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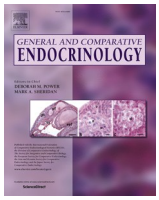

Timing of puberty in F1-generation hatchery-produced greater amberjack (*Seriola dumerili*)

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ABSTRACT

We evaluated the onset of puberty of first-generation (F1) hatchery-produced greater amberjack (*Seriola dumerili*) reared in sea cages for 5 years. Fish were sampled every year in June, at the expected peak of the spawning period in the Mediterranean Sea. No sexual dimorphism in body weight was observed in the study. The ovaries of 1 and 2-year-old (yo) females consisted of primary oocytes only, while at the age of 3-yo early vitellogenic (Vg) oocytes were also identified, but with extensive follicular atresia. At the age of 4-yo, late Vg oocytes were observed, but again extensive follicular atresia characterized the ovaries of 50 % of females. At the age of 5-yo, follicular atresia of Vg oocytes was very limited. In males, gametogenesis was evident already in 1- and 2-yo fish, and 100 % of sampled 3-yo males produced collectable viable sperm. Plasma testosterone (T), 17β-estradiol (E2), and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) remained similar in 3 – 5-yo females, with T and E2 levels being highest in females in advanced vitellogenesis or with significant follicular atresia, compared to immature females. In males, plasma T declined over the years, while 11-ketotestosterone (11-KT) and 17,20β-P were highest in 4 and 5-yo males, with spermatozoa motility characteristics being improved from the 4th year onwards. The administration of GnRHa implants to 5-yo fish induced only two spawns, albeit no fertilized eggs were obtained. The results indicate that hatchery-produced greater amberjack males mature well and within the same age observed in the wild, however with smaller gonad size. On the contrary, females mature later than in the wild, also with a smaller gonad size. Spawning in response to GnRHa treatment was not effective, suggesting that Mediterranean hatchery-produced broodstocks may be dysfunctional, and further research is needed to document any improvement as the fish get older, or to determine if the results may be related to the specific stock of fish.

1. Introduction

Puberty has been defined as the transitional period occurring after sex differentiation, during which the brain-pituitary-gonad (BPG) axis of immature individuals acquires competence and functionality (Taranger et al., 2010). In teleosts, spermatogenesis in males (Almeida et al., 2016; Schulz and Miura, 2002) and vitellogenesis in females (Patiño and Sullivan, 2002; Sullivan and Yilmaz, 2018) are mostly used to mark the onset of puberty (Schulz and Nóbrega, 2011), whereas its ending point would be the acquisition of the ability to reproduce for the first time, *i.e.* to produce fertile gametes (Okuzawa, 2002).

Exogenous factors, such as temperature, photoperiod and nutrition, as well as endogenous factors, such as somatic growth and sex steroid hormones, have been proposed to stimulate the BPG axis during puberty (Migaud et al., 2010; Mylonas et al., 2010; Zohar et al., 2010). The alteration of one or more of these factors may lead to a shift in the timing of puberty (Avella et al., 2012; Passini et al., 2019; Rodríguez et al., 2000). Acquiring insights into the process of puberty and the age at which a species acquires full reproductive functionality is of primary importance for the commercial farming of any species. The onset of puberty is one of the most significant stages in the species' lifecycle, and under aquaculture conditions, precocious or late occurrence of puberty can impact greatly commercial production -both positively and negatively. For instance, in the well-established aquaculture species the European seabass (*Dicentrarchus labrax*) (Felip et al., 2006; Papadaki et al., 2005) and Atlantic salmon (*Salmo salar*) (Crouse et al., 2022),

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precocious maturation can inhibit growth, increase the size of the gonad and affect negatively the organoleptic properties of the flesh, thus leading to economic losses at harvesting (McClure et al., 2007). On the contrary, in species with late puberty, such as the Atlantic bluefin tuna (*Thunnus thynnus*) (Berkovich et al., 2013; Corriero et al., 2003) and striped bass (*Morone saxatilis*) (Holland et al., 2000), maintaining a broodstock for several years before it reaches maturity might build up excessive costs (*i.e.* feeding, labor and facilities) (Higuchi et al., 2017), and also delay the implementation of selective breeding programs.

The greater amberjack (*Seriola dumerili*) is a cosmopolitan species, distributed in temperate and subtropical waters (Jerez et al., 2006; Marino et al., 1995; Nyuji et al., 2016). Because of its numerous attractive flesh characteristics (Alexi et al., 2020; Mazzola et al., 2000), greater amberjack is easily marketable and appreciated by consumers worldwide (Nijssen et al., 2019). Therefore, it has gripped the interest of the Mediterranean aquaculture industry. This fish is a seasonal spawner and its reproductive season lies between spring and summer, even though the timing and extension of spawning might differ according to the environmental conditions of different geographical areas (Jerez et al., 2006; Wells and Rooker, 2004). In the Mediterranean Sea, early spawns are recorded at the end of May when the sea surface temperature reaches 19–20 °C, although it is when temperatures increase to 23–24 °C in June-July that gonads are found fully mature in most adult fish, and the spawning season peaks (Pousis et al., 2018).

Although much information on the reproductive physiology of greater amberjack is available in the scientific literature, most of the studies have used fish sampled in the wild or wild-caught captivity-reared fish. Wild-caught greater amberjack breeders adapt to aquaculture conditions and grow rapidly, but they have been shown to exhibit reproductive dysfunctions and hormonal imbalances in both sexes (Corriero et al., 2021a; Corriero et al., 2021b; Fakriadis et al., 2019; Micale et al., 1999; Mylonas et al., 2004). The development of hatchery-produced breeders is essential for the sustainability of the aquaculture industry, as domesticated stocks may be better adapted to the captive environment. In addition, breeding selection programs require hatchery-produced breeders that are able to reproduce under captivity. Therefore, it is imperative to (a) study the timing of puberty of hatchery-produced greater amberjack and (b) evaluate the reproductive function of these fish, when they reach puberty.

Data on sex steroid levels of greater amberjack are limited, focused mainly on describing changes in adult fish during the reproductive season and obtained, once more, mostly from wild or wild-caught reared fish. Moreover, reported steroid levels have been so far restricted to the most common androgens and estrogens, namely testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) (Mandich et al., 2004; Zupa et al., 2017a; Zupa et al., 2017b). Recently, some studies have been conducted on hatchery-produced greater amberjack. For instance, in the Canary Islands (Spain), an F1 broodstock was used in spawning experiments (Jerez et al., 2018), where levels of sex steroids were found to be lower compared to fish in the wild, but similar to captive-reared wild fish. In Greece, sex differentiation was described in a hatchery-produced greater amberjack stock (Papadaki et al., 2021). The authors suggested the involvement of 11-KT as the male-specific hormone participating in the sex differentiation of greater amberjack, and androgens (adrenosterone, androstenedione, testosterone and 11-KT) and progestogens (progesterone and 17,20β-dihydroxy-4-pregnen-3-one) were considered as regulators of the process in both sexes (Papadaki et al., 2021). Here, we were interested in examining the levels of these hormones during the gonadal development of hatchery-produced greater amberjack over the years, and their possible role in the initiation of puberty.

The present work aimed to examine the onset of puberty and the relevant physiological and endocrine changes in a hatchery-produced greater amberjack stock. To do so, biometrical parameters, gonadal development, as well as the hormonal profile of various sex steroid hormones in the blood were examined for five consecutive reproductive seasons, in fish maintained in sea cages. During the last two reproductive seasons, gonadotropin-releasing hormone agonist (GnRHa) was administered in an attempt to induce spawning in 4 and 5-yo fish, and to evaluate reproductive performance.

2. Materials and methods

Ethical statement

The experimental protocol was approved by the National Veterinary Services (AP31326). All procedures involving animals were conducted in accordance with the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), 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).

2.1. Fish and rearing conditions

In June 2017, greater amberjack eggs were obtained from wild-caught breeders induced to spawn with GnRHa implants in Argosaronikos Fish Farm S.A. (Salamina Island, Greece) (Fakriadis et al., 2020a). Eggs were transferred to the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR, Registration NoEL91-BIObr-03, and EL91-BIOexp-04), and hatched larvae were reared until 50 days post-hatching. Then, the fingerlings were divided into two batches. The first one was transferred and maintained in grow-out cages (6 x 6 m) at the pilot sea cage farm of HCMR (Souda Bay, Chania, Crete, Greece, GR94FISH0001) (Papadaki et al., 2021) and was used as the 1-yo population of the present study. The second batch was transferred to Argosaronikos Fishfarms S.A, where it was maintained in sea cages for five years and was used for the subsequent years' samplings. The temperature of surface seawater was measured at least once a week from October 2017 to June 2022, and it ranged between 13.5 and 29 °C during the years (Fig. 1) and between 18.5 and 26 °C in June, during the usual spawning induction period. Fish were fed to apparent satiation with a commercial feed (Vitalis Repro, Skretting, Spain) five times a week.

