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Cyclic Variations of Ovarian Development, Hormones and Sex Related Genes of Rainbow Trout (*Oncorhynchus mykiss*) During Different Growth Stages

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Abstract

This study aimed to investigate the reproductive biology of female rainbow trout (Oncorhynchus mykiss). Mature female (n.80) rainbow trout from different reproductive phases were collected. Through a transmission electron microscope (TEM), it was feasible to examine the oocyte at several developmental stages, namely the perinucleolus stage, cortical alveoli stage, vitellogenic stage, mature stage, and postovulatory stage. Progesterone and estradiol levels were measured at their lowest during the immature and spent phases, while reaching their highest levels during the maturing and mature stages. The total amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) was greater in mature ovaries compared to immature and maturing ovaries. Gene transcripts related to the production of steroids, specifically star, cyp11a1, cyp17a1 and hsd3b were expressed at low levels during the immature stage and showed a significant rise during the mature stage. The ovarian gene expression patterns during oogenesis exhibited distinct dissimilarities. The fatty acid content, essential for the development of fish ovaries, and the sex steroid hormonal profile, which play a key role in regulating oogenesis, are particularly significant. These results will enhance our comprehension of the reproductive biology and sustainable management of rainbow trout in their natural environment.

Introduction

The underlying pattern of reproductive activity must be recognized in order to manage fisheries. The reproductive state of fish may be determined using a variety of approaches, including microscopic gonadal staging, oocyte size distribution, sex steroid evaluation, and gonadal indices (West, 1990). Gonadal analyses are a straightforward way to describe the phases of development in the gonads of fish. The essential sequence of gametogenesis is preserved in teleost (Milton *et al*., 2018). In the case of fishes, significant changes in structure occur throughout oocyte development (Ge, 2005; Sharma *et al*., 2023). Two layers

of cells surround the teleost oocyte: the outer layer of thecal and the inner granulosa. In the mature oocyte stage, the migration and breakage of germinal vesicles, lipid droplet coalescence, and yolk globules are prevalent events (Nagahama, 1983; Yueh and Chang, 2000). The seasonal changes in fish gonads are crucial tools for predicting the reproductive cycle (Sharma *et al*., 2023), therefore, understanding the development of sexual maturity and reproductive cycle in female teleosts is a vital phenomenon (Sivakumaran, 1991). Researchers have found that including the proteins vitelliogenin and zone radiata causes ovarian development in female fish (Wallace, 1985; Celius and Walther, 1998; Sharma and Bhat, 2014; Mohamedien et al. 2023).

During oogenesis, lipids stored in various tissues such as the liver, muscle, and intraperitoneal fat are mobilized and utilized to support the growth and maturation of oocytes. Lipids serve as important energy reserves and structural components necessary for the successful development and maturation of gametes (eggs and sperm) (Abdel-Aziz and El-Nady, 1993; Sargent, 1995; Luzzana *et al*., 1996; Bhat *et al*., 2022; Abduh *et al*., 2021). During the process of reproduction, a higher quantity of energy is expended, and the lipid storage in the liver and muscles is mobilized and transferred to the gonad during fish maturation and spawning (Zaboukas *et al*., 2006; Sutharshiny and Sivashanthini, 2011; Singh *et al*., 2012). Reproduction in fish relies heavily on lipids as the main energy source. Female fish require substantial amounts of lipids for the formation and expansion of eggs during reproduction (Goda *et al*., 2007; Ebrahimnezhadarabi *et al*., 2011; Leng *et al*., 2019; Zhu *et al*., 2020). The arrangement of fish reproductive organs based on their fatty acid composition is an essential characteristic for successful fish reproduction (Tocher, 2010; Anido et al., 2015; Dhurmeea *et al*., 2018). Oocytes often preferentially transport long-chain, polyunsaturated, and unsaturated fatty acids (PUFA and HUFA, respectively) instead of saturated and monounsaturated fatty acids, which are more commonly used for catabolic activity (Henderson, 1996; Tocher, 2010). Lipid deposition, particularly fatty acids, is crucial for reproduction at all stages of fish development due to its vital function in fish metabolism (Ghaedi *et al*., 2016; Huang *et al*., 2010; Ng and Wang, 2011; Dhurmeea *et al*., 2018). The process of gonadal maturation, the quality of eggs, and the development of larvae are all impacted by polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA) (Izquierdo *et al*., 2001; Anido *et al*., 2015). Different sex steroid hormones coordinate and govern reproduction in teleosts. Hormones, including testosterone, 11-ketotestosterone, 17-estradiol, and progesterone, are involved in the reproductive process (Kime, 1993; Barannikova *et al*., 2002; Barannikova *et al*., 2004; Estay *et al*., 2012). Fish reproduction (gonad maturation) is regulated and guided by the brain-pituitary-gonadal axis. Photoperiod, water temperature, eating, and rainfall are all essential elements that cause the brain to secrete hormone-releasing gonadotropin (Rottman *et al*., 1991; Zohar *et al*., 2010). Steroid hormones are said to have a direct function in fish ovarian development (Chen *et al*., 2021). While the highest level of 17-estradiol (E2) is seen during vitellogenesis (Chen *et al*., 2021), the highest level of progesterone is observed during ovulation or spawning. Researchers have found a link between changes in plasma levels of steroids and oocyte development in a variety of teleosts, including salmon, Asian stinging catfish (*Heteropneustes fossilis*), goldeye (*Hiodon alosoides*) and yellow pike (*Stizostedion vitrum*) (Whitehead *et al*., 1983; Lamba *et al*., 1983; Pankhurst *et al*., 1986; Truscott *et al*., 1986; Malison *et al*., 1994).

