Induction of sex inversion and feminization in the protandrous gilthead seabream (*Sparus aurata*) with 17β-estradiol implants, and its effects on egg production and quality

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ABSTRACT

The Mediterranean gilthead seabream (*Sparus aurata*) exhibits protandrous hermaphroditism, with all fish maturing first as functional 2-year-old males, and then 15–80% of these males invert to females prior to their second reproductive year, when 3 years old. For selective breeding programs, fish are selected to contribute to the next generation when they reach harvest size at 18–20 months of age (1+ years old, 400–600 g body weight, b.w.). At this time, all fish are males and cannot be predicted how many and which individuals will invert to females as 3-years old, complicating the management of selective breeding programs. Controlled-release 17β-Estradiol (E2) implants of 0.5 and 1 mg E2 kg^{-1} b.w., administered in three monthly treatments (July–September) induced 89–100% feminization of 2 + −year-old males, compared to spontaneous feminization of only 20–25% in Controls. The resulting 3-year-old E2-feminized females underwent vitellogenesis successfully during the reproductive season and spawned eggs with slightly lower mean relative fecundities (15,200–27,100 eggs day^{-1} kg^{-1} b.w.), but equal fertilization (81–90%) and larval survival (85–94%) compared to the Controls (21,000–32,400 day^{-1} kg^{-1} b.w., 83–85% and 93–97%, respectively). Furthermore, fecundity, fertilization and larval survival of the produced eggs from these E2-feminized females a year later (4-year-old females) were similar to the previous year, and again within the published range of values for the species. The study produced an efficient E2-induced feminization protocol for gilthead seabream, which may facilitate breeding selection programs by eliminating the uncertainty for sex inversion success, thus allowing the reproduction of any selected 3-year-old gilthead seabream as a female.

1. Introduction

Although the gilthead seabream (*Sparus aurata*) is ranked only 33rd among the most reared fish species worldwide (FAO, 2024; Mhalhel et al., 2023), it is the most produced marine species in the Mediterranean region (Pavlidis and Mylonas, 2011; FAO, 2024). It is also cultured in the Red Sea, the Persian Gulf, the Arabian Sea and the temperate regions of the Eastern Atlantic Ocean (Morocco, Spain and Portugal) (FAO, 2024). Its production is based increasingly on genetically improved stocks produced through selective breeding programs

(Robledo et al., 2018), and genetic gains in growth of 5–29% and in disease resistance of approximately 13% per generation have been achieved, with morphology, fillet yield and feed efficiency also being characters of interest (Allal et al., 2025; Basurco et al., 2011; Chavanne et al., 2016; Gjedrem et al., 2012; Gjedrem and Rye, 2018; Janssen et al., 2017; Mhalhel et al., 2023; Vandeputte et al., 2020).

The gilthead seabream is a protandrous hermaphrodite, with individuals functioning as males during their first reproductive maturity at 2 years of age, and transitioning to females one year later, with sex change being variable among different facilities, stocks and years,

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reportedly ranging between 15 and 80% of the population (Bruslé-Sicard and Fourcault, 1997; Mylonas et al., 2011; Sola et al., 2007; Zohar et al., 1978). Commercial broodstocks include fish from different age groups (from 2-year-old males to 8-year-old females) with periodical addition of younger males to maintain a sex ratio of 1 male:2 females, as older males tend to gradually undergo sex inversion (Jerez et al., 2012; Mylonas et al., 2011; Papadaki et al., 2024), and sex ratios of 1:4 (male: female) are not uncommon if the broodstock remains unmanaged (Jerez et al., 2012; Mylonas et al., 2011; Papadaki et al., 2024).

In order to speed up genetic gain through selective breeding, selected gilthead seabream individuals are usually crossed already during their first reproductive maturation, which for male gilthead seabream is at 2 years of age and for females at 3 years of age. Breeding values are assigned and fish are selected to contribute to the next generation when they reach harvest size at 18–20 months of age (1+ years old) at a body weight of 400–600 g. Fish selected to reproduce as males are placed immediately in broodstocks and reproduce as 2-year-old breeders. Fish selected to reproduce as females, on the other hand, are maintained separately for another year-and-a-half until they become 3 years old, and then only the sex-inverted females (15–80% of the population) are placed in the broodstock for selective breeding, while the non-inverted males are discarded, as it is not allowed to cross with females from the same population. A low percentage of spontaneous sex inversion means that only a small number of selected females would be available, resulting in a low number of families produced, hampering the selective breeding program. Consequently, a reliable method to ensure sex inversion and feminization of 100% of the 1 + −year-old individuals selected to reproduce as 3-year-old females, would facilitate the implementation of selective breeding programs in gilthead seabream.

Sex inversion in gilthead seabream is highly influenced by environmental factors, rearing conditions and social interactions (Bruslé-Sicard and Fourcault, 1997; Happe and Zohar, 1988; Holhorea et al., 2023; Ross, 1990; Wong et al., 2006). Sex steroid hormones are the main drivers of sex differentiation in fish and sex inversion in hermaphrodite species, with 17β-estradiol (E2) being the principal estrogen promoting ovarian development and sex change from male to female (Adolfi et al., 2023; Budd et al., 2015; Liu et al., 2017; Mondal et al., 2025; Piferrer, 2001; Todd et al., 2016; Wong et al., 2006). The application of E2 has been effective in inducing feminization in many aquaculture species, with the development of several protocols for both protandrous hermaphrodites (Banh et al., 2021; Fine-Idan et al., 2024) and gonochoristic species (Kabpha et al., 2023; Voorhees et al., 2023). Administration of exogenous E2 via dietary treatment in juvenile green sunfish (*Lepomis cyanellus*) at doses of 100–150 mg E2 kg^{-1} of diet, resulted in 100% feminization (Teal et al., 2023), while 84–86% feminization success has been reported in brown trout (*Salmo trutta*) at E2 doses of 20–30 mg kg^{-1}, respectively (Voorhees et al., 2023). Administration of exogenous E2 via intra-muscular administration of controlled-release implants is considered advantageous in adults, due to the lower amounts of hormone used relative to the amount used in the feed, and the possibility of targeting specific individuals for reproductive management (Passini et al., 2016). Induction of sex inversion and feminization through E2 implantation has been achieved in barramundi (*Lates calcarifer*) at doses of 0.5–1.5 mg E2 kg^{-1} body weight (b.w.) with female percentages reaching 80–85% (Fine-Idan et al., 2024), whereas 100% females were obtained with a dose of 0.5 mg E2 kg^{-1} in 3-year-old common snook (*Centropomus undecimalis*) (Passini et al., 2016).

A previous study in male gilthead seabream reported feminization following dietary or implant treatment (Happe and Zohar, 1988), while significant inhibition of testicular development together with higher proportion of ovarian tissue has been observed after administration through the feed (Condeça and Canario, 1999). The first study (Happe and Zohar, 1988) used 1 mg E2 kg^{-1} feed and implants of 3.6 mg E2 kg^{-1} b.w. resulting only in 89 and 56% feminization, respectively. The second study (Condeça and Canario, 1999) used only pre-pubertal fish (∼90 g b. w., 0+ year old) and administered E2 also in the feed, but the feminized

fish did not reach maturation, as their ovaries contained only primary oocytes. The objective of the present study was to develop a method for sex inversion and feminization of 2 + −year-old males and the production of 100% 3-year-old female gilthead seabream to be used in selective breeding programs, using E2-loaded controlled-release implants, using the lowest amount of exogenous E2 possible.

2. Materials and methods

A preliminary experiment was conducted to determine the optimal dose of E2 required to induce feminization, using 1+ year-old juvenile gilthead seabream, which are smaller and allow for more experimental groups, larger numbers of fish used, as well as lower total amount of hormone (Fig. 1). Then, the main feminization experiment was conducted using the target size fish, which were 2 + −year-old mature
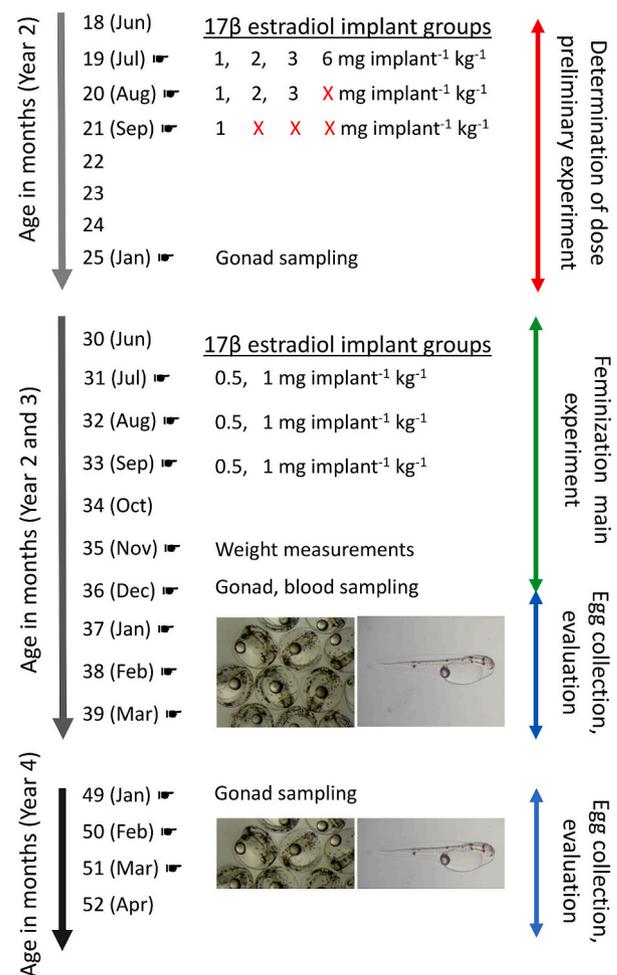

Fig. 1. Schematic representation of the experimental design. The age (in months) of the gilthead seabream used in shown on the left vertical axis (with the corresponding month in parentheses), during the three years of experimentation. The pointing hand indicates a treatment, sampling or other activity. On the right vertical axis, different colors indicate the two different E2-administration experiments (preliminary in red and main in green) and the two spawning and egg evaluation periods (in blue). In the two experiments, fish were given 3 monthly treatments of E2 implants of different doses between Jul-Sept, the feminization success was estimated at the beginning of the spawning period (Dec-Jan) and egg collection was carried out for a part of the spawning season (Dec-Mar). The red "X" indicates that no implantation was done at this time. The differences in the total number of implantations among treatment groups was due to the mortalities that occurred during the E2 treatment (see Fig. 2A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

males, examining both the percentage of fish feminized, as well as their reproductive performance (fecundity, fertilization, and embryo and larval survival) of the sex inverted females for two consecutive reproductive periods, when they were 3 and 4 years old (Fig. 1).

