

# Study of Endocrine Factors Influencing the Reproductive Stages of Indian Salmon, *Eleutheronema tetradactylum*

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## ABSTRACT

*Eleutheronema tetradactylum* is a polynemid fish species commonly known as Indian salmon and locally called as “Kaala” in Tamil. The population of this commercially valuable species and their availability has been drastically reduced and also the knowledge on reproductive biology of fish is minimum. The present study focuses on the influence of endocrine factors such as steroid hormones and peptides towards its reproduction. Based upon the histomorphological scrutiny, it has been understood that Indian salmon breeds during early January and early June as a biannual breeder. Previtellogenic, Vitellogenic and Post-Vitellogenic ovaries were tested for steroids such as Estradiol-17 $\beta$ , Testosterone, Pregnenolone, Progesterone, 17 $\alpha$ -hydroxyprogesterone 20 $\beta$ -hydroxyprogesterone, 17, 20 $\alpha$ -dihydroxyprogesterone and 17,20 $\beta$ -dihydroxy progesterone using HPLC. Alongside, genetic expression of insulin receptor-b (IRb), leptin receptor (LR) and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) were analyzed in the gonads of *E. tetradactylum* using RT-PCR (Real-Time). A Progesterone, precursor of majority of the steroids and a 17 $\alpha$ -hydroxyprogesterone, metabolite of progesterone significantly differed in the stages of ovary. The Expression IRb and LR were observed in both ovary and testis, and 3 $\beta$ -HSD in the previtellogenic ovary. The results suggest that IRb might be playing a significant role in the gonad of male and female salmon. Leptin might play an indirect role in the gonadal maturation. While 3 $\beta$ -HSD expression, signifies the steroidogenic indication in the gonadal development and maturation in *E. tetradactylum*. Identification of IRb in the developing ovary of polynemid species in the present study is the first report. The study highlights the steroids and peptides might have their own role to contribute in the reproduction of *E. tetradactylum* while the synergetic effects of them in the gonads is a question that should be considered in the future.

**Keywords:** Indian Salmon; Gonads; Steroids; Insulin receptor-b; Leptin receptor; 3 $\beta$ -HSD

## INTRODUCTION

Indian Salmon, *Eleutheronema tetradactylum* (Shaw, 1804), is a silvery grey perciform fish of polynemidae family. In concern with the present species, *E. tetradactylum* was once predominant in the Coromandel Coast during 1960s. It is one of the very high commercial and important fish species for Kuwait, India, Thailand, Vietnam, Indonesia, Singapore, Bangladesh, Cambodia, Myanmar, Northern Australia, and Malaysia [1]. The population has drastically reduced and the information of its reproductive biology in Indian waters is sparse. Understanding the reproductive biology of the fishes demands the knowledge of gonadal development and the factor influencing the development. Gonads are the primarily differentiated reproductive organs of testis and ovary. The germinal and endocrine tissue of gonads requires the induction of hormone for their normal development and function [2]. The hormones

required for gonadal development are mostly steroid in nature. In the vertebrates, gonadal steroids are the mediators of gametogenesis [3]. Leptin is a gonadal peptide known to involve in normal gonadal function [4]. Insulin hormone binds and activates the insulin receptor which plays a major role in the reproductive cycle of the fishes. The affinity for the insulin to binds to its receptor varies in different phases of reproductive cycle [5]. 3 $\beta$ -HSD is an enzyme that catalyzes the conversion of 3 $\beta$ -hydroxysteroid into 3 $\beta$ -ketosteroids that leads to the formation of progesterone from pregnenolone and androstenedione from dehydroepiandrosterone [6]. Besides their direct role in reproduction, they have synergetic effects with the sex steroids. Expression of the leptin receptor can be modulated by the steroids [7,8] and on the other hand, steroidogenesis in the gonads is directly linked with Insulin by gonadotropin-like activity [9,10]. Altogether the study aims towards the steroid profiling, and the expression of IRb, LR, and 3 $\beta$ -HSD with respect to the gonadal

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development and also tries to establish a possible synergetic effect of the steroids and peptides with respect to reproduction.

## MATERIALS AND METHODS

### Specimen

The fishes were collected from Neelankarai and Kovalam, Tamilnadu with the help of fishermen. Their body weight (g), total body length were measured and recorded. The gonads were dissected aseptically and its weight (g) was noted. For further analysis, the gonads were fixed in 0.6% saline for steroid analysis, 10% formalin for histology and RNA later for the Gene expression studies.

