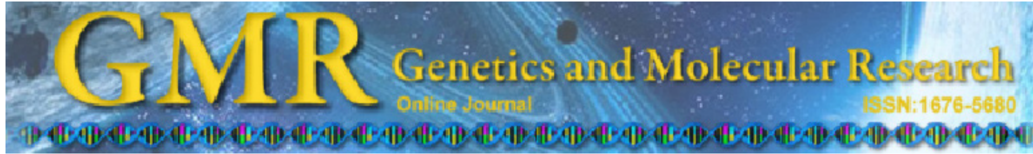

Expression pattern of the zona pellucida 3 (*ZP3*) gene during ovarian development and the location of ZP3 protein in oocytes in a natural, wild triploid crucian carp mutant, *Carassius auratus* var. Pingxiangnensis

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ABSTRACT. *Carassius auratus* var. Pingxiangnensis (designated *CaP*), distributed in the Pingxiang region of Jiangxi Province, China, is a natural, wild triploid crucian carp mutant that has two reproductive development modes: gynogenesis and bisexual reproduction. Little information is available about the expression pattern of the zona pellucida 3 (*ZP3*) gene during ovarian development and the location of the ZP3 protein in oocytes of this fish. In this study, we obtained the full-length cDNA of *ZP3* (*CaP_ZP3*). *CaP_ZP3* contains an open reading frame of 1305 bp that encodes 435 amino acid residues. Real-time polymerase chain reaction (PCR) was used to determine the *CaP_ZP3* mRNA expression levels in the ovary at different stages of maturation. Results revealed high levels of *CaP_ZP3* expression

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in 4- to 8-month-old ovaries (stage II-stage III), with a significant decline in 9- to 12-month-old ovaries (stages IV-stage V). The high levels of *CaP_ZP3* transcripts during the early growth period suggest an important role for *CaP_ZP3* in early oocyte development. In addition, a polyclonal antibody was prepared against CaP_ZP3, and the immunofluorescence localization was determined. CaP_ZP3 protein was detected close to the oocyte plasma membrane. The results also showed that no fluorescent signal was detected in stage I and II oocytes. CaP_ZP3 protein is primarily detected in stage III oocytes, and the protein accumulates as oocytes develop into stage IV oocytes. These results suggested that the transcription and translation of the *CaP_ZP3* gene is asynchronous and that the transcription of the CaP_ZP3 protein occurs prior to its translation in this triploid fish.

Key words: *Carassius auratus* var. Pingxiangnensis; *ZP3*; Expression analysis; Real-time PCR; Immunofluorescence

INTRODUCTION

The zona pellucida (ZP) enclosing vertebrate oocytes is important in the processes of regulating the sperm-egg interaction, preventing polyspermy, and protecting the embryo during the successive stages of development (McLeskey et al., 1998). The ZP is composed of three kinds of glycoproteins encoded by multiple gene families including *ZP1*, *ZP2*, and *ZP3* (Wassarman, 1999, 2008). ZP proteins have a conservative homologous region known as the ZP domain (Bork and Sander, 1992). The ZP domain is thought to be involved in protein-protein interactions and is essential for ZP assembly (Qi et al., 2002). The ZP domain of ZP3 contains eight conserved Cys residues (Bork and Sander, 1992). Furthermore, *ZP3* has been identified to regulate vertebrate fertilization (Barros et al., 1996; Snell and White, 1996), and it serves as a primary sperm receptor and induces the acrosome reaction in mice (Wassarman et al., 2004).

Oocyte growth and development are important issues in fish growth (Lubzens et al., 2010). Oocytes in all teleosts undergo the same basic pattern of growth: oogenesis, primary oocyte growth, cortical alveolus stage, vitellogenesis, maturation, and ovulation (Tyler and Sumpter, 1996). Morphological changes in developing oocytes have been described for several species of teleosts (Chen et al., 2010; Mohamed, 2010). Detailed information about the histological events accompanying ovarian development in *Carassius auratus* var. Pingxiangnensis can be found in reports from Hong (2005) and Wu et al. (2009), where 1- to 2-month ovaries (primordial germ cells), 3-month ovaries (stage I), 4- to 5-month ovaries (stage II), 6- to 8-month ovaries (stage III), nine- to 10-month ovaries (stage IV), and 11- to 12-month ovaries (stage V) were studied and analyzed.

