Providing recombinant gonadotropin-based therapies that induce oogenesis from previtellogenic oocytes to produce viable larvae in a teleost, the flathead grey mullet (*Mugil cephalus*)

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Abstract

Under intensive captive conditions, wild-caught flathead grey mullet (*Mugil cephalus*) females remained arrested in previtellogenic stages of gonadal development and no sperm could be obtained from males. With the aim to induce oogenesis from previtellogenesis to oocyte maturation, induce the release of sperm and obtain fertilized eggs, female and male flathead grey mullet were treated with *Mugil cephalus* single-chain recombinant gonadotropins (rGths), follicle-stimulating (rFsh) and luteinizing (rLh) hormones. In Experiment 1, fish were treated with a weekly dose of rFsh (15 μg kg^{-1}), which in females significantly ($P < 0.001$) increased plasma concentration of 17β-estradiol and induced vitellogenic oocyte growth up to a maximum mean diameter of 425 ± 19 μm after 9 weeks of treatment. 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, from which final oocyte maturation and ovulation was induced with 30 μg kg^{-1} of rLh and 40 mg kg^{-1} of progesterone. Good quality sperm (> 75% motile spermatozoa) was obtained from males in both experiments, and in Exp. 2 the addition of rLh induced the production of higher quantities of sperm that were used to fertilise the eggs. Although fertilisation was low (0.4 %), these fertilized eggs with embryo development produced viable larvae (71% hatching). In comparison, control females remained arrested at previtellogenesis and control males did not produce sperm. The study demonstrated that both rGths are effective to induce the process of oogenesis in female flathead grey mullet and to obtain flowing sperm from males, adding more data to confirm the roles of the Gths in teleost gametogenesis. This is the first report, in a teleost species, of the use of rGths (rFsh and rLh) to induce oogenesis from previtellogenesis through to maturation to obtain eggs and larvae. This advance provides the bases for the development of therapies for the use in

43 the aquaculture of teleost of commercial interest or the conservation of endangered
44 species.

45 ***Keywords:*** *Mugil cephalus*, gametogenesis induction, eggs, rFsh, rLh.

1. Introduction

The flathead grey mullet (*Mugil cephalus*) is a catadromous teleost with a worldwide distribution (between latitudes 40º North and South) (McDonough et al., 2005) that has been cultured for several centuries principally in some Asian countries and around the Mediterranean basin. Many positive attributes of flathead grey mullet culture have made this species a suitable option for aquaculture. Flathead grey mullet has fast growth (approximately 1 kg per year) (FAO, 2019), does not require dietary fish meal and oil and can be reared in a wide range of salinities and culture systems (González-Castro and Minos, 2016). In addition, the final product marketed in various forms has good texture, taste (Yousif et al., 2010) and is an excellent source of omega-3 essential fatty acids (Khemis et al., 2019).

Flathead grey mullet show a wide variation in reproductive strategies and characteristics across the species worldwide distribution. The species is gonochoristic and generally matures at the age of 3 years. In the Mediterranean, the spawning period has been observed from July to October when breeders spawn externally fertilised pelagic eggs in the sea (Whitfield et al., 2012). Females appear to spawn one set of ova a year (Rao and Babu, 2016). However, in captive conditions flathead grey mullet exhibit different degrees of reproductive dysfunctions in both genders. Despite of the species long history of culture, these dysfunctions that have not been overcome, have limited the possibility to close the life cycle and, thus, culture is still based on the capture of wild juveniles (González-Castro, 2016; Yousif et al., 2010) or the induction of oocyte maturation and spawning of wild breeders captured at advanced stages of gonadal development (Abraham et al., 1999; Das et al., 2014; El-Gharabawy and Assem, 2006; Karim et al., 2016; Vazirzadeh and Ezhdehakoshpour, 2014). However, the use of wild caught mature fish is unsustainable as it relies on fisheries that are in decline (González-Castro and

71 Minos, 2016) and offers no possibility to close the life cycle in captivity and make genetic
72 improvements of cultured stocks. Flathead grey mullet held in aquaculture facilities
73 present two types of reproductive dysfunction: arrest in late or early stages of
74 gametogenesis. Arrest in late stages of gametogenesis (maturation and ovulation) has
75 been observed in recently caught wild flathead grey mullet or wild flathead grey mullet
76 that were acclimated to ponds or large tanks (El-Greisy and Shaheen, 2007; Kuo et al.,
77 1973; Yousif et al., 2010). This is the most commonly observed dysfunction in fish and
78 can be controlled by hormonally inducing spawning (Mañanós et al., 2009; Zohar and
79 Mylonas, 2001) as has been achieved for flathead grey mullet with therapies that combine
80 different substances such as: carp pituitary homogenates, steroids, human Chorionic
81 Gonadotropin (hCG), gonadotropin releasing hormone synthetic analogues (GnRHa) and
82 drugs that inhibit dopamine (see review by González-Castro and Minos, 2016). In
83 comparison, in wild and hatchery-reared fish held in intensive culture conditions in the
84 Mediterranean region, a more severe reproductive dysfunction has been observed where
85 development was arrested in the early stages of gametogenesis. The artificial propagation
86 of these fish in intensive culture systems would be a sustainable solution for a consistent
87 supply of juveniles (Yousif et al., 2010). In these intensive conditions, females did not
88 initiate vitellogenesis; remained at the primary growth stage or cortical alveoli stage
89 (present study), or were arrested at early stages of vitellogenesis (Aizen et al., 2005).
90 Males failed to initiate spermiation (De Monbrison et al., 1997; Yashouv, 1969) or
91 produced highly viscous milt that could not fertilize the eggs (Shehadeh et al., 1973).
92 These reproductive dysfunctions may be related to alterations in the endocrine control in
93 the brain-pituitary-gonadal (BPG) axis.

94 In vertebrates, the pituitary gonadotropins (Gths), the follicle-stimulating hormone (Fsh)
95 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 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 final maturation and release (ovulation and spermiation, in females and males, respectively) (Lubzens et al., 2010; Mañanós et al., 2009). The mechanism underlying the reproductive dysfunctions in Mediterranean captive flathead grey mullet has been described as an inhibition by dopamine (DA) on the action of gonadotropin releasing hormone (GnRH) to release Gths in both females (Aizen et al., 2005) and males (Glubokov et al., 1994). Therefore, methods based on the mechanisms controlling gametogenesis are required to induce complete gonadal development, from early stages (i.e. previtellogenesis) through to the late stages. In the case of males, 17α-methyltestosterone (MT) implants enhanced spermatogenesis and spermiation (Aizen et al., 2005; De Monbrison et al., 1997). In females, treatment with GnRH agonist (GnRHa) in combination with a DA antagonist (Aizen et al., 2005) or a single injection of recombinant Fsh produced in the yeast *Pichia pastoris* (Meiri-Ashkenazi et al., 2018) increased the number of vitellogenic 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 pituitary Lh content was low (Yaron et al., 2009), indicating that alternative therapies may be required in these situations.

A strategy to control gametogenesis in flathead grey mullet as in other teleost, which would not require the availability of endogenous Gths from the pituitary, is the long-term use of 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 expression systems, such as the *Drosophila* S2 cell line (Kazeto et al., 2008; Zmora et al., 2007), the yeast *Pichia pastoris* (Aizen et al., 2007; Chen et al., 2012; Kamei

et al., 2003; Kasuto and Levavi-Sivan, 2005; Palma et al., 2018; Sanchís-Benlloch et al., 2017), baculovirus silkworm larvae (Cui et al., 2007; Glubokov et al., 1994; Ko et al., 2007; Kobayashi et al., 2010, 2003; Meri et al., 2000), HEK293 cells (Kazeto et al., 2019) and Chinese hamster ovary (CHO) cells (Chauvigné et al., 2017; Choi et al., 2005; Giménez et al., 2015; Molés et al., 2011; Peñaranda et al., 2018; So et al., 2005). The application of rGths based therapies has shown promise to control gametogenesis in different teleost (Chauvigné et al., 2018, 2017; Giménez et al., 2015; Kamei et al., 2006; Peñaranda et al., 2018) and, therefore, could be an effective method to induce gametogenesis in cultured flathead grey mullet arrested in the early stages of sexual maturation.

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 mullet females that were arrested in previtellogenesis and males that did not have flowing sperm. For this purpose, *Mugil cephalus* rFsh was administered to induce gametogenesis followed by treatments to induce oocyte maturation and ovulation, which were either (a) therapies previously employed in this species such hCG and GnRHa with a DA agonist, or (b) *Mugil cephalus* rLh.

2. Material and methods

2.1. Study animals and maintenance

Flathead grey mullet were used in different experiments to examine the effect of rGth hormone therapies. An *in vivo* dose-response test was carried out for rFsh. Experiment 1 examined the long-term effect of rFsh on vitellogenesis and the use of hormone therapies (hCG or GnRHa with DA agonist) previously used in female flathead grey mullet to

induce final oocyte maturation and ovulation. Experiment 2 examined the effect of a combined rFsh and rLh therapy in females. In order to obtain sperm, males were administered rFsh (Exp. 1) or rFsh in combination with rLh (Exp. 2). The fish used were from two origins, wild fish caught in the Ebro River and fish from a semi-extensive pond fish farm (Finca Veta La Palma, Isla Mayor, Spain). The fish used in the different experiments from both origins had a mean weight of 0.8 ± 0.3 kg when brought to IRTA facilities (Sant Carles de la Ràpita, Spain) and at the start of the experiments a mean weight of 0.9 ± 0.3 kg. All the fish used were fish that were larger than the reported size of first maturity (Whitfield et al., 2012), which indicated the fish had the potential to sexually mature and produce gametes. Individuals used in the *in vivo* dose-response and Experiment 1 were wild-caught flathead grey mullet from the Ebro River held for 7-14 months in IRTA. No fish were used in both experiments. In Experiment 2, the broodstock was formed with wild-caught individuals from the Ebro River reared for 19-21 months, and individuals from the semi-extensive fish farm held for 3 months in IRTA. All fish were tagged intramuscularly with a Passive Integrated Transponder (PIT) tag (Trovan®, ZEUS Euroinversiones S.L. Madrid, Spain) for individual identification. To determine the 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 cm through the gonopore. Individuals were assigned as males if no oocytes were observed in the biopsies. During all experimental procedures, for hormone administration and sampling, fish were first anaesthetised with 73 mg L^{-1} of MS-222 and placed in a tank with 65 mg L^{-1} of MS-222 for manipulation.

One month before each experiment, individuals were transferred to a 10-m^3 tank per experiment to examine individuals held in the same environment. Individuals were held in a recirculating system (IRTAmar®) under natural conditions and were gradually

acclimatized from fresh water to sea water at 36 ‰ to provide the conditions for gonad development, as Tamaru et al. (1994) concluded that the rate of oocyte growth was lower in females maturing in fresh water. To evaluate the *in vivo* dose-response of rFsh, fish were held for 21 days in May when temperature was controlled to 24 ± 1 ºC and photoperiod was natural (14L:10D - light:dark). During Experiment 1, completed from early August to November, water temperature was controlled at 24 ± 1 ºC. Photoperiod was ambient (14L:10D August - 11L:13D October) until October when photoperiod conditions were maintained at 11L:13D until the end of the experiment to maintain the natural environmental conditions for the spawning season and avoid large changes of decreasing day length. The fish did not accept a pelleted broodstock diet and were, therefore, fed daily at 1.5% of the body weight with a soft mixture of 20 % sardines, 20% hake, 15 % mussels, 10 % squid, 10 % shrimp and 25 % a commercial broodstock diet (Mar Vitalis Repro, Skretting, Spain) with 0.1% spirulina. In Experiment 2, completed from the end of July to mid-October, water temperature was also controlled at 24 ± 1 ºC while photoperiod was ambient (from 14L:10D to 11L:13D). Fish were fed a commercial marine fish broodstock diet (Brood Feed Lean, 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. Prior to the experiments, fish had the same feeding regimens and were held in natural conditions of photoperiod and temperature.

