Administration of single-chain recombinant follicle-stimulating hormone (sdrFsh) and luteinizing hormone (sdrLh) stimulates spermatogenesis, but not vitellogenesis in pre-pubertal greater amberjack (*Seriola dumerili*)

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Abstract

A combination of homologous single-chain recombinant follicle-stimulating hormone (sdrFsh) and luteinizing hormone (sdrLh) was administered to 1-year old, pre-pubertal greater amberjack (*Seriola dumerili*), to induce precocious gametogenesis. The minimum effective sdrFsh dose to induce steroidogenic activity was found to be 10 μg kg^{-1}, based on the elevation of plasma testosterone (T) and 11-ketotestosterone (11-KT) in males. The same dose had only a mild effect on plasma T and 17β-estradiol (E2) in females. A combination of sdrFsh/Lh was then administered weekly for 12 weeks, beginning at the expected onset time of gametogenesis in greater amberjack in the Mediterranean Sea (March). Mean plasma T, 11-KT and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) increased significantly in sdrFsh/Lh-treated males compared to controls, and spermatogenesis and spermiation were completed at the end of the 12-week treatment (June), with sperm being collectable from all individuals. The sdrFsh/Lh-treated males produced sperm of comparable or better sperm concentration and motility characteristics than naturally matured hatchery-produced F1 males reared in sea cages, and higher than that of both captive-reared wild fish in the Mediterranean Sea and F1 males from the Atlantic Ocean. On the contrary, females did not respond to the treatment, their plasma sex steroid hormones remained unchanged during the study, and no vitellogenic development was observed at the end of the 12-week sdrFsh/Lh treatment. Results suggest that shortening the time of puberty to 2 years of age can be achieved in males but not in female greater amberjack. Further research should examine the cause of the unresponsiveness of pre-pubertal females to sdrFsh/Lh, and if it may be possible to stimulate oogenesis in older, pre-pubertal females.

Keywords: *Seriola dumerili*, greater amberjack, puberty, gametogenesis, maturation, rFsh, rLh

1. Introduction

Although the greater amberjack (*Seriola dumerili*) has been targeted by the aquaculture sector worldwide, the production of this species lags considerably compared to its congeners; for instance, Japanese yellowtail (*Seriola quinqueradiata*) production in 2021 in Japan was estimated at 132,700 mtn, compared to only 172 and 140 mtn of greater amberjack produced in Greece and Spain, respectively (OECD, 2024). Similarly, the rearing of yellowtail kingfish (*Seriola lalandi*) has expanded in many areas around the world, often using recirculating aquaculture systems (RAS) (Horstmann et al., 2023; Nocillado et al., 2019). The incomplete control of the reproductive cycle of greater amberjack (Corriero et al., 2021a; Fakriadis et al., 2020b; Jerez et al., 2017; Nyuji et al., 2019; Sarih et al., 2018) and its failure to spawn spontaneously in captivity have contributed to the slow incorporation of this species in aquaculture production (Sicuro and Luzzana, 2016). As a result, much effort for this species has been dedicated to developing efficient spawning induction protocols for the industry (Corriero et al., 2021a).

In greater amberjack from the wild, the onset of puberty usually takes place between 3-5 years of age, with females developing reproductively later than males (Corriero et al., 2021a; Harris et al., 2007; Marino et al., 1995). Late reproductive maturation is a beneficial biological characteristic for grow-out, as it allows harvesting the fish before growth is affected negatively by investment of nutrients in gonad development (Zupa et al., 2017b). In comparison, well-established species in Mediterranean aquaculture such as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) reach maturity at a younger age and often precociously under aquaculture conditions (Carrillo et al., 2015; Meiri et al., 2004). On the other hand, the long time required by greater amberjack to reach maturity poses problems in broodstock management; significant investments in infrastructure, human and feed resources become unavoidable before the stock can contribute to the productive

cycle (Taranger et al., 2010). Recently, the first report on the timing of puberty in F1 hatchery-produced greater amberjack maintained in sea cages indicated that, although a very limited fraction of males matured precociously, the totality of the population matured at 3 years of age, while females were identified as spawning-capable for the first time at 5 years of age (Lancerotto et al., 2024), essentially mirroring the maturational patterns observed in the wild. Nonetheless, these captivity-maturing females failed to respond to the established protocol for spawning induction using gonadotropin-releasing hormone agonist (GnRHa) (Fakriadis et al., 2020a; Fakriadis et al., 2020b), whereas the administration of recombinant *Seriola dumerili* follicle-stimulating (sdrFsh) and luteinizing hormone (sdrLh) employed recently enhanced gametogenesis and achieved maturation and spawning of good quality eggs (Lancerotto et al., 2025). Given this recently demonstrated potential of sdrFsh and sdrLh to induce gametogesis, and the long period before natural reproductive maturation (Lancerotto et al., 2024), artificial advancement of puberty could be achieved using sdrFsh/Lh treatment. Advancement of puberty may reduce generation time in breeding selection programs and speed up domestication, which are prerequisites in modern aquaculture for optimizing rearing methods, and improving growth, feed efficiency, disease resistance and final product quality of a species (Boudry et al., 2021) (Teletchea, 2021). Therefore, efforts to shorten generation time in late-maturing species by advancing puberty have become more and more frequent in aquaculture (Banh et al., 2021; Guzmán et al., 2015).

Due to the fundamental role of Fsh and Lh in the regulation of gametogenesis (Levavi-Sivan et al., 2010; Lubzens et al., 2010; Mylonas et al., 2010; Schulz et al., 2010), it is not surprising that their recombinant forms have been tested in different species as a tool to reduce the time necessary to reach the age of first maturity (Moles et al., 2020). The most convincing example is provided by freshwater eels (*Anguilla spp*), due to their permanent

existence in a pre-pubertal state under aquaculture conditions (Vidal et al., 2004). Recombinant Fsh/Lh have been employed extensively in the past two decades; diverse expression systems have been tested (Kazeto et al., 2008; Ohta et al., 2007), both *in vitro* (Nguyen et al., 2022) and *in vivo* (Kobayashi et al., 2010), in males (Penaranda et al., 2018) and females (Nguyen et al., 2020), to investigate the possibility of stimulating reproductive development and inducing maturation. Recently, the induction of vitellogenesis and the achievement of maturation in European eel (*Anguilla anguilla*) was obtained with the administration of rFsh and rLh (Jéhannet et al., 2023). Attempts to induce precocious gonadal development in immature individuals have been conducted also in an increasing number of other species. To mention just a few, in reproductively immature meagre (*Argyrosomus regius*) males, the administration of specific rFsh for six weeks triggered testis growth and enhanced spermatogenesis (Zupa et al., 2023), whereas in immature tiger grouper (*Epinephelus coioides*) females, the repeated administration of rFsh and rLh were successful in inducing ovarian development (Chen et al., 2012).

In the present work, the combination of sdrFsh and sdrLh administration, which has already been used successfully in enhancing gametogenesis and spawning in adult greater amberjack males and females (Lancerotto et al., 2025), was evaluated for its potential in stimulating spermatogenesis and oogenesis in 1-year old, pre-pubertal, hatchery-produced greater amberjack, with the objective of advancing puberty and obtaining viable gametes earlier, for selective breeding programs.

2. Materials and methods

The employed experimental protocol received approval from the National Veterinary Services of the Region of Crete, Hellenic Republic (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 husbandry

At one year of age (June 2021), fish (n = 75) were transferred from sea cages located at the facilities of Argosaronikos Fishfarm S.A. (Salamina Island, Greece), to 2-m^3 indoor tanks at the AQUALABS of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research in Crete, Greece. Tanks were exposed to a natural photoperiod (roof windows) and were supplied with 1-μm filtered borehole seawater (for biosecurity reasons, to avoid pathogens entering the rearing tanks) at a constant temperature of 19 ± 0.5°C. Fish were then used in two consecutive experiments (see sections below) to test single-chain *Seriola dumerili* gonadotropins (sdrGths) produced by Rara Avis Biotec S.L. (Valencia, Spain), following protocols utilized previously for other fish species (Chauvigné et al., 2017; Jéhannet et al., 2023; Penaranda et al., 2018; Ramos-Júdez et al., 2021; Zupa et al., 2023) and presented in detail recently (Lancerotto et al., 2025).