ZP3 cDNA molecules have already been characterized from some fishes, including *Oryzias latipes* (Murata et al., 1995), *Sparus aurata* (Modig et al., 2006), *Danio rerio* (Wang and Gong, 1999; Liu et al., 2006), *Cyprinus carpio* (Chang et al., 1996), *C. auratus gibelio* (Fan et al., 2001), *Cynoglossus semilaevis* (Sun et al., 2010), and *Acipenser sinensis* (Chuang-Ju et al., 2011). These studies have improved our understanding of the structural homology and tissue-specific expression of the *ZP3* gene among fish. However, the expression profile of *ZP3* mRNA during ovarian development and the cellular localization of ZP3 protein in oocytes have not been examined in the special triploid *C. auratus* var. Pingxiangnensis

(*CaP*). Understanding the molecular events of the *ZP3* gene during oogenesis is a key step to determining the secret of “high-quality” eggs for aquaculture. In this study, the full-length cDNA of *CaP_ZP3* was analyzed. In order to elucidate the *CaP_ZP3* mRNA expression pattern during ovarian growth, we investigated the expression level of the *CaP_ZP3* gene in 1- to 12-month-old ovaries. An immunofluorescence assay using a fluorescein isothiocyanate (FITC)-labeled antibody specific to CaP_ZP3 protein was carried out to further determine the cellular localization of ZP3 protein in oocytes in the mutant.

MATERIAL AND METHODS

RNA extraction and SMART cDNA synthesis

One- to 12-month-old triploid fish were collected from the Pingxiang Fisheries Research Institute of Jiangxi Province in China. *CaP* was identified to be triploid *Carassius auratus* by DNA content measurement and chromosome analysis (Hong et al., 2005). Artificial propagation experiments indicated that *CaP* could reproduce by gynogenesis (Hong et al., 2005). Total RNA from the ovary was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA). After the DNase treatment, total RNA (5 μg) extracted from the ovary was used to synthesize the 1st-strand cDNA using the SMART™ cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) (Zeng et al., 2012).

Cloning and sequencing

A pair of degenerate primers was designed according to the conserved region among the known *ZP3* genes that were available from the GenBank database (AF180465, *C. auratus*; Z48974, *C. auratus*; L41636, *C. auratus*; Z48972, *C. carpio*; and L41638, *C. carpio*). A partial cDNA sequence that was homologous to *ZP3* genes in other species was obtained, and some gene-specific primers were designed for rapid amplification of cDNA ends (RACE). The full-length *CaP_ZP3* cDNA was amplified by 3'- and 5'-RACE (Clontech, Mountain View, CA, USA). The amplified cDNA was subcloned into the pMD19-T vector (Takara, Otsu, Japan) and sequenced.

Data and sequence analysis

All of the primers were designed by the Premier 5.0 and Oligo 6.0 software. The glycosylation sites and the putative signal peptide were predicted using YinOYang 1.2 and Signal P at http://www.cbs.dtu.dk/services/SignalP (Nielsen et al., 1997). Multiple alignments were performed with DNASTAR software. Phylogenetic analysis was performed using the MEGA4.1 software by bootstrap analysis.

Real-time polymerase chain reaction (PCR)

Prior to cDNA synthesis, DNase treatment with RQ1 RNase-free DNase (Promega) was carried out to eliminate the genomic DNA contamination following the manufacturer instructions. The RNA was reverse-transcribed using M-MLV Reverse Transcriptase (RT) and oligo(dT)$_{18}$

(Promega) following the manufacturer protocol. Samples were assayed in triplicate, and each experiment was repeated three times. The reaction mixture (25 μL) contained 1X SYBR® Premix EX Taq™ PCR Mix (Takara, Dalian, China), 1 μL template cDNA, and 0.2 μM each primer, with ddH_2O added to reach 25 μL (Zeng et al., 2012). Quantitative real-time PCRs were performed in a 96-well microtiter plate (Applied Biosystems, California, America). The primer pairs, denoted CaP_ZP3.1F and CaP_ZP3.1R (Table 1), were designed to detect the differential expression pattern of CaP_ZP3 mRNA. Primers (CaP_β-actin-F and CaP_β-actin-R) for the detection of the ubiquitously expressed gene β-actin as a control gene are also given in Table 1.

