogenesis, making females eligible for GnRHa induction of spawning and enhancing dramatically sperm quality in males, at the expected natural spawning season of greater amberjack in the Mediterranean Sea. Furthermore, some individuals may complete vitellogenesis and undergo oocyte maturation, ovulation and spawning, even without the use of the spawning induction therapy with GnRHa.

All greater amberjack females treated with sdrFsh/Lh underwent complete vitellogenesis. Moreover, by week 6 at least one female underwent ovulation and spawning even before receiving the GnRHa spawning induction therapy, two females were found to be ovulating at this time, and three more had oocytes initiating maturation. The sdrFsh/Lh treatment was initiated at the time females were expected to have just started vitellogenesis at the end of April, when only a very small fraction of the ovary consisted of early vitellogenic oocytes. The sdrFsh/Lh treatment began with the administration of only sdrFsh, as it is known that vitellogenesis can proceed normally without any addition of Lh in the early stages. In fact, when the reproductive stages of females were reevaluated on week 3, after three injections of sdrFsh, most of the oocytes had already reached a mean diameter of 550 μm, indicating that the fish were in very advanced stages of vitellogenesis, thus corroborating the central role of Fsh in this process in fish (Hara et al., 2016; Sullivan and Yilmaz, 2018). Our results on the ability of Fsh to stimulate