2.2. Test of bioactivity *in vivo*

To test the steroidogenic activity of sdrFsh/Lh on pre-pubertal greater amberjack and to determine their effective dose to be used later in a puberty-advancement experiment, single-chain recombinant follicle-stimulating hormone (sdrFsh) was used in a dose-response experiment conducted in the off-reproduction season (October 2021), when the fish were 16-months-old and had a mean ± SD body weight of 654 ± 14 g. Unsexed fish were assigned to one of four experimental groups (n=12). Although a male: female ratio of 1:1 was expected

157 (Papadaki et al., 2021), the sex of the fish was unknown at the time of treatment, and it was
158 determined at the conclusion of the experiment when they were sacrificed.
159 The fish (individually tagged with a Passive Integrated Transponder, PIT tag) received
160 either one of three dosages of sdrFsh (5 μg kg^{-1}, sdrFsh5; 10 μg kg^{-1}, sdrFsh10; 15 μg kg^{-1},
161 sdrFsh15) dissolved in 2% saline solution or sham injections (saline solution, Control), every
162 7 days over 6 weeks (total of six treatments). At the end of the sdrFsh administration, fish
163 were euthanized with a lethal dose of anesthetic, and body weight (BW, g) and gonad weight
164 (GW, g) were measured to estimate the gonadosomatic index (GSI, %; [GW/BW]x100).
165 After dissection of the gonads, a small fraction was preserved in a fixative solution of 4%
166 formaldehyde:1% glutaraldehyde (4F:1G) for histological analysis. Blood samples were
167 obtained from the caudal vasculature using heparinized syringes every 3 weeks. Blood was
168 centrifuged for 15 min at 5,000 rpm and the supernatant plasma was stored at -80°C until
169 analyzed for plasma levels of sex steroid hormones with liquid chromatography-tandem mass
170 spectrometry (LC-MS/MS).
171
172 **2.2 Stimulation of gametogenesis using sdrFsh/Lh treatment**
173 A combined administration of sdrFsh/Lh was conducted during the reproductive season
174 of greater amberjack in the Mediterranean Sea (March to June, 2022), when pre-pubertal
175 greater amberjack were 20-months-old. The utilized doses for sdrFsh and sdrLh were based
176 on the bioactivity of sdrFsh determined above -assuming that the effective dose of sdrLh
177 would be similar to that of sdrFsh- and the relative doses used in previous works using
178 rFsh/Lh for similar purposes in other fish species (Chauvigné et al., 2017; Jéhannet et al.,
179 2023; Penaranda et al., 2018; Ramos-Júdez et al., 2021; Zupa et al., 2023). The fish were
180 maintained in 2-m^3 tanks supplied with filtered borehole water at a constant temperature of
181 19 ± 0.5°C and exposed to a natural photoperiod (roof windows). As before, unsexed fish

were assigned to the experimental groups, and the sex of the fish was determined at the conclusion of the experiment when the fish were sacrificed or their sex could be identified by obtaining a gonadal biopsy (oocytes or sperm). Two experimental groups were created in different tanks. Fish from the first group (Control, n = 10, 1002 ± 85 g) were given an injection of saline solution, and fish from the second group (sdrFsh/Lh, n = 15, 1026 ± 36 g) were treated with increasing doses of sdrFsh (8, 12 μg kg^{-1}, Week 0,1-11) and sdrLh (10 μg kg^{-1}, week 1-11), at weekly intervals for 12 weeks (**Fig. 1**). Blood samples were collected every 3 weeks, to measure plasma levels of sex steroids using LC–MS/MS.

At the end of the experiment, milt samples and ovarian biopsies (when possible) were collected to assess the reproductive development of the fish, or fish were sacrified and gonads were excised for histological processing. Ovarian biopsies were collected with the use of a catheter inserted at the opening of the genital pore (Pipelle de Cornier, Laboratorie CCD, France). Sperm for sperm quality evaluation was collected by applying abdominal pressure, when possible, or by cannulation of the genital pore when necessary (Pipelle de Cornier, Laboratorie CCD, France). Collected gonads and biopsies from all samplings were preserved in a solution of 4F: 1G for histological analysis. Sperm samples (100-200 μL) were stored at 4°C until analyzed using computer-assisted sperm analysis (CASA, ISAS, Spain). Blood was collected, centrifuged at 5,000 rpm for 15 min at +4°C and the obtained plasma was maintained at -80°C until hormonal analysis.

2.3 Histological analysis

The dissected gonads were dehydrated in a 70-95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraus Kulzer, Germany). Serial sections of 4 μm were obtained using a semi-automatic microtome (Leica RM2245, Germany), and histology slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976)

and examined under a light microscope (50i Eclipse, Nikon, Japan). Eventually, microphotographs of the stained content of the ovary were taken using a digital camera (Progres, Jenoptik AG, Germany).

2.4 Measurement of plasma sex steroids

The extraction and analysis of steroid hormones was performed according to Papadaki et al. (2021) with a few modifications. The following sex steroids were included in the panel of analytes: testosterone (T), 17β-estradiol (E2), 11-ketotestosterone (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) that were 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 four compounds in varying concentrations (10 to 85 pg μL^{-1}) was prepared in methanol:water 1:1 and 10 μL of this solution were added to the serum samples prior to solid phase extraction. Subsequently, the preparation of samples and the analysis of hormones by LC-MS/MS was implemented following the same procedures as those described in our previous study.

2.5 Sperm quality analysis

For Computer Assisted Sperm Analysis (CASA, ISAS, Spain), sperm was activated in seawater containing 2% bovine serum albumin (1:201 or 1:334) to obtain 200–300 cells in the field. A reusable counting chamber with a fixed depth (SpermTrack, ISAS) was used to record spermatozoa movement using a digital camera at 100 frames per second (fps) attached to a light microscope (Primo Star, Zeiss, Germany) under 100× magnification. Spermatozoa movement recording started 15 s after activation and was stopped when less than 5% of the

spermatozoa in the field of view were showing forward motility. The CASA included the following parameters: sperm density (number of spermatozoa ml^{-1} of sperm), duration of forward spermatozoa motility of ≥ 5% of the spermatozoa in the field of view (motility duration, min), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) (μm sec^{-1}), motile cells, progressive cells (> 80% straightness, STR), rapid cells, and STR (%). The software settings were adjusted to 1 to 90 μm for the head area; VCL < 10 μm sec^{-1} to classify a spermatozoon as immotile; and spermatozoa were considered rapid when VCL was higher than 100 μm sec^{-1}.

2.6 Statistical analysis

Differences in the GSI between treatment groups were examined with a Kruskal-Wallis's test. A repeated measures two-way ANOVA (time, treatment) followed by Tukey's test was used to compare the levels of sex steroids in the plasma of fish treated with sdrFsh in the "Dose-response" experiment, and in fish treated with or without sdrFsh/Lh (treatment, time) in the "Stimulation of gametogenesis" experiment. Differences in sperm quality parameters were not analyzed due to the limited number of samples from the Control group (n=1). Results are presented as mean values ± standard error of the mean (SEM), unless mentioned otherwise. In all the statistical tests performed, p-values ≤ 0.05 were considered statistically significant. Statistical analyses and graphics were run using GraphPad Prism 9.4.1 for Mac OS, GraphPad Software, San Diego, California USA, www.graphpad.com.

3. Results

3.1 Dose response of sdrFsh

In the off-reproduction season experiment, males exhibited a dose-dependent response in GSI% and plasma steroid levels when treated with sdrFsh (**Fig. 2**). Significantly increased

GSI values (**Fig. 2A**), and plasma levels of T and 11-KT were evident in fish to which 10 (n=4) and 15 (n=4) μg kg^{-1} of sdrFsh was administered, compared to saline-treated Control fish (**Fig. 2B and C**). In the Control group (n=6), the testes were dominated by spermatogonia, and spermatocytes represented a negligible fraction of the gonadal content (**Fig. 3A**). A more advanced stage of germ cell development was observed in the testes of males from the sdrFsh5 group (n=4), compared to the Control group; other than spermatogonia, also spermatocytes, spermatids and a small fraction of spermatozoa were observed (**Fig. 3B**). The administration of 10 and 15 μg kg^{-1} of sdrFsh stimulated the proliferation and differentiation of germ cells. Sperm was found within the testes, and histology confirmed the presence of cysts in all spermatogenic stages and spermatozoa in the seminiferous tubules (**Fig. 3C and D**).