Recombinant CaP_ZP3 protein expression

A pair of primers (CaP_ZP3F and CaP_ZP3R) (Table 1) were used to amplify a 300-bp cDNA fragment. The amplified fragment was initially cloned into the pGEM-T vector (Promega). The plasmid containing *CaP_ZP3* was cut with *Nco*I and *Hin*dIII and subsequently subcloned into the pET-32α plasmid. Recombinant CaP_ZP3 protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer in *Escherichia coli* BL21(DE3) cells (Mate et al., 2003). The recombinant CaP_ZP3 proteins were purified using nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen, GmbH, Hilden, Germany) as described previously (Kaul et al., 1997).

Preparation of polyclonal antibody and enzyme-linked immunosorbent assay (ELISA)

Table 1. Sequences of the primers used in this study.

Primers	Sequence (5'-3')	Product size (bp)
Cap_ZP3.1F	TGTTCCAGTACCGAGTG	171
Cap_ZP3.1R	CATCCTCCCAAAGTCAGACC	
Cap_β-actin-F	CACTGTGCCCATCTACGAG	200
Cap_β-actin-R	CCATCTCCTGCTCGAAGTC	
Cap_ZP3F	GAATTCCATATGCGCAGCCAAAGTCCAAGC	300
Cap_ZP3R	GGATCCTTAAAGCTTACTGCATTGAACAGCCAC	

Two New Zealand male white rabbits were injected subcutaneously at multiple sites using about 300 μg ZP3 fusion protein emulsified in complete Freund's adjuvant (CFA). After 2 weeks, the rabbits were boosted intramuscularly with an equivalent amount of ZP3 fusion protein in incomplete Freund's adjuvant (IFA). After 2 months, the blood was collected. The reactivity of anti-ZP3 protein antibodies was tested by ELISA (Li et al., 2008).

Immunofluorescence localization

The mature ovaries at different stages were embedded in optimal cutting temperature (OCT) (Reichert-Jung, Heidelberg Germany), and 10-μm-thick sections were obtained by the frozen microtomy method (Liu and Gui, 2005). The cryostat sections were rehydrated in phosphate-buffered saline (PBS) for 10 min and incubated for 1 h with 5% dry milk in PBS at room temperature. The sections were then incubated with the anti-ZP3 protein antiserum (1:100 dilution) overnight at 4°C. After washing with PBS, the sections were incubated for 1 h with a FITC-conjugated secondary antibody, goat anti-rabbit IgG (Zhong Shan Jin Qiao, Beijing, China), and washed

with PBS. Finally, the sections were observed with a fluorescence microscope (Leica, Heidelberg, Germany). Control sections were treated with pre-immune serum as the primary antibody.

RESULTS

Cloning and sequence analysis of the full-length cDNA of *CaP_ZP3*

The cDNA sequence and deduced amino acid sequence of *CaP_ZP3* were shown in Figure 1. Its nucleotide sequence was deposited on GenBank (accession No. JN669426). The full-length *CaP_ZP3* cDNA was 1347 bp (poly (A) tail excluded) and consisted of a 21-bp 5'-untranslated region (UTR), a coding sequence of 1308 bp, and a 56-bp 3'-UTR. The putative signal peptide was located between amino acid positions 1 and 21. The mature peptide contains two putative O-linked glycosylation sites at residues Thr 230 and Thr 237 (Figure 1). The *CaP_ZP3* domain contained eight conserved cysteine residues, which is consistent with other corresponding regions in fish (Figure 2).

