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Providing recombinant gonadotropin-based therapies that induce complete gametogenesis and produce viable larvae from an immature teleost, flathead grey mullet (*Mugil cephalus*)

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Abstract

Under intensive captive conditions, wild-caught flathead grey mullet (*Mugil cephalus*) females remain arrested at early gonad development and males do not produce sperm. With the aim to induce gametogenesis and obtain fertilized eggs, adult female and male grey mullet were treated with *M. cephalus* single-chain recombinant gonadotropins (rGths), follicle-stimulating (rFsh) and luteinizing (rLh) hormones. In Experiment 1, a weekly dose of rFsh (15 µg kg^{-1}) significantly ($P < 0.001$) increased plasma concentration of 17β-estradiol in females and induced oocyte growth up to a maximum diameter of 425 ± 19 µm after 9 weeks of treatment. In males, spermiogenesis (> 75% motile spermatozoa) was induced after 5 weeks to produce low quantities (29.3 ± 6.1 µL) of viscous sperm. However, the application of therapies based on human chorionic gonadotropin (hCG) or gonadotropin releasing hormone agonist (GnRHa) were ineffective to complete oocyte maturation. In Experiment 2, fish were treated with weekly injections of both rFsh and rLh at different doses (from 2.5 to 12 µg kg^{-1}). Oocyte diameter reached 609 ± 5 µm prior to oocyte maturation and ovulation, which was induced with 30 µg kg^{-1} of rLh and 40 mg kg^{-1} of progesterone. Higher quantities of good quality sperm (92.7 ± 24.7 µL) were obtained after rLh treatment of males and used to fertilise the eggs. Although the percentage of fertilisation was low (0.4 %), these eggs were able to produce viable larvae (71% hatching rate). In comparison, control groups remained arrested as immature fish. The study demonstrated that both rGths are effective to induce the entire process of gametogenesis in sexually immature male and female grey mullet, which confirmed the central roles of the Gths in teleost gametogenesis. This advance provides the bases of a therapy for the use in the aquaculture of teleosts of commercial interest or the conservation of endangered species.

Keywords: *Mugil cephalus,* flathead grey mullet, gametogenesis induction, eggs, larvae, rFsh, rLh.

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1. Introduction

40 The flathead grey mullet (*Mugil cephalus*) is a catadromous teleost with a worldwide distribution (between latitudes 40º North and South) [1] that has been cultured for several 42 centuries principally in some Asian countries and along the Mediterranean basin. Many positive attributes of grey mullet culture have made this species a suitable option for 44 aquaculture. Grey mullet has fast growth (0.75 - 1 kg per year) [2], does not require dietary fish meal and oil and can be reared in a wide range of salinities and culture systems 46 including polyculture [3]. In addition, the final product marketed in various forms has good texture, taste [4] and is an excellent source of omega-3 essential fatty acids [5].

48 Although this species has a long history of culture, it exhibits different degrees of reproductive dysfunctions in both genders under captive conditions. These dysfunctions 50 have limited the possibility to close the life cycle and, thus, culture has been based on the capture of wild juveniles [4] or induced spawning of wild breeders [6 - 10]. Two types of 52 reproductive dysfunction have been described and can be placed in two categories; arrest in the late stages or early stages of gametogenesis. Arrest in late stages of gametogenesis 54 (maturation and ovulation) has been observed in recently caught wild mullet or wild mullet that were acclimated to ponds or large tanks [4, 11, 12]. This is the most commonly 56 observed dysfunction in fish and can be controlled by hormonally inducing spawning [13, 14], as has been achieved for flathead grey mullet [4, 6 - 11]. However, the spawning 58 induction of these wild fish arrested in the late stages of gametogenesis does not represent a sustainable solution for mullet culture. The more severe reproductive dysfunction when 60 development is arrested in the early stages of gametogenesis has been observed in wild and hatchery reared fish held in intensive culture conditions in the Mediterranean region. 62 Females did not initiate vitellogenesis; remained at the primary growth stage or cortical alveoli stage (present study), or were arrested at early stages of vitellogenesis [15]. Males 64 failed to initiate spermiation [16, 17] or produced highly viscous milt that could not fertilize the eggs [18]. These reproductive dysfunctions may be related to alterations in 66 the endocrine control in the brain-pituitary-gonadal (BPG) axis.

In vertebrates, the pituitary gonadotropins (Gths), the follicle-stimulating hormone (Fsh) 68 and luteinizing hormone (Lh), are generally accepted to be the central components of the BPG axis in the control of gonad development. Current knowledge in teleost suggest that 70 the major role of Fsh is to promote gametogenesis from early stages through to late stages (vitellogenesis in females and spermatogenesis in males), while Lh is involved in gamete 72 maturation and release (ovulation and spermiation, in females and males, respectively) [14, 19]. The mechanism underlying the reproductive dysfunctions in Mediterranean 74 captive mullets has been described as an inhibition by dopamine (DA) on the action of gonadotropin releasing hormone (GnRH) to release Gths in both females [15] and males 76 [20]. Therefore, methods based on the mechanisms controlling gametogenesis are required to induce complete gonadal development, from early stages through to the late 78 stages. In the case of males, 17α-methyltestosterone (MT) implants enhanced spermatogenesis and spermiation [15, 17]. In females, treatment with GnRH agonist 80 (GnRHa) in combination with a DA antagonist [15] or a single injection of recombinant Fsh produced in the yeast *Pichia pastoris* [21] increased the number of vitellogenic 82 females by promoting the release of Gths from the pituitary. However, hormonal therapies to enhance endogenous Lh release have been observed to be less effective when the 84 pituitary Lh content was low [22], indicating that alternative therapies may be required in these situations.

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86 An alternative strategy to control gametogenesis in mullet as in other teleost, which would not require the availability of endogenous Gths from the pituitary, is the long-term use of
88 recombinant Fsh and Lh (rFsh and rLh, respectively). This approach is nowadays possible through the production of large amounts of species-specific rGths in heterologous
90 expression systems, such as the *Drosophila* S2 cell line [23, 24], the yeast *Pichia pastoris* [25 - 30], baculovirus silkworm larvae [31 - 36], HEK293 cells [37] and mammalian
92 Chinese hamster ovary (CHO) cells [38 - 43]. The application of rGths based therapies has shown promise to control gametogenesis in different teleost [41 - 45], and therefore
94 could be an effective method to induce gametogenesis in cultured mullet arrested in the early stages of sexual maturation.

96 The present study aimed to use homologous single-chain rGths produced in CHO cells as the basis of a long-term hormone therapy to obtain viable offspring from flathead grey
98 mullet that were arrested in early gametogenesis. For this purpose, *M. cephalus* rFsh was administered to induce gametogenesis in immature females and males followed by
100 treatments to induce oocyte maturation, ovulation and spermiation, which were either (a) therapies previously employed in this species such as human chorionic gonadotropin
102 (hCG) and GnRHa, or (b) *M. cephalus* rLh.

2. Material and methods

104
2.1. Study animals and maintenance

Flathead grey mullet were used in two experiments to examine the effect of rGth hormone
106 therapies. Experiment 1 examined the long-term effect of rFsh on gametogenesis and was followed by Experiment 2 that examined the effect of a combined rFsh and rLh therapy.
108 Experiment 1, used wild-caught mullet from the Ebro River reared in 10-m³ tanks for 7-9 months in IRTA facilities (Sant Carles de la Ràpita, Spain). In Experiment 2, the
110 broodstock was formed with wild-caught individuals reared for 19-21 months and individuals reared for 3 months in IRTA that were obtained from semi-extensive pond
112 culture in the fish farm Finca Veta La Palma (Isla Mayor, Spain).

One month before each trial, individuals were transferred to two 10-m³ tanks in a
114 recirculating system (IRTAmar®) under natural conditions and were gradually acclimatized from fresh water to sea water at 36 ‰ to provide the conditions for gonad
116 development, as Tamaru *et al*. [46] concluded that the rate of oocyte growth was lower in females maturing in fresh water. During Experiment 1, completed from August to
118 November, water temperature in the tanks was controlled at 24 ± 1 ºC. Photoperiod was ambient until October, when a constant 11L:13D (light:dark) was maintained through to
120 the end of the experiment to avoid large changes of decreasing day length. Individuals were fed five days a week on a soft mixture of 20 % sardines, 20% hake, 15 % mussels,
122 10 % squid, 10 % shrimp, spirulina and 25 % a commercial broodstock diet (Mar Vitalis Repro, Skretting, Spain). In Experiment 2, completed from the end of July to mid-
124 October, water temperature was also controlled at 24 ± 1 ºC while photoperiod was ambient. Fish were fed a commercial marine fish broodstock diet (Brood Feed Lean,
126 Sparos, Portugal) during five days a week at a daily rate of 1.5% of the body weight and two days a week with mussels and polychaetes. To evaluate *in vivo* dose-response of rFsh,
128 fish were treated during May and held for 21 days when temperature was controlled to 24 ± 1 ºC, photoperiod was natural and feeding was as described for Experiment 2. Prior to
130 the experiments, fish had the same feeding regimens and were held in natural conditions of photoperiod and temperature.

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132 The present experimental study has been approved by IRTA's Ethics Committee for Animal Experimentation and the Animal Experimentation Commission from the Local
134 Government (*Dpt. de Territori i Sostenibilitat from the Generalitat de Catalunya*). The study was conducted in accordance with the European Union, Spanish and Catalan
136 legislation for experimental animal protection (European Directive 2010/63/EU of 22 September on the protection of animals used for scientific purposes; Spanish Royal
138 Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado (BOE), 2013; Catalan Law 5/1995
140 of June 21th, for protection of animals used for experimentation or other scientific purposes and Catalan Decree 214/1997 of July 30th for the regulation of the use of
142 animals for the experimentation or other scientific purposes). During all experimental procedures, for hormone administration and sampling, fish were first anaesthetised with
144 73 mg L^{-1} of MS-222 and placed in a tank with 65 mg L^{-1} of MS-222 for manipulation. An overdose of MS-222 (250 mg L^{-1}) was the method used for the euthanasia of two fish
146 in the present study. Death was confirmed by decapitation according to Directive 2010/63/EU guidelines.

