Reproductive performance and related gene expression in female Siamese fighting fish, *Betta splendens* (Regan, 1910) treated with Homoeopathy medicine Oophorinum

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Abstract

The study investigated the reproductive performance and relative gene expression in *Betta splendens* treated with an Oophorinum-incorporated diet. The experiment consists of four treatments with replicates at varying levels of the Oophorinum-incorporated diet. Treatments: T1 (without oophorinum), T2 (1% oophorinum), T3 (2% oophorinum), and T4 (3% oophorinum). The subadult female fishes (n = 20) were stocked for 30 days. The result shows that GSI was higher in T4 (8.79%), followed by T3 (8.24%) and T1 (7.77%). Fecundity was higher in T2, followed by T4, and ova diameter was 50–800 µm among the treatments. The large-size ova were higher in T4 (57.82%) and lower in T1 (10.39%). Smaller-sized ova were higher in T1 (89.61%) and lower in T4 (42.18%). The estradiol-17β and progesterone levels were maximum in T4 and minimum in T1. Histology of the liver sample in T1 showed an elevated number of vacuoles in the hepatocytes. The expression of the vitellogenin gene and β-actin showed high intensity in the liver tissue of T4 and T3, and a very faint band was observed in T1 and T2. In conclusion, oophorinum in diets at 3% shows better results, and incorporating oophorinum in ornamental fish diets could increase reproductive performance without adverse effects.

Keywords: *Betta splendens;* Homoeopathy; Gene expression; Oophorinum; Vitellogenesis

Introduction

A common species of freshwater aquarium fish, *Betta splendens* (Regan, 1910), called as Siamese fighting fish is a member of the family, Osphronemidae, has its huge flowing fins, vibrant colour spectrum and affordable commercial value. Genetic manipulation of *Betta* spp is facilitated by their reproductive biology that provides valuable resources for the effective use of genetic tools in animal model (Palmiotti et al.,2023). Aquacultural homoeopathy and use of natural products like active coal feed supplement (Ostrenko et al, 2021) has grown quickly in part, due to the misuse of strong medications that have been shown to cause significant negative effects, such as hormones, antibiotics and disinfectants. Homoeopathy is an eco-friendly alternative in aquaculture to improve various products and health aspects (Manuel *et al*., 2018). Homeopathic medications have no negative side effects, activate the body's natural healing processes that treats each animal as an individual to stimulate their healing ability and works with their body to relieve symptoms, restore itself and improve their overall health (Rath et al, 2018).

The economic viability of fish larval production is determined by its reproductive performance. Further, reproductive function, spawning frequency, egg count, and hatching rate are critical for improving the production of cultured fish in captivity. The proficient understanding of broodstock fish gonad maturation and spawning induction techniques, as well as effective egg fertilization are necessary (Mylonas *et al*., 2010). The approaches for induced breeding of captive fishes are not identified in most tropical cultured fishes (Moorhead & Zeng, 2010). Meanwhile, research has been done on the development and improvement of natural inducing methods for ornamental fish species, such as substitutes for induced spawning (Hill *et al*., 2005). Asian fish breeders have successfully developed methods that stimulate spawning conditions in a few freshwater species using carp pituitary gland extract, Human Chorionic Gonadotropin, Ovaprim, Ovatide, Synahorin, Pimozide, II-Desoxycorticosterone – acetate, Antiestrogen Tamoxifen and other synthetic hormones (Panda, 2016). The reproductive performance of female *B channoides* using the hormone Oodev (Pregnant Mare Serum Gonadotropine + Dopamine Antagonist) was investigated (Permana *et al.,* 2023). The masculinization effect of letrozole (a non-steroidal aromatase inhibitor) in different doses (mg/kg feed) incorporated into diet and fed to *B splendens* was evaluated (Katare *et al*., 2015). The effect of partial or complete replacement of live feed with formulated feed on the reproductive performance, growth, gonad weight and fecundity of *B splendens* were investigated (James and Sampath, 2004; Mandal *et al*., 2010; Srikrishnan *et al*., 2017).

Sarcodes from healthy endocrine glands which is taken from sheep or cows, have been used in homoeopathy to be effective medications for ovulation and gonadal maturation in livestock. The sarcode-based Oophorinum has been utilized with great effectiveness to induce the follicle-stimulating hormone (FSH), estrogen and progesterone are present in the ovarian extract. FSH is involved with the beginning of gonadal maturation or vitellogenesis, whereas LH is involved ovulation (Levavi-Sivan *et al*., 2010). According to Verma and Induvaid (1997) the Oophorinum has a carbohydrate content ranging from 8 to 95%. The structure of the glycoprotein Oophorinum is Pyroglu-His-TrySer-Tyr-Gly-Leu-Arg-Pro-Gly. Research on the final gonadal maturation and spawning in fish has not been conducted using Oophorinum, even though fish breeders and researchers have successfully developed methods that use homoeopathic medicines like *Natrum muriaticumin* to stimulate spawning conditions in a few freshwater ornamental fish species (Sathyaprakesh, 1986; Mitra and Raizada, 1986; Visakan *et al*., 2005; Sudha and Gogul, 2014; Premdass *et al*., 2014). The present study aimed to investigate the reproductive performance of female Siamese fighting fish *Betta splendens* treated with graded levels of Oophorinum and its related gene expression.

Materials and methods

Experimental fishes

Sub-adult female Siamese fighting fish *Betta splendens* were procured from commercial ornamental fish suppliers and conditioned in 10-litre tanks. An oviparous group of sub-adult virgin female *B. splendens* were selected with the age group of 120 dph (day of post hatch) given in Figure1., excluding infected fishes.

Design of experiment

Four treatments each with three replicates following a completely randomised design with varying levels of Oophorinum incorporated feed-fed groups. The four treatments were named T1 (without oophorinum), T2 (1% oophorinum), T3 (2% oophorinum) and T4 (3% oophorinum). In each replicate 20 number of fishes were stocked. The duration of the experiment was 30 days.

