Aquaculture Research

Aquaculture Research, 2012, 1–11 doi: 10.1111/j.1365-2109.2012.03153.x

Molecular mechanism of P450c17-II (17, 20-lyase) regulating gonad development in female *Cynoglossus semilaevis*

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Abstract

Cytochrome P450c17 (*CYP17*, 17α-hydroxylase/17,20-lyase) is a critical enzyme in the production of androgens and estrogens in vertebrates. A 2102 bp full-length cDNA of P450c17-II (*CYP17A2*) has been isolated from the ovary of half-smooth tongue sole, *Cynoglossus semilaevis* which encodes 524 amino acids. The putative P450c17-II enzyme shares higher sequence identity with those of teleosts than with P450c17-I of vertebrate. The similarity between the two types of tongue sole P450c17 was 48%.Semi-quantitative RT-PCR analysis of spatial expression showed the enzyme was specifically expressed in the ovary and the head kidney. However, temporal expression shows that P450c17-II can be found in the brain. Furthermore, temporal expression pattern of P450c17-II in ovary and brain revealed developmental stage-dependency, and ovary P450c17-II expressed remarkably throughout the whole reproductive cycle. Otherwise, the expression pattern of P450c17-II in head kidney indicated negative ovary development-dependence. In addition, combined with our data on P450c17-I, T and E_2 levels, the results further endorse the critical role of P450c17-II during shift in steroidogenesis, suggesting that P450c17-I and -II may act together to this physiological process. Based on the present study, we indicate an important role for P450c17-II during ovarian development.

Keywords: half-smooth tongue sole, P450c17-II, gene expression, steroidogenic shift

Introduction

Cytochrome P450c17 (*CYP17*), possessing 17α-hydroxylase and 17,20-lyase activities, is critical to the synthesis of C18,C19 and C21steroids in fish head kidney and gonad. Once it was thought that there was only one P450c17 until a novel P450c17 was identified (Zhou, Wang, Kobayashi, Yano, Paul-Prasanth, Suzuki, Sakai & Nagahama 2007; Zhou, Wang, Shibata, Paul-Prasanth, Suzuki & Nagahama 2007). Being boundary, the former one was named P450c17-I, and the later one was named as P450c17-II. Known to date, P450c17-II cDNA has been cloned in several species of fish, such as tongue sole *Cynoglossus semilaevis*, tilapia *Oreochromis niloticus*, medaka *Oryzias latipes*, zebrafish *Danio rerio*, fugu *Takifugu rubripes*, three spined stickleback *Gasterosteus aculeatus* and barfin flounder *verasper moseri*, Japanese flounder *Paralichthys olivaceus*(Zhou *et al.* 2007; Jin, Wen, He, Li, Chen, Zhang, Chen, Shi, Shi, Yang, Qi & Li 2011).

The 17α-hydroxylase activity of P450c17 is indispensible for the biosynthesis of progestogen (17α, 20β- dihydroxy- 4- pregnen-3-one; 17α, 20β-DHP) and adrenal glucocorticoids (cortisol), while 17,20-lyase activity is responsible for the sex steroids (androgen and oestrogen). In general opinion, the vertebrate gonads experience a steroidogenic shift from oestrogens to progestogens during the transition from the follicular phase to luteal/maturation phase. In fish, during the maturation of oocytes, postvitellogenic follicles have to synthesize 17α, 20β-DHP to complete the final

physiological maturation. It is reported that an obvious change of steroidogenic pathways from E_2 to 17α, 20β-DHP has occurred during the ovarian development (Kagawa, Young & Nagahama 1983; Nagahama, Goetz & Tan 1986; Kanamori, Adachi & Nagahama 1988; Shoonen, Lambert, Penders, Van Roosmalen, Van der Hurk, Goos & Van Oordt 1989; Suzuki, Asahina, Tamaru, Lee & Inano 1991; Nagahama, Yoshikuni, Yamashita, Tokumoto & Katsu 1995; Nagahama 1997; Joy, Senthilkumaran & Sudhakumari 1998; Planas, Athos, Goetz & Swanson 2000; Zhou,Wang, Shibata *et al.* 2007; Zhou,Wang, Kobayashi *et al.* 2007; Sreenivasulu & Senthilkumaran 2009). Furthermore, 17α-hydroxy progesterone is synthesized by hydroxylase activity of P450c17, whereas lyase activity acts to the downstream of ovarian growth and maturation in fish. Thus, the regulation of P450c17 activities may be a cardinal factor for the postvitellogenic follicles developing to final maturation stage. And Zhou *et al.* also suggested that P450c17-I is responsible for the synthesis of estradiol-17β (E_2) during oocyte growth, whereas P450c17-II is required for the production of 17α, 20β-DHP in the period of oocyte maturation (Zhou *et al.* 2007). In the present study, the temporal expression of ovarian P450c17-II shows that P450c17-II and -I may act together to this steroidogenic shift, to stimulate the production of maturation-inducing hormone.