The procedures used were evaluated by the Ethics and Animal Experimentation Committee (CEEA) of IRTA and the Catalan Government Commission of Animal Experimentation as Animal Experimentation Project 10997 and was authorized with ID 7YBYJ1T92. The study was conducted in accordance with the European Union, Spanish and Catalan legislation for experimental animal protection (European Directive 2010/63/EU of 22nd September on the protection of animals used for scientific purposes;

195 Spanish Royal Decree 53/2013 of February 1st on the protection of animals used for
196 experimentation or other scientific purposes; Boletín Oficial del Estado (BOE), 2013;
197 Catalan Law 5/1995 of June 21th, for protection of animals used for experimentation or
198 other scientific purposes and Catalan Decree 214/1997 of July 30th for the regulation of
199 the use of animals for the experimentation or other scientific purposes).
200

201 **2.2. Cloning of *M. cephalus* Gths β and α subunits for rGths production**

202 The pituitary gland was removed from one sacrificed female, frozen in liquid nitrogen,
203 and stored at -80ºC. Total RNA was purified using the GenElute™ mammalian total RNA
204 miniprep kit (Sigma-Aldrich) according to the manufacturer's instructions, and cDNA
205 synthesis was performed with 1 µg of total RNA following the manufacturer's
206 instructions of the 3' RACE kit (Invitrogen). Polymerase chain reaction (PCR) was
207 carried out as indicated in the 3' RACE kit using partially degenerated forward primers
208 for the Fshβ or α subunits, the common abridged universal amplification primer (AUAP)
209 as reverse primer, and the EasyA™ high-fidelity PCR cloning enzyme (Agilent
210 Technologies, Santa Clara, CA, USA). The forward primer for each gene covered the
211 translation initiation codon ATG and was designed based on sequences available in the
212 GenBank repository for *Epinephelus coioides* (AY186242), *Oreochromis niloticus*
213 (AY294015), *Dicentrarchus labrax* (AF543314), *Acanthopagrus schlegelii* (AY921613),
214 *Maylandia zebra* (XM_004558042), *Fundulus heteroclitus* (M87014), *Oryzias latipes*
215 (AB541981), *Sparus aurata* (AF300425), *Amphiprion melanopus* (EU908056),
216 *Chrysiptera parasema* (KM509061), and *Kryptolebias marmoratus* (EU867505). For
217 Fshβ, the forward primer was 5'-ATGCAGCTGGTTGTCATGGYAGC-3', whereas for
218 the α subunit the primer was 5'-ATGGGCTCMNTGAAAYCHVCTG-3. The Lhβ
219 subunit was cloned using a degenerate forward primer covering the central region of the

RNA (5'- CAAYCAGACRRTDTCTCTRGA), designed based on teleost sequences publically available (*E. coioides*, AY186243; *Oreochromis niloticus*, AY294016; *Dicentrarchus labrax*, AF543315; *Acanthopagrus schlegelii*, EF605276; *Maylandia zebra*, XM_004553532; *Pundamilia nyererei* XM_005741532; *Fundulus heteroclitus*, M87015; *Cyprinodon variegatus*, XM_015404196; *Oryzias latipes*, AB541982; *Kryptolebias marmoratus*, XM_017431834; *Poecilia reticulata* XM_008429103; *Nothobranchius furzeri*, XM_015975766; *Xiphophorus maculatus*, XM_005816155), and the reverse AUAP primer. The 5' end of the cDNA was further amplified using RACE (5' RACE kit, Invitrogen) and specific primers. In all cases, 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 sequencing on ABI PRISM 377 DNA Analyser (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The nucleotide sequence corresponding to the full-length Lhβ, Fshβ and α subunit cDNAs were deposited in GenBank with accession numbers MF574169, MF574168 and MF574167, respectively. Single chain recombinant *M. cephalus* rFsh and rLh were produced by Rara Avis Biotec S.L. (Valencia, Spain) using in-house technology. Briefly, CHO cells where transfected with expression constructs encoding fusion proteins containing the entire coding sequence of *M. Cephalus* Fshβ (GenBank accession nº MF574168) or Lhβ (GenBank accession nº MF574169) subunit, the 28 carboxyl-terminal amino acids of the hCG β subunit as a linker, and the mature sequence of the *M. cephalus* glycoprotein hormone α subunit (GenBank accession nº MF574167). The secreted recombinant hormones were subsequently purified from the culture medium by ion exchange chromatography, concentrated (rFsh at 12 μg mL^{-1} and rLh at 8 μg mL^{-1}) and stored at -80ºC until use.

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-estradiol (E_2) production, to determine the minimum effective dose and optimal dosing schedule, intramuscular injections to administer different rFsh doses (3, 6, 9, 12 and 15 μg kg^{-1}) were given to flathead grey mullet females that had ovaries in previtellogenesis (five fish per dose group). Control females (n = 5) were injected with CHO conditioned culture medium (1 mL $fish^{-1}$). The mean body weight was 0.9 ± 0.3 kg. Blood samples (0.40 mL) were collected before injection (day 0) and at different days (1, 3, 6, 9, 13, 17, 21 days) after injection.

2.4. Experiment 1. Long-term rFsh therapy

In Experiment 1, twenty-six flathead grey mullet were used in the trial. Nine females and three males (mean ± SD body weight 1 ± 0.3 and 0.9 ± 0.1 kg; mean ± SD standard length 41.4 ± 4.1 and 40.8 ± 2.4 cm, respectively) received the gonadotropic treatment and 11 females and three males (mean ± SD body weight 1 ± 0.2 and 0.9 ± 0.1 kg; mean ± SD standard length 42 ± 4.1 and 41.3 ± 1.5 cm, respectively) were set as controls. The total biomass was 24.7 kg. Only three males were selected for each group, as only six males were available. The fisheries capture to form the broodstock was biased towards females as has been observed in other studies (Rao and Babu, 2016). The fish in the treatment group were administered rFsh followed by either hCG alone (El-Gharabawy and Assem, 2006; Yousif et al., 2010) or GnRH combined with DA antagonist (Aizen et al., 2005).

2.4.1 Stage 1. Long-term rFsh administration

Individuals belonging to the gonadotropic treatment group (both males and females) received weekly intramuscular injections of specific flathead grey mullet rFsh at a dose of 15 µg kg^{-1} for 11 weeks (Fig 1). The rFsh dose applied was chosen according to the dose with highest potency on E_2 induction in the *in 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 senegalensis*) using recombinant Gths produced in CHO cells. Chauvigné et al. (2017) described that a dose of 12 - 17 µg kg^{-1} rFsh was effective in stimulating spermatogenesis, 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 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 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) per female were measured *in situ* and samples were fixed for histology. In parallel, males received a gentle abdominal pressure to check the presence of milt.

2.4.2 Stage 2: Completion of oocyte growth and maturation induction in females

This second stage of the experiment investigated the effects of different hormones used as a source of Lh or to induce endogenous Lh release to complete oocyte growth and induce maturation in females that were previously treated with rFsh to induce vitellogenesis. Five females were not used in the second stage and 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 vitellogenesis. One female was treated with the GnRHa des-Gly10, [D-Ala6]-gonadotropin releasing

hormone (product code L4513, Sigma, Spain) in combination with Metoclopramide (MET) (product code M0763, Sigma, Spain), a dopamine antagonist, according to the Aizen et al. (2005) 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 administered 22.5 h apart. Three females received weekly consecutive injections of hCG (Veterin Corion, DIVASA-FARMAVIC S.A, Barcelona) at increasing doses (1000, 2000, 6000, 12000 IU kg^{-1}) in combination with the rFsh treatment (15 μg kg^{-1}) (Supplementary Fig 1A). Dosage of hCG were in the range of previous studies on flathead grey mullet maturation (El-Gharabawy and Assem, 2006; Yousif et al., 2010) and other fish species (Mañanós et al., 2009). Weekly samples of oocytes and blood (0.40 mL) were obtained.

2.5. Experiment 2. Combined rFsh and rLh therapy

A total of twenty-four flathead grey mullet were used in Experiment 2. Females with a body weight of 0.9 ± 0.1 kg (mean ± SD) and standard length of 38.5 ± 3.1 cm, and males with 0.6 ± 0.1 kg and 33.3 ± 1.2 cm received the rGths treatment, while females with a body weight of 0.8 ± 0.1 kg and standard length of 39.5 ± 1.3 cm and males with 0.8 ± 0.1 kg and 38.6 ± 2.7 cm were used as controls. The total biomass was 20 kg. Although all females were at previtellogenesis, two-thirds of the females had perinucleolar primary growth oocytes as the most advanced stage of gonadal development (5 in control group and 6 in treated group) and one-third of the females presented cortical alveoli oocytes (2 were in the control group and 3 in the gonadotropin treated group) and were randomly distributed between treated and control groups. The females that were at advanced stages in previtellogenesis originated from a semi-extensive culture and had less time in intensive captive conditions (3 months).

317 The aim of the administration pattern in this experiment was to simulate natural increases
318 and decreases of gonadotropins in the bloodstream of individuals according to their
319 suggested regulatory role in gamete development (Levavi-Sivan et al., 2010). Initial
320 administration of rFsh followed by a gradual increase of rLh as gametogenesis progresses
321 and subsequent decline of rFsh.
322
323 **2.5.1. Females**
324 Initially, all nine females received increasing doses of rFsh, 6 μg kg^{-1} (week 0) and 9 μg^{-}
325 ^{1} kg (week 1) before the dose was fixed at 12 μg kg^{-1} rFsh per week. A maximum 12 μg
326 kg^{-1} dose was selected for long-term treatment based on Experiment 1 and the *in vivo*
327 dose-response study. From the 4th week onwards, females (n = 8) also received a weekly
328 administration of rLh at increasing doses (2.5, 4, 6 μg kg^{-1}). When vitellogenesis arrived
329 to advanced stages (week 9), weekly rFsh dose was decreased to 4 μg kg^{-1} while rLh dose
330 was increased (9 and 12 μg kg^{-1}) (Fig 2 and Supplementary Fig 1B). At this point (week
331 8 and onwards), treatments were adjusted accordingly to oocyte diameter of each
332 individual fish to ensure females at the same stage of vitellogenesis received the same
333 rGth treatment. When females presented oocytes ≥ 550 μm, no more rFsh was
334 administered and consecutive doses starting with 9 and maintaining 12 μg kg^{-1} rLh were
335 administered every 3 days (see summary in Table 1 and detail in Supplementary Fig 1B).
336 The aim of this increased frequency of administration was to maintain high levels of rLh
337 in the bloodstream, based on the half-life (shorter than rFsh) described for rLh produced
338 in CHO cells and administered to Senegalese sole (Chauvigné et al., 2017). Doses for rLh
339 were assigned according to other studies on the use of rLh produced in CHO cells for
340 vitellogenesis induction (Giménez et al., 2015) or spermatogenesis (Chauvigné et al.,
341 2017) in other fish species. When the most developed oocytes reached a diameter ≥ 600

342 µm or did not show further growth, females were considered to have completed
343 vitellogenic growth and, therefore, were ready for maturation and ovulation induction. To
344 induce oocyte maturation, ovulation and spawning, females were administered higher
345 doses of rLh (15 or 30 µg kg^{-1}) combined with 40 mg kg^{-1} of progesterone (Prolutex,
346 IBSA Group, Italy) administered 24 h after the rLh (Table 1). Three females received 15
347 µg kg^{-1} of rLh and five females received 30 µg kg^{-1}.