Experimental diet preparation

The feed ingredients as per the feed formulation given in the following table 1, were selected and an experimental diet with 40 % crude protein and 6% fat content was prepared according to Prabu *et al*. (2018) with minor modifications. After drying the feed pellets of 2 mm size, according to the mouth size of the fish, 200 µ crumbled feed was prepared by crushing and sieving. Then, Oophorinum was added at the rate of 1%, 2% and 3% of the diet as a top coating and mixed well by tumbling the feed in the closed glass container and one

diet was kept as the control without the addition of Oophorinum. All 4 diets were stored in closed containers during the feeding experiment, given in Figure 2.

Fish rearing and sampling

The fish were fed to apparent satiation twice daily at 10.00 hrs and 16.00 hrs. The water in the experimental tanks was changed every alternate day to maintain the optimum water quality parameters. After the completion of 30 days of the experiment, some of the fish from each treatment group were randomly sampled and anaesthetized with clove oil (50 µL L^{-1}). After tranquilization, the fishes were sacrificed and tissue samples were collected for reproductive performance study, hormonal assay, histology of ovary & liver and gene expression study, given in Figure 3, 4, 5.

Tissue homogenate preparation for hormonal assay

The ovaries from the experimental fishes were removed carefully and weighed. It was homogenized with phosphate buffer saline solution in an Eppendorf tube using a tissue homogenizer. The homogenate was centrifuged at 10000 rpm for 10 min at 4 °C in a cooling centrifuge. The supernatant was collected and used for quantifying the level of reproductive hormones such as estradiol-17β and progesterone from the fishes of different treatments were assessed by Radio Immunosorbent assay at Sundaram Arulrhaj Hospital (SAH) Diagnostic Centre, Thoothukudi, Tamil Nadu.

Reproductive performance analysis

The effect of oophorium dosage on the reproductive performance of female Siamese fighting fish was assessed through microscopic observation of the size of ova, oocyte developmental stages through histology section, fecundity, number of eggs spawned and hatching percentage. Reproductive parameters are as follows:

Gonadosomatic index (GSI): It is used as an indicator of the degree of gonadal development. After sampling, female fish from control and treatment (Oophorinum incorporated diet-fed fishes) weighed on a weighing balance and the ovary weight to the nearest 0.01 g to calculate the GSI. The ovaries were removed and weighed in an electrical monopan balance and the gonadosomatic index (GSI) was computed according to the formula of Dahlgren (1979); GSI (in %) = wet weight of gonad/wet weight of the fish x 100.

Oocyte diameter: Oocytes will be fixed in 5% neutral buffered formalin and 30 oocytes from the anterior, middle and posterior ovary of each female fish were measured using ocular micrometry within the same day. The diameter of the oocytes was measured to the nearest 0.01mm. The mean diameter of 100 oocytes represents the oocyte diameter of fishes.

Fecundity: Exactly 0.1 g of the ovary weighed, fixed in 5% neutral buffered formalin and counted individually on the same day of sampling. Fecundity is the product of the total number of oocytes and the weight of the entire ovary, divided by the body weight of each fish. Fecundity was noted down for fishes from each replicate and a comparison was done to find out fecundity according to weight of the female fish. $F = n \times G/ g$

Where “F” is fecundity, “n” is the average number of eggs, “G" is the weight of the gonads and “g" is the weight of the sample.

Histology

Liver tissues were fixed in 10% Neutral Buffered Formalin (NBF) for histological examination. The formalin-fixed tissues were cut into pieces of 2-3 mm thickness and washed thoroughly with water for several hours before placing them in ascending grades of alcohol for dehydration. The dehydrated tissues were cleared in turpentine oil and embedded in paraffin wax. Sections of 4-5 µm thickness were made from paraffin blocks and stained with haematoxylin and eosin (Lillie and Fulmer, 1976).

Sample collection for RNA isolation

Immediately after purchase, a few fishes for tissue sample collection for RNA extraction. A few fishes were anaesthetized with clove oil (50 µL L^{-1}) and the tissue samples such as liver and ovary (20 mg of tissue) were immediately taken in sterile Eppendorf tubes containing 0.5 mL Trizol reagent for RNA isolation.

Tissue samples kept in Trizol were completely homogenized using a handheld homogenizer and waited for 5 minutes at room temperature. To this 0.15 mL of chloroform was added and shaken vigorously for 15 sec and stood for 5 minutes at room temperature. The mixture was centrifuged at 12000 rpm for 15 min at 4 °C. Collect the colourless aqueous top phase which contains RNA in a fresh tube and add 0.25 ml of 2-propanol and mix well. It was allowed to stand for 5-10 minutes and centrifuge for 15 minutes at 12000 rpm. Decant the supernatant and add 500 µl of 75% ethanol and centrifuge at 7500 rpm for 5 min at 4 °C. Discard the supernatant and air dry the pellet for about 5 minutes. Add an appropriate volume (20-40 µl) of RNAase-free water or DEPC-treated water to dilute the pellets. The concentration of RNA was quantified in a Biophotometer. Simultaneously, ran about 2-3 µl of RNA sample in agarose gel electrophoresis (2%) to confirm its integrity.

cDNA synthesis

The RNA samples were converted into cDNA by the following method. In a PCR tube add 2-5 µg template RNA, oligo $(dt)_{18}$ 1 µl and remaining with DEPC treated water to make up to 12 µl. This mixture is mixed gently and incubated at 65 °C for 5 minutes in a Thermocycler. Then the following components were added to the above mixture, 5× reaction mixture 4 µl, Ribolock RNase inhibitor 1 µl, 10 mM dNTP mix 2 µl, and Revert Aid H minus M-MuLV reverse transcriptase 1µl, so that the total volume becomes 20 µl. After brief

mixing in mini-centrifuge, the mixture was incubated at 42 °C for 60 minutes and the reaction was terminated by heating at 70 °C for 10 minutes in Thermocycler. The cDNA is stored at -20 °C for use as a template in polymerase chain reaction (PCR).

Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method for the detection, separation, identification and quantification of nucleic acids. To visualize PCR products, 1.5% agarose gel was prepared using 1× TBE buffer by adding 2-3 µl of Ethidium Bromide. The gel was placed in the buffer tank filled with 1× TBE buffer. Carefully load 4 µl of PCR product after mixing with 1 µl of loading dye into the well. Connected the electrodes to the power pack and electrophoresed at 85 volts until the dye reached three-fourths of the gel length. The gel was viewed under UV light in a transilluminator and the image was recorded using the Gel documentation system (BIORAD GEL DOCUMENTATION SYSTEM, Manufacturer details) using PD Quest software and an image of a gel was captured.

Gel elution

The bands obtained in the gel were cut and DNA eluted using a quick gel extraction kit and DNA was collected in 1.5 mL Eppendorf tubes. The DNA fragment from the agarose gel was incised with a clean, sharp blade with minimal UV light exposure to avert DNA damage. Weigh the gel slice in a 1.5 mL tube and add 3 volumes of QG buffer to 1 volume of gel. Incubate the tube in a water bath at 50 °C for 10 minutes with repeated vortex once every 2-3 minutes to dissolve the gel completely. Add 1 gel volume of isopropanol to the sample mix well and pour the sample into a spin column provided with a 2 ml collection tube. Centrifuge the column at 10000 rpm for 1 minute to enable binding of DNA and discard flow through. Place the spin column back into the collection tube add 500 µl of QG buffer and centrifuge for 1 minute. Decant the flow through place the column back into the same tube and wash by adding 750 µL of buffer PE and centrifuge for 1 minute at 10000 rpm. Discard the flow-through and place the column into a new 1.5 mL microcentrifuge tube. Elute the bound DNA by adding 20 µl of buffer EB to the Centre of the membrane and centrifuge the column for 1 minute. For increased DNA yield, let the column stand for 4-5 minutes before centrifuge.

Sequencing and Sequence analysis

The gel-eluted DNA products were sequenced using universal primers M13 through the Sanger dideoxy sequencing method in PISBI TEK INDIA Private Ltd, Trichy, Tamil Nadu. The sequence was analyzed using BLAST (Basic Local Alignment Search Tool) software in the NCBI (National Center for Biotechnology Information) GenBank nucleotide database for finding homology with other sequences. The gene runner software was used to translate the sequence into a protein sequence to analyse the amino acid sequence for the presence of functional domains of the VTG gene. The sequence details were prepared using Sequin Version 15.10 software to submit to NCBI and the GenBank Accession number is yet to be received.

Semi-quantitative RT-PCR analysis of expression of VTG gene in liver

The semi-quantitative analysis of *the B splendens* VTG gene from liver tissues was studied by Reverse transcriptase-PCR (RT-PCR) analysis.

Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16 in which data were subjected to one-way ANOVA and Duncan's multiple range tests were used to determine the significant differences between the means. Comparisons were made at the 5 % probability level.

Results

Reproductive performance

The details of the reproductive performance of *B splendens* are given in the table.2 The gonad weight was found higher in T3 and T4, respectively. GSI was found higher in T4 with 8.79% followed by T3 (8.24%) and lowest in T1 (7.77%).

Fecundity and egg size

The fecundity of female fishes from the different treatments and the measured ova diameter were listed in the following table 3. The fecundity was higher in the T2 treatment followed by T4 treatment. The ova/egg diameter among the treatments was in the range of 50-800 µm given in figures 6. The large size ova above 450 µm were higher in the T4 treatment with a percentage of 57.82 % and lower in T1 showing just 10.39 %. Smaller sized ova below 450 µm were higher in T1 treatment (89.61%) and lower in T4 treatment which had 42.18%, respectively.

Hormone level

Reproductive hormones such as estradiol-17β and progesterone were studied among the treatments given in table 4. The level of estradiol-17β and progesterone showed an increasing trend as the dose of oophorinum increased in the experimental feed. The maximum value of estradiol-17β and progesterone was noticed in the T4 group and the minimum value was witnessed in the control (T1).

Histology of liver:

The histological observations of liver samples of experimental fishes were depicted in the following figures**.7.** The results indicate that the oophorinum stimulates the synthesis of vitellogenin and the figures did not show any vacuoles or lesions in the T3 and T4 group. However, the T1 group showed an elevated number of vacuoles in the hepatocytes.

Sequence analysis of vitellogenin gene

The PCR product was sent for sequencing and the sequence was obtained in a chromatogram and the obtained sequence was confirmed as vitellogenin gene. The BLAST search in the NCBI GenBank database with *B splendens* partial vitellogenin gene sequence resulted in 19 hits for the vitellogenin gene. Among the hits, those sequences which exhibited more than 75% of query coverage and identity were listed in the below table. *B splendens* partial vitellogenin gene showed maximum homology (99%) with *Colisa fasciata* vitellogenin gene, followed by (80%) with *Thunnus thynnus*, followed by (80%) with *Morone saxatilis.*

Sequence homology analysis of *B splendens partial* vitellogenin gene using BLAST

Accession No.	Description	Query coverage (%)	Max. identity (%)
GU727852.1	*Colisa fasciata* vitellogenin A mRNA, partial cds	100	99.15
FJ743688.1	*Thunnus thynnus* vitellogenin mRNA, complete cds	100	80.6
HQ846509.1	*Morone saxatilis* vitellogenin Aa mRNA, complete cds	100	80.11
JQ283441.1	*Dicentrarchus labrax* vitellogenin Aa (vtgAa) mRNA, complete cds	100	80.05
DQ020120.1	*Morone americana* vitellogenin A mRNA, complete cds	100	79.88

