Identification and expression analysis of *sox* family genes in common carp: *sox30* and *sox19* regulate gonadal function

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CERTIFICATE

This is to certify that the thesis entitled ***"Identification and expression analysis of sox family genes in common carp: sox30 and sox19 regulate gonadal function"*** submitted by Ms. **Anitha Arumugam** bearing registration number **15LAPH08** in partial fulfillment of the requirements for award of **Doctor of Philosophy** in the School of Life Sciences is a bona fide work carried out by his under my supervision and guidance.

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Parts of this thesis have been:

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DECLARATION

I, **Anitha Arumugam** hereby declare that this thesis entitled "***Identification and expression analysis of sox family genes in common carp: sox30 and sox19 regulate gonadal function***" submitted by me under the guidance and supervision of **Prof. B. Senthilkumaran** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

Date: 23-12-2020

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Anitha Arumugam

Table of contents

Abbreviations

3RWGD	– third round of whole genome duplication
11-KT	– 11-ketotestosterone
11-OHT	– 11β-hydroxytestosterone
17α,20β-DP	– 17α,20β-dihydroxy-4-pregnen-3-one
aa	– amino acid
ad4bp/sf-1	– adrenal 4 binding protein/steroidogenic factor-1
amh	– anti-Mullerian hormone
amhr2	– anti-Mullerian hormone receptor type 2
amhy Y	– chromosome-linked anti-Mullerian hormone
ar	– androgen receptor
atm	– Ataxia telanglectasia mutated
COG	– clusters of orthologous groups
CPCSEA	– Committee for the Purpose of Control and Supervision on Experiments on Animals
creb	– cAMP-responsive element binding protein
Ct	– cycle threshold
ctnnbip1	– catenin beta interacting protein 1
cux1	– cut like homeobox 1
cux2a	– cut like homeobox 2a
cxcl12	– C-X-C motif chemokine ligand 12
cxcr4	– C-X-C motif chemokine receptor 4
cyp11	– cytochrome P450, family 11
cyp11b1	– cytochrome P450, family 11, subfamily b, polypeptide 1
cyp17	– cytochrome P450 17-hydroxylase/lyase
cyp19a1	– cytochrome P450, family 19, subfamily a, polypeptide 1

dax – dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X

dax1 – dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X, gene 1

DDRT-PCR – Differential display reverse transcription-PCR

DEG – differentially expressed genes

DMEM – Dulbecco's modified Eagle's medium

dmrt1 – doublesex and mab-3 related transcription factor 1

DMY/dmY – DM-domain gene on the Y chromosome

dph – days post hatch

E_2 – estradiol-17β

eSRS21 – eel spermatogenesis related substances 21

egr2b – early growth response 2b

EIA – enzyme immunoassay

Er – estrogen receptor

fam101b – family with sequence similarity 101, member B

fam192a – family with sequence similarity 192, member A

fam210b – family with sequence similarity 210, member B

fbox43 – F-box only protein 43

fem1b – fem-1 homolog B

ff1b – Fushi tarazu factor 1b

ff1d – Fushi tarazu factor 1d

fgfr1a2 – fibroblast growth factor receptor 1-A-like

foxk2 – forkhead box k2

foxl2 – forkhead box L2

FSH – follicle stimulating hormone

fshr – follicle stimulating hormone receptor

fstl – follistatin

fstl3 – follistatin-like 3 (secreted glycoprotein)

fzd8 – frizzled class receptor 8

ftz-f1 – Fushi tarazu factor 1

gata4 – GATA binding protein 4

gata6 – GATA binding protein 6

GC – granulosa cells

gdf9 – growth differentiation factor 9

GEO – Gene Expression Omnibus

gnrh – gonadotropin releasing hormone

GO – gene ontology

gsdf – gonadal somatic cell-derived factor

GTH – Gonadotropin hormone

hCG – human chorionic gonadotropin

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMG – high mobility group

HRP – horseradish peroxidase

hsd11b – hydroxysteroid 11-β dehydrogenase

hsd11b2 – hydroxysteroid 11- beta dehydrogenase 2

hsd17b – hydroxysteroid 17- β dehydrogenase

hsd17b2 – hydroxysteroid 17- beta dehydrogenase 2

hsd20b – hydroxysteroid 20- β dehydrogenase

hsd20b2 – hydroxysteroid 20- beta dehydrogenase 2

hsd3b – hydroxy- Δ-5-steroid dehydrogenase, 3β

hsd3b7 – hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7

hsd11b1 – hydroxysteroid 11-beta dehydrogenase 1

IAEC – Institutional Animal Ethics Committee

IGF1 – insulin-like growth factor-1

igf1ra – insulin-like growth factor 1a receptor

IHC – immunohistochemistry

ipo4 – importin 4

KASS – KEGG automatic annotation server

KEGG – Kyoto encyclopedia of genes and genomes

kiss2 – kisspeptin2

KPO_4 – potassium phosphate buffer

KVAFSU – Karnataka Veterinary Animal and Fisheries Sciences University

L15 – Leibovitz-15

LH – luteinizing hormone

lhcgr – luteinizing hormone/choriogonadotropin receptor

lhr – luteinizing hormone receptor

mapk – mitogen-activated protein kinase

MDHT – 17α–methyl-di-hydroxy-testosterone

mis – Mullerian inhibiting substance

MS222 – ethyl 3-aminobenzoate methanesulfonate

msl1b – male-specific lethal 1 homolog b

MT – 17αmethyltestosterone

NGS – next generation sequencing

NR5A1 – nuclear receptor subfamily 5 group A member 1

ORF – open reading frame

PBS – phosphate-buffered saline

pcna – proliferating cell nuclear antigen

pdgf – platelet-derived growth factor

PEI – polyethylenimine

PFA – paraformaldehyde

PGC – primordial germ cells

plzf – promyelocytic leukemia zinc finger

pou5f1 – POU class 5 homeobox 1

pou5f3/oct4 – POU domain, class 5, transcription factor 3/octamer-binding transcription factor 4

PPA – protein phosphatase 2A

prlhr2a – prolactin releasing hormone receptor 2a

qPCR – quantitative PCR

RA – retinoic acid

RAR-RXR – retinoic acid receptor/retinoid x receptor heterodimers

RNA-Seq – RNA sequencing

rspo1 – R-spondin 1

RT – room temperature

sdY – sexually dimorphic on the Y chromosome

siRNA – small interfering RNA

smad3b – SMAD family member 3b

sox – SRY-box

srd5a1 – steroid 5 α-reductase 1

SRY – sex-determining region Y

SSR – simple sequence repeat

Star – steriodogenic acute regulatory protein

sycp1 – synaptonemal complex protein 1

sycp3 – synaptonemal complex protein 3

T – testosterone

tac3a – tachykinin 3a

TC – theca cells

tcf3a – transcription factor 3a

TGF-β – transforming growth factor- *β*

TSGD – teleost-specific genome duplication

wnt – wingless-type MMTV integration site family

wt1 – Wilms' tumor suppressor 1

zar1 – zygote arrest 1

zp2 – zona pellucida sperm-binding protein 2

General Introduction

Sex determination/differentiation in fish

Sex determination/differentiation and gonadal development are central processes in sexually reproducing living organisms. A bipotential gonad develops into a testis or an ovary, termed as sex determination is governed by genetic factors, the dose of gene, and time. Following which, hormones secrete and the gonad grows phenotypically different in both the sexes termed as sex differentiation (Hughes, 2001; Short, 1998). Teleost (bony fishes) exhibit various mechanisms of sex determination/differentiation, acquiring recognition in scientific research (Fig. 1). In fishes, sex determination could be polygenic or monogenic with determinants in autosomes or sex chromosomes (Devlin and Nagahama, 2002). In fishes, *DMY* was the first identified sex determining gene (Matsuda *et al*., 2002) in the Japanese medaka, *Oryzias latipes* also named as *dmrt1Y* (Nanda *et al*., 2002). Subsequently, a series of sex determination/differentiation and gonadal development related genes have been reported in different fishes like *sdY* in rainbow trout (*Oncorhynchus mykiss)*, *amhy* in pejerrey (*Odontesthes hatcheri*), *dmrt1* in the African catfish (*Clarias gariepinus*), *amhr2* in fugu (*Takifugu rubripes*), and *gsdf* in (*O. luzonensis*) medaka (Chen *et al*., 2014; Hattori *et al*., 2012; Kamiya *et al*., 2012; Myosho *et al*., 2012; Raghuveer and Senthilkumaran, 2009; Yano *et al*., 2012). Teleost reproduction involves unisexuality/hermaphroditism/gonochorism modes of sex differentiation (Dipper and Pullin, 1979; Schartl *et al*., 1995; Warner, 1984). The direct development of testis or ovary from an undifferentiated gonad is termed gonochorism (Sadovy and Shapiro, 1987). Hermaphroditism comprises of synchronic (both gonads are simultaneously active) and sequential, which is subdivided into protogynic (female to male sex change) and protandric (male to female sex change).

Fig. 1 Summary of endogenous mechanisms and external variables influencing different types of sex differentiation utilized by major orders of fish. Licensed for reuse from Elsevier; Devlin and Nagahama, 2002. Aquaculture 208, 191–364.

Fish gonadal differentiation

In teleost, the bipotential gonad differentiates into ovary or testis through ontogenesis involving PGC and somatic cells (Meijide *et al*., 2005). During embryogenesis, totipotent blastomeres lead to the formation of PGC, which differentiates further and develops into a functional ovary/testis. The maternal cytoplasmatic components (germ plasma) specify PGC formation (Knaut *et al*., 2000). The formed PGC migrate along dorsal mesentery of the intestine with peritoneal hedge besides genital crest region and later situated beneath mesonephric ducts (Molyneaux and Wylie,

2004; Patiño and Takashima, 1995). The gonadal somatic components are of embryonic origin (Hoar, 1969).

Ovarian differentiation and development

Generally, in gonochoristic fish ovarian differentiation starts earlier than testicular differentiation. Ovarian differentiation consists of (germ cell proliferation) mitosis, meiosis, and (arrangements of somatic cells) cavity formation (Nakamura *et al*., 1998). The gonads destined to become ovary have more germ cells in undifferentiated stage (Nakamura *et al*., 1998), thus higher quantity of germ cells prior to meiosis is an indicate of ovary. The increase in germ cell mitosis leads to formation of characteristic clusters, followed by meiosis (Strüssmann *et al*., 1996). Besides in females, germinal and somatic differentiations occur instantaneously leading to the formation of follicles, containing oocytes with an internal GC and external TC (Nagahama, 1983). Teleostean ovary (Fig. 2) could be cystovarian (membrane bound) or gymnovarian (ovarian lamellae is open to abdominal cavity) by nature (Kagawa, 2013).

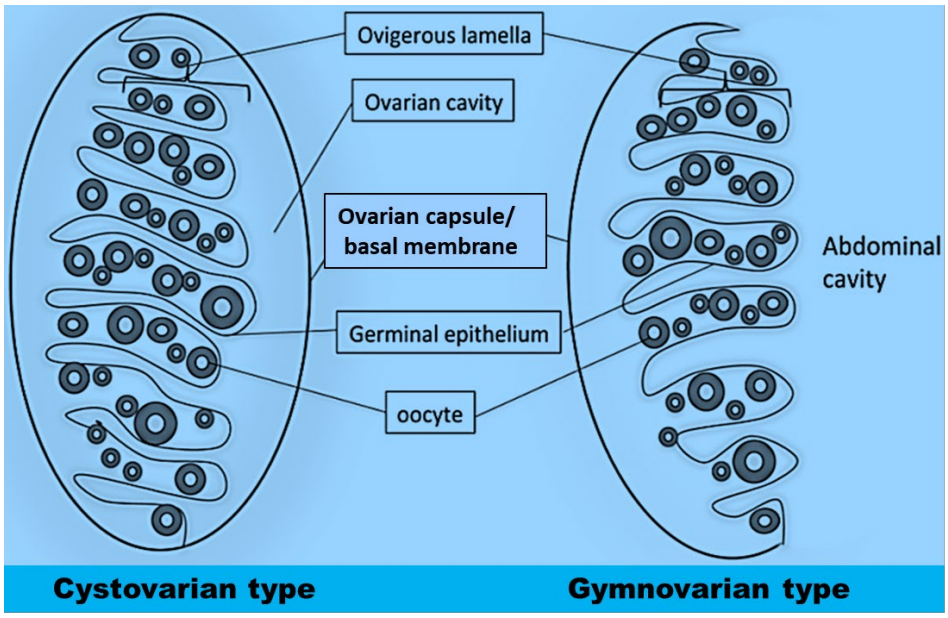

Fig.2 Types of teleostean ovary adapted and modified with permission from Aqua-BioScience Monographs; Kagawa, 2013. Aqua-Bio Sci. Monogr. 6, 99–127.

The germ cells in different stages are present in adult fishes (Nakamura *et al*., 2011) distinct to mammals. The ovarian structure with GC enveloping oocytes and outer TC over the basal membrane is maintained through follicular growth (Patiño and Takashima, 1995; Wallace and Selman, 1990). The ovarian follicle grows with distinctive previtellogenic and vitellogenic stages, during which nutrients for oocyte development are stored. Accumulation of lipid droplets in ooplasm occurs in previtellogenesis. During vitellogenesis, *vtg* (phospholipid-rich yolk protein precursor) produced in liver is transported via blood to the ovary gets accumulated in the oocytes. Post vitellogenesis the oocyte maturates and ovulates, controlled by hormones (Nath and Maitra, 2001; van–Bohemen *et al*., 1982; Wallace, 1985).

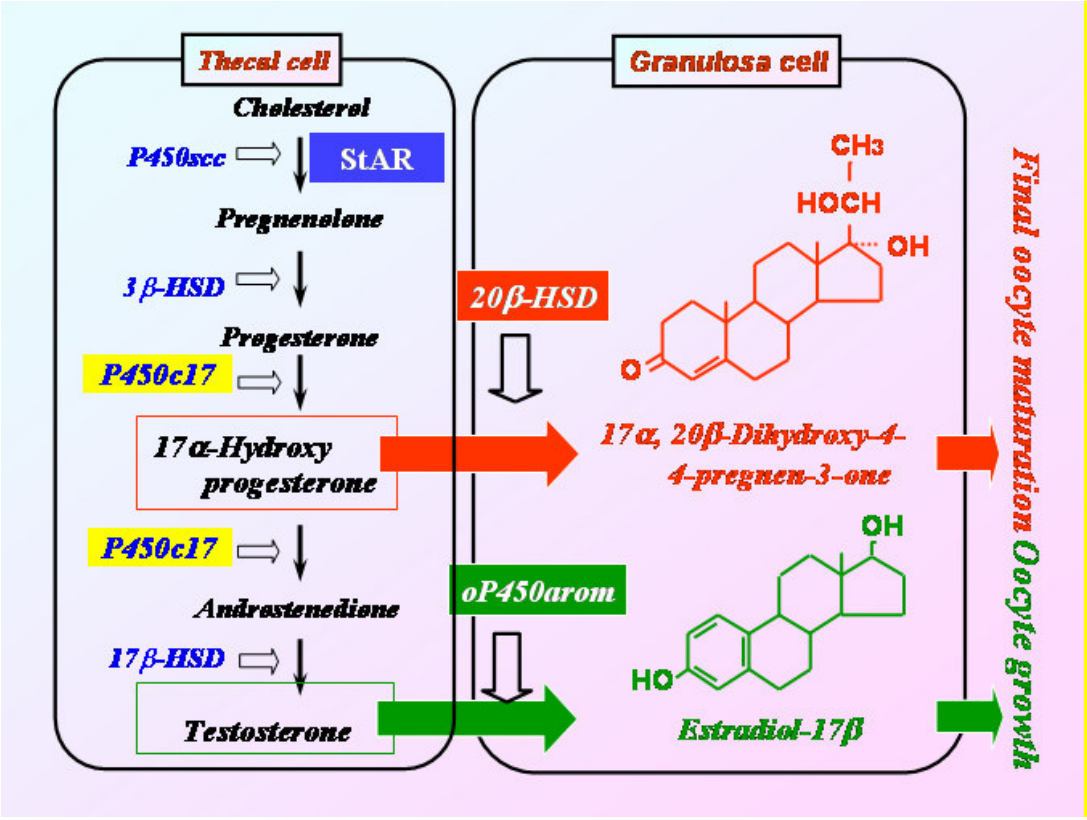

Fig. 3 Schematic representation of two-cell type model in the shift in steroidogenesis occurring in teleost ovarian follicles prior to oocyte maturation. Licensed for reuse from Elsevier; Senthilkumaran *et al*., 2004. Mol. Cell. Endocrinol. 215, 11–18.

Nagahama (1997) projected the two-cell model in teleost ovary, wherein LH regulates conversion of progesterone to 17α-hydroxyprogesterone by *cyp17* in TC, which passes to GC through the basal lamina and by the action of *hsd20b* converts to 17α, 20β-DP (Gur *et al*., 2002; Nagahama, 1997; Sherwood *et al*., 2000). Besides, in TC GTH stimulates steroidogenic pathway in which cholesterol under the action of various enzymes gets converted to T and by *cyp19a1a* in GC into E_2 during oocyte growth (Senthilkumaran *et al*., 2004), whereas 17α, 20β-DP is synthesized during final oocyte maturation (Fig. 3). Further, catechol estrogens are important for maturation of oocyte in the catfish, *C. batrachus* and *Heteropneustes fossilis* (Senthilkumaran and Joy, 2001). The role of various genes involved in oocyte maturation of teleost bony fishes are has been reviewed extensively (Senthilkumaran, 2011).

Testicular differentiation and development

In general, the gonads intended to become testis remains undifferentiated for a longer time and soma differentiates prior to migration of germ cells (Patiño and Takashima, 1995). In 'differentiated' gonochorists, if the germinal and somatic elements stay quiescent for more time than those gonads are assumed to become testis. During testicular differentiation, development of a lobed structure (Patiño *et al*., 1996) was evident in channel catfish (*Ictalurus punctatus*). In guppy (*Poecilia reticulata*) testis differentiation is identified by stromatic cell aggregation which later becomes sperm ducts in addition to interstitial tissues (Nakamura *et al*., 1998). Testicular differentiation is also signaled by sperm duct formation, developing lobule appearance, blood vessels occurrence in stroma (Rasmussen *et al*., 2006). Spermatogenesis controlled by intrinsic and extrinsic factors marks the transformation of (diploid) spermatogonia into (haploid) sperms. Sertoli cells are prime in regulating spermatogenesis and testis development, controlling germ cell development to spermatozoa and seminiferous tubules environment (Nóbrega *et al*., 2009).

Spermatogenesis comprise of (mitotic) spermatogonial, meiotic and spermiogenic phases. In mitotic phase, the gonad progresses from undifferentiated to differentiating spermatogonia stage. In meiotic phase, diploid to haploid conversion of spermatogonia results in primary and secondary spermatocytes. Lastly, spermatids mature giving rise to motile spermatozoa (Schulz *et al*., 2010). Spermatogenesis process is continuous in few species and discontinuous (superimposing cycles) in other fishes (Billard, 1986). Cystic spermatogenesis is observed in fishes (Fig. 4). Each cyst consists of germ cell clone surrounded by Sertoli cells, which produce factors/hormones for development of germ cell and production of sperm (Matta *et al*., 2002; Schulz *et al*., 2005). In fishes, spermatogonia do not bind with basal lamina and acrosome is absent, contrasting to mammals (Schulz *et al*., 2010). Spermiation, release of milt (sperm fluid) into water occurs in natural habitat, while in laboratory an abdominal massage causes milt expulsion. Spermatogenesis period varies from 4 – 21 days under different environmental conditions (Schulz *et al*., 2010).

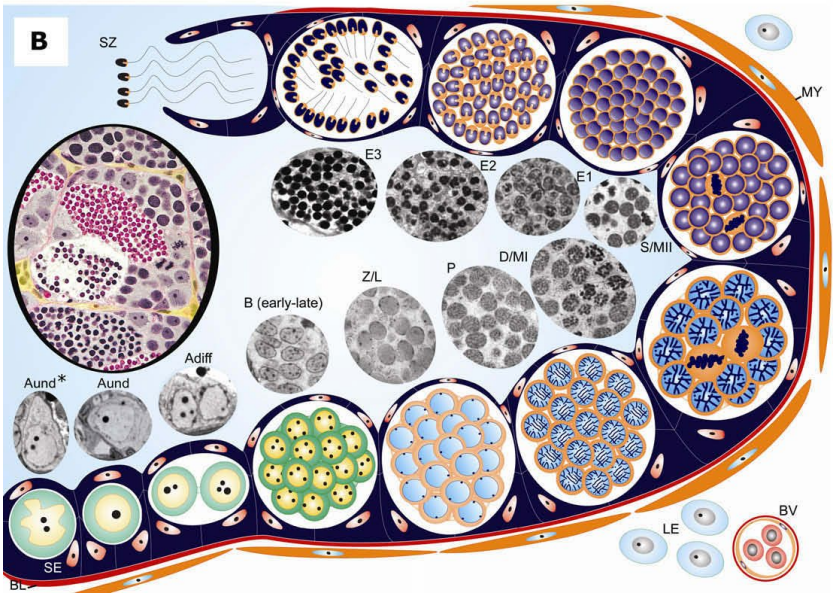

Fig. 4 Schematic representation of cystic spermatogenesis in fish. Licensed for reuse from Elsevier; Schulz *et al*., 2010. Gen. Comp. Endocrinol. 165, 390–411.

Endocrine control of sex differentiation

Endocrine regulation of sex differentiation (Fig. 5) is well documented in fishes (Bieniarz and Epler, 1992; Nagahama, 1994).

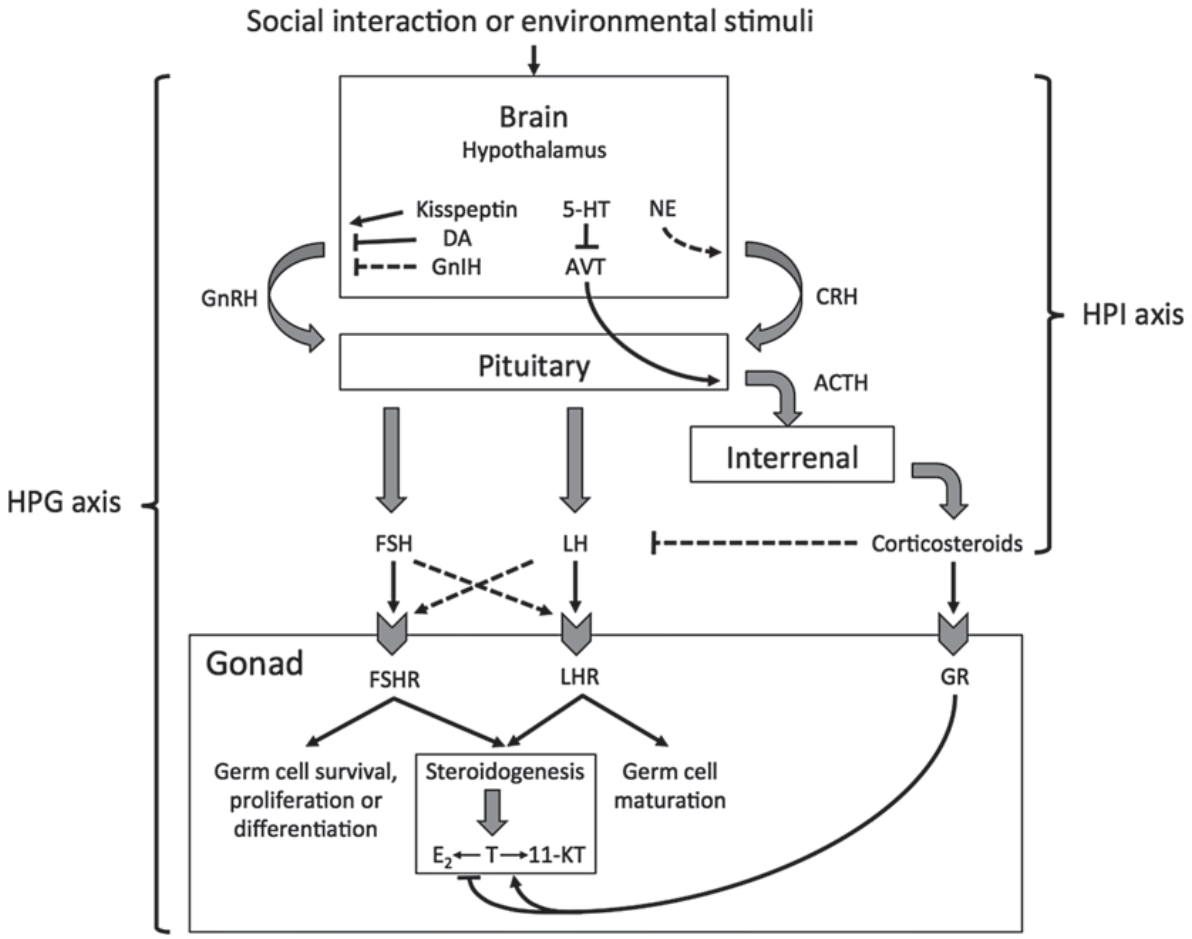

Fig. 5 Representation of neuroendocrine regulation of steroidogenesis in teleost. Solid lines denote interactions in fish, and dashed lines denote interactions in non-teleost models. ACTH-adrenocorticotropic hormone, AVT-arginine vasotocin, CRH-corticotropin releasing hormone, DA-dopamine, E_2-estradiol-17β, GnIH-gonadotropin inhibitory hormone, GnRH-gonadotropin releasing hormone, FSH-follicle stimulating hormone, FSHR-FSH receptor, LH-luteinizing hormone, LHR-LH receptor, MIH-maturation inducing hormone, NE-norepinephrine, T-testosterone, 5-HT-serotonin, 11-KT, 11-ketotestosterone. Licensed for reuse from S. Karger AG, Basel; Todd *et al*., 2016. Sex. Dev. 10, 223–241.

Sex differentiation occurs in coordination with steroidogenesis (von Hofsten and Olsson, 2005). Though nonsteroids (IGF and somatotropins) act upon gonads (Duan *et al*., 1993; Kagawa *et al*., 1995), the most studied are (Van der Kraak *et al*., 1990) the sex steroids. E_2 (Yamamoto, 1969) and 11-KT (Todo *et al*., 1999) are the potent estrogen and androgen respectively, in fishes. Furthermore, maturation induction by 17α, 20β-DP and significance of several hormones have been reported in fish (Kime, 1993). Fishes display sexual plasticity controlled by genetic as well as environmental factors (Devlin and Nagahama, 2002; Nakamura *et al*., 1998). Exogeneous hormonal treatments have been proven to reverse sex in several teleost (Billard *et al*., 1982; Gomelsky *et al*., 1994; Olito and Brock, 1991).

Molecular mechanisms involved in fish sex differentiation

Several genetic elements like transcription factors, signaling molecules and other factors also influence teleostean gonadal differentiation and development as reviewed by Sudhakumari and Senthilkumaran (2013). *dmrt1* was reported to play a crucial role in catfish testicular differentiation (Raghuveer and Senthilkumaran, 2009). Analogous to *amh*, eSRS21 was observed in eel testis (Miura *et al*., 2002). *amh* transcripts were reported in both the gonads of zebrafish and medaka (Klüver *et al*., 2007; Rodríguez-Marí *et al*., 2005). An essential role of *ad4bp/sf-1* in testicular development has been shown in catfish (Murugananthkumar and Senthilkumaran, 2016). In zebrafish (*Danio rerio*) *ad4bp/sf-1* homologs (*ff1b* and *ff1d*) were expressed in Sertoli and Leydig cells (Chai and Chan, 2000). Similar genes were reported in medaka (Watanabe *et al*., 1999), chum salmon (*O. keta*) and (Higa *et al*., 2000) sockeye salmon (*O. nerka*). Significant role of *gata4* in testicular growth was showcased in catfish (Murugananthkumar and Senthilkumaran, 2016). *dax1* was reported (Wang *et al*., 2002) with significant expression during sex differentiation of the Nile tilapia (*Oreochromis niloticus*). In zebrafish and medaka isoforms of *wt1* was evident

in gonads (Klüver *et al*., 2009; Perner *et al*., 2007). In catfish (Murugananthkumar and Senthilkumaran, 2016), *wt1* regulates *gata4* and *ad4bp/sf-1* by influencing steroidogenesis. *wt1* was also reported in zebrafish mesoderm (von Hofsten and Olsson, 2005). In teleost, *foxl2* is abundantly expressed in ovary (Liu *et al*., 2007), specifically in GC of previtellogenic and vitellogenic follicles (Wang *et al*., 2004). *foxl2* modulates *ad4bp/sf-1* and ovarian aromatase in the Nile tilapia (Wang *et al*., 2007). Besides, *foxl2* might be a marker of (female) brain and ovarian development in catfish (Sridevi and Senthilkumaran, 2011). Androgens and estrogens are produced by steroidogenesis and the balance between them is vital for proper sexual development. *cyp19a1* is crucial for ovarian development in the Nile tilapia (Chang *et al*., 2005) and catfish (Chourasia and Joy, 2008; Rasheeda *et al*., 2010). Aromatase, the product of *cyp19a1* directs T to E_2 conversion and is decisive in regulating their balance (Fig. 6). In teleost, genetic regulation of gonadal steroidogenesis was reviewed (Rajakumar and Senthilkumaran, 2020).

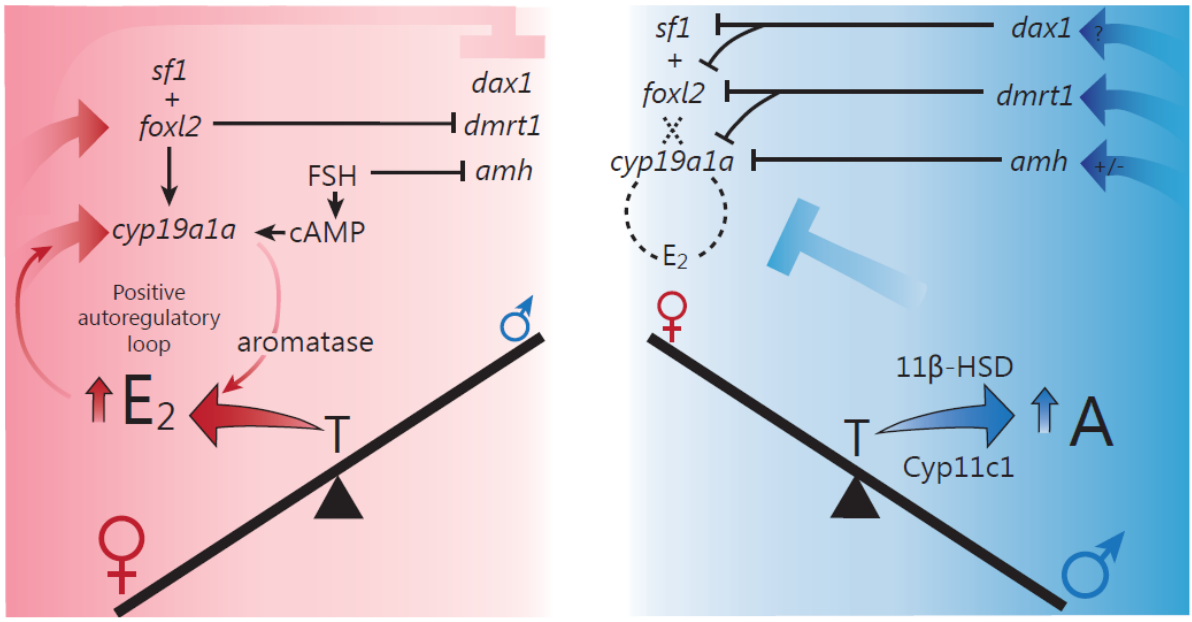

Fig. 6 Antagonistic sex-specific gene networks maintain sexual fate in fishes by promoting either an estrogenic or androgenic environment. Licensed for reuse from S. Karger AG, Basel; Todd *et al*., 2016. Sex. Dev. 10, 223–241.

SOX genes

SOX genes have been reported to play significant role in various processes such as chondrogenesis, organ growth, neural crest cell development, cell-type specification, central nervous system neurogenesis, germ layer construction and oligodendrocyte development (Betancur *et al*., 2010; Hong *et al*., 2005; Kiefer, 2007, Wegner, 1999). In addition, *SOX* transcription factors are also important for sex determination, gonadal growth and development (Graves, 1998).

Table 1. Classification of *SOX* genes

Subgroup	**Gene**
SOXA	*SRY*
SOXB1	*SOX1*
	SOX2
	SOX3
	SOX19
SOXB2	*SOX14*
	SOX21
SOXC	*SOX4*
	SOX11
	SOX12
SOXD	*SOX5*
	SOX6
	SOX13
SOXE	*SOX8*
	SOX9
	SOX10
SOXF	*SOX7*
	SOX17
	SOX18
SOXG	*SOX15*
SOXH	*SOX30*
SOXI	*SOX31*
SOXJ	*SOXJ*
SOXK	*SOX32*

SOX family genes are specific to animals and constitute to HMG superfamily, encoding ~80 aa DNA-binding domain with a conserved "RPMNAFMVW" motif (Bowles *et al*., 2000). They bind

to "(A/T)(A/T)CAA(A/T)G" sequence and classified (Zhang *et al*., 2018) into subgroups A–K (Table1). *SOX* genes also share structural domains distant from HMG among subgroup (Koopman *et al*., 2004). SOX protein expression is dynamic as well as tissue-specific (Cui *et al*., 2011). The target specificity is accomplished by complicated mechanisms involving coordinated cell-specific transcription along with spatio-temporal expressions (Kamachi *et al*., 1999, 2000; Wilson and Koopman, 2002). *SRY*, male determining factor in mice and human, was the first (Sinclair *et al*., 1990) documented *SOX* family gene (Gubbay *et al*., 1990).

Most *sox* genes are present in fish. s*ox9* is a requisite for testis development in teleost. Two *sox9* forms (Chiang *et al*., 2001) was identified in teleost like fugu, zebrafish, catfish, rice field eel, and the Japanese medaka, reported to have risen during whole genome duplication (Klüver *et al*., 2005). *sox9a* plays a role in testis differentiation while *sox9b* is essential during ovarian differentiation (Raghuveer and Senthilkumaran, 2010). *sox3* (Rajakumar and Senthilkumaran, 2014) regulates testicular development and recrudescence and binds to *hsd11b* promoter in catfish (Rajakumar and Senthilkumaran, 2016) and initiates testicular differentiation (Takehana *et al*., 2014) in the Indian rice-fish (*O. dancena*). The involvement of *sox5* in germ-cell regulation of medaka (Schartl *et al*., 2018) was evident. In medaka, *sox14, sox10, sox11, sox2*, and *sox7* were found in ovary, while *sox17*, *sox21* and *sox4* were expressed in both gonads (Cui *et al*., 2011). Galay-Burgos *et al*. (2004) have reported the evidence of *sox2*, *sox4, sox1*, *sox3*, *sox14*, *sox10*, *sox9*, *sox31*, and *sox17* in both testis and ovary of the European sea bass (*Dicentrarchus labrax*). In rice field eel (*Monopterus albus*) *sox14*, *sox1 and sox4* were evident (Zhou *et al*., 2002). Significance of *sox2* during testis development was reported in (Kumar *et al*., 2020) gold fish (*Carassius auratrus*) and in (Patra *et al*., 2015) the Indian major carp (*Labeo rohita*). Yu *et al*. (2018) observed that *sox8* is testis-biased while *sox2*, *sox7* and *sox10* were ovary-biased in the

Japanese flounder (*Paralichthys olivaceus*). In zebrafish and medaka (Voldoire *et al*., 2017) duplicated *sox* (*sox11, sox10, sox9, sox4* and *sox8*) genes were observed during embryogenesis. Kanda *et al*. (1998) detected *sox24* maternal transcripts in rainbow trout. *sox8* was evident in both the gonads of platyfish (*Xiphophorus maculatus*) while *sox9*, *sox11* and *sox4* were expressed only in testis (Voldoire *et al*., 2017). In zebrafish *sox9*, *sox11* and *sox9* were present in testis and ovary, whereas *sox4* was observed only in testis. On the other hand, in medaka *sox9* was expressed in both the gonads, while *sox11* and *sox4* were expressed in testis (Voldoire *et al*., 2017). In channel catfish, Zhang *et al*., (2018) observed *sox11*, and *sox3* in both ovary and testis, while *sox17*, *sox4*, and *sox9* were found only in testis. *sox* genes were expressed in large yellow croaker (*Larimichthys crocea*) during embryogenesis (Wan *et al*., 2019). In the Chinese sturgeon, (*Acipenser sinensis*) *sox1*, *sox14*, and *sox2*, were expressed in both the gonads, in ovary *sox3*, *sox21*, and *sox12* were evident while, in testis *sox18*, *sox5*, and *sox11* were observed (Yang *et al*., 2020).

Despite these findings, the significance of *SOX* family genes during sex determination/differentiation, gonadal development and function is yet to be understood in teleost. Hence, this thesis work intended to identify and examine the function of *SOX* family genes apart from those whose roles have been well-known. The recognition of gonadal DEG is important to understand teleostean gonadal differentiation process and a means to acquire *SOX* family genes.

Methods to identify DEG to obtain *SOX* genes

Conventionally, DEG were identified using qPCR. RNA fingerprinting with amplified restriction fragment polymorphism was used to view DEG (Bachem *et al*.,1996). Using DDRT-PCR, low transcript, DEG, and alternate splice variants could be detected (Steinau and Rajeevan, 2009). Though the technique is cost-effective, still very time consuming and laborious. Another means of DEG identification is subtractive hybridization. The above-mentioned techniques are difficult with

requirement of larger preliminary material (Diatchenko *et al*., 1999). Though microarray enables DEG identification with the real-time analysis of whole genome, the prerequisite of prior sequence data limits its usage (Yang, 2005). High-throughput sequencing is gaining popularity in gene expression quantification (Mortazavi *et al*., 2008). Further, RNA-Seq enables '*de novo*' transcriptome reconstruction (Costa-Silva *et al*., 2017) without reference sequence information. RNA-Seq is data highly reproducible, quantifies unidentified transcripts expression and also identifies isoforms (Habegger *et al*., 2010). In view of the above, the current study planned to perform gonadal transcriptome analysis using RNA-Seq to identify DEG.

Common carp

The common carp (henceforth referred to as carp), *Cyprinus carpio* has more (~50) *sox* genes than other fishes (Xu *et al*., 2014) being a suitable animal model for this study. Carp, belongs to the Cypriniformes (order) and Cyprinidae (family). *C. carpio*, is a differentiated gonochoristic teleost species following primary gonochoric pattern of sex differentiation (Devlin and Nagahama, 2002; Komen *et al*., 1992). They are found in several (olive green silvery yellow/silvery grey) colors, often used as ornamental fish in ponds of tourist attractions. Carp is a one of the major freshwater food fish in India, thus economically important. Further, carps are extensively researched (Hulata, 1995; Horvath and Orban, 1995) with its genome partially sequenced (Xu *et al*., 2014). This research thesis explored potential genes involved in carp sex differentiation to focus on *sox* family. Carps follow a seasonal reproductive cycle in the Southern part of India, spawning biannually during monsoon and winter (Routray *et al*., 2007). Four distinct phases of reproductive cycle of carp are preparatory, pre–spawning, spawning, and post–spawning (Fig. 7).

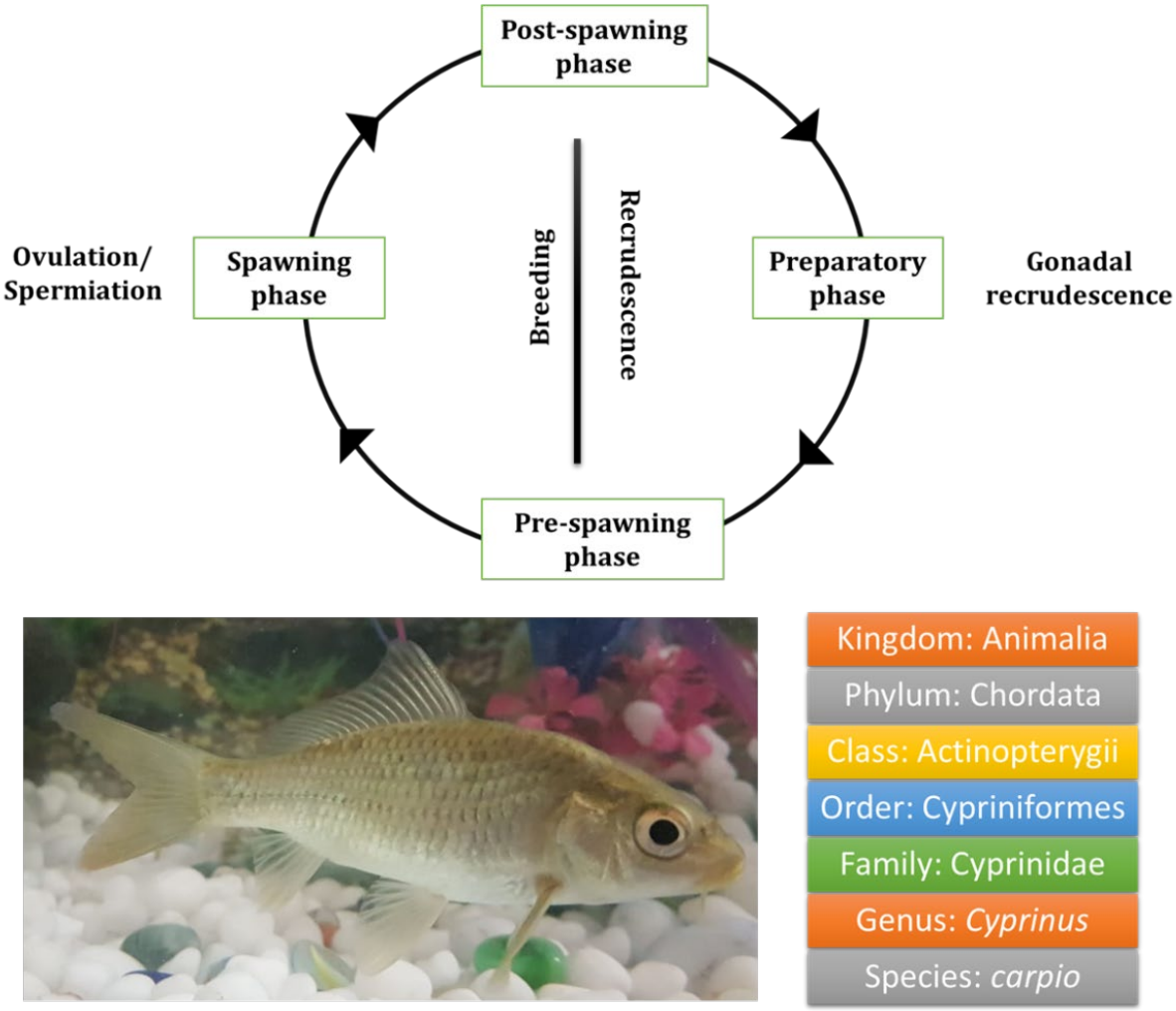

Fig. 7 Carp

Considering these, the present Ph.D. thesis work aimed to identify *sox* family genes involved in carp gonadal development by using a transcriptome analysis for DEG among the gonads. Nearly ten genes belonging to the *sox* family were identified from carp gonadal transcriptome. Expression profiling of *sox* family genes was done in different tissues and in gonads during the distinct phases of the reproductive cycle. To elucidate the significance of few genes of *sox* family, gene silencing using siRNA and its effect on other transcription factors, steroidogenic enzymes and certain gonad-related genes was also analyzed.

The findings of the present study are compiled into three major chapters for the Ph.D. thesis entitled, "**Identification and expression analysis of *sox* family genes in common carp: *sox30* and *sox19* regulate gonadal function**" with a general introduction and a consolidated summary as below mentioned.

Chapter 1 Identification of differentially expressed genes in gonads of the common carp by RNA sequencing-based transcriptome analysis: Expression profiling of *sox* family genes

Chapter 2 Significance of *sox30* along with *sox9a/b* in testicular steroidogenesis

Chapter 3 Understanding the role of *sox19* in ovarian function

Each chapter has separate bibliography in order to correlate the findings with earlier reports. All the abbreviations used in the thesis were listed and hence the same has been used without abbreviating again in chapters as wel as general introduction.

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Chapter 1

Identification of differentially expressed genes in gonads of the common carp by RNA sequencing-based transcriptome analysis: Expression profiling of *sox* family genes

ABSTRACT

Carp is a world-wide freshwater fish of eutrophic waters. *C. carpio*, have various reproductive traits, including early sexual maturity, that may make them excellent, large, realistic, aquaculture model species. In the present work, *de novo* assembly of gonadal (testicular and ovarian) transcriptomes from juvenile carp was performed to identify genes involved in gonadal development. A total of 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, were obtained from the immature testicular and ovarian transcriptomes, respectively. About 84,367 unigenes were constructed after removing redundancy involving the representation of transcripts in both gonadal transcriptomes. GO (39,171 unigenes), COG analysis (6,651 unigenes), and KAAS analysis (4,783 unigenes) were performed to identify potential genes along with their functions. Furthermore, 18,342 (testis) and 8,693 (ovary) SSRs were identified. Nearly 809 genes related to reproduction were identified, the sex-wise expression pattern of genes related to steroid synthesis, endocrine regulation, germ cell maintenance, and other factors related to gonadal differentiation was observed. About 298 DEG were identified, of which 171 and 127 genes were up-regulated in testis and ovary, respectively. qPCR was performed to validate the differential expression of selected genes in the testis and ovary. Interestingly, several genes belonging to the *sox* family were obtained from the transcriptome. Expression profiling of *sox* family genes namely, the tissue distribution and reproductive phase analysis were performed, which warranted the importance to analyze the significance of *sox30* and *sox19* in gonadal function. The present study identified certain important genes/factors involved in the gonadal development of *C. carpio* which may provide insights into the understanding of sex differentiation and gonadal development processes.

