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Expression and localization of vitellogenin genes (VTG) and receptor (VGR) in the gonad development of silver pomfret *Pampus argenteus**

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Abstract Vitellogenesis is the main event of oocyte growth in oviparous animals, which is mainly manifested by the accumulation of vitellogenin (VTG). The accumulation of vitellogenin depends mainly on the absorption of exogenous vitellogenin, which enters oocyte through endocytosis mediated by its receptor (VGR). We investigated the expression and localization of VTG and VGR during gonad development of *Pampus argenteus*. The qPCR results show that *vtgs* were not expressed in male fish, but in the ovary and liver of female fish; the expression levels went up at first and then down. The expression levels of *vgr* in the testis were low and only 1%–3% of that in ovary. ELISA results show that during the ovarian development of *P. argenteus*, VTG in liver, serum, and ovary all showed a trend from increasing to decreasing. However, VTG in liver peaked in Stage IV, and in serum and ovary peaked in Stage V, reflecting changes in the characteristics of VTG in the liver (synthesis), blood (transport), and ovaries (accumulation). During gonad development, VGR in the ovaries first increased and then decreased, reaching a peak in Stage V, in contrast to *vgr* mRNA expression. The VGR content in the testis was extremely low and stable, consistent with *vgr* mRNA. Immunohistochemistry results show that the location and intensity of VTG and VGR positive signals were synchronized with the changes of their protein content, which revealed that VTG was mainly synthesized in the liver cytoplasm, secreted into the blood, and transported to ovary in Stage III. VGR is highly expressed in oocytes in Stage II. In Stage III, a large amount of VTG reaches the ovary, when VGR begins to translate and is subsequently transported to the plasma membrane of the oocyte. Therefore, the positive signal of VGR was stronger near the plasma membrane of oocytes in Stages I and II. By using qPCR, ELISA, and immunohistochemistry, the synthesis, transport, and accumulation of vitellogenin were elucidated and the mechanism of its endocytosis on egg membrane mediated by VTG during the development of *P. argenteus* was revealed preliminarily.

Keyword: *Pampus argenteus*; vitellogenin (VTG); vitellogenin receptor (VGR); vitellogenesis

1 INTRODUCTION

Vitellogenin (VTG) is a high-molecular-weight (200–600 kDa) glycophospholipid protein and a precursor of yolk protein (YP). Vitellogenin is the main nutrient source for embryogenesis and early larval development in oviparous animals (Wallace, 1985). There are three types of vitellogenin in acanthomorpha: VtgAa, VtgAb, and VtgC (Hiramatsu et al., 2002b). VtgAa and VtgAb are composed of lipovitellin heavy chain (LvH), phosvitin (Pv), lipovitellin light chain (LvL), β′-component (β′), and C-terminal peptide (CT) in series, which comprises a complete VTG. Due to the lack of Pv, β′, and CT in the structure, VtgC is an incomplete VTG (Matsubara et al., 2003; Hiramatsu

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et al., 2005). Vitellogenesis is the main event of oocyte growth in oviparous animals, mainly manifested in an accumulation of vitellin, which is the material basis to ensure that oviparous animals achieve a high survival rate of offspring. In most oviparous animals, the accumulation of vitellin in oocytes mainly depends on the absorption of exogenous vitellogenin, which enters oocytes through endocytosis mediated by the specific membrane receptor vitellogenin receptor (VGR) (Patiño and Sullivan, 2002; Reading et al., 2009; Lubzens et al., 2010).

The vitellogenin receptor is a specific receptor for vitellogenin located on the egg membrane and belongs to the low-density lipoprotein receptor superfamily (LDLR). It is also called "LR8" because it has eight ligand-binding repeats in the amino acid sequence (Hiramatsu et al., 2004). Its ligand-binding site is located in the LvH domain of vitellogenin. At present, rainbow trout (Perazzolo et al., 1999), white bass (Hiramatsu et al., 2004), *Thunnus thynnus* (Pousis et al., 2012), and *Oncorhynchus clarki* (Mizuta et al., 2013), the basic process of fish VGR-mediated Vtgs endocytosis has been reported as follows: (1) estrogen secreted from follicles before vitellogenesis (17 β-Estradiol) stimulates the liver to synthesize VTG; (2) in the early stage of vitellogenesis, VGR is transported to the cell membrane of oocyte; (3) during vitellogenesis, VTG is transported to the ovaries, passes through the interstitial space in the radial zone after passing through the follicular cell layer, and is specifically bound by VGR on the egg membrane; (4) the complex of Vtgs and its receptor VGR passes through the cage protein and enters the cytoplasm by the nest; (5) Vtgs are enzymatically hydrolyzed and stored in the yolk granules, while VGR returns to the egg membrane to prepare for subsequent binding.

Pampus argenteus is a warm temperate pelagic cluster fish and an important fish in the maritime industry China (Lan et al., 2018). However, due to marine pollution and overfishing, the wild population of *P. argenteus* has been rapidly depleted. Although techniques and methods of industrialized seedling raising and breeding of *P. argenteus* have been developed in recent years, significant problems exist in artificial breeding practices, including the low egg quantities of parent fish, congenital undernutrition of yolk sac larva, which leads to stunting, and even death. These issues seriously restrict the improvement of the breeding quantity and quality of *P. argenteus*. Therefore, in this study, we studied the expression levels and distribution characteristics of Pa-VTGs (*Pa*) and Pa-VTG (*Pa*) during the process of gonad development in artificially cultivated *P. argenteus* and analyzed the relationship between *Pa*-VTGs and *Pa*-VGR in the process of gonadal development. We provide a preliminarily discussion of vitellogenogenesis and *Pa*-VGR-mediated endocytosis mechanism in *P. argenteus*, which is important for understanding the mechanism underlying egg maturation. The aim of this study is to address the lack of fundamental research data on gonadal development and egg maturation in *P. argenteus* and provide a theoretical basis for a better regulation of *P. argenteus* reproduction.

2 MATERIAL AND METHOD

2.1 Experimental fish and sample collection

Artificial cultivation of *P. argenteus* was obtained from *P. argenteus* Breeding Demonstration Base from 2017 to 2018 (Ningbo, China). Healthy individuals with Stages I–VI gland development were selected as experimental animals, and samples of the blood, brain, gill, heart, kidney, esophageal sac, stomach, intestines, muscle, liver, ovaries, and testis were collected for RNA or protein extraction. In addition, a part of the liver, ovaries, and testis was fixed in Bouin's solution for immunohistochemistry.

2.2 Primer design, synthesis, and sequencing

The core sequences of *Pa-vtgs* and *Pa-vgr* were derived from the whole genome of *P. argenteus* (unpublished data). The specific primers for *Pa-vtgs* and *Pa-vgr* were designed using Primer Premier software (version 5.0) (Table 1) and synthesized by Beijing Genomics Institute (Shanghai, China) for semi-quantitative RT-PCR and real-time quantitative PCR (qPCR).

2.3 Total RNA extraction and synthesis of the first strand of cDNA

Total RNA extraction was performed using TRIzol Reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The integrity and concentration of the RNA were detected by 1% agarose gel electrophoresis and a nucleic acid protein concentration analyzer. First-strand cDNA was synthesized using the PrimeScript® RT reagent Kit (TaKaRa, Japan). All cDNAs were stored at -20 °C.

2.4 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out according to the instructions of the 2×Power Taq PCR MasterMix (Bioteke, Beijing, China). β-Actin was

Table 1 Oligonucleotide primers used in this study

Primer name	Sequence reported in 5′→3′	Annealing temperature (T_m)	Amplification efficiency (%)
vtgAa-F	AAGAAAGAGCCCAGAATGAT	52	97.9
vtgAa-R	CTTGTCTCAAACAGGACGATA		
vtgAb-F	TGAATATGCCAACGGAGTC	55	99.9
vtgAb-R	TCAGCCTTTGCGTCCTC		
vtgC-F	TGGACCTGCCCTTTAGTA	53	98.9
vtgC-R	TTAATGCTGCCTGGGTG		
vgr-F	TCAAGGAATGCGATGTAAACG	57	99.5
vgr-R	CGCACTTGTATCCGCCCTTT		
β-actin-F	TGCTGCCTCCTCTTCTTCCC	57	97.8
β-actin-R	CCGCAGGACTCCATACCAA		

selected as the internal reference gene (Table 1). PCR was performed using each tissue cDNA (dilution 1∶25) as template and performed as follows: 94 °C for 5 min and 35 cycles (β-Actin: 25 cycle) at 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products and DNA markers (TaKaRa, Japan) were separated by electrophoresis through 1% agarose gel electrophoresis, and the gels were visualized under UV light.

2.5 Real-time quantitative PCR (qPCR)

qPCR was carried out according to the instructions of the UltraSYBR Mixture kit (Cwbio, Beijing, China), with three parallel analysis samples per group. After the reaction, the amplification and dissolution curves of qPCR were determined. β-actin (Actb) was used as a positive control. The result was analyzed using the $2^{-\Delta\Delta Ct}$ method, while statistical analysis of the data was performed using SPSS software (version 20.0), where $P<0.05$ was considered significant.

2.6 Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out according to the instructions of the fish vitellogenin (VTG)/receptor (VGR) ELISA detection kit (Qiaodu, Shanghai, China). For VTG determination, the serum sample volume was 5 μL. Liver and gonad tissue samples were homogenized in advance. These were diluted once, and added 10 μL; for VGR determination, the final volume was 20 μL. The volume of the sample diluent added was also changed appropriately. However, the total amount of the solution to be tested in each well at 50 μL was unchanged. Statistical analysis of the data was carried out with SPSS 20.0, where $P<0.05$ was considered significant.