### Gonadal histology

The dissected gonads fixed in 10% formalin was sectioned in a microtome at a thickness of 0.4 to 0.5 microns and stained with haematoxylin and eosin stain. The histoarchitecture of the various stages of gonads were observed at 10x and 40x magnification under the light microscope.

### Analysis of gonadal steroid

The gonads were homogenized with 0.6% saline using mortar and pestle. The homogenate was vortexed with cyclohexane and ethyl acetate (1:1). The supernatant was collected and air-dried. The residue was diluted with 50 µl of ethanol and injected in High-Performance Liquid Chromatography (HPLC) (ShimadzuADvp) with reverse phase C18 column (ODS column) and 20 µl sample injection loop. The photodiode array (PDA) and UV-visual detectors were used to identify the steroids without any ambiguity. The standard steroids used for the analysis are Estradiol-17β, Testosterone, Pregnenolone, Progesterone, 20β-hydroxyprogesterone, 17α, 20α-dihydroxyprogesterone and 17α, 20β-dihydroxyprogesterone. (Sigma).

### Gene expression studies

Total RNA was extracted from the gonads of *E. tetradactylum* using Trizol method. Gel electrophoresis was performed using 1.2% agarose gel; the trace of double band was seen in the gonad sample when viewed under UV trans-illuminator (JH Biotech) and was quantified using a micro-spectrophotometer (Bio-Rad).

### cDNA synthesis

cDNA was synthesized using M-MLV Reverse transcriptase enzyme (cDNA synthesis kit, GCC Biotech). The following are the mixture of components used for the preparation; 10X M-MLV RT buffer towards cDNA synthesis: Oligo (dT), DNTP, total RNA, RNAase inhibitor, M-MLV RT.

## Polymerase Chain Reaction (PCR)

The primers of Leptin, Insulin receptor b, and 3β-HSD were designed from closely related species (Table 1). Amplification of genes was performed for 39 cycles; the cyclic steps include initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 10 seconds, and annealing temperature at 57°C for 30 seconds.

## RESULTS

A-Pre-vitellogenic ovary (100x) having several nucleoli arranged at the peripheral of nucleus; B-Vitellogenic ovary (100x) showing the yolk accumulation in the cytoplasmic region of ovarian follicle; C-Mature ovarian follicle (100x) showing the cortical alveolus and prominent well grown oocyte.

### Analysis of gonadal steroid

Histological observations have demarked the stages of ovary which has helped to distinguish three different stages (Figure 1). In the present study, reproductive steroids were identified in all the three stages of gonads. When compared with the standard steroids (Table 2), the authenticity of each steroid was cross-referred with the spectrum (Figure 2). Progesterone and 17α-hydroxyprogesterone are shown to be present in all the stages of ovarian development (Figure 3) and the highest level of progesterone was observed in post-vitellogenic period whereas, 17α-hydroxyprogesterone was observed in the vitellogenic period (Table 3).

### Gene expression studies

The presence of band observed under the UV transilluminator, signifies the expression of insulin receptor b and leptin receptor in both the ovary and testis, and a prominent expression of 3β-HSD was observed in the ovary of *E. tetradactylum*. The purity of 3β-HSD expression yielded to get sequenced. The partial 3β-HSD obtained from the present study showed the 733bp.

### 3β-HSD gene sequence

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5'TGGACATATGCCATGCGGATGGTTTCAATGGAAGGC-
GGCTGGGTACCTGGGCTTTCGGGGCGAAGGATATT-
TATGGAAAAGGTTGGCGGGTTAATTTAGGGCATT-
GAAGAAAGGAATCCAGAAAGGGGAAGGGG-
TAATGAAGAAGGCACGGGGGGGAGCTTAAAT-
CAATCCTGGGGAAAGTGGGAAAATTTTATGTG-
GTCACCCCCCCCCCGCGGTTTGGCCCCCCTTT-
GTACCCAAACATTGTGGCACGTGGCCAAC-
CGCTGGCGGTAGGGGCCCTGATTATCTGCC-
GTCCCCCCACCTTCCCTCCGTTTGGCACGGGC-
CGTCCCCTCAAAGCCCCCCCCCGAATTGCT-
GACCGCTCAAGACCACGGCTGCGCTCGTTAC-
GGGACTTTCCCAACCTTTCACGACACGAAT-
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**Table 1:** The forward (F) and reverse (R) oligonucleotide primers used for analysis.

