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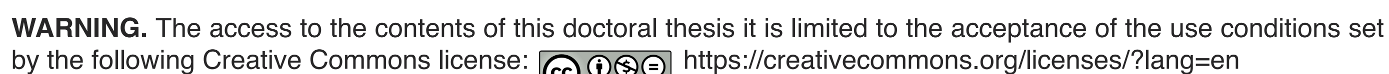

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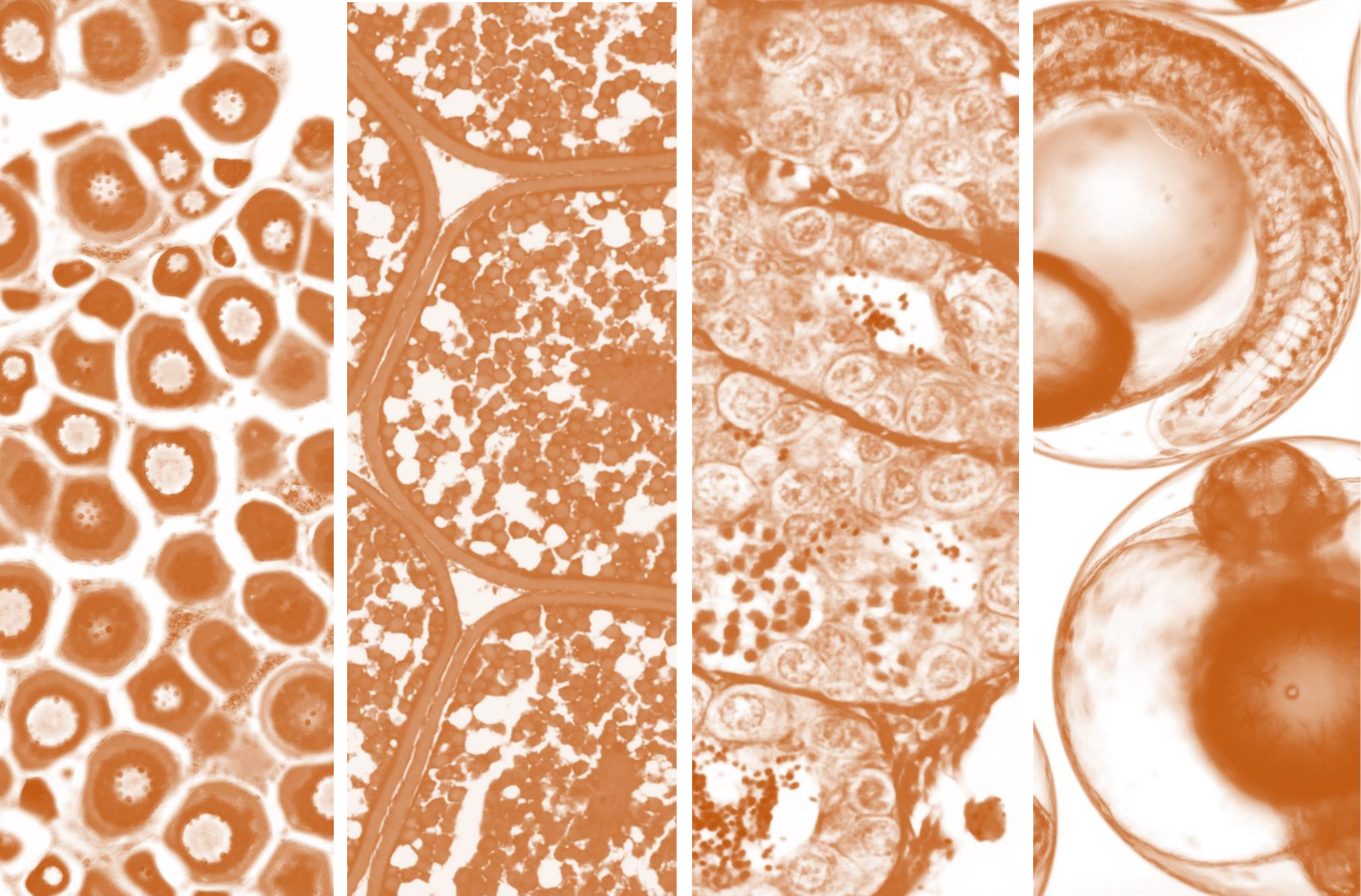

Control of reproduction in the flathead grey mullet *Mugil cephalus*

Broodstock management, hormonal therapies and transcriptomic signature

PhD thesis

2021

Sandra Ramos Júdez

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Control of reproduction in the flathead grey mullet *Mugil cephalus*: Broodstock management, hormonal therapies and transcriptomic signature

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for obtaining the title of Doctor of Philosophy (PhD) in Aquaculture

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A mis padres

Abstract

The flathead grey mullet (*Mugil cephalus*) is a good candidate for the diversification of aquaculture species due to its adaptability to different culture conditions, ecological profile, and the market potential of its high added-value products. The future development of this species relies on the control of reproduction in captivity, especially in intensive conditions, to provide hatchery-reared fry and not depend on the capture of wild fry or the induction of wild mature individuals. The control of reproduction has been complicated by the severe reproductive dysfunction found in intensive captive conditions; females remain arrested at previtellogenesis or early-vitellogenesis, and males do not produce sperm or barely produce a drop of highly viscous milt. Therefore, with the main goal to control the reproduction of this species in captivity, the present thesis focused on the application of different hormonal approaches to solve the reproductive dysfunction including examining the endocrinology and transcriptome in control and treated fish. In addition, other aspects related to broodstock management were examined; the timing of gonadal development and spawning in a natural population, lipid and fatty acid changes during gonadal development and how to provide adequate broodstock nutrition, considering the species feeding habits, when diets do not exist.

To identify the onset of vitellogenesis and the spawning season of wild flathead grey mullet in the Western Mediterranean, which would serve as an indicator of the timing of the reproductive dysfunction in captivity, forty-four wild females were sampled in October and November 2018 and from February to October 2019. Macroscopic, histological, and biometric analysis (gonadosomatic index, GSI%) were performed. According to the results, vitellogenesis had started in early August, and the spawning season occurred between September and October.

To identify the nutritional requirements of the flathead grey mullet broodstock, the seasonal changes in lipid and fatty acid composition of muscle, liver and ovary of wild flathead grey mullet females were characterized. Samples were obtained at: (i) previtellogenesis (n = 7), (ii) early-vitellogenesis (n = 6), (iii) late-vitellogenesis (n =7), and

(iii) post-spawning period (n =7). Throughout ovarian development, total lipid content was low and constant in the muscle (3.85 - 4.92 %), high and constant in the liver (18.46 - 22.62 %), and increased during gonadal development (4.90 - 34.59 %) to decrease after spawning. During vitellogenesis, percentage of total saturated fatty acids deposits, mainly of the palmitic acid (16:0), decreased in the ovary suggesting their catabolization for oocyte formation. Contrarily, percentage of total mono-unsaturated fatty acids, principally of the palmitoleic acid (16:1) and the heptadecenoic acid (17:1) which is of bacterial origin and acquired through the diet, significantly increased in ovaries and might contribute to embryo reserves. There was a mobilization of n-3 polyunsaturated fatty acids (PUFA), especially of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), from the liver. Percentage of PUFA, mainly of EPA, DHA, and arachidonic acid (20:4n-6, ARA) which was accumulated at high levels in the ovary, significantly decreased in the ovaries with advancing vitellogenesis.

To describe the optimal feed characteristics and feeding habits of wild-caught flathead grey mullets maintained in an intensive culture system, the behavioural responses to pellets and the preferred feeding area were studied. In a first experiment, the optimal pellet size was defined according to the attractiveness and acceptability of the different diameters (2, 4, 6, 8 mm) that were dropped in a random sequence (143 ± 19 pellets / diameter) into the tank. In a second experiment, two pellet types, floating or sinking, were offered simultaneously in the water column: at the surface, mid-water column and bottom of the tank. Larger pellets (6 and 8 mm) were more attractive (lower reaction time, high percentage of capture), but the small to medium-sized pellets (2 and 4 mm) were the highest consumed. Optimal pellet diameters for juveniles (~ 360 g) were 2 and 4 mm, while for adults (~ 930 g) was 4 mm. Flathead grey mullet preferred to feed in the water column and the bottom rather than in the surface; therefore, the recommendation would be sinking or slow-sinking pellets.

Several experiments were conducted with different hormonal schemes to induce or enhance gametogenesis, spermiation, and final oocyte maturation and ovulation. Broodstock included wild-caught flathead grey mullets and from a semi-extensive culture brought to intensive conditions. Hormonal treatments consisted, on one hand, of an acute treatment with two intramuscular injections: recombinant follicle-stimulating

hormone (Fsh) (5 µg kg^{-1}) produced in *Pichia pastoris* expression system, and a dopamine antagonist (15 mg kg^{-1}), as it is reported to exist a dopaminergic inhibition of sexual maturation, together with 17α-methyltestosterone (MT) implants (6.7 to 11.6 mg kg^{-1}) in males. This treatment did not induce vitellogenesis, and females (n = 9) at the end of the experiment were in previtellogenesis as the control group (n = 5) that was treated with saline solution. Four out of six treated males produced milt (10 – 200 µL), while the two control males did not spermiate.

On the other hand, different long-term treatments were tested consisting of weekly injections of rFsh (doses from 4 to 15 µg kg^{-1}) and /or recombinant luteinizing-hormone (rLh) (doses from 2.5 to 24 µg kg^{-1}) produced in CHO (Chinese Hamster Ovary) cells. To induce oocyte maturation and ovulation when vitellogenic growth was completed, injections of rLh (15 or 30 µg kg^{-1}) with Progesterone (P_4) (40 mg kg^{-1}) (rLh + P_4), priming and resolving rLh (30 µg kg^{-1}) (rLh + rLh), or priming and resolving P_4 (40 mg kg^{-1}) (P_4 + P_4) were given to females. The weekly application of recombinant gonadotropins (rGths) showed different results in females: (1) the rFsh application during eleven weeks induced vitellogenesis (n = 9 females) to a maximum oocyte diameter of 425 ± 19 µm, (2) initial rFsh application, followed by a combination of rFsh and rLh, and mainly rLh at advanced vitellogenesis, induced the completion of vitellogenic growth to ~600 µm in the 100 % of females that received the complete treatment (total 29 females from two experiments). Regarding male development: (1) rFsh application during eleven weeks induced two out of three males to produce viscous milt, while (2) 100 % (4 males in one experiment and 9 in other) spermiated with higher fluent milt when treated with a combination of rFsh and rLh. Regarding oocyte maturation (OM) and ovulation, only those females that received the highest rLh dose (30 µg kg^{-1}) presented OM. In one experiment (n = 5 females), rLh + P_4 induced ovulation in 80 % females with the 60 % having a low percentage of fertilized eggs (0.4 % eggs with embryo) after artificial fertilization. In other experiment, rLh + P_4 (n = 9 females) and rLh + rLh (n = 6) induced 100 % ovulation with 89 % and 100 % spawning success, respectively. The eggs collected from the tanks presented 63 ± 21% fertilization with embryo development and 58 ± 23 % hatching. The treatment P_4 + P_4 (n = 6) had a lower ovulation success (50 %) and spawning success (17 %) with no fertilized eggs. Controls did not show further gonadal

development from initial stages. Altogether, the present results confirmed the possibility of controlling reproduction of flathead grey mullet from early gametogenesis to the completion of maturation and fertilized tank spawning using exclusively rFsh and rLh.

To describe the differential expression of genes and molecular pathways in the transcriptome amongst different stages of ovarian development induced with the treatment of rFsh and rLh, repeated ovarian samples were collected by cannulation from the same five females at four sampling points/stages of development; from initial arrested gonad before rGths application (previtellogenesis) (Stage I), from early-to-mid-vitellogenic oocytes after rFsh administration (Stage II), from late secondary-growth oocytes after combined treatment with rFsh and rLh (Stage III), and from full-grown oocytes after rLh administration (Stage IV). The RNASeq libraries were constructed for all the stages, sequenced on an Illumina HiSeq4000, and a *de novo* transcriptome assembly was constructed, which was constituted of 287,089 transcripts after filtering. Differentially expressed genes (DEGs) were identified during development and characterized the functional properties of DEGs by comparison with the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes databases. Upregulated DEGs from Stage I to II were enriched in pathways related to steroidogenesis and reproductive development, such as *steroid biosynthetic process (GO:0006694)* and *response to estradiol (GO:0032355)*. From Stage II to III, pathways related to the production of energy, such as *lysosome (GO:0005764)*, or with the incorporation of lipoproteins into oocytes, such as *regulation of low-density lipoprotein receptor binding (GO:1905599)*, were enriched of upregulated DEGs. In the transition from Stage III to IV there was an enrichment in the *C-21 steroid hormone biosynthetic process* pathway, indicating a preparation of the oocyte towards maturation. Overall, the enriched molecular pathways and DEGs described during the induced vitellogenesis of flathead grey mullet with rFsh and rLh were typical of natural oogenesis reported for other fish species.

To sum up, this thesis has increased knowledge about different aspects for the proper management of flathead grey mullet broodstock, has successfully applied a treatment with rFsh and rLh that, together with the evaluation of the transcriptomic changes at ovarian level, have permitted to have full control of the flathead grey mullet reproduction in intensive conditions with the production of viable eggs and larvae. In

addition, these findings raise the possibility of using the treatment of rFsh and rLh in species that present similar reproductive disorders in aquaculture, the aquarium industry and for the conservation of endangered species.

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CHAPTER I

General Introduction

CHAPTER I:

General Introduction

1. A global overview of aquaculture production

Aquaculture refers to the farming of aquatic organisms including fish, molluscs, crustaceans, and aquatic plants. During the last 20 years aquaculture has been one of the food production sectors with the fastest growth in the world. The sector contribution to global finfish and shellfish production has increased from 25.7 % in 2000 to account for about 47.8 % of the total production in 2019 [62], and 52 % of production destined for human consumption [64]. The average world growth rate was 5.8 % per year from 2001 to 2010, however, it has slowed gradually to 4.5 % in the last years (2011 to 2019). In 2019, the world aquaculture finfish and shellfish production (excluding algae and ornamental shells and pearls) achieved a maximum of 84.3 million tons; 56.3 million tons of fish — 47.3 million tons from freshwater aquaculture and 9 million tons from marine and brackishwater aquaculture—, 17.5 million tons of molluscs and 10.5 million tons of crustaceans [62]. At the regional level, aquaculture represented the 60.3 % of finfish and shellfish total production in Asia, 19.2 % in North and South America, 19.1 % in Europe, 18.4 % in Africa, and 11.3 % in Oceania [62]. The aquaculture production is mainly concentrated in Asia (88.3 %) with China as the largest producer holding more than the half (56.1 %) of world production [62]. Nowadays, despite of the great diversity in the total species raised (> 600 species), finfish aquaculture is dominated by a small number of species with the 20 most important species representing the 83.6 % of total production. These 20 most important finfish species are principally inland species, i.e., cyprinids, tilapia, salmon, catfish, and trout [64].

Seventy-five percent of finfish aquaculture in Europe is concentrated in Norway (56.1 %), the United Kingdom (7.8 %), Greece (4.1 %), Spain (3 %), Italy (1.9 %), and France (1.8 %) [62]. In 2019, European finfish aquaculture production was dominated by the Atlantic salmon (*Salmo salar*) (1,721,809 tons), rainbow trout (*Oncorhynchus mykiss*) (329,751 tons), common carp (*Cyprinus carpio*) (168,351 tons), gilthead seabream (*Sparus*

aurata) (91,091 tons) and sea bass (*Dicentrarchus labrax*) (82,758 tons) [62]. This actual European finfish aquaculture production is mainly sustained by carnivorous species. Nowadays, approximately 12 % of world fish production (including fisheries) is reduced to fishmeal and fish oil to produce aquafeeds or used as raw material for direct feeding in aquaculture [64]. The availability of fishmeal and fish oil is a limiting factor in which the production of carnivore species mostly depends on. The supply of fishmeal and oil, however, has been projected to diminish in the long-term because of, for example, the higher pressure by society to improve aquaculture sustainability, and the increasing fishing costs and production [244] which could be linked to the reduction of natural fish stocks —34.2 % of species have already been declared as overfished [64]—. Although the aquaculture production of species that require little or no dietary fish meal is increasing in freshwater aquaculture, for example, with carps and tilapia [64], it is important to direct the focus of research on new feed sources and / or diversify the species that are cultured with, in particular, herbivorous or omnivorous marine species, such as the flathead grey mullet (*Mugil cephalus*), to make aquaculture sustainable for the future.

2. The flathead grey mullet (*Mugil cephalus*)

Out of the 26 genera and 80 species belonging to the family Mugilidae [58], only three species are of aquaculture importance [46]. The most commonly cultured mullet species, the flathead grey mullet, is considered to be a potential candidate for the diversification of European marine aquaculture since it has good characteristics; can be reared in seawater, brackish water and freshwater, also in various aquaculture systems [195], and has fast growth (~ 1 kg per year) [63] converting food efficiently to body mass [59]. Flathead grey mullet is a highly marketable fish, a high-quality source of protein and has good flesh properties [122]. Moreover, the salted and dried roe *bottarga* from gravid females is considered a pricey delicacy (>100€ kg^{-1}) in the southern Mediterranean and Asia [59], adding value to the culture of this species which is contemplated to be inexpensive and resource-efficient [20]. Besides, it is an excellent candidate for sustainable and environmentally friendly aquaculture that have been reared with fishmeal-free feed [77,132] as the species natural diet is omnivorous detritivore [34]. In relation to these attributes

for aquaculture, Nash and Shehadeh postulated in 1980, "the Mugilidae have the brightest future of all marine and brackishwater finfish in the developing technology of aquaculture".

2.1. Biology and characteristics

Flathead grey mullets are members of the Order Mugiliformes, Family Mugilidae, which are Actinopterygian teleost. *Mugil cephalus* consists of a globally distributed complex of 14 parallel mitochondrial lineages (cryptic species) [54] found in coastal temperate and tropical waters between latitude 42 ºN and 42 ºS following a discontinuous distribution [82] (**Fig 1**). In general, each lineage has a regional distribution but different lineages can coexist at a single locality, and, therefore, the delimitation is still challenging [54]. Even though considered a marine species, flathead grey mullets are euryhaline and can be found throughout the full range of estuarine salinities [195]. Flathead grey mullets are demersal fish [121] that commonly inhabit water depths of 20 m but can be found offshore or in deeper waters [272].

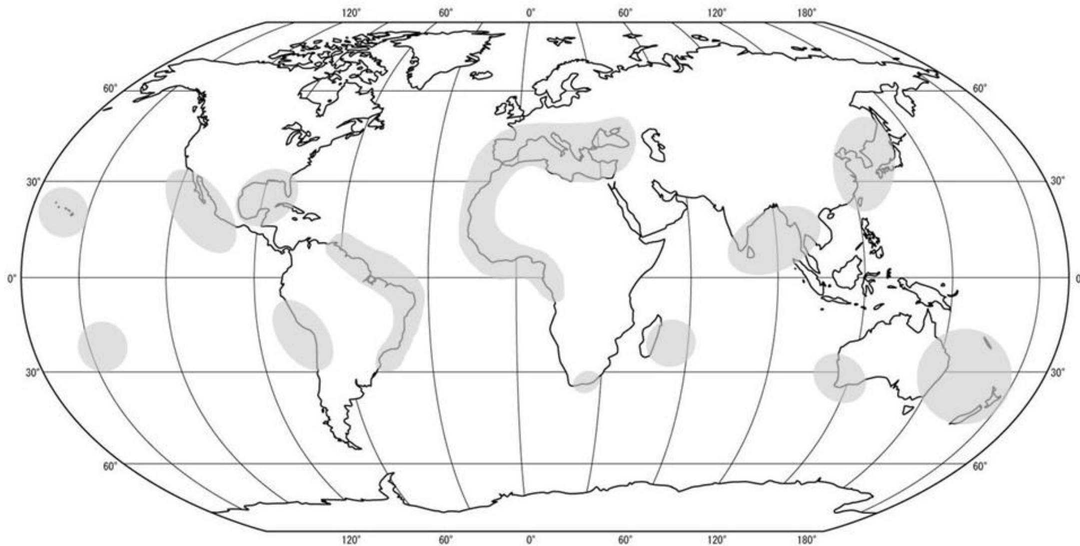

Figure 1. Global distribution records of flathead grey mullet (*Mugil cephalus*). Modified from Whitfield *et al*. (2012).

Flathead grey mullets are ray-finned fish with a subcylindrical body shape, oval in cross-section with a smoothly curving profile, and uniform in appearance (**Fig 2A**).

Flathead grey mullets are usually greyish-green or blue dorsally with silvery flanks and horizontal dark stripes, and absent lateral line. The ventral side is pale or yellowish. The species can reach a length of up to 120 cm making it the largest mullet species [82]. It is a gonochoric species, with separated sexes, but there is no external sexual dimorphism [83]. Flathead grey mullets are characterized by their broad head which is dorsally flattened and their thick, soft, transparent adipose eyelid which seems to be the most developed within mullet species (**Fig 2B, 2C**). The adipose eyelid covers most of the eye and has a vertical elliptical opening. This species has two separated dorsal fins, the first has four spines and the second has one spine and eight branched rays. Pelvic fins are sub-abdominal with one spine and five branched rays. The anal fin has three spines and eight branched rays. However, spine and ray counts cannot be used to differentiate *M. cephalus* from other mugilids. The scales are typical percomorph type and are cycloid in the early juveniles becoming later ctenoid [82]. The species possess an oral and branchial filter-feeding mechanism with gill rakers and denticulate pharyncobranchial organ used for filtration of ingested material [34].

The species is mainly diurnal. Larvae are zooplanktivorous, and juveniles and adults are primarily detritivores and benthic microalgal feeders, ingesting and filtering organic matter, although they can feed on invertebrates and plankton. Algae also forms part of their diets while living in freshwater [34].

The size at sexual maturity recorded for this species is 25 - 30 cm standard body length (SL) for males and 27 - 35 cm SL for females which would correspond to approximately 3 years old. Spawning season differs according to the geographical locations but avoids extreme water temperatures —under 17 ºC and over 28 ºC —; for instance, in the eastern Mediterranean it is between June and October coinciding with the warmest months (20 ºC – 28 ºC) while in the Atlantic coast of South Carolina (USA), within October and April coinciding with the coldest months (20 ºC – 25 ºC) [272]. Adult flathead grey mullets migrate in large schools from inshore waters and estuaries to the sea for spawning in a single spawn per season. Fecundity is high and a review by González-Castro and Minos (2016) estimates a normal range of 500,000 to 3,000,000 eggs female^{-1} depending on the adult size which would correspond to 1,473,488 eggs in individuals of 1 kg according to the formula of *Ln Fecundity = 6.95 + 1.05 (Ln BW without*

the ovaries) established by Mcdonough *et al*. (2003). Flathead grey mullet is oviparous; pelagic eggs are released into the water and fertilized. When the larvae reach 16 - 20 mm, migrate back to rivers and estuaries [272].

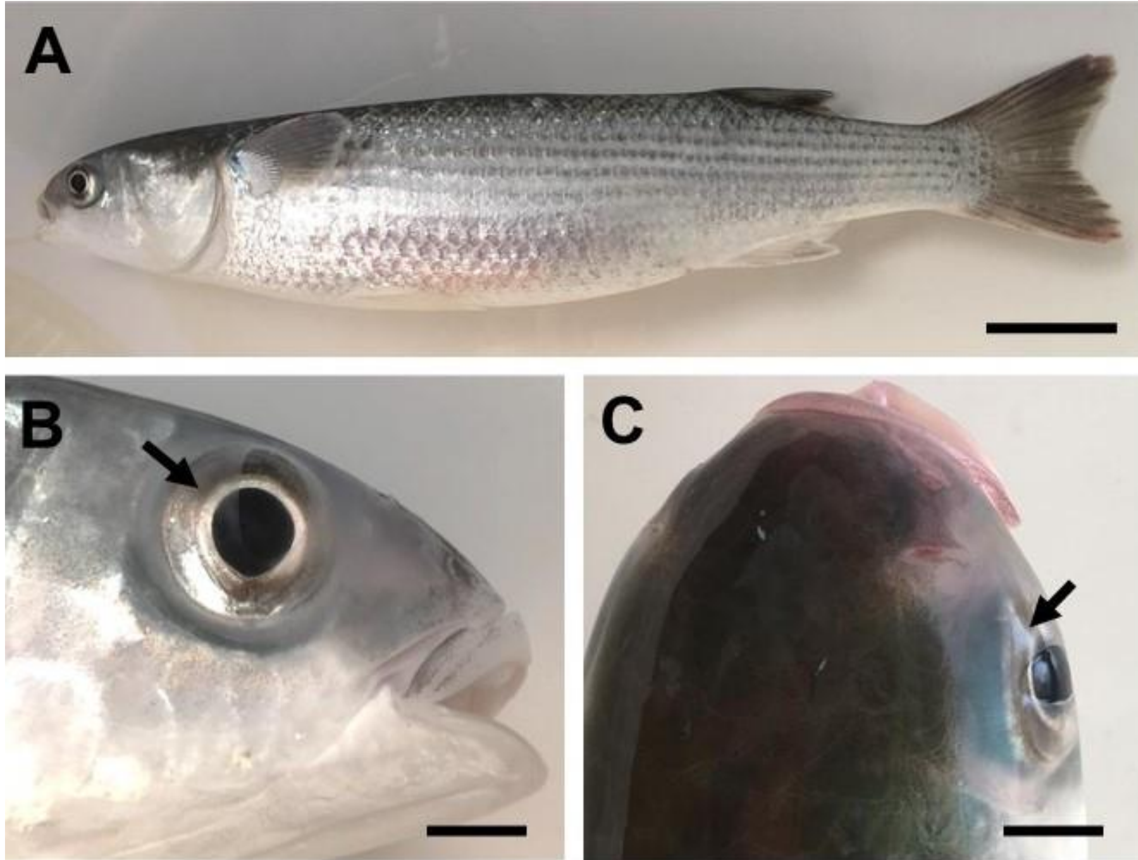

Figure 2. Pictures of (A) a flathead grey mullet (*Mugil cephalus*) from Ebro Delta canals, Spain, (B) and (C) detail of the transparent adipose eyelid which covers part of the eye and has a vertical elliptical opening. Scale Bars = (A) 5 cm, (B, C) 0.5 cm.

2.2. State of flathead grey mullet culture

The flathead grey mullet has a long production history and has been of major importance in several countries. For instance, Ancient Egyptian hieroglyphics show locals farming mullets around 4,300 years ago [46]. First flathead grey mullet production data records were published in 1950 with 1040 tons; the 10 % of which was produced in Europe and the 90 % in Asia, with more than half of the total production from China [62]. Despite the great enthusiasm for mullet culture in the 70s and 80s, it never underwent the development expected in those years. Most probably because of the difficulties

closing the life cycle in captivity and that the performed experiments were not applied at a commercial scale [46]. The highest production levels were reached in the late 90s with the intensification of cultures [46] with a peak of 9585 tons in 1997 [62]. Since then, flathead grey mullet production has never reached that level. In 2019, world flathead grey mullet production was 6124 tons, with an average annual growth of 0.05% in the last decade (2009 - 2019). In 2019, Asia held more than the 90% of world-wide flathead grey mullet aquaculture production. The seven countries with the highest production were: Taiwan (2182 tons), Israel (2147 tons), China (984 tons), Singapore (500 tons), Greece (251 tons), Tunisia (247 tons), and Saudi Arabia (60 tons) [62].

The actual production of flathead grey mullet is still largely dependent on wild captures [6,34,281]. The experience with wild seed collection, and the high seed production costs have hindered the development of commercial mullet hatcheries. Besides that, egg supplies for hatcheries are mostly obtained from captured wild breeders at post-vitellogenesis that are induced to spawn [1,49,55,116,260]. However, the capture of wild seed and the fishing activity are unreliable and unpredictable practices, as wild juveniles / broodstock are available seasonally for a limited time. These activities are also unsustainable and together with land reclamation of lagoons, brackish water lakes, and agricultural and industrial pollution, have led to the collapse of stocks in several areas but mainly in the Western Central Atlantic, the Mediterranean and the Black Sea [78,122,281]. However, in captive conditions and notably under intensive culture conditions which would represent a sustainable solution for a consistent supply of eggs, larvae, and juveniles, flathead grey mullet breeders do not spawn spontaneously. For instance, in the Mediterranean intensive conditions, although some females manage to go through vitellogenesis, the vast majority remain arrested at previtellogenesis or at the early stages of vitellogenesis [6] and males produce no sperm or low volumes of highly viscous milt which causes a reduction in the dispersal of the spermatozoa in the water and hence reduces the sperm fertilization capacity [6,173,279]. Therefore, a high priority for flathead grey mullet culture is the development and application of protocols for the control of reproduction in captivity, especially in intensive culture.

3. Reproduction in Teleosts

The reproductive cycle is a chain of successive processes that start from immature germ cells to the formation of mature gametes, egg and spermatozoa, with the aim of obtaining a fertilized egg and the development of an embryo. Gametes develop from primordial germ cells (PGC) that migrate to the germinal epithelium, where the gonad is formed, during embryonic development. The PGC then proliferate by mitosis until their differentiation into oogonia or spermatogonia, in females and males, respectively. In the last mitotic division, cells enter into meiosis initiating gametogenesis [104]. The reproductive cycle can be separated in two relevant phases; gametogenesis with gonadal growth, and maturation that culminates in ovulation or spermiation and spawning [152,155,231].

There is a high variety of reproductive strategies among fish, including viviparity —the guppy (*Poecilia reticulata*)—, sequential hermaphroditism —the protandrous gilthead seabream or the protogynous orange spotted grouper (*Epinephelus coioides*)—, simultaneous hermaphroditism —the black hamlet (*Hypoplectrus nigricans*) —[19]. However, the great majority of fish are gonochoristic and oviparous, present separate sexes, external fertilization and embryonic development [19,104,152].

3.1. Gonadal development

3.1.1. Ovarian development: oogenesis, maturation and ovulation

The ovary of female fish is a bilateral organ in the abdominal cavity. The ovaries are projected through a pair of oviducts that connect to the genital papilla opening to the exterior [155]. There are two types of ovaries: cystovarian and gymnovarian. The cystovarian ovary, which is present in many teleosts, is surrounded by an ovarian capsule formed of somatic tissue. The gymnovarian, characteristic of salmonids and eels, does not have part of the ovarian capsule and ovulated eggs are released into the abdominal cavity [110].

The germinal unit of the ovary (follicle) consists of an oocyte englobed by two layers of somatic follicular cells; an inner layer of granulosa cells and an outer layer of

theca cells separated by a thin basal membrane [104]. These follicular cells offer structural and functional support, mediate the entrance of molecules, synthesize hormones and factors necessary for the differentiation, growth and survival of the oocyte. A thick acellular envelop, the zona radiata, surrounds the oocyte, to which the granulosa cells are directly attached, and will constitute the egg chorion [155].

Before the start of the reproductive cycle, the oogonia populations in the ovary proliferate through mitotic divisions. At a certain time, some oogonia enter into meiosis and become primary oocytes, which are arrested at prophase I. Oogenesis (**Fig 3**) is commonly classified into primary (PG) and secondary growth (SG). The primary oocytes go through a PG phase or previtellogenesis, which is characterized by a size increase, the presence of pale material in the cytoplasm and the formation of the follicle. At PG, two oocyte types are observed: the nucleolus stage and the perinucleolar nucleolus stage. The first one is characterized by centrally-located germinal vesicle with a single nucleolus, and the second, by the growth of the oocyte and the presence of multiple peripheral nucleoli situated around the internal membrane of the germinal vesicle. Oocytes can remain at this stage all the entire juvenile period. After it, the SG follows, and can be divided in three developmental stages: cortical alveolus stage, vitellogenesis, and oocyte maturation (OM). The cortical alveolus stage is characterized by the occurrence of cortical alveoli, that have a role in the prevention of polyspermy during fertilization of the oocytes, and lipids droplets in the ooplasm leading to a significant increase in oocyte diameter. However, not all species present cortical alveoli. In the vitellogenesis stage, the oocyte increases in size as the cytoplasm is filled with yolk granules, formed principally with vitellogenin (VTG), the storage protein in fish oocytes [140,263–265], together with other molecules, such as carbohydrates and lipids [110]. Yolk oocytes reach their maximum size, becoming full-grown oocytes before OM, when the resumption of meiosis occurs. The oocyte advances to metaphase II, the first polar body is released and the oocyte becomes a secondary oocyte [184]. During the maturation process, the germinal vesicle migrates towards the periphery of the oocytes with the formation, in some species, of large oil droplets and the coalescence of yolk. Final OM is characterized by the germinal vesicle completely migrated and the dissolution of the nuclear membrane, a process called germinal vesicle break down (GVBD). At this point, the mature oocyte remains arrested

until hydration, that happens just prior to ovulation and spawning. In this process, the oocyte incorporates water due to a modification of the ionic composition of the cytoplasm. The hydration is principally relevant in fishes producing pelagic (buoyant) eggs with a 2 or 3-fold increase in oocyte volume. At ovulation, the follicle layers surrounding the oocyte break and release the ova into the lumen of the ovary. In oviparous species, spawning occurs right after ovulation since there is a window of viability of ovulated eggs, that varies amongst species. Once spawned, the empty follicular envelops remain in the ovary and form the postovulatory follicles (POFs) that are reabsorbed a few days after ovulation [27,152,155,178]. Before and after ovulation, atretic oocytes can also be found. These are oocytes that interrupted the process of vitellogenesis or OM because of different factors in the reproductive environment such as starvation, temperature changes and stress [152] that induce changes in the hormonal regulation [155].

The underlying oocyte recruitment pattern into SG varies in relation to the spawning characteristics of a species. Ovarian development has been classified in synchronous, group-synchronous and asynchronous. In fish with synchronous ovaries, all oocytes start vitellogenesis and advance synchronously through further stages of development. Total spawners semelparous species such as the coho salmon (*Oncorhynchus kisutch*) that participate in one reproductive cycle and then die, do not maintain a reserve of PG oocytes and synchronously recruit all oocytes into SG. In fish with group-synchronous ovaries, at minimum two populations of oocytes are present in the ovary throughout the reproductive season (i.e., one population of PG and one or more populations of developing oocytes). These fish can be separated into single-batch and multiple-batch spawners. Single-batch spawners with group-synchronous ovaries undergo OM and ovulate once per spawning season [19,155,178,253] such as the striped bass (*Morone saxatilis*), the rainbow trout [253] and the flathead grey mullet [136]. On the contrary, multiple-batch spawners ovulate and spawn several times within the course of a few weeks such as the European sea bass [155] and the greater amberjack (*Seriola dumerili*) [180]. In fish with asynchronous ovaries, oocytes of all stages of development are present without a dominant population. Several batches are recruited into OM, and ovulation

and spawning are almost daily during the annual spawning season such as the gilthead seabream [288] and the Japanese yellowtail (*Seriola quinqueradiata*) [269].

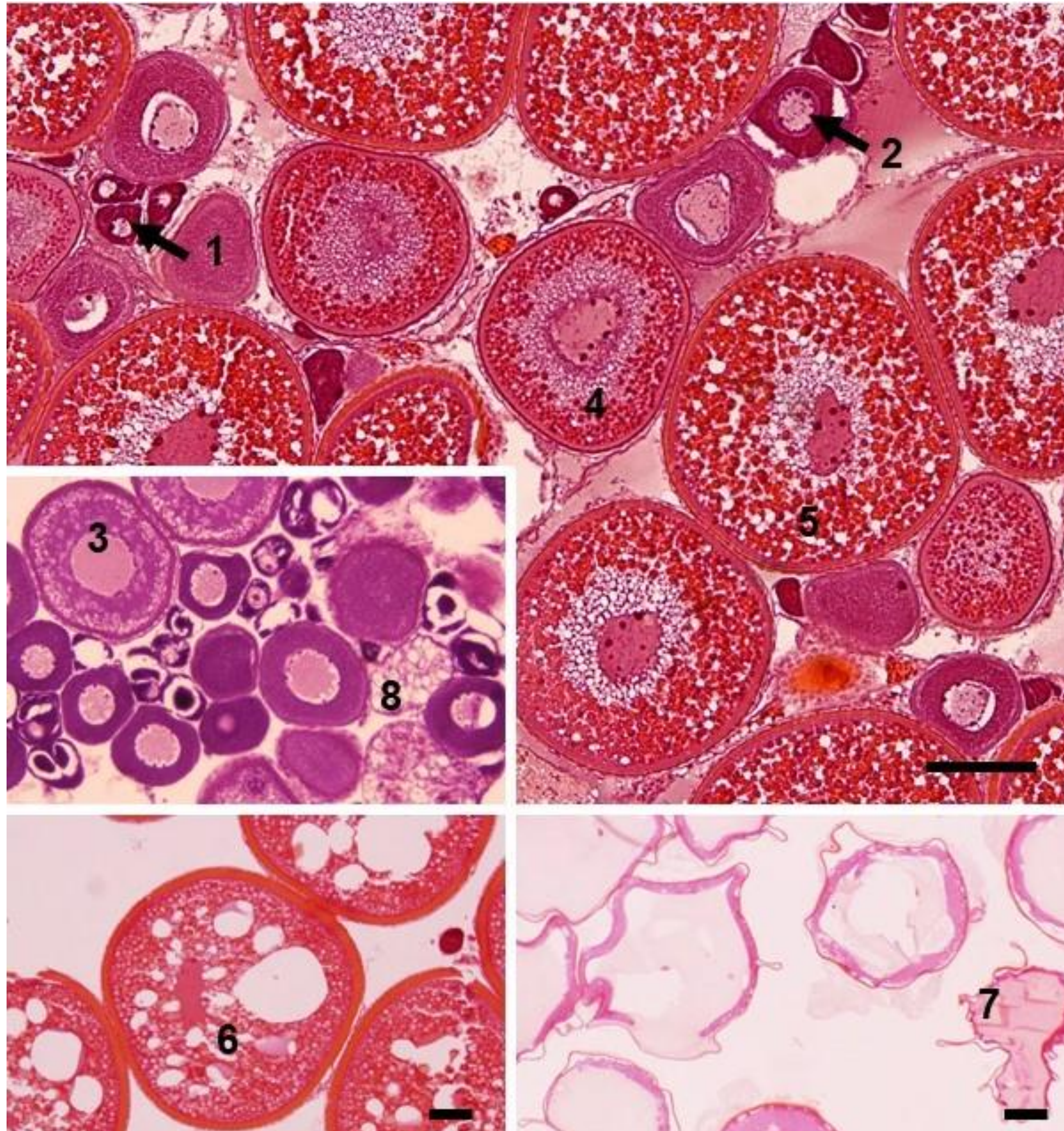

Figure 3. Ovarian development of flathead grey mullet (*Mugil cephalus*) females. Photomicrographs of histological oocytes sections stained with hematoxylin and eosin. Previtellogenic oocytes at nucleolus stage (1) and at perinucleolar nucleolus stage (2), oocytes at secondary growth at cortical alveolus stage (3), early vitellogenesis (4) and advancing in vitellogenesis (5), at maturation stage (6), hydration (7), and atresia (8). Scale bars = 100 µm.

3.1.2. Testicular development: spermatogenesis, maturation and spermiation

The testes are generally comprised by a pair of elongated lobes separated by a septum between them or independent. A vas deferens leaves the mesodorsal surface of each testicle to reach the urogenital pore located between the anus and the urinary tract. The testes are formed by germinal and somatic tissue [18]. The somatic tissue of the testes forms the seminiferous tubules and the supporting connective tissue. There are specialized somatic cells, the Sertoli and Leydig cells, that offer structural support to the germinal cells and have an endocrine role in the production of the necessary hormones for germ cell differentiation, development and survival [231]. The Sertoli cells envelop a clone of germ cells at the same stage of development to form units called cysts or spermatocysts [18]. The total of all the spermatocysts forms the germinal epithelium of the testes. The Sertoli cells are attached to a basement membrane, which separates the germinal epithelium from the interstitial compartment. The interstitial compartment is formed by somatic tissue, in which the Leydig cells are situated, between the seminiferous tubules.

Two types of testes exist according to the structure of the germinal epithelium: the tubular and the lobular testes that differentiates between unrestricted and restricted. The tubular type presents a branched structure with the tubules forming loops at the testes periphery and connect with the efferent ducts, as in the rainbow trout. The lobular type present lobules that have a blind end in the periphery of the testes. In the unrestricted type, spermatocysts in different stages are found along the lobules and the spermatozoa are released into the lobular lumen that is in continuity with that of the efferent ducts, as in the perch (*Perca flavescens*) [256] and the flathead grey mullet [133]. In the restricted type, spermiation takes place in the testicular lobules close to the efferent ducts, as in the guppy [256].

Spermatogenesis (**Fig 4**) is the process in which single diploid spermatogonial stem cell (spermatogonia) transforms into a spermatozoa with fertilization capability. It starts with the mitotic proliferation of the spermatogonia, through a self-renewal process. This number of mitotic divisions differs within species. During this phase, the population of spermatogonia in the testes increases in number and the result divisions remain together in the spermatocyst. At a point, some spermatogonia proceeds towards meiosis,

in which the spermatogonia differentiate into spermatocytes that go through two meiotic divisions followed by spermiogenesis, in which haploid spermatids are obtained and differentiate into flagellated spermatozoa [149,169]. Maturation occurs when spermatozoa migrate along the efferent duct and obtains the ability to fertilize —capacity of motility— [168]. Simultaneously, the efferent duct produces a high amount of fluid —sperm hydration—, leading to the formation of the milt, the fluid that contains spermatozoa in suspension [155,231]. There are two types of spermatogenesis, cystic or semi-cystic. In the cystic type, which is the most common in fishes, spermatogenesis is completed within the spermatocyst, that releases the flagellated spermatozoa into the testicular lumen. In the semi-cystic type, the spermatocyst releases the spermatids into the lumen where they complete spermatogenesis and are transformed into spermatozoa [161] such as in the Senegalese sole (*Solea senegalensis*) [71].

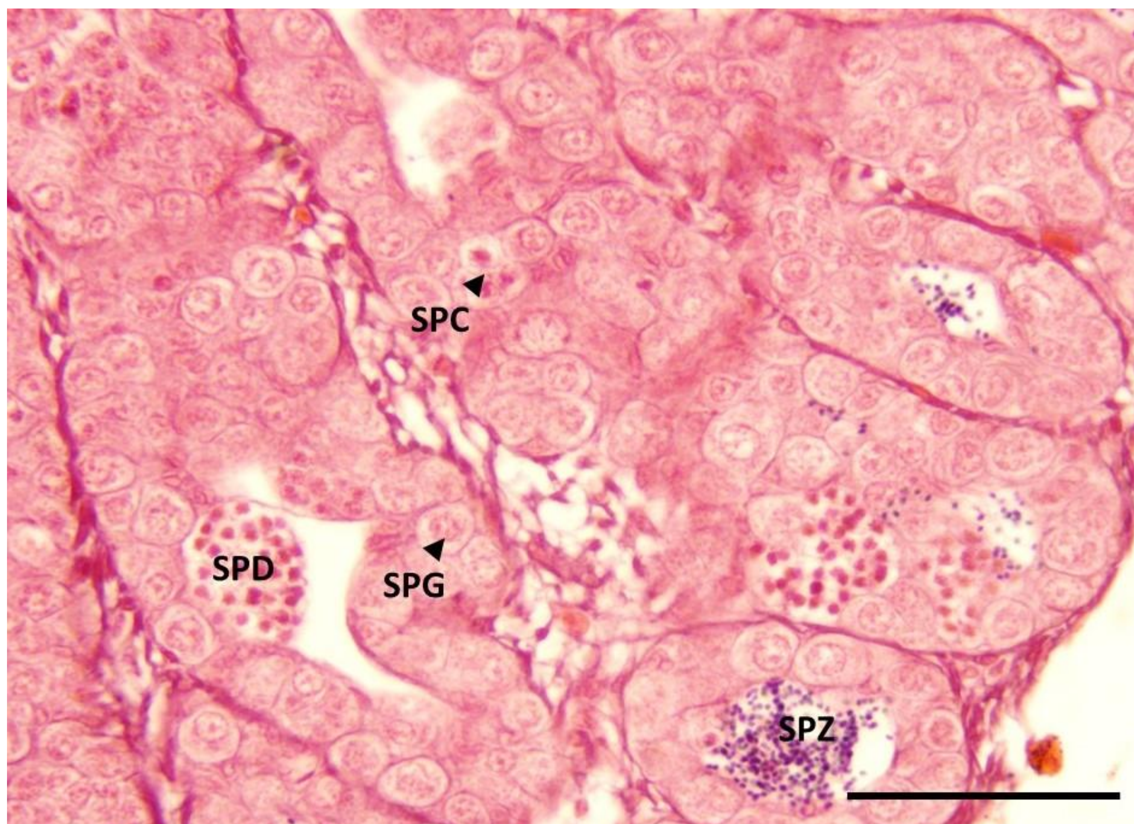

Figure 4. Photomicrograph of a histological section of flathead grey mullet (*Mugil cephalus*) testes during spermatogenesis stained with hematoxylin and eosin. SPG, spermatogonia; SPC, spermatocyte; SPD, spermatid; SPZ, spermatozoa. Scale bar = 50 μm.

3.2. Hormonal control of reproduction

Maturation in teleost is marked by vitellogenesis in females and spermatogenesis in males. It depends on internal stimuli (genetic factors, maturation age, metabolism, energy stores, etc.) and external variables such as temperature, photoperiod, water salinity, presence of a potential mate, etc. The neuro-endocrine regulation of reproduction is mediated by the hypothalamus-pituitary-gonad (HPG) axis (**Fig 5**) and the liver [19,155,178]. The detection of the adequate cues stimulate the synthesis and secretion of neuropeptides by the hypothalamus, mainly gonadotropin releasing hormone (GnRH) [236], and monoamines, principally dopamine (DA) [52], that regulate the activity of endocrine cells in the pituitary gland. GnRH stimulates gonadotropic cells in the pituitary gland to produce and release two gonadotropins (Gths) into the bloodstream, the follicle-stimulating hormone (Fsh) and the luteinizing hormone (Lh), that control reproduction in fish [19,155,178]. The Fsh and Lh belong to the glycoprotein hormone family and are heterodimeric glycoproteins formed by a common α subunit and a specific β subunit [210]. Both gonadotropins target the gonads, stimulating the secretion of specific sex steroids and growth factors with an important role in the regulation of reproduction. In addition to the direct role of sex steroids in gonadal development, the steroids also provide feedback on the brain-pituitary level, mainly through the dopaminergic system, and thus, regulate GnRH secretion [155,251].

The function of Fsh and Lh is mediated by specific membrane gonadotropin receptors in the somatic cells that surround the oocyte or male germ cells [24], the follicle-stimulating hormone receptor (*fshr*) and the luteinizing hormone receptor (*lhcgr*) which can be two, *lhcgr1* and *lhcgr2*, in some fish species [162]. The differential regulation of each oocyte generation may be attributed to differences in the hormone binding that results in receptor activation [259]. The *fshr* and *lhcgr* appearance increase or decrease parallelly to the appearance of the gonadotropin hormones in the bloodstream. Although several fish species present ligand-receptor promiscuity [5], there are two principal described roles for gonadotropins; Fsh regulates gametogenesis whereas Lh regulates gamete maturation and spawning [278].

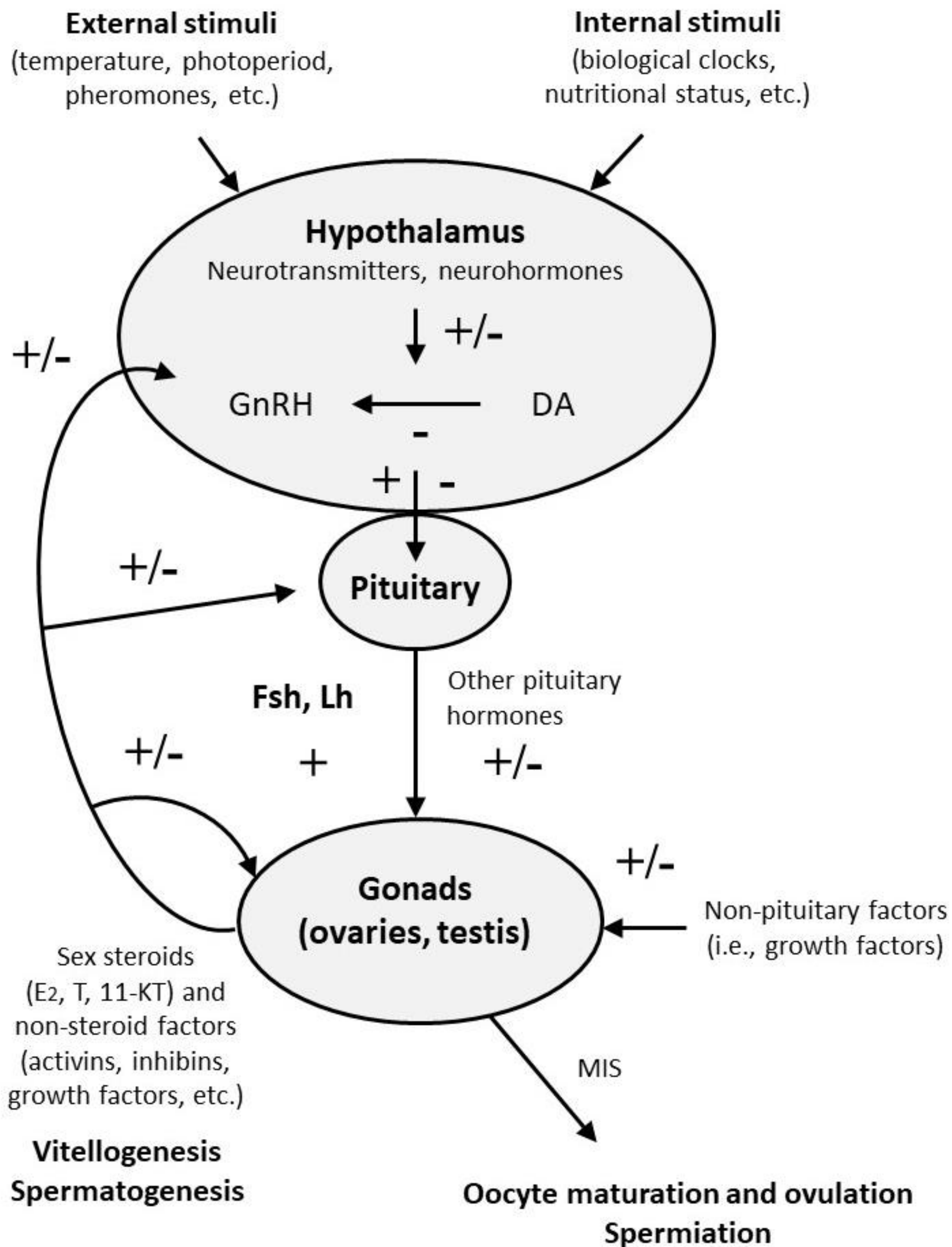

Figure 5. Schematic representation of the hypothalamus–pituitary–gonad (HPG) axis in teleost, its major components and stages, and its endocrine and environmental control. Modified from Weltzien *et al*. (2004). DA, dopamine; E_2, 17β-estradiol; Fsh, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; Lh, luteinizing hormone; MIS; maturation inducing steroid; T, testosterone; 11-KT, 11-ketotestosterone.

3.2.1. Oogenesis and maturation regulation

Previtellogenesis is a hormone-independent phase; is independent of pituitary control, do not rely on Gths effect. After it, the SG is characterized by the control of Gths. In fishes with synchronous ovarian development, two separate roles have been suggested for Fsh and Lh as fish undergo well-defined metabolic changes during vitellogenesis that are different from those occurring during final oocyte maturation and spawning. On the contrary, in fish with asynchronous ovarian development, the role of Fsh in vitellogenesis is not that clear and it is possible that Lh also plays a role. Indeed, it has been shown that both Fsh and Lh are able to stimulate follicle cells to produce 17β-estradiol (E_2) *in vitro*, the principal sex steroid involved in vitellogenesis. In fishes with synchronous ovarian development, theca cells of the follicle respond to Fsh by the transcription and enzyme activity of cytochrome P450c17I (*cyp17a1*) to produce testosterone (T), which is then aromatized into estrogens, primarily E_2, by the transcription and enzyme activity of P450 ovarian aromatase (*cyp19a1*) in granulosa cells [110]. Gonadal E_2 induces the synthesis and release of VTG by the liver and regulates its accumulation in the oocyte [152]. When vitellogenesis is completed, Lh secretion and an increased expression of the *lhcgr* in ovarian follicles induce a shift in the steroid production from T and E_2 towards the synthesis of the maturation inducing steroid (MIS) in granulosa cells [8,184,251]. This is correlated with the down-regulation of *cyp17a1* and *cyp19a1* expression, the up-regulation of the P450c17-II isoform (*cyp17a2*) that lacks the lyase activity and has 17αhydroxylase activity and, after it, the activity of the 20b-hydroxysteroid dehydrogenase enzyme [110,251]. There are two major MIS identified in fish, 17α,20β,dihydroxy-4-pregnen-3-one (17,20β-P or DHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), but probably one of them is the predominant MIS for a determinate species. The action of MIS on OM is also mediated by the complex interaction of different factors, including prostaglandins, insulin-like growth factors, activin B and other signal transduction pathways. During OM, the oocyte first gets the competence to mature, that includes the production of the necessary factors for MIS synthesis under Lh stimulation and the capacity to respond to MIS. After, Lh-induced MIS secretion from granulosa cells acts over membrane receptors in the oocyte to undergo final coalescence of yolk granules, GVBD and the resumption of meiosis [152,184].

3.2.2. Spermatogenesis and maturation regulation

Sex steroid hormones control the process from the stem-cell renewal of spermatogonia to sperm maturation. Before initiation of spermatogenesis, spermatogonial stem-cell renewal appears to be regulated by E_2. The initiation of spermatogenesis through the proliferation of spermatogonia toward meiosis is mediated by the production of 11-ketotestosterone (11-KT), the principal androgen in teleost, under the stimulation of Sertoli cells by Gths, mainly Fsh. The action of 11-KT regulating testicular growth might be mediated by growth factors such as insulin-like growth factor-I (IGF-I) and activin B also produced by Sertoli cells. Levels of Fsh are high during early spermatogenesis and through testicular growth, but decrease after spawning. On the contrary, while Lh is low at early spermatogenesis, it increases during spermiation and reaches a maximum during the spawning season. Through Lh stimulation, 17α-hydroxyprogesterone produced in Leydig cells is converted to MIS in the spermatozoa. The MIS, suggested to be 17,20β-P or 20β-S, induces an increase in the pH of seminal plasma, which permits the acquisition of spermatozoa motility. In males, androgen production remains high through the entire period of sexual maturation, even while MIS levels are high [18,178,231,232,256].