148 2.2. Recombinant Gths production and biological activity

2.2.1. Cloning of *M. cephalus* Gths β and α subunits

150 The pituitary gland was removed from sacrificed fish, frozen in liquid nitrogen, and stored at -80ºC. Total RNA was purified using the GenElute™ mammalian total RNA miniprep
152 kit (Sigma-Aldrich) according to the manufacturer's instructions, and cDNA synthesis was performed with 1 µg of total RNA following the manufacturer's instructions of the
154 3'end RACE kit (Invitrogen). Polymerase chain reaction (PCR) was carried out as indicated in the 3'end RACE kit using partially degenerated forward primers for the Fshβ
156 or α subunits, the common abridged universal amplification primer (AUAP) as reverse primer, and the EasyA™ high-fidelity PCR cloning enzyme (Agilent Technologies, Santa
158 Clara, CA, USA). The forward primer for each gene covered the translation initiation codon ATG and was designed based on sequences available in the GenBank repository
160 for *Epinephelus coioides* (AY186242), *Oreochromis niloticus* (AY294015), *Dicentrarchus labrax* (AF543314), *Acanthopagrus schlegelii* (AY921613), *Maylandia*
162 *zebra* (XM_004558042), *Fundulus heteroclitus* (M87014), *Oryzias latipes* (AB541981), *Sparus aurata* (AF300425), *Amphiprion melanopus* (EU908056), *Chrysiptera parasema*
164 (KM509061), and *Kryptolebias marmoratus* (EU867505). For Fshβ, the forward primer was 5'-ATGCAGCTGGTTGTCATGGYAGC-3', whereas for the α subunit the primer
166 was 5'-ATGGGCTCMNTGAAAYCHVCTG-3. The Lhβ subunit was cloned using a degenerate forward primer covering the central region of the RNA (5'-
168 CAAYCAGACRRTDTCTCTRGA), designed based on teleost sequences publically available (*E. coioides*, AY186243; *O. niloticus*, AY294016; *D. labrax*, AF543315; *A.*
170 *schlegelii*, EF605276; *M. zebra*, XM_004553532; *Pundamilia nyererei* XM_005741532; *F. heteroclitus*, M87015; *Cyprinodon variegatus*, XM_015404196; *O. latipes*,
172 AB541982; *Kryptolebias marmoratus*, XM_017431834; *Poecilia reticulata* XM_008429103; *Nothobranchius furzeri*, XM_015975766; *Xiphophorus maculatus*,
174 XM_005816155), and the reverse AUAP primer. The 5' end of the cDNA was further amplified using RACE (5'end RACE kit, Invitrogen) and specific primers. In all cases,
176 the PCR products were cloned into the pGEM-T Easy vector (Promega Biosciences, LLC, San Luis Obispo, CA, USA) and sequenced by BigDye Terminator Version 3.1 cycle
178 sequencing on ABI PRISM 377 DNA Analyser (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The nucleotide sequence corresponding to the full-length Lhβ, Fshβ

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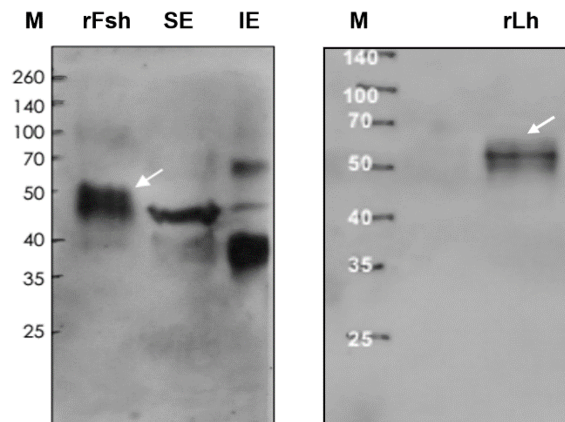
180 and α subunit cDNAs were deposited in GenBank with accession numbers MF574169, MF574168 and MF574167, respectively.

182 2.2.2. Production of *M. cephalus* rFsh and rLh

Recombinant *M. cephalus* rFsh and rLh were produced by Rara Avis Biotec S.L. 184 (Valencia, Spain) as described in previous studies [42, 47]. Briefly, the constructs that encoded a fusion protein containing the coding sequence of the *M. cephalus* Fshβ subunit 186 (GenBank accession no. MF574168) or Lhβ (GenBank accession no. MF574169), the 28 amino acids of the carboxyl-terminal sequence of the hCG β and the sequence of the *M.* 188 *cephalus* glycoprotein hormone α subunit (GenBank accession no. MF574167) were transfected to CHO cells. After a suspension culture of 120 h, the secreted recombinant 190 hormones in the culture medium were purified by ion exchange chromatography and subsequently concentrated. A semiquantitative Western blot was used to quantify the 192 hormones (Fig 1) by means of the use of antibody against European seabass (*D. labrax*) glycoprotein α subunit raised in rabbit. Recombinant gonadotropin hormones were stored 194 at -80 ˚C until use.



196 **Fig 1. Western blot analyses of culture medium from CHO cells expressing M. cephalus rFsh and rLh.** Protein expression of rFsh and rLh was assessed by reaction with a rabbit anti-European seabass α- 198 subunit antisera after culture medium precipitation with ethanol and resuspension in phosphate buffered saline to a 10x of protein concentration. Thick positive bands (indicated by arrows) with an apparent 200 molecular mass from approximately ~60 kDa to ~45 kDa were considered to be rFsh and rLh secreted in the medium. M, Molecular weight markers (kDa); SE, soluble cellular extract; SI, insoluble cellular extract.

202 2.2.3. *In vivo* dose-response of rFsh on female steroid production

To evaluate the biological potency of rFsh produced in CHO cells in inducing 17β- 204 estradiol (E_2) production and to determine the minimum effective dose and optimal dosing schedule, a single intramuscular injection of different rFsh doses (0, 3, 6, 9, 12 206 and 15 µg kg^{-1}) were administered to immature grey mullet females (five fish per dose group) (mean body weight 0.9 ± 0.3 kg). Blood samples (0.40 mL) were collected before 208 injection (day 0) and at different days (1, 3, 7, 9, 13, 17, 21 days) after injection. Control females were injected with CHO conditioned culture medium (1 mL $fish^{-1}$).

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210 **2.3. Experiment 1. Long-term rFsh therapy**

In Experiment 1, the recombinant hormone, rFsh, was used in combination with other
212 hormone therapies, GnRHa and hCG, that had been successfully used in earlier studies to
induce final maturation in grey mullet [4, 7, 15].

214 **2.3.1 Stage 1. Induction of gametogenesis in males and females**

Twenty-six immature adult flathead grey mullet were used in the trial. To determine the
216 sex of individuals, a sample of gonadal biopsy was obtained through slight suction with
a plastic catheter (1.67 x 500 mm; Izasa Hospital, Barcelona) inserted approximately 5
218 cm into the gonopore. Individuals were assigned as males if traces of testicular cells were
obtained and / or no oocytes were observed in the biopsies. Twelve and fourteen
220 individuals were randomly assigned to two treatment groups. Nine females and three
males (mean ± SD body weight 1 ± 0.3 and 0.9 ± 0.1 kg; mean standard body length 41.4
222 ± 4.1 and 40.8 ± 2.4 cm, respectively) to the treatment group and 11 females and three
males (mean ± SD body weight 1 ± 0.2 and 0.9 ± 0.1 kg; mean standard body length 42
224 ± 4.1 and 41.3 ± 1.5 cm, respectively) to the control group. Only three males were selected
for each group, as only six males were available. The fisheries capture to form the
226 broodstock was biased towards females as has been observed in other studies [48].
Individuals belonging to the treatment group received weekly intramuscular injections of
228 specific grey mullet rFsh at a dose of 15 µg kg^{-1} for 11 weeks (Fig 2). The rFsh dose
applied was chosen according to the dose with highest potency on E_2 induction in the *in*
230 *vivo* dose-response study. The dose and the time frame of administration were also
selected based on the results obtained in a previous study on Senegalese sole (*Solea*
232 *senegalensis*) using recombinant Gths produced in CHO cells. Chauvigné *et al.* [42]
described that a dose of 12 - 17 µg kg^{-1} rFsh was effective in stimulating spermatogenesis,
234 while the hormone was detectable in the bloodstream for approximately seven days. The
control fish were injected in the same manner as rFsh treated fish, but with CHO
236 conditioned culture medium (1 mL $fish^{-1}$). Fish were sampled before the first injection
and on different weeks before receiving the corresponding weekly injection. At
238 fortnightly intervals, blood samples (0.40 mL) from the caudal vein and oocytes through
cannulation were obtained. The diameter of the largest oocytes (n = 20) were measured
240 *in situ* and samples were fixed for histology. In parallel, males received a gentle
abdominal pressure to check the presence of milt.

242 **2.3.2 Stage 2: Induction of oocyte maturation in females**

This second stage of the experiment investigated the effects of different hormones to
244 induce oocyte maturation in adult females that were treated with rFsh to induce
vitellogenesis to advanced stages. Five females were not used in the second stage and
246 rFsh administration was stopped, although oocyte changes were assessed until the end of
the experiment. Stage 2 focused on the four fish with the most advanced stages of
248 vitellogenesis. One female with a mean maximum oocyte diameter of 539 ± 5 µm was
treated with GnRHa in combination with Metoclopramide (MET), a dopamine antagonist,
250 according to Aizen *et al.* [15] protocol, which consisted of a priming (GnRHa 10 µg kg^{-1}; MET 15 mg kg^{-1}) and a resolving (GnRHa 20 µg kg^{-1}; MET 15 mg kg^{-1}) injection
252 administered 22.5 h apart. Three females that reached a mean maximum diameter of 450
± 10, 450 ± 9 and 470 ± 8 µm after rFsh treatment, received weekly consecutive injections
254 of hCG at increasing doses (1000, 2000, 6000, 12000 IU kg^{-1}) in combination with the
rFsh treatment (15 µg kg^{-1}). One of those females that received hCG injections, was also
256 treated with one injection of 9000 IU kg^{-1} hCG and 25 mg kg^{-1} of progesterone (Prolutex,