| Gene         | Sequence (5'-3')                                    | Accession no | References               |
|--------------|---|--------------|--------------------------|
| <i>Insrb</i> | F: GACTGATTACTATCGCAAGGG<br>R: TCCAGGTATCCTCCGTCCAT | EU447178     | Toyoshima et al., (2008) |
| <i>Lepr</i>  | F: CTCCAGTGACGAAGGCAACTT<br>R: GGGAAGGAGCCGGAATGT   | NM_001113376 | Tang et al., (2007)      |
| <i>hsd3b</i> | F: CAGCCAGACCAAAAAGGAAG<br>R: TCCCACGTACACAGGATTGA  | HQ 6809831   | Mishra et al., (2016)    |



Figure 1: Cross sections showing the three stages of ovary of *E. tetradactylum*.

Table 2: The Standard steroids used as reference in HPLC to detect the presence of ovarian steroids in the Indian Salmon, *E. tetradactylum* as base line data.

| Standard steroids                | RT (min) | Max $\lambda$ (nm) | Peak area at 244 nm (for 20 $\mu$ g) |
|----------------------------------|----------|--------------------|--------------------------------------|
| Estradiol-17 $\beta$             | 8.19     | 205, 279           | 63740830                             |
| Testosterone                     | 9.31     | 242                | 74713375                             |
| Pregnenolone                     | 30.10    | 197                | 27794628                             |
| Progesterone                     | 25.91    | 242                | 62317092                             |
| 17 $\alpha$ -OHprogesterone      | 11.44    | 244                | 28583889                             |
| 20 $\beta$ -OHprogesterone       | 27.27    | 245                | 40900467                             |
| 17,20 $\alpha$ -diOHprogesterone | 8.56     | 244                | 48225488                             |
| 17,20 $\beta$ -diOHprogesterone  | 9.63     | 245                | 37110508                             |