Table 1. A summary of the induction protocol administered to flathead grey mullet (*Mugil cephalus*) females in Experiment 2 to induce oogenesis and oocyte maturation and ovulation. Includes origin of fish, previtellogenic stage at the beginning of the experiment, individual oocyte diameter before inducing oocyte maturation (mean ± SEM) and egg fecundity data. W, wild-caught individuals from the Ebro River reared for 19-21 months; SE, individuals from a semi-extensive fish farm held for 3 months in IRTA facilities (Sant Carles de la Ràpita, Spain); PGpn, perinucleolar primary growth oocyte; SGca, cortical alveoli step; OM, oocyte maturation. PGpn, perinucleolar primary growth oocyte; SGca, cortical alveoli step; SGfg, full-grown secondary growth oocytes.

Fish No.	Fish Origin	Previtellogenic oocyte stage at the start of the experiment	Induction of oogenesis		Max. oocyte diameter before OM induction (µm)	Induction of maturation and ovulation			Total eggs	Fecundity (eggs kg^{-1} bw)	Fertilisation (%)
			Treatment before < 550 µm oocyte diameter	Treatment at ≥ 550 µm oocyte diameter (* in Fig. 2)		Full-grown secondary-growth oocytes (SGfg) (▲ in Fig. 2)					
						rLh (µg kg^{-1}) t = 0	P (mg kg^{-1}) t = 24	PGF2α (µg kg^{-1}) t = 39			
1	W	PGpn	Combined rFsh and rLh treatment (see Fig. 2)	Doses of 9 to 12 µg kg^{-1} rLh (no rFsh applied)	619 ± 7	15	40	-	-	-	-
2	SE	SGca			627 ± 8	15	40	-	-	-	-
3	SE	SGca			625 ± 8	30	40	18.75	801,913	832,723	Not used
4	W	PGpn			603 ± 10	30	40	-	974,928	574,500	0.1
5	SE	SGca			608 ± 8	30	40	-	754,774	676,320	0.31
6	W	PGpn		No treatment was applied as fish developed from < 550 µm to SGfg	610 ± 6	15	40	-	-	-	-
7	W	PGpn			598 ± 7	30	40	-	891,600	888,047	0.81
8	W	PGpn			605 ± 4	30	40	-	-	-	-

After the application of rLh to the GtHs-treated group to induce oocyte maturation, ovulation and spawning, females were placed in a separate 10 m^3 tank with spermiating males from the rGths treated group (n = 4). 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 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 spawning for rGth treatments. Latency time reported for flathead grey mullet after resolving doses from other hormone treatments varies from 17 to 48 hours at 22 - 25 ºC (El-Gharabawy and Assem, 2006; Yousefian et al., 2009). One female (female 3, 30 μg kg^{-1} rLh + 40 mg kg^{-1} progesterone in Fig 2 and Table 1) that had oocytes ≥ 600 μm earlier (week 9) than the other females, developed a large swollen belly without ovulation and was administered 18.75 μg kg^{-1} of prostaglandin F2α (PGF2α) (VETEGLAN, Laboratorios Calier, S.A., Spain) 39 hours after the rLh administration. The other seven females (females 1, 2, 4 - 8 in Fig 2 and Table 1) did not receive PGF2α and were checked and/or stripped as there was no natural spawning. Four females ovulated and were stripped, one female (female 3) 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 4 weeks of rFsh treatment).

2.5.2. 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 (Chauvigné et al., 2018; Peñaranda et al., 2018) (Fig 2).

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 4 weeks of hormone treatment).

2.5.3. *In vitro* fertilisation

For the *in vitro* fertilisation, sperm was obtained from three males prior to fertilization procedures, diluted 1:4 in the extender solution Marine Freeze® (IMV Technologies, France) that showed the best results for sperm conservation in a marine species (González-López et al., 2020) and stored at 4ºC for one hour before use. The eggs from each female (n = 4) were stripped and total volume registered. Aliquots of 0.5 mL of eggs (~1200 eggs) from three females (females 4, 5 and 7 that received 30 μg kg^{-1} rLh + progesterone) 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 presence and the percentage of eggs fertilised was calculated as the number of eggs with live

405 embryos/number of eggs used for the *in vitro* fertilisation. Eggs with embryonic
406 development were transferred individually into individual wells filled with sterile
407 seawater in a 96 well plate and incubated (24ºC). To evaluate the quality of the eggs with
408 embryo, the hatching success was calculated as the number of hatched larvae / number of
409 eggs with embryos 22-hours post fertilisation. Larvae were checked daily until all hatched
410 larvae had died and percentage survival on each day was calculated as the number of live
411 larvae on the day / total number of larvae that hatched. A subsample of ~one-third of
412 fertilised eggs and larvae were used for taking measurements and afterwards returned to
413 the incubation.

414

415 **2.6. Plasma steroid analysis**

416 Blood samples were centrifuged at 3,000 rpm at 4 ºC for 15 min and the plasma stored at
417 -80 ºC until steroid analysis. Plasma levels of E_2 and 11-ketotestosterone (11-KT) were
418 measured for females and males, respectively, and were analysed using a commercially
419 available enzyme immunoassay (EIA) kits (Cayman Chemical Company, USA). Steroids
420 were extracted with methanol, which was evaporated and extracts were re-suspended 1:10
421 in the EIA buffer.

422

423 **2.7. Histological observations and classification of developing ovaries**

424 Ovarian biopsy samples were preserved in Bouin's fluid, dehydrated through an ethanol
425 series and embedded in paraffin. Histological sections (3 µm) were stained with
426 hematoxylin and eosin (Casa Álvarez, Spain). To examine ovarian development, oocytes
427 sections were observed under a light microscope (Leica DMLB, Houston, USA).
428 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 each week. Oocyte developmental stage was based on the identification of structures, morphological changes and increasing oocyte diameter. Oocytes were classified as: *multiple nucleoli step of primary growth (PGmn)* characterised by small oocytes with multiple nucleoli situated within the germinal vesicle, *perinucleolar step of primary growth (PGpn)*, step after the PGmn in which the nucleoli are located around the internal membrane of the germinal vesicle, *cortical alveoli step (SGca)*, determined by the presence of small oil droplets and granular vesicles "*cortical alveoli*" in the peripheral ooplasm, *early secondary growth (SGe)*, with the appearance of yolk globules and with this the initiation of vitellogenesis, *secondary growth (SG)* corresponding to mid- to late- vitellogenesis when oocytes reached ≥ 400 μm (Greeley et al., 1987), *full-grown secondary growth oocytes (SGfg)* when vitellogenesis was completed and oocytes reached their maximum diameter prior to maturation, *oocyte maturation stage (OM)*, with the identification of coalesced oil droplets and the displacement of the germinal vesicle to the ooplasm periphery and some hydration and coalescence of yolk globules, and *ovulation stage (OV)*, when one large yolk globule is observed (Lubzens et al., 2010). Atresia was identified by the hypertrophy of granulosa cells, the loss of the individuality of yolk globules and the dissolution of their content (Valdebenito et al., 2011).

2.8. Sperm collection and evaluation

Sperm samples were collected in a 1 mL syringe avoiding the contamination by faeces, urine and / or sea water. Approximately 1 μL of sperm was placed on a microscope slide beside 0.2 mL of sea water, mixed to activate the spermatozoids and immediately (first 10 seconds) observed through a microscope at 100x magnification (Zeuss Microscopes).

453 The assessment of the milt quality was estimated by the percentage of motile spermatozoa
454 and by the total duration of the movement from sperm activation until all forward
455 movement of spermatozoa stopped. The observations were made in triplicate and the
456 percentage of motile spermatozoa was classified into different motility scores: 0 for no
457 motile sperm, 1 for > 0 – 25 % of sperm with progressive movement, 2 for > 25 % - 50
458 % of sperm with progressive movement, 3 for > 50 – 75 % and 4 for > 75 % of sperm
459 with progressive movement (Mañanós et al., 2009). For those samples in Exp. 2 with a
460 motility score of 4 and manageable sperm volumes (≥ 100 µL) (n = 10), sperm quality
461 was also evaluated using a CASA system (Wilson-Leedy and Ingermann, 2007). For this,
462 0.5 µL of diluted sperm (1/4 in Marine Freeze®) were dropped on the centre of a slide
463 and activated using 20 µL of sea water. A 1 µL sample containing the activated
464 spermatozoa was pipetted into an ISAS counting chamber (Integrated Sperm Analysis
465 System, Spain). The tracks of the activated spermatozoa were recorded through a bright
466 field equipped video microscope at 200x magnification (Olympus BH Microscope and
467 DMK 22BUC03 Camera with 744×480 "0.4 MP" resolution at 60 FPS, The Imaging
468 Source Europe GmbH, Bremen, Germany). The video sections from 15 to 17 s after
469 activation were transformed to image sequences using VIRTUALDUB 1.9.11
470 (virtualdub.org) free software. The spermatozoa in each field were selected by adjusting
471 the grayscale threshold through Image J software (https://imagej.nih.gov/ij/). The
472 following sperm quality parameters were determined: (1) sperm motility (%), (2) sperm
473 velocity (µm s^{-1}): the curvilinear velocity (VCL), straight-line velocity (VSL) and average
474 path velocity (VAP), (3) sperm movement trajectory: path linearity of actual sperm track,
475 LIN = VSL/VCL x 100), path wobble (deviation from average path, WOB = VAP/VCL x
476 100), and path straightness (linearity of the average path, STR = VSL/VAP x 100). All
477 parameters were evaluated in triplicate for each sperm sample.

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 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 mL of sperm (spz mL^{-1}). Quantification of spermatozoa was conducted using ImageJ software.

2.8. Statistical analysis

Shapiro-Wilk and Levene tests were used to check the normality of data distribution and 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 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 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 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 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 for pairwise comparisons. The data from the two experiments was compared with a 3-way-ANOVA with the independent variables, experiment, week of experiment and treatment (control vs rGths) for the dependent variables, oocyte diameter and volume of sperm. There were no significant differences amongst control groups and week 0 (before rGth application) between experiments indicating that rGth treatments could be compared between the two experiments. Analyses were performed using SigmaPlot version 12.0

(Systat Software Inc., Richmond, CA, USA). Significance was set at $P < 0.05$. Data is presented as mean ± standard error (SEM) unless indicated otherwise.

3. Results

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

There were no significant increases from the E_2 basal values after the application of doses of 0 (Control), 3, 6 and 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 rFsh produced significant increases in E_2 levels on 3 to 6 days after the injection with respect to basal levels. The administration of 15 µg kg^{-1} of rFsh produced a significant increase in E_2 levels on day 3 after the injection, which was the highest average level of E_2 observed. 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.