Phylogenetic analysis of CaP_ZP3

```
1      ATTGTGTTAACGTTTGTCAGG---------------------------------------
22     ATGGGTCTTTTGCAATATGTGTTAGTGCTGGTTGTGCTTGTGGTGTTTGATCTGAAGAAT
1      M  G  L  L  Q  Y  V  L  V  L  V  V  L  V  V  F  D  L  K  N
82     GCTTTTGGAAGTTTGAGATCCAGTCAAAGTCCAAAAAGCAAGAAGCATCAATCATATCCA
21     A  F  G  S  L  R  S  S  Q  S  P  K  S  K  K  H  Q  S  Y  P
142    GCTTCCAGAGTGCCTGTTTCTTCTCAAGTGCTCGGAAACGCTTTGCAGAAGGCTTCTCTG
41     A  S  R  V  P  V  S  S  Q  V  L  G  N  A  L  Q  K  A  S  L
202    TCTCAGAGTCTTGACTACAGAGGATTTGCACAAGAGCCTCTTGGTCTTCAGGAGAAGCAG
61     S  Q  S  L  D  Y  R  G  F  A  Q  E  P  L  G  L  Q  E  K  Q
282    GTGTTGCAGGGTCCAGTGAAGCCTTTGGACTGGAGGTTTCCCACTGTTCCAGAAGTGCCG
81     V  L  Q  G  P  V  K  P  L  D  W  R  F  P  T  V  P  E  V  P
322    AGTGAGATGGCGGTGGACTTCCATTTGAGGCAACCTGTGACTCCCAGTAGTGTAGCTATT
101    S  E  M  A  V  D  F  H  L  R  Q  P  V  T  P  S  S  V  A  I
382    CAATGCGGTGAGAACCGGGTTCATGTGGAGGTACAGCAGGACTTGTTTAGCAATGGTGAA
121    Q  C  G  E  N  R  V  H  V  E  V  Q  Q  D  L  F  S  N  G  E
442    CTGATCCAGCCATCTGGTCTGACTTTGGGAGGATGTCCTGTTGTCGGTTTGGTCCCAGGC
141    L  I  Q  P  S  G  L  T  L  G  G  C  P  V  V  G  L  V  P  G
502    TCTAAGGTGCTCTTCTTTGAGAATGAACTGCAGGACTGCAACAGTGTCTTGATGATGACC
161    S  K  V  L  F  F  E  N  E  L  Q  D  C  N  S  V  L  M  M  T
562    AAGGATGAGCTTGTCTACACCTTTGCCCTTACCTACACTCCTGAGGCGTTTGCTGGCACT
181    K  D  E  L  V  Y  T  F  A  L  T  Y  T  P  E  A  F  A  G  T
622    CCGATTACCCGTGCAGGTGGTGCAGTTATTGGAGTTCAATGCCACTATCAAAGGTTTCAA
201    P  I  T  R  A  G  G  A  V  I  G  V  Q  C  H  Y  Q  R  F  Q
682    AATGTCAGCAGTAGTGCCTTGAAGCCAACTTGGGTCCCTTATGCCTCAACGGAGGCTGGT
221    N  V  S  S  S  A  L  K  P  T  W  V  P  Y  A  S  T  E  A  G
742    GAAGAAGTCTTGGTGTTCTCCCTGAAGCTCATGACTGATGACTGGTCCTATGAGAGGCCT
241    E  E  V  L  V  F  S  L  K  L  M  T  D  D  W  S  Y  E  R  P
802    TCAAACTCTTACTTCCTGGGTGACGTTATTAATGTTGAGGCATCTGTGAAGGTATACAAC
261    S  N  S  Y  F  L  G  D  V  I  N  V  E  A  S  V  K  V  Y  N
862    CACGTCCCTCCGCGTGTGTTTGTGGACAGCTGTGTGGCCACCCAAGTACCTGATGTGAAC
281    H  V  P  P  R  V  F  V  D  S  C  V  A  T  Q  V  P  D  V  N
922    GCCCTTCCGAGATATTTGTTCATTGAGAATCATGGATGTCTTGTGGATGCCAAGGTCACC
301    A  L  P  R  Y  L  F  I  E  N  H  G  C  L  V  D  A  K  V  T
982    GCTTCCAGCTCGCGCTTCATGCCTCGATCCCAGGAAGACAAAATCCGGTTCCAGCTGGAG
321    A  S  S  S  R  F  M  P  R  S  Q  E  D  K  I  R  F  Q  L  E
1042   GCCTTCATGTTCCAGGGGGGATCCAGTCCTTCTATTTACATGACGTGTGTTTTGAAGGCC
341    A  F  M  F  Q  G  G  S  S  P  S  I  Y  M  T  C  V  L  K  A
1102   ACTCTTGCTTTTGCACCTAGTGACGCGCTCCACAAATCCTGTTCCTTTGCCAATGGGTGG
361    T  L  A  F  A  P  S  D  A  L  H  K  S  C  S  F  A  N  G  W
1162   CTTGCTGCTGATGGGAACAACCAGGTTTGTGGTTGCTGTGACTCAACATGTGGTCCTGAT
381    L  A  A  D  G  N  N  Q  V  C  G  C  C  D  S  T  C  G  P  D
1222   GGTGGAACTGCTGCTTCTCCTTTTGGAGGCCTTCGGTGGGAAGGGAAGGCCTCGCTCGGT
401    G  G  T  A  A  S  P  F  G  G  L  R  W  E  G  K  A  S  L  G
1282   CCTGTAGTGGTTCAAGAGCACAAGAAGACTTTAGCTGGTCTTCAATAAATGTGGGGGAGC
421    P  V  V  V  Q  E  H  K  K  T  L  A  G  L  Q  *