2.1. Ethical approval

The experiments were carried out at the AQUALABS facilities of the Hellenic Centre for Marine Research (HCMR), Crete, Greece, a registered facility for experimental animal maintenance and use (Registration No EL91-BIObr-03 and EL91-BIOexp-04). Ethical approval of the experiments was obtained from National Veterinary Services under permit number 255356 (APA: 6Λ4Σ7ΛΚ-ΩΜΥ). All procedures involving animals were conducted in accordance with the "Guidelines for the treatment of animals in behavioural research and teaching" (Anonymous, 1998), the "Ethical justification for the use and treatment of fish in research: An update" (Metcalfe and Craig, 2011), and the "Directive, 2010/63/EU of the European Parliament and of the Council of 22 September, 2010 on the protection of animals used for scientific purposes"(EU, 2010).

2.2. Implant preparation

Controlled-release implants were developed using a 15% solution of Ethylene-Vinyl Acetate polymer (EVAc) dissolved in MeCl2, mixed with inulin (Sigma, Germany) and E2 (Sigma, Germany). The solution was placed on ice to avoid evaporation, homogenised with a tissue grinder (RZR 2020, Heidolph, Germany) and subjected to ultrasound (UP200S, Dr. Hielscher GmbH, Germany) before being placed in an aluminium cast (50x50x4 mm) at −80 °C. The resulting solid plate was transferred to a − 80 °C freezer for 1 h and then to a − 20 °C freezer for 3 days for evaporation of the MeCl2. After that, it was placed in a vacuum desiccator at 25 °C for 2 days to remove any moisture. The 2 mm diameter implants obtained after punching with a Keyes dermal punch (Miltex GmbH, Germany) were stored at −20 °C until use. For the preparation of the implants, the amount of hormone per implant was calculated based on an average fish body weight of 200 g for the preliminary and 800 g for the main experiment, so that 1 implant would be administered to each fish. Thus, implants containing 0.2, 0.4, 0.6 and 1.2 mg E2 each were prepared for doses 1, 2, 3 and 6 mg kg^{-1}, respectively, for the preliminary experiment, while implants of 0.4 and 0.8 mg E2 each were prepared for doses 0.5 and 1 mg kg^{-1}, respectively, for the main experiment. The release of the implants was evaluated in vivo using 1 + −year-old gilthead seabream, and it was demonstrated that plasma E2 levels peaked on days 7 and 14, while on day 28 they were only slightly higher than before E2 implant administration (data not shown).

2.3. Determination of optimal E2 dose – preliminary trial

The experimental design included three monthly administrations of E2 implants applied to 1 + −year-old, juvenile gilthead seabream (198 ± 3 g b.w.) in 5 groups ($n = 18$), a Control and four E2 doses (1, 2, 3 and 6 mg kg^{-1}), in a preliminary trial to determine the optimal E2 treatment dose. The five groups were stocked in separate 2-m^3 tanks with a well-water supply (2000–2250% water renewal day^{-1}) under natural photoperiod and constant well-water temperature (19.7 ± 0.3 °C). Temperature, dissolved oxygen and pH were measured weekly. Fish were fed manually to apparent satiation with industrial feed (Plus 4.5 and 6 mm, IRIDA SA) three times weekly.

On day 0 (19/7/2022), E2 implants of different doses were administered intramuscularly (on the side of the fish, 3–4 scale rows below the dorsal fin) with a 12-G syringe, whereas control fish remained untouched. This procedure was to be repeated twice more with a single E2 implant administered every 28 days, based on the in vivo release evaluation of the implants (see Section 2.2.). Due to heavy mortality in the 6 mg E2 kg^{-1} group after the first implantation, this treatment was

discontinued. For the same reason, the 3 and 2 mg E2 kg^{-1} treatments were discontinued after the second implantation, and only the 1 mg E2 kg^{-1} treatment received all three planned implants, one every 28 days.

During the spawning period (10/1/2023), the feminization evaluation sampling took place. Abdominal pressure was applied to all fish in order to check for spermiation of the males in the population, and then all fish were sacrificed and examined for sex macroscopically. Gonads were extracted and fixed for histological examination.

2.4. Feminization using E2 implants – main experiment

Based on the results of the preliminary study above, 1 mg E2 kg^{-1} was selected as the optimal dose to induce feminization with a treatment duration of three months. A second dose of 0.5 mg E2 kg^{-1} was also administered, in order to examine the potential of a lower hormone usage and to minimize the mortalities associated with the E2 treatment.

In June 2023, 130 2 + −year-old, male gilthead seabream were maintained in outdoor tanks supplied with flow-through seawater from a well. Ten fish were sacrificed before the initiation of the experiment, and their livers were collected for the calculation of hepatosomatic index (HSI, [liver weight / body weight] x 100). Pieces of livers and gonads were fixed in 4% formalin: 1% glutaraldehyde solution until histological processing (McDowell and Trump, 1976), as described later.

On 25–26/7/2023, individually tagged fish with a Passive Integrated Transponder (PIT), were weighted (662 ± 7 g b.w.) and transferred to six 2-m^3 tanks ($n = 20$ per tank) with well-water supply (1600–2500% water renewal day^{-1}), under natural photoperiod and constant well-water temperature (19.4 ± 0.1 °C). Temperature, dissolved oxygen and pH were measured weekly. Six groups of fish were used to conduct the experiment: 2 Control groups (Control A and Control B, n = 20 each), 2 groups treated with the low E2 dose of 0.5 mg kg^{-1} (0.5 mg A and 0.5 mg B, n = 20 each) and 2 groups treated with the high E2 dose of 1 mg kg^{-1} (1 mg A and 1 mg B, n = 20 each). Intramuscular administration of E2 implants started on 31/7/2023 (Day 0) and continued until the completion of a total of three implantations (one every 28 days), after anaesthetizing the fish completely with clove oil (Mylonas et al., 2005), while fish from the Control groups were sham-implanted with an empty 12-G syringe.

During the experiment, fish were fed manually three times a week until apparent saturation with industrial feed (Vitalis Cal 9 mm, Skretting, Norway) from 1/7/2023 until 17/11/23. The feed was subsequently replaced by Ruby 8 mm (IRIDA SA, Greece) from 18/11/23 until 11/12/2023, in order to provide a fortified feed with additives that strengthen the immune system against the endoparasite *Enteromyxum leei* (Myxosporea, see the Results section).

Three months after the first implantation (1/11/2023), body weight was recorded in order to examine any treatment effect on growth. Blood samples were also collected from 5 randomly chosen individuals from each tank, in order to measure plasma sex steroid concentrations during the gametogenesis period. After centrifugation for 10 min at 3234 xg at 4 °C (Centrifuge 5804R, Eppendorf), the plasma was aliquoted and stored at -80 °C until processing.

On 11/12/2023, at the onset of the natural spawning period, all fish (now 3 years old) were examined for sex inversion and reproductive stage, and body weight was again recorded. Abdominal pressure was applied to all fish to check for spermiation of the males in the populations. Ovarian biopsies were obtained from all females using a plastic catheter (Pipelle de Cornier, Laboratoire CCD, France) inserted in the genital pore, by applying gentle suction and the collected biopsies were examined under the microscope. Blood samples were also collected from the same 5 fish per replicate treatment group sampled previously (1/11/2023) using heparinized syringes, and were stored on ice until separation of the plasma as above. After blood collection, these 5 fish, plus five more fish were sacrificed (n = 10 per treatment) for the collection of gonads and livers for histological analysis. Gonad and liver samples for histological examination were also collected from any mortalities that

occurred during the experiment.

2.5. Histological analysis

Fixed gonad and liver samples were dehydrated in a 70–96% ethanol series and embedded in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany). Successive 4 μm thick sections were obtained with a semi-automatic microtome (Leica RM 2245, Germany) and the histology slides were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA)(Bennett et al., 1976). The dried covered slides were examined under a light microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

To assess the effect of hormonal treatments on liver histology, three randomly selected tissue sections were examined for each fish, and all sections were included in the assessment. Fatty degeneration was scored using a four-point semi-quantitative system based on the proportion of hepatocytes affected [0 = absent (<5%), 1 = mild (5–25%), 2 = moderate (25–50%), 3 = severe (>50%)]. Scoring was performed by a trained histopathologist, without knowing the treatment group of each fish. Similarly, all mortalities that occurred during the experiment were also examined histologically; however, these samples were not included in the semi-quantitative analysis, because they were not collected immediately post-mortem, and tissue autolysis may have affected lesion interpretation. For this reason, only samples processed under controlled and equivalent conditions were used for scoring and group comparison of liver morphology.

The classification of the ovarian samples during the spawning period was based on the most developed oocyte stage observed in the biopsy and histological samples. Immature ovarian tissue consisting exclusively of primary oocytes (po) and oogonia was designated as "po". Ovaries with oocytes in vitellogenesis (Vg) were designated as "Vg" and ovaries with oocytes in early maturation or germinal vesicle breakdown were designated as "OM". Finally, ovulating females having ovulated oocytes or post-ovulatory follicles, POFs were designated as 'OV'.

2.6. Evaluation of reproductive/spawning performance

During the 11/12/2023 sampling, some females were selected from each treatment group/tank and placed in five 5-m^3 tanks ($n = 8$ per tank) to form spawning broodstocks. Two broodstocks were prepared from each of the 0.5 and 1 mg E2 groups. Since only 8 females in total were found in the two Control groups, only one spawning broodstock was prepared from Control fish. The selected females (based on ovarian biopsies) were fully vitellogenic, while those with immature ovaries were excluded. Some eggs were produced by the Control group B prior to this sampling, but no egg collection was conducted, since these experimental tanks could not be fitted with an egg collector.