3.2. Experiment 1: Effect of long-term rFsh therapy in female development

3.2.1. Stage 1: Gametogenesis induction

Weekly injections of 15 µg kg^{-1} rFsh during eleven weeks to previtellogenic females generated 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 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 administration induced a significant increase of oocytes diameter ($P < 0.001$) (Fig 5A)

and vitellogenic growth (Fig 6) compared to the control group. At the beginning of the treatment all females presented oocytes at the PGpn (mean maximum diameter = 97 ± 4 µm) (Fig 6) with the exception of one female assigned to the rFsh-treated group that presented oocytes at PGmn. After 5 weeks of treatment, all rFsh-treated females (89%) except one had vitellogenic oocytes (Fig 7A). In addition, some traces of atresia appeared in some females. In the two subsequent revisions (weeks 7 and 9), SG oocytes were the most abundant with a maximum size of 425 ± 19 µm in diameter (Fig 5A). After 9 weeks of treatment, the proportion of atresia observed in the vitellogenic ovaries increased from 3 to 24 % (Fig 7A). The female that at the start of the experiment before any treatment had oocytes at PGmn was delayed compared to other females and only developed to SGe after 11 weeks of treatment. Therefore, of the nine treated females all (100%) developed from previtellogenic oocyte stages to vitellogenesis and eight (89%) developed to late vitellogenic stages of oocyte development. In comparison, the oocytes of all (100%) untreated females remained at primary growth during the entire experiment (Figs 5A, 6A).

3.2.2. Stage 2: Completion of oocyte growth and maturation

Histological examination of the oocytes after each treatment (GnRHa+MET or hCG) did not show variations in oocyte morphology although a significant increase in oocyte diameter was observed in the female injected with GnRHa+MET protocol (Table 2). The injections of hCG at doses of 1000, 2000, 6000, 12000 IU kg^{-1} combined with 15 µg kg^{-1} rFsh did not completed oocyte growth and oocyte maturation. High E_2 levels were 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 significantly different from E_2 levels (391.4 ± 56.5 pg mL^{-1}) during weeks 4 - 10 (Stage

1) in the same group. When rFsh administration for five females was ceased from week 11 onwards, the vitellogenic oocytes underwent atresia and after five weeks, only previtellogenic oocytes were observed.

Table 2. Effects of treatments applied to flathead grey mullet (*Mugil cephalus*) females to induce completion of oocyte growth and oocyte maturation 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 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 µg kg^{-1}); hCG (IU 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	437 ± 6 [a]
3	450 ± 9 [a]	-	-	1000, 2000, 6000, 12000	422 ± 8 [b]
4	470 ± 8 [a]	-	-	1000, 2000, 6000, 12000	490 ± 8 [a]

3.3. Experiment 2: Effect of combined rFsh and rLh therapy in female development

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 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 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 Supplementary Fig 1B) 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 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 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 the mean diameter of the most advanced oocytes compared to Exp 1 (Fig 5A vs 5B). As vitellogenesis progressed, oocytes at different developmental stages were present at the same time in the ovaries of rGths-treated females (Fig 7) but the size variation of the vitellogenic oocytes was reduced as the ovary developed. The progress in oogenesis in response to treatment was slightly different amongst females, which reached a ≥ 550 µm oocyte diameter at different time points between week 8 and 11. Full-grown oocytes were obtained in all eight (89%) females and the oocyte size (mean diameter of 609 ± 5 µm) became uniform as expected for isochronal spawning fishes. In comparison, all (100%) control females showed no oocyte growth or development as in Exp 1.

The three females that received 15 µg kg^{-1} rLh followed by 40 mg kg^{-1} of progesterone did not respond to the treatment and no significant increase in oocyte diameter was observed. Only those females that received 30 µg kg^{-1} of rLh followed by 40 mg kg^{-1} of progesterone (n = 5), presented oocyte maturation (OM), hydration and ovulation. Five females showed the initiation of OM indicated by oil globule coalescence and germinal vesicle migration after 24 h from rLh injection. From these females, female 3 had not ovulated 39 hours after rLh administration when an injection of PGF2α was administered. The PGF2α appeared to 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 not fully hydrated (Fig 8A). Three females, females 4, 5 and 7, which were checked at 48 ± 0.5 hours from rLh injection, ovulated (Fig 8B) and after

stripping, eggs were used for *in vitro* fertilisation (Table 1). 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 presented oocytes in OM. Therefore, of the nine females, eight (89%) terminated vitellogenesis to stage immediately prior to OM, five (56%) were induced with 30 μg kg^{-1} of rLh + progesterone and 100% of these five advanced to OM, four (80%) ovulated and three (60%) had a low percentage of viable eggs according to the percentage of fertilisation.

3.4. Male development

Control males did not produce milt neither in Experiment 1 (n = 3) nor in Experiment 2 (n = 4). In Experiment 1, in the first revision after five weeks of rFsh treatment, two of three (66.6%) males produced sperm that coincided with an increase in 11-KT levels (P = 0.043, α = 0.05, statistical power = 0.66) (Fig 9). The production of sperm was prolonged for 6 weeks, but sperm was highly viscous and sperm volumes were low (29.3 ± 7.1 μL), which made it difficult to manipulate. The mean sperm concentration was 4.6 ± 1.5 10^{10} spermatozoa mL^{-1}, the motility grade recorded was 4 (> 75% sperm with progressive movement) and the mean motility duration was 40 ± 2 seconds with no significant differences among individuals between weeks.

In Experiment 2, along the course of the treatment, all treated males (n = 4) produced sperm, which also coincided with an initial significant increase in 11-KT levels in the treated group (P = 0.006, α = 0.05, statistical power = 0.97) (before treatment: 2.2 ± 0.8; after 4 weeks: 10.5 ± 2.2 ng mL^{-1}) in comparison with the control group (before treatment: 0.7 ± 0.3; after 4 weeks: 0.5 ± 0.2 ng mL^{-1}). The inclusion of rLh in Exp 2, significantly (P <0.001) increased the volumes of sperm obtained in comparison to Exp 1. In

618 Experiment 2, sperm could be obtained by applying abdominal pressure to treated males
619 after 3 weeks of treatment (50% of males), 4 weeks (75 %) and from the fifth week to the
620 end of the treatment (100 %). From the third week of treatment to the fifth, first traces of
621 sperm (mean 30.3 ± 12.3 µL) were highly viscous with a significantly higher
622 concentration of spermatozoa (mean 2.1 ± 0.2 10^{11} spz mL^{-1}) and a motility score of 2 to
623 4 (25 to > 75% motility). After six weeks, higher quantities of sperm where obtained
624 (242.5 ± 70.9 µL) coinciding with a previous increase in rLh administration, which
625 decreased (68.7 ± 13.7 µL) afterwards. Viscosity and spermatozoa concentration (2.3 ±
626 0.8 10^{10} spz mL^{-1}) significantly decreased compared to the first weeks sperm was
627 obtained. Motility score was 4 for all males until the end of the treatment. Mean duration
628 of sperm motility was 89 ± 14 seconds during the 6 weeks that sperm was collected.

629 Assessment by CASA of the 10 samples collected from all four males with high motility
630 score and ≥ 100 µL volume showed a mean motility percentage of 74 ± 0.01 %, VCL of
631 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
632 %, WOB of 93.5 ± 0.1 % and STR of 97.9 ± 0.7 %.

633

634 **3.5. *In vitro* fertilisation**

635 The 0.5 mL aliquots of stripped eggs (1224 ± 150 eggs) were fertilised by mixing with
636 60 µL (20 µL/male) of pooled diluted stripped sperm (sperm 1:4 in Marine Freeze®) (3.8
637 ± 0.8 10^{9} spz mL^{-1}). The mean sperm to egg ratio at fertilisation was 189,521 ± 23,541
638 spermatozoa egg^{-1}. After an incubation period of 22 - 23 hours (24ºC), mean embryo
639 percentage survival was 0.4 ± 0.2 % (n = 3 females). At this age, the head region had
640 formed and dark pigments covered almost all of the embryo and the oil globule (Fig 10A).
641 Although, 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. Hatching percentage of the fertilized eggs, observed at 39 - 40 hours after fertilisation, was 70.8 ± 20 % (Fig 10B). *Mugil cephalus* larvae at 1 dph had developing eye lens and a reduced yolk sac diameter (Fig 10C). At 2 dph the yolk and oil globule were still present, but mouth parts were completely formed with upper and lower jaws opened (Fig 10D). Survival percentage of larvae decreased to 38.6 ± 22 % at 1 day post-hatching (dph) and continued decreasing to 4.1 ± 1.4 % (2 and 3 dph) until zero (4 dph).

4. Discussion

The present study shows that rFsh drives oogenesis from early to late gonad developmental stages in female flathead grey mullet, that rLh is influential to achieve oocyte maturation and ovulation and that rGths can be used to produce milt from male flathead grey mullet. These findings are significant to both demonstrate the accepted roles of the Gths in teleost reproductive development and to provide advances for the control of reproduction in teleost species that experience reproductive dysfunctions early in the maturation process.

Flathead grey mullet is a species that exhibits severe reproductive dysfunctions in captivity that threatens the sustainability of its culture making it mostly dependant on wild captures. Despite of the present study being timed to coincide with the natural reproductive period, no reproductive development was observed in control females that remained arrested in previtellogenesis (primary growth or cortical alveoli stage) and no sperm was obtained from control males when abdominal pressure was applied. All of the control fish had sufficient size, 35 - 49 cm for females and 32 - 42 cm SL for males, and

condition to mature according to reported sizes of maturity; 27 - 35 cm standard length for females and 25 - 30 cm for males (Whitfield et al., 2012). The present study, encountered a more severe reproductive dysfunction than has been observed in other studies (Aizen et al., 2005). The severity of the reproductive dysfunction, highlights that in the present study, the application of rGths was critical in stimulating reproductive development in female fish and availability of sperm in males.

The hormone therapy to control the progress of oogenesis was initiated with the application of rFsh. The administration of different doses of rFsh to examine the biological activity of this recombinant hormone, obtained a significant and prolonged (3 - 6 days) increase of E_2 after injection 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 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 Exp 2) to female flathead grey mullet. In the present study, the rFsh-mediated 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 by the liver (Lubzens et al., 2010). In both experiments, oocytes grew from previtellogenic perinucleolar stage and/or cortical alveoli stage to advanced vitellogenic stages after rFsh administration. This oocyte growth was observed in eight (89%) of the nine treated females in both experiments. There was some variation in individual responses that ranged from a few more advanced females to two females (one in each experiment) that did not reach vitellogenic stages in the 4 - 5 week-period. Despite of this variation, the present study presents a considerable advance to successfully induce oogenesis in 89% of experimental fish with the application of rFsh in a teleost. The fact

690 that the rFsh doses including lower rFsh doses in Exp 2 were sufficient to induce
691 vitellogenesis may indicate that rFsh doses could be refined for future inductions.

692 The present study also provides evidence that Fsh is the major hormone to initiate
693 vitellogenesis in teleosts. To date, no study has demonstrated that the exogenous
694 application of just Fsh promotes the initiation of vitellogenesis and development through
695 to late vitellogenic stages and that development progressed correctly to provide oocytes
696 for the formation of viable eggs and larvae. The central role of Fsh in fish vitellogenesis
697 is accepted (Lubzens et al., 2010; Mañanós et al., 2009) based on parallels drawn with
698 other taxa, the synchronised increase in plasma Fsh and oocyte development found in
699 many fish species, genomic approaches such as gene knockout to define Gths pathways
700 (Zhang et al., 2015) and that rFsh induced partial development of vitellogenesis (Kazeto
701 et al., 2008; Nguyen et al., 2020; Palma et al., 2018; Sanchís-Benlloch et al., 2017).
702 However, some criticisms can be made as, many differences in the control in reproduction
703 exist between taxa, synchronised increases in Fsh and oocyte development do not
704 necessarily indicate cause – effect, vitellogenesis although delayed proceeded when the
705 Fshb gene was knocked out to make Fsh-deficient zebrafish (*Danio rerio*) (Zhang et al.,
706 2015) and previous studies did not induce the entire process of vitellogenesis (Kazeto et
707 al., 2008; Nguyen et al., 2020; Palma et al., 2018; Sanchís-Benlloch et al., 2017).
708 Therefore, the present study has added clear evidence to demonstrate the accepted
709 function of Fsh by reporting in a teleost species that rFsh successfully induced the process
710 of vitellogenesis from previtellogenic stages to advanced stages from which fertilised
711 eggs and larvae were obtained.

