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Induction of Spawning in Captive Female Thicklip Grey Mullet (*Chelon labrosus*) Treated with Different GnRHa Delivery Systems

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Abstract

Thick-lipped grey mullet (*Chelon labrosus*) has important characteristics that make it a promising candidate species for diversifying Mediterranean aquaculture. However, spontaneous spawning in captivity has not been documented, mainly due to failure of females to spawn, highlighting the need for further research on reproduction control. This study evaluated the efficacy of GnRHa administration, using repeated intramuscular injections or slow-release Ethylene–Vinyl Acetate (EVAc) implants combined with a dopamine antagonist (metoclopramide, Met), in terms of spawning performance and egg quality. Three groups were established: (a) saline injection (0.9% NaCl; Saline-INJ), (b) GnRHa [Des-Gly^{10}, D-Ala^{6}-ProNEth^{9}-mGnRHa] injection at 10 μg kg^{-1} BW (GnRHa-INJ), and (c) EVAc implant containing GnRHa at 50 μg kg^{-1} bw (GnRHa-IMP). Over four weeks, Saline-INJ and GnRHa-INJ females received weekly saline or GnRHa, respectively. GnRHa-INJ and GnRHa-IMP females also received weekly Met (15 mg kg^{-1} bw). GnRHa induced 11 spawns (1,768,680 eggs), nearly triple the Saline-INJ group (4 spawns, 394,400 eggs). Daily relative fecundity (DRF) and fertilization success were highest in GnRHa-INJ (56,982 eggs kg^{-1} day^{-1}; 59.7%), followed by GnRHa-IMP (20,375; 18.8%) and Saline-INJ (13,061; 9.1%). Multiple injections showed a trend toward higher spawning performance and egg quality compared to implants, although variability was high and further replication is needed. Nevertheless, optimizing both GnRHa delivery methods could further enhance their effectiveness while maintaining operational benefits for aquaculture.

Keywords: *Chelon labrosus*; thicklip grey mullet; GnRHa; metoclopramide; spawning induction; broodstock management; reproduction; aquaculture

Key Contribution: (1). Administration of GnRHa + metoclopramide (Met) induced multiple spawning about three times more than the controls. (2). Weekly GnRHa + Met injections yielded higher relative fecundity and significantly higher fertilization success than GnRHa implants and control-treated females. (3). Physical handling of females (blood collection, ovarian biopsy) resulted in fewer spawns and lower total fecundity. (4). Spontaneous

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spawning at the population level was observed; the first recorded in captivity at 16.2–18 °C and 32–33 psu, suggesting seasonal temperature cues and salinity thresholds.

1. Introduction

Grey mullets are considered among the important species for coastal aquaculture, primarily due to their euryhaline nature, which allows them to grow rapidly under a wide range of salinity conditions and corresponding environments—from brackish to hypersaline waters. As such, they can be reared in coastal facilities using brackish or hypersaline water, as well as in inland earthen ponds supplied with freshwater [1–3]. Grey mullets play a key ecological role in ecosystem energy flow, as they occupy one of the lowest trophic positions in the fish food web, deriving their energy primarily from detritus [4].

A native species of the Mediterranean and eastern Atlantic, the thicklip grey mullet (*Chelon labrosus* Risso, 1827) can be found in a range of environments, such as freshwater ponds and reservoirs, coastal lagoons, river estuaries, and marine habitats. Due to its highly appreciated flesh quality, this species has historically been targeted in coastal fisheries and extensive aquaculture making it a commercially valuable species [5,6]. Like other grey mullets, it displays an omnivorous feeding behaviour in its early stages of development before gradually shifting towards a herbivorous diet as it ages [7,8]. In addition, it readily accepts formulated feeds, displays strong adaptability, and achieves high growth rates under captive conditions [9], making it a promising candidate for commercial aquaculture with substantial potential for sustainable production [10]. Nevertheless, the success of sustainable aquaculture of this species also depends on a thorough understanding of its reproductive strategy, food preferences, and feeding behavior [7,8,10–15]. These aspects are essential for developing appropriate protocols for large-scale production.

Regarding its reproduction, to the best of our knowledge, spontaneous spawning of thicklip grey mullet has never been documented in captivity. Although encouraging results have been obtained in the semi-intensive production of hatchery-reared juveniles [1], large-scale aquaculture of this species remains underdeveloped. Current research and commercial practices still depend largely on the capture and rearing of wild broodstock and juveniles, largely because females do not release eggs naturally in captivity. Consequently, there is no reliable reproductive control protocol to secure consistent egg production for large-scale fry production.

In fishes that exhibit reproductive dysfunctions in captivity, such as those that fail to spawn, these issues are typically managed through hormonal therapies administered to the broodstock. Such treatments may involve the use of pituitary extracts rich in gonadotropins, purified gonadotropins (GtHs), or gonadotropin-releasing hormone (GnRH) analogues [16,17]. In females, hormonal therapies using gonadotropin-releasing hormone analogues (GnRHa) have been successfully applied in many fish species [18], particularly those that spawn multiple times, whether they exhibit group-synchronous or asynchronous oocyte development. The administration of GnRHa has proven effective through single or multiple injections or by using sustained-release delivery systems, with high success in inducing spawning. It has also played a crucial role in developing more reliable and less technically demanding protocols for reproduction control in captive fish species [16,19,20]. However, while the administration of GnRHa via a single injection has proven effective in inducing spawning in mullet species [21–24], we are not aware of any studies that have used repeated injections or slow-release delivery systems for this purpose.

The inhibitory role of dopaminergic activity in grey mullet reproduction has long been recognized [9]. In females, spawning induction often requires overcoming this inhibition

by administering high doses of progestogens, carp pituitary extracts (CPE), or human chorionic gonadotropin (hCG) [25]. Alternatively, dopaminergic inhibition can be bypassed by combining GnRHa with a dopamine antagonist, such as domperidone, metoclopramide, pimozide, or haloperidol.

Efforts to control thicklip grey mullet reproduction in captivity mainly focused on early spawning induction trials and broodstock management [21,23,26], as well as studies on embryonic development and early larval rearing [1,6,27,28]. Specifically, a two-step protocol combining gonadotropins (CPE or hCG) with LHRHa analogues has proven to be the most effective method for spawning thicklip grey mullet. For instance, Ref. [21] reported successful spawning induction of thicklip grey mullet using CPE as a priming dose (7 mg kg^{-1} BW) followed by an LHRHa resolving dose (50 μg kg^{-1} bw). Similarly, Ref. [23] successfully induced spawning using a protocol involving priming injections of hCG (5,000–10,000 IU kg^{-1} bw) followed by resolving injections of hCG (10,000 IU kg^{-1} bw) in combination with LHRHa (100–200 μg kg^{-1} bw). These findings highlight the efficacy of combined gonadotropin–GnRHa approaches; however, the reliance on high hormone doses illustrates the importance of developing more efficient and sustainable reproductive control protocols.

Alternatively, a single-step administration of a DA with GnRHa, even at low doses, has proven highly potent in inducing spawning of flathead grey mullet (*Mugil cephalus*) in captivity. For instance, Ref. [22] treated female flathead grey mullet with GnRHa (slow-release implants at 10 mg kg^{-1} bw) in combination with metoclopramide (15 mg kg^{-1}), achieving high ovulation and spawning success rates without any priming dose of gonadotropins (hCG or CPE). Given the demonstrated efficacy of DA–GnRHa combinations in inducing spawning without the need for high doses of gonadotropins, this approach could provide a cost-effective and welfare-friendly alternative to conventional two-step hormonal protocols. This is particularly relevant since spontaneous spawning of thicklip grey mullet has not been reported in captivity, and no reliable reproductive control protocol currently exists to ensure sufficient egg production for large-scale fry production.

