Large-scale tissue-specific and temporal gene expression profiles in Pengze crucian carp

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ABSTRACT. In the present study, the tissue-specific and temporal gene expression profiles of four catalogues of gonadal development-related genes (sex differentiation-related, steroid receptor, steroidogenic, and structural genes) were detected in nine tissues and during 11 successive developmental stages in the Pengze crucian carp (Pcc) (a triploid mono-female gynogenic fish). The results showed that these target genes exhibited overlapping distributions in various tissues, with the exception of Pcc-vasa and Pcc-cyp17a1. Gene expression profiling of the developmental stages showed that all of the target genes simultaneously reached peak expression levels at 40 and 48 days post hatching (dph). Both 40 and 48 dph appeared to be two key time points associated with the process of

Pcc gonadal development. These data will provide a clear understanding of gene expression patterns associated with the gonadal development-related genes of this gynogenic teleost.

Key words: Gene expression; Gonadal development; Gynogenic Pengze crucian carp; Tissue-specific; Temporal

INTRODUCTION

Pengze crucian carp (*Carassius auratus* var. Pengze; Pcc) can be activated using heterologous sperm from other fish species without nuclear decondensation via artificial propagation methods. It is a naturally gynogenic species that is composed of all-female individuals, and it was identified as a triploid fish. Studies of diploid fish [e.g., zebrafish, medaka, fathead minnow, black porgy (*Acanthopagrus schlegelii*, Wu et al., 2010), rainbow trout, and Atlantic salmon] have demonstrated that fish sex differentiation is oriented towards one of the sexes, and it is largely dependent on the levels of endogenous sex steroid hormones (testosterone for T, 11-ketotestosterone for 11-KT, and 17β-estradiol for E2). Therefore, a change in the balance of steroid hormones can shift the differentiation pathway. E2 plays a vital role in the complete sex reversal of the proandrous hermaphrodite black porgy (Wu et al., 2010). Moreover, lower E2 and high T levels could promote testicular differentiation in hermaphroditic groupers (Zhou and Gui, 2010). Recently, additional research indicated that endogenous E2 is more important for fish sex differentiation (Li et al., 2013a). The longer life cycle and sexual maturation time for triploid fish, compared to the small diploid fish models, implie important differences in the fluctuation of hormone levels, Vtg synthesis, and gonadal maturation (Jensen et al., 2001).

A positive correlation exists between endogenous E2 and aromatase (*cyp19a1a*) gene expression in teleosts. Furthermore, some genes associated with sex differentiation are involved in endogenous E2 production and the gene regulation/modulation of aromatase activities. Anti-Müllerian hormone (*amh*), a member of the transforming growth factor β family, is produced directly by the ovarian follicles (Rodríguez-Marí et al., 2005). Dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X-chromosome gene 1 (*dax1*), an unusual member of the nuclear receptor superfamily, is essential for normal testicular development, and it influences male fertility (Li et al., 2013a). Doublesex and mab-3 related transcription factor 1 (*dmrt1*) is the sex determination gene in reptiles and birds, and its expression was predominantly detected in testes as compared to ovaries (Kobayashi et al., 2008). Forkhead box L2 (*foxl2*) contains a fork-head DNA-binding domain, and it may play a role in ovarian development and function maintenance (Hale et al., 2011; Raghuveer et al., 2011). Nuclear receptor subfamily 5, group A, member 1d (also called steroidogenic factor 1d, *nr5a1b*) helps control gene modulation that is related to the development of gonads (ovaries and testes) and adrenal glands (von Hofsten et al., 2005). SF1 protein controls sexual development in the embryo and at puberty. SRY-box containing gene 9a (*sox9a*) plays an important role in the growth and development process, especially regarding sex determination in male fish (Rodríguez-Marí et al., 2005; Kobayashi et al., 2008). Vasa (*vasa*) is an essential gene for germ cell development, which belongs to the DEAD-box family.

Until recently, two estrogen receptors (ERs) were found in teleosts [ERα (encoded by *esr1*) and ERβ (encoded by *esr2*)], which play vital roles in the fish reproductive system via estrogen signaling pathways (Segner et al., 2013). The androgen receptor (AR, *ar*), a member

of the steroid receptor superfamily, is activated by binding to androgenic hormones, testosterone, and dihydrotestosterone. Among the biochemical pathways in the process of steroidogenesis, steroidogenic acute regulator protein (*star*) takes charge of the regulatory step during acute steroid production for the transfer of cholesterol into pregnenolone across the mitochondrial membrane, which is mediated by the cytochrome P450-mediated side-chain cleavage enzyme (P450scc, *cyp11a1*). 3β-hydroxysteroid dehydrogenase (*3bhsd*) is a key enzyme that catalyzes the synthesis of potent steroid hormones (Arukwe et al., 2008). Both hydroxylase and lyase activity of cytochrome 17α-hydroxylase/17, 20-lyase 1 (*cyp17a1*) are required for the synthesis of testosterone (Sampath Kumar et al., 2000). Furthermore, 11β-hydroxysteroid dehydrogenase 2 (*11bhsd2*) converts 11β-hydroxytestosterone to 11-KT, and 11-KT is the main androgen found in the majority of fish species.

Structural genes may also play a potent role in gonadal development (Wang and Lou, 2006), and a correlation between elevated vitellogenin (VTG) levels and the presence of intersex gonads exists (Jobling et al., 1998). A series of VTG synthesis, maturation, processing (including possible cleavage into yolk proteins, lipovitellin heavy/light chains, and phosvitin), and the activities of three zona pellucida proteins (ZP1-ZP3, the main components of mature oocyte cells) was demonstrated in a female-specific manner between the embryonic and late gonadal development periods (Modig et al., 2006).

In our previous studies, the cDNAs of four catalogues were isolated, including sex-differentiation related genes (*amh*, *dax1*, and *cyp19a1a* in Li et al., 2013a; *dmrt1* in Zheng et al., 2014), steroid receptor genes (*esr1*, *esr2,* and *ar* in Zheng et al., 2013), steroidogenic genes, and structural genes (Zheng Y, Chen JZ, Bing XW, Yang YP, et al., unpublished results). Gene expression profiles in different genders (Hale et al., 2011), tissues, and developmental stages (Ijiri et al., 2008; Shi et al., 2013) may have dual functions in sex determination and differentiation. The present study detected all four gene catalogues associated with tissue-specific and temporal distributions in Pcc. The results will provide a foundation for further investigations of the molecular mechanisms associated with estrogen signaling and steroidogenesis in Pcc.