712 The biological activity of rFsh applied to females of other fish species has been previously
713 studied, but most studies have focused on *in vitro* approaches for receptor-binding
714 capacity (So et al., 2005) and steroidogenic potency (Kasuto and Levavi-Sivan, 2005;

715 Meri et al., 2000; Zmora et al., 2007) or *in vivo* short-term effects (Kazeto et al., 2008;
716 Ko et al., 2007; Kobayashi et al., 2006, 2003; Molés et al., 2011) rather than *in vivo* long-
717 term effects on gonadal development. When rFsh produced in other heterologous systems
718 than CHO cells were tested in long-term treatments in different fish species, more time,
719 dose and/or number of administrations were required to reach a less advanced stage of
720 ovary development than in the present study. For instance, after 60 days of treatment with
721 injections at 10-day intervals of rFsh produced in *P. pastoris* (10 - 20 µg kg^{-1}) immature
722 yellowtail kingfish oocytes developed to cortical alveoli stage (Sanchís-Benlloch et al.,
723 2017). Weekly injections for 8 weeks at 100 µg kg^{-1} to juvenile grouper (*Epinephelus*
724 *fuscoguttatus*) also induced development to the cortical alveoli stage (Palma et al., 2018).
725 Recombinant Fsh produced in *Drosophila* S2 cell line (100 µg kg^{-1}) induced early
726 vitellogenesis in the Japanese eel after 56 days of treatment with a weekly administration
727 (Kazeto et al., 2008) and rFsh (500 µg/kg) produced in HEK293 cells induced initial oil
728 droplet stage in previtellogenic yellow shortfinned eels (*Anguilla australis*) after three
729 weeks (Nguyen et al., 2020). These comparisons between the present study and other
730 studies suggest a higher biological potency of rFsh produced in CHO cell lines as
731 previously reported in some species (Molés et al., 2011).

732 Nevertheless, the administration of only rFsh in Exp 1 failed to complete oocyte growth
733 as although oocytes developed until mid to late secondary growth, the cells appeared to
734 be arrested in this stage and subsequently, a substantial number of atretic cells were found
735 in the later weeks (weeks 9 - 11). These results agree with previously described E_2 roles
736 that did not include OM (Lubzens et al., 2010), but differ from those obtained by Das et
737 al. (2014) who induced OM in *Mugil cephalus* post-vitellogenic oocytes that were
738 incubated *in vitro* with E_2. The fact that completion of oocyte growth could not be
739 achieved using only rFsh suggested that, as previously described, OM and ovulation are

Lh-dependent (Lubzens et al., 2010; Nagahama and Yamashita, 2008). According to Nagahama and Yamashita (2008), secretion of Lh from the pituitary coincides with a switch in the gonad steroidogenic pathway from the production of predominantly E_2 during vitellogenesis to the production of progestin-like steroids, the maturation-inducing steroids (MIS). The MIS bind to oocyte membrane-specific receptors to activate the maturation promoting factor (MPF) that induces germinal vesicle breakdown and OM (Lubzens et al., 2010). Therefore, in Stage 2 of Exp 1 and in Exp 2, we focused on the use of exogenous sources of Lh receptor agonists or hormones that may trigger the release of Lh from flathead grey mullet pituitary with the aim to complete oocyte growth and induce OM.

The application of hormone treatments (GnRHa+MET or hCG) in Stage 2 of Exp 1 failed to induce oocyte growth and OM. The oocytes remained arrested in the secondary growth stage of development with mean oocyte diameters of 425 ± 19 µm and an increasing incidence of atresia. It appeared that the developmental stage of the oocytes was not sufficient to respond to the hormone treatments that have been successful in a wide range of species that were arrested at a later developmental stage close to OM (Malison et al., 1998; Mañanós et al., 2009). Other studies on *Mugil cephalus* have recommended an oocyte diameter > 550 µm before OM and ovulation induction (Aizen et al., 2005; El-Gharabawy and Assem, 2006; Vazirzadeh and Ezhdehakoshpour, 2014). However, a wide range of other possible contributing factors can be cited, such as pituitary Lh content may have been low, the follicles were not receptive at the time of hormone application and did not stimulate the switch in gonad steroidogenic pathway to MIS or that the administration of rFsh complicated the switch as agonists of the Lh receptor also stimulated the Fsh receptor (Chauvigné et al., 2012; So et al., 2005). However, considering that the hormone treatments used in Stage 2 of Exp 1 were applied to few

765 fish, no conclusion can be drawn other than oocyte development was arrested with the
766 application of only rFsh and no further development was observed.

767 In contrast, in Experiment 2, the co-administration of rLh at advanced stages of
768 vitellogenesis induced the completion of oocyte growth to a mean size of 609 ± 5 µm in
769 eight (89%) of the nine treated females. Experiment 2 compared to Exp 1 (arrest at oocyte
770 diameters of 425 ± 19 µm with only rFsh), appeared to show that the addition of rLh was
771 required to increase maximum oocyte diameter to a diameter (>550 µm) that represents
772 the completion of oocyte growth and a diameter from which OM has been observed to
773 progress (Tamaru et al., 1993; Yousefian et al., 2009, present study). These observations
774 indicate that the completion of vitellogenesis could be dependent on Lh, which is a role
775 often associated to Fsh. However, some caution is needed in comparing these groups in
776 different experiments as different rFsh doses were used and the two experiments had
777 slightly different conditions. In Exp 1, the rFsh dose was higher (15 µg kg^{-1}) compared to
778 Exp 2 that used a lower rFsh dose (12 µg kg^{-1}). The higher rFsh dose in Exp 1 did not
779 induce the completion of vitellogenesis and may have been detrimental as an increasing
780 percentage of atresia was observed when the ovary was arrested in late vitellogenesis. In
781 comparison, in Exp 2 the lower dose of rFsh in combination with increasing doses of rLh
782 induced the completion of vitellogenesis. The different experimental conditions appeared
783 not to affect maturational development as control groups in both experiments remained
784 in previtellogenesis with no significant difference. The dosage and the time interval of
785 rLh treatment applied to induce OM were based on previous studies (Chauvigné et al.,
786 2017). However, since the half-life of rLh in plasma has not been determined in flathead
787 grey mullet, the most efficient hormone treatment (dose and timing) remain to be
788 established. In relation to the induction of OM and ovulation, the rationale behind the
789 treatment of rLh plus progesterone, a precursor of maturation-inducing steroids, was to

induce the Lh-mediated up-regulation of genes associated with these processes and to avoid potential substrate-limiting factors for MIS synthesis. In Exp 2, only the five fish receiving the highest rLh dose (30 µg kg^{-1}) with progesterone proceeded to OM compared to three fish that received a lower rLh dose (15 µg kg^{-1}) with progesterone that did not develop to OM. This indicated 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 bitterling (*Rhodeus ocellatus ocellatus*) (Kobayashi et al., 2006), common carp (*Cyprinus carpio*) (Aizen et al., 2017) and Malaysia catfish (*Hemibagrus nemurus*) (Salwany et al., 2019). However, the present study cannot confirm if a unique injection of rLh could have completed OM and ovulation without the need of progesterone application. Further work is required to fully understand the roles and administration of rFsh, rLh and progesterone to successfully execute the steroid switch to induce OM and ovulation. The mean fecundity of the four (44%) females that were successfully induced with rLh and progesterone to complete OM and ovulation was 742,900 ± 71,840 eggs kg^{-1} bw (~ 855,800 eggs $female^{-1}$), which was within the range previously reported for *M. cephalus*, 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 (González-Castro and Minos, 2016). The fecundity obtained, the dynamics of oocyte development and characteristics of stripping all the eggs were consistent with reports that flathead grey mullet produce one set of ova a year (Whitfield et al., 2012; Rao and Babu, 2016).

Regarding males, the rGths treatments induced the production of milt for fertilisation procedures. The 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 in Exp 2, both significantly increased the levels of 11-KT, which is the major androgen responsible for testicular development (Aizen et al., 2005; Chauvigné et

al., 2012; Mañanós et al., 2009; Schulz et al., 2010). In comparison, no sperm could be obtained from males in control groups. Other studies have induced or increased the production of milt obtained by abdominal pressure in sexually immature Japanese eel (Hayakawa et al., 2008; Kamei et al., 2006; Kobayashi et al., 2010) and European eel (Peñaranda et al., 2018) and mature Senegalese sole (Chauvigné et al., 2018, 2017) after gonadotropin administration. The administration of rFsh alone induced the production of low milt volumes, whilst the additional administration of rLh increased milt volumes and decreased spermatozoa 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 (Peñaranda et al., 2018) and similarly the addition of rLh increased volumes and decreased spermatozoa concentration. The little seminal fluid produced in the present experiments could 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}) (Ramachandran and Natesan, 2016). Nevertheless, the rGth treatments provided sperm for fertilisation procedures even though the number of males in the study was low. Curiously, the present study also indicated that there may be a sex specific contrast in the effect of rFsh, as in males rFsh alone induced the production of mature spermatozoa compared to females in which rFsh alone did not induce mature gamete production, and ovaries were arrested in late vitellogenesis and atresia was observed. However, further studies are required to examine and determine the existence of this sex specific difference and clarify the interactions amongst rGth levels and receptors or the mechanisms that may be responsible.

After hand stripping gametes (3 females and 3 males) and *in vitro* fertilisation, 0.4% of eggs developed embryos. The low percentage of eggs developing an embryo may be related to *in vitro* fertilisation procedures. The morphological aspect of the eggs appeared

840 normal with the exception that 28% of the eggs had multiple oil droplets. In flathead grey
841 mullet, the manual pressure of artificial stripping increased the frequency of multiple oil
842 droplets (Kuo et al., 1973) and multiple oil droplets were related with low egg survival
843 (Nash and Shehadeh, 1980). Another aspect related to bad egg quality and *in vitro*
844 fertilisation procedures is overripening (Ramos-Júdez et al., 2019). After ovulation, there
845 is a period of egg ripeness with optimal viability after which the eggs overripen, losing
846 quality and viability. This period of optimal egg quality for stripping varies among
847 species, with temperature, between different stocks, holding conditions, hormone
848 induction treatments and ideally should be defined for each situation (Ramos-Júdez et al.,
849 2019). For example, latency to obtain good quality eggs can be as long as 5 - 15 days over
850 a temperature range of 10 – 17 ºC for rainbow trout (*Oncorhynchus mykiss*) (Samarin et
851 al., 2008), 3 hours in meagre (*Argyrosomus regius*) at 18 ºC (Ramos-Júdez et al., 2019)
852 but only 30 min in white bass (*Morone chrysops*) at 22 °C (Mylonas et al., 1996). For the
853 present treatment in *M. cephalus*, the timing of ovulation and optimal egg quality has not
854 been previously defined. However, latency times have been reported for flathead grey
855 mullet using carp pituitary extracts with hCG or GnRHa (Karim et al., 2016), hCG (Kuo
856 et al., 1973) and pituitary glands combined with synahorin and vitamin E (Liao, 1975)
857 and times ranged from 30 to 48 hours after the initial priming dose and 12 to 26 h after
858 the resolving dose. In the present study, eggs were stripped at 40 h and 48 ± 0.5 h from
859 rLh administration (16 h and 24 h from progesterone). The female stripped at 40 h was
860 induced to ovulate with PGF2α and the stripped eggs had vitellogenin and oil in the
861 process of coalescing apparently not having completed maturation and hydration when
862 the oocytes were ovulated. On the contrary, at 48 ± 0.5 h after the rLh injection, low
863 fertilisation percentages were obtained. This was at the limit of the period of good egg
864 quality that has been found with 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. However, it cannot be discounted that the egg quality was low due to aspects of the rGth induction protocol. Therefore, further studies to determine the timing of ovulation and the window of good egg quality are crucial to determine the quality of eggs that can be achieved with rGth based therapies.