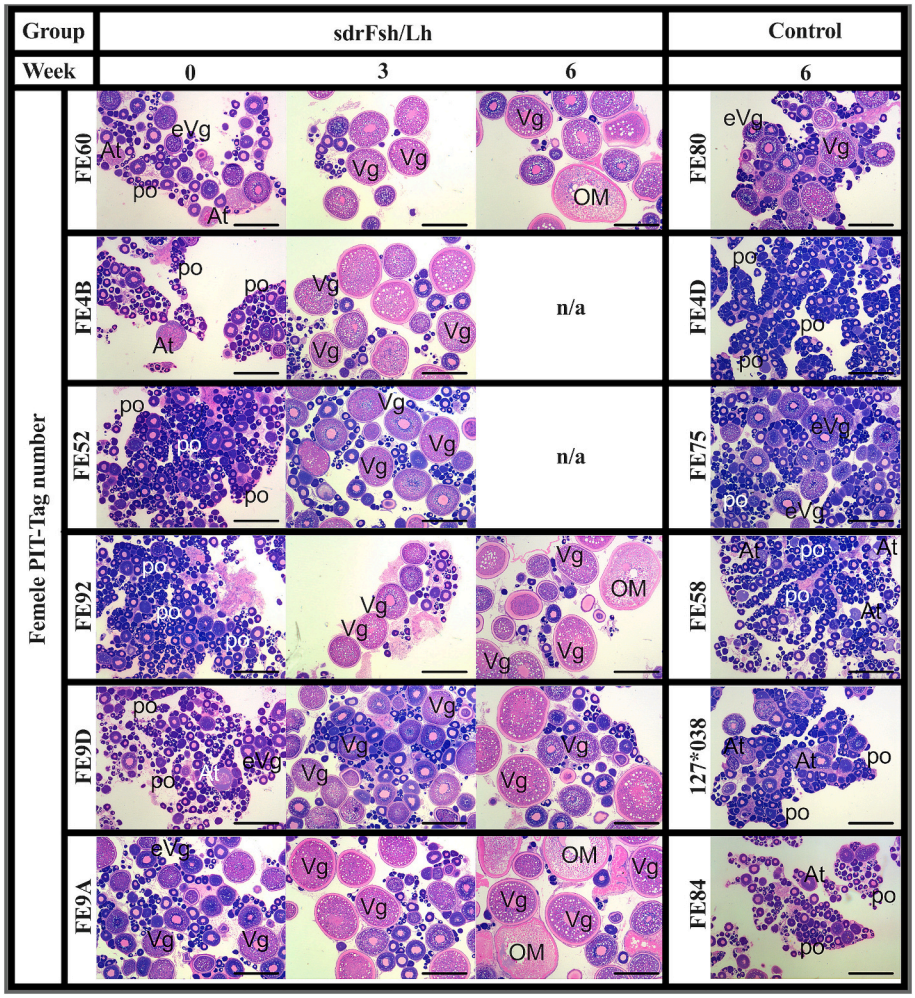

Fig. 8. Microphotographs of ovarian biopsies (histological sections) from individual hatchery-produced female greater amberjack, *Seriola dumerili* in the Control group (sampled on week 6) and the group treated with sdrFsh/Lh (sampled on weeks 0, 3 and 6). Females from each group (n = 6) are indicated with their corresponding PIT-Tag and their oocytes and ova are labeled as primary oocytes (po), oocytes in early vitellogenesis (eVg), oocytes in advanced vitellogenesis (Vg), oocytes in maturation (OM), oocytes undergoing follicular atresia (AT) and ova/ eggs (E). Bars = 500 μm.

vitellogenesis also agree with those obtained in flathead grey mullet, a species that also exhibits dysfunction in undergoing gametogenesis in captivity (Ramos-Júdez et al., 2021; Ramos-Judez et al., 2022). In this species, previtellogenic females treated only with rFsh completed vitellogenesis (Ramos-Júdez et al., 2021). However, obtaining females with oocytes able to undergo maturation and ovulation, required the combined administration of rFsh and rLh in the final stages of vitellogenesis (Ramos-Júdez et al., 2021; Ramos-Judez et al., 2022). For this reason, in the present experiment, sdrLh was administered concomitantly with the 5th administration of sdrFsh, when oocytes achieved a diameter of ≥550 μm. Vitellogenesis continued to be supported by the sdrFsh treatment and soon after the first sdrLh treatment we obtained a small spawn, indicating that some oocytes began undergoing maturation, followed by ovulation and spawning.

Testosterone levels are reported to increase in female fish during the phase of vitellogenesis and, by the activity of aromatase, T is later converted to E2, which in turn acts at the hepatic level promoting the production of vitellogenin and, therefore, oocyte growth (Levavi-Sivan et al., 2010; Sullivan and Yilmaz, 2018). However, the measured mean T and E2 levels did not increase significantly in the present study, with the exception of E2 at the last sampling, despite the histological evidence of significant progress of vitellogenesis already during week 3 of the experiment. Mean T levels were also the same in sdrFsh/Lh-treated and Control fish at the last sampling in week 6, whereas plasma E2 levels were significantly different for the first time. The appearance of a significant increase in plasma E2 levels only at week 6, while vitellogenesis was obviously supported strongly for all 6 weeks, was puzzling at first. On further examination of the results, we realized that although all previous samplings and hormone treatments were done exactly every 7 days, the last sampling at week 6 -concomitantly with the GnRHa implantation of eligible females- was done only 5 days after the previous sdrFsh treatment. Most likely, the steroidogenic action of sdrFsh declined a few days after each administration of the treatment, resulting in plasma E2 levels returning to pre-treatment levels, while the process of vitellogenesis by the liver and sequestration by the developing oocytes proceeded continuously. The steroidogenic activity and the highest elevation in plasma levels of E2 after treatments with sdrFsh/Lh in other fish were demonstrated to occur within 24 h in the cinnamon clownfish, *Amphiprion melanopus* (Kim et al., 2012) and the Manchurian trout, *Brachymystax lenok* (Ko et al., 2007); in addition, in rainbow trout, *Oncorhynchus mykiss* E2 was able to induce production and release of vitellogenin within 48 h (Arukwe et al., 2001). Furthermore, the treatment 5 days prior to the final sampling at week 6, of sdrLh in addition to sdrFsh -for the first time- might have resulted in even higher steroidogenesis and production of E2 than after previous treatments with only sdrFsh. Taken together, this information may explain why we were not able to detect increasing plasma levels of E2 earlier than week 6, even though sdrFsh/Lh-treated fish completed vitellogenesis, in contrast with

