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Recurrent hormone treatment alters growth, haematology, and gonadal maturation in the endangered spiny eel (*Mastacembelus armatus*)

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ABSTRACT

The freshwater Spiny eel (*Mastacembelus armatus*) is an endangered species native to South and Southeast Asia, with declining populations due to habitat degradation and reproductive failure in captivity. This study evaluated the effects of repetitive hormone treatment using Compound S-GnRHa on the growth performance, hematological parameters, and gonadal development of *M. armatus* over a 90-day experimental period. Sixty wild-captured fish were divided into hormone-treated and control groups, with the hormone group receiving intramuscular injections at 20-day intervals. Results showed that repeated hormone injections significantly enhanced the gonadosomatic index (GSI) and fecundity (1865.66 ± 230.22 eggs), while fish in control group failed to reach full sexual maturity. Histological analysis revealed progressive oocyte development from early perinucleolar stages to final maturation in females, and advanced stages of spermatogenesis in males from the hormone-treated group. However, hormone treatment negatively affected somatic growth and induced hematological instability, reflected by reduced red blood cell (RBC) and white blood cell (WBC) counts, hemoglobin, and cholesterol levels. Despite its effectiveness in promoting reproductive maturation, repetitive hormone administration may generate physiological stress and growth suppression. These findings demonstrate that synthetic gonadotropin-releasing hormone analog (S-GnRHa) stimulation holds promise for ex-situ breeding programs while underscoring the importance of minimizing trade-offs between maturation and health. Further studies should focus on optimizing hormone dosages, reducing stress through environmental enrichment, and exploring alternative maturation induction methods to support conservation and aquaculture of the endangered spiny eel.

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Introduction

The Spiny eel, *Mastacembelus armatus*, is a ray-finned fish species of the Mastacembelidae family, native to Southeast Asia's river systems. This species is also commonly referred to as the Zig Zag Eel. Previously, this species was predominant across the country, particularly in muddy areas of shallow beels and boro-paddy fields, especially in Sylhet, Mymensingh, and Tangail districts (Mollah *et al*., 2013). Its breeding period in Kishoreganj, Bangladesh has been recorded from April to June and studies have been identified this fish as a single spawner (Ali *et al*., 2013). *M. armatus* is highly regarded as a table fish due to its tasty flesh, unique flavor, distinct texture, and high levels of protein, oil, and vitamin C (Bogard *et al.*, 2015). The caloric content of its flesh is 303 Cal/100 g, significantly higher than the 110 Cal/100 g found in average fish (Nasar, 1997; Mollah *et al*., 2013). Additionally, it is favored as an aquarium fish for its appealing color patterns and is in high demand among aquarium enthusiasts. It has been reported as an indigenous ornamental fish exported from India to other countries (Gupta and Banerjee, 2012). Over the past two decades, it is believed that the population of this species has decreased by more than 50%, leading to its classification as endangered (Ahmed, 2015; IUCN, 2015). Consequently, there is a need to protect this species from extinction by developing breeding and culture techniques. Few systematic studies have been conducted on the biology and culture potential of this fish (Narejo, 2003; Rahman *et al*., 2004). Given these considerations, *M. armatus* could be a promising candidate for aquaculture, particularly in Bangladesh. However, its gonadal development is hindered in captivity. Initial attempts at induced breeding techniques for *M. armatus* by Mollah *et al*. (2013) were not efficacious.

European eel do not naturally mature in captivity, necessitating the use of long-term hormonal treatments to induce maturation (Asturiano *et al*., 2005). Hormones act as chemical messengers that facilitate communication between different cell types, enabling them to identify their roles and functions through receptors, which are specialized protein structures for molecular recognition. Synthetic hormones are lab-created compounds that closely mimic, however, are not identical to, the molecular structure and shape of natural hormones in the body. The use of external hormones is the only effective method for inducing maturation in European eels (Asturiano *et al*., 2006).

