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A role for cytochrome P450 17α-hydroxylase/c17-20 lyase during shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation☆

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ABSTRACT

Cytochrome P450 17α-hydroxylase/c17-20 lyase (P450c17) is regarded as one of the key enzymes involved in the steroidogenic shift that occurs prior to oocyte maturation in teleosts. Role of P450c17 in the shift in steroidogenesis during oocyte maturation is a contentious issue even after identification of a novel type of P450c17 that lacks lyase activity. To understand the role of P450c17 in steroidogenic shift explicitly, a full length cDNA encoding *p450c17* from ovary of air-breathing catfish, *Clarias gariepinus* was cloned. *p450c17* transiently expressed in COS-7 cells converted progesterone to androstenedione through 17α-hydroxyprogesterone and catfish *p450c17* was found to be expressed ubiquitously with relatively higher levels in gonads, brain, kidney and gills. Immunocytochemical analysis revealed the presence of P450C17 in follicular layer of ovarian follicle, interstitial cells and spermatocytes of testis. *p450c17* expression and ratio of lyase to hydroxylase was high in preparatory and pre-spawning phases of ovary and low in spawning phase. Expression of *p450c17* correlated well with testicular recrudescence with maximum expression in preparatory and spawning phases. Neither protein expression nor lyase/hydroxylase activity changed significantly during hCG-induced oocyte maturation, *in vitro* and *in vivo* though mRNA levels increased. These results tend to suggest that the ovarian follicles attains capacity to produce maximum precursor steroid levels before spawning that might contribute to the shift in steroidogenesis.

1. Introduction

Cytochrome P45017α-hydroxylase/c17-20 lyase (P450c17) is a microsomal enzyme that catalyzes two distinct activities; the 17α-hydroxylase activity converts pregnenolone or progesterone (Prog) to 17α-hydroxypregnenolone or 17α-hydroxyprogesterone (17α-OHP) and c17-20 lyase activity breaks the c17-20 bond of c21 steroids 17α-hydroxypregnenolone or 17α-OHP to produce dehydroepiandrosterone (DHEA) or androstenedione (AD), respectively [1]. Thus, P450c17 controls an important branch point in steroid hormone biosynthesis leading to the production of three classes of main steroid hormones namely glucocorticoids, mineralocorticoids and precursors of sex steroids [2].

P450c17 catalyzes two mixed function oxidase reactions utilizing cytochrome P450 oxidoreductase and microsomal electron transfer system. These reactions require NADPH and molecular oxygen [2]. Lyase activity is regulated by a set of modulators in tissue dependent manner. In mammals, modulators of lyase activity include electron-donating redox partners such as P450 reductase [3]. Cytochrome b5 has been shown to stimulate lyase activity by acting as an allosteric facilitator [4]. Moreover, serine/threonine phosphorylation of P450c17 increases the affinity of enzyme for redox partners [5,6] and is implicated in adrenarche, polycystic ovarian syndrome and associated insulin resistance in humans [5].

cDNA encoding P450c17 has been cloned from several higher vertebrates [2 and references therein] and P450c17 expression is regulated by gonadotropins [7], adrenocoriticotropin [8], growth factor such as TGF and IGF-I [9,10], activin, inhibin [11] and anti-Müllerian hormone [12]. On the other hand, hormonal regulation of P450c17 in lower vertebrates including teleosts remains to be clarified. Recent report in frog implicated a role for P450c17 in sex differentiation [13].

During oocyte maturation, a shift in steroidogenesis from estradiol-17β to 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) is demonstrated for several teleost species [14,15] and reports do exist on high expression of 20β-hydroxysteroid dehydrogenase [16,17], enzyme that produces 17α,20β-DP. However, it is possible that *p450c17* might play a crucial role in shift in steroidogenesis by controlling the availability of precursor steroids. Though there are reports available on the cloning and expression of *p450c17* from different fish species [18–22], none of these reports neither recorded

☆ *Note*: As per nomenclature, teleost P450c17 cDNA was represented as *p450c17* (lower case, italics) and protein as P450C17 (upper case, no italics). In general it is referred as P450c17 throughout the text. Catfish *p450c17* nucleotide sequence has been submitted to GenBank under the accession no. FJ790422.

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changes in expression pattern and protein level/activity during steroidogenic shift nor provided convincing claim of regulation of lyase activity. But very recently, a novel form of *p450c17* that lacks lyase activity has been identified in tilapia (a fortnight breeder) and medaka (a daily breeder) whose differential expression pattern was proposed to be important during shift in steroidogenesis [23,24]. However, many teleost species that reproduce annually (more importantly fresh water habitats) posses only single form of *p450c17* (*p450c17-I*) and its role during meiotic maturation is a contentious issue. Against this backdrop, present study is designed to delineate contribution of *p450c17* expression and activity in steroidogenic shift. *p450c17* was cloned and characterized from the ovarian follicles of air-breathing catfish, *Clarias gariepinus*. Expression of *p450c17* during hCG-induced oocyte maturation, *in vitro* and *in vivo* was analyzed by quantitative real-time RT-PCR and Western blot methods. In addition, expression in testicular recrudescence, immunolocalization and tissue distribution pattern was analyzed. Results from this study provide interesting information about the role of *p450c17* in steroidogenic shift during oocyte maturation.

2. Materials and methods

2.1. Animals

Adult catfishes weighing about 400–500 g were purchased from local fish market (Hyderabad, India) and acclimated for 2–3 weeks before experimentation by maintaining in aquarium tanks filled with filtered tap water under normal photoperiod and ambient temperature ($26 \pm 2\,^{\circ}C$). Fish were fed with minced goat liver *ad libitum* during acclimation and experimentation. Wild caught fish of both sexes shows annual breeding pattern with four broadly distinguishable phases, namely preparatory, pre-spawning, spawning and post-spawning/regressed were [25]. Spawning phase usually lasts for three months i.e. from June to August and some times extend up to October. The preparatory phase lasts from mid-February to end of May and fish usually caught around late November to January are in resting to early preparatory phase. To correlate *in vitro* versus *in vivo* results, catfishes offer an excellent model system in which the induced-breeding facilitates collection of naturally matured oocytes *in vivo*. Moreover, shift in steroidogenesis in terms of plasma estradiol-17β to 17α,20β-DP is known for catfish species [15,26].