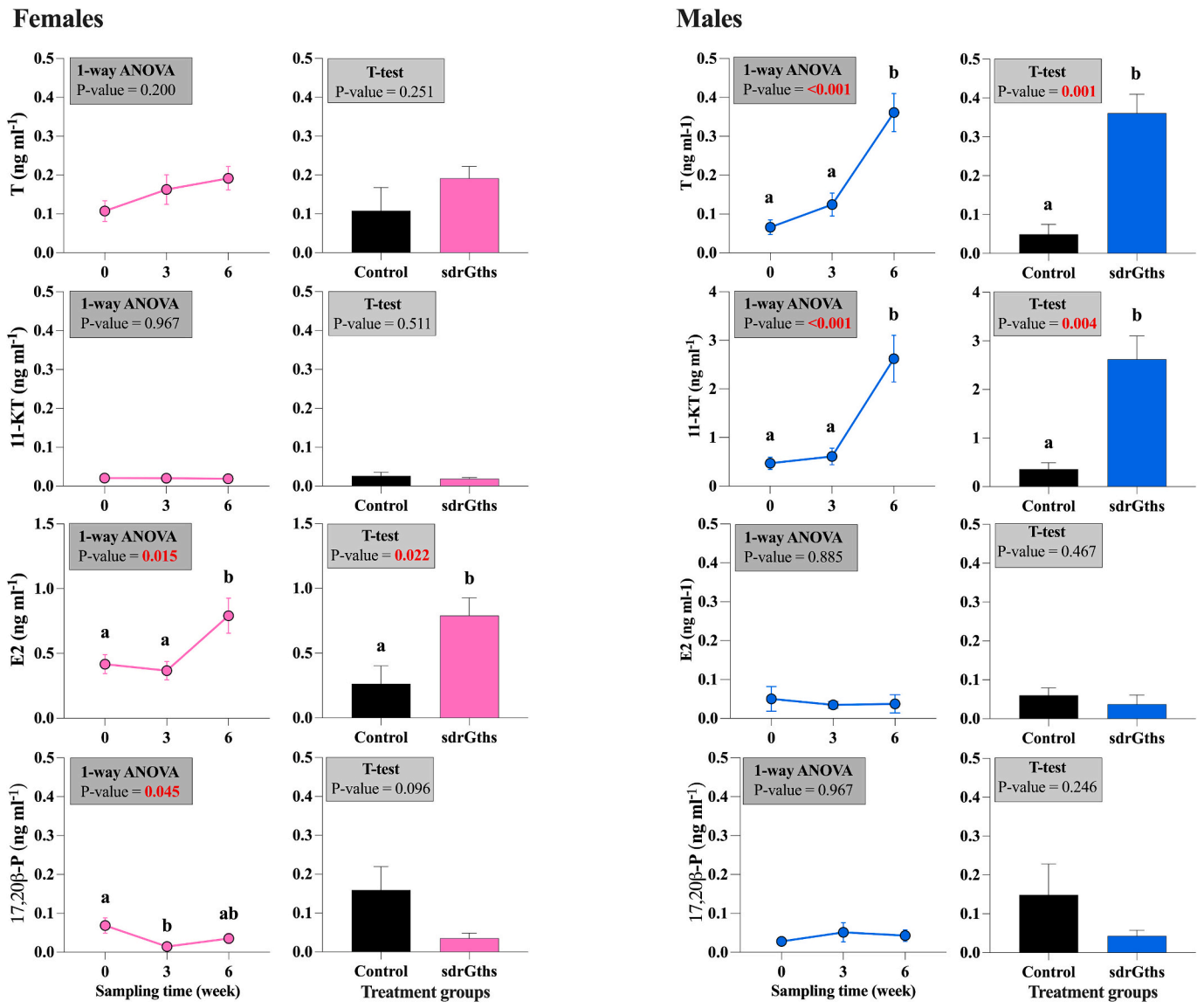

Fig. 9. Mean (± SEM; n = 6 females, n = 6 males) plasma hormone levels of testosterone (T), 11-ketotestosterone (11- KT), 17β-estradiol (E2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in 5-yo hatchery-produced greater amberjack, *Seriola dumerili* in response to six weekly treatments with sdrFsh/Lh for the enhancement of gametogenesis. For each sex, the left graphs show the plasma levels at 0, 3 and 6 weeks after weekly sdrFsh/Lh treatment (Control fish remained undisturbed, without intervention at weeks 0 and 3). The right graphs show the plasma levels of Controls and sdrFsh/Lh-treated fish at week 6, the time of evaluation for GnRHa administration to induce spawning. Letter superscripts indicate differences over time in the sdrFsh/Lh group (one-way ANOVA, Tukey HSD, $P < 0.05$) and between the two treatment groups at week 6 (t-Test, $P < 0.05$).

the absence of further gonadal development in the Control group.

In another study with wild-caught, captive-reared 4-yo greater amberjack, it has been shown that plasma Fsh and Lh vary seasonally (Nyuji et al., 2016). Plasma Fsh levels were relatively lower than Lh and had their annual maximum in August after the spawning season, while they remained unchanged during the process of vitellogenesis between March and June. On the contrary, plasma Lh levels were many-fold higher than Fsh, and increased gradually between winter and summer, reaching maximal levels during the second part of the process of vitellogenesis, when females with fully vitellogenic oocytes were present. In the present study, the