MATERIAL AND METHODS

Animals and sampling

The eggs of mature gynogenetic Pcc (weight: 450 g) were inseminated with the sperm of the red common carp (*Cyprinus carpio* var. red style, weight: 600 g) to activate eggs during artificial propagation experiments. The collection of parental fish, spawning and spermiation, fish culturing in glass tanks, feeding, and management followed the methods of Zheng et al. (2013, 2014). All fish were euthanized before sampling using 0.1% 2-phenoxyethanol (Sigma-Aldrich, USA). Nine tissues (brain, eye, gill, hepatopancreas, intestine, kidney, muscle, ovary, and spleen) were sampled from one-year-old adult female Pcc (length: 21 ± 2.1 cm; weight: 226 ± 23.2 g; N = 9), and were subsequently used to detect mRNA tissue distribution. RNA samples from 11 developmental stages were collected every four days from 20 until 60 days post hatching (dph) (N = 9 fish/stage). To investigate tissue-specific and temporal mRNA expression patterns of the target genes, all of the samples were frozen in liquid nitrogen and kept individually in frozen tubes at -80ºC until use. Fish were incubated at 24.0° ± 0.5ºC with a photoperiod of 14 h:10 h (light:dark) in 125-L glass tanks with dechlorinated tap water. After 5 dph, fish larvae were fed *Artemia* nauplii

at a rate of 0.1% body weight per day. During the experimental period, the water conditions were set as follows: pH: 7.0 ± 0.5 units; dissolved oxygen: 7.21 ± 0.13 mg/L; total phosphate: 2.19 ± 0.11 mg/L; total nitrogen: 0.54 ± 0.10 mg/L; ammonia nitrogen: 0.42 ± 0.06 mg/L; Mg: 28.45 ± 0.12 mg/L; Ca: 46.43 ± 0.27 mg/L. All of the protocols involving the use of animals were in accordance with approved guidelines of the Animal Care and Use Committee of the Northwest A&F University (Approval No.: 2011ZX08008-002).

RNA isolation and reverse transcription (RT)

Adult female fish samples at different developmental stages were homogenized in Trizol reagent (Invitrogen, USA), and total RNA was extracted and treated with RNase-free DNase I (Fermentas, Canada) to remove genomic DNA contamination as previously described (Zheng et al., 2013). The quality of total RNA was verified on 1% agarose gels via visual inspection of 18S and 28S rRNA bands and by A_{260nm}/A_{280nm} ratios measured with a Nanodrop spectrophotometer (Thermo Electron Corporation, USA). Complementary DNAs (cDNAs) were synthesized from 3 μg total RNA with M-MLV reverse transcriptase and oligo$(dT)_{18}$ primer in a 20-μL final volume.

qRT-PCR and reference gene selecting

qRT-PCR was performed using a CFX96 thermocycler (Bio-Rad, USA) and a SYBR Premix ExTaq II kit (TaKaRa, Japan). The qRT-PCRs were conducted in a final 25-μL volume using 1X SYBR *Premix Ex Taq*™, 0.4 μM each primer, and 2.5 μL RT reaction solution. Cycling parameters were as follows: initial denaturation at 95ºC for 30 s; 40 cycles of denaturation at 95ºC for 5 s and annealing at 60ºC for 30 s. Each individual sample was run in triplicate. A melting curve analysis was performed at the end of each PCR thermal profile to verify the specificity of each amplicon. Analyses of SYBR green I density and determination of threshold cycle (C_t) values were carried out using the CFX Manager software (Bio-Rad, USA). The efficiency (*E*) of each PCR was determined based on the slope generated by a 10-fold diluted cDNA series with five dilution points that were measured in triplicate, and the $E = 10^{(-1/slope)}$ equation was used. The selection of reference genes was based on the methods of Zheng et al. (2013).

Tissue-specific and temporal distributions of Pcc target genes

The mRNA tissue-specific and temporal distributions of four catalogue genes were performed using qRT-PCR. Primers for qRT-PCR are shown in Table 1. The qRT-PCR primers for those genes were designed to avoid the amplification of genomic DNA by spanning intron/exon boundaries, and the mRNA amounts were normalized to the most reliable reference gene. The relative mRNA levels in diverse tissues and stages were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analyses

All data are reported as means ± standard deviation. Data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) prior to any additional analysis. Prior to the analyses, data were log transformed to meet one-way analysis of variance

(ANOVA) assumptions of normality and variance homoscedasticity when necessary. In data sets where there was a significant difference, a Kruskal-Wallis analysis and the Dunn post hoc test were conducted ($P < 0.05$ for significance).

Table 1. Primers for selected reference and gonadal development-related *Carassius auratus* var. Pengze (Pcc) genes.

