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Cloning and expression of P450c17-I (17α-hydroxylase/17,20-lyase) in brain and ovary during gonad development in *Cynoglossus semilaevis*

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Abstract Cytochrome P450c17 (CYP17, 17a-hydroxylase/17,20-lyase) is a critical enzyme in the production of androgens and estrogens in vertebrates. A 2,469 bp full length cDNA of P450c17-I (*CYP17A1*) has been isolated from the ovary of half-smooth tongue sole, *Cynoglossus semilaevis* which encodes 509 amino acids. Additionally, a relatively shorter cDNA (1,742 bp), a likely result of polyadenylation, was also found. The putative P450c17-I enzyme shares high sequence identity with that of the fathead minnow (73%), zebrafish (71%), the Japanese eel (70%), catfish (70%), tilapia (79%), three-spined stickleback (81%), medaka (79%), dogfish (60%), chicken (65%), rat (47%), and human (49%). Semi-quantitative RT–PCR analysis of spatial expression showed the enzyme was predominantly expressed in the ovaries and the brain. P450c17-I was also detected in the stomach, intestine, gill, spleen, kidney, and head kidney, albeit weakly. Further examination of temporal expression pattern of P450c17-I in ovary and brain revealed developmental stage-dependency. In addition to this our data on T and E2 levels further endorse the critical role of P450c17-I during shift in steroidogenesis. Based on the present study we indicate an important role for P450c17-I during ovarian development. However, further studies are needed at transcriptional regulation level for deeper insights into the physiological functions of P450c17-I.

Keywords Half-smooth tongue sole · P450c17 · mRNA expression · Physiological function · Testosterone · Estradiol-17β

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Introduction

In vertebrates, a serial action of the steroidogenic enzymes is responsible for the pathways of steroid hormone synthesis. One of the enzymes critical to steroidogenesis is Cytochrome P450c17 (*CYP17*), a single microsomal enzyme and having a unique character—two activities, namely 17α-hydroxylase and 17,20-lyase (Miller et al.1997). After cholesterol is catalyzed by the cholesterol side-chain cleavage enzyme (P450scc), pregnenolone and progesterone undergo 17α-hydroxylation and proceed down the C21, 17-hydroxy pathway to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively. Then these substrates undergo cleavage of the C17-20 bond to yield the C19 sex steroid and adrenal cortisol precursors dehydroepiandrosterone (DHEA)

and androstenedione (Halm et al. 2003). Thus the nature of P450c17 activity is fundamental important in reproductive biology, and it is indispensible for the biosynthesis of sex steroids.

Known to date, there are two types of Cytochrome P450c17s (P450c17-I and P450c17-II) encoded by two different genes. The novel type, P450c17 is termed as P450c17-II (Zhou et al. 2007b), while the original one as P450c17-I. Prior to the reporting of P450c17-II, the P450c17 (P450c17-I) cDNA has been cloned in several species of fish, such as the rainbow trout *Oncorhynchus mykiss* (Sakai et al. 1992), the Japanese eel *Anguilla japonica* (Kazeto et al. 2000), the fathead minnow *Pimephales promelas* (Halm et al. 2003), the rice field eel *Monopterus albus* (Yu et al. 2003), zebrafish *Danio rerio* (Wang and Ge 2004), and the dogfish *Squalus acanthias* (Trant 1995). However, in flatfish including the half-smooth tongue sole *Cynoglossus semilaevis*, P450c17-I cDNA and its function has not been investigated yet.

The gonads and the head kidney (the piscine counterpart of the mammalian adrenal) are major organs for P450c17 gene expression in both the cartilaginous and bony fishes (Halm et al. 2003; Kazeto et al. 2000; Sakai et al. 1992; Trant 1995; Wang and Ge 2004). Besides, P450c17 has also been detected in other non-classical steroidogenic tissues in fish, including the brain (Halm et al. 2003; Yu et al. 2003; Wang and Ge 2004), the gastrointestinal tract, liver and gill (Wang and Ge 2004), indicating that steroidogenic activities are present in these tissues. Also, P450c17 seems to follow a similar distribution pattern in other high vertebrates (Compagnone et al. 2000; Matsunaga et al. 2001; Yu et al. 2002; Zwain and Yen, 1999; Dalla Valle et al. 1995; Katagiri et al. 1998; Vianello et al. 1997). However, the origin of androgens in the female brain is highly controversial, but is important for their effects on behavior and development (Stacey and Kobayashi 1996).

In the ovary of teleosts, it is well established that estradiol-17β (E_2) and 17α, 20βdihydroxy-4-pregnen-3-one (17α, 20β-DHP, the oocyte maturation-inducing hormone) are essential for oocyte growth (mainly vitellogenesis) and final oocyte maturation, respectively (Nagahama 1997; Nagahama et al. 1995). Previous studies on steroid synthesis have indicated that P450c17 can regulate the levels of the steroid precursors, testosterone (T) and 17α-hydroxyprogesterone, responsible for the synthesis of E_2 and 17α, 20β-DHP respectively (Senthilkumaran et al. 2004). Therefore, it has been thought that P450c17 may be a cardinal factor in the change of steroidogenic pathway from E_2 in the vitellogenic follicles to 17α, 20β-DHP in the full-grown follicles (Nagahama et al. 1995; Nagahama 1997; Planas et al. 2000; Sreenivasulu et al. 2005; Sreenivasulu and Senthilkumaran 2009). Furthermore, it has been indicated that P450c17 expression is dependent on the ovarian developmental stage in the fathead minnow (Halm et al. 2003), rainbow trout (Sakai et al. 1992), and the Japanese eel (Kazeto et al. 2000).

The half-smooth tongue sole *C. semilaevis* is a native commercially important marine flatfish in China with great aquacultural potential. The females of the species grow 2–3 times faster than their male counterparts. In the present study, *C. semilaevis* P450c17-I cDNA was first isolated for further insight into the regulation of steroid hormone synthesis in the female *C. semilaevis*. The physiology function of P450c17-I is discussed, combined with serum steroid hormone level (E_2 and T), the spatial and temporal expression of P450c17-I gene, as well as gonadosomatic (GSI) and hepatosomatic (HSI) indices. The study aims to explore the role of P450c17-I in the reproductive cycle of female *C. semilaevis* for further study on hormonal-induced spawning as an oxytocin. The results are expected to come handy in the efficient manipulation of *C. semilaevis* during its hatchery and husbandry operations.