2.2. Blood, gonad sampling, and hormonal spawning induction

Fish were sampled once a year in June, when the natural reproductive season of greater amberjack in the Mediterranean basin reaches its peak (Fakriadis et al., 2020a; Mandich et al., 2004; Marino et al., 1995; Pousis et al., 2019), over a period of five years (2018–2022). In June 2018, 17 fish of the 1-yo population were anesthetized with phenoxyethanol for blood collection and then killed in an overdose of the anesthetic bath. Total length (TL, cm) and body weight (BW, g) were measured, and gonads were collected for histology. After sacrificing the fish, macroscopical examination of the gonads allowed for sex identification in 15 of the collected fish (8 males and 7 females), the blood samples of which were used for subsequent plasma sex steroid concentration measurements (Papadaki et al., 2021).

From 2019 to 2021, prior to each sampling, 2—3-, and 4-yo fish were sedated at a dose of 0.01 ml l^{-1} clove oil in a 15-m^3 sedation sack placed inside the sea cage and then transferred individually in a 1 m^3-tank at a dose of 0.03 ml l^{-1} of clove oil for full anesthesia (Mylonas et al., 2005). Biopsies were collected to sex the fish before dissection, in order to obtain an equal number of specimens of both sexes. Once the established number of fish (n = 6) for each sex was obtained, subsequent fish of the same sex were transferred back to the cage. Blood samples were collected, and BW, TL, and gonad weight (GW, g) were measured after euthanizing with a lethal dose of anesthetic, followed by a post-cranial section of the spinal cord and dissection of the fish. The GW was used later to calculate the gonadosomatic index (GSI; [GW/BW] x100). Due to severe weather conditions, in 2019 the sampling was interrupted as

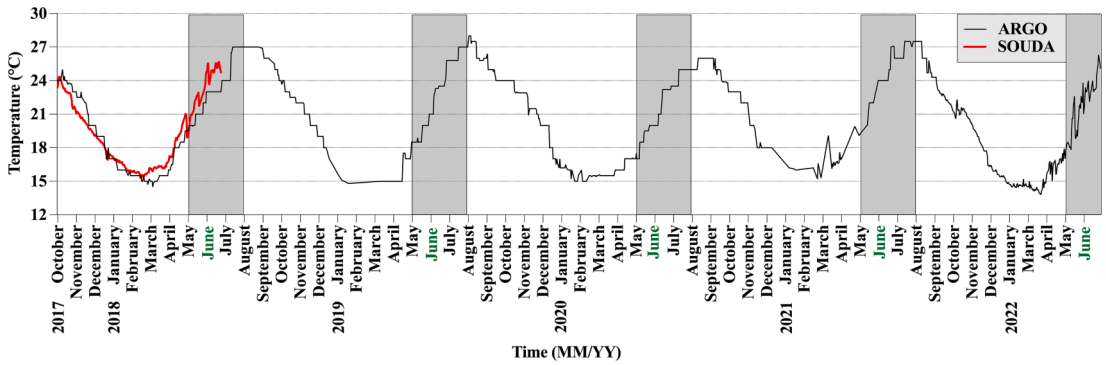

Fig. 1. Water temperature profile (°C) in the sea cages where the two populations of hatchery-produced greater amberjack were maintained during the time of the experiment (SOUDA, 2017–2018 in Souda Bay, Crete, Greece; ARGO, 2017–2022 in Argosaronikos Fish farms S.A., Salamina Island, Greece). Shaded sections mark the expected period of reproductive maturation and spawning of greater amberjack in the Mediterranean Sea. Fish were sampled every year in June at the expected peak of the spawning season.

the operations were conducted on a platform nearby the cages. At this sampling point, it was not possible to collect blood, and a reduced number of samples was obtained (n = 2 males, n = 4 females) for histological evaluation of the gonads. Data for TL and GSI of 5-yo fish were not obtained in 2022, since no fish were sacrificed at this stage. It was considered unnecessary for the purpose of the study, and there was a need to maintain an adequate number of breeding individuals for the future commercial purposes of the company.

From the third year of age (2020), fish were evaluated for spawning eligibility. For spawning induction, only females having fully vitellogenic oocytes of > 600 μm in diameter were used, and males that were in spermiation and sperm could be obtained for evaluation. Ovarian biopsies were collected using a catheter (Pipelle de Cornier, Laboratorie CCD, France) inserted into the ovary through the genital pore, and then applying gentle suction. The biopsies were evaluated (a) under a microscope at 4x and 10x magnification in order to select females for spawning induction and then (b) through histological processing (see later). Collected gonads and biopsies were preserved in a solution of 4 % formaldehyde: 1 % glutaraldehyde for histological analysis. Stripping of sperm using abdominal pressure was not possible due to the thick muscular abdominal structure of greater amberjack (Fakriadis and Mylonas, 2021). Therefore, sperm for quality evaluation was also collected using a biopsy catheter (Pipelle de Cornier, Laboratorie CCD, France) inserted into the genital pore and then applying gentle suction. Sperm samples (100–200 μL) were stored at 4 °C until analyzed using computer-assisted sperm analysis (CASA, see later).

At each sampling time, from the third to the fifth year of the study, blood was collected from the caudal vein using heparinized syringes and after collection, it was centrifuged at 4500 rpm for 15 min at + 4 °C. The obtained plasma was maintained at −80 °C until hormonal analysis using liquid chromatography tandem mass spectroscopy (LC-MS/MS).

2.3. Spawning induction

Some 4-yo and 5-yo fish were considered capable of spawning and were selected according to their reproductive stage, as explained above, for spawning induction. They were administered implants of ethylene–vinyl acetate copolymer (EVAc) (Mylonas et al., 2007; Zohar and Mylonas, 2001) loaded with Des-Gly^{10}, D-Ala^{6}-Pro-NEth^{9}-mGnRHa (H-4070, Bachem, Switzerland). After GnRHa treatment, fish were transferred to a 25-m^3 (2021; females: n = 4, 9.4 ± 0.9 kg; males: n = 4, 8.1 ± 0.3 kg) or a 75-m^3 (2022; females: n = 5, 10.7 ± 0.9 kg; males = 7, 10.3 ± 0.7) round tank for spawning, and reproductive performance was evaluated (see later). The effective GnRHa dose was 69 ± 11 μg GnRHa kg^{-1} in 2021 and 66 ± 4 μg GnRHa kg^{-1} in 2022. The tanks were covered and were supplied with surface seawater at ambient temperature (Fig. 1) at a water renewal of 400–500 % day. The fish were exposed to a natural photoperiod through large windows in the building where the 25-m^3 tank was located, or through openings in the plastic cover of the outside 75-m^3 tank.

2.4. Histological analysis

The dissected gonads of all the sampled fish were dehydrated in a 70–95 % ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraus Kulzer, Germany). Serial sections of 4 μm of the gonadal tissue were obtained using a semi-automatic microtome (Leica RM2245, Germany). Histology slides were stained with methylene blue/azure II/basic fuchsin (Bennet, 1976) and examined under a light microscope (50i Eclipse, Nikon, Japan). Eventually, microphotographs of the stained tissues were taken using a digital camera (Progres, Jenoptik AG, Germany).

2.5. Measurement of plasma sex steroids

The extraction and analysis of steroid hormones were performed according to Papadaki et al. (2021) with a few modifications. The following sex steroids were included in the panel of analytes: T, estrone (E1), E2, 11-KT and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P). Furthermore, instead of N,N-dimethyl-L-phenylalanine, 13C-labelled estradiol, testosterone, and progesterone (>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 hormones. A mixture of those compounds in varying concentrations (10 to 85 pg μL 1) was prepared in methanol: water 1:1 and 10 μL of this solution was 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.

2.6. Sperm quality analysis

For sperm quality analysis using CASA (ISAS, Spain), milt from each sampled male (3-yo in 2020, n = 2; 4-yo in 2021, n = 10; and 5-yo in 2022, n = 8) was activated in seawater (1:201 or 1:334) containing 2 % bovine serum albumin to obtain 200–300 cells in the field. A re-usable counting chamber with a fixed depth (SpermTrack) was used to record spermatozoa movement using a digital camera at 100 frames per second (fps) attached to a light microscope (Zeiss Primo Star, Germany) under 100 × magnification. The analysis included the following parameters: sperm density (number of spermatozoa ml^{-1} of milt), duration of forward spermatozoa motility until 5 % of the spermatozoa in the field of view were motile (motility duration, min), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) (μm/sec),

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 to classify a spermatozoon as immotile; and spermatozoa were considered rapid when VCL was higher than 100 μm/sec.

2.7. Reproductive performance evaluation

Following spawning induction in 4-yo and 5-yo greater amberjack, fish were kept in the tank for 14 days for spawning. A semi-conical egg collector tank (1–2 m^3) fitted with a 300-μm mesh cylinder was connected to the water overflow of the broodstock tanks and checked twice a day (8.00 a.m. and 8.00p.m.). Egg production was evaluated through the estimation of daily fecundity (number of eggs) and fertilization success (%). Spawned eggs were taken from the egg collector and placed into a 10-l bucket, from which a sub-sample of 10 ml was taken with a plastic pipette for egg counting and quality estimation. All eggs contained in the sub-sample were counted using a stereoscope (Zeiss Stemi 305, Germany) and checked for fertilization success immediately after collection. Fertilized eggs are then incubated in a 1-m^3 conical incubator supplied with sand-filtered (10 μm) and UV-treated surface seawater at ambient temperature (Fig. 1).

