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Reproductive Management and Larval Culture of Flathead Grey Mullet (Mugil cephalus)

Giancarlo Enrique Cerrud Barría

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Reproductive Management and Larval Culture of Flathead Grey Mullet (*Mugil cephalus*)



Giancarlo Enrique Cerrud Barría

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Faculty of Biosciences

Department of Animal Biology, Plant Biology, and Ecology In collaboration with: *Institut de Recerca i Tecnologia Agroalimentàries (IRTA)*

Reproductive Management and Larval Culture of Flathead Grey Mullet (*Mugil cephalus*)

Thesis Submitted by

Giancarlo Enrique Cerrud Barría

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Aquaculture

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Bellaterra, 2026

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&
Institute of Agrifood Research and Technology (IRTA)

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DEDICATION

"*I am grateful to God for allowing me to reach this important stage of my academic life and for guiding my path.*

Finally, I would like to express my deepest gratitude to my parents, Mary Barría and Ezequiel Cerrud, for their unwavering support and encouragement—not only throughout this research, but throughout my entire life and career. I am equally thankful to my entire family—siblings, uncles, and relatives—for their constant support and encouragement.

With all my love, you are my strength and my greatest source of joy".

Giancarlo Cerrud Barría

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ABSTRACT

The flathead grey mullet (*Mugil cephalus*) is a euryhaline species with a strong sustainability profile and high market value, particularly females, which are prized for their roe, known as bottarga in Mediterranean regions. Due to its biological and economic characteristics, this species represents a promising candidate for aquaculture diversification. However, the future development of mullet aquaculture largely depends on achieving reproductive control under intensive conditions, which has been limited by severe reproductive dysfunctions; females frequently experience reproductive arrest at previtellogenic or early vitellogenic stages, while males either fail to produce sperm or release only small quantities of highly viscous milt. Technologies and protocols are needed to secure this reproductive control and achieve reliable out-of-season reproduction under intensive captive conditions. This would enable year-round production of high-quality eggs and larvae, reducing reliance on wild fry capture or on wild-caught mature broodstock. This thesis aimed to address these limitations by evaluating and improving recombinant gonadotropin (rGths) protocols for out-of-season spawning. Particular emphasis was placed on optimising rFsh administration during vitellogenesis and on assessing broodstock performance across three culture systems: earth ponds, recirculating aquaculture systems (RAS), and biofloc technology (BFT). Additionally, the potential of rFsh to induce early puberty in males was investigated to better understand its role in regulating spermatogonial proliferation, spermatogenesis progression, and testicular apoptosis.

Out-of-season spawning was successfully induced three months before the natural reproductive period. Eight females and seven males were divided into rGths-treated and control groups. Treated males completed spermiation, and females completed vitellogenesis and spawning in June–July 2023, whereas control fish showed no reproductive progression. Larvae were reared under different stocking densities and tank conditions. Survival at 42 days post hatch reached 14.13 ± 5.2%, and modelling predicted that optimal stocking densities could yield up to 3 juveniles/L. Larvae reared in black tanks exhibited significantly greater growth compared to those reared in grey tanks ($P = 0.01$). These results indicate that rGths protocols are effective for inducing out-of-season spawning and support their potential for reliable juvenile production under intensive aquaculture conditions.

Dose–response effects of rFsh on vitellogenesis were evaluated using four doses (3, 6, 9, and 12 µg/kg). Oocyte diameter increased significantly with increasing rFsh dose ($P < 0.001$), demonstrating a clear dose-dependent effect. Higher doses produced greater total fecundity, although individual variability limited statistical significance. Follicular atresia analysis revealed that higher rFsh doses reduced early-stage follicular degeneration, suggesting a protective role of rFsh during oocyte development.

The influence of culture systems on natural gonadal maturation was also investigated. First-maturation individuals failed to achieve reproductive maturation in all culture systems, exhibiting low gonadosomatic index, small oocyte diameter, and low steroid hormone concentrations. The fatty acid analyses revealed nutritional deficiencies, likely limiting reproductive development. Although BFT showed some potential in wild-caught adults, overall results indicate that environmental and nutritional limitations prevent spontaneous reproductive maturation under intensive culture conditions.

Finally, the role of rFsh in initiating puberty in juvenile males was evaluated. A single rFsh injection induced spermatogenic progression in 40% of treated individuals, accompanied by increased 11-ketotestosterone levels and histological evidence of spermatogenic advancement. Germ cell survival remained high, indicating that rFsh stimulates spermatogenesis without inducing widespread apoptosis.

Overall, the results of this thesis indicate that recombinant gonadotropin protocols represent an effective tool for overcoming reproductive dysfunction in captive mullet. These findings represent an important step towards sustainable hatchery-based production of mullet, supporting the diversification and sustainability of marine aquaculture.

Key words: Broodstock management, recombinant gonadotropins, reproductive control, larval development, aquaculture, *Mugil cephalus.*

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CHAPTER I



GENERAL INTRODUCTION



AQUACULTURE STATUS

Aquaculture is a technology-based activity that involves the controlled cultivation of aquatic organisms by managing key physiological processes, including reproduction, nutrition, growth, and survival. Through the controlled culture of commercially valuable species, aquaculture aims to enhance production efficiency and contribute to resource sustainability (Montanari *et al*., 2009).

In 2022, total fisheries and aquaculture production reached approximately 223.2 million tonnes, with world capture fisheries contributing about 92.3 million tonnes (41.1%), and global aquaculture accounting for 130.9 million tonnes (58.6%) (FAO, 2024) Asia was the leading region in global aquaculture production in 2022, accounting for 91.4% of total output, followed by Latin America and the Caribbean at 3.3%, Europe at 2.7%, Africa at 1.9%, Northern America at 0.5%, and Oceania at just 0.2% (FAO, 2024). Per capita consumption and population growth have increased in recent decades and are expected to continue increasing, reaching 21.5 kg in 2030, up from 20.6 kg in 2021. Asia represented 71% of consumers, followed by Europe (10%), Africa (8%), Northern America (5%), Latin America and the Caribbean (4%), and Oceania (1%) (FAO, 2024). Although fisheries provide approximately half of the production, capture fishing has declined by 2.3% in 2021 compared to 2019. This decline is mainly due to overfishing across most fishing grounds and the imminent threat of extinction facing several endangered species (Dulvy *et al.*, 2021). Consequently, aquaculture is expected to play an increasingly important role in meeting future demand for fish and seafood, including both inland and marine production systems. Within this broader context, marine aquaculture has contributed to increasing the supply of marine species for human consumption; however, it represents only one component of total global aquaculture production, rather than the dominant production system (FAO, 2024).

Finfish dominated global aquaculture production in 2022, accounting for 65.6% of world aquaculture production. Crustaceans (23.1%) and mollusks (12%) accounted for smaller shares of global production, while other aquatic animals accounted for 1.1%. By production area, inland aquaculture accounted for the largest percentage of global aquaculture production at 62.4%, while marine and coastal aquaculture accounted for 37.4%. This distribution highlights

that although marine aquaculture plays an important role, global aquaculture production remains predominantly inland-based (FAO, 2024).

Currently, the world's most important marine species are the common ray-finned fishes (*Actinopterygii)* (2,937,708.79 tonnes), which include the species pampano (*Trachinotus ovatus;*2,507,032.01 tonnes), European seabass (*Dicentrarchus labrax;*1,742,941.41 tonnes), gilthead seabream (*Sparus aurata;*1,634,580.32 tonnes) and Japanense amberjack (*Seriola quinqueradiata;* 1,028,712.85 tonnes) FAO, 2024. However, these species pose important challenges, particularly regarding seed production and nutritional requirements (Tacon and Metian, 2008). The main raw materials for their nutrition are fishmeal and fish oil, derived from a substantial portion of the world's fisheries. To produce this vital raw material, it is necessary to use whole fish species, especially small pelagic species such as anchovies (FAO, 2024). Unfortunately, due to global climate change, obtaining fish species for oil and fishmeal production for aquaculture is becoming increasingly difficult (Barange *et al.*, 2018). Therefore, diversification toward alternative species, such as mullet that do not have high nutritional requirements, represents an important strategy. Mullet is the most commercially important species within the family Mugilidae and is likely the most commercially viable omnivorous marine species, with minimal dietary fatty-acid requirements.

According to documents published by the FAO, 2024, fourteen significant mugilidae species are subject to capture and aquaculture worldwide, including abu mullet (*Liza abu*), bluespot mullet (*Moolgarda seheli*), bobo mullet (*Joturus pichardi*), flathead grey mullet *(Mugil cephalus*) (hereafter referred to as "mullet"), golden grey mullet (*Liza aurata*), Klunzinger's mullet (*Liza klunzingeri*), leaping mullet (*Liza saliens*), lebranche mullet (*Mugil liza*), mountain mullet (*Agonostomus monticola*), parassi mullet (*Mugil incilis*), redlip mullet (*Liza haematocheila*), thicklip grey mullet (*Chelon labrosus*), thinlip grey mullet (*Liza ramada*), and white mullet (*Mugil curema*).

The total capture of Mugilidae began in 1950 at 62,067 tonnes and reached 819,176 tonnes by 2023. Similarly, mullet aquaculture began in the 1950s, with production of 1,980 tonnes, increasing to 369,493 tonnes in 2023 **(Fig. 1)**.

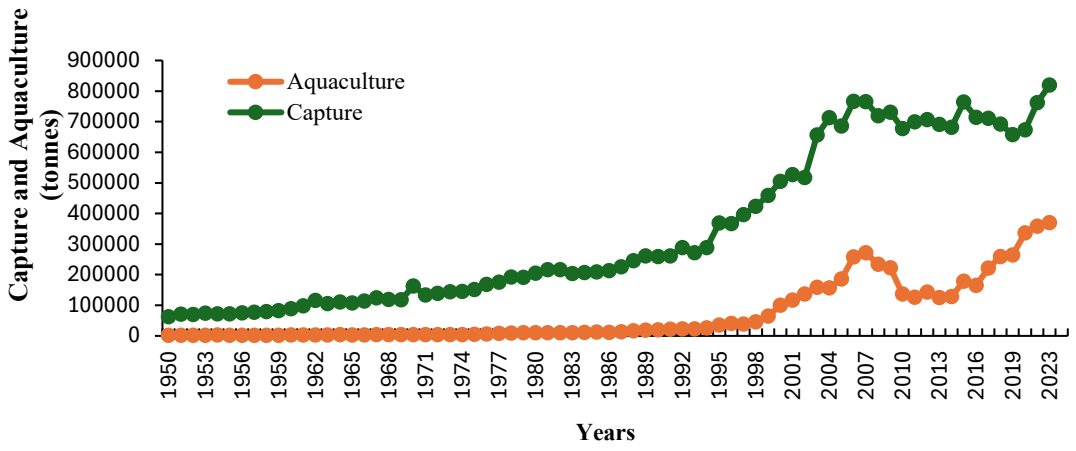


Figure 1. Capture and aquaculture production over the years for all mullets. The green line represents capture, while the orange line shows aquaculture production.

Several countries have historically participated in the capture and production of mullet. In 1950, the main countries involved in its capture were Japan (8,000 tonnes), Taiwan Province of China (3,000 tonnes), Venezuela (4,000 tonnes), Greece (1,500 tonnes), the Republic of Korea (1,000 tonnes), and the Socialist Federal Republic of Yugoslavia (100 tonnes) **(Fig.2)**. By 2023, capture activities were distributed among numerous countries, the most significant contributors being: China (69,611 tonnes), Mauritania (9,880 tonnes), the Republic of Korea (4,490 tonnes), the United States (3,000 tonnes), Venezuela (1,855 tonnes), Ecuador (1,601.15 tonnes), Greece (1,284.3 tonnes), New Zealand (868.4 tonnes), Taiwan Province of China (609.61 tonnes), Libya (460 tonnes), the Russian Federation (261 tonnes), Portugal (230 tonnes), Tunisia (201 tonnes), Senegal (113 tonnes), Colombia (110.23 tonnes), and France (105.3 tonnes) **(Fig.2)** (FAO, 2024).

Aquaculture production in mullet began in 1950 in China's Hong Kong SAR (500 tonnes), Taiwan Province of China (435 tonnes), and Italy (105 tonnes) **(Fig.2)**. By 2023, aquaculture production was represented by Israel (2,050 tonnes), Taiwan Province of China (1,759.66 tonnes), Singapore (352.84 tonnes), China's Hong Kong SAR (327.28 tonnes), Tunisia (211.84 tonnes), and French Guiana (200 tonnes) **(Fig.2)** (FAO, 2024). Aquaculture practices can be categorized into four distinct farming systems: extensive and semi-extensive (both typically

inland), and intensive farming systems, which may be located either inland or in marine environments.

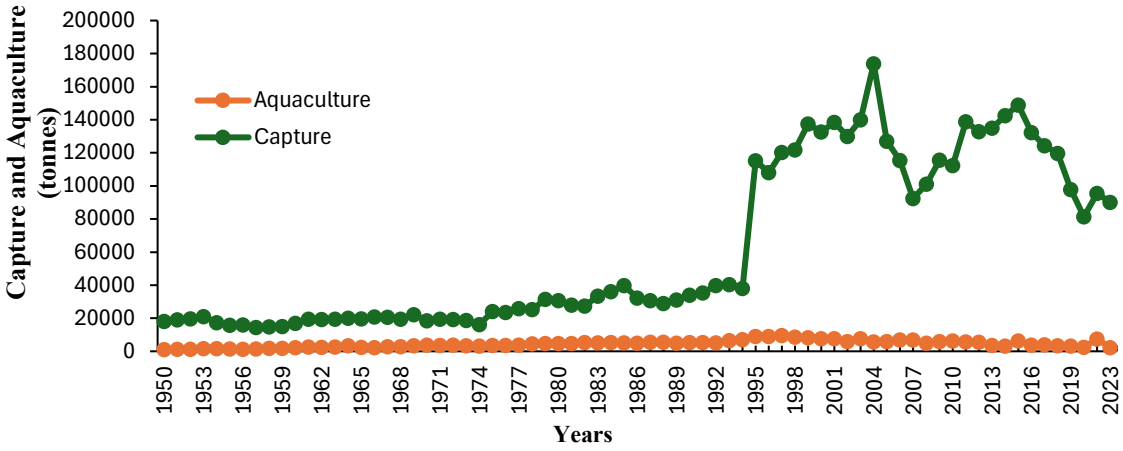


Figure 2. Capture and aquaculture production over the years for *Mugil* cephalus. The green line represents capture, while the orange line shows aquaculture production.

The flathead grey mullet (*Mugil cephalus*)

The mullet is an ecologically adaptable species found in a variety of habitats, but primarily in coastal waters in tropical and subtropical regions. In the western Atlantic, it ranges from Nova Scotia, Canada to Brazil, including the Gulf of Mexico. Notably, it is absent in the Bahamas and the Caribbean. In the eastern Atlantic, the species range spans from the Bay of Biscay (France) to South Africa, including the Mediterranean Sea and the Black Sea. In the eastern Pacific, the species is found from southern California to Chile **(Fig.3)** (FAO, 2004).

This species is native to 110 countries and islands. In Africa, it is found in Algeria, Angola, Cameroon, Comoros, Côte d'Ivoire, Egypt, Gabon, Gambia, Ghana, Guinea, Guinea-Bissau, Kenya, Libya, Madagascar, Mauritania, Mauritius, Mayotte, Morocco, Mozambique, Namibia, Rodriguez, Réunion, Sao Tome and Principe, Senegal, Seychelles, Sierra Leone, Somalia, South Africa, Tanzania, and Tunisia.

In Asia, its native range includes the Andaman Islands, Bahrain, Bangladesh, Cambodia, Chagos Islands, China, Christmas Island, Cyprus, Georgia, Hong Kong, India, Indonesia, Iran, Iraq,

Israel, Japan, Kazakhstan, South Korea, Kuwait, Lebanon, Malaysia, Myanmar, the Ogasawara Islands, Oman, Pakistan, the Philippines, Qatar, Saudi Arabia, Syria, Taiwan, Thailand, Turkey, the United Arab Emirates, Vietnam, and Yemen.

In Europe, *Mugil cephalus* is native to Albania, Bulgaria, France, Greece, Italy, Malta, Portugal, Romania, Russia (introduced), Serbia, Slovenia, Spain, the United Kingdom, and Ukraine. In Africa, it is reported from Cape Verde.

In North America, the species occurs in Canada, the United States of America and Mexico, and throughout Central America in Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama. In Caribbean islands, the species occurs in Trinidad and Tobago, Antigua and Barbuda, Barbados, Cuba, Dominica, the Dominican Republic, Grenada, Haiti, Jamaica, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, and Saint Vincent and the Grenadines. In South America, it may also occur in Guyana and Suriname.

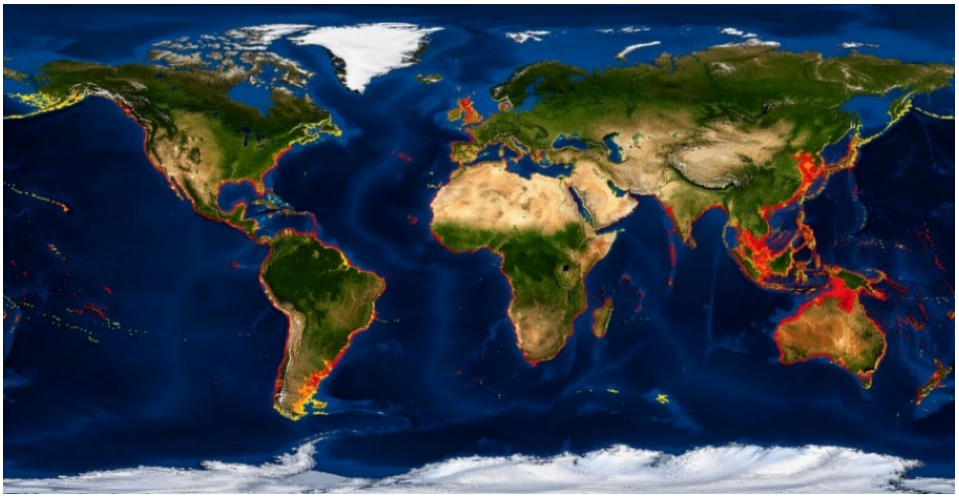


Figure 3. Distribution of range of colours. The distribution range is color-coded based on habitat suitability, which can be considered occurrence probabilities. The red color indicates high suitability with a probability ranging from 0.80 to 1.00, whereas the yellow color corresponds to low suitability with a probability ranging from 0.01 to 0.19 (Froese and Pauly, 2025).

The mullet is considered a cosmopolitan species found in the coastal waters of tropical, subtropical, and temperate regions worldwide. Throughout the species extensive distribution, the species has been recognised as an economically significant euryhaline and eurythermal

species, with considerable potential for aquaculture diversification in regions such as: the Mediterranean, the Republic of Korea, Taiwan, and South Africa. Key attributes that make mullet suitable for aquaculture include good adaptation to captivity, rapid growth (Whitfield *et al.*, 2012), omnivorous and detritivorous feeding habits (Vinatea *et al*., 2018). Furthermore, the high value of products processed from the mullet, including "bottarga" (the roe from fully mature females), which commands a market price exceeding 200 euros per kilogram (Besbes *et al.*, 2020).

The main morphological traits of mullet include a subcylindrical body shape, distinguished by an oval cross-section and a gently curved profile. The species exhibits a dorsal coloration ranging from blue to greyish-green, silvery flanks with dark longitudinal bands, and lacks a lateral line. The ventral surface exhibits a pale yellowish coloration. The fish sizes are generally medium to large, growing up to 120 cm in length, though most are closer to 30 cm (Crosetti and Blaber, 2016). Mullet are sexually dimorphic, with individuals developing as either males or females (González-Castro and Minos, 2015).

The mullet are characterised by a pyramidal head that is dorsally broad and nearly flat, with a gentle curvature. Laterally, the snout appears pointed, while in dorsal view, it presents a broad profile. The mouth is slightly subterminal, extending just above the anterior margin of the eye (Saleh, 2008). The eyes are large and positioned anteriorly, partially covered by a prominent adipose eyelid that forms a vertical slit, revealing only a portion of the pupil. The fish has two dorsal fins: the first one has four spines, and the second one has a single spine and eight branched rays. The pelvic fins are subabdominal in location and have one spine and five branched rays. The anal fin has two to three spines that are set very close together, followed by eight or more branched rays that are distinctly visible. The last ray terminates at a sharp point, but it is not as pronounced as the corresponding ray on the second dorsal fin, which is directly opposite. (Crosetti and Blaber, 2016). Mullet cannot be distinguished from other mugilids by fin spine and fin ray counts. This species is an oral and gill-based filter feeder that utilizes gill rakers and a denticulate pharyngobranchial organ to entrap and macerate particles for feeding (Cardona, 2016).

Mullet larvae are primarily zooplanktivorous, whereas juveniles and adults exhibit omnivorous feeding habits. Additionally, their diet may include invertebrates and plankton. In freshwater environments, algae also represent an important component of their diet (Cardona, 2016).

Sexual maturity in this species is typically attained at standard lengths of 20–30 cm in males and 27–30 cm in females, which generally corresponds to an age of around 3 years. The timing of the spawning season varies by geographic location, but it generally occurs within a temperature range that excludes extremes, avoiding waters colder than 17°C and warmer than 28°C (Whitfield *et al.*, 2012). During the reproductive season, adult mullets migrate from estuarine habitats to the sea to carry out a single spawning event.

Reproductive control in flathead grey mullet (*Mugil cephalus)*

Fish reproduction is often seasonal, with spawning occurring during specific periods of the year. This process requires prolonged gonadal development, beginning with immature germinal cells and culminating in mature gametes, ultimately leading to the propagation of a new generation. This comprehensive process, known as gametogenesis, includes oogenesis in females and spermatogenesis in males (Díaz and Neira, 2005). The reproductive cycle of both sexes is characterised by two distinct phases: gonadal growth and development (gametogenesis) and maturation (ovulation/spermiation), culminating in spawning (Mañanós *et al*., 2008). In fish, this intricate process is initiated with photoperiodic and water temperature variations, which are received as photic and thermal information through the eyes and other glands (Díaz and Neira, 2005). The pineal gland detects light stimuli and transmits them to the hypothalamus, which, in turn, regulates the pituitary gland through activation by gonadotropin-releasing hormone and inhibition of dopamine.

The pituitary gonadotropins (Gths) and receptors in the gonads are key to conveying the hormonal signals released by the pituitary as part of the brain-pituitary-gonad (BPG) axis. These Gths belong to the glycoprotein hormone family and are heterodimeric glycoproteins composed of a common α subunit and a hormone-specific β subunit linked non-covalently (Schulz *et al.*,

2001; Levavi-sivan *et al.*, 2010; Crosetti, 2015). The synthesis and regulation of Gths by the pituitary gland are primarily controlled by the neuropeptide gonadotropin-releasing hormone (GnRH). However, several other factors also contribute to this regulation, including kisspeptins, dopamine (Da), gonadotropin-inhibitory hormone (GnIH), neuropeptide Y (Npy), serotonin, leptin, glutamate, γ-aminobutyric acid (Gaba), norepinephrine (Ne), secretoneurin, ghrelin, and pituitary adenylate cyclase-activating peptide (Pacap). Hypothalamic neurohormones, mainly neuropeptide gonadotropin-releasing hormone (GnRH) and Kisspeptins (encoded by the Kiss1 gene), regulate the reproductive axis via the pituitary gland through the activation of the process of puberty by inducing the production of follicle-stimulating hormones (Fsh), which initiate gametogenesis. The Fsh is crucial in the initial phases of gamete development. In males, it supports the onset of spermatogenesis, while in females, it facilitates the initiation of oogenesis. These processes are mediated through the stimulation of key steroid hormones, notably 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) (Degani, 2020). In females, Fsh stimulates 17β-estradiol production, which acts on the liver receptor to initiate and incorporate vitellogenin into developing eggs (Díaz and Neira, 2005). In males, Fsh promotes the proliferation of Sertoli cells to maintain normal spermatogenesis (Ozaki *et al*., 2006; Chauvigné *et al.*, 2012; Crosetti, 2015). The Lh levels increase during the later stages of gametogenesis to stimulate final maturation, oocyte maturation, ovulation in females, and spermiation in males (Mañanós *et al*., 2008).

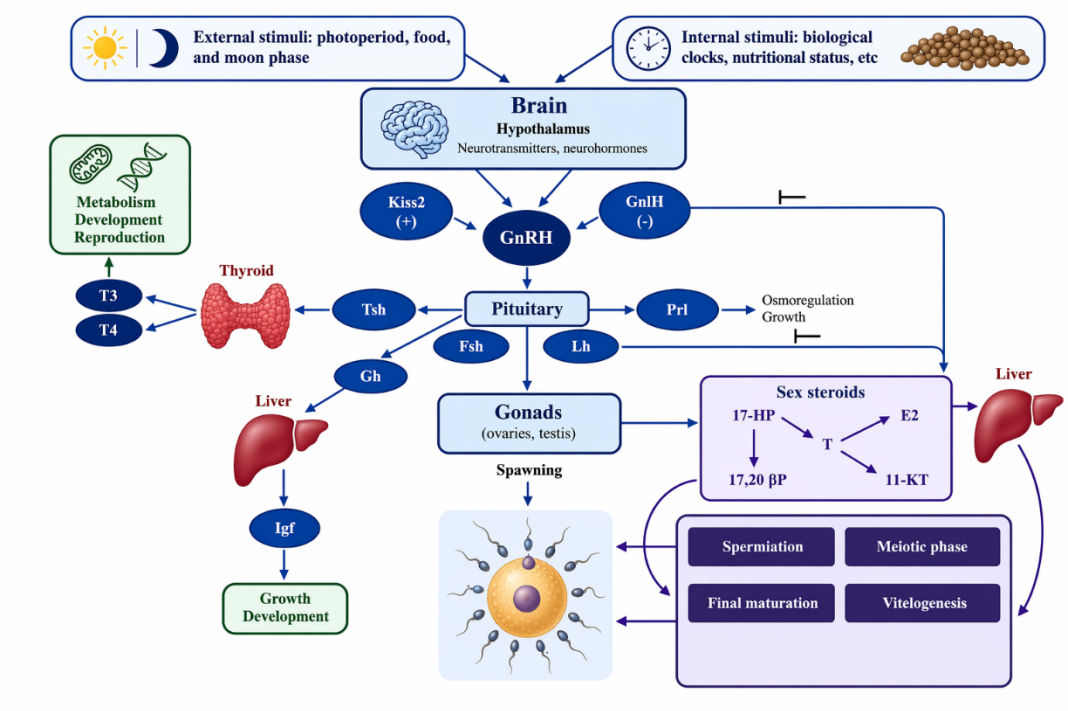


Figure 2. Brain-pituitary-gonad (BPG) Axis. This figure illustrates the intricate interactions among the brain, pituitary, and gonad (BPG). Key components include Gonadotropin-releasing hormone (GnRH), Follicle-stimulating hormone (Fsh), Luteinizing hormone (Lh), Prolactin (Prl), Growth hormone (Gh), Kisspeptin (Kiss2), Gonadotropin inhibitory hormone (GnIH), Growth factors (Igf), Thyroid-stimulating hormone (Tsh), Triiodothyronine (T3), and Tetraiodothyronine (T4). The secretion of GnRH acts upon a specific population of gonadotropic cells within the pituitary gland, instigating the release of Lh and Fsh. Additionally, the pituitary gland serves as the site of synthesis, storage, and release for Gh, Tsh, and Prl. Functioning as a transducer, the pituitary gland orchestrates various endocrine functions through its secretions, encompassing reproduction, osmoregulation, growth, and metabolism (Rodríguez Gabilondo *et al.*, 2021).

Spawning History of flathead grey mullet (*Mugil cephalus*)

Aquaculture in the Mediterranean has seen an increasing focus on species diversification. Traditionally, the cultivated species were carnivorous, such as seabream (*Sparus aurata*), greater amberjack (*Seriola dumerili*), and meagre (*Argyrosomus regius*), which require high-protein diets (Kuo *et al.*, 1973). However, omnivorous species are gaining attention for their lower requirements for fishmeal and fish oil, with mullet emerging as a promising candidate. However, breeding mullet presents two major reproductive challenges: the inability to complete gametogenesis and failure of final oocyte maturation (Shehadeh *et al.*, 1973a). These issues are related to disruptions in the hypothalamus-pituitary-gonadal (HPG) axis (Zohar and Mylonas, 2001) and are influenced by the specific conditions of semi-intensive and intensive aquaculture

systems. In semi-intensive systems, males often reach sexual maturity but produce viscous, non-functional milt (Shehadeh *et al*., 1973b), while females complete vitellogenesis but fail to achieve final oocyte maturation, ovulation, and spawning (Kuo *et al.*, 1974a, 1974b; Pien and Liao, 1975). In intensive systems, the dysfunction is even more severe, with females unable to progress beyond early stages of vitellogenesis, and males do not produce any fluent sperm (Ramos-Júdez *et al.*, 2021, 2022b). These failures to achieve reproductive success across different environments highlight the complex multifactorial nature of reproductive dysfunction in mullet under captive conditions. This emphasises the need for integrated biotechnological strategies. Early advances in hormone-induced spawning established foundational knowledge of oocyte maturation and gamete viability (Abraham and Yashouv, 1966; Shehadeh and Ellis, 1970; Shehadeh *et al.*, 1973a). However, variability in outcomes from different hormonal protocols reflects the species' high sensitivity to both endocrine signals and environmental factors (Lee *et al.*, 1988). Cost-effective approaches—such as combining carp pituitary extract (CPE) with luteinizing hormone-releasing hormone analogs (LHRH-a)—have shown potential for scalable application (Lee *et al*., 1988), although reproductive responses remain inconsistent. Environmental manipulation, particularly through controlled photoperiod and temperature, has proven essential in promoting oocyte development (Kuo *et al*., 1974b), while androgen treatments have supported male sexual maturation (Weber and Lee, 1985). More recently, fertilisation and hatching success rates have been greatly improved by using refined hormonal therapies, such as dopaminergic antagonists and GnRHa implants (Aizen *et al.*, 2017; Besbes *et al.*, 2020; Vallainc *et al.*, 2021).

Despite these advances, hormone therapies are not yet fully applicable in intensive aquaculture systems (Besbes *et al.*, 2020). The reliance on wild-caught broodstock in semi-intensive systems presents logistical and sustainability concerns, leading to the need for more reliable and reproducible approaches. While intensive aquaculture systems offer better broodstock control, they have historically faced severe reproductive dysfunction in mullet.

Recent efforts have addressed this challenge by using weekly recombinant follicle-stimulating hormone (rFsh) and luteinizing hormone (rLh) injections over 13 weeks, aligned with the natural spawning season. This approach successfully regulated gametogenesis and yielded viable

gametes, achieving fertilisation rates of 63 ± 21% and hatching rates of 58 ± 23% (Ramos-Júdez *et al.*, 2021, 2022b). Recombinant gonadotropins (rGths) have been successfully used to overcome reproductive dysfunctions in adult cultured fish, and rFsh has proved to stimulate spermatogenesis in Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2018), European eel (*Anguilla anguilla*) (Peñaranda *et al.*, 2018) and flathead grey mullet (*Mugil cephalus*) (Ramos-Júdez *et al.*, 2021, 2022b).

Larval Culture and Live Food

The mullet larviculture process faces several significant challenges that impact survival and growth rates. One of the primary issues identified is the high mortality rate during early life stages, particularly between 2–4 days post-hatch (after yolk sac absorption) and again around 8–11 days post-hatch. The latter phase is often linked to mechanical damage from tank surfaces and the larvae's natural migration behaviour, which makes them prone to injury (Kuo *et al.*, 1973; Nash *et al.*, 1974). A better understanding of the behaviour exhibited by these larvae is also needed to tailor the design of the tanks to provide a suitable environment that reduces mortalities during the early stages.

Feeding is vital to the survival of mullet larvae, as they rely on exogenous food sources such as rotifers and *Artemia* (Nash *et al.*, 1974; Eda *et al.*, 1990; Abraham *et al.*, 1999; Vallainc *et al.*, 2021; Chhaba, 2022). Research highlights the importance of timing when introducing live foods, which must be enriched with essential nutrients, DHA derived from algae to ensure proper growth (Watanabe *et al.*, 1983; Eda *et al.*, 1990; Tamaru *et al.*, 1993). Optimising these feeding strategies, including the timing and enrichment of *Artemia nauplii*, is vital for improving survival rates and accelerating development. Thus, the consistency and quality of feeding protocols is critical for successful mullet larviculture.

Another key factor is maintaining optimal environmental conditions, such as temperature, salinity, and dissolved oxygen levels (Sylvester *et al.*, 1975; Sylvester and Nash, 1975; Kraul, 1983; Walsh *et al.*, 1991; Yousif *et al.*, 2010). Mullet larvae require a salinity of 32 – 36 ppt and

a temperature of 22 to 24ºC for optimal growth. Also, stable oxygen levels are essential for maintaining larval health and development (Murashige *et al.*, 1991; Sylvester *et al.*, 1975).

Despite efforts to optimise practices, survival rates in mullet larviculture are often low, typically ranging between 1% – 4% after 30 days (Oz *et al.*, 2022). Further research is required to address this issue and improve survival, including genetic selection and optimization of feeding regimes and environmental controls.

Another key issue to consider is sustainability in mullet farming; production still depends on wild-caught fry, which is not sustainable in the long term to meet increasing demand. Developing reliable breeding programs and refining hatchery techniques to reduce dependence on wild stock is essential (Gisbert *et al.*, 2016). Sustainable practices, including artificial spawning and optimised hatchery conditions, will be crucial for maintaining a steady supply of fry for aquaculture operations (Ramos-Júdez *et al.*, 2022; Cerrud-Barría *et al.*, 2026).

General problems in aquaculture

One of the main challenges in aquaculture is achieving reliable control of gametogenesis and improving reproductive performance under intensive culture conditions. While recombinant hormones therapy has shown potential for success in addressing these challenges, its application remains limited. Continued exploration and development of these hormonal treatments are crucial for overcoming reproductive dysfunction, particularly in intensive aquaculture systems.

Low survival and growth rates in certain aquaculture species are another significant issue. To address this, researchers need to focus on refining feeding strategies, optimizing environmental conditions, and gaining a better understanding of larval behaviour. These improvements are crucial for the efficient and sustainable development of aquaculture practices. Specifically, reducing reliance on wild-caught fry and improving hatchery success are critical steps forward. Ultimately, the future of aquaculture lies in continuously enhancing feeding protocols and overall cultivation practices for species such as the mullet.

OBJECTIVES AND HYPOTHESES

The flathead grey mullet (*Mugil cephalus*) (hereafter referred to as "mullet") has become increasingly relevant as a candidate for aquaculture diversification due to their favorable ecological characteristics, broad adaptability to diverse farming conditions, and significant commercial importance in several countries. Additionally, the female mullet's gonads offer considerable added economic value, particularly in markets where they are considered a delicacy.

Despite their potential, the main bottleneck to the expansion of mullet aquaculture is insufficient reproduction in captivity. Currently, this aquaculture industry depends on capturing wild juveniles, which is unsustainable in the long term and therefore cannot be used to scale up production. This research aimed to contribute to this transition by exploring key aspects of reproductive control and larval rearing. The outcomes were expected to support the development of improved breeding protocols and enhance the reliability of seed production in aquaculture systems.

General Objective

To achieve reliable reproductive control of mullet under intensive culture conditions, including the extension of the natural spawning period.

Specific Objectives

i. To improve the reproductive performance of mullet in captivity using recombinant hormones (rGths) outside their natural spawning season, and to evaluate egg quality and viable juvenile production from rGth-induced spawning **(CHAPTER II)**.
ii. To evaluate the effects of different doses of recombinant follicle-stimulating hormone (rFsh), specifically 3, 6, 9, and 12 µg/kg — on the vitellogenesis stage and overall fecundity **(CHAPTER III)**.

iii. To assess the impact of three different aquaculture systems (biofloc technology, earthen ponds, and recirculating aquaculture systems) on the reproductive performance of mullet **(CHAPTER IV)**.

iv. To determine the effects of recombinant follicle-stimulating hormone (rFsh) on the onset of puberty and testicular development in juvenile male mullet, with particular emphasis on the role of follicle-stimulating hormone in spermatogenesis dynamics, progression of spermatogenesis, and apoptotic processes during the pre-pubertal period (**CHAPTER V)**.

The study tested the following hypotheses:

(i) Long-term administration of rGths outside of the natural reproductive season induces gonadal development and spawning in captive mullet.

(ii) Increasing doses of rFsh influence the vitellogenesis stage and fecundity in female mullet.

(iii) Culture system type affects gonadal maturation, fecundity, and gamete quality in mullet broodstock.

(iv) Recombinant gonadotropin treatment, particularly recombinant follicle-stimulating hormone (rFsh), stimulates puberty onset, testicular development, and spermatogenesis in juvenile mullet.



CHAPTER II

RECOMBINANT GONADOTROPIN INDUCED OUT-OF-SEASON SPAWNING AND LARVAL CULTURE OF FLATHEAD GREY MULLET (*MUGIL CEPHALUS*)

1. Introduction

The flathead grey mullet (*Mugil cephalus*) (hereafter referred to as “mullet”) is distributed in both temperate and tropical waters in the Pacific, Indian, and Atlantic Oceans (extending from Bay of Biscay to the entirety of the Mediterranean and Black Sea) (FAO, 2009). Over this distribution, mullet has been recognised as an economically important euryhaline and eurythermal species with potential for aquaculture diversification in the Mediterranean and other regions, such as the Republic of Korea, Taiwan, and South Africa. The species positive attributes for aquaculture include good adaptation to captivity, rapid growth (Whitfield *et al.*, 2012), omnivorous and detritivore feeding habits (Vinatea *et al.*, 2018), and a valued food market including "bottarga" (the roe of fully mature females) with a market value exceeding 200 euros per kilogram (Besbes *et al.*, 2020).

However, a bottleneck for mullet aquaculture development is reproductive control in captivity. The mullet is a species that exhibits gonochorism, with defined male and female sexes. The timing of maturational development and the natural spawning season vary with environmental conditions in each region (FAO, 2009). In the Western Mediterranean, gametogenesis was from July to September, and spawning occurred during September and October (Katselis *et al.*, 2005; Lamraouhi *et al.*, 2022; Ramos-Júdez *et al.*, 2023). Mullet reproductive control has been studied in a range of systems, including earth and rubber-lined ponds (Kuo and Nash, 1973; Liao, 1975; Weber and Lee, 1985; Lee *et al.*, 1987; Lee and Tamaru, 1988; Tamaru *et al.*, 1989; Karim *et al.*, 2016; Besbes *et al.*, 2020), large tanks (Shehadeh *et al.*, 1973a; Shehadeh *et al.*, 1973b; Kuo *et al.*, 1974a; Lee and Weber, 1986; Lee *et al.*, 1987; Yousif *et al.*, 2010; Vazirzadeh and Ezhdehakoshpour, 2014; Sukumaran *et al.*, 2021) and more recently, intensive recirculating aquaculture systems (Ramos-Júdez *et al.*, 2021, 2022b, 2022a). Depending on the region and the broodstock holding system, two reproductive dysfunctions have been reported. Generally, when held in larger semi-intensive systems, mullet exhibited a reproductive dysfunction in the late stages of maturation. The males matured but produced viscous milt that failed to fertilize eggs (Shehadeh *et al.*, 1973c), while the females completed vitellogenesis, but did not undergo oocyte maturation, ovulation, and spawning (Kuo *et al.*, 1974a, 1974b; Liao, 1975). In intensive systems, mullet exhibited a more severe reproductive dysfunction where the females fail to progress through the vitellogenesis phase, and males fail to generate fluid sperm (Ramos-Júdez

et al., 2022b). These two types of reproductive dysfunctions were described and classified by Zohar and Mylonas, (2001). The dysfunctions have been attributed to stress and absence of environmental signals from the captive conditions of the broodstock holding system, which cause alterations in the endocrine control of the brain-pituitary-gonadal (BPG) axis. The pituitary gland produces two gonadotropin forms (Gths, the most important hormones in fish reproduction), follicle-stimulating hormone Fsh, and luteinizing hormone Lh (Díaz and Neira, 2005). In teleosts, Fsh has been described to promote gametogenesis from early to late stages. In females, Fsh stimulates the production of 17β-estradiol, which acts on hepatic nuclear estrogen receptors to initiate vitellogenin production for the developing oocytes. In males, it stimulates Sertoli cell proliferation to maintain quantitatively normal spermatogenesis. The Lh levels increase during the later stages of gametogenesis to stimulate final maturation, oocyte maturation, and ovulation in females and spermiation in males (Mañanós *et al.*, 2008). In semi-intensive systems, mullet maturation was arrested in the late stages as Lh was probably not released to continue with oocyte maturation and spermiation. In intensive systems, mullet maturation was arrested in the early stages, as both Fsh and Lh were probably not released to advance beyond the early stages of gametogenesis. Both dysfunctions have been overcome with hormonal treatments.

Many studies have focused on mullet arrested in the late stages of maturation, obtained from both semi-intensive systems and wild-caught fish. Attempts to reproduce mullet were initiated in Taiwan in 1964 (Liao, 1975). Various hormone therapies have been successful, such as human chorionic gonadotropin (HCG) (Kuo *et al.*, 1974a, 1974b), salmon gonadotropin (SG-G100) alone (Shehadeh *et al.*, 1973b; Kuo *et al.*, 1974b; Kuo and Nash, 1975) or combined with HCG or deoxycorticosterone (Kuo and Watanabe, 1978), gonadotropin-releasing hormone analogue (GnRHa) alone or combined with carp pituitary extract (CPE) (Lee *et al.*, 1987) or human chorionic gonadotropin (HCG) combined with a second and third injection of GnRHa (des Gly^{10}[$D\text{-}Ala^{6}$] GnRHa ethylamide) (Meseda and Samira, 2006), a single dose of the GnRHa preparation, leuprorelin acetate (ENANTONE®, Takeda Italia S.p.a., $C_{59}H_{84}N_{16}O_{12}$, amino acid sequence H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu- Arg-Pro-NHEt) (Vallainc *et al.*, 2021) or a GnRHa implant in combination with dopamine antagonists (Domperidone -5 mg/kg) (Aizen *et al.*, 2005). These studies demonstrated that the dysfunction in the late stages of maturation can

be overcome with a range of different hormonal therapies. However, the use of semi-extensive systems or wild-caught fish has logistical and sustainability problems for aquaculture, and the experimentation with a wide range of therapies also indicates that improved results on these therapies are desirable.

The use of intensive aquaculture systems provides fuller control of broodstock, but as mentioned, mullet exhibited a severe reproductive dysfunction that has only recently been overcome with weekly injections of recombinant hormones (rFsh and rLh) to regulate gametogenesis over a long-term 13-weeks period that coincided with the natural spawning period of mullet (Ramos-Júdez *et al.*, 2021, 2022b). This study demonstrated the ability to induce vitellogenesis and spermatogenesis to produce viable gametes that had 63 ± 21% fertilisation and a 58 ± 23% hatching rate to produce large numbers of viable larvae. Although rGths have been used to control gametogenesis in other fish species such as in Japanese eels (*Anguila japonica*) (Kazeto *et al.*, 2008; Kobayashi *et al.*, 2010), Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2018), Malaysia catfish (*Hemibagrus nemurus*) (Salwany *et al.*, 2019), Macquarie perch (*Macquaria australasica*) (Nocillado *et al.*, 2023) and European eel (*Anguilla anguilla*) (Jéhannet *et al.*, 2023a), these studies did not demonstrate that viable gametes and larvae from rGths treatment can form the bases for juvenile aquaculture production.

Considering this captivity-induced reproductive dysfunctions encountered to control mullet reproduction and the hormonal induction therapies applied to obtain spawning, no work has been published to control mullet reproduction to obtain out-of-season spawning. To obtain out-of-season spawning, the entire reproductive process must be displaced to a different part of the year, with the aim of obtaining eggs at dates outside of the natural spawning period (Bromage *et al.*, 2001). Commonly, environmental cues of photoperiod and temperature are manipulated to displace the entire reproductive process, and hormonal therapies are applied when the fish have matured to advanced stages of sexual maturity (Bromage *et al.*, 2001). The development of recombinant gonadotropin therapies (Chauvigné *et al.*, 2018; Ramos-Júdez *et al.*, 2021, 2022b; Jéhannet *et al.*, 2023a; González-Cid *et al.*, 2024) opens the window to control the entire process of reproduction out-of-season without the necessity to control environmental parameters months or even years before the period of gametogenesis.

Recombinant hormones have shown promising results, but further work is needed to demonstrate that rGths can successfully induce out-of-season spawning and produce the quality of eggs and larvae, that will guarantee the supply of high-quality seed with high survival for growing out in fish farms at different times of the year. The principal aim of this study was to induce spawning outside the natural spawning period and further characterise the quality of gametes for larval culture of mullet.

2. Materials and methods

2.1. Broodstock and maintenance

An experiment was conducted between March 20th and July 31st, 2023, to induce gametogenesis in mullet using recombinant hormones. The experiment involved 15 mullet randomly selected from a stock of 38 wild adult mullet, with an average body weight of 1.53 ± 0.28 kg and a mean standard length of 48.4 ± 4.94 cm introduced into IRTA´s facilities (IRTA Aquaculture, Centre de La Rápita, Spain) between 2015 and 2018. Each fish was tagged intramuscularly with a Passive Integrated Transponder (PIT) tag (Trovan®, ZEUS Euroinversiones S.L. Madrid, Spain).

The selected breeders were placed in a 10 m^3 tank with a natural photoperiod from natural daylight increasing from LD12:12 in March to LD14:10 in July (L: light; D: dark), a constant temperature of 24 ºC maintained with a heat pump, a salinity of 36 ‰, and 6.5 ppm of dissolved oxygen in a recirculation system (IRTAmar®). The tank had both surface and bottom water outlets that were adjustable to control the volume of water exiting the tank from each outlet. The surface water outlet was adjusted to facilitate that spawned eggs in water exiting the tank were collected in a 500 µm mesh basket. The fish were fed daily once with a commercial Repro Sole diet (5 mm) (Sparos, Portugal), at 1.5% body weight. The diet contained 60% protein, 13% fat, 0.5% fibre, 7% ash, 1.2% phosphorus, and 0.9% calcium.

Ethics. The research was carried out in adherence to the following regulations: the Spanish Royal Decree 53/2013 of February 1st, which also concerns the protection of animals used for scientific purposes; the Catalan Law 5/1995 of June 21st, which pertains to safeguarding animals used for scientific purposes; and the Catalan Decree 214/1997 of July 30th, which governs the regulation of such animals. The European Directive 2010/63/EU was implemented on September 22nd, 2010. The Catalan Government and the Committee of Ethics and Experimental Animals (CEEA) of IRTA evaluated the procedures used in this study, which were authorized with authorisation of experimental project number 12016, expedient number FUE-2023-02998468 and ID NT0G72MXQ. ARRIVE guidelines were adhered to by the authors.

2.2. Induction with recombinant hormones

The breeders were randomly divided into two groups: rGth-treated and control. Four females and three males (1.52 ± 0.37 kg/body weight and 48.6 ± 4.4 cm standard length) received the rGths while four females and four males (1.62 ± 0.26 kg/body weight and 49.3 ± 4.9 cm standard length) were administered saline solution and served as controls. Throughout the experiment, the fish were anesthetised with MS-222 at a dose of 73 mg/L for weekly sampling and the application of hormone and saline control treatments. The recombinant hormones (rFsh and rLh) used were produced using Chinese hamster ovary (CHO) cells from isolated Gth-coding cDNAs of mullet by Rara Avis Biotec S.L., Valencia, Spain (Ramos-Júdez *et al.*, 2022a).

2.2.1. Induction of vitellogenesis and final maturation in females

The eight females selected for the study had oocyte diameters of 105.8 ± 5.81 µm. The treatments of the females initiated on staggered dates, every two weeks a pair of one rGth female and one control female received the first recombinant hormones and saline respectively. The treatment of each pair commenced on March 20th, April 3rd, April 17th, and May 2nd, 2023. This programmed the spawning of the females to be every two weeks to facilitate live food production for larval rearing. The rGth were administered to mullet following the protocol described by Ramos-Júdez *et al.* (2022b). The objective was to stimulate the transition from previtellogenesis (oocyte diameter of approximately 100 µm) to vitellogenesis (oocyte diameter

exceeding 200 µm), the completion of vitellogenesis (600 µm), oocyte maturation, ovulation and finally spontaneous tank spawning of fertilised eggs. The administration of weekly intramuscular dosages of rFsh was escalated from 6 µg/kg to 9 µg/kg and finally to 12 µg/kg. The administration of rFsh at 12 µg/kg was sustained throughout vitellogenesis. As vitellogenesis progressed and oocytes reached a mean diameter ≥300 µm, the females received additional weekly doses of recombinant luteinizing hormone (rLh): starting 2.5 µg/kg until (≥ 400 µm), followed by an increase to 4 µg/kg and 6 µg/kg. As oocyte diameter approached 500 µm, weekly dosages rFsh were decreased to 4 µg/kg, while rLh dose was increased to 9 µg/kg. The combined regimen of 4 µg/kg rFsh and 12 µg/kg rLh was continued weekly until the completion of vitellogenic development **(Fig. 1)**. The completion of vitellogenesis was established as when the most advanced oocytes had a mean diameter of approximately 600 µm. The final administration of rLh was given at this stage, with two injections of 30 µg/kg separated by a 24-hour interval. The control group received saline injections at a dosage of 1 mL over the same thirteen-week period **(Fig. 1)**.

A 1.67 mm (5ch) x 500 mm plastic cannula was used to collect ovarian samples from each fish (weeks 0, 4, 7, 9, 11, 12, 13, and 14) and the diameter of the 20 most advanced oocytes was measured using a Zeiss Axiostar microscope at a magnification of 40X. Clearing solution (6 mL absolute ethanol, 3 mL formalin, 2 mL glycerol) was used to clear the 20 most advanced oocytes (Ramos-Júdez *et al.*, 2019) and the position of the germinal vesicle was registered. Part of the oocyte sample was preserved in Bouin`s solution and stored in 70% ethanol until histological analysis. From week 0, 4, 7, 9, 11, and 13 in females, blood samples were collected immediately before the injection of saline or hormones.

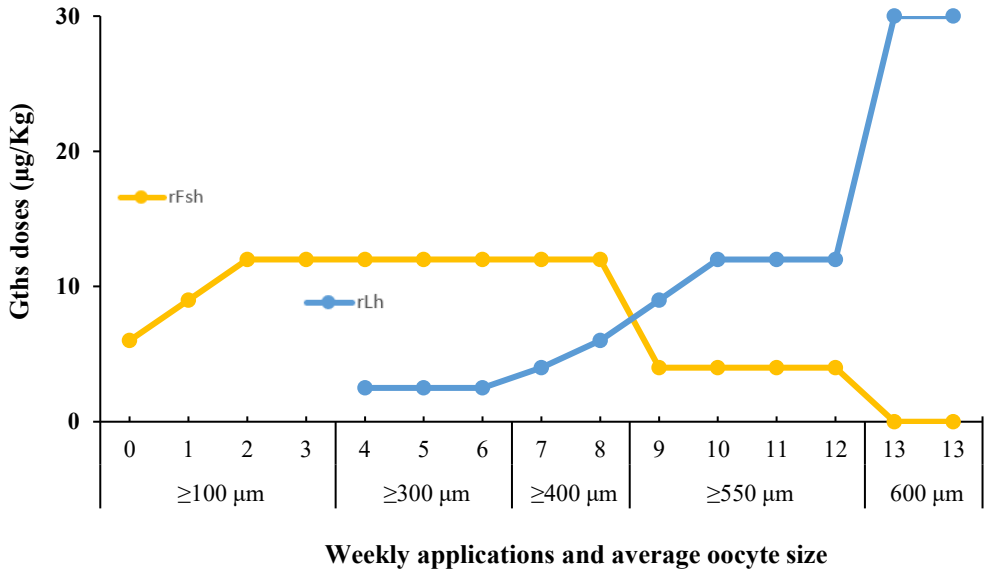


Figure 1. Protocol for administration of recombinant hormones to four females at weekly intervals. The protocol was applied according to Ramos-Júdez *et al.*, (2022b). Weekly doses of 6, 9, and 12 µg/kg rFsh were applied to induce previtellogenesis (~100 µm oocyte diameter) to vitellogenesis (> 200 µm) and vitellogenic growth was maintained with a dose of 12 µg/kg 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 rLh was maintained until females presented ≥ 400 µm oocytes and was raised to 4 and 6 µg/kg. At ~500 µm diameter, weekly rFsh doses were reduced to 4 µg/kg whereas rLh was increased to 9 µg/kg. A combination of 4 µg/kg rFsh and 12 µg/kg rLh per week were administered until vitellogenic growth was completed (~600 µm). The final administration of rLh was given at this stage, with two injections of 30 µg/kg separated by a 24-hour interval. Each point corresponds to a weekly administration. The yellow line represents rFsh doses, and the blue line represents rLh.

2.2.2. Induction to spermatogenesis and final maturation in males

The induction of gametogenesis in males started four weeks (week 4) after the females **(Fig. 2)**. A total of seven males (without sperm presence), were randomly divided into two groups: rGths (n = 3) and control (n = 4). The degree of spermiation was estimated based on four categories: 0 (no presence of sperm when performing the massage), 1 (presence of sperm, but poor fluidity, impossible to obtain a sample), 2 (fluid) and 3 (very fluid, pressure not necessary).

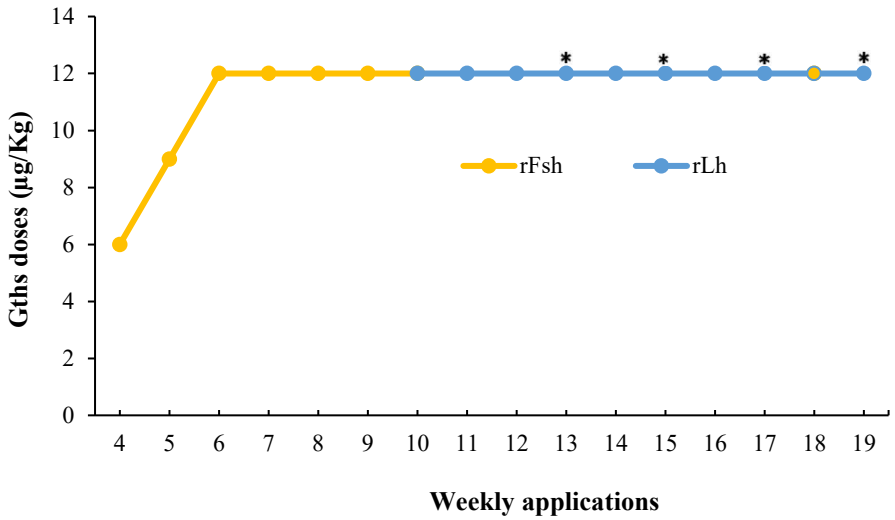


Figure 2. Illustrates the weekly administration of recombinant hormones therapy to the males (*n* = 3), following the protocol detailed by Ramos-Júdez *et al.* (2022b). The initiation of rFsh therapy for males initiated four weeks after the females (week 4), starting with 6 µg/kg, followed by an increase to 9 µg/kg in week 5 and 12 µg/kg from weeks 6 to 9, while rLh therapy initiated from week 10-19 at 12 µg/kg**.** On week 18, a dose of rFsh at 12 µg/kg was administered together with the rLh dose at 12 µg/kg for two males and 24 µg/kg for one male, as sperm motility in the three males was 50%. The asterisks-marked weeks (*) correspond to female spawning (week 13, 15, 17 and 19). Each data point depicted on the charts corresponds to a specific induction week. The orange line represents rFsh doses, while the blue represents rLh.

The same three rGths-treated males were used for all four spawning events **(Fig. 2)**, with each female from the rGths group. Sperm samples were collected in weeks 18 and 19. From weeks 4, 9, 13, 17, and 19 in males, blood samples were collected immediately before the injection of saline or hormones.