Materials and methods

The experimental fish

One-hundred *C. semilaevis* on an average weighing around 500.3 g (±30.5 g) each, were reared in a pond at a commercial fish farm (Laizhou, Shandong, PRC). The fish were fed a commercially prepared diet at 1.5–3.0% of body weight per day and reared in natural sea water under controlled conditions (20 ± 0.5°C; ≥4 mg l^{-1} O_2; 14:10 h light: dark cycle). In all, sampling was conducted four times after a span of every 3 months, and on an average, 10 specimens were randomly sampled each time. Following sampling, fish were acclimatized for 3–4 days in natural sea water under controlled conditions in the laboratory.

Gonadosomatic index and hepatosomatic index and ovarian developmental phases

Fish were sacrificed by an overdose of MS-222 (Sigma, St. Louis, MO). Firstly, their wet (body) weights were recorded followed by dissecting them to obtain their gonads (ovaries) and liver. The weights of the ovaries and liver were recorded for computing the GSI and HSI, respectively: GSI = [gonad weight/(body weight − viscera weight)] × 100, HSI = [liver weight/(body weight − viscera weight)] × 100.

The ovaries were sectioned in two parts, one part was fixed in Bouin's solution for hematoxylin and eosin (HE) dyeing so as to identify the ovarian developmental stages and the other part was snap-frozen in liquid nitrogen and subsequently stored at −80°C for RNA preparation. Stomach, intestine, gills, heart, spleen, kidneys, head kidneys, brain, and muscle were also collected, processed and stored in a similar fashion.

The ovaries fixed in Bouin's solution were sectioned at 5–7 μm followed by HE staining. Four phases of ovarian development were identified: perinucleolus stage (PN), primary yolk stage (PY), tertiary yolk stage (TY), and atretic follicles stage (AF). Photomicrographs of HE-stained *C. semilaevis* ovaries are illustrated in Fig. 1.

Steroid radioimmunoassay

Blood samples were withdrawn from the caudal vessel after anesthetizing the fish with MS-222. Blood was allowed to clot at 4°C for 4–6 h and the serum was obtained by centrifuging at 16,000*g* for 8 min. The serum was stored at −40°C for the steroid analysis. Serum testosterone (T) and estradiol-17β (E_2) levels were measured by I^{125} radioimmunoassay (RIA) kits from Diagnostic Products Corporation (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd). The bind rate is highly specific with an extremely low crossreactivity to other naturally occurring steroids, less than 0.1% to most circulating steroids. Precision of the batch is 7.4 and 7.7%for testosterone assay and the estradiol-17β assay,

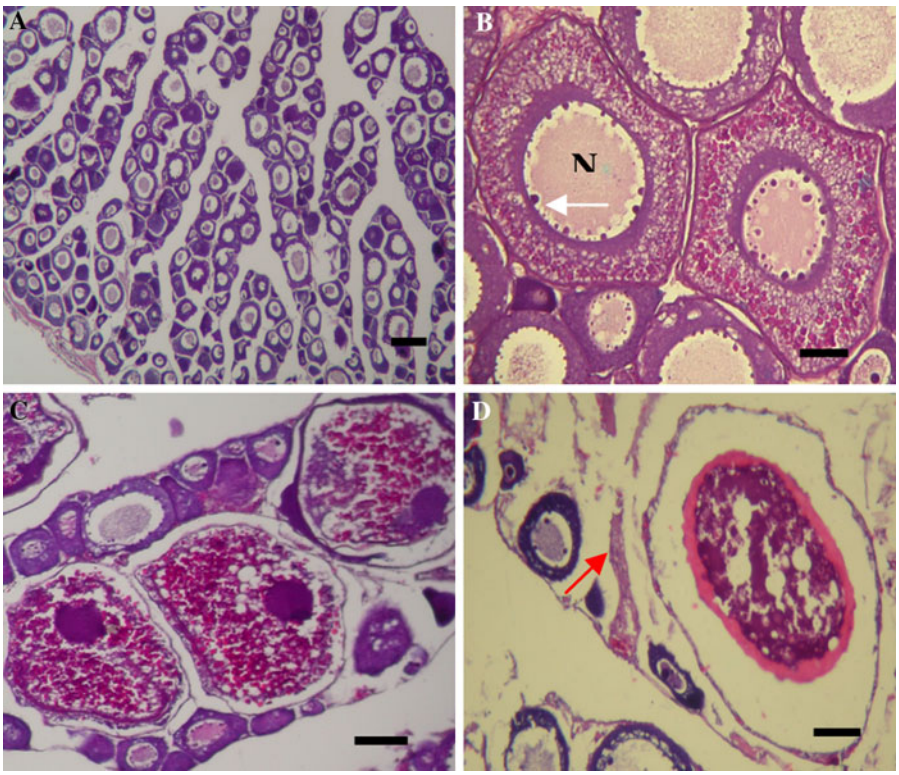

Fig. 1 Photomicrographs of *C. semilaevis* oocytes. **a** Perinucleolus stage oocyte (*PN*); **b** primary yolk stage oocyte (*PY*); **c** tertiary yolk stage oocyte (*TY*) and **d** atretic follicle stage oocyte (*AF*). *Nu* nucleolus, *AF* atretic follicle

respectively. Any samples with coefficients of variation higher than 10% were excluded in the analysis. The assay sensitivity reached to 1.9 ng/dl and 2.1 pg/ml for T and E_2 by the kit protocol, respectively. This steroid RIA was optimized by He et al. (2008).

RNA extraction

Total RNA was extracted using Trizol® reagent (RNA Extraction Kit, Invitrogen, USA) according to the manufacturer's protocol. The concentration of the total RNA was quantified on a UV spectrophotometer (Ultrospec-2100Pro, Amersham) and its integrity was confirmed by agarose-gel electrophoresis.

First strand cDNA synthesis

A 2 μg total RNA was mixed with 0.5 μg random primer and diluted with sterile deionized water to a final volume of 13 μl. The mixture was heated at 70°C for 5 min, and immediately cooled on ice followed by addition of 5 μl 5× reaction buffer, 5 μl 2.5 mM dNTP, 1 μl RNase inhibitor (TAKARA, Japan), 200 units of M-MLV RT (Promega,USA). The mixture was incubated at 37°C for 60 min and inactivated at 95°C for 5 min. The synthesized cDNA was stored at −20°C for later use.