Repetitive hormone treatment is a technique for artificially inducing maturation through the repeated administration of hormones. There is limited research on the laboratory rearing techniques (Mollah *et al*., 2013), morphometric and meristic measurements (Mahmud *et al.*, 2018), and reproductive biology (Narejo *et al.*, 2003; Rahman *et al*., 2006; Alam *et al*., 2020) of *M. armatus*. Previous attempts for domestication and induced breeding of *M. armatus* with

low to high doges of PG extract found no survivability of larvae despite successful breeding (Mollah *et al*., 2013). While repetitive hormone treatment has been successful in European eels (Gallego *et al*., 2012), no studies have explored its application in freshwater spiny eel. GnRHa stimulate the release of gonadotropins, thereby accelerating gonadal maturation in teleost fishes (Zohar and Mysonas, 2001). However, repeated hormonal administration may impose physiological stress that can influence growth performance and hematological parameters. Therefore, evaluating both reproductive benefits and potential health trade-offs is essential. Building on previous research on Spiny eel, the current study aimed to acclimate *M. armatus* to captive conditions and apply repetitive hormone treatment to assess its impact on gonadal development.

Materials and Methods

Collection of experimental fish

Live spiny eels (60 individuals) were collected from the Ujandhanu river in Kishorganj district. Fish were collected from different portion of the river through local fishermen. The number of sample size was limited since it is an endangered species, and he collection of live specimens poses a significant challenge owing to their scarce availability in natural habitats. The collected fish were transported to hatchery of the Faculty of Fisheries, Gazipur Agricultural University, and was released in two separate cisterns (5'×6'). Low density was maintained and constant supply of aeration was ensured. Small pieces of PVC pipe were used in the cistern to facilitate the fish for hiding. The fish were handled carefully throughout the collection operation to reduce stress of fish.

Design of experiment

After 1 week of acclimatization, the collected mature spiny eels were randomly distributed into four concrete cisterns, with 15 fish stocked in each cistern. Experimental cistern was a concrete rectangular tank measuring 2.08 m × 1.52 m × 0.52 m. The average body weight of the fish was 45.2 ± 3.1 g and total length was 28.6 ± 2.4 cm. Two experimental treatments with one replication each were applied, with two cisterns serving as the control group and two subjected to repetitive hormonal treatment, considering the endangered status of the species and ethical limitations on sample size. Fish in the treatment group received intramuscular injections of S-GnRHa at 5, 10, 20, and 30 mg/kg body weight for the first, second, third, and fourth doses, respectively at 20-day intervals for a period of three months. Control fish were handled similarly; however, did not receive hormone injections. As spiny eel is a nocturnal and cryptic species that prefers low-light conditions. All cisterns were covered with cork sheets to maintain darkness and protect the fish from direct sunlight. A continuous flow-through system was maintained in each cistern to facilitate a daily water exchange of approximately 10–20%. Fish were fed live tubificid worms and chopped shrimp twice daily with the amount of 1-2% of their body weight in the early morning and evening.

Hormone preparation and administration by injection

The compound S-GnRHa (Ovupin) hormone, commercially produced by Ningbo Fonland Trading Co. Ltd. in China, was purchased from a local dealer. A 0.85% NaCl solution was prepared and autoclaved. Then, 10 mL of the solution was transferred into the powdered hormone vial and mixed thoroughly. The hormone solution was then ready for administration. The fish were removed from the cistern using a scoop net, and their head regions were wrapped with a wet and soft cloth. Based on the body weight of each fish, the required volume of the hormone solution was drawn into a graduated 3.0 mL syringe. The hormone was then intramuscularly injected at a 45° angle below the dorsal region, between the lateral line. After injection, 10% povidone-iodine was applied to the injection site as an antiseptic. The fish were then released, and continuously monitored. Repetitive hormone treatment was administered at 20-day intervals throughout the experiment, resulting in a total of four hormone doses. Both male and female fish were treated with compound S-GnRHa at progressively increasing doses of 5, 10, 20, and 30 mg/kg body weight for the first, second, third, and fourth administrations, respectively. Fish were compared between the control and hormone-treated groups before the third dose of injection based on various parameters. The final sampling was conducted 90 days after the start of the experiment.

