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Effect of exogenous hormone treatments on spermatogenesis in male grey mullet out of the spawning season

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Abstract

The aim of the present study is to investigate the effects of human chorionic gonadotropin (hCG), carp pituitary extract (CPE), melatonin, and adrenal gland extract (AE) administered either separately or in combination on the induction of testicular maturation and spermatogenesis in the thin-lipped grey mullet, *Liza ramada*, out of its spawning season. The results revealed that testes obtained from fish out of their reproductive season have empty seminiferous tubules with germ cells or Sertoli cells markers barely expressed and a low gonadosomatic index (GSI). A significant increase in GSI levels was observed by different hormone treatments. As predicted, hormone treatments showed an obvious stimulation of germ cells proliferation and testicular maturation as indicated by the immunohistochemical expression of germ cells and Sertoli cells markers with the highest values achieved by hCG/AE treatment. We conclude that hormone treatments may be a potential strategy for the induction of spermatogenesis and testes maturation.

Keywords: hormones, grey mullet, spermatogenesis, testes

1. Introduction

Grey mullet is one of the most widely distributed food fish species in the world [1]. The thin-lipped grey mullet, *Liza ramada*, is an euryhaline species cultured in fresh water, brackish water or seawater, however they spawn only in salt water. Mulletts exhibit a restricted spawning season, they mature only once per spawning cycle and spawn only during the season. However, in captivity, most fish species exhibit reproductive dysfunctions, which are thought to be caused by the stress associated with intensive culture conditions and the absence of adequate environmental cues [2]. The artificial induction of spawning in grey mullet has attracted more and more attention in the past decade due to their commercial importance and great economic significance. In many cases, the application of exogenous hormone treatments is an effective therapy to stimulate reproduction and induce spawning in captive fish [3]. The first methods employed freshly ground pituitaries collected from reproductively mature fish, which contained gonadotropins (mainly LH) and induced steroidogenesis and gonadal maturation [2]. Administration of pituitary gland (PG) and human chorionic gonadotropin (hCG) are the most common strategies used for inducing or maintaining spermatogenesis in many fish species. Tang [4] had reported spawning induction by the injection of PG extract. Moreover, PG and hCG have been used to induce spermiation in mullet *Mugilcephalus* [5], Japanese eel *Anguilla japonica* [6] and bream *Abramisbrama* [7]. Carp-PG and gonadotropin-releasing hormone agonist (GnRHa) treatments were also effective in enhancing milt production in fish [8, 2]. However, Mylonas *et al.* [3] reported some factors that should be considered when choosing spawning induction:

- The developmental stage of the gonads at the time the hormonal therapy,
- The type of hormonal therapy,
- The possible stress induced by the manipulation necessary for the hormone administration and
- In the case of artificial insemination, the latency period between hormonal stimulation and stripping for in vitro fertilization [3].

The present study was aimed to analyze the effect of in vivo hormonal treatment either alone or in combination of different hormones on testicular maturation and spermatogenesis in thin-lipped grey mullet, *Liza ramada*, out of the spawning season.

2. Materials and Methods

2.1 Animals

Fish were collected during the spawning season (December 2013-January 2014). Adult male samples of *Liza ramada* (32-35 cm in total length and weight 369- 511 g) were collected from Shalatayat culture ponds in Port Said, Egypt and were reared in outdoor earthen ponds at Sahl El-Hussinia (30°57'26"N, 32°4'3"E) aquaculture farm until April 2014 where sampling started. Fish were maintained under natural temperature (22 - 32 °C) and natural light (13L /11D) for the entire duration of the experiments. Fish were distributed in eight partitions separated by nets (25 m³, 5mx5mx1m each). Water parameters were as follows: dissolved oxygen content (5 mg O₂/L), total nitrogen as NH₄Cl (3.4 to 5.1 mg/L), total alkalinity (690-700 mg/L), total hardness (30-37 mg/L), total dissolved solids (6.20-6.59 ppt), electric conductivity (E.C) (17.9-23.3 mV) and pH value (8.07).