4. Control of reproduction in aquaculture

The control of reproduction in aquaculture, and particularly in intensive systems (tanks, raceways and cages), is needed to provide good quality gametes for a massive production of larvae, and to facilitate the implementation of genetic breeding programs to maintain traits of commercial interest [147]. The first step in developing a protocol to control reproduction in a fish species is the knowledge of its reproductive strategy and reproductive cycle, i.e., size at first maturity, reproductive endocrinology, spawning behaviour and egg parameters. In addition, the identification of the optimal environmental conditions, i.e., photoperiod, temperature, substrate and social conditions, required for a species to undergo maturation and advance to late stages of gametogenesis or spawning is crucial in order to create adequate culture conditions that leads to reproductive development. The ultimate factor to reproduction is an appropriate

nutrition [155,181]. Fish maintained in intensive fish culture systems are mostly dependent on the provision of nutritionally complete pelleted diets [141]. Deficient diets, and primarily, inadequate lipids and fatty acids in diets, could not fulfill the nutritional requirements in breeders, and thus, influence broodstock reproductive success, i.e., fertilization success, hatching, and survival of larvae [103,157,273]. Lipids and in particular fatty acids in diet represent the main energy substrate in fish reproduction [109,250]. Indeed, determinate fatty acids are precursors of physiologically active molecules that are directly linked with the reproductive development [250]. Besides, it is not only dietary deficiencies that affect spawning quality, but also food restriction [65] due to, for example, poorly accepted food has serious effects on reproduction.

When an adequate diet has been supplied and eaten by fish held in intensive conditions, the environment may become a factor that limits the progress of reproduction. For some species, it is impossible to control all the environmental parameters for the correct progression of reproduction. Captivity on its own can cause stress as the adequate environmental conditions that lead to reproduction are missing in a captive environment [155,178]. Stress can affect the HPG axis [230], and thus, the endocrine regulation of reproduction provoking reproductive dysfunctions in cultured fish. The reproductive problems experienced by the species will depend on the species itself and the culture conditions. Females show three types of reproductive dysfunctions: (i) at early stages of development, i.e., previtellogenesis or vitellogenesis, such as the freshwater eels [197] and the flathead grey mullet [6]; (ii) at OM, in which post-vitellogenic oocytes cannot undergo OM and become atretic, as observed in the meagre (*Argyrosomus regius*) [179]; and (iii) at the spawning time, in which there is no spontaneous spawning and ovulated oocytes remain in the ovarian or abdominal cavity like in salmonids (*Onchorhynchus* and *Salmo spp.*) [181]. On the other hand, male dysfunctions are most commonly linked with a reduction in the production of sperm volume and milt fluidity [155,289] but can also present an arrest in early spermatogenesis as eels [111,207]. In some cultured fishes a proper control of environmental factors or the use of them, mainly of photoperiod and temperature, may be enough to obtain spawns [26], while in other fish, because it is impractical or does not overcome the dysfunction, it is necessary to apply hormonal therapies.

4.1. Endocrine manipulations of fish reproduction

The development and application of hormonal therapies for the treatment of reproductive disorders in cultured fish not only have permitted to reproduce in captivity several fish species that show any reproductive problem, but also to improve the reproductive performance of broodstock. According to the reproductive problems presented by females and males, the hormonal approach should be different [155,181,289].

4.1.1. Hormonal therapies for females

Hormone therapies have been applied to females in relation to the three types of reproductive dysfunctions. The use of hormones has been most successful for overcoming the dysfunction (ii) where females do not undergo OM. In these females that complete vitellogenesis, but post-vitellogenic oocytes do not proceed to OM, it is considered that the failure is due to the lack of release of Lh from the pituitary. Therefore, manipulations of reproduction might be done by the use of exogenous hormonal sources that act on the gonad, or by the use of commercial synthesis of agonists of GnRH (GnRHa), with or without a dopamine antagonist (DA), that induces the release of Lh stores from the pituitary. The application of DA, i.e., pimozide, domperidone and metoclopramide, is to block the action of dopamine on the brain which inhibits the GnRH-stimulated production and release of Lh. Dopamine seems to have a strong effect in freshwater species and to be weak or nonexistent in seawater species [155]. The flathead grey mullet held in captivity appears to have a strong inhibition of basal and GnRH-stimulated release of gonadotropins caused by DA presence [6,79]. Among the hormonal treatments that act at the gonad level, we find the maturation-inducing steroid (17,20β-dihydroxy-4-pregnen-3-one, DHP) that is commonly used in the Japanese eel (*Anguilla japonica*) [111], and preparations that stimulate the Lh receptor such as: the carp pituitary extract (CPE) and salmon pituitary extract (SPE), which are purified extracts obtained from the pituitaries of mature fish during the spawning season and contain mainly Lh; the chromatographic purification of fish pituitary Gths; the purified human Chorionic Gonadotropin (hCG) [155,178,289]; and recombinant Gths, such as the recombinant hCG [177] or species-specific recombinant Lh (rLh) that are produced by introducing the protein DNA

sequence into a plasmid, which transfers the sequence into an expression system — cultured cells of another organism, such as yeast, mold, insect or mammalian cells— to produce glycosylated proteins [143,170].

In captive females exhibiting inhibition of early gonadal development, hormonal treatments are required to stimulate vitellogenesis from previtellogenesis or to complete vitellogenesis, so that the oocytes can then undergo OM and ovulation in response to another hormonal therapy. Since vitellogenesis is a process that takes a long time, from weeks to months, therapies are not usually applied and they are still under investigation. The use of GnRHa is not common, it was shown to enhance vitellogenesis in some species such as the flathead grey mullet [6,173] or the milkfish (*Chanos chanos*) [142] but does not initiate or promote it [93,98], with very few exceptions such as the red sea bream (*Pagrus major*) [135]. Dopamine antagonists, alone or combined with GnRHa, have also shown to mainly enhance vitellogenesis [6] and to initiate it in some exceptions such as in the mosquitofish (*Gambusia affinis*) [17]. In comparison, successive injections of pituitary gland extracts as SPE, in the Japanese eel [197] or the European eel (*Anguilla Anguilla*) [205,206], and hCG, in the basa catfish (*Pangasius bocourti*) [30] or the blue-spotted grouper (*Epinephelus fario*) [138] have proven effective in inducing or enhancing vitellogenesis. With the sequencing of fish cDNAs that code for gonadotropin subunits [143], a new approach has been developed through the production of recombinant Fsh (rFsh) and rLh in heterologous systems, which have been demonstrated to be successful to induce initial stages of vitellogenesis in different teleost such as the sea bass and eels [75,119,190]. Nevertheless, to date rFsh and rLh therapy has not been successfully used for the completion of vitellogenesis and studies are required to obtain viable eggs and larvae to finally apply them in the aquaculture industry [170].

In the case of females with inhibition of spawning, hormonal treatments are not essential to obtain eggs because they can be stripped. However, the application of hormonal treatments has been used to synchronize ovulation, reduce the spawning season, and increase success, i.e., production of more eggs, higher fertilization, and hatching percentages [178]. For example, the administration of GnRHa emulsified in Freund's incomplete adjuvant to rainbow trout synchronized ovulation and shortened the reproductive period in comparison with control group [9].

4.1.2. Hormonal therapies for males

Hormonal therapies are applied in males to induce and enhance spermatogenesis (spermiogenesis and spermiation), and / or to increase the volume of sperm produced and extend the spermiation period. In those males that are arrested at early spematogenesis, long-term treatments during some weeks have to be used in order to induce spermiogenesis and spermiation [178] since it is a process that takes from weeks to months [231]. Treatments to address this problem by inducing full spermatogenesis and the production of spermatozoa with spermiation include hCG, as for the Japanese eel [111]; homologous rFsh and rLh, as for the European eel [207] or the Senegalese sole [38]; and GnRHa alone or combined with DA, as in the basa catfish (*Pangasius bocourti*) [29].

On the other hand, in those males that produce low quantities of sperm and it is very viscous or it is needed to extend the spermiation period, for example, because females present a longer spawning season or are batch-spawners, treatments are focused on stimulating further the process of spermatogenesis to enhance the production of spermatozoa and to increase the seminal fluid. Implants of 17-methyltestosterone, which is a synthetic androgen, are commonly used to accelerate spermatogenesis in species that do not achieve full maturation, such as the flathead grey mullet [6] or the common snook (*Centropomus undecimalis*) [204]. As the low sperm and seminal fluid production is mainly related to low levels of Lh in the bloodstream during the spermiation period [289], treatments are focused on: the use of exogenous hormones that act directly at the level of the gonad to stimulate the Lh receptor; or the use of GnRHa, with or without a DA, to stimulate the liberation of Gths from the pituitary. Treatments that stimulate the Lh receptor can be CPE or SPE, specially in cyprinids; hCG, which is successful in a wide range of species [178]; rhCG, which has been used to induce spermiation in the European eel becoming a cheaper alternative to the routinely hCG treatment [70]; and species-specific rLh that has been successful in several species [170]. Although in some species an acute treatment with a simple injection has proven effective to enhance spermiation, treatments with multiple injections or controlled-release delivery systems have shown to be more effective over a prolonged period [178]. For example, a GnRHa implant was more effective in enhancing milt production and

extending the spermiation period in meagre than two injections of GnRHa given in twelve days of difference [61].

4.1.3. Pros and cons of each hormonal preparation

A high variety of hormones are available as treatments to control fish reproduction, and each hormonal approach presents advantages or disadvantages. On one hand, preparations that act at the gonad level — CPE, SPE, purified Gths, hCG, DHP and rGths— present rapid action and do not require an active pituitary containing gonadotropins. Among these preparations, CPE, SPE, and hCG have been extensively used in hormonal manipulation of reproduction in fish for decades and are commercially available throughout the world. However, CPE and SPE involve difficulties in calculating the doses to administer as they are obtained from pituitaries that may have variable quantity of gonadotropins, might also suppose a disease transmission threat and their use can be restricted to phylogenetically-related fishes because of the species-specificity of fish gonadotropins. The use of fish purified Gths has advantages over pituitary extracts, as permits accurate dosing because of the calibration of the preparations, and reduces the disease risk. However, its use is not as extensive and has been limited to salmon and carp species, as only purified Gths preparations for these fish are available in the market. The routine use of hCG for human assisted reproduction and veterinary purposes has favored the increased use of this preparation over the purified Gths in fish, although these last ones would be physiologically more convenient. In addition, hCG has been reported to induce immune responses in the treated fish not allowing repeated treatments [155,178]. The use of DHP, for example, is limited to the period in which oocytes acquire the ability to respond to it, and it requires the combined application of other hormones as SPE as priming injections to provide the oocyte with this ability [110]. Regarding rGths, the use of rFsh and rLh is quite recent and they are not easily available. In fact, most of the rFsh and rLh are produced for scientific purposes and for a limited number of species as they are species-specific [170]. However, because of their specificity, assure no contamination with other pituitary glycoproteins [170] and have been reported

to have high potency and high success in the species in which have been tested [3,37–39,75,119,128,130,190,207,223]. In comparison, recombinant hCG has not been used much in fish [177].

On the other hand, the application of GnRHa-based therapies has important advantages over preparations that act the gonad level, as GnRHa acts at a higher level of the HPG axis and stimulates the release of the endogenous Gths as well as other pituitary hormones that may be important to the reproductive function (i.e., growth hormone, insulin-like growth factors, prolactin, and thyroid hormones), and thus, providing a better integration of the reproductive process. The use of GnRHa do not suppose a disease transmission threat, do not induce immune responses in the treated fish allowing repeated treatments and are not species-specific. In addition, GnRHa preparations are also available in a variety of hormone-delivery systems [289] such as implantable cylindrical pellets of cholesterol [270] or ethylene-vinyl acetate (EVAc) [176], injectable biodegradable microspheres using co-polymers of lactic acid and glycolic acid or a co-polymer of fatty acid dimer and sebacic acid [178]. The advantage of the use of a delivery system is that permits to reduce the repetitive handling that might be stressful to the breeders when multiple injections are required, such as in fishes with multiple spawns during a long-term period (multiple-batch group-synchronous and asynchronous fish), or when the breeders are difficult to manipulate because they are very large or are kept outdoors in large facilities [182,289]. Nevertheless, GnRHa can be less potent at the beginning and the end of the breeding season when the natural pool of Fsh and Lh hormones are low [155].

Objectives and Hypotheses

The flathead grey mullets are among the best candidates for culture-based production with regard to their ecological profile, adaptability to different culture conditions, their commercial importance in some countries and the potential processing of highly value-added product. The future development of this species is dependent on controlling reproduction in captivity, shifting from capture of wild fry to supplying hatchery-reared fry for stocking, and to address certain nutrition issues to describe the nutritional requirements of this species, among them, the development of artificial feeds for an optimal gonadal development. Therefore, the present thesis addressed a wide variety of different aspects related to reproduction to achieve the following general and specific objectives:

General objective

To achieve full control of reproduction of the flathead grey mullet (*Mugil cephalus*) in intensive culture conditions.

Specific objectives

(i) To identify the onset of vitellogenesis and the spawning season of wild flathead grey mullet in the western region of the Mediterranean (CHAPTER II).

(ii) To characterize and describe the variation in the lipid and fatty acid content of the gonads, liver and muscle of wild mature females of flathead grey mullet at different stages of the reproductive cycle in an attempt to provide a better understanding of the lipid requirements of this species for the production of high-quality eggs (CHAPTER II).

(iii) To describe the optimal feed characteristics and feeding habits of wild-caught flathead grey mullets maintained in an intensive culture system (CHAPTER III).

(iv) To address the reproductive dysfunctions of this species in intensive culture — induce vitellogenesis, oocyte maturation and ovulation in females, induce and enhance spermatogenesis and spermiation in males, synchronizing both sexes development to obtain viable fertilized eggs and larvae— with acute and long-term hormonal treatments that include species-specific rFsh and / or rLh, among other hormones (**CHAPTER IV**, **CHAPTER V** and **CHAPTER VI**).

(v) To describe the differential expression of genes and molecular pathways in the transcriptome amongst different stages of ovarian development, induced with a treatment of rFsh and rLh, to provide a higher resolution on the reproductive and endocrine processes induced with recombinant gonadotropins in the flathead grey mullet (**CHAPTER VII**).

The hypotheses that arise from the previous objectives are the following:

(i) The spawning season of the flathead grey mullet in the Western Mediterranean is close to the breeding season in the nearest eastern regions of the Mediterranean, i.e., Greece and Tunisia. Therefore, it should spawn close to or during August to October [272].

(ii) The flathead grey mullet stores lipids primarily in the liver, such as other demersal species [45]. The fatty acid profile of tissues and the seasonal changes associated to gonadal development are similar to other omnivorous species, such as the white sea bream (*Diplodus sargus*) [208].

(iii) The flathead grey mullet, as a filter-feeding omnivorous species [34], preferentially selects smaller pellet items than carnivorous species as, for example, the bivalve-eater gilthead seabream (*Sparus aurata*) that possess a well-developed chewing apparatus [13].

(iv) The flathead grey mullet feed predominantly in the bottom of the tank, according to the foraging feeding behavior of the species [34].

(v) Long-term treatments are necessary to address the reproductive dysfunction flathead grey mullets present in captivity. The long-term application of species-specific rFsh and rLh induces gonadal development in both males and females through to the production of viable gametes and larvae and permits full control of reproduction.

(vi) The application of rFsh and / or rLh at different stages of ovarian development leads to the upregulation and downregulation of different transcripts involving molecular pathways that are observed to support vitellogenesis in other fish species.

CHAPTER II

Seasonal changes in ovarian development and in lipid and fatty acid composition of muscle, liver and ovary of wild flathead grey mullet

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Seasonal changes in ovarian development and in lipid and fatty acid composition of muscle, liver and ovary of wild flathead grey mullet

1. Introduction

The flathead grey mullet (*Mugil cephalus*) has a cosmopolitan world distribution and a high demand for human consumption in the Mediterranean region, Asia, and the United States of America [46]. This mugilid fish presents high growth rates in captivity and can be cultured in different salinities [195]. The positive market and culture attributes and, in particular, the omnivorous diet of this species [34] indicate the potential of the species for aquaculture diversification. Consequently, there is an increasing interest to develop intensive aquaculture production [281]. Nevertheless, there are limiting factors for scaling up industrial production caused by different bottlenecks, such as reproduction disfunctions in captivity [83] and juvenile availability [222,281]. There is also a lack of knowledge on the seasonality of reproductive development in different regions, and the nutrient requirements of breeders across all regions implies that artificial feeds for broodstock have not been developed. Mullets are usually produced in semi-intensive polyculture systems with other fish where they constitute less than twenty percent of the reared stocks. Therefore, feeding of this species is commonly done on chicken manure, pellets that target other cultured species, food leftovers, or detritus [222].

Nutrition plays a substantial role in reproductive development. The lipid and fatty acid composition of the broodstock diet have been identified as the major metabolic energy resource that determines the successful reproduction and survival of offspring [109,250]. Some fatty acids, such as highly unsaturated fatty acids and particularly arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA), are not only essential components of the gametes, but also precursors of physiologically active molecules such as prostaglandins and other eicosanoids that are directly linked with reproductive development and success [250]. The use of inadequate

diets for breeders did not fulfil the species' nutritional requirements and, thus, influenced the reproductive success [103,157,273] and offspring survival [212]. Differences in broodstock nutrition has been shown to affect gamete fertilization, hatching, and survival of larvae in a wide range of fish species, i.e., freshwater eels (*Anguilla spp.*) [96], Senegalese sole (*Solea senegalensis*) [174], common sole (*Solea solea*) [203], gilthead seabream (*Sparus aurata*) [66], Japanese flounder (*Paralichthys olivaceus*) [69], red seabream (*Pagrus major*) [268], yellowtail seriola (*Seriola quinqueradiata*) greater amberjack (*Seriola dumerili*) [228], Atlantic halibut (*Hippoglossus hippoglossus*) [163], and mangrove red snapper (*Lutjanus argentimaculatus*) [57].

Lipids can be acquired (i) directly from food, (ii) *de novo* synthesized in the gonads, or (iii) mobilized from storage tissues to the gonads [273]. Therefore, the lipids dynamics through the reproductive cycle are related to their functions in reproduction [196]. However, to our knowledge, the importance of different tissues for lipid storage and the changes in fatty acid composition that follows the seasonal changes in lipid mobilization and deposition related to reproduction of the flathead grey mullet have not been established. Besides, the spawning season has not been determined in the Western Mediterranean as it has been determined for the Eastern Mediterranean regions —Turkish, Egyptian, Greek, and Tunisian coasts—, the Black Sea, the Aegean Sea [272], the Atlantic Ocean —USA [165], Mauritanian, and Moroccan coasts—[272], the Gulf of Mexico [100], the Indian Ocean —Indian [136], Sri Lanka, and South African coasts—, and the Pacific Ocean —Australian coast [272]—.

The main purpose of this study was to identify the spawning season of flathead grey mullet in the western area of the Mediterranean and to describe total lipid content and fatty acid composition and the process of their allocation during ovarian development in the muscle, liver, and gonads in an attempt to provide a better understanding of the species lipid requirements for reproductive development.

2. Material and methods

2.1. Fish samples

Wild flathead grey mullets (total n = 69) were obtained from fishermen between October 2018 and October 2019. No male fish was obtained between February and September 2019 and, therefore, males were not included in the present study. On the contrary, no female fish was captured between December 2018 and January 2019 —the winter time—, and males presenting immature testes with 0.04 ± 0.05 % GSI were captured instead. Finally, wild female fish (total n = 44) obtained in October and November 2018 and between February and October 2019 were included in the study. Samplings were collected and processed once a month (mid-month), except during July to October that samples were obtained twice a month (first and third weeks of the month). Fish were caught in the Ebro Delta canals (Spain) and the Western Mediterranean (Subarea 37.1.1 of FAO) that comprises the waters to the north of the Ebro Delta, between the Spanish mainland and Sardinia —without including the north-western Gulf of Lion—, and were kept on ice until during transport to the laboratory and while being processed. Each fish was measured (standard length, SL; fork length, FL; and total length, TL) to the nearest 0.5 cm and weighted to the nearest 1 g with an electronic balance (Cobos Precision, Spain). Whole liver and ovary weights were also recorded to the nearest 0.1 g (Mettler Toledo, Spain). Two condition indices, the gonadosomatic index (GSI) and the hepatosomatic index (HSI), common metrics of reproductive allocation and reproductive condition in fisheries biology, were estimated as follows: (W_g or W_l / W) x 100, where W_g and W_l correspond to the gonad and liver weights respectively, and W to the body weight. Sections of the ovaries were taken from the anterior, middle, and posterior parts of the right and left lobe and preserved in a Bouin's solution for later histological examination.

For lipid and fatty acids analysis, ~5-g samples of gonads, liver, and muscle from directly under the dorsal fin were collected. Each sample was stored at -20ºC until further analysis. A total of 27 flathead grey mullet females were selected at four different phases of the reproductive cycle for lipid and fatty acid analysis; at previtellogenesis (n = 7; 1659 ± 337 g BW; 47.5 ± 2.5 cm SL), early-vitellogenesis (n = 6; 2522 ± 437 g; 52.6 ± 3,3 cm),

late-vitellogenesis (n = 7; 2435 ± 477 g; 50.6 ± 3.9 cm), and in the post-spawn period (n = 7; 2256 ± 520 g; 52.9 ± 5.2 cm).

2.2. Lipid content and fatty acid analysis

Total lipids were extracted from samples by homogenization in chloroform/methanol (2:1, v:v) using the method of Folch *et al*. (1957) using a double extraction and were quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) at -20 ºC prior to fatty acids transmethylation. Fatty acids were methylated following the acid-catalyzed transmethylation method used by Christie (1982). Methyl esters were extracted twice using isohexane diethyl ether (1:1, v:v), purified on TLC silica plates (Macherey-Nagel Düren, Germany) and quantified through gas-liquid chromatography analysis on a Thermo TraceGC (Thermo Fisher, Spain) fitted with a Thermo TR-FAME capillary column (30 m × 0.25 mm id; Thermo Scientific, Spain), using a two-stage thermal gradient from 50 ºC (injection temperature) to 150 °C after ramping at 40 ºC min^{-1} and holding at 250 ºC after ramping at 2 ºC min^{-1}. Helium (1.2 mL min^{-1} constant flow rate) was used as the carrier gas, and on-column injection and flame ionization detection at 250 ºC were used. Peaks of each fatty acid were identified and quantified according to the response to the internal standard, 21:0 fatty acid, added before transmethylation.

To obtain the percentage of total lipids, ~200 mg of each sample was weighted to the nearest 0.01 mg (KERN & SOHN GmbH, Germany) before and after being dried at 100 ºC (Memmert, Germany) for 24 h, and the water percentage was calculated to obtain the dry weight of samples. Total lipids percentages were obtained by dividing the total lipid weight by the dry weight of samples multiplied by 100.

2.3. Histological analysis

Ovarian samples were dehydrated in gradually increasing ethanol (76 % to 96 %) and xylene solutions and embedded in paraffin. Sections of 3 µm were obtained and

stained with hematoxylin and eosin (Casa Álvarez, Spain). Sections were examined under a light microscope (Leica DMLB, Houston, USA). The histological classification of flathead grey mullet ovaries followed the classification described by Greeley *et al*. (1987). The ovaries were classified according to the most advanced oocyte stage present in the ovary: previtellogenesis, ovaries with small oocytes in chromatin nucleolar and perinucleolar stages (typically without yolk or lipids droplets); early-vitellogenesis, recruitment of previtellogenic oocytes into vitellogenesis by yolk accumulation; late-vitellogenesis, with large vitellogenic oocytes as recruitment into vitellogenesis had ceased indicated by the absence of early stage vitellogenic oocytes; and post-spawn ovaries, when spawning is complete and ovaries present postovulatory follicles (POFs) and previtellogenic oocytes.

2.4. Statistical analysis

Data are presented as mean ± standard deviation (SD) unless otherwise noted. All variables were checked for normal distribution with the Shapiro-Wilk test and homogeneity of the variances with the Levene test. Total lipids (%) data was 1/x transformed, and GSI data was transformed by square root to follow normality. Data were analysed by one-way analysis of variance (ANOVA) to determine differences between different stages of development, followed by Holm Sidak's multiple comparisons. All statistical analyses were performed using SigmaPlot v12.0 (Systat Software Inc., Richmond, CA, USA). Significance was set at $P < 0.05$.

3. Results

3.1. Biometric data

A total of 44 females were collected and stages of ovary development were determined; 18 females were at previtellogenesis, six females were at early-vitellogenesis, 13 females were at advanced vitellogenesis, and seven were at post-spawning period (**Table 1**). Previtellogenic females were obtained from middle October 2018 to early August 2019, had GSIs ≤ 1 %, and small pink ovaries (**Fig 1A**) filled with previtellogenic oocytes (**Fig 2A**). Females at early-vitellogenesis were collected from early August to

early September and had slightly higher GSIs; however, this time, ovaries turned to yellowish pink (**Fig 1B**) because of the recruitment of oocytes into yolk stages in the ovaries (**Fig 2B**). Females at late-vitellogenesis were first collected at middle August, together with some females at early-vitellogenesis, to middle September. Females at late-vitellogenesis had significant larger ovaries represented by a GSI value of 15.5 ± 3.4 % (ranging from 11 to 22.6 %) ($P < 0.001$). These ovaries had a yellow to orange color (**Fig 1C**) due to the abundance of oocytes at late-vitellogenesis stage (**Fig 2C**). Females with opaque pink color ovaries with a red hue from extensive vascularization (**Fig 1D**) were also obtained at the middle of September and through to mid-October. These females presented POFs (**Fig 2D**) indicating post-spawning period, together with variable atresia and a batch of previtellogenic oocytes. The GSI values in this post-spawn ovary presented a sharp decrease to 1.8 ± 0.9 %. By examining the GSIs per month (**Fig 3**), the low GSIs indicated that the gonadal resting period was from October through to July, gonadal growth was during August and September, and spawning was mainly in October.

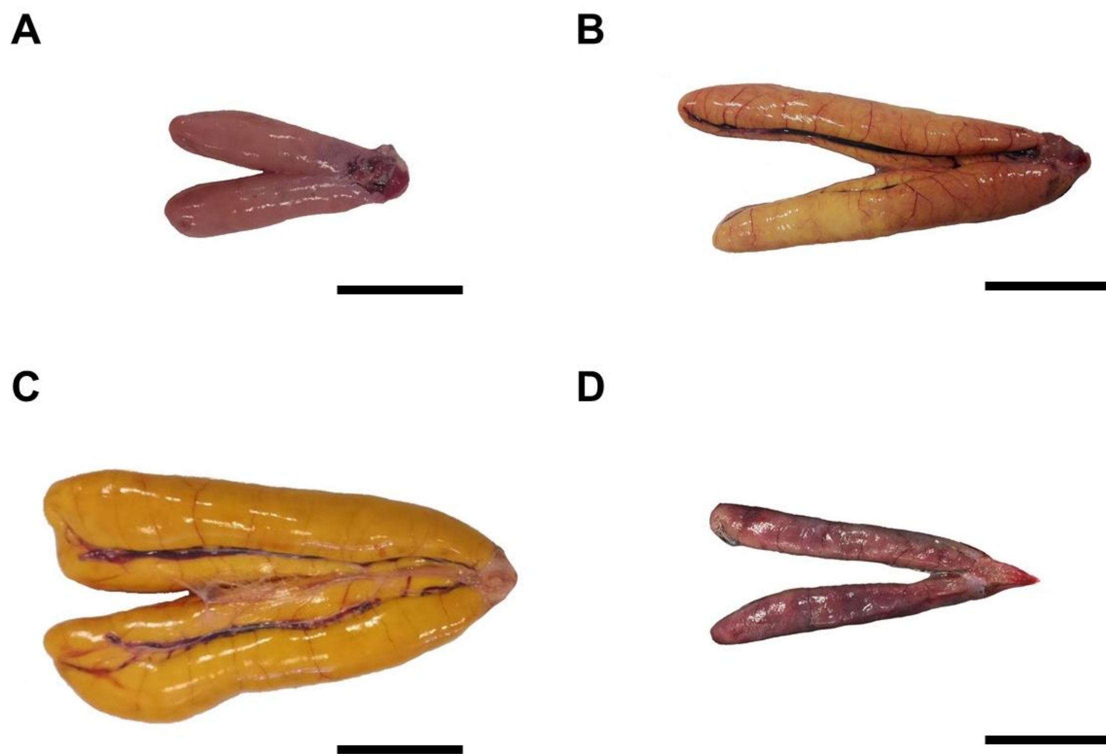

Figure 1. Macroscopic appearance of flathead grey mullet (*Mugil cephalus*) ovaries at different stages of maturity; (A) previtellogenic gonads, (B) gonads at early-vitellogenesis, (C) gonads at late-vitellogenesis, and (D) post-spawning ovaries. Scale bar: 5 cm.

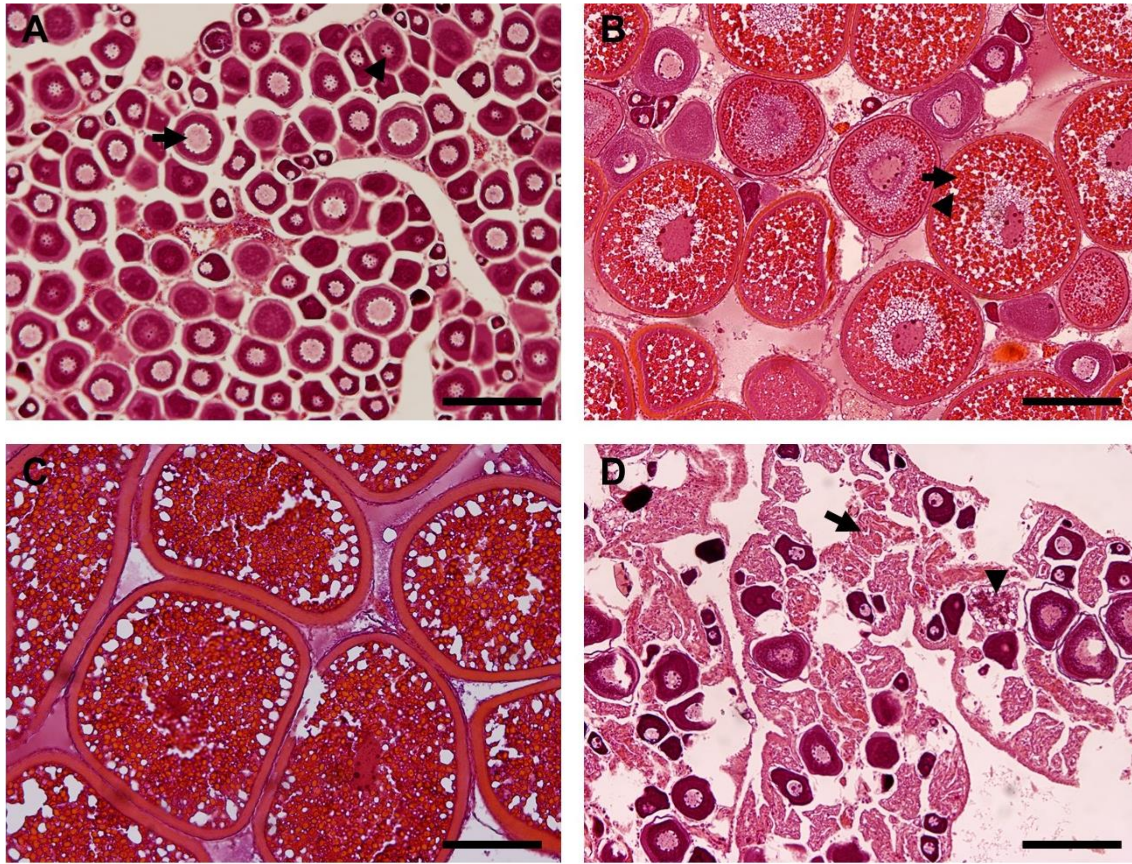

Figure 2. Light microscopic photograph of *Mugil cephalus* ovary at different maturity stages; (A) previtellogenic ovary rich in chromatin nucleolar oocytes (arrowhead) and perinucleolar oocytes (arrow), (B) early-vitellogenic ovary with the inclusion of lipid droplets (arrowhead) and yolk granules (arrow) into oocytes, (C) late-vitellogenic ovary with maximum size of lipid droples and thickening of vitelline membrane, and (D) post-spawning ovary presenting post-ovulatory follicles (arrow), previtellogenic oocytes, and atresia (arrow head). Scale bar: 200 µm.

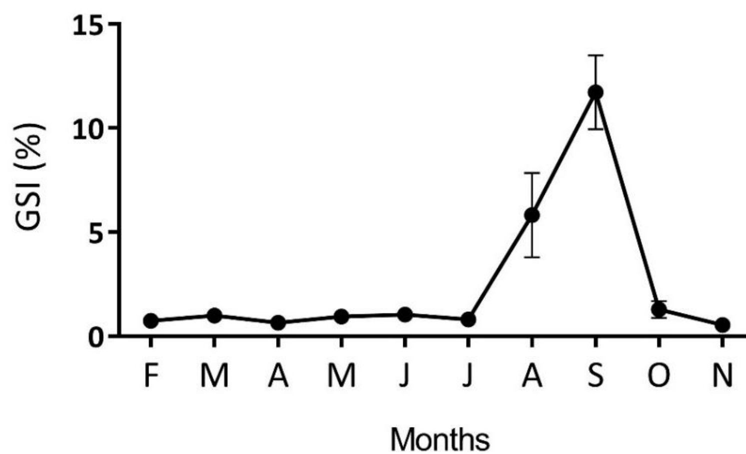

Figure 3. Monthly variation from February to November in the gonadosomatic index (GSI) of flathead grey mullet (*Mugil cephalus*) females (n = 44) collected in the Ebro Delta canals (Spain) and in the Western Mediterranean. Data is presented as mean ± SEM.

Table 1. Biometric data of *Mugil cephalus* (n = 44) captured in the Western Mediterranean. Values are mean ± SD. Values with different superscripts within rows are statistically different.

Morphometry	Maturation stage			
	Previtellogenesis (n = 18)	Early-vitellogenesis (n = 6)	Late-vitellogenesis (n = 13)	Post-spawning (n = 7)
Body weight (g)	1591.7 ± 423.9^{a}	2521.7 ± 436.6^{b}	2334.3 ± 388.8^{b}	2256.5 ± 520.7^{b}
Standard length (cm)	46.1 ± 4.4^{a}	52.6 ± 3.3^{b}	50.3 ± 2.9^{b}	52.9 ± 5.2^{b}
Fork length (cm)	50.2 ± 4.7^{a}	57.0 ± 3.8^{b}	54.5 ± 3.3^{b}	57.6 ± 5.6^{b}
Total length (cm)	53.9 ± 6.0^{a}	61.2 ± 3.6^{b}	59.2 ± 3.6^{b}	62.4 ± 5.9^{b}
Gonadosomatic index (GSI %)	0.8 ± 0.3^{a}	4.2 ± 3.6^{a}	15.5 ± 3.4^{b}	1.8 ± 0.9^{a}

3.2. Total lipid content in tissues at different ovarian development

The lipid content in the muscle, liver, and ovaries of female flathead grey mullet breeders ranged from 3.85 % - 4.92 %, 18.46 % - 22.62 %, and 4.90 % - 34.59 %, respectively, at different ovarian development stages (**Fig 4A**). The highest lipid content was found in the gonads, followed by the liver and the muscle. During ovarian development, total lipid content in the muscle and liver did not significantly differ. However, HSI % was significantly higher (P = 0.003) during vitellogenesis and at a post-spawning period compared to previtellogenesis (**Fig 4B**). As ovaries of flathead grey mullet developed, total lipid content in the ovaries changed significantly (P < 0.001) with the lowest values obtained at previtellogenesis. There was a significant (P < 0.001) increase through vitellogenesis with a peak in late-vitellogenesis and a significant decrease (P = 0.014) at the post-spawning period. The lipid accumulation in the gonads followed the pattern of GSI (**Fig 4B**), which significantly increased (P < 0.001) at late-vitellogenesis and decreased afterwards. Four out of seven females presented lipid accumulation in the shape of perivisceral fat at previtellogenesis, whereas only one presented perivisceral fat at early-vitellogenesis (results not shown).

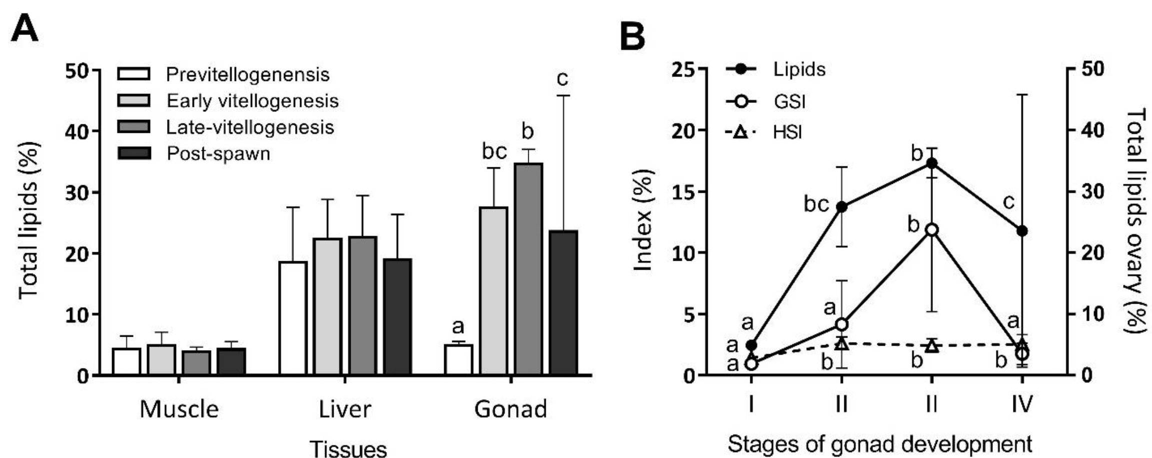

Figure 4. Variation in (A) total lipid content (%) in the muscle, liver and ovaries of the flathead grey mullet (*Mugil cephalus*), and (B) gonadosomatic index (GSI) and hepatosomatic index (HSI) respect to total lipids in ovaries of female breeders across maturity stages of gonad development; (i) previtellogenesis (n = 7), (ii) early-vitellogenesis (n = 6), (iii) late-vitellogenesis (n = 7), and (iv) post-spawn (n = 7). Data is presented as mean ± SD. Different letters show significant differences (P < 0.05) in each tissue along gonadal development.

3.3. Fatty acid composition at different ovarian developmental stages

3.3.1. Muscle

Percentage of total saturated fatty acids (ΣSFA), dominated by 16:0, mono-unsaturated fatty acids (ΣMUFA), and Σn-3 and Σn-6 polyunsaturated fatty acids (PUFA), represented mainly by 22:6n-3 (DHA), 20:5n-3 (EPA) and 20:4n-6 (ARA), exhibited no significant differences through ovarian development and remained constant (**Fig 5**, **Table 2**). Only 22:5n-3 presented a significant decrease (P = 0.002) through ovarian development and at the post-spawning period. No significant differences were found in the DHA/EPA ratio, whereas the ARA/EPA ratio significantly increased (P = 0.002) at late-vitellogenesis and post-spawn.

3.3.2. Liver

As the ovaries developed from previtellogenesis to late-vitellogenesis, the percentage of ΣSFA in the liver showed a significant increase (P = 0.02), whereas the content of ΣMUFA remained unchanged (**Fig 5**, **Table 3**). Total n-6 PUFA did not show any change whereas Σn-3 PUFA levels significantly decreased (P = 0.024) from previtellogenesis to late-vitellogenesis due to a decrease in EPA, 22:5n-3 and DHA. The reduction of EPA values was significant (P = 0.001) at late-vitellogenesis and after spawning and, thus, it affected the DHA/EPA and ARA/EPA ratios that increased accordingly (**Table 3**). DHA values significantly decreased at early vitellogenesis and showed an increasing trend towards late-vitellogenesis and after spawning but with no significant differences.

3.3.3. Gonad

The percentage of ΣSFA significantly decreased in the ovaries from previtellogenesis to late-vitellogenesis and after spawning (P = 0.001) (**Fig 5**, **Table 4**). The most noteworthy decrease in individual fatty acids observed during vitellogenesis is due to a downturn in 16:0 that remained low after spawning. On the contrary, ΣMUFA values significantly increased (P < 0.001) along vitellogenic development, mainly due to a rise in 16:1, 17:1, 18:1n-9 (oleic acid), and 18:1n-7 fatty acids, and then decreased again after spawning. The most noticeable increases were in 16:1 and 17:1 content. The 16:1

fatty acid was not present in previtellogenic ovaries and appeared during vitellogenesis. Additionally, the 17:1 rose approximately 5-fold in late-vitellogenesis with respect to previtellogenesis. Total n-6 PUFA significantly decreased ($P < 0.001$) during vitellogenesis and increased after spawning following the same trend of ARA levels (**Table 4**). Total n-3 PUFA content, including the major fatty acids 22:5n-3, EPA and DHA, decreased during gonadal development. The percentages of 22:5n-3 and DHA increased again after spawning, while EPA values remained low after spawning. Therefore, the DHA/EPA and ARA/EPA ratios increased significantly during the post-spawning period.

Table 2. Fatty acid composition (% of total fatty acids) of *Mugil cephalus* female muscle at different maturation stages (n = 6 – 7 females per stage). Values are mean ± SD. Fatty acids with < 0.5 % are excluded. Values with different superscripts within rows indicate significant differences in fatty acids % between different maturation stages (previtellogenesis, early-vitellogenesis, late-vitellogenesis and post-spawning). Values without superscripts within rows indicate there is no significant difference in fatty acids % between different maturation stages.

Fatty acid	Previtellogenesis	Early-vitellogenesis	Late-vitellogenesis	Post-spawning
14:0	1.57 ± 0.69	2.1 ± 1.36	1.81 ± 1.12	1.21 ± 0.99
15:0	0.95 ± 0.34	1.92 ± 1.6	3.67 ± 2.5	3.7 ± 2.89
16:0	22 ± 3.16	22.2 ± 3.23	21.17 ± 1.14	20.79 ± 3.67
18:0	7.21 ± 1.84	6.8 ± 2.47	7.85 ± 1.5	8.98 ± 1.75
ΣSFA	**31.93 ± 2.34**	**33.02 ± 2.67**	**34.69 ± 2.23**	**34.83 ± 4.06**
16:1	7.44 ± 4.78	9.77 ± 6.01	6.66 ± 3.32	5.11 ± 4.65
18:1n-9	4.02 ± 1.12	3.77 ± 1.28	4.74 ± 0.75	4.92 ± 1.5
18:1n-7	3.4 ± 0.65	3.33 ± 1.2	4.74 ± 1.31	4.55 ± 1.64
20:1	0.07 ± 0.09	0.03 ± 0.08	0.99 ± 2.05	0.13 ± 0.09
ΣMUFA	**15.09 ± 6.41**	**17.05 ± 6.8**	**16.37 ± 2.99**	**14.74 ± 7.15**
18:2n-6	1.07 ± 0.54	1.77 ± 1.23	0.68 ± 0.49	0.68 ± 0.6
18:3n-6	1.62 ± 0.93	1.78 ± 0.38	1.26 ± 0.38	1.05 ± 0.32
20:4n-6 ARA	7.34 ± 2.04	6.99 ± 1.38	7.75 ± 1.19	9.14 ± 2.64
22:4n-6	0.86 ± 0.25	0.72 ± 0.26	0.91 ± 0.22	0.97 ± 0.32
22:5n-6	1.5 ± 0.59	1.12 ± 0.4	1.73 ± 0.45	1.66 ± 0.63
Σn-6 PUFA	12.38 ± 2.07	12.38 ± 1.79	12.28 ± 1.7	13.49 ± 3.07
18:3n-3	0.4 ± 0.2	0.65 ± 0.52	0.28 ± 0.2	0.26 ± 0.12
18:4n-3	0.89 ± 0.59	1.37 ± 1.02	0.85 ± 0.64	0.6 ± 0.31
20:4n-3	0.66 ± 0.24	0.52 ± 0.22	0.33 ± 0.2	0.35 ± 0.21
20:5n-3 EPA	11.14 ± 2.32	10.83 ± 1.98	8.23 ± 2.08	8.73 ± 3.28
22:5n-3	7.34 ± 1.62^{a}	5.52 ± 1.33^{b}	4.85 ± 0.58^{b}	4.9 ± 1.01^{b}
22:6n-3 DHA	14.33 ± 4.77	11.79 ± 5.75	14.07 ± 3.49	13.53 ± 5.56
Σn-3 PUFA	35.13 ± 7.55	31.23 ± 5.36	28.8 ± 4.6	28.57 ± 7.63
ΣPUFA	**47.51 ± 8.57**	**43.61 ± 4.98**	**41.08 ± 5.57**	**42.06 ± 9.8**
DHA/EPA	1.27 ± 0.26	1.14 ± 0.62	1.79 ± 0.51	1.57 ± 0.42
ARA/EPA	0.66 ± 0.14^{a}	0.66 ± 0.19^{a}	0.99 ± 0.28^{b}	1.1 ± 0.23^{b}
Total FA (mg g^{-1} lipids)	521.64 ± 37.26	507.4 ± 91.57	485.3 ± 54.77	425.17 ± 81.76

Table 3. Fatty acid composition (% of total fatty acids) of flathead grey mullet female liver at different maturation stages (n = 6 – 7 females per stage). Values are mean ± SD. Fatty acids with < 0.5% are excluded. Values with different superscripts within rows indicate significant differences in fatty acids % between different maturation stages (previtellogenesis, early-vitellogenesis, late-vitellogenesis and post-spawning). Values without superscripts within rows indicate there is no significant difference in fatty acids % between different maturation stages.

Fatty acid	Previtellogenesis	Early-vitellogenesis	Late-vitellogenesis	Post-spawning
14:0	0.75 ± 0.25	1.62 ± 0.51	1.12 ± 1.06	1.02 ± 0.48
15:0	0.82 ± 0.3	2.6 ± 3.14	2.65 ± 1.53	2.57 ± 1.36
16:0	20.48 ± 1.63	26.71 ± 5.43	24.33 ± 7.57	23.15 ± 8.11
18:0	8.23 ± 3.11	8.51 ± 3.27	11.68 ± 3.75	10.42 ± 3.35
ΣSFA	**30.64 ± 4.18^{a}**	**39.52 ± 5.91^{ab}**	**40.26 ± 5.58^{b}**	**37.75 ± 7.06^{ab}**
14:1	4.12 ± 5.8	11.21 ± 4.26	7.35 ± 5.34	7.25 ± 5.31
16:1	3.8 ± 3.84	1.85 ± 2.48	2.45 ± 1.71	2.22 ± 1.07
18:1n-9	4.52 ± 3.64	6.46 ± 3.39	7.3 ± 2.16	7.26 ± 4.93
18:1n-7	8.6 ± 4.32	7.62 ± 1.08	9.3 ± 3.13	7.2 ± 2.52
ΣMUFA	**20.74 ± 12.57**	**24.07 ± 4.72**	**26.89 ± 10.25**	**21.93 ± 9.73**
18:2n-6	1.13 ± 0.84	1.14 ± 0.72	0.8 ± 0.95	0.55 ± 0.91
20:4n-6 ARA	5.97 ± 1.9	5.68 ± 4.57	6.25 ± 2.49	5.78 ± 3.45
22:4n-6	0.69 ± 0.17	0.84 ± 0.79	0.76 ± 0.22	0.81 ± 0.56
22:5n-6	0.54 ± 0.11	0.48 ± 0.28	0.66 ± 0.37	0.7 ± 0.4
Σn-6 PUFA	9.26 ± 1.64	8.88 ± 5.74	9.64 ± 2.98	8.58 ± 4.98
18:3n-3	0.74 ± 0.94	0.43 ± 0.45	0.08 ± 0.13	0.11 ± 0.14
18:4n-3	0.75 ± 0.39^{ab}	1.15 ± 1.13^{a}	0.23 ± 0.41^{ab}	0.08 ± 0.2^{b}
20:4n-3	0.72 ± 0.56	0.57 ± 0.32	0.21 ± 0.2	0.29 ± 0.44
20:5n-3 EPA	8.64 ± 1.62^{a}	6.51 ± 3.7^{a}	2.25 ± 1.08^{b}	2.74 ± 1.73^{b}
22:5n-3	6.95 ± 2.22^{a}	4.96 ± 2.64^{ab}	2.97 ± 1.13^{b}	4.13 ± 2.17^{ab}
22:6n-3 DHA	17.09 ± 7.52^{a}	6.16 ± 1.99^{b}	8.75 ± 4.91^{ab}	12.86 ± 7.94^{ab}
Σn-3 PUFA	35.35 ± 7.85^{a}	20.07 ± 8.08^{b}	14.49 ± 6.52^{b}	20.31 ± 11.34^{ab}
ΣPUFA	**44.6 ± 8.64^{a}**	**28.95 ± 12.29^{ab}**	**24.13 ± 9.26^{b}**	**28.89 ± 15.45^{ab}**
DHA/EPA	1.9 ± 0.72^{a}	1.82 ± 2.05^{a}	4.33 ± 1.8^{b}	4.8 ± 0.95^{b}
ARA/EPA	0.69 ± 0.16^{a}	1.26 ± 1.39^{ab}	2.57 ± 0.89^{b}	2.35 ± 1.42^{b}
Total FA (mg g^{-1} lipids)	540.09 ± 28.94	691.89 ± 106.26	663.89 ± 75	607.08 ± 53.06

Table 4. Fatty acid composition (% of total fatty acids) in flathead grey mullet female ovary at different maturation stages (n = 6 – 7 females per stage). Values are mean ± SD. Fatty acids with < 0.5% are excluded. Values with different superscripts within rows indicate significant differences in fatty acids % between different maturation stages (previtellogenesis, early-vitellogenesis, late-vitellogenesis and post-spawning). Values without superscripts within rows indicate there is no significant difference in fatty acids % between different maturation stages.

Fatty acid	Previtellogenesis	Early-vitellogenesis	Late-vitellogenesis	Post-spawning
14:0	0.49 ± 0.07^{a}	0.94 ± 0.19^{b}	0.52 ± 0.15^{a}	0.31 ± 0.11^{c}
15:0	0.63 ± 0.3	1.91 ± 1.97	2.5 ± 1.61	1.99 ± 1.05
16:0	19.14 ± 1.98^{a}	13.07 ± 2.09^{b}	8.65 ± 0.58^{c}	9.28 ± 4.22^{c}
18:0	9 ± 1.28^{a}	5.39 ± 1.77^{b}	6.33 ± 2.4^{ab}	8.39 ± 3.05^{ab}
ΣSFA	**29.32 ± 1.85^{a}**	**21.31 ± 3.32^{b}**	**18 ± 3.74^{b}**	**19.98 ± 7.61^{b}**
16:1	0.0 ± 0.0^{a}	11.93 ± 2.93^{b}	8.7 ± 4.11^{c}	3.96 ± 2.41^{d}
17:1	2.94 ± 0.5^{a}	11.34 ± 3.8^{bc}	14.65 ± 5.99^{c}	7.48 ± 7.79^{ab}
18:1n-9	3.45 ± 0.41^{a}	5.9 ± 2.95^{ab}	8.14 ± 2.23^{b}	6.57 ± 2.6^{ab}
18:1n-7	4.69 ± 0.68^{a}	5.27 ± 0.8^{ab}	9.21 ± 1.4^{c}	6.86 ± 2.36^{b}
ΣMUFA	**11.43 ± 0.72^{a}**	**31.8 ± 9.57^{b}**	**39.08 ± 9.37^{b}**	**24.49 ± 14.92^{ab}**
18:2n-6	0.43 ± 0.19	1.8 ± 1.39	0.88 ± 1.25	0.72 ± 1.07
18:3n-6	0.98 ± 0.35	3.11 ± 0.63	3.86 ± 0.89	2.85 ± 1.87
20:4n-6 ARA	12.26 ± 2.28^{a}	4.8 ± 1.82^{b}	4.25 ± 0.59^{b}	10.35 ± 4.98^{a}
22:4n-6	1.21 ± 0.34^{abc}	0.82 ± 0.36^{ab}	0.76 ± 0.12^{b}	1.89 ± 0.95^{c}
22:5n-6	0.91 ± 0.34^{abc}	0.63 ± 0.22^{ab}	0.72 ± 0.23^{b}	1.37 ± 0.61^{c}
Σn-6 PUFA	15.78 ± 2.9^{ab}	11.16 ± 3.55^{bc}	10.46 ± 1.48^{c}	17.16 ± 4.9^{a}
18:3n-3	0.17 ± 0.18^{a}	0.69 ± 0.34^{b}	0.39 ± 0.4^{b}	0.35 ± 0.51^{b}
18:4n-3	0.31 ± 0.18^{a}	0.89 ± 0.26^{b}	0.61 ± 0.24^{bc}	0.43 ± 0.22^{ac}
20:4n-3	0.36 ± 0.1^{a}	0.76 ± 0.34^{b}	0.45 ± 0.22^{ab}	0.31 ± 0.27^{a}
20:5n-3 EPA	12.38 ± 1.61^{a}	8.06 ± 3.53^{b}	3.7 ± 1.63^{c}	4.04 ± 1.03^{c}
22:5n-3	7.28 ± 1.12^{a}	6.21 ± 2.31^{ab}	3.9 ± 0.73^{b}	7.79 ± 2.74^{a}
22:6n-3 DHA	17.33 ± 2.25^{a}	8.43 ± 4.27^{b}	6.81 ± 1.26^{b}	13.59 ± 4.75^{a}
Σn-3 PUFA	38 ± 3.16^{a}	25.36 ± 8.47^{b}	15.98 ± 2.72^{c}	26.6 ± 6^{b}
ΣPUFA	**53.78 ± 1.45^{a}**	**36.53 ± 9.09^{b}**	**26.44 ± 3.62^{c}**	**43.76 ± 9.57^{b}**
DHA/EPA	1.43 ± 0.33^{a}	1.41 ± 1.16^{a}	2.16 ± 0.9^{ab}	3.42 ± 1.05^{b}
ARA/EPA	1.02 ± 0.28^{a}	0.78 ± 0.55^{a}	1.32 ± 0.5^{a}	2.64 ± 1.31^{b}
Total FA (mg g^{-1} lipid)	452.63 ± 90.36	548.52 ± 90.49	589.84 ± 56.15	456.37 ± 107.09

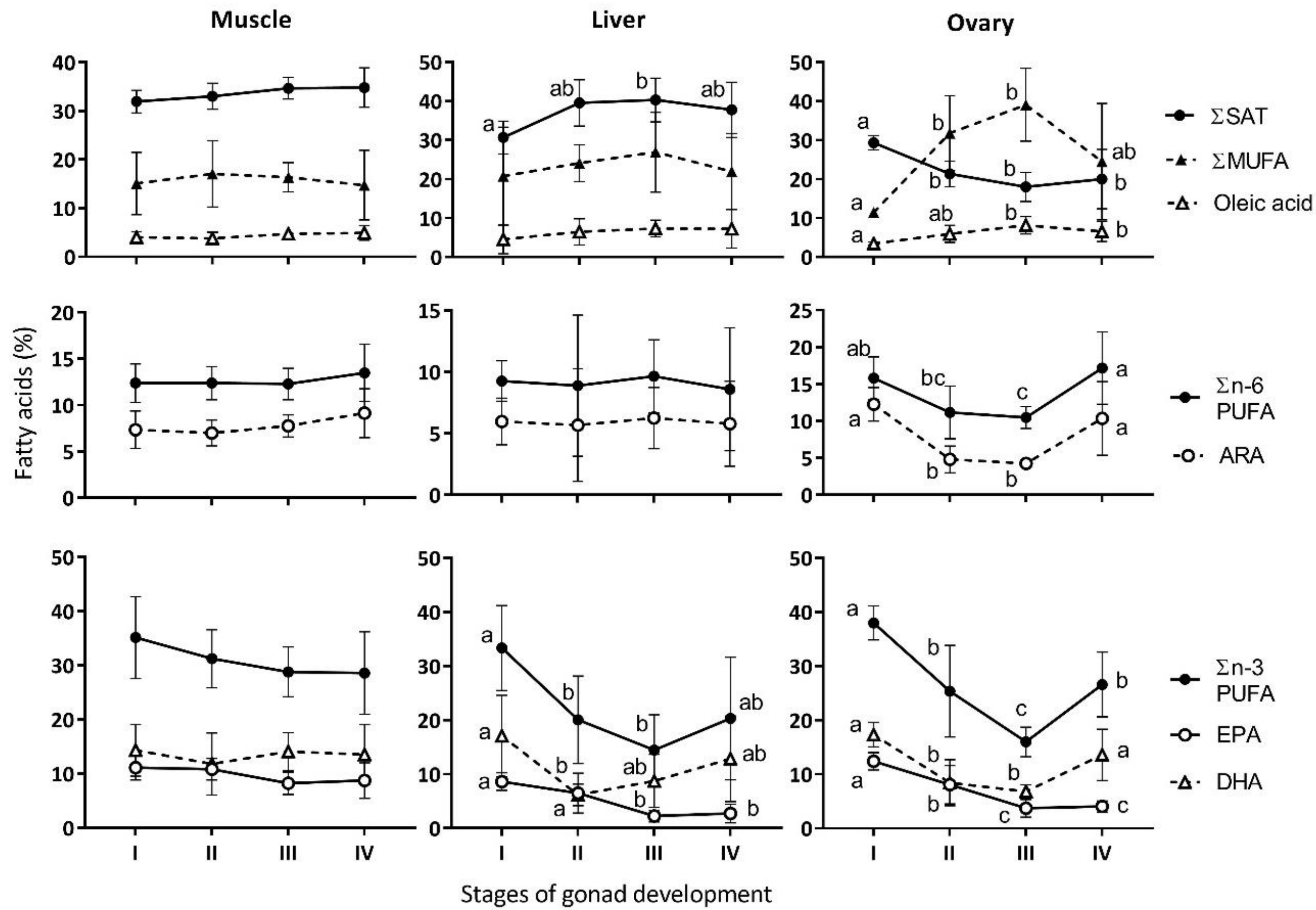

Figure 5. Total fatty acid content (%) of the most representative groups (ΣSFA, ΣMUFA, oleic acid, Σn-6 PUFA, ARA, Σn-3 PUFA, EPA, DHA) in the muscle, liver and ovaries of the flathead grey mullet (*Mugil cephalus*) female breeders at different stages of gonad development: (i) previtellogenesis (n = 7), (ii) early-vitellogenesis (n = 6), (iii) late-vitellogenesis (n = 7), and (iv) post-spawning (n = 7). Data is presented as mean ± SD. Different letters show significant differences ($P < 0.05$) in each fatty acid percentage through gonadal development.