dose of sdrFsh was increased during the first 3 weeks, and sdrLh was not administered until very late in the process, well after the fish reached advanced stages of vitellogenesis. Nevertheless, the overall profile and combination of sdrFsh/Lh was similar to what has been seen in nature, with steady levels of Fsh during vitellogenesis and increasing levels towards the completion of the process. Therefore, we believe that the employed protocol simulated a "natural-like" profile of the necessary endocrine changes occurring during the reproductive season of greater amberjack in the temperate zone, promoting not only vitellogenesis, but also maturation and ovulation to some extent.

Although reproductive dysfunctions reported previously in male greater amberjack are significant (*i.e.* low quantity and high variability of sperm quality) (Fakriadis et al., 2020b), spermatogenesis is usually completed, sperm is released during spawning and fertilization is achieved, albeit with relatively low fertilization success relative to other cultured fishes (Fakriadis et al., 2020b; Jerez et al., 2018; Mylonas et al., 2004). In the present study, fish from both the Control and sdrFsh/Lh groups completed spermatogenesis and spermiation, and sperm could be collected, in agreement with previous studies in captivity (Fakriadis and Mylonas, 2021). Collecting sperm through stripping is not, usually, possible in greater amberjack in captivity due to the relatively small seminiferous tubules and testes, and the low amount of sperm produced (Zupa et al., 2017a). In addition, the thick muscular tissue surrounding

