

# Effect of Clomid and Hormonal Priming on Maturation and Spawning of *Labeo Rohita* and *Oreochromis niloticus*

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## Abstract

Effect of priming on the maturation and spawning were tested in two fish species using gonadotropin-releasing hormone (GnRH), carp pituitary homogenate (CPH) and clomiphene citrate (clomid). The fish were injected with a dose of GnRH, CPH and clomid, calibrated to contain 0.3ml/kg, 7 mg/kg and 70mg/kg respectively in three split doses (two priming doses and one final dose). Two priming doses enhanced the maturation and spawning in fish as evident from hormone assay, histology and gamete analysis. Among the inducing agents, two priming doses of GnRH showed 94% maturation and higher GSI (5.31 $\sigma$ ;18.5 $\varphi$ ) than CPH and clomid injected rohu. Subsequently, successful spawning recorded supported by elevated levels of FSH and LH in blood. Priming with CPH and clomid showed better maturation rate than GnRH injected Nile tilapia. The sperm count of tilapia administered with CPH and clomid was 3.7 $\pm$ 0.72 $\times$ 10<sup>9</sup> and 4.1 $\pm$ 0.86 $\times$ 10<sup>9</sup> respectively. The efficacy of hormonal priming in synchronising the gamete development in rohu and tilapia was further evident from the increased number of synchronised oocyte development. This study may help in developing strategies for broodstock and hatchery management of carps and tilapias.

## Introduction

Indian major carps and tilapia gained prominence in aquaculture due to their high demand and production potential as food fish. Carps accounted for nearly 25% of global aquaculture production in 2018. Similarly, tilapia became the second major produced fish after carps in the aquaculture sector. Farmed tilapia accounted for 5.27% of global aquaculture production in 2018, more than double the 2.38% in 1998 (Miao and Wang, 2020). The production of carps and tilapia is supported by the supply chain in the form of stocking materials such as fry and fingerlings sourced from hatcheries. Over the last three decades, there has been a remarkable increase in fish-seed production in many parts of world including

India using different inducing agents (Brzuska, 2021; Szabó et al., 2019; Kucharczyk et al., 2008; Routray et al., 2007). The demand for fry and fingerlings increased many folds due to the expansion of aquaculture (Rahman et al., 2013). In many parts of the Indian subcontinent, the seed production of carps involves either GnRH based hormone administration or hypophysation where fish pituitary gland homogenate is injected to sexually mature fishes during monsoon season under favorable conditions.

However, one of the major issues is the non-availability of matured fishes (carps) in the spawning season in adequate numbers. Although induced spawning is practiced in carps, the maturation of gonads is known to be influenced by various factors during

development. Moreover, the gonads show variations in maturation stages resulting in non-synchronous spawning and low fertilization. So, there is a necessity to improve the reproductive potential and induce synchronicity of spawning in carps and tilapia by hormonal priming or any other means as there are reports of unpredicted spawning response and non-synchronous oocyte production in tilapia also (Azevedo et al., 2021). In many species, sophisticated protocols are followed to induce gonad development and synchronise the spawning process through hormone treatment (Mylonas et al., 2010). Depending on the species and the stage of gametogenesis, a single exogenous hormone stimulation can often successfully induce maturation (Routray et al., 2007). However, a final hormone dose is required to facilitate the spawning process. The beneficial impacts of priming hormones before administering a final ovulatory dose increase the egg numbers and egg quality in fish (Crim et al., 1988). In some species, ovulation did not occur without priming (Bailey and Cole, 1999). Chemicals (hormones) of natural and synthetic origin are reported to interact with the endocrine system and alter germ cell generation and proliferation in fish (Routray et al., 2011). These germ cells play an essential role in gonadal development and gametogenesis (Zhao et al., 2017). The reproductive success of any species mainly depends on gamete quality (Routray et al., 2007). Carp pituitary homogenate (CPH) was the foremost hormone cocktail used in fish reproduction and effective in artificial breeding (Hu et al., 2020). In addition, gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus targets the pituitary and modulates gonadotropin's release, including FSH and LH, which play a key role in early gonad development and final gamete maturation (Kanda, 2019). There are some other synthetic agents, such as clomiphene citrate (commonly called clomid; chemical formula 2-[p-(2-chloro-1,2-diphenylvinyl) phenoxy] triethylamine citrate (1:1) approved in 1967 for the use of human gonadal dysfunctions (Kaminetsky and Hemani, 2009) and also reported to be helpful in inducing ovulation in teleost (Ueda and Takahashi 1977a; Pandey and Hoar, 1972; Breton et al., 1975). The clomid acts on hypothalamo-hypophyseal axis to stimulate gonadotropin secretion, which ultimately increases the sex hormones in fish (Kapur and Toor, 1979). These hormone priming effects were primarily designed to bypass unknown environmental cues necessary for maturation, which are often required to

complete spermiation and vitellogenesis in males and females, respectively.

In the present study, it was tested the hypothesis that by applying a priming dose of the three chemicals viz. CPH, GnRH and clomid prior to spawning season may improve the reproductive potential of rohu and tilapia leading to maturation and synchronized spawning. The efficacy of priming dose was assessed in terms of gonad development, sperm count, Gonado somatic index (GSI), maturation rate, gonad histology and hormone levels.

## Materials and Methods

### Collection and Rearing of Spawners

For this study, 240 numbers (120 ♂ and 120 ♀) of rohu, *L. rohita* (2-year old; male: 1023±65.23g; female: 1268±84.23g) and 240 numbers (120 ♂ and 120 ♀) of tilapia, *O. niloticus* (7-months old; male: 257±19.6g; female: 232±28.6 g) were obtained from the Carp and Tilapia Breeding Unit of ICAR-CIFA, Bhubaneswar, India. All the brood fish were transported in the fish hammock (Routray et al., 2007). After the initial acclimation for fifteen days, males and females of rohu and tilapia were stocked equally (1:1) in control and three treatment groups (20 ♂ and 20 ♀ in each pond, n=40). Experimental fish were reared in 12 earthen ponds (0.05 ha area, size: 25 m x 20 m, 1 m depth) in triplicate each for three months (May to July) in the farm facility of ICAR-CIFA (Lat. 20.2961° N, long. 85.8245° E) following standard husbandry procedures (Routray et al., 2007). The physico-chemical parameters of pond water such as pH (digital pH meter), dissolved oxygen (mg/l) (Winkler's method), NH<sub>4</sub>-N and NO<sub>3</sub>-N (mg/l) (spectrophotometry) and hardness (ppm) (test kit) were measured every week following (APHA, 1998) are shown in Table 1. Fish were fed with floating pellet feed (30% crude protein and 4% crude fat) ad libitum twice a day (09:00 AM and 04:00 PM).