2.7 Immunohistochemistry

The streptavidin-biotin complex method was used for immunohistochemistry. The fixed samples were cut into 5-μm sections using conventional paraffin sectioning methods. The slices were then baked at 60 °C in a constant temperature oven for 20 min, followed by xylene dewaxing and gradient alcohol dehydration. Next, citrate buffer (pH 6.0) was added and the samples were heated (95−98 °C) for 10 min to restore the antigen. Then, 3% hydrogen peroxide methanol was added for 10 min to block endogenous catalase, followed by the addition of 5% BSA at 37 °C for 30 min to block non-specific protein binding sites. The primary antibody (rabbit anti-VTG antibody (medaka) (Boaosen, Beijing, China) and rabbit anti-VGR antibody (chicken) (Boaosen, Beijing, China)) was incubated overnight at 4 °C. The secondary antibody (goat anti-rabbit (H+L) IgG) was incubated for 30 min at 37 °C. Using an immunohistochemistry kit and DAB display kit (Boster, Wuhan, China), SABC was added to the slides at 37 °C for 30 min and DAB stain for 5 min, followed by hematoxylin counterstain. After the alcohol and xylene were dehydrated, the sections were mounted in neutral resin and observed under a light microscope.

3 RESULT

3.1 Expression analysis of multiple *Pa-vtgs* and *Pa-vgr* mRNA

3.1.1 Expression analysis of *Pa-vtg* and *Pa-vgr* in different tissues

In this study, we divided the gonad development (testis (Fig.1) and ovary (Fig.2)) of *P. argenteus* into Stages I–VI according to the method of Wang et al. (2017).

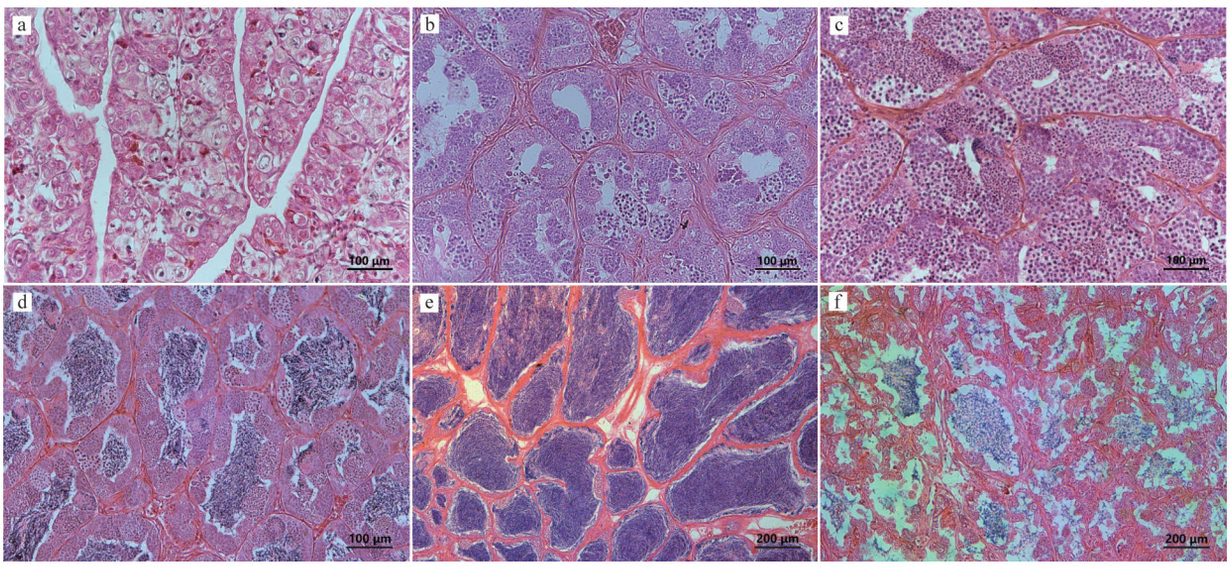

Fig.1 Microscopic testis structure of *P. argenteus*

a. testis in Stage I (×400); b. testis in Stage II (×400); c. testis in Stage III (×400); d. testis in Stage IV (×400); e. testis in Stage V (×200); f. testis in Stage VI (×200).

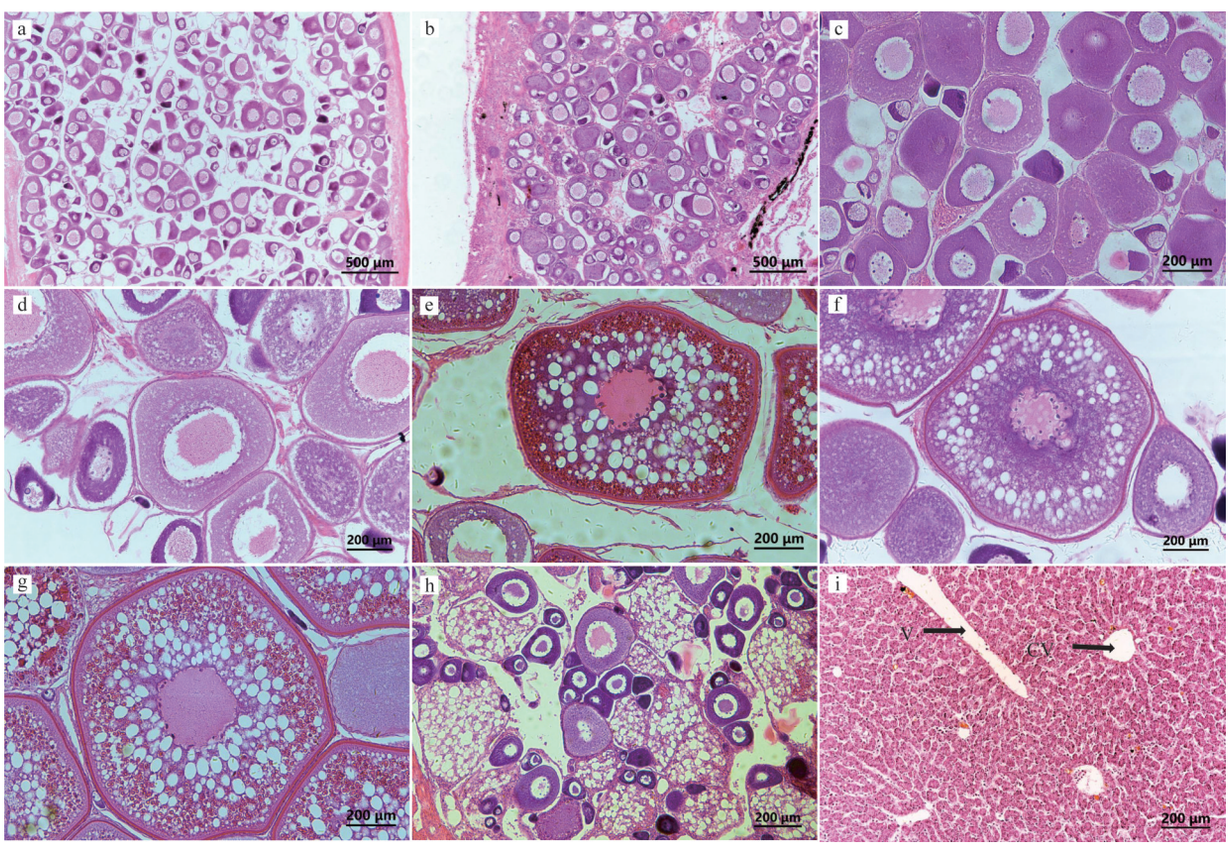

Fig.2 Microscopic ovary structure of *P. argenteus*

a. ovary in Stage I (×100); b. ovary in Stage II (×100); c. early oocytes in Stage III (×200); d. late oocytes in Stage III (×200); e. early oocytes in Stage V (×200); f. late oocytes in Stage V (×200); g. ovary in Stage V (×200); h. ovary in Stage VI (×200); i. liver (×200). CV: central vein; V: vein.

Semi-quantitative RT-PCR was used to detect the expression of *vtgs* (*vtgAa*, *vtgAb*, and *vtgC*) and *vgr* mRNA in the IV Stage of male and female *P. argenteus* tissues (brain, gills, heart, kidney, esophageal sac, stomach, intestine, muscle, liver, and gonads). The results show that *vtgAa* and *vtgC* were not

expressed in male *P. argenteus*, while *vtgAb* was slightly expressed in the brain (Fig.3b). *vtgAa*, *vtgAb*, and *vtgC* were found to be expressed in multiple tissues in female *P. argenteus*, especially in the liver and ovary. The expression bands in the liver were brighter, with the *vtgAb* band being the brightest (Fig.3a). *vgr* mRNA was expressed in all tested tissues of both female and male *P. argenteus*, especially in ovary with high expression (Fig.3a–b).