4. Discussion

The reproductive season for the flathead grey mullet in the Western Mediterranean extends from September through October as determined by GSI values and the histological confirmation of the reproductive stage. The available data on flathead grey mullet sexual maturity in the Mediterranean are scarce and refer to different studies of wild fish sampled in the coast of Tunisia and Algeria. The data from these areas are highly variable; females from 27 cm SL to 40 cm TL [7,227] were considered to have reached first sexual maturity. The reported worldwide size of maturity also ranges widely: from 23 cm to 41 cm SL [164,272]. In the present study, the size of sampled females ranged from 40 to 59.5 cm SL (50.5 to 71 cm TL) and, therefore, correspond to a size at which flathead grey mullet females are reproductively active adults. Ovarian recrudescence in the flathead grey mullet occurs during mid-summer in Ebro Delta canals and waters of Western Mediterranean as indicated by oocyte development. The first date that showed the initiation of oocyte development —oocytes containing yolk droplets and, thus, vitellogenesis— was observed in early August and was prolonged to early September. Further gonadal development was well described by the GSI values, thus, females at advanced vitellogenesis observed from middle August to mid-September, showed values increasing from 4.2 ± 3.6 % to 15.5 ± 3.4 % and decreasing after spawning (1.8 ± 0.9 %). In fully mature flathead grey mullet females, GSI values are very variable worldwide with different maximums. The highest GSI obtained in the present study was 22.6 % whereas from the Mediterranean Sea was reported to be around 25 % [11,227]. The lowest GSI value was obtained in the waters of Korea with 5.32 % [124], while the highest GSI value, close to 40 %, was obtained in the Gulf of Mexico [100]. The presence of females with flaccid ovaries and prominent blood vessels with postovulatory follicles and atresia indicated the end of the spawning season. The capture of those females in mid-September was the evidence that spawning occurred during September, and females caught in mid-October showed that spawning may still occur during this month. These results suggest that the spawning season in the Western Mediterranean takes place mainly from September to October. According to different studies, the breeding of flathead grey mullets in some eastern regions of the Mediterranean overlaps with that of our study. However, it seems that flathead grey mullet populations present earlier spawning periods from the eastern to

the western areas of the Mediterranean. For instance, flathead grey mullet breed from June to August in Turkey, June to September in Egypt, and August to October in Greece and Tunisia [272].

Regarding lipid accumulation, flathead grey mullet females showed different body compositions according to the seasonal increase of GSI during vitellogenesis. Muscle had a lower lipid content, and no changes were observed through the different stages. Lipid content observed in muscle (3.85 - 4.92 %) is in the range of previous analysis on mullet flesh. Data from the Mediterranean corresponds to samples collected on the Turkish coast with 2.1 ± 0.1 % lipid content in fillets. Nevertheless, it is reported that flathead grey mullet is among the fish showing the greatest variability in lipid content depending on geographic location, with more than a 10-fold range of values; therefore, comparisons between samples from different regions are limited [122]. On the contrary, high lipid values (18.46 - 22.62 %) were present year-round in the liver. Although no clear trend of liver lipids utilization was observed during the reproduction period, HSI % increased during vitellogenesis, suggesting that changes in lipid content were happening following gonadal development. It is possible that there were differences in the different lipid fractions —the neutral, comprising triacylglycerols and wax esters, and the polar, consisting of membrane glycolipids and phospholipids— through gonadal development that were not shown in the total. For example, ratios of lipid classes (neutral:polar) accumulation varied during gonadal development according to the utilization of lipid reserves in white seabream (*Diplodus sargus*) [208]. In gonads, total lipid content increased from 4.90 % to 34.59 % during vitellogenesis as expected to contribute to egg reserves [250]. The reported lipid content concerning raw roe of flathead grey mullet is scarce and, similar to the flesh, shows a high variation according to the geographical location. It ranges from 13.1 to 23.3%, being the lowest in a lagoon of Turkey [122]. According to the present results, it is apparent that the flathead grey mullet makes little use of muscle tissue as a lipid depot, such as other demersal species [45], as low lipids accumulated in the muscle. These suggest that, during gonadal development, endogenous lipids from the muscle are not used for gonadal growth. Hence, mobilization of liver lipids, dietary uptake, and depletion of intraperitoneal fat during vitellogenesis,

mainly constituted by SFA (16:0) and MUFA (16:1 and 18:1n-9) (results not shown), must be responsible for the accumulation of lipid reserves in the ovaries.

Profiles of the fatty acid composition of tissues varied significantly within gonadal development. While there was no substantial evident change in the muscle fatty acids profile, it was clearly observed in the liver and the ovaries. The saturated fatty acid composition of muscle, liver, and ovaries was determined by the palmitic acid 16:0, which values were similar to those previously reported in fillet and raw roe [234]. During vitellogenic development, the percentage of ΣSFA increased in liver, mainly of 16:0, which would serve as a lipid depot for energy metabolization. On the contrary, the percentage of ΣSFA in ovaries decreased during vitellogenesis, which may indicate the requirement of saturated fatty acids in energy metabolism during gonad development with a substantial decrease in the percentage of 16:0. Henderson, Sargent and Hopkins (1984) reported that 16:0 fatty acid is the main source of energy metabolism for breeders, especially during the egg production period.

While mono-unsaturated fatty acids constituted half of the saturated fatty acids in ovaries at previtellogenesis, they turned to the double during vitellogenesis. The continuous accumulation of ΣMUFA in ovaries during gonadal development (up to 39.08 ± 9.37 %) and the posterior decrease once spawned, demonstrated the importance of MUFAs on the formation of embryo reserves. Our reported levels of MUFAs agree with published data for flathead grey mullet raw roe in other locations (13 - 42 %) [122]. Among MUFAs, 16:1 (palmitoleic acid), 17:1 (heptadecenoic acid), 18:1n-9 (oleic acid), and 18:1n-7 were preferentially accumulated in the flathead grey mullet ovaries throughout vitellogenesis. The accumulation of oleic acid in fish gonads during gonadal development has been widely reported in other fish species [208]. The predominance of palmitoleic acid in flathead grey mullet eggs has been previously reported and attributed to the fish diet [234]. Palmitoleic acid was also the main MUFA for flathead grey mullet muscle, contributing to 35 - 47 % of total MUFAs, contrary to most fish species in which oleic acid is the most represented MUFAs in muscle. High levels of 16:1 in the flesh have also been reported by Argyropoulou *et al*. (1992), Özogul and Ozogul (2007), and Sengör *et al*. (2003). Palmitoleic acid is produced by desaturation of 16:0 palmitic acid. The decrease in the percentage of 16:0 in the ovaries could have led to the production of palmitoleic acid,

which high levels are characteristic of freshwater fish [10,199], correlating with the habitat occupation of the flathead grey mullet, which is a catadromous marine fish that often enter estuaries and freshwater environments [272].

One interesting feature of the MUFA was the noticeably high levels of 17:1. The presence of 17:1 heptadecenoic acid —an odd chain fatty acid— has been previously reported as a trace element in flathead grey mullet muscle (< 0.1 – 1.94%) [199,234] and raw caviar (< 0.1 %) [234], and in tissues of other fish species, such as in muscle, liver, and gonads of the Pacific herring (*Cuplea harengus pallasi*) (≃ 1 %) [99], but has not been observed in other omnivorous species such as the white sea bream (*Diplodus sargus*) [208]. In the present study, though, while 17:1 is not present either in muscle or liver, it shows up to 14.65 ± 5.99 % levels in ovaries during vitellogenesis with a notable increase from immature stages. This observation suggests that the accumulation of this particular fatty acid in the ovaries depends mainly upon the fish diet, reflecting an active feeding behaviour during reproductive development. The relatively high level of fatty acids with an odd number of carbons likely originates from bacteria [199,219,234]. The flathead grey mullet is omnivore and detritivore, feeds on epiphytic and benthic microalgae, macrophyte decaying detritus, and inorganic sediment [34], and has been observed to feed also on the bacterial scum of *Anabaena spp* [48]. The presence of 17:1 indicates the relevance of bacteria in the diet as a source of lipids. Its substantial increment during vitellogenesis suggests the importance of this fatty acid as a reservoir for future embryonic development, which, to our knowledge, has not been previously reported in this species.

In female fish, it has been assumed that PUFA are involved in the physiological reproductive processes; play an essential role in the development of gonads, the formation of gametes, and the formation of cell membrane structures or regulate ion channels at the cell membrane [250]. In this study, the high levels of PUFA (53.78 ± 1.45 %) accumulated in the ovaries at previtellogenesis and the subsequent decrease during ovarian development (down to 26.44 ± 3.62 %) reflects the importance of these fatty acids as an energy reserve for metabolization during gonad development. The source of total PUFA variation in flathead grey mullet ovaries at different reproductive stages was due to the difference in Σn-6 fatty acids, mainly of ARA, and Σn-3 fatty acids, mainly of

EPA and DHA. The decrease of Σn-3 PUFA fatty acids, especially EPA, 22:5n-3, and DHA, in the liver during vitellogenesis further suggests the importance this tissue has as a fatty acid reserve in support of the reproductive effort in this species.

Polyunsaturated fatty acids DHA, EPA, and ARA are essential fatty acids in marine fish. Docosahexaenoic acid and EPA have a structural role in membrane phospholipids [131] and are a source of metabolic fuel for reproduction [97]. Eicosapentaenoic acid and ARA are precursors for eicosanoids, including prostaglandins, thromboxanes, and leukotrienes, which are involved in steroidogenesis, oocyte maturation, and ovulation [250]. In marine fish, ARA and EPA compete for the enzymes that regulate the eicosanoid production [102]; however, ARA forms more biologically active prostaglandins than EPA [94]. Different levels of ARA and EPA have been shown to influence prostaglandin production in wild and cultured Senegalese sole [193,194]. Higher levels of DHA, ARA, and EPA were found in the present study compared to muscle and raw caviar samples from flathead grey mullet in Sengör *et al*. (2003). Docosahexaenoic acid and ARA levels in muscle samples reported in the present study were also greater than in fillets samples from Özogul and Özogul (2007). The high levels of ARA found in gonads and the decrease in percentage during gonadal maturation show the important role this fatty acid plays in the reproductive function of this species. The degree of difficulty to obtain essential ARA differs among species, habitat, and food sources. High levels of ARA were also found in other fish species exhibiting a demersal omnivore feeding strategy [53] or low trophic demersal feeding flatfish [192]. The ARA/EPA ratio in the liver showed an increasing trend, which would indicate that the utilization of EPA in the liver was higher than that of ARA during ovarian development in the female. A relatively higher DHA/EPA ratio (greater than 2-3) was obtained in the liver and gonads at late vitellogenesis, which would represent selective catabolism and utilisation of EPA relative to DHA in fatty acid oxidation producing energy for oogenesis. During the post-spawning period, high ratios were also obtained as EPA reserves were reduced and DHA remained relatively conserved in comparison.

In summary, the spawning season of the flathead grey mullet in the Western Mediterranean lays within September and October. This study also showed that gonadal recrudescence and maturation are associated with increases in gonadal size, lipid

accumulation and changes in the fatty acid composition during gonadal development. Therefore, fatty acid composition profiles reflect the different compositions of muscle, liver, and gonads and would help to develop adequate diets for breeders. However, the present results only characterize flathead grey mullet breeders from the Western Mediterranean due to the high variation fatty acid composition reported for this species among different locations [219].

CHAPTER III

Feeding habits and the influence of pellet diameter on the feeding responses of the flathead grey mullet in captivity

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Feeding habits and the influence of pellet diameter on the feeding responses of the flathead grey mullet in captivity

1. Introduction

Mullet species are of great ecological and economic importance for both fisheries and aquaculture around the world, which is mainly concentrated in the Mediterranean, Black Sea region and South East Asia [46]. There is increasing interest in the intensive culture of mullets as it is necessary to diversity marine aquaculture products away from carnivorous fish, to supply demand in local market where mullet is appreciated and to reduce fishing pressure on mullet species [281]. Mullets present good biological characteristics and potential for aquaculture. Positive aquaculture characteristics include, the euryhaline capabilities of many mullets, the fast growth (~ 1 kg per year) (FAO, 2019), the high efficiency in converting food to body mass [59] and the omnivorous diet [34]. The euryhaline capabilities enable that flathead grey mullets (*Mugil cephalus*) are cultured in freshwater, brackish water, and marine water aquaculture facilities [195] and the omnivorous diet [34] indicates the species high potential for culture with diets that do not contain fishmeal and fish-oil. In the Mediterranean region, in addition to flathead grey mullet, other species being cultured are: thicklip grey mullet (*Chelon labrosus*), golden grey mullet (*Liza aurata*), thinlip mullet (*Liza ramada*), and leaping mullet (*Liza saliens*) [46].

The expansion of flathead grey mullet aquaculture depends on the development of intensive-farming techniques for which, current limitations include diet formulation, characteristics such as pellet size and feeding practices, i.e., how, when and where to deliver feed to optimize feeding efficiency. It is important to develop feeds that are readily accepted to meet fish nutritional requirements for maintenance, growth and guarantee the developmental competence of breeders and offspring [178]. The flathead grey mullet and many species from the Mugilidae family have been described as omnivorous [34,105]. This omnivorous classification has focused research on the growth performance of fry and juveniles using feeds formulated with vegetable protein sources [77,132,252], which will allow aquaculture to be a sustainable activity reducing and / or

eliminating fishmeal and fish oil from the diets. Despite of the recent interest in Mugilidae diets, the feeding responses of mullets concerning pellet characteristics have received no attention in comparison to other cultured species. As a consequence, there is a lack of commercial pelleted feed for flathead grey mullet juveniles or breeders which, additionally, seem to reject commercially produced pellets during acclimatisation once captured from the wild. Efforts to maximise feed utilisation in mullet species fed artificial feeds in intensive aquaculture should take into account the species feeding behaviour and the characteristics of the pellet.

The physical characteristics of pellets, such as size (shape, diameter and length), colour (contrast), texture (hardness) and density (sinking rate; floating, slow sinking, or fast sinking) that offers a different distribution of the feed into the water, determine the ability of fish to detect the pellet, capture the pellet, and once captured, the acceptance to ingest the feed [243]. Fish species could be reluctant to eat pellets with characteristics that make them not perceived as a desirable food item [243] and do not match with the type of feeding habits the species occupies; surface, surface/column, column feeder or benthic/bottom feeder [108,211]. For instance, catfish, salmon, and shrimp require floating, slow-sinking, or fast-sinking feeds, respectively [141]. In addition, pellet characteristics such as size can directly influence growth, as described for Atlantic salmon (*Salmo salar*) [267] and Nile tilapia (*Oreochromis niloticus*) [12]. Therefore, providing an adequate pellet particle presented according to the species feeding habits will improve the feed acceptance, growth and development of flathead grey mullet in culture.

The present study aimed to identify pellet characteristics to improve delivery of diets to flathead grey mullet by determining the appropriate pellet size accepted by juveniles and adults that meet feeding habits in intensive captive conditions.

2. Material and methods

2.1. Experimental animals

Wild flathead grey mullets were captured from Ebro River and brought to IRTA facilities (Sant Carles de la Ràpita, Spain) and held for 16-17 months before the experiments. Fish were stocked in 10 m^3 tanks. During the first year, the fish did not

accept pellets and were fed at 1.5% of the body weight a soft mixture of 20% sardines, 20% hake, 15% mussels, 10% squid, 10% shrimp and 25% of a ground commercial diet (Skretting, Spain) with 0.1% spirulina. After one year and before the present experiment, fish were presented different sizes of pellet from a commercial on-growing diet for sea bream (*Sparus aurata*) (Skretting, Spain) at 1.5% body weight. Twelve juveniles and twelve breeders were selected to examine pellet size preference and returned back to the main tank. A total of 21 fish were then selected to evaluate the feeding habits of this species. In both tests, fish was allowed to acclimatize to the tanks for 7 days prior to the start of the experiment. Throughout the acclimatization period, fish were fed with polychaetes (TOPSY Bait, Netherlands). Forty-eight hours prior to the behavioral tests the fish were not fed to increase their potential activity and appetite.

The study was performed 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; Spanish Royal 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 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 animals for the experimentation or other scientific purposes).

2.2. Preferred pellet diameter

In order to record the response of the flathead grey mullet to pellets of different diameters, three fish were placed per tank in a total of four circular tanks of 2000 L (1.7 m diameter x 1 m depth). The test was performed with twelve juveniles (mean weight: 365.50 ± 36.90 g; mean standard length: 28.80 ± 0.84 cm) and twelve adults (937.49 ± 146.54 g; 40 ± 1.12 cm) separately. In the case of the juveniles, three different diameters (2, 4 and 6 mm) were tested and in the case of the adults, four (2, 4, 6 and 8 mm). The pellets were all the same commercial on-growing diet for sea bream (*Sparus aurata*) (Skretting, Spain).

In each tank, a feeding tube was positioned just below the water surface to introduce the pellets and guide them into the water. The part of the tank that had the

feeding tube was curtained with black plastic to screen the fish from any movement or disturbance caused by introducing the pellets. The responses to pellets were recorded with a video system. A video camera (Camera KPC-SN505U, Korea Technology and Communications, Seoul, Korea) encased in a waterproof housing (Integraqua Technology, A Coruña, Spain) was placed inside the water to show a direct view of the entrance of the pellets into the tank and allow observations of the full water column in the tank from surface to bottom (**Fig 1A**). The camera was connected to a video recorder (Presentco, Xmotion HD Enterprise 08 Video Recorder, Rister, Barcelona, Spain) and responses were recorded. Pellets were randomly dispensed individually through the tube (143 ± 19 pellets / diameter) at time intervals of 50 s. The test was repeated on three different days at 9:00 h, as the morning was found to be the peak of flathead grey mullet feeding activity, which was diurnal [44].

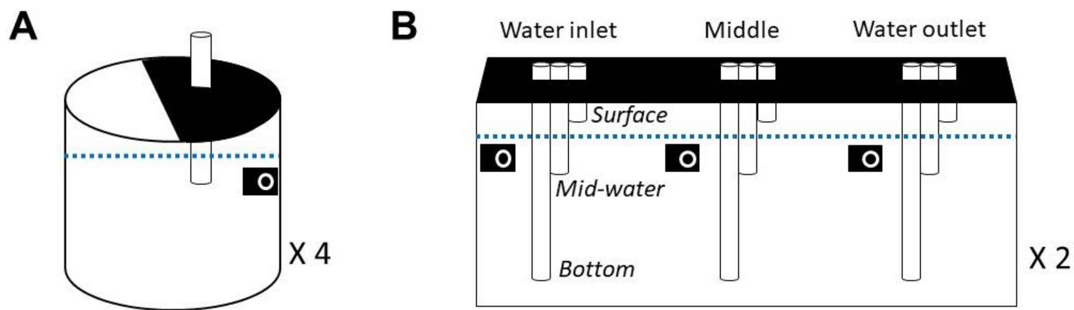

Figure 1. Schematic representation of the tank systems set-up for A) the pellet diameter preference test and B) evaluation of feeding habits. In the first trial, four fiberglass tanks (2000 L each) were prepared with three individuals per tank for adults and juveniles. In the second trial, two 10 m^3-tanks were used with 15-16 fish each. Each feeding area (water inlet, middle, water outlet of the tank) was coupled with three tubes with different depths to allow the presentation of food in three levels; surface, mid-water column and bottom. Dotted line indicates water limit. Dark areas represent the part of the tank that was curtained with black plastic to avoid the disturbance of fish caused by the introduction of pellets. Dark square with white circle indicates the camera position.

Different behavioural responses and measures were made from the video recordings. The responses evaluated are based on studies by Stradmeyer (1989) and Smith *et al*. (1995) and are related to the parameters that influence the final success of feeding; the detectability or attractiveness and acceptability of the food item. Linked to the detectability and attractiveness two parameters were registered: (i) the percentage

reaction (rapid movement of the head towards the pellet) or no-reaction of the fish and (ii) the time elapsed since the pellet entered the water and the first catch. Linked to acceptability, the following parameters were registered: (i) the percentage of pellets ingested or rejected —those spat out and not recaptured for ingestion— and (ii) the handling time (time elapsed between the first capture and its final ingestion). A preference index (scale from 0 to 1) was calculated dividing the total number of pellets ingested by the number of times the pellet was captured; some pellets were captured more than once as the pellet was spat out and captured a second time (recaptured) or more. The preference index was applied only in those pellets that were ingested. Those pellets with an index close to 0 would be less acceptable than those with an index close to 1. An index of 1 would indicate that all the pellets were ingested when first captured.

2.3. Feeding habits

Thirty-one individuals (mean weight: 1044.82 ± 388.92 g; mean standard length: 41.27 ± 3.89 cm) were split in two groups (n = 15, n = 16) and placed in two rectangular 10 m^3 tanks of 6.1 x 2.1 x 1.2 m. Three feeding areas were designated in the tanks; water inlet, the middle and water outlet of the tank and a video camera was placed in each feeding area to record the activity as described in the previous trial. In each feeding area, three polyethylene tubes of different lengths were placed to guide pellets into the water column to different depths: surface, in which the tube was placed above the water surface; mid-water column, in which the tube was positioned approximately 25 cm below the water surface to have the pellets fall and appear in the water column; and the bottom, the tube reaching the bottom of the tank (see schematic representation in **Fig 1B**). The part of the tank that had the opening of the feeding tubes was curtained with black plastic to avoid disturbance of fish by the introduction of pellets. The tank was illuminated with a strip of LEDs that were positioned in the middle of the tank from the inlet to the outlet and programmed to switch on and off at sunrise and sunset with a gradual increase in intensity. The LEDs were set to emit blue light and the intensity at the water surface increased from 10 lux at the inlet and outlet to 30 lux in the central area of the tank. The lux at the feeding points was approximately, inlet 25 lux, middle 30 lux and outlet 25 lux.

Pellets with the same nutritional formula and the same commercial dietary presentation (Optiline, Skretting, Spain) fabricated for rainbow trout (*Oncorhynchus mykiss*), but with different characteristics were used depending on the depth of the tube in the water column. Floating 4-mm pellets (Optiline AE, Skretting, Spain) were administered through the tube at the surface, and non-floating 4-mm pellets (Optiline AE Ouro, Skretting, Spain) were administered through the tubes positioned mid-water column and the bottom. Five minutes before the feeding activity, a vibrating alarm was presented in both tanks. Subsequently, pellets were administered in the three feeding areas (inlet, middle and outlet of the tank) in one different tube depth (surface, mid-water column or bottom) per feeding area. In this way, the presentation of pellets at three depths were made at the same time with each depth in a different feeding area. Pellets were dropped into the tubes in the following sequence: bottom – mid-water column – surface, in order that pellets were presented to the fish at the same moment. The same pattern of administration (depth and area) was followed for seven days and on the eighth day, it was randomly changed. This sequence was repeated four times over 28 days. This set up ensured that each feeding area in both tanks had periods when feed was administered at each water column depth.

The distribution of feeding fish in the tank was determined by quantifying the number of fish feeding at each feeding point where feed was introduced. The feeding points were a combination of area (inlet, middle and outlet of the tank) and depth at which the pellets were presented (surface, mid-water column and bottom of the tank). The feeding activity was considered to last a total of 25 s, as this was the time that the pellets in the water column had not yet reached the bottom and during which most of the pellets were eaten. Each day, the number of individuals feeding at each feeding point were counted each 5 s during the 25 s feeding period. The proportion of fish feeding at each feeding point was calculated by dividing the number of fish feeding at a feeding point by the total number of fish feeding at the three feeding points on the specific day.

2.4. Statistical analysis

The differences between responses to different pellet diameters were evaluated by means of a two-way repeated measures (RM) ANOVA with normal distributed data

(Shapiro-Wilk test) and equal variance (Levene test), considering the tanks as subjects and the diameter and the days on which the test was performed as factors. Pairwise statistical analysis was performed with the Holm-Sidak *post hoc* test. The Friedman non-parametric test followed by Wilcoxon test with the Bonferroni correction was applied when the data did not pass the normality test. Feeding habits data did not met normality assumptions. Therefore, the non-parametric Scheirer-Ray-Hare test was applied in each tank. The test is an extension of the Kruskal-Wallis test and represents the alternative to two-way ANOVA [241]. Proportion of feeding fish was the dependent variable and the independent variables were feeding area (inlet, middle and outlet) and depth (surface, mid-column and outlet). The Scheirer-Ray-Hare test was done by applying a two-way ANOVA on ranked data. The H statistic was computed by dividing the Sum of Squares (SS) for each factor and interaction by the total Mean Square (MS). The significance of H was tested as a chi-square variable with the degrees of freedom of the SS being tested. The Dunn's *post hoc* test of pairwise multiple comparisons based on rank sums was performed.

A P value of < 0.05 was set to indicate significant differences. Data is presented as mean ± standard deviation (SD) if not indicated otherwise. Statistical analyses were performed using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA) and MS Excel was used to calculate the H statistic and the significance of H.

3. Results

3.1. Pellet diameter preference

There was no significant effect of the day in which the test was performed on the reaction and ingestion percentages of different pellet diameter in adults or juvenile fish. However, the day in which the test was performed had a significant effect on the manipulation time of pellets in the adults (P = 0.001), as a quicker ingestion was showed in the first day, and on the response time in the case of juveniles (P = 0.005) with a slower response on the first and second days.

The diameter of the pellets had an influence on the reaction of the adult individuals ($P < 0.001$) (**Fig 2A**). The adult flathead grey mullet detected the larger pellets

more easily (P = 0.002), although the response time did not significantly differ between the diameters presented (9.7 ± 2.1 s on average) (**Table 1**). Nevertheless, the initially most attractive sizes were not those consumed once captured, since the diameter was critical in their ability to consume the pellets (P < 0.001). The 4 mm pellets were consumed in a higher proportion followed by the 2- and 6-mm pellets (**Fig 2A**). The decrease in the preference index from the 2, 4, 6 to the 8-mm pellets (**Table 1**) indicated that smaller pellets tended to be rejected least often. The preference index's value for the 8 mm pellets was 0.07 as just one pellet was ingested and this pellet was captured several times before being swallowed. The handling time of the pellets before ingestion was significantly different depending on the pellet diameter (P < 0.001) (**Table 1**). The smaller the pellet, the shorter the observed manipulation time. Pellets of 2 mm required 2.5 ± 2.4 s to be ingested, 4-mm pellets required 18.7 ± 4.8 s, 6-mm were eaten in 36.5 ± 9.9 and the only 8-mm pellet consumed was consumed after 100 s of manipulation.

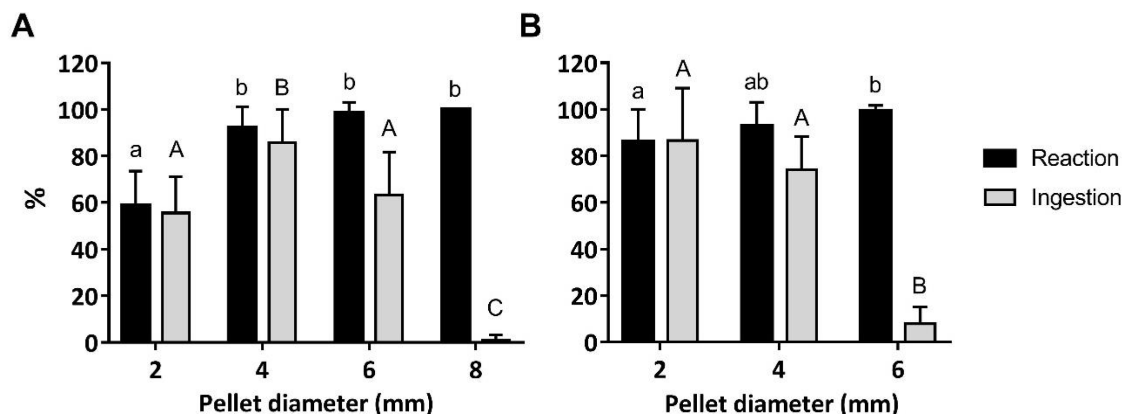

Figure 2. Percentage reaction to pellets and percentage ingestion of pellets (mean ± SD) of different diameters by (A) adult and (B) juvenile flathead grey mullet (*Mugil cephalus*). Three flathead grey mullets were held in each of four tanks that were tested on three days and a percentage reaction / ingestion to 143 ± 19 pellets / diameter was calculated for each tank on each day (n = 12). Different lowercase letters indicate significant differences (P < 0.05) in reaction and capital letters in ingestion. Pairwise comparisons were performed by the Holm-Sidak *post-hoc* test after two-way RM ANOVA or the Wilcoxon test with the Bonferroni correction after the Friedman test in no normally-distributed data.

Table 1. Reaction and handling time (s) of different pellets diameter and Index of preference for adult and juvenile flathead grey mullet (*Mugil cephalus*). Reaction time refers to the time that fish detects the pellet; handling time refers to the total manipulation time by the fish from the first capture of the pellet until ingestion. The preference index was calculated dividing the total number of pellets ingested by the number of times the pellet was captured (scale from 0 to 1, from less acceptable to direct ingestion). Different letters indicate significant differences ($P < 0.05$). N (in brackets) represents the number of tanks and days in which data was available following the examination of the responses to 143 ± 19 pellets introduced per each diameter in adults and juveniles.

Pellet diameter (mm)	Mean reaction time (s) per tank per day		Mean handling time (s) per tank per day		Mean Preference Index per tank per day	
	Adults	Juveniles	Adults	Juveniles	Adults	Juveniles
2	12.5 ± 6.7^{a} (12)	7.0 ± 2.9^{a} (12)	2.5 ± 2.4^{a} (12)	6.2 ± 2.8^{a} (12)	1.00 ± 0^{a} (12)	0.98 ± 0.04^{a} (12)
4	10.1 ± 4.7^{a} (12)	5.1 ± 1.6^{ab} (12)	18.7 ± 4.8^{b} (12)	19.1 ± 8.4^{b} (12)	0.80 ± 0.12^{b} (12)	0.79 ± 0.16^{a} (12)
6	7.9 ± 5.0^{a} (12)	3.9 ± 1.2^{b} (12)	36.5 ± 9.9^{c} (12)	39.6 ± 9.4^{c} (7)	0.58 ± 0.17^{c} (12)	0.41 ± 0.30^{b} (7)
8	8.5 ± 4.8^{a} (12)	-	100 (1)	-	0.07 (1)	-

In juvenile individuals, significant differences were found amongst the percentage reaction to 2-, 4- and 6-mm pellet diameters (P = 0.01) (**Fig 2B**). Although the juvenile mullet exhibited a high percentage of detection of the three pellet sizes, the reaction to 2 mm pellets was lower than to 6 mm pellets. Flathead grey mullet also took a significantly longer time to respond to the smaller pellet diameters (**Table 1**). The time of response was significantly different (P = 0.01) between the biggest (6 mm, 3.9 ± 1.2 s of response) and smallest pellets (2 mm, 7.0 ± 2.9 s), while there was no significant difference between intermediate pellets and biggest. On the other hand, smallest and intermediate pellets (2 mm and 4 mm) were consumed at a significantly higher proportion (**Fig 2B**) and their preference index values were closer to 1 (**Table 1**). The largest diameter (6 mm) was more likely to be rejected. The mean time for pellets to be eaten varied significantly with the diameter (*P* < 0.01). Ingestion of the pellets took significantly longer with increasing pellet diameter, from 6.2 ± 2.8 s, 19.1 ± 8.4 s to 39.6 ± 9.4 for 2-, 4- and 6-mm pellet diameter, respectively.

3.2. Feeding habits

The distribution of fish during the feeding activity significantly depended on the feeding area (inlet, middle and outlet of the tank) (*P* < 0.001) and the depth in the water column where the food was presented (surface, mid-water column and bottom) (P = 0.009) in both tanks. There was no significant interaction between both factors indicating that the presence of mullet feeding at the surface, in mid-water column or at the bottom did not depend on the feeding area; the inlet, middle or outlet. A significantly higher proportion of fish fed in the middle of the tanks (64 ± 4 % and 63 ± 6 % in tanks 1 and 2, respectively) (mean ± SEM) in comparison with the inlet (15 ± 2 % and 29 ± 6 %) and outlet (21 ± 3 % and 4 ± 2 %) (*P* < 0.001) (**Fig 3A**). Regarding the distribution of fish in the water column (**Fig 3B**), tank 1 showed a significantly higher proportion of fish exhibiting feeding activity in the mid-water column (52 ± 5 %) (*P* < 0.001), and tank 2, both in the mid-water column (36 ± 6 %) and in the bottom (47 ± 8 %) (*P* < 0.001). Significant lower proportion of fish was found feeding in the water surface (20 ± 3 % and 13 ± 4 % in tanks 1 and 2, respectively) in comparison with the depth where the fish fed the most in each tank (*P* < 0.001).

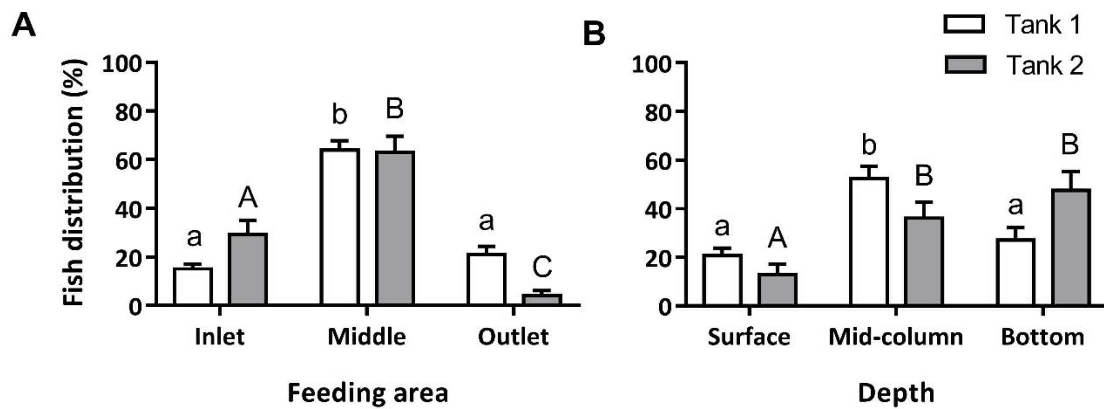

Figure 3. Proportion of fish (mean ± SEM) observed to be feeding in (A) each feeding area (water inlet, middle or water outlet) and in (B) each depth where the food was presented (surface, mid-water column or bottom). Analysis was performed with the non-parametric Scheirer-Ray-Hare with feeding area and depth as factors followed by a Dunn's *post-hoc* test. Different small letters represent significant differences ($P < 0.05$) between feeding areas and depths in tank 1, and different capital letters represent significant differences in tank 2. N per each feeding area and depth is 28 days.

4. Discussion

The feeding responses of flathead grey mullet recorded in this study reveals the effects of the pellet diameter on the feeding activity and manifests a pattern of feeding habit preferences for this species. The tests were performed in groups to favor responsiveness of the fish for the feed, as flathead grey mullet appear to have a quicker response to feeding in the presence of a feeding group than when isolated [198]. Isolated individuals could become stressed [200] and reduce feed intake [202]. Pellets with different diameters have a different detectability / attractiveness and acceptability that influence the final success of feeding. The percentage of pellets detected and the response time to the pellet represent measures of the pellet detectability and attractiveness. The percentage of pellets consumed and the number of captures followed by rejection (index preference) are measures of the pellet acceptability.

Both juveniles (~360 g) and adults (~930 g) of flathead grey mullet responded with higher percentages of responses and a shorter or quicker response time towards the largest and intermediate pellets compared to the smallest pellets. However, the pellet diameters that appeared to be at first the more attractive, were more frequently rejected

and were not ingested immediately once captured. The larger pellets were less often ingested and the time from capture to ingestion was longer. It is possible that those larger pellets that were finally ingested had softened because of longer contact with water and some manipulation in the mouth of the mullet. Therefore, the present study suggests that the optimum diameter, indicated by those diameters least often missed or rejected and therefore, optimal for feeding, would be 2 and 4 mm for juveniles of ~360 g and 4 mm for adult breeders weighing ~930 g. These results manifest that flathead grey mullet would require pellets of smaller diameter compared to what is expected in other fish species of the same size. If we compare with the manufacturer recommendations for gilthead seabream (*Sparus aurata*), larger diameters are recommended for individuals of similar sizes; 4 to 6-mm pellet diameter for seabream juveniles from 71 to 500 g of weight and 8 mm pellet diameter for seabream > 500 g [13]. The ability of the sea bream to ingest pellets of larger diameter could relate to this species well-developed chewing apparatus enabling bream to break the pellet into smaller pieces [13]. In contrast, the flathead grey mullet has been observed to capture the food and keep it in the oral cavity or spit it out and recapture before the pellet was consumed. Mullet did not chew the food item; therefore, the pellets were not observed to be broken into smaller particles. Besides, the teeth of flathead grey mullets are described to be weak and their mandibles are thought to favor the ingestion of sand/mud and organic matter and to select the particles for their final ingestion [34]. Although flathead grey mullets are mainly detritivores and usually feed on plant materials, algae, dinoflagellates, or diatoms, bigger items such as crustaceans, annelids, fish parts, insect parts have also been identified in their stomach contents [105]. It may be possible that flathead grey mullet could eat bigger diameter pellets, but softer than the ones tested in the present study. Soft pellets of a given diameter have been more acceptable in several species [126] including salmon juveniles [243]. Therefore, it would be interesting to test pellets of different hardness and evaluate the relationship between the hardness of the pellet, the diameter, and acceptability.

In regard to flathead grey mullet feeding habits, the present study identifies that mullet exhibited a distribution throughout the water column, including the water surface and the bottom, while feeding. Nevertheless, it was detected that mullet have a preference for feeding in the water column and the bottom rather than in the surface.

This result matches with the natural feeding habit of the species, which feeds on suspended plant materials and forages the bottom [34]. The same feeding habits were identified by Jimenez-Rivera *et al*. (2021) in juveniles held in tanks, in contrast with Ghion (1986) which identified that although eating in all parts of the tank, the feeding increased when juveniles were fed at the surface. According to the present results, slow-sinking or fast-sinking feeds would meet the preferred feeding habits in the flathead grey mullet. The present study also detected a preference for feeding location in the tank; the middle of the tank. There are several possible explanations for this preference as the tank environment was not completely uniform. For example, the inlet area had more disturbance from the incoming water and the outlet area may have had slightly lower water quality. In addition, the blue light intensity emitted by the LEDs was slightly higher in the middle of the tank than at the ends (inlet and outlet) and might have affected motivation for feeding in these areas.

In conclusion, the present study has identified the preferred characteristics of pellets in terms of size; diameter (2 and 4 mm for juveniles and 4 mm for adults), and density (sinking or slow-sinking pellets) according to the feeding responses and feeding habits of juveniles and adults of flathead grey mullet (feeding in the water column and the bottom). The present study provides a basis for the future development of an optimal pelleted diet for this species in intensive culture conditions.

CHAPTER IV

Application of protocols based on the rFsh hormone, produced in the *Pichia pastoris* expression system, to induce vitellogenesis and spermiation

CHAPTER IV:

Application of protocols based on the rFsh hormone, produced in the *Pichia pastoris* expression system, to induce vitellogenesis and spermiation

1. Introduction

In the flathead grey mullet (*Mugil cephalus*), dopamine appears to be responsible for the inhibition of basal and GnRH-stimulated release of gonadotropins (Gths) from pituitary [6,79]. Aizen *et al*. 2005 demonstrated that a single injection of domperidone (Dom), an antagonist of dopamine D2 receptors, was effective in removing the dopaminergic inhibition, thereby inducing the release of Gths and completing vitellogenesis in females. Meiri-Ashkenazi *et al*. 2018 increased the number of vitellogenic females administrating an injection of a dopamine receptor antagonist (Metoclopramide, personal communication Dr. H. Rosenfeld) and of a species-specific recombinant follicle-stimulating hormone (rFsh) produced in the yeast *Pichia pastoris* to stimulate gonads recrudescence directly. This hormonal treatment, also applied to males, synchronized breeders development with the obtention of spermiating males and fully mature females suitable for the spawning induction trials [166]. On the other hand, the use of 17α-methyltestosterone (MT) administered via ethylene-vinyl acetate copolymer (EVAc) slow-release implants is a methodology that has also been previously used in flathead grey mullet males to increase the number of spermiating males [6,173]. The administration of rFsh and Dom, in females, and also MT implants in males is an established protocol successfully used in the DIVERSIFY project in the Israel Oceanographic Limnological Research Institute (IOLR) (Eilat, Israel) to obtain mature flathead grey mullet.

In the present study, therefore, as a first step to control reproduction in flathead grey mullet breeders held in intensive culture conditions, the rationale behind the proven successful protocol that induces gametogenesis to the obtention of spermiating males and mature females was followed. Both males and females received a single injection of

Mugil cephalus-specific rFsh produced in the yeast *Pichia pastoris* together with a dopamine antagonist, i.e., Metoclopramide (MET), with the aim to stimulate ovaries / testis and the liberation of the pituitary blockage. In males, MT implants were administered in addition to rFsh and the dopamine antagonist.

2. Material and Methods

2.1. Study animals and maintenance

Flathead grey mullet broodstock, originally caught from the Ebro Delta and from a semi-extensive pond fish farm (Finca Veta La Palma, Isla Mayor, Spain), were reared in IRTA Sant Carles de la Ràpita (Spain) for a period of over 19-21 and 3 months, respectively. All fish were intramuscularly PIT-tagged (Trovan®, ZEUS Euroinversiones S.L. Madrid, Spain) for identification once in the facilities. Fish was sexed the previous year to this experiment and at the moment of arrival in the case of those coming from the semi-extensive pond fish farm. As flathead grey mullets do not display sexual dimorphisms, if an ovarian biopsy was obtained, the fish were classified as females and fish that could not be biopsied were considered as males. One month before the hormonal induction, individuals were transferred to a 10-m^3 tank. Fish were held in a recirculating system (IRTAmar®) and were gradually acclimatized from fresh water to sea water at 36 ‰ as flathead grey mullet spawns in seawater [6,247]. Temperature was controlled and maintained at 24ºC and photoperiod was ambient (from 14L:10D to 11L:13D; light:dark). 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 (Sariego Intermares, Spain) and polychaetes (TOPSY Bait, Netherlands). During the year prior to the experiment, all fish had the same feeding regimens and were held in natural conditions of photoperiod and temperature.

2.2. Hormonal treatment

Flathead grey mullet females and males were randomly selected from the broodstock for the experiment. Sixteen females (mean ± SD body weight 0.9 ± 0.1 kg; standard length 38.5 ± 3.1 cm) were checked at the beginning of the experiment to evaluate the initial maturity status of females in the broodstock. The maturity status of these females was evaluated through ovarian biopsies. The diameter of the 20 largest and most advanced oocytes were recorded *in situ* through a miscroscope (Zeuss Microscopes) and samples were kept for histology procedures. Fourteen different females from the same broodstock were used for the experiment; nine females (1.1 ± 0.4 kg; 41.3 ± 5.5 cm) received the hormonal treatment and five females (0.90 ± 0.4 kg; 39.7 ± 6.1 cm) were set as controls. These females used in the present experiment were not canulated in order to reduce the handling stress. The maturity status of eight males (0.7 ± 0.1 kg; 36 ± 3.5 cm) from the broodstock was examined. Maturation was evaluated by the release of sperm upon application of gentle abdominal pressure and spermiation stage was determined on a scale from 0 to 3 (0 = not fluent, 1 = fluent but little sample can be obtained, 2 = fluent, 3 = very fluent). Eight different males from the same broodstock were used in the experiment; six males (0.6 ± 0.2 kg; 33.3 ± 4.3 cm) received the hormonal treatment and two males (0.5 ± 0.2 kg; 33.3 ± 2.5 cm) were set as controls. However, as with the females, males used in the experiment were not stripped at the beginning of the treatment in order to reduce the handling stress. Treated individuals were stocked together while fish checked for maturity were used in a different experiment (Experiment 2, CHAPTER V).

The experimental period was from the end of July to the beginning of October in order to coincide with the reported natural spawning season of this species in the Mediterranean area [272]. The hormonally-treated females and males were intramuscularly injected with 15 mg kg^{-1} of Metoclopramide (MET) (Sigma, Spain) and 5 µg kg^{-1} of rFsh, produced in *Pichia pastoris* expression system provided by the IOLR (Eilat, Israel). The control fish were injected with 0.5 mL of saline solution. Two weeks later, males were injected with MT (Sigma) loaded on EVAc slow-release implants at 5 mg $implant^{-1}$ prepared at the Hellenic Center for Marine Research, Institute of Aquaculture (Iraklion, Crete, Greece). Males < 1 kg received one implant of 5 mg MT and males ≥ 1 kg-weight

received two implants. Final MT doses ranged from 6.7 to 11.6 mg kg^{-1}. A large bore syringe as used for the implants was used to inject saline in control males. Four weeks later, males received another MT administration following the same procedure. At the end of the experiment, coinciding with the beginning of October (ten weeks from the start of the experiment), the maturity status of all hormonally-treated and control males and females were revised and determined as previously described (**Fig 1**). For manipulation procedures, 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.

The broodstock was handled in agreement 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; Spanish Royal 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 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 animals for the experimentation or other scientific purposes).

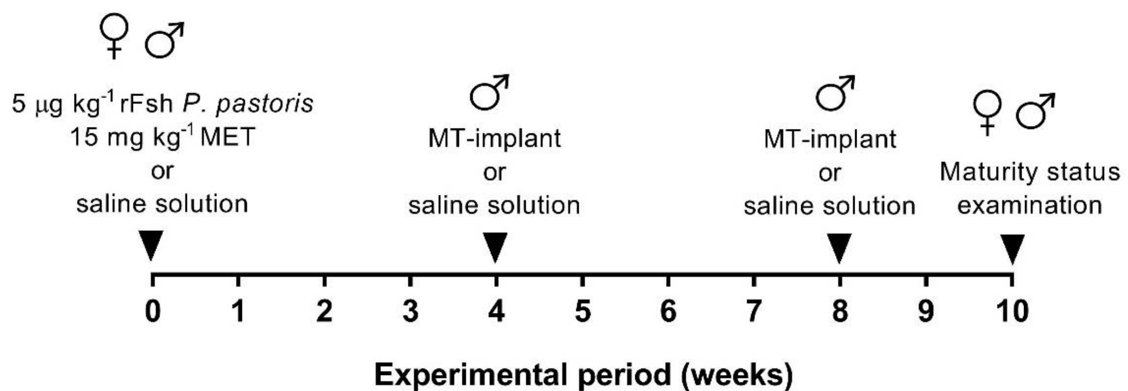

Figure 1. Schematic representation of the hormonal treatment applied to males (n = 6) and females (n = 9). Control individuals (n = 2 males, n = 5 females) received 0.5 mL of saline each time the hormonally-treated group received any treatment.

2.3. Histological observations and classification of maturity status

Ovarian biopsy samples were kept in Bouin's fluid for 24 h, dehydrated through an ethanol and xylene series and embedded in paraffin. Histological sections (3 µm) were

stained with hematoxylin and eosin (Casa Álvarez, Spain). Oocytes sections were observed under a light microscope (Leica DMLB, Houston, USA) and were classified as previtellogenic oocytes, which includes primary growth oocytes and cortical alveoli oocytes.

2.4. Sperm collection and evaluation

Milt samples were collected with a 1 mL-syringe avoiding contamination by faeces, urine and/or sea water. Approximately 1 µL of milt was placed on a slide and mixed with 0.2 mL of sea water for activation. The percentage of motile spermatozoa (motility) and duration of motility were assessed by the observation of the activated spermatozoa through a microscope at 100x magnification (Zeuss Microscopes) in triplicate. The percentage of motility was classified into different scores: 0 for no motile spermatozoa (spz), 1 for > 0 – 25 % of spz with progressive movement, 2 for > 25 % - 50 %, 3 for > 50 – 75 % and 4 for > 75 % of spz with progressive movement [155].

2.5. Statistical analysis

The normality of data distribution was checked by Shapiro-Wilk test. As normality test failed, differences in oocyte diameter were examined by Kruskal-Wallis One Way Analysis of Variance on Ranks. Data are presented as mean ± SD.

3. Results

The revision of the initial maturity status of the female breeders at the beginning of the experiment showed that females in the broodstock were in previtellogenesis (**Fig 2A**). The revision of the final maturity status in both hormonally-treated and control females at the end of the experimental period also showed that these females were at previtellogenesis (**Fig 2B**, **2C**). No significant differences were found in oocyte diameter between the hormonally-treated (94 ± 10 µm) and control (93 ± 5 µm) females at the end of the experiment, nor with the oocyte diameter of females revised at the beginning of the experiment (106 ± 54 µm) (P = 0.852).

In the case of males, 100 % of the males that were checked at the beginning of the treatment did not present milt upon application of abdominal pressure (**Fig 3**). Neither did the control males by the end of the experiment. Four out of the six males (66.6 %) that were hormonally treated did produce milt. Two of the males had a spermiation stage of 1 (a viscous drop of sperm of 10 - 20 μL) and two had stage 2, with 100 and 200 μL of fluid sperm. The motility score of all sperm samples was 4 (> 75 % of sperm with movement) and motility duration was 102 ± 24 s.

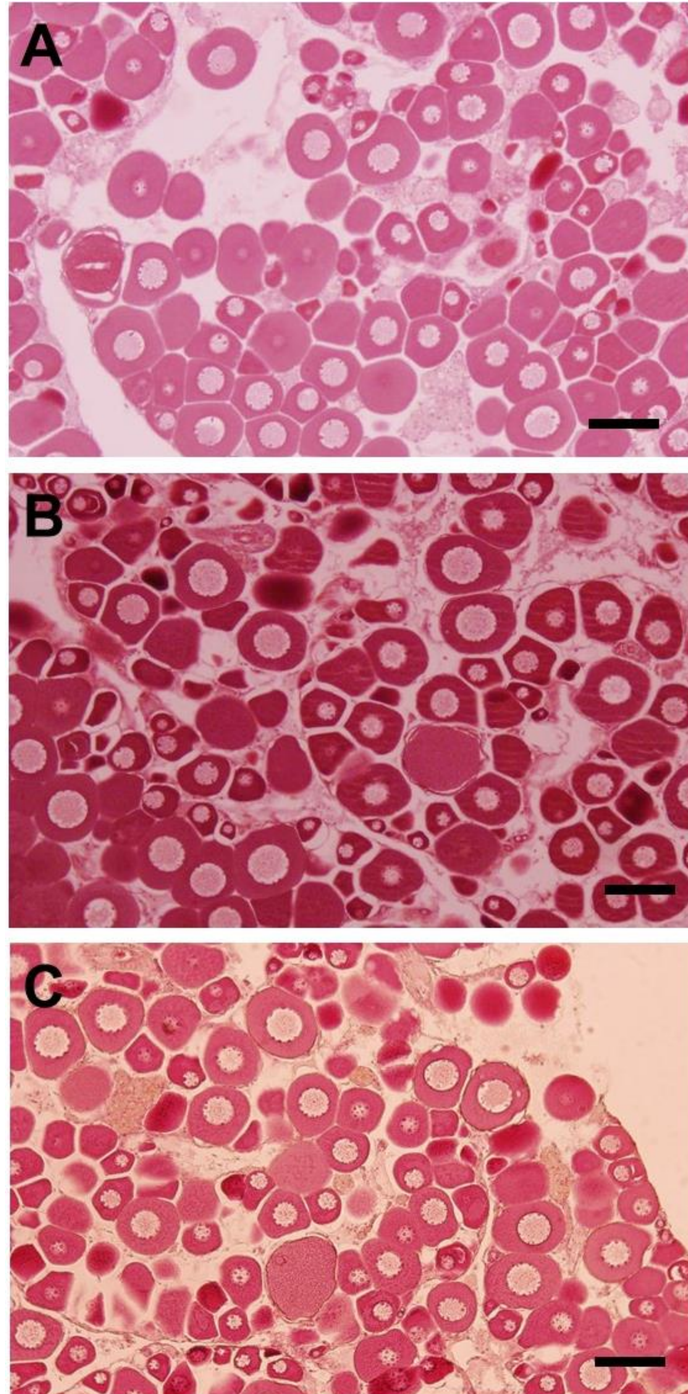

Figure 2. Histological sections of ovarian samples in flathead grey mullet (*Mugil cephalus*). Samples from (A) females from the same broodstock as the females used in the experiment revised at the beginning of the experiment, (B) hormonally-treated females at the end of the experimental period and (C) control females at the end of the experimental period. Scale bar: 100 µm.

