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Evaluation of Gonadal Gene Expression in Himalayan Snow Trout *Schizothorax plagiostomus* during Annual Reproductive Cycle

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Abstract

The snow-trout *Schizothorax* is a prevalent food fish found in the cold water bodies of Hindukush- Karakoram- Himalayan mountains/foothills and is one of the major sources of animal protein. During the past decade, there has been a rapid fall in the *Schizothoracine* population due to climate change with unrestricted urbanization and other anthropogenic interventions. Therefore, an in-depth understanding of the annual gonadal development in these fish taxa becomes critically essential for designing appropriate/suitable conservation strategies. Like most freshwater teleosts, hill-stream fishes are typical seasonal breeders. In the current study, the season-dependent developmental gene expression was examined in adult *S. plagiostomus* gonads (both testes and ovaries) obtained from a natural habitat at Garhwal, Uttarakhand, India. A total of six putative genes (three testicular and three ovarian) were investigated in three different developmental stages, keeping the *β-actin* gene as the endogenous reference. Our data suggested that *Dmrt1* and *Gdnfr-α* mRNAs were highly elevated during the pre-spawning stage, gradually down-regulated during the spawning stage, and almost undetectable at the post-spawning stage. However, in contrast, *Fgf11* was found to be augmented during the spawning stage. On the contrary, $Foxl_2$ and *Runx1* transcripts were elevated during pre-spawning and spawning stages compared to those found during post-spawning stages. Finally, *Gdf9* mRNA was found to be maximal at the pre-spawning stage and subsequently diminished during the spawning stages. This is the first comparative gonadal transcriptomic data showing the season-dependent developmental gene expressions critically regulating the optimal gametogenic drive/output in any Schizothoracine fish. This study provides fundamental information regarding the gonadal biology of *Schizothorax*, which will be supportive of designing suitable artificial breeding/conservation programs for this species in the future.

Keywords: Gene Expression, Gonads, Ovary, Schizothorax, Testis

1. Introduction

The fishes, commercially valuable and developmentally diverse taxa[1,2] contribute at least 15% of the overall animal protein consumption per person globally[3]. However, during the recent past, a severe decline in the freshwater fish population has been recorded globally due to uncontrolled anthropogenic interferences[4]. Therefore, there has been an urgent need to develop new breeding and/or conservation strategies to cater to the increasing market demand for the products, maintaining the ecological balance intact.

Schizothorax, generally reported as snow trout/ minnows (order Cypriniformes, family Cyprinidae, subfamily Schizothoracinae), are found in the cold-water bodies of Hindukush-Karakoram-Himalayan mountains/ foothills (at the elevation upto 3000 meters above the sea level) and recognized as a major source of animal protein covering more than 2400 km of Central Asia (ranging from Iran, Kazakhstan, Tajikistan, Uzbekistan, Afghanistan,

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Pakistan) and Indo-(Kashmir, Ladakh, Himachal, Uttarakhand, Sikkim, Assam and Arunachal Pradesh) -Qinghai-Tibetan plateau to Nepal, Bhutan, China up-to Myanmar[5-7]. For the sub-family Schizothoracinae, almost 100 species are reported under 15 genera[8]. However, despite having such a broad distribution pattern with significant economic importance, *Schizothorax* remains a highly neglected Himalayan ichthyo-fauna. Furthermore, an alarming decline has been observed in this fish population due to a significant rise in anthropogenic activities (e.g. -habitat destruction, dam and hydroelectric power projects, road/railway construction/ expansion, water pollution, etc) and climatic change[9]. Therefore to restore this species, it is important to study the cellular and molecular basis of the breeding biology of Schizothoracine fishes. It is to be anticipated that fish spawning patterns vary depending on the geographical region and biological habitat[10]. Among hill-stream teleosts like golden mahasheer *Tor putitora,* extended day length and elevated temperatures are considered the most critical factors for gonadal maturation and subsequent spawning in the wild[11-13]. We have recently reported the differential histological architecture of the developing gonads of *S. plagiostomus* during annual reproductive cycles[14,15]. However, the annual variations in gonadal gene expression are unexplored in this species.

Critical spermatogenic genes [like *Doublesex and mab-3 related transcription factor 1* (*Dmrt1,* critical for testicular differentiation), *GDNF family receptor alpha* (*Gdnfr-α,* prominent marker of spermatogonial stem/ progenitor cells) and Fibroblast family growth factor family 11 (*Fgf11,* essential for cellular differentiation and patterning) etc. have shown periodic expression patterns concerning season-dependent testicular maturation. For example, *Dmrt1* plays a critical role in metazoan testicular development and spermatogenesis[16]. The *Dmrt* gene family comprises many paralogous transcription factors sharing a highly conserved DNA binding domain[17]. In fishes such as Nile tilapia *Oreochromis niloticus*, Japanese rice-fish medaka *Oryzias latipes*, rainbow trout *Oncorhynchus mykiss* and Yangtze catfish *Silurus meridonalis, Dmrt* gene-family shows male restricted/ biased expression pattern. In *O. latipes, Dmrt1bY* / *Dmy* (the mammalian *Dmrt1*- orthologous gene in the Y chromosome) is directly involved in male sexual development[18], displaying all the characteristics of being the master regulator of sex determination[19]. A testicular stage-specific expression of the *Dmrt* gene family is found in spermatogonia and spermatocytes of African sharp-tooth catfish *Clarias gariepinus,* Zebrafish *Danio rerio* and Atlantic cod *Gadus morhu.* The *Fgf family* (including *Fgf11*) plays an essential role in controlling cellular proliferation and differentiation. In Zebrafish, *D. rerio, Fgf24* regulates early somatic gonadal morphogenesis and *Fgf24* mutants are found to develop as sterile adults[20]. In mammals, *Fgf9* is critically essential for early testicular development suppressing *Wnt4* signalling[1]. In *O. latipes*, *Fgf* expression has been found predominantly in testicular Sertoli cells suggesting a critical role in teleostean spermatogenesis[21]. On the other hand, *GDNFr-α* is expressed by undifferentiated spermatogonial stem/ progenitor cells. In *O. mysis*, *GDNFr-α* has been shown to express in all types of A spermatogonia (Asg). In the maturing testes, the Asg shows strong signals and these signals dramatically decreased with the progression of the germ cell development, whereas *GDNFr-α* dramatically decreased in type B spermatogonia and became undetectable in spermatids[22].

