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Research Article

Effects of ovulin or pituitary extract treatments on spermatological, hematological, and biochemical responses of male *Cyprinus carpio* var. Sazan

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Abstract

The present study aimed to compare the effects of pituitary extract or Ovulin (containing s-GnRH and anti-dopamine) injection on spermatological parameters, hematological, and serum biochemical characteristics of male *Cyprinus carpio* var. Sazan. The male broodstocks were caught from the Caspian Sea and transferred to the Sijeval Center of Fish Propagation and Reconstruction of Genetic Reserves of Bony Fish in Bandar-e-Turkmen, Iran. After acclimation, the fish were divided into two experimental groups: one injected with pituitary extract (PT; 2 mg/kg) and the other with Ovulin (OV; 0.25 mL/kg). The blood samples were taken from the fish before and 12 hours after the injections (analyzed by two-way ANOVA); the sperm samples were also collected 12 hours after the injections (analyzed by t-test). The results showed that OV treatment significantly increased sperm pH, motility duration, and spermatocrit percentage, compared to PT treatment. The serum cortisol level was significantly increased in OV treatment after the injection. The serum glucose concentration and erythrocyte count were significantly increased after the injections, whereas the serum cholesterol, total protein, and sodium concentrations significantly decreased. The blood hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin were significantly decreased after the injection. There were no significant changes in blood leukocyte counts, serum potassium, calcium, albumin, triglyceride, and blood mean corpuscular hemoglobin concentration among the treatments (p>0.05). In conclusion, OV treatment serves as a beneficial alternative to PT extract for stimulating final spermatozoa maturation in male Sazan, without causing any health issues.

Introduction

Common carp, *Cyprinus carpio*, inhabits various geographical areas, including the Caspian Sea, where it is locally referred to as Sazan and serves as a vital food source for local communities. However, its population in the Caspian Sea has decreased due to overfishing and the deterioration of spawning habitats, particularly in the southern regions and their tributaries (Ghelichpour *et al*., 2013). Consequently, efforts are needed to rehabilitate and restock Sazan populations in the Caspian Sea. Currently, a stock rehabilitation program is underway that involves releasing artificially propagated fry into the tributaries of the Caspian Sea. This process starts with collecting broodstocks from the sea, followed by artificial propagation (Hosseini and Hoseini, 2012). Given the limited availability of Sazan broodstocks, achieving high success rates in propagation is essential. To enhance this success, broodstocks are stimulated using exogenous hormonal induction to promote final maturation and maximize the release of reproductive products.

The oldest hormone used for artificial induction in fish is pituitary (PT) extract, which contains gonadotropins that facilitate final maturation at the gonadal level (Mechaly *et al*., 2024). However, this extract has several drawbacks, including high cost, limited availability, inconsistent hormonal composition, and the presence of substances not suitable for reproduction (Mohammad Nejad *et al*., 2022). As a result, various synthetic alternatives have been commercially developed. These alternatives typically include gonadotropin-releasing hormone analogs that act at the PT level (Schneider *et al*., 2006). Additionally, they may contain other effective compounds, such as anti-dopamine agents, which are necessary for successful reproduction in certain fish species. These synthetic products are cost-effective, readily available, durable, and provide consistent results. Examples of these commercially used materials include Ovulin (OV), Ovaprim, Ovatide, human chorionic gonadotropin (HCG), Ovopel, Dagin, and Aquaspawn (Zohar *et al*., 2022).

In addition to facilitating reproduction, hormone therapy has various effects on fish. For instance, following the injection of pituitary extract in common carp, there was a notable increase in serum glucose, triglycerides, leukocyte counts, and the percentage of blood neutrophils, which varied depending on the gender of the broodstock (Baghizadeh and Khara, 2015). Immune responses have also been observed in gilthead seabream, *Sparus aurata*, treated with 17-beta estradiol or testosterone (Cuesta *et al.,* 2007). Female bennei, *Barbus sharpeyi*, displayed varying cortisol responses when induced with carp PT extract alone, or in combination with ovaprim, with the latter leading to lower cortisol levels (Mohammadian *et al.,* 2015). Increasing doses of GnRH (Shokr, 2020) or gonadotropins (Shokr, 2015) resulted in hepatic and renal damage—evidenced by elevated blood transaminase enzymes, uric acid, and creatinine levels—as well as stress, indicated by rises in cortisol and glucose in African catfish, *Clarias gariepinus*, and Nile tilapia, *Oreochromis niloticus*, respectively. Ovulin contains a salmon GnRH analogue and a dopamine