The *C. semilaevis* is a native commercially flatfish in China, having great aquacultural potential. The females grow 2–3 times faster than their male counterparts. Furthermore, the weight of ovary is 200–900times higher than the weight of testis. Thus, for better serving the breed of *C. semilaevis*, it is essential to research the female reproductive physiological mechanism. The study aims to investigate the role of P450c17-II in the reproductive cycle of female *C. semilaevis*, as to explore how P450c17 (P450c17-I, P450c17-II) act in the switch of steroidogenic shift.

Materials and methods

The experimental fish

Fish, sampling methods and the biological index were the same as Chen *et al.* (Chen, Wen, Wang, He, Zhang, Chen, Jin, Shi, Shi, Yang, Li, Qi & Li 2010) described. During 1 year, 10 individuals were randomly sampled each time, and there are four times with a period of every 3 months.

Gonadosomatic index and hepatosomatic index and ovarian developmental phases

The Gonadosomatic index (GSI) and hepatosomatic index(HSI) were calculated, and the relationship between GSI/HSI and the ovary developmental phase were seen in Chen *et al.* (Chen *et al.* 2010).

Four phases of ovarian development were also identified, namely perinucleolus stage (PN), primary yolk stage (PY), tertiary yolk stage (TY) and atretic follicles stage (AF). The four phase's photomicrographs of HE-stained *C. semilaevis* ovaries were shown in Chen *et al.* (Chen *et al.* 2010).

Steroid radioimmunoassay

The fish were anaesthetized with MS-222, and blood was withdrawn from the caudal vessel to obtain serum. Serum testosterone (T) and E_2 levels were measured by I^{125} radioimmunoassay (RIA) (He, Wen, Dong, Wang, Chen, Shi, Mu, Yao & Zhou 2008). The relationship between serum steroid hormone level and the ovary developmental phase was shown in Chen *et al.* (Chen *et al.* 2010).

RNA extraction and cDNA synthesis

Total RNA was extracted according to the manufacturer's protocol (Invitrogen, San Diego, CA, USA), and was purified using DNase I (TAKARA, Tokyo, Japan). Subsequently, reverse transcription was carried out using a M-MLV First Strand cDNA Synthesis Kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instruction. The synthesized cDNA product was kept at −20°C for later use.

Rapid amplification of cDNA ends

A core fragment, 554 bp in length was obtained from *C. semilaevis* ovary, using a pair of degenerate primers (CH1, CH2; Table 1) designed by CODEHOP (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). The PCR amplification was performed 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 (*T*a), and 35 s at 72°C with a range of

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

Primer	Sequence(5′-3′)
CH_1	GGCGGCCTGGTGgayathtwycc
CH_2	TGGTGGATGGCCcacatrttnac
GSP_{1-1}	CTCCAAATGCCTCGGCTGCGGTC
GSP_{1-2}	AACCTGTTGACCTTGCTGTTTGACC
GSP_2	GATCCGTCCAGTCAGCCCAGTCC
SP_1	AACTGAAGGACTGCATCTAC
SP_2	TGTTCACTGCCCGTTCAC

*T*a from 60°C to 50°C, decreasing by 1°C each cycle and followed by an additional 22 cycles at a Tn of 50°C, with the PCR finally terminating with an extension of 10 min at 72°C.

The 5′ and 3′ Rapid amplification of cDNA ends (RACE) were performed with Advantage II RACE system (Clontech, Washington, DC, USA) in accordance with the manufacturer's protocol. The Gene specific primers (GSPs) were seen in Table 1. A quantity of 1 μg total RNA from *C. semilaevis* fresh ovary was used as the template for the full-length amplification. The PCR product of the expected length was separated on 1% agarose gel and purified with TIANgel midi Purification Kit (Tiagen, Beijing, China). The purified fragments were then cloned into pMD19-T vector (TAKARA, Japan) followed by propagation in *E. coli* DH5α and subsequently sequenced on an ABI3730XL sequencer (Applied Biosystems, Foster City, CA, USA).