On the other hand, expressions of essential oogenic genes [like *Fork-head domain-containing protein* (*Foxl*$_2$, critical for ovarian differentiation and estrogen production), *Runt-related transcription factor* (*Runx1,* crucial for ovarian differentiation) and *Growth/ differentiation factor 9* (*Gdf9,* essential for oocyte development and maturation) etc.] also show seasonal fluctuations correlating with annual ovarian cycle. For example, *Foxl*$_2$ has been shown to determine the ovarian function. *Foxl*$_2$ along with nuclear receptor 5A (*Nr5a1*), functions as a transcriptional regulator of *Cyp19a1a*, playing an important role in the biosynthesis of Estrogen (E_2)[23]. In Japanese grenadier anchovy or Chinese tapertail anchovy *Coilia nasus,* the ovarian *Foxl*$_2$ expression is found to be at a low level during stage I and II and peaks at stage III, then gets down-regulated slowly up to the attainment of the resting stage[24]. However, *Cyp19a1a* expression gets augmented during the onset of stage I (in March), then maintains such elevated levels during the subsequent stages before reaching the second peak in the mature stage and then slowly gets diminished from pre-spawning to the resting stage[24]. In Celebes medaka, *O. celebensis, Foxl*$_2$ mRNAs get localized in ovarian follicular cells (mainly in pre-vitellogenic and vitellogenic follicles) and gradually show decline during follicular maturation[25]. *Runx1* induces female developmental programming and pre-granulosa cell specification/differentiation[26]. Notably, during early

gonadal differentiation, an evolutionarily conserved ovary-enriched expression of *Runx1* has been observed from *O. mykiss* to red-eared slider turtles up to mammals (human and goat)[27]. Finally, *Gdf9* expression is mainly found in ovaries and plays a pivotal role in oocyte development/maturation along with BMP15[28]. Previous studies in mammals[29,30], chicken[31] and multiple fish species[32-36] indicate such high expressions of *Gdf9* and *Bmp15* during the primary and early secondary stages of oocyte development, followed by a reduction during later stages of oocyte maturation[37]. Immunohistochemical analyses have revealed robust *Gdf9* expression in oocytes at the end of the primary growth phase, prior to vitellogenesis. In the yellow fat-tailed scorpion *Androctonus australis, Gdf9* mRNA levels get elevated progressively in pre-vitellogenic oocyte growth stage[36] whereas in *D. rerio* the level of *Gdf9* mRNA is maximal in the PG follicles and gradually gets diminished with follicle maturation[32].

In this current study, such season-dependent developmental variations in gene expressions were examined in adult *S. plagiostomus* gonads.

2. Materials and Methods

Three prominent/distinct developmental stages of this fish were selected based on typical cellular organizations, viz., mature/ developing *pre-spawning* stage (having undifferentiated spermatogonia for testes or maturing follicles with numerous cortical alveoli for ovaries), ripe/gravid *spawning-capable stage* (meiotic spermatid, spermatozoa for testes or secondary/tertiary vitellogenic oocytes for ovaries) and depleting/spent *post-spawning stage* (some left-over spermatozoa for testes or atretic follicles/ oocytes for ovaries). A total of 30 testes and 36 ovaries (covering all 3 stages) were collected from freshly captured/ live adult *S. plagiostomus* and 3 different spermatogenic (*Dmrt1, Gdnfr-α, Fgf11*) and oogenic (*Foxl*$_2$, *Runx1* and *Gdf9*) genes were evaluated in testicular and ovarian samples, respectively, using semi-quantitative RT-PCR analyses.

2.1 Sample Collection

The gonadal tissues (from total live/freshly captured 15 males and 18 females of adult *S. plagiostomus*) were obtained from two different river streams namely,

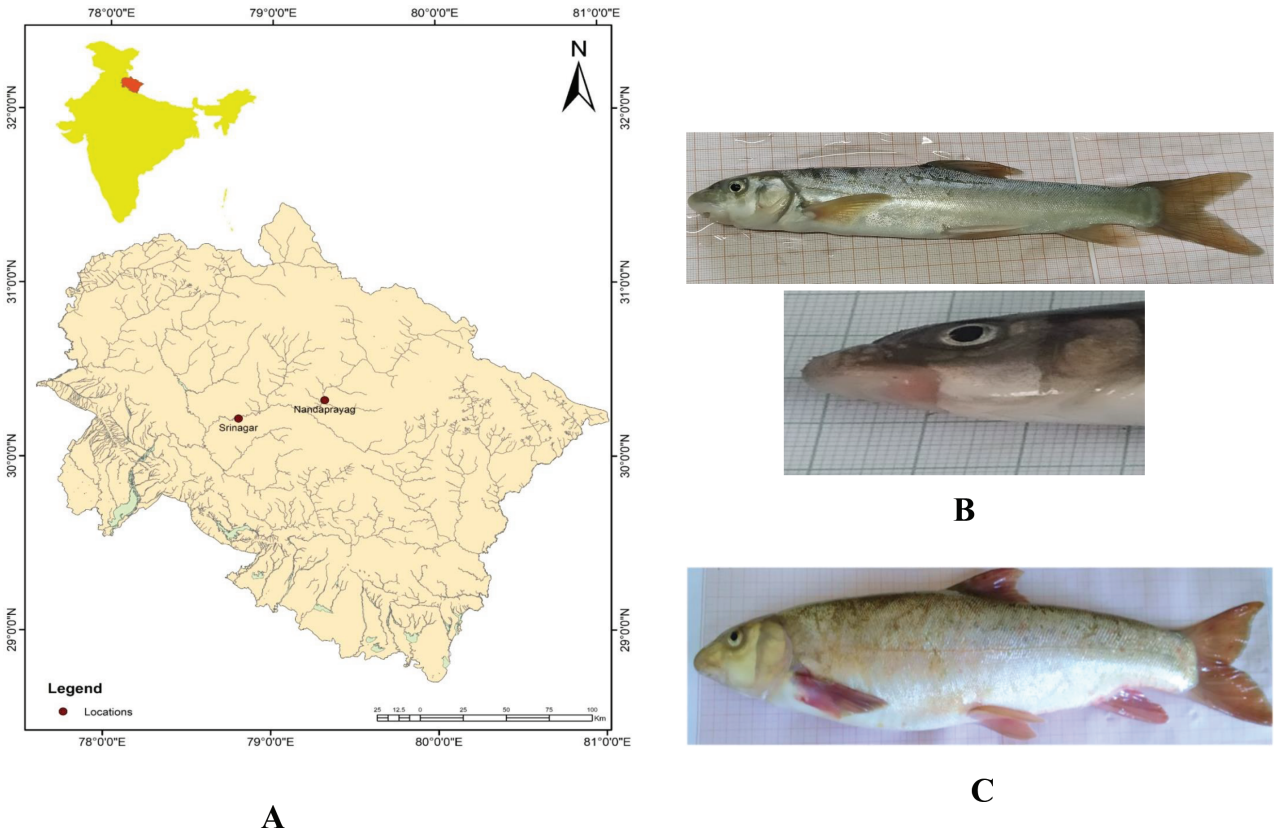
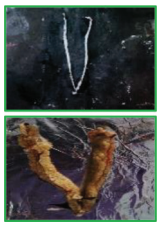
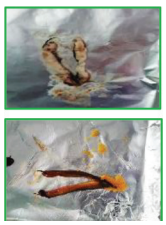
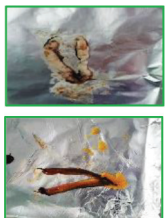


Figure 1. Sample collection sites **(A)** and representative images of adult male **(B)** and adult female **(C)** *S. plagiostomus*.