2.8. Statistical analysis

Differences in BW, TL, and GSI were tested using a two-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. Due to high variability among individuals as well as between the different classes of age, in order to avoid type I errors due to the violation of the assumption of homogeneity of variances, hormonal profiles of sex steroids over time in females and males, as well as levels of steroids by reproductive stage, were analyzed using Welch's Analysis of Variance (ANOVA) followed by Dunnett's T3 post hoc test. Sperm quality parameters were analyzed using Kruskal-Wallis followed by Dunn's post hoc test. 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, https://www.graphpad.com.

3. Results

3.1. Growth and gonadal development

The BW of the fish increased during the first 4 years of the study, but remained unchanged in the 5th year, with no differences detected between sexes (Fig. 2a). The mean (±SEM) final BW of 5-yo males and females reached 10,300 ± 736 g and 10,670 ± 869 g, respectively. The TL of males and females also did not differ, and increased significantly for the first 4 years (Fig. 2b). The GSI increased significantly over time with mean values of 2.3 ± 0.5 % and 3.0 ± 1.2 % in 4-yo males and females, respectively (Fig. 2c).

In 1-yo fish, ovaries contained a predominant population of primary oocytes with oogonia interspersed among them (Fig. 3a and 5a). One year later, all 2-yo female gonads were found to be constituted by primary oocytes at the perinuclear stage (Fig. 3b and 5a). At 3 years of age, the gonadal development of females was more variable (Fig. 3c-d, and 5a). Primary oocytes were the only germ cell population in 17 % of the sampled females, whereas oocyte growth reached early vitellogenesis in 33 % of the females, exhibiting negligible levels of atresia (Fig. 3c and 5a). The remaining 3-yo females showed oocytes in extensive atresia surrounding primary and cortical alveoli oocytes (Fig. 3d and 5a). Following an earlier classification (Hunter and Macewicz, 1985), yolked oocytes at the alpha (α) stage of atresia were visible throughout the histological sections. Nuclear disappearance, coalescence of yolk material, and fragmentation of zona radiata were distinctive signs of the process. A comparable, yet not identical, reproductive status was found in the 4-yo females. A high percentage (33 %) did not initiate vitellogenesis at all, whereas only 17 % were found to be in an advanced stage of vitellogenesis (Fig. 3e and 5a). The remaining 50 % of the females presented diffuse atretic activity among vitellogenic follicles (Fig. 5a). Along with α-stage atretic oocytes exhibiting disrupted membranes and disorganization of cytoplasmic contents, some oocytes underwent complete degradation entering β-stage atresia (Fig. 3f). At this stage, atretic follicles appeared as masses of unorganized vacuoles with completely digested membranes, surrounded by blood vessels. Eventually, in all 5-yo females all stages typical of vitellogenesis were detected (Fig. 3g-h, and 5a). At this age, atresia was present only at low and physiological levels (Corriero et al., 2021b).

Regarding the males, 25 % of dissected testes from 1-yo fish were immature, containing only spermatogonia (Fig. 4a and 5b). Early (Fig. 4a) or advanced spermatogenesis was detected in a few cysts in the remaining 1-yo fish, with spermatocytes, spermatids and spermatozoa all being detected in the gonads together with spermatogonia (Fig. 5b). Although in 2-yo males spermatogenic activity was present, spermatogonia still constituted the main population of germ cells in 50 % of the males (Fig. 4b and 5b). On the contrary, the testicular lumen of the remaining 50 % of males was found filled with large numbers of spermatozoa as well as many spermatocysts with developing germ cells (Fig. 4c and 5b). Both in 3-yo and 4-yo males, the testes exhibited a testicular lumen filled with spermatozoa (Fig. 4c and d), with 50 and 35 % of males, respectively, being in an arrested state of spermatogenesis with only a few residual developing spermatocysts in the germinal epithelium (Fig. 4d and 5b).

3.2. Sex steroid plasma levels

Significant changes in the sex steroid profile of both sexes were found over the course of the experiment (Figs. 6 and 7). Differences were detected in the levels of E1 in females and T, 11-KT, E2, and 17,20β-P in males. In females, the highest levels of E1 were in 3- and 4-yo fish and decreased sharply in 5-yo females. In males, T, and E2 reached maximum concentrations in 3-yo fish, dropping thereafter in 5-yo fish. On the contrary, plasma levels of 11-KT and 17,20β-P reached maximum concentrations in 4- and 5-yo males, at the time of the greatest number of males in full spermiation. Plasma levels of sex steroids were also examined in relation to the reproductive stage of females, regardless of their age, due to the variability observed in the reproductive stage among similarly aged individuals. The concentration of T values rose markedly in atretic females, while being similar to vitellogenic individuals (Fig. 8a). Plasma E2 concentration of vitellogenic and atretic females was significantly higher than immature ones (Fig. 8b).

3.3. Sperm quality evaluation

In 3-yo males, spermatozoa concentration ranged between 45 – 51x10^9 szoa ml^{-1}, being on average 48 ± 4x10^9 szoa ml^{-1}, and was statistically lower when compared to 4-yo males, of which spermatozoa concentration values varied between 65 – 144x10^9 szoa ml^{-1} with a mean value of 95 ± 9x10^9 (Fig. 9a). Spermatozoa concentration was slightly lower in 5-yo males. There was a large individual variability observed in the measured sperm quality parameters among fish of the same age class, with percentages of motile, progressive and rapid cells differing at times by 40 %, although no statistically significant differences in the mean values were found among years (Fig. 9b). The ability of the progressive cells to move on a straight trajectory was less variable, with mean STR being 89 ± 5.3 % in 3-yo males, and 91 ± 0.8 % or 93 ± 0.8 % in 4- and 5-yo fish, respectively. Still, no significant difference was observed among different aged males (Fig. 9b). Finally, the parameters related to the velocity of the analyzed milt, such as VCL, VSL, and VAP, also did not differ significantly among years (Fig. 9c).

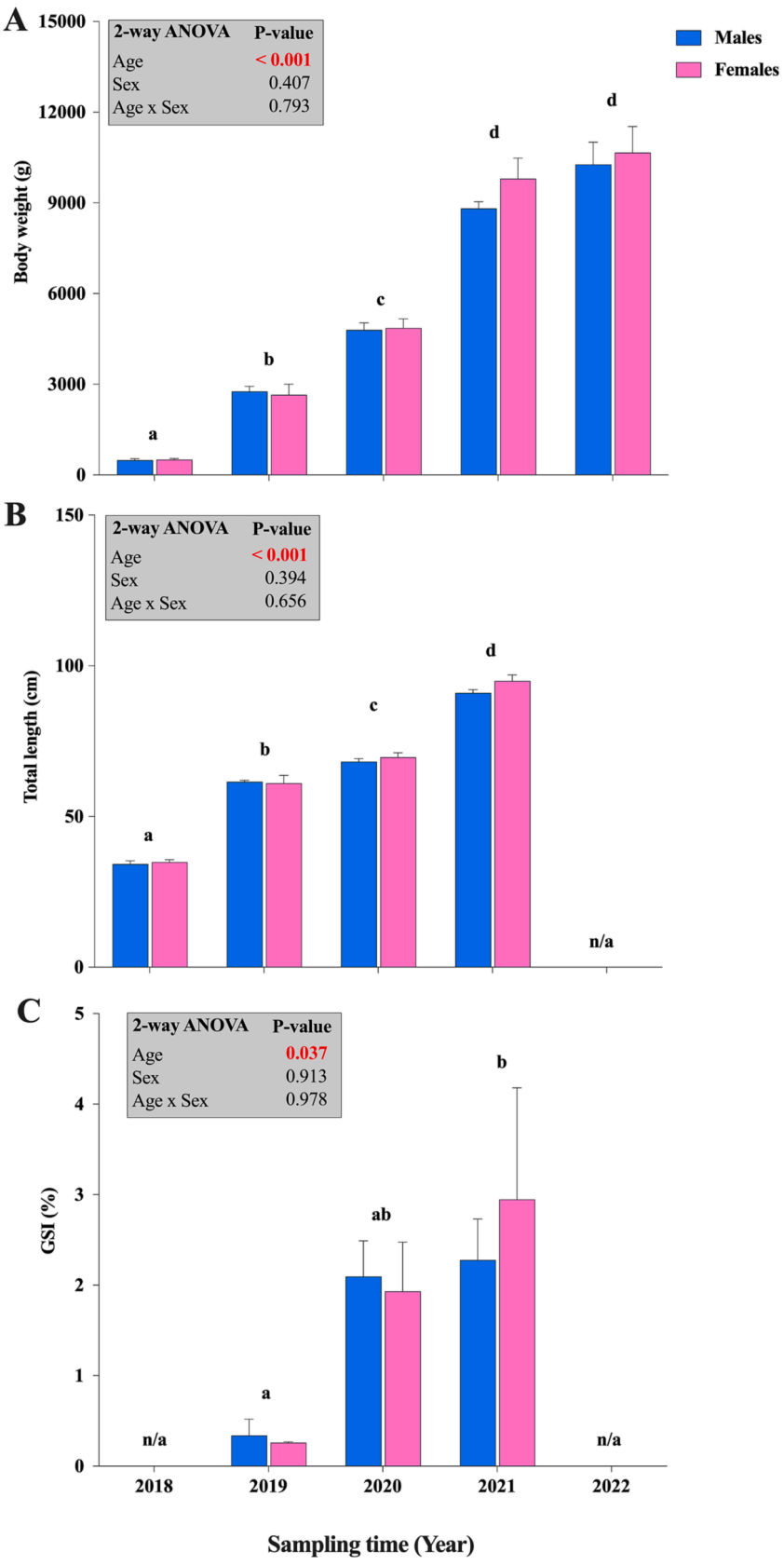

Fig. 2. Mean (±SEM) **A.** body weight (BW), **B.** total length (TL) and **C.** gonadosomatic index (GSI) of hatchery-produced greater amberjack males and females reared in sea cages from 1 to 5 years of age (2018–2022). Fish were sampled every year in June at the expected peak of the spawning season (See Fig. 1). Different lowercase letters indicate statistically significant differences between years (two-way ANOVA, Tukey HSD, $P < 0.05$). n/a: no data obtained.