Rapid amplification of cDNA ends

To obtain the core fragment, a pair of degenerate primers (CH_1, CH_2; Table 1) were designed using CODEHOP, amplifying approximately 702 bp product from the tongue sole ovary. PCR reaction was carried out using touchdown PCR with initial denaturation at 94°C for 5 min followed by 10 cycles each of 35 s at 94°C, 35 s at the annealing temperature (Tn), and 35 s at 72°C with a range of Ta from 65 to 55°C, decreasing by 1°C each cycle and followed by an additional 22 cycles at a Tn of 55°C, with the PCR finally terminating with an extension of 10 min at 72°C.

Table 1 Primer sequences for cloning tongue sole P450c17-I and for gene expression analysis

Primer	Sequence (5′–3′)
CH_1	ATCTTCCCCTGGCTGcarathttycc
CH_2	TCTTGGCCAGG GCCtcnccanrrca
GSP_{1-1}	CTCTGCACGTGGTCGCTGTAGTC
GSP_{1-2}	GGAGAGGACGGAGCACAGAGTCTTG
GSP_2	CGCTTCATTGACAGTAAAGGAACAGG
SP_1	GGCCTGCGTCTTCTAAGG
SP_2	ATGATGGCCCATTTAAGAAC

Gene specific primers (GSPs) for 5′and 3′ Rapid amplification of cDNA ends (RACE) were designed by using the sequence of the previous PCR product. RACE was carried out utilizing the Advantage II RACE system (Clontech, USA) according to the manufacturer's protocol. A 1 μg of total RNA from tongue sole ovary was used as template for the synthesis of 5′and 3′ RACE ready cDNA. GSPs were used individually with the universal primer mix supplied in the kit for both 5′and 3′ RACE. The GSPs are mentioned in Table 1. The PCR product of the expected length was separated on 1% agarose gel and purified with TIANgel midi Purification Kit (Tiagen, China). The purified fragments were then cloned into pGM-T vector (Tiangen, China) followed by propagation in *E. coli* DH5α and subsequently sequenced on an ABI3730XL sequencer.

RT–PCR analysis

Semi-quantitative RT–PCR assays were performed to evaluate the level of mRNA expression of P450c17-I. A TY stage of the ovarian sample was used for the spatial distribution of tissue expression of *CYP17A1*. Total RNA of the ovaries, liver, stomach, intestine, gills, heart, spleen, kidneys, head kidneys, brain and muscles were treated as described above. Four fish were chosen for investigating the temporal expression of P450c17-I during each ovarian developmental stage (PN, PY, TY and AF). Total RNAs of ovaries and brain were prepared as described above. The first-strand cDNAs were used as templates for PCR with specific primers (SP_1, SP_2; Table 1), which amplified a PCR product of 269 bp.

Semi-quantitative RT–PCR was carried out in a Biometra Tpersonal Cycler (Germany). Initial denaturation was performed at 94°C for 5 min, followed by 30 cycles each at 94°C for 35 s, 57°C for 35 s, 72°C for 35 s or 18 cycles each (for 18S rRNA, Sun et al. 2008) at 94°C for 35 s, 61°C for 35 s,72°C for 35 s, and a final extension at 72°C for 10 min. The 18S rRNA are considered as suitable internal control genes other than β-actin (Schmittgen and Zakrajesk 2000; Goidin et al. 2001). To confirm the specificity

of RT–PCR amplification, the RT–PCR products were purified from the gel and submitted for sequencing. Electrophoretic images and the optical densities of amplified bands were analyzed using Quantity Onev4.62 software (Bio-Rad, America).

Sequence analysis and Statistics

Sequence alignments and comparisons of homology between various P450c17 cDNA sequences were performed using the European Molecular Biology Open Software Suite (EMBOSS). The alignment of P450c17-I amino acid was carried out using the Clustalw programs and the phylogenetic tree was constructed using the neighbour-joining method of Mega 4.0. Statistical analyses consisted of analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's *t*-test. Statistical significance was inferred at $P < 0.05$.

Results

Cloning of P450c17-I full-length cDNA from the ovary of half smooth tongue sole

P450c17-I cDNA cloned from the ovary of half smooth tongue sole was 2,469 bp with two potential polyadenylation signals (AATAAA) in the 3′-untranslational region (GenBank accession number: EU580533). An alternative 1,742 bp transcript with a variation in the 3′-end was also cloned (GenBank accession number: GU338001, that will be released soon), as the result of polyadenylation.

The open reading frame for P450c17-I was 1,527 nucleotides long and encoded 509 amino acids. The putative half-smooth tongue sole enzyme shared high identity with its counterparts of the fathead minnow (73%), zebrafish (71%), the Japanese eel (70%), catfish (70%), tilapia (79%), three spined stickleback (81%), medaka (79%), dogfish (60%), chicken (65%), rat (47%), and human (49%; Chung et al. 1987; Halm et al. 2003; Kazeto et al. 2000; Namiki et al. 1988; Ono et al. 1988; Sreenivasulu and Senthilkumaran 2009; Trant 1995; Wang and Ge 2004; Zhou et al. 2007a; Zhou et al. 2007b; Fig. 2). The three conserved functional domains of P450c17 (Ono et al. 1988), consisting of Ono sequence [I], Ozols' tridecapeptide region [II] and haeme-binding region [III]), can be identified in half-smooth tongue sole, and they shared higher identity than the other parts of the enzyme (Fig. 2).

To identify the evolutionary position of tongue sole P450c17-I, a phylogenetic tree was constructed with other 11 animals, including teleosts, cartilaginous fish, birds and mammals (Fig. 3). As expected, all the teleosts obviously formed one branch, whereas the cartilaginous fish, birds, mammals were separated into three groups each, respectively. Half smooth tongue sole P450c17-I was found to belong to the teleost branch, and was most close to the tilapia P450c17-I.

Spatial distribution of tissue-specific expression of *P450c17-I* gene

P450c17-I gene expression in different tissues of TY stage females by RT–PCR (Fig. 4) showed the gene to be expressed abundantly in the ovaries and followed by the brain. However, the gene was also found to be expressed in the stomach, intestine, gills, spleen, kidneys, and the head kidneys. No expression was evident in the liver, heart and muscles.