2.2. Molecular cloning of catfish p450c17

A set of degenerate primers was designed by aligning the existing sequences of teleost *p450c17* to clone a cDNA fragment from the ovarian follicles of catfish. Using these degenerate primers, a cDNA of 911 bp was amplified by RT-PCR and cloned in pGEM-T Easy vector (Promega, Madison, WI, USA) and subsequently sequence was determined bi-directionally. The identity of amplified partial cDNA was analyzed by BLAST search. RNA-ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA) was used to clone the 5′ and 3′ end sequences of the catfish *p450c17*. The gene specific primers for 5′ and 3′ RACE are listed in Table 1. Preparation of 5′ and 3′ cDNA (RACE) templates and RT-PCR amplifications were done as per the manufacturer's instructions and different sized PCR amplicons were cloned into pGEM-T Easy vector and subsequently sequence was determined through ABI PRISM sequencer (Applied Biosystems, Foster, CA, USA).

2.3. Genomic Southern analysis

Genomic DNA from the ovarian follicles was prepared using genomic DNA preparation kit (Bangalore Genei, Bangalore, India). Twenty-five micrograms of genomic DNA was digested separately with *Bam*HI, *Hind*III, *Pst*I, *Kpn*I, and *Eco*RI. Restriction digested DNAs were then electrophoresed on 0.8% agarose gel and transferred on to positively charged nylon membrane (Amersham, Buckinghamshire, England) by capillary transfer. After cross-linking, the membrane was hybridized overnight with ^{32}P-labeled 450 bp partial cDNA of *p450c17*. Following high stringency washes, membranes were analyzed using phosphorimager (GE healthcare).

Table 1
Primers used for degenerate PCR, RACE and real-time RT-PCR.

S. no.	Primer	Sequence 5′ to 3′	Purpose
1	*p450c17*-DFI	ASCTGCARMAGAARTAYGG	PCR-cloning
2	*p450c17*-DR1	CACYTCYCTGATRGTGGCYTC	PCR-cloning
3	*p450cl7*-GSPI	GACGCTAAGATTGGGAGGGACAGG	3′ RACE
4	*p450c17*-GSP2	CTGGAAGCCACTATCAGAGGTG	3′ RACE
5	*p450c17*-GSP3	TAGCAGGACCTCCTTTGCATGGTG	5′ RACE
6	*p450c17*-GSP4	AATGTCTCCGTATTTCTTCTGCAG	5′ RACE
7	*p450c17*-RTF	CCATGGCTCCAGCTCTTTCC	Real-time PCR
8	*p450cl7*-RTR	CAGTAAGACCAACATCCTGAGTGC	Real-time PCR
9	*β-Actin*-RTR	ACCGAATGCCATCACAATACCAGT	Real-time PCR
10	*β-Actin*-RTF	GAGCTGCGTGTTGCCCCTGAG	Real-time PCR

2.4. Northern blot analysis

Total RNAs from ovary and testis were prepared using Trireagent (Sigma, St. Louis, MO, USA) and 25 μg of total RNA was separated on a 1% denaturing formaldehyde–agarose gel and transferred onto a positively charged nylon membrane (Amersham). The membrane was hybridized under high stringency conditions with partial cDNA fragment of *p450c17* (450 bp) labeled with ^{32}P-dCTP by random primer labeling kit (PerkinElmer, Boston, MA, USA). After overnight hybridization, the membrane was washed with 2×SSC, 1×SSC, 0.1×SSC containing 0.1% SDS at 60 °C each for 10 min. The signals were detected by exposing on to X-ray film (Kodak) with an intensifying screen for 5–6 days.

2.5. Production of rabbit anti-catfish P450C17 antiserum

To produce catfish P450C17 antigen, catfish P450C17 was over expressed in *E. coli* using pET28a expression system. A 1.365 kb NdeI–XhoI fragment of catfish ovarian *p450c17* cDNA lacking the region encoding the N-terminal 56 amino acids was inserted into the NdeI and XhoI sites of vector pET28a. The expression constructs were verified by restriction analysis and checked for cloning artifacts, if any by nucleotide sequencing. The induction of recombinant protein was verified by 12% SDS-PAGE and confirmed by Western blot analysis with monoclonal anti-His antibody. Recombinant protein was then separated on 12% SDS-PAGE and gels were stained in ice-cold 0.25 mM KCl and 1 mM dithiothreitol. Protein from thin slices of gel pieces containing over expressed P450C17 was recovered and used as an antigen to raise antibody in rabbits as described earlier [27]. Polyclonal antiserum was collected and IgG fraction was purified by affinity chromatography using Protein A-CL agarose column (Bangalore Genei). Further, antibody was characterized by employing dot blot and Western blot methods.

2.6. Functional characterization of catfish p450c17 expressed in mammalian cells

COS-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected using Tfx20 (Promega) with either pcDNA3.1 (Mock) or pcDNA3.1 containing cDNA encoding catfish *p450c17*. After 24 h, fresh medium was added and 50,000 cpm/well of ^{3}H Prog (Amersham) was added. Cells were then incubated at 37 °C and medium was collected after 4 h. Steroids in the medium were extracted twice with 3 ml of diethyl ether,

separated on thin layer chromatography plates using benzene:acetone (4:1, v/v) solvent system and analyzed using phsophorimager. The signals were identified based on the Rf values of standards. Steroid metabolites were extracted and quantified by liquid scintillation counting.

2.7. hCG-induced oocyte maturation, in vitro and in vivo

In vitro oocyte maturation studies were carried out by killing the fish (decapitation) and about 100 oocytes with centrally located germinal vesicles were incubated in triplicate in catfish oocyte incubation medium [28] with 100 IU/ml of hCG (Pubergen, Uni-Sankyo, Hyderabad, India). Controls were treated with saline. Follicles were collected at different time points and used for real-time RT-PCR, Western blotting and enzyme activity measurement. Dead eggs were identified based on their color change and were removed with a sterile pipette. Average death rate under these conditions was about 8–10%. Experiment was repeated three times independently with different batch of catfish.

For *in vivo* oocyte maturation studies, fish were injected intraperitoneally with hCG (1000 IU/kg body mass) and about 250–300 follicles at different time points were collected by gently stripping from ovipore. For controls, same procedure was carried out except injection with physiological saline. Each group consisted of three to five fishes and experiment was repeated thrice in the spawning period (August–October) of two consecutive years. Dosages for both *in vitro* and *in vivo* oocyte maturation were used based on standardization in our laboratory.