The aim of the present study was to evaluate the efficacy of GnRHa administration combined with metoclopramide using two different delivery systems (weekly injections versus slow-release EVAc implants) on the spawning performance and egg quality of thicklip grey mullet. To our knowledge, this is the first systematic comparison of injection vs. implant delivery of GnRHa in this species.

2. Materials and Methods

2.1. Broodstock Maintenance and Sampling Groups Formation

The experimental trial was carried out at the facilities of a private fish farm, operating in Variko lagoon (Regional Unit of Pieria, Region of Central Macedonia, Northern Greece). Thicklip grey mullet (*C. labrosus*) broodstock were transferred in mid-January from a seawater channel within the lagoon, in close proximity to the fish farm facility, where they had been maintained for over one year under natural photoperiod and temperature conditions, and fed daily to apparent satiation with a commercial diet. Each fish was subcutaneously tagged with a passive integrated transponder PIT tag (12 mm, 125 kHz; AVID Identification Systems Ltd., Lewes, UK). In mid-March, females with oocytes > 500 μm and minimal atresia, and spermiating males were selected for the experiment. Selected fish were first sedated in tank with 2-phenoxyethanol (0.08 mL L^{-1}) and then deep-anaesthetized in a transfer bath (0.1 mL L^{-1}) prior to handling. A total of 31 females (>6 years old; mean body weight, BW = 1.1 kg) and 29 males (mean BW = 0.865 kg) were distributed among three treatment groups (Saline-INJ, GnRHa-INJ, GnRHa-IMP) each containing two replicate populations (sampled and unsampled) maintained in six 3.5 m^3

tanks. Unsampled populations (three tanks) received treatment but not sampled. Sampled populations (three tanks) received treatment and underwent blood collection, and ovarian biopsy (females) or sperm collection (males), (Figure 1).

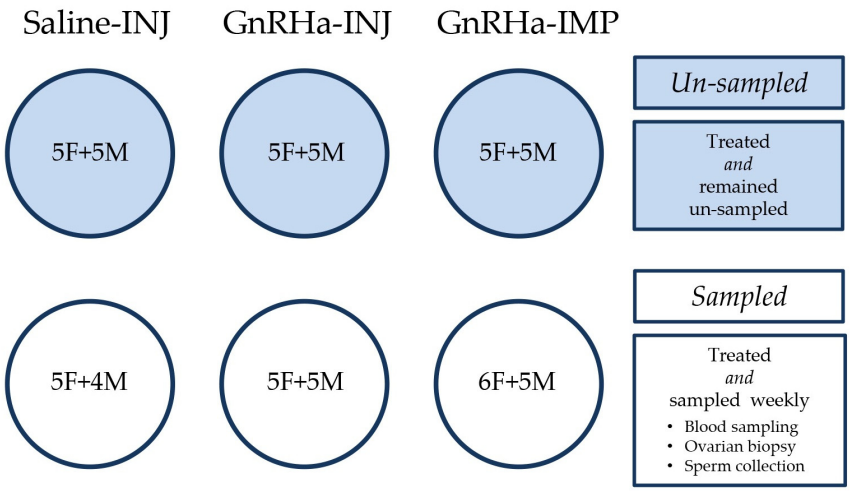

Figure 1. Experimental setup showing number of tanks, number of female and male thicklip grey mullet broodstock treated, treatment groups (Saline-INJ, GnRHa-INJ, GnRHa-IMP) and replicate populations per treatment (Unsampled, Sampled).

Fish were maintained under natural photoperiod and temperature and were fed to apparent satiation once daily, six days per week, with a mixed diet of marine grower pellets (Biomar, Velestino, Greece), frozen squid, and deep-water rose shrimp. Salinity, temperature, and dissolved oxygen concentrations were monitored three times per week. Throughout the experimental period, salinity and water temperature averaged 32.6 ± 0.71 psu and 15.8 ± 1.96 °C, respectively; dissolved oxygen was maintained at 7.5 ± 0.5 mg L^{-1}, and pH was 8.00 ± 0.2.

2.2. Experimental Design and Hormonal Treatment

To evaluate the effectiveness of a GnRH analog (GnRHa) on spawning induction, three experimental groups of females were treated: (a) a single intramuscular injection of physiological saline (0.9% NaCl, 1 mL; Saline-INJ), (b) a single IM dose of GnRHa (Des-Gly^{10}, d-Ala^{6}-ProNEth^{9}-mGnRHa; 10 μg kg^{-1} bw; GnRHa-INJ), and (c) a subcutaneous Ethylene–Vinyl Acetate (EVAc) implant containing the same GnRHa (50 μg kg^{-1} bw; GnRHa-IMP). Females in the GnRHa-INJ and the Saline-INJ groups were subsequently treated once a week with a single injection of GnRHa (10 μg GnRHa kg^{-1} bw) or saline, respectively, for four weeks post treatment (D7, D14, D21, D28). Males in each group received the same respective treatments. The EVAc implants were loaded with a high dose of GnRHa (50 μg kg^{-1}) to ensure sustained release over time, since they were applied only once (Day 0), during the four-week experimental period. In contrast, the injections contained a lower GnRHa dose (10 μg kg^{-1}) to provide weekly stimulation.

Additionally, at each treatment date, GnRHa treated females (GnRHa-INJ and GnRHa-IMP groups) also received a single intramuscular injection of the dopamine antagonist, metaclopramide (Met) at a dose of 15 mg kg^{-1} bw.

2.3. Handling and Sampling Schedule

Prior to handling, fish were fasted for 48 hrs. On each sampling date, fish were first tranquilized initially in their tank (0.08 mL L^{-1} 2-phenoxyethanol) and then transferred to a bath for deep sedation (0.1 mL L^{-1} 2-phenoxyethanol) to minimize handling stress. In

each treatment group, only females from the sampled population were subjected to weekly handling, while the unsampled population was left unhandled. Evaluations involved blood collection for steroid hormone measurement, ovulation assessment, and ovarian biopsy to determine oocyte diameter and oogenesis stage. This design allowed assessment of potential handling effects on reproductive performance. Five sampling events were conducted (D0, D7, D14, D21, D28).

2.4. Blood Collection

Blood samples (~1 mL) were collected weekly from the caudal vein using a sterile 1 mL syringe fitted with a 23G needle and immediately transferred to microcentrifuge tubes kept on ice. Blood samples were centrifuged at 3000 g for 10 min and the collected plasma was stored at −20 °C until sex steroid hormone (testosterone, T and 17β-estradiol, 17β-E2) analysis.

2.5. Steroid Hormones Analysis

Plasma steroids from each fish were quantified using previously described enzyme-linked immunoassays (ELISA) [29–31], with minor modifications and reagents supplied by Cayman Chemical Company (Ann Arbor, MI, USA). For steroid extraction, 200 µL of plasma were extracted twice with 2 mL diethyl ether. Extraction was performed using vigorous vortexing (Vibramax 110, Heidolph, Schwabach, Germany) for 3 min. Following the decantation of the organic phase, the supernatant was dried under a nitrogen stream using a Reacti-vap III (Pierce, Rockford, IL, USA) and the residue was reconstituted in assay buffer for ELISA analysis.