qRT Primer	Sequence (5' to 3')	Length (bp)
Pcc-*ef1a*-F	TGGAGGTATTGGAACTGTGCC	422
Pcc-*ef1a*-R	CAGATTTGAGAGCCTTGGGGT	
Pcc-*tubulin*-F	CCAGGGCTGTGTTTGTAGACC	144
Pcc-*tubulin*-R	CAATAGTGTAGTGTCCACGGGC	
Pcc-*foxl2*-F	TTTATTTCAGTCAAAAGTCTCGTC	124
Pcc-*foxl2*-R	CAAAGCCATTGCGTCATC	
Pcc-*nr5a1b*-F	GATAAAACCCAGAGGAAACGCT	354
Pcc-*nr5a1b*-R	GCTGGAAGGATGCGAGGA	
Pcc-*sox9a*-F	CTGAGGACGGCAGTGAGCA	452
Pcc-*sox9a*-R	GCTCTTGCTCTCCAGGGCT	
Pcc-*vasa*-F	TACAGGACCCAAGGTTGTCTATG	234
Pcc-*vasa*-R	TGTTTCTGGACAGGAGTAGGCT	
Pcc-*esr1*-F	TACGGCATCAGTAAATCGGGT	111
Pcc-*esr1*-R	GTGCTCCATTCCTTTGTTGCTC	
Pcc-*er alpha2*-F	TAGGCAGCCCTCGCATCTT	155
Pcc-*er alpha2*-R	TGCCTGCTGAGAAGATACCACA	
Pcc-*esr2a*-F	TATCATTATGGTGTGTGGTCGTG	254
Pcc-*esr2a*-R	ATCCTGCCAGAGAATCGTGTC	
Pcc-*esr2b*-F	CACACTCTACGCCGTCTCTGC	134
Pcc-*esr2b*-R	CCCTAACAGCTTTGCATGAGTAA	
Pcc-*ar*-F	ATGGCAGGTTTGATGGAGGG	125
Pcc-*ar*-R	TCCACAAGTGAGGGCTCCATA	
Pcc-*3bhsd* -F	GAGGAATGGAATCCGCAATG	271
Pcc-*3bhsd* -R	CAGAAGTGAGAAGGGCAGAACG	
Pcc-*11bhsd2*-F	GTTTGGCATCATACGGGGC	322
Pcc-*11bhsd2*-R	TGGGGTTGAGGAGAGAGGAGT	
Pcc-*cyp11a1*-F	AGGAGCCCCGAAGGAAAC	139
Pcc-*cyp11a1*-R	ACGACCCATAGCGTACAGACC	
Pcc-*cyp17a1*-F	GGCTGCAGATATTCCCAAATAAAG	144
Pcc-*cyp17a1*-R	TCAGAAGTGCGTCCAAGAGGT	
Pcc-*star*-F	CCACATCCGAAGAAGAAGC	130
Pcc-*star*-R	CTGGTTACTGAGGATGCTGAT	
Pcc-*vtg B*-F	AGCGCACCCTGAGAGCACTC	180
Pcc-*vtg B*-R	AGCCGCCTTCAACATTTCACGT	
Pcc-*zp2*-F	TTCTCTGCACTTAACCTTCAC	214
Pcc-*zp2*-R	CTGCTCACCATCACGCT	

RESULTS

Tissue-specific distributions of sex differentiation-related genes

The tissue distributions of the sex differentiation-related genes in diverse tissues of one-year-old adult female Pcc are shown in Figure 1. In general, mRNA expressions of Pcc-*foxl2* and Pcc-*nr5a1b* in the muscle were significantly higher than those in other tissues (Figure 1A). The highest expression of Pcc-*nr5a1b* transcripts was found in brain and hepatopancreas tissues (Figure 1B). The Pcc-*sox9a* transcripts in the hepatopancreas, intestine, and kidney tissues were significantly higher than those in spleen (Figure 1C). The detected Pcc-*sox9a* transcripts in brain, muscle, and ovary tissues were significantly higher than those in eye and gill tissues. Pcc-*vasa* transcripts were significantly higher in ovary tissues (followed by gill and hepatopancreas tissues) compared to eye, muscle, and spleen tissues. Several tissues, including brain, intestine, and kidney tissues, showed trace expression of Pcc-*vasa* transcripts (Figure 1D).

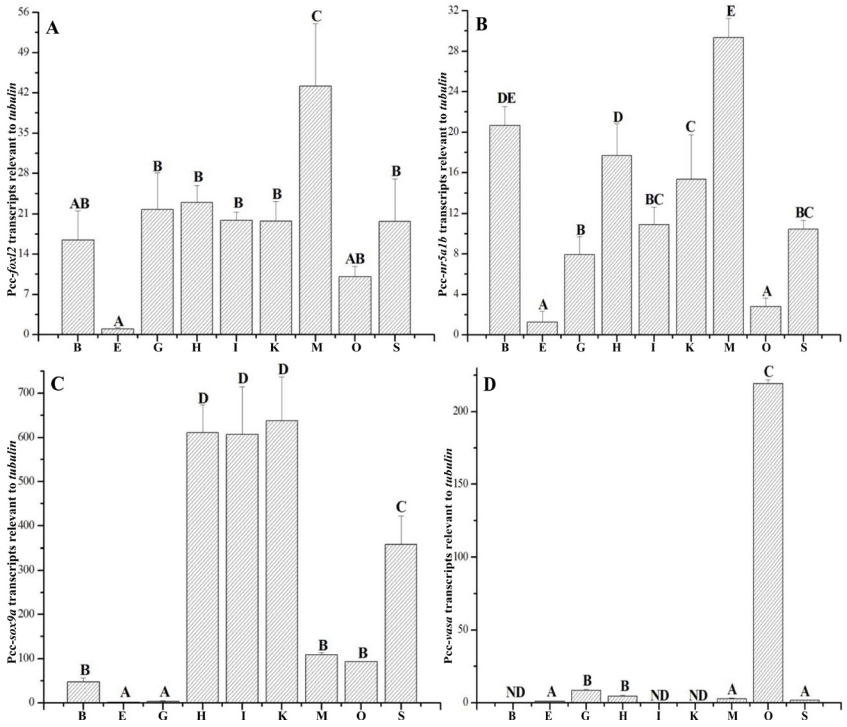

Figure 1. Tissue-specific gene expression profiles of sex differentiation-related genes in nine different tissues. **A.** Pcc-*foxl2*, **B.** Pcc-*nr5a1b*, **C.** Pcc-*sox9a*, and **D.** Pcc-*vasa*. B = brain, E = eye, G: gill, H = hepatopancreas, I = intestine, K = kidney, M = muscle, O = ovary, S = spleen, and ND = not detected. The values were calibrated with the internal control *tubulin*. Each value is reported as means ± SD (N = 9 for each value). The different capital letters indicate highly significant differences ($P < 0.05$).

Tissue-specific distributions of steroidogenic genes

The brain showed higher amounts of Pcc-*3bhsd* transcripts than other tissues (Figure 2A). Moreover, the expression levels in spleen and intestine tissues exhibited significantly higher expression compared with other tissues except for both the hepatopancreas and kidney. The expression of Pcc-*11bhsd2* transcripts in the muscle and spleen tissues was significantly higher than that found in brain tissues (moderately expressed), and those expressed in gill and intestine tissues were significantly higher than those in eye, hepatopancreas, and kidney tissues (Figure 2B). The expression of Pcc-*cyp11a1* transcripts in the hepatopancreas and spleen tissues was significantly higher than that observed in other tissues, with the exception of muscle and ovary tissues (Figure 2C). The Pcc-*cyp11a1* transcripts in the remaining tissues, including gill, intestine, and kidney, were significantly higher than those detected in brain tissues (Figure 2C). Pcc-*cyp17a1* was also predominantly expressed in the ovary compared to other tissues. However, several tissues, such as brain, intestine, kidney, muscle, and spleen, showed trace expression of Pcc-*cyp17a1* transcripts (Figure 2D). The expression of Pcc-*star* transcripts in muscle and spleen tissues was significantly higher than that observed in other tissues (Figure 2E). The ranking order of Pcc-*star* transcripts was hepatopancreas, gill, ovary, kidney, intestine, brain, and eye tissues.