2.2 Experimental protocol

Experiment 1: The effects of a single hormone, namely hCG, melatonin, and carp pituitary extract (CPE) on spermatogenesis were examined. The dosages of each hormone are shown in Table 1. Hormones were injected into the dorsal muscular of each fish.

Experiment 2: The effects of a combination of hCG with other hormones, namely CPE, melatonin, and adrenal gland extract (AE) were investigated. The combination of CPE with melatonin was also tested. The dosages of hormones are shown in Table 1. Hormones were injected into the dorsal muscular of each fish.

2.3 Testes histology

Testes were quickly removed 24 hours post-injection, weighed, and fixed overnight in 4% paraformaldehyde. Then, the tissues were dehydrated in graded series of ethanol and finally embedded in paraffin. Sections (3 μm thick) were deparaffinized, hydrated, and stained with hematoxylin and eosin.

2.4 Gonadal growth

The length and weight of control and hormone-treated fish were registered at the end of the treatment period. The gonadosomatic index (GSI) was determined as Gonad weight/body weight×100.

2.5 Immunohistochemistry

Immunohistochemical analyses for Stella and Sox9 were performed to detect the germ and Sertoli cells respectively, using the streptavidin–biotin technique. Sections were deparaffinized, hydrated, and the antigens were unmasked by incubating the sections in buffered citrate (pH 6.0) for 15 minutes at 105 °C. The endogenous peroxidase was blocked by incubating sections in methanol containing 0.3% H₂O₂ for 20 min. After blocking with normal goat serum for 1 hour, the sections were incubated at 4 °C overnight with either rabbit anti-Stella (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or with rabbit anti-Sox9 (dilution 1:200; Abcam). Then the sections were washed with PBS three times

for five minutes each and treated with biotin conjugated goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 minutes at room temperature and then with streptavidin biotin complex for 30 minutes at room temperature. Next the sections were incubated with 3, 3'-diaminobenzidine tetrahydrochloride solution containing 0.006% H₂O₂ until the stain developed and then were counterstained with hematoxylin. The specificity of the staining was controlled by processing adjacent sections without primary antibody and served as negative controls.

2.6 Statistical analysis

All data were expressed as mean ± SE. The statistical significance was analyzed by Student's *t*-test or ANOVA. Values of *p*<0.05 were considered significant.

3. Results

3.1 Effects of single-hormone treatments on the histological structure of the testes

Histological examinations showed that the testes of *Liza ramada* out of their spawning season consisted of looser and empty seminiferous tubules with thick walls (Fig. 1Aa). Melatonin-treated testes showed seminiferous tubules in which the predominant cells were mainly spermatogonia and primary spermatocytes (Fig. 1Ab). Spermatogonia appeared as large cells with faintly stained cytoplasm and large nuclei with a prominent deeply stained nucleolus. The primary spermatocytes showed lesser amounts of cytoplasm and the nucleus was more deeply stained with hematoxylin than the spermatogonia. In hCG-treated *Liza ramada*, the testes consisted of more abundant seminiferous tubules with a relatively thin wall. Within the tubules, different spermatogenic cell lineages were observed (spermatogonia, primary and secondary spermatocytes) with the appearance of a few spermatids next to the wall of the tubules. The secondary spermatocytes, which resulted from the division of the primary spermatocytes, are smaller in diameter with few cytoplasm, and a darker chromatin material within the nucleus than the primary spermatocytes. They are observed in large number within cysts. The spermatids are smaller than the secondary spermatocytes with scant cytoplasm and dense elongated or elliptical nuclei. Furthermore, clusters of resting or residual spermatogonia with electron dense nuclei and voluminous cytoplasm were observed in the central region of the testes (Fig. 1Ac). Testes of fish treated with CPE showed the presence of cell clusters close to tunica albuginea that consisting mainly of primary spermatocytes. Moreover, a few seminiferous tubules containing secondary spermatocytes were observed. Secondary spermatocytes are relatively smaller than primary spermatocytes with dark staining nuclei, often occupying the central area of the testes. Clusters of resting or residual spermatogonia in the central region of the testes were also observed (Fig. 1Ad).