Relationship between GSI, HSI and the ovarian developmental phase

Changes in GSI and HSI of female tongue sole are shown in Fig. 5. According to the data, the average GSI of female tongue sole was 1.55 ± 0.45 during PN, increasing gradually to 1.84 ± 0.51 during the PY. The GSI peaked to 4.89 ± 3.88 ($P < 0.05$) during the TY and finally dropped markedly to 2.36 ± 1.28 during AF ($P < 0.05$). On the other hand HSI averaged 0.85 ± 0.13 during the PN, dropping markedly to 0.66 ± 0.09 during the PY ($P < 0.05$), then increased rapidly to 0.85 ± 0.10 during TY ($P < 0.05$), and finally appreciated to 0.98 ± 0.24 during the AF.

Relationship between serum steroid hormone level and the ovary developmental phase

Changes in the serum E_2 and T values vis-à-vis ovarian developmental stage are shown in Fig. 6. The average of serum E_2 level in female tongue sole during PN was 21.97 ± 6.95 pg/ml, and increased remarkably to 42.82 ± 21.58 pg/ml during PY ($P < 0.05$), then peaked to 66.19 ± 23.07 pg/ml

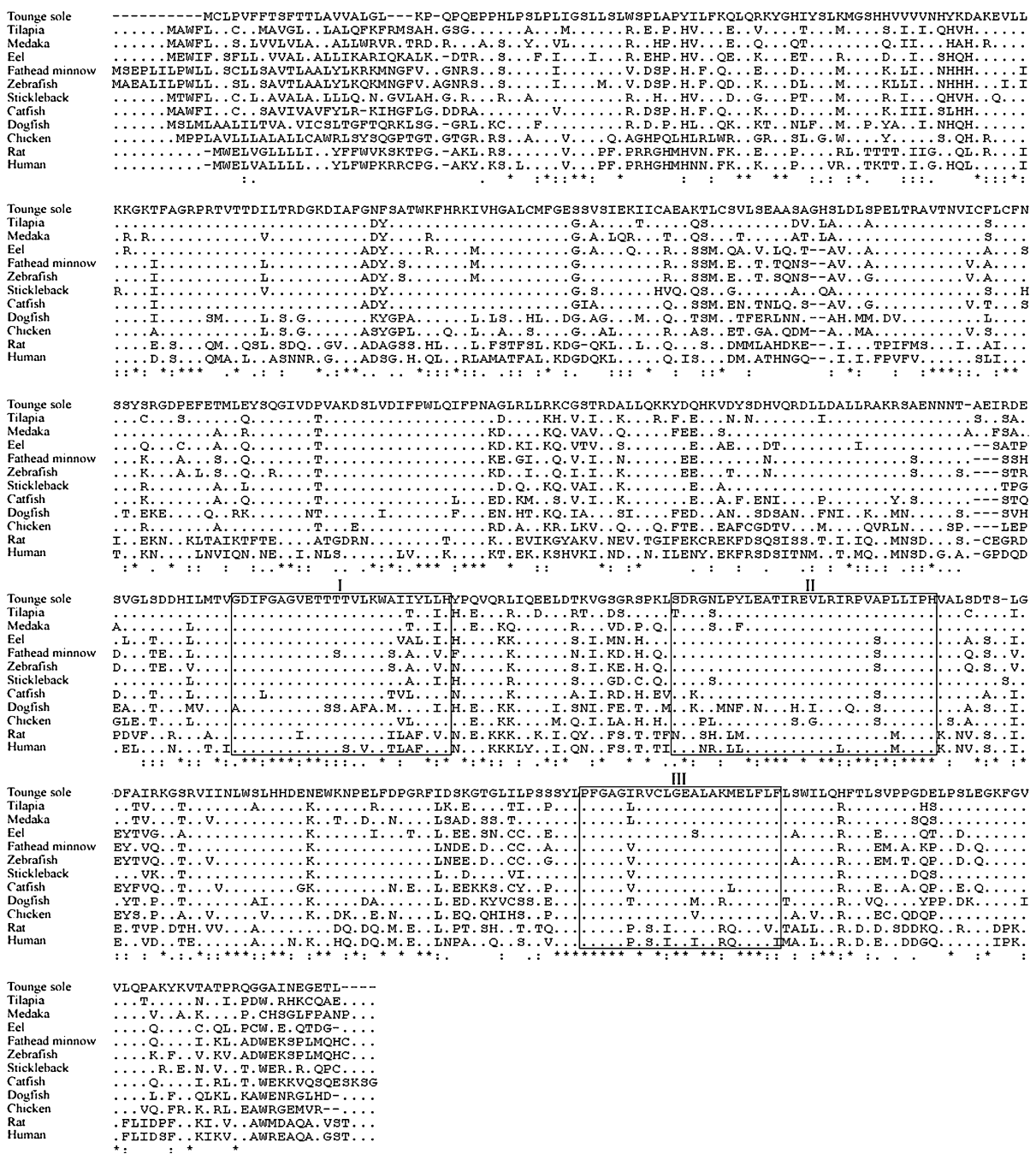

Fig. 2 Alignment of tongue sole P450c17I amino acid sequence with other 11 animals' P450c17I amino acid sequence. The functional (*conserved*) regions of P450c17I are *boxed* (*I* Ono-sequence, *II* Ozols-tridecapeptide region, and *III* haem-binding region)

($P < 0.05$) during TY, and finally fell to 52.39 ± 21.60 pg/ml during AF.

Average serum concentration of T during PN was 1.87 ± 0.75 ng/dl, which dropped down to undetectable levels during the PY. During the TY the value increased rapidly to 15.16 ± 8.11 ng/dl ($P < 0.05$), but dropped markedly to 8.38 ± 3.63 ng/dl during AF ($P < 0.05$).