Fertilised flathead grey mullet egg diameter has been reported to vary from 0.65 - 1.08 mm differing with different geographical areas (González-Castro and Minos, 2016). 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 ‰. 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 (González-Castro and Minos, 2016). 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 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 the second-day, 36 hours post-hatch, before the completion of yolk sac absorption (Abraham et al., 1999).

In conclusion, the present study reports that treatment with rGths (rFsh and rLh) was able to induce oogenesis from previtellogenesis to produce eggs and larvae in a teleost. These advances in the control of the reproductive process using rGths, and particularly the induced initiation of vitellogenesis, development through to late stages with rFsh and the completion of oocyte growth with rLh offer further data about the roles of the Gths in teleost oogenesis. A refined protocol 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 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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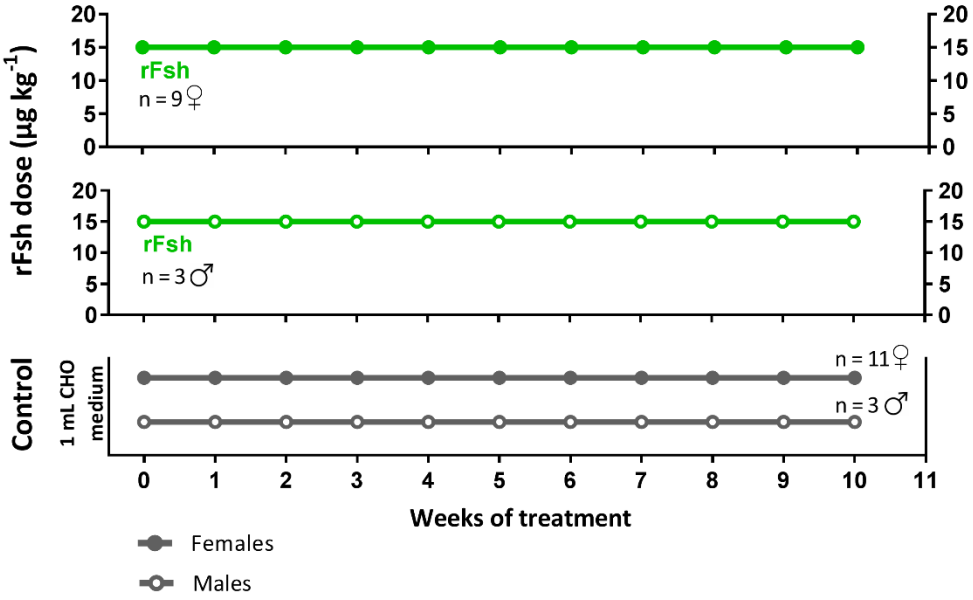

Figure 1. Schematic representation of the protocol administered to flathead grey mullet (*Mugil cephalus*) in Stage 1 in Experiment 1.

Flathead grey mullet females (n = 9) and males (n = 3), received weekly doses of intramuscular injections of rFsh during 11 weeks. Control individuals (n = 11 females, n = 3 males) received weekly injections of CHO conditioned culture medium (1 mL fish^{-1}). Information about Stage 2 can be found in the text or Supplementary Figure 1A.

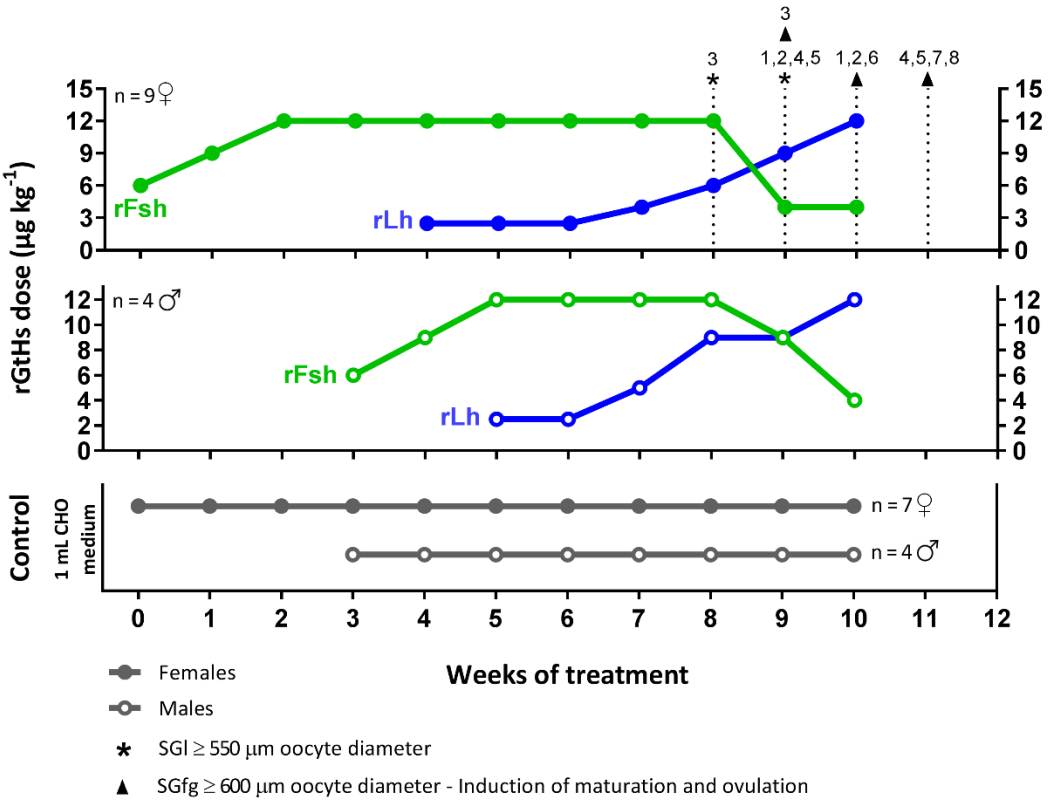

Figure 2. Schematic representation of the weekly administration to flathead grey mullet (*Mugil cephalus*) in Experiment 2.

Initial increasing administration of rFsh was followed by a gradual increase of rLh as gametogenesis progressed and, after this, by a subsequent decline of rFsh in both females (n = 9) and males (n = 4). Males followed a shortened program in order to synchronise development of both sexes. The aim of the rGths administration pattern was to simulate increases and decreases of Fsh and Lh in the bloodstream in accordance to their proposed regulatory role in gamete development (Levavi-Sivan et al., 2010). An asterisk indicates when numbered females presented ≥ 550 µm oocytes and, therefore, rLh was administered every three days. A triangle indicates when females were considered to have completed vitellogenic growth (≥ 600 µm oocyte diameter or maximum diameter achieved). At this point, females were administered higher doses of rLh (15 or 30 µg kg^{-1}) combined with 40 mg kg^{-1} of progesterone administered 24 h after to induce oocyte

1178 maturation, ovulation and spawning (see details in Table 1). The individuals in control
1179 groups (n = 7 females, n = 4 males) underwent the same number of intramuscular
1180 administrations as treated individuals, but with CHO culture medium (1 mL $fish^{-1}$).
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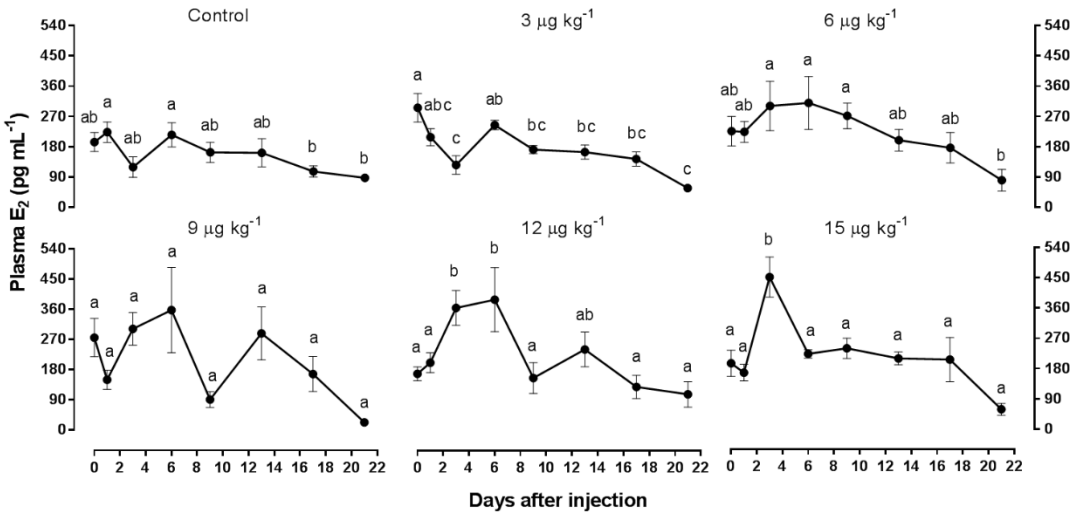

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1184 **Figure 3. Mean (± SEM) plasma E_2 levels of female flathead grey mullet (*Mugil*
1185 *cephalus*) before (day 0) and after (day 1, 3, 6, 9, 13, 15 and 21 days) the rFsh**
1186 **injection.** Females (n = 5/group) received a single injection of rFsh at doses 3, 6, 9, 12
1187 or 15 µg kg^{-1} and an injection of 1 mL $fish^{-1}$ CHO conditioned culture medium for control.
1188 Different letters indicate significant differences ($P < 0.05$) over time within each dose.