1 INTRODUCTION

Carp belonging to the family Cyprinidae is native to western Asia and was translocated or introduced throughout the world. Certain native wild populations were considered vulnerable to extinction in 2008 by the International Union for Conservation of Nature. The carp has become one of the most important food fishes with many strains and varieties in different parts of the world (Xu *et al*., 2012). There are abundant strains and local populations of carp found in India, including many hybrid populations. The Indian carp spawn in two peak breeding periods, from January to March and from July to August. Furthermore, at different stages, the gonads show discrete changes during the reproductive cycle. Due to its economic and ecological importance, different genetic and genomic studies were performed in the carp (Ji *et al*., 2012; Kongchum *et al*., 2010). Despite the reports being available on different aspects of reproduction and breeding (Routray *et al*., 2007), the molecular mechanisms underlying gonadal maturation and breeding of carp in tropical or subtropical climates have not been clearly understood. In comparison with the bisexual reproduction in higher vertebrates, teleosts have different approaches for sexual reproduction, like unisexuality (Schartl *et al*., 1995), hermaphroditism (Warner, 1984), and gonochorism (Dipper and Pullin, 1979). Besides, there are two mechanisms of sex determination, genetic and environmentally regulated (Janzen, 1995). Teleost fishes are an excellent model for studying the evolution of sex chromosomes as they have a broad range of sexual developmental systems, with an absence of morphologically distinct genders in most species. A master set of sex determination and gonadal phenotype-related genes have been identified in multiple fish species (Chen *et al*., 2014; Hattori *et al*., 2012; Kamiya *et al*., 2012; Matsuda *et al*., 2002; Myosho *et al*., 2012; Nanda *et al*., 2002; Raghuveer and Senthilkumaran, 2009; Yano *et al*., 2012). Several conserved genes playing critical roles in the sexual development of mammals were also analyzed in fish, including

ad4bp/sf-1, *dax1*, *foxl2*, *gata4*, *mis*, *sox3*, *sox9a*, *sox9b*, *StAR, wt1, cyp11b1*, *cyp17*, *cyp19a1*, *hsd3b*, and *hsd11b* (Raghuveer *et al*., 2011; Sandra *et al*., 2010). These genes act together in a complex network to direct gonadal development. Besides, hormones and various biological pathways, such as estrogen signaling pathway, steroid hormone biosynthesis (Baroiller *et al*., 1999), *TGF-β* signaling pathway (Liu *et al*., 2017), and *wnt* signaling pathway (Amberg *et al*., 2013; Prathibha and Senthilkumaran, 2017) also play a vital role in the gonadal differentiation. Although much is known about the process of sex differentiation in fish, the precise mechanisms of sex differentiation, as well as those involved in primary sex determination, remain undefined in several teleosts (Devlin and Nagahama, 2002). Fish gonadal sex differentiation shows varied strategies and physiological regulations compared to mammals (Baron and Guiguen, 2003). The differentiation of bi-potential gonad to either testis or ovary depends on the regulation of the steroidogenic pathway. In lower vertebrates, sex steroids (androgens and estrogens) play a critical role in gonadal differentiation (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). However, the mechanism of action of these steroids on gonad sex differentiation is not well understood. Unlike sex-determining systems, the genes involved in gonadal differentiation appear to be relatively conserved. Nevertheless, studies on the functional connections of these genes to relevant pathways are minimal in fish. Novel sex-related genes are required to elucidate the complex mechanisms of gonadal development in fishes. Over the last decade, NGS technologies have provided operative tools for high throughput sequencing, which has enhanced the efficacy and speed of gene discovery. Compared to whole-genome sequencing, NGS via RNA-Seq affords a cost-effective approach to retrieve transcriptome sequences and molecular markers that can reveal DEG associated with phenotypic traits and gender. DEG between testis and ovary gives prospective information to recognize and understand the molecular basis of gonadal development

and growth. Groups of gonadal development-related genes have been revealed by scrutiny of sex-biased transcriptomes in several species including, as examples, gilthead sea bream (Calduch-Giner *et al*., 2013), the Nile tilapia (Tao *et al*., 2013), the Japanese flounder (Zhang *et al*., 2016), the Russian sturgeon, *A*. *gueldenstaedtii* (Hagihara *et al*., 2014), the Southern bluefin tuna, *Thunnus maccoyii* (Bar *et al*., 2016), and the yellow catfish, *Pelteobagrus fulvidraco* (Lu *et al*., 2014). The transcriptome of carp (Ji *et al*., 2012) has been sequenced and assembled employing NGS platforms, and differences between the ovary and testis transcriptomes of adult carp from the China's yellow river have been explored using suppression subtractive hybridization (Chen *et al*., 2015). However, ideally, such differences will be fully explored only by using a comprehensive set of transcriptomes from every tissue through each life stage across every major habitat (Ji *et al*., 2012). Furthermore, complete annotation and functional validation of the carp genome or transcriptome have not been done concerning gonadal stage and function. Hence, in this study, gonadal transcriptomes of juvenile carp were sequenced to obtain a critical overview of genes potentially involved in gonadal growth and development. Besides, DEG and biological pathways were also identified by comparing testis and ovary transcriptomes. These data provide information on marker genes found expressed in gonads sex-wise at specific reproductive stages, genes related to steroid synthesis, endocrine regulation, germ cell maintenance, and other factors, which are crucial for acquiring valuable insights into mechanisms of gonadal differentiation in fish. The present study also identified a major group of reproduction-related genes, potential players in gonadal development, and characterized SSRs to be employed for genetic improvement purposes. The large number of reproduction-related genes identified in this study also provide direction for future genetic and genomic research in carp.

2 MATERIALS AND METHODS

2.1 Animals and sampling

Carps used for this study were reared at the aquaculture facility of KVAFSU, Bangalore, India. Nearly 2,000 offsprings were raised in a 2000 m^2 pond and fed four times daily under a standard feeding regime (3% feed/bodyweight ratio daily). The oxygen level was retained at 3 mg/L or above. Animals were procured and during acclimatization, fishes were maintained under natural photo-thermal conditions by feeding commercial carp food *ad libitum* and kept in 1 m^3 tanks with filtered tap water, until experimentation. Fifteen juvenile carps, 110 to 130 dph were randomly selected and gonadal tissues were dissected out. A portion of the gonadal tissue was fixed in Bouin's fixative (15:5:1, saturated picric acid, formaldehyde, and glacial acetic acid) for histological observation, and the remaining tissue was snap-frozen in liquid nitrogen and stored at -80°C for RNA-Seq. To perform tissue distribution analysis, various tissues from adult carp (n=5), males (muscle, liver, testis, kidney, and brain), and females (ovary) were collected during the late preparatory phase of the reproductive cycle, snap-frozen in liquid nitrogen and stored at -80°C. Carps follow a seasonal reproductive cycle in the Southern part of India, spawning biannually during monsoon and winter (Routray *et al*., 2007). Gonads from adult carp were collected (n=5) during the seasonal reproductive cycle flanking preparatory, pre-spawning, spawning, and resting phases for quantifying gene expression. Following anesthetization with 100 mg/L of MS222 (Sigma, MO, USA) in mild ice-cold water, fishes were sacrificed, tissues were collected and kept at -80 °C until experimentation. Fish sampling was performed following the general procedures and with the endorsement of the IAEC, University of Hyderabad (CPCSEA, Inst. Reg# No#151/1999 dt.22.07.1999).

2.2 Histology

The male and female gonads of juvenile carp, 110 to 130 dph were fixed in Bouin's solution, dehydrated, and embedded in paraplast (Sigma). Sections of 5 μm were cut using a microtome (Leica, Wetzler, Germany), stained with hematoxylin-eosin and photomicrographs were taken using Olympus CX41 bright field light microscope (Olympus, Tokyo, Japan).

2.3 Total RNA isolation and cDNA library construction

The gonads of the juvenile carp were staged by histology. Total RNA was prepared from immature gonad tissue (testis and ovary) samples of carp individually using TRI reagent (Sigma). The concentration and integrity of RNA were examined with Ribogreen method using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA with RIN values >7.0 were selected. Equal amounts of the high quality RNA from gonads were then pooled together for mRNA isolation, cDNA synthesis, and sequencing. Library preparation was performed following NEB Next Ultra RNA Library Prep Kit (New England Biolabs, USA) following the manufacturer's instructions. In brief, one μg of total RNA was used to isolate mRNA using NEB Next Poly (A) mRNA Magnetic Isolation Module which was time-dependently fragmented using RNA binding buffer provided in the kit. Then first strand cDNA was prepared using ProtoScript II Reverse Transcriptase, following which the second strand cDNA was synthesized, purified using AMPure XP Beads, end-repaired using NEBNext End Prep Enzyme Mix, and NEBNext Adaptor ligated using Blunt/TA Ligase. These adapter-ligated fragments were subjected to 12 cycles of enrichment and barcoding and the products were purified using Ampure XP beads.

2.4 Sequencing and assembly

Sequencing was done using Illumina Next Seq 500 Sequencer with 75 PE chemistry. The paired-end reads obtained after massive parallel sequencing were subjected to several filters to obtain the

potential reads that are specific to identify the transcripts. First, a quality check using FASTQC was performed. The low quality reads (mean Phred_score<20) were removed and the reads with adapter sequences were trimmed. If the length of the trimmed read was below 50 bp, then such reads were discarded. All processed reads were then assembled into transcripts without any reference (*de novo*) using Trinity software (trinityrnaseq20140413p1).

2.5 Functional annotation and ontology

The homology search was done for assembled transcripts' sequences against *T. rubripes*, *Danio rerio*, and (partially annotated) *C. carpio* protein sequences downloaded from the UniProt database (http://www.uniprot.org/uniprot/). NCBI-blast2.2.29 was used for homology search between the sequences. BLASTX program was used to perform similarity searches. Gene annotation was assigned to the RNA-Seq transcripts based on the top BLAST hit. GO annotation analysis was then performed. The annotation results were categorized into biological process, molecular function, and cellular components. KEGG pathways were assigned to those assembled contigs using the online KAAS server (http://www.genome.jp/tools/kaas). COG annotation for transcripts of testis and ovary were based on homology (https://www.ncbi.nlm.nih.gov/COG/).

2.6 Identification of DEG

The reads for both gonadal tissues were separately aligned to the unigene sequences of *C. carpio* and read count profiles were generated. DESeq "R" package was used for differential gene expression. The package DESeq provides methods for testing for differential expression by use of the negative binomial distribution and a shrinkage estimator for the distribution's variance. The threshold for the P-value was set at 0.05. DEG between male or female gonads were identified.

2.7 SSRs

SSRs were identified by mapping all clean reads to the assembled transcript reference using the MIcroSAtellite identification tool (MISA, version 1.0, http://pgrc.ipkatersleben.de/misa/). The minimum repeat number used for every unit was ten for mononucleotide, six for dinucleotide, five for tri-, tetra-, penta- and hexa-nucleotide microsatellites.

2.8 Experimental validation by qPCR

Few candidate DEG were validated by qPCR to verify the expression profile obtained from the transcriptome data. The tissue distribution pattern of different transcripts was analyzed using the SYBR Green detection method. For this, total RNA was extracted from different tissues (brain, kidney, liver, muscle, ovary, and testis) of adult carp using the TRI (Sigma) method. Following DNase I treatment, cDNA synthesis (OneScript® Plus cDNA Synthesis Kit, Applied Biological Materials Inc., BC, Canada) was carried out as described in the manufacturer's manual. Each reaction was performed in triplicate for five distinct biological samples using gene-specific primers designed cautiously to prevent the amplification of genomic DNA with one of the primers straddling the exon-exon boundary. The gene-specific primers (Table 1) were designed based on the sequences obtained from transcriptome analysis and the NCBI GenBank database. The manufacturer's manual was shadowed for amplification (Power SYBR™ Green PCR Master Mix, Thermo Fisher Scientific, MA, US) in ABI Step One Plus real time PCR (Thermo Fisher Scientific). Ct value was estimated from the exponential phase of the PCR amplification and the gene expression was normalized using *18S rRNA* (reference gene) to obtain ΔCt (Ct of target gene - Ct of endogenous control). From the preliminary experiments of this study, as per the validation done based on Radonic´ *et al*. (2004), *18S rRNA* (FJ710827.1) normalization was found to be efficient and constitutive with the lowest transcription range compared with other reference genes

such as *β-actin* (M24113.1) and *gapdh* (JX244278.1). For estimating fold changes in gene expression, $2^{-\Delta Ct}$ was used.

Table 1: Primers used for qPCR

S. No.	Gene name/symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
1	*18s rRNA*	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG
2	*ad4bp/sf-1*	CGGACCTGGAAGAGTTGT	GGTTCTCTTGGCATGCAG
3	*amh*	CCGTGATGAGCAAAGGACACC	CAAAGAGACAATGTTTGCAC
4	*dax*	CCAGATGTTGCAGGGCTGC	CCTCCATGTTGACAGCGCC
5	*dmrt1*	GCAGCCCAGGTGGCGTTA	CGTGCTGTCAGTGTGCCCTC
6	*egr2b*	GACGGCAGGAGGAGAGT	TCAACCAGGAAGCTGGTC
7	*gata6*	CCCTGCCGACATGTATCAGAC	GGCTGCGACGACACGCTG
8	*fbox43*	CTACCGGTTCGGCCAGGTGTC	GTGTGTGTGTGCGGCGTGCG
9	*foxk2*	GGGCTGGCAAAACTCGAT	GCTGGAGCGCTCCTGGAT
10	*mapk*	TGGGAACCCCTTCTCTGG	CAATGGTGTGTTCCCGCT
11	*sox5*	GGACAGCCTGGTGGAGAAAG	CTGCTGTTGTCGTGCAATC
12	*sox6*	CCGGGGAAGGAACAGCAAC	GTGCCTGCTCCTCATAATATG
13	*sox9a*	GGTGAACAACGGCCAGAG	GACTGGCCTGAGTGCTCG
14	*sox9b*	GAACGGCCAGAGCGAGAG	GACCCTGGGACTGACCTG
15	*sox11*	GCACCAAGTGGAATAAACACCTG	CTGATTTTCTTGCTGCTAGTCAC
16	*sox13*	GGCCAAAGACGAAAGACGG	GGCCGGGGTTTGTATTTGTAG
17	*sox18*	GATGCTGGGTCAATCCTGG	CGATGTGGCGAGTAGGCATC
18	*sox19*	GGCACAGGAGAACCCCAAAATG	CTTCATGAGGGCTTTGGTC
19	*sox21*	GGTAAGGTTCATGTTGAGCTTTG	CATTACTCAATGCTCGGGTATC
20	*sox30*	CAGAGCAGTCTAAATTCTCCAGC	GGCTAGAAGAGCTTGAACATGG
21	*zp2*	CCCTGTGGATGAGTCTTC	GTACCATCTGCTGGGGTC
22	*fzd8*	CGCGCTGCCGAGGATGAGTA	GCTGCTTCACCTCCGCTATG
23	*wt1*	CCAGGAATCAGGGTTACGGCAC	GCCTGACTTCCTGTACAGCTG

3 RESULTS

3.1 Histological observation

The maturation level of gonads was histologically determined. Upon observation, the testis (Fig. 1A) showed the presence of primary spermatogonia, secondary spermatogonia, and spermatids/sperms indicating the progression from meiotic to spermiogenic phase of

spermatogenesis. The ovary showed several nucleoli appearing at the periphery of the nucleus indicating the perinucleolar stage (Fig. 1B) of primary oocyte growth.

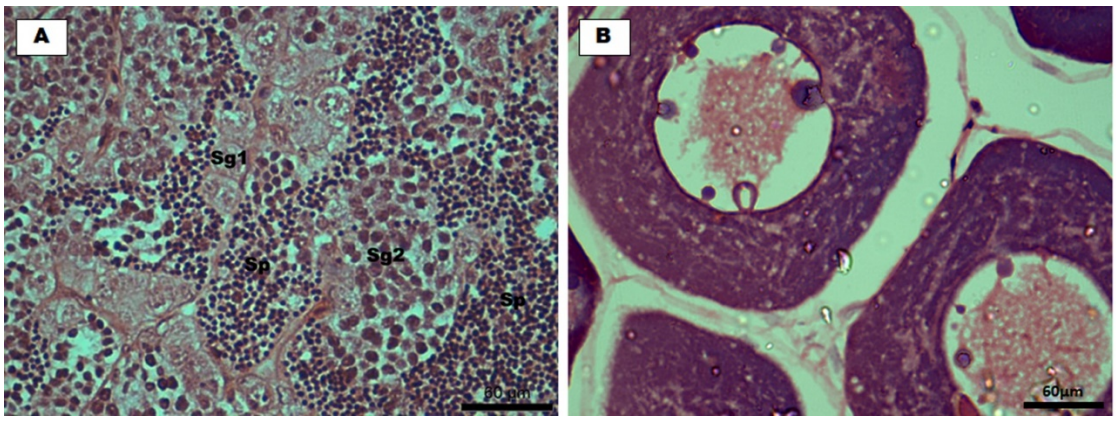

Fig. 1 Histology of gonads A-Immature testis showing Sg1: Primary spermatogonia, Sg2: Secondary spermatogonia, Sp: Sperms/spermatids; B-Immature ovary at perinucleolar-oil droplet stage (Scale: 60 µm).

3.2 Sequencing and reads assembly

A total of 17.06 and 14.87 million paired-end reads with 94.1% and 93.6% high quality bases (Phredscore > 20) were generated from high throughput sequencing of the testis and ovary cDNA libraries, respectively. The transcriptome sequences generated from this study have been deposited in NCBI's GEO Series accession number GSE112157. Nearly, 81,757 and 43, 257 transcripts with average lengths of 769 and 856 bp, and N50 of 1364 and 1453 bp, were obtained after *de novo* assembly for testis and ovary, respectively (Table 2). From these, 84,367 unigenes were constructed after removing redundancy which was a representation of all testicular and ovarian transcriptomes. The total size of the transcriptome was 62.9 and 37 Mbp for testis and ovary, respectively. All transcripts longer than 201 bp were selected and nearly 18,472 and 12,574 transcripts longer than 1000 bp of testis and ovary, respectively were obtained. About, 62,231

unigenes (50%) showed significant hits compared to known proteins. Of these, only 39,171 unigenes could be fully annotated with the available GO data.

Table 2. Summary of immature *C. carpio* gonadal transcriptome.

Transcriptome Assembly	**Testis**	**Ovary**
Transcripts generated	43,257	81,757
Maximum transcript length	14,732 bp	14,333 bp
Minimum transcript length	201 bp	201 bp
Average transcript length	856 bp	769 bp
Total transcripts length	37 Mbp	62 Mbp
Transcripts > 200 bp	43,257	81,757
Transcripts > 500 bp	21,462	34,010
Transcripts > 1000 bp	12,574	18,472
Transcripts > 10 Kbp	2	3
N50 value	1453 bp	1364 bp
Number of reads used	1,38,84,178	1,01,99,309
Total number of reads	1,62,67,344	1,39,16,372
Percentage of reads used	85.35	73.29

3.3 Unigene annotation

The unigenes with counterparts in public protein databases were annotated with GO, which offers a dynamically organized vocabulary and classified relationships to symbolize information regarding the categories of Biological process, Cellular component, and Molecular function. Analysis of GO term distribution showed that 'regulation of transcription, DNA templated [GO: 0006355]', 'integral component of membrane [GO: 0016021]', and 'ATP binding [GO: 0005524]'

were the most common annotation terms within the three GO categories, respectively. For the Biological process, 'regulation of transcription' (1745 unigenes) and 'transcription' (1668 unigenes) were the predominant groups. Under the Cellular component, the 'integral component of membrane' (12405 unigenes) represented the most abundant classification followed by 'nucleus' (6951 unigenes). Under the Molecular function, 'ATP binding' (6821 unigenes) and 'zinc ion binding' (4686 unigenes) represented nearly half of the category (Fig. 2).

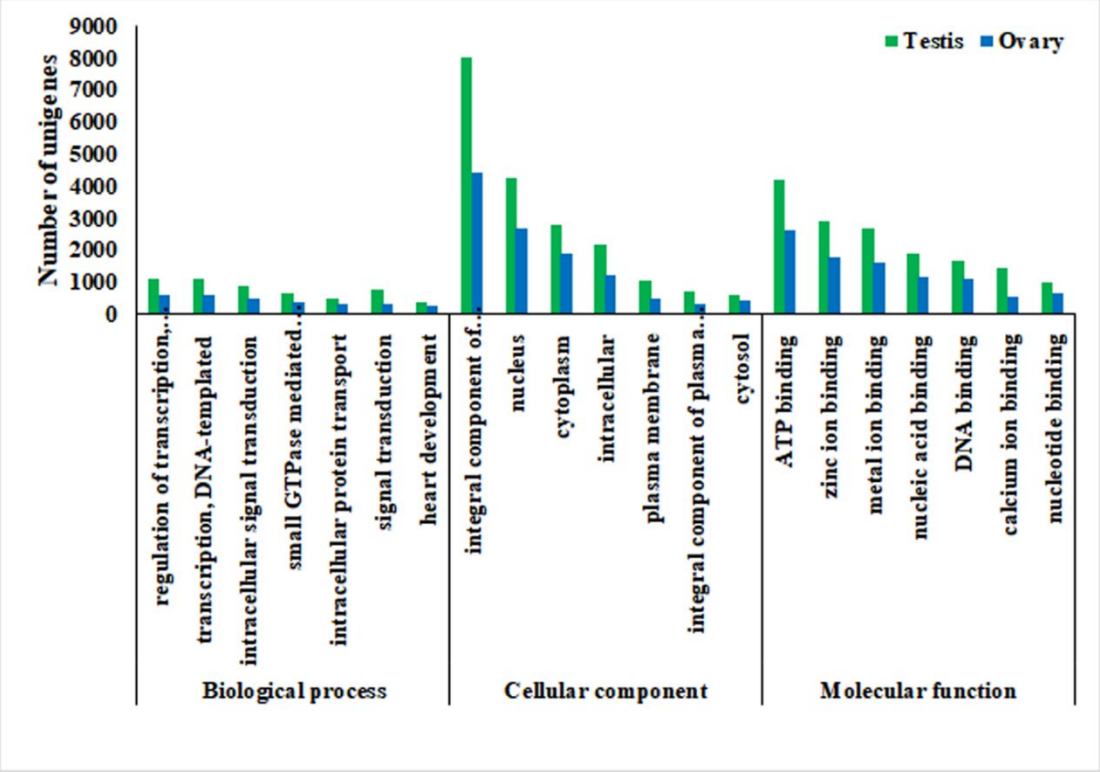

Fig. 2 GO analysis of the *C. carpio* testicular and ovarian transcriptomes of immature carp. The distribution of GO terms portrayed in the three categories: Biological process, cellular component, and molecular function.

To conduct further functional prediction and classification, all unigenes were compared with proteins from the COG database. In total, 6,651 unigenes were classified into 26 categories (Fig. 3). The top three classifications are general function prediction only (1089 unigenes), signal

transduction mechanisms (813 unigenes); and translation, ribosomal structure, and biogenesis (760 unigenes). Following the most abundant three groups were post translational modification, protein turnover, chaperones (674 unigenes), and amino acid transport and metabolism.

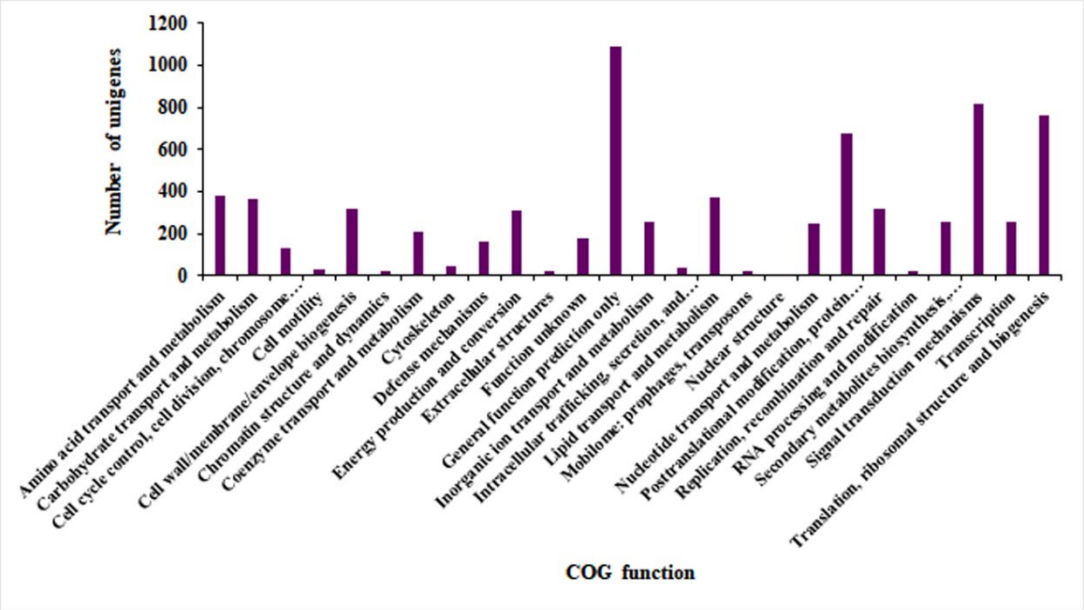

Fig. 3 COG classification of *C. carpio* testicular and ovarian transcriptomes of immature carp.

KAAS was used to perform pathway analysis for functional categorization. Enzyme Commission (EC) numbers were assigned with 3,012 enzyme codes for 4,782 unigenes. Pathways were categorized into 6 main groups (Table 3), metabolism (1440 unigenes), organismal systems (175 unigenes), environmental information processing (917 unigenes), genetic information processing (1511 unigenes), cellular processes (697 unigenes), and human diseases (42 unigenes). The majority of the mapped unigenes represented (Fig. 4) signal transduction (13.1%, 627 unigenes), followed by folding, sorting, and degradation (11.7%, 563 unigenes) and translation (11.2%, 538 unigenes). KAAS annotation identified 96 genes related to reproduction, distributed in 7 pathways (Table 4). These genes covered the major processes of reproduction, including the *wnt* signaling

pathway, GnRH signaling pathway, steroid biosynthesis, oocyte meiosis, steroid hormone biosynthesis, neuroactive ligand-receptor, and insulin signaling pathway.

Table 3. KAAS mapping for the gonadal transcriptome of *C. carpio.*

KAAS categories represented	Number of KO	Unique sequences
Metabolism		
• Amino acid metabolism	104	140
• Biosynthesis of other secondary metabolites	1	1
• Carbohydrate metabolism	126	183
• Energy metabolism	105	170
• Glycan biosynthesis and metabolism	128	174
• Lipid metabolism	145	217
• Metabolism	119	180
• Metabolism of cofactors and vitamins	76	109
• Metabolism of other amino acids	16	47
• Metabolism of terpenoids and polyketides	18	25
• Nucleotide metabolism	123	188
• Xenobiotics biodegradation and metabolism	5	6
Cellular Processes		
• Cell growth and death	93	142
• Cell motility	37	60
• Cellular community	44	84
• Transport and catabolism	226	411
Organismal Systems		
• Circulatory system	22	30
• Development	5	16
• Endocrine system	40	67

• Immune system	42	56
• Sensory system	5	6
Human Diseases		
• Endocrine and metabolic diseases	4	7
• Infectious diseases	19	35
Genetic Information Processing		
• **Folding, sorting, and degradation**	327	563
• **Replication and repair**	120	149
• **Transcription**	143	261
• **Translation**	339	538
Environmental Information Processing		
• **Membrane transport**	19	22
• **Signal transduction**	371	627
• **Signaling molecules and interaction**	190	268
Total	**3012**	**4782**

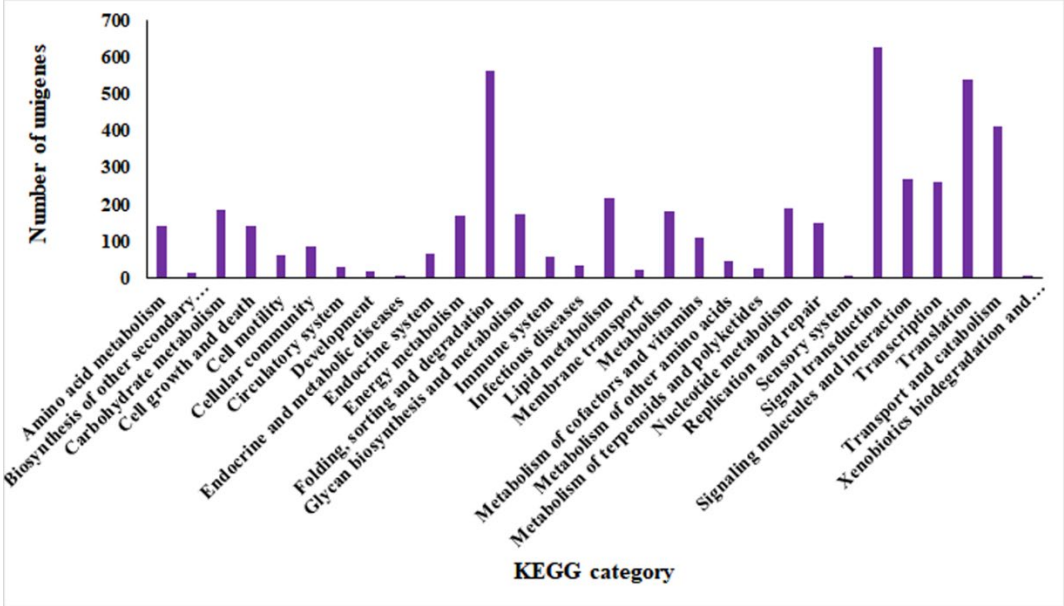

Fig. 4 KEGG categorization of *C. carpio* testicular and ovarian transcriptomes of immature carp.

Table 4: Reproduction-related pathways identified in carp gonadal transcriptome.

Description	**Number of genes in the pathway**
Wnt signaling pathway	47
Steroid biosynthesis	14
Oocyte meiosis	12
Insulin signaling pathway	10
Steroid hormone biosynthesis	8
GnRH signaling pathway	3
Neuroactive ligand-receptor interaction	2

3.4 Identification of SSRs

A total of 18,342 and 8,693 SSRs were finally obtained from the testis and ovary, respectively. Within SSRs in the testis, the most abundant type of repeat motif was mono (10938), di-nucleotide repeats (3748), followed by tri (2127), quadra- (194), penta- (6), and hexa-nucleotide (4) repeat units (Fig. 5). Within SSRs in the ovary, the most abundant types of repeat motif were mono (4805) and di-nucleotide repeats (1919), followed by tri- (1298), quadra- (87), penta- (13), and hexa-nucleotide (1) repeat units (Fig. 6A). The most abundant repeat motif in SSRs (Fig. 6B) was A/T (18115), followed by AC/GT (3792), AG/CT (1603), AT/AT (1281), and ATC/ATG (787).

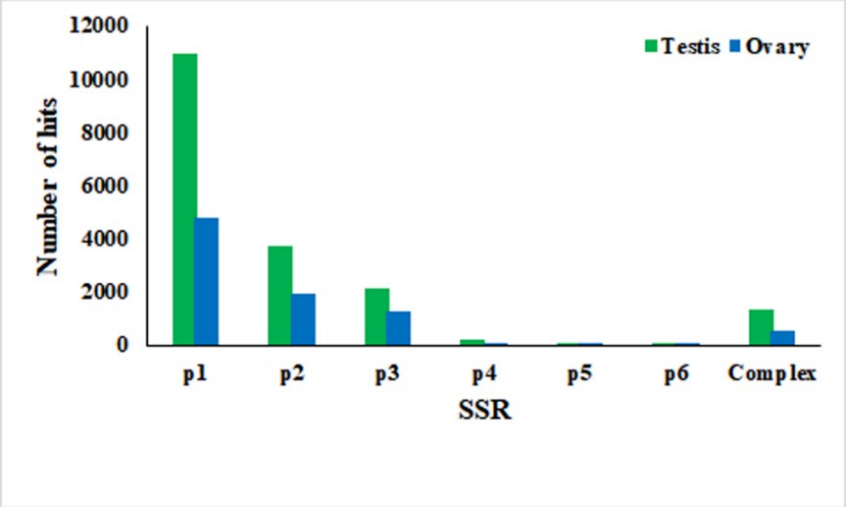

Fig. 5 Distribution of SSRs identified from carp testis and ovary transcriptomes of immature carp.

A

Description	Ovary	Testis
Total number of sequences examined	43257	81757
Total size of examined sequences (bp)	37049686	62930191
Total number of identified SSRs	8693	18342
Number of SSR containing sequences	7419	15115
Number of sequences containing more than 1 SSR	1078	2594
Number of SSRs present in compound formation	570	1326

B

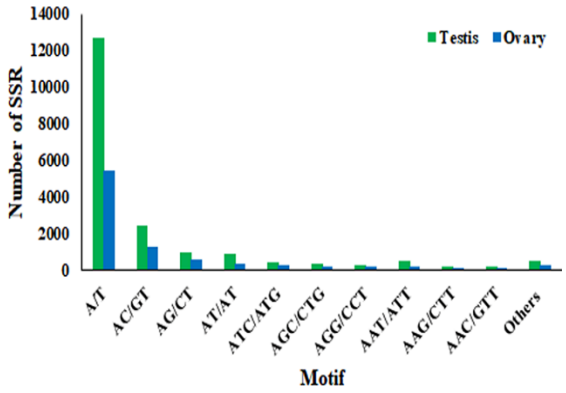

Fig. 6 A. Statistics of SSRs from the gonadal transcriptome of immature *C. carpio*. B. Frequency distribution of SSRs based on motif sequence types.

3.5 DEG

Differential expression between testis and ovary were analyzed to identify the genes involved in sex differentiation. Based on the applied criteria (twofold or greater change and p <= 0.05), 171 unigenes were showing higher expression in the testis than in the ovary, and 127 unigenes showed higher expression in the ovary than in the testis. There was no significant difference in the

expression of most unigenes between testis and ovary. To visualize comprehensive similarities and differences in gene expression between testis and ovary, a scatterplot was generated using R package and clear differences in gene expression between testis and ovary could be observed (Fig.7). The DEG report has been uploaded in NCBI's GEO GSE112157.

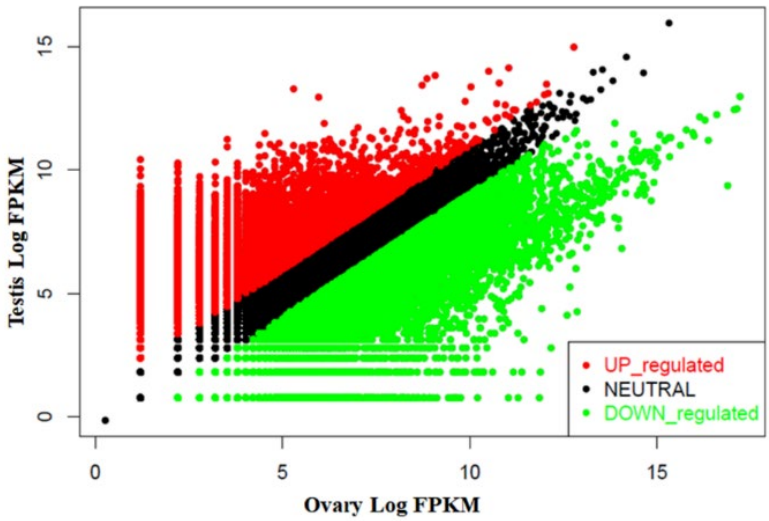

Fig. 7 Analysis of the DEG between the (immature) testis and ovary from the gonadal transcriptome of *C. carpio.* FPKM (fragments per kilobase of transcript per million mapped reads).

3.6 DEG validation in gonads

Twelve DEG such as *amh*, *egr2b*, *dax1*, *dmrt1*, *fbox43*, *foxk2*, *fzd8*, *gata6*, *mapk*, *sox9a*, *wt1*, and *zp2* related to sex differentiation, were chosen to verify the changes in their expression levels in gonads. Expression of *amh*, *dax1*, *dmrt1*, *gata6*, *sox9a*, and *wt1* was higher in testis, whereas *egr2b*, *fbox43*, *foxk2*, *fzd8*, *mapk*, and *zp2* were found to be elevated in the ovary, (Fig. 8). These results reiterate the differential gene expression pattern observed in gonadal transcriptome analysis.

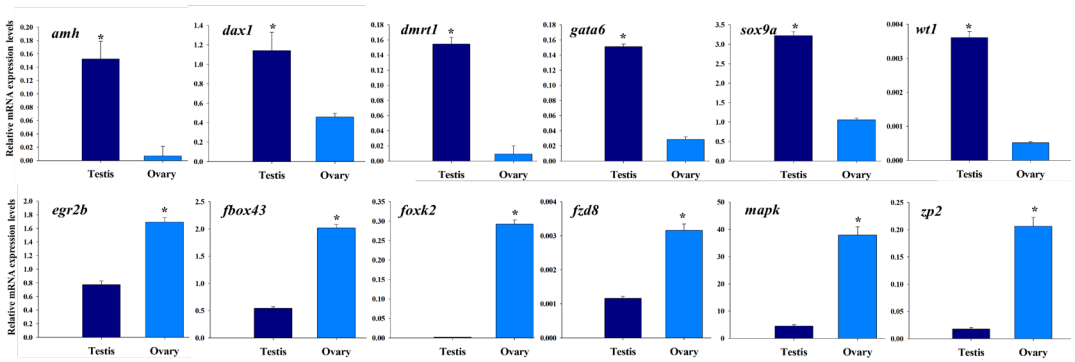

Fig. 8 Validation of immature *C. carpio* gonadal tissue transcriptome results by qPCR using 12 selected DEG in testis and ovary. qPCR fold changes are normalized by changes in 18SrRNA values ($P < 0.05$; ANOVA followed by Student–Newman–Keuls' test). All data from qPCR studies were expressed as mean ± SEM.

3.7 Identification of reproduction-related genes

Nearly, 809 genes related to reproduction were identified by literature supported searching. The gonadal transcriptome data was used to profile the expression of several reproduction-related genes. Sex steroid synthesis related genes such as *star*, *hsd3b7*, *hsd17b*, *hsd20b2*, *cyp19a*, and *er* showed higher expression levels in the ovary than in the testis. The exceptions are *hsd11b*, and *ar* which showed higher expression in testis (Fig. 9A). Genes involved in various gonadotropin pathway receptors such as *lhcgr*, *fgfr1a2*, *tac3a*, *notch2,* and *fshr*, *prlhr2a*, *kiss2* were moderately over-expressed in the testis and ovary, respectively (Fig. 9B). Some of the genes involved in germ cell maintenance were highly expressed in the ovary, namely *vasa*, *pou5f1*, *ipo4*, *pcna*, and *notch1* and other germ cell-related genes like *sycp3*, *piwi*, *plzf*, *cxcr4*, and *cxcl12* showed higher expression in testis (Fig. 9C). Another set of genes that were found to be over-expressed in testicular tissue included various factors related to gonadal differentiation such as *igf1ra*, *wt1*, *rspo1*, *ctnnbip1*, *tcf3a*, *msl1b*, and *atm* while *fem1b* and *smad3b* showed higher expression in the ovary (Fig. 9D).

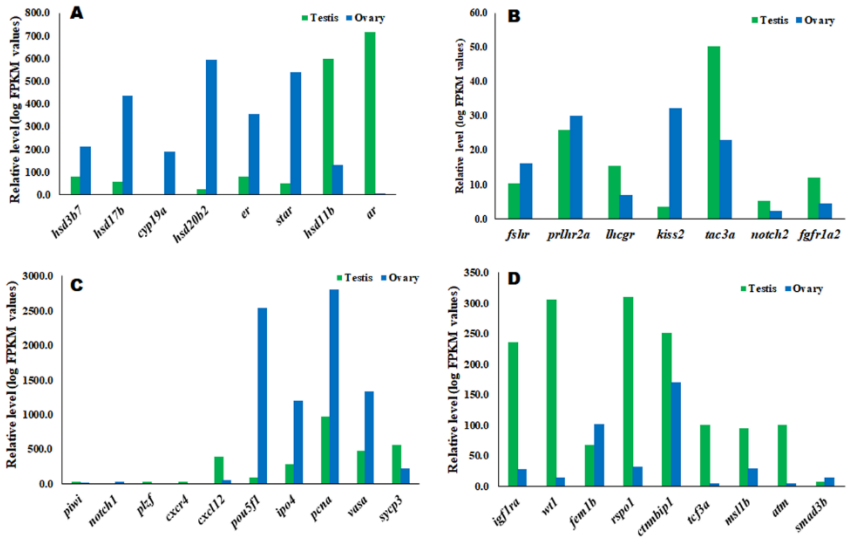

Fig. 9 Relative levels of transcripts of genes potentially involved in gonadal differentiation in juvenile carp (A-steroid synthesis, B-endocrine regulation, C-germ cell maintenance, D-other factors).

3.8 Identification and expression profiling of *sox* family genes

About ten genes, a representative from each of the sub-group (B-H) of the *sox* family were also obtained from the transcriptome analysis and few of them showed differential expression between testis and ovary (Fig. 10).

Sub-group	*Gene*
soxB1	*sox19*
soxB2	*sox21*
soxC	*sox11*
soxD	*sox5*
	sox6
	sox13
soxE	*sox9a*
	sox9b
soxF	*sox18*
soxH	*sox30*

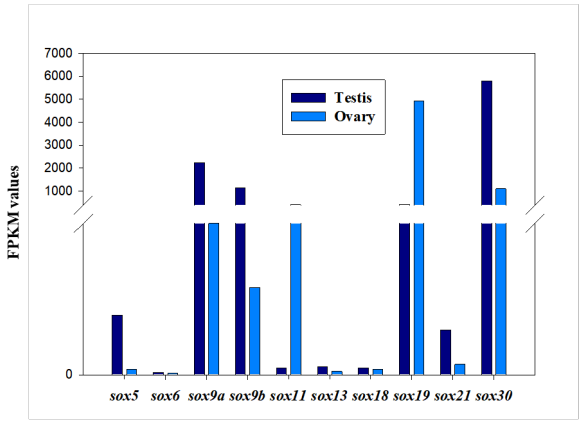

Fig. 10 Relative levels of transcripts of *sox* family genes obtained from the transcriptome.

Primers were designed based on the sequences obtained from transcriptome and qPCR was performed. Tissue distribution analysis revealed that the expression of *sox* family genes was ubiquitous in the tissues (n=5) analyzed (Fig. 11). The expression of *sox5* was high in gonads, while *sox6*, *sox13*, *sox18*, and *sox21* showed varied expression in the tissue distribution analysis. The expression of *sox9a/b* was high in the brain followed by the testis, whereas the predominant expression of *sox30* was evident in the testis followed by the ovary. The expression of *sox19* was high in the brain followed by the ovary, while *sox 11* showed higher expression in the ovary as compared to other tissues analyzed.

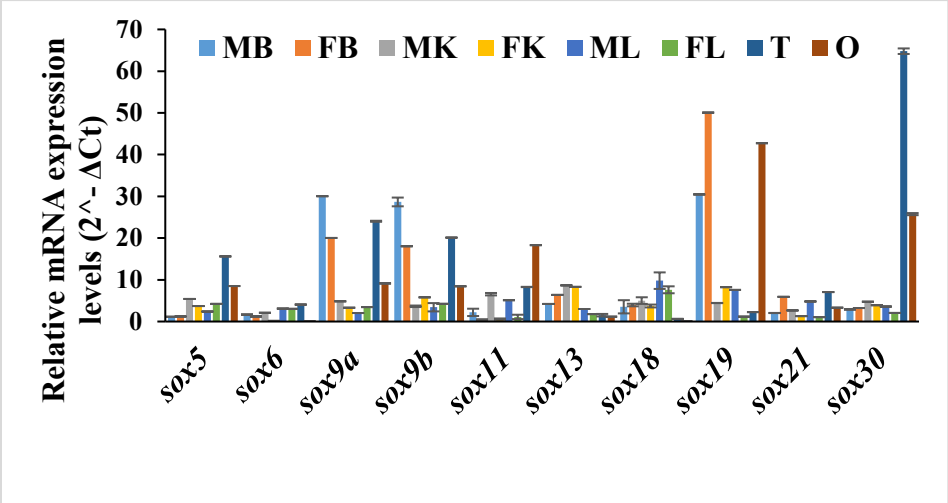

Fig. 11 Tissue distribution analysis of *sox* family genes. Abbreviations: MB-male brain, FB-female brain, MK-male kidney, FK-female kidney, ML-male liver, FL-female liver, T-testis, O-ovary. All other details are the same as Fig. 8.

Analysis through the testicular reproductive cycle of carp displayed significant expression of *sox30* during pre-spawning ($P < 0.05$) followed by spawning in comparison with the post-spawning phase (Fig. 12). Notable expression of *sox5*, *sox9a/b* was observed in pre-spawning and spawning phases.

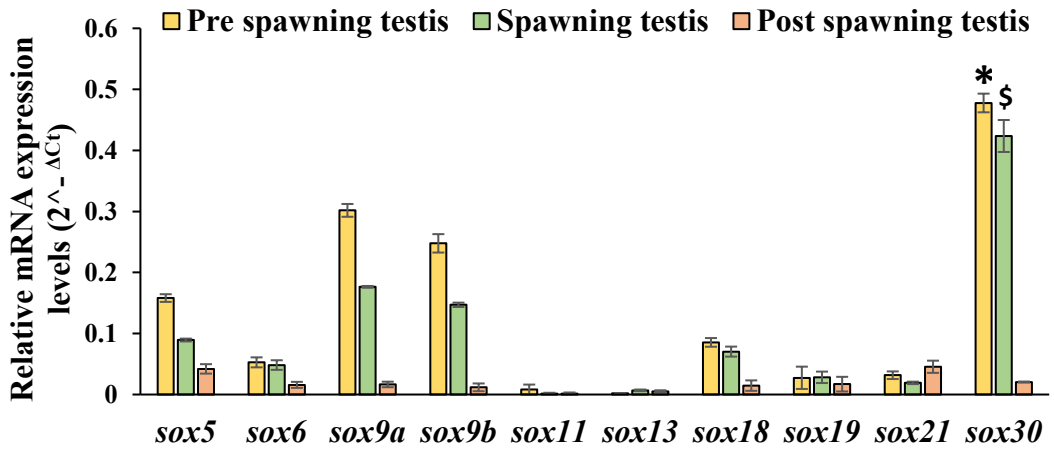

Fig. 12 Testicular reproductive phase analysis of *sox* family genes. All other details are the same as Fig. 8.

Analysis through the ovarian reproductive cycle of carp exhibited significant high expression of *sox19* in pre-spawning ($P < 0.05$) and spawning, and minimal expression during the post-spawning phase (Fig. 13). Notable expression of *sox11 and sox30* was observed in pre-spawning and spawning phases.

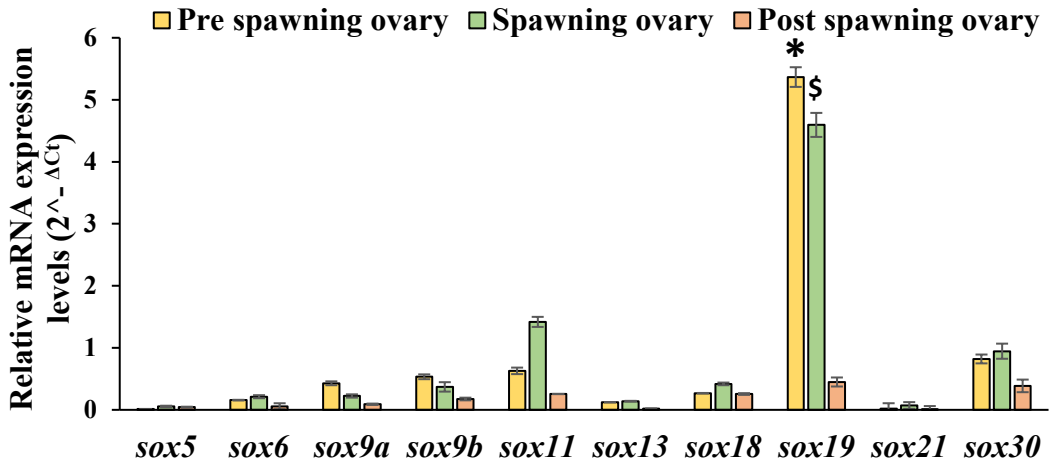

Fig. 13 Ovarian reproductive phase analysis of *sox* family genes. All other details are the same as in Fig. 8.

4 DISCUSSION

With the advancement of NGS technology, the information on DEG between testis and ovary is growing, and these genetic databases offer libraries facilitating the identification of corresponding genes in other fish species. Though the available gonadal transcriptomic data continues to increase, there is a need for novel sex-related genes to understand the convoluted process of gonadal development in fishes. To gain insight into the molecular fundamentals of gonad development and growth, in this study, the gonadal transcriptome of *C. carpio* was obtained after *de novo* assembly of RNA-Seq data from developing testis and ovary of immature fish. Several new players showing dimorphic gene expression were identified. Candidate genes for sex differentiation and development, showing differential gene expression, were further tested to validate our findings. Further, the major group of reproduction-related genes identified were similar with previous reports indicating that these genes are functional in fishes. The data obtained in this study adds to the transcriptomic profile available for the family Cyprinidae.