In recent years, there have been advancements in teleost biological knowledge, including the creation of genomic techniques. In teleost fish, gametogenesis is mediated by pituitary gonadotropins, which cause steroid formation in the gonads. The majority of large-scale genomic investigations were conducted on zebrafish. Understanding the physiological processes controlling ovarian development requires knowledge of how steroidogenic proteins (steroidogenic enzymes and *Star*) are regulated at the molecular level in the ovarian steroidogenic pathway. In fishes, *StAR* mRNA levels in follicles significantly increased during final oocyte maturation and ovulation, indicating that increased *StAR* gene expression is necessary for the pre-maturational surge in *17,20β-P* (Kusakabe *et al*., 2002). Similar to star, higher *3-hsd* gene expression is related to the follicle's ability to produce a significant amount of *17,20β-P* in a short amount of time during maturation and ovulation (Kumar *et al*., 2000). Average expression patterns of *cyp11a*, *cyp17a1*, *hsd3b*, and *hsd17b1* in eel ovaries showed a consistent rise in correlation with gonad size (Matsubara *et al*., 2019).

Rainbow trout is indeed an exotic fish species known for thriving in cold-water environments. This makes it well-suited for regions like Jammu and Kashmir, which have suitable climatic conditions for cold-water fish farming. Currently, the suboptimal egg quality, reduced fertilization rate, and decreased hatching rate of cultured female rainbow trout are still impeding the advancement of cold-water fish farming in Jammu and Kashmir. In order to examine the attributes of hormonal alterations, fatty acid composition, and sex-related genes throughout the development of female fish gonads, and to comprehend the underlying mechanism of its endocrine control, it is crucial to gather sufficient data on rainbow trout reproduction. The results of this research are expected to be significant for the healthy growth of rainbow trout (*O. mykiss*) farming in Kashmir. The data obtained will serve as a reference for understanding and improving the reproduction of rainbow trout in the region, ultimately aiding in the development of sustainable and successful aquaculture practices.

Materials and Methods

Collection of Fish Samples

The current study obtained 80 samples of sexually mature female rainbow trout weighing around 500 ± 50 g from Verinag hatchery in Jammu and Kashmir. These fish were first-time spawners and were in various breeding phases. Fish specimens in various reproductive stages were sampled then the fish were euthanized with the dosage of clove oil (0.20 mL of clove oil per 500 mL of water). Subsequently, they were measured, weighed, and dissected, and the gonads were extracted. Immediately following dissection, the recently obtained samples were sent to the Department of Zoology, Kurukshetra University, Kurukshetra, where they underwent analysis for a variety of biological tests.

Ultrastructure Studies

Transverse sections of fish gonads were obtained for the study. The tissue sections were fixed for 24 hours in a solution containing 2.5 percent glutaraldehyde in 0.2M phosphate buffer saline at a pH of 7.2. This step is crucial for preserving the cellular structure and preventing degradation of the sample. Glutaraldehyde is a common fixative that cross-links proteins and stabilizes cellular structures (Zamboni, 1976). After this sample preparation, the tissues were usually dehydrated through a series of alcohol washes and then embedded in a resin, such as epoxy resin, which is subsequently polymerized (Luft, 1961). Once the resin blocks were prepared, ultrathin sections (around 50-70 nanometers thick) were cut using an ultramicrotome and mounted on TEM grids. Finally, these ultrathin sections were then mounted on 100 mesh grids and stained with uranyl acetate and lead citrate (Reynolds, 1963). Sections were photographed under M-10 Phillips.

Total Lipid Extraction

Lipids were isolated from the ovaries (gonad, 5 gm) using the method of (Bligh and Dyer, 1959). A solution containing dichloromethane, methanol, and water at a ratio of 10:20:7.5 ml was added to the tissue. The mixture was shaken periodically and then allowed to sit overnight in a separator funnel. The addition of 10 ml of dichloromethane and salty water (9 g sodium chloride L^{-1}) resulted in the separation of the mixture into two distinct phases. The extract was afterwards acquired using a rotary evaporator from the lower dichloromethane procedure and transferred into pre-weighed glass vials.

Fatty Acid Analysis

Methyl ester fatty acids (FAMEs) were derived from total lipids using acid-catalyzed transesterification (Christie and Han, 2012). The analysis of fatty acids was conducted using gas chromatograph mass spectrometry (GC-MS). An analysis of the GC-MS was conducted using a gas chromatograph equipped with a mass detector and a capillary column measuring 30m in length, with a diameter of 0.25 mm and a particle size of 0.25 μm. A total of 0.5 milliliters was extracted and subsequently evaporated, resulting in a final volume of 0.5 microliters. The injection temperature is set to 280°C, the quadruple temperature was set to 150°C, the flow rate of helium was set to 1.5 ml/min, and the temperature of the ion source was at 230°C. The injection was executed using a 1 µL volume in the splitless mode. The apparatus was initially heated to a temperature of 90°C and maintained at that temperature for a duration of 5 minutes. The temperature was thereafter increased at a rate of 10 degrees Celsius per minute. The chemicals in tissue samples were subjected to electron ionization at 70 eV to get their mass spectrum. The detector was set to run in scan mode (AMU) from 60 to 800 atomic mass units. The identification relied on comparing the chemical structure and mass spectrum data with reference compounds in the NIST database.