>5’CCTTCAGCTCAACATCAAGAAGACGCAGAATGTCTACGAGTTGCTAGAGGCCGGAGCTCAGGG
CGTGTGCAAAACCATCTACGCCATCGCTGAGGATGAGAAAGCTGATCGCTTCCTTCTGACAAAGAC
CAGAGATCTGAACCATTGCCAGGAGGCAATCGTCATGGACATCGGCCTCGCATACACTGAGATAT
GCGCAAAGTGCCGGGAGGATTCAAAGAACGTGAGAGGAGCCACGGCCTACAGCTACGTCCTAAA
GCCGGTGGCAGGCAGCATTCTTATCATGGAGGCTAGCGGCACCGAGCTGATCCAGTTCTCCCCTTT
CAGTGAGATGAACGGAGCGGCTCAGATGGAGACAAAGCAATCCCTGGTCTTCGTTGAGACTAGAG
CAGCCGCTGTTGTGCCCGTGGAGGCCGAGTACCTTCACCGAGGGTCTATCAAGTATGAGTTTTCCA
CTGAGCTTATACAGACACCCATTCAGCTCATTAAGATGACCAACGCACAGGCCCAGATTGTGGAA
GCTCTGAACCATTTGGTCACCTACTACGTGGAGAGAGTCTACGAGAATGCCCCTCTGAAGTTTTTG
GAGCTCGTCCAGCTCCTCCGTGCAGCACAGTTTGAAGATCTAGAAATGATCTGGAGCCGCTACAAA
ACCAAACCTGCTTATAGGCAGTCGATCTTGGACGCGGCCCCCGTGATTGGGACTCATGTAGCTCTG
AGACTCATCAAGGATAAATTCTTGGCCAACGAGATGACGGCTGTTGAAGCGGCTCACGCTTTGATT
ACAGCTGTTCATATGGTGGCGGCAAACACTGAGGCCATAGAGCTGGTCAAAGCTTTAGCAGCACA
TAATAAAATAGTGGAGAACCCCGTCCTGCGTGAAATCGTCCTCGTGGGCTACGGTACGATGGTTTC
CAAACGCTGCGCTGCAGAGGCTGTTTGTCCTGCTGAACTTATAAAGCCCATCCATGAGCTTCTTGC
AAATGCTGTTGCTAAGGATAACACCGAAGACATCATACTGCTCCTCAAGGTTCTGGGTAACGCTGG
CCATCCGAGCAGCCTAAAGTCCATCACCAAAATCCTGCCCATACATGGCACAGCAGCTGCGTCCAT
GCCCACAAGAGTCCACGTCGCTGCCATCTTGGCTTTGCGAAATCTTGCGAAAAAGGAGCCCAAAA
TGATTCAGGAATTGGCTCTTCAGCTCTACATGGACAAGTCCCTGCACCCGGAGCTCCGCATGCTTG
CTTGCATAGCGATGTTCGAGACCAAGCCTCCCATGGGTCTGGTGACAGCTCTTGCCAATATTGTGA
AGACGGAGGAGAACCTGCAGGTGGCGAGCTTCACATATTCCCACATGAAGTCCCTGTCCAGGAGC
ACCATCCACGCCTCAGTTGCTGCAGCTTGCAACGTTGCCGTCAAAATCCTGAGCCCAAGGCTGGAC
AGACTGAGCCTACGTTTCAGCAAAGCCATCCACATGGTCATCTATAATAGTCCTTTGATGCTCGGT
GCCGCTGCCAGCGCCTTCTACATCAACGATGCTGCCACCATCCTGCCCAGATCCATCGTTACCAAG
GCCAGTGCCTACATTGCTGGTGCTGCCGCTGATGTTCTGGAGATCGGGCTGAGAGCCGAGGGAATC
CAGGAGGCACTTCT-3’

Nucleotide sequence of *Betta splendens* vitellogenin gene

>5’_LQLNIKKTQNVYELLEAGAQGVCKTIYAIAEDEKADRFLLTKTRDLNHCQEAIVMDIGLAYTEICA
KCREDSKNVRGATAYSYVLKPVAGSILIMEASGTELIQFSPFSEMNGAAQMETKQSLVFVETRAAAVV
PVEAEYLHRGSIKYEFSTELIQTPIQLIKMTNAQAQIVEALNHLVTYYVERVYENAPLKFLELVQLLRAA
QFEDLEMIWSRYKTKPAYRQSILDAAPVIGTHVALRLIKDKFLANEMTAVEAAHALITAVHMVAANTE
AIELVKALAAHNKIVENPVLREIVLVGYGTMVSKRCAAEAVCPAELIKPIHELLANAVAKDNTEDIILLL
KVLGNAGHPSSLKSITKILPIHGTAAASMPTRVHVAAILALRNLAKKEPKMIQELALQLYMDKSLHPEL
RMLACIAMFETKPPMGLVTALANIVKTEENLQVASFTYSHMKSLSRSTIHASVAAACNVAVKILSPRLD
RLSLRFSKAIHMVIYNSPLMLGAAASAFYINDAATILPRSIVTKASAYIAGAAADVLEIGLRAEGIQEALL
_3’

Deduced amino acid sequence of *Betta splendens* vitellogenin gene

Table 1. The experiment feed Ingredients composition (dry weight basis)

Ingredients	Inclusion (g)
Wheat gluten	**30**
Soya flour	**300**
Squid meal	**20**
Clam meal	**20**
Meat and Bone meal	**60**
Groundnut oil cake	**60**
Shrimp meal	**80**
Fish meal	**145.5**
Wheat powder	**185**
Fish oil	**25**
Vitamin	**20**
Mineral	**20**
SMB	**1**
BHT	**1**
Methionine	**4**
Lysine	**6**
Vitamin C	**2.5**
Lecithin	**10**
DCP	**5**
Salt	**5**
Total	**1000**

SMB- sodium meta bisulphate; BHT-Butylated hydroxyl toluene; DCP- Dicalcium phosphate