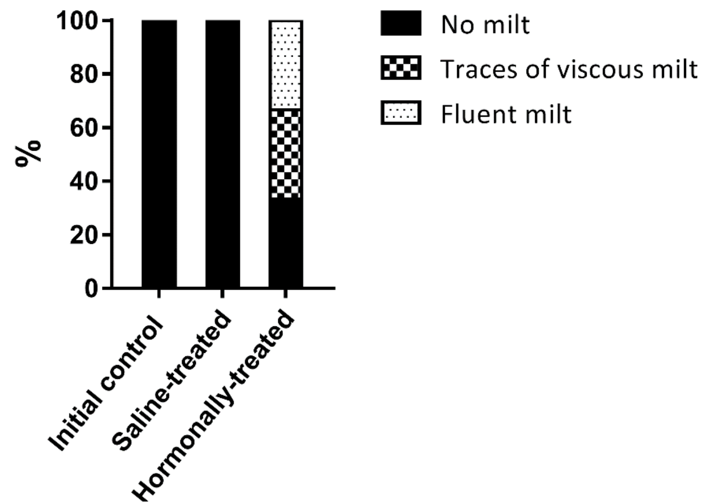

Figure 3. Percentage of males in each spermiation stage at the beginning of the experiment and at the end of the experiment; after treatment with rFsh produced in *Pichia pastoris*, metoclopramide and 17α-methyltestosterone (hormonally-treated) or with saline solution (saline-treated).

4. Discussion

The administration of 5 μg kg^{-1} of species-specific rFsh produced in *Pichia pastoris* and 15 mg kg^{-1} of MET did not lead to the development of vitellogenesis of wild flathead grey mullet females that have been in captivity for 3 to 21 months. At the end of the experiment, the fish were in previtellogenic stages of development. Samples from females in the broodstock taken at the beginning of the experiment indicated that all females in the broodstock were previtellogenic at the start of the experiment and that treated females remained previtellogenic at the end of the experiment. The results of the present study are contrary to previous results with the use of a dopamine antagonist [6] or in addition with rFsh [166] in flathead grey mullets in which fish were described to mature and were successfully induced to spawn. However, the difference in results with these studies can perhaps be explained by differences in the stock of mullets and the holding conditions, both before and during the experiments. For instance, the stock in Aizen *et al*. (2005) was formed with bigger individuals; 5- to 8-year-old females ≥ 2 kg of weight and 3- to 5- year-old males of around 1 kg that were held at 40 ppt. Actually, Aizen *et al*. (2005) described that a proportion of these females (< 20 %) were observed to mature without the need of any hormonal treatment while in the present study no control fish matured. It has been reported that at least two to three years of acclimatization are

needed for flathead grey mullet females [16] and other mullet species [50,175] to develop mature oocytes and respond to spawning induction treatments. Therefore, it is possible that some differences exist between studies in the time that breeders have been in captivity before the application of treatments. If a process of domestication favors maturational development in this species, different hormonal treatments are required for wild adult fish or from semi-extensive cultures brought into an intensive system for less than at least two years.

In the case of males, the treatment with rFsh, MET and MT administered via EVAc slow-release implants induced the production of milt in the 66.6 % of males. Although good quality of sperm was obtained (≥ 75% of motility), low quantities of viscous sperm were collected from some males. In contrast, Aizen *et al*. (2005) reported that 100 % of treated males presented sperm 6 weeks after MT-treatment (coinciding with the maturity revision in the present study). In this regard, there is no evidence that the priming treatments of rFsh and MET had any added effect on the increase of spermiating males at this point of revision. Aizen *et al*. 2005 also noted the presence of a few males with small amount of milt at this revision and it was not until the next checking (8 weeks after MT treatment) that all males had white fluent milt. It might be possible that a further sampling of males in the present study could have led to the detection of more spermiating males. On the other hand, it is also possible that the factor of domestication may have had an effect on the responsiveness of males to the treatment. In fact, Aizen *et al*. (2005) reported to observe traces of viscous milt in control males along the experimental period whereas control males in the present study did not present milt upon examination.

In conclusion, under the described conditions, the application of the protocols based on the single administration of rFsh with a dopamine antagonist (and MT in males) did not stimulate oogenesis and stimulated a low production of sperm. Therefore, alternative therapies are needed to stimulate vitellogenesis and increase the number of running males with fluent milt in order to achieve the control of reproduction in broodstocks formed by wild breeders or from semi-extensive cultures that have been held in intensive conditions for a short period of time, i.e., for less than 21 months.

CHAPTER V

Providing recombinant gonadotropin-based therapies that induce oogenesis from previtellogenic oocytes to produce viable larvae in the flathead grey mullet

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1. Introduction

The flathead grey mullet is a gonochoristic species and generally matures at the age of three years. In the Mediterranean, the spawning period has been observed from June to October when breeders spawn externally fertilized pelagic eggs in the sea [272]. Females appear to spawn one set of ova a year [216]. 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 [34,281] or the induction of oocyte maturation and spawning of wild breeders captured at advanced stages of development [1,49,55,116,260]. However, the use of wild caught mature fish is unsustainable as it relies on fisheries that are in decline [83] and offers no possibility to close the life cycle in captivity and make genetic improvements of cultured stocks. Flathead grey mullet held in aquaculture facilities present two types of reproductive dysfunction: arrest in late or early stages of gametogenesis. Arrest in late stages of gametogenesis (maturation and ovulation) has been observed in recently caught wild flathead grey mullet or wild flathead grey mullet that were acclimated to ponds or large tanks [56,137,281]. This is the most commonly observed dysfunction in fish and can be controlled by hormonally inducing spawning [155,289] as has

been achieved for flathead grey mullet with therapies that combine different substances such as: carp pituitary homogenates, steroids, human Chorionic Gonadotropin (hCG), gonadotropin releasing hormone synthetic analogues (GnRHa) and drugs that inhibit dopamine [83]. In comparison, in wild and hatchery-reared fish held in intensive culture conditions in the Mediterranean region, a more severe reproductive dysfunction has been observed where development was arrested in the early stages of gametogenesis. The artificial propagation of these fish in intensive culture systems would be a sustainable solution for a consistent supply of juveniles [281]. In these intensive conditions, 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 [6]. Males failed to initiate spermiation [173,279] (present study) or produced highly viscous milt that could not fertilize the eggs [235]. These reproductive dysfunctions may be related to alterations in the endocrine control in the brain-pituitary-gonadal (BPG) axis.

In vertebrates, the pituitary gonadotropins (Gths), the follicle-stimulating hormone (Fsh) 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) [152,155]. 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 [6] and males [79]. 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 [6,173]. In females, treatment with GnRH agonist (GnRHa) in combination with a DA antagonist [6] or a single injection of recombinant Fsh produced in the yeast *Pichia pastoris* [166] 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 [277], 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 [119,287], the yeast *Pichia pastoris* [4,41,115,118,201,225], baculovirus silkworm larvae [47,79,127,129,130,167], HEK293 cells [120] and Chinese Hamster Ovary (CHO) cells [38,42,75,172,207,240]. The application of rGths based therapies has shown promise to control gametogenesis in different teleost [37,38,75,114,207] 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 as hCG and GnRHa with a DA agonist, or (b) *Mugil cephalus* rLh.

2. Material and methods

2.1. Study animals and maintenance

Flathead grey mullets 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 [272], 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 freshwater. 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.

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; Spanish Royal 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 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 animals for the experimentation or other scientific purposes).

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

The pituitary gland was removed from one sacrificed female, frozen in liquid nitrogen, and stored at -80ºC. Total RNA was purified using the GenElute^{TM} mammalian total RNA miniprep 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 3' RACE kit (Invitrogen). Polymerase chain reaction (PCR) was carried out as indicated in the 3' RACE kit using partially degenerated forward primers for the Fshβ or α subunits, the common abridged universal amplification primer (AUAP) as reverse primer, and the EasyA^{TM} high-fidelity PCR cloning enzyme (Agilent Technologies,

Santa 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 for *Epinephelus coioides* (AY186242), *Oreochromis niloticus* (AY294015), *Dicentrarchus labrax* (AF543314), *Acanthopagrus schlegelii* (AY921613), *Maylandia zebra* (XM_004558042), *Fundulus heteroclitus* (M87014), *Oryzias latipes* (AB541981), *Sparus aurata* (AF300425), *Amphiprion melanopus* (EU908056), *Chrysiptera parasema* (KM509061), and *Kryptolebias marmoratus* (EU867505). For Fshβ, the forward primer was 5′-ATGCAGCTGGTTGTCATGGYAGC-3′, whereas for the α subunit the primer was 5′-ATGGGCTCMNTGAAAYCHVCTG-3. The Lhβ 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 or Lhβ 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. 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 -80 ºC 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 and determine the minimum effective dose and optimal dosing schedule, intramuscular injections of 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. 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 [216]. The fish in the treatment group were administered rFsh (Stage 1 of Exp 1) followed by either hCG alone [55,281] or GnRH combined with DA antagonist [6] (Stage 2 of Exp 1).

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, Annex I**). Dosage of hCG were in the range of previous studies on flathead grey mullet maturation [55,281] and other fish species [155]. Weekly samples of oocytes and blood (0.40 mL) were obtained.

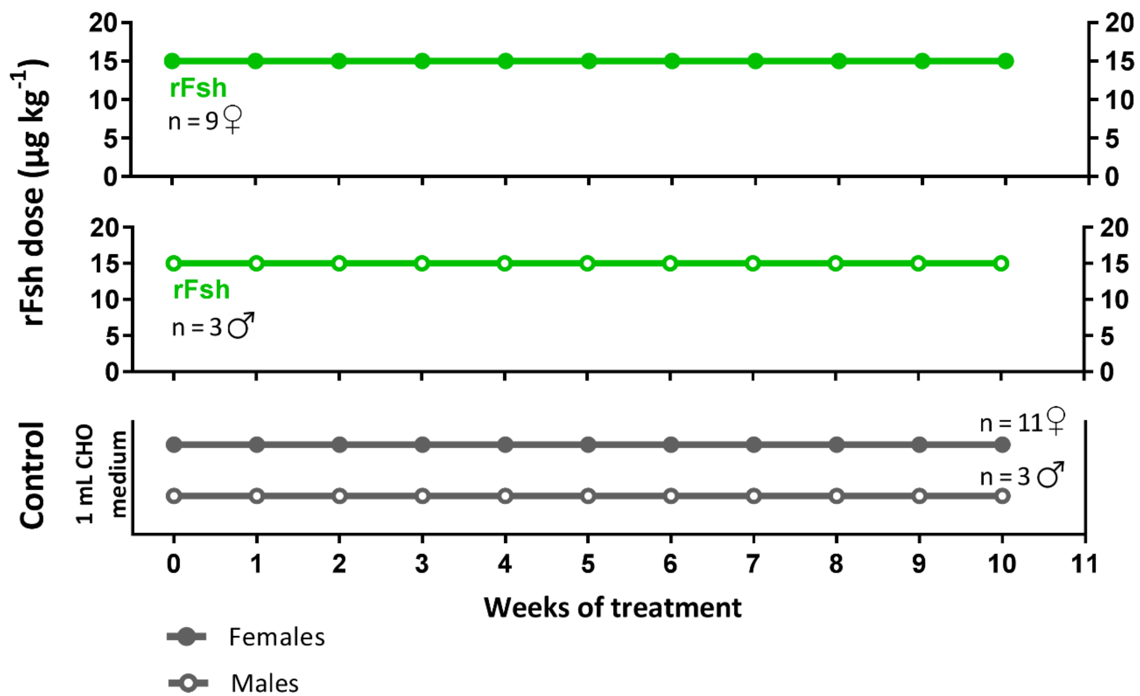

Figure 1. Schematic representation of the protocol administered to flathead grey mullet (*Mugil cephalus*) in Stage 1 in Experiment 1. Flathead grey mullet females (n = 9) and males (n = 3), received weekly doses of intramuscular injections of rFsh during 11 weeks. Control individuals (n = 11 females, 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 (Annex I).

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. 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). The aim of the administration pattern in this experiment was to simulate natural increases and decreases of gonadotropins in the bloodstream of individuals according to their suggested regulatory role in gamete development [143]. Initial administration of rFsh followed by a gradual increase of rLh as gametogenesis progresses and subsequent decline of rFsh.

2.5.1. Females

Initially, all nine females received increasing doses of rFsh, 6 µg kg^{-1} (week 0) and 9 $µg^{-1}$ kg (week 1) before the dose was fixed at 12 µg kg^{-1} rFsh per week. A maximum 12 µg kg^{-1} dose was selected for long-term treatment based on Experiment 1 and the *in vivo* dose-response study. From the 4th week onwards, females (n = 8) also received a weekly administration of rLh at increasing doses (2.5, 4, 6 µg kg^{-1}). When 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}) (**Fig 2** and **Supplementary Fig 1B, Annex I**). At this point (week 8 and onwards), treatments were adjusted accordingly to oocyte diameter of each individual fish to ensure females at the same stage of vitellogenesis received the same rGth treatment. When females presented oocytes ≥ 550 µm, no more rFsh was administered and consecutive doses starting with 9 and maintaining 12 µg kg^{-1} rLh were administered every 3 days (see summary in **Table 1** and detail in **Supplementary Fig 1B, Annex I**). The aim of this increased frequency of administration was to maintain high levels of rLh in the bloodstream, based on the half-life (shorter than rFsh) described for rLh produced in CHO cells and administered to Senegalese sole [38]. Doses for rLh were assigned according to other studies on the use of rLh produced in CHO cells for vitellogenesis induction [75] or spermatogenesis [38] in other fish species. When the most developed oocytes reached a diameter ≥ 600 µm or did not show further growth, females were considered to have completed vitellogenic growth and, therefore, were ready for maturation and ovulation induction. To induce oocyte maturation, ovulation and spawning, females were administered higher doses of rLh (15 or 30 µg kg^{-1}) combined with 40 mg kg^{-1} of Progesterone (Prolutex, IBSA Group, Italy) (P_4) administered 24 h after

the rLh (**Table 1**). Three females received 15 μg kg^{-1} of rLh and five females received 30 μg kg^{-1}.

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 [55,280]. One female (female 3, 30 μg kg^{-1} rLh + 40 mg kg^{-1} P_4 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).

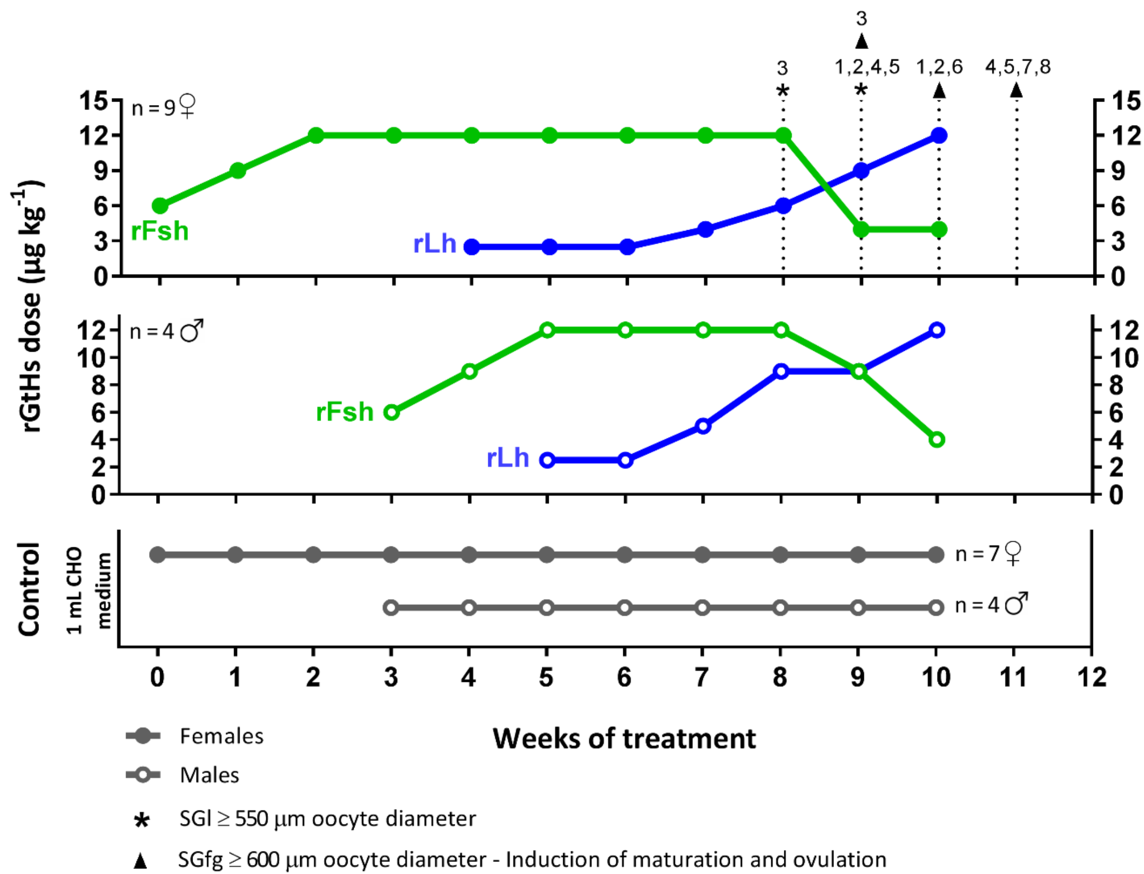

Figure 2. Schematic representation of the weekly administration to flathead grey mullet (*Mugil cephalus*) in Experiment 2. Initial increasing administration of rFsh was followed by a gradual increase of rLh as gametogenesis progressed and, after this, by a subsequent decline of rFsh in both females (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 [143]. 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 maturation, ovulation and spawning (see details in Table 1). The individuals in control groups (n = 7 females, n = 4 males) underwent the same number of intramuscular administrations as treated individuals, but with CHO culture medium (1 mL fish^{-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.

			1	2	3	4	5	6	7	8
	Fish No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	Fish Origin		W	SE	SE	W	SE	W	W	W
	Previtellogenic oocyte stage at the start of the experiment		PGpn	SGca	SGca	PGpn	SGca	PGpn	PGpn	PGpn
Induction of oogenesis	Treatment before < **550 μm** oocyte diameter		Combined rFsh and rLh treatment (see Fig. 2)							
Induction of oogenesis	Treatment at ≥ **550 μm** oocyte diameter (* in Fig. 2)		**Doses of 9 to 12 μg kg^{-1} rLh** (No rFsh applied)					**No treatment was applied as fish developed from < 550 μm to SGfg**		
Induction of oogenesis	Max. oocyte diameter before OM induction (μm)		619 ± 7	627 ± 8	625 ± 8	603 ± 10	608 ± 8	610 ± 6	598 ± 7	605 ± 4
Induction of maturation and ovulation	Full-grown secondary- growth oocytes (SGfg) (▴ in Fig. 2)	**rLh** (μg kg^{-1}) t =0	15	15	30	30	30	15	30	30
Induction of maturation and ovulation		**P_4** (mg kg^{-1}) t =24	40	40	40	40	40	40	40	40
Induction of maturation and ovulation		**PGF2α** (μg kg^{-1}) t =39	-	-	18.75	-	-	-	-	-
	Total eggs		-	-	801,913	974,928	754,774	-	891,600	-
	Fecundity (eggs kg^{-1} BW)		-	-	832,723	574,500	676,320	-	888,047	-
	Fertilization (%)		-	-	Not used	0.1	0.31	-	0.81	-

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 [37,207] (**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 fertilization

For the *in vitro* fertilization, 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 [84] 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 + P_4) were each fertilized 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 fertilizations). 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 fertilization, all eggs were checked for embryo presence and the percentage of eggs fertilized was calculated as the number of eggs with live embryos/number of eggs used for the *in vitro* fertilization. Eggs with embryonic development were transferred individually into individual wells filled with sterile seawater in a 96 well plate and incubated (24ºC). To evaluate the quality of the eggs with embryo, the hatching success was calculated as the number of hatched larvae / number of eggs with embryos 22-hours post fertilization. Larvae were checked daily until all hatched larvae had died and percentage survival on

each day was calculated as the number of live larvae on the day / total number of larvae that hatched. A subsample of ~one-third of fertilized eggs and larvae were used for taking measurements and afterwards returned to the incubation.

2.6. 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 analyzed using a commercially available enzyme immunoassay (EIA) kits (Cayman Chemical Company, USA). Steroids were extracted with methanol, which was evaporated and extracts were re-suspended 1:10 in the EIA buffer.

2.7. Histological observations and classification of developing ovaries

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 hematoxylin and eosin (Casa Álvarez, Spain). To examine ovarian development, oocytes sections were observed under a light microscope (Leica DMLB, Houston, USA). 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)* characterized 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, *late-secondary growth (SGl)* corresponding to mid- to late- vitellogenesis when oocytes reached ≥ 400 µm [86]; *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 [152]. Atresia was identified by the hypertrophy of granulosa cells, the loss of the individuality of yolk globules and the dissolution of their content [257].

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). The assessment of the milt quality was estimated by the percentage of motile spermatozoa 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 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 % of sperm with progressive movement, 3 for > 50 – 75 % and 4 for > 75 % of sperm with progressive movement [155]. For those samples in Exp 2 with a motility score of 4 and manageable sperm volumes (≥ 100 µL) (n = 10), sperm quality was also evaluated using a CASA system [274]. For this, 0.5 µL of diluted sperm (1/4 in Marine Freeze®) were dropped on the center 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 (Integrated Sperm Analysis System, Spain). The tracks of the activated spermatozoa were recorded through a bright field equipped video microscope at 200x magnification (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 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 were selected by adjusting the grayscale threshold through Image J software

(https://imagej.nih.gov/ij/). The following sperm quality parameters were determined: (1) 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: path linearity of actual sperm track, LIN = VSL/VCL x 100), path wobble (deviation from average path, WOB = VAP/VCL x 100), and path straightness (linearity of the average path, STR = VSL/VAP x 100). All 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.

2.9. 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 Exp 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.

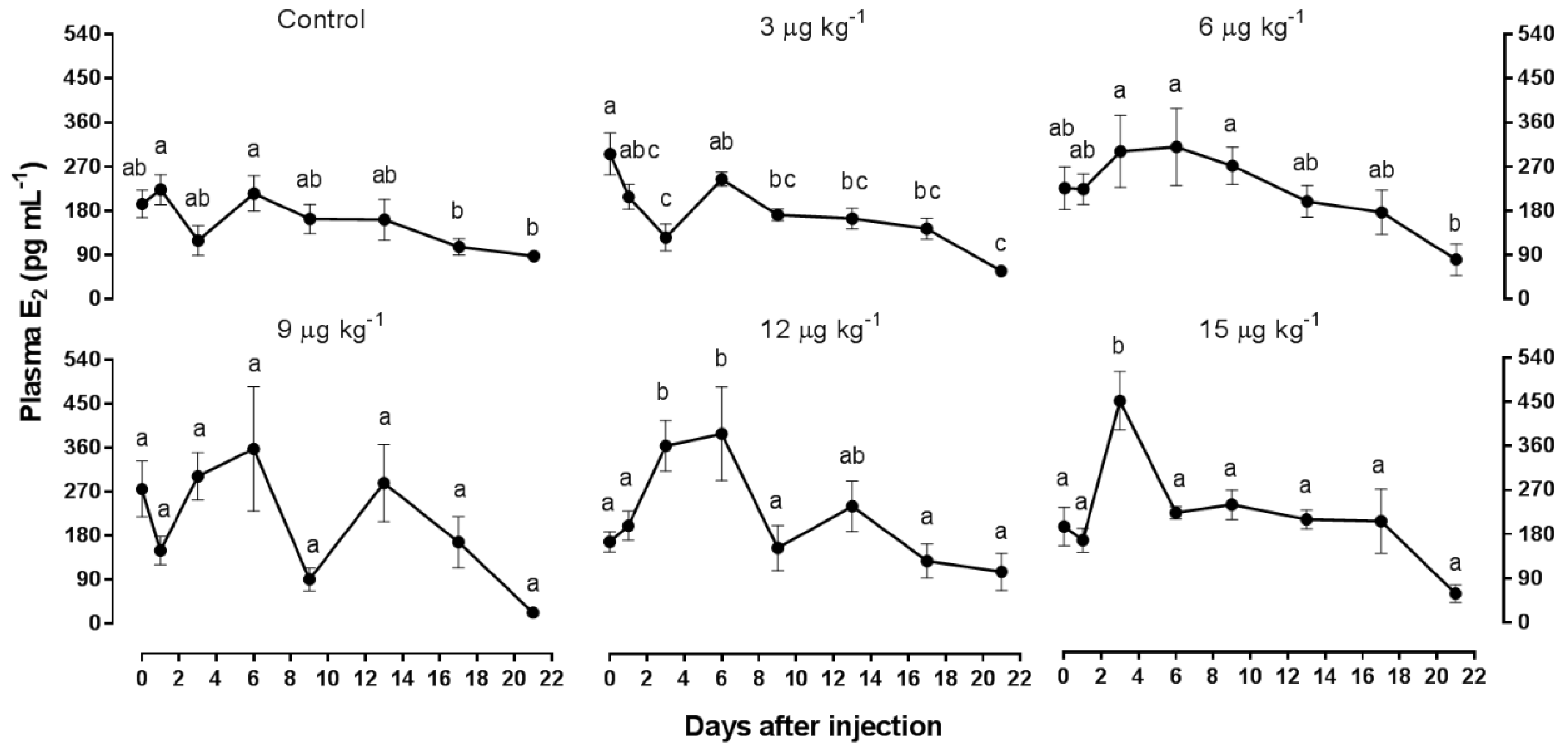

Figure 3. Mean (± SEM) plasma E_2 levels of female flathead grey mullet (*Mugil cephalus*) before (day 0) and after (day 1, 3, 6, 9, 13, 15 and 21 days) the rFsh injection. Females (n = 5/group) received a single injection of rFsh at doses 3, 6, 9, 12 or 15 µg kg^{-1} and an injection of 1 mL $fish^{-1}$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

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), SGl 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 (**Fig 5A**, **6A**).

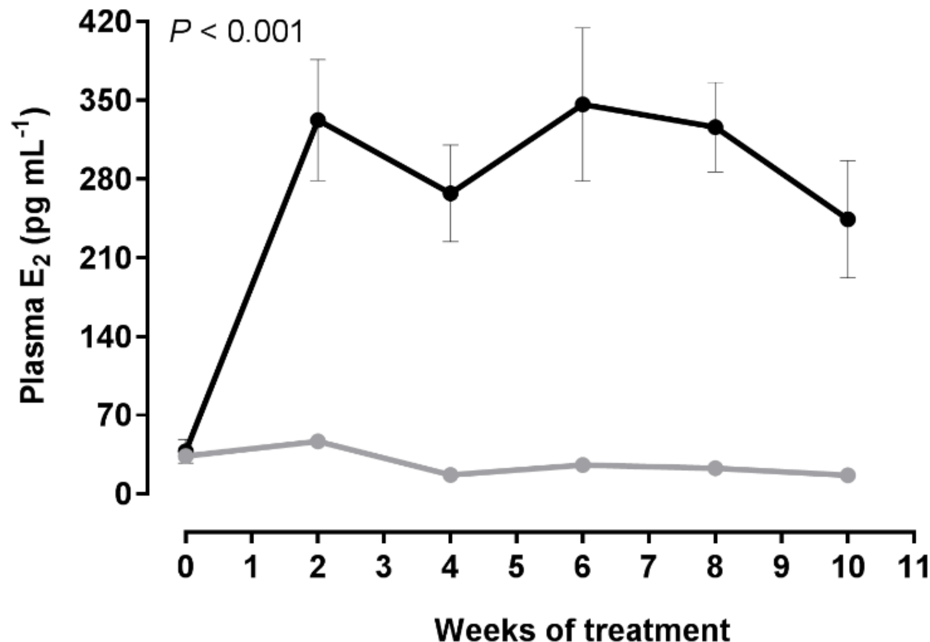

Figure 4. Mean (± SEM) plasma E_2 levels of rFsh-treated and control flathead grey mullet (*Mugil cephalus*) females (n = 9-11) in Experiment 1. Treated females received weekly injections of rFsh (15 µg kg^{-1}) and control females of CHO conditioned culture medium (1 mL $fish^{-1}$). There were significant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

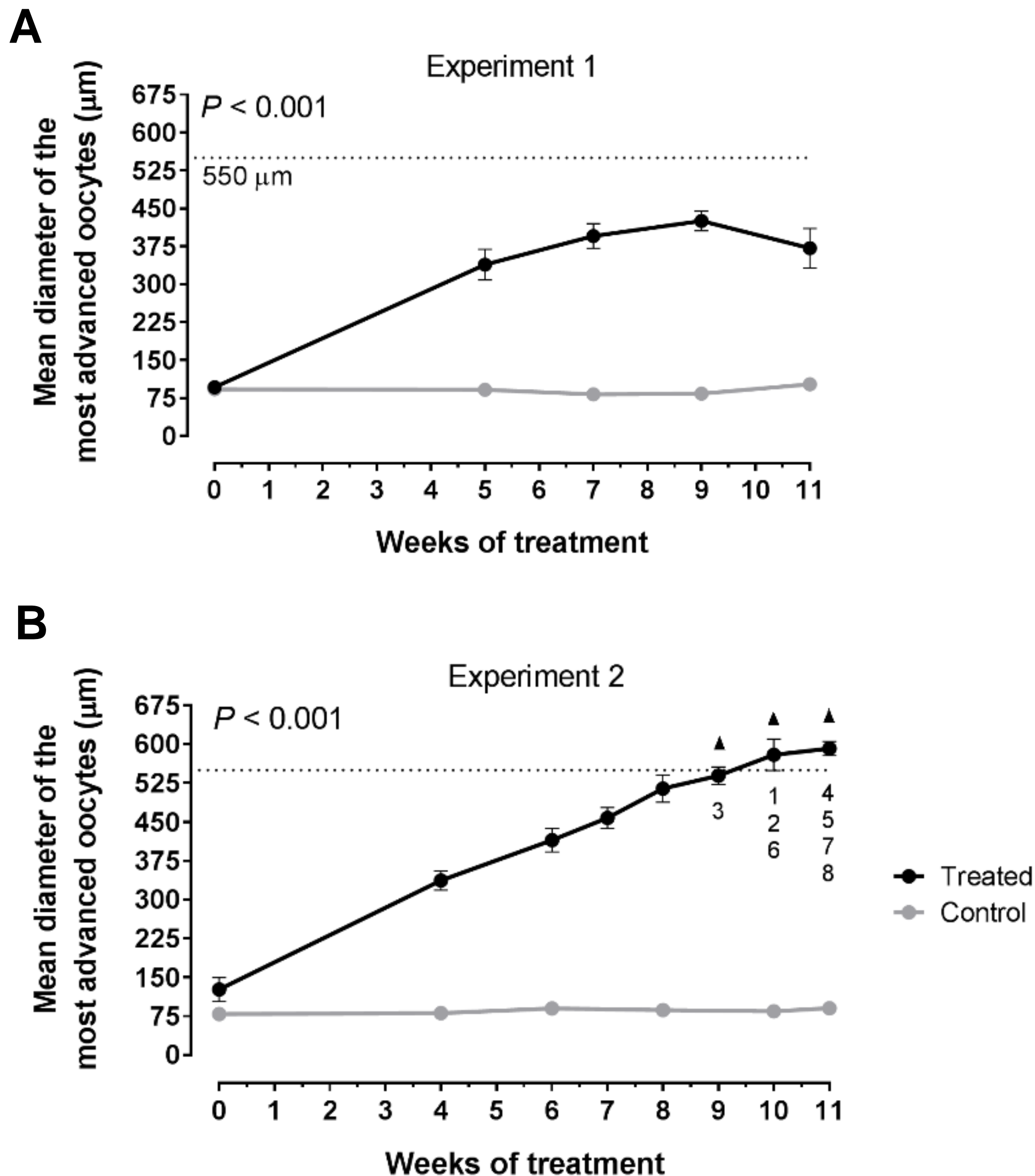

Figure 5. Mean (± SEM) oocyte diameter of the most developed oocytes in wet mounts from rFsh treated and control flathead grey mullet (*Mugil cephalus*) females. (A) Experiment 1, females treated (n = 9) with a weekly 15 µg kg^{-1} rFsh administration or CHO conditioned culture medium (control, n = 11) during 11 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 females checked twice in the same week were the mean of both revisions. Triangles show the moment when numbered females (see Fig 2 and Table 1) were selected for maturation and ovulation induction. There were significant 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.

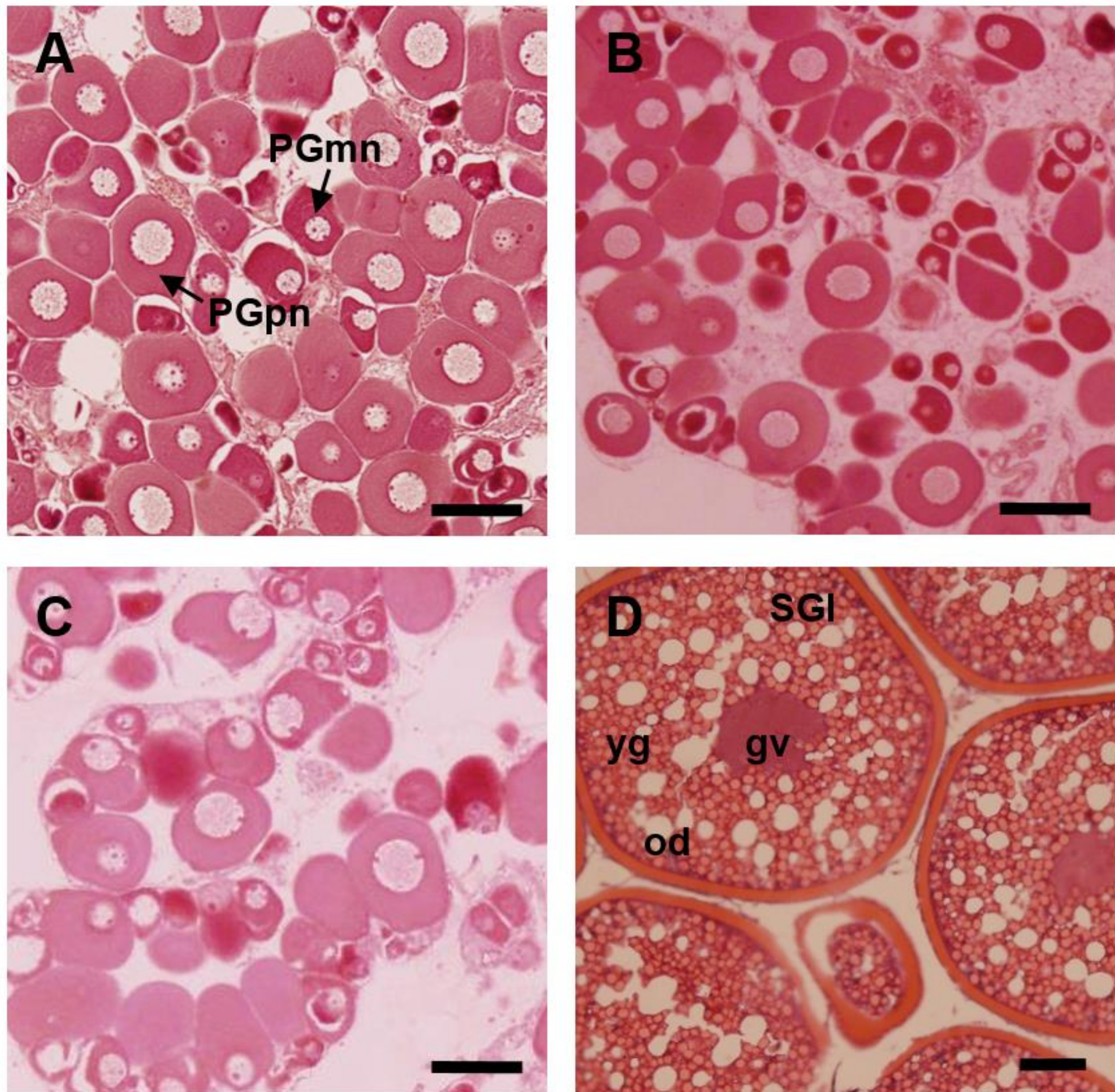

Figure 6. Effects of long-term treatment of rFsh on ovarian development in previtellogenic flathead grey mullet (*Mugil cephalus*) *in vivo*. Histological sections stained with hematoxylin and eosin show oocytes samples from (A) initial control fish, (B) rFsh-treated fish before treatment, (C) control 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.

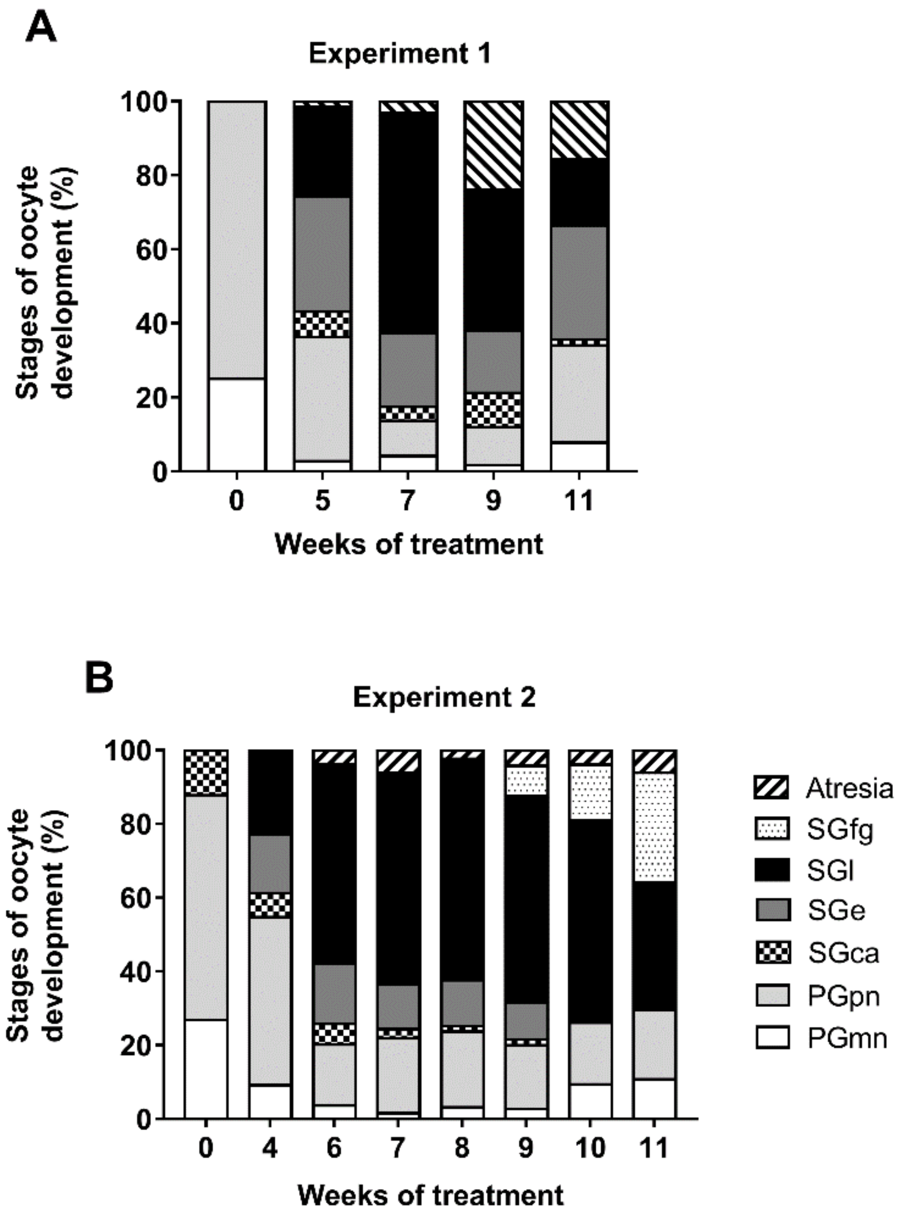

Figure 7. Temporal weekly evolution of percentage frequency of oocyte developmental stages observed in rGth-treated flathead grey mullet females (*Mugil cephalus*). (A) Experiment 1 with weekly 15 µg kg^{-1} rFsh administration to the treated group (n = 9) during 11 weeks. (B) Experiment 2 with the administration 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.

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, Annex I**) 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 P_4 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 P_4 (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 3**). 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 + P_4 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.

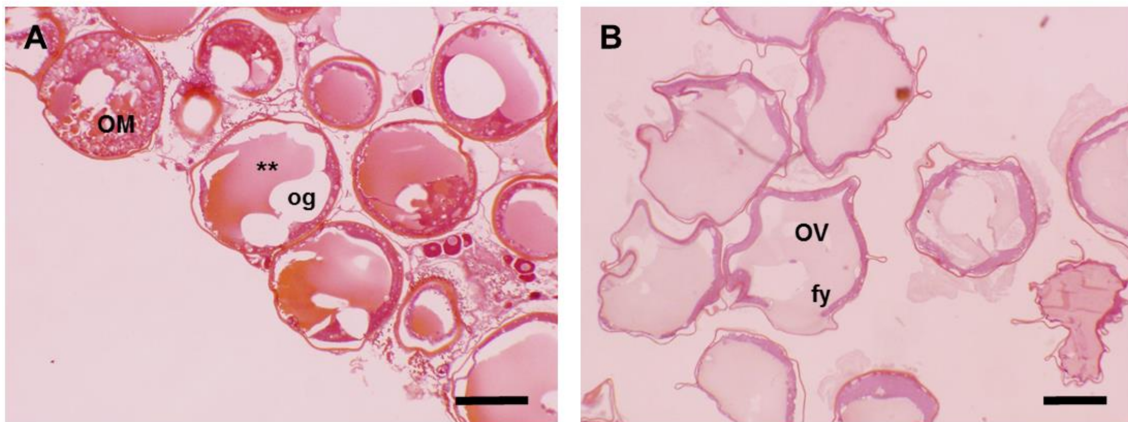

Figure 8. Oocyte maturation and hydration stages for treated flathead grey mullet females (*Mugil cephalus*) in Experiment 2. (A) Ovulated eggs from female 3 at 40 hours after 30 µg kg^{-1} of rLh injection (16 hours from 40 mg kg^{-1} progesterone) and 1 hour from 18.75 µg kg^{-1} PGF2α injection. Oocytes 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.

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.

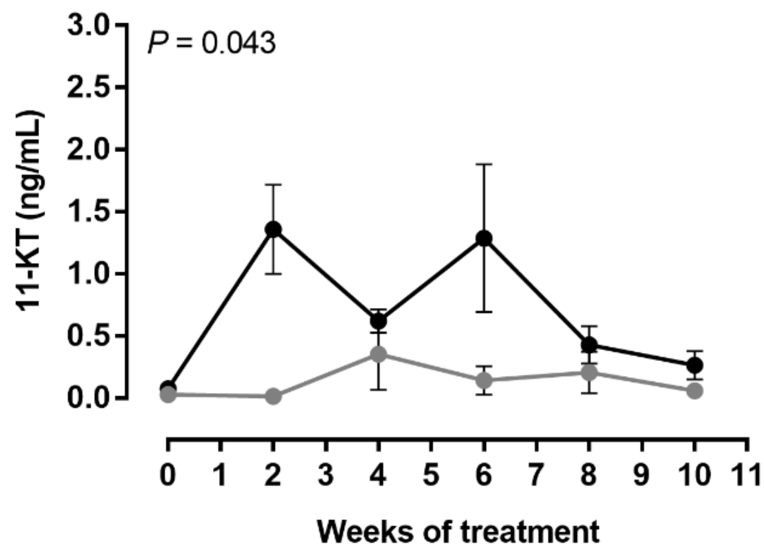

Figure 9. Mean (± SEM) plasma 11-KT levels of rFsh-treated (15 µg kg^{-1}) flathead grey mullets males and controls (n = 3-4). Treated males received weekly injections of rFsh (15 µg kg^{-1}) and control males of CHO conditioned culture medium (1 mL $fish^{-1}$). There are significant differences among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

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

Assessment by CASA of the 10 samples collected from all four males with high motility 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 %, WOB of 93.5 ± 0.1 % and STR of 97.9 ± 0.7 %.

3.5. *In vitro* fertilisation

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 ± 0.8 10^9 spz mL^{-1}). The mean sperm to egg ratio at fertilisation was 189,521 ± 23,541 spermatozoa egg^{-1}. After an incubation period of 22 - 23 hours (24ºC), mean embryo percentage survival 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 10A**). 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).

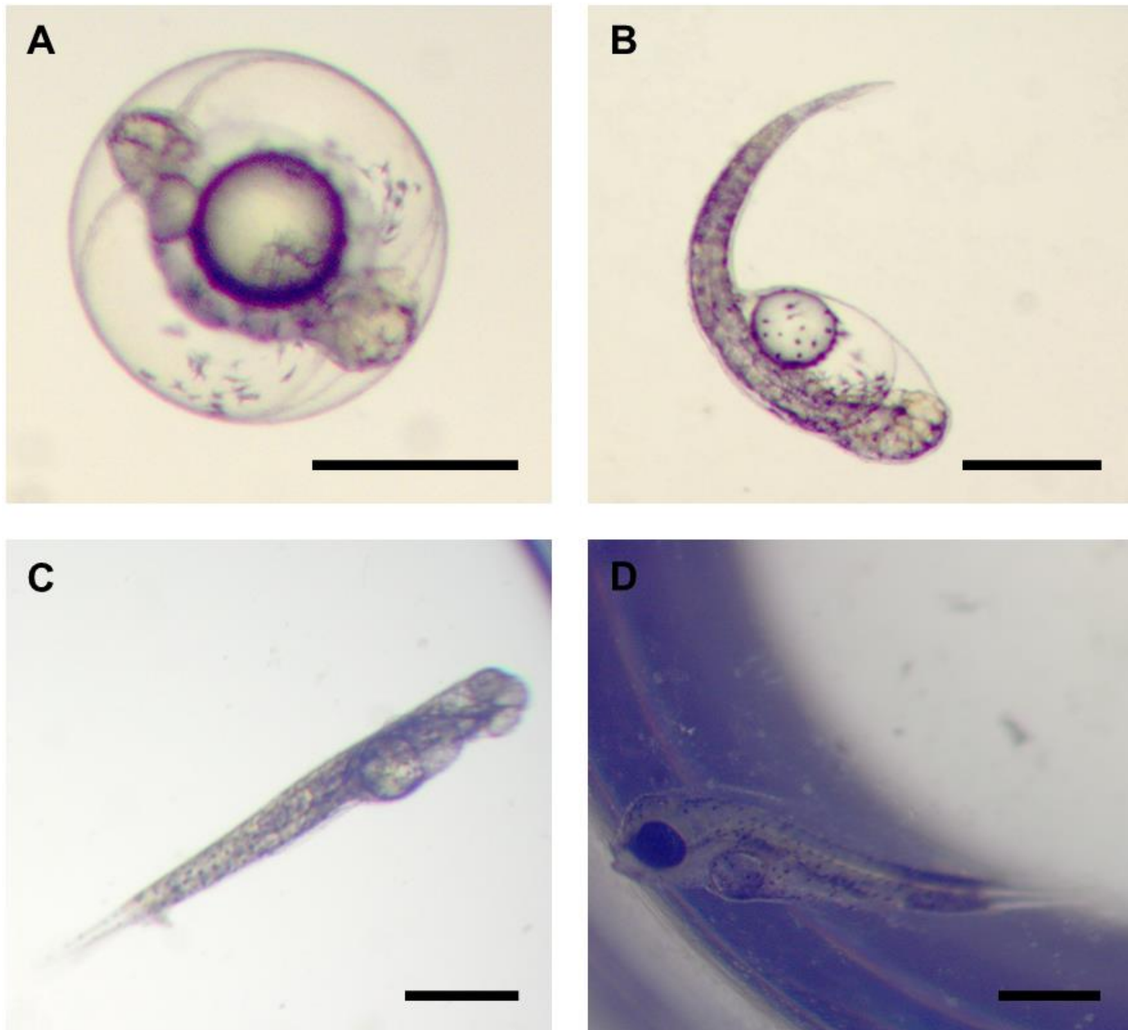

Figure 10. Developing *Mugil cephalus* embryos and larvae from Experiment 2. (A) Embryo at age 22 h post-fertilisation with head region formed and dark pigments covering almost all the embryo and on the oil globule. (B) Hatching at age of 40 hours post-fertilisation. (C) Larva after 1 dph. A decrease 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.

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 [6] that threatens the sustainability of its culture making it mostly dependent on wild captures [281]. 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 [272]. The present study, encountered a more severe reproductive dysfunction than has been observed in other studies [6]. The severity of the reproductive dysfunction, highlights that in the present study, the long-term 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 [152]. 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 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 inductions.

The present study also provides evidence that Fsh is the major hormone to initiate vitellogenesis in teleosts. To date, no study has demonstrated that the exogenous application of just Fsh promotes the initiation of vitellogenesis and development through 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 is accepted [152,155] based on parallels drawn with other taxa, the synchronised increase in plasma Fsh and oocyte development found in many fish species, genomic approaches such as gene knockout to define Gths pathways [284] and that rFsh induced partial development of vitellogenesis [119,190,201,225]. However, some criticisms can be made as, many differences in the control in reproduction exist between taxa, synchronised increases in Fsh and oocyte development do not necessarily indicate cause – effect, vitellogenesis although delayed proceeded when the Fshb gene was knocked out to make Fsh-deficient zebrafish (*Danio rerio*) [284] and previous studies did not induce the entire process of vitellogenesis [119,190,201,225]. Therefore, the present study has added clear evidence to demonstrate the accepted function of Fsh by reporting in a teleost species that rFsh successfully induced the process of vitellogenesis from previtellogenic stages to advanced stages from which fertilised eggs and larvae were obtained.

The biological activity of rFsh applied to females of other fish species has been previously studied, but most studies have focused on *in vitro* approaches for receptor-binding capacity [240] and steroidogenic potency [118,167,287] or *in vivo* short-term effects [119,127–129,172] rather than *in vivo* long-term effects on gonadal development. When rFsh produced in other heterologous systems 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 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 yellowtail kingfish oocytes developed to cortical alveoli stage [225]. Weekly injections for 8 weeks at 100 µg kg^{-1} to juvenile grouper (*Epinephelus fuscoguttatus*) also induced development to the cortical alveoli stage [201]. Recombinant Fsh produced in *Drosophila* S2 cell line (100 µg kg^{-1}) induced early vitellogenesis in the Japanese eel after 56 days of treatment with a weekly administration [119] and rFsh (500 µg kg^{-1}) produced in HEK293 cells induced initial oil droplet stage in previtellogenic yellow shortfinned eels (*Anguilla australis*) after three weeks [190]. These comparisons between the present study and other studies suggest a higher biological potency of rFsh produced in CHO cell lines as previously reported in some species [172].

Nevertheless, the administration of only rFsh in Exp 1 failed to complete oocyte growth as although oocytes developed until mid to late secondary growth, the cells appeared to be arrested in this stage and subsequently, a substantial number of atretic cells were found in the later weeks (weeks 9 - 11). These results agree with previously described E_2 roles that did not induce OM [152], but differ from those obtained by *Das et al*. (2014) who induced OM in *Mugil cephalus* post-vitellogenic oocytes that were incubated *in vitro* with E_2. The fact that completion of oocyte growth could not be achieved using only rFsh suggested that, as previously described, OM and ovulation are Lh-dependent [152,184]. 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 [152]. 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, which have been successful in

a wide range of species that were arrested at a later developmental stage close to OM [154,155]. Other studies on *Mugil cephalus* have recommended an oocyte diameter > 550 µm before OM and ovulation induction [6,55,260]. However, a wide range of other possible contributing factors can be cited, such as Lh pituitary 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 [39,240]. However, considering that the hormone treatments used in Stage 2 of Exp 1 were applied to few fish, no conclusion can be drawn other than oocyte development was arrested with the application of only rFsh and no further development was observed.

In contrast, in Experiment 2, the co-administration of rLh with rFsh at advanced stages of vitellogenesis induced the completion of oocyte growth to a mean size of 609 ± 5 µm in eight (89%) of the nine females treated. Experiment 2 compared to Exp 1, appeared to show that the addition of rLh was required 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 progress [246,280]. The increase in oocyte diameter and advance in development obtained in Experiment 2 compared to Experiment 1 (arrest at oocyte diameters of 425 ± 19 µm with only rFsh), indicated that the completion of vitellogenic growth was dependent on Lh, which has not been previously described. Some caution is needed in comparing groups in different experiments that had slightly different conditions, but there were no differences between control groups in the two experiments indicating that experimental conditions had similar effects on maturational development. The dosage and the time interval of rLh treatment applied to induce OM were based on previous studies [38]. However, since the half-life of rLh in plasma has not been determined in flathead grey mullet, the most efficient hormone treatment (dose and timing) remain to be established. In relation to the induction of OM and ovulation, the rationale 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 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 P_4 proceeded to OM compared to three fish that

received a lower rLh dose (15 µg kg^{-1}) with P_4 that did not develop to OM. This indicated that rLh dosage has a relevant effect and that high doses were required. Recombinant Lh has been previously successfully used to induce OM and ovulation in bitterling (*Rhodeus ocellatus ocellatus*) [128], common carp (*Cyprinus carpio*) [3] and Malaysia catfish (*Hemibagrus nemurus*) [223]. 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 [83]. 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 per year [216,272].