Hormonal Analysis

The analysis of sex steroid hormones was conducted using commercially available ELISA kits from Cayman Chemical Company, USA. The determination of sex steroids was conducted using the techniques and methods specified in the assay kit published in references (Cuisset *et al*., 1994; Nash *et al*., 2000). For the purpose of hormone extraction, a volume of 50 µl of plasma was utilized from each sample. The plasma underwent extraction using diethyl ether, ethyl acetate/hexane (50:50), and methylene chloride in order to isolate progesterone (17,20-dihydroxy-4-pregnen-3-one, 17,20-DP) and estrogen (17-Estradiol). The organic extracts were dried using a nitrogen stream gas for evaporation. The pellets generated were dissolved in 0.5 ml of ELISA buffer. Standards for the analysis of 17,20-DP and 17b-estradiol were developed. The plate was prepared and samples, together with the reference absorbance, were measured at a wavelength of 412 nm using a microplate reader.

Gene Expression and Analysis by RT qPCR

RNA extraction and cDNA synthesis

The preserved ovarian fragments (5 fish ovarian samples from each developmental stage) were removed from the RNA Later solution. To do this, they were typically blotted on Whatman #1 filter paper. Blotting can help remove excess liquid and prepare the tissue for further processing. For follicle wall-enriched tissues, prior to applying Tri Reagent, 500 µl of processed tissue was briefly homogenized with a pestle. Extractions were done using normal protocols for phenol/chloroform.

Total RNA and subsequent synthesis of first-strand cDNAs was carried out by following the method described by (Matsubara et al., 2003) For the intact ovarian fragment comparison, total RNA (5 µg) was pooled from 12 fish samples in different breeding stages viz. early development, vitellogenin and mature fish ovary as reported by ultrastructural analysis. However, prior work in coho salmon has shown that the findings of quantitative PCR produced when utilizing mRNA as the template and normalizing to reference genes best reflect transcript levels on a per-follicle basis.

Real-Time Quantitative PCR (QPCR)

Real-time quantitative PCR was set up for the amplification of target and reference genes with Fast Start DNA Master SYBR Green I mix (Bio-Rad, USA) on Real Time Thermocycler (Biorad CFX96™, C1000™). The selected genes, their sequence of primers, and the length of gene fragments amplified are shown in Table 1. The thermal cycling temperature was set: 15 min at 95°C for denaturation, followed by 40 cycles at 95°C for 15-s for denaturation, and then at 60°C for 30 s for an annealing-elongation phase. In order to validate amplicon specificity, a melting curve study was conducted after amplification. All of the samples were run in replicates. By creating standard curves for all the genes studied, the efficiency of primers was verified. For confirmation of amplicon specificity, the dissociation curve was used. The relative levels of expression of each sample were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relative gene expression was expressed in terms of fold change. Data were calibrated using the geometric mean of elongation factor 1 alpha (eef1a), which remained constant throughout the main stages of oogenesis in salmon and also reported in tilapia. Elongation factor 1 alpha is a highly conserved protein involved in protein synthesis, and its expression levels are often relatively stable across different developmental stages, the reliability and interpretability of gene expression data, contributing to our understanding of the molecular basis of reproductive development in rainbow trout ovary

Statistical Analysis

The data were analyzed using SPSS software version 16 (SPSS Inc., Chicago, IL) and GraphPad Prism (version 5.01). The statistical errors were represented as the mean value plus or minus the standard error of the mean (SEM). Mean variation was assessed using one-way analysis of variance (ANOVA), followed by Duncan's New Multiple Range Test (DMRT). The Tukey test was employed to examine the mean gonad fatty-acid composition across different phases of maturation. The statistical significance level is $P<0.05$.

Results

During the present investigation on ultrastructural, hormonal, free fatty acid composition and gene expression in the ovaries of rainbow trout (*O. mykiss*) were analyzed.

Ultrastructural Analysis of Rainbow Trout Ovary

Ultrastructural analysis of perinucleolar stage oocytes revealed the presence of numerous spherical bodies of varied sizes. The small sized nucleolini were observed adjacent to nuclear membrane, whereas the large sized were extruded out into the cytoplasm and were unevenly distributed in perinuclear zone. The ooplasm appeared less electron dense heterogeneous granular structure with numerous cell organelles like mitochondria and Golgi lying within (Figure 1a). Ultrastructural analysis revealed that in rainbow trout ovary a large number of cortical alveoli were appeared throughout the oocyte. The alveolus appeared as big organelles throughout the cytoplasm. Few electron dense mitochondria, endoplasmic reticulum and Golgi bodies were distributed throughout the oocyte (Figure 1b). Electron microscopic analysis revealed that the oocyte in vitellogenesis stage attained maximum growth and development and it was due to the accumulation of yolk protein. Female specific protein, vitellogenin was synthesized during this stage. Due to the accumulation of yolk proteins and cortical alveoli the size of the oocyte increased as compared to the earlier stages (Figure 1c). During the maturation stage the yolk globules continued to coalesce and lost their peripheral attachments and thus were observed freely in the oocyte. During this stage the oocyte became denser as lipid and yolk globules coalesced (Figure 1d). In zona pellucida the pore canals contained microvilli and were perpendicular to its surface. Initial formation of the zona pellucida occurred at the early stage of cortical alveoli

Table 1: List of primers and product size of selected genes for sequencing

Gene	Primers	Product Size
Elongation factor-1α (ef1a)	CCCCTGGACACAGAGATTTCATC AGAGTCACACCGTTGGCGTTAC	473 bp
Star	F: GAATGCGATGGTGGCCATTC R: ACCTTGTCTCCATTCGCCTG	563 bp
cyp11a1	F: TCTTCCAACGTTCCAGTCGG R: ACGCTCCCCATACAACACAG	528 bp
cyp17a1	F: GGAGGAACTGGACAGTGTGG R: ATCTCCATCTTAGCCAGCGC	580 bp
hsd3b	F: TGCTCTGTGTGCTCAGATGG R: CAGCTCTCCATTGGCCTGAA	509 bp

stage, with the deposition of a homogenous layer which was designated as zona pellucida interna (ZP1). As the cortical alveoli stage advanced the zona pellucida formed a trilaminar appearance, which was denoted by zona pellucida interna (ZP1), zona pellucida middle layer (ZP2) and zona pellucida externa (ZP3) in the vitellogenic stage (Figure 1e). Under electron microscopic analysis, postovulatory follicles were the prominent structures in the rainbow trout ovary. Postovulatory follicles showed irregular shape in the nucleus with many folds and were with heterogenous electron density (Figure 1f).