2.2.3. Induced spawning

The fish that were induced to spawn were transferred to a 10 m^3 spawning tank, isolated from the main group under the same conditions (temperature, water replacement, and light condition). The ratio was 1 female with 3 males, for all four spawning events. After spawning, both the female and male were then relocated to the main tank. The following spawning data was collected latency period, floating eggs weight, total number of eggs, percentage of fertilised eggs and percentage of hatched eggs.

2.3. Egg disinfection and incubation

The eggs were transferred to IRTA´s larviculture facility that consisted of a recirculation system equipped with tronco-conical tanks (100 L in black and 500 L in black and grey colours). The floating eggs were disinfected using 350 parts per million (ppm) of hydrogen peroxide (48%) for 10 minutes with continuous aeration. Following disinfection, eggs were incubated in 500L tank under controlled conditions in low lighting: continuous aeration, 22 °C, 6 mg/L and 37.8‰ for temperature, oxygen, and salinity, respectively. A water flow rate of 0.7 L/min was used and a central mesh (150 μm) was used to prevent eggs leaving the tank.

After one hour of incubation, a sample of eggs was collected and eggs were individually placed (one egg per well) in two 96-cell well microplates filled with sterile seawater and incubated at 24 °C (Giménez *et al.*, 2006; Shields *et al.*, 1997). Daily evaluations of the microplates were conducted to assess the hatching rates, survival of larvae under starvation conditions and determine the ultimate day of survival. Percentage survival of larvae was calculated using the number of living larvae and the number larvae that hatched.

2.4. Larval rearing

After hatching, the larvae from female 1, were stocked into 100 L tanks at different densities (16, 32, and 51 larvae/L) with four replicates for each treatment. In parallel, female 1 eggs were stocked into two grey 500 L tanks at a density of 40 eggs/L. Larvae of females 2 and 3 were stocked in two black 500 L tanks and two grey 500 L tanks at a density of 30 larvae/L.

The physico-chemical parameters used for larval rearing were: 22 °C, 6 mg/L and 37.8‰ for temperature, oxygen, and salinity, respectively. The larval rearing protocol was similar to that described by Vallainc *et al.*, (2021). Microalgae mix of *Tetraselmis Chuii* and *Isochrysis galbana* was added to the larval culture tank. Rotifers (*Brachionus rotundiformis),* were offered from 2 to 22 dph **(Fig. 3)** at a density of 4 rotifers/mL enriched for 2 hours at 0.15 grams of Multigain (BioMar, France, containing 17% protein, 44% fats, 7% ash, 0.5% fibre, 5.1% vitC, 0.58% vitE, 150 IU/g, 30 IU/g, 20 mg/kg Cu, 17% n-3 HUFA, 15% DHA, 1.5% EPA and 0.05% ARA) per million rotifers. *Artemia* nauplii were supplied from 11 to 16 dph, and *Artemia* enriched with 0.5 grams of Multigain/million were supplied from 17 to 34 dph. The proportion of rotifers and

Artemia was day 2-7: 100% rotifers; 8-10 days: 98.8 % rotifers and 1.2 % *Artemia*; days 11-14: 97.6% rotifers and 2.4% *Artemia* nauplii; days 15-17: 95.2 % rotifers and 4.8% *Artemia* nauplii; days 18-21: 93.0% rotifers and 7.0% *Artemia*; 22 days: 90.9% rotifers and 9.1% *Artemia*, 23 days: 100% *Artemia*. The specified portion was provided twice daily as part of the feeding regiment.

Gemma Wean (0.1 mm) artificial diet (Skretting) was used for weaning from 21 to 42 dph. The central mesh of the tanks was changed as larvae and live feed-increased in size, starting with 150 µm mesh from day 2 to 12 dph, followed by 300 µm mesh between 12 to 17 dph, and finishing with 500 µm from day 18 to 42 dph.

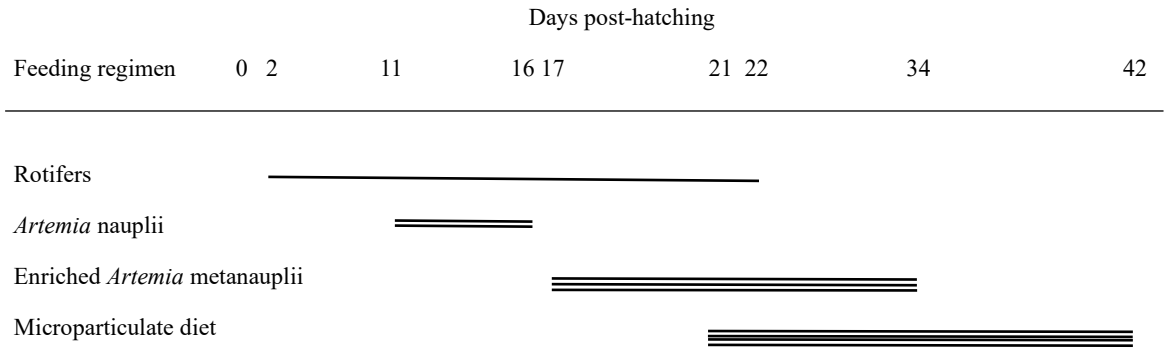


Figure 3. Feeding regimen conditions of mullet larvae cultured in different densities (16, 32 and 51 larvae/L). Rotifers were offered from 2 to 22 dph (one line); *Artemia* nauplii was included in the diet from 11 to 16 dph (double line); *Artemia* was added from 17 to 34 dph (triple line) and microparticulate diet was provided from 21 to 42 dph (four-line).

Larvae were collected on days 0, 3, 5, 10, 14, 20, 30 and 42 anesthetised with MS-222 and the standard length measured with a digital camera connected to an image analyser (SIS Gmbh, Germany). Photos were also taken to check larval growth signs like type of food, swimming bladder, and pigmentation.

2.5. Histological analysis

Oocyte samples were collected using a cannula at various time points 0, 4, 9, 13 for both control and rGths group. An additional time point at week 14 (24 hours after the first injection of rGths at week 13) was included for the rGths treated females. All samples were fixed in Bouin´s solution (comprising Formaldehyde, Picric Acid and Acetic Acid) and dehydrated using a series of graded ethanol baths. The samples were incorporated into paraffin and cut into 3 µm sections

that were dried and stained with Mayer-Bennett hematoxylin and Floxina / Eosina (Casa Álvarez, Spain). Finally, the slides were examined under a light microscope (Leica DMLB, Houston, USA) at magnifications of 600X and 1000X and photographs taken for analysis of oocyte developmental stage. Oocytes were classified as previously described by Ramos-Júdez et al. (2021) based on the oocyte diameter (measured with ImageJ) and cell morphology. Briefly, oocytes were distinguished by the following size and cellular characteristics early perinucleolar (27-70 µm) and late perinucleolar (70-110 µm) with the presence of multiple nucleoli in the germinal vesicle, cortical alveoli (110-203 µm) with the presence of granular vesicles and small oil droplets in the peripheral ooplasm, early vitellogenesis (203-360 µm) with initial presence of yolk granules and late vitellogenesis (361-510 µm) with the cytoplasm full of yolk granules. Oocyte maturation stage (OM), when the nucleus was situated to one side of the oocyte and oil droplets were coalescing; these characteristics indicated the beginning of germinal vesicle migration (GVM). Oocytes exhibiting hypertrophy and structural disintegration were in atresia. The stage of maturation of females was ascertained based on the presence of the most developed stage of oocytes. The percentage of oocytes in different phases within the ovaries was determined by selecting and classifying at least fifty oocytes at random from each female.

2.6. Sperm quality evaluation

Sperm samples were collected and analysed on week 18 and 4 days after week 19. For all samples abdominal massage was applied to facilitate the collection of sperm into a 1 mL syringe avoiding contamination from urine, water, or faeces. Immediately after collection, a drop of sperm was activated with 20 µL of seawater, and sperm quality was examined using a Zeiss Axiostar microscope (40X). The percentage motility was estimated in units of 10. In addition, immediately after sperm collection on week 19, a sperm sample was stored in an eppendorf tube diluted 1:10 (1 part sperm + 9 parts diluent) in Marine Freeze® (IMV Technologies, L'Aigle, France) and samples were maintained at 4ºC until evaluation. Sperm was activated by pipetting 2 µL of the diluted sperm sample into an Eppendorf with 500 µL seawater (Ramos-Júdez *et al.*, 2022). 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), (2) sperm motility (%), (3) rapid progressive sperm (%), and (4) sperm velocity (μm/s): 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 and fast progressive sperm to have a straightness (SRT = VSL/VAP x 100) of > 80% and a VCL of > 80 μm/s (Ramos-Júdez *et al.*, 2022b).

2.7. Plasma steroid analysis

Blood samples were kept on ice until being centrifuged at 3000 RPM at 4 °C for 15 minutes. Plasma was collected into an Eppendorf tube and stored at -80 °C until analysis. Plasma levels of 17β-estradiol (E_2), testosterone 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 (E2) or 1:100 (11-KT) in the EIA buffer (Ramos-Júdez *et al.*, 2022b).

2.8. Statistical analysis

Data is expressed as the mean ± standard deviation (SD) unless stated as standard error mean (SEM). Shapiro–Wilk and Levene tests were used to check the normality of data distribution and homogeneity of variance, respectively. Kruskal-Wallis test, followed by Dunn's pairwise comparison, were used to compare oocyte diameter between rGth treated and control group within a week and between weeks. In addition, the Mann-Whitney test was employed to compare the egg and oil globule diameter between females, and the effect of tank colour on larval total length. An ANOVA and Holm-Sidak test were utilised to compare the larval length and oil globule diameter in the larvae between females, and a Kruskal-Wallis test was used to compare the hatching survival percentage in a 96-well cell between females and larvae survival

at various densities. Two-way RM ANOVA with pair- wise comparison by the Holm–Sidak test was used to determine statistical difference in variables of plasma levels of E_2, T and 11-KT between rGths and control group. The proportions statistical test was used to compare the proportions of oocytes with or without variable germinal vesicle migration (oocyte nucleus position) at 13 weeks within the rGths group. Different correlations were realized between percentage hatching in 96-well plates, survival on days 3, 5, and 10 dph, and the parameters oocyte diameter and percentage of oocytes with germinal vesicle migration, using data from 2020 (Ramos-Júdez *et al.*, 2022b) and 2023 (present study) obtained under the same induction protocol and conditions. Statistical analyses were performed with SPSS software version 20.0 (Armonk, NY: IBM Corp). Significant differences were detected at a significance level of $P < 0.05$.

3. Results

3.1. Induced vitellogenesis and steroidogenesis

All four females (100%) treated with rGths completed gametogenesis in week 13 (594.5 ± 19 µm). Oocytes developed from previtellogenic and early vitellogenic phases to the completion of vitellogenesis and oocyte maturation. Fertilised spawning were obtained 44 to 68 hours after the first 30 µg/kg rLh injection. In comparison, all four control females showed limited development and remained in the pre-vitellogenesis phase throughout the experiment and consequently were not induced to spawn (**Fig. 4**).

The spawning events were on different dates with the staggered application of the rGth treatments: the first on June 21st (female 1), the second on July 5th (female 2), the third on July 19th (female 3), and the fourth on August 2nd (female 4) **(Table 1)**.

Table 1. **Spawning data for mullet induced with recombinant hormones (rFhs and rLh). Data are expressed as the mean ± standard deviation (SD).** [1] Weight per female in kg. [2,3] Oocyte diameter in each female in weeks 12 and 13 (Dunn's test). [4] Position of the germinal vesicle in 20 late vitellogenic oocytes (GV_central and GV_migrated) (proportions test). [5] Latency period, time interval between the first rLh injection for final induction and the spawning. [6] Weight of floating eggs in grams. [7] Total eggs per female in each spawning. [8] Relative fecundity: total eggs per kg of body weight in each spawning event. [9] Fertilisation rate: the percentage of eggs successfully fertilised. [10-13] Kruskal-Wallis test did not identify any statistically difference in hatching in the 96-well plate and survival percentages (3, 5 and 10 dph) between each spawning of rGths group. [14] Hatching in incubation tanks (%): larvae at 0 dph. Different letters indicate statistical differences amongst spawns for the parameter, which was determined with the statistical test indicated in brackets.

Spawning date	June 21st, 2023	July 5th, 2023	July 19th, 2023	August 2nd, 2023
Weight of female (kg)[1]	1.00	2.07	1.85	1.83
Oocyte diameter (µm) week before final induction (week 12) [2]	564±15[a]	595±23[b]	582±18[b]	584±21[b]
Oocyte diameter (µm) at final induction (week 13)[3]	589±13[a]	609±16[b]	595±19[a,b]	585±19[a]
GV_Central/GV_migrated [4]	19/5[a]	17/13[b]	2/21[c]	16/16[b]
Latency period (hours)[5]	44:57	45:25	44:35	68:00
Weight of floating eggs (g)[6]	550.4	468.1	419.4	197.4
Total eggs per female[7]	1,024,172	847,973	659,790	378,845
Relative fecundity (Total eggs per kg of body weight) [8]	1,024,172	409,648	356,643	207,019
Fertilisation rate [9]	97	96	71	0
Hatching 96-well plate (%)[10]	96.13 ±1.42	82.05 ±13.72	66.58±12.27	0
% survival. 3 dph[11]	63.81 ±16.51	93.17 ±4.89	51.87±42.3	0
%survival. 5 dph[12]	57.33 ±14.59	30.09 ±5.11	49.49±43.00	0
% survival 10 dph[13]	43.56 ±13.26	15.82 ±7.81	4.32±4.17	0
Hatching in incubation tanks (%)[14]	92.65 ±0.31	80.40±2.5	70.07±9.45	0

At the start of the experiment (week 0), rGths **(Fig. 8 b)** and control **(Fig. 8 a)** groups had 100% of females in previtellogenesis with oocytes in the perinucleolar **(Fig. 7)**. There was no significant differences in the mean initial diameter of the largest oocytes between groups (control = 108 ± 17 µm, rGth-treated =103 ± 12 µm,). A significant increase in oocyte diameter was observed in week 4 in the rGth treated group ($P = 0.001$) (control = 92.6 ± 22 µm, rGth-treated = 319.5 ± 37.6 µm,) **(Fig. 4)**. The oocyte diameter of the group treated with rGth increased significantly each week ($P < 0.001$) **(Fig. 7 a, Fig. 8 d, f)**. Oocytes in the rGth-group were aligned by week of treatment and the progression of this growth was represented using a second order polynomial (quadratic) equation (*Oocyte diameter* = 106.57 + 53.35 *week* − 1.226 $week^2$; $R^2 = 0.991$) **(Fig. 4)**. When oocyte maturation was induced (week 13), the rGths treated females exhibited an average oocyte diameter of 594.5 ±19 µm that was statistically

significantly larger (*P = 0.001*) compared to the control group (153 ± 76 µm oocyte size) **(Fig. 4)**.

In comparison, the control group females exhibited little development in diameter of the largest oocytes, remaining in the previtellogenic perinucleolar phase (early and late) during most of the experiment. No significant changes were observed in three females with oocyte diameter ranging between 102 ± 11 µm- and 128 ± 39 µm **(Fig. 7 b, Fig. 8 c, e)**. However, one female showed a development from the previtellogenic stage to the initiation of vitellogenesis (161± 16 to 277 ± 18 µm) with a significant increase in oocyte diameter from week 11 to week 13 (*P=0.001*) **(Fig. 7 b).**

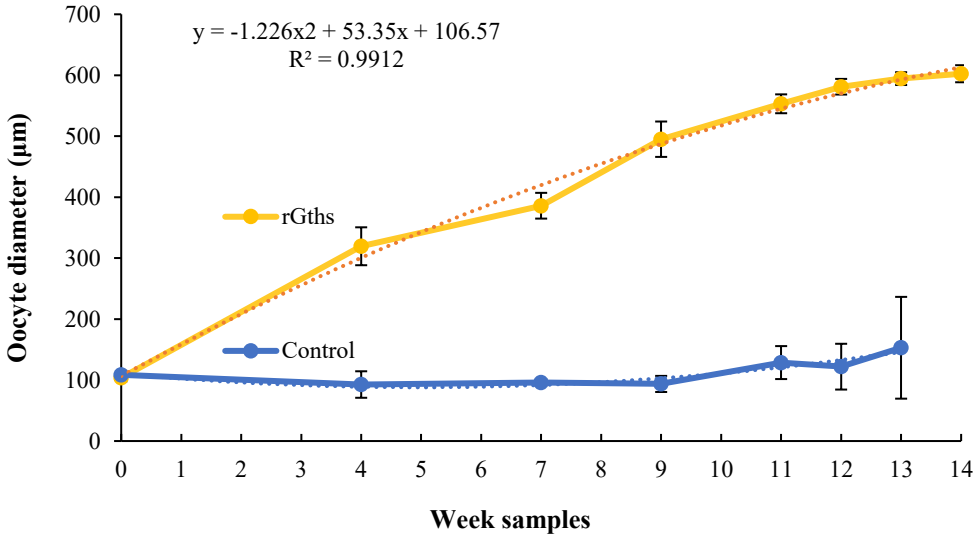


Figure 4. Mean ± SD oocyte diameter of the four females on weeks 0, 4, 7, 9, 11, 12,13, and 14 (24 hours after the first injection of rGths at week 13) for the rGths and control groups. The continuous orange line in the graph indicates rGths while the blue line is for the control group. Dunn's pairwise comparison, were used to compare oocyte diameter between rGth treated and control group within a week and between weeks. Oocytes in the rGth-group were aligned by week of treatment and the progression of this growth was represented using a second order polynomial (quadratic) equation (Oocyte diameter = 106.57 + 53.35 week − 1.226 week^2; R^2 = 0.99).

The increase in oocyte diameter in the rGth treated females was accompanied with an increase in steroids. Regarding E_2 concentrations, there was no significant difference (*P*= 0.344) in the initial levels (week 0) between the control and rGths group (0.375 ± 0.06 ng/mL and 0.392 ± 0.07 ng/mL, respectively). However, at week 13, E_2 levels were significantly higher (*P = 0.017*) in females treated with rGths (3.10 ± 1.17 ng/mL) compared with females in the control group

(0.78 ± 0.24 ng/mL), which showed no significant variation in E_2 during the experiment **(Fig. 5)**.

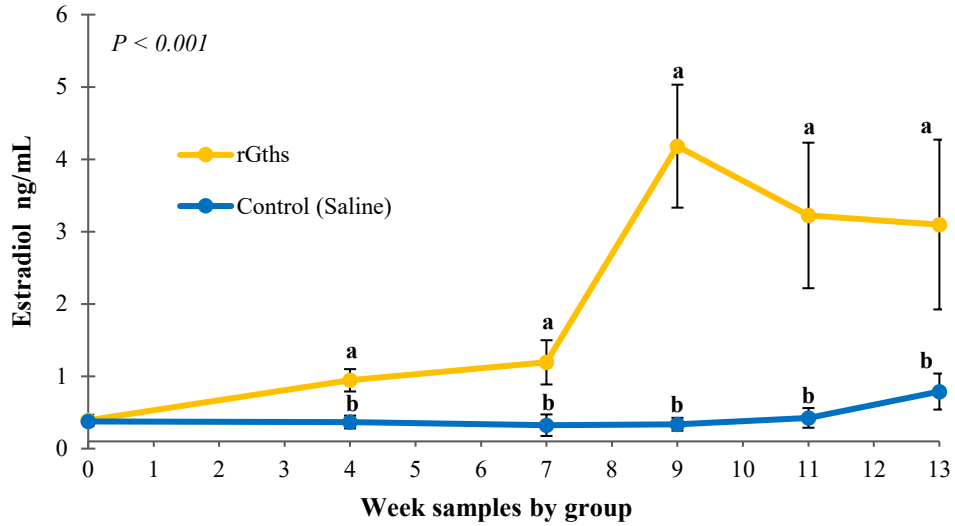


Figure 5. Effect of rGths treatment (rFsh and rLh) and saline (control) on plasma estradiol levels (Mean and SD) in female mullet. The results showed a significant difference between the rGths and the control group (*P < 0.003)* from week 4 until week 13 (*P < 0.017)* Holm–Sidak test at the post hoc. The orange line represents the rGth, while the blue line represents the control group. Different letters indicate statistical differences in plasma E_2 levels between rGths and the control group.

Initial testosterone levels (week 0) exhibited no significant difference *(P = 0.758)* between the control (0.199 ± 0.05 ng/mL) and rGths groups (0.203 ± 0.10 ng/mL). On week 4 a small, but significant increase in T levels (*P = 0.019)* was shown for rGths (0.533 ± 0.189 ng/mL) in comparison with the control group (0.216 ± 0.06 ng/mL) **(Fig. 6)**.

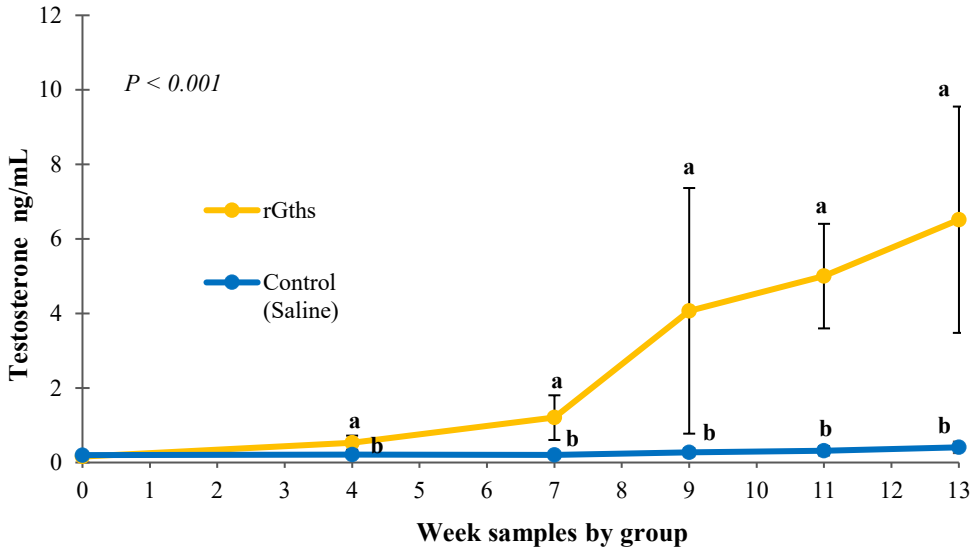


Figure 6. Effect of rGths treatment (rFsh and rLh) and saline (control) on the plasma T levels (mean ± SD) in the female mullet. A significant difference was found between the rGths and the control group from week 4 (*P = 0.019)* until week 13 (*P = 0.004)* onwards using the Holm correction at the post hoc test. The orange line represents the rGths, while the blue line represents the control group. Different letters indicate statistical differences in plasma T levels between rGths and the control group.

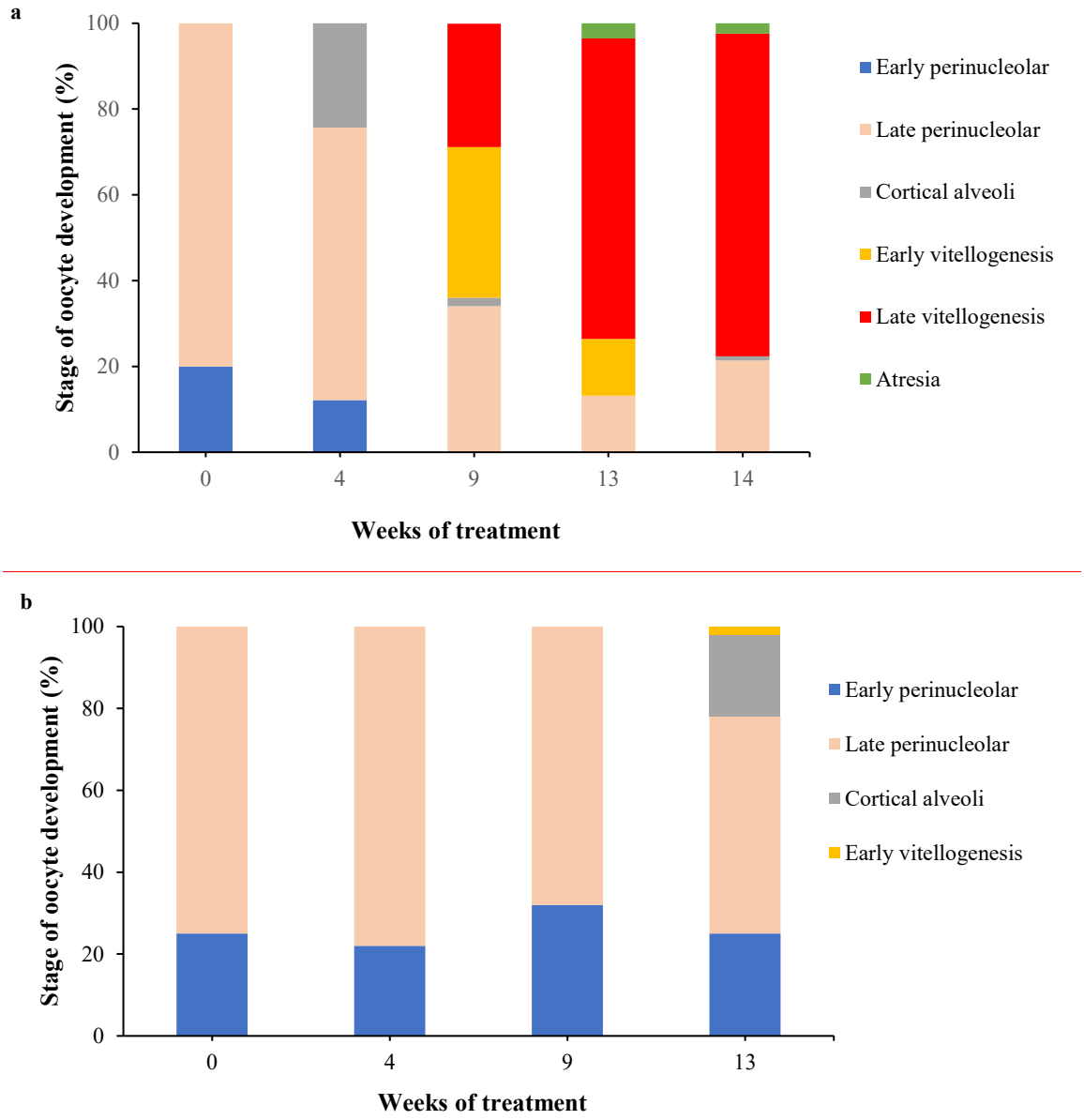


Figure 7. Oocyte development stages in female mullet for the rGths-treated (Fig. 7a) and control-saline (Fig. 7b) groups represented by percentage frequency. 50 to 100 random oocytes were analysed and categorized into distinct stages per female during weeks 0,4,9,13, and 14 (24 hours after the first injection of rGths at week 13). Oocytes were extracted through cannulation and fixed in Blouin's solution for histology analysis. The classification comprised the following: Early perinucleolar (27-70 µm), Late perinucleolar (70-110 µm), Cortical alveoli (110-203 µm), Early vitellogenesis (203-360 µm), Late vitellogenesis (361-510 µm), Atresia.

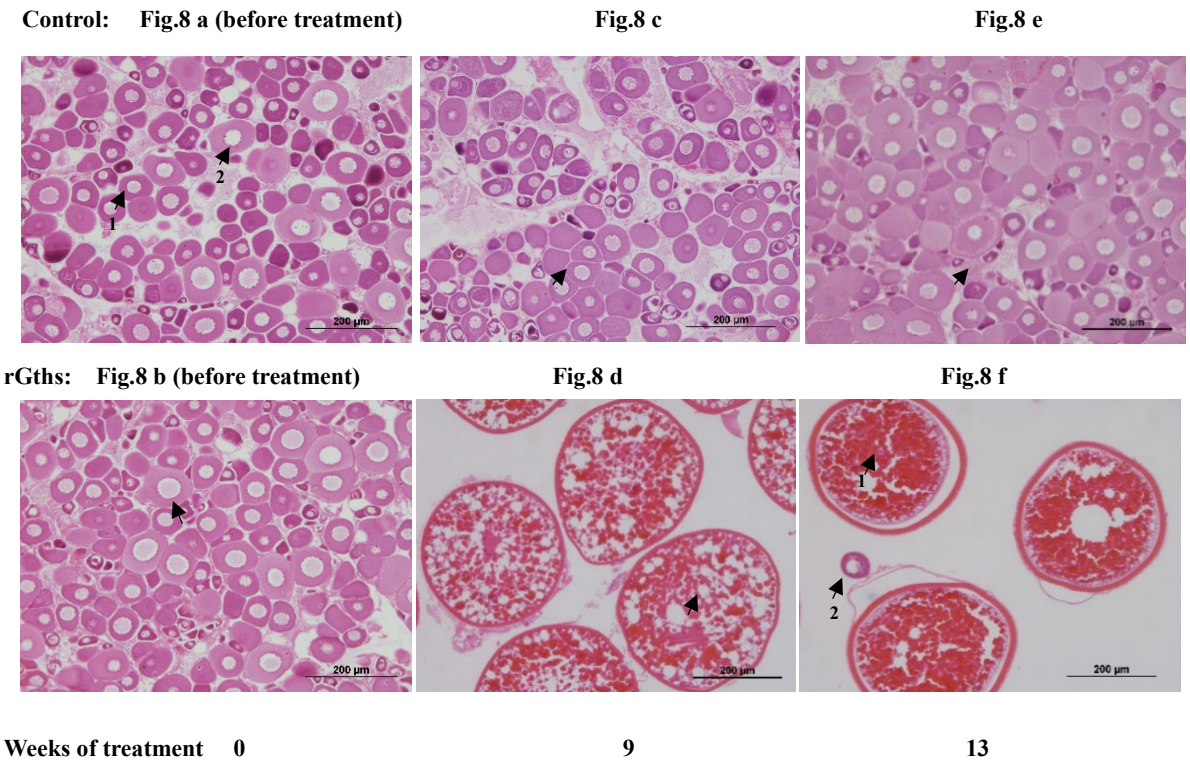


Figure 8. Oocyte development stages in female mullet for the rGths-treated and control-saline groups at week 0, 9, and 13. Fig. 8a and b (before treatment) for both control and rGths group showed equal stages: early perinucleolar (1) and late perinucleolar (2) at week 0. Fig. 8c (control) was in the perinucleolar stage, while Fig. 8d (rGths) was in early vitellogenesis at week 9 of rGths treatment. Fig. 8e (control) was observed in late perinucleolar. Fig. 8f, females in the rGths-treatment group were observed in late vitellogenesis stage (1) and a second group of oocytes in the early perinucleolar (2). Scale: 200 µm.

3.2. Induced spermatogenesis and steroidogenesis

All three (100%) of the males treated with rGths advanced to stages of spermatogenesis with fluid sperm (spermiation index 2 and 3) and were available for spawning with induced females. In comparison, no spermiation was observed in the control males. The protocol for males began on week 4 after initiating treatment with females (week 0). At the beginning of male treatment, all individuals, in both groups (rGths and control), exhibited the same spermiation index= 0, indicating the absence of sperm when performing abdominal massage and no statistical difference ($P = 0.379$). The first males with fluid sperm (index= 2), were observed on the week after the fifth rGths injection (week 9 of the experiment). From week 6 to 17 all three males had

sperm index 2 or 3. On week 18, the fluid sperm index of one male dropped to 1. After injection with rGths, the fluid sperm index increased to 2 in all males in week 19. Plasma 11-KT levels were significantly higher ($P = 0.006$) in males treated with rGths on week 9 (0.70 ± 0.36 ng/mL before treatment; 2.47 ± 0.94 ng/mL after 5 weeks) and onwards (27.46 ± 24.28 ng/mL week 19), compared to males in the control group that exhibited no significant differences in plasma 11-KT during the experiment (0.59 ± 0.30 ng/mL before treatment; 0.70 ± 0.30 ng/mL week 19) **(Fig. 9)**.

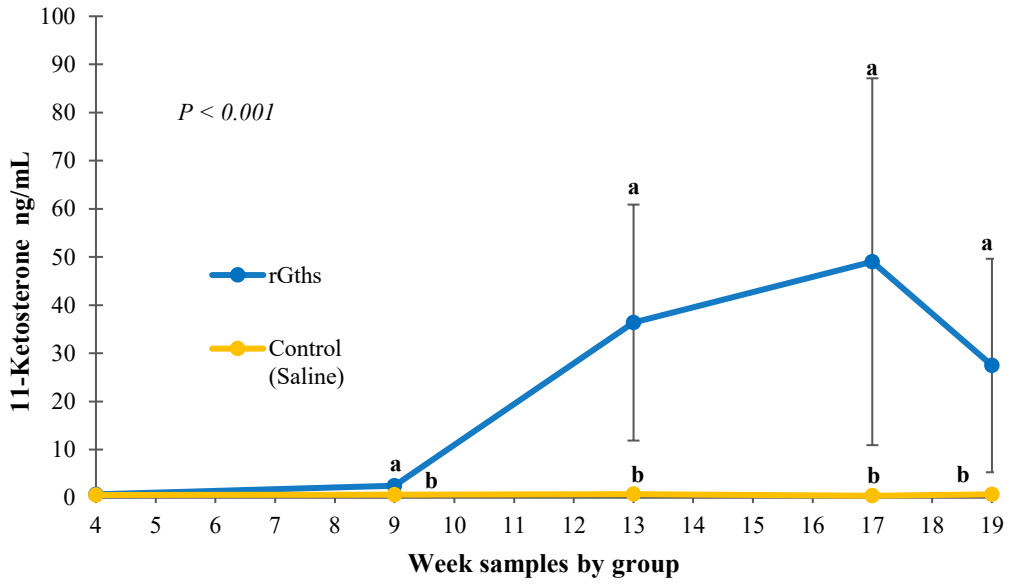


Figure 9. Effect of rGths treatment (rFsh and rLh group) and saline (control) on plasma 11-ketotestosterone (11-KT) levels (mean and SD) in the mullet male. There was a significant difference between the rGths and the control group ($P = 0.006$) at week 9 until the end of the experiment ($P < 0.001$ at week 19). The orange line represents the rGths, while the blue line represents the control group. Different letters indicate statistical differences in plasma 11-KT levels between rGths and the control group.

On week 18, an rFsh dose was administered together with the rLh dose, due to low sperm motility on week 18 (50% motility in samples activated and examined by microscope). This dose was considered necessary to increase sperm motility and fluidity for the final spawning of female 4 (August 2nd, 2023) on week 19. The assessment of sperm during week 19 (4 days after rGth application) exhibited high motility (activated under microscope) and > 100 µL volume from all three males. The analysis by CASA showed 79.09 ± 12.26% motility, 2.86 ± 0.38 x 10^{11} cells/mL concentration of spermatozoids, 94.15 ± 34.16 µm/s VCL, 116.06 ± 17.46 µm/s VAP, 104.36 ± 15.09 µm/s VSL, 78.71 ± 6.08 % LIN, 87.75 ± 6.70 % WOB, and 88.20 ± 0.7% STR.

3.3. Induced spawning and egg and larval quality

All females treated with rGths completed vitellogenesis and were induced to spawn on week 13, while all males treated with rGths exhibited fluid sperm production and were stocked with females for spawning. Statistical differences (Dunn´s test) were observed in the mean oocyte diameter between females at the time of spawning induction **(Table 1, Fig. 10).** Female 2 (July 5th, 2023) (609 ± 16 µm) had significantly larger oocytes compared to both female 1 (June 21st, 2023) (589 ± 13 µm, *P = 0.004*) and female 4 (August 2nd, 2023) (585 ± 19 µm, *P = 0.001*). Oocytes from female 3 (July 19th, 2023) (595 ± 19) were not statistically different compared to other females.

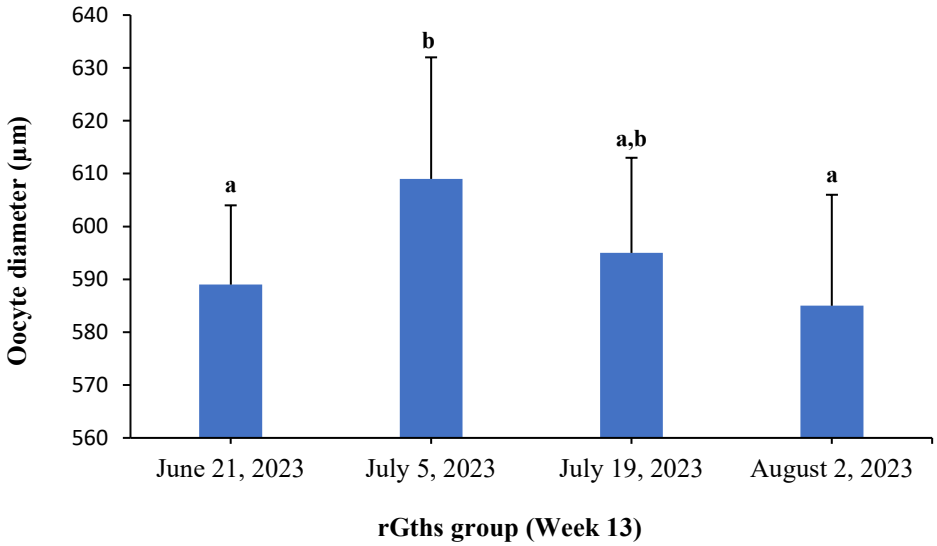


Figure 10. Average oocyte diameter for four mullet females on week 13 prior to spawning induction. Different letters indicate statistical differences indicated by Dunn's statistical comparison. Female 2 (July 5th, 2023) had significantly larger oocytes than females 1 (June 21st, 2023) and 4 (August 2nd, 2023), while female 3 (July 19th, 2023) was intermediate with no difference compared to other females.

Female 1 exhibited the highest fecundity of 1,024,172 eggs/kg of body weight, while the other females' fecundity ranged between 409,648 and 207,019 eggs/kg. Female 4 had the highest interval of time between the final injection and the spawning (68:00 h), while latency in the other females ranged from 44:35 hours to 45:25 hours **(Table 1)**. The eggs spawned by female 4 were not fertilised and fecundity was the lowest with the longest time between the final induction and the spawning. Furthermore, female 1 (96.13 ± 1.42%) had the highest hatching rate in the 96-well plates, followed by females 2 (82.05 ± 13.72%) and 3 (66.58 ± 12.27%).

Nevertheless, there were no statistically significant variations (*P = 0.156*) detected in the hatching rate between females. Female 1 had the highest survival rate, but there was no statistically significant difference (*P = 0.489*) between females **(Fig. 11)**.

The eggs presented a mean diameter of 0.80 ± 0.02 mm, whereas a homogeneous yolk mass at the posterior part of the yolk sac (oil droplet) measured 0.31 ± 0.02 mm. Female 1 exhibited a smaller diameter of eggs and oil droplets (0.79 ± 0.02 mm and 0.29 ± 0.02 mm) than female 2 (0.81 ± 0.01 mm and 0.32 ± 0.009 mm). There were no differences in larval length, which was measured at 0, 3, 5, 10, and 14 dph **(Table 2)**.

Table 2. Total length (mm) of mullet larvae in different times 0, 3, 5, 10- and 14 days post-hatching for each female.

Dph	Female 1 (mm, TL)	Female 2 (mm, TL)	Female 3 (mm, TL)
0	2.67 ± 0.16	2.76 ± 0.10	2.67 ±0.07
3	2.80 ± 0.08	2.74 ± 0.06	2.73 ±0.08
5	2.77 ±0.11	2.81 ± 0.11	2.67± 0.10
10	3.20±0.11	2.82 ±0.19	2.87 ±0.26
14	3.51±0.26	3.63 ±0.22	2.99 ±0.20

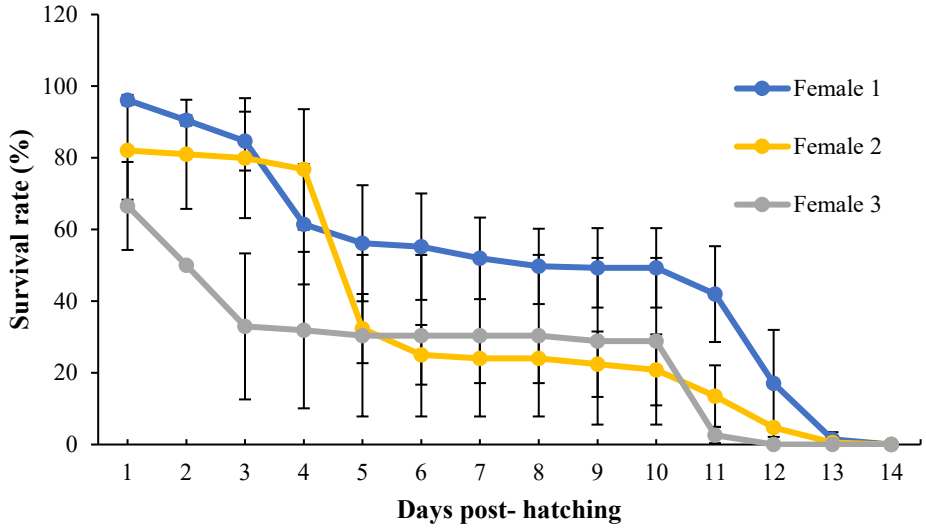


Figure 11. Survival percentages average ± SD in the 96-well plate in the females 1, 2, and 3 by 14 days. Each line represents a female (blue: female 1; orange: female 2, and grey: female 3). The Kruskal-Walli's test = 1.431 gl=2 *P=0.489* determined no significant differences between females and survival rate.

3.4. Larvae survival at different densities and tank colour

The final survival rates ± standard error (SE) at 42 dph was 5.24 ± 4.15 % at 51 larvae/L, 8.44 ± 5.3 % at 32 larvae/L and 14.13 ± 5.2 % at 16 larvae/L. The final survival as number of larvae/L ± standard error (SE) at 42 dph were 2.26 ± 0.41 from 16 larvae/L, 2.70 ± 0.84 from 32 larvae/L

and 2.67 ± 1.05 from 51 larvae/L (Fig. 12). There was no statistical (*P = 0.146*) differences in survival as a percentage or number of larvae/L amongst the initial stocking densities tested. The final survival larvae/L and different densities were represented by a second-order polynomial (quadratic) equation: (*Survival larvae* = -0.0021 *densities*^{2} + 0.1598 *densities*; R^2 = 0.915) **(Fig.12).** The horizontal and vertical intercepts with the peak of the second-order polynomial (quadratic) relationship **(Fig. 12, blue line)** showed that a density of 38 larvae/L was predicted to obtain a maximum survival density of 3 larvae/L at 42 dph.

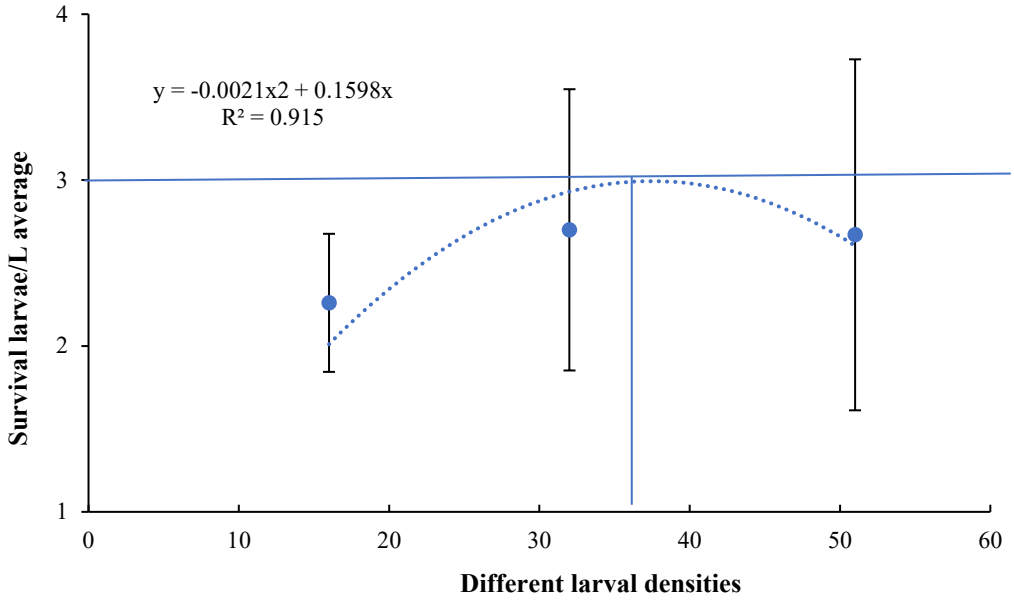


Figure 12. Final survival larvae/L average ± standard error (SE) in different stocking densities (16 larvae/L; 32 larvae/L and 51 larvae/L) at 42 days post-hatch. The final survival larvae/L were 2.26 ± 0.41 from 16 larvae/L, 2.70 ± 0.84 from 32 larvae/L and 2.67 ± 1.05 from 51 larvae/L. The Kruskal-Walli's test = 3.846 gl=2 *P*=0.146 determined no significant differences between different densities and survival larvae/L at 42 days post-hatch. A second-order polynomial quadratic equation: (*Survival larvae* = -0.0021 *densities*^{2} + 0.1598 *densities*; R^2 = 0.915) between survival larvae/L rate and days after hatching was determined. The blue lines show the horizontal (3 survival larvae/L) and vertical (38 density larvae/L) intercepts with the peak of the second-order polynomial (quadratic) line representing the relationship between larval survival and stocking density.

In the two grey 500 L larval rearing tanks stocked at a density of 40 eggs/L, high larval mortality was observed at 11 dph. A total of 193 juveniles were collected from the grey tanks at 34 dph, resulting in a larval survival rate of 0.48%. Female 2 exhibited substantial larval mortality at 11 dph when cultured in grey and black 500 L tanks at a density of 30 larvae/L. At 20 dph, few to no larvae were observed and this larval culture was terminated with 0% survival. On the other hand, larvae obtained from the female 3, reared under the same conditions, had a mean final

larval survival of 0.21% in the black tanks and 0.54% in the grey tanks at 42 dph. The total length of larvae, in the black tanks was significantly larger than that of larvae in the grey tanks from 20 dph to 42 dph ($P = 0.01$). Furthermore, a positive correlation ($R^2 = 0.99$, $P = 0.02$) between size (TL, mm) and days after hatching was observed **(Fig. 13)**.

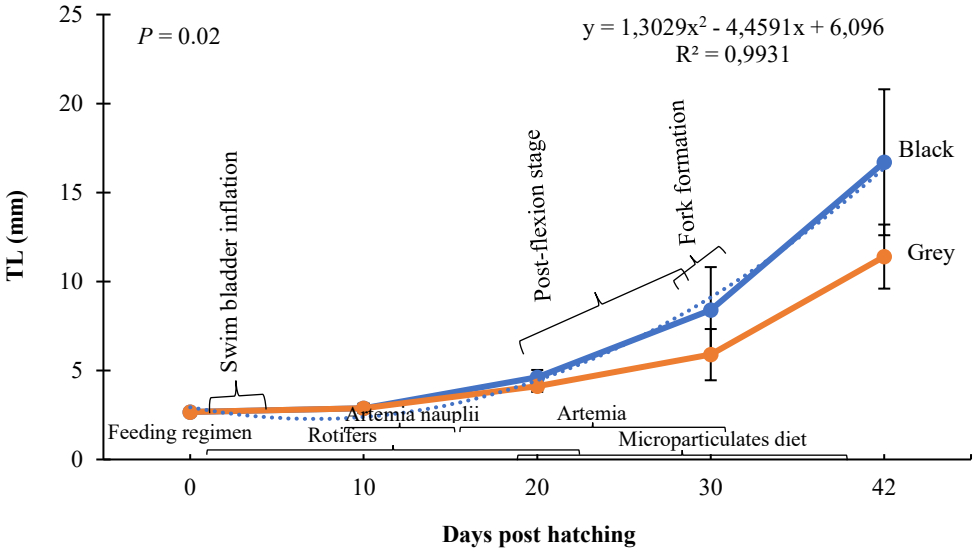


Figure 13. Mean total length of larvae (n=15) on 0, 3, 5, 10, 14-, 20-, 30- and 42-days post hatching in the black and grey tank. U. Man-Whitney statistical test showed differences on days 20, 30, and 42 dph ($P = 0.01$). The blue line in the graph indicates black tank larvae with R^2: 0.99 while the orange line, represents grey tank larvae with an R^2: 0.98. Feeding regimen conditions of mullet larvae cultured in two different colour tanks. Rotifers were offered from 2 to 22 dph; *Artemia* nauplii was included in the diet from 11 to 16 dph; *Artemia* was added from 17 to 32 dph and a microparticulates diet was provided from 21 to 40 dph.

Pigmented eyes were first observed between 1 and 2 dph. The mouth was open, and the upper and lower mandible were fully developed at 3 dph (2.75 ± 0.07 mm TL, **Fig. 14 D**). At this stage, the larvae had depleted the majority of the yolk reserves, leaving only the oil globule **(Fig.14 E)**. The duration of the pre-flexion stage was from 4 to 19 dph **(Fig. 14 F)**, during which the oil droplet and yolk were fully absorbed at 11 dph. Swim bladder development commenced between days 4 and 6 (2.75 to 3.1 mm TL, **Fig. 13**) and is evident ventrally below the notochord. Throughout the process of swim bladder formation and enlargement, larval swimming activity (resting at the surface and/or feeding on rotifers) increased **(Fig. 14 F)**. The larvae underwent tail flexion until they reached a length of 3.7 mm. However, the post-flexion stage persisted for multiple days (20-30 dph, 4.6-8.3 mm TL, **Fig. 13 and 14 G**), during which time the caudal fin

and fork were fully developed. As of the conclusion of the experiment (day 42), every fish exhibited adult-like characteristics and was classified as a juvenile. The developmental stages of mullet, from larvae to juveniles, are illustrated in Figures 13 and 14.

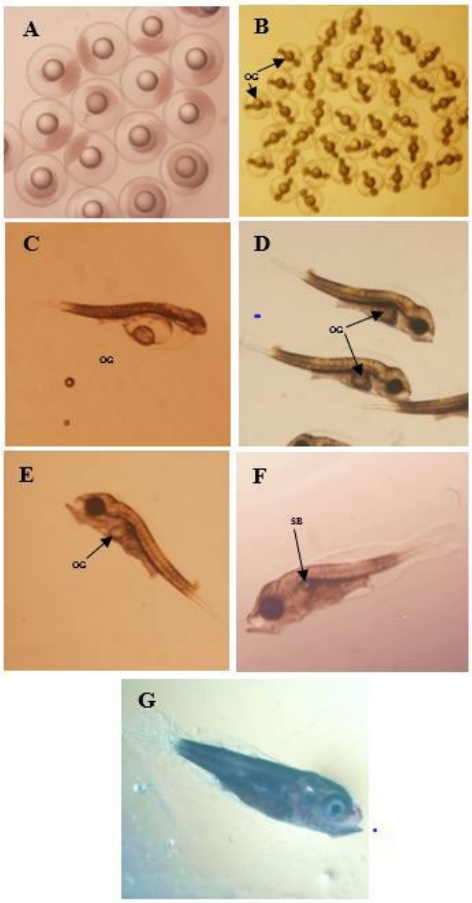


Figure 14. Mullet eggs, embryos, larvae. (A) Eggs in formation (blastoderm) (mean egg diameter 0.80 ± 0.02 mm), (B) Embryo with a developed head region and a dark pigment that covers the majority of the body and the oil globule (OG), (C) newly hatched larvae (mean TL 2.67 ± 0.16 mm), (D) At 3 dph, larvae have fully developed mouthparts that are functional for consuming rotifers (mean TL 2.74 ± 0.06 mm), (E) at 5 dph, oil globules are still present among the larvae (mean TL 2.81 ± 0.11 mm), (F) At 11 dph, the larvae have fully assimilated the oil globule and developed a hyperinflated swim bladder (SB) (mean TL 3.20 ± 0.11), (G) larvae at 20 dph (mean TL 4.62 ± 0.41 mm).

The mortality events were recorded at various time intervals in this study. The first mortality period occurred with the development of the swim bladder, between 5 and 6 dph. The second event with high mortalities was documented at 11 dph, which corresponded to the complete assimilation of the oil globule, larvae with fully developed swimming ability, and the start of the *Artemia nauplii* feeding phase. The final mortality period occurred between 17 and 18 dph,

which coincided with a change in the artemia feeding phase when the switch was made from *Artemia nauplii* to enriched *Artemia metanauplii*.

3.5. Correlation between oocyte characteristics and egg quality

In the current study, a smaller oocyte size at induction and a lower percentage of oocytes with germinal vesicle migration (GVM) coincided with higher larval hatching and survival rates **(Table 1)**. The data set was expanded by combining data from 2020 (Ramos-Júdez *et al.*, 2022b) with 2023 (present study) to analyse correlations between female maturational stage and egg and larval quality. The results demonstrated positive, negative, and non-significant correlations **(Table 3)**. The highest, very good negative correlation (R = **- 0.98**) was found between the percentage of GVM and hatching rate in 96-well plates. Good negative correlations were observed between GVM (%) and both total larvae (R = **- 0.69**) and survival at 10 dph in 96-well plates (R = **- 0.79**). A good positive correlation (R = **0.56**) was observed between GVM and total number of eggs. However, poor or no correlation (R < 0.41) was observed between all other combinations of parameters.

Table 3. Correlation coefficients (R) between oocyte characteristics and 96-well plate egg and larval quality parameters from 2020 and 2023 data.

Variables	Total eggs	Hatching (96-well plate)	Total larvae	3 dph	5 dph	10 dph	Oocyte diameter (µm)
Oocyte diameter (µm)	-0.25	0.07	-0.07	0.06	-0.28	-0.04	
GV migrated (%)	0.56	**-0.98**	**-0.69**	-0.24	-0.41	**-0.79**	-0.12

A significant finding in this study was the strong negative correlation between GVM (%) and hatching in 96-well plates **(Table 3)**. This is particularly relevant as the successful induction of spawning in captivity is a critical phase of the hormone induction protocol for mullet. As indicated by this correlation, females with GVM below 20% achieved a 97% of hatching rate in 96-well plate **(Fig. 15)**. This relationship was modelled by the equation: (*Hatching 96-well plates* = - 0.3649 *GV migrated* % + 99.525; R^2 = 0.96, P < 0.001), indicating an inverse

relationship where an increase in the hatching rate in 96-well plates corresponds to a decrease in GVM.

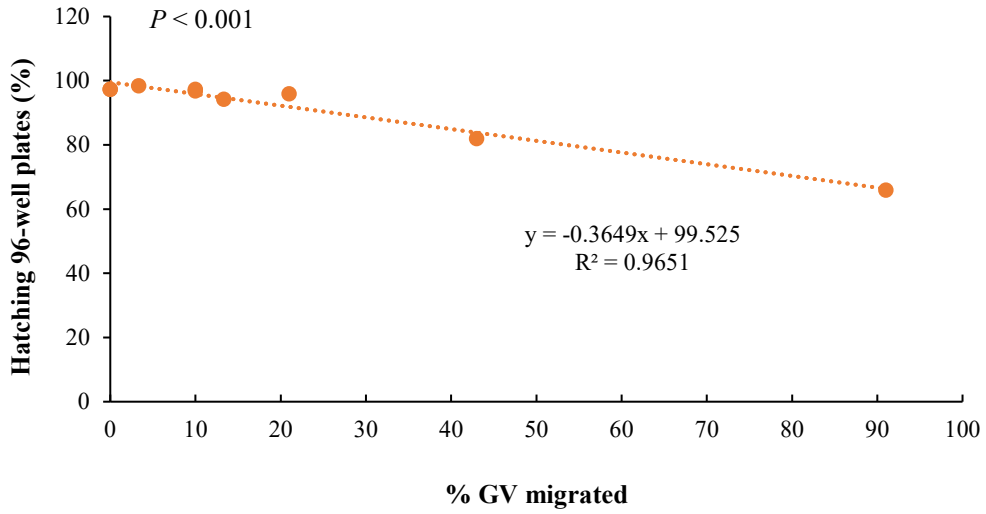


Figure 15. A negative correlation between GVM % and hatching rate in 96-well plates defined by the equation: (*Hatching rate 96-well plates* = - 0.3649 GVM% + 99.525; $R^2 = 0.96$, *P < 0.001*). This suggests an inverse relationship where an increase in the hatching rate in 96-well plates corresponds to a lower GVM %.