Table 1

Months	July 2023	August 2023	September 2023	October 2023	November 2023	December 2023
Status of Gonadal Maturity	Developing / Mature Phase 	Capable of Spawning/ Ripe/ Gravid/ Phase 	Active Spawning Phase 	Active Spawning Phase 	Depletion/ Regressing/ Spent Phase 	Depletion/ Regressing/ Spent Phase 
Average Rainfall Srinagar (in mm) Uttarakhand	312.85±1.5	190.52±2.7	164.18±3.5	35.49±1.9	2.05±1.3	00
Max- Min Temperature Srinagar °C (30.2247°N, 78.7986°E) Uttarakhand	16-13	17-15	17-16	16-12	13-8	9-8
Sunrise-Sunset Srinagar (30.2247°N, 78.7986°E) Uttarakhand	5:21 am 7:19 pm	5:40am-6:57 pm	5:57 am-6:22 pm	6:15 am- 5:45 pm	6:38 am- 5:19 pm	7:01am-5:17pm
Average Rainfall Nandaprayag (30.3320°N, 79.3205°E) (in mm) Uttarakhand	703.38	445.43	211.94	93.45	2.29	0.00
Temperature at Nandaprayag, °C (30.3320°N, 79.3205°E) Uttarakhand	14-11	15-13	16-12	16-13	11-7	8-6
Sunrise-Sunset at Nandaprayag (30.3320°N, 79.3205°E) Uttarakhand	5:19 am-7:17 pm	5:38 am-6:56 pm	5:55 am-6:20 pm	6:13 am-5:43 pm	6:36 am-5:17 pm	7:07 am-5:15 pm

Nandakini (at Nandaprayag-Chamoli, 30.3320°N, 79.3205°E) and Alaknanda (at Srinagar-Pauri Garhwal, 30.2247°N,78.7986°E), Uttarakhand, India. The sample collection sites and representative fish images are illustrated in Figure 1 (A-C). Table 1 summarizes the environmental/ climatic parameters [*viz*., day length (timing of sunrise and sunset), maximal and minimal temperature, and mean rainfall[38] noted during sample collection from the two different sites during July-December 2023.

2.2 Species Identification

S. plagiostomus samples were identified and screened/ differentiated from other two sympatric species (*S. richardsonii* and *S. progastus*) based on our previous experience[14,15,39,40] and as per reported morphometric features[41-43]. For each set and sex, around 5-6 adult *S. plagiostomus* (50-100 g) were obtained during June-July (pre-spawning), Sep-Oct (spawning) and November-January (post-spawning) having comparable / closest gonado-somatic indices (GSI) values. Gonads having uniform/comparable GSI values (for each stage and sex) were dissected out from the fishes and pooled to make one set (for one stage and one sex). Similarly, two more sets for two stages (a total of three sets per sex) were prepared on different calendar dates. All gonads were saved either in fixative [2.5% paraformaldehyde with

2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 2-3 hr at 4°C] for histological studies or in TRIzol (at -20°C) for subsequent gene expression analyses, respectively.

2.3 Histological Analyses

Gonads were obtained from live/freshly captured adult *S. plagiostomus* during three different reproductive stages (i.e., mature/pre-spawning/developing, ripe/gravid/spawning, and post-spawning/depletion/spent) and fixed using 2.5% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 2-3 hr at 4°C as reported earlier[44]. Transmission electron microscopy (TEM) of processed gonadal tissues was carried out with Morgagni 268D Transmission Electron Microscope (FeiCo, The Netherlands) as described by us[14,45] at AIIMS, New Delhi, India.

2.4 Semi-Quantitative RT-PCR Analyses

Total RNAs were extracted from the gonadal samples of each set (having 3 or more individual fishes for each stage per sex) adopting the phenol-chloroform method. The quantities and the qualities (ratio $A_{260/280}$) of the isolated RNAs were evaluated by NANODROP ONEC (Thermo Scientific). The cDNAs were then synthesized using a Hi-cDNA synthesis kit from HIMEDIA (HiGenoMB) following the manufacturer's protocol. The cDNAs were stored at -20°C for further use. The cDNAs were amplified further with primer using 2X PCR TaqMixture from HIMEDIA (HiGenoMB). The primers of 6 gonadal genes were designed using NCBI BLAST (https://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHome). The thermal regime and primer sequences used for validating the genes are shown in Table 2. After the PCR amplification, the PCR products were loaded in 2% agarose gels for electrophoresis using GENECO-H-MIDI (GENAXY), visualized and gel images captured by Hi-UV™ Duo (HiMedia).

2.5 Data Representation and Statistical Analyses

The TEM images shown in this study represent at least three independent samples (for all stages and sex) performed on different calendar dates, and each image was captured from 10 random fields per sample.

In all RT-PCR experiments, one set of data has been generated from three different developmental/maturational stages. Each stage included 3-5 pooled testes and 4-6 pooled ovaries. Three such sets were prepared on different calendar dates and used to interpret the data. Data were expressed as means± SEM. Statistical analyses of data were performed on the non-parametric Student's t-test for comparison between two groups or one-way analysis of variance followed by Dunnett's test for comparison between multiple groups. Statistical tests were conducted using GraphPad Prism 5.01 (GraphPad Software, La Jolla, Calif., USA). *P-values* <0.05 were considered statistically significant.

3. Results

3.1 Differential Gonadal Morphology

The annual alterations in gonadal morphology have been shown in Figure 2, with three distinct developmental/maturational stages/phases.

Figure 2A represents the pinkish-red coloured pre-spawning/developing testes. Figure 2B shows the white-coloured, enlarged pre-spawning/developing ovaries with distinct eggs (mean diameter 1.13±0.09 to 1.2±0.16 mm) discernible by the naked eye. Figure 2C illustrates large and firm ripe/spawning capable testes where milt was freely oozing after applying gentle abdominal pressure from the urogenital papilla. Figure 2D represents the gravid/spawning capable ovaries that have attained maximal weight, filled by reddish-yellow coloured mature yolky ova (mean diameter 1.7±0.12 to 2.003±0.012 mm) with massive vasculature. Figure 2E shows light brown colored thin, slender, flaccid post-spawning/depleted testes. Figure 2F depicts blackish-yellow coloured, shrunken, flaccid post-spawning/spent ovaries with some left-over mature yolky eggs (mean diameter 1.1±0.14 to 1.18±0.20 mm).