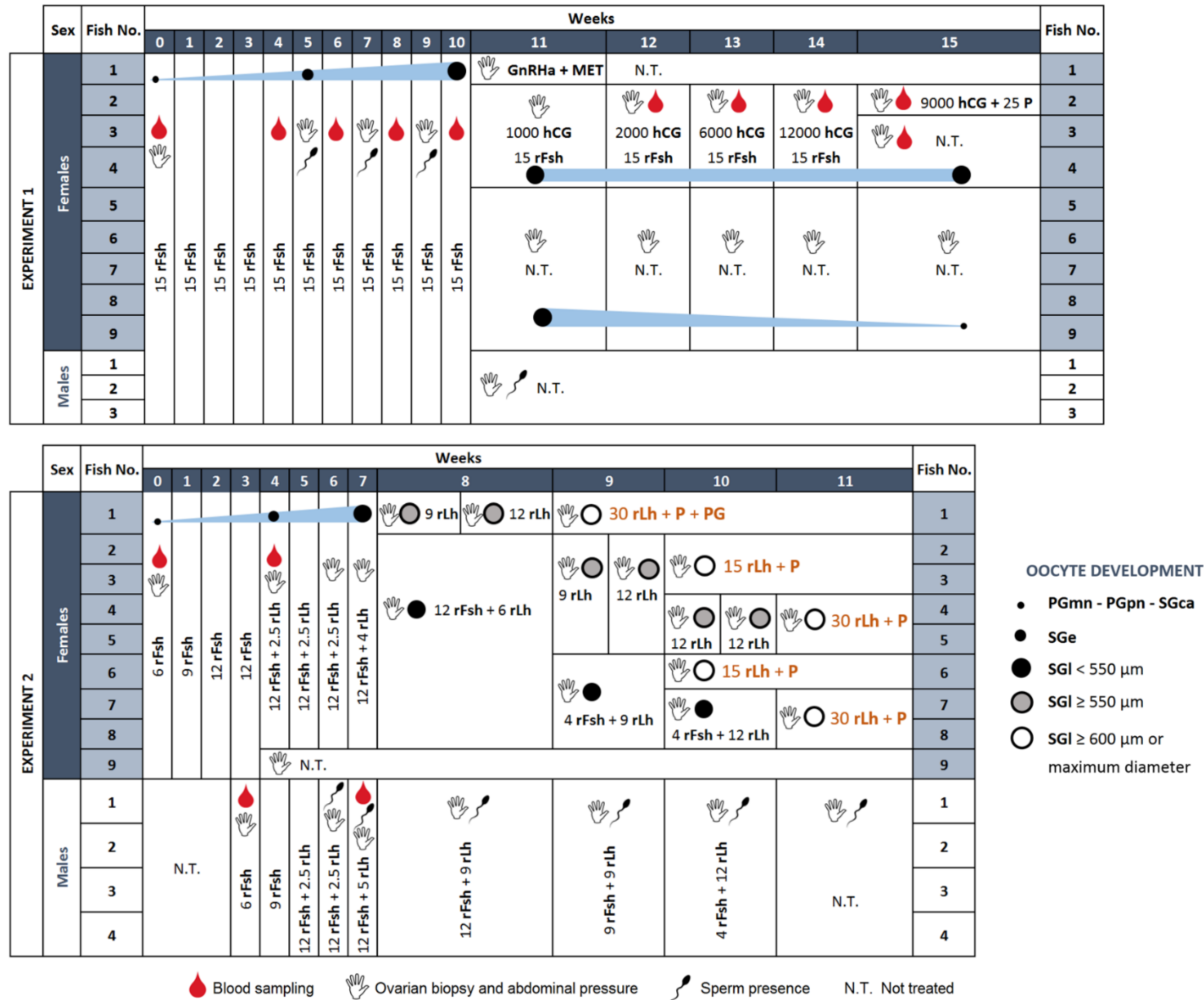

Fig 2. Schematic representation of the induction protocols administered to flathead grey mullet (*M. cephalus*). Upper figure represents Experiment 1 and lower figure, Experiment 2. Columns represent weeks of each experiment and rows represent the different fish. In Experiment 1, adult grey mullet females (n = 9) and males (n = 3), received weekly doses of intramuscular injections of rFsh. From 11 weeks onwards, the females with the most advanced stages of vitellogenesis received different weekly treatments. Female 1 received a GnRHa + MET protocol consisted of a priming (GnRHa 10 µg kg^{-1}; MET 15 mg kg^{-1}) and a resolving (GnRHa 20 µg kg^{-1}; MET 15 mg kg^{-1}) injection administered 22.5 h apart [15], whilst females 2 - 4 were administered increasing doses of hCG (plus 25 mg kg^{-1} of progesterone "P" in one case) in addition to rFsh. In Experiment 2, females (n = 9) received increasing doses of rFsh, and from the 4th week combined with increasing doses of rLh, followed by a decrease in rFsh. When females presented ≥ 550 µm oocytes rLh was administered every three days (weeks 9 and 10 for females 2 - 5). When the most developed oocytes reached a diameter of ≥ 600 µm, females were administered higher doses of rLh 15 µg kg^{-1} (females 2, 3 and 6) or 30 µg kg^{-1} (females 1, 4, 5, 7 and 8) combined with a 40 mg kg^{-1} injection of P administered 24 h after the rLh injection to induce oocyte maturation, ovulation and spawning. Female 1 was also administered 18.75 µg kg^{-1} of prostaglandin F2α (PG) 39 hours after the rLh injection. In experiment 2, males (n = 4) initiated rFsh treatment on week 3 and were administered a similar, but shortened program of increasing rFsh dose followed with a combined increasing rLh before decreasing rFsh. Doses of rFsh and rLh are expressed in µg kg^{-1} and doses of hCG in IU kg^{-1}. A hand symbol represents when ovarian biopsies or abdominal massage for sperm were made, red drops represent blood sampling, a spermatozoa represents when males had flowing sperm and circles represent the different stages of oocyte development encountered; small black is primary growth to cortical alveoli stage, medium black is early secondary growth (vitellogenesis), large black is late secondary growth < 550 µm oocyte diameter, large grey is late secondary growth ≥ 550 µm diameter and large white is late secondary growth ≥ 600 µm diameter or maximum diameter achieved.

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258 IBSA Group, Italy). Dosage of hCG were in the range of previous studies on grey mullet maturation [4, 7] and other fish species [14]. Weekly samples of oocytes and blood (0.40
260 mL) were obtained and the presence of sperm was assessed.

2.4. Experiment 2. Combined rFsh and rLh therapy

262 A total of twenty-four adult flathead grey mullet were used in Experiment 2. Fish were sexed as in Experiment 1. Females at a body weight of 0.9 ± 0.1 kg (mean ± SD) and
264 standard length of 38.5 ± 3.1 cm, and males at 0.6 ± 0.1 kg and 33.3 ± 1.2 cm formed the treatment group, while females at a body weight of 0.8 ± 0.1 kg and standard length of
266 39.5 ± 1.3 cm and males at 0.8 ± 0.1 kg and 38.6 ± 2.7 cm were used as controls. In this trial, 2/3 of the females were immature with previtellogenic ovaries, while 1/3 were at the
268 beginning of secondary growth (with cortical alveoli stage oocytes). All females were randomly distributed between treated and control groups. The aim of the administration
270 pattern was to simulate natural increases and decreases of gonadotropins in bloodstream of individuals according to their suggested regulatory role in gamete development [49].
272 Initial administration of rFsh followed by a gradual increase of rLh as gametogenesis progresses and subsequent decline of rFsh.

274 2.4.1. Females

Initially, all nine females received increasing doses of rFsh, 6 μg^{-1} kg (week 0) and 9 μg^{-1} kg (week 1) before the dose was fixed at 12 μg kg^{-1} rFsh per week (Fig 2). A maximum
276
12 μg kg^{-1} dose was selected for long-term treatment based on Experiment 1 and the *in*
278 *vivo* dose-response study. From the 4th week onwards, females (n = 8) were also administered a weekly injection of rLh at increasing doses (2.5, 4, 6 μg kg^{-1}). When
280 vitellogenesis arrived to advanced stages (week 9), weekly rFsh dose was decreased to 4 μg kg^{-1} while rLh dose was increased (9 and 12 μg kg^{-1}). At this point (week 8 and
282 onwards), treatments were adjusted accordingly to oocyte diameter of each individual fish. When females presented oocytes ≥ 550 µm, oocytes were considered to have
284 completed vitellogenic growth, therefore, no more rFsh was administered and consecutive doses starting with 9 and maintaining 12 μg kg^{-1} rLh were administered every 3 days. The
286 aim of this increased frequency of administration was to maintain high levels of rLh in the bloodstream, based on the half-life described for rLh produced in CHO cells and
288 administered to Senegalese sole [42]. When the most developed oocytes reached a diameter ≥ 600 µm or did not show further growth, females were administered higher
290 doses of rLh (15 or 30 μg kg^{-1}) combined with a 40 mg kg^{-1} injection of progesterone (Prolutex, IBSA Group, Italy) administered 24 h after the rLh injection to induce oocyte
292 maturation, ovulation and spawning (see schematic representation of the experimental setup in Fig 2). Three females received 15 μg kg^{-1} of rLh and five females received 30 μg
294 kg^{-1}.

After the application of rLh, females were placed in a 10 m^3 tank with spermiating males.
296 Surface out-flow egg collectors were placed to receive eggs from the tanks and were checked for eggs regularly. The fish were also observed frequently (from outside of the
298 tank), for swelling of the abdomen (hydration) in females and the initiation of courtship behaviour. These frequent checks were made as there is no established latency time of
300 spawning for rGth treatments. Latency time reported for grey mullet after resolving doses from other hormone treatments varies from 17 to 48 hours at 22 - 25 ºC [7, 50]. One
302 female (female 1, 30 μg kg^{-1} rLh + 40 mg kg^{-1} P in Fig 2) that had oocytes ≥ 600 µm earlier (week 8) than the other females, developed a large swollen belly without ovulation
304 and was administered 18.75 μg kg^{-1} of prostaglandin F2α (VETEGLAN, Laboratorios

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Calier, S.A., Spain) 39 hours after the rLh injection. The other seven females (females 2 - 8 in Fig. 2) did not receive prostaglandins and were checked and/or stripped as there was no natural spawning. Four females ovulated and were stripped, one female (female 1) at 40 h and three (females 4, 5 and 7) at 48 ± 0.5 h after the rLh injection. Total number of eggs (fecundity) was estimated by counting the number of eggs in triplicate in a subsample of 500 µl.

The seven females in the control group underwent the same number of intramuscular injections as treated females but with CHO culture medium (1 mL fish^{-1}). Females were sampled for oocyte tissue (weeks 0, 4, 6, 7, 8, 9, 10, 11, immediately before hormone administration) and blood (week 0 – before treatment, week 4 – after 3 weeks of rFsh treatment).

2.4.2. Males

The treatment of males in the rGth group (n = 4) initiated three weeks after the females in order to synchronise development of both sexes and have sperm and eggs available at the same time for fertilisation. The same rFsh doses were applied as for females and the dose range of rLh was fixed accordingly to other studies in male spermatogenesis and spermiogenesis [43, 45].

The four males in the control group were treated as previously reported for control groups. Males were sampled for sperm (weeks 3, 6, 7, 8, 9, 10 and 11 of the experiment) and blood (week 3 – before treatment, week 7 – after 3 weeks of hormone treatment).

2.4.3. *In vitro* fertilisation

For the *in vitro* fertilisation, sperm from three males was obtained, diluted 1:4 in the extender solution Marine Freeze® (IMV Technologies, France) that showed the best results for sperm conservation in a marine species [51] and stored at 4ºC to fertilise the eggs. The eggs from each female (n = 3) were stripped and total volume registered. Aliquots of 0.5 mL of eggs (~1200 eggs) from each female were each fertilised in triplicate with a pool of 60 µL of diluted sperm (20 µL from each of the three males, ~190,000 spermatozoa egg^{-1}) (3 females x 3 triplicates = 9 fertilisations). The diluted sperm was pipetted directly onto the 0.5 mL of eggs in a 100 mL beaker and immediately activated by mixing the eggs and sperm with 5 mL of clean tank water. After 5 minutes, the beaker was filled to 100 mL with clean tank water and placed in a temperature-controlled incubator (24ºC) to incubate the eggs. Twenty-two hours after fertilisation, all eggs were checked for embryo development and the percentage of eggs fertilised was calculated as the number of eggs with live embryos/number of eggs used for the *in vitro* fertilisation. Eggs with embryonic development were transferred individually into individual wells filled with sterile seawater in a 96 well plate and incubated (24ºC). The hatching success was calculated as the number of hatched larvae/live 22-hours embryos. Larvae was checked every 24 h until all hatched larvae had died and larval survival rate was calculated as the number of live larvae / larvae that hatched. A subsample of ~1/3 fertilised eggs and larvae were used for taking measurements and afterwards returned to the incubation.

2.5. Plasma steroid analysis

Blood samples were centrifuged at 3,000 rpm at 4 ºC for 15 min and the plasma stored at -80 ºC until steroid analysis. Plasma levels of E_2 and 11-ketotestosterone (11-KT) were measured for females and males, respectively, and were analysed using a commercially available enzyme immunoassay (EIA) kits (Cayman Chemical Company, USA). Steroids

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were extracted with methanol, which was evaporated and extracts were re-suspended 1:10
352 in the EIA buffer.