Regarding males, the rGth treatment 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 significantly increased the levels of 11-KT, which is the major androgen responsible for testicular development [6,39,155,231]. In comparison, no sperm could be obtained from males in control groups. Other studies have induced or increased the production of milt in sexually immature Japanese eel [95,114,130] and European eel [207] and mature Senegalese sole [37,38] 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 [207] 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) [213]. Nevertheless, the rGth

treatment 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 rGths 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 normal with the exception that 28% of the eggs had multiple oil droplets. In flathead grey mullet, the manual pressure of artificial stripping increased the frequency of multiple oil droplets [137] and multiple oil droplets were related with low egg survival [188]. Another aspect related to bad egg quality and *in vitro* fertilisation procedures is overripening [215]. After ovulation, there is a period 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 temperature, between different stocks, holding conditions, and hormone induction treatments, and ideally should be defined for each situation [215]. For example, latency to obtain good quality eggs can be as long as 5 - 15 days over a temperature range of 10 – 17 °C for rainbow trout (*Oncorhynchus mykiss*) [224], 3 hours in meagre (*Argyrosomus regius*) at 18 ºC [215], but only 30 min in white bass (*Morone chrysops*) at 22 °C [183]. For the present treatment in *M. cephalus*, the timing of ovulation and optimal egg quality has not been previously defined. However, latency times have been reported for flathead grey mullet using carp pituitary extracts with hCG or GnRHa [116], hCG [137] and pituitary glands combined with synahorin and vitamin E [146]. Latency times ranged from 30 to 48 hours after the initial priming dose and 12 to 26 h after the resolving dose. In the 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 PGF2α and the stripped eggs had yolk and oil in the process of coalescing apparently not having completed maturation and hydration when the

oocytes were ovulated. On the contrary, at 48 ± 0.5 h after the rLh injection, low fertilisation percentages were obtained. This was at the limit of the period of good egg 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 [83]. 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 [83]. 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 [1].

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.

CHAPTER VI

Recombinant Fsh and Lh therapy for spawning induction of previtellogenic and early spermatogenic arrested flathead grey mullet

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1. Introduction

Intensive aquaculture is looking for ways to improve reproductive control to ensure the supply of fry for large-scale commercial production. The development of culture protocols will not only ensure a consistent and sustainable supply for grow-out operations, but will also allow for genetic improvements through selective breeding. The success of culture protocols will, in turn, alleviate the fishery pressure on stocks of natural populations that in many cases are compromised. However, to develop aquaculture production of some species, it is necessary to apply exogenous hormonal therapies to control the reproductive cycle.

Two stages of the reproductive cycle, gametogenesis (oogenesis and spermatogenesis) and maturation (oocyte maturation and spermiation) are controlled by different reproductive hormones produced in the pituitary and gonad, i.e., gonadotropin hormones (Gths) and steroids [155]. Hormone therapies based on gonadotropin releasing hormones and luteinizing hormone receptor agonists (human chorionic gonadotropins or pituitary extracts) are commonly used to control the maturation phase, while hormonal control of gametogenesis is rarely used in the aquaculture industry [181]. The use of relatively new recombinant gonadotropin hormones (rGths), the recombinant follicle-stimulating (rFsh) and luteinizing hormones (rLh), can open new strategies in aquaculture to treat reproductive disorders and develop out-of-season breeding programs [170]. To this end, different *in vivo* treatments have been developed, mainly focused on final maturation and spermiation/ovulation stages by single or double rGths injections [3,128]. However, fish species arrested at the early stages of the reproductive cycle require control of gametogenesis, with long-term treatments of repeated injections that maintain elevated plasma levels of specific Gths [170]. In the case of males, different

successful long-term approaches have been described for immature European eel (*Anguilla anguilla*) [207] and mature Senegalese sole (*Solea senegalensis*) [37,38]. In the case of females, it has been more difficult to define similar long-term treatments to produce viable gametes from females arrested prior to vitellogenesis. A significant advance was achieved with the long-term treatment of previtellogenic flathead grey mullet (*Mugil cephalus*) females with rFsh and rLh to successfully complete vitellogenesis [214] (Experiment 2, CHAPTER V). However, after maturation induction with rLh and Progesterone (P_4), females held with spermiating males failed to spawn spontaneously. Therefore, gametes were stripped and artificially fertilised, and a low percentage of fertilisation (<1%) was obtained, which questioned the viability of the process for aquaculture purposes. However, despite of the low fertilisations, the study demonstrated the possibility of using rFsh and rLh to induce oogenesis from previtellogenesis to obtain eggs and larvae in intensive conditions and encouraged further research to improve the results obtained.

The flathead grey mullet, has a worldwide distribution in tropical, subtropical and temperate waters [164], tolerance to wide ranges of salinities [195], excellent flesh quality [122], and high growth rates [63]. It represents an important species and a potential candidate in the diversification of aquaculture products mainly in the Mediterranean area, the Southeast of Asia, Taiwan, Japan and Hawaii [83]. In the Mediterranean, flathead grey mullet spawn from July to October [272], however, breeders held in intensive conditions show reproductive dysfunctions; males rarely produce fluent milt [6,173,279] and females are found to be arrested at previtellogenesis [214] or early stages of vitellogenesis [6].

The objective of the present study was to demonstrate that long-term treatment with rGths, rFsh and rLh, can induce gametogenesis in males that present no spermiation or viscous milt and in females arrested at early stages of gametogenesis, and induce spawning off eggs with high fertilization percentages that provide viable larvae. The necessity of rLh to induce oocyte maturation and spawning in females was evaluated by the application of rLh or P_4 as priming and resolving doses. As a final step, the eggs obtained were used to carry out a preliminary larval rearing trial using a mesocosm technique to check larval development and growth.

2. Material and methods

2.1. Study animals and maintenance

Flathead grey mullet broodstock was formed with individuals, originally obtained from the Ebro River (Spain) or from a semi-extensive pond fish farm (Finca Veta La Palma, Isla Mayor, Spain), which had been held for 1.5 to 3.5 years in IRTA facilities (Sant Carles de la Ràpita, Spain). Thirty females with weights ranging from 0.9 to 2.4 kg and standard length (SL) from 37 to 53 cm, and fifteen males ranging from 0.7 to 1.3 kg and 34 to 43.5 cm SL were used. All fish were larger than the reported SL for first maturation in this species (27 - 35 cm SL for females, 25 - 30 cm SL for males [272]). To identify the fish, each individual was tagged intramuscularly with a Passive Integrated Transponder (PIT) tag (Trovan®, ZEUS Euroinversiones S.L. Madrid, Spain). The sex of the individuals was determined by the presence of sperm after abdominal massage, or by the presence or absence of oocytes obtained through slight suction with a 1.67-mm plastic catheter inserted through the genital opening. Fish were maintained in 10 m^3 covered tanks in a recirculating system (IRTAmar®) supplied with 36 ‰ salinity water under natural conditions of light and controlled winter temperatures (≥ 14ºC) during the last year. Before the study, conducted from early August to early November, the selected individuals were transferred to another 10 m^3 tank; water temperature was controlled at 23.1 ± 0.2 ºC and photoperiod was ambient (14L:10D - 11L:13D). Fish were fed 5 days a week at the rate of 1.5 % of their body weight with a mix of two commercial marine fish diets; 90 % mix of Le-2 and Le-5 Europa RG (Skretting, Spain) and 10 % Brood Feed Lean (Sparos, Portugal). During all experimental procedures, for hormone administration and sampling, fish were anaesthetised with 73 mg L^{-1} of MS-222. When required for the study, males were euthanised with an overdose of MS-222 (250 mg L^{-1}) and death was confirmed by a cut in the gills to exsanguinate the fish.

The study was conducted in accordance with the European Directive 2010/63/EU of 22nd September on the protection of animals used for scientific purposes; the Spanish Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; the Catalan Law 5/1995 of June 21th, for protection of animals used for experimentation or other scientific purposes and the

Catalan Decree 214/1997 of July 30th for the regulation of the use of animals for the experimentation or other scientific purposes. The procedures used were evaluated by IRTA´s Committee of Ethics and Experimental Animal (CEEA) and the Catalan Government and were authorized with ID V7MH4802M.

2.2. Hormonal induction

2.2.1. Induction of vitellogenesis

Females were assigned randomly to rGth and control groups taking care to have similar distribution of females in different initial maturation stages in both groups. The control group (total n = 9) was formed with 6 females in previtellogenesis (5 in primary growth and 1 in cortical alveoli step) and 3 in early-vitellogenesis. A total of 21 females were assigned to receive the rGth treatment; 12 females initially were in previtellogenesis (8 in primary growth and 4 in cortical alveoli stage) and 9 in early-vitellogenesis. Those females in early-vitellogenesis had longer time in intensive captive conditions (> 2.25 years) although not all females that were held for this time had started vitellogenesis.

Single chain recombinant *Mugil cephalus* rFsh and rLh produced in Chinese Hamster Ovary (CHO) cells were purchased from Rara Avis Biotec S.L. (Valencia, Spain). *Mugil cephalus* rFsh was supplied with a concentration of 12 µg mL^{-1} and rLh with concentration of 8 µg mL^{-1}. A methodology based on the protocol described by Ramos-Júdez *et al*. (2021) was followed. The pattern of application of rFsh and rLh aimed to mimic the physiological variations of Fsh and Lh associated with natural reproductive development; initially only rFsh was administered during the early stages of gametogenesis and, subsequently, a decrease in rFsh with an increase of rLh to regulate advanced gametogenesis [152]. The protocol was applied according to ovarian development (**Fig 1**). Increasing weekly doses of 6, 9 and 12 µg kg^{-1} rFsh were administered intramuscularly to induce previtellogenesis (~100 µm oocyte diameter) to vitellogenesis (> 200 µm). Weekly doses were maintained at 12 µg kg^{-1} during vitellogenesis. As vitellogenesis progressed and when mean diameter of the most developed oocytes was ≥ 300 µm, females received additionally a weekly administration of rLh at rising doses. A dose of 2.5 µg kg^{-1} rLh was maintained until females entered into

late-vitellogenesis (≥ 400 µm) [86] and was then increased to 4 and 6 µg kg^{-1}. At an oocyte diameter of ~ 500 µm, weekly rFsh doses were reduced to 4 µg kg^{-1} while rLh was increased to 9 µg kg^{-1}. A combination of 4 µg kg^{-1} rFsh and 12 µg kg^{-1} rLh per week was administered until vitellogenic growth was completed. The completion of oocyte growth was determined when oocytes were deemed approaching maturation; microscopic examination showed that the most developed oocytes were nearing 600 µm in diameter. The nine females in the control group were also manipulated each week and were injected with saline solution (1 mL) a total of twelve times.

Assessment of gonadal development was undertaken on alternate weeks by ovarian biopsies obtained by slight suction through a plastic cannula. Fresh ovarian samples were examined under a microscope (×40 magnification), to measure the mean diameter of the largest most advanced oocytes (n = 20 per female), and fixed for histology. Blood samples were obtained before the initial treatment (week 0) and when vitellogenic growth was completed in the treated group and at the end of the experiment in the control group.

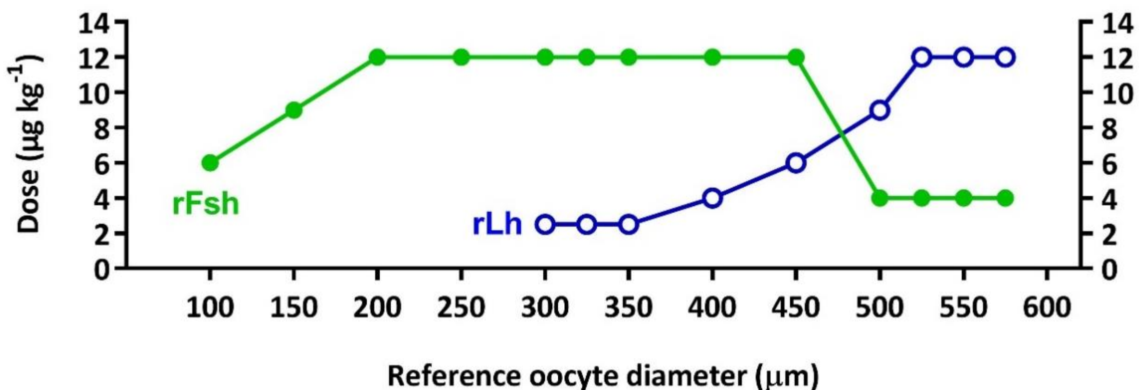

Figure 1. Schematic representation of the rFsh and rLh treatment applied to flathead grey mullet females (n = 21). The protocol was applied according to the development of female gonads determined by ovarian biopsies. Weekly doses of 6, 9 and 12 µg kg^{-1} rFsh were applied to induce previtellogenesis (~100 µm oocyte diameter) to vitellogenesis (> 200 µm) and vitellogenic growth was maintained with a dose of 12 µg kg^{-1} per week. When the mean diameter of the largest oocytes was ≥ 300 µm, in addition to rFsh, rLh was administered in increasing doses. A dose of 2.5 µg kg^{-1} rLh was maintained until females presented ≥ 400 µm oocytes and was raised to 4 and 6 µg kg^{-1}. At ~500 µm diameter, weekly rFsh doses were reduced to 4 µg kg^{-1} whereas rLh was increased to 9 µg kg^{-1}. A combination of 4 µg kg^{-1} rFsh and 12 µg kg^{-1} rLh per week were administered until vitellogenic growth was completed (~600 µm). Each point corresponds to a weekly administration. This scheme represents the longest pattern of administration of those females that required a total of thirteen weeks to complete vitellogenic growth.

2.2.2. Hormonal administration in males

In parallel, males were assigned randomly to rGth and control groups taking care to have similar distribution of males in different initial maturation stages in both groups. The control group (total n = 6) was formed with 5 males with no presence of sperm and 1 with a presence of sperm (sperm index 1, presence of sperm, but not fluid, see below). A total of 9 males were assigned to receive the hormonal treatment; 7 males with no presence of sperm and 2 with presence of sperm. Males treated with rGths were split into two groups that received the same treatment, but at different times in the experimental period. The reason was to assure the availability of spermiating males for spawning induction when the females completed vitellogenic growth. Group 1 of males (n = 4 treated, n = 3 control) initiated the treatment on week 1 and the Group 2 (n = 5 treated, n = 3 control) on week 3 (**Fig 2**). The aim of the treatment was to apply rGths according to their described role in spermatogenesis [231]. Increasing doses of rFsh (6, 9 and 12 µg kg^{-1}) were administered at early spermatogenesis and high levels (12 µg kg^{-1}) during testicular growth (**Fig 3**). No rLh was administered during early spermatogenesis, both rFsh and rLh (12 µg kg^{-1} of both rFsh and rLh) during the middle stages of testicular growth, and only rLh (12 µg kg^{-1}) was administered at late stages to induce sperm maturation [231]. Group 1 received the treatment for a total of 12 weeks whereas Group 2 for 10 weeks. At week 9, Group 1 received a dose of 12 µg kg^{-1} rFsh instead of rLh as a reduction in the spermiation stage was observed (see 3.2 section). Doses were in the range of previous studies with males using rGths produced in CHO cells [37,38,207,214]. Control males were injected with 1 mL of saline solution per week.

In order to evaluate the progression of maturation, at the beginning of the treatment and on a weekly basis, a gentle squeeze on the ventral abdomen toward the urogenital opening was applied to release the sperm. Spermiation stage was determined on a scale from 0 to 3 (0 = not fluent, 1 = fluent but no sample can be obtained, 2 = fluent, 3 = very fluent). By this method, only mature cells are released together with the seminal plasma. Therefore, to determine the stage of development in males, some males were sacrificed at the beginning (n = 2) and the end of the treatment (n = 2 per group) and the testes were removed for histological procedures and the measurement of gonadosomatic index (GSI: testes weight/fish weight x 100). Towards the end of the

experiment (weeks 10 and 13), sperm samples from running males were collected, total volume recorded and stored at 4ºC for sperm quality analysis. Blood samples were also taken from treated and control males each two weeks of the treatment.

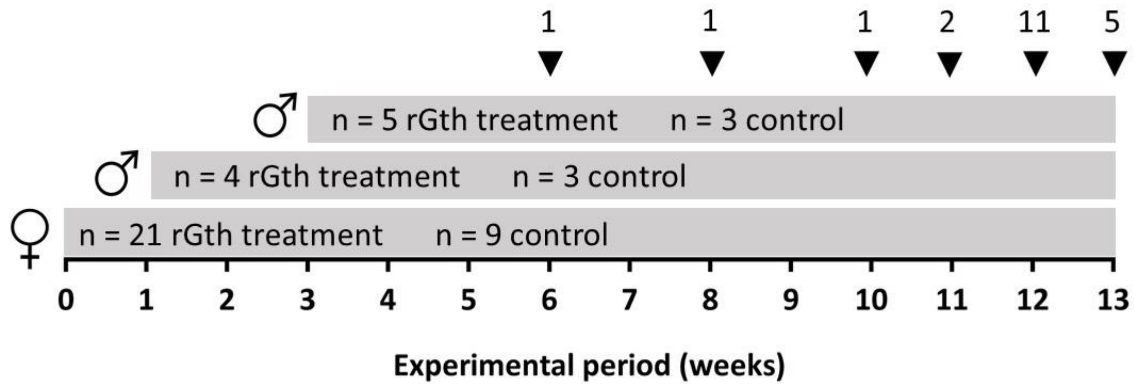

Figure 2. Overall scheme of the experiment. Females receiving the rGth treatment were weekly injected until completion of vitellogenic growth which took place in a maximum period of thirteen weeks, following thirteen administrations. Arrow heads indicate the moment in which individual females were induced to spawn (number of females indicated by the number on the top). Control females received a total of twelve saline injections. Group 1 of males started the treatments (rGth treatment and saline) on week 1, whereas the Group 2 on week 3.

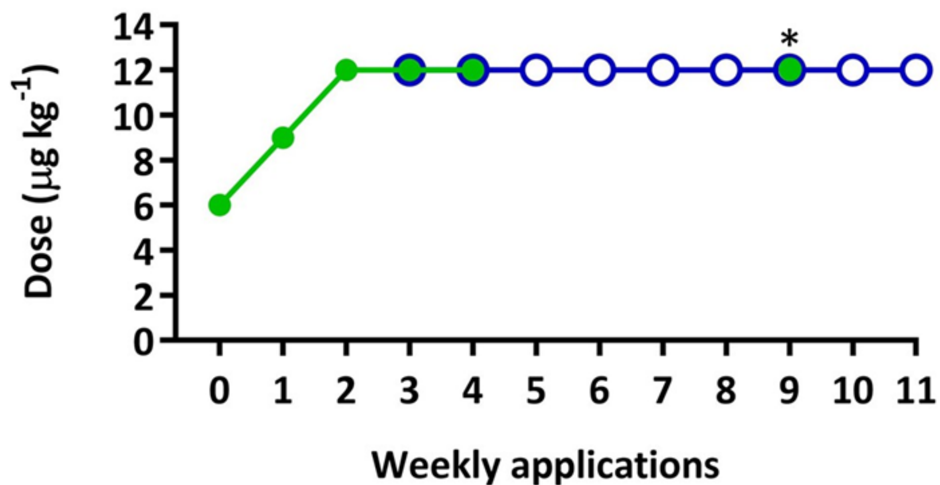

Figure 3. Diagram of the hormonal treatment of flathead grey mullet males. Green indicates rFsh administration and blue indicates rLh administration. Asterisk indicates the moment that Group 1 received 12 μg kg^{-1} of rFsh instead of rLh.

2.2.3. Oocyte final maturation and spawning induction

Three different treatments were followed for maturation and spawning induction. Females that completed vitellogenic growth (as determined by gonadal biopsy) and males with running milt (spermiating stage 2 or 3) were selected for spawning induction. The individuals were separated from the main group and stocked in a separate 10-m^3 tank per treatment. The three separate tanks had the same conditions as the main holding tank. One male in each spawning tank received a dose of 24 μg kg^{-1} of rLh while the others followed the hormonal treatment previously described, receiving 12 μg kg^{-1} of rLh. The selected females were injected intramuscularly with either: (i) priming rLh and resolving Progesterone (P_4) (Prolutex, IBSA Group, Italy) as in Ramos-Júdez *et al.* (2021) (rLh + P_4), (ii) rLh for priming and resolving injections (rLh + rLh), or (iii) P_4 for priming and resolving injections (P_4 + P_4) (doses in **Table 1**). Priming and resolving injections were administered 24:05 ± 0:40 h apart. Ovarian samples were taken with a cannula and examined before each administration. Maturation and ovulation induction of females from each treatment group was staggered on different consecutive days in order to separate the different spawning events. The sex ratio was 1:2 or 1:3 (female:male) per spawning event depending on availability of males. After all the spawning events, males were returned back with the main group to the initial tank. In the cases where females ovulated and showed a swollen belly, but did not release the eggs, manual stripping was applied.

Parallelly, oocytes obtained from cannulation from females (n = 6) that had received a priming injection of rLh (30 μg kg^{-1}) were incubated *in vitro* with different hormones. A total of 58.7 ± 29.9 oocytes were incubated per well of a 96-well plate with 200 μL of Leibovitz's L-15 medium, with either no hormone (control), rLh (concentrations of 400, 200, 100, 50 and 10 ng mL^{-1}), rFsh (400, 200, 100, 50 and 10 ng mL^{-1}) or P_4 (4000, 1000, 500 and 50 ng mL^{-1}). Each treatment was applied in triplicate to oocytes from each female. After 48 hours of incubation at 21ºC, the follicles were examined under a binocular microscope and oocytes without the follicular layer (ovulated) and intact follicles (un-ovulated) were counted for each well.

Table 1. Treatments to induce maturation and spawning in flathead grey mullet (*Mugil cephalus*) females and the results obtained. Latency period indicates the time (h) between resolving injection and spawning; Ovulation success (%) indicates the number of females that ovulated after the resolving injection divided by the total number of injected females; Spawning success (%) indicates the number of females that spawned naturally in the tank after the resolving injection, divided by the total number of injected females. Data are expressed as mean ± standard deviation. Oocyte diameter, total eggs per female and fecundity between the three treatments were compared by one-way ANOVA while latency period, percentages of initial fertilization, embryo survival, hatching and hatching in 96-well plate between the successful treatments (rLh + rLh and rLh + P_4) were compared through a t-student test. Values with equal superscripts within rows are not statistically different.

Group	rLh + P_4	rLh + rLh	P_4 + P_4
Treatment Priming injection	30 μg kg^{-1} rLh	30 μg kg^{-1} rLh	40 mg kg^{-1} P_4
Treatment Resolving injection	40 mg kg^{-1} P_4	30 μg kg^{-1} rLh	40 mg kg^{-1} P_4
Oocyte diameter (μm)	606 ± 10^a	602 ± 6^a	600 ± 7^a
No. of injected females	9	6	6
No. of spawned females	8	6	1
Latency period (h)	16:36 ± 1:56[a]	16:51 ± 2:22[a]	24:30:00
Ovulation success (%)	100	100	50
Spawning success (%)	89	100	17
Total eggs per female	1,760,100 ± 821,102[a]	1,631,889 ± 954,138[a]	1,280,833 ± 966,42[a]
Fecundity (egg kg^{-1} bw)	1,194,637 ± 635,747[a]	1,205,1387 ± 487,002[a]	694,529 ± 526,457[a]
Initial fertilization (%)	49 ± 23^a (8 min – 80 max)	60 ± 16^a (39 min – 84 max)	0
Embryo survival (%)	56 ± 22^a (15 min – 80 max)	73 ± 21^a (42 min – 90 max)	0
Hatching (%)	57 ± 21^a (25 min – 88 max)	61 ± 30^a (23 min – 90 max)	-
Hatching 96-well plate (%)	95 ± 4^a	97 ± 1^a	-

2.3. Egg collection and incubation

Surface out-flow egg collectors (mesh size of 500 μm) were placed to receive eggs from the tanks and were frequently inspected for eggs. When spawning was observed, eggs were transferred into a 10-L bucket. The number of eggs per spawn (fecundity) was estimated by counting the eggs in three subsamples. A sample of eggs (n = 50 – 100) was observed under a microscope and the percentage (%) fertilization for each batch of

spawned eggs was determined by calculating the % of eggs that reached the 2- to 16-cell stage. Eggs for incubation were collected after careful agitation of the eggs in the 10-L bucket. The eggs were incubated at a density of 13,230 ± 7,273 eggs L^{-1} in 30-L incubators with the same conditions as the broodstock holding tanks. Each incubator was supplied with an air stone, placed down in the centre, to maintain the eggs in suspension and prevent accumulation of the eggs at the surface or bottom of the incubator. The number of eggs in each incubator was estimated by mixing the incubator homogenously and taking three 100 mL samples and counting the eggs in each sample.

The eggs were left one day to develop and survival rate (percentage of eggs with embryos) was estimated as for percentage fertilization with a sample of eggs (n = 50 – 100) taken from the incubator. The following day, the number of hatched larvae in each incubator was estimated volumetrically as for the eggs. In parallel to the 30-L incubators, eggs were transferred into individual wells filled with sterile seawater in a 96-well cell culture plate (EIA plate) and placed in a refrigerated incubator at 21ºC in duplicate for each spawn and revised daily until the last larva died, to estimate hatching rate and larval survival under starvation as in Giménez *et al*. (2006). Percentage of survival was calculated as the number of larvae alive / total hatched larvae.

A preliminary trial was made to examine the larval growth and development. The trial did not focus on survival as the facilities and staff were not available to provide optimal conditions for the larvae. Larval rearing was carried out using mesocosm conditions [51], with low larval density in a large tank (6 larvae L^{-1}, 1500 L tank) under more natural or, at least, less strict conditions than those used in intensive rearing, and using an endogenous bloom of wild marine zooplankton, mostly harpacticoid copepods, together with periodic addition of rotifers and *Artemia nauplii*. The trial was carried out from November 11th to December 18th 2020 using larvae 4-days post hatch (dph) that hatched on November 7th, from a spawn obtained with the rLh + rLh spawning treatment. The larvae were stocked in a 1500 L tank at 20ºC, 12hL:12hD photoperiod and fed on rotifers for 26 days (4 to 30 dph) followed by newly hatched *Artemia nauplii* (24 - 39 dph). Phytoplankton (a mixture of *Tetraselmis suecica* and *Isochrysis galbana*) was added every day in order to maintain a green medium, and every two days the rotifer (rot) concentration was assessed to maintain a density of 5 rot mL^{-1}. *Artemia nauplii* were

added when the larvae reached 4.3 mm TL (23 dph) being the main food for larvae after 5 mm TL as suggested by Hagiwara *et al*. (1992).

Ten larvae arbitrarily chosen were sampled at 4, 6, 9, 11, 17, 23, 27, 32, 37 and 39 dph and anaesthetised with MS-222. Standard length was measured using a digital camera connected to an image analyser (AnalySIS, SIS Gmbh, Germany). Photographs were also used to estimate the presence or absence of food in the gut and to examine swim bladder inflation as well as other indicators of larval development.

2.4. Histological analysis

Ovarian samples obtained by cannulation and testis portions were preserved in Bouin's fluid for 24 h and stored in 70 % ethanol until processed. The dehydrated tissues were embedded in paraffin and 3 µm sections cut. The testes portions (from the anterior, middle and posterior part) were oriented to obtain horizontal sections. Cut sections were stained with hematoxylin and eosin (Casa Álvarez, Spain) for morphological evaluation. The slides were examined under a light microscope (Leica DMLB, Houston, USA).

Oocytes were classified as previously described by Ramos-Júdez *et al*. (2021). Oocytes were classified as previtellogenic, with primary growth (PG) oocytes which presented multiple nucleoli situated in the germinal vesicle, or with cortical alveoli (SGca) oocytes that had small oil droplets and granular vesicles in the peripheral ooplasm. The incorporation of yolk globules indicated vitellogenic stages: early secondary growth (SGe), late-secondary growth (SGl) when oocytes reached ≥ 400 µm [86] and full-grown secondary growth oocytes (SGfg) when the vitellogenic growth was completed with the fusion of yolk granules and thickening of vitelline membrane. Oocytes were classified as oocyte maturation stage (OM) when oil droplets were coalescing and the nucleus was positioned to one side of the oocyte, indicating the initiation of the germinal vesicle migration (GVM). Oocytes with disintegrating structure and hypertrophy were in atresia [257]. Maturation stage of females was determined according to the most developed stage of oocytes present. Additionally, the percentage of oocytes in different stages in the ovaries among weeks was calculated through the identification of ≥ 50 random oocytes per female each week.

To evaluate testes samples, the number of spermatogonia (SPG), spermatocytes (SPC), spermatids (SPD) and spermatozoa (SPZ) were scored in 12 seminiferous tubules randomly selected from different areas (anterior, middle and posterior) per sample and the percentage abundance of each germ cell type was determined.

2.5. Sperm quality evaluation

Sperm samples from running males were collected for quality evaluation on week 10 and week 13 at the end of the experimental period. Samples were collected in a 1 mL syringe avoiding the contamination by urine, faeces and water. Sperm was divided into two aliquots, one was maintained as undiluted sperm and one was diluted 1:10 (1 part sperm + 9 parts diluent) in Marine Freeze® (IMV Technologies, L'Aigle, France) extender [84] and both samples were maintained in Eppendorf tubes at 4ºC until evaluation. Sperm was activated by pipetting 5 µL of the sperm sample (undiluted or diluted) into an Eppendorf with, depending on the concentration of the sperm, 195, 295, 495 or 995 µL of sea water. Immediately, the Eppendorf was agitated to thoroughly mix, then 2 µL containing activated sperm was pipetted into an ISAS counting chamber (Integrated Sperm Analysis System, Spain), and videos of tracks of the activated spermatozoa were recorded 15 s after activation with the CASA system SCA-VET-01 (Microptic, Barcelona, Spain). Videos were recorded using a digital camera (Basler Ace ACA1300-200UC, Basler AG, Ahrensburg, Germany) connected to an optical phase-contrast microscope (Nikon Eclipse Ci, Tokyo, Japan) with ×10 negative phase contrast objective. The following sperm parameters were determined: (1) sperm concentration (spz mL^{-1}), (2) sperm motility (%), (3) rapid progressive sperm (%), and (4) sperm velocity (µm s^{-1}): the curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP). The CASA program was set to classify motile sperm to have a VCL of > 25 µm s^{-1} and fast progressive sperm to have a straightness (SRT = VSL/VAP x 100) of > 80% and a VCL of > 80 µm s^{-1}. All samples were analysed on the day of the collection and 48 h after collection. Samples collected at the end of the experiment (week 13) were also analysed on days 1, 4, 6, 8, 11, 13 and 15 after collection.

2.6. Plasma steroid analysis

Blood samples were collected and centrifuged at 3,000 rpm at 4 ºC for 15 min and the plasma stored at -80 ºC until analysis. Plasma levels of 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) were analysed using commercially available enzyme immunoassays (EIA) (Cayman Chemical Company, USA). Steroids were extracted with methanol that was evaporated and extracts were re-suspended 1:10 (E_2) or 1:100 (11-KT) in the EIA buffer.

2.7. Statistical analysis

Data is expressed as the mean ± standard deviation (SD). A Chi-square test was used to examine the distribution between groups of fish that had different maturational stages at the start of the experiment, and whether fish that ovulated or spawned had a determinate maturity status at the beginning of the experiment. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and homogeneity of variance, respectively. Mann-Whitney U test or Kruskal-Wallis test, followed by Dunn's pairwise comparison, were used in non-normally distributed data to compare oocyte diameter between treated and control group within a week and between weeks during the experiment, respectively. One-way ANOVA followed by Holm-Sidak *post hoc* test was used to separately examine differences in the independent variables of diameter of full-grown oocytes, percentage of OM, total eggs per females, and fecundity between the three spawning treatments. Variances across the groups were not equal for the GSI data in males, which was log-transformed and groups were compared using the Brown-Forsythe test and Games-Howell *post-hoc* multiple comparisons test. Spawning data from rLh + rLh and rLh + P_4 spawning treatments (i.e., latency period, fertilization and hatching percentages) was compared using a t-student test. Differences in percentage of OM and oocyte diameter before and after the priming or resolving injections, and percentage ovulation of oocytes incubated *in vitro* were examined by one-way repeated measures (RM) ANOVA with individual females as the subject. Two-way RM ANOVA with pairwise comparison by the Holm-Sidak test was used for comparing E_2 and 11-KT between weeks and treatment groups. Sperm quality parameters were compared with a

one-way and two-way RM ANOVA. In the one-way RM ANOVA, the male was the subject, day of storage the independent variable and sperm quality parameters the dependent variables. In the two-way RM ANOVA, the male was the subject, time of storage (0 or 48h) and sample dilution (undiluted or Marine Freeze®) were the independent variables and sperm quality parameters the dependent variables. Significant differences were detected at a significance level of $P < 0.05$. Statistical analyses were performed with SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA), with the exception of the Brown-Forsythe test that was conducted with SPSS software version 20.0 (Armonk, NY: IBM Corp).

3. Results

3.1. Induction and completion of vitellogenesis

Induction and completion of vitellogenesis (603 ± 8 μm oocyte diameter) from previtellogenic and early vitellogenic females was achieved with 100 % success in the rGth-treated group; as observed for all 12 previtellogenic females and 9 early vitellogenic females. No females from the control group completed oocyte growth (**Fig 4A**).

At the beginning of the experiment, females in different stages of ovarian development were evenly distributed between the control and treatment groups (χ^2 = 0.824; gl = 2; *P* = 0.662). Control (**Fig 5A**) and rGth (**Fig 5B**) groups had 62 ± 7 % of females in previtellogenesis (from which the 61 ± 7 % and 23 ± 16 % were at primary growth and at cortical alveoli, respectively) and 38 ± 7 % in early-vitellogenesis, with no significant difference in mean initial diameter of the largest oocytes (control = 164 ± 82 μm, rGth-treated = 172 ± 72 μm). Although some females had started vitellogenic growth, at week 0 the ovaries had principally PG oocytes, some cortical alveoli oocytes with very few oocytes in early-vitellogenesis and some atresia (**Fig 5C**, **5D**, **6A**).

Previtellogenic females at PG from the control group did not show further development during the experiment (**Fig 5A**, **6D**). The number of early-vitellogenic females in the control group decreased during the experiment (**Fig 5A**), concomitantly

there was an increase in the occurrence of follicular atresia (**Fig 5C**). In addition, no significant differences in oocyte diameter were observed amongst weeks in the control group (**Fig 4A**). On the other hand, the hormonal treatment induced a significant (P < 0.001) growth of oocyte diameter. The rGth-treated group showed a significant weekly increase in oocyte diameter (P < 0.001) (**Fig 4A**) and by the fourth week of treatment all (100 %) females had progressed to vitellogenesis (**Fig 5B**). At this point, a clear clutch of vitellogenic oocytes was the most abundant in the ovaries (**Fig 5D**, **6B**). From the sixth week onwards, different females completed oocyte growth (**Fig 6C**) with the majority completing gonadal development in week 12 and 13. To define the pattern of oocyte growth in the rGth-group, oocytes were aligned from the completion of vitellogenic growth and the growth was represented by a second order polynomial (quadratic) equation $y = 113.9 + 53.62\,x - 1.234\,x^2$ ($R^2 = 0.9625$) (**Fig 4B**). A greater increase in oocyte diameter was observed between weeks (injections) in earlier stages, with ~ 50 µm per week, while approximately three weeks (injections) were required to grow the last 50 µm to complete vitellogenic growth. Once calculated the oocyte volume ($V = 4/3\ \pi r^3$), conversely, showed a greater weekly increase (more than 5 times greater) in the later stages of development compared to the early stages (results not shown).

Concerning E_2 levels, no significant differences were observed between control and treated group at the beginning of the experiment (0.67 ± 0.71 ng mL^{-1} and 0.68 ± 0.46 ng mL^{-1}, respectively), whereas significant higher levels of E_2 (P < 0.001) were observed when the treated group completed vitellogenic growth (1.53 ± 0.70 ng mL^{-1}) in comparison with no changes in controls (0.52 ± 0.25 ng mL^{-1}) at the end of the experiment.

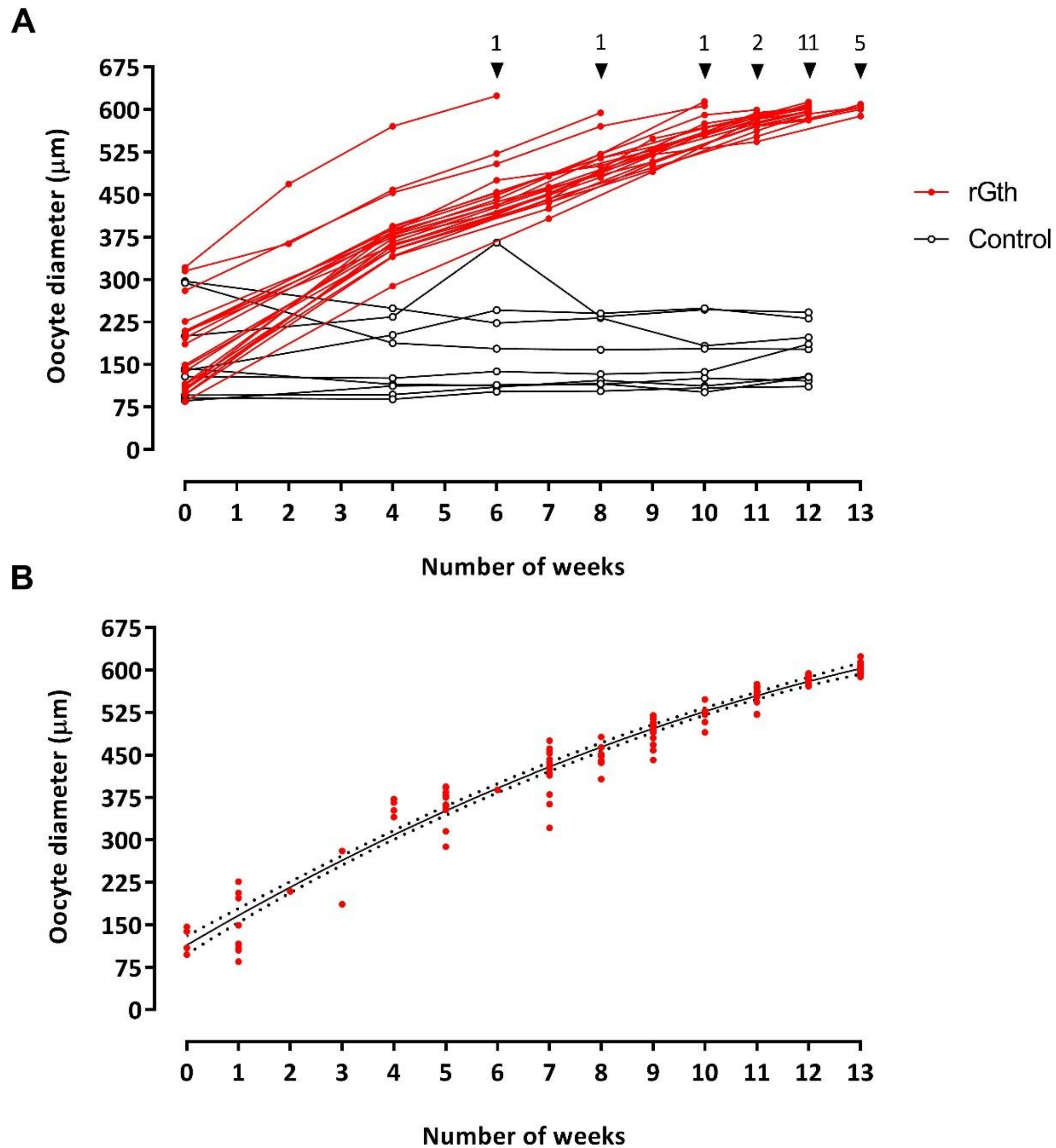

Figure 4. Mean oocyte diameter of the largest oocytes (n = 20 per female) in wet mounts in: (A) each female (represented by a line) that received the rGth treatment (n = 21) and females that received saline injections (control) (n = 9). Triangles show the moment and the number of females that completed vitellogenic growth and were selected for maturation and ovulation induction. And in (B) rGth-treated females aligned from the completion of vitellogenic growth, the moment that were selected for maturation and spawning induction. The growth of oocytes in represented by a second order polynomial (quadratic) equation (R^2 = 0.9625).

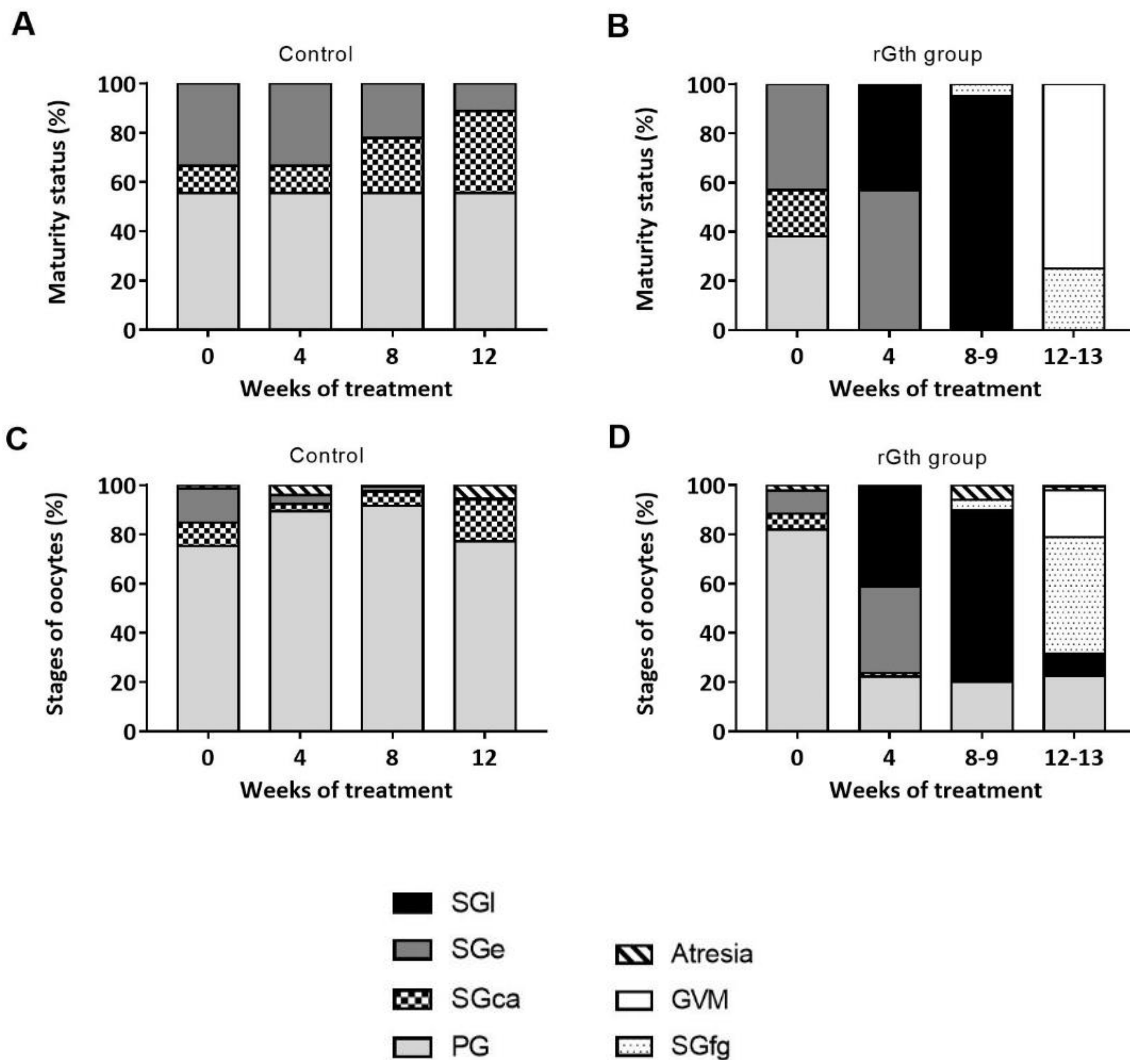

Figure 5. Percentage of females in different maturity stages of gonadal development in control (A) and rGth-treated group (B), and evolution of percentage frequency of oocyte developmental stages observed in control (C) and rGth-treated group (D) in different weeks of the experimental period. The maturity status of females was determined by the most advanced oocyte stage present in the samples. To calculate the percentage of each oocyte stage, a total of ≥50 random oocytes per female were classified and proportions were estimated. Each bar section represents the mean percentage of oocytes per stage from females for each week. PG, primary growth oocyte; SGca, cortical alveoli step; SGe, early secondary growth; SGl, late secondary growth oocyte (> 400 μm); SGfg, full-grown secondary-growth oocytes; GVM, initial germinal vesicle migration.

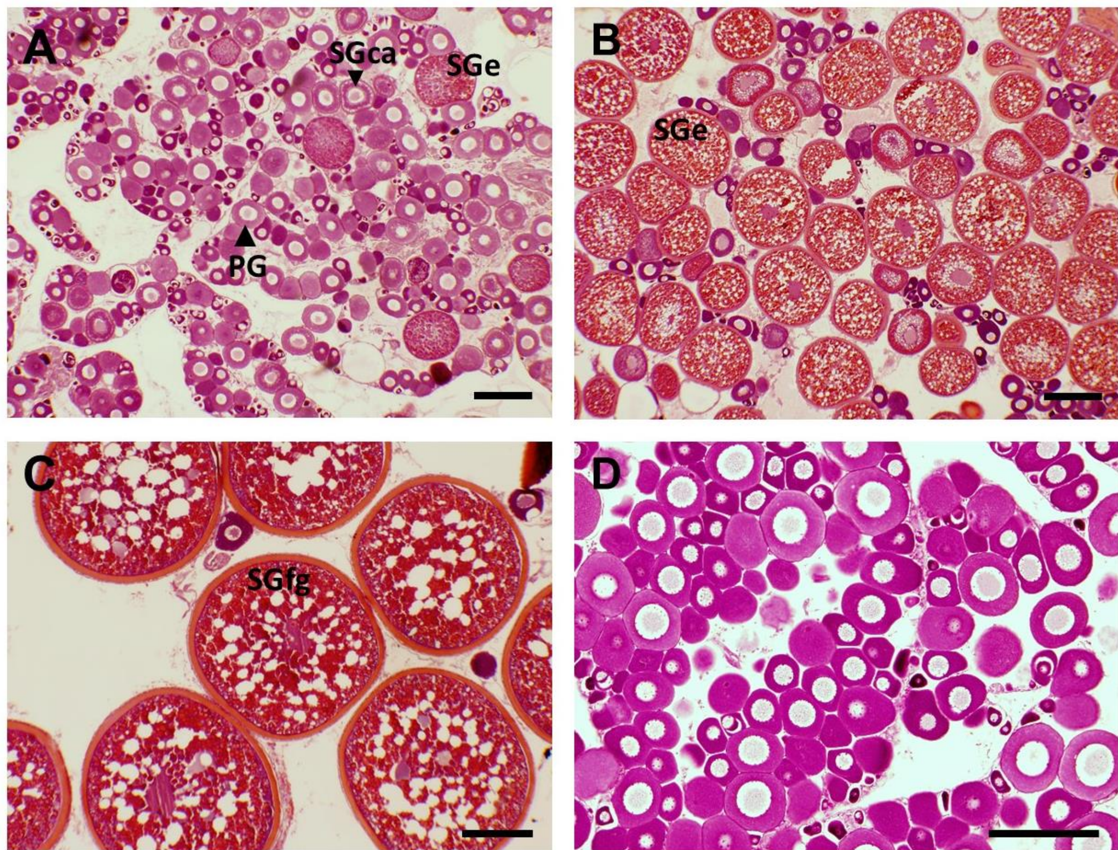

Figure 6. Histological photographs of representative ovarian biopsies from flathead grey mullet females (*Mugil cephalus*) during the experimental period. (A) A female in early-vitellogenesis (SGe) with mainly primary growth oocytes, some cortical alveoli stage (SGca) and only a small percentage of oocytes in SGe at the beginning of the experiment, (B) a female in SGe after rFsh treatment with a clear clutch of oocytes recruited into vitellogenesis, (C) a rGth-treated female with full-grown oocytes (SGfg), and (D) a control female in previtellogenesis by the end of the experimental period. Scale bar: 200 µm.

3.2. Induction of spermatogenesis and spermiation

Spermiation was induced in 100 % (n = 9) of the males in the rGth-treated group. All males advanced from a sperm index of 0 or 1 at the start of the experiment, indicating no sperm sample could be obtained, to a sperm index of 2 or 3 indicating males had fluent or very fluent sperm. All control males were in sperm index 0, no presence of sperm at the end of the experiment.

At the beginning of the experiment, males with or without presence of sperm were evenly distributed between the control and treatment groups ($\chi^2 = 0.156$; gl = 1; $P = 0.693$). Only 20 % (3 out of 15 individuals; 1 from the control group and 2 from the

rGth group) presented traces of high viscous milt, but not sufficient to obtain a sample (**Fig 7**). The histological evaluation of the testes from two fish in which no milt was obtained after abdominal pressure at the beginning of the experiment, showed that one male presented only SPG within the seminiferous tubules, while the other male presented many SPG and some SPC, SPD and SPZ, but many tubules did not have the central lumen formed and those that had, presented very few spermatozoa (**Fig 8A**, **8B**). During the experimental period, males in the control group did not produce fluent milt and only the same one male out of six (17%) produced a small drop of viscous milt within different weeks. At the end of the experiment, the testis from two control males —the male that in previous weeks presented viscous milt and one male that never had milt— contained tubules with a higher number of SPC than the initial situation, but there was no presence of SPD or SPZ (**Fig 8C**, **8D**). In contrast, running males with fluent white milt were observed in the rGth-group. After five weeks of treatment (three weeks of rFsh treatment and two weeks of combined rFsh and rLh treatment) eight out of nine (89 %) males presented milt; either viscous traces (33 %) or fluent milt (56 %) (**Fig 7**). With the application of rLh, the number of males with fluent milt increased. By seven weeks, 100 % of the males were spermiating with fluent milt and males maintained fluent milt until the end of experiment (week 12), with the exception of week 9 when one male produced viscous milt. However, after the application of rFsh in Group 1 and continuing the rLh treatment in Group 2, fluent milt was produced again in the following week. In clear contrast to the control males, in the histology from two rGth-treated males at the end of the experiment, the sperm ducts of the rGth-group were completely filled with spermatozoa (**Fig 8E**, **8F**) and sperm volumes ranged from 0.25 to 2.89 mL. In addition, GSI values reflected the growth of the testis in rGth-treated males, which had significantly higher (P = 0.026) GSI compared to control group at the end of the experiment. Males before the hormonal treatment (n = 2) and control males at the end of the treatment (n = 2) showed thin undeveloped testes with GSI of 0.10 ± 0.05 % and 0.06 ± 0.01 %, respectively, while rGth-treated males at the end of the experiment (n = 2) presented well developed white testes with 5.35 ± 1.25 % GSI. Taken together, males with sperm index 0 and 1 had germ cells predominantly at stages of SPG and SPC in undeveloped testes (GSI $\lesssim$ 0.1) and the rGth treatment induced testes growth (5.35 ± 1.25 % GSI) and

development of germ cells to complete spermatogenesis and provide testes full of spermatozoa and fluent spermiation (index 2 and 3).

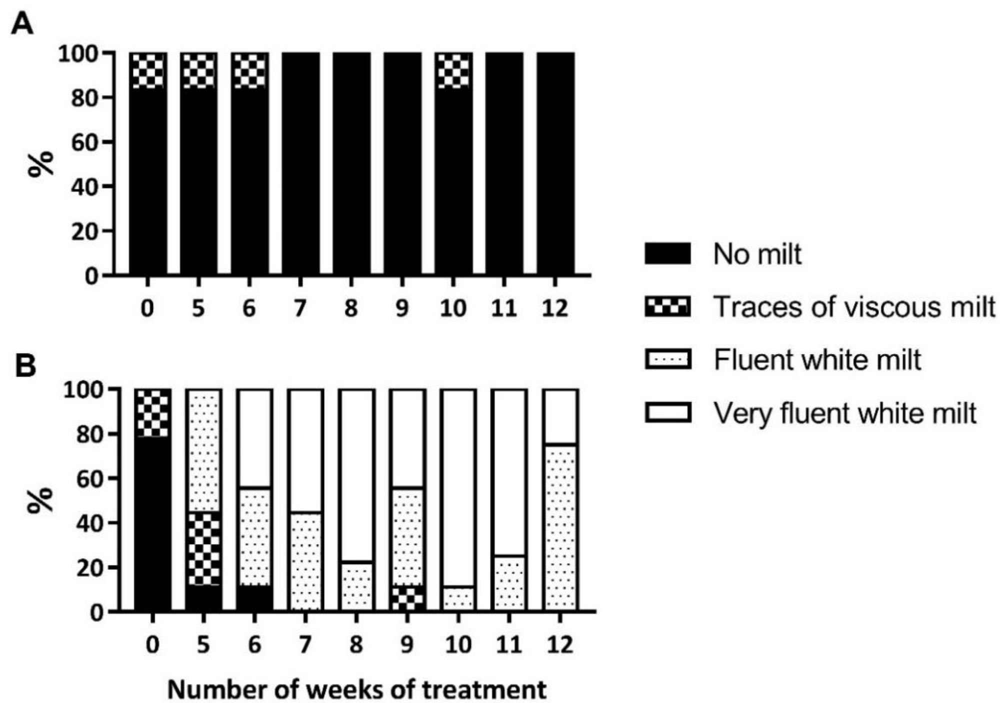

Figure 7. Percentage of no-spermiating and different degrees of spermiating males in (A) control (n = 6) and (B) rGth group (n = 9) during the treatment period. Males were classified according to the presence of milt; no milt (index 0), traces of viscous milt from which no sample could be obtained (index 1), fluent white milt (index 2) and very fluent white milt (index 3). Data from Group 1 (n = 4 rGth-treated males, n = 3 control) from the first application (week 0) to the last checking (week 12) was combined with data from Group 2 (n = 5 rGth-treated males, n = 3 control) from week 0 to 10 in order to present the results.