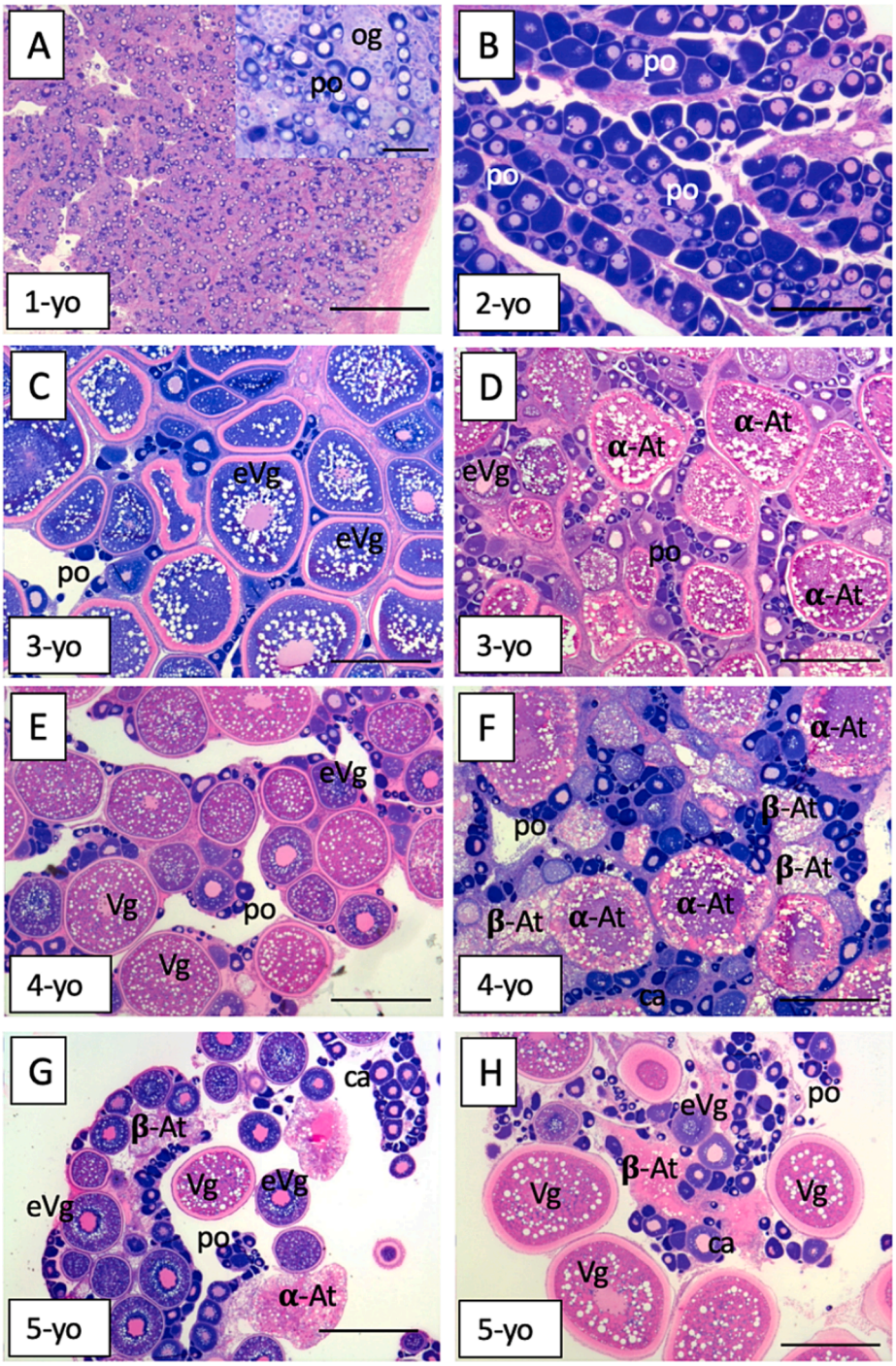

Fig. 3. Microphotographs of histological sections from ovaries of hatchery-produced greater amberjack males and females reared in sea cages from 1 to 5 years of age (2018–2022) at representative stages of reproductive development. Fish were sampled every year in June at the expected peak of the spawning season (See Fig. 1). **A.** Ovary of 1-year-old (yo) female having mostly oogonia (og) and primary oocytes (po) in the lamellae. **B.** Ovary of 2-yo female filled with po. **C.** Ovary of 3-yo female having early vitellogenic oocytes (eVg). **D.** Ovary of 3-yo female with extensive stage α-atresia (α-At) of vitellogenic follicles. **E.** Ovary of 4-yo females, showing eVg and advanced Vg oocytes (Vg). **F.** Ovary of 4-yo females with massive presence of α- and β-atresia (β-At) of vitellogenic follicles. **G.** Ovary of 5-yo female having oocytes at all stages of gametogenesis, with isolated α-At follicles and oocytes at the cortical alveoli stage (ca). **H.** Ovary of 5-yo female with β-At vitellogenic follicles. The scale bars indicate 500 μm (**A-H)** and 100 μm (**A**. insert).

3.4. Spawning performance

In 4-yo fish, no spawning was observed after treatment with GnRHa. The next year, a total of two spawning events were recorded in 5-yo breeders (Fig. 10). The first spawn occurred 72 h after the GnRHa administration, while the second occurred 7 days later. Total production was 389,000 eggs, with a daily relative fecundity of 6,710 and 488 eggs kg^{-1} female BW in the first and the second spawn, respectively. Fertilization success was 0 % in both spawns (Fig. 10), therefore no egg incubation was attempted.

4. Discussion

Following the description of the process of sex differentiation

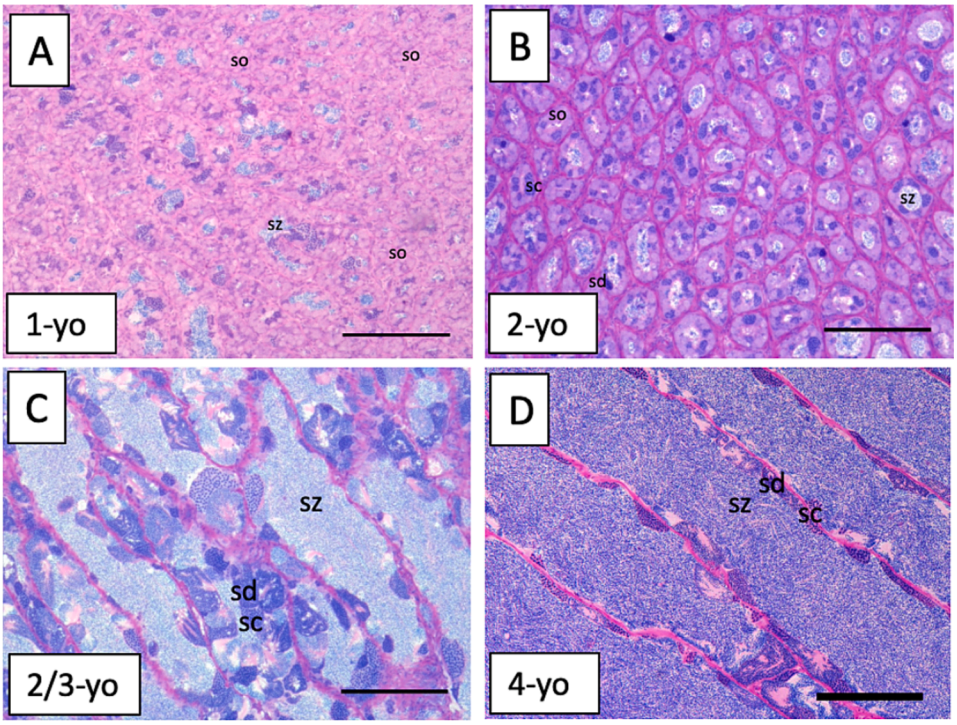

Fig. 4. Microphotographs of histological sections from testes at representative stages of reproductive development, of hatchery-produced greater amberjack males reared in sea cages from 1 to 5 years of age (2018–2022). Fish were sampled every year in June at the expected peak of the spawning season (See Fig. 1). **A.** Testicular section of 1-year-old (yo) immature male. Spermatogonia (so) are the main cell population in the testes, with the occasional presence of more advanced spermatocysts, as well as free spermatozoa (sz). **B.** Early gametogenesis in a 2-yo males. Different germ cell stages, including so, spermatocytes (sc), spermatids (sd), and sz were equally present in the testicular lobules. **C.** Advanced spermatogenesis in 2-yo and 3-yo males, with testes displaying many luminal sz and active sperm cysts in the germinal epithelium. **D.** Arrested spermatogenesis in a 4-yo male, with vast numbers of spermatozoa present in the lumen, and only a few residual spermatocysts in the germinal epithelium. Black scale bar = 500 μm.