### Hormones and Drugs Administration

CPH: The pituitary glands were collected from Indian major carp, *Labeo catla* available locally. The foremost part of the skull was removed with a knife to get the pituitary. The pituitary gland is left behind on the base of the skull. Collected pituitary glands were homogenized in sterilized distilled water. The pituitary homogenate was prepared following the method

**Table 1.** Physico-chemical parameters of water in the experimental tanks used to rear *L. rohita* and *O. niloticus*. Data shown as Mean ± SEM

Treatments	Water Temperature (°C)	pH	Dissolve oxygen (mg/l)	Ammonia (mg/l)	Nitrate (mg/l)	Hardness (ppm)
Control	32.25±1.26	7.5±0.12	5.2±0.02	0.005±0.002	0.001±0.00	122±0.04
CPH	33.12±1.56	7.7±0.02	5.5±0.03	0.008±0.001	0.001±0.00	110±0.09
GnRH	32.12±1.6	7.9±0.23	5.1±0.02	0.007±0.001	0.002±0.00	112±0.07
Clomid	32.27±1.75	7.8±0.01	5.3±0.01	0.006±0.002	0.001±0.00	130±0.09

described by Thomas et al. (2003). The CPH was administered to females and males as two priming doses with 15 days interval. GnRH: Synthetic hormone GnRH (Salmon GnRH-A (20mcg) + domperidone (10mg)) under trade name ovaprim, (Suyog Pharmaceuticals Pvt. Ltd) was administered to females and males as two priming doses with 15 days interval. The clomiphene citrate powder (Merck, India) was dissolved in distilled water and the solution was prepared by following the Kapur & Toor (1979) protocol in which 2 drops of Tween 80 (emulsifier) was used to stabilise the solution.. This solution was 10-fold diluted with distilled water then the precipitate was mixed to maintain consistency for serial dilution. A NaCl solution (0.6% saline) was added to make a 100 mM vehicle for the drug and administered to females and males as two priming doses at 15 days intervals. The fish were injected with a dose of GnRH, CPH and clomid, calibrated to contain 0.3ml/kg, 7 mg/kg and 70mg/kg respectively in three split doses (two priming doses and one final dose). The detailed dose of hormones and drug administration to males and females as priming doses are shown in Table 2.

### Experimental Design

The present study has four experimental groups, including the control and all three treatment groups were in triplicate. The hormone and drug were administered intraperitoneally to males and females of rohu and tilapia as priming doses prior to spawning month. We used two priming doses at an interval of 15 days, i.e. 1<sup>st</sup> dose on 16<sup>th</sup> of May and 2<sup>nd</sup> dose on 1<sup>st</sup> of June. Different treatments were used in the experiment, along with priming doses to males and females of rohu and tilapia. The final dose with respective drugs for spawning induction was given during 16<sup>th</sup> June early hours of morning (08:00 AM) in all the treatments. The experimental design is represented in Figure 1.

### Hormone Analysis

To avoid stress and ease of handling, fishes were anesthetized by 2-phenoxyethanol (0.1 ml/l) with aeration (MP Biomedicals, LLC France). For hormone studies, each time 3-5 ml of blood samples were collected from the caudal vein of six males and six females of rohu and tilapia in a non-heparinized syringe for serum. The serum was attained after centrifugation (4500 x g for 10 min) and stored at -20°C until further analysis. The serum FSH and LH levels were determined using ELISA Kit No. 500710 and ELISA Kit No. 500720 (Cayman Chemical, Michigan, USA). The absorbance was measured at 450 nm using an ELISA reader within 30 min.

### Assessment of Gonad Somatic Index (GSI)

For the assessment of GSI, six males and six females each in rohu and tilapia were collected

randomly by drag netting at the end of the experimental study. All the fish were sacrificed humanely to remove the gonads. GSI was calculated following the below formula:

$$\text{Gonado Somatic Index (GSI\%)} = \frac{\text{Gonad Weight (g)}}{\text{Body Weight (g)}} \times 100$$

### Maturation, Spawning Induction, Sperm Count and Fecundity

After 6h of final injection dose, the fishes were anesthetized with 2-phenoxyethanol for estimating the maturation of males and females. A ventral incision was made to expose gonads for determination of gonadal maturation rate by physical observation of gonads (i.e., the presence of eggs or milt) and the final maturation rate (%) was estimated by the following formula:

$$\text{Maturation rate(\%)} = \frac{\text{No. of fish matured}}{\text{sample size (n)}} \times 100$$

At around the same time, milt was collected after dissection of the testes and provided sufficient enough headspace for oxygen in the collection tubes (15 ml). The collected milt was maintained at 4°C. The sperm sample was prepared as described in Verma et al. (2009). The sperm count was done by diluting it 1000 times with an Extender C (Gupta et al., 1995) and adding 20 µl of mixture to the Neubauer haemocytometer and observed under a phase contrast microscope at ×400 magnification and expressed as number of spermatozoa/ml. Fecundity is estimated by the gravimetric method as per the protocol of Hunter et al. (1989). Three cross-sectional samples were taken from the anterior, middle and posterior portions of the two lobes of each ovary. The eggs in each of the three sections were counted and then the mean number of eggs was calculated.

### Fertilization Rate and Hatching Rate

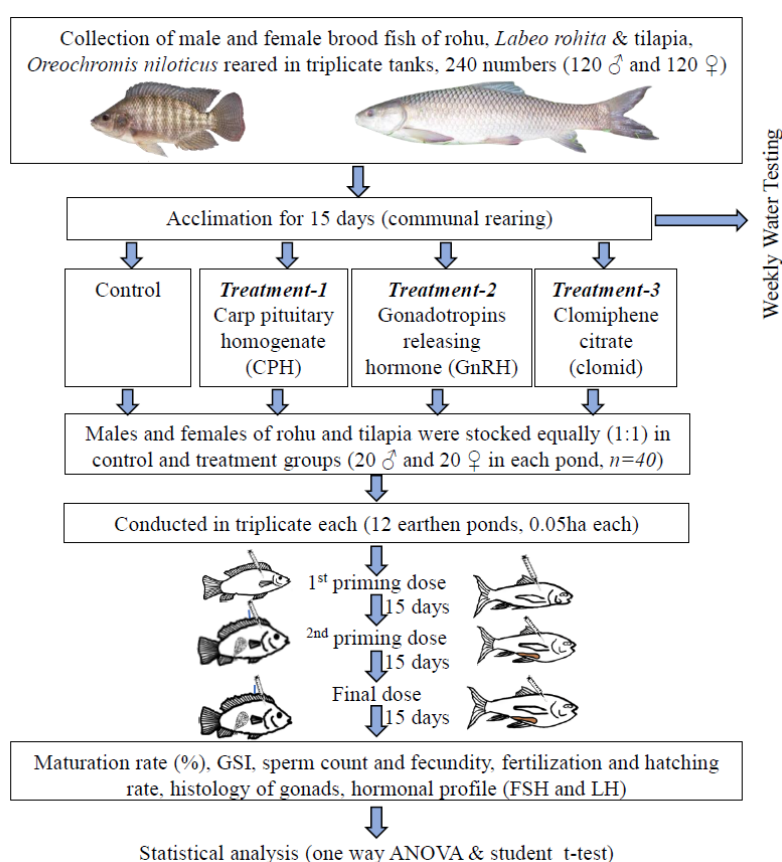
The fertilization rate and hatching rate was calculated by examining a minimum of three samples from each treatment. The fertilized eggs were easily identified from the unfertilized eggs by the presence of a transparent shell with a dark grey or black spot within the egg shell, while the unfertilized eggs were opaque. The fertilized eggs were counted by visual observations and was determined by using the formula:

$$\text{Fertilization rate} = \frac{\text{No. of fertilized eggs in sample taken}}{\text{Total No. of eggs in sample taken}} \times 100$$