2.6. Histological observations and classification of developing ovaries

354 Ovarian biopsy samples were preserved in Bouin's fluid, dehydrated through an ethanol series and embedded in paraffin. Histological sections (3 μm) were stained with
356 hematoxylin and eosin (Casa Álvarez, Spain). To examine ovarian development, oocytes sections were observed under a light microscope (Leica DMLB, Houston, USA).
358 Quantification of the percentage of oocytes in different stages in the ovaries among weeks was made by the identification of 50 - 100 random oocytes per female. Oocyte
360 developmental stage was based on the identification of structures, morphological changes and increasing oocyte diameter. Oocytes were classified as: *multiple nucleoli step of*
362 *primary growth (PGmn)* characterised by small oocytes with multiple nucleoli that were not situated in the periphery of the germinal vesicle, *perinucleolar step of primary growth*
364 *(PGpn)*, with the nucleoli located around the internal germinal vesicle membrane, *cortical alveoli step (SGca)*, determined by the presence of small oil droplets and granular vesicles
366 "cortical alveoli" in the peripheral ooplasm, *early secondary growth (SGe)*, with the appearance of yolk globules and with this the initiation of vitellogenesis, *late secondary*
368 *growth (SGl)*, when oocytes reached ≥ 450 μm, *oocyte maturation stage (OM)*, with the identification of coalesced oil droplets and the displacement of the germinal vesicle to the
370 ooplasm periphery and some hydration and coalescence of yolk globules, and *ovulation stage (OV)*, when one large yolk globule is observed [3, 19, 52].

372 ### 2.7. Sperm collection and evaluation

Sperm samples were collected in a 1 mL syringe avoiding the contamination by faeces,
374 urine and / or sea water. Approximately 1 μL of sperm was placed on a microscope slide beside a 0.2 mL of sea water, mixed to activate the spermatozoids and immediately (first
376 10 seconds) observed through a microscope at 100x magnification (Zeuss Microscopes). The assessment of the milt quality was estimated by the percentage of motile spermatozoa
378 and by the total duration of the movement from sperm activation until all forward movement of spermatozoa stopped. The observations were made in triplicate and the
380 percentage of motile spermatozoa was classified into different motility scores: 0 for no motile sperm, 1 for > 0 – 25 % of sperm with progressive movement, 2 for > 25 % - 50
382 % of sperm with progressive movement, 3 for > 50 – 75 % and 4 for > 75 % of sperm with progressive movement [53]. For those samples in Exp. 2 with a motility score of 4
384 and manageable sperm volumes (≥ 100 μL) (n = 10), sperm quality was also evaluated using a CASA system [54]. For this, 0.5 μL of diluted sperm (1/4 in Marine Freeze®)
386 were dropped on the centre of a slide and activated using 20 μL of sea water. A 1 μL sample containing the activated spermatozoa was pipetted into an ISAS counting chamber
388 (Integrated Sperm Analysis System, Spain). The tracks of the activated spermatozoa were recorded through a bright field equipped video microscope at 200x magnification
390 (Olympus BH Microscope and DMK 22BUC03 Camera with 744×480 "0.4 MP" resolution at 60 FPS, The Imaging Source Europe GmbH, Bremen, Germany). The video
392 sections from 15 to 17 s after activation were transformed to image sequences using VIRTUALDUB 1.9.11 (virtualdub.org) free software. The spermatozoa in each field
394 were selected by adjusting the grayscale threshold through Image J software (https://imagej.nih.gov/ij/). The following sperm quality parameters were determined: (1)
396 sperm motility (%), (2) sperm velocity (μm s^{-1}): the curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP), (3) sperm movement trajectory:
398 path linearity of actual sperm track, LIN = VSL/VCL x 100), path wobble (deviation from

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average path, WOB = VAP/VCL x 100), and path straightness (linearity of the average
400 path, STR = VSL/VAP x 100). All parameters were evaluated in triplicate for each sperm sample.

402 Sperm concentration was also recorded for each sperm sample used. In this case, sperm was diluted 1/1000 and 10 µL were pipetted into a THOMA cell counting chamber where
404 it was allowed to settle for 10 min, and then, was observed under the microscope at 100x magnification. The estimated densities are expressed as the number of spermatozoa per
406 mL of sperm (spz mL^{-1}). Quantification of spermatozoa was conducted using ImageJ software.

408 **2.8. Statistical analysis**

Shapiro-Wilk and Levene tests were used to check the normality of data distribution and
410 variance homogeneity, respectively. Oocyte diameter data (Stage 1 from Exp. 1 and Exp. 2), E_2 levels (Stage 1 from Exp. 1 and 2) and 11-KT levels (Exp. 1) were normalised with
412 the ln log transformation. For oocyte diameter, E_2 levels and 11-KT levels (Stage 1 from Exp. 1 and Exp. 2) a two-way repeated-measures (RM) ANOVA followed by Dunnett's
414 test was used to compare to the control, which was the control group and week 0 of treatment. A t-student was used to compare oocyte diameter before and after the Stage 2
416 treatments in Experiment 1. Differences in weekly E_2 levels in Stage 2 (Exp. 1) treatments were examined by one-way RM ANOVA. Statistical differences in the dose-response test
418 and in sperm characteristics (density, duration) among weeks were examined by a one-way repeated-measures analysis of variance (ANOVA) followed by the Holm-Sidak test
420 for pairwise comparisons. Analyses were performed using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA). Significance was set at $P < 0.05$. Data is presented
422 as mean ± standard error (SEM) unless indicated otherwise.

3. Results

424 **3.1. *In vivo* dose-response of rFsh on female steroid production**

There were no increases from the E_2 basal values after the application of doses of 3, 6 and
426 9 µg kg^{-1} of rFsh (Fig 3). A great individual variation in magnitude of response was observed when a dose of 9 µg kg^{-1} was administered. The administration of 12 µg kg^{-1} of
428 rFsh produced significant increases in E_2 levels on 3 to 6 days after the injection. The highest average levels of E_2 were obtained 3 days after the injection of 15 µg kg^{-1}.
430 Therefore, the doses of 12 to 15 µg kg^{-1} of rFsh were the most effective to stimulate E_2 production and were considered the most appropriate for the induction experiments.

432 **3.2. Experiment 1: Effect of long-term rFsh therapy**

3.2.1. Female development

434 **3.2.1.1. Stage 1: Gametogenesis induction in females**

Weekly injections of 15 µg kg^{-1} rFsh during eleven weeks to immature females generated
436 a significant increase (2 - 10 weeks) in the plasma levels of E_2 compared to the control group ($P < 0.001$) (Fig 4). Among the untreated females (control), plasma E_2 levels
438 remained unchanged at basal levels during the experimental period (0 - 10 weeks). *In situ* and histological observation of oocytes obtained by cannulation indicated that rFsh
440 induced a significant increase of oocytes diameter ($P < 0.001$) (Fig 5A) and vitellogenic growth (Fig 6) compared to the control group.

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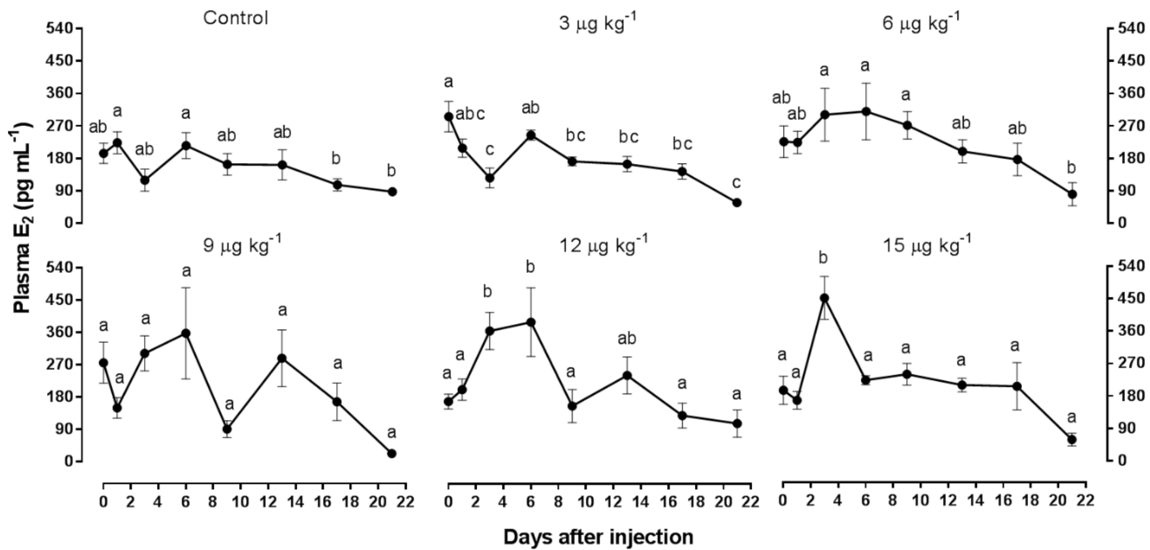

Fig 3. **Mean (± SEM) plasma E_2 levels of female flathead grey mullet (*M. cephalus*) before (day 0) and after (day 1, 3, 6, 9, 13, 15 and 21 days) the rFsh injection.** Females (n = 5/group) received a single injection of rFsh at doses 3, 6, 9, 12 or 15 µg kg^{-1} and an injection of 1 mL $fish^{-1}$ CHO conditioned culture medium for control. Different letters indicate significant differences ($P < 0.05$) over time within each dose.

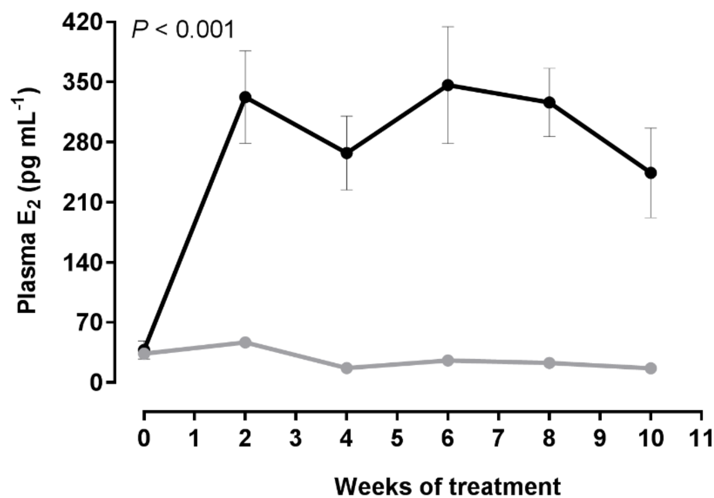

Fig 4. Mean (± SEM) plasma E_2 levels of rFsh-treated and control flathead grey mullet (*M. cephalus*) females (n = 9-11) in Experiment 1. Treated females received weekly injections of rFsh (15 µg kg^{-1}) and control females of CHO conditioned culture medium (1 mL $fish^{-1}$). There were significant differences among treatments (two-way repeated measures ANOVA, $P < 0.001$).