Table 2. Gonad weight (g) and GSI (%) values in different treatment groups

Parameter	T1	T2	T3	T4
Length (cm)	3.75±0.12	3.95±0.15	4.025±0.09	4.1±0.18
Weight (g)	0.79±0.04	0.91±0.06	1.00±0.07	0.90±0.06
Gonad weight	0.061±0.008	0.076±0.005	0.081±0.008	0.079±0.008
GSI (%)	7.77±0.17	8.11±0.34	8.24±0.23	8.79±0.27

Table 3. Details of measured ova diameter

Ova dia (µm)	T1	T2	T3	T4
50	150	100	84	48
100	83	44	11	44
150	3	0	10	12
200	60	52	52	38
250	6	0	3	17
300	50	78	26	35
350	44	3	25	15
400	44	80	33	15
450	30	13	25	26
500	19	110	94	66
550	1	39	69	76
600	1	59	47	94
650	0	5	10	15
700	0	2	10	27
750	0	0	2	1
800	0	0	0	2
Fecundity	491	585	501	531
% of ova below 450 µm	89.61	61.02	48.70	42.18
% of ova above 450 µm	10.39	38.98	51.30	57.82

Table 4. Reproductive hormone levels

Parameter	T1	T2	T3	T4
17β estrodiol (pg/ml)	878.8±42.1	1056.5±39.89	1184.0±78.4	1235.4±72.3
Progesterone (ng/ml)	<0.25±0.01	0.29±0.02	0.30±0.01	0.32±0.01

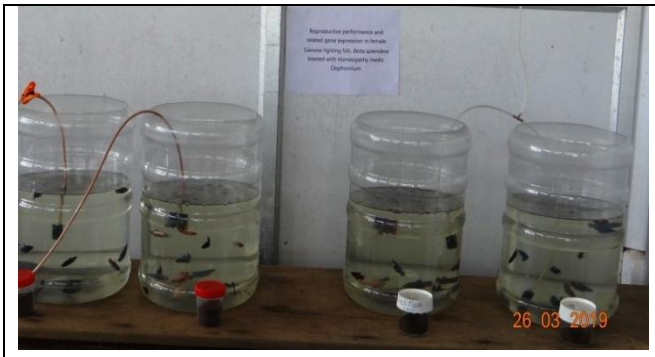


Fig 1. Experimental setup; female ***B splendens***

Fig 2. Oophorinum incorporated feed

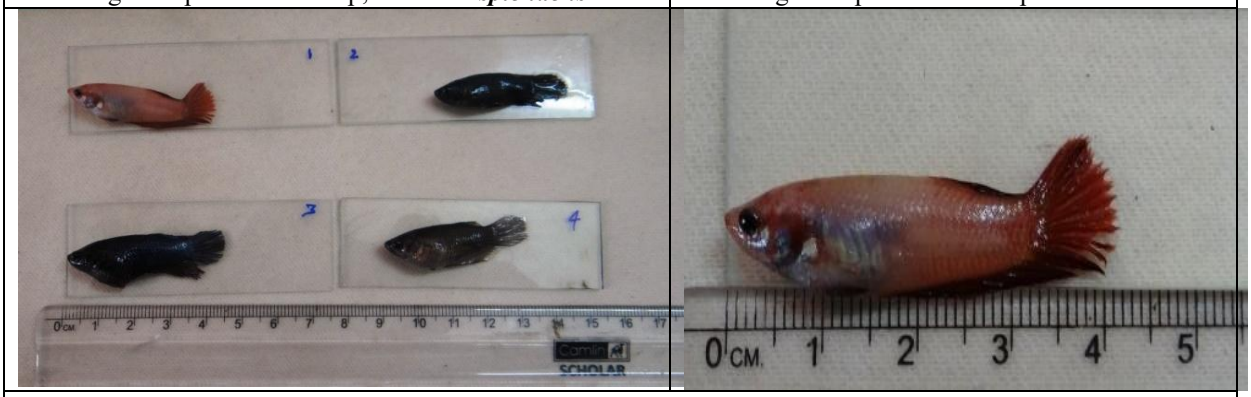
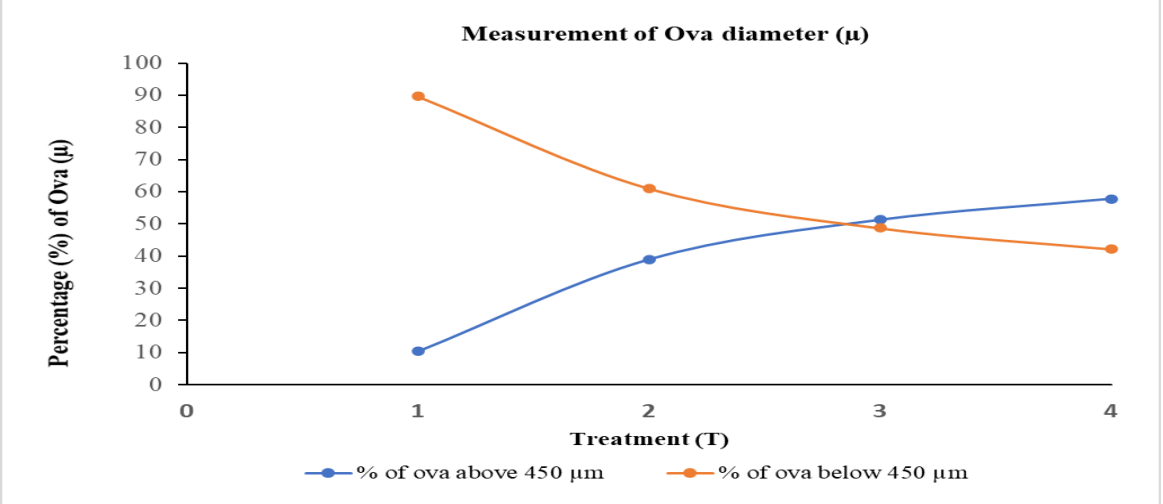