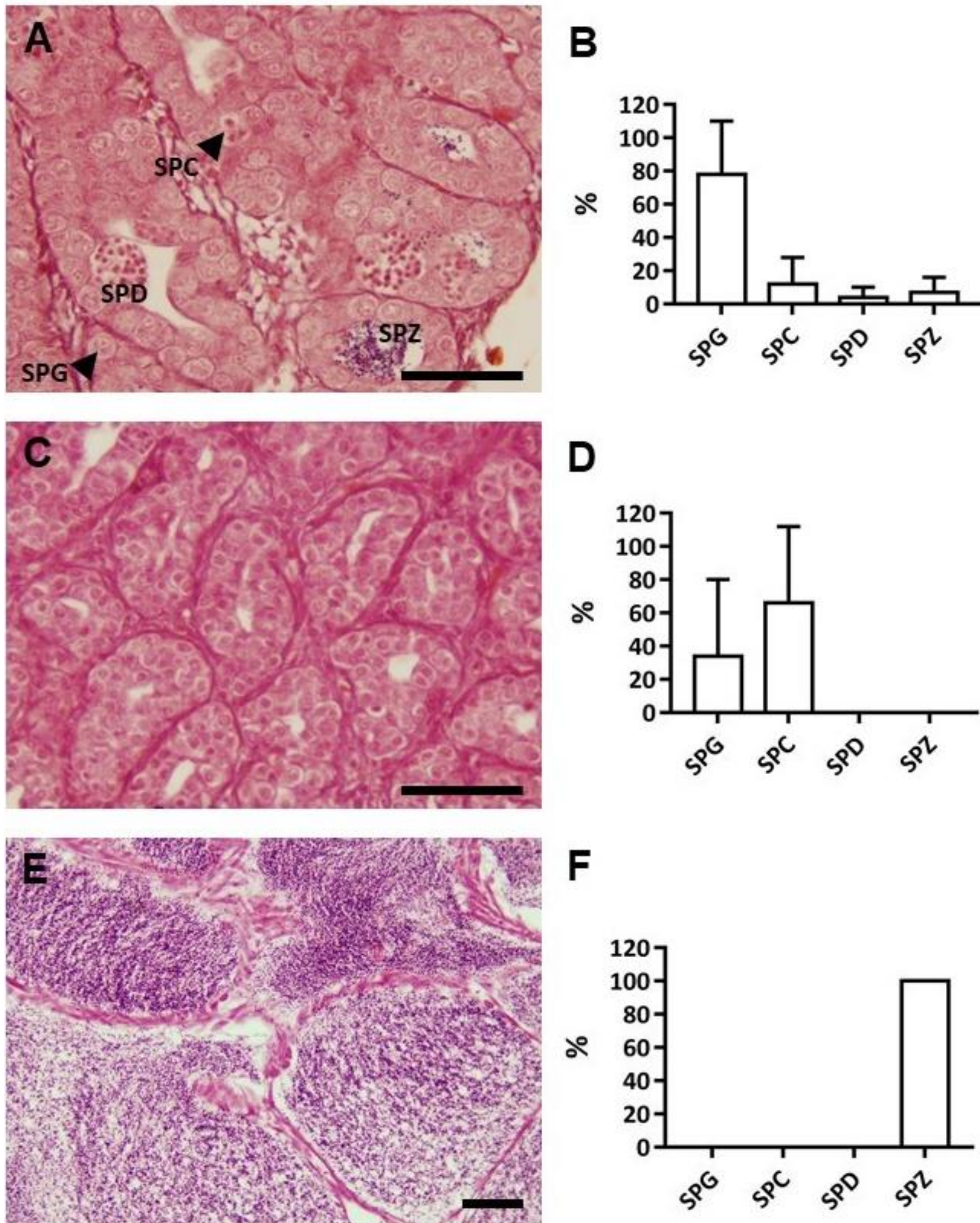

Figure 8. Histological sections of testis and the percentages of the spermatogenesis developmental stages at the beginning of the study (A and B, n = 2) and at the end of the experimental period in control group (C and D, n = 2) and rGth-treated group (E and F, n = 2). SPG, Spermatogonia; SPC, Spermatocyte; SPD, Spermatid; SPZ, Spermatozoa. Scale bars = 50 μm.

In regard of steroid changes, rGth treatment had a significant effect in the increase of 11-KT levels (P < 0.001) compared to the control group (**Fig 9**) that was maintained without significant changes. In the rGth group, increasing values of 11-KT were obtained within the course of the experiment coinciding with the availability of spermiating males, with the maximum at eight weeks and a decrease afterwards.

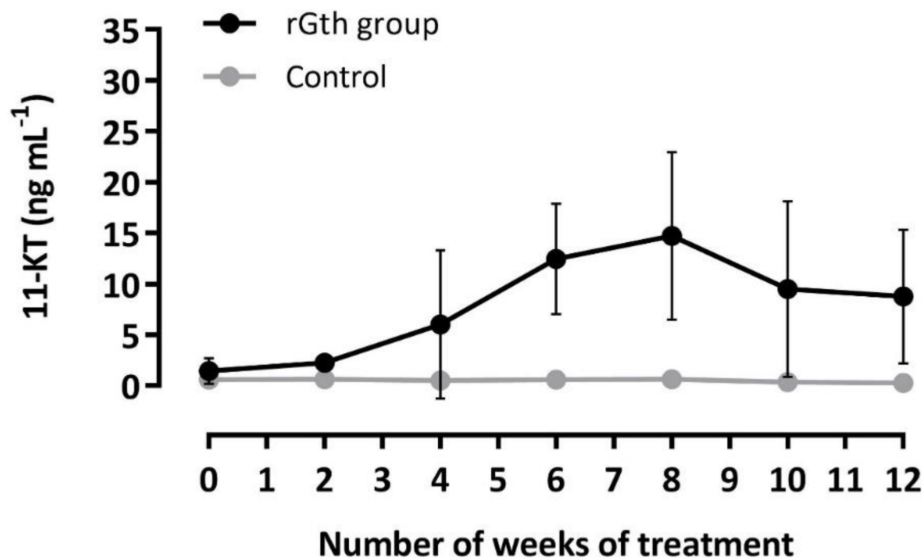

Figure 9. Effect of rFsh and rLh treatment (rGth group) and saline (control) on 11-ketotestosterone (11-KT) levels (mean ± SD) in the flathead grey mullet (*Mugil cephalus*). Data from Group 1 (n = 4 rGth-treated males, n = 3 control) from the first application (week 0) to the last checking (week 12) was combined with data from Group 2 (n = 5 rGth-treated males, n = 3 control) from week 0 to 10 in order to present the results. Two-way RM ANOVA showed a significant effect of the rGths treatment on the production of 11-KT (P < 0.001).

Regarding sperm quality, on the day of collection (0h), the sperm diluted 1:10 in Marine Freeze® had the following mean characteristics: motility of 58 ± 22 %, head size of 13 ± 5 μm^2, 107 ± 24 $\mu m\ s^{-1}$ VCL, 92 ± 29 $\mu m\ s^{-1}$ VAP, 70 ± 31 $\mu m\ s^{-1}$ VSL, 67 ± 12 % STR, 57 ± 16 % LIN, and 79 ± 11% WOB. Whilst 20 ± 16 % of the motile spermatozoa were fast progressive that had velocity of 149 ± 17 $\mu m\ s^{-1}$ VCL, 140 ± 20 $\mu m\ s^{-1}$ VAP, 129 ± 21 $\mu m\ s^{-1}$ VSL, 92 ± 2 % STR, 86 ± 7 % LIN, and 93 ± 6% WOB. The variation amongst the nine males was wide, with sperm motility % ranging from 19 to 89 %. The mean concentration was 7.59 x 10^{10} spermatozoa mL^{-1} or 15.56 x 10^{10} spermatozoa kg^{-1}. The sperm diluted in Marine Freeze® had significantly higher motility than undiluted sperm

on the day of collection (0 h) and 48 h after collection (**Fig 10**). The motility of undiluted sperm decreased significantly from day 0 to 48 h after collection, compared to diluted sperm that maintained similar motility. The sperm collected and diluted in Marine Freeze® at the end of the experiment (week 13) maintained similar motility for 6 days of storage at 4ºC (tested days 0, 1, 2, 4 and 6) with variation from 41.1 ± 20.0% to 70.2 ± 17.3% before decreasing significantly from day 4 (70.2 ± 17.3%) to day 8 (11.7 ± 5.1%), whilst day 6 was intermediate (41.1 ± 20.0%).

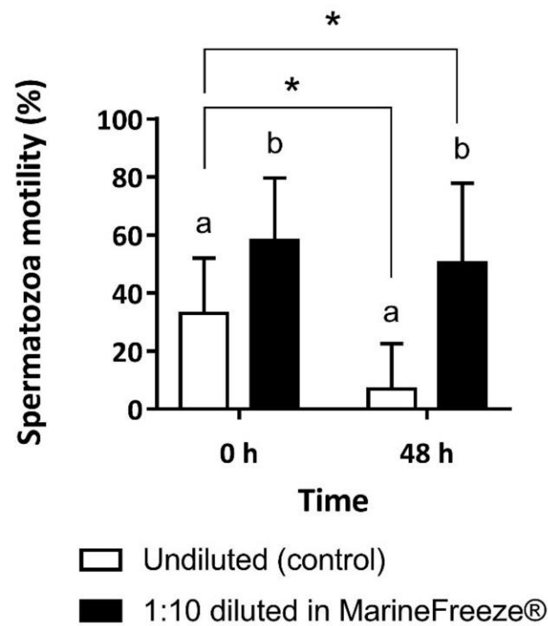

Figure 10. Percentage motility of sperm samples collected from rGth-treated males at the end of the experimental period (week 10 and 13). A two-way RM ANOVA was performed followed by the Holm-Sidak *post hoc* test with males as subjects, time of storage (0 or 48h) and sample dilution (undiluted or 1:10 diluted in Marine Freeze®) as the independent variables, and percentage motility as the dependent variables. Different letters indicate significant differences between undiluted and diluted samples within the same time of evaluation, while asterisks show significant differences between evaluation time.

3.3. Oocyte final maturation and spawning induction

Females that had completed vitellogenic growth —available only from the rGth group— were selected for spawning induction following three different treatments. Gonad samples were examined before the priming injections and after 24:05 ± 0:40 h,

just prior to the resolving doses. Ovaries from chosen females were composed by SGfg with a mean diameter of 603 ± 8 μm and the 76 % (16 of 21) of the females presented 17 ± 23 % of oocytes that had already initiated OM with the migration of germinal vesicle (**Fig 5D**); the nucleus had moved off-center and there was a small degree of fusion of yolk granules and lipid droplets, but with no single large yolk mass. No significant differences in oocyte diameter or the percentage of OM were found between females that received different spawning treatments. After the priming injections (24 h), 100 % of the females that received rLh had entered into OM with most of the oocytes (96 ± 9 %) in late GVM with yolk coalescence (**Fig 11**). Meanwhile, those that received P_4 did not show a significant increase in GVM percentage (35 ± 35 %) respect to initial stage (12 ± 5 %) thereby showing that most of the oocytes were retained at the secondary growth stage.

The maturity status females had at the beginning of the experiment had no effect on the proportion of females that ovulated or spawned at the end (χ^2 = 1.150; gl = 2; P = 0.563 0.001; χ^2 = 2.149; gl = 2; *P* = 0.342). In the rLh + P_4 group, 100 % of the females ovulated and eight out of nine females (89 %) spawned in the tank 16:36 ± 1:56 h after the resolving injection. In the rLh + rLh group, 100 % of the females (n = 6) ovulated and spawned 16:51 ± 2:22 h after the resolving injection. Meanwhile, three out of six fish (50 %) under P_4 + P_4 treatment ovulated but only one (17 %) actually liberated some eggs (177,667 eggs) 24:30 h from the administration of the resolving dose. The average number of eggs spawned and female fecundity for each hormone treatment were similar (**Table 1**). Those females that did not ovulate in the P_4 + P_4 treatment group, did not show a significant increase in OM after the second P_4 (**Fig 11**). The eggs from the females that ovulated and did not spawn were stripped by applying gentle abdominal pressure to liberate all ovulated eggs from the female. The stripped eggs did not have the appearance of viable eggs and formed a dense globular mass with almost no ovarian fluid.

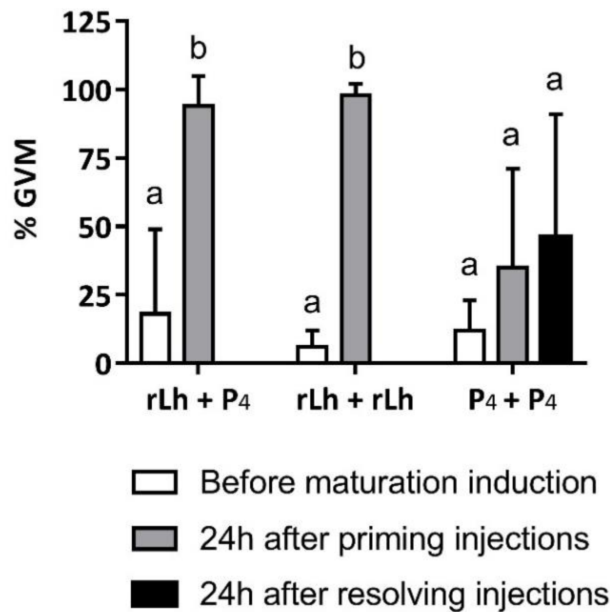

Figure 11. Percentage of oocytes at maturation stage (OM) with germinal vesicle migration (GVM) before OM induction, ~24 h after the priming injections and ~24 h after the resolving injections. Treatments applied to induce OM and spawning were (i) priming 30 µg kg^{-1} rLh and resolving 40 mg kg^{-1} Progesterone (P_4) (rLh + P_4), (ii) 30 µg kg^{-1} rLh as priming and resolving injections (rLh + rLh) or (iii) 40 mg kg^{-1} P_4 as priming and resolving injections (P_4 + P_4) given 24:05 ± 0:40 h apart. Different letters indicate significant differences at different timing of the inductions within a same treatment following one-way RM ANOVA.

The *in vitro* incubation of oocytes that had initiated OM confirmed the *in vivo* ovulation and spawning. The highest percentages of ovulation (> 50 %) were obtained from oocytes treated with P_4 (4000, 1000, 500 and 50 ng mL^{-1}) or 100 ng mL^{-1} of rLh (**Fig 12**). All oocytes treated with P_4 and oocytes treated with 400, 200 and 100 ng mL^{-1} of rLh had significantly (P < 0.05) higher percentages (> 34 %) of ovulation than untreated oocytes (control) and oocytes treated with rFsh or 10 ng mL^{-1} of rLh (< 8%). Oocytes treated with 50 ng mL^{-1} of rLh had a percentage of ovulation (21.3 ± 18.5 %) that was intermediate between the highest and lowest ovulation groups.

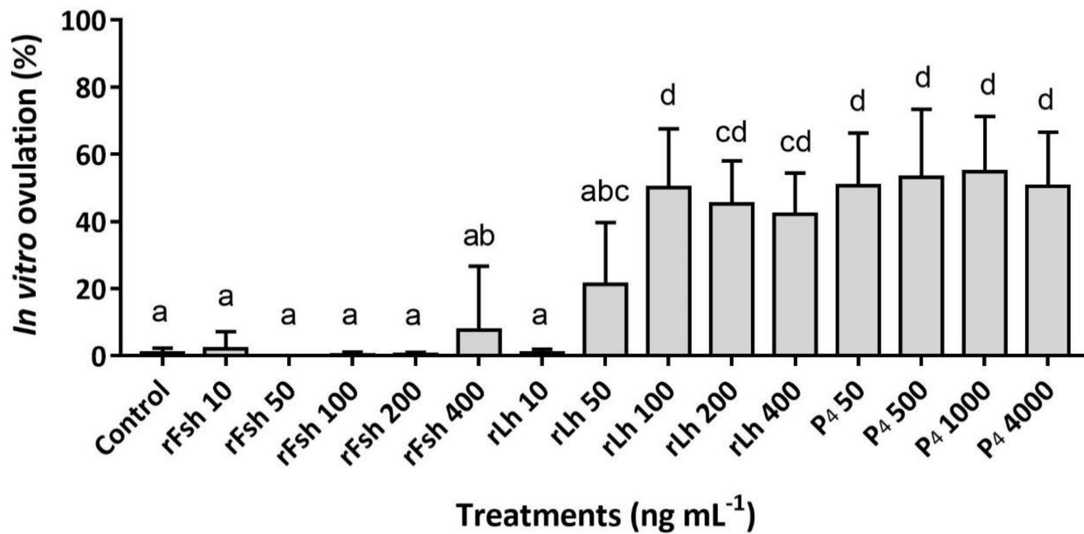

Figure 12. *In vitro* percentage of ovulation (mean ± SD) of oocytes in OM with different effectors and doses. Statistical differences between treatments were examined by a one-way RM ANOVA with individual females as subjects (3 replicates per individual, 6 individuals, n = 18 wells per hormone concentration). rFsh, recombinant follicle-stimulating hormone; rLh, recombinant luteinizing hormone; P_4, Progesterone.

3.4. Egg and larval quality

No significant differences were found in latency, egg production and quality parameters; fecundity, percentages of fertilization, embryo survival or hatching, in those females that received rLh as priming dose and either rLh or P_4 as resolving injections (**Table 1**). There were also no differences between females that were at different maturity stage at the beginning of the experiment. Mean total fecundity was 1,738,798 ± 868,950 eggs per female (relative fecundity was 1,245,600 ± 552,117 eggs kg^{-1} bw) with 54 ± 21 % fertilization. On the contrary, spawned eggs from one female that received P_4 + P_4, were not fertilised (0% fertilization).

No differences were observed in egg and larval quality between rLh + rLh and rLh + P_4 groups. The mean embryo survival and hatching percentages were 64 ± 22 % and 57 ± 24 %, respectively. Hatching in EIA 96-well plates was 96 ± 3 %. Eggs with an embryo measured 0.83 ± 0.02 mm in diameter (**Fig 13A**) and larvae length at hatching was 1.86 ± 0.14 mm total length (TL). Larval survival percentages in the 96-well plates at 21 ºC

were 85 ± 18 % at 2 dhp, 67 ± 18 % at 5 dhp, 55 ± 17 % at 9 dph and decreased until 8 ± 11 % on 12 dph. By 14 dph, all larvae were dead.

Larvae used for the preliminary larval culture trial measured 2.1 ± 0.05 mm TL at hatching showing a homogeneous yolk mass with a round oil droplet at the posterior part of the yolk sac (**Fig 13B**). The eyes started to be pigmented between 1 and 2 dph (**Fig 13C**). Upper and lower jaws were well-developed and the mouth was open from 3 dph (2.93 ± 0.08 mm TL, **Fig 13D**, **Fig 14**) when the larvae had consumed most of the yolk reserves and only the oil globule remained (**Fig 13E**). The pre-flexion stage lasted from 4 to 19 dph (**Fig 13F**) with yolk and oil droplet completely absorbed (11 dph). Swim bladder formation started around day 4 - 6 (2.9 - 3.11 mm TL, **Fig 14**) visible ventrally beneath the notochord. The swimming activity of the larvae increased during the formation and enlargement of the swim bladder although several larvae either swam at the bottom of the tank, or remained floating in the water surface without moving and/or feeding on rotifers due to a hyperinflation of the swim bladder (**Fig 13G**). Most of these larvae died and, in order to reduce this mortality, light intensity was reduced to 500 lux from 9 dph until the end of the rearing trial. The tail flexion was completed when the larvae reached 3.7 mm length whereas the post-flexion stage was extended during several days (20 - 30 dph, 4.3 - 5.3 mm TL, **Fig 14**) until the caudal fin and fork were completed. At the end of the trial (day 39) all the fish looked like adult individuals and were considered juveniles (**Fig 13J**). Development of *Mugil cephalus* larvae and post-larval stages to juvenile are shown in Fig 13 and Fig 14.

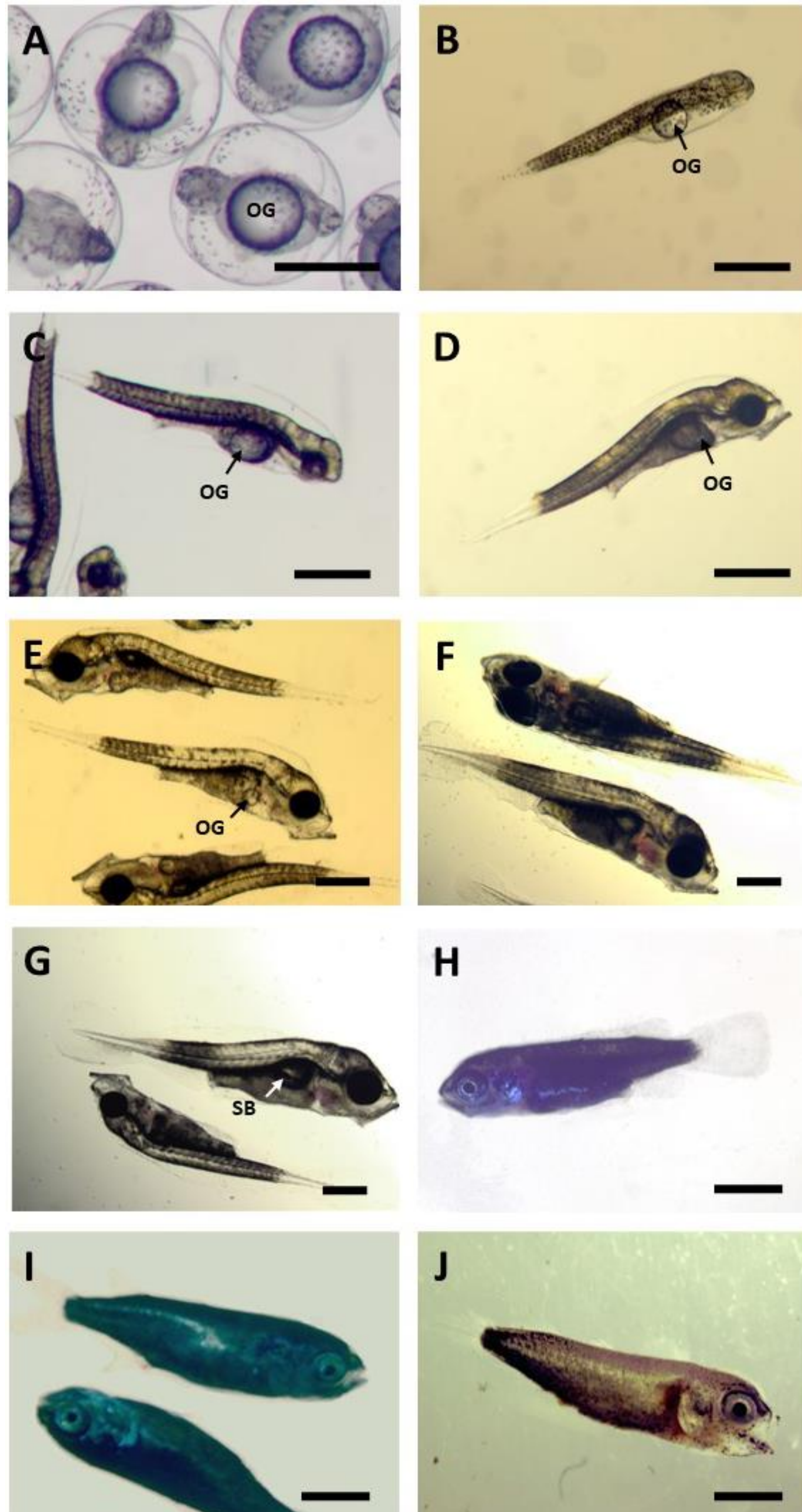

Figure 13. *Mugil cephalus* embryos, larvae, post-larval and juvenile stages. (A) Embryo with head region formed and dark pigment covering almost all the body and the oil globule (OG), (B) newly hatched larvae, (C) larvae at 2 dph with eyes already pigmented, (D) larvae at 3 dph with the mouth parts completely formed and functional, (E) larvae at 9 dph with the oil globule still present, (F) larvae at 19 dph with the oil globule completely absorbed and (G) with a hyperinflated swim bladder (SB), (H) post-larvae at 32 dph, (I) post-larvae at 37 dph, (I) juvenile at 39 dph. Scale bar: 500 µm in A, B, C, D, E, F and G; 1 mm in H, I, and J.

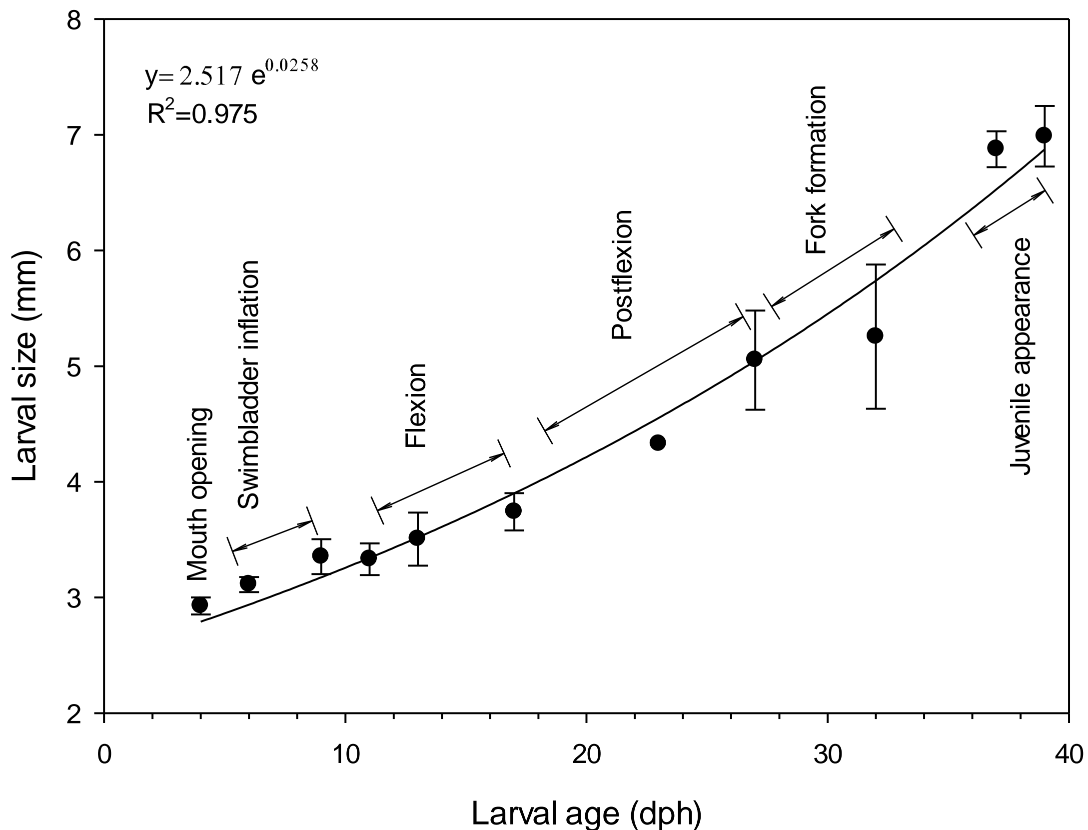

Figure 14. Growth performance of flathead grey mullet (*Mugil cephalus*) larvae in terms of total length (mm, mean ± SD), in relation to time (days post hatch, dph) showing the timing of appearance of the main developmental stages.

4. Discussion

Flathead grey mullet (*Mugil cephalus*) held in intensive captive conditions experience reproductive dysfunctions early in the maturation process. In the present study, both males and females needed to be assisted to induce vitellogenesis, oocyte maturation, ovulation, enhance spermatogenesis, spermiation and spawning. The present results showed that rFsh and rLh can be used as a reliable method to induce and complete oogenesis from previtellogenesis, produce milt and induce spontaneous voluntary tank spawning in 100 % of experimental fish to provide viable, good quality eggs and larvae.

Observing the ovarian developmental stage at the start of the experiment, a range of dysfunctions were found in females; fish were arrested at stages that ranged from previtellogenesis as in Ramos-Júdez *et al*. (2021) through to early vitellogenesis as reported in other Mediterranean areas [6]. Nevertheless, in the present study, the ovaries of the early-vitellogenic females at the beginning of the experiment had few vitellogenic oocytes that were not distributed homogeneously, being a clutch of previtellogenic oocytes the most abundant. The females that received the rFsh and rLh treatment, as previously described by Ramos-Júdez *et al*. (2021), showed a uniform clutch of abundant oocytes recruited into vitellogenesis and followed the typical ovarian development in this species. *Mugil cephalus* has group synchronous ovary development with one clutch of oocytes maturing annually following a single spawning episode [136]. The rGth hormonal administration was 100 % successful in inducing vitellogenesis from previtellogenesis (12 of 12 females) and early vitellogenesis (9 of 9 females) to complete vitellogenic growth. In comparison, control females that were at early vitellogenesis remained without advances in gonad development during the experiment and presented atresia, which may indicate a lack of stimulation of the vitellogenic oocytes to develop further [152]. Previtellogenic control females did not show further development. This observation is in clear contrast with Aizen *et al*. (2005), in which up to 20 % of control females developed mature oocytes without hormonal stimulation. The completion of vitellogenesis in rGth-treated females was accompanied by an increase in plasma E_2 levels, that was not observed in controls, showing the gonadotropic stimulation of the ovary by rGths. Ramos-Júdez *et al*. (2021), using the same rGths, obtained an almost identical result with an increase in plasma E_2 levels and eight out of nine (89%) of treated females completing oogenesis.

The rGth treatment in males induced and enhanced spermatogenesis and spermiation as 100 % of treated males produced fluent milt for 8 weeks or more. In comparison, control group males remained with no sperm or viscous sperm during the experiment. The histological examination of males at different stages of spermiation showed that initial or control captive males that presented no sperm or a little drop of viscous sperm had undeveloped testes with a low GSI ($\lesssim$ 0.1 %), predominantly SPG and SPC and few or no SPD or SPZ. In comparison, the histological examination showed rGth-

treated males had well developed testes with a significantly higher GSI (5.35 ± 1.25 %), that was 50 times higher than control males, and testes were filled entirely with SPZ and no SPG or SPC. Although there was significant growth, some caution is perhaps required in extrapolating the histology from two fish to the other individuals in the control and rGth groups. Few males were available and the n for histology was reduced to ensure sufficient males for rGth treatment and spawning. All the evidence indicates that the rGths have induced spermatogenesis of large numbers of germ cells from early stages (SPG and SPC) through to SPZ. Clearly more work is required for confirmation of this rGth action and a specific study on the direct effect of each rGth on testes development and in the pattern of administration has to be performed to consolidate conclusions. However, other studies, as suggested in the present study, have successfully induced spermatogenesis with rGths, i.e. in sexually immature European eel [207], immature Japanese eel [95,114,130] and mature Senegalese sole [37,38]. Peñaranda *et al*. (2018) represents a specific study that tested different combinations in the application of homologous rFsh and rLh (produced by Rara Avis Biotec, S. L. as in the present study) in immature European eel that led to different testis development including different degrees of spermiation. The biological effects of rGths on males were also evaluated through plasma 11-KT levels, which is the major androgen responsible for testicular development [6,39,155,231]. The rGth treatment significantly increased the levels of 11-KT in the plasma of treated males compared to control males. The concentration of plasma 11-KT in treated males increased gradually as maturity progressed shown by the presence of sperm and the increased fluidity of milt obtained in all treated males from 5 weeks onwards. The levels of 11-KT measured, from 0.1 to 28 ng mL^{-1}, were in the range of levels previously measured in flathead grey mullet males treated with 17α-methyltestosterone implants to enhance spermatogenesis and spermiation [6].

Higher quantities of fluent milt were obtained (0.25 to 2.89 mL) in the present study compared to Ramos-Júdez *et al*. (2021) (max ~ 0.25 mL). The increased sperm production may be due to the different pattern and dosage of rGths administration, which included a longer period and higher doses of rLh. The quality of the sperm was variable amongst the nine rGth-treated males, but had adequate mean sperm quality parameters of motility and velocity. The volume of sperm obtained was amongst the

highest reported for wild mature *Mugil cephalus*, such as 0.1 to 2 mL [213], and the concentration was two powers to ten (10^{10}) higher than previously reported (10^8) [213], indicating that a higher degree of dilution during spermiation, which is attributed to the action of Lh [232], would be desirable. The extender Marine Freeze® was effective to maintain sperm quality during 6 days of cold 4 ºC storage, which also indicated the sperm was of good quality.

From an applied point of view, male development was synchronised with the female's completion of vitellogenesis making them available for the spawning events. Selected males had fluid sperm and were administered either 12 or 24 μg kg^{-1} rLh to stimulate spermiation and reproductive behaviour. Regarding females, three treatments were applied to induce oocyte maturation, ovulation and spawning. The application of 30 μg kg^{-1} rLh as priming injection induced oocyte maturation with the migration of the germinal vesicle in all 100% of females when revised before the application of the resolving dose (~24 h after the priming dose). The following resolving injections of 40 mg kg^{-1} P_4 or 30 μg kg^{-1} rLh induced 100 % of the fish to ovulate, and 89 % (P_4 resolving) or 100 % (rLh resolving) of the fish to tank spawn, with a mean percentage fertilization of 54 ± 21 % indicating the success in the application of both treatments. The *in vivo* success of the resolving doses was confirmed by the success of both P_4 and rLh to induce *in vitro* ovulation after the priming dose of rLh. The *in vitro* test indicated the importance of the selection of a correct rLh dose for the induction of ovulation, as the response of oocytes was different depending on the rLh concentration in the media. Otherwise, low P_4 doses were as effective for inducing ovulation as high doses, which suggests that a refinement in the P_4 dose applied *in vivo* would be possible. On the contrary to females that received rLh as a priming injection, females that received priming P_4 had not initiated OM ~24 h after the priming dose. The P_4 resolving dose had no effect on 50 % of females, whilst the other 50 % of the females ovulated, and from this, just one female (17%) spawned eggs that had no fertilization. Although P_4 as priming and resolving treatment did induce ovulation, all the maturation and ovulation process was concentrated in less than 24 h after the resolving dose was applied. In comparison, females that received rLh as priming completed OM and ovulation during 40:45 ± 2 h; initiated OM after the priming dose, and completed OM, ovulation and spawning after the resolving dose. It is

possible that ovulation was induced by the double P_4 even though OM had not been properly induced, indicating the importance in this process of Lh or Lh-induced factors as previously indicated by Ramos-Júdez *et al.* (2021). In that study, only the females that received the highest priming rLh dose (30 µg kg^{-1}) proceeded to OM compared to females that received a lower priming rLh dose (15 µg kg^{-1}), which did not develop to OM. Taken together, the action of the hormones appears to confirm described roles [152,155], as high doses of rLh were required to induce oocyte maturation and either rLh or P_4 were needed after the priming rLh dose to induce ovulation and spawning.

The two spawning treatments, rLh + rLh and rLh + P_4, induced spontaneous tank spawning of large numbers of fertilised eggs. Therefore, the rGth treatment did not only induce gametogenesis development, but also the reproductive behaviour of both sexes to achieve a successful courtship that led to spawning and successful fertilisation of liberated gametes. The presence of good quality males in the tank with fluent milt may also have been a decisive factor for spawning success. Besides, a proper male to female sex ratio could also have been important. In the present study, male to female ratios of 2:1 or 3:1 were placed together and typical mating behaviour —swimming close to the female, pushing the abdomen with their head and body, ceasing to swim momentarily [272]— was observed by males when females had swollen bellies. In addition, the data from the present study indicate that high-quality eggs up to 80% fertilization can be obtained through the induction of oogenesis by rFsh and rLh in previtellogenic females. Retrieving good quality floating eggs from females that spawned with treated males, contrasts notably with the report by Ramos-Júdez *et al.* (2021) in which no spontaneous spawning was observed after priming rLh and resolving P_4 spawning treatment. Ramos-Júdez *et al.* (2021) did not observe spawning behaviour in rGth treated fish and, therefore, gametes were stripped and artificially fertilised to obtain 0.4 % fertilisation. Therefore, the present study would suggest that other factors such as a delay in the stripping of the eggs coinciding with the process of egg overripening, as stated in Ramos-Júdez *et al.* (2021), may have resulted in the low fertilization percentages in that study rather than the application of the rGth treatment *per se*.

Comparisons of spawning success (number of fish that spawned from total injected), fertilization rates and fecundities in other studies with flathead grey mullets are

limited owing to differences in methodology and initial gonadal development stage of individuals. A few studies have attempted to enhance vitellogenesis, i.e., with 5 mg kg^{-1} Domperidone (Dom) or in combination with implants of 10 µg kg^{-1} gonadotropin-releasing hormone agonist (GnRHa), in which lower rates of fully mature females were obtained (50 - 85 %) [6] compared to the present study (100 %). Many other studies worked directly with fully mature females and applied different treatments to induce oocyte maturation and spawning. For example, Aizen *et al*. (2005) applied GnRHa (10 µg kg^{-1} priming, 20 µg kg^{-1} resolving) combined with Metoclopramide (15 mg kg^{-1} priming and resolving), El-Gharabawy and Assem (2006) injected 20 to 70 mg kg^{-1} carp pituitary extract or 10,000 IU $fish^{-1}$ hCG as priming and one or two resolving injections of 100 - 200 µg kg^{-1} GnRHa, while Besbes *et al*. (2020) treated with a priming dose of 10,000 IU kg^{-1} hCG and resolving of 10,000 IU kg^{-1} hCG and 200 µg kg^{-1}. Those treatments respectively resulted in: (i) 85 % spawning success with low (< 40 %) to high (< 90 %) fertilization percentages and fecundities of 1,649,000 eggs kg^{-1} bw, (ii) 40 % spawning success with 75 to 80 % fertilization and fecundities of 1,395,000 eggs kg^{-1} bw, and (iii) 100 % success with 63 % fertilization but low fecundities of 418,945 eggs kg^{-1} bw. In general, these studies showed a highly variable spawning success and / or variable fertilization percentages whilst the present study presents a reliable spawning success, from 85 to 100% depending on the spawning treatment, with one of the highest fecundities of 1,245,600 ± 552,117 eggs kg^{-1} bw obtained from females that were successfully induced.

Regarding egg quality of fertilised eggs incubated in 96 well plates, 96 ± 3% of eggs with embryos hatched indicating a high egg quality. Moreover, larvae survived as long as 13 days at 21 ºC without exogenous feeding which is considered a great improvement compared to the previous study by Ramos-Júdez *et al*. (2021), in which larvae survived no longer than 4 dph at 24 ºC. Not only larvae survived longer without the application of external feeding, but also larvae reared using mesocosm conditions demonstrated the potential to develop until juveniles, indicating that eggs and larvae obtained after the induction of gametogenesis with rFsh and rLh could supply a hatchery. Indeed, larval development and growth in our study, using mesocosm rearing conditions, was very similar to other published studies either using intensive or extensive conditions

[1,16,55,148]. Most of these studies, emphasized the effects of algal addition to the rearing tanks (green water technique) as the best way to optimize larval feeding on rotifers due to its effect not only in facilitating the contrast but also improving the nutritional state and / or health of the rotifers [245]. The exponential growth recorded in the present study was similar to what Besbes *et al*. (2020) described, with larvae measuring 2.1 mm TL at hatching, and 2.96 mm TL at 4 dph, followed by a stagnated growth between 4 dph and 11 dph when the larvae reached 3.5 mm TL. Growth was accelerated from 13 dph to 35 dph when they reached 5 mm TL and then until day 39 when they reached almost 7 mm TL. Therefore, it was promising that the larvae reared in the mesocosms demonstrated similar growth and development as other studies on flathead grey mullet larvae, indicating the potential for hatchery production of larvae from adults that had gametogenesis-induced with rGths.

Two critical periods have been described during larval rearing of flathead grey mullet [101]. One at 2 - 3 dph due to the yolk sac resorption, a decrease in lipid reserves and an increase in specific gravity of the larvae, sinking to the bottom of the tank and gradually perish [160], and the other, at 8 -11 dph during swim bladder formation with an excessive inflation, especially in intensive ("artificial") rearing systems [187,189]. These periods would justify the higher mortalities from 85 ± 18 % survival at 2 dph to 55 ± 17 % at 9 dph, and 8 ± 11 % at 12 dph observed in larvae maintained in 96-well plates without exogenous feeding. Although survival was not the aim of the larvae rearing in mesocosms, the main mortality problem encountered was swimbladder hyperinflation. The larvae affected (**Fig 13G**) remained floating in the water surface without moving and / or feeding on rotifers. This over inflation has been observed in other marine fish larvae such as in meagre (*Argyrosomus regius*) larvae [258], and is often associated with stressful conditions such as high light intensity, the use of long photoperiod, too early introduction of prey during larval rearing, or high larval density [87,220,261] that induce the larvae to gulp too much air in the water surface inducing the hypertrophy of the swim bladder.

In conclusion, the approach described in the present study to induce oogenesis from previtellogenesis or early vitellogenesis to the completion of oocyte growth and spawning using single chain recombinant gonadotropins (rFsh and rLh) produced in CHO

cells, offers replicability and guarantees a high success in spawning and high egg quality in flathead grey mullet. It was significant that in addition to inducing maturation from early gametogenesis through to the production of viable male and female gametes, the rGths have also induced the processes and cascade of hormones and pheromones that control reproductive behaviour and successful courtship in both females and males. From an applied point of view, the present protocol provides full control of reproduction with long-term weekly rGth administration. The protocol provided high fecundities from flathead grey mullet females (~ 1,700,000 eggs female^{-1}), with fertilisation and hatching of ~ 50 % of the spawned eggs. These fecundities, indicate that the induction of 6 - 7 females (~1 kg) per season could permit a hatchery production of ~ 1 million fry, based on survivals reported in the literature. This would reduce the need of many breeders and the quantity of hormones used. Besides, the present protocol can probably be applied to develop out-of-season spawning in flathead grey mullet and other species with similar reproductive dysfunctions in captivity.

CHAPTER VII

Transcriptome analysis of flathead grey mullet *Mugil cephalus* ovarian development induced by recombinant gonadotropins, rFsh and rLh

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1. Introduction

The development of eggs, oogenesis, is the process by which the female gametes develop from oogonia, the precursor of oocytes, to matured and fertilizable ova. In teleost, as in other vertebrates, oogenesis is controlled by the brain-pituitary-gonad axis, wherein the pituitary glycoprotein hormones, gonadotropins (Gths) play a crucial regulatory role. Two types of Gths, the follicle-stimulating hormone (Fsh) and the luteinizing hormone (Lh), are produced and released into the bloodstream by the pituitary gland under the stimulation of the hypothalamus-produced gonadotropin-releasing hormone (GnRH) [156]. The Gths bind to their cognate receptors in the gonads and regulate oogenesis mainly through the stimulation of gonadal steroidogenesis, which is the ultimate mediator of the different developmental stages. In broad terms, the principal role of Fsh is to promote the onset of vitellogenesis through the stimulation of steroidogenesis in the follicular cells —hepatic synthesis of vitellogenins and uptake by the oocyte to be processed into yolk globules [152,218]—. Whereas, Lh is mostly involved in regulating the late stages of oogenesis, including the final gamete maturation and ovulation [152].

The development so far to produce recombinant Gths (rGths), recombinant Fsh (rFsh) and Lh (rLh), through expression of their cDNAs in heterologous eukaryotic systems such as yeast, insect cells, and mammalian cell lines have helped to determine the specific functionality of Gths [143,170]. Under the study of their function, rGths have been tested as therapeutic agents to overcome problems halting oocyte development in several fish species [3,75,119,127,170,190,201,223,225]. Similarly to many wild animals held in captivity, many fish of commercial interest to the aquaculture industry exhibit reproductive dysfunctions as a result of the captive environment. Different failures during oogenesis

development can occur in females, with a dysfunction in the release of Gths as the main endocrine cause of gonadal arrest [178].

The flathead grey mullet (*Mugil cephalus*) is an important marine fish species in Asian and Mediterranean countries [83] with a capture of 111,932 tons and a production of 6124 tons in 2019 [63]. The most severe dysfunction this species shows when maintained in captivity, is that females remain arrested at previtellogenesis and do not undergo vitellogenesis [214] and, consequentially, aquaculture production is based mainly on the capture of wild juveniles or broodstock, which is unsustainable in the long term [281]. Therefore, artificial hormone manipulation is needed to force the mullet previtellogenic gonad to enter into vitellogenesis, complete oocyte development to maturation and ovulation and obtain eggs and larvae. Recently, the induction of vitellogenesis to produce fertilized eggs that developed to larvae has been possible by means of a therapy with the use of homologous single-chain rFsh and rLh produced in Chinese Hamster Ovary (CHO) cells, whereas untreated females remained at previtellogenic gonadal arrest [214] (Experiment 2, CHAPTER VI). Provided the rGth approach was successfully used to induce and complete oogenesis, the study of stage-specific molecular variations underlying the ovarian development under the stimulation by rGths might provide insights into their direct role in the flathead grey mullet oogenesis.

To date, large-scale studies for the transcriptome of the teleost ovary have improved the knowledge of the molecular and cellular mechanisms of this complex process in fish by examining the transcriptomic signalling during the reproductive cycle [22,25,36,80,125,153,226,248,262]. Moreover, recent studies applying RNA-sequencing (RNA-Seq) that provide with a larger gene-diversity coverage have elucidated the mechanisms of ovary differentiation [31,91] and transcriptomic changes during different stages of ovarian development in a natural breeding season in non-model organisms [76,158,217].

In this context, the aim of this work was to unravel the transcriptomic changes that occur in the ovary of female flathead grey mullet during rGth-induced vitellogenesis. For this, we used an RNA-Seq approach and *de novo* transcriptome assembly of samples collected at previtellogenic arrested stage and at different points during the ovarian development under the stimulation of rGths from the study developed by Ramos-Júdez *et al.* (2021) (Experiment 2, CHAPTER VI). These data constitute a resource for elucidating

the molecular mechanisms that underlie ovarian development induced by rGths which will help to improve induction protocols and facilitate the breeding of flathead grey mullet and other species with similar reproductive dysfunctions.

2. Material and methods

2.1. Animals and experimental design

Five flathead grey mullet (*Mugil cephalus*) females (mean ± SD, 915 ± 126 g initial body weight; 38 ± 3 cm standard length) that were arrested at early stages of gonad development (previtellogenesis) were selected from an experimental group and were sampled to follow ovarian development induced with species-specific rGths as described in Ramos-Júdez *et al.* (2021) (Experiment 2, CHAPTER VI). Briefly, the fish were weekly treated with increasing doses of rFsh —from 6, 9 to 12 μg kg^{-1} — and after four weeks, rFsh was combined with rLh —increasing doses of 2.5, 4 to 6 μg kg^{-1}—. Then, when oocyte diameter was ≥ 550 μm, only rLh —9 and 12 μg kg^{-1}— was administered on a three-day-basis to complete oocyte growth to ~ 600 μm. Broodstock origin and culture conditions are further detailed in Ramos-Júdez *et al.* (2021) (Experiment 2, CHAPTER VI).

Repeated ovarian samples were collected by cannulation from the five females at four sampling points during the induced gonadal development; (1) before rGth treatment from initial arrested gonad (Stage I), (2) after four weeks of rFsh administration (Stage II), (3) after the combined treatment with rFsh and rLh (Stage III), and (4) after rLh treatment (Stage IV). At each sampling procedure, fish were first placed in a tank with 73 mg L^{-1} of MS-222 (Sigma-Aldrich, Spain) and moved to a recipient with 65 mg L^{-1} of MS-222 for manipulation.

2.2. Ovarian biopsy collection

Biopsy samples were aspirated into a plastic cannula inserted through the urogenital pore and were thoroughly divided into three portions. One portion was expelled into 2 mL Eppendorf tubes containing RNAlater® (Sigma-Aldrich, Spain) held at 4 ºC before storage at -80 ºC until further processing. The second portion was used for *in situ* measurement of the diameter of the largest oocytes (n = 20) by light microscopy (Zeuss Microscopes) and the third portion was fixed in Bouin's solution for 24 h and storage in 70% ethanol for histological analysis.

2.3. Histological analysis

Ovarian samples fixed in Bouin's solution and kept at 70% ethanol, were dehydrated in ascending grades of ethanol, embedded in paraffin, then sectioned at 3 µm thickness and stained with hematoxylin and eosin (Casa Álvarez, Spain). The histological slides were observed under a light microscope (Leica DMLB, Houston, USA). The percentages of different stages of oocyte development were calculated by counting 100-150 oocytes in each gonad sample. Maturation stage of females was determined according to the most developed and abundant stage of oocytes present.

2.4. RNA extraction, library preparation and sequencing

Total RNA was extracted from the twenty ovarian samples using the RNeasy® extraction kit (Qiagen) following the manufacturer's recommendations that includes an on-column DNase digestion to remove gDNA from total RNA preparations. The amount of isolated RNA was measured by spectrophotometry (NanoDrop® ND-2000, Thermo Scientific™) and its integrity was assessed through agarose gel electrophoresis (2%) according to Masek *et al*. (2005). Transcriptome libraries were constructed from the five biological replicates for each state (Stage I-1/2/3/4/5, Stage II-1/2/3/4/5, Stage III-1/2/3/4/5 and Stage IV-1/2/3/4/5). Libraries were created with the Illumina TruSeqTM RNA Sample Preparation Kits following the manufacturer's protocol and sequenced as 150 bp paired-end reads in one lane of a HiSeq 4000 TM performed by the Norwegian Sequencing Centre (Oslo, Norway).

2.5. *De novo* transcriptome assembly and quantification

Raw reads quality control was performed using FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and were preprocessed through a pipeline using Trimmomatic v0.39 [23] to remove reads containing adapters and low-quality reads with parameters set to "ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:2:keepBothReads SLIDINGWINDOW:4:15 LEADING:10 TRAILING:10 MINLEN:75 AVGQUAL:30". Then, all gonadal stages trimmed reads were assembled

together to obtain a single transcriptome. Trinity software v2.8.5 was used for *de novo* assembly with default parameters settings (Kmer = 25) [85,89]. Assembled transcriptome completeness was assessed with BUSCO v3.1.0 [238] using the vertebrate orthologs database as reference. Trimmed reads of each gonad sample were mapped back separately to the assembly with Bowtie2 and the calculation of relative abundances was performed by RSEM through a Trinity script [144]. Gene expression given as Fragments Per Kilobase of transcript per Million mapped read (FPKM) was calculated and transcripts with less than 1 FPKM were excluded. All computations were performed at the high-performance computing bioinformatics platform of HCMR (Crete, Greece).

2.6. Transcriptome functional annotation

The assembled transcripts were functionally annotated using Trinotate pipeline v3.2.1 with e-value cut-off of 10^{-5} (https://trinotate.github.io/). TransDecoder v5.5.0 (http://transdecoder.github.io) was run to predict coding peptide sequences within the transcripts and transform the longest open reading frame (ORF) of 100 amino acids or more into peptide sequences. The sequence similarity of the assembled transcripts with more than 1 FPKM was evaluated using BLASTX (NCBI-blast 2.9.0+) against diverse databases (UniProtKB/SwissProt database, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO)). The TransDecoder peptide sequence file for final candidate longest-ORF was searched for amino acid sequence homologies using BLASTP (NCBI-blast 2.9.0+). In order to identify conserved protein families among the predicted peptide sequences, HMMER hmmscan (v3.3) [67] was used for protein domain identification against Protein family (Pfam) database. In addition, SignalP (v4.1) [209] was used to predict the presence of signal peptides, and the TMHMM (v2.0) [134] was used to predict transmembrane helices within the predicted ORFs. All the outputs from BLASTX, BLASTP, HMMER, THMM and SignalP were loaded into the Trinotate SQLite database and generated a flat file report containing all annotation information for each contig.

2.7. Quantification and analysis of differentially expressed genes (DEGs)

Count data matrix from the filtered transcriptome was constructed and imported in R v3.6.1. Genes with less than thirty reads in all samples were excluded prior to differential expression analysis to improve the statistical power. To visually explore the global gene expression pattern in the samples, a principal component analysis (PCA) was performed on the normalized counts after the variance stabilizing transformation (VST) [151] (including all the genes which sum of counts in each row >30). Differential expression analysis was performed on raw reads by DESeq2 v1.26.0 under Bioconductor package, which uses the Benjamini and Hochberg (1995) method for multiple testing correction of the P-values obtained by the Wald test. Pairwise differential expression analyses were performed between gonadal stages (Stage I vs II, Stage II vs III, Stage III vs IV, Stage I vs III and Stage I vs IV) with special attention to comparison of gonadal stages in time lap sequence (Stage I → II, Stage II → III, Stage III → IV). Genes with an adjusted P-value < 0.05 were considered to show statistically significant differential expression. Analyses were performed within the R statistical environment.

To obtain the DEGs that were specifically expressed or shared between determinate stages, up/down-regulated Venn diagrams were produced for (1) the DEGs obtained from the comparisons in time-lap sequence (Stage I - II, Stage II – III, and Stage III – IV), and (2) the DEGs obtained from the comparisons of Stage I with II, III and IV.

2.8. Enrichment analysis of DEGs

To gain an insight into the biological roles of the significant up- or downregulated genes in ovarian stages in time-lap sequence (Stage I - II, Stage II - III, Stage III – IV), GO and KEGG pathway enrichment analysis were performed. For this, the enrichment factor was calculated as: the number of differentially expressed genes in a specific GO term or KEGG pathway divided by the total number of genes in this GO term or KEGG pathway. The statistic of enrichment, which is the probability of observing *k* or more significantly expressed genes in the pathway by chance, was calculated as the sum of the mass functions —following a hypergeometric equation— for each gene count that is greater than or equal to the number of genes observed [117], as follows:

$$P(x \geq k) = \sum_{k=0}^{x} \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

Where K is the total number of significantly differentially expressed genes, k is the number of significantly differentially expressed genes in a specific pathway, n is the total number of genes in the pathway, and N is the total number of genes.

GO terms and KEGG pathways of DEGs with P-value < 0.05 were considered to show statistically significant enrichment.

3. Results

3.1 Stages of ovarian development

To identify differentially expressed genes during ovarian development induced by rGths in *Mugil cephalus*, samples of ovarian tissue were obtained throughout the treatment course. The histological sections showed that the flathead grey mullet had a group-synchronous ovary under the stimulation of rGths, which means that there were oocytes at different stage of development at a time with the prevalence of one cluster of oocytes that was recruited into vitellogenesis and advanced synchronously through further stages of development.

Females before hormone treatment (Stage I) were arrested at previtellogenesis (before the appearance of yolky oocytes); three females had oocytes at primary growth and two females presented some oocytes that had initiated the secondary growth phase with the accumulation of cortical alveoli around the periphery of the oocyte and inward to the nucleus (mean diameter of the most developed oocytes: 100 ± 17 µm) (**Fig 1A**, **1B**). Samples collected after rFsh treatment (Stage II) were undergoing vitellogenesis and presented, together with previtellogenic oocytes, an abundant clutch of yolky oocytes at early-to-mid vitellogenesis with maximum diameter of 323 ± 46 µm (**Fig 1C**, **1D**). Samples obtained after combined treatment of rFsh and rLh (Stage III) mostly presented oocytes at late-vitellogenesis with maximum diameter of 559 ± 15 µm (**Fig 1E**, **1F**), whereas samples collected after rLh (Stage IV) presented full-grown vitellogenic oocytes with a maximum diameter of 603 ± 3 µm (**Fig 1G**, **1H**).