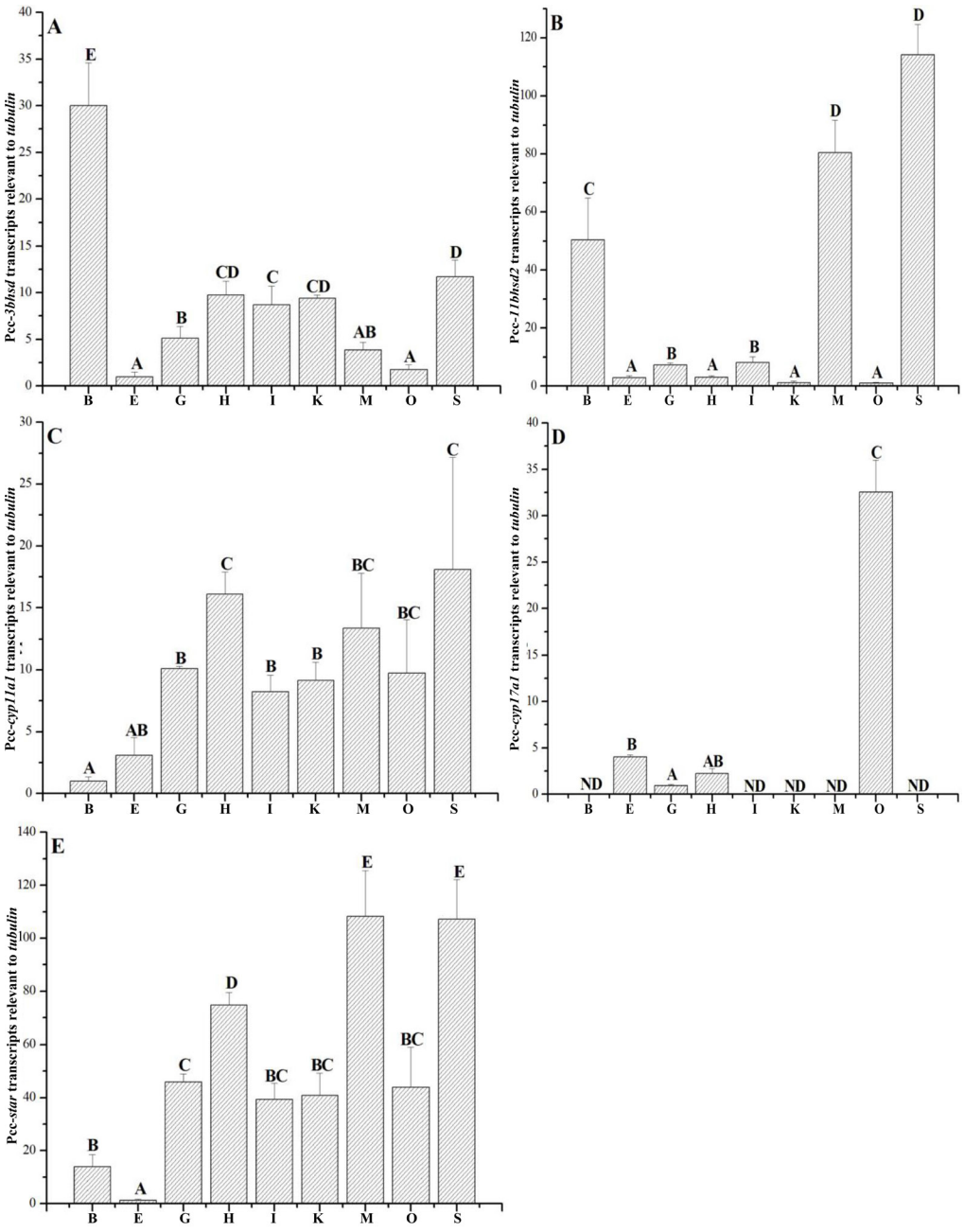

Figure 2. Tissue-specific gene expression profiles of steroidogenic genes in nine different tissues. **A.** Pcc-*3bhsd*, **B.** Pcc-*11bhsd*, **C.** Pcc-*cyp11a1*, **D.** Pcc-*cyp17a1*, and **E.** Pcc-*star*. The different capital letters indicate highly significant differences ($P < 0.05$).

Tissue-specific distributions of Pcc-*zp2*

The Pcc-*zp2* transcripts in the spleen were significantly higher than those in gill, hepatopancreas, intestine, and kidney tissues (Figure 3). Those in brain and ovary tissues were significantly higher than those in eye and muscle tissues.