2.6. Assessment of Ovulation and Ovarian Biopsy

Females were examined for ovulation by gentle pressure to the abdominal region to detect the release of eggs. In non-ovulating females, ovarian biopsies were obtained to measure oocyte diameter and a portion was fixed for histological analysis. Ovarian biopsies were obtained with the use of a Pipelle de Cornier catheter. A wet mount of the biopsy was examined immediately under a stereoscope for a preliminary evaluation of oogenesis stage. A second subsample was fixed in Gilson's fluid for image-analysis measurement of oocyte diameter (n = 50 largest oocytes of the pooled ovarian biopsies from two females per sampling day per treatment), and a third was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing and analysis. For histology, the ovarian biopsies were dehydrated through graded ethanol solutions (70–96%) and embedded in methacrylate resin (Technovit 7100®, Wehrheim, Germany). Sections of 4 µm thickness were obtained using a microtome (Leica RM 2245, Nussloch, Germany), stained with a combination of methylene blue-azure II-basic Fuchsin, and examined under a light microscope (Olympus BX 40, Tokyo, Japan). Images (Figure 2) were captured with a microscope digital camera (MIchrome 5 Pro; Tucsen Photonics Co., Ltd., Fuzhou, China).

2.7. Egg Collection, Fecundity and Fertilization Rate

Egg collectors were continuously connected to the broodstock tanks throughout the experiment. Spawned eggs were collected every morning into a 10 L bucket, for quantifying fecundity (egg production) and evaluating fertilization success. Total daily fecundity was estimated by counting the total number of eggs in three 10 mL sub-sample, after gentle aeration/stirring for homogenization. Fertilization success was evaluated at the same time by calculating the number of viable eggs with respect to the total number of eggs collected.

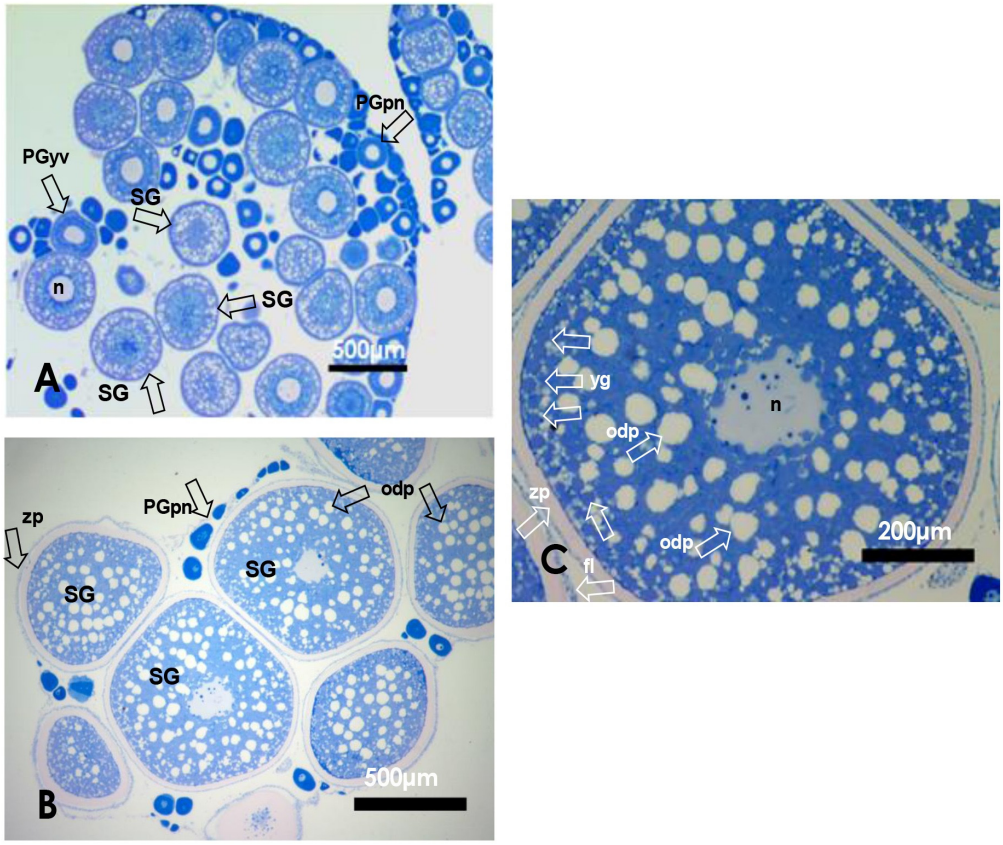

Figure 2. Ovarian sections of *Chelon labrosus*. (**A**): ovary at vitellogenesis phase. Primary growth phase oocyte at the perinucleolus step (PGpn), primary growth phase oocyte at yolk vesicle step (PGyv), secondary growth stage oocyte (SG). (**B**,**C**): secondary growth phase oocyte (SG) with oil droplet plates (odp) and acidophilic yolk granules (yg), follicular layer (fl), nucleus (n), and zona pellucida (zp).

2.8. Statistical Analysis

Data were checked for normality using the Shapiro–Wilk test. A Scheirer–Ray–Hare test (non-parametric two-way ANOVA) was used to assess the effects of the main factors (sampling time/day, hormonal treatment) and their interaction on each repeatedly measured parameter. A Kruskal–Wallis test (non-parametric one-way ANOVA for independent samples) was applied to evaluate the effect of hormonal treatment (one factor) on independently measured spawning performance variables. Where omnibus tests were significant, post hoc pairwise comparisons were conducted using Dunn's test and p values were adjusted with Holm's step-down correction. Results considered significant at a = 0.05. Analyses were performed using IBM SPSS Statistics v 28 [32] and R v 4.5.1 [33] using the packages FSA v 0.10.0 [34] and rcompanion v 2.5.0 [35].

3. Results

3.1. Oocyte Growth

At the beginning of the experiment (D0, 17 March), all females, whether Saline-INJ or GnRHa treated, were in advanced vitellogenesis, with mean oocytes diameter at similar sizes ranging from 0.884 to 0.893 mm (p = 0.465, Figure 3B). The Scheirer–Ray–Hare analysis on oocyte diameter showed that all three sources of variation (treatment, day, and treatment × day) were highly significant (treatment, H(2) = 61.19, $p < 0.001$; day, H(4) = 191.39, $p < 0.001$; treatment × day interaction, H(8) = 109.40, $p < 0.001$), indicating that oocyte growth was influenced not only by the hormonal treatment but also by the

sampling day and the interaction between the two variables. The significant treatment × day interaction suggested that the pattern of oocyte growth over time differed among the three treatment groups.