(Papadaki et al., 2021), here we continued with the monitoring of the onset of puberty in first-generation (F1) hatchery-produced greater amberjack reared in sea cages. We then evaluated their response to GnRHa-induction of spawning using previously established methodologies for wild-caught captive-reared broodstocks (Fakriadis et al., 2020a; Fakriadis et al., 2020b; Mylonas et al., 2010). Spawning attempts were carried out in June, based on previous findings indicating that greater amberjack in the Mediterranean Sea reaches maximal reproductive development and spawn between May and July (Pousis et al., 2018; Zupa et al., 2017b).

The onset of puberty and the age of first maturity of hatchery-produced greater amberjack has not been described so far, and it is essential for building up a reliable aquaculture production strategy for this species. According to the existing data, the greater amberjack is a late-maturing species with relative plasticity in the timing of puberty, which may depend on sampling location and environmental conditions (Harris et al., 2007; Kozul et al., 2001; Marino et al., 1995). Moreover, due to the different criteria adopted to define the first age of maturity, there are noticeable discrepancies in the published information. For instance, specimens from the Southeastern U.S.A. Atlantic coast and the Gulf of Gabes with developing, ripe or spent gonads were all considered sexually mature (Harris et al., 2007; Sley et al., 2014). In addition, considering mature females, individuals with the most advanced oocytes being at the cortical alveoli stage, likely produced bias in estimating the numbers of spawning-capable individuals, and the age and size of first maturity.

In our study, no sexual dimorphism in growth rate was found until the fish's 5th year of life, in agreement with the work conducted on hatchery-produced greater amberjack during their first year of life (Papadaki et al., 2021). Sexual size dimorphism was reported in wild greater amberjack from the Western Atlantic and was attributed either to a longer life span in one of the sexes (Thompson et al., 1999), or to an adaptation of mature females to maximize fecundity (Harris et al., 2007). Therefore, contradictory information exists on sexual dimorphism of size, which may be related to differences between rearing in the wild and in captivity.

As regards gonadal development, a steady increase over the years was evident in the GSI of both sexes with no differences between them, even though complete sexual maturation was achieved in different ages in the two sexes. Comparing our results with fish of a similar age and BW (≥9 kg) sampled during the spawning period (June-July) in the wild, the mean GSI values of both males and females of the present study (~3%) were lower than fish in the wild (≥4.5 %), but higher than wild-caught captive-reared individuals (~1%) (Zupa et al., 2017a; Zupa et al., 2017b). This was a positive result, suggesting that the growth of the gonad in F1 individuals was better than in wild-caught juveniles reared in captivity, perhaps due to better adaptation of the hatchery-produced individuals.

The histological assessment of reproductive development in the present study showed that Vg oocytes appeared first in 3-yo females, similar to results obtained from the wild, as well as with captive-reared individuals (Kozul et al., 2001), but in a larger fraction of individuals than in the wild (Marino et al., 1995). Unfortunately, extensive follicular atresia was also observed in 50 % of the ovaries of both 3- and 4-yo females. The same condition was reported in two different studies on captive-reared wild fish (Micale et al., 1999; Zupa et al., 2017b); the authors reported that follicular atresia became the dominant process visible once the oocytes reached the late stage of secondary growth (vitellogenesis). In some teleost fishes, an incomplete gametogenic cycle might occur, referred to as a "dummy run" (Holland et al., 2000; Papadaki et al., 2018), before puberty and the completion of reproductive maturation. This may happen in wild fish due to a multiplicity of

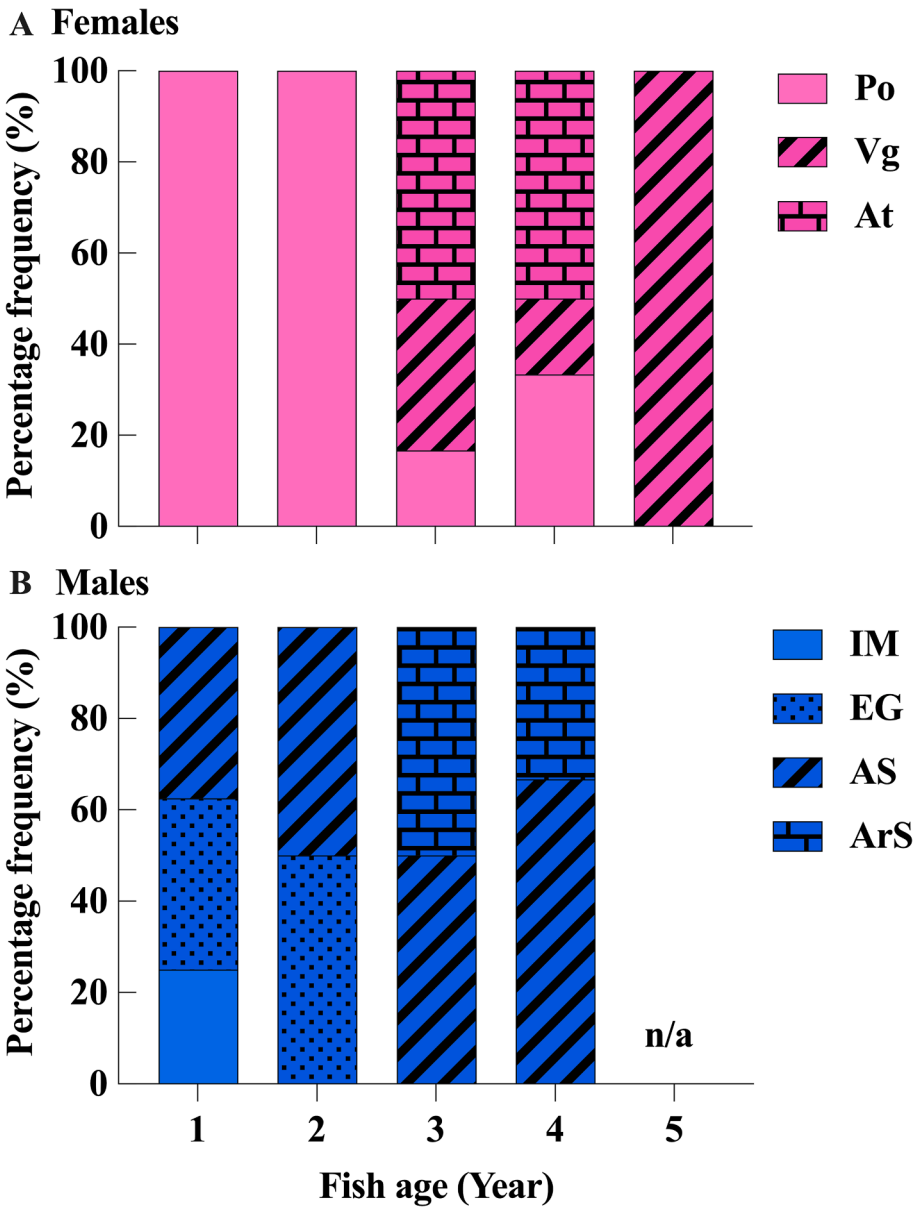

Fig. 5. Percentage frequency (%) of gonadal maturation stages of hatchery-produced greater amberjack sampled in June of each year (2018–2022, See Fig. 1). **A.** Females of 1 and 2 years of age had immature ovaries containing only primary oocytes (Po). At 3 and 4 years of age, females at the stage of advanced vitellogenesis (Vg) or with extensive follicular atresia (At) were also present. **B.** Males showed variable stages of spermatogenesis in their testes, from the first year of the study. In addition to immature males (IM) with testes filled with spermatogonia, some males were in early spermatogenesis (EG) with an equal presence of all germ cell stages (*i.e.* spermatogonia, spermatocytes, spermatids, and spermatozoa) in the testes. At 3 and 4 years of age, all males were either at the advanced spermatogenesis stage (AS) with large numbers of spermatozoa in the testicular lumen, as well as many spermatocysts with developing germ cells or were at the arrested spermatogenesis stage (ArS), with testes filled with luminal spermatozoa and very few residual spermatocysts. n/a: no data obtained.

factors, such as poor environmental conditions and availability of food, while in hatchery-reared fish, as most likely in this study, it may happen due to partial incompetence of the BPG axis (Corriero et al., 2021b; Zmora et al., 2014). Eventually, late Vg oocytes were found in the ovaries of 5-yo females that were considered spawning-capable and eligible for hormonal induction, in the present study. Although follicular atresia was still present, its frequency was considered typical of multiple spawners during the spawning season (Bromley et al., 2000; Corriero et al., 2021b), suggesting that these F1 females finally reached full reproductive capacity.