3.2 Testicular Ultra-Structure

Developing stage: This pre-spawning stage was observed during monsoon between July-August. As represented in Figure 3A, the conspicuous presence of primary spermatogonia (Sg1), and primary spermatocytes (Sc1) was found in the testes. Notably, the testicular lumina showed all the spermatogenic stages except spermatid (Spt) and/or spermatozoa (Sz).

Ripe stage: This was recognized as the primary/principal spawning/breeding stage that happened to be during September-October. As displayed in Figure 3B, both primary (Sc1) and secondary spermatocytes

Table 2. Thermal regime and Primer sequences

S. No.	Gene name	Forward Primer	Reverse Primer	Product length	Thermal regime	
1	Beta actin	CATCTACG AGGGTTACGCCC	GTGGTCTCAT GGATACCGCA	342	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-65°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles
2	*Dmrt1*	GTTTACTT TGATGCTGCAG	CAACTGTC CCAGAGCCAC	223	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-52°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles
3	GDNF-family receptor alpha	GCGGCAGT TATACAAAGGG	GACTGTGTG AAGGCGAACG	183	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-54.4°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles
4	*Fgf11*	CTGAGGCTC GGGTCTTTTTTC	CCACCAAA CGCAAGCC	128	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-54.4°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles
5	*Foxl2*	GAGAAGAAC AAAAAAGGATGG	CCGGTAGTT GCCCTTTTC	159	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-52.3°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles
6	*Gdf9*	GATTCATTTC GCTTGTTTGG	GGTAATGTTTA ACAAAGC ATTG	148	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-52°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles
7	*Runx1*	CAAAGATGC TGTTGGAGGCC	GAATCAGCG GATGAGACCG	155	Initial denaturation-94°C for 2min Denaturation-94°C for 45 sec Annealing-59.3°C for 45 sec Extension-72°C for 45 sec Final extension-72°C for 7 min 4°C for ∞	45 cycles

(Sc2) were visible at this stage. The testicular lumina was fairly expanded as lobules where active spermatogenesis continued, possessing abundant Spt and Sz. Notably, all spermatogenic stages were discernible here, with a predominance of round (RSpt) and elongated spermatid [(ESpt) with flagella (F) and middle piece (Mp)].

Depletion stage: After spawning (releasing Sz) the testes undergo an active regression stage. Histological data shown in Figure 3C identified the presence of Sc1/Sc2 containing abundant RSpt and ESpt. Some left-out/residual Sz were also present in the lumen of the lobules but without milt. The tunica albuginea turned thick, with wide inter-lobular space having scattered Sc/Spt.

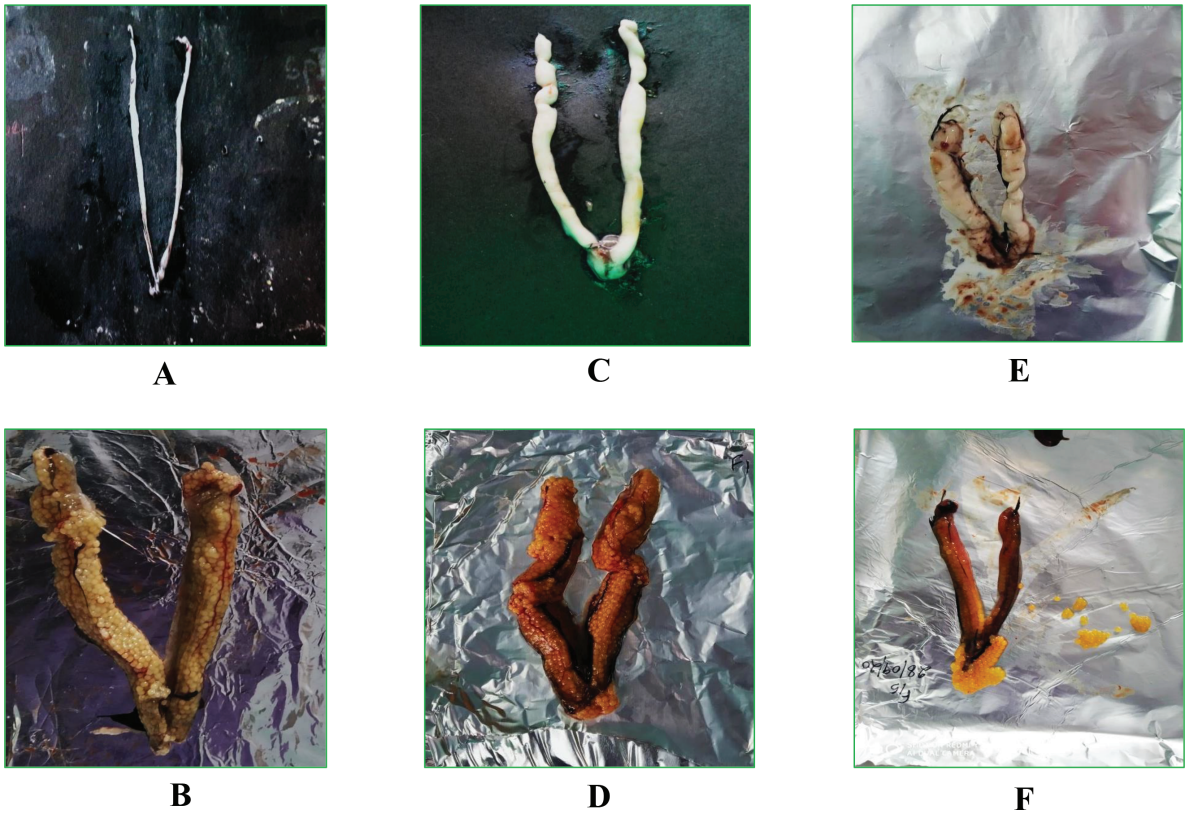


Figure 2. The seasonal variations of gonadal (A, C, E for testes and B, D, F for ovary) morphology during annual reproductive cycles. **A, B** - Mature/Pre-Spawning/ Developing testis and ovary, respectively; **C, D** - Ripe/Spawning-Capable testis and ovary, respectively; **E, F** - Post- Spawning/Regressing/Spent testis and ovary, respectively.