RT-PCR analysis

Semi-quantitative RT-PCR assays were applied to evaluate the spatial expression of P450c17-II. A female fish of TY stage was chosen for the tissue spatial distribution of *CYP17A2*, and during each ovarian developmental stage (PN, PY, TY and AF), four fish were chosen for the temporal expression of *CYP17A2*. It was the same fish described in Chen *et al.* (Chen *et al.* 2010), so was the cDNA template. A couple of specific primers (SP1, SP2; Table 1) were designed to amplify a PCR product of 324 bp.

Semi-quantitative RT-PCR was done in a Biometra Tpersonal Cycler (Goettingen, Germany), and PCR programme was initial denaturation 94°C 5min, followed by 34 cycles each at 94°C for 35 s, 62°C for 35 s, 72°C for 35 s or 18 cycles each (for 18S rRNA, internal control gene; Sun, Zhang, Qi, Wang, Chen, Li & Zhong 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. To confirm the specificity of RT–PCR amplification, its PCR products were sequenced. Electrophoretic images and the optical densities of amplified bands were analysed using Quantity Onev4.62 software (Bio-Rad, Hercules, CA, USA).

Sequence analysis and Statistics

Sequence alignments and comparisons of homology among various P450c17 cDNAs were performed using the European Molecular Biology Open Software Suite (EMBOSS; TUCOWS.COM CO., Mountain View, CA, USA). The alignment of P450c17-II amino acid was carried out using the Clustalw programmes and the phylogenetic tree was reconstructed using the neighbour-joining method of Mega 4.0. Statistical analyses (ENOM, INC., Providence, RI, USA) 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 the full-length cDNA of P450c17-II from *Cynoglossus semilaevis* ovary

The full length of P450c17-II cDNA from the ovary of *C. semilaevis* was 2102 bp (GenBank accession number: EU732518), and its open reading frame was 1572bp, encoding 524 amino acids. Similar to *C. semilaevis* P450c17-I cDNA sequence, two potential polyadenylation signals (AATAAA) were also found in the 3′-untranslational region, but any other alternative transcript was not obtained.

The putative tongue sole P450c17-II enzyme shared high homology with P450c17 enzymes of other species, e.g. barfin flounder P450c17-II (83%), medaka P450c17-II (79%), tilapia P450c17-II (78%), three spined stickleback P450c17-II (72%), Fugu rubripes P450c17-II(72%), zebrafish P450c17-II (67%); three spined stickleback P450c17-I (51%), barfin flounder P450c17-I (50%), tilapia P450c17-I (50%), chicken P450c17-I (50%), Fugu rubripes P450c17-I (49%), zebrafish P450c17-I (49%), dogfish P450c17-I (48%), tongue sole P450c17-I (48%), medaka P450c17-I (47%), human (42%), rat P450c17-I (40%; Chung, Picado-Leonard, Haniu, Bienkowski, Hall, Shively & Miller