3.2 Effects of double-hormone treatments on the histological structure of the testes

As shown in Fig. 1Ae, testes of *Liza ramada* injected with hCG in combination with melatonin consisted of numerous tubules containing different cell lineages and the majority of cells within the tubules were spermatogonia and primary spermatocytes with few secondary spermatocytes. The walls of seminiferous tubules were thin and few clusters of residual spermatogonia were observed in the central region of the testes. In fish treated with a combination of hCG and AE,

numerous seminiferous tubules were observed containing mainly secondary spermatocytes and few primary spermatocytes. Moreover, cysts containing spermatids were frequently observed in the hCG/AE-treated fish (Fig. 1Af) compared with fish treated with hCG only (Fig. 1Ac). The testes of fish treated with a combination of CPE and melatonin revealed the presence of numerous seminiferous tubules in which secondary spermatocytes are the predominant cells with few primary spermatocytes and spermatids (Fig. 1Ag). In the case of fish treated with an hCG/CPE combination, the testes were similar to the CPE-treated group. However, numerous seminiferous tubules consisting mainly of secondary spermatocytes with few spermatogonia, primary spermatocytes and spermatids were observed. Few clusters of small-sized primary spermatocytes were observed beneath the tunica albuginea. Furthermore, few clusters of residual spermatogonia in the central region of the testes were noticed (Fig. 1Ah).

3.3 Effects of hormone treatments on GSI

In the present study, the GSI was evaluated. The values of GSI showed remarkable changes in all treated groups compared with fish out of spawning season (Fig. 1B, $P < 0.001$ by ANOVA and $P < 0.001$ by Student's *t*-test for each hormone-treated group vs. out of season). Furthermore, there was a significant difference in GSI in fish treated with a combination of hormones compared with single hormone treatments, whereas the combination of hCG/melatonin and CPE/melatonin significantly increased the GSI compared with the group treated with melatonin only ($P < 0.001$ by Student's *t*-test vs. melatonin group). Moreover, CPE/melatonin- and CPE/hCG-treated groups showed a higher GSI value compared with the group treated with CPE only ($P < 0.05$ and $P < 0.001$ by Student's *t*-test for CPE/melatonin and CPE/hCG vs. CPE only respectively). In addition, treatment with the hCG/CPE combination significantly increased the GSI compared with hCG only ($P < 0.001$ by Student's *t*-test). However, no change in GSI was observed in hCG/melatonin or hCG/AE combinations compared with hCG only (Fig. 1B).

3.4 Effects of hormone treatments on the expression of germ cells marker

The effect of hormone treatments on primordial germ cells marker expression was investigated using the anti-Stella antibody, a specific marker for primordial germ cells. Testes of fish out of spawning season failed to show expression for germ cells (Fig. 2A). However, germ cells marker expression was significantly increased in all hormones treated groups as indicated by the percentage of area fraction (Fig. 2B, $P < 0.001$ by ANOVA). Furthermore, double-hormone treatments significantly increased the primordial germ cells marker expression compared with single hormone treatments ($P < 0.001$ by Student's *t*-test for hCG/melatonin, hCG/AE, hCG/CPE and CPE/melatonin vs. hCG only, melatonin only or CPE only) with the maximum increase in the hCG/CPE-treated group ($6.03 \pm 0.4\%$, Fig. 2B).

3.5 Effects of hormone treatments on the expression of Sertoli cell marker

Sertoli cells marker Sox 9 was utilized to investigate the effects of hormone treatments. As shown in Fig. 3A, testes obtained from fish out of spawning season showed almost no expression of Sertoli cells marker. However, they were significantly increased in all hormone-treated groups (Fig. 3B,

$P < 0.001$ by ANOVA). Moreover, double-hormone treatment shows a higher percentage of expression as indicated by area fraction compared with single-hormone treatments ($P < 0.001$ by Student's *t*-test for hCG/melatonin, hCG/AE, hCG/CPE and CPE/melatonin vs. hCG only, melatonin only or CPE only, Fig. 3B).