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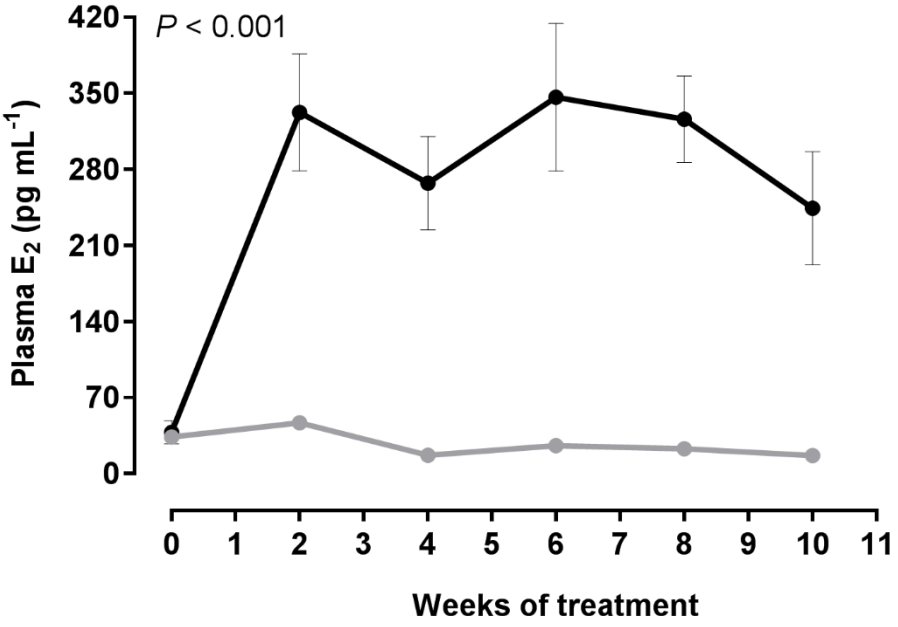
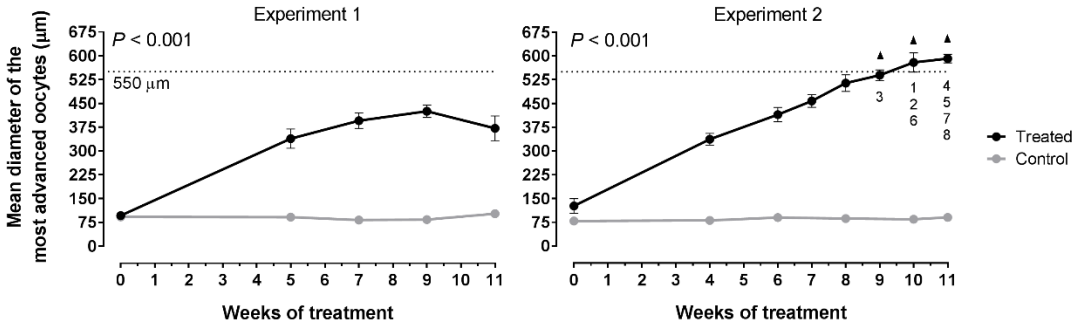


Figure 4. Mean (± SEM) plasma E_2 levels of rFsh-treated and control flathead grey mullet (*Mugil cephalus*) females (n = 9-11) in Experiment 1. Treated females received weekly injections of rFsh (15 µg kg^{-1}) and control females of CHO conditioned culture medium (1 mL $fish^{-1}$). There were significant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



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1198 **Figure 5. Mean (± SEM) oocyte diameter of the most developed oocytes in wet**
1199 **mounts from rFsh treated and control flathead grey mullet (*Mugil cephalus*)**
1200 **females.** (A) Experiment 1, females treated (n = 9) with a weekly 15 µg kg^{-1} rFsh
1201 administration or CHO conditioned culture medium (control, n = 11) during 11 weeks.
1202 (B) Experiment 2, females treated (n = 9) with initial increasing doses of rFsh followed
1203 by increases in rLh and subsequent rFsh decrease or CHO conditioned culture medium
1204 (control, n = 7). Values used for females checked twice in the same week were the mean
1205 of both revisions. Triangles show the moment when numbered females (see Fig 2 and
1206 Table 1) were selected for maturation and ovulation induction. There were significant
1207 differences between treated and control groups (two-way repeated measures ANOVA, *P*
1208 < 0.001). Dotted line indicates oocyte size recommended for the hormonal induction of
1209 oocyte maturation.

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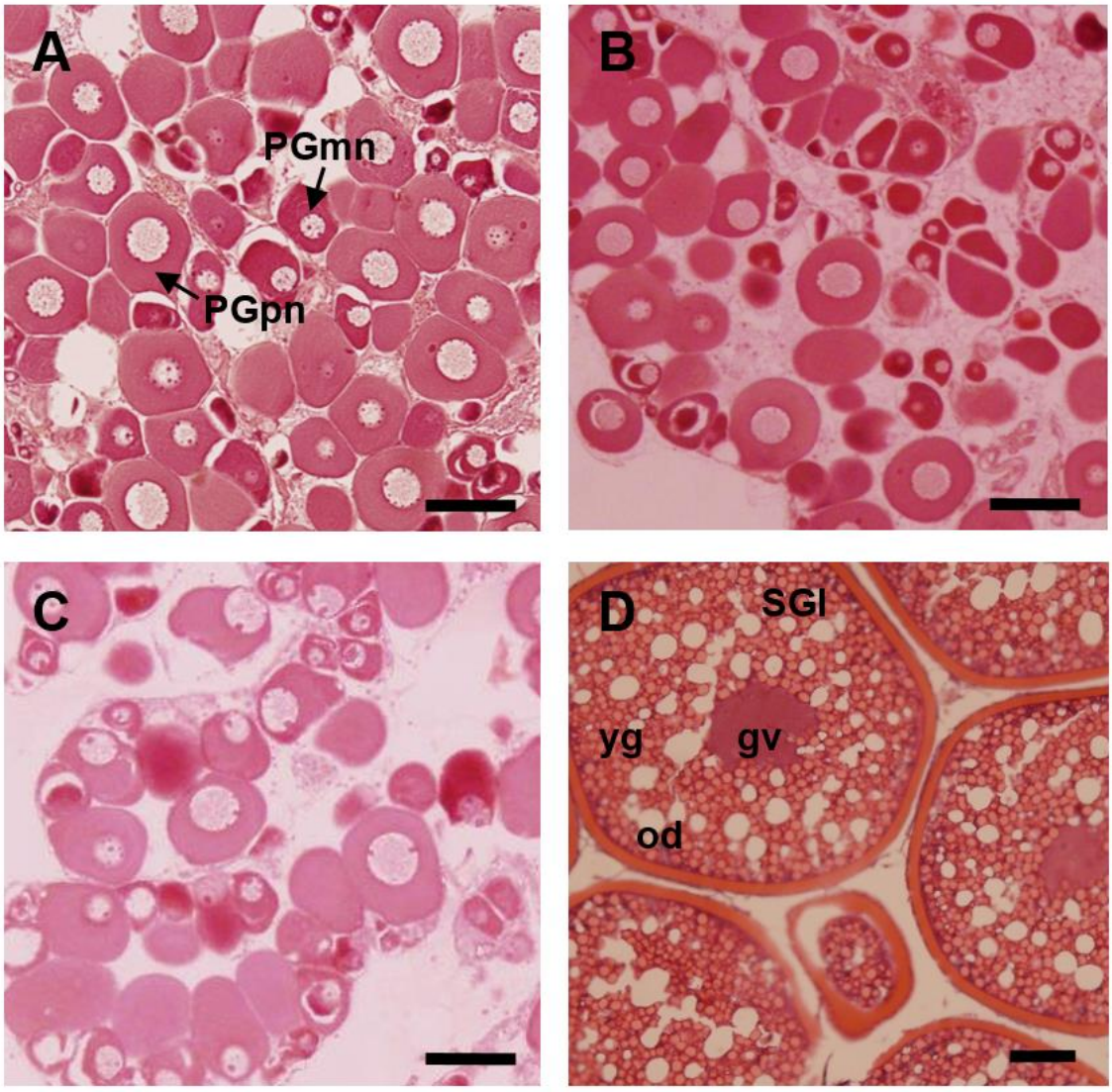


Figure 6. Effects of long-term treatment of rFsh on ovarian development in previtellogenic flathead grey mullet (*Mugil cephalus*) *in vivo*. Histological sections stained with hematoxylin and eosin show oocytes samples from (A) initial control fish, (B) rFsh-treated fish before treatment, (C) control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

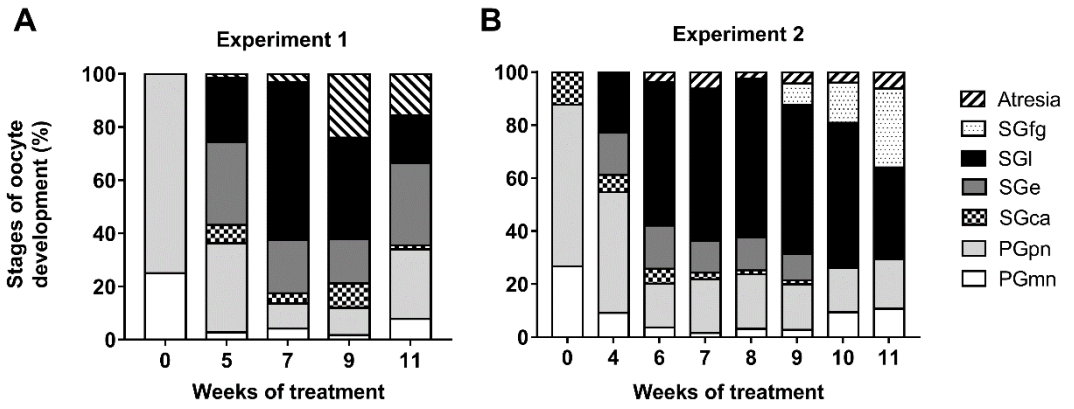


Figure 7. Temporal weekly evolution of percentage frequency of oocyte developmental stages observed in rGth-treated flathead grey mullet females (*Mugil cephalus*). (A) Experiment 1 with weekly 15 µg kg^{-1} rFsh administration to the treated group (n = 9) during 11 weeks. (B) Experiment 2 with the administration of initial increasing doses of rFsh followed by increases in rLh and subsequent rFsh decrease (n = 9). Shaded bar sections represent the mean percentage of oocytes per stage from all females for each week. A total of 50 to 100 random oocytes were classified per female and percentage of each oocyte stage calculated per female. Oocytes were obtained from each female by cannulation and fixed in Bouin's solution for histology for examination and classification. PGmn, 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-growth oocytes.

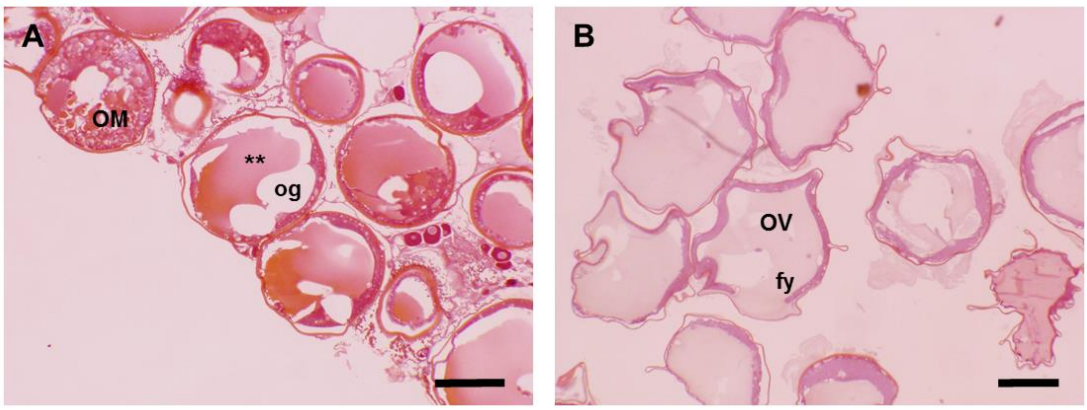


Figure 8. Oocyte maturation and hydration stages for treated flathead grey mullet females (*Mugil cephalus*) in Experiment 2. (A) Ovulated eggs from female 3 at 40 hours after 30 µg kg^{-1} of rLh injection (16 hours from 40 mg kg^{-1} progesterone) and 1 hour from 18.75 µg kg^{-1} PGF2α injection. Oocytes in maturation: yolk globules coalesce and fuse to form a one large globule (**). Central oil globule 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). 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. Scale bar: 500 µm.