At the beginning of the treatment all individuals, but one presented oocytes at the PGpn (mean maximum diameter = 97 ± 4 µm) as the most developed stage (Fig 6). Just one female assigned to the rFsh-treated group presented most developed oocytes at PGmn. After 5 weeks of treatment, all rFsh-treated females (89%) but one responded to rFsh and had vitellogenic oocytes. These vitellogenic females had populations of oocytes at different stages of development: PGpn, PGca, SGe and SGl with the most abundant stage being secondary growth oocytes (Fig 7A). In addition, some traces of atresia appeared in some females. In the two subsequent revisions (weeks 7 and 9), SGl oocytes were the

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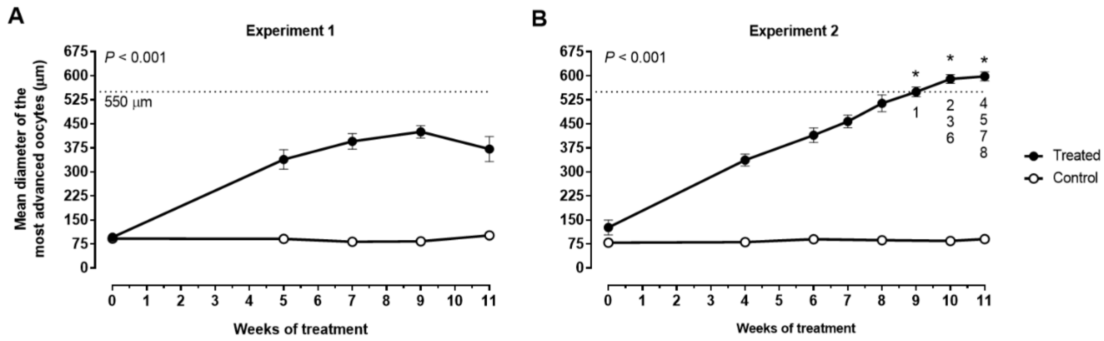

460

Fig 5. Mean (± SEM) oocyte diameter of the most developed oocytes in wet mounts from rFsh treated 462 **and control flathead grey mullet (*M. cephalus*) females.** (A) Experiment 1, females treated (n = 9) with a weekly 15 µg kg^{-1} rFsh administration or CHO conditioned culture medium (control, n = 11) during 11 464 weeks. (B) Experiment 2, females treated (n = 9) with initial increasing doses of rFsh followed by increases in rLh and subsequent rFsh decrease or CHO conditioned culture medium (control, n = 7). Values used for 466 females checked twice in the same week were the mean of both revisions. Asterisks show the moment when numbered females (see Fig 2) were selected for maturation and ovulation induction. There were significant 468 differences between treated and control groups (two-way repeated measures ANOVA, $P < 0.001$). Dotted line indicates oocyte size recommended for the hormonal induction of oocyte maturation.

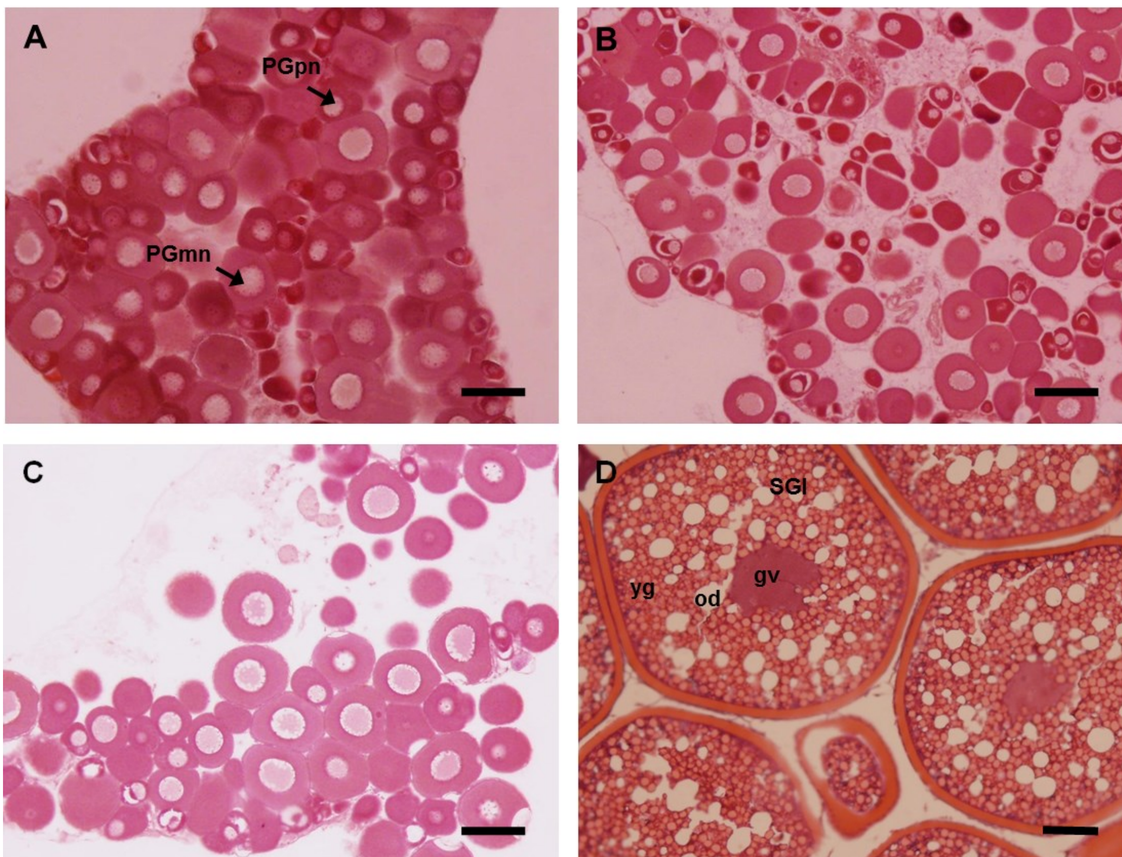

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Fig 6. Effects of long-term treatment of rFsh on ovarian development in previtellogenic flathead grey 472 **mullet (*M. cephalus*) *in vivo*.** Histological sections stained with hematoxylin and eosin show oocytes samples from (A) initial control fish, (B) rFsh-treated fish before treatment, (C) control fish after 7 weeks 474 and (D) rFsh-treated fish after 7 weeks of treatment (weekly 15 µg kg^{-1} rFsh). gv, germinal vesicle; nu, nucleolus; od, oil droplets; PGpn, perinucleolar primary growth oocyte; PGmn, multiple nucleoli primary 476 growth oocyte; SGl, late secondary growth oocyte; yg, yolk globules. Scale bar: 100 µm.

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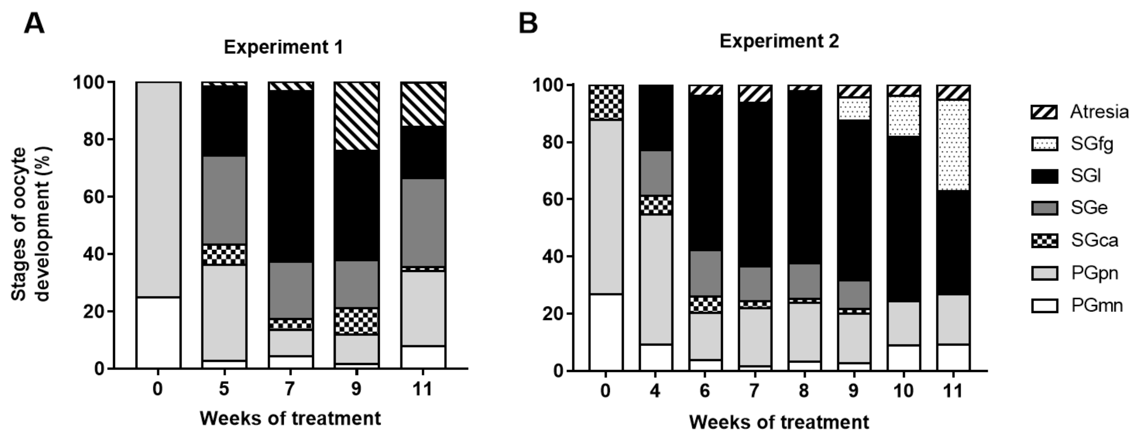
most abundant with a maximum size of 425 ± 19 µm in diameter (Fig 5A). After 9 weeks
478 of treatment, the proportion of atresia observed in the vitellogenic ovaries increased from three to 24 % and after 11 weeks of treatment the proportion of SGl oocytes decreased
480 while population of oocytes at SGe increased (Fig 7A). The female that at the start of the experiment before any treatment had oocytes at PGmn was delayed compared to other
482 females and only developed to SGe after 11 weeks of treatment. In comparison, the oocytes of all (100%) untreated females remained at primary growth (perinucleolar stage)
484 during the entire experiment (Figs 6, 7A). When rFsh administration for five females was ceased from week 11 onwards, the morphology of the ovary returned to primary growth
486 oocytes after five weeks.



488 Fig 7. Temporal weekly evolution of percentage frequency of oocyte developmental stages observed in rFsh treated flathead grey mullet females (M. cephalus). (A) Experiment 1 with weekly 15 µg kg-1 rFsh
490 administration to the treated group (n = 9) during 11 weeks. (B) Experiment 2 with the administration of initial increasing doses of rFsh followed by increases in rLh and subsequent rFsh decrease (n = 9). PGmn,
492 multiple nucleoli step of primary growth; PGpn, perinucleolar primary growth oocyte; SGca, cortical alveoli step; SGe, early secondary growth; SGl, late secondary growth oocyte; SGfg, full-grown secondary-
494 growth oocytes.

3.2.1.2. Stage 2: Induction of oocyte maturation

496 None of the treatments (priming and resolving GnRHa and MET injections, doses of 1000, 2000, 6000, 12000 IU kg^{-1} hCG combined with 15 µg kg^{-1} rFsh, and 9000 IU kg^{-1}
498 hCG with 25 mg fish^{-1} of progesterone) induced oocyte maturation. Histological examination of the oocytes after each treatment did not show variations in oocyte
500 morphology or stage although a significant low increase in oocyte diameter was observed in the female injected with GnRHa+MET protocol (Table 1). High E_2 levels were
502 maintained during the period of weekly hCG injection (week 12: 186.5 ± 20.6, week 13: 258.3 ± 35.1, week 14: 241.1 ± 42.1 and week 15: 184.5 ± 30.8 pg mL^{-1}) that were not
504 significantly different from E_2 levels (391.4 ± 56.5 pg mL^{-1}) during weeks 4 - 10 (Stage 1) in the same group.

506 **3.2.2. Male development.**

Two of three (66.6%) rFsh-treated males produced sperm after five weeks of treatment
508 that coincided with an increase in 11-KT levels ($P = 0.043$, $\alpha = 0.05$, statistical power = 0.66). One male that did not produce flowing sperm was humanly sacrificed with an
510 overdose of MS-222 on week 9, had a gonad somatic index (GSI) of 0.17 and spermatozoa

258 **Table 1.** Effects of treatments applied to flathead grey mullet (*M. cephalus*) to induce oocyte maturation and ovulation in Stage 2 from Experiment 1. Differences (t-student, $P < 0.05$) between maximum oocyte diameter (mean ± SEM) reached with rFsh treatment at Stage 1 and final oocyte diameter after corresponding treatments are indicated by
260 different letters for each female.