The fertilised eggs after 24±2 hours in hatching pool develops itching moment with in the egg shell and after 48±3 hours of fertilization, the egg shell was detached. In determining hatching rate, the samples were collected from the hatching pool and the total numbers of fertilized eggs in the sample and along with

**Table 2.** Details of treatment with doses of priming agents (CPH, GnRH and clomid) used in the experiment

Treatments	1 <sup>st</sup> Priming dose to males	1 <sup>st</sup> Priming dose to females
Control	0.5% NS	0.5% NS
CPH	1 mg/Kg	2 mg/Kg
GnRH	0.05ml/Kg	0.1ml/Kg
Clomid	10 mg/Kg	20 mg/Kg
2 <sup>nd</sup> Priming dose to males		2 <sup>nd</sup> Priming dose to females
Control	0.5% NS	0.5% NS
CPH	1 mg/Kg	2 mg/Kg
GnRH	0.05ml/Kg	0.1ml/Kg
Clomid	10 mg/Kg	20 mg/Kg
Final dose to males		Final dose to females
Control	0.5% NS	0.5% NS
CPH	5 mg/Kg	7 mg/Kg
GnRH	0.2 ml/Kg	0.4 ml/Kg
Clomid	50 mg/Kg	80 mg/Kg



**Figure 1.** Schematic depiction of the experimental design to study the effect of clomid and hormonal priming on maturation and spawning of *Labeo rohita* and *Oreochromis niloticus*.

the hatchlings were counted by visual observations. The hatching rate was determined using the following formula:

$$\text{Hatching rate} = \frac{\text{No. of hatchlings in sample taken}}{\text{Total No. of fertilized eggs in sample taken}} \times 100$$

**Gonadal Histology for Light Microscopy**

To check the maturation and GC status through histological studies, every time, the middle portion of gonads of either male or female was excised, sliced and

immediately fixed in 10% neutral buffered formalin. After dehydrating through an ascended sequence of ethanol, tissues were cleared in xylene and embedded in paraffin wax. Tissue sections of 5-6µm thickness were cut using a semi-automated microtome (Leica, Germany), stained with hematoxylin, and counterstained with eosin (Merck, India Ltd). The histological changes were photographed using a photomicroscope (Nikon, Japan). The changes in exposed sections of gonads were compared between the two spawners during different seasons.

**Statistical Analysis**

The data obtained from the present study were statistically analysed by using SPSS version 22 (IBM, USA). One-way ANOVA and students t- test was used to determine significance between the means. The values were expressed as mean ± SE and each treatment was conducted in duplicates. The values were considered significant at a  $P < 0.05$ .

**Results**

**Maturation Rate**

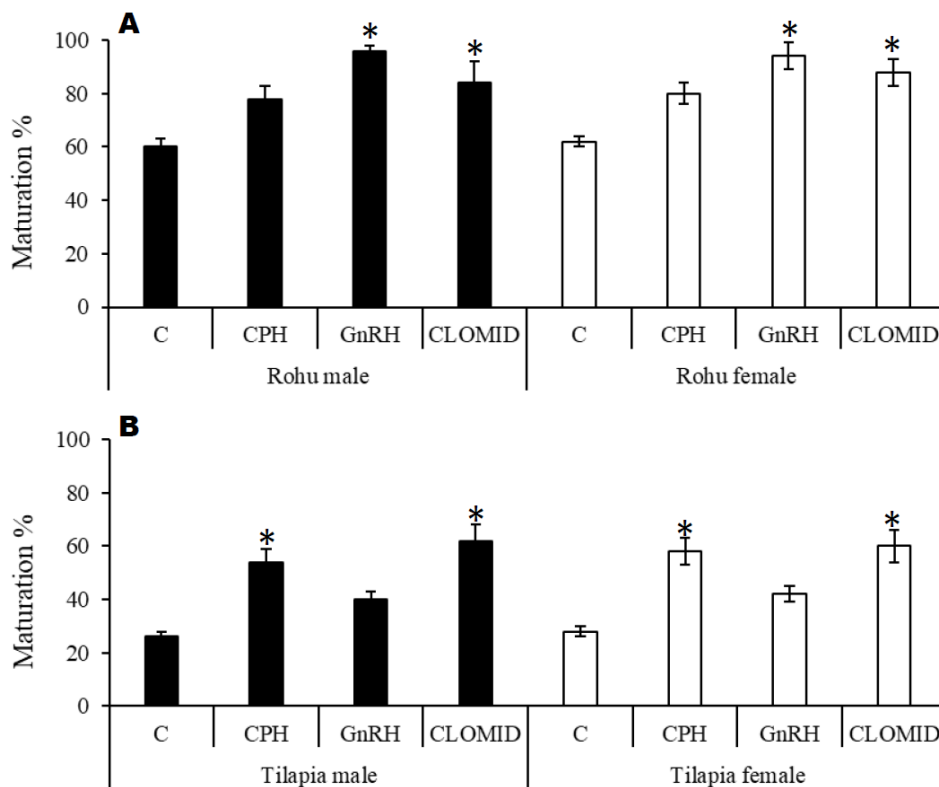
The effects of different priming agents on the maturation pattern of rohu and tilapia are shown in Figure 2. All the tested agents viz. CPH, GnRH and clomid showed a significant higher maturation in both sexes of rohu and tilapia when compared with the control. It was observed that administration of two doses of priming agents resulted in increasing the maturation rate in this study. In rohu, GnRH and clomid were more effective in attainment of maturity than CPH. However, in case of tilapia, it was the CPH and clomid that helped in attainment of more maturity than GnRH. The maximum maturation percentage in rohu males and females primed with GnRH were 96% and 60% respectively. The maturation rate of rohu males and females primed with clomid were 84% and 88% respectively, whereas, 78%

and 80% of maturation in male and females were noticed with CPH (Figure 2A).

Tilapia males and females also positively responded to two priming doses of CPH, GnRH and clomid in the present study. There were no significant differences between the maturation rate of tilapia primed with CPH and clomid. The maximum maturation of males (62%) and females (60%) of tilapia was observed with priming of clomid and a similar maturation in males (54%) and females (58%) primed with CPH was obtained. A lower maturation rate of males (40%) and females (42%) of tilapia was observed when primed with GnRH. A very low maturation rate was observed in the control group when compared with treated ones (Figures 2B).

**Gonad-somatic Index**

The GSI of rohu and tilapia was measured at the end of experimental period. The administration of two priming doses of hormones in treatment groups showed a significant increase in GSI of rohu and tilapia (Figure 3). GnRH and clomid treated rohu showed significantly higher GSI than the fishes treated with CPH. However, in case of tilapia, it was the CPH and clomid that helped in attainment of maximum GSI than GnRH. The maximum GSI of males and females primed with GnRH were  $5.31 \pm 1.41$  and  $18.53 \pm 2.1$  respectively. Similarly, the rohu males and females primed with clomid had a GSI of



**Figure 2.** Changes in maturation rate (%) in both sex of rohu, *Labeo rohita* and Nile tilapia, *Oreochromis niloticus* induced with (two doses) and without priming dose of CPH, GnRH and Clomid. A- male and female of rohu; B- male and female of tilapia. Data shown as mean value ± SE (n=6), asterisks indicate significant values ( $P < 0.05$ ).