Biochemical Changes in Rainbow Trout Ovary

Progesterone (17,20β-dihydroxy-4-pregnen-3-one, 17α,20β-DP)

Progesterone (17α,20β-DP) estimation was found to be maximum during the months from November to January which was the mature stage of the fish. The 17α, 20β-DP level calculated was 0.645±0.17 ng/ml (Figure 2). Thereafter, it declined in the spent stage, which lasted from February to mid-March. The calculated value of 17α,20β-DP during spent phase was 0.15±0.09 ng/ml (Figure 2). After spent stage, fish entered into immature stage and level of 17α,20β-DP increased to 0.224±0.20 ng/ml. The 17α,20β-DP level increased dramatically from immature stage to maturing stage which lasted from June to September. The level of 17α,20β-DP was 0.517±0.16 ng/ml (Figure 2). A significant positive relationship was found between progesterone content and different developmental stages of fish (P<0.05).

Estrogen (17β-Estradiol)

The serum 17β-Estradiol levels exhibited seasonal changes and remained low during the immature stage which started from mid-March to May. The 17β-Estradiol level during immature stage was 0.272±0.023 ng/ml (Figure 3). Soon after immature stage fish entered into maturing stage and estrogen level increased dramatically in this stage. The peak level of 17β-Estradiol was during June to September. The calculated 17β-Estradiol level was 0.825±0.023 ng/ml (Figure 3). During the mature stage the 17β-Estradiol level began to fall as compared to maturing stage. The 17β-Estradiol level was 0.795±0.021 ng/ml (Figure 3). The spent stage lasted from mid-February to March and the 17β-Estradiol level fall dramatically as compared to previous stages. The 17β-Estradiol levels obtained was 0.171±0.07 ng/ml (Figure 3). A significant positive relationship was found between 17β-Estradiol content and different developmental stages of fish (P<0.05). Several peaks of 17,20β-dihydroxy-4-pregnen-3-one and 17β-Estradiol levels were recorded during the annual reproductive cycle (P<0.05). During different reproductive stages progesterone and estrogen were positively correlated with each other.

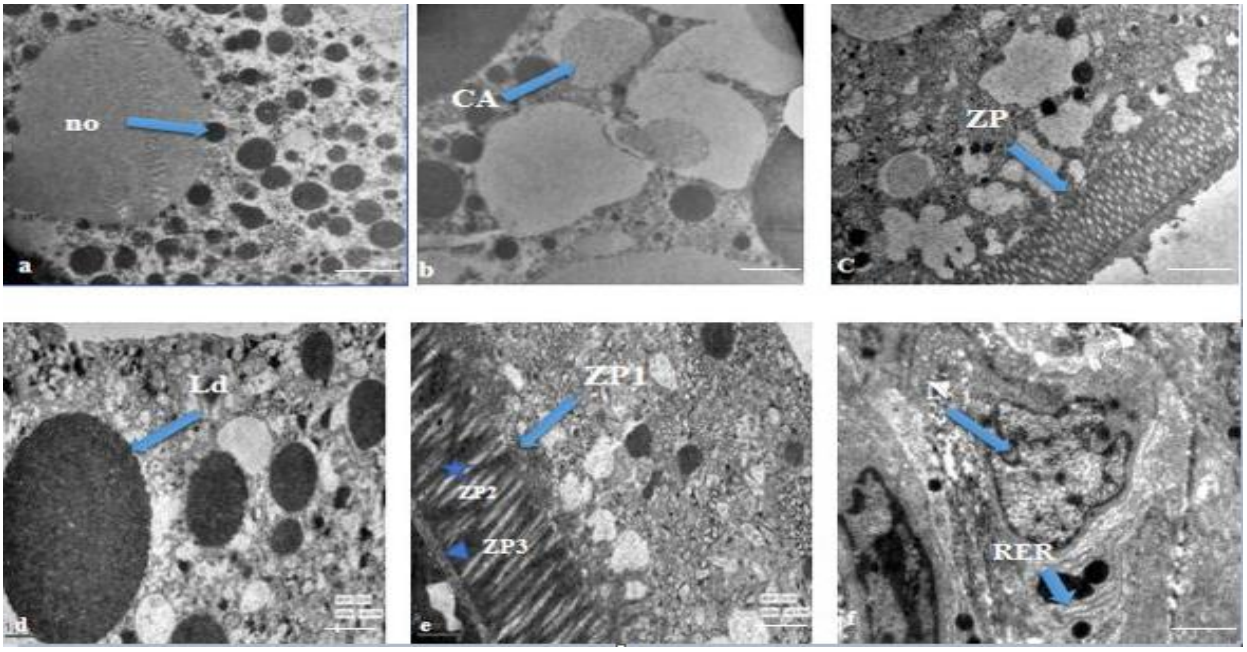

Figure 1. Transmission electron micrograph showing follicle **(a)** at perinucleolus stage. Note the presence of nucleolus (no) of various sizes surrounding the nucleus, **(b)** cortical alveolus stage (CA) with a number of lipid droplets and mitochondria, **(c)** vitellogenic stage with a number of cortical alveoli, and thick developed zona pellucida (ZP) with microvilli and pores, **(d)** maturation stage with a number of large sized lipid droplets (Ld), **(e)** maturation stage with mitochondria, and glycogen bodies and thick developed zona pellucida interna (ZP1), Zona pellucida middle layer (ZP2) and zona pellucida externa (ZP3) with microvilli and pores, **(f)** oocyte in postovulatory follicles stage showing large indented nucleus (N), condensation of nuclear membrane, electron dense nucleolus, abundant RER, mitochondria, thin layer of envelope (scale bar 10 μm)