Monitoring Water Quality and Environmental Parameters

Physico-chemical parameters of the water were regularly monitored. Water temperature (°C) was measured using a digital thermometer, while dissolved oxygen levels were determined with a digital DO meter (Hach Co., Colorado, USA). Water pH was assessed using a digital pH meter (Hach Co., Colorado, USA), and ammonia concentration (mg/L) was analyzed using a HANNA instrument test kit. Water temperature ranged from 24–28 °C, Dissolved oxygen ranged from 6.5–8.2 mg L^{-1}, and Ammonia levels remained below 0.05 mg L^{-1}. A natural photoperiod of approximately 12 h light: 12 h dark was maintained to simulate natural environmental conditions.

Growth performance

The total length of each fish (measured from the snout to the caudal tip) was determined using a standard measuring scale in centimeters. The weight of each fish was measured using a digital weighing balance.

Average weight gain (AWG) = Mean final body weight – Mean initial body weight.

Specific growth rate (%/day) = (Ln W2 – Ln W1) / (T2 - T1) × 100

Where, W1 = Initial live body weight of fish (g) at time T1 (day), W2 = Final live body weight (g) of fish at time T2 (day), T2 - T1 = No. of days of the experiment.

Analysis of hematological parameters

Blood cells (RBC, WBC) were counted using an improved Neubaur hemocytometer (Shah and Altindağ, 2004). Blood was diluted 1:200 with Hayem's fluid (Mishra *et al*., 1977). Erythrocytes were counted in the loaded hemocytometer chamber and total numbers were recorded as 10^6 mm^{-3} (Wintrobe, 1967). For WBC, blood was diluted 1:20 with Turk's diluting fluid and placed in a hemocytometer. Four large (1 sq mm) corner squares of the hemocytometer were counted under the microscope (Olympus Corporation,Tokyo,Japan) at 40X. The total number of WBC was calculated in mm^3 $x10^3$ (Wintrobe, 1967). Glucose, hemoglobin and cholesterol were determined with an EasyTouch GCHb Blood Glucose/ Cholesterol/Hemoglobin Multi-Function Test Kit (3 in 1). Type ET-301. MCV, MCH, MCHC, PCV were determined in the laboratory by using Automatic Hematology Analyzer (VSI-38).

Estimation of gonadosomatic index (GSI)

The value of GSI was calculated using the following formula:

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

Estimation of fecundity

Fecundity of the fish was estimated using the gravimetric method. Sub-samples of 1 g were taken from the anterior, middle, and posterior regions of the ovary. The absolute number of eggs in each sub-sample was counted, and the average number of eggs per gram of ovarian tissue was calculated. A known weight (1gm) of ovarian tissue was used to determine the egg count, and total fecundity was then extrapolated based on the total weight of the ovary.

The fecundity of the fish was determined by using following formula:

$$F = N \times \frac{Gonad\ weight(GW)}{Body\ weight(BW)}$$

Where, F= fecundity, and N = the number of eggs in the sample.

Histological analysis

The gonad and liver of *M. armatus* were collected and preserved in 10% buffered formalin for histological analyses. The samples were coronally sectioned, dehydrated in ethanol of different concentration, cleaned in xylene and embedded in paraffin according to standard histological procedures. The histological process was done manually. Five paraffin sections (12 µm thickness) of each sample were stained with hematoxylin–eosin by standard protocol. The stained sections were mounted on the glass slide with DPX and covered by coverslips, and studied under a compound microscope (Model: XSB 301B). The photographic records were done simultaneously for future documents of the study.

Statistical analysis

Throughout the experimental period, all data were systematically recorded and stored in a computer spreadsheet. Data are presented as mean ± standard error (SE). Statistical

analyses were performed using Statistix 10 software. Prior to analysis, data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was checked using Levene's test. Differences between control and hormone-treated groups were analyzed using one-way analysis of variance (ANOVA). When significant differences were detected, mean separation was performed using Tukey's post hoc test. A significance level of $P < 0.05$ was applied.