The next day (12/12/2023), spermiating males from a 2-year-old stock were added to each tank ($n = 8$), following body weight recording (584 ± 12 g b.w.) and individual PIT tagging (AVID, Uckfield, East Sussex, UK). The five breeding groups were maintained under natural photoperiod with well seawater supply (700–760% water renewal d^{-1}) at a constant temperature (19.4 ± 0.1 °C). Fish were fed manually to apparent satiation with industrial feed (Ruby 8 mm, IRIDA SA) three times weekly. Temperature, dissolved oxygen and pH were measured weekly.

Spawning and egg production performance were monitored daily, from 13/12/2023 until 13/3/2024, even though the expected spawning season for gilthead seabream extends well into May. This was done since we were not interested in documenting the total annual egg production of the E2-treated females, but simply to confirm that spawning and egg production were similar to Control females. From a passive collector, positioned at the tank's surface outflow, eggs were collected every morning and were transferred into a 10-L bucket with a net. A 10-mL sample was then obtained and eggs were observed and counted under

a stereoscope to estimate fecundity and fertilization success. Fecundity was calculated as the total number of eggs collected, while fertilization success was determined by dividing the number of fertilized eggs by the total number of eggs in the 10-mL sample and multiplying by 100. Relative fecundity was expressed as fecundity kg^{-1} female body weight.

Egg and larval quality, including 1-day embryo survival, hatching success and 5-day larval survival, was evaluated through individual egg incubation in 96-well microtiter plates, based on the method of Panini et al. (2001). The quality assessment occurred in the mid-reproductive period (January) with three weekly measurements, using two replicates per spawning tank to confirm the viability and survival of eggs from E2-treated females. Survival to each developmental stage was calculated based on Mylonas et al. (2004), by dividing with the live embryos at the previous developmental stage.

Four females from the Control and the two E2-treated groups (0.5 mg and 1 mg E2) were maintained separately for an additional year, with males ($n = 4$ males) under the same culture conditions, in order to re-evaluate sex, egg production, and egg quality parameters of the 4-year-old sex reversed females. On 29/1/2025, during the natural spawning period, all fish were examined for sex and reproductive stage, and body weight was recorded. Ovarian biopsies were obtained from females as mentioned earlier (section 2.4) and spawning and egg production were monitored daily from 30/1/2025 until 26/3/2025. The quality assessment of eggs and larvae was conducted in mid-February and March with three weekly measurements, using two replicates per spawning tank. This was done to confirm once again that spawning, egg production, viability and survival of larvae from E2-treated females from one year ago were similar to Control. On the last sampling (26/3/2025) females were examined again for reproductive stage and body weight was again recorded.

2.7. Plasma sex steroid measurement

The extraction and analysis of plasma E2, testosterone (T) and 17α,20β-dixy- droxy-4-pregnen-3-one (17,20β-P) with liquid chromatography tandem mass spectrometry (LC-MS/MS) were performed according to Papadaki et al. (2021), with some modifications. For better quality control and more accurate quantification of the hormones, an internal standard of 13C-labeled E2, T, cortisol and progesterone (purity >98%) purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA) was used instead of N,N dimethyl-*L*-phenylalanine. A mixture of these four compounds at different concentrations (10 to 85 pg μL^{-1}) was prepared in methanol: water 1:1 and 10 μL of this solution were added to the plasma samples prior to solid phase extraction.

2.8. Statistical analysis

Mean HSI values among treated (0.5 and 1 mg E2) and Control groups before and after the completion of the E2 treatments were compared using one-way ANOVA followed by Tukey's test, whereas mean plasma sex steroid levels of E2-treated females (groups 0.5 and 1 mg E2) between the gametogenesis and spawning periods were examined using repeated measures (RM) two-way ANOVA (time, E2 dose) followed by Tukey-adjusted estimated marginal means (emmeans) post-hoc test. Due to the almost complete lack of females in the Control groups ($n = 1$) collected for blood analysis, only females from the E2-treated groups (0.5 and 1 mg E2) were included in the sex steroid hormone data analysis. Differences in mean daily relative fecundity and fertilization percentage among treatment groups (Control, 0.5 mg and 1 mg E2) over time for the two spawning seasons monitored (3 and 4-year-old females) were examined using two-way ANOVA followed by Tukey's test. Mean body weight of 3 and 4-year-old females, and mean 1-day embryo survival, hatching success and 5-day larval survival among treatment groups of 3-year-old females were tested using RM two-way ANOVA, followed by Tukey-adjusted emmeans post-hoc test. Prior to applying parametric tests (ANOVA), the assumptions of normality and

homogeneity of variances were tested using Shapiro-Wilk and Levene's tests (car package, Fox and Weisberg, 2019), respectively, and Mauchly's test (ez package, Lawrence, 2025) was used for RM two-way ANOVA sphericity test when needed. In case of violation of the assumptions of normality or homogeneity of variance, appropriate data transformations, including logarithmic or arcsine square root transformations, were applied prior to conducting parametric analyses. Results are expressed as means ± standard error of the mean (SEM) unless reported otherwise. The significance threshold for all statistical tests was set at $p \leq 0.05$ and analyses were conducted using R (version 4.3.1, R core Team, 2022) within the RStudio statistical software (Rstudio Team, 2022).

3. Results

3.1. Determination of optimal E2 dose – preliminary trial

Administration of E2 implants to 1+ year-old, juvenile gilthead seabream resulted in mortalities in the 2, 3 and 6 mg E2 kg^{-1} treatment groups, already beginning after the first E2 implantation and reaching a total of 42, 61 and 56%, respectively (Fig. 2A). Due to the heavy mortalities, the 6 mg E2 kg^{-1} treatment was discontinued after the first implantation, while the 3 and 2 mg E2 kg^{-1} treatments were discontinued after the second implantation. The mortality resulting from the lower E2 dose of 1 mg E2 kg^{-1} was only 6% after three monthly implant administrations.

Regarding feminization success, at the onset of the spawning period (10/1/2023) when the fish were 2 years old and would be naturally mature males, the 1 mg E2 kg^{-1} treatment achieved 88% feminization after three implant administrations (Fig. 3A). Lower feminization of 55% and 57% was achieved in the 2 and 3 mg E2 kg^{-1} treatments, respectively, while no females were identified in the 6 mg E2 kg^{-1} treatment or in the Control group. One individual was not assigned a sex, because it did not produce sperm during the sperm assessment and no gonadal tissue was found in the histological sample, most likely due to a sampling error (Sex not identified, Fig. 3A). The feminized females had gonads with immature ovarian tissue consisting either exclusively of primary oocytes (po), or mainly po and some Vg oocytes (data not shown). In all female gonads, a limited portion of immature testicular tissue consisting exclusively of spermatogonia was detected in the periphery of the gonad. The males, as expected for 2-year-old gilthead seabream, had mature testes with spermatozoa, releasing various amounts of sperm upon application of gentle abdominal pressure (data not shown).

3.2. Feminization using E2 implants – main experiment

All 2 + −year-old fish grew significantly during the study from a mean body weight of 662 ± 7 g in July to 876 ± 15 g in December (data not shown), without any differences among treatment groups (RM two-way ANOVA, Tukey HSD, $p < 0.001$). While no mortalities were observed in the Control or the 0.5 mg groups, 5% and 0% of the replicated populations treated with 1 mg E2 died 14 days after the second implantation while 5% and 10% of the 1 mg E2 treated fish died 11 days after the third implantation (Fig. 2B). Fifteen days after the completion of the E2 treatments (90 days after first E2 implantation), some additional mortalities occurred in the Control A group (10%) and in the 1 mg B group (15%), caused by the endoparasite *Enteromyxum leei* (Myxosporea), responsible for enteritis and weight loss in affected fish, unrelated to the E2 treatment (Fig. 2B). The sex of these fish was evaluated and was included in the final results, with the exception of one female that was not included in the stage of maturation data (Fig. 7), because it died well before the sampling for maturation stage in December.

Mean HSI of the 1 mg E2-treated fish that died during the experiment was 2.8 ± 0.2% and was higher than the corresponding HSI of fish sacrificed before the onset of the E2 treatment or at during the spawning period, regardless of treatment (one-way ANOVA, Tukey HSD, p < 0.001, Fig. 4). Although the HSI of the E2-treated groups at the spawning period showed a trend of slightly higher mean values than Controls at both sampling times, no statistically significant differences were detected.

Fish sampled prior to the beginning of the experiment and Control fish sampled at the spawning period exhibited extensive lipoid degeneration (Fig. 5A), while E2-treated fish exhibited normal liver morphology with minimal or no fat accumulation (Fig. 5B). Specifically, the livers of all non-treated fish at all times were characterized by fatty degeneration, which was severe in the majority of the cases (80%). In contrast, the livers of the E2-treated groups showed only mild to moderate fatty degeneration. A Kruskal–Wallis test revealed a statistically significant difference among the treatment groups, **H(3) = 20.85, P < 0.001**, indicating that hepatic lipid accumulation was strongly reduced by the E2 treatment (Data not shown). Histological examination of liver samples from deceased fish in the E2-treated groups revealed relatively preserved liver morphology, with mild to moderate lipid infiltration. However, many hepatocytes in these fish contained eosinophilic hyaline intracytoplasmic inclusions, which may indicate different pathological processes including drug toxicity (Fig. 5C and D).