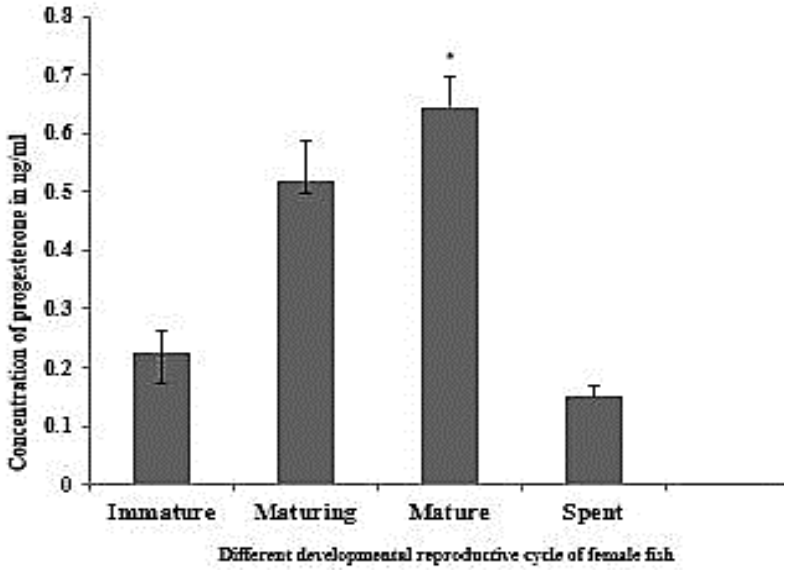

Figure 2. Plasma levels of progesterone in female rainbow trout (*O. mykiss*) at different developmental stages. Each bar represents the Mean ± SEM (n=20 per reproductive stage) with P<0.05.

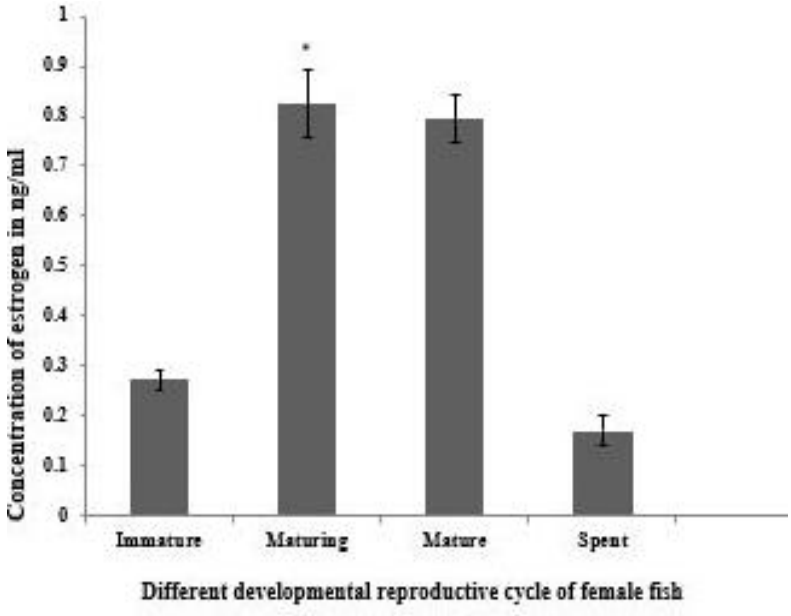

Figure 3. Plasma levels of estrogen in female rainbow trout (*O. mykiss*) at different developmental stages. Each bar represents the Mean ± SEM (n = 20 per reproductive stage) with P<0.05.

Ovarian Fatty Acid Composition

The total lipid level changed according to gonad development. During immature stage 18.41 (% in dry weight) was reported while in maturing and mature stage, lipid content level was 21.43 and 28.92 (% in dry weight) respectively. A significant increase in lipid content was found in mature stage of the fish. The fatty acid composition of rainbow trout ovary is presented in Table 2, 21 different fatty acids were reported viz C14-C22:6. On average in the mature stage of the ovary saturated fatty acids constitute 27.6±3.4, monounsaturated fatty acids constitute 17.2±4 while polyunsaturated fatty acids compose of 46±8.3. Gonad fatty acid composition varied within each maturity stage. Compared to the maturation and mature stage, the concentrations of total saturated fatty acids (∑SFA) in gonads were lower in the immature stage, which is mostly due to differences in C16, C17 and C18, which make up the maturity of fatty acids during different stages of ovarian development. But ∑SFA ovary did not change significantly during development (P>0.05) (Table 2).