inhibitor that promotes final maturation at doses of 0.5 mL/kg for females and 0.25 mL/kg for males. Limited research on the effectiveness of Ovulin in Sazan broodstocks has primarily examined the effects of different doses on the fish, with studies assessing hematological and serum biochemical responses to various doses in female Sazan (Mohammad Nejad *et al.,* 2022). However, similar data for males is lacking. Consequently, this study aimed to evaluate the effects of an effective dose of Ovulin (0.25 mL/kg body weight) on spermatological, hematological, and serum biochemical parameters in male Sazan, comparing the results to those from broodstocks stimulated with 2 mg/kg of pituitary extract.

Materials and methods

Experimental fish

The research was carried out in the spring of 2020 at the Sijeval Center for Fish Propagation and Genetic Reserve Reconstruction of Bony Fish in Bandar-e-Turkmen, Golestan province, Iran. A total of 24 Sazan carp broodstocks were sourced from the southeastern Caspian Sea and transported to the center in a vehicle equipped with oxygen tanks. Upon arrival, the male fish (8 specimens) were separated, and their weight (ranging from 1240 to 1800 g) and length (between 41.8 and 54.2 cm) were recorded. The age of the fish was determined using their scales, indicating they were between 3 to 5 years old. The fish were then acclimatized to their new environment for two weeks without being fed. During this period, water temperature, dissolved oxygen levels, and pH were monitored using a digital device (Model HI98199; Hana Ins., USA), yielding values of 21±2°C, 6.0±0.3 mg/L, and 7.7±0.2, respectively.

Hormone injection

The PT extract for common carp was obtained from a local market, while OV was acquired from Ningbo Sansheng Pharmaceutical Company Ltd. (China). Following the acclimation period, the fish were divided into two groups, each housed in separate 2000-L tanks for one week. Subsequently, one group received an injection of PT extract, while the other was injected with OV. For the injections, individual fish were caught and placed in a 200-L tank containing clove extract (0.2 g/L) for anesthesia. The injections were administered peritoneally beneath the base of the pectoral fin at doses of 2 mg/kg for PT extract and 0.25 mL/kg for OV. The PT extract dosage was standard in hatcheries involved in stock rehabilitation programs, while the OV dosage was based on previous research (Mohammad Nejad *et al*., 2022).

Blood samplings

Blood samples were collected twice: once prior to hormone injection and again 12 hours post-injection, after the fish had completed spermiation. The fish were caught and anesthetized in a 200-L tank containing 2 g/L clove extract (Hoseini and Ghelichpour, 2012). Blood was drawn via caudal puncture into heparinized tubes. After sampling, the fish were marked with colored wool threads and returned to their respective tanks for post-injection sampling and sperm collection.

Sperm collection and analysis

After the post-injection blood sampling, sperm samples were taken by stripping the fish abdomen from the head to tail and collected in a syringe (Hajirezaee *et al.*, 2010). The sperm samples were collected in sterile tubes and used for pH, motility, and spermatocrit determination. The sperm pH was immediately determined by a digital pen-pH-meter (HI98115 – GroLine, Hana Co., USA). In order to calculate the period of sperm motility, a drop of sperm was poured on a slide under a microscope. To start the spermatozoa motion, a drop of water was poured on them and the motility time was measured using a chronometer until 90-95% of the cells became immobile (Billard *et al*., 1977). Spermatocrit was determined using a microcentrifuge (7 min; 10000 rpm).

Blood and serum analysis

The blood erythrocytes (RBC) and leukocytes (WBC) were determined using the Dacie diluting solution, as suggested before. Hemoglobin (Hb) levels were measured using a commercially available kit from Zistchem Co. (Tehran, Iran) and a spectrophotometer (Yousefi *et al.*, 2022a). Hematocrit (Hct) percentages were calculated by micro-centrifuging the blood samples (Abbasi *et al.*, 2023). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the formula developed by Blaxhall (1972). The differential WBC count was performed by preparing a blood smear and staining it with Giemsa, following the method described by Blaxhall (1972).