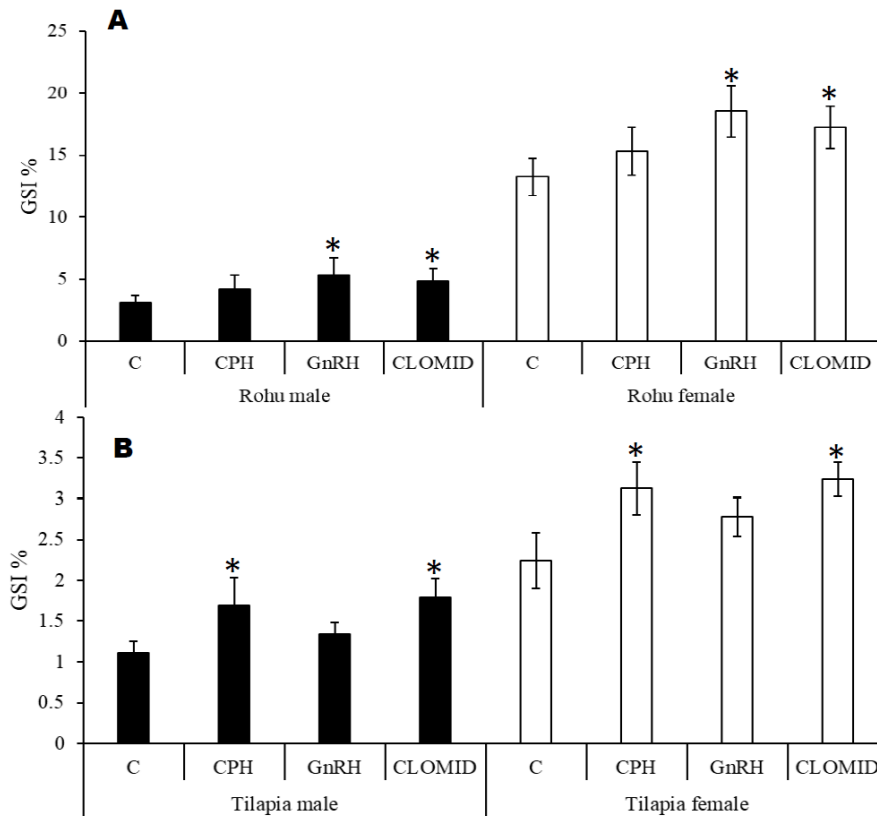
4.87±0.95 and 17.23±1.7 respectively, whereas the CPH injected males showed GSI of 4.21±1.12 and 15.3±1.9. The control group without any hormonal priming in rohu males and females showed a GSI of 3.12±0.52 and 13.24±1.5 respectively (Figure 3A).

Tilapia males and females also showed a significant increase in the GSI with priming of hormones whereas low GSI was marked in control (non-priming group). Priming with clomid to tilapia males and females resulted in a maximum GSI value of 1.79±0.23 and 3.24±0.32 respectively. Similarly, priming with CPH to male and female tilapia showed a GSI of 1.67±0.34 and

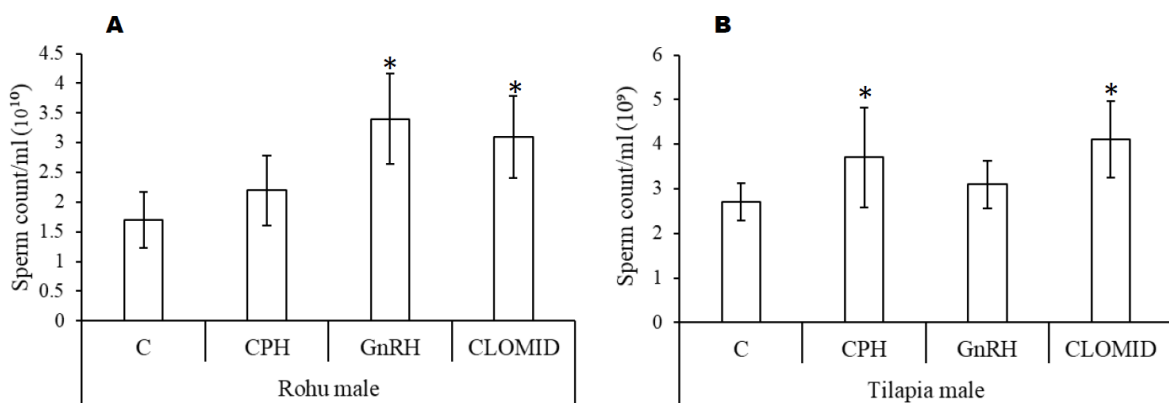
3.12±0.21 respectively. The GnRH administered males and females displayed a GSI of 1.34±0.14 and 2.78±0.24. In the control group, males and females showed significantly lower GSI of 1.12±0.14 and 2.24±0.34 than the hormonal primed groups (P<0.05) (Figure 3B).

**Sperm Count**

The effects of different priming agents on the sperm count of rohu and tilapia are shown in Figure 4. In rohu, the maximum sperm yield of 3.4×10<sup>10</sup> sperm/ml was obtained in GnRH primed group. Similarly, the



**Figure 3.** Changes in GSI (%) in both sex of rohu, *Labeo rohita* and Nile tilapia, *Oreochromis niloticus* induced with and without priming dose of CPH, GnRH and Clomid. A- male and female of rohu; B- male and female of tilapia. Data shown as mean value± SE (n=6), asterisks indicate significant values (P<0.05).



**Figure 4.** Changes in sperm count of rohu, *Labeo rohita* and Nile tilapia, *Oreochromis niloticus* without priming (Control) and with priming groups (CPH, GnRH and clomid). A- male rohu; B- male tilapia. Data shown as mean value± SE (n=6), asterisks indicate significant values (P<0.05).

priming with clomid to rohu males showed a sperm count of  $3.1 \times 10^{10}$  numbers/ml. The priming of CPH showed  $2.2 \times 10^{10}$  spermatozoa/ml and the control group without priming showed  $1.7 \times 10^{10}$  spermatozoa/ml (Figure 4A). In tilapia, the priming of clomid and CPH resulted in sperm yield of  $4.1 \times 10^9$  and  $3.7 \times 10^9$  sperm/ml respectively. There is no significant difference between clomid and CPH treated groups. The priming of GnRH showed a significantly reduced sperm yield  $3.1 \times 10^9$  sperm/ml ( $P < 0.05$ ) than other hormone treatment groups. The lowest sperm count of  $2.7 \times 10^9$  sperm/ml was observed in the control group (Figure 4B).