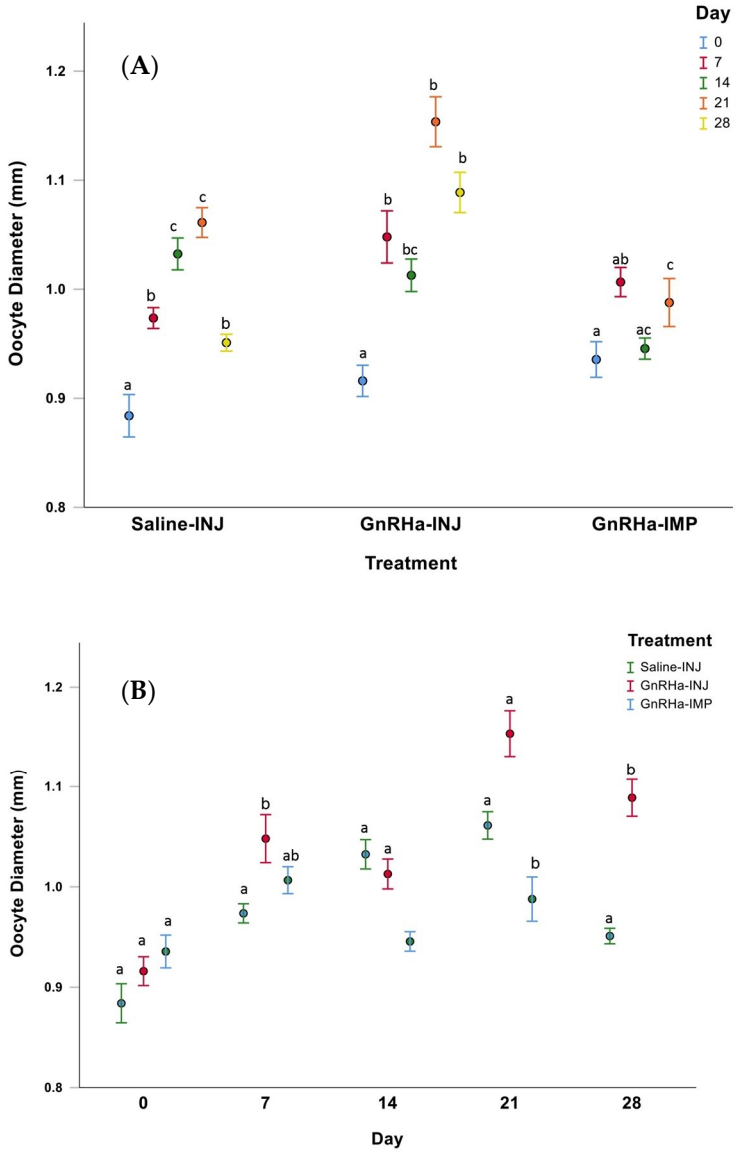

Figure 3. (**A**): Mean (±SE) values of diameter of the largest 50 oocytes from ovarian biopsies according to day (0–28) for saline-injected (Saline-INJ), GnRHa-injected (GnRHa-INJ) and GnRHa-implanted (GnRHa-IMP) female individuals. (**B**): Mean (±SE) of oocyte diameter according to treatment on each sampling day. Different lowercase letters indicate significant differences among sampling days at each treatment (**A**) or among treatments at each day (**B**), at significance level a = 0.001.

Indeed, in Saline-INJ females, an initial increase followed by a notable reduction in oocyte size was detected on D28, marking a regression phase toward the end of the experimental period (Figure 3A), indicating a return toward smaller diameters late in the trial. A similar pattern was observed in GnRHa-IMP females, where oocyte diameter peaked at D7 and then subsequently declined (D14, D21) to levels comparable to those recorded on D7, suggesting a transient maturation response and indicating limited or incomplete progression of oocyte development (Figure 3A). In contrast, GnRHa-INJ females maintained larger diameters beyond D7, with sustained or further increases through D14 and D21, whereas GnRHa-IMP showed no significant change among D0–D14 and Saline-INJ regressed by D28 (Figure 3A).

At specific timepoints, the GnRHa-treated females (GnRHa-IMP & GnRHa-INJ) exhibited significantly larger oocyte diameters than Saline-INJ females (e.g., D7 and D28; $p < 0.001$, Figure 3B), indicating a stimulatory effect of GnRHa on oocyte growth. However, the mean oocyte diameter of GnRHa-IMP females was significantly lower than those of Saline-INJ and GnRHa-INJ females at Days 14 & 21 (Figure 3B). This oocyte growth pattern implies that the injection-based GnRHa protocol (i.e., the GnRHa-INJ treatment) was more effective in promoting and sustaining oocyte development, in comparison to both the implant-based treatment (GnRHa-IMP) and the control group (Saline-INJ, Figure 3B).

3.2. Reproductive Performance (Spawns, Fecundity and Fertilization Success) Between Unsampled and Sampled Populations

Across the three experimental populations (Saline-INJ, GnRHa-INJ, and GnRHa-IMP), a total of 15 spawning events were recorded over a 22-day period, yielding 2,163,080 eggs in total (Table 1). Unsampled females spawned more frequently (11 vs. 4 spawns) and produced more eggs (1,636,900 vs. 526,080 eggs) than the sampled females (Table 1). Although the number of spawnings and the total fecundity (TF) were nearly twice as high in the unsampled populations, the values for daily fecundity (DF) and daily relative fecundity (DRF) were only slightly higher. For instance, DF was 148,818 in unsampled females compared to 131,520 eggs day^{-1} in sampled ones, while DRF values were 25,416 and 24,826 eggs day^{-1} kg^{-1}, respectively. Thus, the higher fecundity in the unsampled groups was mainly due to their increased spawning frequency, as they spawned nearly twice as often.

Table 1. Number of spawns, days of spawns (in parenthesis), total fecundity (TF), mean (±SD), values of daily fecundity (DF), daily relative fecundity (DRF) and fertilization success of sampled or unsampled experimental populations of female thicklip grey mullet treated with either saline injections (Saline-INJ) or GnRHa injections (GnRHa-INJ) or GnRHa implants (GnRHa-IMP). Different superscript letters within a column indicate significant differences among treatments ($\alpha = 0.05$).

Sampling Procedure	Treatment	Spawns	Fecundity (nb of Eggs)	DF (Eggs Day^{-1})	DRF (Eggs Day^{-1} kg^{-1})	Fertilization Success (%)
Unsampled populations	Saline-*INJ*	4 (days: 15, 17, 18, 19)	311,900	77,975 ± 39,141 ^{a}	12,556 ^{a} ± 6303	2.9 ^{a} ± 1.8
	GnRHa-*INJ*	2 (days: 13, 14)	558,300	279,150 ± 169,150 ^{a}	51,408 ^{a} ± 31,151	69.4 ^{b} ± 18.6
	GnRHa-*IMP*	5 (days: 4, 7, 14, 17, 18)	766,800	153,360 ± 135,452 ^{a}	25,307 ^{a} ± 22,352	12.8 ^{a} ± 5.0
	Total	11	1637,000	148,818 ± 67,198	25,416 ± 11,450	19.5 ± 8.3
Sampled populations	Saline-*INJ*	1 (day 13)	82,500	82,500 ± 0	15,082 ± 0	82.6 ± 0
	GnRHa-*INJ*	1 (day 22)	357,000	357,000 ± 0	68,130 ± 0	40.3 ± 0
	GnRHa-*IMP*	2 (days: 4, 7)	86,580	43,290 ± 40	8046 ± 7	2.9 ± 1.8
	Total	4	526,080	131,520 ± 75,726	24,826 ± 14,529	32.2 ± 19

In contrast, fertilization success was lower in unsampled populations (19.5%) compared to sampled ones (32.2%), mainly due to the notably low success in unsampled Saline-INJ females (2.9%, Table 1).

3.3. Comparing Data Within Unsampled Populations

When comparing egg production within the unsampled populations, it was evident that the GnRHa-treated females (both GnRHa-INJ and GnRHa-IMP) exhibited higher performance than the Saline-Treated Control (STC) females (Table 1), indicating the effectiveness of GnRHa treatment in enhancing reproductive output.