2.8. Real-time RT-PCR

Expression of *p450c17* was analyzed by real-time RT-PCR with total RNA isolated from the ovarian follicles collected at different durations of hCG-induced oocyte maturation *in vitro* and *in vivo*. In addition, total RNA was isolated from gonads at different stages of ovarian and testicular cycles. Five micrograms of total RNA was reverse transcribed using random hexamers and MMLV-reverse transcriptase (Invitrogen). The cDNA corresponding to 5 μg of reverse-transcribed RNA served as templates for each of triplicate PCR reactions (25 μl) using power SYBR Green PCR master mix (Applied Biosystems). The PCR amplifications and fluorescence detection were performed with the ABI Prism Sequence Detector 7500 under the manufacturer's universal thermal cycling conditions. *p450c17* and *β-actin* were amplified in separate reactions using the same pool and quantity of cDNA. Transcript abundance of *p450c17* was normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained for spawning phase using the formula $2^{-\Delta\Delta CT}$.

2.9. Western blot analysis

Ovarian follicular homogenate was prepared and 150 μg of protein was separated on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Pall life sciences), blocked with 5% (w/v) skimmed milk and then reacted with anti-cfP450C17 antibody for 1 h at room temperature. After washing, membranes were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Bangalore Genei) secondary antibody. Following washing, blots were developed with BCIP-NBT (Bangalore Genei).

2.10. Enzyme assay

17α-Hydroxylase and c17-20 lyase activities of catfish ovarian P450C17 were assayed as described by Zhang et al. [5]. Ovarian follicular microsomes were prepared by homogenizing 500 mg of tissue in 3 ml of 0.25 M sucrose/5 mM EDTA pH 7.4, clearing debris at 9000 × *g* for 20 min, and centrifuging at 105,000 × *g* for 1 h. The crude microsomal pellet was washed in 0.1 M phosphate buffer pH 7.4/0.1 mM EDTA; microsomes were harvested at 105,000 × *g* for 1 h, resuspended in 200 μl of 0.1 M phosphate buffer pH 7.4/0.1 mM EDTA/20% (v/v) glycerol. P450 activity was measured by incubating 100 μg of microsomal protein and 1 nmol of [^{3}H] Prog in 0.2 ml of 0.1 M phosphate buffer pH 7.4, 1 mM $MgCl_2$, 0.4 mM $NADP^+$, 5 mM glucose-6-phosphate and 0.2 unit of glucose-6-phosphate dehydrogenase at 37 °C for 1 h. Steroids were extracted and analyzed by TLC as mentioned above.

2.11. Immunocytochemistry

Immunocytochemistry was carried out as described by Swapna et al. [25]. In brief, sections were deparaffinized and then blocked with 10% normal goat serum for 1 h at room temperature. The primary antibody was applied to the sections and incubated for 1 h at room temperature in a humid chamber. Following incubation with primary antibody, sections were incubated either with FITC (for immunofluorescence) or horse radish peroxidase labeled secondary antibody (Bangalore Genei), washed with PBS and developed using commercially supplied 3′ 3′ diaminobenzidine for 3–10 min. Images were taken with a Motic light microscope fitted with Motic digital camera. Pre-immune serum/pre-absorbed antibody as well as secondary antibody alone was used as negative controls.

2.12. Data analysis

All the data was expressed as mean ± SEM. Statistical significance among the groups was tested by ANOVA followed by Student Newman–Keuls' test using Sigmastat 3.1 software. Differences among groups were considered at $P < 0.05$.

3. Results

3.1. Molecular cloning of catfish ovarian p450c17

Using a set of degenerate primers, a partial cDNA of 911 bp was isolated by RT-PCR and sequence identity was confirmed by BLAST search. Full-length cDNA of *p450c17* was then obtained through 5′ and 3′ RACE approaches using gene specific primers designed from partial cDNA fragment. The cloned full length cDNA encoding *p450c17* from the ovary of air-breathing catfish was 2042 bp in length with a 431 bp 3′ un-translated region (UTR) and 66 bp 5′ UTR. The 3′ UTR has three polyadenylation signals. The open reading frame was 1542 bp long encoding a putative enzyme of 514 amino acids. The sequence data has been submitted to gene bank. ClustalW multiple alignment demonstrated the presence of signature domains including heme-binding region, Ozol's tridecapeptide and Ono sequence were well conserved in catfish P450c17 and all these signature domains showed high homology to that of *p450c17-I* of other teleosts. In contrast, catfish *p450c17* showed considerable difference in amino acid sequence in Ono sequence and Ozol's tridecapeptide of *p450c17-II* while heme-binding region seems to be more or less similar in both forms of *p450c17* (Fig. 1). Phylogenetic analysis revealed that catfish *p450c17* had about 60–87% homology with other teleostean *p450c17-I*. In contrast, it exhibited only 41–43% homology to that of teleostean *p450c17-II* (Fig. 2).

3.2. Genomic Southern and Northern blot analyses

Southern blot analysis of genomic DNA prepared from ovarian follicles identified single band in all the restriction digests when probed with a partial cDNA fragment that possess heme-binding