### Fecundity

The fecundity of rohu females was high in hormonal primed groups compared to control groups (Figure 5). The maximum fecundity was observed in GnRH injected fish with  $2.9 \times 10^5$  eggs/kg body weight followed by the clomid injected fish with fecundity of  $2.6 \times 10^5$  eggs/kg body weight. The CPH injected fish showed a fecundity of  $1.4 \times 10^5$  eggs/kg whereas the control group displayed the lowest fecundity  $0.85 \times 10^5$  eggs/kg (Figure 5A). In tilapia, the fecundity was high in clomid and CPH injected groups viz.  $3.5 \times 10^3$  eggs/kg and  $3.4 \times 10^3$  eggs/kg respectively. The GnRH injected fish had a fecundity of  $2.5 \times 10^3$  eggs/kg and the control groups showed a fecundity of  $2.1 \times 10^3$  egg/kg (Figure 5B).

### Fertilization Rate and Hatching Rate

The priming dose of GnRH administered to rohu showed the highest fertilization rate of 92.1% and a statistically similar fertilization rate of 88.7% was obtained by use of clomid (Figure 6A). The priming dose of CPH and the control group showed 78.6% and 69.7% of fertilization rate respectively. The GnRH primed dose performed better for fertilization of eggs. As the hatching rate of eggs are concerned, priming dose of GnRH resulted in highest percentage with 91.3%. The rate of fertilization/hatching of rohu in different priming groups can be put in the order as GnRH > clomid > CPH > control. In tilapia, the priming of clomid and CPH resulted in maximum fertilization rate of 86.8% and 81.5% respectively. The lowest fertilization rate of 58.34% was observed in the control group. Similarly, the maximum hatching rate was observed in clomid and CPH treated groups in tilapia (Figure 6B). The rate of fertilization/hatching of tilapia in different priming groups can be put in the order as clomid > CPH > GnRH > control.

### Histology

Gonad histoarchitecture of both the sex of rohu and tilapia were studied after the experimental period in the treatment groups (GnRH, CPH and clomid) and control group. It was evident that the priming of

hormones resulted in synchronicity in gamete development in males and females of rohu and tilapia. The testis and ovary histology in rohu and tilapia treated with two priming doses of hormones showed an active spermatogenesis with spermatozoa filled in seminiferous tubules and active oogenesis with tertiary oocytes filled in ovary whereas the histoarchitecture of control group showed partially filled spermatozoa and oocytes at various developmental stages. The administration of two priming doses of GnRH to rohu males helped in activation of final gametogenesis and many spermatozoa can be seen in the seminiferous tubules. Similarly, the ovary of rohu revealed the presence of closely packed tertiary oocytes that are ready to ovulate. The two priming doses of clomid and CPH to rohu males showed more occurrence of secondary spermatocytes and spermatids respectively whereas the two priming doses of clomid and CPH to rohu female showed loosely arranged tertiary oocytes with scattered yolk granules. Further, the presence of primary, secondary, tertiary oocytes was also visible in the histology. The rohu males and females in control group displayed different stages of gametogenesis and less pronounced oocytes at various stages of development (Figure 7).

In CPH and clomid administered groups of tilapias, the histology of testis revealed active spermatogenesis and the seminiferous tubules filled with spermatozoa whereas the control group displayed gametes at different stages of spermatogenesis. Similarly, the histology of tilapia females primed with CPH and clomid showed mainly the matured tertiary oocytes showing a synchronous maturation pattern. However, the control and GnRH groups showed all the development stages of oocytes (Figure 8).

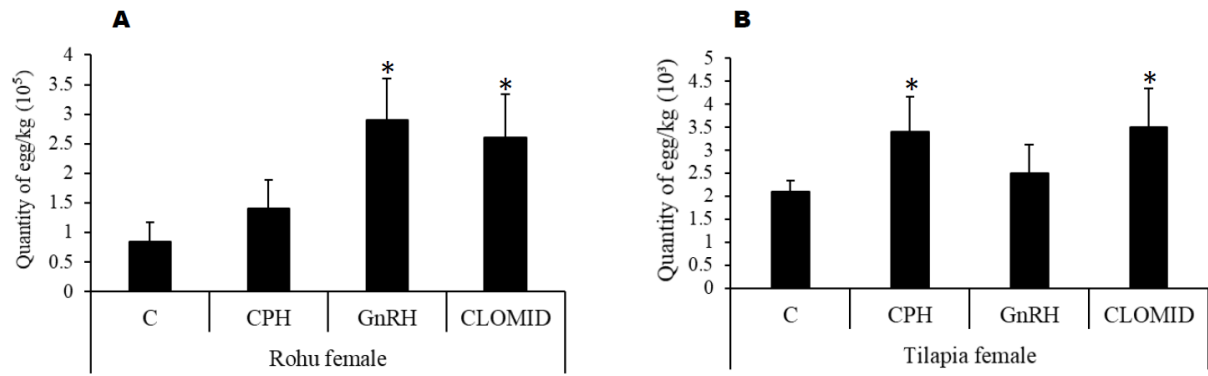
### Hormone Profile of Rohu and Tilapia

The effects of different priming agents on the FSH and LH levels of rohu and tilapia is shown in Figure 9. All three tested priming agents showed higher levels of FSH and LH than the control group. In rohu males, the highest levels of FSH ( $0.8 \pm 0.14$  mIU/ml) and LH ( $1.2 \pm 0.1$  mIU/ml) were observed in GnRH treated groups. Similarly, in rohu females, the highest levels of FSH ( $0.82 \pm 0.15$  mIU/ml) and LH ( $1.04 \pm 0.1$  mIU/ml) were observed in GnRH treated fish. In tilapia males, the highest levels of FSH ( $2.57 \pm 0.43$  mIU/ml) and LH ( $8.13 \pm 1.12$  mIU/ml) were observed in CPH treated groups. Similarly, in tilapia females, the highest levels of FSH ( $2.73 \pm 0.36$  mIU/ml) and LH ( $8.8 \pm 1.37$  mIU/ml) were observed in CPH treated fish. The lowest FSH and LH levels were noticed in control group in both sex of rohu and tilapia (Figure 9).

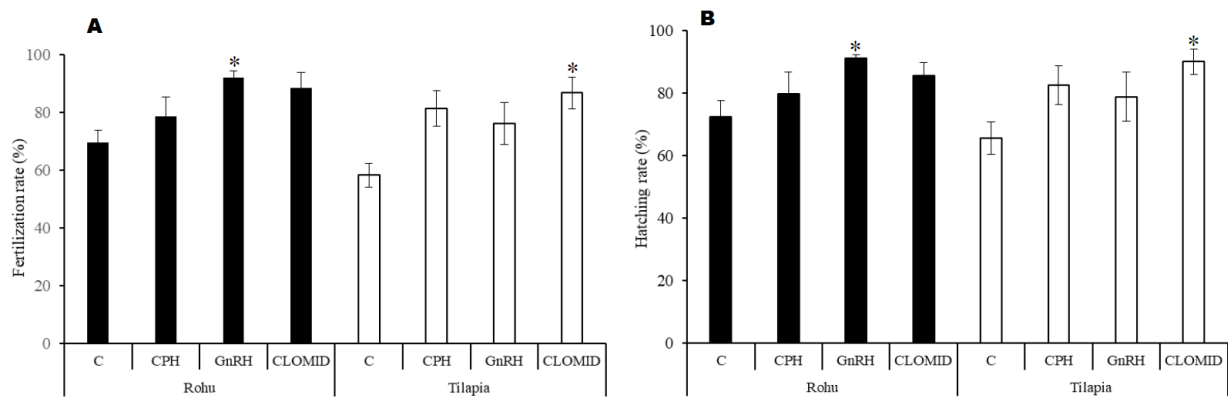
### Discussion

Overall, the present study reveals that maturation and subsequent spawning could be successfully