Results and Discussion

Growth performance

The initial weights of the spiny eel were similar between the control (325.06±4.08 g) and hormone treatment (340.2±5.71 g) groups, indicating comparable starting conditions. However, by the end of the experiment, the control group exhibited a substantially higher final weight (356.10±1.63 g) compared to the hormone-treated group (343.7±2.61 g) (Table 1).

Average weight gain was markedly greater in the control group (31.98±2.24 g) than in the hormone-treated group (3.5±0.19 g), demonstrating a significant reduction in growth following hormone administration. This trend was further reflected in the specific growth rate, which was much higher in the control group (0.113±0.020) than in the hormone-treated group (0.010±0.003).

A previous study on the effect of shelter and stocking density on the growth of *M. armatus* in cisterns revealed the highest mean weight gain of 14.10±0.90 g and SGR ranged from 1.37-1.79 %. (Narejo *et al*., 2003), which supports the weight gain of the control treatment in the present study but significantly differed from the hormone-injected group. Alit *et al*. (2023) found reduced growth in *M. armatus* reared in plastic tanks, suggesting stress conditions in confined areas.

These present results indicate that hormone treatment did not enhance, and in fact significantly suppressed, somatic growth in *M. armatus* under the conditions tested. This pattern is consistent with the findings in other eel species, where certain hormone treatments, particularly those aimed at inducing sex differentiation or maturation can negatively

Table 1. Initial weight, final weight, weight gain, average weight gain and specific growth rate of *M. armatus* in two different treatments

Growth parameters	Control	Hormone treatment
Initial weight(g)	325.06±4.08	340.2 ±5.71
Final weight(g)	356.10±1.63	343.7 ±2.61
Average weight gain (g)	31.98±2.24[a]	3.5 ±0.19[b]
Specific growth rate (%per day)	0.113±0.020[a]	0.010±0.003[b]

Values in the same row having different subscripts are significantly different (P<0.05).

impact growth performance, possibly due to metabolic trade-offs or physiological stress associated with hormone exposure (Hwang *et al.*, 2024).

Overall, the data suggest that while hormone treatments may be effective for reproductive manipulation, they may have adverse effects on growth in Spiny eel, highlighting the importance of balancing reproductive and growth objectives in aquaculture and conservation programs (Hwang *et al.*, 2024).

Haematological values of M. armatus

Hormone application led to significant changes in several haematological parameters of *M. armatus* compared to the control group (Table 2). Red blood cell (RBC) and white blood cell (WBC) counts were significantly lower in the hormone-treated group (RBC: 1.81±0.17 $\times10^6$/mm^3; WBC: 51.53±3.38 $\times10^3$/mm^3) than in controls (RBC: 2.94±0.34 $\times10^6$/mm^3; WBC: 82.15±4.56 $\times10^3$/mm^3). Hemoglobin concentration and cholesterol levels also decreased significantly following hormone treatment (hemoglobin: 7.7±1.04 g/dl vs. 10.2±2.36 g/dl; cholesterol: 165.5±13.25 mg/dl vs. 209.6±10.15 mg/dl). In contrast, mean corpuscular volume (MCV) was significantly higher in the hormone-treated group (172.8±7.48 µm^3) compared to controls (113.1±5.20 µm^3). No significant differences were observed in packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), or glucose levels between the two groups. These results indicate that hormone treatment alters specific haematological parameters, particularly those related to erythrocyte and leukocyte profiles, in *M. armatus*.