In F1 hatchery-produced males, on the other hand, testes with spermatocysts showing spermatozoa were found since the first year of the study and increased gradually over time. However, males were considered fully mature when they were 3-yo, when there was evidence of large numbers of free spermatozoa in the testicular lumen (Almeida et al., 2016). While in the Mediterranean Sea the age of first sexual maturity for wild males was considered to occur in the 4th year of life (Marino et al., 1995), in the Atlantic Ocean 99 % of 3-yo individuals completed gametogenesis (Harris et al., 2007), corroborating our results. To date, from the limited data available on the characteristics and the quality of sperm in greater amberjack, what does seem evident is that captive conditions also hamper the development of the testes, leading to a consequent reduction of sperm quality (Zupa et al., 2017a). Therefore, starting from the age of first maturity that was identified as the 3rd year of life in the present study, milt was also collected to further evaluate the reproductive capacity of F1 hatchery-produced greater amberjack. A recent study provided valuable information on spermiation and sperm quality in wild-caught greater amberjack reared in sea

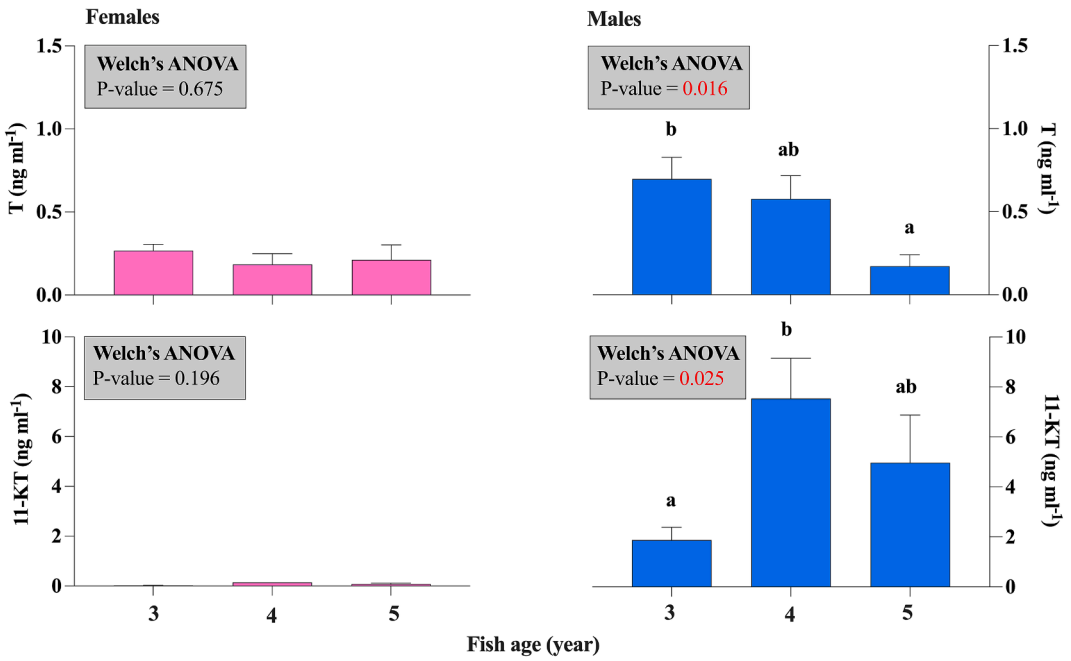

Fig. 6. Mean (±SEM) plasma sex steroid hormone levels of testosterone (T), and 11-ketotestosterone (11- KT) of hatchery-produced greater amberjack (3–5 years old) reared in sea cages and sampled in June of each year (2020–2022, See Fig. 1). Different lowercase letters indicate statistically significant differences (Welch's ANOVA, Dunnett's T3 HSD, $P < 0.05$).

cages before being transferred to tanks during the reproductive period (Fakriadis and Mylonas, 2021). In that study, before males were treated for spawning induction, mean values for velocity-related parameters -namely, VCL and VSL- did not exceed 100 and 70 μm/sec, respectively. In comparison, sperm collected from F1 males in the present study had mean VCL and VSL higher than 120 and 100 μm/sec, respectively. Likewise, the mean values of VAP of F1 males surpassed those of captive-reared wild males recorded during the spawning season (Fakriadis and Mylonas, 2021; Zupa et al., 2017a). Regarding the mean values of motile, progressive and rapid cells, as well as for STR, no evidence of differences was found among fish of different age classes. As much as it may appear as a contradiction when compared to captive-reared wild fish, 3-yo males displayed similar values, while on the other hand, the percentage of spermatozoa classified as progressive and rapid from 4- and 5-yo F1 males was two-fold higher than wild-caught captive-reared males (Fakriadis and Mylonas, 2021). The limited number of sperm samples available from 3-yo males might be accountable for this discrepancy and might have made existing changes in sperm quality parameters in relation to age in the current study, undetectable. Finally, concerning the concentration of spermatozoa, in 3-yo males it was comparable to those of captive-reared wild fish, but increased in the following reproductive seasons and surpassed that of captive-reared wild fish in other studies (Fakriadis and Mylonas, 2021; Fakriadis et al., 2020b; Zupa et al., 2017a) as well as of that of F1 males from the Atlantic Ocean (Jerez et al., 2018). Unfortunately, no data on sperm quality parameters of greater amberjack or any other congener are available from the wild, making it impossible to determine the optimal values for these studied parameters presented here.

The acquisition of competence of the BPG axis is pivotal to reaching sexual maturity, as its full activation leads to the production of gonadal steroids, which are responsible for the initiation and completion of the gametogenic cycle (Carrillo et al., 2009; Chakraborty et al., 2011; Lubzens et al., 2010; Nagahama and Yamashita, 2008; Taranger et al., 2010). Therefore, steroids such as E2, T, 11-KT, 17,20β-P and 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) have been used as reliable markers to discern the reproductive status in fish (Mylonas et al., 2013; Zohar and Mylonas, 2001), including the greater amberjack (Mandich et al., 2004). However, the same seasonal elevations, both in amplitude and duration, observed in plasma levels of sex steroids in wild greater amberjack (Mandich et al., 2004; Zupa et al., 2017b) and its congener yellowtail kingfish (*Seriola lalandi*) (Poortenaar et al., 2001) were not evident in captive-reared wild-caught greater amberjack, in which farming conditions seemingly affected gonadal steroidogenesis (Zupa et al., 2017b). Likewise, in the monitored F1 females, overall low and unchanged levels of T and E2 were found, aligned with those of captive-reared fish from previous studies (Jerez et al., 2018; Nyuji et al., 2016; Zupa et al., 2017b). Also, plasma levels of 17,20β-P were found to be a good indicator of oocyte maturation in the yellowtail kingfish and in wild greater amberjack (Mandich et al., 2004; Poortenaar et al., 2001). However, in F1 greater amberjack in the Atlantic Ocean, plasma levels of 17,20β-P remained unchanged after GnRHa induction of spawning (Jerez et al., 2018). In the examined F1 females of the present study, no differences in the plasma levels of 17,20β-P were observed over the years. These results may be because of the rapid metabolism of this steroid (Scott et al., 2010), or due to the fact that at the sampling time, no fish were observed to have oocytes undergoing oocyte maturation.

To further expand our investigation, plasma levels were also correlated with specific ovarian stages rather than solely with the age of females. Elevated T and E2 concentrations were evident in females undergoing vitellogenesis compared to less developed females, indicating that although the recorded concentrations were considerably lower than in wild fish (Mandich et al., 2004), they were sufficient in stimulating and sustaining vitellogenesis. Opposite to the well-known and acknowledged role of E2 and 11-KT in the gametogenic and maturational process of fish, few studies have dealt with the role of intermediate metabolites of the steroidogenic pathway. For instance, in the congeneric Japanese yellowtail (*Seriola quinqueradiata*), it was reported that the synthesis of E2 passed through the conversion of Δ4 (Rahman et al., 2002), while in two other teleost species, the red seabream (*Pagrus major*) and the bambooleaf wrasse (*Pseudolabrus sieboldi*) the main substrate for the biosynthesis of E2 came from E1 via Adrenosterone conversion, instead of T (Ohta et al., 2001; Ohta et al., 2002). Although it remains to be verified in greater amberjack, a more complex than a T-to-E2 pathway might explain our results. In fact, even though the levels