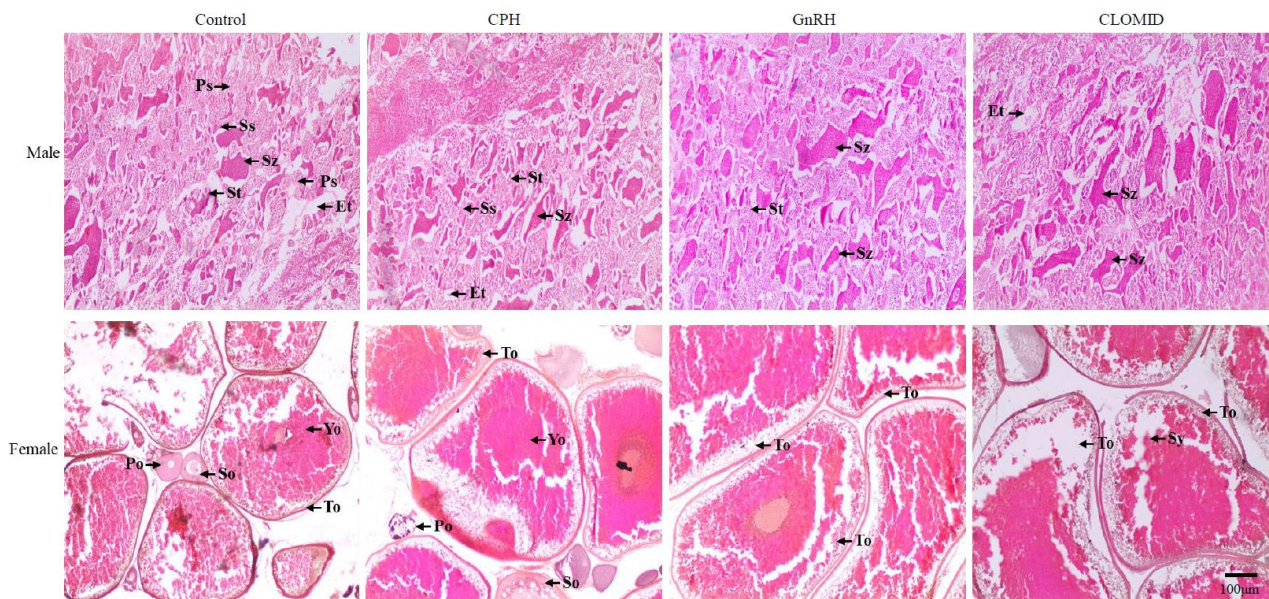




**Figure 5.** Changes in fecundity of rohu, *Labeo rohita* and Nile tilapia, *Oreochromis niloticus* without priming (Control) and with priming groups (CPH, GnRH and CLOMID). A- female rohu; B- female tilapia. Data shown as mean value  $\pm$  SE (n=6), asterisks indicate significant values (P<0.05).

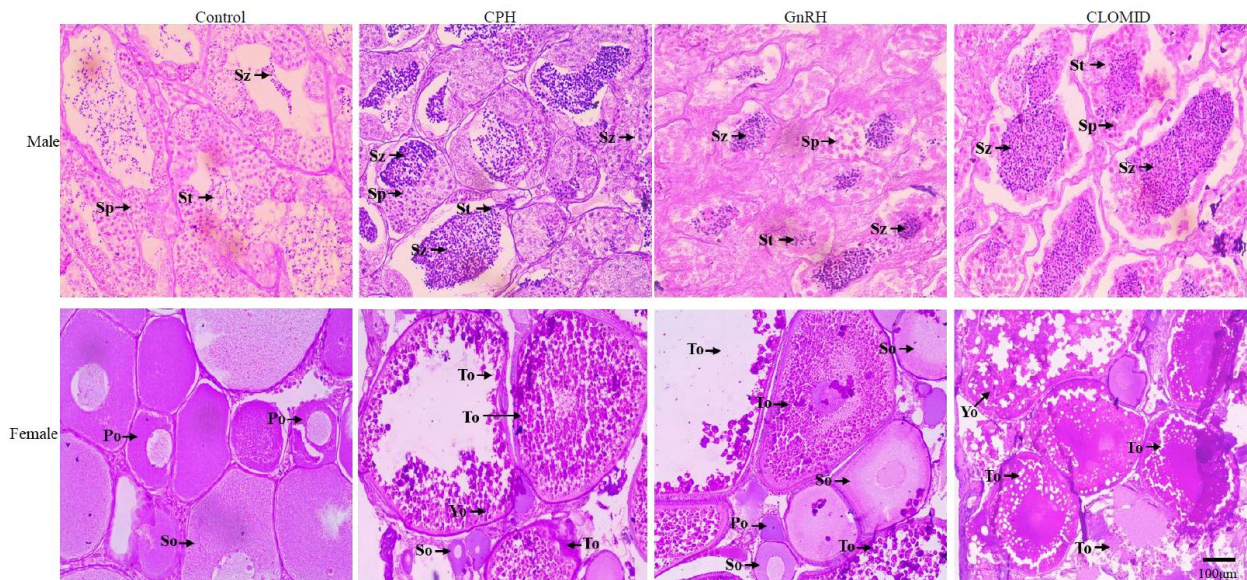


**Figure 6.** Changes in fertilization rates and hatching rates of rohu, *Labeo rohita* and Nile tilapia, *Oreochromis niloticus* without priming (Control) and with priming groups (CPH, GnRH and CLOMID). A- Fertilization rates; B- hatching rates. Data shown as mean value  $\pm$  SE (n=6), asterisks indicate significant values (P<0.05).