The GnRHa–INJ treated females, which had only two spawns compared to the four spawns of the STC group they produced an almost two times higher total fecundity, with an almost four times higher DF (279,150 vs. 77,975 eggs day^{-1} in STC group) and DRF values (51,408 vs. 12,556, eggs day^{-1} kg^{-1}, Table 1). Similarly, the GnRHa-IMP treated females, despite performing less effectively that the GnRHa-INJ females, they had an almost 2.5 times higher total fecundity than STC females with two times higher DF and DRF values (153,360 and 25,307 eggs day^{-1} kg^{-1}, respectively) compared to STC females. Regarding the fertilization success, the eggs produced by the GNRHa-INJ females had a significantly higher fertilization rate value (69.4%, $p < 0.001$) compared to the other two groups, whereas the fertilization success in the GnRHa-IMP females, although higher (12.8%), was not significantly different from that of the Saline-INJ females (1.8%, $p = 0.27$).

3.4. Spawning Kinetics According to the Treatment

The spawning patterns varied across treatments (Figure 4). Saline-INJ females spawned five times between days 13–19 post-treatment (third week). Fecundity ranged from 5300 to 189,000 eggs, with the highest output and fertilization success on day 13 (189,000 eggs; 82.6%). Three spawning events of GnRHa-INJ females were recorded on days 13, 14, and 22, corresponding to 6–7 days after the second injection and 1 day after the third. Fecundity ranged from 110,000 to 448,300 eggs, with fertilization success exceeding 40% (88%, 50%, 40.3%). Two spawning events of GnRHa-IMP females occurred on days 4 and 7 (94,250 and 53,130 eggs), both with very low fertilization rates (5.0% and 3.4%). Additional events took place on days 14, 17, and 18, coinciding with the other groups. The day 17 spawn yielded 694,000 eggs (29.8% fertilization), the highest single-day egg production in the study.

3.5. Total Egg Production Data

Saline-INJ females spawned exhibiting the lowest—though not statistically significant—daily relative fecundity (13,061 eggs kg^{-1} day^{-1}, $p = 0.097$, Table 2). However, they achieved a marginally non-significant intermediate fertilization success (18.8%, $p = 0.059$, Table 2).

GnRHa-INJ females spawned earlier than Saline-INJ females, with spawning events occurring during the second and fourth weeks (6 and 7 days after the second injection and 1 day after the third injection). These females achieved the highest daily relative fecundity (56,982 eggs kg^{-1} body weight, $p = 0.097$), along with a significantly higher fertilization success (59.7%, $p = 0.034$; Table 2). The final spawning event of this group was recorded 24 h after the fourth injection (D22, 15 April).

Also, the GnRHa-IMP females spawned earlier than the Saline-INJ females. They spawned primarily during the first (4 out of 7 events) and second weeks post-treatment, exhibiting an intermediate egg production ($p = 0.55$ compared to Saline-INJ females) and the significantly lowest fertilization success (9.1%, $p = 0.012$; Table 2).

3.6. Steroid Hormone Levels

The analysis of T plasma levels revealed no significant differences between treatment groups ($p = 0.153$), suggesting that the type of treatment did not significantly influence T levels. However, there was a significant effect of day ($p = 0.011$), indicating that T levels varied over time regardless of treatment. In fact, levels were highest on day 0, ranged similarly up to day 21 and significantly decreased on day 28 ($p = 0.009$, Figure 5A). The interaction between treatment and day was not significant, demonstrating that temporal patterns in T levels were similar across all treatment groups ($p = 0.99$).

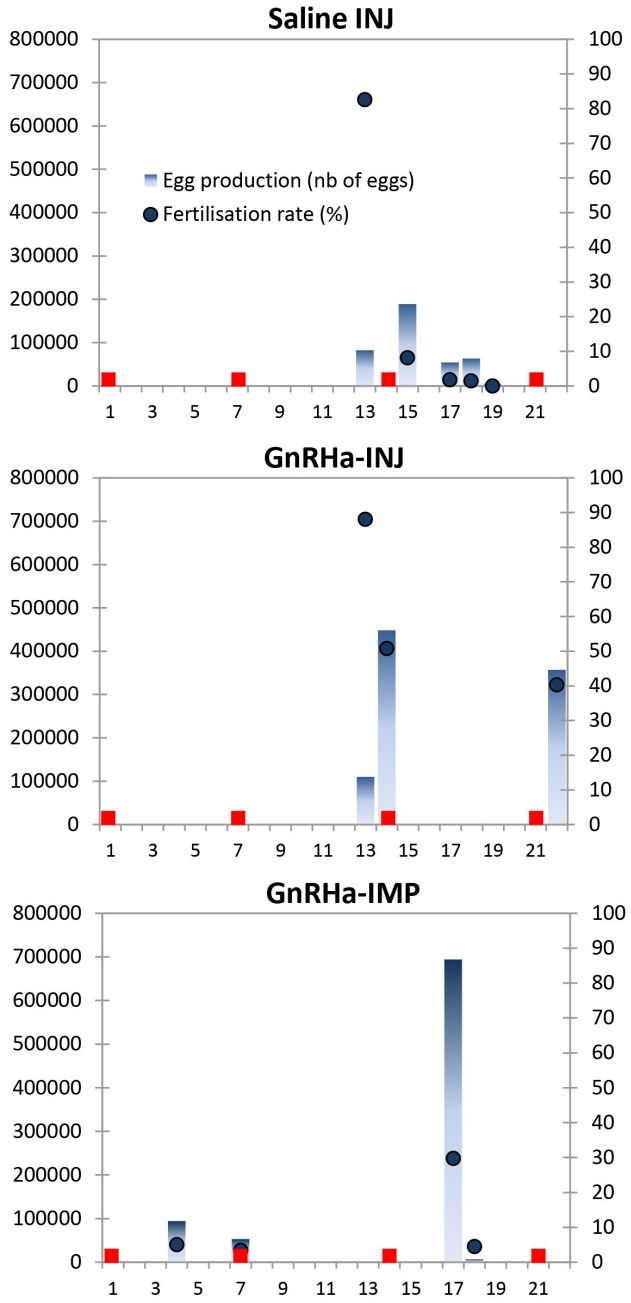

Figure 4. Daily fecundity (number of eggs) and fertilization success of saline-injected control (Saline-INJ), metoclopramide-injected GnRHa-injected (GnRHa-INJ) and metoclopramide-injected GnRHa-implanted (GnRHa-IMP) female thicklip grey mullets. The red square indicates the day of injection: saline in the Saline-INJ group, metoclopramide in both GnRHa groups, and GnRHa in the GnRHa-INJ group.

Similarly to T levels, a significant effect of day on 17β-E2 concentrations was also revealed ($H = 34.16$, $p < 0.001$). The 17β-E2 levels were significantly decreased by day 7 ($p < 0.001$), reaching consistently low levels through day 28 (Figure 5B), suggesting a stabilisation of 17β-E2 levels during the later stages ($p \geq 0.207$). The effect of treatment ($p = 0.069$), but also the effect of the treatment × day interaction ($p = 0.094$), was not significant, suggesting that the temporal pattern of 17β-E2 concentrations was similar across all treatment groups.

Table 2. Number of spawns, days of spawns (in parenthesis), total fecundity (TF), mean (±SD) values of daily fecundity (DF), daily relative fecundity (DRF) and fertilization success of female thinlip grey mullet treated with saline injections (Saline INJ) or GnRHa injections (GnRHa-INJ) or GnRHa implants (GnRHa-IMP). Different superscript letters within a column indicate significant differences among treatments ($\alpha = 0.05$). Note: Day numbers are relative to D0 = 17 March; therefore, D22 = 8 April and D28 = 14 April.