1342   AAACTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 1. Nucleotide and deduced amino acid sequence of *CaP_ZP3*. The amino acids of the signal peptide are boxed. The amino acids of the *CaP_ZP3* domain are underlined. Two putative O-linked glycosylation sites at residue Thr230 and Thr237 are shaded. Consensus polyadenylation signals AATAAA are italic and bold.

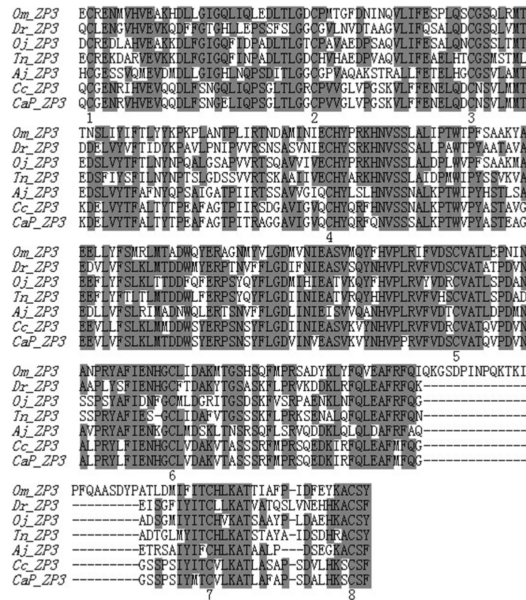

Figure 2. Protein sequence alignment of the CaP_ZP3 domain of *CaP* in comparison with other fish species. *Oncorhynchus mykiss* (Om_ZP3, Q9I9M6), *Danio rerio* (Dr_ZP3, Q5TYP2), *Oryzias javanicus* (Oj_ZP3, Q5EC18), *Tetraodon nigroviridis* (Tn_ZP3, Q4RJR6), *Anguilla japonica* (Aj_ZP3, Q9YIB1), *Cyprinus carpio* (Cc_ZP3, Q92027), *CaP* (CaP_ZP3, AEQ76844). Points (---) are inserted to optimize the alignment of the sequences. The relatively conserved amino acids are shaded. The 8 Cys residues in ZP3 domains are shaded and numbered accordingly.

As shown in Figure 3, there were three independent clusters in the phylogenetic tree. The 1st cluster has only one branch, namely, CsZP3 (*C. semilaevis* ZP3). The 2nd cluster contains mainly some higher vertebrates including the ZP protein of frog, chick, mouse, and human. The 3rd cluster consists of the ZP3 from some fish species, and the *CaP_ZP3* is clustered in close proximity to the ZP3s of *C. auratus* and *C. carpio* (Figure 3).

CaP_ZP3 mRNA expression during ovarian development

To investigate the developmental behavior of *CaP_ZP3* mRNA during ovarian development, we examined the expression patterns of *CaP_ZP3* mRNA in ovaries that were one to 12 months old. Real-time PCR analysis revealed that the transcripts of *CaP_ZP3* were broadly distributed in the 12 tested samples. We found that the *CaP_ZP3* mRNA expression level was extremely low in the 1- and 2-month-old ovaries. Using the 3-month-old *CaP_ZP3* expression value as a calibrator to determine the relative expression levels of other months, the highest *CaP_ZP3* mRNA expression level was seen in the 4-month-old ovary (about 28.17-fold), followed by the 5-month-old ovary (10.27-fold), 6-month-old ovary (7.87-fold), 7-month-old ovary (3.88-fold), and 8-month-old ovary (2.99-fold) (Figure 4). A low expression level was detected in the 9-month-old ovary (about 1.13-fold), 10-month-old ovary (about 1.25-fold), 11-month-old ovary (about 0.79-fold), and 12-month-old ovary (about 0.66-fold) (Figure 4). The specificity of

the real-time PCR products from the 12-month-old ovary was verified by sequencing.

Immunolocalization of CaP_ZP3

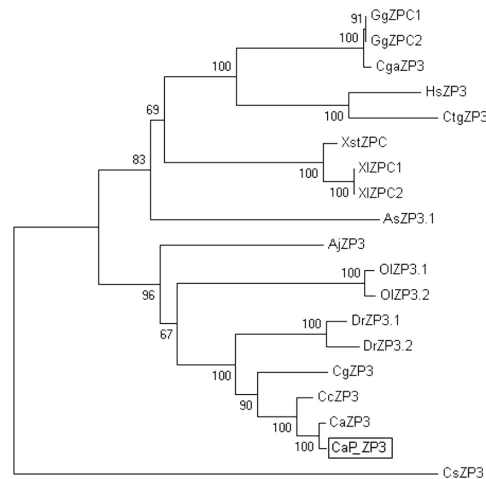