As compared to immature and maturing gonads, total monounsaturated fatty acids (∑MUFA) were also substantially higher in mature gonads. The most substantial monounsaturated fatty acids found in higher concentrations were C16:1 and C18:1. However ∑MUFA in ovary did not change significantly during development (P>0.05) (Table 2). There was a significant

Table 2. Fatty acid profiles (mg/g) in the gonads of *O. mykiss* during different developmental stages of the fish. Different letters within the same row represent significant differences, with a significance level of (P<0.05)

Fatty acid class	Immature stage	Maturing stage	Mature stage
Number of samples	**17**	**18**	**18**
14:0	0.4±0.1	1.2±0.29	1.3±0.34
15:0	0.6±0.2	1.1±0.3	`1.5±0.45
16:0	14.2±0.91	15.3±1.23	16.6±1.46
17:0	0.8±0.2	1.2±0.31	1.4±0.24
18:0	5.9±0.49	6.2±1.2	6.6±0.93
∑SFA	**22.2±1.9[a]**	**25.2±3.32[a]**	**27.6±3.4[a]**
14:1	0.2±0.03	0.4±0.03	0.6±0.2
15:1	0.3±0.02	0.5±0.1	0.8±0.3
16:1	3.4±0.4	4.1±0.5	5.6±1.3
17:1	0.5±0.03	0.5±0.1	1.2±0.3
18:1	4.7±0.5	5.3±1.2	6.8±1.2
20:1	0.8±0.3	1.1±0.3	1.2±0.4
22:1	0.6±0.1	0.7±0.2	0.9±0.3
∑MUFA	**10.6±1.35[a]**	**12.8±2.4[a]**	**17.2±4[a]**
18:2	1.2±0.2	1.4±0.5	2.1±0.5
18:3	0.3±0.02	0.6±0.1	0.9±0.2
20:2	1.1±0.4	1.7±0.6	2.2±0.7
20:3	0.2±0.01	0.3±0.01	0.6±0.02
20:4 (AA)	3.2±0.4	4.6±0.6	5.8±1.2
20:5 (EPA)	4.4±0.8	6.3±0.9	8.4±1.2
22:5	1.5±0.2	2.3±0.7	3.2±0.7
22:6 (DHA)	13.5±1.8	17.4±2.4	22.6±3.6
∑PUFA	**25.5±3.9[a]**	**34.9±5.9[a]**	**46±8.3[b]**
DHA/EPA	3.06±1.5[a]	2.8±1.1[a]	2.7±1.6[a]
DHA/AA	4.2±1.2[a]	3.8±0.6[a]	3.9±0.7[a]

difference (P<0.05) in total ∑SFA and ∑MUFA during different developmental stages. Polyunsaturated fatty acids constitute major %age of fatty acids throughout ovarian growth. ∑PUFA in ovary revealed a significant increase during development (P<0.05) (Table 2) Among PUFA, DHA constitutes about 45% of total Polyunsaturated fatty acids. It increases significantly during the mature stage of the ovary (P<0.05). The other important Polyunsaturated fatty acids which were higher in the mature stage of the ovary were Arachidonic acid (20:4, AA) and Eicosapentaenoic acid (20:5, EPA). The ratio of DHA/EPA and DHA/AA was higher in the immature stage as compared to the maturation and mature stages, while the ratio of EPA/AA was significantly similar in all stages.

Gene Expression Analysis

During the perinucleus (PN) and cortical alveoli (CA) stage, analysis of mRNA in rainbow trout ovarian follicles has shown lower but detectable *star* mRNA levels. *Star* mRNA levels increased during the vitelligenic stage (VIT) and were higher in mature stage (MAT) (Figure 4). The relative gonadal size was expressed by ovarian *cyp11a* mRNA levels. The ovarian *cyp11a1* transcript in rainbow trout was found to be very minimal during the PN and CA stage and then increased in VIT stage, thereafter it showed a 3-fold increase in MAT stage of the ovary (Figure 4). Regarding *cyp17a1* expression, the peak expression was detected in the MAT stage, it begins to increase from PN and CA to MAT stage significantly. Like other genes, increased *3ß-hsd* gene expression was observed around the time of MAT with a minimum level during the PN and CA (Figure 4). The expression level of all the genes were lower during the PN and CA stage.

Discussion

During the present study, ultrastructural analysis of perinucleolar stage oocytes revealed the presence of numerous spherical bodies of varied sizes. The nucleus was massive and had a uniform electron density. Thiry and Poncin (2005) reported an increase in the number of nucleoli in *Barbus barbus*. This rise suggests the activation of protein synthesis as oogenesis or ovary development progresses. According to Rao *et al*., (2009) during the peri-vitelliogenic stage of *Epinephelus diacanthus*, the nucleus grew in size and the number of nucleoli around the nucleus rose significantly. Earlier electron microscopic investigations established that cortical alveoli have an electron dense core and a homogeneous electron-lucent content during the oocyte's alveoli stage (Inoue *et al*., 1987; Ohta *et al*., 1990). In terms of fine morphology, the majority of teleosts shared the structure and function of several cytoplasmic organelles and their arrangement Ohta *et al*., 1990).

During the vitelliogenic stage, a trilaminar appearance of thick zona pellucida, identified by ZP1,