At the onset of the spawning period, 100 and 95% of the replicated populations treated with 0.5 mg E2 were females, while 100 and 89% of
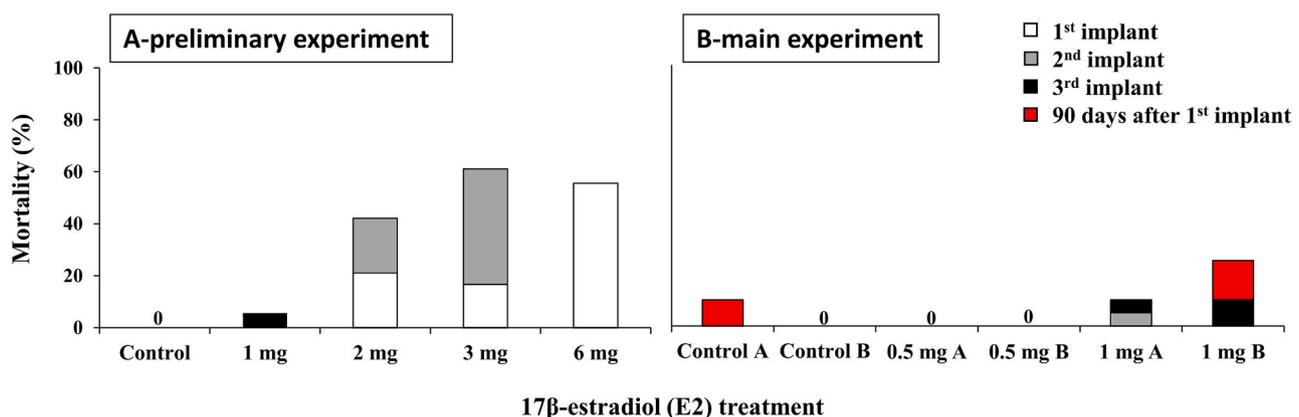

Fig. 2. A. Mortality of 1 + −year-old, juvenile gilthead seabream after each E2 implant (given every 28 days) of different doses during the determination of optimal E2 dose - preliminary experiment ($n = 1$ tank per treatment). Due to the heavy mortality in the 6 mg E2 kg^{-1} group after the 1st implantation, this dose was discontinued. Similarly, the 3 and 2 mg E2 kg^{-1} doses were discontinued after the 2nd implantation, and only the 1 mg E2 kg^{-1} treatment received all three planned implants. **B.** Mortality of 2 + −year-old, male gilthead seabream ($n = 2$ tanks per treatment) after each E2 implant (given every 28 days) of different doses, and 90 days into the experiment due to a parasite infection, during the feminization using E2 implants – main experiment. Both replicates are shown.
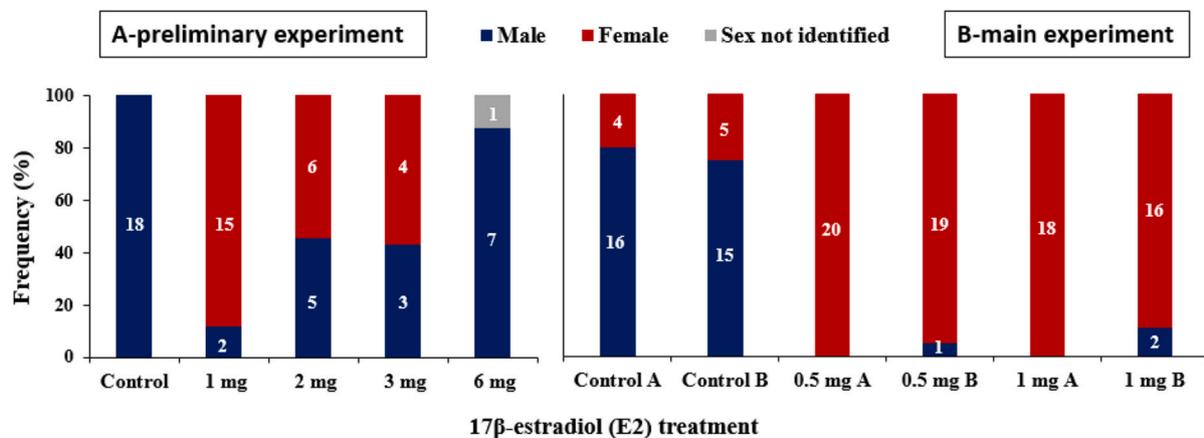
Fig. 3. A. Relative occurrence of male and female 2-year-old gilthead seabream after E2 treatment (n = 1 tank per treatment, 18 fish initially in each tank) at the spawning period sampling (10/1/2023) of the determination of optimal E2 dose - preliminary experiment (See Fig. 1). Sex identification was done by macroscopic and histological evaluation of the excised gonads. The numbers inside the bars indicate the individuals of each sex at the sampling time. The differences in the total number of individuals among treatment groups was due to the mortalities that occurred during the E2 treatment (see Fig. 2A). **B.** Relative occurrence of male and female 3-year-old gilthead seabream after E2 treatment (n = 2 tanks per treatment, 20 fish initially in each tank) at the spawning period sampling (11/12/2023) during the feminization using E2 implants – main experiment (See Figs. 1 and 2B). Both replicates are shown.
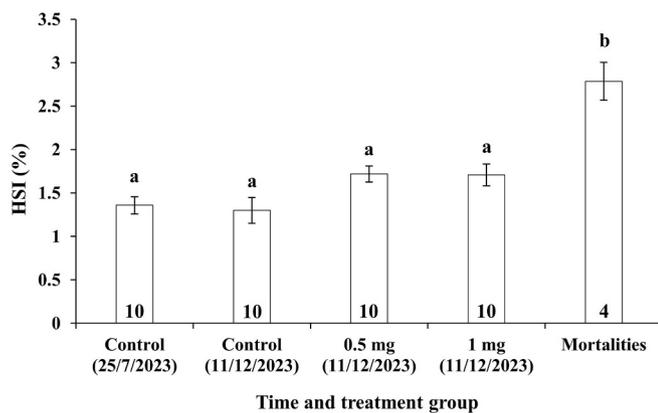

Fig. 4. Mean (±SEM) hepatosomatic index (HIS) of the Control group at the initial (25/7/2023) and final (11/12/2023) samplings, of the two E2-treated groups (0.5 mg and 1 mg E2) at the final sampling and of the mortalities that occurred in the 1 mg E2 group from the E2 treatment. Numbers inside the bars indicate the number of samples contained for each mean. Letter superscripts indicate statistically significant differences among groups (one-way ANOVA, Tukey HSD, $p < 0.001$).

the replicated populations treated with 1 mg E2 were females (Fig. 3B). The corresponding values in the replicated Control groups were only 20 and 25%. All males identified in both E2-treated and Control groups were found to be spermiating (data not shown).

Histological examination of the initial Control samples in July and of mortalities resulting from the E2 treatment revealed bisexual gonads, with predominantly ovarian tissue, consisting of po, and with the testicular tissue located peripherally and characterized by spermatogonia (Sg) (Fig. 6A). In females at the spawning period sampling, histological analysis of ovarian biopsies revealed gonads with po, Vg oocytes (Fig. 6B) or oocytes in oocyte maturation (OM, Fig. 6C), and ovulated (OV) eggs, with some follicular atresia in the gonads. Females (ovaries) from the three treatment groups were divided into four categories (po, Vg, OM, OV), according to the most advanced reproductive development stage (Fig. 7). Notably, a higher number of Vg females were observed in the 0.5 and 1 mg E2 treatment groups (Fig. 7), in contrast to the Control group where most females were classified as OM or OV. In addition, increased atresia was observed in histology samples of females in the E2-treated groups compared to Control, which was

particularly evident in the ovaries classified as Vg (not shown). Male gonads had mainly free spermatozoa (Sz) while testicular tissue contained also some spermatogonia (Sg) and spermatocytes (Sc) (Fig. 6D). All testes were found to retain immature ovarian tissue on the dorsal side, confined around the central cavity consisting of few layers of po and oogonia (not shown).

There was a significant increase in the concentrations of E2 and T in E2-treated females in the spawning period, with T levels also being higher in 0.5 mg than in 1 mg E2-treated females (RM two-way ANOVA, Tukey HSD, $p < 0.05$, Fig. 8A and B). In addition, higher levels of 17,20b-P were found in the 0.5 mg E2 treatment group in both sampling times (RM two-way ANOVA, Tukey HSD, $P = 0.025$, Fig. 8C).

3.3. Egg production and quality from feminized 3-year old females

Egg collection begun 1 day after the establishment of the spawning stocks for the Control tank (13/12/2023) and 2 days later (14/12/2023) for the 0.5 and 1 mg E2 broodstocks (Fig. 9). As mentioned earlier (Section 2.5) some fertilized eggs were found in one of the Control replicates already a few weeks before the establishment of the spawning stocks (data not collected), but since the tanks at that time were not fitted with egg collectors, daily fecundity and fertilization success could not be determined.