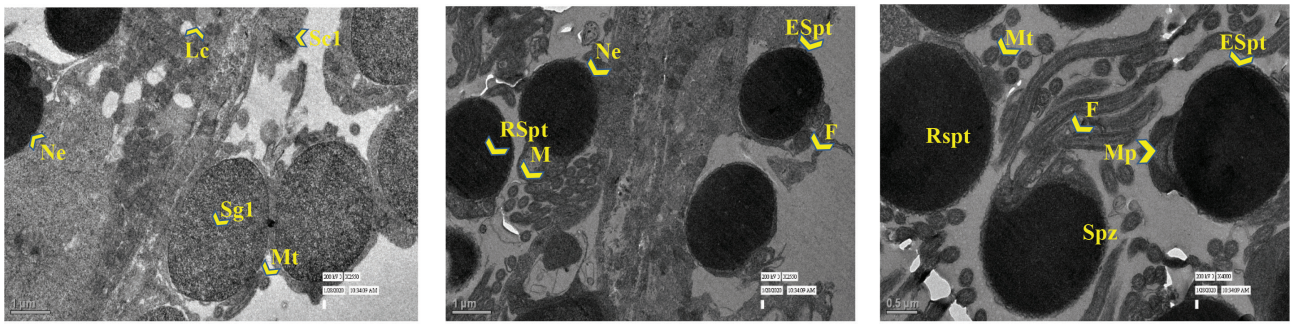


Figure 3. Testicular ultra-structure of *S. plagiostomus*. **A-C** - Each TEM image is representative of **A** - Pre-Spawning, **B** - Spawning, **C** - Post Spawning, testes. Sg1: Primary Spermatogonia, Ne: Nuclear envelope Lc: Leydig cell, Mt: Microtubule, RSpt; Round Spermatid, F: Flagella Spz: Spermatozoa, Mp: Middle piece, Ne: Nuclear envelope, M: Mitochondria. A scale bar is given in each image.

3.3 Ovarian Ultra-Structure

Mature stage: This stage was found during the monsoon between July-August. As illustrated in Figure 4A, primary, vitellogenic oocytes (Vtg1, Vtg2) were clearly evident.

Gravid stage: This has been recognized as the major/ principal spawning/breeding phase observed during September-October. The histological images depicted in Figure 4B revealed the presence of secondary and tertiary vitellogenic oocytes (Vtg2 and Vgt3).

Spent stage: This is found during the onset of winter between November-December, when the ovaries showed post-spawning regression. Figure 4C displays the presence of Vitellogenic Atretic Follicles (VAF) and Atretic Oocytes (AO).

3.4 Testicular Gene Expression

The representative (one of the three independent experiments) gel images shown for *Dmrt-1*, (Figure 5A) were normalized with housekeeping *ß –actin* (Figure 5B). The relative expression of *Dmrt1* (Figure 5C) was highest during the developing stage and the lowest during the depleted stage. The representative gel images for *Fgf11* (Figure 5D) and housekeeping *ß –actin* (Figure 5B) are shown here. The relative expression of *Fgf11* (Figure 5E) was minimal during the developing and depleted stages and was found to be the highest at the spawning stage. The representative gel images are shown for *GDNFr-α* (Figure 5F) with housekeeping *ß –actin* (Figure 5B). The *GDNFr-α*

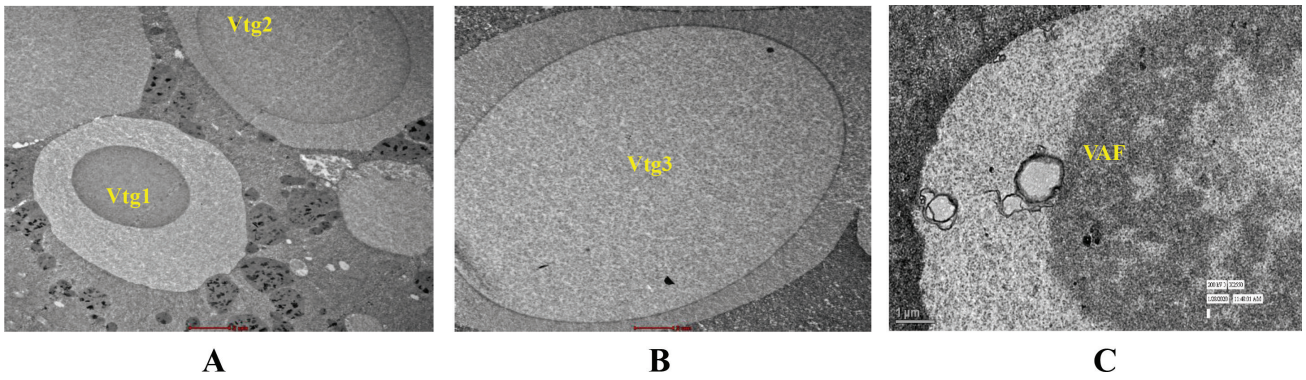


Figure 4. Ovarian ultra-structure of *S. plagiostomus*. **A-C** - Each TEM image is representative of **A** - Pre-Spawning, **B** - Spawning, **C** - Post-Spawning, ovaries. Vtg1, primary vitellogenic oocytes; Vtg2, secondary vitellogenic oocytes, Vtg3, tertiary vitellogenic oocytes, VAF, vitellogenic atretic follicle. A scale bar is given in each image.

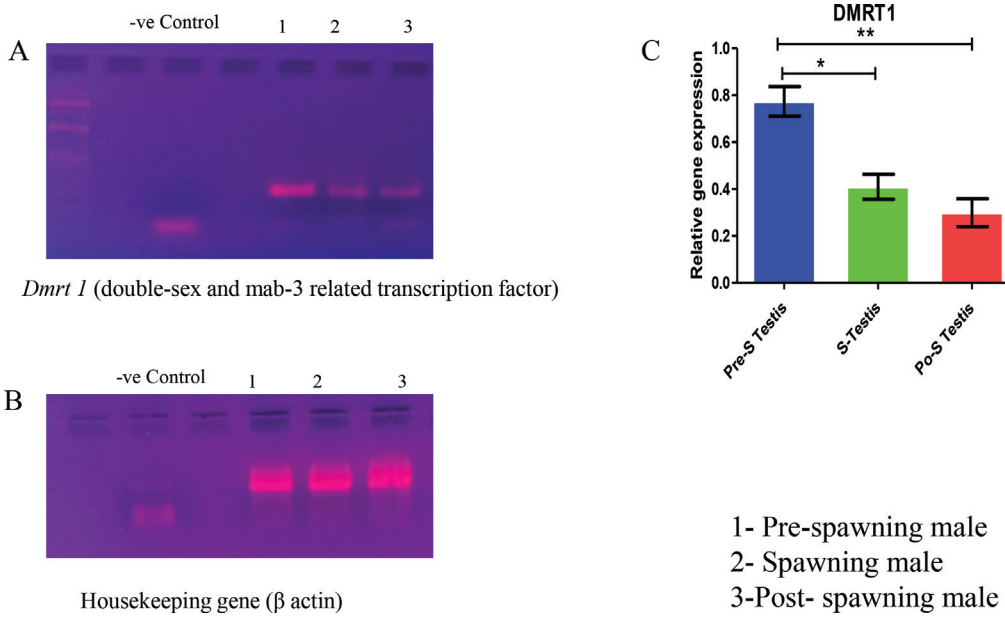


Figure 5. Testicular gene expression by semi-qualitative RT-PCR analysis. **A** - Representative gel image of *Dmrt 1*, **B** - Representative gel image of Housekeeping gene *ß-actin* (1- Pre-spawning male, 2- Spawning male, 3-Post- spawning male), **C** - Relative expression of *Dmrt1*. **D** - Representative gel image of *Fgf11*, (1- Pre-spawning male, 2- Spawning male, 3-Post-spawning male), **E** - Relative expression of *Fgf11*. **F** - Representative gel image of *GDNF-rα*, (1- Pre-spawning male, 2- Spawning male, 3-Post- spawning male), **G** - Relative expression of *GDNF-rα*