4. Discussion

In the present study, both female and male mullet were induced with rGths to mature and spawn in June – July, a period outside the natural spawning season of wild populations, which typically occurs in September — October in the Western Mediterranean. All four females were during a 13-week period induced from the early stages of pre-vitellogenesis to complete maturation and ovulation. Over a 5-week period, all three rGth treated males developed from having no sperm present to producing and releasing sperm with spermiation sustained for 10 weeks. Three females spawned with males to give fertilised eggs that were incubated and larvae cultured. Juveniles were obtained with a maximum survival of 14.1 ± 5.2% to 42 days post hatch (final experiment day). In contrast, control females and males remained in the early stages of gametogenesis and did not produce viable gametes, highlighting the success of out-of-season induction with rGths.

In the captive environment, particularly in the Mediterranean region, reproductive dysfunctions where mullet maturation and development are arrested during the primary growth phase of gametogenesis present a significant impediment to mullet aquaculture. Therefore, in an intensive aquaculture environment, as in the present study, both female and male mullet required assistance to induce critical phases of maturation: vitellogenesis, oocyte maturation, ovulation, spermatogenesis, and spermiation. We confirmed the benefit of rFsh and rLh for inducing complete vitellogenesis (Ramos-Júdez *et al*., 2021, 2022b, 2022a) and for the first time, used rGths to advance maturation 2-3 months compared to the natural spawning period. Out-of-season spawning has significant advantages for the aquaculture industry as it gives control of the timing of the production cycle to program production to meet market demand (Bromage *et al.*, 2001). Maturational development in fish is widely understood to be controlled by environmental signals that activate the brain-pituitary-gonad axis to stimulate the gonadotropin cascade of hormones and maturational progress (Bromage *et al.*, 2001; Levavi-sivan *et al.*, 2010; Hatef and Unniappan, 2019). Generally, out-of-season spawning is achieved by controlling environmental cues such as photoperiod and temperature. This is one of the first studies to use hormonal control to achieve a substantial advance to the spawning period. The natural period for maturation and spawning of mullet in the Western Mediterranean was gametogenesis during July-August-September and spawning during September-October (Ramos-Júdez *et al.*, 2023). In the present study, we induced gametogenesis during March-June and spawning during June-July. The photoperiod was not manipulated and mullet were induced during the natural increase from 12:12 light:dark (LD) to 14:10 LD. This was the opposite to the photoperiod during the natural period of gametogenesis (July to September), which decreased from 14:10 LD to 12:12 LD (Ramos-Júdez *et al.*, 2023). Few studies have explored the interaction between photoperiod and hormonal manipulations. A study on Eurasian perch (*Perca fluviatilis*) found that constant darkness disrupted hormonally induced ovulation and reduced ovulation rates (Żarski *et al.*, 2021). However, no disruption in hormonally induced gametogenesis was observed in the present study compared to previous ones, despite the opposite photoperiod to the natural change experienced during gametogenesis. It is worth noting that the temperature was manipulated to be at a constant 24ºC, similar to the temperature during the natural period of gametogenesis. Temperature has been described to have two controlling influences over the timing of maturation (a) as a cue for the timing of maturation and (b) as a factor that affects the kinetics

of the biological processes (Bromage *et al.*, 2001; Akhtar *et al.*, 2018; Alix *et al.*, 2020). Therefore, the rationale behind establishing 24ºC was to ensure the normal progress of kinetics of the biological processes of maturation. While the increase to 24ºC in March may have been perceived as a cue for maturation, the control group showed no initiation in gonadal development, whereas only the rGths-treated group progressed. Combined temperature and hormonal control has also been used to control maturation of European eels with an increase of 2°C combined with weekly CPE injections (Vílchez, 2012) or constant 16°C, constant darkness and weekly rGth injections (Jéhannet *et al.*, 2023a).

The out-of-season induced maturation (present study) was similar to the changes registered when induced in-season (Ramos-Júdez *et al.*, 2021, 2022b). The out-of-season hormonally induced oogenesis was successful for all four rGths females that exhibited oocyte development with significant growth and a uniform number of oocytes advancing through the different maturational phases, reaching a fully grown vitellogenic oocyte diameter of 600 µm that were ovulated for spawning. The oocyte development confirmed that mullet are characterised by group synchronous ovary development and a single annual spawning event (Wallace and Selman, 1981), as observed in other studies on mullet (Ramos-Júdez *et al.*, 2021, 2022b, 2023; Castellanos-Juárez *et al.*, 2024). The increase in oocyte diameter was accompanied with significant increase in E_2 and T, as has been registered in response to the administration of rGths in many species, such as goldfish (*Carrassius auratus*) (Kobayashi *et al.*, 2006), spotted scat (*Scatophagus argus*) (Zhang *et al.*, 2017), European eels (Jéhannet *et al.*, 2023b) and short-finned eel (*Anguilla australis*) (Nguyen *et al.*, 2020). No significant changes in plasma steroid levels were observed in control fish. The present study adds to the growing body of literature demonstrating that an adequate application of rGths induces oogenesis.

During the experimental period, the males were induced from no presence of sperm to having fluent sperm 2-3 months before the natural spawning period. Demonstrating how as in females, rGth hormone treatment can advance maturation under an increasing photoperiod. The production of fluent sperm, completion of spermatogenesis, spermiation, and an increase in 11-KT, commenced in all rGths-treated males at week 9 of the experiment (5 weeks of male rGth treatment) with a significant increase in week 13 with rLh treatment. Spermiation and fluent

sperm was maintained with the application of rGth during a 10-week period. The control group did not exhibit fluent sperm throughout this study. However, at week 18 rGth treated males exhibited a drop in sperm production and a dose of rFsh (12 µg/kg) was applied with rLh (12 µg/kg - one male or 24 µg/kg two males) to improve spermiation for week 19 (final spawning-female 4), which coincided with a decrease in 11-KT plasma levels from 49.02 ng/mL (week 17) to 38.24 ng/mL (week 18). The present study contributes to a large and growing number of other studies that have reported induced spermiation accompanied with a significant elevation of 11-KT concentration using rGths treatment such as Ramos-Júdez *et al.*, (2022b) in mullet, Hayakawa *et al.*, (2009) in Japanese eel (*Anguilla japonica*), García-López *et al.*, (2009) in zebrafish (*Danio rerio*), Chauvigne *et al.*, (2017) in flatfish Senegalese sole, and Zhang *et al.*, (2017) in spotted scat *(Scatophagus argus*). Additional studies in sexually immature European eels showed robust evidence that rGths induce spermatogenesis and spermiation (Peñaranda *et al.*, 2018).

The mature males and females were induced with rLh to spontaneously spawn. The males were administered a single injection of 12 µg/kg (one male) or 24 µg/kg (two males) and placed with the female. The females were administered two injections of 30 µg/kg rLh separated by 24 hours to effectively induce maturation and spawning in all treated females with an oocyte diameter of around 600 µm at week 13. All combinations of males and females (100%) spawned. However, the eggs from female 4 were not fertilised. Female 4 presented small oocytes 585 µm when induced, 50% GVM and no increase in oocyte diameter during the week prior to final induction (585 ± 19 µm in week 13) compared to week 12 (584 ± 21 µm) and an extended latency period between the final induction and spawning which, produced 100% non-fertilised eggs. In comparison oocyte size was the same or larger, % GVM was lower and oocyte diameter increased during the final week in the three females that produced fertilised spawns. It cannot be discounted that the males failed to participate in the courtship and spawning as female 4 did ovulate, but egg release was delayed by more than 24 hours, and the eggs may have been overripe when released. However, this was one female and the success rate for fertilised spawning was 75%. Similar success was obtained with the same protocols with in-season spawning of mullet (Ramos-Júdez *et al.*, 2021, 2022b). A range of other studies in the natural spawning season have induced oocyte maturation, ovulation, and spawning in mullet. One other

study used hormones to improve the vitellogenesis phase, Aizen *et al.*, (2005) reported a lower rate of mature females (50-85%) using implants with GnRHa at 10 mg/k in combination with dopamine antagonists (domperidone -5 mg/kg). The vast majority of studies utilised mature fish in the late stages of vitellogenesis captured from their natural habitats with an oocyte diameter of 570 to 650 µm. To stimulate egg maturation and spawning, mullet were confined and subjected to various hormonal treatments, including injection with carp pituitary extract (CPE, 20 and 70 mg/kg) or human chorionic gonadotropin (HCG, 10.000 IU/fish) followed by a second or third injection of LHRH-a (100-200 µg/kg) (Meseda and Samira, 2006); two-step injection of HCG (10000 IU) plus HCG+LHRH-a (10000 IU + 200 µg/kg) (Besbes *et al*., 2020) and an injection of 200 µg/kg GnRHa (Vallainc *et al*., 2021). The results of these previous studies have been variable with a spawning percentage of 40, 100, and 100% and fertilisation rate of 75–80, 63, and 83 ± 14%, respectively. This variation underscores the remarkable efficacy of the rGth protocol reported in the present study, as well as in the studies by Ramos-Júdez *et al.*, (2021; 2022b), where 100% of the females treated with rGths successfully completed gametogenesis. This is the third study (present study, Ramos-Júdez *et al.*, 2021; 2022b) by the authors group to induce spawning in mullet with the successful rGth protocol. All females (100% of 34 females) in these studies treated with combined rFsh and rLh completed oogenesis. All males treated with combined rFsh and rLh completed spermiation (100% of 16 males). Having achieved 100% completion of gametogenesis in both female and male mullet the next critical step was to induce spawning. In these three studies all mullet administered the double rLh induced spawning protocol for females combined with a single rLh injection for males have spawned (100% of 10 females induced with males). However, the eggs from one female in the present study were not fertilised (90% fertilised spawning of 10 females). Similar success to induce gametogenesis with rGths has been described in European eel (Jéhannet *et al.*, 2023a). A 100% efficacy of these treatments is quite exceptional and can provide a reliability that would enable the aquaculture sector to change practices and reduce the number of breeders maintained for years at enormous cost. Generally, a hatchery may hold 50% more breeders than are required for the hatchery´s egg production (Moretti *et al.*, 2005). This excess of breeders must be grown to maturity during a number of years and this has a high cost in feed, installations and energy. Although, the rGth treatments are generally recognised as costly, holding and

treating a minimal number of breeders may be more economical than holding a large number of breeders through to natural maturation.

After successfully inducing spawning in aquaculture, ensuring high egg quality becomes the next major challenge. Several studies have identified eggs size, buoyancy, lipid droplets and blastomere morphology as egg quality parameters (Aristizabal *et al.*, 2009; Kohn and Symonds, 2012). Egg fertilisation and hatching rates are significant indicators of egg quality; however, the most reliable indicators may be the larval survival rate and the production of healthy fry (Harmin and Crim, 1992). In this respect, the three spawns that were fertilised, had good quality eggs with fertilisation percentage from 71 – 97% and hatching percentage from 66 to 96%. In addition, egg morphology was normal with an average egg diameter of 800 µm and the oil globule of 310 µm. Similar egg diameter and oil globule reported by Meseda and Samira, (2006) were 870 ± 30 µm and 350 µm, respectively. Furthermore, larval survival under starvation was 13 days at 21 °C. Egg quality parameters have also been related to both ova and sperm quality, which is crucial for successful fertilisation (Duncan *et al.*, 2013; Ramos-Júdez *et al.*, 2019), in addition egg quality is related to the protocol used to induce spawning (Mañanós *et al.*, 2008; Duncan *et al.*, 2013). The critical factors of a hormonal induction protocol that influence egg quality are the stage of ovarian development at the time of hormone administration, as well as the hormone dosage (Mañanós *et al.*, 2008; Duncan *et al.*, 2013). The stage of ovarian development, which is assessed by oocyte diameter and the position of the germinal vesical (nucleus) has an optimal window for hormonal induction. Inducing at a ovarian development stage that is too early may lead to lower fecundity or no spawning, while inducing too late can result in reduced egg quality (Lee *et al.*, 1986; Berlinsky *et al.*, 1996, 1997; Mugnier *et al.*, 2000; Ibarra-Castro and Duncan, 2007; Duncan *et al.*, 2013). Similarly, administering a hormone dose below the optimal level can reduce fecundity or fail to induce spawning, while higher doses may compromise egg quality (Barbaro *et al.*, 1997; Garcia, 1989; Ibarra-Castro and Duncan, 2007). To further complicate this relationship, an interaction has been described in which, generally, the earlier the stage of ovarian development, the higher the dose of hormone required to obtain good egg quality (Kuo and Nash, 1975; Harvey and Hoar, 1979; Ibarra-Castro and Duncan, 2007). The present study unveils a very good negative correlation (R^2 = - 0.98) between percentage of germinal vesicle migration (GVM) and hatching rate in 96-well plates based on

spawning data from 2023 (present study) and 2020 (Ramos-Júdez *et al.*, 2022). In the present report we have identified that the optimal timing for the final induction was when less than 21% of germinal vesicles had migrated. This protocol refinement is crucial for achieving the highest hatching rates, with 97% hatching rate being an expected outcome. The strong correlation observed indicates GVM% was a reliable predictor of egg quality during the final induction with a doble 30 µg/kg rLh injection, allowing for more effective broodstock management. However, the interaction previously described where optimal hormone induction dose decreases with the advance of ovarian stage of development (Kuo and Nash, 1975; Harvey and Hoar, 1979; Ibarra-Castro and Duncan, 2007) indicates that the result can be interpretated to show that the present protocol of a double 30 µg/kg rLh injection should be applied to oocytes in an early stage of development prior to germinal vesicle migration initiates and that the doses should be lower for oocytes that have an advanced stage of ovarian development with higher percentages of GVM. Therefore, it is possible that reducing the doses of rLh for the final injection in oocytes with greater GVM might lead to higher hatching rates. Furher work is required to adequately explore this relationship for the application of rLh to mullet.

The newly hatched larvae in this study measured 2.67 ± 0.11 mm, which falls within the range of 1.8 to 2.75 mm observed in other studies (Nash *et al.*, 1974; Kuo *et al.*, 1973; Eda *et al.*, 1990; Abraham *et al.*, 1999; Loi *et al.*, 2020; Meseda and Samira, 2006); 2.3± 0.11 mm (Yousif *et al.*, 2010; Vallainc *et al.*, 2021). The larvae measured 2.75 ± 0.11 mm at 5 dph, which differs from the 3.5 ± 0.2 mm reported by Besbes *et al.*, (2020). Larvae at 20 dph measured 4.62 ± 0.41 mm of density of 16 larvae/L with *Tetraselmis chuii* and *Isochrysis galbana* (microalgae mix), while larvae at 18 dph measured 6.3 ± 2 mm by Besbes *et al.*, 2020. These results suggest that the green method, using the microalga *Chlorella minutissima* at densities ranging from 10,000 to 80,000 cells/ml, produces larvae with greater total length (LT) when reared at 100 larvae/L.

Regarding larval growth in production systems, larvae cultured in the black 500 L tanks exhibited a higher growth rate (16.70 ± 0.05 mm) compared to those in the grey tanks (11.40 ± 2.80 mm) at 42 dph. Nevertheless, this improved growth in the black tank may be attributed to better food availability as the lower survival rate in the black tanks (0.21%) compared to the grey tanks (0.54%) at 42 dph may have resulted in less competition for resources. However, a

similar growth rate was observed in the study by Kuo *et al.* (1973), which reported a total length (LT) of 17.69 mm at 42 days post-hatching (dph) using wild zooplankton as feed. The highest final survival rate in this study was 14.13 ± 5.2% in 100-litre tanks at a density of 16 larvae per litre. Other studies have also reported low survival rates, such as 1.2%, 1.5%, and 4.35% at 30 dph (Oz *et al.*, 2022), and between 10% and 19% at 60 dph (Vallainc *et al.*, 2021). These studies followed similar larval rearing protocols, with the main difference being the type of microalgae used — specifically *Nannochloropsis sp.* The relationship between initial stocking density and number of juveniles produced per litre was described by a second-order polynomial (quadratic) equation that showed the culture system could produce 2 – 3 juveniles per litre with initial stocking densities of 15 – 50 larvae/litre. At the lower initial densities, the survival was highest and as initial stocking densities increased the survival decreased, but with an increase to a peak of three juveniles per litre at an initial stocking density of 38 larvae per litre. The lower production of larvae with high initial density in this system could be linked to various environmental factors, such as salinity, temperature, pH, dissolved oxygen levels, and water quality (Saraswathy *et al.*, 2015; Mariu *et al.*, 2023). Additionally, water quality is influenced by biological, physical, and chemical factors, all of which are crucial for growth, survival, and production in the hatchery (Saraswathy *et al.*, 2015). Larval production in any hatchery is highly vulnerable to environmental issues (Ramos *et al.*, 2006, 2009). Overall, low survival was more common in the present study, especially in the 500L tanks. The exact cause of these low survivals cannot be defined as there were many variables. Egg quality may have been a factor and as discussed larval survival was variable amongst females. Although these small larval rearing tanks give better control and replication for experiments it is generally accepted that large tanks give more stable rearing conditions that can give increased survival and consequentially large tanks are often used in a commercial hatchery (Moretti *et al.*, 2005). One of the primary issue contributing to the low survival in this study may be the lack of a standardised protocol for seed production in this species (Quirós-Pozo *et al.*, 2023). Despite numerous efforts, further research is needed in this area. Larviculture protocols involve several critical factors, including the amount of microalgae to be supplied, light intensity, larval density, water quality (de Jalón *et al.*, 2007; Rodríguez Pesantes *et al.*, 2020), artificial diets (de Azevedo Manhães *et al.*, 2024), eggs quality, and the ideal timing for transitioning from rotifers to *Artemia*. Despite of these poor results, it should be acknowledged that good survivals (14.13 ±

5.2%) were obtained in the present study and these clearly indicate that the rGth spawning protocols can give good larval survival. Therefore, future studies must focus on egg quality and larval rearing protocols to improve the consistency of results from different spawns obtained with the rGth protocol.

5. Conclusions

The present study provides the first evidence that recombinant gonadotropins (rGths) can be used to induce gametogenesis, maturation and spawning in a teleost species out of season. Egg and larval quality were sufficient to show that, with further refinement, these protocols can be used by the aquaculture industry as a basis for juvenile production. The high efficacy of induced maturation, over 90% observed in the present and previous studies that developed the protocol, indicate that it offers an attractive alternative for breeder management. Therefore, the findings of this study hold crucial implications for aquaculture, as the culture of this species relies heavily on wild juvenile resources, with hatchery-produced juveniles being scarce.

CHAPTER III.

IMPACT OF RECOMBINANT FOLLICLE-STIMULATING HORMONE ON VITELLOGENESIS AND FECUNDITY IN FLATHEAD GREY MULLET (*MUGIL CEPHALUS*)



1. Introduction

In teleost fish and other vertebrates, the brain-pituitary-gonad (BPG) axis regulates reproduction function (Liao, 1975; Rodríguez Gabilondo *et al.*, 2021). In females, Fsh stimulates gametogenesis by promoting the development of oocytes from pre-vitellogenesis stages through early, mid, and late stages of vitellogenesis. The Fsh stimulates the production of 17β-estradiol, which acts on hepatic nuclear estrogen receptors to initiate vitellogenin production and transport to the developing oocyte. In contrast, Lh levels rise during late-stage gametogenesis, stimulating final maturation, such as oocyte maturation and ovulation (Lubzens *et al.*, 2010; Reading *et al.*, 2017). Alterations in the environment affect the BPG axis to modify the release of these two gonadotropins, which could result in reproductive dysfunctions in females during the vitellogenesis phase (Zohar and Mylonas, 2001; Mylonas *et al.*, 2010).

Reproductive dysfunctions in teleost fish have been broadly categorized into three main types (Zohar and Mylonas, 2001; Mylonas *et al.*, 2010): (1) failure to initiate or complete vitellogenesis, (2) failure to undergo final oocyte maturation despite completing vitellogenesis, and (3) disruptions in spawning behaviour or ovulation despite successful oocyte maturation. The severity and type of dysfunction often depend on rearing conditions and the species' degree of domestication. In flathead grey mullet (*Mugil cephalus*) (hereafter referred to as “mullet”) specifically, depending on the aquaculture system, two predominant types of dysfunctions have been identified: In semi-intensive systems, females typically complete vitellogenesis but fail to achieve final maturation and spawning (Kuo *et al.*, 1974a; Liao, 1975). In contrast, in intensive systems, females exhibit a more severe dysfunction, failing to initiate vitellogenesis or arrest development in the early stages of vitellogenesis (Ramos-Júdez *et al.*, 2021, 2022b).

Reproductive dysfunctions are a significant challenge in aquaculture, affecting a wide range of fish species, including the meagre (*Argyrosomus regius*) (Duncan *et al.*, 2023), Japanese eels (*Anguila japonica*) (Kazeto *et al.*, 2008; Kobayashi *et al.*, 2010), Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2018), Malaysia catfish (*Hemibagrus nemurus*) (Salwany *et al.*, 2019), and Macquarie perch (*Macquaria australasica*) (Nocillado *et al.*, 2023). These reproductive obstacles limit the potential for sustainable reproduction and harvesting of these species, ultimately endangering the development and sustainability of aquaculture. The main

bottlenecks are not only biological; they also include the need to identify species with low fish-oil and fishmeal requirements, which are considered highly unsustainable from an environmental perspective. In this aspect, mullet is emerging as a candidate for the diversification of aquaculture. Additionally, mullet is euryhaline, eurythermal, low trophic level (Lebreton *et al.*, 2011), and high economic value of mullet females, particularly for "bottarga" (the roe of fully mature females), exceeding 200 euros per kilogram (Besbes *et al.*, 2020).

Despite the many advantages of mullet, reproductive dysfunctions represent a major bottleneck in aquaculture. Females in intensive systems frequently experience issues during vitellogenesis, which have been associated with insufficient gonadotropin production by the pituitary gland (Ramos-Júdez *et al.*, 2021, 2022b). Overcoming these biological limitations is essential to unlocking the full potential of this species in aquaculture systems and meeting economic and sustainability goals.

Vitellogenesis is a vital process in oocyte growth, in which egg yolk proteins are produced and accumulated in the ooplasm. During this process, the oocyte selectively absorbs vitellogenin via receptor-mediated endocytosis, converts them into yolk proteins, and stores these proteins in structures such as yolk granules, globules, or platelets (Reading *et al.*, 2017). However, the challenges are not limited to vitellogenesis but also extend to overall fecundity, as reproductive impairments reduce the quantity and quality of viable eggs produced.

The reproductive maturation at puberty (first sexual maturation) or during an annual reproductive cycle is a complex process regulated by activation of the brain-pituitary-gonad (BPG) axis. In teleosts, this process occurs in response to a combination of internal (body size, energy reserves, nutritional status) and external cues (photoperiod, temperature, social interactions) that stimulate the brain to activate secretion of Fsh from the pituitary (Rodríguez Gabilondo *et al.*, 2021). The Fsh initiates the recruitment of previtellogenic oocytes into the vitellogenic pathway (Tyler and Sumpter, 1996; Murua and Saborido-Rey, 2003). This initial Fsh pulse is particularly critical, as it determines not only the timing of reproductive onset but also the magnitude of oocyte recruitment, thereby establishing the potential fecundity for that reproductive cycle (Reading *et al.*, 2017). Hence, understanding the mechanisms controlling the

onset of reproductive maturity is crucial to develop efficient intervention strategies to overcome reproductive failure in intensive culture.

Vitellogenesis kinetics refer to the rate of oocyte enlargement and the length of the vitellogenic phase. This process is primarily regulated by Fsh, which stimulates the production of 17β-estradiol (E_2) in ovarian follicles through the two-cells system involving the theca and granulosa follicle layers (Lubzens *et al.*, 2010; Reading *et al.*, 2017). In this two-cell system, androgen production occurs in theca cells through P450c17α1 (cyp17a1) activity, while estrogen synthesis takes place in granulosa cells via P450 aromatase (cyp19a1), which catalyzes the conversion of testosterone into E_2 under Fsh stimulation (Lubzens *et al.*, 2010; Reading *et al.*, 2017). The released E_2 acts on hepatic estrogen receptors to induce vitellogenin (Vtg) synthesis in the liver, which is then transported via the bloodstream to the ovary (Tyler and Sumpter, 1996). The rate of Vtg incorporation into oocytes, and consequently the growth rate of vitellogenic oocytes, is directly proportional to plasma Fsh and E_2 levels (Lubzens *et al.*, 2010). In species with asynchronous or group-synchronous ovarian development, the duration of vitellogenesis can range from weeks to months, depending on environmental conditions, nutritional status, and endocrine regulation (Mylonas *et al.*, 2010). Insufficient Fsh levels during this phase can prolong the vitellogenic period or result in incomplete oocyte development, leading to reproductive failure (Zohar and Mylonas, 2001).

Control of fecundity defined as the number of oocytes that complete vitellogenesis and are available for spawning, is determined during the recruitment phase, when a cohort of previtellogenic oocytes transitions into the vitellogenic phase (Tyler and Sumpter, 1996; Murua and Saborido-Rey, 2003). This recruitment event represents an important regulatory step in determining reproductive success. The number of oocytes recruited into vitellogenesis is depends upon: (1) the size of the standing stock of previtellogenic oocytes available for recruitment, (2) the sensitivity of these oocytes to Fsh stimulation, and (3) the plasma levels of Fsh at the time of recruitment (Murua and Saborido-Rey, 2003; Lubzens *et al.*, 2010). In determinate spawners, fecundity is fixed prior to the spawning season, thereby recruitment only occurs once, early in the reproductive cycle. In contrast, indeterminate spawners are able to continuously recruit oocytes, thus multiple recruitment events throughout the spawning season

are possible. (Murua and Saborido-Rey, 2003). In species with reproductive dysfunctions in captivity, both kinetics and fecundity can be compromised due to impaired gonadotropin production. In mullet held in intensive systems, females remain arrested at previtellogenesis or early vitellogenesis due to insufficient Fsh production (Aizen *et al.*, 2005; Ramos-Júdez *et al.*, 2021, 2022a). Similarly, reproductive dysfunctions associated with inadequate gonadotropin secretion have been documented in other cultured teleosts, including Japanese eels (*Anguila japonica*) (Kazeto *et al.*, 2008), Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2018), and meagre (*Argyrosomus regius*) (Duncan *et al.*, 2023). Understanding these regulatory mechanisms is essential for developing effective hormonal therapies to overcome reproductive failures in intensive aquaculture systems.

Exogenous hormones have been effectively used to stimulate maturation and induce spawning in fish in captivity. Attempts to induce maturation in mullet were initiated in Hawaii in 1973 (Shehadeh *et al*., 1973) and continue to this day. Various hormone therapies have successfully induced spawning in semi-intensive systems, such as salmon gonadotropin (SG-G100) applied in two doses 24 hours apart, which obtained spawning with a fecundity of 648 ± 62 eggs/grams body weight (bw) (Shehadeh *et al.*, 1973), carp pituitary extract (CPE), or human chorionic gonadotropin (HCG) combined with a second and third injection of GnRHa (des Gly10[D-Ala6] GnRHa ethylamide) induced spawning with a fecundity of 1395.3 ±190 eggs/grams bw (Meseda and Samira, 2006), a single dose of the GnRHa preparation, leuprorelin acetate (ENANTONE®, Takeda Italia S.p.a., C59H84N16O12, amino acid sequence H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu- Arg-Pro-NHEt) have achieved a fecundity between 842 to 704 eggs/grams bw (Vallainc *et al.*, 2021).

The more recent study, which employed an intensive system using rGths protocol (rFsh and rLh), induced spawning with a fecundity of 694 ± 187 eggs/grams bw during the final induction with 30 µg/kg of rLh and 40 mg/kg of progesterone (Ramos-Júdez *et al.*, 2021). When the same protocol was applied with a key modification in the final induction, using only recombinant follicle-stimulating hormone (rFsh), the fecundity was 1,205 ± 487 eggs/grams bw (Ramos-Júdez *et al.*, 2022b) subsequent study in 2024 using the same rGths protocol (Ramos-Júdez *et al.*, 2021, 2022b) obtained four induced spawning events with fecundities (eggs/grams bw) of

1,024; 409; 356, and 207 (Cerrud-Barría *et al.*, 2026). These studies demonstrated the first evidence that achieving reproduction in intensive systems is possible using rGths. However, the high cost of recombinant hormone production represents a major economic barrier for commercial aquaculture applications. Optimising rFsh dosage is therefore critical to maintain reproductive efficiency while reducing production costs. Given that Fsh regulates both the recruitment of oocytes into vitellogenesis (Tyler and Sumpter, 1996; Murua and Saborido-Rey, 2003) and the kinetics of oocyte growth (Lubzens *et al.*, 2010) rFsh dosage during early vitellogenesis may potentially maintain fecundity and development while minimizing hormone use. The present study aimed to determine the optimal rFsh dose administered during the vitellogenesis phase, starting from the previtellogenic stages of oocyte development, to maximise fecundity in mullet females while improving protocol cost-effectiveness.

2. Materials and methods

2.1. Broodstock and maintenance

The experiment was conducted from April 25th to July 9th, 2024, to evaluate the effect of four different rFsh doses (3, 6, 9, and 12 µg/kg) on the kinetics and fecundity of vitellogenesis in mullet. Twenty-four females were randomly selected from a stock of 192 mullet introduced into IRTA facilities in 2020. Prior to the experiment, mullet were held in fiberglass tanks under natural environmental conditions (41°36′46″N, 2°10′11″E). For identification, each mullet was tagged intramuscularly with a Passive Integrated Transponder (PIT) tag (Trovan®, ZEUS Euroinversiones S.L., Madrid, Spain). The selected breeders (mean ± SD body weight: 0.78 ± 0.12 kg; standard length: 36.2 ± 1.8 cm) were randomly assigned to four experimental groups of six females each, corresponding to the different rFsh doses.

The experimental groups were placed in a 10 m³ rectangular fibreglass tank (6.1 × 2.1 × 1.2 m) under natural photoperiod from natural daylight, increasing from LD12:12 on April 25th, 2024, to LD14:10 on July 9th, 2024 (L: light; D: dark), with constant temperature of 24°C and salinity of 36‰ in a recirculation system (IRTAmar®) at IRTA La Ràpita, Spain. The tank had both surface and bottom water outlets that were adjustable to control the volume of water exiting the

tank from each outlet. Fish were fed daily with a commercial Repro Sole diet (5 mm) (Sparos, Portugal) at 1.5% body weight. The diet contained 60% protein, 13% fat, 0.5% fibre, 7% ash, 1.2% phosphorus, and 0.9% calcium.

Ethics. The research was carried out in adherence to the following regulations: the Spanish Royal Decree 53/2013 of February 1st, which also concerns the protection of animals used for scientific purposes; the Catalan Law 5/1995 of June 21st, which pertains to safeguarding animals used for scientific purposes; and the Catalan Decree 214/1997 of July 30th, which governs the regulation of such animals. The European Directive 2010/63/EU was implemented on September 22nd, 2010. The Catalan Government and the Committee of Ethics and Experimental Animals (CEEA) of the Institute of Agrifood Research and Technology (IRTA) evaluated the procedures used in this study, which were authorized with authorisation of experimental project number 12016, expedient number FUE-2023-02998468, and ID NT0G72MXQ. ARRIVE guidelines were adhered to by the authors.

2.2. Induction with recombinant gonadotropin

For rFsh treatment and sampling during the experiment, fish were anaesthetised with MS-222 at a dose of 73 mg/L. The rFsh used was produced using Chinese hamster ovary (CHO) cells and mullet gonadotropin (Gth)-coding cDNAs by Rara Avis Biotec S.L., Valencia (Ramos-Júdez *et al.*, 2022).

2.2.1. Induction of intermediate vitellogenesis in females

The twenty-four female mullet selected for the study had a mean ± SD oocyte diameter of 130.8 ± 22 µm. All females received weekly injections of rFsh at the assigned dose (3, 6, 9, or 12 µg/kg) from May 7th, 2024, until July 2nd, 2024. The objective was to evaluate the effect of different doses of rFsh on the transition from previtellogenesis (oocyte diameter of approximately 100 µm) to mid / late vitellogenesis (oocyte diameter exceeding 300-400 µm).

Weekly intramuscular doses of rFsh were administered to four experimental groups: group 3 (3 µg/kg), group 6 (6 µg/kg), group 9 (9 µg/kg), and group 12 (12 µg/kg) for eight weeks. During the experiment, vitellogenesis was monitored by examining the most advanced oocyte in ovarian

biopsies. A 1.67 mm (5ch) x 500 mm plastic cannula was used to collect ovarian samples from each fish. Using a Zeiss Axiostar microscope at a magnification of 40X, the diameter of the 20 most advanced oocytes was measured at weeks 0, 4, 6, and 8, while 100 randomly selected oocytes were measured at week 9. When vitellogenesis had advanced to intermediate vitellogenesis (week 9) with a clear cohort of induced oocytes (300-400 µm) females were sacrificed, and the following parameters recorded: ovaries dissected (initial, middle, and final) for fecundity calculations (pre-weight sub-samples of ovary, (Murua *et al.*, 2003), body weight, gonadosomatic index (GSI), macroscopic evaluation of gametes, blood samples for steroid analysis, and samples fixed for histology.

A portion of the oocyte sample was collected using a cannula (week 0, 4, 6, 8) and by dissection (week 9-final samples). These samples were preserved in Bouin´s solution (comprising formaldehyde, picric acid, and acetic acid) and stored in 70% ethanol for histological analysis. Blood samples were collected prior to hormone administration at week 0, 4, 8, and 9.

2.3. Histological analysis

Oocyte samples fixed in Bouin´s solution (week 0, 4, 6, 8, and 9) were dehydrated with a series of graded ethanol baths. The samples were embedded in paraffin and cut into 3 µm sections that were dried and stained with Mayer-Bennett hematoxylin and Floxina/Eosina (Casa Álvarez, Spain). Finally, the slides were examined under a light microscope (Leica DMLB, Houston, USA) at magnifications of 600x and 1000x to analyse the oocyte developmental stage, capture images and measure oocyte diameter (oocyte classification by diameter: perinucleolar (27-110 µm), cortical alveoli (110-203 µm), early vitellogenesis (203-250 µm), middle vitellogenesis (250-320 µm), and late vitellogenesis (>320 µm), using ImageJ software) Oocytes were classified as previously described by Ramos-Júdez *et al.* (2021). Previtellogenic oocytes in the primary growth (PG) stage were distinguished by the presence of multiple nucleoli in the germinal vesicle of perinucleolar oocytes, while cortical alveoli (AC) oocytes exhibited granular vesicles and small oil droplets in the peripheral ooplasm. Early (VE), middle (VM), and late vitellogenesis (VL) were identified when oocytes reached sizes greater than 203 µm, and full-grown late vitellogenesis when vitellogenin growth was completed with the fusion of yolk

granules and thickening of the vitelline membrane, were indicated by the incorporation of yolk globules. Furthermore, the percentage of oocytes in each developmental stage was determined over the experimental period by randomly selecting at least fifty oocytes from each female at each sampling point.

2.4. Plasma steroid analysis

Blood samples from females in rFsh treatment groups were collected at various time points (week 0, 4, 6, 8 and 9). Samples were kept on ice until centrifuging at 3000 rpm at 4 °C for 15 minutes. Plasma was collected into Eppendorf tubes and stored at -80 °C until analysis. Plasma levels of 17β-estradiol (E_2) and testosterone were analysed using commercially available enzyme immunoassays (EIA) (Cayman Chemical Company, USA). Steroids were extracted with methanol, which was subsequently evaporated, and extracts were resuspended 1:10 (E_2) or 1:10 (T) in the EIA buffer (Ramos-Júdez *et al.*, 2022a).

2.5. Fecundity analysis

Fecundity was estimated from 24 ovary sub-samples (six individuals per treatment dose) collected at week 9 of treatment and preserved in a buffered 4% formaldehyde solution (100 mL formalin-36% formaldehyde, 900 mL distilled water, 4 g $NaH_2PO_4 \cdot H_2O$ and 6 g Na_2HPO_4).

Fecundity was estimated using two complementary approaches:

1) Gravimetric Method (Hunter *et al.*, 1989): This method involved counting and measuring all oocytes in the ovary sub-samples, corresponding to six individuals from each of the four-dose treatments.
2) Stereometric Method (Emerson, 1990): This method involved counting and measuring all oocytes from several histological images of the ovaries of each individual in the experiment.

Gravimetric Method

Sub-samples were collected from excised ovaries at week 9 of the treatment and preserved in a buffered 4% formaldehyde solution. For each fish, the sub-sample was taken from the central region of the right ovary, with a weight of approximately 0.09 – 0.10 grams, which was accurately weighed and recorded. The coefficient of variation (CV) for the number of oocytes/g weight was less than 5% (Kjesbu, 1989).

Oocytes from the sub-samples were separated using a series of 100 - 300 µm mesh size sieves, and a jet of water was used to disaggregate oocytes and strip connective tissue. The separated oocytes were photographed under a binocular stereoscope, and fecundity was calculated by counting and measuring all oocytes in the sub-sample using an image analysis macro in ImageJ (Schindelin *et al.*, 2012). Oocytes with a mean diameter smaller than 100 µm were excluded, as they corresponded to the primary growth stage (based on prior histological measurements (Murua *et al.*, 2003). The number of oocytes in secondary growth stages (vitellogenic stages) was then multiplied by the sub-sample weight and the total fixed ovary weight of each female to estimate the total number of mature oocytes per ovary, i.e., potential fecundity (F).

Fecundity (F) was calculated as the product of gonad weight and oocyte density per unit weight (Murua *et al.*, 2003), using the following formula:

$$F = \frac{\left[\sum_i \frac{Oi}{Wi}\right]}{n} * W_{ovary}$$

Where:

Oi: number of oocytes in sub-sample i

Wi: weight of sub-sample i

W_{ovary}: total weight of the ovary

n: number of sub-samples

Stereometric Method

Ovaries excised and preserved at week 9 of treatment were processed for histological analysis. Six non-overlapping histological images were obtained per ovary, and oocyte diameters were measured to the nearest 0.001 mm using STERapp (Mbaidin *et al.*, 2021).

Oocytes were processed through STERapp to select oocytes for developmental stages and identify those with a visible nucleus for diameter measurement. Diameter measurements were grouped by size class using the results file generated by STERapp, and frequency distribution, mean nucleus diameter, and a linear regression between oocyte and nucleus size were performed using these data.

Based on these outputs, the method estimated stage-specific and total oocyte areas, relative areas, and object densities per fish. Ovary volume was estimated using regression equations and corrected for tissue shrinkage. Statistical moments (M1, M3, K) were computed to support the estimation of fecundity. Both potential fecundity and realized fecundity (excluding atretic oocytes) were calculated for each individual.

2.6. Gonadosomatic Index (GSI)

Final samples were collected at week 9. Total body weight and total length were recorded. During dissection, the ovaries of each female were carefully removed and weighed. The GSI was calculated (Afonso-Dias *et al.*, 2005).

$$GSI = (\text{Gonad weight (GW)} \times 100) / \text{Body weight (BW)}$$

2.7. Statistical analysis

Data were expressed as mean ± standard error (SE) or standard deviation (SD) as indicated. The Shapiro-Wilk and Levene tests were used to check the normality of data distribution and homogeneity of variance, respectively.

Oocyte development and steroid analysis. The least significant difference (LSD) test was used to compare oocyte diameter between rFsh treatment groups at weeks 0, 4, 6, 8, and 9. Two-way repeated measures (RM) ANOVA with pairwise comparison by the Holm-Sidak test was used to determine significant differences in plasma levels of 17β-estradiol (E_2) and testosterone between groups over time.

Gonadal regional analysis. One-way ANOVA was used to compare statistical differences among gonadal regions (anterior, middle, and posterior) within each treatment. Dunn's test was used for pairwise comparisons of the middle gonadal region between treatments at week 9.

Histological and stereological analysis. The proportions Z-test with Bonferroni correction (adjusted $P = 0.008$) was used to compare the percentage of oocytes in different development phases (perinucleolar, cortical alveoli, early vitellogenesis, middle vitellogenesis, and late vitellogenesis) between treatments. The same test was applied for ovarian atresia analysis, comparing the percentages of cortical alveoli, vitellogenic, and atretic oocytes among treatments.

Fecundity and oocyte measurements. One-way ANOVA was performed to assess differences in total fecundity and oocyte density among treatment groups. One-way ANOVA followed by Tukey's post-hoc test was used to compare oocyte size distribution (mean oocyte diameter) obtained from mesh sieve analysis (200 - 300 µm). Analysis of covariance (ANCOVA) was performed to evaluate the effect of fish body length (as covariate) on total fecundity and oocyte density, with treatment as the main factor.

Data transformations and GSI analysis. Total lipids (%) were transformed using reciprocal transformation (1/x), while GSI data were square root transformed to achieve normality. One-way ANOVA was performed to assess differences in GSI and total lipids among treatments, followed by Holm-Sidak post-hoc test for multiple comparisons. All statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $P < 0.05$ for all tests.

3. Results

3.1. Induced vitellogenesis

The females treated with different rFsh doses (3, 6, 9, and 12 µg/kg) responded with ovarian development from previtellogenic stages to mid- and late vitellogenesis stages **(Fig.5)**. The four rFsh treatments showed different patterns of oocyte diameter development **(Fig. 1)**.

At the start of the experiment, all groups had 100% females in previtellogenesis with a stage of primary growth **(Fig. 1, week 0)**, and there were no significant differences in the mean initial diameter of the largest oocytes among groups (group 3 =122 ± 9.1 µm, group 6 =133 ± 9.5 µm, group 9 = 133 ± 12.6 µm, and group 12 = 135 ± 7.89 µm, mean ± SE). However, a significant increase in oocyte diameter (mean ± SE) was observed in week 4 in groups 6, 9, and 12, (206 ± 10.5 µm, 230 ± 12.2 µm, 229 ± 13.1 µm while group 3 did not (153 ± 19.2 µm).

The oocyte diameter of all groups increased significantly each week (4, 6, 8, and 9) ($P < 0.001$). The mean oocyte diameter of group 3 females was significantly lower than groups 6, 9, and 12 from week 4 until week 9 **(Fig. 1)**.

The growth progression of oocytes in groups 6, 9, and 12 was aligned by the week of treatment using a second-order polynomial (quadratic) equation ($Oocyte\ diameter\ (\mu m) = -7.2857week^2 + 98.448week + 44.267$; $R^2 = 0.978$) **(Fig. 1)**. Similarly, the growth of oocytes in group 3 was aligned with the weeks of treatment and modelled using another second-order polynomial (quadratic) equation ($Oocyte\ diameter\ (\mu m) = 1.5832week^2 + 2.5261week + 120.84$; $R^2 = 0.978$).

At the final sampling point (week 9), the group exhibited an oocyte diameter (mean ± SE) of 333 ± 12.09 µm (group 6), 347 ± 7.86 µm (group 9), 370 ± 12.52 µm (group 12), which was statistically significantly larger ($P < 0.001$) compared to the group 3 (262 ± 31.2 µm oocyte diameter) **(Fig. 1).**

One female from the 12 µg/kg group exhibited atypical oocyte development and was excluded from all analyses as an outlier from week 5 onwards. At week 4, this female showed an oocyte

diameter of 234 ± 3.77 µm, indicating initial vitellogenic development. However, oocyte diameter subsequently decreased significantly, returning to previtellogenic diameters at weeks 6, 8, and 9 (166 ± 3.34 µm, 129 ± 4.77 µm, and 104 ± 2.95 µm, respectively; $P = 0.001$). This abnormal regression was not observed in any other individual. Additionally, one female from the 3 µg/kg group died (jumped from the tank) after sampling at week 8 and was excluded from week 9 analyses. Final sample sizes were $n = 5$ for groups 3 and 12 µg/kg, and $n = 6$ for groups 6 and 9 µg/kg.

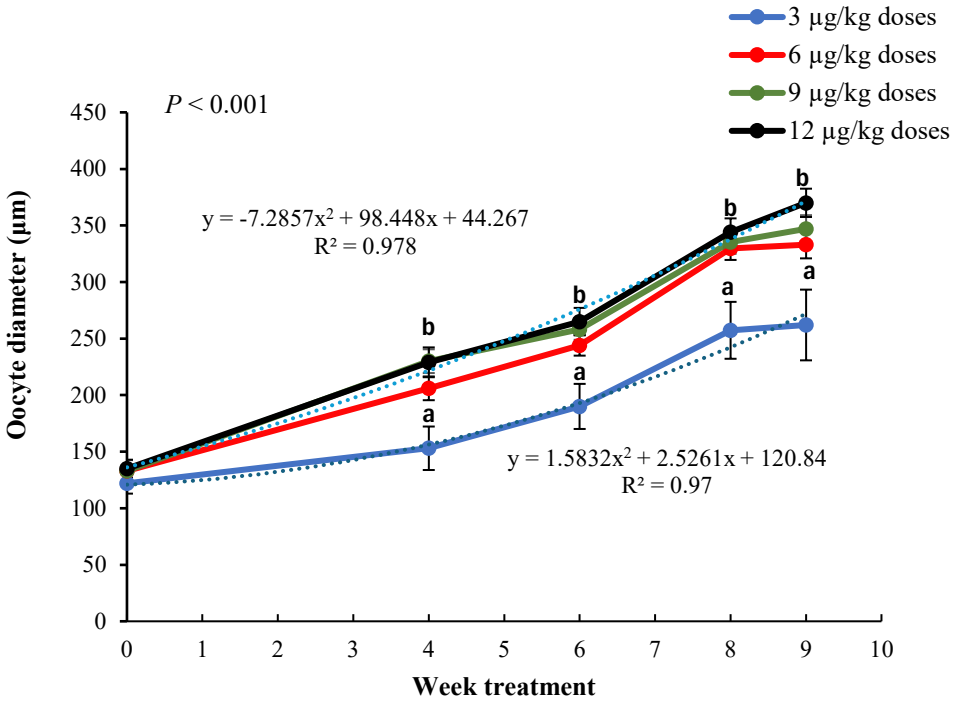


Figure 1. Oocyte diameter (mean ± SE; 20 most advanced oocytes) of the four rFsh treatments on weeks 0, 4, 6, 8, and 9. The continuous blue line in the graph indicates rFsh dose at 3 µg/kg ($n = 6$; $n = 5$ week 9), red line: 6 µg/kg ($n = 6$), green line: 9 µg/kg ($n = 6$) and black line:12 µg/kg ($n = 6$; $n = 5$ from week 5-9). The Least Significant Difference (LSD) test was used to compare oocyte diameter between rFsh-treated groups.

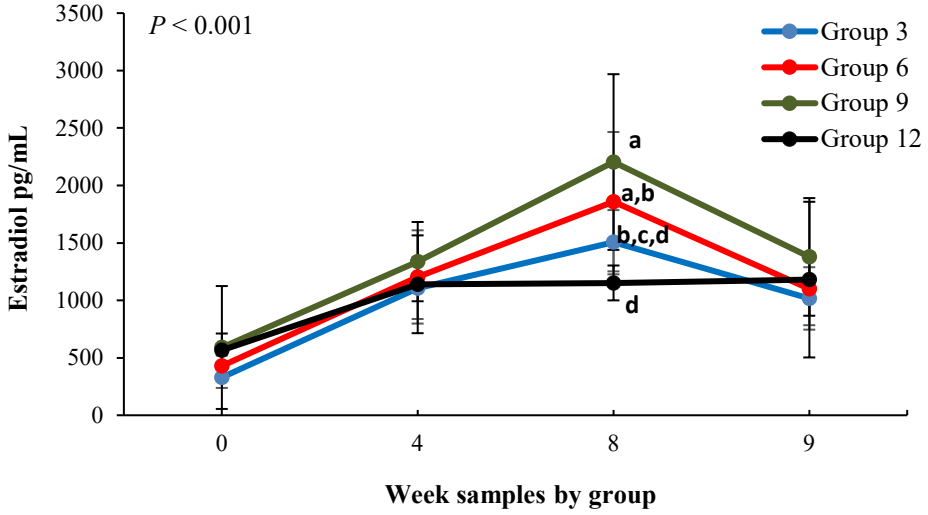


Figure 2. Effect of four rFsh treatments on plasma estradiol levels (mean and SD) in female mullet. The outlier is a female in group 12 from week 8 (mean and SD): 374.47 ± 18.51 pg/mL. The Holm–Sidak test was used to determine statistical differences in plasma 17β-estradiol (E2) levels between groups. The continuous blue line in the graph indicates rFsh dose at 3 µg/kg (n = 6; n = 5 week 9), red line: 6 µg/kg (n = 6), green line: 9 µg/kg (n = 6), and black line:12 µg/kg (n = 6; n = 5 from week 5-9).

The increase in oocyte diameter observed in females was associated with increased steroid levels from week 0 to week 8 in groups 3, 6, and 9 ($P < 0.05$). Plasma estradiol (E_2) concentrations increased significantly from baseline (week 0) to week 4 in all treatment groups (groups 3, 6, 9, and 12; $P < 0.001$), indicating initial vitellogenic response to rFsh treatments. From week 4 to week 8, E_2 levels continued to increase significantly in groups 3, 6, and 9 ($P < 0.05$), reaching peak concentrations at week 8. In contrast, group 12 exhibited no significant increase from week 4 to week 8 ($P > 0.05$). From week 8 to week 9, E_2 concentrations decreased significantly in groups 3, 6, and 9 ($P < 0.01$), consistent with late vitellogenic or pre-maturational stages, while group 12 showed no significant change ($P > 0.05$).

Regarding estradiol (E_2) concentrations at week 8, there was a significant difference ($P<0.05$) in the levels between groups 3 and 9 (mean ± SD plasma E_2 1,506.33 ± 279.70 pg/mL and 2,204.17 ± 764.33 pg/mL, respectively). Group 12 (mean ± SD plasma E_2 1,151.60 ± 151.66 pg/mL) was also significantly different ($P < 0.05$) from the groups 6 (1,858.83 ± 606.14 pg/mL) and 9 (2,204.17 ± 764.33 pg/mL). However, on weeks 0, 4, and 9, E_2 levels were not significantly different between any group ($P > 0.05$) **(Fig. 2)**.

Plasma testosterone (T) concentrations increased significantly from baseline (week 0) to week 4 in all treatment groups (3, 6, 9, and 12 µg/kg; $P < 0.001$), indicating an initial vitellogenic response to rFsh treatments. From week 4 to week 8, T levels continued to increase significantly in the 6, 9, and 12 µg/kg groups ($P < 0.05$), reaching peak concentrations at week 8. In contrast, the 3 µg/kg group exhibited no significant increase from week 4 to week 8 ($P > 0.05$). From week 8 to week 9, T concentrations decreased significantly in all treatment groups ($P < 0.01$). Between-group comparisons revealed that T levels differed significantly only between the 3 and 9 µg/kg groups at weeks 8 and 9 ($P < 0.05$; **Fig. 3)**.

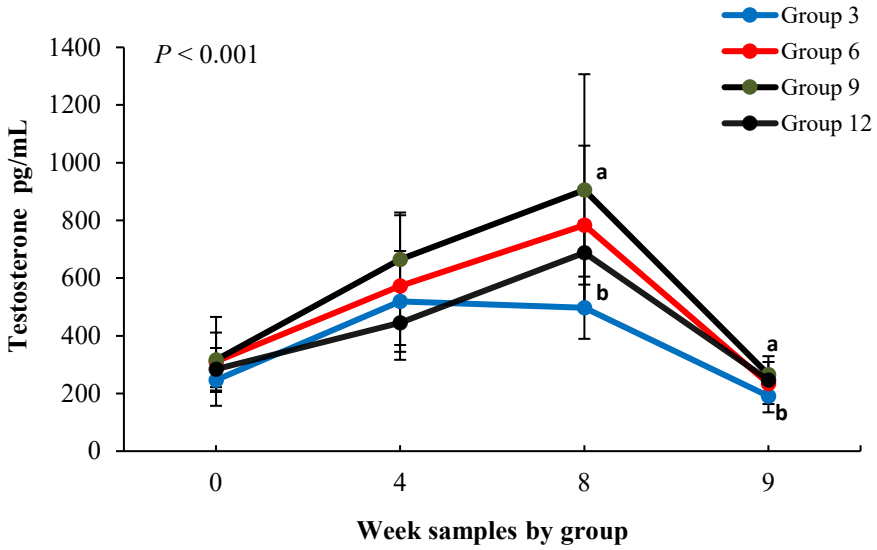


Figure 3. Effect of four rFsh treatments on plasma testosterone levels (mean and SD) in female mullet. The Holm–Sidak test was used to determine a statistical difference in the variables of plasma levels of testosterone (T) between groups. The continuous blue line in the graph indicates rFsh dose at 3 µg/kg ($n = 6$; $n = 5$ week 9), red line: 6 µg/kg ($n = 6$), green line: 9 µg/kg *(*$n = 6$) and black line:12 µg/kg ($n = 6$; $n = 5$ from week 5-9).

3.2. Gonadal regions

In the final samples at week 9, three parts from the gonadal regions (initial, middle, and final) were collected in each fish for all groups (3, 6, 9, and 12). A random selection of 50 - 60 oocytes from each gonadal region was analysed using ImageJ based on histological sections. No statistical differences were found between gonadal regions within each treatment, group 3: $P > 0.954$, group 6: $P > 0.709$, group 9: $P > 0.973$, and group 12: $P > 0.891$ **(Supplementary Figure 1).**

The middle gonadal part (mean and SE) was used to compare differences between treatments at week 9. Significant differences were observed between group 3 (192 ± 7.88 µm) and all other groups (6: 228 ± 5.5 µm, 9: 249 ± 6.2 µm, and 12: 250 ± 7.9 µm) as well as between group 6 (228 ± 5.5 µm) and groups 9 (249 ± 6.2 µm) and 12 (250 ± 7.9 µm). No statistical difference was observed between group 9 and group 12. Different letters indicate statistically significant differences ($P < 0.001$; **Fig. 4**).

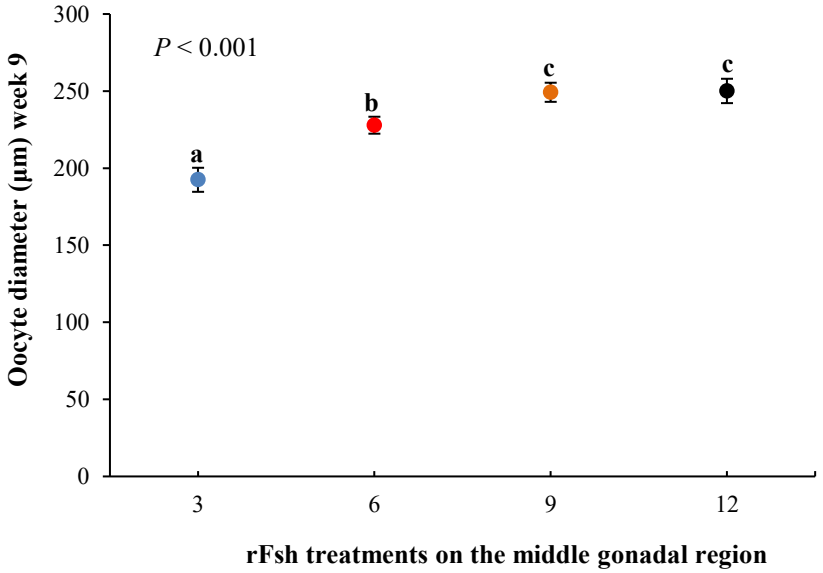


Figure 4. **Average ± SE oocyte diameter on histological sections (50-60 randomly selected) in the gonadal middle regions using ImageJ for each treatment at week 9.** Each colour represents a specific rFsh treatment: blue circles (A): 3 µg/kg (n = 5), red circles (B): 6 µg/kg (n = 6), green circles (C): 9 µg/kg (n = 6), and black circles (D):12 µg/kg (n = 5). Duncan´s post hoc test revealed significant differences between group 3 µg/kg and all other groups, as well as between the 6 µg/kg and 9 µg/kg and 12 µg/kg groups. No statistical difference was observed between the 9 µg/kg and 12 µg/kg groups. Different letters indicate statistically significant differences ($P < 0.001$).