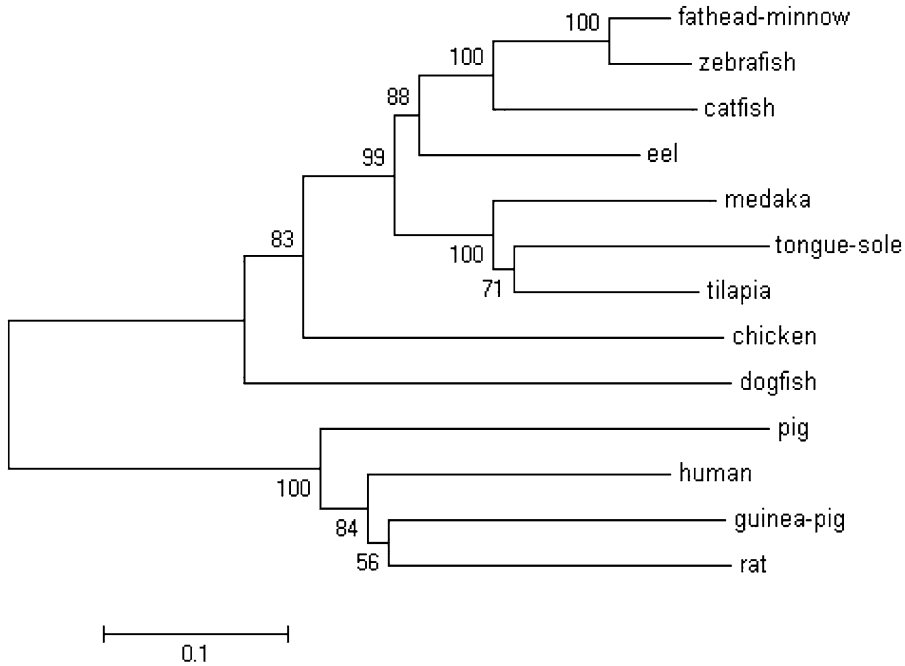

Fig. 3 Phylogenetic tree for P450c17I amino acid sequence of teleost fish, cartilaginous fish, birds, and mammals. The GenBank accession numbers are followed: Fathead minnow: CAC38768; Zebrafish: NP_997971; Catfish: ACT88154; Eel: AAR88432; Medaka: BAA13253; Tongue sole: ACB70405; tilapia:BAF75924; Chicken: P12394; Dogfsh: Q92113; Pig: NP_999593; Human: NP_000093; Guinea pig: Q64410; Rat: NP_036885

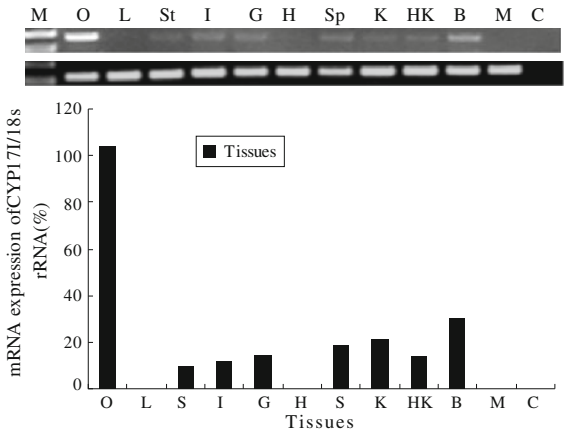

Fig. 4 mRNA expression of *CYP17I* gene in various tissues of female *C. semilaevis*. *G* gonad, *L* liver, *St.* stomach, *I* intestine, *G* gill, *H* heart, *S* spleen, *K* kidney, *HK* head kidney, *B* brain, *Mu* muscles, *C* control, and *M* marker

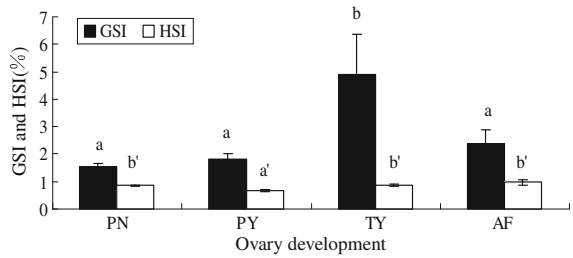

Fig. 5 Gonadosomatic (*GSI*) and Hepatosomatic (*HIS*) indices of female *C. semilaevis*. Values are expressed as mean ± standard error of mean. *Different letters* indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test)

Semi-quantitative RT–PCR analysis of expression at the ovary developmental phase

The temporal expression of the *CYP17A1* gene in ovaries and brain samples are shown in Fig. 7 during different ovary developmental phase. It was apparent that the 18S rRNA was far more expressed than *CYP17A1* even though the 18S rRNA cDNAs were only amplified for 18 PCR cycles, while *CYP17A1* cDNAs were amplified for 30 cycles. This is indicative of the fact that the ovarian *CYP17A1* expression continuously rises throughout the ovarian development, except for a slight drop during the PY stage. During the PN, PY and TY stages, no significant difference in *CYP17A1* expression was observed,

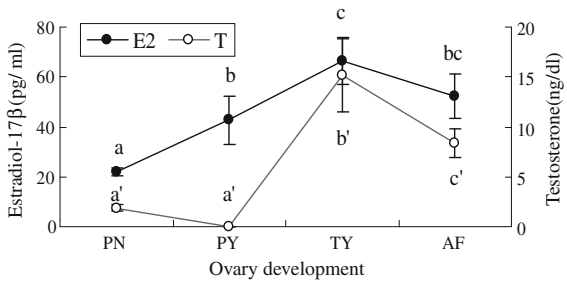

Fig. 6 Serum 17β-Estradiol and testosterone levels in female *C. semilaevis*. Values are expressed as mean ± standard error of mean. *Different letters* indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test)

whereas during the AF stage, the expression of *CYP17A1* was remarkably up-regulated and reached the highest level ($P < 0.05$). A similar pattern was observed with the brain *CYP17A1* expression during PN, PY and TY stages, but during the AF stage, the gene expression dropped sharply to the lowest level ($P < 0.05$). However, significant difference of the brain *CYP17A1* expression was observed between PY and TY stage, not seen in those of the ovary *CYP17A1* expression. During the TY stage, brain *CYP17AI* expression was 3.1-fold higher than the PY stage, and 4.4-fold higher than that at AF stage.

Discussion

In the present study, a full length cDNA encoding P450c17-I was cloned from half-smooth tongue sole ovary. The cloned P450c17-I cDNA was 2,469 bp in length, encoding 509 amino acids. A relative shorter 1,742 bp cDNA was also obtained, different from the longer one at 3′-untranslated region, but having the same open reading frame. In both sequences, polyadenylation signals were found, pointing to the notion that the longer sequence presumably underwent polyadenylation to become the shorter one. Similar type of P450c17-I cDNA polyadenylation signals were reported in the rice field eel (Yu et al. 2003) and the zebrafish (Wang and Ge 2004).

The half smooth tongue sole P450c17-I shared a high homology with other teleosts, followed by birds, cartilaginous fish, and mammals. In accordance with the identity, the phylogenetic tree indicated half smooth tongue sole belonged to the teleost branch. Similar to other animals' P450c17-I, half smooth tongue sole P450c17-I was found to possess three functional domains which were well conserved than the rest parts of the sequence.