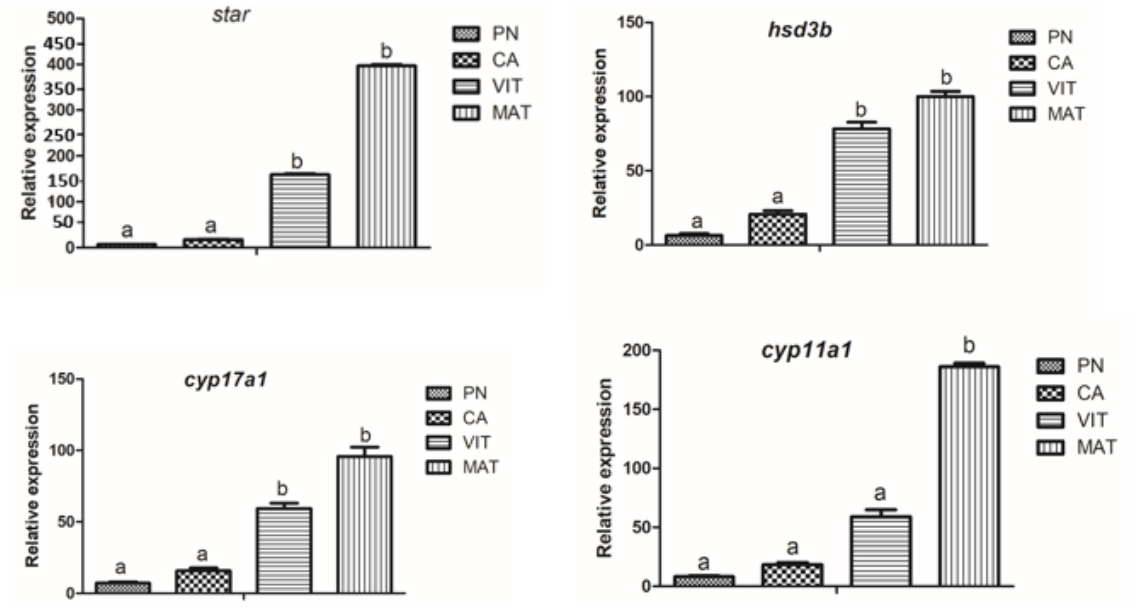

Figure 4. Expression profiles of genes linked to steroidogenesis during the various developmental processes of the ovary of the *O. mykiss.* The amounts of messenger RNA were measured by qPCR and the data was normalized to ef1a. (PN) represents perinucleus, (CA) represents cortical alveoli, VIT represents (vitelliogenesis) and (MAT) represents mature stage. Each bar represents the Mean + SEM of 5 fish per stage. Different letters represent significant differences, with a significance level of ($P<0.05$).

ZP2, and ZP3 was recorded. Mohamedien et al. (2023) have also reported an increase in the thickness of zona pellucida in zebra fish ovary which are in the agreement of the present results. Additionally, Gopalakrishnan (1991) recorded an increase in the quantity of yolk globules in the cytoplasm, rough endoplasmic reticulum, and thick zona radiata of grey mullet (*Mugil cephalus*) in vitelligenic oocytes. Initially, the yolk granules developed in the core of the oocyte; however, as the oocyte matured, these yolk globules aggregated and traveled to the oocyte's periphery (Abdalla and Cruz-Landim, 2003). Rao *et al*. (2009) reported that during the vitelliogenic stage of spinycheek grouper (*Epinephelus diacanthus*), yolk globules comprised the majority of the cytoplasm and oocytes had a dense aggregation of mitochondria around the zona radiata. Fine morphological investigation indicated that the oocyte grew in size throughout the maturation period. At the mature stage, the yolk globules consolidate and are seen freely within the egg. Lipid droplets combine to create yolk granules of various sizes; these yolk globules are spread throughout the ooplasm. Abascal and Medina (2005) reported comparable alterations in Atlantic bluefin tuna (*Thunnus thynnus*) throughout the mature stage. Hydration during final maturation resulted in a significant increase in oocyte volume.

In the female ovary of rainbow trout, polyunsaturated fatty acids (PUFAs) constituted the bulk of the fatty acid reservoir, followed by saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). PUFAs act as structural factors in the formation of bio-membranes in developing oocytes (Mourente *et al*., 2002; Sargent, 1995). Both DHA and EPA levels were considerably greater in mature blue tuna ovaries (Ortega and Mourente, 2010), DHA also showed increased trend in Cobia fish during mature stage (Asmanik *et al*., 2020) which is consistent with the current findings. Fatty acid composition in the gonad tissues of rainbow trout has been reported to change during different developmental stages of ovary and testis (Yıldız *et al*., 2020; Bhat *et al*., 2022) which is in agreement with the results of the present study. For ovarian dentex (*Dentex dentex*), the predominant polyunsaturated fatty acids (PUFA) were docosahexaenoic (DHA, 22:6 n-3) and eicosapentaenoic (EPA, 20:5 n-3), whereas the predominant n-6 (PUFA) acids were linoleic (18:2 n-6) and arachidonic acids (20:4 n-6) acids (Ismail *et al*., 2016). The fatty acids 16:0 and 18:1 are generally catabolic in nature and are used for energy needs (Ostaszewska, 2005). All of these fatty acids were consumed in high concentrations throughout fish growth and development and were rapidly catabolized by mitochondria (Henderson, 1996; Asmanik *et al*., 2020). Thus, the high values for C16:0 and C18:1 in the ovarian sample show the energy metabolism required for gonad development. C16:0 and C18:0 were the most prevalent saturated fatty acids in fish ovaries. Certain fatty acids are kept, processed, and used preferentially in fish gonads (Ng and Wang, 2011). The results obtained in a present study regarding fatty acid composition changes in gonad tissues of rainbow trout are in line with findings reported in previous studies involving Indian fish species (Jakhar *et al*., 2012). Furthermore, Varljen *et al*. (2003) demonstrated that these fatty acids are required for two banded sea bream (*Diplodus vulgaris*).