3.3. Histological analysis

Histological analysis was performed for each treatment using four-week samples **(Fig. 5: sections a, b, c, and d).** The perinucleolar phase was observed in all groups prior to rFsh treatment **(Fig. 5, section a), and a** proportions Z-test with Bonferroni correction ($P = 0.008$) showed no statistical differences among the groups ($P > 0.008$).

At week 4 and 6, the proportions test showed a statistically significant difference between group 3 and other groups in the perinuclear and middle vitellogenesis phases **(Fig. 5, sections b and**

c; *P* < 0.008). Similarly, at week 9, a significant difference was observed between group 3 and other groups in the late vitellogenesis phase ($P < 0.008$).

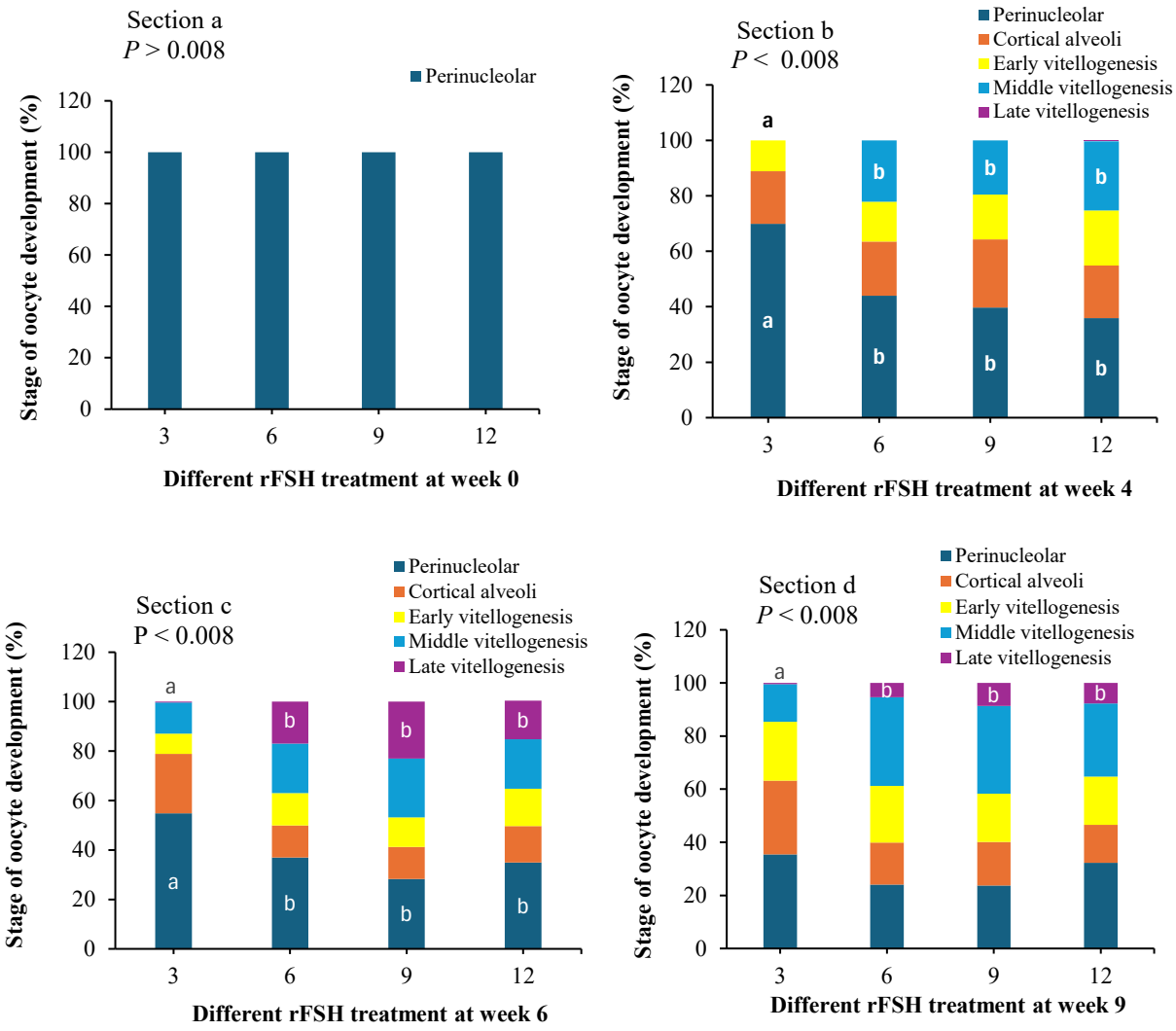


Figure 5. Oocyte development stages identified in histological sections from the mullet females in the four rFsh-treated groups on week 0 (section a), week 4 (section b), week 6 (section c), and week 9 (section d) are represented by average percentage frequency. A total of 100-180 random oocytes were analysed and categorized into distinct stages per female during weeks 0, 4, 6, and 9. Oocytes were extracted via cannulation and fixed in Blouin´s solution for histological analysis in each female, and results were presented as averages per treatment group (weeks 0, 4, 6) and by dissection (week 9 final samples). The classification comprised the following: Perinucleolar, Cortical alveoli, Early vitellogenesis, Middle vitellogenesis, and Late vitellogenesis. phases. A two-sample proportion Z-test with Bonferroni correction ($P = 0.008$) indicates statistical differences (different letters) between rFsh treatments and the stage of oocyte development (%), week 0: $P > 0.008$, week 4: $P < 0.008$, week 6: $P < 0.008$, week 9: $P < 0.008$. The absence of letters indicates no statistical differences.

3.4. Gonadosomatic Index (GSI)

The GSI was calculated in each treatment (Afonso-Dias *et al.*, 2005). At week 9, the gonadosomatic index (GSI) and oocyte diameter (20 most advanced fresh oocyte) reveal a positive correlation, as represented using a second-order polynomial (quadratic) equation: (*Oocyte diameter* = -12.133GSI^2 + 94.493 *GSI* + 182.8; R^2 = 0.96) **(Fig. 6).**

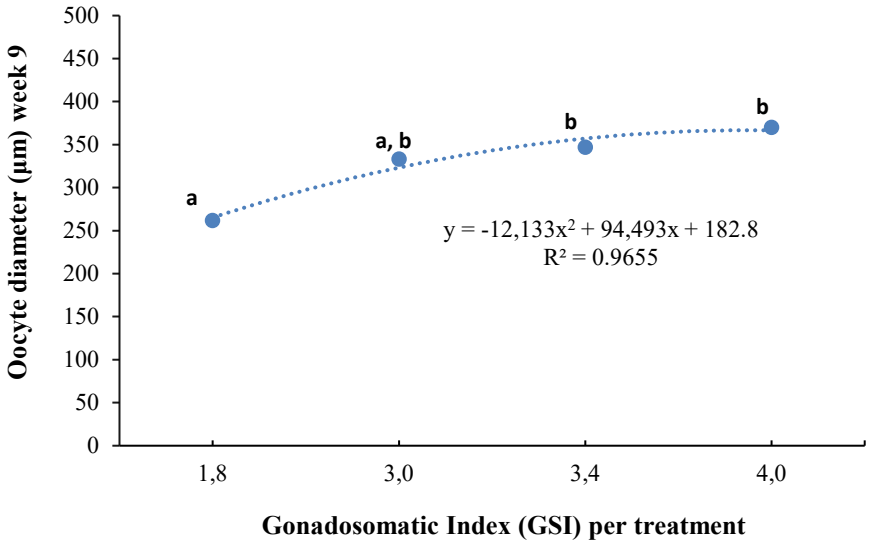


Figure 6. The positive correlation between Gonadosomatic index (GSI) and oocyte diameter (20 most advanced fresh oocytes) was represented using a second-order polynomial (quadratic) equation: *Oocyte diameter* = -12.133GSI^2 + 94.493 *GSI* + 182.8; R^2: 0.96. A positive correlation suggests that a higher GSI is associated with a larger oocyte diameter (more mature), which occurs with increasing rFsh doses. Holm–Sidak post hoc test ($P < 0.05$) indicates statistical differences (different letters) among the 3 (GSI:1.8) with 9 (GSI: 3.4), and 12 (GSI: 4.0) groups.

The average gonadosomatic index (GSI) was calculated for each treatment and analysed statistically. Group 3 showed a GSI of 1.8, which was significantly lower than that of group 9 (GSI: 3.4) and group 12 (GSI: 4.0), but not significantly different from group 6 (GSI: 3.0). The outlier is a female from week 9, treated with 12 µg/kg, showing a fresh oocyte diameter of 76 ± 16.81 µm (average ± SD) and 0.6 of GSI.

3.5. Total fecundity and oocyte density

Total fecundity estimated by the whole mount method showed higher values in groups 6, 9, and 12 compared to group 3 **(Table 1)**. Mean fecundity was similar in group 12 (92,227 ± 67,695

oocytes), group 9 (79,273 ± 53,729 oocytes) and group 6 (80,472 ± 65,007 oocytes), while group 3 showed substantially lower fecundity (25,115 ± 16,004 oocytes) **(Fig. 7)**. Despite the three-fold increase in mean fecundity between group 3 and higher-dose treatments, no statistically significant differences were observed among treatments (ANOVA: $P = 0.24$). A post-hoc power analysis indicated limited statistical power to detect treatment effects ($1-\beta \approx 0.53$), suggesting that the experimental design had a reduced capacity to detect differences among treatments.

Oocyte density analysis showed a similar pattern **(Table 1)**, with group 6 exhibiting the highest mean density (40,976 ± 8,149 oocytes/grams), followed by group 9 (33,320 ± 8,768 oocytes/grams), group 12 (35,761 ± 13,053 oocytes/grams), and group 3 (21,617 ± 13,174 oocytes/grams). However, these differences were not statistically significant ($P > 0.05$) **(Fig. 8)**.

The fish body length had no significant effect on either total fecundity ($P = 0.99$) or oocyte density ($P = 0.14$), indicating that the observed differences in reproductive parameters were primarily attributable to rFsh treatment rather than individual size variation.

Table 1. Total fecundity and oocyte density (average ± SD) by treatment using whole mount method. One-way ANOVA showed no significant differences among treatments, fecundity ($P = 0.249$). Oocyte density showed a trend toward significance ($P = 0.05$). Sample size per group: 3 ($n = 6$; $n = 5$ week 9), 6 ($n = 6$), 9 ($n = 6$) and 12 ($n = 6$; $n = 5$ from week 5-9).

Treatment (µg/kg)	Total fecundity (mean ± SD)	Oocyte density (mean ± SD oocytes /g)	*n*
3	25,115 ± 16,004	21,617 ± 13,174	5
6	80, 472 ± 65,007	40,976 ± 8,149	6
9	79,273 ± 53,729	33,320 ± 8,768	6
12	92,227 ± 67,695	35,761 ± 13,053	5

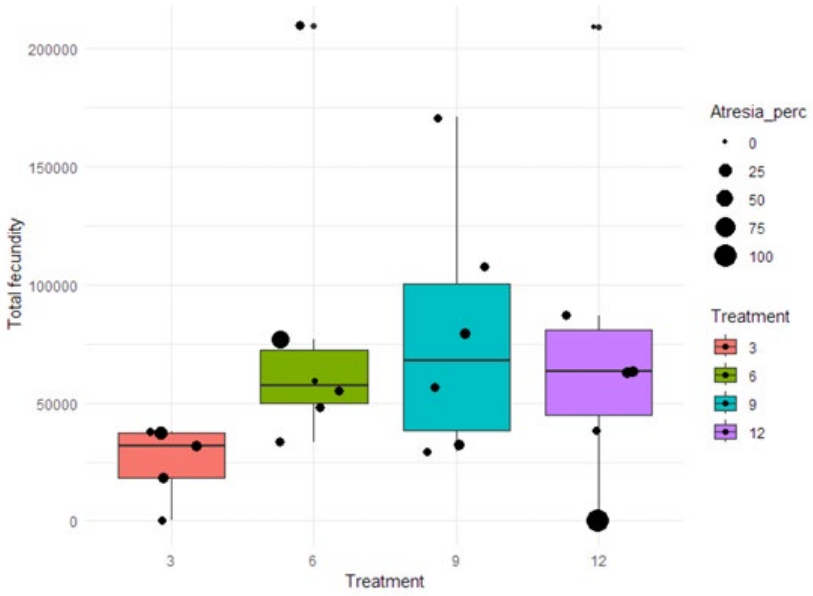


Figure 7. Total fecundity and atresia percentage by rFsh treatment. Boxplots show the distribution of total fecundity (number of oocytes per female) for each treated group (3, 6, 9, and 12 µg/kg rFsh) at week 9. Each colour represents a specific rFsh treatment: orange box: 3 µg/kg (n = 5), green box: 6 µg/kg (n = 6), cyan box: 9 µg/kg (n = 6) and purple box:12 µg/kg (n = 5). Bubble size represents the percentage of atretic oocytes for each individual female. Box boundaries represent the 25th and 75th percentiles, the line inside represents the median, and whiskers extend to 1.5× the interquartile range. One-way ANOVA showed no significant differences among treatments (P = 0.249).

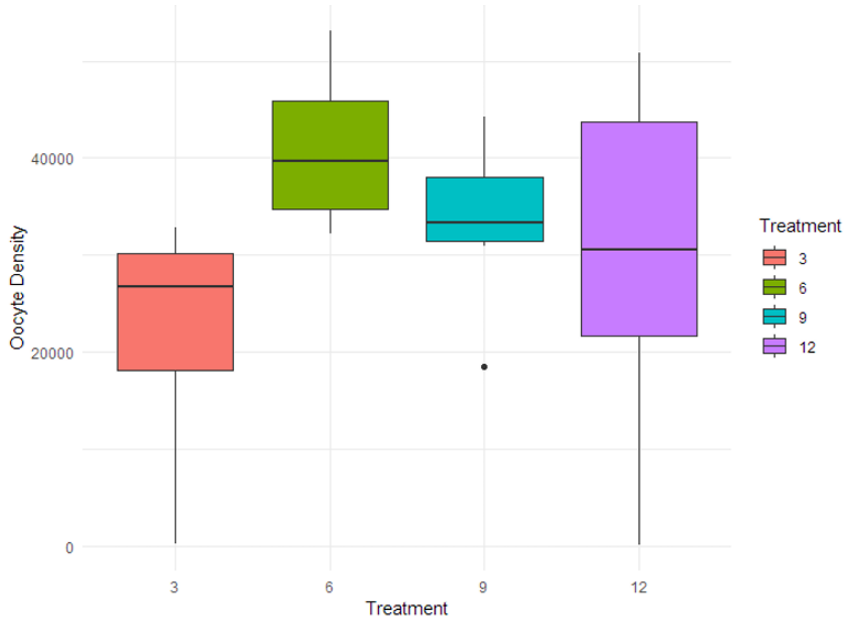


Figure 8. Oocyte density by rFsh treatment. Boxplots showing oocyte density (oocytes/grams of ovarian tissue) for each treated group (3, 6, 9, and 12 µg/kg rFsh) at week 9. Each colour represents a specific rFsh treatment: orange box: 3 µg/kg (n = 5), green box: 6 µg/kg (n = 6), cyan box: 9 µg/kg (n = 6) and purple box:12 µg/kg (n = 5). Box boundaries represent the 25th and 75th percentiles, the horizontal line represents the median, and whiskers extend to 1.5× times the interquartile range. One-way ANOVA showed no significant differences among treatments (P = 0.13).

3.6. Oocyte size distribution

Analysis of oocytes retained in 200 and 300 µm mesh sieves revealed significant differences in mean oocyte diameter among all treatment groups **(Fig. 9),** and all pairwise comparisons were statistically significant ($P < 0.001$). Mean oocyte diameter increased progressively with rFsh dose: group 3 exhibited the smallest oocytes (~300 µm), followed by group 6 (~320 µm), group 9 (~360 µm), and group 12 (~380 µm). Even between the two highest doses, a significant difference persisted (group 12 vs. group 9), confirming a dose-dependent effect of rFsh on oocyte growth across the tested dose range.

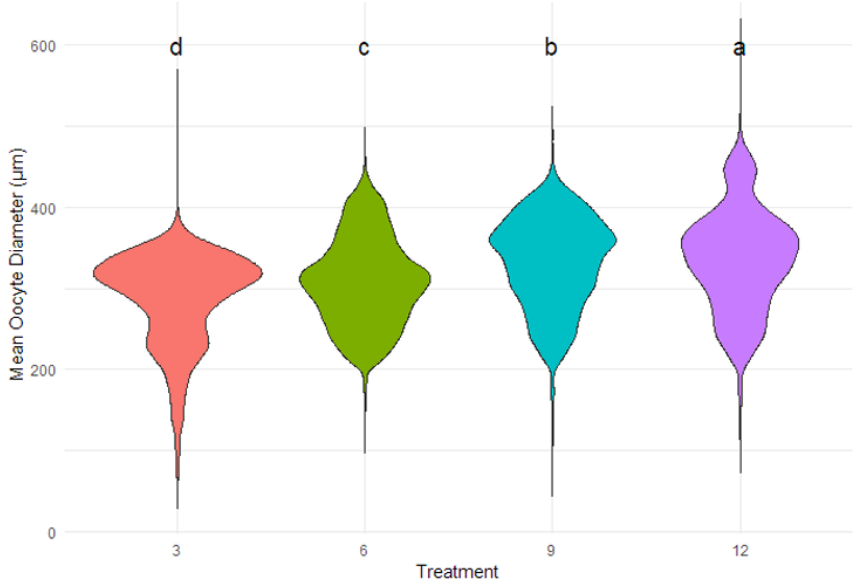


Figure 9. Dose-dependent effect of rFsh on oocyte diameter distribution. Violin plots show the complete distribution of mean oocyte diameter (µm) of oocytes retained in 200 - 300 µm mesh sieves across treatments (3, 6, 9, and 12 µg/kg rFsh) at week 9. The shape and position of each violin illustrate how rFsh dose affects both the central tendency and variability of oocyte diameter. Treatment 3 (d) produced the smallest oocytes with a narrow distribution, while treatment 12 (a) produced the largest oocytes with greater diameter variability. All pairwise comparisons were significant (Tukey's HSD, $P < 0.001$), indicating a dose-dependent stimulation of oocyte growth.

3.7. Ovarian atresia

Stereological analysis of ovarian sections at week 9 revealed differences in oocyte composition among treatments **(Table 2)**. Group 3 showed 36% of oocytes in the cortical alveoli stage, 54% in vitellogenic stages, and 10% atretic oocytes. In contrast, groups treated with higher rFsh doses

(6, 9, and 12 µg/kg) showed more advanced oocyte development, with 76%, 86%, and 85% of oocytes vitellogenic, respectively. Also, lower proportions of cortical alveoli oocytes, 9%, 6%, and 7%, respectively.

Notably, group 6 exhibited the highest atresia rate (15%), significantly higher than group 3 (10%; $P < 0.001$). In contrast, groups 9 and 12 maintained lower atresia levels (8% each), which were not significantly different from group 3. The significantly elevated atresia in group 6, combined with a high fecundity coefficient of variation (81%, **Table 1**), suggests that this dose may represent a threshold at which individual responses diverge, with some females progressing optimally while others experience increased follicular loss.

Table 2. Oocyte developmental stage composition by rFsh treatment. Values represent the percentage of oocytes at each developmental stage determined by stereological analysis at week 9. Atresia data from stereological quantification. Sample size: $n = 5$ for treatment 3 and 12 µg/kg; $n = 6$ for treatments 6 and 9 µg/kg.

Treatment (µg/kg)	Cortical alveoli (%)	Vitellogenic (%)	Atretic (%)	Total (%)
3	36	54	10	100
6	9	76	15	100
9	6	86	8	100
12	7	85	8	100

3.8. Comparison of fecundity estimation methods

Total oocyte counts from stereological analysis of histological sections at week 9 (excluding primary growth oocytes) were as follows: group 3 (636 oocytes), group 6 (604 oocytes), group 9 (677 oocytes), and group 12 (584 oocytes) **(Table 3)**. These counts represent the sum of all secondary growth oocytes (cortical alveoli, Vit 1, Vit 2, and Vit 3) identified across six non-overlapping microscopic fields/ovary.

Stereological counts (584 - 677 oocytes across six histological fields/ovary) were substantially lower than whole mount fecundity estimates at week 9 (25,115 - 92,227 oocytes/female). This reflects the respective scales of sampling of the two techniques (six microscopic fields versus physical disaggregation and counting of a sub-sample of ovarian tissue weighting ~0.09 grams). Both methods are complementary—stereology characterises ovarian composition and quality

(developmental stage distribution and atresia (**Table 2 and 3**), while whole mount quantifies absolute fecundity **(Table 1)**.

Table 3. Stereological oocyte counts by developmental stage and treatment (excluding primary growth oocytes). Values represent total counts across six non-overlapping histological fields/ovary at week 9. Sample size: *n* = 5 for treatments 3 and 12 µg/kg; *n* = 6 for treatments 6 and 9 µg/kg.

Treatment (µg/kg)	Cortical alveoli	Vit 1	Vit 2	Vit 3	Total
3	252	97	138	149	636
6	65	108	217	214	604
9	47	83	252	295	677
12	44	38	128	374	584

4. Discussion

This study, conducted from April 25th to July 9th, 2024, evaluated the effects of four different rFsh doses on ovarian development of female mullet and demonstrated that rFsh influenced vitellogenesis and fecundity. The treatments were administered over a nine-week period, transitioning the females from the early stages of pre-vitellogenesis to the vitellogenesis phase. During the first four weeks, groups 6, 9, and 12 increased oocyte diameters, while group 3 did not exhibit significant changes. Statistical differences in oocyte diameter were observed between group 3 and the other groups from week 4 to week 9. By the end of week 9, groups 6, 9, and 12 had oocytes with significantly larger diameters than group 3. However, one female from group 12 between weeks 6 and 9 was identified as an outlier. This female initially advanced from the pre-nuclear phase (week 0) to the cortical alveoli-early vitellogenesis phase (week 4), but subsequently regressed to the pre-nuclear phase between weeks 6 and 9, suggesting an abnormal pattern of ovarian development.

In intensive captivity environments, particularly in the Mediterranean region, reproductive dysfunctions where mullet development becomes arrested during the primary growth phase of gametogenesis. Therefore, in an intensive aquaculture environment, such as the present study, female mullet required hormonal induction to facilitate critical transitions from pre-vitellogenesis to vitellogenesis, as demonstrated by Ramos-Júdez *et al.* (2021, 2022a, 2022b) and Cerrud-Barría *et al.* (2026). For the first time, this study evaluated different rFsh doses and their impact on vitellogenesis and fecundity, demonstrating their ability to stimulate vitellogenic

progression and oocyte growth during the early perinuclear to late vitellogenesis phase. The rFsh application increases the oocyte diameter by promoting the accumulation of lipid droplets (Dunning *et al.*, 2014; Ramos-Júdez *et al.*, 2021, 2022a, 2022b). The accumulation of lipid droplets has been associated with elevated estradiol levels (Kitano *et al.*, 2022; Ramos-Júdez *et al.*, 2021, 2022a, 2022b). In contrast, our study showed an increase in estradiol levels up to week 8. However, by week 9, estradiol levels decreased in groups 3, 6, and 9, while in group 12, there was remain stable from week 4 to week 9. The decline (group 3, 6, and 9) or no difference (group 12) may be linked to the absence of Lh, as only rFsh was administered, which could have limited the full hormonal regulation required for sustained estradiol production (Filicori, 1999).

This decrease of estradiol levels also could be due to desensitisation of Fsh receptors in the granulosa cells in the ovary after prolonged exposure to rFsh, which impacts estradiol production (Dierich *et al.*, 1998; Schipper *et al.*, 1998) or individual factors that may influence the response to hormonal treatment. These results suggest that week 8 appeared to represent the peak endocrine response to rFsh treatment **(Fig. 4)**, when the estradiol levels were high (group 3, 6 and 9), while higher rFsh doses (group 12) significantly affected estradiol levels.

The study also analysed the effects of rFsh doses on the different regions of the ovary in four treatments. There were no significant differences between the initial, middle, and final sample regions of the ovary. Mullet exhibits a group-synchronous ovarian development pattern, characterised by a dominant cohort of oocytes during the reproductive season and progress in a coordinated manner toward final maturation and spawning, resulting in a relatively homogeneous ovarian developmental stages (Wallace and Selman, 1981). In comparison, tilapia species (Cichlidae) exhibit group-asynchronous ovarian development pattern, characterised by the presence of multiple cohorts of oocytes at different developmental stages, allowing repeated spawning events across the year (Yoshida *et al.*, 2015).

Morphometric analysis of oocyte diameter from the middle gonadal region on histological sections (50-60 oocytes randomly selected/treatment) using ImageJ at week 9 showed significant differences between the lowest dose treatment (3 µg/kg) and higher dose treatments (6, 9, and 12 µg/kg). However, the key finding in this part of the study was the absence of any

difference between groups 9 and 12 in oocyte diameter (249 ± 6.2 µm and 250 ± 7.9 µm, respectively), as well as in the histological analysis of the final sample (development phase of oocyte, week 9). This suggests that a dose of 9 µg/kg (group 9) can result in a high quantity of oocytes during late vitellogenesis without issues, unlike the high doses of rFsh in group 12, which showed an outlier. This result is also supported by the GSI index analysis, in which group 9 shows no significant differences relative to group 12, indicating that dose 9 may achieve the same percentage of reproductive maturity as dose 12, potentially reducing hormone use and associated costs. Furthermore, estradiol and testosterone level also show that group 9 showed the highest mean steroid concentrations, with 2,204.17 ± 764.33 pg/mL of estradiol and 905.29 ± 401.55 pg/mL of testosterone in week 9, compared to the other treatments. The observed increase in testosterone can be attributed to the action of recombinant rFsh, which stimulates its synthesis in the theca cells. Hence, testosterone is subsequently aromatised into estradiol (E_2) by the granulosa cells of the ovarian follicle. In turn, the elevated plasma estradiol levels, promote vitellogenesis in the liver—a process mediated by membrane receptors that recognise and enable the uptake of yolk precursors into the oocytes (Valdebenito, 2008).

The analysis of total fecundity using the whole mount method revealed a complex picture of the rFsh dose-response relationship. Although groups 6, 9, and 12 exhibited nearly three times the mean fecundity (76,000-80,000 oocytes) of group 3 (25,000 oocytes), the large variation among individuals, especially in groups 6 and 12, resulted in no statistically significant difference (P = 0.249). This high variability in fecundity contrasts sharply with the highly significant differences in oocyte diameter across all treatment groups, suggesting that, while rFsh consistently promotes oocyte growth (Lubzens *et al.*, 2010; Reading *et al.*, 2017), its effect on the total number of recruited oocytes is more variable among individuals. Similar inter-individual variability in response to gonadotropin treatments has been reported in other teleost species, including Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2018), and Japanese eel (*Anguilla japonica*) (Kazeto *et al.*, 2023).

The higher fecundity coefficient of variation observed in group 6 (~81%) compared to groups 3, 9, and 12 (64%, 68%, and 73%, respectively) may reflect individual variation in ovarian response to rFsh or different levels of follicular atresia at the time of treatment (Mylonas *et al.*,

2010; Corriero *et al.*, 2021b). Stereological analysis revealed significant differences in follicular atresia among treatments. Group 6 exhibited significantly elevated total atresia (15%, $P < 0.001$) compared to control group 3 (10%) and higher-dose groups 9 and 12 (8% each). This elevated atresia in group 6, combined with high inter-individual variation in fecundity (CV = 81%), suggests that 6 µg/kg represents a threshold dose where individual responses diverge. Notably, analysis of early-stage follicular degeneration showed that alpha atresia decreased progressively with increasing rFsh dose (26 follicles in group 3 to 16 in group 6, 8 in group 9, and 6 follicles in group 12 (Fisher's Exact Test, $P < 0.0001$), indicating a protective effect of higher rFsh doses against early follicular degeneration. Follicular atresia is a normal component of fish oogenesis, involving apoptosis, autophagy, and heterophagy (Corriero *et al.*, 2021b), and can be influenced by environmental factors, stress, and hormonal imbalances (Mylonas *et al.*, 2010; Zohar and Mylonas, 2001; Habibi and Andreu-Vieyra, 2007). Abnormal rates of follicular atresia increase the level of infertility and may cause spawning mortality in wild and cultured fish populations (Corriero *et al.*, 2021a).

The dose-dependent increase in oocyte diameter, confirmed by highly significant differences in all pairwise comparisons ($P < 0.001$), demonstrates that rFsh effectively stimulates vitellogenin uptake and oocyte growth across the dose range tested (Lubzens *et al.*, 2010; Ramos-Júdez *et al.*, 2022a). In teleost fish, Fsh promotes the production of 17β-estradiol through steroidogenic pathways in both Leydig cells and granulosa cells, as both gonadotropin receptors (Fshr and Lhcgr) are expressed in interstitial cells (García-López *et al.*, 2010; Zhang *et al.*, 2015). Then, estradiol acts on hepatic nuclear estrogen receptors to initiate vitellogenin production and its transport to the developing oocyte (Reading *et al.*, 2017). Interestingly, studies in Japanese eel have demonstrated that Fsh-treated groups had higher fecundity than Lh-treated groups, even though differences in steroidogenesis between treatments were minimal (Nyuji *et al.,* 2024), suggesting that mechanisms regulating fecundity in teleosts extend beyond steroid-mediated pathways. However, the observation that oocyte diameter continues to increase significantly even between 9 and 12 µg/kg (difference: 3.8 µm), while fecundity and atresia rates remain similar, suggests that these higher doses primarily enhance individual oocyte quality rather than quantity. This finding aligns with our earlier observation that group 9 achieved GSI values similar to those of group 12 without outliers, supporting the conclusion that 9 µg/kg is the most

effective dose under the conditions of this study, balancing oocyte growth, fecundity, and treatment reliability.

The lack of a significant effect of fish length on fecundity ($P = 0.998$) and on oocyte density ($P = 0.142$) suggested that the observed differences were driven by hormonal treatment, rather than size-dependent differences in reproductive potential. This finding supports the conclusion that the treatment effects were due to hormonal stimulation and that the rFsh protocols can be used across all female size ranges in this study (0.78 ± 0.12 kg), yielding similar results. While body size is often correlated with fecundity in fish (Murua *et al.*, 2003), and our results indicate that within the size range tested, hormonal regulation was the predominant factor determining reproductive output.

Comparing our fecundity results with those of previous studies using different induction protocols highlights the variability in mullet reproduction. Traditional methods using CPE or HCG combined with GnRHa achieved fecundities of approximately 1,395 eggs/grams body weight in semi-intensive systems (Meseda and Samira, 2006), whereas recent studies using rGThs in intensive systems reported fecundities of 694 -1,205 eggs/grams body weight (Ramos-Júdez *et al.*, 2021, 2022b). Converting our whole-mount fecundity data to eggs/grams body weight yields estimates of approximately 32 eggs/grams for group 3 and 103, 102, and 118 eggs/grams for groups 6, 9, and 12, respectively. These values are lower than previously reported, which may reflect methodological differences in fecundity estimation (Hunter *et al*., 1989; Murua *et al.*, 2003), the exclusive use of rFsh without final maturation induction with rLh (Ramos-Júdez *et al.*, 2022b), or the specific developmental stage at which measurements were taken (intermediate to late vitellogenesis rather than post-ovulation). In mullet, Lh levels rise during late-stage gametogenesis to stimulate final maturation, including oocyte maturation and ovulation (Lubzens *et al.*, 2010; Ramos-Júdez *et al.*, 2021), processes that were not induced in the present study. Genetic studies in zebrafish have demonstrated that loss of Fsh significantly retards follicle growth, particularly at the primary growth-previtellogenic transition, while Lh is crucial for final maturation (Chu *et al.*, 2014; Zhang *et al.*, 2015). This suggests that additional oocyte recruitment or further oocyte hydration might occur during Lh-induced final maturation, potentially increasing final fecundity values.

Methodological comparison between whole mount and stereological approaches revealed expected differences in absolute fecundity estimates, with stereological counts (636-677 oocytes counted across histological fields) approximately 25 – 40 times lower than whole mount estimates (25,115 - 92,227 oocytes/female). The large difference is attributable to the fact that stereological sampling estimates total ovarian content from a small number of histological sections (6 fields/individual in our study), whereas the whole-mount method physically counts all oocytes in larger tissue subsamples. While stereological analysis provides valuable information about oocyte developmental stage composition and spatial distribution within the ovary, whole-mount counting is considered more accurate for absolute fecundity estimation (Hunter *et al.*, 1989; Murua *et al.*, 2003).

The choice of size threshold for oocyte inclusion represents another critical methodological consideration. In this study, oocytes <100 μm (primary growth stage) were excluded from fecundity counts following established protocols (Murua *et al.*, 2003). However, as indicated by our stereological data, which show substantial numbers of cortical alveoli oocytes across treatments (44 – 252/sampled fields), the transition from primary to secondary growth is gradual, and threshold selection can significantly influence fecundity estimates.

5. Conclusions

This study demonstrates that rFsh can effectively induce vitellogenesis in mullet under out-of-season captive conditions. The optimal dose that achieved substantial vitellogenic development while minimizing follicular atresia and hormone costs was 9 μg/kg, with no outliers (one female in the 12 μg/kg treatment). Higher doses (9-12 μg/kg) significantly reduced early follicular degeneration (alpha atresia), though group 6 showed elevated total atresia (15%) and high inter-individual variability (CV = 81%), suggesting that this dose represents a threshold at which individual responses diverge. However, fecundity in this study remains a bottleneck in mullet reproduction under intensive captivity, as evidenced by high inter-individual variability and lower fecundity values than in previous studies.



CHAPTER IV.

INFLUENCE OF THREE CONTRASTING ENVIRONMENTS ON THE REPRODUCTIVE DEVELOPMENT OF FLATHEAD GREY MULLET (*MUGIL CEPHALUS*)

1. Introduction

In teleost fish and other vertebrates, the brain-pituitary-gonad (BPG) axis regulates reproduction (Liao, 1975; Rodríguez Gabilondo *et al.*, 2021). The brain controls the release of gonadotropins (Gths), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which act on the gonads to regulate reproductive development (Lubzens *et al*., 2010; Levavi-Sivan *et al.*, 2010). Follicle-stimulating hormone and Lh are important for the reproductive process and are produced by the pituitary gland (Marques *et al.*, 2000; Duncan *et al.*, 2013). This hormone mainly stimulates gametogenesis by promoting the development of oocytes in the early, middle, and late stages. In females, Fsh stimulates the production of 17 β-estradiol (E_2), which acts on hepatic nuclear estrogen receptors to initiate vitellogenin production and transport to the developing oocyte. Luteinizing hormone levels rise during late-stage gametogenesis, stimulating final maturation, such as oocyte maturation, ovulation, and spermiation (Lubzens *et al.*, 2010; Reading *et al.*, 2017). Alterations in the environment affect the BPG axis to modify the release of these two gonadotropins. Under suboptimal environmental conditions, Gth release may be reduced, which can result in reproductive dysfunctions in females during vitellogenesis and in males during spermatogenesis (Zohar and Mylonas, 2001; Mylonas *et al.*, 2010).

The location and environmental conditions play a crucial role in the reproductive cycle (Sukumaran *et al.*, 2021) as well as the maintenance of manageable groups of adult specimens of flathead grey mullet (*Mugil cephalus*) (hereafter referred to as "mullet") in captivity conditions. Over the years, the reproductive control of mullet has been studied across a variety of systems, including earth and rubber-lined ponds (Kuo and Nash, 1973; Liao, 1975; Weber and Lee, 1985; Lee *et al.*, 1987; Lee and Tamaru, 1988; Tamaru *et al.*, 1989; Karim *et al.*, 2016; Besbes *et al.*, 2020), large tanks (Shehadeh *et al.*, 1973a; Shehadeh *et al.*, 1973b; Kuo *et al.*, 1974; Lee and Weber, 1986; Lee *et al.*, 1987; Yousif *et al.*, 2010; Vazirzadeh and Ezhdehakoshpour, 2014; Sukumaran *et al.*, 2021) and more recently, intensive recirculating aquaculture systems (Ramos-Júdez *et al.*, 2021, 2022a, 2022b, 2023).

In this study, three rearing systems—high-density polyethene-lined outdoor ponds (earth pond) with a sand substrate bottom**,** recirculating aquaculture systems (RAS)**,** and biofloc technology (BFT)**—**were selected to evaluate how different environmental conditions influence the

reproductive phase of mullet. Each environmental condition has particular attributes and handling approaches that warrant its use in the experiment.

Polyethylene-lined earth ponds (earth pond) are semi-intensive aquaculture systems that have aspects of a natural environment facilitating reproductive behaviour and reducing stress. These ponds maintain stable water quality, supported by sediment interactions that buffer pH, oxygen, and nitrogen levels. Additionally, the presence of natural food sources such as plankton and benthic organisms improves broodstock nutrition and enhances gonadal development (Kuo and Nash, 1973). Due to their simplicity and low operating costs, these ponds are still widely used for the long-term maintenance of brood fish. However, while semi-natural conditions may support gonadal maturation, the inability to observe spawning events or effectively collect eggs necessitates the transfer of broodstock to more controllable environments. This capture and handling process may induce stress that can arrest reproductive development in the final stages of oocyte maturation, making hormonal induction necessary to complete spawning. In this context, earth pond serves as a baseline system, representing the most natural but least controllable environment for achieving full reproductive control.

Recirculating Aquaculture Systems (RAS) are intensive systems and are designed to optimise space and water use through the continuous recirculation of water via mechanical and biological filtration, often including UV sterilisation. These systems allow for precise environmental control, which is critical for manipulating the reproductive cycle (Losordo *et al.*, 1999; Verdegem *et al.*, 2006). Other features offered by biofilters include ammonia control, oxygen regulation, and temperature control, which provide a more suitable environment for hormone induction. Previous studies by Ramos-Júdez *et al.*, (2021, 2022a, 2022b) have shown that weekly injections of recombinant hormones (rFsh and rLh) for the RAS over 13 weeks can effectively induce vitellogenesis and spermatogenesis, leading to viable gametes and successful spawning. RAS is included in this study due to its demonstrated efficacy in reproductive manipulation under intensive, standardised conditions, which are crucial for scalable, year-round production.

Biofloc Technology (BFT) is a newly emerging environmental concept based on microbial processes for water quality management and environmental impact control. It relies on the

addition of biodegradable carbon sources to stimulate the growth of heterotrophic bacteria, which assimilate nitrogenous waste and convert it into microbial biomass (Vinatea *et al.*, 2018)**.** This biomass serves as an additional food source, reducing the dependency on formulated high-protein feed (Avnimelech, 1999; Hargreaves, 2006). While BFT has been extensively studied for juvenile and grow-out stages (Vinatea *et al.*, 2018), its potential for reproductive control in fish and shellfish remains largely unexplored (Ogello *et al.*, 2021). Its inclusion in this study aims to evaluate whether BFT could serve as a sustainable and cost-effective alternative to RAS and ponds for broodstock management. Furthermore, these systems typically have advanced environmental controls, including pH regulation, foam fractionators, solid clarifiers, carbon dioxide removal, and continuous oxygen supply (De Schryver *et al.*, 2008; Ekasari *et al.*, 2015; Vinatea *et al.*, 2018)

Compared to RAS or BFT, earth pond requires minimal infrastructure and energy input but offers less control over environmental variables. Meanwhile, RAS provides full control but at higher operational costs. BFT may represent a compromise between sustainability and performance, offering some level of water quality control and nutritional benefits without extensive infrastructure. These comparisons are essential to understand how environmental conditions shaped by each environmental effect mullet reproduction. For example, Kuo and Nash (1973) maintained three-year-old mullet in an earth pond until sexual maturity. During the natural spawning season (December to February), the broodstock were transferred to an intensive environment and induced with hormones, successfully producing fertilised eggs. More recently, (Ramos-Júdez *et al.*, 2021, 2022a, 2022b) demonstrated the effectiveness of recombinant hormones to induce maturation in mullet held in RAS. The RAS environment maintained high levels of water quality to ensure high welfare standards for the mullet, and the recombinant hormones were used to synchronise gametogenesis, resulting in fertilisation rates of 63 ± 21% and hatching rates of 58 ± 23%. These results validate the potential of controlled systems and hormone therapies to improve reproductive efficiency and larval output.

Despite its potential, BFT remains under-researched in terms of its impact on fish reproduction, particularly for species like mullet. While preliminary studies have shown promising results in species such as tilapia (*Oreochromis niloticus*) (Avnimelech, 2007; Emerenciano *et al.*, 2012),

further research is necessary to assess its specific effects on adult mullet reproduction. It is crucial to determine whether BFT can match or even surpass the reproductive outcomes typically achieved in more conventional systems, such as RAS or earth pond. As BFT continues to show promise in improving water quality and microbial dynamics, additional studies will be essential to fully understand its potential in optimising reproductive success across various aquaculture systems.

The present study aims to evaluate the influence of three different rearing environments—earth pond, RAS, and BFT—on the reproductive phase of mullet, providing insight into the most effective and sustainable strategies for broodstock management.

2. Materials and methods

2.1. Broodstock

The experiment was conducted from July 10th to October 2nd, 2024. BFT maturation began on May 28th, 2024. The study aimed to investigate the effect of three culture environments—earth ponds, RAS, and BFT—on the reproductive cycle of mullet at three sampling points: initial, mid-point, and final. The experiment involved two different stocks of mullet. The principal stock comprised 196 first-maturation mullet, originating from wild fry introduced into the IRTA facilities in 2020 from Delta Ebro, and the second stock of wild-caught adults from L´Encanyissada, Finca Veta la Palma (Coria del Río), and Wild-Sant Jaume Enveja.

In November 2023, the mullet were divided and placed into two environments: the main stock was divided in two, 98 individuals in a flow-through tank, and 98 individuals in an earth pond treatment. Additionally, the wild-caught adult mullet was maintained in an earth pond treatment. All treatments were exposed to natural environmental conditions (40°37′41.7″N, 0°39′37.9″E), with a mean temperature of 24.43 ± 3.87 °C, dissolved oxygen levels of 5.69 ± 2.62 mg/L, a pH of 7.93 ± 0.15, and salinity of 36.8 ± 1.46 ‰.

At the start of the experiment on July 10th, 2024, from the 98 fish in the flow-through tank, 36 individuals were allocated to the RAS system, and 36 to the BFT system. The 98 fish in the earth pond remained in the same treatment. Additionally, all systems included 8 wild-caught adult mullet (4 males and 4 females). Each treatment comprised two replicates, with each replicate consisting of 18 first-maturation individuals and 4 wild-caught adults (2 females and 2 males sourced from the earth pond). Thus, each treatment had a total of 44 individuals (22 individuals/replicate). The average weight and standard length of first-maturation individuals were: 805.00 ± 80.88 grams and 39.86 ± 1.49 cm in the earth pond, 809.00 ± 84.45 grams and 37.73 ± 2.17 cm in the RAS, and 811.00 ± 86.76 grams and 37.86 ± 1.35 cm in the BFT. Similarly, the average weight and standard length of wild-caught adults were 1758.63 ± 485.47 grams and 49.50 ± 4.43 cm in the earth pond, 1556.25 ± 515.53 grams and 48.91 ± 5.54 cm in the RAS, and 1550.88 ± 456.70 grams and 51.50 ± 4.12 cm in the BFT treatment. To identify the fish in the experiment, each mullet was tagged intramuscularly with a Passive Integrated Transponder (PIT) tag (Trovan®, ZEUS Euroinversiones S.L. Madrid, Spain). The fish were fed at 1.5% of the biomass per day, split into two meals, with a commercial Repro Sole diet (5 mm) (Sparos, Portugal). The diet contained 60% protein, 13% fat, 0.5% fibre, 7% ash, 1.2% phosphorus, and 0.9% calcium.

2.2. Three different environments

Earth pond: The 44 selected breeders were housed in two earth pond, each measuring 22 m x 7 m with a depth of 1 m and maintained under natural photoperiod conditions. These ponds were lined with 1.5 mm thick polyethylene (PE) sheets, incorporating both inlet and outlet connections within the liner. Each pond was designed with a gentle slope towards the drainage point and had a bottom layer consisting of 15-20 cm of sand.

The natural photoperiod followed seasonal daylight changes, starting from LD 14:10 (L: light; D: dark) on July 10th, 2024 (initial sample), and gradually decreasing to LD 11:13 by October 2nd, 2024 (final sample). Salinity levels were 36.8 ± 1.46 ‰, and the average water temperature from July to October was 25.6 ± 2.30 °C (range: 21.2 °C to 28.8 °C) **(Fig. 1)**. The water flow

rate was 3,967 L/h in each earth pond. Additional water quality parameters were monitored, including dissolved oxygen.

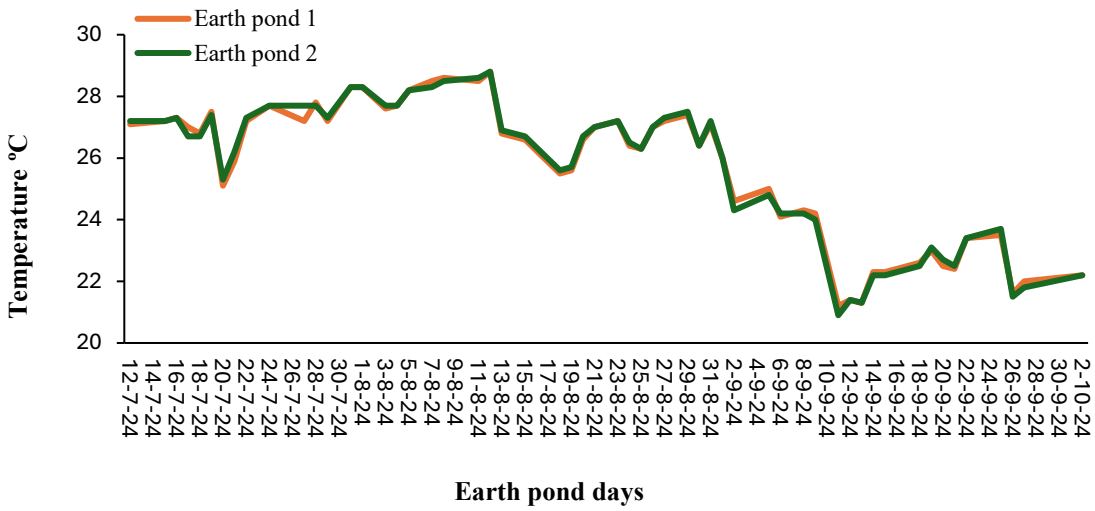


Figure 1. Temperature variation (°C) in two earth pond (pond 1 and pond 2) over an 80-day period, from July 12th, 2024, to October 2nd, 2024. The orange and green lines represent earth pond 1 and earth pond 2.

Recirculation aquaculture systems (RAS) IRTAmar®: the 44 selected breeders were placed in two 10 m3 rectangular fibreglass tanks (6.1 x 2.1 x 1.2 m) under a natural photoperiod. The tanks were equipped with both surface and bottom water outlets, each independently adjustable to regulate the volume of water discharged from each level, ensuring optimal flow dynamics and solid waste removal. The dissolved oxygen (DO) levels in the tanks were continuously monitored in real time using sensors. In the case of a critical drop in oxygen levels, emergency diffusers were automatically activated, and a warning was sent to the staff via SMS. The total water flow rate was 6,000 L/h, with an external water renewal rate of 10%, equivalent to 600 L/h of new water input.

The natural photoperiod followed seasonal daylight changes, decreasing from LD 14:10 on July 10th, 2024 (initial sampling) to LD 11:13 on October 2nd, 2024 (final sampling). a salinity of 36.8 ± 1.46 ‰ and the average temperature from July to October was 26.81 ± 2.06 °C (min: 23.2 °C, max: 29.6 °C) **(Fig 2.)**. Other water quality parameters were recorded for the RAS: ammonium, nitrite, pH, and dissolved oxygen concentration.

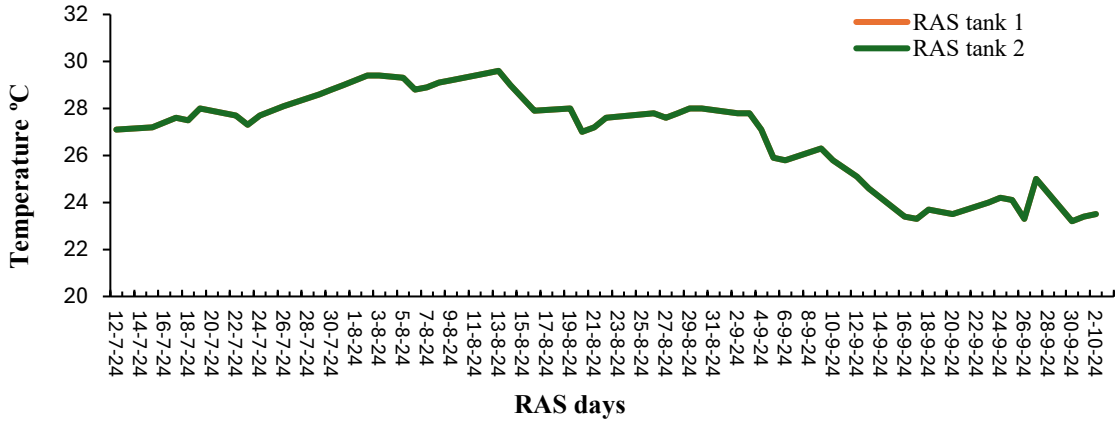


Figure 2. **Temperature variation (°C) in two RAS (tank 1 and tank 2) over an 80-day period, from July 12th, 2024, to October 2nd, 2024.** The orange line representing RAS tank 1 overlaps the green line for tank 2, indicating that both tanks had nearly identical temperatures throughout the period.

Biofloc technology (BFT): the 44 selected breeders were placed in two 10 m^3 rectangular fibreglass tank (6.1 x 2.1 x 1.2 m) with a natural photoperiod. Biofloc formation resulted from food inputs and limited water exchange in the tanks, which encouraged the proliferation of microorganisms and the development of biofilm. The activity of heterotrophic bacteria was enhanced by the daily addition of anhydrous glucose with 46% organic carbon. The amount of carbohydrate required was calculated following the C: N ratio of 20:1, as suggested by Avnimelech (1999). In instances where total ammonium levels surpassed 5 mg/L, 50% of the tank water was replaced. When total solids concentration exceeded 500 mg/L, clarifiers with a capacity equal to that of the culture tanks were activated using an air-lift system, following the recommendations of Luo *et al.* (2014) for tilapia BFT treatment.

The natural photoperiod followed seasonal daylight changes, decreasing from LD 14:10 on July 10th, 2024 (initial sampling) to LD 13:11 on August 21st, 2024 (mid-point sampling). The BFT treatment was initiated 43 days (On May 28th, 2024 - BFT maturation phase) prior to the start of the experiment (July 10th, 2024). During the June to August period at IRTA La Ràpita, Spain, the average temperature was 27.49 ± 2.57 °C (ranging from 21.8 °C to 30.1 °C) **(Fig. 3)**, and the salinity averaged 33.84 ± 4.23 ‰ (ranging from 25.71 ‰ to 42.98 ‰). Other water quality

parameters were recorded specifically for the BFT: dissolved oxygen, alkalinity, pH, ammonium, and nitrite levels.

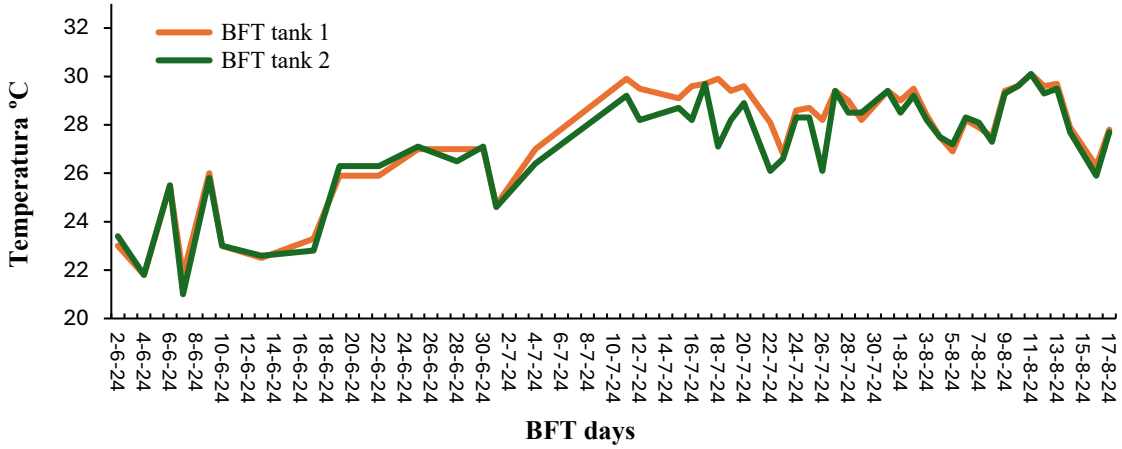


Figure 3. Temperature variation (°C) in two BFT over an 80-day period, from June 2nd, 2024, to August 17th, 2024. The green and orange lines represent tank 1 and tank 2.

2.3. Assessment of reproductive stage

To evaluate the reproductive status of the first-maturation individuals, three sampling points were established. The initial sampling was conducted at the start of the experiment and included fish from the flow-through and earth-pond treatments. This was followed by two subsequent samplings-mid-point and final- carried out after fish were assigned to their respective treatments (RAS, BFT, and earth pond) **(Fig. 4)**.

Initial Sample – July 10th, 2024:

At the start of the experiment, twelve fish were randomly selected and sacrificed from flow-through (before being distributed to RAS and biofloc) and earth pond treatments. These fish were randomly selected in addition to the 44 individuals assigned to each treatment. Data collected included body weight, total length, gonadosomatic index (GSI), fresh gamete characterisation, blood samples for steroid and biochemical analyses, and gonads preserved for histological examination.

Mid-point Sample – August 21st, 2024:

Twelve fish from RAS and earth pond were randomly selected and sacrificed. For the BFT, all but one of the 44 fish died in the days before the fish were sampled. One replica could not be sampled. In the second replica, the fish had died more recently, and one live male, four dead males, and five dead females were examined. The same parameters recorded during the initial sampling were collected: body weight, total length, GSI, fresh gamete characterisation, blood samples for steroid and biochemical analyses, and gonads fixed for histology.

Final Sample – October 2nd, 2024:

Twenty-four mullet were sampled in each treatment, with data collected on body weight, total length, fresh gamete characterisation, blood samples for steroid and biochemical analyses, and fresh gametes preserved for histological analysis.

Gamete evaluation procedures

In females, gamete samples were obtained using a 1.67 mm (5 ch) × 500 mm plastic cannula to extract ovarian fluid. The diameters of the 20 most advanced oocytes were measured under a Zeiss Axiostar microscope at 40X magnification. In males, spermiation was assessed by applying gentle abdominal pressure to check for the presence or absence of sperm.

Wild-caught adults were evaluated for weight and length at the beginning of the experiment. At the mid-point sampling, only one female and one male which had died in the BFT tank were assessed for fresh gametes and GSI. During the final sampling, gamete samples were collected from females using a cannula and from males via abdominal massage. Blood samples for steroid and biochemical analyses, as well as gonad fixation for histological analysis, were not performed at any sampling point, and no fish were sacrificed.