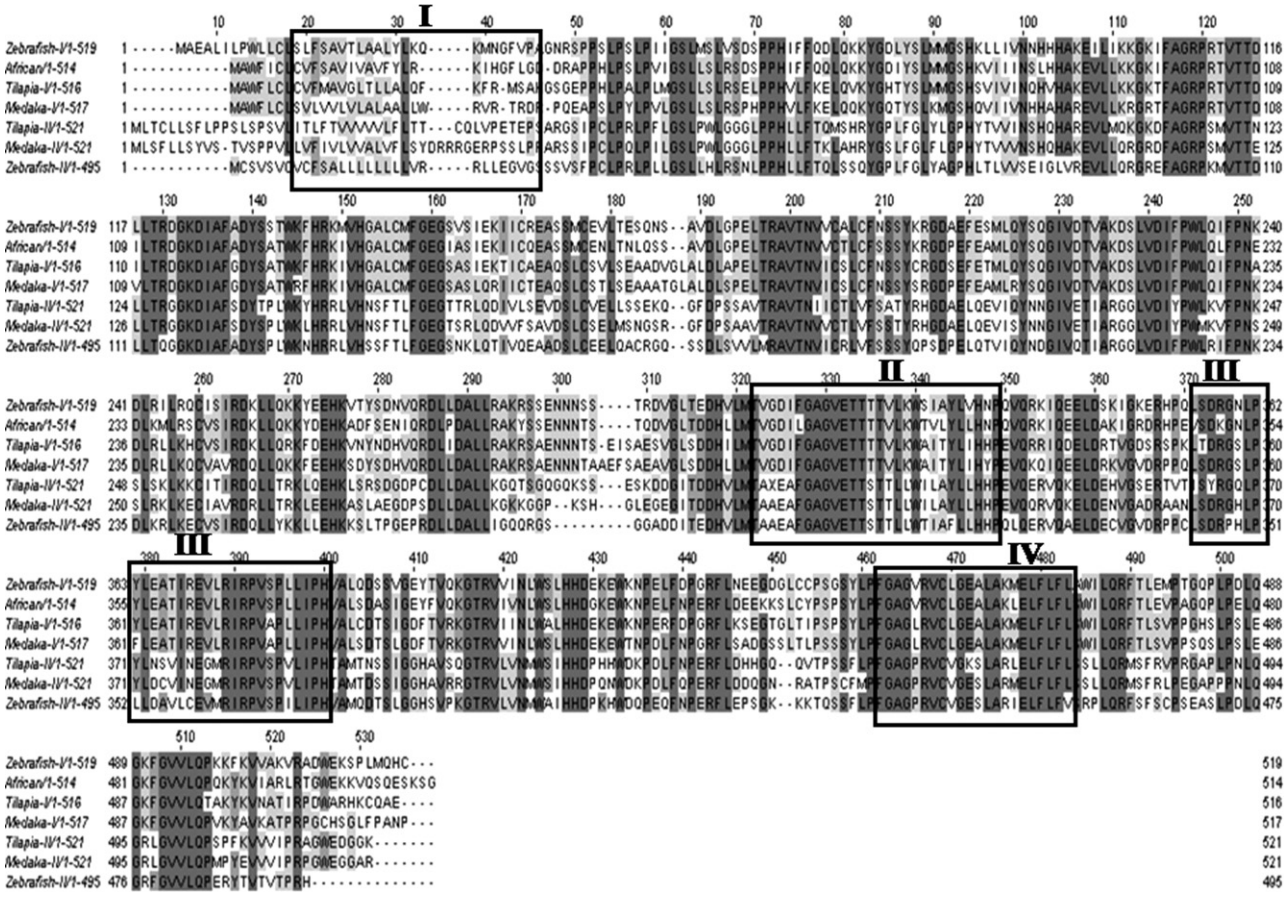

Fig. 1. Alignment of deduced amino acid sequence of catfish *p450c17* with those of *p450c17-I* and *p450c17-II* of other teleosts by ClustalW multiple alignment. Conserved domains are shown in rectangles. I: putative membrane spanning region, II: the P450c17 specific Ono sequence, III: Ozols tridecapeptide regions, IV: the heme-binding region (accession no.: Catfish FJ790422; Tilapia-I AB292401; Tilapia-II EF423917; Zebrafish-I AY281362; Zebrafish-II EF624003; Medaka-I NM_001105094; Medaka-II EF429318).

region (Fig. 3A). Northern blot analysis identified a single transcript of about ~2.1 kb both in testis and ovary (Fig. 3B).

3.3. Functional characterization in COS-7 cells

A cDNA corresponding to the open reading frame of catfish *p450c17* was obtained by PCR and cloned in to mammalian expression vector pcDNA3.1. COS-7 cells transfected with *p450c17* expression construct was able to convert Prog to AD through the intermediate 17α-OHP demonstrating that the cDNA product indeed possess both hydroxylase and lyase activities (Fig. 4). Similar results were obtained with HEK293 cells after *p450c17* expression (data not shown).

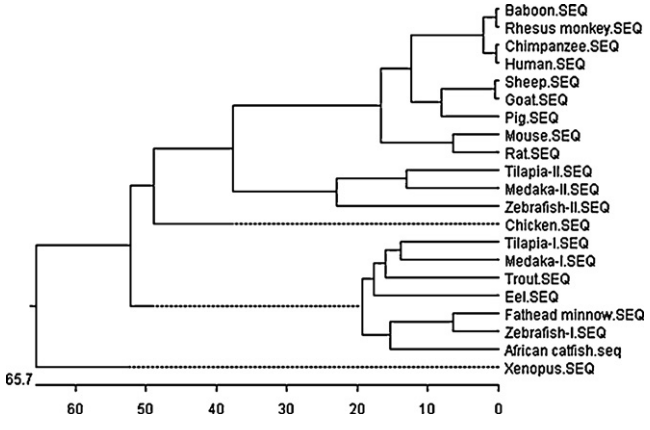

Fig. 2. Phylogenetic tree showing the evolutionary relationship of catfish *p450c17*. Residue substitution was shown in *x*-axis (please refer Fig. 1 for accession numbers of teleost *p450c17*s. Accession no.: Trout NM_001124747; Eel AY489619; Rhesus monkey NM_001040232; Pig NM_214428; Goat AF251387; Chimpanzee NM_001009052; Baboon AF297650; Sheep AF251388; Chicken M21406; Human M14564; Mouse NM_007809; Rat M31861; Frog AF325435).

3.4. Tissue distribution and immunocytochemical localization in gonads

Using RT-PCR analysis, *p450c17* expression was detected in several tissues other than gonads including brain, gill, liver, intestine, kidney, heart and muscle. The expression was relatively higher in gonads, brain, kidney and spleen (Fig. 5). Catfish P450C17 antibody characterization details are presented in Fig. 6. Immunocytochemical localization of P450C17 demonstrated immunoreactivity (ir-) in interstitial cell layer and spermatocytes of testis (Fig. 7A–C). This was also evident with immunofluorescence method. In ovary, ir- was observed in the follicular layer of previtellogenic, vitellogenic and post-vitellogenic oocytes (Fig. 7E–I).