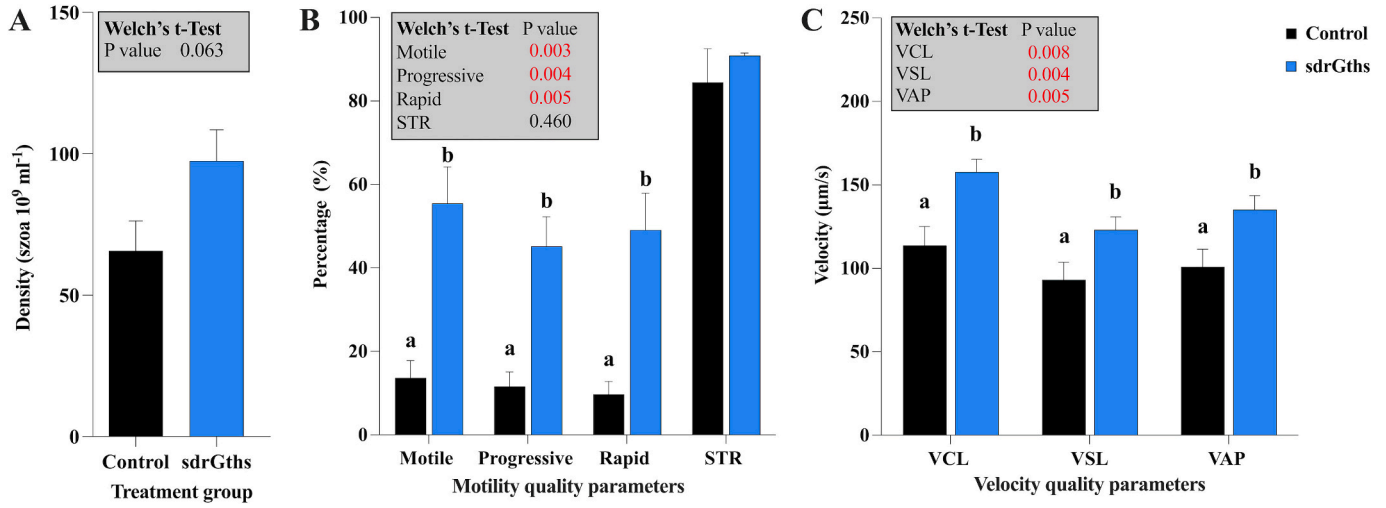

Fig. 10. Mean (± SEM, n = 6) sperm quality parameters of hatchery-produced male greater amberjack in response to six weekly treatments with sdrFsh/Lh for the enhancement of gametogenesis. Sampling was done only once at week 6 (6 June 2022), just prior to GnRHa administration for spawning induction. **A.** Spermatozoa (szoa) density (x10^9 szoa ml^{-1}). **B.** Percentage (%) of motile szoa, progressive szoa, rapid szoa and straightness of the trajectory (STR). **C.** Curvilinear (VCL, µm sec^{-1}), straight line (VSL, µm sec^{-1}) and average path velocity (VAP, µm sec^{-1}). Numbers inside the columns indicate the sample size. Letter superscripts indicate differences between treatment groups in each analyzed parameter (Welch's t-Test, P < 0,005).