Mean daily relative fecundity of the Control group during the monitoring period was $32 \pm 1.7 \times 10^3$ eggs kg^{-1} female b.w. and the fertilization success ranged between 28 and 100% with a mean value of $85 \pm 1.1\%$. Concomitantly, mean daily relative fecundity of the E2-treated broodstocks ranged between $15 \pm 1.1 \times 10^3$ and $27 \pm 1.4 \times 10^3$ eggs kg^{-1} b.w., with mean fertilization ranging between $81 \pm 1.2\%$ and $90 \pm 1.2\%$, varying between 35 and 100% during the monitoring period. Higher mean daily fecundities were recorded in December and January, with lower values in March, with the Control broodstock exhibiting slightly, but significantly higher values than both of the E2-treated broodstocks during the monitoring period (two-way ANOVA, Tukey's HSD, $p < 0.001$, Fig. 10A). Conversely, mean fertilization displayed the opposite trend, with the lowest values occurring in December and the highest ones in February and March (two-way ANOVA, Tukey's HSD, $P = 0.014$, Fig. 10B) without any difference among groups. Furthermore, there were no significant differences among treatment groups in 1-day embryo survival, hatching success or 5-day larval survival, with the latter being significantly lower than the other two embryo development parameters (RM two-way ANOVA, Tukey's HSD, $P =$
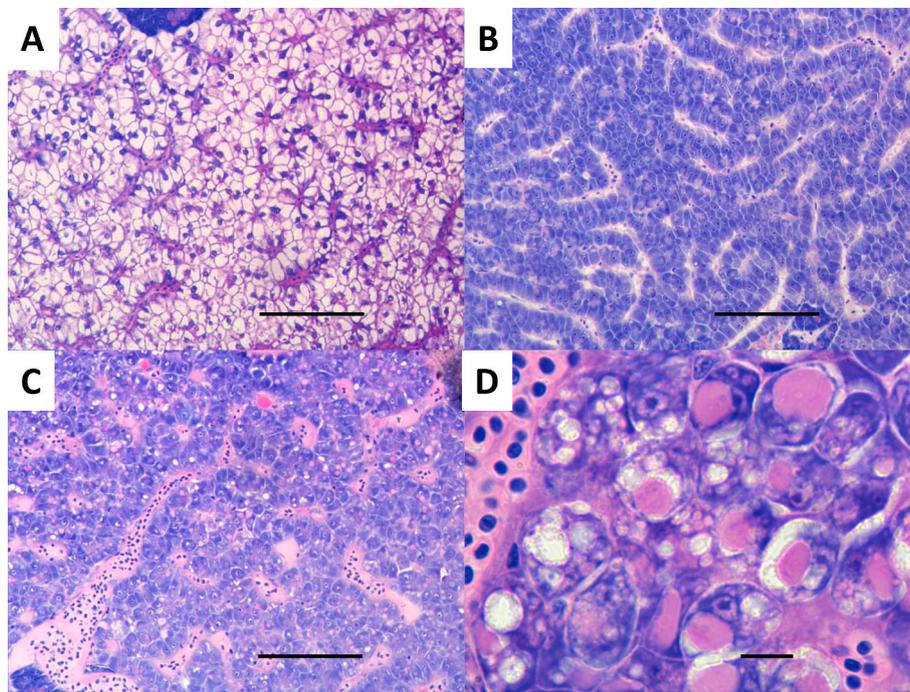
Fig. 5. A. Representative liver section from Control fish at the initial (25/7/2023) and spawning period sampling (11/12/2023) of the E2-feminization experiment. There was extensive lipoid degeneration characterized by clearly demarcated, fatty vacuoles in the cytoplasm of hepatocytes pushing the round nuclei externally. **B.** Liver section from a fish treated with E2, sampled on 11/12/2023. Hepatocytes exhibit a normal morphology, with no evidence of fat accumulation. **C.** Liver section from a mortality during the E2 treatment (1 mg E2). Hepatocytes retain a relatively normal appearance with minimal lipid vacuolization. However, many hepatocytes contain eosinophilic hyaline intracytoplasmic inclusions, visible in higher magnification (**D**). Scale bars: 100 μm in panels A, B, and C, and 20 μm in panel D.
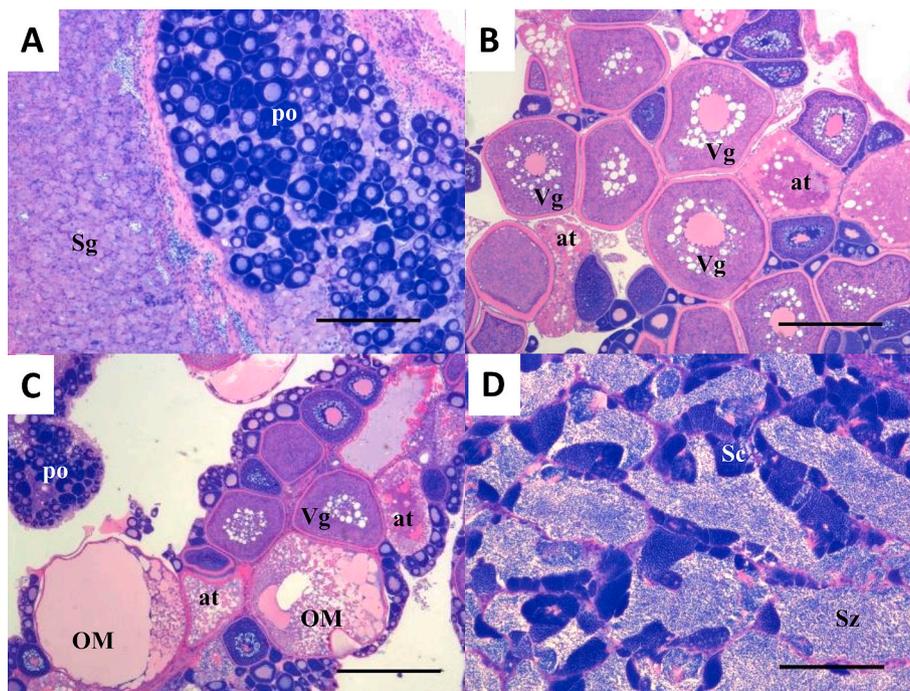

Fig. 6. Representative microphotographs of histological sections of gonads from 2 + −year-old gilthead seabream. **A.** At the beginning of the E2 feminization experiment (initial sampling, 25/7/2023), where both immature male tissue with spermatogonia (sg) and immature female tissue with primary oocytes (po) could be found in the gonad. **B and C.** Three-year-old female gonads at the spawning period sampling (11/12/2023) containing vitellogenic oocytes (Vg) and oocytes at maturation (OM) and atretic oocytes (at). **D.** Mature 3-year-old male at the spawning period sampling (11/12/2023) with spermatocytes (sc) and spermatozoa (Sz). The developmental stages of photographs B, C and D were found in all E2-treated and Control groups (See Fig. 7). Scale bars: 200 μm in panels A and D and 500 μm in panels B and C.
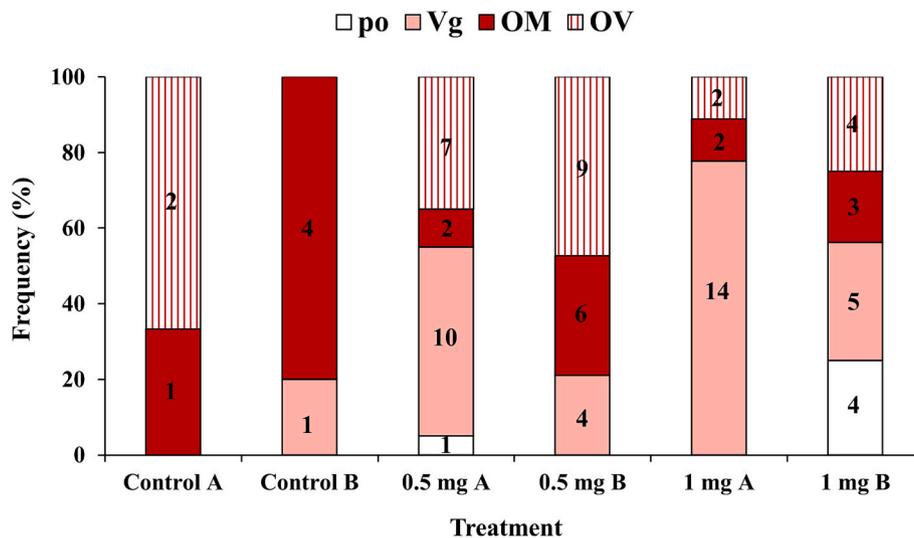
0.005, Fig. 11).

Fig. 7. Percentage of female gonadal maturation stages of 3-year-old gilthead seabream sampled at the spawning period sampling (11/12/2023), in response to E2 treatment (n = 2 tanks per treatment, 20 fish initially in each tank) at two doses (0.5 and 1.0 mg E2). Classification was performed after histological examination of gonadal biopsies (see Fig. 6). The four categories were: immature ovaries with primary oocytes (po), vitellogenic oocytes (Vg), oocytes undergoing maturation (OM) and ovulated eggs (OV). Numbers inside the columns indicate the females classified in each gonadal category at the sampling time. Both replicates are shown.
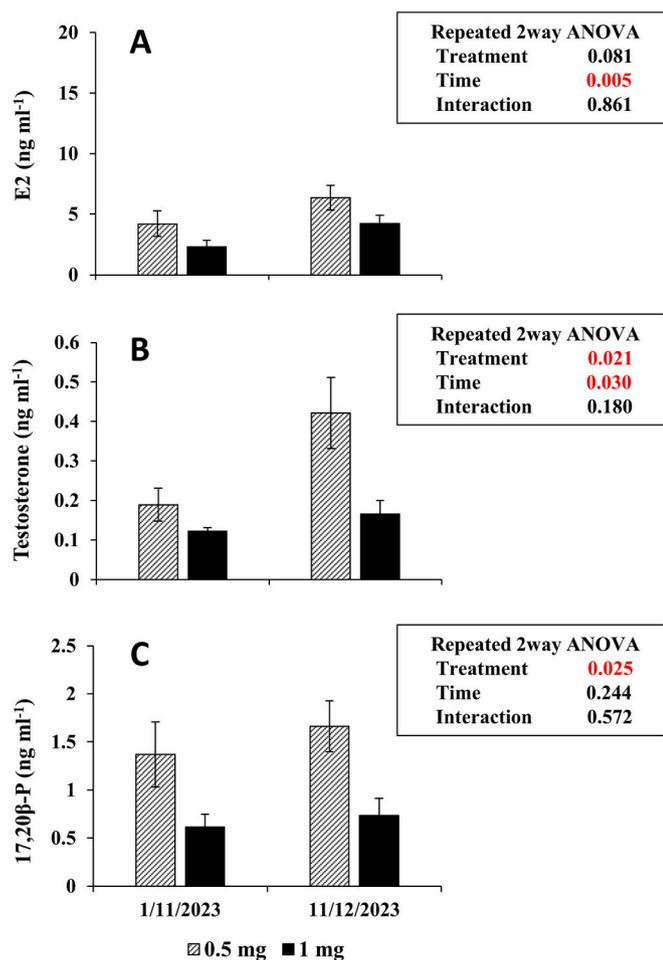

Fig. 8. Mean (± SEM) plasma hormone levels in response to E2 treatment, of female gilthead seabream ($n = 9$ fish in group 0.5 mg, $n = 10$ fish in group 1 mg) at the period of gametogenesis (1/11/2023) and spawning (11/12/2023). **A.** 17β-estradiol (E2). **B.** Testosterone (T). **C.** 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), repeated measures two-way ANOVA, Tukey's HSD, $P < 0.05$.

3.4. Egg production and quality from feminized 4-year old females

In the following year de Contreras-García et al. (2025), all 4-year-old fish that had undergone sex inversion through E2 treatment (both 0.5 and 1.0 mg E2) remained females. At the time of sampling during the spawning season (29/1/2025), ovaries of all females contained oocytes in maturation (OM) and ovulated eggs (OV) (data not shown). During the spawning monitoring season (29/1/2025 to 26/3/2025), all groups exhibited a significant reduction in body weight (RM two-way ANOVA, Tukey HSD, $P < 0.001$, Table 1), while no differences were detected among treatment groups ($P = 0.085$). The mean daily relative fecundity was higher in February compared to March, while the Control group produced again slightly, yet significantly more eggs than the 0.5 mg E2 group, but not the 1.0 mg E2 one (two-way ANOVA, Tukey's HSD, $P < 0.05$, Table. 1). In contrast, mean fertilization did not differ significantly among treatments or time (two-way ANOVA, Tukey's HSD, $P > 0.05$). Finally, no significant differences were detected among treatments in 1-day embryo survival, hatching success or 5-day larval survival, with the latter being significantly lower than hatching (RM two-way ANOVA, Tukey HSD, $P = 0.045$).