Fish No.	Max. oocyte diameter reached with rFsh at Stage 1 (µm)	Priming GnRHa (µg kg^{-1}); MET (mg kg^{-1})	Resolving GnRHa (µg kg^{-1}); MET (mg kg^{-1})	Weekly rFsh (15 µL kg^{-1}); hCG (IU kg^{-1})	hCG (IU kg^{-1}); progesterone (mg kg^{-1})	Final max. oocyte diameter at Stage 2 (µm)
1	539 ± 5 ^{a}	10; 15	20; 15	-	-	569 ± 10 ^{b}
2	450 ± 10 ^{a}	-	-	1000, 2000, 6000, 12000	9000; 25	470 ± 6 ^{a}
3	450 ± 9 ^{a}	-	-	1000, 2000, 6000, 12000	-	422 ± 8 ^{b}
4	470 ± 8 ^{a}	-	-	1000, 2000, 6000, 12000	-	490 ± 8 ^{a}

Table 2. Individual flathead grey mullet (*M. cephalus*) oocyte diameter of most advanced stages (mean ± SEM), hormone treatment and egg fecundity data in Experiment 2.
262 PGpn, perinucleolar primary growth oocyte; SGca, cortical alveoli step.

Fish No.	Most developed oocyte stage	Max. oocyte diameter before induction (µm)	rLh (µg kg^{-1}) (t = 0)	Progesterone (mg kg^{-1}) (t = 24)	Prostanglandins (mg kg^{-1}) (t = 25)	Total eggs	Fecundity (eggs kg^{-1} bw)	Fertilisation rate (%)
1	SGca	625 ± 8	30	40	18.75	801,913	832,723	Not used
2	PGpn	619 ± 7	15	40	-	-	-	-
3	SGca	627 ± 8	15	40	-	-	-	-
4	PGpn	603 ± 10	30	40	-	974,928	574,500	0.1
5	SGca	608 ± 8	30	40	-	754,774	676,320	0.31
6	PGpn	610 ± 6	15	40	-	-	-	-
7	PGpn	578 ± 7	30	40	-	891,600	888,047	0.81
8	PGpn	605 ± 4	30	40	-	-	-	-

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258 were observed in the histology. Control group males did not produce sperm. The production of sperm was prolonged for 4 and 6 weeks in the two spermiating males but
260 sperm volumes were low (29.3 ± 6.1 µL). Sperm was white in colour and highly viscous, which made it difficult to manipulate. The mean sperm concentration was 4.6 ± 1.5 10^{10}
262 spermatozoa mL^{-1} and no significant differences were observed among individuals between weeks. The motility grade recorded was 4 (> 75% sperm with progressive
264 movement) and the mean motility duration was 40 ± 2 seconds with no significant differences between weeks.

266 **3.3. Experiment 2: Effect of combined rFsh and rLh therapy**

3.3.1. Female development.

268 Histology showed that at the beginning of the treatment, those females from semi-extensive culture and with less time in intensive captive conditions (3 months) had
270 oocytes in cortical alveoli stage (2 were in the control group and 3 in treated group, Table 2), while the wild individuals with 12+ months in captivity had primary growth
272 perinucleolar stage as the most advanced stage of gonadal development (5 in control group and 6 in treated group, Table 2).

274 As in Experiment 1, the administration of rFsh significantly ($P < 0.001$) increased the production of E_2 (week 0: 123.9 ± 27.4; week 4: 458.7 ± 113 pg mL^{-1}) compared to the
276 control group (week 0: 95.6 ± 21.5; week 4: 81.1 ± 18.7 pg mL^{-1}). This increase in E_2 levels in Exp 2, was achieved despite of using a lower and increasing dose during the first
278 weeks (Fig 2). After the first 4 weeks of treatment, all but one female (89 %) had vitellogenic oocytes. The treatment of the delayed non-vitellogenic female (female 9 in
280 Fig 2) was stopped, even though the diameter of the most developed oocytes had increased significantly from week 0 (89 ± 2 µm) to week 4 (167 ± 3 µm). Oocyte growth
282 of all other females followed the same pattern as observed in Exp 1 during the first 7 weeks of treatment (Fig 7). However, during the following weeks, with the administration
284 of rLh, the proportion of atresia was reduced (week 9 = 4%) in comparison with Exp 1 (24%) in which just rFsh was administered. The inclusion of rLh in Exp 2 also increased
286 the mean diameter of the most advanced oocytes compared to Exp 1 (Fig 5A vs 5B). The response to treatment was different between females, which reached a ≥ 550 µm oocyte
288 diameter at different time points between week 8 and 11. Full-grown oocytes obtained prior to maturation induction had a mean diameter of 609 ± 5 µm. As in Exp 1, all (100%)
290 control females showed no oocyte growth or development.

Only those females (n = 5) that received 30 µg kg^{-1} of rLh followed by 40 mg kg^{-1} of
292 progesterone, presented oocyte maturation (OM), hydration and ovulation. Four females showed the initiation of OM indicated by oil globule coalescence after 24 h from rLh
294 injection. From these females, female 1 had not ovulated 39 hours after rLh administration when an injection of prostaglandin F2α was administered. The prostaglandin appeared to
296 induce ovulation and, one hour after administration, poor quality eggs were stripped that were not used for fertilisation. Posterior histological analysis showed that the eggs were
298 not fully hydrated (Fig 8A and Table 2). Three females, females 4, 5 and 7, which were checked at 48 ± 0.5 hours from rLh injection, had ovulated (Fig 8B) and eggs were used
300 for *in vitro* fertilisation (Table 2). The mean relative fecundity was 742,900 ± 71,840 eggs kg^{-1} bw. Female 8 did not ovulate and at 48 ± 0.5 hours after rLh administration only
302 presented oocytes in OM.

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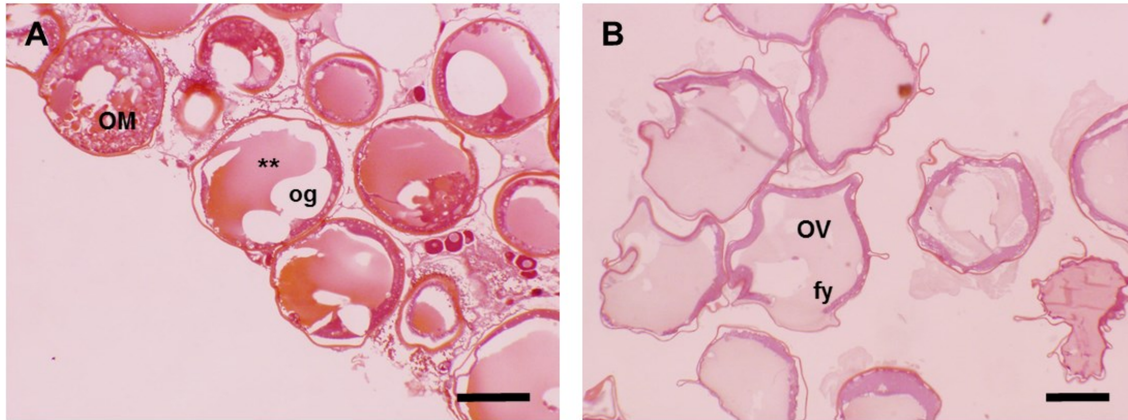

304 **Fig 8. Oocyte maturation and hydration stages for treated flathead grey mullet females (*Mugil cephalus*) in Experiment 2.** (A) Ovulated eggs from female 1 at 40 hours after 30 µg kg^{-1} of rLh injection
306 (16 hours from 40 mg kg^{-1} progesterone) and 1 hour from 18.75 µg kg^{-1} prostaglandin F2α injection. Oocytes in maturation: yolk globules coalesce and fuse to form a one large globule (**). Central oil globule
308 displaces the germinal vesicle into an eccentric position. (B) Ovulated eggs from three females (females 4, 5 and 7) at approx. 48 hours after 30 µg kg^{-1} of rLh injection (24 hours from 40 mg kg^{-1} progesterone).
310 Oocytes have undergone hydration after completion of germinal vesicle breakdown with homogenous fluid yolk. fy, fluid yolk; og, oil globules; OM, oocyte maturation; OV, hydrated oocytes at ovulation stage.
312 Scale bar: 500 µm.

3.3.2. Male development.

314 Along the course of the treatment, all males (n = 4) produced sperm which also coincided with an initial significate increase in 11-KT levels in the treated group (P = 0.006, α =
316 0.05, statistical power = 0.97) (week 3: 2.2 ± 0.7; week 7: 10.5 ± 2.2 ng mL^{-1}) in comparison with the control group (week 3: 0.7 ± 0.3; week 7: 0.5 ± 0.2 ng mL^{-1}).
318 Spermiating males were observed at week 6 (50% of males), week 7 (75 %) and from week 8 to 11 (100 %). First traces (38.7 ± 14.4 µL) were white and highly viscous (week
320 6 to 8) as described in Exp 1 with a motility score of 2 to 4 (25 to > 75% motility). At week 9 higher quantities of sperm where obtained (242.5 ± 70.9 µL) that decreased (68.6
322 ± 21.7 µL) at weeks 10 and 11. Viscosity decreased and motility score was 4 for all males until the end of the treatment. Sperm concentration of the first samples (from weeks 6, 7
324 and 8) (mean 2.1 ± 0.2 10^{11} spz mL^{-1}) was significantly higher (P < 0.001, α = 0.05, statistical power = 1.0) than the later weeks (9, 10 and 11) (2.3 ± 0.8 10^{10} spz mL^{-1}). Mean
326 duration of sperm motility was 89 ± 14 seconds during the 6 weeks that sperm was collected. Significant differences were found in duration of sperm motility between week
328 6 (154 ± 38 s) and weeks 7, 8 and 10 (mean 67 ± 4 s). One male that was humanly sacrificed on week 10 had a GSI of 1.81 and histology showed the testes were full of
330 spermatozoa.

Assessment by CASA of the 10 samples collected from all four males with high motility
332 score and ≥ 100 µL volume showed a mean motility percentage of 74 ± 0.01 %, VCL of 90.7 ± 3.3 µm s^{-1}, VAP of 84.6 ± 5.5 µm s^{-1}, VSL of 83.4 ± 6.9 µm s^{-1}, LIN of 91 ± 0.5
334 %, WOB of 93.5 ± 0.1 % and STR of 97.9 ± 0.7 %.

3.3.3. *In vitro* fertilisation.