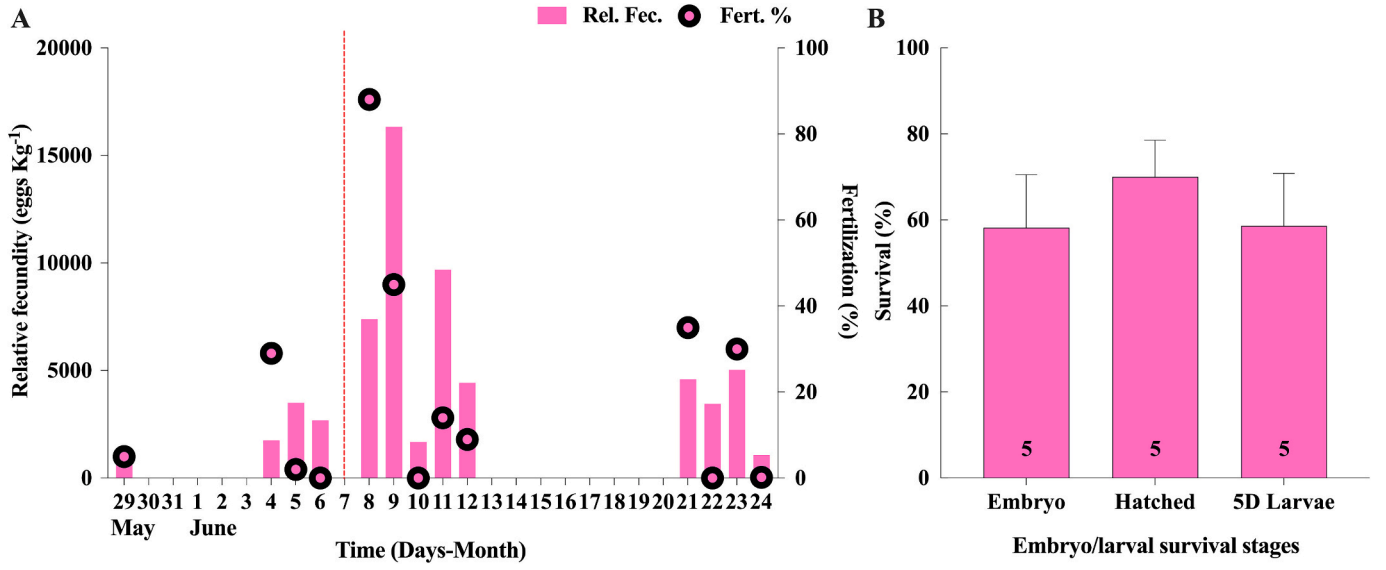

Fig. 11. **A.** Daily relative fecundity (bars, eggs kg^{-1} female) and fertilization success (circles, %) of hatchery-produced greater amberjack, *Seriola dumerili* in response to six weekly treatments with sdrFsh/Lh (beginning on 27 April 2022) for the enhancement of gametogenesis and induced to spawn with a GnRHa implantation (6 June 2022). The dotted red line separates the egg production before and after the GnRHa administration. **B.** Mean (± SEM) embryo survival 24-h after spawning, hatching success and 5-day larval survival of egg batches having >25% fertilization success (n = 5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the abdomen prevents the application of adequate pressure to the testes (Fakriadis and Mylonas, 2021). There was no attempt -as it would not be possible- to measure the amount of sperm produced by the males in the present study, therefore we do not know if there was an increase in sperm volume due to the sdrFsh/Lh treatment. Nevertheless, the fact that we could strip some sperm from one of the males treated with sdrFsh/Lh may suggest that some increase in sperm volume was achieved. Furthermore, there was a tendency towards higher spermatozoa density in sdrFsh/Lh-treated males (P = 0.06) suggesting an increase in spermiation and spermatozoa production. More clear results have been obtained in a similar study with Senegalese sole, where histological evidence indicated that combined rFsh/rLh treatments enlarged the seminiferous tubules and efferent duct, resulting in a doubling of sperm collection when compared to the control group (Chauvigné et al., 2022). Likewise, similar results were obtained also in juvenile meagre treated with homologous arrFsh (Zupa et al., 2023). Based on our results, it is interesting to speculate that the lack of clear evidence of sperm volume increase (*i.e.* stripping of sperm with abdominal pressure) by the sdrFsh/Lh treatment in the present study might be explained by (a) the difficulty in applying adequate external pressure to the testes and (b) the short treatment with sdrLh before sampling -only a single administration the week before- which did not allow for adequate action of the increased testicular steroid hormones (*i.e.* T and 11-KT), which are driven by Lh and necessary to induce sperm hydration (Schulz et al., 2010). The elevated plasma T and 11-KT induced by the combined sdrFsh/Lh treatment has been also reported in other fish species (Chauvigné et al.,

2012; Kamei et al., 2003; Penaranda et al., 2018). The fact that both hormones increase gradually during the study period, with a many-fold increase in the last sampling (week 6) that took place 5 days after the 1st sdrLh treatment (week 5), underlines the significant role of this pituitary hormone in sex steroid hormone production in the testes, and its role in the last stage of the reproductive cycle in males, namely spermiation and spawning.