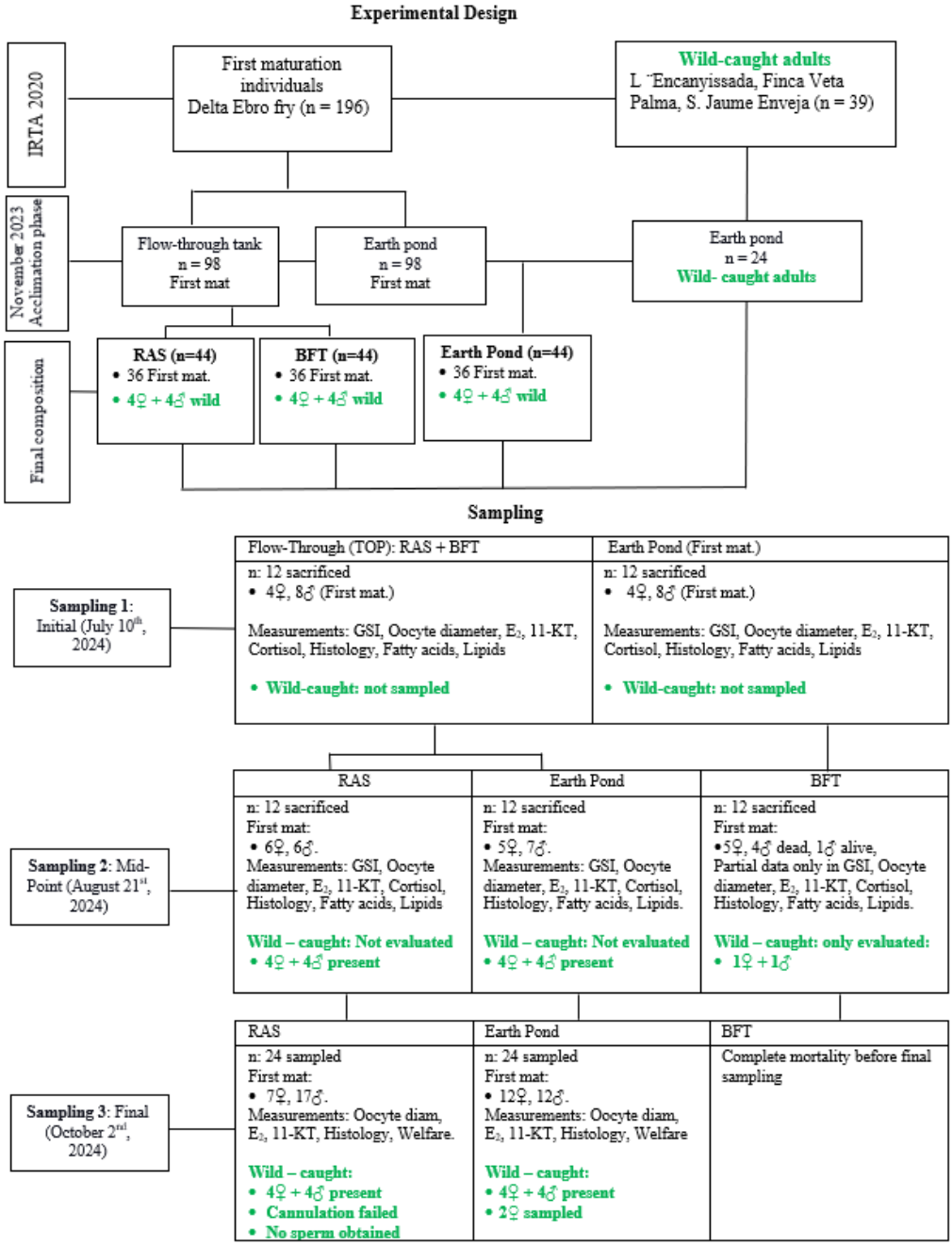


Figure 4. Experimental design and sampling timeline for reproductive development evaluation in mullet under three aquaculture systems. Fish originated from two groups: first-maturation individuals (*n* =196, Delta Ebro fry) and wild-caught adults (L'Encanyissada, Finca Veta la Palma, Sant Jaume Enveja). In November 2023, first-maturation fish were distributed into flow-through tank (*n* = 98) and earth pond (*n*=98 plus wild-caught adults). On July 10th, 2024, flow-through fish were redistributed into RAS (*n* = 36) and BFT (*n* = 36), while earth pond fish remained in situ (*n* = 98). Wild-caught adults from earth pond were distributed across all three treatments (4♀+4♂ per treatment). Each treatment comprised two replicates of 22 fish (18 first-maturation + 4 wild-caught). Three sampling events occurred: initial (July 10th), mid-point (August 21st: After 6 weeks in treatments), and final (October 2nd After 12 weeks in treatments – Not sacrificed). At mid-point, *Amyloodinium* sp. caused high mortality in BFT. By final sampling, all BFT fish had died; wild-caught adults in BFT were not sampled at final time point due to complete prior mortality.

2.4. Histological analysis

Oocyte and testis samples collected at the initial, mid-point, and final sampling periods were fixed in Bouin´s solution and dehydrated using a series of graded ethanol baths. The samples were embedded in paraffin and cut into 3 μm sections that were dried and stained with Mayer-Bennett hematoxylin and floxina/eosina (Casa Álvarez, Spain). Finally, the slides were examined under a light microscope (Leica DMLB, Houston, USA) at magnifications of 600x and 1000x to analyse the oocyte and testes developmental stage, measurements of diameter, and image capture.

Oocytes were classified according to Ramos-Júdez *et al.* (2021), based on both oocyte diameter (measured using ImageJ) and cellular morphology. Briefly, oocytes were categorized by the following size and cellular characteristics:

- Early perinucleolar (27-70 μm): characterised by a single nucleolus in the germinal vesicle.
- Late perinucleolar (70-110 μm): multiple nucleoli in the germinal vesicle.
- Cortical alveoli (110-203 μm): presence of granular vesicles and small oil droplets in the peripheral ooplasm.
- Early vitellogenesis (203-360 μm): initial presence of yolk granules in the ooplasm.
- Mid-vitellogenesis (361-510 μm): progressive accumulation of yolk granules throughout the cytoplasm.
- Late vitellogenesis (>510 μm): cytoplasm full of yolk granules.
- Oocyte maturation stage (OM): nucleus was situated to one side of the oocyte, and oil droplets were coalescing; these characteristics indicated the beginning of germinal vesicle migration (GVM).
- Atretic oocytes: hypertrophic oocytes exhibiting signs of structural disintegration.

The stage of maturation of females was ascertained based on the presence of the most developed stage of oocytes. The percentage of oocytes in different phases within the ovaries was determined by selecting and classifying at least fifty oocytes at random from each female. In

males, testis development was histologically classified in SPG (spermatogonia), SPC (spermatocyte), SPD (spermatid), and SPZ (spermatozoa).

2.5. Plasma steroid analysis

Blood samples were collected at three sampling time points (initial, mid-point, and final). Samples were kept on ice and then centrifuged at 3000 rpm at 4 °C for 15 minutes. Plasma was collected into an Eppendorf tube and stored at -80 °C until analysis. Plasma levels of 17β-estradiol (E_2), 11-ketotestosterone (11-KT), and cortisol were analysed using commercially available enzyme immunoassays (EIA) (Cayman Chemical Company, USA). Steroids were extracted with methanol, which was evaporated, and extracts were re-suspended 1:2 (E_2) and 1:50 (11-KT) in the EIA buffer (Ramos-Júdez *et al.*, 2022b).

2.6. Gonadosomatic Index (GSI)

Initial and mid-point samples were taken from the sacrificed fish. Total body weight and length were recorded. During dissection, the ovaries of each female were carefully removed and weighed. The GSI was calculated as follows (Afonso-Dias *et al.*, 2005).

GSI= Gonad weight (GW) x 100/ Body weight (BW)

2.7. Mullet welfare

Mullet welfare was evaluated at both the mid-point and final sampling phases in fish reared under Recirculating Aquaculture Systems (RAS) and earth pond conditions. The protocol followed the model proposed in the welfare quality project (Canali and Keeling, 2009; Reenen et *al.*, 2024), which considers four welfare domains. The optimal welfare indicators (OWIs) selected for mullet were based on MedAid deliverable 4.5 (Roque *et al.*, 2020).

The assessment included qualitative variables to evaluate key welfare principles, including eye injuries, skin lesions, fin damage, overall body colouration, and the presence of deformities, condition index for the good food principle, and parameters of water quality were recorded (see section 2.1: 3 paragraph) for the good housing principle. For this, each fish was given a score of

0-2 for each parameter mentioned based on a simple ordinal scale: a score of 0 was assigned to animals with no visible lesions, 1 to those with a single lesion, and 2 to individuals exhibiting two lesions within the same variable.

Blood plasma parameters related to welfare were measured at the mid- and final points using an Abbott i-STAT Handheld Blood Analyser (supplier details). The parameters measured included sodium (Na^+), potassium (K^+), chloride (Cl^-), total carbon dioxide (TCO_2), blood urea nitrogen (BUN), creatinine (Crea), glucose (Glu), ionized calcium (iCa), anion gap (AnGap), hematocrit (Hct), hemoglobin (Hb), pH, partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3^-), base excess (BE), oxygen saturation (sO_2), total carbon dioxide (TCO_2), and lactate (Lac).

2.8. Lipid content and fatty acid analysis

Total lipids were extracted from the samples by homogenisation in chloroform/methanol (2:1, v/v) following the method of Folch *et al.*, 1957, using a double extraction procedure. Lipid content was quantified gravimetrically after evaporation of the solvent under nitrogen flow, followed by overnight vacuum desiccation. Extracted lipids were stored in chloroform: methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) at −20 °C until fatty acid transmethylation.

Fatty acid methyl esters were prepared following the acid-catalyzed transmethylation method described 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 internal diameter; Thermo Scientific, Spain). The analysis employed a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C/min and holding at 250 °C after ramping at 2 °C/min. Helium (1.2 mL/min constant flow rate) was used as the carrier gas, and on-column injection and flame ionisation detection at 250 °C were used. Peaks of each fatty acid were

identified and quantified according to the response to the internal standard, 21:0 fatty acid (Sigma-Aldrich, Spain, Ref. H5149), added before transmethylation.

To determine the percentage of total lipids, approximately 200 mg of each sample was weighed to the nearest 0.01 mg (KERN & SOHN GmbH, Germany) before and after drying at 100 °C (Memmert, Germany) for 24 h. Moisture content was calculated to determine the dry weight of samples. Total lipid content was expressed as a percentage by dividing the total lipid weight by the sample dry weight and multiplying by 100. An estimation of the total fatty acids accumulated in the entire gonad was calculated by multiplying the fatty acid concentration (mg per mg of wet tissue) by the total fresh gonad weight (mg).

2.9. Ethics.

This research was carried out in accordance with the following regulations: Spanish Royal Decree 53/2013 of February 1st, concerning the protection of animals used for scientific purposes; Catalan Law 5/1995 of June 21st, which pertains to safeguarding animals used in scientific research; and Catalan Decree 214/1997 of July 30th, which regulates the use of such animals. All procedures were reviewed and approved by the Catalan Government and the Committee of Ethics and Experimental Animals (CEEA) of the Institute of Agrifood Research and Technology (IRTA). The study was authorized under experimental project number 12016, with expedient number FUE-2023-02998468 and project ID NT0G72MXQ. ARRIVE guidelines were adhered to by the authors.

3. Statistical analysis

Data were expressed as the mean ± standard deviation. Shapiro–Wilk and Levene tests were used to check the normality of data distribution and homogeneity of variance, respectively. The proportions Z-test with Bonferroni correction ($P = 0.008$) was used to compare the proportions of oocytes between treatments across the different development phases (perinucleolar, cortical alveoli, early vitellogenesis, middle vitellogenesis, and late vitellogenesis) identified through histological analysis and quantified using ImageJ. A two-way repeated-measures ANOVA (RM

ANOVA), followed by pairwise comparisons using the Holm–Sidak test, was used to assess differences in oocyte diameter between rearing systems (RAS vs. earth pond) and sampling times (initial, mid-point, and final). The same analysis was applied to evaluate differences in plasma levels of 17β-estradiol (E_2), 11-KT, and cortisol between the two rearing treatments and across sampling times.

A two-way repeated measures ANOVA (RM ANOVA), followed by pairwise comparisons using the Holm–Sidak test, was used to assess statistical differences in sodium, glucose, lactate levels, and condition index between environments (RAS vs. earth pond) in the same phase and between sampling phases (mid-point vs. final) for each sex. The same analysis was applied to evaluate differences in GSI % between the two-rearing treatments in initial and mid-point. Chi-square test (χ^2) was conducted to compare proportions between and within sampling events in the RAS and earth pond treatments. Analyses were restricted to these treatments due to mortality in the BFT group.

4. Results

4.1. Samples

In this experiment, sampling was conducted on the reproductive development at three points—initial, mid-point, and final—across different treatments in two mullet groups (first-maturation individuals and the wild-caught adults).

4.1.1. The first maturation individuals

At the start of the experiment on July 10th, 2024 (initial sample), the average weight and standard length of the first-maturation individuals (36 fish/treatment) was 805 ± 80.88 grams and 39.86 ± 1.49 cm in the earth pond, 809 ± 84.45 grams and 37.73 ± 2.17 cm for the RAS, and 811 ± 86.76 grams and 37.86 ± 1.35 cm in the BFT, with no significant differences between groups.

During the initial sampling conducted on July 10th, 2024, twelve fish were sacrificed from the flow-through tank (representing RAS and BFT conditions) and twelve from the earth pond. The

gonadosomatic index (GSI) of first-maturation females showed no significant differences between the flow-through tank (0.58%, n = 4) and the earth pond (0.624%, n = 6) **(Fig. 5)**. Similarly, no significant differences in male GSI were observed among treatments, with values of 0.256% (n = 8) in the flow-through tank and 0.052% (n = 6) in the earth pond **(Fig. 6)**.

At the mid-point, August 21st, 12 individuals from both the RAS and earth pond treatments were sacrificed for evaluation. However, high mortality in the BFT due to *Amyloodinium* sp. infection limited data collection, and only one BFT tank was evaluated. For the BFT, four dead males, one live male, and five dead females were examined. The GSI in females did not show any significant differences between BFT (GSI% = 0.65, n = 5), RAS (GSI% = 0.52, n = 6), and earth pond (GSI% = 0.52, n = 5) **(Fig. 5)**. Similarly, the GSI of males did not differ significantly between treatment, with BFT (males dead GSI% = 0.326 and males live GSI% = 0.105; n = 5), RAS (GSI% = 0.089, n = 6), and earth pond (GSI% = 0.098, n = 7) **(Fig. 6)**. The high mortality observed in the BFT was linked to an outbreak of *Amyloodinium* sp., a parasitic dinoflagellate responsible for velvet disease. The surviving mullet (n = 1 male) was heavily infected, with 107 *Amyloodinium* sp. organisms observed on a single gill filament **(Fig. 7)**. The earth pond was also affected by this parasite; however, in this case, the parasite was effectively controlled through three treatments of hydrogen peroxide at 150 ppm, administered every three days.

At the final sampling on October 2nd, no fish were sacrificed, and only oocyte diameter, steroids, and histological analyses were realized on 24 fish from each treatment.

The oocyte size, at the start of the experiment, 100% of the females from both the tank (flow-through tank) and the earth pond treatments were in the previtellogenic stage, corresponding to the primary growth phase of ovarian development **(Fig. 8, initial)**. There were no significant differences in mean oocyte diameter between treatments at any of the sampling points — initial, mid-point, and final. Specifically, the mean oocyte diameters were as follows: initial sample: flow-through tank (n = 4) = 90.47 ± 15.47 µm, earth pond (n = 6) = 87.16 ± 14.48 µm; mid-point samples: RAS (n = 6) = 90.3 ± 14.59 µm, earth pond (n = 5) =101.16 ± 28.72 µm); BFT (n = 2 of 5) = 104.58 ± 20.63 µm and final samples: RAS (n = 7) =104 ± 14.15 µm, earth pond (n = 8 of 12) =102.03 ± 17.72 µm) **(Fig. 9)**.

However, when comparing sampling points within the same treatment, a small but statistically significant increase in oocyte diameter was observed in both the mid-point and final samples compared to the initial samples. In the RAS **(Fig. 9)** a significant increase was found between the initial- flow-through tank (90.47 ± 15.47 µm) and final-RAS (104 ± 14.15 µm) samples ($P < 0.001$). Whereas in the earth pond **(Fig. 9)**, statistically significant increases ($P < 0.001$) were observed across the initial, mid-point, and final samples (87.16 ± 14.48 µm; 101.16 ± 28.72 µm; and 102.03 ± 17.72 µm, respectively). Oocyte diameter data obtained from a mid-point sample of two deceased fish in the BFT (104.58 ± 20.63 µm) were excluded from intergroup comparisons. Histological analysis in reared females revealed no differences in oocyte developmental stage, as all were in the previtellogenic phase across the initial, mid-point, and final samples of the experiment **(Fig. 8: initial, mid-point, and final).**

Steroid levels were absent across all treatments, and there was no increase in oocyte diameter in females. No statistically significant differences in E_2 were observed between earth pond and RAS over time. However, within the earth pond, a decrease in E_2 was observed between the initial and final samples **($P = 0.018$) (Fig. 10)**. In the RAS, E_2 concentrations showed values of 1.00 ± 0.46 ng/mL in the initial samples-flow-through tank ($n = 4$) 0.32 ± 0.07 ng/mL in the mid-point samples-RAS ($n = 6$), and 0.43 ± 0.40 ng/mL in the final samples-RAS ($n = 7$). In earth pond, the E_2 levels were 0.93 ± 0.32 ng/mL in the initial samples ($n = 6$), 0.49 ± 0.38 ng/mL in the mid-point samples ($n = 5$), and 0.32 ± 0.16 ng/mL in the final samples (n =10 of 12) **(Fig. 10)**. Two samples from the final sampling of the earth pond treatment (one replicate) were excluded due to estradiol concentrations of 7.4 and 4.2 ng/mL, which was considered outlier relative to the other individuals in the group. Although both samples showed elevated estradiol (E_2) levels, histological analyses revealed immature oocytes at the previtellogenic stage.

The hormone level of 11-KT in males did not show statistically significant differences between earth pond and RAS over time. However, within the RAS, small significant increases in 11-KT were observed between the initial-flow through tank and mid-point-RAS **($P = 0.042$)**, followed by a significant decrease between the mid-point-RAS and final samples-RAS **($P < 0.001$) (Fig. 11)**. The hormone level of 11-KT for the RAS, showed values of 0.60 ± 0.18 ng/mL in the initial

samples-flow-through tank (n = 7 of 8), 1.45 ± 0.64 ng/mL in the mid-point-RAS (n = 6), and 0.34 ± 0.29 ng/mL in the final samples-RAS (n = 17). One initial sample from the flow-through tank was excluded from the analysis due to an 11-KT level of 2.10 ng/mL, which was considered an outlier relative to the other individuals in the group. In earth pond, the 11-KT levels were 0.39 ± 0.23 ng/mL in the initial samples (n = 6), 0.36 ± 0.30 ng/mL in the mid-point (n = 7), and 0.12 ± 0.11 ng/mL in the final samples (n = 12) **(Fig. 11)**. The BFT includes data from a single male fish, with an 11-KT concentration of 0.02 ng/mL. Histological analysis of males revealed consistent developmental stages—characterized by the presence of spermatogonia (SPG)—across the initial, mid-point, and final sampling in all treatments. Additionally, no sperm was detected through abdominal massage in any environment at any sampling point.

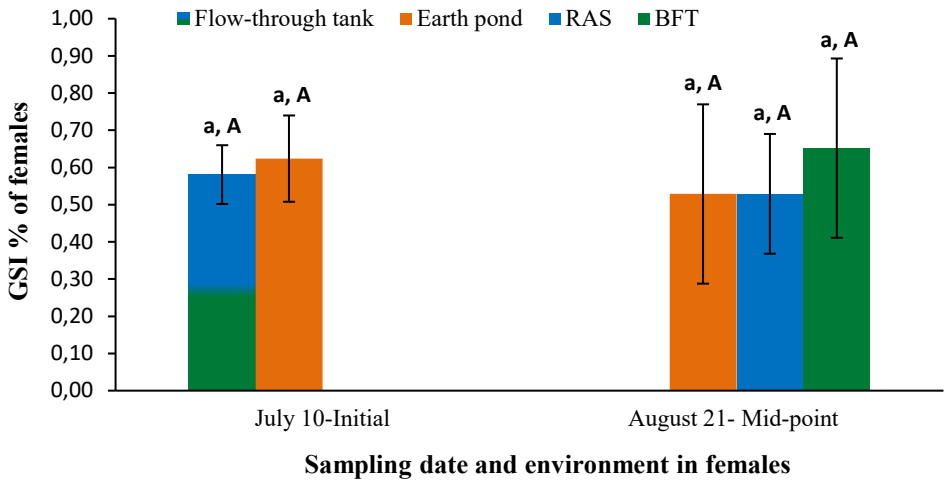


Figure 5. Mean GSI (%) of females from each treatment group. Females were randomly selected and sacrificed, including both the initial (RAS-BFT: flow-through tank-n = 4 and earth pond-n = 6) and mid-point samplings (RAS-n = 6; BFT-n = 5 and earth pond-n = 5). The BFT was represented by five dead females. The Holm–Sidak test showed no statistically significant differences between treatments or within the same treatment (BFT, RAS, and earth pond). The flow-through tank at the initial point represented both RAS and BFT. Green and blue bars represent flow-through tank, orange bars represent earth pond, blue bar represents RAS, and green bar represents the BFT. Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

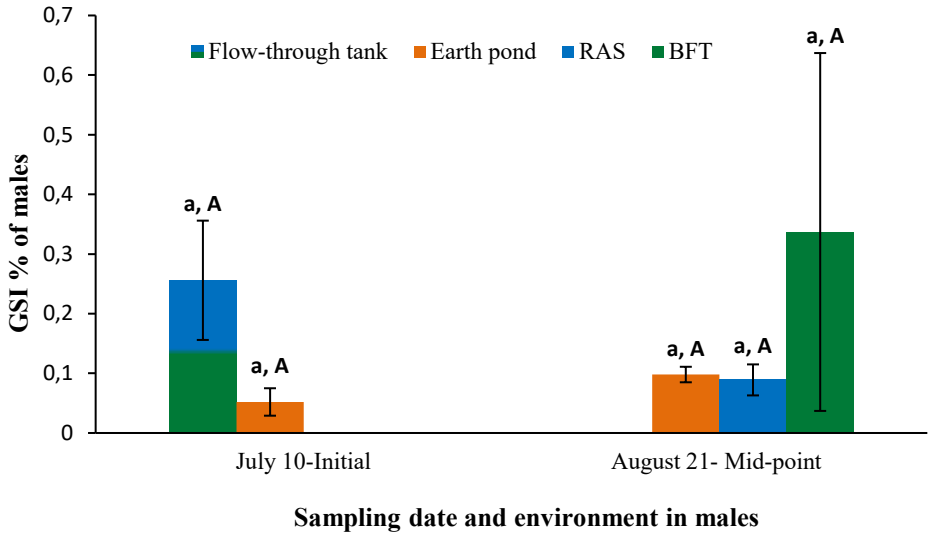


Figure 6. GSI (%) in males from each treatment that were randomly selected and sacrificed, including both the initial (RAS-BFT: flow-through tank-*n* = 8 and earth pond-*n* = 6) and mid-point samplings (RAS-*n* = 6, BFT-*n* = 5, and earth pond-*n* = 7). The BFT was represented by four dead males, one live male. Holm–Sidak test showed no statistically significant differences between BFT, RAS, and earth pond treatment. The flow-through tank at the initial point represented both RAS and BFT. Green and blue bars represent flow-through tank, orange bars represent earth pond, blue bar represents RAS, and green bar represents the BFT. Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

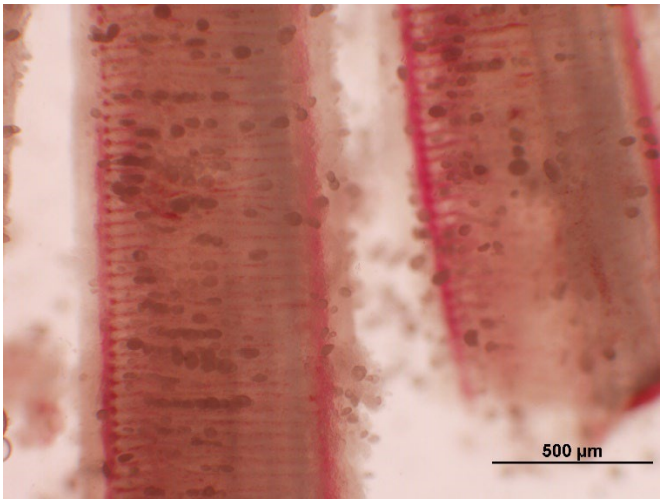


Figure 7. *Amyloodinium* sp., in the gills displayed dark spots as well as haemorrhagic areas and showed excessive mucus production. Scale bars: 500 µm. A total of 107 *Amyloodinium* sp. organisms were observed on a single gill filament of the only live individual.

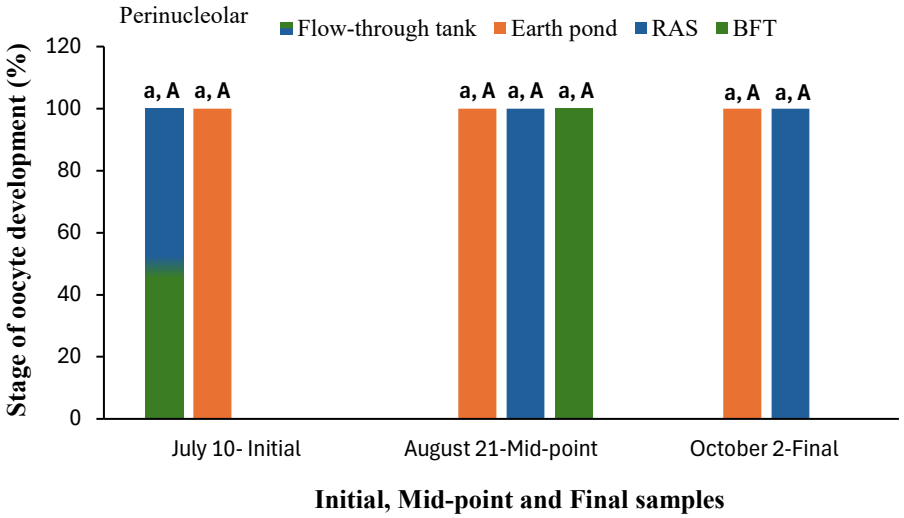


Figure 8. Stage of oocyte development (%) measured in females from each treatment, including initial (RAS-BFT: flow through tank -*n* = 4 and earth pond-*n* = 6), mid-point (RAS-*n* = 6, BFT-*n* = 2 and earth pond-*n* = 5), and final (RAS-*n* = 7 and earth pond-*n* = 8), represented by percentage frequency 50 to 100 random oocytes were analysed and categorized into distinct stages/female. The proportions Z-test with Bonferroni correction ($P = 0.008$) did not show significant differences among treatments. The flow-through tank at the initial point represented both RAS and BFT. Green and blue bars represent flow-through tank, orange bars represent earth pond, green bar represents the BFT, and blue bar represents RAS. The classification comprised the following: Perinucleolar. Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

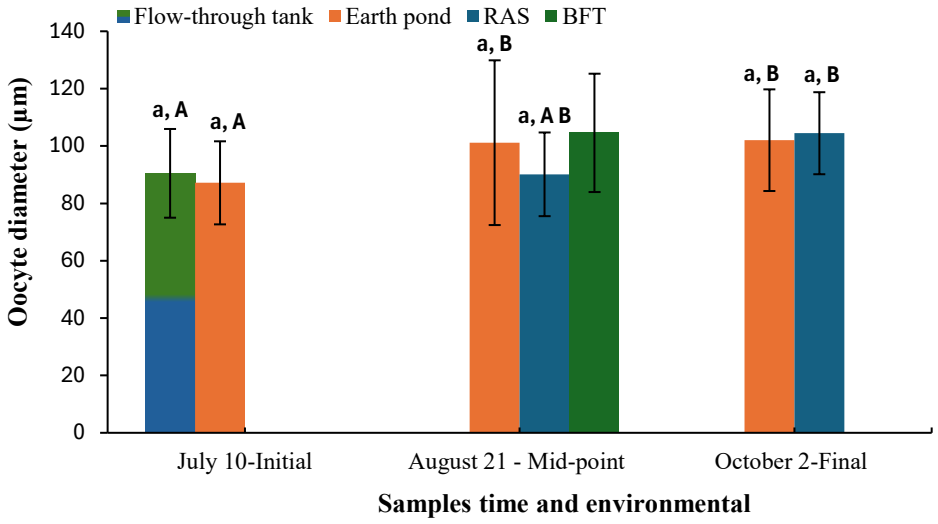


Figure 9. Average ± SD oocyte diameter in females from each treatment, including initial (RAS-BFT: flow-through tank-*n* = 4 and earth pond-*n* = 6), mid-point (RAS-*n* = 6, BFT-*n* = 2, and earth pond-*n* = 5), and final (RAS-*n* = 7 and earth pond-*n* = 8). BFT mid-point was represented by two females (104.58 ± 20.63 µm) and were excluded from intergroup comparisons. Flow-through tank at the initial point represented both RAS and BFT. Green and blue bars represent flow-through tank, orange bars represent earth pond, green bar represents the BFT, and blue bar represents RAS. Holm–Sidak test showed no statistically significant differences in oocyte diameter between RAS and earth pond at any of the sampling points (initial, mid-point, and final). However, when comparing sampling points within the same treatment, the RAS showed a statistically significant increase from the initial (90.47 ± 15.47 µm) to the final (104 ± 14.15 µm) samples ($P < 0.001$). In the earth pond, significant differences ($P < 0.001$) were observed between the initial, and mid-point, final samples (87.16 ± 14.48 µm; 101.16 ± 28.72 µm; and 102.03 ± 17.72 µm, respectively). Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

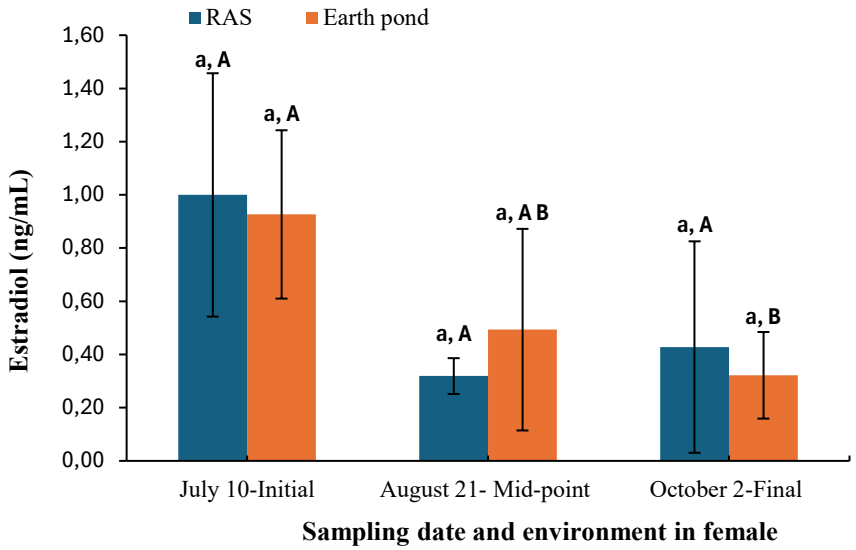


Figure 10. Effect of treatment on plasma E_2 (mean and SD) levels in females, including initial (flow-through tank-*n* = 4 and earth pond-*n* = 6), mid-point (RAS-*n* = 6 and earth pond-*n* = 5), and final samples (RAS-*n* = 7 and earth pond-*n* = 10 of 12). Blue bars represent RAS; orange bars represent earth pond. The Holm–Sidak test showed a small, significant decrease within the earth pond between the initial and final samples ($P = 0.018$). Two final samples from the earth pond were excluded due to estradiol concentrations of 7.4 and 4.2 ng/mL. Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

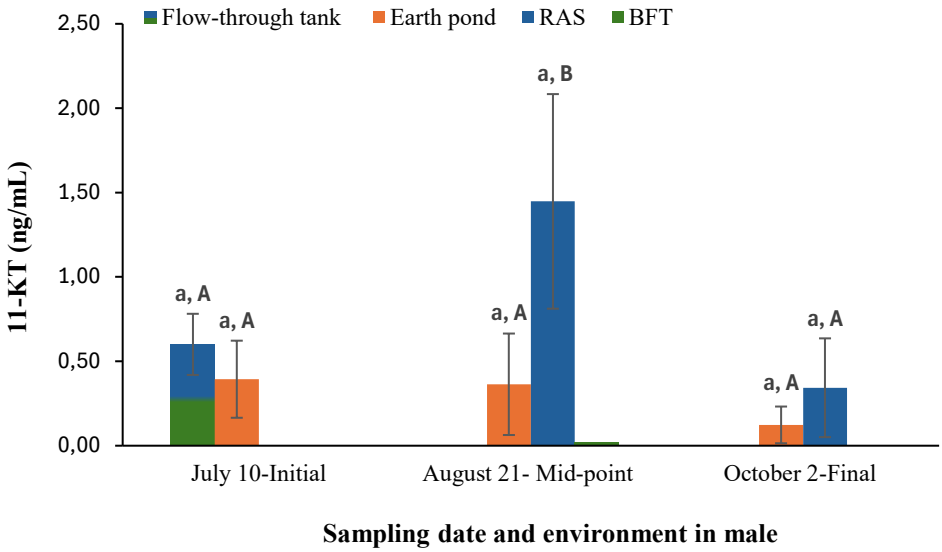


Figure 11. Effect of treatment on 11-KT (mean and SD) in males, including initial (flow-through tank-*n* = 7 of 8 and earth pond-*n* = 6), mid-point (RAS-n = 6, BFT-*n* = 1 and earth pond-*n* = 7), and final samples (RAS-*n* = 17 and earth pond-*n* = 12). Blue bars represent RAS, orange bars represent earth pond, and green bars represent BFT. The Holm–Sidak test showed a small, significant increase within the RAS between the initial and mid-point samples ($P = 0.042$) and a significant decrease between the mid-point and final samples ($P < 0.001$). One initial sample from the flow-through tank was excluded from the analysis due to an 11-KT level of 2.10 ng/mL. The BFT includes data from a single fish, with an 11-KT concentration of 0.02 ng/mL. Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

4.1.2. The wild-caught adults

Regarding the wild-caught adults, each treatment included eight individuals (4 males and 4 females). At the initial sampling (July 10th, 2024), the earth pond treatment had an average weight of 1758.63 ± 485.47 grams and a standard length of 49.50 ± 4.43 cm; the RAS had an average weight of 1556.25 ± 515.53 grams and a standard length of 48.91 ± 5.54 cm; and the BFT had an average weight of 1550.88 ± 456.70 grams and a standard length of 51.5 ± 4.12 cm, with no significant differences between groups.

Individuals from the RAS and earth pond were not evaluated at the mid-point sampling (August 21), and only BFT individuals were assessed due to *Amyloodinium* sp.-related mortality. However, the four adult fish in one BFT treatment tank were found dead, and advanced decomposition occurred during sampling. In the remaining BFT tank, only two of the four fish could be sampled: one female with a gonad weight of 281.66 grams (GSI of 14.17%) and one mature male with a gonad weight of 12.68 grams (GSI of 1.40%). Nevertheless, due to the condition of the fish at the time of discovery, it was not possible to collect blood or oocyte samples for physiological and reproductive analysis from BFT wild-caught adult fish.

At the final sampling (October 2nd), it was not possible to obtain samples from females in the RAS by cannulation, nor to obtain sperm from males by abdominal massage. In the earth pond, the eight individuals, no males had sperm, and only two females could be sampled: one was in early vitellogenesis with an average oocyte diameter of 230 ± 20 µm, and the other was in late vitellogenesis with an average oocyte diameter of 546 ± 41 µm. The latter was injected with 0.94 µg/kg rFsh and 2.8 µg/kg rLh to induce final maturation. Nonetheless, the hormone treatment was not continued due to no cannulation in females (final induction dose) and no spermiation in males. No blood samples were collected, nor were histological analyses performed on these fish at any sample point.

4.2. Welfare

4.2.1. Water quality

On May 28th, 2024, the BFT treatment was initiated without animals present, allowing the environment to stabilize before the introduction of mullet. After 43 days, the fish were introduced into the system. During this time, various water quality parameters were monitored to ensure optimal conditions for the BFT treatment. The recorded water quality parameters in the BFT were as follows: dissolved oxygen at 6.20 ± 0.70 mg/L (min: 4.12 and max: 8.05), temperature at 27.49 ± 2.57 °C (min: 21.8 °C and max: 30.1 °C), alkalinity at 213.74 ± 28.13 mg $CaCO_3$/L (min: 160 and max: 286), pH of 8.02 ± 0.18 (min: 7.19 and max: 8.33), ammonium concentration of 1.36 ± 1.41 mg/L (min: 0 and max: 7), and nitrite levels of 0.033 ± 0.040 mg/L (min: 0 and max: 0.23) **(Fig. 12-17).** In contrast, the recirculating aquaculture (RAS) showed lower levels of ammonium (0.30 mg/L) and nitrite (0.03 mg/L), with a pH of 7.8, temperature of 26.81 ± 2.06 °C (min: 23.2 and max: 29.6), and dissolved oxygen concentration of 6.10 ± 1.08 mg/L (min: 5.2 and max: 7.6) **(Fig. 18).** Meanwhile, the earth pond presented a temperature of 25.62 ± 2.30°C (min: 21.2 and max: 28.8) and a dissolved oxygen level of 5.06 ± 1.24 mg/L (min: 3.5 and max: 8.21) **(Fig. 19-20)**.

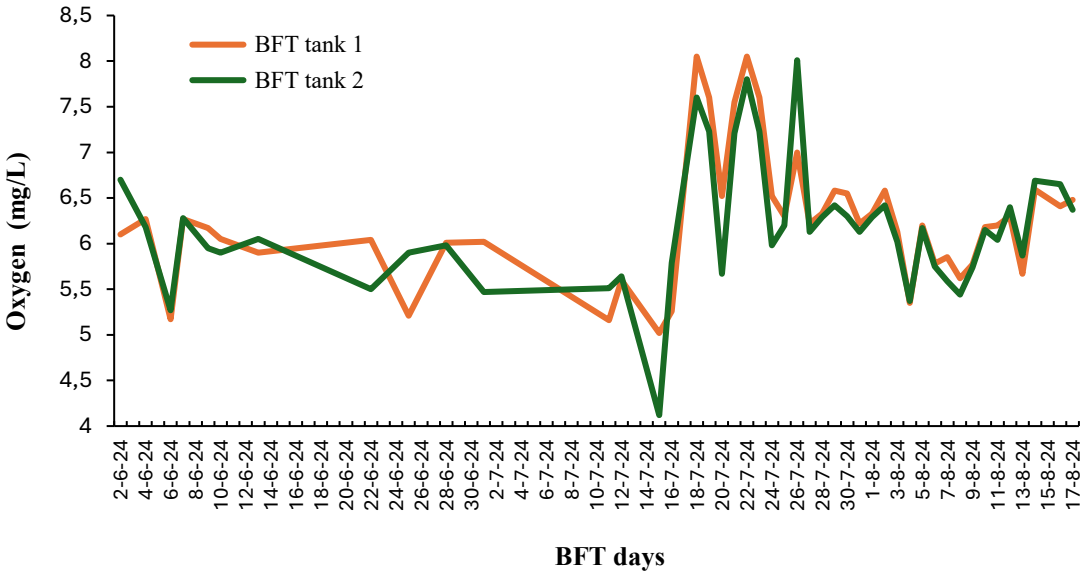


Figure 12. Oxygen concentration (mg/L) in two tanks for the BFT over an 80- day period, from June 2nd, 2024, to August 17th, 2024.

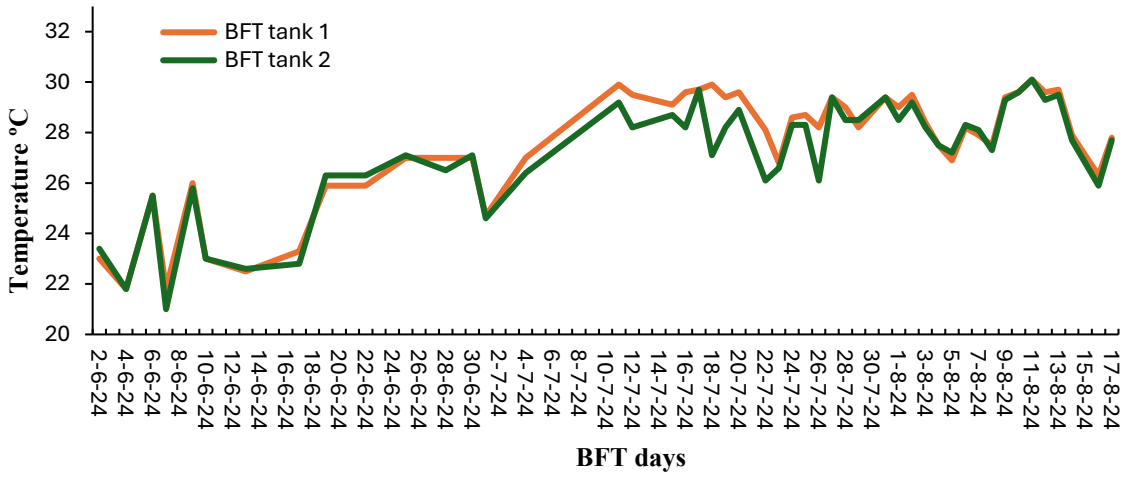


Figure 13. Temperature (ºC) in two tanks for the BFT over an 80- day period, from June 2nd, 2024, to August 17th, 2024.

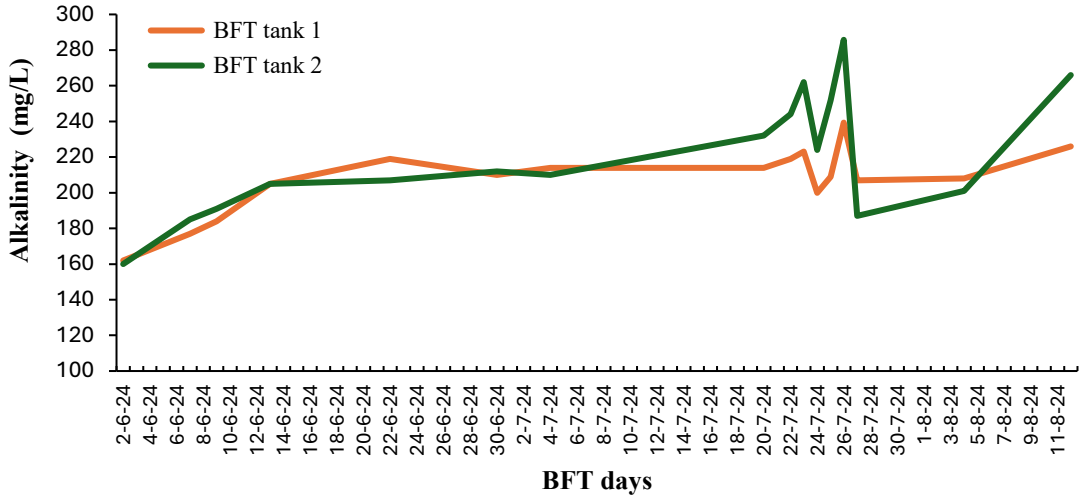


Figure 14. Alkalinity concentration (mg/L) in two tanks for the BFT over an 80- day period, from June 2nd, 2024, to August 17th, 2024.

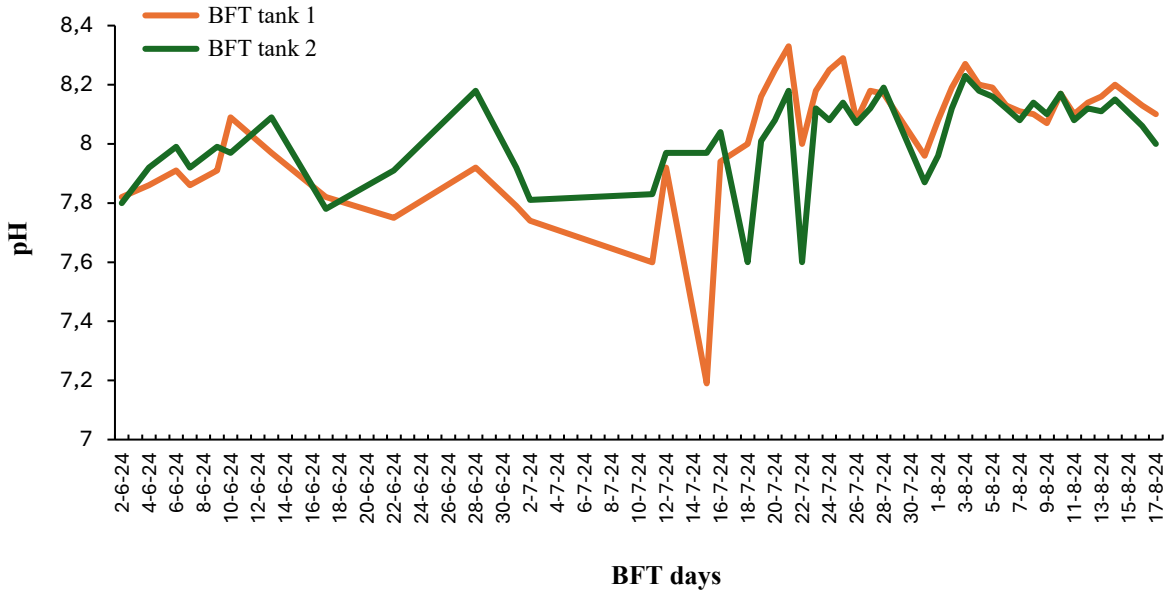


Figure 15. pH in two tanks for the BFT over an 80- day period, from June 2nd, 2024, to August 17th, 2024.

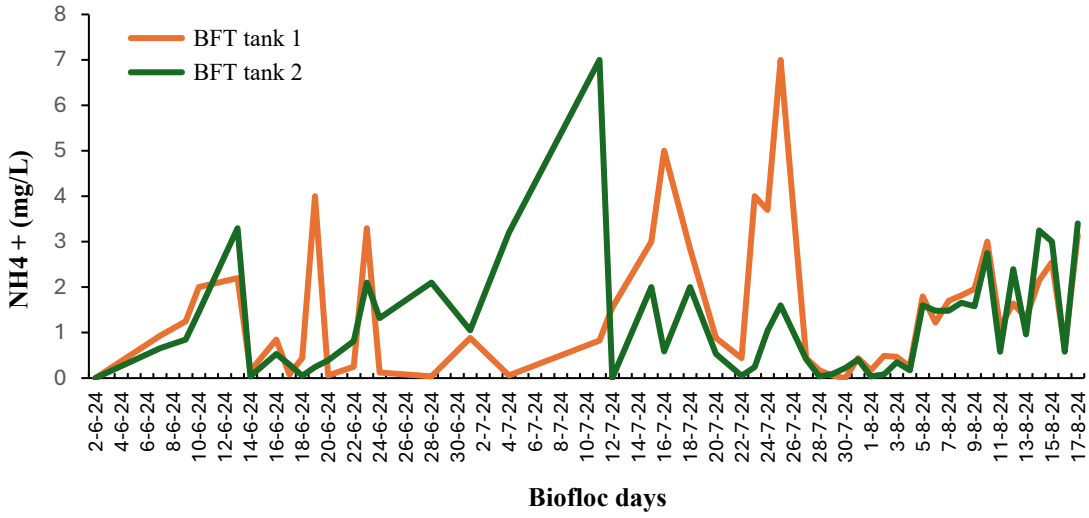


Figure 16. Ammonium concentration in two tanks for the BFT over an 80-day period, from June 2nd, 2024, to August 17th, 2024.

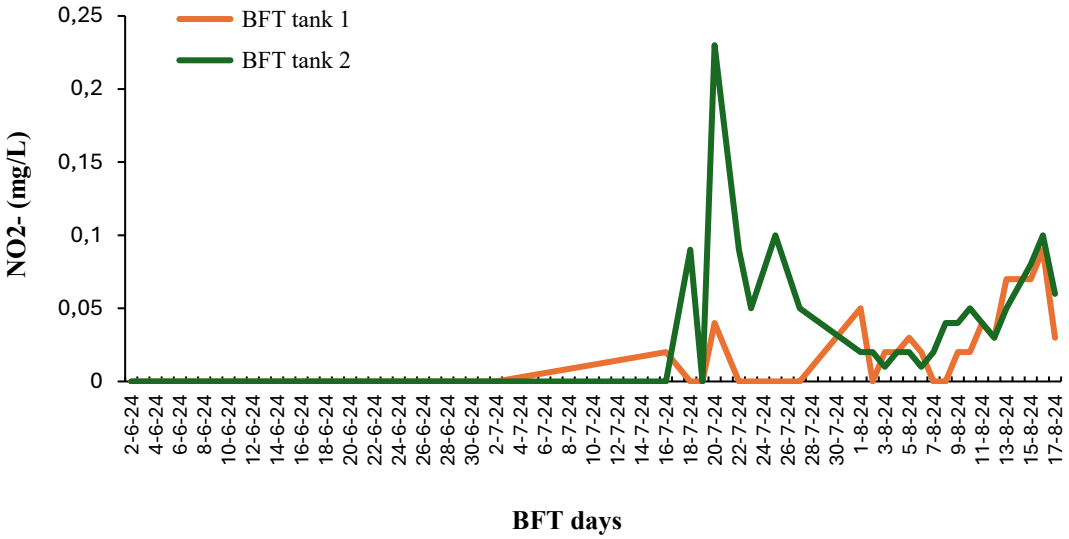


Figure 17. Nitrite concentration in two tanks for the BFT over an 80- day period, from June 2nd, 2024, to August 17th, 2024.

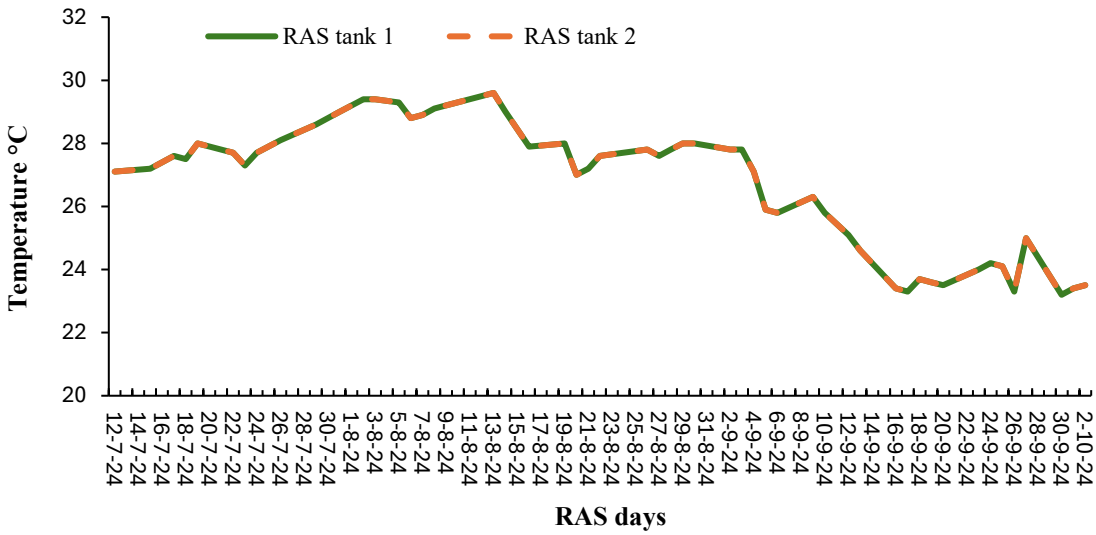


Figure 18. Temperature variation (°C) in two RAS systems over 80 days, from July 12th, 2024, to October 2nd, 2024. The green and orange lines represent tank 1 and tank 2 in the RAS, respectively. The temperature was the same in the two tanks, and the yellow line is obscured by the green line.

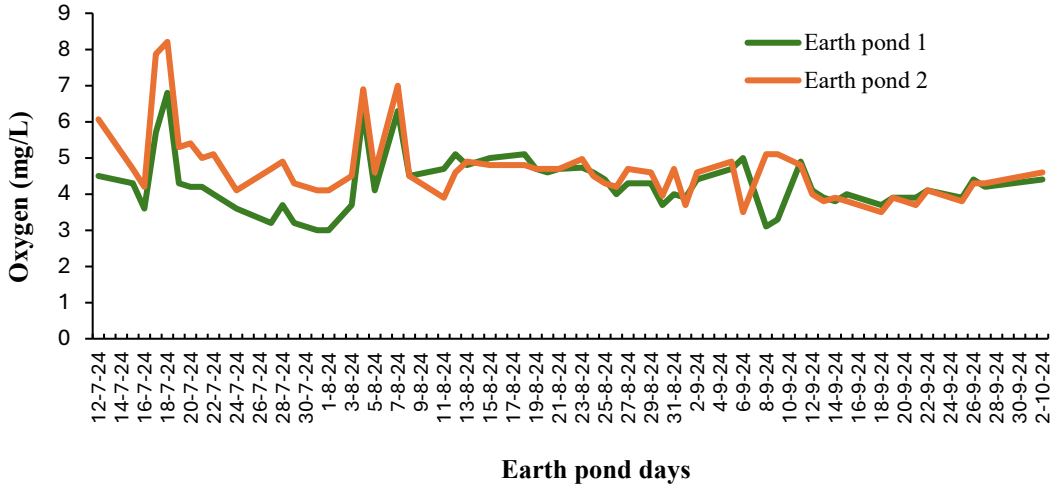


Figure 19. Oxygen variation (mg/L) in two earth ponds over an 80-day period, from July 12th, 2024, to October 2nd, 2024. The yellow and green lines represent earth pond 1 and earth pond 2, respectively.

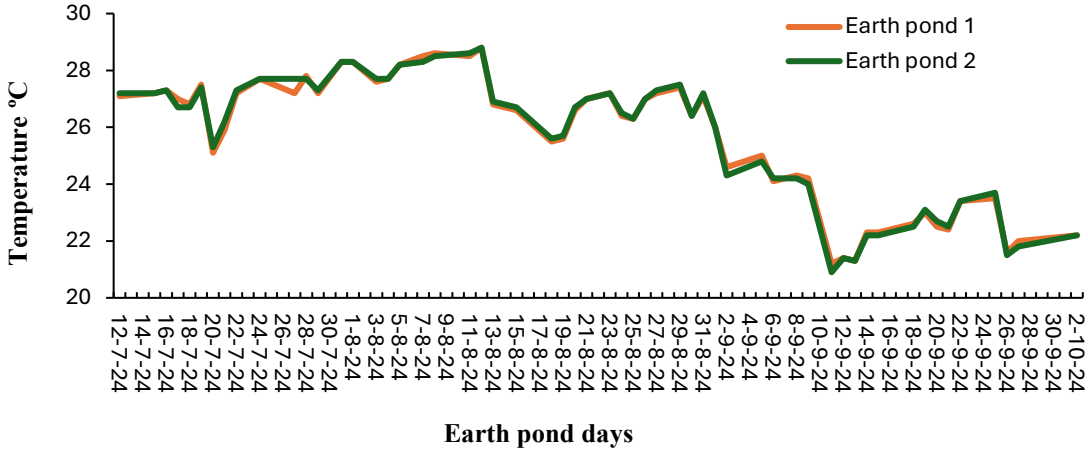


Figure 20. Temperature variation (°C) in two earth ponds over an 80-day period, from July 12th, 2024, to October 2nd, 2024. The yellow and green lines represent earth pond 1 and earth pond 2, respectively.

4.2.2. Mullet welfare

In this experiment, mullet welfare was assessed during both the mid-point and final samples for the RAS and earth pond treatments. The evaluation included variables such as: eye injuries, skin lesions, fin damage, overall coloration, and deformities. Statistical analysis revealed significant differences only between the mid-point (n = 12) and final (n = 24) samples in the earth pond for skin lesions (P = 0.03) and fin lesions (P = 0.04) **(Fig. 21)**.

For the mid-point sampling **(Fig. 21)**, the only significant difference between RAS (n = 12) and earth pond (n = 12) was observed for skin lesions (P = 0.04). In contrast, the final sampling **(Fig. 21)** showed significant differences in fin lesions (P = 0.02) and overall coloration (P = 0.04) between RAS (n = 24) and earth pond (n = 24).