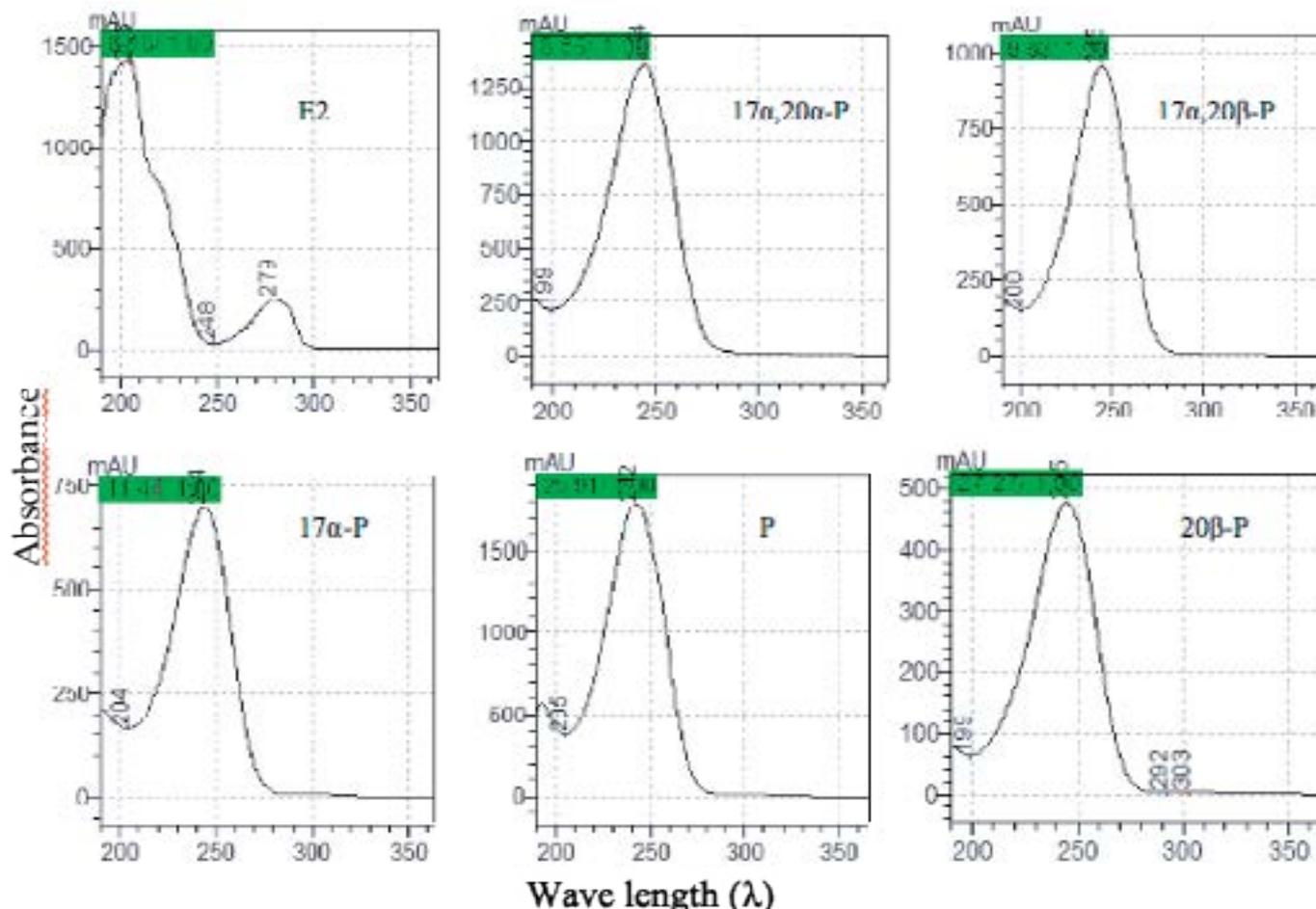


Figure 2: The UV-Visual spectrum of reference steroids showing the retention time and maximum absorbance ( $\lambda_{max}$ ). (E2:-Estradiol-17 $\beta$ ; 17 $\alpha$ , 20 $\alpha$ -P:-17 $\alpha$ , 20 $\alpha$ -diOH-progesterone; 17 $\alpha$ , 20 $\beta$ -P:-17 $\alpha$ , 20 $\beta$ -diOH-progesterone; 17 $\alpha$ -P:-17 $\alpha$ -OH-progesterone; P:-Progesterone; 20 $\beta$ -P:-20 $\beta$ -OH-progesterone)

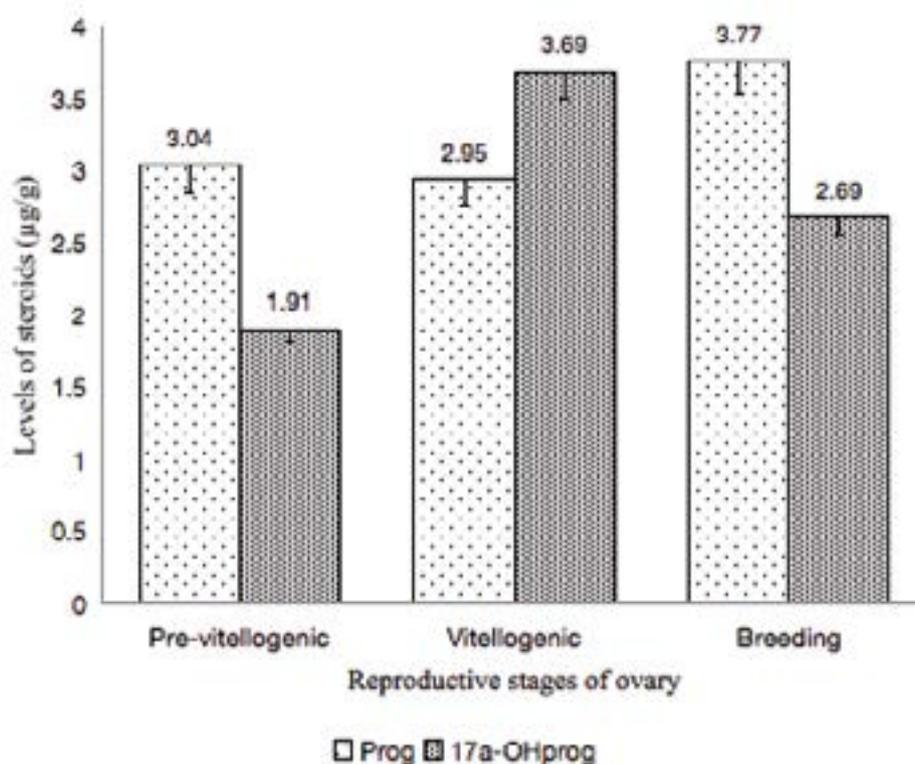


Figure 3: Levels of steroids present in the three different stages of ovary of *E. tetradactylum*.