4. Discussion

The thin-lipped grey mullet, *Liza ramada* is a seasonal breeder that migrates to the sea for spawning in the spawning season (December- January) [9]. The process of spermatogenesis involves spermatogonial multiplication, differentiation, entry of mature spermatogonia into meiosis and their eventual metamorphosis into spermatids and spermatozoa [10]. From a physiological point of view, hormones play an important role in testes maturation and spermatogenesis, and exogenous hormone treatments can be used to influence reproduction in fish aquaculture [11]. Few trials have been conducted using hormone therapy for the induction of gonadal maturation in male fish, yielding limited results. In the present study, we examined the effectiveness of different types of hormones administered either individually or in combination, on the induction of testes maturation and spermatogenesis. Gonadal maturation in teleost fish is mainly regulated by the brain-pituitary-gonadal axis, which can directly or indirectly stimulate the release of hormones required for successful spermiation [2]. The role of pituitary gonadotropins in teleost spermatogenesis has been reported in eel, catfish and salmon [12, 13].

Over the past years, hCG that acts directly at the level of the gonads [2] has been increasingly used for the induction of spawning in many fish species. In male eels, induction of spermatogenesis by the injection of hCG has been reported [14, 15]. Moreover, hCG and pituitary gland hormones also been used to induce spermiation in Japanese eel [6] and mullet [5] and a single hCG injection induced a 13 fold increase in stripped sperm volume in *Pangasius bocourti* [16]. Our histological examinations revealed that hCG injections increased the number of seminiferous lobules in fish out of their reproductive season. Furthermore, different spermatogenic cell lineage including spermatogonia, and primary and secondary spermatocytes were observed within the lobules. Moreover, hCG treatments significantly increased GSI levels compared with the untreated fish indicating the increase in testes weight ($P < 0.001$ by Student's *t*-test). This was accompanied with the elevation in Stella area fraction indicating the presence of a higher number of primordial germ cells (Fig. 2Ac). We also demonstrated that CPE, a pituitary extract obtained from carp, increased GSI levels and Stella protein expression as well (Fig. 1Ad and Fig. 2Ad). It has been reported that CPE treatments have induced an increase in volume as well as an increase in the quantity of sperm cells in *Mystus nemurus* [8]. In addition, both PG and hCG treatments induced testes hydration and facilitated semen collection from testes of African catfish [17]. Sertoli cells are believed to provide nutrition to the developing spermatogenic cells. Based on the function of Sertoli cells, the immunohistochemical expression of Sox9, a direct target of the testicular sex-determining gene, was investigated [18]. It was found that it is up-regulated in the developing Sertoli cells and plays a crucial role in testicular development [19, 20]. We demonstrated that testes of fish out of the reproductive season show almost no expression of Sox9 proteins (Fig. 3). However, immunohistochemical expression of the Sox9 protein significantly increased after hCG or CPE injections

($P < 0.001$ by Student's *t*-test). An increase in Sox9 mRNA in testicular slices treated with hCG in vitro at 12 and 24 hour time points has been reported, suggesting the role of gonadotropins in Sox9 regulation [19]. In the present study, the effects shown by injections of hCG on GSI levels and Stella and Sox9 expression were further increased by the injection of hCG in combination with other hormones like melatonin, AE or CPE with the maximum values obtained by the hCG/CPE combination. The results indicate that hormonal combination treatments may represent an effective way to induce testes maturation and spermatogenesis in *Liza ramada*.

It has been reported that melatonin is an important factor in the regulation of testicular development [21]. Melatonin may act in the reproductive axis through a direct action on the Gonadotropin-releasing hormone gene expression, which would increase the levels of the luteinizing hormone (LH) [22].