3.5. Stage dependent expression of p450c17 in ovary and testis

p450c17 transcript was highly expressed in preparatory and prespawning phases where as a low level of expression was noticed in spawning and regressed phases (Fig. 8A). Western blot analysis of protein expression in these phases of ovarian follicles was in accordance with mRNA levels (Fig. 8C). Consistent with expression, the ratio of lyase to hydroxylase activity was high in preparatory

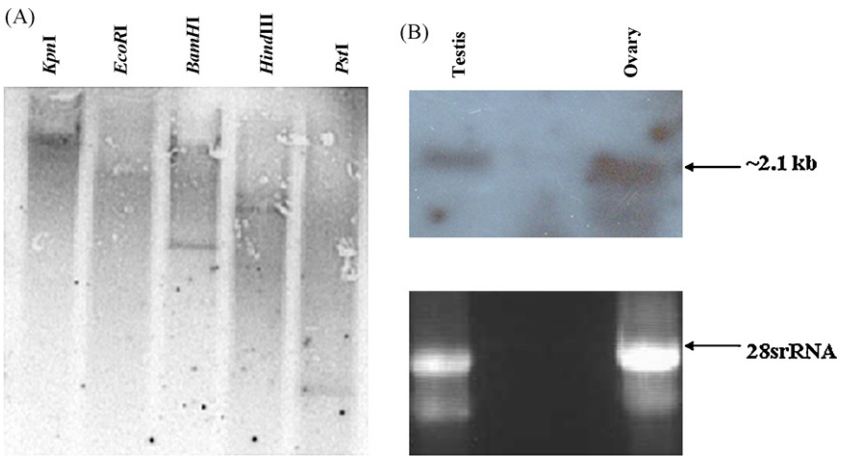

Fig. 3. (A) Genomic Southern analysis of catfish ovarian follicles probed with a partial cDNA lacking sites for the enzymes used in digestion. 1 kb DNA ladder was used to identify the size of signals. (B) Northern blot analysis of 25 μg of total RNA from catfish ovary and testis. RNA ladder was used to identify the size of bands.

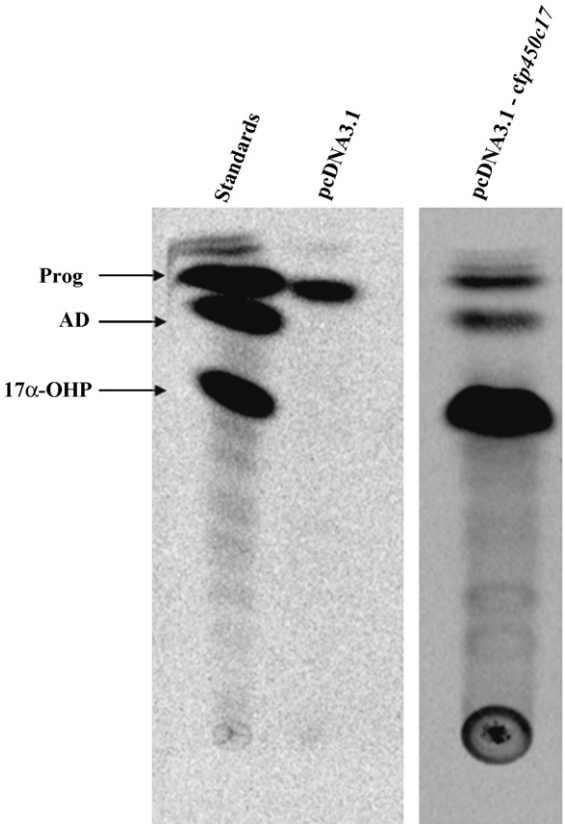

Fig. 4. Autoradiogram showing the 17α-hydroxylase and c17-20 lyase activities of catfish *p450c17* transiently expressed in COS-7 cells.

and pre-spawning phases while it was very low in spawning and regressed phases (Fig. 8D). Real-time RT-PCR analysis demonstrated that the expression of *p450c17* was high in preparatory and spawning phases of testicular cycle while it was low in pre-spawning and regressed phases (Fig. 8B).

3.6. *p450c17 expression and activity during hCG-induced oocyte maturation, in vitro and in vivo*

There was significant increase in the expression of *p450c17* by 2 h after treatment with hCG both *in vitro* and *in vivo* compared to saline-treated controls (Fig. 9A and B). On the contrary, Western blot analysis revealed no changes in protein levels of P450C17 (Fig. 9C and D). Concomitantly, no significant difference was noticed between the ratio of lyase to hydroxylase activity (Fig. 9E and F).

4. Discussion

In the present study, we used hCG-induced oocyte maturation both *in vitro* and *in vivo* to delineate the role of *p450c17* in steroidogenic shift. To achieve this, a full-length cDNA encoding *p450c17* was isolated from catfish ovarian follicles. The catfish *p450c17* has high homology to that of *p450c17-I* cDNA sequences reported in other teleosts and their counterparts in higher vertebrates. The close relationship of P450c17s among these species is likely to be common. In general, P450 enzymes exist as single gene in multiple species mediating multiple enzymatic steps [29] and multiple forms of P450c17 have not been found in mammals and other lower vertebrates except in few teleost species in which a second form of *p450c17* that lacks the lyase activity [23,24]. Since we used partial cDNA fragment of *p450c17* spanning the common heme-binding region as probe in genomic Southern analysis, our identification of single copy gene for *p450c17* seems to be reasonable. COS-7 cells

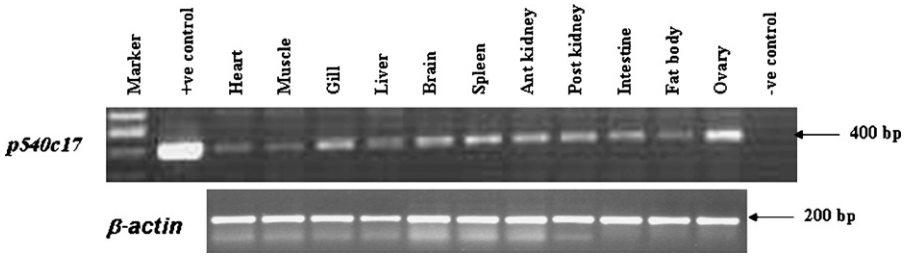

Fig. 5. RT-PCR analysis of spatial expression pattern of catfish *p450c17* in different tissues. Plasmid clone containing catfish *p450c17* was used as positive control (+ve ctl.). Negative control (−ve ctl.) contains no cDNA template. Ant. kidney, anterior kidney; post. kidney, posterior kidney.