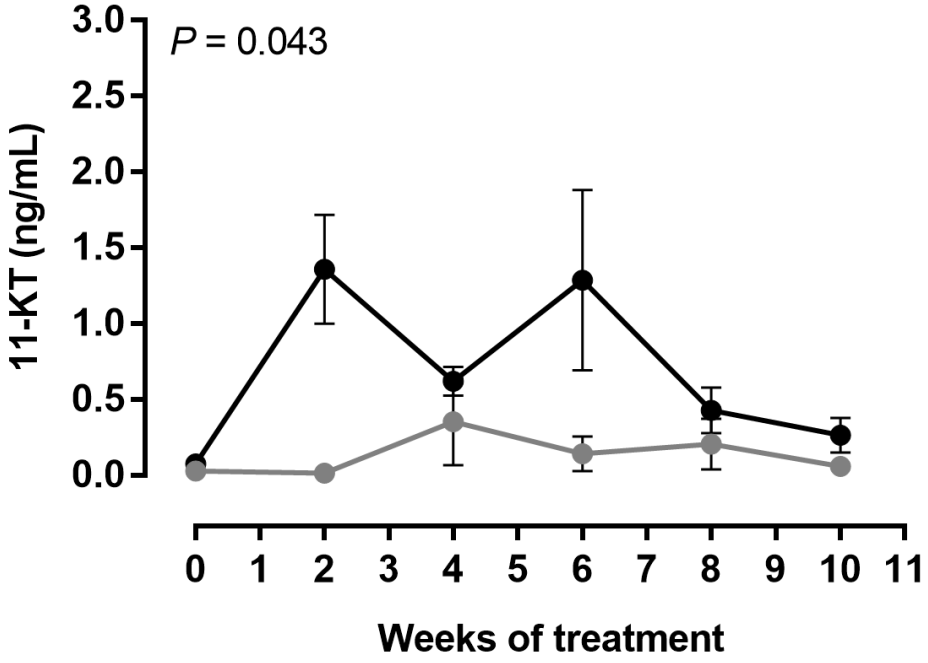


Figure 9. Mean (± SEM) plasma 11-KT levels of rFsh-treated (15 µg kg^{-1}) males flathead grey mullets and controls (n = 3-4). Treated males received weekly injections of rFsh (15 µg kg^{-1}) and control males of CHO conditioned culture medium (1 mL $fish^{-1}$). There are significant differences among treatments (two-way repeated measures ANOVA, $P = 0.043$, $\alpha = 0.05$, statistical power = 0.66).

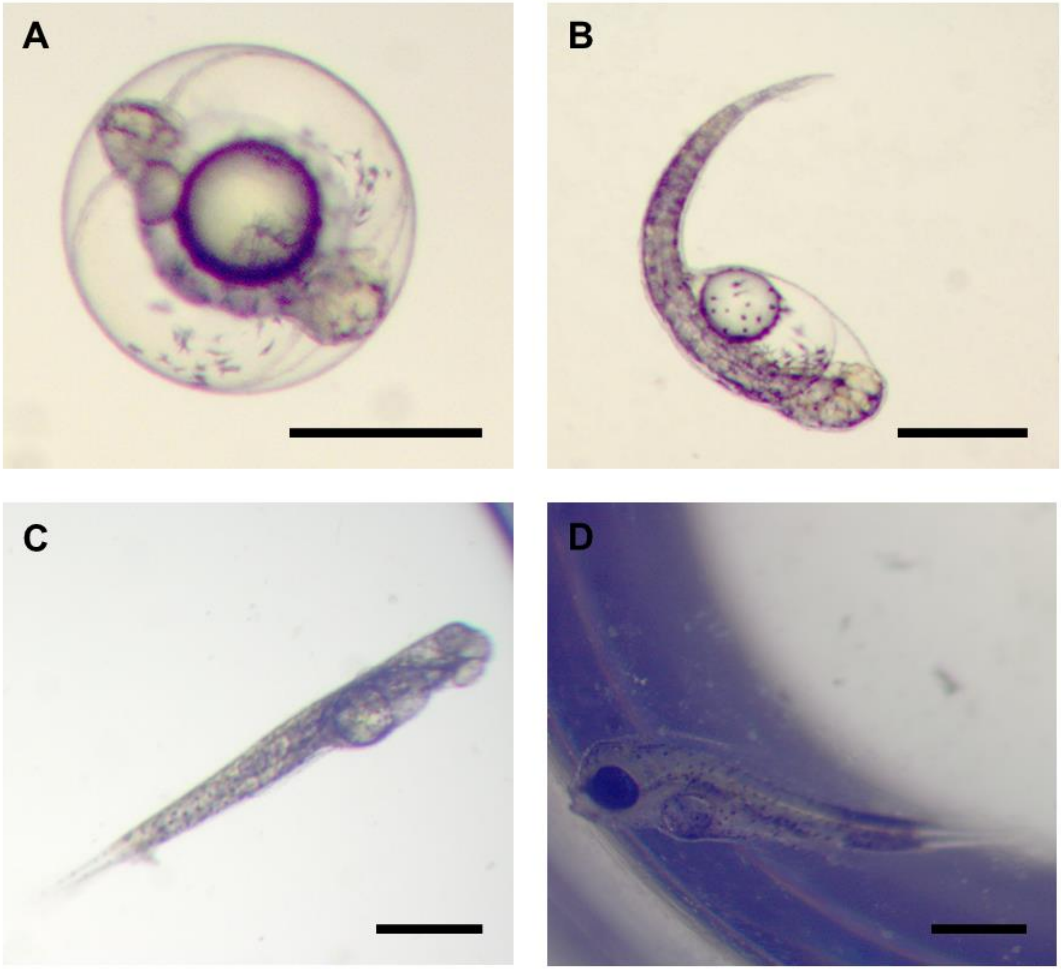
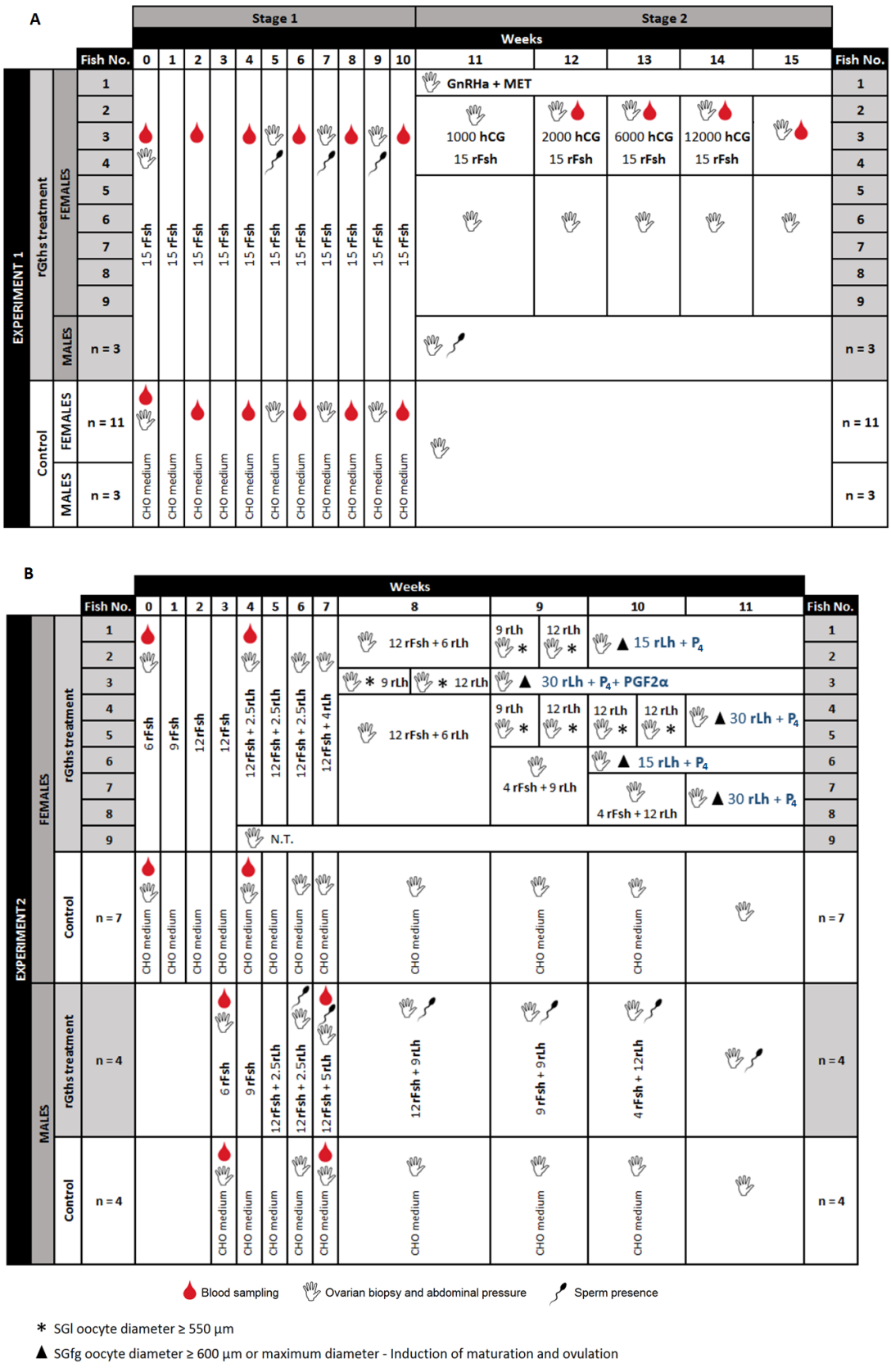


Figure 10. Developing *Mugil cephalus* embryos and larvae from Experiment 2. (A) Embryo at age 22 h post-fertilisation with head region formed and dark pigments covering almost all the embryo and on the oil globule. (B) Hatching at age of 40 hours post-fertilisation. (C) Larva after 1 dph. A decrease in yolk sac was observed and the eye lens formed. (D) Larva after 2 dph with well-developed eye, with mouth parts formed and opened. Oil globule was still present. Scale bar: 500 µm.



Supplementary Figure 1. Detailed schematic representation of the protocol of

administration in flathead grey mullet (*Mugil cephalus*) in (A) Experiment 1 and (B) Experiment 2.

Columns represent weeks of each experiment and rows represent the different fish. In (A) Experiment 1, females (n = 9) and males (n = 3), received weekly doses of intramuscular injections of rFsh. Control individuals (n = 11 females, n = 3 males) received weekly injections of CHO conditioned culture medium (1 mL $fish^{-1}$) during 11 weeks. 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 (Aizen et al., 2005), whilst females 2 - 4 were administered increasing doses of hCG in addition to rFsh. In (B) Experiment 2, females (n = 9) received increasing doses of rFsh, and from the 4^{th} 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. When the most developed oocytes reached a diameter of ≥ 600 µm, females were administered higher doses of rLh, 15 µg kg^{-1} (females 1, 2 and 6) or 30 µg kg^{-1} (females 3, 4, 5, 7 and 8) combined with 40 mg kg^{-1} of progesterone (P_4) administered 24 h after the rLh injection to induce oocyte maturation, ovulation and spawning. Female 3 was also administered 18.75 µg kg^{-1} of PGF2α 39 hours after the rLh injection. 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 progesterone in mg 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, an asterisk

1287 shows when females had oocytes ≥ 550 µm and a triangle shows the moment when
1288 females had oocytes ≥ 600 µm and maturation and ovulation was induced.