Contrary to the males, no differences were found in GSI% of females between groups (Control, n = 6; sdrFsh5-10-15, n = 8) (**Fig. 2D**). Ovarian lamellae were filled with oogonia, and primary oocytes were found in females from all groups (**Fig. 4A-D**). On week 3, significantly increased plasma levels of T were detected in females treated with 10 μg kg^{-1} of sdrFsh, while on week 6 increased plasma T was found for females treated either with 10 or 15 μg kg^{-1} of sdrFsh (**Fig. 2E**). No differences were detected in the plasma levels of E2 of any of the sdrFsh-treated fish during the whole experiment (**Fig. 2F**).

3.3 Stimulation of gametogenesis using sdrFsh/Lh treatment

At the end of the 12th week experiment during the reproductive season of greater amberjack in the Mediterranean Sea, histology revealed the presence of almost exclusively spermatogonia in the testes of most Control fish (**Fig. 5A**), with sparse presence of spermatocytes at different stages of development. On the other hand, it was possible to collect sperm upon abdominal pressure application in 100% of sdrFsh/Lh-treated males

(n=8)(**Fig. 5B**), therefore the fish were not killed for histological evaluation. Only a single male (33%) of the Control males matured precociously and produced collectible sperm. All the analyzed sperm quality parameters (spermatozoa concentration, percentage motile spermatozoa and spermatozoa velocity) were found to be similar in all fish, although it was not possible to compare sperm quality statistically between the sdrFsh/Lh and Control groups, since sperm could be collected from only one Control male (**Fig. 6 A-C**). The small number of Control males compared to the sdrFsh/Lh in the experiment was coincidental and due to the unavoidable use of unsexed pre-pubertal 1+-year old fish for the experiment.

Contrary to males, the combined administration of sdrFsh/Lh for twelve weeks, did not stimulate vitellogenesis, and no differences were found between females from the sdrFsh/Lh (n=7) and the Control group (n=7) in ovarian development (**Fig. 5C and D**). The collected biopsies had primary oocytes <150 μm in diameter (data not shown).

Significant changes in the sex steroid profile of males were found, as the administration of sdrFsh/Lh induced an increase in the plasma levels of T, 11-KT and 17,20β-P (**Fig. 7**). Both T and 11-KT increased gradually during the time of the experiment, reaching their highest levels on week 12. On the other hand, plasma 17,20β-P in fish from the sdrFsh/Lh group increased significantly only at week 9. In females, in general there were no significant differences in plasma sex steroid hormone levels during the experiment, apart from a small, yet significant, increase in plasma 17,20β-P during week 6 (**Fig. 7**).

4. Discussion

Using recombinant greater amberjack single-chain sdrFsh and Lh, we have recently enhanced gametogenesis, and induced maturation and spawning in adult, 5-year-old reproductively dysfunctional hatchery-produced greater amberjack held in tanks (Lancerotto et al., 2025). In the present study, we tested the same recombinant hormones in stimulating

307 gametogenesis in pre-pubertal fish and demonstrated that it is possible to advance puberty in
308 males, but not females, and have 100% spermiating fish when they are 2 years old. In
309 agreement with other studies conducted on immature male teleosts (Hayakawa et al., 2009;
310 Penaranda et al., 2018; Zupa et al., 2023), the administration of sdrFsh alone was sufficient
311 for stimulating steroidogenic activity, testicular growth and spermatogenesis in a dose-
312 dependent manner. In 18-month-old males of the congeneric yellowtail kingfish, treatment
313 with recombinant yellowtail kingfish Fsh (rytkFsh) also induced steroidogenesis *in vitro*, and
314 induction of gametogenesis in males was achieved (Sanchis-Benlloch et al., 2017).
315 Surprisingly, there was no significant increase in GSI after six weekly treatments of rytkFsh,
316 while in the present study males treated with 10 and 15 μg kg^{-1} sdrFsh had 12 and 14-fold
317 higher GSI values, respectively, compared to the males from the Control group. In the
318 European seabass, it has been demonstrated that different expression systems used to produce
319 rFsh and rLh could result in differences in the stability and half-life of the hormones, making
320 them best suited either for *in vitro* or *in vivo* applications (Moles et al., 2011). Therefore, this
321 discrepancy in results between greater amberjack and yellowtail kingfish that were similarly
322 treated with recombinant hormones might be due to the different expression systems used to
323 produce the specific rGths.
324 The stimulation of spermatogenesis described in the dose-response experiment was
325 conducted during the non-reproductive season for this species. In teleost fishes living in
326 temperate waters, such as the greater amberjack (Corriero et al., 2021a), day length variations
327 constitute the fundamental cue for the activation of the Brain-Pituitary-Gonad (BPG) axis
328 (Migaud et al., 2010). Also, studies conducted on *Seriola spp*. suggested that photoperiod is
329 the main environmental cue stimulating and driving reproductive development (Mushiake et
330 al., 1994; Nyuji et al., 2018), if temperature is maintained above the minimum values
331 appropriate for fish undergoing gametogenesis (Mushiake et al., 1998). Although these

studies were focused on females, it is reasonable to consider that both sexes would respond in the same way to photoperiodic cues. Overcoming this environmental cue, differentiation of germ cells in pre-pubertal male yellowtail kingfish was attained during the non-breeding season when both photoperiod and temperature were suboptimal for reproductive development, after peripheral administration of kisspeptins, in particular *kiss2* (Nocillado et al., 2013), which was shown also in European seabass to control the secretion of Fsh and Lh (Espigares et al., 2015). Therefore, it is reasonable to assume that 16-month-old males have the ability to respond to sdrFsh, even if exposed to non-optimal light conditions since the water temperature was maintained constant at values that are typically found in the Mediterranean Sea during spring when gametogenesis takes place (Fakriadis and Mylonas, 2021; Mandich et al., 2004). Under these conducive conditions, fish not only responded quickly to the sdrFsh treatment, but they reached an advanced-stage of gametogenesis, and spermatozoa were plentiful in the testicular lumen. While the first experiment was conducted only to verify the responsiveness of pre-pubertal fish to the sdrGths, such a response to the sdrFsh during the non-breeding season might imply that this approach could be further implemented to control the reproductive cycle of greater amberjack, reducing the reliance on the seasonal variations of environmental cues, in order to stimulate reproductive development "off-season" in this species, in situations that photoperiod could not be controlled, such as when large breeders are reared in sea cages or outdoor tanks.

By administering both sdrFsh and sdrLh to pre-pubertal, 20-month-old male greater amberjack during the natural reproductive period (March–June), we induced full sexual maturation in 100% of treated males when the fish were 2 years old. The first age of male maturity has been reported between the third (Harris et al., 2007; Lancerotto et al., 2024) and fourth year of life (Marino et al., 1995). Obviously, this approach for the induction of precocious puberty in males 1-2 years earlier is preferable than relying on the natural

occurrence, which, nevertheless, is possible yet very limited for commercial purposes (Lancerotto et al., 2024; Marino et al., 1995). It is especially worth mentioning also, that fluent milt could be obtained via abdominal pressure from all sdrFsh/Lh-treated males, contrary to what was observed in 16-month-old fish treated only with sdrFsh in the present study, but also in other studies with adult greater amberjack. Under captive conditions, collecting fluent milt through abdominal pressure in this species is rare (Fakriadis and Mylonas, 2021; Mylonas et al., 2004), partly because of the thick abdominal musculature of the species, but also because males exhibit reduced gonadal development and smaller seminiferous lobules (Zupa et al., 2017a). Moreover, inconsistent and low sperm output (Fakriadis and Mylonas, 2021), associated to lower plasma levels of 11-KT and 17,20β-P (Zupa et al., 2017b) have also been reported. In other species, such as meagre and Senegalese sole, rGths have proven effective in promoting steroidogenesis, gonadal development, and enlarging seminiferous and efferent duct tubules (Chauvigné et al., 2022; Zupa et al., 2023). However, when looking carefully in meagre treated only with rFsh, spermiation was not attained (Zupa et al., 2023). This is not surprising, considering the key role of the other Gth, namely Lh, in stimulating steroidogenesis and sperm hydration, and its involvement in the conversion of spermatids to spermatozoa (Schulz et al., 2010). Nevertheless, in adult breeders, although fertilized eggs could be obtained during spawning, milt could not be collected by stripping, even after receiving injections of both sdrFsh and sdrLh (Lancerotto et al., 2025). The reason for this difference between the latter and the present work likely resides in the different protocol of administration of sdrLh, which in the present study it started already on week 1 of the combined sdrFsh/Lh treatment, well before the expected period of spermiation. In fact, adult male breeders received only one sdrLh injection before they were allowed to spawn with induced females, which was deemed insufficient in this species to stimulate hydration (Lancerotto et al., 2025). While we could

not perform histology to avoid killing the fish in the present study, altogether, the present results suggest that the early and combined administration of sdrFsh/Lh, was critical to achieving full maturation and the release of fluent milt, with gentle abdominal pressure, for the first time in our experiments (Fakriadis and Mylonas, 2021; Fakriadis et al. 2020b).