336 The 0.5 mL aliquots of stripped eggs (1224 ± 150 eggs) were fertilised by mixing with 60 µL (20 µL/male) of pooled diluted stripped sperm (sperm 1:4 in Marine Freeze®) (3.8
338 ± 0.8 10^9 spz mL^{-1}). The mean sperm to egg ratio at fertilisation was 189,521 ± 23,541

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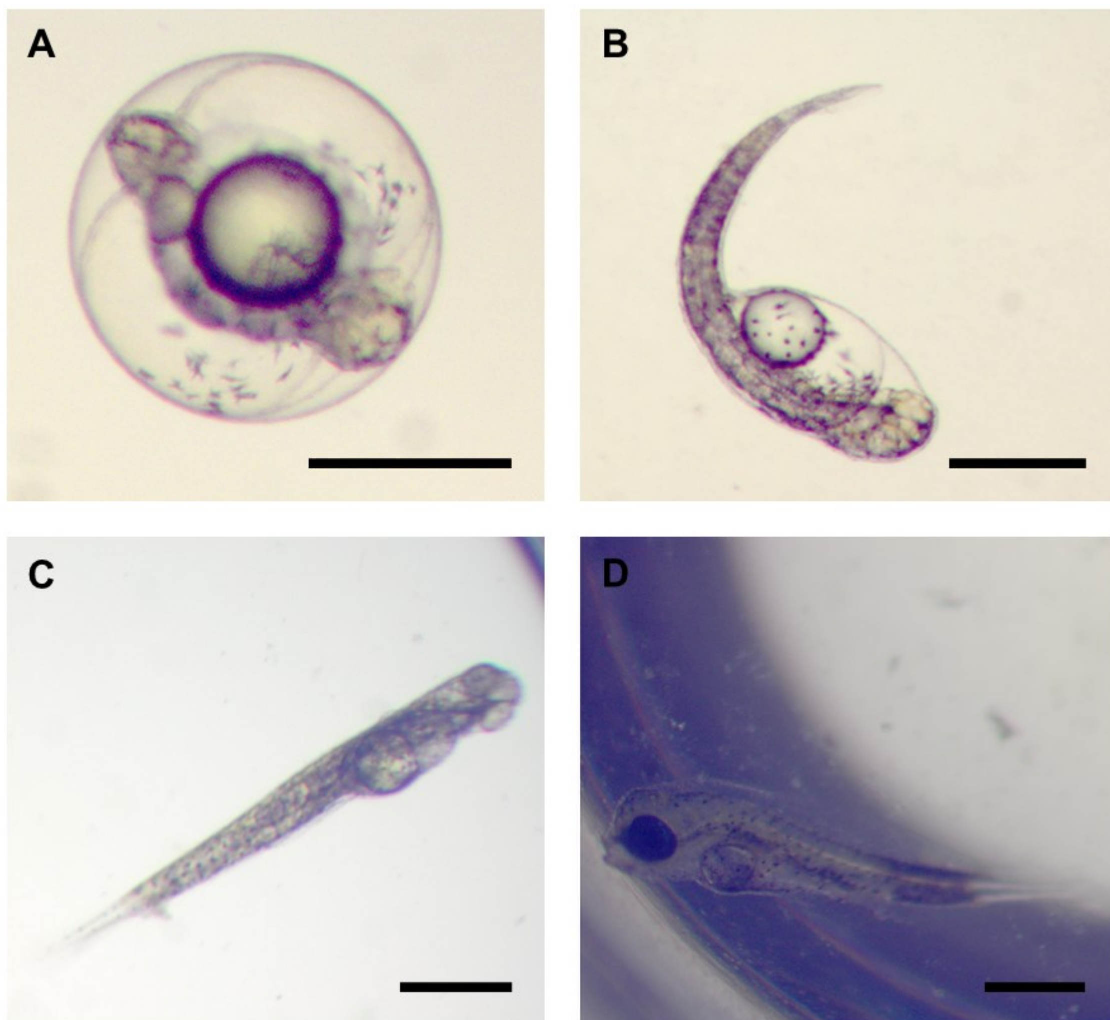
spermatozoa egg^{-1}. After an incubation period of 22 - 23 hours (24ºC), mean embryo
340 survival rate was 0.4 ± 0.2 % (n = 3 females). At this age, the head region had formed and dark pigments covered almost all of the embryo and the oil globule (Fig 9A). Although,
342 a single oil yolk globule was noticed in the majority of embryos, 28 % of the examined eggs presented multiple oil droplets. Mean fertilised egg diameter was 844 ± 4 µm.
344 Hatching rate, observed at 39 - 40 hours after fertilisation, was 70.8 ± 20 % (Fig. 9B). Survival rate of larvae decreased to 38.6 ± 22 % at 1 day post-hatching (dph). *M. cephalus*
346 larvae at 1 dph had developing eye lens and a reduced yolk sac diameter (Fig 9C). Survival rate of larvae decreased to 4.1 ± 1.4 % after 2 and remained the same to 3 dph.
348 At 2 dph the yolk and oil globule were still present, but mouth parts were completely formed with upper and lower jaws opened (Fig 9D). In the last revision, corresponding to
350 4 dph the survival rate was zero.



352 **Figure 9. Developing *Mugil cephalus* embryos and larvae from Experiment 2.** (A) Embryo at age 22 h post-fertilisation with head region formed and dark pigments covering almost all the embryo and on the
354 oil globule. (B) Hatching at age of 40 hours post-fertilisation. (C) Larva after 1 dph. A decrease in yolk sac was observed and the eye lens formed. (D) Larva after 2 dph with well-developed eye, with mouth parts
356 formed and opened. Oil globule was still present. Scale bar: 500 µm.

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358 **4. Discussion**

The present study shows that rFsh drives gametogenesis from early to late gonad
360 developmental stages in both female and male mullet, and that rLh was influential to
achieve oocyte maturation and gamete release (ovulation and spermiation). These
362 findings are significant to both demonstrate the accepted roles of the Gths in teleost
reproductive development and to provide tools for the control of reproduction in teleost
364 species that experience reproductive dysfunctions early in the maturation process.

Flathead grey mullet is a species that exhibits severe reproductive dysfunctions in
366 captivity that threatens the sustainability of mullet culture making it mostly dependant on
wild captures. In the present study, all control fish remained arrested and gametogenesis
368 did not progress although it was timed to coincide with the natural reproductive period.
No control females developed further than primary growth or cortical alveoli stage and
370 no control males produced sperm during the experimental periods. All of the control fish
had sufficient size, 35 - 49 cm for females and 32 - 42 cm SL for males, and condition to
372 mature according to reported sizes of maturity; 27 - 35 cm standard length for females
and 25 - 30 cm for males [55]. The present study, encountered a more severe reproductive
374 dysfunction than has been observed in other studies [15]. The severity of the reproductive
dysfunction, highlights that in the present study, the application of rGths was critical in
376 stimulating reproductive development in the experimental fish.

The hormone therapy to control the entire process of gametogenesis was initiated with
378 the application of rFsh. The administration of different doses of rFsh to examine the
biological activity, obtained a significant and prolonged (3 - 6 days) increases of E_2 after
380 doses of 12 - 15 µg kg^{-1}. The increase in plasma E_2 levels reflected the gonadotropic
stimulation of the ovary by rFsh produced in the CHO system. The potent activity was
382 further confirmed by the significant increase in E_2 plasma levels in relation to the weekly
administration of rFsh (15 µg kg^{-1} in Exp 1 and increasing doses 6, 9 and 12 µg kg^{-1} in
384 Exp 2) to female grey mullets. The plasma levels of E_2 at week 4 after rFsh application
were 272 ± 43 pg mL^{-1} in Exp 1 and 458.7 ± 113 pg mL^{-1} in Exp 2. It should be noted
386 that in both experiments, plasma E_2 was measured seven days after the application of rFsh
as blood sampling was immediately before the weekly rFsh injection. The plasma levels
388 seven days after rFsh application were similar to the natural E_2 peak of 312 ± 12 pg mL^{-1} in *M. cephalus* [56], while other studies applying rFsh induced plasma levels of ~ 200
390 pg mL^{-1} 24 h after injection in rainbow trout (*Oncorhynchus mykiss*) [33] and ~ 4000 pg
mL^{-1} in goldfish (*Carassius auratus*) [35]. In the present study, the rFsh-mediated
392 increase of E_2 plasma levels in females appeared to stimulate oocyte growth by the
accumulation of lipid globules and yolk droplets, as E_2 stimulates vitellogenin synthesis
394 by the liver [19]. The fact that the rFsh doses including lower rFsh doses in Exp 2 were
sufficient to induce vitellogenesis may indicate that rFsh doses could be refined for future
396 inductions.

In both experiments, oocytes grew from immature perinucleolar stage and/or cortical
398 alveoli stage to vitellogenic stages after 4 - 5 weeks of rFsh administration. However, the
response exhibited some variation from a few more advanced females to two females (one
400 in each experiment) that did not reach vitellogenic stages in the 4 - 5 week-period. The
delay in response of one female from Exp 1 could be attributed to the initial earlier stage
402 of development (primary growth in the multiple nucleoli) at the beginning of the
treatment. However, the other female did not initiate from an earlier stage of
404 development, making it unclear if the variation in response was related to initial stage of
development or a natural variation in the response to rFsh. The biological activity of rFsh

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406 applied to females of other fish species has been previously studied, but most studies have focused on *in vitro* approaches for receptor-binding capacity [39] and steroidogenic
408 potency [23, 27, 31] or *in vivo* short-term effects [24, 33 - 35, 40] rather than *in vivo* long-term effects on gonadal development. When rFsh produced in other heterologous systems
410 than CHO cells were tested in long-term treatments in different fish species, more time, dose and/or number of administrations were required to reach a less advanced stage of
412 ovary development than in the present study. For instance, after 60 days of treatment with injections at 10-day intervals of rFsh produced in *P. pastoris* (10 - 20 µg kg^{-1}) immature
414 yellowtail kingfish oocytes developed to cortical alveoli stage [29]. Weekly injections for 8 weeks at 100 µg kg^{-1} to juvenile grouper (*Epinephelus fuscoguttatus*) also induced
416 development to the cortical alveoli stage [30]. Recombinant Fsh produced in *Drosophila* S2 cell line (100 µg kg^{-1}) induced early vitellogenesis in the Japanese eel after 56 days of
418 treatment with a weekly administration [24] and rFsh (500 µg/kg) produced in HEK293 cells induced initial oil droplet stage in previtellogenic yellow shortfinned eels (*Anguilla*
420 *australis*) after three weeks [57]. These comparisons between the present study and other studies suggest a higher potency of rFsh produced in mammalian cell lines as previously
422 reported in some species [40, 49].

The present study also provides evidence that Fsh is the major hormone to initiate
424 vitellogenesis in teleosts. To date, no study has demonstrated that the exogenous application of just Fsh promotes the initiation of vitellogenesis and development through
426 to late vitellogenic stages and that development progressed correctly to provide oocytes for the formation of viable eggs and larvae. The central role of Fsh in fish vitellogenesis
428 is accepted [14, 19] based on parallels drawn with other taxa, correlations across many fish species between plasma Fsh and oocyte development, genomic approaches such as
430 gene knockout to define Gths pathways [58] and evidence that rFsh induced partial development of vitellogenesis [26, 59, 60]. However, some criticisms can be made as,
432 many differences in the control in reproduction exist between taxa, correlations do not necessarily indicate cause - effect and vitellogenesis although delayed proceeded when
434 the Fshb gene was knocked out to make Fsh-deficient zebrafish (*Danio rerio*) [58]. Therefore, the present study has added clear evidence to demonstrate the accepted
436 function of Fsh by reporting for the first time in a teleost species that rFsh successfully induced the entire process of vitellogenesis from previtellogenic stages to late stages from
438 which fertilised eggs and larvae were obtained.