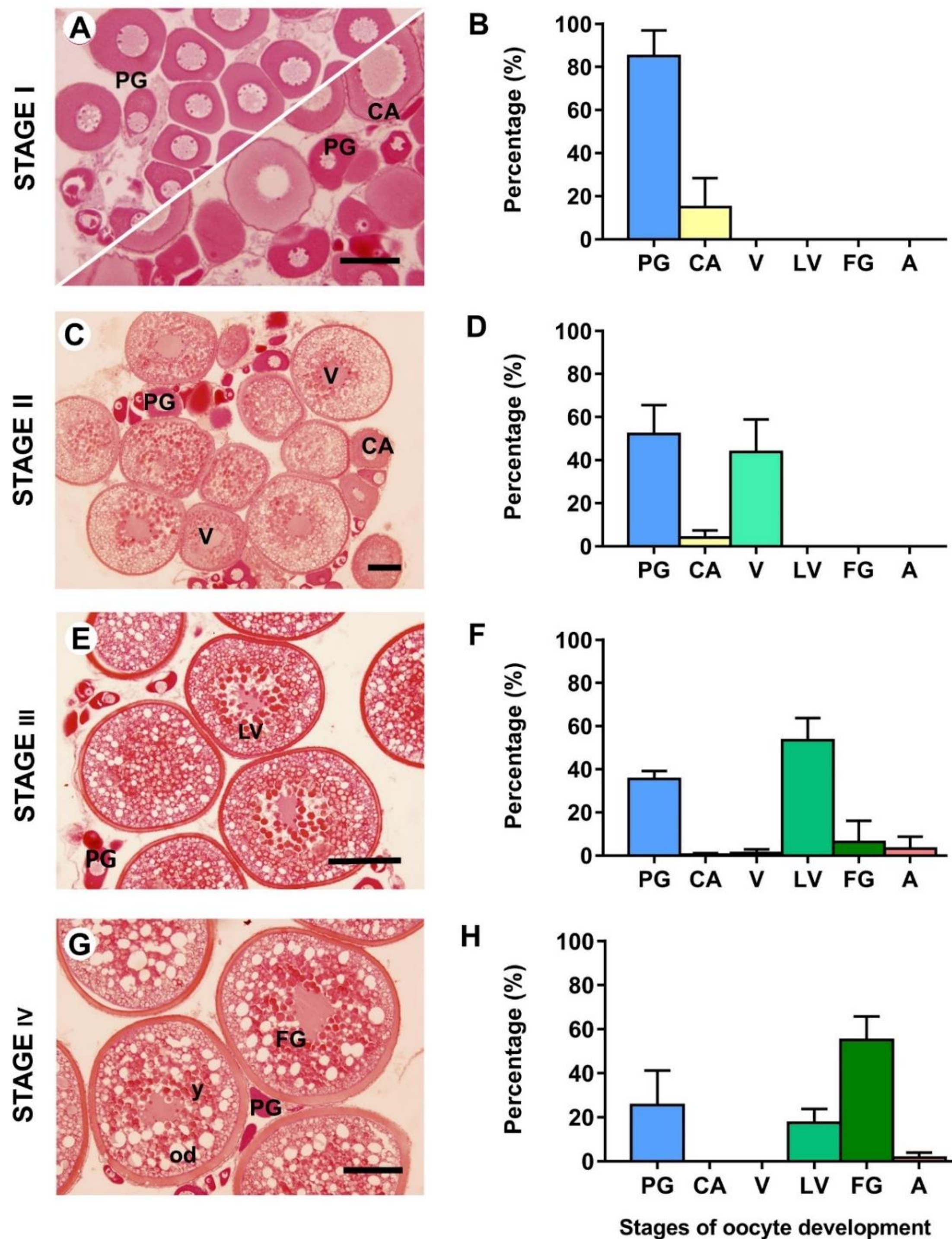


Figure 1. Development of the grey mullet (*Mugil cephalus*) ovaries representing the four points at which samples used for transcriptome sequencing were collected. Light micrographs (A, C, E, G) of oocytes stained with hematoxylin-eosin are representative of the fish used. Frequency of oocytes in the ovary at each sampling point (B, D, F, H) at different developmental stages are presented as the mean ± SD (n = 5 females per stage). (A) Previtellogenic ovary; (C, E and G) vitellogenic ovary. PG, primary growth oocytes; CA, cortical alveolus stage; V, early-to-mid vitellogenesis; LV, late-vitellogenesis; FG, full-grown oocyte; A, atresia; od, oil droplets; y, yolk. Scale bars correspond to 100 µm (A, C) and 200 µm (E, G).

3.2 Quality control, trimming and *de novo* assembly

Illumina HiSeq 4000 paired-end sequencing generated a total of 614,942,156 raw paired reads (307,471,078 read pairs) of which 506,875,944 paired reads were maintained after trimming (82.43 %) (**Table 1**). High quality reads from all stages reads were *de novo* assembled via the Trinity program, which produced initial 513,643 transcripts with an average contig length of 919.18 nucleotides (nt), N50 value of 1,561 nt while the average GC content was 43.61 %. BUSCO revealed an 86.4% of transcriptome completeness (**Table 2**). Moreover, an average of 89.68 % of the reads were successfully back-mapped on the assembled transcriptome (**Table 1**). Transcripts with an expression value of FPKM ≥ 1 were filtered and constituted the final assembly of 287,089 transcripts. This set of filtered transcripts had an average size of 798.43 nt, N50 value of 1,539 nt and an average GC content of 44.13 %.

3.3 Transcriptome annotation

The SwissProt, GO and KEGG databases were employed for annotation of the 287,089 sequences. The BLASTx against the Swiss-Prot databases resulted in 58,306 (20.3 %) transcript gene assignments using $1e^{-5}$ as the e-value cutoff. A total of 57,021 (19.9 %) sequences had a match against the GO database, of which 50,268 (88.2 %) representing biological processes (BC), 53,992 (94.7 %) associated with cellular components (CC) and 48,190 (84.5 %) matching molecular function (MF). A total of 51,237 (17.8 %) sequences were associated to a KEGG pathway.

TransDecoder resulted in 58,370 predicted peptides of which 42,762 (73.3 %) presented blastp hit. This assembly resulted in 37,768 transcripts with predicted Pfam domains, 9886 transcripts with predicted transmembrane helices, 4748 transcripts with a signal peptide cleavage site, and 23,365 transcripts with GO (Gene Ontology) annotations from Pfam-A hits.

Table 1. Overview of RNA-Seq reads and mapping back to the assembled transcriptome.

Sample	Total raw paired reads	Total trimmed paired reads	Trimmed reads ratio (%)	Total mapping ratio (%)
Stage I - 1	35,289,446	28,688,398	81.29	92.01
Stage I - 2	28,957,550	22,272,550	76.91	91.70
Stage I - 3	26,395,762	21,502,258	81.46	91.36
Stage I - 4	35,329,986	29,616,570	83.83	90.44
Stage I - 5	31,666,710	26,290,908	83.02	91.19
Stage II - 1	27,951,938	23,088,548	82.60	91.32
Stage II - 2	27,696,758	23,183,714	83.71	90.82
Stage II - 3	27,949,986	23,023,802	82.38	89.74
Stage II - 4	29,931,924	24,872,658	83.10	89.87
Stage II - 5	33,041,106	27,564,656	83.43	90.55
Stage III - 1	32,244,488	26,676,528	82.73	89.86
Stage III - 2	32,054,802	26,656,800	83.16	89.79
Stage III - 3	36,213,184	30,209,676	83.42	89.47
Stage III - 4	31,790,304	26,881,922	84.56	89.75
Stage III - 5	28,951,286	22,595,958	78.05	90.14
Stage IV - 1	27,270,110	22,687,576	83.20	87.87
Stage IV - 2	24,525,510	20,159,616	82.20	89.15
Stage IV - 3	31,695,898	26,355,426	83.15	80.80
Stage IV - 4	32,273,266	26,954,152	83.52	89.67
Stage IV - 5	33,712,142	27,594,228	81.85	88.10
Total	614,942,156	506,875,944	-	-

Table 2. BUSCO metrics for the transcriptome assembly produced by Trinity.

Complete (%)	Duplicated (%)	Fragmented (%)	Missing (%)
86.4	53.4	9.6	4

3.4. Identification of DEGs

The PCA picture (**Fig 2**) of the Euclidean distance of VST counts showed that samples tended to group together according to the stage, with remarkable scattering of some samples from Stage I. On this general overview, the component 1 of the PCA explained 55 % of the variance, while the component 2 contributed to 11 % of the variance. The separation within samples obtained at Stage I might be related to the different gonad development at which females were arrested; the five females were at previtellogenesis, but three females only had primary growth oocytes (blue points on the left of **Fig 2**), while two females had some oocytes with cortical alveolus (blue points on the right of **Fig 2**). While there was an obvious separation between samples obtained at Stage I and Stage IV, samples from Stages II and III grouped together. The fact that not all the oocytes were at the same stage of development at a time, as previously described, may contribute to the partial overlap of the molecular signatures of samples obtained at different but consecutive sampling points.

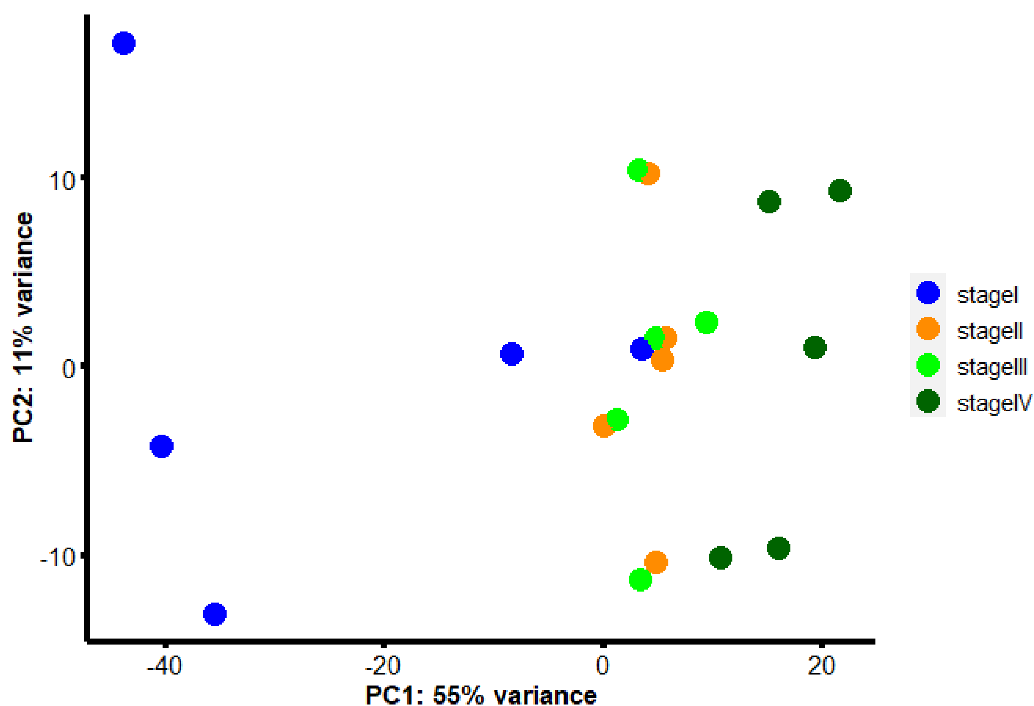


Figure 2. Principal component analysis of sample-to-sample Euclidean distances following VST transformation of gene counts in samples from four points of sampling: at previtellogenic arrested gonadal development before rGth treatment (Stage I - blue); at early-to-mid vitellogenesis after 4 weeks of rFsh treatment (Stage II - orange); at late-vitellogenesis obtained after the combination of rFsh and rLh (Stage III – light green); and full-grown oocytes after the application of rLh to induce the latest stages of oocyte growth (Stage IV – dark green).

A total of 8954, 1113 and 1587 DEGs were found in the comparisons of Stages I – II, II – III, and III – IV, respectively. The analysis of DEGs throughout oogenesis (**Fig 3**) showed that 6147 genes were significantly up-regulated from previtellogenesis (Stage I) to early-to-mid vitellogenesis induced with rFsh treatment (Stage II); 814 genes were up-regulated from Stage II to advanced vitellogenesis (Stage III) obtained after the rFsh and rLh combined treatment, while 994 genes were up-regulated in the transition from Stage III to full-grown oocytes (Stage IV) after rLh application. The corresponding numbers for downregulated genes were 2807, 299, and 593, respectively. Throughout oogenesis more transcripts of genes were upregulated than downregulated and smaller differences in number of DEGs were observed in consecutive stages of development or sampling points rather than in separated developmental stages. The largest difference was detected from previtellogenesis (Stage I) to full-grown oocytes (Stage IV) with 23,169 upregulated and 4965 downregulated DEGs, and the smallest difference was observed between the vitellogenic stages II and III.

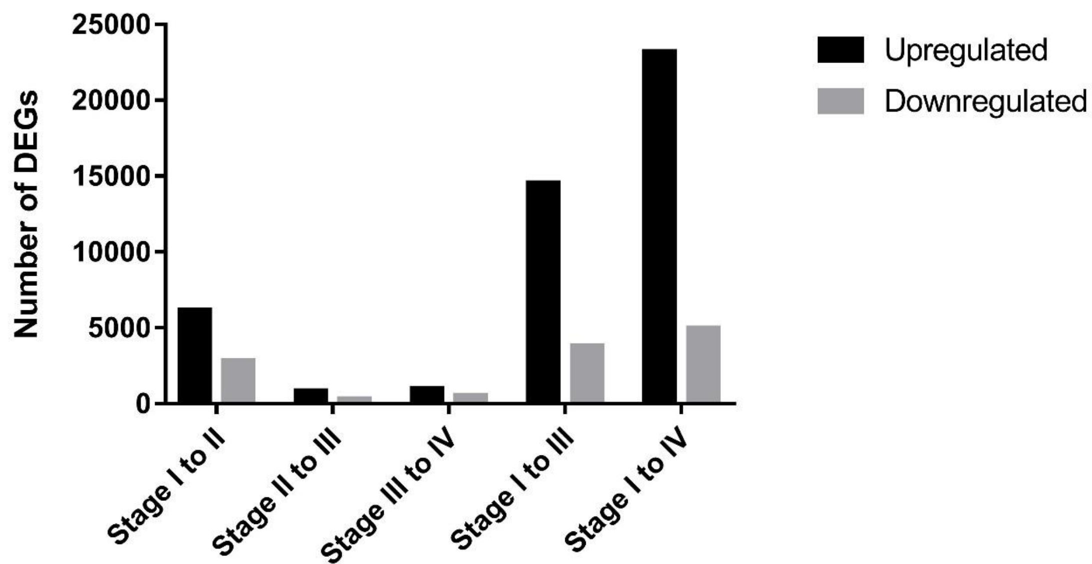


Figure 3. Number of up- and downregulated differentially expressed genes (DEGs) between different sampling points that show different stages of oogenesis induced by recombinant gonadotropins, recombinant follicle-stimulating (rFsh) and luteinizing hormones (rLh), in flathead grey mullet. Stage I, previtellogenesis; Stage II, early-to-mid-vitellogenesis induced with rFsh; Stage III, advanced vitellogenesis after combined treatment with rFsh and rLh; Stage IV, full-grown oocytes after rLh treatment.

According to Venn diagrams, a number of DEGs were only specifically expressed between two developmental stages but not between others. Almost all DEGs obtained in the comparisons of consecutive stages of gonadal development (Stage I - II, Stage II – III, and Stage III – IV) were stage-specific (**Fig 4A, 4B**). Among the DEGs obtained in the comparison of Stage I with II, III and IV, there were 163, 1646, and 10602 stage-specific DEGs with up-regulation in Stage I to II, I to III and I to IV, respectively (**Fig 4C**), and there were 282, 613, and 1508 stage-specific DEGs with down-regulation in Stage I to II, I to III and I to IV, respectively (**Fig 4D**).

A detailed analysis of DEGs (**Fig 5**) identified steroidogenic-related genes that were upregulated at different stages during vitellogenesis: acute regulatory protein (*star*), the 3β-hydroxysteroid dehydrogenase (*hsd3b*), the ovarian form of Cytochrome P450 aromatase (*cyp19a1*), P450 17-Alpha-Hydroxylase/17,20 Lyase **(***cyp17a1*) and estrogen receptors (*esr1* and *esr2*). Also, a wide set of genes related with the insulin-like growth factor (IGF) system (*igf1*, *igf1r*, *igf2*), transforming growth factors (*tgfb1*, *tgfbr1*), and others factors, such as bone morphogenic proteins (*bmp2*, *bmp6, bmp7, bmpr2*) were upregulated during vitellogenesis. Several genes related to lipid metabolism (*fads6*) and transport (*vldlr*, *fapd6, fapd7, fapd1, apoeb, lrp1, lrp2, lrp5, star3, star5*), to the electron transport chain and oxidative phosphorylation (*cyc, cycb, cox2, cox3*), cytoskeletal-related transcripts such as collagen (*col11a1, col12a1*), and lysosomal cathepsins (*ctsc, ctsd, ctsk*) were also identified. The pattern of expression of gonadotropins receptors (*fshr* and *lcghr*) was also identified.

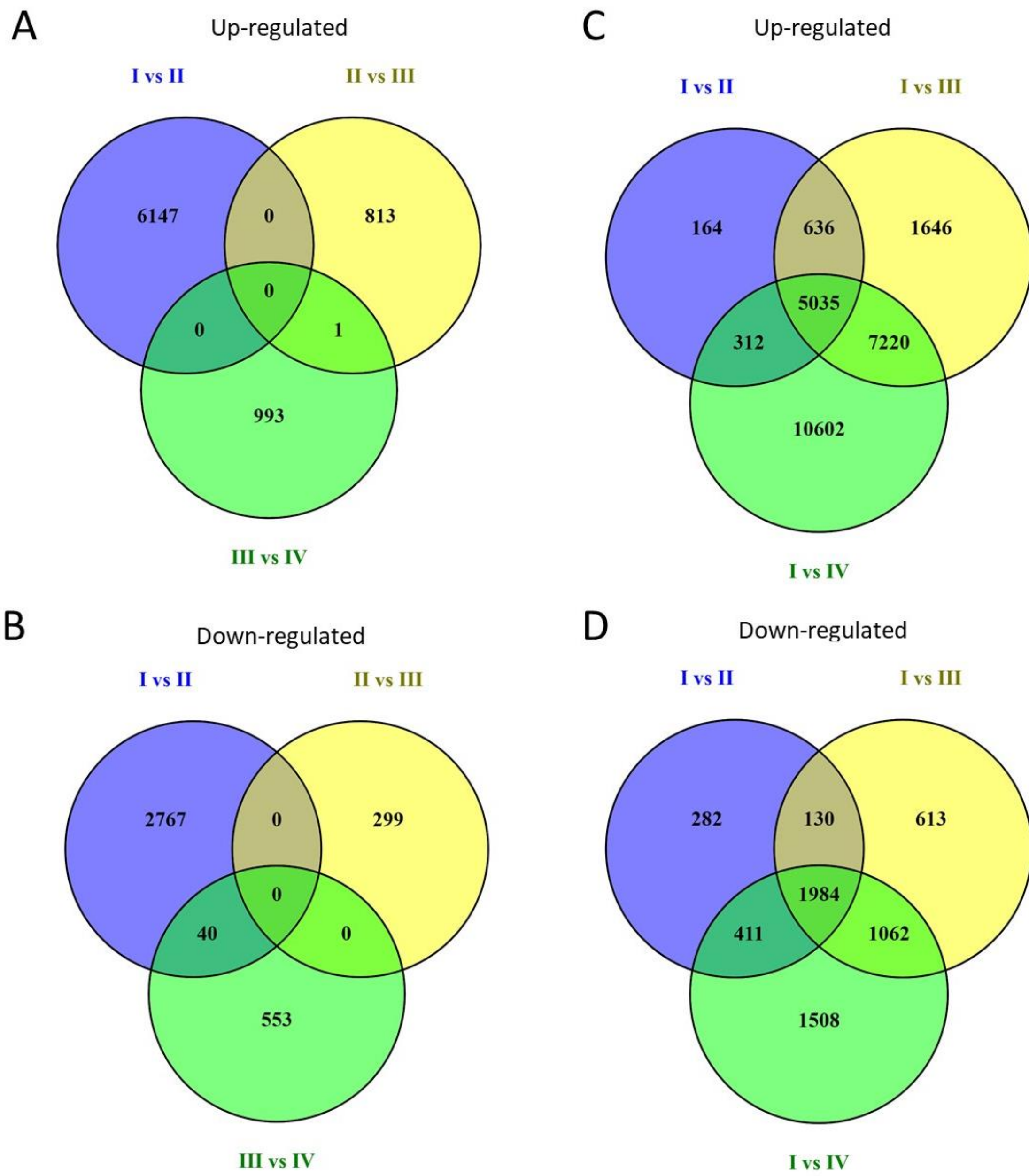


Figure 4. Venn diagrams of up/down-regulated DEGs for (A, B) the comparisons among Stage I with II, II with III, and III with IV, and (C, D) for the comparisons between Stage I with II, III and IV.

Figure 5. Matrix of a set of genes that vary in expression in different stages of ovarian development. Presence of a green or red box indicates up- or down-regulation, respectively, while grey indicates no significant change.

3.5. Gene ontology (GO) functional analysis of DEGs

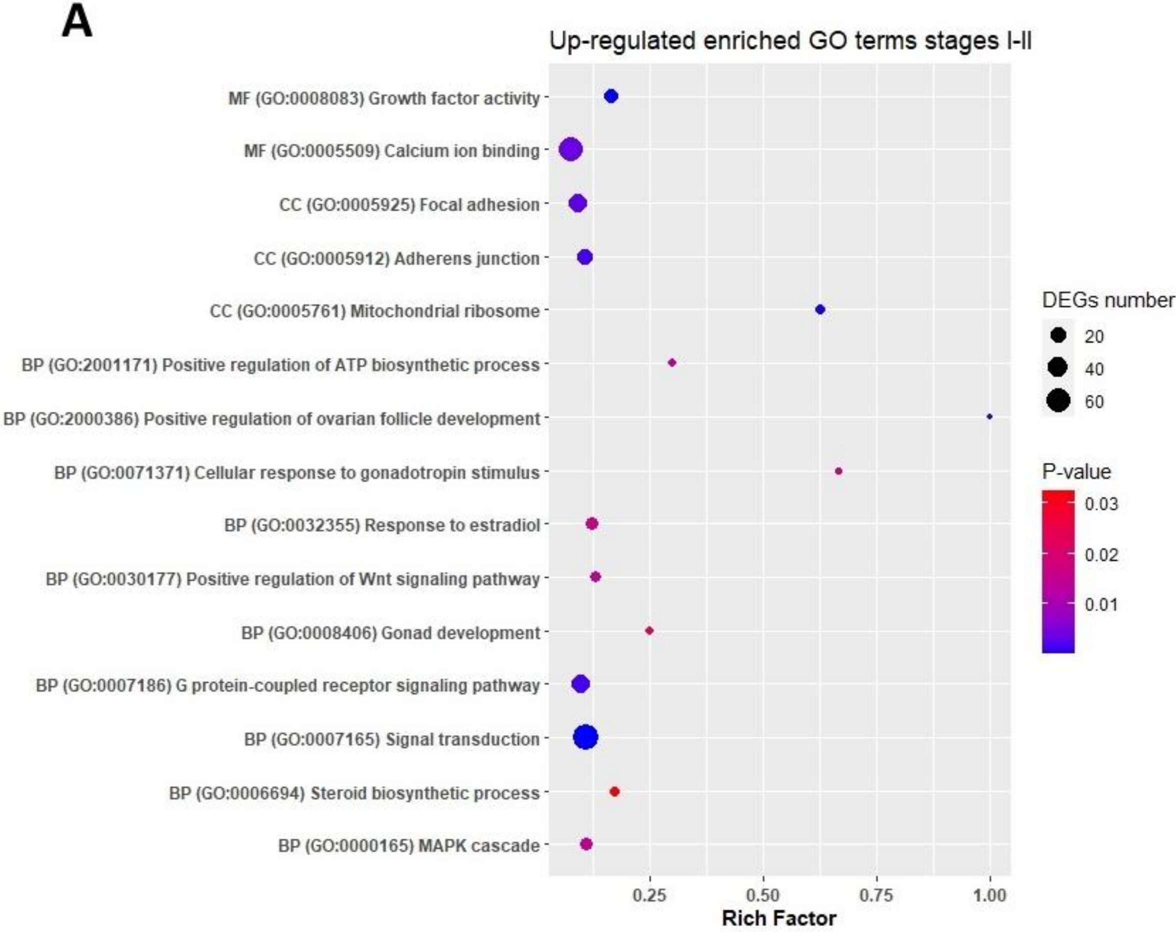
In brief, DEGs from previtellogenesis (Stage I) to early-to-mid vitellogenesis (Stage II), after rFsh administration, were significantly enriched to 1890 GO terms and classified into categories of BP with 1169 GO terms, CC with 307 GO terms, and MF with 413 GO terms. By analysing the significant enriched terms, we found several closely associated with gonadal development. Some of the significant upregulated DEGs were attached to GO terms (**Fig 6A**) related to BP that were *cellular response to gonadotropin stimulus (GO:0071371)*, *gonad development (GO:0008406), positive regulation of ovarian follicle development (GO:2000386)*, *response to estradiol (GO:0032355), G-protein-coupled signalling pathway (GO: 0007186)*, *positive regulation of ATP biosynthetic process (GO:2001171)*, and *positive regulation of Wnt signaling pathway (GO:0030177)*. In the CC category, genes were significantly enriched with the terms of *adherens junction (GO:0005912)* and *focal adhesion (GO:0005925)*, and in the category of MF, genes were significantly enriched with the terms of *growth factor activity (GO:0005509)* and *calcium ion binding (GO:0005509),* among others. The terms of *gonadotropin secretion (GO:0032274)*, *regulation of follicle-stimulating hormone secretion (GO:0046880)*, *response to follicle-stimulating hormone (GO:0032354)*, *3-beta-hydroxy-delta5-steroid dehydrogenase activity (GO:0003854)*, *lipid binding (GO:0008289)* and *glycerophospholipid biosynthetic process (GO:0046474)* were enriched from upregulated genes but not showing a strong significance ($P \geq 0.05$). These pathways should not be ignored because of their importance in the response of the ovary to gonadotropic hormones and their involvement in vitellogenesis. On the contrary, some of the significant enriched downregulated GO terms (**Fig 7A**) related to BP were *negative regulation of electron transfer activity (GO:1904733)*, *mitochondrial electron transport, ubiquinol to cytochrome c (GO:0006122)* and *glycosphingolipid metabolic process (GO:0006687)*. Downregulated terms related to CC were *cytochrome complex (GO:0070069)* and to MF, *carbonyl reductase (NADPH) activity (GO:0008670)*.

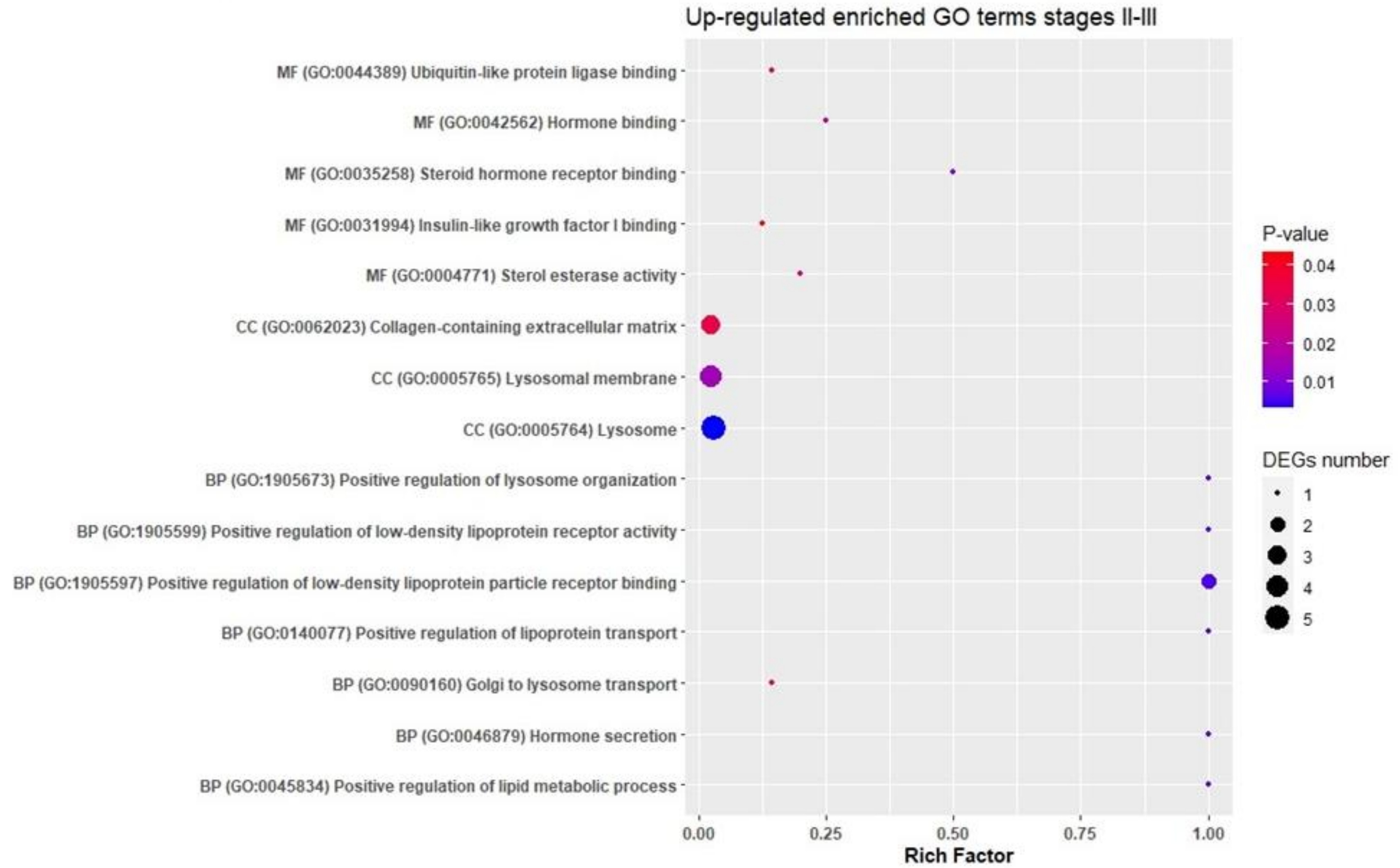
Differential expressed genes from early-to-mid vitellogenesis (Stage II) to late-vitellogenesis (Stage III), were significantly enriched to 170 GO terms and classified into categories of BP with 119 GO terms, CC with 17 GO terms, and MF with 34 GO terms. Some of the significant enriched upregulated GO terms (**Fig 6B**) related to BP were

positive regulation of lipid metabolic process (GO: 0045834), *positive regulation of low-density lipoprotein particle receptor binding (GO:1905597)* and *Golgi to lysosome transport (GO:0090160)*. In the CC category were *lysosome (GO:0005764)* and *collagen-containing extracellular matrix (GO:0062023),* and in the MF category: *steroid hormone receptor binding (GO:0035258)* and *Insulin-like growth factor I binding (GO:0031994)*.

Differential expressed genes between late-vitellogenesis (Stage III) and full-grown oocytes (Stage IV), were significantly enriched to 197 GO terms and classified into categories of BP with 125 GO terms, CC with 24 GO terms, and MF with 48 GO terms. Some of the significant upregulated DEGs were attached to GO terms (**Fig 6C**) related to BP were *regulation of behaviour (GO:0050795)*, *sexual reproduction (GO:0019953)*, *embryonic morphogenesis (GO:0048598)* and *C-21 steroid hormone biosynthetic process (GO:0006700).* Some of the downregulated enriched GO terms (**Fig 7C**) related to BP were *positive regulation of cell-cell adhesion (GO:0033630)*, *collagen fibril organization (GO:0030199)* and *estrogen biosynthetic process (GO:0006703),* and to CC, *tight junction (GO:0070160)*.

Figure 6. Dot plot showing some relevant significantly enriched Gene Ontology biological processes (BP), cellular components (CC) and molecular functions (MF) from upregulated DEGs in (A) Stage I to II, (B) Stage II to III, and (C) Stage III to IV. Rich factor is the ratio of the differentially expressed gene number to the total gene number in a certain GO term. The colour and size of the dots represent the range of the P-value and the number of DEGs mapped to the indicated GO term, respectively.




B
Up-regulated enriched GO terms stages II-III
MF (GO:0044389) Ubiquitin-like protein ligase binding
MF (GO:0042562) Hormone binding
MF (GO:0035258) Steroid hormone receptor binding
MF (GO:0031994) Insulin-like growth factor I binding
MF (GO:0004771) Sterol esterase activity
CC (GO:0062023) Collagen-containing extracellular matrix
CC (GO:0005765) Lysosomal membrane
CC (GO:0005764) Lysosome
BP (GO:1905673) Positive regulation of lysosome organization
BP (GO:1905599) Positive regulation of low-density lipoprotein receptor activity
BP (GO:1905597) Positive regulation of low-density lipoprotein particle receptor binding
BP (GO:0140077) Positive regulation of lipoprotein transport
BP (GO:0090160) Golgi to lysosome transport
BP (GO:0046879) Hormone secretion
BP (GO:0045834) Positive regulation of lipid metabolic process
0.00
0.25
0.50
0.75
1.00
Rich Factor
P-value
0.04
0.03
0.02
0.01
DEGs number
1
2
3
4
5

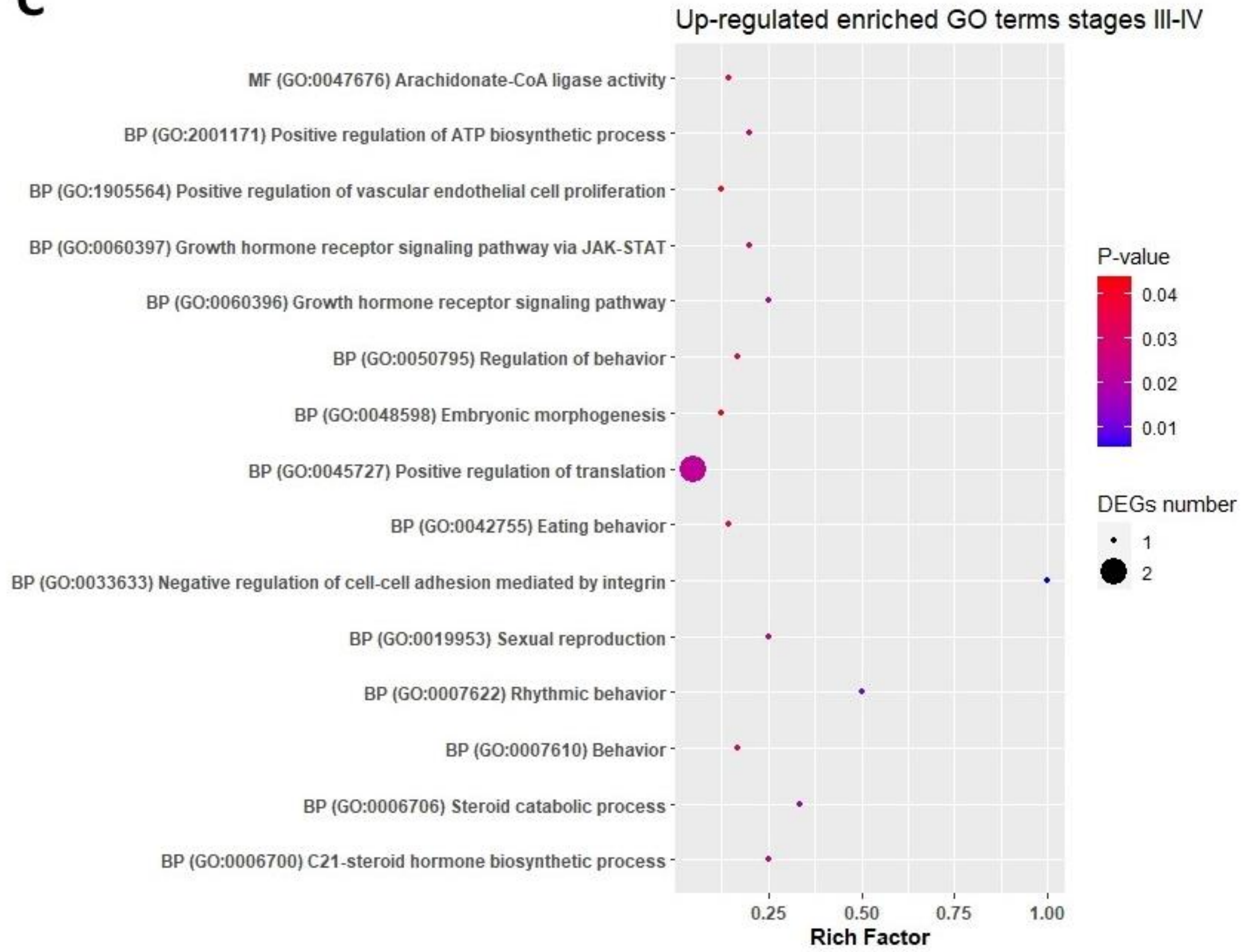
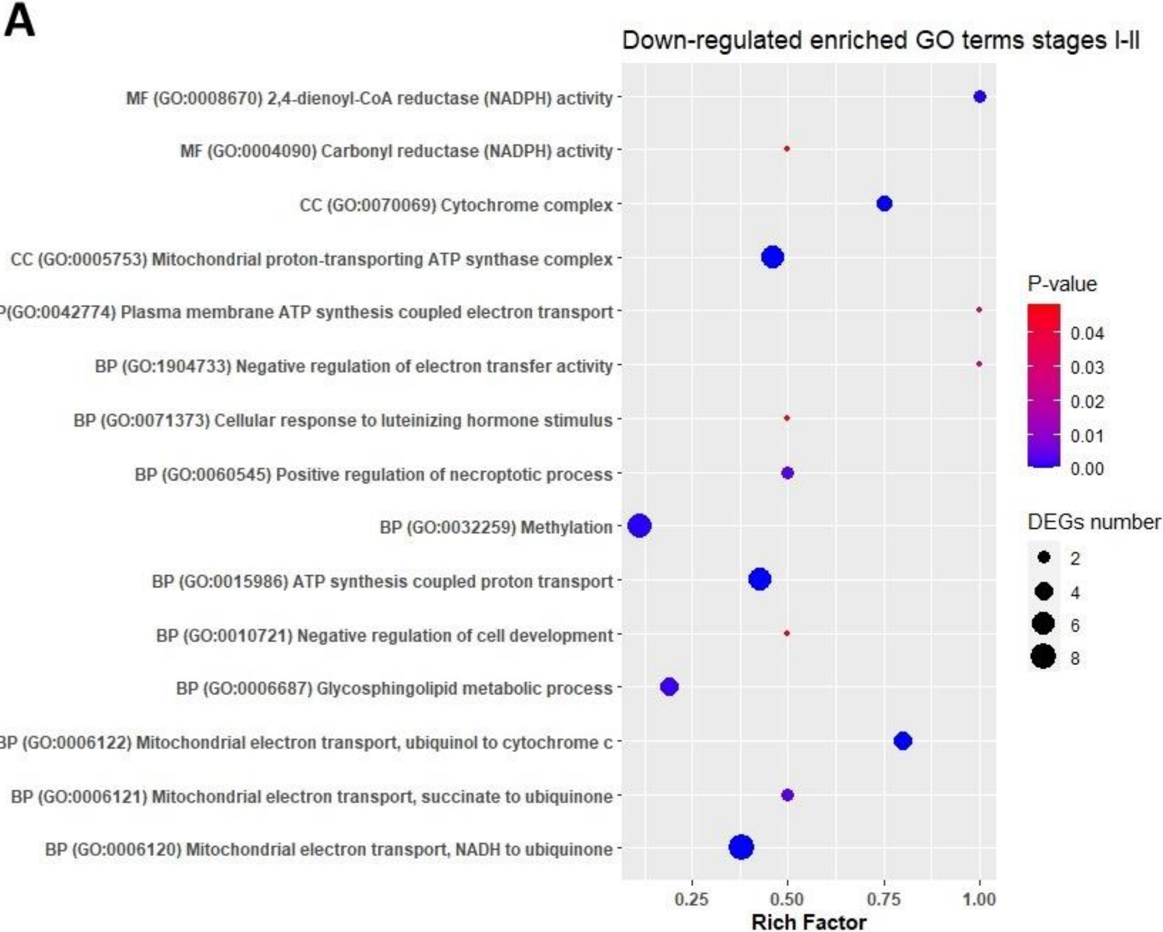
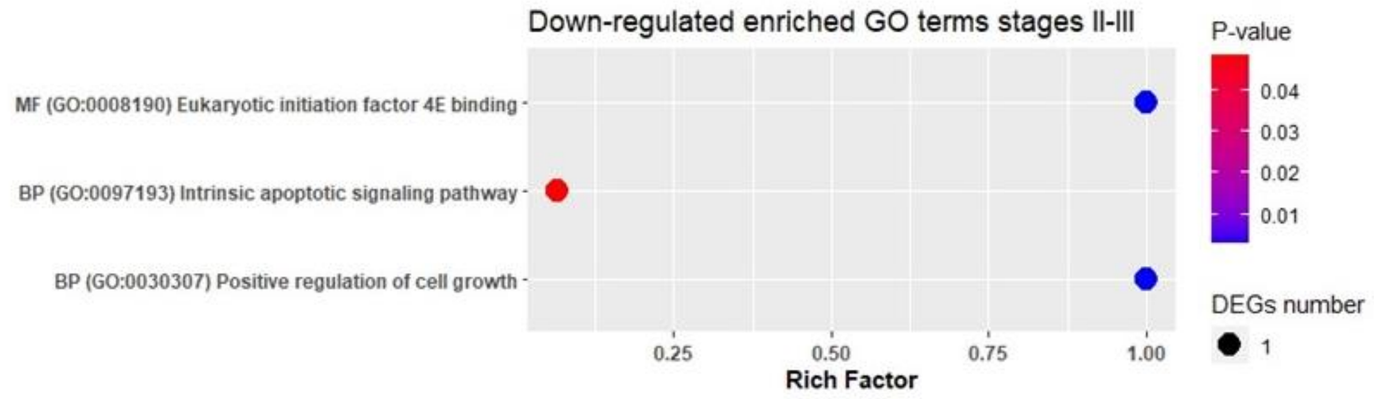
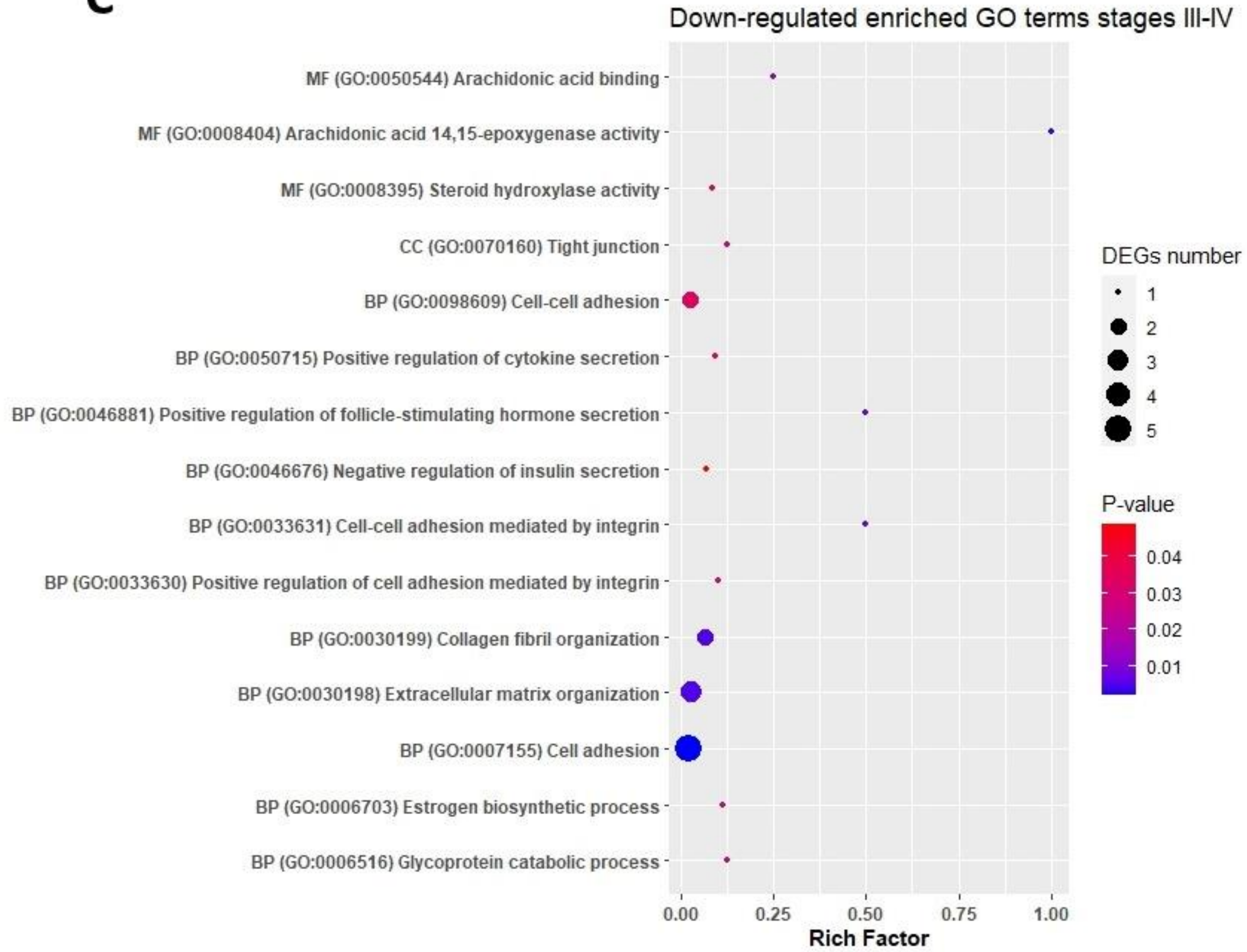

C
Up-regulated enriched GO terms stages III-IV
MF (GO:0047676) Arachidonate-CoA ligase activity
BP (GO:2001171) Positive regulation of ATP biosynthetic process
BP (GO:1905564) Positive regulation of vascular endothelial cell proliferation
BP (GO:0060397) Growth hormone receptor signaling pathway via JAK-STAT
BP (GO:0060396) Growth hormone receptor signaling pathway
BP (GO:0050795) Regulation of behavior
BP (GO:0048598) Embryonic morphogenesis
BP (GO:0045727) Positive regulation of translation
BP (GO:0042755) Eating behavior
BP (GO:0033633) Negative regulation of cell-cell adhesion mediated by integrin
BP (GO:0019953) Sexual reproduction
BP (GO:0007622) Rhythmic behavior
BP (GO:0007610) Behavior
BP (GO:0006706) Steroid catabolic process
BP (GO:0006700) C21-steroid hormone biosynthetic process
0.25
0.50
0.75
1.00
Rich Factor
P-value
0.04
0.03
0.02
0.01
DEGs number
1
2

Figure 7. Dot plot showing some relevant significantly enriched Gene Ontology biological processes (BP), cellular components (CC) and molecular functions (MF) from downregulated DEGs in (A) Stage I to II, (B) Stage II to III, and (C) Stage III to IV. Rich factor is the ratio of the differentially expressed gene number to the total gene number in a certain GO term. The colour and size of the dots represent the range of the P-value and the number of DEGs mapped to the indicated GO term, respectively.



B





C
Down-regulated enriched GO terms stages III-IV
MF (GO:0050544) Arachidonic acid binding
MF (GO:0008404) Arachidonic acid 14,15-epoxygenase activity
MF (GO:0008395) Steroid hydroxylase activity
CC (GO:0070160) Tight junction
BP (GO:0098609) Cell-cell adhesion
BP (GO:0050715) Positive regulation of cytokine secretion
BP (GO:0046881) Positive regulation of follicle-stimulating hormone secretion
BP (GO:0046676) Negative regulation of insulin secretion
BP (GO:0033631) Cell-cell adhesion mediated by integrin
BP (GO:0033630) Positive regulation of cell adhesion mediated by integrin
BP (GO:0030199) Collagen fibril organization
BP (GO:0030198) Extracellular matrix organization
BP (GO:0007155) Cell adhesion
BP (GO:0006703) Estrogen biosynthetic process
BP (GO:0006516) Glycoprotein catabolic process
0.00
0.25
0.50
0.75
1.00
Rich Factor
DEGs number
1
2
3
4
5
P-value
0.04
0.03
0.02
0.01

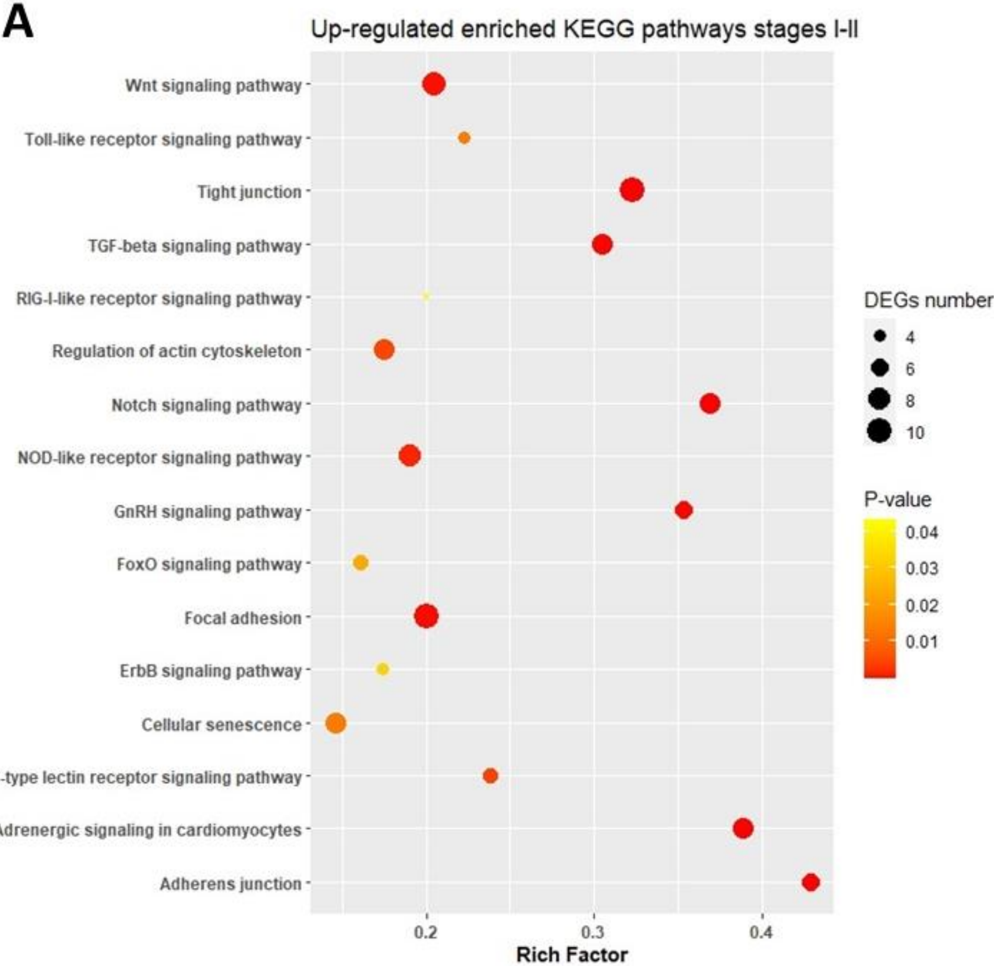
3.6. KEGG pathway enrichment analysis of DEGs

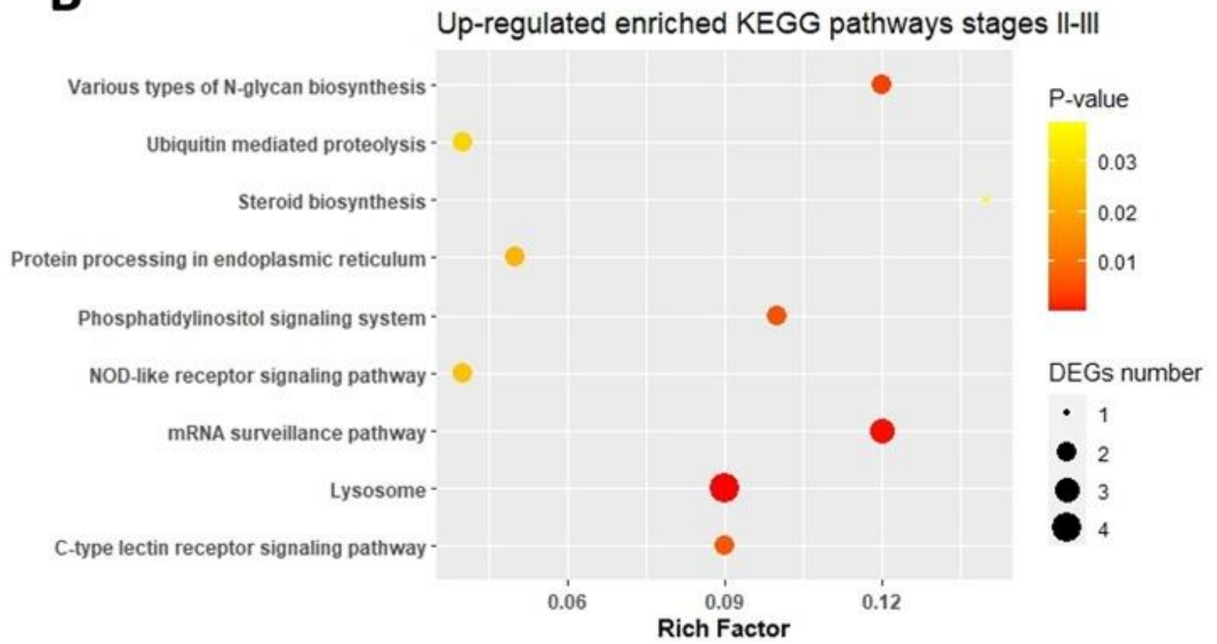
The highest number of significantly enriched KEGG pathways of the differential expressed genes occurred in the transition from Stage I (previtellogenesis) to II (early-to-mid vitellogenesis), with 105 upregulated DEGs enriched in 17 pathways, and 191 downregulated DEGs enriched in 33 pathways. Among them, *GnRH signaling pathway* was enriched by the higher expression of 6 genes (**Fig 8A**). The enriched downregulated pathways related to ovarian development were *steroid biosynthesis*, *oocyte meiosis* and *progesterone-mediated oocyte maturation* (**Fig 9A**).