Contrary to the lack of clear evidence of an increased spermatozoa and sperm production, sperm quality was significantly and dramatically enhanced by the combined sdrFsh/Lh treatment. Both the percentage of motile spermatozoa and their velocity were significantly enhanced. Our results are in agreement with other studies of aquaculture species, in which males exhibit reproductive dysfunctions and their sperm quality was enhanced after treatment with specific rFsh/Lh, such as the Japanese eel (Ohta et al., 2017) and Senegalese sole (Chauvigne et al., 2018). The observed mean spermatozoa motility parameters in the present study were even higher than values reported of a stock of the same origin and age maintained in sea cages at the same time (Lancerotto et al., 2024). In the latter study of 5-yo hatchery-produced males maintained in sea cages, the mean percentage of motile cells (*versus* the present study) was reported to be 40 ± 6.4% (*vs* 56 ± 8.7%), while the progressive and rapid cells were 33 ± 5.3 (*vs* 45 ± 7.1%) and 35 ± 6.4% (*vs* 49 ± 8.8%), respectively. Not only the sdrFsh/Lh-treatment has proved to be effective in increasing motility, but also velocity-related parameters were significantly enhanced in comparison to the Control group; furthermore, mean values of VCL, VSL, and VAP of spermatozoa from milt of sdrFsh/Lh-treated males were found to be 50, 70 and 60% higher than those of wild-caught captive-reared males maintained in sea cages (Fakriadis and Mylonas, 2021). The results demonstrated the effectiveness of the single-chain sdrFsh/Lh designed and produced for the present study in not only enhancing spermatogenesis and spermiation, but in improving significantly the quality of the produced sperm. Among the parameters that are known to be reliable indicators of the fertilization potential of good quality sperm, percentage spermatozoa motility and velocity are the most important (Kowalski and Cejko, 2019).

Spawning before the GnRHa administration resulted in very low fecundity -we assume from only a single female- and very low or 0% fertilization. Both fecundity and fertilization success improved after the GnRHa induction, although 0%-fertilization spawns were also produced. It is interesting to wonder whether the initial low fertilization success was due to low spermiation and sperm release at the time, low spermatozoa motility characteristics, or low fertilization capacity of these first eggs that were released. In other established fish in the Mediterranean aquaculture, such as the gilthead seabream, *Sparus aurata*, white seabream, *Diplodus sargus*, and sharpsnout seabream, *Diplodus puntazzo* it is very well described that the spawning season starts with both low fecundity and low fertilization success (Mylonas et al., 2011; Papadaki et al., 2024; Papadaki et al., 2008). As discussed earlier, sperm quality in terms of percentage motility and spermatozoa velocity was higher in sdrFsh/Lh-treated males compared to Controls (present study), as well as compared to both wild-caught, captive-reared (Fakriadis and Mylonas, 2021) and hatchery-produced males (Lancerotto et al., 2024) maintained in sea cages during the year. Nevertheless, to our knowledge, there is no existing literature describing sperm characteristics of greater amberjack captured in the wild during the spawning season and it is difficult to hypothesize if the low fertilization of many spawns in the present study, was due to lower sperm quality of greater amberjack in captivity.