Figure 3. Phylogenetic analysis based on the ZP3 amino acid sequences of various vertebrates including fish, amphibian, and mammals. Numbers at tree nodes refers to percent bootstrap values after 1000 replicates. The CaP_ZP3 is boxed in the phylogenetic tree. *Carassius auratus* (CaZP3, AAD53946), *Carassius auratus* var. Pingxiangnensis (CaP_ZP3, AEQ76844), *Cyprinus carpio* (CcZP3, CAA88836), *Carassius gibelio* (CgZP3, AAD53947), *Acipenser sinensis* (AsZP3.1, AEM43808), *Cynoglossus semilaevis* (CsZP3, ABY81291), *Anguilla japonica* (AjZP3, BAJ61008), *Danio rerio* (DrZP3.1, NP_001103211; DrZP3.2, NP_001020357), *Oryzias latipes* (OlZP3.1, AAM91819), *Oryzias sinensis* (OlZP3.2, AAV34196), *Xenopus tropicalis* (XstZPC, NP_988853), *Xenopus laevis* (XlZPC1, AAB39079; XlZPC2, AAH72326), *Gallus gallus* (GgZPC1, AAV35184; GgZPC2, BAA83418), *Callipepla gambelii* (CgaZP3, ABW87653), *Cricetulus griseus* (CtgZP3, EGW01840), *Homo sapiens* (HsZP3, CAA40095).

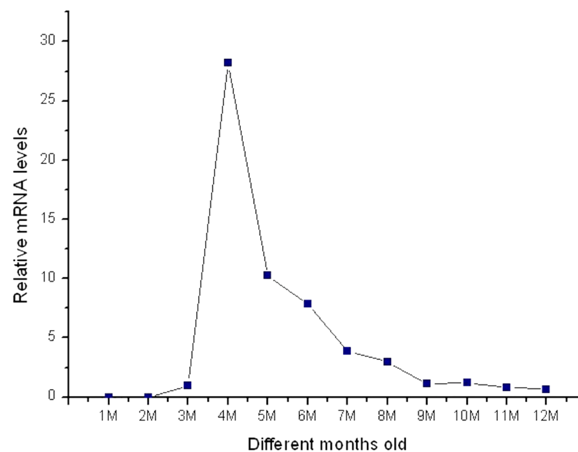

Figure 4. Analyzing the expression of *CaP_ZP3* mRNA by real-time PCR. The mRNA expression of *CaP_ZP3* was measured at every month of one year. The relative mRNA expression of *CaP_ZP3* was calculated by $2^{-\Delta\Delta CT}$ method using β-actin as a reference gene. Data are reported as mean relative expression ± standard deviation for three replicate real-time reactions from pooled tissue of five individual ovaries at each month point.

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In this study, an approximately 31-kDa recombinant CaP_ZP3 protein was obtained. We used ELISA to test the reactivity of the anti-ZP3 protein antiserum. The polyclonal antibody to CaP_ZP3 was used for the immunofluorescence staining to determine the CaP_ZP3 localization in oocytes. The expression of CaP_ZP3 was visualized as green fluorescence, and its localization was shown in Figure 5. The CaP_ZP3 protein was located close to the oocyte plasma membrane. The anti-ZP3 antiserum stained the early stage III oocytes, and intense staining of ZP3 was observed in stage IV oocytes. However, it was not seen in stage I and stage II oocytes.

DISCUSSION

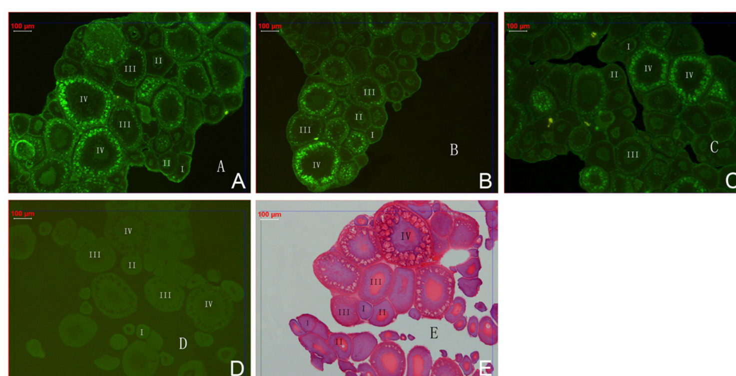

Figure 5. Immunofluorescent staining of CaP_ZP3 protein expression in ovary. Frozen ovarian sections from CaP were probed with a monoclonal antibody to CaP_ZP3 protein followed by anti-rabbit IgG-FITC. CaP_ZP3 proteins exist closest to the oocyte plasma membrane (**A.-C.** Immunofluorescent sections. **D.** Control section. **E.** Hematoxylin and eosin stain section). Different stages of oocyte were marked by roman numeral.

In this study, we successfully isolated and sequenced ZP3 from the special crucian carp mutant, *C. auratus* var. Pingxiangnensis. The deduced amino acid sequence of *CaP_ZP3* shared a high homology with other known *ZP3* genes from *C. auratus* or *Cyprinus carpio*. The alignment of the *CaP_ZP3* domain with that of other fish species indicated that the ZP domains are highly conserved. All eight of the Cys residues of the ZP domain are highly conserved among the fish species that we examined. The phylogenetic analysis showed that CaP_ZP3, CaZP3 (*C. auratus*, AAD53946), and CcZP3 (*C. carpio*, CAA88836) were in the same branch clade. Overall, the primary structures of ZP3 proteins are highly conserved in fish species as revealed by sequence analysis. It has been reported