Table 2. Haematological parameters of freshwater spiny eel, *M. armatus* in different treatments

Treatment	Control (Final)	Hormone treatment (Final)
RBC (10^6 /mm^3)	2.94±0.34^a	1.81±0.17^b
WBC (10^3 /mm^3)	82.15±4.56^a	51.53±3.38^b
PCV%	33.2±0.75^a	31.2±0.31^a
MCV (µm^3)	113.1±5.20^a	172.8±7.48^b
MCH (pg)	34±2.00^a	42.5±2.91^a
MCHC (g/dl)	30.1±0.45^a	24.6±0.56^a
Glucose (mg/dl)	33.0±2.71^a	31.2±5.33^a
Cholesterol (mg/dl)	209.6±10.15^a	165.5±13.25^b
Hemoglobin (g/dl)	10.2±2.36^a	7.7±1.04^b

Within the same column, values sharing the same subscript are not significantly different ($P > 0.05$), whereas values with different subscripts are significantly different ($P < 0.05$). RBC = Red blood cell; WBC = White blood cell; PCV = Packed cell volume; MCV = Mean cell volume; MCH = Mean corpuscular hemoglobin; MCHC = Mean cell hemoglobin

The haematological parameters of *M. armatus* showed significant alterations following hormone treatment compared to the control group. Notably, both red blood cell (RBC) and white blood cell (WBC) counts were significantly reduced in the hormone-treated group. Lower RBC (1.81±0.17 $\times 10^6/mm^3$) and hemoglobin (7.7±1.04 g/dl) values suggest a possible suppressive effect of repetitive hormone administration on erythropoiesis or increased hemolysis, which may compromise the oxygen-carrying capacity of the blood. Similarly, the marked decrease in WBC (51.53±3.38 $\times 10^3/mm^3$) could indicate immunosuppression or altered leukopoiesis, potentially increasing susceptibility to infections.

Despite these reductions, packed cell volume (PCV), mean corpuscular hemoglobin (MCH), and glucose levels did not differ significantly between groups, indicating that some aspects of blood physiology remained stable. However, mean corpuscular volume (MCV) was significantly elevated in the hormone-treated group (172.8±7.48 μm^3), suggesting the presence of larger, possibly immature erythrocytes, which may reflect a compensatory response to anemia. The decrease in mean corpuscular hemoglobin concentration (MCHC) and cholesterol levels in the hormone-treated group further supports the notion of altered erythrocyte integrity and metabolic shifts under hormone influence.

The observed haematological changes are consistent with findings in other teleosts, where exogenous hormone administration can disrupt normal haematopoiesis and metabolic homeostasis (Sakae *et al*., 2017). These results highlight the need for careful monitoring of blood health during repetitive hormone treatments, as such interventions, while beneficial for inducing gonadal development and breeding, may have unintended physiological consequences. Further research is warranted to elucidate the mechanisms underlying these changes and to optimize hormone treatment protocols for the conservation and aquaculture of this endangered species.

Gonadal development of M. armatus

Ovary morphology

The ovaries of *M. armatus* appeared ribbon-like and asymmetrical, even under laboratory conditions, with variable coloration depending on the maturation stage (reddish-brown to yellow). These characteristics matched those noted by Walsh *et al*. (2003) in *Anguilla reinhardtii* and were consistent with typical ovary lobation described in spiny eels (Rahman, 2004).

The testes were elongated and whitish, positioned lateral to the kidneys. Connection via mesorchium and multi-lobular structure agreed with prior anatomical accounts in *M. cuchia* and *M. armatus* from Gupta and Banerjee (2016).

Gonado-somatic index (GSI) and fecundity of M. armatus

The Gonado-Somatic Index (GSI) showed significant variation between hormone-

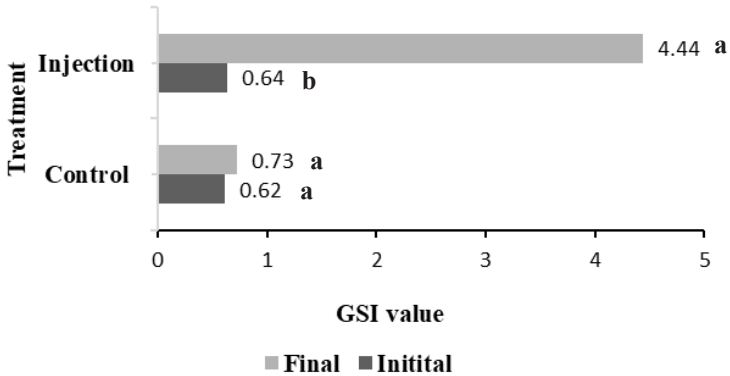

Figure 1. Mean % of Gonado-somatic index (GSI) of female of *M. armatus.* Different letters on the graph show the significant difference ($p < 0.05$) between treatments.