Besides enhancing the maturation via the pituitary-hypothalamus-gonad axis, melatonin also acts directly on the testes through Leydig cells [23] and stimulates spermatogenic activity by increasing the sensitivity of Leydig cells to the LH [24]. In the present study, we found that fish treated only with melatonin showed an increase in GSI levels and expression of germ cells and Sertoli cells markers, compared with the untreated fish ($P < 0.001$ by Student's *t*-test). The injection of melatonin in combination with hCG or CPE further potentiated the effects observed by melatonin only ($P < 0.001$ by Student's *t*-test), indicating a positive effect of melatonin in enhancing spermatogenesis in *Liza ramada*. Based on the results of the present study, we conclude that the administration of different hormone treatments may be an effective method for enhancing the maturation of testes and spermatogenesis for fish in captivity.

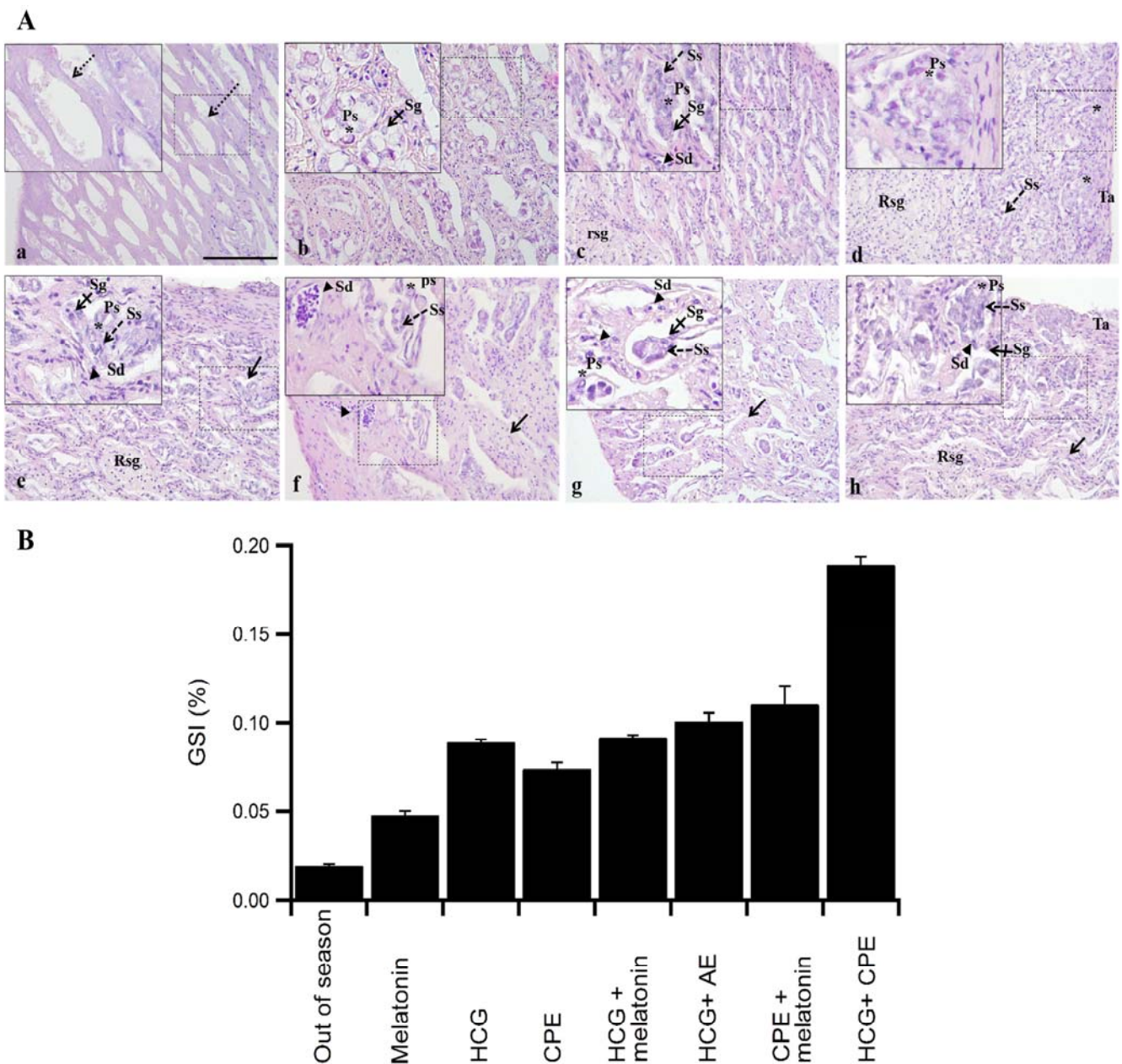


Fig 1: A: Light microscopic photographs of hematoxylin and eosin (HE) stained cross sections of the testes of *Liza ramada* out of spawning season (a) with empty testicular lobules (dotted arrow), or