Nevertheless, the administration of only rFsh in Exp 1 failed to complete oocyte growth
440 as although oocytes developed until late secondary growth, the cells appeared to be arrested in this stage and subsequently, a substantial number of apoptotic cells were found
442 in the later weeks (weeks 9 - 11). These results agree with previously described E_2 roles that did not include OM [19], but differ from those obtained by Das *et al.* [61] who
444 induced OM in *M. cephalus* post-vitellogenic oocytes that were incubated *in vitro* with E_2. The fact that OM could not be achieved using only rFsh suggested that, as previously
446 described, OM and ovulation are Lh-dependent [19, 62]. According to Nagahama and Yamashita [62], secretion of Lh from the pituitary coincides with a switch in the gonad
448 steroidogenic pathway from the production of predominantly E_2 during vitellogenesis to the production of progestin-like steroids, the maturation-inducing steroids (MIS). The
450 MIS bind to oocyte membrane-specific receptors to activate the maturation promoting factor (MPF) that induces germinal vesicle breakdown and OM [19]. Therefore, in Stage
452 2 of Exp 1 and Exp 2, we focused on the use of exogenous sources of Lh receptor agonists or hormones that may trigger the release of Lh from mullet pituitary with the aim to
454 complete oocyte growth and induce OM.

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In Experiment 1, none of the hormone therapies, GnRHa with MET, hCG alone or in
456 combination with progesterone induced the completion of vitellogenesis or maturation. Despite of the hormone treatments, the arrested oocyte diameter and morphology
458 indicated that vitellogenic growth was not completed and the oocyte did not enter maturation. Oocyte diameter was arrested at 425 ± 19 µm, compared to >550 µm that
460 appears to indicate complete vitellogenic growth and below which OM was not observed [50, 63, present study). The GnRHa with MET protocol used, was that described by Aizen
462 *et al*. [15] for grey mullet, where GnRHa acts on the pituitary to stimulate the production and secretion of endogenous gonadotropins, which in turn induces steroidogenesis in the
464 gonad, while MET removes the dopaminergic inhibition. However, in the present study, although there was a significant increase in oocyte diameter, the increase was not
466 sufficient and there was no progress to OM, thus, the pituitary appeared not to respond. One explanation would be that the pituitary Lh content was low and insufficient for the
468 induction of maturation. In carp species, when pituitary Lh content was low at the end of the spawning season, hormone therapies that induced the release of endogenous Lh were
470 observed to be less effective [22]. The Lh receptor agonist, hCG, is widely used and has been employed to induce spawning in many cultured species [14, 64] including grey
472 mullet with a dose range from 1000 to 30,000 IU kg^{-1} [7, 9, 65]. The advantage of hCG is that it generally binds to the same receptors as Lh in the gonad to stimulate
474 steroidogenesis and thus, does not dependent on the release of endogenous pituitary Lh [14]. The ineffectiveness of hCG in the present study may be related to a number of
476 aspects. The mean higher oocyte diameter of the individuals that received the hCG injections did not reach the recommended size of previous studies for maturation
478 induction (>500 µm) in *M. cephalus* [7, 8, 15] and, possibly, the oocytes and follicles were not receptive at the time of hCG application. Another consideration is that hCG did
480 not stimulate the switch in gonad steroidogenic pathway to MIS, as high E_2 levels were still produced in the late vitellogenic stage even with the application of progesterone, a
482 precursor for MIS production [66]. The administration of rFsh may have complicated the switch as agonists of the Lh receptor also stimulated the Fsh receptor [39, 47] and high
484 levels of rFsh may have affected this action of hCG. However, although the action of co-administration of hCG and rFsh can be questioned, when rFsh administration was ceased,
486 females entered into regression.

In contrast, in Experiment 2, the co-administration of rLh with rFsh at advanced stages
488 of vitellogenesis induced the completion of oocyte growth (mean 609 ± 5 µm). Experiment 2 compared to Exp 1, appeared to show that the addition of rLh was required
490 to increase maximum oocyte diameter to a diameter (>550 µm) that represents the completion of oocyte growth and a diameter from which OM has been observed to
492 progress [50, 63, present study]. In comparison, in Exp 1 when rFsh alone or in combination with hCG was administered oocyte development was arrested in late
494 vitellogenesis and did not reach diameters that indicate the completion of vitellogenic growth. These observations, indicate that the completion of vitellogenic growth was
496 dependent on Lh, which has not been previously described. It should also be highlighted that in Exp 2 vitellogenic growth was completed both with and without the application of
498 rFsh. The dosage and the time interval of rLh treatment applied to induce OM were based on previous studies [42]. However, since the half-life of rLh in plasma has not been
500 determined in grey mullet, the most efficient hormone treatment (dose and timing) remains to be established. In relation to the induction of OM and ovulation, the rationale
502 behind the treatment of rLh plus progesterone, a precursor of maturation-inducing steroids, was to induce the Lh-mediated up-regulation of genes associated with these
504 processes and to avoid potential substrate-limiting factors for MIS synthesis. In Exp 2,

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only fish receiving the highest rLh dose (30 µg kg^{-1}) proceeded to OM and ovulation.
506 This demonstrated that rLh dosage has a relevant effect and high doses were required. Recombinant Lh has been previously successfully used to induce OM and ovulation in
508 bitterling (*Rhodeus ocellatus ocellatus*) [35], common carp (*Cyprinus carpio*) [67] and Malaysia catfish (*Hemibagrus nemurus*) [68]. However, the present study cannot confirm
510 if a unique injection of rLh could have completed OM and ovulation without the need of progesterone application. The competence to undergo OM and, subsequently, ovulation
512 required rLh, however, it appeared that there were additional requirements such as other hormones or a specific controlled interaction amongst rLh, rFsh and Gth receptors. The
514 mean fecundity recorded in the present study was 742,900 ± 71,840 eggs kg^{-1} bw (~ 855,800 eggs $female^{-1}$), which was within the range previously reported for *M. cephalus*,
516 from 500,000 to 3,000,000 eggs $female^{-1}$, that shows variation in relation to fish size and the technical procedures employed for egg collection [3].

518 Regarding males, the rGths induced spermatogenesis and spermiation in immature specimens in intensive captivity to provide milt for fertilisation procedures. The
520 biological effects of rGths were evaluated through plasma 11-KT levels and by the presence of milt after abdominal pressure. The rFsh treatment in Exp 1 and rFsh with rLh
522 in Exp 2, both increased the levels of 11-KT, which is the major androgen responsible for testicular development and increased plasma levels reflect the onset of gametogenesis
524 [14, 15, 47, 69]. Moreover, males in control groups did not spermiate and spermiating males were only obtained with rGth treatment. Other studies induced spermatogenesis
526 and spermiogenesis with long-term rGth administration. For example, in immature Japanese [36, 44, 70] and European eel [43] and in mature Senegalese sole [42, 45]. The
528 administration of rFsh alone induced the production of low milt volumes, whilst the additional administration of rLH increased milt volumes and decreased spermatozoa
530 concentration probably due to a stimulation of the production of seminal fluid. The induction of spermiation by rFsh alone has also been demonstrated in the European eel
532 [43] and similarly the addition of rLh increased volumes and decreased spermatozoa concentration. However, the little seminal fluid produced in both experiments could
534 explain the higher sperm concentrations observed (in the range of 10^{10} and 10^{11} spz mL^{-1}) with respect to that previously reported for this species (10^{8}) [71]. Nevertheless, the
536 rGth treatments provided spermiating males and, thus, sperm for fertilisation procedures even though the number of males in the study was low. The present study also indicated
538 that there was a gender specific contrast in the action of rFsh alone as males completed a reduced production of spz compared to females that were arrested in late vitellogenesis
540 and did not complete vitellogenic growth.

After hand stripping gametes and *in vitro* fertilisation, 0.4% of eggs developed embryos.
542 The low percentage of eggs developing an embryo may be related to *in vitro* fertilisation procedures. The morphological aspect of the eggs appeared normal with the exception
544 that 28% of the eggs had multiple oil droplets. In grey mullet, the manual pressure of artificial stripping increased the frequency of multiple oil droplets [11] and multiple oil
546 droplets were related with low egg survival [72]. Another aspect related to bad egg quality and *in vitro* fertilisation procedures is overripening [73]. After ovulation, there is a period
548 of egg ripeness with optimal viability after which the eggs overripen, losing quality and viability. This period of optimal egg quality for stripping varies among species, with
550 temperature, between different stocks, holding conditions, hormone induction treatments and ideally should be defined for each situation [73]. For example, latency to obtain good
552 quality eggs can be as long as 5 - 15 days over a temperature range of 10 - 17 °C for rainbow trout (*Oncorhynchus mykiss*) [74], 3 hours in meagre (*Argyrosomus regius*) at

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554 18 ºC [73] but only 30 min in white bass (*Morone chrysops*) at 22 °C [75]. For the present treatment in *M. cephalus*, the timing of ovulation and optimal egg quality has not been 556 previously defined. However, latency times have been reported for grey mullet using the hormone treatments, carp pituitary extracts with hCG or GnRHa [9], hCG [76] and 558 pituitary glands combined with synahorin and vitamin E [77] and times ranged from 30 to 48 hours after the initial priming dose and 12 to 26 h after the resolving dose. In the 560 present study, eggs were stripped at 40 h and 48 ± 0.5 h from rLh administration (16 h and 24 h from progesterone). The female stripped at 40 h was induced to ovulate with 562 PG-F2α and the stripped eggs had vitellogenin and oil in the process of coalescing apparently not having completed maturation and hydration when the oocytes were 564 ovulated. On the contrary, at 48 ± 0.5 h after the rLh injection, low fertilisation rates were obtained. This was at the limit of the period of good egg quality that has been found with 566 other hormone treatments (30 to 48 hours), which may indicate that the 48-h stripping time was late and that the eggs were undergoing overripening. Determining the timing of 568 ovulation and the window of good egg quality are crucial to obtain high fertilisation rates and, therefore, further studies are required to determine the quality of eggs that can be 570 achieved with rGth based therapies.

Fertilised grey mullet egg diameter has been reported to vary from 0.65 - 1.08 mm 572 differing with different geographical areas [3]. In the present study, the fertilised eggs ranged in diameter from 0.82 to 0.88 mm at a temperature of 24°C and salinity of 36 ‰. 574 Hatching was 39 - 40 hours after fertilisation at 24 ºC, which is in agreement with previous reports of hatching time: 34 - 38 h at 22 - 24.5 ºC and 49 - 54 h at 22.5 - 23.7 ºC 576 [3]. High mortalities were found at two and three days post hatching (dph), which coincides with the period that mouth, upper and lower jaws opened although the yolk sac 578 was still present. These high mortalities were probably due to starvation as no food was offered and survival depends on the availability of external food organisms to larvae on 580 the second-day, 36 hours post-hatch, before the completion of yolk sac absorption [6].

In conclusion, the present study reports that treatment with rGths is able to induce full 582 gametogenesis and the production of viable eggs and larvae in a teleost. The control of the entire reproductive process using rGths, and particularly the initiation of 584 vitellogenesis and development through to late stages with rFsh, offer further confirmation of the central roles of the Gths in teleost gametogenesis. A refined protocol 586 based on the present study could provide full reproductive control of flathead grey mullet held in intensive aquaculture facilities. In addition, these findings raise the possibility of 588 using the rGth treatments for species that present similar reproductive disorders in aquaculture, the aquarium industry and for the conservation of endangered species.

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