Additionally, when analysing plasma levels of sodium (Na), glucose, and lactate across different treatments, sampling points, and sexes, no significant differences in sodium levels were observed between males and females for the RAS and earth pond treatments during the mid-point and final sampling. However, in the final sample, both males (n = 9) and females (n = 8) reared in earth pond showed a significant increase in plasma sodium levels compared to those in the RAS treatment (males-n = 17 and females-n = 6) **(Fig. 22)**. Specifically between treatments within each sampling point, sodium concentrations reached 172 ± 8.3 mmol/L in males (n = 9 of 12) and 167 ± 5.4 mmol/L in females (n = 8 of 12) from the earth pond treatment, while values were significantly lower in the RAS treatment 154 ± 5.45 mmol/L for males (n =17) and 151 ± 3.2 mmol/L for females (n = 6) (P < 0.001). Comparing sodium levels between sampling points within each treatment, a significant increase was observed from the mid-point (n = 6) to the final (n = 17) samples for the RAS males (P = 0.048, from 147 ± 2.4 to 154 ± 5.45 mmol/L) and earth pond males (P < 0.001, from 155 ± 6.7 (n = 6) to 172 ± 8.3 (n = 12) mmol/L). Among females, this increase (mid-point to final samples) was only significant in the earth pond treatment (P = 0.021), rising from 156 ± 2.08 (n = 4) to 167 ± 5.4 (n = 8) mmol/L. The BFT treatment includes data from a single fish, with a sodium concentration of 141 mmol/L **(Fig. 22)**.

Regarding glucose levels, during the mid-point and final samples, males reared in the RAS treatment (mid-point: 64 ± 10.87 mg/dL (n = 6) and final:121 ± 17.44 mg/dL (n = 17) showed significantly lower glucose concentrations compared to males in the earth pond (mid-point: 119 ± 15.2 mg/dL (n = 6) and final:176 ± 48.90 mg/dL (n = 12) **(*P* = 0.008, Fig. 23)**. When comparing between phases, a significant increase in glucose levels was observed in males from the mid-point (119 ± 1.87 mg/dL) to the final phase for the RAS (121 ± 17.44 mg/dL; P = 0.002). The BFT treatment includes data from a single fish, with a glucose concentration of 35 mg/dL **(Fig. 23)**.

In terms of lactate levels, males reared in earth pond showed higher concentrations compared to those in the RAS treatment at both the intermediate and final sampling. Specifically, at the mid-point, lactate levels reached 7.61 ± 2.34 mmol/L (n = 7) in the earth pond treatment vs. 4.15 ± 1.05 mmol/L (n = 6) in the RAS treatment (***P* = 0.007**, **Fig. 24)**. At the final phase in male, values were 5.47 ± 1.92 mmol/L (n = 7) in earth pond and 3.02 ± 1.49 mmol/L (n = 13) for the RAS (P = 0.038). In females at the final sample, values were 6.70 ± 1.85 mmol/L (n = 9) in earth pond and 1.59 ± 0.79 mmol/L (n = 5) for the RAS (P < 0.001). The BFT treatment includes data from a single fish, with a lactate concentration of 7.22 mmol/L **(Fig. 24)**.

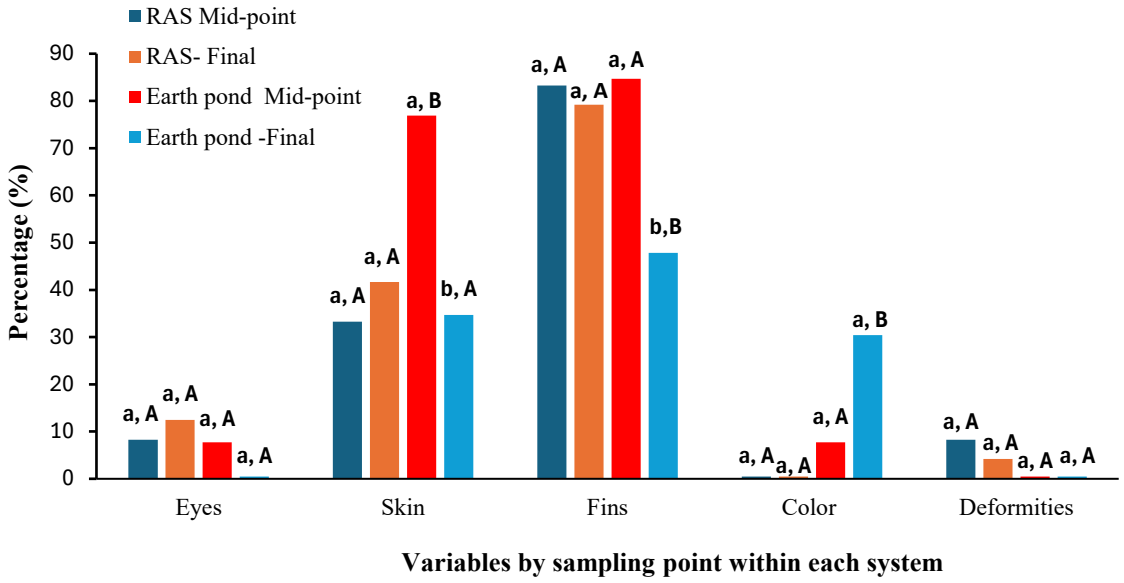


Figure 21. Percentage of external abnormalities observed in mullet at mid-point and final sampling from two treatments: recirculating aquaculture systems (RAS) and earth pond. RAS samples are represented by blue bars (mid-point-n =12) and orange bars (final-n = 24), while earth pond samples are shown by red bars (mid-point-n = 12) and light blue bars (final-n = 24). The variables assessed include abnormalities in eyes, skin (scale lesions), fins, color, and deformities. Statistical analysis was performed using Chi-square test (P = 0.05). Significant differences were detected within the earth pond treatment between mid-point and final sampling for skin (P = 0.03) and fin lesions (P = 0.04). In the mid-point sample, skin (scale) lesions between RAS and earth pond were significantly different (P = 0.04). The final sampling showed significant differences in fin lesions (P = 0.02) and overall coloration (P = 0.04) between RAS and earth pond. Different lowercase letters indicate significant differences between different points within each treatment. Different capital letters indicate significant differences between treatments within each sampling point.

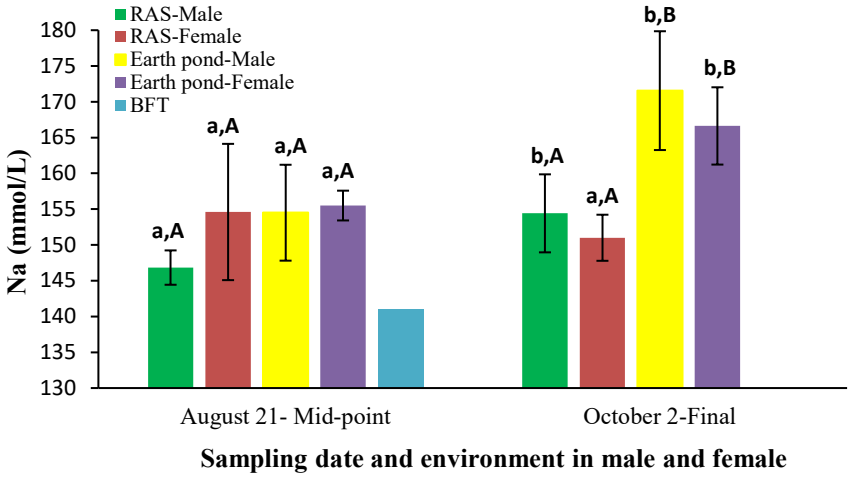


Figure 22. Effect of RAS and earth pond treatment on plasma sodium levels (mean ± SD) in male and female mullet at the mid-point (August 21st, 2024) and final (October 2nd, 2024) sampling. Males are represented by green bars for RAS (mid-point-*n* = 6 and final-*n* = 17), yellow bars for earth pond (mid-point-*n* = 6 and final-*n* = 9 of 12), and a blue bar for BFT (mid-point-*n* = 1), while females are shown in red bars for RAS (mid-point-*n* = 5 and final-*n* = 6) and purple bars for earth pond (mid-point-*n* = 4 and final-*n* = 8 of 12). The Holm–Sidak test was used to determine statistical differences in sodium levels both between treatments (RAS vs. earth pond) and between sampling points (mid-point vs. final) for each sex. At the final sampling, both males and females reared in earth pond showed significantly higher plasma sodium levels compared to those in the RAS ($P < 0.001$). When comparing sodium levels between sampling points, a significant increase from mid-point to final sampling for the RAS males ($P = 0.048$) and in earth pond males ($P < 0.001$). Among females, this increase was only significant in the earth pond treatment ($P = 0.021$). Different lowercase letters indicate significant differences within each treatment at the same sample point. Different capital letters indicate significant differences in the same treatments between sampling points. The BFT treatment (blue bar) includes data from a single male, with a sodium concentration of 141 mmol/L.

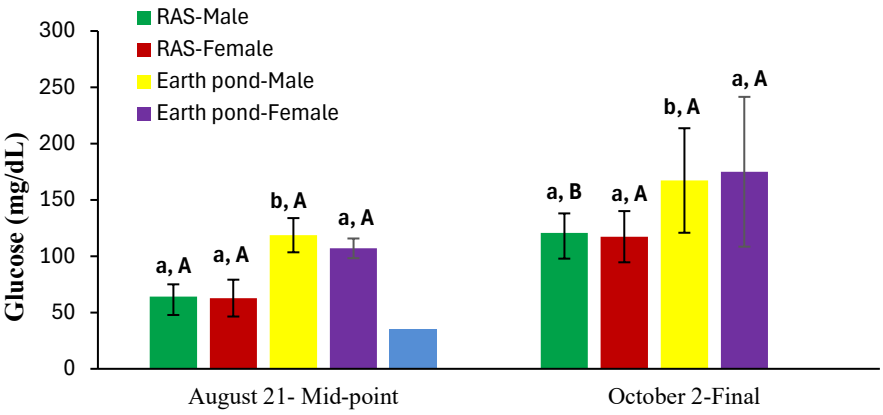


Figure 23. Effect of RAS and earth pond treatment on plasma glucose levels (mean ± SD) in male and female mullet at the mid-point (August 21st, 2024) and final (October 2nd, 2024) sampling. Males are represented in green bars RAS (mid-point-*n* = 6 and final-*n* = 17), yellow bars earth pond (mid-point-*n* = 6 and final-*n* = 12), and blue bar BFT (mid-point-*n* = 1), while females are shown in red bars RAS (mid-point-*n* = 5 and final-*n* = 6) and purple bars earth pond (mid-point-*n* = 4 and final-*n* = 12). The Holm–Sidak test was used to determine statistical differences in sodium levels both between treatments (RAS vs. earth pond) and between sampling (mid-point vs. final) for each sex. In the mid-point and final samples, males reared in the RAS treatment showed significantly lower glucose concentrations than males in the earth pond ($P = 0.008$). In the RAS, a significant increase in glucose levels was observed in males from the mid-point to the final sample ($P = 0.002$). Different lowercase letters indicate significant differences within each treatment at the same sample point. Different capital letters indicate significant differences in the same treatments between sampling points. The BFT treatment (blue bar) includes data from a single male, with a glucose concentration of 35 mg/dL.

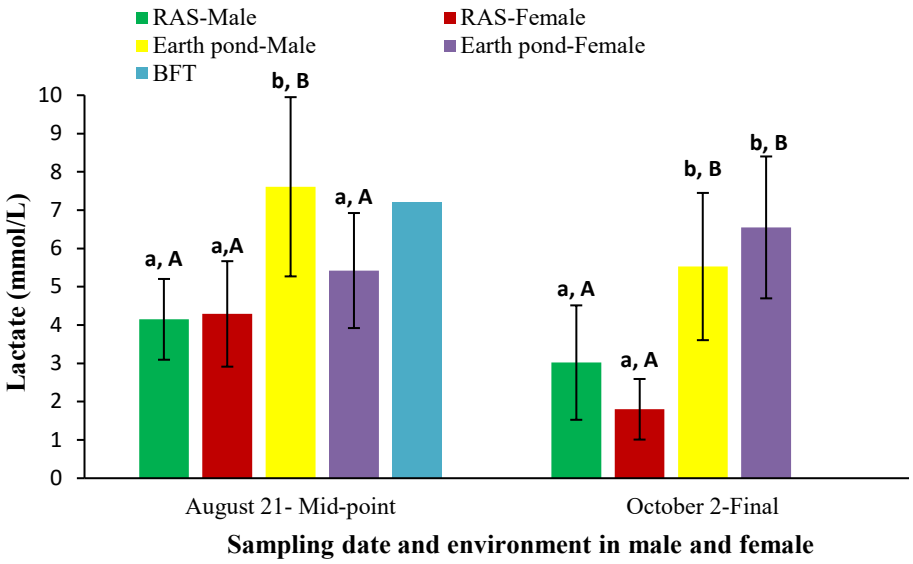


Figure 24. Effect of RAS and earth pond treatments on plasma lactate levels (mean ± SD) in male and female mullet at the mid-point (August 21st, 2024) and final (October 2nd, 2024) sampling. Males are represented in green bars RAS (mid-point-*n* = 6 and final-*n* = 13), yellow bars earth pond (mid-point-*n* = 7 and final-*n* = 7), and blue bar BFT (mid-point-*n* = 1), while females are shown in red bars RAS (mid-point-*n* = 6 and final-*n* = 5) and purple bars earth pond (mid-point-*n* = 5 and final-*n* = 9). The Holm–Sidak test was used to determine statistical differences in lactate levels both between treatments (RAS vs. earth pond) and between sampling points (mid-point vs. final) for each sex. Males reared in earth pond showed higher concentrations compared to those in the RAS treatment at both the mid-point ($P = 0.007$) and final sampling phases ($P = 0.038$). Females reared in earth pond showed higher concentrations compared to those in the RAS treatment in final sampling ($P < 0.001$). Different lowercase letters indicate significant differences within each treatment at the same sample point. Different capital letters indicate significant differences in the same treatments between sampling points. The BFT treatment (blue bar) includes data from a single male, with a lactate concentration of 7.22 mmol/L.

Regarding cortisol levels in male, during the initial (0.15 ± 0.05 ng/mL - *n* = 4), mid-point (0.13 ± 0.10 ng/mL - *n* = 6) and males reared in the RAS treatment showed significantly lower cortisol concentrations compared to RAS in the final sample (0.25 ± 0.09 ng/mL - *n* = 17) (***P* < 0.001, Fig. 25**). When comparing between treatments in males, a significant increase in cortisol levels was observed for the RAS (*n* = 17) compared to earth pond (*n* = 12) in the final samples ($P = 0.005$). The BFT treatment includes data from a single male, with a cortisol concentration of 0.14 ng/mL (**Fig. 25**). An outlier was identified in the RAS treatment at the final sampling point: a male with a cortisol concentration of 0.644 ng/mL.

Regarding cortisol levels in female, during the initial (0.12 ± 0.02 ng/mL - *n* = 2) and mid-point (0.10 ± 0.03 ng/mL - *n* = 6) phase, females reared in the RAS showed significantly lower cortisol concentrations compared to RAS in the final sample (0.34 ± 0.10 ng/mL - *n* = 7) (***P* < 0.001,**

Fig. 26). When comparing between treatments, a significant increase in cortisol levels was observed for the RAS (n = 7) compared to earth pond (n = 12) in the final samples (***P* < 0.001, Fig. 26**). No significant differences in cortisol levels were observed between males and females within the RAS treatment at the initial, mid-point, or final sampling points. Similarly, no sex-based differences were detected in the earth pond treatment at any sampling point.

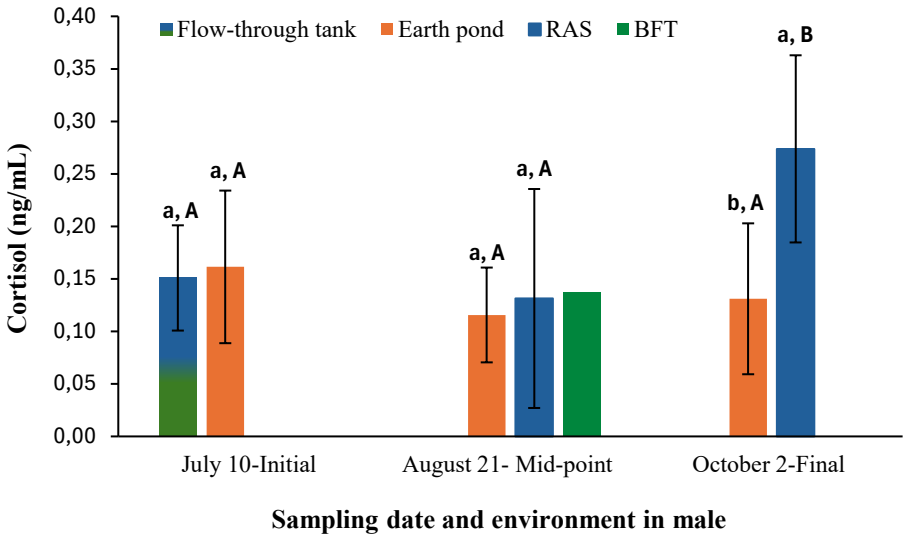


Figure 25. Effect of environment on cortisol levels (mean ± SD) in male mullet over time: initial (July 10th, 2024), mid-point (August 21st, 2024), and final (October 2nd, 2024). Blue and green bars represent flow-through tank at the initial point represented both RAS and BFT (initial-n = 4), orange bars represent earth pond (initial-n = 5, mid-point-n = 7, and final-n = 12), blue bars represent RAS (mid-point-n = 6 and final-n = 17), and the green bar (n =1) represents the BFT treatment. The Holm–Sidak test revealed a small but significant increase in cortisol levels within the RAS between initial, mid-point, and final samples (P < 0.001). At the final sampling point, cortisol levels were significantly higher for the RAS compared to earth pond (P = 0.005). Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point. The BFT (green bar) consists of a single male, with a cortisol concentration of 0.14 ng/mL at mid-point. An outlier was identified in the RAS at the final sampling: a fish with a cortisol concentration of 0.644 ng/mL, and this data point was excluded from the analysis. Green and blue bars represent flow-through tank, orange bars represent earth pond, green bar represents the BFT, and blue bar represents RAS.

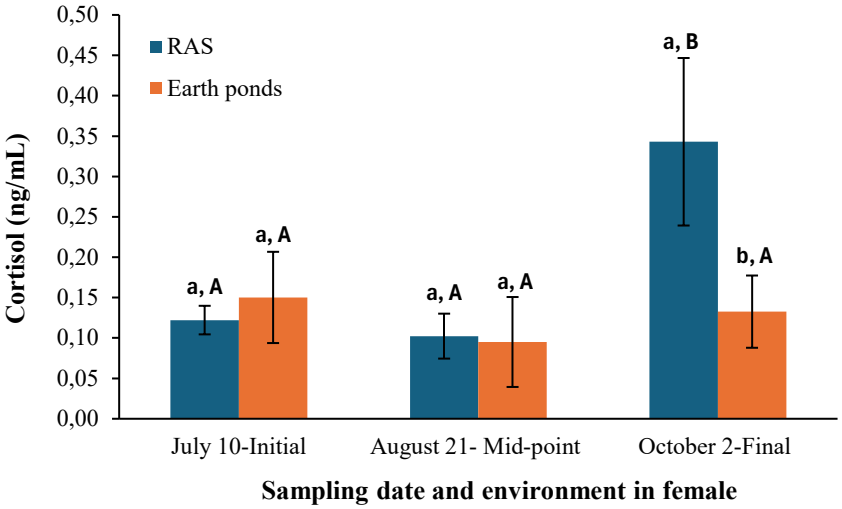


Figure 26. Effect of environment on cortisol levels (mean ± SD) in female mullet over time: initial (July 10th, 2024), mid-point (August 21st, 2024), and final (October 2nd, 2024). Blue bars represent RAS (initial-*n* = 2, mid-point-*n* = 6 and final-*n* = 7), orange bars represent earth pond (initial-*n* = 6, mid-point-*n* = 5 and final-*n* = 12). The Holm–Sidak test revealed a small but significant increase in cortisol levels within the RAS between initial, mid-point, and final samples ($P < 0.001$). At the final sampling point, cortisol levels were significantly higher for the RAS compared to earth pond ($P < 0.001$). Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

The condition factor was also measured during the mid-point and final samplings in both treatments **(Fig. 27),** the RAS and the earth pond. At the mid-point sampling, the condition factor was higher ($P < 0.001$) in the RAS (n =12) (K = 1.77) compared to the earth pond (n =12) (K = 1.56). However, in the final sampling, the condition factor in the RAS ($P < 0.001$) (K = 1.39) (n = 24) and earth pond ($P = 0.01$) (K = 1.44) (n = 24) was lower than in the mid-point sampling in the RAS (K = 1.77) (n = 12) and earth pond (K = 1.56) (n = 12).

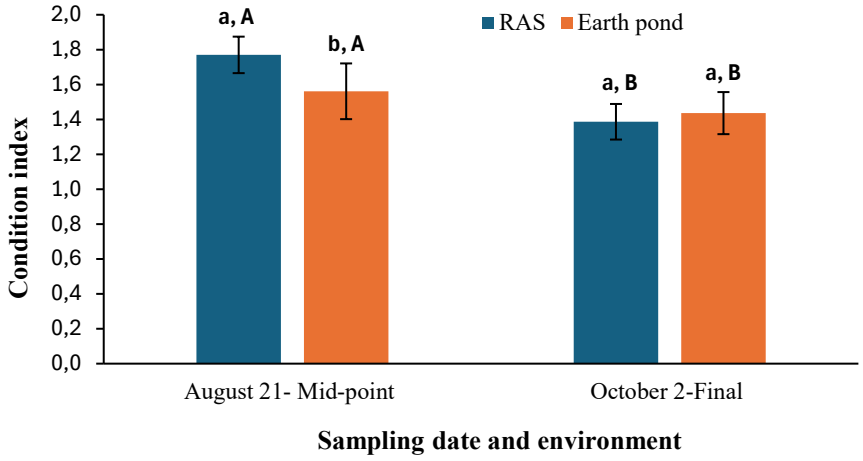


Figure 27. Effect of environment on condition index (mean ± SD) in mullet over time: mid-point (August 21st, 2024), and final (October 2nd, 2024). Blue bars represent RAS (mid-point n = 12 and final- n = 24), orange bars represent earth pond (mid-point n = 12 and final- n = 24). At the mid-point sampling, the condition factor was higher (P <0.001) in the RAS (K = 1.77) compared to the earth pond (K = 1.56). In the final sampling, the condition factor in the RAS (P <0.001) (K = 1.39) and earth pond (P = 0.01) (K = 1.44) was lower than in the mid-point sampling in the RAS (K = 1.77) and earth pond (K = 1.56). Different capital letters indicate significant differences between sample points within each treatment. Different lowercase letters indicate significant differences between treatments within each sampling point.

4.3. Gonad Analysis

Fatty acid analysis was conducted on female mullet from both treatments (earth pond and RAS) at both sampling points (initial and mid-point). However, for males, this analysis was performed only at the mid-point, as no male samples were available at the initial sampling.

The percentages of total saturated fat, total n-6 PUFA, and total PUFA showed no significant differences either between sampling points within each treatment or between treatments at each sampling point in female mullet **(initial and mid-point; Table 1, Fig. 28)** and male mullet **(mid-point; Table 2, Fig. 28)** (P > 0.05).

In contrast, the percentage of total monounsaturated fat in females varied significantly both across sampling points within treatments and between treatments at each sampling point (P < 0.05) **(Table 1, Fig. 28)**. However, in males **(Table 2, Fig. 29)** no significant differences in monounsaturated fat were observed between treatments (earth pond: 352 ± 22.54 µg FA/mg

lipid (n = 7) and RAS: 361 ± 20.96 µg FA/mg lipid (n = 6)) at the mid-point. In total, monounsaturated fat for the BFT was 316.9 µg FA/mg lipid (n =1).

At each sampling point, female mullet in the RAS treatment showed a significantly higher percentage of total monounsaturated fat compared to those in the earth pond treatment. At the initial sampling, total monounsaturated fat for the RAS females reached 242 ± 46.2 µg FA/mg lipid (n = 4), while significantly lower values were recorded in the earth pond treatment (166 ± 18.9 µg FA/mg lipid, n = 6; P = 0.003). At mid-point sampling, the RAS treatment again showed higher levels (293 ± 52.2 µg FA/mg lipid, n = 6) than the earth pond group (231 ± 55.3 µg FA/mg lipid, n = 5; P = 0.007). When comparing total monounsaturated fat between sampling points within each treatment, a significant increase was observed in both systems. For the RAS, values rose from 242 ± 46.2 µg FA/mg lipid at the initial sampling (n = 4) to 293 ± 52.2 µg FA/mg lipid at mid-point (n = 6; P = 0.003). Similarly, in the earth pond group, levels increased from 166 ± 18.9 µg FA/mg lipid (n = 6) to 231 ± 55.3 µg FA/mg lipid (n = 5; P = 0.007).

Regarding total n-3 PUFA, significant differences between treatments were observed at each sampling point in both females **(initial and mid-point; Table 1, Fig. 28)** and males **(mid-point; Table 2, Fig. 29)** (P < 0.05). Additionally, significant differences across sampling points within each treatment were found only in females from the earth pond treatment (P < 0.05) **(Table 1, Fig. 28)**. At the initial sampling, total n-3 PUFA in earth pond females reached 97.5 ± 9.58 µg FA/mg lipid (n = 6), while significantly lower values were recorded in the RAS treatment (81.2 ± 8.69 µg FA/mg lipid, n = 4; P < 0.044). At mid-point sampling, the earth pond treatment again showed higher levels (102 ± 22.1 µg FA/mg lipid, n = 5) than the RAS treatment (81.3 ± 7.31 µg FA/mg lipid, n = 6; P < 0.014). At the mid-point sampling in males **(Table 2, Fig. 29)**, the earth pond treatment again showed higher levels (90.6 ± 14 µg FA/mg lipid, n = 7), compared to the RAS treatment (75.4 ± 13.65 µg FA/mg lipid, n = 6; P = 0.031). In total, n-3 PUFA for the BFT was 53.2 µg FA/mg lipid (n =1).

Table 1. Fatty acid composition (µg FA/mg lipid: mean ± SD) in female mullet from earth pond and RAS treatments at initial and mid-point samplings, and from BFT treatment at mid-point only. Different lowercase letters indicate significant differences ($P < 0.05$) between treatments within the same sampling point, while capital letters indicate significant differences between sampling points within the same treatment using Holm–Sidak test ($P < 0.05$). Sample sizes: earth pond initial ($n = 6$), RAS initial ($n = 4$), earth pond mid-point ($n = 5$), RAS mid-point ($n = 6$).

	Initial sample		Mid-point sample	
Fatty acid	Earth pond	RAS	Earth pond	RAS
14:0	3.71 a, A	6.81 b, A	8.51 a, B	8.67 a, A
15:0	0 a, A	23.8 b, A	0 a, A	0 a, B
16:0	88, 4 a, A	80.9 a, A	102 a, A	117 a, B
17:0	2.13 a, A	7.99 a, A	3.22 a, A	1.89 a, A
18:0	48.9 a, A	23.9 b, A	36.6 a, B	27.5 b, A
20:0	1.48 a, A	1.42 a, A	1.89 a, A	2.04 a, A
22:0	0	0	0	0
23:0	0	0	0	0
24:0	0	0	0	0
Total saturated	145 a, A	145 a, A	152 a, A	156 a, A
14:1	0	0	0	0
15:1	8.23 a, A	5.04 a, A	6.44 a, A	4.43 a, A
16:1	20.1 a, A	36.2 b, A	41.8 a, B	47.9 a, B
17:1	2.12 a, A	0.434 b, A	3.19 a, A	0 b, A
18:1n-9	109 a, A	165 b, A	139 a, A	193 b, A
18:1n-7	16.6 a, A	20.6 b, A	22.1 a, B	23.8 a, B
20:1	12.1 a, A	15.2 a, A	15.2 a, A	19.3 b, A
22:1	0	0.863	4.46	5.99
24:1	0	0	0	0
Total monounsaturated	166 a, A	242 b, A	231 a, B	293 b, B
18:2n-6	71.6 a, A	96.2 b, A	69.9 a, A	96.2 b, A
18:3n-6	1.50 a, A	2.09 a, A	2.13 a, A	2.37 a, A
20:2n-6	12.2 a, A	11.2 a, A	8.97 a, B	9.14 a, A
20:3n-6	0	0	0	0
20:4n-6 ARA	33.4 a, A	17.3 b, A	22.4 a, B	12.9 b, A
22:2n-6	0	0	0	0
Total n-6 PUFA	119 a, A	127 a, A	104 a, A	121 a, A
18:3n-3	6.28 a, A	11.5 b, A	8.62 a, A	13.0 b, A
18:4n-3	0.398 a, A	0.896 a, A	2.08 a, B	1.80 a, A
20:3n-3	0. 270 a, A	1.03 a, A	0 a, A	0.643 a, A
20:4n-3	0	0	0	0
20:5n-3 EPA	21.6 a, A	10.4 b, A	23.5 a, A	10.3 b, A
21:5n-3	0	0	0	0
22:5n-3	17. 2 a, A	12.6 b, A	22.0 a, B	12.7 b, A
22:6n-3 DHA	52.7 a, A	44.4 a, A	46.8 a, A	43.7 a, A
Total n-3 PUFA	97.5 a, A	81.2 b, A	102 a, A	81.3 b, A
Total PUFA	217 a, A	210 a, A	204 a, A	203 a, A

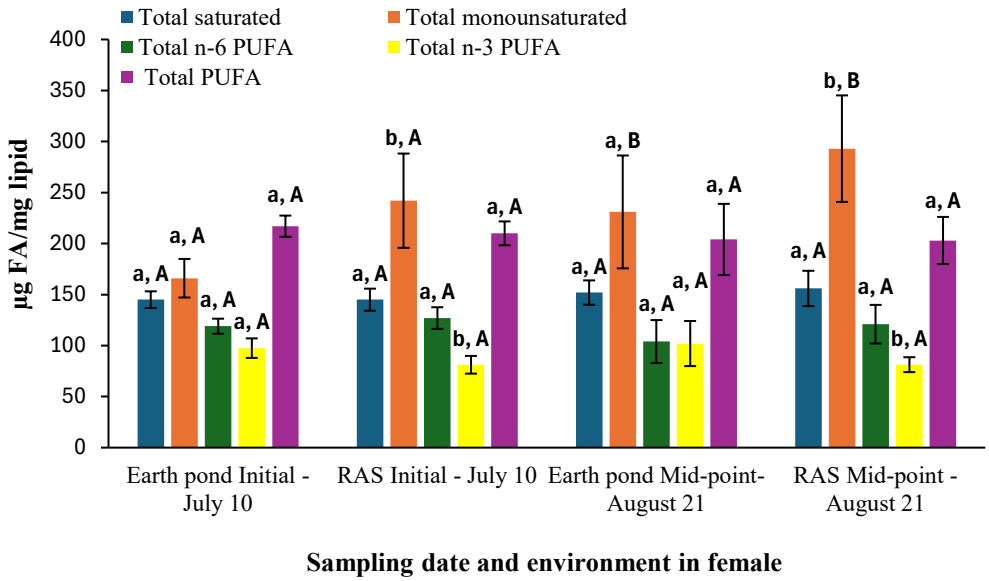


Figure 28. Fatty acid composition (µg FA/mg lipid: mean ± SD) in female mullet from earth pond and RAS treatments at initial and mid-point samplings, and from BFT treatment at mid-point only. Bar colours represent different lipid categories in female mullet across treatments and sampling points: blue (total saturated fat), orange (total monounsaturated fat), green (total n-6 PUFA), yellow (total n-3 PUFA), and purple (total PUFA). Data are shown for earth pond (initial and mid-point), RAS (initial and mid-point). Sample sizes: earth pond initial (n = 6), RAS initial (n = 4), earth pond mid-point (n = 5), RAS mid-point (n = 6). Different lowercase letters indicate significant differences ($P < 0.05$) between treatments within the same sampling point, while capital letters indicate significant differences between sampling points within the same treatment using Holm–Sidak test ($P < 0.05$).

Table 2. Fatty acid composition (µg FA/mg lipid: mean ± SD) in male mullet from earth pond and RAS treatments at mid-point sampling. Different lowercase letters indicate significant differences ($P < 0.05$) between treatments at mid-point sampling using U. Mann-Whitney test ($P < 0.05$). BFT data are shown for descriptive purposes only ($n = 1$) and were not included in the statistical analysis. Sample sizes at mid-point: earth pond ($n = 7$), RAS ($n = 6$) and BFT mid-point ($n = 1$).

Fatty acid	Earth pond Mid-point	RAS Mid-point	BFT Mid-point
14:0	15.7 a	10.4 b	3.1
15:0	0.183 a	0.00 a	0.0
16:0	129 a	139 a	112,1
17:0	3.88 a	2.28 b	2,0
18:0	20.7 a	19.9 a	20,9
20:0	2.54 a	2.35 a	2,5
22:0	0	0	0,0
23:0	0	0	0,0
24:0	0	0	0,0
Total saturated	172 a	174 a	140,5
14:1	0	0	0,0
15:1	0	0	0,0
16:1	70.8 a	65.0 a	48,8
17:1	2.46 a	0. 0 b	0,0
18:1n-9	221 a	236 b	224,2
18:1n-7	29.3 a	30.5 a	24,2
20:1	22.7 a	22.7 a	19,6
22:1	5.41 a	6.05 a	0,0
24:1	0	0	0,0
Total monounsaturated	352 a	361 a	316,9
18:2n-6	113 a	119 a	115,4
18:3n-6	3.20 a	3.24 a	2,1
20:2n-6	9.59 a	8.87 a	7,9
20:3n-6	0	0	0,0
20:4n-6	6.01 a	3.55 b	2,8
22:2n-6	0	0	0,0
Total n-6 PUFA	132 a	135 a	128,1
18:3n-3	15.4 a	15.5 a	12,6
18:4n-3	3.41 a	2.25 b	1,7
20:3n-3	0.283 a	1.41 b	0,0
20:4n-3	0	0	0,0
20:5n-3	16.0 a	7.49 b	5,8
21:5n-3	0	0	0,0
22:5n-3	21.4 a	14.7 b	10,5
22:6n-3	34.2 a	34.1 a	22,6
Total n-3 PUFA	90.6 a	75.4 b	53,2
Total PUFA	223 a	210 a	181,3

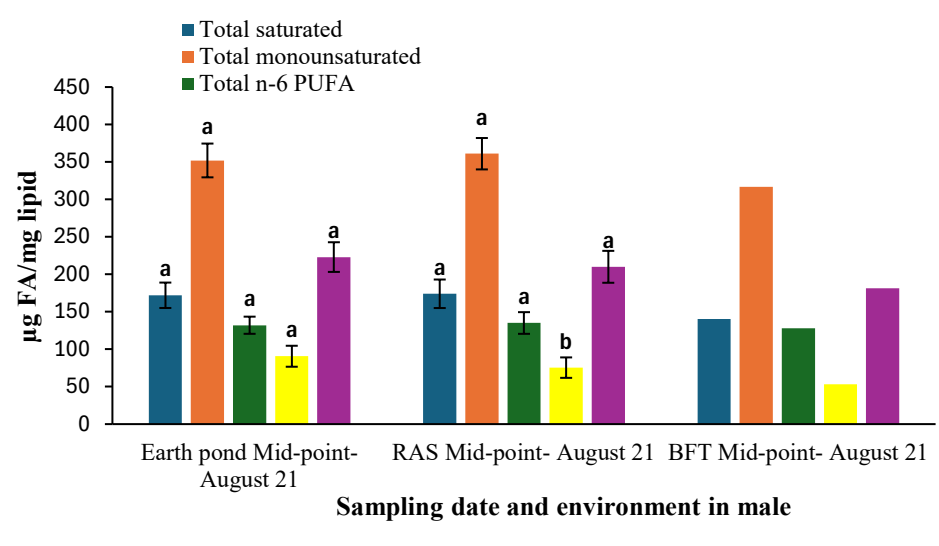


Figure 29. Fatty acid composition (µg FA/mg lipid: mean ± SD) in male mullet from earth pond and RAS treatments at mid-point sampling. Bar colours represent different lipid categories in male mullet across treatments in mid-point: blue (total saturated fat), orange (total monounsaturated fat), green (total n-6 PUFA), yellow (total n-3 PUFA), and purple (total PUFA). Sample sizes: earth pond mid-point (n = 7), RAS mid-point (n = 6) and BFT (mid-point). Different lowercase letters indicate significant differences (P < 0.05) between treatments at mid-point sampling using U. Mann-Whitney test (P < 0.05). BFT data are shown for descriptive purposes only (n = 1) and were not included in the statistical analysis.

Lipid analysis in female **(Table 3, Fig. 30)** revealed higher values in the RAS (n = 4) treatment compared to earth pond (n = 6) in initial sample, with increases observed in lipid weight (9.88 ± 3.62 mg vs 4.17 ± 0.51 mg, P = 0.012), the percentage of lipids in the LH fraction (3.95 ± 1.38% vs 1.76 ± 0.19%, P = 0.016), and phosphatidylserine (PS) content (16.8 ± 5.21% vs 8.13 ± 0.89%, P = 0.002). When comparing lipid analysis between sampling points within each treatment in female, a significant increase was observed in the earth pond treatment for both % lipids (LH) and % lipids (PS). Specifically, the percentage of lipids in the LH fraction increased from 1.76 (initial sample, n = 6) to 3.5 (mid-point sample, n = 5) (P = 0.045), while the percentage of phosphatidylserine (PS) rose from 8.13 (initial sample, n = 6) to 15.1 (mid-point sample, n = 5) (P = 0.010).

In males **(Table 4, Fig. 31)**, the lipid weight and percentage of lipids in the LH fraction revealed higher values in the RAS (n = 6) treatment compared to earth pond (n = 7) in mid-point, with increases observed in lipid weight (22.0 ± 14.3 mg vs. 11.1 ± 4.01 mg, P = 0.004), the percentage of lipids in the LH fraction (49.4 ± 12.2 % vs. 36.2 ± 12 %, P = 0.007). Although the BFT treatment was excluded from statistical analysis due to a single sample, it exhibited markedly

higher values for lipid weight (17.4 mg), % LH lipid (49), and % PS content (83.2) compared with the other treatments.

Table 3. Lipid weight, % lipids (LH), % lipids (PS) in female mullet from earth pond and RAS treatments at initial and mid-point samplings (mean ± SD). Different lowercase letters indicate significant differences ($P < 0.05$) between treatments within the same sampling point, while capital letters indicate significant differences between sampling points within the same treatment using Holm–Sidak test ($P < 0.05$). Sample sizes: earth pond initial ($n = 6$), RAS initial ($n = 4$), earth pond mid-point ($n = 5$), RAS mid-point ($n = 6$).

	Initial sample		Mid-point sample	
	Earth pond	RAS	Earth pond	RAS
Lipid weight (mg)	4.17 a, A	9.88 b, A	6.55 a, A	10.5 a, A
% lipids (LH)	1.76 a, A	3.95 b, A	3.5 a, B	4.66 a, A
% lipids (PS)	8.13 a, A	16.8 b, A	15.1 a, B	17.5 a, A

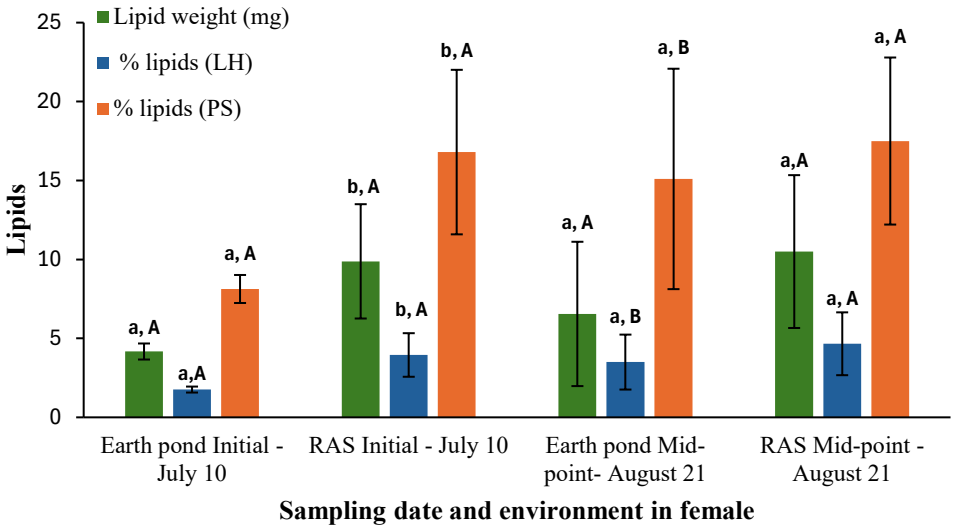


Figure 30. Lipid weight (mg), % lipids (LH), % lipids (PS) in female mullet from earth pond and RAS treatments at initial and mid-point samplings (mean ± SD). Bar colours represent different lipid categories in female mullet across treatments in initial and mid-point: green (lipid weight -mg), blue (% lipids LH), and orange (% lipids PS). Different lowercase letters indicate significant differences ($P < 0.05$) between treatments within the same sampling point, while capital letters indicate significant differences between sampling points within the same treatment using Holm–Sidak test ($P < 0.05$). Sample sizes: earth pond initial ($n = 6$), RAS initial ($n = 4$), earth pond mid-point ($n = 5$), and RAS mid-point ($n = 6$).

Table 4. Lipid weight (mg), % lipids (LH), % lipids (PS) in male mullet from earth pond, RAS and BFT treatments (mean ± standard deviation). Different lowercase letters indicate significant differences ($P < 0.05$) between treatments at mid-point sampling using U. Mann-Whitney test ($P < 0.05$). BFT data are shown for descriptive purposes only ($n = 1$) and were not included in the statistical analysis. Sample sizes at mid-point: earth pond ($n = 7$), RAS ($n = 6$), and BFT mid-point ($n = 1$).

	Earth pond Mid-point	RAS Mid-point	BFT Mid-point
Lipid weight (mg)	11.1 a	22.0 b	17.4
% lipids (LH)	36.2 a	49.4 b	49.0
% lipids (PS)	71.4 a	67.8 a	83.2

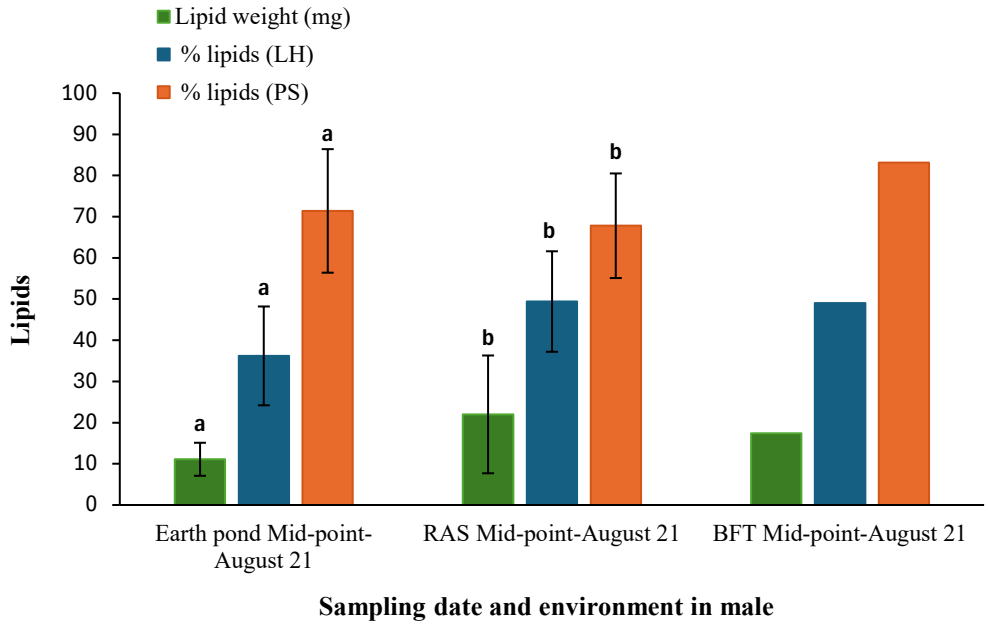


Figure 31. Lipid weight (mg), % lipids (LH), % lipids (PS) in male mullet from earth pond and RAS treatments at mid-point sampling (mean ± SD). Bar colours represent different lipid categories in male mullet across treatments in mid-point: green (lipid weight-mg), blue (% lipids LH), and orange (% lipids PS). Different lowercase letters indicate significant differences ($P < 0.05$) between treatments at mid-point sampling using U. Mann-Whitney test ($P < 0.05$). BFT data are shown for descriptive purposes only ($n = 1$) and were not included in the statistical analysis. Sample sizes at mid-point: earth pond ($n = 7$), and RAS ($n = 6$), and BFT mid-point ($n = 1$).

5. Discussion

This study assessed the reproductive maturation and welfare of mullet, focusing on two biologically distinct mullet groups: first-maturation individuals and wild-caught adults. Fish were maintained under three aquaculture systems with different environmental and management characteristics: biofloc technology (BFT), recirculating aquaculture systems (RAS), and earth pond. Overall, the results highlight the complex interaction between environmental conditions,

physiological stress responses, nutritional status, and reproductive development in captive mullet.

Reproductive Maturation and Stress

First-maturation individuals failed to complete reproductive development in all treatments. They exhibited physiological signs of prolonged activation of the hypothalamic-pituitary-interrenal (HPI) axis, consistent with sustained or repeatedly triggered stress responses, as evidenced by significant, progressive increases in cortisol over time, particularly in the RAS treatment ($P < 0.001$). Although absolute cortisol values measured with the Abbott i-STAT analyser were lower than those reported in literature using radioimmunoassay (RIA) methods (Barthel *et al*., 2003), the consistent temporal increase indicates sustained HPI axis activation rather than a single acute stress event (Wendelaar Bonga, 1997; Schreck, 2010). Previous studies have shown that portable point-of-care blood analysers such as the i-STAT may yield hormone values that differ from laboratory-based immunoassays, highlighting the importance of interpreting cortisol dynamics primarily through temporal trends rather than absolute concentrations (Stoot *et al.,* 2014).

Recent species-specific research on mullet juveniles (Linares-Cordova *et al*., 2024) has demonstrated the existence of proactive and reactive stress coping styles, characterised by intrinsic differences in endocrine and metabolic responses to stress. Proactive individuals exhibit lower cortisol and glucose elevations following stress exposure compared with reactive individuals. Consequently, part of the variability observed in cortisol and metabolic biomarkers in the present study may reflect intrinsic coping-style differences rather than a uniform stress condition across all individuals. This finding emphasises the importance of considering species-specific physiological variability when interpreting stress responses in mullet.

Metabolic biomarkers further support the presence of stress-related physiological disturbance. Plasma glucose and lactate concentrations were particularly elevated in earth pond fish, suggesting repeated exposure to acute stress events associated with handling and capture procedures such as trawl netting (Barthel *et al.*, 2003; Pottinger *et al.*, 2000). Although baseline glucose values for unstressed mullet are not well established, the persistence of elevated glucose

concentrations across successive sampling events suggests a reduced capacity for physiological recovery following repeated challenges. Sustained hyperglycemia in teleosts is commonly associated with cortisol-mediated gluconeogenesis and glycogenolysis, which mobilise energy reserves in response to stressors (Wendelaar Bonga, 1997; Huntingford *et al*., 2006).

Despite favorable somatic condition (K = 1.39 – 1.77), these fish appeared to exhibit limited physiological recovery following repeated stressors, which may have contributed to altered activation of the brain–pituitary–gonad (BPG) axis and, consequently, to reduced vitellogenesis and gametogenesis (Ibarra-Zatarain *et al*., 2019). Elevated lactate concentrations also indicate increased anaerobic metabolism associated with intense physical activity during capture or handling procedures, further suggesting that management practices may contribute to metabolic disturbance in aquaculture systems. Under normal conditions, an increase in oocyte diameter is accompanied by a significant rise in estradiol (E_2) levels, a trend widely observed following administration of recombinant gonadotropins (rGths) in various teleost species, including goldfish (*Carassius auratus*) (Kobayashi *et al.*, 2006), spotted scat (*Scatophagus argus*) (Zhang *et al.*, 2017), European eel (*Anguilla anguilla*) (Jéhannet *et al.*, 2023a), short-finned eel (*Anguilla australis*) (Nguyen *et al.*, 2020), and flathead grey mullet (*Mugil cephalus*) (Cerrud-Barría *et al.*, 2026). Similarly, spermiation events—accompanied by significant increases in 11-KT concentrations—have been reported following rGths treatment in mullet (*Mugil cephalus)* (Ramos-Júdez *et al.*, 2022b), Japanese eel (*Anguilla japonica*) (Hayakawa *et al.*, 2009), zebrafish (*Danio rerio*) (García-López *et al.*, 2009), Senegalese sole (*Solea senegalensis*) (Chauvigne *et al.*, 2017), and spotted scat (*Scatophagus argus)* (Zhang *et al.*, 2017). However, in the present study found that first-maturation females failed to elevate E_2 or advance past the previtellogenic stage, and males failed to show sustained increases in 11-KT and undergo spermatogenesis/spermiation. This suggests that the endocrine system activation observed in the present study was insufficient to overcome the inhibitory effects of chronic stress and/or poor environmental conditions. In contrast, wild-caught adults exhibited more advanced gonadal development, particularly within the biofloc technology (BFT) system, where females attained gonadosomatic index (GSI) values as high as 14.17%. Morphological examination of the gonads from females in the earth pond indicated two females in early vitellogenesis with mean oocyte diameters of 230 ± 20 µm, and one female in late vitellogenesis with a mean oocyte diameter of

546 ± 41 µm. Females from the RAS could not be cannulated for oocyte collection, nor could males from the RAS be stripped for sperm collection by abdominal massage, and the single male from the BFT treatment could not be sampled in this way either. These findings suggest that environmental complexity, prior reproductive experience, and a fully mature endocrine system collectively facilitate enhanced reproductive activation in adult mullet (Espino Barr *et al.*, 2016; Boubekeur and Ramdane, 2021).

Overall, these findings indicate that reproductive failure in captive mullet is not driven by a single limiting factor but rather by a mismatch between the fish´s endogenous endocrine status and external environmental conditions.

Environmental Influence and Welfare

The RAS system maintained stable physicochemical parameters (oxygen ~6.1 mg/L, pH ~7.8) and supported good somatic condition (K = 1.77). However, the progressive increase in cortisol levels observed suggests that one or a combination of confinement, environmental parameters, and environmental monotony induced sustained stress and compromised reproductive success (Amponsah *et al.*, 2021; McConnachie *et al.*, 2012).

The earth pond offered a more natural environment, but fish were subject to variable water quality and physical trauma. External scratches and injuries were common and likely sustained during collection or in contact with abrasive surfaces. These factors contributed to elevated metabolic stress indicators (Barthel *et al*., 2003) and may negatively affect both welfare and reproductive potential, despite otherwise acceptable water quality.

The BFT system provided a microbe-rich environment containing bioactive molecules that could promote reproductive processes (Avnimelech, 2007; Emerenciano *et al*., 2012). However, BFT also faced challenges such as ammonia spikes (up to 7 mg/L) and pathogen outbreaks (*Amyloodinium* sp.), highlighting the need for careful microbial and water quality management (Vinatea *et al*., 2018). The BFT water's pH and alkalinity showed no decline, and no acidification was evident. This is likely due to the strong buffering capacity, as indicated by the alkalinity near 250 mg/L and the water hardness near 650 mg/L. This showed that there was sufficient buffer capacity to maintain system balance despite the high microbial activity

expected in BFT systems. Despite these challenges, the BFT treatment supported reproductive maturation in wild-caught adults, likely due to its microbial diversity and bioactive compounds, which create an enriched, semi-natural environment (Avnimelech, 2007; Emerenciano *et al.*, 2012).

In general, wild-caught adults with breeding experience were better able to undergo reproductive development than captive-reared animals, particularly in the enhanced environment of the BFT systems. BFT has been widely recognised as an innovative aquaculture approach capable of improving productivity in several cultured species, including tilapia (Avnimelech, 2007), common carp (*Cyprinus carpio*) (Najdegerami *et al*., 2016), and African catfish (*Clarias gariepinus*) (Yusuf *et al*., 2015). The effect of BFT on the reproductive performance of broodstock has been studied in a few aquatic animals. These studies suggest that BFT supports gonadal development and ovarian maturation of broodstock, synergizing reproductive processes as observed in shrimp (Emerenciano *et al*., 2012). However, the effects of BFT on fish and shellfish reproduction remain incompletely understood, and success depends strongly on rigorous environmental and sanitary management (Ogello, 2021; Khanjani *et al*., 2024).

However, as indicated under IRTA conditions, the BFT systems did not fully stabilise to establish mature BFT, and this resulted in ammonia peaks that may have contributed to disease issues. To establish BFT, a defined carbon source, anhydrous glucose, needs to be added daily, representing 43.05% of the carbon (personal communication, Dr. Luis Vinatea, Universidad Federal de Santa Catarina, Brazil). Vinatea suggested that, to stabilize BFT in IRTA, different C:N ratios (i.e., 20:1, 30:1, and 40:1) should be tested to determine which best promotes heterotrophic bacterial growth for the further establishment of chemolithoautotrophic bacteria. Similarly, Vinatea *et al*. (2018), under similar water quality conditions, observed that heterotrophic bacteria did not consistently dominate the floc bacterial community in the present study. Anhydrous glucose was applied in the current study at a C:N ratio of 20:1, as suggested by Avnimelech (1999). Future studies should aim to define the C:N ratios required to establish BFT under IRTA conditions.

This further suggests that the dissociation between positive body growth and reproductive failure indicates that traditional husbandry parameters are inadequate for evaluating welfare or reproductive status. Therefore, integrated welfare assessment combining hormonal, behavioural, and environmental metrics is critical (Cavallino *et al.*, 2023; Bhat *et al.*, 2025).

Nutritional Factors

Lipids from dietary sources, gonadal de novo synthesis, or storage tissues are vital for reproduction (Wiegand, 1996; Tocher, 2003). The lipid and fatty acid composition of broodstock diets is a critical metabolic resource influencing reproductive success and offspring viability (Izquierdo *et al*., 2001; Johnson, 2009).

Analysis of gonadal fatty acids revealed critical nutritional deficiencies in captive fish, notably the near absence of bacterial-derived heptadecenoic acid (17:1), which was found to be present at high levels in wild mullet ovaries (Ramos-Júdez *et al*., 2023), suggesting that this fatty acid may be essential for ovarian development in this species. Wild mullet obtain this fatty acid naturally by feeding on bacterial-rich substrates (Darnell, 1958), whereas commercial diets may not fully replicate this natural nutritional profile.

RAS fish had higher total lipid weight, but the fatty acid composition was poor with regard to n-3 PUFAs, including DHA, EPA, and ARA. These fatty acids are vital components of gamete membranes and precursors of steroidogenic eicosanoids essential for ovulation (Tocher, 2003). Earth pond fish had higher n-3 PUFA levels, which may still be insufficient to fully support vitellogenesis.

Nutritional deficits and chronic stress may have acted in concert to suppress activation of the BPG axis and to prevent reproductive development despite adequate somatic growth (Sánchez-Velázquez *et al.*, 2024). Similar reproductive impediments of sub optimal nutrition and stress have been reported for a number of teleost species, including European eel (*Anguilla anguilla*) (Heinsbroek *et al.*, 2013), Senegalese sole (*Solea senegalensis*) (Morais et *al.*, 2014), gilthead seabream (*Sparus aurata*) (Fernández-Palacios *et al.*, 1995), Japanese flounder (*Paralichthys olivaceus*) (Furuita *et al.*, 2000), and Atlantic halibut (*Hippoglossus hippoglossus*) (Mazorra *et*

al., 2003)—where poor nutrition and stress reduce gamete quality, fertilisation rates, and larval survival.

6. Conclusions

In this study, wild-caught adults from earth ponds were close to reaching reproductive maturity, and adults from the biofloc technology (BFT) system exhibited similar or better reproductive status. These observations suggest that, within these environments, the interplay of environmental parameters, stress levels, and nutritional status was generally sufficient to support reproductive activation. Conversely, first-maturation individuals appear to require more specific, possibly more stringent, conditions to successfully complete their reproductive development. Accordingly, it is recommended that captive breeding programs implement integrated, stage-specific management approaches that encompass environmental enrichment, carefully tailored nutritional support, and thorough welfare assessment to optimise reproductive outcomes. Moreover, further research on hormonal and behavioural dynamics is warranted to refine breeding protocols and alleviate stress, particularly in complex culture systems such as: BFT and earth pond, which offer promising benefits through enriched, more naturalistic rearing environments.