treated with different hormones (b-h), namely melatonin (b), HCG (c) carp pituitary extract (CPE) (d), and combination of HCG/melatonin (e), HCG/adrenal gland extract (AE) (f), CPE/melatonin (g) and HCG/CPE (h). Spermatogonial cells (Sg, tailed-arrow), primary spermatocytes (Ps, asterisk), secondary spermatocytes (Ss, dashed arrow), spermatid (Sd, arrow head), tunica albuginea (Tg), residual spermatogonia (Rsg) and seminiferous tubules (solid arrow) are indicated. Scale bar 100 μ m. B: Gonadosomatic index (GSI, %) in male *Liza ramada*.

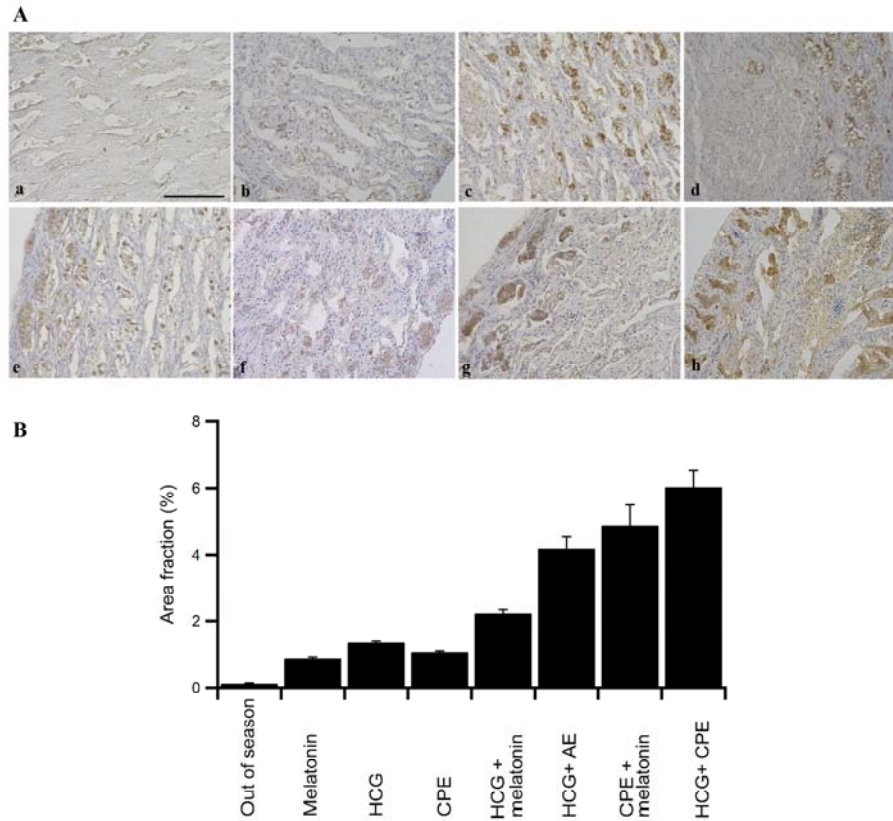


Fig 2: A: Immunohistochemical staining of cross sections of the testes of *Liza ramada* with primordial germ cells marker, anti-Stella antibody. Testes of fish out of the reproductive season (a), or treated with melatonin (b), HCG (c) or carp pituitary extract (CPE) (d) or HCG/melatonin (e), or HCG/adrenal gland extract (AE) (f), or CPE/melatonin (g) or HCG/CPE (h). Scale bar 100 μ m. B: Graphs showing the percentage area of immunopositive cells for stella in control fish (out of spawning season) compared to different hormonal treatments. $n = 3$. Values are shown as the means SE.