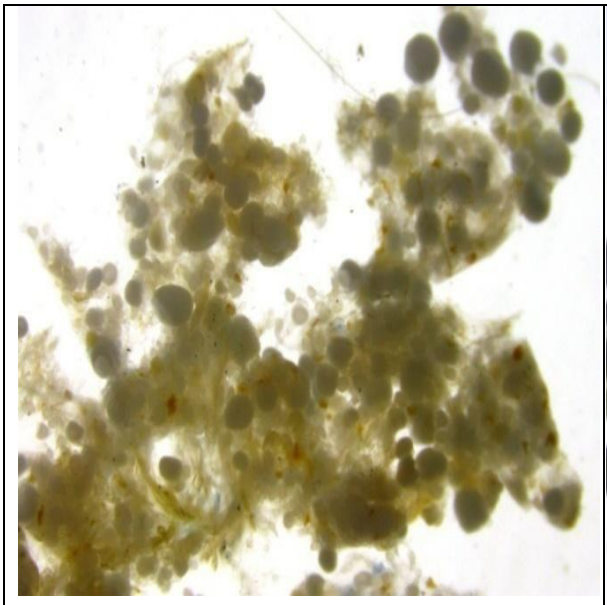
Fig.3. Final length (cm) of the experimental fishes

Fig.4 Growth of Ovary in different treatments

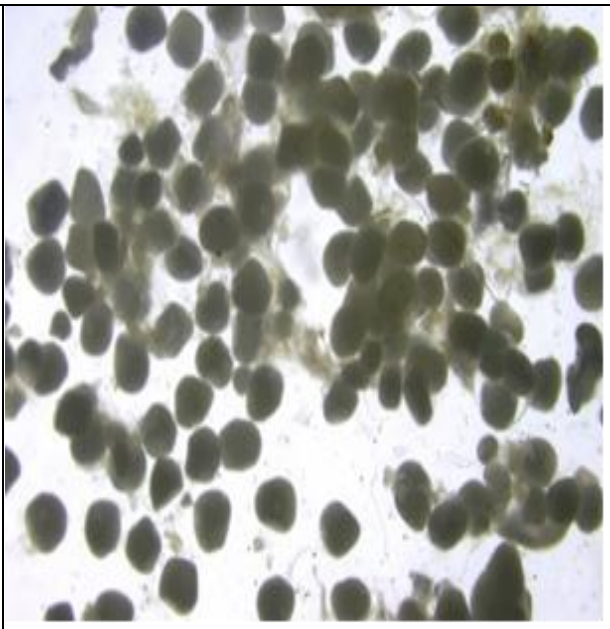
Fig.5 Dissection & removal of ovary after 30 days