The fact that the administration of sdrFsh/Lh allowed 100% of the treated males to release milt, as opposed to solely one spermiating male in the Control group, was *per se* an indication of the efficacy of the given therapy in inducing successful spermatogenesis and spermiation. Based on the available literature on milt quality of adult greater amberjack (Fakriadis and Mylonas, 2021; Fakriadis et al. 2020b) and the very limited data from a precociously matured male here, sdrFsh/Lh-treated males produced sperm of comparable or better characteristics. For example, the spermatozoa concentration in the current study was 2-fold higher than in 3-year-old, but was comparable to that of 4- and 5-year-old F1 hatchery-produced males reared in sea cages (Lancerotto et al., 2024) and higher than that of both captive-reared wild fish (Fakriadis and Mylonas, 2021) and F1 males from the Atlantic Ocean (Jerez et al., 2018). As regards spermatozoa motility (motile, progressive and rapidly moving spermatozoa percentage) and spermatozoa velocity characteristics (VCL, VSL and VAP), the sperm of the sdrFsh/Lh-treated males were comparable to those of F1 hatchery-produced adult males reared in sea cages (Lancerotto et al., 2024) and higher than those of wild-caught, captive-reared adult males evaluated before spawning induction (Fakriadis and Mylonas, 2021). Therefore, our results suggest that the sdrFsh/Lh treatment was physiologically sound and conferred the ability to produce functional sperm to pre-pubertal males when they were 2 years old.

Describing further the effect of sdrFsh/Lh treatment in pre-pubertal greater amberjack males, an elevation of plasma androgens was achieved a few weeks after treatment, as reported in other species (Chauvigné et al., 2012; Kamei et al., 2003). The administration of

sdrFsh alone increased plasma androgen levels in a dose-dependent manner, outside the reproductive season and was sufficient to stimulate steroidogenesis here, confirming the central role of Fsh during gametogenesis in teleosts (Kamei et al., 2006; Levavi-Sivan et al., 2010; Schulz et al., 2010). Further, with the additional administration of sdrLh, the plasma levels of T and 11-KT increased strongly over time reaching values >5-fold higher than those treated only with sdrFsh, and the hormone levels were comparable to those of mature adult wild fish (Zupa et al., 2017b). Moreover, although in terms of absolute mean values the difference between sdrFsh/Lh-treated and Control fish was < 0.15 ng ml^{-1}, plasma levels of 17,20β-P exhibited a marked increase and peaked after 9 weeks of treatment with sdrFsh/Lh. Members of the genus *Seriola* were categorized in a group of fish in which despite the existence of seasonal variations in plasma 17,20β-P, the absolute concentrations remain relatively low (Fakriadis et al., 2024; Poortenaar et al., 2001; Zupa et al., 2017b) compared to other teleosts (Scott et al., 2013; Scott et al., 2010). Apparently, even a seemingly trivial increase in this sex steroid might exert a significant control on the BPG axis, as reported already for greater amberjack males during sex differentiation (Papadaki et al., 2021). In many male teleosts, 17,20β-P is the maturation-inducing steroid (Scott et al., 2010), and its rise is associated with testicular hydration and the increase in the amount of releasable milt (Schulz et al., 2010); 17,20β-P was also found to up-regulate the gene encoding for 11β-HSD, a key enzyme controlling the testicular production of 11-KT (Ozaki et al., 2006), which is known for being the main spermatogenesis-inducing steroid (Schulz et al., 2010). Considering the temporal profile of plasma sex steroid in pre-pubertal greater amberjack males, with the increased 17,20β-P on week 9 followed by a strong elevation of 11-KT concentration on week 12, the results obtained were congruent with the ability of the treated males to release milt upon the application of abdominal pressure, further confirming the

effectiveness of the given treatment in driving fish through maturation 1-2 years before natural puberty.

Contrary to the males, pre-pubertal greater amberjack females <2 years-old showed complete refractoriness to sdrFsh/Lh, and we did not observe either somatic growth of the ovary (*i.e.* increase in GSI) or vitellogenic development of the oocytes. Similarly, rFsh given to 18-month-old female yellowtail kingfish also failed to stimulate the initiation of vitellogenesis (Sanchis-Benlloch et al., 2017). In other fishes, a single injection of rFsh in Manchurian trout (*Brachymystax lenok*) was reported to increase GSI in immature females, and vitellogenic oocytes could be identified three days after administration (Ko et al., 2007).

The lack of response that we observed might be attributed to the longer time required for greater amberjack females to reach the first age of maturity compared to males (Kozul et al., 2001; Lancerotto et al., 2024; Marino et al., 1995; Micale et al., 1999) and the consequential inability of the gonad to perceive the hormonal stimulus (Fontaine et al., 2020; Okuzawa et al., 2002), as acquisition of competence by the different components of the BPG axis occurs only near the natural age of puberty (Kumakura et al., 2003). Two indirect indications seem to support this hypothesis. The first is the occurrence in greater amberjack of incomplete annual gametogenic cycles, interrupted abruptly by the insurgence of extensive follicular atresia before reaching maturity (Lancerotto et al., 2024; Micale et al., 1999), which is recognized as an indicator of incomplete competence of the BPG axis (Corriero et al., 2021b), and has been termed as a "dummy run" in other species (Holland et al., 1998). The second evidence is provided by 5-year-old mature greater amberjack females treated with sdrFsh/Lh, in which the completion of vitellogenesis, oocyte maturation, ovulation and spawning was obtained in conditions that usually prevent reproductive function (Lancerotto et al., 2025). Although vitellogenesis is dependent on the interaction between Gths and their receptors in the ovaries, the latter might have a more significant control over this process, as

456 recently demonstrated in Pacific bluefin tuna (*Thunnus orientalis*) (Higuchi et al., 2024). In
457 fact, while the deletion of the gene for the *Fsh-β* subunit delayed but did not arrest follicular
458 growth (Zhang et al., 2015b), the deletion of the gene encoding the Fsh-receptor (*fshr*) in
459 zebrafish (*Danio rerio*) (Zhang et al., 2015a) and medaka (*Oryzias latipes*) (Kitano et al.,
460 2022) prevented ovarian development in both species, probably due to the strong
461 constitutional nature of its expression during follicular development (Kwok et al., 2005).
462 Also in greater amberjack, results from a study on the seasonal variation of Gths and their
463 receptors revealed that an increased *fshr* expression in the ovary could be more relevant for
464 supporting vitellogenesis than the elevation of Fsh (Nyuji et al., 2016). Moreover, in the
465 gonads of immature Atlantic salmon (*Salmo salar*), expression of *fshr* was almost 3-fold
466 lower in the females (Andersson et al., 2009), which could explain why in the present study
467 males responded to sdrFsh/Lh, while females did not. It is plausible that physiologically low
468 expression -if not a lack- of *fshr* in the ovary of pre-pubertal female greater amberjack could
469 explain their failure to respond to the combined sdrFsh/Lh treatment in our study.
470 As expected from the lack of stimulation of oogenesis, plasma levels of sex steroids did
471 not show a great increase in response to sdrFsh/Lh. Very small increases were observed in T
472 and E2 levels in the sdrFsh dose-response experiment, and a small and transient elevation of
473 17,20β-P occurred on week 6 of our combined sdrFsh/Lh stimulation experiment.
474 Testosterone levels increase in mature female teleosts during vitellogenesis, T is converted to
475 E2 through the activity of the aromatase enzyme in the follicular cells, and released E2 acts at
476 the hepatic level to promote the production of vitellogenin, which is then sequestered from
477 the bloodstream into the developing oocytes (Levavi-Sivan et al., 2010; Sullivan and Yilmaz,
478 2018). The lack of stimulation of ovarian steroidogenesis in the present study may further
479 support the conclusion that pre-pubertal female greater amberjack during their 2nd year of life

were not competent yet to respond to the sdrFsh/Lh treatment, possibly due to absence of the *fshr* in the ovary.