Table 3: Presence of different steroids at the reproductive stages of ovary of *E. tetradactylum*.

| Standard steroids       | Stage-I ovary (PV) | Stage-II ovary (V) | Stage-III ovary (POV) |
|-------------------------|--------------------|--------------------|-----------------------|
| Estradiol-17β           | Nd                 | nd                 | nd                    |
| Testosterone            | Nd                 | nd                 | nd                    |
| Pregnenolone            | +                  | +                  | nd                    |
| Progesterone            | +                  | +                  | +                     |
| 17α-OHprogesterone      | +                  | +                  | +                     |
| 20β-OHprogesterone      | Nd                 | +                  | nd                    |
| 17,20α-diOHprogesterone | Nd                 | nd                 | nd                    |
| 17,20β-diOHprogesterone | Nd                 | nd                 | nd                    |

PV- Previtellogenic Period; V- Vitellogenic Period; POV- Post Vitellogenic Period of Ovary.

The '+' ve indicates the presence of the steroid and 'nd' indicates the non-detectable range or absence of the steroids, since the sensitivity of HPLC of detection is >10 ng.

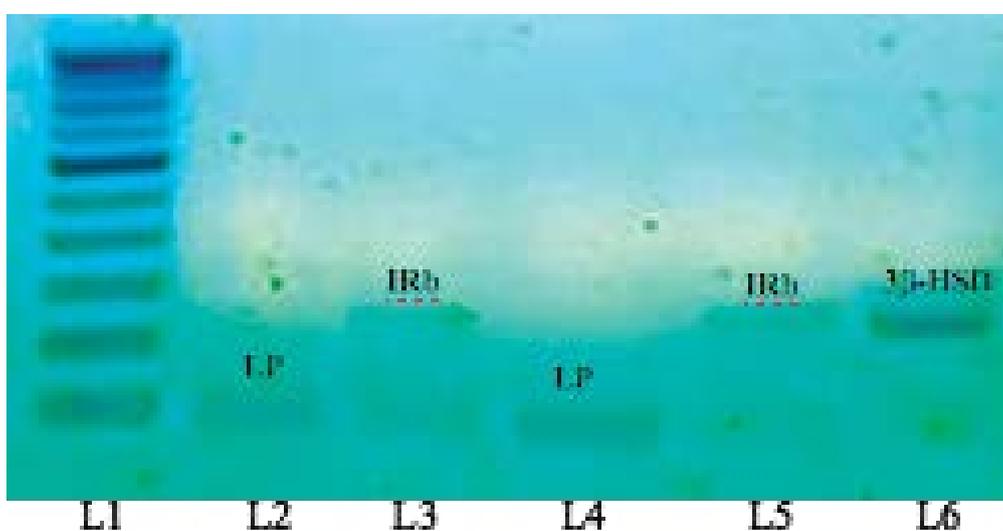


Figure 4: Agarose gel electrophoresis showing the expression of genes in the gonads of *E. tetradactylum*. L1-lane one showing the 1 kb ladder; L2-expression of leptin receptor (LP) in ovary; L3-expression of insulin receptor b (IRb) in ovary; L4-expression of leptin receptor b (LP) in testis; L5-expression of leptin receptor (LP) in testis; L6-expression of 3β-hydroxysteroid dehydrogenase (3β-HSD).

GAAAAATTTTTTTTAAACACCTGACCCCGAGTTCCCA-CAGGGGGGGGGCCCCCCCCCCCCCAGTCTCTACTT-GTCCTAAAGTGGGAAGGAAGTTCCTCCCGCTGTCCTAAT-TAAACCACCTTTCCCGGCGTTAGTGCGGACCTAGT-CATAGTCATTTTGAGTTGAACCATGATCGCGTAGTCCCA-CCCTCGGTTCGAGTGATCGAGTGGCCTGCTCCTCTAG-TATCTTCTGTATCCCATCGTCTATTGCGGCATAGTT-TACGCAATTATTAACAAGTGATATAATCTAGATT-GCTCCCTACGCCTTCGCTCATCACTGAC<sup>3' ~ 733</sup>

## DISCUSSION

The reproductive study of the fishes demands the knowledge of gonadal development and the molecular changes that take place during the development stages. The oogenesis in sardines was found to have a series of changes occurring in the oocytes during the development. Histological study on ovary of zebrafish reveals that the oocytes development happens in four stages namely primary growth, cortical alveolus, vitellogenic and mature oocytes [11]. The histomorphological changes observed in the different stages of the gonads of *E. tetradactylum* are very much similar to the related species this paves a way to classify them stage-wise demarcation. The different seasons have their effect on the histological and histochemical structure of the gonads of the catfish [12]. The histological knowledge gained through the present study reveals that the *E. tetradactylum* breeds twice a year, i.e., depending upon the season, the histology of the gonads has shown changes at the end of summer, when rainy season starts at the end of May when the temperature drops from 38°C to 26°C; similarly the temperature drops at the end of December due to winter season from 32°C to 20°C. Hence, Indian salmon breeds during July to August and January to February in the Chennai coast.