CHAPTER V.

RECOMBINANT FOLLICLE STIMULATING HORMONE PROMOTES SPERMATOGONIAL PROLIFERATION AND SPERMATOGENESIS ONSET IN JUVENILE FLATHEAD GREY MULLET (*MUGIL CEPHALUS*)



1. Introduction

Follicle-stimulating hormone (Fsh) plays a fundamental role in fish spermatogenesis, with particular importance during the spermatogonial phase (Schulz *et al.*, 2010a; Xie *et al.*, 2020). This phase encompasses two distinct mitotic processes: slow self-renewal to sustain the stem cell pool, and rapid proliferation of cells committed to differentiation and subsequent entry into meiosis (Clermont, 1972; Miura and Miura, 2001). Fsh stimulates spermatogonial mitosis by modulating sex steroid and growth factor production, though these pathways show variation across different species (Schulz *et al*., 2010b; De Rooij and Griswold, 2012; Xie *et al.*, 2020) Fsh stimulates spermatogonial mitosis by modulating sex steroid and growth factor production, although these pathways show variation across different species (Schulz *et al*., 2010b; De Rooij and Griswold, 2012; Xie *et al.*, 2020). Generally, Fsh stimulates spermatogenesis indirectly by promoting androgen synthesis and secretion (Schulz *et al.*, 2010a). However, research in zebrafish (*Danio rerio*) (Crespo *et al.*, 2016), rainbow trout (*Oncorhynchus mykiss)* (Sambroni *et al.*, 2013) and Atlantic salmon (*Salmo salar*) (Andersson *et al.*, 2024) has demonstrated that Fsh can also act directly on germ cell development, independently of androgen signalling, including Igf3, Amh, Wnt, and Hedgehog (Crespo *et al.*, 2016; Xie *et al.*, 2020). Studies across different teleost species indicate that Fsh plays a role in sexual differentiation, especially when undifferentiated gonads develop into testes or ovaries (Moles *et al.*, 2007; Taranger *et al.*, 2010), and also influences when puberty begins (Taranger *et al.*, 2010; Schulz *et al.*, 2019).

Over recent years, the use of recombinant gonadotropins (rGths) has gained importance for reproductive control in aquaculture. In addition to resolving reproductive dysfunctions that frequently affect captive broodstock, these treatments have been tested to induce earlier maturation and shorten production cycles in different species, with variable results (Kamei *et al.*, 2006; Mazón *et al.*, 2014; Sanchís-Benlloch *et al.*, 2017; Zupa *et al.*, 2023; Lancerotto *et al.*, 2025a; Ventriglia *et al.*, 2025a). In 20-month-old pre-pubertal male greater amberjack (*Seriola dumerili*), rGths treatment induced the production of high-quality strippable sperm 1–2 years earlier than under natural conditions (Lancerotto *et al.*, 2025b). Similarly, pre-pubertal meagre (*Argyrosomus regius*) exhibited initial spermatogenic activity following six weeks of rFsh administration and produced spermatozoa (Zupa *et al.*, 2023). The combined administration of rFsh and rLh to these pre-pubertal meagre successfully induced

spermiogenesis and spermiation, resulting in viable sperm production (Ventriglia *et al.*, 2025a). These experiments demonstrated that rFsh clearly stimulates spermatogonial proliferation and progression toward meiotic stages; however, its role in promoting spermatogonial self-renewal remained unclear, likely owing to the extended interval between treatment and tissue sampling (Zupa *et al.*, 2023; Ventriglia *et al.*, 2025b). Additionally, these meagres in pre-pubertal exhibited substantial germ cell apoptosis, predominantly affecting committed spermatogonia, and while rFsh treatment decreased germ cell death, this protective effect was lost when treatment ceased, with apoptosis returning to baseline levels (Ventriglia *et al.*, 2025b).

Recently, research with species-specific flathead grey mullet (*Mugil cephalus*) (hereafter referred to as "mullet") recombinant gonadotropins (rGths), has demonstrated effectiveness in resolving the severe reproductive failures characteristic of adult post- puberty breeders of this species held under intensive conditions (RAS system), enabling the production of high-quality fertilised eggs (Ramos-Júdez *et al.*, 2021, 2022b; Cerrud-Barría *et al.*, 2026). However, the effects of rGths on pre-pubertal mullet have not been researched. Three main objectives guided this study: i) to evaluate whether recombinant gonadotropin treatment could accelerate the onset of puberty in this species; ii) to gain deeper insights into how follicle-stimulating hormone influences spermatogonial self-renewal, proliferative activity, and progression through spermatogenesis; iii) to expand current understanding of testicular apoptosis during the pre-pubertal period in teleosts.

2. Materials and methods

2.1. Fish maintenance

An experiment was conducted from 16th to 23rd May 2024 to evaluate rFsh effects on spermatogonial proliferation and the onset of spermatogenesis in juvenile mullet. Twenty-eight males were randomly selected from a stock of 192 mullet that were grown from wild mullet fry introduced into IRTA facilities in 2020. Prior to the experiment, mullet were held in fibreglass tanks under natural environmental conditions (41°36′46″N 2°10′11″E). For identification, each mullet was tagged intramuscularly with a Passive Integrated Transponder (PIT) tag (Trovan®,

ZEUS Euroinversiones S.L., Madrid, Spain). The selected juveniles (mean ± SD body weight: 338.1 ± 47.1 grams; standard length: 31.3 ± 1.5 cm) were randomly assigned to three experimental groups control day 0 group (named C-0; N = 9); control day 7 group (named C-7; N = 9); and rFsh-treated group (named rFsh-T; N=10). The experimental groups were placed in a 10 m^3 rectangular fibreglass tank (6.1 × 2.1 × 1.2 m), LD12:12 on May 16th, 2024 (L: light; D: dark), with constant temperature of 24°C and salinity of 36‰ in a recirculation system (IRTAmar®) at IRTA La Ràpita, Spain.

The tank had both surface and bottom water outlets that were adjustable to control the volume of water exiting each outlet, with a 20% daily renewal of the system's water. Fish were fed daily with a commercial Repro Sole diet (5 mm) (Sparos, Portugal) at 1.5% body weight. The diet contained 60% protein, 13% fat, 0.5% fibre, 7% ash, 1.2% phosphorus, and 0.9% calcium.

Ethics. The research was carried out in adherence to the following regulations: the Spanish Royal Decree 53/2013 of February 1st, which also concerns the protection of animals used for scientific purposes; the Catalan Law 5/1995 of June 21st, which pertains to safeguarding animals used for scientific purposes; and the Catalan Decree 214/1997 of July 30th, which governs the regulation of such animals. The European Directive 2010/63/EU was implemented on September 22nd, 2010. The Catalan Government and the Committee of Ethics and Experimental Animals (CEEA) of the Institute of Agrifood Research and Technology (IRTA) evaluated the procedures used in this study, which were authorized with authorisation of experimental project number 12016, expedient number FUE-2023-02998468 and ID NT0G72MXQ. ARRIVE guidelines were adhered to by the authors.

2.2. Sampling and recombinant hormone induction

All fish were fasted for 24 h prior to injection and tissue collection. Before euthanasia, blood was drawn from each individual and maintained on ice until centrifugation (3000 RPM, 4 °C, 15 min). The resulting plasma was transferred to Eppendorf tubes and frozen at -80 °C for subsequent analysis. Fish were then euthanized using an excess dose of MS-222 anesthetic, followed by brain destruction via pithing to collect gonadal tissue.

First sample: On 16th May 2024, 9 fish of the C-0 group were sacrificed and sampled (Table 1), and 9 fish of the C-7 group were given a single 0.35 mL injection of saline solution (C-7 group) or a single injection of single-chain rFsh at the dose of 12 µg/kg (rFsh-T group).

Second sample: On May 23rd, 2024, sampling was conducted on both control specimens (C-7 group) and rFsh-treated fish (rFsh-T group). Biometric measurements were recorded for each individual, including total length (TL, cm), body mass (BM, grams), and gonad mass (GM, grams). These measurements were used to calculate the gonadosomatic index (GSI) as GSI = 100 × GM/BM (Table 1). Subsequently, small sections of testicular tissue were excised from each fish and preserved in Bouin's fixative for subsequent histological and immunohistochemical examination.

The fish were anesthetised with MS-222 at a dose of 73 mg/L. The rFsh used was produced using Chinese hamster ovary (CHO) cells and isolated Gth-coding cDNAs of mullet by Rara Avis Biotec S.L., Valencia (Ramos-Júdez *et al.*, 2022b).

Table 1. Morphometric parameters and gonadosomatic index (GSI) of juvenile male mullet used in the experiment.

Fish group	Total length (cm)	Body mass (grams)	Gonad mass (grams)	GSI
C-0	33.0	366.3	0.03	0.01
	30.0	288.6	0.06	0.02
	31.5	365.7	0.10	0.03
	30.0	288.9	0.05	0.02
	31.0	327.4	0.10	0.03
	31.0	310.9	0.08	0.03
	29.0	276.3	0.10	0.04
	31.0	291.3	0.13	0.04
	31.5	290.0	0.24	0.08
C-7	33.0	383.6	0.16	0.04
	33.5	398.2	0.15	0.04
	31.5	389.1	0.19	0.05
	31.0	303.6	0.13	0.04
	31.0	324.6	0.18	0.06
	32.5	371.2	0.25	0.07
	33.5	373.4	0.22	0.06
	30.0	284.3	0.13	0.05
	31.0	285.8	0.12	0.04
rFsh-T	33.5	451.0	0.26	0.06
	28.0	301.8	0.17	0.06
	30.5	331.4	0.22	0.07
	33.0	389.9	0.28	0.07
	33.0	424.5	0.34	0.08
	29.0	281.4	0.33	0.12
	31.0	367.5	0.29	0.08
	32.0	341.4	0.15	0.04
	30.0	314.4	0.12	0.04
	30.0	344.8	0.10	0.03

2.3. Plasma steroid analysis

Plasma levels of 17β-estradiol (E_2), 11-KT were analysed using commercially available enzyme immunoassays (EIA) (Cayman Chemical Company, USA). Cortisol was analysed directly from undiluted plasma using a commercially available EIA kit (Demeditec Diagnostics GmbH, Germany). Steroids were extracted with methanol, which was evaporated, and extracts were re-suspended 1:4 (E_2) and from 1:50 (control groups) to 1:200 (rFsh-treated group) for 11-KT in the EIA buffer (Ramos-Júdez et al., 2022b).

2.4. Histology, immunohistochemistry and identification of apoptotic germ cells

Testis samples were fixed, dehydrated through ascending ethanol concentrations, cleared with xylene and embedded in paraffin. Sections of 4 μm were cut and stained with haematoxylin-eosin (H-E) for histological examination or subjected to immunohistochemical procedures and apoptotic detection.

Undifferentiated type A (Aund) spermatogonia—stem cells with self-renewal capacity in the germinal epithelium—were detected by immunohistochemistry against Nanos2, a zinc-finger RNA-binding protein essential for maintaining the spermatogonial stem cell pool. Mitotic activity in germ cells was assessed by immunohistochemistry using the marker Proliferating Cell Nuclear Antigen (PCNA), an auxiliary protein of DNA polymerase delta whose levels increase during late G1 and S phases, making it a reliable marker of cell proliferation.

Both Nanos2 and PCNA immunostaining procedures were essentially the same, except that antigen retrieval was required only for Nanos2 detection. This step consisted of heating sections in a microwave oven at 750 W for 20 min in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0). Before applying the primary antibodies, sections were treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. After this, sections were incubated with normal horse serum (NHS; Vector, Burlingame, CA, USA) for 30 min, followed by overnight incubation at 4°C in a moist chamber with either polyclonal anti-Nanos2 antibodies (Abcam, Cambridge, UK; ab76568) or monoclonal anti-PCNA antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Both antibodies were diluted in phosphate-buffered saline (PBS; 0.01 M, pH 7.4, 0.15 M NaCl) supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, Milan, Italy), at working concentrations of 1:25 and 1:50 for Nanos2 and PCNA, respectively. The avidin-biotin-peroxidase complex (ABC) technique was used for visualisation, employing the Vectastain Universal Elite Kit (Vector). Peroxidase activity was detected after a 10 min incubation with the 3,3'-diaminobenzidine (DAB) substrate (Vector). Specificity controls consisted of replacing primary antibodies with NHS or PBS alone.

Early apoptosis was assessed by immunohistochemical detection of Caspase-3—a heterodimeric protease of the peptidase C14A family that functions as the main executioner

enzyme in apoptosis. The procedure followed that described for Nanos2, except that citrate buffer (0.01 M, pH 6.0) was used instead of Tris-EDTA for antigen retrieval. Sections were then incubated overnight at 4°C with polyclonal anti-Caspase-3 antibodies (Bio-Techne, Minneapolis, USA; AF835) at a 1:150 dilution.

DNA fragmentation associated with late apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique with an in-situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany). Before adding the reaction mixture, sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 8 min. Terminal deoxynucleotidyl transferase was diluted 1:2 in the manufacturer's reaction buffer, and the signal was developed using a ready-to-use NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) solution (Roche Diagnostics).

2.5. Seminiferous tubule diameter and quantification of germ cell proliferation

For each testis, diameter measurements were obtained from a minimum of ten seminiferous tubules using the same histological preparations (hematoxylin-eosin). Concerning cell quantification, five randomly chosen areas were evaluated in each testicular section to determine two parameters: the number of undifferentiated type A spermatogonia (Aund) showing positive PCNA labeling (expressed as cells/mm² of germinal epithelium), and the number of spermatogonial cysts displaying positive labeling for the same marker (expressed as cysts/mm² of germinal epithelium). All measurements were performed by analyzing microphotographs captured with a DFC 420 digital camera (Leica, Cambridge, UK) mounted on a DIAPLAN light microscope (Leitz, Wetzlar, Germany) using the Leica Application Suite image analysis software (version 3.3.0, Cambridge, UK).

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (SD) as indicated. Shapiro-Wilk and Levene tests were used to assess the normality of the data distribution and the homogeneity of variance, respectively.

GSI analysis were transformed using the arcsine transformation, as pertinent to proportions (Sokal and Rohlf, 1970). An Outlier in 11-KT measurements was identified using Z-score analysis and excluded from further analyses.

The 11-KT/E_2 ratio showed extreme variability attributed to very low or near-zero E_2 concentrations in certain samples. To address this, a constant equivalent to the 5^{th} percentile of all E_2 measurements ($\varepsilon = 0.024$) was added to the ratio before applying a log-transformation (Aitchison, 1982).

One-way ANOVA followed by Tukey's post-hoc test was used to compare differences in GSI, seminiferous tubule diameter, density of anti-PCNA positive A_{und} spermatogonia and spermatogonial cysts, 11-KT/E_2 ratio and cortisol plasma concentration.

All statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $P < 0.05$ for all tests.

3. Results

3.1. Gonadosomatic Index (GSI)

The gonadosomatic index (GSI) and gonad mass (grams) reveal a positive correlation, as represented using a second-order polynomial (quadratic) equation: *Gonad mass (grams)* = $13{,}761GSI^2 + 2{,}6132\ GSI$; $R^2 = 0.99$ **(Fig. 1).**

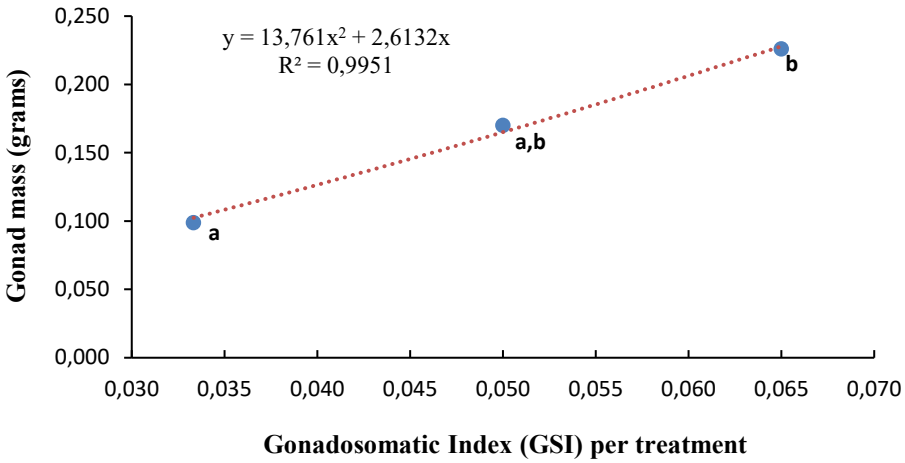


Figure 1. The positive correlation between gonadosomatic index (GSI) and gonad mass (grams) reveals a positive correlation in juvenile mullet, which was represented using a second-order polynomial (quadratic) equation: *Gonad mass (grams)* = 13,761*GSI*² + 2,6132 *GSI*; R² = 0.99. GSI values differed significantly between rFsh-T and C-0 treatments ($P < 0.05$), whereas both control groups and the rFsh-T versus C-7 comparison showed no statistical differences ($P = 0.07$ and $P = 0.34$, respectively). Different letters represent statistically significant differences (ANOVA; $P < 0.05$).

GSI values in the analysed fish varied from 0.01 to 0.12, showing no statistical differences between the two control groups ($P = 0.07$). Fish from the rFsh-T group exhibited significantly higher GSI compared to the C-0 group ($P < 0.05$), while no significant difference was observed when compared to the C-7 group ($P = 0.34$) **(Fig. 1).**

3.2. Histological

Histological analysis of the control group showed that seminiferous tubules contained large single spermatogonia with euchromatic nuclei, which are morphologically consistent with undifferentiated spermatogonia **(Fig. 2a, b, c)**. Differentiated spermatogonial cysts were scarce, and meiotic cells or sperm were not observed. Seminiferous tubules varied in size across individuals: some had barely visible lumens **(Fig. 2b)** while others showed well-developed lumens **(Fig. 2c)**. The single spermatogonia were labeled with anti-Nanos2 antibodies **(Fig. 2d)**, confirming they were undifferentiated stem spermatogonia.

Among rFsh-group, testicular development was variable. Six fish showed similar histology to controls **(Fig. 3a)** and were classified as non-responsive. Four fish had more advanced

spermatogenesis with differentiated spermatogonia and spermatocytes **(Fig. 3b)**, and were classified as responsive to rFsh. Seminiferous tubule diameter was significantly larger in responsive fish compared to controls and non-responsive fish **(Fig. 4)**.

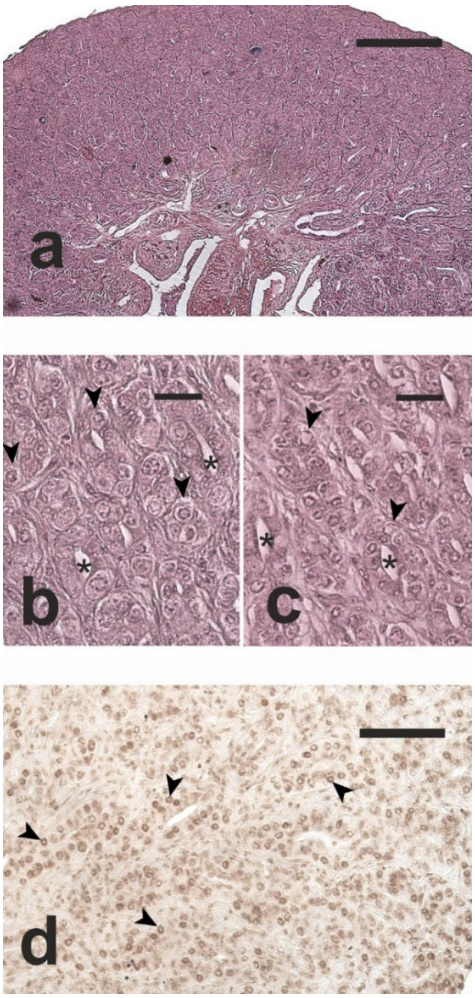


Figure 2. Testicular histology of juvenile mullet from control groups. a) Inactive testis from a non-treated control male collected at the start of the trial (C-0), displaying no signs of spermatogenic activity. H-E stain. b) Testicular section from a C-0 fish showing seminiferous tubules with isolated undifferentiated spermatogonia and either closed or barely visible luminal spaces. H-E stain. c) Seminiferous tubules from a saline-injected control fish sampled at day 7 (C-7), containing isolated undifferentiated spermatogonia and small luminal areas. H-E stain. d) Testicular section from a C-0 male immunolabeled with anti-Nanos2, revealing brown-stained cytoplasm in isolated undifferentiated spermatogonia. Scale bars: 200 µm (a), 20 µm (b, c), 50 µm (d). Arrowhead indicates undifferentiated spermatogonium; asterisk marks seminiferous tubule lumen.

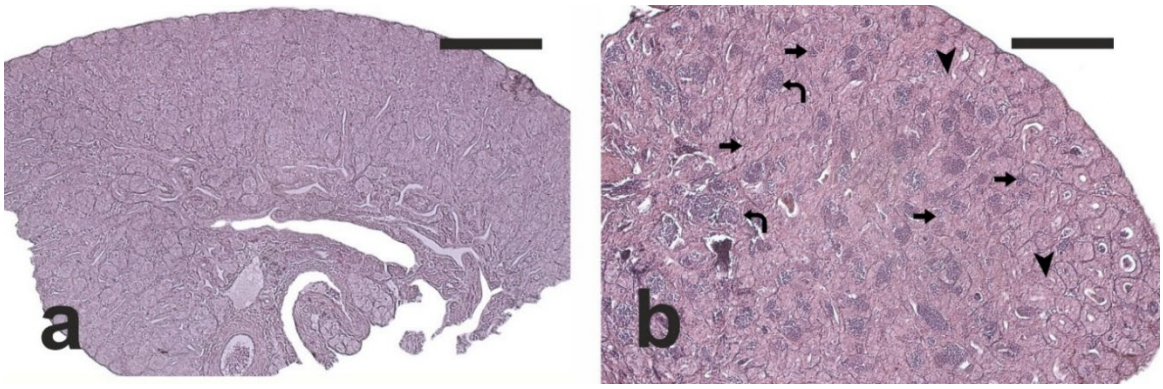


Figure 3. Testicular sections from rFsh-treated juvenile mullet. a) Inactive testis from a rFsh-treated fish (rFsh-T) that did not respond to hormonal treatment, with no evidence of spermatogenic progression. b) Testis from a rFsh-responsive fish (rFsh-T) displaying seminiferous tubules with germ cells at various stages of development. H-E stain. Scale bars: 200 µm. Arrowhead indicates undifferentiated spermatogonium; arrow marks spermatogonial cyst; curved arrow points to primary spermatocyte cyst.

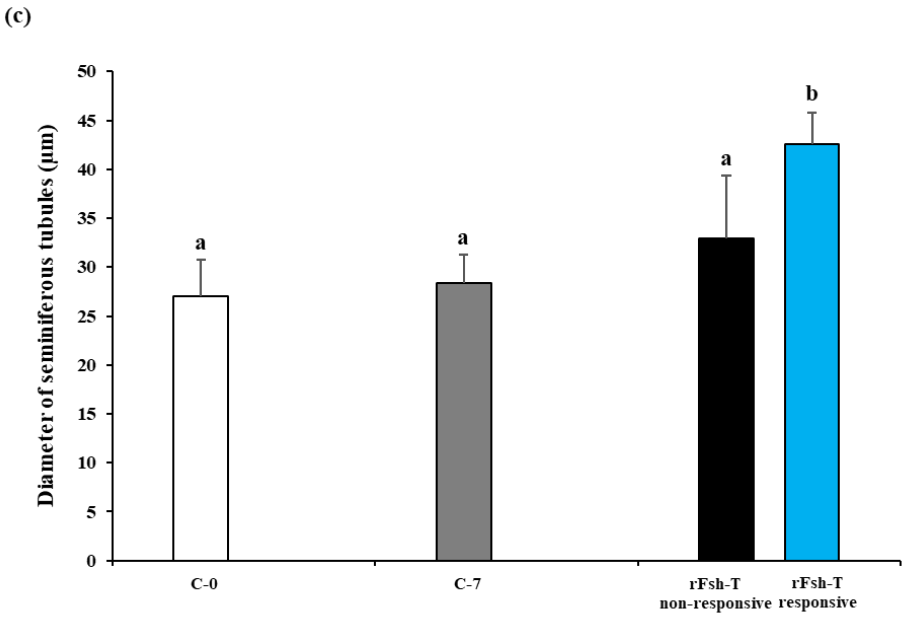


Figure 4. Seminiferous tubule diameter (mean ± SD) in juvenile mullet. C-0: non-treated control males collected at the start of the trial. C-7: saline-injected control males sampled at day 7. rFsh-T: males sampled seven days following rFsh treatment. Letters denote significant statistical differences (ANOVA; $P < 0.05$). rFsh-T fish were categorized as either responsive or non-responsive according to the developmental stages of germ cells present in testicular tissue.

PCNA immunolabeling revealed positive nuclei in certain large isolated spermatogonia, presumably undifferentiated, as well as in groups of more differentiated spermatogonia arranged within small cysts **(Fig. 5a, b)**. Additionally, primary spermatocyte cysts showing PCNA-positive nuclei were detected in two rFsh-T fish **(Fig. 5c)**.

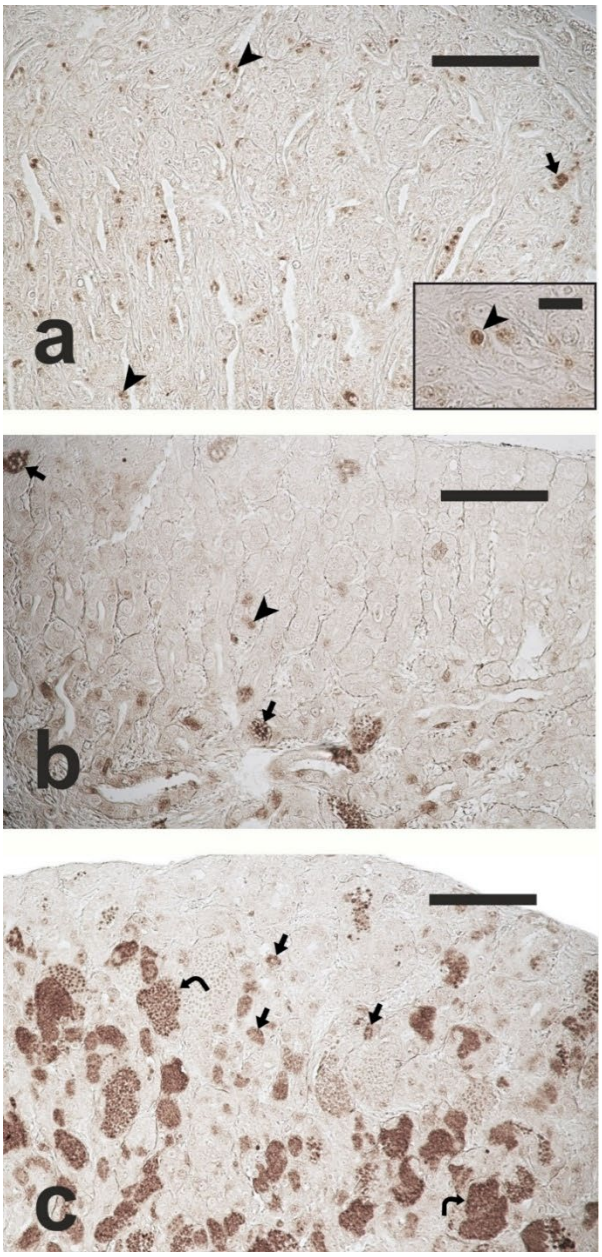


Figure 5. Testicular sections from juvenile mullet immunolabeled with anti-PCNA. a) Non-treated control male (C-0) displaying scattered PCNA-positive spermatogonia. The inset shows a high-magnification detail of a large, isolated PCNA-positive spermatogonium. b) Saline-injected control fish at day 7 (C-7) with PCNA-positive isolated spermatogonia and spermatogonial cysts. c) Responsive rFsh-treated fish (rFsh-T) exhibiting PCNA-positive spermatogonia and spermatocytes. Scale bars = 100 µm (a, b, c); 20 µm (inset in a). Brown staining indicates anti-PCNA immunopositive nuclei. Arrow indicates anti-PCNA-positive spermatogonial cyst; arrowhead marks anti-PCNA-positive single type Aund spermatogonium; curved arrow points to anti-PCNA-positive primary spermatocyte cyst.

Quantification of proliferating germ cells revealed that rFsh-T fish had significantly fewer isolated undifferentiated spermatogonia than both control groups ($P < 0.05$) **(Fig. 6a)**. On the

other hand, responsive rFsh-T fish showed significantly more proliferating differentiated spermatogonia compared to controls and non-responsive rFsh-T fish ($P < 0.05$ in all cases) **(Fig. 6b)**.

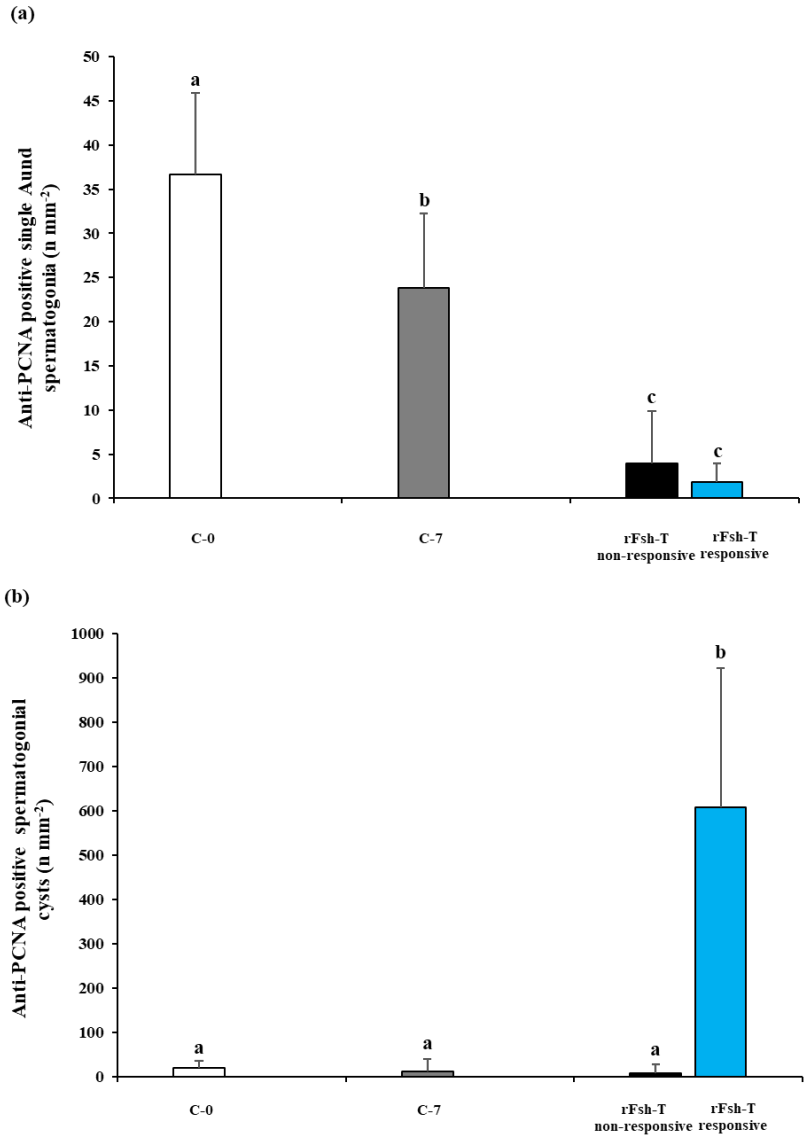


Figure 6. PCNA-positive germ cells density (mean ± SD) in juvenile male mullet from different treatment groups: non-treated controls (C-0), saline-injected controls (C-7), and rFsh-treated fish (rFsh-T, classified as non-responsive or responsive). (a) PCNA-positive single type Aund spermatogonia. (b) PCNA-positive spermatogonial cysts. Letters indicate significant differences among groups (ANOVA; $P < 0.05$).

Both TUNEL assay and anti-Caspase-3 immunostaining showed minimal apoptotic germ cell loss in all three groups, except for one rFsh-T fish that displayed scattered apoptotic spermatogonia and Sertoli cells **(Fig. 7)**.

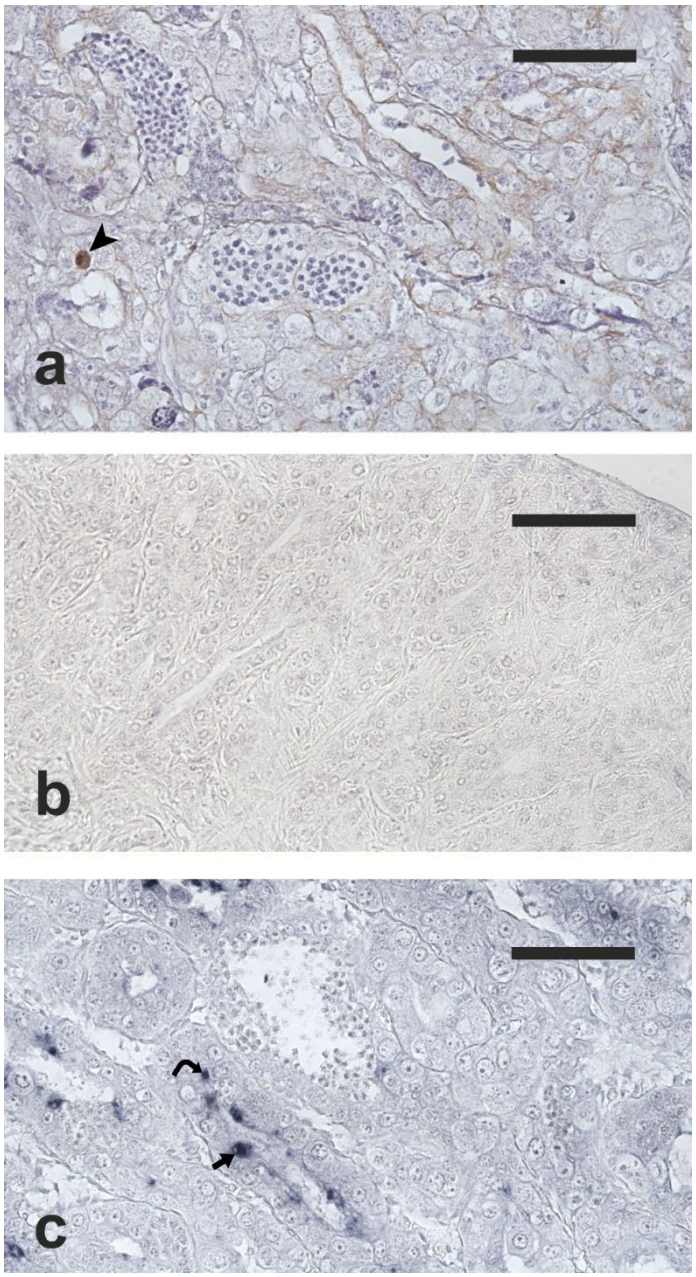


Figure 7. Testicular sections from juvenile mullet. (a) Testis from a responsive rFsh-T fish immunolabeled with anti-Caspase-3. (b) Testis from a non-treated control fish (C-0) showing negative TUNEL staining. (c) Testis from a responsive rFsh-T fish with apoptotic cells-stained dark blue by TUNEL method. Scale bars = 50 µm. Arrow indicates TUNEL-positive spermatogonium; arrowhead marks anti-Caspase-3-positive spermatogonium; curved arrow points to TUNEL-positive Sertoli cell.

Plasma 11-KT levels showed no significant differences between C-0 and C-7 groups ($P = 0.12$). However, fish that responded successfully to rFsh treatment displayed significantly higher plasma 11-KT concentrations compared to non-responsive fish ($P < 0.05$) **(Fig. 8)**.

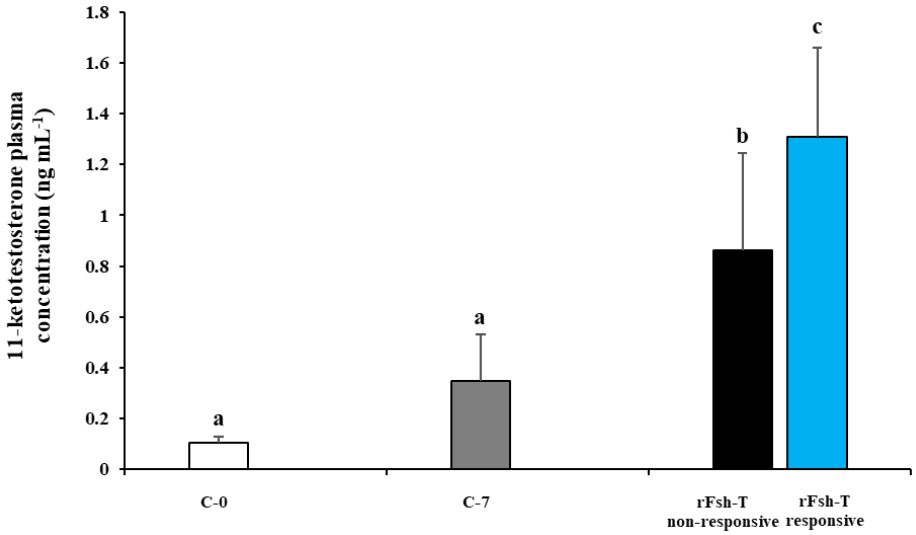


Figure 8. Effect of rFsh treatment and saline (control) on plasma 11-ketotestosterone (11-KT) levels (mean ± SD) in juvenile mullet. C-0: non-treated control fish; C-7: saline-injected fish sampled at day 7; rFsh-T: rFsh-treated fish. Based on testicular histology, rFsh-treated fish were categorized as either responsive or non-responsive. Letters indicate significant differences among groups (ANOVA; $P < 0.05$).

The 11-KT/E_2 ratio differed significantly between the two control groups ($P < 0.05$). Responsive rFsh-T fish displayed significantly elevated 11-KT/E_2 ratios relative to both control groups ($P < 0.01$). While responsive rFsh-T fish had higher 11-KT/E_2 ratios than non-responsive individuals, this difference did not reach statistical significance, likely due to the small sample size **(Fig. 9)**.

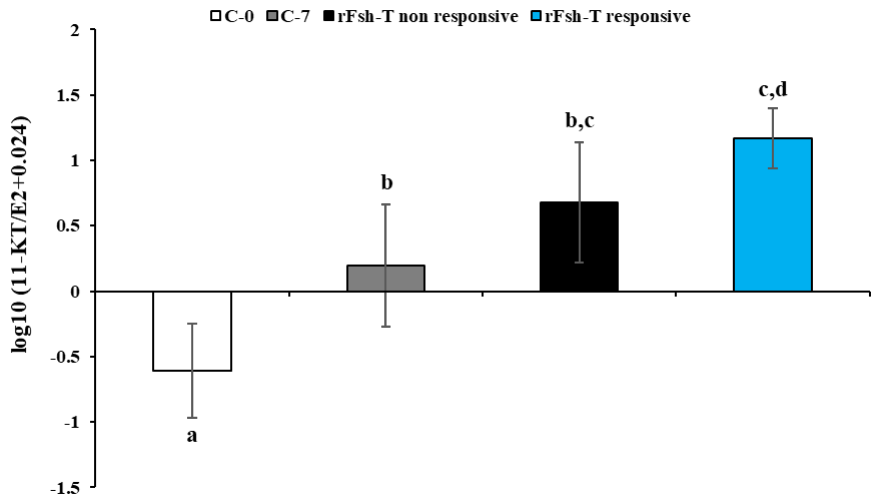


Figure 9. Effect of rFsh treatment and saline (control) on ratio plasma 11-ketotestosterone/17β-estradiol (11-KT/E_2) in juvenile mullet. C-0: non-treated control fish; C-7: saline-injected fish sampled at day 7; rFsh-T: rFsh-treated fish. Based on testicular histology, rFsh-T fish were categorized as either responsive or non-responsive. Letters indicate significant differences among groups (ANOVA; $P < 0.05$).

Regarding plasma cortisol, the C-0 group displayed significantly elevated levels compared to C-7 and rFsh-T fish ($P < 0.05$) **(Fig. 10)**, indicating that fish gradually adapted to experimental procedures with reduced stress over time.

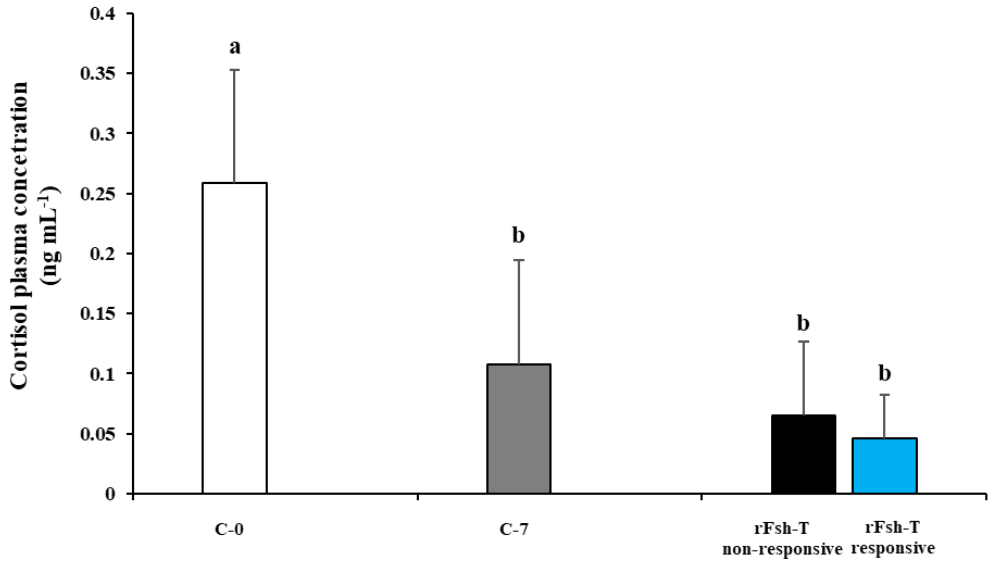


Figure 10. Effect of rFsh treatment and saline (control) on plasma cortisol levels (mean ± SD) in juvenile mullet. C-0: non-treated control fish; C-7: saline-injected fish sampled at day 7; rFsh-T: rFsh-treated fish. Based on testicular histology, rFsh-T fish were categorized as either responsive or non-responsive. Letters indicate significant differences among groups (ANOVA; $P < 0.05$).

4. Discussion

In recent years, recombinant gonadotropin technology has become increasingly valuable for aquaculture applications, including overcoming reproductive dysfunctions in adult fish (Hayakawa *et al.*, 2009; Ramos-Júdez *et al.*, 2022b; Lancerotto *et al.*, 2025; Cerrud-Barría *et al.*, 2026) and the stimulation of gametogenesis in pre-pubertal individuals (Kamei *et al.*, 2006; Mazón *et al.*, 2014; Sanchís-Benlloch *et al.*, 2017; Zupa *et al.*, 2023; Ventriglia *et al.*, 2024; Lancerotto *et al.*, 2025). Previous studies using the single-chain recombinant *Mugil cephalus* Fsh (rFsh) produced in CHO cells showed that its combination with recombinant Lh (rLh) effectively stimulates vitellogenesis, oocyte maturation, spermiation, and spawning in captive adult mullet (body mass ≥ 0.9 kg, standard length ≥ 34 cm) (Ramos-Júdez *et al.*, 2021, 2022b; Cerrud-Barría *et al.*, 2026). Under intensive rearing conditions, captive mullet females fail to

complete vitellogenesis while males do not produce fluid sperm (Ramos-Júdez *et al.*, 2022a). To overcome this arrested reproductive state, a biphasic recombinant gonadotropin strategy consisting of an initial phase of rFsh administration followed by combined rFsh and rLh treatment proved essential. When implemented in the Western Mediterranean, this protocol successfully triggered gametogenesis throughout July-September and spawning during September-October, coinciding with the natural breeding season (Ramos-Júdez *et al.*, 2023). Notably, the identical treatment regimen also stimulated gametogenesis from March to June and spawning in June-July, thereby achieving reproductive success three months prior to the typical spawning (Cerrud-Barría *et al.*, 2026). While these protocols have proven highly effective in adult mullet with arrested gametogenesis, their application in immature fish remains largely unexplored. The present study investigated the response of juvenile mullet (~300 grams body mass) with recently differentiated gonads to recombinant gonadotropin treatment. Unlike the captive adults, where the brain-pituitary-gonadal (BPG) axis is functional but suppressed, juveniles possess an BPG axis that is not yet fully operational. Consequently, a simplified single-dose rFsh protocol (12 μg/kg) was tested rather than the biphasic approach required for adults. Results revealed a partial response, with 40% of treated juveniles exhibiting activation of early spermatogenesis but not reaching spermiation or expressible milt—contrasting sharply with the 100% response rate and complete reproductive maturation observed in adults receiving the two-phase rFsh/rLh protocol (Ramos-Júdez *et al.*, 2022b; Cerrud-Barría *et al.*, 2026), although the latter was a long term 13-week rGth protocol.

Inducing sexual maturation in genuinely pre-pubertal fish presents challenges distinct from reactivating suppressed reproduction in adults. Our experimental population consisted of small fish (~300 grams) whose reproductive systems were fundamentally immature; some individuals had not completed gonadal sex differentiation and therefore required exclusion. Thus, the limited response observed here likely reflects the developmental status of the reproductive axis rather than a lack of hormonal sensitivity per se. The partial activation induced by rFsh alone- initiation but not completion of spermatogenesis- suggests that more comprehensive stimulation may be required. In this context, exploring the combined administration of rFsh and rLh in juvenile fish warrants further investigation, as it may promote complete sexual maturation at

earlier phases and potentially shorten the time required to obtain reproductively competent broodstock under captive conditions.

Environmental stress from intensive culture conditions and physical handling during treatment administration may have contributed to the observed variability in response. While previous research has shown cortisol can spike dramatically under stressful conditions—reaching 537 ± 53 ng/mL in confined *Leuciscus cephalus* after just 4 hours and approximately 700 ng/mL in salmonids within an hour of handling (Pottinger *et al.*, 1999, 2000)—the levels we measured in juvenile mullet remained considerably lower. Both control and treated groups exhibited a downward trend in cortisol over the seven-day experimental window, indicating successful adjustment to the holding conditions. This acclimation likely resulted from either lower fish density or more stable environmental parameters. The relevance of this stress reduction cannot be overstated, given that reproductive processes in teleosts are highly sensitive to cortisol—both short-term spikes and prolonged elevation can suppress gonadal function and spawning performance (Schreck, 2010; McConnachie *et al.*, 2012). Notably, rFsh-treated juveniles maintained relatively low cortisol concentrations throughout, suggesting the hormone successfully triggered spermatogenesis without interference from chronic stress. Additionally, the absence of a treatment-induced cortisol spike indicates that the injection procedure itself didn't induce physiological stress that might have undermined reproductive responses. From an applied perspective, these observations highlight why stress management matters in production settings: elevated cortisol under high-density recirculating conditions frequently disrupts reproduction (Amponsah *et al.*, 2021), making pre-treatment acclimation a critical component of hormone-based breeding protocols.

The experimental design placed hormone-treated and control fish in shared tanks for the entire seven-day period, a design choice that warrants consideration from a methodological standpoint. Published literature indicates that fish can release steroid hormones into their aquatic environment, where neighboring individuals may absorb them (Sorensen and Goetz, 1993; Vermeirssen and Scott, 1996). Additionally, spermatogenically active males produce reproductive pheromones that potentially stimulate endocrine changes in nearby conspecifics (Kobayashi *et al.*, 2002; Sorensen and Hoye, 2007). However, evidence from mullet studies

suggests minimal impact under typical experimental settings. Both Ramos-Júdez *et al.* (2022) and Cerrud-Barría *et al.* (2026) co-housed control fish with rGth-treated individuals and detected no plasma 11-KT increase in controls, demonstrating that hormone transfer through water or pheromonal stimulation did not materialise despite proximity. Our data tell a similar story. Control fish sampled at day 7 (C-7) had numerically higher 11-KT than those sampled initially (C-0), but statistical analysis revealed no significant difference. What matters most is that testicular histology showed absolutely no spermatogenic activation in controls—meaning that even if some subtle endocrine shifts occurred through indirect mechanisms, the complete spermatogenic program required direct exogenous gonadotropin input. This interpretation gains strength when considering that 60% of fish that were actually injected with rFsh still didn't develop active spermatogenesis, demonstrating that spermatogenic readiness—not mere hormone availability—represents the critical limiting factor. Taken together, these results validate our experimental framework and demonstrate that shared housing did not undermine the control condition.

In sexually immature juveniles, the brain-pituitary-gonad axis has not yet reached full functionality (Carrillo *et al.*, 2009; Taranger *et al.*, 2010), and the testicular germ cell compartment is dominated by undifferentiated or stem spermatogonia (Schulz *et al.*, 2010b). These cells remain in a relatively inactive state during this phase, undergoing mostly self-renewal divisions without actively initiating spermatogenesis (Schulz *et al.*, 2010a; Lacerda *et al.*, 2014; Xie *et al.*, 2020). This characteristic makes immature fish an appropriate model for studying how Fsh regulates the balance between spermatogonial self-renewal and differentiation. Thus, in addition to evaluating the feasibility of advancing puberty in farmed mullet, the present experiment sought to improve our understanding of Fsh effects on juvenile testicular tissue.

Fsh is recognised as a key hormone for initiating spermatogenesis in fish, playing a crucial regulatory role during the early developmental stages of male germ cells (Ohta *et al.*, 2007; Schulz *et al.*, 2010). Research using zebrafish testicular tissue cultures has revealed that this hormone acts through multiple cell types: Leydig cells support spermatogonial self-renewal via Wnt5a signaling and promote both differentiation and proliferation via insulin-like peptide 3

(Insl3) (Xie *et al.*, 2020). In Sertoli cells, Fsh triggers insulin-like growth factor 3 (IGF3) production, which subsequently drives spermatogonial renewal and meiotic progression through IGF receptor pathways (Nóbrega *et al.*, 2015). Studies using both cultured tissues (Xie *et al.*, 2020) and live animals (Mazón *et al.*, 2014) have confirmed that Fsh promotes spermatogenesis primarily by boosting androgen production. This steroid-dependent mechanism was clearly demonstrated in immature Japanese eels, where blocking steroid synthesis with trilostane completely prevented Fsh from inducing spermatogenesis. Different steroids regulate distinct aspects of spermatogenesis in fish—there's general consensus that E_2 controls self-renewal of spermatogonia, while 11-KT drives their proliferation toward meiotic entry (Miura *et al.*, 1991; Miura and Miura, 2001; Schulz *et al.*, 2010b). Looking at the testicular tissue we examined, several features suggested these gonads had only recently undergone sexual differentiation. They were extremely small with a thread-like morphology, and the germinal epithelium consisted primarily of Nanos2-positive spermatogonia that showed relatively little proliferative activity based on PCNA staining. When we did find proliferating cells, most appeared to be isolated spermatogonia—a pattern consistent with undifferentiated stem cells. We occasionally observed cysts with small groups of dividing cells (differentiated spermatogonia), but these were uncommon.

A week after administering rFsh, several changes became apparent. Relative gonad weight increased, plasma 11-KT concentrations rose, and the 11-KT/E2 ratio climbed. These endocrine shifts coincided with dramatic changes in spermatogonial activity—the density of proliferating differentiated spermatogonia jumped roughly one hundred-fold. At the same time, we saw the opposite pattern in undifferentiated cells: dividing single spermatogonia dropped to less than one-seventh of baseline levels. The treatment essentially appeared to push cells toward differentiation and meiotic progression while reducing self-renewal activity.

These observations align with previous findings in pre-pubertal sea bass (Mazón *et al.*, 2014) and meagre (Zupa *et al.*, 2023; Ventriglia *et al.*, 2025b, 2025a). Those studies similarly reported that recombinant gonadotropin treatment reduced early-stage spermatogonia (designated StgA in sea bass, type Aund in meagre) while increasing more advanced stages (StgA1 or type Adiff). In contrast, in vitro studies have shown that Fsh promotes simultaneous spermatogonial self-

renewal and differentiation (Nóbrega *et al.*, 2015; Xie *et al.*, 2020). These divergent findings likely reflect fundamental differences between simplified in vitro culture systems and the complex hormonal and paracrine interactions present in intact testes.

Strong evidence from both laboratory and animal experiments indicates that E_2 stimulates spermatogonial renewal, though it does not drive rapid proliferation or meiotic entry (Miura and Miura, 2003; Schulz *et al.*, 2010a). After rFsh treatment in our experiment, we detected a steroidogenic shift favoring 11-KT production over E_2, reflected in the elevated 11-KT/E_2 ratio. This hormonal rebalancing may have diminished E_2's contribution to self-renewal-promoting factors too far toward the latter. Whatever the mechanism, our results raise an important concern: prolonged rFsh treatment intended to induce early puberty or resolve reproductive dysfunction could exhaust the spermatogonial stem cell pool, leading to sterility.

Germ cell death through apoptosis is a normal part of fish reproduction, occurring throughout all three spermatogenic phases: the spermatogonial stage, meiosis, and spermiogenesis (Billard and Escaffre, 1969; Young and Nelson, 2001; Prisco *et al.*, 2003; Chaves-Pozo *et al.*, 2005; Schulz *et al.*, 2005; Nóbrega, 2006; Almeida *et al.*, 2008; Corriero *et al.*, 2009; Zupa *et al.*, 2013, 2014, 2017; Zakariah *et al.*, 2020; Ventriglia *et al.*, 2025a, 2025b). Gonadotropins appear to regulate this process in teleosts and other seasonal breeders, though we still lack detailed information about how apoptosis patterns differ across spermatogenic stages. Studies on seasonal spawners provide interesting insights. In species like Atlantic bluefin tuna, swordfish, and greater amberjack, testicular apoptosis remains very low during the intense spermatogonial proliferation that marks the beginning of reproductive recrudescence—presumably because rising Fsh levels stimulate androgen production (Corriero *et al.*, 2007; Zupa *et al.*, 2013, 2017). But these same species show increased apoptosis during the meiotic phase, when spermatocytes dominate the germinal epithelium and 11-KT (the main fish androgen) reaches peak plasma concentrations (Corriero *et al.*, 2007; Zupa *et al.*, 2013, 2017).

Captivity-induced reproductive dysfunction is associated with high testicular apoptosis (Zupa *et al.*, 2013, 2017; Ventriglia *et al.*, 2024), which makes sense given that this condition involves reduced pituitary gonadotropin secretion (Zohar and Mylonas, 2001). Similarly, when

continuous light exposure reduced androgen levels in Atlantic cod *(Gadus morhua),* apoptotic germ cells increased (Almeida *et al.*, 2009).

Our untreated juvenile mullet showed no apoptotic markers—neither early signals detected by anti-Caspase-3 immunostaining nor advanced signs revealed by TUNEL—in spermatogonial germ cells. After rFsh treatment, only one fish showed apoptotic cells. This fits with extensive evidence that stem cells possess various protective mechanisms against apoptosis (Miura *et al.*, 1991; Miura and Miura, 2001; Nóbrega *et al.*, 2015; Xie *et al.*, 2020).

However, this finding appears to contradict earlier work with pre-pubertal meagre experiencing abortive spermatogenesis. Those fish—which showed signs of spermatogenic activation but couldn't complete the process (likely their first attempt at spermatogenesis)—exhibited high testicular apoptosis, mainly affecting spermatogonia. Treating these fish with rGths reduced germ cell death (Zupa *et al.*, 2023), but the effect reversed once treatment stopped (Ventriglia *et al.*, 2025a, 2025b). Our current findings support a recently proposed model of teleost germ cell apoptosis (Ventriglia *et al.*, 2025b). According to this framework, stem spermatogonia experience negligible apoptosis, whereas germ cell death increases sharply during the first, incomplete spermatogenic cycle in pre-pubertal fish. At this developmental stage, gonadotropin levels may be sufficient to trigger spermatogonial proliferation and differentiation but insufficient to fully support meiotic entry and spermatogenic completion—resulting in the selective loss of differentiated germ cells.