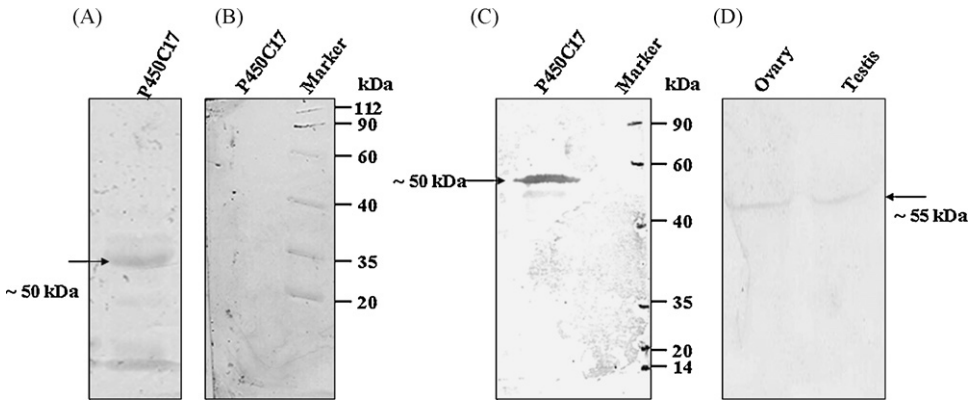

Fig. 6. Western blot analysis demonstrating catfish anti-rabbit P450C17 antibody. (A) Anti-His antibody detecting catfish recombinant P450C17. (B) Detection of recombinant P450C17 with pre-immune serum. No signal was observed. (C) Catfish P450C17 antibody detecting recombinant P450C17. (D) Catfish P450C17 antibody detecting in ovary and testis.

transfected with catfish *p450c17* cDNA converted exogenous Prog to 17α-OHP and AD that is in accordance with previous reports in which only single form of *p450c17* was identified [18,20]. Consistent with cDNA cloning and genomic Southern analysis, a single transcript was detected both in ovary and testis.

Positive correlation of *p450c17* expression with gonadal cycle in catfish shares similarity with other teleosts like trout [18], eel [20] and channel catfish [26]. However, there was no obvious association of *p450c17* expression with ovarian development in fathead minnow [21] while the expression of *p450c17* was high throughout the

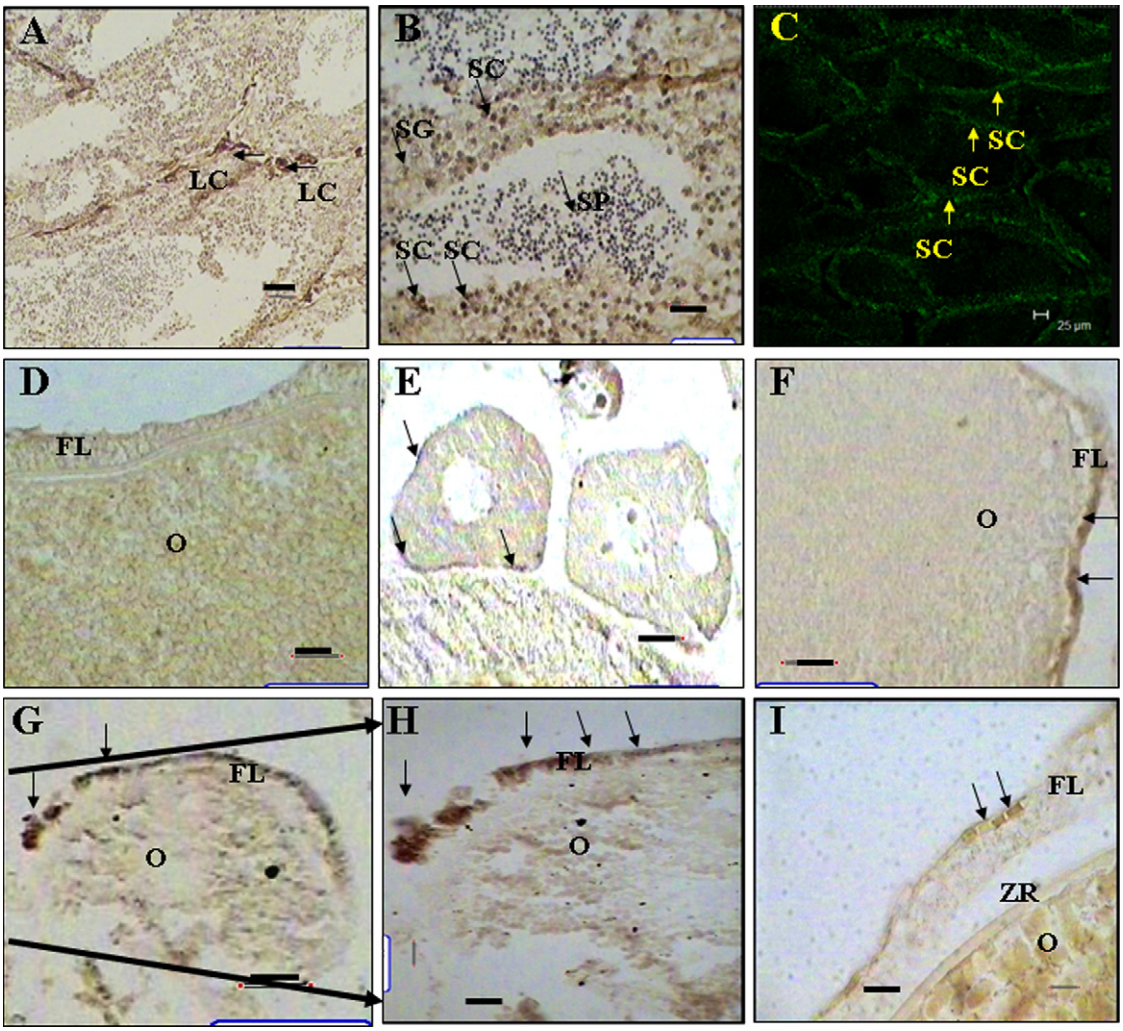

Fig. 7. Immunolocalization of P450c17 in catfish gonads. (A and B) Testicular sections detected with DAB and counterstained with hematoxylin. (C) Immunofluorescence detection in testis section. LC-Leydig cell, SG-Spermatogonia, SC-Spermatocytes, SP-Spermatids. (D) Pre-absorbed antibody showed no ir- however, DAB background was seen in yolk granules of post-vitellogenic oocyte. (E) Pre-vitellogenic, (F) vitellogenic, (G) post-vitellogenic, (H and I) are higher magnification pictures. O-Ooplasm, ZR-Zona radiate, FL-Follicular layer. Bar in (A–C) represents 25 μm and in (D–I) 50 μm. In DAB stained sections different filters were used for the better contrast.