In many fish species, a common reproductive dysfunction of females involves the failure of late-vitellogenic oocytes to undergo oocyte maturation and ovulation due to a blockage in the BPG axis, and lack of pituitary Lh release, the latter being necessary for the acquisition of maturational competence by the late-vitellogenic oocytes (Kagawa et al., 1998) as well as for the maturation, ovulation and spawning (Nagahama and Yamashita, 2008). To overcome this type of dysfunctions, treatments based on GnRHa administered in various ways (Mylonas et al., 2010; Mylonas and Zohar, 2001) act at the pituitary level stimulating the release of the endogenous Lh that accumulates in the pituitary during vitellogenesis. Such therapies have been administered successfully in greater amberjack females throughout the world (Fakriadis et al., 2020a; Fernández-Palacios et al., 2015; Nyuji et al., 2019). However, when trying to induce a hatchery-produced greater amberjack broodstock of the exact origin and age as the present broodstock, that was reared in sea cages and considered to be eligible for GnRHa induction of spawning based on their oocyte stage of development, the given GnRHa was not very effective (Lancerotto et al., 2024). The latter results suggested the presence of perhaps a more severe reproductive dysfunction in hatchery-produced greater amberjack than the one common in wild-caught broodstocks (Corriero et al., 2021a; Fakriadis et al., 2020b), that is the failure of late-vitellogenic oocytes to acquire maturational competency. The successful gametogenesis of the hatchery-produced greater amberjack in the present study using sdrFsh/Lh, while the fish were reared in tanks of a relatively small volume, presenting sub-optimal conditions for reproductive development (Fakriadis et al., 2020b) demonstrates the potential of sdrFsh/Lh in providing a powerful tool for the control of a very significant reproductive dysfunction in greater amberjack. In doing so, it made previously ineligible females able to respond to established spawning induction therapies using GnRHa, and produce significant numbers of fertilizable eggs.

Comparing the reproductive performance of this first study of its kind in any *Seriola spp.*, with results obtained in other studies of spawning induction in greater amberjack makes our results encouraging. During the whole experiment, a total relative fecundity of 62,786 eggs kg^{-1} was recorded, compared to 10,000–200,000 eggs kg^{-1} in other studies (Fakriadis et al., 2019; Fakriadis et al., 2020a; Fakriadis et al., 2020b). In regards to the egg quality and larval survival, embryo survival 24 h after spawning and the hatching success were lower than those obtained from eggs obtained from both natural and induced spawning of the same species in the Canary Islands (Sarih et al., 2018). On the other hand, egg/larval survival were comparable to previous studies conducted on wild-caught fish, maintained in identical tanks, to which GnRHa was administered for spawning induction in the form of either liquid injections or solid implants (Fakriadis et al., 2019). Finally, larval survival until yolk sack absorption in the present study was between 3-fold and 7-fold higher than wild-caught captive-reared fish induced to spawn with GnRHa (Fakriadis et al., 2019; Sarih et al., 2018), underlying the fact that the combined sdrFsh/Lh therapy constitutes a physiologically sound approach for the enhancement of gametogenesis in hatchery-produced greater amberjack.

5. Conclusions

Combined treatment of hatchery-produced greater amberjack with 7 weekly injections of combinations of sdrFsh/Lh, beginning at the stage of early vitellogenesis, was highly effective in enhancing vitellogenesis in females and making the fish eligible for spawning induction with established GnRHa-based protocols. Furthermore, the sdrFsh/Lh treatment was also effective in initiating the process of oocyte maturation in females and spawning of fertile eggs in at least one female. In males, it stimulated spermatogenesis and enhanced spermiation, and resulted in the increased production of spermatozoa and the significant improvement of spermatozoa motility percentage and velocity characteristics. Optimizing the dosage and timing of administration might further increase the treatment's efficacy and reduce its cost, resulting in the development of a very useful protocol for enhancing gametogenesis and improving spawning performance of greater amberjack in aquaculture, and enabling the implementation of selective breeding programs to improve desirable phenotypic traits.

CRediT authorship contribution statement

Stefano Lancerotto: Writing – original draft, Methodology, Investigation, Formal analysis. **Ioannis Fakriadis:** Writing – review & editing, Resources, Project administration, Investigation, Formal analysis, Conceptualization. **Maria Papadaki:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Ignacio Giménez:** Writing – review & editing, Resources, Project administration, Formal analysis, Data curation, Conceptualization. **José Vicente Roig Genovés:** Writing – review & editing, Formal analysis, Data curation. **Constantinos C. Mylonas:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ignacio Giménez and José Vicente Roig Genovés are associated with the biotech company Rara Avis Biotec, S. L., which produced the recombinant gonadotropins employed in this study.

Data availability

Data will be made available on request.

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