Overall, these results indicate a stage-dependent regulation of germ cell survival in juvenile mullet. While stem spermatogonia appear to be largely protected from apoptosis, differentiated spermatogonia require adequate and sustained Fsh and androgen support to survive and progress through spermatogenesis. In pre-pubertal fish, gonadotropin stimulation may be sufficient to initiate spermatogonial differentiation but insufficient to maintain meiotic progression, resulting in selective loss of differentiated germ cells. This framework reconciles the absence of apoptosis in untreated juveniles with abortive spermatogenesis in immature teleosts, highlighting the importance of developmental stage and hormonal context in recombinant gonadotropin strategies.

5. Conclusions

This study demonstrates for the first time that rFsh can effectively stimulate testicular development in pre-pubertal mullet, triggering spermatogonial proliferation toward meiotic entry and elevating 11-KT secretion in fish with recently differentiated, quiescent gonads. Our findings highlight the importance of implementing pre-selection programs in aquaculture based on morphometric and endocrine parameters to improve treatment success. In this study, only 4 out of 10 individuals in the rFsh group responded to treatment, resulting in 40% efficacy. This relatively low response rate suggests that individual variability plays a crucial role in hormonal responsiveness, underscoring the need for improved selection criteria before treatment.

Future research should focus on understanding the mechanisms underlying reproductive dysfunction in pre-pubertal mullet, including the potential benefits of combined rFsh/rLh protocols and determining the optimal timing of hormonal interventions to maximise reproductive success in aquaculture programs. Additionally, this study revealed that early spermatogonia are not subject to apoptosis in mullet juveniles, thereby contributing to a better understanding of testicular apoptosis patterns in seasonally breeding fish species.

CHAPTER VI. GENERAL DISCUSSION

The present thesis aimed to improve knowledge and control of reproduction in flathead grey mullet (*Mugil cephalus*) (hereafter referred to as "mullet") under intensive aquaculture conditions. One of the main challenges facing mullet aquaculture is reproductive control. This thesis evaluated the use of endocrine tools based on recombinant gonadotropins to induce reproduction out of the natural spawning season in the Western Mediterranean.

The results demonstrated also that these protocols can successfully induce gametogenesis and spawning in captive broodstock, providing a sufficient quality of eggs and larvae to be used for hatchery production of juveniles (CHAPTER II), to understand the role of the recombinant Fsh in vitellogenesis to improve hormonal protocols (CHAPTER III), and compared the broodstock performance of earth pond, RAS, and BFT cultured fish (CHAPTER IV). The role of recombinant Fsh in inducing pre-pubertal male reproductive development was also studied (CHAPTER V). The results of these studies provide an integrated perspective for advancing mullet broodstock management and reproductive control in aquaculture.

The mullet has recently been identified as a species with significant potential in aquaculture intensification and diversification, due to its omnivorous feeding habits, adaptability, and high market value. Most aquaculture-dominant marine fish species are carnivorous, whereas mullet can make good use of food at the bottom of the food web and have broad environmental tolerance, making them particularly interesting from sustainability perspectives (Whitfield *et al.*, 2012; Cardona, 2016). As aquaculture rapidly grows and becomes increasingly constrained by the surrounding environment, there will be a strong need in the future to use its organic and inorganic inputs sustainably. Hence, from a sustainability perspective, it is urgently necessary to identify aquaculture species that can efficiently utilize low-trophic-level organic and inorganic inputs to produce valuable protein and lipids for human consumption (FAO, 2024). One major impediment to mullet aquaculture expansion is the difficulty in controlling reproduction under captive conditions to provide eggs, larvae, and ultimately juveniles for grow-out.

The reproductive dysfunctions observed in mullet appear to be linked to the species' natural history and ecology. In the wild, mullet undertake reproductive seasonal migrations and are subjected to progressive changes in temperature, salinity, photoperiod, and hydrological dynamics, all known to play an important role in eliciting the activation of the brain-pituitary-gonadal (BPG) axis and the subsequent gametogenesis (Bromage *et al*., 2001; Zohar and Mylonas, 2001). Under captive conditions, these signals are either absent or greatly simplified, which may prevent the proper integration of environmental and endocrine stimuli required for full reproductive maturation (Rodríguez Gabilondo *et al.*, 2021).

Similar observations have been described for other migratory teleosts that do not reproduce spontaneously in captivity (Zohar and Mylonas, 2001). For example, in European eel (*Anguilla anguilla*) (Jéhannet *et al.*, 2023a); Japanese eel (*Anguilla japonica*) (Kazeto *et al.*, 2008; Kobayashi *et al*., 2010), where reproduction does not take place in captive animals, and these animals require repeated hormonal injections to induce gametogenesis and spawning. Comparable limitations have also been reported in thinlip grey mullet (*Chelon ramada*). Hence, it can be hypothesized that the reproductive dysfunctions in captivity reflect a general problem in migratory fish that may require changing, but precise environmental cues that are not present in intensive rearing conditions (Aarestrup *et al*., 2009).

This interpretation is further supported by the comparison between captive-reared and wild broodstock. In the present study, none of the fish maintained exclusively under intensive captive conditions were able to complete the reproductive cycle, regardless of the system used. In contrast, some wild-caught individuals were able to reach more advanced reproductive stages, particularly in the biofloc technology (BFT) system, where females attained gonadosomatic index (GSI) values as high as 14.17%. Morphological examination of the gonads from females in the earth pond indicated two females in early vitellogenesis with mean oocyte diameters of 230 ± 20 µm and one female in late vitellogenesis with a mean oocyte diameter of 546 ± 41 µm. This difference points towards the importance of previous environmental exposure and reproductive experience in establishing a functional endocrine axis (Espino Barr *et al.*, 2016; Boubekeur and Ramdane, 2021). It is therefore likely that endocrine maturation in mullet

depends not only on current environmental conditions but also on the individual's physiological history.

The application of recombinant gonadotropins in this thesis demonstrated that endocrine stimulation can effectively overcome this limitation. Recombinant hormones have been used in aquaculture to induce reproduction in fish species that are reproductively dysfunctional by providing a more natural and directed endocrine signal for gametogenesis (Molés *et al*., 2020). For examples, in Japanese eels (*Anguila japonica*) (Kazeto *et al.*, 2008; Kobayashi *et al.*, 2010), Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2018), Malaysia catfish (*Hemibagrus nemurus*) (Salwany *et al.*, 2019), Macquarie perch (*Macquaria australasica*) (Nocillado *et al.*, 2023) and European eel (*Anguilla anguilla*) (Jéhannet *et al.*, 2023a), and mullet (Ramos-Júdez *et al.*, 2021, 2022b). The success of these treatments in inducing full reproductive development in captive mullet highlights their potential as a key tool for broodstock management.

One of the most relevant findings of this thesis was the successful induction of spawning outside the species' reproductive season. In the Western Mediterranean, mullet spawn between September and October after a gametogenesis period that takes place between July and September (Ramos-Júdez *et al.*, 2023). In the present study, the developed hormonal protocol induced gametogenesis between March and June and spawning between June and July, altogether advancing the reproductive cycle by two to three months. These results show that the reproductive cycle of mullet can be modified. In contrast, the hormonal protocols developed in this study advanced gametogenesis to March–June and spawning to June–July. This shift of several months demonstrates that reproductive timing in mullet is not rigidly fixed, but can be modulated through endocrine intervention. From a production perspective, this represents a significant advantage, as it allows hatcheries to decouple juvenile production from natural seasonal constraints.

Although environmental manipulation, particularly through photoperiod and temperature control, has been successfully used in some species to alter reproductive timing (Bromage *et al.*, 2001; Al-Emran *et al.*, 2024), these approaches appear insufficient in mullet (Ramos-Júdez *et al*., 2022). Even under different culture systems, reproductive development remained incomplete in first-maturation fish. This suggests that, in species with pronounced reproductive

dysfunction, direct endocrine intervention may be more effective than attempting to replicate complex and multifactorial environmental conditions.

Recombinant gonadotropins have also been used in other fish species that suffer reproductive dysfunction in captivity. In female, recombinant hormones have been successfully employed to induce oogenesis, including vitellogenesis and final oocyte maturation, as demonstrated in species as the European eel (*Anguilla anguilla*) (Jéhannet *et al.*, 2023a), Japanese eel (*Anguilla japonica*) (Kazeto *et al.*, 2008; Kobayashi *et al*., 2010) and mullet (Ramos-Júdez *et al.*, 2021, 2022). In males, recombinant gonadotropins also promote spermatogenesis, including spermatogonial proliferation, meiosis, and spermiogenesis as reported in Senegalese sole (*Solea senegalensis*) (Chauvigné *et al.*, 2017, 2018), European eel (*Anguilla anguilla*) (Peñaranda *et al.*, 2018), meagre (*Argyrosomus regius*) (Zupa *et al.*, 2023a,b; Ventriglia *et al.*, 2025a,b), Japanese eel (*Anguilla japonica*) (Kazeto *et al.*, 2008; Kobayashi *et al*., 2010) and mullet (Ramos-Júdez *et al.*, 2021, 2022).

Nevertheless, the gametogenesis can be induced by hormonal treatment in most species, obtaining viable offspring remains a considerable challenge. This indicates that the induction of gonadal development does not necessarily guarantee full reproductive competence, and that further optimisation of hormone doses, treatment duration, and administration protocols is required to improve egg quality, fertilisation success, and larval survival.

The induction of reproductive development in juvenile fish using rFsh represents another important contribution of this thesis. In many aquaculture species do not undergo sexual maturation until after their first years in culture, which extends production time and incurs broodstock costs. Therefore, inducing and advancing the onset of puberty has been an area of interest in recent years (Kamei *et al.*, 2006; Mazón *et al.*, 2014; Sanchís-Benlloch *et al.*, 2017; Zupa *et al.*, 2023; Ventriglia *et al.*, 2024; Lancerotto *et al.*, 2025). In species such as meagre (*Argyrosomus regius*), greater amberjack (*Seriola dumerili*) and red spotted grouper (*Epinephelus akaara*), hormonal and environmental approaches have variable result (Moles *et al*., 2007; Bo Oh *et al*., 2018; Zupa *et al*., 2023a, 2023b; Lancerotto *et al*., 2025a, 2025b). The partial induction of the initiation of spermatogenesis observed in juvenile mullet using rFsh

treatment suggests that endocrine stimulation could represent a promising strategy to advance puberty and improve broodstock availability.

Nonetheless, captive-reared broodstock continued to experience reproductive dysfunction. None of the fish maintained exclusively in captivity matured reproductively in any of the culture systems examined here (i.e., earth ponds, RAS, BFT) whereas several wild broodstock did reach advanced reproductive stages. This strongly suggests that reproductive experience in a natural environment, or age and size, plays a crucial role in the development and activation of the brain-pituitary-gonadal (BPG) axis. A lack of prior reproductive experience may result in an immature or unresponsive endocrine system that can not properly activate gametogenesis under captive conditions.

Reproductive dysfunction in captive animals is not uncommon in marine fishes and has been linked to environmental, nutritional, and physiological factors (Migaud *et al.*, 2013; Teletchea and Fontaine, 2014; Volkoff, 2024). Environmental factors such as photoperiod, temperature changes, water current, and habitat complexity play a role in the endocrine regulation of reproduction and can be difficult to simulate in intensive aquaculture systems. Nutritional factors can also play a role, especially if broodstock diets lack the natural fatty acid profile of wild diets (Izquierdo *et al.*, 2001; Fernandez-Palacios *et al.*, 2014). Physiological stress can also play an important role in captive reproduction. Chronic stress can alter reproductive endocrinology by inhibiting gonadotropin-releasing hormone (GnRH) secretion and subsequent gonadotropin production (Schreck, 2010; Pankhurst and Munday, 2011). The elevated cortisol levels in RAS fish suggest that prolonged stress from intensive culture conditions may underlie endocrine disruption and reproductive dysfunction.

From an aquaculture perspective, a reliable protocol for reproductive control would allow consistent hatchery production of mullet. Reproductive manipulation to produce viable offspring under controlled conditions would alleviate the dependence on capturing wild fry, and promote the culture of mullet as a new candidate for aquaculture.

In summary, this thesis provides compelling evidence for the successful use of recombinant gonadotropins to control the reproductive development of mullet from the initial stages through

spawning. This achievement in inducing gametogenesis, spawning, and producing viable juveniles under captivity is considered a major breakthrough in the management of mullet broodstock and an important contribution towards the establishment of mullet aquaculture. These findings demonstrate that reproductive dysfunction in mullet is primarily associated with incomplete activation of the reproductive endocrine axis under captive conditions, and that this limitation can be overcome through targeted endocrine intervention.

CONCLUSIONS

Recombinant gonadotropins

- The use of species-specific recombinant gonadotropins successfully induced complete reproductive development and spawning outside the natural reproductive season in a marine teleost, demonstrating their effectiveness as endocrine tools for overcoming reproductive dysfunction in captivity.

- Dose–response analysis identified 9 µg/kg rFsh as an effective dose for promoting vitellogenesis while reducing hormone usage compared to higher doses. These findings show that hormonal protocols may be optimised to improve both biological efficiency and cost-effectiveness under controlled conditions.

- Higher doses (12 µg/kg) resulted in increased early atresia (15%) and greater inter-individual variability (CV = 81%), indicating that excessive stimulation reduces reproductive consistency and highlighting the importance of dose optimisation.

- The optimisation of hormonal protocols through dose-response analysis implies biological and economic benefits, such as biological performance, avoiding excessive stimulation, and cost-effectiveness under controlled conditions.

- The rGths protocol enabled controlled induction of reproductive development, spawning, egg, and larvae that can form the basis for juvenile production under captive conditions. These findings support the potential of recombinant gonadotropins as a practical tool for broodstock management and represent an important step toward improving reproductive reliability and facilitating closure of the production cycle in this species.

The captivity problem

- First-maturation fish did not initiate reproductive development across the three culture systems evaluated (earth pond, RAS, and BFT), and activation of the reproductive endocrine system was observed. This reproductive dysfunction during early maturation represents a significant limitation to complete the aquaculture life cycle under captive conditions.

- Post-mortem analysis of wild-caught adults maintained in BFT systems showed advanced gonadal development, with one female reaching a GSI of 14.17% and one male reaching 1.40%. Our results show that the BFT system has the potential to promote complete reproductive development in mullet.

- The reproductive progression observed in wild-caught broodstock demonstrates that endocrine maturity and reproductive history determine reproductive competence, rather than age or somatic condition alone.

Culture systems: stress, welfare, and practical realities

- Elevated glucose and lactate levels revealed sustained metabolic stress in captive fish, particularly in earth pond, indicating physiological conditions that can directly interfere with reproductive processes.

- Cortisol levels remained within expected physiological ranges, indicating that cortisol alone is not a principal indicator of chronic stress in this species. These results demonstrate that metabolic indicators are more sensitive and informative markers of stress under intensive aquaculture conditions.

- The broodstock diets used failed to reproduce the lipid composition and essential fatty acid profiles observed in wild mullet. These nutritional constraints represent a critical and often-overlooked limitation for mullet captive broodstock.

Puberty induction in juveniles

- The administration of recombinant Fsh stimulated testicular development in 40% of pre-pubertal male grey mullet, promoting spermatogonial proliferation and increasing circulating 11-KT levels in individuals with immature gonads.

- The partial response observed across individuals demonstrates that endocrine responsiveness is intrinsically heterogeneous, reflecting differences in physiological readiness within the population.

- The apparent resistance of early spermatogonial stages to apoptosis indicated that reproductive dysfunction in non-responsive individuals is driven by upstream regulatory mechanisms rather than irreversible germ cell loss. These demonstrate that rFsh stimulation alone is insufficient to achieve full reproductive maturation in pre-pubertal male grey mullet.

- Administration of recombinant Fsh promotes spermatogonia proliferation and can regulate the levels of specific hormones in individuals with immature gonads. Furthermore, the endocrine response to rFsh stimulation can vary depending on the physiological characteristics of individuals within a population and the regulators of biochemical mechanisms.

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APPENDIX I.

PRODUCTION OF THE FIRST LIVE FEED (*BRACHIONUS ROTUNDIFORMIS*) FOR MULLET LARVAE.

1. Introduction

Larviculture is one of the main challenges in expanding marine fish farming, as it relies heavily on auxiliary cultures (Hamre *et al.*, 2013). From an evolutionary perspective, most larvae depend on live prey because they do not adapt to inert or dry diets. Even when they do accept inert diets, their digestive systems are still underdeveloped, with low enzymatic activity that prevents them from properly digesting formulated feeds (Rønnestad *et al.*, 2013; Melaku *et al.*, 2024).

Rotifer culture is one of the most extensively studied live prey systems worldwide due to several key factors, including their small size (130–320 µm), ease to enrich to obtain a high nutritional value, rapid reproduction rate, and slow swimming behavior (Yoshimatsu and Hossain, 2014). Additionally, rotifers can be mass-cultured under controlled conditions (Chhaba, 2022). There are two main types of rotifers: *Brachionus plicatilis* (L-strain) and *Brachionus rotundiformis* (S-strain) (Papakostas *et al.*, 2007). Rotifers play a crucial role in the early development of cultured larval fish and crustaceans, serving as the primary live feed (Yoshimatsu and Hossain, 2014; Jana, 2024). Importantly, the rotifers can be enriched with essential nutrients, such as highly unsaturated fatty acids (HUFAs), which makes them an ideal prey for larval fish, which require specific dietary components for proper growth and survival (Kotani, 2017).

The growth of hatcheries and the increasing demand for aquaculture production have led to a rising daily need for rotifers, creating a bottleneck that limits the sector's industrialisation. This

unpredictability forces hatcheries to maintain multiple simultaneous cultures, including backup systems, to ensure a stable and sufficient food supply for larvae (Candreva' *et al.*, 1996). To achieve stable and efficient production, various culture systems have been developed, each with its advantages and challenges. The batch system, one of the most traditional, involves harvesting multiple tanks at scheduled intervals. However, this approach requires more space and labour and can result in unstable and unpredictable outcomes, as well as risks of protozoan contamination (Dhert *et al.*, 2001).

These systems utilise microalgae, yeast and specially designed diets as a food source, either solely or in combinations, without continuous water flow (Lubzens, 1987). A more efficient alternative is the semi-continuous culture system, in which a portion of the rotifer population is removed and replaced daily with fresh food and clean water. This method enables more stable production, better control over population dynamics, and regular harvesting at predetermined intervals (Navarro and Yufera, 1998; Kostopoulou *et al.*, 2012). On the other hand, more advanced recirculating systems optimise resource use and provide greater control and stability in production (Suantika *et al.*, 2001, 2003). In Europe, the most common method for rotifer culture is the batch (also referred to as a discontinuous system), where rotifers undergo periodic water renewals and are fed microalgae, mixtures of microalgae and yeast, or specific rotifer diets (Suantika *et al.*, 2000).

To address these limitations without relying on continuous recirculation systems, ozone use has emerged as a promising strategy to improve water quality before the start of culture. Ozone is a powerful oxidizing agent that, when applied at low, controlled concentrations, can reduce organic load, decrease microbial activity, and prevent contamination without harming rotifers. Unlike continuous ozone applications or complex water treatment systems, a single ozone dose before introducing the culture could offer a practical and cost-effective solution to stabilise the initial system of conditions. In this context, the objective of this study was to evaluate the effect of a single ozone application at the beginning of the culture period on the stability and productivity of rotifers in a traditional semi-continuous culture system. This approach aims to improve initial water quality, reduce environmental stress, and promote higher rotifer density and yield throughout the culture.

The main challenge of this study was to overcome instability in rotifer culture by using an artificial diet as a substitute for microalgae. Therefore, the design of this research focuses on developing a stable, controllable cultivation method that addresses common instability issues in traditional semi-continuous rotifer culture systems.

Therefore, the main objective of this research was to evaluate the most appropriate rotifer culture system to employ when environmental fluctuations compromise water quality, in order to maintain stable and efficient production.

2. Materials and Methods

2.1. Rotifer cultures

The rotifer (*Brachionus rotundiformis*) was maintained from April 29th, 2023, to October 10th, 2023, and from October 8th, 2024, to October 22nd, 2024. The rotifer strain used in both experiments (2023 and 2024) was provided by the company AVRAMAR Spain Aquaculture S.L., located at Paseo Alameda 353, and introduced into the IRTA facilities in April 2023 and October 2024.

Prior to the start of the study in July 2023, a stock of 100 million rotifers—received on April 29th, 2023—was maintained in a 300 L tank under controlled conditions: temperature at 24 °C using a thermostat heater (Eheim, Germany, which is made of laboratory-grade glass and includes an adjustable thermostat with a temperature range of 18–34 °C and an accuracy of ±0.5 °C), oxygen at 10 mg/L, and salinity at 28‰ measured using YSI probe (professional plus: multiparameter instrument). The rotifers were cultured until reaching a density of 250 million. On July 13, 2023, a total of 232 million rotifers were harvested and divided into two groups: one assigned to the control tank and the other to the ozone-treated tank.

2.2. Experimental design

In 2023, a comparison was conducted between water used for the control group and the same water that was ozone-treated for the treatment group, as detailed below:

- **Control Group (no Ozone)**: The water was treated using a system consisting of sand filtration, string-wound cartridge filter (10, 5, and 1 μm), activated carbon (Micro 10 is a thermally activated carbon made from coconut shell. Being plant-based, it contains no metals or contaminants found in mineral carbons), and ultraviolet (UV) light.
- **Ozone Group**: The water underwent the same treatment as the control group (paper filter, thread filter, activated carbon, and UV light), with the addition of ozone. Ozone was applied at a concentration corresponding to an oxidation-reduction potential (ORP) of 400 mV (millivolts) for 10 minutes, following a two-hour aeration process. Ozone (O_3) was generated using an ozonator (Sander Ozonisator 200, Sander Aquarientechnik, Germany).

Salinity levels were maintained at 27‰ in both experimental groups, increasing to 28‰ by the end of the culture period. The temperature was controlled between 23.5 and 24°C using a thermostat heater. Dissolved oxygen levels averaged 11.21 ± 1.55 mg/L, and pH was at 7.98 ± 0.17, monitored using a YSI probe.

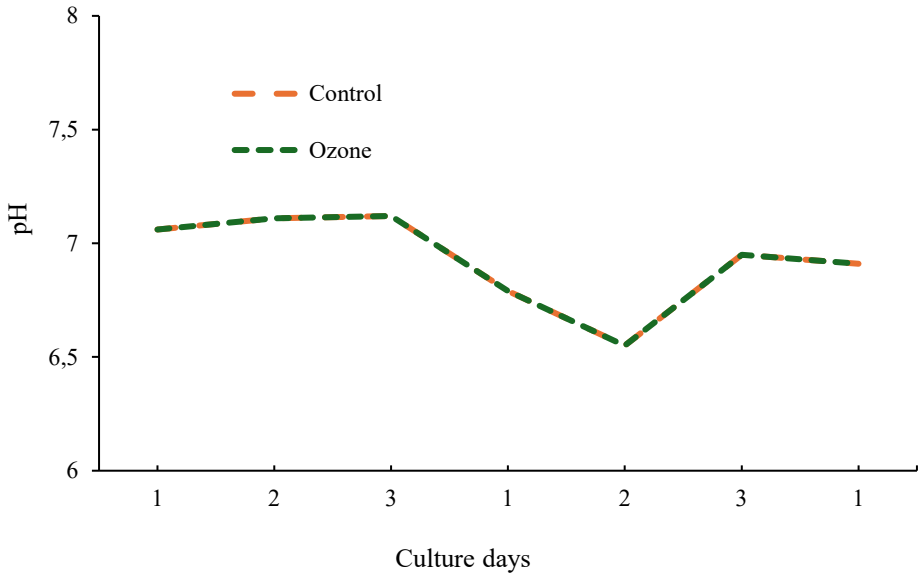


Figure 1. **pH in semi-continuous rotifer culture in two tanks (Control vs Ozone) over 7 days of experiment.** U. Mann-Whitney test revealed not statistically significant difference between treatments. In the graph, the dashed green line represents the ozone treatment, while the orange dashed line represents the control.

Rotifers were fed daily with yeast at a rate of 0.30–0.40 grams per million individuals. Each day, the tanks were purged and replenished with 10–15 litres of seawater. A complete harvest of the tanks was performed every three days, following a semi-continuous culture system.

Toxic free ammonia in the culture water was neutralised by adding ClorAm-X every day, which reacts with toxic ammonia to form non-toxic compounds while releasing H^+ ions, leading to a decrease in pH. To counteract the pH drop, 5 grams of sodium bicarbonate were added to the ClorAm-X solution (at a rate of 0.25 grams per 10^6 rotifers) as a buffering agent every day (Bentley *et al.*, 2008). The solution was prepared in 10 litres of water and administered in four doses throughout the day.

Although **ClorAm-X** is a commercial product with a proprietary formula (based on modified sodium bisulfite or sodium thiosulfate), its general mode of action in neutralizing **toxic free ammonia (NH_3)** can be represented as follows:

NH_3+ClorAm-X→Non-toxic compounds + H^+

The experiment conducted in 2024 (17/09/2024 to 08/10/2024) was carried out in two tanks under identical conditions. The water used for rotifer culture underwent treatment through sand filtration, string-wound cartridge filter (10, 5, and 1 μm), activated carbon (Micro 10 is a thermally activated carbon made from coconut shell. Being plant-based, it contains no metals or contaminants found in mineral carbons), ultraviolet (UV) light, and ozonation. Both tanks received the same initial ozonation treatment, consisting of an oxidation-reduction potential (ORP) of 400 mV applied for 10 minutes, followed by two hours of aeration.

Salinity levels were maintained at 27‰ in one tank group and 28‰ in the other, from the beginning to the end of the culture period. The temperature was controlled between 23.5 and 24°C using a thermostat heater. Dissolved oxygen levels averaged 12.21, and pH was maintained at 7.98 ± 0.17, monitored using a YSI probe.

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Also in 2024, a rotifer RAS (Recirculating Aquaculture System) was implemented to reduce labor in rotifer culture. Ozone (O3) was produced by an ozonator (Sander Ozonisator 200, Sander Aquarientechnik, Germany) connected to a protein skimmer in the RAS, and controlled using an MC510 PRO Digital ORP controller (250–300 mV). The rotifers were fed with fresh baker's yeast at 0.35–0.40 g yeast per million rotifers **(Fig. 2)**.

The system consisted of two main units: a rotifer culture tank and a settlement (sedimentation) tank. The rotifer culture tank**,** where the organisms were maintained and fed, and a sedimentation tank connected in series. Water from the rotifer tank flowed by gravity into the

sedimentation tank, allowing uneaten feed and solid waste to settle at the bottom. From there, the partially clarified water was directed to a protein skimmer**,** where fine suspended particles and dissolved organic matter were removed. After skimming, the treated water was recirculated back into the rotifer tank, completing the loop. This configuration ensured continuous removal of waste, stabilisation of water quality, and reduced contamination risk while maintaining a constant culture environment.

Water quality parameters, including salinity (28.74 ± 0.03‰) and temperature (23.75 ± 1.98 °C, maintained by a thermostatic regulator), were carefully controlled throughout the trial. Daily purging of both tanks was carried out to further stabilise water quality.

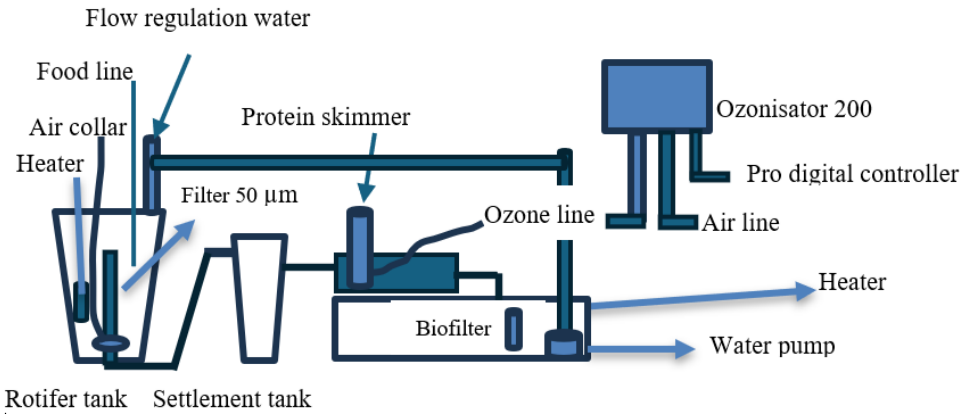


Figure 2. Schematic overview of the recirculation system.

Water quality parameters, including salinity (28.74 ± 0.03‰) and temperature (23.75 ± 1.98 °C), were carefully controlled using a thermostatic regulator throughout the trial. Daily purging was performed in each tank to maintain water quality.

2.3. Fertility

The % fertility (F) of the rotifers was estimated daily in all systems by following the formula:

$$F\ (\%) = \frac{Nt}{N} \times 100$$

where, F= fertility (%), Nt= rotifers with at least one egg, N= total number of rotifers.

In the 2023 experiment (from July 14th to July 26th), fertility in both the control and ozone-treated groups was measured daily as the percentage of egg-bearing females.

In the 2024 experiment (from September 17th to October 8th), fertility in the two ozone-treated tanks was assessed as the percentage of egg-bearing females during the final nine days of the trial.

2.4. Statistical analysis

Data are expressed as the mean ± standard deviation. Shapiro–Wilk and Levene tests were used to assess the normality of the data distribution and the homogeneity of variance, respectively.

An independent-samples U. Mann-Whitney test was used to compare the growth rate and fertility of the rotifers within and between systems. Statistical analyses were used with SPSS software version 20.0 (Armonk, NY: IBM Corp). Significant differences were detected at a significance level of $P < 0.05$.

3. Results

In the 2023 experiment (conducted from July 14th to July 26th), water parameters were closely monitored throughout the trial. Salinity (27‰ to 28‰) and water temperature (23.5 °C to 24 °C) were maintained within controlled limits. Dissolved oxygen levels averaged 11.09 ± 1.49 mg/L in the control group and 11.32 ± 1.67 mg/L in the ozone-treated group **(Fig. 3)**. The pH values

were consistent across both groups, with a mean of 6.97 ± 0.23. No statistically significant differences were observed between the groups in terms of salinity, temperature, dissolved oxygen, or pH.

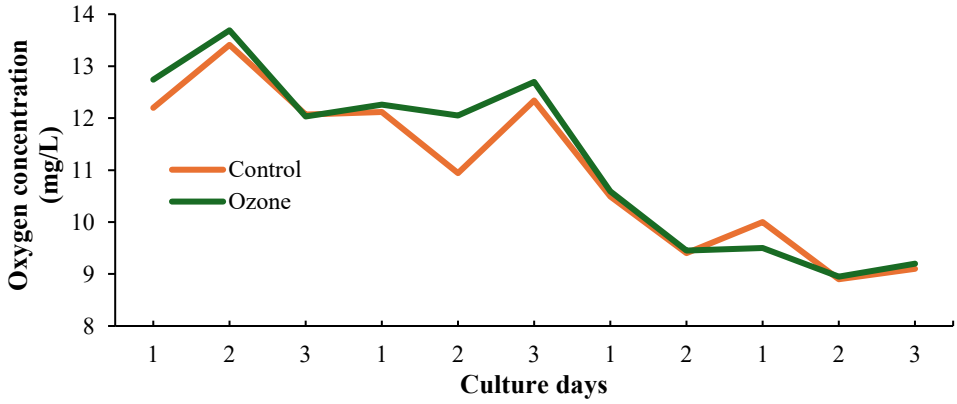


Figure 3. Oxygen concentration (mg/L) in a semi-continuous rotifer culture in two tanks (control vs ozone) over 12 days of experiment. An independent samples U. Mann-Whitney test revealed no statistically significant difference between treatments. In the graph, the continuous green line represents the ozone treatment, while the orange line represents the control.

The cultures were maintained in a semi-continuous culture system, with complete harvests performed every three days **(Fig. 4)**. Each culture cycle started on day 1, and populations were counted, harvested, and re-inoculated every three days. This procedure was repeated across four consecutive cycles **(Table 1).**

Table 1. Rotifer counts (rotifer/mL) in control and ozone treatments. The culture started on day 1 and was maintained for a total of 12 days, consisting of four consecutive 3-day cycles. In each cycle, populations were counted daily, harvested, and re-inoculated at the end of the 3-day period.

Days	Control	Ozone
1	645	640
2	424	590
3	502	620
1	481	702
2	525	722
3	534	790
1	632	906
2	612	984
3	493	850
1	599	877
2	770	1071
3	775	1192

Daily measurements of rotifer density revealed that the control group, which was initiated with 645 rotifers/mL (180 litres), reached an average of 582.66 ± 110 individuals/mL and ended with 775 rotifers/mL (250 litres) after 12 days **(Table 1)**. In contrast, the ozonated treatment started with 640 rotifers/mL (180 litres), reached a significantly higher average density of 814.41 ± 192.62 rotifers/mL, and peaked at 1,192 rotifers/mL (250 litres) by the end of the experiment **(Fig. 4, table 1)**. The comparison revealed a statistically significant difference in rotifer density between the control and ozone treatments.

Fertility (%) was determined daily by microscopic observation and expressed as the ratio of egg-bearing rotifers (Nt) to total rotifers counted (N), multiplied by 100. The average number of eggs was 151 ± 42.03 eggs/mL in the control and 199 ± 41.18 eggs/mL in the ozone treatment **(Fig. 5)**. In contrast, fertility expressed as the percentage of egg-bearing females showed averages of 25.95 ± 5.14% in the control and 24.94 ± 4.43% in the ozone treatment **(Fig. 6)**. A statistically significant difference was observed between treatments for eggs/mL ($P = 0.019$, Fig. 5), whereas no significant difference was detected in fertility (%) ($P > 0.05$, Fig. 6).

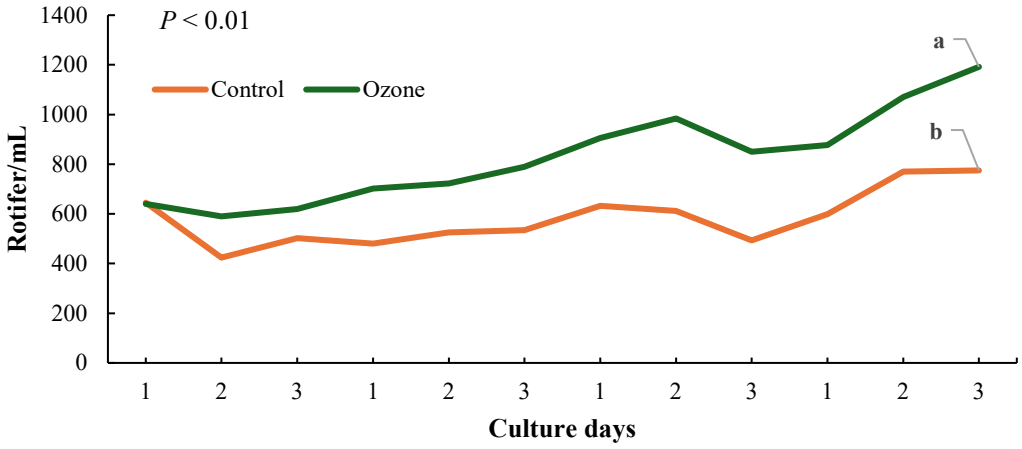


Figure 4. Semi-continuous rotifer culture (individuals/mL) over 12 days under control and ozone treatments. U. Mann-Whitney test revealed a statistically significant difference between the control and ozone treatments. In the graph, the continuous green line represents the ozone treatment, while the orange line represents the control treatment.

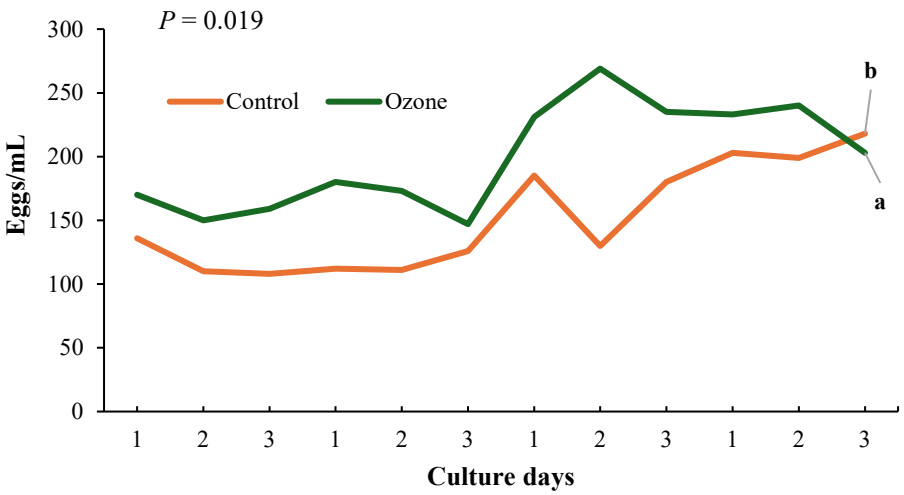


Figure 5. Semi-continuous rotifer culture eggs/mL over 12 days under control and ozone treatments. U. Mann-Whitney test revealed a statistically significant difference between the control and ozone treatments. In the graph, the continuous green line represents the ozone treatment, while the orange line represents the control treatment.

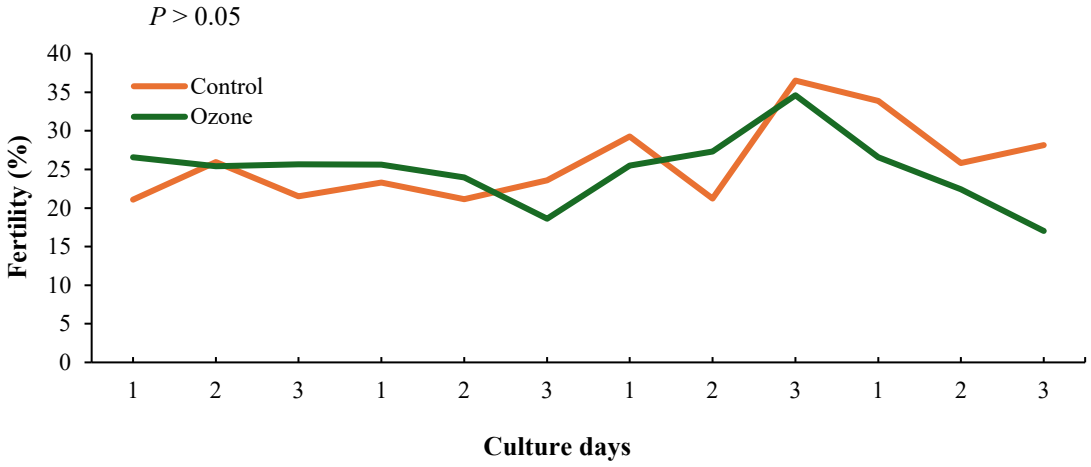


Figure 6. Semi-continuous rotifer culture fertility rotifer culture (egg/mL) over 12 days under control and ozone treatments. U. Mann-Whitney test revealed no statistically significant difference between the control and ozone treatments. In the graph, the continuous green line represents the ozone treatment, while the orange line represents the control treatment.

In the experiment conducted in 2024 (17/09/2024 to 08/10/2024), water quality parameters were carefully controlled throughout the trial. Salinity, temperature, pH, and dissolved oxygen showed no significant differences. Daily measurements of rotifer density revealed that in Tank 1, the culture started with 410 rotifers/mL (130 litres), reached an average of 583.66 ± 175

rotifers/mL, and ended with 810 rotifers/mL (300 litres) after 22 days. In Tank 2, the initial density was 510 rotifers/mL (130 litres), with an average of 655.04 ± 224.72 rotifers/mL and peaked at 1,013 rotifers/mL (300 litres) by the end of the experiment. No significant differences in rotifer density were observed between the two ozone-treated tanks **(Fig. 7)**.

The percentage of fertility was determined daily by microscopic observation and expressed as the ratio of egg-bearing rotifers (Nt) to total rotifers counted (N), multiplied by 100, and was assessed during the last nine days of the experiment. Tank 1 showed an average fertility of 19.40 ± 8.64 %, while Tank 2 had 20.70 ± 5.91 %. No statistically significant differences were observed between the two tanks **(Fig. 8)**.

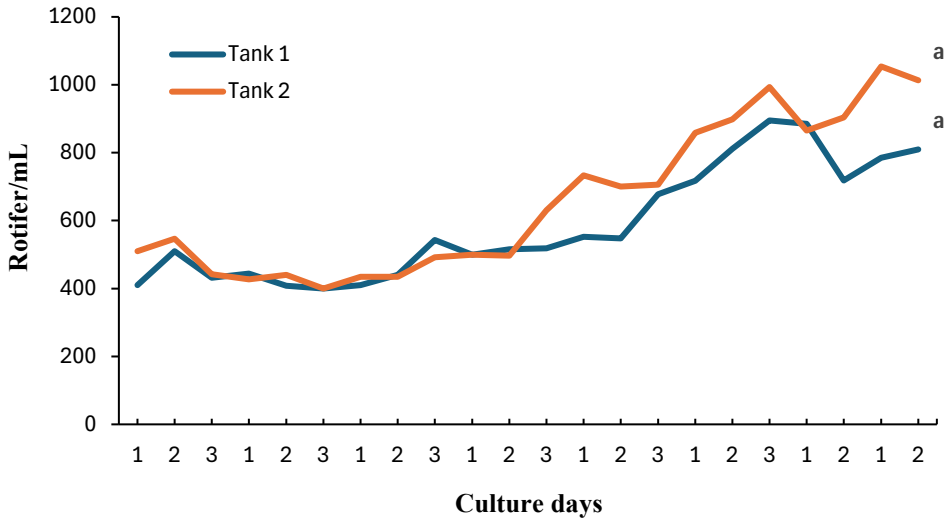


Figure 7. Semi-continuous rotifer culture (individuals/mL) over 22 days under Ozone treatments. U. Mann-Whitney test revealed no statistically significant difference between the tanks' Ozone treatments. In the graph, the continuous blue line represents tank 1, while the orange line represents tank 2.

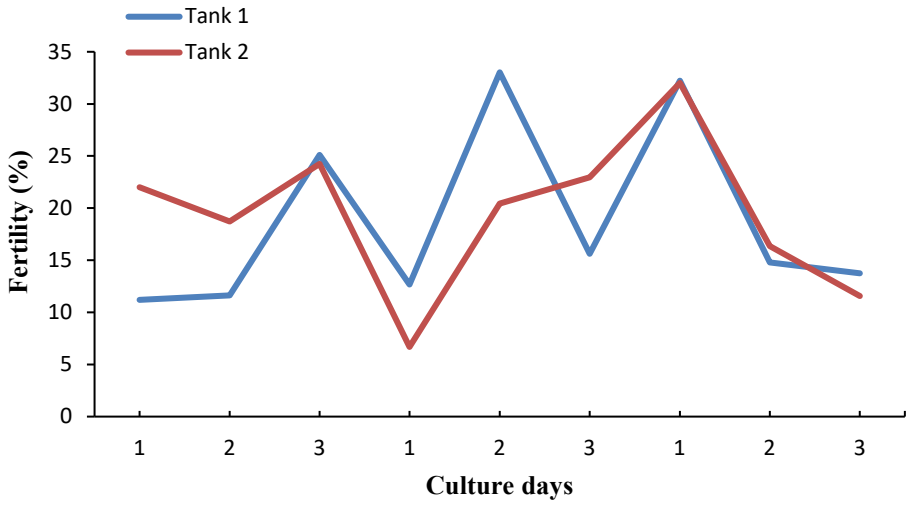


Figure 8. Semi-continuous rotifer culture (individuals/mL) during the last nine days of the experiment. U. Mann-Whitney test revealed no statistically significant difference between the tanks' Ozone treatments. In the graph, the continuous blue line represents tank 1, while the orange line represents tank 2.

On day 20 of the rotifer culture with ozone, corresponding to October 6, 2024, we observed a slight decrease in culture density. In response, a complete cleaning of the rotifer tanks was carried out, and the cleaning frequency was increased to every two days. As a result, we decided to implement a Recirculating Aquaculture System (RAS) to help reduce the workload associated with culture maintenance.

Daily measurements of rotifer density revealed that in the RAS tank, the culture started with 100 rotifers/mL (300 L), reached an average of 265.85 ± 180 rotifers/mL, and ended with 644 rotifers/mL (300 L) over 7 days **(Fig. 9)**. Water quality parameters, including salinity (28.74 ± 0.03‰) and temperature (23.75 ± 1.98 °C), were carefully controlled throughout the trial. Dissolved oxygen levels were 8.00 ± 0.05 mg/L, Ammonium: 0.5-1 mg/L, Nitrite (NO_2^-) 0.16 - 0.20 mg/L, Nitrate (NO_3^-) 5 mg/L, pH: 7.95. The rotifers were fed with fresh baker´s yeast at 0.40 g yeast / million rotifers. For fertility, measured as the percentage of egg-bearing females, daily assessments averaged 24.71 ± 6.26%.

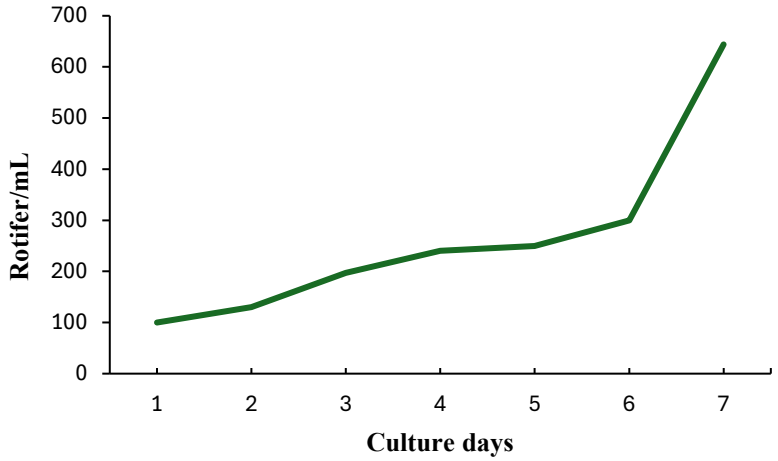


Figure 9. RAS system in rotifer culture (individuals/mL) over 7 days under Ozone+ treatments.

4. Discussion

This rotifer study was conducted in two separate phases: the first in July 2023 and the second from September to October 2024. The objective was to evaluate the stability and productivity of rotifers in a traditional semi-continuous culture system between April (spring) and October (autumn). Both trials were conducted during the summer in the western Mediterranean region, a season characterised by elevated temperatures and fluctuations in oxygen levels that frequently hinder the stable production of live feed in aquaculture facilities. Identifying resilient culture systems under such conditions is essential to maintain a reliable supply of rotifers for hatchery operations.

Agricultural practices—especially rice cultivation during the summer months—can significantly affect specific isolated aquatic ecosystems in this region. The intensive input of nutrients, primarily from fertilisers, commonly leads to eutrophication, characterised by excessive growth of algae and aquatic plants (Szpakowska *et al.*, 2022). As this organic matter decomposes, it drastically reduces dissolved oxygen levels in the water, creating hypoxic or even anoxic conditions that degrade water quality and threaten aquatic life. Additionally, the presence of agrochemicals such as herbicides and pesticides can disrupt the reproductive capacity and population dynamics of sensitive aquatic organisms, ultimately disturbing the

ecological balance (Guo and Chen, 2015; Moreira *et al.*, 2016). Among the various bioindicators used to assess aquatic ecosystem health, rotifers have gained particular attention due to their wide geographic distribution, ease of cultivation, and rapid response to environmental changes. These microscopic zooplankton play a key role in aquatic food webs and have proven to be effective tools in environmental toxicity testing (Oh *et al.*, 2017).

The rotifers biological traits—such as parthenogenetic reproduction, which enables the use of genetically uniform test populations, and their ability to quickly reach high densities—make them ideal candidates for ecotoxicological assessments (Hagiwara *et al.*, 2007; Kotani *et al.*, 2005). Numerous studies have demonstrated that the composition and abundance of rotifer communities can provide valuable insights into the trophic status of aquatic environments, particularly for classifying systems as mesotrophic, eutrophic, or hypertrophic—especially in ponds and similar water bodies (Stamou *et al.*, 2022). Furthermore, rotifer populations are known to respond rapidly to changes in nutrient concentrations, pollutant levels, and habitat conditions (Choi and Kim, 2020; Muñoz-Colmenares *et al.*, 2021; Kolarova and Napiórkowski, 2024). Rotifers are frequently used in toxicity tests involving a range of harmful substances such as: heavy metals, pesticides, and naturally occurring toxins.

In the 2023 experiment, rotifers were fed with fresh baker's yeast at a concentration of 0.30 to 0.40 gram/million individuals. This study found that the treatment without ozone application resulted in a final population of 193 million rotifers in 250 litres of water (775 rotifers/mL) after 12 days of culture. In contrast, the ozone-treated group also fed with the same baker´s yeast, showed a higher rotifer density from the beginning, reaching 298 million in 250 litres of water (1,192 rotifers/mL). This difference may be related to variations in water quality, potentially influenced by the onset of summer and nearby agricultural activities. In this context, ozone has been proposed as a promising tool to enhance water quality in aquaculture systems (Spiliotopoulou *et al.*, 2018). Ozone (O_3) is a gas composed of three oxygen atoms, colorless and odorless, known for its strong oxidative capacity. This property allows it to break down complex organic compounds into simpler, harmless substances such as carbon dioxide, water, and other stable molecules. Its interaction with pesticide residues facilitates their decomposition

into non-toxic elements, thereby reducing the risk of contamination in aquatic farming environments (Aidoo *et al.*, 2023).

Fertility obtained in both the ozone-treated and non-ozone-treated groups showed no statistically significant differences, with a combined average of 25.44 ± 4.78%. In both cases, the rotifers were fed exclusively with fresh baker's yeast, yielding results very similar to those reported by Rojo-Cebreros *et al.* (2017), where fertility was 21.5 ± 8.43%, and the rotifers were fed with the microalga *Nannochloropsis sp.* (Rojo-Cebreros *et al.*, 2017).

Parameters such as ammonia, dissolved oxygen, and pH were within acceptable ranges in the experiments conducted in 2023 and 2024. Ammonium concentration has been shown to significantly influence both the reproductive performance and lifespan of rotifer cultures, and in this experiment, it was controlled by adding ClorAm-X (De Araujo *et al.*, 2001; Bentley *et al.*, 2008). ClorAm-X is an alkali metal formaldehyde bisulfate that binds to un-ionized ammonia, forming an aminomethane sulfonate salt and thereby reducing total ammonia nitrogen (TAN). This compound is non-toxic and does not disrupt the nitrogen cycle. This approach enabled ammonium levels (NH_4^+) to remain below the critical toxicity threshold (< 1 mg/L).

Temperature and salinity are critical environmental factors that significantly influence the reproduction of *Brachionus rotundiformis.* According to Lee *et al.*, 2022, fecundity is markedly reduced at a salinity of 35 ‰ combined with a temperature of 15 °C. Further studies conducted in 2022 indicate that salinity exerts a greater impact on rotifer culture performance than temperature (Fielder *et al.*, 2000). However, previous studies have shown that reproductive rates are higher at temperatures between 30 and 35 °C (Hagiwara *et al.*, 2001), compared to those observed at 25 °C (Assavaaree *et al.*, 2003). In our experiment, salinity was reduced from 35 ‰ to 27–28 ‰, while temperature was maintained between 23.5 and 24 °C. This temperature range was selected to align with the rearing requirements of *Mugil cephalus* larvae, for which the rotifers were cultured as live feed. Under these conditions, *B. rotundiformis* exhibited a satisfactory reproductive rate, even when fed exclusively with yeast at concentrations ranging from 0.30 to 0.40 grams/million individuals.

Microalgae used as feed for rotifers generally leads to better results compared to yeast in rotifer culture (Das *et al.*, 2021; Hosain *et al.*, 2024; Khan and Rahman, 2025). In batch systems fed with fresh baker's yeast, reported rotifer densities range from 150 to 400 individuals/mL (Hirata, 1980; Lubzens, 1987). However, these relatively low densities were often unreliable, and cultures frequently collapsed, mainly due to the deterioration of water quality (Hirayama, 1987). However, one challenge with microalgae is that production often requires more manual labor. Even so, several studies have shown that feeding rotifers with microalgae can lead to 10–25% higher production compared to using powdered yeast (øie *et al.*, 1994). Das *et al.*, (2021) found that rotifers fed *Nannochloropsis sp*. showed greater population growth and higher egg production compared to those fed baker's yeast or powdered spirulina. This improved performance may be related to the smaller cell size of *Nannochloropsis sp*. (approximately 2–5 μm), which could make it easier for rotifers to consume and digest (Hoff and Snell, 1993).

In a rotifer culture maintained under RAS (Recirculating Aquaculture System) conditions with ozone treatment, population density increased significantly over 7 days. The culture was initiated with 30 million rotifers in 300 litres (equivalent to 100 individuals/mL) and reached a final count of 193 million rotifers (644 individuals/mL). Throughout the experiment, rotifers were fed exclusively with fresh baker's yeast. Other studies have reported significantly higher densities, starting with 500 rotifers per milliliter and reaching, by day 14, concentrations of up to 15,200 rotifers/mL in Experiment I and 20,500 rotifers/mL in Experiment II (Suantika *et al.*, 2001). In our case, during the ozone treatment, the oxidation-reduction potential (ORP) ranged from 210 to 250 mV in the protein skimmer observed in MC510 PRO Digital ORP controller, whereas in the study by Suantika *et al.*, (2001), the values were considerably higher, ranging from 310 to 360 mV. Although we attempted to increase the ORP in our system, we were unable to achieve this goal. One possible explanation is that the fresh baker's yeast did not settle properly in the sedimentation tank and remained suspended in the system. As fresh baker's yeast passed through the protein skimmer, it may have reacted more intensely with the ozone, thereby reducing the efficiency of the oxidation process. Despite this limitation, the use of ozone in the recirculating system proved beneficial, as it improved water quality (Suantika *et al.*, 2001) and reduced the need for frequent cleaning in comparison with the traditional-cultured rotifer system.

During the rotifer culture, water quality parameters were maintained within optimal ranges for optimal rotifer development. Ammonium (NH_4^+) concentrations ranged from 0.5 to 1 mg/L (Hamre *et al.*, 2008), nitrite (NO_2^-) ranged from 0.16 to 0.20 mg/L (Dhert *et al.*, 2001), and nitrate (NO_3^-) remained at 5 mg/L, while pH stabilised at 7.95 ± 0.15 (Das *et al.*, 2021). These values are consistent with ideal conditions to ensure the health, reproduction, and stability of rotifer populations. Therefore, future studies should focus on optimising water quality and Recirculating Aquaculture System (RAS) conditions, with particular emphasis on ozone treatment protocols, to enhance the consistency and reliability of rotifer culture outcomes, especially under scenarios where external factors may negatively impact water quality.

5. Conclusions

The present study demonstrated that initial ozone treatment in semi-continuous rotifer cultures resulted in a 20% increase in population density compared to non-ozonated controls. This enhancement is likely attributable to improved water quality through ozone's oxidative capacity, particularly under conditions susceptible to environmental perturbations. Furthermore, integrating ozone treatment into an RAS significantly accelerated population growth during shorter culture periods. Given the critical role of rotifers as first-feeding prey in marine larviculture, ozone-enhanced RAS represents a promising approach to improve production efficiency, culture stability, and biosecurity in hatchery operations.

ANNEX I

CHAPTER III. IMPACT OF RECOMBINANT FOLLICLE-STIMULATING HORMONE ON VITELLOGENESIS AND FECUNDITY IN FLATHEAD GREY MULLET (*MUGIL CEPHALUS*).

Supplementary Figure 1-Gonadal regions: Average ± SE oocyte diameter in different gonadal regions for each treatment at week 9. A random selection of 50-60 oocytes from each gonadal region was analysed. Each colour and letter represents a specific rFsh treatment: blue circles (A): 3 µg/kg, red circles (B): 6 µg/kg, green circles (C): 9 µg/kg, and black circles (D):12 µg/kg. ANOVA results showed no statistical difference between gonadal regions within each treatment: A: $P \geq 0.954$, B: $P \geq 0.709$, C: $P \geq 0.973$, D: $P \geq 0.891$.