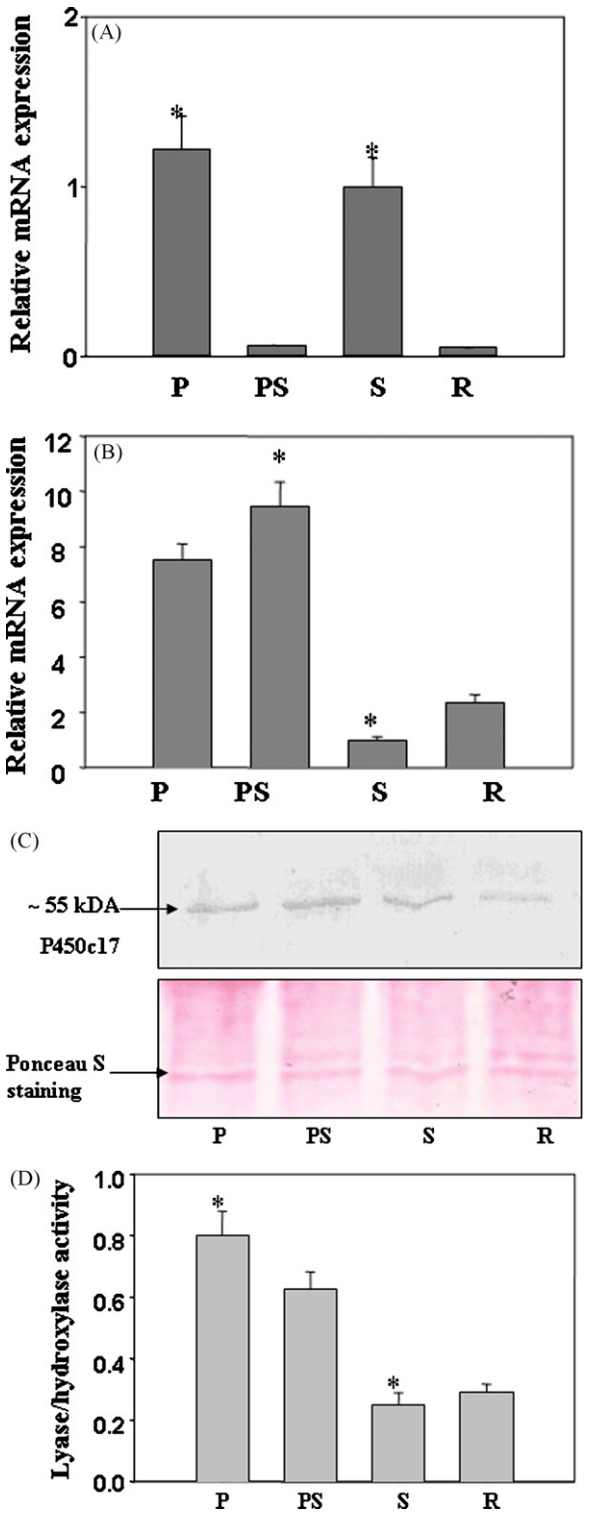

Fig. 8. Real-time RT-PCR analysis of *p450c17* expression in catfish (A) ovarian and (B) testicular cycles. (C) Representative Western blot showing P450C17 expression in different stages of ovary. Ponceau S staining was used to depict equal loading (lower panel). (D) Lyase to hydroxylase ratio in different stages of ovarian cycle. P, preparatory; PS, pre-spawning; S, spawning; R, regressed. *Indicates the significance ($n = 3$, $P < 0.05$, ANOVA).

developmental stages of ovarian follicles without any significant difference among the developmental stages in zebrafish [22]. The discrepancy in expression pattern of *p450c17* among these species could be attributed to the sensitivity of the techniques used or the varied pattern of reproductive cycles of zebrafish and fathead minnow compared to eel, trout and catfish. On the other hand, in tilapia and medaka *p450c17-I* expression peaks around mid-vitellogenic stage and declines by maturational stage while *p450c17-II* expression was maximum during maturation [23,24]. Consistent with mRNA, protein levels and ratio of lyase to hydroxylase activity in different stages of follicle development (present study) corroborates to *p450c17* expression in eel and channel catfish [20,26]. To our knowledge, present study was first of its kind to correlate expression analysis to enzyme activity for *p450c17*. The higher expression of *p450c17* during early and mid-stages of follicular development is presumed to be important for production of higher levels of Δ^4 steroids and is supported by the correlation between expression and enzyme activity [26]. Intriguingly, a negative correlation was identified with testicular development in fathead minnow [21] while we observed maximum expression of *p450c17* in preparatory and spawning phases of catfish testis. The expression pattern of *p450c17* in catfish testis matches with levels of Δ^4/sex steroids in preparatory and 17α,20β-DP levels in spawning phase (new reference).

Consistent with previous reports in mammals, birds and fishes, we could also detect *p450c17* transcript in many tissues of catfish. P450c17 was originally thought to be present exclusively in gonads and adrenals, later on mRNA, protein and/or activity were found in several other tissues [22]. In agreement with its widespread localization, P450c17 is a potent oxidant and catalytic reactions other than hydroxylation and lyase have been suggested [30]. Ying et al. [31] reported that P450c17 also functions as squalene monooxygenase involved in cholesterol biosynthesis.

The 17α-hydroxylase activity of P450c17 is necessary for synthesis of cortisol in adrenal while the lyase activity becomes important for production of sex steroids in gonads [2]. The mechanism behind organ-specific differential actions of P450c17 was attributed to post-translational modification regulations such as abundance of electron-donating partner P450 oxidoreductase, cytochrome b5 and ser/thr phosphorylation in mammals [5]. Alternatively, the presence of P450c17 isoenzymes has been proposed to explain differential actions of P450c17. In teleost ovary, lyase activity is required for the production of estrogens during growth phase while hydroxylase activity is necessary for the production of 17α,20β-DP during maturational phase [14]. Until recently [23,24], only one form of *p450c17* that is highly homologous to mammals has been found in few fish species and it has been thought that the similar mechanism found in mammalian adrenals were responsible for these differential actions of *p450c17* [20,22,17]. In the present study neither protein levels nor enzyme activity showed significant changes to hCG both *in vitro* and *in vivo* although mRNA levels increased. The significance of *p450c17* mRNA rise after treatment with hCG is not clear at present. But the increase in *p450c17* mRNA levels could be attributed to the increase in intracellular cAMP levels with hCG as observed in rat Leydig cells [32]. It is also plausible that the increase in *p450c17* transcripts during hCG-induced oocyte maturation might have higher input to steady levels of P450C17 protein. This contention needs further evaluation. Similarly in eel, *p450c17* mRNA levels increased gradually throughout the artificial induction of gonadal development [20]. In contrast, the cultured zebrafish ovarian follicles did not respond to either hCG or activin [22]. In the case of tilapia and medaka, differential expression patterns of two forms of *p450c17* are presumed to be important during final oocyte maturation [23,24]. All the previous reports, including the recent identification of *p450c17-II* [23,24] did not analyze enzyme activity and regulation of lyase activity