injected and control groups. At the beginning of the experiment, the mean GSI for females was 0.64 ± 0.05 in the hormone-treated group and 0.62 ± 0.04 in the control group. By the end of the 90-day trial, GSI values increased significantly ($p < 0.05$) in hormone-injected fish, reaching 4.44 ± 0.28, whereas the control group exhibited only a marginal rise to 0.73 ± 0.09 (Fig. 1). These findings align with previous observations that *M. armatus* does not mature spontaneously under captive conditions unless induced by hormonal treatment (Mollah *et al*., 2013). Such differences highlight the positive effect of repetitive hormonal application on ovarian development. In this study, the GSI increased significantly due to hormonal stimulation. These findings are consistent with earlier reports that GSI in *M. armatus* may range from 0.44 ± 0.06 to 14.40 ± 1.48 in various environmental and physiological conditions (Mollah *et al.*, 2013). Female fish exhibited significantly higher GSI values compared to males in the hormone-treated group ($P < 0.05$).

Fecundity estimation was only successful in hormone-treated fish, averaging 1865.66 ± 230.22 eggs per female. In contrast, the control group did not reach sexual maturity and thus fecundity could not be determined. This supports prior findings that *M. armatus* rarely reaches maturation unaided in captivity (Serajuddin and Pathak, 2012). Captive rearing combined with repetitive hormonal administration likely triggered gonadal development sufficient for gamete release and oocyte maturation in this study.

Historical accounts have reported much higher fecundity ranges (2235–19493 eggs) in *M. armatus* under natural conditions (Mollah *et al*., 2013; Serajuddin and Pathak, 2012), however, they also underscore geographical and environmental variability. The comparatively low fecundity observed in

this study likely results from the combination of captivity-related stress and repeated hormonal intervention.

Ovarian histology

Histological analysis verified the morphological changes occurring in ovarian development across treatment groups. In the control group, ovaries remained at the early perinucleolar stage for most of the experiment (Fig. 2A). In contrast, hormone-injected fish reached the late perinucleolar stage prior to the third injection (Fig. 2B), and advanced to yolk vesicle stage by the experiment's conclusion (Fig. 2C). Notably, fish that received four hormone doses demonstrated final maturation characterized by complete yolk granule aggregation and the absence of the nucleus typical features of fully matured oocytes (Fig. 2D).

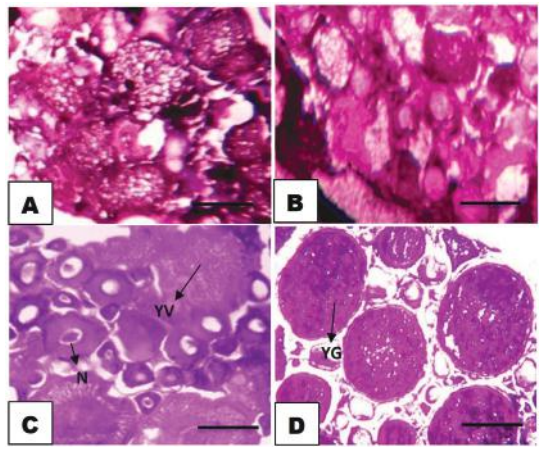

Figure 2. Transverse sections through ovaries of *M. armatus* illustrating oogenesis. (A) Early perinucleolar stage oocytes, (B) Late perinucleolar stage, (C) Yolk vesicle stage oocyte, (D) Final maturation stage YG: Yolk Granule, YV: Yolk vesicle, N: Nucleus. (Haematoxylin - Eosin Staining). Scale bar, 100µm of each section.