4.1 GO, COG, KEGG

One of the essential features of transcriptome analysis is to associate individual sequences and related expression information with biological functions. The juvenile stage is transcriptionally active as the bipotential gonad differentiates into a specific and functionally active gonad, either testis or ovary. GO, COG, and mapped unigenes were majorly involved in translational machinery, indicating that juveniles are at a crucial phase of gonadal development. The KAAS pathway analysis is based on sequence similarities and accommodates bi-directional best hit information, enabling it to attain a high degree of accuracy in comparison to a manually curated KEGG GENES database. Hence, KASS can facilitate research on the relationship between different genes obtained from the transcriptome (Moriya *et al*., 2007). Transcripts encoding enzymes or other proteins

involved in reproductive pathways, for example, *hsd3b*, GnRH, serine/threonine-PP2A, corticosteroid *hsd11b*, sterol 14-demethylase, lanosterol synthase, and many more were detected. Pathway analysis mapped some of the genes involved in the *wnt* signaling pathway, oocyte meiosis, and steroid hormone biosynthesis, all of which play a pivotal role in sex differentiation and gonadal development (Baroiller *et al*., 1999; Prathibha and Senthilkumaran, 2017). Recognizing these pathways permits the analysis of reproduction mechanisms in *C. carpio*, although most of the genes present in the putative KEGG pathways that were identified were not found in the present study.

4.2 SSRs

SSRs or microsatellites are polymorphic regions existing in genomic DNA consisting of 2 to 6 bp repeated core sequences (Queller *et al*., 1993) and important to perform research on evolution, molecular ecology, quantitative trait loci analysis, genetic diversity, gene mapping, population genetic analysis comparative genomics, and marker-assisted selective breeding. The SSRs identified in the current study, provide a valuable resource for molecular research in carp.

4.3 DEG

Studies have suggested that the sex-biased genes, primarily or absolutely expressed in one sex, drive the phenotypic differences in males and females (Assis *et al*., 2012; Ellegren and Parsch 2007) and cause the phenotypic sexual dimorphism in zebrafish (Small *et al*., 2009). In juveniles, a group of genes is differentially expressed to fulfil the requirement of gonadal development. A total of 298 genes showed significant differences between the testicular and ovarian transcriptomes, including 171 and 127 genes showing higher expression in the testis and ovary, respectively. The higher number of testis versus ovary up-regulated genes found in the carp was in accordance with the result obtained in the Nile tilapia (Tao *et al*., 2013). The histological

observations depict (Fig 1A) active stages of spermatogenesis in the testis with evidence of the presence of spermatocytes and spermatids in the spermatogenic cysts, and the increase in the mass of the testis during this time is largely due to materials synthesized locally coinciding with our transcriptome data showing a greater number of transcripts and DEG in testis. On the other hand, the ovary (Fig 1B) was observed to be in late primary oocyte growth or early secondary growth before the onset of vitellogenesis. Possibly, at this stage, the intense transcriptional activity in the oocyte subsides as it transits into the main phase of growth, which is due to the acquisition of materials (such as neutral lipid precursors and yolk proteins) produced in other tissues due to which the number of transcripts and DEG may be lesser in the ovary in comparison to the testis. Overall, the difference of gene numbers between females and males might imply that male gonadal development may require a larger number of early expressing transcripts compared with females in teleosts, triggering an early testis upregulated gene expression pattern before any female pattern emerges. DEG were investigated exposing several genes that might be enriched in sex-related biological pathways. Among DEG, a few candidate genes like *amh*, *dmrt1*, *sox9a*, associated with testicular differentiation (Kamiya *et al*., 2012; Kobayashi *et al*., 2008; Nagahama 2005; Raghuveer and Senthilkumaran, 2009; Raghuveer and Senthilkumaran, 2010a), *dax1* and *gata6* involved in gonadogenesis (Liu *et al*., 2016; Wang *et al*., 2002), *wt1* regulating SRY (Hossain and Saunders, 2001), *zp2* and *mapk* playing roles in oocyte development (Chang *et al*., 1997; Ponza *et al*., 2011), *foxk2* involved in gonadal development (Yuan *et al*., 2014), *fbox43* having a role in oocyte meiosis (Tung *et al*., 2005), *fzd8* receptor of *wnt* signaling (von Schalburg *et al*., 2006) and *egr2b* expressed in murine granulosa cells (Jin *et al*., 2016) were selected and their differential expression was validated by qPCR to indicate their critical role in gonadal development. The previous study by Chen *et al*. (2015) also identified differential expression of *wt1* and *zp3* in testis and ovary of

adult carp using suppression subtractive hybridization. Thus, *wt1*, *zp2*, and *zp3* show dimorphic expression from the juvenile stage and is consistent up to adulthood.

4.4 Identification of new players from DEG

New players like *cux1*, *dmrt2a*, *fam192a*, *fstl3*, *retinoic acid receptor RXR-beta-A*, *stathmin*, *sycp1,* and *zar1* also showed dimorphic gene expression pattern. CDP/Cut (CCAAT displacement protein) is a transcription factor involved in the regulation of cell growth and differentiation-related genes (Nepveu, 2001). Vanden Heuvel *et al*. (1996) reported that *cux1* represents an example of a transcription factor that undergoes testis-specific alternative splicing during spermatogenesis. Previous studies have reported *cux* is required for reproductive functions and its genetic ablation results in reduced male fertility (Luong *et al*., 2002) and impaired lactation (Tufarelli *et al*., 1998) in mice. A dimorphic expression pattern of *cux2a* was observed, indicating its role in the reproduction of fishes as well.

Gene expression of *dmrt1* is associated with testicular development in many species (Raghuveer and Senthilkumaran, 2009). Differential gene expression pattern of *dmrt2a* was observed, coinciding with the previous report by Peng *et al*. (2016) in which *dmrt2b* showed dimorphic expression pattern, suggesting these new players' *dmrt2a* and *dmrt2b* might potentially be associated with sex differentiation in carp.

A recent transcriptomic study on developmental gonads in protandrous black porgy, *Acanthopagrus schlegelii* (Zhang *et al*., 2018) reported up-regulated expression of *fam101b* in ovary coinciding with *fam192a* and *fam210b* showing dimorphic expression in carp, elucidating their importance in ovarian development.

FSTL is an FSH-suppressing protein that is produced in the pituitary and upon binding to *activin*, neutralizes the FSH stimulatory actions of *activin* (Nakamura *et al*., 1990). *FSTL*, which encodes

a TGF-β superfamily binding protein, inhibits the formation of the XY-specific coelomic vessel in XX gonads, a feature normally associated with testis differentiation (Yao *et al*., 2004). *fstl* displayed early sexually dimorphic expression profile in rainbow trout (Vizziano *et al*., 2007). Incidentally, *fstl3* showed a dimorphic expression pattern, revealing its crucial role in carp gonadal differentiation.

RA is an active derivative of vitamin A that diffuses through tissues and binds to heterodimers of the nuclear receptors RAR-RXR, which recognize RA-response elements in DNA to control the expression of RA-target genes (Duester 2008). RA signaling stimulates differentiation of spermatogonial germ cells and induces meiosis in male but not female gonads (Duester 2013). RA receptor RXR-beta-A showed differential gene expression in juvenile carp coinciding with the report of Rodríguez-Marí *et al*. (2013) in zebrafish suggesting that the continuous availability of retinoic acid to germ cells in bipotential gonads prohibits the sexually dimorphic onset of meiosis and is consistent with the initial development of meiotic oocytes in juvenile teleosts.

Stathmin is a neuron-enriched gene mediating tubulin depolymerization (Sobel *et al*., 1989). Studies demonstrated that maternal *stathmin* accumulates in oocytes and is redistributed in pre-implantation embryos (Koppel *et al*., 1999). Chu *et al*. (2006) observed an intense expression of *stathmin* in the ovary of tilapia. The dimorphic expression pattern of *stathmin* observed in the present study indicates its functional significance in the gonads of carp.

Sycp3 is a meiosis-specific component of the synaptonemal complex, required for the synapsis of homologous chromosomes, and is a marker of meiosis in mammals (Page *et al*., 2006). Its importance in gametogenesis and fertility has been studied in a few teleost species such as medaka and zebrafish (Iwai *et al*., 2006; Ozaki *et al*., 2011). Laldinsangi and Senthilkumaran (2018) demonstrated that *sycp3* has a potential role in the development and maintenance of testicular

function in catfish. The dimorphic expression pattern of *sycp1* revealed its crucial role during the gonadal development of carp as well.

Ovary-specific maternal factor, *zar1* plays an essential role during the oocyte-to-embryo transition (Wu *et al*., 2003). Expression of *zar1* was identified in gonads of carp, coinciding with a recent transcriptome study in tilapia (Tao *et al*., 2018) that reported a correlation of *zar1* with the expression of genes previously known to be involved in sex differentiation. Further investigations including functional analysis of these genes in female and male fishes are needed to substantiate our findings.

4.5 Genes related to reproduction

A large number of genes (809) related to reproduction have been identified by gonadal transcriptome analysis. Corticosteroids (*hsd3b7*, *hsd17b*, *hsd11b*) and their receptors (*ar* and *er*) play a crucial role in the regulation of reproduction by acting as transcription factors in somatic cells of gonads (Milla *et al*., 2009). Steroid synthesis gene *hsd3b7*, expressed in Leydig cells and responsible for the biosynthesis of steroids, mainly progesterone and testosterone, was moderately over-expressed in the ovary. Besides, *hsd17b*, which regulates the concentration of biologically active androgens and estrogens and is involved in the synthesis of E_2, was also over-expressed in the ovary. Other steroid-related genes like, the sterol transfer-coding gene *star*, genes coding for *er* and *cyp19a1* (Barney *et al*., 2008) which is responsible for E_2 biosynthesis in females, followed similar expression patterns as reported in transcriptome analysis of the Russian sturgeon (Hagihara *et al*., 2014). On the other hand, *hsd11b*, an enzyme which converts gonad maturation inhibiting cortisol or corticosterone into inactive cortisone or 11-dihydrocorticosterone, respectively, and the gene coding for *ar* showed higher expression in testis, like that of the expression pattern in the Nile Tilapia gonadal transcriptome analysis (Tao *et al*., 2013). Key marker genes of germ cells

namely, *vasa*, *pou5f1* (Raghuveer and Senthilkumaran, 2010b; Wang *et al*., 2011) playing role in stem cell maintenance and regulation, and *pcna*, playing a crucial role in meiosis (Miura *et al*., 2002) were over-expressed in the ovary, indicating their germ cell regulation in the female ovary. Other germ cell maintenance genes such as *piwi*, *notch1*, *plzf*, *cxcr4*, *cxcl12*, *ipo4*, and *sycp3* were moderately over-expressed in testis like that of the transcriptome of Southern bluefin tuna (Bar *et al*., 2016). The expression pattern of other genes/factors related to gonadal differentiation like *hsd20b2, lhcgr*, *fgfr1a2*, *tac3a*, *notch2, fshr*, *prlhr2a*, *kiss2*, *igf1ra*, *wt1*, *rspo1*, *ctnnbip1*, *tcf3a*, *msl1b*, *fem1b*, *smad3b*, and *atm* between testis and ovary were comparable to the transcriptome analysis of previous reports (Bar *et al*., 2016; Hagihara *et al*., 2014; Shi *et al*., 2015; Wang *et al*., 2017).

4.6 Expression analysis of *sox* family genes

Genes of the *sox* family are involved in the regulation of various developmental processes such as the germ layer formation, organ development, and cell type specification (Wegner, 1999). Several reports have shown the essentiality of *sox* family genes namely *sox3/5/9a/9b* in differentiation, growth, and development of gonads in teleost (Raghuveer and Senthilkumaran, 2010; Rajakumar and Senthilkumaran, 2014; Schartl *et al*., 2018; Takehana *et al*., 2014). However, the role of other genes belonging to the *sox* family is poorly understood. The abundant expression of *sox30* in testis and *sox19* in the ovary from expression profiling of the ten *sox* genes in the tissue distribution and reproductive phases, indicated for a detailed study of *sox30* and *sox19*, which is described in the following chapters (2 and 3).

5 CONCLUSION

Overall, analysis of carp gonadal transcriptome using NGS RNA-Seq technology identified diverse regulatory mechanisms/ pathways of sexual development and provided a valuable resource

for evaluating differential gene expression patterns. The dimorphic expression of sex-related genes offers additional insights into gonadal differentiation. In addition, results from this study warrant the analysis of *sox30* and *sox19* role in gonadal function.

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Chapter 2

Significance of *sox30* along with *sox9a/b* in testicular steroidogenesis

ABSTRACT

Expression of transcription factors is crucial for the regulation of steroidogenesis and gonadal development in fish. Sox proteins regulate gene expression of various events related to vertebrate reproduction. This study reports the role of *sox30* and its influence on *sox9a/b* in regulating testicular steroidogenesis of the carp, *C. carpio*. Tissue distribution showed predominant expression of *sox30* in gonads, while gonadal ontogeny indicated significant dimorphic expression of *sox30* from 120 dph. Higher *sox30* transcripts during the spawning season, an elevation of *sox30* after hCG induction, and 11-KT treatment authenticate gonadotropin dependency. Treatment of MDHT to juvenile carp for mono-sex induction, vis-à-vis elevated *sox30* expression. Sox30 protein was detected abundantly in spermatocytes and spermatid/sperm of carp testis. Transient silencing of *sox30* using siRNAs decreased *sox9a/b* expression, leading to downregulation of certain molecule/factor, transcription factor, germ/stem cell marker, and steroidogenesis-related enzyme genes. Serum T and 11-KT decreased significantly upon transient silencing of *sox30, in vivo*. Concomitantly, a reduction in testicular microsomal Hsd11b activity was observed. These results demonstrate the influence of *sox30* as well as *sox9a/b* in the regulation of testicular steroidogenesis in carp.

1 INTRODUCTION

Steroidogenesis drives reproduction by promoting growth, differentiation, and maturation of a bipotential gonad into the testis or ovary and is regulated differently by multi-tiered modulation of promoter motif of transcription factors influencing steroidogenic enzyme genes to eventually govern enzyme activity (Rajakumar and Senthilkumaran, 2020). Transcription factors like *ad4bp/sf-1*, *creb*, *foxl2*, *sox3* and *wt-1* play a regulatory role in fish steroidogenesis. Further, the influence of transcription factors, sex steroids, steroidogenic enzymes, sex steroid receptors,

germ/stem cell markers, growth factors, and other molecules in the gonadogenesis of teleost has been reviewed (Devlin and Nagahama, 2002). *SOX* family of transcription factors are implicated in regulating many biological functions, including reproduction (Wegner,1999). SOX proteins are specific to animals and constitute to HMG superfamily, encoding ~80 aa DNA-binding domain further classified into subgroups A–K (Zhang *et al*., 2018a). In fish, s*ox9* is a requisite for testis development with two forms identified in teleost like fugu, zebrafish, catfish, rice field eel, and the Japanese medaka (Raghuveer and Senthilkumaran, 2010). *sox3* (Rajakumar and Senthilkumaran, 2014) regulates testicular development and recrudescence in catfish and initiates testicular differentiation (Takehana *et al*., 2014) in the Indian rice-fish. The involvement of *sox5* in germ-cell regulation of medaka (Schartl *et al*., 2018) was evident. In mammals, evidence for the presence of *SOX4*, *SOX5*, *SOX6*, *SOX7*, *SOX17*, *SOX20,* and *SOX22* in testis has been reported (Wegner, 1999). Whereas, in teleost having more (~50) *sox* genes than other vertebrates (Zhang *et al*., 2018b), the involvement of other genes belonging to the *sox* family in gonadogenesis has seldom been reported. In this regard, the carp comprising more *sox* genes than other fish species (Zhang *et al*., 2018b) would be an appropriate model for investigation. The significance of *sox9a/b* in the testicular development of carp have been reported earlier (Du *et al*., 2007; Guo *et al*., 2010). A previous study on the transcriptome-based analysis from our laboratory demonstrated that *sox* genes of carp presented stage-specific and/or sex-dimorphic expression during gonadal development (Anitha *et al*., 2019). Among which, *sox30* of subgroup H was predicted to have higher expression in testis warranting in-depth analysis. *SOX30*, primarily isolated from mouse and human (Osaki, 1999) was thought to be specific in mammals (Koopman *et al*., 2004), while later evidence documented its presence throughout the animal kingdom (Han *et al*., 2010). Recent reports (Yu *et al*., 2018; Wei *et al*., 2016) stated the absence of *sox30* in zebrafish, spotted gar

(*Lepisosteus oculatus*), the Japanese medaka, tongue sole (*Cynoglossus semilaevis*), fugu, the Japanese flounder, and carp. Contending these, the present study is the first to provide evidence for the presence of *sox30* in carp. *SOX30* has been proven to be critical for mammalian and teleostean spermatogenesis (Han *et al*., 2010; Zhang *et al*., 2018a). However, its influence on the regulation of testicular steroidogenesis was never clarified. Early sexual maturation accompanied by seasonal spawning makes carp (Basavaraju *et al*., 2002) an ideal model to understand the role of *sox30* during gonadogenesis. This work reports cloning, expression analysis, and localization of *sox30* in comparison with *sox9a/b* in carp. Gonadotropin regulation on *sox9a/b* and *sox30* was assessed during the natural spawning cycle, artificial induction, and also in mono-sex induced carp. The current report provides substantial evidence to denote *sox30* as a novel regulator of carp testicular steroidogenesis by transient silencing.

2 MATERIALS AND METHODS

2.1 Fish sampling

Procuring and rearing of carps followed by sampling were done as described earlier in chapter 1. Initially, to clone *sox9a/b* and *sox30*, adult carp (~1-year, ~30–40 cm, ~400-700 g) testis was used. In carp, PGC divide rapidly from 28 to 63 dph and differentiate into oogonia and spermatogonia around 70 and 100 dph, respectively (Komen *et al*., 1992). Different ages of carp, 0, 40, 80, 120, 160 and 200 dph (~40 mm–20 cm, ~0.2–300 g) were collected (n=5~10) for gonadal ontogeny studies. Total RNA was prepared from 0 (whole trunk), 40 (mesonephric gonadal complex), 80 to 200 dph (testis/ovary) samples. For localization analysis, tissues were fixed using 4% PFA-PBS (0.01M). Mono-sex induction was performed at KVAFSU and those carps were procured for experimentation.

2.2 Cloning of *sox9a/b* and *sox30* from adult carp testis

Gene-specific primers were designed for *sox30* with the sequence data available in NCBI's GEO (GSE112157), a transcriptome study conducted earlier in carp from our laboratory. Cloning of *sox9a/b* was done as detailed (Sudhakumari *et al*., 2017) to compare their expression with *sox30*. Total RNA (TRI reagent, Sigma) isolation, followed by DNase I treatment and cDNA synthesis (OneScript® Plus cDNA Synthesis Kit, Applied Biological Materials Inc., BC, Canada) were carried out as described in the manufacturer's manual. PCR amplification with designed primers (Table 1) using Taq 2X master mix (New England Biolabs Inc., MA, US) was done under standard PCR conditions. No isoforms of *sox30* were recognized as amplicons displayed a specific single explicit band. To verify the presence of paralogs of *sox30* if any, primers (Table 1) were designed by aligning neighboring exons with a flanking variable intron in the conserved HMG domain. A single band was observed in the genomic DNA PCR indicating the absence of any paralogs. Following gel purification and ligation (pGEM®-T easy vector, Promega, WI, US), the positive clones were ascertained by DNA sequencing using the dideoxy method. Lasergene software (DNASTAR, WI, US) was employed in the sequence alignment.

Table 1. List of primers used in the study.

S. No.	Gene name/symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number
1	*18s rRNA*	GCTACCACATCCAAGGAAGGCAG2C	CGGCTGCTGGCACCAGACTTG	FJ710827.1
2	*ad4bp/sf-1*	CGGACCTGGAAGAGTTGT	GGTTCTCTTGGCATGCAG	GSE112157
3	*amh*	CCGTGATGAGCAAAGGACACC	CAAAGAGACAATGTTTGCAC	KU168255.1
4	*ar*	GATGGCAGGTTTGATGGAG	GAGATAATTCTGCTTCCCCTCAG	KU168254.1
5	*cyp11*	TCAACCAAGCGGACCACTGT	CACCTCCAGCCATCAGCTC	GSE112157
6	*cyp17*	GCAGCACTGTATCTCAGAAG	GGCACGCGTCAGCTCCGGTG	GSE112157
7	*dax*	CCAGATGTTGCAGGGCTGC	CCTCCATGTTGACAGCGCC	GSE112157
8	*dmrt1*	GCAGCCCAGGTGGCGTTA	CGTGCTGTCAGTGTGCCCTC	KF713504.1
9	*fshr*	CTGACTGTTCCCAGGTTTTTAATG	GCATGGCGTAAGTGATGGTG	MH726214.1
10	*gata4*	CAGAGACGACTCTCTGCTTCCAG	CCCCGTGGAGCTTCATGTAGAG	GSE112157
11	*gnrh*	GGTGGAAAGAGAAGCACTGGTG	GTTTTTAAACTCTTCCTCGTCTG	AY189960.1
12	*gsdf*	CTGTGTGTCGTCGCCGTTC	CACTGCTCACGTAAATGATGC	GSE112157

13	*hsd3b*	GGCTTTTCTGTTCATGCCTG	CACGCGTCAGCTCCGGTGCC	GSE112157
14	*hsd11b*	ATCACAGGGTGCGACTCGGGTTTCGGG	CGGCTGAGTGATGTCCACCTGA	GSE112157
15	*hsd17b*	GACATCCTGGTGTGTAATGCAGG	CTGCCTGTGACCAGGATCCGT	GSE112157
16	*hsd20b*	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA	GSE112157
17	*kiss2*	GTGGAGCGGAGGCAGTTTG	CAGGAAAAGCATCATTGGCAGC	JQ715608.1
18	*lhr*	CCGCTGCAAACTGACCGTCC	GTATAGACAGATAGTTCGCCG	MH726213.1
19	*pou5f3/oct4*	CCGAAAACCCTCAGGATATGTAC	CACACACGTACTACATCTCTCTCC	GSE112157
20	*pdgf*	GGGAAACTGGCGGATCGAG	GCGCACAAACTCACTTTCATAG	GSE112157
21	*piwi*	GGTAAAGATTGGGGAAAGAG	CTCCATCAAAGGTGTGTGC	JF505506.1
22	*sox5*	GGACAGCCTGGTGGAGAAAG	CTGCTGTTGTCGTGCAATC	GSE112157
23	*sox6*	CCGGGGAAGGAACAGCAAC	GTGCCTGCTCCTCATAATATG	GSE112157
24	*sox9a*	GGTGAACAACGGCCAGAG	GACTGGCCTGAGTGCTCG	GSE112157
25	*sox9b*	GAACGGCCAGAGCGAGAG	GACCCTGGGACTGACCTG	AY956415.1
26	*sox11*	GCACCAAGTGGAATAAACACCTG	CTGATTTTCTTGCTGCTAGTCAC	GSE112157
27	*sox13*	GGCCAAAGACGAAAGACGG	GGCCGGGGTTTGTATTTGTAG	GSE112157
28	*sox18*	GATGCTGGGTCAATCCTGG	CGATGTGGCGAGTAGGCATC	KY860090.1
29	*sox19 qPCR*	GGCACAGGAGAACCCCAAAATG	CTTCATGAGGGCTTTGGTC	GSE112157
30	*sox21*	GGTAAGGTTCATGTTGAGCTTTG	CATTACTCAATGCTCGGGTATC	GSE112157
31	*sox30* qPCR	CAGAGCAGTCTAAATTCTCCAGC	GGCTAGAAGAGCTTGAACATGG	MT193291
32	*sox30* ORF	ATGGATAAACACCCCAAACTGAGA	CTAGTCTCCATAATGGAGGCAATAT	MT193291
33	*sox30* genomic PCR	CTGAGCTTCACTCAGCCGCC	GCTTCTTCTGCTCTTCAGACAG	MT193291
34	*srd5a1*	CTATAAGATACCTAGAGGGGGC	GAGATACCACTTGTGGTGGG	GSE112157
35	*star*	GGAGCTCCCTTCTCAGCAG	CTGTCTTCTGTTCCAGCATC	GSE112157
36	*sycp3*	GCTGGAAAGATTTGGTGCGG	CTCCTGCTCCTCAGACTTC	MN447718.1
37	*vasa*	CCATGGTGATCGGGAGCA	GTTCCCACAGCGTCCGGT	AF479820.2
38	*wt1*	CCAGGAATCAGGGTTACGGCAC	GCCTGACTTCCTGTACAGCTG	GSE112157

2.3 Multiple sequence alignment and phylogenetic analysis

Following ClustalW alignment of cloned carp Sox30 and its vertebrate counterparts with aa sequences available in GenBank, the phylogenetic tree was constructed. Multiple sequence alignment was done by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and displayed using Jalview 2.8. The phylogenetic tree was constructed and presented using PhyML (http://www.phylogeny.fr/simple_phylogeny.cgi). Bootstrapping with 1000 replicates delineates the integrity of every clade.

2.4 qPCR

Total RNA and cDNA from all samples were prepared as detailed earlier. Real-time primers (Table1) were designed and qPCR was done as described earlier in chapter 1. Relative expression of genes between control and treated groups was calculated using $2^{-\Delta\Delta Ct}$. Log copy number analysis was performed to analyze the differences in the concentration of *sox9a/b* and *sox30* as explained by Mamta *et al*. (2014).

2.5 Polyclonal antibody generation for Sox30

For antibody generation, an antigenic peptide, MSHYEDLRQEAPVQNC was synthesized commercially using carp Sox30 aa sequence in conjugation with keyhole limpet hemocyanin carrier protein (Sigma). Six-weeks-old (n=2) Swiss albino male mice used for antibody generation were handled and maintained following the norms of IAEC (UH/IAEC/BSK/2018-I/28) and CPCSEA. During experimentation, mice were fed commercial food pellets, *ad libitum*. Pre-immune sera were collected by retro-orbital puncture before administration of the peptide dissolved using 0.01 M PBS. Primary (200 µg of antigenic peptide combined with Freund's complete adjuvant) and two (14 days apart) boosters (100 µg of antigenic peptide combined with Freund's incomplete adjuvant) were injected subcutaneously. Blood was collected for serum separation and then used for IHC and western blot as a Sox30 antibody source.

2.6 Western blot

Sox9 and Sox30 proteins were detected in carp testis upon western blotting with anti-SOX9 antibody (Santa Cruz Biotechnology, Texas, US) and carp anti-Sox30 polyclonal antibody as described by Gupta and Senthilkumaran (2020). SOX9 antibody rose against 22-62 aa near N-terminus of SOX9-human origin showed 81% and 90% homology with N-terminal region of carp Sox9a and Sox9b proteins, respectively (Fig. 1). In brief, the protein was prepared from adult carp

testis and intestine with RIPA (0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris HCl [pH 8], 0.1% SDS, and 0.1% Triton X–100) buffer separately and quantified using Bradford's method. About 20 µg of protein run in 10% polyacrylamide gel was transferred to nitrocellulose membrane (Pall Life sciences, NY, US), incubated overnight with respective diluted antibody (1:1000 for Sox30; 1:100 for SOX9) and probed using HRP-conjugated goat anti-mouse antibody (1:5000; Bangalore Genei, Bengaluru, India) incubation at RT for 1 h. Specific bands were detected with a chemiluminescent reagent (G-Biosciences, MO, US). Anti-mouse α–tubulin monoclonal antibody was used as a reference control for both testis and intestine protein fractions. Image J (NIH) software was used for densitometry analysis.

```
sox9Homosapiens          PSPTMSEDSAGSPCPSGSGSDTENTRPQENTFPKGEPDLKK---   41
sox9aCyprinuscarpio      PSPSMSEDSAGSPG-SGSGSDTENTRPAENSLIAPDGTLGEFKK   43
                         ***:*********  ************ **::   :  * :

sox9Homosapiens          PSPTMSEDSAGSPCPSGSGSDTENTRPQENT--FPKGEPDLKK    41
sox9bCyprinuscarpio      PSPSMSEDSAGSPCPSGSGSDTENTRPEEHLGEFKKDEDKF--    41
                         ***:************************:*:   * *.* .:
```

Fig. 1. Multiple sequence alignment results of the SOX9-human N-terminal region showing 81% and 90% homology with the N-terminal region of carp Sox9a and Sox9b proteins, respectively.

2.7 IHC

Sox9 and Sox30 proteins were localized, as described in Laldinsangi and Senthilkumaran [26]. In brief, tissues were fixed using 4% PFA-PBS, embedded (optimum cutting temperature compound, Leica Microsystems) in cryomold (Tissue–Tek, AJ Alphen aan den Rijn, The Netherlands) and sectioned (Cryostat, Leica CM1850). Initially, the sections were blocked using 10% of goat serum (Bangalore Genei) and incubated at 4 °C either with SOX9 (1: 50) or Sox30 (1: 100) antibody or pre-adsorbed antibody along with respective excessive antigen (negative control) for overnight. The sections were probed using HRP-conjugated goat anti-mouse antibody (1:1000) at RT for 2 h, the next day. VECTASTAIN® Elite ABC (Vector Laboratories, CA, US) incubation was done

for 30 min before developing them with commercially available 3,3′-diaminobenzidine and H_2O_2 as substrate. After development, DPX was used to mount the sections. DM6 B upright microscope fitted with a camera (DFC 4500, Leica Microsystems, Germany) and Leica Application Suite X software was used to assess the sections. Pre-adsorbed antisera with an excess of commercially synthesized peptides (antigen) of Sox30 (MSHYEDLRQEAPVQNC) and Sox9 (CPSGSGSDTENTRPQE) were used as their respective negative controls for IHC.

2.8 *In vitro* culture of testicular slices

Testicular slice culture was performed as outlined by Raghuveer and Senthilkumaran (2010). Testis from carp in the late preparatory phase was dissected, sliced (McIIwain tissue chopper, Vibratome, Ted Pella Inc., CA, US) and cultured at 20-22 °C in a tissue culture plate with 2 ml of L15 culture medium, 10 mM HEPES and antibiotics (streptomycin, 0.1 mg/ml; penicillin, 100 IU/ml) supplementation. Testicular slices were treated using 100 IU/ml of hCG (Uni-Sankyo Pvt. Ltd., Hyderabad, India) or (control) physiological saline. At short intervals of 0, 3, 6, and 12 h, tissue was collected and washed using ice-cold PBS. Likewise, testicular slices were also treated with 100 ng/ml (dose tested with pilot experiments) of 11-KT (Sigma) to study its effect on *sox9a*/*b* and *sox30* expression. 11-KT was initially dissolved using absolute ethanol, completely air-dried to remove any traces of alcohol, and reconstituted with the L15 medium for experimentation. Total RNA was isolated using TRI reagent followed by cDNA synthesis and quantification as described earlier.

2.9 *In vivo* induction by hCG

During the preparatory phase, laboratory acclimated adult male carp (n=5) weighing about 400–500 g was briefly anesthetized with MS222. The ventral portion of the carp was sterilized with alcohol, a single dosage of hCG (1000 IU/Kg body weight) was gradually injected into the testis

directly using a sterilized 1 ml syringe (Gupta and Senthilkumaran, 2020). For control fish, physiological saline was injected. Subsequently, fishes were kept in circulatory water for a few minutes at ambient temperature to regain from anesthetization. Testis was collected at intervals of 12 and 24 h from control and treated fish for expression analysis. Total RNA and cDNA from all samples were prepared as mentioned earlier.

2.10 Mono-sex induction

Mono-sex induction was performed at KVASFU by following established protocols (Basavaraju *et al*., 2008; Wang *et al*., 2008). In brief, 50 dph carp larvae were fed hormone incorporated diet for 50 days. Hormones, MDHT (Sigma) at 50 mg/Kg feed for masculinization, and E_2 (Sigma) at 150 mg/Kg feed for feminization were given to produce the mono-sex population. The feed required for the study was prepared as described (Guerrero and Shelton, 1974). The treatments were given regularly and, fish samples were collected at the end for analysis.

2.11 Culture of carp primary testicular cells (mixed)

Testicular mixed cell culture was prepared as described by Murugananthkumar and Senthilkumaran (2016). Adult male carp was anesthetized to dissect out testis, and disinfected, also severed into small portions in the L15 culture medium. Following, incubation with 0.005% DNase and 0.25% collagenase type I, the slices were pressed to obtain cell suspension, further filtered using a 40 mm cell strainer, and centrifuged at 100×g, to obtain pellet which was resuspended in DMEM. Almost 1×10^6 cells were cultured separately in a 24-well plate comprising DMEM, antimycotic-antibiotic, 1×Glutamax, 10% fetal bovine serum and maintained for 24 h in 5% CO_2 at 30 °C. Cell viability was assessed with trypan blue staining.

2.12 *In vitro* treatment of *sox30*-siRNA in carp primary testicular culture

Custom synthesized *sox30* oligomer (antisense: 5'-rGrCrCrUrUrUrUrGrArUrArGrArArArCrUrGrUrUrUrUrGrUrUrCrU-3'; sense: 5'-rArArCrArArArArCrArGrUrUrUrCrUrArUrCrArArArArGGC-3') siRNAs were commercially manufactured (Integrated DNA Technologies, Iowa, US). The siRNA for *sox30* was designed spanning only a few base pairs of the HMG domain to ensure specificity. The siRNA in combination with branched PEI (Sigma) was dissolved using sterilized HEPES-NaCl (pH 7.4), as detailed by Höbel and Aigner (2010), and used in teleost (Gupta and Senthilkumaran, 2020; Laldinsangi and Senthilkumarn, 2018; Murugananthkumar and Senthilkumaran, 2016). The mixture was incubated at RT for 20 min for the formation of the siRNA-PEI complex. About, 1×10^6 cells of carp testicular cell culture were maintained as earlier described. Before treatment, the culture medium was removed, followed by the addition of 100 µl OPTI-MEM® I (Thermo Fisher Scientific) solution. Concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 20, and 50 ng/ml of antisense *sox30*-siRNA and 10 ng/ml of sense *sox30*-siRNA complexed with branched PEI were prepared. Aliquots of 25 µl containing antisense *sox30*-siRNA-PEI/ sense *sox30*-siRNA-PEI (negative control)/ only PEI (control) was added and the cells were incubated for 12 h. Following treatment, siRNA-PEI mixture, and OPTI-MEM® I was removed, replenished using fresh culture medium, and maintained for 24 h. Cells were rinsed with PBS and harvested by trypsinization (0.5% Trypsin-EDTA) for total RNA isolation using TRI reagent, followed by cDNA synthesis and relative expression analysis, as detailed earlier. Treatment was validated using MISSION® siRNA Fluorescent Universal Negative Control #1 (Sigma). The treated cells were captured using the IX81 Olympus microscope (Olympus Corporation) and assessed by Cell Sens dimension software.

2.13 *In vivo* treatment of *sox30*-siRNA in adult male carp

Specific approvals from the IAEC (UH/IAEC/SB/2019-I/04) and Institutional Bio-Safety Committee (IBSC No. BSK–N–15-July2019) were obtained for siRNA treatments. Gene silencing using siRNA was done (Höbel and Aigner, 2010) and used in teleost (Gupta and Senthilkumaran, 2020; Laldinsangi and Senthilkumarn, 2018; Murugananthkumar and Senthilkumaran, 2016). Adult male carp, in the pre-spawning phase, was anesthetized, and about 100 µl of 100 ng/ml of antisense *sox30*-siRNA-PEI complex dissolved in HEPES buffer was injected directly into the testis, and fish were kept in circulatory water for a few minutes at ambient temperature to regain from anesthetic effect. Similarly, in treatments with sense *sox30*-siRNA-PEI (negative control), only PEI and control groups were done. Four fish groups (control, only PEI, antisense *sox30*-siRNA-PEI, and sense *sox30*-siRNA-PEI) were kept in separate tanks of 50 L capacity, continuously aerated, and replenished with fresh water. At days 0, 2, 4, 6, and 8, fishes (n=5/group) were sacrificed to dissect out the testis for analysis. Total RNA from PEI, control, antisense *sox30*-siRNA-PEI, and sense *sox30*-siRNA-PEI treated samples was prepared and analyzed, as earlier. There was no mortality in any of the treatment groups throughout the experiment.

2.14 EIA of androgens

Serum samples were obtained from control and *sox30*-siRNA treated fish on day 4 post-treatment. Carp serum T and 11-KT levels were valued using Immunotag fish EIA kits (G-Biosciences) adopting the manufacturer's protocol. The sensitivity for T and 11-KT measurements is 6.92 and 0.28 pg/ml, respectively, and the assay was validated as described (Swapna *et al*., 2006). Assays were performed for each independent sample (n=5) in triplicates with appropriate dilutions.

2.15 Estimation of Hsd11b enzyme activity in testis

Enzyme activity of Hsd11b was measured as explained by Rasheeda *et al*. (2010). In brief, microsomal fractions were prepared by homogenizing 100 mg of control and *sox30*-siRNA treated testicular tissue in 1 ml of 0.1 M KPO_4 buffer, followed by centrifugation and ultra-centrifugation at 9000×g for 20 min and 105000×g for 1 h, respectively at 4 °C. The microsomal pellet was rinsed once and dissolved with 500 µl of 0.1 M KPO_4 buffer, 20% (v/v) glycerol, and 0.1 mM EDTA. About 250 µg of testicular microsome, was added to 1 ml of assay medium comprising of 100 µM NAD^+ and 2 nM 11-OHT incubated at 37 °C in a shaker water bath for 60 min. The reaction was interrupted by the addition of ice-cold diethyl ether. For extraction of steroids, diethyl ether was used, the organic layer was then dried under N_2 gas and dissolved with 100 µl of EIA buffer. All incubates were analyzed in triplicates. The quantity of 11-KT formed was estimated with Immunotag fish 11-KT-EIA kit, as detailed earlier. Heat-denatured microsome was employed as a negative control for assay validation.

2.16 Statistics

Densitometry analysis of images was performed using ImageJ software. All qPCR representations were denoted as mean ± Standard Error of the Mean (SEM) which qualified both normality and homogeneity tests. ANOVA, followed by posthoc tests were performed for statistical calculation with SigmaPlot 14.0 software (Systat Software Inc., Chicago, IL, US). For each test, P-value < 0.05 was treated to be statistically significant.

3 RESULTS

3.1 Cloning of *sox9a/b* and *sox30*

Upon cloning, partial cDNAs of *sox9a* (~147 bp), *sox9b* (~336 bp), and *sox30* ORF (~1170 bp) encoding a putative protein of ~389 aa with a molecular weight of ~44 kDa, comprising HMG box

domain were obtained. The *sox30* ORF nucleotide and aa sequence have been submitted to GenBank (MT193291).

3.2 Homology

The identity of carp Sox30 was compared with its vertebrate counterparts. Multiple sequence alignment revealed that carp, *C. carpio* Sox30 displayed much identity at the HMG box domain region (Fig. 1). Phylogenetic analysis showed that *C. carpio* Sox30 is closely related to *Plecoglossus altivelis* and formed a separate clade (Fig. 2).

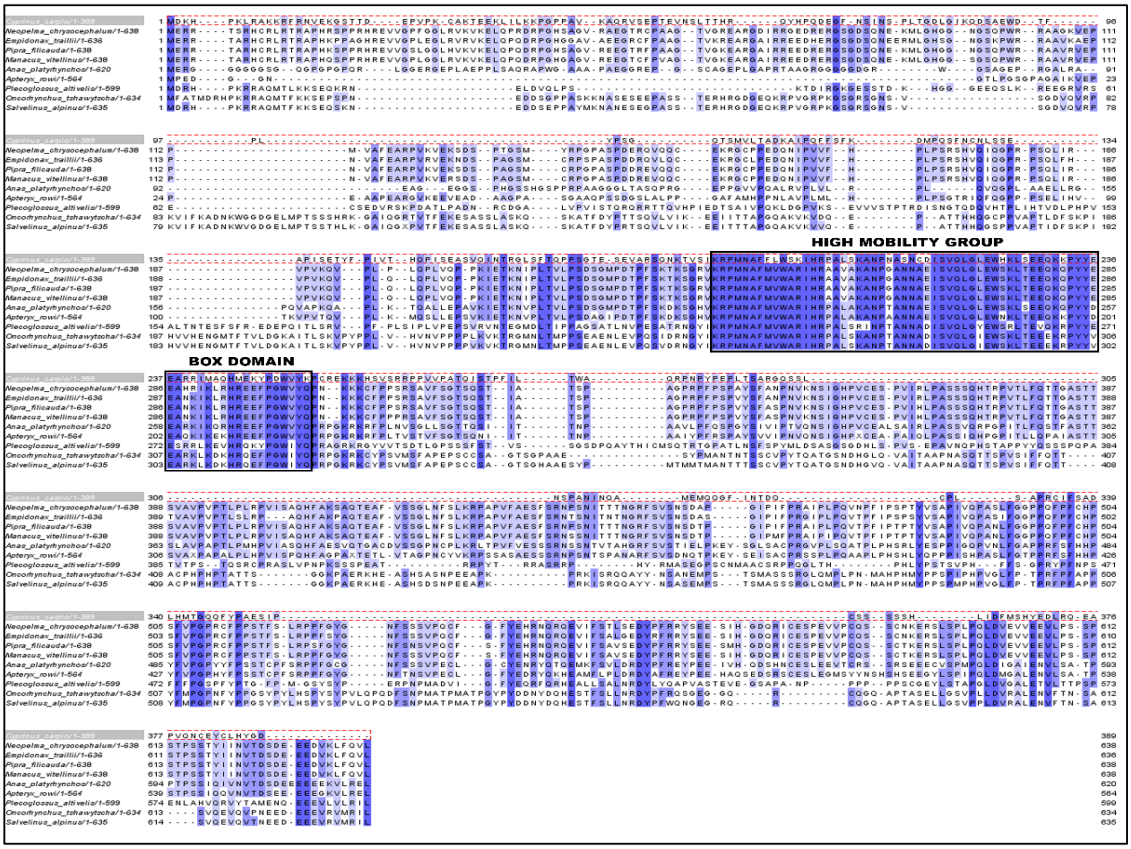

Fig. 1 Multiple sequence alignment of carp Sox30 with other vertebrate counterparts: *Anas platyrhynchos* (XP_005015498.2), *Apteryx rowi* (XP_025919212.1), *C. carpio* (MT193291), *Empidonax traillii* (XP_027741383.1), *Manacus vitellinus* (XP_017928916.1), *Neopelma chrysocephalum* (XP_027538170.1), *Oncorhynchus tshawytscha* (XP_024289861.1), *Pan*

troglodytes (NM001280287.1), *Pipra filicauda* (XP_027574476.1), *Plecoglossus altivelis* (AHK05944.1), *Salvelinus alpinus* (XP_023992871.1).

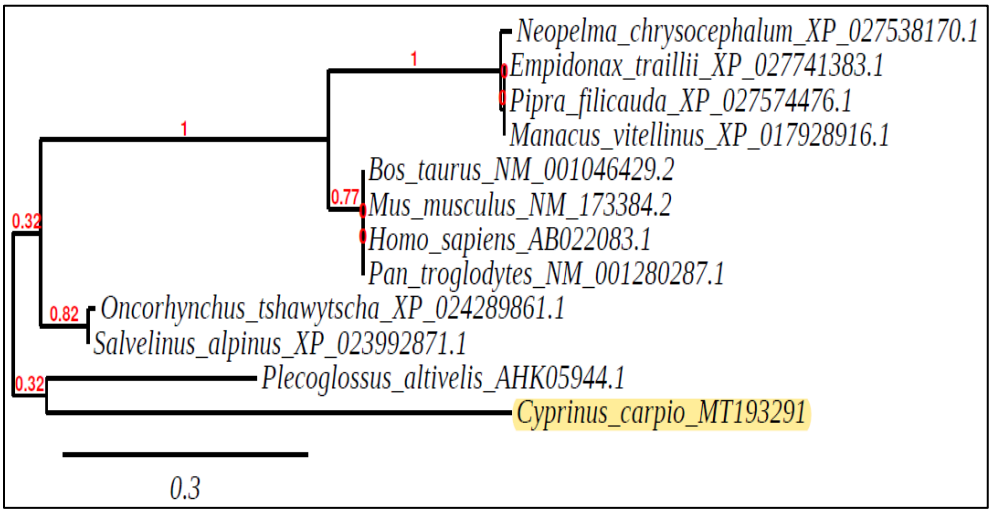

Fig. 2 Phylogenetic analysis of carp Sox30 with other vertebrates. The phylogenetic tree was generated using http://www.phylogeny.fr/simple_phylogeny.cgi. GenBank accession numbers of Sox30 sequences used are as follows: *Bos taurus* (NM_001046429.2), *Homo sapiens* (AB022083.1), *Mus musculus* (NM_173384.2), *Pan troglodytes* (NM_001280287.1). All other details are the same as Fig. 1.

3.3 Expression analysis of *sox9a/b* and *sox30*

Transcript copy number analysis was performed to compare the expression of *sox9a/b* and *sox30*. Tissue distribution analysis during the late preparatory phase of the reproductive cycle revealed that the expression of *sox9a/b* and *sox30* was ubiquitous in the tissues (n=5) analyzed with negligible expression in the intestine. The expression of *sox9a/b* was high in the brain ($P < 0.05$) followed by the testis (Fig. 3A, B), while the predominant expression of *sox30* was evident in the gonad of both female ($P < 0.05$) and male ($P < 0.05$) carp (Fig. 3C). Gonadal ontogeny showed a high expression of *sox9a/b* during 40 dph ($P < 0.05$), which later had varied expression in both female and male gonads through gonadal development till maturity (Fig.3D, E). On the other hand, *sox30* displayed significant dimorphic expression ($P < 0.05$) between female and male gonads

from 120 dph onwards until adulthood (Fig. 3F). Analysis through the testicular reproductive cycle of carp displayed significant expression of *sox9a/b* at preparatory ($P < 0.05$), modest expression in pre-spawning and spawning, and minimal expression during resting (Fig. 3G, H). However, high levels of *sox30* were observed during pre-spawning followed by spawning and preparatory in comparison with the resting phase (Fig. 3I).