that the O-linked oligosaccharides of ZP3 play an important role in the recognition and adhesion of sperm and eggs (Kinloch et al., 1995; Litscher and Wassarman, 1996). Additionally, the number of O-linked glycosylation sites in the ZP3 proteins seemed to vary between different fish species. There is only one site that is common in carp (Chang et al., 1996), there are five sites in gibel carp (Fan et al., 2001), and there are no sites in zebrafish (Wang and Gong, 1999). CaP_ZP3 was found to have two O-linked glycosylation sites. We suspect that the difference in the number of glycosylation sites in the ZP3 protein might be related to different sperm-egg recognition mechanisms. However, further studies are required to determine whether CaP_ZP3 in the go-

nad is involved in controlling gynogenesis in this fish.

As a major component of the ZP, the amount of *CaP_ZP3* mRNA in oocytes is unusually high (Chang et al., 1996). In mammals, ZP3 is indispensable for early ovarian development; in its absence, the zona matrix does not form and mice are infertile (Liu et al., 1996; Rankin et al., 1996). A major objective of this study was to determine the expression of *CaP_ZP3* in ovaries of different developmental stages. We found that ovaries of all developmental stages contain *ZP3* mRNA, and early stage ovaries have a higher expression level than the later stage ovaries. However, *ZP3* mRNA is indeed present in the later ovary stages, although its expression level was much weaker than that of the early ovary stages. These facts suggest that *ZP3* transcription begins in primitive ovary stages and persists in ovaries to the late developmental phases. In the ovaries that were 4-8 months old (stage II-stage III), the predominant presence and high expression of *CaP_ZP3* suggests an important role for ZP3 during early ovarian development in this species. During the later period, when ovaries were 9-12 months old (stage IV-stage V), there is a dramatic decline in the abundance of *CaP_ZP3* transcripts, and the level falls to less than 3% of the peak in mature eggs. Similar to *CaP*, ZP transcript levels in zebrafish are abundantly transcribed during oogenesis, where 10.3% of total transcripts expressed in the ovary code for proteins of the *ZPA-ZPC* families (Zeng and Gong, 2002). Consistent with this, Chang et al. (1996, 1997) used Northern blot analysis and found carp *ZP2* and *ZP3* mRNAs in all stages of oocytes, with decreasing levels from early to late stage. In mouse, the expression of all three *ZP* genes is precisely regulated during oogenesis and restricted to a 2-week growth phase, when they represent together approximately 1.5% of the total polyA$^+$ RNA. However, in the later stages of oogenesis, their abundance declines, and each zona transcript is present in ovulated eggs at less than 5% of its maximal level (Roller et al., 1989; Epifano et al., 1995). These analogous expression patterns suggest that *ZP3* genes are active during oogenesis, particularly during the early stages