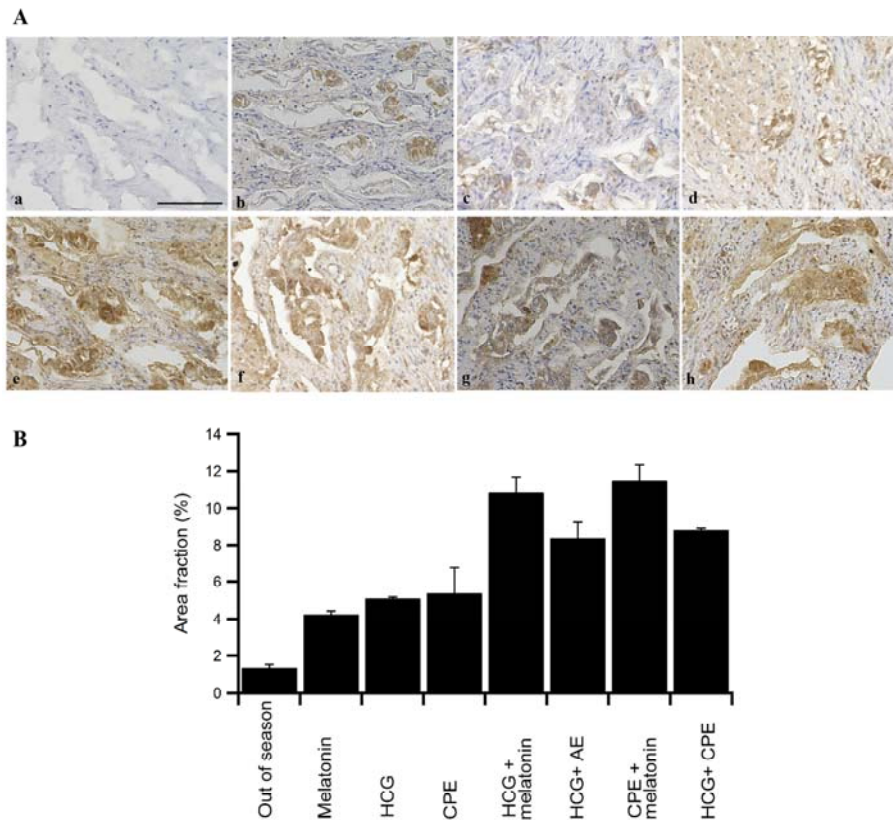


Fig 3: A: Immunohistochemical staining of cross-sections of the testes of *Liza ramada* with anti-Sox 9 antibody. Testes of fish out of the reproductive season (a), after receiving single -hormone treatment with melatonin (b), HCG (c) or carp pituitary extract (CPE) (d) and double-hormone treatment with HCG/melatonin (e), HCG/adrenal gland extract (AE) (f), CPE/melatonin (g) or HCG/CPE (h). Scale bar 50 μ m. B: Graphs showing the percentage of area of immunopositive cells for SOX9 in control fish (out of spawning season) compared to hormone-treated fish. $n = 3$. Values are shown as the means SE.

Table 1: Doses of hormone treatments.

Group	Treatment	Injection
1	HCG	4500 IU/kg
2	Melatonin	3 mg/1ml saline/Fish
3	CPE	3 carp pituitary extract/1ml saline/Fish
4	HCG+ melatonin	4500 IU/kg+ 3 mg melatonin/1ml saline/Fish
5	HCG+ CPE	4500 IU/kg+ 3 carp pituitary extract/1ml saline/Fish
6	HCG+ AE	4500 IU/kg+ 3 adrenal gland extract/1ml saline/Fish
7	CPE+ melatonin	3 carp pituitary extract/1ml saline/Fish+ 3 mg melatonin/1ml saline/Fish

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6. Disclosure

The authors declare no conflicts of interest, financial or otherwise.

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