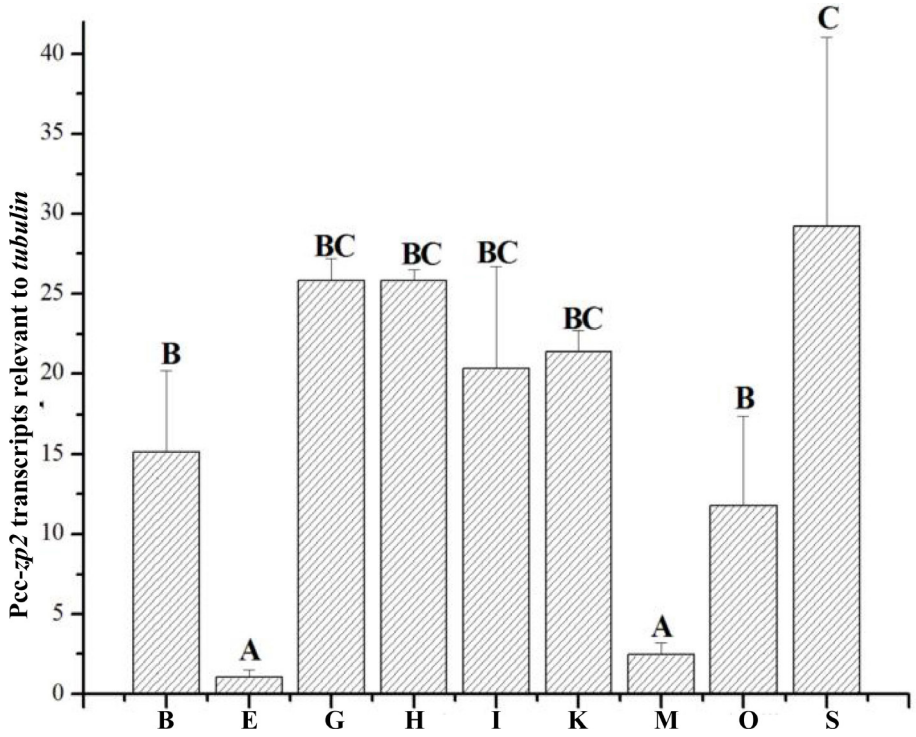

Figure 3. Tissue-specific gene expression profiles of Pcc-*zp2* in nine different tissues. The different capital letters indicate highly significant differences ($P < 0.05$).

Temporal distributions of sex differentiation-related genes

The transcript level of Pcc-*foxl2* was lowest at 24 dph, and it peaked at 48 dph (Figure 4A). The mRNA level of Pcc-*foxl2* persistently increased from 44 to 60 dph, and the Pcc-*nr5a1b* level at 48 dph was significantly higher than that observed at other developmental stages, with the exception of those detected at 44 dph (Figure 4B). The transcript levels of Pcc-*sox9a* were relatively stable during the developmental stages, with significantly higher expression at 40 and 48 dph ($P < 0.05$) compared to other stages (Figure 4C). The Pcc-*sox9a* transcripts at 40 dph were significantly higher than other stages, with the exception of those detected at 48 dph. The transcript level of Pcc-*vasa* was lowest at 20 dph, and it reached the first and second peaks at 24 and 56 dph, respectively (Figure 4D). The Pcc-*vasa* transcripts at 28 dph were significantly higher than those detected at other stages, with the exception of those at 24 and 56 dph.

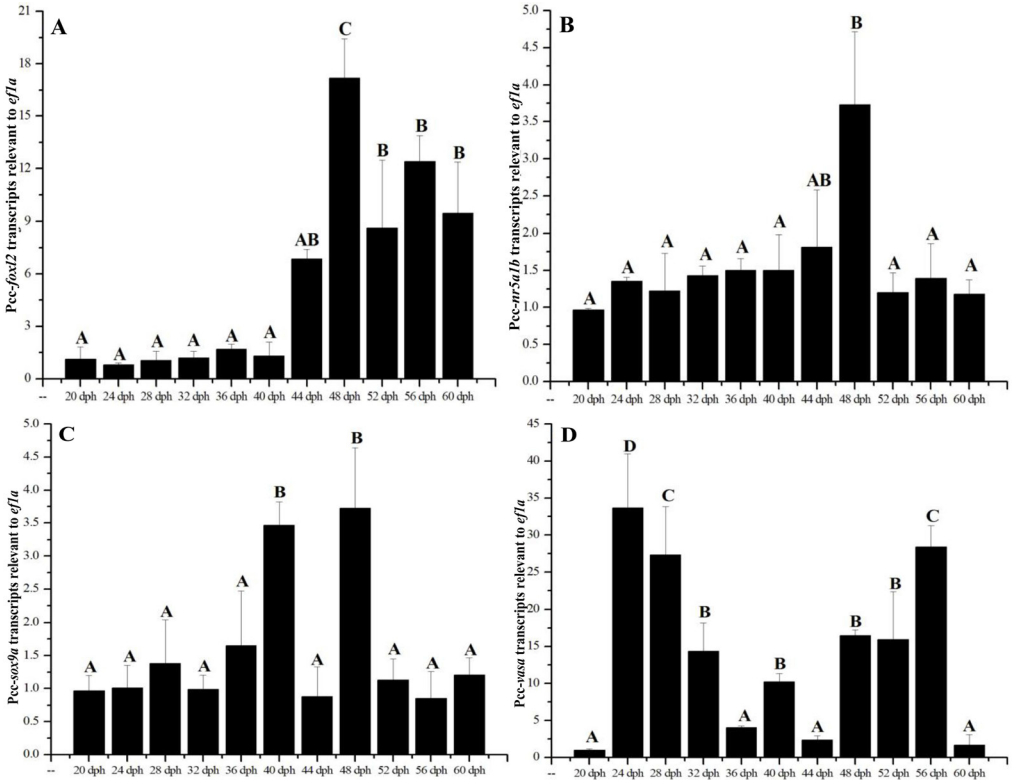

Figure 4. Temporal gene expression profiles of sex differentiation-related genes at 11 different developmental stages. **A.** Pcc-*foxl2*, **B.** Pcc-*nr5a1b*, **C.** Pcc-*sox9a*, and **D.** Pcc-*vasa*. The values were calibrated using the internal control *ef1a*. Transcript abundance is expressed relative to that of the 20 dph stage, and each value is reported as means ± SD (N = 9 for each value). The different capital letters indicate highly significant differences ($P < 0.05$).

Temporal distributions of steroid receptor genes

The transcript level of Pcc-*esr1* peaked at 48 dph ($P < 0.05$, Figure 5A), and its expression was significantly higher than that observed at the second stage rank (24, 32, 40, and 52 dph) and the third stage rank (20, 28, 36, 44, 56, and 60 dph). The highest expression of Pcc-*er alpha2* was found at 56 dph, and trace expression was detected at 24, 28, and 36 dph (Figure 5B). The Pcc-*er alpha2* transcripts at 32 and 40 dph were significantly higher than those at 48 and 60 dph, and it was also significantly higher than those observed at 20, 44, and 52 dph. The transcript levels of Pcc-*esr2a* were relatively stable during the developmental stages, with significantly higher expression at 56 and 60 dph ($P < 0.05$) compared to those at 32, 40, and 48 dph (Figure 5C). Moreover, all of the measurements were significantly higher than those at 20, 24, 28, 36, 44, and 52 dph. The transcript levels of Pcc-*esr2b* showed significantly higher expression at 32 and 40 dph ($P < 0.05$) compared to those observed at 20, 24, 28, and 48 dph, and all of the measurements were significantly higher than those at 36, 44, 52, 56, and 60 dph (Figure 5D). The transcript level of Pcc-*ar* at 40 and 48 dph was significantly higher than those in the second rank (32 and 56 dph), the third rank (20, 24, 28, 52, and 60 dph), and the forth rank (36 and 44 dph; Figure 5E).