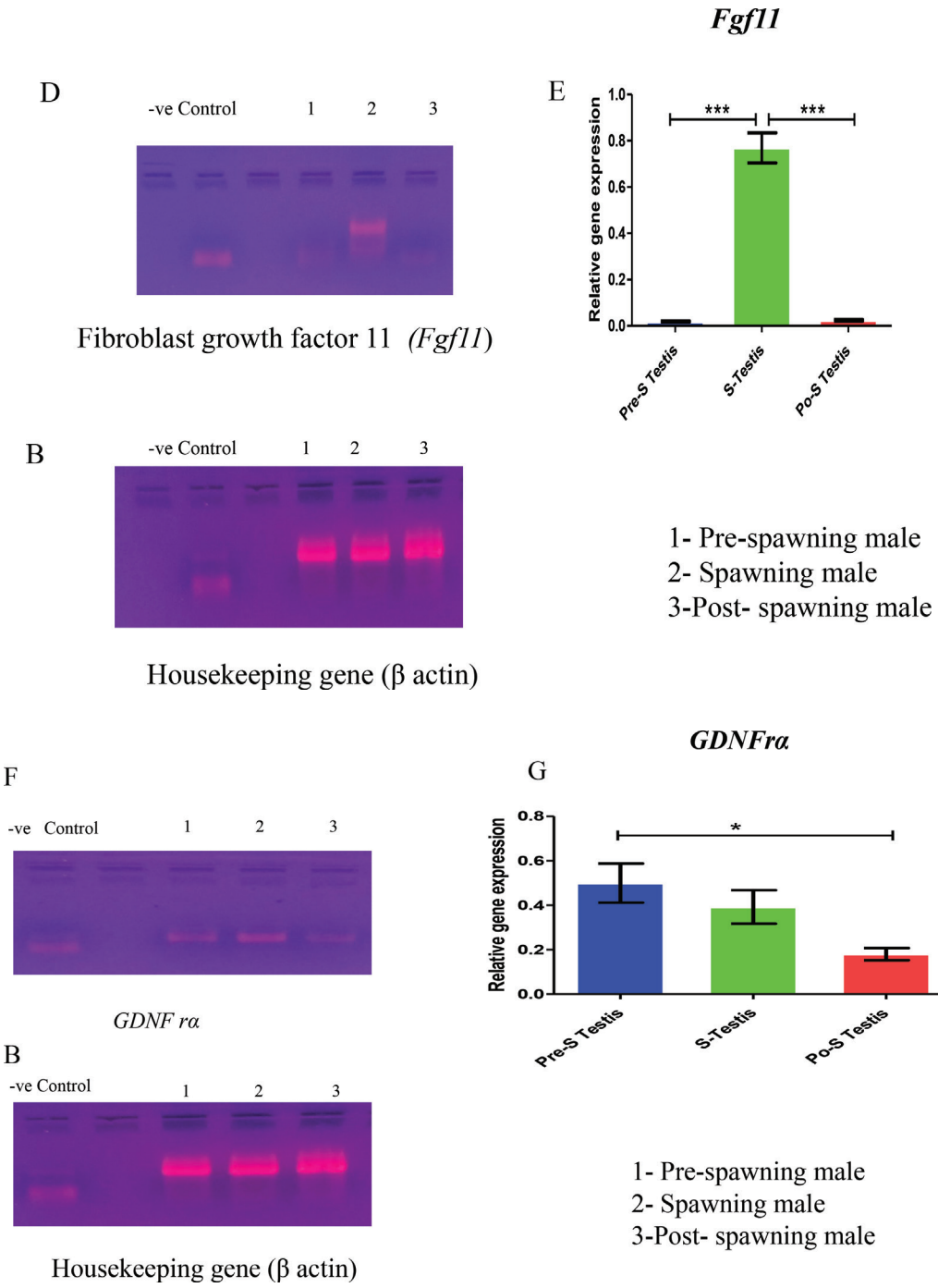


Figure 5. Continued...

(Figure 5G) was predominantly expressed during the developing stage, followed by a progressive decline during the spawning and the depleted stages, respectively.

3.5 Ovarian Gene Expression

The representative (one of the three independent experiments) gel images are shown for *Foxl*$_2$ (Figure 6A) with *ß –actin* (Figure 6B) as the housekeeping gene. The relative expression of *Foxl*$_2$ (Figure 6C) was the highest during the pre-spawning and maintained (with a slight decline) during the gravid/pre-spawning and the lowest during the post-spawning stage (Figure 6B). The relative expression of *Runx1* (Figure 6E) was elevated during the mature and gravid stage and subsequently got down-regulated at regressive spent

ovaries. The representative gel images are shown for *Gdf9* (Figure 6F) with *ß –actin* (Figure 6B) as the endogenous control. The relative expression of *Gdf9* (Figure 6G) showed that this transcript was predominantly expressed at the mature stage followed by a progressive decline during the gravid and spent stages, respectively.

4. Discussion

This present study focused on the seasonal changes in gonadal gene expression during the annual reproductive cycle of *S. plagiostomus*. The Schizothoracine fishes evolved from primitive species like Tibetan barbine teleosts during