3.1.2 Expression analysis of *vtgs* mRNA in liver and ovary in different stages of females *P. argenteus*

Furthermore, qPCR was used to detect the expression levels of *vtgs* (*vtgAa*, *vtgAb*, and *vtgC*) mRNAs in the liver and ovaries at different developmental Stages (I–VI) of female *P. argenteus*. The results show that with ovary development, the relative expression of the three *vtgs* mRNAs tended to increase at first and then decrease in the liver of female *P. argenteus* (Fig.4a). In Stage I, the relative expression of the three *vtg* mRNAs was low, but they were significantly different from each other. In Stage II, the expression of *vtgAb* and *vtgC* mRNAs increased significantly, whereas in Stage III, the expression of *vtgAb* mRNAs increased significantly. The expression reached its peak first, which was approximately four times that of Stage II. At this time, the expression of *vtgAa* mRNAs also increased significantly, which was about 4 times that of Stage II and the expression of *vtgC* mRNAs was relatively stable. In Stage IV, the expression of *vtgs* in the liver was generally higher, and the expression of *vtgAa* and *vtgC* mRNAs both reached a peak at this time, which was significantly different from that in Stage III. However, the expression of *vtgAb* mRNAs began to decrease significantly, which was the same as the expression of *vtgAa* mRNAs in the same period. The expression levels of the three *vtgs*

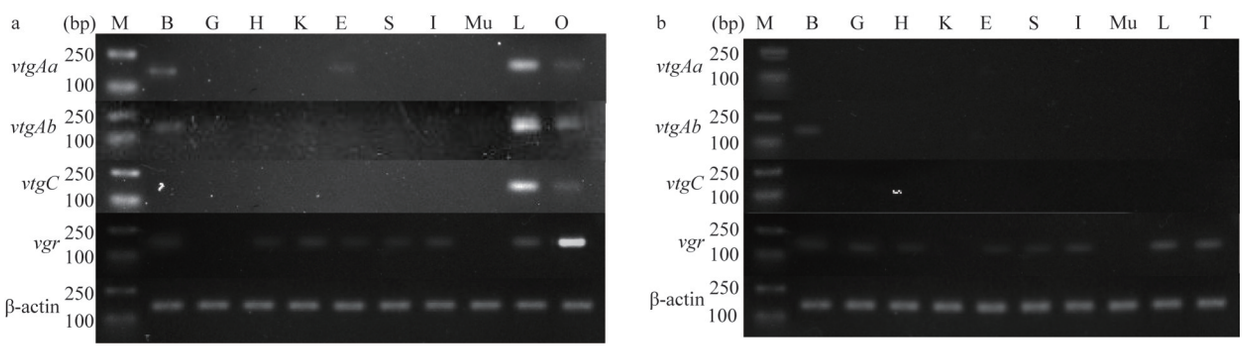

Fig.3 Semi-quantitative RT-PCR analysis of *vtgs* and *vgr* expression in different tissues of *P. argenteus*

a. female; b. male; M: DNA mark 2000; B: brain; G: gill; H: heart; K: kidney; E: esophageal sac; S: stomach; I: intestines; Mu: muscle; L: liver; O: ovary; T: testis.

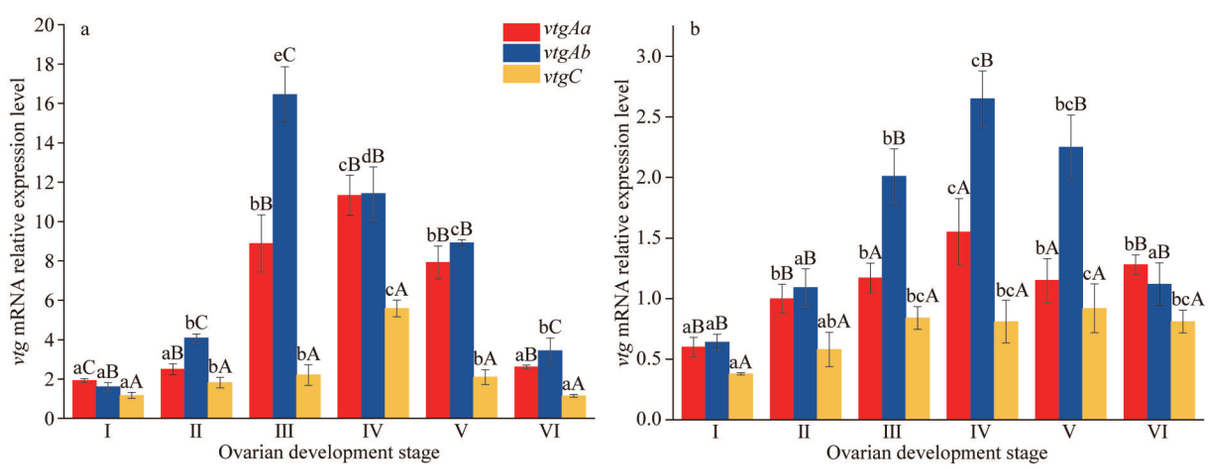

Fig.4 Relative expression of *vtg* mRNA in the liver and ovaries in different stages of females *P. argenteus*

qPCR analysis of mRNA expression of three *vtgs* in the female liver (a) and the ovaries (b) during ovarian development of *P. argenteus*. Different superscript lowercase letters indicate that the relative expression levels of the same gene were significantly different in different periods of the same tissue ($P<0.05$), and different capital letters indicate that the relative expression levels of different *vtg* in the same tissue at the same stage were significantly different ($P<0.05$).

continued to decrease significantly in Stages V and VI , and the expression levels of the three *vtgs* mRNAs in Stage VI were essentially equivalent to that in Stage II. During ovary development, the ratio of the relative expression levels of the three *Pa-vtgs* mRNAs in the liver was *vtgAa*∶*vtgAb*∶*vtgC*=18∶23∶7.

In the different stages of ovary development in female *P. argenteus*, the relative expression of the three *vtgs* mRNAs in the ovary also increased first and then decreased (Fig.4b). In Stage I, the relative expression of the three *vtgs* mRNAs was the lowest in *vtgC*. In Stage II , the expression of *vtgAa* and *vtgAb* mRNAs increased significantly, which was still significantly higher than the expression of *vtgC* mRNAs in the same period. In Stage III, the relative expression of the three *vtgs* mRNA continued to increase significantly, of which *vtgAb* mRNAs had the most significant increase. In Stage IV , the expression of *vtgA*a and *vtgAb* mRNAs continued to increase, and reached their peaks. In Stage V , the expression of *vtgAa* and *vtgAb* mRNAs decreased significantly, while the expression of *vtgC* mRNAs reached the peak, which was twice as high as that in Stage I and less than half of that in the same period for *vtgAb*. In Stage VI , the expression of *vtgAb* mRNAs dropped significantly by about half, while the expression of *vtgAa* rebounded slightly. The relative expression ratio of the three *vtg* mRNAs in the ovary of *P. argenteus* was *vtgAa*∶*vtgAb*∶*vtgC*=6∶9∶4.

We further compare the expression of *vtgAa* (Fig.5a), *vtgAb* (Fig.5b), and *vtgC* (Fig.5c) mRNA in ovary and liver separately during the different stages of ovary development in female *P. argenteus*. The result show that the expression levels of *vtgAa*, *vtgAb*, and *vtgC* in liver were higher than those in ovary. In addition, we also compared the relative

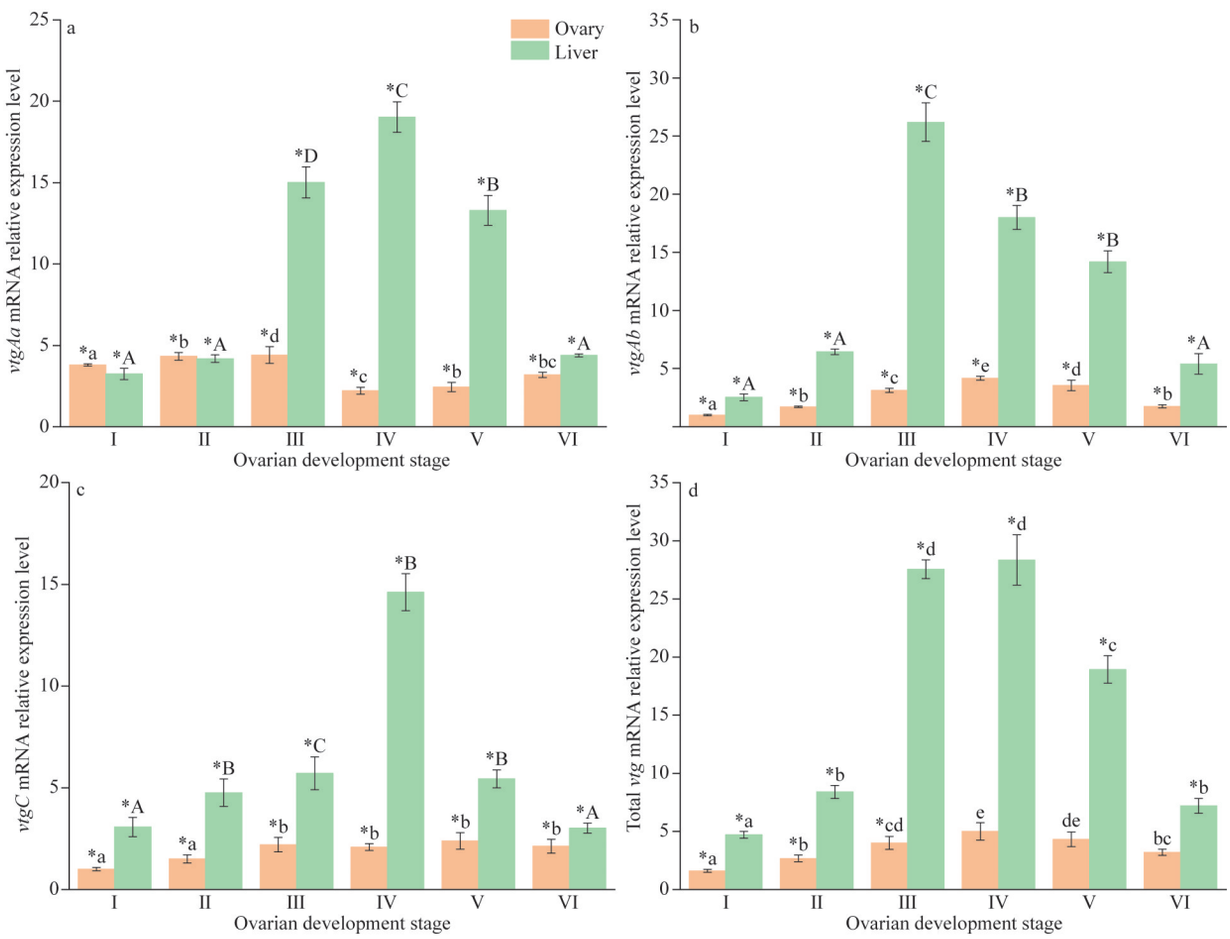

Fig.5 Relative expression of *vtgs* mRNA in the female liver and ovary during ovarian development of *P. argenteus*