In conclusion, this study demonstrated the ability of a combined treatment of sdrFsh/Lh to stimulate spermatogenesis in pre-pubertal greater amberjack males, resulting in the production of strippable, high quality sperm, 1-2 years before the expected age of first reproductive maturation. Therefore, such treatment may be used commercially to advance puberty and reduce generation time in breeding selection programs. On the contrary, sdrFsh/Lh was ineffective in pre-pubertal females and further work is needed to investigate the reason for this failure and the possibility of advancing puberty in older juvenile greater amberjack (*e.g.* 3-4 years old) that would be closer to their natural age of reproductive maturation.

492 **Figure Legends**
493
494 **Fig. 1** Schematic representation of the sdrFsh/Lh experiment for the induction of
495 gametogenesis, using 20-month-old, pre-pubertal greater amberjack. Fish were given the
496 sdrFsh/Lh treatment every 7 days for a total of 12 injections (↓). Fish were bled every three
497 weeks to evaluate the plasma levels of sex steroids (🩸). The reproductive stage of the fish
498 was evaluated on week 12, seven days after receiving the last injection (🔬).
499
500 **Fig. 2** Mean (± SEM) gonadosomatic index (GSI, %) of 16- month-old, pre-pubertal male
501 (Saline-Control, n = 6; sdrFsh5-10-15, n = 4) and female (Saline-Control, n = 6; sdrFsh5-10-
502 15, n = 8) greater amberjack sacrificed at the end of the 6-week sdrFsh dose-response
503 experiment (**A** and **D**). Mean (± SEM) plasma levels of testosterone (T), 11-ketotestosterone
504 (11- KT) and 17β-estradiol (E2) of pre-pubertal greater amberjack males (**B** and **C**), and
505 females (**E** and **F**) at weeks 3 and 6 after sdrFsh treatment. Letter superscripts indicate
506 significant differences in GSI among treatment groups (Kruskal-Wallis's test, $P \leq 0.05$), and
507 among treatments in sex steroid hormone levels (two-way, Repeated Measures ANOVA,
508 Tukey HSD, $P \leq 0.05$)
509
510 **Fig. 3** Microphotographs of histological sections from testes of 16-month-old, pre-pubertal
511 greater amberjack males at the end of the sdrFsh dose-response experiment on week 6 (Fig.
512 2). **A.** Testicular section of a male from the Control group. Spermatogonia (so) are the main
513 cell population in the testes. **B.** Testis in early spermatogenesis from the sdrFsh5 group.
514 Different germ cells stages, *i.e.* so, spermatocytes (sc), spermatids (sd) and spermatozoa are
515 found in the testicular tubules. **C** and **D.** Advanced spermatogenesis in males from the
516 sdrFsh10 and sdrFsh15 groups, with luminal spermatozoa (sz) and active spermatocysts in
517 the germinal epithelium. Black scale bar = 100 μm.

Fig. 4 Microphotographs of histological sections from ovaries of 16-month-old, pre-pubertal-greater amberjack females at the end of the sdrFsh dose-response experiment on week 6 (Fig. 2). Primary oocytes (po) constituted the main cell population of the ovary in the females of all groups. **A**. Control group. **B**. sdrFsh5. **C**. sdrFsh10. **D**. sdrFsh15 group. Black scale bar = 100 μm.

Fig. 5 A. Microphotograph of histological sections from testes of 20-month-old, pre-pubertal greater amberjack males from the Control group on week 12, at the end of the experiment on the use of sdrFsh/Lh to induce gametogenesis. Spermatogonia (so) are the only cell population in the testes. **B.** Sperm release upon abdominal pressure from a male of the sdrFsh/Lh group on week 12. **C** and **D.** Microphotographs of histological sections from greater amberjack females from the Control and sdrFsh/Lh groups, respectively, on week 12. Primary oocytes (po) constituted the main cell population of the ovary in all females. The black scale bars indicate 100 (A) and 200 (C,D) μm

Fig. 6 Mean (± SEM) spermatozoa concentration ($\times 10^9$ ml^{-1}) **(A)**, percentage (%) of motile, progressive, and rapid cells and straightness (STR), (**B**) Curvilinear (VCL, μm sec^{-1}), straight line (VSL, μm sec^{-1}) and average path velocity (VAP, μm sec^{-1})(**C**) of sperm samples collected from greater amberjack males, 12 weeks after treatment with sdrFsh/Lh (n = 8 out of 8) or saline (Control, n = 1 out of 3) in the experiment to induce gametogenesis in 20-mo-old pre-pubertal fish. The sampling was done at the onset of the expected spawning season (June), at which time the fish were 24-mo-old. Statistical evaluation of sperm quality

parameters -obtained with computer assisted sperm analysis (CASA)- between treatments was not done, due to the limited number of sperm samples from the Control group (n=1).

Fig. 7 Mean (± SEM) plasma levels of testosterone (T), 11-ketotestosterone (11- KT), 17β-estradiol (E2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) of 20-month-old, pre-pubertal greater amberjack males (Control, n = 3; sdrFsh/Lh, n = 8) (**A, C, E** and **G**) and females (Control, n = 7; sdrFsh/Lh , n = 7) (**B, D, F** and **H**) treated with weekly injections of sdrFsh/Lh for 12 weeks. Letter superscripts indicate significant differences among sampling times within treatment group (two-way, Repeated Measures ANOVA, Tukey HSD, $P \leq 0.05$). Asterisks indicate significant differences between treatments within sampling time (two-way ANOVA, Tukey HSD, $P \leq 0.05$)

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Statements & Declarations

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Competing Interests

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: Ignacio Giménez and José Vicente Roig Genovés are associated with the biotech company Rara Avis Biotec, S. L., which produced the recombinant gonadotropins (sdrFsh/Lh) used in this study.

CRediT authorship contribution statement

Stefano Lancerotto: Data curation, Formal analysis, Investigation, Visualization, Writing- original draft, Writing- review & editing. **Ioannis Fakriadis:** Investigation, Writing- review & editing. **Maria Papadaki:** Investigation, Writing-Original draft preparation, Writing- review & editing. **Irini Sigelaki:** Investigation. **Ignacio Giménez:** Funding acquisition, Conceptualization, Visualisation, Methodology, Writing- review & editing. **Jose Vicente Roig Genoves:** Writing – review & editing, Data curation. **Andrea Meloni:** Investigation. **Constantinos C. Mylonas:** Funding acquisition, Conceptualization, Methodology, Investigation, Resources, Writing- original draft, Writing-review & editing.

Data availability

Data will be made available on request.