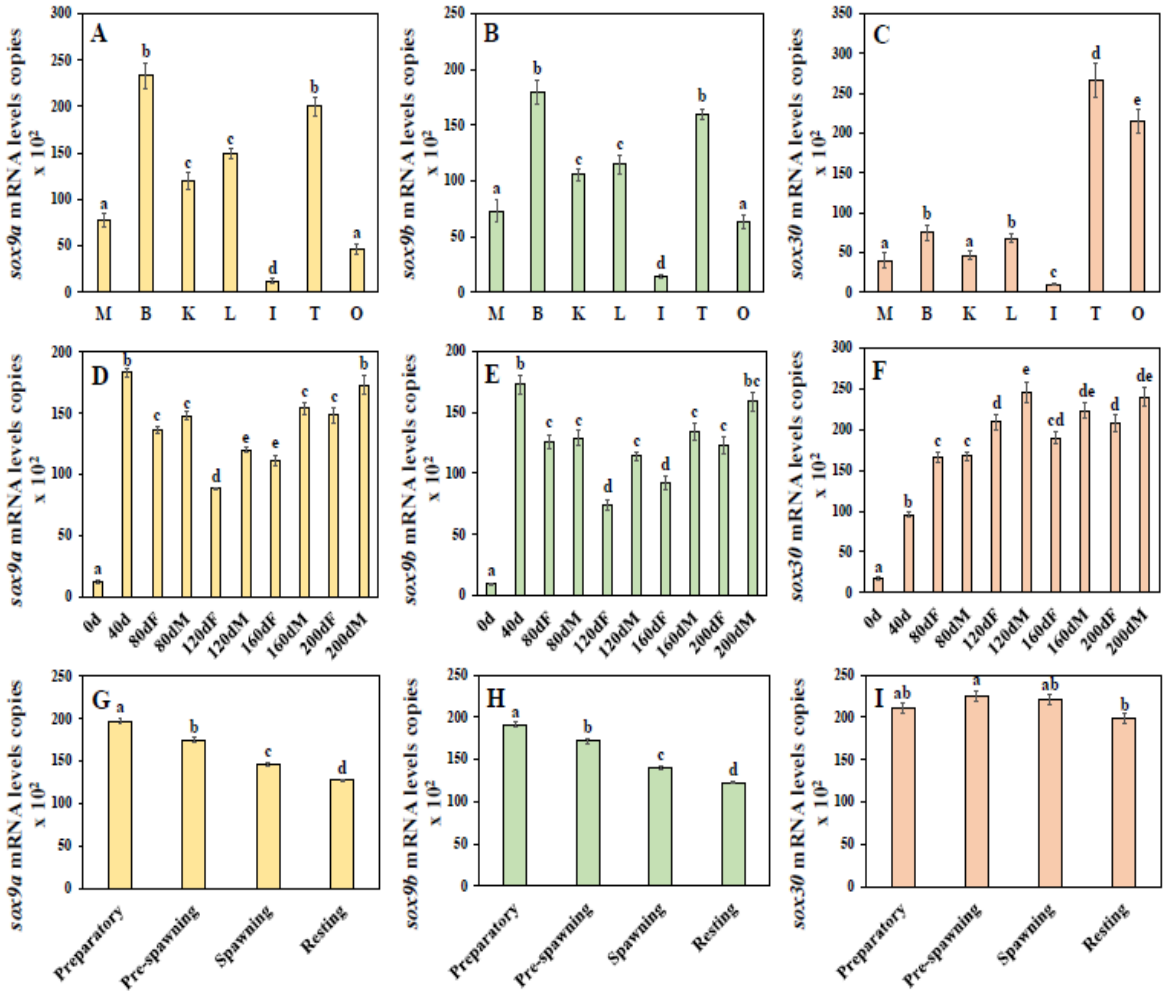

Fig. 3 Expression profiling of *sox* genes: Tissue distribution [*sox9a* (A), *sox9b* (B), *sox30* (C)], gonadal ontogeny [*sox9a* (D), *sox9b* (E), *sox30* (F)] and testicular reproductive phase [*sox9a* (G), *sox9b* (H), *sox30* (I)] analysis using absolute quantification (copy number) in carp. Data (n=5) were expressed as mean ± SEM ($P < 0.05$; One-way ANOVA followed by Tukey test). Means with different alphabets differ significantly while means with similar alphabets did not show any

significance. Abbreviations: M-muscle, B-brain, K-kidney, L-liver, I-intestine, T-testis, O-ovary, d-days post hatch, F- female, M-male.

3.4 Immunolocalization and Western blot of Sox9 and Sox30

Sox9 immunoreactivity was detected in the spermatogonia of carp testis (Fig. 4A, B) while the absence of immunoreactivity in the control (pre-adsorbed antibody) confirm the antibody specificity (Fig. 4C). Western blot presented a sharp band of Sox9 protein ~50 kDa (Fig. 4D) in testis, revealed to be highly specific by the absence of signal in the negative control (intestine). Sox30 antisera elicited immunoreactive signals in spermatocytes and spermatid/sperm (Fig. 4E-G), but not in spermatogonia of the testis. As expected, in control of the pre-adsorbed antibody with excessive Sox30 antigen, no positive signal was evident (Fig. 4H). Further, a positive band ~44 kDa (Fig. 4D) corresponding to Sox30 was observed in testis but not in the negative control (intestine) by western blot, validating antibody specificity. Neither Sox9 nor Sox30 immunoreactive signals were seen in the supporting cells of the testis.

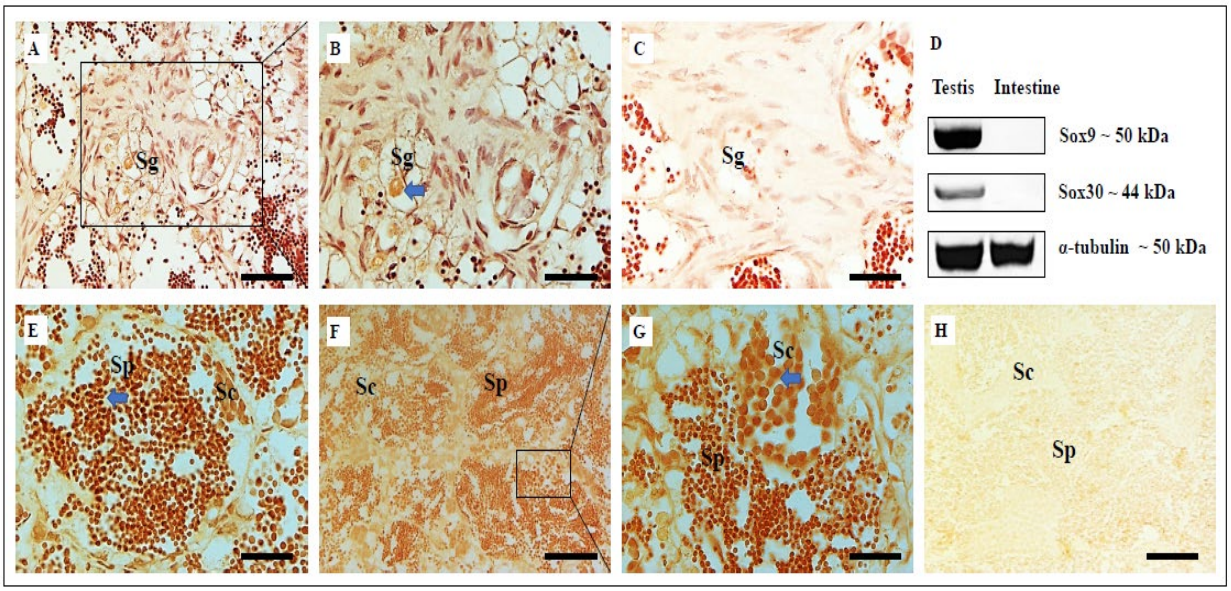

Fig. 4 Localization and western blot of Sox9 and Sox30 proteins in adult carp testis. Immunoreactivity of Sox9 (A, B) and Sox30 (E-G) in testis, whereas their respective negative controls (pre-adsorbed antibody with excess antigen) showed no signal (C, H). Western blot

analysis of Sox9 and Sox30 proteins displayed bands of ~50 kDa and ~44 kDa respectively, in testis while in the negative control (intestine) no bands were observed (D). Abbreviations: Sg: spermatogonia, Sc: spermatocytes, Sp: spermatid/sperm. The scale bar indicates A, E: 30 µm; B, C, G: 10 µm; F, H: 50 µm.

3.5 Effects of hCG and 11-KT

Induction with hCG at the preparatory phase of the carp testicular reproductive cycle enhanced the expression of *sox9a/b* and *sox30*. In both *in vivo* (Fig. 5A-C) and *in vitro* (Fig. 5D-F) treatments (n=5), prominent expression of *sox9a/b* and *sox30* was observed after 12 h ($P < 0.05$) of induction. Progressive increase of expression was evident with time upon induction from 12 to 24 h. The influence of hCG on androgen induction in testis is well known. 11-KT being a potent androgen in fish [33], its effect on *sox9a/b* and *sox30* expression was studied. *In vitro* study (Fig. 5G-I), showed a substantial elevation ($P < 0.05$) of *sox9a/b* and *sox30* at 12 h in comparison with control.

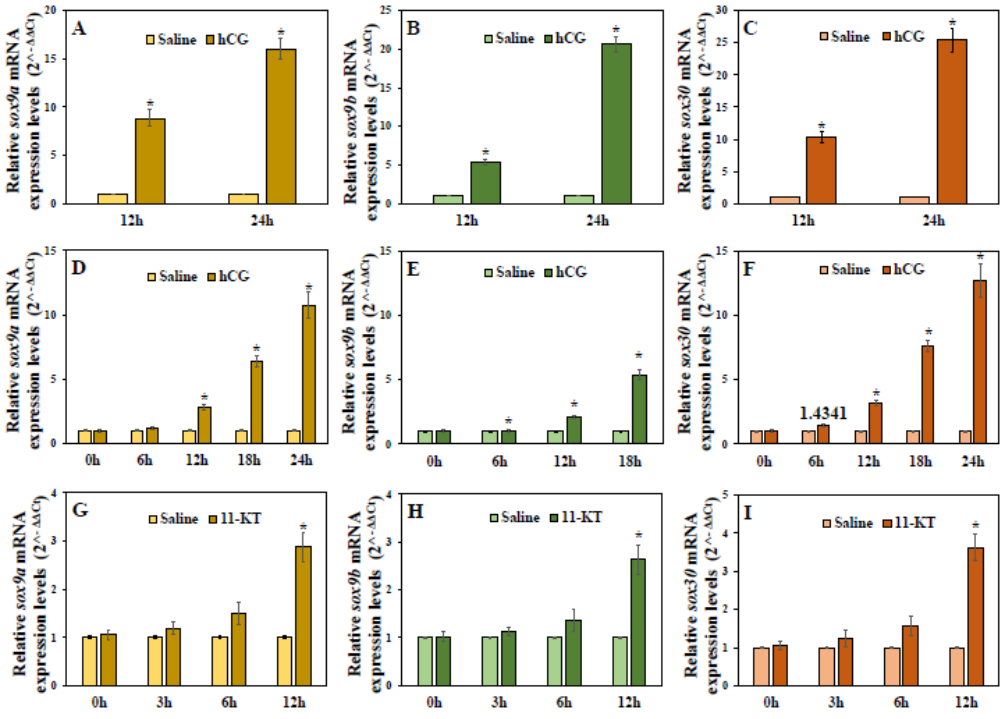

Fig. 5 Relative gene expression after hCG (1000 IU/Kg body weight) *in vivo* [*sox9a* (A), *sox9b*(B) and *sox30*(C)], hCG (100 IU/ml) *in vitro* in testicular slices [*sox9a* (D), *sox9b*(E) and

sox30(F)] and 11-KT (100 ng/ml) *in vitro* in testicular slices [*sox9a* (G), *sox9b* (H) and *sox30* (I)] treatments at different time intervals compared with control (saline) in carp. Data (n=5) were expressed as mean ± SEM (* P < 0.05; One-way ANOVA followed by Dunnett's method). Abbreviation: h-hour.

3.6 Effects of mono-sex induction by MDHT and E_2 treatments

The expression levels of *sox9a/b* and *sox30* were compared in mono-sex-induced carp. MDHT treatment for 50 days increased the expression of *sox9a/b* and *sox30* significantly (P < 0.05) in testis (Fig. 6A) during the gonadal development of carp. *kiss2* and *gnrh* expression were determined in the brain while expression of *fshr* and *lhr* was determined in the gonads of mono-sex-induced carp.

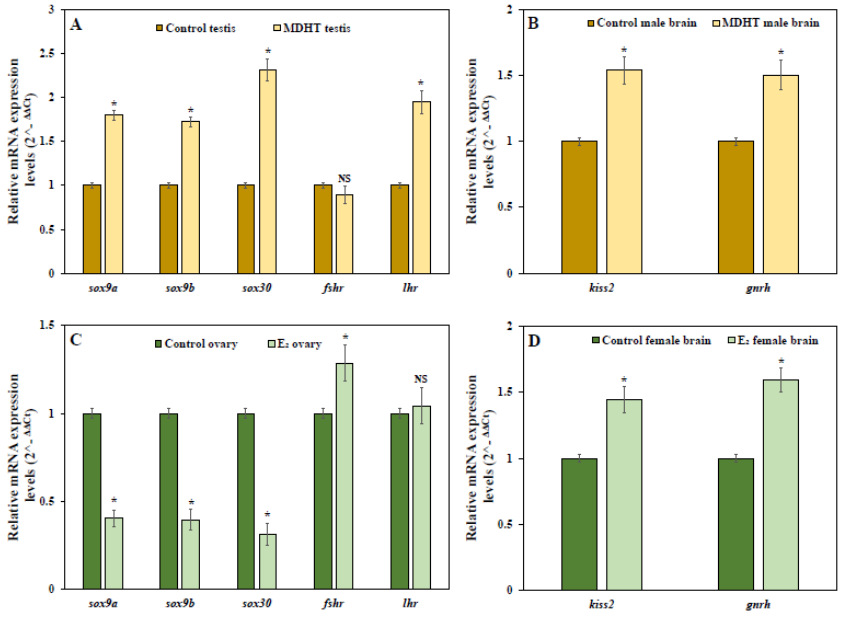

Fig. 6 Relative expression of genes (*sox9a*, *sox9b*, *sox30*, *fshr*, and *lhr*) in mono-sex-induced carp gonads (A, C). Relative expression of genes (*kiss2* and *gnrh*) in mono-sex-induced carp brain (B, D). NS- not significant. Data (n=5) were expressed as mean ± SEM (* P < 0.05; One-way ANOVA followed by Dunnett's method).

The expression of *lhr* was significantly ($P < 0.05$) high, while *fshr* expression was comparable to control in MDHT treated testis (Fig. 6A). The expression of *kiss2* and *gnrh* was elevated in the brain of MDHT (Fig. 6B) treated carp. E_2 treatment decreased the transcript levels of *sox9a/b* and *sox30* in the ovary (Fig. 6C). *fshr* expression was significantly ($P < 0.05$) high, while *lhr* expression was comparable to control in E_2 treated ovary (Fig. 6C). The expression of *kiss2* and *gnrh* was elevated in the brain of E_2 (Fig. 6D) treated carp.

3.7 Silencing of *sox30*, *in vitro*

In carp testicular culture (n=5), PEI complexed with antisense *sox30*-siRNA significantly ($P < 0.05$) reduced *sox30* expression compared to control and after sense *sox30*-siRNA treatments (Fig. 7A). Besides, the expression of *sox30* post-siRNA treatment exhibited dosage-related downregulation. Expression of *sox30* significantly ($P < 0.05$) declined to 88% after treatment with 10 ng/µl of *sox30*-siRNA in comparison with control, hence this dose was chosen for later on experiments. Transient silencing of *sox30* displayed a significant reduction ($P < 0.05$) in the transcript levels of certain transcription factors (Fig. 7B), few germ/stem cell markers, and other factors (Fig. 7C) crucial for testicular function, and steroidogenesis-related genes (Fig. 7D). Relative mRNA levels of *ad4bp/sf-1*, *gata4*, *wt1*, *sox5*, *sox9a/b*, *dmrt1*, *sycp3*, *vasa*, *pou5f3/oct4*, *gsdf*, *pdgf*, *amh, star*, *cyp11*, *cyp17*, *hsd3b*, *hsd17b*, *hsd20b*, *hsd11b*, and *srd5a1* showed a decline, while *dax* and *piwi* did not show any significant change and *ar* showed a significant up-regulation when compared to control. The uptake of siRNA was confirmed by fluorescent signals evident in carp primary testicular culture treated with control siRNA (Fig. 7E and F).

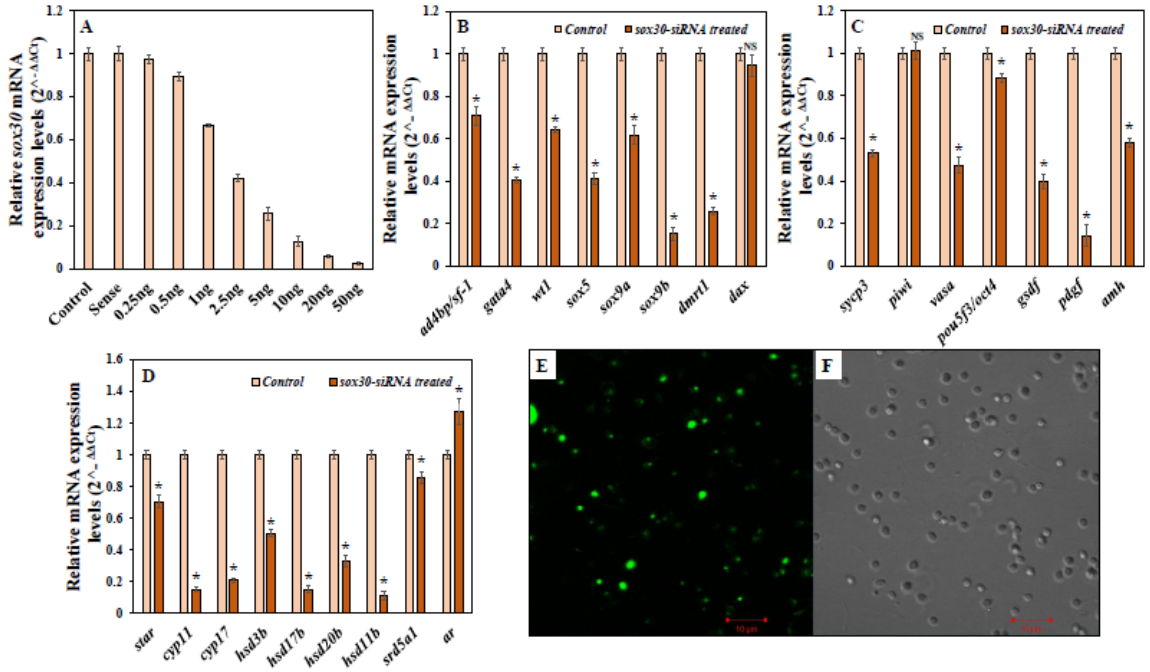

Fig. 7 Relative expression of (A) *sox30* 24 h after *in vitro* PEI mediated *sox30*-siRNA treatment (control, 10 ng/µl of sense *sox30*-siRNA and various doses of antisense *sox30*-siRNA) in carp testicular culture (mixed; n=5). Relative expression of (B) transcription factors, (C) germ/stem cell marker genes and other factors, and (D) steroidogenesis related genes between control and 10 ng/µl of antisense *sox30*-siRNA treated carp testicular culture after 24 h of treatment (* $P < 0.05$; One-way ANOVA followed by Dunnett's method). NS-not significant. Representative (E and F) confocal microscope images [E (fluorescent), F (phase-contrast)] 24 h after *in vitro* PEI mediated transfection of MISSION siRNA fluorescent universal negative control conjugated with 6-FAM on carp testicular culture (mixed).

3.8 Transient silencing of *sox30*, *in vivo*

Treatment of antisense *sox30*-siRNA-PEI complex significantly downregulated ($P < 0.05$) the expression of *sox30* indicating that siRNA complexed with PEI was capable of silencing *sox30* in carp testis (Fig. 8A). On the other hand, only PEI or sense *sox30*-siRNA-PEI complex treatments showed no effect in terms of *sox30* expression. Expression of *sox30* was quantified in testis on days 0, 2, 4, 6, and 8 post-treatment along with corresponding control at every time point (Fig.

8A). The antisense *sox30*-siRNA-PEI complex treatment decreased the expression of *sox30* ($P < 0.05$) on days 2 and 4 post-treatment with 44 and 90% reduction, respectively. Further, *sox30* expression decreased to 37% on day 6 and 10% on day 8 indicating that transcript levels might restore to normal as reported earlier (Laldinsangi and Senthilkumaran, 2018; Murugananthkumar and Senthilkumaran, 2016). The antisense *sox30*-siRNA-PEI sample on day 4 showed a 90% decrease in *sox30* expression and hence that time-point was chosen as a treatment sample for later on experiments. Sox30 protein levels reduced similar to that of *sox30* transcripts while there was no change in α–tubulin employed as a control (Fig. 8B). A substantial decrease in Sox9 protein levels was observed after siRNA treatment, as opposed to the control (Fig. 8B). The band intensity was measured using ImageJ software and normalized with α–tubulin (Fig. 9). There were no significant changes in the expression of *sox11*, *sox13*, *sox18*, *sox19*, and *sox21* in the *sox30*- siRNA treated group (Fig. 10) demonstrating the specificity of the siRNA used in this study. Further, no significant phenotypic changes were observed in the histological images between control and siRNA injected testis (Fig. 11) proving the transient nature of the PEI siRNA treatment. Besides, it might be difficult to detect any change in the spermatogenesis process as the testis was at the late spermatogenesis stage. However, molecular changes similar to the *in vitro* treatment were observed upon *in vivo* transient silencing. The expression of transcription/other factors namely, *ad4bp/sf-1*, *gata4*, *wt1*, *sox5*, *sox9a/b*, *dmrt1*, *gsdf*, *pdgf*, *amh* (Fig. 8C), *star* and steroidogenic enzyme genes *cyp11*, *cyp17*, *hsd3b*, *hsd11b*, *hsd17b*, *hsd20b*, *srd5a1* (Fig. 8D) showed a decline on day 4 post-*sox30*-siRNA treatment but the expression levels were comparable to that of control on day 8 post-*sox30*-siRNA treatment, further substantiating the transient nature of siRNA silencing. There was no significant change on day 4 and day 8 in the expression of *dax*, while *ar* expression showed a significant up-regulation on day 4, reverting to normal levels on day 8.

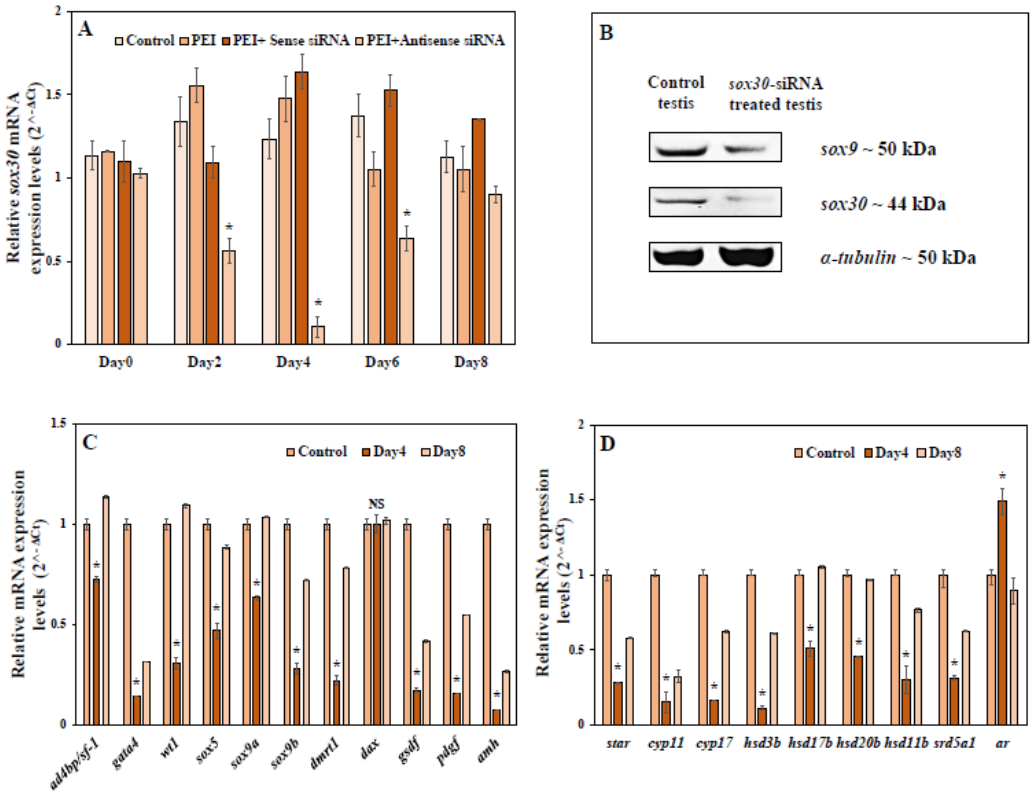

Fig. 8 Relative expression of *sox30* (A) post i*n vivo* PEI mediated *sox30*-siRNA treatment in adult (n=5) carp (*, P < 0.05; One-way ANOVA followed by SNK test). (B) Western blot of Sox9 and Sox30 proteins on day 4 in control and *sox30*-siRNA treated carp testis. Relative expression of various (C) testis-related and (D) steroidogenesis-related genes/factors on day 4 in control and *sox30*-siRNA treated carp testis (* P < 0.05; Mann–Whitney test One-way ANOVA on ranks followed by SNK test). NS-not significant.

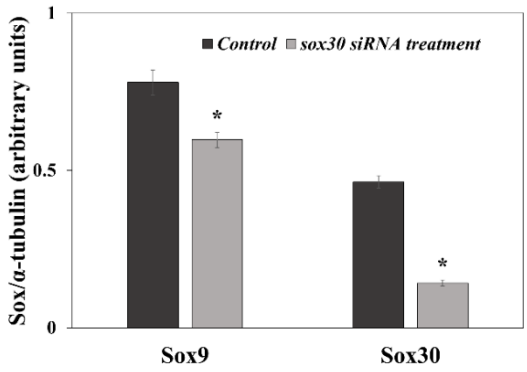

Fig. 9 Densitometry of western blot of Sox9 and Sox30 proteins on day 4 in control and *sox30*-siRNA treated carp testis. The protein band intensities from three independent experiments

(mean ± SEM) were measured using ImageJ software and normalized with the α-tubulin (* P < 0.05; One-way ANOVA followed by Tukey test).

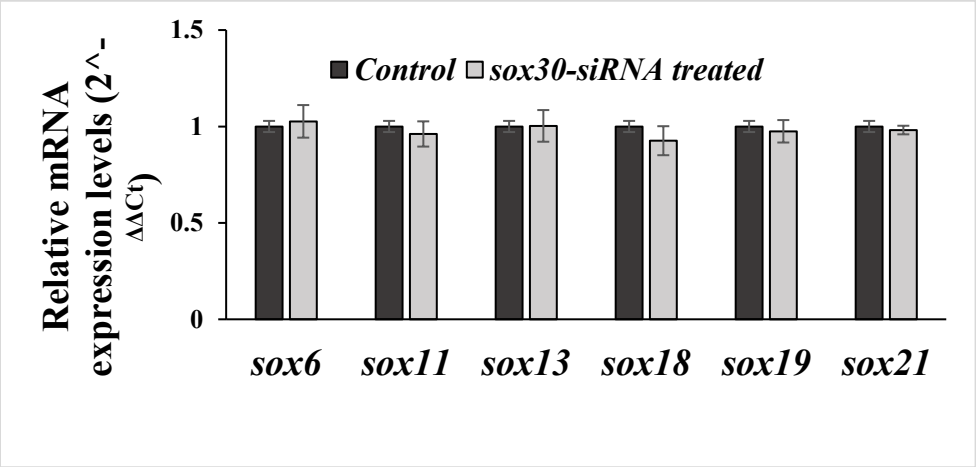

Fig. 10 Relative expression of *sox* genes on day 4 between control and *sox30*-siRNA treated carp testis. No significant difference was observed in any of the groups.

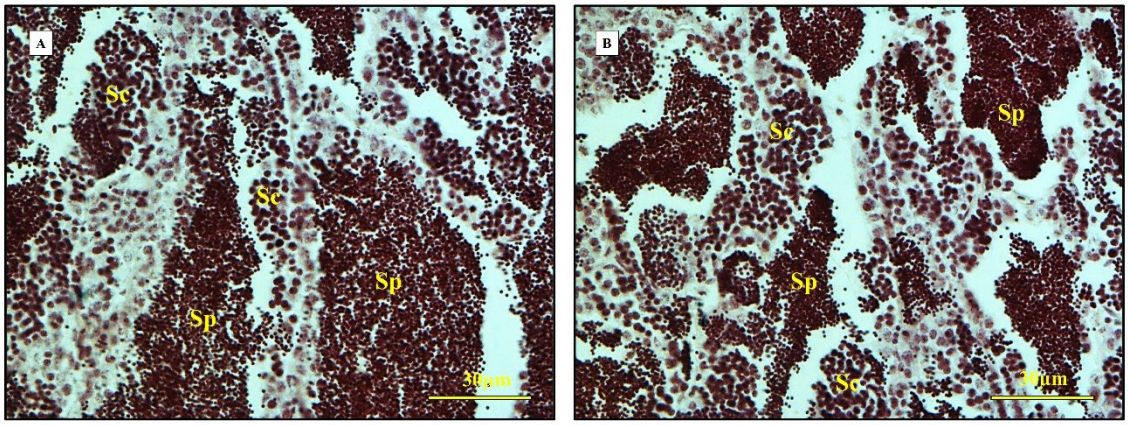

Fig. 11 Representative histological images of carp testis on day 4 (A) control and (B) *sox30*-siRNA treatment. Abbreviations: Sc: spermatocytes, Sp: spermatid/sperm.

Serum levels of T (Fig. 12A) and 11-KT (Fig. 12B) declined significantly (P < 0.05) on day 4 post *sox30*-siRNA treatment. Enzyme activity of Hsd11b significantly (P < 0.05) reduced on day 4 post *sox30*-siRNA treatment (Fig. 12C).

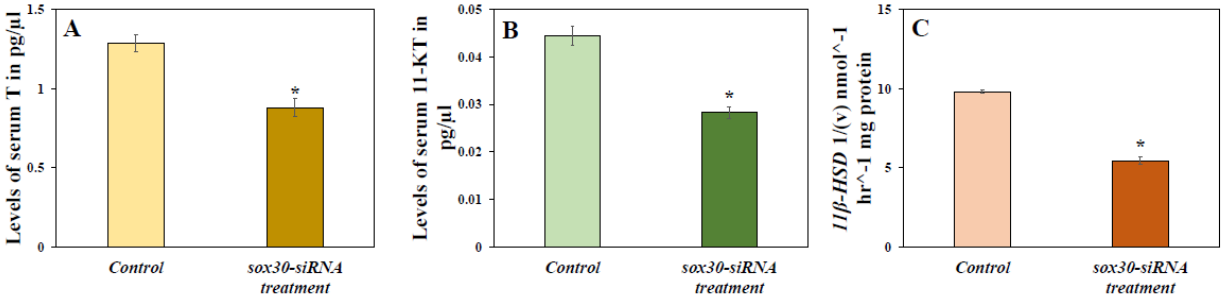

Fig. 12 Changes in the levels of serum (A) T and (B) 11-KT on day 4 in the control and *sox30*-siRNA treated carp. (C) Changes in enzyme activity of Hsd11b on day 4 in the control and *sox30*-siRNA treated carp testis. Data (n=5) were expressed as mean ± SEM. (* $P < 0.05$; Student's t-test).

4 DISCUSSION

The presence of *sox30* in teleost (Han *et al*., 2010) and its vital role in mammalian spermiogenesis (Zhang *et al*., 2018a) have been well documented. However, the significance of *sox30* in testicular steroidogenesis remains unexplored in lower vertebrates including teleost. The current study reports the cloning of *sox30* from adult *C. carpio* testis, and cloning partial cDNAs of *sox9a/b*, whose roles have been earlier demonstrated in carp (Du *et al*., 2007). Expression of s*ox9a/b* and *sox30* during gonadal development, changes in distinctive phases of the testicular reproductive cycle, post-11-KT treatment, after hCG induction, in mono-sex-induced carp, and the repercussions of *sox30*-siRNA silencing, were highlighted to demonstrate their dominant role in testicular steroidogenesis vis-à-vis testis function.

4.1 Cloning, expression analysis, and localization

An earlier study from our laboratory employing a comparative transcriptomic strategy identified key genes involved in sex- differentiation, revealing the existence of *sox30* in carp (Anitha *et al*., 2019). Using gene-specific primers, ORF of *sox30,* and partial cDNA fragments of *sox9a* and

sox9b were obtained in the current study. The expression of *sox30* was elevated in the testis followed by the ovary in contrast to somatic tissues (Wei *et al*., 2016), while *sox9a/b* showed higher expression in the brain followed by the testis. Upon quantification, *sox30* displayed predominantly higher expression in the testis in comparison to *sox9a/b*. A previous study (Han *et al*., 2010) in the Nile tilapia reported the expression of *sox30* from 10 dph, earlier than the morphological gonadal differentiation period (about 25 dph). However, in this study, *sox30* displayed significant dimorphic expression at 120 dph during the onset of testicular growth in carp (Komen *et al*., 1992). Further, expression of *sox30* was high in mature testis as reported in mice (Feng *et al*., 2017) implicating its role in maturation as well. The present study quantified the expression of *sox9a/b* and *sox30* at distinctive phases of the carp testicular reproductive cycle. Higher expression of *sox9a/b* was observed during preparatory and pre-spawning, marking their role during the initiation of spermatogenesis. *sox30* expression was abundant in pre-spawning, characterized by the predominance of spermatogonia and spermatocytes and in the spawning phase, corresponding to spermiogenesis. This sign that in carp, the expression of *sox30* was high during spermatogenesis and spawning/spermiation as observed in mice (Zhang *et al*., 2018a). Seasonal changes in *sox30* expression may signify its role during testicular maturation. Sox9 was found in spermatogonia while Sox30 was localized in spermatocytes and spermatid/sperm as previously reported in the Nile tilapia (Han *et al*., 2010) and mice (Han *et al*., 2014).

4.2 Influence of gonadotropin

Steroidogenesis is strictly regulated by gonadotropins (Payne and Youngblood, 1995) and in fact, gonadotropins are well known to stimulate steroid synthesis in teleost gonads (Devlin and Nagahama, 2002). The influence of gonadotropin induction in carp has been testified (Yu *et al*., 1986). There was a substantial increase in *sox9a/b* and *sox30* mRNA transcripts post-hCG

induction, validating the regulatory role of gonadotropin on *sox30* expression. LH-like hCG is proven to bind to LH receptors (Vischer *et al*., 2003) expressed by Leydig cells to induce androgen synthesis and also to act upon Sertoli cells to regulate spermatogenesis (Skinner, 1991).

4.3 Effect of hormone treatment

It is known that hCG induces T and 11-KT production in fish (Wade and Van der Kraak, 1991). Having studied the leverage of gonadotropins on *sox30*, the influence of androgen on gene expression is yet another exhilarating area to study. The expression of *sox9a/b* and *sox30* was found to be elevated after *in vitro* 11-KT treatment in carp testicular slices. In fish, treatment with sex steroids during the critical window of sexual differentiation can alter the developmental processes of gonads (Devlin and Nagahama, 2002). Reports suggest that 40-50 days of treatment in carp (Hulak *et al*., 2008) with different supplementations to feed can result in complete sex-reversal. An all-male population of blue tilapia, *O*. *aureus* was produced with 17 α-ethynyltestosterone treatment (Guerrero, 1975). Exposure of methyltestosterone to catfish during gonadal differentiation resulted in the male population (Raghuveer & Senthilkumaran 2009). Expression of *sox9a/b* and *sox30* was elevated after MDHT but not after E_2 treatment. In teleost, during sex-inversion/differentiation, gender-specific sexual differences are contingent on the sequential presence of brain and/or gonadal sex differences under the impact of gonadal factors to hormonal response (Senthilkumaran *et al*., 2015). Gonadotropin regulation during natural and artificial induction was observed. Further, to clarify the role of the brain-pituitary gonadal axis during mono-sex induction, expression levels of gonadotropin receptors were studied. Therefore, the expression of *kiss2* and *gnrh* was determined in the brain, which showed their higher expression in both E_2 and MDHT treatments. However, the expression of *fshr* was higher in E_2 and *lhr* was higher in MDHT treated gonads. Our results were comparable to that of the previous reports (Cabas

et al., 2013) in gilthead seabream, *Sparus aurata L.* and (Hu *et al*., 2011) orange-spotted grouper *Epinephelus coioides*. In most gonochoristic teleost, *lhr* expression was high during the late stages of gonadal development, vital for spawning and spermiation (Levavi-Sivan *et al*., 2010). The current study showed the transcript levels of *lhr* was significantly high after MDHT treatment, suggesting the involvement of LH signaling in triggering mono-sex induction and subsequent testis development in carp.

4.4 Transient silencing of *sox30*

Most knockdown studies to investigate the significance of *sox30* during gametogenesis were done in mammals however, this report is the first of its kind to show the transient silencing of *sox30* in any lower vertebrate. To further elucidate the role of *sox30* in gonadogenesis, the effect of PEI mediated transient silencing of the *sox30* was studied. Previous reports delineated that synthetic siRNA induces gene silencing, concerning numerous developmental processes (Gupta and Senthilkumaran, 2020; Murugananthkumar and Senthilkumaran, 2016). Transient silencing using *sox30*-siRNA did not impact phenotypic changes in carp testis as reported earlier (Laldinsangi and Senthilkumaran, 2018). Delivery of *sox30*-siRNA resulted in decreased expression of the regulators of gonadogenesis, namely *ad4bp/sf-1, gata4,* and *wt1* (Murugananthkumar and Senthilkumaran, 2016), demonstrating *sox30* might affect the expression of these genes. In vertebrates, *ad4bp/sf-1* is an important steroidogenic factor required for the basal expression of steroidogenic enzymes (Morohashi and Omura, 1996). An *in vitro* study by Sakai *et al*. (2008) reported that *sox30* is a more effective regulator of *ad4bp/sf-1* than *sox9*. Bai *et al*. (2008) reported a *sox30* binding site in the promoter motif of *ad4bp/sf-1* in mice. In mammals, it is well established that *sox9* transactivates *ad4bp/sf-1* gene expression (Shen and Ingraham, 2002) to achieve tissue-specific gene expression. Similarly, *sox30* might regulate *ad4bp/sf-1* expression in fish. Besides,

Chen *et al*. (2018) showed differential expression of *gata2* in the *SOX30* knockout mice line. In this study, transient silencing of *sox30* resulted in the downregulation of transcription factors crucial for testicular development namely, *dmrt1* (Raghuveer and Senthilkumaran, 2009), *sox5* (Schartl *et al*., 2018), and *sox9a/b* (Raghuveer and Senthilkumaran, 2010). Zhang *et al*. (2018a) have reported evidence of the *SOX30* binding site in *DMRTA2* of mice. In the Nile tilapia, *dmrt1* directly binds to a putative cis-regulatory element within the *sox30* promoter (Tang *et al*., 2019). Feng *et al*. (2017) have shown the downregulation of *SOX5* in *SOX30* knock-out mice. Though there were no reports about the relationship between *sox30* and *sox9*, the presence of *sox30* DNA binding domain ACAAT (Osaki *et al*., 1999) in the *sox9* promoter regions of *Sebastes schlegelii* (GenBank: KJ624401.1[unpublished]) warrants such a perspective. This report is the first to show the reduction of *sox9a/b* upon transient silencing of *sox30* in carp, indicating either a direct or indirect regulatory loop among the *sox* genes. Gonadogenesis is influenced by several other factors such as *amh* (Rodríguez-Marí *et al*., 2005), *gsdf* (Shibata *et al*., 2010), *PDGF* (Gnessi *et al*., 1995). Gamete production by meiosis is dependent upon crucial germ cell governing genes like *vasa*, *sycp3,* and *pou5f3/oct4* (Laldinsangi and Senthilkuamaran, 2018; Rodríguez-Marí *et al*. 2013). Silencing of *sox30* also affected the expression of *amh*, *gsdf*, *pdgf*, *vasa*, *pou5f3/oct4,* and *sycp3* in this study as observed in mice (Cheng *et al*., 2018; Zhang *et al*., 2018a). The production of testicular androgen is governed by steroidogenesis. In teleost, studies (Socorro *et al*., 2007; Wang and Orban, 2007) have reported the direct participation of 11-OHT and 11-KT in the differentiation and development of testis. Previous reports have shown the significance of *hsd11b* during testicular development and recrudescence in teleost (Rasheeda *et al*., 2010). In particular, *sox3* has been proven as a transcriptional activator of the *hsd11b* gene by binding to two of its specific promoter motifs in catfish (Rajakumar and Senthilkumaran, 2016), suggesting that genes from the

sox family may regulate *hsd11b* expression, and hence testicular steroidogenesis, in teleost. Concomitantly, transient knockdown of *sox30* resulted in decreased T, 11-KT, Hsd11b enzyme activity of and downregulation of *star* and steroidogenesis-related genes, *srda51*, *hsd3b*, *hsd11b*, *hsd17b,* and *hsd20b*. Supporting the above contention, Chen *et al*., (2018) have observed differential expression of HSD3B in SOX30 knockout mice. Further, transient knockdown of *sox30* exhibited overexpression of *ar*, the nuclear receptor for 11-KT in teleost (Todo *et al*., 1999). Reports have suggested that *ar* overexpression could be a potential mechanism for hypersensitivity due to low androgen (Kawata *et al*., 2010).

4.5 sox9a/b vs *sox30*

The most interesting aspect of this study was attempting to signify the role of *sox30* in comparison with *sox9a/b*. The dominance of *sox30* over *sox9a/b* expression was observed in adult testis. Evidence of dominant expression of *sox30* during spermatogenesis and especially in spermiogenesis and high expression of *sox9a/b* at the initial stages of spermatogenesis signifies their role in gamete maturation. Incidentally, the influence of gonadotropin, 11-KT, and mono-sex induction was similar in *sox9a/b* and *sox30*. Transient silencing of *sox30* decreased *sox9a/b* levels hinting that *sox30* may act as a regulating factor for *sox9a/b*, either directly or indirectly.

5 CONCLUSION

The current study reveals that *sox30* transcripts are predominantly expressed in the testis in comparison to the ovary and might regulate testicular growth as well as maturation during the reproductive cycle. Overexpression of *sox30* upon mono-sex induction indicates an important role during testicular development. Transient silencing of *sox30* influenced steroidogenic enzyme, and other factor genes as well as sex steroid levels. Overall, the present study demonstrated the significance of *sox30* in testicular steroidogenesis vis-à-vis testis function of carp by influencing

sox9a/b, either directly or indirectly, along with other male-related genes as evidenced by expression analysis and transient gene silencing.

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Chapter 3

Understanding the role of *sox19* in ovarian function

ABSTRACT

In teleost, ovarian steroidogenesis governed by the neuroendocrine system is also regulated by several transcription factors of gonadal origin. Investigating the synchronized interactions between the transcriptional and the hormonal factors is vital to comprehend the mechanisms that lead to gonadal differentiation. This study signifies the role of *sox19* in regulating ovarian steroidogenesis of carp, *C. carpio*. Tissue distribution displayed the predominant expression of *sox19* in brain and ovary, and gonadal ontogeny showed overexpression of *sox19* at 80 dph. Higher *sox19* mRNA expression during the spawning phase, an elevation of *sox19* post hCG induction substantiate gonadotropin dependency. E_2 treatment to 50 dph carp for inducing mono-sex, elevated *sox19* expression substantially. Sox19 protein was observed in GC of follicular layer in carp ovary. Higher *sox19* expression was detected in isolated GC and TC, *in vitro*. Transient gene silencing with *sox19*-siRNA caused downregulation of certain genes related to *wnt*-signaling, transcription factors, and steroidogenic genes related to ovary. Serum E_2 and 17α, 20β-DP reduced significantly post *sox19* silencing*, in vivo*. Concomitantly, a decrease in aromatase activity was detected post *sox19*-siRNA treatment, *in vivo*. This study demonstrates the impact of *sox19* in the regulation of carp ovarian growth and steroidogenesis.

1. INTRODUCTION

Fish gonadogenesis and steroidogenesis are highly synchronized processes, in which several transcription factors and hormones are involved. The developmental pathways leading to steroid synthesis in gonadal cells require a composite regulation of multiple genes (Swain and Lovell-Badge, 1999) intricated in the differentiation of steroid secreting cells. Transcription factors regulating the expression of steroidogenic enzymes are a great deal of interest. The most researched are those that regulate *cyp19* as it plays a decisive role in fish sex differentiation.

Members of the *sox* family of transcription factors play a variety of roles during various developmental processes (Uy *et al*., 2012). Extensive studies on *sox* family genes have been conducted in many teleost (Hett and Ludwig, 2005; Koopman *et al*., 2004; Galay-Burgos *et al*., 2004; Zhou *et al*., 2002). *sox* genes are sub grouped into A–K based on HMG box sequence homology (Bowles *et al*., 2000, Zhang *et al*., 2018). Group B1, comprising three genes in mammals (*SOX1/2/3*), also has an additional *sox19*, not represented at all in mammals (Schepers *et al*., 2002). Interestingly, *sox19* was only found in the teleost genome such as half smooth tongue sole, fugu, pufferfish, the Nile tilapia, zebrafish, and carp (Koopman *et al*., 2004; Zhang *et al*., 2018). Reports suggest that *sox19* combines certain features of *sox2* and *sox3* and could have evolved through an ancient fish-specific duplication of *sox3* (Koopman *et al*., 2004). SOX proteins bind to the similar targets (Wegner, 1999). Precision in selection of target gene is thought to be executed via a combinatorial machinery involving communication with other cell-specific transcription factors along with spatio-temporal expressions (Kamachi *et al*., 1999, 2000; Wilson and Koopman, 2002). Strikingly, when HMG of *SRY* was substituted with either of *SOX3*/*SOX9*, *SRY* retained male determination ability in transgenic mice (Bergstrom *et al*., 2000) proving that *SOX3*, *SOX9*, and *SRY* target similar genes. In amphibians, *sox3* was reported to play a significant role in ovarian differentiation by binding and activating *cyp19a1a* promoter (Oshima *et al*., 2009). Navarro-Martín *et al*. (2012) have proposed a possible role of *sox19* in ovarian differentiation of the European sea bass. In light of the above, in this study the possibility that a fish specific, group B1 *sox* gene, such as *sox19* was analyzed to understand its role, if any during ovarian steroidogenesis. For which, carp which follows a seasonal spawning cycle and attains early gonadal maturation was chosen instead of typical annual breeders. Expression profiling of *sox19* in adult tissues, as well as in ovary during natural spawning cycle, after hCG induction, post mono-

sex-induction, localization and analysis of *sox19* regulation on ovarian steroidogenesis by transient gene silencing were performed to test impact of *sox19* in teleost.

2. MATERIALS AND METHODS

2.1 Fish sampling

Procuring and rearing of carp were done as described earlier in the chapter 1. Sampling for tissue distribution, gonadal ontogeny, localization and mono-sex induction were followed as described in chapter 2.

2.2 Cloning of *sox19* from adult carp ovary

Following the procedure described in chapter 2, primer designing (Table1) and cloning of *sox19* from adult carp ovary was performed.

Table 1. List of primers used in the study.

S. No.	Gene name/symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number
1	*18s rRNA*	GCTACCACATCCAAGGAAGGCAG2C	CGGCTGCTGGCACCAGACTTG	FJ710827.1
2	*ad4bp/sf-1*	CGGACCTGGAAGAGTTGT	GGTTCTCTTGGCATGCAG	GSE112157
3	*erα*	GCCCACAAACTCTCACCCATG	CCAGTGCCTAAGATCTGC	AB334722.1
4	*erβ1*	GGGTATGTTTTGGCCAGTTG	CTGCCGTATCATCAACAGGGAGC	GSE112157
5	*erβ2*	GCCCACAAACTCTCACCCATG	CCAGTGCCTAAGATCTGC	KU205262.1
6	*ctnnb1*	CAATCTGACTTGATGGAGC	GGTCTGCCACCTGCTCAG	GSE112157
7	*cyp11*	TCAACCAAGCGGACCACTGT	CACCTCCAGCCATCAGCTC	GSE112157
8	*cyp17*	GCAGCACTGTATCTCAGAAG	GGCACGCGTCAGCTCCGGTG	GSE112157
8	*cyp19a1a*	GGTCCTTGTTTCTTCCTGGG	CAAGATGAGAGTTTCCTCACC	DQ534411.1
9	*fzd*	CGCGCTGCCGAGGATGAGTA	GCTGCTTCACCTCCGCTATG	GSE112157
10	*foxl2*	GCGTCTCACGCTGTCCGG	GCCGGTAGTTGCCCTTCT	KP764768.3
11	*fstl*	GAGCAATGCAAGCCTCATAAG	CATAACACACAACTGGGCTGGC	GSE112157
13	*hsd3b*	GGCTTTTCTGTTCATGCCTG	CACGCGTCAGCTCCGGTGCC	GSE112157
15	*hsd17b*	GACATCCTGGTGTGTAATGCAGG	CTGCCTGTGACCAGGATCCGT	GSE112157
16	*hsd20b*	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA	GSE112157
21	*rspo1*	GAGACGAATAAGCACTGAAG	GTGTGCATTTGTTCATATCACG	GSE112157
22	*sox5*	GGACAGCCTGGTGGAGAAAG	CTGCTGTTGTCGTGCAATC	GSE112157
23	*sox6*	CCGGGGAAGGAACAGCAAC	GTGCCTGCTCCTCATAATATG	GSE112157
24	*sox9a*	GGTGAACAACGGCCAGAG	GACTGGCCTGAGTGCTCG	GSE112157
25	*sox9b*	GAACGGCCAGAGCGAGAG	GACCCTGGGACTGACCTG	AY956415.1

26	*sox11*	GCACCAAGTGGAATAAACACCTG	CTGATTTTCTTGCTGCTAGTCAC	GSE112157
27	*sox13*	GGCCAAAGACGAAAGACGG	GGCCGGGGTTTGTATTTGTAG	GSE112157
28	*sox18*	GATGCTGGGTCAATCCTGG	CGATGTGGCGAGTAGGCATC	KY860090.1
29	*sox19 qPCR*	GGCACAGGAGAACCCCAAAATG	CTTCATGAGGGCTTTGGTC	GSE112157
32	*sox19* ORF	ATGTACAGCATGATGGAG	TCAGATATGAGTGAGGGGAAC	GSE112157
33	*sox21*	GGTAAGGTTCATGTTGAGCTTTG	CATTACTCAATGCTCGGGTATC	GSE112157
34	*sox30*	CAGAGCAGTCTAAATTCTCCAGC	GGCTAGAAGAGCTTGAACATGG	MT193291
35	*star*	GGAGCTCCCTTCTCAGCAG	CTGTCTTCTGTTCCAGCATC	GSE112157
36	*wnt4*	TCCACCGACATGTGTGCATC	ATCATAATTTGTTAACAAAT	GSE112157
37	*vtg*	CCTTGTTGCAAACAATGTGG	GTGCCAACAGAAGGAAGAGC	AF414432.1

2.3 Multiple sequence alignment and phylogeny

Multiple sequence alignment and phylogenetic analysis of deduced *sox19* aa with its counterparts was done as demonstrated in chapter 2.