The expression of *vtgAa* (a), *vtgAb* (b), *vtgC* (c), and total *vtg* mRNA (d) in ovary and liver separately during the different stages of ovary development in female *P. argenteus*. Different superscript lowercase letters indicate that the relative expression levels of the same gene were significantly different in different stage of ovary (P<0.05), different capital letters indicate that the relative expression levels of the same gene were significantly different in different stage of liver, and * indicates that the relative expression level of the same gene is significantly different in during the same stage of different tissues (P<0.05).

expression of total *vtg* mRNA in the liver and ovaries of *P. argenteus* (Fig.5d). The results show that with the development of the ovary, the expression of total *vtg* mRNA in both the liver and ovary tended to increase at first, and then decreased and reached a peak in Stage IV. However, there was no significant difference in the expression of total *VTG* in Stage III. In contrast, the range of changes in the liver was even greater. At each developmental stage, the expression of total *vtg* in the liver was significantly higher than that in the ovary, with the highest reaching up to seven times in Stage III. In general, the expression of total *vtg* in the liver was approximately 20% of that in the ovary.

3.1.3 Comparison of *vgr* mRNA expression in the ovaries and testis of *P. argenteus*

qPCR was used to detect the level of *vgr* mRNA expression in the ovaries and testes of *P. argenteus* (Fig.6). The results show that with the development of the ovary, the relative expression of *vgr* mRNA in the ovaries tended to decrease at first, and then increase. From Stages I to II, the expression of *vgr* mRNA increased slightly and reached a peak in Stage II . In Stage III, the expression of *vgr* decreased significantly until Stage V, where the expression level of *vgr* reached the trough value. By Stage VI, the expression level of *vgr* had increased significantly.

In the different stages of testis development in *P. argenteus* males, the expression of *vgr* mRNA reached its peak in Stage III. However, its expression was extremely low compared to that in the ovaries during the same period.

3.2 Expression analysis of VTG and VGR protein in *P. argenteus*

3.2.1 Expression analysis of VTG in liver, serum, and gonads of *P. argenteus*

The expression levels of VTG protein in the liver, serum, and gonads at different stages of male and female gonadal development (I–VI) were detected using ELISA. The results show that the VTG content in the liver, serum, and testis of the male *P. argenteus* remained around a value 0, and there was no effective VTG content (data not shown).

In the different stages of ovarian development in female *P. argenteus*, the content of VTG in the liver, serum, and ovary fluctuated within a range of 81.56–996.54 ng/mL, with changes in the liver, serum, and ovary tending to increase at first before subsequently decreasing. The lowest values were observed in Stage I (Fig.7). In the liver, the content of VTG reached its peak in Stage IV, which was 1.28 times higher than that in Stage III. In Stage V, the content of VTG began to decrease, but it was still significantly higher than that in Stage III. In the serum, the VTG content was significantly different at each stage. The level of VTG in the serum was only observed in Phase I, reaching a peak value before significantly decreasing in Stage VI to a level that was significantly lower than the level in Stage III. In the ovary, the content of VTG was also in Stage V,

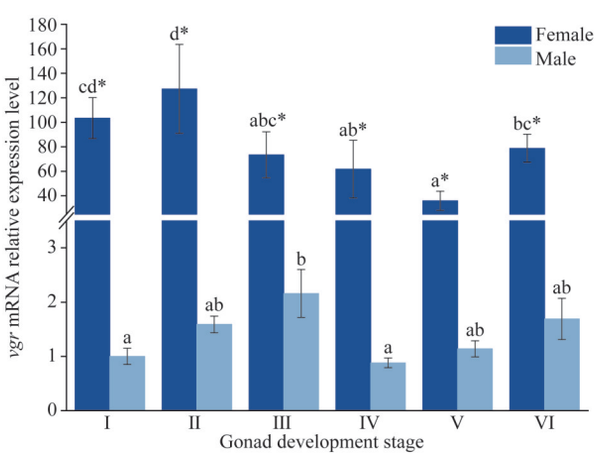

Fig.6 Relative expression of *vgr* mRNA using qPCR in the ovary and testis of *P. argenteus*

Different superscript lowercase letters indicate that the relative expression levels of the same gene were significantly different in different stage of the same tissue (P<0.05). * indicates that the relative expression level of *vgr* is significantly different in different tissues during the same stage (P<0.05).

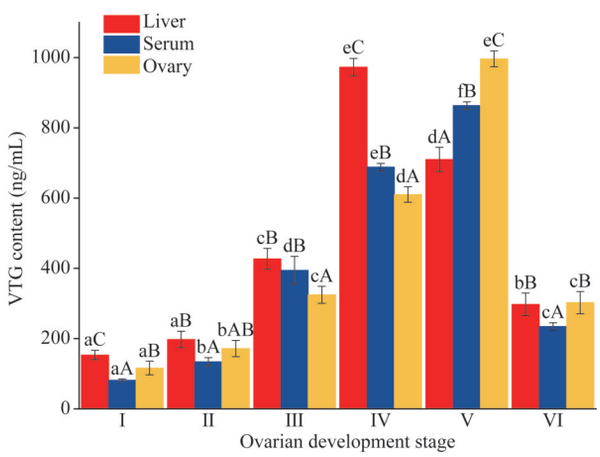

Fig.7 The VTG content in liver, serums and ovary during ovarian development of *P. argenteus*

Different superscript lowercase letters indicate that the VTG content in the same tissue at different ovarian development stage is significantly different (P<0.05), different superscript capital letters indicate that the VTG content in different tissues during the same ovarian development stage is significantly different (P<0.05).

where it reached its peak, which was equivalent to the level in the liver in Stage IV, before decreasing significantly in Stage VI to a level that was the same as in Stage III.

Among the three tissues, when the VTG content in Stages I and II was liver>ovary>serum; in Stages III and IV, liver>serum>ovary; in Stage V, ovary>serum>liver; and in Stage VI, ovary>liver>serum.

3.2.2 Expression analysis of VGR in different gonads development stages of *P. argenteus*

ELISA was used to detect the expression levels of VGR protein in the different stages of gonad development (I–VI) in female and male *P. argenteus*. The results show that the content of VGR in the ovaries of female development was significantly different at each developmental stage, whose content fluctuated in a range of 43.75–564.58 ng/mL (Fig.8). In Stage I, the content of VGR was the lowest, and reached its peak in Stage V. The protein content in Stage VI decreased significantly. In the male testes, the content of VGR was stable between 33.33 and 43.75 ng/mL, and there was no significant difference between various developmental stages. The levels were equivalent to the VGR content of the ovary in Stage I.

3.3 Immunohistochemistry

3.3.1 Protein localization of VTG in the liver and gonads of *P. argenteus*

Immunohistochemical analysis was used to detect the location of VTG protein in the liver and gonads of different gonad development Stages (I–VI) of male and female *P. argenteus* (Figs.9–11) The results show that no positive signal (brown-yellow) of VTG was detected in the liver or testis at different gonad development Stages (I–VI) of male *P. argenteus* (Figs.9a–l, 11b, e, h, k, n, q).

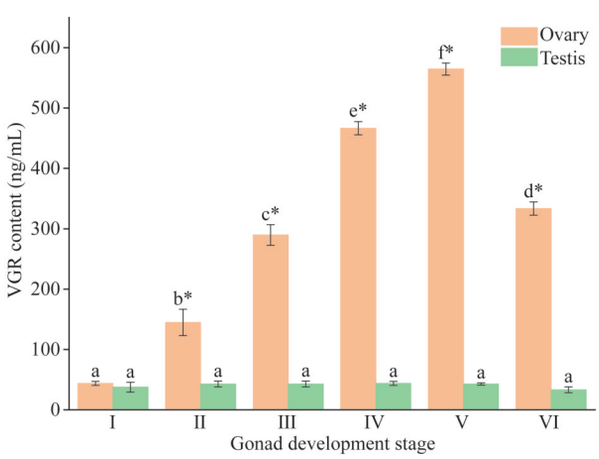

Fig.8 The VGR content in ovary and testis during gonadal development of *P. argenteus*

Different superscript lowercase letters indicate that the VTG content in the same tissue at different ovarian development stage is significantly different (P<0.05). * indicates that the content of VGR is significantly different in different tissues during the same stage (P<0.05).

In the different stages of ovarian development in female *P. argenteus*, positive signals of VTG were detected in the liver (Fig.10a–n). In Stage I, there was a positive signal of VTG in the cytoplasm of hepatocytes, but there was no positive signal of VTG on blood cells in the veins (Fig.11b). In Stage II, a stronger VTG-positive signal was observed in the cytoplasm of hepatocytes, while a small amount of VTG-positive signal was observed in venous blood cells (Fig.9d). In Stage III, a strong positive VTG signal was observed in the cytoplasm of hepatocytes, and more VTG-positive signals were observed in venous blood cells (Fig.10f & g). In Stage IV, a strong positive VTG signal was observed in the cytoplasm of hepatocytes, and a large number of VTG-positive signals were observed in the cytoplasm of hepatocytes (Fig.10i & j). In Stage V, the VTG-positive signal in the cytoplasm of hepatocytes was weaker, and the VTG-positive signal in venous blood cells was reduced (Fig.10l). In Stage VI, the VTG-positive signal in the cytoplasm of hepatocytes continued to weaken, and the VTG-positive signal in venous blood cells decreased (Fig.10n).