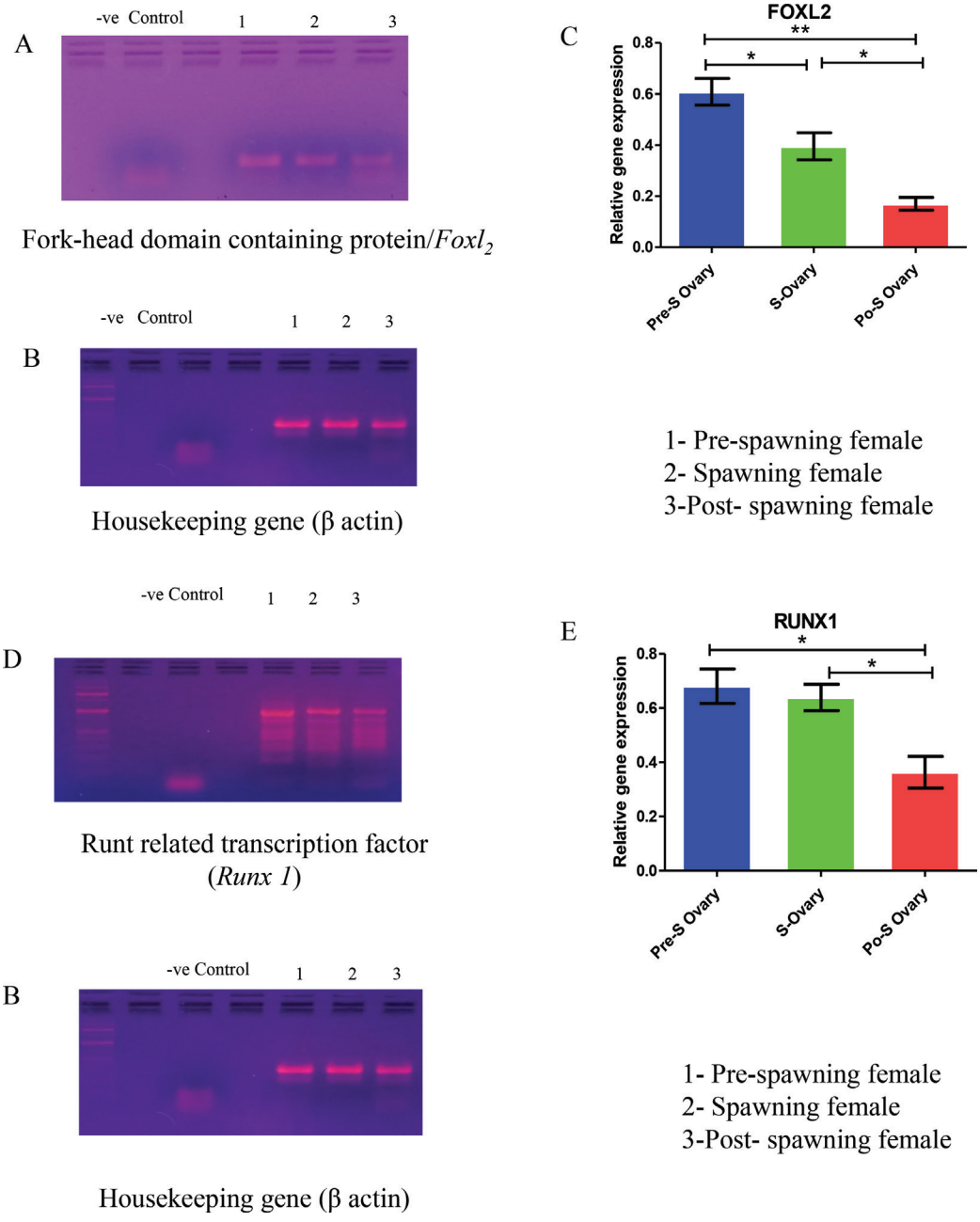


Figure 6. Ovarian gene expression by semi-qualitative RT-PCR analysis. **A** - Representative gel image of *Foxl2*, **B** - Representative gel image of Housekeeping gene *ß-actin* (1- Pre-spawning male, 2- Spawning male, 3-Post- spawning male), **C** - Relative expression of *Foxl2*. **D** - Representative gel image of *Runx1*, (1- Pre-spawning male, 2- Spawning male, 3-Post-spawning male), **E** - Relative expression of *Runx1*. **F** - Representative gel image of *Gdf9*, (1- Pre-spawning male, 2- Spawning male, 3-Post- spawning male), **G** - Relative expression of Gdf9.

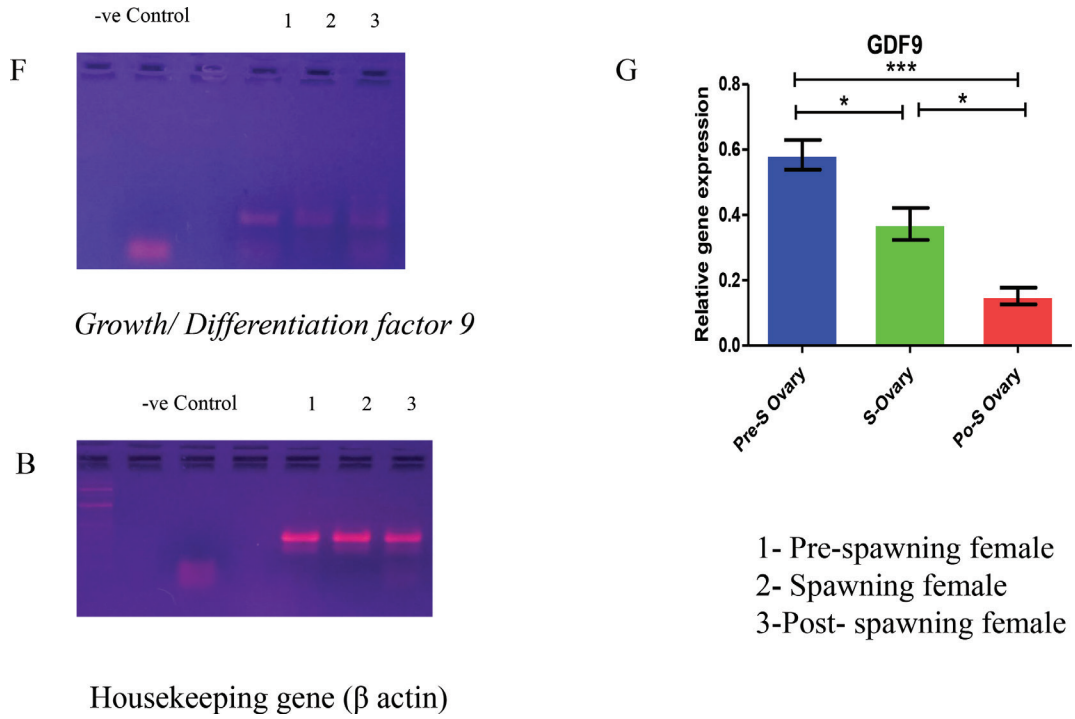


Figure 6. Continued...

the late Tertiary period[46-48] and have migrated to the Central Asian plain (Hindukush-Karakoram-Himalayan ranges at present) and thereafter got isolated due to the upliftment of the Indo-Himalayan and Qinghai-Tibetan mountains (He *et al.,* 2004)[8]. Schizothoracine fishes thereby share a broad range of habitats throughout Indo-China, and substantially support the local rural economy[9]. However, *Schizothorax* remains a highly neglected ichthyo-fauna[49]. Notably, out of seven major Schizothracine species (*S. niger, S. labiatus, S. curvifrons*, *S. esocinus*, *S. progastus, S. richardsonii* and *S. plagiostomus*) found in Indo-Himalayan region, *S. plagiostomus* was selected as the model organism for this study due to its wide distribution and abundance [from Afghanistan, Pakistan, India (Kashmir, Ladakh, Himachal, Uttarakhand, Sikkim, Assam, and Arunachal), Nepal, Bhutan to Qinghai- Tibetan plateau, China (River Yangtze) and Myanmar] with high economic importance[50,51].