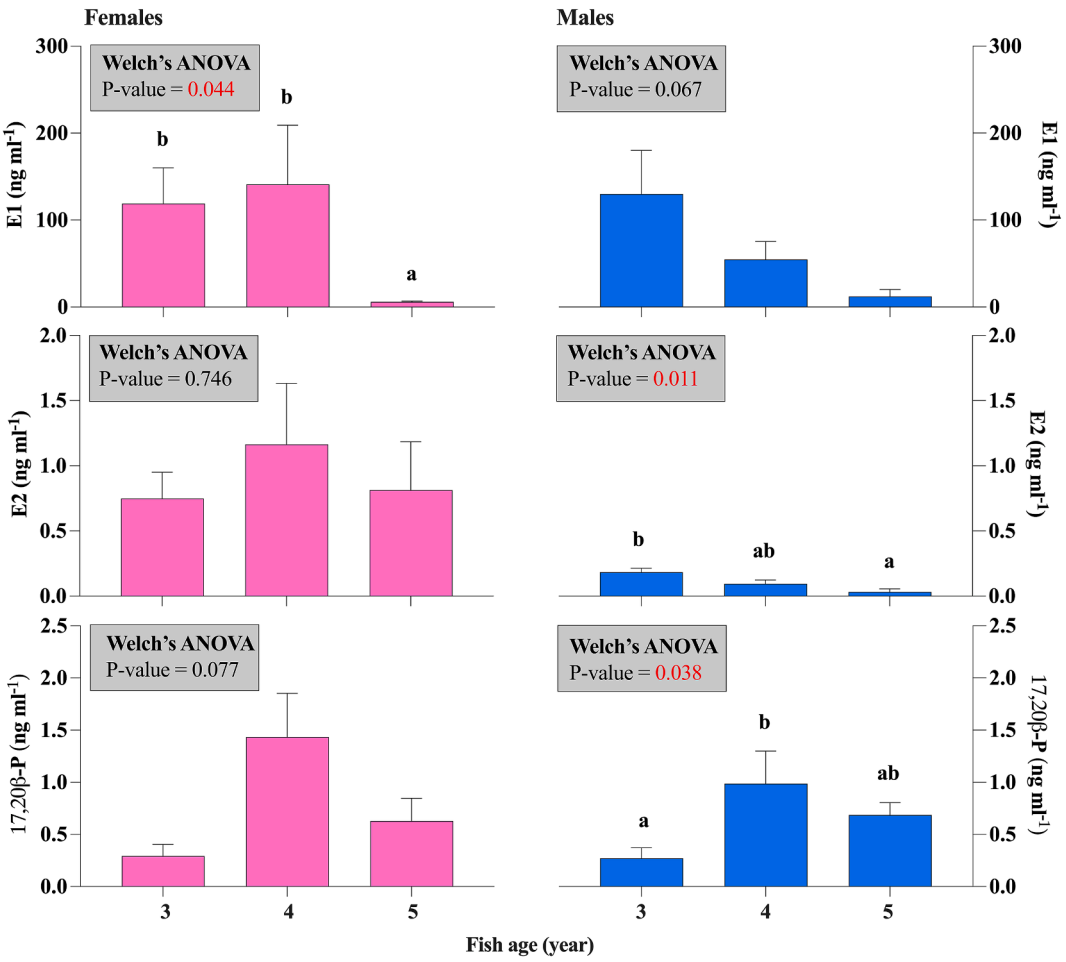

Fig. 7. Mean (±SEM) plasma sex steroid hormone levels of estrone (E1), 17β-estradiol (E2), and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) of hatchery-produced greater amberjack (3–5 years old) reared in sea cages and sampled in June of each year (2020–2022, See Fig. 1). Different lowercase letters indicate statistically significant differences (Welch's ANOVA, Dunnett's T3 HSD, $P < 0.05$).

of T in F1 females were low and unchanged over the years, it was when all females were found in advanced vitellogenesis, at five years of age, that a concomitant reduction in the concentrations of E1 was evident. Furthermore, not only the steroidogenic pathway might differ during gametogenesis among teleost fish, but also specific differences in the development of the BPG axis do exist (Okuzawa, 2002). For instance, in the black carp (*Mylopharingodon piceus*) the acquisition of competence of the pituitary and gonads to respond to hormonal stimulation was demonstrated to occur in synchrony in the two organs (Gur et al., 2000). In red seabream, on the other hand, the administration of GnRHa increased plasma levels of luteinizing hormone (Lh), but did not cause a gonadal response in 12-month-old fish, while the same treatment induced vitellogenesis and ovulation in 16-month-old fish, suggesting distinct timing of acquisition of competency for pituitary and gonads (Kumakura et al., 2003). Considering all these data together, the present results indicate a partial lack of competence of the BPG axis resulting in reduced aromatization of precursors of E2 and the progression of vitellogenesis in young females of this species.

The main steroids controlling spermatogenesis in fishes are 11-KT and its precursor T (Schulz and Miura, 2002; Schulz et al., 2010). In wild greater amberjack in the Mediterranean, the highest recorded levels of T were found in males with mature testes having sperm cysts in advanced spermatogenesis, as well as free spermatozoa in the luminal space, whereas 11-KT reached maximum values when the testicular lobules were greatly enlarged and entirely occupied by spermatozoa (Mandich et al., 2004). In F1 males here, the mean T plasma concentration was lower than in wild individuals (Mandich et al., 2004), but comparable with those of both captive-reared and hatchery-produced breeders examined during the reproductive season elsewhere (Jerez et al., 2018; Zupa et al., 2017b). Furthermore, T plasma levels decreased gradually over consecutive reproductive seasons, concomitantly with increasing concentrations of 11-KT; these levels of 11-KT were two- to four-fold higher compared to captive fish and resembled those found in the wild during the reproductive season (Mandich et al., 2004; Zupa et al., 2017b). We conducted samplings at yearly intervals, thus it was not possible to monitor seasonal variations of steroids in relation to gonadal development. Even so, plasma levels of T and 11-KT were at the highest concentration in 3- and 4-yo males, which exhibited an analogous reproductive stage to males in the wild that were categorized as ripe, suggesting the correct progression of the gametogenic process. In addition, a prominent role in both sperm maturation and motility is played by progestins (Schulz et al., 2010; Tubbs and Thomas, 2008). In adult greater amberjack (Mandich et al., 2004), as well as in yellowtail kingfish (Poortenaar et al., 2001), low levels of 17,20β-P were found and were associated to a partial inability to reach full maturation, as evidenced by the lack of sperm hydration and the ability to obtain sperm by abdominal pressure, and the presence of milt of high density and low GSI (Zupa et al., 2017a). The generally low concentration of 17,20β-P in the plasma of F1 males seemingly reflected these previous results, and when lower GSI values and higher sperm density than those of wild fish were also considered, it may conceivably point out to a partial inability to produce expressible milt and also explain the absence of fertilization during the spawning events.

Due to the scarcity of spontaneous spawning in cultured greater

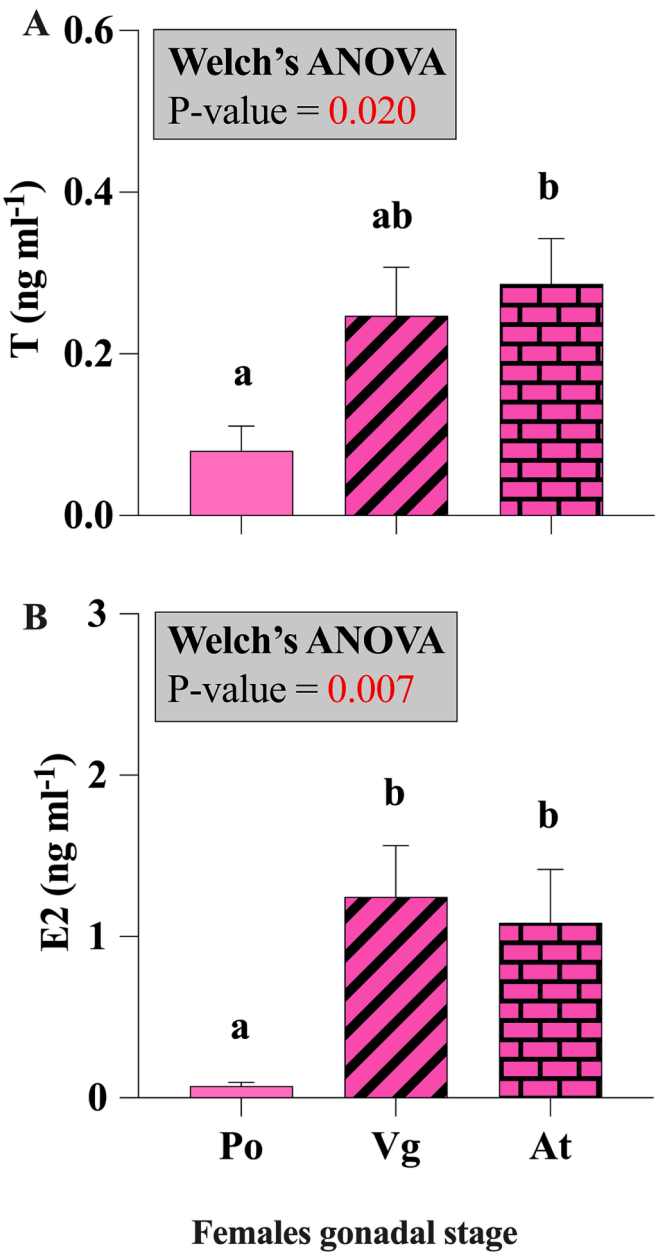

Fig. 8. Mean (±SEM) plasma concentration of the sex steroid hormone **A**. Testosterone (T), and **B**. 17β-estradiol (E2) of hatchery-produced female greater amberjack (3–5 years old) according to the stage of their ovaries (see Fig. 5), *i.e.* primary oocytes (Po), advanced vitellogenesis (Vg) or presence of extensive follicular atresia (At). Different lowercase letters indicate statistically significant differences (Welch's ANOVA, Dunnett's T3 HSD, $P < 0.05$).