Treatment	Spawns (Days)	TF (nb of Eggs)	DF (Eggs Day^{-1})	DRF (Eggs Day^{-1} kg^{-1})	Fertilization Success (%)
Saline-INJ	5 (13, 15, 17, 18, 19)	394,400	78,880 (30,331) ^{a}	13,061 (4908) ^{a}	18.8 (16.0) ^{a}
GnRHa-INJ	3 (13, 14, 22)	915,300	305,100 (101,047) ^{a}	56,982 (18,828) ^{a}	59.7 (14.5) ^{b}
GnRHa-IMPL	7 (4, 7, 14, 17, 18, 4, 7)	853,380	121,911 (95,650) ^{a}	20,375 (15,749) ^{a}	9.1 (3.9) ^{a}
Total	15	2,163,080	144,205 (51,933)	25,258 (8985)	22.9 (7.7)

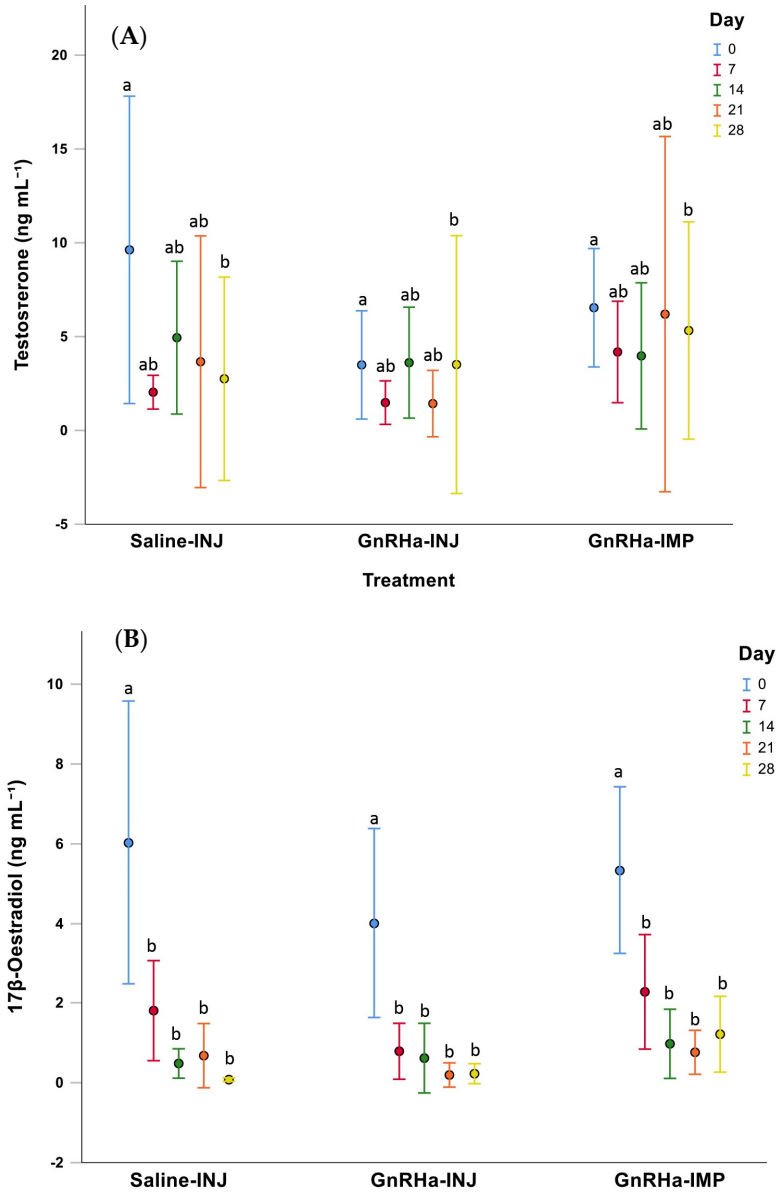

Figure 5. Mean (±SE) values of testosterone (**A**) and 17β-oestradiol (**B**) concentrations according to Day (0, 7, 14, 21, 28) in saline-injected (Saline-INJ), GnRHa-injected (GnRHa-INJ) and GnRHa-implanted (GnRHa-IMP) female individuals. Different letters denote significant differences among days within treatment (Dunn's test, $\alpha = 0.05$).

4. Discussion

The present study demonstrated that administration of GnRHa and metoclopramide (GnRHa | Met) in captive female thicklip grey mullet (*C. labrosus*) successfully induced multiple spawning, producing nearly three times as many spawns than those observed in control females (11 vs. 4 spawns). Multiple GnRHa injections triggered a rapid spawning response, yielding 51,408 eggs day^{-1} kg^{-1} with 69.4% fertilization success. Compared with slow-release implants, this method produced higher fecundity and significantly better fertilization, indicating improved control over egg production.

Species with group-synchronous ovarian development (such as the thicklip grey mullet) exhibit relatively long ovulatory intervals—for example, 2–10 days in meagre, *Argyrosomus regius* [36] and 1–2 weeks in European sea bass *Dicentrarchus labrax* [37]. When GnRHa injections are timed to match this ovulatory rhythm (e.g., every 7 days in meagre [38] and 7–14 days in European sea bass [39], they are often more effective at inducing oocyte maturation and spawning—sometimes outperforming sustained-release implants—likely because they better mimic the natural surges in plasma LH that accompany the maturation–ovulation cycle of each successive oocyte batch [38].

GnRHa successfully induced spawning under both delivery protocols when preceded by weekly injections of metoclopramide (Met, 15 mg kg^{-1} bw). This is the first report describing successful spawning induction in thicklip grey mullet using GnRHa, particularly in combination with a dopamine antagonist (DA). Injections of Met have also been successfully used to induce spawning in flathead grey mullet (*M. cephalus*). For example, priming injections of Met (15 mg kg^{-1} bw) combined with GnRHa (10 µg kg^{-1} bw), followed by resolving injections of Met (15 mg kg^{-1} bw) and GnRHa (20 µg kg^{-1} bw), successfully induced spawning in *M. cephalus* [22]. Furthermore, priming injections of CPE (20 mg kg^{-1} bw) followed by resolving injections of GnRHa (200 µg kg^{-1} bw) combined with Met (20 mg kg^{-1} bw) successfully induced spawning in *M. cephalus* [40].

In the present study, Met injections were synchronised with the GnRHa administration; all females treated with GnRHa—whether by injection or implant—received weekly Met injections. Such synchronisation may have played an important role in enhancing the effectiveness of GnRHa in inducing spawning. In a preliminary trial aiming to induce spawning in thicklip grey mullet, domperidone (Dom) was used as a DA (10 mg kg^{-1} bw) in a single injection at the beginning of an experiment of GnRHa delivery either via weekly injections or as slow-release implants. The single administration of Dom failed to induce spawning in any of the GnRHa-treated groups, suggesting that in a delivery system involving multiple GnRHa injections, only concurrent repeated DA administration may be effective in inducing spawning (personal observation). The favourable reproductive response observed—using weekly Met injections concurrently with GnRHa administration in this study—supports the presence of a strong dopaminergic inhibitory mechanism. Nevertheless, further experimental confirmation of this dopaminergic inhibition could be obtained by monitoring post-treatment plasma LH levels in females treated for spawning under optimal environmental conditions.