Serum separation was done by centrifugation at 4°C (3000 g; 7 min) and the obtained materials were kept at -20°C until analysis (Rajabiesterabadi *et al.*, 2020). Serum cortisol levels were assessed using a commercially available kit (Monobind Co., CA 92630 USA) at a wavelength of 450 nm and a temperature of 20°C. This kit has been previously utilized and validated for cortisol analysis in various fish species (Mirzargar *et al.*, 2022; Taheri Mirghaed *et al.*, 2022; Yousefi *et al.*, 2022b) and is based on the competitive ELISA method with a detection range of 0-500 ng/mL (sensitivity of 3.6 ng/mL). Serum glucose, total protein, albumin, cholesterol, triglyceride, and calcium levels were measured using commercial biochemical kits (Zist Chem Co., Tehran, Iran), employing the glucose oxidase method (wavelength 546 nm; detection range 5-400 mg/dL; sensitivity 5 mg/dL), Biuret method (wavelength 546 nm; detection range 0.5-6 g/dL; sensitivity 0.005 g/dL), bromocresol green method (wavelength 620 nm; detection range 0.5-6 g/dL; sensitivity 0.003 mg/dL), colorimetric-enzymatic method (wavelength 505 nm; detection range 15-1000 mg/dL; sensitivity 1.33 mg/dL), colorimetric-enzymatic method (wavelength 505 nm; detection range 30-700 mg/dL; sensitivity 5.6 mg/dL), and arsenazo method (wavelength 660 nm; detection range 5-15 mg/dL; sensitivity 0.14 mg/dL), respectively (Hoseini *et al.*, 2016; Taheri Mirghaed *et al.*, 2018; Yousefi *et al.*, 2018). Serum sodium and potassium concentrations were determined using a flame-photometer, as suggested by Mazandarani *et al.* (2017).

Statistical analysis

Spermatological parameters before and after the hormone injections were compared by t-test. After confirming normality and variance homogeneity, the blood and serum parameters were subjected to two-way repeated measure ANOVA to find the significant effects of sampling time (before-after injection) and hormone type (PT vs. OV). When there was an interaction effect of the sampling time and hormone type, pair comparisons were conducted by Duncan test, among the treatments. Data are expressed as mean ± standard deviation and alpha was set at 0.05. The statistical analyses were performed in SPSS v. 26.

Results

Results showed that the fish injected with OV had significantly higher sperm pH, motility duration, and spermetocrit percentage, compared to those injected with PT (Fig. 1).

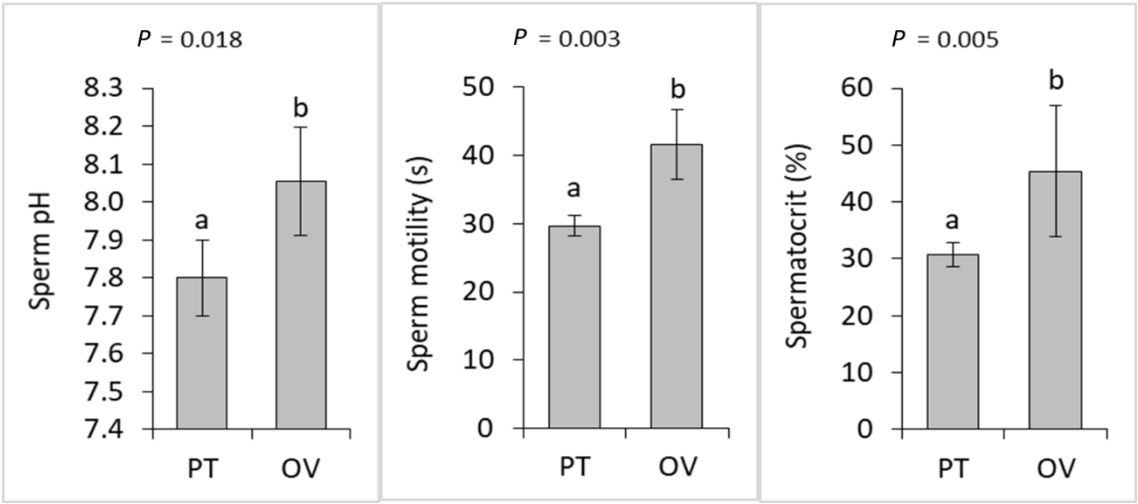

Figure 1: Sperm pH and motility, and spermatocrit percentage of the male Sazan treated with either PT or OV. Different letters above the bars show significant differences between the treatments (mean ± SD; n = 12; t-test).