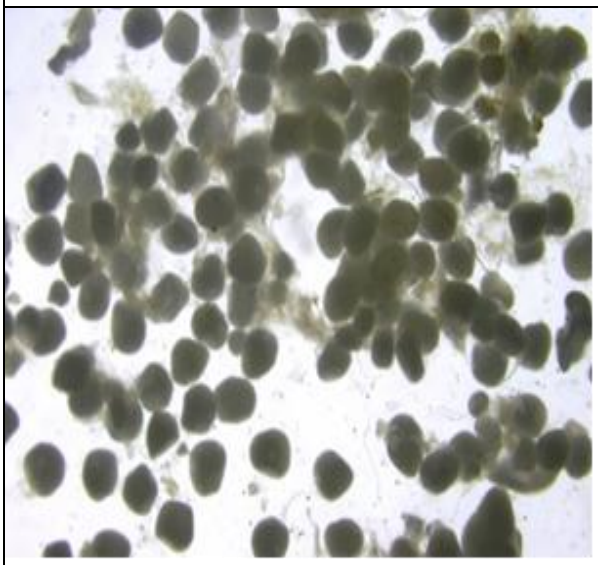
Graph 1. Percentage (%) of ova size



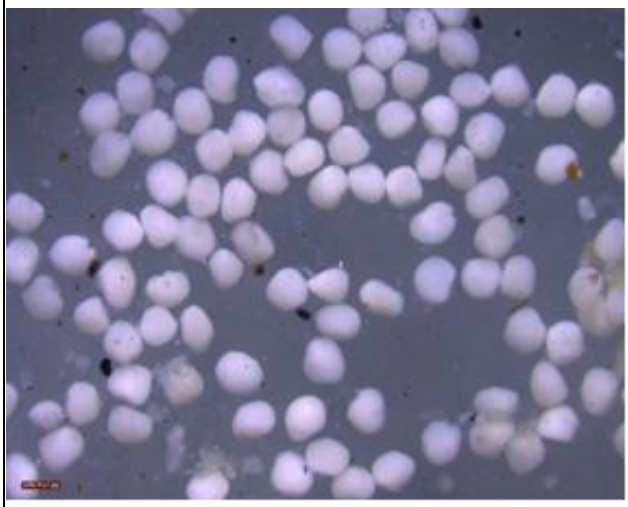
T 1



T 2

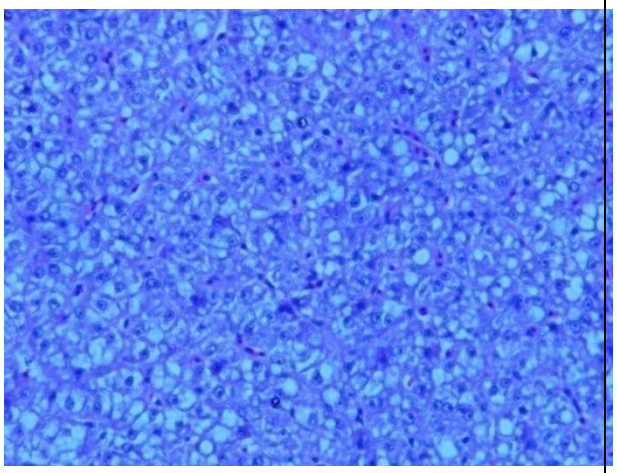


T 3

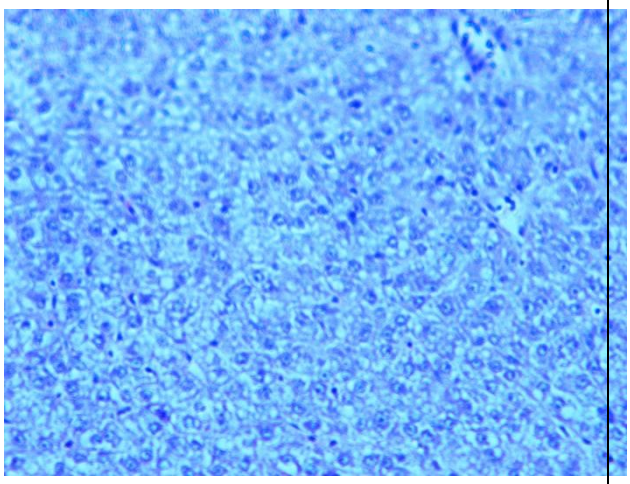


T 4

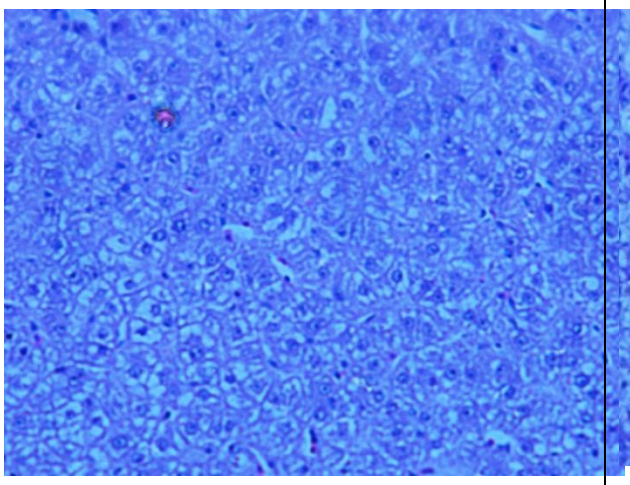
Fig 6. Observation of ova in control & treatments under Leica™ stereo zoom microscope (2X) (T1, T2, T3, T4)



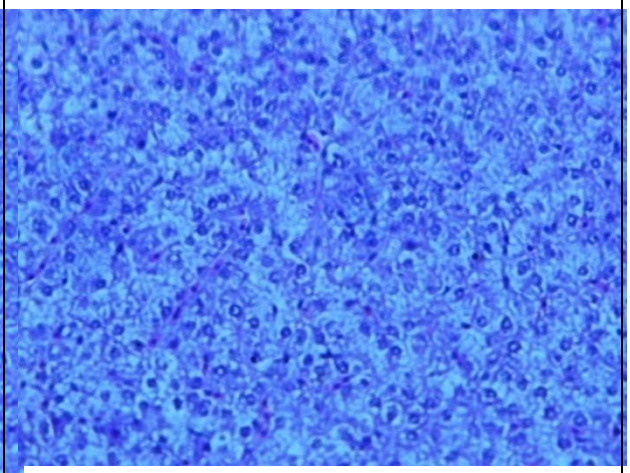
Histological observation of T1 group (50X)



Histological observation of T2 group (50X)



Histological observation of T3 group (50X)



Histological observation of T4 group (50X)

Fig 7. Histological observations of liver tissues of female *B. splendens*

Discussion

Reproductive performance of the brood fish is most often the deciding factor on the profitability of hatchery fish seed production. It requires competent knowledge in gonad maturation and spawning induction techniques of broodstock fish and successful egg fertilization procedures. The improvement in the spawning and standardization of doses, it would be possible to meet a larger requirement of quality fish seed production for aquariculture industries besides synthetic compounds. In the present study, graded levels of Oophorinum incorporated feed were fed and assessed the oocyte maturation and its related gene expression in female Siamese fighting fish.

Feed formulation and Oral administration of Oophorinum

In the present study, Oophorinum were incorporated at the rate of 1%, 2% and 3% of the diet as a top coating and mixed well by tumbling the feed and fed apparent satiation to the sub adults of female *B splendens*, twice a day. The study observation shows that fishes accept the Oophorinum incorporated feed readily. Permana *et al*., (2023) given encapsulated Oodev to the live feed (zooplankton *Moina* sp.) to increase the reproductive performance of female *B. channoides* by oral application. Kavumpurath and Pandian, (1993) done oral administration of estrogens in *B splendens* for sex reversal. Katare *et al*., (2015) found the masculinization effect of letrozole, incorporated into diet (mg/kg feed) and fed to *B splendens.* In this experiment, diet with higher levels of total crude protein (40 %) and (6%) fat was formulated. Pellet feed with 35% protein content, on dry weight basis was formulated and prepared for *B splendens* (James, and Sampath, 2002). Experimental diet was formulated using both plant and animal-based protein sources to contain (34.07 ± 0.39 %) crude protein

(CP) of pellet size (2mm) feed for *B splendens* (Mandal *et al*., 2012). Fish fed high protein diets (35%) had a greater ovary weight and gonadosomatic index than those fed low protein diets (5%) (Shim et al., 1989). Feeding *B. splendens* with 35% CP feed recorded better growth and spawning performance (James and Sampath, 2003). The reproductive cycle of fighter fish was short and should contain high protein. Adult Artemia contains high protein (56.5%) fed females *B splendens* developed gonad earlier, attaining the spawning stages on 37th day. The total amount of fat per dry weight is 20-27% required for adult females *B splendens* (Srikrishnan *et al*, 2017). James, and Sampath, (2002) found that fish fed with pelleted feed did not sexually mature and spawn. This may be due to several reasons, differ in the palatability and attractant of given feed in that experiment.