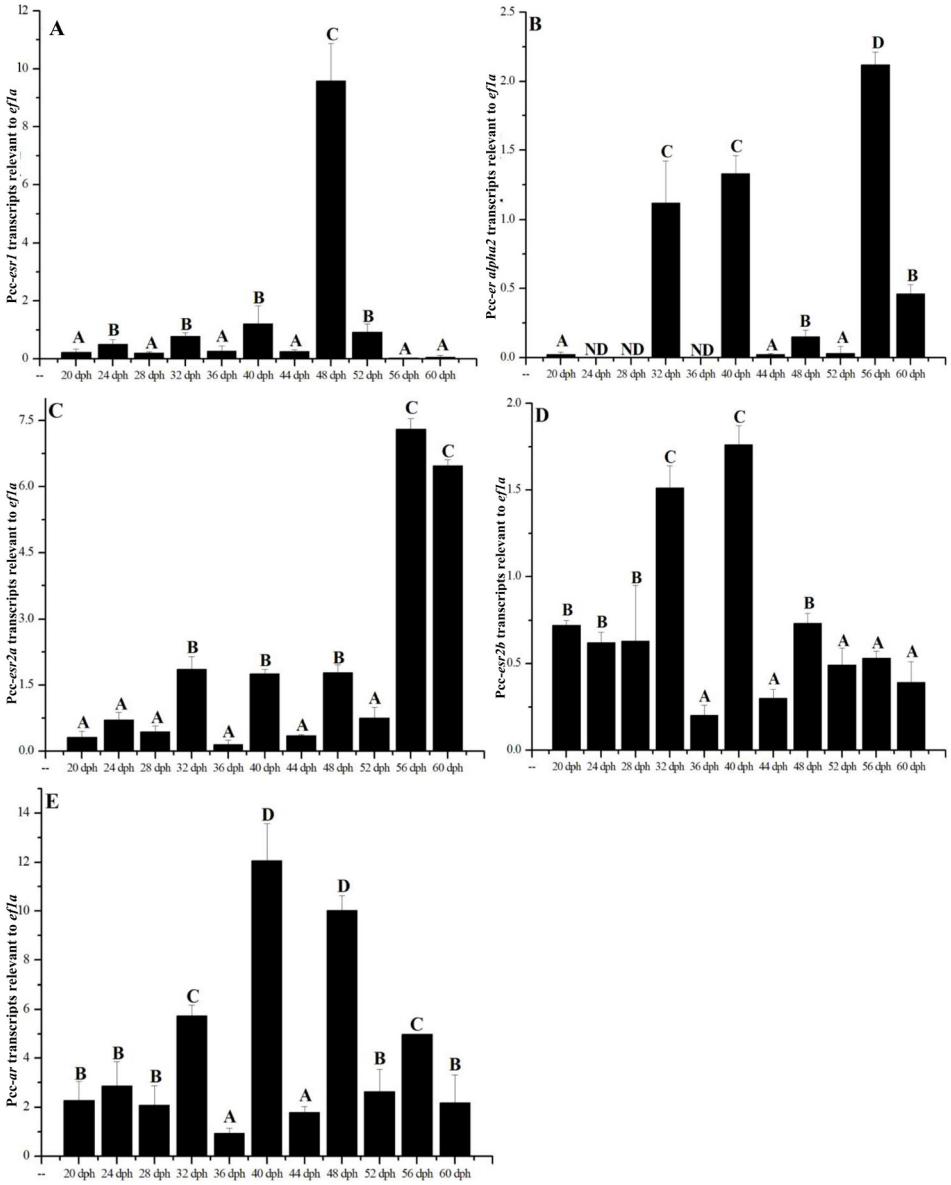

Figure 5. Temporal gene expression profiles of steroid receptor genes at 11 different developmental stages. **A.** Pcc-*esr1*, **B.** Pcc-*er alpha2*, **C.** Pcc-*esr2a*, **D.** Pcc-*esr2b*, and **E.** Pcc-*ar*. The different capital letters indicate highly significant differences ($P < 0.05$).

Temporal distributions of steroidogenic genes

The transcript level of Pcc-*3bhsd* peaked at 20 dph, and expression at 48 dph was significantly higher than those observed at other stages, with the exception of 56 dph (Figure 6A). The highest expression of Pcc-*11bhsd2* was found at 60 dph, and those at 48 dph ranked second.

Moreover, Pcc-*11bhsd2* expression at 40 dph was significantly higher than the remaining stages, with the exception of those at 56 dph (Figure 6B). The Pcc-*cyp11a1* transcripts at stages 44 to 60 dph exhibited significantly higher expression than those at stages 24 to 40 dph (Figure 6C). The transcript levels of Pcc-*cyp17a1* were relatively stable during the developmental stages, with significantly higher expression at 48 and 60 dph ($P < 0.05$) (Figure 6D). The transcript level of Pcc-*star* exhibited significantly higher expression at stages 48 to 60 dph, and the levels peaked at 60 dph. Moreover, Pcc-*star* expression was significantly higher than levels observed at other stages, with the exception of 20 and 44 dph (Figure 6E).