In the different stages of ovarian development in female *P. argenteus*, positive VTG signals were also detected in the ovaries (Fig.12b, e, h, k, n, & q). In Stage I, weak VTG-positive signals were detected in the entire ovary, including ovarian connective tissue, the cytoplasm, and plasma membrane of Phase I oocytes (Fig.12b). In Stage II, a small amount of VTG-positive signals were observed in the cytoplasm of Stage II oocytes. However, the VTG-positive signal in the whole ovary was still weak (Fig.12e). In Stage III, a strong VTG-positive signal was observed in the cytoplasm, plasma membrane, and plasma membrane of Phase III oocytes (Fig.12h). Up until Stage IV, the VTG-positive signal was mainly concentrated near the follicular membrane and plasma membrane of the Phase IV oocyte, but not at the radioactive membrane, and only a small amount of positive signals were observed in the cytoplasm (Fig.12k). In Stage V, the positive signals of VTG were more concentrated and intense, and a large number of these signals were detected near the follicular membrane and cytoplasm membrane of Phase V oocytes (near the cytoplasm) (Fig.12n). In

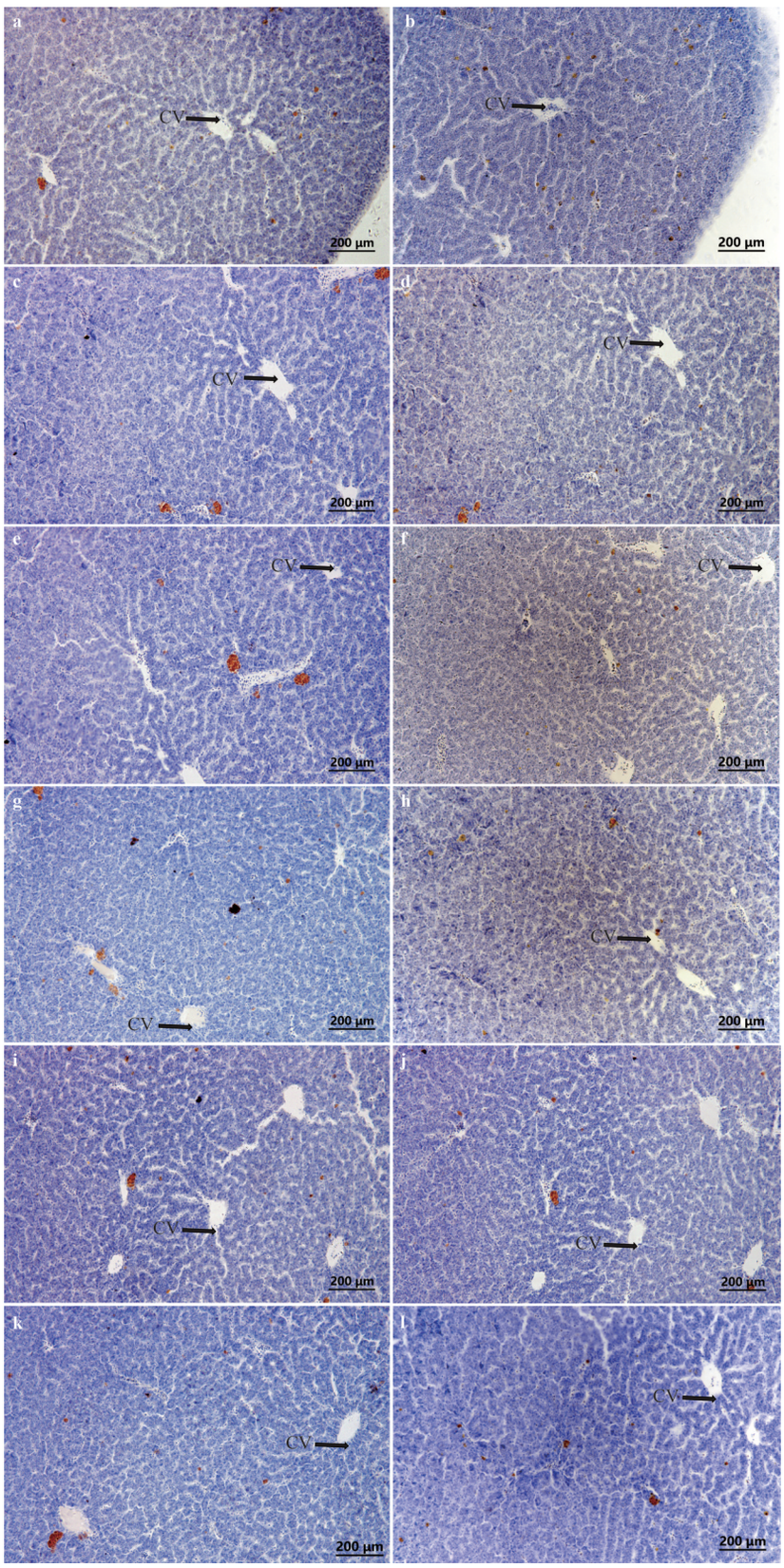

Fig.9 Immunohistochemistry of VTG in liver during different development in male *P. argenteus*

Negative control in liver in Stages I(a), II (c), III (e), IV (g), V (i), VI (k) of testis (×200); immunohistochemistry of VTG in liver in Stages I (b), II (d), III (f), IV (h), V (j), VI(i) of testis (×200). CV: central vein.

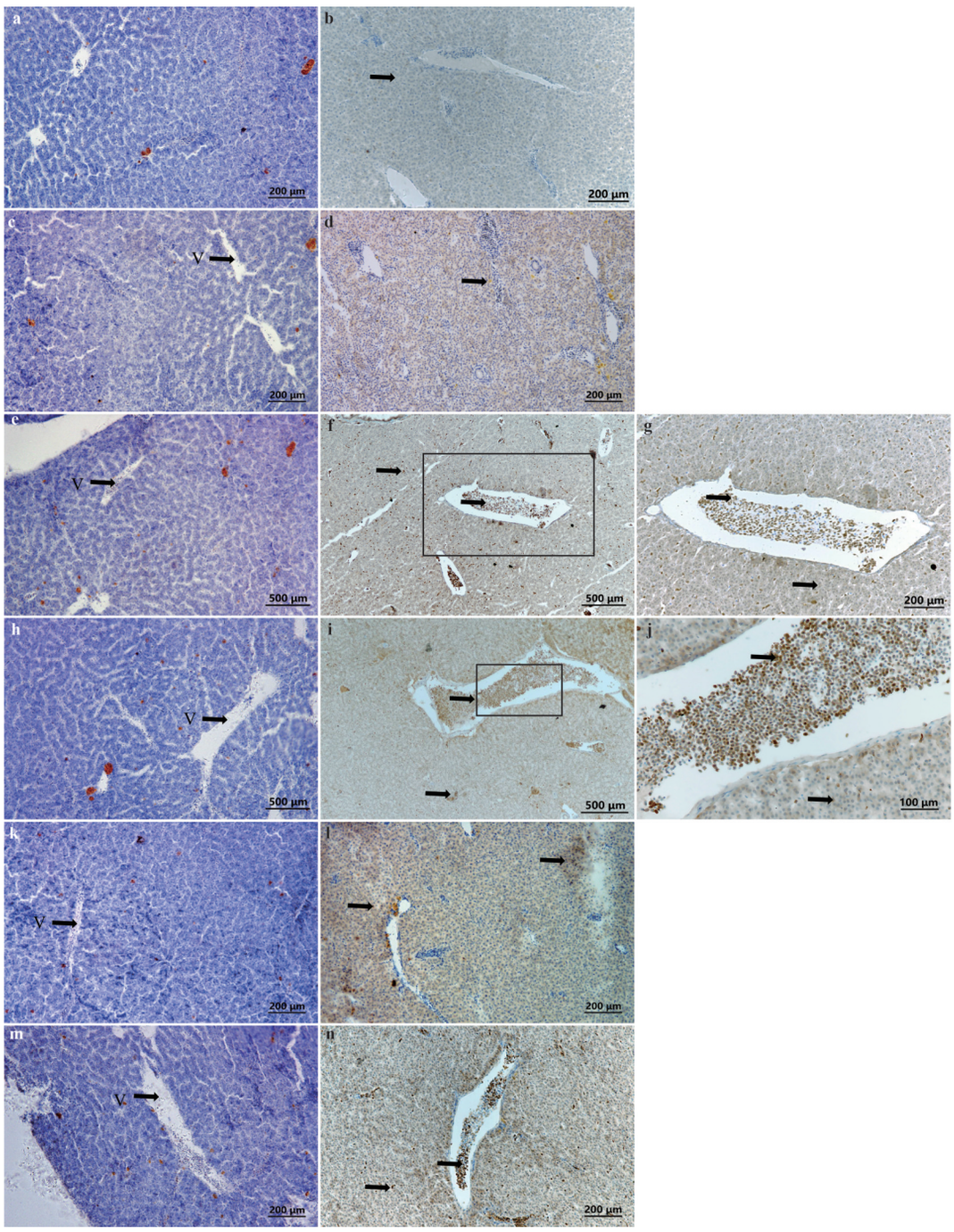

Fig.10 Immunohistochemistry of VTG in liver during different development in female *P. argenteus*

a. negative control in liver in Stage I (a) (×200), II (c) (×200), III (e) (×100), IV (h) (×100), V (k) (×200) of ovary; b. immunohistochemistry of VTG in liver in Stage I of ovary (×200), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes; d. immunohistochemistry of VTG in liver in Stage II of ovary (×200), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes; f. immunohistochemistry of VTG in liver in Stage III of ovary (×100), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes and venous blood cells; g. the partial enlargement of the box in (f), showing that immunohistochemistry of VTG in liver in Stage III of ovary (×200), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes and venous blood cells; i. immunohistochemistry of VTG in liver in Stage IV of ovary (×100), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes and venous blood cells; j. the partial enlargement of the box in (i), showing that immunohistochemistry of VTG in liver in Stage IV of ovary (×400), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes and venous blood cells; l. immunohistochemistry of VTG in liver in Stage V of ovary (×200); m. immunohistochemistry of VTG in liver in Stage IV of ovary (×200), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes; n. immunohistochemistry of VTG in liver in Stage V of ovary (×200), arrows indicate the positive signal of VTG in cytoplasm of hepatocytes and venous blood cells. V: vein.