Figure 1

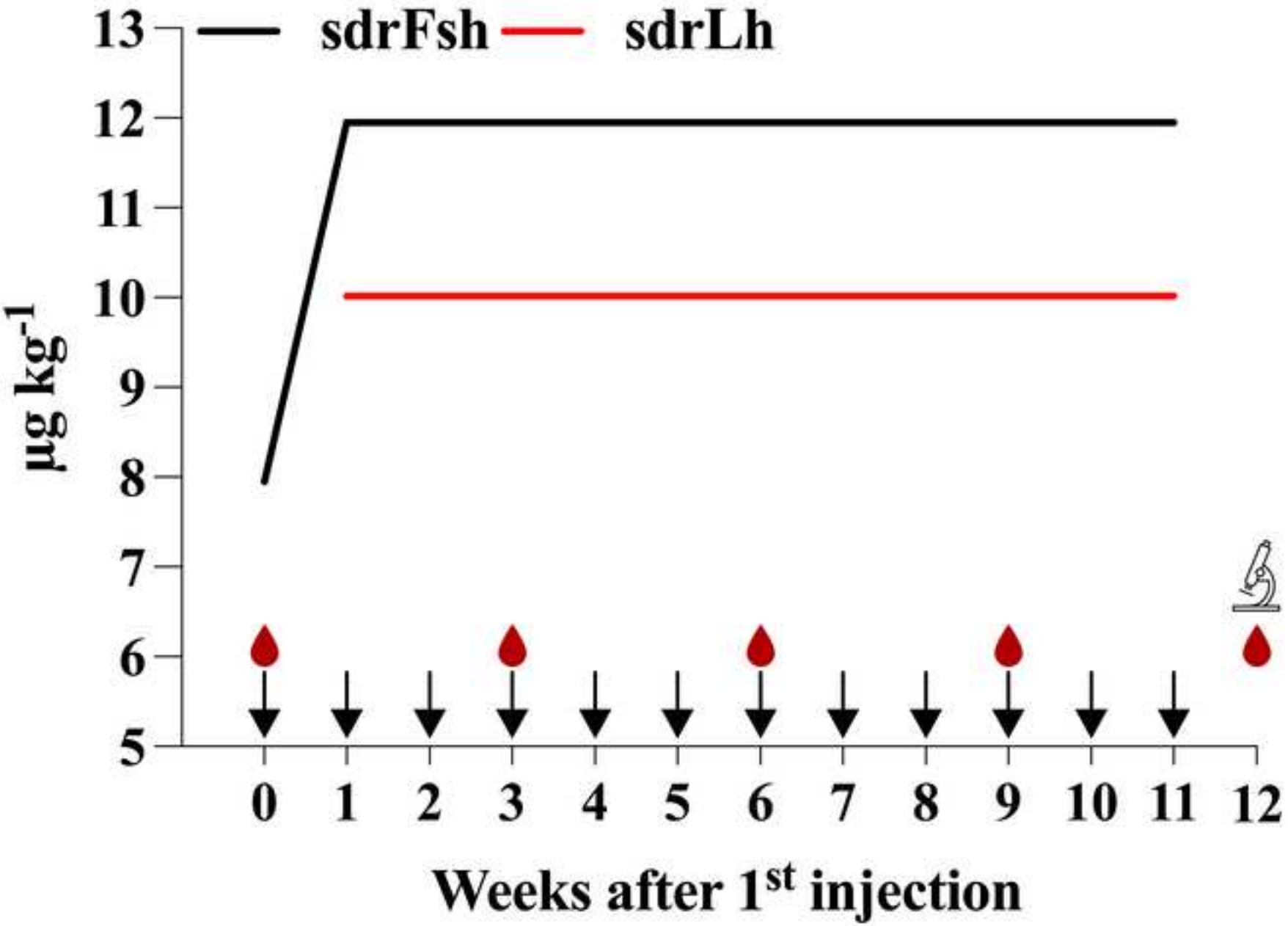

Figure 2

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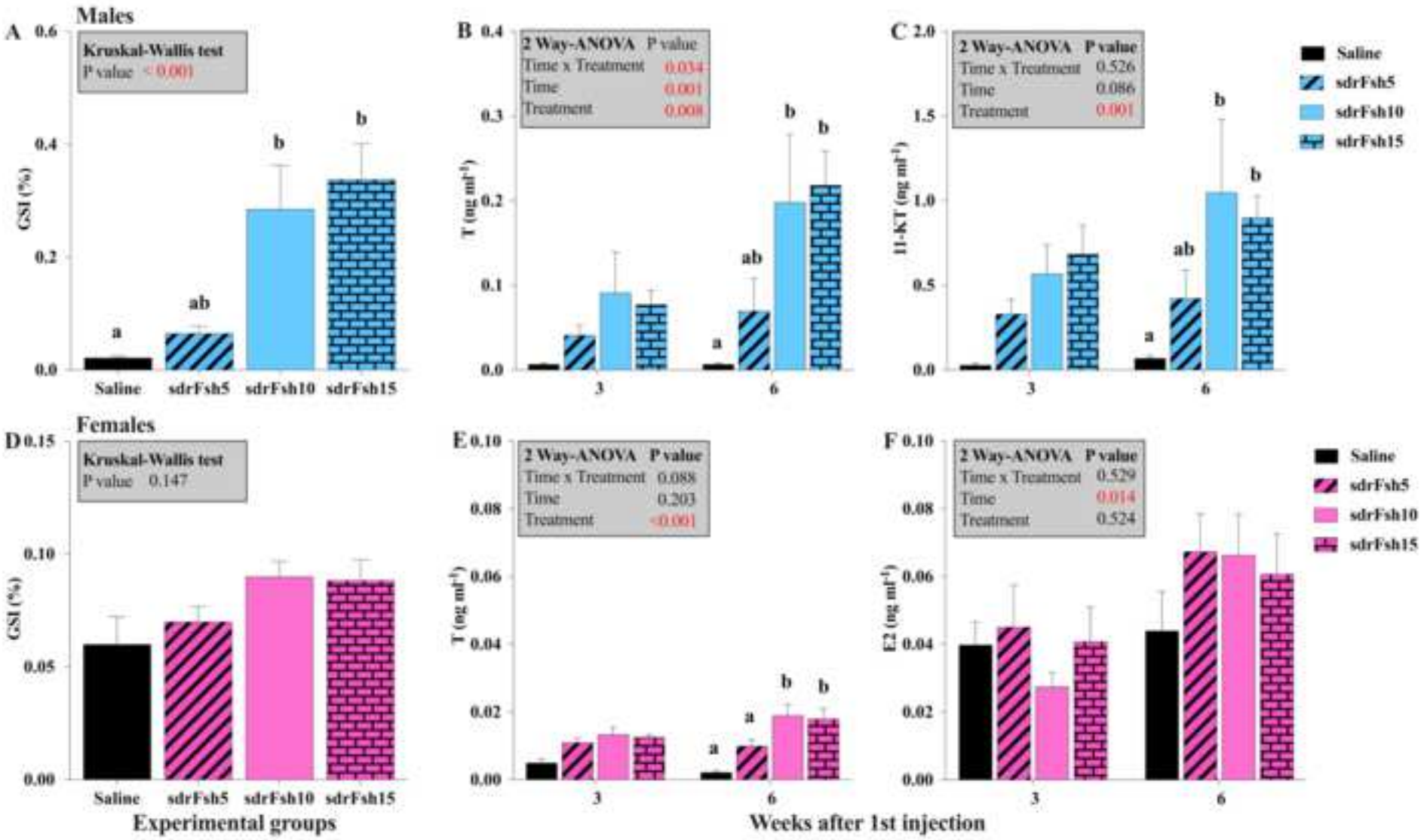