2.4 qPCR

Total RNA isolation, reverse transcription, real-time primer design (Table 1) and qPCR were done as detailed in chapter 2.

2.5 Polyclonal antibody generation for Sox19

Following the procedure explained in chapter 2, using an antigenic peptide GSKTSCPPGGDSMDKC Sox19 antibody was generated.

2.6 Western blot

For validating anti-carp Sox19 antibody, western blot was done in ovary and muscle samples as mentioned in chapter 2.

2.7 IHC

IHC was employed to localize Sox19 in adult carp ovary following description of chapter 2.

2.8 *In vitro* hCG induction

The *in vitro* hCG induction was performed by following the procedure of Gupta and Senthilkumaran (2020). Ovaries from adult female carp during preparatory phase were dissected,

oocytes were collected and (triplicate) placed in oocyte incubation medium (Senthilkumaran and Joy, 2001) containing 100 IU/mL of hCG (Pubergen, Uni-Sankyo, Hyderabad, India) or control (fish physiological saline). The oocytes were taken during 0, 6, 12 and 24 h post hCG induction followed by total RNA isolation, cDNA synthesis and proceeded for qPCR analysis. During the course of the experiment, average death was about 8–10 % and from time to time by observing color changes the dead eggs were carefully removed using a sterile pipette.

2.9 *In vivo* hCG induction

During the preparatory phase hCG injection was given to adult female carp as detailed in chapter 2.

2.10 Mono-sex induction

Mono-sex induced samples were obtained as mentioned in chapter 2.

2.11 Aromatase assay

The aromatase activity in carp gonads was assayed shadowing Rasheeda *et al*. (2010). In brief, microsomes from gonads were obtained by homogenizing 2 g of tissue with 2 mL of 100 mM KPO_4 (pH 7.4) and clearing debris by centrifuging at 9000 × *g* at 4 °C for 20 min. Then, ultracentrifugation at 105,000 × *g* at 4 °C for 1 h (Optima™ Max-XP Tabletop Ultracentrifuge, Beckman Coulter, USA) was done to obtain the microsomal pellet, which was rinsed and reintroduced in 500 µl of KPO_4/ 20% glycerol/ 0.1 mM EDTA (v/v). For measuring aromatase, 300 µg of microsomal protein along with 0.6 µM of 1β- [^{3}H] androstenedione (Amersham) in 500 µL reaction constituting 10 U of glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate, 1 mM β-NADP+,1 mM EDTA and 100 mM KPO_4 was incubated for 1 h in a shaker incubator at 37 °C. To terminate the reaction, 10 % chilled trichloroacetic acid with 20 mg dextran-layered activated charcoal/ mL was added. Following centrifugation at 1000 × *g* at 4 °C for 10 min, the supernatant was added to 3 mL of cocktail-T (SRL Chemicals, Mumbai, India) contained in

scintillation vials to measure radioactivity (Wallac 1409-liquid scintillation counter, Pegasus Scientific Inc., USA). The experiment was done in triplicate for five different biological samples. The results were expressed as mean ± standard error of the mean and the data was evaluated using One-way ANOVA followed by post hoc tests with P-value < 0.05 considered to be statistically significant.

2.12 Isolation of GC and TC from adult carp ovary

The isolation of GC and TC was performed as described (Benninghoff and Thomas, 2006) with some modifications (Paul *et al*., 2010). Adult carp ovaries were dissected and placed into ice-cold Idler's medium followed by frequent gentle pipetting to separate the mature oocytes. Adult female carps were dissected and ovaries placed in ice-cold Idler's medium (pH 7.4) comprising penicillin (100 IU/mL) and streptomycin (100 µg/mL). The experiment was performed at RT using sterile reagents and equipment in a HEPA-filtered laminar. Individual ovarian follicles were carefully separated using sterile forceps, kept in 50 mL conical tubes and then washed two times with two volumes of Idler's medium. To separate out the individual ovarian follicles gentle pipetting was done repeatedly during each wash. Subsequently, the follicles were digested by 0.1% collagenase type-I in Idler's medium for 30 min with constant moderate mixing on a rotatory shaker. After incubation, vigorous mixing accompanied by frequent pipetting was done several times to obtain the follicular cells from oocytes. Then, filtration using 100 µm nylon strainer was done to collect the disassociated follicular cells from other cells in the medium. Following centrifugation of the cell suspension at 500 × *g* for 5 min, the resultant pellet was resuspended in 3 mL of DMEM. This cell suspension was layered on a single density Percoll layer (9 volume of Percoll to 1 volume of 8 % NaCl) diluted with 1 volume of DMEM and centrifuged at 2800 × *g* for 20 min. At the Percoll and media interface, a compact layer of GC and TC are formed, which were collected by aspiration

using a big gauge needle and syringe. The isolated cells were then washed with new medium, centrifuged for 5 min at 500 × *g* to obtain the pellet and resuspended in 5 mL of fresh cell-culture medium. The cell density was noted using hemocytometer and the cell viability mostly above 90% was assessed by trypan blue staining method. Both the isolated GC and TC as well as other ovarian cells collected were immediately processed for total RNA isolation using TRI reagent followed by cDNA synthesis and quantification as described earlier. The aromatase assay and qPCR were performed for validation of GC and TC isolation.

2.13 *In vitro* treatment of sox19-siRNA in isolated GC and TC

The *in vitro* siRNA treatment was done as detailed by Gupta and Senthilkumaran (2020) with minor modifications. The isolated GC and TC were grown in cell-culture plates comprising 25 mL of M199 medium complemented with 0.25 % HEPES buffer solution, 10 % FBS, and antimycotic-antibiotic, for 24 h at 37 °C in 5 % CO_2 incubator with 95 % water-saturated atmosphere. Custom synthesized oligomers of *sox19* (sense: 5-rGrGrArCrArArArGrUrGrArArGrCrGrUrCrCrCrArUrGrAAT-3' and antisense: 5-rArUrUrCrArUrGrGrGrArCrGrCrUrUrCrArCrUrUrUrGrUrCrCrArU-3') siRNAs were produced commercially (Integrated DNA Technologies, Iowa, US). The *sox19*-siRNA was designed straddling limited base pairs from the HMG domain to warrant specificity. The siRNA combined with branched PEI (Sigma) was dissolved in sterilize HEPES-NaCl (pH 7.4) and incubated for 20 min at RT for siRNA-PEI complex formation, as mentioned by Höbel and Aigner (2010) and used in carp (Anitha and Senthilkumaran, 2020). About, 1×10^5 cells were placed in 24-well plates and maintained as mentioned. Before treatment, the culture medium was removed, followed by the addition of 100 µl OPTI-MEM® I (Thermo Fisher Scientific). Antisense *sox19*-siRNA (1, 5, 10, and 50 ng/mL) and sense *sox19*-siRNA (10 ng/mL) complexed with branched PEI were prepared.

Aliquot part 25 µL comprising only PEI (control)/ sense *sox19*-siRNA-PEI (negative control)/ antisense *sox19*-siRNA-PEI was added along 1 µl of Lipofectamine™ 2000 transfection reagent and the cells were incubated for 12 h. After treatment, the siRNA-PEI-lipofectamine-OPTI-MEM® mixture was withdrawn and the cells were cultured for 24 h with fresh culture medium. Following PBS rinse the cells were collected by trypsinization (0.5 % Trypsin-EDTA) for total RNA isolation, cDNA synthesis and qPCR, as earlier. Fluorescent Universal Negative Control MISSION® siRNA #1 (Sigma) was employed for validation and the treated cells were observed under the IX81 Olympus microscope (Olympus Corporation) and evaluated with Cell Sens dimension software.

2.14 *In vivo* treatment of *sox19*-siRNA in adult female carp

To understand the specific role of *sox19* in ovarian function, *in vivo* transient silencing of *sox19* by ovary–targeted injection was performed in female carp as described earlier in chapter 2.

2.15 EIA

Four days post *sox19*-siRNA *in vivo* treatment, the serum was obtained from the treatment groups. Carp serum E_2 and 17α, 20β-DP levels were valued using EIA kits (Cayman, USA) implementing the producer's instructions. The sensitivity for E_2 and 17α, 20β-DP measurements was 20 pg/mL and 4 pg/mL, respectively. Anti-E_2 had 12 %, 10 % and 0.3 % cross-reactivity with estrone, estradiol-17-glucuronide and estriol, respectively. Anti-17α, 20β-DP had minimal cross-reactivity of 0.004 % and 0.01 % with 17α, 20α-dihydroxy progesterone and 20β-hydroxyprogesterone, respectively. Assays were done for every sample (n=5) in triplicates with the proper dilutions, whenever required.

2.16 Statistics

ImageJ (NIH) software was employed for all densitometry analysis. SigmaPlot 14.0 (Systat Software Inc., Chicago, USA) was employed for all statistical analysis. qPCR results were indicated as mean ± Standard Error of the Mean (SEM) after qualifying in normality and homogeneity tests. ANOVA, trailed by post hoc tests were performed for statistical assessment with P-value < 0.05 as statistically significant.

3. RESULTS

3.1 Cloning and sequence analysis

The *sox19* ORF of ~879 bp which encodes a putative protein of ~292 aa with a deduced ~32 kDa molecular weight was cloned from adult carp ovary. Sequence analysis exposed the existence of the HMG box domain. The homology of carp Sox19 was equated with its vertebrate counterparts. Multiple sequence alignment exhibited that carp, *C. carpio* Sox19 showed similarity at the HMG box domain region (Fig. 1).

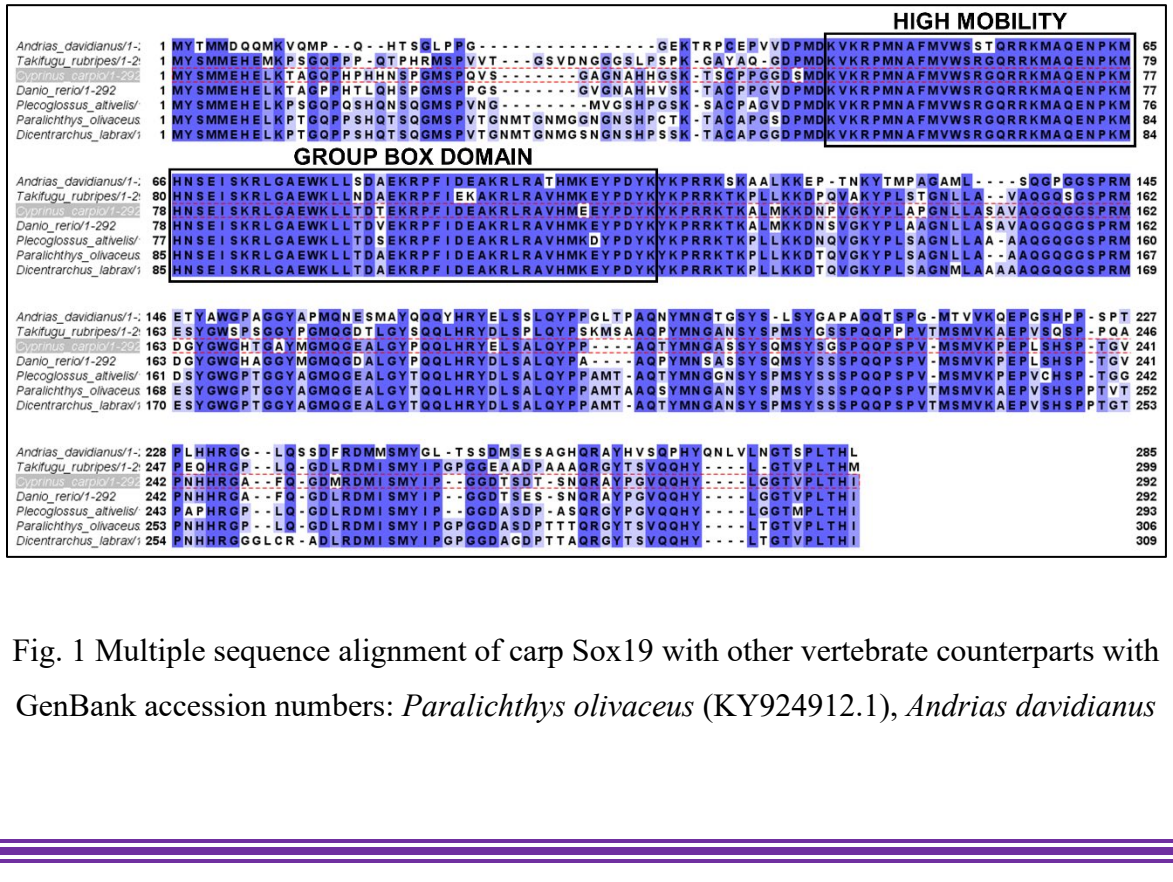

Fig. 1 Multiple sequence alignment of carp Sox19 with other vertebrate counterparts with GenBank accession numbers: *Paralichthys olivaceus* (KY924912.1), *Andrias davidianus*

(KJ623265.1), *Takifugu rubripes* (AY277954.1), *Dicentrarchus labrax* (JN251020.1), *Plecoglossus altivelis* (KF730866.1), *Danio rerio* (AB242332.1) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) alignment.

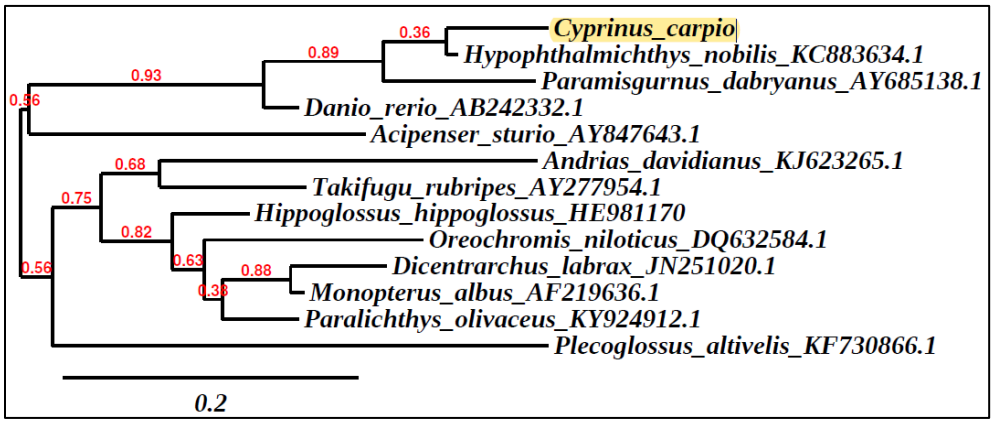

Fig. 2 Phylogenetic analysis of carp Sox19 with other vertebrates with GenBank accession numbers: *Acipenser sturio* (AY847643.1), *Oreochromis niloticus* (DQ632584.1), *Hippoglossus hippoglossus* (HE981170), *Monopterus albus* (AF219636.1), *Paramisgurnus dabryanus* (AY685138.1) using PhyML (http://www.phylogeny.fr/simple_phylogeny.cgi). All other details are the same as Fig. 1.

Phylogenetic analysis revealed that *C. carpio* Sox19 is more homologous with Sox19 of *Hypophthalmichthys nobilis* and Sox19 of *Paramisgurnus dabryanus* and formed a separate clade (Fig. 2).

3.2 Expression of *sox19*: tissue distribution, gonadal ontogeny, and ovarian reproductive phases

Tissue distribution showed abundant ($P < 0.05$) *sox19* expression in brain and ovary in comparison to other tissues analyzed with negligible expression in muscle (Fig. 3A). Varied levels of *sox19* mRNA were observed during gonadal ontogeny, with significantly higher expression ($P < 0.05$), around 80 dph (Fig. 3B), an important period in carp ovarian differentiation. qPCR analysis during

the ovarian reproductive phases showed significant ($P < 0.05$) high expression of *sox19* in pre-spawning followed by spawning phase, with moderate expression in preparatory and a minimal expression in post spawning (Fig. 3C).

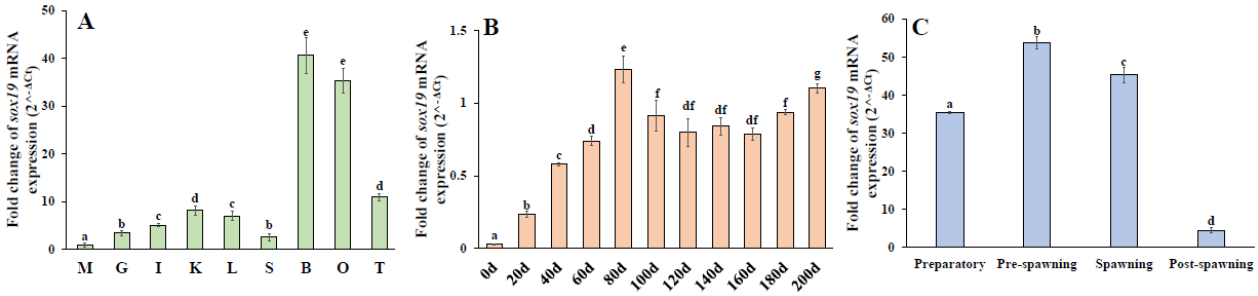

Fig. 3 Expression analysis of *sox19*: (A) Tissue distribution, (B) gonadal ontogeny and (C) ovarian reproductive phases in carp. Data (n=5) were expressed as mean ± SEM ($P < 0.05$; One-way ANOVA followed by Tukey test). Means with different alphabets differ significantly while means with similar alphabets did not show any significance. Abbreviations: M-muscle, G-gill, I-intestine, K-kidney, L-liver, S-spleen, B-brain, O-ovary, T-testis, d-days post hatch. 0d (whole trunk), 20-60d (mesonephric gonadal complex), 80–200d (ovary) samples.

3.4 Western blot and IHC of Sox19

A positive band of *C. carpio* Sox19 ~32 kDa was observed in protein obtained from the ovary (Fig. 4A) while no signal was seen in the negative control (muscle). Immunolocalization revealed Sox19 protein signals in GC of the follicular layer in post vitellogenic oocytes (Fig. 4B), while immunoreactivity was not evident in the pre-adsorbed antibody control (Fig. 4C) with excess of Sox19 antigen, proving the specificity. Further, immunoreactivity was observed specifically in the GC of the follicular layer (Fig. 4D-F).

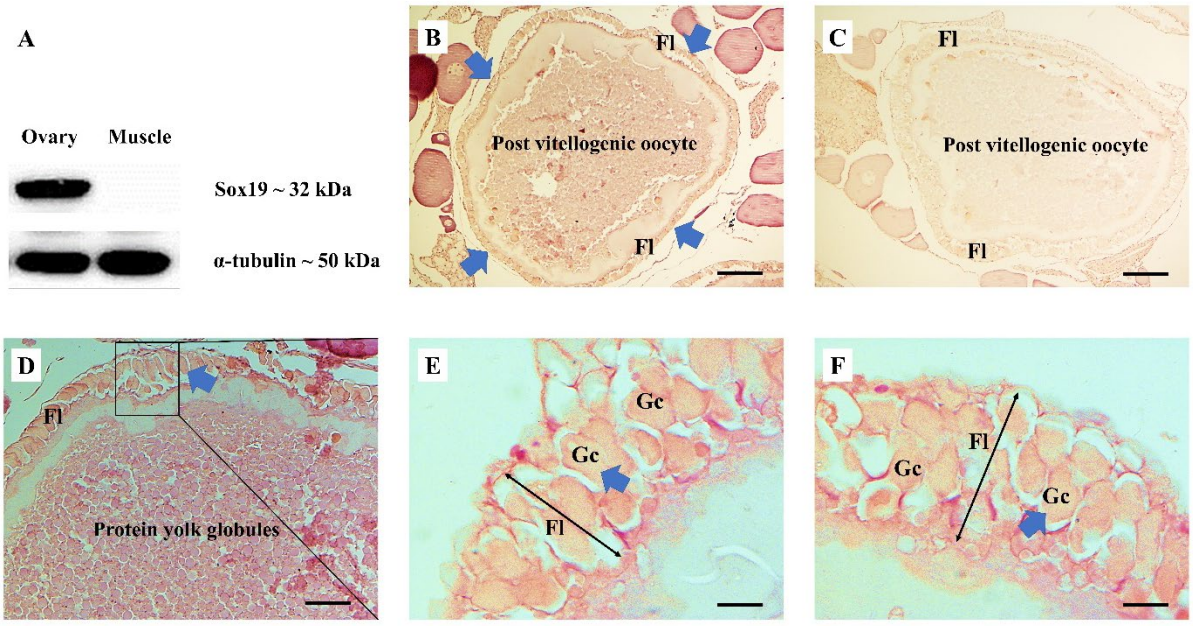

Fig. 4 Western blot and IHC of Sox19 protein in adult carp ovary. (A) Western blot analysis of Sox19 protein displayed band of ~32 kDa in ovary while in the negative control (muscle) no band was observed. Immunoreactivity of Sox19 (B) was seen in in the follicular layer of mature oocyte, whereas the negative control (pre-adsorbed antibody with excess Sox19 antigen) showed no signal (C). Sox19 was localized in granulosa cells of the follicular layer (D-F). Abbreviations: Fl: follicular layer, Gc: granulosa cells. The scale bar indicates B, C: 50 µm; D: 30 µm; E, F: 10 µm.

3.5 Effect of hCG on *sox19* mRNA expression, *in vitro* and *in vivo*

Induction with hCG during the preparatory phase of the carp ovarian reproductive cycle modulated the expression of *sox19*. In both *in vitro* (Fig. 5A) and *in vivo* (Fig. 5B) treatments (n=5), significant ($P < 0.05$) increase in *sox19* mRNA expression was observed after 12 h and maximum 24 h post hCG induction.

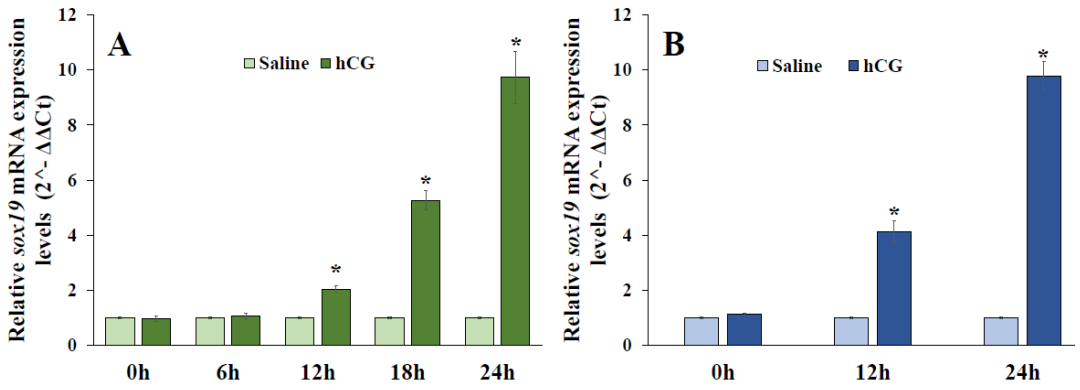

Fig. 5 Relative mRNA expression of *sox19* after hCG induction (A) *in vitro* (100 IU/ml) and (B) *in vivo* (1000 IU/Kg body weight) compared with control (saline) in adult carp ovary at different time intervals. Data (n=5) were expressed as mean ± SEM (* $P < 0.05$; One-way ANOVA followed by Dunnett's method). Abbreviation: h-hour.

3.6 Effect of mono-sex induction treatments

Aromatase activity was measured in the mono-sex-induced carp gonads. There was an increase in activity of aromatase in the ovary of E_2 treated carp (Fig. 6A) while there was a reduction in aromatase activity in the testis of MDHT treated carp (Fig. 6B). E_2 treatment increased the expression of *sox19* significantly ($P < 0.05$) in ovary (Fig. 6C) during the gonadal development of carp.

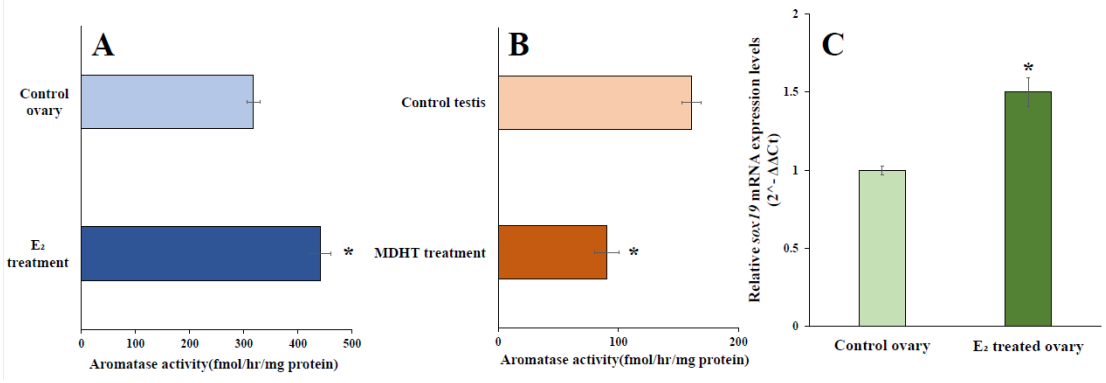

Fig. 6 (A) Assessment of aromatase activity in control and E_2 treated carp ovary. (B) Assessment of aromatase activity in control and MDHT treated carp testis. (C) Relative expression of *sox19*

mRNA in expression control and E_2 treated carp ovary. Data (n=5) were expressed as mean ± SEM (* P < 0.05; One-way ANOVA followed by Dunnett's method).

3.7 Aromatase assay and expression analysis in isolated GC and TC

The GC and TC were isolated from the adult carp ovary. Aromatase activity was assessed to validate the isolated cell population. Elevated aromatase activity was evident in GC and TC enriched cell population in comparison to other cell (yolk granule, yolk vesicle, cortical alveoli, stroma cells, and cortical vesicle) population (Fig. 7A).

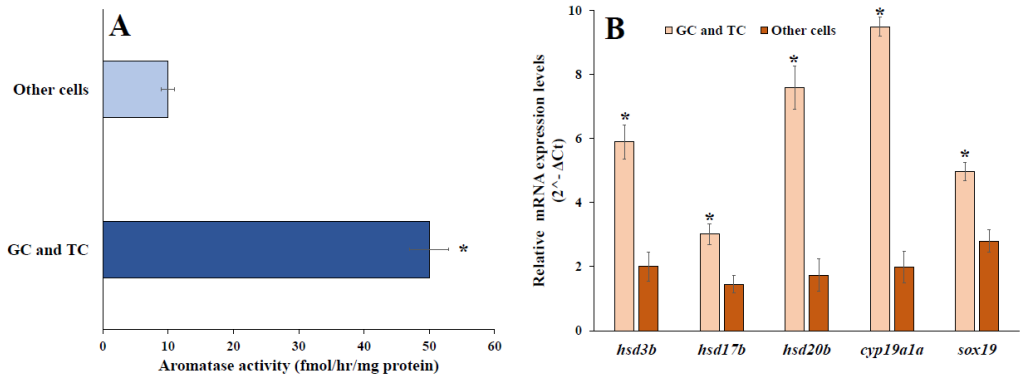

Fig. 7 (A) Assessment of aromatase activity in isolated GC and TC cells in comparison with other cell population. (B) Relative mRNA expression of genes (*hsd3b*, *hsd17b1*, *hsd20b*, *cyp19a1a*, and *sox19*) in isolated GC and TC enriched population compared to other cell population. Data (n=5) were expressed as mean ± SEM (* P < 0.05; One-way ANOVA followed by Tukey test).

In addition, qPCR analysis of follicular layer marker steroidogenic enzyme genes like *hsd3b*, *hsd20b*, *hsd17b1*, and *cyp19a1a* were estimated. In the GC and TC enriched cell population the expression levels of *hsd3b*, *hsd17b1*, *hsd20b*, *cyp19a1a*, and *sox19* were found to be upregulated (Fig. 7B).

3.8 Silencing of *sox19*, *in vitro*

The isolated GC and TC carp cells were cultured and subjected to *sox19*-siRNA (n=5) treatments. Fluorescent signals were evident in control siRNA validating siRNA uptake by cells (Fig. 8A).

The *sox19* mRNA expression was similar in the control (only PEI) and sense *sox19*-siRNA treatment groups. However, in the antisense *sox19*-siRNA treatments, there was a significant ($P < 0.05$) dose-dependent reduction in the *sox19* mRNA expression levels (Fig. 8B). The expression of *sox19* reduced to about 87 % upon treatment with 10 ng/µl of *sox19*-siRNA as compared to control, hence this dose as well as sample was selected for further analysis. Treatment of isolated GC and TC cell culture with antisense *sox19*-siRNA significantly ($P < 0.05$) decreased the transcript levels of *hsd3b*, *hsd17b*, *hsd20b*, and *cyp19a1a* (Fig. 8C).

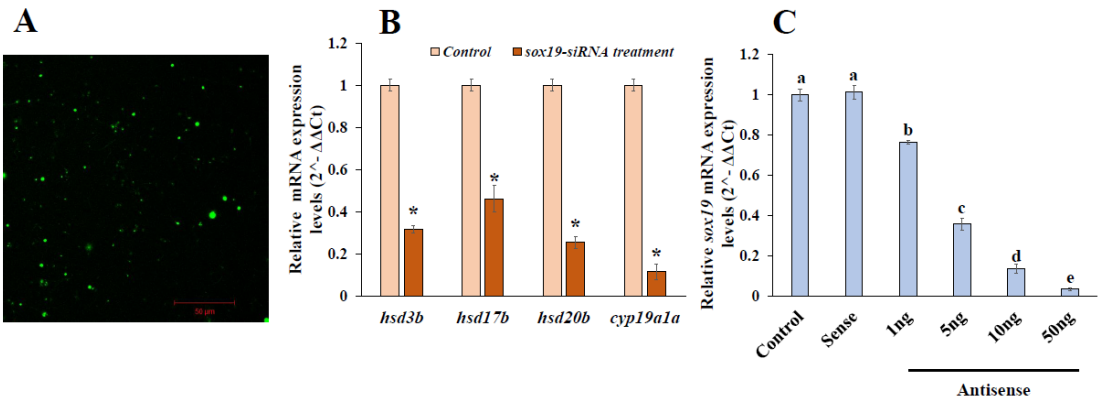

Fig. 8 (A) Representative fluorescent image 24 h after *in vitro* PEI mediated treatment of MISSION siRNA universal negative control conjugated with 6-FAM to check the efficiency in carp cell culture. (B) Relative *sox19* mRNA expression 24 h after *in vitro* PEI mediated *sox19*-siRNA treatment in carp GC and TC culture (n=5). (C) Relative expression of genes (*hsd3b*, *hsd17b*, *hsd20b*, and *cyp19a1a*) between control and 10 ng/µl of antisense *sox19*-siRNA groups in carp GC and TC culture after 24 h of treatment (* $P < 0.05$; One-way ANOVA followed by Dunnett's method).

3.9 Transient silencing of *sox19*, *in vivo*

In vivo treatment of antisense *sox19*-siRNA-PEI complex significantly reduced ($P < 0.05$) the expression of *sox19* demonstrating transient gene silencing of *sox19* in carp ovary (Fig. 9A). In contrast, PEI only or sense *sox19*-siRNA-PEI complex treatments showed no significant difference

in *sox19* mRNA expression. Expression of *sox19* was measured in ovary on days 0, 2, 4, 6, 8 and 10 post-siRNA treatment and compared with equivalent control during all time periods (Fig. 9A). In the antisense *sox19*-siRNA treatment group the expression of *sox19* significantly ($P < 0.05$) reduced to 43, 90, 46, and 17 % on days 2, 4, 6 and 8 respectively. There was no significant change in *sox19* mRNA expression on day 10 proving that transcript levels might return to normal due to transient nature (Anitha and Senthilkumaran, 2020; Laldinsangi and Senthilkumaran, 2018; Murugananthkumar and Senthilkumaran, 2016).

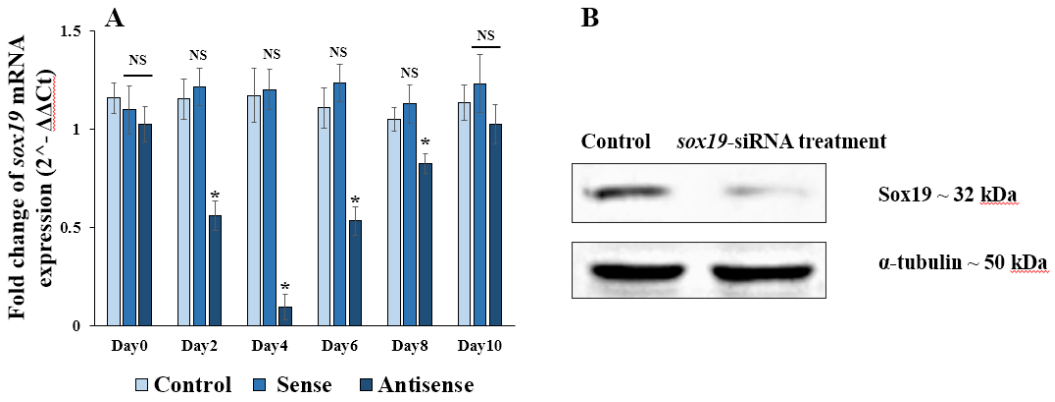

Fig. 9 (A) Relative *sox19* mRNA expression post i*n vivo* PEI mediated *sox19*-siRNA treatment in adult (n=5) carp ovary (*, $P < 0.05$; One-way ANOVA followed by SNK test). (B) Western blot analysis of Sox19 protein on day 4 in control and *sox19*-siRNA treated carp ovary.

The antisense *sox19*-siRNA treatment on day 4 showed a 90 % decline in *sox19* mRNA expression and henceforth, experiments were performed with this as treatment sample. Western blot analysis showed reduced Sox19 protein levels in *sox19*-siRNA treated ovary (Fig. 9B). The *sox19*-siRNA treatment did not affect the mRNA expression of *sox5*, *sox6*, *sox9a*, *sox9b*, *sox11*, *sox13*, *sox18*, *sox21*, and *sox30* (Fig. 10A) claiming the specificity of siRNA designed. The transient nature of the PEI mediated siRNA treatment is reinforced with nonexistence of noticeable phenotypic

modifications by histological analysis in *sox19*-siRNA treated (Fig. 10B) and control ovary (Fig. 10C).

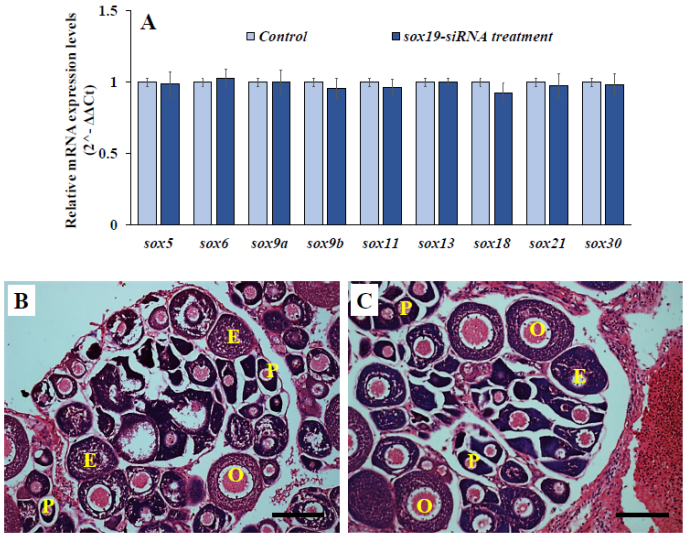

Fig. 10 (A) Relative expression of *sox* genes on day 4 between control and *sox19*-siRNA treated carp ovary. No significant difference was observed in any of the groups. Representative histological images of carp ovary on day 4 (B) control and (C) *sox19*-siRNA treatment. Abbreviations: P: perinucleolus stage, O: oil droplet stage, E: early vitellogenic stage. The scale bar indicates C, D: 30 µm.

Nevertheless, siRNA treatment imparted molecular variations. There was a significant downregulation in transcript levels of ovary-related factors/genes namely, *foxl2, vtg, fstl, ad4bp/sf-1*, and genes related to *wnt* signalling like *ctnnb1*, *rspo1*, *wnt4*, and *fzd* (Fig. 11A), as well as steroidogenesis related genes such as *star*, *cyp11*, *cyp17*, *hsd3b*, *hsd17b*, *hsd20b*, *cyp19a1a,* and *erα*, while *erβ1* and *erβ2* displayed a significant upregulation (Fig. 11B) on day 4 post-*sox19*-siRNA treatment. The transient nature of siRNA silencing was proven further with the mRNA transcript levels reverting to near normal on day 10 post-*sox19*-siRNA treatment (Fig. 11C).

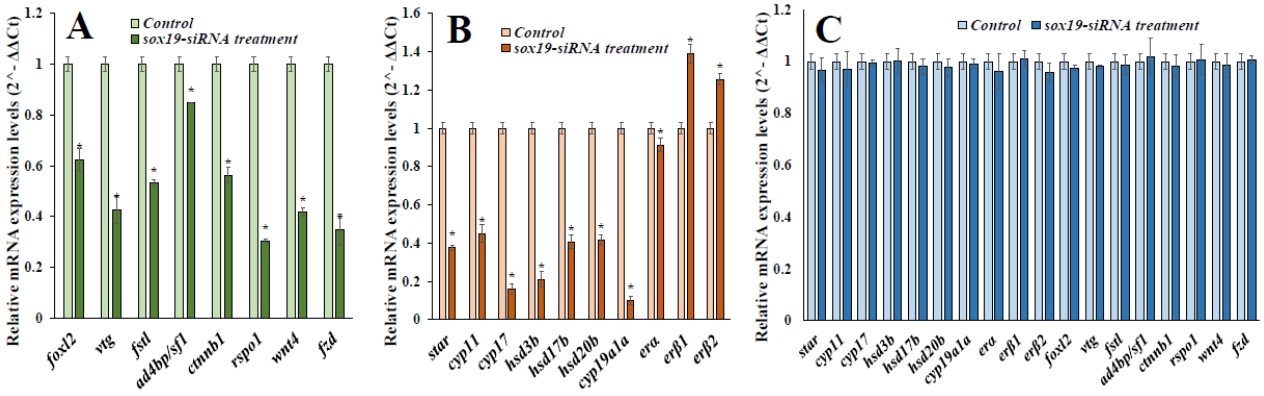

Fig. 11 Relative mRNA expression of (A) ovary-related, (B) steroidogenesis-related genes/factors on day 4 in control and *sox19*-siRNA treated carp ovary (* $P < 0.05$; Mann–Whitney test One-way ANOVA on ranks followed by SNK test). (C) Relative mRNA expression of ovary-related and steroidogenesis-related genes/factors on day 10 in control and *sox19*-siRNA treated carp ovary. No significant difference was observed in any of the groups.

Serum E_2 (Fig. 12A) and 17α,20β-DP (Fig. 12B) levels decreased significantly ($P < 0.05$) on day 4 post *sox19*-siRNA treatment, with concomitant increase in serum T levels (Fig. 12C). Aromatase activity declined significantly ($P < 0.05$) on day 4 post *sox19*-siRNA treatment (Fig. 12D).

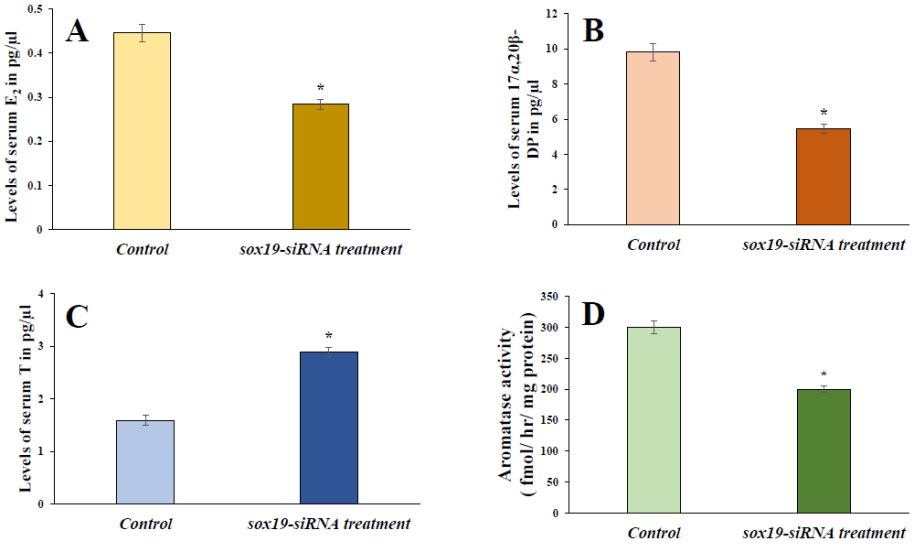

Fig. 12 Changes in the levels of serum (A) E_2, (B) 17α, 20β-DP (C) T on day 4 in the control and *sox19*-siRNA treated carp. (D) Changes in aromatase activity on day 4 in the control and *sox19*-

siRNA treated carp ovary. Data (n=5) were expressed as mean ± SEM. (* $P < 0.05$; Student's t-test).

4. DISCUSSION

The current study, reports the cloning of carp *sox19*, its expression during initial ovarian differentiation, variations in different phases of the ovarian reproductive cycle, post hCG induction and after E_2 treatment. This work is the first to elucidate the significance of *sox19* in ovarian steroidogenesis in *C. carpio* by transient gene silencing. *sox19* belongs to subgroup B1, comprising transcriptional activators and was initially reported in zebrafish to contain highly conserved motifs apart from the HMG (Vriz and Lovell-Badge, 1995). Most multigene families are discovered in mammals, and later found to consist more members in fish genomes (Wittbrodt *et al*., 1998), however *sox19* is absent in mammals (Schepers *et al*., 2002) and specific to fish, evident in rice field eel (Liu and Zhou, 2001), fugu (Koopman *et al*., 2004), the European Atlantic sturgeon, *A. sturio* (Hett and Ludwig, 2005), the turbot, *Scophthalmus maximus* (Viñas *et al*., 2012), the European sea bass (Navarro-Martín *et al*., 2012), and carp (Zhang *et al*., 2018). Significance of *sox19* during neurogenesis in turbot (Taboada *et al*., 2018) and embryogenesis in the large yellow croaker, *Larimichthys crocea* (Wan *et al*., 2019) and the Japanese flounder (Yu *et al*., 2018) have been reported. In the European sea bass, importance of *sox19* in ovarian differentiation was suggested (Navarro-Martín *et al*., 2012). Hence, this study intended to explore the role of *sox19* in ovarian function of carp. The ORF of *sox19* was cloned using gene-specific primers based on the nucleotide sequences obtained from a previous study on gonadal transcriptome analysis of carp (Anitha *et al*., 2019). Tissue distribution in adult carp revealed higher expression of *sox19* in brain and ovary comparable to the results obtained previously in the European sea bass (Navarro-Martín *et al*., 2012), the Chinese sturgeon (Yang *et al*., 2020), the Nile tilapia (Wei *et al*., 2016), channel catfish (Zhang *et al*., 2018) and the Japanese flounder (Yu *et al*., 2018). In teleost, the genes

expressed during the gonadal differentiation govern gonadal development and the associated mechanisms. Ovary related genes like *cypa19a1a* and *foxl2* show abundant expression from 30–80 dph in yellow river carp (Jiang *et al*., 2020). The current study showed dominant *sox19* expression from 80 dph onwards, the period of initial ovarian differentiation in carp (Komen *et al*., 1992). There was only a minimal expression of *sox19* during the course of ovarian development in the Nile tilapia (Wei *et al*., 2016) and *sox19* expression was evident after 150 dph the period of sex differentiation in the European sea bass (Navarro-Martín *et al*., 2012). Carp is a natural breeder following a seasonal spawning pattern (Basavaraju *et al*., 2002). The *sox19* transcript levels were high during pre-spawning and spawning, the phases which are regulated by hormones (Martyniuk *et al*., 2009; Senthilkumaran *et al*., 2004). These results indicate the significance of *sox19* in carp ovarian growth and development. It is well known that the brain–pituitary–gonad axis regulates gonadal growth and maturation by signaling a cascade of hormones and their receptors. During the spawning season, several factors/genes are differentially expressed under the regulation of gonadotropin (McLaren, 1988; Jørgensen *et al*., 2008; Raghuveer *et al*., 2011; Shi *et al*., 2013). In teleost, ovary differentiates, develops, follicular layer endures widespread differentiation and proliferation known as folliculogenesis, to attain final oocyte maturation (Senthilkumaran *et al*., 2004). The follicular layer comprising of an outer TC and inner GC is under the strict regulation of gonadotropin (Young and McNeilly, 2010) which by activating several transcription factors modulate ovarian growth and oocyte maturation by a shift in steroidogenesis (Senthilkumaran *et al*., 2004). Incidentally, in the current study Sox19 protein was detected in the GC of follicular layer, indicating that *sox19* might play a role in ovarian steroidogenesis under the influence of gonadotropin. LH and hCG share same receptor (Vischer *et al*., 2003), the exogenic exposure of hCG might induce a steroidogenic shift promoting ovarian maturation in teleost (Kumar *et al*.,

2007). The vitality of hCG in carp ovarian steroidogenesis has been proven both *in vitro* and *in vivo* (Cao *et al*., 2014; Paul *et al*., 2010). The over expression of *sox19* post hCG induction in both *in vitro* and *in vivo* correlates the regulation of *sox19* expression by gonadotropin. Gonadotropin regulates estrogen in carp (Yaron, 1995). Having observed that gonadotropin controls *sox19*, further illustration on the impact of estrogen on gene expression was studied. E_2, is the potent estrogen vital for ovarian differentiation and development in teleosts (Piferrer and Guiguen, 2008). Exogenous estrogen and pseudo-estrogens treatments stimulated sex reversal resulting in mono-sex population in several fishes (Chang and Lin, 1998; Kobayashi and Iwamatsu, 2005; Raghuveer *et al*., 2005; Yamamoto and Matsuda, 1963) including carp (Gimeno *et al*., 1998). Parenthetically, in the current study, higher expression of *sox19* was observed post E_2 treatment, affirming the regulation of *sox19* by both gonadotropin and estrogen, the major contributors of ovarian differentiation and development in teleost. Furthermore, Sox19 protein was localized in the GC of the follicular layer and *sox19* mRNA transcript levels were high in the GC and TC enriched cell population. GC and TC cells are the most probable sites of steroid production in teleost (Nagahama, 1983). In order to know the specific function of *sox19* in carp oogenesis, gene silencing using siRNA treatment was performed *in vitro*, in the isolated GC and TC cell culture. After treatment with *sox19*-siRNA, there was a down regulation in the transcripts of steroidogenic enzyme genes, namely *hsd3b*, *hsd17b*, *hsd20b*, and *cyp19a1a* an indicative of *sox19* significance in carp steroidogenesis. In teleost, gonadotropins are well known to stimulate steroid synthesis (Devlin and Nagahama, 2002). Steroidogenic genes are essential for teleostean ovarian development and maturation (Raghuveer and Senthilkumaran, 2012; Rajakumar and Senthilkumaran, 2014; Rasheeda *et al*., 2010; Senthilkumaran *et al*., 2004; Senthilkumaran *et al*., 2009; Sreenivasulu and Senthilkumaran, 2009; Yamaguchi *et al*., 2007; Yoshiura *et al*., 2003).