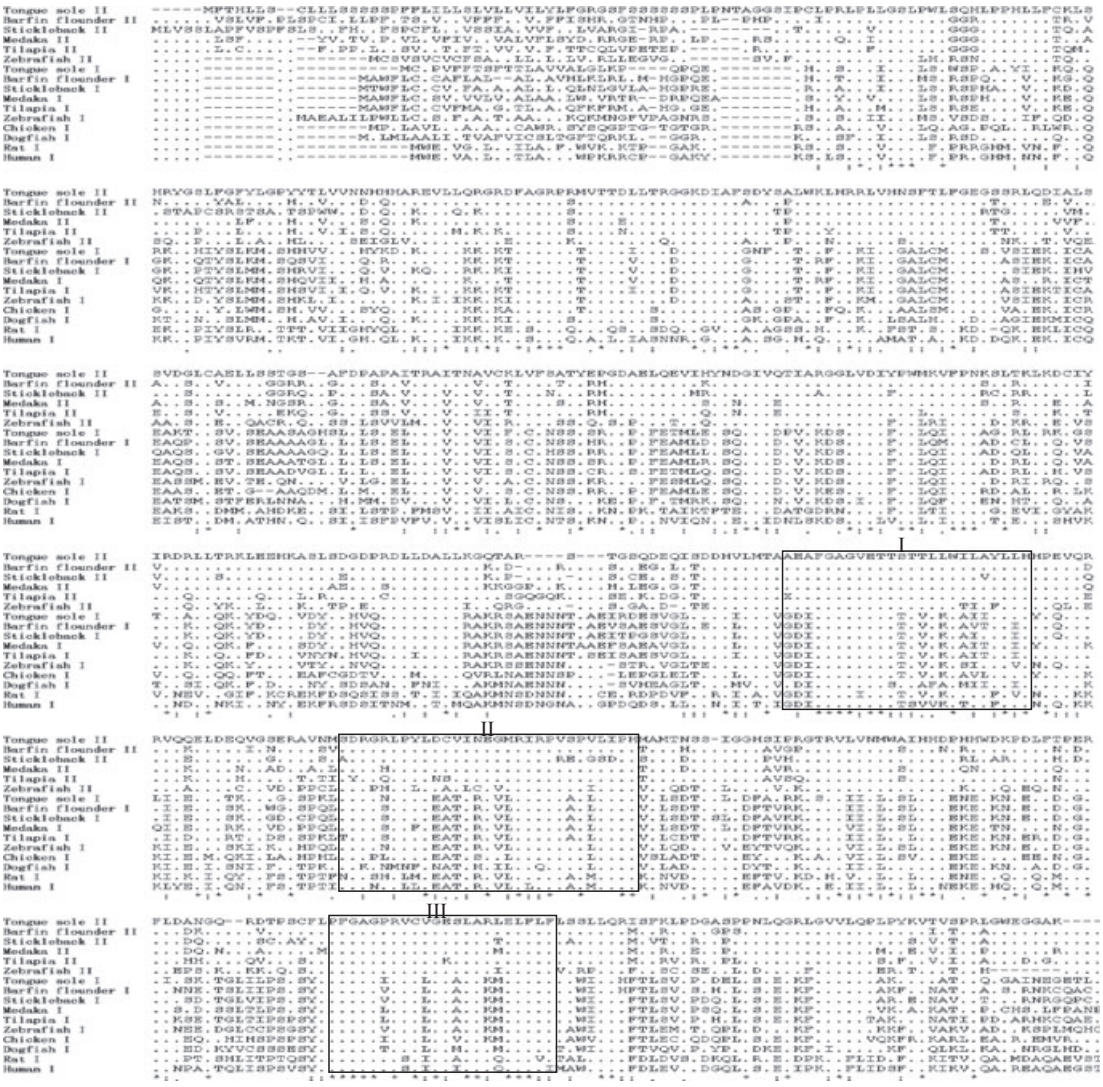

Figure 1 Alignment of tongue sole P450c17-II amino acid sequence with P450c17s amino acid sequences of other 15 animals'. The functional (conserved) regions of P450c17-II are boxed (I: Ono-sequence; II: Ozols_ tridecapeptide region; and III: haem-binding region).

1987; Namiki, Kitamura, Buczko & Dufau 1988; Ono, Iwasaki, Sakamoto & Mizuno 1988; Trant 1995; Wang & Ge 2004; Zhou,Wang, Shibata *et al.* 2007; Zhou,Wang, Kobayashi *et al.* 2007; Fig. 1). The three conserved functional domains of P450c17 were also founded within P450c17-II, as well as tongue sole P450c17-I, including Ono-sequence [I], Ozols' tridecapeptide region [II] and heme-binding region [III] and they were highly conseved between P450c17-I and -II. These three domains are, respectively, considered as the signature sequence of the P450c17s (Ono *et al.* 1988; Wang & Ge 2004), the putative steroid-binding domain of cytochrome P450 family (Ozols, Heinemann & Johnson 1981; Zuber, John, Okamura, Simpson & Waterman 1986) and the binding site for haem-iron which mediates the catalysis of the substrate binding to P450c17 (Gotoh, Tagashira, Iizuka & Fujii-Kuriyama 1983; Miller 2005).

A phylogenetic tree was constructed based on the amino acid sequences of vertebrate P450c17-I and -II, including teleosts, cartilaginous fish, birds and mammals (Fig. 2). As expected, P450c17-II of tongue sole, barfin flounder, three spined stickleback, medaka, tilapia, fugu rubripes clustered into