Estrogen, progesterone, and testosterone all play a critical role in gonadal development (Lubzens *et al*., 2010; Mylonas *et al*., 2010; Soranganba and Singh, 2019). Numerous studies have examined seasonal

variations in the profiles of steroid hormones and gonad development in a variety of fish species (Scott *et al*., 1984; Matsuyama *et al*., 1988; Soranganba and Singh, 2019). 17α,20β-DP levels were found to be highest during the mature stage, which corresponds to the gonadosomatic index. Numerous researchers have demonstrated that progesterone is responsible for the oocyte's ultimate maturation in fish (Kagawa *et al*., 1981). Progesterone levels have been observed to rise throughout the spawning or mature stage (Kagawa *et al*., 1981, Zohar and Billard, 1984). Scott *et al*. (1984) revealed that the maximum quantity of progesterone produced by rainbow trout during ovulation. 17,20-dihydroxy-4-pregnen-3-one has been found as a maturation-inducing steroid in a variety of fish species during oocyte maturation (Petrino *et al*., 1993). Hobby *et al*. (2000) revealed that progesterone levels were highest in female fish during maturation and ovulation. Alvarado *et al*. (2015) reported that plasma progesterone levels in Southern hake (*Merluccius australis*) reached a maximum at the mature stage. In male rainbow trout, Bhat et al., (2022) have reported highest levels of testosterone during mature stage of rainbow trout, testosterone and progesterone both are reported to be important for gonadal development viz testis and ovary respectively.

The 17β-Estradiol hormone was shown to fluctuate throughout the various developmental stages of fish in the present study. In some fishes, a rise in estradiol during the vitelliogenic stage and a drop during the maturation stage was recorded (Kagawa *et al*., 1984; Sakai *et al*., 1988). 17β-Estradiol levels have been reported to peak during the vitelliogenesis stage (Chen et al., 2021) and then decline during the ovulation stage in a variety of fish, including whitespotted char (*Salvelinus leucomaenis*) (Kagawa *et al*., 1981), *Acheilognathus rhombea* (Shimizu *et al*., 1985), grey mullet (*Mugil cephalus*) (Tamaru *et al*., 1991), Japanese pilchard (*Sardinops melanostictus*) (Cisneros, 2007). Similar findings were reported in present investigations. Unal *et al*. (2005) recorded the highest level of estrogen in pearl mullet (*Chalcalburnus tarichi*) during the vitelliogenic stage. Both gonadosomatic index and estrogen levels in blood plasma rose as the oocyte matured, confirming the significance of estrogen in vitelliogenesis and oocyte maturation in teleost (Patino and Sullivan, 2002; Adebiyi *et al*., 2013).

As shown by the increase in *star* mRNA, the increase in estradiol levels recorded may be a result of the increased availability of cholesterol, a precursor to steroids. Similar increases in plasma estradiol levels have been seen in investigations in common carp (Mandich et al., 2007), goldfish (Hatef *et al*., 2012), and zebrafish (Fang *et al*., 2016). As with *star*, increased *3-hsd* gene expression is connected with the follicle's capacity to produce considerable amounts of *17,20-P* in a very short period of time during maturation and ovulation. By contrast, *3-hsd* mRNA levels remained largely consistent in catfish ovarian follicles during ovarian recrudescence, vitellogenesis, and maturity (Kumar *et al*., 2000). Steroidogenesis-related transcript levels for the *star*, *cyp11a1*, *cyp17a1*, and *hsd3b* were found to be lowest at the PN and CA stage and highest during the mature stage. These patterns did not correspond to the complicated alterations seen during oogenesis in other salmonids (Nakamura *et al*., 2005; Kusakabe *et al*., 2002) *Cyp11a, cyp17a1, hsd3b, and hsd17b1* average expression patterns in the ovaries of eels exhibited a steady increase in correlation with the size of gonads (Matsubara *et al*., 2019).

On the basis of mRNA levels, previously, it was found that advanced phases of oogenesis in Japanese eels resulted in a significant rise in ovarian *cyp19a* expression (Ijiri *et al*., 2003). Increased expression of *cyp17a1* has been seen in channel catfish during early gonadal recrudescence and early vitellogenesis. However, after vitellogenesis was complete, transcript levels quickly fell (Kumar *et al*., 2000). Similarly, *cyp17a1* transcript levels were much greater during the 'developing' stage of oogenesis in Atlantic croaker ovaries (*Micropogonias undulatus*) than during the spawning stage (Nunez and Applebaum, 2006). *Cyp17a1* mRNA levels continued to grow throughout vitellogenesis in the Japanese eel, *Anguilla japonica* (Matsubara *et al*., 2003). The present research found similar findings for *cyp17a1* in rainbow trout ovaries. The increase in all steroidogenic gene transcripts seen throughout the mature stage implies a broad shift in steroidogenic activity.

Conclusions

In conclusion, the present study confirmed that female rainbow trout follow the general pattern related to the structure of the ovary. The hormonal profile results generated are indeed expected to be used as important initial information for the induced breeding of rainbow trout from Indian water. The progressive rise in fatty acid content as the fish matures is a unique observation in this species. The expression patterns of genes involved in steroidogenesis, namely *star*, *cyp11a1*, *cyp17a1*, and *hsd3b*, were significantly increased throughout the mature stage, suggesting their important role in oocyte development. The study's results are anticipated to provide valuable information about the timing of spawning in rainbow trout. This information is essential for aquaculture production and management, as it can help optimize breeding practices and maximize reproductive success.

Ethical Statement

According to the rules set by the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA) in 2007, ethical permission is not necessary for conducting experiments on fish. The experiments were done according to precise protocols. Furthermore, all procedures involving fish in the study

were conducted in accordance with European Union Directive no: 2010/63.

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Author Contribution

Conceptualization, R.A.B. and O.S.K methodology, R.A.B.; software, F.F., O.S.K; validation, R.A.B., J.K, F.F. formal analysis, R.A.B., F.F., investigation, R.A.B. resources, R.A.B, and F.F.; data curation, R.A.B.; writing original draft preparation, R.A.B., J.K; writing review and editing, R.A.B., F.F., V.P., O.S.K; C.G; G.P. visualization, F.F., O.S.K; supervision, R.A.B., F.F. project administration R.A.B., F.F. and O.S.K. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The author(s) declare 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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