By RT–PCR, we detected a predominant P450c17-I expression in the ovary, in accord with the results in birds and fish (Freking et al. 2000; Halm et al. 2003;

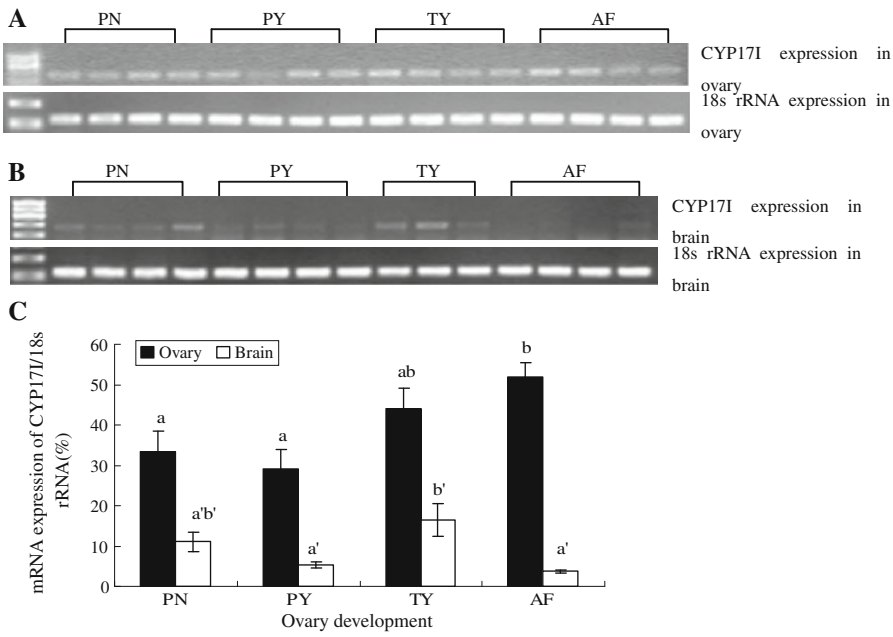

Fig. 7 mRNA expression of CYP17I in ovary and brain at different ovary development stage. Densitometric analyses of the mRNA expression of CYP17I. 'mRNA expression of CYP17I/18s rRNA (%)' indicates the ratio obtained from the density of the band of CYP17I mRNA expression standardized with that of the18s rRNA mRNA expression level

Kazeto et al. 2000; Ono et al. 1988; Sakai et al. 1992; Trant 1995; Yu et al. 2003; Wang and Ge 2004), indicating that the half smooth tongue sole ovary is the main tissue for P450c17-I expression, and P450c17-I plays a central role in the biosynthesis of sex-steroids.

Interestingly, a strong signal was first found in the brain, just 3.5-fold lower than the ovary. In previous studies, brain P450c17 gene expression was also detected in some species of fish, including fathead minnow (Halm et al. 2003), rice field eel (Yu et al. 2003), and zebrafish (Wang and Ge 2004). However, in fathead minnow, the expression level in the brain was at least 30-fold lower than that in the gonads, while a very faint band in the rice field, and lower level than ovary and kidney in the zebrafish. This discrepancy may be related to the ovarian developmental stages, supported by the temporal mRNA expression of P450c17-I (data discussed below), suggesting that the steroids regulation is dependent on the axis of brain–pituitary–gonad.

Besides, the mRNA expression of P450c17-I was also present in a variety of other tissues, such as head kidney, stomach, intestine, gill, spleen, kidney, Although the levels in these tissues were generally low compared with ovary and brain. As another classic steroidogenic tissue, a weak band was detected in the head kidney (equivalent to the adrenal cortex of mammals) and kidney. Similar result has been reported in the zebrafish (Wang and Ge 2004), suggesting that the head kidney in teleosts has been able to produce P450c17 protein that is involved in the biosynthesis of cortisol and 17α, 20β-DHP (Wang and Ge 2004; Kobayashi et al. 1998; Sangalang and Freeman 1988). Another phenomenon of the spatial distribution attracting our focus is the P450c17-I expression in the gill of half smooth tongue sole, as well as the zebrafish (Wang and Ge 2004).This is consistent with previous study that P450 aromB was present in the gill (Dalla et al. 2002), and gill cells had the potential to convert the testosterone to 5α-dihydrotestosterone and androstenedionein rainbow trout (Leguen et al. 2000). Our evidence for the expression of P450c17-I in the gill showed the possibility that androgen and estrogen may be partially produced from the gill via P450c17 activities.

It is a well known fact that both E_2 and 17α, 20β-DHP are essential for oocyte growth and final oocyte maturation, respectively in the teleosts (Nagahama et al. 1995; Nagahama 1997). Whereas the production of testosterone—precursor of E_2—needs both 17α-hydroxylase and 17,20-lyase activities of P450c17 (with the assistant of 17β-HSD proceeding down the E_2 biosynthesis pathway), 17α-hydroxyprogesterone—precursor of 17α,20β-DHP—only requires the 17α-hydroxylase activity. However, both steroidogenic pathways commence with progesterone and then branch off into two after the production of 17α-hydroxyprogesterone. An obvious change of steroidogenic pathways from E_2 to 17α, 20β-DHP has been reported during the ovarian development (Kagawa et al. 1983; Kanamori et al. 1988; Nagahama et al. 1986; Nagahama et al. 1995; Nagahama 1997; Joy et al. 1998; Planas et al. 2000, Shoonen et al. 1989; Suzuki et al. 1991; Sreenivasulu and Senthilkumaran 2009) implicating the role of P450c17 in this steroidogenic pathway switch. Moreover, previous study has shown that P450c17 expression appears to ovary developmental stage-dependence in some species of teleost fish (Kazeto et al. 2000; Sakai et al. 1992; Kumar et al. 2000). For example, the mRNA level of P450c17 became abundant with the later ovarian development while its expression was not detected in early ovarian development in rainbow trout (Sakai et al. 1992) and the Japanese eel (Kazeto et al. 2000). Similar result was seen in our present study supported by the P450c17-I temporal expression in half smooth tongue sole. To large extent, the signal of P450c17-I expression increased as the ovary developed, but was sequential present with significant variation throughout all the ovarian developmental stage investigated. Consistent with that in half smooth tongue sole ovary, the expression levels of P450c17 was detected in all developmental stages of follicles (Kumar et al. 2000; Halm et al. 2003; Wang and Ge,2004). However, in zebrafish ovary, P450c17 expression showed little stage-dependent variation as demonstrated by both Northern blot analysis and RT–PCR (Wang and Ge 2004). Our data from half smooth tongue sole ovary suggests that P450c17-I may regulate the level of androgen and estrogen to stimulate the oocyte growth and maturation. Additional evidence to support this hypothesis is that in present study the expression pattern of P450c17-I was found to strongly correspond to the serum T and E_2 levels, particular to the serum levels of T. As the ovary developed (GSI trended to increase), the