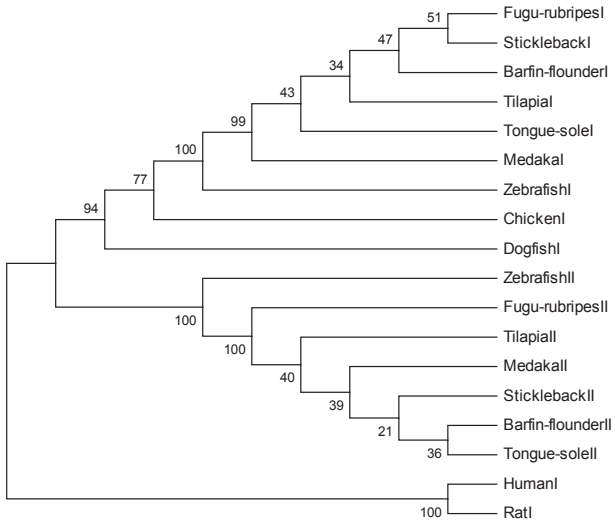

Figure 2 Phylogenetic tree for P450c17-II amino acid sequence. The GenBank accession no. are followed: Tongue sole I, II: 241669074, 268054455; Barfin flounder I, II: 242308763, 242308769; Fugu rubripes I, II: 157412300, 157412294; Medaka I, II: 157311679, 148612550; Tilapia I, II: 156151272, 148612548; Stickleback I, II: 156152290, 156152296; Zebrafish I, II: 170172551, 157785674; Dogfish I: 999088; Chicken I: 50053695; Rat I: 6978731; Human I: 4503195.

one clade, which was distinct from the P450c17-I clades. Tongue sole P450c17-II was found to belong to the teleost P450c17-II clade, and was most close to the barfin flounder P450c17-II, which accorded with the evolution history.

Tissue distribution of P450c17-II gene

The P450c17-II gene expression in different tissues of TY stage females by RT–PCR (Fig. 3) showed to be tissue-specific, merely expressed in ovary and head kidney. The same event existed in the males (data unpublished). As two classic steroidogenic tissues, the expression level of head kidney P450c17-II was much higher than that of ovary P450c17-II. Otherwise, brain P450c17-II expression was not detected. However, the temporal expression of brain P450c17-II gene can be detected throughout the whole ovarian development (seen below, the reasons shown in discussion).

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

The temporal expression of the P450c17-II gene in ovaries and that in brain samples are shown in Fig. 4 during different ovary developmental phases. The expression level of 18S rRNA was much higher than P450c17-II even though the amplification cycles for 18S rRNA were 18 cycles, while the amplification cycles for P450c17-II were 34 cycles. The Densitometric analyses of P450c17-II gene expression was described as the ratio obtained from the density of the band of P450c17-II mRNA expression standardized with that of the 18S rRNA mRNA expression level. During the PN, PY and TY stages, the ovarian P450c17-II expression pattern was similar to that of the CYP17A1, tending to rise with the ovarian development(during the TY stage, the ovarian P450c17-II expression reached the highest level and was about threefold higher than the PN stage and PY stage, $P < 0.05$), whereas

Figure 3 mRNA expression of P450c17-II in various tissues of the female *Cynoglossus semilaevis.* O: ovary; L: liver; St: stomach; I: intestine; Gi: gill; H: heart; Sp: spleen; K: kidney; Hk: head kidney; B: brain; Mu: muscle; M: Marker; N: negative control.

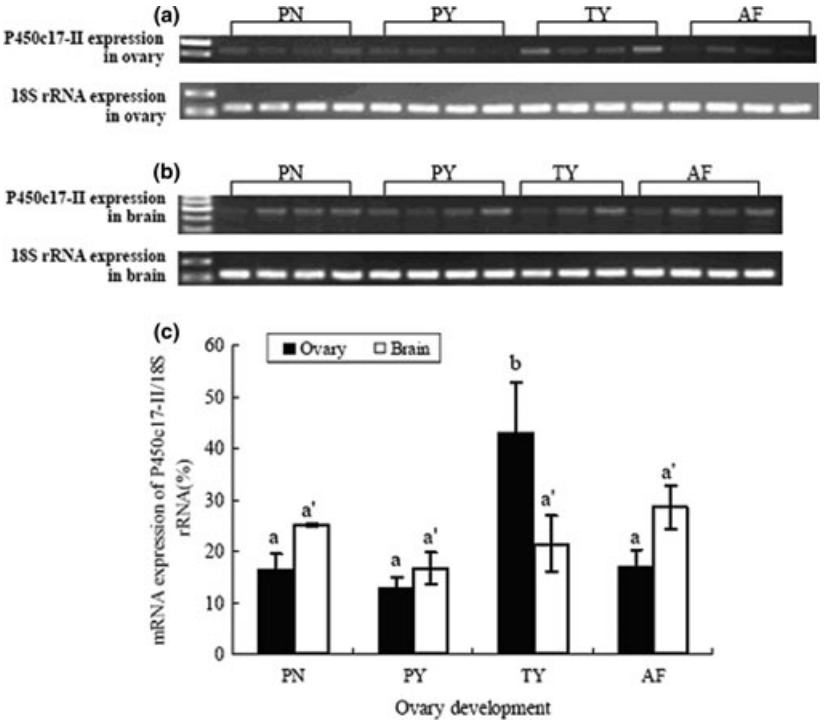

Figure 4 P450c17-II expression in ovary and brain at various development phase. Densitometric analyses of the mRNA expression of P450c17-II. 'mRNA expression of P450c17-II/18S rRNA (%) 'indicates the ratio obtained from the density of the band of P450c17-II mRNA expression standardized with that of the 18S rRNA mRNA expression level.