Spawning of the thicklip grey mullet has previously been successfully induced using progesterone [25,26], and exogenous gonadotropins, i.e., human chorionic gonadotropin, hCG [27,28], and LHRHa [23,41]. For example, Ref. [23] successfully induced spawning using hCG (5000–10,000 IU kg^{-1} bw) as a priming injection followed by a resolving injection of hCG (10,000 IU kg^{-1} bw) and LHRHa (100–200 µg kg^{-1} bw). Similarly, Ref. [21] achieved successful induction with CPE at a priming dose (7 mg kg^{-1} bw) followed by a resolving dose of LHRHa (50 µg kg^{-1} bw). However, these protocols required substantially higher hormone doses (in mg or thousands of IU) compared to low doses of Met (15 mg kg^{-1}) and GnRHa (10 and 50 µg kg^{-1}) used in the present study. Beyond dose efficiency, GnRHa

administration in the form of multiple injections provides greater scheduling flexibility (e.g., weekly pulses aligned with ovulatory rhythms) and is generally more effective in species with group-synchronous ovarian development, such as the thicklip grey mullet, than exogenous gonadotropins. Similarly, GnRHa implants, by reducing the frequency of handling, are highly effective for the prolonged spawning induction and egg production, particularly in species with asynchronous ovarian development. Also, the use of exogenous gonadotropins is constrained by batch-to-batch variability in potency and by biosafety risks related to potential pathogen transmission [17,18].

Under the multiple-injection GnRHa protocol, female thicklip grey mullets in this study achieved a daily relative fecundity of 51,408 up to 68,130 eggs kg^{-1} bw. These values are substantially lower than 494,655 eggs kg^{-1} BW reported after administration of 10,000 IU hCG as a priming dose and 10,000 IU hCG plus 100 μgLHRHa kg^{-1} bw as a resolving dose to a single female thicklip mullet of 1.690 kg [23] and lower than the potential fecundity value of 372,000 to 745,000 eggs kg^{-1} BW reported for captive females induced to spawn [9]. The daily relative fecundity values of the spontaneously spawning females in this study (12,556 eggs kg^{-1} BW; unsampled Saline-INJ populations) were also lower than the species' potential fecundity in captivity. However, optimisation of the spawning induction protocol could increase both the number of spawnings and total fecundity. Such optimization may involve testing the efficacy of higher doses of GnRHa, such as triple-dose injections (30 μg GnRHa kg^{-1} bw were successful in *M. cephalus* [22], as well as higher DA doses (30 mg kg^{-1} in *M. cephalus*, [22]. Furthermore, the effectiveness of more frequent DA | GnRHa administrations (e.g., every three days instead of weekly injections) could be investigated. In the case of Senegalese sole, a multiple spawner fish with group-synchronous ovarian development, tri-daily GnRHa administrations have been shown to alter steroid hormone concentrations, influencing final oocyte maturation and ovulation more markedly than weekly administrations [42].

The results of this study showed a fertilization success of 69.4% following multiple GnRHa injections of unsampled (i.e., not biopsied) females, although this value represents the mean of only two spawnings from unsampled populations. Overall, fertilization rates were variable. In GnRHa–treated females, higher fecundity tended to coincide with higher fertilization success, whereas in control females, fertilization success appeared random with respect to fecundity. In any case, the fertilization success achieved from repeated injections of GnRHa was not significantly higher than that obtained with CPE or hCG and LHRHa in priming and resolving doses (0–63% in [15]; up to 88% in [13], but it was less variable.

In contrast to the Mediterranean variety of the flathead grey mullet (*M. cephalus*), which typically exhibits limited ovarian maturation and spermatogenesis in captivity [22,43], both female and male thicklip grey mullet in the present study achieved full gonadal development from mid-March to mid-April. Females had large vitellogenic oocytes (>800 μm), and all males were in full spermiation (unpublished data). Control females spawned spontaneously, releasing approximately 394,400 eggs over five spawning events (from March 19 to April 8), during which salinity was 32 to 33.5 psu and temperature gradually increased from 14 to 18 °C (Figure 6). Notably, during this same period, most of the spawnings of GnRHa-INJ-treated females, as well as some spawnings of GnRHa-IMP-treated females, were also recorded. These constitute the first documented spontaneous spawns from captive populations (apart from two isolated cases reported previously [21,44]). As temperature is a key regulator of final oocyte maturation and spawning in fish [45], the spring warming—potentially surpassing a species-specific minimum threshold [46]- may be linked to these spontaneous spawns (Figure 6). Salinity, however, is also well known to have a significant role in reproduction in euryhaline fish species, such as thicklip grey mullet. In the flathead grey mullet, salinities between 13 and 35 psu promoted ovarian

maturation and spawning while enhancing egg quality. In contrast, insufficient freshwater conditions resulted in lower fertilization rates, fewer mature females, and a delay in oocyte growth [47]. In the thinlip grey mullet (*Liza ramada*), salinity plays a critical role in oocyte hydration during final oocyte maturation (FOM) and spawning, with egg buoyancy rising with increased salinity [48]. Likewise, salinity influences the onset of spawning in female sea bass (*Lateolabrax japonicus*), with both the quantity and quality of produced eggs being higher in low-salinity seawater than in normal seawater [49]. Ovarian development in black bream (*Acanthopagrus butcheri*) proceeded normally over a salinity range of 5–35 psu, showing little effect of salinity, whereas fertilization rate and egg/larval viability were highly salinity-dependent, with poor success at 5 psu and optimal results at 20–35 psu [50].

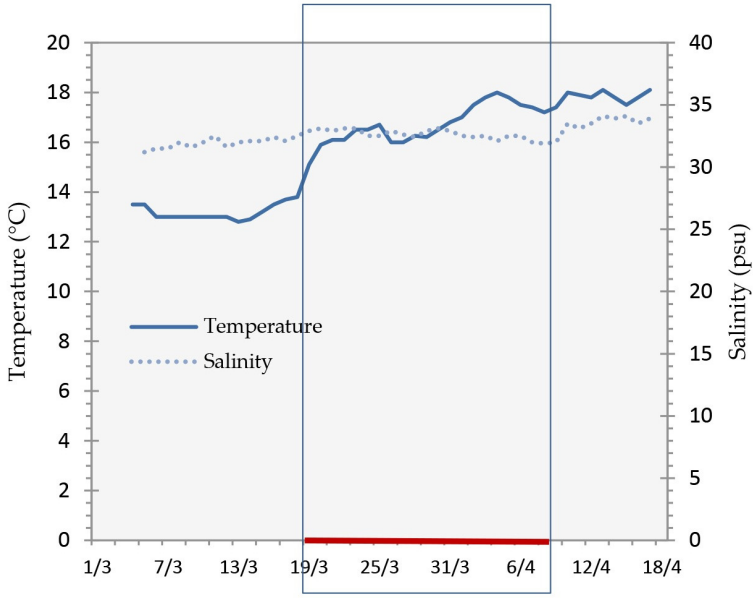

Figure 6. Daily fluctuations in water temperature (°C) and salinity (psu) from early March to mid-April. The experimental period (17 March–14 April) is indicated by the red line.