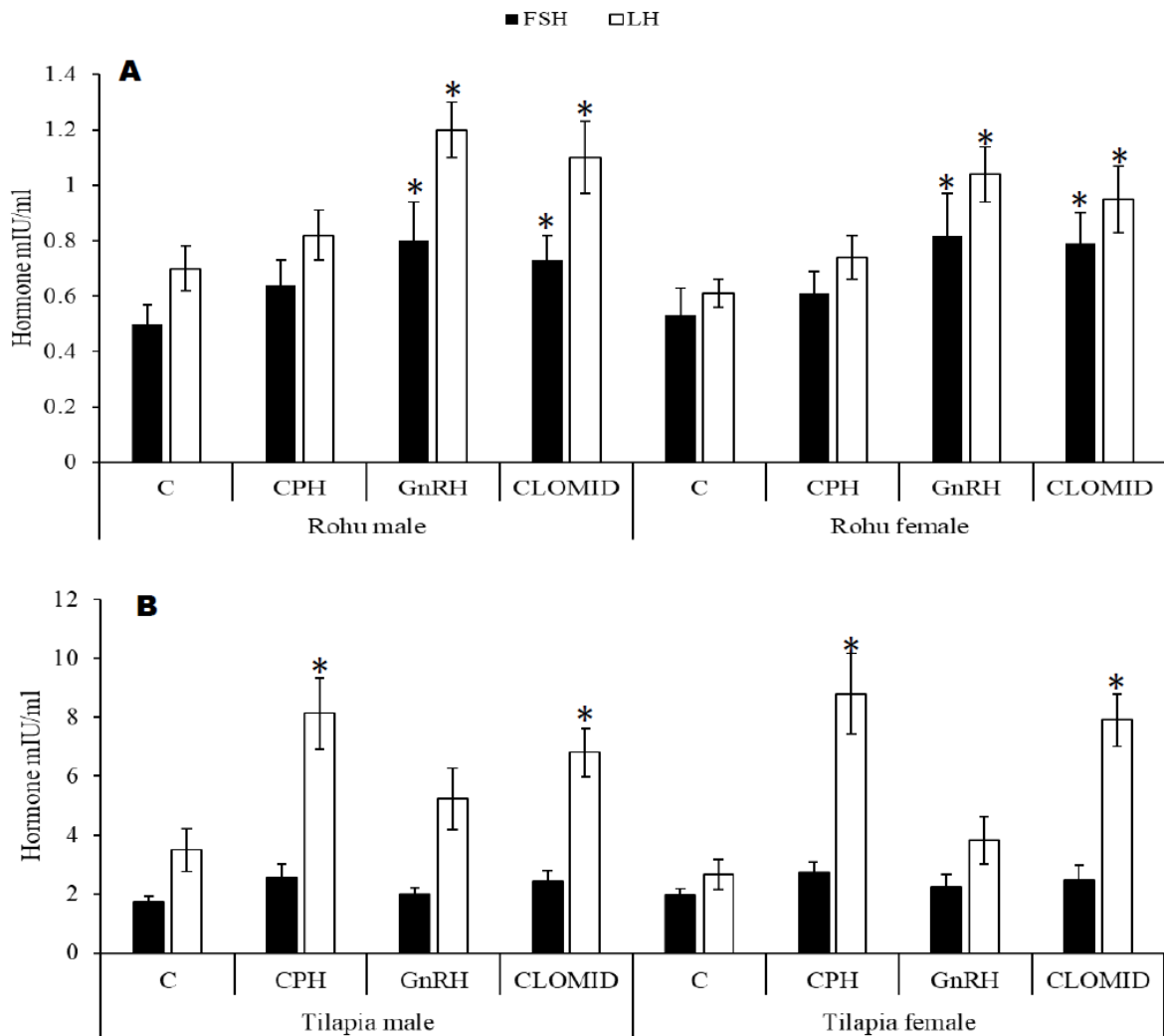


**Figure 7.** Changes in gonad (testis and ovary) histology of rohu, *Labeo rohita* without priming (Control) and with priming groups (CPH, GnRH and CLOMID), all sections were stained with hematoxyline-eosin. Et- empty seminiferous tubule; St- spermatids; Ps- primary spermatocytes; Ss- secondary spermatocytes; St- spermatids; Sz- spermatozoa; Po- primary oocyte, So- secondary oocytes, To- tertiary oocyte, Yo- yolk granules and Sy- scattered yolk granules. Bar = 100  $\mu$ m.





**Figure 8.** Changes in gonad (testis and ovary) histology of Nile tilapia, *Oreochromis niloticus* without priming (Control) and with priming groups (CPH, GnRH and CLOMID), all sections were stained with hematoxyline-eosin. St- spermatids; Sp- spermatocytes; Sz- spermatozoa; Po- primary oocyte, So- secondary oocyte, To- tertiary oocyte and Yo- yolk granules. Bar = 100 µm.



**Figure 9.** Changes in FSH and LH levels of Nile tilapia, *Oreochromis niloticus* and rohu, *Labeo rohita* without priming (Control) and with priming groups (CPH, GnRH and CLOMID). A- male and female of rohu; B- male and female of tilapia. Data shown as mean value  $\pm$  SE ( $n=6$ ), asterisks indicate significant values ( $P<0.05$ ).

performed with two priming doses of CPH, GnRH and clomid in rohu and tilapia. The effect of priming on the reproduction of these two species could be modulated to arrive at successful maturation, spermiation and ovulation. As hypothesized, the lowest maturation rate was observed in fishes that did not receive any priming dose (control). The primary use of the exogenous hormone in induced spawning is frequently believed to help in reproduction of species in captivity (Routray et al., 2007). There is an increased understanding of exogenous hormones to alter or synchronize the spawning time of species that do mature and produce gametes in captivity (e.g., tilapia, common carp) (Huang et al., 2021; Donaldson and Hunter, 1983). In the present study, the maturation response to two priming doses of GnRH in rohu was above 90% in both males and females. Elakkanai et al. (2015) reported that in Indian major carps the spawning response was highest for GnRH injected fish when compared to the CPH injected fish. Das et al. (2016) and Rath et al. (2007) investigated a relative efficacy of CPH and GnRH based synthetic hormones on the induced breeding performance of Indian major Carps (IMC), which support our present findings. Kucharczyk et al. (2020) investigated spawning effectiveness of different artificial inducing agents like hCG, CPH and GnRH in combination with dopamine inhibitors to ide (*Leuciscus idus*) and found less effective was by inducing with CPH. Similarly, Kucharczyk et al. (2021) reported in ruffe (*Gymnocephalus cernua*) in which greatest hatching was shown in hcG and lowest in CPH injected fish. However, it is pertinent to inform the readers that the earlier studies mentioned above did not tried priming doses. Here, the clomid injected fish showed an increased maturation rate above 84% in rohu. Worthington et al. (1983), Pandey and Hoar, (1972) and Pandey et al. (1973) reported that clomid is effective in inducing spawning in gravid roach and gold fish respectively. Present studies are also in line with the above reports. There are few reports regarding the exogenous hormone treatment in tilapia (Fernandes et al., 2013; Senthilkumaran et al., 2002; and Owusu-Frimpong 2008). Here, present results were varying in terms of maturation with different priming agents. This may be attributed due to their different chemical nature and inducing capacity. However, these priming agents worked successfully in inducing maturation at different levels in the tested fish. Another important point that needs to be informed is that, as the luteinizing hormone content in CPH is not known exactly, the dose used in this study could have been smaller than the minimum level required for inducing maturation in female rohu. Similar results were also reported by Fernandes et al., 2013. It is also reported that GnRH and clomid differ in their effectiveness for inducing gonadotropin release and utilize different receptors (Pandey and Hoar, 1972).

The GSI values of rohu male and female obtained in this study showed a significant increase in the priming groups. The highest female GSI was observed in GnRH primed fish whereas the lowest GSI was observed in

control group (without priming). This increased GSI was associated with the heavier weight of ovaries which contained the fully matured eggs (Shinkafi and Ipinjolu, 2012). Pereira et al. (2018) reported that there is an increase in GSI of *Leporinus elongatus* by the use of CPH and mGnRH analogue combined with dopamine receptor. Similarly, the highest GSI in males is showed in GnRH injected fish and the clomid also showed a significant increase in GSI. Ueda and Takahashi (1977a and 1977b) reported that the clomid exerts a positive response in the gonad maturation in both males and females of loach and gold fish respectively. Contrary to our findings Chaudhuri (1976) reported that the clomid injected in two doses at 8-28 mg/kg did not responded for spawning in rohu, this may be due to the dose used and interval between doses.