There were interaction effects of hormone treatment and sampling time on serum cortisol and glucose levels. Serum cortisol levels in PT-1, PT-2, and OV-1 were similar and significantly lower than that of OV-2. Serum glucose levels significantly increased after either PT or OV injection (Fig. 2).

Serum total protein and cholesterol significantly decreased after the hormone injections, but the type of hormone had no significant effects on these parameters. Also, the type of hormone and sampling time had no significant effects on the serum albumin and triglyceride levels (Table 1).

The hormone type and sampling time had no significant effects on the serum potassium and calcium levels. However, the serum sodium concentration significantly decreased after the hormone injections, but the hormone type had no significant effects on these parameters (Table 2).

The hormone type and sampling time had no significant effects on the blood WBC, granulocyte, and agranulocyte percentages (Table 3).

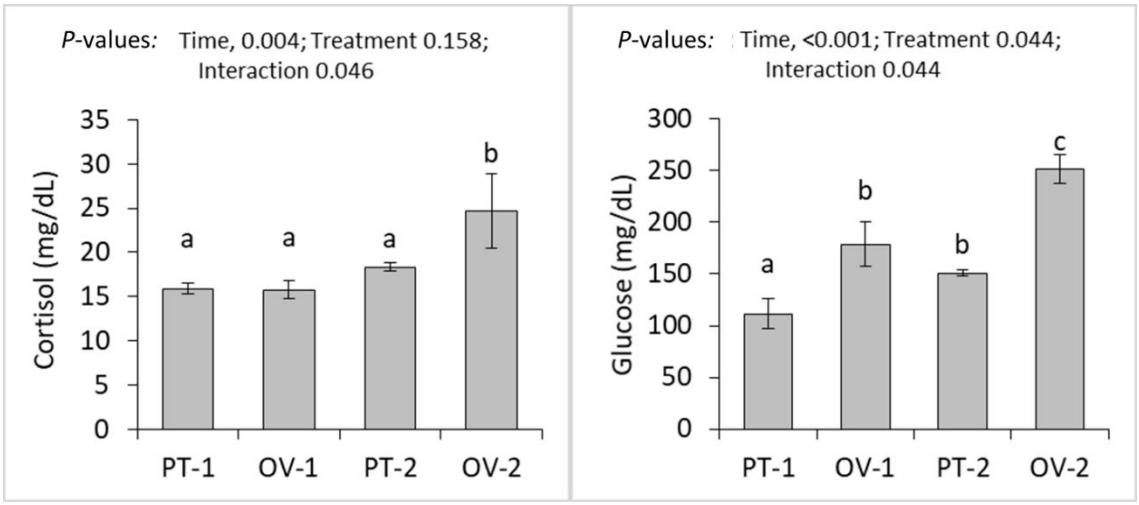

Figure 2: Serum cortisol and glucose levels in the male Sazan treated with either PT or OV. Different letters above the bars show significant differences between the treatments (mean ± SD; n = 12; two-way ANOVA, Duncan). PT-1: before injection of PT; OV-1: before injection of OV; PT-2: after injection of PT; OV-2: after injection of OV.

Table 1: Serum total protein, albumin, triglyceride, and cholesterol levels in the male Sazan treated with either PT or OV (mean ± SD; n = 12; two-way ANOVA).

		Total protein (g/dL)	Albumin (g/dL)	Triglyceride (mg/dL)	Cholesterol (mg/dL)
Before injection	PT	3.85 ± 0.07	1.80 ± 0.00	78.0 ± 1.41	288 ± 14.8
	OV	3.82 ± 0.08	2.50 ± 0.60	141 ± 53.7	299 ± 70.9
After injection	PT	3.65 ± 0.07	1.60 ± 0.00	67.0 ± 1.41	274 ± 7.07
	OV	3.60 ± 0.06	1.67 ± 0.19	105 ± 34.4	257 ± 67.6
Sig.	Time	<0.001	0.116	0.054	0.006
	Treatment	0.432	0.074	0.168	0.959
	Interaction	0.776	0.304	0.254	0.078

PT: pituitary extract; OV: Ovulin

Table 2: Serum sodium, potassium and calcium levels in the male Sazan treated with either PT or OV (mean ± SD; n = 12; two-way ANOVA).