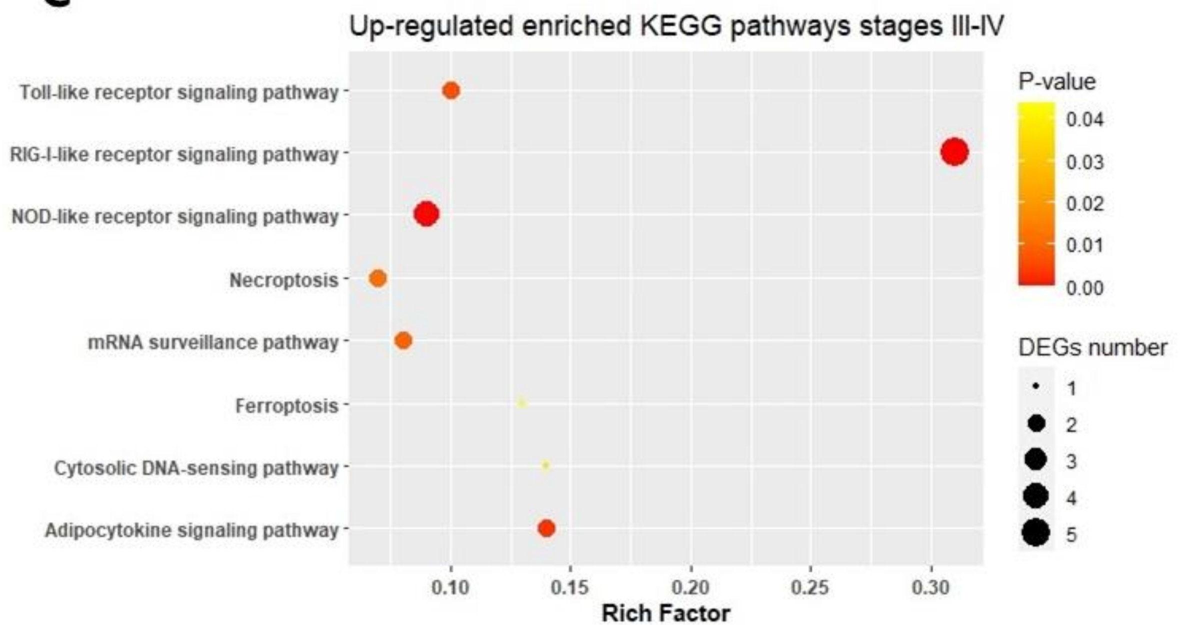
The transition from Stage II to III (late-vitellogenesis) was represented with the lowest number of significant enriched pathways, with only 20 upregulated DEGs annotated in nine pathways. Among them, the pathways of *lysosome*, *various types of N-glycan biosynthesis*, *protein processing in endoplasmic reticulum* and *steroid biosynthesis* (**Fig 8B**).

There were 19 upregulated and 13 downregulated DEGs annotated in nine and eight significantly enriched pathways, respectively, in the transition from Stage III to IV (full-grown oocytes) (**Fig 8C, 9B**). *Steroid biosynthesis*, *tight junction* and *focal adhesion* were significantly down-regulated.

Figure 8. KEGG pathway enrichment analysis of upregulated differently expressed genes between (A) Stages I and II, (B) Stages II and III, and (C) Stages III and IV. Rich factor is the ratio of the differentially expressed gene number to the total gene number in a certain pathway. The color and size of the dots represent the range of the P-value and the number of DEGs mapped to the indicated pathways, respectively.




B
Up-regulated enriched KEGG pathways stages II-III
Various types of N-glycan biosynthesis
Ubiquitin mediated proteolysis
Steroid biosynthesis
Protein processing in endoplasmic reticulum
Phosphatidylinositol signaling system
NOD-like receptor signaling pathway
mRNA surveillance pathway
Lysosome
C-type lectin receptor signaling pathway
0.06
0.09
0.12
Rich Factor
P-value
0.03
0.02
0.01
DEGs number
1
2
3
4


C
Up-regulated enriched KEGG pathways stages III-IV
Toll-like receptor signaling pathway
RIG-I-like receptor signaling pathway
NOD-like receptor signaling pathway
Necroptosis
mRNA surveillance pathway
Ferroptosis
Cytosolic DNA-sensing pathway
Adipocytokine signaling pathway
0.10
0.15
0.20
0.25
0.30
Rich Factor
P-value
0.04
0.03
0.02
0.01
0.00
DEGs number
1
2
3
4
5

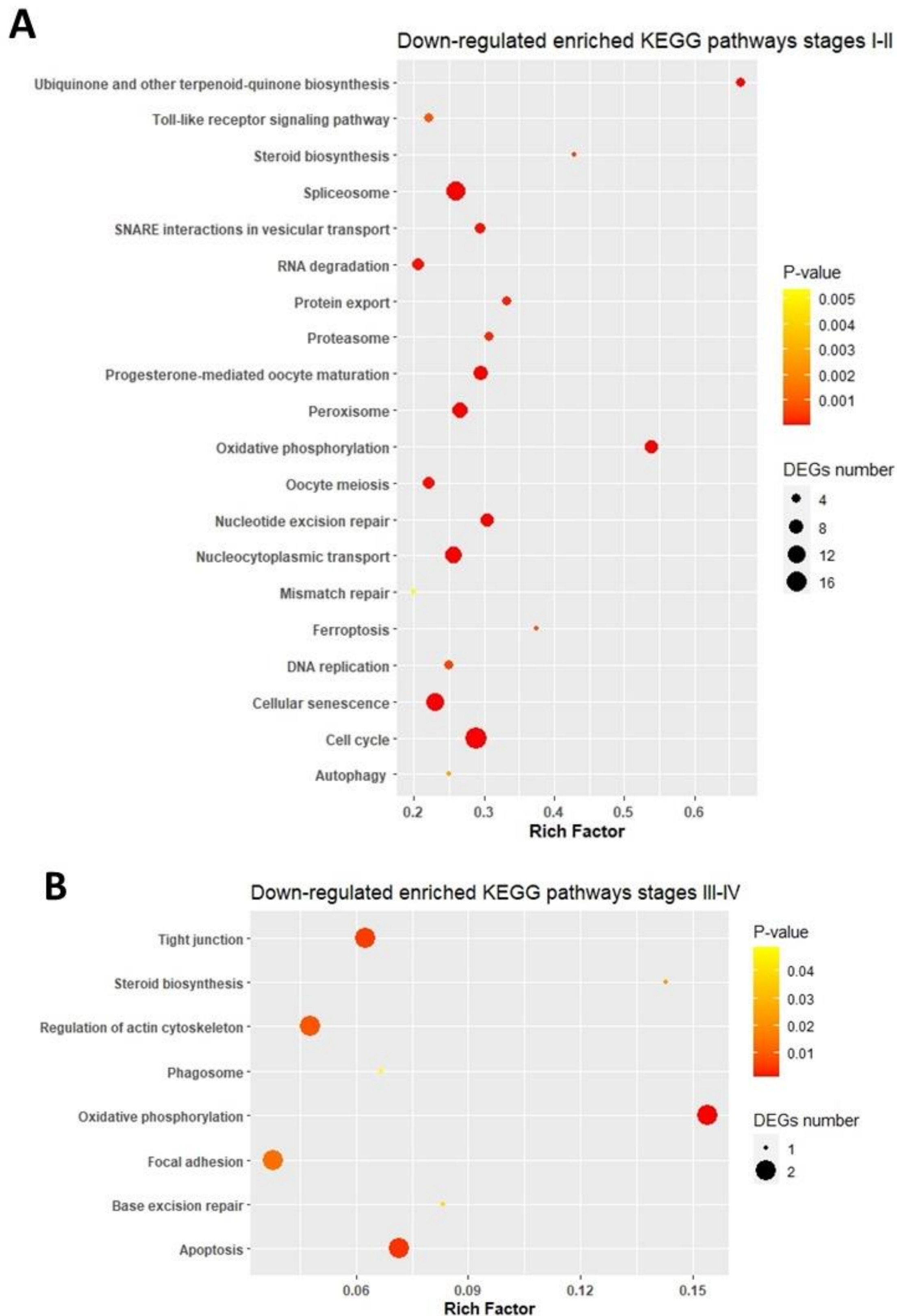


Figure 9. KEGG pathway enrichment analysis of down-regulated differently expressed genes between (A) Stages I and II (top 20 terms), and (B) Stages III and IV. Rich factor is the ratio of the differentially expressed gene number to the total gene number in a certain pathway. The color and size of the dots represent the range of the P-value and the number of DEGs mapped to the indicated pathways, respectively.

4. Discussion

In recent decades, the flathead grey mullet (*Mugil cephalus*) has been identified as an important species for the diversification of European aquaculture, however, the culture of *M. cephalus* is facing several problems, such as breeding problems. Flathead grey mullet females, for example, remain arrested at early gametogenesis, mainly at previtellogenesis. The reproductive dysfunction that females present in intensive conditions has been overcome with the application of recombinant gonadotropins (rGths). Treatment with rFsh and rLh in female flathead grey mullet induced vitellogenesis to the completion of oocyte growth, that finally produced fertile eggs [214]. Recombinant Gths have also been shown to induce gametogenesis in other species [75,119,127,190,201]. Considering these advances and the potential use of rFsh and rLh in other aquaculture species with similar reproductive disorders or to develop out-of-season breeding programs [170], it is important to clarify the molecular regulation of mechanisms between the different stages of rGths-induced ovarian development. The technique of RNA-Seq is both accurate and sensitive [266], therefore, in the present study we attempted to elucidate the ovarian transcriptome of *M. cephalus* using the RNA-Seq method and *de novo* assembly.

In this study, ovarian biopsies of flathead grey mullet at gonadal arrested stage and at different stages through ovarian-induced development with rFsh and rLh were used for transcriptome analyses. In total of 287,089 transcripts with an expression value of FPKM ≥ 1 constituted the final assembly in the present study. The average transcript length of the assembled transcripts was 798.43 nucleotides (nt) which is comparable to other previously reported assembled fish ovarian *de novo* transcriptomes, i.e., 727 nt [32]. Before running DEG analysis, a PCA was performed and the overall variance explained by the first principal component best discriminated the different stages of ovaries with 55% of variance. Previtellogenic samples in cortical alveolus stage formed a clade with vitellogenic samples which could be related to the fact that all belong to the secondary growth stage [152]. There was also an overlap of some molecular signatures between the closest stages that could be due to the presence of oocytes at different stages at the same time in the flathead grey mullet ovary. Although this molecular overlap existed, the stages were incremental and were characterized by visual changes in the overall

appearance of the histological sections. This partial overlap has been described also in other fish species that have simultaneously oocytes at different developmental stages, i.e., have group-synchronous ovarian development, such as the Largemouth bass (*Micropterus salmoides*) [158].

Analysis of DEGs during ovary development revealed that the number of DEGs between Stage I - II, I - III and I - IV were notably greater that between II - III and III - IV, indicating that many similar genes are expressed in the vitellogenic stages II, III and IV. This was in accordance with the results observed in Venn diagrams. A large proportion of DEGs between Stage I - II, Stage I - III and Stage I - IV were overlapping, suggesting they were specifically involved in the developmental processes at the Stage I. The present study identified a greater number of significantly up-regulated DEGs thought vitellogenesis than down-regulated. The observation of a upregulation of genes throughout vitellogenesis implies that mRNAs are actively accumulated [125]. Contrary trend for the total number of DEGs between stages of ovarian development have been reported in the Largemouth bass [158] and in the Atlantic Cod (*Gadus Morhua*) [125], where more genes were downregulated than upregulated during gonadal development.

Changes from previtellogenesis (Stage I) to vitellogenesis (Stages II, III and IV)

Histological sections showed that females were arrested at previtellogenesis (Stage I). With the application of rFsh (Stage II), there was an increase of oocytes size with an accumulation of lipid droplets which is characteristic of vitellogenesis [152]. It is well known that 17β-estradiol (E_2) production in follicular cells and its release in the bloodstream is the main response upon circulation of Fsh. The release of this steroid leads to the hepatic vitellogenin synthesis, that is incorporated into oocytes and degraded to produce egg yolk proteins [152], such as vitellin and phosvitin, which are stored in the oocyte [92]. Related to these events, a dominant feature from previtellogenesis to early-to-mid vitellogenesis was the enrichment of GO terms and pathways of upregulated genes related to the ovarian response to rFsh and to reproductive development. For example, pathways of *cellular response to gonadotropin stimulus (GO:0071371)*, *steroid biosynthetic process (GO:0006694)*, *steroid hormone receptor activity (GO:0003707)*, and *positive regulation of ovarian follicle development*

(GO:2000386) were significantly enriched. We found that rGths upregulated the expression of the follicle stimulating gonadotropin receptor *fshr* in early-to-mid vitellogenesis (Stage II) and at late-vitellogenesis (Stage III), in which vitellogenesis was progressing, compared to previtellogenesis (Stage I), as previously described in other species [72,76]. In addition, genes such as *star* and *hsd3b* [72,76,125] that belong to the downstream cascade of enzymes involved in steroidogenesis, which refers to the transformation of cholesterol into steroid hormones, were upregulated. For example, *star* mediates cholesterol transfer in the mitochondria [242] —a rate-limiting step in steroidogenesis [249]—, and *hsd3b* converts pregnenolone into progesterone —both precursors of most steroid hormones—. Although no significant upregulation was observed for other steroidogenic-related genes, i.e., *cyp17a1* that converts progesterone and pregnenolone to precursors for the synthesis of androgens and estrogens [81] and *cyp19a1* which is involved in the conversion of androgens to E_2 [112,255], at early-to-mid vitellogenesis (Stage II) compared to previtellogenesis, they were significantly upregulated at late-vitellogenesis (Stage III). This increase in *cyp19a1* and *cyp17a* expression reflected the increased steroidogenic activity of the ovary as oogenesis progressed given the involvement of steroids in regulating the production of vitellogenin by the liver. A possible reason for the absence of upregulation of these steroidogenic-related genes at early-to-mid vitellogenesis (Stage II) respect to previtellogenesis (Stage I), may be the great abundance of previtellogenic oocytes in early vitellogenic ovaries. The higher expression profile of nuclear estrogen receptors 1 and 2, *esr1* and *esr2*, during induced vitellogenesis, from early-to-mid vitellogenesis to full-grown vitellogenic oocytes respect to previtellogenesis, may indicate the involvement of estrogens in oocyte development. Several studies have demonstrated the role of estrogen receptors in early oocyte development, favoring the change from the primary into the secondary oocyte growth phase, as well as during late-vitellogenesis [72,185]. The application of rFsh also enhanced the mRNA levels of genes enriched in *growth factor activity (GO:0008083)* such as the gene encoding for the transforming growth factor beta 1 (*tgfb1*), and others factors, such as bone morphogenic proteins 2 and 6 (*bmp2* and *bmp6*), that were further upregulated during vitellogenesis under rGth stimualtion. As previously suggested for other species, in addition to the accepted role on ovarian steroidogenesis, Fsh seems to regulate genes associated with ovarian cell, growth differentiation and survival [88,139,186,221].

There was an upregulated enrichment of *glycerophospholipid biosynthetic process (GO:0046474)* and a downregulated enrichment of *glycosphingolipid metabolic process (GO:0006687)* during early-to-mid vitellogenesis (Stage II) with respect to previtellogenesis (Stage I), that implies a degradation of lipids for storage [125]. Several genes associated with the production of vitellogenin and the transport or endocytosis of vitellogenin and other very low-density lipoproteins (*lrp1 lrp5, lrp2, apoeb*) were upregulated during vitellogenesis. The involvement of upregulated *fads6* and *fabph, fabp6, fabp7, apoeb* implied an active lipid metabolism and cholesterol metabolism, respectively. These findings add data to the reported role of Fsh in the process of yolk uptake during vitellogenesis in *M. cephalus* and in teleost [88,123,171,254]. Throughout vitellogenesis, the expression of ovarian mRNA levels of genes encoding IGF-binding proteins (*igf2*, *igf1r*, *igf1*) also increased, which reflects the possible involvement of the IGF-system in lipid accumulation in the oogenesis in flathead grey mullet under the stimulation by gonadotropins. High ovarian levels of *igf1* and *igf2* during the accumulation of lipids have been previously described in other fish species [33,72,125].

Focal adhesion was a GO term and KEGG pathway significantly enriched from previtellogenesis to early-to-mid vitellogenesis. Focal adhesion results in a connection mediated by cells to the extracellular matrix (ECM) which is formed of collagen, glycosaminoglycans, proteoglycans, etc [286]. Besides, *adherens junction* and *tight junction* were both enriched in the transcriptome. The enrichment of theses pathways indicated that cell connection occurs, and that oocyte communicates with its surrounding cells, such as granulosa and theca cells, for the transport of small molecules that contain metabolites, information and nutrients, and regulate oocyte growth and development [286]. The *regulation of actin cytoskeleton* pathway was also enriched in early-to-mid vitellogenesis (Stage II) compared to previtellogenesis (Stage I). It has been described that actin cytoskeleton has an important function in the transport of oocyte-specific RNA [286]. Two signalling pathways, *Gonadotropin-releasing hormone (GnRH) signalling pathway* and *Wnt signalling pathway,* and *MAPK cascade (GO:0000165)* were also enriched. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) by pituitary gonadotropes [152]. It also regulates the MAPKs activities, serine/threonine protein kinases that act as a

component of signalling transduction in the regulation of cell growth, cell differentiation and cell cycle [233]. The *Wnt signalling pathway* is constituted by glycoproteins that regulate cell differentiation and oocyte survival [145]. The enrichment of these pathways, that has been described in other species ovary transcriptomes, are important for the maintenance of the physiological activity of the ovary [106,286] and necessary for normal fertility [21]. Related to this, the higher expression of forkhead box L2 (*foxl2*) in early-to-mid vitellogenesis and along vitellogenesis respect to previtellogenesis implies that ovary maintenance events were taking place [106].

A noteworthy aspect of the transition from previtellogenesis (Stage I) to early-to-mid vitellogenesis (Stage II) was the downregulation of genes enriched in the *cytochrome complex (GO:0070069)*. Contrarily to other species, in which mRNA levels of genes involved in mitochondrial respiration and oxidative phosphorylation were upregulated in early-vitellogenesis respect to previtellogenesis [150,158,276], in the present study cytochrome c and b transcripts were downregulated. However, a high expression of cytochrome c was still present in all stages, and thus, oxidative phosphorylation required for ATP production was present. On the other hand, the neurotransmitter receptor *npy1r*, a member of the G-protein-coupled receptor family was up-regulated during vitellogenesis (Stage II, III and IV) compared to previtellogenesis (Stage I), as in the Atlantic cod vitellogenic follicles [125]. The neurotransmitter of neuropeptide Y (NPY) is described to be an important regulator of energy homeostasis in fish and mammals [275]. This upregulation together with the positive enrichment of the *G protein-coupled receptor signalling pathway (GO: 0007186)* that forms part of the nervous system signalling, may indicate activation of processes important for energy uptake and oocyte growth during vitellogenesis [125].

<u>*Changes from early-to-mid (Stage II) to late-vitellogenesis (Stage III)*</u>

Physiological processes that occur from Stages I to II, III and IV differed greatly, which likely explains the large difference in the transcriptome sequencing results for these comparisons. On the other side, lesser DEGs were observed between II and III. Some of the upregulated enriched terms both in GO terms and KEGG pathways on the transition from early-to-mid vitellogenesis (Stage II) to late-vitellogenesis (Stage III) were

those related to the *lysosome*, enriched by the presence of cathepsins, and *Ubiquitin-mediated proteolysis*. Among all the cellular processes that the lysosome is involved in, i.e., cholesterol homeostasis, tissue remodeling, cell signaling, macromolecules degradation etc., several authors have described a relationship with ovarian development, with a role in the degradation of vitellogenin [35], the precursor of vitellin which is an important source of nutrients in gonadal development. It has been also suggested that lysosomes are involved in energy redistribution and that the activation of lysosomes implies the production of energy for reproduction [283]. Therefore, it could be hypothesized that lysosomes were related to the production of energy during *M. cephalus* rGths induced vitellogenesis, such as in the shrimp (*Macrobrachium nipponense*) and other fishes [283].

Changes at late-vitellogenesis (Stage III) and full-grown oocytes (Stage IV)

In the transition from late-vitellogenesis (Stage III) to full-grown oocytes (Stage IV), there was a downregulation of genes related to *tight junction*, *focal adhesion*, *actin cytoskeleton* and *cell-cell adhesion (GO:0007155),* among others. The downregulation of DEGs in cell-cell adhesion pathways once completed vitellogenic growth and the upregulation of an arrestin (*arr3*) at late-vitellogenesis and full-grown oocytes compared to previtellogenesis, previously described to be involved in the meiotic arrest relieve in *Xenopus laevis* and mouse [125], suggest that final maturation is approaching and signals associated with late-meiotic steps might be activated. In addition, the luteinizing hormone receptor (*lcghr*) was upregulated at late-vitellogenesis and full-grown oocytes compared to previtellogenesis. A similar pattern of expression has been previously described in the European sea bass (*Dicentrarchus labrax*) in which expression increased at advanced vitellogenesis to later peak during maturation [72]. The upregulation of *lcghr* together with the downregulated enrichment in the *estrogen biosynthetic process (GO:0006703)* and the upregulated enrichment in the *C-21 steroid hormone biosynthetic process (GO:0006700)* indicates a preparation of the oocyte towards maturation. During oocyte maturation, ovarian steroidogenesis shifts from the synthesis of estrogens E_2 to a C-21 derived steroid, the maturation-inducing steroid 17α, 20β-dihydroxy-4-pregnen-3-one [152].

In addition, the upregulation of enriched pathways as *embryonic morphogenesis (GO:0048598)* at full-grown oocytes might be related to the future development of the egg. For example, the upregulation of genes such as the iInositol Monophosphatase 1 (*impa1*), which is related to osmoregulation [60,113], could be preparing the oocyte for the marine environment [125], where it will be released and fertilized. Surprisingly, several paths related to behavior were enriched from upregulated genes, such as *sexual reproduction (GO:001995)* and *behaviour (GO:0007910)* which leads to speculate that together with the multiple genes necessaries to complete follicular development to be followed by oocyte maturation and ovulation, the application of rGths at this stage promoted the expression of genes that might coordinate daily rhythms in physiology and reproductive behaviour.

To sum up, RNA-Seq and bioinformatics tools were used to evaluate the ovarian transcriptome development under rGths induction in the flathead grey mullet. The present study described enriched paths with genes being differentially expressed in ovary as induced vitellogenesis progressed. Whether the described molecular patterns are exactly the same to those of the flathead grey mullet natural cycle without the use of external recombinant Fsh and Lh cannot be concluded. However, the paths and genes described were typical of natural oogenesis in other fish species, and vitellogenesis was proven to be successfully induced with the final production of fertile eggs [214]. These data will serve as a platform for studies that aim to understand the molecular basis of stage-specific physiological events during rGths-induced vitellogenesis in the ovary of teleost. The description of molecular mechanisms involved in gonadal development under rGths treatment has not only basic interest but also is of practical relevance for fish aquaculture breeding programs.

CHAPTER VIII

Overall Discussion

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Overall Discussion

The present thesis has determined different aspects of the flathead grey mullet (*Mugil cephalus*) broodstock management to finally have control of the reproduction of this species in intensive conditions. It has studied the reproductive cycle of this species according to wild females development in the Western Mediterranean (**CHAPTER II**), addressed nutritional issues required for the adequate maintenance of breeders in captivity (**CHAPTER II** and **CHAPTER III**), tested different treatments for the induction and to enhance gametogenesis (**CHAPTER IV**, **CHAPTER V** and **CHAPTER VI**) to finally establish a reliable hormonal therapy based on the use of recombinant gonadotropins (rGths), specifically of recombinant follicle-stimulating (rFsh) and luteinizing homones (rLh), that permitted to synchronize gonadal development in flathead grey mullet males and females, and to induce spawning obtaining high-quality gametes and larvae. Moreover, the changes presented at the transcriptome level in the ovary during the induced vitellogenesis by rFsh and rLh that lead to the production of fertilizable eggs were evaluated (**CHAPTER VII**).

Regarding the natural development of wild flathead grey mullet females in the Western Mediterranean, the determination of the onset of ovarian development —at least early August—, and the spawning season —September to October— by the near year-round evaluation of the gonadosomatic index (GSI %) together with the histological and macroscopic evaluation of the ovaries in **CHAPTER II**, served as a guide for the detection of the moment in which gonadal development of this species was arrested when held in intensive conditions. By the time that wild flathead grey mullet females had started vitellogenesis, revealed by the presence of a predominant clutch of yolky oocytes and a slight increment of the GSI % —from 0.8 ± 0.3 % to 4.2 ± 3.6 %—, their counterparts in captive conditions —for three months to 3.5 years in IRTA facilities— presented only previtellogenic oocytes (**CHAPTER IV**, **CHAPTER V** and **CHAPTER VI**) or had a low percentage (<10 %) of early-vitellogenic oocytes surrounded by a predominant clutch of previtellogenic oocytes (**CHAPTER VI**). To our knowledge, the gonadal arrest observed in

females in the present thesis is the most severe described in intensive conditions for this species. Although Aizen *et al*. (2005) also reported females arrested at early stages of gametogenesis, the 20 % of females that did not receive hormonal stimulation were fully mature by the end of the experimental period. In comparison, no maturational advances were observed in control fish in the experiments in the present thesis. All fish used were larger than the reported standard length for first maturation in this species (27 - 35 cm) [272], indicating all fish had the age and size to mature. In addition, all experiments were timed to coincide with the described natural spawning season of flathead grey mullet in Western Mediterranean in CHAPTER II i.e., from the end of July to the beginning of October in CHAPTER IV, from early August to November in Experiment 1 from CHAPTER V, from the end of July to mid-October in Experiment 2 from CHAPTER V, and from early August to early November in CHAPTER VI.

In terms of nutritional issues, on the one hand, the lack of commercial pelleted feed for mullets and the observed rejection of commercially produced pellets during acclimatization once captured from the wild, lead to the evaluation of feeding responses to different sizes of pellets and the species feeding behaviour in terms of the occupation of the tank during the feeding activity. The present thesis demonstrated the requirement of smaller pellets in this species compared to individuals from the same size of other species, such as carnivorous species as the gilthead seabream (*Sparus aurata*) [13], for which there are commercially available diets. In addition, the flathead grey mullet seemed to be a column and bottom feeder as preferentially distributed in the water-column and the bottom of the tanks. On the other hand, as previously discussed, the absence of studies describing the specific nutritional requirements for flathead grey mullet breeders could lead to the provision of inadequate diets with limited or inadequate amounts of specific fatty acids that could negatively affect the reproductive outcome in captivity [103,157,273] as some fatty acids mediate a wide range of aspects of reproductive development [109,250]. For example, the Senegalese sole (*Solea senegalensis*) that presented low arachidonic acid (20:4n-6, ARA) content had a lower ARA/EPA ratio and showed reproductive dysfunctions [192]. In CHAPTER II, the flathead grey mullet was described to mobilize/use during ovarian development: lipids stored in the liver, mainly of n-3 polyunsaturated fatty acids, especially eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3,

DHA), lipids in perivisceral fat, and lipids obtained directly from feeding intake for ovarian development. The following fatty acids were relevant due to the high levels presented at different moments during gonadal development: the palmitic acid (16:0), which was suggested to be used as metabolic energy in the ovary during vitellogenesis as generally in fish [97], the palmitoleic acid (16:1) and the heptadecenoic acid (17:1) that increased by 5-fold at least during vitellogenesis and were proposed to be part of the embryo reserves for future development. Among the polyunsaturated fatty acids, which have an essential role in the development of gonads, the formation of gametes and cell membrane structures [250], ARA presented high levels in gonads that decreased in percentage during vitellogenesis. However, one limitation in this study was that total lipids were examined instead of examining the changes in total lipids and fatty acids in the different lipid fractions (neutral and polar) which have different functions [250]. The neutral fraction comprises triacylglycerols and wax esters and provides energy, and the polar fraction has a more structural function as it consists of membrane glycolipids and phospholipids. For instance, the pattern of fatty acids accumulation in the fractions and the neutral:polar lipid ratio has been reported to change during the maturation period [208] and between wild and cultured fish [192]. The study of separate fractions could have provided a deeper comprehension of the lipids function during the reproductive cycle.

As the fatty acid profile of wild flathead grey mullet females was determined, which hypothetically contain the desired nutritional composition for breeders, future studies could focus on the study of mature wild flathead grey mullet broodstock maintained or entirely reared in captivity to determine the importance in the reproductive outcome of this species of certain dietary fatty acids, such as the relevance of the 17:1 fatty acid, that is from bacterial origin [199,219,234] and was highly accumulated in vitellogenic ovaries. Besides, pellets that fulfil the requirements of breeders for proper reproductive success could be formulated. Overall, the description of lipids and fatty acids requirements together with the feeding responses to a determined pellet size in juveniles and, principally, in breeders, permitted to sequentially introduce changes during the broodstock management labours. In fact, the identification of the adequate pellet size aided the acclimation to pelleted feed of the wild-caught individuals in captivity which was crucial for the proper management of the broodstock. At first (**Experiment 1**,

CHAPTER V), wild-caught fish did not accept a pelleted broodstock diet and were, therefore, fed 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. After that, the incorporation of manufactured Senegalese sole (*Solea Senegalensis*) broodstock diets of small pellet size (Europa RG from Skretting (Spain) and Brood Feed Lean from Sparos (Portugal)) resulted in a high acceptance and consumption. The broodstock diet for Senegalese sole, a carnivorous species [282], was selected because of the unavailability of broodstock diets for omnivorous species. Moreover, has been reported that the digestive characteristics of Senegalese sole, i.e., residual acid digestion and proteolysis in stomach and a long intestine, are more closely related to those usually found in fish with omnivorous feeding habits [282]. Even though the selected diet was designed for breeders and to favour the correct development of gametes, a lack of some fatty acids, specifically of 17:1, which is not present in Senegalese sole gonads [192], could have had an effect on the reproductive success, i.e., fertilization success, hatching, and survival of larvae of the flathead grey mullet. It seems, however, that a good nutrition was used to enable the breeders to successfully mature and produce viable eggs given the quality of spawns obtained in CHAPTER VI. Spawns had 54 ± 21% of fertilization, 58 ± 23 % of hatching percentages, and larvae survived up to 13 days post-hatching without external feeding. However, we cannot conclude that the feeding with a diet for Senegalese sole broodstock could have an effect on the further development of larvae.

Regarding the reproductive development of this species in intensive conditions, different reproductive dysfunctions were observed in males and females within chapters even though all experiments started by mid-summer —late-July or early August— and same holding conditions were used —tanks were filled with 36 ppt seawater, about 23 - 24ºC of water temperature and photoperiod from 14L:10D later July and early August to 11L:13D late October and early November—. Males developmental stage was evaluated by determining the spermiation index upon abdominal pressure (CHAPTERS IV to VI) and by GSI% and histological evaluation (CHAPTER VI). In CHAPTER IV and CHAPTER V, males were not spermiating at the beginning of the treatments, whereas 20 % (3 out of 15 individuals) presented traces of high viscous milt in CHAPTER VI. The examination of those males that presented sperm showed undeveloped testes with a low GSI (≲ 0.1 %) with

mainly spermatogonia and spermatocytes and few or no spermatids or spermatozoa. Females were monitored by measuring the diameter of the most developed oocytes obtained through ovarian biopsies and were found to be arrested at stages ranging from previtellogenesis (**CHAPTERS IV** to **VI**) to early vitellogenesis (**CHAPTER VI**). The main reason that may explain the differences in gonadal arrest is the time individuals were kept in captivity in the different chapters. In **CHAPTER IV** and **CHAPTER V**, individuals were initially caught from the Ebro Delta (Spain) or obtained from a semi-extensive pond fish farm (Finca Veta la Palma, Isla Mayor, Spain) and reared in IRTA Sant Carles de la Ràpita for over seven to 21 months or three months, respectively. In **CHAPTER VI**, individuals were held for 18 months up to 3.5 years in IRTA facilities despite sharing the same origin. Although a correctly designed experiment was not conducted to study the effect of the time in captivity on reproductive dysfunctions, we observed that only females that had remained more time in intensive conditions (> 2 years) presented early-vitellogenic oocytes. However, some of those females (> 2 years in captivity) were also at previtellogenesis as the females held for less time (< 2 years). It is well-known that the stress produced by captivity or suboptimal environmental conditions can lead to a lack of vitellogenesis, oocyte maturation, ovulation, and / or spawning [155,178]. According to other studies, mullets require approximately two to three years to develop mature oocytes once in captivity [15,50,175]. Therefore, acclimatization to the captive environment may favour reproductive development in flathead grey mullet and may explain the advance in gonadal development of those females with more time in captivity. Whether acclimation and domestication of the species affects or not in the arrested stage of gonadal development could be a topic for future investigations.

Concerning the different hormonal treatments administered to females, there was a variable success in induction and completion of vitellogenic growth. The administration in **CHAPTER IV** of rFsh produced in the yeast *Pichia pastoris* (5 μg kg^{-1}) and metoclopramide (MET) (15 mg kg^{-1}), a dopamine antagonist (DA), previously used by Aizen *et al.* (2005) and Meiri-Ashkenazi *et al.* (2018), did not lead to the development of vitellogenesis and flathead grey mullet females remained arrested at previtellogenesis. In contrast, the treatment with weekly doses of rFsh (from 4 μg kg^{-1} to 12 μg kg^{-1}) and rLh (from 2.5 μg kg^{-1} to 12 μg kg^{-1}) produced in Chinese Hamster Ovary cells in **Experiment**

2 of CHAPTER V and in CHAPTER VI, induced the completion of vitellogenic growth in the 100 % of females that received the complete treatment (29 females). The breeders used for CHAPTER IV and CHAPTER V were obtained from the same broodstock and experiments were performed simultaneously; therefore, there is an evident success of the weekly rFsh and rLh treatment over the two-injection treatment of rFsh and DA on the previtellogenic-arrested flathead grey mullet. The previously reported successes in inducing and completing vitellogenesis in the flathead grey mullet with a treatment with a DA antagonist [6], or combined with rFsh produced in *P. pastoris* [166], compared to the unsuccessful application in the present thesis could be due to differences in the stock of breeders and the holding conditions. The reproductive dysfunction that females presented in IRTA installations appeared to be more severe than that reported by Aizen *et al.* (2005) and Meiri-Ashkenazi *et al.* (2018). Aizen *et al.* (2005) described that a proportion of control females (< 20 %) were observed to mature without the need for any hormonal treatment, while in the present study, no control fish matured. However, we cannot conclude that the treatment of rFsh produced in *P. pastoris* with a DA antagonist would not be effective on fish that already presented some early vitellogenic oocytes as in CHAPTER VI. In any case, the experiment with an injection of rFsh and antidopaminergic combined with the performance of control individuals in the present thesis, that remained arrested in previtellogenesis or developed atresia of the vitellogenic oocytes, suggested that therapies with few injections that rely on the antidopaminergic action to stimulate the pituitary liberation of gonadotropins were not sufficient to stimulate long term gonadal development. We hypothesize that the pituitary content of the Gths in the individuals was low and not sufficient for an effective liberation of Fsh and Lh to induce gonadal development.

The induced-vitellogenesis by rFsh and rLh seemed to be a longer process than the natural vitellogenic development of wild females in the Western Mediterranean. Considering that vitellogenesis had initiated early in August and post-spawn females were observed in mid-September, natural vitellogenesis may take around one and a half months to occur. The weekly rGth treatment required from six to thirteen weeks (1.5 to 3.25 months) to complete vitellogenesis from early arrested stages, with most females completing vitellogenic growth (~ 600 µm oocyte diameter) around the eleventh or

twelfth week of treatment. Aizen *et al*. (2005), that enhanced vitellogenesis in flathead grey mullet females with a DA antagonist, also required a longer time to complete vitellogenesis, precisely 99 days (~14 weeks, 3.5 months). This indicates that there is still work to do to adjust hormonal therapies to meet the duration of the natural oogenesis. Moreover, it is not known how the more extended period of induced vitellogenesis could affect the future development of offspring. The shortening of induced-vitellogenic period under rGths treatment would possibly reduce the amount of rGths required, reduce samplings and handling of fish, and thus, reduce the stress in fish, which can have adverse effects on gonadal development [155,289].

Regarding the requirements of Lh to complete vitellogenic growth in the flathead grey mullet, it is suggested in CHAPTER V that contrarily to what is reported as the two different roles for Fsh and Lh in fishes with synchronous ovarian development —Fsh stimulates vitellogenic growth until the completion of oocyte growth and Lh controls the oocyte maturation processes [152]—, the flathead grey mullet needs Lh to complete vitellogenesis. The administration of only rFsh in Experiment 1 of CHAPTER V failed to complete oocyte growth, and oocytes only developed until mid-to late-secondary growth (425 ± 19 µm), and after that, atretic cells were found. In Experiment 2 of CHAPTER V, the co-administration of rLh with rFsh, and/or the single administration of rLh at advanced stages of vitellogenesis induced the completion of vitellogenic growth (~ 600 µm). Slightly different experimental conditions were followed in the experiments that although appeared not to affect maturational development, caution is needed when comparing these results. On the other hand, the transcriptomic evaluation of changes between developmental stages in induced vitellogenesis (CHAPTER VII), indicated that there was an upregulation of the follicle-stimulating hormone receptor (*fshr*) from previtellogenesis to early-to-mid vitellogenesis (after rFsh application) and late-vitellogenesis (after rFsh and rLh application), whereas there was no significant difference with full-grown oocytes (after rLh application), and that the luteinizing hormone receptor (*lhcgr*) was significantly upregulated from previtellogenesis to late-vitellogenesis and full-grown oocytes. The increased presence of *lhcgr* at advanced stages of vitellogenesis may indicate a higher dependence and responsiveness to Lh to achieve the completion of vitellogenesis. However, we still consider that further work would be required to fully

understand the roles of Fsh and Lh in the flathead grey mullet, and specifically the moment in which Lh seems to have an important role in vitellogenic progression.

What was evident from the present thesis results (**CHAPTER V** and **CHAPTER VI**) was that rLh was required to stimulate oocyte maturation once females completed vitellogenic growth. In addition, **CHAPTER VI** showed that rLh could induce ovulation once oocyte maturation had started. However, the applied doses in both scenarios seem to be crucial. Regarding oocyte maturation (OM) induction, in the *in vivo* study in **CHAPTER VI**, 30 µg kg^{-1} rLh induced OM with the migration of the germinal vesicle in all 100% of females (n = 15) when revised 24 h after the application. On the contrary, females that received priming 40 mg kg^{-1} of Progesterone (P_4) had not initiated OM 24 h after the priming dose, and although an additional P_4 administration (resolving) did induce ovulation, all the maturation and ovulation process was concentrated in less than 24 h obtaining bad egg quality, indicating the relevance of Lh or Lh-induced factors in this process. Moreover, in **CHAPTER V** only the females that received the highest priming rLh dose (30 µg kg^{-1}) (four out of five females) proceeded to OM compared to females that received a lower priming rLh dose (15 µg kg^{-1}) (n = 3 females), which did not develop to OM. Regarding ovulation induction, in the *in vitro* study in **CHAPTER VI**, incubation with doses of 10 and 50 ng mL^{-1} induced a lower proportion of ovulation, while higher doses with an optimum at 100 ng mL^{-1} induced the highest percentages of ovulation. The cruciality of the rLh doses on OM and ovulation induction is in agreement with the study of Aizen *et al*. (2017), which observed a different effect of several rLh doses on the spawning success in the common carp (*Cyprinus carpio*), and the highest rLh dose applied (350 µg kg^{-1}) gave the best results. Recombinant Lh has been reported to have high success in inducing OM and ovulation in other species; for example, a dose of 50 µg kg^{-1} rLh induced OM in the malaysia catfish (*Hemibagrus nemurus*) [223] and 20 µg g^{-1} induced ~70% ovulation in the bitterling (*Rhodeus ocellatus*) [128].

Concerning the stimulation of testes development, all treatments in males (**CHAPTER IV**, **CHAPTER V** and **CHAPTER VI**) effectively increased the percentage of spermiating males; however, some treatments presented better results. The treatment with rFsh produced in *P. pastoris* (5 µg kg^{-1}), MET (15 mg kg^{-1}), and 17α-methyltestosterone (MT) (6.7 to 11.6 mg kg^{-1}) administered via EVAc slow-release

implants in CHAPTER IV induced four out of six spermiating males producing low quantities (10 – 200 µL) of either viscous or fluent milt. Although low sperm quantities were also obtained in CHAPTER V under rGths stimulation (from 30.3 ± 12.3 µL to 242.5 ± 70.9 µL), 100% of males were induced to spermiate. The higher success of rGths in inducing spermiation suggests a more potent effect over MT treatment in individuals kept in intensive conditions from three to 21 months. In contrast, Aizen *et al.* (2005) described that MT treatment induced 100% spermiating flathead grey mullet males with the production of fluent milt. However, some control males that received no treatment also presented viscous milt along the experimental period. Contrarily, no control males presented milt in CHAPTER IV nor in CHAPTER V, indicating a more severe dysfunction in male individuals in IRTA facilities. However, whether MT treatment could have stimulated spermatogenesis and spermiation in breeders kept in captivity for a longer time (up to 3.5 years), such as in CHAPTER VI, cannot be determined, as some control males (~20 %) presented traces of high viscous milt without hormonal treatment. In relation to the observed male development, although CHAPTER VI showed that the application of rGths provided males with sufficient sperm to fertilize the eggs, a specific study on the direct effect of each rGth on testes development such as that developed by Peñaranda *et al.* (2018) in European eel testing different combinations and doses of rFsh and rLh would be interesting.

The correct selection of an induction procedure is crucial; the election of the inadequate dosage might have significant consequences on gamete quality [178]. In addition, the handling of fish for the application of hormonal treatments can lead to stress in individuals that can also have adverse effects on the gonadal development [155,289]. One of the limitations during the selection of the rGths doses and the timing of administration in the present thesis was that it was not possible to determine the actual levels of gonadotropins in the bloodstream of individuals. Therefore, as 17β-estradiol (E_2) is known to be produced by the follicle under the stimulation of Fsh [152], doses in females were set by measuring the levels of E_2 in plasma several days after the application of different doses of rFsh (3, 6, 9, 12 and 15 µg kg^{-1}) (CHAPTER V). The timing of administration was decided according to the remaining presence of high levels of induced E_2. The optimal situation would have been to have developed a specific ELISA

for flathead grey mullet rGths as has been possible for several species [2,28,40,120,191,229,237] and examine the half-life [37] of both rGths in the bloodstream of both sexes. Although it was possible to set the rFsh dosage according to the induced levels of E_2 in females, it would be more challenging and possibly inadequate to apply the same procedure for rLh. Therefore, it would be recommendable to develop an ELISA for rLh to refine the administered doses.

Even though we cannot conclude that the doses of rGths utilized in the present thesis are optimal to induce spermatogenesis and spermiation together with vitellogenesis, oocyte maturation, and ovulation, we demonstrated that the rGth treatment itself did not have a negative effect on egg quality and that high motile sperm can be obtained. Furthermore, the normal progression of vitellogenesis was obtained with the evaluation of the ovarian transcriptome, which showed that enriched molecular pathways and differential expressed genes described during the induced vitellogenesis of flathead grey mullet with rFsh and rLh are typical of natural oogenesis and have been previously reported for other fish species [106,125,158,276,285]. For instance, upregulated genes in the transition from previtellogenesis to early-vitellogenesis in response to rFsh (such as *fshr*, *star* and *hsd3b*) were significantly enriched in GO terms and KEGG pathways directly related to steroidogenesis and reproductive development, such as *steroid biosynthetic process (GO:0006694)*, *response to estradiol (GO:0032355)*, *and positive regulation of ovarian follicle development (GO:2000386)* (**CHAPTER VII**). These results further agreed with the morphological changes observed in oocytes in histological samples and in the surge of E_2 in the plasma of females from which the samples used for the RNA-Seq analysis were taken (levels at previtellogenesis = 123.9 ± 27.4 pg mL^{-1}; levels at early-vitellogenesis: 458.7 ± 113 pg mL^{-1}) (**Experiment 2, CHAPTER V**).

Although the present thesis showed the possibility to culture juvenile-like flathead grey mullet from the spawns obtained, the definition and application of protocols that ensure good survivals of larvae were not available to effectively evaluate the larvae's potential for culture and make further conclusions on their viability to obtain the number of juveniles that production demands. However, we have described that the quality of the egg following induced spawning did not appear to be impaired and that the embryo and emergent larvae were of good quality and identical in all respects to

other studies [16,83], including timing and characteristics of the initial larval development through to a juvenile.

The success of the application of rFsh and rLh in controlling reproduction in the flathead grey mullet producing viable offspring has been proven in the present thesis. It may serve as a reference point for the development of protocols for other species with similar reproductive dysfunctions, which until recently, to the best of our knowledge, have not been developed. Regarding the future application of the present protocol in the flathead grey mullet, the major concern for the aquaculture industry will be the costs associated with the obtention of recombinant gonadotropins, together with the work and personnel necessary to carry out the weekly procedures. Although we have not reduced the costs in the production of fry to make it economically more viable than the current practises that involve the capture of wild fry or the induction of wild mature individuals, the present thesis offers a possibility not to have to rely on these practices which are not sustainable and are unstable considering the decline in wild *M. cephalus* populations [281] and the variable success of current treatments to induce maturation and ovulation [6,16,55], and would permit to develop selective breeding programs. In addition, the present protocol offers replicability and, as concluded in CHAPTER VI, the number of individuals required to produce ~ 1 million fry could be reduced to the induction of 6 - 7 females (~1 kg) per season, provided the high fecundities obtained (~ 1,700,000 eggs female^{-1}), and the fertilisation and hatching of ~ 50 % of the spawned eggs. The future reduction in the production costs of rGths would be linked to the demand from industry, not only for the flathead grey mullet, but also for other species, or to the biotechnological development of cost-effective ways of production.

Overall, this thesis has identified different aspects for the proper management of flathead grey mullet broodstock, and has successfully applied rFsh and rLh, together with the evaluation of the transcriptomic changes at the ovarian level, to have full control of the flathead grey mullet gonadal development. The lipids and fatty acid profile description will permit the formulation of pelleted diets for flathead grey mullet broodstock, facilitating the management of this species in captivity. The transcriptomic data will serve as a platform for studies that aim to understand the molecular basis of stage-specific physiological events during rGths-induced vitellogenesis in the ovary of

teleosts. There is also future practical research to further improve and refine the present protocol, apply it outside of the natural maturation period, see the possibility of obtaining year-round spawns in the flathead grey mullet, and apply long-term rGth treatments in other fish species arrested at previtellogenesis not only for aquaculture, but also for conservation purposes.

CONCLUSIONS

Conclusions

Maturation and lipid and fatty acid changes in the wild flathead grey mullet:

- The gonadal resting period of wild flathead grey mullet females caught in the Western Mediterranean was from October through to July, recrudescence had initiated at early August and spawning occurred during September and October.

- During vitellogenesis progression, the flathead grey mullet of Western Mediterranean utilized lipids from three sources: (1) from the liver, which mainly involved the mobilization of n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA); (2) from the diet; and (3) by the depletion of intraperitoneal fat.

- Percentage saturated fatty acid reserves, especially of the palmitic acid (16:0), significantly decreased in the ovary during vitellogenesis.

- Mono-unsaturated fatty acid reserves in the ovary, principally of the palmitoleic acid (16:1) and the heptadecenoic acid (17:1) which is of bacterial origin and acquired through the diet, significantly increased during oocyte development.

- During the progression of vitellogenesis, PUFA significantly decreased as a percentage of total fatty acids in the ovaries. The reduction in PUFA was due to the decrease in Σn-6 fatty acids percentage, mainly of arachidonic acid (20:4n-6, ARA) which was accumulated at high levels in the ovary, and a decrease in Σn-3 fatty acids percentage, mainly of EPA and DHA.

Feed characteristics and feeding behavior:

- Optimal pellet diameters for flathead grey mullet juveniles (~ 360 g) were 2 and 4 mm, while for adults (~ 930 g) was 4 mm.

- Sinking or slow-sinking pellets would be recommended as flathead grey mullet preferred to feed in the water column and the bottom rather than in the surface.

Hormonal treatments:

- Treatment with 5 µg kg^{-1} of recombinant follicle-stimulating hormone (rFsh) produced in *Pichia pastoris* expression system combined with 15 mg kg^{-1} of metoclopramide, a dopamine antagonist, and 6.7 to 11.6 mg kg^{-1} of 17α-methyltestosterone administered via ethylene-vinyl acetate copolymer slow-release implants, stimulated spermiation in four out of six males with a low production (10 – 200 µL) of sperm.

- Treatment with rFsh and recombinant luteinizing hormone (rLh) produced in Chinese Hamster Ovary cells was able to induce oogenesis from previtellogenesis and early vitellogenesis through to oocyte maturation and ovulation in the 100 % of females that received the complete treatment.

- Treatment with rFsh and rLh enhanced spermatogenesis and induced spermiation in the 100 % of males with the production of fluent milt.

- The treatment with rFsh and rLh permitted the full control of reproduction in the flathead grey mullet: gave the possibility to synchronize both sexes development, to induce courtship behavior and tank spawning, to produce high egg quality and commercially valid numbers of viable larvae.

Transcriptome evaluation of induced vitellogenesis:

- The enriched molecular pathways and differential expressed genes described during the induced vitellogenesis of flathead grey mullet with rFsh and rLh were typical of natural oogenesis reported for other fish species.

- The application of rGths to induce vitellogenesis in the flathead grey mullet induced a great number of differentially expressed genes during vitellogenesis, and more genes were up-regulated than down-regulated as vitellogenesis progressed from previtellogenesis through to full-grown oocytes.

- The application of rFsh to females in previtellogenesis that lead to early-to-mid vitellogenesis, induced the upregulation of genes significantly enriched in GO terms and KEGG pathways related to cholesterol metabolism, ovarian steroidogenesis and reproductive development, ovarian growth and differentiation, and involved in the maintenance of the physiological activity of the ovary. The application of rFsh also upregulated genes enriched in pathways related to lipid metabolism and lipid accumulation in the oocytes, in processes important for energy uptake, and in cell-to-cell adhesion pathways.

- The application of rFsh and rLh at early-to-mid vitellogenesis that lead to late-vitellogenesis, induced the upregulation of genes significantly enriched in GO terms and KEGG pathways related to lysosomes activity.

- The application of rLh at late-vitellogenesis that lead to full-grown oocytes, induced: the downregulation of genes significantly enriched in the synthesis of estrogens and in cell-to-cell adhesion pathways; and the upregulated gene enrichment in the synthesis of C-21 steroids; processes linked with the preparation of the oocyte for maturation.

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ANNEX I

Supplementary Figures

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 [6], 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 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. 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 shows when females had oocytes ≥ 550 µm and a triangle shows the moment when females had oocytes ≥ 600 µm and maturation and ovulation was induced.

A

			Fish No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Fish No.
				Stage 1											Stage 2					
				Weeks																
EXPERIMENT 1	rGths treatment	FEMALES	1	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	15 rFsh	GnRHa + MET					1
			2–4												1000 hCG 15 rFsh	2000 hCG 15 rFsh	6000 hCG 15 rFsh	12000 hCG 15 rFsh		2–4
			5–9																	5–9
		MALES	n = 3																	n = 3
	Control	FEMALES	n = 11	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium						n = 11
		MALES	n = 3																	n = 3

B

			Fish No.	0	1	2	3	4	5	6	7	8	9	10	11	Fish No.
				Weeks												
EXPERIMENT 2	FEMALES	rGths treatment	1–2	6 rFsh	9 rFsh	12 rFsh	12 rFsh	12 rFsh + 2.5rLh	12 rFsh + 2.5rLh	12 rFsh + 2.5rLh	12 rFsh + 4 rLh	12 rFsh + 6 rLh	9 rLh * / 12 rLh *	▲ 15 rLh + P_4		1–2
			3									* 9 rLh / * 12 rLh	▲ 30 rLh + P_4 + PGF2α			3
			4–5									12 rFsh + 6 rLh	9 rLh * / 12 rLh *	12 rLh * / 12 rLh *	▲ 30 rLh + P_4	4–5
			6										4 rFsh + 9 rLh	▲ 15 rLh + P_4		6
			7–8											4 rFsh + 12 rLh	▲ 30 rLh + P_4	7–8
			9					N.T.								9
		Control	n = 7	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium		n = 7
	MALES	rGths treatment	n = 4				6 rFsh	9 rFsh	12rFsh + 2.5rLh	12rFsh + 2.5rLh	12rFsh + 5rLh	12rFsh + 9rLh	9 rFsh + 9rLh	4 rFsh + 12rLh		n = 4
		Control	n = 4				CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium	CHO medium		n = 4

Blood sampling — Ovarian biopsy and abdominal pressure — Sperm presence

* SGI oocyte diameter ≥ 550 µm

▲ SGfg oocyte diameter ≥ 600 µm or maximum diameter - Induction of maturation and ovulation

ANNEX II

Publications and Participation in Conferences

Publications from the thesis

Ramos-Júdez, S., Chauvigné, F., González-López, W. Á., Rosenfeld, H., Cerdà, J., Giménez, I., Duncan, N. 2021. Providing recombinant gonadotropin-based therapies that induce oogenesis from previtellogenic oocytes to produce viable larvae in a teleost, the flathead grey mullet (Mugil cephalus), *Aquaculture* (536), 736418. ISSN 0044-8486. https://doi.org/10.1016/j.aquaculture.2021.736418. **(Peer-reviewed article)**

Ramos-Júdez, S., Giménez, I., Gumbau-Pous, J., Arnold-Cruañes, L. S., Estévez, A., Duncan, N. 2021. Recombinant Fsh and Lh therapy for spawning induction of previtellogenic and early spermatogenic arrested teleost, the flathead grey mullet (*Mugil cephalus*), *bioRxiv 2021.09.29.462352*. https://doi.org/10.1101/2021.09.29.462352. **(Preprint article)**

Ramos-Júdez, S., Duncan, N. 2021. Feeding habits and the influence of pellet diameter on the feeding responses of the flathead grey mullet (*Mugil cephalus*) in captivity; *bioRxiv 2021.07.07.451406*. https://doi.org/10.1101/2021.07.07.451406. **(Preprint article) (Under review in Animal Feed Science and Technology)**

Participation in conferences

Ramos-Júdez, S., Giménez, I., Gumbau-Pous, J., Arnold-Cruañes, L. S., Estévez, A., Chauvigné, F., Cerdà, J., Duncan, N. Implementation of recombinant gonadotropin therapies to control maturation from early gametogenesis through to spawning of commercially valid numbers of viable larvae of flathead grey mullet *Mugil cephalus*. Oral presentation in Aquaculture Europe 2021, Madeira, Portugal, October 4 – 7, 2021.

Ramos-Júdez, S., Manousaki, T., Danis, T., Angelova, N., Tsakogiannis, A., Giménez, I., Duncan, N., Tsigenopoulos, C. Transcriptome analysis of *Mugil cephalus* ovarian

development induced by recombinant gonadotropins. Poster presentation in Aquaculture Europe 2021, Madeira, Portugal, October 4 – 7, 2021.

Ramos-Júdez, S., González, W., Duncan, N.J. La influencia del diámetro del pellet en las respuestas de alimentación en el múgil (*Mugil cephalus*) juvenil y adulto. Poster presentation in XVII National Aquaculture Conference, Cartagena, Spain, May 7 - 10, 2019.

Ramos-Júdez, S., Giménez, I., Chauvigné, F., González, W., Cerdà, J., Duncan, N.J. Inducción del proceso completo de maduración sexual en el múgil (*Mugil cephalus*) bajo condiciones de cautividad mediante gonadotropinas recombinantes: de estados inmaduros a la obtención de esperma, huevos y larvas viables. Oral presentation in XVII National Aquaculture Conference, Cartagena, Spain, May 7 - 10, 2019.

Ramos, S., Chauvigné, F., González, W., Rosenfeld, H., Cerdà, J., Giménez, I., Duncan, N.J. Recombinant follicle-stimulating hormone induces vitellogenesis and spermatogenesis in flathead grey mullet (*Mugil cephalus*). Poster presentation in the 11th International Symposium on Reproductive Physiology of Fish (ISRPF - 2018), Manaus, Brazil, June 3 - 8, 2018.