amberjack (Jerez et al., 2006; Sarih et al., 2018) and the reported reproductive dysfunctions in this species (Corriero et al., 2021a; Corriero et al., 2021b; Pousis et al., 2018; Zupa et al., 2017b), the production of fertilized eggs still remains one of the main bottlenecks for the expansion of its culture. Given the extensive observations that greater amberjack fail to undergo oocyte maturation, ovulation, and spawning reliably in land-based tanks (Fakriadis et al., 2020a; Fakriadis et al., 2020b; Jerez et al., 2018; Sarih et al., 2018), in the present study 4- and 5-yo fish were induced to spawn following the established methodology for wild-caught, captive-reared individuals of this species. In captive-reared greater amberjack broodstocks, induction of spawning through GnRHa administration has been effective in increasing egg production (Fakriadis et al., 2019; Fakriadis et al., 2020a; Fakriadis et al., 2020b; Fernández-Palacios et al., 2015). Similarly, in F1 individuals from the Atlantic Ocean, GnRHa administration induced successfully multiple spawning over a prolonged period of time, using either sustained-release implants or multiple injections (Jerez et al., 2018; Sarih et al., 2018). In contrast, in the present study treating F1 breeders from the Mediterranean Sea with GnRHa implants was not successful, since only two spawns were obtained from probably only one 5-yo female in a period of 15 days, with no fertilized eggs, whereas no spawns at all were obtained from 4-yo fish. This dichotomy in results might be explained by a combination of different factors. The main goal of the present study was to identify the first age of maturation of hatchery-produced greater amberjack and evaluate their reproductive capacity. It is customary to define individuals as sexually mature using the presence of vitellogenic oocytes in the ovary as a criterion of spawning capability. This has also been done previously with greater amberjack (Marino et al., 1995), thus, the 4- and 5-yo F1 females examined in the present study were considered to have completed puberty. However, although the precise age of F1 breeders used from the Atlantic Ocean was not clearly defined, the authors indicated that greater amberjack induced to spawn in May 2015 were born between 2005 and 2009, which implies that the youngest fish was at least 6-yo (Jerez et al., 2018). In many teleost species, young individuals might undergo gonadal development, but eventually skip the reproductive season and reabsorb their Vg follicles, in order to redirect and reinvest the energy in somatic growth (Rideout and Tomkiewicz, 2011). Therefore, due to the extensive follicular atresia recorded in the ovary of 4-yo females, and the poor reproductive output (*i.e.* fecundity and fertilization) after the GnRHa therapy in 5-yo females, it seems doubtful that F1 generation Mediterranean greater amberjack can be induced to spawn successfully at this age. On the contrary, natural spawning was reported for 2-yo hatchery-produced F1 breeders produced and reared in Japan (Kawabe et al., 1996), as well as for 3-yo females in the Canary Islands (Sarih et al., 2018). This vast discrepancy in the age of maturity and spawning capability might result from the worldwide distribution of this species and the existence of distinct genetic populations (Gold and Richardson, 1998; Hasegawa et al., 2020; Šegvić-Bubić et al., 2016) adapted to different environmental conditions -varying between temperate and sub-tropical regions. Therefore, further investigation is necessary, in order to examine if the genetic differences that can be identified in stocks of greater amberjack inhabiting different geographical areas, such as the Mediterranean Sea and the Atlantic Ocean (Šegvić-Bubić et al., 2016), may correlate with the observed differences in reproductive function, already reported and proposed earlier (Corriero et al., 2021a).

5. Conclusions

The results obtained in the present study indicate that male hatchery-produced F1 greater amberjack mature as 3-yo as observed in wild males, albeit with smaller gonad size. On the contrary, females seem to mature at a later age than in the wild, also with a smaller gonad size. Although 5-yo females have a normal ovarian histological appearance based on ovarian biopsies, spawning in response to GnRHa treatment was not effective, contrary to what has been shown with wild-caught captive-reared broodstock, but also with hatchery-produced broodstocks from the Atlantic Ocean. More studies are necessary to evaluate (a) if reproductive function may improve with age, (b) if different hatchery-produced broodstocks may perform better reproductively and to establish an effective protocol for spawning induction of hatchery-produced greater amberjack from the Mediterranean Sea.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

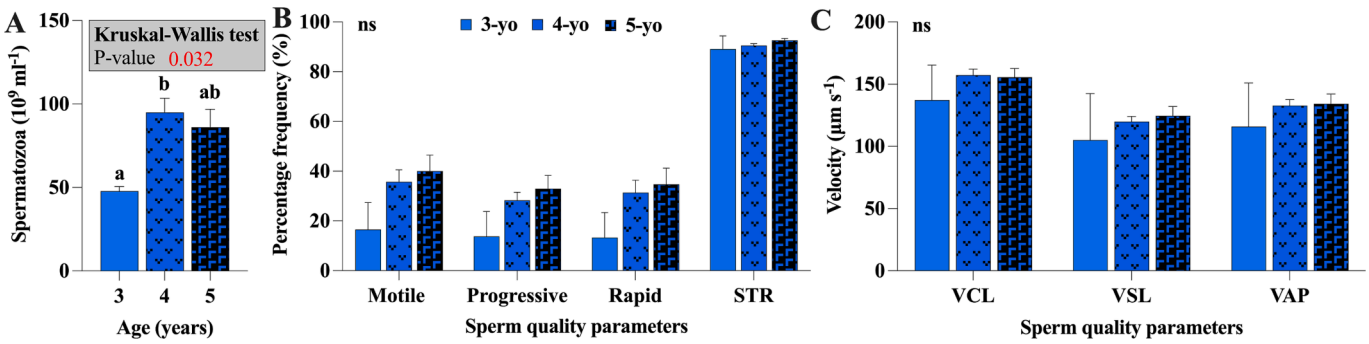

Fig. 9. Mean (±SEM) sperm quality parameters of hatchery-produced male greater amberjack (3–5 years old) reared in sea cages and sampled in June of each year (2020–2022, See Fig. 1). In 2021 and 2022, the sampling was done during the selection for spawning induction. **A.** Sperm density ($\times 10^9$ szoa ml^{-1}), **B.** Percentage (%) of motile cells, progressive cells, rapid cells and straightness (STR). **C.** Curvilinear (VCL, μm/sec), straight line (VSL, μm/sec) and average path velocity (VAP, μm/sec). Different lowercase letters indicate statistically significant differences (Kruskall-Wallis test, Dunn's HSD, $P < 0.05$). ns: not significant.

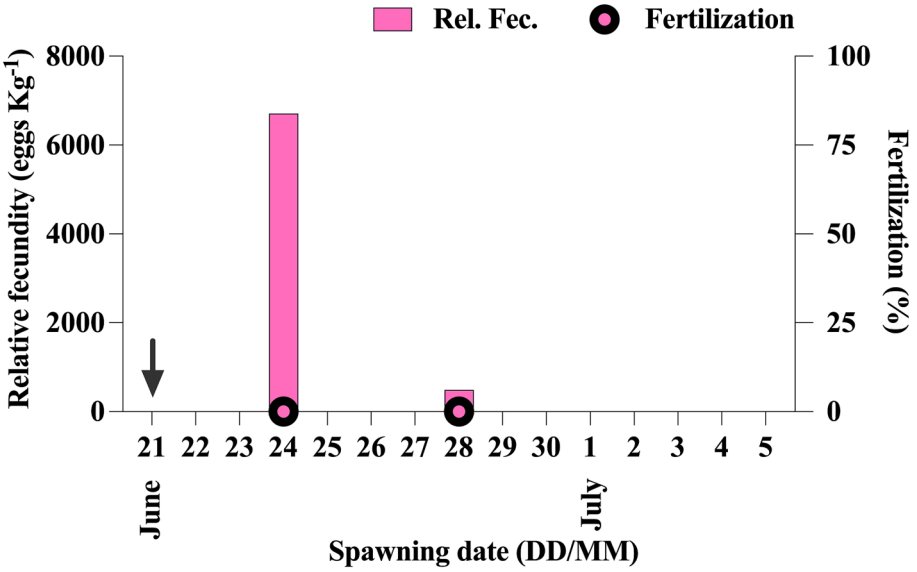

Fig. 10. Daily relative fecundity (bars, eggs kg^{-1} female) and fertilization success (circles, %) of 5-yo hatchery-produced greater amberjack induced to spawn with GnRHa implants in 2022. The black arrow indicates the time of GnRHa administration.

Data availability

Data will be made available on request.

Acknowledgements

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