Similar to flathead grey mullet [51], male thicklip grey mullet have been shown to have a considerable inhibition of sperm motility within a salinity range of 9 to 18 psu. This may indicate a key threshold for effective reproduction [44]. It is reasonable to hypothesise that both salinity and temperature may play significant roles in regulating the sexual maturity and spawning of *C. labrosus*, given the species' euryhaline capacity [52,53], its widespread occurrence in both freshwater and brackish environments [3], and the evidence that salinity is a key determinant of habitat selection for this species [54,55]. In the wild, adult populations of thicklip grey mullet spawn offshore in open seawater, where salinity values typically exceed 38 psu [3]. If this ecological requirement also applies under captive conditions, adjusting salinity to levels above 30–33 psu, in combination with appropriate temperature, may enhance the likelihood of spontaneous spawning in females. Further research is warranted to elucidate more precisely the role of salinity and temperature in the reproductive physiology of the thicklip grey mullet.

The markedly lower number of spawns in sampled females compared to unsampled ones likely reflects handling-induced stress during induced spawning. Routine manipulations such as anesthesia, blood sampling, ovarian biopsies, and repeated injections are known to elevate cortisol, which disrupts the reproductive endocrine axis and reduces fecundity and egg quality [56–58]. Our findings, showing reduced spawning and total fecundity in frequently handled females, are consistent with this mechanism and align with reports from other cultured species, where pre-spawning stressors impaired reproductive

performance through cortisol-mediated disruption [58–60]. Cortisol interferes with reproductive function by suppressing the release of GnRH or gonadotropins and by directly inhibiting the steroidogenic capacity of the gonads [57,61,62]. Another possible mechanism involves alterations in the binding characteristics of sex steroid-binding proteins thereby further affecting steroid availability [63]. Our data indicate that frequent handling (i.e., repeated blood collection and gonad biopsies) of sampled females was associated with substantial reductions in both the number of spawns and total fecundity.

Specifically, for thicklip grey mullet [44] reported that >50% of captive females reared in tanks in Cantabria (N. Spain) failed to complete ovarian development from mid-season onwards (during the first captivity season from March to April) due to repeated handling, i.e., the stress associated with fortnightly gonadal cannulation (ovarian biopsy every two weeks). However, given that only a single spontaneous, fertilized spawn was reported in their trial, Ref. [44] also argued that optimizing environmental variables (e.g., photoperiod, temperature, salinity) may help overcome this failure of females to complete final oocyte maturation, ovulation, and spawning. In the present study, by contrast, we observed no evidence of oocyte regression on either macroscopic or microscopic examination of weekly ovarian biopsies used to monitor ovarian development, despite the higher frequency of ovarian biopsies applied. On the contrary, all females remained in excellent reproductive condition throughout the spawning season, with large vitellogenic oocytes (0.80–1.15 mm), without any sign of ovarian regression.

Acute physical handling has been reported to impair spawning performance via cortisol-mediated suppression of gonadal steroidogenesis. In vivo, acute stress elevates plasma cortisol followed by a rapid decline in plasma testosterone—and in some cases 17β-oestradiol—without concomitant changes in maturational gonadotropin. The steroid decreases can occur within 1–3 h of the stressor and constitute a recognized mechanism by which stress compromises spawning performance, often increasing follicular atresia and reducing egg/larval viability in many non-salmonids [61]. Maintaining cortisol within an optimal physiological range—the "Goldilocks" zone—is critical for successful completion of FOM and spawning. Deviations from this optimal cortisol range, or disruption of the complex hormonal interplay by handling stress or hormonal interventions, have been associated with impaired reproductive function, reduced fecundity, and poor egg quality in teleosts [64].

The fact that plasma levels of T and 17βE2 did not increase following the administration of GnRHa and instead showed a progressive decrease during subsequent samplings is notable. In other marine fishes with group-synchronous oocyte development, including *M. cephalus* [22], Senegalese sole *Solea senegalensis* [42], and European sea bass *Dicentrarchus labrax* [65], the 17βE2 levels typically raise prior to ovulation—whether natural or GnRHa-induced—signaling the recruitment of vitellogenic oocytes into FOM and spawning. For example, in Senegalese sole, a marked increase in 17βE2 accompanied by a decline in T—was observed shortly before multiple highly fertile spawning events [42]. Such surges have been reported to occur very shortly after GnRHa administration in many fish species (18 h after treatment in captive white bass, *Morone saxatilis*; 1–2 dpt in stripped trumpeter *Latris lineata*; 2–3 dpt in *Solea senegalensis*; 2–3 dpt in *S. senegalensis*, 4 dpt in female yellowtail flounder, *Pleuronectes ferrugineus* [17,42,66]. In female thicklip grey mullet examined in this study, the consistently low plasma concentrations of E2 and T observed during weekly sampling, both in controls and in GnRHa-treated groups, are most likely attributable to the transient nature of E2 surges, which typically last fewer than seven days and therefore escaped detection under a weekly sampling regime. In many fish species, brief, elevations in plasma T and E2 following GnRHa administration have been reported to be followed by sharp declines, concomitant with increases in plasma progestogens [17]. This pattern

suggests that the reduced E2 and T levels recorded here may reflect a steroidogenic shift toward progestogen production, which is closely associated with the stimulation of OM and spawning [66]. Nonetheless, only future studies employing higher-resolution sampling capable of capturing the fine-scale prespawning dynamics of steroid fluctuations will be able to definitively clarify their role in the initiation of final oocyte maturation and spawning.

The paucity of additional spawnings and the lack of highly fecundity spawnings in GnRHa-induced females may therefore reflect environmentally suboptimal conditions and their systemic effects on physiology beyond the gonads (e.g., energy allocation, osmoregulation, neuroendocrine sensitivity), which limited recruitment of otherwise healthy oocytes (as was seen macro- and microscopically) into FOM and spawning. Male factors (e.g., sperm availability, quality or reproductive behaviour) are unlikely to have constrained outcomes, as almost all males were spermiating, a 1 M:1 F sex ratio was maintained in all treatment groups, and the males of the GnRHa treatment groups were also treated with GnRHa at doses known to enhance spermiation and maintain high sperm fertilizing ability.

5. Conclusions

Weekly GnRHa and metoclopramide injections reliably induced multiple spawnings in captive thicklip grey mullet (*Chelon labrosus*), producing 11 spawns versus 4 in controls. Daily relative fecundity reached 51,000–68,000 eggs kg^{-1} with a 69% fertilization rate, both significantly higher than those achieved with GnRHa implants. Due to the species' group-synchronous ovulatory rhythm, timed injections are probably a better way to mimic natural LH surges than slow-release implants. Nevertheless, fecundity was below the values reported from traditional spawning induction methods. The low levels of steroid hormones (T, 17βE2) and the presence of healthy vitellogenic oocytes that did not progress to maturation in both control and treated females point to suboptimal environmental conditions as the primary limiting factor for spawning, with handling stress as a secondary contributor. Male constraints appear unlikely under conditions of this study. Our results suggest optimising both dose and frequency (e.g., higher doses of GnRHa | Met or shorter intervals between repeated injections) within temperature-salinity regimes favourable for spawning, while minimising disturbances and verifying endocrine responses to enable scaling of this protocol for commercial application.

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Institutional Review Board Statement: The experimental procedures involving fish were conducted in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes, and the corresponding Greek national legislation (Presidential Decree 56/2013). All activities complied with the principles of the Replacement, Reduction, and Refinement (3Rs) and performed under existing project licences approved by competent Greek authorities. No additional ethical approval was required for this study.

Data Availability Statement: The row data supporting the conclusions of this article will be made available by the authors on request.

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