during the AF stage, the expression of P450c17-II remarkably dropped(about twofold less than the TY stage, $P < 0.05$), while CYP17A1 significantly up-regulated. A similar tendency was observed in the brain P450c17-II expression during PN, PY and TY stages, whereas during the AF stage, the expression of P450c17-II was remarkably increased and reached the highest level. However, no significant difference of the brain P450c17-II expression was observed throughout the ovarian development.

The expression pattern of the P450c17-II gene in female head kidneys was also detected (Fig. 5). The result exhibited that head kidney P450c17-II expressed during all of the various developmental stages, and fluctuated significantly ($P < 0.05$). The expression of P450c17-II was at its peak at the PN stage, then continuously decreased at the stage of PY and TY ($P < 0.05$), followed by marked rise ($P < 0.05$). During the PN stage, head kidney P450c17-II expression was 1.9 fold higher than the PY stage, and 2.87 fold higher than the TY stage. During the AF stage, head kidney P450c17-II expression was up-regulated 2.2 fold higher than the TY stage.

Discussion

In the present study, a full-length cDNA encoding P450c17-II was cloned from *C. semilaevis* ovary. The cloned P450c17-II cDNA was 2, 102 bp in length, encoding 524 amino acids. At 3′-untranslated region, two polyadenylation signals were also found. Similar results could be seen in the rice field eel (Yu, Cheng, Guo, Xia & Zhou 2003), the tongue sole(Chen *et al.* 2010) and the zebrafish (Wang & Ge 2004). The former two species got more than one transcript, but in this article, only one transcript was cloned.

The homology among tongue sole P450c17-II and other vertebrate P450c17s showed that, tongue sole P450c17-II shared higher identity with those of teleosts, followed by teleosts P450c17-I and other species P450c17-I. The similarity between the two types of tongue sole P450c17 was 48%. Consistent with the identity, the phylogenetic tree indicated that tongue sole P450c17-II belonged to the teleost P450c17-II clade, and the latter one was distinct from teleosts P450c17-I clade. This may due to gene

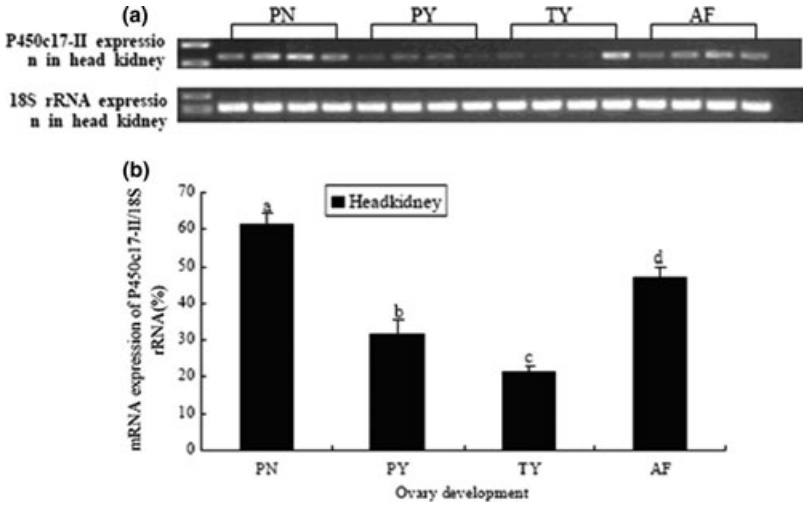

Figure 5 P450c17-II expression in head kidney at various development phase. Densitometric analyses of the mRNA expression of P450c17-II. 'mRNA expression of P450c17-II/18S rRNA (%) 'indicates the ratio obtained from the density of the band of P450c17-II mRNA expression standardized with that of the 18S rRNA mRNA expression level.

tandem duplication and then dispersion during evolution. In common with other animals' P450c17-I, tongue sole P450c17-II was found to possess three functional domains as well, which were well conserved than the other parts of the sequence.