Taken together, over expression of *sox19* after hCG induction may imply the regulatory role of *sox19* in ovarian steroidogenesis by influencing other factors that regulate ovarian development and maturation. To elucidate the role of *sox19* in oogenesis, this is the first report to study the effects of PEI mediated transient silencing of *sox19*, *in vivo*. There was a significant downregulation of *star* and steroidogenic enzyme genes post *sox19*-siRNA treatment. *star* catalyzes the rate limiting step, transport of free cholesterol into mitochondria, the first step in steroidogenesis (Hanukoglu, 1992). The pivotal role of *cyp19a1a* in carp ovarian steroidogenesis and its regulation by transcription factors *ad4bp/sf1* and *foxl2* is well demonstrated in teleost (Wang *et al*., 2007; Yoshiura *et al*., 2003). Parenthetically, *sox19*-siRNA treatment resulted in downregulation of these factors. *Fstl*, a paracrine factor regulates biosynthesis and release of pituitary gonadotropin, gonadal development, and ovulation cycle (Tao *et al*., 2018) and gonadotrophin regulation by activin/follistatin system is well-documented in carp (Fung *et al*., 2017). Vitellogenesis is the process of *vtg* assimilation and processing into yolk globule and *vtg*, a glycoprotein synthesized in the liver of females is estrogen dependent and increases during oocyte growth in carp (Solé *et al*., 2000). Hence, reduction of *fstl* and *vtg* by *sox19*-siRNA in carp could be correlated with the results obtained in hCG and E_2 treatment, indicating the significant role of *sox19* in ovarian growth. The role of *wnt* signaling in gonadal differentiation and development was shown in teleost (Nicol and Guiguen, 2011; Wu *et al*., 2019). Wnt proteins bind to *fzd* receptors over cell surface leading to Wnt/β-catenin pathway activation, dependent on *ctnnb1*. The main components of the canonical Wnt signaling are *wnt4* and *rspo1.* Wnt/ β-catenin signaling was suggested as a "pro-female" pathway regulating gonadal differentiation in zebrafish (Sreenivasan *et al*., 2014). In the Japanese medaka (Zhou *et al*., 2012) and the Nile tilapia (Wu *et al*., 2016) *rspo1* is necessary to trigger ovarian development, playing a decisive role in the ovarian

differentiation. The role of *wnt4* in ovarian function was shown in catfish (Prathibha and Senthilkumaran, 2017). The transient gene silencing of *sox19* altered the transcript levels of *ctnnb1*, *rspo1*, *wnt4*, and *fzd*. In accordance to the results obtained in the current study, regulation of Wnt signaling by Sox proteins has been reported in *Xenopus* (Zorn *et al*., 1999). The hormonal mechanisms monitor oogonium proliferation and oocyte maturation involving gonadotropin, steroids, E_2 and 17α, 20β-DP along with growth factors in teleost (Lubzens *et al*., 2010). The decrease in E_2 and 17α,20β-DP post *sox19*-siRNA treatment could be related to the alteration in transcription factor, steroidogenesis related and wnt signaling genes modulating E_2 and 17α, 20β-DP. It has been shown that in carp, *erβ*s activate at minor concentrations of E_2 compared with *erα* (Katsu *et al*., 2013). Thus, the upregulation of *erβ1* and *erβ2*, and downregulation of *erα* post *sox19*-siRNA treatment might be due to the reduction in E_2 levels. Gonadotropin and *ad4bp/sf1* regulation of aromatase and *cyp19a1a* during oocyte development has been studied in teleost (Moulik *et al*., 2016; Wang *et al*., 2007; Yoshiura *et al*., 2003). Aromatase is crucial for E_2 synthesis during ovarian growth on the other hand, 17α, 20β-DP, the maturation-inducing steroid, is vital for final oocyte maturation in several teleost (Senthilkumaran *et al*., 2004; Senthilkumaran, 2011). Interestingly *sry*/*sox* binding sites were recognized in 5′ contiguous region of *cyp19a* in goldfish (Tchoudakova *et al*., 2001) and tilapia (Chang *et al*., 2005) and in *cyp19a* promoter of the European sea bass (Galay-Burgos *et al*., 2006) and orange-spotted grouper (Zhang *et al*., 2008). In line with the downregulated *cyp19a1a* expression levels, a decrease in aromatase activity was observed after *sox19*-siRNA treatment, implying a role of *sox19* in ovarian steroidogenesis.

5. CONCLUSION

This study reveals that *sox19* is principally expressed in the brain and ovary, regulating ovarian growth besides maturation through the reproductive cycle of carp. The localization of Sox19 in GC, over expression of *sox19* in GC and TC enriched cell population indicated an important role

in oogenesis. The expression of *sox19* is dependent on both gonadotropin and E_2. Present study is the first comprehensive report to implicate *sox19* in ovarian steroidogenesis of carp by transient gene silencing. *sox19* seems to influence ovary-related transcription factors/ genes, steroidogenic enzymes and sex steroids. Overall, the current study demonstrated the significance of *sox19* in early ovarian differentiation, development, and maturation of carp with a role in steroidogenesis.

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Summary

Sex determination/differentiation followed by gonadal growth and development happen during ontogeny in sexually reproducing living organisms. In teleost, gonadal differentiation is under the influence of genetic factors, environmental conditions and sex steroids. The role of *sox* family genes in fish sex differentiation has not been well-characterized. Teleost, the largest group among vertebrates, is inclusive of carp reported to have more *sox* genes than other species. Carp gonadal differentiation is governed by both genetic and environmental factors.

In the present study, with the aim to identify several genes from the *sox* family of transcription factors, gonadal transcriptome analysis using RNA Seq was performed in carp. GO, COG, and KEGG were analyzed to identify various processes/ pathways regulating carp sexual development. Several DEG were identified and validated with qPCR. In addition, many SSR were obtained. Various genes (sex-differentiation, germ-cell maintenance, steroid synthesis, endocrine regulation) and pathways (steroid hormone biosynthesis, *wnt* signaling, oocyte meiosis) related to reproduction were analyzed. About ten genes representing each sub-group of *sox* (*soxB1-sox19, soxB2-sox21; soxC-sox11; soxD-sox5, sox6, sox13; soxE-sox9a, sox9b; soxF-sox18; soxH-sox30*) family were also identified from gonadal transcriptome. Expression profiling, namely tissue distribution and gonadal reproductive cycle analysis were carried out for the ten genes. The results indicated the significance of *sox19* and *sox30* in carp gonadal function, paving way for further studies.

From carp testis, *sox30* ORF and *sox9a/b* partial cDNAs were cloned. Pre-dominant *sox30* expression was seen in testis compared to ovary. *sox9a/b* exhibited significant high expression during 40 dph, while *sox30* showed dimorphic expression at 120 dph during carp testicular differentiation. Testicular reproductive phase analysis, showed that during initial stages of spermatogenesis *sox9a/b* and during spermatogenesis and spermiogenesis *sox30* were highly

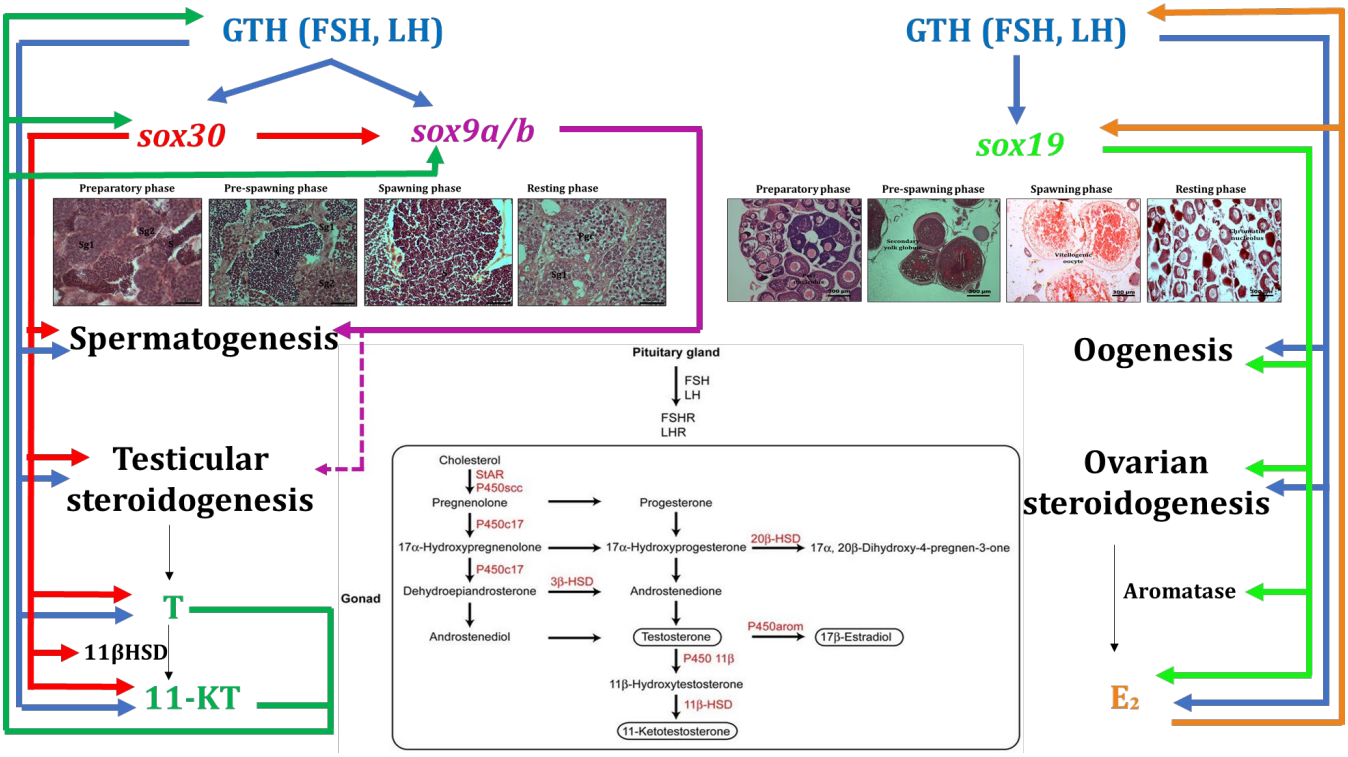
expressed, indicating their role in testicular growth and maturation. Sox30 and Sox9 proteins were detected in spermatocytes/spermatids/sperm and spermatogonia of carp testis, respectively. The effect of 11-KT, gonadotropin, and mono-sex induction was equivalent in *sox9a/b* and *sox30*. Further, *sox30* gene silencing modified transcription factors, steroidogenic genes, sex steroids, and other factors related to testicular development and function. Hsd11b enzyme activity decreased post *sox30* silencing. The present study demonstrated the significance of *sox30* in testicular steroidogenesis vis-à-vis testis function of carp by influencing *sox9a/b*, either directly or indirectly, along with other male-related genes as evidenced by expression analysis and transient gene silencing.

sox19 ORF was cloned from carp ovary. Tissue distribution revealed predominant *sox19* expression in ovary than in testis. *sox19* mRNA expression was high during carp early ovarian differentiation, at 80 dph. Ovarian reproductive cycle analysis revealed significant *sox19* expression during breeding season indicating its role in maturation. Mono-sex induction elevated *sox19* mRNA expression, showing its importance in ovarian development. Sox19 protein was detected in follicular layer accompanied with high expression in isolated GC and TC, show a role in steroidogenesis. Transient *sox19* silencing modulated steroidogenic enzymes, sex steroids, and other ovary-related factors/genes. Aromatase activity reduced post *sox19* silencing. The importance of *sox19* in ovarian differentiation, maturation, and function with a definite role in steroidogenesis was elucidated.

Overall, this thesis work identified ten genes belonging to *sox* family from carp gonadal transcriptome analysis. Expression profiling, warranted further study on *sox30* and *sox19*. Under the control of gonadotropin, *sox30* along with *sox9a/b* regulates spermatogenesis and testicular steroidogenesis by influencing T and 11-KT in carp males. In female carps, *sox19* governed by

gonadotropin and E_2 play a significant role in oogenesis and ovarian steroidogenesis. A figure depicting these findings was shown as a summary.

Figure: Schematic illustration of the significant findings from the research thesis.



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Role of *sox30* in regulating testicular steroidogenesis of common carp

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ABSTRACT

Expression of transcription factors is crucial for the regulation of steroidogenesis and gonadal development in fish. SRY-related box (SOX) proteins regulate gene expression of various events related to vertebrate reproduction. This study reports the role of *sox30* and its influence on *sox9a/b* in regulating testicular steroidogenesis of the common carp, *Cyprinus carpio*. Tissue distribution showed predominant expression of *sox30* in gonads, while gonadal ontogeny indicated significant dimorphic expression of *sox30* from 120 days post hatch. Higher *sox30* transcripts during the spawning season, an elevation of *sox30* after human chorionic gonadotropin induction, and 11-ketotestosterone (11-KT) treatment authenticate gonadotropin dependency. Treatment of 17α–methyl-di-hydroxy-testosterone to juvenile common carp for mono-sex induction, vis-à-vis elevated *sox30* expression. Sox30 protein was detected abundantly in spermatocytes and spermatid/sperm of carp testis. Transient silencing of *sox30* using small interfering RNAs decreased *sox9a/b* expression, lead to downregulation of certain molecule/factor, transcription factor, germ/stem cell marker, and steroidogenesis-related enzyme genes. Serum testosterone and 11-KT decreased significantly upon transient silencing of *sox30, in vivo*. Concomitantly, a reduction in testicular microsomal 11-β hydroxysteroid dehydrogenase activity was observed. These results demonstrate the influence of *sox30* as well as *sox9a/b* in the regulation of testicular steroidogenesis in common carp.

1. Introduction

Steroidogenesis drives reproduction by promoting growth, differentiation, and maturation of a bipotential gonad into testis or ovary and is regulated differently by multi-tiered modulation of promoter motif of transcription factors influencing steroidogenic enzyme genes to eventually govern enzyme activity [1]. Transcription factors like *ad4bp/sf-1*, *creb*, *foxl2*, *sox3* and *wt-1* play a regulatory role in fish steroidogenesis [1]. Further, the influence of transcription factors, sex-steroids, steroidogenic enzymes, sex-steroid receptors, germ/stem cell markers, growth factors, and other molecules in the gonadogenesis of teleost has been reviewed [2]. *SOX* family of transcription factors are implicated in regulating many biological functions, including reproduction [3]. SOX proteins are specific to animals and constitute to HMG superfamily,

Abbreviations: 11-KT, 11-ketotestosterone; 11-OHT, 11β-hydroxytestosterone; aa, amino acid; *ad4bp/sf-1*, adrenal 4 binding protein/steroidogenic factor-1; *amh*, anti-Mullerian, hormone; *ar*, androgen, receptor; CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals; *creb*, cAMP-responsive, element binding protein; Ct, cycle threshold; *cyp11*, cytochromeP450, family 11; *cyp17*, steroidogenic cytochrome P450 17-hydroxylase/lyase; *cyp19a1*, cytochromeP450, family 19, subfamily a, polypeptide 1; *dax*, dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X; DMEM, Dulbecco's modified Eagle's medium; *dmrt1*, double sex and mab-3 related transcription factor 1; dph, days post hatch; E_2, estradiol-17β; EIA, enzyme immunoassay; *fshr*, follicle-stimulating, hormone receptor; *gata4*, GATA, binding protein 4; *gnrh*, gonadotropin-releasing hormone; *gsdf*, gonadal, somatic cell-derived factor; hCG, human chorionic gonadotropin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMG, high mobility group; HRP, horse radish peroxidase; *hsd3b*, hydroxy- Δ-5-steroid dehydrogenase, 3β; *hsd11b*, hydroxysteroid 11-β dehydrogenase; *hsd17b*, hydroxysteroid, 17- β dehydrogenase; *hsd20b*, hydroxysteroid, 20- β dehydrogenase; IAEC, Institutional Animal Ethics Committee; IHC, immuno histo chemistry; *kiss2*, kisspeptin2; KVAFSU, Karnataka Veterinary Animal and Fisheries Sciences University; L15, Leibovitz-15; LH, luteinizing hormone; MDHT, 17α–methyl-di-hydroxy-testosterone; MS222, ethyl 3-aminobenzoate methanesulfonate; ORF, open reading frame; PBS, phosphate-buffered saline; *pdgf*, platelet-derived growth factor; PEI, polyethylenimine; PFA, paraformaldehyde; *pou5f3/oct4*, POU domain, class 5, transcription factor 3/octamer-binding transcription factor 4; qPCR, quantitative real time PCR; RT, room temperature; siRNA, small interfering RNA; *sox*, SRY-box; *srd5a1*, steroid5 α-reductase 1; *SRY*, sex-determining region Y; *star*, steroidogenic, acute regulatory protein; *sycp3*, synaptonemal complex protein 3; T, testosterone; *wt1*, Wilms' tumor suppressor 1.

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encoding ~80 aa DNA-binding domain further classified into subgroups A–K [4]. In fish, *sox9* is a requisite for testis development with two forms identified in teleost like fugu (*Fugu rubripes*), zebrafish (*Danio rerio*), catfish (*Clarias gariepinus*), rice field eel (*Monopterus albus*), and [5] the Japanese medaka (*Oryzias latipes*). *sox3* [6] regulates testicular development and recrudescence in catfish (*C. batrachus*) and initiates testicular differentiation [7] in the Indian rice-fish (*O. dancena*). The involvement of *sox5* in germ-cell regulation of medaka [8] was evident. In mammals, evidence for the presence of *SOX4*, *SOX5*, *SOX6*, *SOX7*, *SOX17*, *SOX20,* and *SOX22* in testis has been reported [3]. Whereas, in teleost having more (~50) *sox* genes than other vertebrates [9], the involvement of other genes belonging to the *sox* family in gonadogenesis has seldom been reported. In this regard, the common carp (henceforth referred to as carp), *Cyprinus carpio*, comprising more *sox* genes than other fish species [9] would be an appropriate model for investigation. The significance of *sox9a/b* in testicular development of carp have been reported earlier [10,11]. A previous study on the transcriptome-based analysis from our laboratory demonstrated that *sox* genes of carp presented stage-specific and/or sex-dimorphic expression during gonadal development [12]. Among which, *sox30* of subgroup H was predicted to have higher expression in testis warranting in-depth analysis. *SOX30*, primarily isolated from mouse and human [13] was thought to be specific in mammals [14], while later evidence documented its presence throughout animal kingdom [15]. Recent reports [16,17] stated the absence of *sox30* in zebrafish, spotted gar (*Lepisosteus oculatus*), the Japanese medaka, tongue sole (*Cynoglossus semilaevis*), fugu, the Japanese flounder (*Paralichthys olivaceus*), and carp. Contending these, the present study is first to provide evidence for the presence of *sox30* in carp. *SOX30* has been proven to be critical for mammalian and teleostean spermatogenesis [4,15]. However, its influence on the regulation of testicular steroidogenesis was never clarified. Early sexual maturation accompanied by seasonal spawning makes carp [18] an ideal model to understand the role of *sox30* during gonadogenesis. This work reports cloning, expression analysis and localization of *sox30* in comparison with *sox9a/b* in carp. Gonadotropin regulation on *sox9a/b* and *sox30* was assessed during natural spawning cycle, artificial induction and also in mono-sex induced carp. Current report provides substantial evidence to denote *sox30* as a novel regulator of carp testicular steroidogenesis by transient silencing.

2. Materials and methods

2.1. Fish sampling

Carps used in the current study were procured from the aquaculture facility of KVAFSU, Bangalore, India, and acclimated in outdoor tanks (6 × 4 × 5[l × b × h] feet) with filtered tap water, under ambient photothermal conditions and fed commercial fish food pellet *ad libitum*. Initially, to clone *sox9a/b* and *sox30*, adult carp (~1-year, ~30–40 cm, ~400−700 g) testis was used. To perform tissue distribution analysis, various tissues from adult carp (n = 5), males (muscle, liver, testis, kidney, and brain) and females (ovary) were collected during the late preparatory phase of the reproductive cycle. In carp, primordial germ cells divide rapidly from 28 to 63 dph and differentiate into oogonia and spermatogonia around 70 and 100 dph, respectively [19]. Different ages of carp, 0, 40, 80, 120, 160 and 200 dph (~40 mm–20 cm, ~0.2–300 g) were collected (n = 5~10) for gonadal ontogeny studies. Fish hatchlings collected for gonadal ontogeny were maintained at ambient temperature (23 ± 3 °C) as described [20]. Total RNA was prepared from 0 (whole trunk), 40 (mesonephric gonadal complex), 80–200 dph (testis/ovary) samples. Carps follow a seasonal reproductive cycle in the Southern part of India, spawning biannually during monsoon and winter [21]. Gonads from adult male carp were collected (n = 5) during the seasonal reproductive cycle flanking preparatory, pre-spawning, spawning, and resting phases for quantifying gene expression. Following anesthetization with 100 mg/L of MS222 (Sigma, MO, US) in mild ice-cold water, fishes were sacrificed, tissues were collected and kept at −80 °C until experimentation. For histology, tissues were fixed using Bouin's fixative (1:5:15, glacial acetic acid, formalin and saturated picric acid) at RT for 12−14 h. For localization analysis, tissues were fixed using 4 % PFA-PBS (0.01 M). Mono-sex induction was performed at KVAFSU and those carps were procured for experimentation. Fish sampling and experimentations were carried out by complying to the norms laid by IAEC, University of Hyderabad (CPCSEA, Inst. Reg. No. 151/1999 dt.22.07.1999).

2.2. Cloning of sox9a/b and sox30 from adult carp testis

Gene-specific primers were designed for *sox30* with the sequence data available in NCBI's Gene Expression Omnibus (GSE112157), a transcriptome study conducted earlier in carp from our laboratory. Cloning of *sox9a/b* was done as detailed by Sudhakumari et al. [22] to compare their expression with *sox30*. Total RNA (TRI reagent, Sigma) isolation, followed by DNase I treatment and cDNA synthesis (OneScript® Plus cDNA Synthesis Kit, Applied Biological Materials Inc., BC, Canada) were carried out as described in the manufacturer's manual. PCR amplification with designed primers (Supplementary Table 1) using Taq 2X master mix (New England Biolabs Inc., MA, US) was done under standard PCR conditions. No isoforms of *sox30* were recognized as amplicons displayed a specific single explicit band. To verify the presence of paralogs of *sox30* if any, primers (Supplementary Table 1) were designed by aligning neighboring exons with a flanking variable intron in the conserved HMG domain. A single band was observed in the genomic DNA PCR indicating the absence of any paralogs. Following gel purification and ligation (pGEM®-T easy vector, Promega, WI, US), the positive clones were ascertained by DNA sequencing using the dideoxy method. Lasergene software (DNASTAR, WI, US) was employed in the sequence alignment.

2.3. Multiple sequence alignment and phylogenetic analysis

Following ClustalW alignment of cloned carp Sox30 and its vertebrate counterparts with aa sequences available in GenBank, the phylogenetic tree was constructed. Multiple sequence alignment was done by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and displayed using Jalview 2.8. The phylogenetic tree was constructed and presented using PhyML (http://www.phylogeny.fr/simple_phylogeny.cgi). Bootstrapping with 1000 replicates delineates the integrity of every clade.

2.4. qPCR

Expression analysis was done by qPCR as described by Murugananthkumar and Senthilkumaran [23]. Total RNA and cDNA from all samples were prepared as detailed earlier. Each reaction was performed in triplicate for five distinct biological samples using gene-specific primers designed cautiously to prevent the amplification of genomic DNA with one of the primers straddling the exon-exon boundary. The gene-specific primers (Supplementary Table 1) were designed based on the sequences obtained from transcriptome analysis and NCBI GenBank database. The manufacturer's manual was shadowed for amplification (Power SYBR™ Green PCR Master Mix, Thermo Fisher Scientific, MA, US) in ABI Step One Plus real time PCR (Thermo Fisher Scientific). Ct value was estimated from the exponential phase of the PCR amplification and the gene expression was normalized using *18S rRNA* (reference gene) to obtain ΔCt (Ct of target gene - Ct of endogenous control). From the preliminary experiments of this study, as per the validation done based on Radonić et al. [24], *18S rRNA* (FJ710827.1) normalization was found to be efficient and constitutive with the lowest transcription range compared with other reference genes such as *β-actin* (M24113.1) and *gapdh* (JX244278.1). For estimating fold changes in gene expression, $2^{-\Delta Ct}$ was used. Relative expression of genes between control and

treated groups was calculated using $2^{-\Delta\Delta Ct}$. Log copy number analysis was performed to analyze the differences in the concentration of *sox9a/b* and *sox30* as explained by Mamta et al. [25].

2.5. Polyclonal antibody generation for Sox30

For antibody generation, an antigenic peptide, MSHYEDLR-QEAPVQNC was synthesized commercially using carp Sox30 aa sequence in conjugation with keyhole limpet hemocyanin carrier protein (Sigma). Six-weeks-old (n = 2) Swiss albino male mice used for antibody generation were handled and maintained following the norms of IAEC (UH/IAEC/BSK/2018-I/28) and CPCSEA. During experimentation, mice were fed commercial food pellets, *ad libitum*. Pre-immune sera were collected by retro-orbital puncture before administration of the peptide dissolved using 0.01 M PBS. Primary (200 μg of antigenic peptide combined with Freund's complete adjuvant) and two (14 days apart) boosters (100 μg of antigenic peptide combined with Freund's incomplete adjuvant) were injected subcutaneously. Blood was collected for serum separation and then used for IHC and western blot as a Sox30 antibody source.

2.6. Western blot

Sox9 and Sox30 proteins were detected in carp testis upon western blotting with anti-SOX9 antibody (Santa Cruz Biotechnology, Texas, US) and carp anti-Sox30 polyclonal antibody as described by Gupta and Senthilkumaran [20]. SOX9 antibody rose against 22–62 aa near N-terminus of SOX9-human origin showed 81 % and 90 % homology with N-terminal region of carp Sox9a and Sox9b proteins, respectively (Supplementary Fig. 1). In brief, the protein was prepared from adult carp testis and intestine with RIPA (0.5 % sodium deoxycholate, 150 mM NaCl, 50 mM Tris HCl [pH 8], 0.1 % SDS, and 0.1 % Triton X–100) buffer separately and quantified using Bradford's method. About 20 μg of protein run in 10 % polyacrylamide gel was transferred to nitrocellulose membrane (Pall Life sciences, NY, US), incubated overnight with respective diluted antibody (1:1000 for Sox30; 1:100 for SOX9) and probed using HRP-conjugated goat anti-mouse antibody (1:5000; Bangalore Genei, Bengaluru, India) incubation at RT for 1 h. Specific bands were detected with a chemiluminescent reagent (G-Biosciences, MO, US). Anti-mouse α–tubulin monoclonal antibody was used as a reference control for both testis and intestine protein fractions. Image J (NIH)

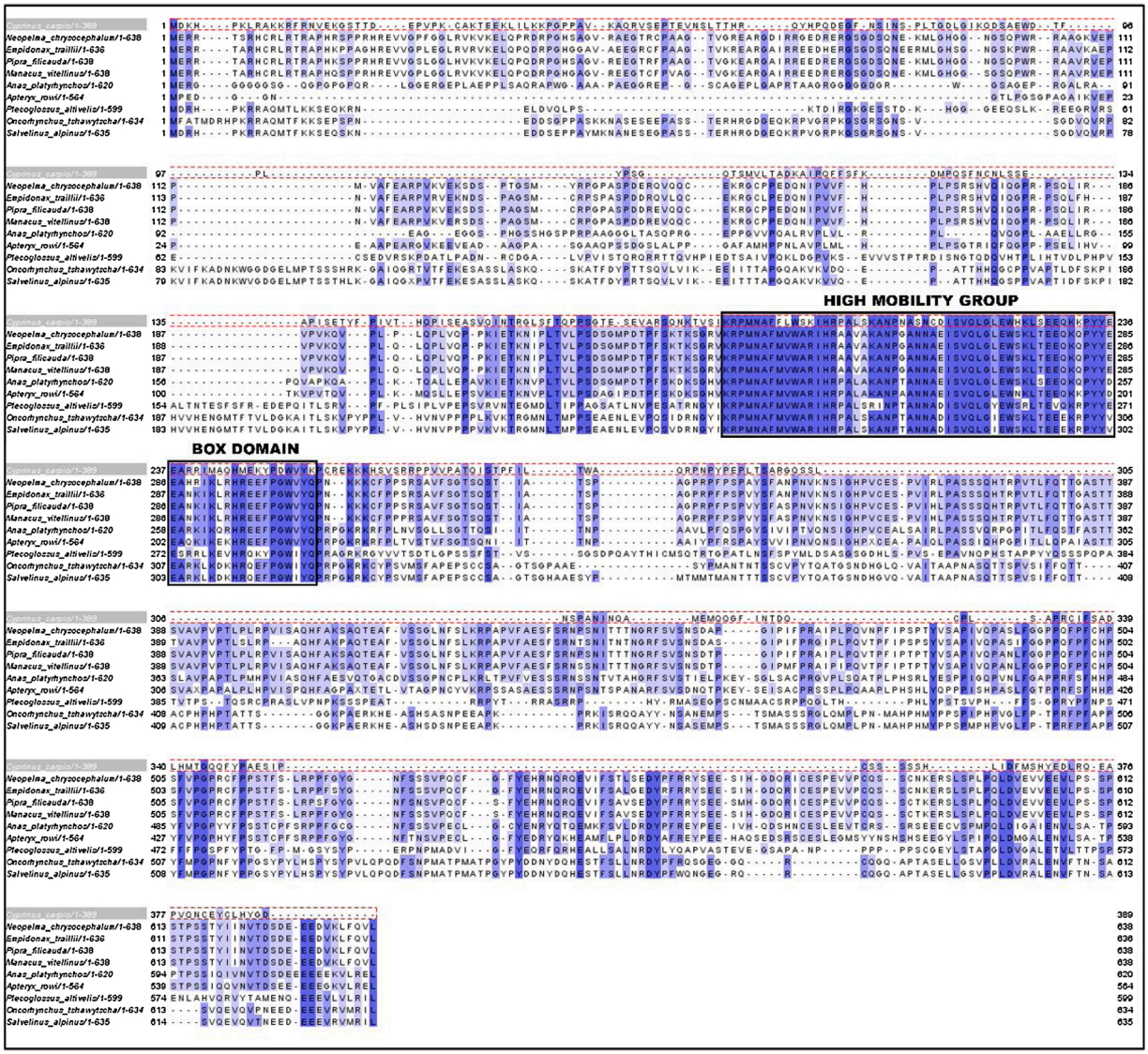

Fig. 1. Multiple sequence alignment of carp Sox30 with other vertebrate counterparts: *Anas platyrhynchos* (XP_005015498.2), *Apteryx rowi* (XP_025919212.1), *Cyprinus carpio* (MT193291), *Empidonax traillii* (XP_027741383.1), *Manacus vitellinus* (XP_017928916.1), *Neopelma chrysocephalum* (XP_027538170.1), *Oncorhynchus tshawytscha* (XP_024289861.1), *Pan troglodytes* (NM001280287.1), *Pipra filicauda* (XP_027574476.1), *Plecoglossus altivelis* (AHK05944.1), *Salvelinus alpinus* (XP_023992871.1).

software was used for densitometry analysis.

2.7. IHC

Sox9 and Sox30 proteins were localized, as described in Laldinsangi and Senthilkumaran [26]. In brief, tissues were fixed using 4 % PFA-PBS, embedded (optimum cutting temperature compound, Leica Microsystems) in cryomold (Tissue–Tek, AJ Alphen aan den Rijn, The Netherlands) and sectioned (Cryostat, Leica CM1850). Initially, the sections were blocked using 10 % of goat serum (Bangalore Genei) and incubated at 4 °C either with SOX9 (1: 50) or Sox30 (1: 100) antibody or pre-adsorbed antibody along with respective excessive antigen (negative control) for overnight. The sections were probed using HRP-conjugated goat anti-mouse antibody (1:1000) at RT for 2 h, the next day. VECTASTAIN® Elite ABC (Vector Laboratories, CA, US) incubation was done for 30 min before developing them with commercially available 3, 3′-diaminobenzidine and H_2O_2 as substrate. After developing, DPX was used to mount the sections. DM6 B upright microscope fitted with a camera (DFC 4500, Leica Microsystems, Germany) and Leica Application Suite X software was used to assess the sections. Pre-adsorbed antisera with an excess of commercially synthesized peptides (antigen) of Sox30 (MSHYEDLRQEAPVQNC) and Sox9 (CPSGSGSDTENTRPQE) were used as their respective negative controls for IHC.

2.8. In vitro culture of testicular slices

Testicular slice culture was performed as outlined by Raghuveer and Senthilkumaran [5]. Testis from carp in the late preparatory phase was dissected, sliced (McIIwain tissue chopper, Vibratome, Ted Pella Inc., CA, US) and cultured at 20−22 °C in a tissue culture plate with 2 mL of L15 culture medium, 10 mM HEPES and antibiotics (streptomycin, 0.1 mg/mL; penicillin, 100 IU/mL) supplementation. Testicular slices were treated using 100 IU/mL [20] of hCG (Uni-Sankyo Pvt. Ltd., Hyderabad, India) or (control) physiological saline. At short intervals of 0, 3, 6, and 12 h, tissue was collected and washed using ice-cold PBS. Likewise, testicular slices were also treated with 100 ng/mL (dose tested with pilot experiments) of 11-KT (Sigma) to study its effect on *sox9a/b* and *sox30* expression. 11-KT was initially dissolved using absolute ethanol, completely air-dried to remove any traces of alcohol, and reconstituted with the L15 medium for experimentation. Total RNA was isolated using TRI reagent followed by cDNA synthesis and quantification as described earlier.

2.9. In vivo induction by hCG

During the preparatory phase, laboratory acclimated adult male carp (n = 5) weighing about 400–500 g was briefly anesthetized with MS222. The ventral portion of the carp was sterilized with alcohol, a single dosage of hCG (1000 IU/Kg body weight) was gradually injected into the testis directly using a sterilized 1 mL syringe [20]. For control fish, physiological saline was injected. Subsequently, fishes were kept in circulatory water for a few minutes at ambient temperature to regain from anesthetization. Testis was collected at intervals of 12 and 24 h from control and treated fish for expression analysis. Total RNA and cDNA from all samples were prepared as mentioned earlier.

2.10. Mono-sex induction

Mono-sex induction was performed at KVASFU by following established protocols [27,28]. In brief, 50 dph carp larvae were fed hormone incorporated diet for 50 days. Hormones, MDHT (Sigma) at 50 mg/Kg feed for masculinization, and E_2 (Sigma) at 150 mg/Kg feed for feminization were given to produce the mono-sex population. The feed required for the study was prepared as described [29]. The treatments were given regularly and, fish samples were collected at the end for analysis.

2.11. Culture of carp primary testicular cells (mixed)

Testicular mixed cell culture was prepared as described by Murugananthkumar and Senthilkumaran [23]. Adult male carp was anesthetized to dissect out testis, and disinfected, also severed into small portions in the L15 culture medium. Following, incubation with 0.005 % DNase and 0.25 % collagenase type I, the slices were pressed to obtain cell suspension, further filtered using a 40 mm cell strainer, and centrifuged at 100 × *g*, to obtain pellet which was resuspended in DMEM. Almost 1×10^6 cells were cultured separately in 24-well plate comprising DMEM, antimycotic-antibiotic, 1 × Glutamax, 10 % fetal bovine serum, and maintained for 24 h in 5 % CO_2 at 30 °C. Cell viability was assessed with trypan blue staining.

2.12. In vitro treatment of sox30-siRNA in carp primary testicular culture

Custom synthesized *sox30* oligomer (antisense: 5′-rGrCrCrUrUrUrUrGrArUrArGrArArArCrUrGrUrUrUrUrGrUrUrCrU-3′; sense: 5′- rArArCrArArArArCrArGrUrUrUrCrUrArUrCrArArArArGGC-3′) siRNAs were commercially manufactured (Integrated DNA Technologies, Iowa, US). The siRNA for *sox30* was designed spanning only a few base pairs of the HMG domain to ensure the specificity. The siRNA in combination with branched PEI (Sigma) was dissolved using sterilized HEPES-NaCl (pH 7.4), as detailed by Höbel and Aigner [30] and used in teleost [20,23,26]. The mixture was incubated at RT for 20 min for the formation of the siRNA-PEI complex. About, 1×10^6 cells of carp testicular cell culture were maintained as earlier described. Before treatment, the culture medium was removed, followed by the addition of 100 µL OPTI-MEM® I (Thermo Fisher Scientific) solution. Concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 20, and 50 ng/mL of antisense *sox30*-siRNA and 10 ng/mL of sense *sox30*-siRNA complexed with branched PEI were prepared. Aliquots of 25 µL containing antisense *sox30*-siRNA-PEI/ sense *sox30*-siRNA-PEI (negative control)/ only PEI (control) was added and the cells were incubated for 12 h. Following treatment, siRNA-PEI mixture, and OPTI-MEM® I was removed, replenished using fresh culture medium, and maintained for 24 h. Cells were rinsed with PBS and harvested by trypsinization (0.5 % Trypsin-EDTA) for total RNA isolation using TRI reagent, followed by cDNA synthesis and relative expression analysis, as detailed earlier. Treatment was validated using MISSION® siRNA Fluorescent Universal Negative Control #1 (Sigma). The treated cells were captured using the IX81 Olympus microscope (Olympus Corporation) and assessed by Cell Sens dimension software.

2.13. In vivo treatment of sox30-siRNA in adult male carp

Specific approvals from the IAEC (UH/IAEC/SB/2019-I/04) and Institutional Bio-Safety Committee (IBSC No. BSK–N–15-July2019) were obtained for siRNA treatments. Gene silencing using siRNA was done [30] and used in teleost [20,23,26]. Adult male carp, in the pre-spawning phase, was anesthetized, and about 100 µL of 100 ng/mL of antisense *sox30*-siRNA-PEI complex dissolved in HEPES buffer was injected directly into the testis, and fish were kept in circulatory water for a few minutes at ambient temperature to regain from anesthetic effect. Similarly, treatments with sense *sox30*-siRNA-PEI (negative control), only PEI and control groups were done. Four fish groups (control, only PEI, antisense *sox30*-siRNA-PEI, and sense *sox30*-siRNA-PEI) were kept in separate tanks of 50 L capacity, continuously aerated and replenished with fresh water. At days 0, 2, 4, 6, and 8, fishes (n = 5/group) were sacrificed to dissect out the testis for analysis. Total RNA from PEI, control, antisense *sox30*-siRNA-PEI, and sense *sox30*-siRNA--PEI treated samples was prepared and analyzed, as earlier. There was no mortality in any of the treatment groups throughout the experiment.

2.14. EIA of androgens

Serum samples were obtained from control and *sox30*-siRNA treated

fish on day 4 post-treatment. Carp serum T and 11-KT levels were valued using Immunotag fish EIA kits (G-Biosciences) adopting the manufacturer's protocol. The sensitivity for T and 11-KT measurements is 6.92 and 0.28 pg/mL, respectively, and the assay was validated as described [31]. Assays were performed for each independent sample (n = 5) in triplicates with appropriate dilutions.

2.15. Estimation of Hsd11b enzyme activity in testis

Enzyme activity of Hsd11b was measured as explained by Rasheeda et al. [32]. In brief, microsomal fractions were prepared by homogenizing 100 mg of control and *sox30*-siRNA treated testicular tissue in 1 mL of 0.1 M KPO_4 buffer, followed by centrifugation and ultra-centrifugation at 9000 × *g* for 20 min and 105,000 × *g* for 1 h, respectively at 4 °C. The microsomal pellet was rinsed once and dissolved with 500 µL of 0.1 M KPO_4 buffer, 20 % (v/v) glycerol, and 0.1 mM EDTA. About 250 µg of testicular microsome, was added to 1 mL of assay medium comprising of 100 µM NAD^+ and 2 nM 11−OHT incubated at 37 °C in a shaker water bath for 60 min. The reaction was interrupted by the addition of ice-cold diethyl ether. For extraction of steroids, diethyl ether was used, the organic layer was then dried under N_2 gas and dissolved with 100 µL of EIA buffer. All incubates were analyzed in triplicates. The quantity of 11-KT formed was estimated with Immunotag fish 11-KT-EIA kit, as detailed earlier. Heat-denatured microsome was employed as a negative control for assay validation.

2.16. Statistics

Densitometry analysis of images was performed using ImageJ software. All qPCR data were denoted as mean ± Standard Error of the Mean (SEM) which qualified both normality and homogeneity tests. ANOVA, followed by posthoc tests were performed for statistical calculation with SigmaPlot 14.0 software (Systat Software Inc., Chicago, IL, US). For each test, P-value < 0.05 was treated to be statistically significant.

3. Results

3.1. Cloning of sox9a/b and sox30

Upon cloning, partial cDNAs of *sox9a* (~147 bp), *sox9b* (~336 bp), and *sox30* ORF (~1170 bp) encoding a putative protein of ~389 aa with a molecular weight of ~44 kDa, comprising HMG box domain were obtained. The *sox30* ORF nucleotide and aa sequence have been submitted to GenBank (MT193291).

3.2. Homology

The identity of carp Sox30 was compared with its vertebrate counterparts. Multiple sequence alignment revealed that carp, *C. carpio* Sox30 displayed much identity at the HMG box domain region (Fig. 1). Phylogenetic analysis showed that *C. carpio* Sox30 is closely related to *Plecoglossus altivelis* and formed a separate clade (Fig. 2).

3.3. Expression analysis of sox9a/b and sox30

Transcript copy number analysis was performed to compare the expression of *sox9a/b* and *sox30*. Tissue distribution analysis during the late preparatory phase of the reproductive cycle revealed that the expression of *sox9a/b* and *sox30* was ubiquitous in the tissues (n = 5) analyzed with negligible expression in the intestine. The expression of *sox9a/b* was high in the brain ($P < 0.05$) followed by the testis (Fig. 3A, B), while the predominant expression of *sox30* was evident in the gonad of both female ($P < 0.05$) and male ($P < 0.05$) carp (Fig. 3C). Gonadal ontogeny showed a high expression of *sox9a/b* during 40 dph ($P < 0.05$), which later had varied expression in both female and male gonads through gonadal development till maturity (Fig.3D, E). On the other hand, *sox30* displayed significant dimorphic expression ($P < 0.05$) between female and male gonads from 120 dph onwards until adulthood (Fig. 3F). Analysis through the testicular reproductive cycle of carp displayed significant expression of *sox9a/b* at preparatory ($P < 0.05$), modest expression in pre-spawning and spawning, and minimal expression during resting (Fig. 3G, H). However, high levels of *sox30* were observed during pre-spawning followed by spawning and preparatory in comparison with the resting phase (Fig. 3I).

3.4. Immunolocalization and Western blot of Sox9 and Sox30

Sox9 immunoreactivity was detected in the spermatogonia of carp testis (Fig. 4A, B) while the absence of immunoreactivity in the control (pre-adsorbed antibody) confirm the antibody specificity (Fig. 4C). Western blot presented a sharp band of Sox9 protein ~50 kDa (Fig. 4D) in testis, revealed to be highly specific by the absence of signal in the negative control (intestine). Sox30 antisera elicited immunoreactive signals in spermatocytes and spermatid/sperm (Fig. 4E–G), but not in spermatogonia of the testis. As expected, in control of the pre-absorbed antibody with excessive Sox30 antigen, no positive signal was evident (Fig. 4H). Further, a positive band ~44 kDa (Fig. 4D) corresponding to Sox30 was observed in testis but not in the negative control (intestine) by western blot, validating antibody specificity. Neither Sox9 nor Sox30 immunoreactive signals were seen in the supporting cells of the testis.

3.5. Effects of hCG and 11-KT

Induction with hCG at the preparatory phase of the carp testicular reproductive cycle enhanced the expression of *sox9a/b* and *sox30*. In both *in vivo* (Fig. 5A–C) and *in vitro* (Fig. 5D–F) treatments (n = 5), prominent expression of *sox9a/b* and *sox30* was observed after 12 h ($P < 0.05$) of induction. Progressive increase of expression was evident with time upon induction from 12 to 24 h. The influence of hCG on androgen induction in testis is well known. 11-KT being a potent androgen in fish

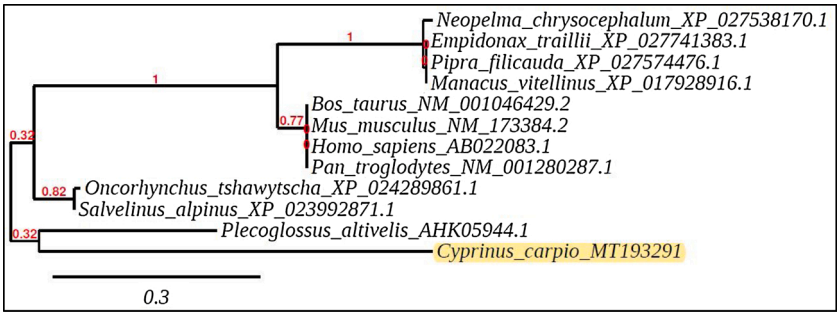

Fig. 2. Phylogenetic analysis of carp Sox30 with other vertebrates. The phylogenetic tree was generated using http://www.phylogeny.fr/simple_phylogeny.cgi. GenBank accession numbers of Sox30 sequences used are as follows: *Bos taurus* (NM_001046429.2), *Homo sapiens* (AB022083.1), *Mus musculus* (NM_173384.2), *Pan troglodytes* (NM_001280287.1). All other details are the same as Fig. 1.