These findings parallel oocyte development reported by others in various teleosts (Chakrabarty and Ray, 2016; Pieterse, 2004; Walsh *et al*., 2003). The acceleration of reproductive stage progression due to hormonal treatments reveals its utility in captive breeding programs of endangered species like *M. armatus*.

Testis histology

Testicular development exhibited a similar trend. In control males, only spermatogonia were observed, indicating immature testis (Fig. 3A). However, by the final trial phase, hormone-injected males developed spermatids, signifying mid-to-late stage spermatogenesis (Fig. 3B).

These results are consistent with the classification of spermatogenesis stages (spermatogonia → spermatocytes → spermatids → spermatozoa) as detailed by Rahman *et al.* (2004) and Ali *et al.* (2018), reinforcing the observation that hormonal intervention effectively induces testis development and male gamete production in captivity.

Hormone application impact

Only a few changes were observed in spiny eels following the injections, and these were not consistent across individuals. After the second dose, a white spot appeared on the head of 30% fishes. It was absent in others and, in most affected individuals, disappeared

within 7 days. Although head-spot disease in fish is generally attributed to specific parasites, the present condition exhibited a distinct presentation. Possible causes include collision with the tank wall or a secondary infection.

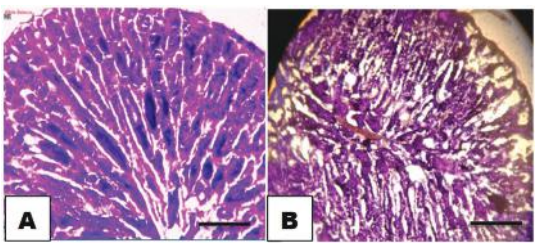

Figure 3. Transverse sections through testes of *M. armatus* in different maturation stages: (A) spermatogonia dominate in immature testis in control, (B) testis increase in size dominated by spermatid in hormone injected fish. Scale bar, 100µm of each section.

Additionally, fishes on which injections were applied showed injection-related injuries. The injection sites turned red or white, and abrasions developed around these areas. Because four doses were administered, the procedure may have been stressful for the fish. The injuries could reflect decreased local immunity due to repeated injections, or they may have resulted from the fish rubbing against tank surfaces or other objects.

Conclusion

The present study demonstrates that repetitive hormone treatment using compound S-GnRHa effectively induces gonadal development and reproductive maturation in the endangered freshwater Spiny eel, *M. armatus*, under captive conditions. A significant increase in GSI and fecundity, along with detailed ovarian and testicular histological development, confirm the efficacy of hormone treatment in advancing oocyte and spermatid maturation stages. However, this improvement in reproductive parameters was accompanied by reduced growth performance and notable alterations in blood parameters, suggesting stress responses and physiological adaptation to the hormonal intervention. These findings support the potential use of repetitive hormone applications as a tool to overcoming the reproductive challenges faced in the ex-situ breeding of *M. armatus*. Nonetheless, the study also highlights the trade-offs between growth and reproductive output, emphasizing the need for a well-balanced aquaculture practice that fosters both health and reproductive efficiency.

Future research should investigate lower or optimized hormone dose regimens, longer acclimation periods, sex-specific protocols, and environmental enrichment techniques to minimize side effects. Additionally, genetic profiling and endocrine analysis may offer deeper insights into the mechanisms regulating induced maturation in spiny eels, thereby contributing to more effective conservation strategies and commercial aquaculture practices.

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Conflict of Interest

The authors confirm that no financial or commercial relationships that might be construed as a potential conflict of interest existed during the study.

Author Contributions

Conceptualization, methodology, formal analysis: M.L.R and S.T; investigation and writing original draft: S.T. and F. A.; writing—review and editing: F. A., S. K. M., M. E. A. and M.L.R.; project administration and supervision: M.L.R; funding acquisition: M.L.R. All authors have read and agreed to the published version of the manuscript.

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