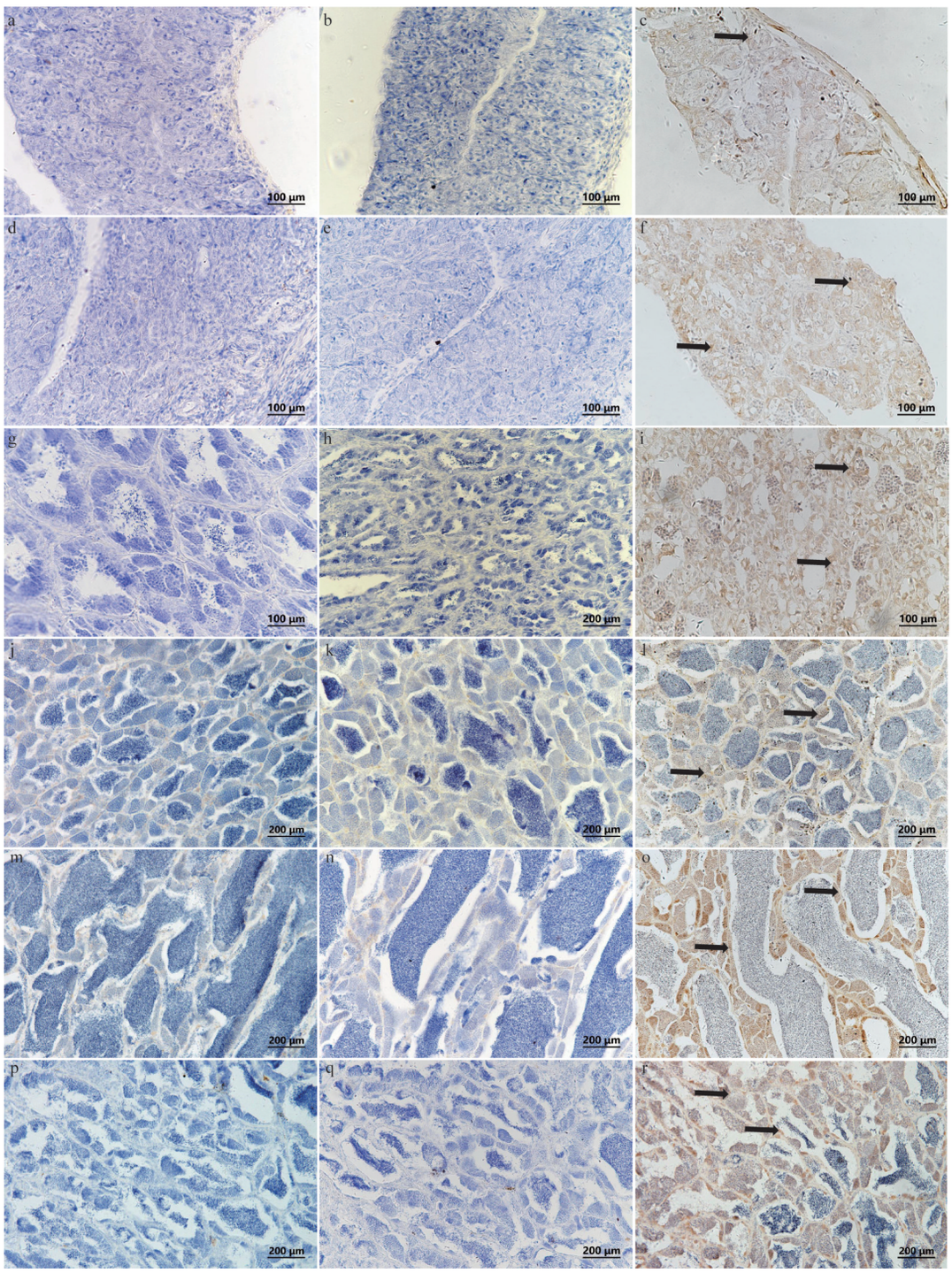

Fig.11 Immunohistochemistry of VTG and VGR in testis during different development in *Pampus argenteus*

Negative control in Stages I (a) (×400), II (d) (×400), III (g) (×400), IV (j) (×200), V (m) (×200),VI (p) (×200) of testis; b. immunohistochemistry of VTG in Stages I (b) (×400), II (e) (×400), III (h) (×400), IV (k) (×200), V (n) (×200), VI (q) (×200) of testis; c. immunohistochemistry of VGR in Stage I of testis (×400), arrows indicate the positive signal of VGR on spermatogonia cytoplasm membrane; e. immunohistochemistry of VTG in Stage II of testis (×400); f. immunohistochemistry of VGR in Stage II of testis (×400), arrows indicate the positive signal of VGR on primary spermatocyte cytoplasm; h. immunohistochemistry of VTG in Stage III of testis (×200); i. immunohistochemistry of VGR in Stage III of testis (×400), arrows indicate the positive signal of VGR on primary spermatocyte cytoplasm; k. immunohistochemistry of VTG in Stage IV of testis (×200); l. immunohistochemistry of VGR in Stage IV of testis (×200), arrows indicate the positive signal of VGR on primary spermatocyte cytoplasm; n. immunohistochemistry of VTG in Stage V of testis (×200); o. immunohistochemistry of VGR in Stage V of testis (×200), arrows indicate the positive signal of VGR on primary spermatocyte cytoplasm; q. immunohistochemistry of VTG in Stage VI of testis (×200); r. immunohistochemistry of VGR in Stage VI of testis (×200), arrows indicate the positive signal of VGR on primary spermatocyte cytoplasm.

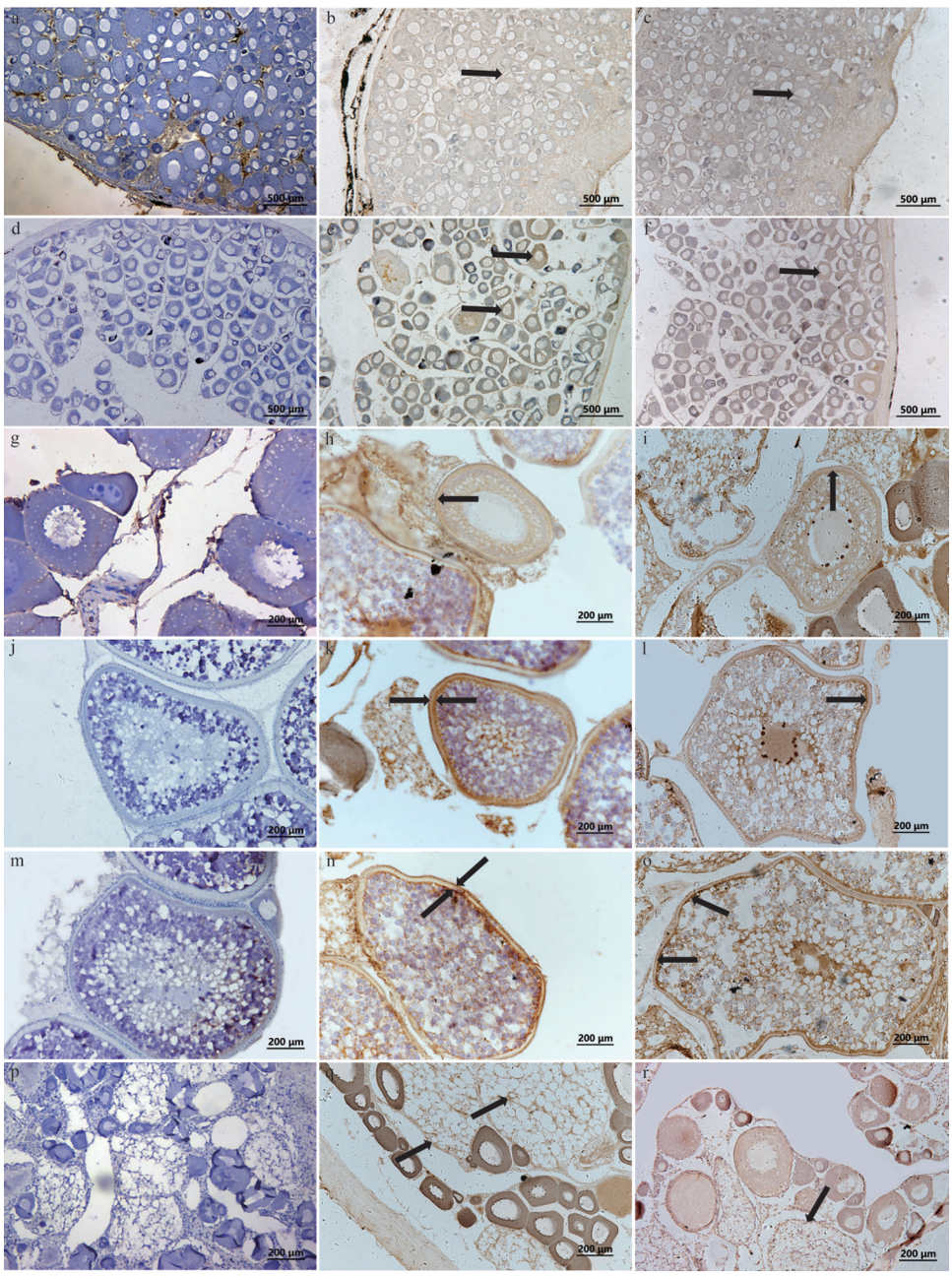

Fig.12 Immunohistochemistry of VTG and VGR in ovary during different developmental stages development in *Pampus argenteus*