By using RT–PCR, we detected a predominant P450c17-II expression in female head kidney, and under the same amplification cycles, the level of head kidney P450c17-II expression was almost close in female and male (data unpublished). The same result occurred in tilapia (Zhou *et al.* 2007). But the tilapia head kidney only expressed P450c17-II and not the P450c17-I. In our study it appeared that tongue sole kidney and head kidney expressed both types of P450c17 (Chen *et al.* 2010). Besides, a hybridization signal of P450c17 was detected in the whole kidney of the zebrafish (Wang & Ge 2004). This may contribute to the biosynthesis of steroids in head kidney, which was equivalent to the adrenal cortex of mammals. Moreover, studies have shown that the head kidney in teleosts has the capability to produce P450c17 protein that is involved in the biosynthesis of cortisol and 17α, 20β-DHP (Sangalang & Freeman 1988; Kobayashi, Nakamura, Kajiura-Kobayashi, Young & Nagahama 1998; Wang & Ge 2004).

As another classic steroidogenic tissue, gonad also expressed P450c17-II, in accordance with *in situ* hybridization and gene expression results of tilapia and medaka (Zhou,Wang, Shibata *et al.* 2007; Zhou,Wang, Kobayashi *et al.* 2007). *In situ* hybridization analysis showed that both medaka P450c17s were expressed in the follicular cells of the ovary and interstitial cells of the testis. These results indicate that tongue sole gonad is another main tissue for P450c17-II expression and P450c17-II plays a central role in the biosynthesis of sex steroids, as well as P450c17-I.

Surprisingly, the tissue distribution of P450c17-II was not present in tongue sole brain, but its temporal expression was examined in the female brain through all the reproductive stages. This discrepancy may be because of the selection of plateau phase and the ovarian developmental stage. Since the concentration of P450c17-II in gonad and head kidney is far more than that in brain, after the selection of plateau phase, the brain amplification product cannot be detected under certain cycles. Furthermore, the female used for tissue spatial distribution was in TY stage, whereas the brain P450c17-II is still low during the TY stage, not up to a higher value or the peak (shown in Fig. 4). The brain P450c17-II expression is detected for the first time.

As is well known, 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

17α-hydroxylase activity of P450c17 is vital to the two physiological events described above (Nagahama & Adachi 1985). The two steroidogenic pathways (E_2 pathway, 17α, 20β-DHP pathway) commence with progesterone and then branch off into two after the production of 17α-hydroxyl progesterone. One branch is responsible for E_2 biosynthesis, undergoing 17, 20-lyase of P450c17 and 17β-HSD; the other is responsible for 17α, 20β-DHP, under the action of 20β-HSD. An obvious change of steroidogenic pathways from E_2 to 17α, 20β-DHP has been reported during the ovarian development (Kagawa *et al.* 1983; Nagahama *et al.* 1986, 1995; Kanamori *et al.* 1988; Shoonen *et al.* 1989; Suzuki *et al.* 1991; Nagahama 1997; Joy *et al.* 1998; Planas *et al.* 2000; Sreenivasulu & Senthilkumaran 2009), implicating the role of P450c17 on the switch point of the two steroidogenic pathways. Moreover, previous study indicated that p450c17 might play a crucial role in shift in steroidogenesis by controlling the availability of precursor steroids (Sreenivasulu & Senthilkumaran 2009). In the present study, the ovarian temporal expression of *C. semilaevis* P450c17-II also supported this statement. The signal of ovarian P450c17-II expression was detected during all developmental stages. As the ovary developed (GSI tended to increase and the serum E_2 increased), the mRNA expression of P450c17-II gradually tended to rise. In TY stage, its expression remarkably increased to the peak, whereas in AF stage, as it significantly decreased, simultaneously the serum levels of E_2 declined, and the expression of P450c17-I remained to accumulate. Thus, we proposed that during TY stage, P450c17-II expression strongly rose and made the ratio of P450c17 lyase to hydroxylase decline, and caused mass production of precursor 17α-hydroxyprogesterone, contributing to the steroidogenic shift towards 17α, 20β-DHP; whereas in AF stage, the continuously elevated P450c17-I was used to enhance the stability and activity of P450c17 protein, continuously acting to the final spawn of unreleased oocytes. Taken together, the temporal expression patterns of tongue sole ovarian P450c17-II and -I suggests that the two genes may act together to the steroidogenic pathway switch, and tongue sole ovarian P450c17-II expression pattern shows to be ovary developmental stage-dependent.