		Sodium (mEq/L)	Potassium (mEq/L)	Calcium (mg/dL)
Before injection	PT	149 ± 0.71	0.35 ± 0.07	4.75 ± 0.87
	OV	148 ± 5.04	0.38 ± 0.15	5.68 ± 0.95
After injection	PT	145 ± 0.71	0.30 ± 0.00	5.45 ± 1.20
	OV	140 ± 2.45	0.45 ± 0.23	6.87 ± 0.62
Sig.	Time	0.002	0.953	0.074
	Treatment	0.312	0.097	0.067
	Interaction	0.200	0.681	0.600

PT: pituitary extract; OV: Ovulin

RBC significantly increased after the injection, whereas, blood Hb, Hct, MCV, and MCH significantly decreased at this time. The hormone type had no significant effects on these parameters. The hormone type and sampling time had no significant effects on the blood MCHC (Table 4).

Table 3: Blood WBC, granulocytes, and agranulocytes in the male Sazan treated with either PT or OV (mean ± SD; n = 12).

		WBC (× 10^3 cell/µL)	Granulocytes (%)	Agranulocyte (%)
Before injection	PT	17.6 ± 0.49	28.0 ± 0.71	72.0 ± 0.71
	OV	17.2 ± 0.18	31.8 ± 5.47	68.2 ± 5.47
After injection	PT	17.6 ± 0.49	26.0 ± 0.71	74.0 ± 0.71
	OV	17.2 ± 0.32	30.4 ± 4.03	69.6 ± 4.03
Sig.	Time	1.000	0.401	0.401
	Treatment	0.097	0.231	0.231
	Interaction	1.000	0.867	0.867

PT: pituitary extract; OV: Ovulin; WBC: white blood cells

Table 4: Blood RBC, Hb, Hct, MCV, MCH, and MCHC in the male Sazan treated with either PT or OV (mean ± SD; n = 12; two-way ANOVA).

		RBC (× 10^6 cell/µL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Before injection	PT	1.18 ± 0.01	13.5 ± 0.21	32.0 ± 0.57	273 ± 3.18	115 ± 1.13	42.1 ± 0.07
	OV	1.18 ± 0.01	14.0 ± 0.35	33.9 ± 1.58	288 ± 14.0	119 ± 2.90	41.5 ± 2.47
After injection	PT	1.20 ± 0.01	12.2 ± 0.14	28.6 ± 0.49	239 ± 5.59	102 ± 0.57	42.8 ± 1.20
	OV	1.20 ± 0.02	13.0 ± 0.62	29.8 ± 2.88	249 ± 26.9	109 ± 4.04	44.3 ± 6.13
Sig.	Time	0.008	<0.001	<0.001	<0.001	<0.001	0.256
	Treatment	0.980	0.099	0.382	0.436	0.069	0.893
	Interaction	0.776	0.339	0.554	0.570	0.241	0.480

PT: pituitary extract; OV: Ovulin; RBC: red blood cells; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

Discussion

The current findings indicate that OV is a more effective inducing hormone than PT extract in Sazan, as evidenced by the enhancements in spermatological parameters. The duration of sperm motility is influenced by various factors, and extended motility periods can enhance the likelihood of successful fertilization (Merino *et al.,* 2023). For instance, the physicochemical properties of semen significantly impact sperm motility. Research has shown that acidic pH levels reduce sperm motility in male Sazan, while increasing the pH gradually boosts both the percentage of motile sperm and the duration of motility, peaking at pH 8.5 (Bastami *et al.,* 2010). Additionally, the energy reserves of sperm play a crucial role in motility duration (Dzyuba *et al.,* 2024). The current study revealed that OV elevated serum cortisol levels in the fish. Cortisol promotes catabolic processes to supply energy resources for host cells (Yousefi *et al.,* 2023). It has been shown that sperm motility declines in environments that hinder respiration or disrupt the citric acid cycle. Conversely, introducing glucose and pyruvate into the medium extended sperm motility, highlighting the significance of energy metabolism in this process (Lahnsteiner *et al.,* 1999). Therefore, it is hypothesized that the increased sperm motility observed with OV treatment may result from optimized sperm pH and enhanced energy reserves.