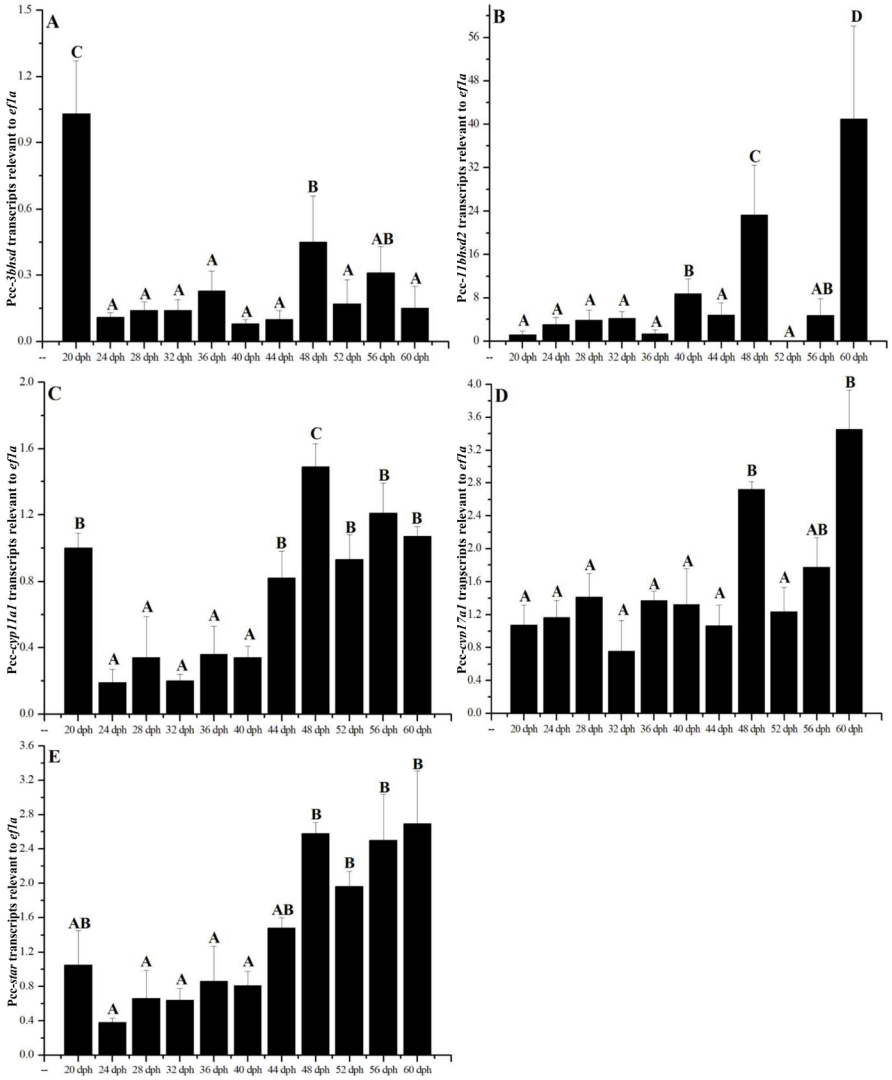

Figure 6. Temporal gene expression profiles of steroidogenic genes at 11 different developmental stages. **A.** Pcc-*3bhsd*, **B.** Pcc-*11bhsd*, **C.** Pcc-*cyp11a1*, **D.** Pcc-*cyp17a1*, and **E.** Pcc-*star*. The different capital letters indicate highly significant differences ($P < 0.05$).

Temporal distributions of structural genes

The transcript levels of Pcc-*vtg B* exhibited significantly higher expression from 48 to 60 dph ($P < 0.05$; Figure 7A) compared to levels measured from 24 to 44 dph. The transcript levels of Pcc-*zp2* exhibited significantly higher expression from 44 to 60 dph ($P < 0.05$; Figure 7B) compared to levels measured from 20 to 44 dph.

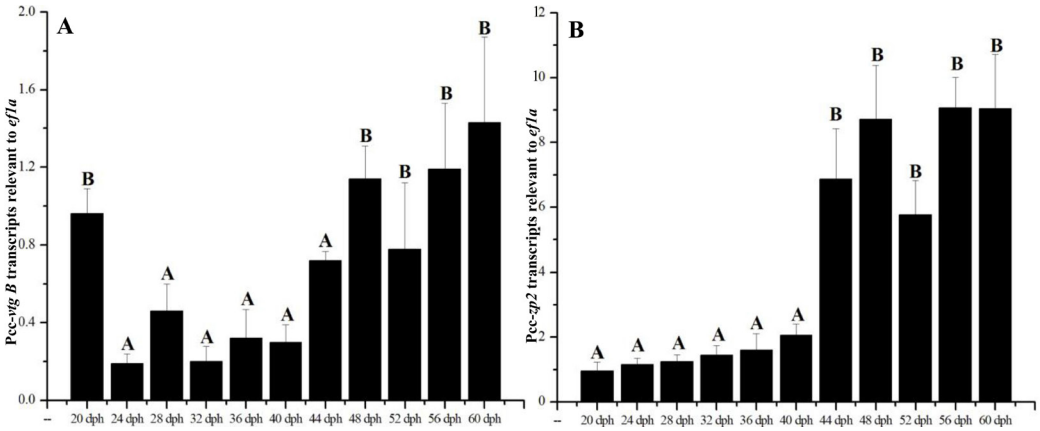

Figure 7. Temporal gene expression profiles of structural genes at 11 different developmental stages. **A.** Pcc-*vtg B* and **B.** Pcc-*zp2*. The different capital letters indicate highly significant differences ($P < 0.05$).

DISCUSSION

Tissue-specific distribution of the target genes

Foxl2 is involved in the regulation of cholesterol and steroid metabolism, apoptosis, reactive oxygen species detoxification, and cell proliferation (Caburet et al., 2012). *nr5a1* is expressed in various tissues, and its transcripts were higher in testes than in ovaries. The results of the present study indicated that Pcc-*foxl2* and Pcc-*nr5a1b* transcripts in both eye and ovary tissues were significantly lower than those detected in other tissues. The actual function of Pcc-*foxl2* and Pcc-*nr5a1b* in gynogenic fish was not determined in the current study. Recently, two subtypes of *sox9* (*sox9a/b*) were found in zebrafish and medaka (Chiang et al., 2001), but reports of gene function in fish *sox9a* are scarce (Rodríguez-Marí et al., 2005). Higher levels of *sox9* were detected in testes than in other tissues, and it could be found in ovary tissues. The present study showed that Pcc-*sox9a* transcripts were detected in most tissues, and levels were significantly higher in kidney, hepatopancreas, and intestine tissues. These results were similar to those observed in other teleosts, which demonstrated the vital role of *sox9* in the development of kidney tissues *vasa* is a biomarker associated with germ cell origination, migration, and proliferation. Zebrafish *vasa* was maternal, and it was persistently expressed during embryonic development. The predominant expression of Pcc-*vasa* transcripts occurred in ovary tissues as compared to other tissues, which suggested the critical role of Pcc-*vasa* in gonadal development.