As steroidogenic organs, the brain is also well known as well as the gonad, adrenal and the placenta. The steroids synthesized by the brain and nervous system were given the name *neurosteroids*. Furthermore, the enzymes responsible for neurosteroid biosynthesis have been identified unequivocally, which were the same as those in the classic steroidogenic tissues (Mellon, Griffin & Compagnone 2001). P450c17 has been found in the brain of rodent embryo (Compagnone, Bulfone, Rubenstein & Mellon 1995), foetuses (Compagnone, Zhang, Vigne & Mellon 2000), newborns (Zwain & Yen 1999) and the adults in mammals (Yu, Romero, Gomez-Sanchez & Gomez-Sanchez 2002), suggesting that P450c17 may involve in the synthesis of neurosteroids. In the present study, we clearly confirmed that P450c17-II expression existed in the female half-smooth tongue sole brain throughout the whole reproductive cycle, and as the ovary developed, the expression of P450c17-II tends to rise but without any significant change, indicating that fish brain may have the ability to produce neurosteroids. In recent reports, brain P450c17 has been examined in other species, including the fathead minnow (Halm, Kwon, Rand-Weaver, Sumpter, Pounds, Hutchinson & Tyler 2003), rice field eel (Yu *et al.* 2003) and zebrafish (Wang & Ge 2004). However, in fathead minnow, brain P450c17 expression has no obvious relation with the ovarian development (Halm *et al.* 2003). In the present study, the brain P450c17-II expression appeared to be ovary developmental stage-dependent, to some extent.

Head kidney of teleost, equivalent to the adrenal cortex of mammals, excretes three types of cortical hormones, namely mineralocorticoids (aldosterone), glucocorticoids (cortisol) and sex steroids. The synthesis of glucocorticoids (cortisol) and sex steroids depends on the hydroxylase activity of P450c17 (Gallo & Civinini 2003). In fish, cortisol can regulate both energy metabolism (under normal and stress environment) and electrolyte balance (Gallo & Civinini 2003), especially important for adapting water environment (Idler & Truscott 1972; Butler 1973; Sandor, Fazekas & Robinson 1976; Henderson & Garland 1980; Donaldson 1981; Hanke & Kloas 1995; Wendelaar Bonga 1997; Mommsen, Vijayan & Moon 1999). There are also many reports on the synthesis of sex steroids in head kidney (Sangalang & Freeman 1988; Schreck, Bradford, Fitzpatrick & Patinõ 1989; Vermeulen, Lambert, Looy & Goos 1994; Vermeulen, Lambert, Teitsma, Zandbergen

& Goos 1995; Kubokawa, Yoshioka & Iwata 2011). Adrenal tissue in African Catfish *Clarias gariepinus* can produce 21steriods, including androstenedione, testosterone, 11-ketotestosterone and 17α, 20β-DHP (Vermeulen *et al.* 1995), whereas the head kidney in Atlantic Salmon *Salmo salar* also has the ability to synthesize 17α, 20β-DHP (Sangalang & Freeman 1988). So, adrenal tissue is presumed another source of sex steroids. In the present study, P450c17-II expression exactly existed in the tongue sole head kidney, and its relationship with gonad development was firstly investigated. The result showed that with ovarian development, head kidney P450c17-II expression tended to decline, suggesting negative ovary development-dependence. Moreover, head kidney P450c17-II expression fluctuated markedly during the whole reproductive cycle. Thus, we presume tongue sole head kidney has the potential to produce cortisol and sex steroids, and the secretion of sex steroids may affect the ovarian development by paracrine.

In sum, a full-length cDNA coding for P450c17-II has been cloned from the *C. semilaevis* ovary. Spatial expression substantiates that as classical steroidogenic tissues, head kidney and gonad are the major tissues for P450c17-II gene expression, while non-classical steroidogenic tissues did not detect its expression, such as stomach, intestine, gill, spleen. The results show that P450c17-II is specifically expressed. Tongue sole P450c17-II expression pattern appears to be ovarian developmental stage-dependent in both ovary and brain, and ovary P450c17-II expressed remarkably throughout the whole reproductive cycle. Up to date, the P450c17-II expression in ovary, brain and head kidney is first linked with serum E_2 and T to work over the relationship between P450c17-II and the ovarian development. The results show that P450c17-II can regulate the level of androgen and oestrogen, and together with P450c17-I, may play a crucial role in the shift of steroidogenic pathway from E_2 pathway to 17α, 20β-DHP pathway by controlling the availability of precursor 17α-hydroxyprogesterone. Our next work is to localize P450c17-II by immunohistochemistry, on the protein level to explore the function of P450c17-II in tongue sole ovarian development and the whole reproductive cycle.

Acknowledgments

This research was supported by National High Technology Research and Development Program of China (2011AA10A403).

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