Negative control in Stages I (a) (×100), II (d) (×100), III (g) (×200), IV (j) (×200), V (m) (×200), and VI (p) (×200) of ovary; b. immunohistochemistry of VTG in Stage I of ovary (×100), arrows indicate the positive signal of VTG in the cytoplasm membrane of Phase I oocytes; c. immunohistochemistry of VGR in Stage I of ovary (×100), arrows indicate the positive signal of VGR in the cytoplasm membrane of Phase I oocytes; e. immunohistochemistry of VTG in Stage II of ovary (×100), arrows indicate the positive signal of VTG in the cytoplasm membrane of Phase II oocytes; f. immunohistochemistry of VGR in Stage II of ovary (×100), arrows indicate the positive signal of VGR in the cytoplasm membrane of Phase II oocytes; h. immunohistochemistry of VTG in Stage III of ovary (×200), arrows indicate the positive signal of VTG in the cytoplasm membrane of Phase III oocytes; i. immunohistochemistry of VGR in Stage III of ovary (×200), arrows indicate the positive signal of VGR in the cytoplasm membrane of Phase III oocytes; k. immunohistochemistry of VTG in Stage IV of ovary (×200), arrows indicate the positive signal of VTG in the cytoplasm membrane and follicular membrane of Phase IV oocytes; l. immunohistochemistry of VGR in Stage IV of ovary (×200), arrows indicate the positive signal of VGR in the cytoplasm membrane of Phase IV oocytes; n. immunohistochemistry of VTG in Stage V of ovary (×200), arrows indicate the positive signal of VTG in the cytoplasm membrane and follicular membrane of Phase V oocytes; o. immunohistochemistry of VGR in Stage V of ovary (×200), arrows indicate the positive signal of VGR in the cytoplasm membrane of Phase V oocytes; q. immunohistochemistry of VTG in Stage VI of ovary (×200), arrows indicate the positive signal of VTG on degenerate oocyte; r. immunohistochemistry of VGR in Stage VI of ovary (×200), arrows indicate the positive signal of VGR on degenerate oocyte.

Stage VI, the VTG-positive signal in the ovary continued to weaken, but it appeared in various locations (Fig.12q).

3.3.2 Protein localization of VGR in the gonads of *P. argenteus*

Immunohistochemistry was used to detect the location of VGR protein in different gonadal developmental Stages (I–VI) of female and male *P. argenteus*. The results show that the testes of *P. argenteus* developed from Stages I to VI, and positive VGR signals were detected in the testes (Fig.11c, f, i, l, o, & r). In Stages I, weak VGR-positive signals were observed in the testis, including the testis connective tissue and the plasma membrane of spermatogonia (Fig.11c). In Stages II, the VGR signal in the whole testis was strengthened, and a small amount of VGR signal was observed in the cytoplasm of primary spermatocytes, which was stronger than that in the connective tissue and plasma membrane of spermatogonia (Fig.11f). In Stages III, the VGR-positive signal in the whole testis appears in spermatogonia and primary spermatocytes, and was very weak in the secondary spermatocytes (Fig.11i). In Stages IV, a VGR-positive signal was observed in the entire testis, with the secondary spermatocytes and sperm stained blue-purple, indicating that there was essentially no positivity for VGR. The positive signal of VGR in spermatogonia and primary spermatocytes was stronger (Fig.11l). In Stages V, the distribution of positive signals showed differences, with secondary spermatocytes, sperm cells, and sperm showing hardly any positive signals; by contrast, the VGR-positive signal in spermatogonia and primary spermatocytes was stronger, with the signal intensity concentrated in the seminal lobules (Fig.12o). In Stages VI, the majority of the spermatozoa were emptied, and a positive VGR signal was observed in the spermatolobular wall, which is composed of spermatogonia and primary spermatocytes (Fig.12r).

The location of VGR protein varies in the different development Stages (I–VI) of ovaries in *P. argenteus* (Fig.12c, f, i, l, o, & r). In Stages I, similar to the results of VTG immunohistochemistry, weak VGR-positive signals were observed in the entire ovary, including in the ovarian connective tissue, cytoplasm, and plasma membrane of oocytes in Phase I; the strength of the signal was not different from that of VTG (Fig.12c). In Stages II, a small amount of VGR-positive signal was detected in the cytoplasm of Phase II oocytes; however, the VGR-positive signal in the whole ovary was weak (Fig.12f), similar to VTG. In Stages III, a VGR-positive signal was observed throughout the ovary, as well as in the cytoplasm, plasma membrane, and follicular membrane of Phase III oocytes (Fig.12i). When developing to Stages IV, VGR-positive signals were mainly concentrated near the plasma membrane, follicular membrane, and cytoplasm of Phase IV oocytes. However, as with VTG, no signal was observed in the radioactive membrane (Fig.12l). In Stages V, the VGR-positive signal in the ovary was more intense, and a large number were detected near the plasma membrane (near the cytoplasm), follicular membrane, and cytoplasm of Phase V oocytes (Fig.12o). The signal distribution range was wider than that of VTG in the same period. In Stages VI, the positive signal of VGR in the ovary continued to weaken. As with VTG, it appeared at various positions in the ovary (Fig.12r).

4 DISCUSSION

Vitellogenin is the precursor of egg yolk protein in oviparous animals and is the most important source of nutrition during embryogenesis and early larval development. The vitellogenin receptor is a specific receptor for vitellogenin that mediates the entry of vitellogenin into oocytes through endocytosis. In this study, three *vtgs* genes (*vtgAa*, *vtgAb*, and *vtgC*) and *vgr* cDNA sequences were retrieved from the gene library of *P. argenteus*, which confirmed the diversity of the *VTG* genes of *P. argenteus* according to the *VTG* "3-gene model" (Finn and Kristoffersen, 2007; Wu et al., 2013; Lubzens et al., 2017).

4.1 Expression and distribution of *vtgs* and *vgr* in male *P. argenteus*

*vtg*s are not expressed in various tissues of male *P. argenteus*, and the expression of *vtgs* and VTG was not detected in the liver and testis of male *P. argenteus* at different stages of testis development. This is consistent with the conclusion that vitellogenin is absent or negligible in male fish (Hara et al., 2016). *VGR* is expressed in most tissues of male *P. argenteus*, and a small amount of *VGR* is expressed in the testes of *P. argenteus*. Its expression did not change with the development of testes. This is consistent with reports on *Oreochromis aureus* (Li et al., 2003), *Morone americana* (Hiramatsu et al., 2004), *Thunnus thynnus* (Pousis et al., 2012), and *Oncorhynchus clarki* (Mizuta et al., 2013). As the

result of immunohistochemistry (IHC), in the early stages of testis development (Stages I and II), VGR was widespread in the testis, spermatogonia, and primary spermatocytes, and the signal in the intercellular substance was relatively strong. When the testis develops into Stage III, the VGR signal was weaker in secondary spermatocytes. After Stages IV, V, and VI, the secondary spermatocytes, sperm cells, and spermids had neither no VGR signal nor a negligible amount, which results in the concentration of VGR signals around the spermatolobule. To sum up, although the *vgr* expression and VGR content of *P. argenteus* are not regulated by gonadal development, the vitellogenin receptor is almost exclusively expressed in early sperm cells. This is a novel discovery regarding the fish vitellogenin receptor, and will require further research to verify and confirm the result. We speculated that the expression of *vgr* in *P. argenteus* is not only involved in reproduction, but may also be involved in other metabolic effects.

4.2 Expression and distribution of vitellogenin and its receptor in female *P. argenteus*

4.2.1 Expression and localization of VTG in the gonad development of *P. argenteus*

The synthesis of vitellogenin in many oviparous animals is under the influence of both exogenous and endogenous synthesis at the same time. Even at different stages of development, the two synthesis modes play different roles, although they are eventually transported by blood to the ovaries, where endocytosis and absorption are mediated by vitellogenin receptors on the oocyte membrane (Chen et al., 1997; Avarre et al., 2003; Tsutsui et al., 2004; Okumura et al., 2007; Zmora et al., 2007). The synthesis of endogenous vitellin refers to its synthesis in oocytes (Tarrant et al., 1999), while the synthesis of exogenous vitellin refers to its synthesis in organs other than the ovary, such as the liver (Chen et al., 1997).

It has long been considered that the liver is the main site of vitellogenin synthesis in fish. Our results confirm that this is also the case for *P. argenteus* (Finn and Kristoffersen, 2007). The results of the tissue distribution study showed that the expression of the three *vtg* genes in the liver of female fish was much higher than that in ovaries or other tissues. This was further confirmed by subsequent analysis of the expression of the liver and ovaries at different stages of ovarian development. In the livers of female *P. argenteus*, the expression level of *vtgAb* was significantly higher than that of *vtgAa* and *vtgC* (18∶23∶7). These results are similar to those of striped bass (Williams et al., 2014) and *Scophthalmus maximus* (Xue et al., 2018) reported in previous studies. In sea teleosts, such as *Verasper moseri* (Matsubara et al., 1999), *Pagrus major* (Sawaguchi et al., 2006), and *Dicentrarchus labrax* (Yilmaz et al., 2016), VtgAb has been reported to be the main vitellogenin. With the development of ovaries, the main products of VtgAb are rarely decomposed and remain relatively intact. VtgAb is the main source of nutrients for larval development.