Cortisol, the primary stress hormone, rises during energy-intensive situations to

stimulate catabolic processes and provide necessary energy for fish under stress (Barton, 2002). Reproduction is energetically demanding, and the final maturation of spermatozoa requires an energy supply from the fish's testes (Rahi *et al.,* 2023). A common effect of elevated cortisol is hyperglycemia, which facilitates glucose delivery to target tissues for energy production via the citric acid cycle and oxidative phosphorylation in mitochondria (Birnie-Gauvin *et al.,* 2023; Chen *et al.,* 2024). This explains the serum glucose levels observed in the present study. Similar findings were reported in male African catfish treated with GnRH (Shokr, 2020) and grass carp, *Ctenopharyngodon idella*, administered HCG, ovaprim, or pregnyl (Metwally and Fouad, 2008; Mousavi and Yousefian, 2012).

A reduction in serum total protein levels, while serum albumin remains unchanged, suggests lower serum globulin levels. Increased energy expenditure can lead to a decrease in new protein synthesis as resources are redirected towards energy production (Jerez-Cepa *et al.,* 2020). Consequently, it can be concluded that hormonal treatments have diminished protein synthesis to allocate energy resources for the maturation of spermatozoa in the testes. Supporting this finding, Shokr (2020) reported a decline in serum total protein levels following GnRH injections in African catfish. Additionally, injections of ovaprim or pregnyl significantly lowered serum total protein levels in male grass carp (Metwally and Fouad, 2008).

Following the injection, there was a decrease in serum sodium concentration. Sodium, the primary cation in fish blood, is tightly regulated within body fluids. Although the precise mechanisms behind this decrease remain unclear, similar findings were reported by Mohammad Nejad *et al.* (2022), who observed a reduction after OV injection in male common carp. However, this minor (yet statistically significant) decrease in serum sodium may not indicate health deterioration in the fish, especially since other ions like potassium and calcium remained stable.

The decline in serum cholesterol post-hormonal injection may be attributed to its use in steroid hormone synthesis. The hormone 17,20β-Dihydroxy-4-pregnen-3-one, which promotes sperm motility and milt production in male fish is derived from cholesterol through a multi-step process and increases during final maturation (Schulz *et al.,* 2010). Therefore, the observed decrease in plasma cholesterol levels following hormonal stimulation could be linked to an increase in 17,20β-Dihydroxy-4-pregnen-3-one production. Similarly, administering 0.2-0.35 mg/kg OV to male wild carp led to a significant reduction in serum cholesterol concentrations (Mohammad Nejad *et al.,* 2022).

An increase in RBC count was noted following the injection, likely due to the effects of testosterone. After the administration of GnRH analogues in fish, testosterone levels rise (Shokr, 2020), and this hormone has been shown to promote erythropoiesis in fish (Pottinger and Pickering, 1987; Soldatov and Kukhareva, 2015). Low hemoglobin (Hb) levels may result from iron deficiency, and RBCs with

lower Hb content tend to be smaller, which accounts for the reductions in MCV and MCH observed in this study. Similar findings were reported in both male and female bighead carp, *Hypophthalmichthys nobilis*, following PT extract injections (Heydari *et al*., 2014). Conversely, male African catfish exhibited significantly elevated RBC, Hct, Hb, MCV, MCH, and MCHC after GnRH injection (Shokr, 2020). These discrepancies highlight the need for further mechanistic studies on this subject.

Conclusion

In this study, OV enhances spermatological parameters compared to PT extract, potentially improving fertilization success. Additionally, hematological and biochemical assessments indicated no significant health issues in fish treated with OV. Given its lower cost and greater availability compared to PT extract, OV is recommended for use in the reproduction and stock rehabilitation programs of Sazan.

Conflicts of interest

The authors declare there is no conflict of interest for this article.

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