of oogenesis. The acellular vitelline envelope develops around the oocyte during the early phases of ovarian development in teleosts. During this period, the oocyte also accumulates RNA (known as maternal RNA) and completes the differentiation of its cellular and noncellular envelopes (Anderson, 1967; Wallace, 1985). In this study, high levels of *ZP3* expression were observed during the early phases of ovarian development (stage II–stage III), which was consistent with results from earlier reports. During the later period of ovary development (9-12 months old; stage IV-stage V), when grown oocytes undergo meiotic maturation and become unfertilized eggs, the growth of the oocyte and formation of the ZP will be accomplished. Therefore, it is reasonable that the level of *ZP3* mRNA falls quite dramatically and maintains a relatively low level in this period.

In this study, we generated antiserum against CaP_ZP3 protein that is specific for CaP_ZP3 protein in oocytes and carried out immunofluorescence assays further determine the localization of ZP3 protein in oocytes of this special mutant fish. The results showed that no staining or very faint staining was detected in the stage I and stage II oocytes with this antiserum. The positive immunostaining signals were primarily detected in the stage III oocytes. Therefore, the translation of CaP_ZP3 protein occurred in stage III oocytes. The high expression levels of *CaP_ZP3* mRNA were observed in stage I and II oocytes and thereafter sharply diminished, in contrast to the observed levels of ZP3 protein. The results indicated that the transcription and translation of the *ZP3* gene in this special triploid fish are asynchronous. Additionally, the translation of ZP3 protein was hysteretic and occurred in stage III oocytes. As the oocyte develops from stage III to stage IV, the ZP3 protein level increases, and the ZP3 content in oocytes increases as vitellogenesis proceeds. ZP proteins appear to be highly conserved to serve as structural components of the

eggshell in different species; their function in sperm recognition does not seem to be conserved (Hyllner et al., 2001). Sperm can penetrate the eggshell anywhere on mammalian eggs, and this is not the case in teleosts, in which sperm must enter through the micropyle (Griffin et al., 1996; Iwamatsu et al., 1997; Hyllner et al., 2001). In our study, the distribution of CaP_ZP3 in oocytes was not restricted to the micropylar region of the egg chorion, but it was also distributed to many sites of the ZP, as observed in mammals. Our results may provide some important clues to further understand whether CaP_ZP3 shares physiological functions with the mammalian homolog, as well as clues to the unknown diverse roles such as the regulation of gynogenesis and bisexual production in the natural triploid crucian carp mutant, *C. auratus* var. Pingxiangnensis.

Conflicts of interest

The authors declare that they have no conflict of interest.

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