During ovarian development in *P. argenteus*, the total *Pa-vtg* mRNA was mainly expressed in the liver, and only a small amount was expressed in the ovary (approximately 20% of that expressed in the liver). The total *Pa-vtg* mRNA expression in the liver tended to increase at first and then decrease, which is similar to that reported in striped bass (Williams et al., 2014), *Oreochromis niloticus* (Luo et al., 2015), and *Scophthalmus maximus* (Xue et al., 2018). During Stages I and II of ovarian development in *P. argenteus* (before vitellogenesis), before oocytes had begun to absorb vitellogenin, the difference between the exogenous expression of *vtg* (liver) and the endogenous expression (ovarian) was not very large (Fig.2c). Thus, the amount of *Pa*-VTG content was as follows: liver>ovarian>serum. By observing the immunohistochemical section of the liver of *P. argenteus*, we found that the liver cells were arranged in a tight and regular manner. Furthemore, brown-yellow particles of different sizes and shades (VTG-positive signals) were observed in the cytoplasm of liver cells, revealing that *Pa*-VTG is synthesized in the cytoplasm of liver cells, which is consistent with reports in most fish (Hara et al., 2016). During vitellogenesis in *P. argenteus*, *VTG* in the liver began to be expressed in large quantities. The level of *VTG* expression in Stage III ovaries was more than three times that of Stage II. In this stage, the expression of *VTG* in the liver and ovaries was very different. Once the endogenous expression of *vtg* was minimized, the liver started to work and the synthesis of VTG was relatively high. The *Pa*-VTG content was as follows: liver>serum>ovary. Due to the significant increase in the protein content of VTG in the liver, the section of liver showed an intense brown. In addition, the veins and blood vessels of the liver in *P. argenteus* were also brown. A similar phenomenon has been reported in zebrafish, which has been proven to occur due to the secretion of a large amount of vitellogenin in blood cells (Li

et al., 2003). In Stage IV, the expression of liver *VTG* and the content of VTG both reached their peak. At this stage, the positive signal was the strongest, the color of liver cells was darkened, and the blood cells in the veins were covered with brown. This is similar to the findings reported for *Xiphophorus helleri* in previous studies (Liu et al., 2011), suggesting that vitellogenin may be synthesized and secreted in large quantities. In Stage V, the expression of *vtg* and VTG in the liver decreased significantly, and the positive signal of VTG was also weakened. The VTG content was as follows: ovarian>serum>liver. This may be due to the completion of the synthesis and accumulation of vitellogenin after negative feedback regulation begins to inhibit the expression of vitellogenin in the liver, which weakens the synthesis of VTG in the liver. At this stage, the serum continues to transport VTG, resulting in a higher VTG content in the serum than in the liver (Luo et al., 2015), which is consistent with reports in other fish, including *Odontobutis potamophilus* (Li et al., 2003) and *O. niloticus* (Luo et al., 2015). We also found that with the growth of oocytes, the content of VTG in the ovaries increased by more than three times from Stages III to V, indicating a rapid rate of yolk deposition. The accumulation of vitellogenin was also observed in the small growth period and the large growth period of the oocytes. The small and large growth periods of oocytes are important periods for their growth, when the accumulation of vitellogenin is used for the growth of oocytes. However, from Stages IV to V, the growth rate of the VTG content was lower than that of Stages III to IV. This is due to the development of the ovary into Stage V, oocytes mature successively. As a result, VTG is decomposed into yolk protein to provide nutrition for ovulation. Reserve participates in embryonic development and larval growth (Li et al., 2003). In Stage VI (ovulation is complete), the expression levels of VTG and VTG in liver were further significantly decreased. At this time, the content of VTG in the liver and ovary is basically the same, which is higher than that in the serum. This may be the result of reproductive physiological regulation of *P. argenteus*, which stores a certain amount of VTG in the body to wait for the next reproduction (Little et al., 2000).

4.2.2 Expression and Localization of VGR in the Gonad Development of *P. argenteus*

In teleosts, VGR is a specific receptor for VTG located on the egg membrane, which mediates the entry of VTG into oocytes through endocytosis and is mainly related to reproduction (Tyler and Lancaster, 1993; Hiramatsu et al., 2002a; Lubzens et al., 2010). Although *Pa-vgr* is expressed in most tissues of female and male fish, it is mainly expressed in the ovaries. This is similar to the results obtained for Clark salmon (Mizuta et al., 2013).

During the ovarian development of *P. argenteus*, the change in the *vgr* expression was found to be in complete contrast to that of *VTG*. At the same time, this is contrary to the change in 17β-estradiol in the serum of *P. argenteus* reported by Wang et al. (2017), indicating that although VGR is related to reproduction, it is not regulated by estrogen (Pousis et al., 2012). Similarly, the change in the expression of *VGR* mRNA in female *P. argenteus* and in the VGR content also showed a diametrically opposing trend, and the strength of the VGR-positive signal was synchronized with the VGR protein content. The relative expression of *VGR* mRNA in female *P. argenteus* reached its peak in Stage II, and was not significantly different from Stage I. On the contrary, the positive signal of VGR was weaker in Stages I and II oocytes, and more positive signals were observed in the cytoplasm of oocytes. In rainbow trout (Hiramatsu et al., 2004) and coho salmon (Luckenbach et al., 2008), VGR has been found to be expressed in large amounts in the ovaries and newly developing oocytes of juvenile fish, which is consistent with the results presented in this study for *P. argenteus*. The expression of *Pa-vgr* reached a trough when the ovary developed to Stage V, but started to rise again after laying eggs. The change in the VGR content and the expression of *VGR* showed a diametrically opposite trend. The VGR content was highest in Stage V. The same phenomenon has also been reported in rainbow trout (Perazzolo et al., 1999) and white bass (Hiramatsu et al., 2004). Pousis et al. (2012) proposed two hypotheses based on the abnormal expression pattern of *vgr* during vitellogenesis: (1) the synthesis time of VGR was earlier than the time when it performs reproductive function; after translation, it is stored in the cytoplasm waiting for vitellogenesis; (2) VGR begins to be transcribed before vitellogenesis, but it is stored in the "RNA pool" of the cell. Until vitellogenesis, a large number of translations begin to take place, resulting in the formation of functional proteins involved in vitellogenin absorption. In this study, *Pa-vgr* was highly expressed in Stages I and II ovaries, indicating that the transcription of *Pa-vgr* occurred in the early stage of oocyte development,

while the content of VGR increased from Stages II to III , and continued into Stage V. The immunohistochemical results also show that a large amount of VGR was translated and transported this period, VGR-positive signals were widely distributed throughout the oocytes. In Stages IV and V, although the VGR-positive signal was stronger near the oocyte plasma membrane, a weak signal was also observed in the cytoplasm, which is not the same as the VTG in the ovary in the same period. Thus, we speculated that VTG was decomposed and stored after entering the cytoplasm of the oocyte, and VGR is the reason for the repeated round trip binding of VTG between the cytoplasm and plasma membrane (Mizuta et al., 2013). These findings indicate that the translation and synthesis of VGR occurs during vitellogenesis, which is in line with the second conjecture of Pousis et al. (2012). In Stages IV and V, the VGR content increased significantly; however, at the same time, the expression level of *VTG* was only 1/3–1/2 of the highest value (Stage III). It is possible that VGR returns to the egg membrane to continue its function after mediating VTG endocytosis, thus completing the cycle of VTG absorption, rather than relying on the continuous transcription and translation of *VGR* (Perazzolo et al., 1999; Hiramatsu et al., 2004). After spawning, although the VGR content was decreased, the expression level of *VGR* increased in preparation for the next vitellogenesis (Mizuta et al., 2013).

Pousis et al. (2012) studied wild and farmed *T. thynnus* and found that although the farmed *T. thynnus* had higher levels of *VTG* expression, the wild population had more *VGR* expression. Insufficient levels of VGR expression can reduce the number of eggs, and even cause infertility in insects. Later, Pousis et al. (2012) conducted a bait experiment, through which they proposed that a culture system with high levels of nutrition (fatty acids and amino acids) could improve the expression of *VGR* and the absorption capacity of oocytes to vitellogenin and other lipoproteins without affecting the expression level of *VTG*, thus improving the fertility of the parents. A comparison of VGR expression between wild and cultured silver pompanos will need to be conducted in the future to gain novel insights into the artificial breeding of *P. argenteus*.

5 CONCLUSION

This study on the expression and localization of VTG and its receptor VGR during gonad development in *P. argenteus* show that Vtgs were not expressed in male fish but concentrated in liver and a little in ovary, indicating that liver is the main synthesis site of *VTG* in *P. argenteus*. The expression of *VGR* was low in the testis and the expression pattern was opposite to that of *VTG* that increased first and then fell. During the ovarian development of *P. argenteus*, VTG in liver peaked in Stage IV, and in serum and ovary peaked in Stage V, reflecting the changes in the characteristics of VTG in the liver (synthesis), blood (transport), and ovaries (accumulation). However, VGR in the ovaries first increased and then decreased, reaching a peak in Stage V, in contrast to *VGR* mRNA expression. The location and intensity of VTG and VGR positive signals were synchronized with the changes of their protein content, which revealed that VTG was mainly synthesized in the liver cytoplasm, secreted into the blood, and transported to ovary in Stage III. VGR was highly expressed in oocytes in Stage II. In Stage III, a large amount of VTG reached the ovary, when VGR began to translate and was subsequently transported to the plasma membrane of the oocyte. In brief, in this study, we preliminarily elucidated the synthesis, transport, and accumulation of vitellogenin, and the process mechanism of its endocytosis on egg membrane mediated by VTG during the development of *P. argenteus*. In the future, the specific mechanism needs to be further studied.

6 DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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