In tilapia, there are no reports on GSI of matured fish after priming or administration of exogenous hormones. In our results, the CPH injected fish showed high GSI in males and females whereas the clomid injected fish also showed a similar increase in GSI. The increased GSI values in both sexes of this species during the matured stage was due to the increase in gonad weight at that stage, compared to the other stages of gonad development. Pandey et al. (1973) reported that clomid at low dose of 0.1mg/kg has shown a positive response in gonad maturation and subsequent spawning in gold fish. There are reports in which female tilapia showed an increase in GSI when injected with CPH rather than with GnRH (Fernandes et al., 2013). There are reports that *Heteropneustes fossilis* injected with clomid, accelerated the synthesis of gonadotropins via hypothalamus which showed a positive response in ovarian development and successive ovulation (Singh and Singh, 1976).

In the present study, the fecundity of female rohu was different among all the treatments because of the varied potency of the exogenous hormones used and including the priming doses in early maturation stages. The rohu which is injected with GnRH showed increased fecundity when compared to all other treatments. This is similar to the reports of Brzuska and Adamek (1999) where *Silurus glanis* responded positively with ovaprim rather than CPH. Ali et al. (2015) reported that silver carp injected with ovaprim showed more fecundity than the other synthetic hormones. Fernandes et al., 2013 also reported an increased fecundity in tilapia using CPH and HCG. The tilapia females are multiple spawners and the highest fecundity was observed in clomid injected fish. To the best of our knowledge, this is the first report of hormonal priming in tilapia. There is significant increase in the synchronicity of egg maturation using CPH and clomid.

Here, the sperm count in male rohu was highest in GnRH treated group that is similar to the results obtained by other workers (Routray et al., 2007). The sperm concentration has been conventionally used for the assessment of sperm quality. It is a crucial parameter, which increases the success rate of

fertilization and is a characteristic feature of a species (Agarwal and Raghuvanshi, 2009). The highest sperm density of rohu injected with GnRH ( $3.4 \times 10^{10}$  sperm/ml) and a similar one with clomid injected fish ( $3.1 \times 10^{10}$  sperm/ml) was seen here. Verma et al. (2009) reported that the rohu sperm count was  $2.7 \times 10^{10}$  sperm/ml. Lukola and Sundqvist (1986) reported in other lab animals (mink males) having azoospermia testis injected with clomid improved the sperm density by 50% and Corrada et al. (2004) reported improved sperm density in male dogs. Berlinsky et al. (1997) reported that summer flounder induced with GnRH and CPH is ineffective in spermiation.

Fertilization rates of fish are determined to check whether oocytes that were subsequently fertilized formed into a diploid zygote whereas hatching rates indicates the live spawn produced. The fertilization rate and hatching rate indicates the status of wellbeing of the broods used in breeding and the efficiency of hormones used for maturation and spawning (Kuo et al., 1979). In the present study, the GnRH primed rohu showed the highest fertilization rate of 92.1% and hatching rate of 91.3%, whereas the control group without any priming dose of hormone showed very low fertilization rate 69.7% and hatching rate 72.6%. Nandeesh et al. (1990) reported that induced breeding of rohu with ovaprim-c resulted in 82% of fertilization rate which is lower than our present results with GnRH (ovaprim). This increase in fertilization rate with intervention of two priming dose of hormones might have stimulated the hypothalamus and in response to this stimulus. It is reported that FSH priming enhanced nuclear and cytoplasmic maturation of oocytes in vitro in other primate animals (Schramm and Bavister, 1994). This is, to our knowledge, the first report in which tilapia responded to priming dose of clomid with a fertilization rate of 86.8% and hatching rate of 90.1%.

Histological study of rohu and tilapia gonads revealed interesting facts that confirmed the maturation and spawning induction activity of CPH, GnRH and clomid. The gonads of males and females of rohu before the priming dose administration showed few secondary oocytes and more primary oocytes, and seminiferous tubules with few spermatids in dividing stages. However, after the hormone and drug administration, higher number of tertiary oocytes filled with yolk granules and seminiferous tubules filled with spermatozoa could be seen in all the treatments. Kumar and Chandrasekhar (1980) reported that clomid at different dose (25 $\mu$ g and 50 $\mu$ g) induced early spawning in *Cyprinus carpio* that were further confirmed by histological evidences showing full ripe oocytes. Here, the formation of yolk granules within the oocytes is a clear sign of maturation process. In tilapia, there is a considerable change in the oocyte development after priming with CPH, GnRH and clomid. Tilapia is believed to be an asynchronous spawner, however, after the priming dose administration, the CPH and clomid treated fish showed a group synchronous maturation

behavior of oocytes that was evident in the gonad histology. The priming of GnRH to tilapia females resulted in an asynchronous type of oocyte development i.e., the presence of primary and secondary oocytes with fewer tertiary oocytes were visible in the gonad histology.

Several studies have reported the physiological roles of FSH and LH and their regulation in a variety of fish species. In fish, FSH plays an important role, mostly involved in promoting early gonadal development and growth, whereas LH is involved in regulating the last stage of gametogenesis, including the final gamete maturation and release (ovulation and spermiation) (Zhang et al., 2015). The rohu injected with GnRH and clomid showed significantly higher levels of LH and FSH that indicates their significant role in gonadal growth as well as in final maturation process. Gen et al. (2003) reported that in red sea bream during the spawning season there is an increased LH level which corroborates our finding in rohu. Breton et al. (1975) reported that the dosage of 1mg/kg clomid was the best to induce high GtH levels which is similar to those present during ovulation in goldfish and carp. Similarly, in tilapia a higher level of FSH and LH was observed in fish treated with CPH and clomid. Billard and Peter (1977) reported that clomid injected to gold fish showed an increased level of gonadotropin hormones.

## Conclusion

In conclusion, we provide first hand report of priming using different agents for achieving better maturation in two types of fish species such as rohu and tilapia. Among the priming agents, GnRH and clomid were efficient in inducing higher gonad maturation in rohu. However, the CPH and clomid was most suitable for inducing maturation in tilapia. The maturation status of both fish was confirmed here through hormone assay and histology that proved our hypothesis. Further, we reported a synchronous type of maturation of gametes through priming with different agents in a batch spawners like tilapia where asynchronous gametes development takes place. Therefore, priming is an effective strategy for getting full potentiality in maturation by igniting the germ cells to enter into development process.

## Ethical Statement

Prior to the experimental design and initiation, the ethical clearance of the Institute Animal Ethical Committee was also obtained

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### Author Contribution

B. Panda and P. Routray: Conceptualization, Data curation, Formal analysis, Writing – original draft. O. Gopikrishna: Data curation, Methodology, Investigation, ms preparation. D.K. Verma: Data curation and Experiment setup. L. Samanta: Supervision and Data analysis. P. Routray: Funding acquisition, Investigation, Supervision, Writing - review & editing.

### Conflict of Interest

The authors declared that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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