4. Discussion

Controlled-release E2 implants at a low dose of 0.5 mg E2 kg^{-1} b.w. induced almost 100% feminization of $2 + -$year old males, compared to spontaneous feminization of only 20–25% in Controls. The resulting 3-year-old, E2-feminized females underwent vitellogenesis successfully and spawned eggs with slightly lower mean fecundities, but equal fertilization and larval survival compared to the Controls. The study produced an efficient E2-induced feminization protocol for gilthead seabream, which may facilitate breeding selection programs by eliminating the uncertainty of sex inversion and feminization success in 3-year-old gilthead seabream. The developed protocol can be tested immediately in the gilthead seabream aquaculture industry, as it was already employed in farm conditions with similar results at the same time (CC Mylonas, unpublished data).

Treating $1 + -$year-old juvenile gilthead seabream prior to the gametogenesis period in July–September with 0.5–1 mg E2 kg^{-1} in controlled-release implants (3 times every 28 days) induced almost 100% feminization. These doses are significantly lower that what has been used in some other fishes (Banh et al., 2021; Fine-Idan et al., 2024;
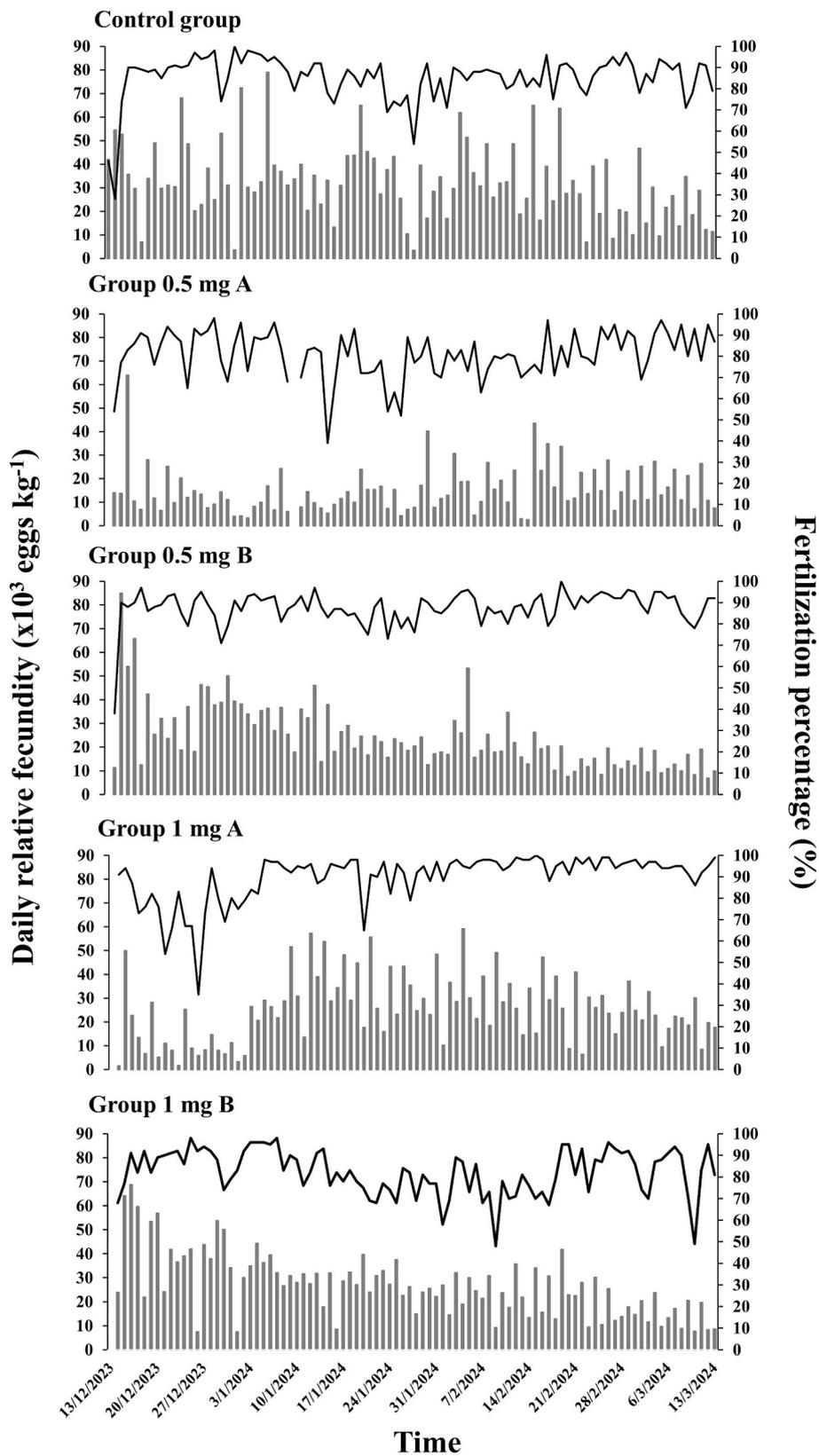
Fig. 9. Daily relative fecundity (x10³ eggs kg⁻¹ female body weight, grey bars) and fertilization success (%, black line) of the five spawning groups of gilthead seabream prepared with the 3-year-old females of the Control and the E2-treated groups (0.5 mg and 1 mg).
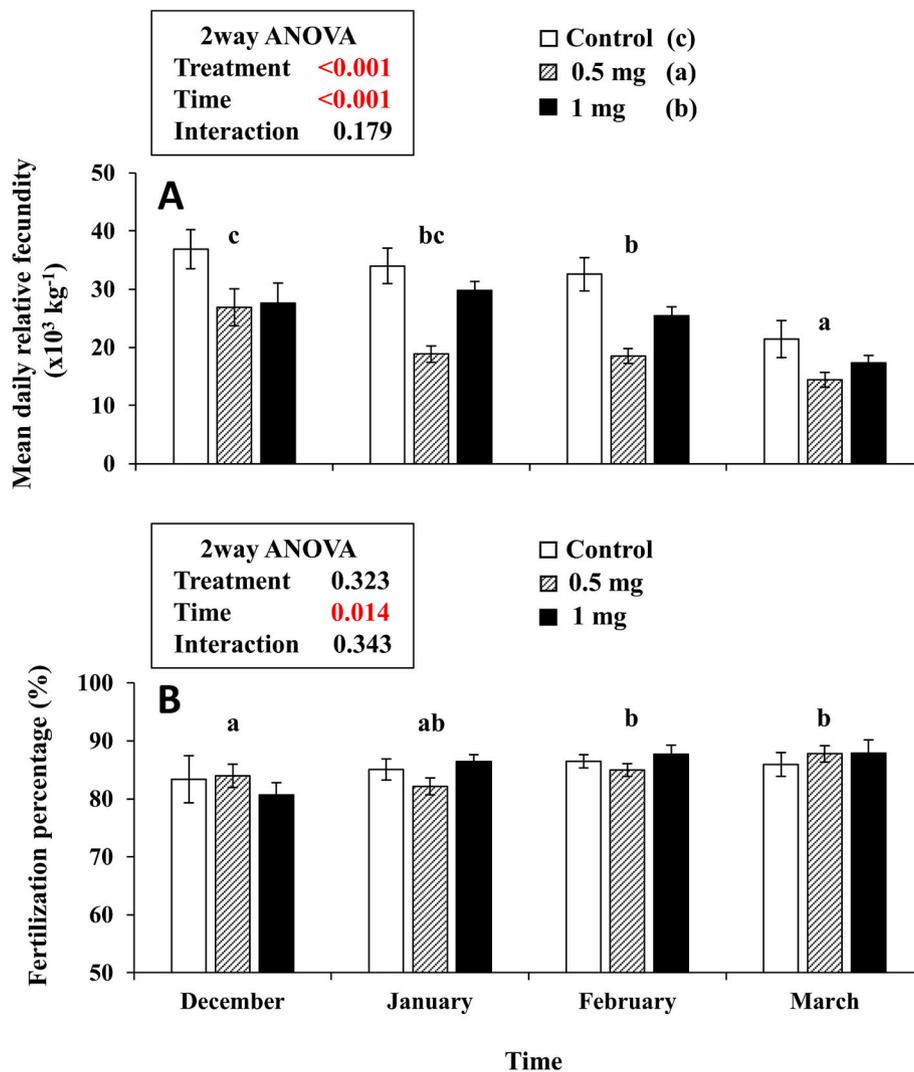
Fig. 10. Mean (± SEM) **A.** Daily relative fecundity (×10³ eggs kg⁻¹ female body weight) and **B.** Fertilization (%) of gilthead seabream spawning groups prepared with the 3-year-old females of the Control and the E2-treated groups (0.5 mg and 1 mg). Letter superscripts above the bars indicate statistically significant differences among months and superscripts beside the legend show significant differences among treatments (when they existed) (repeated measures two-way ANOVA, Tukey's HSD, P < 0.05).

Guppy et al., 2022; Jaime et al., 2023; Passini et al., 2016; Voorhees et al., 2023), as well as in gilthead seabream where 3.6 mg E2 kg⁻¹ resulted in only 56% feminization (Happe and Zohar, 1988). Hormonal treatments for sex inversion have been used recently in a number of studies, involving both protandrous (Banh et al., 2021; Contreras-García et al., 2025; Fine-Idan et al., 2024; Jaime et al., 2023; Passini et al., 2016) and protogynous hermaphroditic fishes (Kobayashi et al., 2021; Nakamura et al., 2003; Park and Im, 2011; Sarter et al., 2006), as well as gonochoristic species (Abdollahpour et al., 2024; Li et al., 2019; Teal et al., 2024, Teal et al., 2023). The success and extend of the sex inversion induction depends on different factors, such as the sex steroid used, the dose and administration method, the age/size of the fish, and the timing and duration of application (Mondal et al., 2025). Preferably, hormonal treatments should be administered during the non-reproductive period, when gonads retain their sexual plasticity, in the case of gilthead seabream during the period of late summer – early autumn (Condeça and Canario, 1999; Mylonas et al., 2011). In the present study, the monthly application (3×, July–September) of E2 implants was successful when given in the non-reproductive period (Mylonas et al., 2011), being completed just prior to the natural onset of gametogenesis, both in 2 + −year-old previously male gilthead seabream and in 1 + −year-old juveniles expected to become males. These

results point to the high plasticity of the gilthead seabream gonad during the non-reproductive period, when testes convert transiently to bisexual gonads (Mylonas et al., 2011), in anticipation of the final decision of what sex to develop, which depends both on body size and the social structure of the population (Bruslé-Sicard and Fourcault, 1997; Happe and Zohar, 1988; Holhorea et al., 2023; Ross, 1990; Wong et al., 2006).