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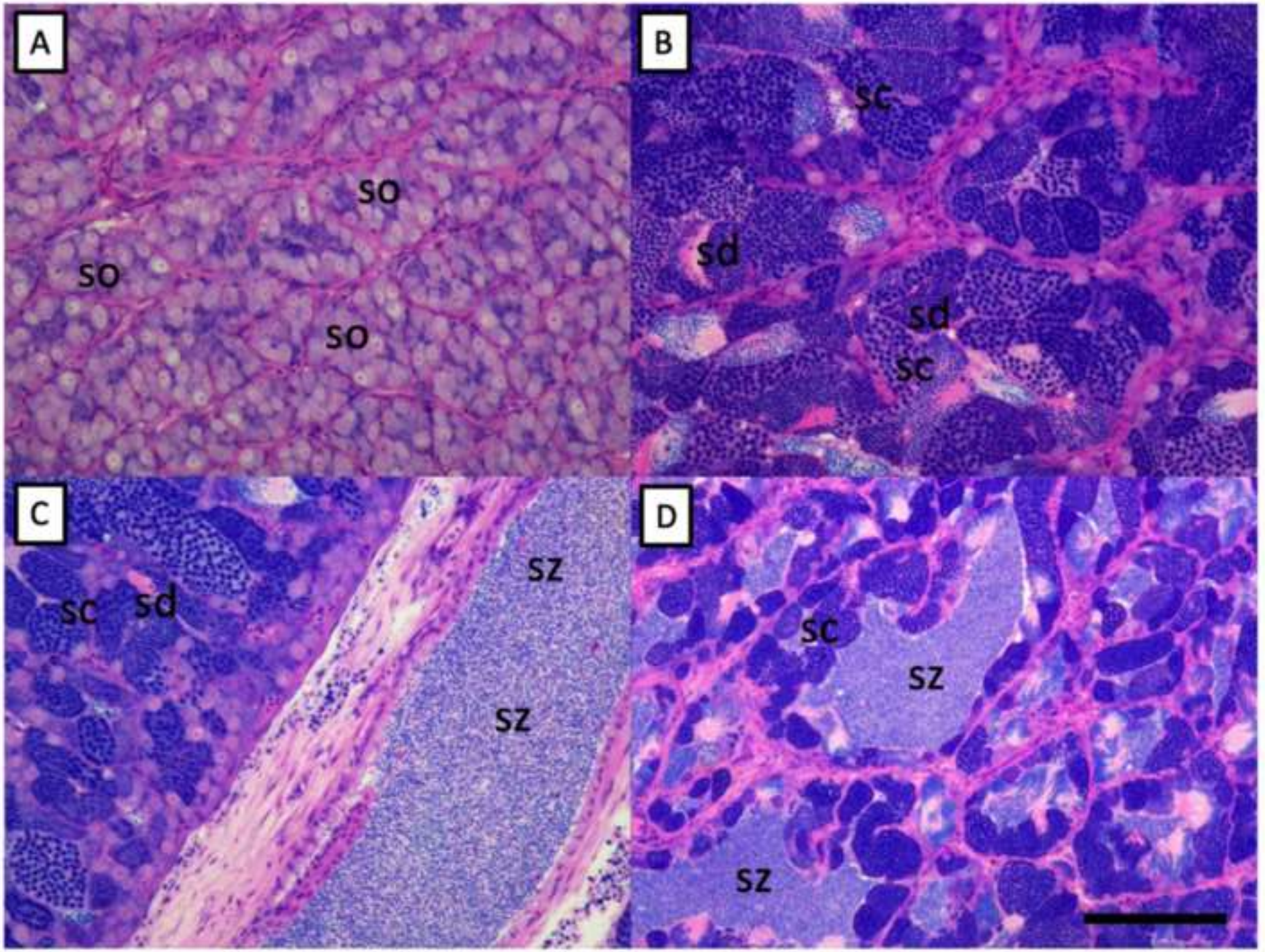

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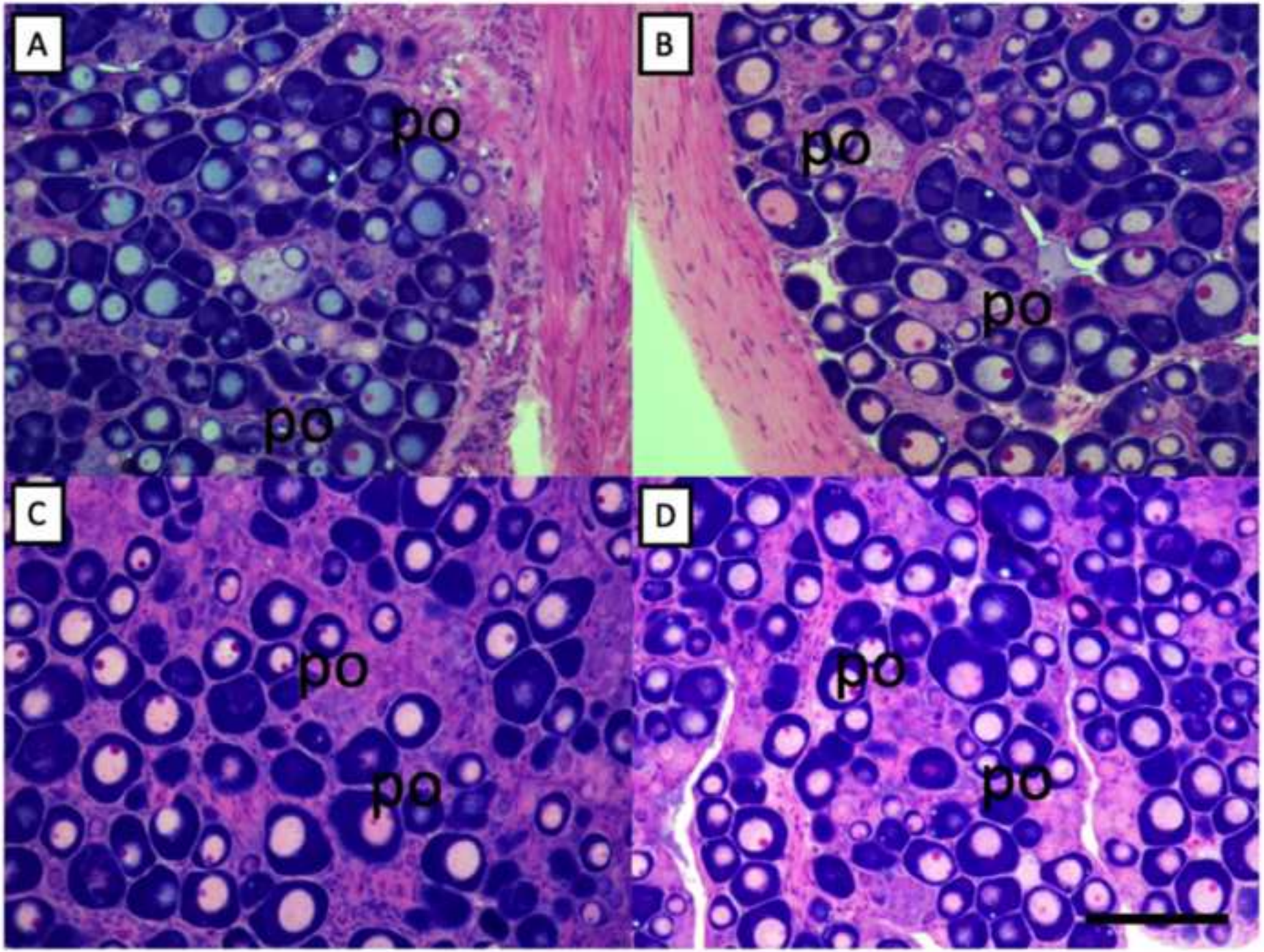

Figure 5

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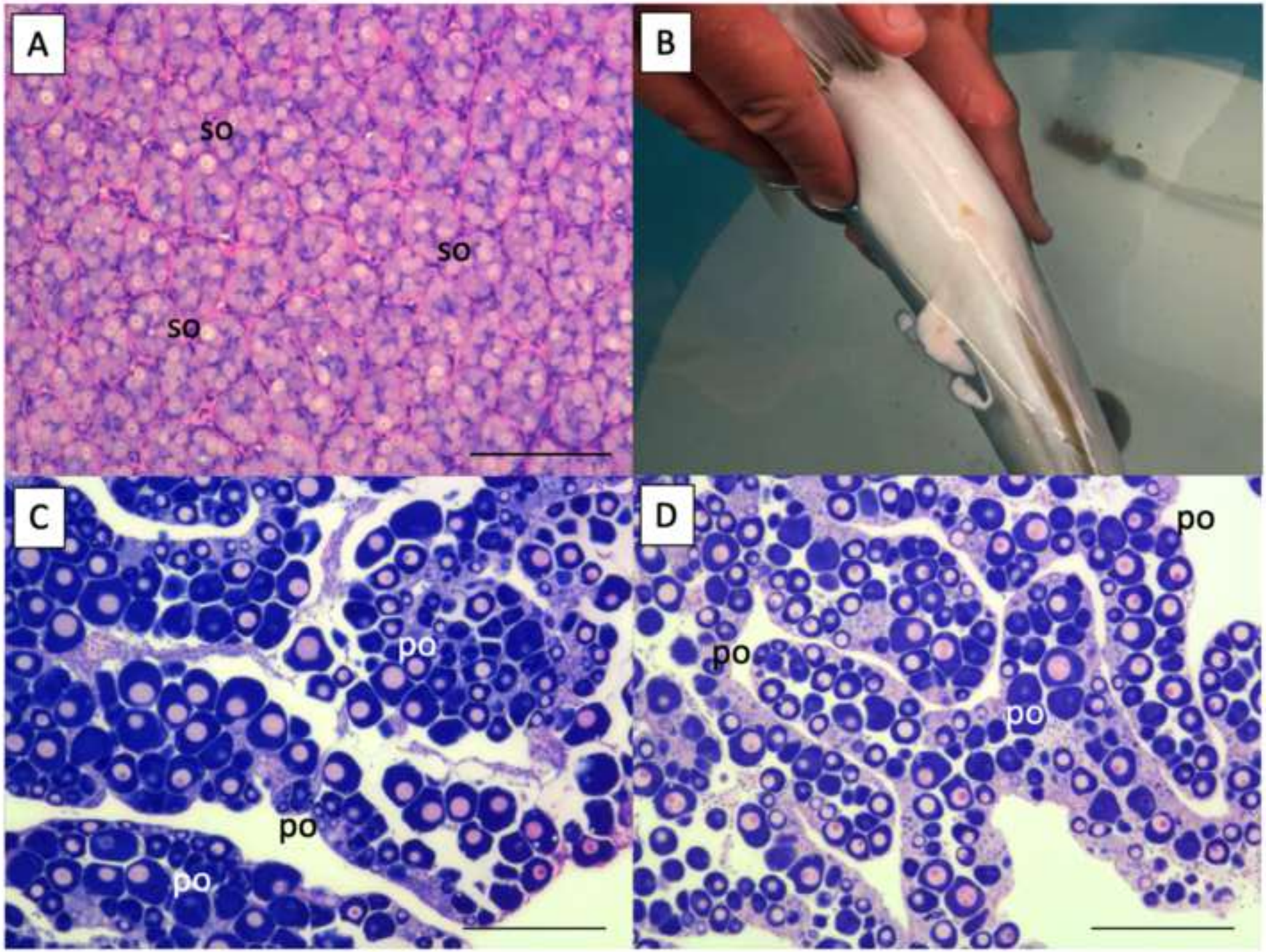