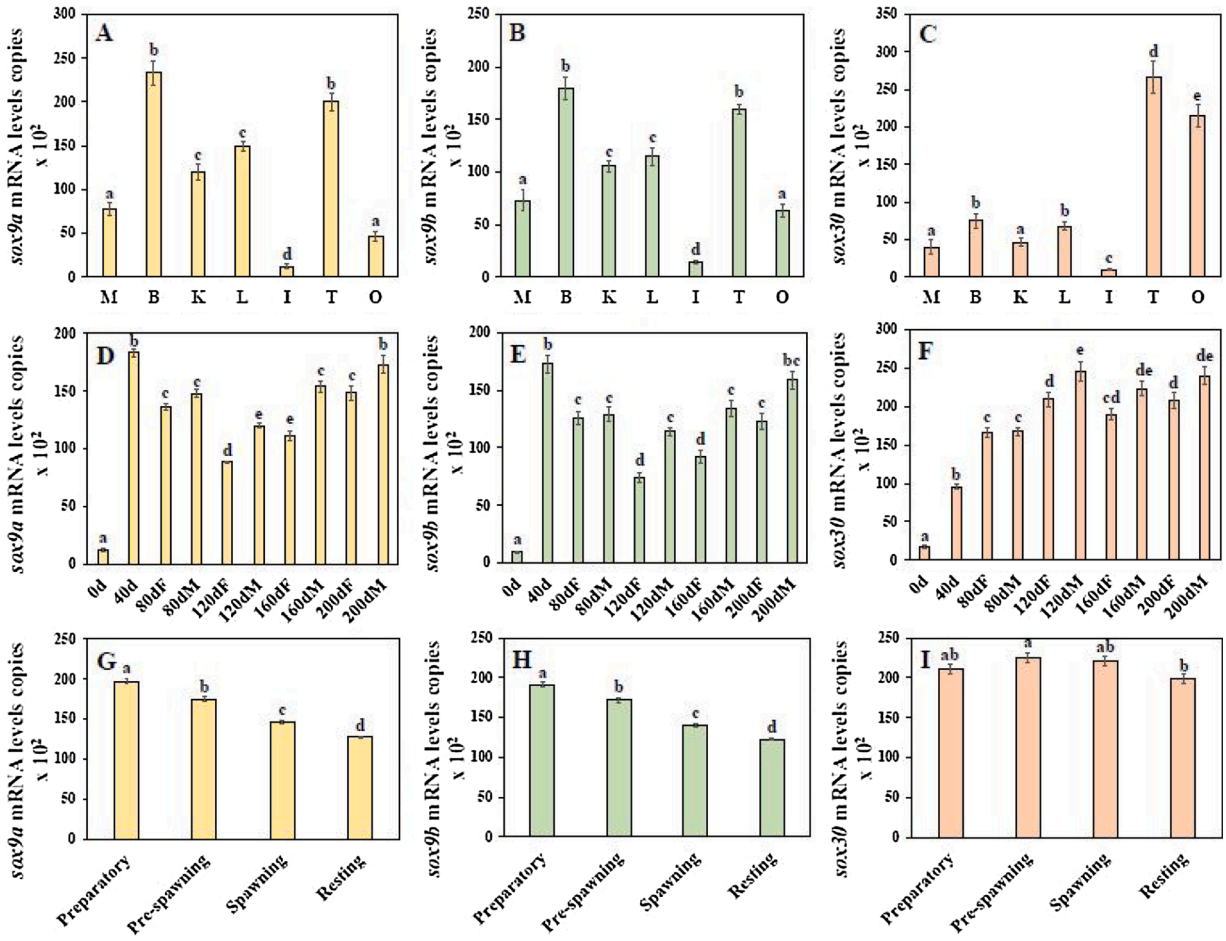

Fig. 3. Expression profiling of *sox* genes: Tissue distribution [*sox9a* (A), *sox9b* (B), *sox30* (C)], gonadal ontogeny [*sox9a* (D), *sox9b* (E), *sox30* (F)] and testicular reproductive phase [*sox9a* (G), *sox9b* (H), *sox30* (I)] analysis using absolute quantification (copy number) in carp. Data (n = 5) were expressed as mean ± SEM ($P < 0.05$; One-way ANOVA followed by Tukey test). Means with different alphabets differ significantly while means with similar alphabets did not show any significance. Abbreviations: M-muscle, B-brain, K-kidney, L-liver, I-intestine, T-testis, O-ovary, d-days post hatch, F- female, M-male.

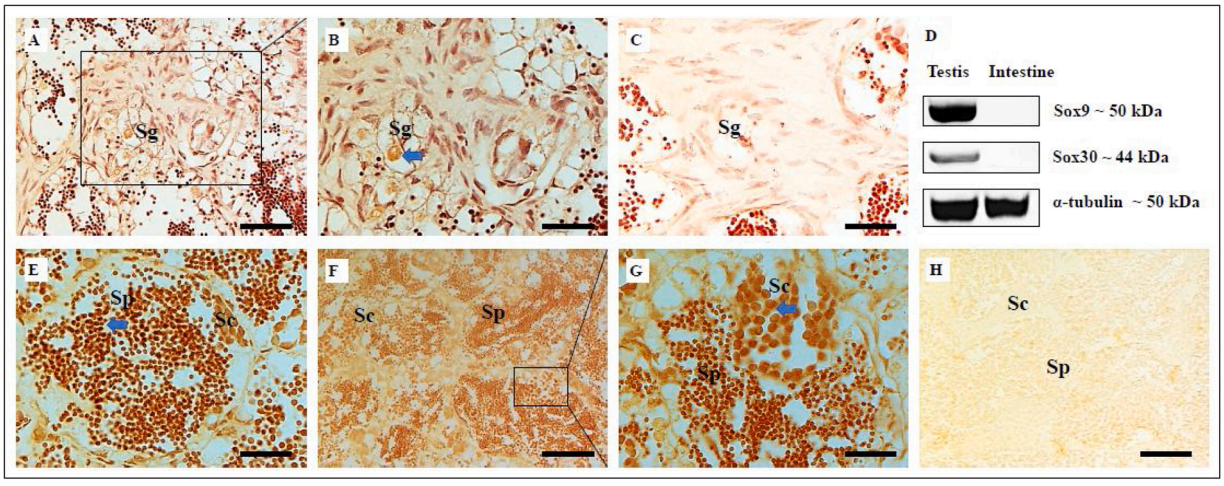

Fig. 4. Localization and western blot of Sox9 and Sox30 proteins in adult carp testis. Immunoreactivity of Sox9 (A, B) and Sox30 (E–G) in testis, whereas their respective negative controls (pre-adsorbed antibody with excess antigen) showed no signal (C, H). Western blot analysis of Sox9 and Sox30 proteins displayed band of ~50 kDa and ~44 kDa respectively, in testis while in the negative control (intestine) no bands were observed (D). Abbreviations: Sg: spermatogonia, Sc: spermatocytes, Sp: spermatid/sperm. The scale bar indicates A, E: 30 μm; B, C, G: 10 μm; F, H: 50 μm.

[33], its effect on *sox9a/b* and *sox30* expression was studied. *In vitro* study (Fig. 5G–I), showed a substantial elevation ($P < 0.05$) of *sox9a/b* and *sox30* at 12 h in comparison with control.

3.6. Effects of mono-sex induction by MDHT and E_2 treatments

The expression levels of *sox9a/b* and *sox30* were compared in mono-

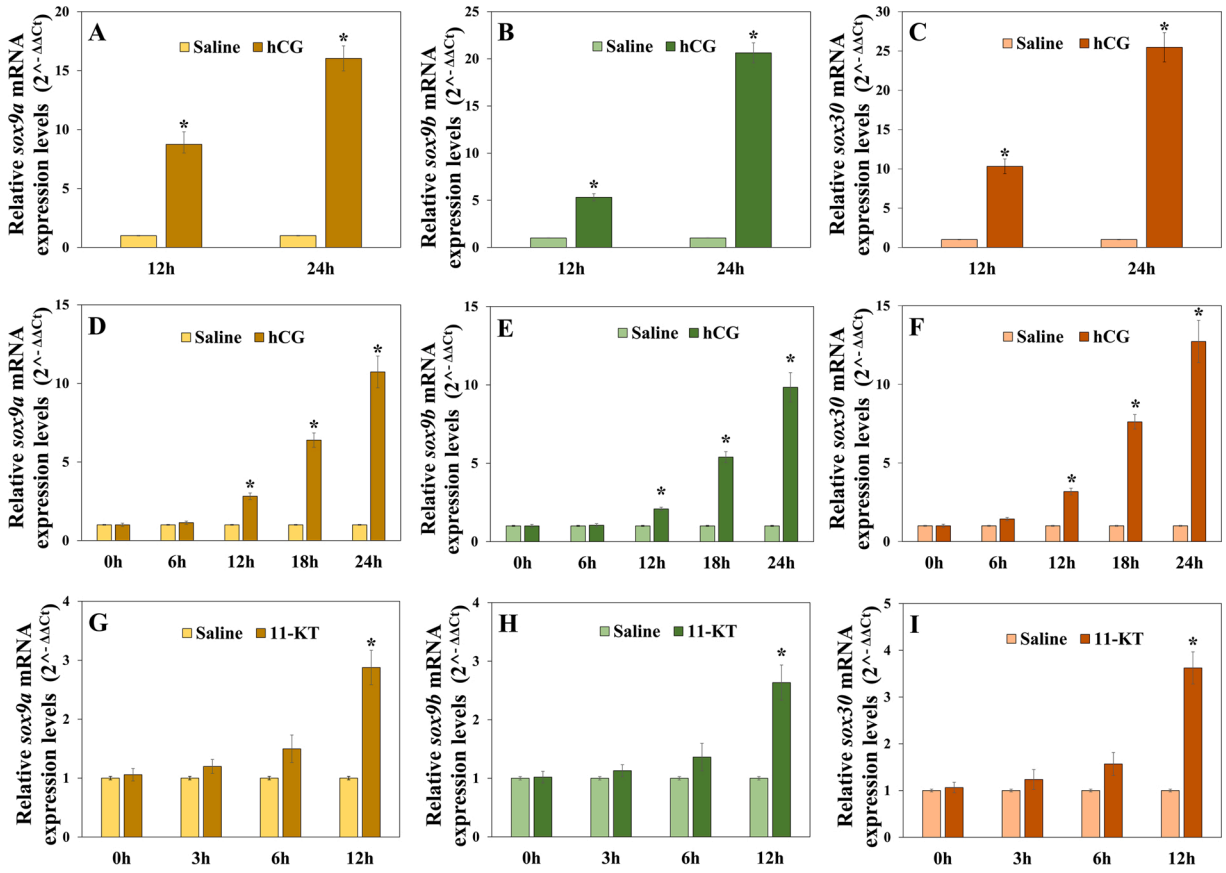

Fig. 5. Relative gene expression after hCG (1000 IU/Kg body weight) *in vivo* [*sox9a* (A), *sox9b*(B) and *sox30*(C)], hCG (100 IU/mL) *in vitro* in testicular slices [*sox9a* (D), *sox9b*(E) and *sox30*(F)] and 11-KT (100 ng/mL) *in vitro* in testicular slices [*sox9a* (G), *sox9b* (H) and *sox30* (I)] treatments at different time intervals compared with control (saline) in carp. Data (n = 5) were expressed as mean ± SEM (* $P < 0.05$; One-way ANOVA followed by Dunnett's method). Abbreviation: h-hour.

sex-induced carp. MDHT treatment for 50 days increased the expression of *sox9a/b* and *sox30* significantly ($P < 0.05$) in testis (Fig. 6A) during the gonadal development of carp. *kiss2* and *gnrh* expression were determined in the brain while expression of *fshr* and *lhr* was determined in the gonads of mono-sex-induced carp. The expression of *lhr* was significantly ($P < 0.05$) high, while *fshr* expression was comparable to control in MDHT treated testis (Fig. 6A). The expression of *kiss2* and *gnrh* was elevated in the brain of MDHT (Fig. 6B) treated carp. E_2 treatment decreased the transcript levels of *sox9a/b* and *sox30* in the ovary (Fig. 6C). *fshr* expression was significantly ($P < 0.05$) high, while *lhr* expression was comparable to control in E_2 treated ovary (Fig. 6C). The expression of *kiss2* and *gnrh* was elevated in the brain of E_2 (Fig. 6D) treated carp.

3.7. Silencing of sox30, in vitro

In carp testicular culture (n = 5), PEI complexed with antisense *sox30*-siRNA significantly ($P < 0.05$) reduced *sox30* expression compared to control and after sense *sox30*-siRNA treatments (Fig. 7A). Besides, the expression of *sox30* post-siRNA treatment exhibited dosage-related downregulation. Expression of *sox30* significantly ($P < 0.05$) declined to 88 % after treatment with 10 ng/μL of *sox30*-siRNA in comparison with control, hence this dose was chosen for later on experiments. Silencing of *sox30* displayed a significant reduction ($P < 0.05$) in the transcript levels of certain transcription factors (Fig. 7B), few germ/stem cell markers and other factors (Fig. 7C) crucial for testicular function, and steroidogenesis-related genes (Fig. 7D). Relative mRNA levels of *ad4bp/sf-1*, *gata4*, *wt1*, *sox5*, *sox9a/b*, *dmrt1*, *sycp3*, *vasa*, *pou5f3/oct4*, *gsdf*, *pdgf*, *amh*, *star*, *cyp11*, *cyp17*, *hsd3b*, *hsd17b*, *hsd20b*, *hsd11b*, and *srd5a1* showed a decline, while *dax* and *piwi* did not show any significant change and *ar* showed a significant up-regulation when compared to control. The uptake of siRNA was confirmed by fluorescent signals evident in carp primary testicular culture treated with control siRNA (Fig. 7E and F).

3.8. Transient silencing of sox30, in vivo

Treatment of antisense *sox30*-siRNA-PEI complex significantly downregulated ($P < 0.05$) the expression of *sox30* indicating that siRNA complexed with PEI was capable of silencing *sox30* in carp testis (Fig. 8A). On the other hand, only PEI or sense *sox30*-siRNA-PEI complex treatments showed no effect in terms of *sox30* expression. Expression of *sox30* was quantified in testis on days 0, 2, 4, 6, and 8 post-treatment along with corresponding control at every time point (Fig. 8A). The antisense *sox30*-siRNA-PEI complex treatment decreased the expression of *sox30* ($P < 0.05$) on days 2 and 4 post-treatment with 44 and 90 % reduction, respectively. Further, *sox30* expression decreased to 37 % on day 6 and 10 % on day 8 indicating that transcript levels might restore to normal as reported earlier [23,26]. The antisense *sox30*-siRNA-PEI sample on day 4 showed a 90 % decrease in *sox30* expression and hence that time-point was chosen as a treatment sample for later on experiments. Sox30 protein levels reduced similar to that of *sox30* transcripts while there was no change in α–tubulin employed as a control (Fig. 8B). A substantial decrease in Sox9 protein levels was also observed after siRNA treatment, as opposed to the control (Fig. 8B). The band intensity was measured using ImageJ software and normalized with α–tubulin (Supplementary Fig. 2). There were no significant changes in the expression of *sox11*, *sox13*, *sox18*, and *sox21* in the *sox30*- siRNA treated group (Supplementary Fig. 3) demonstrating the specificity of the siRNA used in this study. Further, no significant phenotypic changes were

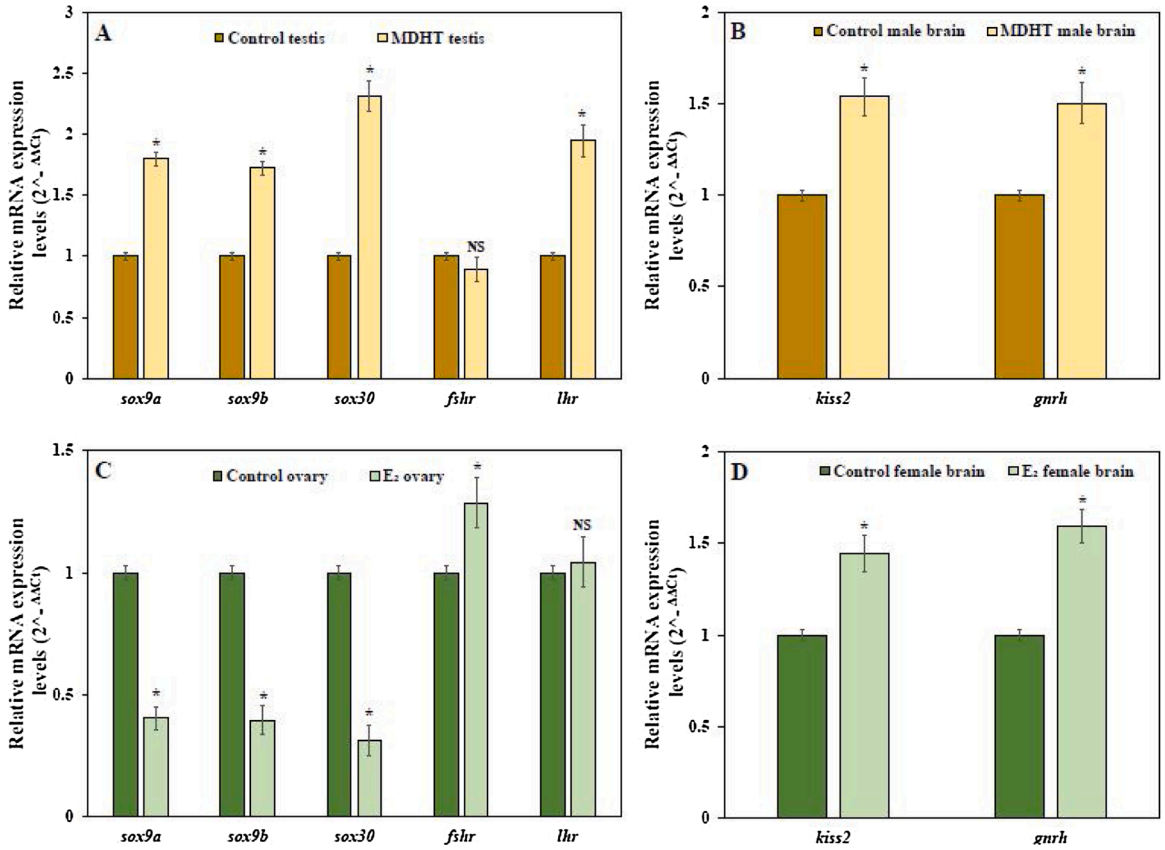

Fig. 6. Relative expression of genes (*sox9a*, *sox9b*, *sox30*, *fshr*, and *lhr*) in mono-sex-induced carp gonads (A, C). Relative expression of genes (*kiss2* and *gnrh*) in mono-sex-induced carp brain (B, D). NS- not significant. Data (n = 5) were expressed as mean ± SEM (* $P < 0.05$; One-way ANOVA followed by Dunnett's method).

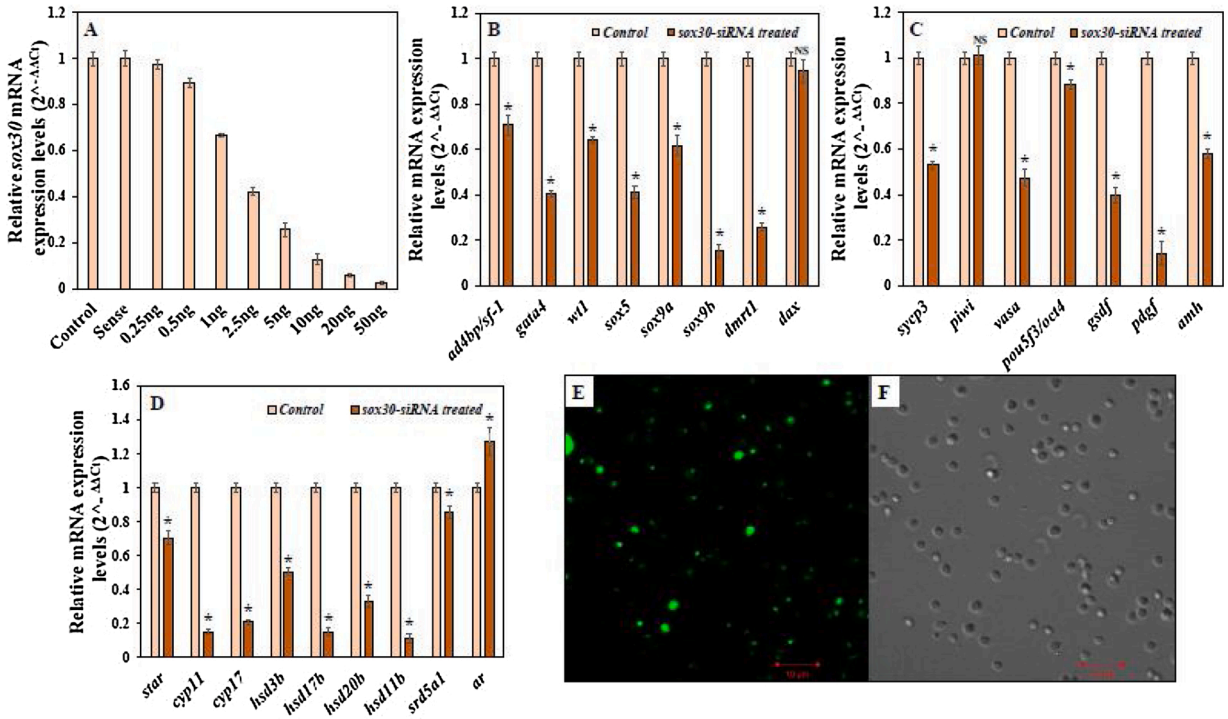

Fig. 7. Relative expression of (A) *sox30* 24 h after *in vitro* PEI mediated *sox30*-siRNA treatment (control, 10 ng/μL of sense *sox30*-siRNA and various doses of antisense *sox30*-siRNA) in carp testicular culture (mixed; n = 5). Relative expression of (B) transcription factors, (C) germ/stem cell marker genes and other factors, and (D) steroidogenesis related genes between control and 10 ng/μL of antisense *sox30*-siRNA treated carp testicular culture after 24 h of treatment (*$P < 0.05$; One-way ANOVA followed by Dunnett's method). NS-not significant. Representative (E and F) confocal microscope images [E (fluorescent), F (phase-contrast)] 24 h after *in vitro* PEI mediated transfection of MISSION siRNA fluorescent universal negative control conjugated with 6-FAM on carp testicular culture (mixed).

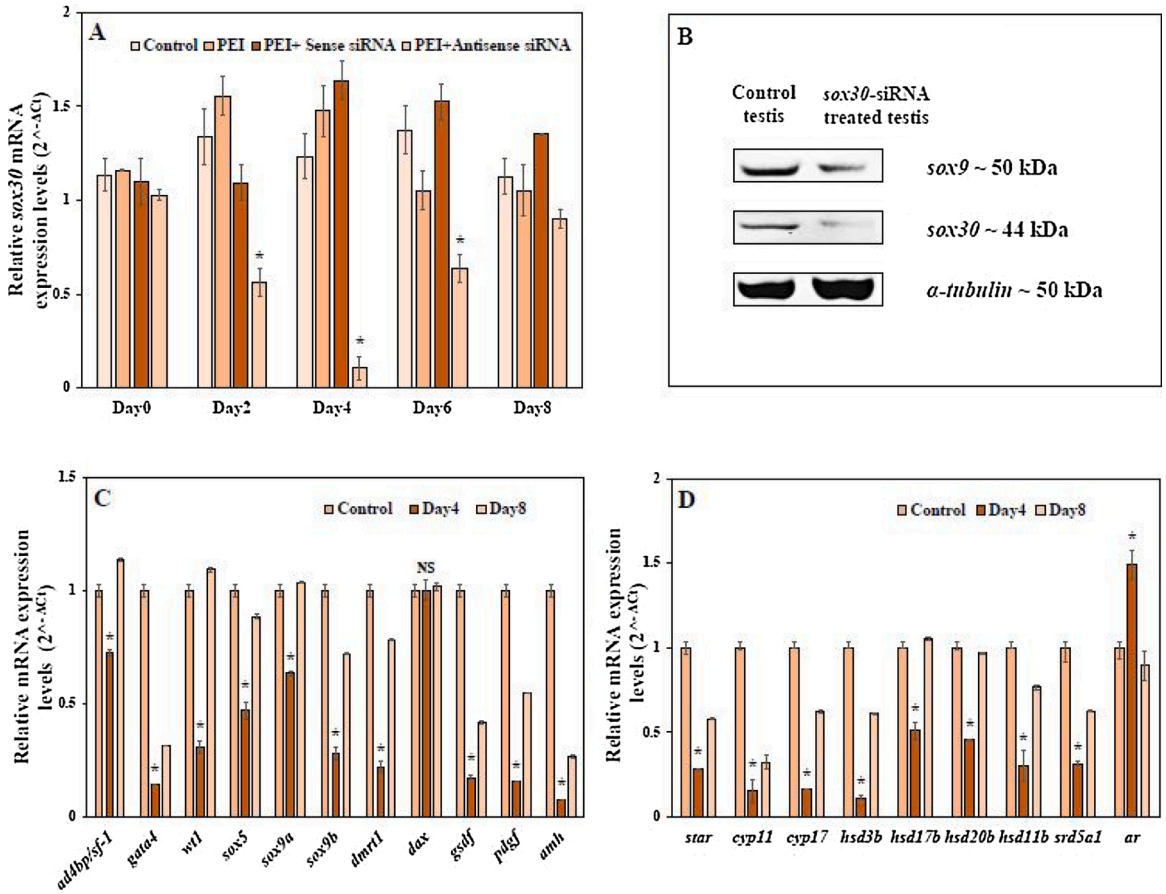

Fig. 8. Relative expression of *sox30* (A) post i*n vivo* PEI mediated *sox30*-siRNA treatment in adult (n = 5) carp (*, P < 0.05; One-way ANOVA followed by SNK test). (B) Western blot of Sox9 and Sox30 proteins on day 4 in control and *sox30*-siRNA treated carp testis. Relative expression of various (C) testis-related and (D) steroidogenesis-related genes/factors on day 4 in control and *sox30*-siRNA treated carp testis (*P < 0.05; Mann–Whitney test One-way ANOVA on ranks followed by SNK test). NS-not significant.

observed in the histological images between control and siRNA injected testis (Supplementary Fig. 4) proving the transient nature of the PEI siRNA treatment. Besides, it might be difficult to detect any change in the spermatogenesis process as the testis was at the late spermatogenesis stage. However, molecular changes similar to the *in vitro* treatment were observed upon *in vivo* transient silencing. The expression of transcription/other factors namely, *ad4bp/sf-1*, *gata4*, *wt1*, *sox5*, *sox9a/b*, *dmrt1*, *gsdf*, *pdgf*, *amh* (Fig. 8C), *star* and steroidogenic enzyme genes *cyp11*, *cyp17*, *hsd3b*, *hsd11b*, *hsd17b*, *hsd20b*, *srd5a1* (Fig. 8D) showed a decline on day 4 post-*sox30*-siRNA treatment but the expression levels were comparable to that of control on day 8 post-*sox30*-siRNA treatment, further substantiating the transient nature of siRNA silencing. There was no significant change on day 4 and day 8 in the expression of *dax*, while *ar* expression showed a significant up-regulation on day 4, reverting to normal levels on day 8.

Serum levels of T (Fig. 9A) and 11-KT (Fig. 9B) declined significantly (P < 0.05) on day 4 post *sox30*-siRNA treatment. Enzyme activity of Hsd11b significantly (P < 0.05) reduced on day 4 post *sox30*-siRNA treatment (Fig. 9C).

4. Discussion

The presence of *sox30* in teleost [15] and its vital role in mammalian spermiogenesis [4] have been well documented. However, the significance of *sox30* in testicular steroidogenesis remains unexplored in lower vertebrates including teleost. The current study reports the cloning of *sox30* from adult *C. carpio* testis, and cloning partial cDNAs of *sox9a/b*, whose roles have been earlier demonstrated in carp [11]. Expression of

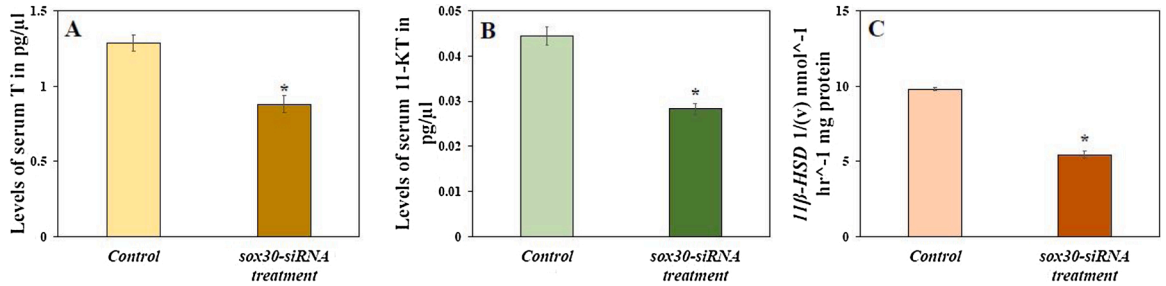

Fig. 9. Changes in the levels of serum (A) T and (B) 11-KT on day 4 in the control and *sox30*-siRNA treated carp. (C) Changes in enzyme activity of Hsd11b on day 4 in the control and *sox30*-siRNA treated carp testis. Data (n = 5) were expressed as mean ± SEM. (* P < 0.05; Student's t-test).

sox9a/b and *sox30* during gonadal development, changes in distinctive phases of the testicular reproductive cycle, post-11-KT treatment, after hCG induction, in mono-sex-induced carp, and the repercussions of *sox30*-siRNA silencing, were highlighted to demonstrate their dominant role in testicular steroidogenesis vis-à-vis testis function.

4.1. Cloning, expression analysis, and localization

An earlier study from our laboratory employing comparative transcriptomic strategy identified key genes involved in sex- differentiation, revealing the existence of *sox30* in carp [12]. Using gene-specific primers, ORF of *sox30,* and partial cDNA fragments of *sox9a* and *sox9b* were obtained in the current study. The expression of *sox30* was elevated in testis followed by the ovary in contrast to somatic tissues [17], while *sox9a/b* showed higher expression in the brain followed by the testis. Upon quantification, *sox30* displayed predominantly higher expression in the testis in comparison to *sox9a/b*. A previous study [15] in the Nile tilapia (*Oreochromis niloticus*) reported the expression of *sox30* from 10 dph, earlier than the morphological gonadal differentiation period (about 25 dph). However, in this study, *sox30* displayed significant dimorphic expression at 120 dph during the onset of testicular growth in carp [19]. Further, expression of *sox30* was high in mature testis as reported in mouse [34] implicating its role in maturation as well. The present study quantified the expression of *sox9a/b* and *sox30* at distinctive phases of the carp testicular reproductive cycle. Higher expression of *sox9a/b* was observed during preparatory and pre-spawning, marking their role during the initiation of spermatogenesis. *sox30* expression was abundant in pre-spawning, characterized by the predominance of spermatogonia and spermatocytes and in the spawning phase, corresponding to spermiogenesis. This sign that in carp, the expression of *sox30* was high during spermatogenesis and spawning/spermiation as observed in mice [4]. Seasonal changes in *sox30* expression may signify its role during testicular maturation. Sox9 was found in spermatogonia while Sox30 was localized in spermatocytes and spermatid/sperm as previously reported in the Nile tilapia [15] and mice [35].

4.2. Influence of gonadotropin

Steroidogenesis is strictly regulated by gonadotropins [36] and in fact, gonadotropins are well known to stimulate steroid synthesis in teleost gonads [2]. The influence of gonadotropin induction in carp has been testified [37]. There was a substantial increase in *sox9a/b* and *sox30* mRNA transcripts post-hCG induction, validating the regulatory role of gonadotropin on *sox30* expression. LH-like hCG is proven to bind to LH receptors [38], expressed by Leydig cells to induce androgen synthesis and also to act upon Sertoli cells to regulate spermatogenesis [39].

4.3. Effect of hormone treatment

It is known that hCG induces T and 11-KT production in fish [40]. Having analyzed the leverage of gonadotropins on *sox30*, the influence of androgen on gene expression is yet another exhilarating area to study. The expression of *sox9a/b* and *sox30* was found to be elevated after *in vitro* 11-KT treatment in carp testicular slices. In fish, treatment with sex-steroids during the critical window of sexual differentiation can alter the developmental processes of gonads [2]. Reports suggest that 40–50 days of treatment in carp [41] with different supplementations to feed can result in complete sex-reversal. An all-male population of blue tilapia (Tilapia aurea) was produced with 17 α-ethynyltestosterone treatment [42]. Exposure of methyltestosterone to catfish during gonadal differentiation resulted in the male population [43]. Expression of *sox9a/b* and *sox30* was elevated after MDHT but not after E_2 treatment. In teleost, during sex-inversion/differentiation, gender-specific sexual differences are contingent on the sequential presence of brain and/or gonadal sex differences under the impact of gonadal factors to hormonal response [44]. Gonadotropin regulation during natural and artificial induction was observed. Further, to clarify the role of the brain-pituitary gonadal axis during mono-sex induction, expression levels of gonadotropin receptors were studied. Therefore, the expression of *kiss2* and *gnrh* was determined in the brain, which showed their higher expression in both E_2 and MDHT treatments. However, the expression of *fshr* was higher in E_2 and *lhr* was higher in MDHT treated gonads. Our results were comparable to that of the previous reports [45] in gilthead seabream (*Sparus aurata* L.) and [46] orange-spotted grouper (*Epinephelus coioides*). In most gonochoristic teleost, *lhr* expression was high during late stages of gonadal development, vital for spawning and spermiation [47]. The current study showed the transcript levels of *lhr* was significantly high after MDHT treatment, suggesting the involvement of LH signaling in triggering mono-sex induction and subsequent testis development in carp.

4.4. Transient silencing of sox30

Most knockdown studies to investigate the significance of *sox30* during gametogenesis were done in mammals however, this report is the first of its kind to show the transient silencing of *sox30* mRNA in any lower vertebrate. To substantially elucidate the role of *sox30* in gonadogenesis, the effect of PEI mediated transient silencing of the *sox30* was studied *in vivo* and *in vitro*. Previous reports delineated that synthetic siRNA induces gene silencing, concerning numerous developmental processes [20,23]. Transient silencing using *sox30*-siRNA did not result in phenotypic changes in carp testis as reported earlier [26]. Delivery of *sox30*-siRNA resulted in decreased expression of the regulators of gonadogenesis, namely *ad4bp/sf-1, gata4,* and *wt1* [23], demonstrating *sox30* might affect the expression of these genes. In vertebrates, *ad4bp/sf-1* is an important steroidogenic factor required for the basal expression of steroidogenic enzymes [48]. Bai et al. [49] reported a *sox30* binding site in the promoter motif of *ad4bp/sf-1* in mice. In mammals, it is well established that *sox9* transactivates *ad4bp/sf-1* gene expression [50] to achieve tissue-specific gene expression. Similarly, *sox30* might regulate *ad4bp/sf-1* expression in fish. Besides, Chen et al. [51] showed differential expression of *gata2* in the SOX30 knockout mice line. In this study, transient silencing of *sox30* resulted in the downregulation of transcription factors crucial for testicular development namely, *dmrt1* [43], *sox5* [8], and *sox9a/b* [5]. Zhang et al. [4] have reported evidence of the SOX30 binding site in *DMRTA2* of mice. In the Nile tilapia, *dmrt1* directly binds to a putative cis-regulatory element within the *sox30* promoter [52]. Feng et al. [34] have shown the downregulation of SOX5 in SOX30 knock out mice. Though there were no reports pertaining to relationship between *sox30* and *sox9*, the presence of *sox30* DNA binding domain ACAAT [13] in the *sox9* promoter regions of *Sebastes schlegelii* (GenBank:KJ624401.1[unpublished]) warrants such a prospective. This report is the first to show the reduction of *sox9a/b* upon transient silencing of *sox30* in carp, indicating either a direct or indirect regulatory loop among the *sox* genes. Gonadogenesis is influenced by several other factors such as *amh* [53], *gsdf* [54], and *PDGF* [55]. Gamete production by meiosis is dependent upon crucial germ cell governing genes like *vasa*, *sycp3,* and *pou5f3/oct4* [26,56]. Silencing of *sox30* also targeted to affect the expression of *amh*, *gsdf*, *pdgf*, *vasa*, *pou5f3/oct4,* and *sycp3* in this study as observed in mice [4,51]. The production of testicular androgen is governed by steroidogenesis. In line with this, Sakai et al. [57] reported that *sox30* is an effective regulator of *ad4bp/sf-1*. In teleost, studies [58,59] have reported the direct participation of 11-OHT and 11-KT in the differentiation and development of testis. Previous reports have shown the significance of *hsd11b* during testicular development and recrudescence in teleost [32]. In particular, *sox3* has been proven as a transcriptional activator of the *hsd11b* gene by binding to two of its specific promoter motifs in catfish [60], suggesting that genes from the *sox* family may regulate *hsd11b* expression, and hence testicular steroidogenesis, in

teleost. Concomitantly, transient knockdown of *sox30* resulted in decreased T, 11-KT, Hsd11b enzyme activity and downregulation of *star* and steroidogenesis-related genes, *srda51*, *hsd3b*, *hsd11b*, *hsd17b* and *hsd20b*. Supporting the above contention, Chen et al. [51] have observed differential expression of HSD3B in SOX30 knockout mice. Further, transient knockdown of *sox30* exhibited overexpression of *ar*, the nuclear receptor for 11-KT in teleost [33]. Another study in mice has suggested that *ar* overexpression could be a potential mechanism for hypersensitivity due to low androgen [61].

4.5. sox9a/b vs sox30

The most interesting aspect of this study was attempting to signify the role of *sox30* in comparison with *sox9a/b*. The dominance of *sox30* over *sox9a/b* expression was observed in adult testis. Evidence of dominant expression of *sox30* during spermatogenesis and especially in spermiogenesis and high expression of *sox9a/b* at the initial stages of spermatogenesis signifies their role in gamete maturation. Incidentally, the influence of gonadotropin, 11-KT, and mono-sex induction was similar in *sox9a/b* and *sox30*. Transient silencing of *sox30* decreased *sox9a/b* levels hinting that *sox30* may act as a regulating factor for *sox9a/b*, either directly or indirectly.

5. Conclusion

The current study reveals that *sox30* transcripts predominantly expressed in the testis in comparison to the ovary and might regulate testicular growth as well as maturation during the reproductive cycle. Overexpression of *sox30* upon mono-sex induction indicates an important role during testicular development. Transient silencing of *sox30* influenced steroidogenic enzyme, and other factor genes as well as sex-steroid levels. Overall, the present study demonstrated the significance of *sox30* in testicular steroidogenesis vis-à-vis testis function of carp by influencing *sox9a/b*, either directly or indirectly, along with other male-related genes as evidenced by expression analysis and transient gene silencing.

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CRediT authorship contribution statement

Arumugam Anitha: Formal analysis, Writing - original draft, Writing - review & editing, Validation, Methodology, Data curation, Visualization. **Balasubramanian Senthilkumaran:** Formal analysis, Writing - review & editing, Conceptualization, Investigation, Supervision, Validation, Visualization, Funding acquisition, Resources, Project administration.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2020.105769.

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Gonadal transcriptome analysis of the common carp, *Cyprinus carpio*: Identification of differentially expressed genes and SSRs☆

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ABSTRACT

Common carp (*Cyprinus carpio*) is a world-wide freshwater fish of eutrophic waters. *C. carpio*, have various reproductive traits, including early sexual maturity, that may make them excellent, large, realistic, aquaculture model species. In the present work, *de novo* assembly of gonadal (testicular and ovarian) transcriptomes from juvenile common carp was performed to identify genes involved in gonadal development. A total of 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, were obtained from the immature testicular and ovarian transcriptomes, respectively. About 84,367 unigenes were constructed after removing redundancy involving representation of transcripts in both gonadal transcriptomes. Gene ontology (39,171 unigenes), clusters of orthologous group's analysis (6651 unigenes) and Kyoto encyclopedia of genes, and genomes automatic annotation server analysis (4783 unigenes) were performed to identify potential genes along with their functions. Furthermore, 18,342 (testis) and 8693 (ovary) simple sequence repeats were identified. About 298 differentially expressed genes were identified, of which 171 and 127 genes were up-regulated in testis and ovary, respectively. Quantitative real-time reverse transcription PCR was performed to validate differential expression of selected genes in testis and ovary. Nearly 809 genes related to reproduction were identified, sex-wise expression pattern of genes related to steroid synthesis, endocrine regulation, germ cell maintenance and others

Abbreviations: ad4bp/sf-1, adrenal 4 binding protein/steroidogenic factor-1; *amh*, anti-Mullerian hormone; *amhr2*, anti-Mullerian hormone receptor type 2; *amhy*, Y chromosome-linked anti-Mullerian hormone; *ar*, androgen receptor; *atm*, Ataxia telanglectasia mutated; COG, clusters of orthologous groups; Ct, cycle threshold; *ctnnbip1*, catenin beta interacting protein 1; *cux1*, cut like homeobox 1; *cux2a*, cut like homeobox 2a; *cyp11b1*, cytochrome P450, family 11, subfamily b, polypeptide 1; *cyp17*, steroidogenic cytochrome P450 17-hydroxylase/lyase; *cyp19a1*, cytochrome P450, family 19, subfamily a, polypeptide 1; *cxcl12*, C-X-C motif chemokine ligand 12; *cxcr4*, C-X-C motif chemokine receptor 4; *dax1*, dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; DEG, differentially expressed genes; *dmrt1*, doublesex and mab-3 related transcription factor 1; *DMY*, Y-specific DM-domain; dph, days post hatch; *egr2b*, early growth response 2b; *er*, estrogen receptor; *fam101b*, family with sequence similarity 101, member B; *fam192a*, family with sequence similarity 192, member A; *fam210b*, family with sequence similarity 210, member B; *fbox43*, F-box only protein 43; *fem1b*, fem-1 homolog B; *fgfr1a2*, fibroblast growth factor receptor 1-A-like; *foxk2*, forkhead box k2; *foxl2*, forkhead box L2; FSH, follicle stimulating hormone; *fshr*, follicle stimulating hormone receptor; *fstl3*, follistatin-like 3 (secreted glycoprotein); *fzd8*, frizzled class receptor 8; *gata4*, GATA binding protein 4; *gata6*, GATA binding protein 6; *gdf9*, growth differentiation factor 9; GnRH, gonadotropin-releasing hormone; GO, gene ontology; *gsdf*, gonadal somatic cell-derived factor; *hsd3b*, hydroxy-delta-5-steroid dehydrogenase, 3 beta; *hsd3b7*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 *hsd11b1* hydroxysteroid 11-beta dehydrogenase 1; *hsd11b2*, hydroxysteroid 11- beta dehydrogenase 2; *hsd17b2*, hydroxysteroid 17- beta dehydrogenase 2; *hsd20b2*, hydroxysteroid 20- beta dehydrogenase 2; *igf1ra*, insulin-like growth factor 1a receptor; *ipo4*, importin 4; KASS, KEGG, automatic annotation server; KEGG, Kyoto encyclopedia of genes and genomes; *kiss2*, kisspeptin2; *lhcgr*, luteinizing hormone/choriogonadotropin receptor; *mapk*, mitogen-activated protein kinase; *mis*, Mullerian inhibiting substance; *msl1b*, male-specific lethal 1 homolog b; NGS, next generation sequencing; *pcna*, proliferating cell nuclear antigen; *plzf*, promyelocytic leukemia zinc finger; *prlhr2a*, prolactin releasing hormone receptor 2a; PPA, protein phosphatase 2A; *pou5f1*, POU class 5 homeobox 1; qRT-PCR, quantitative real time reverse transcription PCR; RA, retinoic acid; RAR-RXR, retinoic acid receptor/retinoid x receptor heterodimers; *rspo1*, R-spondin 1; sdY, *sexually dimorphic on the Y chromosome*; *smad3b*, SMAD family member 3b; *sox9a*, SRY-box 9a; *SRY*, sex-determining region Y; SSR, simple sequence repeat; *StAR*, steriodogenic acute regulatory protein; *sycp1*, synaptonemal complex protein 1; *sycp3*, synaptonemal complex protein 3; *tac3a*, tachykinin 3a; *tcf3a*, transcription factor 3a; *TGF-β*, transforming growth factor-beta; *wnt*, wingless-type MMTV integration site family; *wt1*, Wilms tumor 1; *zar1*, zygote arrest 1; *zp2*, zona pellucida sperm-binding protein 2

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factors related to gonadal differentiation was observed, and expression analysis of *nanos*, *ad4bp/sf-1,* and *gdf9* was performed. The present study identified certain important genes/factors involved in the gonadal development of *C. carpio* which may provide insights into the understanding of sex-differentiation and gonadal development processes.

1. Introduction

The common carp, *Cyprinus carpio* belonging to family Cyprinidae is native to western Asia and was translocated or introduced throughout the world. Certain native wild populations were considered vulnerable to extinction in 2008 by the International Union for Conservation of Nature. The common carp has become one of the most important food fishes with many strains and varieties in different parts of the world (Xu et al., 2012). There are abundant strains and local populations of common carp found in India, including many hybrid populations. The Indian common carp spawn in two peak breeding periods, from January to March and from July to August. Furthermore, at different stages, the gonads show discrete changes during the reproductive cycle. Due to its economic and ecological importance, different genetic and genomic studies were performed in the common carp (Ji et al., 2012; Kongchum et al., 2010). In spite of the reports being available on different aspects of reproduction and breeding (Routray et al., 2007), the molecular mechanisms underlying gonadal maturation and breeding of carp in tropical or subtropical climates has not been clearly understood. In comparison with the bisexual reproduction in higher vertebrates, teleosts have different approaches for sexual reproduction, like unisexuality (Schartl et al., 1995), hermaphroditism (Warner, 1984) and gonochorism (Dipper and Pullin, 1979). In addition, there are two mechanisms of sex-determination, genetic and environmentally regulated (Janzen, 1995). Teleost fishes are an excellent model for studying the evolution of sex chromosomes as they have a broad range of sexual developmental systems, with an absence of morphologically distinct genders in most species. A master set of sex-determination and gonadal phenotype-related genes have been identified in multiple fish species, including, *amhy* in *Odontesthes hatcheri*, *amhr2* in *Takifugu rubripes*, *dmrt1* in *Cynoglossus semilaevis* and *Clarias gariepinus, DMY* and *dmrt1* in *Oryzias latipes*, *gsdf* in *O. luzonensis*, and *sdY* in *Oncorhynchus mykiss* (Chen et al., 2014; Hattori et al., 2012; Kamiya et al., 2012; Matsuda et al., 2002; Myosho et al., 2012; Nanda et al., 2002; Raghuveer and Senthilkumaran, 2009; Yano et al., 2012). Several conserved genes playing critical roles in sexual development of mammals were also analyzed in fish, including *ad4bp/sf-1*, *dax1*, *foxl2*, *gata4*, *mis*, *sox3*, *sox9a*, *sox9b*, *StAR, wt1, cyp11b1*, *cyp17*, *cyp19a1*, *hsd3b*, and *hsd11b* (Raghuveer et al., 2011; Sandra et al., 2010). These genes act together in a complex network to direct gonadal development. In addition, hormones and various biological pathways, such as estrogen signaling pathway, steroid hormone biosynthesis (Baroiller et al., 1999), *TGF-β* signaling pathway (Liu et al., 2017), and *wnt* signaling pathway (Amberg et al., 2013; Prathibha and Senthilkumaran, 2017) also play a vital role in the gonadal differentiation. Although much is known about the process of sex-differentiation in fish, the precise mechanisms of sex-differentiation as well as those involved in primary sex-determination remain undefined in several teleosts (Devlin and Nagahama, 2002). Fish gonadal sex-differentiation shows varied strategies and physiological regulations compared to mammals (Baron and Guiguen, 2003). The differentiation of bi-potential gonad to either testis or ovary depends on the regulation of steroidogenic pathway. In lower vertebrates, sex-steroids (androgens and estrogens) play a critical role in gonadal differentiation (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). However, the mechanism of action of these steroids on gonad sex-differentiation is not well understood. Unlike sex-determining systems, the genes involved in gonadal differentiation appear to be relatively conserved. Nevertheless, studies on the functional connections of these genes to relevant pathways are minimal in fish. Novel sex-related genes are required to elucidate the complex mechanisms of gonadal development in fishes. Over the last decade, NGS technologies have provided operative tools for high throughput sequencing, which has enhanced the efficacy and speed of gene discovery. Compared to whole genome sequencing, NGS via RNA-Seq affords a cost effective approach to retrieve transcriptome sequences and molecular markers that can reveal DEG associated with phenotypic traits and gender. DEG between testis and ovary gives a prospective information to recognize and understand the molecular basis of gonadal development and growth. Groups of gonadal development-related genes have been revealed by scrutiny of sex-biased transcriptomes in several species including, gilthead sea bream (Calduch-Giner et al., 2013), the Nile tilapia (Tao et al., 2013), the Japanese flounder (Zhang et al., 2016), the Russian sturgeon (Hagihara et al., 2014), Southern bluefin tuna (Bar et al., 2016), and yellow catfish (Lu et al., 2014). The transcriptome of common carp (Ji et al., 2012) has been sequenced and assembled employing NGS platforms, and differences between the ovary and testis transcriptomes of adult common carp from the China's yellow river have been explored using suppression subtractive hybridization (Chen et al., 2015). However, ideally such differences will be fully explored only by using a comprehensive set of transcriptomes from every tissue through each life stage across every major habitat (Ji et al., 2012). Furthermore, complete annotation and functional validation of the common carp genome or transcriptome have not been done with reference to gonadal stage and function. Hence, in this study, gonadal transcriptomes of juvenile common carp were sequenced to obtain a critical overview of genes potentially involved in gonadal growth and development. In addition, DEG and biological pathways were also identified by comparing testis and ovary transcriptomes. These data provide information on marker genes found expressed in gonads sex-wise at specific reproductive stages, genes related to steroid synthesis, endocrine regulation, germ cell maintenance and others factors, which are crucial for acquiring valuable insights into mechanisms of gonadal differentiation in fish. The present study also identified a major group of reproduction-related genes, potential players in gonadal development and characterized SSRs to be employed for genetic improvement purposes. The large number of reproduction-related genes identified in this study also provide direction for future genetic and functional genomic research in common carp.