Steroidogenic genes were found in different tissues (e.g., *11bhsd2* transcripts accumulated in tilapia testis and ovary), and these genes were also found in the testis, liver, kidney,

and gill tissues of catfish (Rasheeda et al., 2010). *cyp11b1* and *cyp11a2* (homologs of *cyp11a1* gene copies) played important roles in androgen synthesis (Parajes et al., 2013), and they are associated with sex differentiation in teleosts (Hsu et al., 2009; Evans and Nunez, 2010). Pcc-*3bhsd* and Pcc-*11bhsd2* transcript levels were significantly higher in brain than in other tissues. Expression levels of Pcc-*cyp11a* and Pcc-*star* in gill, intestine, and muscle tissues were just as high as those observed in kidney and ovary tissues. Moreover, Pcc-*cyp11a1* and Pcc-*star* transcripts were significantly higher in hepatopancreas, muscle, and spleen tissues. *cyp17a1* transcripts were found in gonads of medaka, tilapia, zebrafish, and barfin flounder (Jin et al., 2012), and transcripts were also found in brain and gonadal tissues of half-smooth tongue-sole (Chen et al., 2010) and in both kidney and gonad tissues of Korean rockfish (Mu et al., 2013). *cyp17a1* was found to be a key factor associated with mature oocyte cells, and Pcc-*cyp17a1* transcripts were only found in ovary, hepatopancreas, eye, and gill tissues in the present study, which demonstrated that steroidogenic genes were involved in gonadal development of gynogenic Pcc-*zp*, a female-specific gene associated with ovarian development, was present in ovary tissues before being transported to hepatopancreas tissue (Modig et al., 2006), which contains hepatic-specific and ovarian-specific expression patterns (Wu et al., 2012). Pcc-*zp2* was highly significant in brain, hepatopancreas, kidney, ovary, and spleen tissues, and these results were the same as those reported in the rare minnow. Ovarian-specific *zp2* was found in zebrafish, crucian carp, and goldfish (Mold et al., 2001; Shi et al., 2013), and *zp* was found in both the hepatopancreas and ovary tissues of medaka and pufferfish (Kanamori et al., 2003). The results of the present study suggested that Pcc-*zp2* is associated with the ovarian/hepatic-shared expression pattern.

Time of sexual differentiation in gynogenic Pcc

The sexual differentiation times of zebrafish, *Clarias gariepinus*, and gynogenic Pcc are 19, 30-40, and approximately 20 dph, respectively (Raghuveer et al., 2011). This information revealed the formation of early testes at 10 dph in zebrafish (Jørgensen et al., 2008) and intratesticular efferent duct immersion at 25 dph in tilapia (Kobayashi et al., 2008), which were checked via histological observation. With respect to the time of sexual differentiation for Pcc, it was difficult to define an accurate time without histological verification (even though we attempted to do so in the present study). However, steroidogenic genes were associated with the synthesis of endogenous E2, which guides sexual differentiation. The present study showed that the expression levels of steroidogenic genes (together with sex differentiation-related, steroid receptor, and structural genes), were significantly higher at 40 and/or 48 dph than at other time points, with the exception of Pcc-*vasa*, Pcc-*er alpha2*, Pcc-*esr2a*, Pcc-*3bhsd*, and Pcc-*11bhsd2*. *3bhsd* is associated with the conversion from pregnenolone into progesterone (Arukwe et al., 2008; Evans and Nunez, 2010). Moreover, it is predominantly expressed in the mitochondrion, which is active in the early developmental stages of fish. The amount of steroidogenic genes persistently increased from 44 to 60 dph, which suggested that steroidogenesis was active from 44 dph, and 40 and 48 dph were two key time points based on our previous studies (Li et al., 2013a; Zheng et al., 2014).

Temporal distribution of the target genes

Some research focused on the gene expression profiles of gender-specific genes to discover the key time points of sexual differentiation in different teleosts (Rodríguez-Marí et al.,

2005; Jørgensen et al., 2008; Kobayashi et al., 2008). We collected the steroid receptor gene expression data reported in European seabass (Blázquez and Piferrer, 2005), fathead minnow (Filby and Tyler, 2005), goldfish (Nelson and Habibi, 2010), rainbow trout (Hale et al., 2011), rare minnow (Wang et al., 2011), and loach (Zhang et al., 2012), and we found that most steroid receptor genes reached the highest expression levels at different time points. The results of the present study showed that steroid receptor genes simultaneously peaked at 48 dph, and were associated with active steroidogenesis from 48 to 60 dph. Furthermore, we suggested that steroid receptor and steroidogenic genes were associated with the ovarian development of gynogenic Pcc, and that they were involved in the maintenance of endogenous steroid hormones.

Fish sex differentiation is oriented towards one of the sexes, and it is largely dependent on endogenous sex steroid hormone levels. Therefore, a change in the balance of steroid hormones can shift the differentiating pathway. A positive correlation exists between endogenous E2 and *cyp19a1a* gene expression in teleosts (Shanthanagouda et al., 2012). Some transcriptional factors (e.g., sex differentiation-related genes in the present study) were found to mediate endogenous E2 synthesis by directly or indirectly regulating *cyp19a1a* transcripts. *sox9a* and *foxl2* were associated with the battle of sexes and the control of endogenous steroid hormone balance. Recently, *cyp19a1a* was found to be regulated by *dmrt1* and its antagonistic gene, *foxl2* (Li et al., 2013b), and *foxl2* was confirmed as a *cyp19a1a* regulator. In the present study, the sex differentiation-related genes (Pcc-*foxl2*, *nr5a1b*, and *sox9a*) were significantly higher at 48 dph compared with other time points, which indicated that 48 dph was the turning point. The other three genes (Pcc-*amh*, Pcc-*dax1*, and Pcc-*cyp19a1a*) were reported in our previous study (Li et al., 2013a), and the floating changes in these gene expression patterns may be due to different types of reproduction. For example, the diploid zebrafish represents a proterogynous fish that undergoes the conversion from the female to the male phenotype, while the triploid Pcc represents a gynogenic fish.

Conflicts of interest

The authors declare no conflict of interest.

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