mRNA expression of P450c17-I tended to rise, simultaneously the serum levels of E_2 and T appeared to the same phenomenon, during the stages of PN, PY, TY. Interestingly, during the AF stage, the level of P450c17-I mRNA remained to accumulate while the serum level E_2 and T both declined. It has been shown that during final oocyte maturation and ovulation, an elevated P450c17 expression reached to a peak and was linked with the increased production of the maturing inducing hormone 17α, 20β-DHP (Kazeto et al. 2000; Sakai et al. 1992; Sreenivasulu and Senthilkumaran 2009). Taken together, it seems that P450c17-I potentiates during the stages of PY, TY and might influence on the steroidogenic shift during final oocyte maturation and ovulation.

It is now well known that the brain, like the gonad, adrenal and placenta, is a steroidogenic organ. The steroids synthesized by the brain and nervous system, was given the name *neurosteroids.* Furthermore, the enzymes responsible for neurosteroid biosynthesis, has been established unequivocally, which were as same as that in the classic steroidogenic tissues (Mellon et al. 2001). As reported, the enzymes like P450c17, has been found in the brain of rodent embryo (Compagnone et al. 1995), fetuses (Compagnone et al. 2000), newborns (Zwain and Yen 1999) and the adults in mammals (Yu et al. 2002), suggesting P450c17 maybe responsible for the production of neurosteroids. In the present study, we clearly confirmed that P450c17-I expression existed in the female half smooth tongue sole brain throughout the whole reproductive cycle, and changed significantly during the ovary development, suggesting that fish brain may be potential to produce neurosteroids. Similar result has recently been reported in the fathead minnow, although there was no obvious association with the ovarian development (Halm et al. 2003). But in our study, the brain P450c17-I expression appeared to be ovary developmental stage-dependent pattern, to some extent.

In conclusion, a full-length cDNA coding for P450c17-I has been cloned from the *C. semilaevis* ovary. The present study not only substantiates that the ovary are the major tissue for P450c17 gene expression, but also the brain, replacing the kidney. Moreover, our study also detected P450c17-I expression in non-classical steroidogenic tissues, such as stomach, intestine, gill, spleen, but the physiological function in these tissues remains to be studied further. In half smooth tongue sole, the P450c17-I expression pattern appears to be ovarian developmental stage-dependent in both ovary and brain. Furthermore, P450c17-I expressed with significant variation throughout the whole reproductive cycle in half smooth tongue sole ovary and brain. Up to date, the P450c17-I expression in ovary and brain is first linked with serum E_2 and T to work over the relationship between P450c17-I and the ovarian development. The results shows that P450c17-I may regulate the level of androgen and estrogen, and may play a critical role in the shift of steroidogenic pathway from E_2 pathway to 17α, 20β-DHP pathway. Our work is in progress to localize P450c17-I which may form an important basis for supporting the findings on the role of P450c17-I in ovarian development in half-smooth tongue sole fish.

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References

Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE, Miller WL (1987) Cytochrome P450c17 (steroid 17α-hydroxylase/17, 20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. Proc Natl Acad Sci USA 84:407–411

Compagnone NA, Bulfone A, Rubenstein JLR, Mellon SH (1995) Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. Endocrinology 136:5212–5223

Compagnone NA, Zhang P, Vigne JL, Mellon SH (2000) Novel role for the nuclear phosphoprotein SET in transcriptional activation of P450c17 and initiation of neurosteroidogenesis. Mol Endocrinol 14:875–888

Dalla Valle L, Couet J, Labrie Y, Simard J, Belvedere P, Simontacchi C, Labrie F, Colombo L (1995) Occurrence of cytochrome P450c17 mRNA and dehydroepiandrosterone biosynthesis in the rat gastrointestinal tract. Mol Cell Endocrinol 111:83–92

Dalla Valle L, Ramina A, Vianello S, Belvedere P, Colombo L (2002) Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum). J Steroid Biochem Mol Biol 82:19–32

Freking F, Nazairians T, Schlinger BA (2000) The expression of the sex steroid-synthesizing enzymes CYP11A1, 3β-HSD, CYP17, and CYP19 in gonads and adrenals of adult and developing zebra fish. Gen Comp Endocrinol 119:140–151

Goidin D, Mamessier A, Staquet MJ, Schmitt D (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate

dehydrogenase and β-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 295:17–21

Halm S, Kwon JY, Rand-Weaver M, Sumpter JP, Pounds N, Hutchinson TH, Tyler CR (2003) Cloning and gene expression of P450 17α-hydroxylase, 17, 20-lyase cDNA in the gonads and brain of the fathead minnow Pimephales promelas. Gen Comp Endocrinol 130:256–266

He F, Wen HS, Dong SL, Wang LS, Chen CF, Shi B, Mu XJ, Yao J, Zhou YG (2008) Identification of estrogen receptor α Gene polymorphisms by PCR-SSCP and its effect on reproductive traits in Japanese flounder (*Paralichthys olivaceus*). Comp Physiol Biochem Part B 150:278–283

Joy KP, Senthilkumaran B, Sudhakumari CC (1998) Periovulatory changes in hypothalamic and pituitary monoamines following GnRH analogue treatment in the catfish *Heteropneustes fossilis*: A study correlating changes in plasma hormone profiles. J Endocrinol 156:365–372

Kagawa H, Young G, Nagahama Y (1983) Relationship between seasonal plasma estradiol-17β and testosterone levels and in vitro production by ovarian follicles of amago salmon (*Oncorhynchus rhodurus*). Biol Reprod 29:301–309

Kanamori A, Adachi S, Nagahama Y (1988) Developmental changes in steroidogenic response of ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) to chum salmon gonadotropin during oogenesis. Gen Comp Endocrinol 72:13–24

Katagiri M, Tatsuta K, Imaoka S, Funae Y, Honma K, Matsuo N, Yokoi H, Ishimura K, Ishibashi F, Kagawa N (1998) Evidence that immature rat liver is capable of participating in steroidogenesis by expressing 17α-hydroxylase/17, 20-lyase P450c17. J Steroid Biochem Mol Biol 64:121–128