When using sex steroid hormones, one must be aware of possible toxic effects. Increased mortalities were observed 11–15 days after the first implant administration of juvenile gilthead seabream at the doses of 2, 3 and 6 mg kg⁻¹. This mortality period coincides with the maximum release of E2 from the EVAc implants in the blood (7–14 days after treatment, data not shown). Additionally, the increased mortality observed in the 2 and 3 mg E2 kg⁻¹ groups after the second implant administration suggests a cumulative effect of the exogenous hormone. In the main experiment, however, where significantly lower E2 doses were used, mortalities attributed to E2 treatment were decreased (1 mg E2) or eliminated (0.5 mg E2), suggesting that toxic effects were eliminated as well. Similarly, implant administration of 4 and 8 mg E2 kg⁻¹ in common snook resulted in mortalities of 43% and 100%, respectively, with liver tissue damage confirmed through histological analysis, whereas no mortality or toxicity were recorded at lower doses of 0.5 and 1 mg kg⁻¹ (Passini et al., 2016). Nonetheless, the induction of toxicity at
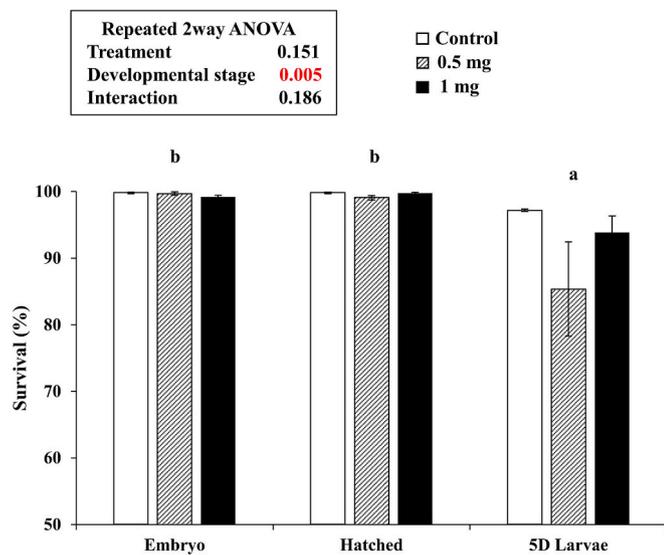
Fig. 11. Mean (± SEM) embryonic and larval survival (%) of eggs produced by 3-year-old gilthead seabream females of the Control and the E2-treated groups (0.5 mg and 1 mg). There were no statistically significant differences among treatments, while differences among the three developmental stages are indicated by different letter superscripts (repeated measures two-way ANOVA, Tukey's HSD, $P < 0.05$).

elevated E2 concentrations depends on the species, the treatment administration method and the rate of E2 release into the bloodstream (Akhavan et al., 2015; Voorhees et al., 2023). Steroid hormones are known to be metabolized in the liver and, in general, exogenous steroids are eliminated within a few days of administration (Piferrer, 2001). However, elevated estrogen concentrations can adversely affect fish survival, as they have been linked to liver toxicity and tissue damage (Elias et al., 2007; Moncaut et al., 2003; Piferrer, 2001; Weber et al., 2003; Zaroogian et al., 2001). A significantly higher HSI was observed in mortalities during the main experiment, with fish from the 1 mg E2 group exhibiting HSI ~2× higher than fish prior to the treatment, in agreement with results in common snook, where HSI was similar among treated groups, but increased dramatically in the mortalities caused by the E2 treatment (Passini et al., 2016). However, in the present study we cannot be sure that the increase in liver size was solely -or at all- due to the E2 treatment and was not the result of dying, since dead fish were collected some time (hrs) after death, whereas samples from the other experimental fish were collected soon after sacrificing the animals.

Histological analysis of gilthead seabream livers before and after E2 treatment showed clear differences between Control and E2-treated fish months after the E2 treatments, and during the spawning period. Control

fish displayed extensive lipoid degeneration, with fatty vacuoles present in hepatocytes, similar to what was observed in the 2 + −year-old males prior to the E2 treatments at the beginning of the experiment. This finding is consistent with earlier studies showing that lipid accumulation is a common response to various stressors in fish, particularly under aquaculture conditions (Maradonna et al., 2015; Naour et al., 2017). In contrast, E2-treated fish had preserved liver structure with significantly less lipid infiltration, suggesting that E2 may play a role in regulating hepatic lipid metabolism. The effects of E2 on liver lipid content in fish appear to depend on factors such as the season and the reproductive stage, with both lipid accumulation and depletion reported under different conditions (De Vlaming et al., 1977b, De Vlaming et al., 1977a). In humans, E2 affects the Toll-like receptor 4 (TLR4) signaling pathway, which plays a role in protecting the liver from injury. Activation of TLR4 triggers the release of pro-inflammatory cytokines and chemokines, worsening liver inflammation and damage. Estradiol reduces TLR4 expression, oxidative stress, and the production of pro-inflammatory cytokines, which helps protect the liver (Khaksari et al., 2024). However, since TLR4 is absent in teleost fish, E2 may act through different mechanisms in these species (Palti, 2011). Exposure of fish to E2 can increase reactive oxygen species (ROS) production and cause DNA damage in the liver (Thilagam et al., 2010). Further research is needed to understand better the potential impact of E2 treatment on fish health, during exposure and afterwards.

In terms of feminization success, our study suggested a more significant role of the duration of the E2 treatment -via the three sequential implants administered- than the E2 dose per se. The dose of 1 mg E2 kg^{-1} implants for 12 weeks induced the highest feminization percentage in juvenile gilthead seabream, followed by the 2 and 3 mg E2 kg^{-1} groups, that were treated for only 8 weeks (2 implants). No feminized females were observed in the 6 mg E2 kg^{-1} group that was treated for only 4 weeks (1 implant). In the main experiment with 2 + −year-old males, where both groups completed their three-implant treatments, feminization reached 100% in one of the two replicates of each treatment group, and only one and two (out of 20) non-inverted males were found in groups 0.5 and 1 mg E2, respectively. Similar results have been reported in several studies using E2 implants (Fine-Idan et al., 2024; Passini et al., 2016) or dietary treatment with E2 (Kabpha et al., 2023; Teal et al., 2024, Teal et al., 2023). Therefore, based on the available information, it appears that it is more effective to use smaller E2 doses for a longer period of time than higher doses for a shorter duration, the latter having more potential of producing deleterious effects and resulting in mortalities of valuable breeders.

Another demonstration of the physiologically appropriate E2 dose used in gilthead seabream was the absence of any negative effect on body weight gain. Surprisingly, these results are not in agreement with studies on E2 dietary treatment of brown trout (Voorhees et al., 2023) or bluegill sunfish (*Lepomis macrochirus*) (Wang et al., 2008) that reported a decrease in body weight gain during the treatment, whereas in a study of

Table 1

Mean (± SEM) of various spawning parameters of 4-year-old gilthead seabream broodstocks with Control and E2-feminized females from 1 year before in Jul-Sept, 2023 (0.5 mg and 1 mg E2 kg^{-1} body weight, b.w.) placed together with males in September 2024 (n = 1 tank per treatment, 4 females in each tank). The parameters include female b.w, daily relative fecundity, and fertilization success, embryonic and larval survival of eggs produced in February and March 2025. Asterisks indicate statistically significant differences between months for b.w. (RM two-way ANOVA, $P < 0.001$) and daily relative fecundity (two-way ANOVA, $P < 0.01$). Different letter superscripts for daily relative fecundity indicate differences among treatment groups at both monthly samplings (two-way ANOVA, Tukey's HSD, $P < 0.05$). For the survival to different developmental stages (24-h embryo, hatching and 5-day larvae), there were no significant differences among treatments (RM two-way ANOVA, $P = 0.167$), but there was a significant difference between developmental stages for all treatments ($P = 0.045$), indicated by different letter superscripts on the headings ($P = 0.045$). Lack of significance among treatment groups is shown by "ns" (not significant) next to the Control values for each parameter ($P > 0.05$).

Time/Treatment	Body weight, b.w. (g)		Daily relative fecundity (x10^3 eggs kg^{-1} b.w.)		Fertilization (%)		24 h viable[ab] (%)	Hatched[b] (%)	5-day live[a] larvae (%)
	January*	March	February*	March	February	March			
Control	1345 ± 93[ns]	1019 ± 41[ns]	35.6 ± 3.13[b]	16.6 ± 1.86	77 ± 3.0[ns]	74 ± 2.4	97 ± 2.9[ns]	99 ± 0.3[ns]	98 ± 1.8[ns]
1 mg E2	1175 ± 121	918 ± 94	26.7 ± 3.46[ab]	15.1 ± 1.97	72 ± 3.8	69 ± 3.1	99 ± 0.3	98 ± 1.7	97 ± 1.7
0.5 mg E2	1020 ± 26	830 ± 25	23.6 ± 2.70[a]	16.0 ± 1.25	79 ± 3.3	74 ± 2.9	95 ± 2.9	98 ± 1.6	88 ± 4.6

red shiner (*Cyprinella lutrensis*) there were no significant effects on growth, regardless of the E2 dose and duration of the treatment (Teal et al., 2024). A dramatic decrease in body weight was observed following the administration of E2 implants in a non-teleost species, the great sturgeon (Akhavan et al., 2015). The effect of E2 on growth, weight gain and mortalities, seems to be related to the dose, species, life stage, administration method, substance and duration of exposure (Abdollahpour et al., 2024). For example, in common snook, no difference in body weight gain was found between Controls and fish treated with 0.5 mg E2 kg^{-1}, and a slight reduction was found in fish given 1.0 mg E2 kg^{-1}, but the E2 dose of 4 mg kg^{-1} resulted in significantly reduced body weight, which was attributed to the administered hormone (Passini et al., 2016). In the study of brown trout, where no mortalities were observed, the reduction in growth was due, at least partly, to a significantly higher feed conversion ratio (FCR) during E2 administration (Voorhees et al., 2023). A similar conclusion may be reached from the studies of great sturgeon (Akhavan et al., 2015) and bluegill sunfish (Wang et al., 2008), where increasing E2 doses resulted in progressively lower weight gain compared to non-treated Controls. In the case of common snook, however, the lower growth was probably due to lower feeding, since heavy mortalities accompanied the high E2 treatment (Passini et al., 2016). Therefore, medium E2 doses that do not cause mortalities may reduce growth due to higher FCR, while high E2 doses affect the health of the fish, reduce appetite and feeding -thus reducing growth- and may result in mortalities. On the other hand, low -and presumably physiological- doses of exogenous E2 do not affect FCR or the growth of the fish, and in some instances they may improve growth, as shown recently in zebrafish (*Danio rerio*) (Abdollahpour et al., 2024).