2. Materials and methods

2.1. Animals and sampling

Common carp used for this study were reared at the aquaculture facility of Karnataka Veterinary Animal and Fisheries Sciences University, Bangalore, India. Nearly 2,000 offsprings were raised in a 2000 m^2 pond and fed four times daily under standard feeding regime (3% feed/bodyweight ratio daily). The oxygen level was retained at 3 mg/L or above. Animals were procured and during acclimatization, fishes were maintained under natural photo-thermal conditions by feeding commercial carp food *ad libitum* and kept in 1 m^3 tanks with filtered tap water, until experimentation. Fifteen juvenile carps, 110 to 130 dph were randomly selected and gonadal tissues were dissected out. A portion of the gonadal tissue was fixed in Bouin's fixative (15:5:1, saturated picric acid, formaldehyde and glacial acetic acid) for histological observation, and the remaining tissue was snap frozen in liquid nitrogen and stored at −80 °C for RNA-Seq. To perform qRT-PCR various tissues from adult female (brain, kidney, liver, muscle, and

ovary) and adult male (brain, kidney, liver, muscle, and testis) fishes were collected, snap frozen in liquid nitrogen and stored at −80 °C. Whenever required gonadal tissues (n = 3) were pooled to obtain one biological sample and necessary experimental sets were chosen according to the analysis. Fish sampling was performed following the general procedures and with the endorsement of the Institutional Animal Ethics Committee, University of Hyderabad (CPCSEA, Inst. Reg# No#151/1999 dt.22.07.1999).

2.2. Histology

The male and female gonads of juvenile common carp, 110 to 130 dph were fixed in Bouin's solution, dehydrated and embedded in paraplast (Sigma). Sections of 5 µm were cut using a microtome (Leica, Wetzler, Germany), stained with hematoxylin-eosin and photomicrographs were taken using Olympus CX41 bright field light microscope (Olympus, Tokyo, Japan).

2.3. Total RNA isolation and cDNA library construction

The gonads of the juvenile common carp were staged by histology. Total RNA was prepared from immature gonad tissue (testis and ovary) samples of carp individually using TRI reagent (Merck, USA; Cat No. T9424). The concentration and integrity of RNA were examined with Ribogreen method using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA with RIN values > 7.0 were selected. Equal amounts of the high quality RNA from gonads were then pooled together for mRNA isolation, cDNA synthesis, and sequencing. Library preparation was performed following NEB Next Ultra RNA Library Prep Kit (New England Biolabs, USA; Cat No. E7530S) following the manufacturer's instructions. In brief, one µg of total RNA was used to isolate mRNA using NEB Next Poly (A) mRNA Magnetic Isolation Module which was time-dependently fragmented using RNA binding buffer provided in the kit. Then first strand cDNA was prepared using ProtoScript II Reverse Transcriptase, following which the second strand cDNA was synthesized, purified using AMPure XP Beads, end-repaired using NEBNext End Prep Enzyme Mix, and NEBNext Adaptor ligated using Blunt/TA Ligase. These adapter-ligated fragments were subjected to 12 cycles of enrichment and barcoding and the products were purified using Ampure XP beads.

2.4. Sequencing and assembly

Sequencing was done using Illumina Next Seq 500 Sequencer with 75 PE chemistry. The paired-end reads obtained after massive parallel sequencing were subjected to several filters to obtain the potential reads that are specific to identify the transcripts. First, a quality check using FASTQC was performed. The low quality reads (mean Phred_score < 20) were removed and the reads with adapter sequences were trimmed. If the length of the trimmed read was below 50 bp, then such reads were discarded. All processed reads were then assembled into transcripts without any reference (*de novo*) using Trinity software (trinityrnaseq20140413p1).

2.5. Functional annotation and ontology

The homology search was done for assembled transcripts' sequences against *T. rubripes*, *Danio rerio*, and (partially annotated) *C. carpio* protein sequences downloaded from UniProt database (http://www.uniprot.org/uniprot/). NCBI-blast2.2.29 was used for homology search between the sequences. BLASTX program was used to perform similarity searches. Gene annotation was assigned to the RNA-Seq transcripts based on the top BLAST hit. GO annotation analysis was then performed. The annotation results were categorized into biological process, cellular components, and molecular function. KEGG pathways were assigned to those assembled contigs using the online KAAS (http://www.genome.jp/tools/kaas). COG annotation for transcripts of testis and ovary were based on homology (https://www.ncbi.nlm.nih.gov/COG).

2.6. Identification of DEG

The reads for both gonadal tissues were separately aligned to the unigene sequences of *C. carpio* and read count profiles were generated. DESeq "R" package was used for differential gene expression. The package DESeq provide methods for testing for differential expression by use of the negative binomial distribution and a shrinkage estimator for the distribution's variance. The threshold for the P value was < = 0.05. DEG between male or female gonads were identified.

2.7. SSRs detection

SSRs were identified by mapping all clean reads to the assembled transcript reference using MIcroSAtellite identification tool (MISA, version 1.0, http://pgrc.ipkatersleben.de/misa/). The minimum repeat number used for every unit was ten for mononucleotide, six for dinucleotide, five for tri-, tetra-, penta- and hexa-nucleotide microsatellites.

2.8. Experimental validation by qRT-PCR

Few candidate DEG were validated by qRT-PCR to verify the expression profile obtained from the transcriptome data. Tissue distribution pattern of different transcripts was analyzed using SYBR Green detection method. For this, total RNA was extracted from different tissues (brain, kidney, liver, muscle, ovary, and testis) of adult common carp using TRI (Sigma) method. qRT-PCR was performed by following

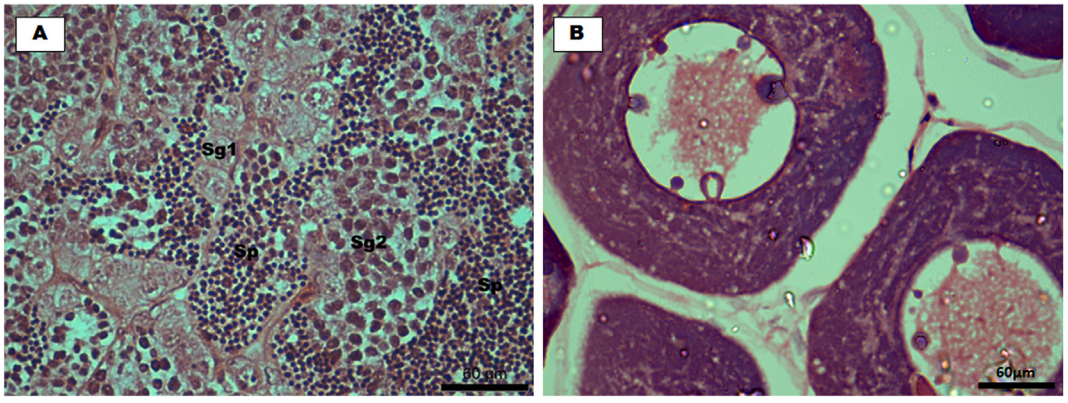

Fig. 1. Histology of gonads A-Immature testis showing Sg1: Primary spermatogonia, Sg2: Secondary spermatogonia, Sp: Sperms/spermatids; B-Immature ovary at perinucleolar-oil droplet stage (Scale: 60 µm).

the method described in Rajakumar and Senthilkumaran (2014). All reactions were performed in triplicate for 3 different samples using specific primers (Table S1). qRT-PCR amplification was carried using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, USA; Cat No. 4367659) in an ABI Step One Plus real time PCR system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The expression was normalized against 18SrRNA expression, used as an endogenous control, generating a ΔCt value (ΔCt = Target Ct −18SrRNA Ct). Relative expression was then calculated according to equation $2^{-\Delta Ct}$.

3. Results

3.1. Histological observation

The maturation level of gonads were histologically determined. Upon observation the differentiating (110 to 130 dph) testis (Fig. 1A) showed presence of primary spermatogonia, secondary spermatogonia and spermatids/sperms indicating the progression from meiotic to spermiogenic phase of spermatogenesis. The juvenile ovary (110 to 130 dph) showed several nucleoli appearing at the periphery of nucleus indicating the perinucleolar stage (Fig. 1B) of primary oocyte growth.

3.2. Sequencing and reads assembly

A total of 17.06 and 14.87 million paired-end reads with 94.1% and 93.6% high quality bases (Phredscore > 20) were generated from high throughput sequencing of the immature testis and ovary cDNA libraries, respectively. The transcriptome sequences generated from this study have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE112157 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE112157). Nearly, 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, and N50 of 1364 and 1453 bp, were obtained after *de novo* assembly for testis and ovary, respectively (Table 1). From these, 84,367 unigenes were constructed after removing redundancy which was a representation of all testicular and ovarian transcriptomes. The total size of the transcriptome was 62.9 and 37 Mbp for testis and ovary, respectively. All transcripts longer than 201 bp were selected and nearly 18,472 and 12,574 transcripts longer than 1000 bp of testis and ovary, respectively were obtained. About, 62,231 unigenes (50%) showed significant hits compared to known proteins. Of these, only 39,171 unigenes could be fully annotated with the available GO data.

3.3. Unigene annotation

The unigenes with counterparts in public protein databases were annotated with GO, which offers a dynamically organized vocabulary and classified relationships to symbolise information regarding the categories of Biological process, Cellular component and Molecular function. Analysis of GO term distribution showed that 'regulation of transcription, DNA templated [GO: 0006355]', 'integral component of membrane [GO: 0016021]', and 'ATP binding [GO: 0005524]' were the most common annotation terms within the three GO categories, respectively. For Biological process, 'regulation of transcription' (1745 unigenes) and 'transcription' (1668 unigenes) were the predominant groups. Under Cellular component, 'integral component of membrane' (12,405 unigenes) represented the most abundant classification followed by 'nucleus' (6951 unigenes). Under Molecular function, 'ATP binding' (6821 unigenes) and 'zinc ion binding' (4686 unigenes) represented nearly half of the category (Fig. 2). To conduct further functional prediction and classification, all unigenes were compared with proteins from the COG database. In total, 6651 unigenes were classified into 26 categories (Fig. 3). The top three classifications are general function prediction only (1089 unigenes), signal transduction mechanisms (813 unigenes); and translation, ribosomal structure, and biogenesis (760 unigenes). Following the most abundant three groups were posttranslational modification, protein turnover, chaperones (674 unigenes) and amino acid transport and metabolism (Table S2).

KAAS was used to perform pathway analysis for functional categorization. Enzyme Commission (EC) numbers were assigned (Table S3) with 3012 enzyme codes for 4782 unigenes. Pathways were categorized into 6 main groups (Table 2), metabolism (1440 unigenes), organismal systems (175 unigenes), environmental information processing (917 unigenes), genetic information processing (1511 unigenes), cellular processes (697 unigenes) and human diseases (42 unigenes). The majority of the mapped unigenes represented (Fig. 4) signal transduction (13.1%, 627 unigenes), followed by folding, sorting and degradation (11.7%, 563 unigenes) and translation (11.2%, 538 unigenes). KAAS annotation identified 96 genes related to reproduction, distributed in 7 pathways (Table 3). These genes covered the major processes of reproduction, including *wnt* signaling pathway, GnRH signaling pathway, steroid biosynthesis, oocyte meiosis, steroid hormone biosynthesis, neuroactive ligand-receptor and insulin signaling pathway (Table S3).

3.4. Identification of SSRs

A total of 18,342 and 8693 SSRs (Table S4) were finally obtained from testis and ovary, respectively. Within SSRs in the testis, the most abundant type of repeat motif was mono (10,938), di-nucleotide repeats (3748), followed by tri (2127), quadra- (1 9 4), penta- (6), and *hexa*-nucleotide (4) repeat units (Fig. 5). Within SSRs in the ovary, the most abundant types of repeat motif were mono (4805) and di-nucleotide repeats (1919), followed by tri- (1298), quadra- (87), penta- (13), and hexa- nucleotide (1) repeat units ((Fig. S1A)). The most abundant repeat motif in SSRs (Fig. S1B) was A/T (18,115), followed by AC/GT (3792), AG/CT (1603), AT/AT (1281) and ATC/ATG (7 8 7).

3.5. DEG

Differential expression between testis and ovary were analysed to identify the genes involved in sex-differentiation. Based on the applied criteria (twofold or greater change and p < = 0.05), there were 171 unigenes showing higher expression in testis than in ovary, and 127 unigenes showed higher expression in ovary than in testis. There was no significant difference in the expression of most unigenes between testis and ovary. In order to visualize comprehensive similarities and differences in gene expression between testis and ovary, a scatterplot was generated using R package and clear differences in gene expression between testis and ovary could be observed (Fig. S2). The DEG report has been uploaded in NCBI's GEO (GEO Series accession number GSE112157).

Table 1
Summary of *C. carpio* gonadal transcriptome.

Transcriptome Assembly	Testis	Ovary
Transcripts generated	43,257	81,757
Maximum transcript length	14,732 bp	14,333 bp
Minimum transcript length	201 bp	201 bp
Average transcript length	856 bp	769 bp
Total transcripts length	37 Mbp	62 Mbp
Transcripts > 200 bp	43,257	81,757
Transcripts > 500 bp	21,462	34,010
Transcripts > 1000 bp	12,574	18,472
Transcripts > 10 Kbp	2	3
N50 value	1453 bp	1364 bp
Number of reads used	1,38,84,178	1,01,99,309
Total number of reads	1,62,67,344	1,39,16,372
Percentage of reads used	85.35	73.29

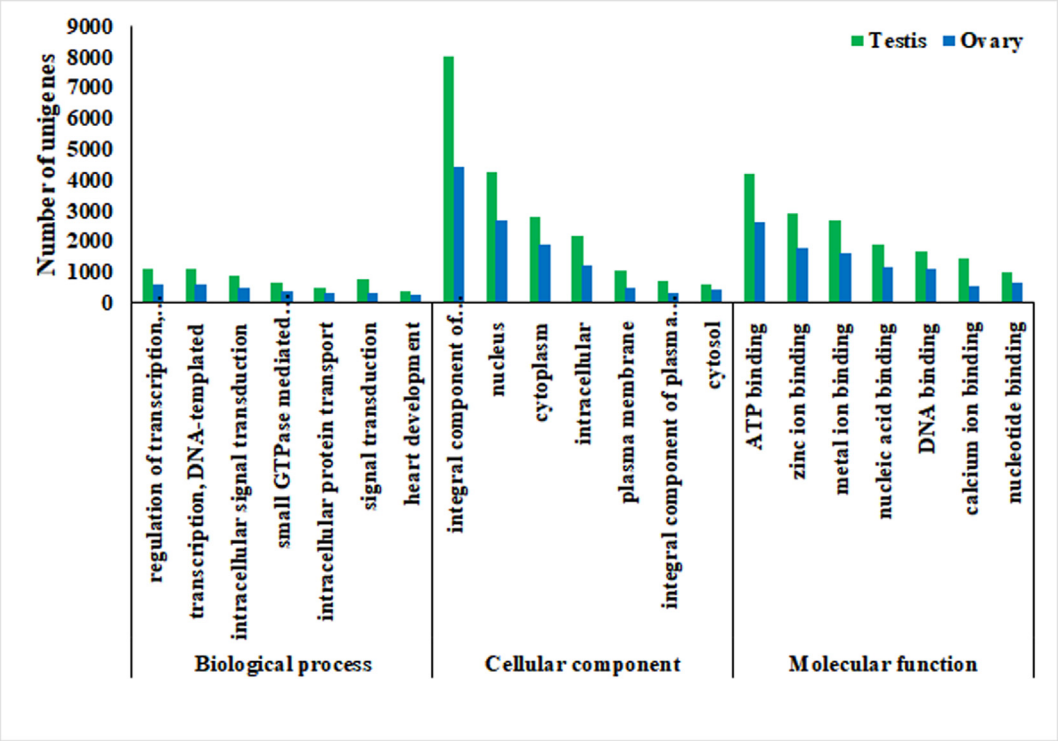

Fig. 2. GO analysis of the *C. carpio* testicular and ovarian transcriptomes of immature carp. The distribution of GO terms portrayed in the three categories: Biological process, cellular component and molecular function.

3.6. DEG validation in gonads

Twelve DEG such as *amh*, *egr2b*, *dax1*, *dmrt1*, *fbox43*, *foxk2*, *fzd8*, *gata6*, *mapk*, *sox9a*, *wt1*, and *zp2* related to sex-differentiation, were chosen to verify the changes in their expression levels in gonads. Expression of *amh*, *dax1*, *dmrt1*, *gata6*, *sox9a*, and *wt1* was higher in testis, whereas *egr2b*, *fbox43*, *foxk2*, *fzd8*, *mapk*, and *zp2* were found to be elevated in ovary, (Fig. 6). These results reiterate the differential gene expression pattern observed in gonadal transcriptome analysis.

3.7. Identification of reproduction- related genes

Nearly, 809 genes related to reproduction were identified by literature supported searching (Table S5). The gonadal transcriptome data was used to profile the expression of several reproduction-related genes. Sex-steroid synthesis related genes such as *StAR*, *hsd3b7*, *hsd17b*, *hsd20b2*, *cyp19a*, and *er* showed higher expression levels in ovary than in testis. The exceptions are *hsd11b*, and *ar* which showed higher expression in testis (Fig. 7A). Genes involved in various gonadotropin pathway receptors such as *lhcgr*, *fgfr1a2*, *tac3a*, *notch2* and *fshr*, *prlhr2a*, *kiss2* were moderately over-expressed in the testis and ovary,

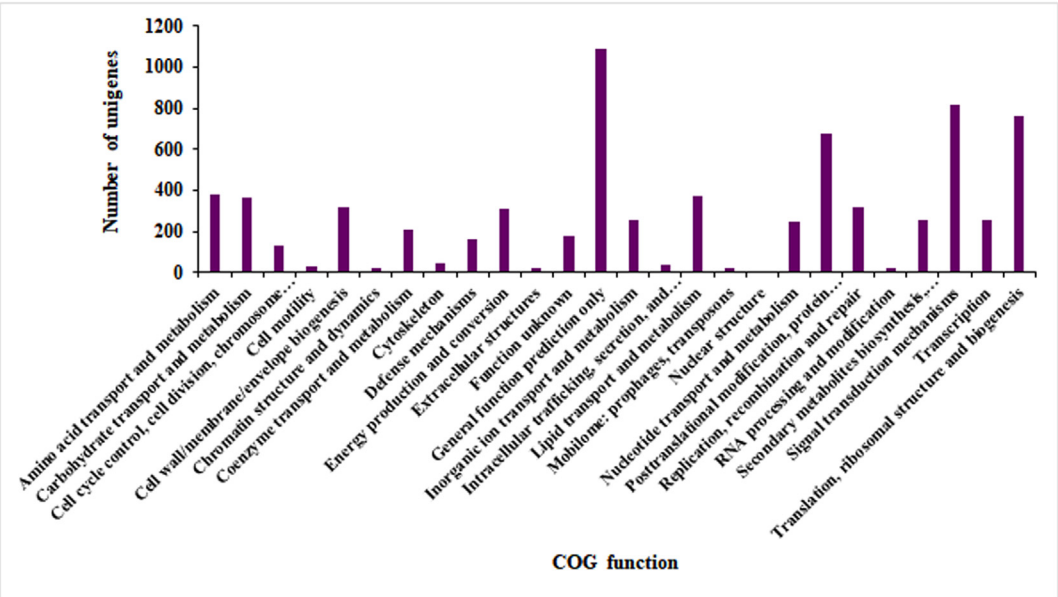

Fig. 3. COG classification of *C. carpio* testicular and ovarian transcriptomes of immature carp.

Table 2
KAAS mapping for gonadal transcriptome of *C. carpio*.

KAAS categories represented	Number of KO	Unique sequences
Metabolism		
● Amino acid metabolism	104	140
● Biosynthesis of other secondary metabolites	1	1
● Carbohydrate metabolism	126	183
● Energy metabolism	105	170
● Glycan biosynthesis and metabolism	128	174
● Lipid metabolism	145	217
● Metabolism	119	180
● Metabolism of cofactors and vitamins	76	109
● Metabolism of other amino acids	16	47
● Metabolism of terpenoids and polyketides	18	25
● Nucleotide metabolism	123	188
● Xenobiotics biodegradation and metabolism	5	6
Cellular Processes		
● Cell growth and death	93	142
● Cell motility	37	60
● Cellular community	44	84
● Transport and catabolism	226	411
Organismal Systems		
● Circulatory system	22	30
● Development	5	16
● Endocrine system	40	67
● Immune system	42	56
● Sensory system	5	6
Human Diseases		
● Endocrine and metabolic diseases	4	7
● Infectious diseases	19	35
Genetic Information Processing		
● Folding, sorting and degradation	327	563
● Replication and repair	120	149
● Transcription	143	261
● Translation	339	538
Environmental Information Processing		
● Membrane transport	19	22
● Signal transduction	371	627
● Signaling molecules and interaction	190	268
Total	**3012**	**4782**

Table 3
Reproduction-related pathways identified in common carp gonadal transcriptome.

Description	Number of genes in the pathway
Wnt signaling pathway	47
Steroid biosynthesis	14
Oocyte meiosis	12
Insulin signaling pathway	10
Steroid hormone biosynthesis	8
GnRH signaling pathway	3
Neuroactive ligand-receptor interaction	2

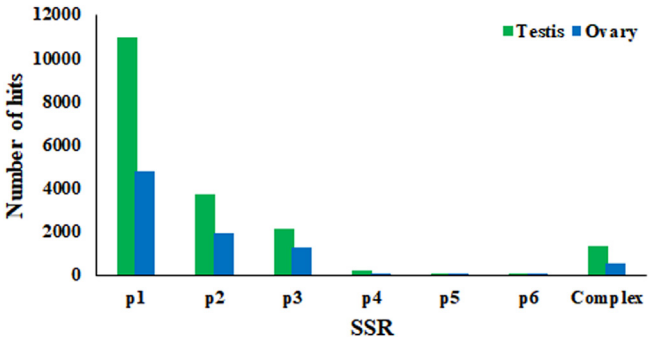

Fig. 5. Distribution of SSRs identified from common carp testis and ovary transcriptomes of immature carp.

respectively (Fig. 7B). Some of the genes involved in germ cell maintenance were highly expressed in ovary, namely *vasa*, *pou5f1*, *ipo4*, *pcna*, and *notch1* and other germ cell related genes like *sycp3*, *piwi*, *plzf*, *cxcr4*, and *cxcl12* showed higher expression in testis (Fig. 7C). Another set of genes that were found to be over-expressed in testicular tissue included various factors related to gonadal differentiation such as *igf1ra*, *wt1*, *rspo1*, *ctnnbip1*, *tcf3a*, *msl1b*, and *atm* while *fem1b* and *smad3b* showed higher expression in ovary (Fig. 7D).

Candidate genes with well-known functions, like *nanos*, germline gene, autosomal *ad4bp/sf-1* and ovary specific *gdf9*, were selected for tissue distribution analysis. Expression of *nanos* was high in brain and kidney followed by testis and ovary (Fig. 8) while *gdf9* was found to be high in ovary followed by brain, testis, kidney, muscle and liver (Fig. 8).

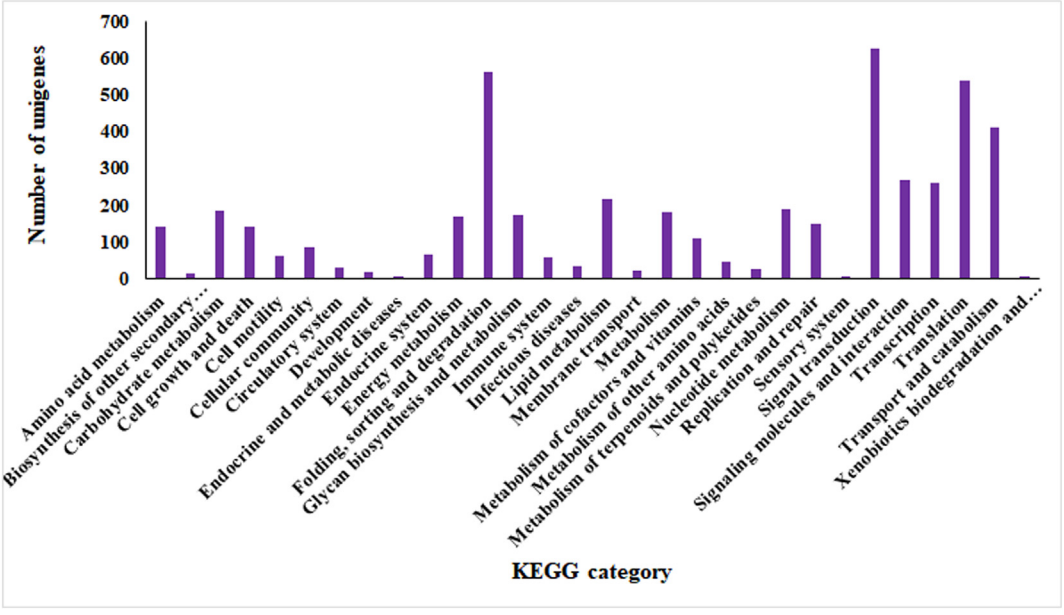

Fig. 4. KEGG categorization of *C. carpio* testicular and ovarian transcriptomes of immature carp.

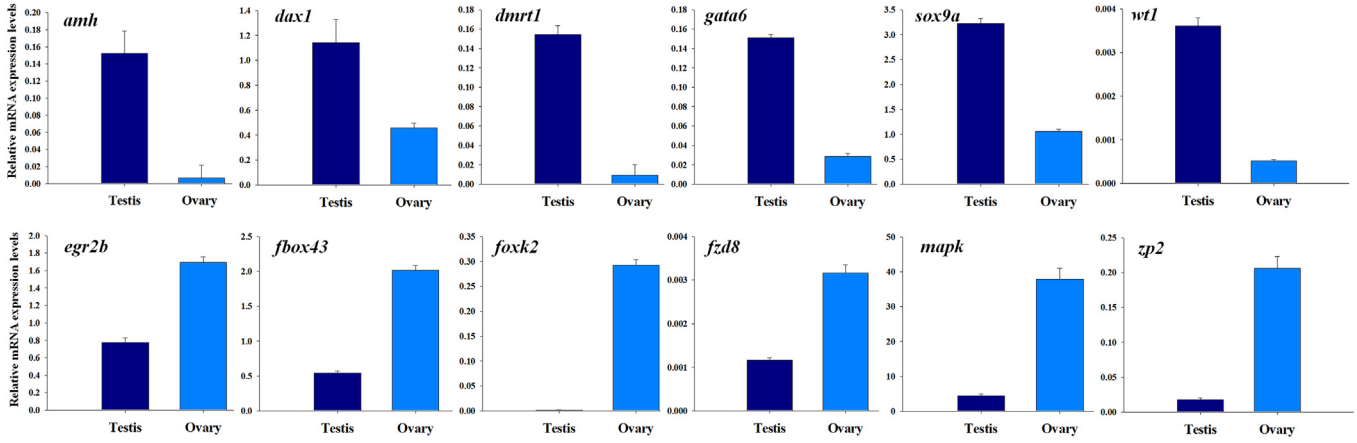

Fig. 6. Validation of immature *C. carpio* gonadal tissue transcriptome results by qRT-PCR using 12 selected DEG in testis and ovary. qRT-PCR fold changes are normalized by changes in 18SrRNA values ($P < 0.05$; ANOVA followed by Student–Newman–Keuls' test). All data from qRT-PCR studies were expressed as mean ± SEM.

Expression of *ad4bp/sf-1* was found to be high in testis, followed by kidney, brain, and ovary based on the qRT-PCR analysis (Fig. 8).

4. Discussion

With advancement of NGS technology, the information on DEG between testis and ovary is growing, and these genetic databases offer libraries facilitating the identification of corresponding genes in other fish species. Though the available gonadal transcriptomic data continues to increase, there is a need for novel sex-related genes to understand the convoluted process of gonadal development in fishes. To gain insight into the molecular fundamentals of gonad development and growth, in this study, the gonadal transcriptome of *C. carpio* was obtained after *de novo* assembly of RNA-Seq data from developing testis and ovary of immature fish. Several new players showing dimorphic gene expression were identified. Candidate genes for sex-differentiation and development, showing differential gene expression, were further tested to validate our findings. Further, the major group of reproduction-related genes identified were similar with previous reports indicating that these genes are functional in fishes. The data obtained in this study adds to the transcriptomic profile available for the family Cyprinidae.

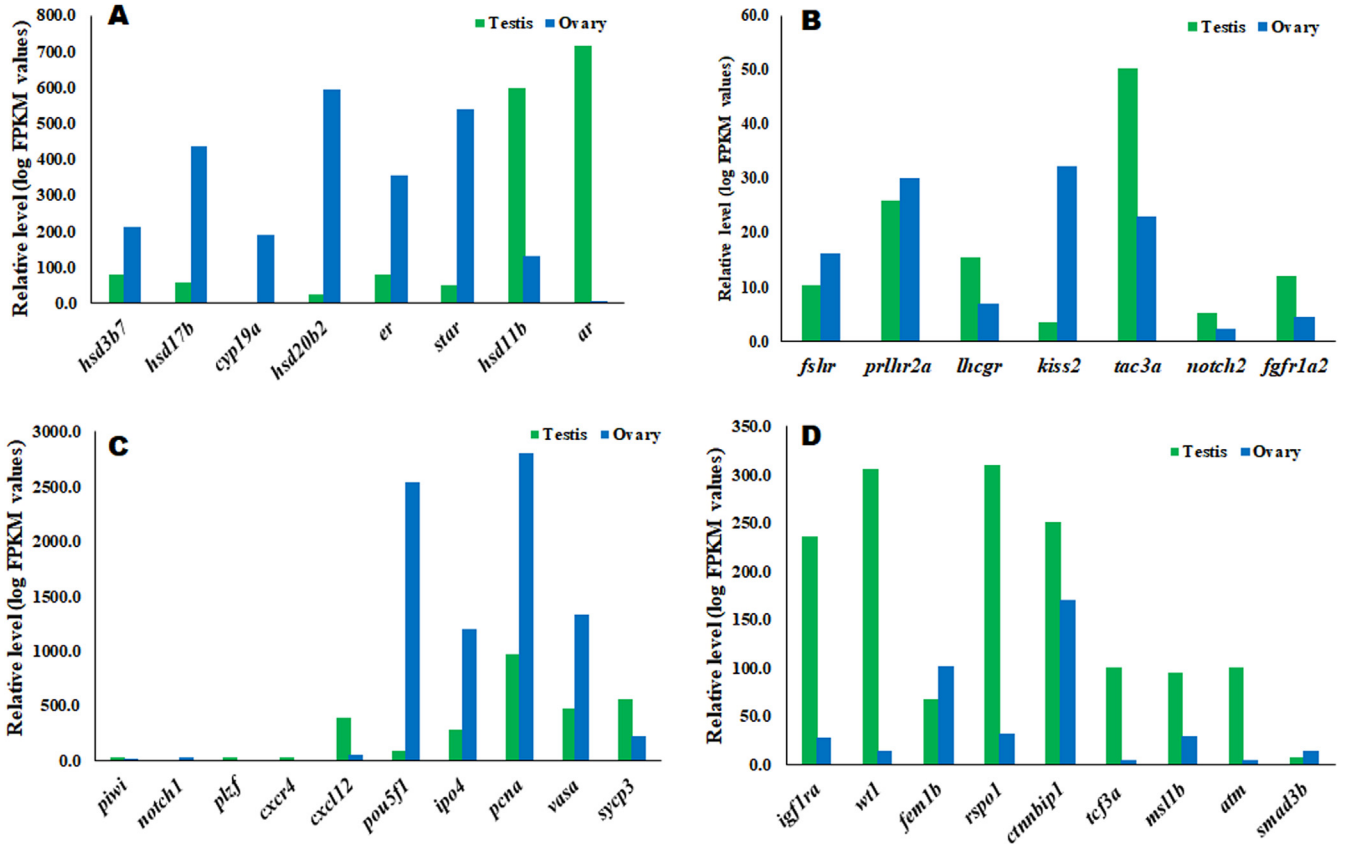

Fig. 7. Relative levels of transcripts of genes potentially involved in gonadal differentiation in juvenile common carp (A-steroid synthesis, B-endocrine regulation, C-germ cell maintenance, D-other factors).

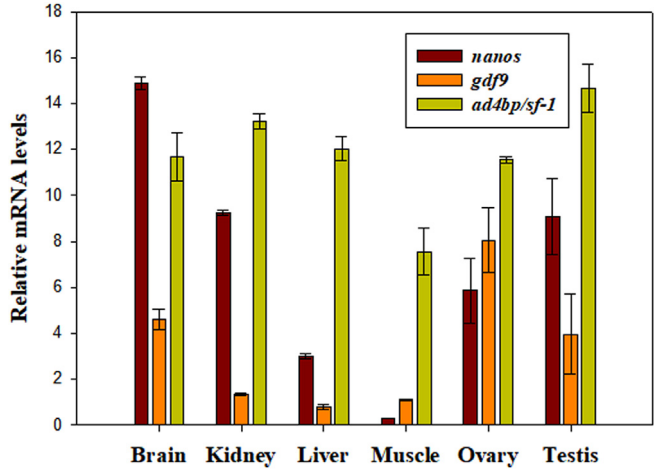

Fig. 8. Tissue distribution analysis of *nanos, gdf9* and *ad4bp/sf-1* in common carp. All other details are same as Fig. 6.

4.1. GO, COG, KEGG

One of the essential features of transcriptome analysis is to associate individual sequences and related expression information with biological functions. The juvenile stage is a transcriptionally active stage as the bipotential gonad differentiates into a specific and functionally active gonad, either testis or ovary. GO, COG and mapped unigenes were majorly involved in translational machinery, indicating that juveniles are at a crucial phase of gonadal development. The KAAS pathway analysis is based on sequence similarities and accommodates bi-directional best hit information, enabling it to attain a high degree of accuracy in comparison to a manually curated KEGG GENES database. Hence, KASS can facilitate research on the relationship between different genes obtained from the transcriptome (Moriya et al., 2007). Transcripts encoding enzymes or other proteins involved in reproductive pathways, for example, *hsd3b*, GnRH, serine/threonine-PP2A, corticosteroid *hsd11b*, sterol 14-demethylase, lanosterol synthase and many more were detected. Pathway analysis mapped some of the genes involved in the *wnt* signaling pathway, oocyte meiosis and steroid hormone biosynthesis, all of which play a pivotal role in sex-differentiation and gonadal development (Baroiller et al., 1999; Prathibha and Senthilkumaran, 2017). Recognizing these pathways permits the analysis of reproduction mechanisms in *C. carpio*, although most of the genes present in the putative KEGG pathways that were identified were not found in the present study.

4.2. SSRs

SSRs or microsatellites are polymorphic regions existing in genomic DNA consisting of 2 to 6 bp repeated core sequences (Queller et al., 1993) and important to perform research on evolution, molecular ecology, quantitative trait loci analysis, genetic diversity, gene mapping, population genetic analysis comparative genomics, and marker-assisted selective breeding. The SSRs identified in the current study, provide a valuable resource for molecular research in common carp.

4.3. DEG

Studies have suggested that the sex-biased genes, primarily or absolutely expressed in one sex, drive the phenotypic differences in males and females (Assis et al., 2012; Ellegren and Parsch 2007) and cause the phenotypic sexual dimorphism in zebrafish (Small et al., 2009). In juveniles, a group of genes are differentially expressed to fulfil the requirement of gonadal development. A total of 298 genes showed significant differences between the testicular and ovarian transcriptomes, including 171 and 127 genes showing higher expression in testis and ovary, respectively. The higher number of testis versus ovary up-regulated genes found in the common carp was in accordance with the result obtained in *Oreochromis niloticus* (Tao et al., 2013). The histological observations depict (Fig. 1A) active stages of spermatogenesis in testis with evidence of the presence of spermatocytes and spermatids in the spermatogenic cysts, and the increase in mass of the testis during this time is largely due to materials synthesized locally coinciding with our transcriptome data showing more number of transcripts and DEG in testis. On the other hand, the ovary (Fig. 1B) was observed to be in late primary oocyte growth or early secondary growth prior to the onset of vitellogenesis. Possibly, at this stage the intense transcriptional activity in the oocyte subsides as it transits into the main phase of growth, which is due to acquisition of materials (such as neutral lipid precursors and yolk proteins) produced in other tissues due to which the number of transcripts and DEG may be lesser in ovary in comparison to testis. Overall, the difference of gene numbers between female and male might imply that male gonadal development may require a larger number of early expressing transcripts compared with females in teleosts, triggering an early testis upregulated gene expression pattern before any female pattern emerges. DEG were investigated exposing a number of genes that might be enriched in sex-related biological pathways. Among DEG, a few candidate genes like *amh*, *dmrt1*, *sox9a*, associated with testicular differentiation (Kamiya et al., 2012; Kobayashi et al., 2008; Nagahama 2005; Raghuveer and Senthilkumaran, 2009; Raghuveer and Senthilkumaran, 2010a), *dax1* and *gata6* involved in gonadogenesis (Liu et al., 2016; Wang et al., 2002), *wt1* regulating SRY (Hossain and Saunders, 2001), *zp2* and *mapk* playing roles in oocyte development (Chang et al., 1997; Ponza et al., 2011), *foxk2* involved in gonadal development (Yuan et al., 2014), *fbox43* having a role in oocyte meiosis (Tung et al., 2005), *fzd8* receptor of *wnt* signaling (von Schalburg et al., 2006) and *egr2b* expressed in murine granulosa cells (Jin et al., 2016) were selected and their differential expression was validated by qRT-PCR to indicate their critical role in gonadal development. The previous study by Chen et al. (2015) also identified differential expression of *wt1* and *zp3* in testis and ovary of adult common carp using suppression subtractive hybridization. Thus, *wt1* and *zp2*, *zp3* show dimorphic expression from juvenile stage and is consistent up to adulthood.

4.4. Identification of new players from DEG

New players like *cux1*, *dmrt2a*, *fam192a*, *fstl3*, *retinoic acid receptor RXR-beta-A*, *stathmin*, *sycp1* and *zar1* also showed dimorphic gene expression pattern. CDP/Cut (CCAAT displacement protein) is a transcription factor involved in the regulation of cell growth and differentiation-related genes (Nepveu, 2001). Vanden Heuvel et al. (1996) reported that *cux1* represents an example of a transcription factor that undergoes testis-specific alternative splicing during spermatogenesis. Previous studies have reported *cux* is required for reproductive functions and its genetic ablation resulted in reduced male fertility (Luong et al., 2002) and impaired lactation (Tufarelli et al., 1998) in mice. Dimorphic expression pattern of *cux2a* was observed, indicating its role in reproduction of fishes as well.

Gene expression of *dmrt1* is associated with the testicular development in many species (Raghuveer and Senthilkumaran, 2009). Differential expression pattern of *dmrt2a* was observed, coinciding with previous report by Peng et al. (2016) in which *dmrt2b* showed dimorphic expression pattern, suggesting these new players' *dmrt2a* and *dmrt2b* might potentially be associated with sex differentiation in common carp.

A recent transcriptomic study on developmental gonads in protandrous black porgy (Zhang et al., 2018) reported up-regulated expression of *fam101b* in ovary coinciding with *fam192a* and *fam210b* showing dimorphic expression in common carp, elucidating their importance in ovarian development.

Follistatin is a FSH-suppressing protein that is produced in the

pituitary and upon binding to *activin*, neutralizes the FSH stimulatory actions of *activin* (Nakamura et al., 1990). Follistatin, that encodes a TGF-β superfamily binding protein, inhibits formation of the XY-specific coelomic vessel in XX gonads, a feature normally associated with testis differentiation (Yao et al., 2004). Follistatin displayed early sexually dimorphic expression profile in rainbow trout (Vizziano et al., 2007). Incidentally, *fstl3* showed dimorphic expression pattern, revealing its crucial role in common carp gonadal differentiation.

RA is an active derivative of vitamin A that diffuses through tissues and binds to heterodimers of the nuclear receptors RAR-RXR, which recognize RA-response elements in DNA to control the expression of RA-target genes (Duester 2008). RA signaling stimulates differentiation of spermatogonial germ cells and induces meiosis in male but not female gonads (Duester 2013). RA receptor RXR-beta-A showed differential gene expression in juvenile carp coinciding with report of Rodríguez-Marí et al. (2013) in zebrafish suggesting that the continuous availability of retinoic acid to germ cells in bipotential gonads prohibits the sexually dimorphic onset of meiosis and is consistent with the initial development of meiotic oocytes in juvenile teleosts. *Stathmin* is a neuron-enriched gene mediating tubulin depolymerization (Sobel et al., 1989). Studies demonstrated that maternal *stathmin* accumulates in oocytes and is redistributed in pre-implantation embryos (Koppel et al., 1999). Chu et al. (2006) observed intense expression of *stathmin* in ovary of tilapia. Dimorphic expression pattern of *stathmin* observed in the present study indicates its functional significance in gonads of common carp.

Sycp3 is a meiosis-specific component of the synaptonemal complex, required for the synapsis of homologous chromosomes, and is a marker of meiosis in mammals (Page et al., 2006). Its importance in gametogenesis and fertility has been studied in a few teleost species such as medaka and zebrafish (Iwai et al., 2006; Ozaki et al., 2011). Laldinsangi and Senthilkumaran (2018) demonstrated that *sycp3* have a potential role in the development and maintenance of testicular function in catfish. Dimorphic expression pattern of *sycp1* revealed its crucial role during gonadal development of common carp as well.

Ovary-specific maternal factor, *zar1* plays an essential role during oocyte-to-embryo transition (Wu et al., 2003). Expression of *zar1* was identified in gonads of carp, coinciding with a recent transcriptome study in tilapia (Tao et al., 2018) that reported correlation of *zar1* with the expression of genes previously known to be involved in sex differentiation. Further investigations including functional analysis of these genes in female and male fishes are needed to substantiate our findings.

4.5. Genes related to reproduction

A large number of genes (8 0 9) related to reproduction have been identified by gonadal transcriptome analysis. Corticosteroids (*hsd3b7*, *hsd17b*, *hsd11b*) and their receptors (*ar* and *er*) play crucial role in the regulation of reproduction by acting as transcription factors in somatic cells of gonads (Milla et al., 2009). Steroid synthesis gene *hsd3b7*, expressed in Leydig cells and responsible for biosynthesis of steroids, mainly progesterone and testosterone, was moderately over-expressed in ovary. In addition, *hsd17b*, that regulate the concentration of biologically active androgens and estrogens and involved in synthesis of estradiol, was also over-expressed in ovary. Other steroid related genes like, the sterol transfer coding gene *StAR*, genes coding for *er* and *cyp19a1* (Barney et al., 2008) which are responsible for estradiol-17β biosynthesis in females, followed similar expression pattern as reported in transcriptome analysis of the Russian sturgeon (Hagihara et al., 2014). On the other hand, *hsd11b*, an enzyme which converts gonad maturation inhibiting cortisol or corticosterone into inactive cortisone or 11-dihydrocorticosterone, respectively and gene coding for *ar* showed higher expression in testis, as that of the expression pattern in the Nile Tilapia gonadal trancriptome analysis (Tao et al., 2013). Key marker genes of germ cells namely, *vasa*, *pou5f1* (Raghuveer and Senthilkumaran, 2010b; Wang et al., 2011) playing role in stem cell maintenance and regulation, and *pcna*, playing crucial role in meiosis (Miura et al., 2002) were over-expressed in the ovary, indicating their germ cell regulation in the female ovary. Other germ cell maintenance genes such as *piwi*, *notch1*, *plzf*, *cxcr4*, *cxcl12*, *ipo4*, and *sycp3* were moderately over-expressed in testis like that of the transcriptome of Southern bluefin tuna (Bar et al., 2016). The expression pattern of other genes/factors related to gonadal differentiation like *hsd20b2, lhcgr*, *fgfr1a2*, *tac3a*, *notch2, fshr*, *prlhr2a*, *kiss2*, *igf1ra*, *wt1*, *rspo1*, *ctnnbip1*, *tcf3a*, *msl1b*, *fem1b*, *smad3b*, and *atm* between testis and ovary were comparable to the transcriptome analysis of previous reports (Bar et al., 2016; Hagihara et al., 2014; Shi et al., 2015; Wang et al., 2017).

4.6. Expression analysis of candidate genes

Few candidate genes including *nanos* (germline), *ad4bp/sf-1* (autosomal) and *gdf9* (ovary specific) were selected for further expression analysis. The germline gene, *nanos*, is a RNA binding protein that plays significant roles in early development and, more precisely, in primordial germ cell development (Tsuda et al., 2003). Tissue distribution analysis revealed high expression of *nanos* in brain and kidney followed by testis and ovary, indicating a plausible role for this gene in various physiological processes including gonadal development. Incidentally, *gdf9*, belonging to the TGF-β superfamily was highly expressed in ovary followed by brain, testis, kidney, muscle, and liver, suggesting that it plays a role in ovarian development and differentiation (McGrath et al., 1995). The orphan nuclear receptor, *ad4bp/sf-1* is an essential factor for mammalian sexual differentiation, endocrine organogenesis and steroidogenesis (Morohashi et al., 1992). In teleosts, *ad4bp/sf-1* is critical for transcriptional regulation of *cyp19a1a* (Hu et al., 2001; Wang et al., 2007; Yoshiura et al., 2003) and other genes encoding steroidogenic enzymes (Nakamoto et al., 2012; Val et al., 2003). The qRT-PCR analysis revealed expression of *ad4bp/sf-1* to be high in testis, followed by kidney, brain, and ovary evidencing the importance of *ad4bp/sf-1* in testicular differentiation. Taken together, present study provides an array of marker genes to understand the molecular basis of sex-differentiation and gonadal development in teleosts.

5. Conclusion

Overall, analysis of common carp gonadal transcriptome using NGS RNA-Seq technology identified diverse regulatory mechanisms/ pathways of sexual development and provided a valuable resource for evaluating differential gene expression patterns. The dimorphic expression of sex-related genes offers additional insights into teleostean gonadal differentiation.

Conflicts of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary data

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