Kazeto Y, Ijiri S, Todo T, Adachi S, Yamauchi K (2000) Molecular cloning and characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. Gen Comp Endocrinol 118:123–133

Kobayashi T, Nakamura M, Kajiura-Kobayashi H, Young G, Nagahama Y (1998) Immunolocalization of steroidogenic enzymes (P450scc, P450c17, P450arom, and 3β-HSD) in immature and mature testes of rainbow trout (*Oncorhynchus mykiss*). Cell Tissue Res 292:573–577

Kumar RS, Ijiri S, Trant JM (2000) Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. Biol Reprod 63:1676–1682

Leguen I, Carlsson C, Perdurand E, Pruner P, Part P, Crdvedi JP (2000) Xenobiotic andsteroid biotrans—formation activities in rainbow trout gill epithelia cells in culture. Aquat Toxicol 48:165–176

Matsunaga M, Ukena K, Tsutsui K (2001) Expression and localization of cytochrome P450 17α-hydroxylase/c17, 20-lyase in the avian brain. Brain Res 899:112–122

Mellon SH, Griffin LD, Compagnone NA (2001) Biosynthesis and action of neurosteroids. Brain Res Rev 37:3–12

Miller WL, Auchus RJ, Geller DH (1997) The regulation of 17, 20 lyase activity. Steroids 62:133–142

Nagahama Y (1997) 17α, 20β-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. Steroids 62:190–196

Nagahama Y, Goetz FW, Tan JT (1986) Shift in steroidogenesis in the ovarian follicles of goldfish (*Carassius auratus*) during gonadotropin-induced oocyte maturation. Dev Growth Differ 28:555–561

Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T, Katsu Y (1995) Regulation of oocyte growth and maturation in fish. Curr Top Dev Biol 30:103–145

Namiki M, Kitamura M, Buczko E, Dufau ML (1988) Rat testis P-450 (17)α cDNA: the deduced amino acid sequence, expression and secondary structural configuration. Biochem Biophys Res Commun 157:705–712

Ono H, Iwasaki M, Sakamoto N, Mizuno S (1988) cDNA cloning and sequence analysis of a chicken gene expressed during the gonadal development and homologous to mammalian cytochrome P-450c17. Gene 66:77–85

Planas JV, Athos J, Goetz FW, Swanson P (2000) Regulation of ovarian steroidogenesis in vitro by follicle-stimulating hormone and luteinizing hormone during sexual maturation in salmonid fish. Biol Reprod 62:1262–1269

Sakai N, Tanaka M, Adachi S, Miller WL, Nagahama Y (1992) Rainbow trout cytochrome P-450c17 (17α-hydroxylase/17, 20-lyase)-cDNA cloning, enzymatic properties and temporal pattern of ovarian P-450c17 mRNA expression during oogenesis. FEBS Lett 301:60–64

Sangalang GB, Freeman HC (1988) In vitro biosynthesis of 17α, 20β-dihydroxy-4-pegnen-3-one by the ovaries, testes, and head kidneys of the Atlantic salmon *Salmo salar*. Gen Comp Endocrinol 69:406–415

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69–81

Senthilkumaran B, Yoshikuni M, Nagahama Y (2004) A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol Cell Endocrinol 215:11–18

Shoonen WGEJ, Lambert JGD, Penders MT, Van Roosmalen ME, Van der Hurk R, HJTh Goos, Van Oordt PGWJ (1989) Steroidogenesis during induced oocyte maturation and ovulation in the African catfish, *Clarias gariepinus*. Fish Physiol Biochem 6:91–112

Sreenivasulu G, Senthilkumaran B (2009) A role for cytochrome P450 17α-hydroxylase/c17–20 lyase during shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. J Steroid Biochem Mol Biol 115:77–85

Sreenivasulu G, Swapna I, Rasheeda MK, Ijiri S, Adachi S, Thangaraj K, Senthilkumaran B (2005) Expression of 20β-hydroxysteroid dehydrogenase and P450 17α-hydroxylase/c17–20 lyase during hCG-induced in vitro oocyte maturation in snake head murrel Channa striatus. Fish Physiol Biochem 31:227–230

Stacey N, Kobayashi M (1996) Androgen induction of male sexual behaviors in female goldfish. Horm Behav 30:434–445

Sun Y, Zhang Q, Qi J, Wang Z, Chen Y, Li C, Zhong Q (2008) Cloning and Expression Analysis of DMRT 1 Gene in *Cynoglossus semilaevis*. J Wuhan Univ (Nat Sci Ed) 54:221–226

Suzuki K, Asahina K, Tamaru CS, Lee CS, Inano H (1991) Biosynthesis of 17α, 20β-dihydroxy-4-pregnen-3-one in the ovaries of gray mullet (Mugil cephalus) during

induced ovulation by carp pituitary homogenates and an LHRH analog. Gen Comp Endocrinol 84:215–221

Trant JM (1995) Isolation and characterization of the cDNA encoding the spiny dogfish shark (*Squalus acanthias*) form of cytochrome P450c17. J Exp Zool 272:25–33

Vianello S, Waterman MR, Dalla Valle L, Colombo L (1997) Developmentally regulated expression and activity of 17α-hydroxylase/C-17, 20-lyase cytochrome P450 in rat liver. Endocrinology 138:3166–3174

Wang Y, Ge W (2004) Cloning of zebrafish ovarian P450c17 (CYP17, 17α-hydroxylase/17, 20-lyase) and characterization of its expression in gonadal and extra-gonadal tissues. Gen Comp Endocrinol 135:241–249

Yu L, Romero DG, Gomez-Sanchez CE, Gomez-Sanchez EP (2002) Steroidogenic enzyme gene expression in the human brain. Mol Cell Endocrinol 190:9–17

Yu H, Cheng H, Guo Y, Xia L, Zhou R (2003) Alternative splicing and differential expression of P450c17 (CYP17) in gonads during sex transformation in the rice field eel. Biochem Biophys Res Commun 307:165–171

Zhou LY, Wang DS, Shibata Y, Paul-Prasanth B, Suzuki A, Nagahama Y (2007a) Characterization, expression and transcriptional regulation of P450c17-I and -II in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun 362:619–625

Zhou LY, Wang DS, Kobayashi T, Yano A, Paul-Prasanth B, Suzuki A, Sakai F, Nagahama Y (2007b) A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology 148:4282–4291

Zwain IH, Yen SS (1999) Dehydroepiandrosterone: biosynthesis and metabolism in the brain. Endocrinology 140:880–887