Based on the gonadal histology of juvenile gilthead seabream, the primary effect of E2 was the arrest of development of the testicular tissue at the early spermatogenesis stage, and the subsequent increase of ovarian tissue with the multiplication of oogonia and the production of primary oocytes, but no vitellogenesis was observed during the expected spawning season (December to April). Condeça and Canario (1999) reported similar results in gilthead seabream after the administration of E2 in the diet for 14 weeks, and a delay of spermatogenesis has been reported also in other fishes (Chang et al., 1994, 1995; Länge et al., 2001). The results were different when E2 was administered to 2 + −year-old males (which are mature individuals) that may undergo sex inversion and feminization naturally prior to the reproductive season at the age of 3 years. These fish were not only feminized, but they progressed successfully through vitellogenesis and spawned viable eggs during the expected spawning season when 3 years old. Similarly, when 6-month-old male barramundi were induced to change sex (Banh et al., 2021), only immature females were produced, whereas when using 18-month-old (Guppy et al., 2022) or 20-month-old individuals (Fine-Idan et al., 2024), vitellogenesis was achieved and the fish were induced to spawn successfully. These results highlight the fact that although E2 can induce successfully sex inversion and development of ovarian tissue (primary oocytes), the fish must first reach a certain age/size to be able to then undergo vitellogenesis and spawn successfully (Akhavan et al., 2015; Guppy et al., 2022). Therefore, E2 treatment prior to the gametogenesis period in gilthead seabream induces sex inversion and feminization, but the following progression to vitellogenesis, maturation and spawning takes place spontaneously (in the absence of exogenous E2) and depends on the age of the fish, and not simply on the presence of an ovary.

Plasma levels of E2 showed a significant increase between the pre-spawning and spawning period in E2-feminized females, in accordance to the onset of vitellogenesis, which is linked with endogenously elevated plasma E2 (Akhavan et al., 2015; Guiguen et al., 1993; Wong et al., 2006). We do not have any reference female plasma hormone data from Controls, due to the very limited spontaneous sex inversion and feminization. Based on other studies, however, our E2-feminized females had 2-3× higher plasma E2 levels compared to spontaneously feminized fish during the spawning season (Jerez et al., 2006; Simó-Mirabet et al., 2018) and about 20% higher plasma levels compared to spawning females (Meiri et al., 2002). Regarding plasma levels of T, although differences were detected among the treatment groups and over time, they were lower (García Hernández et al., 2020) or similar to the natural levels observed in female gilthead seabream during the reproductive season (Jerez et al., 2006) and to those observed in broodstocks after removal of the male individuals (Meiri et al., 2002). Plasma levels of 17,20β-P were typical of females during the reproductive period (Forner-Piquer et al., 2019). Concomitant increases of E2 and decreases of T plasma levels were observed in great sturgeon adult females treated with E2 to induce maturation (Akhavan et al., 2015), and E2 treatment was shown to reduce expression of male-related genes in barramundi (Banh et al., 2021). In the present study, it was not possible to attribute the differences in plasma T and 17,20β-P levels between the 0.5 and 1 mg E2 groups to the differences in E2 dose administered earlier. A more likely explanation can be found in the slightly more advanced stage of gonadal maturation of the 0.5 mg E2 females compared to the 1 mg E2 females, since more females contained oocytes at the stage of ovulation and oocyte maturation in the low dose (24/39 females) compared to the high one (11/34 females), as discussed further in the next paragraph. Higher T and 17,20β-P plasma levels occur during the spawning season and 17,20β-P was associated with the process of oocyte maturation and ovulation in gilthead seabream (Gothilf et al., 1997; Jerez et al., 2006; Meiri et al., 2002) as in many fishes (Adolfi et al., 2023; Nagahama and Yamashita, 2008; Soranganba and Singh, 2019). So, the differences observed in steroid levels between the two E2 doses used, were most likely a result of the gonadal stage of the fish at the time of sampling, and not a direct result of the exogenous E2 treatment itself, which was completed more than a month before.

Further to the differences in the reproductive stages between females of the two E2 treatment groups at the onset of the reproductive season in December, there were also differences between Controls and E2-treated groups. This effect was presumably due to the absence or extremely low numbers of males in the E2-treated groups, as a result of the very high feminization success. In the Controls, the few spontaneously sex-inverted females had ovulated eggs or oocytes in maturation in their ovaries, and spawning was observed from the beginning of November. On the other hand, the E2-treated groups contained many females with vitellogenic oocytes as their most advanced stage of development, and some of them had only primary oocytes in their ovaries. Previous studies on gilthead seabream have shown that socio-sexual stimuli during the reproductive period can trigger endocrine responses that affect reproductive function (Meiri et al., 2002). In the latter study, removal of males from a breeding stock resulted rapidly in reduced egg production and increased occurrence of follicular atresia, as it was observed in the present study. So, it is reasonable to conclude that the differences in reproductive maturation status between Controls and E2-treated females at the onset of the spawning season was due to the absence of males in the stocks of the latter, and not due to the method that feminization was achieved. This interpretation is further supported by the similarities in reproductive stages observed among these broodstocks during the next year, when they were 4 years old and all broodstocks were maintained with equal number of males throughout the entire pre- and reproductive season (see later).

The E2-feminized, 3-year-old females spawned volitionally after the addition of 2-year-old males in December. Egg production in all broodstocks and mean fertilization were within the normal range reported in other studies (Jerez et al., 2012; Mylonas et al., 2011; Papadaki et al., 2024), though there were significant differences in fecundity between Controls and E2-sex-inverted females. Females from the E2 treatments showed lower daily relative fecundity, with differences existing also between the two doses. However, the values were well within the range of 11,400–36,600 eggs kg^{-1} reported by other studies, as variations do exist between broodstocks and in the same broodstock from year to year (Jerez et al., 2012; Papadaki et al., 2024). The reduced fecundity may be attributed to the fact that these females did not start

spawning as soon as they were physiologically ready, since males were absent (or in extremely low numbers) in their tanks. This absence of males, prevented females from undergoing oocyte maturation, ovulation and spawning when post-vitellogenic batches of oocytes appeared in the ovaries, and resulted in follicular atresia of the vitellogenic oocytes, as discussed earlier and shown also previously (Meiri et al., 2002).

In the next spawning season, when the same Control and E2-feminized females were 4 years old, they matured and spawned again with similar values of mean daily relative fecundity compared to the previous year, but with slightly lower fertilization success. The evaluation of reproductive performance was carried out only for the middle period of spawning (February and March) when the peak of egg production takes place (Papadaki et al., 2024). Again, there were differences in relative fecundity between Control and E2-feminized females, although this time only the 0.5 mg E2 dose had significantly lower mean values. Apparently, E2-induced feminization had some negative effect on fecundity, which we cannot explain at this stage, but without any effect on the quality of the eggs (embryonic survival, hatching and 5-d larval survival). Still, the observed mean daily fecundities in both 3- and 4-year-old females were well within the reported values in other studies (Jerez et al., 2012; Papadaki et al., 2024). In barramundi, studies evaluating reproduction of E2-feminized females following spawning induction with Gonadotropin Releasing Hormone agonist (GnRHa) implants (Guppy et al., 2022) or GnRHa injection (Fine-Idan et al., 2024) resulted in high fecundity, but lower fertilization (25–35%) compared to eggs observed from spontaneous spawning (86–98%), but there was no evaluation of Control versus E2-feminized females. The results of the present study demonstrate the physiologically sound use of the E2 administration, in relation to long term reproductive function of gilthead seabream, albeit with a slight reduction in fecundity.

5. Conclusion

The present study established an effective protocol for achieving almost 100% feminization of $2 + -$year-old gilthead seabream males through three monthly administrations of E2 implants at a dose of as low as 0.5 mg kg^{-1} b.w., with very little mortality. This method may be used in commercial hatcheries to ensure that all individuals selected to participate in a selective breeding program as 3-year-old females, will have successfully undergo sex inversion from males to females, and will reproduce with good fecundity, fertilization and larval survival characteristics. In commercial applications, it is advisable to introduce the $1 + -$year-old males selected into the E2-treated $2 + -$year-old gilthead seabream stock (expected to develop into females) as soon as the last implantation is completed, so that the broodstock will begin producing fertilized eggs as soon as vitellogenesis is completed. We believe that this effective and practical protocol for sex inversion in the protandrous gilthead seabream, has a strong potential for application to other protandrous fish species and preliminary studies have been performed already by our lab with sobaity bream (*Sparidentex hasta*), another fish from the Sparidae family (unpublished data).

CRediT authorship contribution statement

Katerina Samiotaki: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Maria Papadaki:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Irini Sigelaki:** Project administration. **Evangelia Manini:** Visualization, Investigation. **Sabrin Hmida:** Visualization, Investigation. **Pantelis Katharios:** Writing – original draft, Methodology, Investigation. **Maja Ruetten:** Writing – original draft, Methodology, Investigation. **Stelios Karapanagiotis:** Investigation. **Constantinos C. Mylonas:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there are no financial interests/personal relationships that may be considered as potential competing interests in this study.

Data availability

Data will be made available on request.

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