Steroidogenesis is the process of synthesizing the steroid hormones from the precursor cholesterol. Gonadal steroids are the major regulators of sex differentiation and gametogenesis in fishes [3]. The gonads and the adrenal gland are the primary site of steroid hormone synthesis. In steroidogenesis, the conversion of cholesterol to pregnenolone was catalyzed by C20-22 lyase enzyme. While 3β-HSD and CYP17 enzymes catalyze the conversion of pregnenolone to progesterone and 17α-hydroxypregnenalone to 17α hydroxy progesterone. In the present study, progesterone and 17α-hydroxyprogesterone was observed in the stage (pre-vitellogenic, vitellogenic, post-vitellogenic stage of ovary). The result suggests that the conversion of pregnenolone to progesterone and 17α-hydroxypregnenalone to 17α-hydroxyprogesterone are occurring at a faster rate, so pregnenolone was not observed in the sample. In most of the salmonid, progesterone is the predominant maturation inducing steroids except in winter flounder [13]. In the present study, a high level of progesterone in the post-vitellogenic ovary gives a clue that it may act as a maturation inducing steroid or it may lead to the synthesis of maturation inducing steroids in the ovary of *E. tetradactylum*. The plasma levels of gonadal steroids were closely correlated with the ovarian development in *Rutilus* [14]. The progesterone and 17α-hydroxyprogesterone were observed in the various stages of the ovary (Figure 4) in the present study on Indian Salmon. The level of progesterone was found to be elevated at a specific time in the reproductive cycle of the skate and this signifies the role of progesterone in the ovulation of skate [15]. In the present study on *E. tetradactylum*, IRb was found to be expressed in both the ovary and tes-

tis. It indicates that the IRb may have a role in lipid catabolism in gonad like that of zebrafish because IRb is more likely to promote lipid catabolism in zebrafish [16]. The insulin receptor is activated by binding to insulin hormone. In hermaphrodite fish, *Sparus aurata* insulin-like growth factor receptor type I was expressed in all developmental stages of gonads and their level of expression varies with different gonadal somatic index [17] so this signifies that IRb plays a major role in the gonadal development of *E. tetradactylum*.

The leptin gene in fishes shows higher similarity with the mammalian leptons [18]. The lack of expression of the fish leptin gene in adipose tissue makes a difference from the mammalian leptin. In Blunt Snout Bream, Leptin receptor and its ligand expression varies with male and female and increasing level of expression in brain during the early stages of ovary plays a significant role in the onset of puberty [19]. The leptin expression is up-regulated during sexual maturation was observed in the male Atlantic salmon and also in few other teleost species [20]. Leptin mRNA of fishes is primarily expressed in the liver [18,21]. For many fishes, the liver is the site where the leptin expression is more but in zebrafish, leptin was highly expressed in gonads [22]. Similar to zebrafish, Leptin gene was highly expressed in the gonads of *E. tetradactylum* in the present study this signifies that leptin may play an indirect role in the gonadal maturation, but the actual role of leptin in the gonad is unknown.

3β-HSD is an important enzyme that catalyzes the progesterone synthesis. The inhibition of 3β-HSD is that of endocrine disruption will lead to the reproductive dysfunction in fishes [23]. The elevated androgens are important for the spawning activity in territorial male fish but in females, the endocrine activity is more closely related to the stage of ovarian development [24,25].

## CONCLUSION

In the present study, 3β-HSD was expressed in the ovary which confirms the active synthesis of progesterone in the ovary of *E. tetradactylum* which denotes the 3β-HSD has a very important role in the oogenesis and spermatogenesis which leads to the synthesis of final maturation inducing steroid, 17α, 20β-dihydroxyprogesterone as well as 17α, 20β,21-trihydroxyprogesterone. Based on the present investigation, there is very limited literature available on the molecular aspects of *E. tetradactylum*, this study will help in conducting the further experiments which will help us to take it to the next level for conserving this commercially important species.

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