We collected 30 testicular and 36 ovarian samples for three distinct developmental/maturational stages (at least three from each stage) of *S. plagiostomus* from river Alaknanda at Srinagar and river Nandakini at Nandprayag of Garhwal Himalaya, Uttarakhand, India. The initial screening of each fish was performed based on our past experience as reported[14,15,39,45] according to the established morphological/morphometric features[41,42].

The annual gonadal cyclicity of these fishes mainly consists of five distinct developmental/maturational phases: immature/resting/dormant (April-May), maturing/preparatory/regenerating (May-June), mature/developing/pre-spawning (June-July) ripe/gravid/actively spawning (September-October and February-March) and post-spawning/ spent/depleted (November-January)[40,52]. Since gonads during the resting/dormant and/or regenerating/preparatory stages remain very tiny/ fragile and do not exhibit any external (to the naked eye) sign of sexual dimorphism, these stages were not deployed for this study. It is pertinent to note here that *S. plagiostomus* found in Garhwal Himalaya shows two distinct spawning periods, principal (September-October) and residual (February-March)[14,39]. Although the spermatogenic output in terms of GSI values or sperm density is comparable in both stages, female fertility potential (in terms of fecundity, GSI values) was found to be the highest during the principal breeding season[14,39]. We have recorded most possible environmental/climatic parameters (like, time of sun-rise and sun-set, i.e., mean photo-period, maximum and minimum temperature, mean rainfall, etc.) during July-August (collection of pre-spawning samples), September-October (collection of spawning samples) and post-spawning, November-December (collection of post-spawning samples). Notably, as found in this study,

in most freshwater teleosts[53], optimal photo-period, water temperature and rainfall collectively play a critical role in fixing the timing of onset of gonadal maturation and, therefore, determine the spawning period and success in *S. plagiostomus*.

Our TEM data confirm the gonadal maturational status in three different stages in both sexes, where the subsequent gene expression analyses were performed. For example, pre-spawning testes showed dominance of undifferentiated spermatogonia, whereas pre-spawning ovaries were filled with primary/secondary vitellogenic oocytes containing numerous cortical alveoli. Spawning-capable testes possessed meiotic spermatid, and spermatozoa whereas secondary/tertiary vitellogenic oocytes were found in spawning ovaries. Finally, some left-out/residual spermatozoa were observed in post-spawning testes, and atretic follicles/oocytes were discernible in post-spawning ovaries. These data are consistent with what we have recently observed and reported for *S. plagiostomus*[14,39].

To determine the seasonal variations in gonadal gene expression, key spermatogenic (*Dmrt1, Gdnfr-α, Fgf11*) and oogenic (*Foxl*$_2$, *Runx1,* and *Gdf9*) genes were selected based on their critical involvement in gonadal maturation and consequent germ cell development. However, it is essential to note here that since the primary aim of this study was focused on the gonadal development during the annual reproductive cycle, other seasonal and/or circadian clock genes [viz., *Period2* (*Per2*) and *Cryptochrome 1a* (*Cry1a*), etc., too showed a periodic/rhythmic expression profile] have not been investigated.

Our RT-PCR data revealed an elevation in *Dmrt1* expression during the pre-spawning and spawning stages. Such stage-dominated expression pattern is consistent with *C. gariepinus*[54]. Notably, in gonochoristic annual breeders (such as *O. mykiss*) an elevated *Dmrt1* expression has been observed during preparatory and pre-spawning stages followed by a gradual decrease during spawning/spermiation. This further indicates that *Dmrt1* may have an important role during testicular recrudescence, particularly during spermatogenesis[17]. *Dmrt1* expression gets elevated further from larval development to the juvenile stage in snow-trout *S. kozlavi*[55]. All these findings directly support our present observation. We found a prominent augmentation of *Fgf 11* during the spawning stage, indicating its importance during cellular differentiation. *Fgf 11* operates via the MFR1 receptor and plays a significant role in male germ cell differentiation[56] indirectly supporting our findings. Finally, we noticed *GDNFr-α* expression to be enriched in pre-spawning testis as compared to the rest of the two subsequent stages. Being a dominant *GDNFr-α* marker for undifferentiated spermatogonial cells, the pre-spawning testes were found to be the principal source for these cells[57,58] corroborating our results.

On the other hand, for ovarian genes, our data revealed highest and lowest expression of *Foxl*$_2$ found during the pre-spawning and post-spawning phases, respectively. However, the *Foxl*$_2$ expression was found to be maintained in spawning ovaries, but the degree of expression varied among individual ovarian samples. This pattern is consistent with what prevails in *C. gariepinus* where higher expression of *Foxl*$_2$ has been detected in the ovary and brain during the pre-spawning phase[59]. Notably, in the snow-trout *S. kozlovi*, *Foxl*$_2$ expression is stable (independent of different temperature fluctuations) and restricted to the brain, gills, and gonads[60]. We found that the expression of *Runx1* was higher during pre-spawning and spawning ovaries than post-spawning ovaries. During early gonadal differentiation, an evolutionarily conserved ovary-enriched expression of *Runx1* has been observed from *O. mykiss* to red-eared slider turtles up to mammals (human and goat)[27]. Finally, *Gdf9* expression was found to peak during the pre-spawning phase and minimal during the post-spawning phase. In the European seabass *Dicentrarchus labrax, Gdf9* and *Bmp15* transcripts are shown to be elevated during pre-vitellogenesis but declined dramatically at the onset of vitellogenesis to reach their lowest levels[33]. This profile too gets correlated with our data. However, in snow-trout *S. prenanti, Gdf9* is expressed in the ovary's Cortical Alveoli (CA) stage[61]. Overall, the seasonal variations of gonadal expression patterns of the genes examined herein showed more or less conserved profiles, as shown by most freshwater teleost species.

5. Conclusion

In summary, our data indicate male-biased expressions of *Dmrt1*, *Gdnfr-α* and *Fgf11* in *S. plagiostomus*. Similarly, expression patterns of key ovarian genes e.g.-*Foxl*$_2$, *Runx1* and *Gdf9* - were also found to be functionally relevant. Most importantly, all these gonadal genes further showed a robust phylogenetic uniformity with other teleostean (or even mammalian) species. To the best of our knowledge, this study is the first to explore the stage-specific and

season-dependent gonadal gene expression in any Schizothoracine fish. This study will surely improve the molecular understanding of the gonadal annual cyclicity of *S. plagiostimus*. Future studies will deploy high-throughput next-generation RNA sequencing to generate a sex- and season-dependent gonadal transcriptional atlas in this species.

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