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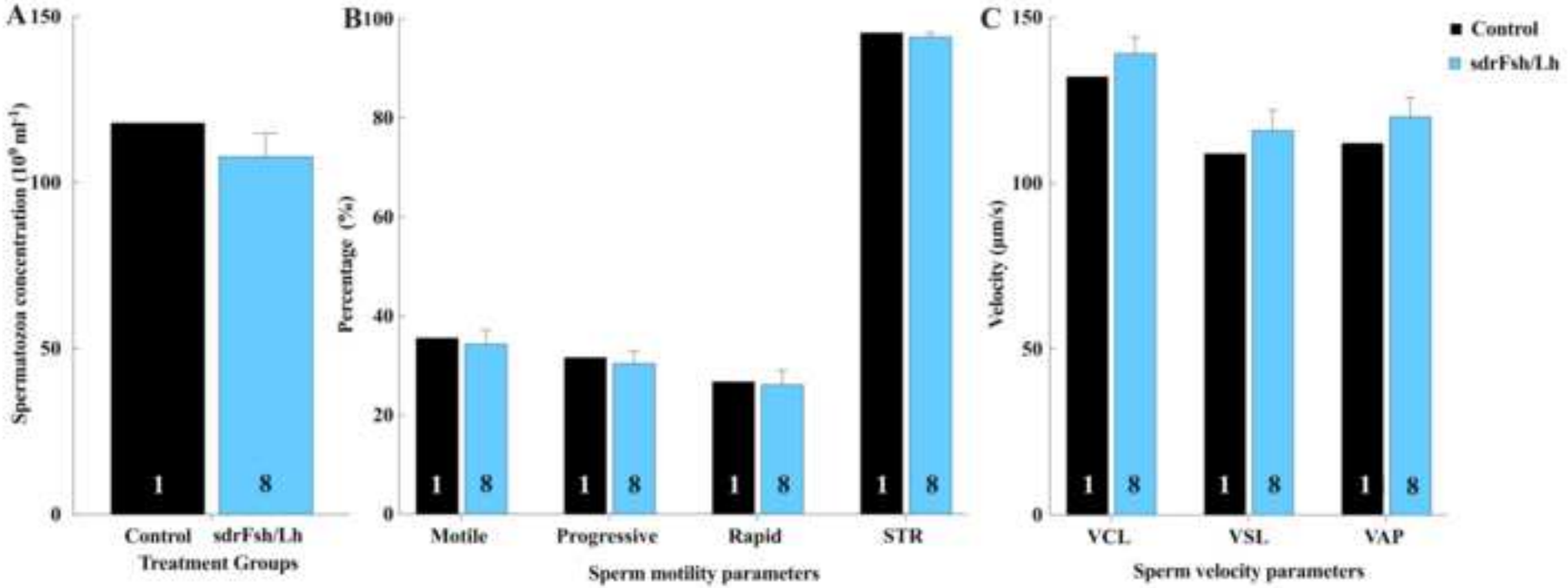

Figure 7

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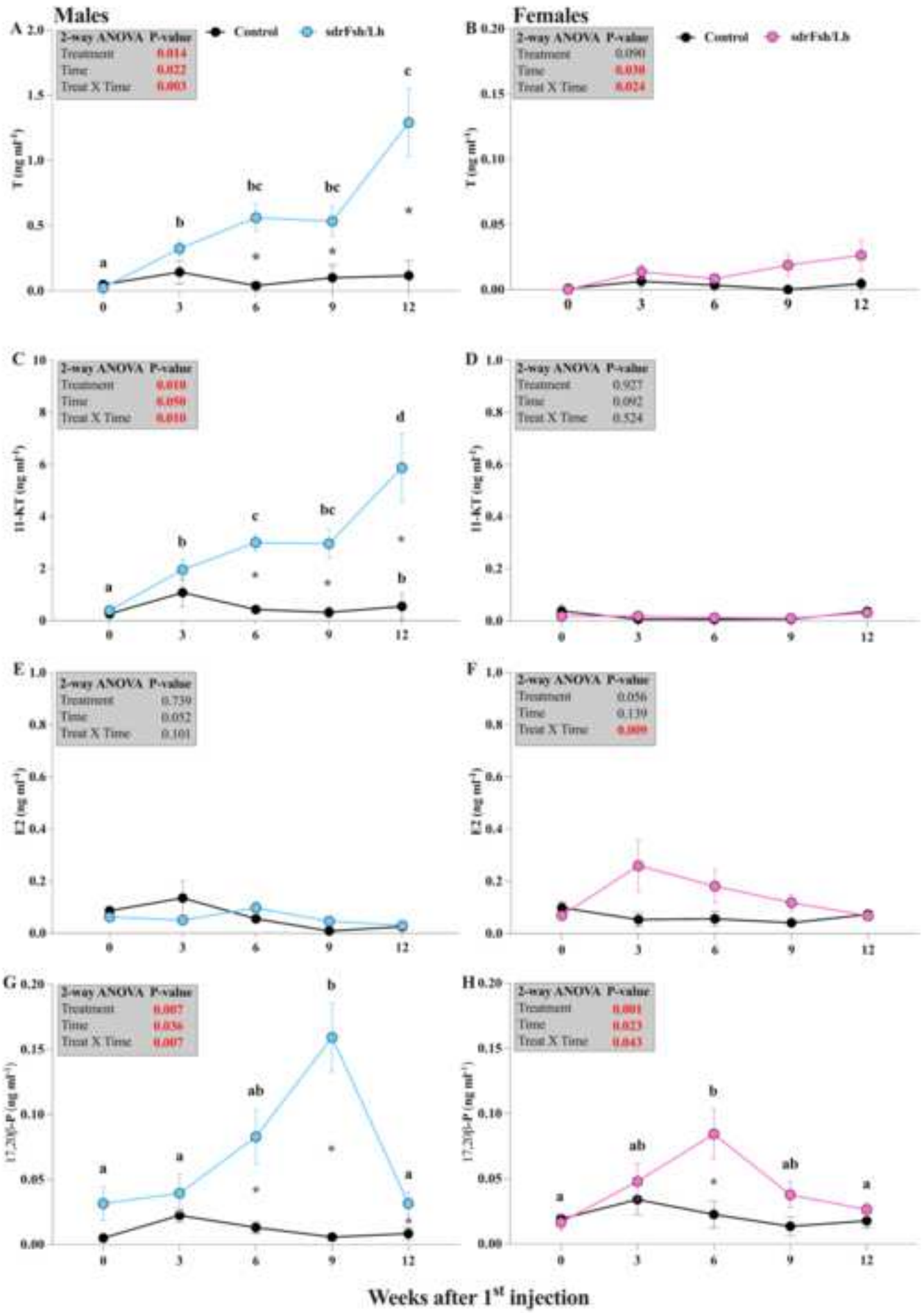

Declaration statement

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