Reproductive performance analysis

In the present study, the reproductive performance of *B splendens* especially, GSI % (8.79±0.27) was found higher in 3% of Oophorinum incorporated diet-fed treatments (T4) than in the control groups (T1) and total number of ova counted were 501(T3) and 531(T4) on 30th day of the experiment. The fecundity was higher in the T2, followed by the T4. The ova diameter among the control and treatments was in the range of 50-800 µm. The large size ova were higher in the T4 with 57.82 (%) and lower in T1, 10.39%. Smaller sized ova were higher in the T1 (89.61%) and lower in the T4, 42.18%. The higher percentage (%) of ova above 450 µm were recorded in T4 (57.82 %) and 51.30 % (T3) shown in Graph 1. The gonad developed significantly ($p<0.05$) in fishes given at twice meal per day reached sexual maturity on day 35. This result elicited the maximum growth and reproductive performance is considered optimal for *B. splendens* and (James, and Sampath, 2004). The more frequent feeding resulted in a higher proportion of oocytes, gonad weight (158 mg wet weight) and gonadosomatic index. The breeding performances were improved at the later part of their maturity as the *B. splendens* became more aged (Mandal et al., 2010). Female *B. splendens* fed with liver had significantly high gonadosomatic index (GSI). Fish fed with pelleted feed developed gonad after 79th days, the ovary weight 9±0.7 (mg wet weight) and gonadosomatic index (%) 3.20±0.2 in *B splendens*. Feed types influenced significantly the feed intake, growth, ovary weight and fecundity in *B. splendens*. (James and Sampath, 2002).

Reproductive hormone levels

The reproductive hormones such as estradiol-17β and progesterone, were studied among the treatment groups. There was a good correlation between estradiol-17β levels and GSI, which is indirect evidence of the synthesis of vitellogenic proteins. The level of estradiol-17β and progesterone showed an increasing trend as the dose of oophorinum increased in the experimental feed. The maximum value of estradiol-17β and progesterone was noticed in the T4 group and the minimum value observed in the control (T1). Kagawa et al., (1983) reported the similar observation during gonadal maturation in female goldfish, *Carassius auratus.* The present study shows that oophorinum incorporation elevated the fecundity and increased the ova diameter of female fishes from the different treatments. Oophorinum-treated groups showed increased number of larger size ova than the control group. This result is supported by Wang et al. (2008), who found that estradiol-17β enhances the egg size and fecundity in bluegill sunfish *Lepomis macrochirus.* In ovarian cells of adult female fishes, the external application of stimulating agents leads to the synthesis and secretion of 17β estradiol and consequently increases the synthesis of vitellogenin in oviparous fish species (Shappell *et al*., 2010; Dammann *et al*., 2011). This accord with the present results where Oophorinum addition in the diet of Siamese fighting fish elevated the reproductive hormones such as estradiol-17β and progesterone significantly than the values witnessed in the control group. Tirado et al. (2017) revealed that the administration of tilapia pituitary extract and estradiol-synthetic enhanced the level of vitellogenin in Nile tilapia fish *Oreochromis niloticus*. A similar observation noticed in the present study through the level of vitellogenin gene expression in the experimental fishes treated with varying levels of oophorinum.

Histological observations of liver samples

In the current study, histological observations of liver samples of experimental fishes indicate that the oophorinum stimulates the synthesis of vitellogenin which was evident from the intact hepatocytes without any vacuoles and lesions. The histological observations of liver samples of female fishes indicated that the oophorinum treatment (T2, T3 & T4) stimulates the process of vitellogenesis, which noticed by its normal hepatocytes. However, the T1 group showed elevated vacuoles in the hepatocytes.

Expression of vitellogenin gene

Expression of vitellogenin gene from liver tissues of female *Betta splendens* indicated that 3% oophorinum incorporated treatment showed higher expression in terms of band intensity followed by 2% oophorinum incorporated treatment and a very faint band was observed control and 1% oophorinum incorporated treatment which was normalized in the presence of β actin, a house keeping gene. This is in agreement with Lim et al. (1991) who revealed that the administration of stimulating hormones such as estradiol elevates the vitellogenin gene expression in a teleost fish, *Oreochromis aureus.* The BLAST sequence search in the NCBI GenBank database with *Betta splendens* partial vitellogenin gene sequence resulted in 19 hits for the vitellogenin gene. Among the hits, those sequences which exhibited more than 75% of query coverage and identity were listed. *Betta splendens* partial vitellogenin gene showed maximum homology (99%) with *Colisa fasciata* vitellogenin

gene, followed by (80%) with *Thunnus thynnus*, followed by (80%) with *Morone saxatilis.* The expression of the vitellogenin gene and β actin (housekeeping gene) from female *Betta splendens* showed high intensity in liver tissues of 3% oophorinum incorporated treatment followed by 2% oophorinum incorporated treatment and a very faint band was observed in control and 1% oophorinum incorporated treatment.

Conclusion

Homoeopathy is a sustainable alternative that can be used in the aquaculture sector to enhance growth, reproduction, immune booster and various productive factors. The present study found that GSI %, ova count and ova size were increased in 3% oophorinum incorporated treatment. The level of estradiol-17β and progesterone showed an increasing trend as the dose of oophorinum increased in the experimental feed. Histological observations of liver samples of experimental fishes indicate that the oophorinum stimulates the synthesis of vitellogenin. Further, the expression of vitellogenin gene and β actin (housekeeping gene) from liver tissues of female *Betta splendens* showed that high intensity in liver tissues of 3% oophorinum incorporated treatment in this study. After analyzing all the parameters and results of this investigation revealed that Oophorinum at the dose of 3% shows better reproductive performance in female *B splendens* than the control group. This observation suggests that incorporation of oophorinum in the fish broodstock diet could increase reproductive performance without any adverse effects.

Author statement

Research involved with fresh water ornamental fish. During the study, gonad, ova and liver samples were collected from the experimental fishes after proper anaesthetization using standard protocol. This research article does not contain any activities that are against to ethics.

Data availability

Data will be shared based on the request and research interest**.**

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