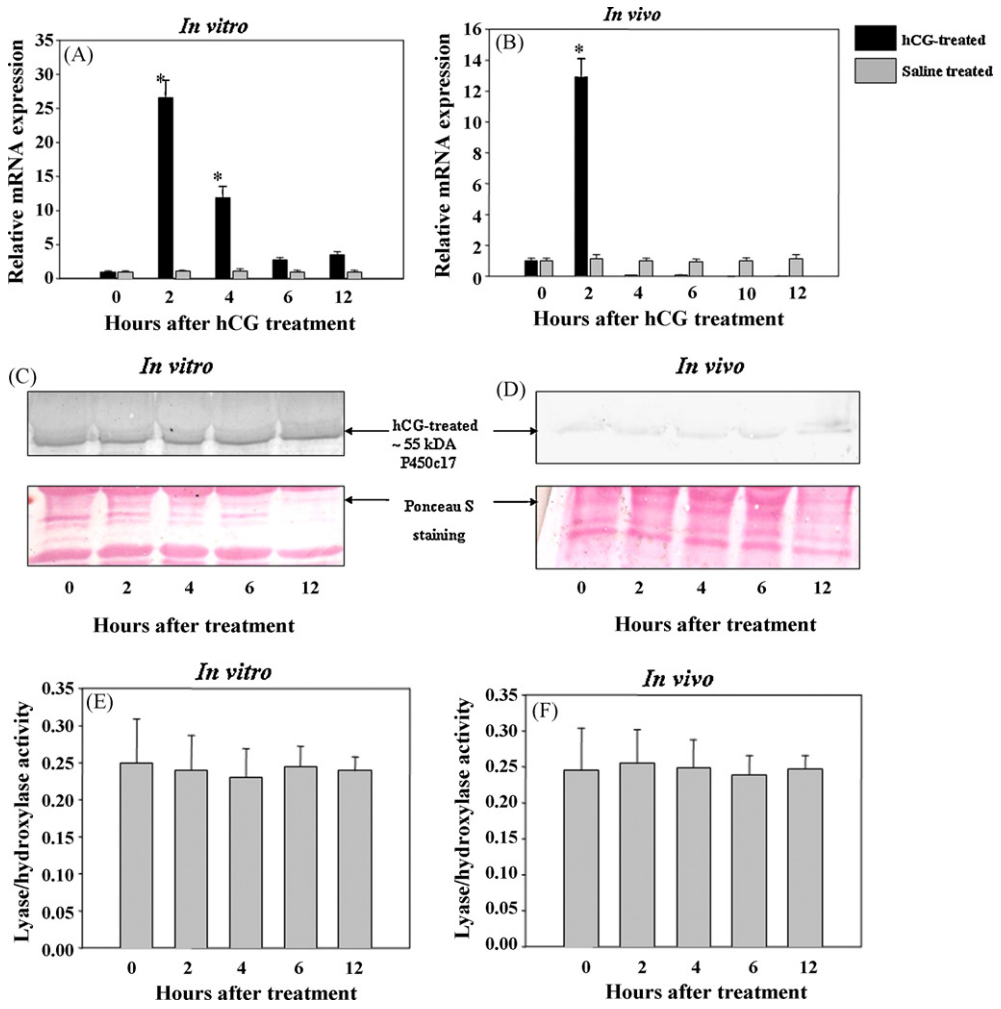

Fig. 9. *p450c17* expression and activity during hCG-induced oocyte maturation. Real-time RT-PCR analysis of *p450c17* expression, *in vitro* (A) and *in vivo* (B). Western blot analysis of P450C17, *in vitro* (C) and *in vivo* (D). Ponceau S staining was used to depict equal loading (lower panel). Changes in lyase to hydroxylase ratio, *in vitro* (E) and *in vivo* (F). *Indicates the significance ($n = 3$, $P < 0.05$, ANOVA).

in detail. Present study attempted to seize this fact and indicated that increase in *p450c17* transcripts need not follow with elevated protein level or enzyme activity during final oocyte maturation. Further immunocytochemical localization of P450C17 in the follicular layer of oocytes at different stages supported its importance in gonadal recrudescence. Localization of P450C17 also provides additional evidence for the two-cell type model proposed by Nagahama [14].

Based on the reports in mammals [5,6,27], it seems compulsory to gain knowledge both at the level of mRNA as well as protein and enzyme activity owing to the complexity of regulation of these enzymes. However, the second form of *p450c17*, unique to two teleost species might have been evolved from the fish specific genome duplication [23,24]. Hence, differential actions of *p450c17* with the presence of multiple forms cannot be generalized to all teleosts. Though present study categorically demonstrated differential *p450c17* expression versus activity during final oocyte maturation, regulation of lyase activity in those teleosts possessing single form of *p450c17* continues to be an issue which needs to be resolved. Further analysis assessing the regulation of lyase activity with reference to P450 oxidoreductase and cytochrome b5 might provide interesting clues about the regulation of *p450c17* in teleosts possessing single form of *p450c17*.

In conclusion, a single form of *p450c17* that is homologous to *p450c17-I* has been cloned from the ovarian follicles of catfish. *p450c17* mRNA, protein levels and activity were found to be high during preparatory and pre-spawning stages of follicle development while testis had maximum expression during preparatory and spawning stages. During hCG-induced oocyte maturation both *in vitro* and *in vivo*, neither protein level nor ratio of lyase/hydroxylase activity changed significantly although there was an increase in mRNA levels by 2 h after induction with hCG. Taken together, it seems that *p450c17* potentiates during preparatory and pre-spawning/spawning phases which might exert an influence on the shift in steroidogenesis during ovarian and testicular recrudescence.

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